

2. 28-day repeated dose toxicity study in young rats (young rat study)

Five to six week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed following the last treatment (the scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals was six for each sex/dose for both scheduled-sacrificed and recovery cases.

The doses were selected in the same way as the newborn rat studies. In the 12-day dose-finding study for 2TBP, ataxic gait was observed at 300 mg/kg and more, and increase in relative liver and kidney weight at 500 mg/kg. For DTBP, with 14-day administration, the death of one of the four females, various changes in some blood biochemical parameters, increase in relative liver weights and light gray macules on kidneys were found at 500 mg/kg. Increase in serum phospholipid and relative liver weights were also demonstrated in the 100 mg/kg group. Based on the results, the doses were determined at 0, 4, 20, 100, or 500 mg/kg/day for 2TBP and at 0, 5, 20, 75, or 300 mg/kg/day for DTBP. Recovery groups were set at 0, 100, 500 mg/kg/day for 2TBP and 0, 300 mg/kg/day for DTBP.

During the study, rats were examined for general condition, 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).

Statistical analysis

Data for body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological, blood biochemical findings (except for differential leukocyte count), and organ weights were analyzed by the 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 homogeneous or for qualitative urinalysis data and differential leukocyte count, the data were analyzed using Steel's multiple comparison tests (Steel 1959), or tests of the Dunnett type (Hollander & Wolfe 1973) ($P < 0.01$ or 0.05). For reflex ontogeny, and physical and sexual development parameters in the newborn rat studies, the χ^2 -test (Fisher 1922) was conducted ($P < 0.01$ or 0.05).

RESULTS

2-tert-butylphenol (2TBP)

Newborn rat study

Various clinical signs such as decrease in locomotor activity, ataxic gait, deep respiration, and muscle weakness were observed throughout the dosing period in the 200 mg/kg group, as shown in Table 1. With 60 mg/kg, transient decrease in locomotor activity was noted on the first dosing day limited to only one of 12 males. Body weights were lowered by 8–17% from dosing day 7 through to the end of the dosing period in males and to recovery-maintenance day 14 in females given 200 mg/kg. At the scheduled sacrifice, there were no hematological changes at any dose, but blood biochemical examination of the 200 mg/kg group showed increases in γ -GTP in both sexes and total protein in males. In addition, significant increase in relative liver weights was noted in 9% of the females in the 60 mg/kg group and in 21–23% of both males and females in the 200 mg/kg group. On histopathological examination, slight hypertrophy of centrilobular hepatocytes was found in one female of the 60 mg/kg group, and in four males and three females from the 200 mg/kg group. During the recovery-maintenance period, no clinical signs were observed and the lowered body weights showed a tendency for recovery. In parameters for physical and sexual development and reflex ontogeny, no definitive changes were detected. At the end of the recovery-maintenance period, no chemical-related changes, also in urinalysis data, were found in any dose group.

The results of the newborn rat study of 2TBP are summarized in Table 2. Since clinical signs and histopathological changes in the liver were observed in the 60 mg/kg group, the NOAEL was concluded to be 20 mg/kg/day.

Young rat study

Ataxic gait were observed sporadically during the dosing period in nine males and 12 females, and decrease in locomotor activity in two females from the 500 mg/kg group. During the dosing period, there were no changes in body weight, food consumption, and urinalysis data. At the scheduled sacrifice, hematological and blood biochemical examination also showed no changes. Eighteen to 19% increases were found in relative liver weights of both sexes receiving 500 mg/kg, but no histopathological changes in liver were observed at any dose. No chemical-related changes were noted during and at the end of the recovery period.

Table 1 Clinical signs observed during the dosing period in the newborn rat study of 2-tert-butylphenol

	Dose (mg/kg/day)			
	0	20	60	200
No. animals (Male/Female)	12/12	12/12	12/12	12/12
No. animals with clinical signs				
Decrease in locomotor activity	0/0	0/0	1†/0	12/12
Ataxic gait	0/0	0/0	0/0	4/6
Deep respiration	0/0	0/0	0/0	12/12
Tremors	0/0	0/0	0/0	2/4
Muscle weakness	0/0	0/0	0/0	12/12
Emaciation	0/0	0/0	0/0	2/2
Pale skin	0/0	0/0	0/0	4/2

†Observed only on the first dosing day.

Table 2 Summary of the results of the newborn and young rat study of 2-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	20	60	200	
Clinical signs	–	M: Decrease in locomotor activity	Various†	
Body weight changes	–	–	8–17%↓	
Blood biochemical changes	–	–	GTP↑, M: TP↑	
Changes in relative organ weights	–	F: Liver 9%↑	Liver 21–23%↑	
Histopathological findings in liver				
– Slight centrilobular hypertrophy of hepatocytes	–	M: 0/6, F: 1/6	M: 4/6, F: 3/6	
Young rat study				
Dose (mg/kg/day)	4	20	100	500
Clinical signs	–	–	–	Ataxic gait F: Decrease in locomotor activity
Body weight changes	–	–	–	–
Blood biochemical changes	–	–	–	–
Changes in relative organ weights	–	–	–	Liver 18–19%↑
Histopathological findings	n.d.	n.d.	n.d.	–

Statistically significant increases ($P < 0.05$) in body weights, blood biochemical parameters and relative organ weights are shown as ↑, while decreases are shown as ↓. Data on histopathological findings are given as no. of animals with the findings/no. of animals examined, according to sex. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. No chemical-related changes were observed in developmental parameters (conducted only in newborn rat study), urinalysis (only in young rat study), and hematological parameters. †Decrease in locomotor activity, ataxic gait, deep respiration, tremors, muscle weakness, emaciation, and pale skin were observed, as shown in Table 1. GTP, γ -GTP; TP, total protein; –, no change; n.d., not determined.

A summary of the results of the young rat study of 2TBP is given in Table 2. The NOAEL was concluded to be 100 mg/kg/day, at which no changes were observed.

2,4-di-*tert*-butylphenol (DTBP)

Newborn rat study

Two males and one female of the 300 mg/kg group were found dead on dosing days 3, 4, and 7. In this group, decrease in locomotor activity (12 males and 12 females), bradypnea (10 males and 10 females), and hypothermia (one male) were observed from the first dosing day, but then the incidence decreased, with disappearance after dosing day 7. Body weights of the 300 mg/kg group were lowered by 15–25% in males and by 9–20% in females during the dosing period, compared with the control values. There were no definitive changes in parameters for physical development and reflex ontogeny in any dose group. At the scheduled sacrifice, blood biochemical examination showed an increase in total bilirubin and a decrease in the A/G ratio in both sexes, an increase in γ -GTP in males, and an increase in total protein and BUN in females of the 300 mg/kg group. In the 300 mg/kg group, there was a 39–51% increase in relative liver weights, a 37–41% increase in relative kidney weights in both sexes, and a 24% decrease in relative spleen weights in males. In the 40 mg/kg group, 14% increases in relative weight of liver were found in females. On histopathological examination, various changes were observed in livers and kidneys in the 300 mg/kg group, as shown in Table 3. Furthermore, periportal fatty degeneration of hepatocytes was evident in one female given 40 mg/kg, and basophilic tubules in kidneys in one animal of each sex receiving 40 mg/kg and one control group male. Regarding

parameters of sexual development, a slight delay in preputial separation was noted in the 300 mg/kg group (the incidences were 0/5, compared with 2/6 in the control group at PND 42 [recovery-maintenance day 21]; 0/5, 3/6 at PND 43; 2/5, 5/6 at PND 44; 2/5, 6/6 at PND 46; 4/5, 6/6 at PND 47; and 5/5, 6/6 at PND 48). During this observation period, body weights were lowered by approximately 10% in males given 300 mg/kg than control levels, which was not statistically significant. In the last week of the recovery-maintenance period, there were no chemical-related changes on urinalysis in any dose group. At the end of the recovery period, changes noted in the scheduled-sacrifice group were not observed except for histopathological changes in the kidneys, significant in the 300 mg/kg group (Table 3).

A summary of the results of the newborn rat study of DTBP is shown in Table 4. Since fatty degeneration of hepatocytes and increase in liver weight were demonstrated at 40 mg/kg, the NOAEL was concluded to be 5 mg/kg/day.

Young rat study

No chemical-related changes were found in general condition, body weight, and food consumption at any dose. On urinalysis at the fourth week of dosing, an increase in urine volume, and a decrease in specific gravity and osmotic pressure were noted in both sexes of the 300 mg/kg group. At the scheduled sacrifice, hematological examination showed a decrease in hemoglobin and hematocrit, an increase in segmented neutrophils in females, and prolongation of PT and APTT in males at 300 mg/kg. On blood biochemical examination, there was an increase in total bilirubin in males given 300 mg/kg, and an increase in total cholesterol and phospholipid in females given 75 mg/kg and above. For organ weights, there were

Table 3 Histopathological findings for the newborn rat study of 2,4-di-*tert*-butylphenol

Dose (mg/kg/day)	Grade	Scheduled-sacrifice group				Recovery-maintenance group†	
		0	5	40	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	5/6	6/6	5/5
Liver							
– Fatty degeneration of periportal hepatocytes	+	0/0	0/0	0/1	0/0	0/0	0/0
	++	0/0	0/0	0/0	3/4	0/0	0/0
	+++	0/0	0/0	0/0	2/2	0/0	0/0
Kidneys							
– Basophilic tubules	+	1/0	n.d.	1/1	4/4	0/0	3/0
– Granular casts	+	0/0	n.d.	0/0	4/2	0/0	0/0
– Cystic dilatation of collecting tubules	+	0/0	n.d.	0/0	0/0	0/0	5/4
	++	0/0	n.d.	0/0	3/4	0/0	0/0
	+++	0/0	n.d.	0/0	2/2	0/0	0/0
– Cellular infiltration of neutrophils	+	0/0	n.d.	0/0	2/1	0/0	1/0
	++	0/0	n.d.	0/0	1/1	0/0	1/0
	+++	0/0	n.d.	0/0	1/1	0/0	0/0

†No histopathological examination was conducted at 5 and 40 mg/kg in the recovery-maintenance group. +, mild; ++, moderate; +++, marked; n.d., not determined.

increases in relative liver weights by 40–43% in both sexes given 300 mg/kg, and by 13% in females receiving 75 mg/kg. On histopathological examination, mild to marked changes in livers and kidneys were observed in both sexes from the 300 mg/kg group, as shown in Table 5. At the end of the recovery period, the increase in total cholesterol and phospholipid and renal histopathological changes observed in the scheduled-sacrifice group remained significant in the highest-dose group (Table 5).

The results of the young rat study are summarized in Table 4. Based on increase in the relative liver weights with some changes in blood biochemical parameters in females given 75 mg/kg, the NOAEL was concluded to be 20 mg/kg/day.

DISCUSSION

During development, many rapid and complex biological changes occur, which can have profound consequences on sensitivity to the effects of exogenous chemicals (Scheuplein *et al.* 2002). Although the neonatal body at birth is reasonably well prepared for the abrupt changes associated with parturition, and most functional systems possess a significant portion of their adult capacity (Dourson *et al.* 2002), it is known that the various functions remain immature in early postnatal period and that some organs and tissues, especially in the nervous, immune and reproductive systems, continue to develop after birth (NAS 1993). Therefore, it is important to evaluate toxic effects by exposure to chemicals during the early postnatal period as well as the fetal period for comprehensive risk assessment. However, economic issues and lack of human resources, arising from practical difficulties regarding protocols, have hindered routine implementation of toxicity studies using newborn animals. Our series of comparative analyzes on susceptibility of the newborn are therefore of particular importance for risk assessment.

In the present study on 2TBP and DTBP, there were no clear differences in toxicity profiles between the newborn and young rats in either case. For 2TBP, clinical signs such as a decrease in locomotor activity and ataxic gait, and effects on liver such as an increase in organ weight were observed. In the DTBP case, hepatic and renal toxicity (histopathological changes, increase in organ weight, etc.) were noted. As a characteristic effect of DTBP on male sexual development, slight delay in preputial separation was also observed in the newborn rat study. Preputial separation, an androgen-dependent process which is an early marker of puberty, represents a reliable non-invasive indicator of chemical-induced perturbation of male pubertal development in the rat (Gaytan *et al.* 1988). However, it is known that decreased body weights can result in non-specific delay in puberty (Ashby & Lefevre 2000). Since DTBP lowered body weights in the period of observation of preputial separation and there were no DTBP-related changes in weights or histopathology of the testes and epididymides, well known to be essentially androgen-dependent, no specific effect on male sexual development could be concluded in the present study. As for NOAELs of both chemicals, clear differences were observed between newborn and young rats, with values of 20 and 5 mg/kg/day in newborn rats, and 100 and 20 mg/kg/day in young rats for 2TBP and DTBP, respectively. Therefore, the susceptibility was four- to five-fold higher in newborn than in young rats.

Our previous analysis of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005) showed dose-response curves to be very different between newborn and young rats. The same was recently reported for the widely used organophosphorus insecticide, chlorpyrifos (Zheng *et al.* 2000), as well as pyrethroid insecticides (Shafer *et al.* 2005). These data showed the importance of estimating unequivocally toxic levels (UETLs), defined for our comparative toxicity analysis as equivalent toxic doses inducing clear toxicity, including death, clinical toxic signs,

Table 4 Summary of the results of the newborn and young rat study of 2,4-di-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	5	40	300	
Death	–	–	M: 2/12, F: 1/12	
Clinical signs	–	–	Decrease in locomotor activity bradypnea, hypothermia	
Body weight changes	–	–	9–25%↓	
Urinalysis	n.d.	n.d.	n.d.	
Hematological changes	–	–	–	
Blood biochemical changes	–	–	Various†	
Changes in relative organ weights	–	F: Liver 14%↑	Liver 39–51%↑, Kidney 37–41%↑ M: Spleen 24%↓	
Histopathological findings	–	F: Fatty degeneration in liver	Various changes in liver and kidney‡	
Developmental parameters	–	–	Slight delay in preputial separation	
Young rat study				
Dose (mg/kg/day)	5	20	75	300
Death	–	–	–	–
Clinical signs	–	–	–	–
Body weight changes	–	–	–	–
Urinalysis	–	–	–	UV↑ SG↓ OP↓
Hematological changes	–	–	–	Various§
Blood biochemical changes	–	–	F: Tcho↑ Pho↑	M: TB↑
Changes in relative organ weights	–	–	F: Liver 13%↑	F: Tcho↑ Pho↑ Liver 40–43%↑
Histopathological findings	n.d.	n.d.	–	Various changes in liver and kidney¶

Data on death are shown as no. of dead animals/no. of animals examined, according to sex. Statistically significant increases ($P < 0.05$) in body weights, urinalysis and blood biochemical parameters, and relative organ weights are shown as ↑, while decreases are shown as ↓. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. †Increase in total bilirubin and decrease in the A/G ratio in both sexes, increase in γ -GTP in males, and increase in total protein and BUN in females were noted. ‡Various changes were observed as shown in Table 3. §Various hematological changes were noted such as decrease in hemoglobin and hematocrit and increase in segmented neutrophils in females and prolongation of PT and APTT in males. ¶Various changes were observed as shown in Table 5. OP: osmotic pressure; Pho: phospholipid; SG: specific gravity; TB: total bilirubin; Tcho: total cholesterol; UV: urine volume; –: no change; n.d.: not determined.

or critical histopathological damage (Koizumi *et al.* 2001). We here tried to apply this UETL approach to the present study. For 2TBP, clinical signs such as decrease in locomotor activity and ataxic gait were noted in most of the animals given 200 mg/kg (newborn rats) and 500 mg/kg (young rats) (Table 2). Furthermore, a 8–17% lowering of body weight was observed at 200 mg/kg in newborn rats, but not in the young rat study. Therefore, equivalent toxic effects to these observed at 500 mg/kg in young rats might be expected to appear at 100–150 mg/kg in newborn animals. The UETLs were concluded to be 100–150 and 500 mg/kg/day in newborn and young rats, respectively. In the case of DTBP, clear toxicity was observed at the top dose of 300 mg/kg in both newborn and young rat studies (Table 4), but the level of severity was very different, for example, deaths were only noted in the newborn cases. It was considered difficult to estimate the UETLs from the results of main studies only. However, the most critical endpoint for toxicity, mortality, was also noted at 100 mg/kg and more, and 500 mg/kg, in the dose-finding studies of newborn and young rats, respectively. Therefore, it would be possible to estimate the appropriate UETLs as the minimum lethal dose by taking the results of the dose-finding

studies into consideration. The UETLs were concluded to be 100 mg/kg/day for the newborn, and 500 mg/kg/day for young rats, at which one out of eight rats was found dead in both cases. These analyzes of UETLs, considering equivalence in toxic degree, showed 3.3–5.0 times higher susceptibility of newborn rats to 2TBP and DTBP than young rats, consistent with our analytical results for NOAELs.

Higher susceptibility of newborn rats was also demonstrated in our previous analyzes of five phenols (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol and 2,4,6-trinitrophenol) (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004), considered mainly due to their poor metabolic and excretory capacity (Horster 1977; Cresteil *et al.* 1986). It has actually been reported that UDP-glucuronyltransferase and sulfotransferase activities, when 4-nitrophenol is used as the substrate, are lower in microsomes prepared from livers of newborn rats, and that the elimination rate of 2,4-dinitrophenol from serum of newborn rabbits is markedly slower than in young adults (Gehring & Buerge 1969; Matsui & Watanabe 1982). Unfortunately, there is no information on the toxicity mechanism and toxicokinetics of both 2TBP

Table 5 Histopathological findings for the young rat study of 2,4-di-*tert*-butylphenol

Dose (mg/kg/day)	Grade	Scheduled-sacrifice group†			Recovery group	
		0	75	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	6/6	6/6
Liver						
– Centrilobular hypertrophy of hepatocytes	+	0/0	0/0	4/4	0/0	0/0
Kidneys						
– Basophilic tubules	+	0/0	0/0	1/4	0/0	3/1
	++	0/0	0/0	4/0	0/0	2/0
	+++	0/0	0/0	1/1	0/0	1/0
– Granular casts	+	0/0	0/0	5/2	0/0	4/0
	++	0/0	0/0	1/1	0/0	0/0
– Proteinaceous casts	+	0/0	0/0	5/1	0/0	2/0
	++	0/0	0/0	1/0	0/0	0/0

†No histopathological examination was conducted for the 5 and 20 mg/kg scheduled-sacrifice groups. +, mild; ++, moderate; +++, marked.

and DTBP; however, the immature functions involved in the toxicokinetics in newborn rats would be implicated in the higher susceptibility, as in the case of five phenols previously analyzed. While there are very little data on toxicokinetics of environmental chemicals in the newborn, relatively plentiful information has been reported in humans for pharmaceuticals which are clinically applied during the early postnatal period. Recently, Ginsberg *et al.* (2002) conducted comparative analysis of pharmacokinetic parameters for 45 drugs in both children and adults, and showed half-lives in children aged two months or under to generally be two-fold longer than in adults.

As for the susceptibility of the newborn to toxicity of chemicals, although it is generally important to take the sensitivity of target organs and tissues themselves (toxicodynamics) into consideration besides toxicokinetics, there are insufficient data on differences between newborn and young/adult animals. For appearance of toxicity, which is the outcome of toxicokinetics and toxicodynamics, some comparative studies have relied on LD₅₀ values (Goldenthal 1971; Sheehan & Gaylor 1990). However, it is not considered that information on acute toxicity at lethal dosage is appropriate when considering the susceptibility of newborn in risk assessment, because dose–response curves could differ, as mentioned above. With prolonged, subtoxic doses, which are basis for TDI or ADI, our series of comparative studies constitute the first systematic assessment, providing an important base for development of new methods of risk assessment of susceptibility of the newborn.

In conclusion, clinical signs and effects on the liver were observed for 2TBP, and hepatic and renal toxicity for DTBP. Although there were no clear differences in toxicity profiles between the newborn and young rats for both chemicals, the toxicity levels differed markedly. The susceptibility of the newborn to these chemicals appears to be 4–5 times higher than that of young animals.

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ORIGINAL ARTICLE

Comparative susceptibility of newborn and young rats to six industrial chemicals

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ABSTRACT To elucidate the comparative susceptibility of newborn rats to chemicals, newborn and young animals were administered six industrial chemicals by gavage from postnatal days (PND) 4 to 21, and for 28 days starting at 5–6 weeks of age respectively, under the same experimental conditions as far as possible. As two new toxicity endpoints specific to this comparative analysis, presumed no-observed-adverse-effect-levels (pNOAELs) were estimated based on results of both main and dose-finding studies, and presumed unequivocally toxic levels (pUETLs) were also decided. pNOAELs for newborn and young rats were 40 and 200 for 2-chlorophenol, 100 and 100 for 4-chlorophenol, 30 and 100 for p-(α,α -dimethylbenzyl) phenol, 100 and 40 for (hydroxyphenyl)methyl phenol, 60 and 12 for trityl chloride, and 100 and 300 mg/kg/day for 1,3,5-trihydroxybenzene, respectively. To determine pUETLs, dose ranges were adopted in several cases because of the limited results of experimental doses. Values for newborn and young rats were thus estimated as 200–250 and 1000 for 2-chlorophenol, 300 and 500 for 4-chlorophenol, 300 and 700–800 for p-(α,α -dimethylbenzyl) phenol, 140–160 and 1000 for (hydroxyphenyl)methyl phenol, 400–500 and 300 for trityl chloride, and 500 and 1000 mg/kg/day for 1,3,5-trihydroxybenzene, respectively. In most cases, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL. An exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.

Key Words: industrial chemicals, newborn rats, susceptibility

INTRODUCTION

In risk assessment of chemicals, the no-observed-adverse-effect-level (NOAEL) determined with repeated dose toxicity studies is generally divided by uncertainty factors (UFs) to obtain the tolerable daily intake (TDI) (Hasegawa *et al.* 2004). UFs include inter- and intraspecies differences, lack of data quality and the nature of observed toxicity. As TDI is an allowable lifetime exposure level for a chemical, at which no appreciable health risk would be expected over a lifetime, the NOAEL must be derived from lifetime exposure studies and appropriate reproductive/developmental studies, or their equivalents. Administration generally starts at the pre-pubertal stage (4–5 weeks old) or with young adults (10–12 weeks old) in rodent studies. Therefore, the suckling phase is the major remaining period where animals are not directly administered to chemicals. If susceptibility of infant animals to chemicals via direct

exposure was evidenced by appropriate comparative studies, the results would preferably be incorporated into the UF as one justification for lack of data quality.

In the latest decade, infant and child health has become a major focus (Landrigan *et al.* 2004), especially since endocrine disrupters became a contentious issue around the world (IPCS 2002). Since there are distinct differences in characteristics from the adult case (Dourson *et al.* 2002), particular attention must be paid to infant and child health. The Japanese government has therefore incorporated the newborn rat study (newborn study) into Existing Chemical Safety Programs as an especial project to comparatively determine susceptibility to 18 industrial chemicals. As the core of this program is to conduct 28-day repeated dose toxicity studies using young rats (young study) with untested chemicals from the existing list, chemicals for newborn studies were selected among the chemicals scheduled for young studies in the same year for the best comparison of data. Furthermore, we have had to newly establish a newborn rat study protocol because of the lack of any standard testing guidelines. Major differences of newborn from young studies are a shorter administration period (18 days only for the suckling phase) and additional examination of early functional, external and sexual development (Koizumi *et al.* 2001). Studies were conducted from 1995 to 1998 and we have already reported the results of comparative analysis for eight chemicals, showing newborn rats to be generally 2–4 fold more susceptible than young rats in most cases on basis of NOAEL and the unequivocally toxic level (UETL), the latter being uniquely defined in this program as doses inducing clear clinical toxic signs, death or critical histopathological damage (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005).

The purpose of this study is to obtain additional information on susceptibility of newborn rats to other chemicals. Here we selected the following six industrial chemicals, mostly phenolic compounds: 2-chlorophenol, 4-chlorophenol, p-(α,α -dimethylbenzyl) phenol (hydroxyphenyl)methyl phenol, trityl chloride and 1,3,5-trihydroxybenzene, because of structural similarity to endocrine-disrupting phenols, bisphenol A (Takahashi & Oishi 2001), and nonylphenol (Lee 1998). These chemicals have been used as an intermediate in dyes and an ingredient in pesticides (2-chlorophenol), an intermediate in dyes, bactericides and an ingredient in cosmetics (4-chlorophenol), an ingredient in surfactants, bactericides, an intermediate in pesticides and plasticizers (p-(α,α -dimethylbenzyl) phenol), an ingredient in resins ((hydroxyphenyl)methyl phenol), an intermediate in medicines (trityl chloride) and an ingredient in medicines, a stabilizer of synthetic rubbers and an adhesive of rubbers (1,3,5-trihydroxybenzene) (Chemical Products' Handbook 2004). Under the same experimental conditions as far as possible, we have examined the repeated dose toxicity of these chemicals in newborn and young rats and compared susceptibility for each. Previously we had applied NOAEL and UETL as estimated doses

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or ranges of doses for comparison of chemical susceptibility, but we have decided to employ the new terminology of presumed NOAEL (pNOAEL) and presumed UETL (pUETL) in their place. As a result, in most cases newborn rats were more susceptible to these industrial chemicals than young rats in terms of both pNOAEL and pUETL.

MATERIALS

2-Chlorophenol (CAS no. 95-57-8, Lot no. OJL-15, purity: 99.49%) was obtained from Inui Corporation and prepared in olive oil; 4-chlorophenol (CAS no. 106-48-9, Lot no. PJF-3, purity: 99.29%) from Inui Corporation and in corn oil; p-(α,α -dimethylbenzyl) phenol (CAS no. 599-64-4, Lot no. 101002, purity: 99.88%) from Sun TechnoChemical Inc. in olive oil; (hydroxyphenyl)methyl phenol (CAS no. 1333-16-0, Lot no. S980013, purity: 99.0% [2,2' isomer 14–18%, 2,4' isomer 44–48%, 4,4' isomer 26–32%]) from Mitsui Chemicals, Inc. in 0.5% CMC-Na solution containing 0.1% Tween 80; trityl chloride (CAS no. 76-83-5, Lot no. 1038, purity: 99.5%) from Kurogane Kasei Co. Ltd. in olive oil; and 1,3,5-trihydroxybenzene (CAS no. 108-73-6, Lot no. OS-12074, purity: 99.9%) from Ishihara Sangyou Co., Ltd. in olive oil. Test solutions were prepared at least once a week and were kept cool and in the dark until dosing. The stability was confirmed to be at least seven days under these conditions. All other reagents used in this study were specific purity grade.

METHODS

All animal studies were performed in five testing laboratories contracted to the Japanese Government, after we approved the test protocol.

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 $24 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 15\%$, a ventilation rate of more than 10 times per hour, and a 12:12 h light/dark cycle. For the studies of newborns, 20 pregnant rats (shipped in at gestation day 14) were allowed to deliver spontaneously. All newborns were separated from dams on postnatal day (PND) 3 and groups of 12 males and 12 females were selected and assigned to each of the four dose groups, including the controls. Twelve foster mothers were selected based on health and nursing conditions, and suckled the four males and four females assigned to each group up to weaning on PND 21 (termination of dosing and autopsy for half of the animals). After weaning, the rest of the animals for the recovery-maintenance group (see Study Design) were individually maintained for nine weeks. In the studies of young, four-week-old male and female rats were obtained and used at ages of 5–6 weeks after acclimation. All animals were allowed free access to a basal diet and water.

Study design (time schedule as described previously [Koizumi et al. 2001])

1. 18-day repeated dose study in newborn rats (newborn study)

In a dose-finding study, chemicals were administered by gastric intubation to newborn male and female rats on PNDs 4–21. Animals were examined for general behavior and body weights during the dosing period, and sacrificed at PND 22 for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, newborn rats (12/sex/dose) were administered chemicals by gastric intubation on PNDs 4–21, the dosage being set on the basis of results of the dose-finding study. On PND 22, half of the animals were sacrificed and the rest were maintained for nine weeks without chemical treatment, and then sacrificed at 12 weeks of age (the recovery-maintenance group). During the study, general behavior and body weight were examined at least once a day and each week, respectively. In addition, 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 and osmotic pressure) was conducted in the late recovery-maintenance period.

At weaning age PND 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, this was conducted at 85 days of age after overnight starvation. Blood was examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count, and for biochemistry (total protein, albumin, albumin/globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase, alanine aminotransferase (ALT), alkaline phosphatase, γ -glutamyl transpeptidase (γ -GTP), calcium, inorganic phosphorus, sodium, potassium and chlorine). Prothrombin time and activated thromboplastin time were examined only in the recovery-maintenance group. The brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, ovaries and uterus were weighed, and these, with other macroscopically abnormal organs, were 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 (young study)

In a dose-finding study, chemicals were administered by gastric intubation to five-week-old male and female rats for 14 days. 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.

In the main study, 5–6 week old male and female rats were given chemicals by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. 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

Quantitative data were analyzed by Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recog-

nized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups. If not homogeneous, the data were analyzed using Steel's multiple comparison test (Steel 1959) or the mean rank test of the Dunnett type (Hollander & Wolfe 1973). For qualitative data such as histopathological findings, the Mann-Whitney's *U*-test (Mann & Whitney 1947) or the Fisher's exact test (Fisher 1973) were performed.

Adoption of pNOAEL and pUETL

NOAEL is a measure used in toxicity studies for the greatest dose at which no adverse effects are observed. No toxicologically meaningful changes are excluded for any grounds, including increase of relative organ weights without any other related changes. As the present purpose was to elucidate susceptibility of newborn rats to chemicals as compared with young rats as accurately as possible, simple application of NOAELs obtained from newborn and young main studies was considered not to be necessarily appropriate even though the dose setting is pertinent. Therefore, we newly defined a pNOAEL as the most likely estimated no-adverse-effect-dose on the basis of data from both main and dose-finding studies. As urinalysis and histopathological examination were not conducted in both dose-finding studies, and the administration period in young dose-finding study was half of the main study, we carefully weighed how the results from the dose-finding study should be taken into account, especially concerning the type of toxicity. In order to consider equivalently toxic intensity doses for newborn and young rats, we also newly defined a pUETL, although this is not without problems given the limited dose points. Therefore, in the most cases, the appropriate pUETL for either newborn or young rats was chosen first, thereafter the matching pUETL or the range of pUETL was speculated to assess equivalent toxicity, considering the entire body of data.

RESULTS

2-Chlorophenol (Table 1)

The newborn investigation was conducted at doses of 0, 20, 100, and 500 mg/kg for the dose-finding and 0, 8, 50, and 300 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 200, and 500 mg/kg for the dose-finding and 0, 8, 40, 200, and 1000 mg/kg for the main study.

Major toxic effects on the central nervous system (CNS) were found in both sexes of newborn and young rats. In the newborn study, tremors appeared within five minutes and disappeared within four hours in most animals at 300 mg/kg. Hypoactivity and an abnormal gait were also observed in a few cases. The histopathological examination showed slight to moderate basophilic renal tubules in more than half the animals of both sexes, without relative kidney weight changes (increase by 8% for males, 4% for females). In addition to these effects, the body weights of both sexes at this dose were transiently decreased. At 50 mg/kg, only one female showed tremors once from 15 to 30 minutes on day nine after the dosing start. There were no chemical-related changes in developmental parameters. In the young study, most animals of both sexes sporadically showed various effects on the CNS such as tremors, hypoactivity, and an abnormal gait within three hours after dosing at 1000 mg/kg. Most animals also exhibited slight centrilobular hypertrophy of hepatocytes, suggesting a compensatory response to a requirement for hepatic metabolism. In the dose-finding study, no toxic signs were observed, but the information was limited because of the small number of animals, the short administration period, and the lack of histopathological examination. There were no chemical-related abnormalities at 200 mg/kg in the main study.

Although the NOAEL was 8 mg/kg/day for newborn rats based on the main study results, this value was concluded to be too low

Table 1 Toxicity findings for 2-chlorophenol in the newborn and young rat main studies

	Newborn study (mg/kg)					Young study (mg/kg)			
	0	20†	50	100†	300	0	200	500†	1000
Male									
General behavior									
Tremors	0/12	0/4	0/12	0/4	11/12	0/12	0/12	0/3	4/12
Hypoactivity	0/12	0/4	0/12	0/4	2/12	0/12	0/12	0/3	8/12
Abnormal gait	0/12	0/4	0/12	0/4	1/12	0/12	0/12	0/3	4/12
Histopathology									
Renal tubules, basophilic	0/6	no data	0/6	no data	4/6	0/6	0/6	no data	0/6
Centrilobular hypertrophy	0/6	no data	0/6	no data	0/6	0/6	0/6	no data	6/6
Female									
General behavior									
Tremors	0/12	0/4	1/12	0/4	12/12	0/12	0/12	0/3	5/12
Hypoactivity	0/12	0/4	0/12	0/4	3/12	0/12	0/12	0/3	5/12
Abnormal gait	0/12	0/4	0/12	0/4	1/12	0/12	0/12	0/3	7/12
Histopathology									
Renal tubules, basophilic	0/6	no data	0/6	no data	5/6	0/6	0/6	no data	0/6
Centrilobular hypertrophy	0/6	no data	0/6	no data	0/6	0/6	0/6	no data	5/6

Only data for items showing change are included in this table. Data are numbers of animals with the change of the total examined. † indicates dose and data from the dose-finding study. All newborn animals died by the 9th dosing day at 500 mg/kg in the dose-finding study. Body weights of both sexes were only transiently, but not finally reduced, at 300 mg/kg in the newborn main study. Clinical signs in newborn rats were not observed at doses of 20 and 100 mg/kg in the dose-finding study.

because of the absence of clinical signs at 20 and 100 mg/kg in the dose-finding study, and only one female showed tremors once at 50 mg/kg in the main study. The pNOAEL for newborn rats was therefore estimated to be 40 mg/kg/day, a little below the 50 mg/kg. For young rats, the pNOAEL can be considered to be 200 mg/kg/day because of the limited information at 500 mg/kg in the dose-finding study. The toxicity at 300 mg/kg for newborn rats seemed to be slightly higher than that at 1000 mg/kg for young rats, because of the transient depression of body weight found limited to the former cases, although the toxicity profile regarding the CNS was very similar in newborn and young rats. The dose for newborn rats showing the same toxic intensity, as that for young rats at 1000 mg/kg, is considered to be slightly lower than 300 mg/kg, at 200–250 mg/kg/day. Therefore, pUETLs of 200–250 and 1000 mg/kg/day may be considered equivalent doses for newborn and young rats, respectively.

4-Chlorophenol (Table 2)

The newborn investigation was conducted at doses of 0, 20, 100, and 500 mg/kg for the dose-finding and 0, 12, 60, and 300 mg/kg for the main study. With young rats doses of 0, 20, 100, and 500 mg/kg were applied in both dose-finding and main studies.

Toxic effects on the CNS were observed in both sexes of newborn and young rats. Most newborn rats at 500 mg/kg in the dose-finding study showed tremors, hypoactivity, bradypnea and hypothermia, and died. All newborn rats at 300 mg/kg exhibited tremors, mostly within 15 minutes to one hour, but these completely disappeared within four hours after dosing. There were no abnormalities at 100 mg/kg in the dose-finding, and 60 and 12 mg/kg in the main study. No developmental abnormalities were observed at any dose in the newborn dose-finding and main studies. In the young study, tremors, tachypnea and salivation were observed from five to 30 minutes after dosing in most animals in

both sexes at 500 mg/kg. There were no other dose-dependent changes at any dose.

The pNOAEL for newborn rats is considered to be 100 mg/kg/day, because CNS toxicity was not observed at 100 mg/kg in the dose-finding study. The pNOAEL for young rats must be set at 100 mg/kg/day, because there were no doses set between 100 and 500 mg/kg. Although the toxicity profile regarding the CNS differed to some extent between newborn rats at 300 mg/kg and young rats at 500 mg/kg with respect to symptom appearance and duration, the same level can be concluded, considering the specific characteristics of the newborn body. Thereby, pUETLs of 300 and 500 mg/kg/day were estimated as appropriate for newborn and young rats, respectively.

p-(α,α -Dimethylbenzyl) phenol (Table 3)

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

No newborn animals died although the body weights of both sexes were transiently lowered at 300 mg/kg (8% maximum decrease). General behavior, functional parameters and urinalysis, hematology and biochemistry data were all within normal ranges except for high urinary volume in males and high BUN in females at 300 mg/kg. The relative kidney weights were increased more than double at 300 mg/kg in both sexes, and dilation of tubules and papillary ducts was observed at relatively high grades in kidneys of both sexes, with no complete recoveries even after a nine-week recovery-maintenance period. Such histopathological change in kidneys was also slightly observed at 100 mg/kg in both sexes. In addition, there were effects on the endocrine systems, despite no effects on sexual differentiation. Absolute testicular weights were reduced by 16% at 300 mg/kg and ovary weights by 26% at 100

Table 2 Toxicity findings for 4-chlorophenol in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)		
	0	60	100†	300	0	100	500
Male							
General behavior							
Tremors	0/12	0/12	0/4	12/12	0/12	0/6	12/12
Tachypnea	0/12	0/12	0/4	0/12	0/12	0/6	11/12
Salivation	0/12	0/12	0/4	0/12	0/12	0/6	9/12
Histopathology							
Kidney	0/6	0/6	no data	0/6	0/6	0/6	0/6
Liver	0/6	0/6	no data	0/6	0/6	0/6	0/6
Female							
General behavior							
Tremors	0/12	0/12	0/4	12/12	0/12	0/6	11/12
Tachypnea	0/12	0/12	0/4	0/12	0/12	0/6	9/12
Salivation	0/12	0/12	0/4	0/12	0/12	0/6	8/12
Histopathology							
Kidney	0/6	0/6	no data	0/6	0/6	0/6	0/6
Liver	0/6	0/6	no data	0/6	0/6	0/6	0/6

Data are numbers of animals with the change of the total examined. All newborn males and 3/4 females died at 500 mg/kg in the dose-finding study. †indicates dose and data from the dose-finding study.

Table 3 Major toxicity findings for p-(α,α -dimethylbenzyl) phenol in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	30	100	300	0	100	300	1000
Male								
Dead or moribund	0/12	0/12	0/12	0/12	0/14	0/7	0/7	3/14
ALT, γ -GTP	/	-	-	-	/	-	-	↑
BUN, Creatinine	/	-	-	-	/	-	-	↑
Relative liver weight	/	-	-	-	/	-	↑	↑
Relative kidney weight	/	-	-	↑	/	-	-	↑
Stomach, hyperplasia	0/6	0/6	0/6	0/6	0/7	0/7	0/7	1/6
Liver, proliferation bile ducts	0/6	0/6	0/6	0/6	0/7	0/7	0/7	6/6
Kidney, regeneration	0/6	0/6	0/6	0/6	3/7	3/7	5/7	6/6
Kidney, dilatation	0/6	0/6	1/6	6/6	0/7	0/7	0/7	6/6
Female								
Dead or moribund	0/12	0/12	0/12	0/12	0/14	0/7	0/7	1/14
ALT, γ -GTP	/	-	-	-	/	-	-	↑
BUN, Creatinine	/	-	-	↑,-	/	-	-	-
Relative liver weight	/	-	-	-	/	-	-	↑
Relative kidney weight	/	-	-	↑	/	-	-	↑
Stomach, hyperplasia	0/6	0/6	0/6	0/6	0/7	0/7	0/7	3/7
Liver, proliferation bile ducts	0/6	0/6	0/6	0/6	0/7	0/7	0/7	7/7
Kidney, regeneration	0/6	0/6	0/6	0/6	0/7	1/7	0/7	7/7
Kidney, dilatation	0/6	0/6	2/6	6/6	0/7	0/7	0/7	4/7

Only critical data are shown in this table. Data are numbers of animals with the change of the number examined. Slashes and bars mean no statistical significance as compared to controls. ↑ indicates significant increase at $P < 0.05$. Relative kidney weights were increased 2.5- and 2.1-fold for males and females at 300 mg/kg in the newborn study. For the young study, 14 males and 14 females (half for examination of recovery) were assigned to each group but 6 males and 7 females at 1000 mg/kg were re-assigned for 28-day examination because of deaths.

and 300 mg/kg. The absolute ovary weights were still lowered by 32% at 300 mg/kg after the recovery-maintenance period. Increased numbers of atretic follicles were found in ovaries of half of the females at 300 mg/kg at the end of the dosing period, and most females continued to show various changes such as decreased numbers of corpora lutea in the ovaries and hypertrophy of endometrial epithelium in the uteri, after the recovery-maintenance period.

In the young study, two males and one female died, and one male was killed in a moribund condition at 1000 mg/kg. The final body weights were reduced by 18%, limited to males. On urinalysis, both sexes showed irregularly sized particles of a black substance, accompanied by 2-4 fold elevation of urine volume. Clear changes of several biochemical parameters such as ALT, γ -GTP, BUN, and creatinine, increases of relative liver and kidney weights, and histopathological changes in the forestomach (squamous hyperplasia), liver (bile duct proliferation), and kidney (regeneration of tubular epithelium and dilatation of tubules) were also observed at 1000 mg/kg. A dose of 300 mg/kg was considered to cause slight toxicity, because the abnormal urinary contents described above were found in half of both sexes and a slightly elevated incidence of mild regeneration of the tubular epithelium was noted in male kidneys. After the two-week recovery period, the pathological changes in male kidneys at 1000 mg/kg continued to be evident. There were no signs of toxicity at 250 and 500 mg/kg in the dose-finding study although the administration period was only half and urinalysis and histopathological examinations were not performed.

The pNOAEL of 30 mg/kg/day for newborn rats is clear and one of 100 mg/kg/day for young rats is reasonable because of slight toxicity at 300 mg/kg in the main study and limited information at 250 mg/kg in the dose-finding study. Toxicity for newborn rats was evident at 300 mg/kg as all animals of both sexes showed histopathological changes in kidneys, with increased relative weights. However, the degree of toxicity for young rats at 1000 mg/kg was obviously much stronger than that of newborn rats at 300 mg/kg, which appeared to be equivalent to doses of 700-800 mg/kg in young rats. Therefore, pUETLs of 300 and 700-800 mg/kg/day may be appropriate for newborn and young rats, respectively. It should be specially noted that this chemical may have endocrine disrupting properties, especially against females, when given only during the suckling phase.

(Hydroxyphenyl)methyl phenol (Table 4)

The newborn investigation was conducted at doses of 0, 20, 60, and 200 mg/kg for dose-finding and 0, 16, 40, and 100 mg/kg for the main study. The young study was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and 0, 8, 40, 200, and 1000 mg/kg for the main study.

Common changes were limited to depression of body weight and death at high doses in newborn and young rats. The highest dose of 100 mg/kg in the newborn main study did not cause any changes, but half the animals at 200 mg/kg in the newborn dose-finding study died, without accompanying liver weight changes in surviving

Table 4 Major toxicity findings for (hydroxyphenyl)methyl phenol in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)			
	0	100	200†	0	40	200	1000
Male							
Dead or moribund	0/12	0/12	3/6	0/12	0/12	0/12	0/12
Final body weight	/	-	↓	/	-	-	↓
Total cholesterol	/	-	↑	/	-	-	↓
Relative liver weight	/	-	-	/	-	-	↑
Stomach, hyperplasia	0/6	0/6	no data	0/6	0/6	0/6	6/6
Liver, centrilobular hypertrophy	0/6	0/6	no data	0/6	0/6	2/6	4/6
Female							
Dead or moribund	0/12	0/12	3/6	0/12	0/12	0/12	1/12
Final body weight	/	-	(↓)	/	-	-	(↓)
Total cholesterol	/	-	-	/	↓	↓	↓
Relative liver weight	/	-	-	/	-	↑	↑
Stomach, hyperplasia	0/6	0/6	no data	0/6	0/6	0/6	6/6
Liver, centrilobular hypertrophy	0/6	0/6	no data	0/6	0/6	0/6	4/6

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 with controls. ↑ indicates significant increase $P < 0.05$. ↓ indicates significant decrease at $P < 0.05$. () indicates that statistical significance was not obtained. Final body weights of surviving newborn males at 200 mg/kg in the dose-finding study were reduced by 30% (14% for females, not significant), respectively. Final body weights of young male rats at 1000 mg/kg in the main study were decreased by 11.8% (5.7% for females, not significant). Increase of relative liver weights was 13% in females at 200 mg/kg, and 16 and 27% in males and females at 1000 mg/kg in the young main study.

animals. There were no chemical-related changes with other examinations, including developmental parameters. In the young study, one female became moribund and the final body weights of males were decreased at 1000 mg/kg. All animals of both sexes at this dose showed squamous hyperplasia of the forestomach or limiting ridge with ulceration, and two-thirds of the animals featured centrilobular hypertrophy of hepatocytes with decrease of total cholesterol (29–51% drop) and increase of relative liver weight. At 200 mg/kg, low incidences of centrilobular hypertrophy in the livers of males and slight increase of liver weights in females with low total cholesterol (45% drop) were found. No toxicity was apparent at 40 mg/kg in the main study. No toxicity was also found at 100 mg/kg in the dose-finding study, but a histopathological examination was not conducted. There were no abnormalities on hematological examination and urinalysis at any dose.

The pNOAEL is considered to be 100 mg/kg/day for newborn rats and 40 mg/kg/day may be appropriate for young rats because of the limited information at 100 mg/kg in the dose-finding study. Although toxicity at 1000 mg/kg for young rats was evident, the dose inducing the same effects in newborn rats was clearly less than 200 mg/kg, because half of the animals died at this dose. We speculate that the dose range for one death in 12 newborn rats would be within 140–160 mg/kg. It is clear that the dose-response curve is much steeper for newborn than young rats. Based on our consideration, pUETLs of 140–160 and 1000 mg/kg/day may be equivalent for newborn and young rats, respectively.

Trityl chloride (Table 5)

The newborn investigation was conducted at doses of 0, 20, 60, 200, and 600 mg/kg for dose-finding and 0, 12, 60, and 300 mg/kg for the main study. The young investigation was conducted at doses

of 0, 30, 100, 300, and 1000 mg/kg for dose-finding and 0, 12, 60, and 300 mg/kg for the main study.

Common effects were observed in livers of newborn and young rats. In the newborn study, increase of relative liver weights were shown at 60 mg/kg and more in both sexes and centrilobular hypertrophy of hepatocytes was noted in 300 mg/kg females. In the dose-finding newborn study, one female died and increase of relative liver weights of both sexes at 600 mg/kg was more evident with low body weights (11.3% drop for males, 13.8% for females). There were no chemical-related changes with other examinations, including developmental parameters. In the young study, both sexes at 60 mg/kg showed a high incidence of centrilobular hypertrophy of hepatocytes with limited increases of relative liver weights (10–14%). At 300 mg/kg, soft feces and mucosal thickening of cecum in most animals were observed in addition to more extensive hepatic changes. Although relative kidney weights were increased at 300 mg/kg in males and 60 and 300 mg/kg in females, there were no renal histopathological findings. Hematological and blood chemical examinations revealed several slight to moderate changes (56% as the maximum) in fibrinogen, ALT, total cholesterol and glucose, as well as prolongation of prothrombin and activated thromboplastin times, at 300 mg/kg.

pNOAELs of 60 and 12 mg/kg/day for newborn and young rats appear appropriate because of the lack of information at higher doses in the dose-finding study, which showed no toxicity but without histopathological examination. The dose of 300 mg/kg in the young main study was a clear toxic level, but intensity was much stronger than that at 300 mg/kg in the newborn main study, while less than that at 600 mg/kg in the dose-finding study. Based on these data, the toxicity with 300 mg/kg for young rats is considered to be within the range with 400–500 mg/kg for newborn rats.

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.

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

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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, tetra-

bromobisphenol 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 TBE. 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 Aspin-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

Susceptibility of newborn rats to 1,3-dibromopropane and 1,1,2,2-tetrabromoethane.

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.

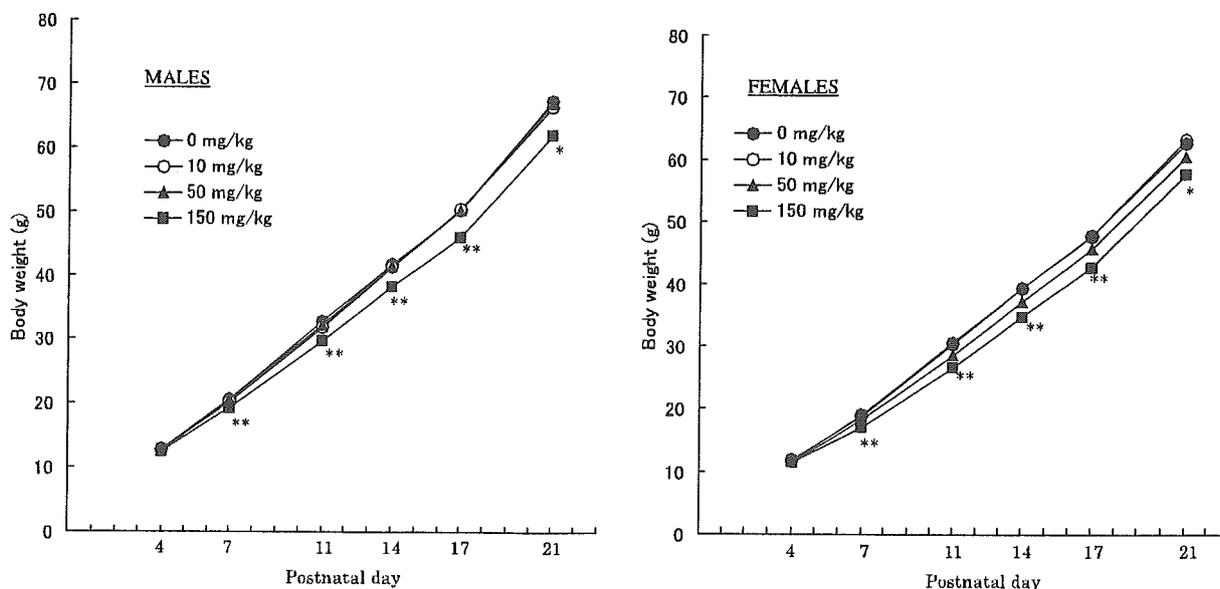


Fig. 1. Body weight curves for the 18-day study of 1,3-dibromopropane in newborn rats.

*: Significantly different from the controls ($p < 0.05$), **: Significantly different from the controls ($p < 0.01$).