

# Carcinogenicity and Chronic Toxicity of 1,4-Dichloro-2-nitrobenzene in Rats and Mice by Two Years Feeding

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**Abstract:** Carcinogenicity and chronic toxicity of 1,4-dichloro-2-nitrobenzene (DCNB) were examined by feeding each group of 50 F344 rats and 50 BDF<sub>1</sub> mice of both sexes a DCNB-containing diet at a concentration of 0 (control), 320, 800 or 2,000 ppm (w/w) for 2 yr. In rats, incidences of hepatocellular adenomas and carcinomas and their combined incidence were increased in the 2,000 ppm-fed males, together with increased incidence of basophilic cell foci in the 800 and 2,000 ppm-fed males. A dose-related increase in combined incidences of renal cell adenomas and carcinomas was noted. Incidence of Zymbal gland adenomas tended to increase in the 2,000 ppm-fed males. In mice, incidences of hepatocellular adenomas in the 800 and 2,000 ppm-fed females and hepatocellular carcinomas in the 2,000 ppm-fed males and in the 800 and 2,000 ppm-fed females were increased. Incidence of hepatoblastomas was increased in all DCNB-fed males and in the 2,000 ppm-fed females. Signs of chronic toxicity were characterized by centrilobular hypertrophy of hepatocytes with nuclear atypia in mice, increased relative liver weight in rats, a dose-related increase in incidences of chronic progressive nephropathy with advanced grades of severity in male rats, and decreased hemoglobin concentration and hematocrit accompanied by increased bone marrow hematopoiesis in female rats. Carcinogenic activity of DCNB was evaluated for the three different tumors, and sensitive signs of the chronic toxicity were discussed.

**Key words:** 1,4-Dichloro-2-nitrobenzene, Hepatocellular tumor, Renal tumor, Zymbal gland tumor, Chronic progressive nephropathy, Rat, Mouse

## Introduction

In recent years, various chloronitrobenzenes including 1,4-dichloro-2-nitrobenzene (DCNB) have been produced in Japan<sup>1,2)</sup>, and the annual production and importation of DCNB were reported to total 2,100 tons in 2000<sup>3)</sup>. DCNB is an organic solid used as an intermediate for *p*-dichloroaniline, which is widely used in the manufacture of dyes and pigments and as an ultraviolet absorber for stabilization of polyolefins<sup>3,4)</sup>. The Organisation for Economic Co-operation and Development (OECD) designated DCNB as a High Production Volume (HPV)

chemical and a Screening Information Data Set (SIDS) chemical for initial assessment of toxicity and exposure, in order to protect human health and the environment from the hazardous chemical<sup>4)</sup>. According to SIDS, exposure to DCNB during synthesis might be excluded, and exposure in the workplace was considered negligible, because DCNB was produced in a closed system<sup>4)</sup>. However, it was reported that workers had contact dermatitis with nitro and amino derivatives of benzene<sup>5)</sup>, and that a total of 52 workers suffered from acute poisoning by aromatic chloro- and nitro-compounds in Japan during the period from 1956 to 1985<sup>6)</sup>. No epidemiological data is available for health risk assessments of workers exposed to DCNB. Several recent *in vitro* studies showed

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that bacterial mutagenicity<sup>7-10</sup>) and mammalian clastogenicity<sup>11, 12</sup>) of DCNB were positive with or without S9 activation. DCNB has been evaluated as one of the existing chemical substances with positive mutagenicity, according to the Technical Guideline of the Japanese Industrial Safety and Health Law, which requires that occupational health countermeasures be taken to protect workers from exposure to those mutagenic substances<sup>13</sup>). No bioassay study of rodent carcinogenicity and chronic toxicity for DCNB has been reported. Carcinogenic potential and classification of DCNB have not been evaluated by the International Agency for Research on Cancer (IARC), the Japan Society for Occupational Health or the American Conference of Governmental Industrial Hygienists.

The present study was undertaken to provide dose-response data of rodent carcinogenicity and chronic toxicity of DCNB for health risk assessments of DCNB-exposed workers. The carcinogenicity and chronic toxicity were examined by feeding F344 rats and BDF<sub>1</sub> mice of both sexes DCNB-containing diets at 3 different concentrations for 2 yr. This paper presented an evaluation of the carcinogenic activity of DCNB for the DCNB-induced tumors, and discussed sensitive signs of chronic toxicity with reference to the prechronic toxicity of DCNB reported previously<sup>14, 15</sup>).

## Materials and Methods

The present study was conducted with reference to the OECD Guideline for Testing of Chemicals 453 "Combined Chronic Toxicity/Carcinogenicity Studies"<sup>16</sup>), and in conformity with the OECD Principle of Good Laboratory Practice<sup>17</sup>). The animals were cared for in accordance with a guide for the care and use of laboratory animals<sup>18</sup>), and the present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

**Test substance:** DCNB of guaranteed grade (> 98.8% pure) was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The DCNB was analyzed for stability by both gas chromatography and infrared spectrometry and for purity by gas chromatography before and after its use. The analyses indicated that the test substance was free from impurities and degradation.

**Animals and husbandry:** Four-week-old F344/DuCrj rats (SPF) and Crj: BDF<sub>1</sub> mice (SPF) of both sexes were obtained from Charles River Japan, Inc (Kanagawa, Japan). After 2-wk quarantine and acclimation periods, the animals were allocated by a stratified randomization procedure into 4 body-weight-matched groups, each comprising 50 rats and 50 mice of both sexes. The animals were housed individually in stainless-steel wire hanging

cages (170 mm [W] × 294 mm [D] × 176 mm [H] for rats and 112 mm [W] × 212 mm [D] × 120 mm [H] for mice) under controlled environmental conditions (temperature of 23.2 ± 0.2 °C and relative humidity of 55 ± 2% with 15 to 17 air changes/h) in barrier system animal rooms. Fluorescent lighting was controlled automatically to provide a 12-h light/dark cycle. All animals had free access to filtered, UV-irradiation-sterilized water supplied by an automatic watering system.

**Diet preparation and feeding:** A diet containing 320, 800 or 2,000 ppm DCNB (w/w) was prepared by mixing DCNB with  $\gamma$ -irradiation-sterilized CRF-1 powdered diet (Oriental Yeast Co., Tokyo, Japan) in a spiral mixer for 20 min, and it was stored at 4 °C until use. The highest dose level of 2,000 ppm was chosen, not to exceed the maximum tolerated dose (MTD), based on our data of both body weight gain and subchronic toxicity from a 13-wk administration study<sup>15</sup>). The criteria of the MTD used in the present study were described in the guidelines of the National Cancer Institute (NCI)<sup>19</sup>) and the IARC<sup>20</sup>). The powdered diet containing DCNB was prepared at an interval of 2 wk during a 2-yr administration period. The feeder filled with DCNB-containing or control diet in the individual cage was exchanged once a week. DCNB concentrations in the powdered diet were determined by gas chromatography, and were found to be 89.7 to 109.1% of the target concentrations at the time of preparation. Those initial concentrations decreased to 87.8 to 92.5% on the 15th day after preparation, when the concentrations at the time of preparation were taken as 100%. The animals were fed the control diet or DCNB-containing diets throughout a 2-yr administration period, starting at age of 6 wk.

**Clinical observations and analysis, and pathological examinations:** The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured once a week for the first 14 wk of the 2-yr administration period and every 4 wk thereafter. All animals underwent complete necropsy. Urinary parameters were measured at the last week of the 2-yr administration period with Ames reagent strips (Multistix for rats and Uro-Labstix for mice: Bayer Health Care LLC, USA). For hematology and blood biochemistry, blood samples were collected under etherization at the terminal necropsy after overnight fasting. Methemoglobin and other hematological parameters were measured with a CO-oximeter (CIBA-CORNING 270, USA) and an Automatic Blood Cell Analyzer (TECHNICON H-1, USA), respectively. Blood biochemical parameters were measured with an Automatic Analyzer (HITACHI 7070, Japan). Organs were removed, weighed and examined for macroscopic lesions. The tissues for microscopic examination were fixed in 10% neutral buffered formalin and

embedded in paraffin. Tissue sections of 5  $\mu\text{m}$  in thickness were prepared, and stained with hematoxylin and eosin (H & E).

Statistical analysis: Incidences of non-neoplastic lesions and urinary data were analyzed by Chi-square test. Incidences of neoplastic lesions were statistically analyzed by Fisher's exact test. A positive trend of dose-response relationship for the neoplastic incidences was analyzed by Peto test<sup>21)</sup>. Body weight, food consumption, and hematological and blood biochemical parameters were analyzed by Dunnett's test. The Kaplan-Meier method<sup>22)</sup> and the log-rank test<sup>23)</sup> were used to test statistical significance of survival rates between any DCNB-fed rat or mice of either sex and the respective control.

## Results

### Rat study

Survival, body weight, food consumption and clinical signs: The Kaplan-Meier survival analysis showed no significant difference in the survival rate between any DCNB-fed group of either sex and the respective control (data not shown).

Growth rates of the DCNB-fed groups of both sexes were suppressed in a dose-related manner (Fig. 1A). Terminal body weights of the 2,000 ppm-fed males and females were decreased by 15% and 20%, compared with the male and female controls, respectively (Table 1A). No difference in food consumption between any DCNB-fed group of either sex and the respective control was found (data not shown). Yellow-colored urine was observed in all the DCNB-fed groups of both sexes throughout the 2-yr administration period.

Organ weights and macroscopic findings: Relative liver weight was increased in all DCNB-fed groups of both sexes (Table 1A). Relative kidney weight was increased in all DCNB-fed males and in females fed 800 and 2,000 ppm. Relative testis weight was apparently increased in the 2,000 ppm-fed rats, although no significant increase in absolute testis weight was found.

In gross findings, incidence of slightly tanned and granular surface in the kidney, indicative of chronic progressive nephropathy (CPN)<sup>24)</sup>, was increased dose-dependently in the DCNB-fed males (control: 7/50, 320 ppm: 10/50, 800 ppm: 27/50, 2,000 ppm: 32/50). Incidence of liver nodules was increased in the 2,000 ppm-fed males (control: 0/50, 320 ppm: 2/50, 800 ppm: 2/50, 2,000 ppm: 8/50).

Hematology, blood chemistry and urinalysis: Significant decreases in hematocrit (Ht) at 2,000 ppm and in hemoglobin concentration (Hb) at 800 and 2,000 ppm were noted in the DCNB-fed females, whereas no hematological parameter were changed in any DCNB-fed males.

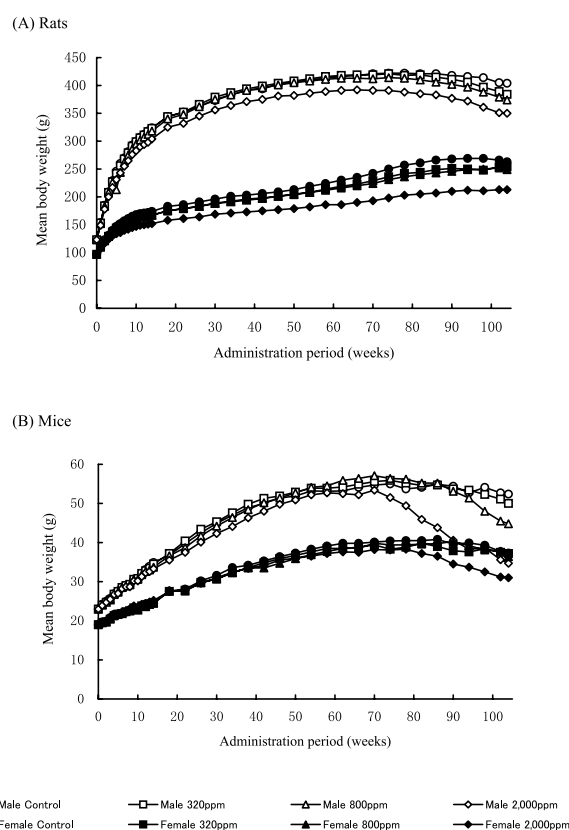


Fig. 1. Growth rate curves of rats (A) and mice (B) fed control diet and DCNB-containing diets at 3 different concentrations for 2 yr.

Methemoglobin level was not increased in any DCNB-fed group of either sex (Table 2A).

Neither AST nor ALT was increased in any DCNB-fed group of either sex (Table 3A).  $\gamma$ -GTP was increased in all DCNB-fed groups of both sexes. Total cholesterol and phospholipids were increased in both 800 and 2,000 ppm-fed males, while those two parameters were increased in all DCNB-fed females. Triglyceride was increased only in males fed 800 and 2,000 ppm. BUN was increased in the 800 and 2,000 ppm-fed males and in all DCNB-fed females. Total protein, albumin and glucose were increased only in the DCNB-fed females.

Positive urinary protein was observed in the 2,000 ppm-fed males. Lowered pH was observed in the 800 and 2,000 ppm-fed males (data not shown). However, the DCNB-fed females did not exhibit any significant changes in those urinary parameters.

Neoplastic and pre-neoplastic lesions: Incidences of hepatocellular adenomas and combined incidences of hepatocellular adenomas and carcinomas were increased in the 2,000 ppm-fed males, along with a significant positive trend of the dose-tumor incidence relationship as indicated by Peto test (Table 4A). Incidence (4%) of hepatocellular carcinomas in the 2,000 ppm-fed males

**Table 1. Absolute and relative organ weights of rats and mice of both sexes necropsied at termination of oral administration of DCNB in feed for 2 yr**

(A) Rats						
Group Name	Control	320 ppm		800 ppm		2,000 ppm
Male						
Number of animals	40	44		41		39
Body weight (g)	384 ± 28	360 ± 48	*	353 ± 22	**	328 ± 25
Liver (g)	10.394 ± 1.540	11.508 ± 2.020	**	11.946 ± 1.759	**	12.361 ± 1.199
Liver (%)	2.716 ± 0.449	3.268 ± 0.848	**	3.397 ± 0.588	**	3.778 ± 0.405
Kidneys (g)	2.634 ± 0.221	2.802 ± 0.399		2.757 ± 0.299		2.853 ± 0.300
Kidneys (%)	0.690 ± 0.086	0.799 ± 0.202	**	0.785 ± 0.120	**	0.873 ± 0.106
Testes (g)	2.769 ± 1.171	2.957 ± 1.333		2.659 ± 1.118		3.137 ± 1.158
Testes (%)	0.720 ± 0.301	0.822 ± 0.360		0.755 ± 0.319		0.954 ± 0.349
Female						
Number of animals	38	35		39		34
Body weight (g)	248 ± 36	238 ± 23		234 ± 32		199 ± 26
Liver (g)	6.317 ± 0.921	6.790 ± 0.930		7.267 ± 0.924	**	7.086 ± 0.923
Liver (%)	2.583 ± 0.413	2.864 ± 0.415	**	3.152 ± 0.502	**	3.572 ± 0.217
Kidneys (g)	1.713 ± 0.138	1.738 ± 0.133		1.766 ± 0.123		1.670 ± 0.113
Kidneys (%)	0.703 ± 0.086	0.733 ± 0.053		0.769 ± 0.118	**	0.849 ± 0.085
(B) Mice						
Group Name	Control	320 ppm		800 ppm		2,000 ppm
Male						
Number of animals	27	35		26		18
Body weight (g)	48.8 ± 6.1	46.8 ± 8.4		41.4 ± 8.4	**	32.0 ± 3.0
Liver (g)	2.168 ± 1.533	2.420 ± 1.014		3.467 ± 1.436	**	5.722 ± 1.957
Liver (%)	4.713 ± 4.288	5.465 ± 3.197		8.976 ± 4.789	**	17.918 ± 5.911
Kidneys (g)	0.612 ± 0.049	0.667 ± 0.230		0.649 ± 0.076		0.675 ± 0.150
Kidneys (%)	1.274 ± 0.191	1.498 ± 0.837		1.602 ± 0.230	**	2.152 ± 0.667
Testes (g)	0.225 ± 0.039	0.215 ± 0.046		0.215 ± 0.038		0.205 ± 0.028
Testes (%)	0.470 ± 0.102	0.469 ± 0.116		0.533 ± 0.109		0.644 ± 0.075
Female						
Number of animals	30	27		28		23
Body weight (g)	34.5 ± 7.2	34.7 ± 5.6		33.8 ± 5.1		28.6 ± 2.9
Liver (g)	1.625 ± 0.820	1.511 ± 0.356		2.028 ± 0.518	**	4.251 ± 1.538
Liver (%)	4.801 ± 2.414	4.437 ± 1.130		6.152 ± 1.882	**	15.195 ± 6.151
Kidneys (g)	0.479 ± 0.169	0.484 ± 0.167		0.459 ± 0.057		0.488 ± 0.103
Kidneys (%)	1.433 ± 0.522	1.430 ± 0.533		1.377 ± 0.179		1.712 ± 0.352

MEAN ± S.D. \* and \*\*: significantly different at  $p \leq 0.05$  and  $p \leq 0.01$  by Dunnett's test, respectively. g: absolute organ weight, %: relative organ weight.

exceeded the upper range of the JBRC historical control data (3 cases [0.2%] in 1,249 male rats in 25 studies, with the maximum incidence of 2%), although no statistical significance was indicated. The hepatocellular tumors were characterized by a lack of the normal lobular architecture, which compressed adjacent hepatic parenchyma. Of those rat hepatocellular tumors, 3 male cases (2 males in the 2,000 ppm group and 1 male in the 800 ppm group) were diagnosed as hepatocellular carcinoma (Fig. 2), because those tumors were composed of cells having

irregular-shaped nuclei with a pseudo-glandular arrangement indicative of pronounced structural atypia. None of those malignant liver tumors metastasized to any other organs. Incidence of basophilic cell foci was increased dose-dependently in the 800 and 2,000 ppm-fed males (Table 4A). However, incidences of basophilic cell foci in the DCNB-fed females and acidophilic cell foci in both DCNB-fed males and females were not dose-related.

Combined incidences of renal cell adenomas and carcinomas were increased in a dose-related manner, as indi-

**Table 2. Hematological analysis of rats and mice of both sexes necropsied at termination of oral administration of DCNB in feed for 2 yr**

(A) Rats					
Group Name	Control	320 ppm	800 ppm	2,000 ppm	
Male					
Number of animals	40	41	41	39	
RBC ( $10^6/\mu\text{L}$ )	8.21 ± 1.25	8.20 ± 1.73	8.62 ± 1.52	8.86 ± 1.69	
Hb (g/dL)	13.6 ± 2.4	13.4 ± 2.7	14.1 ± 2.3	14.1 ± 2.7	
Hematocrit (%)	41.4 ± 6.2	40.9 ± 7.2	42.8 ± 6.0	42.7 ± 7.7	
Methemoglobin level (%)	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.4	0.4 ± 0.3	
WBC ( $10^3/\mu\text{L}$ )	7.37 ± 5.53	10.56 ± 14.39	8.32 ± 10.62	7.08 ± 2.25	
Female					
Number of animals	38	34	38	34	
RBC ( $10^6/\mu\text{L}$ )	8.09 ± 1.30	8.21 ± 1.10	8.03 ± 0.99	7.99 ± 1.51	
Hb (g/dL)	14.6 ± 2.2	14.5 ± 1.6	14.3 ± 1.6	13.6 ± 2.7	**
Hematocrit (%)	43.2 ± 5.8	43.0 ± 3.9	42.3 ± 4.5	40.9 ± 7.1	**
Methemoglobin level (%)	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	
WBC ( $10^3/\mu\text{L}$ )	7.22 ± 28.68	2.82 ± 2.59	3.65 ± 8.71	2.42 ± 1.85	
(B) Mice					
Group Name	Control	320 ppm	800 ppm	2,000 ppm	
Male					
Number of animals	23	32	24	16	
RBC ( $10^6/\mu\text{L}$ )	9.63 ± 1.48	9.30 ± 1.67	9.48 ± 1.60	9.09 ± 1.64	
Hb (g/dL)	13.2 ± 1.8	13.0 ± 2.2	13.2 ± 2.1	12.6 ± 2.1	
Hematocrit (%)	43.1 ± 5.0	42.5 ± 6.8	43.6 ± 6.7	42.2 ± 6.1	
WBC ( $10^3/\mu\text{L}$ )	3.20 ± 1.78	2.87 ± 1.21	3.40 ± 1.52	2.95 ± 1.38	
Female					
Number of animals	23	26	27	22	
RBC ( $10^6/\mu\text{L}$ )	9.12 ± 1.77	9.39 ± 1.86	9.39 ± 0.97	10.44 ± 1.03	**
Hb (g/dL)	13.1 ± 2.0	13.8 ± 2.3	13.6 ± 1.5	14.8 ± 1.3	**
Hematocrit (%)	42.8 ± 5.6	45.2 ± 6.6	44.1 ± 4.2	48.8 ± 4.3	**
WBC ( $10^3/\mu\text{L}$ )	3.67 ± 3.61	3.05 ± 3.24	4.40 ± 6.24	3.56 ± 3.36	

MEAN ± S.D. \* and \*\*: significantly different at  $p \leq 0.05$  and  $p \leq 0.01$  by Dunnett's test, respectively.

RBC: Red blood cell counts, WBC: White blood cell counts, Hb: Hemoglobin concentration.

cated by a significant positive trend of the dose-tumor incidence relationship by Peto test (Table 4A). Incidence (4%) of renal cell adenomas in the 2,000 ppm-fed males exceeded the upper range of the JBRC historical control data, whereas that (2%) of renal cell carcinomas did not (2 renal cell adenomas [0.16%] and 2 renal cell carcinomas [0.16%] in 1,249 males in 25 studies, with the maximum incidence of 2%). Renal tumors of less than 5 mm in size without either evidence of anaplastic feature, prominent cellular pleomorphism or metastasis to other organs were diagnosed as renal cell adenoma. One renal cell carcinoma in the DCNB-fed 2,000 ppm males metastasized to the lung. The incidence of atypical tubule hyperplasias, which are pre-neoplastic, and proliferative lesion in the proximal tubule epithelium<sup>25</sup>, was not increased in any DCNB-fed group (Table 4A).

Incidences of Zymbal gland adenomas in the DCNB-fed males showed a significant positive trend in the dose-tumor incidence relationship by Peto test. The incidence (8%) of this tumor in the 2,000 ppm-fed males exceeded the upper range of the JBRC historical control data (3 adenomas [0.2%] in 1,249 males in 25 studies, with the maximum incidence of 2%), although no statistically significance by Fisher's exact test was indicated. High incidences of interstitial cell tumors were observed in both DCNB-fed and control rats, but those tumor incidences were not increased in a dose-related manner.

Non-neoplastic lesions: Chronic progressive nephropathy (CPN), which is known as a spontaneous disease of aging rats<sup>24</sup>, occurred more frequently in males than in females (Table 5A). Incidences of CPN with marked and severe grades were increased in a dose-related manner in

**Table 3. Blood biochemical analysis of rats and mice of both sexes necropsied at termination of oral administration of DCNB in feed for 2 yr**

(A) Rats					
Group Name	Control	320 ppm	800 ppm	2,000 ppm	
<b>Male</b>					
Number of animals	40	41	41	39	
Total protein (g/dL)	6.5±0.3	6.6±0.4	6.6±0.3	6.5±0.3	
Albumin (g/dL)	3.3±0.2	3.3±0.3	3.3±0.2	3.3±0.2	
A/G ratio	1.1±0.1	1.0±0.1	1.0±0.1	1.0±0.1	
Glucose (mg/dL)	149±17	143±21	148±13	149±23	
Total Cholesterol (mg/dL)	175±50	187±45	219±45	217±51	**
Triglyceride (mg/dL)	80±61	103±79	125±70	146±79	*
Phospholipid (mg/dL)	245±76	267±61	299±55	312±63	**
AST (IU/L)	85±43	243±774	92±50	113±123	
ALT (IU/L)	38±17	70±154	40±15	49±49	
γ-GTP (IU/L)	12±8	25±33	31±24	38±21	**
BUN (mg/dL)	17.2±3.4	20.7±10.9	23.8±5.1	30.7±9.6	**
<b>Female</b>					
Number of animals	38	34	38	34	
Total protein (g/dL)	6.6±0.5	6.7±0.3	6.9±0.4	7.0±0.4	**
Albumin (g/dL)	3.8±0.3	3.9±0.2	4.0±0.3	4.1±0.2	*
A/G ratio	1.4±0.1	1.4±0.1	1.4±0.1	1.4±0.1	
Glucose (mg/dL)	145±18	153±15	153±15	158±11	**
Total Cholesterol (mg/dL)	126±21	149±25	165±25	175±24	**
Triglyceride (mg/dL)	44±31	53±47	55±53	60±113	
Phospholipid (mg/dL)	222±45	251±43	275±40	292±33	**
AST (IU/L)	141±194	116±86	114±61	108±44	
ALT (IU/L)	59±66	54±29	54±28	55±24	
γ-GTP (IU/L)	5±5	7±4	8±3	10±5	**
BUN(mg/dL)	16.3±3.6	17.4±2.0	17.8±2.1	19.4±2.2	**
<b>(B) Mice</b>					
Group Name	Control	320 ppm	800 ppm	2,000 ppm	
<b>Male</b>					
Number of animals	24	32	24	16	
Glucose (mg/dL)	196±34	200±48	183±58	152±26	**
Total Cholesterol (mg/dL)	117±34	153±50	202±78	219±51	**
Triglyceride (mg/dL)	44±22	52±22	41±21	25±11	**
Phospholipid (mg/dL)	213±54	270±84	363±139	380±95	**
AST (IU/L)	159±201	197±509	287±349	990±2,046	**
ALT (IU/L)	112±149	225±490	353±387	1,241±2,112	**
LDH (IU/L)	1,194±3,133	940±2,008	2,214±4,539	9,267±19,122	**
ALP (IU/L)	117±27	253±356	766±900	891±598	**
γ-GTP (IU/L)	3±3	6±10	7±8	22±14	**
BUN (mg/dL)	20.1±2.7	23.9±15.4	22.9±5.6	21.7±3.3	
<b>Female</b>					
Number of animals	23	26	27	22	
Glucose (mg/dL)	161±52	145±59	162±30	140±32	*
Total Cholesterol (mg/dL)	85±72	88±23	96±24	194±75	**
Triglyceride (mg/dL)	32±22	42±33	37±17	27±12	
Phospholipid (mg/dL)	146±77	157±38	191±42	365±138	**
AST (IU/L)	96±55	1,244±5,547	149±83	347±242	**
ALT (IU/L)	40±21	595±2,558	116±49	528±439	**
LDH (IU/L)	366±184	4,245±18,679	515±419	1,796±1,592	**
ALP (IU/L)	152±63	218±171	305±203	1,164±979	**
γ-GTP (IU/L)	3±2	4±5	4±4	27±15	**
BUN (mg/dL)	18.2±9.5	18.1±7.2	16.0±2.4	20.8±4.2	**

MEAN ± S.D. \* and \*\*: significantly different at p ≤ 0.05 and p ≤ 0.01 by Dunnett's test, respectively.

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, γ-GTP: γ-Glutamyl transpeptidase, LDH: Lactate dehydrogenase, ALP: Alkaline phosphatase, BUN: Blood urea nitrogen.

**Table 4. Incidences of neoplastic and pre-neoplastic lesions in rats and mice orally administered DCNB in feed for 2 yr**

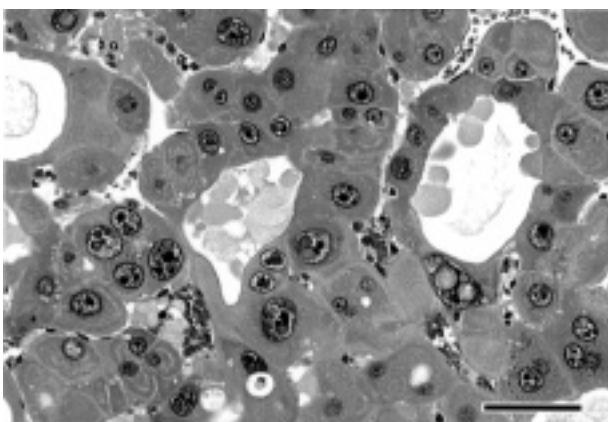
Group Name	Male					Female									
	Control	320 ppm	800 ppm	2,000 ppm	Peto test	Control	320 ppm	800 ppm	2,000 ppm	Peto test					
Number of animals	50	50	50	50		50	50	50	50						
<b>(A) Rats</b>															
Liver															
Hepatocellular adenoma <sup>1)</sup>	0	1	0	6	*	↑ ↑	0	0	0	0					
Hepatocellular carcinoma <sup>2)</sup>	0	0	1	2			0	0	0	0					
1)+2)	0	1	1	8	*	↑ ↑	0	0	0	0					
Basophilic cell foci	21	22	32	40	##		26	20	18	25					
Acidophilic cell foci	14	11	6	18			2	1	1	8					
Kidney															
Renal cell adenoma <sup>3)</sup>	0	0	0	2			0	0	0	0					
Renal cell carcinoma <sup>4)</sup>	0	1	0	1			0	0	0	0					
3)+4)	0	1	0	3		↑	0	0	0	0					
Atypical tubule hyperplasia	0	0	2	2			1	0	0	0					
Zymbal gland															
Adenoma	0	0	0	4		↑ ↑	0	0	0	0					
<b>(B) Mice</b>															
Group Name	Male					Female									
	Control	320 ppm	800 ppm	2,000 ppm	Peto test	Control	320 ppm	800 ppm	2,000 ppm	Peto test					
Number of animals	49 <sup>a)</sup>	50	50	50		50	50	50	50						
Liver															
Hepatocellular adenoma <sup>1)</sup>	17	21	20	16		5	5	17	**	16	**	↑ ↑			
Hepatocellular carcinoma <sup>2)</sup>	15	15	23	31	**	↑ ↑	1	3	15	**	31	**	↑ ↑		
Hepatoblastoma <sup>3)</sup>	1	10	**	12	**	25	**	↑ ↑	0	0	2				
1)+2)+3)	26	34	**	41	**	45	**	↑ ↑	6	8	29	**	39	**	↑ ↑
Acidophilic cell foci	0	2	7	#	11	##		1	7	3	3				

\* and \*\*: Significantly different at  $p \leq 0.05$  and  $p \leq 0.01$  by Fisher's exact test, respectively.

↑ and ↑ ↑: Significantly different at  $p \leq 0.05$  and  $p \leq 0.01$  by Peto test, respectively.

# and ##: Significantly different at  $p \leq 0.05$  and  $p \leq 0.01$  by Chi-square test, respectively.

<sup>a)</sup>: One male control mouse died accidentally during the administration period.



**Fig. 2. Hepatocellular carcinoma in the liver of a male rat fed 2,000 ppm DCNB for 2 yr.**

Bar indicates 100  $\mu\text{m}$ . H & E stain.

the DCNB-fed males. Incidences of urothelial hyperplasia in the pelvis were increased in all DCNB-fed male groups, and mineralization in the papilla were increased in males fed 800 and 2,000 ppm. Increased hematopoiesis of the bone marrow, which was characterized by increased numbers of the hematopoietic cells, occurred in the 2,000 ppm-fed females.

#### Mouse study

Survival, body weight, food consumption and clinical signs: The Kaplan-Meier survival analysis showed no significant difference in the survival rate between any DCNB-fed group of either sex and the respective control (data not shown). However, the survival rates of the 2,000 ppm-fed groups of both sexes tended to be lowered after the 65th wk of the administration period. The num-

**Table 5. Incidences of non-neoplastic lesions in rats and mice orally administered DCNB in feed for 2 yr**

(A) Rats												
Group Name	Male								Female			
	Control	320 ppm	800 ppm	2,000 ppm	Control	320 ppm	800 ppm	2,000 ppm	Control	320 ppm	800 ppm	2,000 ppm
Number of animals	50	50	50	50	50	50	50	50	50	50	50	50
Kidney												
Chronic progressive nephropathy												
Total	46	49 **	50 **	49 **	24	23	32	28				
Slight	26	6	2	1	22	21	28	26				
Moderate	15	27	10	5	2	0	2	2				
Marked	4	14	34	32	0	2	1	0				
Severe	1	2	4	11	0	0	1	0				
Mineralization: papilla	0	2	47 **	48 **	9	9	9	17				
Urothelial hyperplasia: pelvis	1	8 *	36 **	39 **	10	5	15	6				
Bone marrow												
Hematopoiesis: increased	8	10	13	10	5	9	9	14 *				
(B) Mice												
Group Name	Male								Female			
	Control	320 ppm	800 ppm	2,000 ppm	Control	320 ppm	800 ppm	2,000 ppm	Control	320 ppm	800 ppm	2,000 ppm
Number of animals	49 <sup>a)</sup>	50	50	50	50	50	50	50	50	50	50	50
Liver												
Hepatocellular hypertrophy with nuclear atypia: centrilobular	0	38 **	39 **	40 **	0	15 **	29 **	35 **				
Kidney												
Deposit of hemosiderin	1	6	6	25 **	1	0	0	2				
Bone marrow												
Erythropoiesis: increased	7	7	14	23 **	2	2	0	4				

\* and \*\*: Significantly different at  $p \leq 0.05$  and  $p \leq 0.01$  by Chi-square test, respectively.

<sup>a)</sup>: One male control mouse died accidentally during the administration period.

ber of mice that died of liver tumors before the end of the 2-yr administration period was increased, i.e., 7, 8, 11 and 23 males and 0, 3, 4 and 6 females, for the control, 320, 800 and 2,000 ppm-fed groups, respectively. The decrease in the survival rate of the 2,000 ppm-fed groups was due to the increased number of tumor deaths before the end of the 2-yr administration period.

Growth rates of the DCNB-fed groups of both sexes were suppressed in a dose-related manner, and in particular, in the 2,000 ppm-fed male and female groups during the last 30 wk (Fig. 1B). Terminal body weights of the 2,000 ppm-fed males and females were decreased by 34% and 17%, compared with the respective controls, respectively (Table 1B). There was no difference in food consumption between any DCNB-fed group of either sex and the respective control (data not shown). Yellow-colored urine was observed in all the DCNB-fed groups of both sexes throughout the 2-yr administration period.

Organ weights and macroscopic findings: Relative and

absolute liver weights were increased in the 800 ppm- and 2,000 ppm-fed groups of both sexes, compared with the respective controls (Table 1B). Relative kidney weight was increased in the 800 and 2,000 ppm-fed males and in the 2,000 ppm-fed females. Relative testis weight was apparently increased in the 2,000 ppm mice, although no significant increase in absolute testis weight was found.

In gross findings, incidence of liver nodules was increased dose-dependently in the DCNB-fed groups of both sexes (for males, control: 24/49, 320 ppm: 30/50, 800 ppm: 42/50, 2,000 ppm: 46/50, and for females, control: 5/50, 320 ppm: 9/50, 800 ppm: 27/50, 2,000 ppm: 40/50).

Hematology, blood chemistry and urinalysis: Red blood cell counts (RBC), Hb and Ht were increased in the 2,000 ppm-fed females (Table 2B).

AST, ALT, LDH and ALP were increased in the 800 and 2,000 ppm-fed males, but those parameters were

increased in the females fed DCNB at different dose levels (Table 3B). Large values of means and standard deviations of AST, ALT and LDH were observed in the 320 ppm-fed female mouse group, because 1 animal exhibited extremely high values of those parameters.  $\gamma$ -GTP was increased in the 2,000 ppm-fed groups of both sexes. Total cholesterol was increased in all the DCNB-fed males and in females fed 800 and 2,000 ppm. Phospholipid was increased in males and females fed 800 and 2,000 ppm. BUN was increased only in the 2,000 ppm-fed females. Glucose was decreased in males and females fed 2,000 ppm.

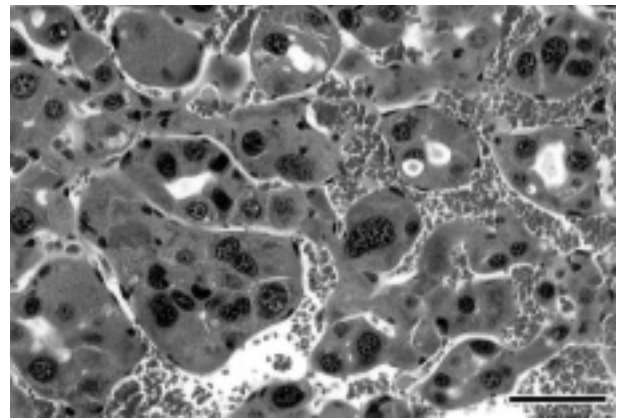
No consistent, dose-related change in the urinary parameters was observed in any DCNB-fed group of either sex (data not shown).

Neoplastic and pre-neoplastic lesions: Increased incidences of hepatocellular adenomas in the 800 and 2,000 ppm-fed females and hepatocellular carcinomas in the 2,000 ppm-fed males and in the 800 and 2,000 ppm-fed females were noted, and those tumor incidences were increased dose-dependently as indicated by Peto test (Table 4B). Incidences of hepatoblastomas were increased in all the DCNB-fed male groups. Although the incidence (4%) of hepatoblastomas in the 2,000 ppm-fed females was not significantly increased, the occurrence exceeded the upper range of the JBRC historical control data (no hepatoblastoma out of a total of 1,048 female mice in 21 studies). The tumor masses were seen in the liver multifocally in DCNB-fed mice, in contrast to the single occurrence in the respective controls. The hepatocellular carcinoma (Fig. 3) was characterized by marked cellular pleomorphism, including the advent of large and irregular-shaped tumor cells, and structure atypia including pseudo-glandular and papillary-like structures associated with sinusoidal dilatation. The hepatoblastoma (Fig. 4) was mostly found within or adjacent to hepatocellular carcinomas or adenomas, composing of smaller, more markedly basophilic, denser in cellularity and more elongated-shaped cells than normal hepatocytes.

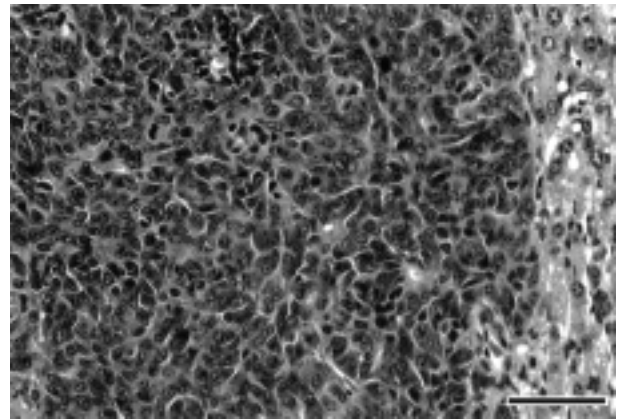
Eighteen cases out of 69 hepatocellular carcinomas and 17 cases out of 47 hepatoblastomas in the DCNB-fed males as well as 4 cases out of the 49 hepatocellular carcinomas in the DCNB-fed females metastasized to the lung.

Incidence of acidophilic cell foci was increased dose-dependently in the 800 and 2,000 ppm-fed males (Table 4B).

Non-neoplastic lesions: Incidences of centrilobular hypertrophy of hepatocytes with nuclear atypia were increased in all DCNB-fed groups of both sexes (Table 5B). Increased incidence of hemosiderin deposit in the kidney occurred in the 2,000 ppm-fed males. Increased erythropoiesis of the bone marrow, which was characterized by



**Fig. 3. Hepatocellular carcinoma in the liver of a male mouse fed 2,000 ppm DCNB for 2 yr.**  
Bar indicates 100  $\mu$ m. H & E stain.



**Fig. 4. Hepatoblastoma in the liver of a male mouse fed 2,000 ppm DCNB for 2 yr.**  
Bar indicates 100  $\mu$ m. H & E stain.

increased number of erythroblast, occurred in the 2,000 ppm-fed males.

## Discussion

### *Carcinogenicity*

This study shows that 2-yr oral administration of DCNB in feed to F344 rats and BDF<sub>1</sub> mice of both sexes produced three kinds of tumors with different weight of evidence of carcinogenic activity. Evidence of the carcinogenic activity of DCNB was weighted, depending on the malignancy of the tumor (carcinoma, hepatoblastoma or adenoma in the present study), a dose-related increase in the tumor incidences by Peto test, an increase in the incidence of malignant or benign tumor by Fisher's exact test or over the JBRC historical control data and metastasis to other organs. In addition, induction of the tumor in the same organ of the two different species (rats and mice) and different sexes (males and females) as well as a cancer-related or pre-neoplastic lesion and mutagenic-

ty of DCNB and its metabolites were also considered.

**Liver tumors:** Induction of hepatocarcinogenicity by DCNB occurred in male rats, male mice and female mice. The hepatocarcinogenicity of DCNB for male rats was evidenced by a dose-related increases in the incidence of benign hepatocellular adenomas, in the combined incidence of hepatocellular adenomas and malignant hepatocellular carcinomas and in incidences of basophilic cell foci, a pre-neoplastic lesion<sup>26</sup>). Notably, the incidence of hepatocellular carcinomas in the 2,000 ppm-fed male rats was increased over the upper range of the JBRC historical control data. These liver tumors were induced only in the male rats but did not metastasize to any other organ. The hepatocarcinogenicity of DCNB for male and female mice was clearly evidenced by dose-related increases in the incidences of malignant hepatocellular carcinomas in male and female mice, in the incidence of malignant hepatoblastomas in male mice, an increase in hepatoblastoma incidence in the 2,000 ppm-fed female mice over the JBRC historical control data, in the incidences of benign hepatocellular adenomas in female mice and incidences of acidophilic cell foci, a pre-neoplastic and proliferative lesion<sup>26</sup>) in male mice. The malignant liver tumors of the DCNB-fed male and female mice metastasized to the lung. The DCNB-induced mouse hepatocarcinogenicity was characterized by marked induction of rarely occurring hepatoblastomas, which had a morphological structure completely different from that of the hepatocellular carcinoma<sup>26</sup>). Rare occurrence of spontaneous hepatoblastomas was indicated by 5 cases out of 1,047 male mice and null case out of 1,047 female mice in the JBRC historical control data. Mouse hepatoblastomas occurred in aged animals, unlike human hepatoblastoma, which primarily occurred in children under 3 yr of age<sup>26, 27</sup>). Treatment of strains of mice with chemicals such as *N*-nitrosodiethylamine<sup>28, 29</sup>) and *N,N*-dimethylformamide<sup>30</sup>) has been reported to increase the incidence of rare hepatoblastomas. The present results can be taken to indicate that while DCNB produced clear evidence of hepatocarcinogenicity for two different species of rats and mice and different sexes of mice, the carcinogenic activity of DCNB was more potent in mice than in rats. In the previous 13-wk study<sup>15</sup>), mouse hepatotoxicity was characterized by hepatocellular death as evidenced by the liver necrosis and the increased serum levels of AST and ALT, and the centrilobular hypertrophy of hepatocytes associated with markedly enlarged cytoplasm, and varying nuclear size and shape, whereas those clear histopathological changes did not occur in rat liver. A genotoxic mode of action is thought to operate in the DCNB-induced hepatocarcinogenicity, as suggested by the results of the following *in vitro* studies. Bacterial mutagenicity of DCNB was positive with *Salmonella typhimurium* TA100 with

and without S9 activation<sup>7, 9, 10</sup>), and with TA98 and TA1538 without S9 activation<sup>8</sup>), and TA1535 without S9 activation<sup>10</sup>). Mammalian clastogenicity was positive with cultured Chinese hamster lung cells in a chromosomal aberration test<sup>11, 12</sup>). Furthermore, it can be inferred that more potent hepatocarcinogenicity found in mice than in rats is due to more likelihood of fixing DCNB-induced cancer-initiating DNA damage into heritable mutations in mice than in rats.

**Kidney tumors:** Induction of renal cell adenomas and carcinomas by DCNB was shown by a dose-related increase in combined incidences of renal cell adenomas and carcinomas and a marginal increase in incidence of benign renal cell adenomas in the 2,000 ppm-fed male rats over the upper range of the JBRC historical control data. Notably, one malignant renal cell carcinoma of a 2,000 ppm-fed male rat metastasized to the lung, but incidence of the malignant renal cell carcinomas in the DCNB-fed male rats did not increase either statistically or over the JBRC historical control data. In addition, the incidence of atypical tubule hyperplasia, which is known as a preneoplastic and proliferative lesion<sup>25</sup>), was not increased in any DCNB-fed groups of male rats. Therefore, the nephrocarcinogenic activity of DCNB in male rats was considered to be marginally increased in a compound-related manner from the above lines of carcinogenic evidence as well as the standpoint of the male rat-specific  $\alpha_{2v}$ -globulin-induced nephropathy in our previous study<sup>15</sup>) and the positive mutagenicity of DCNB and its metabolites reported in literature. The previous 13-wk oral administration of DCNB in feed to male F344 rats increased the incidences of hyaline droplets and granular casts at the renal proximal tubules, both of which were positively stained with  $\alpha_{2v}$ -globulin antibody. In addition, the increased incidence of cytoplasmic basophilia, suggestive of regenerative cell proliferation in the proximal tubular epithelium, was noted in male rats<sup>15</sup>). Those histopathological and immunohistochemical changes in the kidney of male F344 rats were suggestive of  $\alpha_{2v}$ -globulin-induced nephropathy, which caused cell death and cell proliferation due to excessive accumulation of  $\alpha_{2v}$ -globulin in the proximal tubular epithelial cells.  $\alpha_{2v}$ -Globulin-induced nephropathy was reported to occur in male F344 rats exposed to a variety of chemicals including hydrocarbons<sup>31</sup>), unleaded gasoline<sup>32</sup>), 1,4-dichlorobenzene<sup>33</sup>) and *d*-limonene<sup>34</sup>), and was closely associated with the development of renal tumors after the long-term exposure to the chemicals. Swenberg *et al.*<sup>35-37</sup>) proposed a mode-of-action hypothesis for the chemically induced renal tumors by which excessive accumulation of the ligand-bound  $\alpha_{2v}$ -globulin in the tubular epithelial cells caused the cell death and subsequent cell proliferation, leading to development of renal

tumors in male rats. However, it was found in the previous<sup>15)</sup> and present studies that the DCNB-induced  $\alpha_{2v}$ -globulin-induced nephropathy was closely associated with development of the age-related, spontaneous CPN to severer stage, whereas the clear evidence of nephrocarcinogenicity was not obtained in male rats fed DCNB for 2 yr. For risk assessment, it is generally recognized that renal tumors in male rats that are produced by exposure to substances that produce  $\alpha_{2v}$ -globulin nephropathy need not be considered in assessing potential neoplastic health risks to humans, when attributed exclusively to  $\alpha_{2v}$ -globulin accumulation and non-mutagenic substances<sup>38)</sup>. However, the bacterial mutagenicity and mammalian clastogenicity of DCNB was positive with and without S9 activation<sup>7-10)</sup>, as discussed above. Besides, reactive metabolites of glutathione and cysteine conjugates of trichloroethylene, *S*-(1,2-dichlorovinyl)glutathione and *S*-(1,2-dichlorovinyl)-*L*-cysteine, which were biotransformed from the corresponding hepatic glutathione *S*-conjugate by  $\gamma$ -glutamyltranspeptidase and cysteine conjugate  $\beta$ -lyase in the kidney, respectively, were mutagenic with *Salmonella typhimurium* TA2638 in the Ames test<sup>39)</sup>. Elfarrar *et al.*<sup>40)</sup> demonstrated that intraperitoneal administration of *S*-(1,2-dichlorovinyl)glutathione and *S*-(1,2-dichlorovinyl)-*L*-cysteine to male F344 rats induced nephropathy, indicating a crucial role of the renal  $\gamma$ -glutamyltranspeptidase and cysteine conjugate  $\beta$ -lyase in the trichloroethylene-induced nephropathy. Dekant *et al.*<sup>41, 42)</sup> reported that *N*-acetyl-*S*-(1,2-dichlorovinyl)-*L*-cysteine was identified as a urinary metabolite of trichloroethylene, and that thioacylating metabolites of *S*-(1,2-dichlorovinyl)-*L*-cysteine, which were formed by renal cysteine conjugate  $\beta$ -lyase, might contribute to the nephrotoxic and mutagenic effects. Our previous study<sup>43)</sup> demonstrated that DCNB was metabolized and excreted into urine as *N*-acetyl-*S*-(4-chloro-3-nitrophenyl)-*L*-cysteine, suggesting that DCNB was metabolized to the glutathione conjugate in the liver, subsequently to *S*-(4-chloro-3-nitrophenyl)-*L*-cysteine in the kidney. Therefore, it can be rational to infer that reactive and possibly mutagenic metabolites, which are presumably produced from *S*-(4-chloro-3-nitrophenyl)-*L*-cysteine by cysteine conjugate  $\beta$ -lyase in the kidneys contribute to the development of DCNB-induced nephrocarcinogenicity and chronic nephrotoxicity, although the  $\alpha_{2v}$ -globulin-induced nephropathy can not be totally ruled out as a causative factor of nephrocarcinogenicity and nephrotoxicity.

**Zymbal gland tumor:** The induction of the Zymbal gland tumor by DCNB was shown only by a dose-related increase in the incidences of benign Zymbal gland adenomas in the 2,000 ppm-fed male rats and an increase in the tumor incidence over the upper range of the JBRC historical control data. Therefore, the induction of

Zymbal gland tumor by DCNB in male rats was considered to be compound-related, although the increase in the tumor incidence was marginal. NTP reported that 21 chemicals out of a total of 518 substances tested so far for carcinogenicity produced clear evidence of carcinogenicity as shown by increased incidence of Zymbal gland tumors in male rats<sup>44)</sup>. Human relevance and species-specificity of the DCNB-induced Zymbal gland tumors in male rats remain unsolved.

#### *Chronic toxicity*

Chronic toxicity signs of DCNB can be seen in the liver, kidney and blood. The hepatotoxicity was characterized by centrilobular hypertrophy of hepatocytes associated with nuclear atypia in mice, and increased relative liver weight in rats. It is noteworthy that the centrilobular hypertrophy of hepatocytes did not occur in any rat of either sex fed DCNB for 2 yr, although hepatocellular hypertrophy occurred in male and female rats fed DCNB for 13 wk<sup>15)</sup>.

The DCNB-induced renal toxicity was characterized by increased severity and the progression of spontaneous, age-related CPN as evidenced by a dose-dependent increase in incidences of CPN with marked and severe grades of severity in the DCNB-fed male rats. In addition, papillary mineralization, urothelial hyperplasia in the renal pelvis as well as the increased serum levels of BUN were noted in the male rats.

The DCNB-induced hematological toxicity were characterized by the significantly decreased Hb and Ht accompanied by the histopathological change in the increased bone marrow hematopoiesis only in the 2,000 ppm-fed female rats, although the increases in RBC, Hb and Ht and the increased bone marrow erythropoiesis were noted in the 2,000 ppm-fed female and male mice, respectively. It was noteworthy that the methemoglobin level was not increased in any of the rats or mice fed DCNB for 2 yr, whereas the methemoglobin-induced, hemolytic anemia was observed in male rats and mice fed 2,222 ppm DCNB for 13 wk<sup>15)</sup>.

The apparent increase in relative testis weight without any increase in absolute testis of rats and mice fed 2,000 ppm DCNB found in the present study may be attributable not to the DCNB-induced testicular toxicity but to the decreased body weight of the 2,000 ppm-fed animals. Therefore, a 2-yr oral administration of 2,000 ppm DCNB was considered not to induce any sign of testicular toxicity in rats and mice, although testicular toxicity evidenced by the decreased testis weight and the increased incidences of germ cell necrosis was observed in rats fed 2,222 ppm DCNB for 13 wk<sup>15)</sup>.

For mice, hepatocellular hypertrophy and the increased incidence of hepatoblastomas appeared at the same dose

level of 320 ppm. It can not be ruled out that the increased serum levels of AST and ALT and the increased relative liver weight in the DCNB-fed mice might result from the development of liver tumors. The significantly decreased glucose in the 2,000 ppm-fed male and female mice might be related to liver tumor or hepatotoxicity. For rats, however, these hepatotoxic and nephrotoxic signs appeared even at the level of 320 ppm which is much lower than that, 2,000 ppm, causing the significant increases in incidence of liver and kidney tumors. It is suggested that these chronic toxicity signs did not result secondarily from the neoplastic lesions. Since the lowest dose level at which these DCNB-induced hepatotoxic and nephrotoxic signs appeared corresponded to estimated daily intake of 14 DCNB mg/kg body weight per day for male rats, a lowest-observed-effect-level (LOEL) was 14 mg/kg per day for the hepatic and renal endpoints of rats. It is interesting to note that the LOEL value was comparable with the lower confidence limit of the benchmark dose yielding a response with 10% extra risk (BMDL<sub>10</sub>) values of 12.9 mg and 12.0 mg DCNB/kg body weight per day for the hepatic endpoint from the previous 2-wk<sup>14)</sup> and 13-wk<sup>15)</sup> oral administration studies of DCNB in feed, respectively.

### Conclusion

DCNB produced clear evidence of hepatocarcinogenicity in male rats and male and female mice, and equivocal evidence of renal cell tumors and Zymbal gland tumor in male rats. Chronic toxicity of DCNB was characterized by centrilobular hypertrophy of hepatocytes in mice of both sexes, increased relative liver weight in rats of both sexes, CPN with advanced severity in rats and decreased Hb and Ht accompanied by increased bone marrow hematopoiesis in female rats.

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