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Molecular Localization of Copper and Zinc in Rat Fetal Liver in Dietary and Drug-induced Copper Deficiency

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SUMMARY: The teratogenicity of copper deficiency is well known, but underlying mechanisms have not been delineated. One method of studying the biochemical lesions of copper deficiency is the use of chelating drugs with different chemical characteristics. The teratogenicity of a copper deficient diet and of diets containing either D-penicillamine or triethylenetetramine is quite different, although all three diets result in decreased fetal liver copper levels. Feeding D-penicillamine can result in decreased fetal liver zinc, while feeding triethylenetetramine can result in increased fetal liver zinc. The effect of these three diets on fetal liver copper and zinc molecular localization was determined. Gel filtration showed that fetal liver copper and zinc in controls was localized in 3 fractions with MWs of > 50,000 (H), 30,000 (I) and 8-10,000 (L). Independent of dietary treatment, as liver copper diminished, copper was missing first from the L peak, then the I peak and with severe deficiency, from the H peak. Drug induced increases and decreases in fetal liver zinc were reflected in the L peak. These data suggest that the absolute levels of copper in the liver of the term fetus determines the distribution of the element among its binding ligands.

INTRODUCTION: The teratogenic effects of copper deficiency have been established for several species (1,2), but the biochemical lesions underlying the morphological abnormalities produced by its deficiency have not been defined. Copper deficiency can be induced through simple dietary insufficiency, or by feeding drugs that chelate copper and increase its excretion from the body. D-penicillamine (DPA) and triethylenetetramine (TETA) are examples of such drugs (3,4). The teratogenic expression of copper deficiency caused by DPA or TETA is quite different, however, from that caused by dietary copper deficiency. Gross abnormalities are not normally observed in fetuses from dams fed a copper deficient diet during pregnancy (5,6); fetuses from dams fed TETA during pregnancy are often very edematous (4), while fetuses from DPA-fed dams show multiple gross malformations (3). These differences suggest that dietary copper deficiency and the copper chelating drugs may be affecting copper metabolism at different points. While several copper compounds, such as ceruloplasmin, superoxide dismutase and

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metallothionein are known to have important physiological functions (7,8) the effect of copper deficiency during pregnancy on the distribution of copper among different ligands in fetal tissue has not been characterized.

In order to determine the sites of action of these dietary and drug treatments, we have investigated the effects of feeding a copper deficient diet, or diets containing DPA or TETA, on the molecular localization of fetal liver copper. In addition, the molecular localization of fetal liver zinc was studied as the tissue concentration of this element can also be affected by DPA and TETA (3,4).

MATERIALS AND METHODS: Adult female Sprague-Dawley rats (190-200 g) were purchased from a commercial vendor (Simonsen Labs, Gilroy, CA) and housed in stainless steel cages in a temperature and light controlled room (22-23°C, 12 h light/dark cycle). All animals were acclimated for a minimum of 7 days during which time they were fed a complete purified diet containing 6 μg Cu/g and 100 μg Zn/g (control diet). The detailed composition of the diet has been described previously (3). Females were mated overnight with males of the same strain. Mating (day 0 of gestation) was confirmed by the presence of vaginal plugs and sperm positive vaginal smears.

On day 0 of gestation rats were divided into 7 groups and fed throughout gestation the control diet, the control diet containing DPA at 0.17, 0.83 or 1.66% of the diet, TETA at 0.17 or 0.83% of the diet, or a diet deficient in copper (≤ 0.70 μg Cu/g diet). The levels of drugs used were based on previous studies (3,4). On day 21 of gestation dams were killed and litters were removed by C-section. Fetal liver was removed and immediately frozen in liquid nitrogen.

Tissues were homogenized in cold 0.1 M ammonium acetate buffer, pH 6.5. The buffer had been equilibrated with nitrogen in order to minimize oxidative changes of the metalloproteins (9). Homogenates were centrifuged at 10,000 x g for 30 min. at 4°. Supernatants were immediately applied to gel filtration columns (1.6 x 90 cm) equilibrated in the same buffer. The gels (Sephadex G-75; Pharmacia Fine Chemicals, Piscataway, NJ) had been treated previously with sodium borohydride in order to reduce charged groups on the gel matrix which can cause nonspecific absorption of trace elements. With this treatment, recovery from the columns was 95-100% (10). A flow rate of 60 ml/h was used. Chromatographic fractions were analyzed for copper and zinc by atomic absorption spectrophotometry (IL 551, Instrumentation Laboratories, Inc., Wilmington, MA).

ELECTROPHORESIS: Polyacrylamide gel electrophoresis was performed using a continuous system in 0.9 M acetic acid at pH 2.5. The concentration of acrylamide was 8% and that of bisacrylamide was 2% of the total concentration of acrylamide (T_8C_2). Samples (100 μg) were applied and gels were run at 2mA/gel for 3 h at 4°C. Following staining with Amido Black B, gels were destained with 0.9M acetic acid.

RESULTS: Consistent with previous studies, there were no gross abnormalities in the fetuses of the control or copper deficient groups. For both DPA and TETA-fed groups, the frequency of abnormal fetuses increased with higher levels of drugs, but the pattern of abnormalities was quite different between the two drug groups. In fetuses of DPA-treated dams, anomalies included cutis laxa, spina bifida, lung

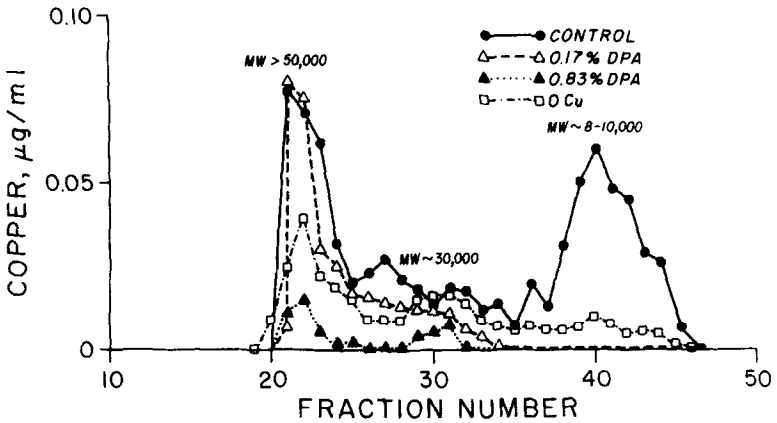

Figure 1. Gel filtration of fetal liver supernatent (25% homogenate) on Sephadex G-75 in 0.1M ammonium acetate buffer at pH 6.5.

dysplasia and abdominal herniations. In contrast, massive hemorrhages and edema were most often observed in the TETA group; abnormalities rarely seen in the DPA fetuses. A detailed description of the abnormalities noted in the DPA and TETA fetuses has been published together with tissue trace element data (3,4). Fetal liver copper concentration was significantly lower in the copper-deficient and drug-treated groups than in controls. Copper levels were lowest in fetuses from dams fed the highest level of the drugs. The concentration of zinc in fetal liver was lower than control level in the high DPA group, and higher than control in the TETA groups, but similar in control and copper-deficient fetuses.

Gel filtration chromatography of liver from control fetuses on Sephadex G-75 resulted in three copper peaks (Figure 1). The molecular weight of the fraction at the first peak was higher than 50,000; the second peak area had a molecular weight of approximately 30,000, and the third peak had a molecular weight of 8-10,000. Most of the copper was found in the third peak, which we have shown by gel electrophoresis to contain metallothionein. We have identified the protein in the second area as copper, zinc-superoxide dismutase with a molecular weight of approximately 32,000.

In fetuses from DPA-fed rats, as liver copper concentration diminished with increasing levels of DPA, the amount of copper in the metallothionein peak decreased first; copper in the superoxide dismutase peak decreased next, and with severe deficiency, the size of the high molecular weight peak decreased (Figure

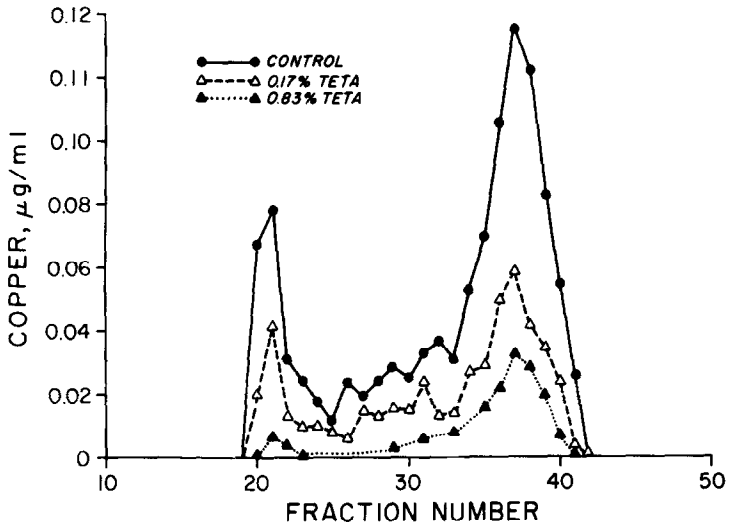

Figure 2. Gel filtration of fetal liver supernatent (25% homogenate) on Sephadex G-75 in 0.1M ammonium acetate buffer at pH 6.5.

1). The chromatogram shown for 0.17% DPA is an example of a particularly low liver copper. Fetuses from dams fed the copper deficient diet had liver copper concentrations similar to those of fetuses from dams fed a 0.17% DPA diet and had similar gel filtration patterns (Figure 1). The effects of TETA feeding were similar to those of feeding DPA with respect to fetal liver copper concentration and molecular localization although the effect on metallothionein copper were not as pronounced (Figure 2).

The molecular distribution of zinc in control fetal liver was similar to that of copper (Figure 3). With increasing levels of DPA fed, the amount of zinc in the metallothionein peak decreased (Figure 3). Zinc distribution in fetal liver from the copper deficient group was similar to that observed for livers of control fetuses (Figure 3). In contrast, with increasing levels of TETA, the size of the metallothionein zinc peak increased (Figure 4). When subjecting the last zinc peak to gel electrophoresis, it was found that the amount of metallothionein increased with the level of TETA fed.

DISCUSSION: The results of this study show that despite considerable differences in the frequency and severity of congenital abnormalities produced by the treatments used, the molecular localization of copper in fetal liver was similar. The localization of the element thus appears to be determined by the absolute

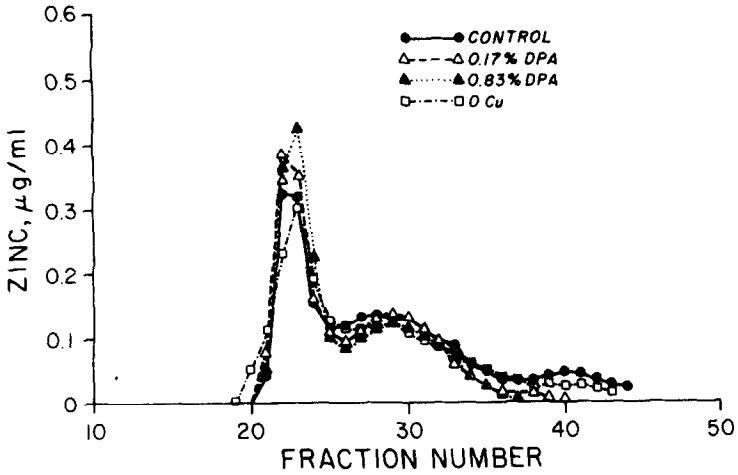

Figure 3. Gel filtration of fetal liver supernatent (25% homogenate) on Sephadex G-75 in 0.1M ammonium acetate buffer at pH 6.5.

amount of copper in the liver, regardless of the mode of induction of copper deficiency. Considering the different chemical properties of the two chelating drugs and the different teratogenicity of the dietary treatments these findings are surprising. The observation of copper localization in 3 peaks is consistent with reports that hepatic cytoplasm labeled with ^{64}Cu or ^{67}Cu from mouse (8,11)

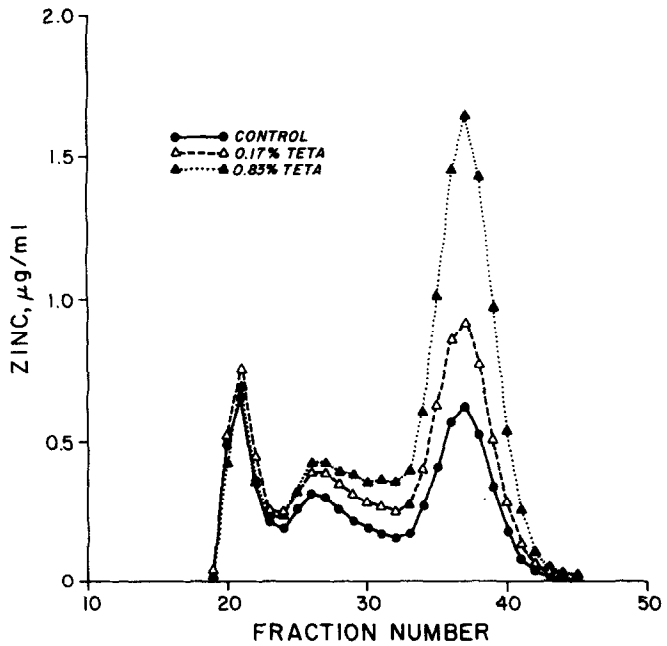

Figure 4. Gel filtration of fetal liver supernatent (25% homogenate) on Sephadex G-75 in 0.1M ammonium acetate buffer at pH 6.5.

and chicken (12) also yielded 3 peaks following chromatography on Sephadex G-75 with approximate molecular weights of ≥ 50,000, 30,000 and 8-10,000. With decreasing concentration of copper in fetal liver, copper was lowest first in the metallothionein peak, second in the superoxide dismutase peak and finally in the peak with a molecular weight higher than 50,000.

These results do not explain the differences in teratogenicity among the dietary treatments, but are consistent with the hypothesis that metallothionein copper accretion occurs after other copper metalloproteins are synthetized and support the idea that metallothionein copper is a storage form of the element. Prohaska (8) has recently reported that hepatic cytosol of copper-deficient suckling mouse pups given an oral dose of ^{67}Cu had a higher amount of ^{67}Cu associated with the superoxide dismutase "peak", and a lower amount associated with the metallothionein peak than did hepatic cytosol of control mouse pups, suggesting that copper repletion of hepatic cytosolic copper ligands in the neonate occurs in the same order as copper is accumulated in fetal liver.

Low concentrations of copper in fetal liver did not lower the amount of zinc in the metallothionein peak, indicating copper does not affect zinc metallothionein synthesis. This observation is not surprising considering the known differences in physical properties of copper metallothionein compared to zinc metallothionein (13-16), and the fact that under normal conditions a cellular accumulation of one metal does not appear to affect the accumulation of the other (17). Although it is important to note that very high concentrations of one metal have been reported to influence the amount of the other bound by metallothionein(s), the mechanisms by which this occurs are not clear (18).

Zinc-metallothionein synthesis in fetal liver, as well as in maternal kidney was affected by feeding TETA, but was not affected in maternal liver (unpublished data). Thus, zinc-metallothionein metabolism may be under different metabolic controls in these tissues, or, alternatively, they may differ in absorption of the drug or drug-metal complex. The mechanisms by which TETA increases fetal liver zinc are under investigation. The observation that TETA differentially affects zinc-metallothionein and copper-metallothionein in fetal liver is consistent with

the observations of Weiner and Cousins (19) on the differential regulation of copper and zinc metabolism in cultured adult rat liver parenchymal cells.

It is possible that the different teratogenic effects of the diets are due in part to their varying effects on zinc metabolism. However, copper supplementation of the drug-containing diets alleviates their teratogenicity (20,21). Fetuses from dams fed copper-supplemented DPA diets had relatively normal levels of liver copper and zinc (20), while those from dams fed copper-supplemented TETA diets had normal copper and very high zinc levels in liver (21). Another explanation for the different teratogenic expression of DPA, TETA, and dietary copper deficiency is that the rate at which copper deficiency is induced in the embryo/fetus may be different among the treatments. This hypothesis is under investigation.

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