The relationship between the zinc status of pigs and the occurrence of copper- and Zn-binding proteins in liver

BY I. BREMNER

Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

(*Received* 9 *April* 1975 – *Accepted* 8 *September* 1975)

1. A study has been made by gel filtration techniques of the soluble copper- and zinc-binding proteins in livers from pigs of different Zn status.

2. The distribution of both Cu and Zn between the three fractions isolated was greatly influenced by the Zn status of the animal. In livers from pigs given a Zn-supplemented diet the proportion of either Cu or Zn found in the fraction with a molecular weight of about 12000 (fraction 3) was a direct function of the total liver concentration of the metal. In livers from pigs given a low-Zn diet, only small amounts of Cu or Zn were present in this fraction, regardless of liver Cu content.

3. These results suggest that Zn may be essential for the stabilization of the metal-binding protein in this fraction.

The results of previous studies on sheep and calves indicated that the distribution of copper and zinc among soluble liver proteins was dependent on both the Cu and Zn concentrations of the livers. The total amount of these metals in a metallothionein-like protein was, however, dependent only on the liver Zn content and it was suggested that Zn, but not Cu, had a functional role in the synthesis or stabilization of this protein (Bremner & Marshall, 1974*a*, *b*). The ability of Zn to induce de novo synthesis of metallothionein in the liver was confirmed in rats which had been injected with Zn (Bremner, Davies & Mills, 1973; Davies, Bremner & Mills, 1973). It was, however, also found that injection of Cu into rats promoted the appearance of a similar Cu-binding fraction by a process requiring active protein synthesis (Bremner & Davies, 1974).

Although the apparent contrast between ruminants and rats in their response to change in liver Cu concentration may merely reflect variations in the experimental procedures used, it is possible that real species differences exist. As pigs, like rats, are much more tolerant of high Cu intakes than are sheep and calves, the relationship between their Cu and Zn status and the distribution of the metals among soluble, liver proteins was studied. The results indicated marked differences between pig and ruminant livers in their capacity to accumulate Cu in the metallothionein-like fraction

EXPERIMENTAL

Animals

Twelve Large White × (Landrace × Large White) boars, aged 11 weeks and weighing about 28 kg, were allocated at random to two treatment groups, each containing six animals. Pigs in treatment group A (low-Zn) were given the basal ration, which contained 25 mg Zn/kg (Table 1), whereas the diet for treatment group B (Zn-supplemented) contained in addition a supplement of 100 mg Zn (as $ZnSO_4.7H_2O$)

Table 1. *Composition (g/kg) of the basal ration given to pigs*

Ground maize	830 (804·9*)
Soya-bean meal	146·5
Dicalcium phosphate	3·5 (28·6*)
Limestone flour	13·5 (13·6*)
Sodium chloride	3·9
Mineral and vitamin supplement†	2·5

* From week 7 onwards.

† The supplement provided (mg/kg diet): retinol 1·20, cholecalciferol 25, riboflavin 2, pantothenic acid 14, nicotinic acid 10, phylloquinone 5, cyanocobalamin 8·5 μg, iron 50, copper (as $CuSO_4.5H_2O$) 200, manganese 25, iodine 2.

/kg. The phytate content of the diet was 8·9 g phytic acid/kg. The pigs were group-fed in wooden pens; in the statistical treatment of the results it was assumed that any possible 'pen' effects were negligible and that the 'within-pen' results were independent. The pigs were weighed weekly. Blood samples were collected for plasma Zn and Cu determinations by atomic absorption spectrometry after 9 weeks and at slaughter after 12 weeks from the start of the experiment.

Additional livers from pigs of similar weight were also obtained from a local slaughterhouse.

Fractionation of livers

Livers were collected immediately after slaughter, and were kept at $-20°$ when it was not possible to process them immediately. Samples (20 g) were homogenized with 2·5 vol. 0·01 M-Tris–acetate buffer, pH 7·4, and centrifuged; the supernatant fractions were separated on Sephadex G-75 (Pharmacia Ltd, Uppsala, Sweden) and analysed as described previously (Bremner & Marshall, 1974*a*). All concentrations refer to the fresh weight of liver.

RESULTS

Although it was hoped that the high phytate content of the diet would restrict the availability of Zn sufficiently to produce a Zn-deficiency state, no clinical signs of this were found in the pigs given the low-Zn diet, even after the dietary Ca and P contents had been doubled to 13·6 and 10·2 g/kg respectively (Table 1). Growth rates were the same as in the Zn-supplemented animals and no parakeratosis of the skin was detected. However, plasma Zn concentrations were reduced to levels characteristic of considerable Zn depletion (Table 2) and this was reflected in the decreased Zn concentration in the livers (Table 3). Liver Cu concentrations were unaffected by dietary treatment, although slight reductions in plasma Cu concentration were found in the pigs given the low-Zn diet (Table 2).

The proportion of Cu recovered in the liver supernatant fraction was inversely related to the liver Cu concentration in the pigs given a Zn-supplemented diet, and ranged from 55 to 28% as liver Cu concentrations increased from about 20 to 200 μg/g; as given by the equation:

$$Y_1 = 59{\cdot}2 - 0{\cdot}15x_1 \text{ (SE of regression coefficient 0·004)},$$

where Y_1 is the proportion of Cu in the supernatant fraction (%) and x_1 is the liver Cu content (μg/g).

Table 2. *Final body-weight (kg) and plasma concentrations of copper and zinc (µg/ml) in pigs given low-Zn (treatment group A) and Zn-supplemented (treatment group B) diets*†

(Mean values with their standard errors for six pigs/group)

	Treatment group A		Treatment group B		
	Mean	SE	Mean	SE	Statistical significance of difference between mean values
Final body-wt	77·1	3·2	75·8	2·6	NS
Plasma Zn concentration (at 9 weeks)	0·30	0·02	1·08	0·06	***
Plasma Cu concentration (at 9 weeks)	1·82	0·08	2·09	0·05	*
Plasma Zn concentration (at slaughter; 12 weeks)	0·31	0·02	1·00	0·03	***

NS, not significant; * $P < 0·05$; *** $P < 0·001$.
† For details of diets, see Table 1 and p. 245.

A typical separation on Sephadex G-75 of the supernatant fraction from the liver of a pig given the Zn-supplemented diet is shown in Fig. 1 *a*.

Three main fractions were obtained. Fraction 1 was a minor Cu-containing component and, as it was eluted at the void volume of the column, had a molecular weight of ⩾75000. At least two Zn-containing components were present in fraction 1, although the relative proportions of these varied in different livers. A small amount of Cu was associated with the higher-molecular-weight component only. Fraction 2 had a molecular weight of about 35000 as estimated from its elution volume (Andrews, 1965; Bremner & Marshall, 1974*a*). It was a minor fraction, and usually contained similar amounts of Cu and Zn. Most of the Cu and about half the Zn in the supernatant fraction was found in fraction 3, which had an approximate molecular weight of 12000 and had a low extinction value at 280 nm. The quantitative distribution of Cu and Zn between these fractions is given in Table 3.

The separation pattern for the livers of pigs given the low-Zn diet was quite different (Fig. 1 *b*, Table 3), with Zn found only in fractions 1 and 2, in the same concentrations as those found in livers of Zn-supplemented pigs. No Zn, and only small amounts of Cu, were present in fraction 3. As the separation pattern for Cu was more diffuse than in normal livers, distinction between fractions 1 and 2 was not always clear. However, the latter was still a minor Cu-binding component and more than 80% of the soluble Cu was always found in fraction 1.

There was no correlation similar to that found in ruminant livers (Bremner & Marshall, 1974*b*) between the total amount of Cu and Zn in fraction 3 and the liver Zn content. Instead, with one exception, the concentration of Cu in fraction 3 was a function merely of the liver Cu concentration in the pigs given the Zn-supplemented diet (Fig. 2) and could best be expressed by the quadratic equation:

$$Y_2 = 0·53x_2 - 0·0013x_2^2 \text{ (SE of regression coefficients 0·029 and 0·00025 respectively; } P < 0·001\text{),}$$

Table 3. *Distribution of copper and zinc among soluble fractions isolated by gel filtration from homogenates of livers* from pigs given low-Zn (treatment group A) and Zn-supplemented (treatment group B) diets† for 12 weeks, and from pigs obtained from a slaughterhouse*

(Mean values with their standard errors, based on a single fractionation of each liver, with six samples each from treatment groups A and B, and fourteen samples from the 'slaughterhouse' group)

	Cu						Zn					
	Treatment group				'Slaughterhouse' group		Treatment group				'Slaughterhouse' group	
	A		B				A		B			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Liver concentration (μg/g fresh liver)	76·9	18·0	94·6	29·1	64·2	11·3	27·3	1·5	49·5	4·3	75·4	21·8
Proportion of liver metal in supernatant fraction (%)	41·0	2·0	45·2	4·3	34·7	3·3	60·8	2·3	66·5	2·9	72·2	2·3
Proportion (%) of soluble metal in supernatant fraction no.‡: 1	80·3	2·3	9·2	4·6	6·9	1·0	83·8	0·5	48·0	8·0	43·6	12·4
2	13·5	2·6	9·0	2·4	16·1	2·0	15·4	0·5	7·8	1·0	6·5	1·8
3	6·2	0·9	81·8	6·2	77·0	2·4	0·9	0·2	44·2	8·9	50·0	14·2
Concentration (μg/g fresh liver) of metal in supernatant fraction no.‡: 1	26·2	6·8	4·2	2·9	1·3	0·1	13·9	0·9	14·7	0·9	22·2	0·8
2	3·4	2·9	2·9	1·0	2·5	0·6	2·6	0·2	2·5	0·2	3·3	0·1
3	2·6	0·68	29·3	6·1	15·3	2·4	0·2	0·05	16·1	3·7	28·8	4·2

* For details of procedures, see p. 246.
† For details of diets, see Table 1 and p. 245.
‡ Approximate molecular wt of the three fractions: 1, ⩾75000; 2, 35000; 3, 12000.

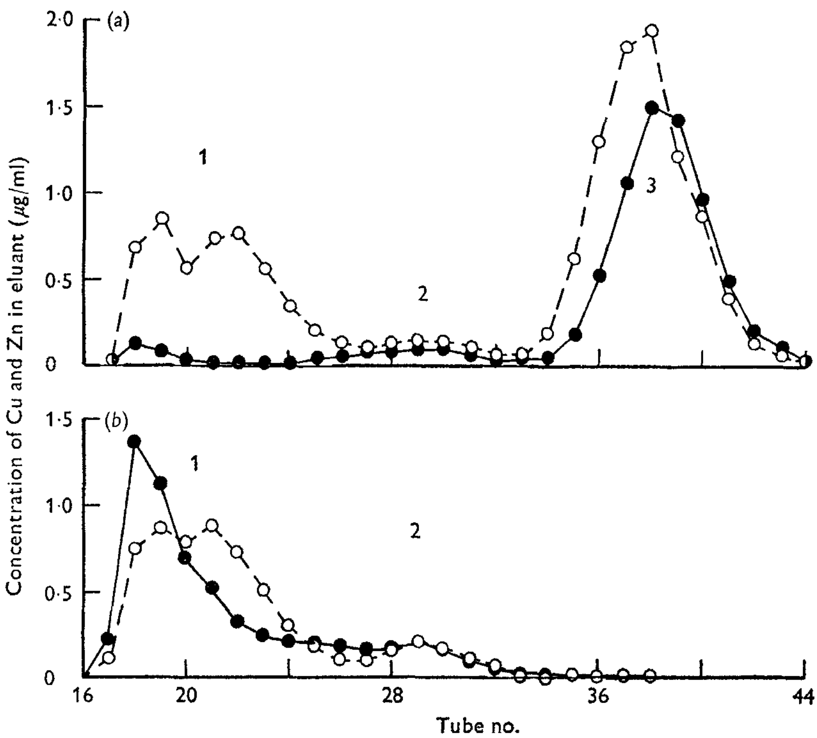

Fig. 1. Fractionation on Sephadex G-75 of supernatant fractions from pig liver homogenate: (*a*) pig given a zinc-supplemented diet with liver copper (●) and Zn (○) concentrations of 34·3 and 51·3 μg/g respectively; (*b*) pig given a low-Zn diet with liver Cu (●) and Zn (○) concentrations of 38·9 and 29·2 μg/g respectively. For details of diets, see Table 1 and p. 245; for details of experimental procedures, see p. 246. Fractions 1, 2 and 3 were contained in tube nos. 17–25, 26–32 and 33–44 respectively; 3·5 ml of eluant was collected in each tube.

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

The concentration of Cu in fraction 3 in the 'exceptional' liver was much lower than that predicted from its liver Cu content of 211 μg/g. However, there was no evidence of a similar 'cut-off' point for production of Cu in this form in the 'slaughterhouse' samples, which covered a much wider range of liver Cu contents. It seems, therefore, that the unusual distribution of Cu in this liver, which was intermediate between that generally found in pigs given the low-Zn and Zn-supplemented diets, may have been a consequence of the relatively low liver Zn concentration (34 μg/g) and marginal Zn status of the pig.

Although the proportion of the liver Cu found in fraction 3 was as predicted from the quadratic equation for some of the 'slaughterhouse' samples analysed, in most instances the amount of Cu isolated in this fraction was only about half that expected. The corresponding regression equation for all these livers was:

$$Y_3 = 0{\cdot}90 + 0{\cdot}224x_3 \text{ (SE of regression coefficient } 0{\cdot}016;\ P < 0{\cdot}001),$$

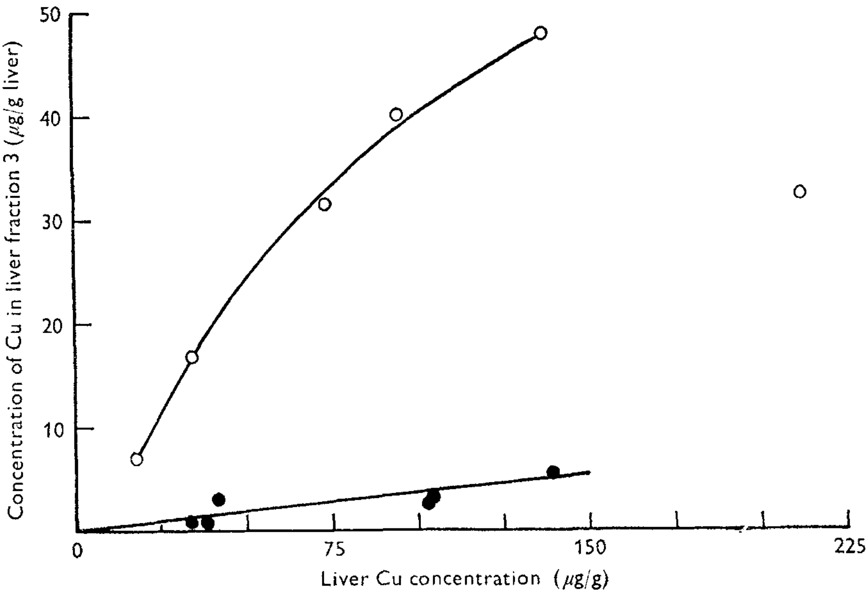

Fig. 2. Relationships between the concentration of copper in liver fraction 3 (for details, see Fig. 1 and p. 247) and that in the whole liver, for pigs given a zinc-supplemented (○) and Zn-deficient (●) diet. For details of diets, see Table 1 and p. 245.

where Y_3 and x_3 are the concentrations of Cu (µg/g fresh liver) in fraction 3 and in the whole liver respectively.

The amount of Zn found in fraction 3 was also proportional to the liver concentration of the metal and could be expressed by the equation:

$$Y_4 = 0{\cdot}76x_4 - 21{\cdot}5 \text{ (SE of regression coefficient 0·20; } P < 0{\cdot}05\text{)},$$

for the pigs given the Zn-supplemented diet; where Y_4 and x_4 are the concentrations of Zn (µg/g fresh liver) in fraction 3 and in the whole liver respectively.

DISCUSSION

The finding that the amount of Cu in fraction 3 of the livers from pigs given a Zn-supplemented diet was a function simply of their Cu content suggests an important distinction between pigs and ruminants in their response to change in liver Cu concentration. In the latter, production of the metallothionein-like protein is related to the liver Zn concentration, although Cu can apparently compete so effectively with Zn for available binding sites that in many instances only the Cu protein is present (Bremner & Marshall, 1974*a*, *b*). A similar relationship was not found in the pig, as at no time was there any evidence of displacement of Zn by Cu from fraction 3. The relationship between the amount of Zn in this form and liver Zn content was similar to that found in rat livers in which synthesis of this protein had been induced by Zn (Davies *et al.* 1973).

Nevertheless, the absence of Cu from fraction 3 in all livers from pigs given the low-Zn diet, regardless of their Cu content, suggests that additional Zn must still be essential for the accumulation of this Cu-binding protein. The absence of growth failure or skin lesions in the pigs implies that they were only marginally Zn deficient, although their plasma Zn concentrations were actually lower than those reported in pigs which were parakeratotic (Lewis, Hoekstra & Grummer, 1957). However, the pigs were still sufficiently Zn deficient to eliminate almost completely the occurrence of the Cu protein and it is interesting that even a slight reduction in liver Zn content in one animal, with no change in plasma levels, partially restricted the accumulation of Cu in this form. The liver distribution of Cu is therefore extremely susceptible to small changes in the Zn status of the pig.

Since the Cu-induced appearance of a similar fraction is unimpaired in Zn-deficient compared with normal rats it seems unlikely that Zn is required to initiate synthesis of the Cu-binding protein (Bremner & Davies, 1974). However, the persistence of Cu in the fraction, several hours after Cu injection, is significantly reduced in the Zn-deficient rats, implying that degradation of the protein may be more rapid in the absence of Zn (I. Bremner & N. T. Davies, unpublished results). This would be consistent with the known stabilizing effect of Zn on various macromolecules (Chvapil, 1973). It is possible, therefore, that in the pig given the low-Zn diet it is the decreased stability of the Zn-free Cu protein which prevents its accumulation in the liver.

The proportion of liver Cu present in fraction 3 was similar to that found in Cu-injected rats (Bloomer & Sourkes, 1973; Bremner & Davies, 1974), confirming that this can represent a major Cu-binding protein. However, at higher liver Cu contents (> 100 μg/g) there was evidence of some limit to the accumulation of Cu in this form, which was in accord with the inverse relationship between Cu solubility and liver Cu content. This is consistent with the progressive saturation of Cu-binding capacity in the cytosol and subcellular organelles which occurs in the livers of rats and sheep (Lal & Sourkes, 1971; Philip, 1973). There is no obvious explanation for the decreased proportion of Cu in fraction 3 in the livers collected from the slaughter-house. Clearly the production of this Cu-binding protein must also be controlled by additional, as yet unknown factors.

No attempt was made to isolate and characterize the Cu and Zn proteins present in fraction 3 and indeed it is not known whether the metals are associated with the same protein. However, as the comparable Zn-binding proteins in calf (Bremner & Marshall, 1974*b*), human (Buhler & Kägi, 1974) and rat liver (Bremner & Davies, 1975) have been unequivocally found to be of the metallothionein type, it is probable that this Zn-binding protein in the pig belongs to the same family. The molecular weight of the protein and the low extinction value at 280 nm are consistent with this. The nature of the Cu protein is more questionable. Liver Cu proteins, with molecular weights of 10000–12000, have been reported in humans (Shapiro, Morell & Scheinberg, 1961), cattle (Evans, Majors & Cornatzer, 1970), sheep and calves (Bremner & Marshall, 1974*b*) and rats (Bloomer & Sourkes, 1973; Bremner & Davies, 1974), but in no instance has the protein been fully characterized. However, the competitive binding between Cu and Zn in fraction 3 of calf liver implied that only one type of protein was present and a high-Zn form was successfully identified as metallothionein (Bremner & Marshall, 1974*b*). The high sulphydryl content of the bovine Cu protein (Evans *et al.* 1970) is also consistent with a metallothionein-like structure and it is significant that the particulate Cu protein of neonatal liver may be a polymeric Cu-rich species of metallothionein (Porter, 1974; Rupp & Weser, 1974).

Final assessment of the importance of these changes in Cu and Zn distribution in pig livers must await the elucidation of the function of these proteins. It has been proposed that the Zn protein is involved in the temporary storage or detoxication of Zn in the rat (Webb, 1972; Bremner *et al.* 1973) and this may apply in the pig also. If the liver Cu protein were essential for Cu to fulfil its biological role, marked differences would be expected in Cu metabolism in Zn-deficient and Zn-sufficient animals. However, only small differences in plasma Cu concentration were noted in the pigs, although increases in the Cu contents of liver and heart of Zn-deficient rats have been reported (Murthy, Klevay & Petering, 1974). It seems more likely that the protein is involved in the storage and perhaps detoxication of Cu, especially as Cu-binding to the protein is often limited in the livers of sheep and calves (Bremner & Marshall, 1974*b*), which are extremely susceptible to Cu poisoning. It might be expected that Zn-deficient pigs may also have a decreased tolerance of Cu: it is significant, therefore, that pigs given high-Cu diets can suffer a conditioned Zn deficiency and that dietary Zn supplementation in these animals decreases the incidence and severity of Cu toxicosis (Suttle & Mills, 1966*a*, *b*).

The author thanks Mr B. W. Young for technical assistance, Dr N. T. Davies for the phytic acid analysis and Mr R. M. J. Crofts for statistical advice.

REFERENCES

Andrews, P. (1965). *Biochem. J.* **96**, 595.
Bloomer, L. C. & Sourkes, T. L. (1973). *Biochem. Med.* **8**, 78.
Bremner, I. & Davies, N. T. (1974). *Biochem. Soc. Trans.* **2**, 425.
Bremner, I. & Davies, N. T. (1975). *Biochem. J.* **149**, 733.
Bremner, I., Davies, N. T. & Mills, C. F. (1973). *Biochem. Soc. Trans.* **1**, 982.
Bremner, I. & Marshall, R. B. (1974*a*). *Br. J. Nutr.* **32**, 283.
Bremner, I. & Marshall, R. B. (1974*b*). *Br. J. Nutr.* **32**, 293.
Buhler, R. H. O. & Kägi, J. H. R. (1974). *FEBS Lett.* **39**, 229.
Chvapil, M. (1973). *Life Sci.* **13**, 1041.
Davies, N. T., Bremner, I. & Mills, C. F. (1973). *Biochem. Soc. Trans.* **1**, 985.
Evans, G. W., Majors, P. F. & Cornatzer, W. E. (1970). *Biochem. biophys. Res. Commun.* **40**, 1142.
Lal, S. & Sourkes, T. L. (1971). *Toxic. appl. Pharmac.* **18**, 562.
Lewis, P. K. Jr, Hoekstra, W. G. & Grummer, R. H. (1957). *J. Anim. Sci.* **16**, 578.
Murthy, L., Klevay, L. M. & Petering, H. G. (1974). *J. Nutr.* **104**, 1458.
Philip, E. (1973). Intracellular distribution of copper in sheep liver and its response to the administration of copper sulphate. MSc Thesis, University of Aberdeen.
Porter, H. (1974). *Biochem. biophys. Res. Commun.* **56**, 661.
Rupp, H. & Weser, U. (1974). *FEBS Lett.* **44**, 293.
Shapiro, J., Morell, A. G. & Scheinberg, I. H. (1961). *J. clin. Invest.* **40**, 1081.
Suttle, N. F. & Mills, C. F. (1966*a*). *Br. J. Nutr.* **20**, 135.
Suttle, N. F. & Mills, C. F. (1966*b*). *Br. J. Nutr.* **20**, 149.
Webb, M. (1972). *Biochem. Pharmac.* **21**, 2751.

Printed in Great Britain