Maternal Hepatic and Embryonic Effects of 1,2,4-Trichlorobenzene in the Rat

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The possible maternal hepatic and reproductive effects of 1,2,4-trichlorobenzene (TCB) were assessed in rats given 0, 36, 120, 360, and 1200 mg/kg/day of TCB on Days 9–13 of gestation. The animals were sacrificed on Day 14 of pregnancy. Maternal deaths (219 rats, 6/6 rats) were recorded in the 360 and 1200 mg/kg/day treatment groups and body weight gain was significantly decreased in the 360 mg/kg/day TCB group. Maternal liver weight, liver/body weight ratio, and hepatic microsomal protein content were unaffected by TCB treatment. Although Day 14 NADPH-cytochrome c reductase activity was affected only at 360 mg/kg/day TCB, the maternal hepatic microsomal cytochrome P-450 content was significantly increased by administration of both 120 and 360 mg/kg/day of TCB. Hepatic microsomal aminopyrine N-demethylase, ethoxyresorufin O-deethylase, and UDP-glucuron transferase activity towards p-nitrophenol were also increased at 120 and 360 mg/kg TCB. Glutathione S-transferase activity to 1-chloro-2,4-dinitrobenzene and 1,2 dichloro-4-nitrobenzene were both increased by pretreatment with TCB. Although pretreatment with 360 mg/kg/day TCB did not increase resorptions, embryolethality, or teratogenicity, embryonic development was significantly retarded by all four growth criteria used (head length, crown–rump length, somite number, and protein content).

INTRODUCTION

1,2,4-Trichlorobenzene is used as a dye carrier, dielectric, solvent, fire retardant, and industrial intermediate (Jones, 1973; Dow Chem. Co., 1975, 1979; Hawley, 1977). It occurs in the environment both from industrial sources and as a breakdown product of other chlorinated organics (Jondorf et al., 1955). Concern about the possible health effects of TCB has risen after TCB was identified in drinking water supplies and waste water (Gaffney, 1976) and in tissues of fresh water fish (Veith et al., 1979).

The number and position of chlorine atoms on the benzene ring influence the lipid solubility, biotransformation, and toxicity of chlorinated benzenes (Allen et al., 1979; Ware and West, 1977; Ariyoshi et al., 1975). The more highly chlorinated benzenes which lack the two adjacent hydrogen atoms required for rapid expoxide formation (Kohli et al., 1976) bioaccumulate in food chains. Chronic toxicity is the primary possible environmental problem with these compounds. In contrast, chlorinated benzenes with two vicinal hydrogens will not bioaccu-
mulate to as great an extent. However, these molecules pose a greater acute toxicity hazard. Acute toxicity is generally inversely related to the degree of chlorination (Allen et al., 1979). Furthermore, the low molecular weight and absence of charge of all chlorinated benzenes should permit substantial placental transport of these lipophilic compounds.

In this study the maternal hepatic and embryonic effects of administration of varying doses of 1,2,4-trichlorobenzene were determined in rats. TCB was administered on Days 9 to 13 of pregnancy, the period of maximal susceptibility to teratogenesis, and experimental animals were sacrificed on the 14th day. Day 14 embryos exposed to TCB in vivo were evaluated for growth and morphological parameters.

METHODS

Experimental animals and dosing. 1,2,4-Trichlorobenzene (Aldrich Chem. Co., >99% pure) dissolved in corn oil (A&P) was orally administered in doses from 36 to 1200 mg/kg/day to pregnant rats on Days 9 to 13 of gestation in a volume of 2 ml/kg. Controls were orally fed corn oil alone. Time-pregnant Sprague-Dawley rats (CD strain, Charles River Breeding Laboratories, Kingston, Mass.) were given food and water ad libitum. The day sperm were detected in the vaginal smear was designated as Day 1 of gestation. Pregnant females were housed two per plastic nestbox cage and six or more rats were used in each TCB dosage group. Standard research laboratory conditions regarding bedding materials, light/dark cycle, temperature, and humidity were maintained throughout the experiment. Animals were killed by decapitation on the morning of Day 14 and their livers were excised and weighed. Hematoxylin and eosin-stained sections of control and treated livers were examined histologically. In some studies, the uteri were removed from 12 pregnant rats in control and 360 mg/kg/day TCB groups and the embryos examined for growth and differentiation parameters (Kitchin et al., 1981). Live embryos were observed with a Wild 7A stereomicroscope and scored as normal or abnormal as compared to corn oil-treated controls. The presence of a beating heart, the somite number, and embryo size were recorded. After homogenization in 50 mM potassium phosphate buffer, pH 7.4, the total embryonic protein content was determined (Lowry, 1951).

Enzyme assays. Five grams of maternal rat liver were homogenized in 20 ml of 0.02 M Tris-HCl buffer (pH 7.5) containing 1.15 M KCl. Washed microsomes were prepared by ultracentrifugation as described by Kitchin and Woods (1978). The high speed supernatant (1 hr 100,000g) was used for glutathione S-transferase determinations. Cytochrome P-450 content was assayed by the method of Omura and Sato (1964) using an Aminco DW-2 spectrophotometer. All enzyme activities were determined at 37°C under conditions of time and protein linearity. NADPH-cytochrome c reductase (LaDu et al., 1971), aminopyrine N-demethylase (Nash, 1973; Lucier et al., 1971), and ethoxyresorufin O-deethylase (Burke and Mayer, 1974) activities were determined by published procedures. The in vitro rates of the glucuronidation of p-nitrophenol and phenolphthalein were determined by substrate disappearance using 0.2% Triton X-100 activated microsomes (Mulder, 1970). Microsomal protein content was determined by the biuret method (Gornall...
et al., 1949). Laboratory chemicals and reagents were obtained from standard commercial sources.

Statistics. Analysis of variance procedures were used to determine statistically significant effects (Goldstein, 1964). Enumerative data were analyzed by the Fishers exact test (Goldstein, 1964). Quantitative data are presented as the mean ± SEM for five to eight independent observations. For statistical purposes, the litter was considered the experimental unit and a P value of 0.05 was considered significant.

RESULTS

All animals that received 1200 mg/kg/day TCB died by the third day of treatment. Administration of 360 mg/kg/day TCB caused some maternal lethality (29 rats) and greatly reduced body weight gain from +37 to −17 grams per rat (Fig. 1). Liver weight, liver/body weight ratio and hepatic microsomal protein content were unaffected by TCB treatment. TCB was a strong hepatic enzyme inducer. Hepatic cytochrome P-450 content was elevated from 0.44 to 0.79 and 1.36 nmol/mg protein by treatment with 120 and 360 mg/kg/day TCB respectively (Fig. 2). NADPH-cytochrome P450 reductase activity was almost doubled at the higher dose of TCB.

Both hepatic microsomal aminopyrine N-demethylase and ethoxyresorufin O-deethylase were induced by administration of 120 and 360 mg/kg/day of TCB to pregnant female rats (Fig. 3). At the higher dose the oxidative enzyme activity in microsomes from TCB-treated dams was approximately double that of controls.

The conjugative enzyme UDP-glucuronyltransferase exhibited a heterogeneous response to TCB. p-Nitrophenol and phenolphthalein were used as representative "late fetal" and "neonatal" isoenzyme substrates, respectively (Wishart et al., 1978). The isoenzyme "late fetal" UDP-glucuronyltransferase was induced by the medium and high dose of TCB while "neonatal" UDP-glucuronyltransferase was not (Fig. 4).

The second conjugative isoenzyme enzyme system examined was hepatic cytosolic glutathione S-transferase. Enzymatic conjugation of 1,2-epoxy-3-(p-nitrophenoxy)propane with glutathione was not affected by prior TCB treatment. At 120 mg/kg/day TCB, glutathione S-transferase activity for 1-chloro-2,4-dinitrobenzene was increased and at the 360 mg/kg/day TCB dose, glutathione S-transferase activity for this substrate as well as for 1,2-dichloro-4-nitrobenzene was increased. Maximal enzyme induction was about threefold above control values for both of these substrates. Hepatic reduced glutathione levels were significantly depressed from 5.05 to 3.19 μmol/g liver by administration of five daily doses of 360 mg/kg/day TCB. In this experiment, about 18 hr elapsed between the last TCB dosing and sacrifice.

Liver histology in the control and 36 mg/kg/day TCB group was unremarkable. At 120 mg/kg/day only one of nine rats showed a slight degree of hepatocellular hypertrophy. Upon histological examination, seven of eight pregnant female rats fed 360 mg/kg/day TCB exhibited moderate hepatocellular hypertrophy. In addition, liver specimens were obtained from three rats which had died after being treated with 1200 mg/kg/day TCB. Although autolysis was evident in two of the
Pregnant female rats were given the indicated dose (mg/kg/day) of 1,2,4-trichlorobenzene on Days 9-13 of gestation by gavage. The body weight gain (gestational Day 14–Day 8) and Day 14 liver weights are expressed as the mean ± SEM for five to eight animals. ***P < 0.001.

Administration of TCB to pregnant dams adversely affected embryonic growth (Table 1). Resorptions, embryolethality, and abnormalities were not increased to a statistically significant extent by TCB treatment. Embryolethality was confined to 3 of the 12 TCB-treated litters. In the TCB group, the two embryonic anomalies three liver specimens, moderate to moderately severe multifocal necrosis was seen in all rats in this treatment group.
Fig. 2. Pregnant female rats were given 1,2,4-trichlorobenzene on Days 9–13 of gestation by gavage. On Day 14 the animals were sacrificed and the hepatic microsomal cytochrome P-450 content and NADPH-cytochrome c reductase activities determined. The data are presented as the means ± SEM for five to eight animals. **P < 0.01, ***P < 0.001.

(from two different TCB-treated dams) observed were misshapen backs. Embryonic growth parameters were all significantly lowered by TCB administration to pregnant rats during organogenesis. Although embryonic head length, crown-rump length, and somite number were decreased by TCB administration by only about 5%, the total embryo protein content was reduced by 23%.
EFFECTS OF 1,2,4-TRICHLOROBENZENE
MATERNAL HEPATIC ENZYME INDUCTION

Fig. 3. Day 14 hepatic microsomal aminopyrine N-demethylase and ethoxyresorufin O-deethylase activities were determined after administration of several different oral doses of 1,2,4-trichlorobenzene to rats on Days 9-13 of gestation. Data are means ± SEM for six to eight animals. *P < 0.05, **P < 0.01.

DISCUSSION

At 120 mg/kg/day TCB, hepatic enzyme induction of cytochrome P-450 levels, aminopyrine N-demethylase, ethoxyresorufin O-deethylase, UDP-glucuronol transferase activity towards the “late fetal substrate” p-nitrophenol and glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene were observed.

At 360 mg/kg/day, some maternal lethality and the expected hepatic glutathione content depression (both at 5 and 18 hr after sacrifice) occurred. The full potency
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UDP GLUCURONYL TRANSFERASE ACTIVITY

Fig. 4. Pregnant female rats were given various doses of 1,2,4-trichlorobenzene during Days 9–13 of gestation by gavage. On Day 14 the animals were sacrificed and the in vitro rates of glucuronidation of p-nitrophenol and phenolphthalein determined. Data are means ± SEM for five to eight animals. *P < 0.05, ***P < 0.001.

of TCB as an hepatic enzyme inducer was obvious at this dosage: the activity of seven out of the nine enzyme activities studied were increased. The results of this study can be compared to those in which phenobarbital and β-naphthoflavone were given to pregnant rats in a previous experiment (Kitchin and Ebron, 1983). Compared to phenobarbital and β-naphthoflavone pretreatment, TCB was a stronger enzyme inducer for five and four of the seven responsive enzymes, respectively. Only in respect to induction of ethoxyresorufin O-deethylase activity was TCB less potent than β-naphthoflavone. A summary of the direction and magnitude of the effects of TCB on maternal hepatic parameters is shown in Table 2. TCB was not found to be significantly embryolethal or dysmorphogenic during embryonic organogenesis (the Day 9–13 period). Embryonic growth retardation
EFFECTS OF 1,2,4-TRICHLOROBENZENE
GLUTATHIONE S-TRANSFERASE ACTIVITY

Fig. 5. Pregnant female rats were given the indicated doses of TCB on Days 9–13 of gestation by gavage. On Day 14 the animals were sacrificed and the hepatic high-speed supernatant glutathione S-transferase activity towards CDNB, DCNB, and ENPP determined. Data are means ± SEM for five to eight independent observations. **P < 0.01, ***P < 0.001.

was caused by 360 mg/kg/day of TCB. Embryonic protein content was 23% lower in embryos of the TCB-treated dams. Further reproductive and developmental studies are required to determine if embryos of TCB treated rats can catch up to normal Day 21 fetal size or if the growth retardation is permanent.

Ariyoshi et al. (1975) fed 250 mg/kg/day TCB to nonpregnant Wistar rats for 3 days and observed increases in liver weight, hepatic microsomal protein, cytochrome P-450, and aminopyrine N-demethylase activity. In adult male rats administration of 40 mg/kg/day of TCB for 14 days increased hepatic microsomal cytochrome P-450, NADPH-cytochrome c reductase, aryl hydrocarbon hydroxylase, and UDP-glucuronyltransferase activity toward the “late fetal” substrate phenol (Carlson and Tardiff, 1976). Their study found TCB induced-hepatic microsomal enzyme induction persisted after a 30-day recovery phase. Both these investigators found TCB to be an extremely potent hepatic enzyme inducer, an observation confirmed in our study. Rats exposed to TCB (25, 100, or 400 ppm) in drinking water did not show decreased fertility, growth, or viability in a multi-generation reproduction study (Robinson et al., 1981). The highest dose for female rats calculated in that study was 53.4 mg/kg/day (Robinson et al., 1981).
## Table 1

Effects of 1,2,4-Trichlorobenzene on Embryonic Growth and Morphogenesis in the Rat

<table>
<thead>
<tr>
<th>Implantations</th>
<th>Resorptions/implantations</th>
<th>Affected litters/embryos</th>
<th>Affected litters/embryos</th>
<th>Abnormalities</th>
<th>Head length (mm)</th>
<th>Crown-rump length (mm)</th>
<th>Somites</th>
<th>Protein (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>14.16 ± 0.31 (12)</td>
<td>9/170</td>
<td>5/12</td>
<td>0/161</td>
<td>0/26</td>
<td>4.72 ± 0.06 (26)</td>
<td>8.17 ± 0.09 (26)</td>
<td>49.6 ± 0.6 (26)</td>
</tr>
<tr>
<td>1,2,4-Trichlorobenzene (360 mg/kg/day)</td>
<td>12.50 ± 0.79 (12)</td>
<td>12/150</td>
<td>7/12</td>
<td>25/138</td>
<td>2/24</td>
<td>4.43 ± 0.10 (24)</td>
<td>7.69 ± 0.17 (24)</td>
<td>47.7 ± 0.6 (24)</td>
</tr>
<tr>
<td>Level of significance</td>
<td>p &lt; .05</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; .001</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; .05</td>
<td>p &lt; .05</td>
</tr>
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</table>

**Note.** Dams were given daily oral doses of vehicle or 360 mg/kg/day TCB on Days 9-13 of gestation. Twelve vehicle and TCB litters were examined. They were sacrificed by ether administration on the morning of Day 14 and the uteri examined for implantations, resorptions, and embryonic parameters. Two embryos, one from each uterine horn, from each female were used in the determination of various embryonic parameters. Quantitative data are expressed as the means ± SEM for the indicated number of embryonic observations. The litter was used as the experimental unit in statistical comparisons of the five embryonic parameters.
### TABLE 2
**SUMMARY OF MATERNAL PARAMETERS**

<table>
<thead>
<tr>
<th></th>
<th>Glutathione S-transferase</th>
<th>UDP-Glucuronyl Transferase</th>
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<tr>
<td></td>
<td>Lethality</td>
<td>Body wt</td>
</tr>
<tr>
<td>Corn oil 36 mg/kg/day TCB</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>120 mg/kg/day TCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>360 mg/kg/day TCB</td>
<td>↓ ↓ ↓</td>
<td></td>
</tr>
<tr>
<td>1200 mg/kg/day TCB</td>
<td>↑ ↑ ↑</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** This figure summarizes the maternal findings. At 1200 mg/kg/day TCB, all animals died and were not evaluated biochemically. Two possibly spurious findings were an increase in glutathione content 18 hr after dosing with 120 mg/kg TCB and a decrease in glutathione S-transferase activity for 1,2-epoxy - 3 - (p-nitrophenoxy)propane (ENPP). These effects were not dose related. The direction of the arrow indicates an increase or decrease from control values. One, two, and three arrows represent $P < 0.05$, 0.01, and 0.001, respectively. The number of experimental animals per group is the same as in Figs. 1–5.
This chronic TCB exposure is less than the 120 and 360 mg/kg/day acute doses used in our study.

To date, the maternal hepatic and embryonic effects of three chlorinated benzenes (1,2,4,5-tetra-, 1,2,3,4-tetra- and 1,2,4-trichlorobenzene) have been determined (Kitchin, 1982; Kitchin and Ebron, 1983; Kitchin and Ebron, in press). Of these three compounds, 1,2,4-trichlorobenzene is the most potent in respect to maternal toxicity, glutathione depletion, hepatic enzyme induction, and embryonic toxicity. Within the chlorinated benzene series, the presence of two vicinal hydrogens increases the rat maternal hepatic and embryonic toxicity. Thus, to date it appears that the same structure–activity relationships useful in predicting the toxicity of chlorinated benzenes to male experimental animals (Allen et al., 1979; Matthews and Kato, 1979) hold for maternal hepatic and embryonic toxicity in rats.

ACKNOWLEDGMENTS

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REFERENCES


