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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 chromatrograph 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 α 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

hexachlorcyclohexan-isomere im menschlichen urin. Z Gesamte Hyg 22:495-498
Fouts JR (1963) Factors influencing the metabolism of drugs in liver microsomes. Ann NY Acad Sci 104:875-880
Gornall AG, Bardawill CJ, David MM (1948) Determination of serum proteins by means of the buiret reaction. J Biol Chem 177:751-766
Hallett DJ, Khera KS, Stoltz DR, Chu I, Villeneuve DC, Trivett GJ (1978) Photomirex synthesis and assessment of acute toxicity tissue distribution and mutagenicity. J Agric Food Chem 26:338-339
Hallett DJ, Norstrom RJ, Onuska FI, Camba ME (1982) Incidence of chlorinated benzenes and chlorinated ethylenes in Lake Ontario herring gulls. Chemosphere 11:277-285
Kacew S, Ruddick JA, Parulekar M, Valli VE, Chu I, Villeneuve DC (1984) A teratological evaluation and analysis of fetal tissue levels following administration of tetrachlorobenzene isomers to the rat. Teratology 29:21-28
Kitchin KT, Ebron MT (1983) Maternal hepatic and embryonic effects of 1,2,4-trichlorobenzene in the rat. Environ Res 31:362-373
Kociba RJ, Leong BKJ, Hefner RE (1981) Subchronic toxicity study of 1,2,4-trichlorobenzene in the rat rabbit and beagle dog. 4:229-249
Mathur SP, Saha JG (1977) Degradation of lindane-(14)C in a mineral soil and in an organic soil. Bull Environ Contam Toxicol 17: 424-430
Oliver BG, Nichol KD (1982) Chlorobenzenes in sediments water and selected fish from Lake Superior Lake Huron Lake Erie and Lake Ontario (USA, Canada). Enivron Sci Technol 16:532-536
Smith EN, Carlson GP (1980) Various pharmacokinetic parameters in relation to enzyme-inducing abilities of 1,2,4-trichlorobenzene and 1,2,4-tribromobenzene. J Toxicol Environ Health 6:737-750
US EPA (1983) Chlorinated benzene aggregates derived from information reported under TSCA section 8(a). Office of Toxic Substances Washington DC
Veith GD, Kuehl DW, Leonard EN, Puglisi FA, Lemke AE (1979) Polychlorinated biphenyls and other organic chemical residues in fish from major watersheds of USA. Pestic Monit J 13:1-11
Villeneuve DC, Khera KS (1975) Placental transfer of halogenated benzenes (pentachloro- pentachloronitro and hexabromo-) in rats. Environ Physiol Biochem 5:328-331
Villeneuve DC, Hierlihy KS (1975) Placental transfer of hexachlorobenzene in the rat. Bull Environ Contam Toxicol 13:489-491
Villeneuve DC, Valli VE, Chu I, Secours V, Ritter L, Becking GC (1979) Ninety-day toxicity of photomirex in the male rat. Toxicology 12:235-250
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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

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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REFERENCES

Ariyoshi T, Ideguchi K. ishizuka Y, Iwasaki K, and Arakaki M (1975a) Relationship between chemical structure and activity. I Effects of the number of chlorine atoms in chlorinated benzenes on the components of drug-metabolizing systems and the hepatic constituents. Chem Pharm Bull (Tokyo) 23:817-823

Ariyoshi T, Ideguchi K, Iwasaki K, and Arakaki M (1975b) Relationship between chemical structure and activity. II Influences of isomers in dichlorobenzene trichlorobenzene and tetrachlorobenzene on the activities of drug-metabolizing enzymes. Chem Pharm Bull (Tokyo) 23:824-830

Becking GC (1973) Vitamin A status and hepatic drug metabolism in the rat. Can J Physiol Pharmacol 51:6-11

Carlson GP (1978) Induction of cytochrome P-450 by halogenated benzenes. Biochem Pharmacol 27:361-363

Carlson GP, Tardiff RG (1976) Effect of chlorinated benzenes on the metabolism of foreign organic compounds. Toxicol Appl Pharmacol 36:383-394

Cochin J, Axelrod J (1959) Biochemical and pharmacological changes in the rat following chronic administration of morphine nalorphine and normorphine. J Pharmacol Expt Therap 125:105-115

Engst von R, Macholz M, Kujawa M (1976) Uber das vorkammen von beta-glucuronidisch gebundenen metaboliten der

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The bone marrow clastogenicity of eight halogenated benzenes in male NMRI mice

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Eight widely used halogenated benzenes, including bromobenzene (BB), chlorobenzene (CB), three isomers of dichlorobenzene (DCB) and three isomers of trichlorobenzene (TCB) were tested for acute toxicity (LD_{50}) and clastogenicity in 8-week-old NMRI mice by intraperitoneal administration. Four doses of each chemical (up to 70% of LD_{50}) were tested for clastogenic activity. Each compound was administered in two equal doses, 24 h apart. Increased formation of micronucleated polychromatic erythrocytes, observed in femoral bone marrow, 30 h after the first injection, was considered to be due to the clastogenic activity of the test compound. All the halogenated benzenes tested were found to be clastogenic ($P < 0.01$). The highest clastogenic activities were induced by *m*-DCB and BB. Among the three isomers of DCB, *m*-DCB significantly ($P < 0.05$) induced more micronuclei than *o*-DCB or *p*-DCB. No significant differences were found between the clastogenic activities of TCB isomers.

Introduction

Halogenated benzenes are widely used in relatively large quantities in various industries. The total production of all chlorinated benzenes in western Europe is estimated to have been more than 208 million kg in 1980, half of which was located in the Federal Republic of Germany (IARC, 1982a). In addition to direct occupational exposure of man, halogenated benzenes are found in most parts of the ecosystem and have been detected in air, soil, food and water (Pellizzari, 1979; Zoeteman *et al.*, 1979; IARC, 1982a; Lai, 1984). Some of these chemicals are not limited to industrial use. For example, dichlorobenzene (*p*-DCB) is used as a fungicide, an air freshener, a moth repellent on clothes, and its crystals can be used to counteract odours in garbage and other refuse (IARC, 1982a; Giavini *et al.*, 1986).

Chlorobenzenes and their metabolites have been detected in human and animal tissues and body fluids indicating the extent of environmental pollution (IARC, 1982a; Jan, 1983). The mean levels of chlorobenzenes in human milk and adipose tissue are found to range from traces to 25 μg/kg in milk and from not detectable to 146 μg/kg in adipose tissue (Jan, 1983). Few biological data relevant to toxicity and genotoxicity of halogenated benzenes are available. Although the hepatotoxic compound, bromobenzene (BB), is found to bind covalently to DNA/RNA to an extent comparable to that of moderately oncogenic substances (Colacci *et al.*, 1985), no data are available on its carcinogenicity. The results of a host-mediated mutagenicity assay on BB were negative (Simmon *et al.*, 1979). No adequate information exists on the carcinogenicity/mutagenicity of chlorinated benzenes (IARC, 1982a). Wester *et al.* (1985) carried out a lifetime carcinogenicity study with a 'mixture' of 11 halogenated hydrocarbons including chlorobenzenes administered to rats in the drinking water, and found no significant carcinogenic effect. The results of some teratogenicity assays with chlorobenzenes were either negative (Hayes *et al.*, 1985), or if positive, they were considered to be the consequence of other factors rather than teratogenic effects (Giavini *et al.*, 1986).

This paper describes an investigation of the effects of a series of halogenated benzenes on the incidence of micronucleus induction within bone marrow polychromatic erythrocytes after intraperitoneal administration to mice.

Materials and methods

Chemicals

Fetal calf serum was obtained from Boehringer Mannheim (Mannheim, FRG). Benzene and halogenated benzenes were purchased from Merck Co. (Hohenbrunn, FRG). All test compounds were of synthetic grade and their purities are given in Table I. *p*-DCB, trichlorobenzene (1,2,3-TCB) and 1,3,5-TCB were supplied as solids which were dissolved in the minimum possible amount of dimethylsulphoxide (Merck, Darmdstadt, FRG).

Animals

Male 8-week-old NMRI mice were obtained from the Zentralinstitut für Versuchstiere (Hannover, FRG). Animals were housed five per cage and were given free access to water and laboratory Altromin chow (Altromin, Lage, FRG).

Micronucleus test

Median lethal doses (LD_{50}) of the test compounds administered by i.p. injection, were conducted according to the standard method (Fowler, 1983) as described elsewhere (Mohtashamipur *et al.*, 1985).

Since the dosing regime of Schmid (1977) for the micronucleus test implies that the highest dose used should be tolerated by the animals, the doses were chosen in such a way that they did not exceed 70% of the LD_{50}. Each single group of five animals was treated by subacute i.p. injection of half of each chosen dosage of a test chemical 24 h before the other half was administered. The chemicals were given in corn oil (when the doses were too small to be injected alone) or alone. The control group of 10 mice received i.p. injections of corn oil only. Benzene, as the mother compound for halogenated benzenes, was chosen as the positive control. The animals were killed by an overdose of ether 30 h after the first injection of the test chemical; the femora were removed and the marrow was suspended in serum. Two smears per femur were prepared and coded. The smears were studied by two different examiners. One-thousand polychromatic erythrocytes per smear were examined for the presence of micronuclei.

Statistical analysis

LD_{50} doses and their 95% confidence limits were calculated using the method of Cavalli-Sforza and Lorenz (1964); significant testing of the micronucleus results was done using the Student's *t* test (Hill, 1967).

Results

The highest toxicity was found with BB and the least toxicity with 1,3,5-TBC, when the median lethal doses were determined (Table I). In general, trichlorobenzenes were less toxic to the animals than dichlorobenzenes (Table I).

All the halogenated benzenes tested were found to be clastogenic ($P < 0.01$) (Table II). The highest clastogenic activities were observed in animals dosed with *m*-DCB and BB. Among the three isomers of DCB, *m*-DCB induced significantly ($P < 0.05$) more micronuclei than *o*-DCB or *p*-DCB (Table II). No significant differences were found between the clastogenic activities of TCB-isomers (Table II).

Discussion

Although the chromosome-damaging effects of benzene in marrow cells of mammals, including man, and the capacity of

Table I. Median lethal doses (LD_{50}) of benzene and eight halogenated benzenes for 25–30 g male NMRI mice by i.p. administration. Purities of the compounds are those given by the manufacturer

Compound	Purity (%)	LD_{50} mg/kg body weight
Benzene	99.7	1714.24 ± 101.37
Bromobenzene	99.0	817.11 ± 50.71
Chlorobenzene	99.0	1355.24 ± 250.55
m-Dichlorobenzene	99.0	1061.84 ± 38.73
o-Dichlorobenzene	99.0	1228.12 ± 262.99
p-Dichlorobenzene	99.0	2000.42 ± 49.70
1,2,3-Trichlorobenzene	99.0	1390.21 ± 225.98
1,2,4-Trichlorobenzene	98.0	1223.25 ± 320.54
1,3,5-Trichlorobenzene	98.0	2259.83 ± 76.96

Table II. Effects of eight halogenated benzenes on frequency of micronucleated polychromatic erythrocytes of femoral bone marrow of male NMRI mice (significant value for all doses: $P < 0.01$). Values represent means ± SD of five individual experiments per dose

Compound	mg/kg body weight	Micronuclei/ 1000 PCE
Vehicle (corn oil)		1.80 ± 0.748
Benzene	264 (2 × 132)	4.40 ± 0.800
	528 (2 × 264)	8.10 ± 0.943
	1056 (2 × 528)	12.40 ± 1.356
	528 (1 × 528)	10.83 ± 1.343
Bromobenzene	125 (2 × 62.5)	3.70 ± 0.640
	240 (2 × 125)	5.50 ± 0.806
	375 (2 × 187.5)	6.50 ± 1.024
	500 (2 × 250)	8.30 ± 1.100
Chlorobenzene	225 (2 × 112.5)	3.10 ± 0.830
	450 (2 × 225)	3.90 ± 0.830
	675 (2 × 337.5)	4.90 ± 0.943
	900 (2 × 450)	7.20 ± 0.871
m-Dichlorobenzene	175 (2 × 87.5)	3.40 ± 0.663
	350 (2 × 175)	4.40 ± 0.916
	525 (2 × 262.5)	6.30 ± 1.100
	700 (2 × 350)	9.20 ± 1.248
o-Dichlorobenzene	187 (2 × 93.5)	3.90 ± 0.943
	375 (2 × 187.5)	4.50 ± 1.204
	562 (2 × 281)	5.44 ± 1.257
	750 (2 × 375)	5.90 ± 1.135
p-Dichlorobenzene	355 (2 × 177.5)	4.00 ± 0.632
	710 (2 × 355)	4.90 ± 1.135
	1065 (2 × 532.5)	6.00 ± 1.000
	1420 (2 × 710)	6.60 ± 0.916
1,2,3-Trichlorobenzene	250 (2 × 125)	4.10 ± 0.943
	500 (2 × 250)	5.20 ± 1.249
	750 (2 × 375)	6.80 ± 0.979
	1000 (2 × 500)	7.30 ± 1.005
1,2,4-Trichlorobenzene	210 (2 × 105)	3.50 ± 0.806
	420 (2 × 210)	4.50 ± 0.806
	630 (2 × 315)	5.80 ± 0.871
	840 (2 × 420)	7.40 ± 0.916
1,3,5-Trichlorobenzene	425 (2 × 212.5)	3.60 ± 0.800
	850 (2 × 425)	4.40 ± 0.916
	1275 (2 × 637.5)	6.60 ± 0.894
	1700 (2 × 850)	7.20 ± 0.979

benzene to induce myelogenous leukaemia and other blood dyscrasias in rodents and supposedly in man have been well documented (Diaz *et al.*, 1980; Meyne and Legator, 1980; Snyder *et al.*, 1980; Goldstein and Snyder, 1982; Harper *et al.*, 1984; Cronkite *et al.*, 1985; Styles and Richardson, 1984; Erexson *et al.*, 1986; Dean, 1985; Infante and White, 1985), little adequate information exists on the carcinogenic/mutagenic effects of halogenated benzenes.

Our data demonstrate the clastogenic activity of these compounds (Table II) and indicate the desirability of further testing of benzene derivatives in order to assess the mutagenic hazard and carcinogenic potential associated with the use of this class of compounds.

The number of human cases of leukaemia associated with DCB-exposure is too small to assess a causal relationship (IARC, 1982b). This observation and the possibility of weak mutagenic activity of chlorinated benzenes (IARC, 1982a) suggest, however, that they may possess the ability to bind to DNA and cause genetic alterations in cells. BB has been found to bind covalently to DNA/RNA in mouse and rat liver cells at rates comparable to those of some moderate oncogenic compounds (Colacci *et al.*, 1985). In this connection, the negative results of Wester *et al.*, 1985) following lifetime carcinogenicity testing of chlorinated benzenes on rat may not be a very reliable assessment of the carcinogenicity of chlorobenzene (CB) isomers. This view is based on a fact that a mixture of 11 halogenated hydrocarbons (including six CB isomers) was tested together rather than testing each compound separately. Although Lai (1984), in a review article, cites a study conducted by the US National Toxicology Program implying that monochlorobenzene is a rat carcinogen, the results of this study do not appear to have been published.

Further studies are in progress to identify the metabolite(s) and the mechanism(s) involved in the induction of clastogenic effects in bone marrow cells of mice by halogenated benzenes.

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References

Cavalli-Sforza,L. and Lorenz,R.J. (1964) Grundbegriffe der Biometrie insbesondere der statistischen Methoden bei der Wertbemessung biologisch wirksamer Substanzen. Gustav Fischer Verlag, Suttgart, pp. 171–177.

Colacci,A., Arfellini,G., Mazzullo,M., Prodi,G. and Grilli,S. (1985) The covalent binding of bromobenzene with nucleic acids. *Toxicol. Pathol.*, **13**, 276–282.

Cronkite,E.P., Drew,R.T., Inoue,T. and Bullis,J.E. (1985) Benzene hematotoxicity and leukemogenesis. *Am. J. Ind. Med.*, **7**, 447–456.

Dean,B.J. (1985) Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.*, **154**, 153–181.

Diaz,M., Reiser,A., Brauer,L. and Diez,J. (1980) Studies on benzene mutagenesis. I. The micronucleus test. *Experientia*, **36**, 297–298.

Erexson,G.L., Wilmer,J.L., Steinhagen,W.H. and Klingerman,A.D. (1986) Induction of cytogenetic damage in rodents after short-term inhalation of benzene. *Environ. Mutagenesis*, **8**, 29–40.

Fowler,J.S.L. (1983) Short-term testing in drug development. In Balls,M., Riddell,R.J. and Worden,A.N. (eds), *Animals and Alternatives in Toxicity Testing*. Academic Press, London, pp.16–23.

Giavini,E., Broccia,M.L., Prati,M. and Vismara,C. (1986) Teratologic evaluation of *p*-dichlorobenzene in the rat. *Bull. Environ. Contam. Toxicol.*, **37**, 164–168.

Goldstein,B.D. and Snyder,C.A. (1982) Benzene leukemogenesis. *Environ. Sci.*

Res., **25**, 2778–2789.
Harper,B.L., Sadagopa Ramanujam,V.C., Gad-El-Karim,M.M. and Legator,M.S. (1984) The influence of simple aromatics on benzene clastogenicity. *Mutat. Res.*, **128**, 105–114.
Hayes,W.C., Hanley,T.R., Gushow,T.S., Johnson,K.A. and John,J.A. (1985) Teratogenic potential of inhaled dichlorobenzenes in rats and rabbits. *Fund. Appl. Toxicol.*, **5**, 190–202.
Hill,A.B. (1967) The 't' test for small numbers. In Hill,A.B. (ed.), *Principles of Medical Statistics*. The Lancet Ltd., London, pp. 146–151.
IARC (1982a) Monographs on the evaluation of the carcinogenic risks of chemicals to humans. *Some Industrial Chemicals and Dyestuffs*. Vol. 29, pp. 213–238.
IARC (1982b) Monographs on the evaluation of the carcinogenic risk of chemicals to humans. *Chemicals, Industrial Processes and Industries Associated with Cancer in Human*. Vol. 29 (Suppl. 4), pp. 108–109.
Infante,P.F. and White,M.C. (1985) Projections of leukemia risk associated with occupational exposure to benzene. *Am. J. Ind. Med.*, **7**, 403–413.
Jan,J. (1983) Chlorobenzene residues in human fat and milk. *Bull. Environ. Contam. Toxicol.*, **30**, 595–599.
Lai,D.Y. (1984) Halogenated benzenes, naphthalenes, biphenyls and terphenyls in the environment: their carcinogenic, mutagenic and teratogenic potential and toxic effects. *J. Environ. Sci. Health*, **2**, 135–184.
Meyne,J. and Legator,M.S. (1980) Sex-related differences in cytogenetic effects of benzene in the bone marrow of Swiss mice. *Environ. Mutagenesis*, **2**, 43–50.
Mohtashamipur,E., Norpoth,K., Woelke,U. and Huber,P. (1985) Effects of ethylbenzene, toluene, and xylene on the induction of micronuclei in bone marrow polychromatic erythrocytes of mice. *Arch. Toxicol.*, **58**, 106–109.
Pellizzari,E.D. (1979) Quantification of chlorinated hydrocarbons in previously collected air samples. *Gov. Rep. Announce. Index (US)*, **79**(8), 68.
Schmid,W. (1975) The micronucleus test. *Mutat. Res.*, **31**, 9–15.
Simmon,V.F., Rosenkranz,H.S., Zeiger,E. and Poirier,A. (1979) Mutagenic activity of chemical carcinogens and related compounds in the intraperitoneal host-mediated assay. *J. Natl. Cancer Inst.*, **62**, 911–918.
Snyder,C.A., Goldstein,B.D., Sellakumar,A.R., Bromberg,I., Laskin,S. and Albert,R.E. (1980) The inhalation toxicology of benzene: incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57BL/6J mice. *Toxicol. Appl. Pharmacol.*, **54**, 323–331.
Styles,J.A. and Richardson,C.R. (1984) Cytogenetic effects of benzene: dosimetric studies on rats exposed to benzene vapour. *Mutat. Res.*, **135**, 203–209.
Wester,P.W., van der Heijden,C.A., Bisschop,A., van Esch,G.J., Wegman,R.C. and de Vries,T. (1985) Carcinogenicity study in rats with a mixture of eleven volatile halogenated hydrocarbon drinking water contaminants. *Sci. Total Environ.*, **47**, 427–432.
Zoeteman,B.C.J., Piet,G.J., Kussmaul,H. and Muehlhausen,D. (1979) Behavior of organic water components during drinking water preparation, especially in groundwater enrichment. *Reinhalt. Wasser*, **1**, 89–106.

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