PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 486–494 (1983)

Differential Regulation of Copper and Zinc Metabolism in Rat Liver Parenchymal Cells in Primary Cultures[1] (41675)

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Abstract. A variety of factors influence copper and zinc metabolism in primary cultures of rat hepatocytes. Pronounced elevations in accumulated ^{64}Cu were produced by incubating cells with either Cu^{2+} or epinephrine with moderate increases produced by incubation with cycloheximide, actinomycin D, or dexamethasone. In addition, copper accumulation was enhanced when cells received culture medium more highly enriched in amino acids and other nutrients. Hepatocytes accumulate greater quantities of ^{65}Zn when incubated with dexamethasone or high extracellular zinc. Higher levels of metallothionein were found in cells incubated with either dexamethasone, zinc, or copper but not with epinephrine. However, epinephrine was effective in elevating ceruloplasmin levels in the cell cytosol. Incubation with dexamethasone or copper-containing medium resulted in elevated ^{3}H-ceruloplasmin in both cytosol and medium. Data presented suggest hormones influence copper and zinc metabolism in liver parenchymal cells in a differential fashion.

Much attention has focused on the role played by the hepatic metal-binding protein metallothionein. In particular, its involvement in the metabolism of zinc (1–4) and copper (5–7) has been investigated. Despite reports that metallothionein provides a common focal point for zinc and copper metabolism and serves as a common binding site (8, 9) regulation of liver copper metabolism may be a distinct and distinguishable process from the process(es) that regulate(s) liver zinc metabolism (10, 11). These differences include: (a) the accumulation characteristics by whole liver or isolated liver cells which differ substantially (10, 12), (b) the difference in half-life, chromatographic behavior, and physical properties of copper-metallothionein compared to zinc-metallothionein (6, 13–15), and (c) under physiological conditions one metal does not seem to affect the cellular accumulation of the other (12).

In this study, we present data on the differential response between copper and zinc metabolism by isolated liver parenchymal cells incubated with copper, zinc, dexamethasone, and epinephrine.

Materials and Methods. *Animals.* Male CD strain rats (Charles River Breeding Laboratory, Wilmington, Mass.) were housed in stainless-steel cages on a 12-hr light/12-hr dark cycle. Commercial diet (Ralston Purina, St. Louis, Mo.) and tap water were fed *ad libitum.* Rats were used for preparation of hepatocytes when they weighed between 200 and 300 g.

Isolation and culture of rat liver parenchymal cells (hepatocytes). Procedures for isolation and monolayer culture of rat hepatocytes have been presented in detail previously (16). Briefly, the peritoneal cavity was opened under pentobarbitol anesthesia (60 mg/kg) and the portal vein and inferior vena cava (caudal to the renal vein) were loosely ligated. A cannula was inserted into the portal vein and secured prior to closing off the vena cava ligature. A slit was made distal to the ligature to allow efflux of perfusate. A series of three buffers were perfused via the cannulation into the liver at 30–40 ml/min. These 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-buffered solutions sequentially contained (i) ethyleneglycol-bis(β-aminoethyl

[1] This work was supported by Grant AM 31651 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases and the New Jersey Agricultural Experiment Station.

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0037-9727/83 $1.50

ether) *N,N'*-tetraacetic acid (EGTA), to remove matrix calcium, (ii) buffer with no additions to wash out residual EGTA, and (iii) collagenase (CLS II, Worthington Biochemical, Freehold, N.J.) for breakdown of intercellular collagen. The liver was then placed in M199 culture medium. Liver cells were dispersed, washed several times by centrifugation at low speed (50*g*) and placed in culture dishes containing 2 ml of M199/10% fetal bovine serum at a concentration of 2×10^6 cells per dish. Parenchymal cells were allowed to selectively attach to the dishes for 1 hr (17) before changing to the desired serum-free incubation medium. Leibovitz L-15 medium was used in all experiments except a study conducted to test the effect of medium formulation on copper accumulation, where Hams F-12, minimal essential medium (MEM), and HWB (modified Waymouth 752/1) were also used. All cultures contained albumin (2 mg/ml), gentamycin (50 μg/ml), glucose (1.5 mg/ml), insulin (1 μg/ml), penicillin (60 μg/ml), sodium bicarbonate (5 m*M*), and streptomycin (100 μg/ml). In experiments requiring labeling of ceruloplasmin with ^{3}H-labeled amino acids, a special L15 medium deficient in leucine, glutamic acid, aspartic acid, glycine, and threonine was utilized. All tissue culture media was supplied by Gibco (Grand Island, N.Y.).

Mineral accumulation in cultured liver cells. Following selective attachment of the parenchymal cells, plates received 4 ml of culture medium containing either 0.5 μCi ^{64}Cu or 0.1 μCi ^{65}Zn (New England Nuclear, Boston, Mass.). Total Cu and Zn concentrations of culture media ranged between 0.1 and 0.7 μg/ml (as determined by atomic absorption spectrophotometry). After maintaining the cells for various times at 37°C in a CO_2-regulated incubator, the medium was removed and the cells were washed 3 times with 10 m*M* Hepes-buffered saline, pH 7.4, containing 10 m*M* ethylenediaminetetraacetate (EDTA) to remove nonspecifically bound metal. Then 1.5 ml of 0.5% deoxycholate was added and the cells were mechanically scraped off the dish surface for measurement of radioactivity by gamma counting (Beckman Gamma 4000 spectrometer). For each time point a replicate set of dishes lacking the isotope was harvested and prepared for protein determination by the microbiuret procedure (18). Each reported value represents a minimum of three replicate plates per experiment which were done in duplicate. For long-term uptake studies, cells received fresh medium containing 0.5 μCi ^{64}Cu and 18 μ*M* copper as $CuSO_4$ at 0, 24, and 48 hr of culture. Concentrations of other additions used are as follows: dexamethasone, 10^{-7} *M;* epinephrine, 10^{-5} *M;* actinomycin D, 10^{-6} *M;* cycloheximide, 0.02 m*M*. Nanomoles of copper or zinc accumulated were calculated from the amount of total ^{64}Cu or ^{65}Zn accumulated and the concentration of zinc or copper in the medium.

Synthesis and isolation of metallothionein. At various times after incubation of cells with either 1.0 μCi ^{64}Cu, 0.1 μCi ^{65}Zn, 1 μCi ^{3}H-(glycine, lysine, serine), or 0.5 μCi [^{35}S]cystine (New England Nuclear) and specified hormones or metals, cultures were washed twice in Hepes-buffered Hanks' salt solution, pH 7.4, and harvested into either 2 or 3 ml of 0.25 *M* sucrose–10 m*M* Tris–acetate (pH 8.6). The cytosol fraction was prepared by homogenization of cells in a Potter–Elvehjem type apparatus and centrifugation of the homogenate at 106,000*g* (average) for 1 hr. For chromatography either 1.5 or 2.5 ml of cytosol was applied to columns (1.5 × 30 cm or 2.6 × 50 cm, respectively) of Sephadex G-75 (Pharmacia Fine Chemicals Piscataway, N.J.) equilibrated with 10 m*M* Tris–acetate, pH 8.6. Fractions (1.5 or 5 ml) were analyzed for ^{65}Zn and ^{64}Cu by gamma counting or for ^{3}H and ^{35}S by liquid scintillation counting.

Synthesis and detection of ceruloplasmin. Detailed methods describing the measurement of rat ceruloplasmin in hepatocyte cultures have been presented earlier (19). Antibodies against purified rat serum ceruloplasmin were raised in New Zealand white rabbits (Davidson Mill Farms, South Brunswick, N.J.). Spent culture medium (1 ml) or cytosol (1 ml) from cells incubated with 20 μCi of ^{3}H-(leucine, glutamic acid, aspartic acid, glycine, threonine) (New England Nuclear) and either 10^{-5} *M* epinephrine, 10^{-7} *M* dexamethasone, or various copper sulfate concentrations were adjusted to 1% deoxycholate, 1% Triton X-100, 25 m*M* NaCl, 5 m*M* $MgCl_2$, and 25 m*M* Tris–HCl, pH 7.4, and incubated with 6.25 μg of carrier rat ceruloplasmin (slight antibody excess as determined by a quantative precipitin curve). After 1 hr at room temperature fol-

lowed by 18 hr at 4°C, the immunoprecipitate was collected by centrifugation and washed 3 times in 1% Triton X-100 containing 0.15 *M* NaCl. The pellet was then dissolved in NaOH and the ^{3}H content was measured by liquid scintillation counting. Electrophoresis and subsequent fluorography of the immunoprecipitate demonstrated that precipitated radioactivity corresponded solely to rat ceruloplasmin (19).

Incorporation of amino acids into total proteins. Liver cells incubated with 0.5 μCi of a complete ^{3}H-labeled amino acid mixture and either cycloheximide, actinomycin, or no addition were washed 3 times at various incubation periods up to 9 hr with 10 m*M* Hepes-buffered saline/10 m*M* EDTA, pH 7.4. The cells were then scraped off the plates into 2 ml 10 m*M* Tris–acetate–0.25 *M* sucrose, pH 8.6, and homogenized. The homogenate was mixed with 10 ml of 10% trichloroacetic acid (TCA) and centrifuged at 30,000*g*. The pellet was dissolved in 4% NaOH and ^{3}H was measured by liquid scintillation counting.

Results. It is clear from data in Fig. 1 that accumulation of ^{64}Cu of hepatocytes varied considerably when different media were used for incubation. The magnitude of accumulation by cells proceeded in the following order: L-15 > HWB > M199 > MEM > F-12. L-15 medium also provided the best conditions for maintenance of liver parenchymal cells for 3–4 days in a serum-free environment. Cell viability dropped significantly after 1.5 days when other culture mediums were used, particularly Hams F-12. L-15 and Hams F-12 have the highest copper concentrations of all the media tested. A pronounced elevation in ^{64}Cu accumulation was produced by repetitive changes of medium (data not shown).

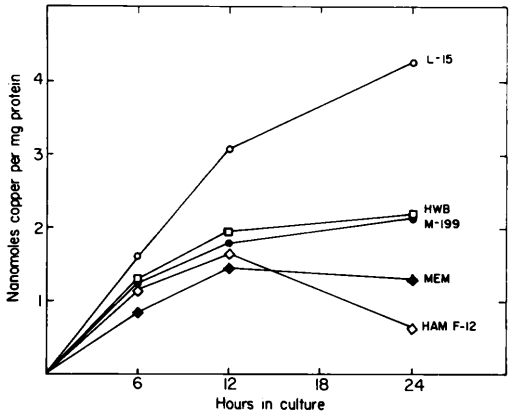

FIG. 1. Copper accumulation by cultured hepatocytes from various commercially supplied culture mediums. Cells were supplemented with 0.5 μCi of ^{64}Cu in 4 ml of either Leibovitz (L15), Waymouth 752/1 (HWB), M199, minimal essential medium (MEM), or Hams-F12 medium containing antibiotics, organic buffers, and albumin as described in the Materials and Methods section. The copper concentration of these media were 2.9, 1.5, 1.5, 1.2, and 2.6 μmole/liter, respectively. Cells were harvested 6, 18, or 24 hr later and radioactivity was measured. Each points represents the mean of three replicate plates.

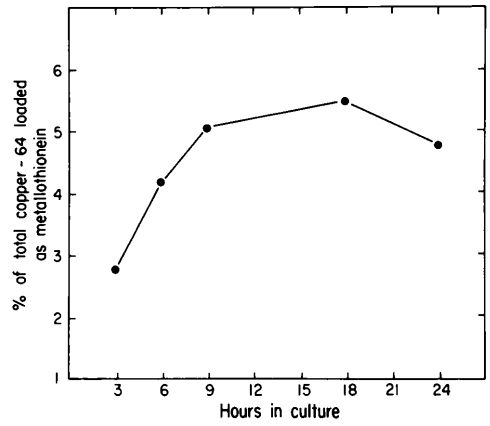

FIG. 2. Metallothionein-bound ^{64}Cu in cytosol from liver parenchymal cells incubated in the presence of 50 μ*M* Cu containing ^{64}Cu. At various times after addition of ^{64}Cu (1.0 μCi) to the hepatocyte cultures, cells from eight plates were pooled into 2 ml of Tris–sucrose buffer. Following homogenization and centrifugation steps as outlined in Materials and Methods, a 1.5-ml aliquot of cytosol was applied to a 1.5 × 30-cm column of Sephadex G-75 and eluted with Tris buffer. ^{64}Cu was measured by gamma counting.

Incubating liver cells with 50 μ*M* copper resulted in changes in the distribution of ^{64}Cu in the cytosol after gel-filtration chromatography. With time, an increased amount of ^{64}Cu appeared in chromatographic fractions which correspond to the co-elution with purified metallothionein (Fig. 2). Also in line with these findings are the results which demonstrate that cells incubated in the presence of 50 μ*M* copper showed highest incorporation of ^{3}H-labeled amino acids into metallothionein between 12 and 18 hr (Fig. 3). Under appropriate conditions a considerable amount of ^{35}S from [^{35}S]cystine was incorporated into metallo-

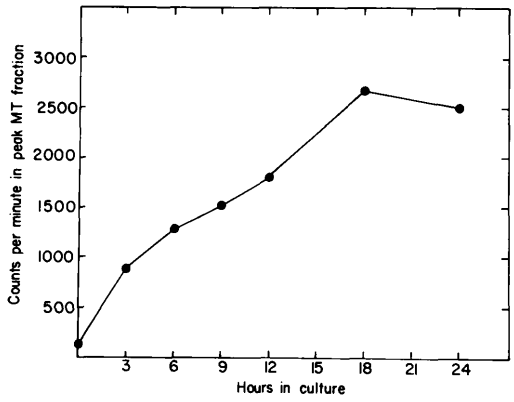

FIG. 3. ^{3}H-labeled metallothionein in cytosol from liver parenchymal cells incubated in the presence of 50 μM Cu in media containing 1 μCi of ^{3}H-(glycine, lysine, and serine). At various times after addition of ^{3}H to the hepatocyte cultures, cells from five plates were pooled into 2 ml of Tris-sucrose buffer. Following homogenization and centrifugation steps as outlined in Materials and Methods, a 2.5-ml aliquot of cytosol was applied to a 2.6 × 50-cm column of Sephadex G-75 and eluted with Tris buffer. ^{3}H was measured by liquid scintillation counting.

thionein when cells were incubated with copper (data not shown).

Enhanced binding of ^{64}Cu to metallothionein in cytosol can be produced by incubation of cells with dexamethasone (Fig. 4). Clearly, the high level of hepatic metallothionein induced via dexamethasone was at the expense of ^{64}Cu binding to the higher-molecular weight constituents. Epinephrine in the incubation medium caused no apparent change in binding of ^{64}Cu to metallothionein.

Despite the elevation in copper associated with metallothionein that was produced as a consequence of dexamethasone treatment, a maximal increase of only 18% in total copper accumulation was observed (Fig. 5). This is quite different from the accumulation of zinc by cells in the presence of dexamethasone (Fig. 6). An increase of approximately 100% in accumulated zinc was evident in hormone-treated cells by 24 hr of incubation. Dexamethasone was also able to initiate a synergistic action on hepatic zinc by raising the extracellular zinc levels (data not shown). Metallothionein-bound zinc increased in cells incubated concurrently with dexamethasone to a far greater extent than in control cells (Fig. 7). No major change in zinc bound to high-molecular weight species were observed.

Inhibiting protein synthesis with both actinomycin D and cycloheximide caused elevations in total accumulated ^{64}Cu (Fig. 8). These altered accumulation profiles appear to be related to the time necessary to see discernable changes in amino acid incorporation into total protein produced by these inhibitors.

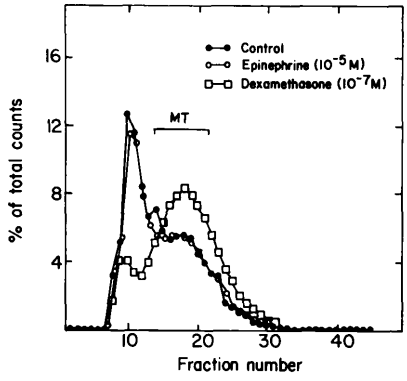

FIG. 4. Isolation of ^{64}Cu-metallothionein (MT) following incubation of liver parenchymal cells with hormones. Cultures received 1.0 μCi of ^{64}Cu and either no addition (●), 10^{-7} M dexamethasone (□), or 10^{-5} epinephrine (○) and were harvested 18 hr later (eight plates/treatment) and subjected to homogenization, centrifugation, and chromatography as described in the legend to Fig. 2.

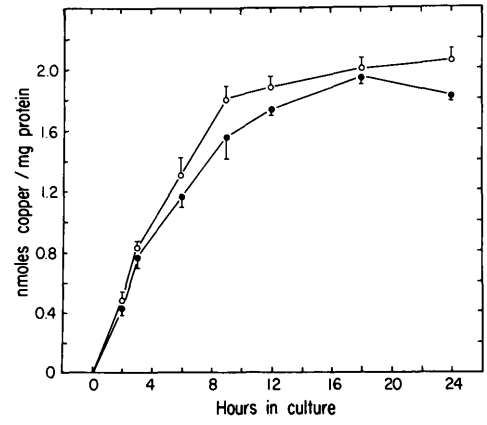

FIG. 5. Copper accumulation of ^{64}Cu by liver parenchymal cells incubated in the presence of dexamethasone. Cells were incubated with 0.5 μCi ^{65}Cu without further additions (●) or with 10^{-7} M dexamethasone (○). At designated intervals cells were harvested and accumulated ^{64}Cu was measured by gamma counting. Each point represents the mean of three replicate plates from duplicate experiments.

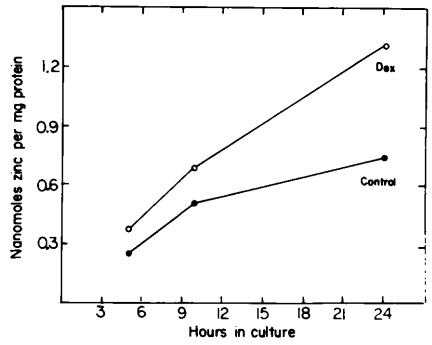

FIG. 6. Zinc accumulation by liver parenchymal cells incubated with dexamethasone. At 5, 10, or 24 hr following incubation with 0.1 μCi ^{65}Zn without (●) or with 10^{-7} M dexamethasone (○), cells were harvested and accumulated ^{65}Zn was measured by gamma counting.

Addition of dexamethasone to cultures caused a 100% increase in secretion of newly labeled ceruloplasmin into the culture medium with a modest elevation (30%) in ceruloplasmin in the cytosol fraction (Table I). Unlike the action of dexamethasone, epinephrine produced large increases in cytoplasmic ceruloplasmin and concurrent increases in accumulated hepatic copper. No enhancement of secreted ceruloplasmin was observed. Elevation of exogenous copper levels to 18 μM caused enhanced ^{3}H-ceruloplasmin in both cell cytosol and spent medium along with a dramatic elevation in accumulation of ^{64}Cu (647%). Clearly, the higher cytosol ceruloplasmin levels produced by epinephrine did not elevate uptake of copper to the extent produced by additional $CuSO_4$, but epinephrine did elevate ^{3}H incorporation into ceruloplasmin in the cytosol to the same extent. Furthermore, incubation of cells with high levels of copper also facilitated enhanced detection of ceruloplasmin in the medium. Increasing the copper concentration of the me-

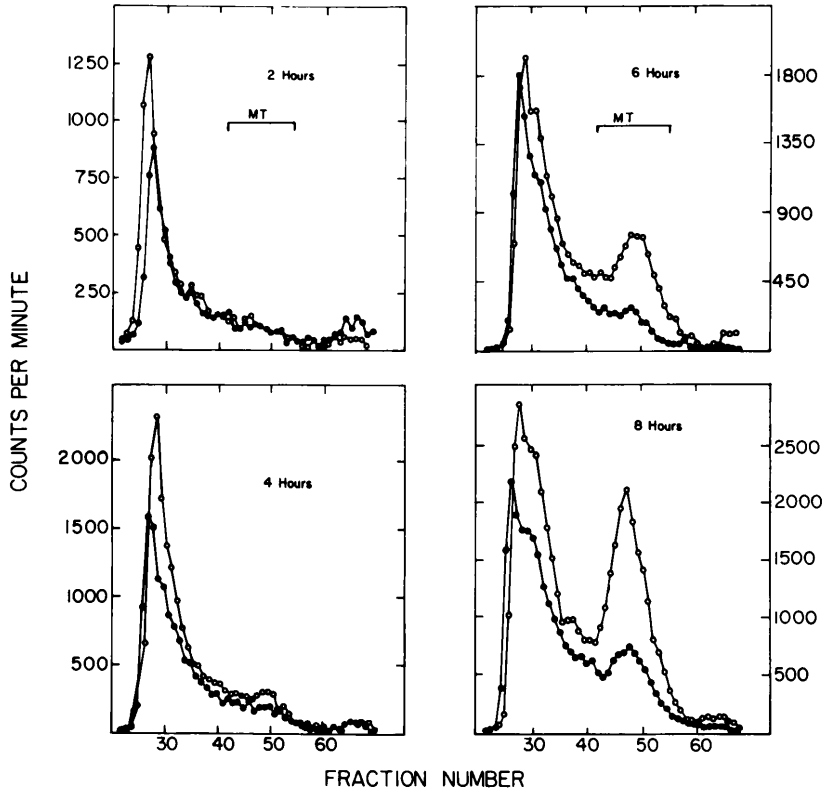

FIG. 7. Appearance of ^{65}Zn-metallothionein in cytosol of dexamethasone-stimulated liver parenchymal cells. Cultures that received 0.1 μCi ^{65}Zn and either no further additions (●) or 10^{-7} M dexamethasone (○) were incubated for 2, 4, 6, or 8 hr. Cells were harvested and metallothionein isolated by treatment as outlined in Fig. 2.

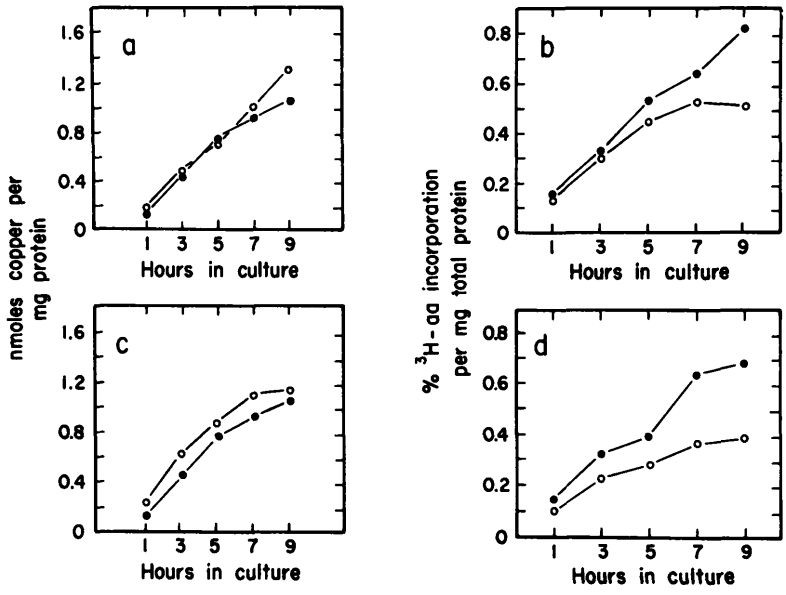

FIG. 8. Effect of actinomycin D and cycloheximide on cellular copper accumulation and incorporation of amino acids into cytosol proteins. Cells supplied with ^{64}Cu (a and c) or ^{3}H-labeled amino acids (b and d) were co-incubated with 1 μM actinomycin D (a and b) (O), 20 μM cycloheximide (c and d) (O), or no additions (controls) (●). Cells were harvested at indicated times and ^{64}Cu accumulation was measured directly by gamma counting. In cultures incubated with ^{3}H-labeled amino acids, intracellular cytosol proteins were precipitated with TCA as described in the Materials and Methods and ^{3}H was measured by liquid scintillation counting.

dium above 10 μM did not increase secretion of ^{3}H-ceruloplasmin into the culture medium (data not shown).

Discussion. These findings further demonstrate the multiplicity of factors which seem to influence hepatic copper. Several of these were found to also produce differential responses of liver cells to the uptake of zinc. Factors which influence either the uptake or eventual accumulation of copper within the liver cell probably are: (i) the form in which copper is presented to the hepatocyte (as free copper, a copper–amino acid complex, or a copper–albumin complex), (ii) the saturation of copper binding sites at the hepatocyte surface, (iii) the intracellular levels of metallothionein, and (iv) the synthesis/secretion profile of ceruloplasmin. Although excretion of copper from the liver cell into the bile is indeed a fifth factor, this aspect of copper metabolism was not investigated in the current study. Increasing the copper concentration of the medium resulted in elevations in accumulated copper, metallothionein, and both intra- and extracellular ceruloplasmin. These results are consistent with animal studies which have examined the effects of copper loading on these aspects of hepatic copper metabolism (5, 7, 8, 20, 45). The magnitude of copper accumulation when hepatocytes are presented with exogenous copper is of particular interest. Increased accumulation with continued incubation may represent saturation of copper binding sites and induction of metallothionein synthesis. However, since copper supplementation of the medium resulted in higher levels of secreted ^{3}H-ceruloplasmin, it might have been expected that only moderate increases in accumulated copper would result due to enhanced copper export, similar to the response we have found upon treatment of hepatocytes with dexamethasone. Although it has been suggested that copper can cause induction of ceruloplasmin in liver (20, 21), it has also been reported that copper may only stabilize ceruloplasmin that is synthesized constitutively (22, 23). This latter possibility may be related to the high intracellular copper levels we observed during copper supplementation of the medium concurrent with high detectable levels of secreted ceruloplasmin.

Unlike what can be shown during copper

TABLE I. EFFECT OF COPPER, DEXAMETHASONE, AND EPINEPHRINE ON ^{64}Cu ACCUMULATION OR ^{3}H-CERULOPLASMIN SYNTHESIS AND SECRETION BY LIVER PARENCHYMAL CELLS CULTURED FOR 24 hr[a]

	Percentage of control levels		
	^{3}H-ceruloplasmin		^{64}Copper
	Cell cytosol	Spent medium	Total cell
Control	100	100	100
Dexamethasone (10^{-7} *M*)	130	200	113
Epinephrine (10^{-5} *M*)	159	94	151
Copper (18 μM)	140	164	647

[a] Ceruloplasmin values were obtained from cells incubated with 20 μCi of a ^{3}H-labeled amino acid mixture described in the Materials and Methods. Labeled ceruloplasmin was immunoprecipitated and measured for ^{3}H content. Copper accumulation was determined in cells that received 0.5 μCi ^{64}Cu as described in Fig. 1.

supplementation, we have observed that dexamethasone treatment results in only moderate increases in accumulated copper. Failla and Cousins have demonstrated that dexamethasone causes dramatic increases in cellular zinc levels (16, 24) (see also Fig. 7). This response can be related to enhanced expression of the metallothionein gene (24, 26). Although we have also shown that dexamethasone increases association of copper with this protein (Fig. 4), it may be that the induction of ceruloplasmin and subsequent secretion of copper–ceruloplasmin due to dexamethasone contributes to a low steady-state intracellular level of copper–metallothionein. Several studies with cultured liver cells have demonstrated the ability of corticosteroids to induce a variety of proteins (27, 28) as well as enhance secretion of proteins from these cells (17, 29, 30). Secreted copper–ceruloplasmin may play a role in the maintenance of normal cell copper content as inferred by our studies using the inhibitors actinomycin D and cycloheximide. These are both known to inhibit metallothionein synthesis (24, 25, 31, 32). They cause elevations in the levels of cell copper, probably through a mechanism which inhibits efflux, viz. copper–ceruloplasmin secretion. Our results are consistent with a previous finding which suggested these inhibitors prevent removal of copper from the liver (33). Further studies are needed to quantitate relative changes in ceruloplasmin synthesis/secretion and biliary copper content with respect to intracellular levels of copper.

Whereas it was observed that incubation of liver cells with exogenous copper caused pronounced copper accumulation, it was also noted that the external milieu of the cell may play an important role in the actual transport of the metal. Our findings (Fig. 1) illustrate that changing the nutrient composition of the culture media produces differential accumulation of copper. Several studies have shown that amino acids, particularly threonine, histidine, and glutamine, are important in binding the free plasma copper pool (approximately 7% of the total blood copper) and that these complexes may represent the metabolically active transport form of this metal into hepatocytes (34–37). Copper uptake was most closely correlated to the threonine concentration of various media. Further support for amino acid-mediated copper uptake comes from studies which have shown that epinephrine elevates amino acid uptake and copper accumulation by isolated rat liver cells (19, 38).

Recently Schmitt *et al.* have examined copper transport by isolated hepatocytes in albumin-free suspension culture (39). They found that histidine did not influence copper transport kinetics and have suggested the metal is transported as the free ion. However, in the presence of albumin, others have found histidine enhances copper uptake (37). Furthermore, Schmitt *et al.* suggest that albumin markedly lowers the availability of free copper ion for transport (39). However, as shown in Fig. 1, different culture media, containing variable amounts of amino acids and copper, but a constant supply of albumin, yielded differing, but substantial levels of copper accumulation. This suggests that under physiological conditions, when the influence of copper binding to albumin in the plasma is a reality, the binding of copper to amino acids may be a primary factor in the transport process. It cannot be ruled out that amino acids help to maintain a constant supply of free copper by preventing copper binding to albumin. It should be pointed out that although the present studies were carried out under more physiological conditions, e.g., in the

presence of serum albumin, accumulation rates were comparable to those Schmitt *et al.* which were obtained without albumin (39). If in fact, amino acids prove to be important in copper transport, it is reasonable to conduct these transport experiments with albumin as a constituent of the culture system, since amino acid transport has been well characterized in cells maintained in albumin-containing media (40). The results of Schmitt *et al.* also differ from those presented here in that they used suspensions of freshly prepared hepatocytes. Our experiments employed liver parenchymal cells that had been purified by selective attachment and cultured for a sufficient period of time to allow for cell repair and equilibration of transport systems (41).

The extent to which metallothionein regulates normal hepatic levels of copper at present remains unclear. Whereas significant correlations between elevations in hepatic zinc levels and metallothionein synthesis under a variety of experimental conditions have been extensively studied (2, 42–44), the relationship of this protein to elevated copper in liver is not well-defined. Although numerous studies have demonstrated *de novo* metallothionein synthesis in liver after copper loading (5, 6, 8, 12, 45, 46), it is clear that other conditions which cause elevated copper (e.g., epinephrine, protein synthesis inhibitors) do not cause apparent increases in hepatic metallothionein. It is probable that accumulated cellular copper under these circumstances is in a form that renders it unavailable for the purposes of metallothionein induction. The data provided in this report raise many new questions as to the mechanisms which may be involved in maintaining normal blood and hepatic copper levels. Indeed, the controls regulating hepatic copper are at least as complex as the mechanisms governing hepatic zinc metabolism.

In summary, it appears that hormones have marked influences on copper and zinc metabolism in liver parenchymal cells. Dexamethasone increased zinc accumulation and binding of some copper and substantial amounts of zinc to metallothionein. Incubation of cells with high levels of copper or zinc increased the cellular metallothionein content. Epinephrine markedly increased copper accumulation, but did not influence the amount of copper bound to metallothionein. Both dexamethasone and epinephrine increased ceruloplasmin synthesis and secretion. Collectively, the results suggest glucocorticoids increase hepatic zinc accumulation through augmented metallothionein synthesis, whereas they increase copper output by increasing secretion as ceruloplasmin.

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Received February 22, 1983. P.S.E.B.M. 1983, Vol. 173.