Degradation of Zinc-Metallothionein in Monolayer Cultures of Rat Hepatocytes[1] (42898)

MING L. CHEN[2] AND MARK L. FAILLA[3]
Vitamin and Mineral Nutrition Laboratory, Agriculture Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

Abstract. **The degradation of zinc-metallothionein (MT) was studied in monolayer cultures of adult rat hepatocytes. Hepatocytes were incubated overnight in serum-free medium containing either [^{35}S]cysteine or [^{3}H]leucine and 100 μM zinc to induce MT synthesis. Total cellular ^{35}S-MT was measured in the heat-stable extract of cell homogenate and quantified by fast protein liquid chromatography. When zinc was removed from the medium, ^{35}S-MT turnover was almost 3-fold faster than that of [^{3}H]Leu protein ($t_{1/2}$ = 11 and 29 hr, respectively). The decrease in the cellular level of ^{35}S-MT reflected degradation since less than 1% of total cellular ^{35}S-MT was secreted into the medium. The rate of MT degradation was inversely proportional to cellular zinc content. In contrast, the degradation of [^{3}H]Leu protein was not affected by changes in cellular zinc concentration. Chloroquine, a lysosomotrophic amine, and tosyl lysine chloromethyl ketone, an inhibitor of trypsin-like neutral protease activity, inhibited ^{35}S-MT degradation by 65% and 50%, respectively, when cells were incubated in medium with 1 μM zinc. Turnover of [^{3}H]Leu protein, but not ^{35}S-MT, was enhanced by insulin deprivation. These data suggest that the degradation of hepatic MT (i) is primarily regulated by cellular zinc content and (ii) occurs in both lysosomal and nonlysosomal compartments.**
[P.S.E.B.M. 1989, Vol 191]

Metallothioneins (MT) are a family of low molecular weight, cysteine-rich, heavy metal-binding proteins. These proteins are believed to be required for the metabolism of essential micronutrients (i.e., zinc and copper) and the detoxification of heavy metals. The synthesis of MT is induced by a variety of factors, including exposure to elevated levels of heavy metals, physiologic stress, and changes in endocrine status, and requires increased transcription of MT genes (1).

In contrast to the detailed information available concerning the structure and synthesis of MT, knowledge of MT degradation is very limited. *In vivo* experiments have suggested that the rate of MT turnover in the cytosol fraction is influenced by the specific metal bound to the polypeptide. The turnover of hepatic copper-MT and zinc-MT ($t_{1/2}$ = 15–20 hr) are significantly faster than that of cadmium-MT ($t_{1/2}$ = 80 hr) (2–6). In such studies, it has been assumed that the disappearance of ^{35}S-labeled MT from the cytosol fraction reflects degradation of this protein by lysosomal proteases since apothionein, zinc-MT and cadmium-MT were rapidly degraded in lysosomal extracts (7). Squibb *et al.* (8) also provided morphologic and biochemical evidence that plasma cadmium-MT was filtered by the glomerulus and subsequently accumulated and degraded in the lysosomes of renal tubule cells. However, these data do not explain the resistance of copper-MT to lysosomal degradation *in vitro* (6). Moreover, the possibility that intracellular MT may be degraded by nonlysosomal proteases has not been critically examined. Feldman *et al.* (7) and Winge and Milkossy (9) reported that zinc-MT and cadmium-MT were partially degraded by neutral proteases *in vitro*. The recent discovery of several cytoplasmic proteases in rat liver (10, 11) provided the impetus to reexamine the subject of MT degradation using monolayer cultures of hepatocytes from adult rat liver.

[1]This work was submitted by M. L. C. in partial fulfillment of the requirements for Ph.D. in Biochemistry and Nutrition at Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

[2] Present address: Geriatric Center, VA Hospital, St. Louis, MO 63125.

[3]To whom requests for reprints should be addressed at USDA, ARS, BHNRC, VMNL, Bldg. 307, Room 205, BARC-East, Beltsville, MD 20705.

Received October 3, 1988. [P.S.E.B.M. 1989, Vol 191]
Accepted January 31, 1989.

0037-9727/89/1912-0130$2.00/0

Data presented below indicate that the micronutrient zinc has an important role in MT degradation and that this protein is degraded in both cytoplasmic and lysosomal compartments of the hepatocyte. Possible mechanisms of MT degradation are discussed.

Materials and Methods

Chemicals and Media. Medium 199 and fetal calf serum were purchased from Gibco Laboratory (Chagrin Falls, OH). Waymouth's MB 752/1 was prepared according to standard formula, except that either cysteine (Cys) or leucine (Leu) were omitted when adding [^{35}S]Cys or [^{3}H]Leu, respectively. Percoll and collagenase were purchased from Pharmacia Inc. (Piscataway, NJ) and Worthington Biochemical (Freehold, NJ), respectively. ^{65}Zn (4.3 mCi/mg), L-[4,5-^{3}H]Leu (5 Ci/mmol), and L-[^{35}S]Cys (>600 Ci/mmol) were obtained from New England Nuclear (Boston, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation and Culture. Male Sprague-Dawley rats (200–250 g) purchased from Charles River Breeding Laboratory (Wilmington, MA) were fed commercial diet (Prolab Rat, Mouse, Hamster 3000 formula; Agway, Inc., Syracuse, NY) and deionized water *ad libitum*. Donor rats were fed overnight and cell isolation was initiated between 0900 and 1000 hr. Liver cells were obtained by the collagenase perfusion technique as previously described (12). Intact hepatocytes were separated from nonparenchymal cells and leukocytes by differential centrifugation and iso-osmotic Percoll density gradient centrifugation (13). Cell viability, as estimated by trypan blue exclusion, was 90–95%. Approximately 2×10^6 purified hepatocytes were added to collagen-coated, multiwell dishes (35 mm^2) and incubated at 37°C in 95% air-5% CO_2. The plating medium consisted of Medium 199 with 5% fetal calf serum, 10^{-7} *M* bovine pancreatic insulin, 50 μg of gentamicin/ml, and 0.5 μg of Fungizone/ml. After 1 hr, nonadherent cells were removed by washing monolayers twice with Puck's balanced salt solution. Monolayers of hepatocytes were then incubated in Waymouth's MB 752/1 medium containing 0.2% bovine serum albumin (BSA), 10^{-8} *M* insulin, 2 μCi of [^{35}S]Cys (20 Ci/mmol)/ml; and 100 μ*M* zinc (as $ZnSO_4 \cdot 7H_2O$) for 20 hr to induce MT synthesis. The morphologic appearance of cells incubated in medium containing 100 μ*M* zinc was identical to that of hepatocytes in medium with basal levels of zinc (1 μ*M*). Also, the activity of lactate dehydrogenase in medium was similar (<5% total activity in well) when cultures were incubated in 1 or 100 μ*M* zinc. These findings suggested that 100 μ*M* zinc was not cytotoxic using the described conditions.

Determination of MT in Hepatocytes. At the time of sample collection, spent medium was removed by aspiration and monolayers were washed twice with ice-cold Puck's solution. Hepatocytes from each dish (six wells) were harvested in 1.5 ml of 10 m*M* Tris-HCl buffer (pH 8.6) containing 0.2% Triton X-100 and 5 m*M* 2-mercaptoethanol. Phenylmethylsulfonyl fluoride (0.01%) and leupeptin (10 μg/ml) were added to the buffer to inhibit cellular proteolytic activity. Cell suspensions were sonicated at 40% output for 30 sec using a microprobe (Microson Instrument, Farmingdale, NY). Microscopic examination of the homogenate showed complete disruption of cells.

In a preliminary experiment, monolayers were incubated overnight in medium containing 100 μ*M* zinc and either 3.2 μCi of [^{35}S]Cys or 0.3 μCi of ^{65}Zn/well. Sonicates from four dishes were pooled and centrifuged at 150,000*g* for 75 min, and the supernatant was fractionated by Sephadex G-75 chromatography as outlined elsewhere (14). In subsequent studies, sonicates were heated at 70°C for 5 min to precipitate ^{35}S proteins with molecular weights higher than MT. Heat-precipitated materials were removed by centrifugation at 10,000*g* for 15 min and the supernatant was concentrated to 0.5 ml by ultrafiltration (Centricon 10; Amicon Co., Danvers, MA). The sample was then applied to a Sephadex G-25 column (9 × 300 mm) to remove the relatively large amount of low molecular weight ^{35}S-containing species. This column was equilibrated and eluted with 0.1 *M* Tris-HCl, 5 m*M* 2-mercaptoethanol (pH 8.6). Fractions (1 ml) were collected at a flow rate of 15 ml/hr and material eluting at 1.0–1.2 × void volume was pooled. Aliquots were analyzed by fast protein liquid chromatography (FPLC; Pharmacia Inc., Piscataway, NJ) equipped with a gel filtration column (Superose 12). After injecting samples (200 μl), the Superose 12 column was washed with 0.1 *M* Tris-HCl (pH 8.6) at a flow rate of 0.2 ml/min. Eluate (1-ml fractions) was transferred to plastic vials containing 10 ml of ScintiVerse II (Fischer Scientific, Fairlawn, NJ) to measure ^{35}S (Beckman LS 3801 Spectrophotometer; Beckman Instrument Co., Fullerton, CA). Quenching was corrected with an automatic external standard. ^{35}S-labeled material eluted from the Superose column in a single symmetrical peak with a mean retention time of 80 min. The elution profile was identical to that of ^{35}S-MT purified from liver of rats injected with cadmium and [^{35}S]Cys (14). In a pilot study, ^{35}S-labeled cellular material collected from Superose 12 was further analyzed by FPLC anion exchange chromatography (Mono Q HR 5/5 column). The mobile phases were 20 m*M* Tris-HCl, 5% glycerol (pH 8.6, Buffer A), and 200 m*M* Tris-HCl, 5% glycerol (pH 8.6, Buffer B). Samples (500 μl) collected at the void volume of the Sephadex G-25 column were injected. After isocratic elution with 6 ml of Buffer A, bound materials were eluted with linear gradient of 20 ml of 20–200 m*M* Tris-HCl (0–100% Buffer B). The flow rate was 1 ml/min and 1-ml fractions were collected. Injected ^{35}S eluted as two peaks with mean retention times of 13 and 20 min. Retention

times for ^{35}S-MT-I and II purified from intact rat liver were also 13 and 20 min, respectively, on Mono Q. Based on heat stability, apparent molecular weight, resolution into two isoproteins, coelution with zinc and responsiveness to cellular zinc status (see below), it was concluded that ^{35}S-labeled material in cellular extracts that eluted from either Superose 12 or Mono Q columns at the indicated times was MT. The amount of ^{35}S-MT is expressed as dpm per mg cell protein. Total cellular protein was measured by a modified Lowry method (15) using BSA as the standard.

To estimate the recovery of ^{35}S-MT after each step, ^{35}S-MT-I or MT-II was added to cell sonicates and samples were processed as described above. The recovery of ^{35}S-MT at each step exceeded 85%, and the total recovery of starting material was 75–80% (Table I). Since the recovery of ^{35}S-MT declined as decreasing amounts of this protein were applied to the column, nonradioactive cadmium-MT (100 μg) and BSA (1 mg) were added to samples prior to heat treatment and ultrafiltration steps, respectively, to minimize nonspecific losses. Attempts to measure cellular MT by the *in vitro* ^{109}Cd binding assay (16) were unsuccessful, because the recovery of ^{109}Cd from Superose 12 and Mono Q columns was only 65% and 50%, respectively (Table I).

Degradation of MT and Non-MT Cellular Proteins. Monolayers of hepatocytes were incubated in medium containing [^{35}S]Cys and 100 μM zinc overnight. After washing twice with Medium 199, monolayers were incubated in fresh Waymouth's MB 752/1 medium containing 0.2% BSA, 10^{-8} M insulin, excess Cys (2 mM), and indicated substances. The basal concentration of zinc in this medium was 1 μM as determined by atomic absorption spectrometry. Cells were harvested at indicated times and the total level of ^{35}S-MT was quantified by FPLC using the Superose 12 column as described above. Attempts to chase low molecular weight ^{35}S (M_r <2000) from hepatocytes by incubation in medium with excess cysteine prior to initiating turnover studies were unsuccessful. Therefore, several pilot studies were performed to determine whether [^{35}S]Cys was being (re)incorporated in MT after transfer of zinc-loaded, [^{35}S]Cys-labeled cultures to low zinc medium. First, monolayers that had been incubated in medium with 100 μM zinc but not [^{35}S]Cys overnight were washed before addition of medium containing 1 μM zinc and [^{35}S]Cys to estimate the level of *de novo* MT synthesis during the 6-hr experimental period. The amount of ^{35}S-MT synthesized was only 8% of the concentration of ^{35}S-MT present in hepatocytes after overnight incubation in medium containing 100 μM and [^{35}S]Cys. Second, addition of cycloheximide (3.4 μM) to low zinc medium did not reduce the level of ^{35}S-MT in zinc-loaded hepatocytes below that in cells incubated in the absence of this inhibitor of protein synthesis. At this concentration of cycloheximide incorporation of [^{35}S]Cys and [^{3}H]Leu into protein was inhibited by >85%. Together, these data indicate that reincorporation of [^{35}S]Cys into MT was minimal during the experimental period.

Since MT does not contain leucyl residues (17), the rate of degradation of [^{3}H]Leu proteins was also determined to provide information on the effects of various treatments on general protein turnover. Thus, replicate monolayer cultures were incubated in medium containing 100 μM zinc and [^{3}H]Leu (10 μCi/nmol) overnight and the degradation of [^{3}H]Leu protein was determined according to the procedure of Hopgood *et al.* (18). Pilot studies demonstrated that the acid-soluble pool of intracellular [^{3}H]Leu was depleted by incubation of monolayers in medium containing 2 mM nonlabeled Leu for 4 hr prior to initiating degradation studies.

The subcellular site(s) of MT degradation was probed by using several chemicals and conditions that modify specific processes in the lysosomal and nonlysosomal pathways of protein degradation. Chloroquine and 3-methyladenine were used to inhibit degradation of proteins in lysosomes (19) and the formation of autophagosomes (20), respectively. *N*-tosyl-lysine chloromethyl ketone (TLCK), an inhibitor of neutral serine and, perhaps, cysteine protease activity, was used to inhibit nonlysosomal proteolysis (21, 22). Insulin deprivation was used to stimulate the lysosomal pathway of protein degradation (23). Indicated chemicals were added to medium containing 1 μM zinc immediately before initiating degradation studies with zinc-loaded hepatocytes that had been labeled with either [^{35}S]Cys or [^{3}H]Leu overnight.

Table I. Recovery (%) of ^{35}S- or ^{109}Cd-labeled Isoforms of MT during Purification[a]

Procedure	Recovery (%)			
	MT-I		MT-II	
	^{35}S	^{109}Cd	^{35}S	^{109}Cd
Heat treatment	98	99	98	99
Ultrafiltration	93	ND[b]	90	ND
Sephadex G-25	95	ND	95	ND
FPLC				
Superose 12	86	56	89	65
Mono Q	89	50	85	50

[a] ^{35}S-MT-I and II were purified from the livers of rats injected (ip) with cadmium and [^{35}S]Cys. One purified protein (100 μg) was added to an aliquot of liver cytosol from control animals. Samples were heated at 70°C for 5 min, the precipitate was removed by centrifugation, and heat-stable proteins were separated from low molecular weights peptides and amino acids by Sephadex G-25 chromatography. Aliquots of Sephadex G-25 eluate were further fractionated by FPLC (Superose 12 and Mono Q columns). In a separate study, ^{109}Cd-MT-I and II were prepared by adding ^{109}Cd to liver cytosol from a zinc-injected rat and purified by DEAE-A25 ion exchange chromatography (14). ^{109}Cd-MT (100 μg) was added to control liver cytosol and analyzed as described above. Values represent means for three to six different preparations.

[b] ND = not determined.

Estimation of Cellular Zinc Status. The zinc content of hepatocytes incubated in 35-mm^2 wells could not be accurately determined by flame atomic absorption spectrometry. Therefore, we estimated the cellular concentration of zinc at the beginning of the experiment from the level of ^{65}Zn accumulated after overnight incubation in medium containing 100 μM zinc and 0.3 μCi of ^{65}Zn (specific activity = 1.5 μCi of ^{65}Zn/μmol of zinc). Changes in cellular zinc content during the 6-hr period of study were determined by measuring both the uptake and efflux of zinc as follows. Overnight cultures were washed twice with Medium 199 containing 10 mM EDTA to remove nonspecifically bound zinc from the cell surface and with two additional washes of Medium 199. One set of cells was harvested in 0.2% Triton X-100 and the level of ^{65}Zn accumulated was measured. The zinc concentration of cell at 0 hr was estimated to be 3.87 ± 0.20 nmol/mg protein (n=15 separate determinations). This value is based on the assumptions that endogenous zinc in freshly isolated cells from chow-fed rats was negligible in comparison to medium zinc and that endogenous zinc readily exchanged with medium zinc during overnight (18–20 hr) incubation. To study ^{65}Zn efflux, the remaining dishes were incubated in fresh medium containing various concentrations of zinc. After 6 hr, medium was removed and monolayers were washed twice with ice-cold Puck's buffer containing 10 mM EDTA to inhibit zinc transport and remove nonspecifically bound zinc from the cell surface. This was followed by two consecutive washes with ice-cold buffer without EDTA. Zinc efflux was calculated as the difference between cellular zinc at 0 and 6 hr. To investigate zinc uptake, monolayers that had been incubated overnight in medium containing 100 μM zinc without ^{65}Zn were washed as described above before the addition of medium containing ^{65}Zn (0.3 μCi) and various concentrations of zinc. The amount of ^{65}Zn accumulated by cells was determined after 6 hr. The net change in cellular zinc during the experimental period was estimated as the difference between uptake and efflux. The zinc content of cells at the end of the experimental period was calculated from the concentration of cellular zinc at 0 hr and the net accumulation/loss of the metal during the 6-hr incubation period.

Statistical Analysis. Data are expressed as mean ± SEM. Each value represents the mean from at least three replicate samples from at least three separate preparations of hepatocytes. One-way analysis of variance and Student-Newman-Keuls multiple range tests were used to test for significant difference ($P < 0.05$) between each treatment group (24).

Results

MT Degradation. In preliminary experiments, monolayers of hepatocytes were incubated in medium containing [^{35}S]Cys and 100 μM ^{65}Zn overnight. The soluble fraction (150,000 g supernatant) contained 86% and 80% of the total cellular ^{35}S and ^{65}Zn, respectively. Fractionation by Sephadex G-75 gel filtration chromatography showed that 10, 12, and 75% of soluble ^{35}S was distributed among high molecular weight proteins (1–2.0 × void volume, V_0), MT (2.0–2.5 × V_0), and low molecular weight species (>2.5 × V_0; M_r <5000), respectively (Fig. 1). The corresponding values for distribution of ^{65}Zn among these pools were 34%, 43%, and 15%, respectively. Thus, 34% of the total cellular zinc was bound to MT. When zinc-loaded hepatocytes were incubated in zinc-deficient (1 μM zinc) medium for 6 hr, cellular ^{35}S and zinc decreased by 13 and 33%, respectively (Fig. 1). More specifically, the levels of ^{35}S proteins, ^{35}S-MT, and low molecular weight ^{35}S-containing species decreased 7%, 35%, and 25%, respectively. The levels of zinc associated with ^{35}S proteins having molecular weights in excess of 10,000, ^{35}S-MT and low molecular weight ^{35}S species (>2.5 × V_0), decreased by 13, 38, and 93%, respectively. Thus, the turnover of ^{35}S-MT was about three times faster than

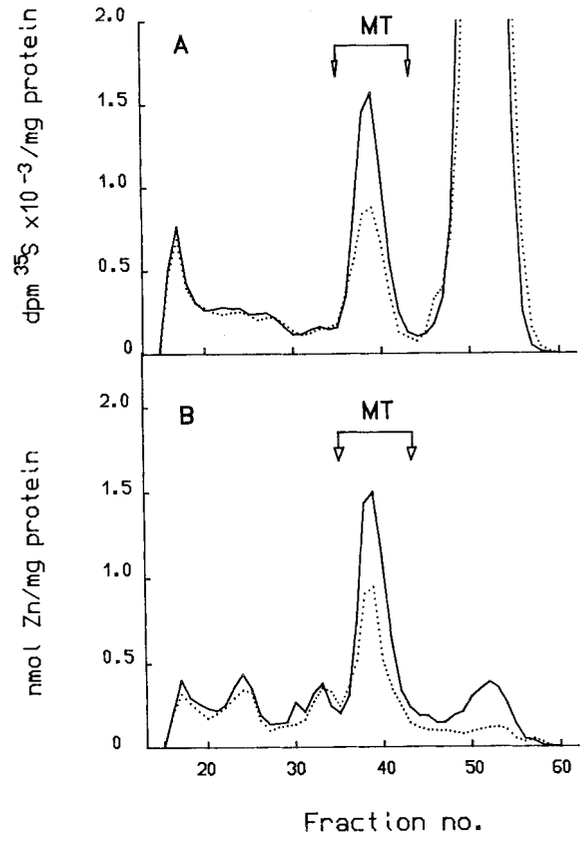

Figure 1. Sephadex G-75 elution profile of soluble fraction of hepatocytes previously labeled with [^{35}S]Cys and ^{65}Zn. Monolayers of hepatocytes were incubated overnight in medium containing [^{35}S]Cys (10 μCi/nmol) and 100 μM zinc (with 0.3 μCi of ^{65}Zn). Cells were washed and reincubated in medium containing nonradioactive Cys and 1 μM zinc for 6 hr. The soluble fraction of sonicates was prepared at 0 (solid line) and 6 (dotted line) hr and analyzed by Sephadex G-75 gel filtration chromatography. The elution profile of ^{35}S and zinc are shown in A and B, respectively. The bracketed areas indicate the position of elution for purified rat liver cadmium, zinc-MT.

general ^{35}S proteins and was accompanied by the loss of zinc from the MT fraction. Also, the low molecular weight pool of zinc was rapidly lost to the medium. Since less than 1% of total cellular MT was present in spent medium after 6 hr, the decrease in cellular ^{35}S-MT reflected degradation of this protein.

Because analysis of cellular MT by Sephadex G-75 gel filtration chromatography required relatively large amounts of material and much time, a method was developed for the rapid analysis of ^{35}S-MT from small amounts of cell material (<5 mg cell protein). Sonicates prepared from ^{35}S-labeled, zinc-loaded hepatocytes were heated and heat-stable proteins were separated from peptides and amino acids by Sephadex G-25 chromatography. Subsequent analysis by FPLC using a Superose 12 column showed that MT was the predominant ^{35}S protein (Fig. 2A). Using this procedure, the cellular level of ^{35}S-MT was found to be 30–40% lower after monolayers were incubated in zinc-deficient medium for 6 hr. Analysis of heat-stable proteins by FPLC using the Mono Q column showed that MT-I and MT-II were synthesized when hepatocytes were incubated in medium with 100 μM zinc and that the levels of both proteins decreased after incubation in zinc-deficient medium (Fig. 2B).

The rate of ^{35}S-MT degradation was determined by periodic harvesting of cultures after transfer of zinc-loaded hepatocytes to zinc-deficient medium. The calculated half-life of ^{35}S-MT was 11 hr (Fig. 3), which was approximately three times shorter than that of [^{3}H]Leu protein ($t_{1/2}$ = 29 hr, Table II).

Effects of Medium Zinc Level on Cellular Content of MT and Zinc. ^{35}S-Labeled, zinc-loaded hepatocytes were incubated in medium containing various concentrations of zinc for 6 hr to evaluate the impact of extracellular zinc concentration on MT degradation. MT degradation was inversely proportional to the concentration of extracellular zinc (Fig. 4). ^{35}S-MT degradation also was inversely proportional to the concentration of zinc in medium containing 3.4 μM cycloheximide (data not shown), demonstrating that the apparent reduction in the rate of MT synthesis in high zinc medium was not due to increased reincorporation of [^{35}S]Cys into synthesized MT. The degradation of [^{3}H]Leu proteins was not affected by extracellular zinc (Fig. 4).

To determine the relationship between medium and cellular concentrations of zinc, the efflux and uptake of this metal were studied. Hepatocytes contained 3.9 nmol zinc/mg cell protein after overnight incubation in medium containing 100 μM zinc. When zinc-loaded hepatocytes were incubated in medium containing various concentrations of zinc for 6 hr, cellular zinc content was correlated with the concentration of this micronutrient in the incubation medium (Fig. 4). At medium zinc levels below 50 μM, the amount of zinc lost from zinc-loaded hepatocytes exceeded that taken

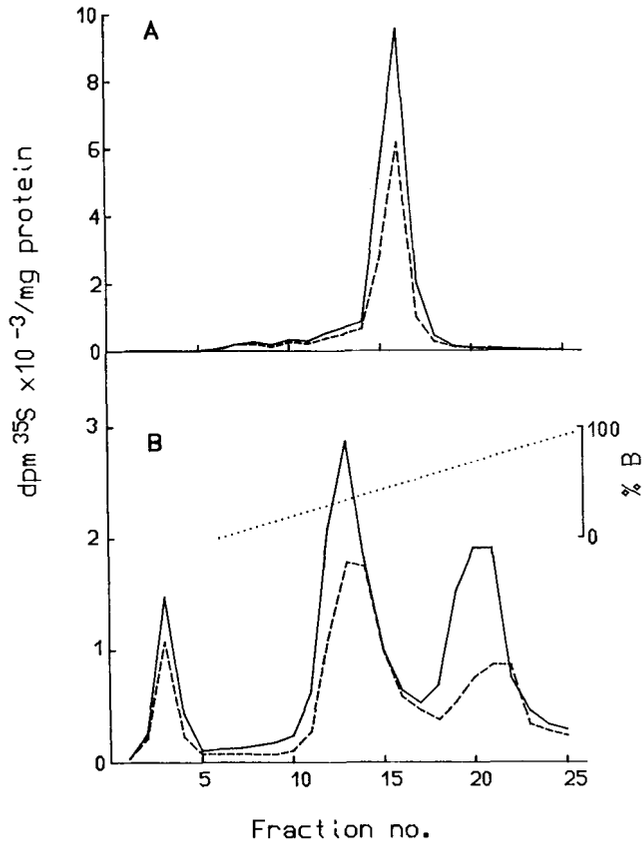

Figure 2. Analysis of heat-stable proteins from ^{35}S-labeled hepatocytes by FPLC. Cell sonicates were prepared from monolayers of hepatocytes incubated overnight in medium with 100 μM zinc and [^{35}S]Cys and then reincubated in medium containing 1 μM zinc and nonradioactive Cys for 6 hr. Heat-stable proteins were collected in the void volume of Sephadex G-25 columns and analyzed using either a Superose 12 (A) or a Mono Q (B) column. Samples were eluted and analyzed as described in the Materials and Methods. Solid line = overnight culture; dotted line = 6-hr incubation in low zinc medium.

up, thereby decreasing total cellular zinc content. The possibility that efflux of zinc from zinc-loaded hepatocytes markedly decreased the specific activity of extracellular ^{65}Zn, especially in medium with low levels of the metal (e.g., 1 μM zinc), was considered. The amount of ^{65}Zn transferred from zinc-loaded hepatocytes to the medium in 6 hr diluted the concentration of zinc in basal medium by only 5%. Together, these results demonstrate that the rate of MT degradation was inversely proportional to the cellular content of zinc.

Subcellular Site(s) of MT Degradation. Exposure of hepatocytes to chloroquine decreased [^{3}H]Leu protein degradation by 41%, demonstrating that the lysosomal pathway contributed to turnover of general cellular proteins under the defined experimental conditions (Table II). Participation of nonlysosomal components in general protein turnover was also demonstrated by the ability of TLCK to inhibit [^{3}H]Leu protein degradation by 17%. Chloroquine and TLCK inhibited ^{35}S-MT degradation by 65% and 51%, respectively, suggesting that this protein was also degraded in both lysosomal and nonlysosomal compartments of the

cell (Table II). Cellular zinc status was slightly lower in hepatocytes treated with chloroquine and TLCK than in control cultures (Table II), showing that the higher levels of ^{35}S-MT in cells treated with these inhibitors were not due to increased cellular retention of the metal.

We next examined the influence of enhanced activity of the macroautophagic arm of the lysosomal pathway on MT degradation by eliminating insulin from medium during the experimental period. As expected, degradation of [^{3}H]Leu protein was increased about 30% when hepatocytes were incubated in insulin-free medium (Table II). Chloroquine, but not TLCK, blocked this response to insulin deprivation (data not shown). In contrast, the degradation of ^{35}S-MT was not affected by insulin deprivation, despite the slightly lower level of zinc in hepatocytes. The degradation of [^{3}H]Leu protein, but not ^{35}S-MT, was also decreased by 3-methyladenine, an inhibitor or autophagosome formation (Table II).

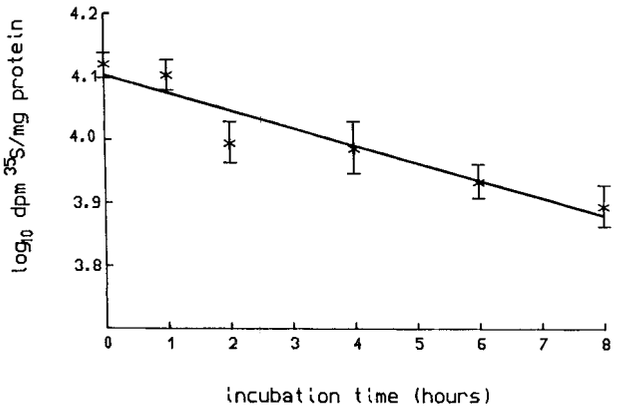

Figure 3. Turnover of ^{35}S-MT in monolayers of hepatocytes. Hepatocytes were incubated in medium containing 100 μM zinc and [^{35}S]Cys for 20 hr. To initiate experiments, fresh medium with 1 μM zinc and excess Cys was added and monolayers were incubated for 0 to 8 hr. The level of ^{35}S-MT in the heat-stable fraction was determined by FPLC using the Superose 12 column. Half-life ($t_{1/2}$) of ^{35}S-MT was calculated from the slope of regression line as outlined by Segal and Kim (25).

Discussion

MT turnover *in vivo* has previously been studied by following the disappearance of ^{35}S-MT from the cytosol fraction of rat liver. However, loss of ^{35}S-MT from hepatic cytosol does not necessarily indicate degradation of the protein. ^{35}S-MT may have been secreted into plasma and bile (26) and transferred to other subcellular compartments (27). Therefore, we measured the level of ^{35}S-MT in heat-stable extracts of whole cell sonicates and in spent medium. The level of intracellular MT decreased 30–40% by 6 hr after transfer of zinc-loaded hepatocytes to zinc-deficient medium. This decrease reflected intracellular degradation, since less than 1% of total cellular MT was secreted.

In this study, MT synthesis was first induced by incubation of cells in medium containing a high con-

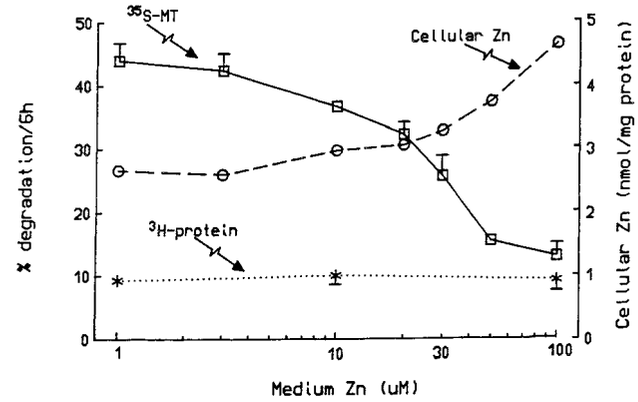

Figure 4. Effect of extracellular zinc concentration on the degradation of ^{35}S-MT and [^{3}H]Leu protein and the cellular level of zinc in monolayer cultures of hepatocytes. Zinc-loaded hepatocytes labeled with either [^{35}S]Cys or [^{3}H]Leu were incubated in medium containing indicated concentrations of zinc. The levels of ^{35}S-MT was assessed using the Superose 12 column. The levels of [^{3}H]Leu proteins were determined as described in Materials and Methods. Data are presented as percentage of the initial cellular level of ^{35}S-MT and [^{3}H]Leu protein that were degraded during the 6-hr incubation period. Cellular zinc levels were calculated by assessing zinc uptake and efflux at each level of medium zinc. The cellular concentration of zinc at the beginning of the experiment was 3.87 ± 0.20 nmol/mg protein. Where absent, SE was less than the height of the datum point.

Table II. Effects of Chloroquine, 3-Methyladenine, TLCK, and Insulin Deficiency on the Degradation of [^{3}H]Leu Protein and ^{35}S-MT and Cellular Zinc Status[a]

Treatment	^{3}H protein (% degradation)	^{35}S-MT	Cellular zinc (nmol/mg protein)
None	9.2 ± 0.1*	29 ± 1**	2.67 ± 0.06**
Chloroquine (1 mM)	5.4 ± 0.4***	10 ± 1***	2.33 ± 0.07*
TLCK (0.5 mM)	7.7 ± 0.2****	14 ± 1*	2.37 ± 0.09*
3-Methyladenine (5 mM)	4.8 ± 0.1***	28 ± 2**	ND[b]
Insulin deficient	12.0 ± 0.2**	30 ± 1**	2.32 ± 0.20*

[a] Hepatocytes were incubated in medium containing 100 μM zinc and either [^{35}S]Cys or [^{3}H]Leu for 20 hr. Intracellular free [^{3}H]Leu was chased from cells exposed to medium containing excess Leu for 4 hr. To initiate experiments, fresh medium containing 1 μM zinc and indicated chemicals were added. Insulin (10^{-8} M) was present unless otherwise indicated. Cellular zinc status was determined in replicate sets of cells as outlined in Materials and Methods. Means in each column with different asterisks are significantly different at $P < 0.05$.
[b] ND = not determined.

centration of zinc. After removal of the inducing metal, degradation of the accumulated protein was examined. When the concentration of extracellular zinc was 1 μM, ^{35}S-MT was degraded rapidly with a half-life of 11 hr; the mean half-life of non-MT cellular proteins was 29 hr. The observed rate of ^{35}S-MT degradation in this study was quite similar to previous studies using HeLa cells (28) and a cadmium-resistant line of Chinese hamster ovary cells (29), viz., 10–12 hr. Kobayashi *et al.* (30) reported that the turnover of ^{35}S-MT in the soluble fraction of zinc-loaded Chang liver cells (40%/3 hr) upon transfer to low zinc medium. Thus, the half-life of ^{35}S-MT in cultured cells appears to be shorter than that of cytoplasmic ^{35}S-MT *in vivo* (2, 3, 5). Similarly, in this and related studies (18, 31) non-MT hepatic proteins were degraded more rapidly ($t_{1/2}$ = 20–32 hr) than in the liver of intact animals ($t_{1/2}$ = 3–4 days) (2, 32). This enhanced rate of general protein degradation in cultured liver cells is probably due to the numerous differences in nutritional and hormonal factors *in vivo* and *in vitro*. Nevertheless, the rate of turnover of zinc-induced ^{35}S-MT is three to four times faster than that of general hepatic protein both *in vitro* ($t_{1/2}$ =11 vs 29 hr) and *in vivo* ($t_{1/2}$ =18–20 hr vs 3–4 days).

To further characterize the degradation of hepatic zinc-MT, the impact of extracellular zinc on both cellular zinc status and MT turnover was determined. The cellular concentration of zinc was correlated with the extracellular level of this micronutrient. For example, cellular zinc declined significantly when hepatocytes were incubated in medium with 1 μM zinc whereas intracellular zinc content was not significantly altered when zinc-loaded monolayers were incubated in medium with 50 μM zinc. The rates of ^{35}S-MT degradation were 3-fold different (15 vs 44%/6 hr) in hepatocytes incubated in medium containing 50 and 1 μM medium zinc, respectively. Enhanced turnover of MT in response to reduction of cellular zinc status has been previously reported. The half-lives of zinc-MT in HeLa cells were 36–38 and 11–12 hr for cultures that were incubated in medium containing 37 μM and 1.5 μM zinc, respectively (28). Similarly, the half-lives of ^{35}S-MT in hepatic cytosol were decreased 50% when rats had previously been fed excessive (2000 ppm) zinc were given a diet containing an adequate (18 ppm) level of the metal (33). The turnover of hepatic cadmium-MT was also faster in rats fed a diet deficient in zinc than with adequate zinc (34).

Maintenance of the native structure of MT is dependent on the presence of bound metal. Conversion of zinc-MT to apo-MT transforms the polypeptide from a highly ordered structure to a random coil conformation (35). Since zinc stabilizes the tertiary structure of MT, it appears that this metal is an important determinant of the rate of MT degradation. This conclusion is consistent with the general concept that metabolites, substrates, coenzymes, and other cofactors affect the rate of degradation of specific proteins via ligand-induced alterations in the conformation of the protein. For example, binding of zinc and iron to carboxpeptidase A and ferritin, respectively, retards the rate of degradation of these polypeptides (36, 37).

It generally has been assumed that lysosomes represent the primary site of MT degradation. The acidic environment within lysosomes facilitates dissociation of zinc and cadmium from thionein (7), thereby increasing susceptibility of the polypeptide to proteolytic attack. In contrast, the copper-thiol bond is acid stable, a property that explains in part the presence of high levels of copper-MT in hepatic lysosomes of Bedlington terriers (38), in individuals with Wilson's disease (39), and in renal lysosomes of diabetic rats (40). The ability of the lysosomotropic amine chloroquine to attenuate MT degradation in hepatocytes supports a role for lysosomes in the turnover of zinc-MT. The insensitivity of ^{35}S-MT turnover to insulin deprivation and 3-methyladenine provides preliminary evidence that MT enters lysosomes by the process of microautophagy (20, 23).

TLCK, a compound that inhibits the activity of serine and some cysteine proteases without affecting lysosomal processes (21, 22), inhibited the degradation of ^{35}S-MT by 50%; this inhibition was 3-fold greater than that observed with [^{3}H]Leu protein. These data provide the first evidence that MT is also degraded in the cytoplasmic compartment of intact cells. This finding is supported by the observations that the neutral serine proteases trypsin, subtilisin, and elastase partially degrade MT *in vitro* (7, 9). Moreover, trypsin-like proteases preferentially catalyze the hydrolysis of peptide bonds in which lysine and arginine contribute the carbonyl group. Both isoforms of rat liver MT contain six to eight lysine residues and the primary sequence of cys-lys-lys-ser (residues 29–32) is found at the hinge connecting A and B domains of MT (17). These lysine residues in the hinge region may be particularly susceptible to cleavage by cytoplasmic endopeptidase(s) with trypsin-like activity. Several serine endopeptidases in rat liver cytosol with trypsin-like activity have been recently characterized (10, 11). The possible role of these enzymes in the cytoplasmic degradation of MT and its derivatives merits investigation.

Recent investigations support the proposal that the degradation of a protein is controlled by both its structural properties and the regulation of the catabolic machinery itself (41). In regards to MT degradation we propose that cellular zinc status is the primary factor affecting the regulation of MT degradation. Binding of the metal is necessary for maintenance of the conformational integrity of this protein. Reduction in the level of extracellular zinc mobilizes labile hepatic zinc

pools, including MT-zinc (42). Such dissociation of zinc would increase cellular levels of "denatured" MT, i.e., partially saturated MT, apo-MT, and, possibly, MT polymers, thereby facilitating cleavage by cytoplasmic and lysosomal proteases. Whether MT uptake into lysosomes is dependent on structural modification and/or limited proteolysis within the cytoplasm requires further investigation.

Support for M. I. C. was graciously provided by the Pratt Animal Nutrition Program at VPI & SU. We greatly appreciate the excellent technical assistance of Karen Seidel and the efforts of Chris Woods in the preparation of the manuscript.

1. Cousins RJ. Absorption, transport and hepatic metabolism of copper and zinc: Special reference to metallothionein and ceruloplasmin. Physiol Rev **65**:238–309, 1985.
2. Feldman SL, Cousins RJ. Degradation of hepatic zinc-thionein following parenteral zinc administration. Biochem J **160**:583–588, 1976.
3. Feldman SL, Squibb KS, Cousins RJ. Degradation of cadmium thionein in rat liver and kidney. J Toxicol Environ Health **4**:805–813, 1978.
4. Bremmer I, Hoekstra WG, Davies NT, Young BW. Effect of zinc status on the synthesis and degradation of copper induced metallothionein. Biochem J **174**:883–892, 1978.
5. Cain K, Holt DE. Metallothionein degradation: metal composition as a controlling factor. Chem-Biol Inter **28**:91–106, 1979.
6. Mehra RK, Bremmer I. Studies on the metabolism of rat liver copper metallothionein. Biochem J **227**:903–908, 1985.
7. Feldman SL, Failla ML, Cousins RJ. Degradation of liver metallothioneins in vitro. Biochim Biophys Acta **544**:638–646, 1978.
8. Squibb KS, Pritchard JB, Fowler BA. Cadmium-metallothionein nephropathy: Relationships between ultrastructural/biochemical alterations and intracellular cadmium binding. J Pharmacol Exp Ther **229**:311–321, 1983.
9. Winge DR, Miklossy KA. Domain nature of metallothionein. J Biol Chem **257**:3471–3476, 1982.
10. Tanaka K, Ii K, Ichihara A, Waxman L, Goldberg AL. A high molecular weight protease in the cytosol of rat liver. I. Purification, enzymological properties and tissue distribution. J Biol Chem **261**:15197–15203, 1986.
11. Hiroi Y, Natori Y. Purification and characterization of a native protease from rat liver cytosolic fraction. J Biochem **102**:985–992, 1987.
12. Failla ML, Cousins RJ. Zinc uptake by isolated rat liver parenchymal cells. Biochim Biophys Acta **538**:435–444, 1978.
13. Kramer BL, Stacker JL, Sawada N, Sattler GL, Stephen-Hsia MT, Pitot HC. Use of low speed, iso-density Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparation. In Vitro Cell Dev Biol **22**:201–211, 1986.
14. Chen ML, Failla ML. Metallothionein metabolism in the liver and kidney of the streptozotocin-diabetic rat. Comp Biochem Physiol **90B**:439–445, 1988.
15. Dulley JR, Grieve PA. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal Biochem **64**:136–141, 1975.
16. Lehman LD, Klaassen CD. Separation and quantitation of metallothionein by high performance liquid chromatography coupled with atomic absorption spectrophotometry. Anal Biochem **153**:305–314, 1986.
17. Winge DR, Nielson KB, Zeikus RD, Gray WR. Structural characterization of the isoforms of neonatal and adult rat liver metallothionein. J Biol Chem **259**:11419–11425, 1984.
18. Hopgood MF, Clark MG, Ballard FJ. Protein degradation in hepatocyte monolayers. Effects of glucagon, adenosine 3′,5′-cyclic monophosphate and insulin. Biochem J **186**:71–79, 1980.
19. Seglen PO, Grinde B, Solheim AE. Inhibition of the lysosomal pathway of protein degradation in isolated rat hepatocytes by ammonia, methylamine, chloroquine and leupeptin. Eur J Biochem **95**:215–225, 1979.
20. Seglen PO, Gordon PB. 3-Methyladenine: Specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA **79**:1889–1892, 1982.
21. McIlhinney A, Hogan BLM. Effect of protease inhibitors on protein degradation in rat hepatoma cells. Biochim Biophys Acta **372**:358–365, 1974.
22. Chida K, Kato N, Kuroki T. Down regulation of phorbol diester receptors by proteolytic degradation of protein kinase C on a cultured cell line of fetal rat skin keratinocytes. J Biol Chem **261**:13013–13018, 1986.
23. Mortimore GE, Poso AR. Lysosomal pathways in hepatic protein degradation: Regulatory role of amino acids. Fed Proc **43**:1289–1294, 1984.
24. SAS Institute. SAS User's Guide. SAS (Statistical Analysis System) Institute. Raleigh, NC, 1979.
25. Segal HL, Kim YS. Glucocorticoid stimulation of the biosynthesis of glutamic-alanine transaminase. Proc Natl Acad Sci USA **50**:912–918, 1963.
26. Bremmer I, Mehra RJ, Morrison JN, Wood AM. Effects of dietary copper supplementation of rats on the occurrence of metallothionein I in liver and its secretion into blood, bile and urine. Biochem J **235**:735–739, 1986.
27. Bremmer I. Involvement of metallothionein in the hepatic metabolism of copper. J Nutr **117**:19–29, 1987.
28. Karin M, Slater EP, Herschman HR. Regulation of MT synthesis in HeLa cells by heavy metals and glucocorticoids. J Cell Physiol **106**:63–74, 1981.
29. Monia BP, Butt TR, Ecker DJ, Mirabelli CK, Crooke ST. Metallothionein turnover in mammalian cells: Implication in metal toxicity. J Biol Chem **261**:10957–10959, 1986.
30. Kobayashi S, Imano M, Kimura M. Induction and degradation of Zn-, Cu- and Cd-thionein in Chang liver cells. Chem Biol Interact **52**:319–334, 1985.
31. Gallo G, Voci A, Schwarze PE, Fugassa E. Effect of tri-iodothyronine on protein turnover in rat hepatocyte primary cultures. J Endocrinol **113**:173–177, 1987.
32. Duncan WE, Offermann MK, Bond JS. Intracellular turnover of stable and labile soluble liver proteins. Arch Biochem Biophys **199**:331–341, 1980.
33. Oh SH, Deagen JT, Whanger PD, Weswig PH. Biological function of metallothionein-IV. Biosynthesis and degradation of liver and kidney metallothionein in rats fed diets containing zinc or cadmium. Bioinorg Chem **8**:245–254, 1978.
34. Held DD, Hoekstra WG. The effects of zinc deficiency on turnover of Cd-MT in rat liver. J Nutr **114**:2274–2282, 1984.
35. Vasak M, Galdes A, Hill HAO, Kagi JHR, Bremmer I, Young BW. Investigation of the structure of metallothioneins by protein nuclear magnetic resonance spectroscopy. Biochemistry **19**:416–425, 1980.
36. Bicknell R, Schaeffer A, Auld DS, Riordan JR, Monnami R, Bertini I. Protease susceptibility of zinc and apocarboxypeptidase. Biochem Biophys Res Comm **133**:787–793, 1985.
37. Munro H, Drysdale JW. Role of iron in the regulation of ferritin metabolism. Fed Proc **29**:1469–1473, 1970.
38. Lerch K, Johnson CF, Grushoff PS, Sternlieb I. Canine hepatic lysosomal copper protein: identification as metallothionein. Arch Biochem Biophys **243**:108–114, 1985.
39. Hanaichi T, Kidokoro R, Hayoshi H, Sakamoto H. Electron

probe x-ray analysis on human hepatocellular lysosomes with copper deposits: Copper binding to thiol-protein in lysosomes. Lab Invest **51**:592–597, 1984.
40. Gassman CA, Failla ML, Osborne SP, Alexander AR. Copper accumulation in the soluble and particulate fractions of renal cortex in the streptozotocin-diabetic rat. Biol Trace Elem Res **5**:475–487, 1983.
41. Beynon RJ, Bond JS. Catabolism of intracellular protein: molecular aspects. Am J Physiol **251**:C141–C152, 1986.
42. Pattison SE, Cousins RJ. Kinetics of zinc uptake and exchange by primary cultures of rat hepatocytes. Am J Physiol **250**:E677–E685, 1986.