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Changes in Selected Indicators of Liver Impairment after Repeated Administration of Mono- and Polybromobenzenes in Mice

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Brominated benzene derivatives have been used as flame retardants during the past 20 years, and have become a potential source of environmental contamination. Hexabromobenzene and its metabolites are present in water, fish, birds, sediments and human tissues (Kashimoto and Tatsukawa 1986; Yamaguchi et al. 1988a; Watanabe and Tatsukawa 1989).

Hexabromobenzene has been the most widely used of the polybromobenzenes. The products of hexabromobenzene debromination (penta-, tetra-, tribromobenzenes) are formed by means of environmental degradation or metabolism. They are more volatile and water soluble than the parent compound (Watanabe and Tatsukawa 1989). Dibromobenzenes found in natural environments may appear as a result of using them as fumigants, additives to cleaning agents, or as half-products in production of pharmaceutical preparations (Colacci et al. 1990). They may also arise from degradation (debromination) of higher brominated benzene derivatives (Yamaguchi et al. 1988b).

Despite wide usage of hexabromobenzene and other aromatic brominated derivatives, we know little about their toxicity. It is known that monobromobenzene administered as a single dose has a high hepatotoxic and nephrotoxic potential and is used therefore as a model substance in research on liver and kidney necrosis (Hanke and Lutz 1986; Lau and Monks 1988). Hepatocyte necrosis is also induced by 1,2- and 1,3-dibromobenzenes in acute intoxication (Szymanska et al. 1996). No data are available on the hepatotoxicity of bromobenzenes following repeated exposures. Porphyrogenic effects have been observed following repeated exposure to chloroderivatives (e.g. hexachlorobenzene). The aim of the present report was to find out if necrotic liver damage, typical for acute intoxication, can be reproduced by repeated exposure to much smaller doses.

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MATERIALS AND METHODS

The experiments were performed on male, BALB'c mice, age 2-3 month, obtained from the Institute of Occupational Medicine in Lódz, Poland. The animals were fed standard Murigram chow (Agripol, Motycz, Poland) and were provided tap water ad libitum. Animals were acclimated to the laboratory two weeks prior to their use in experiments. Exposed mice were housed 20 per cage (size of cages: 30 x 40 x 17 cm), control mice 10 per cage. The animal room had a temperature of 21-23°C and humidity of 55 ± 5 %. In all experiments the Polish rules on the protection of animals were followed (Dziennik Ustaw 1997).

Eight bromobenzenes were administered intraperitoneally in sunflower oil seven daily doses of about 3.5%, 7%, 10.5% and 18% of the respective approximate lethal doses (ALD) (Table 1). The ALD was determined by the method of Deichman-Le Blanc (1943). The administered dose was contained in oil volume of 0.2 ml per 20g of mice (10 ml per kg). All administrations were done in the morning (08:00-09:00 hrs).

Table 1. Approximate lethal dose (ALD) and daily doses of bromobenzenes administered-to mice for 7 days; each group constituted 4-5 animals.

Compound	ALD (mg/kg)	% ALD			
		3.5% (mg/kg)	7% (mg/kg)	10.5% (mg/kg)	18% (mg/kg)
bromobenzene **(BB)** (Fluka 16350)	900	35	65	95	160
1,2-dibromobenzene **(1,2-diBB)** (Fluka 33971)	1100	40	80	120	200
1,3-dibromobenzene **(1,3-diBB)** (Fluka 33980)	750	30	60	80	140
1,4-dibromobenzene **(1,4-diBB)** (Fluka 33990)	2500	90	180	270	450
1,2,4-tribromobenzene **(1,2,4-triBB)** (Aldrich 13,275-6)	2500	90	-	270	450
1,3,5,-tribromobenzene **(1,3,5-triBB)** (Aldrich 14,006-6)	3750	130	-	390	675
1,2,4,5-tetrabromobenzene **(1,2,4,5-tetraBB)** (Aldrich 27,834-3)	3750	130	-	390	675
hexabromobenzene **(HBB)** (Aldrich 10,713-1)	10000	-	-	1200	1800

Intact animals of the same age and sex as the experimental mice constituted the control groups. Two kinds of controls were used: (a) “pure control”, where mice were free of dosing and (b) “oil control” in which sunflower oil only was administered in volume of 0.2 ml per 20g of mice (10 ml per kg). For each bromobenzene species administered at four different dose levels, we included one pure control group and one oil control group (4 animals each). The animals were sacrificed without anaesthesia, by cervical dislocation 24 hours after the administration of the last dose of bromobenzenes. Blood was taken from the heart. Following blood coagulation the serum was obtained by centrifuging at 14000 rpm for 3 minutes.

The following parameters were determined in liver homogenates: the concentration of reduced glutathione (GSH; Sedlak and Lindsay 1968) malondialdehyde (MDA; Mihara et al. 1980) as well as the activity of delta-aminolevulinate dehydratase (ALA-D, EC 4.2.1.24; Berlin and Schaller 1974; Schlick et al. 1983) and delta-aminolevulinate synthase (ALA-S, EC 2.3.1.37; Sassa and Granick 1970). For the determinations, 25% liver homogenates were prepared in phosphate buffer, pH 8.0, using a Universal Laboratory Aid homogenizer, type MPW-309 (Precision Mechanics, Warsaw, Poland). Homogenates were used directly, except for MDA which was diluted 10% prior to analysis.

Serum was assayed for the activity of alanine aminotransferase (ALT, EC 2.6.1.2; Reitman and Frankel 1957; Poznanska and Osinski 1968) and gamma-glutamyltransferase (γ-GT, EC 2.3.2.2; Monotest 10 γ-GT neu from Boehringer-Mannheim). For γ-GT serum samples were stored at -18°C prior to analysis. All other determinations were carried out immediately after the sacrifice of animals.

The results were evaluated using one-way analysis of variance. Accordingly, depending on the outcome, further calculations were made using the parametric procedure (Snedecor and Scheffe tests; Snedecor 1956) or non-parametric methods (Kruskal-Wallis and Conover tests; Conover 1971). The parametric procedure was applied for γ-GT, for all other parameters the non-parametric procedure was applied. The comparisons were made at $\alpha = 0.05$ statistical significance.

Liver histology was evaluated for possible tissue damage using light microscopy. The tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. In some cases, preparations were stained with phosphotungstic acid and haematoxylin by the Van Gieson-method and PAS-reaction (periodic acid-Schiff reaction). To evaluate the relation of steatosis to the exposure of mice a simplified ranking was applied, ranging from 1 (no change) to 4 (steatosis of all lobular zones).

RESULTS AND DISCUSSION

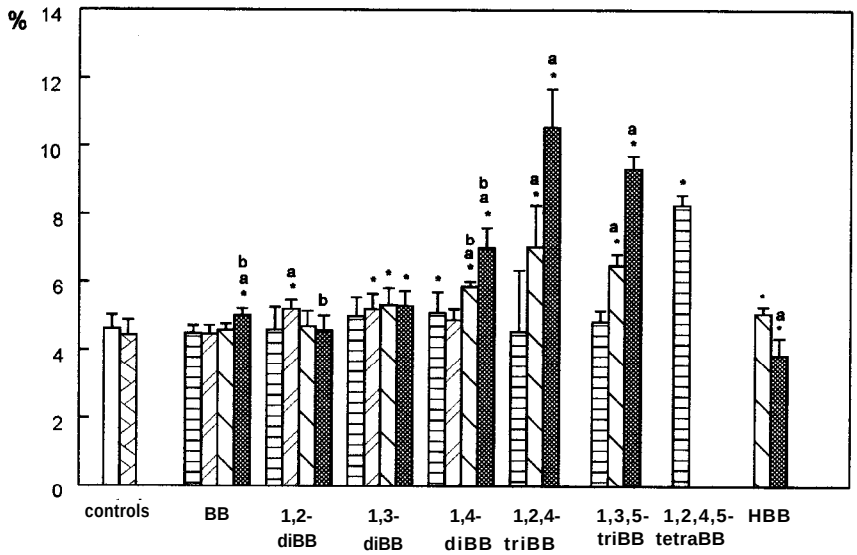

Figure 1. Ratio liver / total body weight (mean ± SE) in mice exposed to selected brominated benzenes for 7 days (expressed in %).

Legends to figures:

pure control | oil control | 3.5% ALD | 7% ALD | 10.5% ALD | 18% ALD

*** - significantly different from control animals, α = 0.05**

a -significantly different from animals administered 3.5% ALD

b - significantly different from animals administered 7% ALD

c - significantly different from animals administered 10.5% ALD

For all parameters (GSH, MDA, ALA-D, ALA-S, ALT, γ-GT, ratio liver/total body weight) determined in control groups, both "pure" and "oil controls", small fluctuations were observed. Therefore the type of control used in calculations was relevant for the outcome. The differences between parameters of different control groups were all insignificant. Also, there were no significant differences between the pooled "pure" and "oil" controls. Therefore, the pooled "pure" controls were taken for comparison with each of the exposed groups. The information on the "oil control" served the purpose of detecting possible artifacts evoked by oil alone.

In order to induce hepatotoxic (necrotic) effects following a single exposure, high doses of bromobenzene (Lau and Monks 1988; Szymanska 1998) or dibromobenzenes (Szymanska et al. 1996) must be applied in excess of 10-20% of the lethal ones. Since environmental exposure usually involves much smaller doses extended in time, we were interested if this effect could be evoked using much smaller doses, administered for 7 days. A 7-day

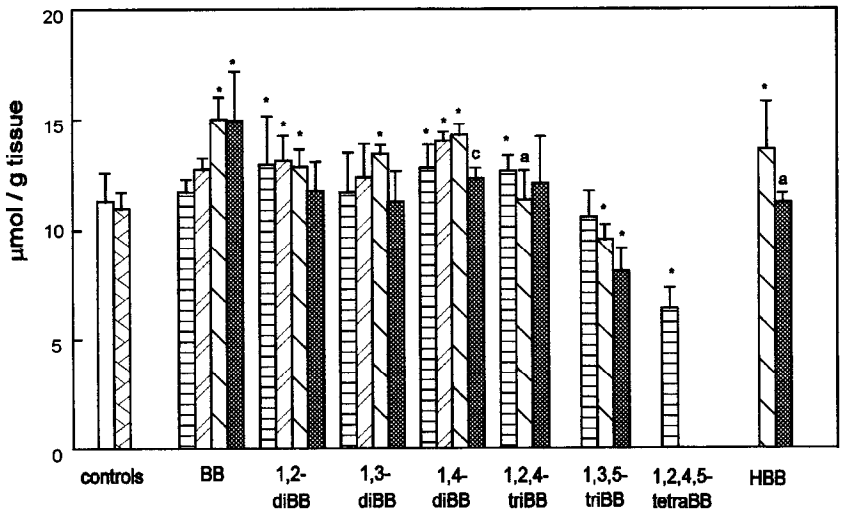

Figure **2.** Mean level (± SE) of GSH after administration of brominated benzenes to mice for 7 days. Legends to figures - see Fig.1.

administration was chosen with individual doses ranging from 3.5 to 18% of ALD, corresponding to cumulative doses of about 25 to 125% of ALD. Gross observations of animals have shown that for 1,2,4-triBB, 1,3,5-triBB and 1,2,4,5-tetraBB diminished mobility was apparent.

Distinct changes could be ascertained for liver/total body weight ratio for all bromobenzenes (Fig.1). An increase in liver weight was noted following repeated doses of bromobenzenes at 10.5% and 18% ALD. Only for 1,2,4,5-tetraBB was there an increase at 3.5% ALD; high mortality occured for this compound at doses above 3.5% ALD (3-5 animals in group; the died animals were not included in the evaluation). For 1,6diBB, 1,2,4-triBB and 1,3,5-triBB the change of liver/body weight ratio was dose dependent. Bromobenzenes had no influence on the enzymatic markers of necrosis, ALT in blood, nor on the histopathological evaluation. The only histopatologic change consisted of distinct steatosis in the peripheral lobular zone (for the histopatologic ranking of steatosis - see Methods). The following mean values were obtained: oil control - 1.5; pure control - 1.7; 1,4-diBB - 2.9; 1,2,4-triBB - 2.4; 1,3,5-triBB - 2.7; 1,2,4,5-tetraBB - 2.8; HBB - 2.3. No dose dependence was found for this parameter irrespective of the chemical administered.

In single exposure studies with bromobenzenes, the necrotic changes are preceeded by a depletion of GSH levels (Szymanska 1998). After 7-day administration of the majority of the examined bromobenzenes an increase in GSH concentration in the liver was observed (Fig. 2). Bromobenzene at the two highest doses (10.5% and 18% of ALD) caused significant increases in GSH levels. Exposure to 1,2-diBB and 1,4-diBB at the lower doses (3.5%, 7%, 10.5% of ALD) induced statistically significant increases in

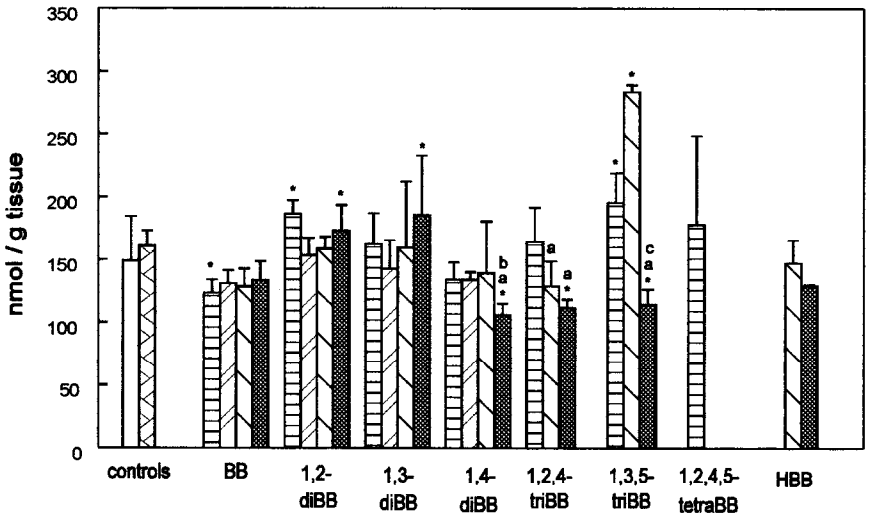

Figure 3. MDA concentration (mean ± SE) following administration of brominated benzenes to mice for 7 days. Legends to figures - see Fig.1.

GSH levels. HBB (1200 mg/kg) also induced elevation of the GSH level. A decrease in GSH concentration was detected after the administration of 1,3,5-triBB (2 higher doses) and 1,2,4,5-tetraBB.

Administration of bromobenzenes resulted in limited changes in hepatic MDA levels, but the alterations were apparently neither unidirectional nor dose dependent (Fig. 3). Increases in MDA levels were noted after 1,2-diBB; and 1,3-diBB, and 1,3,5-triBB administration, whereas decreases occurred after administration of BB, 1,4-diBB, and also the highest dose of 1,3,5-triBB.

Repeated administration of bromobenzenes caused significant changes in the ALA-S activity in the liver (Fig. 4). All administered doses of BB, 1,2,4-triBB and 1,3,5-triBB induced statistically significant increases in ALA-S activity in a dose-dependent manner.

After the administration of BB, ALA-S activity doubled, whereas tribromobenzenes resulted in 2-3-fold elevations compared with controls. Significant increase of ALA-S activity was also observed for the two highest doses of 1,4-diBB (10.5%, 18% of ALD). For 1,4-diBB the lowest dose resulted in the decrease of ALA-S activity whereas for the two highest doses an elevation was apparent. The two other dibromobenzene isomers 1,2-diBB and 1,3-diBB resulted in statistically significant decreases in ALA-S activity.

Hepatic activity of ALA-D showed fluctuations in both directions. The changes were small, 10-20% of the controls, and mostly statistically insignificant (not shown).

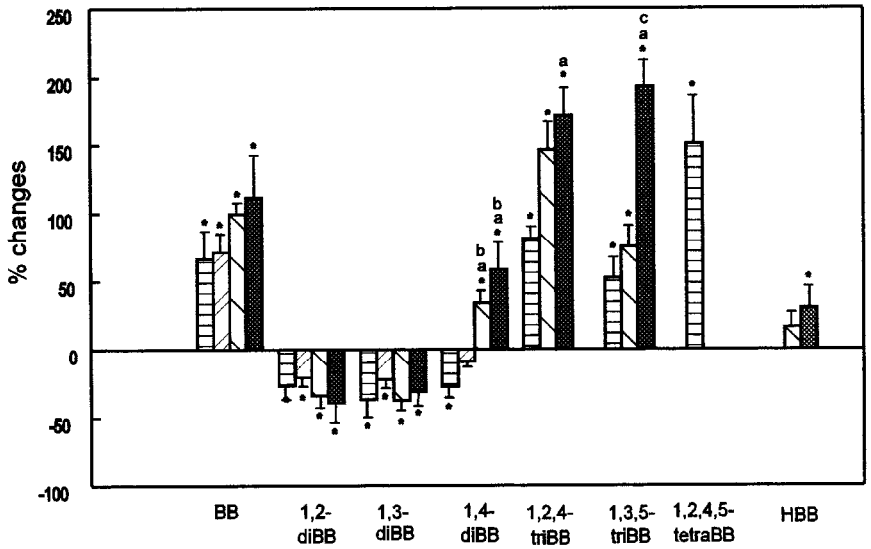

Figure 4. Changes in ALA-S activity in comparison with pure controls following administration of brominated benzenes to mice for 7 days. Legends to figures - see Fig.1.

None of the bromobenzenes affected the activity of ALT suggesting the absence of necrotic changes of hepatocytes; the same applies to γ-GT, except for the highest dose of 1,3,5-triBB, for which a small increase was noted (not shown).

We assume, that under conditions of repeated exposure a shift occurs in the profile of hepatotoxic effects from necrotic changes (in single exposure) to steatosis and porphyrogenic effects. Changes in the activity of ALA-S, a step that may be down-regulated by interactions occurring elsewhere in the chain of heme synthesis, may have some diagnostic value.

Although data on hepatotoxicity of bromobenzenes under conditions of repeated exposure are scarce, the available data are not contradictory to ours. For bromobenzene, lack of effects under repeated exposure is being explained in different ways: (a) by means of analogy with CCl_4, it might be assumed that bromobenzene may impair the system of microsomal enzymes, which, in turn might, prevent the formation of toxic metabolites (Lindstrom and Anders 1977; Guzelian and Swisher 1979; Beyhl and Mayer 1980); (b) repeated administration of bromobenzene may cause a "shift" in the metabolic pathway towards the formation of less hepatotoxic metabolites (e.g. the formation of 2,3-bromobenzene epoxide; Lau and Zannoni 1979, 1981); (c) repeated doses of bromobenzene may by means of inducing microsomal enzymes and GSH levels accelerate the process of bromobenzene metabolism and/or may intensify repair/regeneration processes in the cell (Chakrabarti 1991).

The results presented in this study (no detected necrosis, no increase of ALT and γ-GT, extensive steatosis, increase in ALA-S activity, increase in GSH

concentrations) after repeated exposure to bromobenzenes confirms Chakrabarti's (1991) interpretation.

In the present study we aimed at demonstration that in comparison with a single administration, repeated exposure results in changing the profile of effects. Earlier , the effects of liver impairment such as: necrosis in the liver and the increase in ALT activity in the serum of mice had been observed in our laboratory only after single doses of bromobenzenes (Szymanska 1998). Yet, repeated administration of bromobenzenes for 7 days, resulted in changes of the heme synthesis indicators suggesting porphyrogenic effect induced by these compounds.

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