Distribution of copper and zinc in the liver of the developing sheep foetus

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1. A study has been made of the copper- and zinc-binding proteins in the livers from sheep foetuses of 80–142 d gestational age.
2. Metallothionein was found to constitute the major Zn-binding component in the cytosol at all times and to be identical to Zn-thionein from adult sheep liver.
3. Zn also occurred in two fractions, not normally found in sheep liver, with approximate molecular weights of 28000 and 47000. The relative proportions of these were age-dependent.
4. Between 15 and 35 % of the hepatic Cu, corresponding to most of the Cu in the cytosol, also occurred in the metallothionein-containing fraction.

Normal foetal development in many species is dependent on adequate maternal supply of copper and zinc. For example, the occurrence of enzootic ataxia in lambs (Bennetts & Chapman, 1937) and of congenital malformations in the offspring of Zn-deficient rats (Hurley & Swenerton, 1966) are thought to be a consequence of deprivation in utero of Cu and Zn respectively. Little is known, however, of the foetal accumulation of these metals, except that the proportion occurring in the liver can be considerable and may be dependent on gestational age and species (Pryor, 1964; Widdowson, Dauncey & Shaw, 1974; Williams & Bremner, 1976).

In the bovine and human neonate, much of the hepatic Cu occurs in a specific particulate protein, neonatal hepatic mitochondrocuprein, containing about 40 mg Cu/kg (Porter, 1970) which is thought to be a polymeric form of Cu-rich metallothionein (Porter, 1974). This claim is supported by the observations that Cu-thioneins readily form aggregates (Rupp & Weser, 1974; Bremner & Young, 1976), but it has never been shown that metallothionein occurs in foetal tissues.

Previous studies have shown that the synthesis of this protein in rat liver can be induced by Zn and that the amount of protein present is related to the liver Zn content (Bremner & Davies, 1975). In ruminant liver there is competition between Cu and Zn for binding sites on the protein and the hepatic distribution of the metals is therefore dependent on both the Cu and the Zn content of the liver (Bremner & Marshall, 1974*b*).

As the concentrations of hepatic Cu and Zn in foetal lambs change markedly during their development (Williams & Bremner, 1976) and as the utilization of the metals during early growth may be influenced by the forms in which they are stored, a study has been made of Cu and Zn distribution in the livers from foetal lambs of different ages.

EXPERIMENTAL

Methods and materials

Twenty-three Finn–Dorset Horn ewes in their sixth pregnancy, which were part of a long-term study by Dr J. J. Robinson on the intensive breeding of sheep (Robinson & Ørskov, 1975), were given daily, depending on their foetal burden, 1·2–2·4 kg of the diet of Wainman, Blaxter & Pullar (1970), which contained 8 mg Cu and 43 mg Zn/kg. The ewes were

slaughtered at intervals from 80 d of gestation onwards to 142 d, close to the normal completion of gestation at about 147 d; the foetuses were immediately removed and chilled. As soon as possible thereafter the foetal liver was exposed and a portion (about 10 g) of the right central lobe was removed. This was stored at $-20°$ until it could be processed.

The liver used for isolation of maternal ovine metallothionein was obtained 2 d after parturition from a ewe maintained on a diet containing a supplement of 750 mg Zn (added as $ZnSO_4$)/kg (Suttle & Field, 1968).

The analytical procedures used have been described elsewhere (Bremner & Davies, 1975).

Fractionation of livers

Analytical. Samples (about 10 g) of liver were homogenized with 2·5 vols ice-cold 0·01 M-Tris–acetate buffer, pH 7·4, centrifuged at 75000 ***g*** for 1·5 h and the supernatant liquids fractionated at room temperature on Sephadex G75 (Pharmacia Ltd, Uppsala, Sweden) (900 × 16 mm), using the same buffer as eluent; 3·5 ml fractions were collected.

Preparative. All steps were carried out at 1–4°. Portions of livers from five foetuses, taken from ewes at 112 d of gestation, were homogenized and centrifuged as above. The supernatant liquid (150 ml) was fractionated on a column of Sephadex G75 (550 × 50 mm), eluted with 0·01 M-Tris-acetate buffer, pH 7·4. The fraction eluted between 600 and 900 ml of buffer, which contained the crude metallothionein, was then applied to a column (260 × 25 mm) of DEAE-Sephadex A25 (Pharmacia Ltd) previously equilibrated with the same buffer. The column was eluted with this buffer until no more protein or Zn was removed, and then with 1 l of a linear gradient of 0·01–0·1 M-Tris–acetate buffer, pH 7·4. Only one major Zn-containing fraction was eluted, at about 0·03 M buffer. This was concentrated to 10 ml by ultrafiltration and purified by gel filtration on Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, California, USA) (900 × 26 mm), using 0·01 M Tris–acetate buffer, pH 7·4, as eluent. The main portion of the single Zn-protein obtained was desalted by repeated ultrafiltration and used for characterization of the foetal Zn-thionein.

Maternal ovine Zn-thionein was isolated by the same procedure.

RESULTS

There was a large decrease of 71 % in Zn concentrations in the foetal liver between 80 and 106 d of gestation (Table 1). Concentrations decreased more slowly thereafter to about 36 μg/g at 136 d, when they were close to those in the maternal liver (33 ± 4 μg/g fresh liver), but tended to increase again from then to the end of pregnancy at around 142 d.

The distribution of Zn amongst the soluble proteins separated on Sephadex G75 varied markedly during gestation. For example, in all three livers collected at 80 d and in six of the eight collected at 91 d three main Zn-containing fractions (1, 3 and 4) were isolated (see the example in Fig. 1 *a*). Fraction 1 was eluted at the void volume of the column and the others had approximate molecular weights, as estimated from their elution volumes, of 28000 and 12000 respectively (Andrews, 1965; Bremner & Davies, 1975). In contrast to fraction 1, fractions 3 and 4 did not show much extinction at 280 nm.

The amount of Zn in fraction 3 in the livers from the two other 91 d foetuses and in most of those over 105 d was much less than that in the 80 d samples. However, these livers often contained a significant amount of an additional Zn-containing component, fraction 2, with an approximate molecular weight of 47000 (see the example in Fig. 1 *b*). This was eluted with a major protein fraction, as monitored by its extinction at 280 nm, which was also found, however, in the livers of the younger foetuses.

Fraction 4 was purified on DEAE-Sephadex A25 and Bio-Gel P-10 to give one main Zn-containing component, which was homogeneous on electrophoresis on polyacrylamide

Table 1. *Distribution of copper and zinc in a soluble fraction isolated by gel filtration from homogenates of foetal livers collected from* 80 *to* 140 *d of gestation*

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

Period of gestation (d)	No. of samples	Concentration of Cu (μg/g fresh liver)				Concentration of Zn (μg/g fresh liver)			
		Whole liver		Fraction 4		Whole liver		Fraction 4	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
80	3	16·2	1·4	5·74	0·44	195·3	10·2	96·4	12·8
91	8	14·9	1·4	5·16	0·64	125·4	11·8	75·0	9·6
105–6	3	15·5	2·5	4·67	0·68	56·4	2·7	31·7	2·5
112–3	4	27·1	3·2	5·96	1·00	53·4	7·2	27·6	4·4
121–8	3	22·6	3·4	4·61	0·52	45·5	11·7	15·9	4·2
135–6	5	25·5	2·0	3·93	0·15	36·4	4·8	15·6	3·3
140–2	4	65·0	8·7	10·71	0·87	53·1	18·0	22·4	12·7

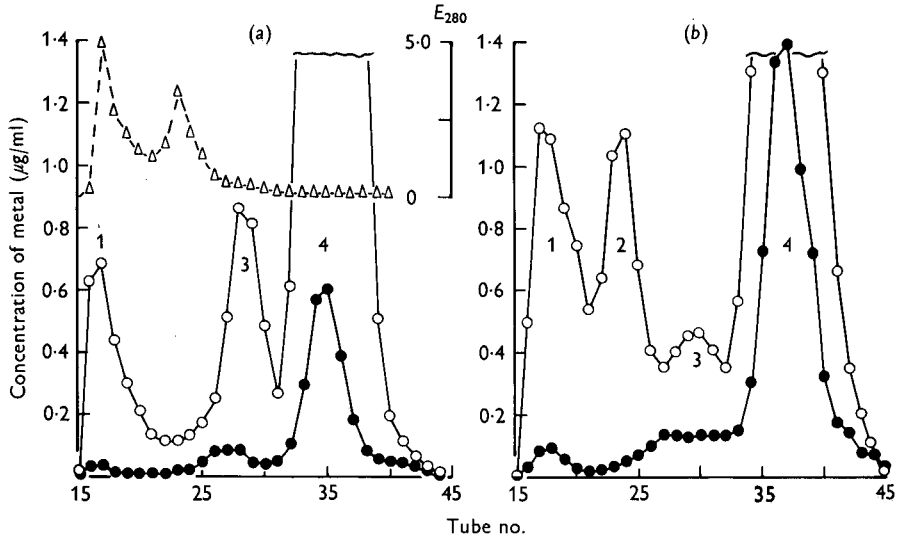

Fig. 1. Separation on Sephadex G75 (Pharmacia Ltd) of supernatant fractions from homogenates of foetal lambs' livers: (*a*) 80 d foetus, with liver zinc (○) and copper (●) concentrations of 17·6 and 16·4 μg/g respectively; extinction at 280 nm (E_{280}) (△) and the positions of fractions 1, 3 and 4 are shown; (*b*) 113 d foetus, with liver Zn (○) and Cu (●) concentrations of 72·7 and 33·9 μg/g fresh weight respectively. Positions of fractions 1–4 are shown. For details of experimental procedures see p. 88.

gels. This had the same mobility relative to a Bromophenol Blue marker, 0·49, as the similar fraction isolated from the liver of a Zn-supplemented ewe. The amino acid composition of these two proteins was virtually identical (Table 2) and typical of Zn-thionein (Bremner & Marshall, 1974*b*) with over 30 % of the amino acid residues accounted for by cysteine and with no aromatic residues present. The Zn content of the foetal protein was 68 mg/g, the protein concentration being calculated from its nitrogen content (× 6·25).

There was a large decrease during gestation in the concentration of metallothionein Zn in fraction 4 (Table 1), but this was always the major form of soluble Zn in the liver. Indeed, metallothionein generally accounted for 40–60 % of the total hepatic Zn. The concentration

Table 2. *Amino acid composition (residues/100 residues) of metallothioneins isolated from livers of adult and foetal sheep*

Amino acid	Adult sheep	Foetal sheep
Lysine	9·8	9·6
Arginine	1·7	1·5
Aspartic acid	5·7	5·9
Threonine	3·1	3·4
Serine	12·7	12·5
Glutamic acid	1·6	1·7
Proline	5·9	6·2
Glycine	9·5	9·9
Alanine	9·7	9·7
Cysteine*	35·4	34·8
Valine	3·0	3·0
Methionine*	1·6	1·7
Leucine	—	—
Isoleucine	0·2	0·2
Phenylananine	—	—

* Cysteine was determined as cysteic acid, and methionine as methionine sulphone.

of Zn in fraction 3 was also related to the liver Zn content, accounting in all samples for about 4 % of the total Zn. On account of its molecular weight of 28000, it was thought that this fraction might have been carbonic anhydrase, as was suggested previously for a minor Zn protein of similar size in rat liver (Bremner & Davies, 1975), but no correlation was found between the concentration of Zn in fraction 3 and the activity of this enzyme.

It was not possible to identify fraction 2 and it did not appear that its concentration was directly related to either liver Zn concentration or gestational age. Two groups of livers seemed to exist, however. In 80 % of the livers from foetuses aged 80–113 d and in 60 % of those at 135–136 d, the mean concentration of Zn in this fraction was $1{\cdot}1 \pm 0{\cdot}1$ (SEM) μg/g fresh liver, equivalent to only $1{\cdot}4 \pm 0{\cdot}2$ % of the total liver Zn. In the remaining thirteen livers, the mean concentration was significantly greater at $5{\cdot}1 \pm 0{\cdot}7$ μg/g liver, equivalent to $9{\cdot}6 \pm 1{\cdot}1$ % of the liver Zn. The mean concentration of Zn in fraction 1, excluded by the column, was $5{\cdot}6 \pm 0{\cdot}3$ μg/g liver and was relatively constant throughout gestation, regardless of the large decrease in liver Zn content in that time.

Despite a fourfold increase in liver Cu concentrations during gestation (Table 1) with most of it occurring in the last few days of pregnancy, the distribution of Cu within the cytosol was relatively constant and over 80 % of the soluble Cu was generally accounted for in fraction 4 (see examples in Fig. 1). Most of the remaining Cu ($0{\cdot}8 \pm 0{\cdot}1$ μg/g liver) was eluted, along with some Zn, between Zn-containing fractions 2 and 3 in a fraction with a molecular weight of about 33000. There was a decrease during gestation in the proportion of the total Cu which was present in the cytosol, from 42·5 % at 80–91 d to 19·6 % at 135–142 d. The concentration of Cu in fraction 4 was therefore relatively constant from 80 to 135 d although it did double to 10·7 μg/g thereafter (Table 1). About 35 % of the total liver Cu was in this fraction up to 106 d but only 15 % from 135 d onwards (Table 1).

DISCUSSION

The distribution of Cu and Zn in the foetal livers differs in several respects from that reported previously for adult sheep (Bremner & Marshall, 1974*a*). The occurrence of Zn in significant amounts in fractions 2 and 3 with molecular weights of about 47000 and 28000 seems to be peculiar to the foetal tissue. It was not possible to identify these proteins but

it is of interest that their relative proportions changed so markedly during development of the foetus. Compared with livers from adult sheep, the mean concentrations of Zn and Cu in fraction 1 of all the foetal livers were small (5·6 and 0·5 μg/g respectively). Thus, only 10 and 1 % of the total hepatic Zn and Cu in the 140 d foetuses were in this form compared with 30 and 10 % respectively in adult sheep with livers of similar Zn and Cu content (51·8 and 69·6 μg/g respectively) (Bremner & Marshall, 1974*a*). The concentrations of Zn, but not of Cu, in the metallothionein fraction were greater by 40 % in the 140 d foetal livers than in those from the same adult sheep. Although the total metallothionein concentration in ruminant liver is related to liver Zn content and independent of Cu concentration (Bremner & Marshall, 1974*b*), it was not possible to determine whether this was also so in the foetal livers.

In previous studies (Bremner & Davies, 1975) it has been suggested that metallothionein is involved in the temporary storage of Zn in the liver, and perhaps other tissues. The present results indicate that this is also the situation in the foetus, as the remarkably high concentrations of Zn in the young foetal liver, similar to those reported in the young human foetus (Widdowson, Chan, Harrison & Milner, 1972), are associated mainly with metallothionein. Indeed it appears that 30–50 % of the Zn in the carcass of the 80–91 d foetal lamb may occur as hepatic Zn-thionein, since 50–90 % of the body Zn in these foetuses is probably in the liver (Williams & Bremner, 1976). The marked decrease in Zn-thionein concentration during gestation is consistent with the mobilization of this Zn reserve in foetal development. It is not clear why liver Zn and Zn-thionein concentrations tended to increase in the last few days of gestation. The apparent, but non-significant increase may merely reflect biological variation or there may possibly be some change in the efficiency of placental Zn transport at this time.

It is interesting that no differences were found in either amino acid composition or electrophoretic behaviour of the Zn-thioneins from the livers of foetal and adult sheep since this supports the claim (Porter, 1974) that neonatal hepatic mitochondrocuprein may be a polymeric species of metallothionein. It was not possible to determine how much of the Cu in the foetal lamb livers was present as mitochondrocuprein, but the decrease in the proportion of Cu found in the cytosol in the older foetuses may be associated with increased conversion of metallothionein into this form.

Although the Zn-protein in fraction 4 was unequivocally identified as metallothionein, insufficient of a Cu-rich preparation was obtained for its characterization. However, as the equivalent fraction in pig liver (Bremner & Young, 1976), and probably in ruminant liver (Bremner & Marshall, 1974*b*) consists of a range of mixed Cu–Zn-thioneins, it seems most likely that Cu is bound in the same way in the foetal livers.

The minor Cu-containing fraction with molecular weight of 33000, eluted between Zn-fractions 2 and 3, was not identified but, by analogy with previous results (Bremner & Marshall, 1974*a*), it seems most likely that it consists mainly of superoxide dismutase (*EC* 1.15.1.1).

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