Cytosolic copper-binding proteins in rat and mouse hepatocytes incubated continuously with Cu(II)

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The proteins that bind copper when it first enters cells are likely to play roles in its intracellular distribution and utilization. When hepatocytes were incubated with $^{64}\text{Cu(II)}$, the time-dependence of the subcellular distribution of ^{64}Cu was consistent with one or more cytosolic proteins distributing copper to the mitochondrial and nuclear fractions. Cytosolic copper was reproducibly distributed among four protein fractions from Sephadex G-150 columns at the earliest time (1 min) and at the lowest concentration used [$2\ \mu\text{M-}^{64}\text{Cu(II)}$] with both rat and mouse hepatocytes. Copper binding to proteins in these fractions was sensitive to copper metabolic status. Hepatocytes from nutritionally copper-deficient rats or neonatal (9–30 days old) developing rats showed an inverse correlation between copper binding to metallothionein and copper binding to proteins in fraction I (~88 kDa apparent) and fraction II (~38 kDa apparent). The distribution of cytosolic ^{64}Cu from the brindled-mouse model of Menkes disease indicated decreased binding by a protein in fraction I. Brindled-mouse hepatocytes also contain decreased levels of a ~55 kDa protein or subunit, which most likely represents a liver-specific secondary response to the primary defect. The results are consistent with one or more copper-binding proteins in fractions I and II having significant functions in intracellular copper metabolism.

INTRODUCTION

Little is known about how copper which enters a cell is subsequently distributed throughout the cell [1,2]. The proteins that bind copper when it first enters cells are likely to play key dynamic roles in its subsequent distribution and utilization. To date, metallothioneins (MT) have received the most attention for possible roles in zinc and copper metabolism [3–5], but it is known that copper also binds to high-molecular-mass cytosolic proteins in most tissues shortly after a copper dose [6–9]. The fact that there are at least three known inherited diseases of copper metabolism (Wilson's disease [2,10], Menkes disease [2,11] and the toxic milk syndrome of mice [2,12,13]) further suggests essential functions in copper metabolism for as yet unidentified proteins.

This paper focuses on identifying the cytosolic proteins which bind copper when it first enters cells. The concentration of extracellular copper varies less during experiments *in vitro* with isolated cells than in experiments *in vivo*. Thus steady-state levels of copper binding to components that may rapidly bind and then rapidly donate copper to other components can be detected. Hepatocytes were chosen for cellular studies because the liver is known to play a central role in whole-body copper homeostasis [14,15], and hepatic copper metabolism seems to be affected in each of the diseases mentioned above [2,14,15]. A possible role for an intracellular hepatic protein(s) in preferential copper uptake by the liver was also suggested [16]. The effects on copper binding to cytosolic proteins of copper deficiency, neonatal development and the X-linked defect in the brindled-mouse model of Menkes disease [2,11,17] were also determined to assess the effect of copper metabolic status on copper binding. The results reported here show that, in addition to MT, one or more copper-binding proteins in Sephadex G-150 fractions, corresponding to apparent molecular masses of ~88 kDa and ~38 kDa at their peaks, bind copper when it first enters hepatocytes.

Copper binding to proteins in these fractions is affected by copper metabolic status, which is consistent with possible functions in cellular copper metabolism.

MATERIALS AND METHODS

Materials

Sodium pentobarbital was from Abbott, Chicago, IL, U.S.A., collagenase (type II) from Cooper Biomedical, Freehold, NJ, U.S.A., Sephadex G-150 from Pharmacia, Piscataway, NJ, U.S.A., and Swim's S-77 medium from GIBCO Laboratories, Grand Island, NY, U.S.A. $^{64}\text{Cu(NO}_3)_2$ (~3 mCi/mg of Cu) was supplied by the Buffalo Materials Research Center at SUNY/ Buffalo. $^{65}\text{ZnCl}_2$ was purchased from New England Nuclear, Boston, MA, U.S.A., and [^{35}S]cysteine was obtained from Amer-sham, Arlington Heights, IL, U.S.A. All other biochemicals were from Sigma, St. Louis, MO, U.S.A.

Animals

Male Sprague–Dawley rats (150–250 g; Blue Spruce, Altamont, NY, U.S.A.) and control mice (C57BL/6J strain; Jackson Laboratory, Bar Harbor, ME, U.S.A.) were maintained on standard rat chow and tap water *ad libitum*. Brindled mice were bred and maintained as previously described [18,19]. Nutritionally copper-deficient rats were obtained by replacing the standard diet (20 p.p.m. of copper) of mothers of 2-day-old rats with deionized water (0.001 p.p.m. of copper), and a copper-deficient diet (0.34 p.p.m.; ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.). Copper-deficient animals were used when they attained a body weight of 300–400 g. Their liver copper levels were decreased by ~40%. Parallel age-matched controls were given an equivalent standard diet and tap water (~0.1 p.p.m. of copper).

Abbreviations used: MT, metallothionein; SOD, superoxide dismutase.

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Hepatocyte isolations

Rat hepatocytes were isolated by perfusion with collagenase as reported previously [20]. These methods yielded cells that were 80–95 % viable by the Trypan Blue exclusion criterion. Viabilities decreased minimally (0–10 %) during the experiments. The procedure described previously [19] for isolating mouse hepatocytes was modified as follows: livers were cleared of blood by perfusion for 10–15 min with Swim's S-77 medium plus 1 mM-EDTA and 207 units of heparin/100 ml. This was followed by collagenase perfusion for about 15–20 min with 20–25 mg of collagenase/50 ml of Swim's S-77 medium containing 2 mM- CaCl_2 . Addition of the Swim's S-77 medium and CaCl_2 helped to increase the yield of viable cells during isolation. The liver was then transferred to fresh Swim's S-77 medium containing 1.2 mM- CaCl_2 in a Petri dish. The cells were released by scraping gently with a plastic scraper. The suspension was filtered through three layers of cheesecloth and allowed to settle at 20 °C for 10 min. The supernatant was aspirated and the cells were resuspended in fresh medium; this was repeated once. Finally, the cells were centrifuged at 30 *g* for 90 s, and the pellet was resuspended in Earle's salts containing Hepes buffer plus glucose (EHG) [20] and 10 % Swim's S-77 medium. The isolated cells had viabilities of 70–85 % by Trypan Blue exclusion. Swim's S-77 medium increased the viabilities of hepatocytes. Under these conditions, loss of viable cells after 6 h incubation was less than 10 %.

Incubations of hepatocytes with $^{64}\text{Cu}(\text{II})$, and isolation of cytosols

The conditions of hepatocyte incubation were similar to those described previously [20]. Cells were incubated in EHG buffer containing 10 % Swim's S-77 medium; 1.5×10^6 cells/ml were used for all incubations. Cells were stored on ice. Hepatocytes were preincubated at 20 °C for 15 min before adding $^{64}\text{Cu}(\text{II})$. Solutions of $^{64}\text{Cu}(\text{II})$ were prepared equimolar with L-histidine in EHG buffer containing 10 % Swim's S-77 medium and added to the cell suspension in equal volume. At these concentrations, histidine and the Swim's S-77 medium, which contains amino acids, but no albumin, did not significantly affect uptake. After incubation, the samples were immediately transferred to equal volumes of ice-cold 0.9 % NaCl containing 10 mM-EDTA and centrifuged at 750 *g* in a Sorvall RC-2B centrifuge at 0–4 °C for 2 min. No effect of EDTA on intracellular copper was detected [20]. The pellet was washed with 2×5 ml of 0.9 % NaCl and finally suspended in 0.25 M-sucrose (1:3 and 1:5, w/v, for rat and mouse hepatocytes respectively). This suspension was homogenized with eight strokes of a motor-driven Teflon homogenizer. The homogenate was centrifuged at 100 000 *g* for 1 h to obtain the cytosol. To obtain whole-organ cytosols, animals were anaesthetized with sodium pentobarbital injected intraperitoneally (0.06 mg/g); 1000 units of heparin was injected into the right ilio-lumbar vein to prevent blood clotting. The liver was cleared of blood by perfusion with ice-cold buffer (142 mM-NaCl, 6.7 mM-KCl and 10 mM-Hepes, pH 7.4) through the portal vein, and the kidney was perfused via the heart. The liver was homogenized in 5 vol., and the kidney in 3 vol. of homogenization buffer (0.25 M-sucrose in 0.05 M-Hepes buffer, pH 7.4). The cytosols (100 000 *g* supernatants) were frozen in liquid N_2 and stored at –70 °C. Protein concentrations were determined by the Lowry method [21] with bovine serum albumin as the standard. ^{64}Cu radioactivity was counted by a model 1282 LKB γ -radiation counter and corrected for decay by using the medium specific radioactivity.

Subcellular distribution of ^{64}Cu

Isolated hepatocytes were incubated with 10 μM - ^{64}Cu ·His (1:1). At the indicated times, equal volumes of EDTA/NaCl were added, and the cells were washed as described above. The cells were centrifuged at 30 *g* for 2.5 min, and subcellular fractions were obtained by sucrose-gradient centrifugation as previously described [20]. Standard enzyme markers were used to determine the purity of each fraction [22]. The results of quadruplicate assays were expressed as specific activity of a marker in a fraction relative to the activity in the total homogenate [22]. By these criteria, the cytosol was the cleanest fraction showing a ~5-fold increase in activity of the marker and negligible activities of the other markers. Mitochondria (heavy mitochondrial fraction) showed a 4-fold increase in marker activity, and small contamination with the plasma-membrane fraction. The microsomal fraction showed the poorest (~2-fold) purification of the marker, and some contamination by the plasma-membrane and lysosomal fractions. The nuclear fraction showed some mitochondrial contamination.

Fractionation on Sephadex columns

Cytosol (1–2 ml) was applied to a Sephadex G-150 column (1.5 cm $\times$ 100 cm) equilibrated with 0.05 M-Hepes/0.1 M-NaCl, pH 7.4. The samples were eluted at 12 ml/h with the same buffer by using a peristaltic pump (model P-1; Pharmacia) at 4 °C; 0.8 ml fractions were collected. Blue Dextran (2000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and cytochrome *c* (12.4 kDa) were used as molecular-mass markers.

Determination of unlabelled (stable) copper

Unlabelled copper was determined by using a Perkin–Elmer model 360 atomic-absorption spectrophotometer equipped with an HGA 2100 graphite furnace. Standards were prepared from an ultra-pure copper standard solution (Alfa Products, Danvers, MA, U.S.A.).

Determination of superoxide dismutase (SOD) activity

Inhibition of xanthine oxidase-coupled ferricytochrome *c* reduction as described by McCord & Fridovich [23] and modified by Salin & McCord [24] was used for the Cu/Zn-SOD assays. Samples (10 μl) of the column fractions were tested for activity, expressed as a decrease in A_{417} .

Incubation of hepatocytes with ^{64}Cu and ^{65}Zn

Hepatocytes were incubated with a solution containing 10 μM - $^{65}\text{Zn}(\text{II})$ and 10 μM - $^{64}\text{Cu}(\text{II})$, prepared equimolar with L-histidine in EHG buffer containing 10 % Swim's S-77 medium, and the cytosols were fractionated as described above. The fractions were counted for ^{65}Zn and ^{64}Cu simultaneously in the γ -radiation counter at different window settings.

RESULTS

Time-dependence of the subcellular distribution of ^{64}Cu

Rat and mouse hepatocytes continue to accumulate ^{64}Cu for about 2 h when incubated with 10 μM - $^{64}\text{Cu}(\text{II})$, which is the K_m for copper transport [19,20]. This was previously shown to reflect net uptake rather than isotopic exchange [20]. The time-dependence of the subcellular distribution of ^{64}Cu was determined to compare the relative rates of copper accumulated in various cell compartments. About half of the total ^{64}Cu accumulated in the cytosol by 2 h was taken up during the first 5 min (Fig. 1), after which steady-state levels were gradually approached. The mitochondrial and nuclear fractions accumulated copper con-

tinuously for at least 90 min (Fig. 1). The rate of accumulation in these fractions was slower than the initial (5 min) rate of accumulation in the cytosol. These results are consistent with one or more cytosolic components delivering copper to the mitochondrial and nuclear fractions. Similar results were obtained with $2 \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$.

^{64}Cu binding to cytosolic proteins

Mouse hepatocytes were incubated with $^{64}\text{Cu}(\text{II})$ for 5 min, and the cytosols were chromatographed on Sephadex G-150 (1.5 cm $\times$ 100 cm) columns. The column yields were 80–100%, and no ^{64}Cu was detected in the ≤ 5 kDa fraction, which indicates that most of the cytosolic copper was bound to proteins. At each of the concentrations examined, four ^{64}Cu -binding protein fractions were detected (Fig. 2). The estimated apparent molecular masses at the peaks of ^{64}Cu binding were ≥ 300 kDa, ~ 88 kDa, ~ 38 kDa and ~ 10 kDa, and these will be referred to as the void volume, fraction I, fraction II and MT fractions respectively. It should be noted that comparable profiles usually could be obtained with Sephadex G-100 (1.5 cm $\times$ 100 cm) columns or Sephadex G-150 (1.5 cm $\times$ 50 cm) columns. However, the resolution of ^{64}Cu -binding proteins was most reproducible with G-150 (1.5 cm $\times$ 100 cm) columns. The same four ^{64}Cu -binding fractions were obtained with rat hepatocytes (Fig. 3). Less ^{64}Cu was reproducibly detected in the MT fraction of mouse than of rat hepatocytes. The relative amounts of copper in the void volume and fraction II were usually greater than in fraction I in the rat cytosols, whereas relatively more ^{64}Cu was reproducibly found in fraction I from the mouse hepatocytes (Figs. 2 and 3). The ~ 10 kDa (apparent) fraction is eluted at the column volume where MT I and II are expected to be eluted, and where immunochemically detected MT is eluted. Cu/Zn-SOD activities of column fractions were measured to determine if cytosolic Cu/Zn-SOD was the major ^{64}Cu -binding component in fraction

Fig. 2. Concentration-dependence of the cytosolic distribution of ^{64}Cu

Mouse hepatocytes were incubated for 5 min in (.....) $2 \mu\text{M}$ -, (—) $10 \mu\text{M}$ - or (---) $20 \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$, as indicated. The cytosols were applied to a Sephadex G-150 (1.5 cm $\times$ 100 cm) column, eluted with Hepes buffer (0.05 M-Hepes/0.1 M-NaCl, pH 7.4) at a flow rate of 12 ml/h; 0.8 ml fractions were collected and analysed for ^{64}Cu . The elution positions of the molecular-mass markers (see the Materials and methods section) are indicated by the arrows at the top of the Figure.

Fig. 1. Time-dependence of the subcellular distribution of ^{64}Cu

Rat hepatocytes were incubated with $10 \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for the indicated times. The subcellular fractions were obtained as described in the Materials and methods section and analysed for ^{64}Cu to determine the total ng of ^{64}Cu per fraction in $\sim 0.25 \times 10^6$ hepatocytes.

II. Maximal Cu/Zn-SOD activity was obtained in fractions eluted after fraction II, and peak activity was detected at about 30 kDa, the approximate molecular mass of Cu/Zn-SOD (Fig. 3). A high percentage of total cytosolic copper was also detected at ~ 30 kDa by flameless atomic absorption. Little, if any, isotopic exchange was detected in the 30 kDa fraction. These results suggest that fraction II contains one or more ^{64}Cu -binding proteins other than Cu/Zn-SOD.

Little correlation was observed between the amount of protein in a fraction as estimated by the A_{280} and the amount of ^{64}Cu bound, i.e. ^{64}Cu binding did not simply increase with increasing protein concentration (Fig. 3). Neither ^{64}Cu -binding fraction I nor fraction II co-eluted with major protein fractions. This suggests at least some protein specificity in ^{64}Cu binding. Also, neither the relative amounts of ^{64}Cu among the four fractions, nor the total amount of ^{64}Cu in the four fractions, changed markedly with increasing time with either mouse or rat hepatocytes. This is consistent with the subcellular-distribution results (Fig. 1).

The cytosolic distribution of ^{64}Cu was also determined after adding ^{64}Cu directly to the cytosols obtained from mouse hepatocytes which had not been incubated with ^{64}Cu . The amount added [$3.2 \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$] was equivalent to the amount of copper in the cytosolic fraction after incubating hepatocytes with $10 \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 5 min. No significant differences in the distribution of cytosolic ^{64}Cu were detected when $^{64}\text{Cu}(\text{II})$ was added to cells or directly to cytosols. Thus, although the total copper in the cytosol is expected to be affected by other cell

Fig. 3. Concentration-dependence of the cytosolic distribution of ^{64}Cu and the distribution of Cu/Zn-SOD

Rat hepatocytes were incubated with (----) $2\ \mu\text{M}$ - or (—) $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 15 min. The cytosols were applied to a Sephadex G-100 ($1.5\ \text{cm} \times 100\ \text{cm}$) column, eluted with Hepes buffer ($0.05\ \text{M}$ -Hepes/ $0.1\ \text{M}$ -NaCl, pH 7.4) at a flow rate of 12 ml/h; 0.8 ml fractions were collected and analysed for ^{64}Cu . Fractions from the $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ experiment were also analysed for A_{280} (—) and for Cu/Zn-SOD activity (●). SOD activity is expressed as the change in A_{417} caused by inhibition of ferricytochrome *c* reduction, as described in the Materials and methods section. The elution positions of the molecular-mass markers (see the Materials and methods section) are indicated by the arrows at the top of the Figure.

compartments, the distribution of cytosolic ^{64}Cu depends solely on the total concentration of copper in the cytosol, and not on any cellular activity requiring intact cells. Whole-organ hepatic cytosols also showed similar ^{64}Cu -binding profiles. Thus the cell isolation procedure did not lead to artifacts, and no copper-binding components were detected in whole-liver cytosols that were not detected in isolated hepatocytes.

Concentration-dependence of ^{64}Cu binding

At $2\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ the cytosolic fraction containing the highest percentage of ^{64}Cu in mouse hepatocytes was fraction II, whereas MT contained the most ^{64}Cu in rat hepatocytes. Some ^{64}Cu was also detected in fraction I at $2\ \mu\text{M}$ with both rat and mouse hepatocytes. Large increases in ^{64}Cu were detected in the void volume, fraction I and fraction II as the incubation concentration was increased from 2 to $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ (Figs. 2 and 3). As the concentration was increased to $20\ \mu\text{M}$, the relative increases in the void volume and fraction I were greater than in fraction II (Fig. 2). At this stage of purification it is difficult to distinguish increased ^{64}Cu binding to specific proteins from non-specific ^{64}Cu binding to an increasing number of proteins.

Zinc-binding fractions

Mouse hepatocytes were incubated with $^{65}\text{Zn}(\text{II})$ and $^{64}\text{Cu}(\text{II})$ concurrently to compare the cytosolic distributions of zinc and copper immediately after they enter the cells. A major ^{65}Zn -binding component(s) was eluted between ^{64}Cu -binding fractions I and II (Fig. 4). Relatively less ^{65}Zn than ^{64}Cu was detected in the void volume. In contrast with ^{64}Cu , ^{65}Zn was also detected in the $\leq 5\ \text{kDa}$ fraction, which indicates possible zinc binding by non-protein ligands. The ^{65}Zn distribution was similar in the absence of ^{64}Cu .

Effects of copper deficiency on the cytosolic distribution of ^{64}Cu

Hepatocytes from copper-deficient rats were incubated with $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 15–90 min. The same four ^{64}Cu -binding fractions were detected with ^{64}Cu -deficient hepatocytes as were observed with normocupric rat or mouse hepatocytes (Fig. 5). Relatively more ^{64}Cu was reproducibly detected in fractions I and II and less in the MT-containing fraction with copper-deficient than with normocupric hepatocytes. In particular,

Fig. 4. Cytosolic distributions of zinc and copper in hepatocytes incubated concurrently with both metals

Mouse hepatocytes were incubated with $10\ \mu\text{M}$ each of $^{65}\text{Zn}(\text{II})$ and $^{64}\text{Cu}(\text{II})$. The cytosols were fractionated on Sephadex G-150 columns ($1.5\ \text{cm} \times 100\ \text{cm}$) and analysed for $^{65}\text{Zn}(\text{II})$ (----) and ^{64}Cu (—) by γ -radiation counting as described in the Materials and methods section. The elution positions of the molecular-mass markers (see the Materials and methods section) are indicated by the arrows at the top of the Figure.

Fraction I was more clearly resolved and contained a higher fraction of the total cytosolic copper with copper-deficient than with normal rat hepatocytes. The increases in the amounts of ^{64}Cu in the void volume and fractions I and II were apparently greater than could be accounted for by the decrease in the MT-containing fraction, suggesting that the increases in ^{64}Cu bound in these fractions were not solely secondary to decreased ^{64}Cu in the MT fraction.

Hepatocytes were also preincubated with [^{35}S]cystine for 1 h, before addition of $^{64}\text{Cu}(\text{II})$. No effect of copper deficiency on ^{35}S labelling was detected. The large amount of ^{35}S label that was detected in the $\leq 5\ \text{kDa}$ fraction (Fig. 5) most likely was due to labelling of low-molecular-mass species, i.e., free cysteine, cystine or glutathione. At 15, 60 and 90 min, ^{35}S was detected in the major protein fractions rather than the major ^{64}Cu -binding fractions; i.e. maximal levels of ^{35}S and ^{64}Cu were reproducibly

Fig. 5. Time-dependencies of the cytosolic distributions of ^{64}Cu and $[^{35}\text{S}]$ cystine in hepatocyte cytosols isolated from copper-deficient rats

Hepatocytes were preincubated with $[^{35}\text{S}]$ cystine (1 mCi/ml) for 60 min and then incubated with $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 15 (—), 60 (---) or 90 (— · —) min. The cytosols were fractionated on Sephadex G-150 (1.5 cm $\times$ 50 cm) columns with 0.05 M-Hepes buffer, pH 7.4, containing 0.1 M-NaCl. The elution rate was 12 ml/h, and 0.8 ml fractions were collected. ^{35}S radioactivity (fine-line curves) was counted in a scintillation counter after the ^{64}Cu (bold-line curves) had decayed. The arrows indicate positions of molecular-mass markers (see the Materials and methods section).

obtained in different fractions, which is also consistent with at least some protein specificity in copper binding. ^{35}S peaks or increases in ^{35}S incorporation with time at the elution volume of MT, which would indicate significant new MT-protein synthesis over the time period examined, were not detected. MT has a high affinity for copper, copper displaces zinc from MT [1], and rapid metal exchange occurs with MT [25]. Thus ^{64}Cu binding to MT most likely represented ^{64}Cu displacement of zinc and isotopic exchange.

Distribution of ^{64}Cu in hepatic cytosols from 9–30-day-old rats

Hepatocytes were isolated from 9–30-day-old rats, and the cytosols were incubated with $^{64}\text{Cu}(\text{II})$ as described in the Materials and methods section. Most of the ^{64}Cu that was detected in the cytosols from 9-day-old animals was bound to MT (Fig. 6a), which is known to be increased in neonatal rats [1,26]. With increasing age, the ^{64}Cu detected in the MT-containing fraction decreased while parallel increases in the amounts of ^{64}Cu associated with ^{64}Cu -binding fractions I and II were detected (Fig. 6b). By age 30 days, the cytosolic distribution of ^{64}Cu was similar to that in adults (Fig. 6a).

Distribution of ^{64}Cu in hepatocyte and renal cytosols from the brindled mice

No significant reproducible differences in the subcellular fractions from control and brindled-mouse hepatocytes were detected when hepatocytes were incubated with $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 5–60 min. Hepatocytes from normal and brindled mice were incubated with $^{64}\text{Cu}(\text{II})$ for 5 min, and the cytosols were applied to Sephadex columns. In the cytosols from the brindled mice, ^{64}Cu -binding fraction I was less well resolved from fraction II than with normal mice (Fig. 7). The profile could only be simulated if it were assumed that ^{64}Cu -binding fraction I was

Fig. 6. Distribution of ^{64}Cu in hepatocyte cytosols from 9–30-day-old developing rats

$^{64}\text{Cu}(\text{II})$ was added to hepatocyte cytosols from rats at ages 9 (—), 21 (---), 24 (— · —) and 30 (—) days. The final concentration was $3.2\ \mu\text{M}$ - ^{64}Cu , which was the concentration of cytosolic copper when hepatocytes from adult mice were incubated with $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 5 min. The cytosols were fractionated on Sephadex G-150 (1.5 cm $\times$ 50 cm) columns at an elution rate of 12 ml/h; 0.8 ml fractions were collected and counted for ^{64}Cu radioactivity. (a) Column distributions. Arrows at the top indicate the elution positions of the markers (see the Materials and methods section). (b) Percentage changes in (●) MT- and (○) non-MT-containing fractions. The ^{64}Cu contents of all fractions in the MT-containing and non-MT-containing fractions were summed and divided by the total cytosolic ^{64}Cu to obtain the percentages.

shifted towards lower molecular masses, as would occur if a ^{64}Cu -binding species in this fraction were missing or defective, and the resulting unbound ^{64}Cu was scavenged by lower-molecular-mass proteins.

The most notable differences between the liver and kidney cytosols from normal mice (or rats) were that no ^{64}Cu was detected in fraction II in the kidney, and kidney cytosols reproducibly contained relatively more ^{64}Cu than did liver cytosols in the MT fraction and fraction I (Fig. 8). The distribution of ^{64}Cu in cytosols from fibroblasts is similar to that in the kidney [16], which further suggests that the copper-binding proteins in fraction II are liver-specific. Consistent with the literature [27], the renal cytosols from the brindled mice contained excess ^{64}Cu bound to MT (Fig. 8). Less ^{64}Cu was detected in fraction I, which could be due to decreased binding to a specific copper-binding protein(s) in this fraction, and/or to a secondary effect of increased ^{64}Cu binding by excess MT.

Fig. 7. Cytosolic distributions of ^{64}Cu in hepatocytes from control and brindled mice

Hepatocytes were incubated with $5\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 5 min. The cytosols (—, control mice; ---, brindled mice) were fractionated on Sephadex G-150 (1.5 cm $\times$ 100 cm) columns eluted with HEPES buffer at a flow rate of 12 ml/h; 0.8 ml fractions were collected and counted for ^{64}Cu radioactivity. The arrow over the brindled-mouse profile indicates the fraction analysed electrophoretically (see Fig. 9); the arrows at the top indicate the elution positions of the markers (see the Materials and methods section).

Fig. 8. Distributions of ^{64}Cu in renal cytosols from control and brindled mice

$^{64}\text{Cu}(\text{II})$ was added directly to the cytosols (—, control mice; ---, brindled mice) obtained from the whole kidney homogenates to give a concentration of $3.2\ \mu\text{M}$. This concentration of copper was equivalent to the amount of copper in the cytosolic fraction of hepatocytes after incubation with $10\ \mu\text{M}$ - $^{64}\text{Cu}(\text{II})$ for 5 min. The cytosols were fractionated on Sephadex G-150 (1.5 cm $\times$ 50 cm) columns eluted with HEPES buffer at a flow rate of 12 ml/h; 0.8 ml fractions were collected and counted for ^{64}Cu radioactivity. Arrows at the top indicate the elution positions of the markers (see the Materials and methods section).

Fig. 9. Densitometric scan of SDS/PAGE gel of Sephadex G-150 fractions from control and brindled mice

The fraction, indicated by an arrow in Fig. 7, from the brindled-mouse hepatocyte sample and a corresponding fraction from the control were analysed by SDS/PAGE, under reducing conditions with silver staining. The dried gels were scanned by a laser densitometer. Only a section of the scan of the control sample (---) which is displaced upward for clarity, is shown, representing a $\sim 55\ \text{kDa}$ protein band (arrow) which is markedly decreased in the brindled-mouse sample (—). The remainder of the scan from control samples was virtually congruent with the brindled-mouse scan.

Components affected in the brindled mice

Individual fractions from the Sephadex columns were analysed by SDS/PAGE. Abnormally low levels of a $\sim 55\ \text{kDa}$ component (protein or subunit) were consistently detected in the hepatocyte cytosols from the brindled mice (Fig. 9). More of this component was detected in fraction II than in fraction I in normal mice. No other significant electrophoretic differences were detected at this level of purification. Consistent with the $\sim 55\ \text{kDa}$ component being a liver-specific protein, it was not detected electrophoretically in equivalent column fractions with renal cytosols from normal mice.

DISCUSSION

Cytosolic ^{64}Cu was reproducibly distributed among four fractions from the Sephadex G-150 columns from both rat and mouse hepatocytes at the shortest incubation times (1 min) and lowest copper concentrations ($2\ \mu\text{M}$) tested. A significant difference between the mouse and rat hepatocytes was that mouse hepatocytes reproducibly contained much less ^{64}Cu bound to MT than did rat hepatocytes. Thus normal mouse hepatocytes represent a particularly sensitive normocupric system for determining which proteins, in addition to MT, bind copper immediately after copper enters a cell. Particularly evident at the lowest incubation concentration used ($2\ \mu\text{M}$) was the relatively high percentage of cytosolic ^{64}Cu that was bound to a component(s) in fraction II. Although ^{64}Cu detected in components of molecular mass near $30\ \text{kDa}$ has usually been assumed to correspond to Cu/Zn-SOD [6,7], the data shown here indicate that ^{64}Cu -binding fraction II of hepatocytes was not exclusively Cu/Zn-SOD.

The copper-binding protein(s) in fraction I was probably not resolved previously because it would be eluted in the void volumes of gel-permeation columns with smaller porosity [6–9]. From the column profiles, the copper-binding protein(s) involved apparently has a lower affinity than the copper-binding compo-

ment(s) in fraction II. However, at least some ^{64}Cu was detected in this fraction in both mouse and rat hepatocytes at the lowest incubation concentration tested. Also, the results do not exclude the possibility of a high flux of ^{64}Cu through a component(s) in fraction I. It should be noted that $2\ \mu\text{M-Cu(II)}$ is similar to the effective non-ceruloplasmin plasma concentration to which hepatocytes are exposed *in vivo*. Albumin is the principal species which carries copper from sites of absorption from the gastrointestinal tract to the liver [1,28]. Albumin-bound copper, which is approx. $1.6\ \mu\text{M}$, has a half-life of about 10 min [28], and a high percentage ($\sim 85\%$) of this copper is taken up by the liver with the same half-time [14,29].

Copper binding, as measured here, to a cytosolic protein could be due to one or more of the following: ^{64}Cu binding to an apo-Cu-binding protein, isotopic exchange of ^{64}Cu for $^{63/65}\text{Cu}$, ^{64}Cu displacing a related metal such as zinc from a zinc-metalloprotein, or non-specific ^{64}Cu binding. Also, ^{64}Cu may bind to a nascent peptide during protein synthesis or post-translationally. Copper incorporation into nascent peptides in fractions I and II seems unlikely, given the small time-dependence of the amounts and distribution of cytosolic copper, and the similar profiles when copper was added to cells or directly to cytosols. Copper displacement of zinc in these fractions is unlikely, because the distributions of ^{65}Zn and ^{64}Cu were clearly different. Since whole-cell net uptake occurred over the time period used in these studies, and 50% of the copper was within the cytosolic fraction [20], at least some copper binding to empty sites in fractions I and II seems likely.

The question of specific versus non-specific copper-binding proteins cannot be fully resolved until specific functions can be assigned to purified specific copper-binding proteins in fractions I and II. However, the amounts of copper bound to proteins which have roles in intracellular copper metabolism are expected to vary with copper metabolic status. ^{64}Cu binding to MT and one or more components in both fractions I and II and the void-volume fraction were clearly affected by copper deficiency. The concomitant decreases in MT-Cu and increases in ^{64}Cu -binding in fractions I and II in copper-deficient rat hepatocytes could be due to: (1) co-ordinate regulation of protein synthesis, (2) secondary effects owing to increases or decreases in ^{64}Cu binding to other Cu-binding proteins, (3) and/or an increased number of empty sites on apo-copper-binding proteins in fractions I and II. Although the functional significance of the changes in ^{64}Cu binding to cytosolic proteins in copper deficiency is not known, more copper is incorporated into ceruloplasmin and less is secreted into bile in copper-deficient rats [30,31]. Moreover, a time-delayed loss of copper that was detected with normocupric hepatocytes was not detected with hepatocytes from copper-deficient mice [19].

The changes detected here during development are consistent with whole-liver studies by Mason *et al.* [26]. Although the concurrent decreases in copper binding to MT and increases in copper binding to higher-molecular-mass proteins could reflect co-ordinate regulation of protein synthesis, the increase in ^{64}Cu binding to components in fractions I and II could also be due to increased availability of ^{64}Cu , owing to decreased MT.

The distribution of ^{64}Cu among cytosolic proteins of hepatocytes from normocupric mice is strikingly similar to the distribution of ^{64}Cu in copper-deficient rat hepatocytes. Like copper-deficient rat hepatocytes, normocupric mouse hepatocytes show less copper binding to MT than do normocupric rat hepatocytes. In both copper-deficient rat hepatocytes and normocupric mouse hepatocytes, relatively more ^{64}Cu is reproducibly bound to components in ^{64}Cu -binding fraction II and especially fraction I. Relatively high levels of ^{64}Cu were also detected in fraction I of normal kidney cytosols, which apparently contain little or none

of the copper-binding component(s) associated with fraction II. Overall, the data are consistent with ^{64}Cu equilibrating among MT and proteins in fractions I and II, with the relative amounts of ^{64}Cu bound to each component being determined by the relative amounts of, especially, MT and the high-affinity ^{64}Cu -binding component in fraction II of hepatocytes. In general, there appears to be an inverse correlation between the amount of ^{64}Cu bound to MT and the amount bound in the other fractions. The relative amount bound to each seems to be determined by copper metabolic status.

The cytosolic distributions of ^{64}Cu in the cytosols from hepatocytes and kidneys from the brindled mice were abnormal. In hepatocytes, the data are consistent with decreased copper binding by a component in fraction I and increased binding by lower-molecular-mass components. The decreased amounts of the $\sim 55\ \text{kDa}$ component that were detected by SDS/PAGE cannot account for the apparent shift of fraction I, because that component was eluted mainly in fraction II. The results suggest that a component in fraction I is either low or defective, which was not resolved by the chromatographic and electrophoretic methods used here. The kinetics of copper transport for both uptake and efflux by brindled-mouse hepatocytes [19] and fibroblasts [32] and Menkes-disease lymphoblasts [33] are normal, which suggests that the primary defect resides with an intracellular component [2]. The fact that specific changes in cytosolic copper binding were detected here is consistent with this concept. The data also suggest that copper-binding proteins other than MT are involved. Menkes disease is an X-linked disease [34], and MT genes are not on the X-chromosome [35,36]. Also, since the levels of MT protein [37] and mRNA [38] are normal in fibroblasts, given the elevated levels of copper that are found in these cells, defects in trans-acting regulators of MT synthesis are unlikely. Increases in MT, mRNA and protein seem to be secondary to the primary defect.

The distributions of ^{64}Cu in hepatocytes isolated from copper-deficient rats and the brindled mice were strikingly different (and different from the species differences), even though they were comparably deficient in copper. In particular, copper-binding to a component(s) in fraction I apparently increased in copper deficiency and decreased in the brindled mice. These results are consistent with results reported by Prohaska [39], and further suggest that the primary defect is expressed in the liver [2,19]. The fact that fraction II was detected in normal liver cells, which express the low-copper-level phenotype in brindled mice and Menkes-disease patients [2,11,40,41], and not detected in the kidney or fibroblasts [16], which express the high-copper-level phenotype [2,11,27,40,41], is consistent with copper levels in the defect being determined by tissue-specific copper metabolic factors [2,19]. If the primary defect in Menkes disease is expressed both in cells which are low in copper and in cells which are high in copper, then low levels of the $\sim 55\ \text{kDa}$ hepatic component most likely represent a secondary defect, because this component was only detected in the liver.

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