Hepatic copper- and zinc-binding proteins in ruminants

1. Distribution of Cu and Zn among soluble proteins of livers of varying Cu and Zn content

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1. A study has been made by gel-filtration techniques of the soluble copper- and zinc-binding proteins in livers from calves and sheep of widely differing Cu and Zn status.

2. Cu and Zn generally occurred together in three main fractions, with approximate molecular weights of > 75000, 35000 and 12000, and Zn also in one other fraction with molecular weight about 65000. The distribution of the metals between these fractions was variable and dependent on both the Cu and Zn status of the animals.

3. Zn was usually absent from the low-molecular-weight fraction in Zn-deficient or high-Cu livers, with Cu also being absent in the former instance.

4. The fraction with molecular weight of 35000 was tentatively identified as hepatocuprein. It generally accounted for only 4% of the total hepatic Cu except in Cu-deficient livers.

5. The possible relationship of these findings to the mutual antagonism between Cu and Zn is discussed.

A major problem in the study of the metabolism of trace elements is still the evaluation of the status of individual animals. Determination of tissue concentrations is not generally satisfactory. Poole (1970) has reported, for example, low liver copper concentrations in clinically normal cattle and regional variations have been shown to occur in what was considered 'normal' Cu concentrations (Hill, Thambyah, Wan & Shanta, 1962). Liver zinc concentrations are not reduced in Zn-deficient rats (Becker & Hoekstra, 1968) although they are significantly related to dietary Zn intake in lambs (Ott, Smith, Stob, Parker, Harrington & Beeson, 1965). Furthermore, conditioned deficiency states which are actually associated with increased tissue concentrations of the 'deficient' element can occur. Thus, liver Zn concentrations are increased in the Cu-induced Zn deficiency of swine (Suttle & Mills, 1966), as are Cu concentrations in rats with molybdenum-induced Cu deficiency (Brinkman, Miller & Engel, 1961). It may be, therefore, that the form in which the elements occur in tissues governs the availability of the metal for various metabolic functions.

The occurrence of a mutual antagonism between Cu and Zn has been ascribed to the isomorphous replacement of the elements in essential metalloproteins (Hill & Matrone, 1970). Evidence has been produced suggesting that Zn (and cadmium) may displace Cu from a Cu-binding protein with a molecular weight of about 10000, isolated from both duodenum and liver of cattle (Evans, Majors & Cornatzer, 1970). This protein may be similar to metallothionein, a Zn- and Cd-binding protein isolated from kidneys and liver of several species (Kägi & Vallee, 1960, 1961; Kägi, 1970). As a result of its high cysteinyl content it has an extremely high affinity for metals and may have an important role to play in the transport and storage of many trace elements (Mills, 1974).

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Little is known of the relative importance of this and of other hepatic Cu- and Zn-binding proteins, such as cytocuprein (Carrico & Deutsch, 1969), in animals of differing Cu and Zn status. The present study is concerned, therefore, with the distribution of Cu and Zn among hepatic proteins in ruminants, with particular emphasis on any changes that might be characteristic of specific deficiency or toxicity states. Evidence has been obtained confirming that a close relationship exists between the protein-binding of Cu and Zn in these livers.

EXPERIMENTAL

Materials. Livers, which were collected immediately after slaughter and stored at −20°, were obtained from animals fed on diets containing varying amounts of Cu and Zn as part of other experiments. Calf livers were obtained from veal calves, including those described in a previous paper (Bremner & Dalgarno, 1973). They are classified in this paper as 'low–normal-Cu' (ten livers) and 'high-Cu' (ten livers), depending on whether the dietary Cu concentration was 0·5 or 5·5 mg/kg dry matter. The dietary iron intake of the calves ranged from 10 to 100 mg/kg dry matter. Sheep livers were obtained mainly from animals fed on a semi-synthetic ration (Mills, Dalgarno, Williams & Quarterman, 1967) with a Zn content of 40 mg/kg ('normal-Zn', five samples) and < 1 mg/kg ('Zn-deficient', three samples). The following materials were used: Sephadex G-75 and blue dextran (Pharmacia Ltd, Uppsala, Sweden), Chelex-100 (Bio-rad Laboratories, Richmond, California). All reagents were analar grade and buffers were purified by passage through Chelex-100 columns.

Analytical methods. Cu and Zn were determined by atomic absorption spectroscopy, either direct using aqueous solutions or after wet ashing of the sample with conc. HNO_3–conc. $HClO_4$–conc. H_2SO_4 (4:2:1, by vol.). All concentrations in liver refer to the fresh weight of the tissue. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin (Fraction V, Sigma Chemical Co.) as standard, and superoxide dismutase activity was determined by the method of McCord & Fridovich (1969).

Fractionation of metalloproteins. Samples (20 g) of each liver were homogenized at 1° in 2·5 vol. (v/w) 0·01 M-Tris–acetate buffer (pH 7·4 or 8·2) using an Ultraturrax homogenizer (Janke and Kunkel KG, Staufen, Germany) and centrifuged for 1 h at 105000*g*. The supernatant fractions were collected and stored at −20°. A portion (3·5 ml) of each fraction was separated on a column of Sephadex G-75 (900 × 16 mm) using 0·01 M-Tris–acetate buffer (pH 7·4 or 8·2), containing 0·1 g/l NaN_3, as eluant. The flow rate of buffer was about 25 ml/h and 5 ml fractions were collected.

RESULTS

Concentration and solubility of Cu and Zn in homogenates

Concentrations of Cu in the livers were typical of those found in Cu-deficient, normal and 'high-Cu' states and Zn concentrations of deficient and normal states (Table 1). Although the Fe intake of the calves was not constant, there were no

Table 1. *Distribution of copper and zinc among fractions isolated by gel filtration on Sephadex G-75 of supernatant fraction of liver homogenate of calves and sheep*

(Mean values with their standard errors, based on a single fractionation of each liver)

Liver	No. of livers	Metal	Concentration (μg/g fresh liver) Mean	SE	Solubility* (%) Mean	SE	Proportion of soluble metal in fraction (%) 1 Mean	SE	2 Mean	SE	3 Mean	SE	Concentration of metal in fraction (μg/g fresh liver) 1 Mean	SE	2 Mean	SE	3 Mean	SE
Calf (low–normal-Cu)	10	Cu	12·8	2·3	40·9	13·6	13·3	1·0	34·3	2·7	51·5	3·3	0·57	0·8	1·46	0·16	24·3	0·51
		Zn	57·1	9·2	59·1	2·6	34·5	3·8	16·2	1·8	48·1	5·2	10·1	0·7	5·0	0·6	20·1	6·3
Calf (high-Cu)	10	Cu	129·0	9·8	30·1	1·5	39·9	5·7	14·8	0·9	46·0	5·7	15·0	2·5	5·6	0·8	17·4	3·0
		Zn	47·2	4·6	41·2	1·6	64·7	2·2	25·9	2·3	4·3	1·9	12·2	1·3	4·7	0·4	1·2	0·7
Sheep (normal-Zn)	5	Cu	69·6	4·8	29·6	0·9	32·0	10·0	14·8	1·4	53·6	10·5	7·0	2·7	3·0	0·2	10·7	2·0
		Zn	51·8	16·9	67·6	3·9	62·2	11·8	13·6	2·2	24·4	13·7	16·1	0·8	3·9	0·6	16·3	13·8
Sheep (Zn-deficient)	3	Cu	64·0	10·4	28·0	2·1	74·3	1·3	13·7	1·9	12·3	0·7	13·4	2·9	2·3	0·2	2·2	0·5
		Zn	19·0	0·1	54·0	1·5	77·0	1·5	21·7	0·3	1·0	1·0	7·9	0·3	2·3	0·1	0·1	0·1

* Solubility is defined as the percentage of the total hepatic metal recovered in the supernatant fraction of a 0·01 M-Tris–acetate (pH 8·2) homogenate after centrifugation at 105000 *g* for 1 h.

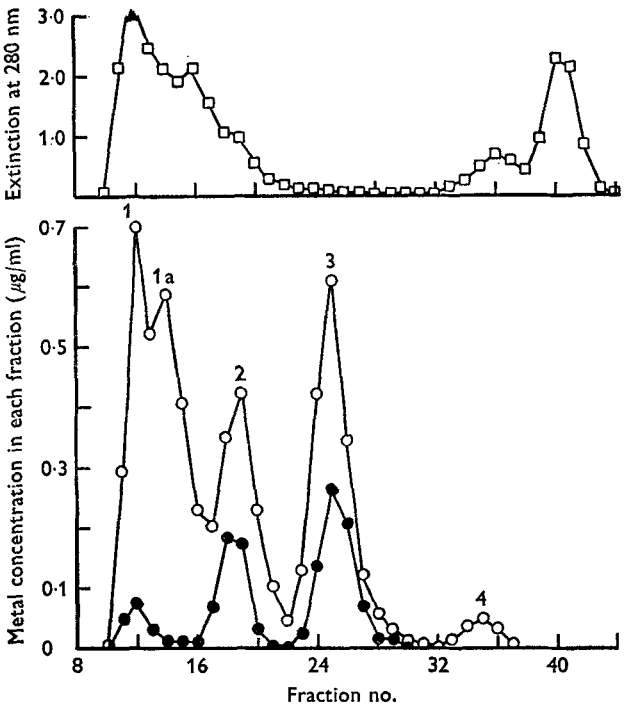

Fig. 1. Fractionation on Sephadex G-75 of supernatant fraction from calf liver with copper and zinc concentrations of 13 and 35 μg/g respectively. Concentrations of Cu (●), Zn (○), extinction at 280 nm and the positions of fractions 1–4 are shown; 5 ml fractions were collected.

indications that this influenced Cu and Zn distribution and results are quoted on the basis of Cu and Zn status only. The solubility of Zn in the liver (i.e. the proportion of the total Zn which was recovered in the supernatant fraction after centrifugation at 105000*g*, with no correction for 'soluble' Zn trapped in the pellet) increased within each group with liver Zn concentration and was usually in the range 40–75 %. It tended to be lower in livers of high Cu concentration. The solubility of Cu was usually around 30 % in livers with Cu concentrations of 40–200 μg/g, but at lower Cu concentrations it increased progressively to about 75 % in severely Cu-deficient livers.

Gel filtration on Sephadex G-75

A typical separation on Sephadex G-75 of the supernatant from a calf liver, with Cu and Zn concentrations of 13 and 35 μg/g respectively, is shown in Fig. 1. Three Cu fractions were obtained, the minor one (1) being eluted with the void volume. Zn was apparently associated with all Cu-containing fractions and in addition a fraction (1a) containing Zn but no Cu was eluted just after the void volume. A minor Zn component (4), of greater elution volume and accounting for < 5 % of the total Zn was occasionally present. Only small amounts of protein, as determined by extinction measurements at 280 nm, were apparently associated with fraction 3 and no Cu or

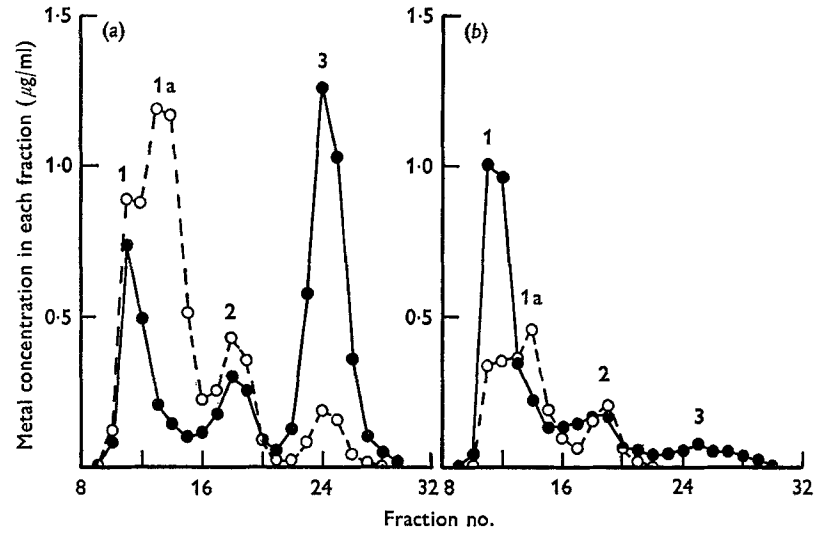

Fig. 2. Fractionation on Sephadex G-75 of supernatant fractions from sheep liver. (*a*) Normal sheep, with liver copper (●) and zinc (○) concentrations of 72 and 40 μg/g respectively. (*b*) Zn-deficient sheep with liver Cu and Zn concentrations of 69 and 19 μg/g respectively. Five ml fractions were collected.

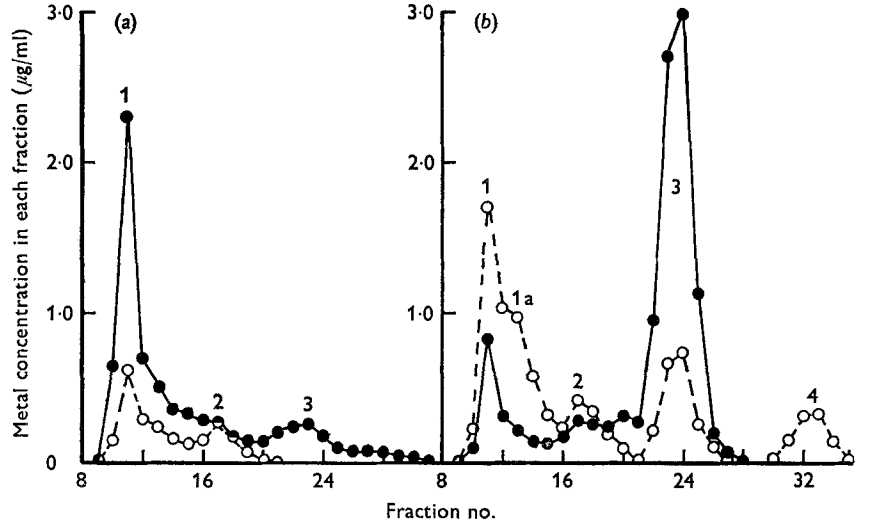

Fig. 3. Fractionation on Sephadex G-75 of supernatants from high-copper calf livers with (*a*) Cu (●) and zinc (○) concentrations of 132 and 31 μg/g respectively and (*b*) Cu and Zn concentrations of 157 and 81 μg/g respectively. Five ml fractions were collected.

Zn were present in the final fractions eluted from the column. Superoxide dismutase activity was associated with fraction 2.

The Sephadex G-75 column was calibrated with the following proteins of known molecular weight, bovine serum albumin (68000), lactoglobulin (36800), chymotrypsin (25700), myoglobin (17200) and cytochrome *c* (12400), as described by Andrews

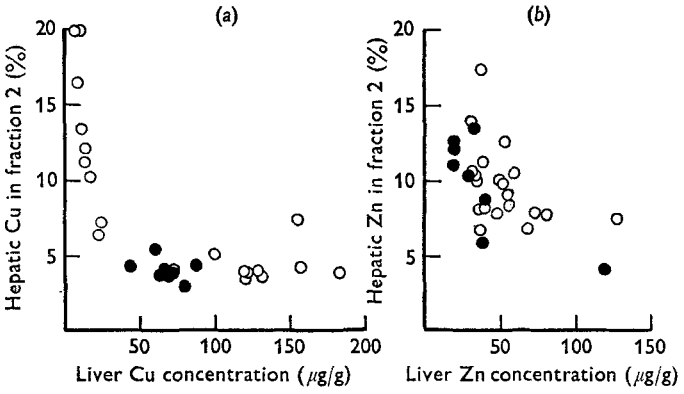

Fig. 4. Relationship between the proportions of (*a*) copper and (*b*) zinc in fraction 2 and the total hepatic concentration of each metal. Results are shown for lambs (●) and calves (○). Fraction 2 was isolated by gel filtration on Sephadex G-75.

(1965). Blue dextran was used to determine the void volume of the column. From their elution volumes fractions 1a, 2 and 3 were found to have molecular weight of around 65000, 35000 and 12000 respectively.

The amounts of Cu and Zn present in each fraction were calculated (fraction 1a included with 1) for all livers and the results expressed both as percentage distribution of the soluble metal among the fractions and as the amount of metal in that fraction/g liver (Table 1). Fraction 3 usually contained the greatest proportion of both Cu and Zn in the normal calf ($P < 0{\cdot}05$) and fractions 1 and 2 the smallest proportion of Cu and Zn respectively ($P < 0{\cdot}01$). There were large variations between individual livers, although the same fractions were present in all samples examined.

Similar variations were found in sheep liver, but there tended to be less Zn in fraction 3 than in 1 (Fig. 2*a*). In one liver (120 μg Zn/g), however, 60% of the total Zn was in fraction 3. The relative amounts of Zn in fractions 1 and 1a (Fig. 2*a*) were usually different from that in calf liver (Fig. 1).

In livers from Zn-deficient sheep the separation pattern was characterized by the virtual absence of Zn and the large reduction in the amount of Cu in fraction 3 ($P < 0{\cdot}01$) (Fig. 2*b*, Table 1). The concentration of Cu in fraction 1 was correspondingly increased in all samples ($P < 0{\cdot}01$).

In calf livers where the Cu concentrations were abnormally high (around 130 μg/g) no consistent pattern of distribution of Cu and Zn was observed. The resolution of the Cu fractions was not as good as previously and it is probable that additional Cu components were present with elution volumes close to that of fraction 2. In some livers, soluble Cu was concentrated mainly in fraction 1 and Zn was completely absent from fraction 3 (Fig. 3*a*), a pattern similar to that found in livers from Zn-deficient lambs. In other livers, however, most soluble Cu occurred in fraction 3, along with up to 10% of the total hepatic Zn (Fig. 3*b*). The distribution in the remaining livers was between these two extremes, with usually very little Zn in fraction 3. The distribution ratio of Cu between fractions 1 and 3 ranged from 0·2 to 5·0. In all instances

the protein distribution, whether measured by the Folin method (Lowry *et al.* 1951) or by extinction at 280 nm, was similar to that shown in Fig. 1.

Fraction 2 was a minor Cu- and Zn-binding component of most livers. The amount of Cu in this fraction in all livers is given by the expression:

$$Y = 0{\cdot}69 + 0{\cdot}038x \text{ (SE of regression coefficient } 0{\cdot}004),$$

where Y and x are the concentrations of Cu (μg/g fresh liver) in fraction 2 and in the whole liver respectively. The proportion of Cu in this form is therefore about 4–5 % except at hepatic Cu concentrations < 25 μg/g, where it increases gradually to more than 20 % (Fig. 4*a*). The amount of Zn in fraction 2 is given by the expression;

$$Y = 2{\cdot}00 + 0{\cdot}049x \text{ (SE of regression coefficient } 0{\cdot}0072),$$

where Y and x are concentrations of Zn (μg/g fresh liver) in fraction 2 and the whole liver respectively. The proportion of Zn in this form, usually 7–13 %, is therefore also inversely proportional to the liver Zn concentration (Fig. 4*b*). The amount of Cu in fraction 2 was independent of liver Zn concentration and vice versa.

DISCUSSION

The large variations observed in the distribution of Cu and Zn among the soluble hepatic proteins are probably a function of the differing concentrations of the metals in the whole liver (Bremner & Marshall, 1974). No direct relationship was found for either metal between the concentration in the main fractions (1 and 3) and that in the liver. However, significant effects of both deficiency and high intake on Cu and Zn distribution in the liver were established. In Cu deficiency the proportion of Cu occurring in fraction 2 was increased. There was thus a tendency to maintain the concentration of Cu in this fraction as the animal became depleted of Cu. Zn distribution was not significantly affected by the low Cu content of the liver.

The changes in Zn distribution in Zn-deficient livers, with the absence of Zn from fraction 3, are of special interest. Changes in liver Zn concentration have generally been ignored in studies of Zn deficiency, but it seems probable that at least part of the slight reduction usually observed may have been associated with the elimination of this particular metal-binding fraction. It is noteworthy that in male rats only < 2 % of the hepatic Zn occurs as the equivalent fraction 3 (Webb, 1972; Bremner, Davies & Mills, 1973) and that in these animals there is little change in liver Zn concentration in Zn deficiency (Becker & Hoekstra, 1968). The associated transfer of Cu from fraction 3 to 1 in Zn-deficient sheep liver is of uncertain significance as there are no reports of disturbances of Cu metabolism in Zn-deficient animals. An antagonistic effect of Cu on intestinal ^{65}Zn uptake in Zn-supplemented but not in Zn-deficient rats has been found, however (G. W. Evans & C. Hahn, unpublished observation). It is suggested that the difference may be connected with the presence of a Zn-binding fraction of molecular weight 10000 in the intestinal mucosa of the Zn-supplemented rats only.

The distribution of Zn in livers of high Cu content, with the absence in most instances of Zn from fraction 3, is very similar to that found in Zn-deficient liver. This

may be relevant to the occurrence of a conditioned Zn-deficiency in swine fed on high-Cu rations (Suttle & Mills, 1966) and with the alleviation of some symptoms of Cu poisoning in sheep by increase in dietary Zn intake (Mills, 1974). The reduction in the solubility of Zn and its concentration in fraction 3 are probably caused by isomorphous replacement of Zn by Cu in fraction 3 (Bremner & Marshall, 1974). Determination of the significance of the variation in the distribution of Cu between fractions 1 and 3 must await detailed chemical and biological studies on these fractions.

It is probable that fraction 2 corresponds to hepatocuprein (Mann & Keilin, 1939). The molecular weight of fraction 2 and bovine hepatocuprein are about 35000 and 33600 respectively, and fraction 2 showed the superoxide dismutase properties characteristic of erythrocuprein (McCord & Fridovich, 1969) the protein from blood identical to hepatocuprein (Carrico & Deutsch, 1969). Furthermore, the frequent occurrence of Cu and Zn in equal amounts in fraction 2 is consistent with the known metal content of hepatocuprein, namely two atoms of Cu and Zn per molecule (Carrico & Deutsch, 1969). Insufficient material was isolated for the purification and full characterization of this fraction and it is possible that Cu and Zn proteins other than the supposed hepatocuprein are also present in our preparations. In species where liver Cu concentrations are generally low, a greater proportion of the hepatic Cu is generally in this form. In rat liver containing approximately 5 μg Cu/g, 45 % of the Cu is in the equivalent fraction 2 (Bremner *et al.* 1973) and in human liver, with a Cu concentration of 10 μg/g, around 65 % of the Cu probably occurs as hepatocuprein (Porter, 1964). Whether or not the superoxide dismutase activity of this fraction has any physiological significance remains to be determined.

No soluble hepatic Cu proteins with molecular weight > 75000 have been isolated previously. Although fraction 1 is normally a minor Cu component in liver, in some instances, as in livers of high Cu and low Zn concentration, it can account for > 70 % of the soluble Cu and may therefore have some storage function. It is not known whether any of the Zn components within fraction 1 have any essential function in Zn metabolism but the equivalent fraction 1a in rat liver has alcohol dehydrogenase activity (Winge & Rajagopalan, 1972). The amount of Zn in fraction 1 does not vary a great deal with increase or decrease in liver Zn concentration, except when liver Cu concentrations are increased.

Fraction 3, which is discussed in more detail in a following paper (Bremner & Marshall, 1974) is similar to metallothionein (Kägi & Vallee, 1960, 1961), a metal-binding protein of high cysteine content. It probably corresponds also to the Cu protein described by Evans *et al.* (1970) and perhaps to that described by Shapiro, Morell & Scheinberg (1961). Although the precise role of this protein, whether in transport, storage or detoxication, has not been established, it may be closely involved in the mutual antagonism between Cu and Zn. Many of the variations in distribution pattern of hepatic Cu and Zn described here can be explained by the apparent changes in the amount of this protein present in the liver (Bremner & Marshall, 1974).

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