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Thirteen-Week Inhalation Toxicity of 2- and 4-Chloronitrobenzene in F344/N Rats and B6C3F1 Mice

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Toxicity studies were performed by exposing F344/N rats and B6C3F1 mice to 2- and 4-chloronitrobenzene (CNB) by whole-body inhalation 6 hr/day, 5 days/week, for 13 weeks. Animals were evaluated for clinical chemistry (rats), hematology (rats), histopathology, and body/organ weights. Exposure concentrations were 0, 1.1, 2.3, 4.5, 9, and 18 ppm for 2-CNB and 0, 1.5, 3, 6, 12, and 24 ppm for 4-CNB. All rats in the 2-CNB study survived until the end of the study. Two male mice in the 18-ppm group in the 2-CNB study, however, died during Week 12; no deaths attributable to 4-CNB exposure occurred in rats or mice. In both studies, the mean body weight gains of exposed animals were similar to those of the respective controls. In rats, inhalation exposure to 2- or 4-CNB resulted in methemoglobinemia leading to a regenerative anemia and a variety of tissue changes secondary to the oxidative erythrocyte injury. In the 2-CNB study, methemoglobinemia resulted in a normocytic, normochromic, responsive anemia, whereas with 4-CNB, the methemoglobinemia was more severe and resulted in a macrocytic, hyperchromic, responsive anemia. Alterations of erythrocyte morphology were observed in both studies; changes included Heinz bodies, poikilocytes, and polychromasia. In rats, both isomers caused increases in serum activities of alanine aminotransferase and sorbitol dehydrogenase and increased bile acid concentrations. Microscopic liver changes included hemosiderin deposition in Kupffer cells (rats and mice exposed to 4-CNB), hepatocytomegaly (mice), and cytoplasmic basophilia (rats). Hepatocellular necrosis and chronic inflammation observed in mice were rather specific to the 2-CNB isomer, as only slight evidence of focal necrosis in the liver was observed in mice exposed to 4-CNB. Splenic lesions included hemosiderin accumulation, capsular fibrosis, and increased hematopoietic cell proliferation. Increased bone marrow hemosiderin and hematopoietic cell proliferation and kidney tubule hemosiderin deposition were also observed. Other findings, attributed to chemical exposure but not to the hematotoxicity, were described. Lesions included hyaline droplet nephropathy and degeneration of the testis in male rats exposed to 4-CNB, inflammation of the harderian gland in rats exposed to 4-CNB, hyperplasia of the nasal cavity epithelium in rats exposed to 2-CNB, and hyperplasia of the forestomach epithelium in mice exposed to 4-CNB; these lesions have not been described previously in studies with these chemicals. Based on the exposure concentrations evaluated, A no-observed-adverse-effect level (NOAEL) for histopathological injury in mice was 4.5 ppm for 2-chloronitrobenzene and 6 ppm for 4-chloronitrobenzene; a NOAEL was not determined for rats.

The nitroaromatic compounds 2- and 4-chloronitrobenzene (CNB) are used in the manufacture of dyes, lumber preservatives, pharmaceuticals, rubber and photographic chemicals, antioxidants, gasoline additives, and corrosion inhibitors (NIOSH/OSHA, 1978). They are produced in large amounts and, in 1990, an estimated 140 million pounds of mixed chloronitrobenzenes were manufactured in the United States (SRI, 1992). Residues of both compounds have been found in the environment (Shafer *et al.*, 1979; Rosen, 1981; Yurawecz and Puma, 1983), and evidence suggests that CNBs are relatively resistant to biodegradation (Anonymous, 1988; Trova *et al.*, 1991).

The general population may come in contact with chloronitrobenzenes through environmental contamination. A greater potential of inhalation or dermal exposure, however, exists for workers producing or handling the bulk chemicals. Methemoglobin formation is a primary effect associated with chloronitrobenzene exposure to humans and animals (Hasegawa and Sato, 1963; Davydova, 1967; Watanabe *et al.*, 1976; Nishida *et al.*, 1982; Ridley *et al.*, 1983; Nair *et al.*, 1986a,b), and toxicity related to workplace exposure has been reported (Renshaw and Ashcroft, 1926; Linch, 1974; Tabuchi *et al.*, 1985). In 1984, NIOSH estimated that 2215 workers in 23 plants were potentially exposed to 2-CNB, and 2948 workers in 30 plants to 4-CNB (NIOSH, 1984). Eight-hour, time-weighted average workplace exposure limits for 4-CNB in air were set at 0.1 ppm (0.64 mg/m^3) by the American Conference of Governmental Industrial Hygienists (ACGIH, 1991) and 1 mg/m^3 by the Occupational Safety and Health Administration (*Fed. Regist.* **39,** 23540, 1974); no exposure limit has been recommended for 2-CNB.

2- and 4-CNB were nominated to the National Toxicology Program for general and reproductive toxicity studies by the U.S. Environmental Protection Agency based upon the

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production volumes, evidence of significant worker exposure, and environmental contamination. Inhalation toxicological data are limited for these compounds for rats (Nair *et al.*, 1986a,b) and there are no data for mice. Thus, the present toxicity studies were conducted to characterize the effects of 2- and 4-CNB after a 13-week inhalation exposure in F344/N rats and B6C3F$_1$ mice. Data from these studies will also be used in the dose selection process for subsequent carcinogenicity studies. Endpoints evaluated included body and organ weights, histopathology, and clinical pathology (rats only). Inhalation was chosen as the route of exposure because 2- and 4-CNB are known to be sufficiently volatile to cause toxicity, and inhalation is a major route of worker exposure. Results of the reproductive studies have been detailed elsewhere (NTP, 1991, 1992a).

MATERIALS AND METHODS

Chemical. 2- and 4-CNB were obtained from the Aldrich Chemical Company (Milwaukee, WI). Chemical analyses (infrared, ultraviolet/visible, and nuclear magnetic resonance spectra) confirmed the identity of the two compounds. Karl Fischer analysis indicated that the chemicals contained less than 0.3% water; purity was approximately 99% for both isomers. Bulk 2-CNB was stored in amber glass containers under a nitrogen headspace at approximately 20°C. Bulk 4-CNB was stored in plastic bags inside metal cans at approximately 20°C. Stability of the bulk chemicals was monitored throughout the studies using gas chromatography; no degeneration was detected.

Animals. Male and female F344/N rats and B6C3F$_1$ mice were obtained from Simonsen Laboratories (Gilroy, CA). Rats and mice were quarantined for 11 to 13 days and were approximately 5- to 6-weeks old when the studies began. Blood samples were collected from rats and mice of each sex 3 weeks after receipt and at the end of the studies. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b); all results were negative.

Groups of 10 rats and 10 mice of each sex (base-study animals) were exposed to 0, 1.1, 2.3, 4.5, 9, or 18 ppm 2-CNB vapor or 0, 1.5, 3, 6, 12, or 24 ppm 4-CNB vapor through whole-body exposures performed 6 hr per day, 5 days per week, excluding weekends and holidays, for 13 weeks. Supplemental groups of 10 rats per sex and exposure level were designated for clinical pathology testing at interim time points. Animals were housed individually, exposed, and maintained in inhalation-exposure chambers produced by Hanford System Division of Lab Products, Incorporated (Aberdeen, MD). Tap water was available *ad libitum* as was pelleted NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA), except during the exposure periods. Animal rooms were maintained with 12 hr of fluorescent light per day.

Vapor generation. 2- and 4-CNB vapors were generated with a rotary evaporation system (Büchi Rotavapor Model EL-131S, Büchi Laboratoriums Technik AG, Flavil, Switzerland). Bulk 4-CNB was transferred into a flask and attached to a vapor generator; bulk 2-CNB was melted by immersion of the storage container in a warm-water bath prior to being transferred to a flask. The flask was immersed in a hot-oil bath and then rotated; a stream of heated nitrogen was metered into the flask. The resulting vapor was forced into a condenser with temperature maintained by a water bath in the 2-CNB studies and by circulating oil in the 4-CNB studies. Because of low volatility and melting points of these compounds, all Teflon (E I. DuPont deNemours and Co., Wilmington, DE) vapor transport lines and all air flows, except the individual chamber dilution air inlet flows, were heated and/or insulated to prevent chemical crystallization and deposition. Basic chamber functions (chemical concentration, airflow, vacuum, temperature, and relative humidity) were computer controlled by a HP 9816 (Hewlett–Packard, Palo Alto, CA), using on-line data collected by HP-85B computers and other data collected by an Intelligent Interface System (Model 53A-IBX; Colorado Data Systems, Englewood, CO). Concentrations of 2- or 4-CNB in the exposure and control chambers, the exposure room, an on-line standard, and nitrogen blank samples were monitored by a HP 5890 gas chromatographic system (Hewlett–Packard) equipped with an electron-capture detector. Samples, collected at the rate of three to four per hr during the 6-hr exposure, were taken from multiple positions via a 12-port stream select valve fed by sampling lines. Calibration of the on-line chamber monitor was based on quantitative analysis of samples taken from the exposure chambers; these samples were analyzed with an off-line gas chromatographic system calibrated with gravimetrically prepared standards for 2- or 4-CNB

Clinical pathology. Clinical pathology evaluations were conducted on 10 rats per sex and exposure concentration during the 13-week studies. In the 2-CNB study, blood for hematology and clinical chemistry evaluations was collected from rats designated for clinical pathology testing on Days 4 and 23 and from base-study rats at study termination, and in the 4-CNB study, from the supplemental rats on Days 3 and 23 and from base-study rats at study termination. At all time points, rats were anesthetized with a CO_2:room air gas mixture, and blood samples were drawn from the retroorbital sinus. Blood for hematology and methemoglobin determinations was placed in tubes containing potassium EDTA as the anticoagulant. Blood for clinical chemistry evaluations was placed in tubes devoid of anticoagulant, allowed to clot at room temperature, and centrifuged, and the serum was separated. All hematological and biochemical analyses were performed on the day of sample collection.

Methemoglobin concentrations were measured within approximately 30 min of sample collection with an IL-CO-Oximeter (Instrumentation Laboratory, Inc., Lexington, MA) calibrated for rat carboxyhemoglobin. An Ortho ELT-8/ds hematology analyzer (Ortho Instruments, Westwood, MA) was used to perform hematology determinations. Variables included hematocrit (Hct), hemoglobin (Hgb) concentration, erythrocyte (RBC) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, and leukocyte (WBC) count. Differential leukocyte counts and morphological evaluation of blood cells were determined microscopically from blood smears stained with Wright–Giemsa. Smears made from whole blood samples incubated with new methylene blue were examined microscopically for the quantitative determination of reticulocytes.

Clinical chemistry variables were measured using an Abbott-VP (Abbott Laboratories, Abbott Park, IL) or a Roche Cobas Fara chemistry analyzer (Roche Diagnostic Systems, Inc, Montclair, NJ). Endpoints included urea nitrogen (UN), creatinine, total protein, albumin, globulin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), sorbitol dehydrogenase (SDH), and total bile acids (TBA) Reagents for assay of SDH activity and TBA concentration were obtained from Sigma Chemical Company (St. Louis, MO); reagents for the other endpoints were obtained from the instrument manufacturers.

Histopathology. At the end of the 13-week studies, all surviving base-study animals were euthanized with CO_2 and necropsied. Body weights and weights of heart, right kidney, liver, lungs, spleen, right testis, and thymus were determined. Organs and tissues were examined for gross lesions and fixed in 10% neutral buffered formalin. A standard battery of 32 tissues and organs (Chhabra *et al.*, 1990) was processed for hematoxylin and eosin-stained histological sections using routine procedures. Complete histopathological examinations were performed on all animals in the vehicle control, the highest exposure groups, and all animals that died prior to study termination. Gross lesions and selected organs were examined for rats and mice in the lower exposure groups.

Statistical analysis of continuous variables. Two approaches were employed to assess the significance of pairwise comparisons between dosed

TABLE 1
Chamber Concentrations of 2- and 4-Chloronitrobenzene in the 13-Week Inhalation Studies in F344/N Rats and B6C3F$_1$ Mice[a]

Target concentration	Rat studies (mean ± SD)	Mouse studies (mean ± SD)
2-Chloronitrobenzene		
0	0.003 ± 0.004	0.003 ± 0.004
1.1	1.11 ± 0.07	1.11 ± 0.07
2.3	2.28 ± 0.13	2.28 ± 0.13
4.5	4.45 ± 0.27	4.45 ± 0.27
9	8.84 ± 0.70	8.84 ± 0.69
18	17.8 ± 1.20	17.8 ± 1.20
4-Chloronitrobenzene		
0	<MDL[b]	<MDL
1.5	1.50 ± 0.10	1.49 ± 0.10
3	2.97 ± 0.21	2.97 ± 0.22
6	5.98 ± 0.35	5.97 ± 0.35
12	12.0 ± 0.96	12.0 ± 0.98
24	23.9 ± 1.71	23.9 ± 1.75

[a] Target and mean concentrations in ppm.
[b] MDL, minimum detectable limit. MDL = 0.01 ppm 2-chloronitrobenzene or 0.015 ppm 4-chloronitrobenzene.

and control groups in the analysis of continuous variables. Organ and body weight data, which are approximately normally distributed, were analyzed using the parametric multiple comparisons procedures of Williams (1971, 1972) or Dunnett (1955). Clinical chemistry and hematology data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparisons methods of Shirley (1977) or Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose–response trends and to determine whether a trend-sensitive test—Williams or Shirley—was more appropriate for pairwise comparisons than a test capable of detecting departures from monotonic dose response—Dunnett or Dunn. If the p value from Jonckheere's test was greater than or equal to 0.10, Dunn's or Dunnett's test was used rather than Shirley's or Williams'.

RESULTS

Chamber concentrations. Mean chamber concentrations, calculated from daily monitoring results, are shown in Table 1. The mean concentrations in all chambers for the 2-CNB studies were between 98 and 101% of the target concentrations. The mean concentrations in all chambers for the 4-CNB studies were between 99 and 100% of the target concentrations. At least 89 and 88% of all individual concentration measurements were within 10% of target concentrations for the 2- and 4-CNB studies respectively (data not shown).

Animals. In rats and mice, the mean body weight gains of animals exposed to either 2- or 4-CNB were equal to or greater than those of the respective controls (Tables 2–5). Two male mice in the 18-ppm group in the 2-CNB study died during Week 12; one male in the 6-ppm group in the 4-CNB study died during Week 8. In rats, there were no chemical-related deaths in either study. There were no clinical signs of toxicity related to 2- or 4-CNB exposure in either species.

2-Chloronitrobenzene. Concentration- and time-dependent increases of methemoglobin concentration occurred in male and female rats exposed to 2-CNB (Table 6). The highest methemoglobin concentrations occurred in the 18-ppm male and female rats at Day 4; these concentrations ameliorated by Day 23 but remained elevated throughout the study. In the lower exposure groups, methemoglobin concentrations increased with time, and, at Week 13, methemoglobin concentrations were elevated in all male and female exposure groups.

Decreases in Hct values, Hgb concentrations, and RBC counts occurred in male and female rats in the top three exposure groups at Week 13 (Table 7). Male rats exposed to 18 ppm were affected at all time points; decreases in these variables, at early time points, also occurred in male rats at lower exposure concentrations, but with less consistency (data not shown). Exposed female rats also were affected at early time points; however, the changes were less consistent than those occurring in similar male exposure groups (data not shown). At Week 13, a hematopoietic response was evidenced by increases of reticulocyte and nucleated RBC numbers (Table 7).

Occasional target cells and schistocytes were observed in the high exposure male and female rats at Day 4. By Day 23, keratocytes, acanthocytes, target cells, spherocytes, stomatocytes, and schistocytes were present in minimally increased numbers. Red cells with cytoplasmic blebs, notched erythrocytes, and eccentrocytes were occasionally seen. Minimal to mild increases of red cell changes, similar to those occurring at Day 23, were observed at study termination; there also was a minimal increase in the numbers of polychromatophilic red cells (Fig. 1).

Serum activities of ALT and SDH were increased in different male and female exposure groups at various time points (Table 8). The most pronounced change occurred in the 18-ppm male and female rats at Day 4. Most male exposure groups also were affected at Day 4. SDH activities were increased in male and female rats in the 9- and 18-ppm groups at most time points. Increased TBA concentrations occurred at Day 4 in male rats in all but the lowest (1.1 ppm) exposure group and in females exposed to 18 ppm.

At necropsy, the observance of a dark spleen in one female and two male rats in the 18-ppm group was the only gross finding attributed to exposure to 2-CNB. In mice, pale discoloration of the liver was noted in one female and six male mice in the 18-ppm group. The spleen was grossly enlarged in three female mice in the 9-ppm group and four females in the 18-ppm group.

Increases of absolute and relative liver weights occurred for rats and mice exposed to 2-CNB. In rats, increases in both variables were significant for male rats exposed to 2.3

TABLE 2
Absolute and Relative Organ Weights for F344/N Rats after a 13-Week Exposure to 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2.3	4.5	9	18
			Male			
Necropsy body wt	334 ± 7	350 ± 7	343 ± 7	349 ± 8	340 ± 7	323 ± 6
Right kidney						
Absolute	1.12 ± 0.03	1.15 ± 0.06	1.18 ± 0.03	1.24 ± 0.03	1.22 ± 0.03	1.23 ± 0.03
Relative	3.36 ± 0.04	3.28 ± 0.15	3.45 ± 0.04	3.56 ± 0.04	3.59 ± 0.04*	3.79 ± 0.04**
Liver						
Absolute	11.82 ± 36	13.10 ± 40*	12.99 ± 0.36*	14.13 ± 0.58**	14.16 ± 0.40**	15.54 ± 0.35**
Relative	35.33 ± 0.58	37.48 ± 1.26	37.81 ± 0.48*	40.40 ± 0.84**	41.67 ± 0.61**	48.07 ± 0.68**
Spleen						
Absolute	0.63 ± 0 016	0.68 ± 0.01[b]	0.65 ± 0.02	0.66 ± 0.02	0.67 ± 0.01	0.75 ± 0.02**
Relative	1.89 ± 0.03	1.93 ± 0.04[b]	1.89 ± 0.03	1.89 ± 0.02	1.97 ± 0.02	2.33 ± 0.04**
			Female			
Necropsy body wt	191 ± 3	188 ± 4	200 ± 4	193 ± 3	196 ± 5	193 ± 4
Right kidney						
Absolute	0.64 ± 0.01	0.64 ± 0.01	0.72 ± 0.05	0.67 ± 0.02	0.70 ± 0.02	0.74 ± 0.03*
Relative	3.36 ± 0.02	3.42 ± 0.08	3.57 ± 0.21	3.44 ± 0.07	3.55 ± 0.05	3.83 ± 0.09**
Liver						
Absolute	6.66 ± 0.19	6.75 ± 0.12	7.40 ± 0.20*	7.61 ± 0.22**	8.59 ± 0.27**	9.77 ± 0.36**
Relative	34.86 ± 0.75	36.00 ± 0.68	36.91 ± 0.61	39.29 ± 0.72**	43.73 ± 0.54**	50.67 ± 1.07**
Spleen						
Absolute	0.42 ± 0.01	0.42 ± 0.01	0.44 ± 0.01	0.46 ± 0.01*	0.47 ± 0.01**	0.54 ± 0.02**
Relative	2.21 ± 0.04	2.24 ± 0.04	2.20 ± 0.03	2.40 ± 0.05*	2.39 ± 0.05*	2.80 ± 0.09**

[a] Body and organ weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body wt (mean ± SE); $n = 10$.
[b] $n = 9$.
* Significantly different ($p < 0.05$) from the control group by Williams' test.
** Significantly different ($p < 0.01$) from the control group by Williams' test.

ppm or greater and females exposed to 4.5 ppm or greater (Table 2). In mice, absolute and relative liver weights increased for male and female mice exposed to 9 or 18 ppm (Table 3). Relative liver weights increased in male mice exposed to 2.3 or 4.5 ppm, and absolute liver weights increased in females in the 1.1-, 2.3-, and 4.5-ppm exposure groups (Table 3). Absolute and relative spleen weights were increased in male rats exposed to 18 ppm and females exposed to 4.5 ppm or greater. Relative right kidney weights of male rats in the 9- and 18-ppm groups and absolute and relative right kidney weights of females in the 18-ppm group were significantly increased (Table 2).

Histopathological lesions related to 2-CNB exposure are listed in Table 9 for rats and Table 10 for mice. A minimal increase of cytoplasmic basophilia of centrilobular hepatocytes was observed in the liver in all male and most female rats in the 9- and 18-ppm groups. In mice, liver lesions were more pronounced and included mild hepatocellular necrosis and mineralization, chronic inflammation, and hepatocytomegaly; the first three were observed primarily in mice exposed to 18 ppm. Liver changes were typically evidenced by foci of hepatocellular mineralization and chronic inflammation in subcapsular locations; in most instances, there was little or no acute hepatocellular necrosis. The chronic inflammation was characterized by fibrosis and accumulations of mononuclear inflammatory cells, including macrophages containing yellow-brown pigment. Hepatocytomegaly occurred in all male and female mice in the 18-ppm groups and, to a minimal degree, in all males exposed to 9 ppm. In the two 18-ppm male mice that died before the scheduled killing, a severe, diffuse sinusoidal congestion with hepatocellular degeneration and necrosis was observed.

Congestion was the only morphological change observed in spleens of rats exposed to 2-CNB. The severity was minimally increased in the 18-ppm male rats, and the incidence was slightly increased in exposed females. In mice, there was an increased hematopoiesis, primarily erythropoiesis, in the red pulp of the spleen; this was a minimal treatment effect in both sexes of mice in the 18-ppm group and, to a lesser degree, in females in the 9-ppm group.

TABLE 3
Absolute and Relative Liver Weights for B6C3F$_1$ Mice after a 13-Week Exposure to 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2.3	4.5	9	18
			Male			
Necropsy body wt	36.7 ± 0.7	37.2 ± 0.3	36.2 ± 0.9	34.9 ± 0.8	36.9 ± 1.0	35.8 ± 1.0[b]
Liver						
Absolute	1.71 ± 0.04	1.84 ± 0.06	1.82 ± 0.06	1.79 ± 0.04	2.03 ± 0.07**	2.28 ± 0.10**[b]
Relative	46.75 ± 0.76	49.30 ± 1.33	50.24 ± 1.22*	51.46 ± 0.82**	54.92 ± 0.83**	63.51 ± 1.46**[b]
			Female			
Necropsy body wt	30.1 ± 0.8	32.6 ± 0.8	34.5 ± 1.1*	33.0 ± 1.0*	34.7 ± 1.2**	33.9 ± 1.3**
Liver						
Absolute	1.47 ± 0.04	1.63 ± 0.04*	1.77 ± 0.05**	1.72 ± 0.05**	1.93 ± 0.05**	2.23 ± 0.07**
Relative	49.00 ± 1.25	49.93 ± 0.97	51.32 ± 0.86	52.31 ± 1.24	56.00 ± 0.97**	66.37 ± 2.15**

[a] Body and liver weights are given in grams; relative liver weights (liver-weight-to-body-weight ratios) are given as mg liver weight/g body wt; mean ± SE; $n = 10$.
[b] $n = 8$.
* Significantly different ($p < 0.05$) from the control group by Williams' or Dunnett's test.
** Significantly different ($p < 0.01$) from the control group by Williams' test.

In the kidney, cytoplasmic pigment within proximal convoluted tubule cells was seen in male rats exposed to 4.5 ppm or greater and female rats exposed to 9 or 18 ppm. All rats in the 18-ppm exposure group were affected. Cytoplasmic pigment was granular and brown in H&E-stained sections. Special stains of selected slides revealed it to be iron-negative and PAS-positive suggesting a lipofuscin pigment. A concentration-dependent increase in the incidence and severity of tubule regeneration also was observed in male rats exposed to 1.1 ppm or greater. No morphological changes consistent with hyaline droplet nephropathy were observed in male rats; the absence of protein droplet accumulation was confirmed by Mallory–Heidenhain special stains. No kidney lesions were observed in the mice.

Hyperplasia/hypertrophy of the respiratory epithelium was an exposure-related effect observed in the nasal cavity of male and female rats exposed to 1.1 ppm or greater. This lesion was restricted to the dorsal meatus and nasoturbinate of the most anterior nasal section (at the level of the incisor teeth). The change was characterized by multifocal infoldings of tall columnar epithelial cells producing a scalloped mucosal surface or small intraepithelial cysts. These invaginations were lined by flattened ciliated cells or goblet-like cells and sometimes contained wispy strands of mucoid material; occasionally, dilated glandular structures with luminal neutrophils were also present in the lamina propria in these areas. This lesion was not observed in the mice.

4-Chloronitrobenzene. In the 4-CNB study, methemoglobin concentrations increased in a concentration- and time-dependent manner (Table 11). Methemoglobin concentrations increased in all male and female rat exposure groups by Day 3 and remained increased throughout the study. Similar to the 2-CNB study, the highest methemoglobin concentration occurred in the top exposure group at Day 3; these concentrations ameliorated by Day 23 but remained elevated throughout the study. In the lower exposure groups, the severity of the methemoglobinemia increased with time.

Decreases in Hct values, Hgb concentrations, and RBC counts occurred in all exposed male and female rats in the 4-chloronitrobenzene study at Week 13 (Table 12). At Day 3, these changes involved most male exposure groups, but, in female rats, decreases in Hct and RBC counts occurred only in the 24-ppm group (data not shown). Decreases in these red cell variables also occurred in all but the lowest (1.5 ppm) male and female exposure groups at Day 23 (data not shown). The red cell indices (MCV, MCH, and MCHC) were increased in male and female rats exposed to 6 ppm and above at Week 13 (Table 12). Elevations in MCV and MCH values also occurred in female rats in all lower exposure groups at Week 13 (Table 12). At the early time points, MCHC was elevated in male and female rats exposed to 24 ppm on Day 3; all three variables were consistently increased in male and female rats in the 12- and 24-ppm groups on Day 23 (data not shown). Reticulocyte counts were significantly increased in male and female rats in the 12- and 24-ppm groups at essentially all time points. With time, reticulocyte counts increased in other male and female rat exposure groups, and, by Week 13, all exposed male and female rats,

TABLE 4
Absolute and Relative Organ Weights for F344/N Rats after a 13-Week Exposure to 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
			Male			
Necropsy body wt	342 ± 4	354 ± 9	347 ± 4	348 ± 6	337 ± 5	346 ± 7
Right kidney						
Absolute	1.14 ± 0.02	1.18 ± 0.03	1.24 ± 0.07	1.19 ± 0.02	1.17 ± 0.02	1.25 ± 0.03*
Relative	3.34 ± 0.06	3.28 ± 0.04	3.56 ± 0.18	3.42 ± 0.05	3.49 ± 0.03	3.62 ± 0.05*
Liver						
Absolute	12.06 ± 0.37	12.68 ± 0.45	12.21 ± 0.28	12.85 ± 0.36	12.43 ± 0.23	14.32 ± 0.37**
Relative	35.19 ± 0.86	35.79 ± 0.58	35.18 ± 0.52	36.89 ± 0.68	36.91 ± 0.40	41.35 ± 0.45**
Spleen						
Absolute	0.64 ± 0.02	0.72 ± 0.02	0.79 ± 0.04*	0.98 ± 0.02**	1.66 ± 0.06**[b]	3.28 ± 0.08**
Relative	1.86 ± 0.04	2.02 ± 0.02	2.28 ± 0.11**	2.82 ± 0.05**	4.92 ± 0.15**[b]	9.48 ± 0.17**
Right testis						
Absolute	1.37 ± 0.05	1.41 ± 0.03	1.37 ± 0.03	1.40 ± 0.03	1.35 ± 0.03	1.06 ± 0.07**
Relative	4.01 ± 0.16	3.99 ± 0.04	3.95 ± 0.07	4.03 ± 0.05	4.01 ± 0.05	3.07 ± 0.21**
			Female			
Necropsy body wt	192 ± 5	195 ± 5	202 ± 6	196 ± 4	197 ± 6	200 ± 2
Right kidney						
Absolute	0.66 ± 0.02	0.67 ± 0.02	0.70 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.76 ± 0.01**
Relative	3.46 ± 0.09	3.45 ± 0.06	3.46 ± 0.09	3.54 ± 0.09	3.52 ± 0.07	3.80 ± 0.06**
Liver						
Absolute	5.92 ± 0.24	6.21 ± 0.20	6.71 ± 0.28*	6.79 ± 0.24**	6.84 ± 0.23**	7.70 ± 0.13**
Relative	30.89 ± 0.87	31.96 ± 0.95	33.19 ± 0.86	34.65 ± 0.72**	34.80 ± 0.82**	38.63 ± 0.74**
Spleen						
Absolute	0.39 ± 0.01	0.45 ± 0.01	0.56 ± 0.02*	0.84 ± 0.03**	1.49 ± 0.06**	3.10 ± 0.09**
Relative	2.06 ± 0.05	2.31 ± 0.07	2.80 ± 0.10	4.32 ± 0.14**	7.61 ± 0.34**	15.55 ± 0.53**

[a] Body and organ weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body wt (mean ± SE); $n = 10$, except 1.5 and 3 ppm female rats ($n = 9$).
[b] $n = 9$.
* Significantly different ($p < 0.05$) from the control group by Williams' or Dunnett's test.
** Significantly different ($p < 0.01$) from the control group by Williams' test.

except the 1.5-ppm female group, exhibited increased reticulocyte counts (Table 12). Increased numbers of nucleated RBCs accompanied the elevations in reticulocyte counts in exposed male and female rats at most time points.

Concentration- and time-dependent alterations of RBC morphology, similar to those which occurred in the 2-CNB study, were more prominent than in the 2-CNB study. Alterations were observed in the high-exposure rats at Day 3, were present with increased frequency at Day 23 and, at study termination, were markedly increased. Red cell morphology changes were also present in lower exposure groups; severity decreased with decreasing concentration. Red cell morphological alterations included cytoplasmic blebs consistent with Heinz bodies, notched red blood cells, eccentrocytes, keratocytes, schistocytes, spherocytes, stomatocytes, dacryocytes, elliptocytes, acanthocytes, target cells, and polychromasia. The cytoplasmic blebs were round-to-irregular, 1- to 3-μm structures seen as nipples protruding from the cell surface or attached to the surface by a thin cytoplasmic stalk (Fig. 1).

Clinical chemistry changes that occurred in the 4-CNB study are shown in Table 13. As in the 2-CNB study, increases in serum SDH activity and TBA concentration occurred in various male and female exposure groups at different time points. At Day 3, the 6-, 12-, and 24-ppm male and female exposure groups exhibited minimally increased SDH activities. This change occurred in the 12- and 24-ppm exposure groups at Day 23 but was abrogated by Week 13. Minimal increases in TBA concentration occurred in male rats in the 3-, 6-, 12-, and 24-ppm groups at almost every time point. In female rats, TBA concentrations increased in all but the 3-ppm group on Day 3 and in the 12- and 24-ppm groups on Day 23. By Week 13, TBA concentrations in female exposure groups were similar to those of controls. Dissimilar to the 2-CNB study, the activity of ALT decreased in male and female rats exposed to various concentrations of 4-CNB.

TABLE 5
Absolute and Relative Organ Weights for B6C3F$_1$ Mice after a 13-Week Exposure to 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
			Male			
Necropsy body wt	34.9 ± 0.8	36.7 ± 1.0	36.7 ± 0.9	35.3 ± 1.4	35.8 ± 0.5	36.8 ± 0.7
Liver						
Absolute	1.60 ± 0.05	1.68 ± 0.05	1.73 ± 0.04	1.70 ± 0.06	1.76 ± 0.05*	1.87 ± 0.05**
Relative	45.81 ± 1.24	45.77 ± 1.05	47.32 ± 1.09	48.44 ± 1.40	49.21 ± 1.26	50.82 ± 1.27**
Spleen						
Absolute	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.08 ± 0.00**	0.16 ± 0.01**
Relative	1.87 ± 0.07	1.85 ± 0.05	1.83 ± 0.06	1.83 ± 0.07	2.21 ± 0.06*	4.36 ± 0.19**
			Female			
Necropsy body wt	31.5 ± 0.9	32.3 ± 1.1	33.5 ± 1.3	31.1 ± 0.8	33.1 ± 0.8	33.0 ± 0.4
Liver						
Absolute	1.47 ± 0.03	1.54 ± 0.05	1.62 ± 0.05	1.55 ± 0.05	1.76 ± 0.06**	1.89 ± 0.02**
Relative	46.78 ± 0.89	47.72 ± 1.00	48 49 ± 1.02	50.07 ± 1.34*	53.10 ± 0.97**	57.25 ± 0.82**
Spleen						
Absolute	0.09 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.13 ± 0.01**	0.25 ± 0.01**
Relative	2.98 ± 0.16	2.95 ± 0.10	2.93 ± 0.15	3.20 ± 0.09	3.94 ± 0.16**	7.68 ± 0.27**

[a] Body and organ weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body wt; mean ± SE; $n = 10$.

* Significantly different ($p < 0.05$) from the control group by Williams' or Dunnett's test.

** Significantly different ($p < 0.01$) from the control group by Williams' test.

At necropsy, the incidence of enlarged or enlarged and darkened spleens in male and female rats increased in a concentration-dependent manner. Enlarged and darkened spleens were also observed in male and female mice exposed to 24 ppm and female mice exposed to 12 ppm. The kidneys of most female rats exposed to 12 or 24 ppm were darkened.

Absolute and relative spleen weights markedly increased, with increasing concentration, in male and female rats ex-

TABLE 6
Methemoglobin Concentration in F344/N Rats Exposed to 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2.3	4.5	9	18
			Male			
Day 4	0.09 ± 0.01	0.11 ± 0.01	0.12 ± 0.01**	0.17 ± 0.01**	0.24 ± 0.01**	1.14 ± 0.09**
Day 23	0.14 ± 0.02[b]	0.15 ± 0.01*	0.19 ± 0.01**[c]	0.23 ± 0.02**	0.41 ± 0.02**	0.55 ± 0.01**[d]
Week 13	0.15 ± 0.01	0.21 ± 0.01**	0.26 ± 0.01**	0.36 ± 0.01**	0.55 ± 0.01**	0.87 ± 0.02**
			Female			
Day 4	0.09 ± 0.01	0.11 ± 0.01	0.14 ± 0.01**	0.17 ± 0.01**	0.25 ± 0.01**	1.04 ± 0.08**
Day 23	0.16 ± 0.01[c]	0.18 ± 0.01	0.22 ± 0.01**	0.30 ± 0.01**	0.47 ± 0.02**	0.71 ± 0.04**[b]
Week 13	0.19 ± 0.01	0.22 ± 0.01**	0.28 ± 0.01**	0.35 ± 0.01**	0.51 ± 0.01**	0.79 ± 0.03**

[a] Data are given as g/dl; mean ± SE; $n = 10$.

[b] $n = 8$.

[c] $n = 9$.

[d] $n = 6$.

* Significantly different ($p < 0.05$) from the control group by Shirley's test.

** Significantly different ($p < 0.01$) from the control group by Shirley's test.

TABLE 7
Hematology Data for F344/N Rats after a 13-Week Exposure to 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2 3	4.5	9	18
			Male			
Hct (%)	46.5 ± 0.4	46.2 ± 0.2	45 5 ± 0.2*	45.1 ± 0.2**	45.0 ± 0.3**	42.6 ± 0.3**
Hgb (g/dl)	15 0 ± 0.1	14 9 ± 0.1	14.6 ± 0.1*	14.5 ± 0.1**	14.4 ± 0.1**	13.7 ± 0.1**
RBC ($10^6/\mu l$)	9 16 ± 0.08	9.11 ± 0 04	8.99 ± 0.05	8.88 ± 0.05**	8.86 ± 0.06**	8.43 ± 0.07**
MCV (fl)	50.7 ± 0.2	50.8 ± 0.2	50.6 ± 0.2	50.8 ± 0.1	50.7 ± 0.2	50.5 ± 0.2
MCH (pg)	16.4 ± 0.0	16.4 ± 0.1	16.3 ± 0.1	16.3 ± 0.1	16.3 ± 0.1	16.2 ± 0.1
MCHC (g/dl)	32.3 ± 0.1	32.3 ± 0.1	32 1 ± 0 1	32.2 ± 0.1	32.1 ± 0.1	32.1 ± 0.2
Reticulocytes ($10^6/\mu l$)	0.25 ± 0.01	0.28 ± 0.01*	0.28 ± 0.02	0.28 ± 0.01	0.33 ± 0.02**	0.38 ± 0.02**
Nucleated RBC ($10^3/\mu l$)	0.05 ± 0.02	0.05 ± 0 03	0.07 ± 0.03	0.10 ± 0.03	0.15 ± 0 03*	0.16 ± 0.03**
			Female			
Hct (%)	47 9 ± 0.4	46.8 ± 0.2	47.2 ± 0.4	45.9 ± 0.3**	45.0 ± 0.3**	42.6 ± 0.3**
Hgb (g/dl)	15.5 ± 0.1	15.1 ± 0.1**	15.2 ± 0.1	14.7 ± 0 1**	14.3 ± 0.1**	13.4 ± 0.1**
RBC ($10^6/\mu l$)	8.80 ± 0.06	8.58 ± 0.06*	8.64 ± 0.06	8 41 ± 0.06**	8.24 ± 0.05**	7.84 ± 0.05**
MCV (fl)	54.5 ± 0.2	54.8 ± 0 2	54.5 ± 0.2	54.7 ± 0.3	54.6 ± 0.3	54.4 ± 0.3
MCH (pg)	17.6 ± 0.1	17.6 ± 0 1	17.6 ± 0.1	17.5 ± 0.1	17.3 ± 0 0**	17.1 ± 0.1**
MCHC (g/dl)	32 4 ± 0.1	32.2 ± 0.1	32.2 ± 0.2	32.0 ± 0.2	31.8 ± 0.2**	31.5 ± 0.1**
Reticulocytes ($10^6/\mu l$)	0.19 ± 0.01	0.22 ± 0.01	0.21 ± 0.02	0.25 ± 0.02**	0.25 ± 0.01**	0.37 ± 0.02**
Nucleated RBC ($10^3/\mu l$)	0.06 ± 0.03	0.09 ± 0.03	0.09 ± 0.03	0.10 ± 0.04	0.11 ± 0.02	0.19 ± 0.03**

Note. Hct, hematocrit; Hgb, hemoglobin; RBC, erythrocyte count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

[a] Data are given as means ± SE; $n = 10$

* Significantly different ($p < 0.05$) from the control group by Shirley's test.

** Significantly different ($p < 0.01$) from the control group by Shirley's test.

posed to 3 ppm or higher of 4-CNB (Table 4). Absolute and relative liver weights increased for female rats exposed to 6 ppm or greater and for males in the 24-ppm group. In mice, the absolute and relative liver and spleen weights were increased for male and female mice exposed to 12 or 24 ppm 4-CNB (Table 5). Absolute and relative kidney weights increased in male and female rats in the 24 ppm group (Table 4). Absolute and relative right testis weights decreased in the 24-ppm male rats (Table 4).

Morphological changes that were observed for animals exposed to 4-CNB are shown in Table 14 for rats and Table 15 for mice. Multiple effects in the spleen of rats and mice were attributed to exposure to 4-CNB. Congestion of the red pulp was present in all exposed rats of each sex, and the severity of the change increased with increasing exposure concentration. All exposed male and female rats exhibited a minimal increase in hemosiderin pigment, and a minimal to mild increase of hematopoietic cell proliferation was present in most animals exposed to 3.0 ppm or greater. Capsular fibrosis of the spleen occurred with concentration-dependent increases in incidence and severity; this lesion was characterized by focal or multifocal fibrous thickenings of the capsule accompanied by mononuclear inflammatory cell infiltrates. In mice, splenic lesions included minimal to mild congestion, increased hematopoietic cell proliferation, and increased hemosiderin pigmentation. Congestion was observed in all mice in the 24-ppm groups, while increased hematopoietic activity and hemosiderin deposition occurred in all mice exposed to 24 ppm and most mice exposed to 12 ppm. Lower incidences of minimally increased hematopoiesis were observed in female mice at all lower exposure levels.

Hemosiderin deposition in Kupffer's cells was the only chemical-related effect observed in the liver of rats. This pigment was observed as a minimal change in male rats in the 12- and 24-ppm groups, while in females a clear exposure effect was observed in all but the lowest exposure groups. Hemosiderin deposition in Kupffer's cells, the most common finding in the liver of mice, was limited to males and females in the 24-ppm groups. Minimal increases of hepatocyte necrosis and centrilobular cytoplasmic basophilia was observed for a few male mice in the 24-ppm group.

Similar to the 2-CNB study, kidney lesions were observed only in rats exposed to 4-CNB. The nature of the change, however, was different for males and females. In male rats, there was a concentration-dependent increase in the amount of eosinophilic hyaline droplets within the cytoplasm of tubule epithelial cells (hyaline droplet nephropathy); in addition, a few small brown pigment granules were also detected in the tubule epithelial cells in the 12- and 24-ppm groups. In female rats, the primary tubule lesion was accumulation

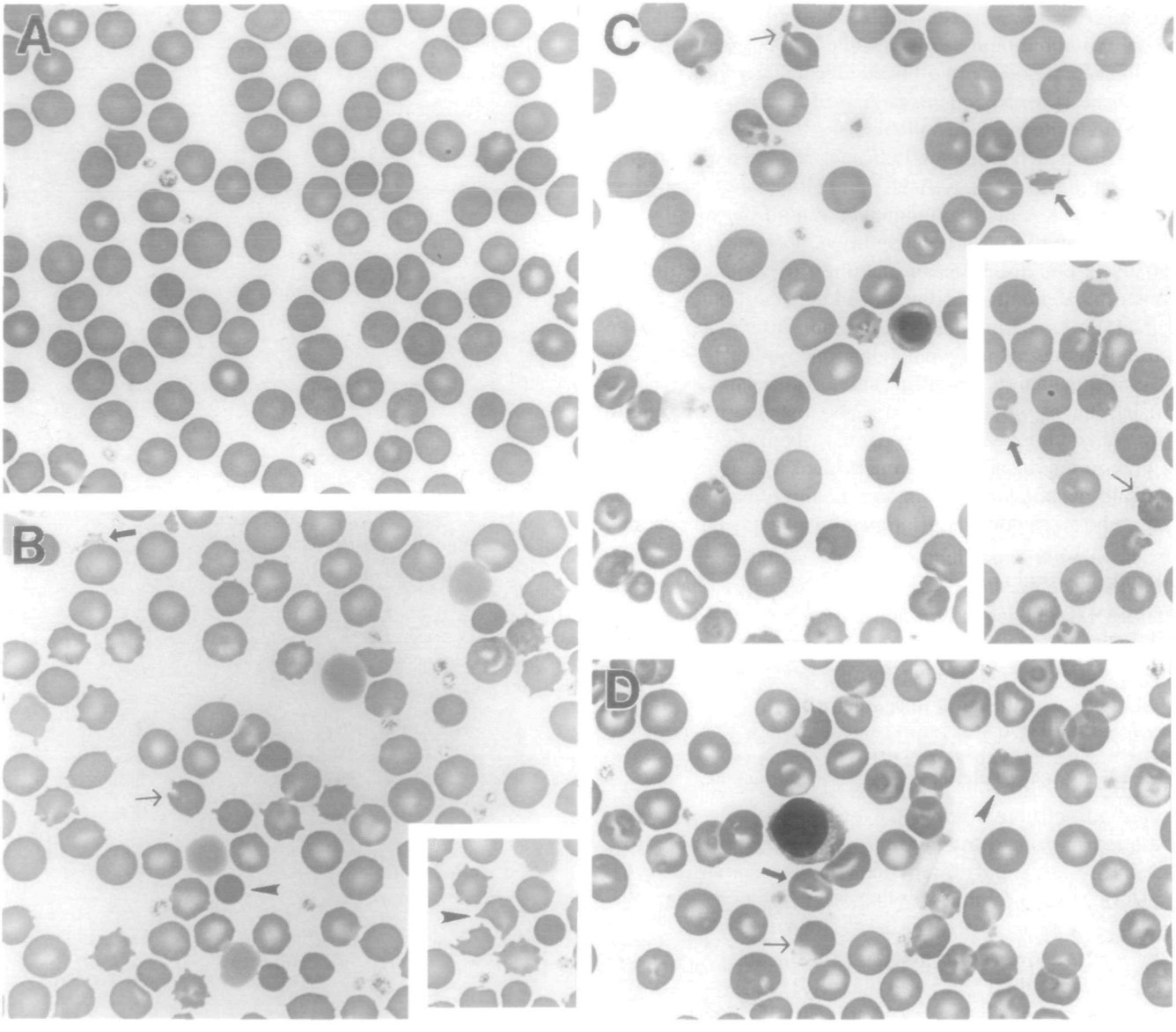

FIG. 1. (A) Normal red cell morphology representative of control rats of both the 2- and the 4-CNB studies. (B–D) Altered red cell morphology in blood of high dose group rats treated with 2-CNB (B) and 4-CNB (C and D). Alterations include, Heinz bodies, ↑ (C and C inset); schistocytes, ↕ (B and C); keratocytes, ↓ (B inset and D); eccentrocytes, ↑ (D); notched erythrocytes, ↑ (B); spherocytes, ↓ (B); microcytes, ↕ (C inset); stomatocytes, ↕ (D); metarubricytes, ↓ (C). Photomicrographs are of Wright's-stained blood films prepared at terminal killing and examined with a light microscope (875×).

of brown pigment granules which occurred to a much greater degree than in males. This pigment was present in all females. Special stains were performed on selected kidney sections to characterize further the tubule pigment. In male rats, Mallory–Heidenhain stains confirmed the presence of protein-positive hyaline droplets; iron stains were negative, and there was an equivocal increase in PAS-positive granules. In contrast, most of the brown tubule pigment granules seen in H&E-stained sections of exposed female rats stained PAS-positive, although the number of iron-positive granules in exposed females was also increased above that in the controls.

Minimal to moderate increases of hematopoietic cell proliferation, which primarily involved erythroid cells, was an exposure-related effect in the bone marrow of rats and mice; this was observed for male and female rats at all exposure levels except 1.5 ppm and for mice in the 24-ppm groups. Increased hemosiderin deposition, also a treatment effect in the bone marrow of mice, was limited to the 24-ppm exposure groups and was minimal in severity.

Squamous cell hyperplasia of the forestomach epithelium was found in 7 of 10 female and 1 of 10 male mice in the 24-ppm groups; this was minimal to moderate in severity and consisted of focal hyperplasia and hyperkeratosis of the

TABLE 8
Clinical Chemistry Data for F344/N Rats Exposed to 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2.3	4.5	9	18
Male						
Alanine aminotransferase (IU/liter)						
Day 4	44 ± 1	48 ± 1	50 ± 2*	53 ± 2**	57 ± 3**	212 ± 19**
Day 23	35 ± 1[b]	33 ± 1	36 ± 1[c]	33 ± 1	39 ± 2	47 ± 3**[d]
Week 13	62 ± 5	57 ± 3	60 ± 4	54 ± 3	49 ± 1*	55 ± 2
Sorbitol dehydrogenase (IU/liter)						
Day 4	10 ± 1	12 ± 0**	14 ± 1**	15 ± 1**	16 ± 1**	34 ± 4**
Day 23	9 ± 0[b]	9 ± 0	10 ± 0[c]	10 ± 0	14 ± 1**	16 ± 1**[d]
Week 13	20 ± 2	20 ± 2	21 ± 3	21 ± 3	22 ± 1	28 ± 2**
Total bile acids (μmol/liter)						
Day 4	19.0 ± 0.9	24.1 ± 2.0	22.7 ± 1.5*	24.7 ± 0.9**	27.2 ± 1.3**	75.4 ± 5.8**[c]
Day 23	17.0 ± 1.3[b]	18.9 ± 1.6	20.5 ± 1.1[c]	18.7 ± 0.8	21.9 ± 2.6	13.9 ± 1.1[e]
Week 13	19.0 ± 0.5	22.0 ± 1.4	22.0 ± 0.7*[c]	22.8 ± 1.8	22.3 ± 0.8*	20.5 ± 0.8
Female						
Alanine aminotransferase (IU/liter)						
Day 4	41 ± 2	40 ± 1	38 ± 1	35 ± 1*	38 ± 1	137 ± 20
Day 23	33 ± 1[c]	33 ± 1	36 ± 1	36 ± 4	35 ± 1	45 ± 3**[b]
Week 13	58 ± 4	50 ± 2	58 ± 3	53 ± 2	47 ± 2	41 ± 2**
Sorbitol dehydrogenase (IU/liter)						
Day 4	9 ± 1	9 ± 0	9 ± 1	9 ± 0	12 ± 1**	20 ± 1**
Day 23	11 ± 1[c]	12 ± 1	12 ± 1	11 ± 1[c]	14 ± 1**	23 ± 2**[b]
Week 13	19 ± 1	18 ± 1	23 ± 1	22 ± 1	24 ± 1*	26 ± 2**
Total bile acids (μmol/liter)						
Day 4	19.3 ± 1.1	20.0 ± 1.2	17.7 ± 0.7	20.4 ± 1.1	21.9 ± 0.9	34.7 ± 3.4**
Day 23	16.4 ± 1.1[c]	12.9 ± 0.6	18.4 ± 3.4	18.6 ± 2.8	16.3 ± 0.6	17.6 ± 0.4[b]
Week 13	24.1 ± 2.6	20.7 ± 2.0	20.6 ± 1.8	19.2 ± 0.9	19.9 ± 1.7	20.5 ± 1.7

[a] Data are given as means ± SE; $n = 10$.
[b] $n = 8$.
[c] $n = 9$.
[d] $n = 6$.
[e] $n = 5$.
* Significantly different ($p < 0.05$) from the control group by Dunn's or Shirley's test.
** Significantly different ($p < 0.01$) from the control group by Dunn's or Shirley's test.

mucosa along the greater curvature of the forestomach between the limiting ridges. Chronic inflammation, consisting of multifocal interstitial aggregates of lymphocytes, was observed in the harderian gland of male and female rats exposed to 24 ppm and females exposed to 12 ppm; this was mild to moderate in severity. All male rats in the 24-ppm group exhibited minimal to moderate testicular atrophy characterized by decreased cellularity of seminiferous tubules.

DISCUSSION

A literature review revealed limited animal toxicological data for these compounds (Hasegawa and Sato, 1963; Davydova, 1967; Watanabe *et al.*, 1976; Nishida *et al.*, 1982; Ridley *et al.*, 1983; Nair *et al.*, 1986a,b). Most of these prior studies were designed to evaluate the effects of single oral or parentral doses of 2- or 4-CNB administered to rats or rabbits; no mouse studies were found. Only one inhalation study was found for each compound (Nair *et al.*, 1986a,b); both studies were limited to 4-week inhalation exposures and only for rats. The results of the present 13-week inhalation studies with 2- and 4-CNB are consistent with findings reported for the aforementioned animal studies. Methemoglobinemia is a primary toxic response, and many of the other lesions described in this report could be explained as secondary to methemoglobin formation and subsequent increases in erythrocyte injury and turnover; lesions include anemia, red cell morphological alterations (e.g., Heinz bodies), and effects on the spleen (hemosiderin accumulation, capsular fibrosis, and increased hematopoietic cell proliferation), liver (Kupffer cell hemosiderin accumulation), and bone marrow (increased hemosiderin and hematopoietic cell proliferation). Other findings, however, including hyaline droplet nephropathy and degeneration of the testis in male

TABLE 9
Incidence and Severity of Selected Lesions in F344/N Rats in the 13-Week Inhalation Study of 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2.3	4.5	9	18
			Male			
Kidney						
Tubule pigment	0	0	0	4 (1.0)	4 (1.0)	10 (1.0)
Tubule regeneration	1 (1.0)	4 (1.0)	6 (1.0)	9 (1.2)	8 (1.0)	10 (1.3)
Liver						
Cytoplasmic basophilia	0	0	0	0	10 (1.0)	10 (1.0)
Nasal cavity						
Respiratory epithelium hyperplasia	4 (1.0)	9 (1.0)	8 (1.0)	10 (1.2)	10 (1.4)	9 (1.1)
Spleen						
Congestion	8 (1.4)	9 (1.6)	10 (1.5)	10 (1.6)	10 (1.4)	10 (1.9)
			Female			
Kidney						
Tubule pigment	0	0	0	0	10 (1.0)	10 (3.0)
Tubule regeneration	0	0	0	0	0	0
Liver						
Cytoplasmic basophilia	0	0	0	0	6 (1.0)	8 (1.0)
Nasal cavity						
Respiratory epithelium hyperplasia	0	8 (1.0)	9 (1.0)	10 (1.1)	9 (1.1)	6 (1.2)
Spleen						
Congestion	4 (1.0)	4 (1.0)	7 (1.0)	3 (1.0)	9 (1.0)	10 (1.0)

[a] $n = 10$; average severity (in parentheses) is based on the number of animals with lesions: 1, minimal; 2, mild; 3, moderate; 4, marked.

TABLE 10
Incidence and Severity of Selected Lesions in B6C3F$_1$ Mice in the 13-Week Inhalation Study of 2-Chloronitrobenzene[a]

Concentration (ppm):	0	1.1	2.3	4.5	9	18
			Male			
Liver						
Cytomegaly	0	0	0	0	10 (1.0)	10 (1.7)
Necrosis/mineralization	0	0	0	0	0	8 (1.9)
Sinusoidal congestion	0	0	0	0	0	2 (4.0)[b]
Chronic inflammation	0	0	0	0	0	5 (2.0)
Spleen						
Hematopoietic cell proliferation	0	0	0	0	0	4 (1.0)
			Female			
Liver						
Cytomegaly	0	0	0	0	0	10 (2.0)
Necrosis/mineralization	0	0	0	0	1 (1.0)	4 (1.2)
Sinusoidal congestion	0	0	0	0	0	0
Chronic inflammation	0	0	0	0	0	1 (1.0)
Spleen						
Hematopoietic cell proliferation	3 (1.0)	0	0	0	10 (1.0)	8 (1.2)

[a] $n = 10$; average severity (in parentheses) is based on the number of animals with lesions: 1, minimal; 2, mild; 3, moderate, 4, marked.
[b] Both incidences were animals that died before the end of the study.

TABLE 11
Methemoglobin Concentration in F344/N Rats Exposed to 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
			Male			
Day 3	0.08 ± 0.00	0.19 ± 0.01**	0.31 ± 0.02**	0.60 ± 0.04**	1.31 ± 0.09**	4.13 ± 0.26**
Day 23	0.13 ± 0.01	0.32 ± 0.02**	0.57 ± 0.02**	0.98 ± 0.03**	1.90 ± 0.07**	2.97 ± 0.23**
Week 13	0.16 ± 0.01	0.50 ± 0.01**	0.73 ± 0.01**	1.22 ± 0.04**	2.08 ± 0.06**	2.96 ± 0.05**
			Female			
Day 3	0.07 ± 0.01	0.20 ± 0.01**	0.38 ± 0.02**	0 72 ± 0.04**	1.79 ± 0 09**	5.90 ± 0.43**
Day 23	0.14 ± 0.01	0.48 ± 0.02**	0.82 ± 0.02**	1.44 ± 0.06**	2.87 ± 0.17**	3.88 ± 0.25**
Week 13	0.16 ± 0.01	0.63 ± 0.03**	0.90 ± 0.03**[b]	1.69 ± 0.04**	2.50 ± 0.09**	2.85 ± 0.07**

[a] Data are given as g/dl; mean ± SE; $n = 10$.
[b] $n = 9$.
* Significantly different ($p < 0.05$) from the control group by Dunn's or Shirley's test.
** Significantly different ($p < 0.01$) from the control group by Dunn's or Shirley's test

rats exposed to 4-CNB, inflammation of the harderian gland in rats exposed to 4-CNB, hyperplasia of the nasal cavity epithelium in rats exposed to 2-CNB, and hyperplasia of the forestomach epithelium in mice exposed to 4-CNB have not been described previously.

In rats, the greatest methemoglobin concentrations occurred in the highest exposure groups (24 ppm 4-CNB and 18 ppm 2-CNB) at the earliest sampling time (Day 3, 4-CNB; Day 4, 2-CNB study), indicating that marked oxidative red cell injury developed rapidly postinhalation exposure. It

TABLE 12
Hematology Data for F344/N Rats after a 13-Week Exposure to 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
			Male			
Hct (%)	46 8 ± 0.3	44.9 ± 0.2**	43.8 ± 0.3**	41 9 ± 0.3**	39.9 ± 0.2**	36.1 ± 0.5**
Hgb (g/dl)	14.9 ± 0.1	14.2 ± 0.1**	13.9 ± 0.1**	13.3 ± 0.1**	13.4 ± 0.1**	12.6 ± 0.2**
RBC ($10^6/\mu l$)	9.00 ± 0.06	8.69 ± 0.04**	8.40 ± 0.04**	7.83 ± 0.04**	7.13 ± 0.06**	5 73 ± 0.07**
MCV (fl)	51.9 ± 0.1	51.7 ± 0.2	52.2 ± 0.1	53.6 ± 0.2**	55.7 ± 0.3**	62.9 ± 0.3**
MCH (pg)	16.5 ± 0.1	16.4 ± 0.1	16.6 ± 0.1	17.0 ± 0.1**	18.8 ± 0.2**	22.1 ± 0.1**
MCHC (g/dl)	31.8 ± 0.1	31 6 ± 0 2	31.8 ± 0.1	31.8 ± 0.1	33.7 ± 0.2**	35.0 ± 0.2**
Reticulocytes ($10^6/\mu l$)	0.17 ± 0.01	0.27 ± 0.02**	0.30 ± 0 02**	0.42 ± 0.02**	0.59 ± 0.03**	0.91 ± 0 03**
Nucleated RBC ($10^3/\mu l$)	0.03 ± 0.01	0.07 ± 0.03	0.10 ± 0.03*	0.23 ± 0.04**	0.50 ± 0.08**	0.93 ± 0 11**
			Female			
Hct (%)	48.7 ± 0.3	44.2 ± 0.4**	42.6 ± 0.3**	41.6 ± 0.3**	39.8 ± 0.3**	34.5 ± 0.5**
Hgb (g/dl)	15.4 ± 0.1	14.2 ± 0.1**	13.6 ± 0.1**	13.7 ± 0.1**	13.7 ± 0.1**	12.3 ± 0.2**
RBC ($10^6/\mu l$)	8.68 ± 0.06	7.77 ± 0.10**	7.41 ± 0.05**	7.00 ± 0.06**	6.36 ± 0.08**	4.87 ± 0.09**
MCV (fl)	55.9 ± 0.1	56.9 ± 0.3**	57.7 ± 0.2**	59.4 ± 0.2**	62.5 ± 0.4**	70.8 ± 0.4**
MCH (pg)	17.8 ± 0.1	18.2 ± 0 2*	18.3 ± 0.1**	19.5 ± 0.1**	21.5 ± 0.2**	25.3 ± 0.2**
MCHC (g/dl)	31.7 ± 0.1	32.0 ± 0.2	31.9 ± 0.1	32.9 ± 0.1**	34.4 ± 0.2**	35.8 ± 0.1**
Reticulocytes ($10^6/\mu l$)	0.17 ± 0.02	0.21 ± 0.01[b]	0.38 ± 0.02**	0.54 ± 0.03**	0.81 ± 0.07**	1.51 ± 0.07**
Nucleated RBC ($10^3/\mu l$)	0.06 ± 0.03	0.18 ± 0.04*	0.29 ± 0.07**	0.82 ± 0.13**	1.29 ± 0.11**	4.96 ± 0.74**

Note. Hct, hematocrit; Hgb, hemoglobin; RBC, erythrocyte count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.
[a] Data are given as means ± SE; $n = 10$, except 3-ppm female rats ($n = 9$).
[b] $n = 9$.
* Significantly different ($p < 0.05$) from the control group by Dunn's or Shirley's test
** Significantly different ($p < 0.01$) from the control group by Dunn's or Shirley's test.

TABLE 13
Clinical Chemistry Data for F344/N Rats Exposed to 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
Male						
Alanine aminotransferase (IU/liter)						
Day 3	44 ± 1	43 ± 1	43 ± 1	47 ± 2	46 ± 1	42 ± 1
Day 23	36 ± 1	33 ± 1*	33 ± 1*	33 ± 1**	32 ± 1**	31 ± 1**
Week 13	58 ± 5	63 ± 5	56 ± 3	54 ± 5	48 ± 1	36 ± 2**
Sorbitol dehydrogenase (IU/liter)						
Day 3	15 ± 0	16 ± 0	14 ± 1	17 ± 0**	20 ± 0**	21 ± 1**
Day 23	15 ± 1	14 ± 0	14 ± 0	16 ± 1	18 ± 1	18 ± 1*
Week 13	24 ± 3	26 ± 3	23 ± 1	25 ± 2	23 ± 1	22 ± 2
Bile acids (μmol/liter)						
Day 3	17.7 ± 0.6	19.7 ± 1.2	22.4 ± 1.4*	26.4 ± 2.1**[b]	30.9 ± 1.3**	24.8 ± 0.9**
Day 23	20.3 ± 1.0	20.8 ± 0.7	21.1 ± 1.1	23.8 ± 1.2*	33.7 ± 2.6**	43.5 ± 4.2**
Week 13	23.2 ± 3.1	26.3 ± 2.2	27.3 ± 2.2*	26.2 ± 1.6*	29.7 ± 1.9**	35.0 ± 3.9**
Female						
Alanine aminotransferase (IU/liter)						
Day 3	39 ± 2	41 ± 1	40 ± 1	40 ± 2	38 ± 1	40 ± 2
Day 23	31 ± 1	34 ± 1	32 ± 1	31 ± 1	31 ± 1	33 ± 1
Week 13	46 ± 2	46 ± 2	49 ± 4	44 ± 1	39 ± 2*	36 ± 2**
Sorbitol dehydrogenase (IU/liter)						
Day 3	15 ± 0	16 ± 1	16 ± 1	17 ± 0**	19 ± 0**	23 ± 1**
Day 23	20 ± 1	19 ± 1	19 ± 1	21 ± 1	23 ± 1**	26 ± 1**
Week 13	19 ± 1	20 ± 1	21 ± 1	21 ± 1	22 ± 1	23 ± 2
Bile acids (μmol/liter)						
Day 3	15.8 ± 1.0	24.3 ± 3.2**	17.9 ± 1.1	22.3 ± 2.8*	23.4 ± 1.3**	24.1 ± 3.4**[b]
Day 23	19.9 ± 1.9	20.6 ± 2.4	18.8 ± 0.8	20.1 ± 1.0	23.9 ± 1.1*	27.8 ± 2.0**
Week 13	22.2 ± 3.8	25.9 ± 3.4	23.1 ± 2.9	34.2 ± 6.8	25.5 ± 3.2	29.6 ± 3.6

[a] Data are given as means ± SE; $n = 10$.
[b] $n = 9$.
* Significantly different ($p < 0.05$) from the control group by Dunn's or Shirley's test.
** Significantly different ($p < 0.01$) from the control group by Dunn's or Shirley's test.

has been shown that methemoglobin concentrations increase, in rats and rabbits, within hours after oral or parentral administration of 2- or 4-CNB (Hasegawa and Sato, 1963; Watanabe *et al.*, 1976; Ridley *et al.*, 1983). In the present studies, the methemoglobin concentrations for the high-exposure animals declined somewhat by Day 23 and remained stable at Week 13; this observation suggests that increased activity of the enzyme systems involved in methemoglobin reduction occurred and could be explained by an absolute increase in enzyme-rich reticulocytes that appeared in response to the anemia. In contrast, the severity of the methemoglobinemia increased with time in the lower exposure groups, suggesting that the red cell injury was not as severe allowing red cells with oxidized hemoglobin to remain in the circulation longer. The 12-ppm 4-CNB group had greater methemoglobin concentrations at Day 3 than the 18-ppm 2-CNB group. The methemoglobin concentration continued to increase with time in the 12-ppm 4-CNB group, while it was abrogated in the 18-ppm 2-CNB group. This difference is unexplained; it does indicate, however, that 4-CNB is a stronger methemoglobin former than 2-CNB.

Both 2- and 4-CNB caused responsive anemias, as evidenced by increases in reticulocyte and nucleated erythrocyte counts and decreases in hematocrit, hemoglobin concentrations, and red blood cell counts. In the 4-CNB study, the anemia was macrocytic and hyperchromic, as indicated by increased mean cell volume and mean cell hemoglobin concentration values, respectively. The macrocytosis was attributed to the increased numbers of larger reticulocytes in the circulation. The hyperchromia indicated increased release of hemoglobin into the plasma and suggested increased red cell fragility that would be consistent with development of a hemolytic anemia. In the 2-CNB study, the anemia was responsive but normocytic and normochromic, not typical of a hemolytic process. Normocytic, normochromic anemias often occur when there is a depression of erythropoiesis and are usually nonresponsive. Evidence of an erythropoietic response in rats exposed to 2-CNB indicates that erythropoi-

TABLE 14
Incidence and Severity of Selected Lesions in F344/N Rats in the 13-Week Inhalation Study of 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
Male						
Bone marrow						
Hematopoietic cell proliferation	0	0	3 (1.0)	10 (1.8)	10 (2.0)	10 (2.8)
Harderian gland						
Chronic inflammation	2 (1.0)	1 (1.0)	1 (1.0)	3 (1.0)	1 (1.0)	8 (2.2)
Kidney						
Hyaline droplet nephropathy	0	8 (1.0)	9 (1.0)	10 (1.0)	10 (1.2)	10 (3.0)
Tubule pigment	0	0	0	0	8 (1.0)	10 (1.0)
Liver						
Hemosiderin	0	0	0	0	9 (1.0)	10 (1.0)
Spleen						
Congestion	0	10 (1.0)	10 (1.4)	10 (1.9)	10 (2.8)	10 (3.0)
Hemosiderin	0	10 (1.0)	10 (1.1)	10 (1.0)	10 (1.0)	10 (1.0)
Hematopoietic cell proliferation	0	0	10 (1.0)	9 (1.0)	10 (1.0)	10 (1.0)
Capsular fibrosis	0	0	4 (1.0)	8 (1.0)	10 (1.8)	10 (2.1)
Testis						
Atrophy	1 (4.0)	2 (1.5)	1 (1.0)	0	1 (1.0)	10 (1.6)
Female						
Bone marrow						
Hematopoietic cell proliferation	0	0	9 (1.2)	10 (2.2)	10 (3.0)	10 (3.8)
Harderian gland						
Chronic inflammation	1 (1.0)	2 (1.0)	4 (1.7)	5 (1.6)	8 (2.2)	10 (3.0)
Kidney						
Hyaline droplet nephropathy	0	0	0	0	0	0
Tubule pigment	0	0	0	10 (1.0)	10 (2.0)	10 (3.0)
Liver						
Hemosiderin	0	0	7 (1.0)	10 (1.1)	10 (1.8)	10 (2.4)
Spleen						
Congestion	0	10 (1.0)	10 (1.4)	10 (1.8)	10 (2.0)	10 (3.0)
Hemosiderin	3 (1.0)	10 (1.0)	9 (1.0)	10 (1.0)	10 (1.0)	10 (1.0)
Hematopoietic cell proliferation	0	0	9 (1.0)	10 (1.0)	9 (1.0)	10 (2.0)
Capsular fibrosis	0	0	2 (1.0)	10 (1.2)	10 (2.3)	10 (2.3)

[a] $n = 10$; average severity (in parentheses) is based on the number of animals with lesions: 1, minimal; 2, mild; 3, moderate, 4, marked.

esis was not suppressed. The anemia that occurred in the 2-CNB study, however, was mild and, therefore, would not be expected to produce a strong stimulus for an erythropoietic response. Reticulocyte numbers increased but were not in sufficient numbers to alter the MCV values; thus, normocytosis occurred. The methemoglobinemia was less severe in the 2-CNB study suggesting less severe oxidative damage to the erythrocytes; less hemoglobin would be released to the circulation resulting in normochromia.

The presence of altered red cell morphology (e.g., Heinz bodies, notched red cells, keratocytes, schistocytes, eccentrocytes, spherocytes, dacryocytes, and acanthocytes) is consistent with erythrocyte damage and is presumed to be related to direct oxidative injury to the red cells by the chemicals or to the pitting function of the spleen. Heinz bodies have been previously reported for rabbits and rats following oral or parentral administration of 2- or 4-CNB (Hasegawa and Sato, 1963; Davydova, 1967; Nishida *et al.*, 1982). The presence of polychromatophilic erythrocytes would reflect the increased numbers of reticulocytes in response to the anemia.

It appears that *in vivo* reduction of the chloronitrobenzenes to chloronitroanilines is required for the formation of methemoglobin. Watanabe *et al.* (1976) gave male Wistar rats a single intraperitoneal dose of 100 μmol/kg 4- or 2-CNB in propylene glycol; 5 hr after dosing, methemoglobin levels were 16.3 and 20.6%, respectively. When the isomers were incubated with hemoglobin *in vitro*, no increase in methemoglobin concentrations occurred for 2-CNB; a minimal increase was reported for 4-CNB. Methemoglobin formation caused by nitrobenzene has been demonstrated to occur only following reduction of nitrobenzene to aniline by gut microflora, and germ-free or antibiotic-treated animals were unable to convert sufficient nitrobenzene to aniline to cause methemoglobin formation (Reddy *et al.*, 1976). Carr *et al.*

TABLE 15
Incidence and Severity of Selected Lesions in B6C3F$_1$ Mice in the 13-Week Inhalation Study of 4-Chloronitrobenzene[a]

Concentration (ppm):	0	1.5	3	6	12	24
			Male			
Bone marrow						
Hyperplasia	0	0	0	0	3 (1.0)	9 (1.2)
Hemosiderin	0	0	0	0	0	10 (1.1)
Red blood cell fragments	0	0	0	0	0	10 (1.0)
Forestomach						
Hyperplasia	0	0	0	0	0	1 (1.0)
Liver						
Hemosiderin	0	0	0	0	0	10 (1.2)
Necrosis	0	0	0	0	1 (1.0)	5 (1.0)
Cytoplasmic basophilia	0	0	0	0	0	4 (1.0)
Spleen						
Congestion	0	0	0	0	1 (1.0)	10 (1.9)
Hemosiderin	0	0	0	0	10 (1.1)	10 (2.0)
Hematopoietic cell proliferation	0	0	0	0	7 (1.0)	10 (2.0)
			Female			
Bone marrow						
Hyperplasia	0	0	0	0	0	10 (1.0)
Hemosiderin	0	0	0	0	0	8 (1.0)
Red blood cell fragments	0	0	0	0	0	9 (1.0)
Forestomach						
Hyperplasia	0	0	0	0	0	7 (2.0)
Liver						
Hemosiderin	0	0	0	0	0	10 (1.0)
Necrosis	0	0	0	0	0	0
Cytoplasmic basophilia	0	0	0	0	0	0
Spleen						
Congestion	0	0	0	0	0	10 (1.9)
Hemosiderin	0	0	0	0	10 (1.1)	10 (2.0)
Hematopoietic cell proliferation	0	1 (1.0)	1 (1.0)	2 (1.0)	9 (1.2)	10 (2.0)

[a] n = 10; average severity (in parentheses) is based on the number of animals with lesions: 1, minimal; 2, mild; 3, moderate; 4, marked.

(1979) demonstrated similar results with 4-CNB; thus, the kinetics of methemoglobin formation may not directly parallel blood concentrations of the chloronitrobenzenes. Except in cats (Thompson *et al.*, 1989), there does not appear to be a great difference in the susceptibility of hemoglobin from different species to be oxidized to methemoglobin; there is a marked difference in the rate at which methemoglobin can be reduced to hemoglobin within the red cell, with the rates in rodents being higher than that observed in erythrocytes of humans (Smith, 1991). Based on this difference, humans may be more susceptible than rats or mice to toxic effects associated with the methemoglobin-producing action of the chloronitrobenzenes.

The liver was a target organ for toxic effects of 2- and 4-CNB in rats and mice, and liver weights increased in both species following 13 weeks of exposure to either chemical. In rats, 2- and 4-CNB caused increased serum activities of alanine aminotransferase and/or sorbitol dehydrogenase suggesting enzyme leakage from hepatocytes which could be attributed to decreased oxygen-carrying capacity of the red cells, related to the methemoglobinemia, resulting in mild hepatocellular hypoxia and increased cell membrane permeability. The observed increases in bile acid concentrations would also support a liver effect and would be consistent with cholestasis and/or hepatotoxicity. Hepatic hemosiderin deposition in Kupffer's cells would be consistent with increased red cell turnover. There also was evidence of cytomegaly in mice and cytoplasmic basophilia in rats, suggesting increased protein production (e.g., induction of metabolizing enzymes). Results of repeated dose disposition and metabolism studies did not, however, suggest a significant enhancement of the rate of metabolism of the chemicals (NTP, 1993). Hepatocellular necrosis and chronic inflammation was observed only in mice and was specific to the 2-CNB isomer, as only slight evidence of focal necrosis in the liver was observed in mice exposed to 4-CNB. Similar

liver lesions were observed for rats and mice after a 2-week exposure to nitrobenzene (Medinsky and Irons, 1985).

Splenic hemosiderin deposition and increased hematopoietic cell proliferation were attributed to erythrocyte injury and increased turnover; additional splenic effects, congestion and capsular fibrosis were also attributed to hemotoxicity. Similar splenic lesions occurred in rats and mice after a 2-week exposure to 10 ppm or greater nitrobenzene (Medinsky and Irons, 1985). The full spectrum of splenic effects (hemosiderin, increased hematopoietic cell proliferation, congestion, and capsular fibrosis) was manifested in rats exposed to 4-CNB and is consistent with that seen in studies of aniline and other nitroaromatic compounds known to produce methemoglobin, in particular *p*-chloroanaline (Chhabra *et al.*, 1991) which would be the presumed metabolite resulting from 4-CNB reduction. Besides these effects, aniline and aniline-related compounds also induce splenic mesenchymal neoplasias in 2-year studies in rats (Stefanski *et al.*, 1990); it could be postulated that the chloronitrobenzenes would produce similar effects in long-term studies.

A clear difference in the toxic effects of the chloronitrobenzenes is the induction of hyaline droplet accumulation in the kidney of male rats exposed to 4-CNB, but not 2-CNB. A similar isomer-specific induction of this lesion was noted with 1,2-dichlorobenzene and 1,4-dichlorobenzene (NTP, 1985, 1987); the 1,4-isomer was an inducer while the 1,2-isomer was not. In addition to hyaline droplets, other pigments accumulated in renal tubule cells of rats exposed to either 2- or 4-CNB. In a 2-week inhalation study using similar exposure concentrations, rats exposed to 4-CNB exhibited granules; the pigment was iron-positive hemosiderin and likely reflected the greater acute erythrotoxicity of 4-CNB relative to 2-CNB (NTP, 1993). In this study, granules were evident in rats exposed to either isomer by Week 13. At this time, the granules are considered to be a lipofuscin-like pigment, based on mostly iron-negative and PAS-positive staining results. The source of this pigment is uncertain. A similar PAS-positive pigment, interpreted to be lipofuscin, was observed in the nitrotoluene studies in rats; these compounds also cause methemoglobin formation, and the pigment was associated with both the *o*- and the *p*-isomers (NTP, 1992b).

The hyperplasia observed in the nasal cavity of rats exposed to 2-CNB was isomer- and species-specific and may represent a local irritant effect that is route-specific. In inhalation studies, hyperplasia of the nasal respiratory epithelium is a common finding in animals exposed to irritants and numerous examples of this phenomenon are reported (Feron *et al.*, 1986). Isomer and species specificity have been observed in studies of other nitroaromatic compounds. For example, in a 2-week inhalation study of nitrobenzene, bronchiolar hyperplasia was observed in B6C3F$_1$ mice exposed to 35 ppm or greater; no respiratory tract changes were observed for F344 rats (Medinsky and Irons, 1985). In studies of *o*-, *m*-, and *p*-nitrotoluenes in rats and mice, nasal cavity lesions developed only in mice exposed to the o-isomer (NTP, 1992b). In rats and mice administered *o*-, *m*-, and *p*-cresols, nasal cavity lesions were observed in rats exposed to the p-isomer (NTP, 1992c). Reasons for isomer and species specificity are unknown, but, they are likely due to the complex factors (e.g., regional distribution of the chemical in the nasal cavity and local tissue susceptibility) affecting the pattern of nasal cavity toxicity by inhaled chemicals (Morgan and Monticello, 1990).

Other organs evaluated in these studies demonstrated isomer- and species-specific pathological changes. An inflammatory lesion in the harderian gland in rats was noted in the 13-week 4-CNB study. This is not a common target organ in short-term toxicity studies, and there is no explanation for this finding. Sialodacryoadenitis virus (SDAV) infection may cause similar lesions, but viral titers to SDAV were negative, and other lesions associated with this infection were not present. Atrophy of seminiferous tubules was noted in rats at the highest exposure concentration of 4-chloronitrobenzene. Similar testicular degeneration in rats occurred in 13-week studies of *o*-, *m*-, and *p*-nitrotoluenes and nitrobenzoic acids (NTP, 1992b,d) and in rats and mice in 2-week inhalation studies of nitrobenzene (Medinsky and Irons, 1985). The observation of hyperplasia of the forestomach epithelium in mice exposed to 4-CNB suggests that oral exposure could have occurred in these inhalation studies, possibly through grooming activities.

In comparing the effects of 2- and 4-CNB described in these studies, it should be noted that the highest exposure concentrations were limited to 18 ppm for 2-CNB and 24 ppm for 4-CNB because of the relatively low vapor pressures of these chemicals. Although exposure concentrations were higher for 4-CNB than for 2-CNB, the degree of red cell and tissue injury was markedly greater with the 4-isomer at similar exposure concentrations as evidenced by the development of a more pronounced methemoglobinemia and responsive anemia and more extensive tissue deposition of hemosiderin with 4-CNB. No microscopic evidence of compensatory hematopoietic cell proliferation was present in rats at 13 weeks in the 2-CNB study, in contrast to the splenic and bone marrow response with 4-CNB. Additionally, except for the respiratory epithelial hyperplasia in the nasal cavity, pathological changes were demonstrated for more tissue types (e.g., kidney, testis, harderian gland, and forestomach) in animals exposed to 4-CNB.

In summary, inhalation exposure of rats and mice to 2- or 4-chloronitrobenzene resulted in methemoglobin formation and oxidative damage to red blood cells, leading to a regenerative anemia and a variety of tissue and biochemical changes secondary to erythrocyte injury. In addition, numerous other lesions considered primary toxic effects occurred

following exposure to both chemicals; these included hyaline droplet nephropathy and degeneration of the testis in male rats exposed to 4-CNB, inflammation of the harderian gland in rats exposed to 4-CNB, hyperplasia of the nasal cavity epithelium in rats exposed to 2-CNB, and hyperplasia of the forestomach epithelium in mice exposed to 4-CNB. A no-observed-adverse-effect level (NOAEL) for rats was not determined, since increases in methemoglobin and histopathological changes occurred at exposure concentrations as low as 1.1 ppm for 2-chloronitrobenzene and 1.5 ppm for 4-chloronitrobenzene in the 13-week studies. A NOAEL for histopathological injury in mice was 4.5 ppm for 2-chloronitrobenzene and 6 ppm for 4-chloronitrobenzene.

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