REGULATION OF TISSUE LEVELS OF METALLOTHIONEIN WITH EMPHASIS ON METALLOTHIONEIN DEGRADATION

by

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(ABSTRACT)

The synthesis and degradation of metallothionein (MT) was studied in streptozotocin-induced diabetic rats and monolayer cultures of adult rat hepatocytes. Elevated levels of MT-I and MT-II were identified in the liver and kidney of untreated diabetic rats. The relative rates of hepatic and renal MT synthesis were significantly higher in STZ-diabetic rats than in controls. The changes in the relative rate of MT synthesis were maximal by 4 and 10 days in liver and kidney, respective, after administration of streptozotocin. The relative rate of cytoplasmic MT turnover was also increased in liver, but largely unaffected in the kidney, of diabetic rats. The altered metabolism of hepatic MT in diabetic rats was attributed primarily to chronic changes in the levels of pancreatic and adrenal hormones in plasma. In contrast, increased synthesis of renal MT in the diabetic rat was due largely to accumulation of excessive dietary Cu in the kidney.

Critical analysis of *in vivo* studies with diabetic rats and other literature revealed that cytoplasmic turnover of MT may not reflect actual degradation of this protein. Therefore, the characteristics of MT degradation in primary cultures of hepatocytes were investigated in subsequent studies. Hepatocytes were incubated in medium containing ^{35}S-cysteine and 100 uM Zn overnight to induce MT synthesis. The level of ^{35}S-MT was quantified in heat stable extracts of cell homogenates by Fast Protein Liquid Chromatography (FPLC). When Zn was removed from medium, the rate of ^{35}S-MT turnover ($t_{1/2}$= 7 hours) was four times faster than general ^{3}H-protein ($t_{1/2}$= 29 hours). This decrease in cellular MT level reflected degradation since less than 1% of cellular MT was secreted. The rate of MT degradation was inversely proportional to cellular Zn status. Cycloheximide, chloroquine and tosyl-lysine chloromethyl ketone (TLCK) inhibited ^{35}S-MT degradation by 33, 65 and 50%, respectively, without affecting cellular Zn status. Degradation of ^{3}H-protein was inhibited by 41, 41 and 16% in the presence of cycloheximide, chloroquine and TLCK, respectively. Removal of insulin increased ^{3}H-protein degradation by 30%, but did not alter ^{35}S-MT degradation. Together, these data suggest that hepatic MT degradation (a) is primarily regulated by cellular Zn status and (b) occurs in both lysosomal and cytoplasmic compartments.

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Finally, I would like to thank my parents and my family members for their love and supportive role during my stay in USA.

DEDICATION

I dedicate this work to my parents - their love and inspiration has and will always accompany me wherever I am.

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CHAPTER I

LITERATURE REVIEW

General characteristics of metallothionein

Metallothioneins (MT) represent a family of low molecular weight proteins with high affinity for several essential micronutrients (i.e., Zn and Cu) and a variety of toxic transition metals (e.g., Cd, Hg and Ag). Comparison of the amino acid composition of MT isolated from different species reveals that its primary sequence is highly conserved (1). MT is a single polypeptide chain with 61 amino acids. At least two isoforms of this protein that differ slightly in amino acid composition and charge are usually present in mammalian tissues. All MT lack aromatic amino acids and contain 25-35 mole percent cysteinyl residues. The position of the cysteinyl residues along the polypeptide chain is invariant and exists in cys-x-cys and cys-cys sequences (2). Heavy metals associate with MT via thiolate bonds with all cysteinyl residues. The relative affinity of metals for MT is as follows : Ag = Cu > Hg > Cd > Zn.

The tertiary structure of MT consists of two domains. The two cluster arrangement of MT has been confirmed by ^{113}Cd-NMR, circular dichroism, proton nuclear magnetic resonance spectroscopy and extended X-ray-absorption fine structure studies (3-8). The A domain is located on the carboxyl-terminal end of polypeptide (residues 31-61) and the B domain contains residues 1-30 (9). Seven atoms of Cd^{2+} or Zn^{2+} are bound by one molecule of apo-thionein. The A and B domains contain 4 and 3 metal ions, respectively, with all metal ions bound to cysteinyl residues. The metal-thiolate cluster in Zn-

or Cd-MT is tetrahedrally coordinated (4,10). In Cu-MT, both domains contain 6 atoms of Cu^{1+} and displays trigonal geometry (11). By using an *in vitro* metal reconstitution system and susceptibility to enzymic digestion, Nielson and Winge (12) demonstrated that Cd and Zn preferentially bind to the A domain and the binding of metal ions within each domain is highly cooperative. The sequence of Cu binding to apo-thionein occurs in the reverse order of Cd and Zn, i.e., Cu^{1+} binds to the B domain first (13,14).

The metal composition of hepatic metallothionein isolated from Cd-exposed animals is heterogenous. It usually contains 2 moles of Zn and 5 moles of Cd per mole of MT. Netteshein et al. (15) have demonstrated that displacement of Zn from Zn_7-MT by ^{113}Cd does not show preferential binding to a specific domain and no cooperativity was observed. By mixing appropriate amounts of Cd_7-MT and Zn_7-MT, the native composition of Cd_5,Zn_2-MT can be obtained *in vitro*. *This suggests that direct metal exchange* between Cd_7-MT and Zn_7-MT leads to formation of Cd_5,Zn_2-MT *in vivo*.

Structure and function of metallothionein genes

The structure and regulation of MT genes have received intensive study (see 16 for review). The functional MT genes from different species have similar structure with two introns and three exons (17,18). Human and rat MT genes are encoded by multigene families.

In addition to the functional MT genes, several pseudogenes have been described. These pseudogenes probably arose from either accumulation of point mutations (19) or insertion of reverse transcripts of MT mRNA into the genome (18,20).

MT synthesis is induced by a variety of factors, including exposure to elevated levels of essential and nonessential heavy metals, physiological stresses, and changes in endocrine status (21-25). In virtually all cases examined, increased synthesis of MT resulted from increased transcription of MT genes (16). Recent studies have shown that MT gene expression is also increased in cells harboring the human ras oncogenes and is activated by protein kinase C (26,27). Furthermore, differential transcriptional activity has been demonstrated for human MT-I_A and MT-II_A genes. The MT-II_A gene is responsive to Cd, Zn and glucocorticoid, while the MT-I_A gene is responsive only to Cd (28).

Analysis of deletion or fusion mutants of MT genes revealed that at least two metal regulatory elements (MREs) and one glucocorticoid regulatory element were located in the 5′-flanking region. The MRE consists of a 12-base-pair sequence. Insertion of this conserved sequence into the promoter region of thymidine kinase gene confers regulation of gene expression by heavy metals (29). It also has been postulated that the glucocorticoid regulatory element of MT gene is a DNA binding site for the glucocorticoid-receptor complex (30). Although trans-acting factors that regulate MT gene transcription

have not been identified, it has been suggested that intracellular factors that induce transcription of these genes are positive activators (31). Sequin and Hamer (32) demonstrated that these trans-acting factors are multiple proteins that bind to MREs of the mouse MT-I gene and can be activated by addition of Cd *in vitro*. In contrast to the observation in mammals, it was found that yeast Cu-thionein acts as a negative autoregulator to thionein genes (33). The mechanism by which these trans-acting factors regulate transcription of MT genes remains unknown.

Functions of metallothionein

Detoxification of heavy metals was the first function proposed for MT. This function was supported after the initial purification of the Cd and Zn binding protein from equine kidney (34). This proposal was supported by the isolation of several Cd resistant mammalian cell lines (e.g. 35,36). The induction of MT synthesis was found to be several-fold higher for Cd-resistant mutants than for wild-type. The increased production of MT in these cell lines is due to gene amplification and is correlated with the decreased toxicity of Cd (36,37). Since high tissue levels of Cd are found only when animals are exposed to toxic environments, it is doubtful that a transient detoxification system would be preserved under the pressure of evolutionary development (38). Therefore, many investigators have not accepted the proposal that the primary function of MT is

detoxification of heavy metals.

Elevation of hepatic levels of Zn and Cu by dietary and parenteral manipulations also markedly increase MT levels (39-42). Furthermore, higher concentrations of MT are present in fetal liver during the third trimester and in the neonatal liver of various animals and humans (43-45). Together, such observations have led to the suggestion that MT functions primarily in Zn and Cu homeostasis. However, Cu-thionein is not required for normal growth of lower eukaryotes, i.e., yeast, in medium containing 0.1 uM Cu (31). More recent studies have provided evidence that this protein also may participate in the intracellular tranport of Zn and Cu, cellular growth, and protection against free radicals (38). Despite numerous studies concerning the protein, the physiological function of MT remains speculative.

Metallothionein metabolism

The synthesis and turnover of MT are subjected to complex hormonal control. Among hormones tested, elevated levels of epinephrine (46), glucagon (47), glucocorticoid (48,49) and dibutyryl cAMP (50) have all been shown to increase hepatic concentrations of MT. Since administration of actinomycin D to animals blocks the induction of MT synthesis in response to these hormones (50), it has been suggested that hormonal modulation of MT level requires transcriptional events (51,52). The glucocorticoid-mediated

induction of MT synthesis has been characterized in greater detail. It has been suggested that glucocorticoid-receptor complex binds to the glucocorticoid regulatory element of MT gene and thus increases its transcriptional efficiency (30). However, the molecular mechanisms by which other hormones induce MT synthesis are unknown. It is apparent that further research is required to explore the metabolic regulation of MT synthesis.

In our laboratory, studies revealed marked alterations in the metabolism of essential trace metals in insulin-deficient diabetic rats. Significant amounts of Zn and Cu accumulated in the liver and kidney of untreated diabetic rats (53,54). Furthermore, the additional complement of Zn and Cu was primarily associated with MT-like proteins. Similar metal accumulation was found in spontaneously diabetic BB Wistar rats (55). Failla and associates (56) have suggested that the accumulation of high levels of Cu in kidney may contribute to the development of renal dysfunction, which is commonly observed in diabetes. Since these diabetic animals are characterized by chronically decreased plasma levels of insulin and elevated levels of glucagon and adrenal hormones (57), it also has been suggested that the changes in Cu and Zn metabolism in untreated diabetic rats were due to endocrine imbalance. Additional studies revealed that the increased levels of Cu and MT-like proteins in diabetic kidney were due in part to hyperglucocorticoidemia (58). Together, these studies have demonstrated the physiological relevance

of in vitro observations that glucagon and glucocorticoid hormones influence various aspects of trace metal and MT metabolism.

In contrast to the plethora of information available concerning MT synthesis, knowledge of MT degradation is very limited. In vivo experiments have suggested that the rate of cytosolic MT turnover is influenced by the species of bound metal. These studies primarily have measured the rate at which ^{35}S-MT disappears from cytosol by using gel-permeation chromatography to quantify cytosol MT levels (59-64). The half-life ($t_{1/2}$) for cytoplasmic ^{35}S-MT is 18-20 hours following i.p. injection of zinc salts to animals. MT-I and -II exhibited similar rates of disappearance from the cytoplasm (59). In contrast, the $t_{1/2}$ of cytoplasmic MT was approximately 80 hours following administration of cadmium salts (60). Intracellular Cd remains bound to MT for much extended periods, presumably because Cd released during MT "turnover" binds to newly synthesized apo-thionein (61). The turnover rate of cytosolic Cu-MT ($t_{1/2}$=15-18 hours) was found to be slightly faster than Zn-MT (64). Whether the measurement of cytoplasmic MT turnover reflects actual degradation of this protein has not been critically addressed. Loss of ^{35}S-MT from cytoplasm might reflect transfer to another cellular compartment, viz., lysosomes (65).

It has been assumed that MT is degraded within lysosomes, since the latter contain an array of hydrolases capable of digesting most cellular macromolecules (3,65). In vitro degradation of MT also has

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Whether this mechanism for the degradation of extracellular MT reflects the fate of intracellular MT is unknown.

Another potential fate of hepatic Cu-MT may be secretion into extracellular fluids, as suggested by Bremner et al. (73). Monomeric and aggregated MT-I were detected by radioimmunoassay in plasma and bile. In addition, various low molecular weight species in bile reacted with anti-MT sera. These may represent partial degradation products of MT. These authors estimated that secreted MT could account for 14% of the turnover of liver MT-I (73,74). The possibility that the secreted protein was partially degraded by extracellular proteases and/or proteases on the trans surface of plasma membrane of vascular endothelial and ductal epithelial cells has not been considered. Obviously, the fate of cytoplasmic MT has not been critically addressed and requires further investigation.

Mechanisms of protein degradation

Current information indicates that cellular proteins are degraded in a heterogenous manner. For example, the regulatory enzymes in rat liver turn over rapidly, thereby enabling the cell to respond to various metabolic demands. The half-lives of these regulatory enzymes (e.g. ornithine decarboxylase and 3-phosphoglycerate dehydrogenase) range from 0.2 to 15 hours (75). The average half-life for rat liver cellular proteins is approximately 3 days. There is no evidence that substrate-specific proteolytic systems

exist. The heterogenous rate of protein turnover may be due to the intrinsic properties of protein. It has been suggested that proteins with short half-lives tend to be more acidic, large and hydrophobic (76). A model proposing that protein turnover is subject to thermodynamic controls has been suggested. It was stated that the susceptibility of a protein to proteolytic attack is dependent on its intramolecular conformational equilibrium; a protein is more susceptible to proteolytic attack in an unfolded state (77). This is supported by the observation that the rate of degradation of oxidatively damaged and, therefore, denatured protein is higher than that of the native protein (78-80). Simlarly, covalent modification of cysteinyl residues (i.e., formation of intramolecular disulfide bonds) of fructose 1,6-bisphosphate aldolase increases susceptibility of this protein to proteolytic digestion (81).

The suggestion that the physiochemical structure of a protein controls its rate of turnover has gained further support from other recent observations. The primary sequences of some short-lived proteins contain regions rich in proline, glutamine, serine and threonine (PEST regions). It has been proposed that such PEST regions impart unusual conformations that render the protein more susceptible to proteolysis (82). Another interesting finding is that the half-life of a protein may depend on the nature of its N-terminal amino acid residue (N-end rule), as in the case of B-galactosidase (83). However, the physiochemical structure of a protein does not

seem to be the sole determinant of its degradation. The stabilizing effects of ligands (e.g., substrates and organic and inorganic cofactors) on protein turnover are well established. For example, mevinolin, an inhibitor of hydroxymethylglutaryl Co-A reductase, decreases the rate of turnover of this enzyme (84). Similarly, the turnover of apo-thionein is much faster than its metal associated holoprotein (66). Other factors affecting protein degradation have been reviewed recently by Beynon and Bond (85).

Mechanisms of intracellular proteolysis

Intracellular proteolytic systems are generally divided into two components, viz., lysosomal and non-lysosomal processes. The lysosomal degradation pathway has received far greater attention than other intracellular protein degradation systems. The lysosomal pathway is often divided into two components with regard to the origin of substrates, i.e., autophagic and heterophagic pathways (86-92 for review). In autophagy, a specialized membrane structure forms around intracellular macromolecules and organelles. These vesicles are called autophagosomes. The origin of this specialized membrane has not been identified, although the Golgi complex may be a source. Autophagosomes subsequently fuse with primary lysosomes and give rise to secondary lysosomes in which protein degradation occurs (Fig. 1.1). Intracellular substrates may enter lysosomal vesicles by invagination of lysosomal membrane. Thus, proteins adsorbed on the

trans surface of the lysosomal membrane are internalized. This processes is referred to as microautophagy (85).

In well nourished cell cultures, there is little evidence of autophagic structures and the rate of proteolysis is relatively low. This basal proteolytic activity is probably due to basal lysosomal activity (microautophagy) or cytoplasmic proteases (87). The enhanced rate of protein degradation in nutrient-deprived cells is due to increased autophagy. Addition of serum, insulin, amino acids or a variety of growth factors inhibits autophagy (93-100). Insulin and amino acids are known to decrease the rate of cellular proteolysis by inhibiting the sequestration of intracellular material into autophagosomes, while glucagon increases the number of autophagosomes formed (96,99). While autophagy seems to be regulated at least in part by hormones, basal proteolysis is relatively unaffected.

Lysosomal and non-lysosomal processes may be distinguished by using lysosomotrophic inhibitors (101). Weak-base amines, e.g., chloroquine, methylamine and ammonia ion, specifically raise the intralysosomal pH thereby providing a suboptimal environment for lysosomal proteases (102,103). Similarly, inhibitors of specific lysosomal enzymes block protein degradation in intact cells. Pepstatin, leupeptin, chymostatin and antipain are commonly used inhibitors for different types of cathepsin proteinases (104,105). 3-Methyladenine inhibits the formation of autophagosomes (106).

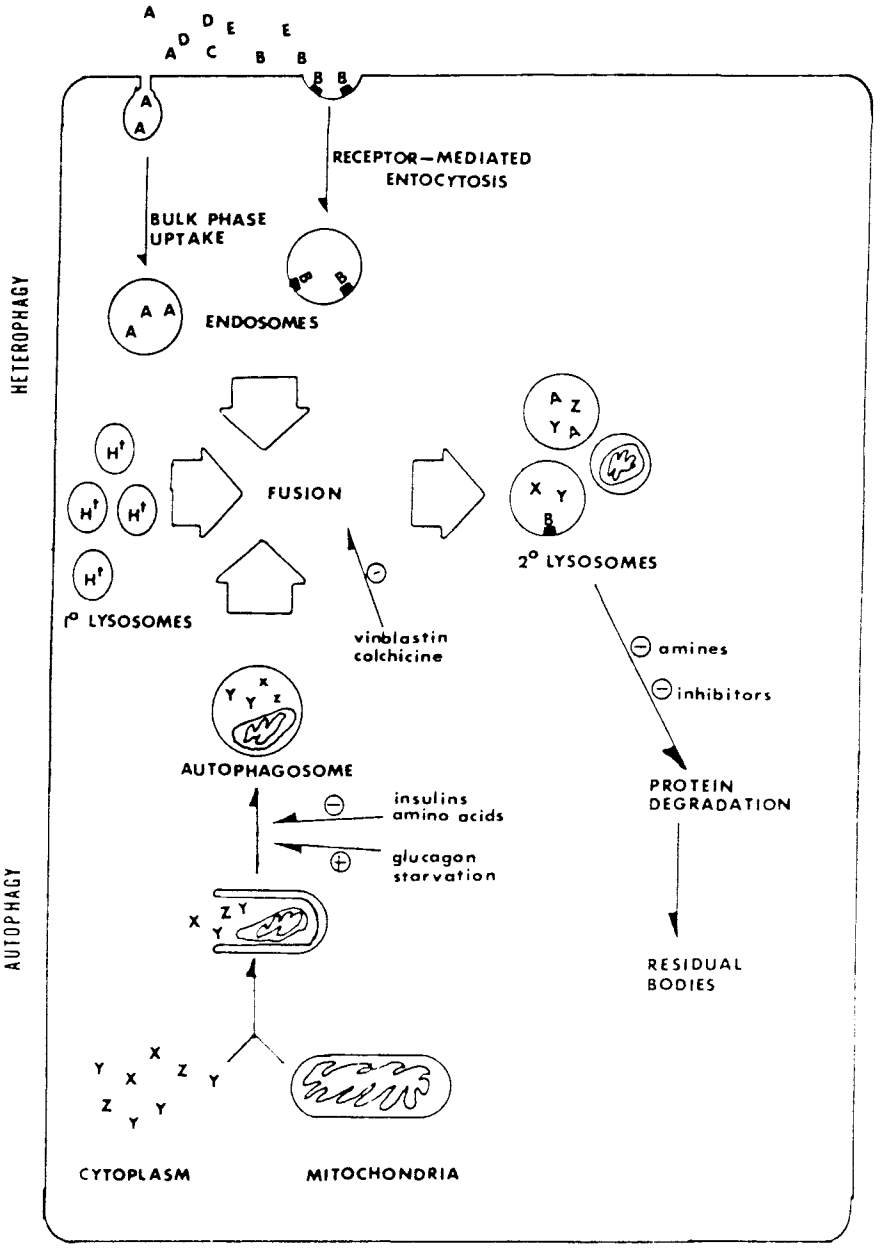

Fig. 1.1 Schematic presentation of autophagic and heterophagic pathways. In autophagy, cellular macromolecules (X,Y,Z) and organelles (i.e., mitochondria) are sequestered into autophagosomes. These vesicles are subsequently fused with primary lysosomes which contain acid hydrolases (e.g. cathepsins). Digestion of macromolecules occurs in the seconday lysosomes. Heterophagy is initiated by formation of pinocytic vesicles containing extracellular materials (A, B, C) within endocytosed plasma membrane. One possible fate of these vesicles (endosomes) is fusion with primary lysosomes to produce secondary lysosomes in which protein degradation occurs.

Vinblastine and colchicine dissociate microtubules, thereby, blocking fusion of autophagosomes and primary lysosomes (107). Recent evidence indicates that chloroquine and ammonia might not inhibit lysosomal degradation completely. Moreover, leupeptin and chymostatin also inhibit certain non-lysosomal proteinases (101). Thus, their effects on lysosomal degradation must be interpretated with reservation.

There are certain similarities between autophagy and heterophagy. Heterophagy is initiated by formation of pinocytic vesicles containing extracellular materials from portions of plasma membrane. These vesicles, referred to as endosomes, move along the cytoskeletal track to their destined target area (Fig. 1.1). One possible fate of endosomes is fusion with primary lysosomes to produce secondary lysosomes in which protein breakdown occurs (108). Although Squibb et al. (72) demonstrated that heterophagy is probably pertinent for degradation of reabsorbed MT in proximal tubule cells, the heterophagic process is not relevant for the degradation of intracellular MT. Assuming that cytoplasmic MT is degraded in lysosomes, this protein will have to enter the lysosomal vesicles via the autophagic process.

In regards to MT degradation, another possibility that does not appear to be previously considered is that the hydrolysis of this metalloprotein occurs partly or completely within the cytoplasmic compartment. Several cytoplasmic catabolic systems have been

described. The ubiquitin-, ATP-dependent proteolytic systems have been characterized in reticulocytes (109). Conjugation of ubquitin to substrate proteins is the essential step in the degradation of these tagged proteins. However, this system has not been found in liver cells. Other ubiquitin-independent, ATP-dependent proteolytic systems recently have been described in mammalian tissues including liver (110,111). Several other serine, cysteine and metal-dependent proteases have been found in rat liver cytosol (112-114). Although proteolytic activity in the cytoplasm is lower than that in lysosomes, cytoplasmic proteolytic activity may provide turnover of specific types of cellular proteins and/or provide the initial event in protein degradation. It has been shown that MT can be separated into its two domains by limited proteolysis in vitro (12). Since MT has been proposed to function as Zn and Cu donor to apo-metalloenzymes (115-117), limited proteolysis of MT by cytoplasmic proteinases may either facilitate the release of bound metals or be the consequence of such a transfer. Thus, the possibility that MT is degraded in the cytoplasmic compartment merits evaluation.

Research objectives

In the initial part of my thesis research, I first purified and characterized the Zn and Cu associated protein from liver and kidney of diabetic rat. Having established that it is indeed MT, I examined

the effects of streptozotocin (STZ)-induced diabetes on the synthesis and turnover of MT in liver and kidney (Chapter II). The relative rates of MT synthesis were increased in the liver and kidney of untreated diabetic rats. I also found that the stress-related induction of hepatic MT synthesis was coordinated with an increased turnover of cytoplasmic MT in the liver, but not in the kidney.

Having realized that the measurement of cytoplasmic MT turnover might not reflect actual degradation of this protein, I subsequently focused on MT degradation in greater detail. An understanding of the characteristics and regulatory aspects of MT degradation is as important as that of synthesis in assessing the physiological function of this protein. Elucidation of the mechanism(s) of MT degradation also may provide insights as to how MT-bound metals are mobilized in vivo. Therefore, the primary objectives of these investigations were two fold: (a) to evaluate the effects of essential trace metal status on MT degradation, and (b) to determine the subcellular site(s) of MT degradation.

Monolayer cultures of rat hepatocytes were chosen as a model system to investigate MT degradation. It is recognized that reincorporation of radiolabelled amino acid is the major obstacle in measuring protein turnover rate in whole animals (76). This problem can often be minimized by following protein degradation in a cell culture system. In addition, investigators interested in the kinetics of Zn, Cu and Cd uptake and effects of hormones on the

uptake and metabolism of these metals have effectively utilized suspension and monolayer cultures of adult rodent hepatocytes for elucidating such information (118-122). Other advantages of this cell system include (a) relative ease of isolating large numbers of one specific type of differentiated cells from the liver, (b) minimal biological variability in replicate cultures from single animal donor, and (c) ability to rapidly alter the extracellular milieu.

I initially studied the effects of hormones and lysosomotropic inhibitors on general protein degradation in monolayer cultures of adult rat liver hepatocytes to develop skills with the in vitro system (Chapter III). Then, the subcellular site(s) of MT degradation and the effects of cellular Zn status and insulin on MT degradation were investigated (Chapter IV).

CHAPTER II

METALLOTHIONEIN METABOLISM IN THE LIVER AND KIDNEY OF THE STREPTOZOTOCIN-DIABETIC RAT

INTRODUCTION

We have previously reported that the metabolism of zinc, copper, iron and manganese is markedly altered in both chemically-induced and spontaneously diabetic rats (55,123). One of the specific changes noted was the presence of significantly elevated levels of a metallothionein (MT)-like, zinc and copper binding protein in liver and kidney of diabetic rats (53-55). Because of the apparent molecular weight of this heavy metal binding protein and the known influences of stress and hormones on MT synthesis (2), we previously proposed that the protein present in high concentration in the tissues of the diabetic rats was MT. The first objective of this study was to purify and further characterize this protein(s) from diabetic liver and kidney. Having established that the zinc and copper binding proteins were indeed MT-I and -II, the effects of the insulin deficiency and dietary copper on MT synthesis and turnover were examined in the streptozotocin-induced diabetic rat.

MATERIALS AND METHODS

Animals and diets

Male rats (Rattus rattus; Sprague Dawley strain) weighing 150-175 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). Insulin-dependent diabetes mellitus was induced by an i.p. injection of streptozotocin (STZ, 90 mg/kg body weight) in 50 mM citrate buffer, pH 4.5. Diabetic rats were polydipsic, polyuric and hyperglycemic, and had a decreased growth rate. Animals were given free access to deionized water and either commercial (Prolab Rat, Mouse, Hamster Formula 3000, Agway Inc., Syracuse, NY) or purified diet. The composition of the purified diet has been reported (53). The commercial diet contained 13 and 76 ppm copper and zinc, respectively. The purified diets contained either 13 or 35 ppm copper and 45 ppm zinc.

Trace metal analysis

The levels of copper and zinc in diets and tissues were determined by atomic absorption spectrophotometric analysis of samples that had been dry ashed at 440^{o}C as described elsewhere (53). Recoveries of these metals from a certified reference material (bovine liver 1577a; National Bureau of Standards, Gaithersburg, MD) with this protocol exceeded 97%. The concentrations of copper and zinc in cytosol, chromatographic fractions and purified protein were

directly measured on samples that were appropriately diluted with 0.1 N ultrapure HCl (Ultrex, J. T. Baker, Phillipsburg, N.J.).

Purification and characterization of metallothionein

Control and diabetic rats (10 days after receiving STZ) were injected i.p. with 25 uCi ^{35}S-cysteine (sp. act. 80 uCi/umol; New England Nuclear, Boston MA) in 0.9% (w/v) saline per 100g body weight. Rats were killed 18 hours later and livers and kidneys were perfused with ice-cold saline to remove residual blood. Tissues were frozen in liquid nitrogen and stored at -70^{o}C. The soluble fraction (cytosol) of these organs were prepared by centrifugation (150,000 x g for 80 min.) of homogenates and fractionated by Sephadex G-75 gel filtration chromatography as previously described (54). Copper, zinc and ^{35}S were measured in each fraction collected from the column. Fractions of a similar elution volume as purified rat liver zinc-MT (i.e., Ve/Vo = 2.0-2.5) were pooled and concentrated by ultrafiltration (YM-2 membrane, Amicon Co., Danvers, MA). The concentrated material was further fractionated by DEAE-Sephadex A25 anion exchange chromatography. After applying the sample, columns (21 x 150 mm) were washed with 2 bed volumes of 20 mM Tris-acetate, pH 7.4, before beginning a linear gradient of 20-200 mM Tris-acetate, pH 7.4 (total volume= 400 ml). Copper, zinc and ^{35}S were measured in each fraction (4 ml). Salt concentrations were estimated from the measured conductance of fractions (Beckman Conductivity Bridge, Model

RC-16B2, Cedar Grove, NJ). Peak fractions containing radioactivity and the trace metals were pooled, dialyzed against 2.5 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol, pH 7.4, lyophilized and stored at -70°C. Before further analysis, lyophilized samples were dissolved in phosphate buffer and fractionated once again by Sephadex G-75 gel filtration chromatography using 2.5 mm phosphate buffer to remove polymers and 2-mercaptoethanol.

In a separate study, control and diabetic rats were injected i.p. with 25 uCi ^{3}H-leucine (sp. act. 80 uCi/umol) per 100 g body weight. Cytosol was prepared and analyzed by gel filtration chromatography and anion exchange chromatography as described above. Reactive sulfhydryl groups of MT isoforms obtained after DEAE-Sephadex A25 chromatography were assayed with 2,2'-dithiodipyridine (124). The level of MT protein was estimated by the method of Lowry et al. (125) using bovine serum albumin as standard. Experimentally determined values were adjusted by dividing these amounts by 2.5 to obtain the actual level of thionein polypeptide (126).

Relative levels of ^{35}S-MT synthesis in liver and kidney

Rats were randomly selected and injected with STZ (90 mg/kg). Because treatment with the diabetogenic drug temporarily decreased food consumption, and fasting affects metallothionein synthesis (39),

control and STZ-diabetic rats were pair-fed for two days after administration of STZ. At indicated times, groups (N=5) of control and diabetic rats were injected i.p. with 15 uCi ^{35}S-cysteine per 100 g body weight between 0800-0900 hour. Food was removed from cages of these rats at 0700 hour. Five hours after injection, rats were killed by decapitation and the organs were perfused, frozen and stored as above. Cytosol was prepared and analyzed by one of two methods. In the first, aliquots of cytosol were fractionated by Sephadex G-75 gel filtration chromatography (22 x 600 mm) and the amounts of ^{35}S, copper and zinc in each fraction were measured. The relative level of ^{35}S-cysteine incorporated into MT was calculated by dividing ^{35}S in fractions that eluted similarily to purified rat liver zinc-MT (Ve/Vo = 2.0-2.5) by ^{35}S present in total cytosol protein (i.e., Ve/Vo = 1.0-2.5). In the alternative procedure, trichloroacetic acid (TCA) was added to cytosol to a final concentration of 2%. Acid precipitated ^{35}S was removed by centrifugation, washed with 2% TCA, solubilized in 1N NaOH and counted by liquid scintillation spectrometry (efficiency of counting was 92%). Pilot studies showed that ^{35}S-MT was not precipitated by 2% TCA. Acid-soluble materials were fractionated by Sephadex G-50 gel filtration chromatography. Columns (9 x 300 mm) were equilibrated and eluted with 100 mM NaCl in 1mM HCl. ^{35}S was measured in collected fractions. The relative amount of MT synthesis was calculated by dividing the amount of ^{35}S that eluted similarly

to purified ^{35}S-MT (Ve/Vo = 1.4-1.8) by the sum of acid-precipitated ^{35}S plus acid soluble materials eluting as ^{35}S-MT. The results with both methods were virtually identical and those presented in Results are values obtained from analysis of intact cytosol by Sephadex G-75 gel filtration chromatography. Cytosol protein was estimated by the microbiuret procedure with bovine serum albumin as standard (127).

Rate of disappearance of ^{35}S-MT from hepatic and renal cytosol

Control and 10 day diabetic rats were injected with 15 uCi ^{35}S-cysteine per 100g body weight as above. After 24 hours, animals received 4 umoles nonradioactive cysteine per 100g body weight to miminize reutilization of ^{35}S-cysteine. Five rats from both groups were killed immediatedly and additional groups (N=5) of control and diabetic rats were killed 36, 48, 72, 96 and 120 hours after injection of ^{35}S-cysteine. Cytosol was prepared from the liver and kidney of each animal and analyzed by Sephadex G-75 gel filtration chromatography as described above. ^{35}S was measured in each fraction and half-life ($t_{1/2}$) values for ^{35}S-MT and ^{35}S-proteins with apparent molecular weights greater than MT were calculated according to Segal and Kim (128).

Data analysis

Values are presented as mean $\pm$ S.E.M. Where appropriate, data

from various treatment groups were analyzed by either Student's t-test or one way analysis of variance and Duncan's multiple range test to determine significant differences at $p < 0.05$.

RESULTS

Purification and characterization of MT in liver and kidney of diabetic rats

The Sephadex G-75 elution profiles of ^{35}S in cytosol prepared from the livers of control and diabetic rats are shown in Figure 2.1A. The amount of ^{35}S associated with species having apparent molecular weights similar to purified zinc-MT (Ve/Vo = 2.0-2.5) was 2.2-fold greater than that in control rats. In contrast, the percentage of ^{35}S in soluble proteins that eluted with apparent molecular weights in excess of 10,000 (i.e., Ve/Vo = 1.0-2.0) was similar in control and diabetic liver. In agreement with earlier reports (53,54), cytosol from diabetic liver contained significantly higher levels of copper and zinc and the majority of the additional complement of these trace metals was associated with MT-like species (Fig. 2.1B).

Total ^{35}S and acid-insoluble ^{35}S were similar in cytosol of diabetic and control rat kidney. However, 1.5-fold more ^{35}S in diabetic renal cytosol eluted at Ve/Vo = 2.0-2.5 compared to control cytosol (Fig. 2.2A). The copper and zinc concentrations in cytosol and the amounts of these trace metals that eluted from Sephadex G-75 at approximately twice void volume were significantly higher for diabetic kidney than for control kidney (data not shown).

MT-like materials were further purified by ion-exchange

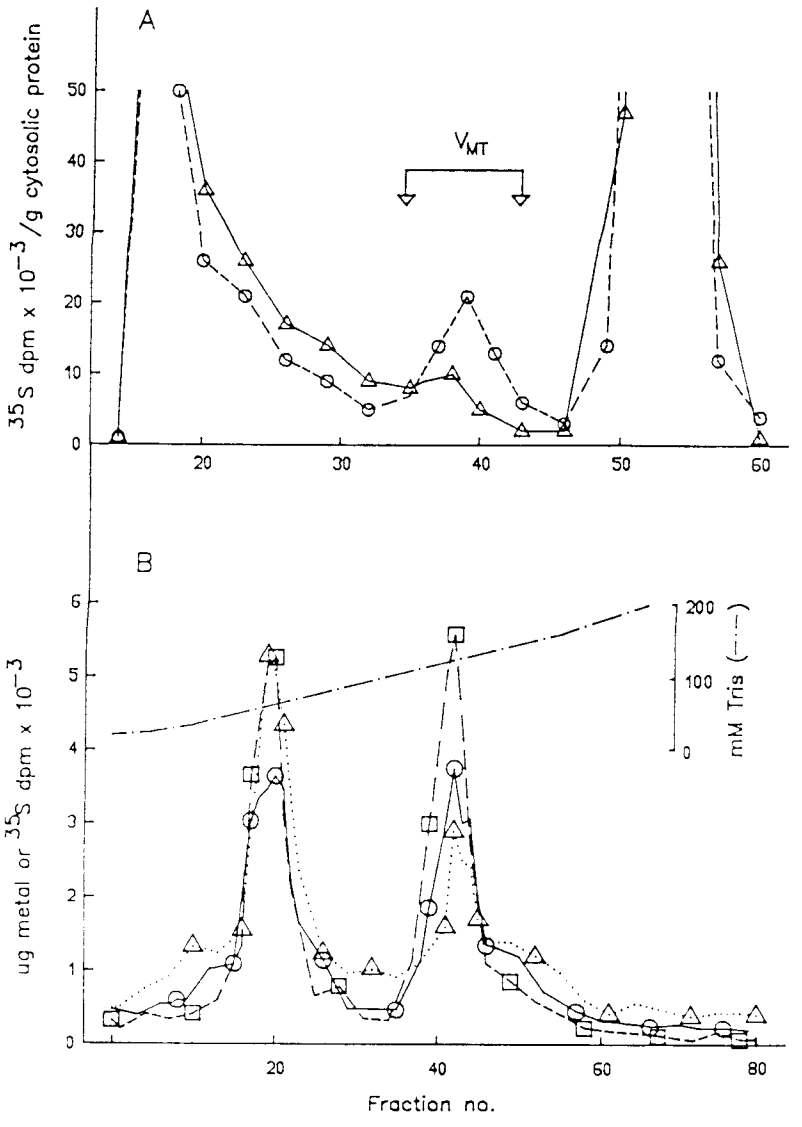

Fig. 2.1 Purification of MT from liver of control and STZ-diabetic rats. Aliquots of cytosol samples from livers of control and diabetic rats were fractionated by Sephadex G-75 chromatography. Eluted fractions that corresponded to purified zinc MT were pooled and analyzed by DEAE-Sephadex A25 chromatography as described in Methods. Panel A shows Sephadex G-75 elution profile of cytoplasmic ^{35}S from livers of control (△) and STZ-diabetic (○) animals. Panel B shows DEAE-Sephadex A25 elution profile for representative samples from STZ-diabetic rats. ^{35}S (○), zinc (□) and copper (△).

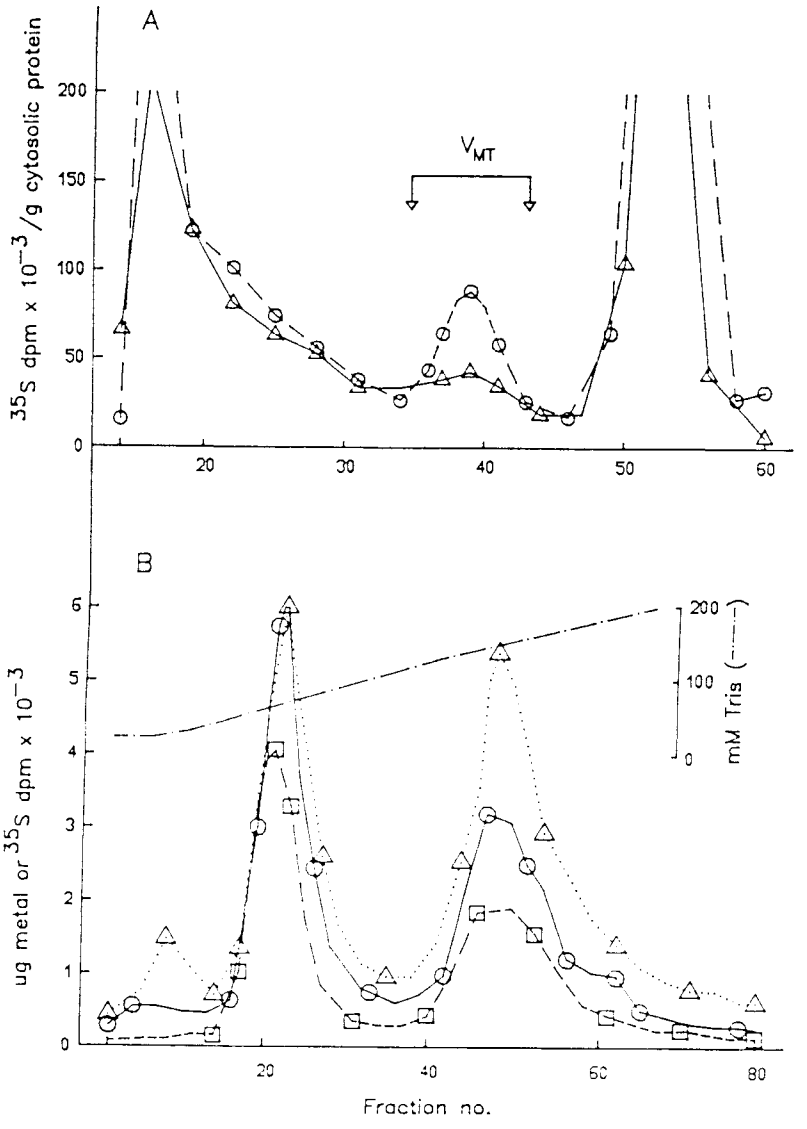

Fig. 2.2 Purification of MT from kidney of control and STZ-diabetic rats. Procedures were identical to that described for hepatic samples in the legend to Fig. 2.1. Representative Sephadex G-75 elution profiles of renal cytoplasmic species containing ^{35}S-cysteine from control (△) and STZ-diabetic (○) rats are shown in panel A. Representative DEAE-Sephadex A25 elution profile of renal sample obtained from STZ-diabetic animals is shown in panel B. ^{35}S (○), zinc (□) and copper (△).

chromatography. The elution patterns shown in Figure 2.1B and 2.2B indicate that ^{35}S, copper and zinc in hepatic and renal samples were present in two forms. These eluted at 65 and 135 mM Tris-acetate; purified rat liver zinc-MT I and II eluted at 70 and 140 mM Tris-acetate, respectively. Approximately 75% of the ^{35}S applied to DEAE-Sephadex column eluted in these two peaks. This indicated that these two MT-like species accounted for the majority of ^{35}S-cysteine that eluted from Sephadex G-75 at approximately twice void volume. Various attempts to assess the purity of the material in two peaks by native and sodium dodecyl sulfate polyacrylamide gel electrophoresis were unsuccessful. Multiple bands were present in gels, even after species that eluted from the ion-exchange column were once again fractionated on Sephadex G-75 to remove polymers. Because anomalous electrophoretic behavior of copper-containing MT has also been reported by others (129), I attempted to dissociate bound copper and block reactive sulfydryl groups by carboxylmethylation. I was unable to completely remove bound copper. Therefore, I directly analyzed the pooled and concentrated material in each peak from the ion-exchange column. The protein and metal content, number of reactive sulfhydryl groups and ratio of sulfhydryl groups to copper and zinc in the peak fractions from diabetic liver and kidney were similar to that of rat liver zinc-MT-I and -II (Table 2.1).

Cytosol from liver and kidney of control and diabetic rats that

Table 2.1 Characteristics of MT purified from liver and kidney of STZ-diabetic rats*.

Organ	Isoprotein	(Cu+Zn)/MT	-SH/metal	-SH/MT
Liver	MT-I	8.6±0.9	2.5±0.4	20.7±1.4
	MT-II	6.5±0.9	3.1±0.8	22.0±0.9
Kidney	MT-I	7.7±0.9	3.2±0.5	24.1±1.7
	MT-II	8.5±0.4	2.4±0.4	22.2±2.4

*Cytosol samples were fractionated by gel filtration and anion-exchange chromatography as described in the legend to Fig. 2.1. Peak fractions containing MT were concentrated and analyzed as described in Methods. Values (mean ± SEM) are expressed on a molar basis and were calculated for samples purified from organs of three separate groups of rats.

had been injected with ^{3}H-leucine was also analyzed by gel permeation and ion-exchange chromatography. Mammalian MT does not contain leucyl residues (1). The relative level of ^{3}H incorporated into cytosol protein that eluted from Sephadex G-75 with apparent molecular weight of 10,000 was lower in the tissues from diabetic rats than control rats. Movever, these ^{3}H-containing species eluted from DEAE-Sephadex as a single peak at a higher concentration of Tris-acetate than the two MT peaks containing copper and zinc (data not shown).

Because of their chromatographic behavior, preferential incorporation of cysteine, and the levels of copper, zinc and sulfhydryl groups, we concluded that the low molecular weight, heavy metal binding proteins present in diabetic liver and kidney were indeed MT. The results in Table 2.2 show the impact of the diabetic condition on the levels of zinc and copper associated with MT-1 and -II in hepatic and renal cytosol. The concentrations of these metals bound to MT-I and -II were significantly higher in cytosol from diabetic liver and kidney than from respective control samples. Renal cytosol contained higher concentrations of zinc and copper bound to MT-I and -II than hepatic cytosol in control and diabetic rats. Finally, the copper-to-zinc ratio for MT-I and -II was usually less than 1 in diabetic and control liver, but approximately 2 for the isoproteins in control and diabetic kidney.

Table 2.2 Concentrations of zinc and copper associated with MT isoproteins in liver and kidney of control and STZ-diabetic rats*.

Tissues	Group	Isoforms	Zn	Cu
			ug as MT/g cytosol protein	
Liver	Control	MT-I	2	1
		MT-II	4	1
	STZ-diabetic	MT-I	47±4	53±5
		MT-II	48±7	30±1
Kidney	Control	MT-I	13,15	23,25
		MT-II	11,13	20,24
	STZ-diabetic	MT-I	82±21	191±36
		MT-II	88±23	219±47

*MT was isolated as described in Methods. Six replicate samples of liver and kidney were analyzed from diabetic rats. MT was isolated from liver and kidney of one or two control animals, respectively. Data are expressed either as the individual value or mean ± S.E.M.

Effect of diabetic condition on the relative levels of MT synthesis

The percentage of ^{35}S incorporated into cytosol proteins (i.e., acid-insoluble ^{35}S) was similar in liver and kidney of control and diabetic rats. The relative level of MT synthesis was determined by quantifying the percentage of ^{35}S-cytosol protein that eluted from Sephadex G-75 as MT. Figure 2.3 shows the effect of duration of the untreated diabetic condition on the relative amount of MT synthesis in liver and kidney. ^{35}S-MT represented approximately 7 and 13% of ^{35}S incorporated into cytosol protein in liver and kidney, respectively, of control rats. Maximal relative levels of MT synthesis were elevated about 3-fold in liver and 2-fold in kidney of diabetic rats. However, the length of time after induction of the diabetic condition required to attain the maximal increase in the relative rate of MT synthesis was only 4 days in liver compared to 10 days in kidney. The level of MT synthesis in the liver and kidneys of control rats was not significantly different during the experimental period (Fig. 2.3).

The effects of dietary copper and dietary composition on MT synthesis in liver and kidney of control and diabetic rats are summarized in Figure 2.4. The relative amount of MT synthesis was greater in liver, but similar in kidney, when diabetic rats were fed purified diet containing 35 ppm copper compared to 13 ppm copper. In contrast, the copper content of the purified diet did not affect MT synthesis in control liver. Substitution of commercial "chow" for

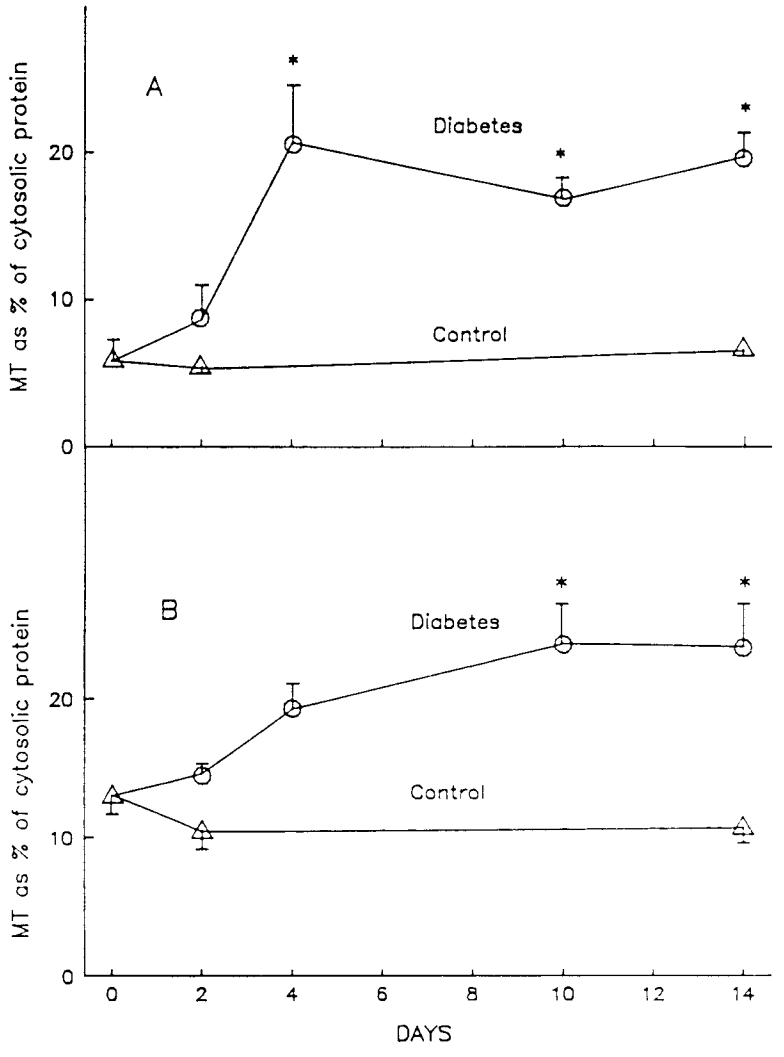

Fig. 2.3 Influence of duration of untreated diabetic condition on relative rates of MT synthesis in liver and kidney. Rats were randomly injected with STZ on day 0 to induce diabetes mellitus. At indicated times, groups (N=5) of control and STZ-diabetic rats were killed 5 hours after i.p. injection of ^{35}S-cysteine. The relative levels of MT synthesis were estimated in liver (panel A) and kidney (panel B) as described in Methods. The presence of an asterisk above the error bar indicates that the means are significantly different from day 0 control ($p < 0.05$). Control (△); diabetic (○).

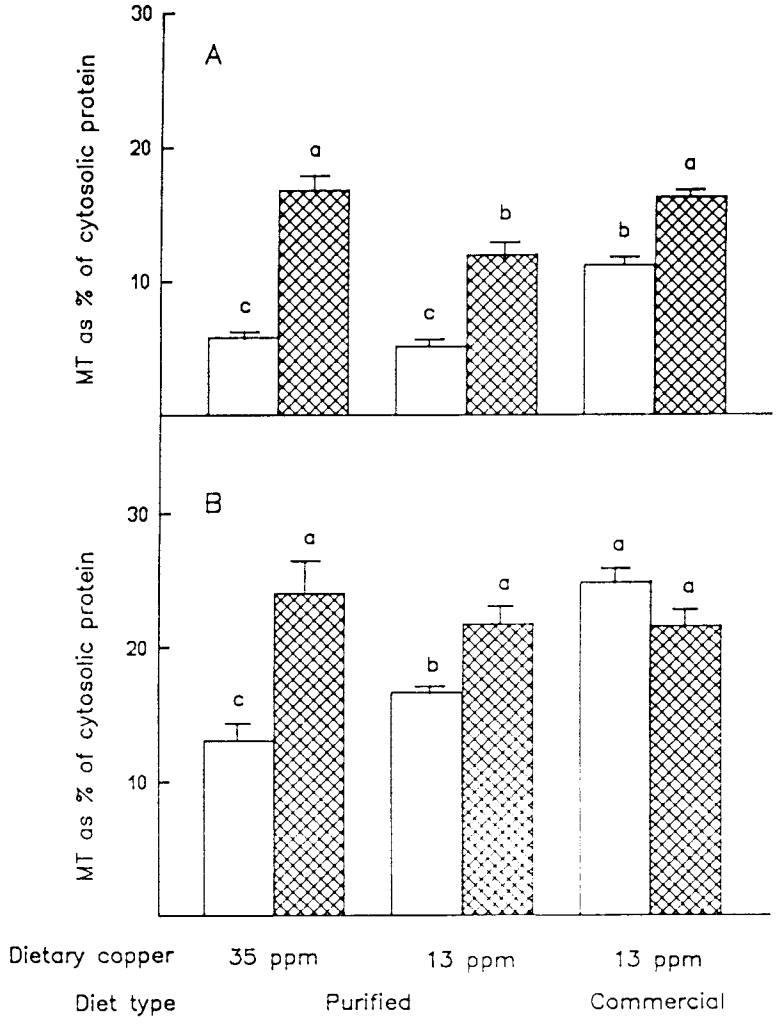

Fig. 2.4 Effect of diabetic condition and diet on relative rates of MT synthesis in liver and kidney. Animals were fed either commercial diet (13 ppm Cu) or purified diet with either 13 or 35 ppm Cu. MT synthesis was determined in the liver (panel A) and kidney (panel B) of control and 10 day STZ diabetic rats as described in Methods. The presence of different letters above error bars in each panel indicates that the means are significantly different ($p < 0.05$). Control (□); diabetic (▨).

purified diet with an identical level of copper (13 ppm) decreased the difference in the relative levels of MT synthesis in hepatic cytosol. Moreover, there was no significant difference in the level of MT synthesis in kidney of control and 10 day diabetic rats fed chow. The relative amount of ^{35}S-cysteine incorporated into hepatic and renal MT was significantly higher in control rats fed commercial diet than in rats fed purified diets.

Effects of diabetic condition on half-life of ^{35}S-MT and ^{35}S-protein in hepatic and renal cytosol

This initial study was aimed at determining the relative rate of removal of ^{35}S-MT from the cytosolic compartment. Whether this measurement represents actual degradation of the polypeptide or its transfer to another cellular compartment (e.g., lysosomes) has not been established (65). The disappearance of hepatic ^{35}S-MT from the cytosol was accelerated by the diabetic condition. The half-life was 1.3 days and 5.1 days for the diabetic and control groups, respectively (Table 2.3). In comparison, the half-life of ^{35}S-cytosol protein was 3.9 and 4.9 days in livers of control and diabetic rats, respectively. In kidney, the half-lives of ^{35}S-MT and cytosol protein were not significantly altered by the diabetic state.

Table 2.3 Influence of diabetic condition on half-life of ^{35}S-proteins and ^{35}S-MT in cytosol from liver and kidney of control and STZ-diabetic rats.*

Tissue	Protein	Control	STZ-diabetic
		Half-life (days)	
Liver	^{35}S-MT	5.1	1.3
	^{35}S-cytosol protein	3.9	4.9
Kidney	^{35}S-MT	2.3	2.6
	^{35}S-cytosol protein	4.6	5.7

*Age-matched control and STZ-diabetic rats were given free access to commercial diet (13 ppm Cu). After 10 days, rats were injected with ^{35}S-cysteine and with non-radioactive cysteine 24 hours later. Sets of animals (N=5) from each group were killed 24, 36, 48, 72 96 and 120 hours after injection of radioisotope. The loss of ^{35}S-MT and ^{35}S-proteins from hepatic and renal cytosol was determined by gel filtration chromatography. Half-life values for the disappearance of ^{35}S from MT and cytosol proteins were calculated as described in Methods.

DISCUSSION

Earlier reports from our laboratory showed that elevated levels of zinc, copper, and a MT-like, heavy metal binding protein were present in the soluble fraction of liver and kidney of chemically-induced and spontaneously insulin-deficient diabetic rats (53-55). As a result of the more detailed studies summarized in this report, I conclude that this protein is indeed MT because its chromatographic behavior, heavy metal binding properties, number of reactive sulfhydryl groups and lack of leucyl residues are similar to that of purified MT (1).

The levels of zinc and copper associated with MT-I and -II were elevated in the liver and kidney of the streptozotocin (STZ)-induced diabetic rats (Table 2.2). STZ-induced diabetes is characterized by a chronically decreased ratio of plasma insulin to glucagon and elevated levels of adrenal hormones (57). It is well recognized that synthesis of hepatic MT is induced by administration of heavy metal salts and by physiological and pharmacological manipulation of endocrine status (2). Among the hormones studied, glucagon, epinephrine, glucocorticoids and dibutyryl cAMP are potent inducers of MT synthesis in rat liver (50). The presence of increased amounts of hepatic MT in animals during episodes of physiological stress, i.e., bacterial infection (41) and fasting (39), supports the central role for hormones as endogenous regulators of MT metabolism. We

previously reported that insulin treatment of STZ-diabetic rats reduced the amount of MT-like proteins in liver towards constitutive (control) levels (53). Therefore, our data strongly suggest that the elevated levels of MT-I and -II in diabetic liver were due in part to chronic endocrine imbalance resulting from STZ-mediated destruction of pancreatic beta cells.

In contrast to their effects on hepatic MT metabolism, pancreatic and adrenal hormones do not seem to significantly influence renal MT status (2). For example, administration of dibutyryl cAMP did not alter the concentration of renal MT (50). Also, dexamethasone, a synthetic glucocorticoid, failed to induce MT synthesis in a cultured cell line derived from rabbit kidney (130). The presence of an increased level of MT in the kidney of the diabetic rat is associated with the accumulation of excess dietary copper in this organ (56,58,131). The concentration of renal copper was 7 fold higher than hepatic copper in STZ-diabetic animals and much of the additional complement of this metal in the cytosol was associated with MT. Hepatic MT was enriched in zinc in the STZ-diabetic rat. Accumulation of copper in the kidney by brindled mice (132) and by rats exposed to cadmium (61) demonstrates that accumulation of copper in this organ is not limited to the diabetic condition. The mechanisms responsible for the preferential accumulation of copper by the kidney in such conditions remain unknown.

Elevated levels of MT in liver and kidney of STZ-diabetic rats

may be primarily due to changes in the rates of synthesis and degradation of this protein. Indeed, MT synthesis was significantly increased in both organs of STZ-diabetic rats fed purified diets (Figs. 2.3 and 2.4). Differential influences of the insulin-deficient diabetic condition on MT synthesis in liver and kidney were found. Maximal relative rates of hepatic and renal MT synthesis were observed 4 and 10 days after treatment with STZ, respectively. The increased synthesis of hepatic MT during the early phase of insulin-deficiency is similar to that previously reported for animals subjected to physiological stresses (e.g. infection and fasting; see 2, for review), suggesting that such alterations are primarily due to disturbances in endocrine status. The delayed enhancement of MT synthesis in diabetic kidney is probably related to the greater length of time between induction of the diabetic condition and the increased absorption and renal accumulation of dietary copper. These changes are correlated with the onset of hyperphagia and hypertrophy of the gastrointestinal tract, which are not evident until 4-5 days after inducing insulin deficiency (58,131). The increased synthesis of MT in diabetic rats exhibited some specificity, since ^{35}S-cysteine incorporation into non-MT proteins in the cytosol from liver and kidney was similar in control and 10 day diabetic rats.

When the copper content of a purified diet was increased almost 3-fold (to 35 ppm), the relative rates of hepatic and renal MT

synthesis in STZ-diabetic rats increased significantly (Fig. 2.4). In contrast, dietary copper did not affect MT synthesis in control rat tissues. This latter observation is consistent with that of Bremner et al. (73). Hepatic MT synthesis was also less pronounced in STZ-diabetic animals fed chow than in those fed purified diet containing an equivalent amount of copper. This difference may be due to the increased bioavailability of the metal from a purified diet (133). Renal MT synthesis was also affected by dietary treatment. The relative rate of renal MT synthesis was slightly lower in 10 day diabetic rats than in control rats fed commercial (chow) diet. This discrepancy may reflect adaptation of chow-fed animals to the diabetic state.

Because the amount of copper associated with renal MT in chow-fed rats was elevated despite the lack of an apparent change in the relative rate of MT synthesis, we investigated the impact of the diabetic condition on the half-life of MT in the cytosol of livers and kidneys of control and diabetic animals fed this diet. The turnover of ^{35}S-cytosol protein in liver and kidney was not significantly altered by treatment with the diabetogenic drug (Table 2.3). This finding is in agreement with others who reported that general protein turnover in liver and kidney of chronically diabetic animals was similar to that in age-matched controls (134,135). The observed half-life of 5 days for cytosolic MT from control liver is consistent with previous reports (61,136). In the STZ-diabetic rat,

the rate of disappearance of ^{35}S-MT from hepatic cytosol was markedly accelerated ($t_{1/2}$= 1.3 days). This enhanced turnover of cytoplasmic MT is similar to that reported by various investigators following the administration of zinc and copper salts (2,65). Sobocinski et al. (41) also found that the rate of disappearance of MT from the cytoplasm was also increased by infection. Thus, stress-related induction of hepatic MT synthesis also appears to be coordinated with an increased rate of removal of this protein from the cytoplasmic compartment. The turnover process may be subject to hormonal control.

The half-life of cytosolic MT in the kidney was not significantly altered in chronically diabetic animals (between 10 and 15 days after onset) fed commercial diet (Table 2.3). This observation was surprising since the relative rate of MT synthesis was also similar in the kidney of control and 10 day diabetic rats fed commercial diet (Fig. 2.4), despite the presence of elevated levels of zinc and copper associated with MT in this organ (Table 2.2). To estimate MT turnover, animals received one injection of ^{35}S-cysteine and the amount of the radioisotope present in MT in the soluble fraction of liver and kidney was determined at various times. This approach is based upon several important assumptions that may not be valid for diabetic kidney. First, reincorporation of the radioisotope must be minimal. Several investigators reported that this assumption is valid in normal animals (59,136). However, renal metabolism is

markedly altered by insulin deficiency (137) and the impact of the diabetic state on cysteine transport and metabolism in the kidney is unknown. Second, we previously reported high levels of copper, possibly in the form of copper-MT, in the lysosomal fraction of the diabetic renal cortex (56). Copper-MT is resistant to lysosomal enzymes in vitro (64,67). Thus, some of the ^{35}S-MT present in the cytosol fraction may have been released from lysosomes during homogenization of frozen tissues. Such factors complicate interpretation of the results for turnover of MT in the kidney of the diabetic rat. Current studies are aimed at clarifying these matters.

In summary, the concentrations zinc and copper bound to MT-1 and -II were markedly elevated in cytosol fraction of liver and kidney from STZ-diabetic rats. The relative rates of MT synthesis and the rate of removal of these proteins from cytosol were increased in liver of STZ-diabetic animals. These changes are probably due to the characteristic alterations in the levels of pancreatic and, perhaps, adrenal hormones in the chemically-induced diabetic animal. The relative rate of renal MT synthesis was also increased in diabetic rats fed a purified diet. This response appears to be modulated by the marked accumulation of dietary copper in the kidney of diabetic rats. The effects of diabetes on the turnover of cytosolic MT in the kidney are inconclusive at this time.

CHAPTER III

EFFECTS OF INSULIN, GLUCAGON AND DEXAMETHASONE ON PROTEIN DEGRADATION IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

INTRODUCTION

In the previous studies with streptozotocin-diabetic rats, I found that the relative rate of MT synthesis and turnover of cytoplasmic MT were both accelerated in liver. The altered metabolism of hepatic MT in diabetic rats was attributed primarily to disturbances in endocrine status (Chapter II). Critical analysis of my data and the literature revealed that cytoplasmic MT turnover might not reflect actual degradation of this protein. Therefore, I decided to investigate this subject in greater detail. The study of protein degradation is difficult in intact animals, since complicating factors, e.g., isotope reincorporation and reutilization of radioisotope by different tissues, often preclude unequivocal interpretation of the results. For this reason, I focused my attention on the use of cultured cells as a model system. The advantages of using cultured cells for studying protein degradation were described in Research Objectives section of Chapter I. Here, I selected monolayer cultures of adult rat hepatocytes as the cellular model because the liver is the central organ involved in short-term control of heavy metal homeostasis.

The rate of general protein degradation has been previously evaluated in perfused rat liver (95), suspension cultures (97) and monolayer cultures (100) of adult rat hepatocytes. These studies have shown that cellular protein breakdown occurs in lysosomal and

non-lysosomal (cytoplasmic) compartments. It has been shown that lack of serum, growth factors, hormones and nutrients increase the rate of protein degradation. This enhanced protein breakdown is solely due to increased lysosomal autophagy (for review, see 85,86). Also, lysosomotropic agents and various types of proteolytic inhibitors have been juidiciously used to differentiate between the lysosomal and cytoplasmic events in protein degradation.

In the studies summarized below, I estimated the rates of cellular proteolysis and characterized the effects of insulin, glucagon and dexamethasone on general protein degradation. The effects of several lysosomotropic amines and cytoplasmic proteinase inhibitors on protein degradation were also investigated. The information on general protein degradation was important for interpreting various characteristics of MT degradation in subsequent studies (Chapter IV).

MATERIALS AND METHODS

Chemicals

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 of Gibco Laboratory except that leucine was omitted. Puck's balanced salt solution consisted of 0.4 g KCl, 0.15 g $MgSO_4.7H_2O$, 8 g NaCl, 0.016 g $CaCl_2.2H_2O$, 1.1 g glucose and 2.38 g HEPES (N-2-hydroxyethyl piperazine-n'-2-ethane sulfonic acid) per liter. Percoll and collagenase were purchased from Pharmacia Inc. (Piscataway, NJ) and Worthington Biochemical (Freehold, NJ), respectively. [4,5-^{3}H]-L-leucine was obtained from ICN Chemical & Radioisotope Inc. (Irvine, CA). Bovine albumin (fatty acid free), lysosomotropic amines, proteinase inhibitors and other chemicals were reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

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 libitium. Animals were maintained on a 12 hour light/12 hour dark cycle (lights on 0600-1800h) in a temperature (21-23^{o}C) and humidity (45-55%) controlled room.

Cell isolation and culture

Donor rats were fed overnight and cell isolation was initiated between 0900-1000 hour. Liver cells were isolated by the collagenase perfusion technique of Seglen (138) as modified by Failla and Cousins (118). Intact hepatocytes were separated from non-parenchymal cells and leukocytes by iso-osmotic Percoll density gradient and differential centrifugation (139). Cell viability, as estimated by trypan blue exclusion, was 90 to 95%. Approximately 2 x 10^6 purified hepatocytes were added to collagen-coated (16 ug/well), multiwell dishes (35 mm^2) and incubated at 37°C in 95% air: 5% CO_2. The plating medium consisted of Medium 199, 5% fetal calf serum (FCS), 10^{-7} M insulin, 50 ug gentamycin/ml and 0.5 ug fungizone/ml. After 1 hour, non-adherent cells were removed by washing monolayers twice with Puck's buffer.

Estimation of protein degradation

To estimate the rate of protein degradation, the procedure of Hopgood et al. (100) was followed with slight modificiations. Monolayers of adult rat hepatocytes were incubated overnight (18 hours) in Waymouth's MB 752/1 medium containing 10^{-8}M insulin, 10 uM Zn ($ZnSO_4.7H_2O$), 0.2% albumin and ^{3}H-leucine (10 uCi/nmol) to label cellular proteins. Monolayers were then extensively washed with M199 medium and ^{3}H-leucine was "chased" by incubation of monolayers with fresh medium containing 1.9 mM non-radioactive

leucine for 4 hours with one change of medium (after 2 hours) during this chase period. This facilitated the efflux of ^{3}H-leucine from the intracellular amino acid pool, thereby minimizing reincorporation of ^{3}H-leucine into newly synthesized proteins during the experimental period.

Experiments were initiated by incubating monolayers in either "basal" or "step-down" medium. The composition of basal medium was the same as "chase" medium which consisted of complete amino acids mixture and a physiological concentration of insulin (10^{-8}M). When indicated, amino acids and insulin were removed from basal medium to stimulate the rate of protein degradation (see Results). This nutritionally deficient medium is referred to as "step-down" medium.

At the completion of the experimental period, cells and medium were collected separately. Monolayers were washed twice with ice-cold Puck's buffered saline before harvesting in 1.5 ml of 0.2% Triton X-100. Cell suspensions were sonicated (Microson Instrument, Farmingdale, NY) at 40% output for 20 seconds using a microprobe. An aliquot of cell homogenate was removed to determine total cellular radioactivity. To another portion of cell homogenate, bovine albumin was added as a carrier protein before the addition of equal volume of 10% (w/v) trichloroacetic acid (TCA). Acid soluble and precipitated materials were separated by centrifugation and an aliquot of supernatant was analyzed to determine the level of acid-soluble radioactivity. Acid-insoluble material was washed twice with 10% TCA

and the final pellet was dissolved in 1 N NaOH. Aliquots were analyzed to determine the amount of radioactivity.

Spent medium was centrifuged at 200 x g for 10 min at 4°C to remove nonadherent cells. Total, acid-soluble and acid-insoluble radioactivity in medium were determined as described for cell homogenates. Radioactive samples were transferred to vials containing liquid scintillation fluid (Scinti Verse II, Fisher, Fair Lawn, NJ) and counted in a Beckman LS 6800 liquid scintillation spectrophotometer. Quenching was corrected with an automatic external standard.

Acid-soluble radioactivity in cells and medium were summed and divided by total radioactivity in the well. Replicate wells were also harvested at zero time and acid-soluble ^{3}H in the well was determined. The increase in relative level of acid-soluble ^{3}H during the experimental period was used to calculate the rate of protein degradation (% degradation/h).

To investigate the effects of hormones, lysosomotropic agents and proteinase inhibitors on general protein degradation, indicated substances were added at the begining of the experimental period, with one exception. That is, monolayers were exposed to dexamethasone during the overnight labelling period and the chase and experimental periods. Stock solutions (1,000X) of insulin and glucagon were prepared in 1.6% glycerol, 0.2% phenol and 0.05% bovine serum albumin, pH 2.0. Lysosomotropic and proteinase inhibitors were

prepared in aqueous medium (Waymouth's MB 752/1), while dexamethasone, pepstatin and TLCK (tosyl-lysine chloromethyl ketone) were dissolved in alcohol (95% v/v). The rate of proteolysis was not affected by the presence of 1% alcohol in the medium.

Estimation of protein synthesis

Monolayers of hepatocytes were incubated in basal medium overnight. Cells were then incubated in medium containing ^{3}H-leucine (20 uCi/nmol) for indicated times. The amount of ^{3}H-leucine incorporated into acid-insoluble fraction per mg of cell protein was determined as described above. The protein concentration was measured by a modified Lowry procedure (140) using bovine serum albumin as standard. To investigate the effects of cycloheximide on protein synthesis, various concentrations of cycloheximide were added to the incubation medium at the begining of the experimental period. The decreased incorporation of ^{3}H-leucine into acid-insoluble fraction of cycloheximide-treated cells was expressed as percent of control. Stock solution of cycloheximide was prepared in Waymouth's MB 752/1 medium.

Statistical analysis

Data are expressed as mean$\pm$S.E.M. Each datum point represents the mean of measurements from at least six separate wells that contained cells from at least two donor rats. One way analysis of variance and

Duncan's multiple range test were used to test for significant differences between treatment groups ($p < 0.05$)

RESULTS AND DISCUSSION

Assessment of protein degradation in hepatocytes.

The levels of radioactivity in acid-soluble and -insoluble fractions of medium and cells during a 6-hour experimental period are shown in Fig. 3.1. Acid-soluble ^{3}H in medium, which reflects exchanged free amino acids resulting from intracellular protein breakdown (141), increased linearly with time. In contrast, acid-soluble ^{3}H within cells, which represents the free leucine pool was only 10% of that in medium after 6 hour. Moreover, the level of acid-soluble ^{3}H in cells gradually decreased with time, suggesting rapid exchange of free leucine between the intra- and extracellular pools. Acid-precipitable ^{3}H in medium remained relatively constant after 1 hour, suggesting minimal incorporation of ^{3}H-leucine into newly synthesized secretory proteins. It is unlikely that the presence of labelled proteins in medium was due to either cell lysis or leaky membranes, since medium contained a low level of lactate dehydrogenase (less than 5% total activity in wells). The level of ^{3}H-labelled cellular proteins decreased slightly with time. Our results are compatible with previous observations with isolated rat hepatocytes (142) and human fibroblasts (98), suggesting that ^{3}H-leucine is an effective radioactive tracer for investigating protein degradation. Therefore, the rate of protein degradation was calculated according to Hopgood

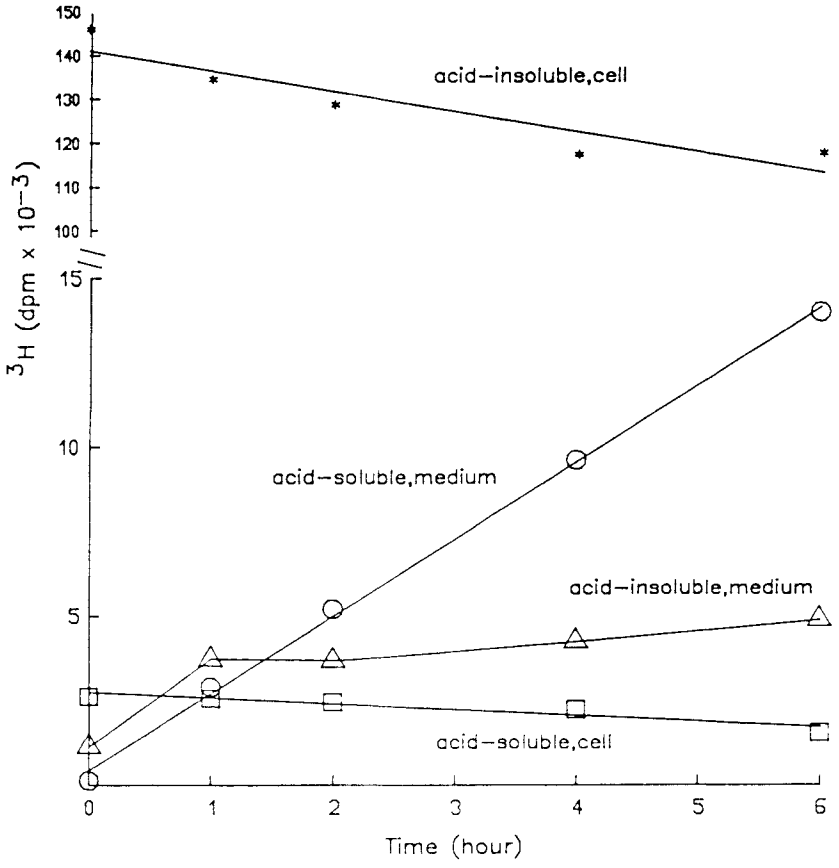

Fig. 3.1 Effect of incubation period on the levels of acid-soluble and acid-insoluble radioactivity in medium and cells prelabelled with ^{3}H-leucine. Hepatocytes were incubated in basal medium (insulin + amino acids) with ^{3}H-leucine for 18 hours to label cellular proteins. Cells were then incubated in medium containing excess leucine to chase intracellular ^{3}H-leucine pool as described in the Materials and Methods. At 0 hour, fresh basal medium was added and acid-soluble and acid-insoluble ^{3}H in medium and cells were measured at indicated times. Each point is the mean value from 6 replicate wells in two separate experiments.

et al. (100).

$$\% \text{ degradation} = \frac{\text{acid-soluble } {}^{3}\text{H in medium \& cells } (t-t_0)}{\text{total } {}^{3}\text{H in medium \& cells } (t)} \times 100\%$$

where t and t_0 indicate the levels of radioactivity at time t and 0 hour, respectively. One major advantage of using this method was elimination of slight variations in the level of cellular protein per well and the absolute amount of ^{3}H-leucine accumulated by cells within and between each experiment.

In a preliminary experiment, hepatocytes were incubated in the "basal medium", i.e., medium containing the complete mixture of amino acids and a physiological concentration of insulin (10^{-8}M). Proteolysis was linear for up to 6 hours at a rate of 1.77%/h (Fig. 3.2). Therefore, a 4-hour experimental period was chosen as the convenient interval in subsequent studies unless otherwise indicated. The proteolytic process was temperature dependent since only minimium degradation (0.09%/h) was detected when cells were maintained at 5^{o}C. Addition of fetal calf serum (2% final concentration) to the incubation medium did not significantly reduce the rate of proteolysis (1.66%/h), suggesting that basal medium provided nutrients and/or factors required for minimal protein turnover in hepatocytes.

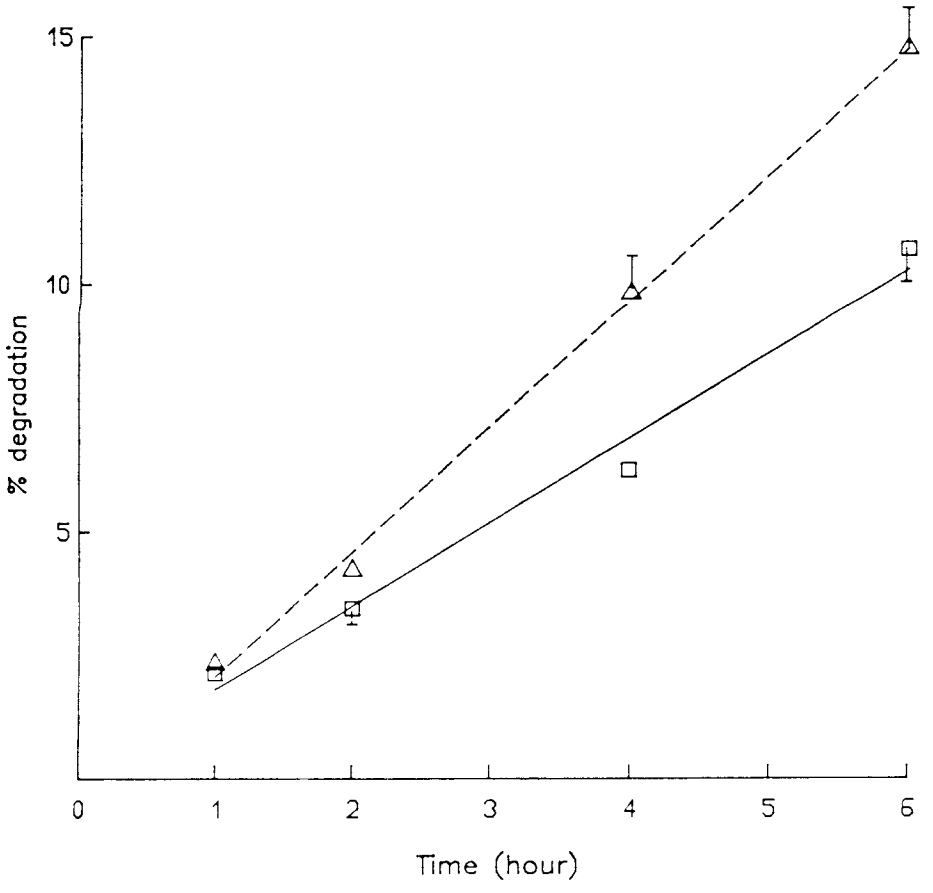

Fig. 3.2 Degradation of ^{3}H-protein in monolayers of adult rat hepatocytes. Monolayers that were previously labelled with ^{3}H-leucine were incubated in basal (□) or step-down (△) medium during the experimental period. The composition of these media has been described in the Materials and Methods. The rate of protein degradation (%) was assessed by monitoring the amount of acid-soluble ^{3}H in medium at indicated time. Each point is the mean value from 6 replicate wells in two separate experiments.

Effects of extracellular amino acids and hormones on protein degradation

Simultaneous removal of insulin and amino acids from incubation medium (step-down) significantly increased the rate of protein degradation (1.3- fold) above basal levels (Table 3.1). The majority (80%) of this enhanced degradation was due to removal of insulin alone. Elimination of only amino acids from the incubation medium did not significantly increase the rate of proteolysis. Hopgood et al. (100) reported that the rate of proteolytic turnover in monolayer cultures of hepatocytes maintained in medium without insulin was 2.5%/h. Addition of insulin (10^{-8}M) to medium decreased the protein degradation rate to a level similar to that observed in the present study. However, both Shworer et al. (96) and Seglen et al. (97) showed that addition of a mixture of amino acids decreased the rate of proteolysis in perfused rat liver and suspensions of freshly isolated hepatocytes, respectively. While these findings appear to contradict the present results, it should be noted that freshly isolated hepatocytes are in a catabolic state with protein degradation rate as high as 4-5%/h (143). The monolayer cultures employed in this study had been incubated overnight in complete medium, which presumbly restores nitrogen balance and maintains protein turnover at a minimal rate. Thus, it is possible that amino acid deprivation would not affect protein degradation in a well nourished cell culture within a short period of time.

Table 3.1 Effects of amino acids (aa) and insulin on protein degradation in monolayer culture of adult rat hepatocytes*.

Experimental medium	% degradation/h
Basal (w/ aa & insulin)	1.77 ± 0.05^{a} (17)
Basal w/o aa	$1.90 \pm 0.12^{a,b}$ (3)
Basal w/o insulin	$2.22 \pm 0.02^{b,c}$ (6)
Step-down (w/o aa & insulin)	2.32 ± 0.10^{c} (15)

*Hepatocytes were incubated in basal medium with ^{3}H-leucine for 18 hours. Cells were then incubated in medium containing excess cold leucine to chase intracellular ^{3}H-leucine as described in the Materials and Methods. To initiate experiments, either fresh basal medium or basal medium without either amino acids and/or insulin was added. The rate of protein degradation was estimated after 4 hours. Data are expressed as mean±SEM. Means with different letters as superscripts are significantly different at $p < 0.05$. The number in parentheses indicates the number of measurements.

To investigate whether higher rate of proteolysis in the absence of insulin was due to enhanced autophagy, the effects of lysosomotropic agents on protein turnover were compared in hepatocytes incubated in basal and step-down media. Chloroquine, methylamine, and ammonium ion each decreased the rate of protein degradation by 40-50% in monolayer cultures of hepatocytes incubated in step-down medium (Table 3.2). Simultaneous addition of chloroquine and NH_4Cl failed to inhibit further proteolysis. Addition of chloroquine to cultures of hepatocytes maintained in basal medium also inhibited protein degradation by 40%. Hopgood et al. (100,144) also reported a 50% inhibition of protein degradation after ammonia was added to monolayers culture of hepatocytes in the presence or absence of insulin.

Assuming that lysosomal degradation was completely inhibited upon exposure to chloroquine, the amine-sensitive (i.e. lysosomal) pathway accounted for protein degradation rates of 0.73% and 1.15%/h in basal and step-down cultures, respectively. That is, removal of insulin and amino acids increased lysosome-mediated turnover of cellular proteins by 1.6-fold with apparently minimal (if any) effect on non-lysosomal processes. Thus, approximately 40% and 60% of hepatic protein turnover was due to lysosomal and non-lysosomal pathway, respectively, in basal cultures. McElligott et al. (145) reported that when rat liver cytosol proteins were microinjected into human fibroblasts, lysosomal and cytoplasmic pathways accounted for the

Table 3.2 Effects of lysosomotropic amines on protein degradation in monolayers of adult rat hepatocytes*.

Experimental medium	Weak-base amines	% degradation/h	% inhibition
Basal	None	1.77 ± 0.05^{A} (17)	-
	Chloroquine (0.1 mM)	1.05 ± 0.02^{B} (6)	41
Step-down	None	2.32 ± 0.01^{a} (15)	-
	Chloroquine (0.1 mM)	1.17 ± 0.02^{b} (6)	49
	Ammonia (10 mM)	1.25 ± 0.07^{b} (6)	45
	Methylamine (10 mM)	1.45 ± 0.05^{b} (6)	37
	Chloroquine+ammonia	1.22 ± 0.07^{b} (6)	47

*The composition of basal and step-down medium has been described in legend to Table 3.1. At the begining of experimental period, fresh basal or step-down medium with indicated substances were added to ^{3}H-labelled monolayers. Data are expressed as mean±SEM. Statistical analyses were made within groups maintained in basal and step-down medium, respectively. Values with different letters as superscripts are significantly different from one another ($p < 0.05$). The number in parentheses indicates the number of measurements.

degradation of approximately 40 and 60% of exogenous material, respectively. By measuring the fractional volumes of lysosomal vacuoles and the rate of proteolysis in perfused rat liver, Schworer et al. (96) reached a similar conclusion. That is, both lysosomal and non-lysosomal routes of protein degradation operate under basal condition. Mortimore et al. (146) suggested that this basal lysosomal activity is probably due to microautophagy.

The effect of lysosomal proteinase inhibitors on the rate of proteolysis was also tested in hepatocytes incubated in step-down medium. Pepstatin (100 ug/ml), an inhibitor of cathepsin D (105), reduced protein degradation by 10% (2.20%/h). The limited effectiveness of this chemical may be due to the brief incubation period since pepstatin uptake by cells is relatively slow (101). In addition, the hydrolytic activity of cathepsin D is greater in hepatic non-parenchymal cells than hepatocytes (147). Leupeptin (50 ug/ml), a strong inhibitor of cathepsin B (104), inhibited protein turnover in the step-down culture by 30% (1.62%/h). These results indicated that pepstatin and leupeptin partially inhibited lysosomal degradation. Such results were expected, since these substances inhibit only specific types of lysosomal hydrolases.

Effects of glucagon and glucocorticoid on protein degradation

Glucagon and glucocorticoids are key hormones in regulating intracellular metabolism in hepatocytes (e.g., see 148 and 149).

Consequently, I tested the effects of glucagon and dexamethasone, a synthetic glucocorticoid, on the rate of protein degradation in hepatocytes (Fig. 3.3). Glucagon (10^{-8}M) stimulated protein turnover by approximately 1.4- and 1.2-fold in the absence and presence of insulin (10^{-8}M), respectively. This glucagon-mediated increase in proteolysis was probably due to enhanced lysosomal degradation, since glucagon has been shown to enhance the formation of autophagosomes (99). In contrast, insulin blocks autophagosome formation (95). This probably serves as the basis for the antagonistic action between insulin and glucagon on protein turnover in hepatocytes.

The rate of protein degradation in both basal or step-down cultures was decreased 12 and 16%, respectively in dexamethasone (10^{-8}M) treated cells (Fig. 3.3). Present data were contradictory to that of Hopgood et al. (144) who reported a 20% increase in protein breakdown in hepatocytes incubated in insulin-free medium containing dexamethasone. However, the maximal effects of dexamethasone-enhanced protein breakdown were observed at a concentration of 10^{-7}M and cells were exposed to this synthetic glucocorticoid for only a brief period (7 hours) in their study. In addition, insulin was absent during overnight incubation of hepatocytes in their study, resulting in a protein degradation rate of 2.5%/h (144) compared to 1.77%/h in the present study (Table 3.1). Husson et al. (150) showed that the activities of urea-cycle

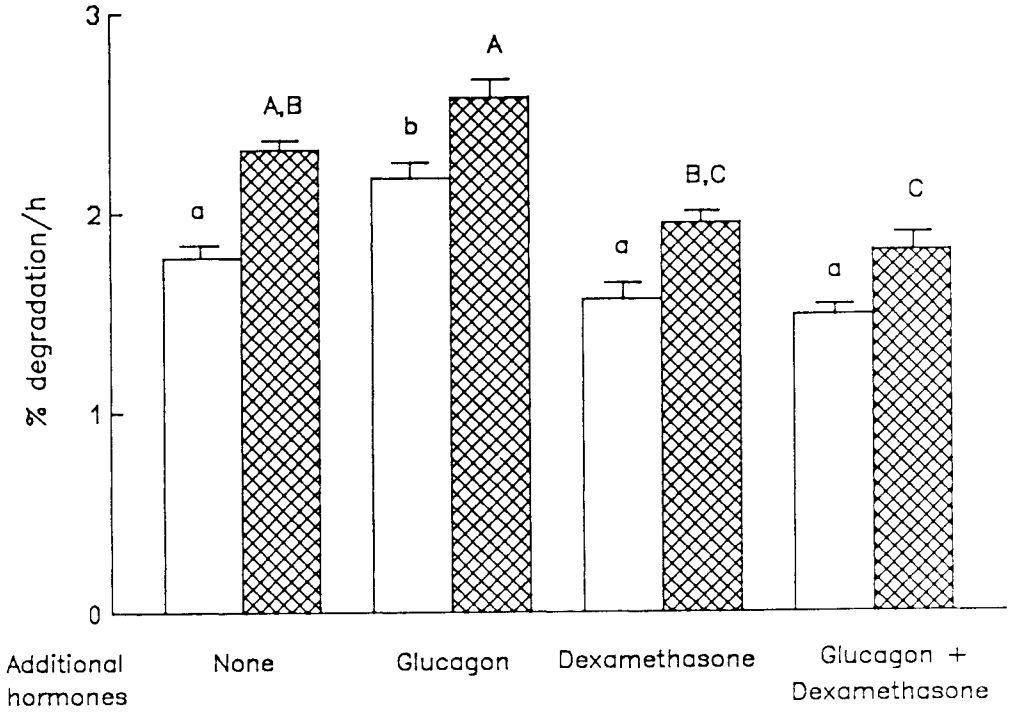

Fig. 3.3 Effects of glucagon and dexamethasone on the rate of protein degradation in monolayers of adult rat hepatocytes. Hepatocytes were previously labelled with ^{3}H-leucine for 18 hours. At the begining of the experimental period, either fresh basal (□) or step-down (⊠) medium containing glucagon (10^{-8}M) was added. In a separate set of preparation, hepatocytes were exposed to dexamethasone (10^{-8}M) during overnight labelling, as well as during the chase and experimental period. The rate of proteolysis was determined as described in the Materials and Methods. Statistical analyses were made within groups maintained in basal and step-down medium, respectively. The difference observed within a group is indicated by the presence of different letters above standard error bars ($p < 0.05$).

enzymes were increased in cultured fetal hepatocytes by dexamethasone treatment, but this activation was inhibited by addition of insulin. It was suggested that enhanced urea-cycle enzyme activities is probably due to increased proteolysis in dexamethasone-treated cells. The integrity of lysosomal membrane in a cultured human diploid cell line (WI-38) was stabilized by addition of hydrocortisone, a glucocorticoid hormone, to culture medium (151). In addition, it has been reported that increase in the rate of protein degradation was more pronounced in myoblasts incubated in medium containing dexamethasone for 4 hours than that of cells exposed to this glucocorticoid for 18 hours (152). This dexamethasone-mediated enhancement of protein degradation in myoblasts was also inhibited by insulin. The interactions between insulin and dexamethasone and the difference in acute and chronic action of dexamethasone on protein turnover were probably the basis for the discrepancies between my observations and those of Hopgood et al. (144).

It was found that addition of glucagon to medium failed to enhance protein degradation when cells had been pretreated with dexamethasone (Fig. 3.3). Laishes and Williams (153) and Schwarze et al. (154) found that addition of insulin and dexamethasone improved attachment, morphology and viability of hepatocytes. Present data suggested that the ability of insulin and dexamethasone to decrease the rate of protein turnover may also contribute to the beneficial

effects of these hormones in primary cultures of hepatocytes.

Effects of cycloheximide and TLCK on protein degradation

To determine the apparent half-life of a protein, many investigators have treated intact animals and isolated cells with cycloheximide, an inhibitor of 80S ribosomal activity, to prevent reutilization of the radiolabelled amino acids. In monolayer culture of hepatocytes, cycloheximide ($\geq$ 3.4 uM) inhibited protein synthesis by 85% (Fig. 3.4). However, cycloheximide also has been found to be an inhibitor of protein degradation (107,155). The rate of protein degradation in hepatocytes incubated in basal medium and basal medium without insulin was inhibited by 0.64 and 0.91%/h, respectively, in the presence of 3.4 uM cycloheximide (Table 3.3). The relative degree of inhibition of proteolysis in cells treated with chloroquine was the same as that of cycloheximide. Addition of chloroquine to cycloheximide-treated cells failed to increase significantly protein degradation, suggesting that cycloheximide selectively inhibited the lysosomal pathway of protein degradation. More specifically, cycloheximide apparently blocks formation of autophagic vesicles, perhaps by increasing the intracellular level of amino acids (87,157).

TLCK (tosyl-lysine chloromethyl ketone) is an inhibitor of serine proteases i.e., proteases with trypsin- and chymotrypsin-like activity (158). The rate of proteolysis was decreased in monolayers

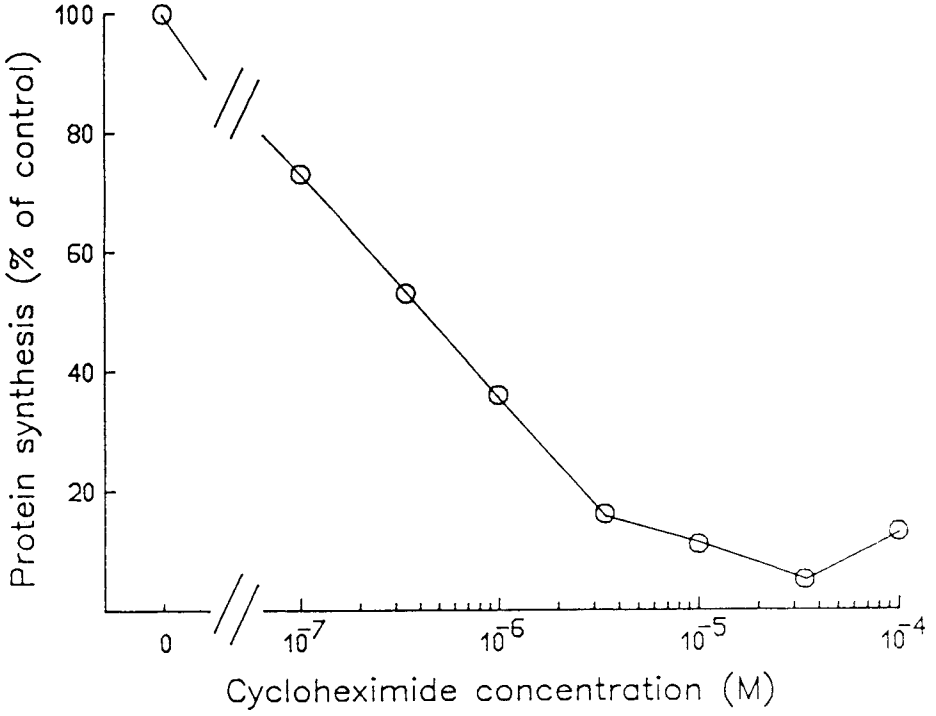

Fig. 3.4 Effects of cycloheximide on ^{3}H-protein synthesis in monolayers culture of hepatocytes. After overnight incubation, hepatocytes were pulsed with ^{3}H-leucine in the presence of indicated concentrations of cycloheximide for 4 hours. Protein synthesis was estimated from the incorporation of ^{3}H-leucine into the acid-insoluble fraction of cells. Inhibition of protein synthesis by cycloheximide was expressed as % of control. Each point is the mean value from 6 replicate wells of two separate experiments.

Table 3.3 Effects of cycloheximide and TLCK on protein degradation in monolayer cultures of adult rat hepatocytes*.

Experimental medium	Inhibitors	% degradation/h		% inhibition
Basal	None	1.54 ± 0.02^{A}	(9)	-
	Chloroquine	0.90 ± 0.06^{C}	(9)	41
	Cycloheximide	0.91 ± 0.04^{C}	(6)	41
	TLCK	1.29 ± 0.04^{B}	(6)	16
Basal w/o insulin	None	1.97 ± 0.08^{a}	(9)	-
	Chloroquine	0.85 ± 0.01^{d}	(9)	57
	Cycloheximide	1.06 ± 0.04^{c}	(6)	46
	TLCK	1.67 ± 0.03^{b}	(6)	15

*Monolayers of hepatocytes were labelled with ^{3}H-leucine for 18 hours. To initiate experiments, either fresh basal medium or basal medium without insulin containing indicated substances was added and incubation was continued for 6 hours. The concentrations of chloroquine, cycloheximide, and TLCK were 1 mM, 3.4 uM and 0.5mM, respectively. Data are expressed as Mean±SEM. Statistical analyses were made within groups maintained in respective medium. Means with different letters as superscripts are significantly different at $p < 0.05$. The number in parentheses indicates the number of separate measurements.

incubated in basal medium containing TLCK by 0.25%/h. Since TLCK also decreased protein turnover by 0.3%/h in step-down medium, this chemical did not seem to interfere with lysosomal degradation. Similar findings were also noted by McIlhinney and Hogan (159).

In summary, primary monolayer cultures of adult rat hepatocytes have been used successfully to monitor the characteristics and the subcellular sites of intracellular protein degradation. It was found that the rate of proteolysis can be modulated by hormones, lysosomotropic agents and proteinase inhibitors. The lysosomal and cytoplasmic pathways of protein degradation were experimentally distinguished. This cellular model provided me with an important tool for investigating the characteristics of MT degradation in subsequent experiments (Chapter IV).

CHAPTER IV

DEGRADATION OF ZINC METALLOTHIONEIN IN MONOLAYER CULTURES OF ADULT RAT HEPATOCYTES

INTRODUCTION

Metallothionein (MT) represents a family of ubiquitous, low molecular weight, cysteine-rich, heavy-metal binding proteins (1). This protein is composed of a single polypeptide chain and at least two distinct isoforms of MT are present in most eukaryotes. Roles of MT in the metabolism of essential micronutrients (i.e., Zn and Cu) and the detoxification of heavy metals have been suggested (38). Most studies on MT metabolism have focused on liver and kidney, the primary sites of heavy metal accumulation. The synthesis of MT is induced by a variety of factors, including exposure to heavy metals, physiological stresses and alteration in endocrine status (2). In virtually all cases examined thus far, increased synthesis of MT resulted from enhanced transcription of MT genes (16).

In contrast to the plethora of information on the structure and synthesis of MT, knowledge of MT degradation is limited. In vivo studies have suggested hepatic Cu- and Zn-MT ($t_{1/2}$= 15-20 hours) turn over significantly faster than Cd-MT ($t_{1/2}$= 80 hours) (59-64). In such studies, it has been assumed that the disappearance of ^{35}S-labelled MT from the cytosol fraction reflects actual degradation of this protein. This conclusion is supported by in vitro studies showing that apothionein, Zn-MT and Cd-MT were degraded in lysosomal extracts (66) and by neutral proteinases (9,66). However, Cu-MT is resistant to lysosomal degradation in vitro

(64,67). In addition, the possibility that loss of MT from cytoplasm in vivo might be due, at least in part, to secretion or transfer to an organelle other than lysosomes, e.g., mitochondria, has not been previously addressed (65,160). It is apparent that the characteristics and regulation of MT degradation remain largely unknown. Information concerning MT degradation is important for a more complete understanding of factors that affect the level of this protein in various tissues. Moreover, elucidation of the function of MT in regulation of essential trace element metabolism will be facilitated by an understanding of MT degradation. With the above in mind, I initiated an investigation of Zn-MT degradation in monolayer cultures of adult rat hepatocytes.

Recent advances in the field of protein degradation indicates that both intrinsic properties of a protein and regulation of the catabolic machinery contribute to the observed heterogenous rate of proteolysis. It appears that the regulation of intracellular catabolic pathways (i.e. lysosomal and non-lysosomal processes) offers coarse control of protein turnover, while the structural characteristics of a protein provide the fine control in determining its rate of degradation (85). Data presented in this study indicate that Zn, which apparently stabilizes the tertiary structure of MT, provides such fine regulation in the degradation of this protein. In addition, it was found that MT is degraded in both cytoplasmic and

lysosomal compartments. Possible mechanisms on MT degradation are discussed.

MATERIALS AND METHODS

Culture and harvesting of hepatocytes

The isolation and purification of adult rat hepatocytes has been described previously (Chapter III). Monolayers of hepatocytes were incubated in Waymouth's MB 752/1 medium containing 0.2% bovine serum albumin (BSA), 10^{-8}M insulin, and 100 uM Zn (as $ZnSO_4.7H_2O$) for 20 hours to induce MT synthesis. After overnight incubation, spent medium was removed by aspiration and monolayers were washed twice with ice-cold Puck's buffer to remove residual medium and detached cells. At the time of sample collection, hepatocytes from each dish were harvested in 1.5 ml 10 mM Tris-HCl buffer, pH 8.6, containing 0.02% Triton X-100 and 5 mM 2-mercaptoethanol (BME). Protease inhibitors, PMSF (0.01%) and leupeptin (10 ug/ml) also were added to the buffer to inhibit cellular proteolytic activity. Cell suspensions were sonicated (Microson Instrument, Farmingdale, NY) at 40% output for 30 seconds using a microprobe. Microscopic examination of the homogenate showed complete disruption of cells.

Determination of cellular MT

1. *In vitro* ^{109}Cd binding assay

Due to limited cellular material obtained from primary culture of hepatocytes, the amount of MT in each sample is relatively low. Two

approaches were developed to quantify the cellular level of MT. In the initial approach, I used ^{109}Cd to label MT *in vitro*. Liver cytosol samples prepared from control and Zn injected rats (1 mg Zn/kg body wt., for 2 consecutive days) were utilized to develop the experimental protocol. The method of Lehman and Klassen (161) was followed with some modifications. First, I determined the amount of Cd required to saturate cytosol MT. Cytosol samples were diluted with 10 mM Tris-HCl, 5% glycerol, pH 7.6 to 1 mg protein/ml. Samples were then incubated with various amount of Cd (as $CdCl_2$) and 0.2 uCi ^{109}Cd for 15 minutes at 0 ^{o}C to permit exchange of ^{109}Cd with Zn associated with MT. Because MT is a heat stable protein, the sample was next heated at 70^{o}C for 5 minutes. The majority of other cellular proteins were precipitated by heating and removed by centrifugation at 10,000 x g, for 15 minutes. The amount of ^{109}Cd in the heat treated supernatant was measured to determine Cd-MT. Results were calculated as mg Cd bound to MT per mg cytosol protein. It was found that MT apparently was saturated in the presence of 2-4 ug Cd per mg cytosol protein. Therefore, 4 ug Cd (0.1 uCi ^{109}Cd/ug Cd) was added to sample containing 1 mg of protein to saturate MT in subsequent analyses. Isoforms of ^{109}Cd-MT were purified from the heat stable fraction by Fast Protein Liquid Chromatography (FPLC, Pharmacia Inc., Piscatway, NJ). The fractionation scheme for FPLC will be described below. Purified isoforms of ^{109}Cd-labelled MT were again subjected to heat treatment and FPLC to determine the

recovery of these proteins during purification procedures (see Results).

2. Measurement of cellular ^{35}S-MT

The second approach taken involved the use of a radioactive amino acid to label MT polypeptide. Monolayers of hepatocytes were incubated in medium containing ^{35}S-cysteine (20 uCi/nmol) and 100 uM Zn for 20 hours. In a preliminary experiment, cell homogenate was centrifuged at 150,000 x g for 75 minutes to remove the particulate fractions. The high speed supernatant was fractionated by Sephadex G-75 chromatography (Pharmacia Inc., Piscatway, NJ). The Sephadex G-75 column (22 x 600 mm) was equilibrated and eluted with 20 mM Tris-HCl, 5 mM BME, pH 8.6. Flow rate was 24 ml/hour and 4 ml fractions were collected (Fig. 4.1). It was found that ^{35}S was distributed among high molecular weight proteins (1.0-2.0 void volume, V_o), MT (2.0-2.5 x V_o) and low molecular weight species (total volume, V_t).

To facilitate the measurement of ^{35}S-MT by FPLC, cell homogenate was heat treated and centrifuged as described above. The ^{35}S-labelled high molecular weight proteins were eliminated by heat treatment and approximately 95% of ^{35}S-MT was recovered in heat stable fractions (Fig. 4.1). Heat-treated supernatant was concentrated by ultrafiltration (Centricon 10, Amicon Co., Danvers, MA) to 0.5 ml. Non-radioactive Cd-MT (100 ug) and BSA (1 mg) were

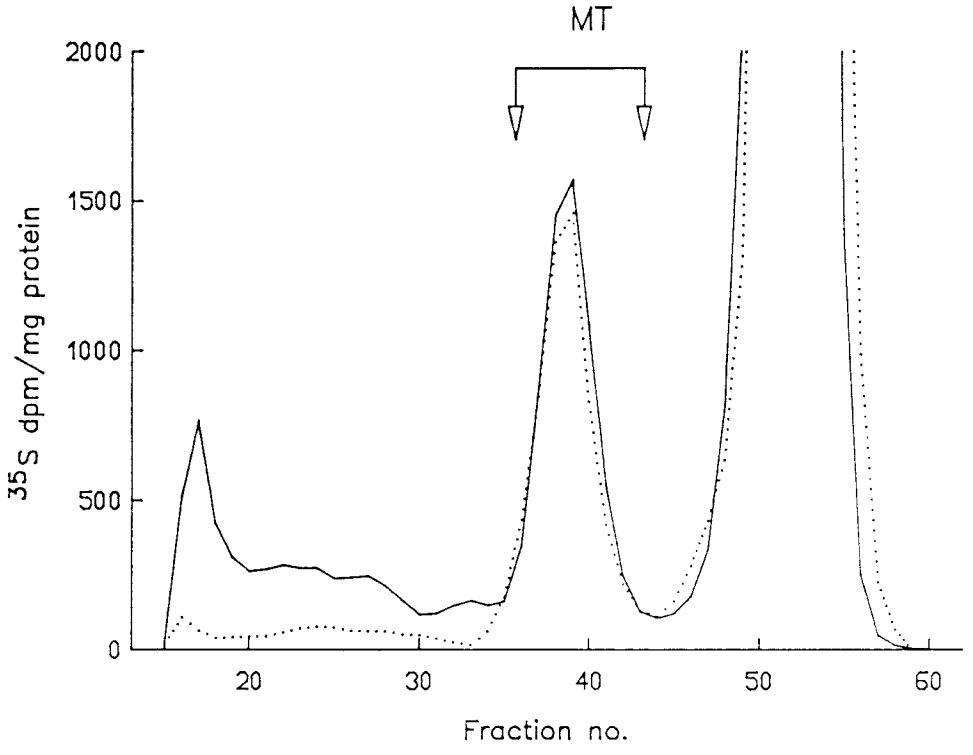

Fig. 4.1 Sephadex G-75 elution profile for samples prepared from monolayers of hepatocytes previously labelled with ^{35}S-cysteine. Monolayers of hepatocytes were incubated in medium containing ^{35}S-cysteine (10 uCi/mmol) and 100 uM Zn overnight. Soluble fraction (——) was prepared by centrifugation of cell homogenate at 150,000 x g for 75 min. Heat stable fraction (·····) of cell homogenate was prepared by heat treatment (70°C, 5 min.) and centrifugation (10,000 x g, 15 min.). Samples were analyzed by Sephadex G-75 chromatography as described in the Materials and Methods.

added to samples before heat-treatment and ultrafiltration steps, respectively, to minimize non-specific loss of ^{35}S-MT. However, the presence of a relatively large amount of low molecular weight ^{35}S-containing species (presumably glutathione) in the heat stable supernate interfered with the analysis of MT by FPLC. Therefore, the concentrated heat stable supernate was next fractionated by Sephadex G-25 chromatography (Pharmacia Inc., Piscatway, NJ) to remove ^{35}S-glutathione. The Sephadex G-25 column (9 x 300 mm) was equilibrated and eluted with 0.1 M Tris-HCl, 5 mM BME, pH 8.6. One milliliter fractions were collected at a flow rate of 15 ml/hour and MT was eluted at the void volume. Fractions containing ^{35}S-MT were pooled and aliquots were analyzed by FPLC.

3. Analysis of MT by FPLC

Chromatography of MT was performed by FPLC equipped with either a gel filtration column (Superose 12) or an anion-exchange column (Mono Q HR 5/5). Sample (200 ul) was injected onto a Superose 12 column equilibrated with 0.1 M Tris-HCl, pH 8.6. MT was eluted at 80 minutes with a flow rate of 0.2 ml/minute and 1 ml fractions were collected. ^{35}S in each fraction was determined by transferring eluate to a plastic vial containing 10 ml scintillation fluid (Scinti Verse II, Fischer, Fairlawn, NJ) and counting in a Beckman LS 3500 spectrophotometer. Quenching was corrected with an automatic external standard. The amount of ^{35}S-MT was expressed as dpm per

mg cell protein. The protein concentration was measured by a modified Lowry method (140) using BSA as the standard.

The mobile phase of Mono Q column consisted of A: 20 mM Tris-HCl, 5% glycerol, pH 8.6; B: 200 mM Tris-HCl, 5% glycerol, pH 8.6. Sample (500 ul) collected from void volume of Sephadex G-25 was injected. After isocratic elution with buffer A for 6 minutes, applied material was eluted with a linear gradient in 20 minutes (0-100%B). The flow rate was 1 ml/minute and 1 ml fractions were collected. MT-I and MT-II were eluted at 13 and 20 minutes, respectively.

In pilot studies, MT-I and MT-II purified from livers of Cd-injected rats (Chapter II) were used to standardize both Superose 12 and Mono Q columns. Elution was followed by monitoring absorbance at 254 and 280 nm and the amount of Cd and Zn in each fraction was determined by atomic absorption spectrophometry. Cd, Zn and A_{254} eluted in identical peaks having minimal absorbance at 280 nm. The chromatographic profiles of Cd-MT purified from intact rat liver and ^{35}S-MT from cultured hepatocytes were identical.

MT degradation

To study the turnover of MT, monolayers of hepatocytes containing ^{35}S-MT were washed twice with Medium 199, then incubated in fresh medium for 6 hours. The experimental medium consisted of Waymouth's MB 752/1, 0.2% BSA, 10^{-8}M insulin, excess cysteine (2 mM) and indicated substances. The concentration of Zn in experimental medium

was 1 uM as determined by atomic absorption spectrophotometry. The level of ^{35}S-MT at 0 and 6 hour was quantified by FPLC as described above. Data are expressed as percent of initial MT degraded per 6 hours.

To probe the subcellular site(s) of MT degradation, the effects of chloroquine and TLCK (tosyl-lysine chloromethyl ketone) on MT degradation were investigated. As disscussed in Chapter III, chloroquine is a lysosomotropic amine and TLCK is an inhibitor of serine proteases. Morever, TLCK does not interefere with the lysosomal pathway. Stock solutions of chloroquine and TLCK were prepared in aqueous medium (Waymouth's MB 752/1) and ethanol (95% v/v), respectively. Cellular protein degradation was not affected by the presence of 1% alcohol. These chemicals were added to the medium at the begining of the experimental period (0 h).

In a parallel set of replicate cultures, monolayers were labelled overnight with ^{3}H-leucine (10 uCi/nmol) and the rate of ^{3}H-protein degradation was determined as described in Chapter III. Brifely, ^{3}H-labelled monolayers were washed and medium containing 2 mM leucine was added for 4 hours to "chase" ^{3}H-leucine from the intracellular amino acid pool. Studies were initated by adding fresh medium containing 2 mM leucine and other indicated substances. The rate of degradation of ^{3}H-protein was estimated by dividing the level of acid-soluble ^{3}H by the amount of total radioactivity in each well. Because MT does not contain leucyl residues (1),

determination of the rate of degradation of ^{3}H-protein (non-MT) provided information on the effects of various treatment on the characteristics on general protein turnover.

MT synthesis

It was assumed that MT synthesis was not completely inhibited by substitution of medium with 1 uM Zn for induction medium, since the half-life of MT mRNA in mouse hepatoma cells was estimated as 2.5 hours (162). The relative degree of MT synthesis was estimated in two ways. First, hepatocytes were incubated with 100 uM Zn overnight. Zn-loaded cells were then incubated in fresh medium containing ^{35}S-cysteine and various concentrations of Zn for 6 hours. The relative level of ^{35}S-MT synthesis was estimated from the amount of radioactivity in heat treated supernate that eluted at the void volume of Sephadex G-25 column. In a second approach, cycloheximide was used to inhibit the incorporation of ^{35}S-cysteine into MT. At 3.4 uM concentration, cycloheximide inhibited protein synthesis by greater than 85% (Fig. 3.4). ^{35}S-MT synthesis was also analyzed in cultures treated with cycloheximide, chloroquine and TLCK. These chemicals were added to Zn-loaded hepatocytes one hour before the addition of ^{35}S-cysteine and incubation was continued for 5 hours. ^{35}S-MT was quantified by Sephadex G-25 chromatography as described above.

Determination of cellular Zn status

The level of Zn in primary cultures of hepatocytes was below the detection limit of the flame atomic absorption spectrophotometry. Although the sensitivity of flameless atomic absorption spectrophotometry is sufficient to determine cellular Zn concentration, normal handling of samples may introduce contamination with exogenous Zn. Therefore, cellular Zn status was estimated by examining the efflux and uptake of ^{65}Zn in monolayers of hepatocytes.

To study Zn efflux, monolayers of hepatocytes were incubated in medium containing 100 uM Zn with ^{65}Zn (0.2 uCi/ml) overnight. Hepatocytes were then washed twice with M199 medium containing 10 mM EDTA followed by two washes of M199 to remove non-specifically bound Zn from cell surfaces. One set of cells was harvested at this time to determine ^{65}Zn content at time 0. The remaining dishes were then incubated in fresh medium containing indicated concentrations of Zn. After 6 hours, medium was removed and monolayers were washed twice with ice-cold Puck's buffer containing 10 mM EDTA to quench Zn transport and remove Zn non-specifically bound to cell surface. This was followed by two consecutive washes with ice-cold buffer without EDTA. Hepatocytes were harvested in 0.2% Triton X-100 and the level of ^{65}Zn retained by cells was determined by gamma ray spectrometry (LKB 1282 Compugamma).

To study Zn uptake, monolayers that had been incubated overnight

in medium containing 100 uM Zn without ^{65}Zn were washed as above before addition of fresh medium with ^{65}Zn (0.2 uCi/ml) and indicated concentrations of Zn for 6 hours. The level of ^{65}Zn in cells was determined as described above.

Zn uptake by hepatocytes was expressed as nmoles per mg of cell protein. Zn efflux was calculated as the difference between cellular Zn concentration at 0 and 6 hour. Therefore, the net change in the amount of Zn transported was estimated from the difference between uptake and efflux. Cellular Zn status at 6 hour was defined as the difference in the cellular concentration of Zn at 0 hour and the amount of Zn secreted or accumulated during the experimental period.

Statistical analysis

Data are expressed as mean$\pm$S.E.M.. Each datum represents the mean value from at least three samples from 2-3 separate cell preparations. One way analysis and Student-Newman-Keuls multiple range test was used to test for significant difference between each treatment group ($p< 0.05$).

RESULTS

Induction and turnover of MT

In preliminary experiments, monolayers of hepatocytes were incubated in medium containing ^{35}S-cysteine and 100 uM Zn (^{65}Zn) overnight. The distribution of intracellular ^{35}S and ^{65}Zn was examined by gel filtration chromatography. Cell homogenates from 4 dishes were pooled and centrifuged (150,000 x g, 75 minutes) to separate particulate from soluble fractions. The resultant supernatant, which accounted for 86% of total cellular ^{35}S, was fractionated on Sephadex G-75 column (Fig. 4.2A). The relative levels of soluble ^{35}S distributed among high molecular weight proteins (1-2.0 x V_o), MT (2.0-2.5 x V_o) and low molecular weight species (V_t) were 10, 12 and 75%, respectively. When Zn-loaded hepatocytes were incubated in Zn-deficient medium (1 uM Zn) for 6 hours, the amounts of ^{35}S-proteins, ^{35}S-MT and low molecular ^{35}S-containing species were 93, 65 and 75% of their initial value, respectively (Fig. 4.2A).

Approximately 80% of total cellular ^{65}Zn was presented in the soluble fraction and 43% of this soluble Zn was associated with MT (Fig. 4.2B). Thus, 34% of the total cellular Zn was bound to MT. This finding is consistent with the postulated role of MT as the storage site for Zn (2). After a 6-hour incubation of Zn-loaded hepatocytes in medium with 1 uM Zn, the levels of Zn associated with

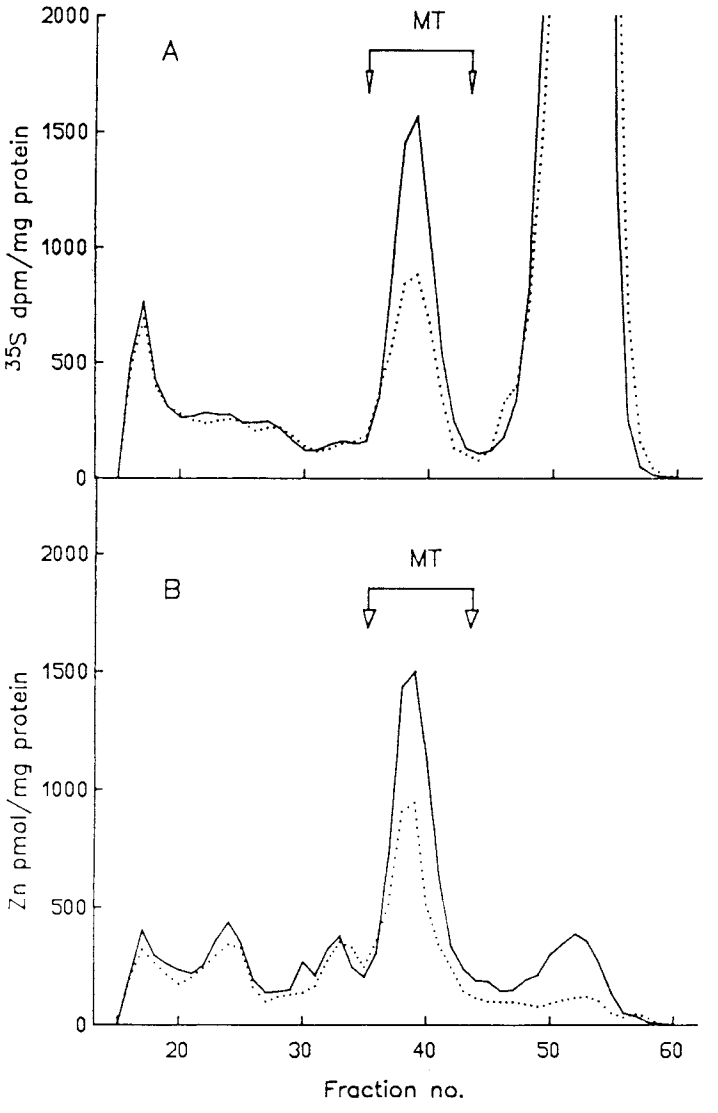

Fig. 4.2 Sephadex G-75 elution profile for soluble fraction prepared from hepatocytes previously labelled with ^{35}S-cysteine and ^{65}Zn. Monolayers of hepatocytes were incubated in medium containing ^{35}S-cysteine (10 uCi/mmol) and 100 uM Zn/^{65}Zn (0.2 uCi/ml) overnight (0h, ——). To study MT turnover, cells were washed and reincubated in medium containing 1 uM Zn for 6 hours (·····). Soluble fraction was prepared by centrifugation of cell homogenate and analyzed by Sephadex G-75 gel filtration chromatography as described in the Materials and Methods. The chromatograms of ^{35}S and Zn are shown in panel A and B, respectively.

^{35}S-MT and ^{35}S-proteins were 62% and 87% of their initial amount, respectively. It is apparent that turnover of ^{35}S-MT was faster than that of ^{35}S-proteins and was accompained by the loss of Zn from the MT fraction. It was also noted that intracellular low molecular weight Zn pool (eluted at V_t) was depleted faster than that of Zn-MT and other Zn associated metalloproteins, perhaps reflecting loss of the metal to the extracellular space. Furthermore, the decrease in intracellular ^{35}S-MT level was not due to secretion of this protein, since only less than 1% of total cellular MT was recovered in the spent medium after 6 hours (Fig. 4.3). Approximately 30% of ^{65}Zn in spent medium was associated with proteins secreted by hepatocytes and/or exogenous BSA, while the majority of extracellular ^{65}Zn eluted at V_t. This low molecular weight pool of Zn was not further characterized.

Determination of MT in hepatocytes by FPLC methods

Due to the limited quantity of material that can be obtained from primary cultures of hepatocytes, the amount of ^{35}S-MT in each sample is relatively low. Conventional chromatographic separation of MT by Sephadex G-75 and DEAE A-25 is a time consuming process that requires relatively large amounts of material. Thus, it was essential to develop a rapid method for isolation and quantitative analysis of ^{35}S-MT from limited amounts of cell material. FPLC seemed to offer a sensitive alternative for the rapid measurement of

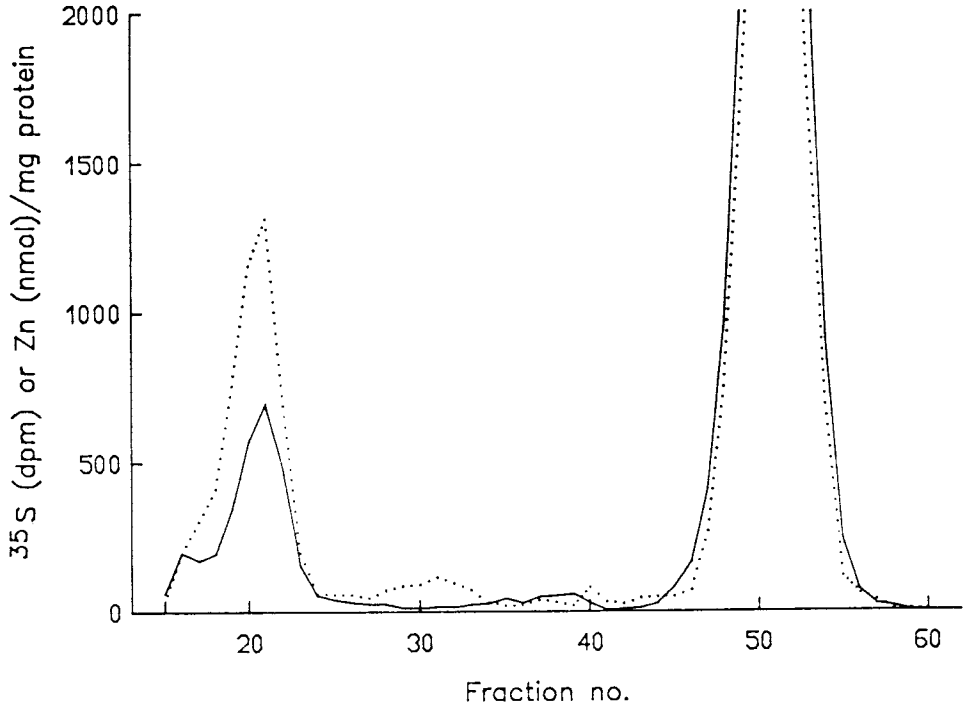

Fig. 4.3 Elution profile of Sephadex G-75 of spent medium. ^{35}S-labeled and ^{65}Zn-loaded hepatocytes were washed and incubated in medium with excess cold cysteine and 1 uM Zn for 6 hours. At the completion of study, medium was collected and centrifuged at 1,000 x g for 10 min. to remove detached cells. Spent medium was concentrated by ultrafiltration (YM-2 membrane) and analyzed by Sephadex G-75 chromatography. Eluate was monitored by measuring ^{35}S (——) and ^{65}Zn (·····).

cellular ^{35}S-MT.

To determine the applicability of FPLC methods, MT-I and MT-II were purified from livers of rats injected with Cd (2mg Cd/Kg B.W.) and ^{35}S-cysteine (30 uCi/100 g B.W.). The purification of ^{35}S-labelled MT-I and MT-II by Sephadex G-75 and DEAE A-25 has been decribed in Chapter II. Purified isoforms of MT (100 ug each) were mixed with cytosol prepared from control rat liver. This sample was subjected to heat treatment, ultrafiltration and chromatography on Sephadex G-25, Superose 12 and Mono Q columns. The recoveries of ^{35}S-MT from each treatment step were greater than 85% and final recovery was 76% (Table 4.1). In a pilot experiment, it was found that the percent recovery of ^{35}S-MT decreased with decreasing amounts of this protein. Addition of exogenous Cd-MT to homogenates prevented non-specific losses of cellular ^{35}S-MT during sample processing. Therefore, 100 ug of non-labelled Cd-MT was routinely added to homogenates prepared from primary cultures of hepatocytes in subsequent studies. In another series of studies, MT was quantified by *in vitro* ^{109}Cd binding assay. It was found that relatively high amounts of Cd bound to Superose 12 and Mono Q resins (Table 4.1). Therefore, it was not possible to quantify MT by *in vitro* ^{109}Cd binding assay.

Cell homogenate prepared from ^{35}S-labelled, Zn-loaded hepatocytes was heat treated and fractionated by Sephadex G-25 chromatography. ^{35}S in fractions that eluted at void volumes of

Table 4.1 Recovery (%) of ^{35}S- or ^{109}Cd-labelled isoforms of MT during purification*.

Purification scheme	Recovery (%) MT-I ^{35}S	MT-I ^{109}Cd	MT-II ^{35}S	MT-II ^{109}Cd
Heat treatment	98	99	98	99
Centricon 10	93	-	90	-
Sephadex G-25	95	-	95	-
Superose 12	86	56	89	65
Mono Q HR 5/5	89	50	85	50

*^{35}S-labelled MT isoforms I and II were purified from the livers of rats injected (i.p.) with Cd and ^{35}S-cysteine. Purified proteins (100 ug) were mixed with liver cytosol from control animals and subjected to heat treatment (70^{o}C, 5 min.). After removal of precipitate, heat stable proteins were separated from small peptides and amino acids by Sephadex G-25 chromatography. Aliquots of Sephadex G-25 eluate were injected into FPLC (Superose 12 and Mono Q columns). In a separate study, isoforms of ^{109}Cd-MT were prepared by adding ^{109}Cd to liver cytosol of Zn injected rat and purified by FPLC. The purified ^{109}Cd-MT was again subjected to heat treatment and FPLC analysis as described in the Materials and Methods. The amount of MT recovered was determined by following either ^{35}S or ^{109}Cd.

the Sephadex G-25 column were pooled and aliquots were injected onto either Superose 12 or Mono Q columns (Fig. 4.4). From the elution profile of Superose 12, it was found that MT is the predominant species (>75%) in the void fraction of Sephadex G-25. ^{35}S-MT content of cells decreased by 40% when monolayers were incubated in Zn-deficient medium for 6 hours. This decrease in the level of ^{35}S-MT was similar to that obtained when samples were analyzed by the traditional approach (Sephadex G-75 chromatography). Thus, fractionation of heat stable material by Sephadex G-25 gel filtration chromatography with subsequent analysis by FPLC provided a rapid and sensitive way to quantify small amounts of cellular MT.

Analysis of the void fraction of Sephadex G-25 by FPLC, using a Mono Q column, revealed the presence of two isoforms of MT (Fig. 4.4). After overnight incubation, the level of MT-I was 1.25-fold higher than that of MT-II in monolayers of hepatocytes. The degradation of MT-II (55%/6h) was greater than that of MT-I (27%/6h) when cells were incubated in medium containing 1 uM Zn.

Estimation of the half-life of MT

To estimate the half-life of MT, cells were first incubated in medium containing ^{35}S-cysteine and 100 uM Zn for 20 hours. After removal of the radiolabelled precursor and the inducing metal, monolayers were harvested periodically and cellular levels of ^{35}S-MT were quantified (Fig. 4.5). The turnover of MT ($t_{1/2}$= 7

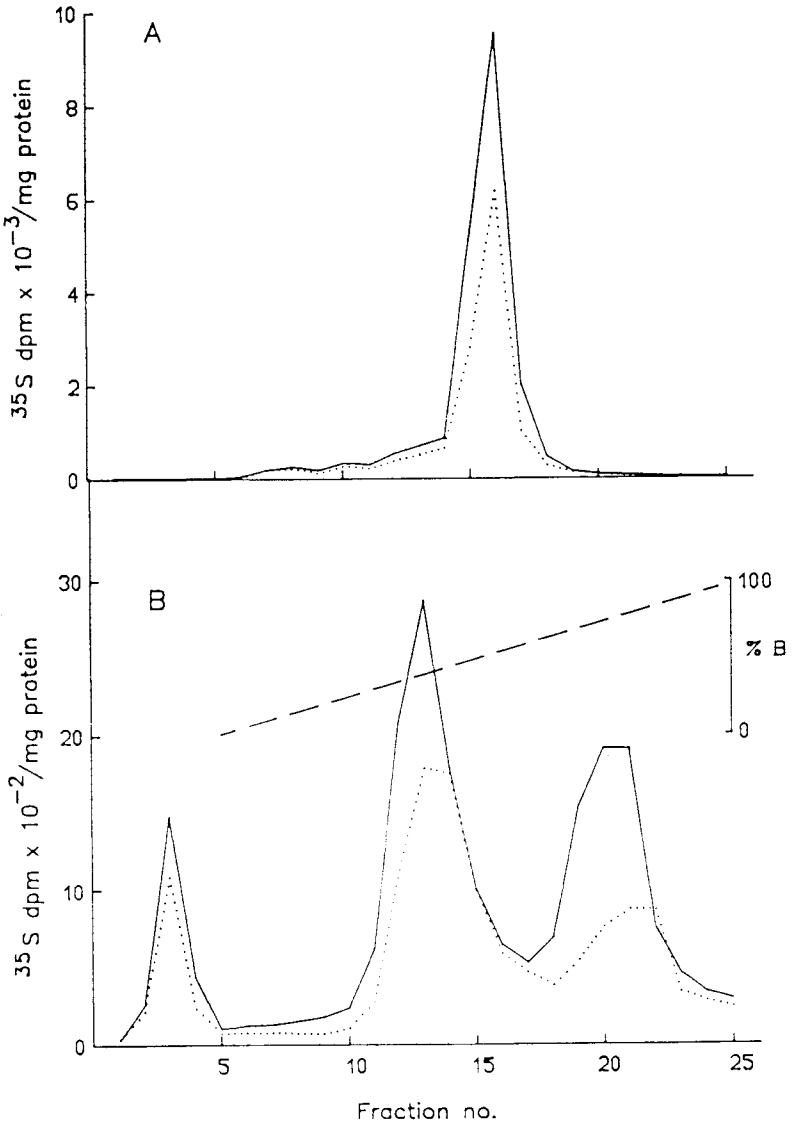

Fig. 4.4 FPLC analysis of heat stable extract from monolayers of hepatocytes incubated in medium with ^{35}S-cysteine and 100 uM Zn. Cell homogenate was prepared from Zn-loaded hepatocytes previously labelled with ^{35}S-cysteine (0 h, ——) and then reincubated in 1 uM medium Zn for 6 hours (.....). After heat treatment, aliquots of the void fraction eluted from Sephadex G-25 was applied to either Superose 12 (A) or Mono Q (B) column. Chromatographic separation using FPLC is described in the Materials and Methods.

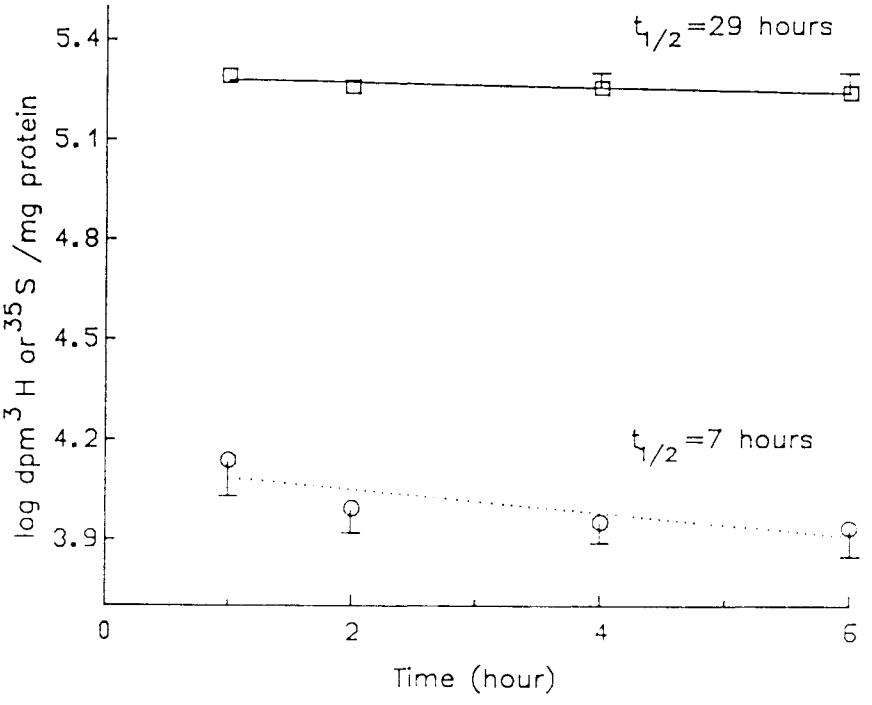

Fig. 4.5 Turnover of ^{35}S-MT and ^{3}H-proteins in monolayers of hepatocytes. Monolayers of hepatocytes were incubated in medium containing 100 uM Zn and either ^{35}S-cysteine (···) or ^{3}H-leucine (—) for 20 hours. To initiate experiments, fresh medium with 1 uM Zn and excess cysteine was added to ^{35}S-labelled cells and incubation was terminated at 1, 2, 4 and 6 hours. For ^{3}H-labelled hepatocytes, a 4-hour chase period was included before initiating experiments. The half-life of proteins was calculated from the slope of regression line as outlined by Segal and Kim (128).

hours) was 4 times faster than ^{3}H-proteins ($t_{1/2}$= 29 hours). Since MT is the predominant Zn-binding species synthesized when cells are incubated with 100 uM Zn, the rapid decrease in cellular MT level after removal of medium Zn suggested a pivotal role for Zn in the regulation of MT degradation.

Accurate determination of the half-life of a protein precludes isotope reincorporation. I previously demonstrated that a 4-hour chase period effectively prevented reutilization of ^{3}H-leucine in hepatocytes (Fig. 3.1). Various attempts to chase ^{35}S from the non-protein pool were unscessful, probably because the primary species of ^{35}S was glutathione instead of cysteine. Thus, reincorporation of ^{35}S-cysteine into MT during the experimental period was a matter of concern. To estimate the contribution of newly synthesized ^{35}S-MT to cellular concentration of this protein, Zn-loaded hepatocytes were incubated in medium containing 1 uM Zn and ^{35}S-cysteine for 6 hours. The amount of ^{35}S-MT synthesized was approximately 8% of the concentration of ^{35}S-MT found in hepatocytes after overnight incubation in medium with 100 uM Zn and ^{35}S-cysteine. Therefore, the half-life of MT, estimated in the present study, does not appear to be markedly overestimated since both Zn and ^{35}S-cysteine were removed during the experimental period. In a separate series of studies, cycloheximide was used to inhibit the reincorporation of ^{35}S-cysteine into MT (see below).

Effects of extracellular Zn concentration on hepatic Zn and MT level

To examine the relationship between medium Zn, cellular Zn and MT degradation, first I investigated the efflux and uptake of Zn by hepatocytes. Hepatocytes accumulated approximately 4 nmol Zn/mg cell protein during overnight incubation with 100 uM medium Zn. When ^{65}Zn-labelled cells were incubated in medium containing various concentrations of Zn for 6 hours, the amount of Zn secreted from cells increased in proportion to the medium concentration of Zn (Fig. 4.6). Probably, this was due to the rapid exchange between extracellular Zn and preexisting intracellular Zn pools. ^{65}Zn uptake was assessed in a parallel series of study. The uptake of Zn by hepatocytes also increased proportionally as the extracellular concentration of Zn increased. When medium Zn was less than 50 uM, the amount of Zn lost from Zn-loaded hepatocytes was greater than the amount of Zn taken up from the medium, thereby resulting in a net decrease in cellular Zn content. Zn balance, i.e., no change in cellular zinc content, was attained at approximately 50 uM medium Zn (Table 4.2).

The efflux of Zn from Zn-loaded hepatocytes may decrease the specific activity of ^{65}Zn in medium. Failure to consider this process may result in underestimation of Zn uptake, especially in medium with low levels of Zn (e.g. 1 uM). Using the amount of ^{65}Zn present in medium (1 uM Zn) after a 6-hour incubation of ^{65}Zn-labelled cells in medium without exogenous ^{65}Zn, the maximal

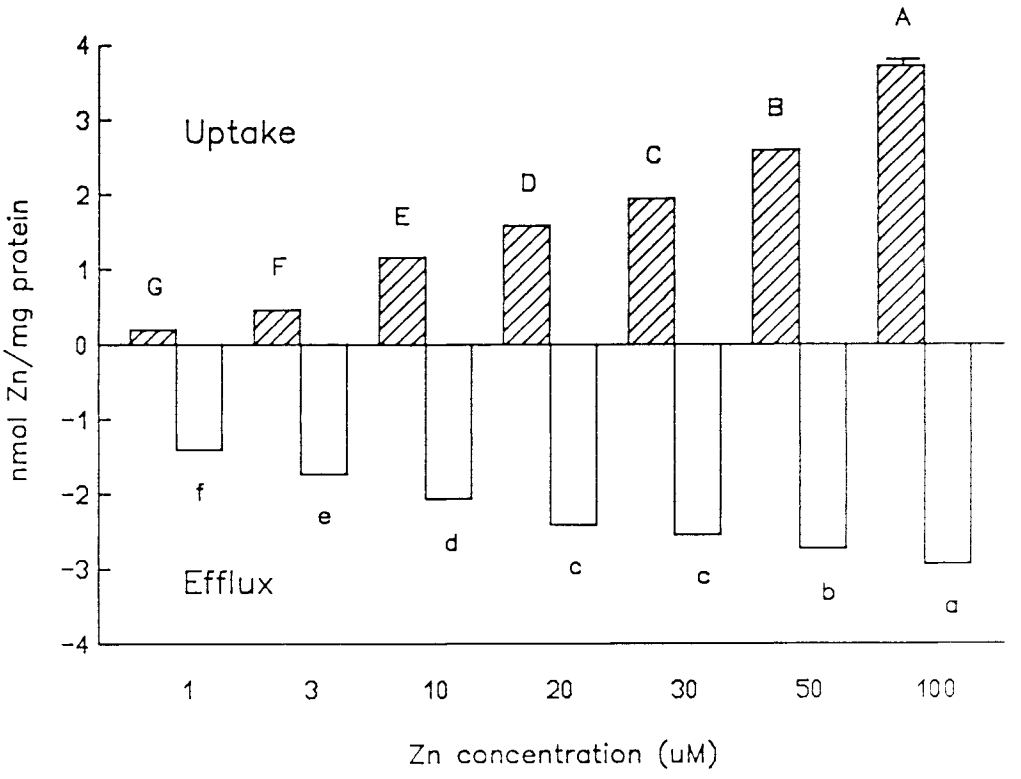

Fig. 4.6 Effects of extracellular concentration of Zn on the uptake and efflux of the micronutrient in monolayers of hepatocytes. Hepatocytes were incubated in medium containing 100 uM Zn with or without ^{65}Zn overnight. The ^{65}Zn-labelled cells were washed and fresh medium containing each of the indicated concentrations of Zn was added. The efflux of Zn from cells was monitored by measuring cellular ^{65}Zn at 0 and 6 hour. In uptake experiments, monolayers incubated in medium without ^{65}Zn overnight were incubated in fresh medium with indicated concentrations of ^{65}Zn. The accumulation of ^{65}Zn by cells was measured after 6 hours. Data are expressed as nmol Zn/mg protein. Means with different letter above/below bars are significantly different at $p < 0.05$. Standard errors of means at each treatment groups were less than 3% of the mean values.

Table 4.2 Effects of medium Zn concentration on cellular Zn status in monolayers of hepatocytes*.

Medium Zn conc. (uM)	nmol Zn/mg protein: Net efflux (-) or uptake (+)	nmol Zn/mg protein: Cellular Zn conc.
1	-1.20 ± 0.06^{e}	2.67 ± 0.06^{E}
3	-1.27 ± 0.08^{e}	2.60 ± 0.08^{E}
10	-0.89 ± 0.04^{d}	2.98 ± 0.04^{D}
20	$-0.81\pm0.03^{c,d}$	$3.06\pm0.03^{C,D}$
30	-0.59 ± 0.06^{c}	3.28 ± 0.06^{C}
50	-0.13 ± 0.10^{b}	3.74 ± 0.10^{B}
100	$+0.78\pm0.15^{a}$	4.65 ± 0.15^{A}

*Cellular Zn concentration and the net change in its efflux and uptake was calculated from data presented in Fig. 4.6. After overnight incubation in medium containing 100 uM Zn (0 hour), hepatocytes accumulated 3.87±0.20 nmol Zn/mg cell protein. Data are expressed as Mean±SEM of six cell samples from two different donor rats. Means with different letters as superscripts are significantly different at $p < 0.05$.

difference between the calculated and experimentally determined cellular concentration of Zn was 0.14 nmol/mg protein (2.81 vs. 2.67 nmoles). When cells were incubated in medium containing 100 uM Zn, the difference between the calculated and experimentally determined level was negligible (4.70 vs. 4.64 nmol/mgprotein). Such calculations fail to account for the dynamic nature of Zn transport. However, the difference between actual and experimentally determined estimates of the impact of extracellular Zn levels on cellular Zn status seem to be minimal and were not considered further when interpreting results.

To evaluate the impact of extracellular Zn concentration on MT degradation, ^{35}S-labelled, Zn-loaded hepatocytes were incubated in medium containing indicated concentrations of Zn for 6 hours. The level of cellular MT was determined by Superose 12 chromatography. The rate of MT degradation was inversely proportional to the concentration of extracellular (Fig. 4.7) and cellular Zn (Table 4.2). In contrast, the degradation of ^{3}H-proteins was not affected by changes in the intracellular levels of Zn (data not shown).

The possibility that higher levels of ^{35}S-MT were present in cultures that had been incubated in medium containing high levels of Zn was due to significant reincorporation of ^{35}S-cysteine into newly synthesized MT required consideration. To circumvent this possibility, cycloheximide (3.4 uM) was added to medium. At this level of cycloheximide, the synthesis of ^{3}H-protein as well as

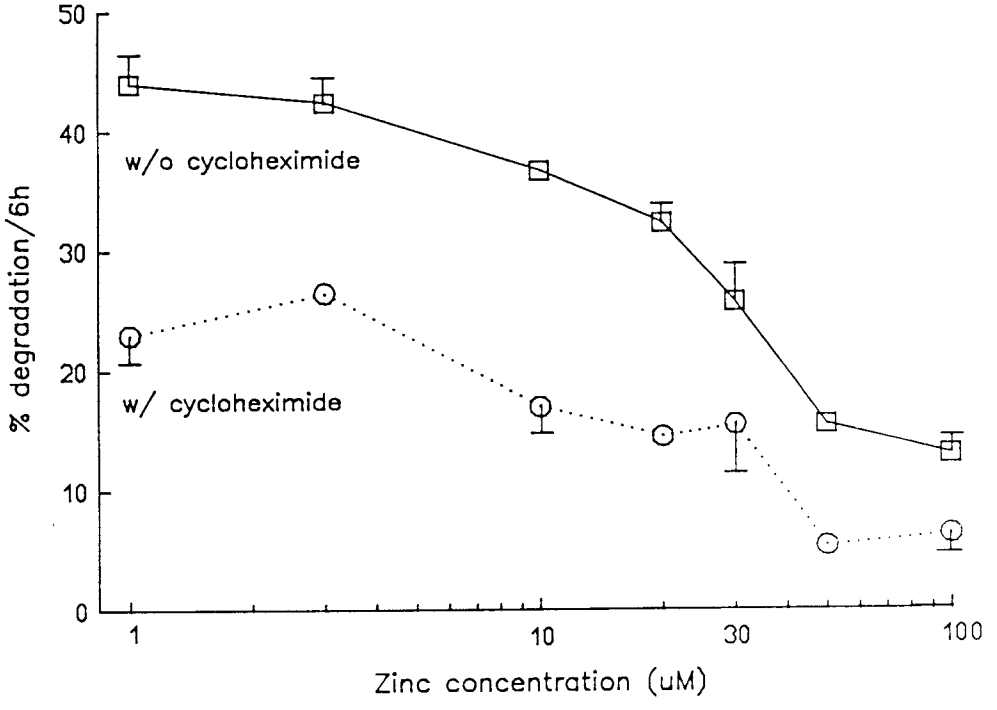

Fig. 4.7 Effects of extracelluar Zn concentration and cycloheximide on the degradation of ^{35}S-MT in primary cultures of hepatocytes. Zn-loaded hepatocytes previously labelled with ^{35}S-cysteine were incubated in medium containing indicated concentration of Zn with or without cycloheximide (3.4 uM). The level of ^{35}S-MT was determined as described in the Materials and Methods. Data are presented as percent of initial MT degraded per 6 hour.

^{35}S-MT in cells incubated in 100 uM medium Zn was inhibited by greater than 85% (data not shown). In the presence of cycloheximide, the rate of MT degradation also was inversely proportional to cellular Zn status. However, cycloheximide attenuated MT degradation at all levels of medium Zn examined (Fig. 4.7), while only slightly affecting the cellular level of the metal (Table 4.3). Consequently, the decrease in MT degradation in cycloheximide-treated cells was not due to increase in retention of celluar Zn. It is more probable that decreased MT degradation was due to cycloheximide-mediated inhibition of lysosomal degradation (Table 3.3).

Effects of chloroquine and TLCK on MT degradation

To probe the subcellular site(s) of MT degradation, the effects of chloroquine and TLCK on MT turnover were investigated. At 1 uM extracellular Zn, cycloheximide, chloroquine and TLCK inhibited MT degradation by 33, 65 and 50%, respectively (Fig. 4.8). Degradation of non-MT protein (^{3}H-leucyl-protein) was inhibited by 41, 41 and 16% in the presence of cycloheximide, chloroquine and TLCK, respectively. At 20 uM medium Zn, the levels of ^{35}S-MT in chloroquine- and TLCK-treated cells at 6 hour were comparable to those at 0 hour, suggesting complete inhibition of ^{35}S-MT degradation (data not shown). Therefore, MT appeared to be degraded in both lysosomal and cytoplasmic compartments.

Experiments were performed to assess whether higher levels of

Table 4.3 Effects of extracellular Zn concentration and cyclohexamide on cellular Zn status*.

Treatment		
Zn (uM)	Cyclohexamide (3.4 uM)	Cellular Zn concentration (nmol/mg protein)
1	-	2.54 ± 0.03^{e}
1	+	2.42 ± 0.02^{f}
10	-	2.75 ± 0.03^{e}
10	+	2.69 ± 0.03^{e}
50	-	3.57 ± 0.11^{c}
50	+	3.29 ± 0.02^{d}
100	-	4.52 ± 0.09^{a}
100	+	4.11 ± 0.15^{b}

*Cellular Zn status was estimated as described in the legend to Fig. 4.6. Cyclohexamide (3.4 uM) was added at the begining of experimental period. Data are expressed as mean±SEM of six samples from two separate cell preparations. Means with different letter as superscript are significantly different at $p < 0.05$.

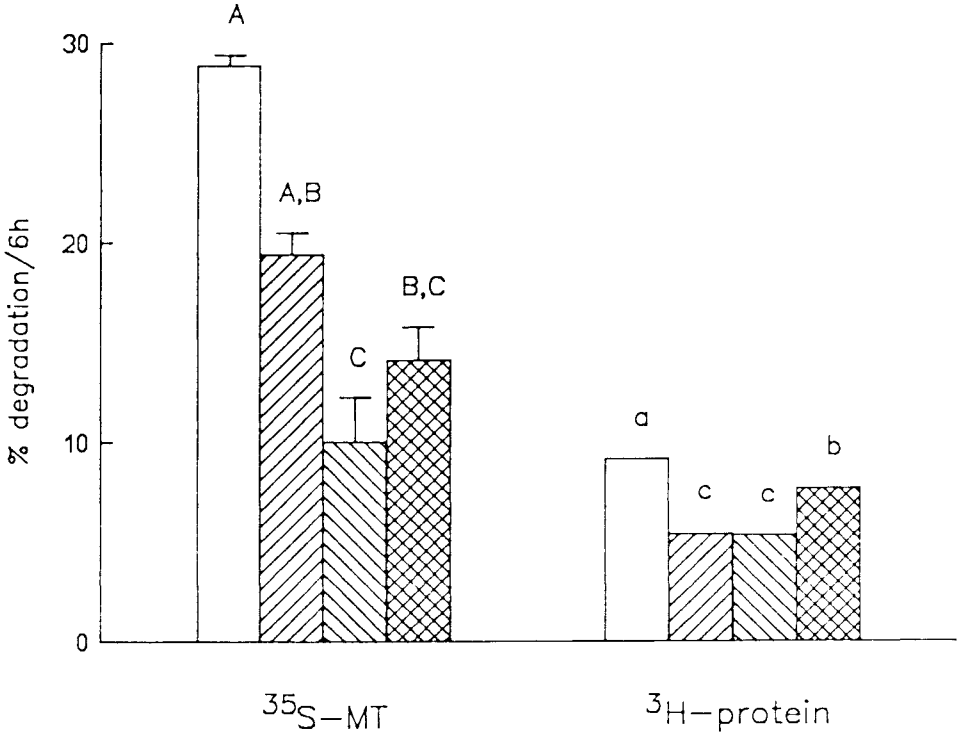

Fig. 4.8 Effects of cycloheximide, chloroquine and TLCK on the rate of ^{35}S-MT and ^{3}H-protein degradation. Hepatocytes were labelled with ^{35}S-cysteine and ^{3}H-leucine in the presence of 100 uM medium Zn for 20 hours. The ^{3}H-labelled cells were exposed to medium containing excess leucine for 4 hours to "chase" the intracellular pool of ^{3}H-leucine. To initiate experiments, fresh medium containing 1 uM Zn (□) or 1 uM Zn with either 3.4 uM cycloheximide (▨) or 1 mM chloroquine (▧) or 0.5 mM TLCK (▩) was added. Degradation of ^{35}S-MT and ^{3}H-proteins was estimated after 6 hours. Standard errors of means in groups of cells labelled with ^{3}H-leucine were less than 3% of the mean values.

^{35}S-MT in hepatocytes treated with chloroquine and TLCK might be due in part to either enhanced synthesis of this protein or increased retention of Zn in cells. ^{35}S-MT and ^{3}H-protein synthesis were actually suppressed by chloroquine and TLCK (73 and 57% of its respective control value). In addition, cellular Zn concentration was slightly, although significantly, decreased in cells treated with chloroquine and TLCK (Table 4.4). These results indicate that the increase in cellular concentration of MT reflected inhibition of degradation of polypeptide.

Effects of insulin on MT degradation

Insulin is known to modulate the lysosomal pathway. Since MT degradation was partially inhibited by chloroquine, I predicted that the rate of MT degradation would be enhanced by removal of insulin from the medium. Indeed, the rate of ^{3}H-protein degradation was increased by 30% when insulin was removed. Chloroquine effectively blocked this enhanced proteolysis by 57% (Fig. 4.9). Surprisingly, the rate of MT degradation was not altered by removal of insulin from Zn-deficient medium. Chloroquine inhibited MT degradation slightly, although not signifcantly, in the absence of insulin. Moreover, cellular Zn status was not affected by removal of insulin (Table 4.4).

Table 4.4 Effects of chloroquine, TLCK and insulin on cellular concentration of Zn in monolayers of hepatocytes incubated at 1 uM medium Zn*.

Treatment	Cellular Zn conc. (nmol/mg protein)
None	2.67±0.06[a]
Chloroquine (1 mM)	2.33±0.07[b]
TLCK (0.5 mM)	2.37±0.09[b]
w/o insulin	2.32±0.20[b]

*Cellular concentration of Zn was assessed as described in the legend to Fig. 4.6. To initiate experiments, fresh medium containing 1 uM Zn and either chloroquine or TLCK was added. In another set of cells, insulin was removed from incubation medium. Data are expressed as mean±SEM of 6 samples from 2 different cell preparations. Means with different letter as superscript are signifcantly different at $p < 0.05$.

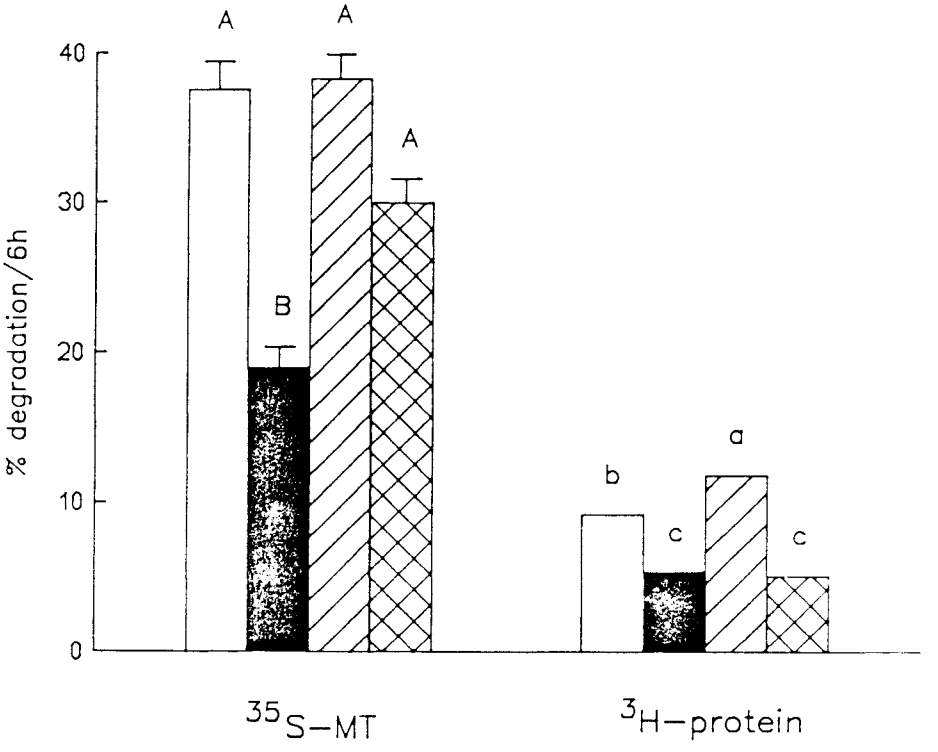

Fig. 4.9 Effects of insulin on the rate of ^{35}S-MT and ^{3}H-protein degradation in monolayers of hepatocytes. Hepatocytes were labelled with ^{35}S-cysteine or ^{3}H-leucine in the presence of 100 uM medium Zn for 20 hours. To initiate experiments, fresh medium containing 1 uM medium Zn and 10^{-8}M insulin in the absence (□) or presence of 1 mM chloroquine (■) was added. In a parallel cell preparation, monolayers were incubated in insulin-deficient medium with (▨) or without chloroquine (▩). The percent degradation of ^{35}S-MT and ^{3}H-proteins was estimated after 6 hours. Standard errors of means in groups of cells labelled with ^{3}H-leucine were less than 3% of the mean values.

DISCUSSION

The rate of induced MT turnover *in vivo* previously has been determined by monitoring disappearance of ^{35}S-MT from the cytosol fraction of rat livers. The half-lives of cytoplasmic MT were estimated as 20, 15 and 80 hours for Zn-, Cu- and Cd-induced ^{35}S-MT, respectively (59-64). However, disappearance of ^{35}S-MT from cytosol does not necessarily indicate degradation of this protein. Loss of cytoplasmic ^{35}S-MT may also reflect secretion of this protein into plasma and bile (73) and transfer to another subcellular compartment, e.g., lysosomes (65,160). To avoid these potential problems, I measured the level of ^{35}S-MT in heat stable extracts of whole cell homogenate and in spent medium in the present study. When Zn-loaded hepatocytes were incubated in Zn-deficient medium, the level of intracellular MT decreased by 40% in 6 hours (Fig. 4.2 & 4.4). This decrease in cellular MT level reflected intracellular degradation, since less than 1% of total cellular MT was secreted during the experimental period (Fig. 4.3).

The basic experimental design of this study was similar to that used in most *in vivo* studies. That is, MT synthesis was first induced by incubation of cells in medium containing a high concentration of Zn and then the rate of degradation of the accumulated protein was examined. The difference between the half-lives of induced and constitutive (basal) MT has been previouly

discussed (Chapter II, 160). When extracellular Zn concentration was 1 uM, ^{35}S-MT was rapidly degraded with a half-life of 7 hours; the half-life of general cellular protein was 29 hours (Fig. 4.5). The observed rate of ^{35}S-MT degradation in the present study was similar to previous studies using HeLa cells (163) and Cd-resistant Chinese hamster ovary cells (164). By measuring total ^{35}S-MT content in cellular lysate, the half-lives of ^{35}S-labelled Zn-MT in these cell lines were estimated at 10-12 hours (163,164). Kobayashi et al. (165) found that the level of ^{35}S-MT in the soluble fraction of Zn-loaded Chang liver cells was decreased faster (40%/3 hour) than that observed in present study.

The half-life of ^{35}S-MT in cultured cells is markedly shorter than that of cytoplasmic ^{35}S-MT *in vivo* (59-61). Similarly, the degradation of general hepatic protein estimated in present (29 hours) and related studies (20-32 hours) (100,166) is also more rapid that those reported in intact animals (3-4 days) (75,167). The basis for the enhanced rate of general protein degradation in cultured liver cells remains unknown (100). Interestingly, the rate of turnover of induced ^{35}S-MT is approximately 4 times faster than that of general hepatic protein both *in vitro* ($t_{1/2}$= 7 vs 29 hours) and *in vivo* ($t_{1/2}$=18-20 hours vs 3-4 days).

To accurately determine the half-life of a protein, reincorporation of radiolabelled amino acid must be minimized. The degree of reincorporation of a radiolabelled precursor is influenced

primarily by two factors, viz., the amount of mRNA being actively translated to produce the protein of interest and the specific activity of the radiolabelled amino acid pool. Yagle and Palmiter (162) estimated the half-life of MT mRNA was approximately 2.5 hours in a mouse liver cell line (Hep 1A) and Karin et al. (168) demonstrated that the amount of translatable MT mRNA in HeLa cells was dependent on the concentration of Zn in the medium. Others have shown that the level of MT mRNA declined rapidly after attaining maximum levels in the livers of animals injected with metal salts (169-171). Therefore, replacement of high Zn medium with medium containing only 1 uM Zn for the experimental period probably resulted in a rapid decline in cellular level of MT mRNA. Although the intracellular ^{3}H-leucine pool was rapidly diluted by incubating monolayers in medium containing excess leucine (Fig 3.1), low molecular weight ^{35}S-containing species were not effectively chased by incubation of hepatocytes in medium containing excess cysteine (Fig. 4.2). This stability of the nonprotein ^{35}S pool was probably due to the high level of glutathione and relatively low concentration of cysteine in hepatocytes (172). The possibility that ^{35}S-MT synthesis continued during the experimental period was considered. I found that the maximal amount of ^{35}S-MT synthesized during incubation of Zn-loaded hepatocytes in medium with 1 uM Zn for 6 hours was only 8% that of the concentration of ^{35}S-MT found in hepatocytes after overnight incubation in medium with 100 uM Zn. The

results suggest that the rate of ^{35}S-MT synthesis declined rapidly after removal of Zn and ^{35}S-cysteine from the medium and that the half-life of MT was not markedly overestimated.

Although the amounts of MT-I and -II induced in hepatocytes incubated in medium with 100 uM Zn overnight were similar, I observed that MT-II was degraded faster than MT-I (Fig. 4.4). Suzuki and Yamamura (173) also reported that the turnover of cytoplasmic MT-II was more rapid than that of MT-I in livers of Cu-injected rats. Others have reported that the turnover rates for both isoforms of MT were similar in liver cytosl of rats exposed to Zn (59,174). In contrast, Cain and Griffiths (175) observed that the half-life of MT-I is shorter than that of MT-II. Mehra and Bremner (64) also found that Zn-MT-I was degraded more rapidly than MT-II in lysosomal extracts. The basis for these discrepancies in the relative susceptibility of MT isoproteins to proteolysis is not apparent.

Having established that MT was indeed degraded in hepatocytes, I next evaluated the impact of cellular Zn concentration on protein degradation. Intracellular Zn was distributed among three distinct groups of soluble species in hepatocytes, viz., high molecular weight proteins (> 10,000 M.W.), MT and low molecular weight species (< 5000 M.W.) (Fig. 4.2). The low molecular weight Zn pool was eliminated when hepatocytes were incubated in Zn-deficient medium for 6 hours and may represent a labile Zn pool that is rapidly exchanged with extracellular Zn (119). The high molecular weight

Zn-metalloprotein pool was relatively unaffected by changes in extracellular Zn levels. Loss of Zn from the MT pool (38%/6 hour) was correlated with degradation of MT (35%/6 hour). This observation is consistent with results from previous in vivo studies showing that the level of Zn in cytosol fraction declines at the same rate as MT (59,63).

Cellular Zn concentrations varied in proportion to the extracellular level of this micronutrient (Table 4.3). MT degradation was inversely proportional to cellular Zn (Fig. 4.7); general protein degradation was not affected by cellular Zn status. The rates of ^{35}S-MT degradation were 15 and 44%/6 hour (apparent $t_{1/2}$ of 20 and 7 hours) in hepatocytes incubated in medium containing 50 and 1 uM medium Zn, respectively (Fig. 4.7). Cellular Zn significantly declined when hepatocytes were incubated in medium with 1 uM Zn, whereas intracellular Zn content was constant in monolayers incubated in medium with 50 uM Zn (Table 4.3). The impact of Zn status on MT degradation has been previously considered (134,163). Karin et al. (163) found that the half-life of Zn-MT in HeLa cells was 36-38 and 11-12 hours when cultures were incubated in medium containing 37 and 1.5 uM Zn, respectively. Oh et al. (136) reported that the half-life of ^{35}S-MT turnover in hepatic cytosol was 1.3 days when rats that had previously been fed 2,000 ppm Zn were fed diet with 18 ppm Zn. In contrast, the half-life of liver cytosol MT was 2.7 days in rats continually fed the high Zn diet.

Similarly, Held and Hoekstra (176) found that the turnover of hepatic Cd-MT was faster in rats fed a Zn-deficient diet than a Zn-adequate diet.

Several investigators (163,165) found that the rate of ^{35}S-MT degradation was biphasic. For example, Karin et al. (163) followed the rate of ^{35}S-MT degradation in HeLa cells incubated in Zn-deficient medium over a period of 16 hours. They found that the rate of ^{35}S-MT degradation during the initial period of incubation (0-8 hours) was 3 times faster than that during the latter half of the incubation period. It is probable that intracellular Zn concentration declined for several hours when cells were transferred from high Zn to low Zn medium and that the rate of MT degradation was retarded once cellular Zn content reached equilibrium with the extracellular Zn pool. The investigators suggested that the rate of MT degradation is attenuated as long as there was sufficient intracellular Zn to saturate the polypeptide. Depletion of cellular Zn accelerated the rate of thionein degradation.

Maintenance of native structure of MT is dependent on the presence of bound metal. Conversion of holoprotein to apothionein results in transformation of the polypeptide from a highly ordered stucture to a random-coil conformation (177). Since Zn stabilizes the tertiary structure of MT, it appears that this cofactor 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 Zn and Fe to carboxypeptidase A and ferritin, respectively, retards the rate of degradation of these polypeptides (178,179).

It has been generally assumed that lysosomes represent the primary site of MT degradation. Indeed, the degradation of various thionein derivatives in lysosomal extracts is rapid (66). Apothionein was digested by lysosomal extracts within 90 minutes, whereas 77 and 46% of Zn- and Cd-MT, respectively, were degraded after 3 hours. Squibb et al. (72) demonstrated that circulating Cd-MT was accumulated and degraded rapidly in the lysosomes of renal tubule cells. However, it is not known whether degradation of exogenous MT reflects the fate of intracellular MT. Others (64,67) have found that Cu-MT was not digested by lysosomal extracts. The minimal degradation of Cu-MT by lysosomal proteases could explain the observations that this protein accumulates in hepatic lysosomes of Bedlington terriers (180), individuals with Wilson's disease (71), human fetuses (69) and in renal lysosomes of diabetic rats (56). It has been suggested that the acidic environment in lysosomes facilitates the release of Zn and Cd, but not Cu, from thionein (65). Dissociation of the metals is associated with a conformational change in thionein which probably increases its susceptibility to proteolytic attack.

I investigated the subcellular site(s) of MT degradation in monolayer culture of hepatocytes. Lysosomotropic amines, e.g., chloroquine, methylamine and ammonia ion, specifically raise intralysosomal pH and decrease the activity of lysosomal acid proteinases (101). I found that chloroquine was the most effective amine in inhibiting the lysosomal pathway of protein degradation in monolayers of hepatocytes (Table 3.2). At 1 uM extracellular Zn, chloroquine inhibited the degradation of ^{35}S-MT and ^{3}H-protein by 65 and 41%, respectively. While cycloheximide is most frequently utilized as an inhibitor of protein synthesis, it also inhibits the lysosomal pathway (Chapter III). In the presence of cycloheximide, the rate of MT degradation was attenuated at all levels of medium Zn examined (Fig. 4.5). Cellular Zn concentration was not affected by treatments with these chemicals (Table 4.3 & 4.4). These data indicate that MT was degraded, in part, in lysosomes of hepatocytes.

The possibility that MT degradation also occurs in a non-lysosomal compartment was investigated. TLCK inhibits the activity of serine and, to a lesser extent, cysteine proteases (181), without affecting lysosomal degradation (Table 3.3; 182,183). The following are included in the family of serine proteases: subtilisin, elastase, trypsin and chymotrypsin. These neutral proteinases have been shown to partially degrade one or more forms of MT (see below). TLCK inhibited the degradation of ^{35}S-MT and ^{3}H-protein by 50 and 16%, respectively, without altering cellular Zn concentration (Table

4.4). These data provide the first in vivo evidence that MT is also degraded in the cytoplasmic compartment. Several previous observations support this finding. Feldman et al. (66) found that apothionein was completely degraded while Zn- and Cd-MT was partially degraded by neutral proteinases, i.e., trypsin and pronase, in vitro. Winge and Miklossy (9) reported that native Cd_5,Zn_2-MT was resistant to neutral proteinases (i.e., subtilisin, trypsin and chymotrypsin). Brief incubation of Cd_5,Zn_2-MT with EDTA removed two Zn ions from the protein. Incubation of the EDTA-treated Cd_5-MT with subtilisin or elastase for 16 hours resulted in partial digestion of MT into its A domain and peptide fragments. This metal-dependent resistance against neutral proteinases also has been observed with yeast Cu-thionein (184). Weser et al. (185) found that yeast Cu-thionein was slightly degraded (15%/12 hour) during incubation of this holoprotein in glutathione-depleted soluble fraction from rat liver.

The 3-fold greater inhibition of MT degradation by TLCK than that of ^{3}H-protein degradation is noteworthy. Trypsin-like proteases catalyze the hydrolysis of peptide bonds in which the residue contributing the carboxyl group to the peptidyl bond is either lysine or arginine. Both isoforms of rat liver MT contain 6-8 lysinyl residues (186). Moreover, the primary sequence of MT at the hinge of A and B domains is cys-lys-lys-ser (residues 29-31). Therefore, it is possible that these lysinyl residues in the hinge region may be

particularly susceptible to cleavage by cytoplasmic endopeptidase(s) with trypsin-like activity. It is conceivable that such cytoplasmic cleavage may indeed be the rate limiting step in intracellular degradation of MT.

Review of available literature indicates that a limited number of cytoplasmic proteolytic systems have been characterized. The ubiquitin-mediated pathway has been extensively studied in reticulocytes, but has not been demonstrated in liver cells (187). Calpains (calcium-dependent papain-like proteinases), a family of cysteine endopeptidases, have been identified in cytosol of liver cells (188,189). It has been suggested that calpains do not have general proteolytic activity, but rather cleave a limited number of substrate proteins, e.g., proteolytic conversion of inactive protein kinase C to its active form in neutrophils (187). Several other cysteine and/or metalloproteinases have been found in liver cytosol (190-192). Recently, a high molecular weight serine endopeptidase with multifunctional catalytic sites has been purified from rat liver cytosol (112). This proteinase has a trypsin- and chymotrypsin-like peptidase activity. Furthermore, this serine endopeptidase has been found in several tissues, suggesting the importance of this enzyme in non-lysosomal protein degradation. This proteinase may participate in the cytoplasmic degradation of thionein polypeptides.

It is well known that cellular proteins are degraded in a heterogenous manner. For example, regulatory enzymes in liver are

degraded more rapidly than "housekeeping" enzymes and structural proteins (75). Fluctuation in the concentration of these regulatory enzymes, largely due to their rapid turnover, enables the cell to rapidly respond to metabolic demands. The mechanisms regulating the degradation of individual protein are presently unknown. However, recent investigations support the proposal that the intrinsic properties of a protein and the regulation of the catabolic machinery itself contribute to the degradation process (85). The lysosomal protein degradation system consists of at least two components: enhanced (i.e. macroautophagy) and basal (i.e. microautophagy) lysosomal pathways. Macroautophagy is primarily controlled by the nutritional status of animals and cells. Anabolic reagents, i.e., insulin, amino acids and growth factors, suppress macroautophagy, whereas glucagon, a catabolic hormone, activates this process (86).

The precise nature of basal lysosomal activity is unknown but probably represents microautophagy. While it has been assumed that intracellular macromolecules are non-specifically sequestered into lysosomes, several examples of selective uptake of cytoplasmic material have been described recently (193,194). Adsorption of a protein onto the outer surface of lysosomal membrane may provide such selectivity. Interactions with the surface of lysosomes would be dependent upon the hydrophobicity and/or the structure of a protein (85). For example, after introduction into fibroblasts, microinjected ribonuclease A was degraded in lysosomes (195). It has

been suggested that a pentapeptide sequence (residues 7-11) of ribonuclease A is required for adsorption onto the trans surface of the lysosomal membrane (193). Ferritin is also known to be degraded by lysosomal proteases. Bridges and Hoffmann (196) found that ascorbate, which does not inhibit lysosomal activity, inhibits the uptake of ferritin into lysosomal vesicles. Decreased degradation of the protein was correlated with the presence of elevated levels of ferritin aggregates in cytosol of ascorbate-treated cells. It was suggested that this structural alteration, i.e., aggregation, retarded uptake of ferritin into lysosomes (197).

Cytoplasmic proteolytic systems also appear to be subjected to regulation. For example, the proteolytic activity of calpain requires the presence of Ca^{2+} and is inhibited by an endogenous inhibitor, calpastatin (189). Calpastatin blocks calpain activity by formation of a proteinase-inhibitor complex. Also, protease La, purified from E. coli, is activated and inactivated by allosteric binding of ATP and ADP, respectively (198).

Finally, the suggestion that the physiochemical structure of a protein affects its turnover rate has received wide support. McLendon and Radany (77) stated that a protein is more susceptible to proteolytic attack when unfolded. In addition, covalent modification of a protein, i.e., oxidation (78-80) and disufide bond formation (81), also have been shown to alter the rate of degradation.

The above observations support the proposal that regulation of

catabolic machinery provides the general or coarse control of overall intracellular proteolysis, whereas the intrinsic properties of a protein affect the specific or fine control of the event. How does this concept relate to my observations concerning MT degradation? Removal of Zn from medium facilitated efflux of cellular Zn and, apparently, the dissociation of Zn from MT. Loss of metal from MT under aerobic conditions has been shown to lead to inter- and intra-molecular disulfide bond formation (160,199,200). Partially saturated MT, apothionein and their aggregates are conformational derivatives of native MT. Alteration in MT strucutre would be expected to increase the susceptibility of the polypeptide to cytoplasmic proteases and, possibly, lysosomal uptake of thionein or its degradation products. Since Zn stabilizes the native structure of MT, I conclude that the availability of this cofactor provides fine control over the rate of MT degradation in hepatocytes.

Most of the experiments presented in this study were conducted in hepatocytes incubated in medium containing a complete amino acid mixture and a physiological concentration of insulin. In this basal medium, chlorquine inhibited cellular proteolysis by about 40%, suggesting that basal lysosomal turnover was responsible for almost half of hepatic protein degradation (Fig. 4.8). Insulin blocks the sequestration of intracellular materials into autophagosomes and consequently attenuates autophagy (95). Removal of insulin from medium increased the rate of ^{3}H-protein degradation by 30% (Fig.

4.9). In contrast, removal of insulin did not affect the rate of ^{35}S-MT degradation nor cellular Zn concentration. This result suggests that MT is selectively retained in the cytoplasmic compartment, even when macroautophagy is initiated. An alternative possibility is that ^{35}S-MT degradation was already maximal in hepatocytes incubated in Zn-deficient medium and that the presence or absence of insulin was insignificant. Similarly, Kobayashi et al. (165) found that the rate of ^{35}S-MT degradation did not increase in Chang liver cells when low Zn medium containing fetal calf serum was replaced with Hanks' balanced salt solution. Removal of serum from culture medium, like insulin, activates macroautophagy.

In summary, observations in the present study support the proposal that cellular Zn 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. A schematic presentation depicting the possible mechanisms of MT degradation is given in Fig 4.10. The intracellular concentration of Zn in monolayers of hepatocytes is controlled largely by the level of this metal in the extracellular environment. Cellular Zn is distributed among metalloproteins, MT and a labile low molecular weight pool. Reduction in the level of extracellular Zn mobilizes cellular Zn to the extracellular space. The level of Zn in the low molecular weight and MT pools is decreased. Consequently, some MT is "denatured" by formation of partially saturated MT, apothionein and,

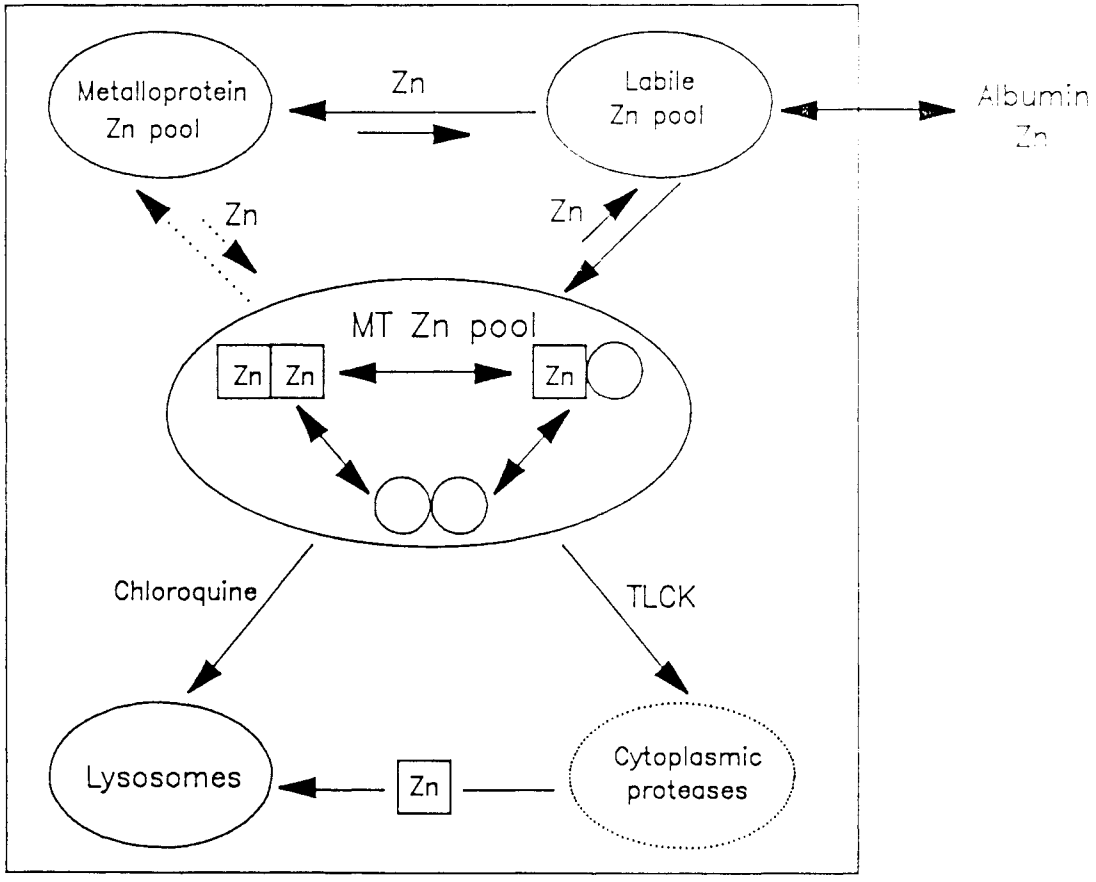

Fig. 4.10 Proposed mechanism for MT degradation in hepatocytes.

possibly, polymers. In addition, transfer of metal from MT to other metalloproteins (115,116,201,202) may normally yield such "denatured" MT. All such denatured MT would be more susceptible to attack by cytoplasmic proteases with trypsin-like activity. Intact MT also may be digested by cytoplasmic proteases, although to a much lesser extent. Limited proteolysis of native MT could produce a stable domain of MT (i.e., Zn_4-A domain). A second, but not exclusive, possibility is that native and/or denatured forms of MT are internalized by either autophagosomes or lysosomes and subsequently degraded therein. The acidic environment of these vesicles facilitates the release of bound metal from MT or its domains, leading to acid protease mediated digestion of this polypeptide.

The proposed scheme fails to address several specific matters. First, what is the relative susceptibility of intact MT, its domain and apothionein to cytoplasmic vs lysosomal degradation? Second, does the release of bound Zn from MT preceed the degradation of this protein or does limited proteolysis of MT stimulate the dissociation of Zn, thereby increasing the susceptibility of MT to further proteolytic attack? Third, is Cu-MT degraded in cytoplasm and, if so, is the process identical to that of Zn-MT? That is, do the strucutral differences in Zn-MT and Cu-MT affect their susceptibility to cytoplasmic proteolysis and/or the uptake by lysosomes? Answers to such questions are necessary to elucidate the specific mechanism(s) of MT degradation.

SUMMARY AND CONCLUSION

The synthesis and degradation of metallothionein (MT) was initally studied in streptozotocin (STZ)-induced diabetic rats. Relative rates of MT synthesis and cytoplasmic turnover of this protein were increased in the liver of untreated diabetic rats. Maximal relative rate of hepatic MT synthesis was observed in the early phase of insulin deficiency, suggesting that the increased rate of synthesis primarily was due to stress-related disturbance in endocrine status. The rate of MT synthesis also was increased in diabetic kidney and was correlated with excessive accumulation of dietary copper in this organ. The relative rate of MT turnover was similar in renal cytosol of control and diabetic rats. From critical evaluation of above data and literature, I concluded that the measurement of cytoplasmic MT turnover might not reflect actual degradation of this protein. Loss of MT from cytosol might also be due to secretion of this protein and/or transfer to another subcellular compartment, e.g., lysosomes. Therefore, I initiated a separate study to more closely investigate the characteristics of MT degradation in monolayer cultures of adult rat hepatocytes.

^{35}S-MT synthesis was induced in hepatocytes by overnight incubation of monolayers in medium containing ^{35}S-cysteine and 100

uM Zn. Total cellular ^{35}S-MT was measured in heat stable extract of cell homogenate and quantified by FPLC. When Zn was removed from medium, ^{35}S-MT ($t_{1/2}$= 7 hours) turnover was faster than general, non-MT ^{3}H-protein ($t_{1/2}$= 29 hours). This decrease in cellular ^{35}S-MT level reflected degradation, since less than 1% of total cellular ^{35}S-MT was secreted during the experimental period. The rate of MT degradation was inversely proportional to cellular Zn status. In contrast, the degradation of ^{3}H-protein was not affected by changes in cellular Zn.

To identify the subcellular site(s) of MT degradation, the effect of chloroquine, a lysosomotropic amine, and TLCK, an inhibitor of cytoplasmic trypsin-like proteases, on ^{35}S-MT content of hepatocytes was investigated. When medium Zn was 1 uM, chloroquine and TLCK inhibited MT degradation by 65 and 50%, respectively. Cycloheximide attenuated the degradation of ^{35}S-MT and ^{3}H-protein by inhibiting autophagy. Removal of insulin increased ^{3}H-protein degradation by 30%, but did not alter significantly ^{35}S-MT degradation. Hepatocyte Zn status was not affected by treatment with these chemicals and hormone.

Together, these data demonstrate that intracellular ^{35}S-MT is rapidly degraded when extracellular level of Zn is decreased. They also suggest that (a) Zn is the primary factor in regulation of MT degradation since this micronutrient is essential to maintain the tertiary structure of MT, (b) both lysosomal and cytoplasmic

proteinases participate in MT degradation and (c) cytoplasmic protease(s) with trypsin-like activity may be responsible for initial cleavage of thionein polypeptide.

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