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Degradation of Hepatic Zinc-Thionein after Parenteral Zinc Administration

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A low-molecular-weight protein, zinc-thionein, a metallothionein, was implicated as having a regulatory function in zinc metabolism. The half-life ($t_{\frac{1}{2}}$) of hepatic zinc-thionein was determined by pulse-labelling with either L-[^{35}S]cystine and/or ^{65}Zn. In two experiments with L-[^{35}S]cystine, the $t_{\frac{1}{2}}$ of zinc-thionein was 18h and 19h. Most of the soluble ^{35}S-labelled hepatic proteins had a $t_{\frac{1}{2}}$ of 4 days. The $t_{\frac{1}{2}}$ of zinc-thionein calculated by using ^{65}Zn was 20h. The close similarity between the calculated and measured $t_{\frac{1}{2}}$ values for zinc-thionein suggests that release of Zn^{2+} from zinc-thionein probably occurs simultaneously with degradation of the protein moiety.

Metallothionein, a soluble cadmium- and zinc-containing protein, was first isolated and characterized from renal cortex (Kagi & Vallee, 1960, 1961; Pulido *et al.*, 1966). Subsequently proteins essentially identical with metallothionein have been identified in tissues from a number of other animal species (Nordberg *et al.*, 1972; Winge & Rajagopalan, 1972; Weser *et al.*, 1973; Cousins *et al.*, 1973). Originally metallothionein was postulated to function as a detoxifying agent for heavy metals, i.e. cadmium and mercury, by sequestering these metals and rendering them biologically inactive (Nordberg, 1972; Weser *et al.*, 1973). However, others have suggested a more general role for this protein in mineral metabolism, especially with regard to zinc (Richards *et al.*, 1974; Chen *et al.*, 1974; Bremner & Davies, 1975; Richards & Cousins, 1975*a*,*b*).

Investigations into the role of metallothionein in zinc metabolism have attempted to correlate the synthesis of zinc-thionein to changes in the zinc status of the animal. Present evidence strongly indicates that zinc injections, as well as changes in dietary zinc, can induce the synthesis of zinc-thionein (Bremner & Davies, 1975; Richards & Cousins, 1975*a*,*b*, 1976). The exact mechanism of the induction process remains unclear; however, zinc-thionein synthesis is blocked by actinomycin D, suggesting the possible involvement of zinc at the transcriptional phase (Richards & Cousins, 1975*b*). In contrast, little attention has been focused on the catabolism of zinc-thionein. If this protein is involved in zinc metabolism, either as a major storage site for zinc or as a component of the homoeostatic control mechanism for total body zinc, or both, then catabolism as well as synthesis must be an important factor in the overall regulation process. Under certain conditions, zinc stored as zinc-thionein could become metabolically available; several groups have shown that zinc bound to metallothionein is indeed mobilized during a metabolic need for zinc (Bremner & Davies, 1975; Richards & Cousins, 1975*b*). This release of zinc from metallothionein may be under some type of control that is sensitive to changes in zinc status.

In the experiments reported here, the degradation of zinc-thionein in the liver of zinc-loaded animals was investigated by the method of pulse-labelling, by using either L-[^{35}S]cystine alone or both L-[^{35}S]-cystine and ^{65}Zn. Our findings indicate that zinc-thionein is degraded more rapidly than the bulk of soluble liver proteins and that the release of zinc from metallothionein occurs concomitantly with the degradation of this protein.

Experimental

Animals

Male Sprague–Dawley-strain rats (Sprague–Dawley Co., Madison, WI, U.S.A.) weighing 180–200g were used in all experiments. All animals were maintained on a commercial diet, which contained 50p.p.m. of zinc (Ralston Purina Co., St. Louis, MO, U.S.A.), and tap water *ad libitum*. The rats were not starved before being used for experiments.

Reagents

All chemicals used were of reagent grade. Buffers and reagents were prepared in resin-deionized water (Ultrapure-Barnstead Co., Framingham, MA, U.S.A.). L-[^{35}S]Cystine (41 or 268mCi/mmol) was diluted to 10μCi/ml with de-ionized water. $^{65}ZnCl_2$ (121mCi/mmol) was diluted to 10μCi/ml with 0.9% (w/v) NaCl. Both radiochemicals were obtained from Amersham/Searle Corp. (Downers Grove, IL, U.S.A.).

Isolation of zinc-thionein

Rats were killed by decapitation and the livers were immediately excised and rinsed in ice-cold 0.9% (w/v) NaCl. All subsequent steps were carried out at 4°C unless otherwise stated. The tissue was weighed, minced, and homogenized in 2vol. of 250mM-sucrose/10mM-Tris/HCl buffer (pH8.6) in a Potter–Elvehjem-type homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 750*g* ($r_{av.}$ 10.88cm) for 15min, and the supernatant was re-centrifuged at 42500*g* for 30min. The pellet was discarded and the postmitochondrial supernatant was centrifuged at 166500*g* ($r_{av.}$ 5.95cm) for 60min in a Beckman type Ti-50 rotor, to obtain the soluble (cytosol) fraction.

A portion (usually 5–6ml) of the soluble fraction obtained from each liver sample was applied to a column (2.6cm×50cm) of Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) equilibrated with 10mM-$(NH_4)_2CO_3$ buffer (pH8.6). The column was standardized with marker proteins (Sigma Chemical Co., St. Louis, MO, U.S.A.) of known mol.wt. [cytochrome *c* (12400), ribonuclease (13700), myoglobin (17200), chymotrypsinogen (25800) and bovine serum albumin (68000)] by the procedure of Andrews (1965). The metallothionein fraction was eluted as previously described (Richards & Cousins, 1975*a*). ^{35}S and/or ^{65}Zn radioactivity was measured with a Beckman liquid-scintillation spectrometer by dissolving a 2ml portion from each 5ml fraction in 15ml of a solubilizing cocktail (Scinti-Verse; Fisher Scientific Co., Pittsburgh, PA, U.S.A.). Fractions were also assayed for E_{280}. When needed, the stable zinc content was determined by atomic absorption spectrophotometry by using a Perkin–Elmer atomic absorption spectrophotometer (model 360) operated with an air/acetylene flame system.

Degradation of L-[^{35}S]cystine-labelled zinc-thionein

The rate constant of degradation and the half-life of zinc-thionein was determined by pulse-labelling the protein with L-[^{35}S]cystine. Rats were given an intraperitoneal injection of 25μmol of zinc (as $ZnSO_4$) in 0.9% (w/v) NaCl. Control animals were given 0.9% (w/v) NaCl alone. Then 2h after these initial injections, each animal received an intraperitoneal injection containing 5μCi of L-[^{35}S]-cystine. Animals were not starved before administration of the labelled amino acid, since starvation alone can result in the induction of zinc-thionein in normal animals (Bremner & Davies, 1975). Animals were killed by decapitation at predetermined times after the zinc dose, and labelled zinc-thionein was isolated as described above. The radioactivity present in the zinc-thionein fractions eluted from the column was summed and expressed as the total ^{35}S c.p.m. in zinc-thionein. The half-life of the protein was calculated by the method outlined by Segal & Kim (1963). To determine the amount of [^{35}S]cystine incorporation into mixed liver proteins, two 25μl portions of each soluble fraction were heated to 90°C with 10% (w/v) trichloroacetic acid. The precipitated material was collected on filter discs and the ^{35}S content measured by liquid-scintillation counting.

Degradation of ^{65}Zn and L-[^{35}S]cystine-labelled zinc-thionein

Rats were initially injected intraperitoneally with 25μmol of zinc [as $ZnSO_4$ in 0.9% (w/v) NaCl] followed immediately by a second intraperitoneal injection of 5μCi of ^{65}Zn. Control animals received only 0.9% (w/v) NaCl before administration of 5μCi of ^{65}Zn. Then 2h after the injections, all animals were given 5μCi of L-[^{35}S]cystine intraperitoneally. The animals were killed at designated time-intervals. Zinc-thionein was isolated as described above. Column fractions were measured simultaneously for ^{65}Zn and ^{35}S content by differential liquid-scintillation spectrometry as described by Cousins *et al.* (1975). Maximal crossover in quenched samples was 12%. The counting efficiency was 28% for ^{65}Zn and 91% for ^{35}S. The radioactivity present in the zinc-thionein fraction for each nuclide was summed and expressed as total c.p.m. in zinc-thionein.

Purification of zinc-thionein

The zinc-thionein fractions, isolated by gel-permeation (Sephadex G-75) chromatography, from animals killed at 24 and 48h after injection in the labelling experiments described above, were pooled and concentrated by ultrafiltration, under N_2 pressure, by using a UM-2 filtration membrane (Amicon Corp., Lexington, MA, U.S.A.). Concentrated samples were applied to columns of DEAE-Sephadex A-25 that were equilibrated with 50mM-Tris/acetate buffer (pH7.4). The columns (1.5cm×30cm) were eluted with 250ml linear gradients of 50–200mM-Tris/acetate buffer (pH7.4); 3ml fractions were collected. A 1ml portion of each eluted fraction was assayed for both ^{35}S and ^{65}Zn. Fractions were monitored for E_{280}. Fractions containing both ^{35}S and ^{65}Zn were pooled and freeze-dried. Electrophoresis in 7% polyacrylamide gels was carried out on the pooled samples by the method of Davis (1964). Gels were stained with Coomassie Blue in 12.5% (w/v) trichloroacetic acid as outlined by Chrambach *et al.* (1967), and sliced and measured for ^{35}S radioactivity as above.

Results

Isolation of zinc-thionein fraction

The synthesis and subsequent degradation of zinc-thionein was followed in animals that were injected

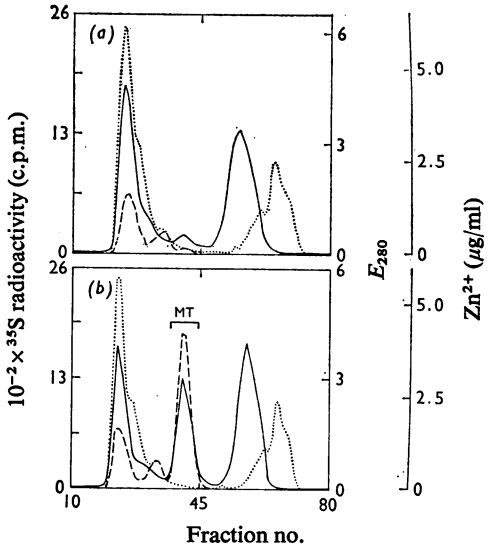

Fig. 1. *Separation on Sephadex G-75 of the cytosol from livers of control (a) and zinc-treated (b) rats*

Rats (180–200g) were given 25μmol of zinc (as $ZnSO_4$) or 0.9% NaCl (control), intraperitoneally, followed 2h later by 5μCi of L-[^{35}S]cystine, intraperitoneally. The animals were killed 24h after the zinc injection. The hepatic cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S c.p.m. (——), zinc (– – – –) and E_{280} (····). The position of zinc-thionein is indicated as MT.

with 25μmol of zinc. Liver proteins were pulse-labelled by using L-[^{35}S]cystine injected 2h after the zinc dose. The elution profiles of liver cytosol from a zinc-treated and a control animal, 24h after administration of either zinc or 0.9% (w/v) NaCl respectively are shown in Fig. 1. Comparison of the two profiles shows a close similarity in the distribution of both ^{35}S and stable zinc among the highest- and lowest-molecular-weight fractions. In the zinc-treated animals there was an additional zinc-containing peak present which had a mol.wt. of 6000–12000. This peak (designated as zinc-thionein) contained more than 60% of the total soluble zinc in the zinc-treated animal. There was also substantial incorporation of labelled cystine into the zinc-thionein region; however, only a slight incorporation of labelled cystine into zinc-thionein was observed in the control animal. In addition, the zinc-thionein region of the profile was found to have minimal E_{280} when compared with other protein fractions. This is a well-known characteristic of metallothionein species (Kagi & Vallee, 1960). It thus appears that the zinc-thionein region, although not homogeneous, does contain predominantly zinc-thionein, since this fraction contains substantial amounts of zinc and incorporated ^{35}S from cystine. The term zinc-thionein fraction, used in subsequent experiments discussed below, will refer to this zinc-thionein region.

Degradation of L-[^{35}S]*cystine-labelled zinc-thionein*

The method of pulse-labelling with L-[^{35}S]cystine was used to follow the degradation of zinc-thionein after a zinc load. Animals were killed 24, 36, 48, 60 and 72h after the zinc injection. The half-life of soluble liver proteins was 4 days in both zinc-treated and control animals. This value agrees quite well with similar estimates for the turnover of liver protein reported by other investigators (Arias *et al.*, 1969; Glass & Doyle, 1972).

To determine the half-life of zinc-thionein, the zinc-thionein fraction from the livers of the animals killed at 24, 36, 48, 60 and 72h after zinc injection was isolated by chromatography. The incorporation of L-[^{35}S]cystine intot his fraction was calculated and was used to determine the half-life from the equations established by Segal & Kim (1963). A ln plot of total c.p.m. in zinc-thionein versus time is shown in Fig. 2. From the slope of this line, the half-life of zinc-thionein was 17.9h.

Degradation of ^{65}*Zn- and* L-[^{35}S]*cystine-labelled zinc-thionein*

To ascertain whether the release of zinc from zinc-thionein occurs concomitantly with degradation of the protein, both L-[^{35}S]cystine and ^{65}Zn were incorporated into zinc-thionein. Elution profiles of

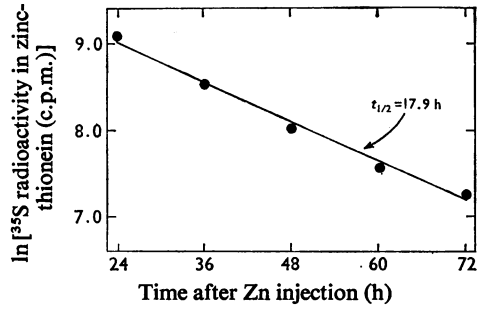

Fig. 2. *Half-life of hepatic zinc-thionein after a pulse-labelling with* L-[^{35}S]*cystine*

Rats were given 25μmol of zinc (as $ZnSO_4$), intraperitoneally, followed 2h later by 5μCi of L-[^{35}S]cystine intraperitoneally. The animals were killed 24, 36, 48, 60 and 72h after the zinc injection. The hepatic zinc-thionein fraction was isolated as described in the Experimental section. A plot of ln(total c.p.m. of ^{35}S in the zinc-thionein fraction) versus time is shown. The half-life is determined from the slope of this line. Each point represents the mean for three animals.

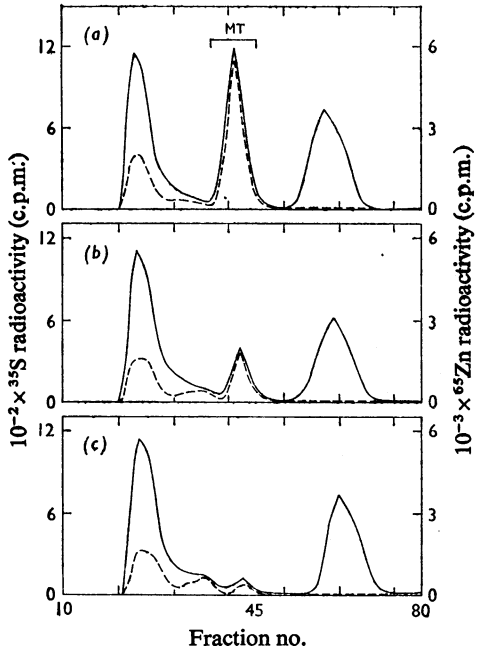

Fig. 3. *Separation on Sephadex G-75 of the cytosol of zinc-treated rats given both* L-[^{35}S]*cystine and* ^{65}Zn

Rats (180–200g) were given 25μmol of zinc (as $ZnSO_4$) and 5μCi of ^{65}Zn (as $^{65}ZnCl_2$), intraperitoneally, followed 2h later by 5μCi of L-[^{35}S]cystine, intraperitoneally. The animals were killed (*a*) 24, (*b*) 48 and (*c*) 72h after the zinc injection. The hepatic cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S c.p.m. (——) and ^{65}Zn c.p.m. (----). The position of zinc-thionein is indicated as MT.

liver cytosol at 24, 48 and 72h after the zinc injection are shown in Fig. 3. A progressive decrease in both ^{35}S and ^{65}Zn content of zinc-thionein was observed. Proteins of high molecular weight, i.e. those eluted at the void volume of the column, showed little change in either ^{65}Zn or ^{35}S content when compared with zinc-thionein.

It appeared from these data that both ^{35}S and ^{65}Zn radioactivity declined together in the zinc-thionein fraction, thus suggesting that the degradation of the protein and release of zinc occur simultaneously. The half-life of ^{65}Zn- and [^{35}S]cystine-labelled zinc-thionein was calculated by using the equation described above. The results of this experiment are shown in Fig. 4. The half-life of the protein, based on the isotopic decay of ^{35}S, was calculated to be 18.7h, which is in agreement with our above findings. The ^{65}Zn-pulse-labelled protein was found to have a half-life of 19.9h. This close similarity tends to support the hypothesis that zinc is released from the protein during the degradation process and not as a separate event.

Purification of zinc-thionein

Although it is clear that zinc-thionein represented the major protein component in the zinc-thionein region, a further purification of zinc-thionein was attempted to clarify the characteristics of this fraction. The zinc-thionein fractions from the previous double-labelling experiment were re-chromatographed on DEAE-Sephadex A-25. The zinc-thionein fraction was resolved into several peaks containing both ^{35}S and ^{65}Zn radioactivity; two small peaks, eluted at the initial gradient concentration, and two major peaks, designated MT-A and MT-B, eluted at higher gradient concentrations (Fig. 5). These two major peaks represent 85–90% of the total radioactivity placed on the column. The initial small peaks also had some E_{280}, whereas there was little or no E_{280} associated with either component A or B. MT-A was the minor of the two components. This relationship also held true when labelled zinc-thionein was obtained from animals killed at 48h.

Disc gel electrophoresis of the MT-A and MT-B components yielded only a single band of radioactivity. Electrophoresis of the initial peak revealed

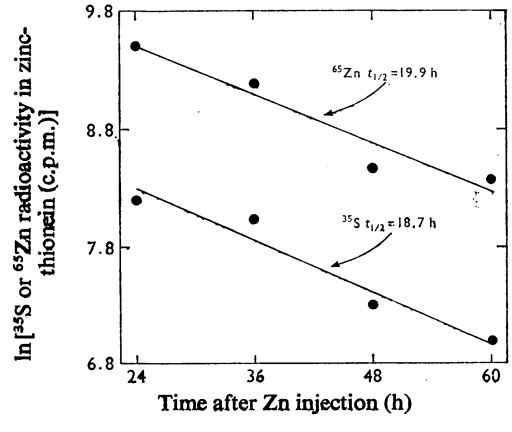

Fig. 4. *Half-life of hepatic zinc-thionein after pulse-labelling with* L-[^{35}S]*cystine and* ^{65}Zn

Rats were given 25μmol of zinc (as $ZnSO_4$), and 5μCi of ^{65}Zn (as $^{65}ZnCl_2$), intraperitoneally, and 2h later all animals received 5μCi of L-[^{35}S]cystine, intraperitoneally. The animals were killed 24, 36, 48 and 60h after the zinc injections. The zinc-thionein fraction was isolated as described in the Experimental section. A plot of ln(total c.p.m. of ^{35}S or ^{65}Zn in the zinc-thionein fraction) versus time is shown. The half-life was determined from the slope of each line. Each point represents the mean for three animals.

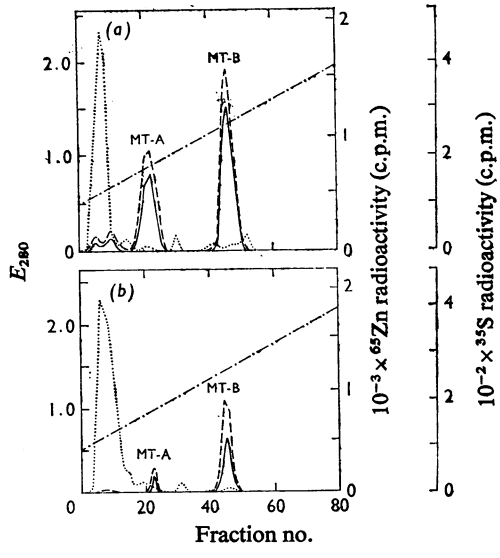

Fig. 5. *Purification of zinc-thionein by ion-exchange chromatography*

The zinc-thionein fractions obtained by chromatography on Sephadex G-75 were subjected to chromatography on DEAE-Sephadex A-25. The column was eluted with a linear gradient (—·—·) of 50–200mM-Tris/acetate (pH7.4). Fractions were assayed for both ^{35}S (——) and ^{65}Zn (----), and for E_{280} (····). (*a*) Zinc-thionein fraction obtained at 24h; (*b*) zinc-thionein fraction obtained at 48h. The position of the two zinc-thioneins are indicated by the symbols MT-A and MT-B.

that it contained the remaining bands found in the original zinc-thionein fraction.

Discussion

Several groups have shown that zinc bound to zinc-thionein can be mobilized. Richards & Cousins (1975*b*) and Bremner & Davies (1975) found that zinc injection resulted in an immediate synthesis of hepatic zinc-thionein. However, after 48h, the zinc content of the zinc-thionein fraction decreased substantially and returned to control values by 3 days. A similar finding was obtained when zinc-loaded rats were placed on a zinc-deficient diet (Bremner *et al.*, 1973; Chen *et al.*, 1975*a*). In zinc-injected and zinc-fed animals, maintained on either a normal diet or a zinc-deficient diet, there was a loss of zinc from zinc-thionein within 48h (Richards & Cousins, 1976). Our own experiments were designed to make these observations quantitative by monitoring the degradation process by the method of pulse-labelling.

Our results indicate that zinc-thionein is a rapidly degraded soluble protein having an approximate half-life of 18–20h. In contrast, the half-life for the bulk of soluble liver proteins is approx. 4 days. Although the half-life of zinc-thionein is short by comparison with that of most soluble proteins, many key regulatory enzymes have been shown to have much shorter half-lives (Schimke, 1970; Goldberg & Dice, 1974). It has been suggested that the short half-life of regulatory enzymes is an important mechanism of metabolic control within cells (Schimke, 1970). How the half-life for zinc-thionein might relate to a control function in zinc metabolism remains unclear.

The loss of zinc from zinc-thionein appeared to occur concomitantly with protein degradation (Fig. 4) and did not occur merely as a release of zinc from the protein, as was suggested for the release of bound iron from ferritin (Mazur *et al.*, 1958). However, a direct transfer of metallothionein-bound zinc to available ligands within the cell cannot be ruled out at present. Previous studies on hepatic catalase, in which the half-life of the enzyme was determined by isotopic decay of δ-aminolaevulinate and labelled amino acids, suggested that the haem group did not dissociate from the enzyme until such time as the entire haem–protein complex was degraded (Poole *et al.*, 1969).

Alternatively, the loss of radioactivity from zinc-thionein with time could have been the result of secretion of the protein from the liver into the blood and subsequent uptake by other tissues. A transport function for the protein has been suggested, at least for cadmium-thionein (Piscator, 1964; Nordberg, 1972). However, we were unable to detect zinc-thionein in the blood, nor was there a corresponding increase of zinc-thionein in the kidneys, the postulated site of uptake (Nordberg, 1972). This evidence, although convincing, does not completely rule out the possible secretion of hepatic zinc-thionein.

Chen *et al.* (1975*b*) determined the half-life of cadmium-thionein in rat liver by pulse-labelling the protein with L-[^{35}S]cystine. They reported a half-life for the cadmium protein of approx. 4 days, which is considerably greater than our calculated half-life for zinc-thionein of 18–20h. The reason for this large difference in half-life is not readily apparent, since it has been shown that cadmium and zinc induce metallothioneins that are electrophoretically indistinguishable (Winge *et al.*, 1975). One suggestion might be that cadmium-thionein may be more slowly degraded, which would allow for additional synthesis of the protein to sequester the released cadmium again. This could account for the low body turnover rate of this metal (Cotzias *et al.*, 1961). This possibility is suggested by the data of Chen *et al.* (1975*b*), which showed essentially no loss of cadmium from labelled metallothionein as a function of time.

Zinc-thionein was found to occur in two different forms separable by ion-exchange chromatography. Of the two forms, that designated MT-B was the

predominant species. These results are in agreement with metallothionein isolated by others (Bremner & Davies, 1975; Winge *et al.*, 1975).

Experiments that utilize pulse-labelling to determine the half-life of a protein are dependent on two key assumptions (Schimke, 1970). First, the radioactivity in the protein sample must be representative of all the protein present in the sample rather than a minor component. Ion-exchange chromatography of the zinc-thionein fraction indicated that 85–90% of the total radioactivity eluted from the Sephadex G-75 columns could be accounted for as electrophoretically pure zinc-thionein. Secondly, the method requires that there be no re-utilization of the radioisotope. This is the major limitation of this method when it is used to determine absolute rates of protein degradation. L-[^{35}S]Cystine (as cysteine) is a re-utilizable amino acid, although the degree of the re-utilization is thought to be less than for most other amino acids (Awwad *et al.*, 1970). Therefore the amount of [^{35}S]cystine incorporated into newly synthesized metallothionein during the time-interval studied should be quite small. In addition, the synthesis of zinc-thionein occurring during this time-interval was found to be quite small after initial zinc administration (K. S. Squibb, S. L. Feldman & R. J. Cousins, unpublished work).

In summary, it appears that zinc-thionein, which may act as a storage protein for zinc in the liver, has a half-life of approx. 18–20h. The release of bound zinc from the protein occurred concomitantly with degradation of the protein and probably not as a result of some other release mechanism. Whether the rate of degradation of this protein can be influenced by factors that can also influence zinc metabolism, i.e. diet, disease, hormones, should be investigated.

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