### 29. Paragraph 43

The phrase '(tectum, tegmentum, and cerebral peduncles)' should be deleted.

### 30. Paragraph 44

The phrase 'typical of the adult brain' is not understandable. Are some words are missing?

### 31. Paragraph 46

The sentence 'While the use . . .' in lines 7–9 can be rewritten more simply. For instance, 'It is preferable that a pathologist who is unaware of the treatment information scores the slides to substantiate the dose–response relationship'.

### 32. Paragraph 48

Delete 'perinatal' in line 1. The name of this guideline is simply developmental neurotoxicity study.

The phrase 'human studies, case reports', is to be changed to 'human epidemiological studies or case reports', since case report is one of the categories of human studies.

### 33. Paragraph 47 after <u>Test report</u>

47 should be 51.

Insert water after diet in the 4th item of Test animals.

The phrase 'reflex ontogeny' in the 9th item of Results must be 'behavioral ontogeny'.

### 34. <u>Literature</u>

Try to unify the style of the reference presentation. In particular, the writing of journal titles should be uniform (e.g. compare 5 and 7 for Environ Health Perspect and italic presentations such as 28 and 32). It is recommended that the abbreviation of journal titles follows the PubMed, NLM style.

The presentation of the authors' names is also confusing (e.g. 5 vs. 9).

The placement of the published year is also variable (e.g. 3, 5 and 12).

Put a space between 18 and 19. Delete one space after 67.

Some good references as background information can be found in Massaro EJ. (2002) Handbook of neurotoxicology. Vols I and II. Humana Press, Totowa. The four papers in vol II (Henck JW, Rice SA, Cappon GD and Stump DG, and Tilson HA) are very valuable.
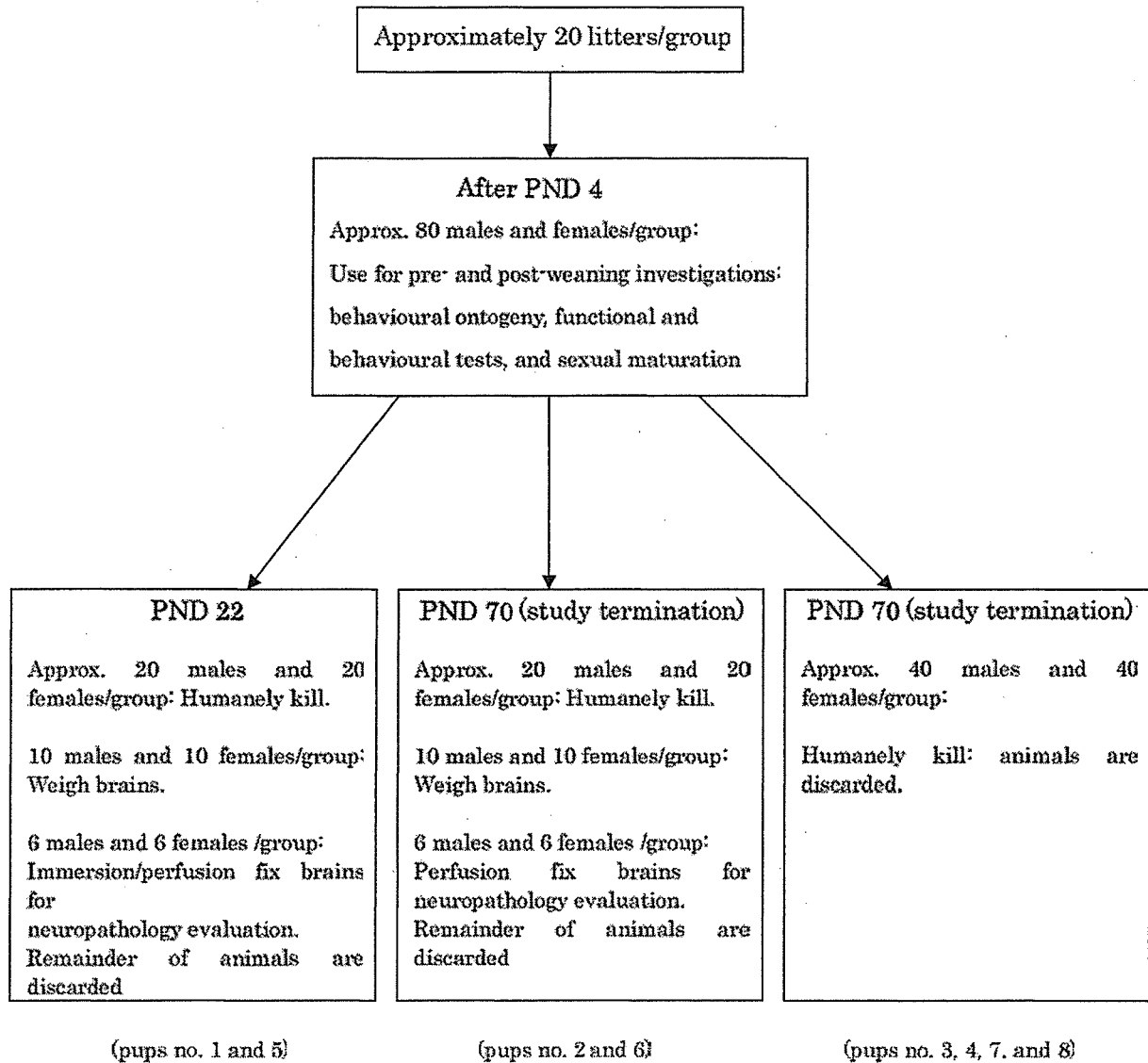
### 35. <u>Appendix A</u>

Totally redraw Fig. 1 according to the description in Tables 2 and 3, and also clarify in the figure legend that this scheme is based on Tables 2 and 3. A suggestion is attached.

## REFERENCES

Organisation for Economic Co-operation and Development (OECD) (1995) *Draft Report of the OECD Ad Hoc Working Group on Reproduction and Developmental Toxicity.* Copenhagen, Denmark, 13–14 June 1995.

Organisation for Economic Co-operation and Development (OECD) (1996) *Final Report of the Consultation Meeting on Developmental Neurotoxicity.* Copenhagen, Denmark, 17–18 June 1996.

Organisation for Economic Co-operation and Development (OECD) (2003) *Report of the Expert Consultation Meeting in Developmental Neurotoxicity Testing.* Washington, US, 23–25 October 2000.

## APPENDIX A

**Fig. 1** Example of the testing scheme for assignment of animals for functional/behavioral tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, and 15. This diagram is based on the description in Tables 2 and 3. (PND = postnatal day).



Approximately 20 litters/group

**After PND 4**

Approx. 80 males and females/group:

Use for pre- and post-weaning investigations:

behavioural ontogeny, functional and

behavioural tests, and sexual maturation

**PND 22**

Approx. 20 males and 20 females/group: Humanely kill.

10 males and 10 females/group: Weigh brains.

6 males and 6 females /group: Immersion/perfusion fix brains for neuropathology evaluation. Remainder of animals are discarded

(pups no. 1 and 5)

**PND 70 (study termination)**

Approx. 20 males and 20 females/group: Humanely kill.

10 males and 10 females/group: Weigh brains.

6 males and 6 females /group: Perfusion fix brains for neuropathology evaluation. Remainder of animals are discarded

(pups no. 2 and 6)

**PND 70 (study termination)**

Approx. 40 males and 40 females/group:

Humanely kill: animals are discarded.

(pups no. 3, 4, 7, and 8)

# SEMI-QUANTITATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF MALE RAT-SPECIFIC $\alpha_{2u}$-GLOBULIN ACCUMULATION FOR CHEMICAL TOXICITY EVALUATION

Masao HAMAMURA[1], Akihiko HIROSE[2], Eiichi KAMATA[2], Koshiro KATOKU[1],
Emiko KUWASAKI[1], Takafumi OSHIKATA[1], Yutaka NAKAHARA[1],
Makoto EMA[2] and Ryuichi HASEGAWA[2]

*[1]Panapharm Laboratories Co., Ltd.,*

*1285 Kurisaki-machi, Uto-shi, Kumamoto 869-0425, Japan*

*[2]National Institute of Health Sciences,*

*1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

**ABSTRACT** — We purified male rat urinary $\alpha_{2u}$-globulin, prepared the antibody in rabbits, and improved an immunohistochemical detection method using this antibody for male rat-specific $\alpha_{2u}$-globulin accumulation appearing as hyaline droplets in the kidneys. Our prepared antibody reacted specifically with $\alpha_{2u}$-globulin in both immunohistochemical and Western blotting analyses, furthermore, and the graded immuno-reactivities on the slide were well associated with computational image analyzing results. Using this method, we retrospectively analyzed the renal sections from the toxicity studies of 12 nephro-toxic chemicals, which had already been conducted under the Japanese Existing Chemicals Survey Program. We demonstrated that the hyaline droplets induced by treatment with 10 chemicals (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene and 3,5,5-trimethylhexan-1-ol) were directly associated with $\alpha_{2u}$-globulin accumulation. This immunohistochemical method is convenient for applying, even retrospectively, paraffin sections from general toxicity studies and could be useful for qualifying male rat-specific hyaline droplets consisting of $\alpha_{2u}$-globulin and renal risk in humans.

**KEY WORDS:** $\alpha_{2u}$-globulin, Immunohistochemistry, Hyaline droplet, Nephrotoxicity

## INTRODUCTION

For risk assessment of chemicals, the most critical data are derived from animal toxicity studies because of a general lack of information on humans. Although all available results from animal studies have been applied to human risk assessment, in principle, exclusion of some specific toxicities, which might not occur in humans, should be taken into account. Among laboratory animals, the rat has been commonly used for toxicity studies, especially sub-acute, long-term or carcinogenicity studies. Nephropathy with hyaline droplets and renal tubular neoplasia caused by chemicals inducing $\alpha_{2u}$-globulin accumulation (CIGA) are con-

sidered to be a male rat-specific toxicity, not occurring in female rats or other animals, including primates. Although low molecular proteins homologous to $\alpha_{2u}$-globulin can be detected in other species, including mice and humans, none of these proteins have been confirmed to bind to CIGA, followed by accumulation of the protein-CIGA complex as in the case of $\alpha_{2u}$-globulin. It is therefore believed that renal toxicity induced by CIGA in male rats is unlikely to occur in humans (Hard *et al.*, 1993).

$\alpha_{2u}$-Globulin was first identified in male rat urine (Roy and Neuhaus, 1966), and had been reported to be a male rat-specific protein with a molecular weight of 18 to 20 kDa. The major source of urinary $\alpha_{2u}$-globulin

is the liver, where $\alpha_{2u}$-globulin mRNA constitutes approximately 1% of the total hepatic mRNA (Sippel *et al.*, 1976; Kurtz and Feigelson, 1977). Neither $\alpha_{2u}$-globulin nor its mRNA is detectable in the female liver (Sippel *et al.*, 1975, 1976; MacInnes *et al.*, 1986). The blood $\alpha_{2u}$-globulin secreted from the liver is freely filtered through the glomerulus, and in mature rats, about two-thirds of the filtered protein is reabsorbed by tubules and the remainder is excreted through the urine (Neuhaus *et al.*, 1981). CIGA binds noncovalently to $\alpha_{2u}$-globulin, and the resulting complex shows less degradability with proteolytic enzymes, causing an accumulation of the complex that is detectable as hyaline droplets with a light microscope. Various chemicals have been suspected of being CIGA based on detection of the evidence for exacerbation of hyaline droplets in renal proximal tubules in male rats, though not in females. Direct evidence for increasing $\alpha_{2u}$-globulin levels has been demonstrated for only a few of these chemicals, however, including 2,2,4-trimethyl-pentane (Stonard *et al.*, 1986; Charbonneau *et al.*, 1987; Lock *et al.*, 1987), decalin (Kanerva *et al.*, 1987), d-limonene (Lehman-McKeeman *et al.*, 1989; Webb *et al.*, 1989), 1,4-dichlorobenzene (Charbonneau *et al.*, 1989), isophorone (Strasser *et al.*, 1988), lindane (Dietrich and Swenberg, 1990), tri- or per-chloroethylene and pentachoroethane (Goldsworthy *et al.*, 1888).

A number of initial safety assessments has so far been conducted for industrial chemicals, including both new and existing chemicals by the Japanese government or the OECD high production volume chemicals programs. Certain chemicals among these industrial chemicals have been suspected of being CIGA. In some cases, however, renal changes in male rats have been assessed as the endpoint for extrapolation to human health risk owing to a lack of direct evidence caused by $\alpha_{2u}$-globulin accumulation, because no antibody against $\alpha_{2u}$-globulin is commercially available for general toxicity studies. Some immunohistochemical $\alpha_{2u}$-globulin analysis methods had already been developed (Burnett *et al.*, 1989; Hashimoto and Takaya, 1992; Caldwell *et al.*, 1999). As these methods required glycolmethacrylate embedding or specific computational analysis, they would be inappropriate for confirming $\alpha_{2u}$-globulin accumulation in routinely conducted guideline-based toxicity studies. We therefore improved an immunohistochemical $\alpha_{2u}$-globulin detection system using paraffin sections, which are generally used for standard toxicity studies. We evaluated the several chemicals suspected of being CIGA, moreover, and indicated the direct evidence caused by

$\alpha_{2u}$-globulin accumulation.

## MATERIALS AND METHODS

### Preparation of anti $\alpha_{2u}$-globulin antibody

$\alpha_{2u}$-globulin as an antigen was obtained from the urine collected from aged male rats, pooled, and used to immunize rabbits. The immunization procedures, including the amount of antigen and immunizing intervals, were determined from the results of a preliminary test referring to the methods of Kurtz *et al.* (1976). The antigen was injected under the skin at a dose of 1 mg/animal (1st injection) or 0.5 mg/animal (2nd and subsequent injections) once at two weeks. Blood sampling was conducted periodically and the antibody titer measured. When the antibody titer level reached a plateau, whole blood was collected and antiserum was obtained from the blood. The antiserum was used for immunohistochemistry and immuno-electron microscopy. For measurement of the $\alpha_{2u}$-globulin content in the urine and tissues, the antibody was purified from the antiserum using a DEAE ionic exchange column after ammonium sulfate precipitation. The singularity of the antibody was confirmed as a single diffuse band of approximately 19 kDa by Western blotting analysis. This study and the following study were carried out in accordance with the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management, etc. of Experimental Animals in Japan.

### Experiment 1 Confirmation of specific reactivity of the antibody to $\alpha_{2u}$-globulin
### 1. Preparation of $\alpha_{2u}$-globulin nephropathy rats

To confirm the specific reactivity of the anti-$\alpha_{2u}$-globulin antibody, we prepared $\alpha_{2u}$-globulin nephropathy rats as follows. Male and female Crj:CD(SD)IGS rats were obtained from Charles River Japan Inc. and used at the age of 11 weeks. d-Limonene (Nacalai Tesque Inc.), a well-known $\alpha_{2u}$-globulin nephropathy inducer, was administered to the rats, consisting of 4 males and 4 females each, for 10 days at doses of 0, 150 and 300 mg/kg/day by gavage using corn oil as a vehicle. The rats were housed individually in stainless steel wire cages in an animal room with a controlled temperature of 24±2°C, humidity of 55±10% and a 12-hr light/dark cycle (lighting from 7:00 to 19:00) and allowed access to food and water ad libitum.

Pooled urine was collected for 24 hr on the day before the start of administration and on Day 9 of administration. After the 10-day administration period,

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Pooled urine was collected for 24 hr on the day before the start of administration and on Day 9 of administration. After the 10-day administration period,

Table 1. Chemical name and effect dose derived from the general toxicity studies.

| Chemical | Test type | Original study doses (mg/kg/day) | Effect doses (mg/kg/day) [a] | | | Original reported NOEL (mg/kg/day) [a] | The selected doses for analyzing (contr./low/high) (mg/kg/day) |
| | | | Histopathological findings | | Non histopathological observations | | |
| | | | AN | Other | | | |
| 1,4-Dibromobenzene | RD | 0/ 4/ 20/100/500 | 20≤/ – | 100≤ | 100≤/ 20≤ | 4 | 0/ 20/500 |
| Dicyclopentadiene | RT | 0/ 4/ 20/100 | 4≤/ – | 20≤ /100 | 20≤ /100 | <4 / 20 | 0/ 4/100 |
| 3,4-Dimethylaniline | RD | 0/10/ 50/250 | 50≤/ – | 250 | 250 / 50≤ | 10 | 0/ 50/250 |
| 1,4-Dicyanobenzene | RD | 0/ 1.25/ 5/ 20/ 80 | 5≤/ – | 20≤ / – | 20≤ | 1.25 / 5 | 0/ 5/ 80 |
| Tetrahydrothiophene-1,1-dioxide | RD | 0/60/ 200/700 | 200≤/ – | – | 700 | 60 / 200 | 0/200/700 |
| 1,3-Dicyanobenzene | RD | 0/ 8/ 40/200 | 8≤/ – | 40≤ / 200 | 40≤ | <8 / 8 | 0/ 8/200 |
| Acenaphthene | RD | 0/12/ 60/300 | 60≤/ – | 300 | 300 / 60≤ | 12 | 0/ 60/300 |
| 3,4-Dichloro-1-butene | RT | 0/ 0.4/ 2/ 10/ 50 | 10≤/ – | 50 | 10≤ / 50 | 2 / 10 | 0/ 10/ 50 |
| 3a,4,7,7a-Tetrahydro-1H-indene | RT | 0/ 67/200/600 | 67≤/ – | 600 | 67≤ / 200≤ | <67 / 67 | 0/ 67/600 |
| 3,5,5-Trimethylhexan-1-ol | RT | 0/ 12/ 60/300 | 12≤/ – | 60≤ | 60≤ | 12 | 0/ 12/300 |
| 2,4-di-tert-butylphenol | RD | 0/ 5/ 20/ 75/300 | – / – | 300 | 300 / 75≤ | 75 / 20 | 0/ –/300 |
| 4-aminophenol | RD | 0/ 4/ 20/100/500 | – / – | 100≤ | 100≤ | 20 | 0/100/500 |

[a] The data were described in a pattern of male/female when the data were different between the male and female.
RD, 28-day Repeat Dose Toxicity Test; RT, Combined Repeat Dose and Reproductive/Developmental Toxicity Test.
AN, α2u-globulin nephropathy including hyaline droplets and subsequent tubular alteration.

$\alpha_{2u}$-globulin antiserum by the above-mentioned protocol. HE-stained sections were used to examine the degree of hyaline droplets and to determine whether or not other findings were present. The degree of occurrence of hyaline droplets was divided into five grades, including none (−), minimal (±, barely detectable minimal appearance), slight (+, multifocal but not dispersed appearance), moderate (++, dispersed appearance over the cortex) and severe (+++, diffused appearance over the whole cortex). The staining sections with PAS, Azan-Mallory and anti-$\alpha_{2u}$-globulin reaction were also graded similarly for positive-stained droplets. In addition, computational image analysis was carried out to verify the above-mentioned grading criteria using three typical immuno-stained samples for each grade. Images including almost all the renal superficial cortex were captured using a light microscope (Olympus BHS) and a digital camera (Olympus DP12). The captured images were measured for positive area using an image analyzing system (C-Imaging System, Compix Inc.), and the positive area (%) was then calculated from the data.

## RESULTS

### Experiment 1 Specific reactivity of the antibody to $\alpha_{2u}$-globulin

On the HE-stained sections of the kidneys, hyaline droplets with round to irregular shapes were observed in the renal proximal tubular epithelium only in males administered d-limonene (Photo. 1a). The hyaline droplets were negative for PAS reaction (Photo 1b) but stained positively with Azan-Mallory staining (Photo 1c). With immuno-staining with the anti-$\alpha_{2u}$-globulin antibody, the hyaline droplets were more clearly stained and more distinguishable than with Azan-Mallory staining (Photo 1d). The hyaline droplets showed a dose-dependent increase on the HE-stained sections (Photo 2, a-c) and positive reactions for hyaline droplets showed a correlational increase with immuno-staining (Photo 2, d-f). Very fine positive granules were also detected on the immuno-stained sections for all the males as background, but no positive reactions were observed in other tissue components. This background was observed generally in male kidneys and was, therefore, excluded from the grading in experiment 2. In the liver, all the males showed a positive reaction for the antibody in centrilobular hepatocytes. The degree of intensity was weaker than in the kidneys, and there was no clear intensification by d-limonene. No positive reaction for

the anti-$\alpha_{2u}$-globulin antibody was detected in the liver or kidneys in any females.

With electron microscopy, electron-dense and irregular-shaped inclusions surrounded by a single membrane were observed as changes corresponding to the hyaline droplets in the renal proximal tubular epithelium, and positive reactions were observed for the antibody with post-embedding method in the inclusions (Photo 3). A similar positive reaction was observed in the lysosomes of the renal tubule epithelium, but no positive reaction was detected in the hepatocytes.

The $\alpha_{2u}$-globulin content in the kidneys of the males was increased dose-dependently by administration with d-limonene (Fig. 1). A dose-dependent but mild increase in $\alpha_{2u}$-globulin content was also observed in the liver of the males. While no dose-dependent increase in the urine was noticeable, a lower molecular type of $\alpha_{2u}$-globulin appeared in the males in the highest dose group, with the $\alpha_{2u}$-globulin type reported as an early marker for $\alpha_{2u}$-globulin nephropathy (Saito et al. 1991).

### Experiment 2 $\alpha_{2u}$-globulin analysis for industrial chemicals

Table 2 indicates the grades of all the samples with respect to hyaline droplets, positive droplets and immunological positive droplets analyzed with HE, Azan-Mallory and anti-$\alpha_{2u}$-globulin antibody staining, respectively. In the controls there was a minimal to moderate amount of hyaline droplets in some animals and consequent variation for Azan-Mallory and anti-$\alpha_{2u}$ -globulin reaction. This variation was due to the arbitrary sampling of specimens, or probably related to the lot of the animals or to the difference of food used in each study. Dose-dependent increases of hyaline droplets in the renal proximal tubular epithelium were, however, confirmed for HE-staining of 10 chemicals suspected of being CIGA (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene, 3,5,5-trimethylhexan-1-ol). This was described in the original reports (Toxicity Testing Reports of Industrial Chemicals), although the occurrence of hyaline droplets varied in shape, size and number/cell with chemicals and showed no clear common features. In the highest dose groups of these chemicals, basophilic tubules, granular casts in the tubules and/or tubular dilatation were intensified or occurred as in the original reports. These changes

showed similar features in spite of the various severity and incidence with the chemicals. In serial sections prepared simultaneously, Azan-Mallory-positive reactions for hyaline droplets were detected dose-dependently in these 10 chemicals. No PAS-positive reaction was detected in any chemical. These staining behaviors of the hyaline droplets were the same as those in the case of d-limonen described above. Immunohistochemical staining using the anti-$\alpha_{2u}$-globulin antibody revealed thoroughly dose-dependent positive reactions for hyaline droplets in all these chemicals. The resulting grades from three types of analysis were the same, demonstrating that a highly positive correlation exists among the three staining methods. As for the remainder not suspected of being CIGA (2,4-di-tert-butylphenol, 4-aminophenol), there was no increase of hyaline droplets or positive immunohis-

tochemical reactions in any dose groups, as well as no stain in either PAS or Azan-Mallory staining. In addition, computational image analysis using three typical immuno-stained sections for each grade (Photo 4) showed a close correlation between the quantitative analysis and semi-quantitative grading (Fig. 2).

## DISCUSSION

Many toxicity studies using laboratory animals have been conducted on environmental and industrial chemicals to ensure their safety or toxicity levels concerning human health. On extrapolating the results to humans, toxic mechanisms that are unlikely to occur in humans should be taken into account. A typical example of such toxicities is $\alpha_{2u}$-globulin-related nephropathy and the consequent renal tumorigenesis in repeated
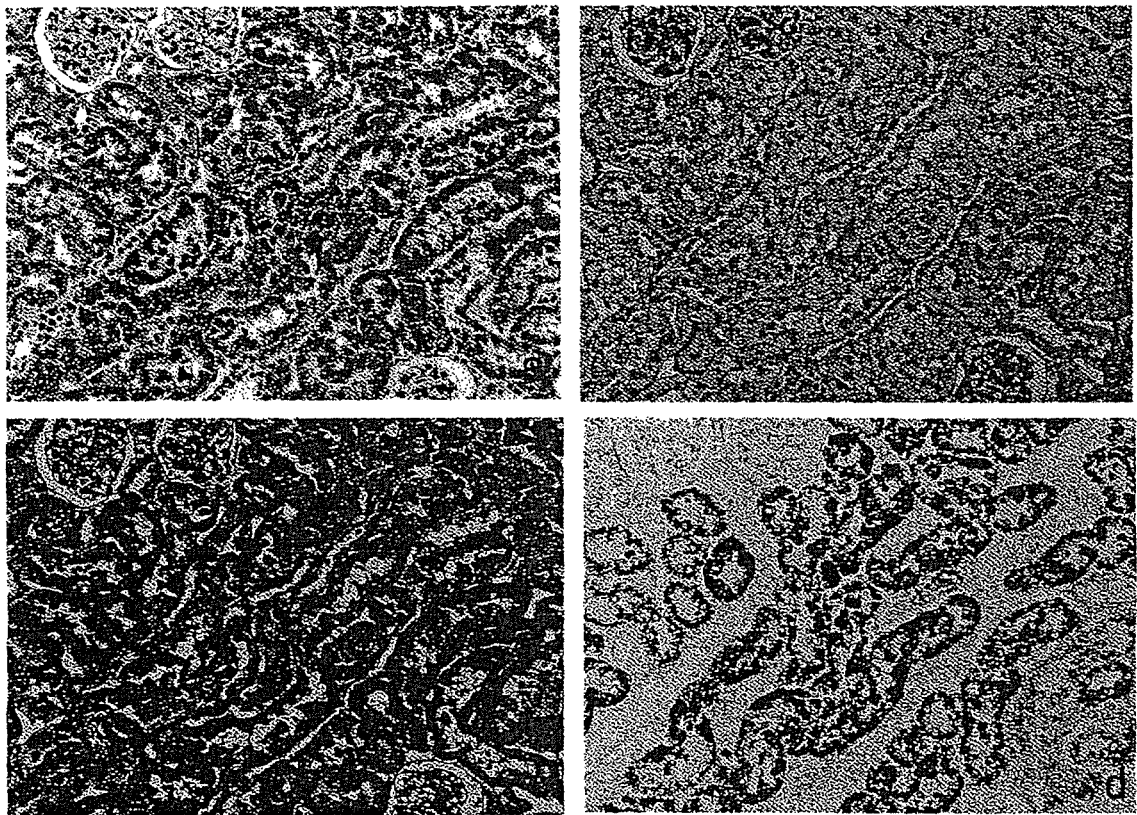


Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti-$\alpha_{2u}$-globulin antibody showed a clear positive reaction consistent with the hyaline droplets (d). Original magnification, ×66.

dose toxicity studies using male rats. This male rat-specific nephrotoxicity is not considered to occur in humans (Hard et al., 1993). To exclude this male rat-specific toxicity from chemical risk assessment, it is necessary to demonstrate properly that such renal tox-

icity results from $\alpha_{2u}$-globulin-CIGA complex accumulation. Detection analysis of $\alpha_{2u}$-globulin in the nephrotoxicity has not been conducted in most conventional toxicity studies, however, especially in sub-acute toxicity screening studies for industrial chemicals. As
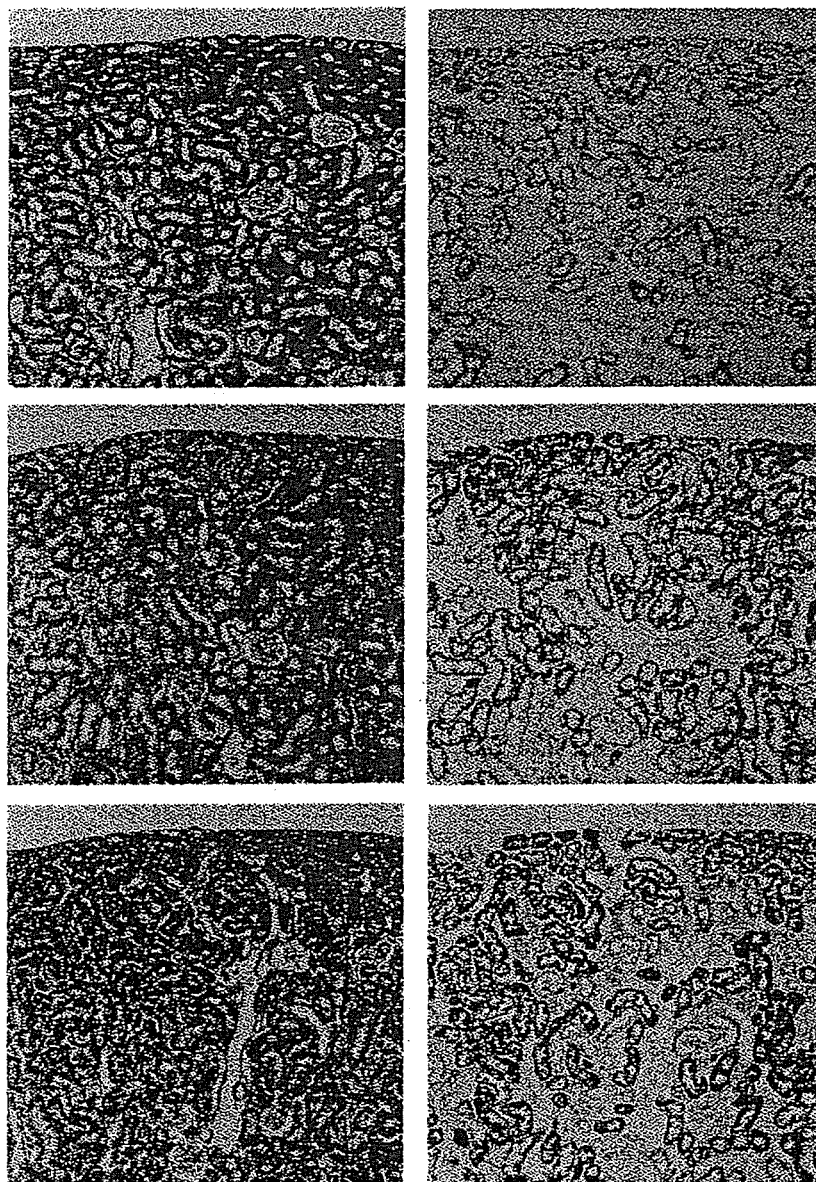
Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of d-limonene(HE, a - c). Positive reaction for the anti-$\alpha_{2u}$-globulin antibody also increased with similar dose dependency (d - f). Original magnification, ×33.
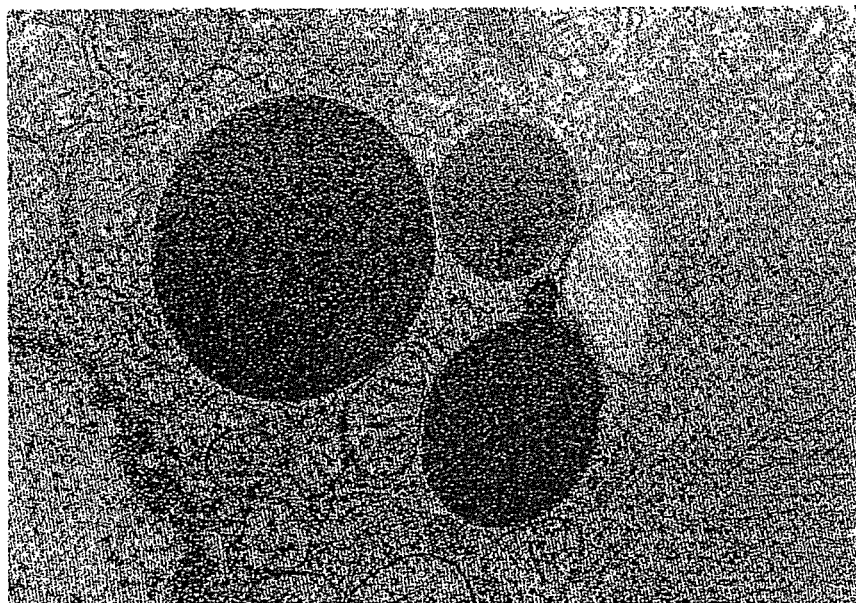
M. HAMAMURA *et al.*



**Photo 3.** Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, ×10,000.
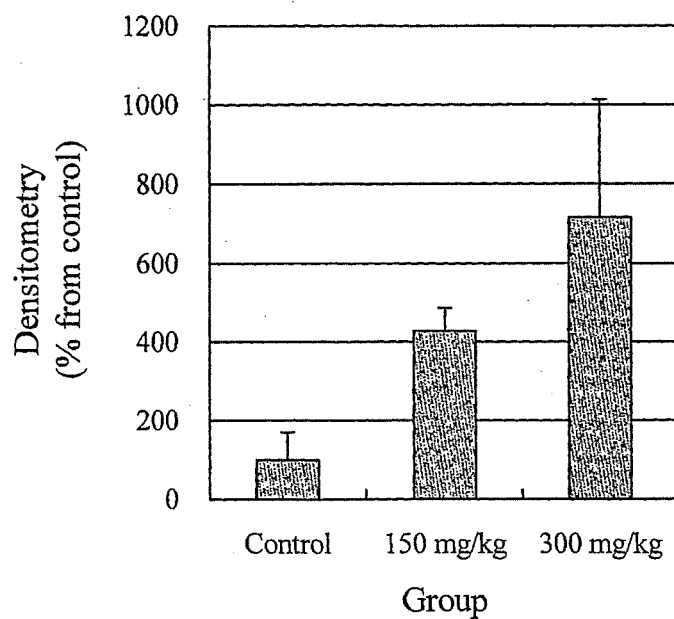


**Fig. 1.** Western blot analysis of $\alpha_{2u}$-globulin in kidney from male rats treated with *d*-limonene.
Results are expressed as mean ± SD (n=4).

an alternative detection method, it is well known that $\alpha_{2u}$-globulin droplets in the kidneys are negative for PAS reaction, but that they are stained positively by Azan-Mallory staining (U.S. EPA, 1991; Alden et al., 1984). Although these additional stainings can distin-guish hyaline droplets resulting from $\alpha_{2u}$-globulin accumulation from those resulting from other causes, these analyses provide only indirect evidence. Direct evidence of $\alpha_{2u}$-globulin accumulation in renal hyaline droplets could be required for appropriate risk assess-

**Table 2.** Grading results of histological/histochemical examination.

| Chemical | Staining | Results Control | Low dose | High dose |
|---|---|---|---|---|
| 1,4-Dibromobenzene | HE [1] | -/-/± | ++/++/+ | ++/+++/+++ |
| | Azan-Mallory [2] | -/-/± | ++/++/+ | ++/+++/+++ |
| | Anti-$\alpha_{2u}$-globulin [2] | -/-/± | ++/++/+ | ++/+++/+++ |
| Dicyclopentadiene | HE | -/-/- | +/++/++ | +++/+++/+++ |
| | Azan-Mallory | -/-/- | +/++/++ | +++/+++/+++ |
| | Anti-$\alpha_{2u}$-globulin | -/-/- | +/++/++ | +++/+++/+++ |
| 3,4-Dimethylaniline | HE | -/-/- | -/-/± | ±/±/+ |
| | Azan-Mallory | -/-/- | -/-/± | ±/±/+ |
| | Anti-$\alpha_{2u}$-globulin | -/-/- | -/-/± | ±/±/+ |
| 1,4-Dicyanobenzene | HE | -/-/- | ±/+/+ | ++/+++/+++ |
| | Azan-Mallory | -/-/- | ±/++/+ | +++/+++/+++ |
| | Anti-$\alpha_{2u}$-globulin | -/-/- | ±/++/+ | +++/+++/+++ |
| Tetrahydrothiophene-1,1-dioxide | HE | +/-/- | +/+/++ | ++/++/++ |
| | Azan-Mallory | +/-/- | ++/+/++ | ++/++/++ |
| | Anti-$\alpha_{2u}$-globulin | +/-/- | ++/+/++ | ++/++/++ |
| 1,3-Dicyanobenzene | HE | -/-/± | +/±/± | ++/++/+++ |
| | Azan-Mallory | -/±/± | +/±/± | ++/+++/+++ |
| | Anti-$\alpha_{2u}$-globulin | -/±/± | +/±/± | ++/+++/+++ |
| Acenaphthene | HE | ±/-/+ | +/-/+ | +/+/++ |
| | Azan-Mallory | ±/-/+ | +/±/+ | +/+/++ |
| | Anti-$\alpha_{2u}$-globulin | ±/-/+ | +/±/+ | +/+/++ |
| 3,4-Dichloro-1-butene | HE | -/-/++ | +/+/± | ++/+/++ |
| | Azan-Mallory | -/-/++ | +/+/+ | ++/+/++ |
| | Anti-$\alpha_{2u}$-globulin | -/-/++ | +/+/+ | ++/+/++ |
| 3a,4,7,7a-Tetrahydro-1H-indene | HE | +/+/++ | ++/++/++ | +++/+++/+++ |
| | Azan-Mallory | +/+/++ | ++/++/++ | +++/+++/+++ |
| | Anti-$\alpha_{2u}$-globulin | +/+/++ | ++/++/++ | +++/+++/+++ |
| 3,5,5-Trimethylhexan-1-ol | HE | -/-/± | +/+/++ | +++/++/+++ |
| | Azan-Mallory | ±/-/± | +/+/++ | +++/++/+++ |
| | Anti-$\alpha_{2u}$-globulin | ±/-/± | +/+/++ | +++/+++/+++ |
| 2,4-Di-tert-butylphenol | HE | -/-/- | | -/-/- |
| | Azan-Mallory | -/-/- | | -/-/- |
| | Anti-$\alpha_{2u}$-globulin | -/-/- | | -/-/- |
| 4-Aminophenol | HE | -/±/- | -/-/- | -/-/- |
| | Azan-Mallory | -/±/- | -/-/- | -/-/- |
| | Anti-$\alpha_{2u}$-globulin | -/±/- | -/-/- | -/-/- |

[1] Grading for hyaline droplets.
[2] Grading for positive droplets.
No PAS-positive reaction for the hyaline droplets was observed in any sample.
Low dose for 2,4-di-tert-butylphenol was not examined.

ment, and a reliable detection method for the existence of $\alpha_{2u}$-globulin is therefore necessary.

Using both immunochemical staining for paraffin-embedded sections and the immuno-electron microscopy technique, we demonstrated that our prepared antibody reacted specifically to $\alpha_{2u}$-globulin in renal hyaline droplets in the male rats administered d-limonene, a well-known $\alpha_{2u}$-globulin nephropathy inducer. The dose-dependent positive immuno-reaction of the antibody in both the tissue sections and the homogenates from d-limonene-treated rat kidneys indicated that the antibody could be applicable for semi-quantitative analysis. In addition, computational image analysis revealed that classical visual microscopic grading was also useful for semi-quantitative analysis of $\alpha_{2u}$-globulin accumulation.

Although immunohistochemical $\alpha_{2u}$-globulin analysis of the glycolmethacrylate-embedded sections

had already been reported by Burnett et al. (1989), our method was advantageous from the standpoint of applicability to the paraffin-embedded sections. The paraffin-embedded specimens were usually prepared and stored for the general toxicity studies. In fact, all the sections used in experiment 2 in this study originated from study specimens which were prepared in the Japanese Existing Chemicals Survey Program conducted previously and stored for a long time. It indicated that our method is applicable to specimens derived directly from ordinary toxicology studies retrospectively. Hashimoto and Takaya (1992) previously investigated the application of $\alpha_{2u}$-globulin immunostaining to paraffin sections by modifying the protocol of Burnett et al. (1989). The protocol includes pronase E treatment owing to enhancement of the antigen reactivity and removal of the non-specific reaction. Our method also includes the pronase E treatment, but
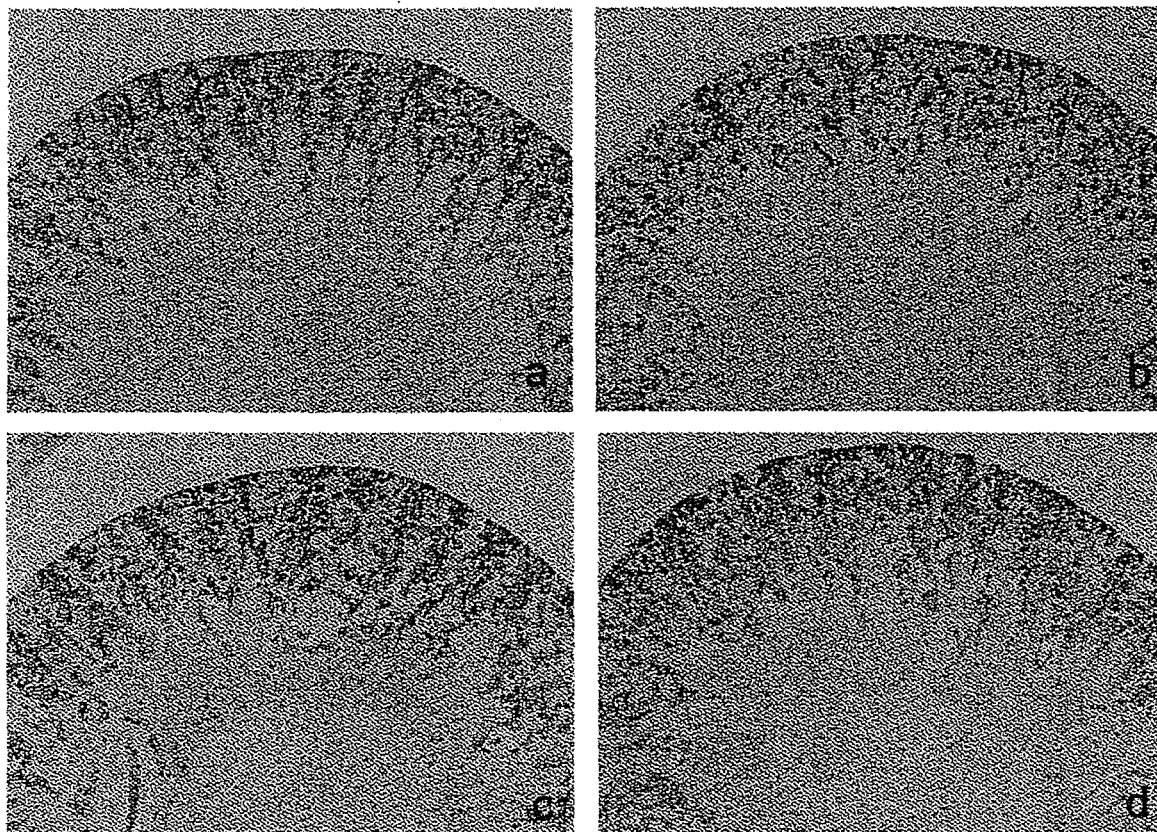


Photo 4. Immunohistochemical features of the anti-$\alpha_{2u}$-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.

Semi-quantitative immunohistochemical analysis of male rat-specific $\alpha_{2u}$-globulin accumulation.
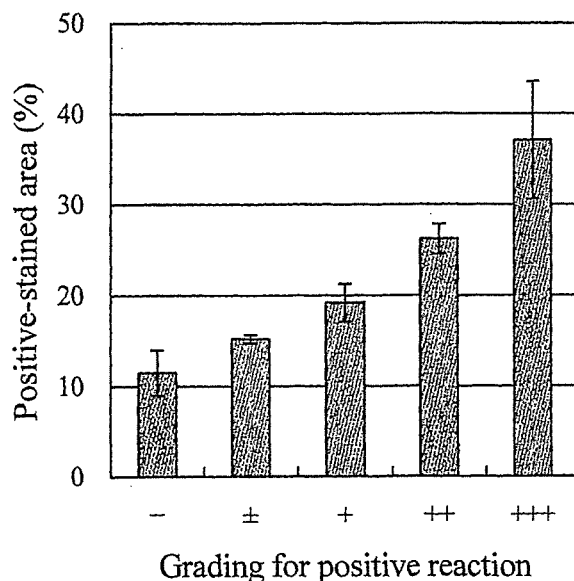


**Fig. 2.** Correlation between semi-quantitative and quantitative analyses for immuno-stained sections.
Results are expressed as mean ± SD (n=3).

the treatment is performed only in order to enhance the antigen activity and not to remove the non-specific reaction. This may suggest that our prepared antibody has a high specificity for $\alpha_{2u}$-globulin. Caldwell *et al.* (1999) had conducted a similar quantitative immunohistochemical $\alpha_{2u}$-globulin analysis, but it seems that the actual analyzed area was limited to narrower fields than in our study.

Urinary immunochemical analysis for detection of $\alpha_{2u}$-globulin accumulation in male rat kidneys has been developed by Saito *et al.* (1996). Although the convenient urinary analysis is sufficient for detecting CIGA, the detectability is weaker than with kidney soluble protein analysis. The aim of the present analysis is not only to detect CIGA, but also to exclude the $\alpha_{2u}$-globulin-induced nephrotoxic effects from risk assessment of chemicals. For 10 chemicals suspected of being CIGA, the occurrence of hyaline droplets in the kidneys with treatment was the lowest endpoint. In the process of evaluating chemical toxicity, if the most sensitive nephrotoxicity is concluded to be a neglected effect for human health, the NOAEL could be set based on other kinds of toxicological effects.

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ORIGINAL ARTICLE

# Comparative susceptibility of newborn and young rats to six industrial chemicals

Ryuichi Hasegawa, Mutsuko Hirata-Koizumi, Mika Takahashi, Eiichi Kamata, and Makoto Ema
*National Institute of Health Sciences, Tokyo, Japan*

**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-trihydroxybenezene, 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 prepubertal 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-trihydroxybenezene, 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

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-($\alpha,\alpha$-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 ± 2°C with a relative humidity of 55 ± 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 animotransferase (ALT), alkaline phosphatase, $\gamma$-glutamyl transpeptidase ($\gamma$-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 homogenous, 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 corpa luteua 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