The Role of Glutathione in Copper Metabolism and Toxicity*

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Cellular copper metabolism and the mechanism of resistance to copper toxicity were investigated using a wild type hepatoma cell line (HAC) and a copper-resistant cell line (HAC<sub>600</sub>) that accumulates copper and has a highly elevated level of metallothionein (MT). Of the enzymes involved in reactive oxygen metabolism, only glutathione peroxidase was elevated (3–4-fold) in resistant cells, suggestive of an increase in the cellular flux of hydrogen peroxide.

A majority of the cytoplasmic copper (>60%) was isolated from both cell lines as a GSH complex. Kinetic studies of <sup>64</sup>Cu uptake showed that GSH bound <sup>64</sup>Cu before the metal was complexed by MT. Depletion of cellular GSH with buthionine sulfoximine inhibited the incorporation of <sup>64</sup>Cu into MT by greater than 50%. These results support a model of copper metabolism in which the metal is complexed by GSH soon after entering the cell. The complexed metal is then transferred to MT where it is stored. This study also indicates that resistance to metal toxicity in copper-resistant hepatoma cells is due to increases in both cellular GSH and MT. Furthermore, it is suggested that elevated levels of GSH peroxidase allows cells to more efficiently accommodate an increased cellular hydrogen peroxide flux that may occur as a consequence of elevated levels of cytoplasmic copper.

Metallothioneins, low molecular weight, cysteine-rich metal-binding proteins, have a central role in hepatic copper metabolism, providing temporary storage for cytoplasmic copper than the nonresistant parental cell line and also contain elevated levels of MT. A copper-glutathione complex, which represented the majority of cytoplasmic copper, could be isolated from both resistant and nonresistant cell lines by anaerobic fractionation of cell lysates. Furthermore copper uptake and incorporation of metal into MT, in vivo, is strongly influenced by GSH levels. The results of these studies support a model of copper metabolism in which the metal is rapidly complexed by GSH after entering the cell and is then transferred to MT. Toxic oxygen species, such as hydrogen peroxide, that may be formed from the oxidation of the GS-Cu complex or Cu-MT (22, 23) are efficiently removed by GSH peroxidase. It is proposed that resistance to copper toxicity is equilibrium with a free copper pool (6). However, free copper would likely be toxic to cells.

Several theories have been proposed to explain the mechanism of cellular copper toxicity. It has been proposed that free copper binds to protein cysteinyl thiol groups thereby inactivating essential enzymes. If oxygen is present, the metal–thiolate bond may be oxidized, irreversibly inactivating enzymes by forming cystine cross-links (7). It has also been suggested that copper interacts with physiologically produced reduced oxygen species, such as superoxide anion and hydrogen peroxide, catalyzing the formation of highly toxic hydroxyl radicals via a Fenton-like reaction (8, 9). Hydroxyl radicals can then go on to inactivate essential enzymes and/or carry out lipid peroxidation reactions, thereby disrupting membranes and organelles (10).

The production of hydroxyl radicals, associated with the cellular accumulation of copper, may occur soon after the metal enters the cell. Although MT is an efficient copper chelator, the accumulation of MT to a level sufficient to inhibit metal toxicity requires several hours after an initial exposure to metal (11–13). Thus, toxic effects of metals may develop and cells become irreparably damaged before MT can be protective.

It has been proposed that GSH is capable of chelating and detoxifying metals soon after they enter the cell (14–18). This proposal is based on observations that the depletion of GSH potentiates metal toxicity in rats (15), mice (17), and cultured cells (14, 16, 18). The importance of GSH in metal detoxification is also supported by its role in the removal of toxic oxygen species (8, 9). Glutathione is also a substrate for GSH peroxidases, enzymes capable of both removing hydrogen peroxide from the cells and repairing peroxidatively damaged membranes (19).

Since GSH may function as an intracellular metal chelator, as well as a substrate for the removal of toxic oxygen species, the relationship between copper, GSH, and Cu-MT was examined using rat liver hepatoma cell lines resistant to the toxic effects of copper (20). These cells accumulate more copper than the nonresistant parental cell line and also contain elevated levels of MT. A copper-glutathione complex, which represented the majority of cytoplasmic copper, could be isolated from both resistant and nonresistant cell lines by anaerobic fractionation of cell lysates. Furthermore copper uptake and incorporation of metal into MT, in vivo, is strongly influenced by GSH levels. The results of these studies support a model of copper metabolism in which the metal is rapidly complexed by GSH after entering the cell and is then transferred to MT. Toxic oxygen species, such as hydrogen peroxide, that may be formed from the oxidation of the GS-Cu complex or Cu-MT (22, 23) are efficiently removed by GSH peroxidase. It is proposed that resistance to copper toxicity is
a consequence of increases in GSH, GSH peroxidase, and MT levels.

**EXPERIMENTAL PROCEDURES**

**Materials**—All media and media supplements were obtained from Grand Island Biological Co. Rabbit MT-L, xanthine, xanthine oxidase, cytochrome c (type VI), cycloheximide, hydrogen peroxide, and BSO were from Sigma. Cumene hydroperoxide, GSH, GSSG, polyethylene glycol 400, 5,5'-dithiobis(2-nitrobenzoic acid), DEM, and 1-chloro-2,4-dinitrobenzene were obtained from Aldrich. GSSG reductase and NAPD were purchased from Boehringer Mannheim. Imidazole and HEPES, Ul tro grade, were from Mallinckrodt Chemical Works and Calbiochem, respectively. [35S]Cysteine (specific activity >1200 Ci/m mol) and L-[U-14C]glutamic acid (specific activity >250 mCi/m mol) were obtained from Amersham Corp. Carrier-free (67Cu) perchloric chloride, in 3 N HCl, was prepared at Los Alamos National Laboratory as described previously (6). At the time of shipment the stock solution contained 0.4 μmol [67Cu] with a specific activity of 150 Ci/μmol.

**Cells and Tissue Culture**—The resistant cell line, HACoom, grown in medium supplemented with 600 μmol copper, and the wild type cell line, HAC, were cultured as described previously (20). A second resistant line HACNM was developed by growing HACoom cells in the absence of added copper for a minimum of 3 months. These cells had previously been shown to maintain resistance to copper toxicity and to produce MT at levels similar to those in the resistant parent, even when grown in the absence of added metal for longer than 1 year (20). The amount of cytoplasmic copper in HACoom and HACNM cells, however, decreased to a level similar to that of wild type cells within 2 weeks after removal from the copper-enriched medium (20). All cell lines were maintained in RPMI 1640 in 10.0 mM HEPES buffer, pH 7.4, supplemented with 10% FBS, nonessential amino acids, 2.0 mM glutamine, penicillin G (10 units/ml), and streptomycin (10 μg/ml), unless otherwise noted.

**Glutathione Assay**—Glutathione was measured in cells grown for 72 ± 4 h. Samples were prepared by washing 4 × 10^6 cells (W × 10^6 cells) three times with PBS (20). Cells were then removed with a rubber policeman and suspended in 10 ml of fresh RPMI 1640. Cell number was determined using a Coulter counter. The remaining cell suspension was centrifuged at 1,000 X g for 10 min and the PBS-washed cell pellet resuspended in 5 ml of 0.05% Polyethylene glycol 400 was added to PBS-washed cells in fresh medium and then incubated for 8 and 3 h, respectively (33, 34). PBS-washed cells were prepared by removing the old medium and then twice rinsing the cells with sterile 37 °C PBS and adding fresh RPMI 1640 containing 1% FBS. To inhibit protein export of PBS-washed cells were incubated with cycloheximide (4.8 μg/ml) for 3 h (35).

Following depletion of GSH or inhibition of protein synthesis, 4 × 10^6 cells of 67Cu (~0.3 μmol), dissolved in serum-free RPMI 1640, pH 7.4, were added to each well containing cells. These were then incubated at 37 °C for 24 h. At the end of the incubation, the cell medium was removed, cells were washed three times with ice-cold PBS, containing 1.0 mM EDTA, and then lysed by adding 200 μl of 1.0 N sodium hydroxide and incubating for 3 h at room temperature. The 67Cu content was determined by measuring the radioactivity in a 100-μl aliquot of the lysate with an LKB Labgamma counter.

**Intracellular Distribution of Copper**—The kinetics of intracellular distribution of copper was analyzed by fitting copper concentrations in subcellular pools to the areas under the respective peaks relative to the total integrated areas under peaks in the chromatogram from the normalized elution profile. The chromatographic data were normalized to account for differences in the amounts of 67Cu and protein applied to the column. The elution volume for superoxide dismutase was determined by chromatographing unlabeled cell lysates or purified superoxide dismutase and analyzing aliquots from each fraction for enzyme activity. The elution volumes for Cu-MT and GS-Cu were identified as described. The percent of 67Cu coeluting with these components was determined from the areas under the respective peaks relative to the total integrated area of the elution profile. The kinetics of copper incorporation into subcellular pools was analyzed by fitting copper concentrations versus time to a pseudo-first order rate equation by an iterated least squares analysis procedure described previously (20). The effect of inhibiting protein synthesis or decreasing the cellular
GSH concentration on copper incorporation into MT was examined. Cells were grown on 35-mm plates as described above. Protein synthesis was inhibited by preincubating cells with cycloheximide (5 μg/ml) for 3 h, or GSH was depleted by preincubating with BSO (5 mM) for 8 h (1.0 mm), prior to Cu addition. Radiolabeled copper (8 × 10^3 cpm) was added to each plate and the cells incubated for 90 min. At the end of this incubation, cell lysates were prepared and the proteins fractionated as described above. As controls, the concentration of GSH was measured after treating cells with BSO or cycloheximide. The levels of protein synthesis in cells treated with cycloheximide or BSO, as described above, were determined by labeling cells for 90 min with [35S]cysteine and measuring the amount of radiolabel incorporated into trichloroacetic acid-precipitable material.

**RESULTS**

**Enzyme Activities of Oxygen-metabolizing Enzymes**—The relative activities of superoxide dismutase, catalase, and GSH peroxidase were compared between wild type and resistant cells to ascertain whether elevated copper levels in resistant cells caused an increased flux of toxic oxygen species (36, 37). Results presented in Table I show that there was no significant difference in superoxide dismutase or catalase activities between the three cell lines. Glutathione peroxidase activity, with hydrogen peroxide as substrate, however, was increased in the resistant cell lines, suggesting an increase in hydrogen peroxide flux.

**Glutathione Content in Wild Type and Resistant Cells**—The content of GSH in wild type and resistant cells was determined. Copper-resistant cells contained higher levels of GSH equivalents (GSH and GSSG) than nonresistant cells (Table II). In addition, HACcooNM cells grown in the absence of added copper for 3–6 months contained more GSH than HACcoo cells maintained in a copper-enriched medium.

Changes in the level of GSH equivalents in the resistant cell lines may be due to a defect in the reduction of GSSG, a change in the conversion of GSH into covalent bound derivatives, an increase in GSH synthesis, or a decrease in GSSG efflux (34). To assess which of these activities may be responsible for the changes observed, the activities of GSSG-reductase, GSH-S-transferase, γ-glutamylcysteine synthetase, and the level of GSSG efflux from cells were determined. No significant differences in enzyme activities between the three cell lines were observed (Table II). However, there was three to seven times less glutathione in the medium in which resistant cells were maintained, compared with wild type cells. These results suggest that the elevated level of glutathione found in resistant cells is due to a decrease in glutathione efflux.

**Identification of Copper- and Glutathione-dependent Enzymes**

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HAC</th>
<th>HACcoo</th>
<th>HACcooNM</th>
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</thead>
<tbody>
<tr>
<td>Superoxide dismutase (n=8)</td>
<td>28.2</td>
<td>23.3</td>
<td>31.2</td>
</tr>
<tr>
<td>Catalase (n=3) (nmol/min/mg protein)</td>
<td>0.32</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>GSH peroxidase (n=8)</td>
<td>41.2</td>
<td>35.0</td>
<td>41.5</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>41.2</td>
<td>35.0</td>
<td>41.5</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>3.9</td>
<td>6.8</td>
<td>16.1</td>
</tr>
</tbody>
</table>

* Significantly different, p < 0.05, from wild type cells.

**TABLE II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HAC</th>
<th>HACcoo</th>
<th>HACcooNM</th>
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<tr>
<td>Glutathione content and activities of glutathione metabolizing enzymes in hepatoma cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione levels and enzyme activities for wild type (HAC) and resistant cells maintained in copper-enriched medium (HACcoo) or nonenriched medium (HACcooNM) were determined as described under &quot;Experimental Procedures.&quot; The values are means ± S.E.</td>
<td></td>
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<table>
<thead>
<tr>
<th>Cell line</th>
<th>HAC</th>
<th>HACcoo</th>
<th>HACcooNM</th>
</tr>
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<tbody>
<tr>
<td>Cellular glutathione (n=8)</td>
<td>8.1±1.7</td>
<td>21.7±9.1</td>
<td>37.6±10.1*</td>
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<tr>
<td>Media glutathione (n=2)</td>
<td>0.49±0.09</td>
<td>0.16±0.03</td>
<td>0.04±0.02*</td>
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<tr>
<td>γ-Glutamylcysteine synthetase (n=6)</td>
<td>3.6±0.7</td>
<td>4.1±0.8</td>
<td>4.3±0.8</td>
</tr>
<tr>
<td>GSH-S-transferase (n=6)</td>
<td>0.38±0.13</td>
<td>0.36±0.11</td>
<td>0.40±0.06</td>
</tr>
<tr>
<td>GSSG reductase (n=6)</td>
<td>17.9±1.7</td>
<td>14.9±5.0</td>
<td>20.4±4.8</td>
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</table>

* Significantly different, p < 0.05, from wild type cells.

**FIG. 1.** Subcellular distribution of glutathione and copper in wild type and copper-resistant cells. HAC (upper panel) and HACcoo (lower panel) cell lysates were prepared in 10.0 mM HEPES buffer, pH 7.4, from ~2 × 10^7 cells as described previously. Aliquots (0.5 ml) were fractionated by gel filtration fast protein liquid chromatography (FPLC) using a Superose 12 column, equilibrated with anaerobic 10.0 mM HEPES, 0.15 M NaCl buffer, pH 7.4. Proteins were eluted at a flow rate of 1.0 ml/min. Fractions (1.0 ml) were collected and then assayed for copper (●) or glutathione (▲). Copper was measured by graphite furnace atomic absorption spectroscopy from 50-μl aliquots diluted to 1.0 ml with distilled-deionized water. Glutathione was determined from 0.5-ml aliquots treated with sodium metaperphosphoric acid and then assayed using the GSSG-reductase system as described under "Experimental Procedures."
cell lysates, the elution volumes for GSH, GSSG, and GS-Cu (GSH:Cu, 5:1) were determined. Reduced glutathione and GSSG elute with $M_0$ values of 2200 and 3100, respectively. GS-Cu, however, elutes with an $M_0$ value of 4500. These results indicate that the 4.5-kDa material, identified in cell lysates from both wild type and resistant cells, that contained both copper and GSH is a GS-Cu complex.

The stoichiometric ratio of GSH to copper in the GS-Cu complex was determined from the integrated areas of the peaks in the chromatogram. For HAC lysates, the GSH:Cu ratio was 1:1, whereas for HAC$_{100}$ lysates the ratio was 2:1, assuming that the GSH coeluting with copper (76-85 ml) was complexed to metal.

When Cu-MT-containing fractions from the gel filtration chromatography of HAC$_{100}$ cell lysates were pooled, concentrated, then fractionated by anion exchange chromatography, three copper-containing proteins were isolated (Fig. 2). The first two proteins, eluting at salt concentrations of 75 and 119 mM, were previously identified as hepatoma Cu-MT-I and Cu-MT-II. The protein eluting at the highest salt concentration, 640 mM, was also shown to be a Cu-MT, based on its copper content (Fig. 2) and its cross-reactivity with anti-rat liver MT antibody (results not shown). Glutathione also coeluted with this last protein, as determined by its activity with GSSG-reductase for which purified Cu-MT, apo-MT, and rabbit Cd/Zn-MT are inactive as substrates. Based on the presence of GSH in the Cu-MT-containing fractions that eluted at high salt concentration, it is proposed that GSH forms a complex with this protein.

**Effect of GSH Depletion and the Inhibition of Protein Synthesis on Copper Uptake**—The effects of DEM, BSO, and cycloheximide on protein synthesis and GSH content in HAC and HAC$_{100}$ cells were examined (Table III). Cycloheximide inhibited protein synthesis by greater than 90%, relative to untreated cells, in both cell lines. It also caused significant increases in the level of cellular GSH. Treatment with BSO or DEM reduced the GSH content in both cell lines by 85%. An increase in [S]cysteine incorporation into proteins was also observed in BSO-treated cells. Treatment with BSO, DEM, or cycloheximide did not affect cell viability within the time period of the experiment, as determined by trypan blue viable staining (20).

Copper content after 30- and 90-min incubations was measured in cells following GSH depletion with BSO or DEM or after inhibition of protein synthesis with cycloheximide (Table IV). No significant differences in $^{67}$Cu uptake between treated and untreated cells in either cell line were observed after 30-min incubations. At longer incubation times, BSO, DEM, and cycloheximide pretreatment inhibited $^{67}$Cu uptake into HAC cells, while $^{67}$Cu uptake decreased only when protein synthesis was inhibited in HAC$_{100}$ cells.

### Table III

<table>
<thead>
<tr>
<th>Cell line and treatment</th>
<th>Glutathione content</th>
<th>[S]Cysteine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAC</td>
<td>None</td>
<td>19.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>36.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>BSO</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>DEM</td>
<td>2.3 ± 0.2</td>
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<tr>
<td>HAC$_{100}$</td>
<td>None</td>
<td>83.0 ± 1.0</td>
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<tr>
<td></td>
<td>Cycloheximide</td>
<td>35.0 ± 1.2</td>
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<tr>
<td></td>
<td>BSO</td>
<td>12.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>DEM</td>
<td>12.5 ± 0.7</td>
</tr>
</tbody>
</table>

* ND, not determined.

### Table IV

<table>
<thead>
<tr>
<th>Cell line and treatment</th>
<th>Copper content</th>
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<tbody>
<tr>
<td></td>
<td>30 min*</td>
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<tr>
<td>HAC</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>91 ± 6</td>
</tr>
<tr>
<td>BSO</td>
<td>108 ± 12</td>
</tr>
<tr>
<td>DEM</td>
<td>116 ± 13</td>
</tr>
<tr>
<td>HAC$_{100}$</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>BSO</td>
<td>119 ± 14</td>
</tr>
<tr>
<td>DEM</td>
<td>112 ± 7</td>
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</table>

* Incubation time.
and coeluted with MT (68–78 ml, peak M_r = 13,000) and GSH (78–89 ml, peak M_r = 4,500) (Fig. 3). The distribution of 67Cu in wild type and resistant cell lysates, however, was considerably different. In HAC cell lysates, 20% of the total radioactivity eluted in the void volume, with 42 and 5% coeluting with MT and GSH, respectively. In HAC_600 cell lysates, 1.4% of the 67Cu eluted in the void volume and 1.8% with GSH, while 82% coeluted with MT. 67Cu-Labeled superoxide dismutase was also identified in HAC_600 cells lysates, eluting between 58 and 62 ml (peak M_r = 32,000) and containing less than 1% of the total eluted 67Cu.

Depleting cells of GSH by treatment with BSO caused the level of copper in MT-containing fractions to decrease by 74% in HAC cells and by 48% in HAC_600 cells, as compared with untreated cells (Fig. 3). This decrease suggests that GSH may function in intracellular copper transport.

The inhibition of protein synthesis in HAC cells reduced the amount of 67Cu observed in MT fractions by 55%, compared with untreated cells (Fig. 3). In contrast, cycloheximide had no effect on the level of copper in MT fractions of HAC_600 cells. An increase in GS-65Cu in the elution profiles of lysates from both cycloheximide-treated cell lines was observed. This change reflects the increase in GSH levels seen when protein synthesis was inhibited (Table III).

Kinetics of Copper Distribution into Subcellular Pools—The time course for the subcellular distribution and complexation of 67Cu by GSH, MT, and superoxide dismutase was determined by fractionating extracts of HAC and HAC_600 cells that had been incubated with 67Cu for different periods of time. Radiolabeled copper was observed at elution volumes corresponding to superoxide dismutase, MT, and GSH in both cell lines.

In HAC cell lysates, 67Cu was observed only as the GS-Cu complex at incubation times less than 1 h (Fig. 4). At incubation times greater than 1 h, radiolabeled copper began to appear in the MT-containing fractions and increased until a steady-state level was reached in which 65% of the total 67Cu applied to the column was bound to MT and 10% was complexed to GSH. These results suggest that after copper enters the cell it is quickly complexed by GSH. The data also implies that copper may be transferred from GSH to MT.

In HAC_600 cells, 85% of the 67Cu was complexed by MT at the earliest incubation time examined (Fig. 5). As the cellular concentration of 67Cu increased, the amount of 67Cu bound to MT increased proportionally, remaining as 85 ± 2% of the total radiolabeled copper present (Fig. 5). In HAC_600 cell lysates less than 2% of the total radiolabel present appeared in the metal is taken up by the resistant cells, it is quickly complexed by GSH. The data also implies that copper may be transferred from GSH to MT.

The rates of uptake and incorporation into Cu-MT was determined. Copper uptake reached steady-state in the HAC_600 cells with a t_1/2 of 0.27 h, while the t_1/2 for 67Cu incorporation into MT was 0.28 h. These values suggest that once the metal is taken up by the resistant cells, it is quickly complexed to MT. Steady-state in either 67Cu uptake or incorporation into MT was not reached in wild type cells during the course of the experiment.
resistant hepatoma cells, however, show a significant increase in total glutathione levels (Table II). A similar increase was previously observed in rats (15) and cadmium/zinc-resistant ovarian carcinoma cells (14) exposed to metal, and in adriamycin-resistant cells (36, 37). It has been suggested that increased GSH peroxidase activity may cause cells to develop higher steady-state levels of GSH (36, 37). This may also, in part, be responsible for elevated GSH levels in copper-resistant hepatoma cells. Copper-resistant cells accumulate glutathione through a decrease in glutathione efflux and not by an increase in GSH synthesis (Table II). In contrast, elevated GSH levels in kidneys from rats exposed to mercury and zinc were caused by increases in GSH synthesis (15).

Copper Metabolism—When HAC cells are depleted of GSH by treatment with either BSO or DEM, 67Cu uptake is inhibited (Table IV), suggesting that GSH is involved in metal uptake (Table III). GSH depletion in HACoo cells, however, has no effect on 67Cu uptake. In these cells, sufficient GSH may remain after BSO or DEM treatment to still function in copper uptake. In lung carcinoma cells it was shown that cadmium uptake was unaffected by BSO treatment (41). In those cells, however, cadmium uptake was inhibited by DEM. Since DEM is a nonspecific thiol-blocking agent, the authors (41) suggested that a thiol-containing protein, and not GSH, was directly involved in metal transport.

Inhibition of protein synthesis in wild type and resistant hepatoma cells causes a decrease of 67Cu uptake (Table IV). In contrast, it was reported that rat hepatocytes and HepG2 cells treated with cycloheximide accumulated more copper than did nontreated cells (5, 42). This accumulation was attributed to a decrease in the synthesis of proteins responsible for copper transport into bile. Since rat hepatoma cell lines in culture lack the bile export system, the amount of metal accumulated is not influenced by this form of copper efflux. The results with hepatoma cell lines suggest that both protein synthesis and GSH are necessary for copper intake.

In addition to serving as a substrate for GSH peroxidase, GSH is shown to function as an intracellular copper chelator. Germann and Lerch (43) hypothesized that GSH may be a copper chelator based on the isolation of GSSG from Neurospora crassa cell lysates and the presence of copper in the breakthrough fraction during the purification of Cu-MT by reverse-phase high pressure liquid chromatography. In the mechanism describing cellular copper metabolism presented in Fig. 6, it is proposed that either Cu(I) is taken up by cells or Cu(II) taken up is quickly reduced to Cu(I). The copper ion is then chelated by GSH forming a GCu-I complex. GCu-I and (GS)n-Cu-I complexes have been tentatively identified in HAC and HACoo cell lysates.

The difficulty in isolating GS-Cu arises from the aerobic lability of copper-thiol complexes (22, 44) and the ability of thiol-containing reducing agents and copper-chelating buffers to dissociate these complexes. Purifications of Cu-MT from copper-loaded cells are commonly performed in Tris or phosphate buffers, both of which can chelate copper (log K values for Cu(II) with Tris and phosphate are 4.0 and 4.7, respectively) (45). Thiol-reducing agents, such as 2-mercaptoethanol or dithiothreitol, are frequently added to the buffers (46, 47). Under these purification conditions, any GCu complex present in cell lysates would likely dissociate. By chromatographing hepatoma cell extracts under anaerobic conditions, using nonchelating buffers and without thiol-reducing agents, an intact GCu complex is isolated. If, however, Tris buffer is substituted for HEPES buffer during gel filtration chromatography, or dithiothreitol is added, the GCu complex is not detected (results not shown).
When wild type cells are incubated with $^{67}$Cu for 30 and 60 min, the majority of the radiolabel is complexed to GSH, and $^{67}$Cu-labeled MT is not detected (Fig. 4). After longer incubation times, however, there is a decrease in the levels of GS-$^{67}$Cu, with a concomitant increase in $^{67}$Cu-labeled MT. These results suggest that copper is transferred from GSH to MT. It should be noted that the concentration of $^{67}$Cu used in these experiments is 1000 times lower than the cellular glutathione level. Therefore $^{67}$Cu is not saturating the GSH pool prior to its complexation by MT. Alternatively, the late appearance of $^{67}$Cu-labeled MT could reflect the time required to accumulate MT after induction by copper. This is unlikely because the concentration of $^{67}$Cu in the uptake medium is four times lower than the amount of copper normally present in HAC growth medium and would not induce de novo MT synthesis.

Depleting HAC and HAC<sub>res</sub> cells of GSH causes a decrease of $^{67}$Cu incorporation into MT (Fig. 3). These results further support the involvement of GSH in copper complexation by MT. The difference in the magnitude of the effect of GSH depletion on $^{67}$Cu incorporation into MT between HAC and HAC<sub>res</sub> cells (74% versus 48%, respectively) may be due to the higher concentration of GSH remaining in the resistant cells following BSO treatment (Table III). Although BSO treatment depletes 85% of the GSH in HAC<sub>res</sub> cells, relative to untreated HAC<sub>res</sub> cells, the amount of GSH remaining is only 40% lower than that in untreated HAC cells.

It has been reported that copper incorporation into MT decreased in cycloheximide-treated rat hepatocytes and HepG2 cells, suggesting that copper is complexed concomitantly with MT synthesis (5, 42, 48). A similar result is obtained with HAC cells; however, there is only a 55% decrease in $^{67}$Cu incorporation into MT (Fig. 3), whereas protein synthesis is inhibited 90% (Table III). Since the half-life of MT in HAC cells is greater than 20 h, the decrease in $^{67}$Cu incorporation is not due to a loss of MT by protein degradation. Inhibiting protein synthesis in HAC<sub>res</sub> cells by 96% has no effect on $^{67}$Cu incorporation into MT. This lack of effect may be due to the higher steady-state concentration of MT maintained in the resistant cells, relative to HAC cells, even when de novo MT synthesis is inhibited. These results indicate that copper is complexed by previously synthesized MT. It is also suggested that MT can chelate copper concomitantly with protein synthesis, because cycloheximide-treated HAC cells show a significant decrease in the level of $^{67}$Cu-labeled MT (Fig. 3).

Based on the appearance of GS-$^{67}$Cu prior to the formation of $^{67}$Cu-labeled MT, the reduction of $^{67}$Cu incorporation into MT when GSH is depleted, and that the inhibition of protein synthesis in HAC<sub>res</sub> cells does not affect $^{67}$Cu complexation by MT, it is proposed that copper is transferred from GSH to MT, possibly via a GS-Cu-MT intermediate (Fig. 6). Glutathione may also function to reduce Cu(I) to Cu(I), preventing the oxidation of the cysteine residues in MT. This would allow all MT cysteine residues to be used exclusively for metal chelation, as described previously (1). Glutathione-bound copper may bind to vacant sites in the protein and/or exchange with MT-bound copper. Other models of cellular copper metabolism proposed the presence of a "free copper" pool, from which MT complexed metal (6). In the present model (Fig. 6) free copper levels are minimal, with the metal largely being complexed to thiol-containing compounds.

It is also proposed that there is an equilibrium between GS-Cu and Cu-MT (Fig. 6). The detection of low levels of GS-$^{67}$Cu in HAC<sub>res</sub> cell lysates (<2%) may be due to the high concentration of MT, which shifts the equilibrium such that most of the metal is bound to the protein. Steady-state levels of $^{67}$Cu-labeled MT and GS-$^{67}$Cu are reached in HAC cells in greater than 6 h. In contrast, steady-state levels in HAC<sub>res</sub> cells are reached in less than 30 min. Again, this difference may be related to the higher concentration of MT in the resistant cells.

The model for copper incorporation into MT, via a GS-Cu complex (Fig. 6), also suggests that GSH is able to remove copper from MT. It was shown by Bremner and co-workers (49) that the half-life for Cu-MT, in vivo, is 12-17 h. In contrast, Cu-MT in vitro was resistant to degradation by

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*J. H. Freedman, unpublished observation.
lysosomal enzymes, unless some of the metal was removed from the protein (50, 51). The authors suggested that an endogenous copper chelator might remove metal from Cu-MT in the cell before lysosomal enzymes can degrade the protein (52). Glutathione may function as such a chelator.

Mechanism of Resistance—Resistance to copper toxicity in hepatoma cell lines is believed to be due to elevated levels of GSH, MT, and GSH peroxidase, relative to nonresistant cells (Tables I and II). High concentrations of GSH allow resistant cells to efficiently remove potentially toxic, free cytoplasmic copper by quickly complexing the metal. The elevated MT concentration increases the metal-binding capacity of the cell and may increase the rate of copper transfer from GS-Cu. Finally, increased GSH peroxidase activity inhibits the production of hydroxyl radical by metabolizing hydrogen peroxide. Thus, resistance to copper toxicity has developed by an amplification of the cells' ability to both sequester copper and to prevent oxidative cellular damage.

Although resistance to copper toxicity in hepatoma cells was induced by adding a single agent, copper, to the growth medium (20), the levels of three different components, GSH, MT, and GSH peroxidase, are amplified. The elevated levels of these components are related to increased levels of cellular copper. The amplification of MT genes in response to metal exposure is well documented (21). An increase in cytoplasmic copper can stimulate hydrogen peroxide production which could lead to an increase in GSH peroxidase. Cellular levels of GSH increase in resistant cells because it is not removed from the cell (Table II). It is possible that complexation of GSH as GS-Cu or Cu-GS-MT inhibits its efflux.

REFERENCES