Metabolism of zinc and copper in the neonate: accumulation of Cu in the gastrointestinal tract of the newborn rat

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1. The concentration of copper in the rat intestine was found to increase rapidly after birth to a maximum greater than 140 μg/g wet weight at 2 d of age and then to decline, at first slowly to 90 μg/g wet weight on day thirteen and then rapidly to 40 μg/g and 3·4 μg/g wet weight on the 15th and 19th day respectively. The intestinal concentration of Zn, which doubled between 1 d prepartum and 2 d post partum, also fell slowly until 10 d of age, but thereafter remained constant.

2. From the 2nd to the 15th day post partum approximately 60% of the total Cu and 50% of the total zinc in the intestine was located in the soluble fraction of the tissue. Most of the Zn in this fraction was bound by proteins of molecular weights greater than 13700 daltons, whereas most of the Cu was present as an extremely polydisperse complex of lower molecular weight. This complex in the intestine of the 5-d-old rat, in contrast with the soluble proteins of higher molecular weight, did not incorporate either ^{3}H or ^{35}S within 4 h of the administration of L-[4,5-^{3}H]leucine and L-[^{35}S]cystine.

3. The loss of Cu from the intestine between the 13th and 15th day of post-natal age occurred mainly from this complex and was accompanied by the transient appearance of Cu in a fraction of low molecular weight.

4. At 21 d of age the soluble fraction of the intestine contained only a small amount of Cu. This was distributed between two protein fractions, one of which contained Zn and appeared to be a metallothionein.

5. The results are discussed in relation to the control of Zn and Cu absorption.

Studies on the accumulation and loss of hepatic zinc and copper metallothioneins during postnatal development in the rat and other mammalian species (Bakka & Webb, 1981; Bakka *et al.* 1981; Mason *et al.* 1981) have indicated that these metalloproteins may function both to regulate the metabolism of the essential metals and to prevent toxic interactions, possibly before the development of intestinal control mechanisms.

In the intestine of the adult animal, metallothionein synthesis appears to regulate the absorption of Zn (Richards & Cousins, 1975, 1976*a*, *b*) and possibly Cu (Evans, 1979), although the work of Hall *et al.* (1979) suggests that this may not be the normal control mechanism for the latter. Nevertheless, homoeostatic mechanisms for the control of the absorption of various metals are known to be poorly developed in the newborn and there is much evidence that non-specific pinocytotic activity accounts for the high efficiency of uptake of both essential (for example iron, Cu, Zn) and non-essential (cadmium, lead, mercury) metals (for example, see Jugo, 1979; Mills & Davies 1979) until the closure of the intestinal mucosa which, in the rat, occurs in the third week after birth (for example, see Jugo, 1977).

The possibility, suggested by these considerations, that the loss of thionein-bound Zn and Cu from the liver of the newborn might be correlated with either the onset of metallothionein synthesis, or the development of other control mechanisms in the intestine, led to the work summarized in this paper, on the uptake, distribution and elimination of Cu and Zn in the intestine of the newborn rat.

EXPERIMENTAL

Animals. Most of the experimental work was done with newborn, random-bred Wistar-Porton rats, from 1st or 2nd litters that were culled to eight at 2 d post partum. Dams were given

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the standard Oxoid 41B diet (Oxoid Ltd., Southwark Bridge Road, London SE1) and tap water *ad lib.* throughout pregnancy and nursing periods. Newborn animals were nursed by their mothers and weaned at 21 d of age. Neonatal rats (5-d-old), that were dosed with L-[^{35}S]cystine (10 μCi/animal) and L-[4,5-^{3}H]leucine (10 μCi/animal) before removal of the gastrointestinal tracts, were the same as those used previously for the isolation of hepatic metallothionein (Mason *et al.* 1981). Foetuses were obtained as described by Samarawickrama (1979).

Methods. Metal analysis and determinations of radioactivity were made as described by Mason *et al.* (1981).

Animals were killed by decapitation and the blood allowed to drain. The entire intestine of each was excised, the first 2 mm was discarded and the contents of the remaining portion were removed gently with a spatula. The tissues from each litter were pooled, frozen in liquid nitrogen and stored at $-20°$. For fractionation, the tissue was thawed, minced and homogenized, usually in 2 or 4 vol. 10 mM Tris-HCl buffer, pH 8·0 (with 0·1 g sodium azide/l) at 0°. In some experiments, 10 mM-ammonium formate, pH 8·0, was used instead of Tris-HCl. The homogenate was centrifuged at 140000 *g* for 45 min at 5° in a Beckman L5-65 centrifuge and a portion of supernatant fraction (equivalent to 1–2 g wet tissue) was fractionated by gel filtration at 4° on a calibrated column (800 × 15 mm) of Sephadex G75 with the appropriate 10 mM buffer as eluant at a flow-rate of 17 ml/h. To permit comparisons between animals of different age, the metal contents of the fractions from these columns were calculated and expressed as concentrations (μg/g wet weight) in the gastrointestinal tract (GIT).

Ion-exchange chromatography was done on a column (140 × 25 mm) of Whatman DEAE-cellulose, previously equilibrated with the homogenization-buffer. After loading, the column was washed with at least 2 vol. of the same buffer before the linear gradient (500 ml; 10–200 mM-Tris-HCl or ammonium formate) was applied. Dithiothreitol, when used, was added to all buffers at 1 mM concentration. For electrophoresis on 7·5% polyacrylamide gels by the method of Davis (1964), column eluate fractions were lyophilized and redissolved in 10 mM-Tris-HCl buffer, pH 7·4.

RESULTS

The total concentration of Cu in the intestine of the rat increased rapidly from approximately 17 μg/g wet weight in the foetal animal at 1 d before parturition to a peak level, greater than 140 μg/g wet weight tissue, by the second day after birth (Fig. 1). Thereafter it declined, at first slowly, to approximately 90 μg Cu/g wet tissue on the 13th day and then more rapidly to 40 μg/g on the 15th, 3·4 μg/g on the 19th and approximately 2 μg/g wet weight tissue on the 21st day. During the same period, the intestinal concentration of Zn increased from approximately 25 μg/g wet tissue at 1 d prepartum to approximately 50 μg/g wet tissue at 2 d post partum and then decreased slowly at a rate not dissimilar to that of Cu (Fig. 1) until the 10th day (Fig. 2). Between 10 and 21 d after birth, however, the concentration of Zn, in contrast with that of Cu, remained essentially constant.

From the 2nd to the 15th day post partum approximately 60% of the total Cu and 50% of the total Zn of the intestine was recovered in the soluble fraction of homogenates of the tissue and the variations in the concentrations of these metals in this fraction largely paralleled those in the whole tissue (Figs. 1 and 2). Gel filtration of the soluble fractions from the early neonatal intestine (i.e. from rats of 2–13 d of age) showed the presence of Cu in two major fractions, one of high molecular weight (V_e/V_0 1) and the second, an extremely heterogeneous polydisperse fraction, of lower molecular weight (V_e/V_0 1·5–2·5, Fig. 3). Most of the Cu was lost from the latter between the 13th and 15th day (Fig. 3, note the difference in scale in the values for the 13- and 15-d-old animals) some apparently

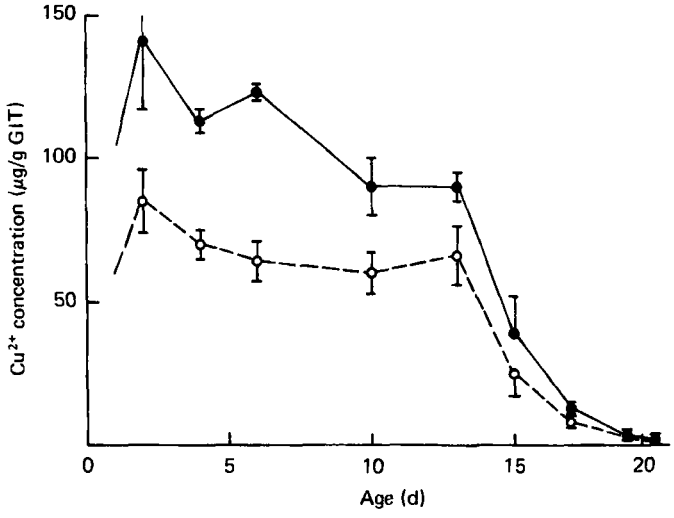

Fig. 1. Concentrations of total copper (●—●) and of soluble Cu (○---○) in the rat intestine in relation to age. The soluble fractions were prepared by centrifugation of homogenates of pooled tissue from individual litters (see p. 392). GIT, gastrointestinal tract. Points are mean values with their standard errors represented by vertical bars.

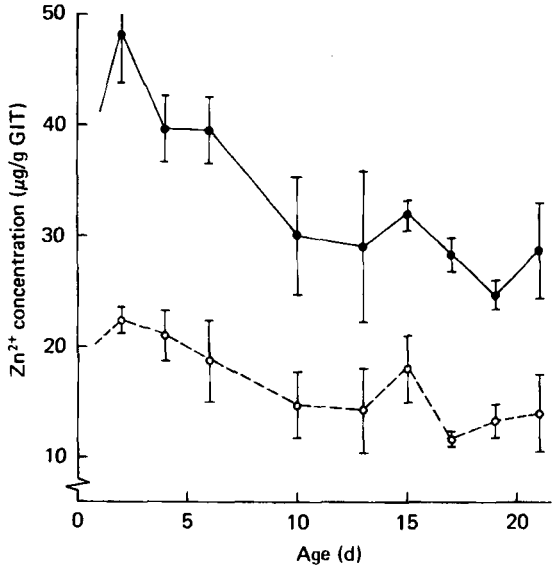

Fig. 2. Concentrations of total zinc (●—●) and of soluble Zn (○---○) in the intestine of the newborn rat in relation to age. The soluble fractions were prepared by centrifugation of homogenates of pooled tissue from individual litters (see p. 392). GIT, gastrointestinal tract. Points are mean values with their standard errors represented by vertical bars.

being transferred to components of very low molecular weight (V_e/V_0 3·0). These components also were eliminated by the 17th day, at which time Cu was distributed throughout the high and intermediate molecular weight range (V_e/V_0 1–2) of the elution profile (Fig. 3). At 21 d of age, when the total intestinal concentration of the metal was low (Fig. 1), Cu in the soluble fraction was associated only with two clearly defined fractions of high (V_e/V_0 1·0)

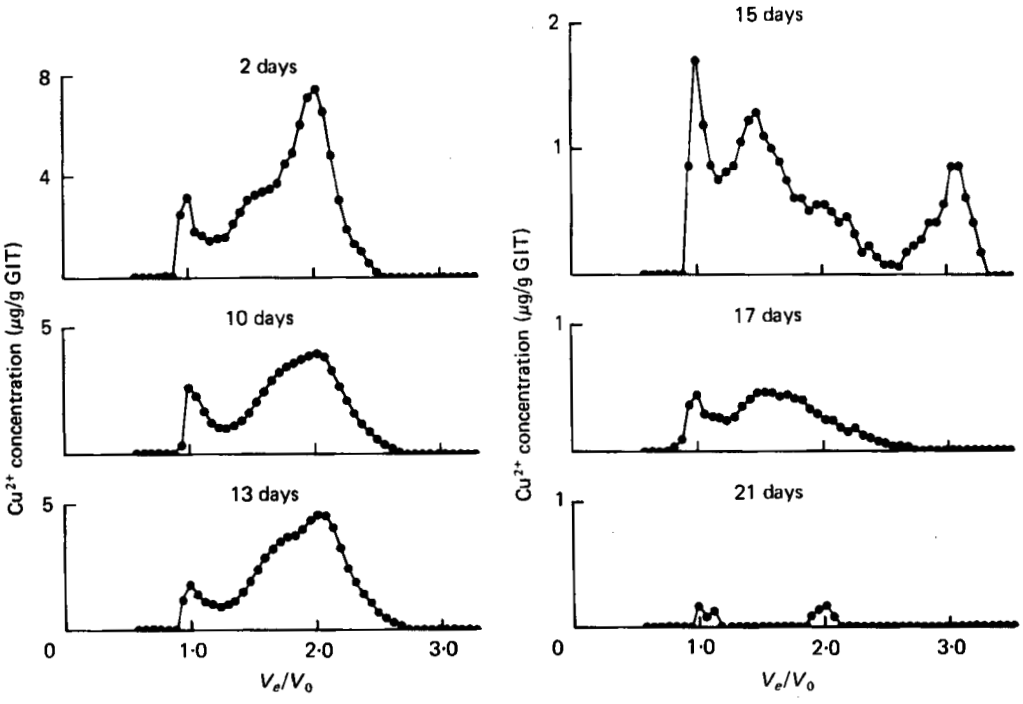

Fig. 3. Relationship between the distribution of copper in the soluble fraction of the rat intestine and age. The soluble fraction from each litter (see p. 392) was fractionated by gel filtration on Sephadex G75. GIT, gastrointestinal tract. Points are mean values with their standard errors represented by vertical bars.

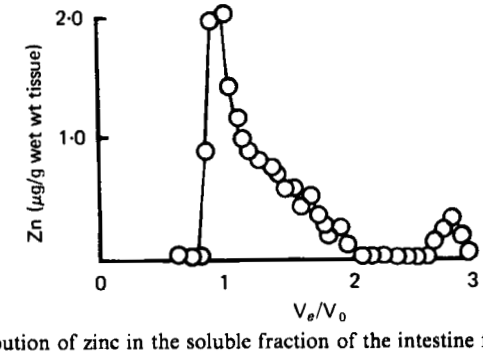

Fig. 4. Distribution of zinc in the soluble fraction of the intestine from the 7-d-old rat.

and low (V_e/V_0 2·0) molecular weight, the latter being similar in its location in the elution profile to metallothionein. This fraction also contained Zn. Between the 2nd and 17th day after birth, however, the presence of Zn-thionein was not detected in the soluble fraction of the intestine (see e.g. Fig. 4). In the early neonate, the distribution of Zn in this fraction appeared to be independent of age. Thus, as shown previously by Hurley *et al.* (1977), at all ages most of the Zn was associated with components of molecular weight greater than that of ribonuclease (13700 daltons), one of the proteins used to calibrate the column, although a smaller amount was present as a low molecular weight species (V_e/V_0 2·75; Fig. 4), possibly the Zn-binding ligand, which is present in rat milk until the 16th day post partum

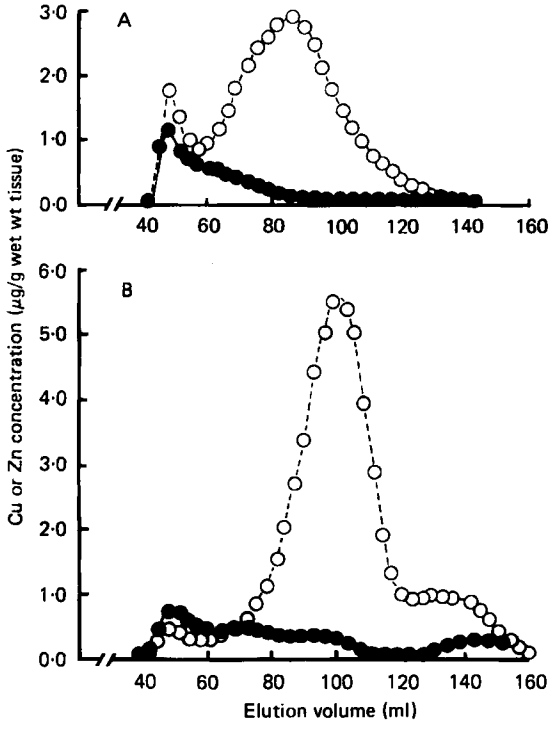

Fig. 5. The effect of dithiothreitol on the separation by gel filtration of the soluble copper complexes of the 5-d-old rat intestine. Soluble fractions were prepared from homogenates of 2·2 and 3·0 g intestinal tissue in 10 mM-Tris-HCl buffer, pH 8·0, and in a 1 mM solution of dithiothreitol in the same buffer respectively, and were applied immediately to Sephadex G75 columns (800 × 15 mm). The control column (A) was equilibrated and eluted with 10 mM-Tris-HCl buffer, pH 8·0, in a nitrogen atmosphere. The second column (B) was eluted under the same conditions, but with the addition of 1 mM-dithiothreitol to the buffer. Elution profiles of Cu (○---○) and zinc (●—●) are shown.

and may be taken up by the intestinal mucosa of the suckling animal (Hurley *et al.* 1977; Duncan & Hurley, 1978).

In common with Cu-thioneins (for example, see Webb, 1979), the low molecular weight polydisperse Cu-complex of the intestinal tract was difficult to isolate and purify on a preparative scale. In the isolation of the complex the time factor seemed to be important, since better results were obtained if the tissue extract was fractionated in 5 ml portions on a number of small Sephadex G75 columns (900 × 15 mm), operated in an N_2 atmosphere, rather than in its entirety on a preparative column (800 × 90 mm). Addition of 1 mM-dithiothreitol to the buffers that were used for the homogenization of the tissue and for elution of the columns yielded much less polydisperse preparations that were eluted at V_e/V_0 values of approximately 2·0 (Fig. 5). In contrast with the authentic metallothionein of the livers of these animals (Mason *et al.* 1981), however, no peaks of radioactivity were associated with the Cu complex, when this was isolated in the presence of dithiothreitol from homogenates of the pooled intestinal tracts of 5 d-old rats at 4 h after the intraperitoneal injection of L-[^{3}H]leucine and L-[^{35}S]cystine (Fig. 6). As both isotopes were incorporated into the high molecular weight components of the soluble fraction and were present in their highest concentrations in the very low molecular weight fraction (Fig. 6), it seems that the absence of incorporation into the Cu-complex cannot be attributed to either a low rate of protein synthesis, or a deficiency of labelled precursors in the intestinal mucosa.

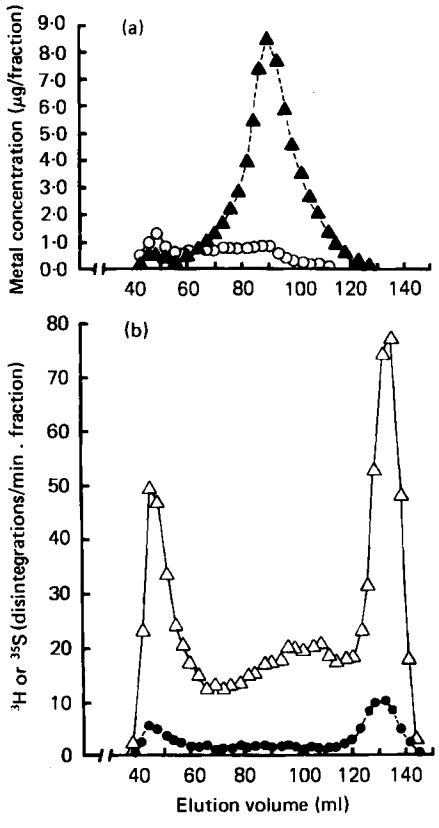

Fig. 6. Distribution of (a) copper and zinc and (b) ^{3}H and ^{35}S in the soluble fraction of the gastrointestinal tract of the 5-d-old rat at 4 h after the intraperitoneal administration of L-[4,5-^{3}H]leucine (10 μCi) and L-[^{35}S]cystine (10 μCi). The tissue (1·8 g) was homogenized in 1 mM-dithiothreitol in 10 mM-Tris-HCl buffer, pH 8·0, and a portion (2·6 ml, equivalent to 0·52 g wet weight tissue) of the soluble fraction was fractionated by gel filtration on a column (800 × 15 mm) of Sephadex G75 with the same buffer solution as eluant and nitrogen as the gas phase. The fractions (3·0 ml) were analysed for Cu (▲---▲), Zn (○—○), ^{3}H (△—△) and ^{35}S (●---●).

Further fractionation of the peak from the Sephadex G75 column by ion-exchange chromatography was unsuccessful. Thus in some experiments, in which 10–200 mM gradients of either Tris-HCl or ammonium formate buffers (pH 8·0) were used in the presence of a constant concentration (1 mM) of dithiothreitol, the Cu-complex remained bound to the column whereas, in others, most of the material was eluted before the gradient was started and only small amounts of Cu were eluted in three peaks at higher ionic strengths (Fig. 7). The observation that, on polyacrylamide gel electrophoresis, two of these had the same mobilities as authentic hepatic Zn-isometallothioneins I and II (Fig. 7), is insufficient proof of identity.

DISCUSSION

The observed increase in the intestinal concentration of Cu from 17 μg/g wet weight at 1 d prepartum to 140 μg/g wet weight at 2 d postpartum corresponds to an increase in the Cu content of the intestine of approximately 26 μg (i.e. from 1·7 to 28·0 μg Cu). With the assumptions that (1) the milk production by the lactating rat during the first 2 d postpartum is approximately 25 ml/d (Mutch & Hurley, 1974) and thus the milk intake/pup in a litter of eight is approximately 3 ml/d and (2) the Cu concentration in rat milk throughout the

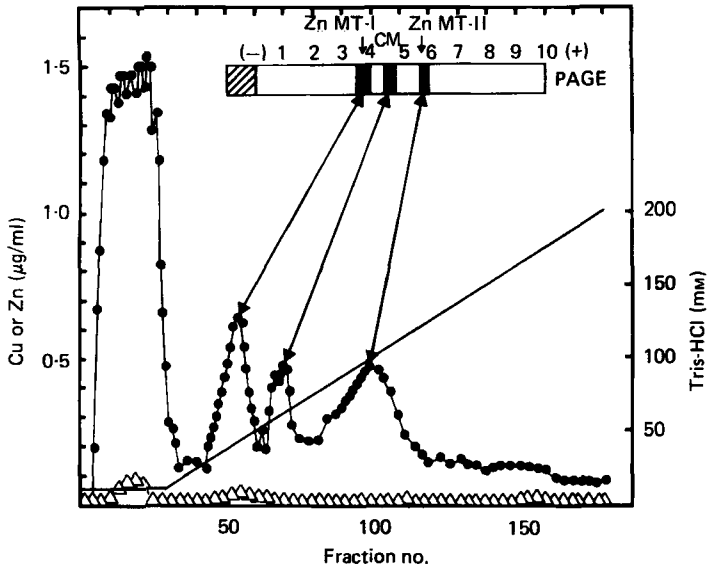

Fig. 7. DEAE-cellulose chromatography of the Sephadex G75 pool of the low molecular weight copper-complex from the gastrointestinal tract of the 5-d-old rat. Fractions from the Sephadex G75 column that contained the Cu complex were pooled and the solution applied to a column (140 × 25 mm) of Whatman DEAE-cellulose, equilibrated with the same buffer (10-mM-Tris HCl, pH 8·0). After loading, the column was washed with several column volumes of the same buffer, after which the linear gradient (500 ml, 10–200 mM-Tris-HCl, pH 8·0) was applied. Fractions (3·0 ml) were collected from the beginning of loading. Fractions nos. 5–33, 44–60, 65–73 and 85–115 were pooled, lyophilized and analysed by disc polyacrylamide gel electrophoresis (PAGE). The results of the PAGE analysis of the three peaks that were eluted from the DEAE cellulose at ionic strengths greater than 10 mM-Tris-HCl are shown. The positions of migration of authentic hepatic Zn-thioneins I and II (Zn MT-I and ZnMT-II respectively) also are shown.

first 3 d of lactation is higher (6·8 ± 2·15 μg/ml) than that (3·5 ± 0·85 μg/ml) at later times (Terao & Owen, 1977), it seems that approximately 60% of the total Cu ingested from the milk (i.e. approximately 42 μg/pup) is retained by the intestine during the first 2 d after birth. Since the Zn concentration in rat milk is approximately 17 μg/ml on the 1st day of lactation (Mutch & Hurley, 1974), similar calculations indicate that the increase in intestinal Zn content from 2·5 μg at 1 d prepartum to 10 μg at 2 d postpartum is much lower (i.e. 7·5 μg) than the probable intake (approximately 100 μg). It appears, therefore, that the neonatal animal has a much higher requirement for Zn than for Cu and, whereas most of the dietary Zn is absorbed, a large proportion of the Cu is retained in the intestine. It is known from the work of Mistiles & Mearrick (1969) that, at 24 h after the intragastric administration of ^{64}Cu to the neonatal (suckling) rat, (1) a major portion of the dose is retained within the ileal mucosa and (2) translocation of ^{64}Cu to the liver and carcass is relatively constant and independent of ileal uptake.

As the intestinal concentration of Cu falls only slowly between the 2nd and 13th day of age, although the body-weight is increasing rapidly (Mason *et al.* 1981), there must be a significant increase in Cu content of the intestine over this period. Between these ages approximately 60% of the total Cu in the tissue can be recovered in the soluble fraction, mainly in association with an extremely polydisperse fraction which, on gel filtration on Sephadex G75, elutes at V_e/V_0 values of from 1·5 to 2·5 (Fig. 3). Although the behaviour of this Cu-complex on ion exchange chromatography (Fig. 7) has some similarities with that of the metallothionein fraction from the livers of copper-injected rats (Bremner &

Young, 1976), its failure to incorporate [^{35}S]cysteine (under conditions that lead to appreciable labelling of the hepatic metallothionein in the same animals; Mason *et al.* 1981) suggests that it is not identical with the intestinal metallothionein of the adult animal, which has been considered to regulate the absorption of Zn (Richards & Cousins, 1975, 1976*a*, *b*) and Cu (Evans, 1979). In this connection it seems unlikely that the uptake of these essential metals would be regulated in the newborn by the same protein. Furthermore, even in the adult animal, Hall *et al.* (1979) find the dietary level of Cu to have no influence on the concentration of metallothionein in the intestinal mucosa and conclude that a mechanism, other than the incorporation of the metal into this protein, is involved in the normal regulation of Cu metabolism.

The absence of significant labelling of the neonatal intestinal Cu complex with either [^{3}H]leucine or [^{35}S]cysteine in vivo possibly suggests that it may be derived from the maternal milk and can be degraded, but not synthesized, in the intestine of the newborn. It is established that macromolecules (immunoglobulins, other proteins and colloids) are absorbed in the newborn by pinocytosis (for example, Halliday, 1955; Clark, 1959) until the closure of the structure of the intestinal mucosa at the end of the 3rd week of life (Halliday, 1956; Clark & Hardy, 1969*a*, *b*; Bainter & Veress, 1970). It is known also that the gastrointestinal absorption of a number of metals (Fe, Cu, Pb, Zn, strontium, Cd and various radioactive nuclides) drops from a high to a low level, characteristic of the adult, at approximately the time of weaning (Forbes & Reina, 1972; Shiraishi & Ichikawa, 1972; see also Mills & Davies, 1979). The high efficiency with which metals are absorbed by the newborn appears to be due to pinocytotic uptake of macromolecular complexes (Mills & Davies, 1979; Camakaris *et al.* 1979).

Jugo (1979) suggests that heavy metals form insoluble colloids at the alkaline pH of the intestinal mucosa of the newborn animal and probably also complexes and chelates with proteins and other macromolecules, which are then taken up pinocytotically. Hurley *et al.* (1977), however, consider that the Zn-binding ligand, which occurs in rat milk, may function in intestinal Zn transport in the neonate and it is possible that a similar mechanism operates in the uptake of Cu. Cousins *et al.* (1978) however, have shown that in the intestine of the adult rat, the low molecular weight Zn complexes, previously assumed to function in Zn transport, are artefacts and originate by proteolytic digestion of metallothionein and other Zn-binding proteins. In newborn rodents the intestinal tract does not achieve the adult configuration until the 3rd week of life (Deren, 1968), at which time the gastric glands assume mature characteristics, the pH of the gastric contents falls to a low level and, in particular, the activities of proteolytic and other hydrolytic enzymes increase markedly (Morris, 1968). As shown in Fig. 3, the loss of most of the Cu from the Cu complex of the rat intestine between the 13th and 15th day after birth seems to be accompanied by the appearance of other Cu-containing components of lower molecular weight, possibly products of enzymic degradation. It should be mentioned, however, that no attempt was made to prevent the access of the pups to the diet provided for the maternal animal. Thus it is also possible that the ingestion of solid food which, as Sasser & Jarboe (1977) have shown, increases the rate of loss of Cd from the intestine, could contribute either directly, or indirectly through associated changes in the bacterial flora, to the loss of Cu. Nevertheless, from the appearance of the intestinal contents, it seems little or none of the maternal diet was ingested by the pups before 15 d of age.

The apparent absence of Zn-thionein from the intestine of the rat before 21 d of age, coupled with previous observations on the time-course of the accumulation and loss of this metallothionein in the liver (Mason *et al.* 1981) supports, but does not prove, the suggestion that the latter organ is of primary importance in the regulation of Zn metabolism before the development of intestinal control mechanisms (for example, Mason *et al.* 1981).

The authors are grateful to Mrs D. Holt for her assistance with certain experiments, the results of which are summarized in Figs. 5 and 6. The work reported was done whilst R. M. was a Visiting Fellow of the New Zealand Medical Research Council on leave of absence from the Toxicology Research Unit, University of Otago Medical School, Dunedin, New Zealand and F.O.B. was on research leave from the Division of Biochemistry, Physiology and Pharmacology, University of South Dakota, School of Medicine, Vermillion, South Dakota 57069, USA.

ADDENDUM

Since the acceptance of this paper, W. T. Johnson and G. W. Evans (*Biochem. biophys. Res. Commun.* 1980, **96**, 10) have described the isolation of a (Zn, Cu)-metalloprotein from the small intestine of the 5-d-old Long-Evans rat and have shown that this is resolved by ion exchange chromatography into two main sub-fractions, the amino acid compositions of which are characteristic of metallothioneins.

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