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copper fibroblast Menkes' disease metallothionein

# Increased Copper Metallothionein in Menkes Cultured Skin Fibroblasts

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## Summary

**Menkes fibroblasts contain a significantly greater amount of cysteine-rich 10,000 dalton copper-binding protein(s) (metallothionein) than normal cells. Mutant fibroblasts incorporated 30 to 40% more tritiated amino acids into 10,000 dalton protein(s) than normal cells. The protein(s) was deficient in aromatic amino acids. The amount of $^{35}$S-cysteine incorporated by the same protein(s) in Menkes fibroblasts was twice that of normal fibroblasts. Comparison of the $^{35}$S:$^{3}$H isotopic ratios of chromatographic fractions of both normal and Menkes cell lysates showed that only the proteins eluted in the 10,000 dalton peak were enriched in $^{35}$S-cysteine, and this ratio was always greater in Menkes than in normal cells. The 10,000 molecular weight $^{35}$S-cysteine- and $^{3}$H-amino acid-labeled peaks coincided with the $^{64}$Cu peak in both cell strains. The copper-labeled peak was always greater in Menkes than in normal cells. No difference in the $^{64}$Cu:$^{35}$S isotopic ratio in the 10,000 dalton peak was observed between normal and Menkes fibroblast strains. This finding shows the direct relationship between the amount of cysteine-rich 10,000 dalton protein(s) and the amount of $^{64}$Cu bound by this protein(s) in both Menkes and normal fibroblasts. DEAE-cellulose ion-exchange chromatography resulted in a further two-fold enrichment of the 10,000 dalton, sulfur-rich proteins that were eluted from the Sephadex G-75 column. Most of the labeled proteins from both normal and Menkes fibroblasts were eluted from the ion-exchange column in a single peak at a chloride concentration of approximately 30 mM. Polyacrylamide disc gel electrophoresis of pooled fractions of the 10,000 dalton proteins eluted from the G-75 column and the DEAE-cellulose ion-exchange column showed no consistent differences in the staining pattern between normal and mutant fibroblast strains. When the acrylamide gels were sliced and subsequently counted for radioactive content, no band showed a further increase in the $^{35}$S:$^{3}$H isotopic ratio when compared to the electrophoresed samples that were eluted from the Sephadex G-75 or the ion-exchange columns. Also, no significant increase in the amount of radioactivity associated with a specific protein band could be demonstrated between the Menkes and the normal fibroblast strains.**

## Speculation

**The increased accumulation and the reduced efflux of copper observed in cultured Menkes fibroblasts is associated with a greater amount of copper metallothionein(s) present in the mutant than in the normal cells. The abnormally high copper concentrations observed in several extra-hepatic tissues of patients with Menkes disease may also relate to the presence in these tissues of increased levels of copper metallothionein(s).**

Danks *et al.* (8) have demonstrated a defect in the intestinal absorption of copper in the X-linked inherited degenerative brain disease described by Menkes *et al.* (17). It has been speculated that most, if not all, symptoms of this disease can be attributed directly or indirectly to a copper deficiency (7). In 1973, Danks *et al.* (9) further showed that the defect in copper absorption lies not simply in the uptake by the gut epithelium, but in the lack of release of copper from these cells.

The finding that cultured skin fibroblasts from patients with Menkes disease accumulate a greater amount of copper than normal cells (11) demonstrates that Menkes fibroblasts express the defect of the disease and has provided an *in vitro* system or the study of this disorder. Subsequently, it was shown that $^{64}$Cu accumulation is greater in Menkes fibroblasts than in normal cells (1, 5). Also, an increased $^{64}$Cu accumulation was observed in cultured amniotic fluid cells from an affected fetus (13). Beratis *et al.* (1) have reported that the efflux of copper is significantly lower in Menkes fibroblasts than in normal cells. Most of the copper accumulated by cultured normal and Menkes fibroblasts was bound to a molecule(s) with a molecular weight of approximately 10,000 daltons (1).

Further studies in our laboratory have demonstrated that the 10,000 molecular weight copper-binding species can also be labeled during short incubation of fibroblast lysates with radioactive copper. In lysates from Menkes fibroblasts, two to three times more copper is bound to this moiety than in lysates prepared from normal fibroblasts. This increased copper binding was not caused by an increased affinity of this molecule(s) for copper in the Menkes cells, nor by the deficiency of a cytoplasmic factor that affects the cleavage of copper from this molecule(s) in the Menkes fibroblasts (15).

The present communication reports the presence in Menkes cultured skin fibroblasts of a greater amount of a 10,000 dalton, copper-binding protein(s) than in normal cells; the properties of this molecule(s) were similar to those ascribed to metallothioneins.

## MATERIALS AND METHODS

Skin fibroblasts derived from three patients with Menkes disease (20) and from four normal subjects (21) were used in the study. Cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 16% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) (22). Cultures were between the fifth and the fifteenth passages. Fibroblasts were transferred to 75 cm$^2$ flasks, and while at growth phase, were incubated in the medium described above to which had been added $^{35}$*S*-L-cysteine-HCl (23) (specific activity, 99.0 mCi/mmole) and a mixture of 15 tritiated amino acids (24) (specific activity, 1.45 to 60 Ci/mmole). Final concentration of these two isotopes in the medium was 0.2 to 1.0 μCi/ml, as indicated. Cells were labeled for 24 to 48 hr.

Experiments were also performed by labeling the fibroblasts with $^{35}$*S*-L-cysteine and the following tritiated aromatic amino acids: *L*-[2,4,6-$^{3}$H]phenylalanine (76 Ci/mmole); L-[5-$^{3}$H]tryptophan (27 Ci/mmole); L-[2,5-$^{3}$H]histidine (60 Ci/mmol); and L-[2,3,5,6-$^{3}$H]tyrosine (82 Ci/mmole) (23). These labeled amino

acids were added to the culture medium at a final concentration of 0.25 μCi each per ml. Cells were labeled for 24 hr.

For harvesting, the radioactive medium was decanted, and the monolayers were washed twice with saline. Cells were removed by trypsinization, pelleted at 1800 × *g* for 10 min, and washed twice with saline. The fibroblasts were used immediately or frozen at −80°C until studied. No differences were found in the parameters examined between freshly prepared and frozen cells. Cells were lysed by 15 cycles of freezing and thawing in an acetone/dry ice bath. The lysates were centrifuged at 2000 × *g*, and the supernatants containing soluble proteins were collected for further investigation. Protein was determined by the method of Lowry *et al.* (16).

The lysates of the $^{35}S$- and $^{3}H$-labeled cells were further labeled with $^{64}Cu$ by incubating the lysates for 30 min at 37°C with carrier-free $^{64}Cu(NO_3)_2$ [specific activity 5.66 μCi/μg (24)]. Gel filtration of the labeled lysates was performed at room temperature on a Sephadex G-75 (25) column (0.9 x 60 cm), equilibrated with 0.01 M Tris-HCl buffer, 0.15 M NaCl, pH 7.0. The column was eluted with the same buffer at a flow rate of 10 to 15 ml/hr, and 1.0 ml fractions were collected. Columns were calibrated using the following molecular weight standards: blue dextran 2000; bovine serum albumin; ovalbumin; chymotrypsinogen A; ribonuclease A (25); and carrier-free $^{64}Cu(NO_2)_3$ (24). Protein in the eluted fractions was monitored by optical density at 280 nm. $^{64}Cu$ content was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer (model 3375) (26) using LSC-Gamma vials (27). $^{35}S$ and $^{3}H$ radioactivity was determined several weeks later, after thorough mixing of an aliquot of sample with 10 ml aqueous counting scintillant (22) in glass scintillation vials. $^{35}S$ and $^{64}Cu$ counts were corrected for radioactive decay where applicable. Recovery of the radioactive isotopes was between 90 and 99%.

The $^{35}S$-labeled 10,000 dalton proteins from both Menkes and normal fibroblasts were further purified using diethylaminoethyl (DEAE)-cellulose ion exchange chromatography. The DEAE-cellulose was equilibrated according to the manufacturer's instructions (28). Before application to the ion-exchange column, the fractions containing the 10,000 dalton proteins that were eluted from the Sephadex G-75 column were concentrated on an Amicon UM-2 membrane (29) and washed free of salt. The concentrated samples were applied to a 1.0 × 8.0 cm DEAE-cellulose column and washed with 15 ml of 10 mM Tris-HCl buffer, pH 8.5. Proteins attached to the resin were subsequently eluted from the column using a 0 to 500 mM linear NaCl gradient at a flow rate of 10 ml/hr. Fractions of 2.0 ml were collected and the $^{35}S$ and $^{3}H$ radioactivities were measured as described.

Acrylamide gel electrophoresis of native 10,000 molecular weight proteins was performed on 10% gels as previously described (10). After electrophoresis, the gels were either stained for protein using Coomassie blue stain or were cut into 2 mm slices. The slices were allowed to swell in 1.0 ml NCS tissue solubilizer (24) at 50°C overnight in tightly sealed glass scintillation vials. Ten ml of organic counting scintillant (23) was added, and after thorough mixing, the vials were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer.

## RESULTS

Menkes and normal fibroblasts grown in medium containing $^{35}S$-cysteine and $^{3}H$-amino acid mix for 48 hr (concentration of radioactivity in the medium for each of the two isotopes was 0.2 μCi/ml) were lysed, and an equal amount of lysate protein from the two strains of fibroblasts was further labeled with $^{64}Cu$ at a ratio of 4.0 mg fibroblast protein to 4.0 μg $^{64}Cu$. Fractionation by Sephadex G-75 chromatography of the same amount of fibroblast protein from both normal and Menkes strains resolved the radioactivity into three major peaks (Fig. 1). The first peak represents large molecular weight components which eluted in the void volume, and contained the majority of soluble fibroblast proteins. The second peak corresponded to an elution volume indicative of a molecular weight of approximately 10,000 daltons. The third

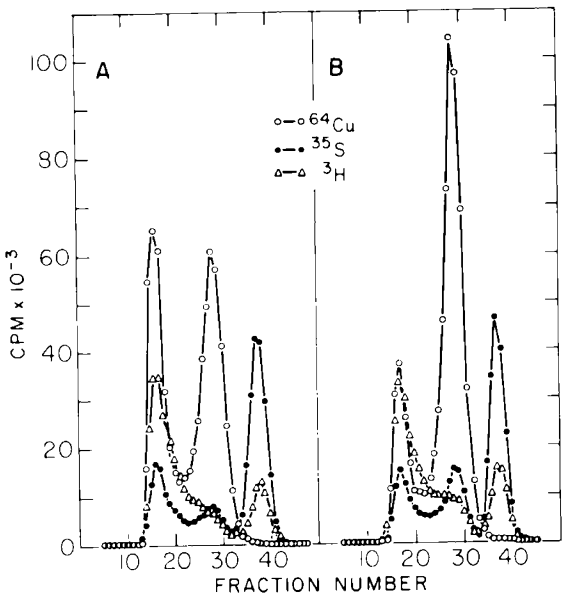


Fig. 1. Sephadex G-75 chromatography of cultured skin fibroblasts labeled in culture with $^{35}S$-cysteine and $^{3}H$-amino acid mix for 48 hr and, after lysis, with $^{64}Cu(NO_3)_2$. *A*, normal fibroblasts; *B*, Menkes fibroblasts.

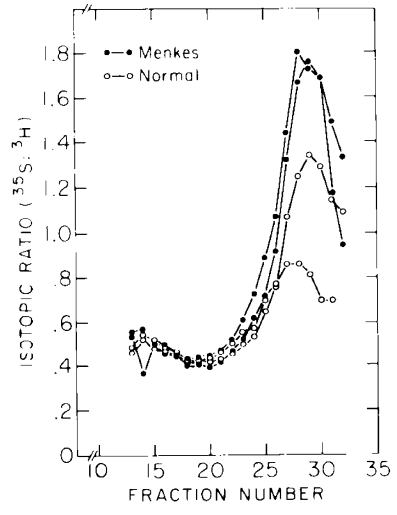


Fig. 2. $^{35}S$:$^{3}H$ isotopic ratios of fractions eluted from the Sephadex G-75 column; the findings from two Menkes and two normal fibroblast strains labeled during culturing are shown.

peak contained free amino acids and very low molecular weight compounds. The proteins prepared from Menkes fibroblasts (Fig. 1*B*) which eluted in the 10,000 molecular weight peak, consistently incorporated greater amounts of $^{35}S$-cysteine than the same proteins from normal fibroblasts (Fig. 1*A*). Menkes fibroblasts also incorporated greater amounts of $^{3}H$-amino acids into the 10,000 dalton proteins than normal fibroblasts. The 10,000 molecular weight $^{35}S$-cysteine- and $^{3}H$-amino acid-labeled peaks coincided with the $^{64}Cu$ peak in both cell strains. This copper-labeled peak was always greater in Menkes than in normal cells.

Comparison of the $^{35}S$:$^{3}H$ isotopic ratios in the chromatographic fractions of both normal and Menkes cells showed that only the proteins eluted in the 10,000 dalton peak (fractions 23 to 32) were enriched in $^{35}S$-cysteine (Fig. 2). No $^{35}S$-cysteine enrichment was observed in the large molecular weight proteins (fractions 13 to 21) in either fibroblast strain. In addition, despite the slight variation in $^{35}S$-cysteine incorporation between individual cases, the $^{35}S$:$^{3}H$ isotopic ratio was always greater in Menkes than in normal cells.

The levels of radioactivity incorporated by the 10,000 dalton proteins in Menkes and normal fibroblast strains is shown in Table 1. Menkes fibroblasts consistently incorporated 30 to 40% more tritiated amino acids than did normal cells. However, the amount of $^{35}$S-cysteine incorporated by the same proteins in the Menkes fibroblasts was twice that the normal fibroblasts, indicating that Menkes fibroblasts contain a significantly greater amount of cysteine-rich 10,000 dalton protein(s) than normal cells.

Figure 3 shows that although the amount of $^{35}$S-cysteine-rich protein(s) was greater in Menkes than in normal cells, no difference in the $^{64}$Cu:$^{35}$S isotopic ratio in the 10,000 dalton peak (fractions 23 to 32) was observed between normal and Menkes fibroblast strains. This finding shows the direct relationship between the amount of cysteine-rich 10,000 dalton protein(s) and the amount of $^{64}$Cu bound by this protein(s) in both Menkes and normal fibroblasts.

Table 2 shows the incorporation of tritiated aromatic amino acids and $^{35}$S-cysteine into 10,000 dalton proteins of Menkes and normal fibroblasts cultured in medium containing a mixture of tritiated aromatic or nonaromatic amino acids and $^{35}$S-cysteine for 24 hr (concentration of radioactivity in the medium 1.0 $\mu$Ci/ml for tritium, and 0.6 $\mu$Ci/ml for $^{35}$S). The amount of tritium label incorporated was reduced by approximately 50% in both normal and Menkes fibroblasts compared to the amount of tritium label incorporated by the cells labeled with the nonaromatic amino acids. The level of $^{35}$S-cysteine incorporation, however, was approximately the same with either tritium-labeled mixture used.

DEAE-cellulose ion-exchange chromatography resulted in a further two-fold enrichment of the 10,000 dalton, sulfur-rich proteins that were eluted from the Sephadex G-75 column. Most of the labeled proteins from both normal and Menkes fibroblasts were eluted from the ion-exchange column in a single peak at a chloride concentration of approximately 30 mM (Fig. 4). More than 95% of the $^{35}$S-labeled proteins and approximately 50% of the $^{3}$H-labeled proteins were recovered in this single major protein peak. The remaining $^{3}$H-labeled proteins were tightly adsorbed to the resin and could not be eluted, even at high NaCl concentrations.

Polyacrylamide disc gel electrophoresis of pooled fractions of the 10,000 dalton proteins eluted from the Sephadex G-75 column showed a large number of protein bands (Fig. 5*A*). Variation of the staining pattern was observed in both Menkes and normal fibroblasts. No consistent differences between normal and mutant fibroblast strains were observed. Figure 5*B* illustrates a reduction in the number of stained protein bands present in the eluates of the ion-exchange column. Again, no consistent differences in the staining pattern could be detected between Menkes and normal cells.

When the acrylamide gels were sliced and subsequently counted for radioactive content, all of the stained protein bands contained $^{35}$S and $^{3}$H radioactivity. However, no band showed a further increase in the $^{35}$S:$^{3}$H isotopic ratio when compared to the electrophoresed samples that were eluted from the Sephadex G-75 or the ion-exchange columns. Also, no significant increase in the amount of radioactivity associated with a specific protein band could be demonstrated between the Menkes and the normal fibroblast strains.

Table 1. *Radioactivity incorporated into the 10,000 dalton copper-binding proteins (metallothionein)*[1]

| Source | Percentage of total radioactivity incorporated: $^{35}$S-Cysteine | $^{3}$H-amino acids |
|---|---|---|
| Menkes[2] | 50.3 ± 6.5[3] | 22.2 ± 3.0 |
| Normal[4] | 26.4 ± 4.8 | 15.7 ± 4.4 |

[1] Cells were labeled for 48 hr; concentration of radioactivity in the medium 0.2 $\mu$Ci/ml.
[2] Three cases; four experiments.
[3] Mean ± S.D.
[4] Three cases; three experiments.

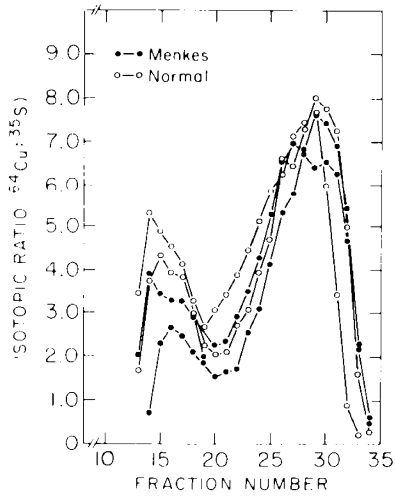


Fig. 3. $^{64}$Cu:$^{35}$S isotopic ratios of the chromatographic fractions studied in Figure 2.

Table 2. *Incorporation of tritiated aromatic amino acids versus nonaromatic amino acids into the 10,000 dalton proteins (metallothionein) of Menkes and normal cultured skin fibroblasts*

| Radiolabeled precursors | Percentage of total radioactivity incorporated: Menkes[1] | Normal[1] |
|---|---|---|
| [$^{3}$H]Aromatic amino acids[2] | 9.6 ± 3.6[3] | 8.0 ± 1.9 |
| $^{35}$S-Cysteine[4] | 41.9 ± 6.0 | 21.6 ± 0.4 |
| [$^{3}$H]nonaromatic amino acids[2] | 18.5 ± 5.2 | 14.0 ± 2.3 |
| $^{35}$S-Cysteine[4] | 47.2 ± 4.3 | 25.8 ± 0.8 |

[1] Two cases; three experiments.
[2] Concentration of radioactivity in the medium 1.0 $\mu$Ci/ml; 24 hr label.
[3] Mean ± S.D.
[4] Concentration of radioactivity in the medium 0.6 $\mu$Ci/ml; 24 hr label.

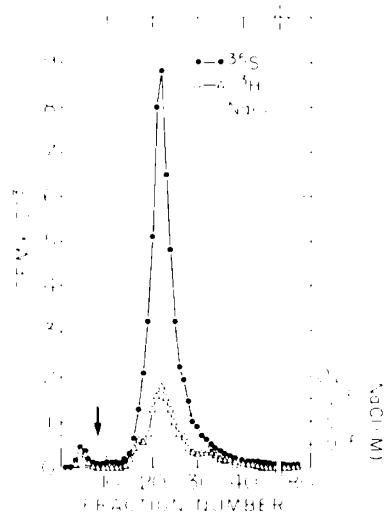

Fig. 4. DEAE-cellulose ion-exchange chromatography of pooled fractions of the 10,000 molecular weight proteins eluted from the Sephadex G-75 column (fractions 23 to 32, Fig. 1). The fibroblasts were derived from a patient with Menkes disease. *Arrow*, application of the NaCl gradient.

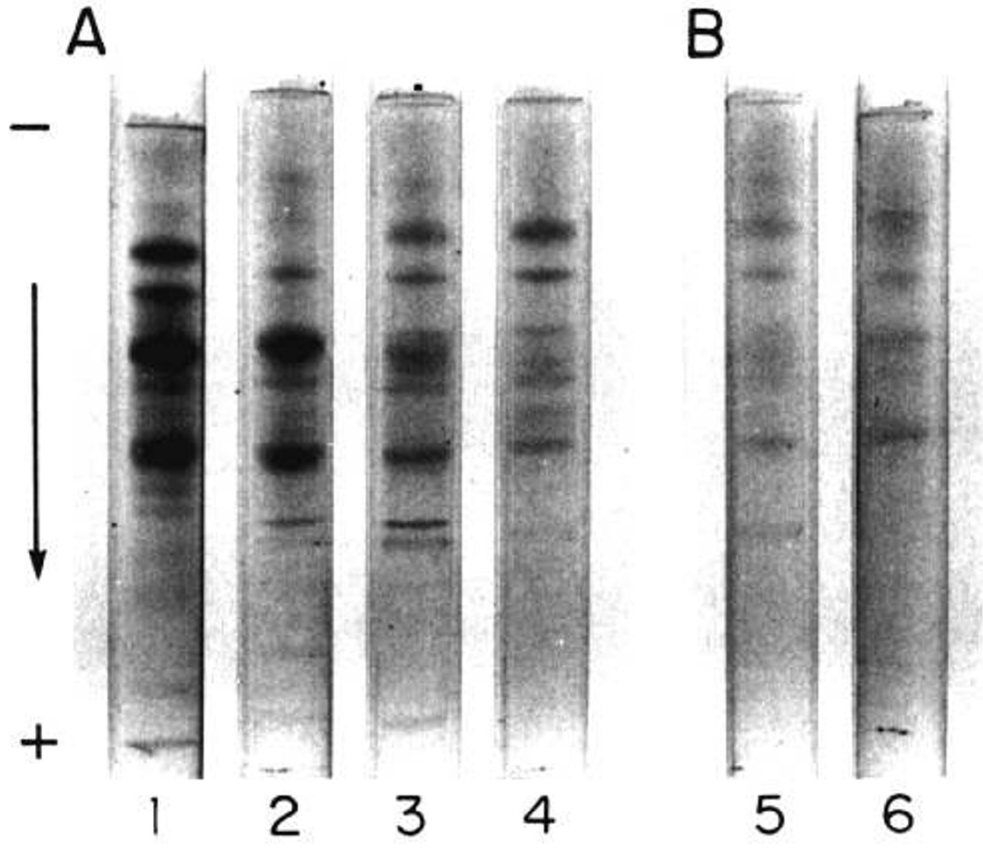


Fig. 5. Polyacrylamide disc gel electrophoresis. Gels stained for protein with Coomassie blue. *A*, pooled fractions of the 10,000 dalton proteins; *1*, *2*, normal fibroblasts; *3*, *4*, Menkes fibroblasts. Samples contained 50 μg protein. *B*, pooled fractions of the eluates of the ion-exchange column (fractions 15 to 30, Fig. 4); *5*, Menkes fibroblasts; *6*, normal fibroblasts. Samples contained 35 μg protein.

## DISCUSSION

The data presented indicate that the increased accumulation of copper observed in Menkes fibroblasts (1, 5, 11) is related to the presence in these cells of a greater amount of copper-binding protein(s) than in normal fibroblasts. This protein(s) had a molecular weight of approximately 10,000 daltons, incorporated large amounts of $^{35}$S-cysteine (see Fig. 2) and was deficient in aromatic amino acids (see Table 2). At least some of the $^{3}$H counts obtained could be attributed to the presence in the 10,000 dalton peak of other contaminating proteins. The 10,000 dalton protein-bound $^{64}$Cu peak always coincided with the $^{35}$S-cysteine-enriched protein peak (see Fig. 1). In addition, there was a direct relationship between the amount of $^{64}$Cu bound and the amount of $^{35}$S-cysteine incorporated by both the normal and the Menkes cells (see Fig. 3). These findings indicate that the cysteine-enriched 10,000 dalton protein(s) is the protein(s) responsible for binding copper in fibroblasts and exhibits the characteristic features of metallothionein, namely a high cysteine content, low levels of aromatic amino acids, and a high affinity for metals (14). In addition, the copper-binding protein(s) appears to be distinct from rat copper-chelatin, primarily on the basis of its behavior or anion-exchange chromatography. Winge *et al.* (19) showed that copper-chelatin isolated from rat liver was tenaciously adsorbed on DEAE-cellulose and was not eluted even at high salt concentrations. The copper-binding protein(s) isolated from fibroblasts, on the other hand, was readily eluted from the ion-exchange column at a salt concentration of approximately 30 mM.

Fibroblast lysates were labeled with $^{64}$Cu in the test tube, rather than by culturing the cells in $^{64}$Cu-supplemented medium. Studies in our laboratory have shown that addition of copper at concentrations as low as 2 μg/ml to the culture medium resulted in a reduction in the rate of DNA synthesis in Menkes fibroblasts, whereas under identical culturing conditions, the rate of DNA synthesis was increased in normal fibroblasts (2). Addition, therefore, of $^{64}$Cu to the culture medium would affect inversely the growth rate of normal and Menkes fibroblasts. Subsequently, this could affect the rate of metallothionein synthesis in the normal and mutant fibroblasts in different ways. It has been demonstrated that metallothionein synthesis can be induced by a number of metals, including copper (3, 4). Chan *et al.* (6) have reported that metallothionein synthesis is not induced by extracellular copper in Menkes and normal cultured skin fibroblasts. These studies were carried out in fibroblasts growing in the presence of $^{64}$Cu (5.5 μg Cu per ml). Although it has been found that this copper concentration does not cause morphologic abnormalities to the fibroblasts (2, 5), it has been demonstrated that even lower concentrations of copper in the culture medium affect the growth of the cells (2). In addition, the total $^{35}$S-cysteine incorporation was used as an indication of metallothionein synthesis (6). However, our studies showed that most of the $^{35}$S-cysteine in the normal cells and approximately half of it in the Menkes cells was incorporated into proteins with a molecular weight greater than 75,000 daltons (see Fig. 1). We have previously demonstrated that the large molecular weight species do not contain aggregates of the 10,000 dalton copper-binding protein (15). Therefore, the question as to whether or not metallothionein biosynthesis can be induced by the presence of copper in the culture medium remains unsettled.

The $^{35}$S-enriched 10,000 dalton protein peak always coincided with the $^{64}$Cu peak in both normal and Menkes cell lysates (see Fig. 1). Additional evidence of the specificity of the $^{35}$S-cysteine for labeling the copper-binding protein(s) is the finding that there was a direct relationship between the amount of $^{35}$S-cysteine incorporated and the amount of $^{64}$Cu bound to the 10,000 dalton protein(s) in both normal and mutant fibroblasts (see Fig. 3). Menkes fibroblasts consistently incorporated twice as much $^{35}$S-

cysteine into the 10,000 dalton protein(s) than normal fibroblasts (see Tables 1 and 2). This finding supports our previous observation that Menkes fibroblasts labeled in culture bound two to three times as much copper to these proteins than similarly labeled normal fibroblasts (1). The same two-fold difference in copper-binding by the 10,000 dalton protein(s) between Menkes and normal fibroblasts was observed when fibroblast lysates were labeled with $^{64}Cu$.

Although the copper-binding proteins were eluted in a single peak from the DEAE-cellulose column, several protein bands were resolved on acrylamide gels. No band showed a significant further increase in the $^{35}S$:$^{3}H$ isotopic ratio compared to the sample subjected to electrophoresis. Whether some or all of the multiple bands reflect heterogeneity of the copper metallothionein in fibroblasts or arise from oxidative changes in the copper protein (12, 18) remains unclear at the present time.

The observed differences in $^{35}S$-cysteine incorporation do not reflect different turnover rates of the metallothionein(s) in the two fibroblast strains. Under the experimental conditions used, the amount of radioactivity incorporated into the 10,000 dalton protein(s) in both Menkes and normal fibroblasts reached a constant value within 24 hr after the addition of $^{35}S$-cysteine (points up to 72 hr were examined; results not presented). This suggests that the turnover rate of the labeled proteins is short relative to the interval studied. Thus, the differences we observed most likely reflect vaying amounts of metallothionein in the two strains and not different rates of synthesis. It is also unlikely that our results are due to a different lability of the copper binding site in the metallothioneins. Studies on the kinetics of copper-binding, as well as on the nature and stability of the copper-metallothionein bond, have shown these to be very similar in Menkes and normal cells (15).

Our findings indicate that there is a greater amount of cysteine-rich copper-binding metallothionein in Menkes fibroblasts than in normal cells. It is not known at the present time whether this increased amount of metallothionein is the primary defect in Menkes disease or whether it is an event secondary to abnormal copper metabolism or copper transport.

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22. Grand Island Biological Co., Grand Island, NY.
23. Amersham Corp., Arlington Heights, IL.
24. New England Nuclear, Boston, MA.
25. Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, NJ.
26. Packard Instruments Corp., Downers Grove, IL.
27. Bicron Corp., Newbury, OH.
28. Whatman Ltd., Clifton, NJ.
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