Subchronic Toxicity of Triethylenetetramine Dihydrochloride in B6C3F1 Mice and F344 Rats

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Triethylenetetramine dihydrochloride (trien-2HCl; CAS No. 38260-01-04), a chelating agent used to treat Wilson's disease patients who are intolerant of the drug of choice, was tested for subchronic toxicity in B6C3F1 mice and F344 rats. Mice and rats received trien-2HCl in the drinking water at concentrations of 0, 120, 600, or 3000 ppm for up to 92 days. Twenty mice and 18 rats of each sex were assigned to each dose group fed either a cereal-based (NIH-31) or a purified (AIN-76A) diet, both containing nutritionally adequate levels of copper. An additional control group of rats and mice received a Cu-deficient AIN-76A diet. This low copper diet resulted in Cu-deficiency symptoms, such as anemia, liver perportal cytomegaly, pancreatic atrophy and multifocal necrosis, spleen hematopoietic cell proliferation, and increased heart weight, together with undetectable levels of plasma copper in rats but not in mice. Trien-2HCl lowered plasma copper levels somewhat (at 600 and 3000 ppm) in rats fed the AIN-76A diet, but did not induce the usual signs of copper deficiency. Trien-2HCl caused an increased frequency of uterine dilatation at 3000 ppm in rats fed AIN-76A diet that was not noted in females fed the Cu-deficient diet. Trien-2HCl toxicity occurred only in mice in the highest dose group fed an AIN-76A diet. Increased frequencies of inflammation of the lung interstitium and liver perportal fatty infiltration were seen in both sexes, and hematopoietic cell proliferation was seen in the spleen of males. Kidney and body weights were reduced in males as was the incidence of renal cytoplasmic vacuolization. There were no signs of copper deficiency in mice exposed to trien-2HCl. The only effect of trien-2HCl in animals fed the NIH-31 diet was a reduced copper level in both rat sexes, noted at 3000 ppm.

Wilson's disease is a rare inherited metabolic disease characterized by an excessive accumulation of copper in certain tissues. Excess copper leads to tissue damage and functional defects such as liver cirrhosis and destruction of motor coordination and renal tubular function. Removal of excess copper by treatment with medical chelating agents can arrest the course of the disease and bring about some structural and functional repair. The major breakthrough in treatment of the disease came with the introduction of penicillamine by Walshe (1956), and this compound still remains the drug of first choice. However, about 10% of patients using penicillamine eventually develop some form of immunological intolerance to the drug (Walshe, 1982). Another chelating agent, triethylenetetramine dihydrochloride (trien-2HCl) has been used successfully in managing the disease in patients who have developed this intolerance to penicillamine (Walshe, 1979, 1982) and is approved by the FDA for use in these patients. The drug may be taken orally in maximum daily adult dosages of 30 mg/kg, taken in two to four divided portions.

Few studies of the toxicity of trien-2HCl have been reported in the peer-reviewed literature. In trying to determine optimal conditions for causing pancreatic acinar atrophy resulting from copper deficiency, Smith et al. (1982) incorporated triethylenetetramine tetrahydrochloride (trien-4HCl) into the diet of male Wistar rats at a concentration of 2000 ppm. When the chelator was administered with standard rat chow for up to 8 weeks, minimal atrophy of the pancreas and minor changes in the lobular architecture of the tissue were noted. However, when administered with a Cu-deficient diet for 6 weeks, pancreatic exocrine atrophy was very marked, in excess of what was found with the Cu-deficient diet alone. Other studies have shown trien-4HCl to be fetotoxic or teratogenic when fed to Sprague-Dawley rats during pregnancy at a level of 1660 ppm (Keen et al., 1983), and trien-2HCl was teratogenic to fetal mouse brain when administered during pregnancy at levels of 6000 or 12,000 ppm in the drinking water (Tanaka et al., 1993). Teratogenicity and effects on the exocrine pancreas possibly are secondary to effects of the drug on copper metabolism.
The purpose of this study was to identify and characterize the possible toxicity of trien-2HCl when administered to B6C3F1 mice and F344 rats for up to 92 days. The intent was to characterize the toxicity in animals fed diets containing nutritionally adequate levels of copper, to compare these to animals fed a low copper diet, and to evaluate the relationship of possible adverse effects to the effect of trien-2HCl on circulating copper levels.

METHODS AND MATERIALS

Experimental design. The experimental design of the current study is presented in Table 1. Trien-2HCl doses were based on preliminary 14-day studies in F344 rats and B6C3F1 mice to determine water concentrations of the test compound that would be acceptable to the animals (Schiefenstein and Greenman, 1984). In these studies rats and mice received dosed water at trien-2HCl concentrations of 0, 3000, 9000, 12,000, 15,000, and 30,000 ppm. Early deaths of mice and rapid weight loss were noted in both species at 30,000 ppm. Soft feces or diarrhea were noted in rats and inhibition of body weight gain or water intake were observed in both species administered trien-2HCl concentrations of 0.3000, 9000, 12,000, 15,000, and 30,000 ppm. Since none of these effects was noted in either species at 3000 ppm, this was selected as the maximum water concentration to be used in the 90-day studies. Thus, rats and mice were administered trien-2HCl in the drinking water at concentrations of 120, 600, or 3000 ppm for a 90- to 92-day period. Dosed animals were fed a cereal-based diet, NIH-31 (Knapka, 1982), or a purified diet, AIN-76A (Ad Hoc, Committee on Standards, 1977, 1980). Controls included undosed fed animals or fed either of these two diets or a Cu-deficient AIN-76A formulation. The purified diet was selected so that a Cu-deficient diet might be formulated and used as a positive control to help distinguish between Cu-deficiency effects and other biological properties of trien-2HCl. The cereal-based diet was used, not because of similarities to the AIN-76A diet in metal ion composition, but to determine the similarity of responses obtained with the AIN-76A diet to those obtained using a standard cereal-based diet used in toxicity studies. All diets were commercially formulated. The copper-adequate AIN-76 diets were formulated by Teklad Test Diets (Madison, WI), and the Cu-deficient diet was formulated by Dyets, Inc. (Bethlehem, PA) by deleting copper carbonate from the AIN-76 mineral mix and using low-copper ingredients.

Animals and animal husbandry. Parent strains of B6C3F1 mice (C57BL/6N females and C3H/HeN males) and the breeding stock of rats (Fischer 344N (F344/N)) used in the studies were obtained from the Veterinary Research Branch, Division of Research Services, National Institutes of Health. F344/N stock and C57BL/6N breeders were obtained in 1979, and C3H/HeN breeders in 1974. B6C3F1 mice and F344/N rats, produced by the National Center for Toxicological Research, were rank-ordered by body weight and randomly allocated to treatment groups in a way that would assure approximately equal body weight distributions in all treatment groups. Four mice or three rats were assigned per cage. Animals were housed in polycarbonate shoebox cages in a conventional animal room. Dosing was started at 35 to 50 days of age. Clean cages containing heat-treated hardwood chip bedding were provided weekly; food and autoclaved, distilled water were supplied ad libitum. Freshly autoclaved water was given weekly to mice, twice weekly to rats. Water consumption was determined by the difference between the weights of the bottles when supplied and when removed from the cage. Water bottles were turned upright whenever racks were moved to minimize leaking. Daily cage checks were made to identify leaking water bottles and to look for dead or moribund animals. Animals were weighed and clinical symptoms recorded weekly. Animals were housed with lighting on a 12-hr light/dark cycle (on at 6 AM, off at 6 PM), temperature controlled at 70 to 75°F, and humidity at ± 10%.

Sexes were kept on separate sides of the same rack (mice) or alternated by row on the same side of the rack (rats). To terminate the studies, dosed water was replaced with control water and animals were fasted overnight before being euthanized.

Dose preparation. Target drinking water concentrations of trien-2HCl were prepared in 10-liter batches by dissolving the appropriate quantity of compound in distilled water. After mixing, the dosed water was delivered to water bottles which were covered with rubber stoppers containing ball-bearing sipper tubes and autoclaved for 30 min at 250°F. After autoclaving, the sipper tubes were covered with sterile plastic caps, and bottles were packed in covered stainless steel carrying cases for storage prior to delivery to the animal rooms.

Chemistry. Triethyleneetetramine dihydrochloride (CAS No. 38260-01-04) was obtained from K and K Greef Chemicals Ltd. (Croydon, England; Batch No. 0344TC, No. 151/0307) and had a mass spectrum identical to a reference material (>99% pure) purchased from another source (Fluka Chemical Corp.). 13C NMR spectra of the compound used in the study indicated a purity greater than 99%. Stability of the compound at ambient temperature was determined in distilled/deionized water at concentrations of 1000, 100,000, and 200,000 ppm. No loss in concentration was detected by 13C NMR or HPLC (conductivity detection) after 36 days of storage (Hansen et al., 1985). Stability of a 3000 ppm solution after autoclaving for 30 min at 250°F was also examined, and no loss of concentration was observed. Dosed water preparations were analyzed routinely by a colorimetric assay of the trien-copper chelate (Hansen et al., 1985). All batches used were within 10% of the target concentration.

Plasma metal analyses were performed by inductively coupled argon plasma–atomic emission spectrometry, using a technique previously described by Blakemore et al. (1984), to obtain the metal quantifications by direct comparison to matrix standards.

For metal analyses, tissue samples were digested by a dry ashing procedure and lyophilized. A National Bureau of Standards standard reference material (bovine liver) was included in the procedure to monitor recovery of the analytes of interest. Tissue metal concentrations were quantified by atomic absorption spectrometric techniques. Furnace or flame atomization of the analytes of interest was used, depending on the amount present. Background correction was used to minimize interference from the substrate.

Diet and water were analyzed for Ca, Cd, Cu, Fe, and Zn as described by Billedeau and Blakemore (1985a,b).

### TABLE 1
Experimental Design of 90-Day Subchronic Studies of Triethyleneetetramine Dihydrochloride in B6C3F1 Mice and F344 Rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>Trien conc (ppm)</th>
<th>No. of animals/sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-31</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>18</td>
</tr>
<tr>
<td>AIN76A</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>18</td>
</tr>
<tr>
<td>AIN76A (low Cu)</td>
<td>0</td>
<td>18</td>
</tr>
</tbody>
</table>

Plasma metal analyses were performed by inductively coupled argon plasma–atomic emission spectrometry, using a technique previously described by Blakemore et al. (1984), to obtain the metal quantifications by direct comparison to matrix standards.

For metal analyses, tissue samples were digested by a dry ashing procedure and lyophilized. A National Bureau of Standards standard reference material (bovine liver) was included in the procedure to monitor recovery of the analytes of interest. Tissue metal concentrations were quantified by atomic absorption spectrometric techniques. Furnace or flame atomization of the analytes of interest was used, depending on the amount present. Background correction was used to minimize interference from the substrate.

Diet and water were analyzed for Ca, Cd, Cu, Fe, and Zn as described by Billedeau and Blakemore (1985a,b).
Necropsy and histopathology. Animals were euthanized with CO₂ and liver, heart, brain, kidneys, adrenals, spleen, and testes or ovaries were weighed. Histopathology was performed on adrenal glands, aorta, brain, colon, coagulating gland, epididymis, esophagus, eyes, gall bladder (mouse), Harderian gland, heart, kidneys, liver, lungs, lymph nodes, sciatric nerve, ovaries, pancreas, parathyroid gland, pituitary gland, prostate, salivary glands, seminal vesicles, small intestine, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid gland, trachea, urinary bladder, uterus, and vagina of control and high-dose animals and on all gross lesions. If lesions were clearly more prevalent in high-dose than in control animals, those organs were examined histopathologically in other dose groups. Tissues were fixed in 10% neutral-buffered formalin, routinely processed and stained with hematoxylin and eosin.

**Hematology and plasma chemistry.** Hematology was done on 10 mice, and hematological and clinical chemistry were examined in 12 rats from each sex per dose group. Since interactions in the absorption and metabolism of Cu, Fe, and Zn are known to exist, plasma levels of these three metals were determined in five mice or six rats per sex per dose group. Liver, aorta, and spinal cord samples from six rats of each sex from control and high-dose groups also were analyzed for the metals. These tissues were analyzed in other dose groups if control and high-dose levels differed. The hematological profile was conducted in an electrostatic flow apparatus (Coulter Counter Model ST90; Coulter Electronics, Hialeah, FL) with a 100 × 75 μm aperture. Blood smears for a leukocyte differential count were air-dried on a glass slide, fixed in methanol, and stained with Wright's stain followed by Giemsa stain on a Hemomatic Model 70 automatic stainer (Gam-Rad, Inc.). Biochemical analyses, performed as described elsewhere (Greenman and Cronin, 1988), included the determination of cholesterol esters, separation and quantification of lipoprotein fractions, determination of total plasma protein and separation and quantitation of protein bands, determination of ceruloplasmin, urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase.

**Statistical analyses.** A univariate analysis of variance model was fit to terminal body weight and organ weight data. The analyses were done by diet and sex. For each diet and sex combination two models were fit to a one-way univariate analysis of variance using a vector of the absolute weight of each organ and the organ weight/body weight ratio, respectively. Within each analysis a multiple comparison test with Bonferroni correction was run to compare doses. The analysis for body weight was done at level alpha. The hypothesis of no dose effect was tested.

With Coulter and differential hematology and clinical chemistry data, two analyses of variance (ANOVA) were performed for each sex, for the AIN-76A diet, and for each variable included in the data set. The first analysis compared untreated control animals to treated animals. The second analysis compared untreated controls to controls fed the Cu-deficient diet. For the animals fed NIH-31 diet only one analysis was performed for each sex, for each variable. Bonferroni tests were used to compare responses from control animals to responses from dosed animals in cases where the ANOVA found a significant dose effect (95% significance level).

For plasma metal concentrations an ANOVA was conducted with the factors sex, diet (AIN-76A or NIH-31), and dose. The error term for tests of these effects and their interactions was the mean square for the variation among animals within each sex—diet—dose group. Duncan's multiple range test was employed to test for differences among dose groups.

Cochran–Armitage test was used to test for dose–response trends of the proportions of animals with various histopathological lesions. Where comparisons of two groups were made, Fisher's exact test was used.

**RESULTS**

**90-Day Study in Mice**

**Body weight.** Trien-2HCl had no effect on body weight gain of mice fed the cereal-based diet and clearly suppressed weight gain only in males fed the purified diet, exposed at the 3000 ppm level. On this diet high-dose males gained 25% less than controls. Mice fed the low copper diet gained somewhat more than Cu-adequate controls fed the AIN-76A diet. Males fed the Cu-deficient diet gained 9% more, females 15% more.

**Water consumption.** In female mice water consumption was not altered by either trien-2HCl exposure or low copper intake, but both sexes fed the NIH-31 diet drank more water than those fed either formulation of the purified diet (Table 2). Male mice receiving trien-2HCl consumed less water than their corresponding controls regardless of the diet. Female mice received somewhat higher daily dosages (mg/kg body wt) of trien-2HCl than their male counterpart; NIH-fed animals received slightly higher daily dosages than AIN-76A-fed animals.

**Clinical symptoms and lethality.** No clinical symptoms were attributable to dosing with trien-2HCl. Only one mouse died during the study, a male receiving 3000 ppm trien-2HCl fed AIN-76A diet (on Day 78).

**Pathology findings.** Multifocal chronic inflammation of the lung interstitium and lung alveolar histocytic infiltration were the most prevalent histologic findings associated with trien-2HCl administration (Table 3). These lesions occurred in high-dose male and female mice fed the AIN-76A diet and were more severe but less frequent in males than in females. They did not occur in mice fed either the NIH-31 or the Cu-deficient diet. Spleen hematopoietic cell proliferation and liver periportal fatty change also were most prevalent in the high-dose AIN-76A-fed animals (Table 3). Furthermore, high-dose males fed AIN-76A diet had a decreased prevalence of kidney cytoplasmatic vacuolization (lipid content) when compared to controls. This cytoplasmic vacuolization is a normal feature of male B6C3F1 mice fed either NIH-31 or AIN-76A diet, but was suppressed by 3000 ppm trien-2HCl only in those fed AIN-76A diet. Controls on the Cu-deficient diet were more similar to normal controls than the high-dose animals in each of these instances.

**Organ weights.** The multiple range test of absolute organ weights to compare doses revealed significant dose–related differences only for the kidneys of male mice fed AIN-76A diet. Absolute kidney weights were lowest in males given 3000 ppm trien-2HCl. For example, the left kidney weighed (g) significantly (p < 0.001) less than in all other treatment groups (control, 0.27 ± 0.033; 120 ppm, 0.27 ± 0.030; 600 ppm, 0.27 ± 0.029; 3000 ppm, 0.24 ± 0.027; low Cu, 0.28 ± 0.023). There were no significant differences in organ/body wt ratios considered to be related to either the low copper diet or trien-2HCl administration.

**Hematology and plasma metals.** Analysis of hematology results revealed few significant differences. These occurred only in males fed the AIN-76A diet. The mean corpuscular
### TABLE 2
**Average Water and Trien-HCl Consumption by B6C3F1 Mice Administered Triethylenetetramine Dihydrochloride in Their Drinking Water for 90 Days**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Dose (ppm)</th>
<th>Chemical Consumption (mg/kg body wt/day)</th>
<th>Water Consumption (g/kg body wt/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>0</td>
<td>174</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>142</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>154</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>148</td>
<td>161</td>
</tr>
<tr>
<td>Low Cu*</td>
<td>0</td>
<td>159</td>
<td>157</td>
</tr>
<tr>
<td>NIH-31</td>
<td>0</td>
<td>187</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>179</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>178</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>162</td>
<td>184</td>
</tr>
</tbody>
</table>

* This diet was the same as AIN-76A, except copper was omitted from the formulation.

### TABLE 3
**Histological Lesions* in B6C3F1 Mice Fed AIN-76A Diet and Given Triethylenetetramine Dihydrochloride in Their Drinking Water for up to 90 Days**

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Trien-HCl Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Lung, chronic interstitial inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Lung, alveolar histocytic infiltration</td>
<td>0</td>
</tr>
<tr>
<td>Spleen, hematopoietic cell proliferation</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Liver, perportal fatty change</td>
<td>3 (1.0)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>Lung, chronic interstitial inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Lung, alveolar histocytic infiltration</td>
<td>0</td>
</tr>
<tr>
<td>Spleen, hematopoietic cell proliferation</td>
<td>3 (1.0)</td>
</tr>
<tr>
<td>Liver, perportal fatty change</td>
<td>0</td>
</tr>
<tr>
<td>Kidney, cytoplasmic vacuolization</td>
<td>20 (2.9)</td>
</tr>
</tbody>
</table>

* Data are presented as number of mice with the lesion (average severity of lesion). Each group had 20 mice.

* Significant at $p < 0.001$ by the Cochran–Armitage trend test.

* Significant at $p < 0.0071$ by the Cochran–Armitage trend test.

* Significant at $p < 0.0024$ by the Cochran–Armitage trend test.

* Significant at $p < 0.0026$ by the Cochran–Armitage trend test.
deficient controls were generally not statistically significant.

in females, the differences between copper-adequate and Cu-
hemoglobin. Although these same tendencies were present
an increase in nucleated RBCs and decreases in hemoglobin,
significant changes included
hematocrit, mean corpuscular volume, and mean corpuscular
In males there were significant changes in
(p < 0.05) changes in hematol-
relative liver weight of males was significantly greater
of these organs were also significantly greater. Furthermore,
weight in females, and increased kidney and testis weights in
were attributable to trien-2HCl treatment in either
AIN-76A diet was 112 g/kg; that of males was 87 g/kg.
were 13, 60, or 323 mg/kg; male dosages were 10,
Daily dosages of trien-2HCl in females fed NIH-31 diet
drank more than males regardless of the diet. On NIH-31 diet, control
males drank 118 g of water/kg body wt daily; control
males drank 94 g/kg. Daily consumption of females fed AIN-
was 87 g/kg.

There were no remarkable diet or dose-related differences
in water consumption. However, female rats drank more
than males regardless of the diet. On NIH-31 diet, control
females drank 118 g of water/kg body wt daily; control
males drank 94 g/kg. Daily consumption of females fed AIN-
was 112 g/kg; that of males was 87 g/kg.

Dietary dosages of trien-2HCl in females fed NIH-31 diet
drew 14, 70, or 352 mg/kg body wt; male dosages were 10,
55, or 276 mg/kg. In females fed AIN-76A diet daily dosages
were 13, 60, or 323 mg/kg; males received 10, 53, or 270
mg/kg daily.

Organ weights. No significant differences in organ weights were attributable to trien-2HCl treatment in either
sex fed either AIN-76A or NIH-31 diet. However, low copper
was associated with significant (p < 0.05) increased
adrenal gland and heart weights in both sexes, increased liver
weight in females, and increased kidney and testis weights in
males. Since low copper animals weighed less than their Cu-
adequate controls, the relative weights (organ/body wt ratios)
of these organs were also significantly greater. Furthermore,
the relative liver weight of males was significantly greater
in deficient than in Cu-adequate rats.

Hematology and clinical chemistry. The low copper diet
resulted in several significant (p < 0.05) changes in hematology parameters. In males there were significant changes in
several RBC parameters, suggesting the presence of hypo-
chromic, microcytic anemia. Significant changes included
an increase in nucleated RBCs and decreases in hemoglobin,
hamatocrit, mean corpuscular volume, and mean corpuscular
hemoglobin. Although these same tendencies were present
in females, the differences between copper-adequate and Cu-
deficient controls were generally not statistically significant.
In addition, there was a tendency toward an increase in the
proportion of polymorphonuclear leucocytes compared to
lymphocytes in Cu-deficient rats of both sexes, but these changes were not always statistically significant. The per-
centage of polymorphonuclear leucocytes was significantly
(p < 0.05) increased in females and the percentage of lymphocytes was significantly (p < 0.05) decreased in males.
Trien-2HCl treatment induced no signs of anemia or other hematologic effects in rats fed either purified or cereal-based
diet.

Among the plasma enzymes, only a few significant differ-
ences were detected. Probably none of these effects are of
biological importance. Aspartate aminotransferase was sign-
ificantly (p < 0.05) lower in Cu-deficient than in Cu-ade-
quate control males fed AIN-76A diet. Also, in NIH-31-fed
males, 3000 ppm trien-2HCl was associated with signific-
antly (p < 0.05) lower ceruloplasmin than that found in
controls (300 ppm, 210 ± 26; control, 293 ± 55 mg/dl).
In females, alanine aminotransferase was signi-
ificantly (p < 0.05) lower in Cu-deficient than in Cu-ade-
quate controls fed AIN-76A diet (30.2 ± 3.5 vs 36.3 ±
5.0 IU/liter, respectively), and 3000 ppm trien-2HCl was
associated with a significant (p < 0.05) rise in this enzyme
compared to Cu-adequate controls and other trien-2HCl
treatment groups (control, 36.3 ± 5.0; 120 ppm, 37.4 ± 2.7;
600 ppm, 36.5 ± 4.8; 3000 ppm, 42.6 ± 3.4 IU/liter).

No differences in plasma cholesterol or protein concentra-
tions were found that could be attributed to trien-2HCl expo-
sure or copper deficiency.

Metals. The Cu-deficient diet reduced plasma copper to
nondetectable levels in nearly all rats of both sexes (Table 4).
Plasma iron levels also were significantly reduced in
these animals. Trien-2HCl significantly depressed plasma
copper levels at 600 and 3000 ppm in males and females
fed the AIN-76A diet but not in rats fed NIH-31 diet. The
effect of trien-2HCl was greater in males than females.

The low copper diet was associated with a significant
reduction in liver copper levels of both sexes (Table 5). A
similar reduction in copper level was not apparent in the
aorta, and in the spinal cord was significant (p < 0.0023)
only in males (Cu-adequate, 4.94 ± 0.94; Cu-deficient, 2.38
± 0.32 μg/g). No distinct associations between copper defi-
cency and iron or zinc levels of liver, aorta, or spinal cord
were found. Trien-2HCl significantly depressed liver copper
at all dose levels in females and at 600 ppm in males fed
AIN-76A diet and at 3000 ppm in both sexes fed NIH-31
diet (Table 5).

Pathology findings. The low copper diet was clearly as-
associated with the appearance of several histopathologic le-
sions in rats. In both sexes, at least half of the animals
fed the Cu-deficient diet exhibited spleen hematopoietic cell
proliferation, liver periportal cytomegaly, and diffuse atro-
phy, and multifocal necrosis of pancreas while none of the
TABLE 4
Plasma Concentrations of Copper, Zinc, and Iron of F344 Rats Administered Triethylenetetramine Dihydrochloride in Their Drinking Water for 90 Days

<table>
<thead>
<tr>
<th>Diet</th>
<th>Sex</th>
<th>Dose (ppm)</th>
<th>Plasma metal concentration (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Copper</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>F</td>
<td>0</td>
<td>0.83 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>0.65 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>0.45 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>0.39 ± 0.11</td>
</tr>
<tr>
<td>Low Cu</td>
<td>F</td>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>NIH-31</td>
<td>F</td>
<td>0</td>
<td>1.40 ± 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>1.10 ± 0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>1.42 ± 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>M</td>
<td>0</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>0.85 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>0.11 ± 0.02^</td>
</tr>
<tr>
<td>Low Cu</td>
<td>M</td>
<td>0</td>
<td>ND*</td>
</tr>
<tr>
<td>NIH-31</td>
<td>M</td>
<td>0</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>0.88 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>0.70 ± 0.09</td>
</tr>
</tbody>
</table>

* Data are reported as means ± standard error of the mean for six rats.

Significantly (p < 0.0001) different from AIN-76A Cu-adequate control when sexes combined.

Significantly (p < 0.0001) different from control for this sex when both diets combined.

Significantly (p < 0.0006) different from control when sexes combined for this diet.

Detected in only one sample.

Significantly (p < 0.05) different from AIN-76A Cu-adequate control

Not detected in any samples.

rats fed Cu-adequate AIN-76A had these lesions. In males fed AIN-76 diets, there also were dramatic rises in the frequency of pancreatic chronic inflammation and adrenocortical vacuolization associated with the low copper diet. Additionally, among rats fed the low copper diet, there were reduced frequencies of hepatic periportal fatty change (in both sexes), uterine epithelial necrosis, and liver coagulative necrosis (in males).

Trien-2HCl had no effects on the histopathology of rats fed NIH-31 diet. Among rats fed AIN-76A, trien-2HCl was significantly associated with the change in frequency of only one lesion in a way similar to what was found in rats fed the low copper diet. In males receiving 3000 ppm trien-2HCl, coagulative necrosis of the liver (2/18) was less frequent than in Cu-adequate controls (10/18) and was absent from all low copper controls.

One lesion, which occurred in AIN-76A-fed females, may be attributed to trien-2HCl exposure, but probably was not caused by its copper depleting action. There was a significant (p < 0.001) dose-related trend toward an increased prevalence of uterine dilatation, especially apparent in the high-dose females (0 ppm = 1/18, 120 ppm = 2/18, 600 ppm = 2/18, 3000 ppm = 8/18, low-Cu = 2/18).

Diet and water analyses. The results of feed and water analyses are summarized in Table 6. Cu, Zn, Fe, Cd, and Ca were all below detection limits in drinking water. Zn levels were very similar in the three diets, but other elements were clearly higher in the cereal-based diet than in either purified diet. The Cu-deficient diet had <0.8 ppm Cu, as anticipated, but it also had a higher calcium content than its Cu-adequate counterpart.

DISCUSSION AND CONCLUSIONS

The current study was designed to distinguish between symptoms of copper deficiency and effects of trien-2HCl not related to the induction of copper deficiency. This objective was achieved in both species, but in quite different ways.
TABLE 5
Liver Concentrations of Copper, Zinc, and Iron of F344 Rats Administered Triethylenetetramine Dihydrochloride in Their Drinking Water for 90 Days

<table>
<thead>
<tr>
<th>Diet</th>
<th>Sex</th>
<th>Dose (ppm)</th>
<th>Copper (µg/ml)</th>
<th>Zinc (µg/ml)</th>
<th>Iron (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adult Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td>F</td>
<td>0</td>
<td>6.12 ± 0.66</td>
<td>38.7 ± 4.6</td>
<td>315 ± 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>3.67 ± 0.14*</td>
<td>26.8 ± 0.4*</td>
<td>366 ± 59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>3.93 ± 0.45*</td>
<td>31.2 ± 0.9</td>
<td>260 ± 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>4.48 ± 0.19*</td>
<td>55.6 ± 3.4*</td>
<td>245 ± 23</td>
</tr>
<tr>
<td>Low Cu</td>
<td>F</td>
<td>0</td>
<td>0.97 ± 0.15*</td>
<td>38.1 ± 3.2</td>
<td>278 ± 26</td>
</tr>
<tr>
<td>NIH-31</td>
<td>F</td>
<td>0</td>
<td>5.96 ± 0.37</td>
<td>43.3 ± 2.5</td>
<td>352 ± 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>5.18 ± 0.42</td>
<td>37.1 ± 1.0</td>
<td>338 ± 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>4.75 ± 0.24</td>
<td>37.4 ± 1.0</td>
<td>324 ± 44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>4.48 ± 0.40*</td>
<td>37.3 ± 1.1</td>
<td>410 ± 47</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>M</td>
<td>0</td>
<td>4.18 ± 0.44</td>
<td>29.4 ± 0.6</td>
<td>195 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>3.62 ± 0.23</td>
<td>25.5 ± 0.5</td>
<td>301 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.52 ± 0.27*</td>
<td>26.2 ± 0.6</td>
<td>269 ± 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>3.45 ± 0.16</td>
<td>40.9 ± 1.9*</td>
<td>218 ± 14</td>
</tr>
<tr>
<td>Low Cu</td>
<td>M</td>
<td>0</td>
<td>0.37 ± 0.05*</td>
<td>29.3 ± 2.3</td>
<td>340 ± 38*</td>
</tr>
<tr>
<td>NIH-31</td>
<td>M</td>
<td>0</td>
<td>4.12 ± 0.2</td>
<td>33.6 ± 1.2</td>
<td>205 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>2.93 ± 0.22</td>
<td>31.1 ± 0.7</td>
<td>192 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>3.38 ± 0.25</td>
<td>34.9 ± 1.7</td>
<td>189 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>3.26 ± 0.18*</td>
<td>33.5 ± 1.1</td>
<td>212 ± 7</td>
</tr>
</tbody>
</table>

* Data are reported as means ± standard error of the mean for six rats.
* Significantly (p < 0.0001) different from AIN-76A Cu-adequate control.
* Significantly (p < 0.0004) different from AIN-76A Cu-adequate control.
* Significantly (p < 0.0012) different from NIH-31 control.
* Significantly (p < 0.0003) different from NIH-31 control.
* Significantly (p < 0.0023) different from AIN-76A Cu-adequate control.
* Significantly (p < 0.0003) different from NIH-31 control when averaged across sexes.

Under conditions of the study, copper deficiency was not induced in B6C3F1 mice either by use of a low copper diet or by exposure to trien-2HCl. Virtually no indications of copper deficiency were present, in terms of either histopathology or circulating or organ levels of copper. Nevertheless, a number of histopathologic changes were clearly associated with trien-2HCl exposure in mice fed AIN-76A diet. These were noted only at 3000 ppm trien-2HCl and included chronic interstitial inflammation and alveolar histocytic infiltration of the lung, spleen hematopoietic cell proliferation,

TABLE 6
Feed and Water Analyses for Selected Metals

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>N</th>
<th>Copper (ppm)</th>
<th>Zinc (ppm)</th>
<th>Cadmium (ppb)</th>
<th>Iron (ppm)</th>
<th>Calcium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-31</td>
<td>4</td>
<td>12.2 ± 4.8°</td>
<td>69 ± 17</td>
<td>105 ± 16</td>
<td>408 ± 42</td>
<td>1.22 ± 0.09</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>2</td>
<td>6.8</td>
<td>47</td>
<td>&lt;50</td>
<td>69</td>
<td>0.50</td>
</tr>
<tr>
<td>AIN-76A°</td>
<td>2</td>
<td>&lt;0.8°</td>
<td>60</td>
<td>&lt;81</td>
<td>77</td>
<td>1.06</td>
</tr>
<tr>
<td>Water</td>
<td>1</td>
<td>&lt;0.02°</td>
<td>&lt;1.0°</td>
<td>&lt;2°</td>
<td>&lt;0.01°</td>
<td>&lt;0.001°</td>
</tr>
</tbody>
</table>

* Means ± SD.
° Copper-deficient diet.
° Lowest detection limits.
liver periportal fatty change, and a reduction in the frequency of kidney cytoplasmic vacuolization. Mice fed NIH-31 diet showed no clear toxic response to trien-2HCl.

The chronic inflammation of the lung interstitium noted in both male and female mice fed AIN-76A diet and receiving 3000 ppm trien-2HCl in our study was the most significant lesion. It clearly was not a response to copper depletion and was not similar to the lung pathology noted in copper-deficient (O'dell et al., 1978) or penicillamine-treated rats (Kilburn and Hess, 1982). Pathology in rats fed a copper-deficient diet has included enlarged lungs with fewer than normal alveoli, but no evidence of edema, pneumonia, leukocytic or lymphocytic infiltration of alveoli, or peribronchial lymphocyte infiltration (O'dell et al., 1978). Hematopoietic proliferation of the spleen noted in mice may have been a secondary response due to the lung pathology, rather than being a response to copper deficiency as it was in the rat studies. The reduction in frequency of kidney cytoplasmic vacuolization in male mice fed AIN-76A diet and 3000 ppm trien-2HCl is of unknown significance. Nevertheless, it is consistent with the reduction in kidney weight also noted in this group. A review of the literature has not shown this observation previously associated with copper deficiency.

The low copper diet did induce signs of copper deficiency in F344 rats that have been previously reported and studied. For example, anemia and effects on heart, liver, spleen, and pancreas are well known responses to copper deficiency (Allen et al., 1982; Fields et al., 1984; Johnson and Hove, 1986; Smith et al., 1982; Underwood, 1971). Furthermore, these signs of copper deficiency in the current study were associated with undetectable levels of circulating copper and low liver copper concentrations.

Although trien-2HCl reduced plasma copper levels in rats fed AIN-76A diet, this reduction was not as severe as in rats fed the Cu-deficient diet, and the evidence of copper deficiency in these animals was not very apparent. In males, trien-2HCl clearly reduced the prevalence of coagulative liver necrosis, as did copper deficiency, but this was the only indication of copper deficiency in trien-2HCl-exposed rats. The increase in uterine dilatation found in female rats exposed to 3000 ppm trien-2HCl was not observed in copper-deficient females and may reflect an altered endocrine milieu since uterine dilatation is a normal phenomenon of the estrous cycle and is known to be sensitive to changes in estrogen exposure (Greenman and Fullerton, 1986). Since trien-2HCl treatment of rats was associated with significant reductions in liver copper concentrations at all doses in rats fed AIN-76A diet and in high-dose rats fed NIH-31 diet, the liver appears to be a more sensitive indicator of the copper depleting action of trien-2HCl than plasma copper levels. Furthermore, the high dose of trien-2HCl did achieve a liver-copper depleting action in rats fed the cereal-based diet even though there was no evidence of copper deficiency in these animals.

That animals fed the two diet types in the present study should respond differently to trien-2HCl is not surprising. The two diets did contain different levels of copper, iron, and calcium. Thus, the response to a chelating agent, such as trien-2HCl, could be quite different for animals fed the two diets due to an interaction between trien-2HCl and the chelation of copper and other metals present at different concentrations in the two diets. In addition, absorption, retention, and distribution of copper are known to be influenced by other inorganic ions (Underwood, 1971). Furthermore, the development of copper deficiency symptoms is highly dependent upon diet composition, particularly the primary carbohydrate source present (Fields et al., 1983, 1984; Johnson and Hove, 1986). Other factors also may be involved. Wattenberg (1971, 1972) has reported quite different levels of mixed function oxidase activity in the lungs and intestine of rodents fed cereal-based and purified diets. Our own laboratory has found that mice fed AIN-76A diet are more sensitive to the action of estriadiol than those fed a cereal-based diet (Greenman and Fullerton, 1986), and this difference appears to be related to the metabolism of estriadiol (Fullerton et al., 1987). Thus, differences between animals fed the two diets may be related to effects on the metabolism of trien-2HCl.

In conclusion, signs of trien-2HCl toxicity were noted only in B6C3F1 mice fed AIN-76A diet and given 3000 ppm trien-2HCl in the drinking water. These toxic signs included inflammation of the lung interstitium, hematopoietic cell proliferation of the spleen, liver periportal fatty infiltration, kidney weight reduction, reduced renal cytoplasmic vacuolization, and body weight gain reduction. These signs do not appear to be related to copper deficiency since most of the usual signs of copper deficiency were not observed. However, it may be important to determine sites of bioaccumulation of this chelating agent before ruling out the possibility that it may bring about localized copper depletion at specific tissue sites. The effectiveness of trien-2HCl in bringing about liver copper depletion was demonstrated at all dose levels in rats fed the purified diet and at 3000 ppm in rats fed the cereal-based diet. Generally, this copper depletion was inadequate to induce overt signs of copper deficiency. However, there was an indication of copper deficiency at 3000 ppm in rats fed the purified diet. The lowest level of the compound causing any effect in rats that probably was not related to its effect on copper levels was 3000 ppm. The response noted at this level (uterine dilatation) was not considered to be of major toxicologic importance.

**ACKNOWLEDGMENTS**

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REFERENCES


