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Age-Dependent Variation of Copper in Tissue and Proteins of Neonatal Rat Small Intestine[1,2] (41011)

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Abstract. Tissue copper levels in the small intestine of neonatal rats increased rapidly following birth, reaching a maximum value of 302.6 ± 22.2 μg/g dry wt on Day 7 and then steadily declined to 6.4 ± 0.5 μg/g dry wt on Day 21. When subjected to gel filtration on Sephadex G-75, high-speed supernatant prepared from the small intestine of rats aged 0 to 21 days eluted as two copper-containing fractions corresponding to molecular weights of 60,000 and 9000 daltons. The copper contents of both fractions varied with age in a manner that correlated significantly with the variations observed in the tissue copper levels. However, orally administered ^{64}Cu was found to be associated primarily with the low-molecular-weight fraction in 5-day-old rats. Furthermore, the amount of ^{64}Cu in the low-molecular-weight fraction did not appear to turnover during the 42 hr following administration of the dose. These results suggest that a portion of the copper ingested by the neonatal rat is initially sequestered in the small intestine by low (9000 daltons) -molecular-weight cystolic proteins either as a mechanism for detoxifying excess copper or as a pool for the slow release of copper and maintenance of copper homeostasis during a period of rapid growth and development.

The essentiality of copper for normal growth and development in young animals and for the maintenance of health in adult animals is well established. However, copper is highly reactive and at excessive levels may impair normal cellular function. Since copper intake may exceed the amount required, biological mechanisms have evolved to eliminate the toxic effects of excess copper while allowing the required levels to manifest their beneficial effects. Such a balance is maintained by homeostatic mechanisms located primarily in the liver and gastrointestinal tract of adult animals (1).

The copper content of milk is higher in rats than in most other mammals. For instance, a mean of 0.62 μg Cu/ml has been reported in human milk at the end of the first week of lactation (2) while rat milk varies during the first 9 days of lactation, decreasing from 6.8 μg Cu/ml for the first 3 days to 3.5 μg Cu/ml for Days 4 through 9 (3). Thus, nursing pups ingest a variable and relatively high amount of copper, which in turn could subject copper homeostasis to unusual stress for several days following birth. High hepatic copper levels (3–5) and slowly increasing ceruloplasmin (3, 6) suggest that such stress does occur in neonatal rats and that the liver has an important role in maintaining copper homeostasis during this period of rapid growth. Little information is available, however, concerning the levels of copper in the gastrointestinal tract or the role of the gastrointestinal tract in maintaining copper homeostasis in the neonatal rat. As part of an ongoing study at this laboratory to elucidate the mechanisms by which the gastrointestinal tract maintains copper balance, this paper presents results from a study of copper levels in the small intestine of neonatal rats and the influence of copper-binding proteins on these levels.

Materials and methods. Litters from Long–Evans dams were culled to eight pups immediately following parturition. The dams were raised and bred in the animal facilities at this laboratory and were main-

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tained on commercial rat chow (Purina)[3] throughout pregnancy and the postpartum period. At the various times reported under Results, the entire small intestine from pylorus to iliocecal valve was excised from each of the eight pups constituting an entire litter, and then rinsed and flushed thoroughly with ice-cold 0.01 *M* Tris–acetate, pH 7.4. Five of the intestines were then individually freeze-dried, weighed, and ashed in 1.0 ml of nitric acid, 0.5 ml of sulfuric acid, and 4.0 ml of 50% hydrogen peroxide in preparation for analysis by atomic absorption spectrophotometry (Varian Techtron Model 1250). The remaining three intestines were pooled, weighed, and homogenized in 10 vol (w/v) of cold, N_2-saturated 0.01 *M* Tris–acetate buffer, pH 7.4, with a motor-driven Potter–Elvehjem homogenizer fitted with a Teflon pestle. The cystolic fraction was obtained by centrifuging the homogenate at 106,000*g* for 1 hr.

Four milliliters of freshly prepared cytosol were applied to a 1.5 × 90-cm column of Sephadex G-75 equilibrated with 0.01 *M* Tris–acetate buffer, pH 7.4. Elution was with this same buffer at a flow rate of 30 ml per hour. Buffer solutions were continually bubbled with N_2 to prevent oxidation of copper-binding proteins, particularly metallothionein (7, 8). The column effluent was monitored continually at 254 nm (LKB Model 8300 Uvicord) and metal analysis was performed directly on each collected fraction (2 ml) by atomic absorption spectrophotometry.

The Sephadex G-75 column was calibrated with the following proteins of known molecular weight (Sigma Chemical Co., St. Louis, Mo.): cytochrome *c* (12,400), α-chymotrypsinogen A (25,000), and ovalbumin (45,000).

Copper-64 was obtained as $Cu(NO_3)_2$ in 1 *N* HNO_3 (New England Nuclear, Boston, Mass.). This was diluted to 5 ml with a solution of 0.005 *M* histidine in 0.9% NaCl and adjusted to pH 7.0 with 3 *N* NaOH. Histidine, because it has been reported to have no effect on the rate of copper absorption from the gastrointestinal tract (9), was chosen as a complexing agent to prevent the precipitation of copper as $Cu(OH)_2$ during pH adjustment. The oral dose administered to rat pups was 10 μg of ^{64}Cu (approximately 40 μCi) in a volume of 0.1 ml. Following the administration of ^{64}Cu, the pups were allowed to stay with their mothers until they were decapitated and their small intestines processed as described above. Radioactivity in fractions (2 ml) from the Sephadex G-75 column was measured in a gamma-well counter (Nuclear Chicago) set at the photopeak for ^{64}Cu.

Results. Values for the levels of copper measured in the small intestines of neonatal rats during their first 3 weeks of life are shown in Fig. 1. For comparison, zinc levels are also shown. Copper levels measured during this time period varied markedly. In pups taken by cesarean section on the 21st day of gestation (Day 0 in Fig. 1), the copper content of the small intestine was 36.2 ± 7.3 μg/g dry wt. Twelve hours following birth, copper levels had risen to 187.6 ± 28.6 μg/g dry wt and continued rising to a maximum of 302.6 ± 22.2 μg/g dry wt on Day 7. Following the 7th day, copper levels steadily declined, reaching a value of 6.4 ± 0.5 μg/g dry wt on Day 21. However, the total copper content of the small intestine remained elevated until Day 12 (inset, Fig. 1). While copper levels showed great variation during the 21 days following birth, zinc content of the small intestine varied little over the 21-day period.

When subjected to gel filtration on Sephadex G-75, cytosol prepared from neonatal small intestine eluted as two prominent copper-containing fractions corresponding to molecular weights of approximately 60,000 (fraction 1) and 9000 daltons (fraction 2). Figure 2 is a typical elution profile for cytosol from 4-day old rats. Of the copper applied to the column (10.3 μg), 60% appeared in fraction 2 while 40% eluted in fraction 1. Total copper

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

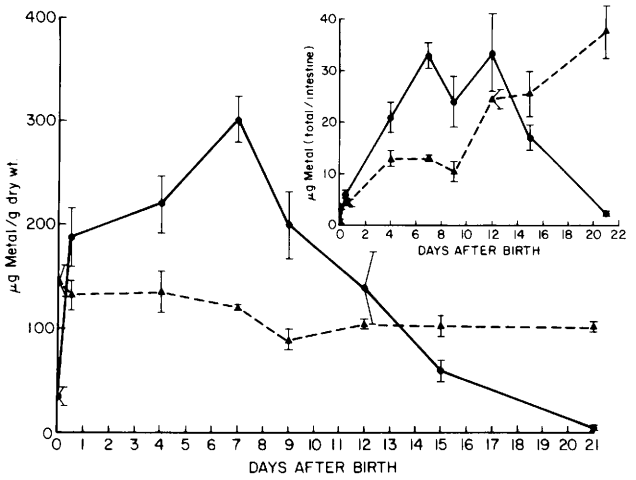

FIG. 1. Copper (●) and zinc (▲) content of neonatal rat small intestine during the 21 days following birth. Each data point represents the mean value ± standard deviation from five individual tissue samples. The zero-day value was obtained from tissue taken from pups delivered on the 21st day of gestation by cesarean section.

recovery from this column averaged 98 ± 23%.

Since the copper content of the neonatal small intestine varied with age, we also investigated the age dependency of the copper content of fractions 1 and 2. As illustrated in Fig. 3, the copper content of fractions 1 and 2, determined from copper in tubes 18–24 and 36–50, respectively, increased rapidly during the 7 days following birth and then declined to nondetectable levels by Day 21. The total copper content of the small intestine correlated positively with the copper contents of fractions 1 and

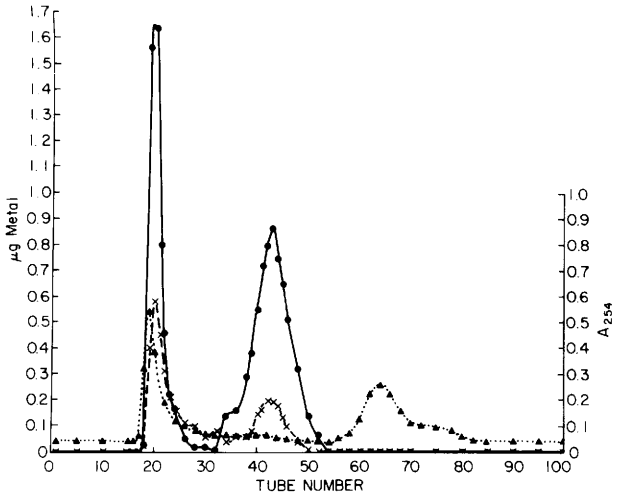

FIG. 2. Sephadex G-75 chromatography of the high speed supernatant prepared from the small intestines of 4-day-old rats. The column (1.5 × 95 cm, V_0 = 38 ml) was operated at 4° and eluted with N_2 saturated 0.01 *M* Tris–acetate, pH 7.4. Two-milliliter fractions were collected and Cu (●), Zn (X), and absorbance at 254 nm (▲) were monitored. Four milliliters of high speed supernatant, representing 0.4 g of tissue, were applied to the column.

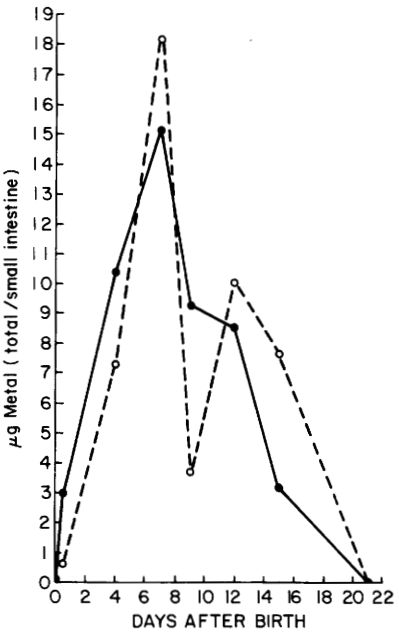

FIG. 3. Copper content, during the 21 days following birth, of fractions 1 (○) and 2 (●) from Sephadex G-75 chromatography of high-speed supernatant obtained from homogenates of small intestine. At each day indicated supernatant applied to the column represents 0.4 mg of tissue and was prepared from the pooled intestines of three pups who were littermates to those represented in the data in Fig. 1.

2 and the sum of the copper contents of fractions 1 and 2. Equations for the regression lines and the corresponding correlation coefficients are

$$y = 1.68x_1 + 7.71, \quad r = 0.83,$$
$$y = 2.08x_2 + 4.56, \quad r = 0.87,$$
$$y = 1.03x_{12} + 4.95, \quad r = 0.89,$$

where y represents the total copper content of the small intestine (values from the inset in Fig. 1), x_1 the copper content of fraction 1, x_2 the copper content of fraction 2, and x_{12} the sum of the copper contents of fractions 1 and 2.

The data shown in Fig. 3 also indicate that the accumulation of copper in fraction 2 may begin sooner following birth than the accumulation in fraction 1. To further investigate this possibility, data were pooled from several experiments in which the copper content of fractions 1 and 2 from rats of less than 5 days of age was measured. As shown in Table I, mean values for the copper content of fraction 1, when compared using Student's t test, show no significant increase between fetal and 12-hr-old rats, but increase significantly in 4-day-old rats. Similar comparison of the copper content of fraction 2 shows significant increases over the fetal value at both 12 hr and 4 days of age.

During the first week after birth, copper accumulated rapidly and maximally in both fractions 1 and 2. However, experiments utilizing ^{64}Cu indicated that fraction 2 contains a protein or proteins that sequester newly ingested copper in the neonatal rat. The elution from Sephadex G-75 of cytosol prepared from the small intestines of 5-day-old rats 2 hr following an oral dose of ^{64}Cu is shown in Fig. 4. Two ^{64}Cu-containing fractions were observed, but the amount of ^{64}Cu in the fractions was disproportionate; fraction 2 contained 60% and fraction 1 only 12% of the radioactivity applied to the column. The elution of a

TABLE I. COPPER CONTENT OF FRACTIONS 1 AND 2 FROM SEPHADEX G-75 CHROMATOGRAPHY

Age	μg Cu/g wet tissue[a]		No. of experiments
	Fraction 1	Fraction 2	
Fetus[b]	2.4 ± 1.9	0.2 ± 0.2	3
12 hr	1.6 ± 1.1[c]	12.2 ± 7.3[d]	3
4 days	10.5 ± 4.1[e]	20.9 ± 4.8[f]	4

[a] Mean value ± standard deviation.
[b] Delivered on the 21st day of gestation by cesarean section.
[c] Not significant compared to fetus.
[d] $P < 0.05$ compared to fetus.
[e] $P < 0.02$ compared to fetus.
[f] $P < 0.01$ compared to fetus.

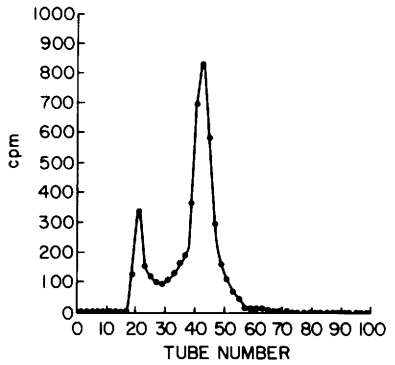

FIG. 4. Isotope elution pattern from a Sephadex 75 column of high-speed supernatant prepared from the small intestines of 5-day-old rats 2 hr following an oral dose of ^{64}Cu. Specific activity of the ^{64}Cu dose was 4 μCi/g.

single dominant radioactive peak is consistent with previous observations regarding the distribution of ^{64}Cu among the soluble duodenal proteins from rats (10) and chicks (11). Furthermore, the amount of ^{64}Cu in fraction 2 remained fairly constant through the 5- to 7-day period following birth. Two hours following the oral administration of ^{64}Cu to 5-day-old pups, the radioactivity in fraction 2 represented 0.50 μg ^{64}Cu/g wet tissue.[4] In littermates to these pups, who also received an identical dose of ^{64}Cu at the same time, the radioactivity in fraction 2 corresponded to 0.49 μg ^{64}Cu/g wet tissue at 17 hr and 0.48 μg ^{64}Cu/g wet tissue at 42 hr following administration of the ^{64}Cu. At the same time, the ^{64}Cu in fraction 1, while remaining lower than that in fraction 2, showed a slight increase from 0.10 μg ^{64}Cu/g wet tissue 2 hr following the dose to 0.18 μg ^{64}Cu/g wet tissue 40 hr later.

Discussion. The studies reported here demonstrate that the small intestines of neonatal rats accumulate considerable amounts of copper during the first 3 weeks of life, reaching a maximum level a week following birth. This observation agrees with that seen in normal mice who achieve maximum levels for gut copper when they are 1–5 days old (10). Furthermore, the change in tissue copper levels paralleled the change in the copper content of two cytosolic protein fractions, one of about 60,000 daltons and the other 9000 daltons.

The two fractions obtained from gel chromatography both contain proteins that affect the tissue copper levels of the small intestine in newborn rats. However, 2 hr following oral administration of ^{64}Cu to 5-day-old rats the low-molecular-weight fraction contained five times the amount of ^{64}Cu present in the high-molecular-weight fraction. This finding suggested that the low-molecular-weight fraction is more active than the higher-molecular-weight fraction in sequestering ingested copper in the neonatal rat small intestine. The low turnover of ^{64}Cu in the low-molecular-weight fraction supports that suggestion.

The presence of a low-molecular-weight copper-binding fraction in intestinal cytosol has been reported for several species (10–13) but, as in this study, the proteins in this fraction were not identified. Although intestinal metallothionein has been implicated in copper absorption (14), evidence also exists describing a markedly different copper-binding protein of about 10,000 daltons in the rat intestine (15). Regardless of the identity of the protein or proteins, our data show that the low-molecular-weight fraction contributes significantly to the tissue level of copper in the small intestine. We suggest that it provides a reservoir for copper presented to the neonatal rat in relatively copper-rich early milk and serves as a mechanism for maintaining homeostasis during a period of rapid growth and development.

Copper in the high-molecular-weight fraction also correlated with tissue copper and varied in a manner similar to the variations observed in the copper content of the low-molecular-weight fraction. However, the increase in the copper content of the high-molecular-weight fraction did not begin until after 12 hr following birth, while the copper in the low-molecular-weight fraction had markedly increased by this time. This time lag might indicate a

[4] Values were obtained from the radioactivity measured in fractions 1 and 2 following Sephadex G-75 chromatography of cytosol prepared from the pooled intestines of three pups in the case of the 2- and 17-hr data and of two pups for the 42-hr data.

dynamic relationship between the two fractions. Exchange of copper between the low- and high-molecular-weight fractions could occur after the copper is first bound by protein of the low-molecular-weight fraction, or the lower-molecular-weight protein could be a precursor for the larger protein. Such relationships appear to exist between cytosolic copper-binding proteins in rat liver (16). It is also possible that the copper in the two fractions is supplied by different sources; that in the low-molecular-weight fraction from ingested copper and that in the high-molecular-weight fraction from circulating copper.

Although the identity and function of the proteins in the two fractions remain to be determined, our data show that they are involved in producing the copper levels observed in neonatal rat small intestine. Our findings also suggest that the neonatal rat may serve as a good experimental model for studying the mechanism of copper transport.

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