Metabolism of zinc and copper in the neonate: accumulation and function of (Zn, Cu)-metallothionein in the liver of the newborn rat

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1. Measurements were made of the hepatic concentrations and contents of total and thionein-bound zinc and copper in late foetal and newborn rats and of the distribution of these metals between the particulate and soluble components of the liver.

2. The decrease in the hepatic concentration of thionein-bound Zn, which occurred with age after birth, was proportional to the increase in liver weight until the 16th day post partum; thereafter it was greater.

3. Throughout the period from birth to 25 d of age the Zn concentration remained constant in the cytosolic non-thionein fraction (i.e. total cytoplasmic Zn − thionein-bound Zn), but decreased in other compartments of the liver.

4. The same constant concentration of cytoplasmic non-thionein-bound Zn also was observed in animals with reduced total hepatic Zn contents, but normal body-weights, and in 20-d-old Zn-deficient pups.

5. The concentration of thionein-bound Cu exhibited two maxima, one at 2 d of age and the second at 14 d.

6. The total hepatic content of Cu increased significantly only between the 6th and 14th day post partum. The age-related variations in Cu contents of the particulate components closely paralleled those in the whole liver, whereas the Cu contents of the cytosolic thionein and non-thionein fractions did not increase appreciably until after the 10th day.

7. It is concluded that the cytosolic non-thionein fraction of newborn rat liver may contain particularly important metabolic sites that require Zn and a major function of Zn-thionein is to regulate the supply of the metal to these sites. As, from birth to 26 d of age, the sum of the concentrations of thionein-bound Zn and Cu was correlated with whole liver Zn, the accumulation of Cu as a soluble metallothionein seems to be a secondary event, dependent on the hepatic Zn concentration.

The metallothioneins are inducible metalloproteins that are synthesized, principally in the mammalian liver, kidney and intestinal mucosa, in response to certain bivalent cations (see, e.g. Kagi & Nordberg, 1979). In adult animals, synthesis and degradation of (zinc, copper)-metallothioneins ((Zn, Cu)-thioneins) appear to provide important regulatory mechanisms in the control of the intestinal absorption or hepatic metabolism, or both, of the essential, but potentially toxic Zn and Cu cations (Webb, 1972; Bremner *et al.* 1973; Bremner & Marshall, 1974; Bremner & Davies, 1975; Richards & Cousins, 1975, 1976*a*, *b*; Evans, 1979; Ohtake & Koga, 1979; Webb, 1979*a*).

In the liver of the foetal rat at, or near term, the concentration of Zn is two- or threefold greater than in the liver of the adult and remains at this level for at least the first 2 weeks of post-natal life (Bergel *et al.* 1957). Much of this Zn is present in the hepatic cytosol as a metallothionein, the concentration of which decreases rapidly after birth to reach the barely-detectable, normal adult level at, or near the age of weaning (Kaszpar *et al.* 1976; Ohtake *et al.* 1978; Bell, 1979; Oh & Whanger, 1979; Webb, 1979*a*; Wong & Klaassen, 1979).

The assessment of the concentration of thionein-bound Cu in noenatal tissues is complicated by the susceptibility of Cu-rich metallothioneins to oxidative polymerization (Rupp & Weser, 1974). The insoluble, sulphur-rich Cu protein in the particulate (mitochondrial-lysosomal) fraction of the livers of newborn animals, for example, is considered by Porter (1974) to be a metallothionein polymer which, as the observations of

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Ryden & Deutsch (1978) suggest, may be formed during the fractionation of the tissue in the absence of reducing agents. Nevertheless, it is known that the hepatic Cu concentration is higher in the rat foetus than in the adult, almost doubles during the first week after birth and then falls to the adult level by 6 weeks of age (Terao & Owen, 1977). Although the time course of hepatic Cu uptake and loss thus differs from that of Zn, much of the soluble Cu in the liver cytosol of the newborn rat also is associated with a low molecular weight protein (Terao & Owen, 1977), previously identified by its properties and amino acid composition as a metallothionein (Evans *et al.* 1975). It has been suggested, therefore, that metallothionein, synthesized in the liver of the late foetal and newborn rat, has a metabolic function and provides a mechanism for the storage of reserves of Cu (Terao & Owen, 1977) and Zn (Webb, 1979*a*; Bell, 1979), which are utilized at later stages of development when growth is rapid and the demands for these metals are particularly high. The present work, which examines in detail the time course of the accumulation, retention and elimination of thionein-bound Zn and Cu in the livers of foetal and newborn rats, was done in an attempt to elucidate this metabolic function.

EXPERIMENTAL

Animals. Female rats of the Wistar-Porton strain were given Oxoid 41B diet (Oxoid Limited, Southwark Bridge Road, London, SE1) and tap water *ad lib.* throughout pregnancy and the nursing period. Unless stated otherwise, first or second litters were culled to eight at 2 d post partum. The newborn pups usually were nursed by their mothers until weaned at 21 d. In some experiments, male and female pups were separated from several litters at 2 d of age and fostered in groups of eight of the same sex. Embryos or foetuses were removed from dams in their second pregnancies on the 12th, 16th, 18th, 20th and 21st day of gestation as described by Samarawickrama (1979).

Analytical procedures. Metallic ions were determined by atomic absorption spectrophotometry, samples of whole tissue and of the subcellular fractions being digested and prepared for analysis by the method of Thompson & Blanchflower (1971). Column eluate fractions were analysed without digestion.

Radioactivity was measured in a Searle Mark III Liquid-Scintillation System, Model 6880, programmed to count both ^{3}H and ^{35}S in the same solution.

Characterization of the low-molecular-weight Zn-, Cu-binding protein of the liver soluble fraction

Each of forty 5-d-old rat pups was given a single intraperitoneal injection of 10 μCi L-[^{35}S]cystine hydrochloride (225 mCi/mmol; The Radiochemical Centre, Amersham, Bucks) and 10 μCi of L-[4,5-^{3}H]leucine (55 Ci/mmol; The Radiochemical Centre) in 0·15 M-sodium acetate buffer, pH 5·4 (0·1 ml). The animals were killed 4 h later, the livers being pooled and separated, as described in the following section, into particulate and soluble fractions. The latter was fractionated by gel filtration on Sephadex G75 (for example, see Webb *et al.* 1979) and the low-molecular-weight fraction (V_e/V_0 1·8–2·2), that contained protein-bound Zn and Cu, was purified further by ion-exchange chromatography on a column (50 × 15 mm) of DE-52 cellulose (Whatman Biochemicals Ltd, Springfield Mill, Maidstone, Kent). This column was eluted with a continuous gradient of 0·01–0·20 M-Tris-HCl buffer, pH 8·0, at a flow rate of 80 ml/h. Eluate fractions (3 ml) were analysed for metallic ions, ^{3}H and ^{35}S.

Effect of age on the distribution of Zn and Cu in the liver of the foetal and newborn rat

Foetuses from at least three pregnant females and newborn from at least three litters and which contained both male and female pups were used at each age. Sex-related variations in hepatic Zn and Cu distributions were determined in pups aged 16, 20 and 24 d. The animals

were killed by decapitation and the blood was drained. The livers were excised, freed from excess blood and weighed. Portions (30–100 mg) of the tissue from either the pooled (foetal) or individual (newborn) livers were analysed for Zn and Cu. The remaining tissue samples from the same litter-mates were pooled, frozen in liquid nitrogen and stored at −20°. For fractionation the tissue was thawed, minced and homogenized in 4 vol. 10 mM-Tris-HCl buffer, pH 8·0. The particulate and soluble fractions were separated from the homogenate by centrifugation at 12000 *g* for 10 min and 100000 *g* for 1 h. The final supernatant solution (equivalent to 1–2 g liver) also was fractionated by gel filtration on a Sephadex G75 column (800 × 15 mm) with 10 mM-Tris-HCl buffer, pH 8·0, at a flow rate of 17 ml/h as eluant.

Subcellular distribution of Zn in the liver of the 14- *and* 26-*d-old rat*

Fresh liver tissue was homogenized in 3 vol. 0·25 M sucrose in 5 mM-Tris-HCl buffer, pH 8·0. The homogenate was fractionated by differential centrifugation to yield the nuclear + cell debris fraction (400 *g*, 10 min), the mitochondrial + lysosomal fraction (12000 *g*, 7 min), the microsomal pellet (100000 *g*, 60 min) and the cytosol (post microsomal supernatant solution). The three crude subcellular particulate fractions were resuspended by homogenization in small volumes of the sucrose-Tris solution and portions of these suspensions were analysed for Zn. The cytosol was fractionated on a column of Sephadex G75.

Effect of litter size on the distribution of Zn and Cu in the liver of the 20-*d-old rat*

Litters, that were born on the same day, were culled to four (small), eight (reference) and thirteen (large) pups/litter at 2 d of age and killed on the 20th day post partum. Particulate and soluble fractions were separated from homogenates of the livers as described previously and analysed for Zn and Cu.

Effect of copper treatment on the distribution of Zn and Cu in the livers of 6- *and* 7-*d-old rats*

Two 5-d-old litters were divided randomly, each into two groups of four animals. Pups of one group from each litter were given single intraperitoneal doses of Cu (2 mg/kg as $CuSO_4 . 5H_2O$) in isotonic saline (9 g sodium chloride/l; 0·1 ml/kg body-weight); those of the other groups were injected with saline only. The litters were killed 24 or 48 h after treatment and the livers removed for fractionation and analysis.

RESULTS

Characterization of the low molecular weight Zn, Cu binding protein of the rat liver soluble fraction

The soluble fraction from the liver of the 5-d-old rat contained approximately 42% of the total Zn (100 μg/g wet weight) and 23% of the total Cu (30 μg/g wet weight). On gel filtration of this fraction the greatest proportion of the Zn (75%) was recovered in a low-molecular-weight fraction (V_e/V_0 2·0), which also contained most (70%) of the soluble Cu (Fig. 1*a*). Animals treated with L-[^{35}S]cystine and L-[4,5-^{3}H]leucine incorporated both amino acids into the high-molecular-weight components of the soluble fraction, but only the ^{35}S label into the low-molecular-weight, metal-containing fraction (Fig. 1*b*). As shown in Fig. 1, the major proportion of the ^{35}S and a significant proportion of the ^{3}H in the soluble fraction were present as small molecular species, presumably the free amino acids.

The low-molecular-weight pool (V_e/V_0 1·8–2·2) from the Sephadex G75 column (Fig. 1) was resolved by chromatography on DEAE-cellulose into two metalloprotein fractions, a characteristic property of rat liver metallothionein (for example, see Kagi & Nordberg, 1979), which eluted at 50 mM and 90 mM-Tris-HCl buffer respectively. The 1st fraction (isometallothionein I) contained more Cu and less Zn than the 2nd fraction (Fig. 2*a*; see

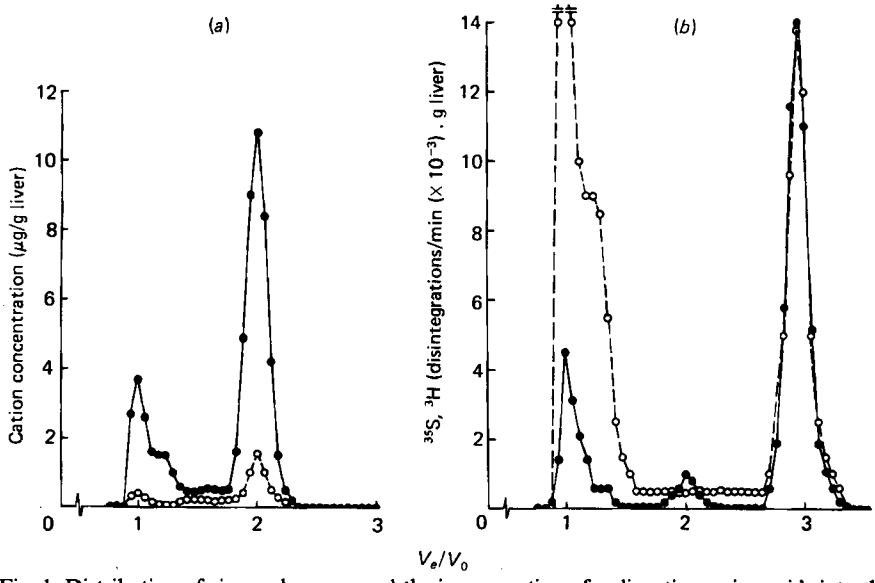

Fig. 1. Distribution of zinc and copper and the incorporation of radioactive amino acids into the liver cytosol of 5-d-old rats. (*a*) Distribution of Zn (●——●) and Cu (○---○); (*b*) distribution of ^{35}S (●——●) and ^{3}H (○---○). Animals were treated with 10 μCi of both L-[^{35}S]cystine hydrochloride and L-[4,5-^{3}H]leucine and killed 4 h later. Liver cytosol was fractionated on Sephadex G75 (800 × 15 mm) with Tris HCl buffer (10 mM, pH 8·0) as eluant.

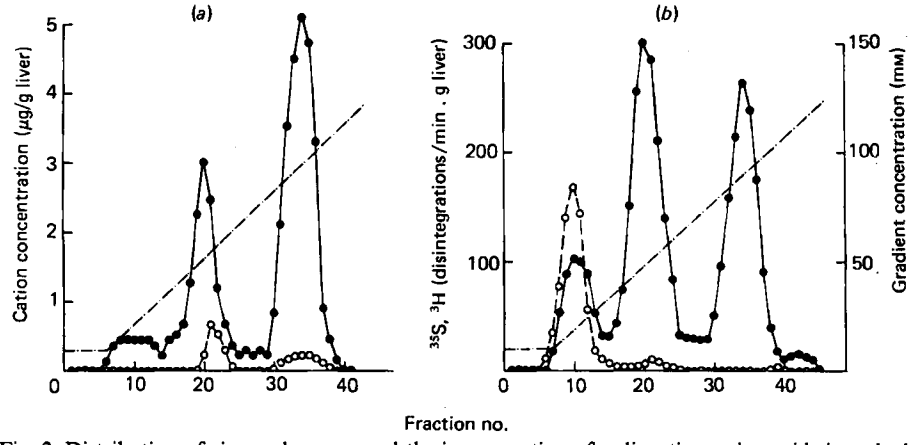

Fig. 2. Distribution of zinc and copper and the incorporation of radioactive amino acids into the low molecular weight fraction (V_e/V_0 1·8–2·2) of the liver cytosol of 5-d-old rats. The Sephadex G75 pool (V_e/V_0 1·8–2·2) (see Fig. 1) was further fractionated by ion exchange chromatography on DEAE cellulose (50 × 15 mm), with a continuous gradient of 0·01–0·20 M-Tris-HCl buffer, pH 8·0. (*a*) (●——●), Zn; (○---○), Cu; (*b*) (●——●), ^{35}S; (○---○) ^{3}H.

also Cain & Holt, 1979). The major peaks of ^{35}S radioactivity were coincident with those of Zn and Cu; these two peaks, however, contained only trace amounts of ^{3}H (Fig. 2*b*). As rat liver metallothioneins contain approximately 28% cysteine residues, but little or no leucine (for example, see Webb, 1979*b*), this double-label-incorporation experiment provides confirmatory evidence that the low-molecular-weight hepatic metalloproteins of the newborn rat are metallothioneins.

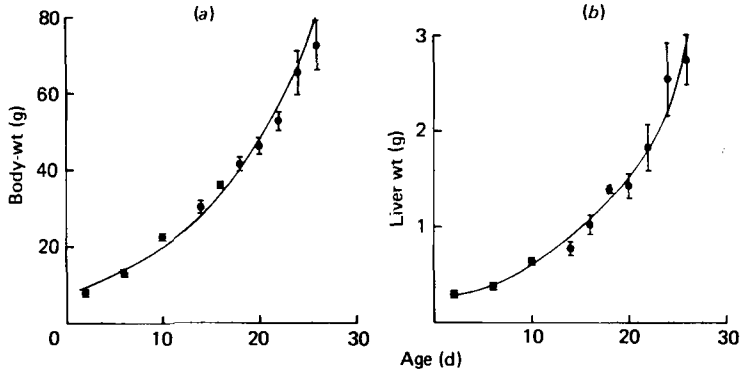

Fig. 3. Relationship between body-weight (*a*) or liver weight (*b*) and age in newborn rats. Points are the mean values with their standard errors represented by vertical bars of at least three litters of eight animals each. The lines are based on the equations log body-wt = 0·0387 × age + 0·896 (*a*) and log liver wt = 0·0412 × age − 0·632 (*b*).

Effect of age on the distribution of Zn and Cu in the liver of the foetal and newborn rat

Zn. As observed previously by Williams *et al.* (1978), the weight of the foetal liver increased rapidly (from 0·05 to 0·36 g) between the 16th and 21st days of gestation and then usually decreased slightly (maximum decrease of 20%) during the first 24 h after birth. Between 2 and 26 d post partum both the average body-weight and liver weight increased as logarithmic functions of age (Fig. 3), the regression equations being:

$$\log \text{body-weight} = (0{\cdot}0387 \pm 0{\cdot}0020) \times \text{age} - (0{\cdot}896 \pm 0{\cdot}034) \quad (r\ 0{\cdot}990;\ n\ 10)$$

$$\log \text{liver weight} = (0{\cdot}0412 \pm 0{\cdot}0015) \times \text{age} - (0{\cdot}632 \pm 0{\cdot}027) \quad (r\ 0{\cdot}995;\ n\ 10)$$

At 12 and 16 d of gestation the concentration of Zn in the foetal liver was less than 25 μg/g wet weight. Thereafter it increased rapidly to 45·1 ± 0·1 μg/g wet weight at 18 d and 91·8 ± 7·6 μg/g wet weight at 21 d (see also Bakka *et al.* 1981). At 2 d after birth the hepatic Zn concentration was above 100 μg/g wet weight tissue but, with increasing liver weight, then declined exponentially until the adult concentration (Bremner *et al.* 1973) was reached between the 20th and 25th days (Fig. 4*a*). Throughout this early period of postnatal growth, the Zn-concentration in the non-thionein fraction of the liver cytosol (Fig. 4*c*), in contrast with that in either the particulate (Fig. 4*b*), or thionein fraction (Fig. 4*d*), remained constant.

The hepatic Zn content, which increased from 1·1 μg at the 16th day of gestation to 33 μg at birth, continued to increase throughout the period from 2 to 26 d post partum (Fig. 5*a*). The thionein-bound Zn content however, remained constant until approximately the 16th day after birth (Fig. 5*d*). Thus the decline in Zn-thionein concentration up to approximately 16 d post partum (Fig. 4*d*) was consistent with the increase in liver weight over this period. After the 16th day, however, the thionein-bound Zn concentration declined more rapidly than the liver weight increased (see Fig. 4*d*).

From the 2nd to the 16th days postpartum there was little change in the appearance of the gel-filtration elution profile of Zn in the liver soluble fraction (Fig. 6). At and between these ages. Zn was present in a high-molecular-weight fraction, that eluted at the void volume and in an incompetely-separated fraction of somewhat lower molecular weight (V_e/V_0 approximately 1·18), as well as in the metallothionein (V_e/V_0 2·0). After 16 d changes occurred in the distribution of Zn. Thus by the 20th day, the Zn-binding fraction (V_e/V_0 1·45), which was first apparent at 16 d, was more clearly defined and, by the 26th day, the shoulder to the high-molecular-weight fraction had increased in concentration and appeared as a

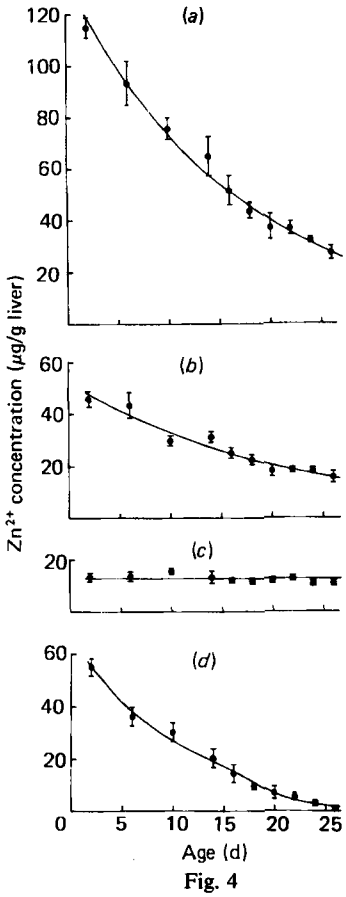

Fig. 4

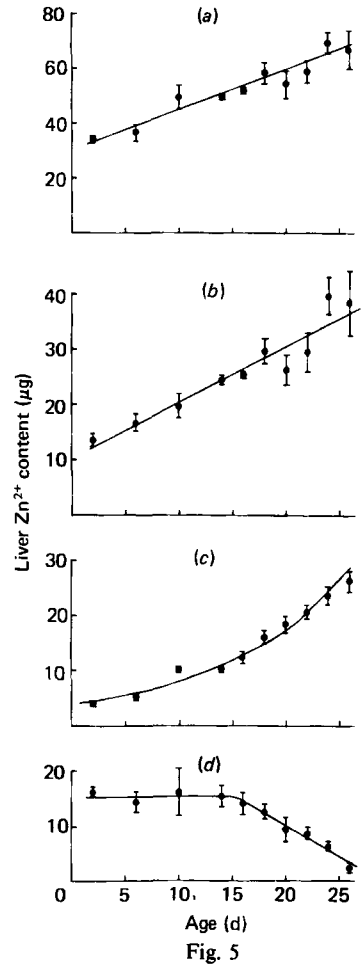

Fig. 5

Fig. 4. Concentration of hepatic zinc (μg/g wet weight liver) as function of age (d) in newborn rats. Litters contained eight pups (male and female) and at least three litters were used at each age. Particulate and cytosolic fractions were prepared by ultracentrifugation of pooled tissue extract from individual litters and the cytosol separated into metallothionein and non-metallothionein components by gel filtration. Points are mean values with their standard errors represented by vertical bars. The lines are based on the equations: (*a*) log total Zn concentration = 2·13 − 0·03 × age (r −0·994); (*b*) log particulate Zn concentration Zn = 1·72–0·02 × age (r −0·976); (*c*) non-thionein Zn concentration (2–26 d) = 12·64 μg Zn/g liver; (*d*) log metallothionein Zn concentration (age 2–16 d) = 1·82 − 0·04 × age (r −0·987), log metallothionein Zn concentration (age 16–26 d), = 2·94 − 0·11 × age (r −0·955).

Fig. 5. Hepatic zinc content as a function of age in newborn rats. Litters contained eight pups (male and female) and at least three litters were used at each age. Particulate and cytosolic fractions were prepared by ultracentrifugation of pooled tissue extract from individual litters and the cytosol separated into metallothionein and non-metallothionein components by gel filtration. Points are mean values with their standard errors represented by vertical bars. The lines are based on the equations: (*a*) total Zn content = 1·41 × age + 30·67 (r 0·960); (*b*) particulate Zn content = 1·03 × age + 10·04 (r 0·951); (*c*) log non-thionein Zn content = 0·034 × age + 0·565 (r 0·988); (*d*) metallothionein Zn content (age 2–16 d) = 15·23 μg Zn, metallothionein Zn content (age 16–26 d) = 32·22 − 1·11 × age (r −0·983).

partially-separated peak (Fig. 6). Bell (1979) also has reported that, as the Zn-thionein peak in the elution profile of the rat hepatic cytosol decreases with age after birth, a Zn-containing peak of higher molecular weight increases in size and, by day 28, has become the major soluble Zn-binding protein of the liver.

Between 14 and 26 d of age the total Zn content of the whole liver increased by 15%,

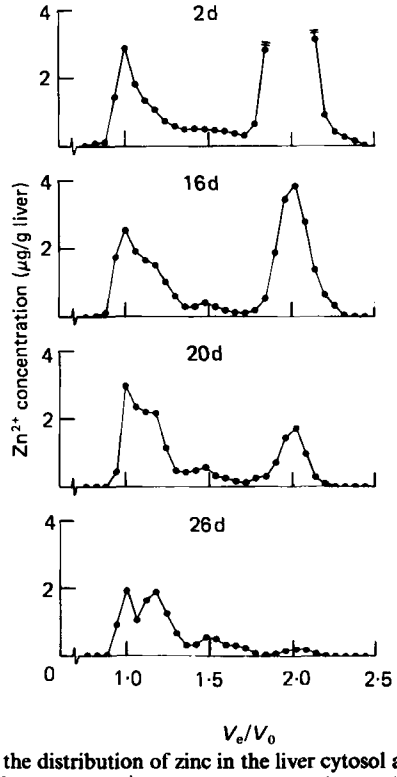

Fig. 6. Relationship between the distribution of zinc in the liver cytosol and age. Cytosol was prepared from pooled liver extract of a single litter by ultracentrifugation and separated on Sephadex G75 (80 × 15 mm) using Tris-HCl (10 mM, pH 8·0) as eluant.

Table 1. *Concentration (a; μg Zn/g. wet weight), content (b; μg Zn) and subcellular distribution of Zn in the liver of the* 14- *and* 20-*d-old rat**

Age (d)	Whole liver		Nuclei + cell debris		Mitochondria-lysosomes		Microsomes		Cytosol: Non-thionein bound		Cytosol: Thionein bound	
	a	b	a	b	a	b	a	b	a	b	a	b
14	61·5	52·5	20·1	17·2	3·1	2·7	5·1	4·4	10·3	8·8	22·9	19·5
26	25·7	60·6	11·1	26·0	1·6	3·8	3·4	7·9	9·0	21·2	0·7	1·6

* Nuclear-cell debris, mitochondrial-lysosomal, microsomal and cytosolic fractions were prepared by differential centrifugation of homogenates of fresh liver, see p. 377.

whereas the contents of the metal in the non-thionein fraction of the cytosol and in the particulate components (nuclei + cell debris, mitochondria + lysosomes and microsomes) increased by 142% and 56% respectively (Table 1). During the same period the content of thionein-bound Zn decreased by 92%. The majority of the additional Zn associated with the particulate components (i.e. 13·6 μg) at 26 d appeared to be located in the nuclei + cell debris (65%) and microsomal (27%) fractions. Despite the appreciable increase in the contents of Zn in these fractions, however, their Zn-concentrations, relative to those at 14 d, were decreased by 45% and 34% respectively.

No significant difference was observed between the total hepatic contents of Zn in male and female rats of the same age, at least between the 16th and 24th days after birth (results not shown). The total concentration tended to be higher in females than in males, but it was not possible to define any difference between either content or concentration in the various cellular compartments.

Cu. The concentration of Cu in the whole liver remained essentially constant (15·5 ± 4·7–16·8 ± 6·5 μg/g wet weight) between the 16th and 21st days of gestation. These values are similar to those reported previously by Terao & Owen (1977) for the hepatic Cu concentration in the rat foetus during the last 5 d *in utero*. After birth the liver Cu increased with age to a maximum of approximately 64 μg/g wet tissue at the 14th day and then declined continuously to approximately 17 μg/g wet tissue on the 26th day (Fig. 7*a*). Similar variations with age were observed in the Cu concentration of both the particulate (Fig. 7*b*) and non-thionein (Fig. 7*c*) fractions. The concentration of thionein-bound Cu which, at 2 d of age, was approximately 9·0 μg/g liver and approximately double that on the 21st day of gestation (4·3 ± 1·6 μg/g liver), however, decreased by approximately 30% between the 2nd and 6th days, then increased, at first slowly and then more rapidly, to a maximum at 14 d and finally fell rapidly to reach negligible levels at 24–26 d of age (Fig. 7*d*).

The total hepatic content of Cu increased significantly only after the 6th day post partum (Fig. 8*a*). The age-related variations in Cu content in the particulate fraction (Fig. 8*b*) closely paralleled those in the whole liver, whereas the contents (and thus the rates of accumulation) of Cu in the non-thionein (Fig. 8*c*) and thionein (Fig. 8*d*) fractions of the cytosol did not increase appreciably until after the 10th day. Also the total content of Cu in the liver appeared to remain essentially constant from 14 to 26 d after birth (Fig. 8*a*), whereas the content of this metal in the metallothionein fraction (Fig. 8*d*) declined, as did the content of Zn (Fig. 5*d*).

In the results of Figs. 7 and 8, the coefficients of variation (SD × 100/mean) associated with many points were large. The extensive scatter of the values was not improved by consideration of male and female litters separately. Nevertheless, it was clear that, although the concentrations of thionein-bound Cu (Fig. 7*d*) were low in relation to those of thionein-bound Zn (Fig. 4*d*) during the early neonatal period, most of the cytosolic Cu, as well as Zn, was present as the metallothionein. Thus, as shown in Fig. 9, only a small amount of Cu was associated with the soluble high-molecular-weight fraction (V_e/V_0 1·0). At 14 d post partum, however, when the cytosolic Cu concentration was maximal (Fig. 7*a*) and the concentration of Cu in the non-thionein components was increased appreciably (Fig. 7*c*), the metal was present in an additional fraction of intermediate molecular weight (V_e/V_0 1·45; Fig. 9) which, as shown previously (Fig. 6), also contained Zn. With the fall in hepatic Cu concentration after 14 d (Fig. 7*a*), an increasing proportion of the soluble Cu was associated with the non-thionein components. Thus, although the concentration in the high-molecular-weight fraction decreased, that in the fraction of intermediate molecular weight (V_e/V_0 1·45) appeared to be less affected and, by 26 d of age, contained the greatest amount of the soluble Cu (Fig. 9).

Effect of litter size on the distribution of Zn and Cu in the liver

As expected from the prevously-mentioned observations and the established correlation between foetal weight and litter size (Webster, 1978), the number of pups in the litter had significant effects on the concentration, content and distribution of Zn in the liver of 20-d-old rats (Table 2). Possibly because of the scatter of the values, even within one litter, and the large SE values, however, no significant difference was observed between the mean total hepatic Cu concentration of pups from small and large litters (t 0·75, df 15, $P > 0·40$) and, in all pups, the liver Cu content increased in proportion to the weight of the organ. Pups

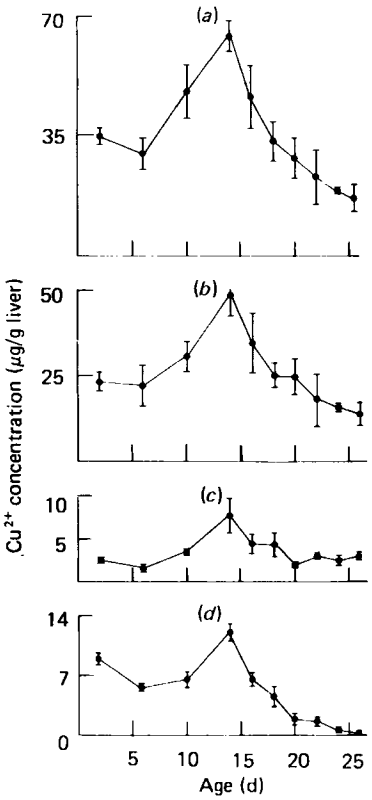

Fig. 7

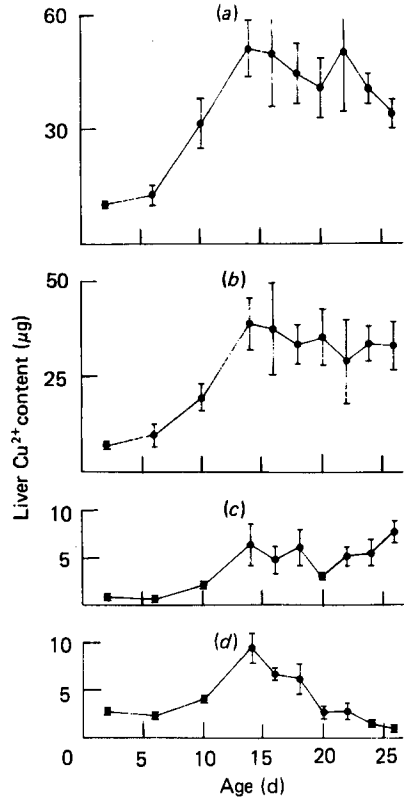

Fig. 8

Fig. 7. Concentration of hepatic copper as a function of age in newborn rats. Litters contained eight pups (male and female) each and at least three litters were used at each age. Particulate and cytosolic fractions were prepared by ultracentrifugation of pooled tissue extract from individual litters and the cytosol separated into metallothionein and non-metallothionein components be gel filtration. (*a*) total; (*b*) particulate; (*c*) non-thionein; (*d*) metallothionein. Points are mean values with their standard errors represented by vertical bars.

Fig. 8. Hepatic copper content as a function of age in newborn rats. Litters contained eight pups (male and female) each and at least three litters were used at each age. Particulate and cytosolic fractions were prepared by ultracentrifugation of pooled tissue extract from individual litters and the cytosol separated into metallothionein and non-metallothionein components by gel filtration. (*a*) Total; (*b*) particulate; (*c*) non-thionein; (*d*) metallothionein. Points are mean values with their standard errors represented by vertical bars. By Student's *t* test, the differences between the mean Cu contents at 2 d ($10{\cdot}23 \pm 0{\cdot}97$ μg Cu/liver, *n* 5) and 6 d ($12{\cdot}73 \pm 2{\cdot}60$ μg Cu/liver, *n* 5) and between 6 d and 10 d ($31{\cdot}44 \pm 6{\cdot}53$ μg Cu/liver, *n* 4) were respectively not significant (*t* 0·90, df 8, $P > 0{\cdot}30$) and significant (*t* 2·66, df 7, $P < 0{\cdot}05$).

in the large litter (*n* 13) were lethargic and their growth was retarded by 4 d (Table 2 cf. Fig. 3). In contrast, animals of the smaller litter (*n* 4) corresponded in body-weight to those of a normal litter (*n* 8) of 24 d of age. The particulate and non-thionein concentrations of Zn in their livers however, were similar to those in animals of the 20-d-old reference litter and were within the 95% confidence limits of the respective concentrations in the control 20-d-old rat (see Fig. 4*b*, *c*). Thus in the pups from the small litter (*n* 4) the hepatic Zn content was disproportionately greater in relation to the observed liver weight (equivalent to that of a 24-d-old animal from a normal (*n* 8) litter). This excess Zn appeared to be located in the particulate fraction, since the cytoplasmic contents of the metal in the livers of pups of the small (*n* 4) and reference (*n* 8) litters (32·8 and 32·1 μg Zn respectively) were the same.

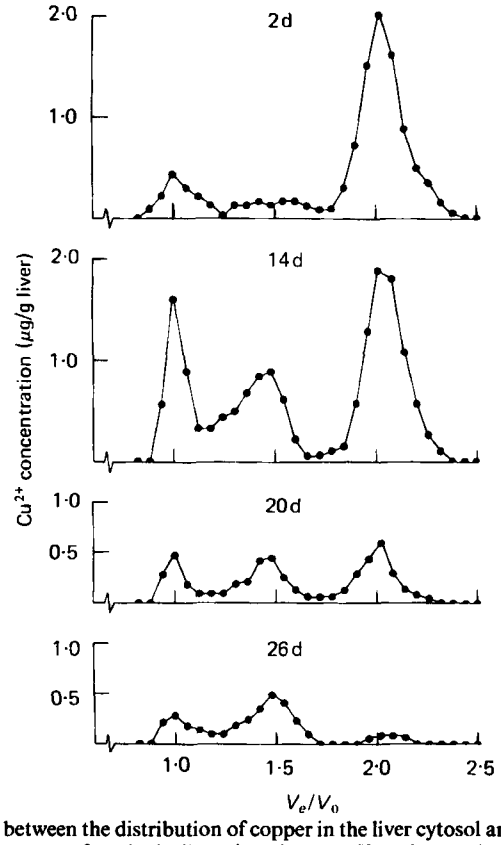

Fig. 9. Relationship between the distribution of copper in the liver cytosol and age. Cytosol was prepared from pooled liver extract of a single litter by ultracentrifugation and separated on Sephadex G75 (800 × 15 mm) using Tris-HCl (10 mM, pH 8·0) as eluant. (●——●), Cu.

Nevertheless, the content of non-thionein-bound Zn in the hepatic cytosol of animals from the small litter was increased to maintain the same Zn concentration in this fraction as in the livers of animals from the reference litter. This increase appeared to be accompanied by a quantitatively similar decrease in Zn-thionein content (Table 2).

In comparison with the reference litter, the hepatic Zn content in pups of the large litter (n 13) was decreased disproportionately in relation to liver weight (Table 2). In these Zn-depleted livers, however, the non-thionein bound Zn was maintained at a concentration similar to, and within the 95% confidence limits (5·10–7·31 μg/g wet weight tissue) for 20-d-old animals. The presence of a significant amount of thionein-bound-Zn (4·4 μg; Table 2) in the livers of these animals suggests that metallothionein does not provide a source of Zn for the particulate fraction, the Zn concentration and content of which appear to reflect the general nutritional depletion.

Effect of Cu-treatment on the distribution of Cu and Zn in the liver of the neonatal rat

The livers of the Cu-treated animals contained more Cu (16·9 μg and 12·15 μg at 24 and 48 h after treatment, respectively) than the controls. This increase in Cu content was significant in the 6-d-old (t 2·72, df 6, $P < 0·05$), but not in the 7-d-old pups (t 0·91, df 6, $P > 0·30$). In both of the treated litters there was no appreciable change in the Cu content of the non-thionein fraction and a greater proportion of the excess Cu was associated with the particulate components than with the cytoplasmic metallothionein (Table 3). As

Table 2. *Effect of litter size on the concentrations (a; μg/g wet weight) and contents (b; μg) of Zn and Cu in the liver of the 20-d-old rat*

(2-d-old litters were culled to four (small), eight (reference) or thirteen (large) pups per litter and were killed on the 20th day post partum (see p. 377). Body-weights, liver weights and hepatic Zn and Cu were determined on all pups and are given as mean values with their standard errors for each litter. Liver subfractions were obtained from the pooled tissue from all pups in each litter)

					Zn											
					Whole liver						Cytosol					
					a		b		Particulate components		Non-thionein bound		Thionein bound			
No. of pups per litter	Body-wt (g)		Liver wt (g)													
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	a	b	a	b	a	b		
4	60·0	2·0	2·180	0·178	34·0	1·1	73·8	5·3	19·0	41·0	12·5	27·2	2·6	5·6		
8	46·0	1·3	1·739	0·057	36·9	1·7	64·0	3·3	18·4	31·9	11·8	20·5	6·7	11·6		
13	33·8	1·3	1·311	0·026	27·0	1·3	35·3	1·4	11·8	15·4	11·8	15·5	3·3	4·4		

					Cu											
					Whole liver						Cytosol					
					a		b		Particulate components		Non-thionein bound		Thionein bound			
No. of pups per litter	Body-wt (g)		Liver wt (g)													
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	a	b	a	b	a	b		
4	60·0	2·0	2·180	0·178	19·1	8·3	38·9	15·2	16·3	32·7	2·0	4·3	0·9	1·9		
8	46·0	1·3	1·739	0·057	18·4	3·5	32·4	7·0	14·5	25·6	2·0	3·6	1·9	3·3		
13	33·8	1·3	1·311	0·026	18·0	4·5	23·2	5·2	15·5	20·0	1·4	1·8	1·1	1·4		

mentioned previously (p. 375), however, this could be due to the formation of insoluble polymers of Cu-rich metallothionein (see e.g. Riordan & Richards, 1980). Nevertheless, as the total hepatic content of soluble thionein-bound metals (Σ μg atom Zn + Cu) was greater in the treated, than in the control rat pups, it seems that the young animal can respond to the influx of Cu into the liver by increased synthesis of cytoplasmic thionein. At 24 h after treatment the total hepatic Cu concentration in the 6-d-old animal (66·17 ± 5·64 μg/g) was similar to that of the normal 14-d-old rat (63·92 ± 4·44 μg/g; Fig. 7*a*). In the latter, however, the concentration of non-thionein-bound Cu in the soluble fraction was greater than at 6 d. Thus the Cu accumulated in the liver of the neonatal rat as a result of normal intake from the maternal milk appears to be distributed differently from that accumulated after a single intraperitoneal injection of $CuSO_4$.

In the 6-d-old rats, treated 24 h previously with Cu, the total hepatic Zn concentrations and contents were increased relative to those of the saline-injected controls, the additional Zn apparently being located mainly in the particulate components (Table 3). The Zn concentration in this fraction, however, was similar to that in animals of a normal litter of the same age and it appeared that, whilst the saline-injected controls of the present experiment had normal liver weights and seemed nutritionally sufficient, they differed from untreated 6-d-old pups in a number of respects. Thus the Zn concentrations in the particulate and metallothionein fractions (26·4 and 22·0 μg Zn/g wet weight liver; Table 3)

Table 3. *Effect of Cu-treatment on the concentrations (a; μg/g wet weight) and contents (b; μg) of Zn and Cu in the livers of 6- and 7-d-old rats*

(Two litters were each divided into two groups of four at 5 d of age. Pups of one group of each litter received single intraperitoneal injections of Cu (2 mg/kg body-weight as $CuSO_4 . 5H_2O$) in isotonic saline (9 g sodium chloride/l). Pups of the other groups (controls) were injected with saline only. Litters were killed 24 h and 48 h after treatment. Body-weights, liver weights and hepatic Zn and Cu were determined on all pups and are given as mean values with their standard errors for each of the four groups. Liver subfractions were obtained from homogenates of the pooled tissue from each group)

						Zn									
						Whole liver						Cytosol			
Age at death		Body-wt (g)		Liver wt (g)		a		b		Particulate components		Non-thionein bound		Thionein bound	
(d)	Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE	a	b	a	b	a	b
6	Saline (controls)	13·36	0·81	0·440	0·021	61·3	2·2	27·0	1·8	26·4	11·7	12·9	5·7	22·0	9·7
6	Cu	12·47	0·75	0·428*	0·046	77·2†	3·3	32·7	2·6	39·0	16·3	13·4	5·7	24·8	10·6
7	Saline (controls)	16·08	0·27	0·457	0·025										
7	Cu	16·19	0·40	0·437	0·016										

						Cu									
						Whole liver						Cytosol			
Age at death		Body-wt (g)		Liver wt (g)		a		b		Particulate components		Non-thionein bound		Thionein bound	
(d)	Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE	a	b	a	b	a	b
6	Saline (controls)	13·36	0·81	0·440	0·021	26·2	6·5	11·9	3·4	17·6	8·1	1·6	0·7	7·1	3·1
6	Cu	12·47	0·75	0·428*	0·046	66·2	5·6	28·8	5·2	43·2	19·0	1·2	0·5	21·8	9·3
7	Saline (controls)	16·08	0·27	0·457	0·025	50·6	12·0	23·3	5·9	39·2	18·1	3·8	1·7	7·7	3·5
7	Cu	16·19	0·40	0·437	0·016	80·3	25·4	35·5	11·9	55·8	24·8	5·4	2·4	19·1	8·3

* Not significantly different from control ($P > 0·05$).
† Significantly different from control ($P < 0·05$).

were low in comparison with the normal levels (Fig. 4) of 40·6 and 38·0 μg Zn/g wet weight liver respectively. Nevertheless the concentration of non-thionein-bound Zn in the soluble fraction of the liver (12·9 μg/g wet weight liver) was similar to that of the normal 6-d-old rat (14·2 μg/g wet weight liver; 95% confidence limits, 12·9–15·4 μg/g wet weight tissue) and thus appeared to be independent of the total hepatic content of Zn. Also, in these saline-treated animals, the thionein-bound Zn seemed to be retained, even though the particulate fraction appeared to be deficient in Zn (Table 3). The reasons for these differences could not be determined.

DISCUSSION

Newborn rats are known to have an extremely high requirement for Zn, particularly throughout the suckling period when the organ and body-weights are increasing rapidly (Sandstead *et al.* 1972; Mutch & Hurley, 1974). During this period, the Zn concentration remains essentially constant in brain, spleen, kidney, lung, heart, muscle (Bakka *et al.* 1981). The decrease in the hepatic content of Zn-thionein in the newborn (Fig. 5*d*), therefore, seems to occur at a time when the demand for Zn must be particularly high. Clearly, the loss of Zn from the hepatic metallothionein fraction between the 16th and the 26th day post partum (i.e. 12 μg; Fig. 5*d*) is insufficient to contribute significantly to the increase in Zn content of other tissues. As, however, a major function of the liver is the storage of metabolites in such physical and chemical forms that can be used by the rest of the body (Doljanski, 1960), the concentration of thionein-bound Zn in the liver of the neonate at any time may reflect the balance between input to and output from a functional pool.

It is possible also that, as the present results (Fig. 4, Table 2) suggest, the hepatic metallothionein provides a major reserve of Zn for the maintenance of a constant concentration of this metal in other cytosolic components of the liver during the accelerated growth period. If, for example, the non-thionein fraction of the cytosol contains particularly important metabolic sites, the intracellular hepatic Zn metabolism may be oriented to the maintenance of optimal Zn concentrations in its constituent metalloproteins. The loss of Zn from metallothionein, which occurs after approximately the 16th day post partum, is concomitant with accelerated liver growth, a decrease in the total hepatic Zn concentration and an approximately equivalent uptake of Zn into the cytosolic non-thionein fraction of the expanding liver.

In contrast, analysis of the Zn depleted livers of 20-d-old pups from large litters (Table 2) and 6-d-old saline-injected animals (Table 3) suggests that the Zn concentration in the particulate components may be regulated by the Zn content of the whole liver and not by the content of Zn-thionein. The observation that all of the additional Zn in the liver of the Cu-treated 6-d-old rat is located in the particulate fraction (Table 3) seems to be in agreement with this hypothesis.

From studies on the livers of newborn rats and adult rats after partial hepatectomy, Ohtake *et al.* (1978) suggest that the concentration of Zn-thionein in the liver is correlated with the regulation of DNA synthesis. The present results (Fig. 4*d*), in apparent agreement with this hypothesis, show that the decrease in hepatic Zn-thionein content occurs at approximately 16–17 d, i.e. when the growth pattern begins to change from increase in cell number to increase in cell size. If, however, Zn-thionein is associated with the regulation of DNA synthesis, high concentrations of this metalloprotein might be expected in most organs of the newborn rat, which also grow by cell proliferation until this age (Enesco & LeBlond, 1962). Evidence for this is lacking. Thus in those organs of the neonate, other than the liver, that have been analysed (e.g. kidney, testes and intestine; Oh & Whanger, 1979; Mason *et al.* 1981; F. O. Brady & M. Webb, unpublished observations), the concentrations of Zn-thionein do not correlate with the rate of DNA synthesis, but are low at birth and increase slowly to reach maximum values after at least 30 d of age.

In vitro both Zn-thionein and Cu-thionein can activate the apoproteins of certain Zn and Cu-dependent metalloenzymes (Udom & Brady, 1980; Geller & Winge, 1980) and thus it is possible that the increase in superoxide-dismutase in newborn rat liver between 5 and 20 d of age (Utsumi *et al.* 1977) might be correlated with the redistribution of Zn and Cu in the hepatic cytosol (for example, see Figs. 5 and 9). Such a function would be in accord with the suggestion of Kojima & Kagi (1978) that metallothionein serves as a reservoir, able to bind and to donate its metal (Zn or Cu) to various metalloproteins. As other organs, in which the pattern of metallothionein accumulation is very different from that in the liver (see p. 387), also acquire adult levels of metalloenzymes at approximately 15 d of age (see e.g. Potter *et al.* 1945; Killewich & Feigelson, 1977), a general role of the metalloprotein as an intracellular metal donor seems unlikely. Whether, however, hepatic metallothionein provides a metal buffer for such syntheses in other tissues remains to be determined. Bell (1979), for example, suggests that metallothionein functions as a general storage site for Zn during perinatal development. Terao & Owen (1977) also consider the possibility that thionein-bound Cu in the liver may provide a reserve of Cu for the critical phase of Cu-dependent myelination in the development of the nervous system in the neonatal rat. The present results, however, suggest that the accumulation of thionein-bound Cu in the hepatic cytosol is a secondary event dependent upon the Zn content of the liver. Thus, combination of the values of Figs. 5 and 9 leads to the equations:

$$Zn_{total} = (1{\cdot}6480 \pm 0{\cdot}0608) \times Zn_{MT} + (0{\cdot}4251 \pm 0{\cdot}0374) \; (r\ 0{\cdot}995)$$

and

$$Zn_{total} = (1{\cdot}4083 \pm 0{\cdot}0681) \times (Zn_{MT} + Cu_{MT}) + (0{\cdot}3830 \pm 0{\cdot}0464) \; (r\ 0{\cdot}991),$$

where the concentrations of total Zn and thionein-bound Zn and Cu are expressed as μg atoms/g wet weight liver. These results suggest, therefore, that in the liver of the neonatal rat, as in calf and sheep liver (Bremner & Davies, 1974), the metallothionein concentration in the cytoplasm is a direct function of the hepatic Zn concentration, although the relative proportions of the two metals in the metalloprotein are dependent on the Cu:Zn value of the tissue. Thus, the primary function of the metallothionein in the liver of the newborn rat may be to provide a regulatory mechanism for the control of potential transient changes in the non-thionein Zn concentration of the hepatic cytosol and as a source of this metal when the need to sustain essential metabolic sites is of prime importance. This mechanism may not operate in other species in which the pattern of the hepatic accumulation of Zn in the foetus and newborn differs from that in the rat (Bakka & Webb, 1981). Nevertheless it is interesting that although the maximum concentration of Zn in the liver of the foetal lamb occurs during gestation, the subsequent changes in the concentrations of thionein-bound and non-thionein-bound Zn (Bremner *et al.* 1973) appear to be similar to those in the newborn rat.

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