Hepatic copper- and zinc-binding proteins in ruminants

2. * Relationship between Cu and Zn concentrations and the occurrence of a metallothionein-like fraction

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1. A metal-binding fraction with a molecular weight of about 12000 in calf and sheep liver has been characterized as a metallothionein-like protein.

2. The combined concentrations of copper and zinc in the fraction (as μg/g liver) are a direct function of liver Zn concentration.

3. The relative proportions of Cu and Zn in the fraction are dependent on the Cu: Zn ratio in the liver.

4. These findings may be relevant to the mutual interaction between Cu and Zn.

In the previous paper (Bremner & Marshall, 1974) the effects of variations in copper and zinc status of ruminants on the distribution of the metals among soluble hepatic proteins were described. Three fractions (1–3), with approximate molecular weights of $\geqslant 75000$, 35000 and 12000, were isolated by gel filtration on Sephadex G-75 but none of these was fully characterized. The distributions of the Cu and Zn were closely related as, for example, Zn was absent from, or present in only low concentration in fraction 3 in high-Cu as well as in Zn-deficient livers. Variable amounts of Cu occurred in this fraction, which is identified in this paper as a metallothionein-like protein. The total amount of Cu and Zn present in fraction 3 has been found to be a function of liver Zn concentration.

EXPERIMENTAL

Analytical methods. Details of the livers and their fractionation on Sephadex G-75 and some analytical methods were described in the previous paper (Bremner & Marshall, 1974). Solutions were concentrated by ultrafiltration under nitrogen in a Diaflo cell, using UM2 filters (Amicon Ltd, High Wycombe, Bucks.). Occasionally further separations were carried out on columns (900 × 16 mm) of Bio-Gel P-10 (Bio-rad Laboratories, Richmond, California) using 0·01 M-Tris–acetate buffer, pH 8·2, as eluant and collecting 2·5 ml fractions. All procedures were carried out at 1°.

Protein concentrations were sometimes also measured by the method of Itzhaki & Gill (1964) and sulphydryl groups by the method of Jocelyn (1962). Polyacrylamide gel electrophoresis was done by the method of Davis (1964). Amino acid analysis was performed in duplicate, using a Locarte analyser (Locarte, Emperors Gate, London), with samples hydrolysed in 6 M-HCl for 24 h after performic acid oxidation (Moore, 1963).

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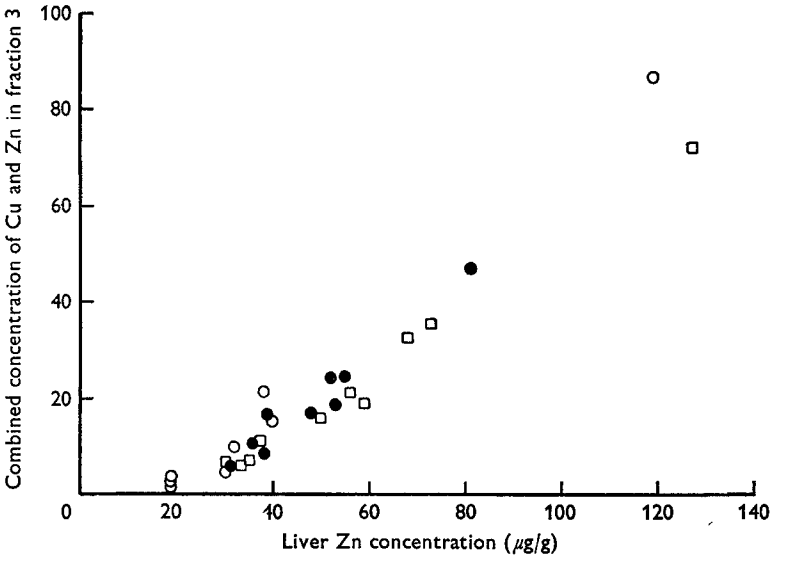

Fig. 1. Relationship between liver zinc concentration and the combined concentration of copper and Zn present in fraction 3 (μg metal/g fresh liver), as isolated by gel filtration on Sephadex G-75 (see p. 293). Samples were obtained from sheep (○) and calves with normal (□) and high (●) liver Cu concentrations.

Addition of cadmium to liver homogenate. Two samples of a normal sheep liver homogenate were treated as follows: (*a*) one sample was centrifuged direct at 105000 *g* for 1 h and a portion (3·5 ml) of the supernatant fraction separated on Sephadex G-75 in the usual way; (*b*) a solution of $CdSO_4$ was added to the other sample to give a Cd concentration of 4 μg/ml. After 1 h the homogenate was centrifuged and fractionated as above.

RESULTS

Concentrations of Cu and Zn in fraction 3

There was no linear relationship between the concentrations of Cu in fraction 3 and in the whole liver and between those of Zn in fraction 3 and the liver. However, the combined concentration of Cu and Zn in this fraction was linearly related to the liver Zn (but not Cu) concentration (Fig. 1) and could be expressed by the equations:

for sheep liver: $Y_1 = 0{\cdot}85x_1 - 16$ (SE of regression coefficient 0·036),
for calf liver: $Y_2 = 0{\cdot}70x_2 - 16$ (SE of regression coefficient 0·032),

where Y_1 and Y_2 are the combined concentrations of Cu and Zn in fraction 3 (μg metal/g fresh liver) and x_1 and x_2 are the liver Zn concentrations (μg/g).

These relationships applied even when Cu or Zn was absent from the fraction, as in Cu-deficient or 'high-Cu' livers. In Zn-deficient livers, with Zn contents of about 16 μg/g, Cu and Zn are absent from fraction 3.

Large variations were found in the relative proportions of Cu and Zn in this fraction. These were dependent on the Cu:Zn ratio in either the whole liver or the

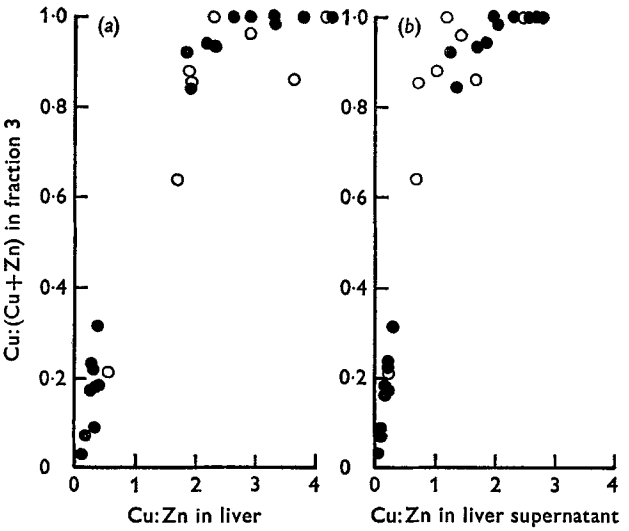

Fig. 2. Relationship between the proportion of binding sites in fraction 3 occupied by copper (i.e. Cu:(Cu+zinc) in fraction 3) and the Cu:zinc ratio (by weight) in (*a*) whole liver and (*b*) liver supernatants. Results are shown for calf (●) and sheep (○) livers. Fraction 3 was isolated by gel filtration on Sephadex G-75 (see p. 293).

supernatant fraction (Fig. 2). The proportion of binding sites apparently occupied in fraction 3 by Cu could be expressed by the equations:

$$(a)\quad \left.\begin{array}{ll} \text{for sheep liver:} & Y_1 = 1{\cdot}02 - 1{\cdot}5e^{-1{\cdot}06x_1}, \\ \text{for calf liver:} & Y_2 = 1{\cdot}02 - 1{\cdot}12e^{-1{\cdot}08x_2}, \end{array}\right\} \text{(residual SD 0·066)},$$

where Y_1 and Y_2 are Cu:(Cu+Zn) in fraction 3 and x_1 and x_2 are the Cu:Zn ratios in the whole liver;

$$(b)\quad \left.\begin{array}{ll} \text{for sheep liver:} & Y_3 = 1{\cdot}01 - 1{\cdot}38e^{-2{\cdot}33x_3}, \\ \text{for calf liver:} & Y_4 = 1{\cdot}01 - 1{\cdot}10e^{-1{\cdot}59x_4} \end{array}\right\} \text{(residual SD 0·047)},$$

where Y_3 and Y_4 are Cu:(Cu+Zn) in fraction 3 and x_3 and x_4 are the Cu:Zn ratios in the supernatant fraction.

It can be seen therefore that when equal amounts of Cu and Zn are present in the supernatant fraction (x_3 and x_4 = 1·0), 80 and 90% of the binding sites in fraction 3 are occupied by Cu in sheep and calf liver respectively. A Cu:Zn ratio of 2·33 and 2·07 is required in the whole liver of sheep and calves for 90% of the binding sites to be occupied by Cu.

Examination of fraction 3

It was thought likely that fraction 3 was similar to the Cd-binding protein, metallothionein, and that it should therefore be possible to displace the Cu and Zn by Cd. Addition of $CdSO_4$ to a single homogenate of lamb liver to give a final concentration of 4 μg Cd/ml caused a redistribution of the Zn and Cu in fraction 3 (Fig. 3). All Zn and part of the Cu were displaced, mainly into fraction 1, by the Cd, about half of which occurred in fraction 3. The associated increase in the extinction at 250 nm in this

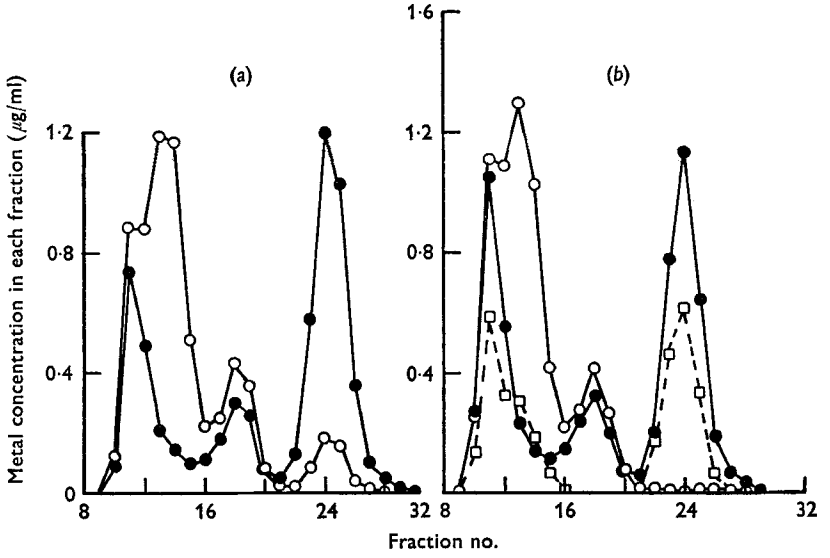

Fig. 3. Fractionation on Sephadex G-75 of supernatant fraction from (*a*) normal sheep liver (*b*) same liver after addition of 4 μg cadmium/ml to liver homogenate. Concentrations of zinc (○), copper (●) and Cd (□) are shown; 5 ml fractions were collected.

fraction was typical of the Cd mercaptide chromophore present in metallothionein (Kägi & Vallee, 1961). The total amount of metal, on a molar basis, in the fraction was unchanged, however, as it also was after addition of excess Cu or Zn to the homogenate. This is consistent with (although not absolute proof of) the absence in the liver of apoprotein corresponding to fraction 3 and with complete saturation of all binding sites in the fraction.

Gel filtration of fraction 3 on Bio-Gel P-10

With further separation on Bio-Gel P-10 of a sample of fraction 3, obtained from calf liver and containing mainly Zn with little Cu, much of the protein was removed from the metal-containing fractions (Fig. 4). From the shape of the Zn peak and the greater elution volume of the Cu it seemed that at least three metal-containing fractions were present. Resolution of these was greatly improved by repeat gel filtration on Bio-Gel P-10. The elution volumes of the re-run fractions (A–C) were 80, 87·5 and 92·5 ml and sufficiently different to support the view that the original fraction was polydisperse. Polyacrylamide gel electrophoresis at pH 8·3 showed that fraction B, the main fraction, was essentially homogeneous. Fraction C contained two components, one with low mobility and one identical to B. Four components were present in fraction A. The Zn:Cu ratios in fractions A–C were > 100, > 100 and 4·8 respectively. All fractions had a high sulphydryl content and a molar ratio of sulphydryl groups: metal of 2·8–3·0. In fraction B the Zn content of the protein was 2·1 %, the protein being measured by the microbiuret method (Itzhaki & Gill, 1964). However, as appropriate standards were not available for protein estimation, this figure must be considered an approximate value only.

The amino acid composition of fraction B is shown in Table 1. Cysteine accounted

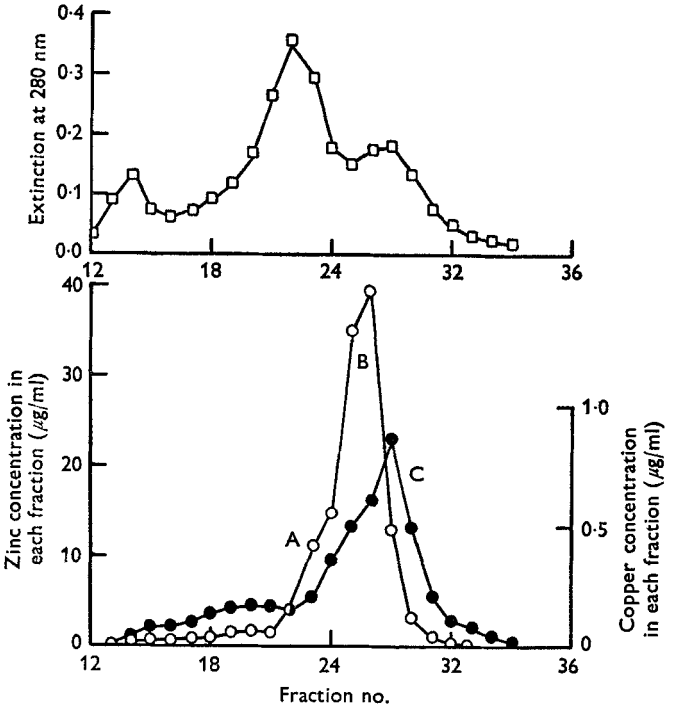

Fig. 4. Fractionation on Bio-Gel P-10 of fraction 3, obtained from calf liver by gel filtration of Sephadex G-75 (see p. 293). Extinction at 280 nm (□), concentrations of zinc (○) and copper (●) and position of subfractions A–C are shown.

Table 1. *Amino acid composition of fraction B* from calf liver and of horse hepatic metallothionein*

Amino acid	Residues†	
	Fraction B	Horse metallothionein‡
Lysine	96	104
Histidine	2	—
Arginine	18	28
Aspartic acid	60	50
Threonine	39	41
Serine	130	115
Glutamic acid	33	40
Proline	62	54
Glycine	101	96
Alanine	91	89
Cysteine	292	332
Valine	27	24
Methionine	20	16
Isoleucine	9	9
Leucine	9	4
Phenylalanine	4	—

* See p. 296.

† Values are expressed per 1000 residues in the molecule. Cysteine was determined as cysteic acid. Fraction B was isolated by gel filtration on Bio-Gel P-10, oxidized with performic acid and hydrolysed for 24 h in 6 M-HCl.

‡ Kägi (1970).

for 29% of the total residues and, with methionine, accounted for all the estimated sulphydryl groups. Only trace amounts of tyrosine and phenylalanine were present, probably derived from the small proportion of contaminant protein. The composition of horse-liver metallothionein is shown for comparison (Kägi, 1970).

DISCUSSION

It is clear that fraction 3 is similar, if not identical, to metallothionein, the Cd-binding protein which may also contain Zn, Cu and mercury, isolated from liver and kidney (Kägi & Vallee, 1960, 1961; Kägi, 1970). The amino acid composition of the principal Zn-'metallothionein' fraction isolated from calf liver is very similar to that of the hepatic Cd–Zn proteins from other species (Table 1) (Kägi, 1970; Nordberg, Nordberg, Piscator & Vesterberg, 1972; Winge & Rajagopalan, 1972). The slightly lower cysteine content may partially arise from the small proportion of contaminant protein present. The similarity of the proteins is supported by the following evidence: (*a*) molecular weight about 12000, compared with about 10000 for the gel-filtration estimate of the Cd protein, (*b*) virtual absence of extinction at 280 nm, consistent with the absence of aromatic amino acid residues, (*c*) increase in extinction at 250 nm on replacement of Zn and Cu by Cd, which is consistent with the formation of the Cd mercaptide chromophore (Kägi & Vallee, 1961), (*d*) high content of sulphydryl groups, (*e*) molar ratio, cysteine residues: metal, approximately 3, (*f*) high metal content of the protein, around 2%, although this is less than some quoted values for the Cd-protein (Kägi & Vallee, 1961). The probable existence of at least three different 'metallo-proteins' in fraction 3 with the same general properties is also consistent with the known heterogeneity of metallothionein (Kägi & Vallee, 1961).

The question of whether the metal-free thionein is normally present in liver can only be satisfactorily resolved by development of a specific assay for it. However, as spectral studies on metallothionein (Pulido, Kägi & Vallee, 1966) suggest that thionein and Cd can recombine to form the native protein, it seems reasonable to suggest that the addition of Cd to a liver homogenate should produce an increase in the metal in the 'metallothionein'-fraction if the apoprotein is present. The failure to detect any such increase in these studies suggests therefore that the apoprotein is absent from the livers. This observation, taken with the absence of the metal-binding fraction in the livers of Zn-deficient animals and the existence of such a close relationship between the concentrations of liver Zn and those of (Cu + Zn) in fraction 3, strongly suggests that Zn must either be involved in the synthesis of the protein or retard its degradation. The former role is supported by recent studies on rat liver which indicate that *de novo* synthesis of the equivalent metal-binding fraction is induced by Zn and inhibited by cycloheximide (Webb, 1972; Davies, Bremner & Mills, 1973). The occurrence of this Zn-binding fraction in male rat liver only after Zn injection or restriction of food intake has also been related to an increase in liver Zn concentration above an apparently critical level (Bremner, Davies & Mills, 1973). This suggests that production of this protein may constitute a temporary means of eliminating potentially toxic concentrations of Zn^{2+}.

As Cu has a greater affinity than Zn for the available binding sites, it is probable that it can compete with and displace Zn from the protein, even after Zn has induced its synthesis. Although the absence of any effect of hepatic Cu on the total metal content of fraction 3 is consistent with a lack of involvement of Cu in the synthesis of the protein in ruminant liver, preliminary results from rats suggest that Cu injection may in some circumstances induce synthesis of a protein of similar molecular weight (Bremner & Davies, 1974). In previous reports on this Cu protein (Evans, Majors & Cornatzer, 1970), it was suggested that it may have a transport function for Cu, but no direct evidence has been produced to substantiate this. It has been postulated that apparently analogous proteins act as detoxifying agents in the metabolism of Zn (Webb, 1972), Cd (Nordberg *et al.* 1972) and Hg (Wisniewska, Trojanowska, Piotrowski & Jakubowski, 1970) and this may also be the position with respect to Cu. Alternatively, the protein may act as a non-essential store for Cu and Zn. However, until the subsequent fate of Cu in this form has been established, this must remain unresolved.

The relationship between the concentrations of Cu and Zn and their distribution among liver proteins may be important in understanding the mutual antagonism between Cu and Zn in animals. A high dietary Zn intake can cause a conditioned Cu deficiency, which may be manifested by increased mortality, growth failure, anaemia (Hill & Matrone, 1970) and as a reduction in the activity of cytochrome oxidase (Van Reen, 1953). Conversely a Zn-deficiency syndrome can result from the use of diets with high Cu contents (Suttle & Mills, 1966). Although the present results do not provide an unequivocal explanation for the interactions between these metals they do demonstrate how changes in hepatic concentration of one can affect the distribution of the other among the hepatic metalloproteins. Such changes might affect excretion or utilization of the metals or (if comparable effects exist in intestine) their absorption. Furthermore, as the thionein-like protein is obviously of key importance in controlling the distribution of Cu and Zn among hepatic proteins, liver Zn concentrations are seen to have an importance not previously recognized. It will be of interest to establish whether the distribution of Cu and Zn proteins in other tissues is in fact similarly controlled.

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