Studies on the appearance of a hepatic copper-binding protein in normal and zinc-deficient rats

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1. A study has been made by gel-filtration techniques of the soluble copper- and zinc-binding proteins in rat liver after both intraperitoneal injection of Cu and dietary Cu supplementation.

2. Liver Cu and Zn concentrations increased after injection of Cu, both metals accumulating in the cytosol, mainly in a fraction with an apparent molecular weight of about 12000.

3. When Zn-deficient rats were injected with Cu, there was little change in liver Zn concentration and the occurrence of Cu in the low-molecular-weight form (about 12000) was more transient. At most periods after injection, Cu accumulated mainly in a fraction with a molecular weight greater than 65000.

4. When the rats were Cu-loaded by dietary supplementation, virtually no Cu or Zn was found in the low-molecular-weight form in Zn-deficient rats, although they were found in the Zn-supplemented animals.

5. The results suggest that Zn is essential for the accumulation of Cu in this form, but not for Cu to stimulate production of the metal-binding protein by a process requiring active protein synthesis.

The results of previous studies on ruminant liver suggested that the occurrence of a metallothionein-like, copper- and zinc-binding fraction was related to the Zn, but not Cu, content of the liver, although both elements apparently competed for binding sites on the protein (Bremner & Marshall, 1974*a*, *b*). This relationship was not found in normal pig liver, as the amount of Cu or Zn present in this form was dependent only on the liver concentration of that metal, with no evidence of competitive binding (Bremner, 1976; Bremner & Young, unpublished results). In the livers from Zn-deficient pigs, however, both the Cu- and Zn-proteins were absent, regardless of the hepatic Cu content, implying that a minimal amount of Zn was in some way essential for the production or accumulation of the metal-binding protein.

Studies of the effects of Zn injection in rats has confirmed that Zn can indeed promote the synthesis de novo or the stabilization of hepatic metallothionein (Bremner & Davies, 1975). Comparable studies have now been made in both normal and Zn-deficient rats on the appearance of an analogous hepatic Cu-binding fraction after a single Cu injection or after long-term dietary Cu supplementation. The results indicate that although Zn is not essential for Cu to stimulate production of this Cu-binding hepatic protein, it is required for accumulation of Cu in this form. A preliminary report of some of this work has been published (Bremner & Davies, 1974).

EXPERIMENTAL

Animals and treatment

Male Hooded Lister rats of the Rowett Institute strain were used. They were caged individually and maintained on the semi-synthetic diet of Williams & Mills (1970), which contained 40 mg Zn and 25 mg Cu/kg. When Zn-deficient or Cu-loaded rats were required, the dietary concentrations were changed to < 1 mg Zn and 200 or 500 mg Cu/kg respectively.

Expt 1. Effects of injection of Cu on hepatic distribution of Cu and Zn

Zn-deficient rats (150 g) were obtained by transferring rats, aged about 60 d, to the low-Zn diet 21 d before the start of the experiment. Several groups of Zn-supplemented (150 g) or Zn-deficient rats, each group containing at least four animals, were injected intraperitoneally with 100 or 300 μg Cu (as cupric sulphate in a solution containing 9 g sodium chloride/l). They were killed by a blow on the head at intervals from 0·5 h to 8 d after the injection, and their livers were removed immediately. Livers were also collected from groups of Zn-supplemented and Zn-deficient control rats which had not been injected with Cu.

Expt 2. Effects of Zn deficiency on hepatic Cu distribution of Cu-loaded rats

Forty-eight rats (75–85 g) were allocated at random to three groups, each of sixteen animals, which were given: group A, the low-Zn, high-Cu diet, *ad lib.*; group B, the Zn-supplemented, high-Cu diet, pair-fed to group A; group C, the Zn-supplemented, high-Cu diet, *ad lib.* The Cu content of the diet was increased from 200 to 500 mg/kg after 5 weeks. Four rats from each group were killed after 3, 5, 8 and 10 weeks on the experimental diets and their livers removed immediately.

Fractionation of livers

Samples of each liver were freeze-dried, digested with conc. nitric acid–conc. perchloric acid–conc. sulphuric acid (5:2:1, v/v), and their Cu and Zn contents determined by atomic absorption spectrophotometry.

The remainders of each liver within the appropriate groups were combined, homogenized with 2·5 vol. (v/w) 0·01 M-Tris–acetate buffer, pH 7·4, centrifuged at 100000 *g* for 1 h, and the supernatant fractions fractionated on Sephadex G-75 (Pharmacia Ltd, Uppsala, Sweden) as described previously (Bremner & Marshall, 1974*a*).

RESULTS

Expt 1. Effect of injection of Cu on hepatic distribution of Cu and Zn

When twelve Zn-supplemented rats were injected with 100 μg Cu and killed after 8 h, variable increases occurred in the hepatic concentrations of both Cu and Zn. The final concentrations (μg/g dry matter (DM)) were: Cu 40·8 ± 3·7, Zn 111·4 ± 4·0; control values for non-injected rats were 22·7 ± 1·6 and 93·3 ± 2·7 for Cu and Zn

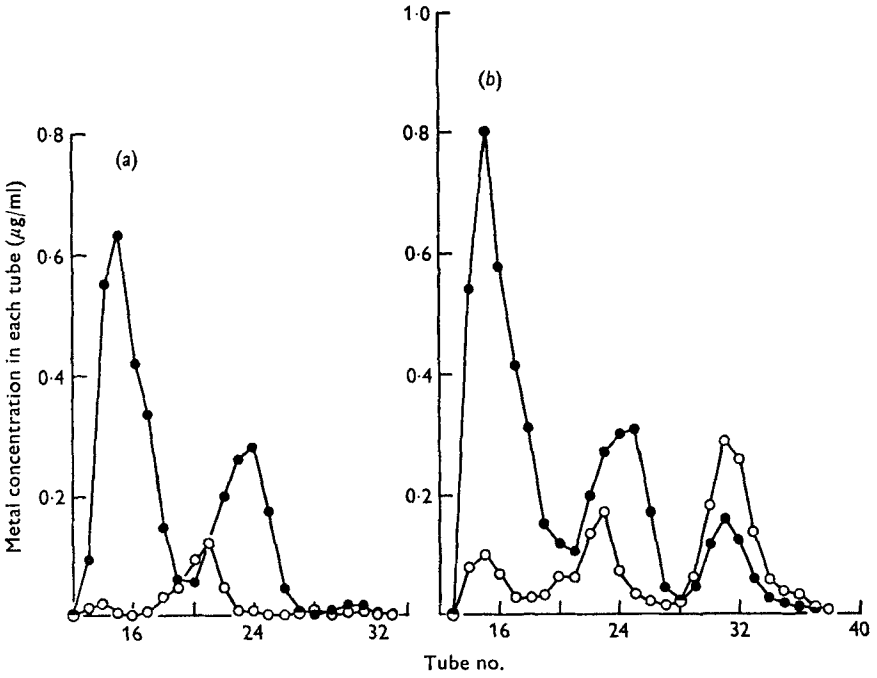

Fig. 1. Expt 1. Fractionation on Sephadex G-75 of the supernatant fractions from the livers of (*a*) control, zinc-supplemented rats and (*b*) Zn-supplemented rats injected with 300 μg copper (as cupric sulphate) 8 h previously. The concentrations (μg/ml) of Cu (○) and of Zn (●) in each tube are shown. Fractions 1–3 were contained in tubes 12–18, 19–27 and 28–37 respectively, and each tube contained 5 ml eluate.

respectively. There was a significant relationship between the hepatic concentrations of the two metals in the injected rats, as expressed by the equation:

$$Y_1 = 0{\cdot}81x_1 - 49{\cdot}5 \quad (\text{SE of regression coefficient } 0{\cdot}14),$$

where Y_1 and x_1 are the liver concentrations (μg/g DM) of Cu and Zn respectively.

The livers were allocated into five groups, according to their Cu and Zn contents and the distribution of the metals in the cytosol was determined by gel filtration on Sephadex G-75. Typical separations for livers from the injected rats with the highest Cu contents, and from control rats are shown in Fig. 1. Three main fractions, 1–3, were collected as described previously (Bremner & Davies, 1975), with approximate molecular weights of > 65000, 35000 and 12000, as estimated from their elution volumes from the column. The most obvious effect of Cu injection was to increase the amount of Cu, and to a lesser extent of Zn, present in fraction 3 and of Cu in fraction 1. There was occasionally a slight increase in the Zn content of fraction 1 but little change in metal-binding contents of fraction 2. It was found that the Cu distribution was not greatly affected by the liver Cu content, as about half the total Cu occurred in fraction 3 in each group of the Cu-injected rats.

In view of the relationship between metal-binding in fraction 3 and liver Zn content found in previous studies (Bremner & Marshall, 1974*b*), a closer study was

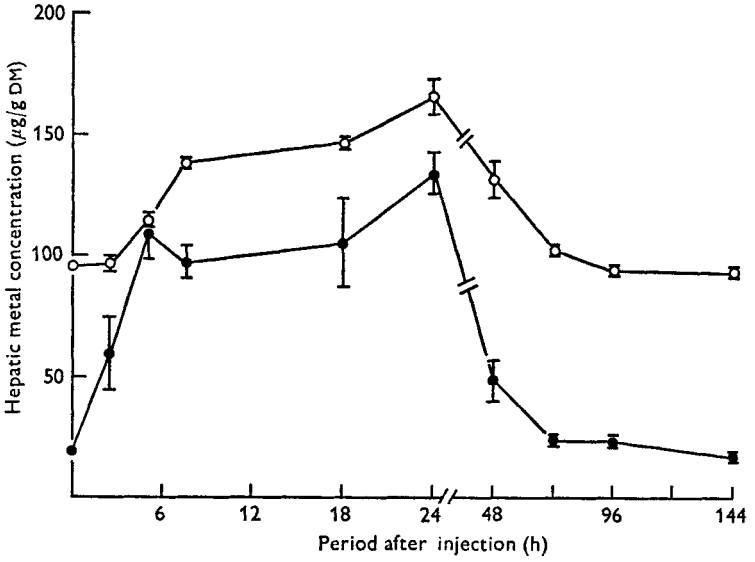

Fig. 2. Expt 1. Change in liver copper (●) and zinc (○) concentrations (μg/g dry matter (DM)) of groups of five Zn-supplemented rats with period (h) after intraperitoneal injection of 300 μg Cu (as cupric sulphate).

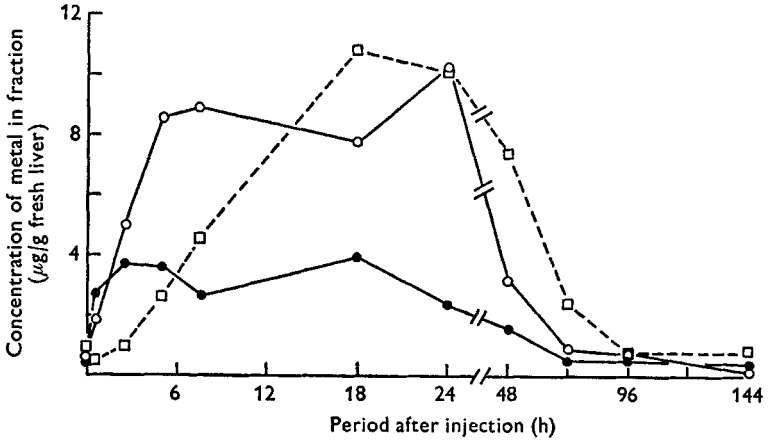

Fig. 3. Expt 1. Changes in the distribution of copper and zinc in fractions from livers of groups of five Zn-supplemented rats with period (h) after intraperitoneal injection of 300 μg Cu (as cupric sulphate). Liver homogenates were centrifuged and then fractionated on Sephadex G-75. The concentrations (μg/g fresh liver) of Cu (○) and Zn (□) in fraction 3 and of Cu in fraction 1 (●) are shown; for details of fractions, see Fig. 1.

made in fifty-five rats of the time-course of changes in the hepatic concentration and distribution of Cu and Zn after injection of 300 μg Cu. Total hepatic Cu concentration increased rapidly in the first 5 h from about 16 to 110 μg/g DM, then more slowly to a maximum of 135 μg/g DM at 24 h (Fig. 2). It decreased thereafter and by 3 d after injection was at 'near-normal' levels. Liver Zn concentration was unchanged in the first 2·5 h at approximately 95 μg/g DM, increased to 115 μg/g DM by 5 h, and increased steadily thereafter to a maximum of 166 μg/g DM at 24 h after

injection. Although the maximum concentrations of Zn and Cu were attained at the same interval after injection the subsequent decrease in Zn concentration was slower than that for Cu. Liver Zn content had returned to 'near-normal' values 3–4 d after injection. There was no consistent significant relationship between the Cu and Zn content of the livers at any interval after injection.

The distribution of soluble Cu and Zn within fractions 1 and 3 is shown in Fig. 3. As only slight increases were found in the total amount of Cu present in fraction 2, usually about 2·9 μg/g fresh liver, these are not shown. The amount of Cu in fraction 1 increased almost tenfold within 2·5 h to 3·7 μg Cu/g fresh liver, but little further change occurred until 18 h after injection, when the concentration started to decrease. Incorporation of Cu into fraction 3 was almost as rapid, and high concentrations were maintained between 5 and 24 h when about 60% of the soluble Cu was in this form. The Cu content of fraction 3 decreased from 10·2 to 3·2 μg/g fresh liver between 1 and 2 d after injection, and was at 'near-normal' levels by 3–4 d. The amount of Zn in fraction 3 was unchanged until about 2·5 h after injection, but increased thereafter up to a maximum of 10·8 μg/g fresh liver at 18 h after injection, whereupon it decreased slowly over the next 3 d to normal levels. The concentrations of Zn in fractions 1 and 2 were relatively unchanged throughout at about 11·7 and 6·0 μg/g fresh liver respectively.

The amount of Cu present in fraction 3 in fifteen groups of livers at 5 h–3 d after injection of 100 or 300 μg Cu was found to be a function of the liver Cu content. This could be expressed by the equation:

$$Y_2 = 0{\cdot}35x_2 - 1{\cdot}28 \quad (\text{SE of regression coefficient } 0{\cdot}032,\ r\ 0{\cdot}95),$$

where Y_2 and x_2 are the concentrations of Cu (μg/g fresh liver) in fraction 3 and the whole liver respectively.

The relationship between the concentration of Zn in whole liver (x_3) and fraction 3 (Y_3) for all rats injected with 300 μg Cu is given by the equation:

$$Y_3 = 0{\cdot}54x_3 - 15{\cdot}8 \quad (\text{SE of regression coefficient } 0{\cdot}073,\ r\ 0{\cdot}91).$$

In an attempt to determine whether the increase in hepatic Zn concentration was essential for the appearance of Cu in fraction 3, similar studies were done using forty-four Zn-deficient rats. It was found that the hepatic Cu uptake after injection of 300 μg Cu (Fig. 4) was more rapid than in Zn-supplemented rats (Fig. 2). Maximum Cu concentrations of 140 μg/g DM were reached within 7·5 h but these decreased thereafter and the concentrations at 24 and 48 h after injection were 70 and 45% of the maximum respectively. Normal values were restored 3 d after injection. Although slight increases in hepatic Zn content of about 30 and 20 μg/g DM occurred after 7·5 and 18 h, at all other intervals after injection Zn contents were similar to those in the non-injected rats.

In contrast to the immediate appearances of Cu in fraction 3 in Zn-supplemented rats, the first appearance of Cu in this fraction in the Zn-deficient animals did not occur until 5 h after injection of Cu, when 28% of the soluble Cu was present in this form (Fig. 5). This proportion increased to 50%, equivalent to 10 μg Cu/g

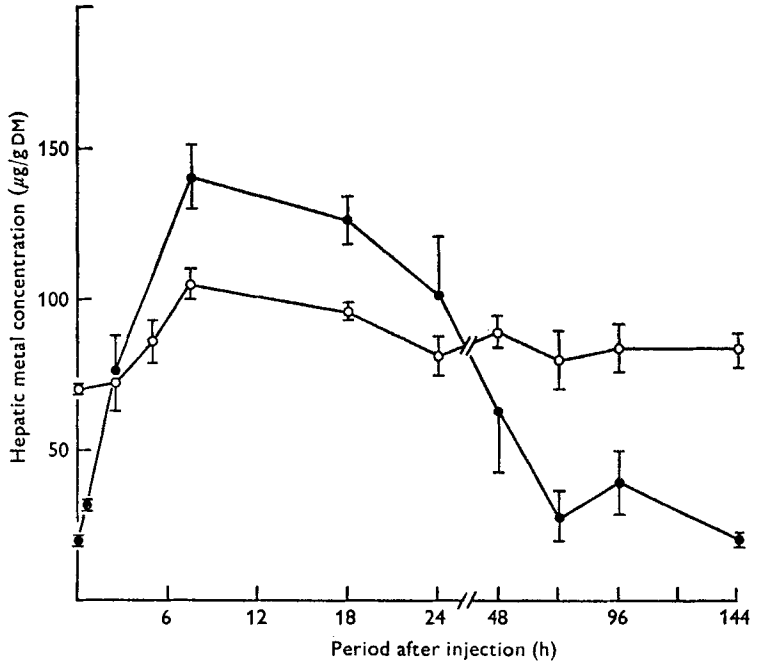

Fig. 4. Expt 1. Changes in the liver copper (●) and zinc (○) concentrations (μg/g dry matter (DM)) of groups of four Zn-deficient rats with period (h) after intraperitoneal injection of 300 μg Cu (as cupric sulphate).

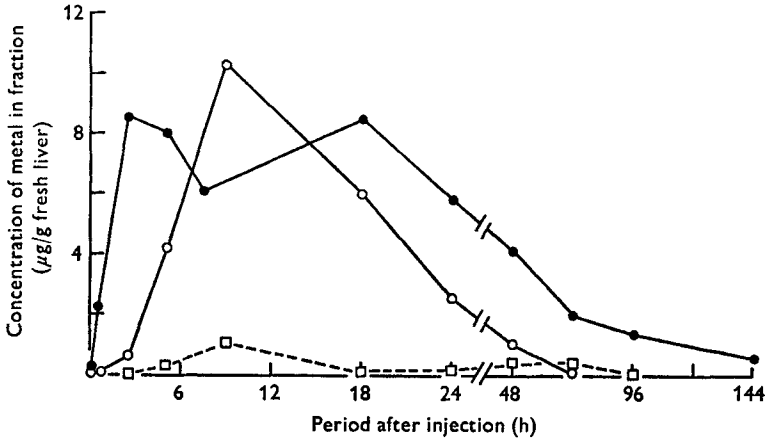

Fig. 5. Expt 1. Changes in the distribution of copper and zinc in fractions from livers of groups of four Zn-deficient rats with period (h) after intraperitoneal injection of 300 μg Cu (as cupric sulphate). Liver homogenates were centrifuged and then fractionated on Sephadex G-75. Concentrations (μg/g fresh liver) of Cu (○) and Zn (□) in fraction 3 and of Cu in fraction 1 (●) are shown; for details of fractions, see Fig. 1.

Table 1. *Expt 1. Effect of simultaneous intraperitoneal injection of cycloheximide (2 mg/kg) and copper (300 μg) on hepatic Cu distribution in zinc-deficient rats*

(Values based on a single fractionation on Sephadex G-75 of combined livers from four rats/treatment)

Treatment	Concentration of Cu (μg/g fresh liver)			
	Whole liver	Fraction 1†	Fraction 2†	Fraction 3†
Control	4·5	0·4	2·16	0·01
+Cu alone*	33·3	7·4	4·55	9·1
+Cu+cycloheximide*	26·6	11·1	2·68	0·9

* Rats were killed 5 h after injection.
† For details of fractionation procedure, see p. 102 and Fig. 1.

fresh liver, over the next 2·5 h but decreased thereafter, the concentration at 24 h being only 25% of the maximum. Compared with the Zn-supplemented rats, proportionally more of the Cu was therefore present in fraction 1 at all intervals after injection, and especially in the periods 0–5 and 18–72 h after injection of Cu. The amounts of Cu in fraction 2 ($2{\cdot}7 \pm 0{\cdot}2$ μg/g fresh liver) and of Zn in fractions 1 ($10{\cdot}7 \pm 0{\cdot}4$ μg/g fresh liver) and 2 ($4{\cdot}2 \pm 0{\cdot}2$ μg/g fresh liver) were relatively unchanged at all intervals after injection. A slight increase in the concentration of Zn in fraction 3 occurred after 7·5 h.

The concentration of Cu in fraction 3 in both Zn-supplemented and Zn-deficient rats decreased at an exponential rate after maximum concentrations had been reached. This could be expressed by the equations:

for Zn-supplemented rats: $Y_1 = 1{\cdot}23 - 0{\cdot}015x$ (SE of regression coefficient 0·0017),

for Zn-deficient rats: $Y_2 = 1{\cdot}29 - 0{\cdot}030x$ (SE of regression coefficient 0·0046),

where Y_1 and Y_2 are $\log_{10}$ concentration of Cu in fraction 3 (μg/g fresh liver) and x is the period (h) after Cu injection. As the slopes of these lines are significantly different ($P < 0{\cdot}025$), it is clear that Cu disappears more rapidly from fraction 3 in the Zn-deficient rat, the half-life being about 10 h compared with 20 h in the Zn-supplemented animals.

When Zn-deficient rats were simultaneously injected with cycloheximide (2 mg/kg body-weight) and Cu (300 μg) and killed after 5 h, the incorporation of Cu into fraction 3 was reduced by 90% (Table 1). The hepatic accumulation of Cu was by comparison only slightly affected, being 75% of that in the non-cycloheximide-treated animals. The dose of cycloheximide used was found in separate studies (Davies, Bremner & Mills, 1973) to be sufficient to inhibit [^{14}C]lysine incorporation into total liver protein by 90%.

Expt 2. Effects of Zn deficiency on hepatic Cu distribution of Cu-loaded rats

Despite the high Cu contents of the diets, increases in liver Cu content were usually small compared with those resulting from injection of Cu. There were no obvious changes in metal distribution in the cytosol within treatment groups when

Table 2. *Expt 2. Distribution of copper and zinc among soluble fractions isolated by gel filtration from liver homogenates of rats given the low-Zn diet (treatment group A), the same quantity (i.e. pair-fed to group A) of the Zn-supplemented diet (treatment group B) and the Zn-supplemented diet* ad lib. *(treatment group C)*

(Mean values with their standard errors, based on a single fractionation on Sephadex G-75 of six groups of two to four livers collected from 3 to 10 weeks after the start of the experiment)

	Cu					Zn				
Treatment group† ...	A	B	C	SE of difference between means	Statistical significance of difference between means	A	B	C	SE of difference between means	Statistical significance of difference between means
Liver concentration (μg/g fresh liver)	7·4	11·0	5·9	3·1	—	24·3	30·0	28·2	1·5	**
Amount (%) of soluble metal in fraction no.‡: 1	41·7	23·7	20·2	6·0	**	66·9	62·2	56·4	1·6	***
2	56·6	37·7	68·5	8·3	—	31·9	32·3	41·6	1·5	***
3	1·7	27·0	11·4	3·7	***	1·2	5·6	2·1	0·9	***
Concentration (μg/g fresh liver) of metal in fraction no.‡: 1	1·9	1·7	0·6	0·5	—	10·3	11·9	9·7	0·5	***
2	2·4	2·8	1·9	0·3	*	4·9	6·2	7·1	0·4	***
3	0·1	2·0	0·3	0·6	**	0·2	1·1	0·4	0·2	***

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

† For details of dietary treatments, see p. 102.

‡ For details of fractionation procedure, see p. 102 and Fig. 1.

liver samples were analysed 3–10 weeks after the start of the experiment, and consequently all results are presented together (Table 2).

The most significant finding was the virtual absence of Cu and Zn from fraction 3 in the livers from the Zn-deficient rats, with less than 2% of the soluble Cu in this form compared with nearly 30% in the pair-fed, Zn-supplemented rats. Cu was evenly distributed between fractions 1 and 3 in the latter animals, whereas in the livers from the Zn-supplemented rats fed *ad lib.* there was less Cu in fraction 3. The amount of Cu in fraction 2 tended to be relatively constant and independent of liver Cu content within treatment groups, and usually accounted for about 30% of the total Cu in the liver.

The only major change in the distribution of Zn was the increased concentration in fraction 3 of the pair-fed, Zn-supplemented rats.

DISCUSSION

The virtual absence of Cu or Zn from fraction 3 of the livers of the Zn-deficient rats given the high-Cu diets, regardless of the liver Cu content, supports the view (Bremner & Marshall, 1974*b*; Bremner, 1976) that Zn must play an essential part in the production of this group of metal-binding proteins. Although these results could indicate that Cu is incapable of stimulating the production of these proteins in a low-Zn situation, the results of acute studies involving injection of Cu suggest that this is not so, as the Cu-binding fractions produced in Zn-supplemented and Zn-deficient rats were apparently the same. However, there were marked differences in the distribution of Cu between fractions 1 and 3 in the two groups of rats, especially in the persistance of Cu in fraction 3 after maximum concentrations had been reached. The more rapid removal of Cu from this form when Zn is absent, as in the Zn-deficient rats, suggests that the presence of Zn may inhibit the degradation of the protein. The absence of hepatic Cu from fraction 3 in the Zn-deficient rats given the dietary Cu supplement could then be a consequence of the decreased stability of the protein in the absence of additional Zn.

Although this can only be unequivocally established by comparison of the turnover rates of the protein in the presence and absence of Zn, it is recognized that the catabolism of many proteins is affected by the supply of various factors which bind to them (Goldberg & Dice, 1974). For example, the susceptibility of superoxide dismutase (*EC* 1.15.1.1) to carboxypeptidase (*EC* 3.4.2.3) digestion in vitro is dependent on the Zn content of the enzyme (Rotilio, Calabrese, Bossa, Barra, Agro & Mondovi, 1972), probably because of the role of Zn in maintaining the conformational stability of this and other Zn enzymes (Drum, Harrison, Li, Bethune & Vallee, 1967). It seems possible that Zn could stabilize the hepatic Cu-protein in fraction 3 in the same way, although at this stage it has yet to be unequivocally established whether Cu and Zn bind to the same protein.

A notable feature of the Cu-injection studies with Zn-supplemented rats is the close connexion between the increase in liver Cu and Zn concentrations. The source of this Zn is not known although it has been found (Bremner & Davies, unpublished

results) that its entry into the liver is not preceded by pancreatic accumulation, as it is after injection of sufficient Zn to produce similar changes in liver Zn content (Davies & Bremner, 1974). The uptake of Zn into fraction 3 after injection of Cu is very similar to that which occurs after injection of cadmium (Nordberg, Piscator & Lind, 1971; Webb, 1972) with resultant formation of metallothionein (Winge & Rajagopalan, 1972). The hepatic responses to injection of Cu and Cd are also similar in that binding of these metals in fraction 3 is preceded to some extent by binding in fraction 1, and administration of cycloheximide inhibits the production of the metal-binding protein in fraction 3 without markedly reducing hepatic accumulation of Cu or Cd (Webb, 1972). In contrast there is no prior binding of Zn in fraction 1 after Zn injection (Bremner & Davies, 1975), and inhibition of protein synthesis reduces both production of Zn-thionein and hepatic accumulation of the metal (Davies *et al.* 1973; Richards & Cousins, 1975).

The inhibitory effect of cycloheximide on the appearance of Cu in fraction 3 suggests either that the process is dependent on active protein synthesis by some inductive mechanism, or that Cu stabilizes a minor apoprotein which is rapidly turning over without necessarily influencing its synthesis de novo. Both explanations are contrary to the claim that the apoprotein is constitutively present in rat liver (Bloomer & Sourkes, 1973). However, our findings that fraction 3 can contain an important Cu-binding protein in rat liver, and our results from the time-course study of the appearance and disappearance of Cu from this fraction after Cu injections, are in general agreement with these of Bloomer & Sourkes (1973). Terao & Owen (1973) have reported that the greatest proportion of injected Cu appears in this fraction within 30 min and persists for only a few hours. However, they injected only tracer amounts of Cu as ^{64}Cu and it is possible that some isotopic exchange may have occurred.

The functional importance of the induced Cu-protein has not yet been established, but it seems likely that it is primarily involved in the rapid hepatic uptake of Cu, perhaps including removal of albumin-bound Cu (Marceau & Aspin, 1973) and temporary storage and detoxication of Cu (Bremner & Davies, 1974). As there are no reports of important disturbances in Cu metabolism of Zn-deficient rats, in which no hepatic Cu occurs in fraction 3, it would appear that the hepatic protein is not essential for Cu to fulfil its metabolic functions.

Hepatic Cu-proteins of similar molecular weight to fraction 3 have been isolated from humans (Shapiro, Morell & Scheinberg, 1961), cattle (Evans, Majors & Cornatzer, 1970) and ruminants (Bremner & Marshall, 1974*b*). In the latter instance competitive binding between Cu and Zn was found to occur and the metal-binding protein was characterized as metallothionein. More recently it has been found that a range of mixed Cu–Zn-thioneins also occurs in the livers of pigs given high-Cu diets (Bremner & Young, unpublished results) and there is some indirect evidence that in Expt 2 at least some of the Cu in rat liver was in this form. When the liver Zn concentration in the rat is increased, as by injection of Zn or restriction of food intake, about 75% of the additional Zn generally occurs as Zn-thionein (Bremner & Davies, 1975). However, this proportion was considerably reduced in the partially-

starved, pair-fed control rats given Cu, especially at the higher liver Cu contents, suggesting that Zn may have been isomorphously replaced on the thionein-protein by Cu, so that at least some of the Cu in fraction 3 is probably present as Cu-thionein. It has been reported also that Cu frequently occurs as a minor component in renal metallothionein (Pulido, Kägi & Vallee, 1966) and it has been suggested that the particulate Cu-protein of neonatal liver may be a polymeric, Cu-rich species of metallothionein (Porter, 1974; Rupp & Weser, 1974).

It is far from certain, however, that this represents the only form of Cu in fraction 3. Since this work was completed, a Cu-protein which differs in several respects from metallothionein has been isolated from the livers of Cu-injected rats (Winge, Premakumar, Wiley & Rajagopalan, 1975). So far this protein, Cu-chelatin, has only been obtained after acute administration of Cu, and it will be of considerable interest to determine whether it occurs also in normal physiological situations.

Important differences occur between ruminants, pigs and rats in the proportion of the hepatic Cu which is normally found in fraction 3, and in the influence thereon of liver Zn content. In all species, negligible amounts of Cu or Zn occur in this form when the animals are Zn-deficient and on long-term dietary experiments (Bremner & Marshall, 1974*a*; Bremner, 1976). In Zn-supplemented pigs fraction 3 usually accounts for 80% of the Cu in the cytosol, whereas in ruminants the proportion can vary from 15 to 70% depending on liver Zn content. In the rat livers proportionately less Cu occurred in this fraction than in the pig livers, but this was increased when liver Zn content increased, as after restriction of food intake. Less is known of species differences in response to injection of Cu. Preliminary studies, involving serial liver biopsies of both Zn-deficient and Zn-supplemented sheep which were injected with Cu, indicate that production of the Cu-binding protein in fraction 3 is independent of Zn status but that, as in the rat, persistence of Cu in this form is reduced in the Zn-deficient animal (Bremner, unpublished results).

These results indicate that considerable differences may occur in the response of animals to acute and long-term exposure to heavy metals, and that the effects can be markedly influenced by the status of the animal with respect to other essential metals. If similar changes also occur in other tissues it is possible that these metal-binding proteins are intimately involved in the mechanism of the interaction between Cu and Zn.

It has now been proved (Bremner & Young, unpublished results) that the Cu in fraction 3 is present as metallothionein.

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