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METABOLISM OF ZINC AND COPPER IN THE NEONATE: CHANGES IN THE CONCENTRATIONS AND CONTENTS OF THIONEIN-BOUND Zn AND Cu WITH AGE IN THE LIVERS OF THE NEWBORN OF VARIOUS MAMMALIAN SPECIES

A. BAKKA* and M. WEBB

Toxicology Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

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Abstract—The livers of newborn rabbits, mice, Syrian and Chinese hamsters contain high cytoplasmic concentrations of metallothioneins. In the rabbit and mouse the hepatic concentrations of these metalloproteins are highest at, or immediately after birth and then decrease rapidly whereas, in the Syrian and Chinese hamster, maximum concentrations are not reached until 4 and 9 days *post partum*, respectively. In the human liver the metallothionein concentration appears to be maximal during gestation and is appreciably less in the newborn than the 33-week foetus. In addition to age-related variations in metal content, the metal composition of the hepatic metallothionein fraction also varies with species; that of the rabbit always contains Zn as principal bound metal, that of the mouse or the Chinese hamster usually contains Zn and Cu in ratios greater than unity, whilst that of the Syrian hamster, at least until 14 days *post partum*, contains more Cu than Zn. In all of these species, as in the human foetus and infant, however the Zn:Cu ratio in the metallothionein varies with age. Values for this ratio, as well as the concentrations of thionein-bound Zn and Cu, in the rodent species seem to be determined by the concentrations of these metals in the whole liver. It is suggested, therefore, that in foetal and newborn animals, thionein synthesis acts as a protective mechanism against excessive amounts of the essential, but potentially toxic Zn and Cu ions, possibly before the development of other control processes.

Metallothioneins, that contain Zn and/or Cu ions as the principal bound cations, have been shown to occur in high concentrations in the livers of newborn pigs [1] foetal and newborn rats [2–6] and both human [7] and lamb [8] foetuses. In the liver of the foetal lamb, the concentrations of thionein-bound Zn and Cu decrease between the 80th and 136th day of gestation and then tend to increase again immediately before parturition [8]. In contrast, in the liver of the foetal rat, the metallothionein concentration is below the limit of detection on the 16th day of gestation and then increases to a maximum at, or immediately after birth [6, 9]. In the newborn, the hepatic concentration of thionein bound Zn then decreases with age to reach the low level, characteristic of the adult, at, or near the time of weaning. The concentration of thionein-bound Cu which, in contrast with that of Zn, does not reach its maximum until about 14 days, also declines to barely detectable levels at about 25 days of age [6]. Whereas Williams *et al.* [10] consider it unlikely that a hepatic store of Zn (and Cu) is laid down (as the metallothionein) during development of the lamb foetus, it has been suggested that the metalloprotein in the liver of the newborn rat provides a reserve of these metals that can be utilized during the period of rapid post natal growth, when the demands for them are particularly high and possibly greater than the supply from the maternal milk [4, 6, 11, 12].

* Fellow of the European Science Exchange Programme; present address: University of Oslo, Akershus Central Hospital, N 1474 Nordbyhagen, Norway.

As part of an investigation of this hypothesis it seemed important to establish whether both the accumulation and subsequent loss of hepatic metallothionein during foetal–neonatal development were common to various species of laboratory animals. The results of such studies on rabbits, two strains of mice and Chinese and Syrian hamsters, are summarized in the present paper. Analyses also are reported on the contents and concentrations of thionein-bound Zn and Cu in the livers of the 33 week and full term human foetus and 9-week old infant.

MATERIALS AND METHODS

Animals. Pregnant LACA albino mice and outbred nude mice were maintained on Oxoid Pasteurized Diet (Oxoid Ltd., Southwark Bridge Road, London, SE1), pregnant HLAC Chinese and Syrian hamsters on Diet PRD (Labsure Animal Foods, Poole, Dorset), supplemented three times and twice per week, respectively, with hay, sunflower seed, wheat, oats, flaked maize and, for the Chinese hamsters, multi-vitamins. Colonies of these animals were maintained in the laboratory. Pregnant New Zealand White rabbits were bought from a commercial breeder. Newborn animals of the different species were kept with their mothers.

Human tissues. Livers from a 33 week old foetus, that probably died from hypoxia due to maternal *ante partum* haemorrhage, a full-term still-birth and an infant, that died from brain damage at 9 weeks, were provided 8–27 hr post mortem by Dr. T. E. W. Goodier, St. Helier's Hospital, Carshalton and Dr. Price Davies, Great Ormond Street Hospital for Sick Children, London. The livers, which appeared normal and showed no evidence of hepatic diseases, were frozen and stored in liquid nitrogen until processed.

Tissue fractionation. Pregnant hamsters were killed by decapitation, the uterus being removed from each *via* a mid-abdominal incision. The uterine horns were cut open, the foetuses removed from the placentae and, after being blotted free from blood, were dissected to isolate the livers. Newborn of all species were killed by decapitation and their livers removed. Portions of each liver were digested and analysed for Cu and Zn as described by Mason *et al.* [6]. The remainder of the tissue was pooled according to age and species and homogenized in 10 mM Tris–HCl buffer, pH 8.0 (2–3 ml/g wet wt tissue) in a glass homogenizer, operated manually. Each homogenate was centrifuged at 4° for 90 min at 105,000 *g* and a suitable volume of the supernatant solution, equivalent to 1–2 g wet wt tissue, was fractionated by gel filtration on a column (90 × 1.6 cm or 80 × 2.5 cm) of Sephadex G75 with the same buffer as eluent. Eluate fractions were monitored for absorbance at 250 nm and analysed for Zn and Cu by atomic absorption spectrophotometry. The metal contents of the metallothionein fraction (Ve/Vo = 2) were calculated from the analytical data.

The crude metallothionein, separated by gel filtration from one sample of human liver (full-term, still-birth) was fractionated further by ion exchange chromatography on a column (30 × 1.6 cm) of DE-52 cellulose (Whatman Biochemicals Ltd., Springfield Mills, Maidstone, Kent) equilibrated with 10 mM Tris–HCl buffer, pH 8.0. After application of the sample, a further 100 ml of this buffer was passed through the column before the linear gradient (400 ml, 10–200 mM Tris–HCl, pH 8.0) was started. Fractions that contained the two isometallothioneins (identified by analysis of the eluate for Zn and Cu) were collected as separate pools. These were transferred to columns of Sephadex-G75, equilibrated with 10 mM ammonium formate buffer pH 8.0. The metalloproteins were eluted from these columns with the same buffer; the appropriate fractions being combined and lyophilized. The solids thus obtained were stored at −20° until analysed.

Analysis of human hepatic metallothioneins. Sulphydryl groups were determined with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in a buffered (pH 8.0) solution of 6M guanidine hydrochloride in 50 mM sodium ethylene diamine tetra-acetate as described by Sokolowski and Weser [13]. Amino acid analyses were made with a BioCal BC 200 Amino Acid Analyzer in conjunction with the ninhydrin system described by Moore [14]. The metalloprotein samples were hydrolysed in either 6N HCl or 0.05% thioglycollic acid in 6N HCl [15] at 108–110 for 24 hr. For the determination of tryptophan by the method of Matsubara and Sasaki [16] the thioglycollic acid concentration was increased to 2% (v/v). Cysteine was determined as cysteic acid after performic acid oxidation [17].

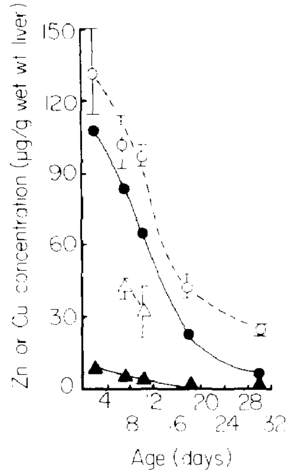

Fig. 1. Changes in the concentrations of total Zn and thionein-bound Zn and Cu with age in the liver of the newborn rabbit. In this and the following figures (Figs. 2–4), total concentrations are given as the means ± S.D. of analyses of tissue from each animal in the litter(s); thionein-bound metals are calculated per g wet wt liver after fractionation and analysis of the pooled residual tissue (see Materials and Methods): ---○---, total Zn; ——●——, thionein-bound Zn; ——▲——, thionein-bound Cu. Total Cu (---△---) was determined at 7 and 10 days only.

RESULTS

The livers of the newborn of all rodent species that were examined contained high concentrations of metallothioneins. These metalloproteins, however, varied greatly between species in content and metal composition in relation to age. Thus the concentration of hepatic metallothionein in the rabbit (Fig. 1) and LAC mouse (Fig. 2) was highest at 2 days after birth (the earliest time at which measurements were made in these species) and then

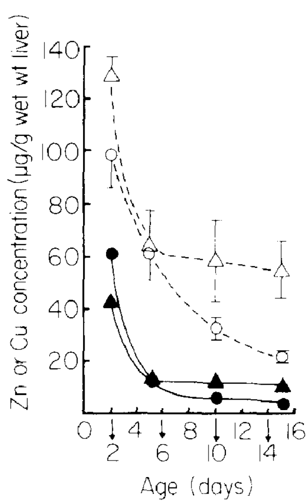

Fig. 2. Changes in the concentrations of total (----) and thionein-bound (———) Zn (○,●) and Cu (△, ▲) with age in the liver of the newborn mouse.

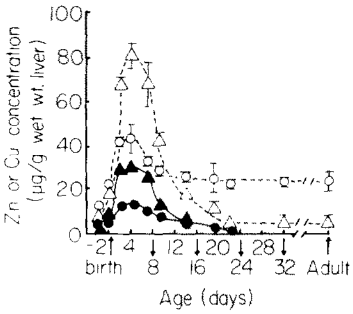

Fig. 3. Changes in the concentrations of total (----) and thionein-bound (———) Zn (○, ●) and Cu (△, ▲) in the Syrian hamster with age from two days *ante partum*.

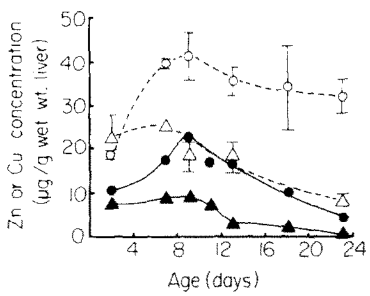

Fig. 4. Changes in the concentrations of total (----) and thionein-bound (———) Zn (○, ●) and Cu (△, ▲) with age after birth in the liver of the Chinese hamster.

decreased rapidly whereas, in Syrian (Fig. 3) and Chinese hamsters (Fig. 4), which have shorter periods of gestation, it did not reach its maximum until 4 or 9 days of age. The results for the nude mice were almost identical with those for the LAC mice and are not shown as a separate figure.

Although the Zn:Cu ratio in the metallothionein fraction of all species changed with age (Fig. 5), the principal bound cation in the hepatic metalloprotein of the newborn rabbit and Chinese hamster was Zn but, in the mouse and Syrian hamster, at least between the 4th and 15th day after birth, it was Cu. At all times, the ratio between the concentrations

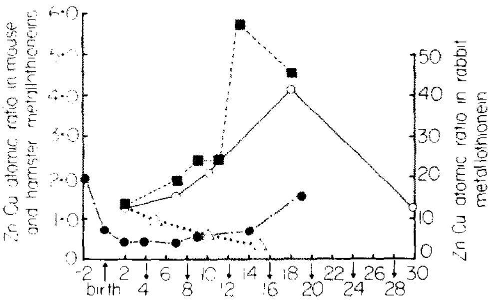

Fig. 5. Age-related variations in the Zn:Cu ratio of the hepatic metallothionein of the newborn rabbit (-○-), mouse (---△---), Syrian hamster (---●---) and Chinese hamster (---■---).

Table 1. Linear regression analysis of the relationship between total zinc and thionein-bound zinc and total copper and thionein-bound copper in the liver of the developing Syrian hamster*

Metal	Interval from 14 days *ante partum*†	a	b	*r*
Zn	16	2.9	6.8	0.97
	21	2.5	11.2	0.92
	23	2.0	15.2	0.87
	34	1.8	17.4	0.84
	adult	1.6	18.6	0.83
Cu	16	2.5	3.0	0.99
	21	2.5	3.9	0.99
	23	2.5	4.2	0.99
	34	2.5	4.3	0.99
	adult	2.5	4.3	0.99

* Values are given for a and b in the equation $y = ax + b$ and for the correlation coefficient (r) at different intervals between 14 days *ante partum* and different ages *post partum*. In the equation, y = total metal (Zn or Cu) concentration in the whole liver and x = concentration of thionein-bound metal (Zn or Cu), the values being taken from Fig. 3.

† The gestational period in the Syrian hamster is 16 days and thus, for example, an interval of 16 days corresponds to the period from 14 days *anti partum* to 14 days *post partum*.

of thionein bound Zn and Cu (Zn_{MT},Cu_{MT}) seemed to be determined by the total concentrations of these metals (Zn_{total},Cu_{total}) and the ratio of these concentrations in the whole liver. Thus comparison of the data of Figs. 2–5 shows that $Zn_{Mt}:Cu_{MT}$ ratios of less than unity were correlated with $Zn_{total}:Cu_{total}$ ratios also less than unity.

In the rabbit between 2 and 30 days of age, the relationship between the concentrations of Zn in the whole liver and the metallothionein fraction was given by the equation:

$$Zn_{total} = 1.1Zn_{MT} + 19.9 \ (r = 0.99)$$

Linear regression analysis of the data for the Syrian hamster (Fig. 3) gave the results summarized in Table 1. These results show a high degree of correlation between total and thionein-bound Zn in the liver from 14 days *ante partum* to 14 days *post partum*. As, after 14 days of age, however, the concentration of thionein-bound Zn had fallen to almost zero and the total liver concentration of Zn remained constant

Table 2. Concentrations of total and metallothionein-bound zinc and copper in human foetal and newborn liver

Source of liver	Whole liver Zn	Whole liver Cu	Metallothionein Zn	Metallothionein Cu
	µg/g wet wt tissue			
Foetus (33 week)	84.9	22.0	60.3	9.7
Full term (still birth)	93.2	37.9	48.4	4.5
9 week old infant	N.D.*	N.D.*	4.2	0.4

* Not determined.

at about 25 μg/g wet wt tissue (Fig. 3), the intercept (b in the regression equation $y = ax + b$) altered towards this latter concentration and both the slope (a) of the regression line and the correlation coefficient (r) decreased progressively with the inclusion of data for animals older than 14 days (Table 1). In contrast with the results for Zn, total liver Cu and thionein-bound Cu were highly correlated at all intervals from 14 days *ante partum* to adulthood (Table 1). As these intervals increased, the intercept (b) also increased and approached the total hepatic Cu concentration of the adult animal. It seems, therefore, that the threshold concentrations of both Zn and Cu, above which thionein synthesis occurs, may be appreciably lower in the newborn than in the adult hamster.

With the Chinese hamster, for which fewer analytical results were available (Fig. 4), the correlations between either total liver Zn and thionein-bound Zn, or total liver Cu and thionein-bound Cu, were less satisfactory. Nevertheless, between 2 and 13 days *post partum*, there appeared to be a significant correlation between total and thionein-bound Zn (i.e. $Zn_{total} = 1.9\,Zn_{MT} + 1.2$; $r = 0.91$). Analysis of the limited data for the LAC-mouse (Fig. 2) for the period between 2 and 15 days *post partum* gave:

$$Zn_{total} = 1.2\ Zn_{MT} + 29.1\ (r = 0.92)\ \text{and}$$
$$Cu_{total} = 2.3\ Cu_{MT} + 32.9\ (r = 1.00).$$

In contrast, analysis of samples of liver tissue from two human foetuses and one newborn infant showed no relationship between the concentrations of thionein-bound Zn and Cu and the total hepatic concentrations of these metals. These results (Table 2), which suggest that the metal content and composition of the human hepatic metallothionein alters with age, both before and after birth, however, were obtained with livers that were removed some time after death from different causes. Nevertheless the crude metallothionein that was isolated by gel filtration from the liver of full-term still-birth (Zn: Cu = 10.4) had an absorption spectrum (max. 215 nm) characteristic of Zn-thionein and showed no evidence of an absorption band, near 255 nm, due to inter- (or intra-) molecular disulphide bonds (see, e.g., [18]). Also incubation (22°) of this metallothionein (70 ng atoms Zn + Cu/ml) with excess Cd

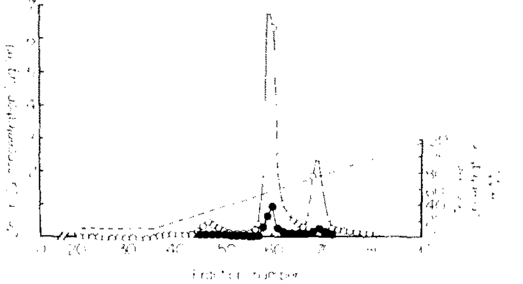

Fig. 6. DEAE–cellulose chromatography of human neonatal liver metallothionein. The metallothionein was isolated by gel filtration of the soluble fraction from the liver of a full-term still-born foetus (see Materials and Methods) and was fractionated on a column (30 × 1.6 cm) of DE-52 cellulose with 100 ml 10 mM Tris–HCl buffer, pH 8.0, followed by a linear gradient of Tris–HCl buffer (----). Elution profiles of Zn (○) and Cu (●) are shown.

Table 3. Amino acid composition of isometallothionein I from the liver of a human full-term, still-born foetus*

Amino Acid	Residues/mole		Per cent of total residues
1/2 Cys.	14.81	(15)	24.4
Asp & Met	3.97	(4)	6.5
Thr.	2.55	(3)	4.2
Ser.	7.48	(7)	12.2
Glu.	5.03	(5)	8.3
Pro.	1.40	(1)	2.4
Gly.	4.69	(5)	7.7
Ala.	5.47	(5)	9.0
Val.	2.09	(2)	3.4
Thr.	1.50	(2)	2.5
Leu.	1.12	(1)	1.8
Tyr.	—†	—	—
Phe.	0.92	(1)	1.5
His.	0.14	—	0.2
Lys.	9.47	(9)	15.5
Arg.	0.27	—	0.4

* The results are calculated on the basis of 61 residues per mole protein and as a percentage of the total residues. The values given are averages for several determinations on 6*N* HCl hydrolysates (see Materials and Methods).

† Not detected.

(210 ng atoms/ml) resulted in a 7-fold increase in absorbance at 254 nm and an absorption spectrum over the spectral range 210–290 nm typical of Cd-thionein [18]. Ion exchange chromotography of the crude metallothionein yielded the isometallothioneins I and II, but in unequal amounts, the isometallothionein I being the predominant form (Fig. 6). These metalloproteins had sulphydryl: metal (Σ Zn + Cu) ratios of 3.12 and 2.84, respectively, i.e. appropriate for native metallothioneins. Furthermore Form I, which was obtained in sufficient quantity for analysis, had an amino acid composition (Table 3) that, apart from the presence of small amounts of histidine and phenylalanine probably indicative of slight contamination by other proteins, was in reasonable agreement with that found by Bühler and Kägi [19] for isometallothionein I of adult human liver and by Ryden and Deutsch [7] for the metalloprotein of human foetal liver. These results suggest therefore, that *post mortem* changes in the composition and metal contents of the metallothioneins of the foetal and newborn human livers, that were used in the present studies, may have been minimal.

DISCUSSION

The hepatic metallothioneins of the foetal lamb and adult sheep have been shown to be identical in amino acid composition [8]. The present results, together with those of Ryden and Deutsch [7], also characterize the low mol. wt (Zn, Cu)-metalloprotein of foetal and newborn human liver as a metallothionein. Evidence that the analogous metalloproteins of the livers of foetal and newborn rats and rabbits are identical with the adult hepatic metallothioneins, of established amino acid composition, has been given by Waalkes and Bell [20] and Mason *et al.* [6]. Furthermore thionein synthesis has been shown to occur in the liver of the Cd-exposed adult mouse [21]. Thus although the low molecular weight Zn- and Cu-binding proteins that, as shown in the present work, occur in the livers of newborn mice and hamsters, have not been identified unequivocally, it seems safe to assume that these cytoplasmic components which, on gel filtration, behave in the same way as those of the rabbit and rat, also are metallothioneins. It seems probable, therefore, that these metalloproteins are normal foetal proteins with important functions in late *antepartal* and early *postpartal* development.

Studies on the accumulation, distribution and age-related loss of Zn and Cu from the livers of late foetal and newborn rats have led to the suggestion that the major function of the metallothionein in this species is to provide a regulatory mechanism to control and maintain the cytosolic non-thionein-Zn concentration [6]. Whilst, however, the hepatic metallothionein of the neonatal rat is predominantly a Zn-metalloprotein and contains only a low, variable amount of Cu [6], the present studies show that the metallothioneins, which occur in high concentrations in the livers of newborn rabbits, Syrian and Chinese hamsters, two strains of mice and man, vary greatly in metal composition and content according to species as well as age. Thus, for example, the hepatic metallothionein of either the newborn rabbit (Fig. 1) or Chinese hamster (Fig. 4) is essentially a Zn-metalloprotein, whereas that of the Syrian hamster (Fig. 3) contains much more Cu than Zn. In all the species of laboratory animals, at least for some days after birth, there seems to be covariance in the concentrations of either metal in the whole liver and in the metallothionein, the age related variations in the Zn:Cu ratio of which (Fig. 5) appear to be determined largely by the Zn:Cu ratio in the liver. With reservations because of the delay in preservation of the tissue, this relationship is not apparent in foetal and newborn human liver (Table 2). It appears from these results (Table 2), however, that in the human foetus, as in the foetal lamb [8], the concentration of the hepatic metallothionein may be maximal during gestation and not at, or after birth as in the rodent. Furthermore, comparison of the present results with those of Ryden and Deutsch [7] for the livers of foetuses of approximately 20 weeks gestational age suggests that the Cu:Zn ratio of the human hepatic metallothionein decreases appreciably with age. It would seem, therefore, that an important function of the hepatic metallothionein in all species may be to protect against excessive amounts of the essential, but potentially toxic Zn and Cu ions, both in the foetus *in utero* and in the newborn, possibly before the development of intestinal control mechanisms. Whilst a specific function of the metalloprotein in the control of hepatic Zn metabolism, irrespective of species, seems unlikely, this hypothesis does not exclude the utilization of the thionein-bound metals in, for example, the synthesis of metalloenzymes.

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