PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 598–605 (1983)

Teratogenesis and Low Copper Status Resulting from Triethylenetetramine in Rats (41693)

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Abstract. The teratogenicity of triethylenetetramine (TETA) was studied using the Sprague–Dawley rat. TETA was fed during pregnancy at levels of 0 (control), 0.17, 0.83, or 1.66% in a complete purified diet. The frequency of resorptions and the frequency of abnormal fetuses at term increased with increasing levels of the drug. Maternal and fetal tissue copper levels were significantly lower in the TETA groups than in controls, with levels decreasing in a dose-related manner. Maternal kidney and fetal liver zinc levels increased within the TETA groups in a dose-related manner. Maternal liver iron was increased in the high-dose group compared to controls. Fetal iron concentration and maternal and fetal manganese level were not significantly affected by the drug. These results show that TETA can be a teratogenic agent. Furthermore, the results suggest that the teratogenicity of the drug may be due in part to induction of copper deficiency, and perhaps through induction of zinc toxicity.

Metal chelating drugs are frequently used clinically to bind metals and enhance their excretion or prevent their absorption. Triethylenetetramine (TETA) is a chelating drug used in the treatment of Wilson's disease, a genetic disorder in which excess copper is deposited in the body. TETA is used primarily when patients have an adverse reaction to D-penicillamine (DPA), the drug most often prescribed for this disease (1, 2). Research in our laboratory has shown that DPA is teratogenic in rats, and causes copper deficiency (3, 4). The teratogenicity of DPA is probably related to the copper deficiency it induces but may also involve other effects stemming from its capacity for sulfhydryl binding. In contrast to DPA, which binds copper through a sulfhydryl group, TETA binds copper through electronegative nitrogens, thus the potential teratogenicity of TETA could be different from that of DPA. The present study was designed to evaluate the teratogenicity of TETA, and to determine its effects on the concentration of several trace elements in maternal and fetal tissues.

Materials and Methods. Virgin female Sprague-Dawley rats weighing 190–200 g were purchased from a commercial source (Simonsen Laboratories, Gilroy, Calif.). All animals were individually housed in suspended stainless-steel cages in a temperature- and light-controlled room (22–23°C, 12 hr light–dark cycle), and were acclimated for a minimum of 7 days during which time they were fed a complete purified diet containing 6 μg copper/g diet, and 100 μg zinc/g diet. The composition of the diet was protein (casein) 30%; fat 8%; cerelose 54.5%; salt mixes 6.0%; and vitamin mixes 1.5%. Details on the salt and vitamin mixes have been previously published (4). Females were mated overnight with males of the same strain fed a pelleted stock diet (Purina Rat Chow, Ralston Purina Co., St. Louis, Mo.). Mating (Day 0 of gestation) was indicated by the presence of vaginal plugs and sperm-positive vaginal smears.

On Day 0 of gestation, rats were divided into four groups and fed either the control diet, or the control diet containing 0.17%, 0.83%, or 1.66% TETA (TETA · 4HCl, 99+% pure, Aldrich Chemical Co., San Leandro, Calif.). The dosages were selected to approximate the range of dosages administered to humans as reported in the literature, and to enable detection of dose-related responses. Daily dosages in humans of 800 mg are common, and dosages as high as 4.5 g have been reported (5). The average human consumes about 400– 500 g of food (dry matter)/day. A dose of 800 mg/day of TETA therefore equals 0.16–0.20% of the diet, while a dose of 4.6 g/day would equal 0.92–1.15% of the diet. For simplicity, the dose-groups will be referred to as low (0.17%), medium (0.83%), and high (1.66%). These designations are not meant to imply that the dosages from which

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they were derived are low, medium, or high dosages in humans, but are intended only as labels of the drug in relation to others in this experiment. Throughout all experiments, daily food intake and weekly weight were recorded.

On Day 21 of gestation the females were anesthetized with ether. Laparotomy was performed and the diaphragm and the lower ribs were cut to allow access to the heart. Blood was collected by cardiac puncture in syringes containing zinc-free heparin (Sigma, St. Louis, Mo.), transferred into centrifuge tubes, and centrifuged for 20 min at 3000*g*. Plasma was removed with transfer pipets (Markson Science Inc., Del Mar, Calif.) and stored in acid-washed plastic vials until analyzed.

The uterus was examined intact and the number of implantation sites was determined by counting metrial nodes. The uterus was then cut open, the live fetuses and their placentas were removed, cleaned of adhering membranes, and weighed. The number of live, dead, and resorbed fetuses was counted. Live fetuses were examined for gross external abnormalities. From each litter, some fetuses were used whole for trace element analysis, some were eviscerated and stored in 70% ethanol, and some were fixed in Bouin's solution. The livers removed from the eviscerated fetuses were stored in acid-washed plastic vials and frozen for trace element analysis. The maternal brain, liver, kidney, uterus, and gastrocnemius muscle were dissected, cleaned of adhering fatty tissue, and stored for trace elements analysis. All tissue samples were placed in acid-washed plastic vials and frozen until analyzed for iron, copper, zinc, and manganese. Trace element concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer Model 370, Norwalk, Conn.), by the method of Clegg *et al.* (6). Using this technique, recovery of added metal was 98–101%.

Assessment of internal malformation. Fetuses fixed in Bouin's solution were examined for gross internal malformations using a modification of Wilson's razor-blade technique (7). Those preserved in 70% alcohol were cleared in 1% potasssium hydroxide, stained with alizarin red, destained in Mall's solution, and stored in 50% glycerol (8) for observation of the skeleton.

Statistical procedures. Statistical significance of the experimental data was established by Student's *t* test (9). Comparisons were made between the individual TETA groups and the control group. Data are shown as the mean ± SEM.

Results. *Reproductive parameters.* Food intake did not differ significantly between rats fed the low or medium amount of TETA and the control group, but food consumption was lower in the high-dose group (Fig. 1). Table I summarizes the effect of TETA on repro-

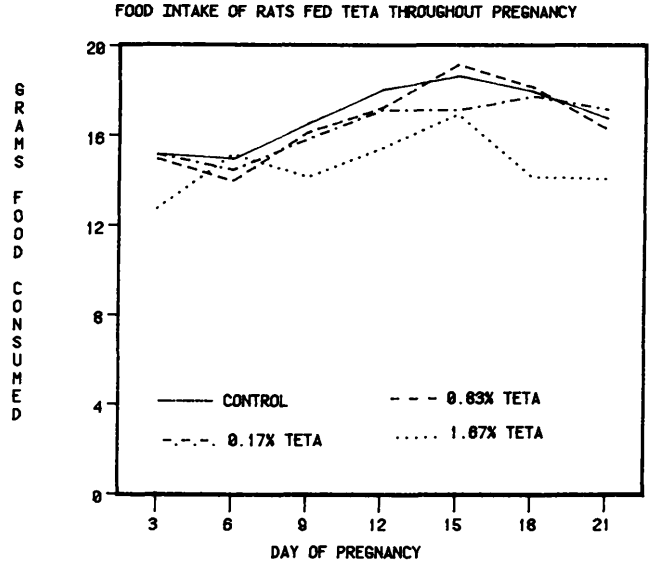

FIG. 1. Mean daily food intake of rats fed triethylenetetramine throughout pregnancy.

TABLE I. REPRODUCTIVE PARAMETERS IN RATS FED TRIETHYLENETETRAMINE DURING PREGNANCY

TETA dose (% of diet) (No. dams)	Weight gain[a] (g)	Average litter size (*N*)	Fetal weight wet weight (g) (No. litters)	Placental weight wet weight[b] (g) (No. litters)	Crown–rump length[c] (cm) (No. measured)
0 (7)	130 ± 8[d]	10.4 ± 0.6	5.16 ± 0.10 (7)	0.51 ± 0.03 (7)	3.9 ± 0.1 (16)
0.17 (5)	131 ± 10	10.4 ± 0.2	5.28 ± 0.15 (5)	0.47 ± 0.02 (5)	4.0 ± 0.1 (16)
0.83 (9)	107 ± 4*	10.3 ± 0.7	5.15 ± 0.11 (11)	0.53 ± 0.03 (9)	3.9 ± 0.0 (29)
1.66 (5)	90 ± 7**	9.6 ± 1.3	4.62 ± 0.11** (5)	0.47 ± 0.02 (5)	3.4 ± 0.1*** (14)

[a] Calculated as weight on Day 20 of gestation minus weight on Day 0 of gestation.
[b] Calculated as the means of the litters.
[c] Length from crown of head to base of tail, measured only in animals fixed in Bouin's solution.
[d] Values expressed as means ± SEM.
* $P < 0.05$ compared to control by Student's *t* test.
** $P < 0.01$ compared to control by Student's *t* test.
*** $P < 0.001$ compared to control by Student's *t* test.

ductive parameters, including materal weight gain during pregnancy, average litter size, fetal and placental weight, and fetal crown–rump length. Dams fed the low TETA dose gained the same weight during pregnancy as controls, while the medium- and high-dose groups gained less. The litters of drug-fed dams were similar in size to those of control dams, although the average fetal weight in the high-dose group was lower than that in controls ($P < 0.01$). Placental weight did not differ significantly among the treatment groups. Crown–rump length was similar among fetuses from control, low, and medium groups, but was significantly less in fetuses from the high-dose group than in controls ($P < 0.001$) (Table I).

Teratological observations. Litter outcome is summarized in Table II, including frequency of resorbed implantation sites, abnormal fetuses, and total implantation sites affected. The frequency of resorptions was higher in the drug-fed groups than in controls (from 5.8 to 18.8%, compared to 0%). There was a striking increase in the frequency of abnormal fetuses corresponding to increasing drug dose (0, 25.6, and 100% compared to 0% in controls). Abnormalities were primarily massive hemorrhaging and edema (Fig. 2). Upon visual inspection of the cleared skeletons, malformations were not consistently noted, although the degree of ossification appeared to be reduced in the caudal vertebrae and phalanges of the high-dose group based on less alizarin-red-stainable material.

Tissue trace element concentrations. The trace element concentrations of maternal and fetal tissues are summarized in Tables IIIA and IIIB.

Maternal tissues. Copper. Liver copper concentration was significantly lower than control in the medium- (73% of control, $P < 0.01$) and high- (38%, $P < 0.001$) dose groups. Plasma copper levels were also sig-

TABLE II. LITTER OUTCOME AFTER FEEDING TRIETHYLENETETRAMINE DURING PREGNANCY

TETA dose (% of diet)	Litters (*N*)	Total sites (*N*)	Total dead (*N*)	Total live (*N*)	Total abnormal (*N*)	Resorbed[a] (%)	Abnormal[b] (%)	Total sites[c] affected (%)
0	7	73	0	73	0	0	0	0
0.17	5	52	3	49	0	5.8	0	5.8
0.83	9	93	7	86	22	8.7	25.6	33.0
1.66	5	48	9	39	39	18.8	100.0	100.0

[a] (Dead/total sites) × 100.
[b] (Total malformed)/(total live) × 100.
[c] (Malformed + dead)/(total sites) × 100.

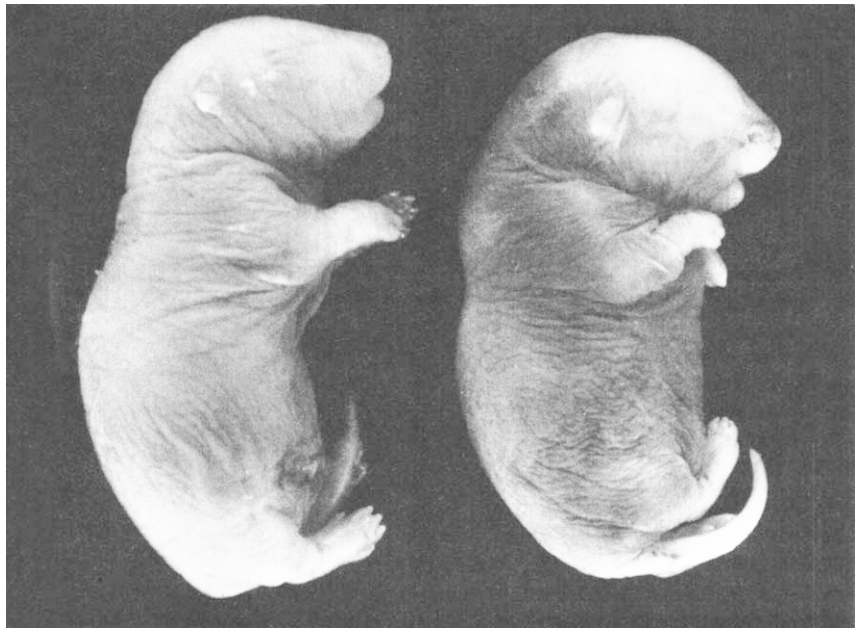

FIG. 2. External appearance of fetuses from dams fed the control diet (left) and 1.66% TETA (right) throughout pregnancy. Note massive hemorrhaging and edema of fetus from drug-treated dam.

nificantly lower than normal in the medium (35% of control, $P < 0.01$), and high (0.04%, $P < 0.01$) groups. Kidney, muscle, brain, and uterus copper concentrations were not significantly affected by TETA.

Zinc. Liver zinc concentration was not significantly affected by TETA. Plasma zinc concentration also did not differ significantly among the groups. However, in the kidney, zinc was higher than in controls in the low- (123%, $P < 0.01$), medium- (182%, $P < 0.001$), and high- (239%, $P < 0.001$) dose groups. Muscle zinc concentration was also higher in the TETA-fed groups than in controls, but the increase was significant only in the medium- ($P < 0.01$) and high- ($P < 0.01$) dose groups. Brain and uterus zinc concentrations were not affected by TETA.

Iron. With the exception of liver, maternal tissue iron was unaffected by addition of TETA to the diet. Liver iron was similar in the control, low- and medium-dose groups, but it was higher in the high-dose group than in controls (272%, $P < 0.001$). Manganese concentrations were not consistently affected by TETA.

Fetal tissues. Copper concentration in whole fetuses was lower than normal in medium- (43%, $P < 0.001$), and high- (15%, $P < 0.001$) dose groups. Liver copper concentration was lower in all TETA groups with 61% (N.S.), 33% ($P < 0.001$), and 2% ($P < 0.001$) of control values in the low-, medium-, and high-dose groups, respectively. Placental copper concentration was lower in all drug-treated groups than in controls; 65% (N.S.), 35% (N.S.), 17% ($P < 0.001$) of control values in the low-, medium-, and high-dose groups, respectively.

Zinc concentration in the whole fetus was 153, 215, and 240% of control values in the low-, medium-, and high-dose groups ($P < 0.001$, all groups). Liver zinc concentration was 165, 235, and 247% of control values in the low-, medium-, and high-dose groups, respectively ($P < 0.001$, all groups). Placental zinc concentration was not affected by TETA treatment.

Iron concentration was not consistently affected by TETA in whole body, fetal liver, or placenta.

Manganese concentration in fetal liver and placenta was not determined. Whole-body manganese concentration increased slightly with the feeding of TETA.

For a limited number of maternal and fetal tissues calcium and magnesium levels were

TABLE IIIA. TRACE ELEMENT CONCENTRATION IN TISSUES FROM RATS FED TRIETHYLENETETRAMINE DURING PREGNANCY

Tissue (*N*)	Fe	Cu	Zn	Mn
Liver				
0 (7)	68.7 ± 9.8[a]	4.57 ± 0.29	24.1 ± 0.9	2.25 ± 0.12
0.17% (5)	72.0 ± 8.9	3.62 ± 0.16[b]	23.1 ± 0.8	2.24 ± 0.17
0.83% (9)	50.0 ± 6.5	3.35 ± 0.23[b]	26.5 ± 0.7	2.48 ± 0.29
1.66% (5)	187.7 ± 22.1[c]	1.75 ± 0.10[c]	26.5 ± 0.4	2.62 ± 0.33
Plasma				
0 (7)	1.5 ± 0.3	1.27 ± 0.11	0.86 ± 0.13	*d*
0.17% (5)	2.34 ± 0.41	0.91 ± 0.22	0.82 ± 0.08	*d*
0.83% (9)	0.75 ± 0.17	0.45 ± 0.07[c]	0.64 ± 0.06	*d*
1.66% (5)	0.99 ± 0.25	0.06 ± 0.03[c]	0.67 ± 0.08	*d*
Kidney				
0 (7)	54.1 ± 5.0	5.76 ± 0.70	22.0 ± 0.9	0.84 ± 0.02
0.17% (5)	61.8 ± 0.8	4.27 ± 0.39	27.1 ± 1.1[b]	0.71 ± 0.04
0.83% (9)	52.8 ± 2.9	5.74 ± 0.43	40.0 ± 2.5[c]	0.82 ± 0.05
1.66% (5)	63.3 ± 2.8	4.19 ± 0.28	52.7 ± 4.2[c]	0.77 ± 0.03
Muscle				
0 (7)	12.6 ± 1.1	1.58 ± 0.28	8.3 ± 0.6	0.08 ± 0.01
0.17% (5)	12.3 ± 0.7	1.30 ± 0.21	9.7 ± 0.8	0.12 ± 0.02
0.83% (9)	12.5 ± 0.8	1.90 ± 0.38	11.2 ± 0.6[b]	0.15 ± 0.02
1.66% (5)	13.8 ± 0.4	1.57 ± 0.45	10.8 ± 0.4[b]	0.20 ± 0.03[b]
Brain				
0 (7)	16.8 ± 0.8	2.49 ± 0.10	11.6 ± 0.2	0.43 ± 0.20
0.17% (5)	16.4 ± 0.8	2.49 ± 0.10	11.6 ± 0.2	0.43 ± 0.04
0.83% (9)	15.0 ± 0.4	2.32 ± 0.05	11.6 ± 0.3	0.49 ± 0.03
1.66% (5)	15.1 ± 1.3	2.32 ± 0.06	11.4 ± 0.7	0.35 ± 0.04

[a] Values expressed as means ± SEM; μg/g wet weight of tissue or μg/ml plasma.
[b] $P < 0.01$ compared to control.
[c] $P < 0.001$ compared to control.
[d] Below detectable limits of flame absorption spectrophotometry.

determined. TETA had no pronounced effect on these elements (unpublished data).

Discussion. The resorption and malformation data presented here show that TETA, like DPA, can be a teratogenic and embryotoxic agent, capable of producing significant fetal mortality and abnormalities. The observation that maternal food intake and weight gain were similar among the various groups rules out the possibility that the teratogenicity was due to a drug-induced suppression of food intake. However, despite similar food intake, maternal weight gain was lower in the groups fed the two higher doses of TETA than in controls. Thus the feeding of TETA apparently results in some energy wasting, which may be a contributing factor to the teratogenicity of this drug. The mechanism underlying the energy wasting is unknown. All dams fed TETA, regardless of dose, appeared healthy and normal throughout the experimental period.

The mechanisms leading to the fetal death and abnormalities caused by TETA are unknown, although the data presented here suggest that copper is involved. There are numerous reports on the teratogenicity of dietary copper deficiency (10). At all of the levels tested, TETA caused low copper concentration in maternal liver and plasma, and this effect was dose-related; tissue copper concentrations were lower in dams receiving higher drug dosages. The relative lack of effect of TETA on copper levels in other maternal tissues was surprising, and is in contrast to the results of feeding DPA (4). On the other hand, the effect of the two drugs on plasma copper is similar,

TABLE IIIB. TRACE ELEMENT CONCENTRATION IN TISSUES FROM RATS FED TRIETHYLENETETRAMINE DURING PREGNANCY

Tissue (*N*)	Fe	Cu	Zn	Mn
Uterus				
0 (7)	43.5 ± 4.1[a]	3.57 ± 0.77	18.5 ± 1.4	*d*
0.17% (5)	39.0 ± 2.3	1.75 ± 0.37	14.1 ± 1.1	*d*
0.83% (9)	38.1 ± 3.7	3.78 ± 0.62	19.5 ± 0.8	*d*
1.66% (5)	53.3 ± 8.4	2.49 ± 0.63	21.8 ± 0.5	*d*
Whole fetus				
0 (7)	48.9 ± 3.4	1.40 ± 0.07	15.5 ± 0.6	0.18 ± 0.02
0.17% (5)	56.3 ± 1.0	1.50 ± 0.20	23.7 ± 1.4[c]	0.19 ± 0.02
0.83% (9)	48.2 ± 3.1	0.60 ± 0.08	33.3 ± 1.4[c]	0.22 ± 0.01
1.66% (5)	48.2 ± 3.6	0.21 ± 0.01	37.2 ± 1.2[c]	0.32 ± 0.04[c]
Fetal liver				
0 (7)	219 ± 19	15.30 ± 1.2	71.6 ± 4.3	*d*
0.17% (5)	205 ± 15	9.40 ± 1.8	118.2 ± 9.3[c]	*d*
0.83% (9)	107 ± 17	5.10 ± 10.[c]	168.5 ± 6.7[c]	*d*
1.66% (5)	200 ± 24	0.30 ± 0.2[c]	196.2 ± 12.3[c]	*d*
Placenta				
0 (7)	78 ± 10	1.70 ± 0.20	11.1 ± 0.6	*d*
0.17% (5)	104 ± 10	1.10 ± 0.20	13.3 ± 0.6	*d*
0.83% (9)	107 ± 11	0.60 ± 0.0[b]	12.4 ± 0.5	*d*
1.66% (5)	80 ± 3	0.30 ± 0.2[b]	8.4 ± 0.1	*d*

[a] Values expressed as means ± SEM; μg/g wet weight.
[b] $P < 0.01$ compared to control.
[c] $P < 0.001$ compared to control.
[d] Below detectable limits for flame atomic absorption spectrophotometry.

indicating that the influence of TETA and DPA on the mobilization of tissue copper is different.

As maternal plasma is the sole source of essential nutrients for the fetus, it could be predicted that reduction of maternal plasma copper concentration would be reflected in the fetus as well. Fetal copper concentrations were in fact consistent with such a reduction, decreasing with increasing levels of drug. It is also important to note that fetuses from dams fed the lowest amount of TETA had low liver copper concentrations, but were not morphologically abnormal. The absence of gross external and internal defects enhances the significance of the low copper concentrations in these animals, as it suggests that the effect of TETA on copper precedes the observation of abnormalities. This finding supports the hypothesis that copper deficiency is related to the teratogenicity of TETA. However, it is also possible that less obvious defects, such as microscopic or ultrastructural anomalies, or biochemical lesions, were present but were not identified by the methods used in this experiment. The three resorptions occurring in the low-dose group were in different litters.

The abnormalities seen in the medium- and high-TETA-dose groups were different from those of fetuses from DPA-fed dams. In the TETA groups, massive hemorrhages and edema were prominent, while DPA produced cutis laxa, spina bifida, abnormal diaphragm, lung dysplasia, and abdominal herniations (4). The biochemical explanation for this differing teratogenicity of the drugs is not known. One possibility is that the two drugs are interfering with copper metabolism at different metabolic points, or at different times during development. An additional explanation is that they affect metals other than copper in different ways. High levels of DPA resulted in low maternal and fetal tissue zinc, which could affect the teratogenicity due to the copper deficiency, as zinc deficiency alone is a potent teratogenic insult (4, 11). In striking contrast to the effects

of DPA, TETA caused increased levels of zinc in fetal liver and maternal kidney. The high liver zinc concentrations seen in fetuses from the TETA-fed dams was surprising, as maternal plasma zinc tended to be lower in the drug-fed groups. The mechanism(s) by which the fetal liver zinc is increased with TETA feeding is unknown, but suggests that either the TETA is effecting a change in plasma zinc distribution which is conducive to fetal liver zinc uptake, or that fetal liver metabolizes TETA-bound zinc in a manner different than that of maternal liver. An effect of TETA on zinc metabolism has been reported previously by Dubois and co-workers (12) who found that urinary zinc output in a young male with Wilson's disease doubled during treatment with the drug. The effect on fetal and postnatal development of the greatly increased fetal liver zinc observed in the fetuses from TETA-fed dams is not known. However, it is known that excess dietary zinc during pregnancy can exacerbate the effects of copper deficiency (13), and presumably this effect is not due solely to interactions at the gastrointestinal tract, suggesting that the high fetal concentrations in the TETA groups are disadvantageous.

We have completed preliminary studies on the molecular localization of zinc and copper in liver from control and TETA fetuses. By use of gel chromatography and gel electrophoresis we have found that the increased zinc observed in fetuses from the TETA-fed dams is primarily associated with the metal-binding protein, metallothionein. This protein normally also binds high amounts of copper in the fetal liver, and it is from this protein that copper is first found to be absent in fetuses from the TETA-fed dams. This protein is thought to serve as a reservoir for zinc and copper for the developing newborn. In fetuses with severe copper deficiency, liver copper was next found to be low in proteins with a molecular weight of approximately 30,000 and finally from proteins with a molecular weight greater than 50,000 (14). The increase in fetal liver zinc, although maternal liver zinc was relatively unaffected, suggests that the metabolism of zinc in the two tissues is quite different, perhaps due to differences in hepatic transport, or metallothionein regulation.

The observation of elevated maternal kidney zinc levels in the TETA groups causes some concern, as Anderson (15) has recently reported that significant increases in kidney zinc concentration can result in renal tubular cell toxicity. These observations suggest that kidney function should be carefully evaluated and monitored in patients receiving this drug. We are currently studying the nephrotoxicity of TETA.

TETA at the high-dose level caused a significant increase in maternal liver iron concentration, presumably due to decreased liver iron mobilization as a function of low plasma ceruloplasmin levels. A similar effect on iron was seen in rats fed DPA (4).

In summary, the results of this study show that TETA is teratogenic at dosages similar to those used clinically. Furthermore, all levels of TETA tested caused low maternal and fetal tissue copper. These data support the hypothesis that the teratogenic effects of TETA are due to the drug-induced copper deficiency, although the effect of TETA on maternal and fetal zinc metabolism may also be a mechanism of TETA teratogenicity. In addition to its effect on tissue copper, TETA caused high concentrations of zinc in maternal kidney and fetal liver, demonstrating the importance of considering the effect of chelating drugs on elements other than the one upon which they are intended to act.

These data detailing the teratogenicity of TETA are in contrast with the recent report by Walshe (2) who observed no malformations in six infants of Wilson's disease patients receiving TETA during pregnancy. A possible explanation for this difference from the present study is that Wilson's disease patients have a high concentration of tissue copper, thus making it unlikely that low plasma copper levels would be induced. Patients who may be given TETA for disorders other than Wilson's disease do not have high tissue copper concentrations and thus may not have similar protection. However, in either Wilson's disease patients or those with other disorders, the effects of high kidney zinc induced by TETA may be detrimental. It should be pointed out that in the paper by Walshe (2) it was stated that TETA had no teratogenic effects in mice; however, as no information on experimental design or data were provided in this paper, we cannot comment on the difference between his results and ours.

We suggest that the plasma copper concentrations of pregnant women receiving TETA

be monitored. The copper status of their infants should also be assessed since inadequate copper stores at birth may compromise normal growth and development. Furthermore, urinary zinc and kidney function should be assessed in all patients who receive the drug.

Supported in part by NIH research Grant HD-02355 from the National Institute of Child Health and Human Development.

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Received September 2, 1982. P.S.E.B.M. 1983, Vol. 173.