Table 5 Major toxicity findings for trityl chloride in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	60	300	600†	0	12	60	300
Male								
Death	0/12	0/12	0/12	0/6	0/12	0/6	0/12	0/12
Final body weight	/	–	–	↓	/	–	–	↓
ALT, Total cholesterol	/	–	–	–	/	–	–	↑
Relative liver weight	/	↑	↑	↑	–	–	↑	↑
Relative kidney weight	/	–	–	–	–	–	–	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	0/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	0/6	no data	0/6	0/6	3/6	6/6
Female								
Death	0/12	0/12	0/12	1/6	0/12	0/6	0/12	0/12
Final body weight	/	–	–	↓	/	–	–	–
ALT, Total cholesterol	/	–	–	–	/	–	–	-,↑
Relative liver weight	/	↑	↑	↑	–	–	↑	↑
Relative kidney weight	/	–	–	–	–	–	↑	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	2/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	4/6	no data	0/6	0/6	5/6	6/6

Only critical data are shown in this table. †indicates a dose from the dose-finding study. Numbers are for animals with the feature in the total examined. Slashes and bars mean no statistical significance as compared to controls. ↑ indicates significant increase $P < 0.05$. ↓indicates significant decrease at $P < 0.05$. Relative liver weights were increased by 11% for males and 8% for females at 60 mg/kg, and 29% for both sexes at 300 mg/kg in the newborn main study and by 44% for males and 46% for females at 600 mg/kg in the newborn dose-finding study. Body weight depression in males (13%) and an increase of relative liver weights (32% for males, 40% for females) were observed at 300 mg/kg in the young main study.

Therefore, pUETLs of 400–500 and 300 mg/kg/day are proposed as appropriate for newborn and young rats, respectively.

1,3,5-Trihydroxybenzene (Table 6)

The newborn investigation was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and at 0, 20, 100, and 500 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 250, 500, and 1000 mg/kg for dose-finding and at 0, 30, 100, 300, and 1000 mg/kg for the main study.

Common changes were observed in the thyroids and liver. The only toxic change in newborn main study was hypertrophy of thyroid follicular cells with increase in relative thyroid weights in both sexes at 500 mg/kg. Increased relative liver weights in females were not accompanied by any histopathological changes. Although decrease of adrenal weight and histopathological alterations such as vacuolization and pigmentation were noted at the end of the dosing and recovery-maintenance periods, these were always slight and not dose-dependent. There were no chemical-related changes with other examinations, including developmental parameters, in newborn rats. In the young study, similar effects on the thyroids and liver were found at 1000 mg/kg, but the incidence of thyroid histopathological changes was slightly less than in newborn animals at 500 mg/kg.

pNOAELs of 100 and 300 mg/kg/day for newborn and young rats can be considered appropriate because of the lack of data with dose settings between 100 to 500 mg/kg in the newborn, and no histopathological examination at 500 mg/kg in the young dose-finding study. The degree of toxicity at 1000 mg/kg for young rats was almost equal to that at 500 mg/kg for newborn rats. Therefore, pUETLs of 500 and 1000 mg/kg/day are proposed as equivalents for newborn and young rats, respectively.

DISCUSSION

More than 100 000 industrial chemicals are now in use around the world and sufficient toxicity information is available for only a small proportion. The Japanese government started the Existing Chemical Safety Program to obtain minimal toxicity data sets from 28-day toxicity studies using young rats for high production volume chemicals lacking toxicity information. For the present six targeted chemicals, we found toxicity information for only two chemicals by literature search. Daniel *et al.* (1993) reported no toxic effects of 2-chlorophenol on oral administration to male and female Sprague Dawley rats at up to 257 mg/kg for 10 days or 150 mg/kg for 90 days. Our results were consistent with their data, as we found no toxicity at 500 mg/kg in young dose-finding study (14 days administration) and at 200 mg/kg in the young study (28 days), while further providing information on CNS effects at higher doses. As for (hydroxyphenyl)methyl phenol, consisting of bisphenol D, E, and F isomers, bisphenol F has been reported to have estrogenic potential evidenced by several *in vitro* and *in vivo* experiments (Hashimoto *et al.* 2001; Yamasaki *et al.* 2002; Stroheker *et al.* 2003). However, we could not establish any such activity in this study. Our results are reasonable because oral administration of bisphenol F increased relative uterus weights only at more than 100 mg/kg, but not 50 mg/kg given during PNDs 22–25 (Stroheker *et al.* 2003), while our highest dose of (hydroxyphenyl)methyl phenol was equivalent to 30 mg/kg of bisphenol F.

Table 6 Major toxicity findings for 1,3,5-trihydroxybenzene in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)		
	0	100	500	0	300	1000
Male						
Relative organ weight						
Liver	/	–	–	/	–	↑
Thyroids	/	–	↑	/	–	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	4/6	0/6	0/6	2/6
Female						
Relative organ weight						
Liver	/	–	↑	/	–	↑
Thyroids	/	–	(↑)	/	–	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	5/6	0/6	0/6	4/6

Only critical data are shown in this table. Slashes and bars mean no statistical significance as compared with controls. ↑ indicates significant increase $P < 0.05$ (except in parentheses where statistical significance was not attained). Numbers are for animals with the feature in the total examined. Increase of relative organ weights at 500 mg/kg in the newborn main study was observed for thyroids (39% for males, 24% for females) and liver (9% for females). Increase of relative organ weights at 1000 mg/kg in the young main study was observed for thyroids (14% for males, 19% for females) and liver (23% for males and 9% for females).

Table 7 Comparative susceptibility of newborn and young rats to the six chemicals

	Newborn study		Young study		pNOAEL	pUETL
	pNOAEL	pUETL	pNOAEL	pUETL	Young/Newborn	Young/Newborn
	mg/kg/day		mg/kg/day			
2-Chlorophenol	40	200–250	200	1000	5.0	4.0–5.0
4-Chlorophenol	100	300	100	500	1.0	1.7
p-(α,α-Dimethylbenzyl) phenol	30	300	100	700–800	3.3	2.3–2.7
(Hydroxyphenyl) methyl phenol	100	140–160	40	1000	0.4	6.3–7.1
Trityl chloride	60	400–500	12	300	0.2	0.6–0.8
1,3,5-Trihydroxybenzene	100	500	300	1000	3.0	2.0

Although there has been no reports for p-(α,α-dimethylbenzyl) phenol, it causes endocrine disruption and possible antiestrogenic activity, when administered to newborn female rats in this study. Therefore, further studies on this chemical should be conducted to elucidate the mechanisms, because the present investigation did not indicate any effects on sexual differentiation such as preputial separation, vaginal opening and the estrous cycle.

For our focus on the comparative sensitivity of newborn and young rats to chemicals, two toxicity endpoints, pNOAEL and pUETL, were newly defined as appropriate, considering the entire data sets from both main and dose-finding studies. We believe that this alternative assessment approach allowed us to make more realistic comparisons between newborn and young rats under the same experimental conditions as far as possible.

The ratios of pNOAELs for chemicals between newborn and young rats may provide an additional UF value in risk assessment according to susceptibility of newborn rats, because regulatory limit values for chemicals to protect public health of humans, including infants, are derived from the division of NOAEL by UFs. The data in Table 7 indicate newborn rats to be 1–5 times more susceptible to four of the tested chemicals, 2- and 4-chlorophenols, p-(α,α-dimethylbenzyl) phenol and 1,3,5-trihydroxybenzene, than young rats in terms of the pNOAELs, similar to the results of previous analyzes of five phenolic chemicals, 4-nitro-, 2,4-dinitro-, 2,4,6-trinitro-, 3-methyl- and 3-amino-phenols (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004). Immaturity in the detoxification potential of phase 1 and phase 2 enzymes in newborn animals may be the major cause of higher toxicity in newborn rats (Rich & Boobis 1997; Gow *et al.* 2001), because these chemical classes are probably direct toxicants. In the case of (hydroxyphenyl)methyl phenol, the pNOAEL (100 mg/kg/day) for newborn rats was 2.5 times higher than that (40 mg/kg/day) for young rats, but it can be speculated that values are in practice rather similar because the toxicity for young rats at the high dose, 200 mg/kg, was only slight (Table 4). As for trityl chloride, newborn rats were obviously less susceptible (0.2 for the pNOAEL ratio). Similar results were

also reported from our previous analysis for bromoalkanes (Hirata-Koizumi *et al.* 2005) and may be explained by mechanisms of action and metabolic characteristics of newborn rats. As this class of chemicals possibly requires metabolism to act as toxicants, the relatively mature metabolic enzyme status of young rats would be expected to provide toxic intermediates by metabolic activation to a greater extent than in newborn rats, as evidenced by data for previously reported chemicals (Onkenhout *et al.* 1986; Kennedy *et al.* 1993). Other compounds such as acetaminophen, bromobenzene, and carbon tetrachloride have also been shown to not produce liver injury in neonatal animals at doses that are hepatotoxic to adults (Gregus & Klaassen 1998).

The ratios of pUETLs, doses inducing the same degree of toxicity in newborn and young rats, were almost the same as for pNOAELs with the direct toxicants, as shown in Table 7. However, newborn rats were considerably more susceptible to (hydroxyphenyl)methyl phenol when considering the pUETL, due to the much steeper dose–response curve in newborn rats, with a 100 mg/kg/day pNOAEL and half the animals dying at 200 mg/kg, compared with a 40 mg/kg/day pNOAEL and only one death in 12 animals at 1000 mg/kg for young rats. Although young rats showed stomach hyperplasia in addition to hepatotoxicity at 1000 mg/kg, the cause of newborn deaths at 200 mg/kg was unclear. With regard to trityl chloride, the pUETL for young rats was almost the same as for newborn although the latter were less susceptible. Such an anomaly has also been found for bromoalkanes previously analyzed. Another example of a chemical for which susceptibility differs at low and high doses is chlorpyrifos, the maximum tolerated dose in 17-day-old rats being reported to be five times less than that in adults following oral exposure (Moser & Padilla 1998), but the differential sensitivity not appearing in low-dose exposure (Pope & Liu 1997). Thus as there are several chemicals of which dose–response curve in newborn rats was obviously steeper than that in young rats, pUETL ratios should be also taken into account for the susceptibility of newborn rats as the second endpoint marker.

In conclusion, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL in most cases. One exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.

ACKNOWLEDGMENT

The authors gratefully acknowledge the financial support of the Office of Chemical Safety, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labor and Welfare, Japan and also deeply appreciate the efforts of the six Japanese contract laboratories in performing the actual animal toxicity studies.

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The Journal of Toxicological Sciences,
Vol.30, No.1, 29-42, 2005

SUSCEPTIBILITY OF NEWBORN RATS TO HEPATOTOXICITY OF 1,3-DIBROMOPROPANE AND 1,1,2,2-TETRABROMOETHANE, COMPARED WITH YOUNG RATS

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(Received July 22, 2004; Accepted December 10, 2004)

ABSTRACT — Newborn rat studies were conducted with oral administration of 1,3-dibromopropane (DBP) and 1,1,2,2-tetrabromoethane (TBE) from postnatal Days 4 to 21 to allow comparison of NOAELs and unequivocally toxic levels with those from 28-day young rat studies starting at 5-6 weeks of age. The unequivocally toxic level was estimated by our specified criteria, requiring simultaneous change of organ weights, histopathology, some biochemical parameters and body weights, because in this study only hypertrophy of hepatocytes was observed as a major histopathological change. DBP caused centrilobular hypertrophy of hepatocytes with alteration in biochemical parameters, as well as lowering of body weights, regardless of sex, in both newborn and young rats. NOAELs and unequivocally toxic levels were considered to be 50 and 150 mg/kg/day in newborn rats and 10 and 250 mg/kg/day in young rats, respectively. In the newborn rat study of TBE, some hepatic effects observed at the top dose of 50 mg/kg were not considered adverse because of the lack of histopathological changes. Significant lowering of body weight was noted at 200 mg/kg in the dose-finding study but histopathological data were not available. In the young rat study, there was no definite toxicity at 6 mg/kg and hypertrophic changes in liver and thyroids without body weight change occurred at 200 mg/kg. There were no clear sex differences in both the newborn and young rat studies. NOAELs were considered to be 50 and 6 mg/kg/day in newborn and young rats, respectively, but unequivocally toxic levels for both rats could not be estimated. Abnormalities of external and sexual development and reflex ontogeny in the newborn were not observed with either chemical. Based on these results, it can be concluded that the target organ of DBP and TBE is the liver in both newborn and young rats, and that while the doses at which toxic signs began to appear are higher in newborn rats, those causing clear toxicity may be paradoxically lower in the newborn case.

KEY WORDS: Toxicity in newborn rats, 1,3-Dibromopropane, 1,1,2,2-Tetrabromoethane

INTRODUCTION

The newborn period is a time of biological changes because birth creates a completely new situation for the offspring. For example, prior to birth, maternal and fetal blood are in close equilibration, and most xenobiotics that cross the placenta to the fetus must shift back to the mother again because the ability of the fetus to dispose of them is extremely immature (Scheuplein *et al.*, 2002). After elimination of compounds across the placenta ceases at birth, metabolic and excretory functions rapidly develop. In the liver, parturition triggers the dramatic development of metabolic enzymes (Alcorn and McNamara, 2002). In man,

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most enzymes have matured to adult activity levels by the first year of life, but cytochrome P450-mediated metabolism, glucuronidation, glutathione conjugation and acetylation are generally deficient in the neonate. Regarding renal clearance, although the adult function is also approached by 1 year of age, the faster development of filtering than absorptive or secretory functions results in a glomerulotubular imbalance. The lack of a balanced detoxication ability during the newborn period would be expected to affect toxicity of chemicals.

For the toxicity evaluation of various kinds of chemicals, repeated dose and reproductive/developmental toxicity studies have been generally conducted. However, the effects of direct exposure to chemicals during the newborn period have not been taken into account. Furthermore, there were no sufficient data on the differences between the newborn and young/adult in the susceptibility to the toxicity of chemicals. Therefore, for the purpose of understanding the sensitivity of the newborn and utilizing it in the toxicity evaluation, we conducted the repeated dose toxicity studies using newborn rats, and analyzed the differences of the sensitivity from that of young rats, which have been recently used to evaluate the chemical toxicity in general. These comparative studies were conducted as a part of an existing chemical testing program of Japan. As the candidate chemicals, phenolic and halogenated compounds were selected among chemicals in this program, considering the potential for endocrine disrupting action in the early development period. Because of no standard experimental protocol, repeated dose toxicity studies in newborn rats were conducted with our newly established protocol (Koizumi *et al.*, 2001), including a detailed examination of early development and a complete toxicity analysis after a sufficient recovery-maintenance period. The results were compared with those of a 28-day repeated dose toxicity study using young rats, which is generally conducted as a screening test in existing chemical testing program in Japan. For more precise comparison, in addition to the no observed adverse effect levels (NOAELs), we estimated unequivocally toxic levels, defined as doses inducing clear toxicity, including clinical toxic signs, death or critical histopathological damage. In order to estimate more appropriate NOAELs and unequivocally toxic levels than those depending on the dosages of main studies, the results of dose-finding studies for each case were incorporated. Earlier, we reported analytical results for five chemicals (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, tetrabromobisphenol A) (Koizumi *et al.*, 2001, 2002, 2003; Fukuda *et al.*, 2004). The susceptibility of newborn rats to the toxicity of the first four was 2 to 4 times higher than that of their young counterparts, although these chemicals had no impact on development in the newborn period and showed similar toxicity profiles in both age groups (mainly effects on the central nervous system). In the case of tetrabromobisphenol A, a specific rather than enhanced renal toxicity was observed in newborn rats.

In the present study, two halogenated alkanes, 1,3-dibromopropane (DBP) and 1,1,2,2-tetrabromoethane (TBE), were chosen as the sixth and seventh chemicals for comparative toxicity analysis, because these two chemicals have similar properties such as analogous chemical structures and hepatotoxicity after hepatic metabolism, and the lower susceptibility of the newborn to these chemicals was expected in preliminary analysis, contrary to all outcomes of previous analyses. There has hitherto been no sufficient information on toxicity of DBP, an intermediate in the production of pharmaceutical agents (Chemical Products' Handbook, 2004), except that the intraperitoneal lowest lethal dose is 750 mg/kg in mice (Sax, 1979). Applications of TBE are various as a fire retardant, in oils and fats, in solvents, for ore dressing, and as a reagent for microscopic examination and as a catalyst (Chemical Products' Handbook, 2004). Regarding its toxicity, inhalation exposure to TBE for 180-184 days (7 hr/day, 5 days/week) caused slight edema and congestion in lungs and slight centrilobular fatty degeneration in the livers of mice, rats, guinea pigs and rabbits at an average concentration of 4 ppm (Hollingsworth *et al.*, 1963). Gavage studies for 3 weeks using F344/N male rats have been conducted on many halogenated ethanes to examine renal toxicity, but all rats administered TBE (214 mg/kg/day and more) died or were killed on becoming moribund by dosing Day 11 (NTP, 1996). Cytoplasmic vacuolization of hepatocytes was observed in these rats. We have conducted the newborn rat studies on DBP and TBE and evaluated the results in comparison with published findings in young rats (MHLW, 2003a, 2003b), in the same manner as for the five chemicals already documented (Koizumi *et al.*, 2001, 2002, 2003).

MATERIALS AND METHODS

Materials

1,3-Dibromopropane (DBP, CAS No. 109-64-8, purity: 99.8%) and 1,1,2,2-tetrabromoethane (TBE,

CAS No. 79-27-6, purity: 99.2%) were obtained from TOSOH CORPORATION (Tokyo, Japan), and dissolved in corn oil and olive oil, respectively. Test solutions were prepared at least once a week and kept cool and in the dark until dosing. The stability was confirmed to be at least 7 days under these conditions. All other reagents used in this study were specific purity grade.

Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at 19-27°C with a relative humidity of 32-75%, a ventilation rate of more than 10 times per hour, and a 12:12 hr light/dark cycle. For 18-day newborn rat studies of DBP and TBE, 20 pregnant rats (gestation Day 14) were purchased for each and allowed to deliver spontaneously. All newborn were separated from dams at postnatal Day 3 (the date of birth was defined as postnatal Day 0), and those with good health without external abnormality were pooled according to sex. Groups of 12 males and 12 females were selected and assigned to each of the 4 dose groups, including the controls, by stratified random sampling based on the body weight. Twelve foster mothers were selected based on health and nursing conditions, and suckled the 4 males and 4 females assigned to each group up to weaning on postnatal Day 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group (see Study design) were individually maintained for 9 weeks. In the 28-day study of young rats, 4 week-old rats were obtained and used at ages of 5-6 weeks after acclimation. All animals were allowed free access to basal diet (CRF-1: Oriental Yeast Co. Ltd., Tokyo, Japan, or LABO MR Stock: Nihon Nosan Kogyo Inc., Yokohama, Japan) and water (tap water or well water treated with sodium hypochlorite).

Study design (Time schedule as reported previously (Koizumi *et al.*, 2001))

1. 18-Day repeated dose study in newborn rats

In a dose-finding study, DBP was administered by gastric intubation to newborn rats (5/sex/dose) from postnatal Days 4 to 21 and TBE from postnatal Days 4 to 20. The dosages were set at 0, 10, 30, 100 or 200 mg/kg/day for DBP and at 0, 12, 50 or 200 mg/kg/day for TBE, based on the results of young rat study, mentioned below. They were examined for general behavior and body weights during the dosing period, and sacrificed at postnatal Day 21 or 22 for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, newborn rats (12/sex/dose) were administered test substances by gastric intubation from postnatal Days 4 to 21. Based on results of the dose-finding study, the dosage was set at 10, 50 or 150 mg/kg/day for DBP and 3, 12 or 50 mg/kg/day for TBE. On postnatal Day 22, 6 males and 6 females in each treated group were sacrificed (the scheduled-sacrifice group) and the rest of animals in all groups (6/sex/dose) were maintained for 9 weeks without chemical treatment and then sacrificed at 12 weeks of age (the recovery-maintenance group). During the study, general behavior, body weight and food consumption (only the recovery-maintenance period) were examined at least once a day. In addition, some developmental parameters were assessed, such as surface righting and visual placing reflex for reflex ontogeny, fur appearance, incisor eruption and eye opening for external development, and preputial separation, vaginal opening and estrous cycle for sexual development. Urinalysis (color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, volume of the urine, osmotic pressure) was conducted in the late recovery-maintenance period.

At weaning age of postnatal Day 22 after the last treatment, blood was collected under anesthesia from the abdomen of all animals in the scheduled-sacrifice group. In the recovery-maintenance group, it was conducted at 85 days of age after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematological parameters such as the red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count. In the recovery-maintenance group, blood was also treated with 3.8% sodium citrate and blood clotting parameters such as prothrombin time and activated thromboplastin time were examined. Serum or plasma from the remaining portions of blood were analyzed for blood biochemistry (total protein, albumin, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen, creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-GTP), calcium, inorganic phosphorus, sodium, potassium, chlorine). Following collection of blood, all animals were

sacrificed by exsanguination, and organs and tissues of the entire body were macroscopically observed. The brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, ovaries and uterus were weighed, and fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides). Paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. All studies were conducted in compliance with the Good Laboratory Practice Act of the Japanese Government.

2. 28-Day repeated dose study in young rats

In a dose-finding study, DBP and TBE were administered by gastric intubation to five-week old rats (5 or 4/sex/dose) for 14 days. The dosages were determined at 0, 20, 60, 200 or 600 mg/kg/day for DBP, and at 0, 10, 20, 50, 100 or 200 mg/kg/day for TBE, based on the results of the preliminary single-dose study. The general behavior, body weight and food consumption were examined, and the animals were sacrificed the day after the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

Referring to the results of the dose-finding study, doses in a main study were set at 10, 50 and 250 mg/kg/day for DBP and at 6, 20, 60 and 200 mg/kg/day for TBP. In the main study, 5-6 week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery groups (0, 50, 250 mg/kg/day for DBP and 0, 200 mg/kg/day for TBE) were maintained for 2 weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals for each sex/dose for both scheduled-sacrifice and recovery cases was 6 for DBP and 5 for TBE. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Parametric data such as body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological and blood biochemical findings, and organ weights were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted for comparison between control and individual treatment groups ($p < 0.01$ or 0.05). If not homogenous, the data were analyzed using Steel's multiple comparison test (Steel, 1959) or the mean rank test of the Dunnett type (Hollander and Wolfe, 1973) ($p < 0.01$ or 0.05). If the number of groups was two, parametric data were analyzed by the F test (Snedecor and Cochran, 1967). When homogeneity was recognized, the Student's *t*-test (Steel and Torrie, 1980) was conducted and if not, the Aspinn-Welch's *t* test (Snedecor and Cochran, 1967) ($p < 0.01$ or 0.05). For histopathological findings, the Mann-Whitney's U test (Mann and Whitney, 1947) or the Fisher's exact test (Fisher, 1973) were performed ($p < 0.01$ or 0.05). In the newborn study, the chi square test (Fisher, 1922) was conducted for physical and sexual development and reflex ontogeny ($p < 0.01$ or 0.05).

Judgment criteria for NOAEL and the unequivocally toxic level

NOAEL is the greatest dose at which no adverse effects are observed. In the case of hepatotoxicity, increased liver weights or changes in biochemical parameters alone are not considered to be adverse effects. The unequivocally toxic level has been used only for our comparative toxicity analysis as a clear toxic dose. However, it is generally not readily definable because it depends on the type of toxicity. In this study, centrilobular hypertrophy of hepatocytes was observed as a major histopathological change with both chemicals. Appearance of hypertrophic hepatocytes may not be considered to be a sign of clear toxicity because it is not usually accompanied by increase in GOT and GPT, typically found with hepatotoxic agents. Therefore, for the special purposes of this study, the unequivocally toxic level was estimated on the basis of concomitant changes in organ weights, histopathology, biochemical parameters and body weights.

RESULTS

1,3-Dibromopropane (DBP)

1. 18-Day study in newborn rats (including the dose-finding study)

In the dose-finding study at doses of 10, 30, 100 and 200 mg/kg, 2 of 5 males and 2 of 5 females of the highest group died on dosing Days 2 to 3, but no

change in general behavior was observed in the others. In the 200 mg/kg group, body weights were also lower by 15-25% than the control values from dosing Day 4 in males and from dosing Day 8 in females. Blood biochemical examination showed a slight increase in total cholesterol in females given 200 mg/kg. For organ weight, increases in relative liver weights were demonstrated in both sexes at 100 mg/kg and more with absolute liver weights in males at 100 mg/kg. Decrease in absolute and relative testis weights were also observed in males of 200 mg/kg group. At autopsy, there were no gross abnormalities except hepatomegaly in all animals, including the dead rats at 200 mg/kg. Based on these results, 10, 50 and 150 mg/kg were selected as the doses for the main study in newborn rats.

In the main study, no change in general behavior was noted during the dosing period in any dose group. Body weights of both sexes given 150 mg/kg were lowered during the dosing period (Fig. 1) and gain was also decreased by approx. 10%. No definitive changes in parameters for external and sexual development and reflex ontogeny were detected in any dose group. At the scheduled sacrifice, blood biochemical examination of the 150 mg/kg group showed increases in γ-GTP in males and total bilirubin in females. There were no dose-related changes in hematological parameters. Significant increase of absolute and relative liver weights was noted in males given 50 mg/kg and in both sexes given 150 mg/kg. The relative liver weights were also increased in females at 10 and 50 mg/kg. Absolute brain weights were lower in both sexes given 150 mg/kg, this being considered due to the lowered body weights. On histopathological examination, hypertrophy of centrilobular hepatocytes was noted in all animals given 150 mg/kg, being mild in 3/6 males and 4/6 females (Table 1). In four of each sex, the endoplasmic reticulum in hypertrophic hepatocytes showed a ground glass appearance. In addition, single cell necrosis was also noted in 3/6 males and 1/6 females at 150 mg/kg. During and at the end of the recovery-maintenance period, the changes observed in scheduled sacrificed group had disappeared.

The results of the dose-finding study and main study of DBP in newborn rats are summarized in Table 2. The NOAEL was concluded to be 50 mg/kg/day because increase in liver weight without biochemical and histopathological changes in this dose of the main study was not considered as an adverse effect. The unequivocally toxic level was concluded to be 150 mg/kg/day, based on increase of liver weight, mild centrilobular hypertrophy of hepatocytes, increase of γ-GTP and total bilirubin, and lowering of body weights at this dose in the main study, taking additional account of the 40% mortality rate at 200 mg/kg in the dose-finding study.

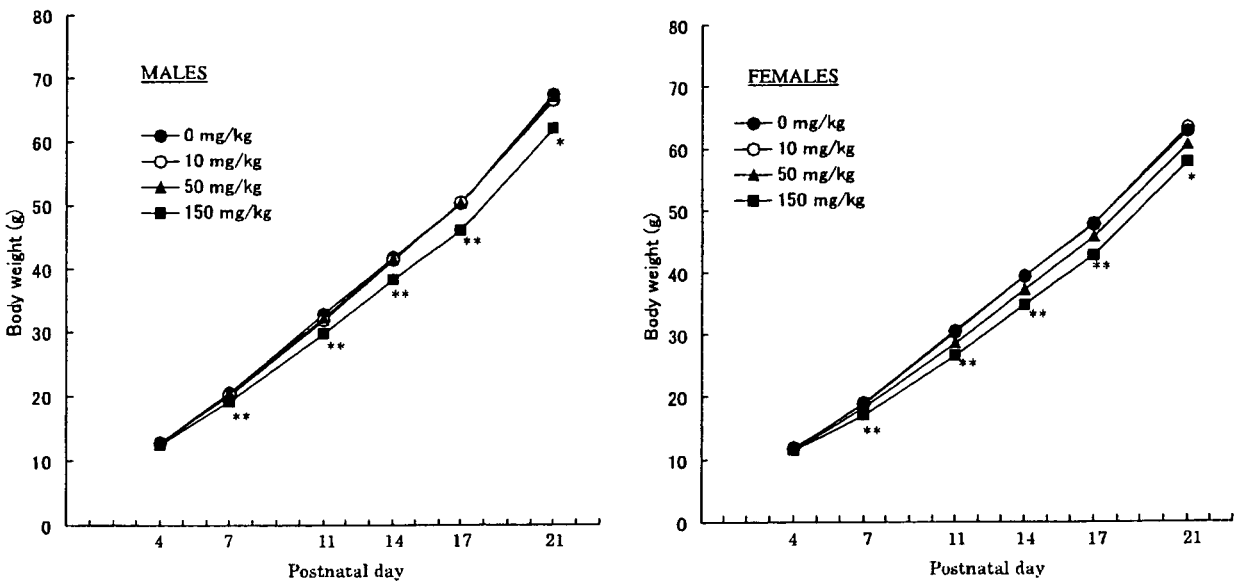

Fig. 1. Body weight curves for the 18-day study of 1,3-dibromopropane in newborn rats.
*: Significantly different from the controls ($p < 0.05$), **: Significantly different from the controls ($p < 0.01$).

2. 28-Day study in young rats (including the dose-finding study)

In the 14-day dose-finding study at doses of 20, 60, 200 and 600 mg/kg, all animals died within 6 days after the first treatment in the highest group. They showed various toxic signs such as decrease in spontaneous movement, oligopnea and adoption of a prone/lateral position. Blood biochemical examination showed increase in total protein in males and in total cholesterol in females at 200 mg/kg. Increase in absolute and relative liver weights was observed in both sexes of the 60 and 200 mg/kg groups and relative liver weights in males of 10 mg/kg. In addition, increase was found in relative kidney weights in males and in absolute and relative kidney and heart weights in females at 200 mg/kg. There were no other dose-related changes evident. Based on the results, 250 mg/kg, at which it was predicted that clear toxic signs would appear, was selected as the top dose for the main study, and by one-fifth division 50 and 10 mg/kg were derived.

In the main study, salivation was observed from dosing Day 12 in 5 to 10 of each sex given 250 mg/kg. In males at this dose, body weights were significantly lowered by approx. 10% from dosing Day 18, in spite of no dose-related change in food consumption. On

Table 1. Histological findings for the liver after 18-day repeat dosing of 1,3-dibromopropane in newborn rats (main study).

	Grade	Dose (mg/kg) 0	10	50	150
Males					
No. of animals examined		6	6	6	6
Liver					
– Single cell necrosis	±	0	0	0	3
– Centrilobular hypertrophy of hepatocytes	±	0	0	0	3
	+	0	0	0	3
					*
Females					
No. of animals examined		6	6	6	6
Liver					
– Single cell necrosis	±	0	0	0	1
– Centrilobular hypertrophy of hepatocytes	±	0	0	0	2
	+	0	0	0	4
					*

±: Slight, +: Mild, *: Significantly different from the control group ($p<0.01$).

Table 2. Summary of the results of the repeated dose studies of 1,3-dibromopropane in newborn rats.

	Dose-finding Study (5 rats/sex/dose)				Main Study (6 rats/sex/dose)		
Dose (mg/kg/day)	10	30	100	200	10	50	150
Toxic Effects							
– Death (No. of dead animals)	0	0	0	2M, 2F	0	0	0
– Body weight	–	–	–	15-25%↓	–	–	10%↓
– Blood biochemical parameters	–	–	–	F: Cho (↑)	–	–	M: GTP↑ F: TB↑
– Relative liver weight	–	–	↑	↑	F: ↑	↑	↑
– Histopathological changes ±	n.d.	n.d.	n.d.	n.d.	0	0	3M, 2F
(No of animals with the findings*) +	n.d.	n.d.	n.d.	n.d.	0	0	3M, 4F

±: Slight change, +: Mild change, M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, –: No change, Cho: Total cholesterol, GTP: γ-GTP, TP: Total protein, n.d.: No available data, *Centrilobular hypertrophy of hepatocytes.

hematological examination at the scheduled sacrifice, slight anemic changes with decrease in Hb and Ht, and an increased reticulocyte ratio were observed in females receiving 250 mg/kg. At 250 mg/kg, many blood biochemical parameters, including total protein, albumin, total cholesterol, triglycerides, phospholipids and total bilirubin, were also increased with an upward trend of GOT and GPT. With 50 mg/kg, slight increase in total protein was only observed in males. Significant increases were found in absolute and relative liver weights of both sexes at 250 mg/kg and in relative liver weights of females at 50 mg/kg. There was also increase in relative heart weights and relative kidney weights in both sexes of the 250 mg/kg group. On histopathological examination, slight to mild centrilobular hypertrophy of hepatocytes was observed at 50 mg/kg and more (Table 3). Perilobular vacuolation of hepatocytes tended to decrease with the dose. Most of the above changes became less prevalent or disappeared during the recovery period. However, body weights remain lower throughout this period in males and the relative liver and heart weights continued to be

Table 3. Histological findings in the repeated dose study of 1,3-dibromopropane in young rats (main study).

		Scheduled-sacrifice group (mg/kg)				Recovery group (mg/kg)		
	Grade	0	10	50	250	0	50	250
Males								
No. of animals examined		6	6	6	6	6	6	6
Liver								
– Centrilobular hypertrophy of hepatocytes	±	0	0	4	2	0	–	0
	+	0	0	0	4	0	–	0
				*				
				**				
– Perilobular vacuolation of hepatocytes	±	0	1	2	5	5	–	6
	+	6	5	4	1	1	–	0
				**				
Spleen								
– Extramedullary hematopoiesis	±	5	–	–	5	6	3	0
	+	0	–	–	1	0	3	6
	++	1	–	–	0	0	0	0
							**	
– Deposits of brown pigment	±	6	–	–	6	6	6	1
	+	0	–	–	0	0	0	5
							**	
Females								
No. of animals examined		6	6	6	6	6	6	6
Liver								
– Centrilobular hypertrophy of hepatocytes	±	0	0	3	2	0	–	0
	+	0	0	0	4	0	–	0
				**				
– Perilobular vacuolation of hepatocytes	±	1	1	4	5	4	–	5
	+	5	5	2	1	2	–	1
				*				
Spleen								
– Extramedullary hematopoiesis	±	6	–	–	5	6	6	4
	+	0	–	–	1	0	0	2
– Deposits of brown pigment	±	6	–	–	5	4	5	1
	+	0	–	–	1	2	1	5

±: Slight, +: Mild, ++: Moderate, *: Significantly different from the control group ($p<0.05$),
**: Significantly different from the control group ($p<0.01$).

high in females at 250 mg/kg. At the same time, decreases in RBC, Hb, Ht and increase in the reticulocyte ratio appeared in males given 250 mg/kg with an increased incidence of extramedullary hematopoiesis and deposits of brown pigment in the spleen (Table 3).

Summary of the results of the dose-finding and main study of DBP in young rats are shown in Table 4. The NOAEL was concluded to be 10 mg/kg/day from the main study, as the 20 mg/kg in dose-finding study was not appropriate because of the lack of histopathological examination. The unequivocally toxic level was concluded to be 250 mg/kg/day, at which increase of liver weight, mild centrilobular hypertrophy of hepatocytes, increase of many biochemical parameters with an upward trend of GOT and GPT, slight anemic effects and lowering body weight were observed in the main study.

1,1,2,2-Tetrabromoethane (TBE)

1. 18-Day study in newborn rats (including the dose-finding study)

In the dose-finding study, when newborn rats were given TBE at 12, 50 and 200 mg/kg, hypoactivity and bradypnea were observed during the dosing period in all animals of the high dose group, the body weights being lowered by 10-20% in both sexes at dosing Days 8 to 17. On blood biochemical examination for this group, slight increase in total bilirubin was found in both sexes. In addition, absolute and relative liver weights were increased in females receiving the 50 mg/kg and both sexes of the 200 mg/kg group, and relative liver weights in females of the 12 mg/kg and males of the 50 mg/kg groups. There were also increases in relative kidney weights of females and decreases in absolute spleen weights of both sexes and relative spleen weights of females at 200 mg/kg. No significant changes were observed on hematological and gross examination. Based on these results, it was predicted that some hepatotoxicity would be observed at 50 mg/kg, which was selected as the top dose in the main study, and 3 and 12 mg/kg were derived by approx. one-fourth divisions.

In the main study, no significant changes were noted in general behavior and body weight (Fig.2). There were also no definitive changes in the parameters for external and sexual development and reflex ontogeny at any dose. At scheduled sacrifice, blood biochemical examination in the 50 mg/kg group showed only a slight increase in total protein in males. There were also increases in absolute and relative liver weights in both sexes, relative kidney weights in males and relative heart weights in females of the 50 mg/kg group. After the recovery-maintenance period, no significant changes were observed in blood biochemical findings and in kidney and heart weights, but the relative liver weights still remained high in males at 50 mg/kg. There were no dose-related changes in food consumption, urinalysis, hematology and histopathology throughout the study, including the recovery-maintenance period.

As shown in summary of the results in Table 5, in the 50 mg/kg group, relative liver weights were increased in both dose-finding and main studies, and total protein was slightly increased only in males of the main study. These changes without histopathological alteration were not considered adverse effects. Therefore, the NOAEL was concluded to be 50 mg/kg/day. Unfortunately, no histopathological changes in the

Table 4. Summary of the results of the repeated dose studies of 1,3-dibromopropane in young rats.

	Dose-finding Study (5 rats/sex/dose)				Main Study(6 rats/sex/dose)		
Dose (mg/kg/day)	20	60	200	600	10	50	250
Toxic Effects							
–Death (No. of dead animals)	0	0	0	5M, 5F	0	0	0
–Body weight	–	–	–	n.d.	–	–	M: 10%↓
–Blood biochemical parameters	–	–	M: TP↑ F: Cho↑	n.d.	–	M: TP (↑)	Many↑
–Relative liver weight		M: ↑	↑	n.d.	–	F: ↑	↑
–Histopathological changes ±	n.d.	n.d.	n.d.	n.d.	0	4M, 3F	2M, 2F
(No of animals with the findings*) +	n.d.	n.d.	n.d.	n.d.	0	0	4M, 4F

±: Slight change, +: Mild change, M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, –: No change, Cho: Total cholesterol, TP: Total protein, Many: Many parameters including Cho, TP, albumin, triglycerides, phospholipids and total bilirubin, n.d.: No available data, * Centrilobular hypertrophy of hepatocytes.

liver were observed at the highest dose of 50 mg/kg in the main study, meaning that the dose setting was not appropriate. Therefore, an unequivocally toxic level could not be estimated. The dose of 200 mg/kg in the dose-finding study was clearly toxic because of effects on the central nervous system (hypoactivity and bradypnea) and lowering of body weight (10-20% reduction), although no histopathological examination was conducted.

2. 28-Day study in young rats (including the dose-finding study)

In the dose-finding study with 14-day exposure at 0, 10, 20, 50, 100 or 200 mg/kg, there were no significant changes in body weight, food consumption and urinalysis at any dose. Hematological examination showed increase in reticulocytes of both sexes at 200 mg/kg, and decrease in Hb in both sexes at 200 mg/kg and in males at 100 mg/kg, as well as Ht in males at 100 and 200 mg/kg and RBC in females at 200 mg/kg. On blood biochemical examination, increases in total

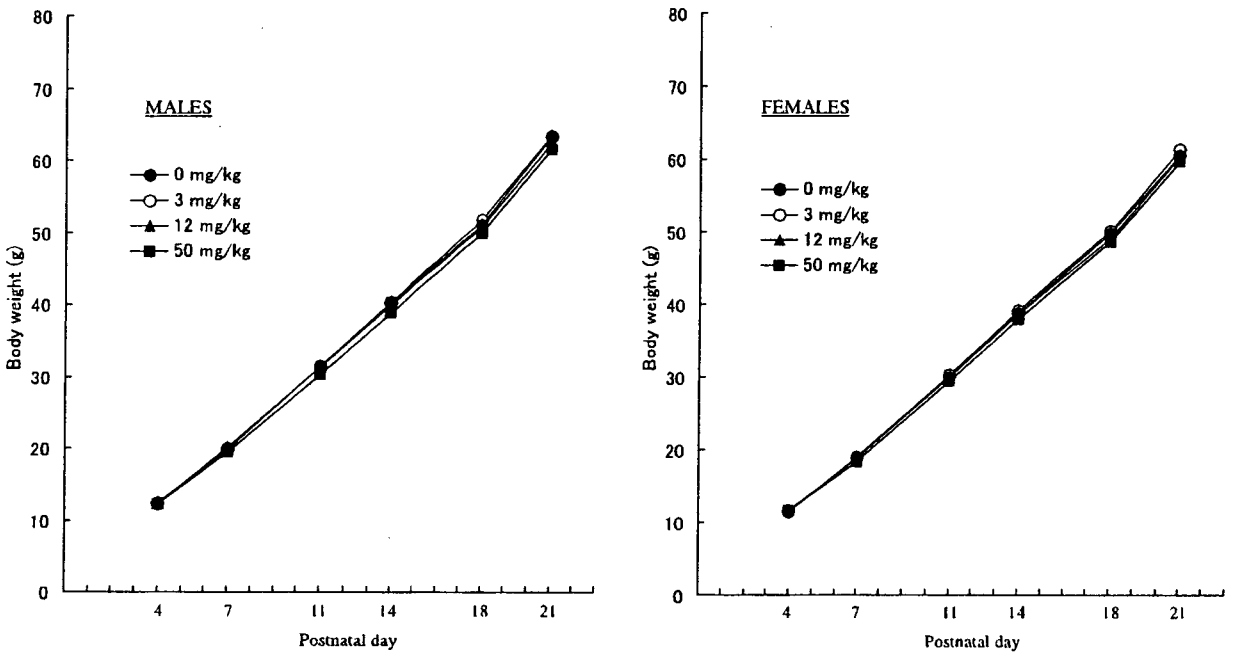

Fig. 2. Body weight curves in the 18-day study of 1,1,2,2-tetrabromoethane in newborn rats.
Not significantly different from the controls.

Table 5. Summary of the results of the repeated dose studies of 1,1,2,2-tetrabromoethane in newborn rats.

	Dose-finding Study (4 rats/sex/dose)			Main Study (6 rats/sex/dose)		
Dose (mg/kg/day)	12	50	200	3	12	50
Toxic Effects						
-Death (No. of dead animals)	0	0	0*	0	0	0
-Body weight	–	–	10-20%↓	–	–	–
-Blood biochemical parameters	–	–	TB (↑)	–	–	M: TP (↑)
-Relative liver weight	F: ↑	↑	↑	–	–	↑
-Histopathological changes (No of animals with the findings)	n.d.	n.d.	n.d.	0	0	0

M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, –: No change, TB: Total bilirubin, TP: Total protein, n.d.: No available data, *Although there were no deaths in this group, hypoactivity and bradypnea were observed in all animals.

cholesterol in both sexes, and total protein and triglycerides in females were noted at 200 mg/kg. In addition, increase in total cholesterol was found in females given 100 mg/kg. There were also increases in absolute liver weight in males at 100 and 200 mg/kg and in females at 200 mg/kg, relative liver weight in both sexes at 50 mg/kg and more, and kidney weights in females at 100 mg/kg and in both sexes at the highest dose. Because of the clear toxic effects, 200 mg/kg was selected as the top dose for the main study, and 60, 20 and 6 mg/kg were derived by one third division.

In the main study, there were no significant changes in body weight and food consumption. At scheduled sacrifice, hematological examination showed decrease in platelet counts in females of 200 mg/kg group. On blood biochemical examination, changes suggestive of effects on the liver, including increase in total protein, albumin, A/G, total cholesterol, were found in both sexes at the highest dose. There were also increases in total protein and albumin in females of the 20 and 60 mg/kg groups and increases in A/G in females of the 60 mg/kg groups. For organ weights, there were increases in absolute and relative liver weights of both sexes given 60 and 200 mg/kg and slight increase in relative liver weights in males given 20 mg/kg. In addition, relative kidney weights were higher in both sexes and absolute kidney weights in females of the 200 mg/kg group. On histopathological examination (Table 6), slight to mild centrilobular hypertrophy of hepatocytes was observed in both sexes given 20 mg/kg and more. In the thyroid, mild hypertrophy of follicular cells was found at 60 mg/kg and 200 mg/kg, and follicles were apt to be miniaturized and colloid to be decreased. At the end of the recovery period, changes observed in the scheduled-sacrifice group remained significant but with a tendency for recovery (total protein, total cholesterol, liver and thyroid weights, centrilobular hypertrophy of hepatocytes (Table 6)).

The results of the dose-finding and main study in young rats are summarized in Table 7. As slight hypertrophy of hepatocytes was observed at 20 mg/kg in the main study, the NOAEL was concluded to be 6 mg/kg/day. The unequivocally toxic level was considered to

Table 6. Histological findings in the repeated dose study of 1,1,2,2-tetrabromoethane in young rats (main study).

		Scheduled-sacrifice group					Recovery group	
	Grade	0	6	20	60	200	0	200
Males								
No. of animals examined		5	5	5	5	5	5	5
Liver								
– Centrilobular hepatocyte hypertrophy	±	0	0	3	4	0	0	3
	+	0	0	0	0	5	0	0
					*			
					**			
– Focal necrosis	±	2	1	3	1	5	1	0
Thyroid								
– Hypertrophy of follicular cells	±	0	0	0	1	4	0	0
Females								
No. of animals examined		5	5	5	5	5	5	5
Liver								
– Centrilobular hepatocyte hypertrophy	±	0	0	3	5	1	0	2
	+	0	0	0	0	4	0	0
					**			
					**			
– Focal necrosis	±	0	0	0	0	1	0	0
Thyroid								
– Hypertrophy of follicular cells	±	0	0	0	2	5	0	0
						**		

±: Slight, +: Mild, *: Significantly different from the control group ($p<0.05$), **: Significantly different from the control group ($p<0.01$).

be more than 200 mg/kg because of the lack of effects on body weights and parameters indicative of hepatotoxicity, such as GOT and GPT. Hypertrophy in the liver and thyroid, and increases in some biochemical parameters at this dose were not considered to be sufficient for a conclusion of toxicity.

DISCUSSION

As with human neonates, the metabolic ability of the newborn rat is known to be extremely immature, with a low cytochrome P450 content (Rich and Boobis, 1997) and a low capacity for glucuronidation (Gow *et al.*, 2001). Therefore, it could be predicted that chemicals directly exerting adverse effects might show stronger toxicity in the newborn than in young/adult rats. As expected, our previous comparative studies demonstrated that the susceptibility to four chemicals (4-nitrophenol, 2,4-dinitorophenol, 3-aminophenol, 3-methylphenol), which may exert toxicity without metabolic activation, was 2 to 4 times greater in the newborn than in young rats (Koizumi *et al.*, 2001, 2002, 2003).

In the present study, DBP and TBE, which differ from the earlier chemicals in requiring biotransformation differently from previous chemicals, were therefore examined. Although hitherto there has been no information on the repeated dose toxicity of DBP, hepatotoxicity with slight centrilobular fatty degeneration or cytoplasmic vacuolization has been already reported for TBE (Hollingsworth *et al.*, 1963; NTP, 1996). The present study showed no effects of either chemical on early development in the newborn, but they caused hepatotoxicity, regardless of sex, in both newborn and young animals. The ratios for NOAELs and unequivocally toxic levels (young/newborn rats) for both chemicals are given in Table 8, the NOAELs for DBP and TBE being considerably higher in newborn than in young rats, so that the latter are clearly more susceptible. Unequivocally toxic levels could not be simply estimated for both chemicals because the hepatic influence observed was only hypertrophy of hepatocytes, usually without increase of GOT and GPT. Therefore, values were estimated on the basis of simultaneous changes of organ weights, histopathology, biochemical parameters and body weights. Based on our specified criteria, the unequivocally toxic level for DBP was in contrast lower in newborn than in young rats. Unfortunately an unequivocally toxic level of TBE could not be estimated for newborn or young rats. However, the dose of 200 mg/kg in the newborn dose-finding study was considered to be sufficiently toxic because of the 10 - 20% lowering of body weights observed, although no histopathology was conducted. The same dose in the young rat main study caused mild hypertrophy of hepatocytes but no change of body weights, was not considered a sufficient toxic level. These results suggest that the unequivocally toxic level of TBE in the newborn might be lower than that in young rats. The reasons for difference in susceptibility presumably lie with metabolic pathways and specific characteristics of newborn animals.

Three studies have demonstrated that DBP is conjugated with hepatic glutathione before or after oxidative biotransformation, leading to urinary excretion of cysteine or mercapturic acid derivatives and exhalation of CO_2 (James *et al.*, 1981, Jones and Wells, 1981, Onkenhout *et al.*, 1986). Activity of the conjugation

Table 7. Summary of the results of the repeated dose studies of 1,1,2,2-tetrabromoethane in young rats.

		Dose-finding Study (4 rats/sex/dose)					Main Study (5 rats/sex/dose)			
Dose (mg/kg/day)		10	20	50	100	200	6	20	60	200
Toxic Effects										
– Death (No. of dead animals)		0	0	0	0	0	0	0	0	0
– Body weight		–	–	–	–	–	–	–	–	–
– Blood biochemical parameters		–	–	–	F: TP↑	M: Cho↑ F: Cho, TG, TP↑	–	F: TP, Alb ↑	F: TP, A/G, Alb ↑	Many↑
– Relative liver weight		–	–	↑	↑	↑	–	M: (↑)	↑	↑
– Histopathological changes	±	n.d.	n.d.	n.d.	n.d.	n.d.	0	3M, 3F	4M, 5F	1F
(No of animals with the findings*)	+	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0	5M, 4F

±: Slight, +: Mild, M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, –: No change, Alb: Albumin, Cho: Total cholesterol, TG: Triglycerides, TP: Total protein, Many: Many parameters including Alb, A/G, Cho and TP, n.d.: No available data, * Centrilobular hypertrophy of hepatocytes.

pathway is supported by a rapid drop in hepatic glutathione level after DBP administration (James *et al.*, 1981). Metabolism via conjugation with glutathione has in fact been indicated in common for dihaloalkanes or dihaloalkenes, such as 1,2-dibromopropane (Zoetemelk *et al.*, 1986), 1,2-dichloropropane (Trevisan *et al.*, 1989), 1,1-dichloroethylene (Jones and Hathway, 1978) and 1,3-dichloropropene (Climie *et al.*, 1979). In the case of 1,2-halogenated ethanes, it is considered that the oxidative metabolites might irreversibly bind to protein and that conjugate derivatives, episulphonium ions, might be responsible for the DNA adduct formation (Shih and Hill, 1981; Ozawa and Guengerich, 1983).

With TBE, Kennedy *et al.* (1993) identified various excretory metabolites after a single oral administration to rats, such as 1,2-dibromoethylene and tribromoethylene in exhaled air and dibromoacetic acid, glyoxylic acid, and oxalic acid in urine. They suggested that a number of metabolic intermediates produced by oxidative biotransformation may be involved in the mutagenicity, hepatotoxicity and nephrotoxicity of the compound. At least, dibromoacetic acid has unequivocal cytotoxicity and mutagenicity (Kargalioglu *et al.*, 2002).

Based on the available information, oxidative biotransformation mediated by cytochrome P450 might be a critical step for the initial hepatotoxic effects of both chemicals. The rate of production of active metabolites, including free radical intermediates, would be expected to be significantly less or negligible in newborn animals at least around 50 mg/kg, at which clearly hepatic changes were observed in young rats for both chemicals, because of their lower content of cytochrome P450 (Rich and Boobis, 1997). This metabolic character for both chemicals as well as the lower blood flow to the liver during the newborn period (Gow *et al.*, 2001) would make a major contribution to the much higher NOAEL in the newborn than in young rats. Similar results have already been demonstrated for aflatoxin B1 (Behroozikha *et al.*, 1992), acetaminophen, bromobenzene and carbon tetrachloride (Gergus and Klaassen, 1998). On the other hand, unequivocally toxic levels for both chemicals appeared to be only 3 to 4 times higher than the NOAELs in newborn rats, in contrast to 25 to >33 times higher in their young counterparts (Table 8). One possible explanation for these differences might be a low capacity for protection against deleterious oxidative stress in the newborn when the toxic chemical burden crosses a threshold in the liver. It has been reported that the content of glutathione and glutathione-*S*-transferase activity in rat liver drops in the early days after birth (Tee *et al.*, 1992).

In our series of comparative studies, the results of the repeated dose toxicity study using newborn rats have been compared with those of routine repeated dose toxicity studies. The routine repeated dose studies have value in identifying target sites for toxicity and providing dose-response information that may be useful for human safety assessment, irrespective of life stage, but the developing period, which could be most vulnerablev to chemical toxicity during life, is not directly evaluated by the studies (Dourson *et al.*, 2002). To compensate for this period, reproductive/developmental toxicity studies that exposed the developing animals via placenta or maternal milk have been conducted. However, the direct exposure to chemicals dur-

Table 8. Comparison of NOAELs and unequivocally toxic levels in newborn and young rats.

	Level (mg/kg/day)		Ratio (young/newborn)
1,3-Dibromopropane			
NOAEL (newborn)	50	× 3	0.2
NOAEL (young)	10		
Unequivocally toxic level (newborn)	150	× 25	1.67
Unequivocally toxic level (young)	250		
1,1,2,2,-Tetrabromoethane			
NOAEL (newborn)	50	× 4*	0.12
NOAEL (young)	6		
Unequivocally toxic level (newborn)	200*	× >33*	>1.0*
Unequivocally toxic level (young)	> 200*		

*: Tentative levels or ratios, due to lack of histology alteration in the newborn and no change in body weight in young rats.

ing the newborn period is not included in these studies, despite the significant possibility that the newborn are exposed to chemicals directly via mouthing toys and household materials, or having chemical-contaminated milk and baby food, and so on. In the routine repeated dose toxicity study, rats at approximately 5-6 weeks of age have generally been used, and this start period is largely a matter of practical convenience and feasibility. Rats much younger than this age, especially newborn rats, are so difficult to handle such as grouping, direct dosing and other testing or observation. Economic issues and lack of the human resource with this technical difficulty make it impossible to subject the newborn rat study to the routine one. Our series of comparative studies are the first systematic study to look into the direct effects of chemicals in newborn animals, and the comparative analysis on the susceptibility of the newborn rats to the toxicity of chemicals with that of young rats would give important information for considering the effects by chemical exposure during the newborn period in risk assessment.

In conclusion, the target organ of DBP and TBE was here found to be the liver in both newborn and young rats, but the doses at which the toxic signs began to appear were higher in newborn rats. In contrast, the doses at which clear toxicity was observed appeared to be lower in the newborn case. However, no special concern with regard to newborn risk is necessary in cases of chemicals which induce toxicity after biotransformation via hepatic cytochrome P450, because the tolerable daily intake (TDI) used for regulation is generally derived from NOAEL in toxicity studies in young/adult animals.

ACKNOWLEDGMENT

The authors gratefully acknowledge the financial support of the Office of Chemical Safety, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labor and Welfare, Japan.

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www.elsevier.com/locate/foodchemtox

Evaluation of developmental toxicity of 1-butanol given to rats in drinking water throughout pregnancy

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Received 21 September 2004; accepted 6 November 2004

Abstract

The objective of this study was to evaluate the developmental toxicity of 1-butanol in rats. Pregnant rats were given drinking water containing 1-butanol at 0.2%, 1.0% or 5.0% (316, 1454 or 5654 mg/kg/day) on days 0–20 of pregnancy. A significant decrease in maternal body weight gain accompanied by reduced food and water consumption was found at 5.0%. No significant increase in the incidence of pre- and postimplantation embryonic loss was observed in any groups treated with 1-butanol. Fetal weight was significantly lowered at 5.0%. Although a significant increase in the incidence of fetuses with skeletal variations and decreased degree of ossification was found at 5.0%, no increase in the incidence of fetuses with external, skeletal and internal abnormalities was detected in any groups treated with 1-butanol. The data demonstrate that 1-butanol is developmental toxic only at maternal toxic doses. No evidence for teratogenicity of 1-butanol was noted in rats. Based on the significant decreases in maternal body weight gain and fetal weight, it is concluded that the no observed adverse effect levels (NOAELs) of 1-butanol for both dams and fetuses are 1.0% (1454 mg/kg/day) in rats.

Keywords: 1-Butanol; Developmental toxicity; Teratogenicity; Fetal abnormality; Rat

1. Introduction

1-Butanol (CAS no. 71-36-3, *n*-butanol; *n*-butyl alcohol), a flammable colorless liquid with a rancid sweet odor, is widely used as an organic solvent and intermediate in the manufacture of other organic chemicals (IPCS/WHO, 1987). Exposure of the general population is mainly through its natural occurrence in food and beverages and its use as a flavoring agent (IPCS/WHO, 1987).

Several reports on the developmental toxicity of 1-butanol are available. Nelson et al. (1989a) reported the results of a developmental toxicity study in which SD rats were exposed to 1-butanol by inhalation for 7 hr/day on days 1–19 of pregnancy at 3500, 6000 and 8000 ppm (equivalent to estimated daily absorbed doses of 350, 600 and 800 mg/kg). They observed maternal deaths at 8000 ppm, decreases in maternal food consumption and fetal weight at 6000 and 8000 ppm, and an increased incidence of rudimentary cervical ribs at 8000 ppm, and concluded that 1-butanol was not a selective developmental toxicant in rats. Nelson et al. (1989b) conducted a behavioral teratology study in which female SD rats were given 1-butanol by inhalation at 3000 or 6000 ppm for 7 hr/day throughout pregnancy (the maternal exposure group); male rats were

Abbreviations: NOAEL, no observed adverse effect level

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doi:10.1016/j.fct.2004.11.003

similarly exposed for 6 weeks and mated to unexposed females (the paternal exposure group), and offspring were behaviorally and neurochemically examined. The data from all tests in their study were within the range of control data in other research conducted by their laboratory. Sitarek et al. (1994) reported a significant increase in the incidence of fetuses with abnormalities after administration of 1-butanol at 0.24–4.0% (300–5000 mg/kg/day) in drinking water during the pre-mating period for 8 weeks and throughout the mating and pregnant period. No maternal toxicity was found at any dose of 1-butanol. The no observed adverse effect level (NOAEL) was not derived from the results of their study, because significant increases in the incidence of fetuses with dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain were found even at the lowest dose (0.24%). They have concluded that 1-butanol is a developmental toxicant and produces anomalies in the skeleton and central nervous system.

The present study was conducted to determine whether or not morphological abnormalities could be produced in fetuses of rats given 1-butanol prenatally and designed to replicate the observations of the study by Sitarek et al. (1994).

2. Materials and methods

This study was performed in compliance with regulatory guidelines (MHW, 1997a) and accordance with the principles for Good Laboratory Practice (MHW, 1997b) and "Guidance for Animal Care and Use" of Ina Research, Inc.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 10 weeks of age and females at 9 weeks of age were purchased from Tsukuba Breeding Center, Charles River Japan, Inc., (Yokohama, Japan). The rats were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum and maintained in an air-conditioned room at 21–25 °C, with a relative humidity of 40–70%, a 12-h light/dark cycle, and ventilation with 16 air charges/hour. Virgin female rats were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 217–273 g and 10–11 weeks of age, were distributed using a computerized randomization procedure (TOXstaff 21 system) into 4 groups of 20 rats each and housed individually.

2.2. Chemicals and dosing

1-Butanol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 1-butanol used in this study was 99.9% pure and a special grade reagent (Lot no. CER5688), and it was kept in a dark place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before and after the study. Rats were given 1-butanol in their drinking water at a concentration of 0 (control), 0.2%, 1.0% or 5.0% on day 0 through day 20 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of 1-butanol in the drinking water on days 0–20 of pregnancy caused decreases in maternal body weight gain and food and water consumption and tended to reduce in fetal weight at 4% and 7% in rats. 1-Butanol was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan). The control rats were given only water. The stability of formulations in a dark and cool place under airtight conditions has been confirmed for up to 3 days. During use, the formulations were maintained under such conditions for no more than 3 days and were 95.7–103.5% of the target concentration.

2.3. Observations

The maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. The pregnant rats were euthanized by exsanguinations under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the numbers of corpora lutea, implantation sites and live and dead fetuses and resorptions were counted. The live fetuses removed from the uterus were sexed, weighed, measured among their crown-rump length, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Dawson, 1926) and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to a free-hand razor-blade sectioning (Wilson, 1973) and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities. The placental weight was also measured.

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. The initial body

weight, body weight gain and food and water consumption of pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, fetal weight and crown-rump length and placental weight were analyzed with Bartlett's test (Snedecor and Cochran, 1980) for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test (Dunnett, 1955) to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the 1-butanol-treated groups was compared with that of the control group with the Dunnett type test. The Dunnett type test was used for the incidences of pre- and postimplantation embryonic loss and fetal anomalies and sex ratio of fetuses to compare the mean rank of groups treated with 1-butanol and that of the control group. The incidence of dams with anomalous fetuses was analyzed by Chi-square test or Fisher's exact test. The significance of differences from the control group was estimated at probability levels of 1% and 5%.

3. Results

Table 1 shows the maternal findings in rats given 1-butanol during pregnancy. No death was found in female rats of any group. All females in all groups became pregnant. The body weight gains on days 0–7 of pregnancy were significantly reduced at 5.0%. The body weight gain during the whole period of pregnancy was also significantly decreased at 5.0%. No significant decrease in the body weight gain was noted at 0.2 or 1.0, except for a transient decrease on days 0–2 of pregnancy at 1.0%. The food consumption on days 0–7, days 7–14, days 14–20 and days 0–20 of pregnancy was significantly lower in the 1.0% and 5.0% groups than the control group. The water consumption on days 0–7 at 1.0 and 5.0% and on days 7–14, days 14–20 and days 0–20 at 5.0% was significantly decreased. The mean daily intakes of 1-butanol were 316 mg/kg for the 0.2% group, 1454 mg/kg for the 1.0% group and 5654 mg/kg for the 5.0% group.

Reproductive findings in rats given 1-butanol during pregnancy are presented in Table 2. No litters totally resorbed were found in any group. No effects of the administration of 1-butanol were observed on the numbers of corpora lutea, implantations, pre- or postimplantation loss, resorptions or dead or live fetuses or sex ratio of live fetuses. The body weights of male and female fetuses were significantly lower in the 5.0% group than in the control group. There was no significant difference in the crown-rump length of male and female fetuses or placental weight between the control and groups treated with 1-butanol.

A summary of morphological findings in live fetuses of rats given 1-butanol during pregnancy is shown in Table 3. One fetus with spina bifida in the control group and one fetus with thread-like tail and anal atresia in the 0.2% group were observed. Skeletal examination

Table 1
Maternal findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of rats	20	20	20	20
No. of pregnant rats	20	20	20	20
No. of dead rats	0	0	0	0
Initial body weight	245 ± 14	247 ± 13	245 ± 11	244 ± 12
Body weight gain during pregnancy (g)[a]				
Days 0–7	44 ± 7	45 ± 7	40 ± 6	20 ± 28**
Days 7–14	40 ± 6	41 ± 5	41 ± 7	42 ± 10
Days 14–20	78 ± 14	82 ± 8	84 ± 7	75 ± 11
Days 0–20	162 ± 19	168 ± 16	165 ± 15	146 ± 16**
Food consumption during pregnancy (g)[a]				
Days 0–7	179 ± 12	180 ± 16	164 ± 12*	138 ± 21**
Days 7–14	193 ± 14	194 ± 17	177 ± 14**	160 ± 11**
Days 14–20	176 ± 14	175 ± 15	161 ± 12**	143 ± 11**
Days 0–20	548 ± 38	548 ± 46	503 ± 34**	441 ± 34**
Water consumption during pregnancy (ml)[a]				
Days 0–7	284 ± 28	305 ± 37	258 ± 29*	175 ± 34**
Days 7–14	318 ± 35	337 ± 48	299 ± 40	239 ± 80**
Days 14–20	328 ± 47	342 ± 47	334 ± 46	256 ± 85**
Days 0–20	930 ± 105	983 ± 126	890 ± 106	669 ± 182**
Mean daily intakes of 1-butanol (mg/kg)[a]	0	316 ± 30	1454 ± 186	5654 ± 1402

*,** Significantly different from the control, *$P < 0.05$ and **$P < 0.01$.
[a] Values are given as the mean ± SD.