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The relationship of excess copper accumulation by fibroblasts from the brindled mouse model of Menkes disease to the primary defect

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Fibroblasts from the brindled mouse model of Menkes disease are known to accumulate excess copper. Most of the copper in the cytosol of these fibroblasts is bound to metallothionein (MT), which is elevated in Menkes or brindled mouse fibroblasts. Copper accumulation by normal fibroblasts containing excess MT was examined to determine if the excess copper accumulation phenotype was secondary to excess MT or associated with the primary defect in fibroblasts from the brindled mice. MT was induced in normal fibroblasts by copper, zinc or dexamethasone to levels comparable with those in brindled mice fibroblasts, as determined by radioimmunoassays. Normal fibroblasts containing excess MT accumulate copper normally, i.e. they do not exhibit the excess copper accumulation phenotype. Consistent with this result, copper efflux from normal fibroblasts containing excess MT was also normal. The data suggest that one function of the protein associated with the primary defect is to help determine how much copper is taken up and retained by fibroblasts and other cell types exhibiting the excess copper phenotype in Menkes disease. The capacity of this protein is apparently exceeded in normal fibroblasts if serum or albumin is not present extracellularly to limit total copper uptake. Consistent with a defect in an intracellular protein, the kinetics of copper transport by brindled mice fibroblasts were found to be normal.

INTRODUCTION

Menkes disease is a fatal, X-linked inborn error of copper metabolism [1–3]. The major symptoms of the disease are associated with defects in connective tissue and impaired brain development. Most of the clinical manifestations of the disease can be rationalized by low activities of known copper-containing enzymes, e.g. lysyl oxidase for the connective tissue symptoms [4,5].

A major breakthrough in research on Menkes disease was the finding by Hunt that the brindled mouse is an animal model of the disease [6]. The brindled mouse mutation is one of five alleles of the mottled locus [6–8]. Brindled mice have virtually identical symptoms and copper abnormalities as Menkes patients. The only apparent difference is that brindled mice survive if a single dose of copper is administered at 7–9 days after birth [9], whereas copper therapy with Menkes patients has been unsuccessful [1]. This may be due to differences in brain development; considerable development occurs after birth in mice.

Whereas the liver and brain are copper-deficient in Menkes disease and in the brindled mice (levels approx. 50% of normal), kidneys and fibroblasts show an approx. 3–4-fold elevation of copper [1,10–12]. Whether or not all cell types express the primary defect is uncertain. Hepatocytes isolated from brindled mice continue to exhibit decreased net copper accumulation [13], and cells with the elevated copper phenotype retain their abnormal properties through several passages in cell culture [14–17]. The simplest hypothesis is that both the low-copper and high-copper cell types express the primary defect and the differences in the effects of the primary defect on copper levels reflect tissue-specific aspects of copper metabolism [3,13].

Copper uptake experiments with fibroblasts from Menkes patients or the brindled mice showed that during prolonged incubations with radiolabelled copper, the diseased cells accumulated much more copper than normal cells [16,18,19]. The increased copper accumulation was due to decreased copper efflux from mutant fibroblasts [16] rather than increased uptake. Similar results were obtained with lymphoblasts from Menkes patients [17]. Most of the radiolabelled copper in the cytosol from these cells is bound to metallothionein (MT) [17,20]. MT levels are elevated in Menkes and brindled mouse fibroblasts and lymphoblasts [17,20]. However, the primary defect does not appear to involve MT, as discussed further below.

This paper examines the kinetics of copper transport by fibroblasts from brindled mice, and the possible role of elevated MT levels in excess copper accumulation. The kinetics of copper transport were found to be normal, consistent with a defective intracellular component. When MT was induced in normal cells to levels comparable with those found in brindled mouse fibroblasts, copper accumulation was normal. This is consistent with excess copper accumulation being directly associated with the primary defect rather than with elevated MT.

EXPERIMENTAL

Animals

Normal control mice (C57BL/6J strain; Jackson Laboratory, Bar Harbor, ME, U.S.A.), brindled male hemizygotes and brindled female heterozygotes (a gift from the late Dr. Robin Bannerman, Department of Medicine, Buffalo General Hospital) were bred and maintained as described previously [13]. Brindled male hemizygotes were treated with copper as described by Mann *et al.* [9], receiving a single injection of 10 μg of Cu/g of body weight at 7–9 days after birth.

Materials

$^{64}Cu(NO_3)_2$ was supplied by the Buffalo Materials Research Center at SUNY/Buffalo. $^{65}ZnCl_2$ was purchased from New England Nuclear, Boston, MA, U.S.A. All other biochemicals were from Sigma, St. Louis, MO, U.S.A.

Abbreviations used: MT, metallothionein; PBS, phosphate-buffered saline.

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Fibroblast cell cultures

Primary cultures of skin fibroblasts were established by standard procedures [21]. The growth medium was Eagle's Minimum Essential Medium (with Earle's salts) supplemented with 10% fetal calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 20 mM-Hepes, pH 7.4. The cells were subcultured on 35 mm plastic tissue culture plates and grown to confluency in 2 ml of growth medium per plate. Experiments were done between the third and twentieth passages.

^{64}Cu(II) incubations

The kinetics of ^{64}Cu transport by normal and brindled mouse fibroblasts were determined as described for mouse hepatocytes [13] or rat fibroblasts [21]. Serum-free media were used, since albumin markedly inhibits copper transport [21]. Fibroblasts were incubated for 10 min in 1 ml of serum-free modified Earle's medium which contained 1.8 mM-$CaCl_2$, 5.4 mM-KCl, 0.83 mM-$MgSO_4$, 1.0 mM-NaH_2PO_4, 96.4 mM-NaCl, 26.2 mM-$NaHCO_3$, 5.55 mM-glucose and 20 mM-Hepes, pH 7.4. Kinetic experiments were done at room temperature in O_2/CO_2 (19:1), with shaking at 60 oscillations/min. After incubation with ^{64}Cu(II) for 30 s, the ^{64}Cu solution was discarded, and the cells were washed once with 2 ml of ice-cold 1 mM-EDTA/0.9% NaCl and then twice with 2 ml of ice-cold phosphate-buffered saline (PBS; 130 mM-NaCl/2.7 mM-KCl/8.0 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4). The entire washing procedure took 30 s. The cells were solubilized in 0.1 M-NaOH and ^{64}Cu radioactivity was counted in a LKB model 1282 gamma counter, correcting for ^{64}Cu decay. Protein was determined by the Lowry method [22]. Kinetic parameters (K_m, $V_{max.}$) were determined by analysing initial velocity (30 s) of copper uptake versus copper concentration data by a non-linear least squares computer program (PENNZYME) as described previously [23].

Fibroblasts were incubated in Minimum Essential Medium plus 10% fetal calf serum at 37 °C in a CO_2 incubator for all experiments involving prolonged incubations with ^{64}Cu(II), unless otherwise indicated. The cells were washed and processed as described above.

Induction of MT in mouse fibroblasts by copper

The medium from confluent cultures was removed and replaced with a medium containing 400 μM-$CuSO_4$ for 48 h. Pilot experiments revealed that this was the highest concentration of copper that did not affect the viability of the cells. The medium containing excess copper was then removed and replaced with medium containing 5 μM-^{64}Cu(II) for copper uptake measurements.

Induction of MT in mouse fibroblasts by zinc

Confluent cultures were incubated with 100 μM-$ZnSO_4$ for 48 h. Histidine was added at a 2:1 molar ratio to prevent the zinc from precipitating in the growth medium. The medium containing excess zinc was removed and replaced with zinc-free medium before incubating with ^{64}Cu(II).

Induction of MT in mouse fibroblasts by dexamethasone

Cells were incubated with 100 nM-dexamethasone in growth medium for 24 h before adding ^{64}Cu(II). Dexamethasone was present throughout the ^{64}Cu uptake measurements, i.e. the medium containing the dexamethasone was not removed prior to adding ^{64}Cu(II).

Quantification of MT

Mouse fibroblasts were grown to confluency on 150 mm x 20 mm tissue culture plates. Cytosol was isolated from single culture dishes of normal or brindled cells, or of normal mouse cells that had been treated with Cu(II), Zn(II) or dexamethasone to induce MT. The medium was discarded, and the cells were washed twice with 15 ml of PBS. Then, 1 ml of ice-cold PBS was added, and the cells were removed from the dish with a rubber policeman. The cytosol was isolated by homogenizing at 0–4 °C with eight strokes of a Teflon pestle (0.08–0.13 mm clearance) and centrifuging at 100000 g for 60 min at 4 °C. [21]. The total amount of MT isoforms I and II was determined by radioimmunoassay [24]. The standard curve was obtained from the effect of a 50/50 (w/w) mixture of rat MT-I and MT-II competing with ^{125}I-labelled rat MT-I. Although the antibody was directed against rat MT, rat and mouse MT have 90% sequence identity [25]. The linearity of response was checked with each sample. These essays were kindly performed by Dr. Justine Garvey of Syracuse University, Syracuse, NY, U.S.A. The results are expressed as ng of total MT/mg of cytosolic protein.

RESULTS

Kinetics of ^{64}Cu(II) transport

The concentration-dependence of initial rates of ^{64}Cu(II) transport by normal and brindled mouse fibroblasts was determined to assess whether the phenotypes of excess copper accumulation and elevated copper levels were related to abnormal copper transport. Both normal and brindled mouse fibroblasts exhibited saturation kinetics for copper transport (Fig. 1). The fit of the experimental data to the K_m and $V_{max.}$ parameters obtained by non-linear regression analyses is consistent with a single transport process in both cases (Fig. 1), and there is no evidence for transport by simple diffusion in either case. No significant differences were observed in the apparent K_m values for copper transport by normal (15.9 $\pm$ 2.4 μM) and brindled (17.5 $\pm$ 0.04 μM) mouse fibroblasts but the $V_{max.}$ obtained with brindled mouse fibroblasts was slightly (30%) lower than normal. This accounts for the difference in the profiles of velocity versus copper concentration in Fig. 1. The relatively small difference obtained in $V_{max.}$ is more likely to be due to experimental variability than to any significant abnormality in copper transport by the brindled fibroblasts. In any event, it is unlikely that a lower than normal $V_{max.}$ could be related to increased copper uptake by the brindled fibroblasts.

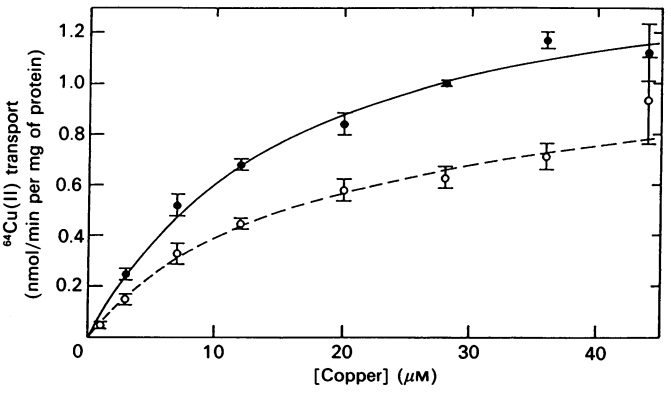

Fig. 1. Kinetics of ^{64}Cu(II) transport by normal and brindled mice fibroblasts

Cells were incubated at room temperature with the indicated concentrations of ^{64}Cu(II) in a serum-free modified Earle's medium for 30 s. The cells were then washed and processed as described in the Experimental section. The curves for normal fibroblasts (●) and brindled mouse fibroblasts (○) represent the theoretical plots calculated from the optimal kinetic parameters obtained from non-linear regression analyses of the data. Each point represents the mean $\pm$ S.D. of triplicates.

Table 1. MT levels in brindled mouse fibroblasts and normal fibroblasts after MT induction

MT synthesis was induced by incubating normal fibroblasts with 400 μM-$CuSO_4$ or 100 μM-$ZnSO_4$ for 48 h or with 100 nM-dexamethasone for 24 h. MT levels were determined by radioimmunoassays. MT levels were also measured after 12 or 24 h incubations with 5 μM-$CuSO_4$ to determine if the MT level changed during the incubation with 5 μM-^{64}Cu(II). Each value is the mean ± S.E.M.

Fibroblast source and treatment	MT (ng/mg of cytosolic protein)
Normal	61 ± 4
Brindled mouse	156 ± 9
Brindled mouse (12 h)	122 ± 8
Brindled mouse (24 h)	165 ± 10
Copper-treated normal	185 ± 9
Copper-treated normal (12 h)	175 ± 9
Copper-treated normal (24 h)	165 ± 10
Dexamethasone-treated	126 ± 8
Zinc-treated	556 ± 36

Copper accumulation by brindled mouse fibroblasts and normal fibroblasts containing excess MT

MT synthesis was induced in normal fibroblasts by incubation with 400 μM-$CuSO_4$ to determine if excess copper accumulation was directly related to elevated MT. The MT level in the copper-treated normal fibroblasts (3-fold elevation) was comparable with the MT level in the brindled mouse fibroblasts (2.6-fold elevation) as determined by radioimmunoassay (Table 1). Copper accumulation by copper-related normal fibroblasts was indistinguishable from copper accumulation by normal fibroblasts, i.e. normal fibroblasts with elevated MT levels do not exhibit the Menkes phenotype (Fig. 2). Radioimmunoassays at 12 and 24 h confirmed that the amount of MT in the copper-treated fibroblasts remained comparable with the MT levels in the brindled mouse fibroblasts throughout the incubation period in 5 μM-^{64}Cu(II) (Table 1).

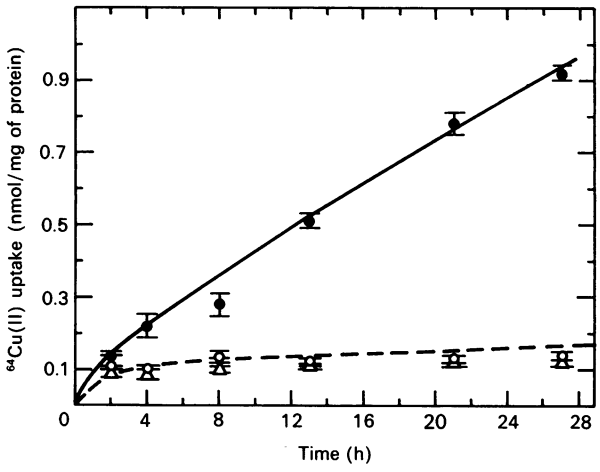

Fig. 2. Time dependence of ^{64}Cu(II) uptake by brindled mice fibroblasts and by normal fibroblasts with normal or elevated MT levels

Normal fibroblasts were incubated in Minimum Essential Medium plus 10% fetal calf serum containing 400 μM-$CuSO_4$, and control and brindled mice fibroblasts were incubated with Minimum Essential Medium plus 10% fetal calf serum for 48 h. After the preincubation, the media were replaced with the same media containing 5 μM-^{64}Cu(II). At the indicated times, the cells were washed and processed as described in the Experimental section to determine copper uptake by normal (○--○), brindled mouse (●) and normal with elevated MT (△) fibroblasts. Each point represents the mean ± S.D. of triplicates.

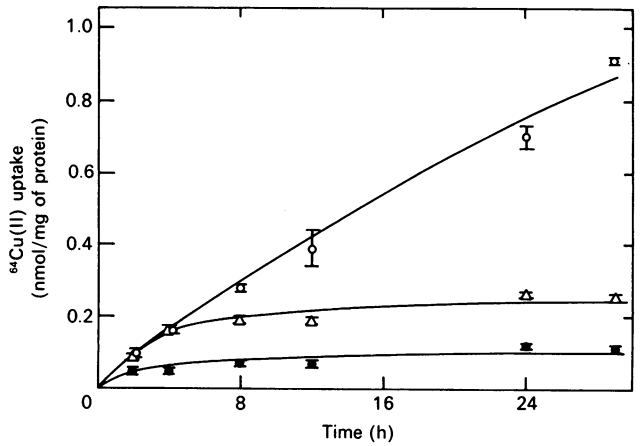

Fig. 3. Time-dependence of ^{64}Cu(II) uptake by normal and brindled mice fibroblasts and by normal fibroblasts after MT induction by zinc

Normal fibroblasts were incubated in Minimum Essential Medium plus 10% fetal calf serum containing 100 μM-$ZnSO_4$, while control and brindled mice fibroblasts were incubated with Minimum Essential Medium plus 10% fetal calf serum for 48 h. After the preincubation, the media were replaced with the same media containing 5 μM-^{64}Cu(II). At the indicated times, the cells were washed and processed as described in the Experimental section to determine copper uptake by normal (●), brindled mice (○) and normal with elevated MT (△) fibroblasts. Each point represents the mean ± S.D. of triplicates.

Copper accumulation by fibroblasts after MT induction by dexamethasone or zinc

MT synthesis was also induced by dexamethasone or zinc to determine if the normal copper uptake observed after MT induction by copper was copper-specific. MT levels in response to dexamethasone (100 nM) were comparable with the MT levels in the brindled mouse fibroblasts, whereas induction by zinc (100 μM) led to somewhat higher MT levels (Table 1). Copper accumulation by the zinc-treated cells (Fig. 3) was significantly higher than by the dexamethasone-treated cells (Fig. 4), and both showed significantly higher copper accumulation than normal fibroblasts. However, under the same experimental conditions, fibroblasts from the brindled mice accumulated much more ^{64}Cu than either dexamethasone-treated or zinc-treated normal fibroblasts.

Efflux of ^{64}Cu(II)

Beratis *et al.* [16] showed that excess net copper accumulation by Menkes fibroblasts was due to impaired copper efflux rather than to excess copper uptake. As expected by the copper accumulation results, efflux from normal fibroblasts with excess MT was normal, indicating that elevated MT levels in normal cells have no effect on copper efflux (Fig. 5). Brindled mouse fibroblasts containing comparable levels of MT exhibit decreased efflux (Fig. 5).

Uptake of ^{65}Zn(II)

The metal specificity of the above effects was assessed by measuring ^{65}Zn uptake. ^{65}Zn uptake rates by normal fibroblasts, normal fibroblasts with excess MT and brindled mouse fibroblasts were the same. Thus excess ^{64}Cu uptake by brindled

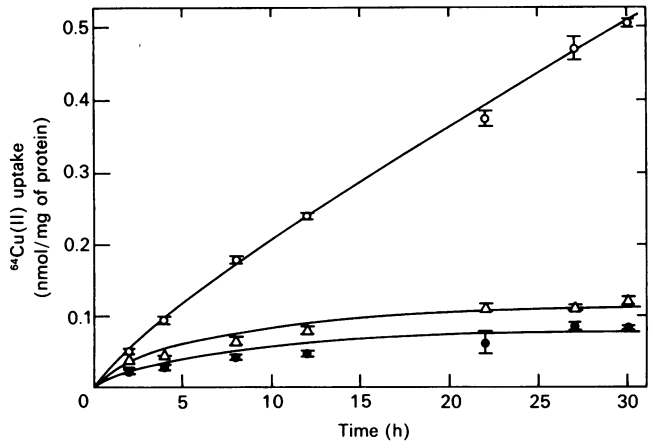

Fig. 4. Time-dependence of ^{64}Cu(II) uptake by normal and brindled mice fibroblasts and by normal fibroblasts after MT induction by dexamethasone

Normal fibroblasts were incubated in Minimum Essential Medium plus 10% fetal calf serum containing 100 nM-dexamethasone for 24 h prior to incubation with 5 μM-^{64}Cu(II). Control and brindled mice fibroblasts were incubated with Minimum Essential Medium plus 10% fetal calf serum for 24 h prior to addition of 5 μM-^{64}Cu(II). At the indicated times, the cells were washed and processed as described in the Experimental section to determine copper uptake by normal (●), brindled mice (○) and normal with elevated MT (△) fibroblasts. Each point represents the mean ± S.D. of triplicates.

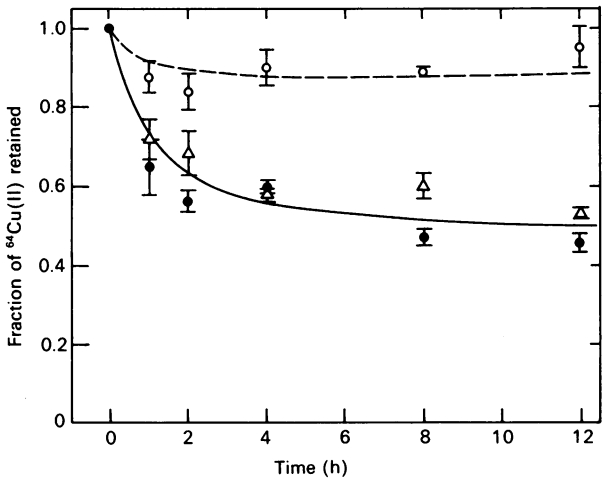

Fig. 5. Efflux of ^{64}Cu(II) from brindled mice fibroblasts and normal fibroblasts with normal or elevated MT levels

Normal fibroblasts were incubated in Minimum Essential Medium plus 10% fetal calf serum containing 400 μM-$CuSO_4$, whereas control and brindled mice fibroblasts were incubated in Minimum Essential Medium plus 10% fetal calf serum for 48 h. After the preincubation, cells were preloaded by incubating with 5 μM-^{64}Cu(II) in Minimum Essential Medium plus 10% fetal calf serum for 12 h. After the preloading period, the media were discarded and the cells were washed once with each of 1 mM-EDTA/0.9% NaCl and PBS. ^{64}Cu(II)-free medium (Minimum Essential Medium plus 10% fetal calf serum) was added, and at the indicated times the cells were washed and processed as described in the Experimental section to determine copper efflux from normal (●), brindled mouse (○) and normal with elevated MT (△) fibroblasts. Each point represents the mean ± S.D. of triplicates.

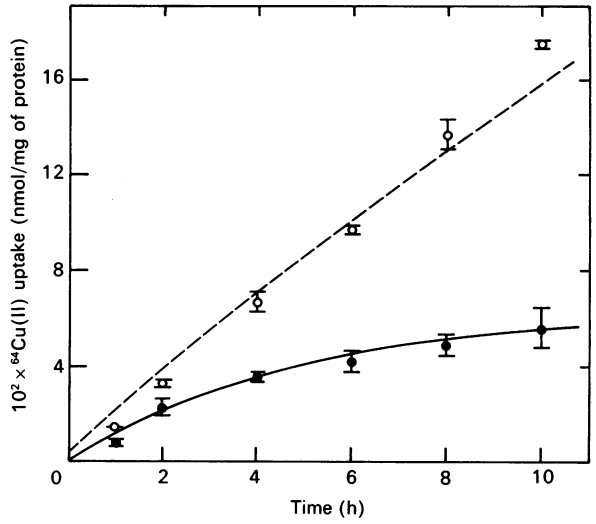

Fig. 6. Effect of albumin on ^{64}Cu(II) uptake by normal and brindled mice fibroblasts

Cells were incubated with 5 μM-^{64}Cu(II) in modified Earle's medium containing 50 μM-albumin. At the indicated times, the cells were washed and processed as described in the Experimental section to determine copper uptake by normal (●) and brindled mouse (○) fibroblasts. Each point represents the mean ± S.D. of triplicates.

mouse fibroblasts is specific for copper, and increased MT levels in normal fibroblasts have no significant effect on zinc uptake.

Copper accumulation in serum-free media

Earlier results showed that serum markedly limited copper accumulation by normal fibroblasts [26]. Normal, brindled and copper-treated normal fibroblasts were incubated in serum-free media to determine if expression of the brindled mouse phenotype depended on the level of copper uptake. All three cell types accumulated more ^{64}Cu than from serum-containing media, and normal and brindled mouse fibroblasts accumulated similar amounts.

Albumin was proposed to be the principal plasma species which limits copper uptake by fibroblasts [26]. Normal and brindled fibroblasts were incubated with 50 μM-albumin and 5 μM-^{64}Cu to determine if limiting copper uptake by albumin alone was sufficient to detect the Menkes phenotype. Excess copper accumulation was exhibited by brindled fibroblasts in the presence of albumin (Fig. 6).

DISCUSSION

The copper transport data indicate that initial rates of copper transport by fibroblasts from the brindled mice are normal. Copper transport, both uptake and efflux, is also normal for hepatocytes from the brindled mice [13] which express the low-copper-level phenotype. Moreover, Herd *et al.* [27] recently reported normal initial rates of both copper uptake and copper efflux by Menkes lymphoblasts, which, like fibroblasts, show excess copper accumulation during prolonged incubations with ^{64}Cu(II) [17]. Thus the primary defect is most probably associated with an intracellular component, as suggested earlier [13].

The total ^{64}Cu accumulated by the brindled mice fibroblasts at 28 h was approx. 0.9 nmol/mg of cell protein (Fig. 2). Since MT binds 12 g-atom of Cu/mol, approx. 150 ng of MT/mg of

cytosolic protein (Table 1) could bind approx. 0.3 nmol. About 25% of the cytosolic Cu in these cells (0.1 nmol) is bound to higher molecular mass cytosolic proteins [26]. The remaining 50% (approx.) of the ^{64}Cu accumulated (0.45 nmol/mg of cell protein) is most probably taken up by particulate cell fractions, as in normal fibroblasts [21].

Several lines of evidence suggest that defects in MT or its synthesis cannot be the primary defect in Menkes disease or in the brindled mice. MT isolated from Menkes fibroblasts has a normal affinity for copper, suggesting that the protein is normal [28]. More directly, none of the structural genes for MT or any *cis*-acting regulatory element can be affected, because Menkes disease is X-linked, and all of the genes for MT are autosomal in both mice [29] and humans [30]. There is also no evidence for abnormal activity of a *trans*-acting factor [31]. Sone *et al.* [32] showed that MT synthesis in Menkes lymphoblasts is normal for the excess copper that they contain. Moreover, Packman *et al.* [33] detected equivalent MT-II mRNA levels in normal and brindled mouse fibroblasts at equivalently higher copper concentrations.

Prior to the results reported here, it was possible that high MT levels and excess copper accumulation were both secondary consequences of the primary defect. For example, a primary defect in a factor involved in intracellular copper utilization could lead to a build-up of non-utilizable copper, which in turn induced MT synthesis. Increased MT synthesis could then lead to increased copper accumulation. Since the results with fibroblasts containing elevated MT show that neither excess copper nor excess MT is sufficient to express the Menkes phenotype, excess copper accumulation by Menkes or brindled mouse fibroblasts must be related directly to the primary defect.

The results of the copper uptake experiments indicate that the protein associated with the primary defect plays some role in determining the amount of copper that is taken up and retained by normal fibroblasts. The fact that copper efflux is specifically affected in brindled mice and Menkes fibroblasts [16,19] suggests that the protein involved mediates copper efflux.

Excess copper accumulation by brindled mouse fibroblasts is detected only under conditions of relatively low copper uptake as occurs when serum or albumin is in the medium. The increased copper uptake observed in serum-free media confirms a role for albumin in limiting copper uptake by extrahepatic cell types [26]. In the absence of serum or albumin, the capacity of the protein associated with the primary defect to regulate net copper uptake is apparently exceeded, which leads to high copper uptake.

Expression of the primary defect in hepatocytes and fibroblasts is seemingly paradoxical, i.e. how can expression of the same primary defect lead to excess copper retention in one cell type (e.g. fibroblasts) and decreased copper retention by another cell type (e.g. hepatocytes)? As postulated earlier, the opposite effects on copper levels may reflect differences in normal copper metabolism in different cell types [13]. In addition to an efflux activity, the protein involved seems to also play a role in delivering copper to cellular sites of copper utilization, as evidenced by low lysyl oxidase and ceruloplasmin activities [1,4,6,34]. The essential difference between hepatocytes and fibroblasts may be that hepatocytes have alternative mechanisms for copper efflux of non-utilizable copper. Whereas copper secretion into bile is the normal mechanism for regulating whole body copper homeostasis [2], Danks and co-workers reported that copper excretion into bile was normal in brindled mice [11]. However, hepatocytes also contain a cytosolic copper-binding protein(s) which is either absent or in low concentration in other cell types [26] and which may mediate copper efflux from hepatocytes. Extrahepatic cell types may have no other mechanism for mediating copper efflux than the mechanism involving the protein associated with the primary defect.

The results reported here may have a bearing on understanding normal cellular mechanisms of controlling net copper accumulation. Although MT has been proposed to play a role in determining net copper and zinc accumulation [35], the results here imply that MT exerts a relatively small effect on copper and zinc uptake, at least at the MT levels examined here. The copper uptake properties of brindled mouse fibroblasts suggest a role for intracellular copper-binding proteins other than MT in determining net copper accumulation by normal cells, as was recently suggested by copper transport and accumulation studies [26].

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