Biochem. J. (1978) **174**, 883–892
Printed in Great Britain

Effect of Zinc Status of Rats on the Synthesis and Degradation of Copper-Induced Metallothioneins

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(*Received 9 January* 1978)

Injection of Zn^{2+}-adequate and Zn^{2+}-deficient rats with Cu^{2+} stimulated the incorporation of L-[^{35}S]cysteine into a low-molecular-weight Cu^{2+}-binding protein in both liver and kidney. No significant incorporation of L-[4,5-^{3}H]leucine into this protein occurred, confirming the previous claim that it was metallothionein and not some other leucine-rich protein. The half-life of the protein was found to be 16.9±1.0 (S.E.)h in the liver of Zn^{2+}-adequate rats but only 12.3±0.5h in Zn^{2+}-deficient animals. The degradation rate of the metallothionein was similar to the rate of disappearance of Cu^{2+} and Zn^{2+} from the protein, indicating that the release of metal from the protein and its catabolism occurred simultaneously. There was no significant difference in the half-lives of the hepatic or renal copper-thioneins in Zn^{2+}-adequate rats.

It has been established that the low-molecular-weight protein metallothionein plays an important regulatory role in the metabolism of Zn^{2+}, particularly in the control of intestinal absorption and hepatic uptake of the metal (Bremner & Davies, 1975; Richards & Cousins, 1975, 1976). Similar roles have been proposed for this protein in the metabolism of Cu^{2+} (Evans, 1973; Bremner & Davies, 1976), and a large proportion of the hepatic Cu^{2+} in pigs (Bremner, 1976; Bremner & Young, 1976*a*) and Cu^{2+}-injected rats (Bremner & Davies, 1976; Bremner & Young, 1976*b*) is generally present in the form of copper–zinc-thioneins. However, only trace amounts of copper-thioneins are present in the livers of Zn^{2+}-deficient animals on long-term experiments, regardless of their Cu^{2+} status (Bremner, 1976; Bremner & Davies, 1976).

The absence of copper-thioneins in these animals was not a consequence of some inability to synthesize the protein, as injection of Cu^{2+} into Zn^{2+}-deficient rats resulted in the rapid incorporation of hepatic copper into metallothionein. It was noted, however, that the elevated concentrations of copper-thionein were maintained for a shorter time in Zn^{2+}-deficient than in Zn^{2+}-adequate rats receiving similar amounts of Cu^{2+}. Moreover the copper-thioneins induced in the Zn^{2+}-deficient rats contained relatively little Zn^{2+} and it was suggested that the presence of Zn^{2+} might stabilize the protein and delay its degradation in the Zn^{2+}-adequate animals (Bremner & Davies, 1976). Provided that the release of the metal from the protein was concomitant with its degradation, as has been reported for Zn^{2+}-induced metallothionein (Feldman & Cousins, 1976), this would explain the lack of any long-term accumulation of copper-thionein in the livers of Zn^{2+}-deficient animals. The determination of the turnover rates of ^{35}S-labelled copper-thioneins in Zn^{2+}-supplemented and Zn^{2+}-deficient rats in the present experiments has confirmed this suggestion.

Although Bremner & Young (1976*b*) were able to isolate and characterize copper-thioneins from the livers of Cu^{2+}-injected rats, others have suggested that Cu^{2+} induces synthesis of a hepatic protein of similar size but different amino acid composition (Evans *et al.*, 1975; Riordan & Gower, 1975; Winge *et al.*, 1975; Irons & Smith, 1977). The fractionation procedures used by these workers were generally inadequate, however, for purification of metallothionein and no attempt was made to prevent oxidative changes occurring during the isolation of the protein (Bremner & Young, 1976*b*; Hartmann & Weser, 1977). These non-thionein proteins could therefore be artifacts which formed during the isolation procedures. In an attempt to distinguish between the thionein and non-thionein proteins without having to purify them rigorously, advantage was taken of the different leucine contents reported for the proteins. Copper-thionein contains less than 1% leucine (Bremner & Young, 1976*a*,*b*), whereas the other proteins contain about 7% leucine residues (Winge *et al.*, 1975; Evans *et al.*, 1975). A comparison was therefore made of the relative incorporation of ^{35}S from [^{35}S]cysteine and of ^{3}H from [^{3}H]leucine into the Cu^{2+}-induced proteins in liver and kidney.

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No significant incorporation of ^3H was found, thus supporting the view that only metallothionein is synthesized in response to Cu^{2+} injection.

A preliminary report on these findings has been published elsewhere (Hoekstra *et al.*, 1978).

Experimental

Materials and methods

All reagents were analytical grade and were dissolved in double-distilled water. Glassware was acid-washed and rinsed with deionized and double-distilled water. L-[^{35}S]Cysteine hydrochloride (62 mCi/mmol) and L-[4,5-^{3}H]leucine (57000mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactivity of samples was measured by using a Packard Tri-Carb model 574 liquid-scintillation spectrometer after dissolution of aqueous solutions (2ml) in 15ml of NE 260 scintillator solution (Nuclear Enterprises Ltd., Edinburgh, Scotland, U.K.). Protein concentrations were measured by the fluorimetric method of Bohlen *et al.* (1973) with bovine serum albumin [fraction V; Sigma (London) Chemical Co., London S.W.6, U.K.] as standard. Fluorescamine (Fluram) was obtained from Hoffmann–La Roche A.G. Diagnostica, Basel, Switzerland. Cu^{2+} and Zn^{2+} concentrations were measured by atomic absorption with a Varian AA5 atomic absorption spectrophotometer (Varian Associates, Walton-on-Thames, Surrey, U.K.).

Animals

Male Hooded Lister rats (Rowett Institute strain) were used. Zn^{2+}-deficient rats were produced by feeding animals aged 60 days and weighing about 120g on the diet of Williams & Mills (1970), which contained 1mg of Zn^{2+}/kg, for 18 days. They then weighed about 150g. Zn^{2+}-supplemented rats received the same diet but with the addition of 40mg of Zn^{2+} (as $ZnSO_4,7H_2O$)/kg from the time of weaning (21 days) until they also weighed 150g. As they were then 9 weeks old, they were 2 weeks younger then the Zn^{2+}-deficient rats at the time of the experiment.

The Zn^{2+}-supplemented and Zn^{2+}-deficient rats were dosed intraperitoneally with 300μg of Cu^{2+} (as $CuSO_4$ in a solution containing 9g of NaCl/litre), followed after 4h by a saline solution containing 16μCi of [^{35}S]cysteine and 40μCi of [4,5-^{3}H]-leucine. Six rats from each group were killed by cervical dislocation and exsanguination at intervals of 6, 10, 17, 24, 36, 48, 72 and 96h after the injection of Cu^{2+}. Four control Zn^{2+}-supplemented and four control Zn^{2+}-deficient rats, which received saline alone instead of the Cu^{2+} injection, were killed at the 24h time interval.

Fractionation of liver and kidneys

The liver and kidneys of the rats were removed immediately and stored at −20°C until required. They were homogenized in 2.5vol. (v/w) of 10mM-sodium phosphate buffer (pH7.4) in an X-1020

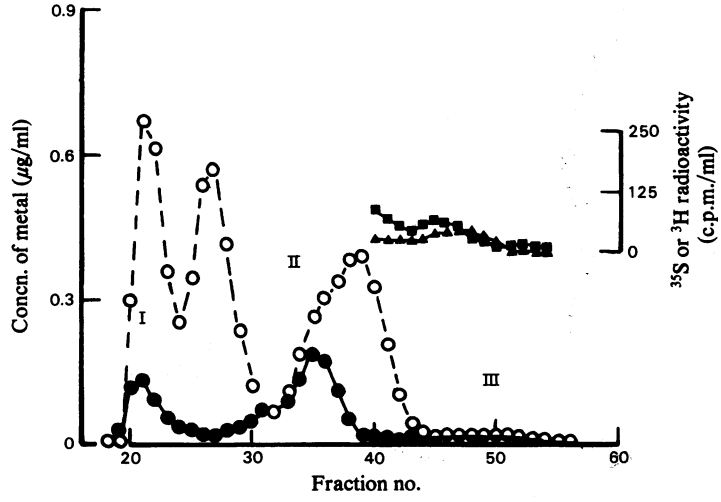

Fig. 1. *Separation on Sephadex G-75 of the hepatic cytosol of control zinc-supplemented rats given* L-[^{35}*S*]*cysteine and* L-[4,5-^{3}*H*]*leucine*

Rats (150g) were given 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine intraperitoneally and were killed 20h afterwards. The hepatic cytosol was fractionated as described in the Experimental section. Each chromatographic fractions was assayed for copper (●), zinc (○), ^{35}S (▲) and ^{3}H (■). The positions of copper-containing fractions I, II and III (metallothionein) are shown.

homogenizer (Scottish Instrument Centre, Edinburgh, Scotland, U.K.). The homogenates were centrifuged immediately at 100000*g* ($r_{av.}$ 7.38cm) for 1h at 1°C in an MSE Superspeed 65 centrifuge. Aliquots (7ml) of the supernatants were then fractionated on columns (900mm × 26mm) of Sephadex G-75 equilibrated with 10mM-sodium phosphate (pH7.4). The columns were eluted with the same buffer at a flow rate of about 30ml/h, 7ml fractions being collected. The Cu^{2+}, Zn^{2+} and protein contents and the ^{35}S and ^{3}H activities of each fraction were determined.

In estimating the degradation rate of proteins, the ^{35}S content of the fraction III eluted from Sephadex G-75 was corrected for the minor contribution from non-thionein proteins. The amount of ^{35}S associated with metallothionein was estimated from the difference between the total area under the third peak as plotted in Figs. 2 and 3 (measured gravimetrically) and the area under the base-line

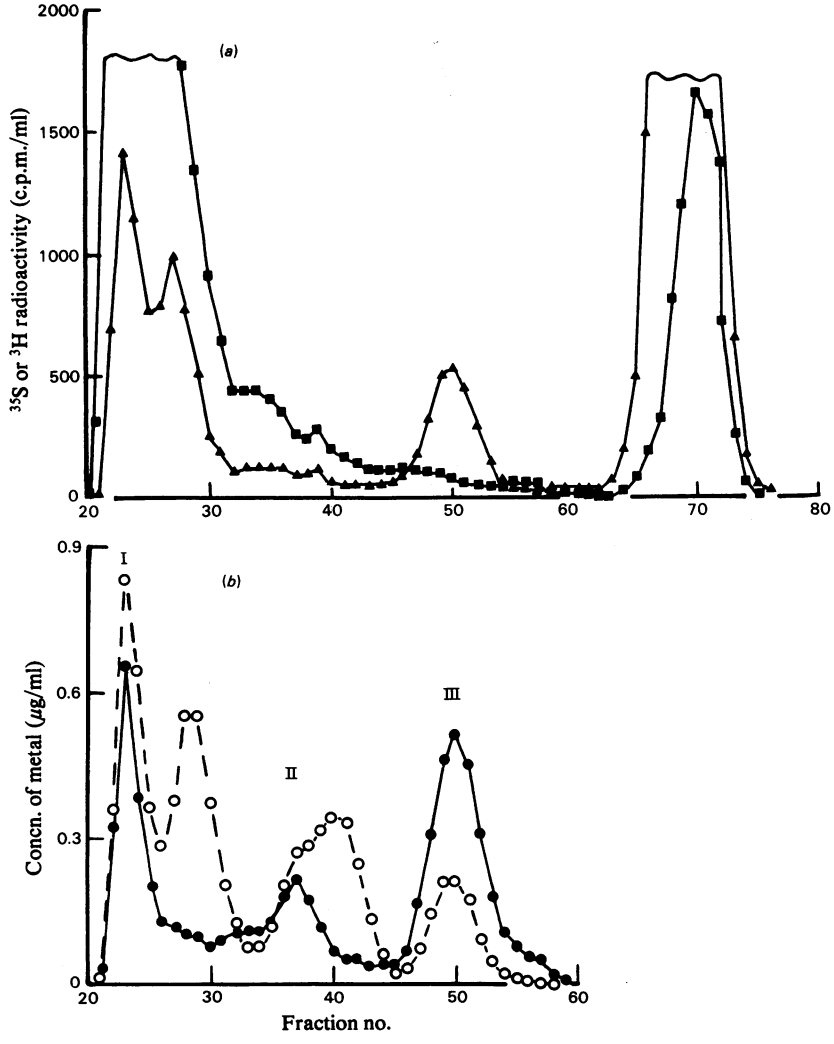

Fig. 2. *Separation on Sephadex G-75 of the hepatic cytosol of zinc-supplemented rats given copper,* L-[^{35}S]*cysteine and* L-[4,5-^{3}H]*leucine*

Rats (150g) were given 300μg of copper (as $CuSO_4$) intraperitoneally followed 4h later by 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine. The animals were killed 6h after injection of the copper and hepatic cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S (▲) and ^{3}H (■) (*a*) and for copper (●) and zinc (○) (*b*). The positions of copper-containing fractions I, II and III (metallothionein) are shown.

portion of the same peak. This base-line portion corresponded closely to the activity present in fraction III from animals which had received no Cu^{2+} injection (Figs. 1 and 3*a*).

Results

Fractionation of liver cytosol on Sephadex G-75

The elution profiles obtained after gel filtration on Sephadex G-75 of the supernatants from the control rats and from those injected 6h previously with 300μg of Cu^{2+} are shown in Figs. 1 and 2 respectively. As reported previously (Bremner & Davies, 1976) most of the Cu^{2+} present in the cytosol from the control rats was eluted with Zn^{2+} in two main fractions, I and II, which had approximate mol.wts. of >75000 and 35000. In the Cu^{2+}-injected rats an appreciable amount of both copper and Zn^{2+} was also present in an additional fraction (III), eluted around tube 50, with an apparent mol.wt. of about 12000. The copper-proteins present in this fraction have previously been characterized as metallothioneins (Bremner & Young, 1976*b*) and will be so called throughout this paper.

An appreciable amount of the ^{35}S from the injected cysteine was associated with the metallothionein fraction in the Cu^{2+}-injected rats, with the elution profiles for the ^{35}S and metal coinciding almost exactly (Fig. 2). In contrast, there was no significant incorporation of ^{3}H from the injected leucine into metallothionein, although a minor ^{3}H peak was eluted before fraction III at about tube 45. This same ^{3}H peak was present in similar amounts in the control rats, but the radioactivity of the ^{35}S present in the metallothionein fraction was extremely low in these animals (Fig. 1). Most of the ^{35}S and ^{3}H in both the Cu^{2+}-injected and control rats was eluted in two main fractions, one eluted near the void volume and the other near the total volume of the column

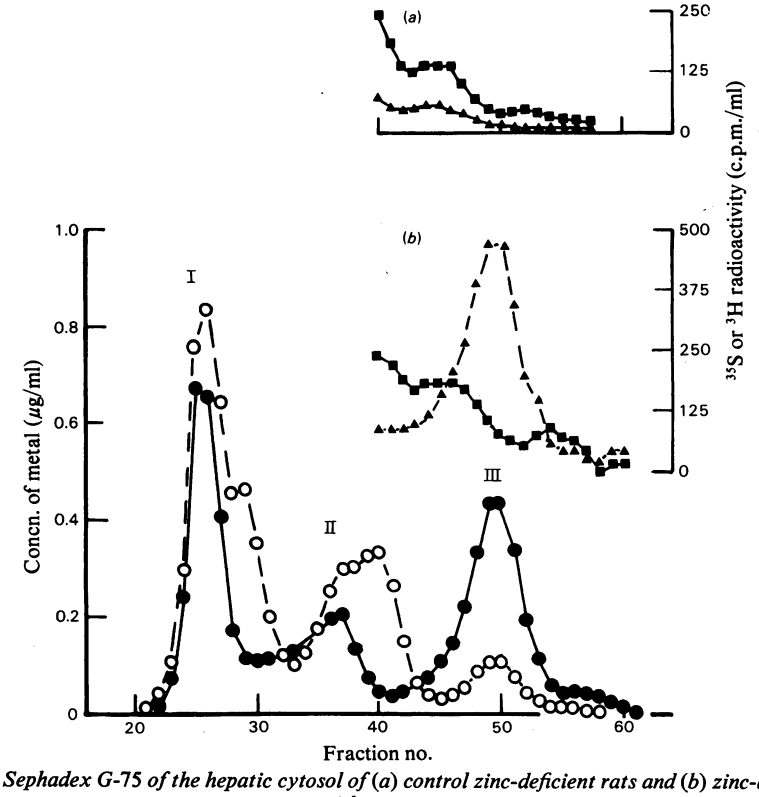

Fig. 3. *Separation on Sephadex G-75 of the hepatic cytosol of (a) control zinc-deficient rats and (b) zinc-deficient rats injected with copper*

Zinc-deficient rats (150g) were given (*a*) a saline solution or (*b*) 300μg of copper (as $CuSO_4$) intraperitoneally. Both sets of rats were then injected after 4h with 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine and were killed 2h later. The hepatic cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S (▲), ^{3}H (■), copper (●) and zinc (○). The positions of copper-containing fractions I, II and III (metallothionein) are shown.

(Fig. 2). The amount of isotope in these fractions was not significantly affected by the Cu^{2+} treatment.

Fractionation of the liver cytosol from the Zn^{2+}-deficient rats gave similar results (Fig. 3). The distribution of Cu^{2+} and Zn^{2+} in the control rats (not shown) was similar to that illustrated in Fig. 1, except that even less of the metal was present as metallothionein. Only trace amounts of ^{35}S and ^{3}H were present in this fraction, although there was again some incorporation of ^{3}H into a minor protein eluted at around tube 45 (Fig. 3*a*), which was present in similar amounts in the liver of the Cu^{2+}-injected rats (Fig. 3*b*). Cu^{2+} injection resulted in significant incorporation of ^{35}S, copper and to a lesser extent of Zn^{2+} into the metallothionein fraction.

Incorporation of copper and zinc into hepatic metallothionein

Maximum Cu^{2+} concentrations of 34.1 ± 0.9 (S.E.) and 36.1 ± 2.7 μg/g wet wt. were attained in the livers of the Zn^{2+}-adequate and Zn^{2+}-deficient rats respectively 10h after Cu^{2+} injection. Zn^{2+} concentrations were also increased after injection of Cu^{2+} with greatest concentrations of 40.9 ± 1.3 and 32.9 ± 1.3 μg/g occurring in the Zn^{2+}-supplemented and Zn^{2+}-deficient rats after 17h. Liver Cu^{2+} and Zn^{2+} concentrations in the non-injected rats were 6.5 and 28.4 μg/g respectively for Zn^{2+}-supplemented and 6.2

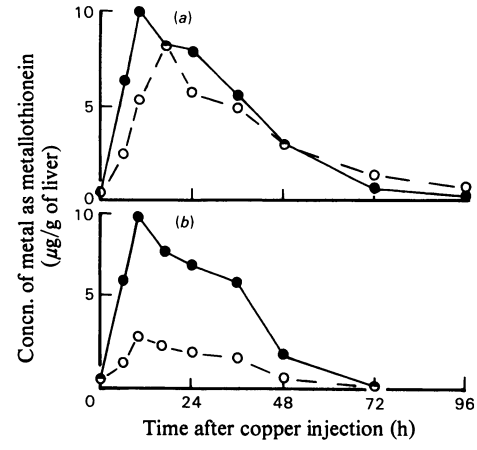

Fig. 4. *Changes in the concentrations of hepatic copper and zinc present as metallothionein after injection of rats with copper*

(*a*) Zinc-supplemented rats (150g) and (*b*) zinc-deficient rats (150g) were injected intraperitoneally with 300μg of copper (as $CuSO_4$). The rats were killed at intervals from 6 to 96h thereafter and the metallothionein-bound metal in the hepatic cytosol was separated as described in the Experimental section. The concentrations of copper (●) and of zinc (○) bound to metallothionein (μg/g of liver) were calculated.

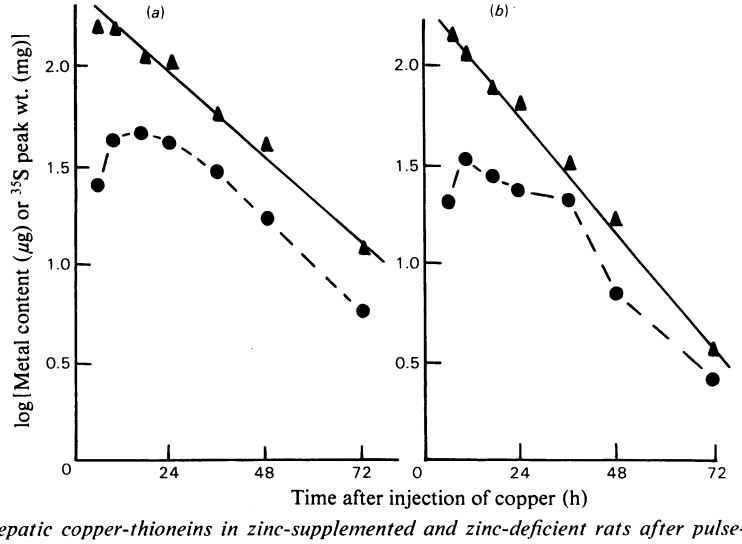

Fig. 5. *Half-life of hepatic copper-thioneins in zinc-supplemented and zinc-deficient rats after pulse-labelling with* L-[^{35}S]-*cysteine*

(*a*) Zinc-supplemented rats (150g) and (*b*) zinc-deficient rats (150g) were injected intraperitoneally with 300μg of copper (as $CuSO_4$), followed after 4h by 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine. They were killed at intervals from 6 to 96h after injection of the copper. The copper-thionein in the hepatic cytosol (7ml) was separated on Sephadex G-75 and then its ^{35}S content estimated as described in the Experimental section. The half-life of the ^{35}S (▲) and of the total metal (copper plus zinc) (●) present as metallothionein were calculated from the plot of the log values against time for (*a*) the zinc-supplemented and (*b*) the zinc-deficient rats.

and 25.0μg/g for Zn^{2+}-deficient animals. The concentrations of copper and Zn^{2+} bound to metallothionein at different times after injection of Cu^{2+} are shown in Fig. 4. Maximum concentrations of thionein-bound copper were attained at 10h and declined gradually thereafter. Zn^{2+} concentrations increased and also decreased more slowly than copper concentrations in the Zn^{2+}-supplemented rats. Because of the smaller increment in thionein-bound Zn^{2+} concentrations in the Zn^{2+}-deficient animals, the copper/Zn^{2+} ratio in metallothionein in these rats was appreciably greater than in the Zn^{2+}-supplemented animals. The mean ratios between 10 and 48h post-injection were 3.1 and 1.3 in the deficient and supplemented rats respectively.

Incorporation of ^{35}S and ^{3}H into metallothionein and other hepatic fractions

As was shown in Figs. 1–3, injection of Cu^{2+} had a marked stimulatory effect on the incorporation of ^{35}S, but not of ^{3}H, into the metallothionein-containing fractions. At 10h after injection of the Cu^{2+}, the specific radioactivity of the ^{35}S present in both fractions I and II from the Sephadex G-75 column was about 200c.p.m./mg of protein, whereas that in the crude metallothionein fraction was 850c.p.m./mg of protein. There was no difference between the Zn^{2+}-supplemented and Zn^{2+}-deficient rats in the specific radioactivities of the three fractions. In contrast the mean specific radioactivity of ^{3}H in the crude metallothionein fraction at this time was about 340c.p.m./mg of protein, which was less by about 35% than that in fractions I and II. This supports the view that Cu^{2+} injection did not promote incorporation of leucine into metallothionein.

There was a steady decrease, at an exponential rate, in the amount of radioactivity present in all protein fractions during the experiment. The half-life of ^{35}S in fraction I, which contained most of the cytosolic protein, was calculated to be 98 and 72h in the Zn^{2+}-supplemented and Zn^{2+}-deficient rats respectively. These values were not significantly different from each other. The decrease in the radioactivity of the [^{35}S]thionein is shown in Fig. 5. From the linear decrease with time in the log value of the radioactivity, the half-life of the protein (±S.E.) in the Zn^{2+}-supplemented and Zn^{2+}-deficient rats was calculated to be 16.9±1.0 and 12.3±0.5h respectively. These values were significantly different from each other.

The amount of metal associated with the protein decreased at similar rates to those for the ^{35}S (Fig. 5).

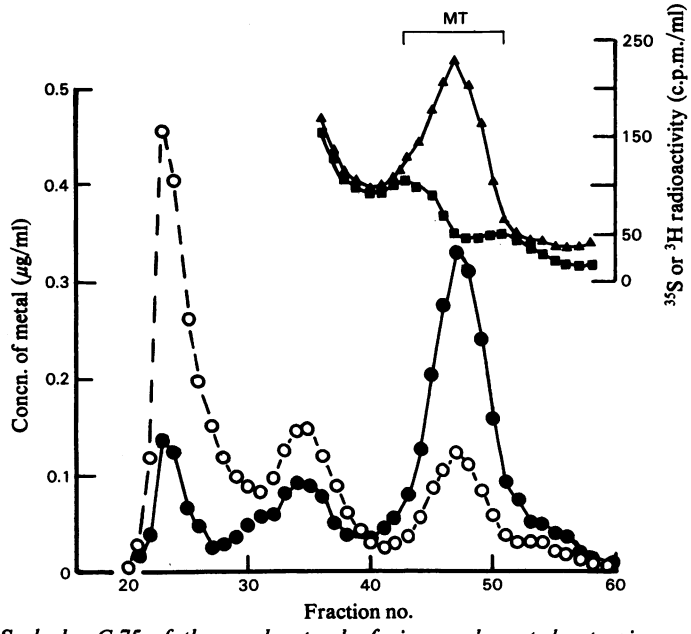

Fig. 6. *Separation on Sephadex G-75 of the renal cytosol of zinc-supplemented rats given copper,* L-[^{35}S]*cysteine and* L-[4,5-^{3}H]*leucine*

Zinc-supplemented rats (150g) were given 300μg of copper (as $CuSO_4$) intraperitoneally followed 4h later by 16μCi of [^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine. The rats were killed after a further 6h and the renal cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S (▲), ^{3}H (■), copper (●) and zinc (○). The position of the metallothionein fraction is shown (MT).

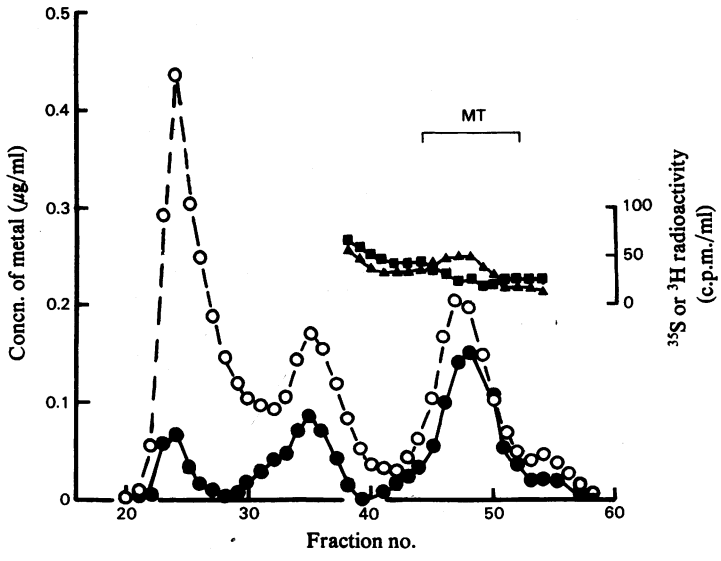

Fig. 7. *Separation on Sephadex G-75 of the renal cytosol of zinc-supplemented control rats given* L-[^{35}S]*cysteine and* L-[4,5-^{3}H]*leucine*

Zinc-supplemented rats (150g) were given 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine intraperitoneally and were killed 6h thereafter. The renal cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S (▲), ^{3}H (■), copper (●) and zinc (○). The position of the metallothionein fraction is shown (MT).

The half-life (±S.E.) for the disappearance of copper plus Zn^{2+} from metallothionein over the time period of 24–72h was 16.4±1.0 and 12.3±1.8h in the Zn^{2+}-supplemented and Zn^{2+}-deficient rats respectively.

Fractionation of kidneys

Injection of Cu^{2+} into the rats resulted in a significant increase in kidney Cu^{2+} concentrations. In the Zn^{2+}-supplemented rats, maximum concentrations of 12.9μg of Cu^{2+}/g wet wt. were attained at 10h post-injection. Concentrations in the control rats were 6.4μg/g.

On separation of the kidney cytosol on Sephadex G-75, three main Cu^{2+}-containing fractions were obtained (Figs. 6 and 7), similar to those reported in liver. After injection of Cu^{2+}, there was an increase in the amount of copper associated with the third of these fractions, which was tentatively identified as metallothionein on the basis of its similar behaviour to the hepatic protein. The concentration of copper present in this form in the Zn^{2+}-supplemented rats was 3.6μg/g of kidney at 10h post-injection. However, exact measurement of the increment in copper-thionein content was difficult because of the relatively high and variable basal concentrations of this renal protein in the control rats (see Fig. 7).

The increase in copper-thionein concentrations after injection of Cu^{2+} was associated with substantial incorporation of ^{35}S from cysteine into the protein

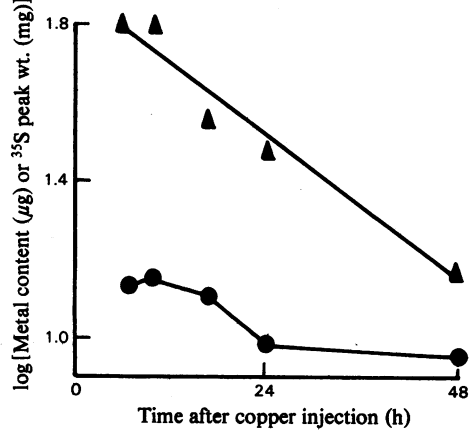

Fig. 8. *Half-life of renal copper-thionein in zinc-supplemented rats after pulse-labelling with* L-[^{35}S]*cysteine*

Rats (150g) were injected intraperitoneally with 300 μg of copper (as $CuSO_4$), followed after 4h by 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine. They were killed at intervals from 6 to 96h after injection of the copper. The copper-thionein in the renal cytosol (7ml) was separated on Sephadex G-75 and its ^{35}S content estimated as described in the Experimental section. The half-life of the ^{35}S (▲) and of the total metal (copper plus zinc) (●) present as metallothionein were calculated from the plot of the log values against time.

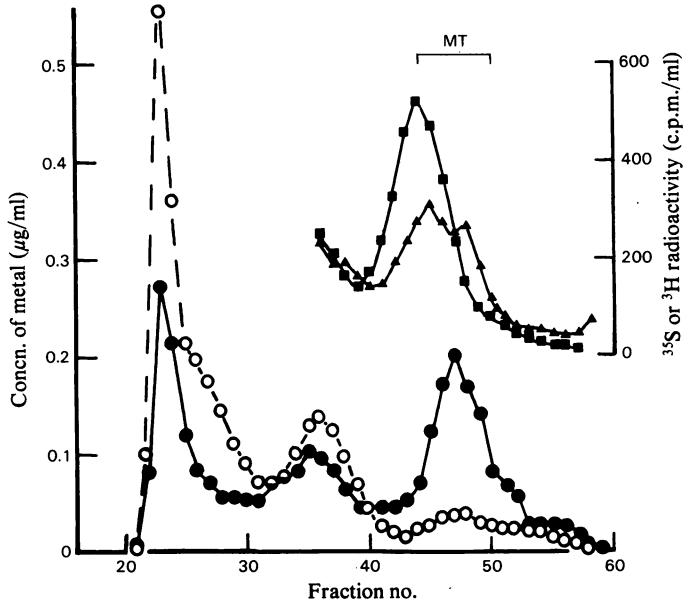

Fig. 9. *Separation on Sephadex G-75 of the renal cytosol from zinc-deficient rats given copper,* L-[^{35}S]*cysteine and* L-[4,5-^{3}H]-*leucine*

Zinc-deficient rats (150g) were given 300μg of copper (as $CuSO_4$) intraperitoneally, followed 4h later by 16μCi of L-[^{35}S]cysteine and 40μCi of L-[4,5-^{3}H]leucine. The rats were killed after a further 6h and the renal cytosol was fractionated as described in the Experimental section. Each chromatographic fraction was assayed for ^{35}S (▲), ^{3}H (■), copper (●) and zinc (○). The position of the metallothionein fraction is shown (MT).

(Fig. 6), with the elution profiles of copper, Zn^{2+} and ^{35}S in this area being similar. The specific radioactivities of the ^{35}S in the three fractions (I–III) obtained from the Sephadex G-75 columns were 554, 548 and 1028c.p.m./mg of protein respectively at 10h post-injection. There was no significant incorporation of ^{3}H from leucine into the metallothionein fraction but a small ^{3}H-containing peak was eluted just before this at tube 43 (Fig. 6).

The degradation rate of the ^{35}S-labelled renal copper-thionein was estimated in the same way as for the hepatic protein, with a correction being made for a small non-thionein component (Fig. 8). The half-life (±S.E.) was 19.7±2.4h in the Zn^{2+}-supplemented rats. No precise estimate could be made of the rate of disappearance of copper plus Zn^{2+} from the protein, but at no time did this exceed the degradation rate of the protein as estimated from the ^{35}S clearance (Fig. 8).

Injection of Cu^{2+} in the Zn^{2+}-deficient rats caused a similar increase in copper-thionein concentrations, from 0.7μg of copper/g of kidney in the control rats to 2.4μg/g in those killed at 10h post-injection and was associated with increased incorporation of ^{35}S into the protein (Fig. 9). However, substantial amounts of both ^{35}S and ^{3}H appeared in a fraction eluted from Sephadex G-75 at tube 46, immediately before the metallothionein and containing no Cu^{2+} or Zn^{2+}. Because of the considerable overlap in the ^{35}S peaks for this fraction and for metallothionein, it was impossible to calculate the degradation rate of the ^{35}S-labelled metallothionein. The half-life of the ^{3}H-rich component was estimated to be 7.5h.

Discussion

Injection of Cu^{2+} into rats has been shown to stimulate the incorporation of both copper and Zn^{2+} into a low-molecular-weight hepatic protein (Bremner & Davies, 1976), which was unequivocally identified as metallothionein (Bremner & Young, 1976*b*). The inhibitory effect of cycloheximide on this process indicated that active protein synthesis was involved (Bremner & Davies, 1976), although Bloomer & Sourkes (1973) suggested that the copper-protein was constitutive. The finding in these studies of increased incorporation of ^{35}S from cysteine into the protein after Cu^{2+} injection strongly supports the view that Cu^{2+} induces synthesis of metallothionein *de novo*.

Premakumar *et al.* (1975) previously demonstrated that Cu^{2+} stimulated the incorporation of ^{3}H from [4,5-^{3}H]lysine into a hepatic protein of roughly

similar size (mol.wt. about 8000) but with different amino acid composition and electrophoretic properties from metallothionein (Winge *et al.*, 1975). Similar claims that Cu^{2+} induces synthesis of a non-thionein protein have been made by others (Evans *et al.*, 1975; Riordan & Gower, 1975; Irons & Smith, 1977), but it is possible that the isolation of these proteins could be a reflection of inadequacies in the purification procedures used (Bremner & Young, 1976*b*; Hartmann & Weser, 1977).

The absence in the experiment reported here of any significant incorporation of ^3H from leucine into the Cu^{2+}-induced protein from either liver or kidney substantiates this view. As the relative radioactivity count rate for ^3H in the cytosolic fractions I and II was at least twice that for ^{35}S, it would appear that the Cu^{2+}-induced protein contained little, if any, leucine. It is significant therefore that rat liver copper-thioneins contain about 30% cysteine but less than 1% leucine residues (Bremner & Young, 1976*a*,*b*). In contrast the non-thionein copper-proteins have been reported to contain 7–14% cysteine and about 6–7% leucine residues (Evans *et al.*, 1975; Winge *et al.*, 1975). It is unlikely that selective loss of leucine residues could have occurred during the partial purification of the copper-protein, as a similar gel-filtration step has been included in the isolation of copper-thionein and of the other proteins. The remote possibility remains that other differences in experimental procedure, such as the strain of rat and doses used, could be responsible for the induction of different copper-proteins in different laboratories. However, this seems unlikely, especially as the antibody to one of the 'non-thionein' proteins has recently been shown to cross-react with cadmium-thionein (Madapallimatam & Riordan, 1977).

Although it was not possible to purify the copper-protein induced in Zn^{2+}-deficient animals because of the development of polydisperse characteristics (Bremner & Young, 1976*b*), the absence of ^3H incorporation into the protein in Zn^{2+}-deficient rats supports the view that this was also copper-thionein. There was no detectable difference between Zn^{2+}-supplemented and Zn^{2+}-deficient rats in the rate at which ^{35}S or copper was incorporated into the protein, with maximum incorporation in both groups of animals occurring after 6–10h. However, there was a significant difference between the two groups in the disappearance rate of isotope and of metal from the protein. The half-life of the copper-thionein, as estimated from the exponential rate of loss of ^{35}S, was about 17h in the Zn^{2+}-supplemented rats but only 12h in the Zn^{2+}-deficient animals. This compared with half-lives of 18–19h for Zn^{2+}-induced hepatic zinc-thionein (Feldman & Cousins, 1976) and of 67h (Shaikh & Smith, 1976) or 100h (Chen *et al.*, 1975) for the Cd^{2+}-induced protein.

The degradation of cadmium-thionein was not associated with the loss of Cd^{2+} from the liver and it appeared that the liberated Cd^{2+} was incorporated into newly synthesized metallothionein (Chen *et al.*, 1975; Shaikh & Smith, 1976). In contrast, the disappearance of metal from the copper-thioneins, as from the Zn^{2+}-induced protein (Feldman & Cousins, 1976), proceeded at the same rate as the degradation of the protein. This suggests that these processes occurred concomitantly and therefore supports the claim (Bremner & Davies, 1976) that the failure of Zn^{2+}-deficient animals to accumulate significant amounts of copper-thionein in their liver, regardless of their Cu^{2+} status, is a consequence of the decreased stability of the copper-thionein in these animals. This could be related to the decreased Zn^{2+} content of the Cu^{2+}-induced metallothionein in these animals. It is perhaps significant that in previous studies (Bremner & Davies, 1976), where a shorter half-life (10h) was found for the disappearance of copper from the protein, even less Zn^{2+} was present in the copper-thionein.

Nothing is known about either the catabolism of metallothioneins, or of the way in which the bound metal influences their susceptibility to degradation. However, the binding of various compounds to proteins can alter their rate of catabolism (Goldberg & Dice, 1974), as is illustrated by the retardation in the degradation of ferritin caused by the binding of Fe^{3+} to the protein (Drysdale & Munro, 1966). The stabilizing effect of Zn^{2+} on copper-thionein could be consistent with one of the functional roles of Zn^{2+} in maintaining the structural integrity of certain zinc-enzymes (Drum *et al.*, 1967) and possibly in preventing oxidation of thiol groups (Chvapil, 1973). It is interesting that the loss of Zn^{2+} which occurred during the purification of renal copper-thioneins from sheep was associated with considerable molecular-weight changes in the proteins, consistent with the occurrence of oxidation and polymerization reactions between copper and exposed thiol groups (Bremner & Young, 1977).

Relatively little attention has been paid in the past to the low-molecular-weight copper-proteins in rat kidney. As shown here, concentrations of these proteins in the kidneys of control animals are generally greater than in liver. They are, moreover, dependent on both the age and sex of the animal, which suggests that production of the renal proteins may be under hormonal control (Bremner *et al.*, 1978). The chromatographic behaviour and amino acid composition of the partially purified renal proteins show clearly that the proteins are copper-thioneins (I. Bremner & B. W. Young, unpublished work) and this is confirmed by the negative results on leucine incorporation reported here.

The rates of synthesis and degradation (as measured

respectively by the uptake and loss of [^{35}S]cysteine) of the hepatic and renal copper-thioneins were similar. It seems therefore that the renal protein was synthesized in the kidneys and not transported in the blood from the liver, as was suggested for cadmium-thionein (Piscator, 1964).

The degradation rate of the hepatic and renal copper-thioneins was considerably greater than that of most of the other cytosol proteins, the half-life of the latter in the Zn^{2+}-adequate rats, 90h, being identical with that reported previously (Glass & Doyle, 1972). These findings emphasize the temporary nature of any storage function which metallothionein may have for copper in these rats. In view of the conclusion that the rate of utilization of copper from this pool is related to the rate of degradation of the protein, it will be of interest to establish whether this is dependent on the Cu^{2+} status of the animal.

No attempt was made to obtain the ^{35}S-labelled copper-thioneins in an absolutely pure state before estimation of the degradation rate. However, the level of ^{35}S incorporation into other proteins within fraction III was found to be low, as is shown by the studies on the control rats, and was corrected for. Furthermore it has been shown in other studies on metallothionein degradation (Chen *et al.*, 1975; Feldman & Cousins, 1976) that practically all the radioactivity in the equivalent fraction III was incorporated into the thionein proteins. It is also possible that the estimates of degradation rate could have been influenced by the reutilization of [^{35}S]-cysteine (Schimke, 1970). However, if this had occurred to an appreciable extent it is doubtful if there would have been such a linear decrease over the period 6–72h in the log of the ^{35}S radioactivity (Fig. 5), or such good agreement between the disappearance rates of metal and ^{35}S.

In conclusion, it has been found that the rate of catabolism of metallothionein is markedly influenced by the nature of the metal bound to it, with the copper-rich protein from Zn^{2+}-deficient rats being degraded most rapidly. Moreover, the release of copper and Zn^{2+} from the protein is concomitant with the degradation of the protein. Finally, the findings on the non-incorporation of leucine into the protein are inconsistent with the induction of synthesis of low-molecular-weight copper-proteins other than metallothionein.

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