A Study of the Copper-Binding Proteins in Liver and Kidney Tissue of Neonatal Normal and Mottled Mutant Mice

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The Cu^{2+} -binding proteins from liver and kidney tissue of 7–8-day-old brindled (Mo^{br}) mice and their normal littermates were compared. (1) Separation over Bio-Gel P-10 showed that the differences in the Cu^{2+} content of mutant tissues were largely associated with a low-molecular-weight protein fraction (mol.wt. 14500). (2) Further purification of this low-molecular-weight fraction by anion-exchange chromatography revealed four subfractions. The Cu^{2+} content of each subfraction reflected the Cu^{2+} status of the tissue of origin; the Cu^{2+} contents of the mutant kidney subfractions were elevated and those of the mutant liver were depressed compared with normal. In contrast, the protein contents of the subfractions were less variable and did not reflect the differing Cu^{2+} contents. (3) Amino acid analysis of the four subfractions from CuCl_2 -treated mutant and normal animals revealed close similarities. The proteins showed high glycine, glutamic acid, serine, alanine and lysine contents and a rather variable cysteine content. Differences were apparent in the normal liver subfractions, which showed a higher cysteine content and lower glutamic acid content than did either the mutant liver or normal and mutant kidney subfractions. These observations, together with the recorded presence of aromatic amino acids, indicated that these proteins are not thioneins.

Menkes' kinky-hair or steely-hair disease was first described by Menkes *et al.* (1962) as an X-linked recessive disorder, characterized by focal cerebral and cerebellar degeneration, pili torti, diminished pigmentation and a tendency to aortic aneurism. Affected male infants fail to thrive, and die in early infancy. More recently, a defect in copper homeostasis has been demonstrated (Danks *et al.*, 1972) in affected infants. The Cu^{2+} concentrations of the brain, liver and serum are considerably decreased, with Cu^{2+} accumulating in the intestinal mucosa and in the kidney (Danks, 1977). Cultured skin fibroblasts also show increased Cu^{2+} retention (Beratis *et al.*, 1978; Horn, 1976), and, in an aborted Menkes'-disease foetus, Heydorn *et al.* (1975) demonstrated Cu^{2+} accumulation in all tissues except liver.

A convenient animal model for this disease was identified in the mottled (Mo) mutant mouse by Hunt (1974). Depending on the particular Mo allele, the severity of the disease ranges from early lethality *in utero* to relatively normal viability and fertility. The brindled (Mo^{br}) allele is perhaps the most useful, since death occurs in mutant hemizygotes at 14 days *post partum*, an approximately equivalent developmental age to the time of death in most Menkes'-disease infants. As in the human disease, the major feature of the neonatal Mo mouse is Cu^{2+} accumulation in the gut and kidney and

reduction elsewhere, although the diminished liver concentration and gut accumulation are largely eliminated in older mice hemizygous for the viable-brindled (Mo^{br}) allele (Hunt & Port, 1979). A study of the cytosol Cu^{2+} -binding proteins has shown that both the accumulation in the gut and kidney and the decrease in the liver are largely associated with a low-molecular-weight protein fraction (mol.wt. 14500) that is apparently synthesized constitutively in neonatal kidney and liver tissue (Hunt & Port, 1979). Further purification of this protein fraction from liver tissue by ion-exchange chromatography has revealed four subfractions, with each mutant subfraction having a diminished Cu^{2+} content.

Cytosol metal-binding proteins were first identified and characterized by Margoshes & Vallee (1957) and Kägi & Vallee (1960), and are generally referred to as metallothioneins in recognition of their high metal and cysteine contents. Bremner & Young (1976) have presented evidence that the low-molecular-weight Cu^{2+} -binding protein present in Cu^{2+} -stimulated adult rat liver is a metallothionein, although non-thionein proteins have been implicated by other workers (Evans *et al.*, 1975; Irons & Smith, 1977; Riordan & Gower, 1975; Winge *et al.*, 1975). At present, it is unclear whether different Cu^{2+} -binding proteins are indeed present or whether the non-thionein proteins reported arise by oxidative degradation of metallothioneins during isolation (Hartmann & Weser, 1977).

Abbreviation used: SDS, sodium dodecyl sulphate.

The purpose of the present work was to characterize further the Cu^{2+} -binding proteins from both normal and *Mo* mutant mice in order to establish their precise role in the aetiology of this disorder. It is also important to establish whether these proteins are similar to the metal-binding proteins previously mentioned, or represent proteins restricted to the neonatal stage of development.

Experimental

Materials

All reagents were analytical grade, and all water was double-distilled. Glassware was soaked in acid and rinsed thoroughly before use.

Protein determination

Protein concentrations were measured by the fluorimetric method of Böhlen *et al.* (1973) as modified by Froehlich & Cunningham (1976), with bovine serum albumin [fraction V; Sigma (London) Chemical Co., London S.W.6, U.K.] as standard. Fluorescamine (Fluram) was purchased from Hoffman-La Roche, Basel, Switzerland.

Copper determination

Cu^{2+} concentrations were measured by flameless atomic absorption spectrometry with a Perkin-Elmer HGA-74 heated graphite furnace attached to a Perkin-Elmer model 303 atomic absorption spectrophotometer equipped with deuterium background correction.

Animals

The 7–8-day-old normal male mice and their mutant (*Mo*^{br}) littermates used were from a random-bred stock kept at Queen Mary College. Animals were maintained on a normal breeding diet (Oxoid) with a Cu^{2+} content of 25 mg/kg. A solution of CuCl_2 at a dose of 0.05 mg/animal was administered intraperitoneally and the animals were killed by decapitation 6 h later. The livers and kidneys were removed, weighed and stored at -20°C until use.

Fractionation of liver and kidneys

Liver tissue and kidney tissue from seven to 16 animals were homogenized in 3–4 vol. (v/w) of 10 mM-Tris/HCl buffer, pH 7.4 at 4°C , and each homogenate was centrifuged at 80000g ($r_{\text{av.}}$ 2.55 cm) for 1½ h at 4°C in an M.S.E. Superspeed 65 centrifuge. The supernatant was removed (any fatty layer being avoided) and where necessary concentrated to approx. 2 ml in an Amicon pressure cell equipped with a UM2 filter.

The supernatant was then applied to a Bio-Gel P-10 column (40 cm × 3.3 cm; void volume 105 ml) and eluted with 10 mM-Tris/HCl buffer, pH 7.4 at 4°C , at a flow rate of 12 ml/h. Fractions of volume 1.6 ml were collected and their Cu^{2+} contents were determined. The Cu^{2+} -containing fractions corresponding to a molecular weight of 14000–15000 were pooled.

The pooled fractions were applied to a DEAE-Sephadex A-25 column (19 cm × 1.5 cm; void volume 15 ml), previously equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, at a flow rate of 7.5 ml/h at 4°C . A gradient was then applied with 100 ml of 10 mM-Tris/HCl buffer, pH 7.4, and 100 ml of 0.2 M-Tris/HCl buffer, pH 7.4, and 2.4 ml fractions were collected. In later experiments, the column was extensively washed with 10 mM-Tris/HCl buffer, pH 7.4, after application of the pooled fractions and before application of the gradient. The fractions were assayed for Cu^{2+} content, and for Tris content by using the Fluram method of Böhlen *et al.* (1973). The relatively low protein content of the fractions does not significantly affect the accuracy of the determination of Tris concentration. The fractions corresponding to the major Cu^{2+} -containing peaks were separately pooled and dialysed for 2 h against 5 litres of double-distilled water to remove most of the Tris. After dialysis, dithiothreitol was added to a final concentration of 0.5–1 mM, and the pooled fractions were freeze-dried.

The freeze-dried preparations were each dissolved in a small volume of double-distilled water and dialysed for 48 h against several changes of water. Cu^{2+} , protein and Tris contents were monitored throughout. Any remaining Tris was removed from the samples by this procedure. The samples were again freeze-dried and stored at -20°C for use in amino acid analysis, dansylation, electrophoresis and thiol-group titration.

For the purpose of calculating recoveries, a record was taken throughout the above procedure of the volumes of the various stages, and a sample at each stage was reserved for Cu^{2+} analysis.

Amino acid analysis

The samples were hydrolysed in 6 M-HCl at 110°C for 24 h and analyses were performed in a Rank-Elmer Chromaspeck instrument. No corrections were made for hydrolytic losses.

Dansylation

Protein for dansylation was dissolved in 200 µl of 20 mM- NaHCO_3 or 20 µl of 0.2 M- NaHCO_3 , mixed with 20 µl of dansyl chloride (2.5 mg/ml of acetone) and incubated for 1 h at 37°C . The solutions were then evaporated to dryness and the protein was hydrolysed with 6 M-HCl at 110°C for 16 h.

Most amino acids could be resolved by using polyamide sheets (BDH Chemicals, Poole, Dorset, U.K.). The first separation was performed with 1.5% (v/v) formic acid. The sheets were dried, then turned through 90°, and a second separation was performed with benzene/acetic acid (9:1, v/v). The sheets were again dried and chromatographed in the same direction with ethyl acetate/methanol/acetic acid (20:1:1, by vol.). The dansyl-amino acids were located under u.v. light and compared with standards.

Amino acids that could not be resolved in this way (cysteine, arginine) were separated by using heat-activated silica sheets with butan-1-ol/pyridine/acetic acid/water (15:10:30:12, by vol.) as solvent.

Electrophoresis

Disc-gel electrophoresis followed the method of Davis (1964). The gel acrylamide concentration was 7.5%.

For SDS/polyacrylamide-gel electrophoresis, protein samples were incubated with 1% SDS and 1% dithiothreitol in 0.625 M-Tris/HCl buffer, pH 6.9, for 40 min at 45°C. Electrophoresis was then carried out as indicated above with gel and buffer systems containing 0.1% SDS.

Coomassie Blue G250 was used to stain both the normal (Diezel *et al.*, 1972) and the SDS/polyacrylamide gels (Weber & Osborn, 1969).

Thiol-group titrations

Copper was first removed with 100% efficiency from the binding protein by solution in 10 mM-EDTA, pH 4.6. Then 0.1 ml of this solution containing the apoprotein was titrated by using the method of Ellman (1959). Cysteine was used as the standard thiol compound.

Results

Liver and kidney copper contents

The cytosol Cu²⁺ contents of liver and kidney tissue from CuCl₂-treated and untreated neonatal *Mo^{br}* males and normal littermates are listed in Table 1. As previously reported (Hunt, 1976), the kidney Cu²⁺ concentrations in untreated mutant animals were elevated approximately 10-fold, whereas in the mutant liver the concentrations were considerably diminished. At 6 h after an intraperitoneal injection of CuCl₂, both tissues showed a considerable increase in Cu²⁺ content. The normal kidney Cu²⁺ concentration was elevated to slightly above that of the untreated mutant kidney, and there was a further increase in Cu²⁺ content of the treated mutant kidney. The liver Cu²⁺ content of the

Table 1. Cu²⁺ contents of liver and kidney supernatant fractions from mutant (*Mo^{br}*) and normal neonatal mice

The values for untreated animals were obtained from a pool of 15 normal and 16 mutant mice. The values for CuCl₂-treated animals are the averages for several pools containing eight to 15 mice. CuCl₂ treatment was carried out as detailed in the Experimental section.

Sample	Cu ²⁺ content of supernatant fraction (μg/g wet wt. of tissue)
Normal liver, untreated	20.4
Normal liver, CuCl ₂ -treated	80.9
Mutant liver, untreated	3.3
Mutant liver, CuCl ₂ -treated	29.3
Normal kidney, untreated	1.4
Normal kidney, CuCl ₂ -treated	20.5
Mutant kidney, untreated	15.4
Mutant kidney, CuCl ₂ -treated	24.6

treated mutant was also considerably increased, reaching a value greater than that of the untreated normal, although still far short of that achieved in the treated normal.

Fractionation of liver and kidney cytosols on Bio-Gel P-10

Figs. 1 and 2 show the Bio-Gel P-10 Cu²⁺ profiles for liver and kidney cytosol proteins from treated and untreated animals. Two major Cu²⁺-containing peaks were detected. The first was eluted with the void volume, i.e. species of molecular weight greater than 20000, and the second at a position corresponding to a molecular weight of 14500 (the small differences in fraction number corresponding to this low-molecular-weight protein peak arise from small changes in column size).

The low-molecular-weight protein peak was barely discernible in the liver profile from untreated mutant animals (Fig. 1a). However, in CuCl₂-treated mutant animals the Cu²⁺ content of this peak was increased to above that found in the untreated normal animal (Fig. 1b).

CuCl₂ treatment of normal and mutant mice also resulted in an increase in kidney Cu²⁺ concentrations (Fig. 2), although that in the normal animal did not approach the extreme values seen in the mutant.

Further purification of Cu²⁺-binding proteins on DEAE-Sephadex A-25

The Cu²⁺-binding proteins were further purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column. Cu²⁺ recoveries after ion-exchange chromatography averaged 95%, indicating that very little Cu²⁺ was lost during separation. The

Fig. 1. Bio-Gel P-10 separation of liver supernatant fraction proteins from 7-8-day-old Mo^{br} male mice and normal littermates

Experimental details are as described in the text. (a) Separation of supernatant-fraction proteins from untreated male mice; (b) separation of supernatant-fraction proteins from $CuCl_2$ -treated male animals. The Cu^{2+} content of each fraction is expressed on the basis of the equivalent of 1 g wet wt. of tissue. $\circ$, Mo^{br} mice; $\bullet$, normal littermates.

Fig. 2. Bio-Gel P-10 separation of kidney supernatant-fraction proteins from 7-8-day-old Mo^{br} male mice and normal littermates

Experimental details are as described in the text. (a) Separation of kidney supernatant-fraction proteins from untreated male mice; (b) separation of kidney supernatant-fraction proteins from $CuCl_2$ -treated male animals. The Cu^{2+} content of each fraction is expressed on the basis of the equivalent of 1 g wet wt. of tissue. $\circ$, Mo^{br} mice; $\bullet$, normal littermates.

Cu^{2+} values given for the profiles are corrected to 100% recovery. The profiles shown in Figs. 3 and 4 were obtained from the pooled 14500-mol.wt. protein fractions from $CuCl_2$ -treated and untreated mutant and normal animals before the procedure was modified to include an overnight wash with starting buffer before application of the gradient. This affected the resolution of the peaks. The profiles, however, clearly showed four subfractions, the first not binding to the column in the presence of 10mM-Tris, and the others being eluted at Tris concentrations of 30, 55 and 100mM. The failure to lose Cu^{2+} from subfraction 1 on dialysis suggests that this Cu^{2+} is protein-bound. Significantly, there was no difference in the number of subfractions present before or after $CuCl_2$ treatment, or in their molarity of elution. Differences were, however, apparent in the Cu^{2+} contents of the individual subfractions from mutant and normal tissues both before and after $CuCl_2$ treatment. In the untreated mutant liver the individual subfractions were barely discernible, and even after $CuCl_2$ treatment the Cu^{2+} contents were

still below those of the normal. No profiles could be obtained from the kidney of untreated normal animals, as there was insufficient Cu^{2+} present in the Bio-Gel fractions. After $CuCl_2$ treatment the Cu^{2+} contents of the normal kidney subfractions approached those of the untreated mutant animal, whereas little further increase in the Cu^{2+} content of the mutant subfractions was apparent.

Since the ion-exchange-chromatography protein profiles obtained from $CuCl_2$ -treated and untreated animals showed only quantitative differences, $CuCl_2$ treatment was used to increase the resolution of the subfractions in all following experiments. The separation of the subfractions was improved by washing the column extensively with starting buffer before application of the gradient. This resulted in a considerable decrease in the size of subfraction 2. A consistent shoulder on the tail of the third peak suggested that this subfraction may be further subdivisible.

Fig. 3. DEAE-Sephadex A-25 separation of the 14500-mol.wt. supernatant fraction of the livers from 7-8-day-old *Mo^{br}* male mice and normal littermates

Experimental details are as described in the text. (a) Separation obtained with the 14500-mol.wt. supernatant fraction of livers from untreated and CuCl₂-treated normal male mice; (b) separation obtained with 14500-mol.wt. fraction of livers from untreated and CuCl₂-treated *Mo^{br}* animals. ----, Concn. of Tris. The 14500-mol.wt. fraction was obtained from the Bio-Gel P-10 separation of the supernatant fraction of livers from CuCl₂-treated and untreated animals. The Cu²⁺ content of each fraction is expressed on the basis of the equivalent of 1 g wet wt. of tissue applied to the column, and the Cu²⁺ values given for the separations are corrected to 100% recovery.

Fig. 4. DEAE-Sephadex A-25 separation of the 14500-mol.wt. supernatant fraction of the kidneys from 7-8-day-old *Mo^{br}* male mice and normal littermates

Experimental details are as described in the text. (a) Separation obtained with the 14500-mol.wt. supernatant fraction of kidneys from CuCl₂-treated normal male mice; (b) separation obtained with the 14500-mol.wt. supernatant fraction of kidneys from untreated and CuCl₂-treated *Mo^{br}* male animals. ----, Concn. of Tris. The 14500-mol.wt. fraction was obtained from the Bio-Gel P-10 separation of the supernatant fraction of kidneys from CuCl₂-treated and untreated animals. The Cu²⁺ content of each fraction is expressed on the basis of the equivalent of 1 g wet wt. of tissue applied to the column, and the Cu²⁺ values given for the separations are corrected to 100% recovery.

Figs. 5 and 6 show the ion-exchange-chromatography protein profiles obtained for kidney and liver tissue from CuCl₂-treated animals. The subfractions were individually isolated and processed as detailed above. Their Tris molarities for elution, Cu²⁺ contents and protein contents are given in Table 2.

The Cu²⁺ contents of the mutant liver subfractions 2, 3 and 4 were considerably lower than the normal, in agreement therefore with the decreased Cu²⁺ content of the 14500-mol.wt. peak eluted from the Bio-Gel P-10 column. In contrast, mutant subfraction 1 yielded a slightly higher Cu²⁺ content

than normal, resulting in an altered Cu²⁺ distribution among the four subfractions in mutant and normal liver, as demonstrated by the percentage values given in Table 3. The estimates for the protein contents of the mutant and normal liver subfractions showed that each subfraction from the mutant contained markedly more protein than did the normal. With the exception therefore of subfraction 1, this resulted in a considerably decreased number of Cu²⁺ ions/molecule of protein for the subfractions from the mutant (Table 3). (These values are based on a protein molecular weight of 12200 derived from amino acid analysis.)

The results for the kidney subfractions showed that the distribution of Cu²⁺ among the four subfractions

Fig. 5. DEAE-Sephadex A-25 separation of the 14500-mol.wt. supernatant fraction of the livers from 7-8-day-old Mo^{br} male mice and normal littermates

Experimental details are as described in the text. In these separations the column was washed extensively with starting buffer before application of the gradient. (a) Separation obtained with the 14500-mol.wt. supernatant fraction of livers from normal mice; (b) separation obtained with the 14500-mol.wt. supernatant fraction of livers from Mo^{br} male animals. ----, Conc. of Tris. The 14500-mol.wt. fraction was obtained from the Bio-Gel P-10 supernatant fraction of the livers from $CuCl_2$ -treated animals. The Cu^{2+} content of each fraction is expressed on the basis of the equivalent of 1 g wet wt. of tissue applied to the column, and the Cu^{2+} values given for the separation are corrected to 100% recovery.

was approximately the same for mutant and normal tissues (Table 3), and that the protein contents were also similar (Table 2). The elevated Cu^{2+} contents of the individual subfractions from the mutant resulted therefore in an elevated number of Cu^{2+} ions/molecule of protein (Table 3). Thus the decreased Cu^{2+} concentrations in the mutant liver and the elevated concentrations in the mutant kidney were not associated with a correspondingly altered amount of binding protein. Rather, it was the amount of Cu^{2+} bound to these proteins that differed.

Fig. 6. DEAE-Sephadex A-25 separation of the 14500-mol.wt. supernatant fraction of the kidneys from 7-8-day-old Mo^{br} male mice and normal littermates

Experimental details are as described in the text. In these separations the column was washed extensively with starting buffer before application of the gradient. (a) Separation obtained with the 14500-mol.wt. supernatant fraction of the kidneys from normal mice; (b) separation obtained with the 14500-mol.wt. supernatant fraction of kidneys from Mo^{br} male animals. ----, Conc. of Tris. The 14500-mol.wt. fraction was obtained from the Bio-Gel P-10 separation of supernatant fraction of kidneys from $CuCl_2$ -treated animals. The Cu^{2+} content of each fraction is expressed on the basis of the equivalent of 1 g wet wt. of tissue applied to the column, and the Cu^{2+} values given for the separation are corrected to 100% recovery.

Dansylation of the major Cu^{2+} -binding proteins

The purity of the four subfractions was examined by dansylation. Not every subfraction from each preparation was examined, but sufficient to confirm that each contained no contaminant proteins with reactive *N*-terminal amino acids. Indeed no *N*-terminal amino acid could be detected for any of the four subfraction proteins, suggesting that the *N*-terminal amino acid residue may be blocked. Such is the case for metallothionein, where the *N*-terminal is *N*-acetylmethionine (Kojima *et al.*, 1976; Huang *et al.*, 1977). Proteins with blocked *N*-terminal amino

Table 2. *Characteristics of the ion-exchange chromatography protein subfractions*

Experimental details are given in the text. All values are averages for two separate experiments, each with seven to 15 CuCl₂-treated animals, except for mutant kidney, where one experiment with 13 CuCl₂-treated animals was performed.

Fractions pooled for analysis				
Subfraction	Normal liver	Mo ^{br} liver	Normal kidney	Mo ^{br} kidney
1	6-65	8-54	9-46	9-52
2	73-87	73-87	85-94	69-83
3	90-106	88-104	95-108	84-101
4	112-124	107-118	116-123	105-114
Molarity of elution (mM-Tris)				
Subfraction	Normal liver	Mo ^{br} liver	Normal kidney	Mo ^{br} kidney
2	35	35	35	40
3	50	55	55	60
4	100	105	100	105
Cu ²⁺ content (μg/g wet wt. of tissue)				
Subfraction	Normal liver	Mo ^{br} liver	Normal kidney	Mo ^{br} kidney
1	5.2	5.9	2.1	6.4
2	2.1	0.9	0.4	1.3
3	8.4	4.5	1.9	4.7
4	4.9	1.7	0.6	1.2
Protein content (μg/g wet wt. of tissue)				
Subfraction	Normal liver	Mo ^{br} liver	Normal kidney	Mo ^{br} kidney
1	192	389	271	312
2	79	133	201	234
3	103	201	273	245
4	81	119	263	183

acids are relatively uncommon, so we conclude that each subfraction contains only Cu²⁺-binding proteins, with no significant contamination.

Electrophoresis of the major Cu²⁺-binding proteins

Conventional and SDS/polyacrylamide-gel electrophoresis was carried out on various samples of the purified Cu²⁺-binding proteins. Both types of gel gave variable results; both occasionally gave multiple bands and the conventional gels sometimes failed to show any stainable material within the gel. Significantly, a densely stained region at the origin of such gels was seen, suggesting that the protein may have formed aggregates too large to penetrate the gel. Aggregation would also account for the multiple bands, which have also been reported by others working with Cu²⁺-binding proteins (Riordan & Madapallimatam, 1976; Hartmann & Weser, 1977). Although dithiothreitol was used to avoid aggregation, a more powerful dissociating agent such as urea may be needed to overcome this problem. There may also be difficulties in staining the Cu²⁺-binding proteins (Irons, 1974).

Amino acid analysis

Amino acid analysis was performed on each of the four subfractions obtained from mutant and normal liver and kidney. The results listed in Tables 4 and 5 reveal that the four subfraction proteins were similar in showing consistently high glycine, glutamic acid, serine and alanine contents, and, unlike metallothioneins (Bremner & Young, 1976), aromatic amino acids were present. Also, there was an unknown amino acid, possibly a derivative of lysine, that was close to and sometimes overlapped lysine in the separation.

Table 3. *Characteristics of the ion-exchange-chromatography protein subfractions*

Values were calculated from results given in Table 2. A molecular weight of 12200 was used for the Cu²⁺-binding proteins, as derived from the amino acid analyses.

Cu ²⁺ content (% of total eluted)				
Subfraction	Normal liver	Mo ^{br} liver	Normal kidney	Mo ^{br} kidney
1	23.7	43.8	42.7	41.5
2	8.6	9.6	9.1	10.6
3	42.3	35.3	38.3	36.2
4	25.5	11.5	9.8	11.7
Cu ²⁺ content (ions/molecule of protein)				
Subfraction	Normal liver	Mo ^{br} liver	Normal kidney	Mo ^{br} kidney
1	5.2	3.2	1.7	3.9
2	5.3	1.4	0.5	1.1
3	15.7	4.4	1.4	3.7
4	11.7	3.1	0.4	1.2

Table 4. *Amino acid analyses of the ion-exchange-chromatography subfractions of Mo^{br} and normal neonatal-mouse liver Cu²⁺-binding proteins*

Experimental details are given in the text. All values are averages of two analyses.

Amino acid	Amino acid composition (percentage composition)							
	Normal liver subfractions				Mo ^{br} liver subfractions			
	1	2	3	4	1	2	3	4
Cys	12.4	5.5	17.0	11.3	3.9	0	7.4	8.8
Met	1.1	1.7	1.0	1.0	1.3	1.9	0.9	0.5
Asp	8.6	8.4	8.2	8.7	8.8	9.7	10.5	10.1
Thr	6.5	4.4	5.6	3.7	5.4	3.5	4.9	3.7
Ser	13.1	13.6	16.7	12.6	11.7	12.6	11.4	10.4
Glu	11.7	19.4	10.1	13.6	16.0	28.6	22.2	21.3
Pro	4.9	Trace	3.1	3.6	3.5	0	3.0	4.7
Gly	10.4	13.5	9.7	9.7	12.6	13.6	11.7	10.8
Ala	9.0	8.8	9.9	9.7	7.8	5.4	8.3	8.8
Val	5.1	5.6	4.1	4.5	4.2	2.9	3.5	3.0
Ile	2.1	Trace	1.0	2.3	2.7	Trace	1.5	0.7
Leu	3.0	1.9	0.9	2.4	4.0	Trace	2.3	2.0
Tyr	1.0	Trace	0.1	1.2	1.3	2.3	0.4	0.5
Phe	2.0	0.6	0.4	1.6	2.0	Trace	1.1	1.2
His	1.0	0.8	0.2	0.9	1.4	Trace	1.4	0.5
Unknown	2.1	3.7	1.1	2.2	3.2	19.7	3.9	4.7
Lys	5.4	12.6	11.2	10.5	8.2		5.5	8.3
Arg	1.2	Trace	Trace	1.1	2.2	Trace	0.6	0.4

Table 5. *Amino acid analyses of the ion-exchange-chromatography subfractions of Mo^{br} and normal neonatal-mouse kidney Cu²⁺-binding proteins*

Experimental details are given in the text. Normal kidney values are averages of two analyses. Only one analysis was performed for mutant kidney.

Amino acid	Amino acid composition (percentage composition)							
	Normal kidney subfraction				Mo ^{br} kidney subfraction			
	1	2	3	4	1	2	3	4
Cys	4.7	Trace	1.9	Trace	6.4	Trace	4.2	Trace
Met	1.3	1.1	1.5	1.5	1.1	Trace	Trace	Trace
Asp	8.3	9.9	11.3	10.3	8.8	9.0	10.7	11.5
Thr	5.3	4.1	4.3	4.4	6.5	4.2	4.8	4.2
Ser	10.8	16.3	13.5	16.1	11.1	11.6	10.1	11.9
Glu	17.2	21.6	23.2	19.9	11.5	22.2	20.0	29.8
Pro	5.8	2.1	Trace	1.5	3.7	Trace	3.9	Trace
Gly	11.6	15.2	13.6	16.7	12.3	23.8	12.5	22.1
Ala	7.2	7.7	7.5	8.1	8.7	10.3	10.9	10.8
Val	4.3	2.6	3.1	3.0	4.8	3.2	3.3	Trace
Ile	2.0	0.9	0.8	0.8	3.3	Trace	1.5	Trace
Leu	2.8	1.7	1.3	1.2	4.6	2.3	3.4	Trace
Tyr	1.3	0.7	1.7	2.0	1.5	2.0	0.9	2.9
Phe	1.7	1.1	1.8	0.7	2.5	Trace	2.1	Trace
His	1.7	1.5	1.3	0.7	1.0	2.3	1.2	Trace
Unknown	6.0	11.4	8.1	9.4	2.8	6.2	3.8	6.8
Lys	7.3	2.4	5.3	4.1	7.3	2.8	6.8	Trace
Arg	1.6	Trace	Trace	Trace	2.2	Trace	Trace	Trace

There was some variability in the contents of other amino acids in the different subfractions. This was especially true for the kidney subfractions, where there were a large number of trace values (see

particularly subfractions 2 and 4 from the mutant). Much of this variability may, however, have arisen as a consequence of the relative scarcity of kidney material for amino acid analysis.

A significant feature was the relatively higher cysteine content and lower glutamic acid content of the normal liver subfractions compared with those of both the mutant liver and the mutant and normal kidney. Also, the Cu²⁺ content of the subfractions appeared to follow the cysteine content.

Thiol-group titrations

Since no corrections were made for hydrolytic losses, thiol-group titrations were performed on two samples. With the normal kidney subfraction 1, the value of 5.2% obtained was close to that obtained by amino acid analysis (4.7%). However, with normal kidney subfraction 4, a wider discrepancy was obtained, with a thiol-group value of 3.9% against only a trace cysteine content. This suggests that the trace or zero estimates for cysteine may have been an underestimate. These thiol-group titrations support, however, the observations from the amino acid analysis of the relatively low cysteine content of these proteins.

Molecular-weight estimation

A molecular weight of 12200 was obtained from these analyses by equating the lower percentage of a residue in a separation to one molecule. Only the separations performed with adequate starting material were used.

Discussion

The present data confirm and extend the observation (Hunt, 1974, 1976) that the Cu²⁺ concentrations are depressed in liver tissue and elevated in kidney tissue of neonatal *Mo^{br}* mice, and that these changes in Cu²⁺ content are associated with differing amounts of Cu²⁺ bound to a low-molecular-weight protein fraction. CuCl₂ injected intraperitoneally resulted in an increased total Cu²⁺ content of liver and kidney tissue, with much of the additional Cu²⁺ being associated with the low-molecular-weight proteins. Proportionately, the increases in the Cu²⁺ content after Cu²⁺ treatment were much greater in the normal kidney and mutant liver. This increase in the Cu²⁺ content of mutant liver 6h after injection of CuCl₂ showed that this tissue was capable of accumulating more Cu²⁺ than was usually seen in the untreated animal. By 3 days after injection, however, the concentration had again fallen (Hunt, 1976).

Further purification of the low-molecular-weight Cu²⁺-binding protein fractions by anion-exchange chromatography revealed at least four subfractions. Identical subfractions were obtained for liver and kidney tissue from both CuCl₂-treated and untreated mutant and normal mice. Major differences were

apparent, however, in the Cu²⁺ content of the subfractions. In the kidney, the subfractions from the mutant each contained proportionately more Cu²⁺ than did those from the normal mice, although the differences were not reflected in differing protein contents. In the liver, the subfractions from the mutant consistently contained more protein than did those from the normal mice, which, together with the depressed Cu²⁺ contents, resulted in a decreased number of Cu²⁺ ions/molecule of subfraction protein.

The four subfractions may represent different isoproteins or the differing anion-exchange characteristics may arise from differing contents of other metals such as Zn²⁺. The differing values for numbers of Cu²⁺ ions/molecule of protein for the same subfractions from mutant and normal tissue clearly indicated that their ion-exchange affinities did not depend on Cu²⁺ content. The amino acid analyses of the different liver and kidney subfractions from CuCl₂-treated mutant and normal animals showed that the proteins were similar in composition, and common features were high glutamic acid, glycine, serine and alanine contents and a rather variable cysteine content. The presence of an unknown amino acid tentatively identified as a derivative of lysine indicated a high lysine content.

Notable differences were apparent in the amino acid compositions of the subfractions from mutant and normal mouse liver. In the subfractions from normal liver, the cysteine content was considerably higher and the glutamic acid content lower. Cherian & Goyer (1978) have suggested that Cu²⁺ can be released by oxidative changes from Cu²⁺-thionein as a Cu²⁺-cysteine complex. The overall yield (82%) of Cu²⁺ from the Bio-Gel fractions to the purified protein suggested that this was not happening here and does not therefore account for the differences between mutant and normal liver, unless similar instabilities occur *in vivo* and are dependent on the Cu²⁺ content of the protein. Certainly the normal liver subfractions had a greater Cu²⁺ content/mol of protein than did the subfractions from either the mutant mouse liver or the normal and mutant mouse kidney, and the Cu²⁺ content of the protein closely followed the cysteine content. On the basis of the low cysteine content and the presence of aromatic amino acids, the present proteins are unlikely to be thioneins (Prinz & Weser, 1975; Bremner & Young, 1976; Huang *et al.*, 1977), although, as with thioneins (Kojima *et al.*, 1976; Huang *et al.*, 1977), a blocked *N*-terminal amino acid is indicated. More work is required to characterize these proteins further. At present, however, the amino acid composition of the Cu²⁺-binding proteins shows closest homology to protein E, described by Riordan & Gower (1975), induced in adult rat liver by subcutaneous injection of CuSO₄ over 6 successive days. The estimated

molecular weight for protein E of 12000 is also similar to our estimate of 12200 for the low-molecular-weight proteins described in the present paper.

The probable identity of the kidney and liver Cu^{2+} -binding proteins makes it unlikely that they represent the *Mo*-gene products. If this were so, it would be difficult to account for the accumulation of Cu^{2+} in the kidney and deficiency in the liver, especially as both proteins are synthesized constitutively in neonatal mice (Hunt & Port, 1979). More probably, these proteins serve a storage function, with the excess Cu^{2+} of the mutant kidney becoming sequestered on these proteins, with the result that the copper status of the mutant animal is adversely affected.

The accumulation of Cu^{2+} in cultured cells from *Mo* mice and Menkes'-disease infants (Goka *et al.*, 1976; Horn, 1976; Danks, 1977; Beratis *et al.*, 1978) and the high concentrations of Cu^{2+} found in all tissues except liver of an aborted Menkes'-disease foetus (Heydorn *et al.*, 1975) suggest that this is a disease of copper accumulation in a largely unavailable form, and that perhaps the deficiency of the liver, brain and other tissues in neonatal mice is a consequence of accumulation elsewhere, although the direct involvement of these tissues in the aetiology of the disorder cannot be ruled out. A further feature of the mouse disorder is that both the decrease in liver Cu^{2+} content and elevation in gut mucosa Cu^{2+} content are restricted to neonatal life (Hunt, 1976; Hunt & Port, 1979). By 21–30 days *post partum* the gut Cu^{2+} concentrations of *Mo*^{br} mice have returned to normal, and at this age the normal liver Cu^{2+} concentrations decline rapidly to a value comparable with that of mutant mice. The disorder may arise therefore from an inability to release Cu^{2+} from binding sites in tissues, such as kidney and gut mucosa (in neonatal mice), where accumulation is seen. The high Cu^{2+} content of the normal neonatal liver may depend on constitutive synthesis of the Cu^{2+} -binding proteins. In the mutant, although Cu^{2+} -binding protein is synthesized at a high rate, there is insufficient Cu^{2+} available for storage in this tissue. An explanation of the rapid fall in Cu^{2+} content of normal liver between 21 and 30 days *post partum* is that the rate of synthesis of Cu^{2+} -binding proteins declines at this age. An age-dependent decrease in the synthesis of a Cu^{2+} -binding protein in the gut, synthesized previously in response to the Cu^{2+} status of the tissue, would effectively eliminate any effect of defective Cu^{2+} release in older mutant mice. In the kidney, this age-related decline in Cu^{2+} -binding protein synthesis does not occur, and Cu^{2+} continues to be sequestered in a non-available form bound to the low-molecular-weight binding proteins.

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