8.7 Developmental and Reproductive Effects

The results of studies concerning the embryotoxicity, fetotoxicity, teratogenicity, and reproductive effects of the chlorobenzenes are presented in Table 24. Effect levels derived in these studies for both mothers and offspring are also presented in this table. In contrast to the lack of information on acute, short-term, long-term, and carcinogenic effects, comparatively more data are available on the potential embryotoxic, fetotoxic, and teratogenic effects of the higher chlorinated benzenes. Furthermore, the TCBs, TeCBs, and PeCB have been better studied in this regard than MCB and the isomers of DCB.

There has been no evidence in any of the studies conducted to date that the chlorobenzenes (mono- to penta-) are teratogenic in animal species. Some relatively minor embryotoxic and fetotoxic effects have been observed for the lower chlorinated benzenes (MCB and DCBs), but only at dose levels that were toxic for the mother. For example, there was a slight delay in skeletal development (ossification) in the fetuses of pregnant rats exposed to 2864 mg MCB/m^3, a dose that induced decreases in body weight gain in the mothers (John et al., 1984). Similarly, the ossification of cervical vertebrae in fetuses of pregnant rats exposed to 2400 mg 1,2-DCB/m^3 was delayed; this dose also induced decreases in body weight gain and food consumption in the mothers (Hayes et al., 1985). However, in both of these studies, there was no convincing evidence of embryotoxic, fetotoxic, or teratogenic effects in rabbits exposed to MCB or 1,2-DCB via inhalation, even at dose levels that were maternally toxic (John et al., 1984; Hayes et al., 1985). In fetuses of pregnant rabbits exposed to 4720 mg 1,4-DCB/m^3, there was an increase in the incidence of retro-oesophageal right subclavian artery, a minor variation in the circulatory system that is often observed in control litters; at this dose, the maternal body weight gain was also decreased (Hayes et al., 1985). In an additional study, Giavini et al. (1986)

administered 1,4-DCB, in corn oil, by gavage, at doses of 250, 500, 750, or 1000 mg/kg body weight per day, between days 6 and 15 of gestation. Effects were noted only at doses greater than 500 mg/kg per day. These included an increased frequency of extra ribs, a reduction in fetal weight, and an increase in skeletal abnormalities. These dose levels also induced decreases in body weight gain and food consumption in the mothers.

There is some evidence that the higher chlorinated benzenes (TCBs, TeCBs, PeCB) are embryotoxic or fetotoxic at dose levels that are not maternally toxic. However, available data are not consistent and the toxicities of the various isomers of the TCBs and TeCBs for the mother and fetus vary considerably. For example, the 1,2,4-isomer was the most maternally toxic of the 3 TCB isomers administered by ingestion to pregnant rats in a study conducted by Black et al. (1988); changes in haematological parameters occurred at doses as low as 150 mg/kg per day. At a lower dose (75 mg/kg), there were mild histological changes in the lenses of pups of exposed mothers; however, these changes were not observed at higher doses (150 or 300 mg/kg) and were unlikely, therefore, to be treatment-related. Kitchin & Ebron (1983a) observed growth retardation in the embryos of pregnant rats administered 360 mg 1,2,4-TCB/kg body weight on days 9-13, a dose that caused some lethality, reduced body weight gain, and produced moderate hepatocellular hypertrophy in mothers.

Although less toxic for the mothers than 1,2,4-TCB, the 1,3,5-isomer of trichlorobenzene induced mild changes in the lenses of pups of

pregnant rats administered doses as low as 150 mg/kg body weight by gavage; there was no significant maternal toxicity at this dose (Black et al., 1988). For the 1,2,3-isomer, there were no effects in offspring at any dose level (up to 600 mg/kg body weight), even though a level of 300 mg/kg was toxic to the mothers. The authors of this study did not discuss the significance of the observed histological changes in the lenses (areas of cellular disorientation and disaggregation with ballooning and granular degeneration) of the pups of mothers administered the 1,3,5-isomer, but concluded that there was no evidence that any of the TCB congeners were teratogenic or fetotoxic.

Kacew et al. (1984) reported that the 1,2,4,5-isomer was the most toxic of the TeCBs for both mothers and fetuses, in a study in which all 3 isomers were administered to pregnant rats by gavage (death of 9/10 animals at 200 mg/kg and increase in serum cholesterol at 50 mg/kg body weight). The toxicity was well correlated with the greater accumulation of 1,2,4,5-TeCB in maternal and fetal tissues. There was a decrease in the number of live fetuses in pregnant rats administered 50 mg 1,2,4,5-TeCB/kg body weight, a dose that induced minor changes (increases in serum cholesterol) in exposed mothers.

Table 24. Developmental and reproductive studies on chlorobenzenes

Compound; Reference	Species[a]	Dose[b,c]	Results[d]	Effect Levels[e]
1,2,3-TCB 1,2,4-TCB 1,3,5-TCB (99.5%) Ruddick et al. (1983); Black et al. (1988)	Sprague-Dawley rat (pregnant); 14/group	1,2,4-TCB: 0, 75, 150, or 300 mg/kg per day; 1,2,3-TCB and 1,3,5-TCB: 0, 150, 300 or 600 mg/kg per day on days 6-15 of gestation; gavage in corn oil	maternal toxicity - reduced body weight gain at 600 mg/kg (1,3,5-TCB), increased liver weight at 600 mg/kg (1,2,3- and 1,3,5-TCB) and 300 mg/kg (1,2,4-TCB), decreased haemoglobin levels and haematocrit, generally at highest dose (all 3 isomers), decreased RBC at 300 mg/kg (1,2,3-TCB), 150 and 300 mg/kg (1,2,4-TCB), and 150 and	1,2,3-TCB: 300 mg/kg per day (F); *600 mg/kg per day (O) (NOEL) 1,2,4-TCB:

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Environmental Contamination and Toxicology

Assessment of Teratogenic Potential of 1,2,3- 1,2,4- and 1,3,5-Trichlorobenzenes in Rats

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Trichlorobenzenes (TrCB) are chemicals that are used as chemical intermediates, dye carriers and as dielectric fluids (U.S. EPA, 1983). They have low water solubility but are freely soluble in organic solvents. Of the isomers (1,2,3-, 1,2,4- and 1,3,5-) 1,2,4-TrCB has been produced in the largest quantities, an estimated annual production of 1.3 - 7 million kg in the U.S. (U.S. EPA, 1983). TrCB have also been shown to arise from the degradation of the pesticide lindane (Mathur and Saha, 1977 and Engst et al., 1976). TrCB are demonstrated environmental contaminants. Gulls sampled from the Great Lakes basin are known to contain residues of TrCB (Hallett et al., 1982) and they were also identified in freshwater fish taken from the Saginaw River, Michigan (Veith et al., 1979) and from the Great Lakes (Oliver and Nichol, 1982).

The present study was initiated to screen the three TrCB congeners for their teratogenic potential and to determine their ability to cross the placenta and accumulate in the fetus.

METHODS AND MATERIALS

Female Sprague-Dawley (175 - 122 g) rats, purchased from Woodlyn Farm Laboratories, Guelph, Ontario were acclimatized for one week prior to mating. Timed pregnancies were obtained by placing two females into a cage overnight containing a male rat. The following morning vaginal swabs were examined to check for mating. The morning on which sperm was detected in the female was designated as day 1 pregnancy. Mated females were randomly assigned to ten groups and housed in wire-top plastic cages containing corn cob bedding. Each group consisted of approximately 14 dams.

Trichlorobenzene congeners (1,2,3-, 1,2,4- and 1,3,5- TrCB, 97-99% pure) purchased from Aldrich Chemical Company (Montreal, Quebec) were recrystallized from ethanol and confirmed as 99.5% pure by gas chromatography. The purified congeners were dissolved in corn oil and administered as follows: 1,2,4- TrCB - 75, 150 and 300 mg/kg

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b.w.; 150, 300 and 600 mg/kg b.w. for both the 1,2,3- and 1,3,5-TrCB. These preparations were administered by gavage (0.5 ml/100 g b.w.) to dams on the 6th to 15th day of gestation. Controls received an equal volume of corn oil. On the 22nd day of gestation, the dams were weighed, anesthetized with sodium pentobarbital and exsanguinated via the abdominal aorta. The uterus was transected anterior to the cervix and removed with the ovaries. Dams were reweighed and their liver, kidney, spleen, heart and brain removed and weighed. The pups were removed and weighed individually.

Maternal blood was examined hematologically using a Baker 7000 blood analyzer. Parameters measured were hemoglobin concentration, hematocrit value, erythrocyte count, total and differential counts of leucocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin.

Serum from each dam was analyzed using a SMA 12/60 microanalyzer (Technicon, Montreal, Quebec). Measured were; sodium, potassium, inorganic phosphorus, total bilirubin, alkaline phosphatase, glutamic oxaloacetic transaminase (GOT), total protein, calcium, cholesterol, glucose, uric acid and lactic dehydrogenase (LDH).

Portions of the central lobe of maternal liver were homogenized in 0.15 M KCl, pH 7.4 (1 g/2.5 ml) and centrifuged at 10,000 x g for 15 min at 0°C. The supernatant obtained was used for enzymatic analysis. Aniline hydroxylase activity (AH) was determined by an automated method based on the procedure of Fouts (1963); aminopyrine-N-demethylase (APDM) was determined by an automated method based on the procedure of Cochin and Axelrod (1959), and protein concentrations were determined by the biuret method of Gornall et al. (1948) as modified by Becking (1973).

At necropsy, portions of maternal tissues were selected for TrCB residue analysis; kidney, brain, spleen, heart, liver and perirenal fat. In addition, one fetus/litter and the liver and brain of a litter mate were removed for TrCB analysis. Tissues were stored frozen at -20°C until analyzed. TrCB congeners were extracted according to a modified method of Hallett et al. (1978). Samples of tissues were homogenized and extracted in 8 mL of acetonitrile: distilled water (1:1). The homogenate was mixed with 4 ml of n-hexane, shaken for 30 min then centrifuged at 1000 x g for 15 min at 22°C. An aliquot of the n-hexane layer was removed and washed with an equal amount of concentrated (95-98% pure) H_2SO_4. The washed solution was analyzed using a Microtech 220 gas chromatograph fitted with a ^{63}Ni electron-capture detector and a glass column (100 cm x 6.25 mm O.D.) packed with 4% SE-30 and 6% QF-1 on Suppleport matrix. Gas chromatographic conditions were: injector temperature, 250°C; oven temperature, 225°C; detector temperature, 325°C; carrier gas, nitrogen and flow rate, 35 ml/min. Recovery ranged from 60-95% depending on the isomer and tissue. The detection limit was 0.01 ppm. Retention times for 1,2,3, 1,2,4- and 1,3,5-TrCB were 0.8, 1.15 and 0.57 min., respectively.

Fetuses were examined grossly at necropsy for birth defects. Only live fetuses were counted and evaluated for terata. Approximately two-thirds of each litter were processed for skeletal examination, the remainder were fixed in Bouin's fluid for visceral examination. The visceral anomalies were searched for by dissecting and razor sectioning. The skeleton anomalies were detected by examining the cleared and stained skeletons stereoscopically.

The histological procedure used to examine the maternal heart, brain, pituitary, eye, thyroid, parathyroid, trachea, bronchi, lung, thymus, stomach, small and large intestine, pancreas, liver, kidney, spleen, adrenal, skeletal muscle, peripheral nerve, skin, bone marrow, ovary, uterus and bladder tissues has been previously described (Villeneuve et al., 1979). In addition, entire fetuses were embedded in paraffin, serial-sectioned, stained and examined microscopically.

The data from organ weight, body weights, hematology and biochemistry were subjected to a simple one way analysis of variance. When significant differences ($p < 0.05$) were indicated, the Duncan's Multiple Range Test (SPSS version 8.1) selected the groups that were significantly different.

RESULTS AND DISCUSSION

Three animals died during the study: one in control group, one dosed with 1,2,4-TrCB (150 mg/kg) and one dosed with 1,2,3-TrCB (300 mg/kg). Deaths were not judged to be treatment related.

None of the animals displayed any signs of toxicity, however mean body weight of the mothers tended to be lower in the high dose treatments. The effect on weight was significant for the 600 mg/kg 1,3,5-TrCB group only (35.4±6.6 g) when compared to controls (53.7±3.1 g).

Table 1 summarizes, numbers of pregnancies, fetal weight, litter size, resorptions and dead fetuses as well as the visceral and skeletal anomalies. A significant increase in the number of resorptions was observed in the group administered 1,3,5-TrCB (300 mg/kg). This was attributed to one animal having 12 resorption sites and was judged not treatment related since similar occurrences were not observed in other animals of that group, nor in the 600 mg/kg group. One dam from the 1,3,5-TrCB (300 mg/kg) group delivered a fetus with micrognathia. No other gross skeletal or visceral abnormalities were observed. A few minor skeletal deviations were present but none had teratological significance.

A significant ($p < 0.05$) increase in the liver weight (as a percent body weight) was observed in 1,2,3- (5.8±0.26%) 600 mg/kg, 124- (5.7±0.1%) 300 mg/kg and 1,3,5-TrCB (5.6±0.1%) 300 mg/kg and (6.4±0.3%) 600 mg/kg when compared to controls (5.1±0.2%).

The wet liver weights of 1,2,4-TrCB 300 mg/kg dosage (15.0±0.4g) and 1,3, 5-TrCB 300 mg/kg (14.8±0.5g) and 600 mg/kg dosages

(16.0±0.7g) were significantly increased ($p < 0.05$). All other organ weights were statistically normal.

ADPM activity was significantly increased at the high dose of 1,2,3-TrCB (600 mg/kg), 27.3±0.9 nano moles formaldehyde/hr/mg protein and the 2 high doses of 1,2,4-TrCB, (150 & 300 mg/kg), 28.9±1 and 28.8±0.9 nano moles formaldehyde/hr/mg protein respectively, compared to control 22.8±0.6. All 3 doses of 1,3,5-TrCB had APDM activity levels greater than the control (25.1±0.6 to 25.5±0.9 nano moles of formaldehyde/hr/mg protein) but the difference was not significant. Treatment did not affect other biochemical parameters.

Hemoglobin concentrations were decreased in animals dosed at the two highest levels of 1,2,3-TrCB (300 and 600 mg/kg), 11.4±0.2 and 11.3±0.2 g/dl respectively, the two highest doses of 1,2,4-TrCB (150 and 300 mg/kg) 11.2±0.1 and 11.3±0.1 g/dl respectively, and the highest dose of 1,3,5-TrCB (600 mg/kg) 10.8±0.2 g/dl compared to control 12.0±0.3 g/dl. Hematocrit levels were decreased by 600 mg/kg 1,2,3-TrCB (31.1±0.6%) 150 and 300 mg/kg 1,2, 4-TrCB (31.2±0.4 and 31.4±0.5% respectively) and at 600 mg/kg 1.3,5-TrCB (29.8±10.5%) compared to control (33.3%).

Mild degenerative renal changes were observed in some rats, however they could not be attributed to treatment. They consisted primarily of multifocal glomerular adhesions with a normal architectural pattern. Mild changes were observed in the thyroid gland of animals dosed with 300 mg/kg 1,2,4-TrCB and the two highest dose groups receiving 1,2,3- and 1,3,5-TrCB. The changes consisted of a reduction of follicle size which was often accompanied by angular collapse. In the highest dose groups of each compound there was increased epithelial height accompanied by cytoplasmic vacuolation. Mild hepatic changes were also observed in the mothers and these were generally restricted to the highest and second highest dose levels for each compound. Changes consisted largely of increased periportal cytoplasmic eosinophilia and mild anisokaryosis of hepatocellular nuclei. In addition increased splenic hematopoesis was observed in some females.

Histological lesions occurred in the lenses of eyes of pups treated with 1,3,5-TrCB (all dose levels) and 1,2,4-TrCB (intermediate dose group). The changes consisted of central areas of cellular disorientation and disaggregation with ballooning and granular degeneration. Autolysis and incomplete preservation made examination of other fetal tissues difficult but there did not appear to be any significant treatment-related changes.

TrCB residues were found only in fat tissues. The 1,2,3-isomer had trace levels at the 2 high doses (means of 0.02 and 0.4 ppm) and 1,3,5-TrCB residues were found in fat at all three dosage levels (means 1.6 low dose to 4.8 ppm high dose). No 1,2,4-TrCB residues were detected in tissues.

Table 1. Fetal data (mean ± S.E.) following oral administration of trichlorobenzene (TrCB) congeners to pregnant rats

	1,2,3-TrCB				1,2,4-TrCB			1,3,5-TrCB		
	Control	150(mg/kg)	300	600	75	150	300	150	300	600
Dams at term/ Inseminated	12/14	10/13	11/13	11/13	11/13	11/13	12/14	12/13	12/13	11/13
Resorption + dead fetuses	0.7±0.2	0.4±0.1	0.4±0.1	0.5±0.2	1.1±0.2	0.6±0.2	0.6±0.3	0.7±0.2	2.0±0.9*	0.4±0.1
Litter size	11.5±1.2	11.5±1.1	13.3±0.6	12.7±1.1	12.1±0.9	13.1±0.5	13.3±0.6	12.5±0.7	11.5±1.0	11.5±1.3
Fetal wt (g)	5.4±0.1	5.4±0.1	5.2±0.1	5.1±0.2	5.3±0.1	5.5±0.1	5.1±0.1	5.4±0.1	5.5±0.1	5.2±0.1
Visceral Observations[a]	31	27	37	37	28	40	42	33	33	31
Skeleton Observations[a]	67	49	61	65	50	76	82[b]	66	55	56
Sternal anomilies[c]	11/3	1/1	1/1	13/3	14/6	11/4	9/7	9/6	5/3	9/5
Wavy ribs[c]	1/1	0	1/1	1/1	1/1	0	0	1/1	0	1/1
Centrum fusion delay	0	0	0	0	0	3/2	1/1	5/3	2/2	0
14th rib	0	1/1	0	0	0	5/5	2/1	2/1	0	3/2
13th rib (short)	0	1/1	0	0	1/1	0	0	0	0	0

a no. of pups examined
b one pup has micrognathia; others normal
c no. of pups affected/no. of litters affected
* significant difference $P \leq 0.05$

There was no evidence that any of the TrCB congeners tested were teratogenic or fetotoxic. This confirms previous findings of Kitchin and Ebron (1983) who also failed to observe any teratogenic effects in rats treated with 1,2,4-TrCB at high levels. In that study all animals in the highest dose group died on the third day and 22% of the dams died in the 360 mg/kg dose group. These authors also reported a significant decrease in embryonic growth prior to term. In a preliminary study, leading up to the study reported here, we found that 1,2,4-TrCB 600 mg/kg caused maternal deaths. Consequently, 300 mg/kg was the highest amount of this isomer administered. Thus our maternal toxicity data, for the 1,2,4- isomer, is in agreement with Kitchin and Ebron's results and suggests that it is more toxic to the dam than the other isomers. The 1,3,5-isomer was the next most potent since it caused decreased maternal body weight gain at 600 mg/kg. It was not possible to compare the feto- toxicity of the three congeners at any of the dose levels since no changes were detected. In terms of the effects observed on liver, both 1,2,4- and 1,3,5-TrCB produced hepatomegaly at levels as low as 300 mg/kg. However, of the two, only the 1,2,4-isomer caused a significant increase in mixed function oxidase activity (APDM). The 1,2,3-isomer also increased APDM activity but only at the high dose level (600 mg/kg). Neither the increased liver weight nor APDM activity could be considered dramatic at any dose levels tested. Increased liver weights were reported in rats and dogs following inhalation of 1,2,4-TrCB at 100 ppm for 7 h/day, 5 day per week for a total of 30 daily exposures (Kociba et al., 1981). Increased liver weight was also observed in male rats dosed orally with 40 mg/kg 1,2,4-TrCB for 14 or 90 days (Carlson and Tardiff, 1976). Numerous investigators have reported that 1,2,4-TrCB is capable of inducing mixed function oxidase activity in rats (Carlson and Tardiff, 1976 Carlson, 1978; Smith and Carlson, 1980). Ariyoshi et al., (1975a,b) reported that 1,2,4-TrCB could stimulate APDM activity and that 1,3,5-TrCB could do the same but to a lesser extent. Our results failed to show that the 1,3,5-isomer could significantly induce APDM activity, but the fact that pregnant animals were used might have some bearing.

The changes observed in the hemoglobin content, and hematocrit of rats treated with all 3 isomers indicated a very mild anemia. Examination of bone marrow demonstrated large numbers of erythroid cells in this tissue. The absence of reticulocytes in the blood smears suggested newly developing cells are not being released into the circulation and we conclude that this was due to ineffective erythropoiesis. No hematological effects of TrCB's have been previously reported.

Histological changes were uniformly mild in all tissues. No TrCB related lesions were found in the kidney and an increased hematopoesis observed in the spleen was likely an attempt to compensate for the mild anemia. The changes detected in the thyroid and liver, while restricted to the high dose levels of each chemical however their severity was not clearly dose related. The major

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lesion found in the fetuses was restricted to the lenses of the eye possibly indicating early cataract development. This observation has not previously been made.

The residue data confirm previous ^{14}C-TrCB pharmacokinetic studies (Chu 1986) showing that none of the isomers accumulate to any great degree, but the 1,3,5-isomer gives higher residues than the other two compounds. Also significant is the fact that the fetus does not bioaccumulate these chemicals. Higher chlorinated benzenes i.e. hexachloro-, pentachloro-, and 1,2,4 - tetrachlorobenzene, do cross the placenta and accumulate rather significantly in the fetus (Villeneuve and Hierlihy, 1975; Villeneuve and Khera, 1975; Kacew et al., 1984).

In summary, none of the TrCB isomers tested in this study produced teratogenic or fetotoxic effects. The most toxic isomer, at least with respect to maternal toxicity was 1,2,4-TrCB. None of the isomers accumulated in maternal or fetal tissue to any significant amount; however, 1,3,5-TrCB did show up in maternal fat samples in low ppm levels.

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