National Toxicology Program
Toxicity Report Series
Number 34

NTP Technical Report
on Toxicity Study of

1-Nitropyrene

(CAS No. 5522-43-0)

Administered by Inhalation
to F344/N Rats

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United States Department of Health and Human Services
Public Health Service
National Institutes of Health

Note to the Reader

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- the National Cancer Institute (NCI) of the National Institutes of Health;
- the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health;
- the National Center for Toxicological Research (NCTR) of the Food and Drug Administration; and
- the National Institute for Occupational Safety and Health (NIOSH) of the Centers for Disease Control.

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The study described in this toxicity study report was performed under the direction of NIEHS and was conducted in compliance with NTP laboratory health and safety requirements. The study met or exceeded all applicable federal, state, and local health and safety regulations. Animal care and use were in accord and compliance with the Public Health Service Policy on Humane Care and Use of Animals.

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NIH Publication 96-3383
April 1996

United States Department of Health and Human Services
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This NTP report on the toxicity study of 1-nitropyrene is based primarily on a 13-week inhalation study that began in August 1991 and ended in November 1991 at Battelle Pacific Northwest Laboratories, Richland, WA.

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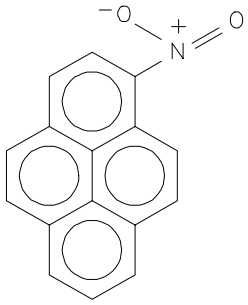
ABSTRACT

1-Nitropyrene



Molecular Formula	$C_{16}H_9NO_2$
CAS Number	5522-43-0
Molecular Weight	247.26
Synonyms	3-Nitropyrene
	Pyrene, 1-nitro

1-Nitropyrene is a by-product of combustion. It is the predominant nitrated polycyclic aromatic hydrocarbon emitted in diesel engine exhaust and has been found at concentrations of up to 57 pg/m^3 in the air over urban and suburban areas.

1-Nitropyrene is detoxified mainly to 1-aminopyrene by nitro reduction. 1-Nitropyrene can also undergo ring oxidation, depending on the concentration of oxygen. Aryl nitrenium ions generated by nitro reduction or K-region nitropyrene epoxides generated by ring oxidation can react with DNA, forming adducts.

1-Nitropyrene was nominated for toxicity study because it is mutagenic, it is found in the environment, and it has potential for human exposure. Administration by inhalation was chosen because humans are exposed to 1-nitropyrene mainly by inhalation. Nose-only inhalation was chosen because whole-body inhalation exposure would require a large quantity of purified 1-nitropyrene that is expensive and difficult to procure. The study was performed in rats because of technical problems with conducting nose-only inhalation studies in mice and because mice are known to be more resistant to 1-nitropyrene toxicity.

In the base study, groups of 10 male and 10 female 7-week-old F344/N rats were exposed to 0, 0.5, 2, 8, 20, or 50 mg/m^3 1-nitropyrene aerosol, 6 hours per day, 5 days per week, for 13 weeks. At 13 weeks, rats were evaluated for histopathology, clinical pathology, and reproductive system effects. In a supplemental evaluation, toxicokinetic effects were assessed in male F344/N rats exposed to 1-nitropyrene for 13 weeks.

All rats survived to the end of the 13-week exposure. For all groups, body weight gains of exposed rats were similar to those of concurrent controls (but lower than those of historical whole body inhalation study control rats); however, liver weights of exposed male rats were higher than those of the controls. There were slight variations in certain hematology and clinical chemistry parameters for some groups, but these were not considered related to 1-nitropyrene exposure. Squamous metaplasia of the respiratory mucosa was observed in the larynx of male rats exposed to 1-nitropyrene at a concentration of 2 mg/m^3 or greater and of female rats at all exposure concentrations. Squamous metaplasia of the bronchial epithelium also occurred in male and female rats in the higher exposure groups. Cytoplasmic alteration of the nasal respiratory epithelium was observed in both sexes exposed to 1-nitropyrene at a concentration of 8 mg/m^3 or greater. No treatment-related effects on sperm motility or vaginal cytology were noted. However, testicular atrophy was observed in all male rats and was considered a secondary effect resulting from the daily confinement within the exposure tubes.

The elimination half-life of 1-nitropyrene in the lungs was about 1 hour for rats exposed to 8 mg/m^3 and 6 hours for rats exposed to 50 mg/m^3. Lung burdens of 1-nitropyrene in rats exposed to 8 mg/m^3 remained the same for the 13-week duration; however, lung burdens in rats exposed to 50 mg/m^3 increased with time indicating that the rats were unable to clear the 1-nitropyrene between exposures. The half-life of 1-nitropyrene in the plasma of rats exposed to 50 mg/m^3 was about 1 hour.

Based on data contained in this report and previously published reports on the genetic toxicity, carcinogenicity, and toxicokinetics of 1-nitropyrene, it is the opinion of the National Toxicology Program (NTP) that 1-nitropyrene has a high likelihood of being carcinogenic to the respiratory tract, particularly under exposure conditions that lead to significant accumulations of 1-nitropyrene in the lungs, and perhaps other organs of F344/N rats.

In summary, nose-only inhalation exposure to 1-nitropyrene for 13 weeks induced squamous metaplasia of the laryngeal and bronchial respiratory epithelium in male and female rats. Cytoplasmic alteration in the nasal respiratory epithelium were also induced in male and female rats. The no-observed-adverse-effect level (NOAEL) for male rats was 0.5 mg/m^3. A NOAEL for female rats could not be determined from these studies.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft report on the toxicity study of 1-nitropyrene on November 29, 1994, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of this NTP study are appropriate and ensure that the toxicity study report presents the experimental results and conclusions fully and clearly.

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SUMMARY OF PEER REVIEW COMMENTS

On November 29, 1994, the draft Technical Report on the toxicity study of 1-nitropyrene received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Committee and associated Peer Review Panel. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.R. Bucher, NIEHS, opened the discussion by noting that at the last meeting (June 21, 1994), the Subcommittee had voted to defer final action on the draft Technical Report of the toxicity study of 1-nitropyrene to allow for obtaining a third external review and to allow for public comment. This report is the first in which the conclusion was drawn that a chemical in an NTP study is a likely carcinogen in the absence of neoplasms.

Dr. P.C. Chan, NIEHS, introduced the toxicity study of 1-nitropyrene by discussing the rationale for study; describing the experimental design for nose-only inhalation studies, including toxicokinetic studies in rats; reporting on compound-related lesions; and relating literature reports on the mutagenicity, carcinogenicity, and DNA-adduct-forming activity of 1-nitropyrene. Based on the findings in this study and in the literature, the conclusion was that sufficient evidence exists that 1-nitropyrene is carcinogenic in rats and mice. Because the International Agency for Research on Cancer (IARC) had determined that 1-nitropyrene is a rodent carcinogen and a possible human carcinogen, and because 2-year nose-only inhalation studies would be very expensive and technically difficult, the NTP decided that 2-year studies would not be conducted.

Dr. Brown asked whether the IARC conclusions were known at the time the NTP study was designed. Dr. Bucher stated that there had been no demonstration that 1-nitropyrene was carcinogenic by itself when administered by inhalation. He stated that the study provided evidence for an appropriate dose range were a 2-year study to be conducted. Dr. Brown commented that Subcommittee members had the opportunity to read the four written reviews and he sensed a consensus for approval for the report and the conclusions reached by the program.

Dr. Ryan moved that the conclusion be accepted as sufficient evidence of carcinogenic activity. Dr. Klaassen seconded the motion, which was accepted unanimously with seven votes.

INTRODUCTION

Physical Properties, Production, Occurrence, and Exposure

1-Nitropyrene is a yellow, needle-like crystalline or prismatic solid with a melting point of 155° C. It is very soluble in diethyl ether and is soluble to a lesser degree in ethanol, benzene, toluene, and tetrahydrofluorenone. 1-Nitropyrene is stable under normal laboratory conditions; however, it undergoes photodecomposition to 2-propanol under ultraviolet/visible light (Stärk *et al.*, 1985). There is no published information available on the boiling point, vapor density, or vapor pressure of 1-nitropyrene (IARC, 1989a).

1-Nitropyrene is a by-product of combustion and is the predominant nitrated polycyclic aromatic hydrocarbon emitted in diesel engine exhaust (Rosenkranz, 1982). It has been extracted at concentrations of 16 to 57 pg/m^3 from particulates in air samples collected in urban and suburban areas in Michigan (Gibson, 1982, 1986). 1-Nitropyrene is also found in coal combustion fly ash, cigarette smoke, cooked meat products, gas burner and kerosene heater emissions (IARC, 1989a), and airplane exhaust (McCartney *et al.*, 1986; Rosenkranz and Howard, 1986). 1-Nitropyrene is formed spontaneously through atmospheric reaction of nitrogen oxide with pyrene in the presence of a trace amount of nitric acid (Pitts *et al.*, 1978; Tokiwa *et al.*, 1987) and by photochemical oxidation of 1-aminopyrene under ultraviolet irradiation (Okinaka *et al.*, 1986).

1-Nitropyrene has reportedly been found in photocopy toners. However, a change in the production process of the carbon black used in photocopy toners has effectively removed the nitropyrenes. Epidemiologic studies of employees of photocopy toner manufacturers (Rosenkranz *et al.*, 1980) and carbon black producers (Robertson and Ingalls, 1980) identified no health effects that were related to exposure to these materials.

Disposition and Metabolism

After radiolabeled 1-nitropyrene was administered to rats intraperitoneally (Ball *et al.*, 1984; Ball and Lewtas, 1985), orally (Dutcher and Sun, 1983), or by intragastric gavage (El-Bayoumy and Hecht, 1984), approximately 70% to 80% of the administered dose was excreted within 4 days of dosing. Excretion via feces was two to three times greater than excretion via urine. The major 1-nitropyrene metabolites identified in the feces included 1-aminopyrene, 1-amino-6-hydroxypyrene, and 1-amino-8-hydroxypyrene; urinary metabolites included

1-amino-3-hydroxypyrene, 1-amino-6-hydroxypyrene, and 1-amino-8-hydroxypyrene (Dutcher and Sun, 1983; Ball *et al.*, 1984; El-Bayoumy and Hecht, 1984). Hydroxy-*N*-acetyl-1-aminopyrenes (6 and 8 isomers) were also identified in the urine and feces (Ball *et al.*, 1984; Ball and Lewtas, 1985; Bond *et al.*, 1986a).

In nose-only inhalation studies in which male and female F344 rats were administered [^{3}H]-1-nitropyrene at 43 ± 8 ng/L for 50 minutes, radioactivity was cleared in less than 1 hour from the respiratory system by direct absorption into the blood followed by biliary excretion (Sun *et al.*, 1983). Biliary excretion was also reported following intravenous administration of [^{3}H]-1-nitropyrene; over 60% of an intravenous dose of 0.3 or 1.2 µmol (74 or 297 µg) 1-nitropyrene was excreted in bile within 24 hours in F344 rats (Medinsky *et al.*, 1985). Bond *et al.* (1986b), after exposing male F344 rats to 50 to 1,100 ng [^{14}C]-1-nitropyrene/L by nose-only inhalation for 1 hour, reported that the amount of radioactivity excreted by the rats via feces was twice as much as that excreted via urine and that the pathway of excretion was independent of exposure concentration. Sun *et al.* (1983) reported that 76% of the radioactivity derived from inhaled [^{3}H]-1-nitropyrene was excreted via urine. This may have been due to $^{3}H_2O$ eliminated in urine as a result of ^{3}H exchange (Medinsky *et al.*, 1985). Urinary and fecal metabolites identified following inhalation exposure to 1-nitropyrene were similar to those found following intraperitoneal or oral administration (Bond *et al.*, 1986b).

Inhaled (nose-only) [^{3}H]-1-nitropyrene condensed onto inert gallium oxide particles (administered to rats at 360 ± 65 ng [^{3}H]-1-nitropyrene/L for 30 minutes) was removed by mucociliary clearance in the upper respiratory airways with subsequent ingestion as well as by absorption into the blood. Evidence of mucociliary clearance included the detection of high levels of radioactivity in the stomachs of exposed rats (Sun *et al.*, 1983). High levels of radioactivity were not observed in the stomachs of rats exposed by nose-only inhalation for 1 hour to aerosols of [^{14}C]-1-nitropyrene coated on diesel exhaust particles (50 to 1,100 ng [^{14}C]-1-nitropyrene/L); however, radioactivity in the lungs of these rats was five times greater 1 hour after exposure and 80 times greater 94 hours after exposure than the levels measured in the lungs of rats exposed to pure [^{14}C]-1-nitropyrene (Bond *et al.*, 1986b).

Following nose-only inhalation exposure of rats to 490 ng [^{14}C]-1-nitropyrene/L for 1 hour, the greatest amounts of radioactivity were retained in the respiratory tract, kidneys, urinary bladder, liver, and alimentary tract (Bond *et al.*, 1986b). Tissue clearance of ^{14}C appeared to be biphasic.

The short-term half-life of ^{14}C in the lungs, liver, and kidneys was 1, 3, and 0.5 hours, respectively, and the long-term half-life was 40, 35, and 120 hours, respectively (Bond *et al.*, 1986b).

After 25 mg 1-nitropyrene/kg was intraperitoneally injected into Wistar rats, *N*-(deoxyguanosin-8-yl)-1-aminopyrene (C8-dG-AP) was found in the kidney, liver, and mammary gland (Hashimoto and Shudo, 1985; Stanton *et al.*, 1985); the same DNA adduct was identified in the lung and liver of $B6C3F_1$ mice following intratracheal instillation of 1 mg 1-nitropyrene/kg body weight (Mitchell, 1988a).

1-Nitropyrene requires metabolic activation by nitro reduction or ring oxidation to react with DNA. Oxygen concentration plays an important role in determining the degree to which each of these activation processes occurs *in vivo*. Under anaerobic conditions, 1-nitropyrene is metabolized by xanthine oxidase, DT-diaphorase, or aldehyde oxidase in mammalian systems or by nitroreductases in bacterial systems through reduction of the nitro group to form, in sequence, the corresponding 1-nitrosopyrene, *N*-hydroxy-1-aminopyrene, and 1-aminopyrene (Figure 1; Saito *et al.*, 1984; Beland *et al.*, 1985; Rosenkranz and Howard, 1986; Wolff *et al.*, 1988). 1-Aminopyrene may be acetylated to *N*-acetyl-1-aminopyrene. The *N*-arylhydroxylamine intermediate formed during the nitro reduction undergoes spontaneous decomposition to form a reactive aryl nitrenium ion (Beland, 1991). The aryl nitrenium ion is capable of covalently bonding to DNA at the C-8 of guanine, forming the C8-dG-AP DNA adduct (Figure 1; Howard *et al.*, 1983a).

In mutagenesis assays with cultured cells deficient in nitroreductases, such as Chinese hamster ovary (CHO) cells (Heflich *et al.*, 1986), Chinese hamster V79 cells (Takayama *et al.*, 1983; Ball *et al.*, 1985), and Chinese hamster lung cells (Nakayasu *et al.*, 1982; Sugimura and Takayama, 1983), positive mutagenesis responses occurred only in the presence of rat liver S9 (Heflich *et al.*, 1990). In contrast, *Salmonella typhimurium* strains TA98 and TA100, due to the presence of nitroreductases, mutate readily when exposed to 1-nitropyrene (Mermelstein *et al.*, 1981).

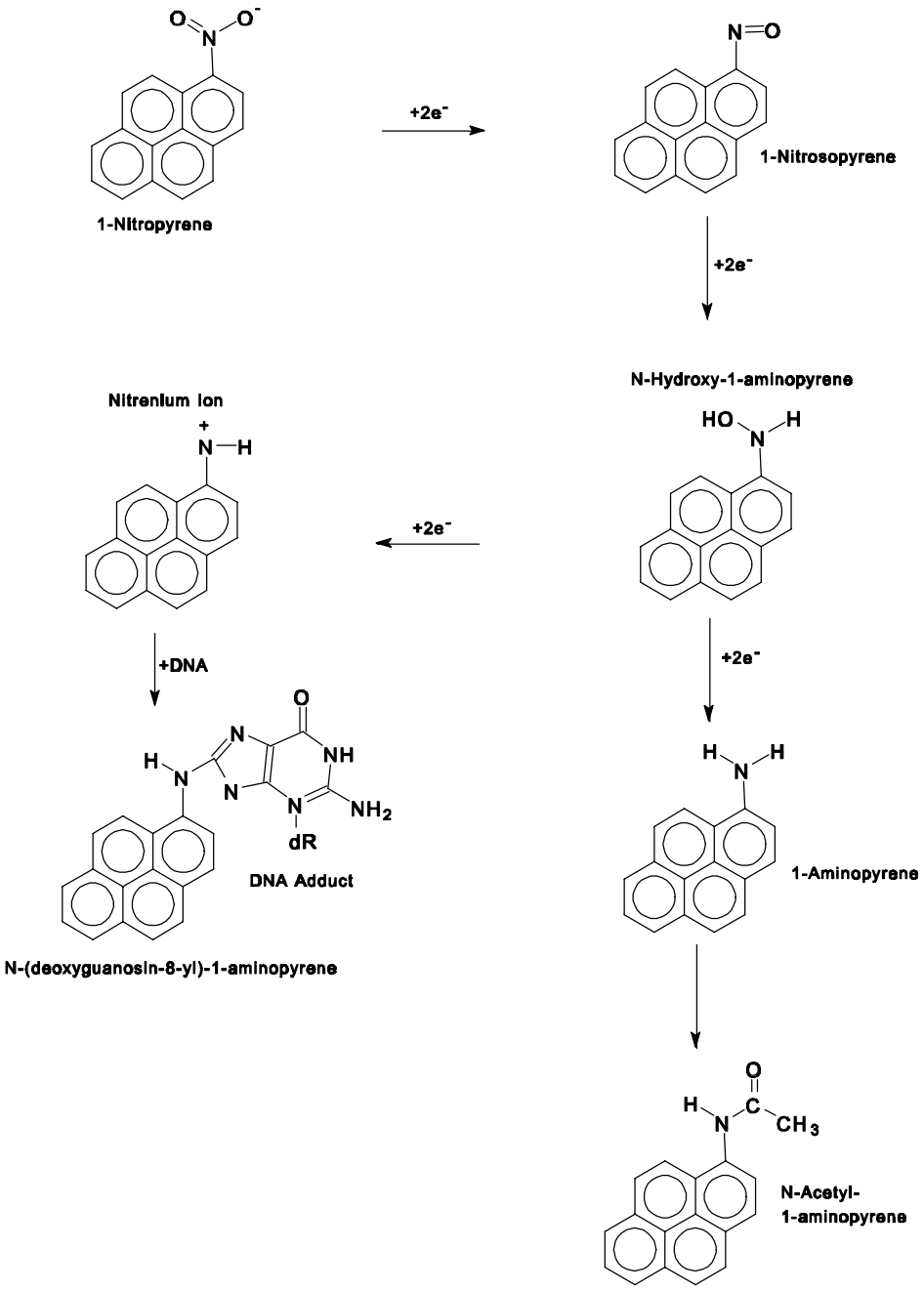

FIGURE 1 Nitro Reduction Pathway for the Cellular Activation of 1-Nitropyrene in Mammalian Cells (Edwards *et al.*, 1986; Adapted from Patton *et al.*, 1986)

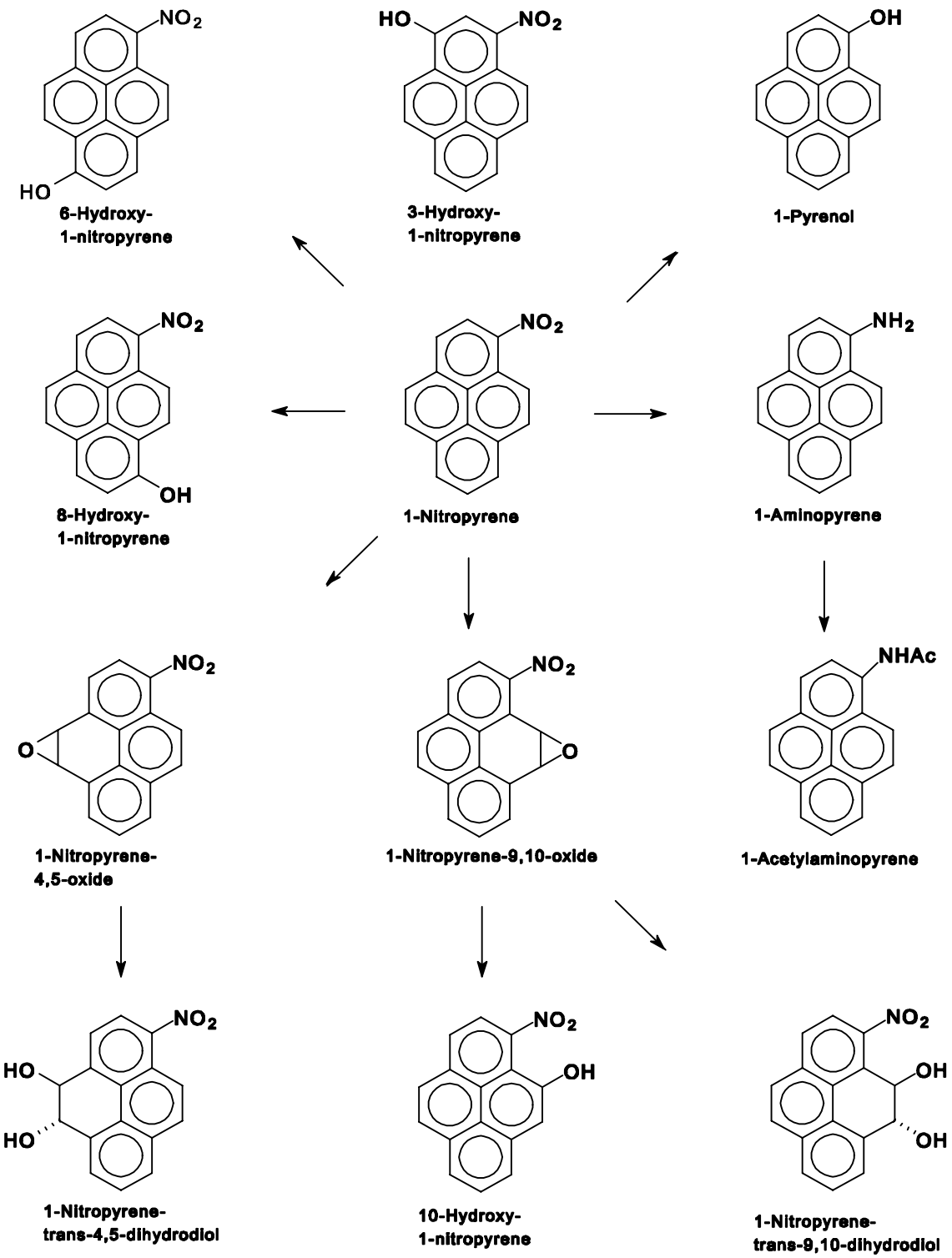

FIGURE 2 Ring Oxidation Pathway for the Cellular Activation of 1-Nitropyrene in Mammalian Cells *in vitro* (Beland, 1991)

Under aerobic conditions, 1-nitropyrene is metabolized by liver cytochrome P_{450} through ring oxidation to a mixture of 3-, 6-, and 8-hydroxy-1-nitropyrene and the K-region nitropyrene epoxides 1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-oxide (Figure 2). The K-region epoxides are capable of forming DNA adducts (El-Bayoumy and Hecht, 1983; Beland *et al.*, 1985; Djurić *et al.*, 1986; Fifer *et al.*, 1986). Pretreatment with 3-methylcholanthrene increased the binding of [^{14}C]-1-nitropyrene metabolites to tissue macromolecules in isolated, perfused, and ventilated rat lungs (Bond and Mauderly, 1984).

Further metabolism of the K-region nitropyrene epoxides to K-region nitropyrene dihydrodiols is catalyzed by the enzyme epoxide hydrolase. Inhibition of epoxide hydrolase by 1,2-epoxy-3,3-trichloropropane enhanced the mutagenicity of 1-nitropyrene at the hypoxanthine-guanine phosphoribosyltransferase locus in CHO cells; the enhanced mutagenicity can be attributed to the build-up of adduct-forming K-region epoxides (Beland, 1991).

King and Lewtas (1993) demonstrated that tracheal epithelial cells isolated from rats, hamsters, and rabbits metabolized 1-nitropyrene *in vitro* by both ring oxidation and nitro reduction mechanisms. In *in vivo* studies in female Sprague-Dawley rats, nitro-reduction appears to be minor (Roy *et al.*, 1989).

Addition of hypoxanthine and bovine milk xanthine oxidase to cultures of human fibroblasts containing 1-nitropyrene resulted in enhanced reduction of 1-nitropyrene to 1-aminopyrene (Howard *et al.*, 1983b). Bovine xanthine oxidase and hypoxanthine also enhanced covalent bonding of 1-nitropyrene to calf thymus DNA (Howard and Beland, 1982); the major DNA adduct formed was identified as C8-dG-AP.

Gut bacteria play an essential role in 1-nitropyrene metabolism and activation. When 1-nitropyrene was administered orally to conventional and germ-free F344 rats, 1-aminopyrene was detected only in the feces of conventional rats (El-Bayoumy *et al.*, 1983). In antibiotic-treated rats, a 50% decrease in covalent binding of radiolabeled 1-nitropyrene to DNA in the lungs was observed (Ayres *et al.*, 1985). Reduction of *N*-hydroxy-1-aminopyrene to 1-aminopyrene is considered to be a detoxifying step, as 1-aminopyrene is nonmutagenic and nonclastogenic in *S. typhimurium* strain TA98 (Matsuoka *et al.*, 1991) and did not bond covalently to calf thymus DNA or Chinese hamster lung fibroblast DNA to form C8-dG-AP (Edwards *et al.*, 1986).

Toxicity

ANIMAL TOXICITY

No toxic effects were observed in male or female F344 rats killed 4 or 14 days after receiving a single oral dose of 5 g 1-nitropyrene/kg body weight (Marshall *et al.*, 1982). However, when [^{3}H]-1-nitropyrene, pure (7 mg/m^3 without SO_2 or 8 mg/m^3 with SO_2) or particle-associated (8 mg/m^3 with or without SO_2), was administered by nose-only inhalation to F344 rats for up to 4 weeks, renal clearance of 1-nitropyrene-derived radioactivity was impaired. These results suggest that the kidney is a target organ (Sun *et al.*, 1985). This was corroborated by Medinsky *et al.* (1988) in a study showing high levels of covalently bound radioactivity in the kidney following inhalation exposure of male F344/N rats to [^{14}C]-1-nitropyrene.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

No information on the effects of 1-nitropyrene on reproduction or development was found in the literature.

TRANSFORMATION STUDIES

Incubation of normal human fibroblasts under anaerobic conditions with various concentrations of 1-nitropyrene led to concentration-dependent increases in anchorage-independent growth in soft agar (Howard *et al.*, 1983b). 1-Nitropyrene also induced dose-dependent increases in transformed colonies in Syrian hamster embryo cells (DiPaolo *et al.*, 1983).

CARCINOGENICITY

The purity of 1-nitropyrene in carcinogenicity studies is critical. Ohgaki *et al.* (1982) reported that when 2 mg 1-nitropyrene in DMSO was subcutaneously injected into 8-week-old male F344/DuCrj rats twice a week for 10 weeks for a total dose of 40 mg, 8 of 17 rats developed tumors at the injection site (one extraskeletal osteosarcoma and seven malignant fibrous histiocytomas). However, Ohgaki *et al.* reportedly observed no tumors at the injection site when 0.2 or 2.0 mg 1-nitropyrene was subcutaneously injected into male F344/DuCrj rats twice a week for 10 weeks for total doses of 4 or 40 mg (Ohgaki *et al.*, 1985). The authors attributed the earlier results to contamination by dinitropyrenes in the 1-nitropyrene preparation. Tokiwa *et al.* (1984) also reportedly observed no tumors at the site of subcutaneous injection in male BALB/c mice receiving 0.1 mg 1-nitropyrene

in DMSO weekly for 20 weeks. In contrast, Odagiri *et al.* (1986) reported induction of mammary gland adenocarcinomas in female F344/Jcl rats receiving 5, 10, or 20 mg/kg 1-nitropyrene (with 0.6% dinitropyrenes) in DMSO and olive oil, by intragastric instillation, twice a week for 55 weeks.

1-Nitropyrene does not act as a skin tumor initiator in mice. Female CD-1 mice receiving a dermal application of 0.1 mg 1-nitropyrene every other day for a total of 10 doses, followed by promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), did not have an increased incidence of papillomas compared to the controls (El-Bayoumy *et al.*, 1982). In addition, the incidence of skin papillomas was not increased in male or female SENCAR mice receiving two dermal applications of 3 mg 1-nitropyrene or a single intraperitoneal dose of 8 mg 1-nitropyrene, followed by promotion with TPA (Nesnow *et al.*, 1984).

1-Nitropyrene is carcinogenic in mice; male mice are more sensitive than female mice. When 1-nitropyrene (>99% pure) was injected intraperitoneally into A/J mice (17 injections in 6 weeks for a total dose of 175, 525, or 1,575 mg/kg), an increased incidence of lung tumors (7/32 control; 22/28, 1,575 mg/kg) was observed in the highest dose group (El-Bayoumy *et al.*, 1984). Newborn male CD mice injected intraperitoneally with 1-nitropyrene (99% pure) on Days 1, 8, and 15 after birth for a total dose of 0, 173, or 692 µg had liver cell tumor incidences of 2/28, 5/34, and 8/29, respectively (Wislocki *et al.*, 1986). Newborn male CD mice injected with 173 µg/kg of the probable proximate carcinogen 1-nitrosopyrene at similar times had a liver cell tumor incidence of 14/31. Newborn female CD mice receiving the same 1-nitropyrene doses as those given to the male CD mice developed no liver cell tumors; however, those receiving 173 µg 1-nitrosopyrene/kg had a liver cell tumor incidence of 3/34. Lung tumor incidences in the dosed mice were similar to those in the controls (Wislocki *et al.*, 1986).

1-Nitropyrene is carcinogenic in rats; Sprague-Dawley rats are more sensitive than CD rats. Hirose *et al.* (1984) reported induction of malignant fibrous histiocytomas (control, 0%; males, 32%; females, 28%) at the site of subcutaneous injection of highly purified 1-nitropyrene (25 mg/kg weekly for 8 weeks) in newborn male and female Sprague-Dawley rats. In addition, an increased incidence of mammary gland tumors (47% vs. 4%) occurred in the dosed females. Similar findings were also reported by El-Bayoumy *et al.* (1988). In contrast, newborn female CD rats receiving subcutaneous injections of 1-nitropyrene in dimethyl sulfoxide (DMSO) weekly for 8 weeks (total dose: 15.5 mg) and weanling female CD rats receiving 1-nitropyrene weekly

for 5 weeks (total dose: 19 mg) did not have increased incidences of mammary gland tumors (King, 1988). Weanling (30-day-old) female CD rats receiving a single injection of 2 µmol (494.5 µg) 1-nitropyrene in the mammary gland also did not have an increased incidence of mammary gland tumors (Imaida *et al*., 1991). On the other hand, El-Bayoumy *et al*., (1995) reported that 30-day-old female CD rats receiving eight weekly doses of 50 µmol 1-nitropyrene developed benign mammary gland tumors.

Thirty-day-old female CD rats intraperitoneally injected with 67 µmol 1-nitropyrene/kg (17 mg/kg) three times per week for 4 weeks did not have a greater incidence of mammary gland tumors than the controls 61 weeks after the first dose was administered (Imaida *et al*., 1985, 1991). However, 30-day-old female CD rats intraperitoneally or subcutaneously injected with 100 µmol 1-nitropyrene/kg (24.7 mg/kg) weekly for 4 weeks had a greater incidence of mammary gland tumors than the controls (59% vs. 37%, respectively) 87 to 90 weeks after the first dose was administered (Imaida *et al*., 1991). The authors explained that 1-nitropyrene is a weak carcinogen, because the mammary gland tumors required a long time to develop, and they ruled out contamination of the 1-nitropyrene by dinitropyrene. Female weanling CD rats receiving 1-nitropyrene (>99.9% pure) intraperitoneally had increased incidences of mammary gland tumors when exposed to 2.5 mg/kg three times per week for 4 weeks (control: 7/31; 2.5 mg/kg: 25/36) or to 25 mg/kg weekly for 5 weeks (control: 11/30; 25 mg/kg: 17/29) (King, 1988). However, a single 1.5 mg dose of 1-nitropyrene injected directly into the lungs of male F344/DuCrj rats did not induce any tumors (Maeda *et al*., 1986).

El-Bayoumy *et al*. (1988) reported induction of mammary gland tumors by 1-nitropyrene (excluding dinitropyrene by GC-MS analysis) following oral administration to newborn female Sprague-Dawley rats at doses of 100 µmol/kg (24.7 mg/kg) or 250 µmol/kg (61.8 mg/kg). Also, Denda *et al*. (1989) reported induction of liver γ-glutamyltranspeptidase (GGT)-positive foci when 1-nitropyrene was administered by intragastric intubation at doses of 100 to 1,000 mg/kg daily for 6 days to 6-week-old male F344 rats. The rats underwent partial hepatectomy 4 hours after the fourth dose of 1-nitropyrene. Two weeks after partial hepatectomy, the rats were placed on a 0.02% 2-acetylaminofluorene (AAF) diet and received a single intragastric dose of carbon tetrachloride 1 week later; AAF feeding was conducted for a total of 2 weeks. The rats were killed 5 weeks after the final dose of 1-nitropyrene. No increase in the incidence of GGT-positive foci was observed in rats receiving a single dose of 1,000 mg 1-nitropyrene/kg and placed on the same regimen (Denda *et al*., 1989).

The reduced acetylated metabolites *N*-hydroxy-*N*-acetyl-1-aminopyrene and *N*-acetyl-1-aminopyrene injected intraperitoneally into 30-day-old female CD rats at doses of 67 µmol/kg (16.6 mg/kg) three times per week for 4 weeks did not induce mammary gland tumors. However, both metabolites induced hepatic neoplasms (Imaida *et al.*, 1991).

GENETIC TOXICITY

1-Nitropyrene has demonstrated genotoxic activity in a wide variety of assays. The genotoxic effects of nitroarenes were reviewed by Rosenkranz and Mermelstein (1983) and Tokiwa and Ohnishi (1986). The most recent review of the genetic toxicity data for 1-nitropyrene is included in a comprehensive review of a group of nitroarenes presented in an IARC Monograph (IARC, 1989a,b). Results of subsequent reported studies have supported the conclusions of these reviews.

1-Nitropyrene induced DNA damage in *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium*. It was mutagenic in *E. coli* and several strains of *S. typhimurium*, inducing both frameshift and base-pair substitution mutations (IARC, 1989a). In addition, urine and bile extracts from male rats receiving 1-nitropyrene intraperitoneally were mutagenic in *S. typhimurium* (IARC, 1989a).

1-Nitropyrene induced gene mutations in human diploid fibroblasts and in a human hepatoma-derived cell line (IARC, 1989a), as well as in mouse lymphoma L5178Y cells (IARC, 1989a) and Chinese hamster ovary cells (Heflich *et al.*, 1990). It also induced DNA single-strand breaks in primary mouse hepatocytes, Chinese hamster ovary cells, and cultured rat hepatoma cells (IARC, 1989a). Unscheduled DNA synthesis was induced in cultured mouse, rat, hamster, and human hepatocytes (Yoshimi *et al.*, 1987; IARC, 1989a; Shaddock *et al.*, 1989) and in cultured human and rat tracheal epithelial cells (IARC, 1989a). Chromosomal aberrations and sister chromatid exchanges (SCEs) were induced in Chinese hamster ovary cells (IARC, 1989a; Matsuoka *et al.*, 1991).

In vivo, SCEs were induced in bone marrow cells of hamsters administered 125 mg 1-nitropyrene/kg and rats administered 0.5 to 5 mg 1-nitropyrene/kg (IARC, 1989a). In addition, the frequency of micronuclei in bone marrow erythrocytes of Chinese hamsters was increased after intraperitoneal injection of 1,000 mg 1-nitropyrene/kg (IARC, 1989a). 1-Nitropyrene

bonded to DNA in target tissues (i.e., liver, lung) of rats and mice treated *in vivo* (IARC, 1989a), and DNA single-strand breaks were observed in lung cells of mice following intratracheal instillation of 1-nitropyrene (Mitchell, 1988b).

Studies were performed with 1-nitropyrene to investigate some of the mechanisms and characteristics involved in the formation of DNA adducts. The molecular mechanisms responsible for 1-nitropyrene mutagenicity involved reductive production of arylhydroxylamines that react with DNA, with or without further esterification or the production of reactive epoxides. DNA adducts were detected by [^{32}P]-postlabeling techniques in the livers of newborn male CD-1 mice receiving three intraperitoneal injections of 1-nitropyrene (total dose of 2,800 nmol) and in the lungs of adult male A/J mice receiving 17 intraperitoneal injections of 1-nitropyrene (total dose of 1,575 mg/kg) (Smith *et al.*, 1990). DNA adducts were also detected in mammary gland tissue and at the injection site in adult Sprague-Dawley rats receiving eight subcutaneous injections of 100 µmol 1-nitropyrene/kg, starting within 24 hours of birth, for a total dose of 800 µmol/kg (Smith *et al.*, 1990).

Study Rationale and Design

1-Nitropyrene was nominated for toxicity testing by the American Federation of State, County and Municipal Employees because it is mutagenic, has been found in the environment (in photocopy toners and diesel exhaust particles), and has potential for human exposure. Inhalation was selected as the route of administration for this study in F344/N rats because human exposure to 1-nitropyrene is mainly through this route and because long-term inhalation studies have not been conducted. Nose-only inhalation was conducted due to the high cost and difficulty involved in procuring a large quantity of purified 1-nitropyrene and to minimize ingestion during exposure. A high dose of 50 mg/m^3 was selected because 1-nitropyrene was not toxic to F344 rats when administered orally at 5 g/kg (Marshall *et al.*, 1982) and because 50 mg/m^3 was considered a reasonable maximal dose for a nose-only inhalation study of 1-nitropyrene, a chemical with low acute toxicity. In the 13-week base study, gross and histopathologic examinations and clinical pathology analyses were performed. In a supplemental evaluation, the toxicokinetics of 1-nitropyrene were assessed in male F344/N rats exposed to the chemical by nose-only inhalation for 13 weeks. The study was performed only in rats because of the limited supply of purified 1-nitropyrene, the unavailability of equipment appropriate for conducting nose-only inhalation

studies in mice, and the reported lower sensitivity of mice to 1-nitropyrene toxicity (El-Bayoumy *et al.*, 1984).

MATERIALS AND METHODS

Procurement and Characterization of 1-Nitropyrene

A single lot of 1-nitropyrene was obtained from Chemsyn Science Laboratories (Lenexa, KS). Lot CSL-89-219-63-6 was used throughout the study.

Initial identity and purity studies were performed on Lot CSL-T-10-85-21-36, which was not used in the study, by Midwest Research Institute (MRI; Kansas City, MO). The chemical, a yellow powder, was identified as 1-nitropyrene by infrared, ultraviolet, and nuclear magnetic resonance (NMR) spectroscopy and by a comparison of sample and literature melting point values. Elemental analyses for carbon, hydrogen, and nitrogen, Karl Fischer water analysis, thin-layer chromatography, and high-performance liquid chromatography (HPLC) indicated an overall purity of approximately 99% for Lot CSL-T-10-85-21-36.

Infrared and NMR spectra of Lot CSL-89-219-63-6 were consistent with the structure of 1-nitropyrene, with literature references (*Sadtler Standard Spectra*; Martin *et al.*, 1965; Pouchert, 1985), and with the spectra of Lot CSL-T-10-85-21-36. Concomitant analysis of the two lots of 1-nitropyrene by HPLC indicated a purity of 100% ± 1% for Lot CSL-89-219-63-6 relative to Lot CSL-T-10-85-21-36, consistent with a purity greater than 99%.

Accelerated stability studies performed by MRI on Lot CSL-T-10-85-21-36 by HPLC indicated that bulk 1-nitropyrene was stable for 2 weeks when stored protected from light at temperatures up to 60° C. At the study laboratory, 1-nitropyrene was stored in the dark at room temperature. The study laboratory reanalyzed the bulk chemical by HPLC before the start of the study and again after the study ended; no degradation of 1-nitropyrene was observed.

Aerosol Generation System

1-Nitropyrene aerosol was produced with a heated nebulizer assembly (Battelle Pacific Northwest Laboratories, Richland, WA) and conveyed by an aerosol distribution system to Cannon flow-past nose-only exposure units where rats were confined in exposure restraint tubes. The nebulizer assembly consisted of a glass tube reservoir, heater, nebulizer, and three-way output adapter with openings into the aerosol output, nitrogen, and aerosol distribution tubes. The output adapter was enclosed in an insulated glass cylinder with an insulated metal cap to reduce heat loss and protect

the aerosol from light. Bulk 1-nitropyrene in the reservoir was melted by the heater (approximately 230° C). Heat transfer to the reservoir and nebulizer was aided by the upward flow of compressed air. Melted 1-nitropyrene flowed upward through a liquid draft tube to the nebulizer head where it met a stream of preheated nitrogen (approximately 130° C) flowing through the nebulizer at 3.5 L/minute. As the aerosol formed, a shroud on the nebulizer head screened the large droplets by impaction and the fine droplets by diffusion, allowing only droplets with diameters of approximately 0.5 to 5 µm to reach the aerosol output tube. 1-Nitropyrene aerosol flowed from the nebulizer to the output adapter and into the distribution system. At the inlet of the distribution system, the droplets were diluted with air and cooled to form an aerosol of solid particles of 1-nitropyrene, which was conveyed to the exposure units.

At each exposure unit location, an Air-Vac® pump (Air-Vac Engineering, Inc., Milford, CT) siphoned material from a six-branch manifold on the distribution line into the exposure unit inlet. The flow rate through each exposure unit was controlled by diluters and the Air-Vac® pumps and was monitored by differential pressure gauges coupled to a Venturi tube that was mounted at the inlet of each unit. In the exposure unit inlet, the aerosol was diluted to the target concentration with conditioned, filtered air; the concentration in each exposure unit was controlled by a meter which regulated the ratio of aerosol to dilution air entering the unit. Unused and exhaust aerosol was removed from the distribution line by a high-efficiency particulate (HEPA) filter (American Air Filter, Louisville, KY). Rats were exposed to the aerosol in confinement tubes that were connected to exposure ports branching from the exposure units. There were 56 exposure ports per exposure unit. Each exposure unit was enclosed in a rigid clear plastic cabinet to prevent contamination of the room by the 1-nitropyrene aerosol.

During the study, nebulizers were cleaned after each exposure and were occasionally replaced; new nebulizers were tested before use to determine the 1-nitropyrene concentration and particle size distribution generated. The Air-Vac® pumps and Venturi tubes were periodically cleaned and the pumps were periodically replaced during the exposure periods to ensure sufficient air flow and uniform exposure. Tests indicated that the pump replacements did not have a significant impact on particle size distribution.

Concentration Monitoring

1-Nitropyrene aerosol concentrations were monitored with three on-line RAM-1 realtime aerosol monitors, or RAMs (MIE, Inc., Bedford, MA). Samples from the exposure units first flowed through sample lines designed to reduce aerosol particle losses due to settling or impaction and then flowed into the RAMs. Results from the RAMs were automatically recorded by an automated data acquisition and control system. An HP85B computer (Hewlett-Packard, Palo Alto, CA) remotely controlled the selection of the correct sample stream and the acquisition of data from each RAM. Each RAM was calibrated daily by correlating the voltages measured by the RAM with 1-nitropyrene concentrations determined by off-line HPLC analysis of exposure unit filter samples; the HPLC was calibrated with gravimetrically prepared standard solutions of 1-nitropyrene.

Mean exposure unit concentrations of 1-nitropyrene during the study were calculated from daily monitoring data (Table 1). The mean concentrations in all exposure units were between 93% and 101% of target concentrations, with relative standard deviations ranging from 13% to 21%. At least 80% of all individual concentration measurements were within 20% of the target concentrations.

TABLE 1 Mean Exposure Unit Concentrations of 1-Nitropyrene in the 13-Week Inhalation Study

Target Concentration (mg/m^3)	Mean ± SD	Target ± RSD[1]	Maximum	Minimum	Samples within Range[2] (%)
0	<MDL[3]	)	<QL[4]	<MDL	)
0.5[5]	0.51 ± 0.09	101.1 ± 17.4	0.97	0.13	80
2.0	1.99 ± 0.41	99.6 ± 20.7	6.93	<QL	81
8.0	7.78 ± 1.05	97.2 ± 13.5	13.0	3.62	89
20	19.9 ± 3.40	99.6 ± 17.0	33.6	6.39	82
50	46.6 ± 6.50	93.1 ± 14.0	68.2	22.1	85

[1] Mean concentration ± relative standard deviation as a percent of target concentration.
[2] A sample was considered to be in range if it was within 20% of the target concentration.
[3] MDL = minimum detectable limit. For the 0, 0.5, and 2.0 mg/m^3 exposure levels, MDL = 0.005 mg/m^3; for the 8.0 mg/m^3 exposure level, MDL = 0.015 mg/m^3; for the 20 and 50 mg/m^3 exposure levels, MDL = 0.083 mg/m^3.
[4] QL = quantitation limit, 0.012 mg/m^3.
[5] The 0.5 mg/m^3 group includes an additional exposure day because of the presence of animals used in the toxicokinetic evaluations.

Due to the high concentrations of 1-nitropyrene required in the distribution lines, slight changes in pump pressure or dilution air flows resulted in significant changes in concentration in the 0.5 mg/m^3 unit and, to a lesser degree, the 2 mg/m^3 unit. Additionally, 1-nitropyrene accumulated in the distribution lines and Air-Vac® pumps and occasionally broke loose and caused brief increases in exposure concentrations.

Exposure Unit Characterization

PARTICLE SIZE DISTRIBUTION

The mass median aerodynamic diameter (MMAD) of the aerosol particles in each exposure unit was measured monthly. Cascade impactor samples (Mercer-style seven-stage impactor; In-Tox Products, Albuquerque, NM) were taken from each exposure unit, and the impactor stages were analyzed for 1-nitropyrene content with HPLC. The relative mass collected on each stage was analyzed by NEWCAS probit analysis. The mean MMADs ranged from 1.6 to 2.1 µm, with geometric standard deviations of 2.2 to 2.5; these results were within the acceptable range of 1 to 3 µm.

Bubbler samples from the 50 mg/m^3 exposure unit were analyzed by HPLC to determine the amount of 1-nitropyrene present in the vapor state relative to the aerosol. HPLC indicated less than 0.2% 1-nitropyrene vapor relative to the aerosol concentration.

CONCENTRATION UNIFORMITY

The uniformity of aerosol concentration within and between exposure ports in each exposure unit was measured with the on-line RAMs before the start of the study. For all exposure units except the 0.5 mg/m^3 unit, the within-port and between-port variability was within the specified limits of ± 5%, and the total port variability was within the specified limits of ± 7%. The 0.5 mg/m^3 unit had a within-port variability of 8.3% and a total port variability of 8.2%, due to concentration fluctuations.

CONCENTRATION BUILDUP AND DECAY

The time following the start of exposure for the 1-nitropyrene aerosol concentration to reach 90% of the final stable concentration in the exposure unit (T_{90}) and the time following the termination of generation for the aerosol concentration to decay to 10% of the stable concentration (T_{10}) were

determined. Measurements were taken without animals present before the start of the study; T_{90} ranged from 1 to 2 minutes and T_{10} was 1 minute. A T_{90} of 5 minutes was used for the study.

STABILITY STUDIES

The stability of 1-nitropyrene in the glass nebulizer reservoir before and after aerosol generation and in the entire aerosol generation system during exposure periods was confirmed by HPLC. Reservoir samples were analyzed by major peak comparison against a reference sample of 1-nitropyrene. Samples were collected from the unoccupied 50 mg/m^3 exposure unit during aerosol generation and from occupied 0.5 and 50 mg/m^3 exposure units during the first and last hours of exposure; the samples were analyzed for impurities by HPLC. The samples from the occupied exposure chambers were screened for 4-nitropyrene; 1,3-, 1,6-, and 1,8-dinitropyrenes; pyrene; 1-hydroxypyrene; 1-aminopyrene; 1,6- and 1,8-pyrenequinones; and 2-nitropyrene. Additional samples were taken from the aerosol generation system and analyzed for the isomer 2-nitropyrene by HPLC under conditions that were more sensitive to 2-nitropyrene. No degradation products of 1-nitropyrene were detected during the study.

Toxicity Study Designs

BASE STUDY

Male and female F344/N rats used in the study were obtained from Taconic Farms (Germantown, NY). The rats were approximately 5 weeks old at receipt and were quarantined for 14 or 15 days. During the quarantine period, rats were acclimated to confinement in the exposure tubes 6 hours per day. Rats were approximately 7 weeks old when the study began. Blood samples were collected from 10 sentinel female rats 3 weeks after receipt and from five male and five female control rats at the end of the study. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b); all results were negative. Additional details concerning the study design are provided in Table 2.

Ten rats of each sex were exposed to 0, 0.5, 2, 8, 20, or 50 mg/m^3 1-nitropyrene aerosol through nose-only inhalation for approximately 6 hours per day, 5 days per week, except weekends and holidays, for 13 weeks. Rats were exposed for at least 2 consecutive days before necropsy.

Rats were housed individually between exposures and in individual exposure tubes during exposures. Softened water (City of Richland) and NIH-07 Open Formula Diet (Zeigler Bros., Inc.,

Gardners, PA) in pellet form were available *ad libitum* except during the exposure periods. The animal room was maintained at 69° to 75° F with 40% to 70% relative humidity and 12 hours of fluorescent light per day.

Complete necropsies were performed on all base-study rats. The heart, right kidney, liver, lungs, right testis, and thymus of each rat were weighed. Organs and tissues were examined for gross lesions and fixed in 10% neutral buffered formalin. Tissues to be examined microscopically were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all rats in the control and 50 mg/m^3 groups. The lungs, nasal cavity, and larynx were identified as target organs and examined, in addition to gross lesions, in all lower exposure groups. For all paired organs (*i.e.*, kidney, ovary, and adrenal gland), samples from each organ are examined. Tissues examined microscopically are listed in Table 2.

Upon completion of the laboratory pathologist's histologic evaluation, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed. Results were reviewed and evaluated by the NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al*. (1985).

SUPPLEMENTAL EVALUATIONS

Clinical Pathology

Clinical pathology evaluations were conducted on all base-study rats at the end of the study. Rats were anesthetized with a 70:30 CO_2:room air gas mixture and blood samples were drawn from the retroorbital plexus. Blood for hematology was placed in Vacutainer® tubes (Becton-Dickinson; Rutherford, NJ) containing potassium EDTA as the anticoagulant. Blood for clinical chemistry evaluations was placed in tubes with separator gel but no anticoagulant and allowed to clot at room temperature; the samples were then centrifuged and the serum was removed. All hematologic and biochemical analyses were performed on the day of sample collection.

Hematology determinations were performed with an Ortho ELT-8/ds Hematology Analyzer (Ortho Diagnostic Systems, Westwood, MA). The parameters evaluated are listed in Table 2. Manual

hematocrit determinations were performed with a Damon/IEC MB microcentrifuge and Damon/IEC capillary reader (International Equipment Company, Needham Heights, MA). Differential leukocyte counts were determined with a Wescor 7100 Aerospray slide stainer (Wescor, Logan, UT). Smears made from blood samples stained with new methylene blue were examined microscopically using a Miller disc for the quantitative determination of reticulocytes.

Clinical chemistry determinations were made with a Roche Cobas Fara Chemistry Analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ) or an Abbott VP Bichromatic Chemistry Instrument (Abbott Laboratories, Irving, TX). The parameters evaluated are listed in Table 2. Reagents for assay of sorbitol dehydrogenase activity were obtained from Sigma Chemical Company (St. Louis, MO); reagents for the other endpoints were obtained from the manufacturer.

Sperm Motility and Vaginal Cytology

At the end of the 13-week study, vaginal cytology and sperm motility evaluations were performed on base-study rats (10 rats per sex) from the 0, 8, 20, and 50 mg/m^3 groups. Methods were those outlined in the National Toxicology Program's Sperm Motility Vaginal Cytology Evaluation protocol (NTP, 1987). Briefly, for the 12 days before sacrifice, the vaginal vaults of 10 female rats per exposure group were lavaged, and the aspirated lavage fluid and cells were stained with Toluidine Blue. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (*i.e.*, diestrus, proestrus, estrus, and metestrus).

Sperm motility was evaluated at necropsy in the following manner. The left testis and epididymis were weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body and weighed. Test yolk buffer was applied to slides, and a small incision was made in the cauda. Then sperm effluxing from the incision were dispersed in the buffer on the slides and the numbers of motile and nonmotile spermatozoa were counted for five microscopic fields per slide by two observers.

Following completion of sperm motility estimates, each left cauda was placed in phosphate-buffered saline solution. Caudae were finely minced and the tissue was incubated and then heat fixed. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, testicular spermatid head count was determined by

removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Toxicokinetics

A preliminary toxicokinetic study was performed to develop and validate analytical methods to be used in the 13-week toxicokinetic study and to estimate the elimination half-life of 1-nitropyrene in the lungs of exposed rats. Twelve supplemental male rats were exposed to 8 mg 1-nitropyrene/m^3 by nose-only inhalation for 6 hours on 1 day. Following exposure, lung tissue samples were collected from two or three rats per time point at five time points (15 minutes and 2, 12, 24, and 72 hours) and analyzed for 1-nitropyrene content.

A 13-week toxicokinetic study was performed to determine the potential for 1-nitropyrene to accumulate in the lungs and plasma of exposed rats and to determine the clearance rate of 1-nitropyrene from the lungs and plasma following the last exposure (Week 13). Supplemental male rats (23 per exposure group) were exposed to 0.5, 8, or 50 mg 1-nitropyrene/m^3 by nose-only inhalation for approximately 6 hours per day, 5 days per week, for 13 weeks. To measure 1-nitropyrene build-up in the lungs and plasma over time, samples were collected within 1 hour after the end of exposure on Days 8 and 36 and at Week 13. At Days 8 and 36, samples were collected from three rats per exposure group; at Week 13, samples were collected from four to seven rats per exposure group. To measure the rate of clearance and half-life of 1-nitropyrene in the lungs and plasma, tissue samples were collected from one to four rats per time point per exposure group at intervals immediately following the last exposure. The samples taken within 1 hour after exposures ended at Week 13 and used to measure 1-nitropyrene accumulation were also used to calculate clearance rate and elimination half-life, along with additional samples taken at later postexposure time points. For the 0.5 mg/m^3 group, samples were collected at 10 minute intervals for up to 90 minutes and at 2 hours. For the 8 and 50 mg/m^3 groups, samples were collected at 10 or 20 minute intervals for up to 1 hour and at 2, 4, 6, 16, and 24 hours.

All samples were analyzed for 1-nitropyrene content except the plasma samples from the 0.5 mg/m^3 group, which were not analyzed due to the low 1-nitropyrene concentrations observed in the plasma of rats in the 8 mg/m^3 group. Clearance rates were modeled assuming first-order clearance. Clearance rate and half-life calculations were based on the following expressions:

$C(t)=C_o e^{-kt}$ and $t_{1/2}=\ln(2/k)$, where t is the time following the end of exposure, C(t) is the lung or plasma concentration at time t, C_o is the lung or plasma concentration at the time exposure was terminated (t=0), k is the first order clearance rate constant, and $t_{1/2}$ is the clearance half-life.

For all toxicokinetic analyses, rats were anesthetized with 70% CO_2 and blood samples were collected from the retroorbital sinus into tubes containing EDTA. The samples were centrifuged, and the separated plasma was stored in polypropylene centrifuge tubes. The rats were then killed with approximately 70% CO_2 and both lungs were removed and frozen in liquid nitrogen. Lungs and plasma were stored at approximately -70° C until analysis.

To prepare lung tissue for analysis, approximately 1 g of tissue was placed in a vial with saturated ammonium chloride, deionized water, and [^{2}H]-1-nitropyrene internal standard. Following homogenization, ethyl acetate was added and the vial was vortexed and centrifuged. The ethyl acetate extract was transferred to an amber glass vial and dried over low heat under a stream of nitrogen. The extract was then reconstituted in 70:30 acetonitrile:water, sonicated, vortexed, and centrifuged to remove solids. The sample was further purified by solid phase extraction and reconstituted in a solution of acetonitrile containing 9-nitrophenanthrene, which was added to deactivate sites on the gas chromatograph column that could react with 1-nitropyrene and the internal standard.

To prepare plasma samples for analysis, [^{2}H]-1-nitropyrene internal standard, potassium sulfate, and benzene were added to 1 mL plasma samples; the samples were then vortexed and centrifuged. The benzene extract was then removed and dried under a nitrogen stream and reconstituted in acetonitrile containing 9-nitrophenanthrene. Lung tissue and plasma samples were analyzed by gas chromatography/mass spectroscopy.

TABLE 2 Experimental Design and Materials and Methods in the 13-Week Inhalation Study of 1-Nitropyrene

EXPERIMENTAL DESIGN

Study Laboratory
Battelle Pacific Northwest Laboratories, Richland, WA

Strain and Species
F344/N Rats

Animal Source
Taconic Farms, Germantown, NY

Size of Study Groups
10 male and 10 female rats per exposure group

Route of Administration
Nose-only inhalation

Exposure Concentrations/ Duration
0, 0.5, 2, 8, 20, or 50 mg/m^3, approximately 6 hours per day, 5 days per week for 13 weeks

Date of First Exposure
Males: 8 August 1991
Females: 9 August 1991

Date of Last Exposure
Males: 5 November 1991
Females: 6 November 1991

Date of Necropsy
Males: 6 November 1991
Females: 7 November 1991

Type and Frequency of Observation
Rats were observed twice daily and were weighed initially, weekly thereafter, and at necropsy. Clinical signs were recorded weekly.

Necropsy and Histologic Examinations
Complete necropsies were performed on all rats from the base study. The heart, right kidney, liver, lungs, right testis, and thymus of each rat were weighed. Histopathologic evaluations were performed on all rats in the control and 50 mg/m^3 groups. The following tissues were examined: adrenal glands, brain (3 sections), clitoral glands, esophagus, eyes (if grossly abnormal), femur and marrow, gross lesions and tissue masses, heart, kidneys, large intestine (cecum, colon, rectum), larynx, liver, lungs, lymph nodes (bronchial, mandibular, mediastinal, and mesenteric), mammary gland, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pharynx (if grossly abnormal), pituitary gland, preputial glands, prostate, salivary glands, seminal vesicle, small intestine (duodenum, jejunum, ileum), spinal cord and sciatic nerve (if neurologic signs were present), spleen, stomach (forestomach and glandular stomach), testes (with epididymis), thigh muscle, thymus, thyroid gland, trachea, urinary bladder, and uterus. The larynx, lungs, nasal cavity, and gross lesions were examined in all lower exposure groups.

Supplemental Evaluations
Clinical Pathology Study:
Blood for hematology and clinical chemistry evaluations was collected from base-study rats at the end of the study. Hematology parameters evaluated included automated and manual hematocrit, hemoglobin concentration, erythrocyte count, reticulocyte count, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, platelet count, and leukocyte count and differential. Clinical chemistry parameters evaluated included urea nitrogen, creatinine, glucose, total protein, albumin, globulin, albumin/globulin ratio, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids.

TABLE 2 Experimental Design and Materials and Methods in the 13-Week Inhalation Study of 1-Nitropyrene (continued)

EXPERIMENTAL DESIGN (continued)

Supplemental Evaluations (continued)
Sperm Motility and Vaginal Cytology Evaluations:
Sperm motility and vaginal cytology evaluations were performed on base-study rats at the end of the study. Rats in the 0, 8, 20, and 50 mg/m^3 groups were evaluated. Male rats were evaluated for necropsy body and reproductive tissue weights, spermatozoal data, and spermatogenesis. Females were evaluated for necropsy body weight, estrous cycle length, and the percent of cycle spent in the various stages.

Preliminary Toxicokinetic Study:
A preliminary toxicokinetic study was performed on 12 supplemental male rats. Rats received a single 6-hour exposure to 8 mg/m^3 1-nitropyrene. At five time points following exposure (15 minutes and 2, 12, 24, and 72 hours), lung tissue samples were collected from two or three rats per time point and analyzed for 1-nitropyrene content. Rate of clearance and elimination half-life of 1-nitropyrene in the lungs were calculated.

13-Week Toxicokinetic Study:
A toxicokinetic study was performed on supplemental rats (23 males per exposure group) exposed to 0.5, 8, or 50 mg/m^3 1-nitropyrene for 6 hours per day, 5 days per week for 13 weeks. To measure the accumulation of 1-nitropyrene over time in the lungs and plasma of exposed rats, lung tissue and plasma samples were collected at time points within 1 hour after the end of exposure at Day 8, Day 36, and Week 13. To measure the rate of clearance and elimination half-life of 1-nitropyrene in lungs and plasma, tissue samples were collected at intervals following the last exposure (Week 13). All samples except plasma samples from rats in the 0.5 mg/m^3 group were analyzed for 1-nitropyrene content.

ANIMAL MAINTENANCE

Time Held Before Study
Males: 14 days
Females: 15 days

Age When Study Began
7 weeks

Age When Killed
20 weeks

Method of Animal Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weight.

Diet
NIH-07 Open Formula Diet (Zeigler Bros., Inc., Gardners, PA) in pellet form and softened water (City of Richland), available *ad libitum* except during exposure periods.

Animal Room Environment
Rats were housed in individual cages between exposures and in individual exposure tubes during exposures. In the animal room, the temperature was maintained at 69° to 75° F with 40% to 70% relative humidity and 12 to 18 room air changes per hour. In the exposure tubes, the temperature was maintained at 72° to 78° F with 30% to 50% relative humidity with an air flow of 22 to 34 L/minute. Fluorescent light was provided for 12 hours per day.

Statistical Methods

ANALYSIS OF CONTINUOUS VARIABLES

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Williams (1971, 1972) or Dunnett (1955). Clinical pathology, spermatid, and epididymal spermatozoal data, which typically have skewed distributions, were analyzed with the nonparametric multiple comparison methods of Shirley (1977) and 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's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose response (Dunnett's or Dunn's test). Trend-sensitive tests were used when Jonckheere's test was significant at a P-value less than 0.1. Average severity values were analyzed for significance using the Mann-Whitney U test (Hollander and Wolfe, 1973). Before analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel and implausible values were eliminated from the analysis.

ANALYSIS OF VAGINAL CYTOLOGY DATA

Because the data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into close conformance with normality assumptions. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for the simultaneous equality of measurements across dose levels.

Quality Assurance

The animal study of 1-nitropyrene was performed in compliance with United States Food and Drug Administration Good Laboratory Practices regulations (21 CFR, Part 58). The Quality Assurance Unit of Battelle Pacific Northwest Laboratories performed audits and inspections of protocols, procedures, data, and reports throughout the course of the study.

RESULTS

13-Week Inhalation Study in F344/N Rats

All rats survived until the end of the study (Table 3). The final mean body weights and mean body weight gains of all exposed groups were similar to those of the controls (Table 3 and Figure 3). There were no clinical signs of toxicity related to 1-nitropyrene exposure.

TABLE 3 Survival and Body Weights of F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene

Concentration (mg/m^3)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls (%)[2]
		Initial	Final	Change	
MALE					
0	10/10	145	276	131	
0.5	10/10	144	273	129	99
2	10/10	144	281	137	102
8	10/10	144	276	132	100
20	10/10	143	279	136	101
50	10/10	143	265	122	96
FEMALE					
0	10/10	107	173	66	
0.5	10/10	111	173	62	100
2	10/10	108	173	64	100
8	10/10	108	169	61	97
20	10/10	108	170	62	98
50	10/10	108	170	62	98

[1] Number surviving at 13 weeks/number of animals per exposure group.
[2] (Exposure group mean/control group mean) x 100.

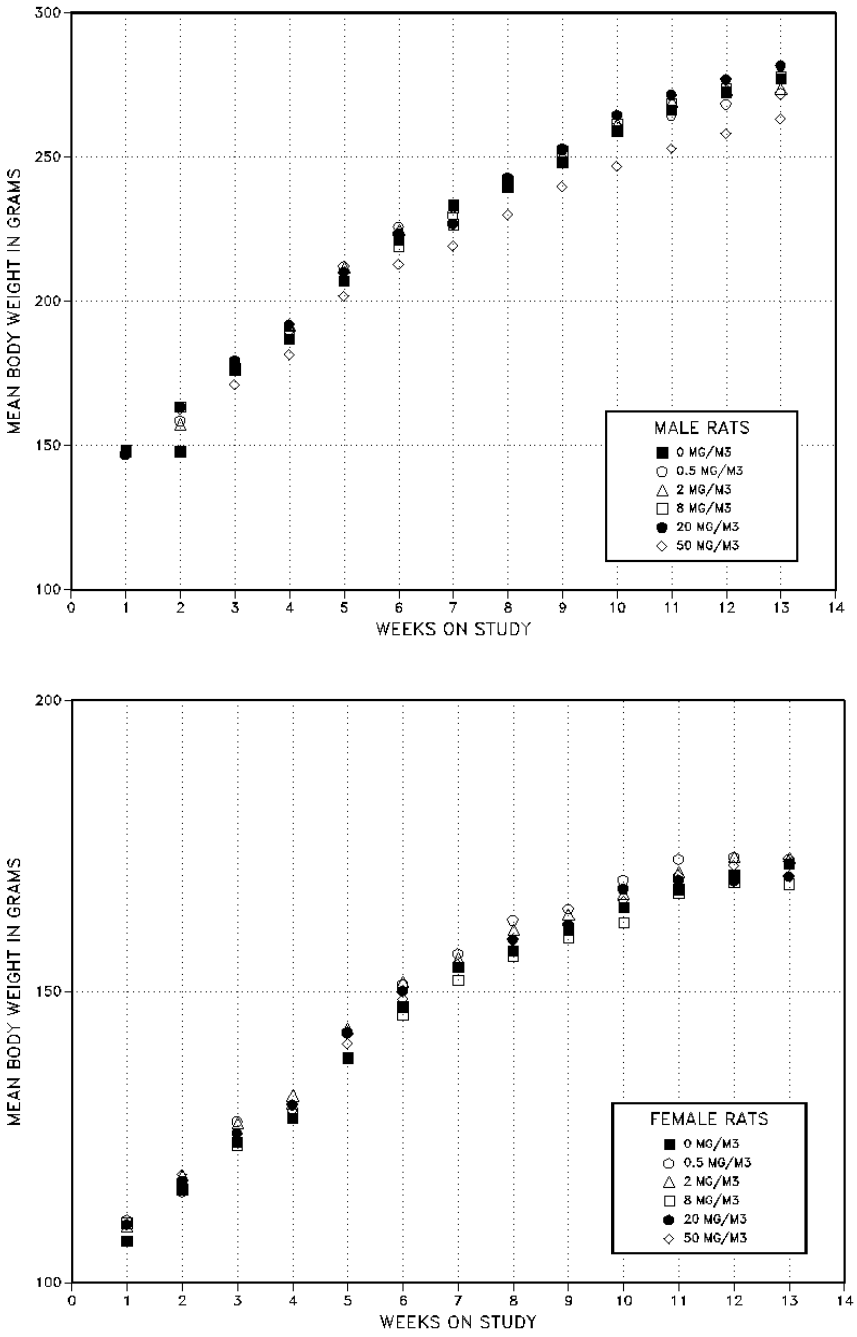

FIGURE 3 Body Weights of F344/N Rats Exposed to 1-Nitropyrene by Inhalation for 13 Weeks

Hematology and clinical chemistry evaluations were conducted at the end of the 13-week study and the results are listed in Tables C1 and C2. The few differences in these parameters were minimal, inconsistent, and not considered related to 1-nitropyrene exposure.

Significant differences in organ weights were limited to male rats (Table 4 and Appendix B) Absolute liver weights were significantly increased in males exposed to 2, 8, or 20 mg/m^3 1-nitropyrene, and relative liver weights were significantly increased at all exposure levels, but a clear dose response was not evident. Absolute and relative lung weights of males in the 2 mg/m^3 group were also significantly increased. No other differences in absolute or relative organ weights occurred in the study.

TABLE 4 Liver and Lung Weights for Male F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	Concentration (mg/m^3)					
	0	0.5	2	8	20	50
n	10	10	10	10	10	10
Necropsy body wt	272 ± 5	269 ± 4	280 ± 6	273 ± 4	277 ± 5	262 ± 5
Liver						
Absolute	8.363 ± 0.142	8.773 ± 0.218	9.922 ± 0.233**	9.357 ± 0.142**	9.621 ± 0.233**	8.816 ± 0.221
Relative	30.80 ± 0.25	32.54 ± 0.50*	35.40 ± 0.40**	34.37 ± 0.49**	34.81 ± 0.72**	33.61 ± 0.42**
Lungs						
Absolute	1.295 ± 0.033	1.421 ± 0.046	1.525 ± 0.075**	1.424 ± 0.051	1.384 ± 0.045	1.354 ± 0.033
Relative	4.78 ± 0.13	5.28 ± 0.17	5.44 ± 0.24*	5.23 ± 0.20	5.00 ± 0.12	5.17 ± 0.12

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test.

Following a single 6-hour inhalation exposure (8 mg/m^3), the terminal elimination half-life of 1-nitropyrene in the lungs of rats was 0.58 hours (Table 5).

TABLE 5 Clearance Rates of 1-Nitropyrene from the Lungs and Plasma of Male F344/N Rats Following 6 Hours or 13 Weeks of Inhalation Exposure[1]

	C_o (µg/g)	k (hours^{-1})	$t_{1/2}$ (hours)	Correlation Coefficient
Lungs				
6-Hour Exposure (8 mg/m^3)	0.20	1.19	0.58	0.97
13-Week Exposure[2] (8 mg/m^3)	0.24	0.69	1.01	0.93
13-Week Exposure[3] (50 mg/m^3)	5.20	0.11	6.12	0.72
Plasma				
13-Week Exposure (50 mg/m^3)	0.03	0.90	0.77	0.77

[1] Clearance of 1-nitropyrene from the lungs or plasma was modeled assuming first-order clearance with the following expressions: $C(t)=C_o e^{-kt}$ and $t_{1/2}=\ln(2/k)$, where
t=time following the end of exposure
C(t)=1-nitropyrene concentration at time t
C_o=1-nitropyrene concentration at the time exposure was stopped (t=0)
k=first order clearance rate constant
$t_{1/2}$=clearance half-life
[2] Final exposure and sample collection on Day 91.
[3] Final exposure and sample collection on Day 90.

In the 13-week study, a notable accumulation of 1-nitropyrene in the lungs of male rats was observed only in the 50 mg/m^3 group (Table 6). In rats exposed to 0.5 or 8 mg/m^3, mean amounts of 1-nitropyrene per gram of lung tissue varied but did not notably increase from Day 8 to Week 13. However, in the 50 mg/m^3 group, mean lung concentration of 1-nitropyrene doubled between Day 8 and Day 36 and again between Day 36 and the end of the study (Table 6).

The mean concentration of 1-nitropyrene in the plasma of rats exposed to 50 mg/m^3 did not consistently increase during the 13-week study (Table 6). In the 8 mg/m^3 group, plasma samples contained less than the experimental limit of quantitation (ELOQ) (5 ng/mL) of 1-nitropyrene, and plasma samples from rats in the 0.5 mg/m^3 group were not analyzed due to the low 1-nitropyrene concentrations observed in the 8 mg/m^3 group.

TABLE 6 Concentrations of 1-Nitropyrene in the Lungs and Plasma of Male F344/N Rats Exposed by Inhalation for 8 Days, 36 Days, or 13 Weeks[1]

	Exposure Concentration		
	0.5 mg/m^3	8 mg/m^3	50 mg/m^3
Lungs			
Day 8	0.010	0.121	1.81
Day 36	0.005[3]	0.077	3.71
Week 13[2]	0.006[3]	0.185[4]	7.91[5]
Plasma			
Day 8	)[6]	)[7]	0.015
Day 36	)[6]	)[7]	0.010[3]
Week 13	)[6]	)[7]	0.026[5]

[1] Each value is the mean 1-nitropyrene concentration (µg/g lung tissue or plasma) of three samples, unless otherwise specified. All samples were collected within 1 hour after the exposures ended on Day 8, Day 36, or Week 13. One sample was analyzed per rat.
[2] Final exposure and sample collection for the 8 and 50 mg/m^3 groups on Days 91 and 90, respectively.
[3] Value for one sample; all other samples contained less than the experimental limit of quantitation (ELOQ), 5 ng/mL.
[4] Mean of five samples.
[5] Mean of four samples.
[6] Plasma samples from rats in the 0.5 mg/m^3 group were not analyzed because of the low 1-nitropyrene concentrations observed in the 8.0 mg/m^3 group.
[7] All samples contained <ELOQ.

Based on 1-nitropyrene concentrations observed in the lung tissue and plasma samples (Table 8) taken at intervals following the end of exposure (Tables 7 and Figure 4), the first-order clearance rate constant (k) and the half-life ($t_{1/2}$) of 1-nitropyrene were calculated for the 6-hour study (lungs only) and the 13-week study (Table 5). The elimination half-life ($t_{1/2}$ = 1.01 hours) of 1-nitropyrene in the lungs of rats exposed to 8 mg/m^3 for 13 weeks was notably longer than the elimination half-life ($t_{1/2}$ = 0.58 hours) in the lungs of rats receiving a single 8 mg/m^3 exposure for 6 hours. For rats exposed to 50 mg/m^3 for 13 weeks, the elimination half-life ($t_{1/2}$ = 6.12 hours) of 1-nitropyrene in the lungs was approximately six times greater than that in the lungs of rats exposed to 8 mg/m^3 for 13 weeks. The half-life ($t_{1/2}$ = 0.77 hours) of 1-nitropyrene in the plasma of rats exposed to 50 mg/m^3 for 13 weeks was considerably shorter than that in the lungs of rats receiving the same exposure (Table 5). The data from rats exposed to 50 mg/m^3 (Table 7) are shown in Figure 4.

TABLE 7 Concentrations of 1-Nitropyrene in the Lungs of Male F344/N Rats Measured at Intervals Following a Single 6-Hour or 13-Week Inhalation Exposure[1]

		Exposure Concentration		
	Time[2]	0.5 mg/m^3	8 mg/m^3	50 mg/m^3
6-Hour Exposure[3]	15 min.		0.138	
			0.102	
	2 hr.		0.022	
			0.018	
	12 hr.		)[4]	
	24 hr.		)[5]	
	72 hr.		)[5]	
13-Week Exposure	20 min.	0.006	0.210	4.98
		<ELOQ	0.239	9.61
		<ELOQ	0.225	12.5
	30 min.	<ELOQ	0.150	
	40 min.	)[5]	0.099	4.54
	50 min.	<ELOQ		
	60 min.	)[5]	0.120	2.10
			0.148	
			0.108	
	70 min.	0.006		
	80 min.	0.007		
	90 min.	<ELOQ		
	2 hr.	)[5]	0.026[6]	3.26
			0.059	
	4 hr.		0.011	2.04
				5.17
				8.11
	6 hr.		0.005	0.994
	16 hr.		)[7]	1.16
				0.351
				0.275
	24 hr.		<ELOQ	0.976
				0.652
				<ELOQ
				0.220

[1] Each value is the 1-nitropyrene concentration (µg/g lung tissue) for one tissue sample, unless otherwise specified. One sample was analyzed per rat.
[2] Number of minutes or hours after the last exposure ended.
[3] For the 6-hour exposure study, rats were only exposed to 8 mg 1-nitropyrene/m^3.
[4] Two samples were analyzed; both contained less than the experimental limit of quantitation (ELOQ), 5 ng/g.
[5] Three samples were analyzed; all contained <ELOQ.
[6] Three samples were analyzed, but one was not used to calculate clearance rate.
[7] Three samples were analyzed; two contained <ELOQ and one was not used to calculate clearance rate.

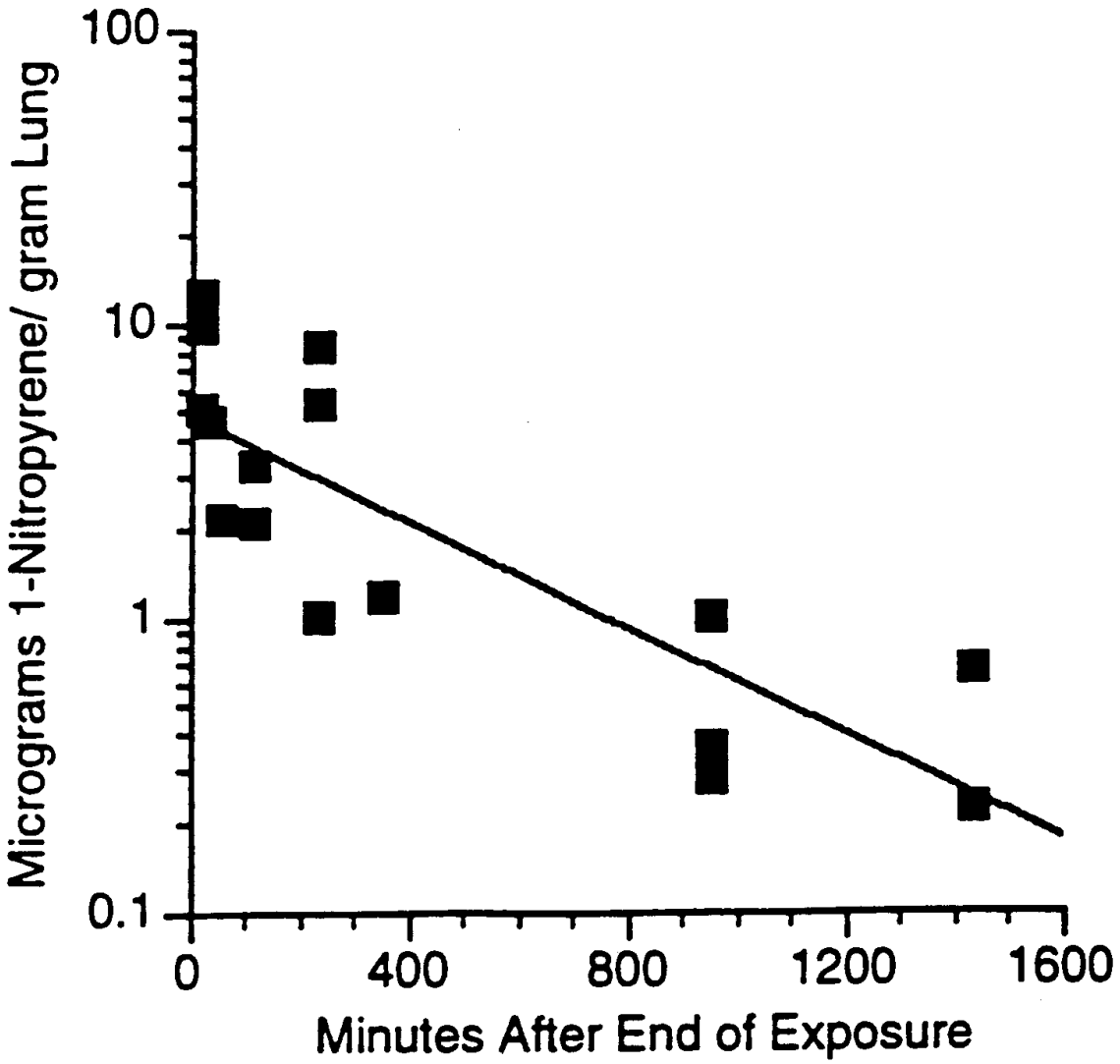

FIGURE 4 Lung Concentrations of 1-Nitropyrene in Rats at Time Intervals after Exposure to 50 mg/m^3 for 13 Weeks.

TABLE 8 Concentrations of 1-Nitropyrene in the Plasma of Male F344/N Rats Measured at Intervals Following 13 Weeks of Inhalation Exposure[1]

		Exposure Concentration[2]	
	Time[3]	8 mg/m^3	50 mg/m^3
13-Week Exposure	20 min.	)[4]	0.018
			0.048
			0.019
	30 min.	<ELOQ	
	40 min.	<ELOQ	0.018
	60 min.	)[4]	0.011
	2 hr.	)[4]	0.006
	4 hr.	<ELOQ	)[5]
	6 hr.	<ELOQ	<ELOQ
	16 hr.	0.014[6]	)[4]
	24 hr.	<ELOQ	)[7]

[1] Each value is the 1-nitropyrene concentration (µg/mL plasma) for one tissue sample, unless otherwise specified. One sample was analyzed per rat.
[2] Plasma samples from rats in the 0.5 mg/m^3 group were not analyzed because of the low 1-nitropyrene concentrations observed in the plasma of rats in the 8.0 mg/m^3 group.
[3] Number of minutes or hours after the last exposure ended.
[4] Three samples were analyzed; all contained less than the experimental limit of quantitation (ELOQ), 5 ng/mL.
[5] Three samples were analyzed; two contained <ELOQ, and one was not used to calculate clearance rate.
[6] Three samples were analyzed; two contained <ELOQ.
[7] Four samples were analyzed; all contained <ELOQ.

There were no gross lesions attributed to the inhalation of 1-nitropyrene. However, microscopic lesions associated with the inhalation of 1-nitropyrene were observed in the larynx, nose, and lungs of male and female rats (Table 9 and Appendix A).

Squamous metaplasia of the larynx occurred in most exposed groups with concentration-dependent increases in incidence and severity (Table 9). This was a focal lesion which occurred in the mucosa of the larynx at the base of the epiglottis. It was characterized by replacement of the normal cuboidal and ciliated columnar cells with well-differentiated stratified squamous epithelium (Plates 1 and 2). Minimal lesions were subtle and focally limited to the central area of the ventral laryngeal floor, while moderate lesions at the higher exposure concentrations were more extensive. Laryngeal squamous metaplasia was observed in males at a concentration of 2 mg/m^3 or greater and in females at all exposure concentrations.

Squamous metaplasia of the bronchus was observed in the lungs of a few male and female rats at the higher exposure concentrations (Table 9). The metaplasia occurred usually at one or more bronchial bifurcation points and consisted of focal replacement of the ciliated

columnar mucosal cells by well-differentiated stratified squamous epithelium (Plates 3 and 4). The affected mucosa was slightly thickened and occasionally keratinized. Squamous metaplasia of the bronchus was a minimal change in all affected animals, regardless of exposure concentration, but had increasing incidence at the higher concentrations. This lesion was present in males at the three highest exposure levels and in females at the two highest levels.

Cytoplasmic alteration of the nasal respiratory epithelium was present in most male and female rats at an exposure concentration of 8 mg/m^3 or greater (Table 9). Cytoplasmic alteration was characterized by the presence of brightly eosinophilic cytoplasmic inclusions within mucosal epithelial cells. This change was minimal to moderate in severity and was most typically observed in the cells lining the nasal septum in the anterior and mid-level nasal sections, with more extensive involvement of the respiratory epithelium in more severe cases. Incidence and severity of this lesion increased with increasing exposure level.

TABLE 9 Incidence and Severity of Respiratory Lesions in F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	Concentration (mg/m^3)					
	0	0.5	2	8	20	50
MALE						
Larynx						
Epiglottis, metaplasia, squamous	0/10	0/10	5/10* (1.0)	10/10** (1.8)	10/10** (2.1)	10/10** (2.5)
Lung						
Bronchus, metaplasia, squamous	0/10	0/8	0/10	1/10 (1.0)	2/10 (1.0)	5/10* (1.0)
Nose						
Respiratory epithelium, cytoplasmic alteration	0/10	0/10	0/10	8/10** (1.4)	10/10** (2.1)	10/10** (2.8)
FEMALE						
Larynx						
Epiglottis, metaplasia, squamous	0/10	4/10* (1.0)	4/10* (1.0)	10/10** (1.7)	10/10** (2.0)	10/10** (2.8)
Lung						
Bronchus, metaplasia, squamous	0/10	0/10	0/10	0/10	2/10 (1.0)	3/10 (1.0)
Nose						
Respiratory epithelium, cytoplasmic alteration	0/10	0/10	0/10	6/10** (1.2)	10/10** (2.2)	10/10** (2.7)

[1] Average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.
* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test.
** Significantly different ($P \leq 0.01$) from the control group by the Fisher exact test.

No treatment-related effects were noted on sperm motility and vaginal cytology (Tables D1 and D2). A lesion of the testis was observed in all male rats, both control and treated. The lesion, diagnosed as atrophy, consisted of partial to total loss of the germ cells. This was a minimal to moderate change depending on the extent of tubule involvement. Hypospermia of the epididymis, characterized by decreased numbers of spermatozoa within the lumens of epididymal tubules, was associated with the testicular atrophy. These lesions were not considered to be chemical-related effect, but rather due to heat or pressure resulting from the daily confinement within the exposure tubes.

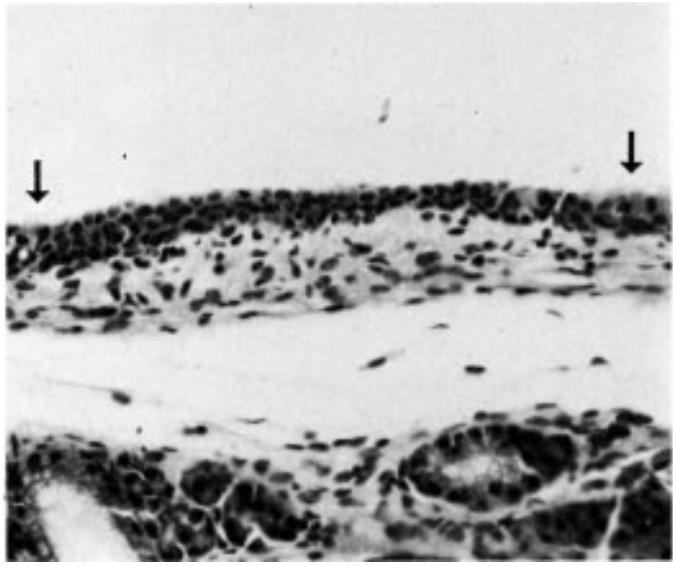

PLATE 1

Normal laryngeal mucosa at the base of the epiglottis from a control male rat showing the mixture of ciliated columnar cells (arrows) and nonciliated, stratified cells. Compare to the exposed male rat in Plate 2. H&E, 260x.

PLATE 2

Laryngeal mucosa from a male rat exposed to 50 mg/m^3 nitropyrene. Note the replacement of the normal epithelium by mild squamous metaplasia, with horizontal orientation of superficial cells. H&E, 260x.

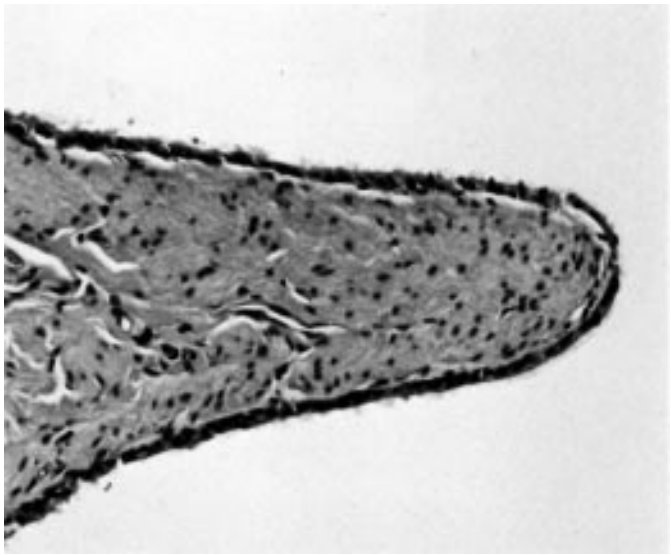

PLATE 3

Normal bronchial mucosa from a control male rat showing the low columnar ciliated cells. Compare to the exposed male rat in Plate 4. H&E, 260x.

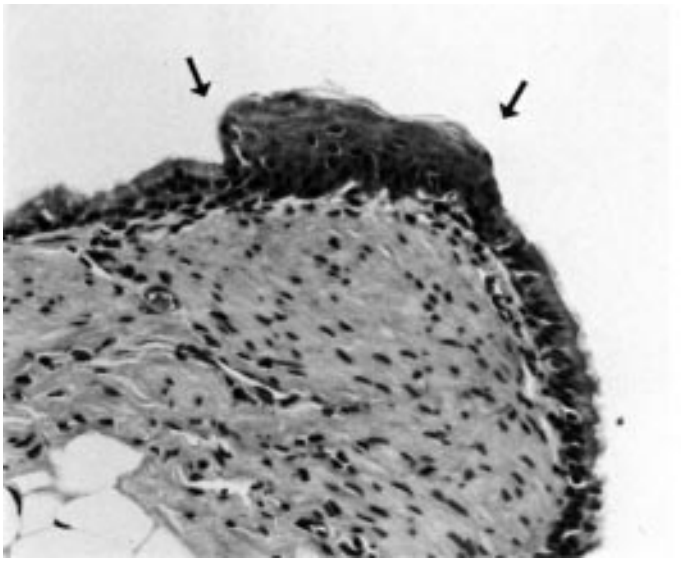

PLATE 4

Bronchial mucosa from a male rat exposed to 50 mg/m^3 nitropyrene. Note the focal replacement of the normal ciliated epithelium by squamous metaplasia (arrows). H&E, 260x.

DISCUSSION

All rats survived to the end of the 13-week study. Body weight gains of exposed groups were similar to controls. However, body weight gains of control F344/N rats in this nose-only inhalation study were significantly lower than those of control F344/N rats in whole-body inhalation studies of other chemicals conducted by the same laboratory (Figure 5). The reduced body weight gains observed in the present study were probably due to the stressful effects of confinement in the exposure tubes. Michajlovskij *et al.* (1988) reported that rats confined in exposure tubes for 150 minutes daily for 7 to 38 days had lower feed and water intake than control rats not confined, with concomitant decreases in body weight and urine output. Excessive heat buildup during exposure tube confinement may have produced the testicular atrophy observed in the present study in control and exposed male rats. Bowler (1972) also reported testicular degeneration in rats following repeated exposures to heat. In one study (Lee *et al.*, 1993), the incidence of spontaneous testicular degeneration in control rats from inhalation studies was more than threefold greater than that seen in control rats from oral toxicity studies and was attributed to stress and immobilization associated with restraint during the exposure period. Histopathologic effects attributable to confinement in exposure tubes were not evident in female rats.

Sporadic changes in hematology and clinical chemistry end points occurred in the 13-week study. However, the changes were not considered to have any biological significance, and their relationship to 1-nitropyrene exposure was unclear.

The half-life of 1-nitropyrene in the lungs of male rats exposed to 8 mg/m^3 for 13 weeks was about 1 hour, whereas that in the lungs of rats exposed to 50 mg/m^3 for 13 weeks was about 6 hours. These data indicate that rats in the 8 mg/m^3 group were able to clear the 1-nitropyrene from their lungs between exposures (18-hour interval), whereas rats in the 50 mg/m^3 group accumulated 1-nitropyrene in their lungs faster than it was cleared during the daily exposure, resulting in an increased lung burden of 1-nitropyrene over time.

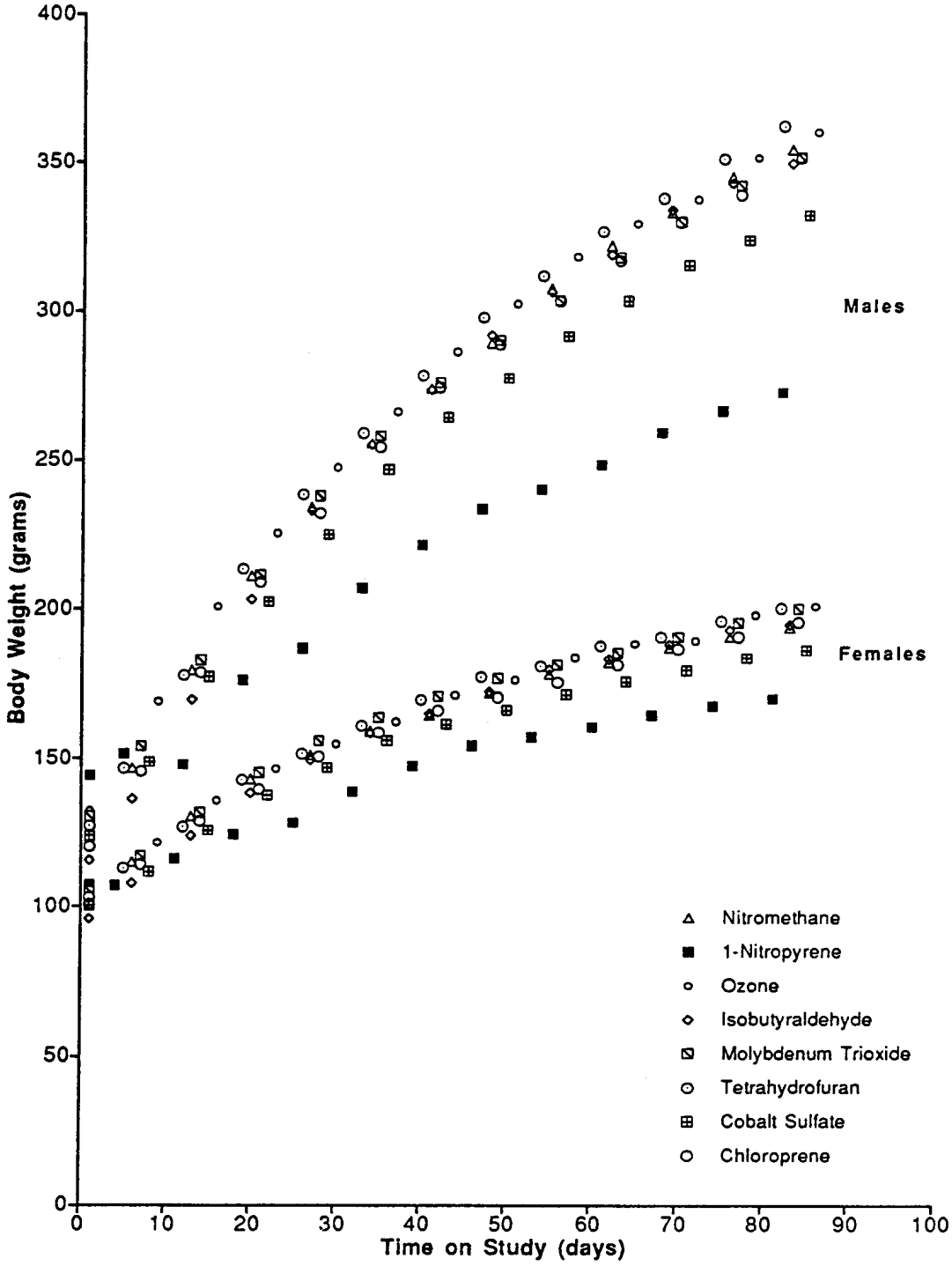

FIGURE 5 Body Weights of Control F344/N Rats in 13-Week Inhalation Studies: Nose-Only Inhalation (1-Nitropyrene) vs. Whole-Body Inhalation (Other Chemicals)

Bond *et al.* (1986a,b) conducted nose-only inhalation studies of [^{14}C]-labeled 1-nitropyrene and reported that radioactivity in the lungs of rats exposed to 50 ng/L for 1 hour was cleared in a biphasic manner with a short-term half-life of 1 hour and a long-term half-life of 100 hours. In the same study, radioactivity in the lungs of rats exposed to 490 ng/L [^{14}C]-1-nitropyrene for 1 hour was cleared with a short-term half-life of 1 hour and a long-term half-life of 40 hours. The chemical identity of the ^{14}C label in the lungs was not determined. The long-term half-life could be the result of some form of tissue binding (Medinsky *et al.*, 1988). In the present study, the elimination half-life of inhaled 1-nitropyrene in the lungs of rats exposed at 8 mg/m^3 for 6 hours was 0.6 hours. No short-term or long-term half-lives were discerned from the present data obtained from gas chromatography/mass spectroscopy analyses; however, only unreacted 1-nitropyrene was detected by this method.

After 13 weeks of nose-only inhalation exposure to 1-nitropyrene, squamous metaplasia of the laryngeal epithelium was observed in male rats exposed to concentrations of 2 mg/m^3 or greater and in female rats exposed to concentrations of 0.5 mg/m^3 or greater. Squamous metaplasia of the bronchial mucosa was also observed in the lungs of males exposed to concentrations of 8 mg/m^3 or greater and of females exposed to concentrations of 20 mg/m^3 or greater. Squamous metaplasia of the larynx and tracheobronchial epithelium is a common response to a variety of inhaled irritants and carcinogens (Renne *et al.*, 1992), such as cigarette smoke (Auerbach *et al.*, 1961), asbestos (Woodworth *et al.*, 1983), and benzo[a]pyrene-ferric oxide (Harris *et al.*, 1972). Squamous metaplasia is considered to be an adaptive response of the respiratory mucosa to chronic irritation. With chronic exposure to carcinogens, squamous metaplasia with cellular atypia (dysplasia) may develop and is considered a precursor to neoplasia (Monticello *et al.*, 1990). The metaplastic lesions of the larynx and bronchus in the current study of 1-nitropyrene did not have dysplastic changes and were not considered necessarily preneoplastic, although the potential for progression with exposures of longer duration must be considered and cannot be ruled out. The stressful experimental conditions and lower body weights may have had an impact on neoplastic progression.

Cytoplasmic alteration (hyaline droplets) of the nasal respiratory epithelium was observed in male and female rats exposed to 1-nitropyrene at concentrations of 8 mg/m^3 or greater. The accumulation of hyaline droplets, which are believed to be proteinaceous secretory material, is a minor, nonspecific degenerative change that can occur spontaneously and can be exacerbated by chemical exposure (Monticello *et al.*, 1990). It is unusual that the lower airway had metaplastic

changes but the nasal cavity was spared. This may be related to the site-specific metabolism of 1-nitropyrene.

The lung is probably a target organ for 1-nitropyrene carcinogenesis following inhalation exposure, as demonstrated by the metabolism of 1-nitropyrene to the reactive intermediate by rat, hamster, and rabbit tracheal epithelial cells (King and Lewtas, 1993) and by rabbit lung slices (Jackson *et al.*, 1985), by the formation of C8-dG-AP adducts in mouse lung following intratracheal instillation of 1-nitropyrene (Mitchell, 1988a), and by the appearance of lung tumors in mice following intraperitoneal administration of 1-nitropyrene (El-Bayoumy *et al.*, 1984). However, in the present study, the only histopathologic changes observed were metaplasia in the epithelium of the larynx and bronchi. Wolff *et al.* (1988) observed no lesions in the lungs of F344 rats after nose-only inhalation exposure to 1-nitropyrene at 7.5 mg/m^3, 2 hours per day, 5 days per week, for 4 weeks. 1-Nitropyrene is considered a weak carcinogen; therefore, a longer period of time may be required for 1-nitropyrene-induced carcinogenesis to manifest (Imaida *et al.*, 1991).

Inhaled 1-nitropyrene has been shown to distribute throughout the body of rats and be metabolized by both nitro reduction and ring oxidation (Ball *et al.*, 1984; El-Bayoumy and Hecht, 1984; Bond *et al.*, 1986c). In mice instilled intratracheally with 1-nitropyrene, C8-dG-AP adducts were identified in the liver and kidney in addition to the lung (Mitchell, 1988a). 1-Nitropyrene intraperitoneally injected into rats has been shown to form C8-dG-AP adducts in the kidney, liver, and mammary gland (Hashimoto and Shudo, 1985; Stanton *et al.*, 1985). Imaida *et al.* (1991) reported induction of neoplastic hepatic lesions in CD rats after intraperitoneal injection of the 1-nitropyrene metabolites *N*-hydroxy-*N*-acetyl-1-aminopyrine or *N*-acetyl-1-aminopyrine at 67 µmol/kg, three times per week for 4 weeks. Denda *et al.* (1989) reported that six daily intragastric intubations of 1-nitropyrene at 100 mg/kg induced γ-glutamyltranspeptidase (GGT)-positive foci in the liver of F344 rats. GGT-positive foci are considered preneoplastic changes. In the present study, 1-nitropyrene was detected in the plasma of rats in the 50 mg/m^3 group; however, no lesions in the liver, kidney, or mammary gland were observed. This may be due to the low concentration of 1-nitropyrene circulating and the short duration of exposure.

Ohgaki *et al.* (1985) reported that 1-nitropyrene containing 0.2% 1,3-dinitropyrene, 0.3% 1,6-dinitropyrene, and 0.3% 1,8-dinitropyrene was carcinogenic and that 1-nitropyrene containing less than 0.05% of each dinitropyrene was not. These authors questioned the purity of the 1-nitropyrene used in a previous study by Ohgaki *et al.* (1982) that reported 1-nitropyrene-induced

carcinogenesis. Beland (1991) demonstrated that 1-nitropyrene used in the studies indeed could be contaminated with dinitropyrenes and again raised the question of whether tumors induced by 1-nitropyrene could be due to the presence of dinitropyrenes. The 1-nitropyrene used in the present study was 99.3% pure, and no impurities greater than 0.1% were detected. Thus, the histopathologic changes observed in rats were considered 1-nitropyrene related.

The International Agency for Research on Cancer (IARC) has concluded that whole diesel engine exhaust is carcinogenic in animals and that 1-nitropyrene, one of the many chemical carcinogens identified in diesel engine exhaust, is a carcinogen in animals and is possibly a 2B carcinogen in humans (IARC, 1989a). A computer program called the Carcinogenicity Prediction and Battery Selection (CPBS) system has been developed to compute the probability that a given chemical is carcinogenic based on the results of short-term tests. According to analyses by the CPBS, values between 0.3 and 0.7 are considered indeterminant. 1-Nitropyrene has a value of 0.4375; therefore, its probability of carcinogenicity is considered inconclusive (Rosenkranz and Howard, 1986) Based on data contained in this report and previously published reports on the genetic toxicity, carcinogenicity, and toxicokinetics of 1-nitropyrene, it is the opinion of the NTP that 1-nitropyrene has a high likelihood of being carcinogenic to the respiratory tract, particularly under exposure conditions that lead to significant accumulations of 1-nitropyrene in the lungs, and perhaps other organs of F344/N rats.

The NTP has elected not to perform a 2-year study of 1-nitropyrene at this time because of technical difficulties and high costs associated with nose-only inhalation and procuring highly purified 1-nitropyrene. If a 2-year study were to be performed, the highest exposure concentration would likely be 50 mg/m^3, based on the lack of significant pathologic or clinical changes in the 13-week study, which could compromise the long-term health of the rats from other than neoplastic effects. It is recognized that a significant accumulation of 1-nitropyrene in the lungs would occur at 50 mg/m^3. In order to provide exposure levels not likely to cause a significant accumulation of 1-nitropyrene in the lungs, lower exposure levels would be based primarily on existing toxicokinetic data.

In summary, nose-only inhalation exposure of rats to 1-nitropyrene for 13 weeks induced squamous metaplasia of the laryngeal and bronchial respiratory mucosa at the bronchial bifurcation in males and females. Cytoplasmic alterations in the respiratory epithelium were also induced in

males and females. The no-observed-adverse-effect level (NOAEL) for male rats was 0.5 mg/m^3. A NOAEL for female rats could not be determined from these studies.

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APPENDIX A

Summary of Nonneoplastic Lesions

TABLE A1 Summary of the Incidence of Nonneoplastic Lesions in Male F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
DISPOSITION SUMMARY						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(2)	(4)	(1)	(1)	(10)
Hepatodiaphragmatic nodule	2 (20%)	2 (100%)	4 (100%)	1 (100%)	1 (100%)	1 (10%)
Cardiovascular System						
Heart	(10)					(10)
Cardiomyopathy	6 (60%)					4 (40%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Epididymis	(9)	(10)	(10)	(9)	(10)	(10)
Hypospermia	9 (100%)	10 (100%)	10 (100%)	9 (100%)	10 (100%)	10 (100%)
Testes	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Mineralization	1 (10%)				2 (20%)	1 (10%)
Hematopoietic System						
Lymph node	(1)		(1)		(1)	
Renal, hemorrhage	1 (100%)		1 (100%)		1 (100%)	
Lymph node, mediastinal	(10)		(1)			(8)
Hemorrhage			1 (100%)			
Thymus	(10)		(1)			(10)
Hemorrhage			1 (100%)			
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						

TABLE A1 Summary of the Incidence of Nonneoplastic Lesions in Male F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene (continued)

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
Respiratory System						
Larynx	(10)	(10)	(10)	(10)	(10)	(10)
Epiglottis, metaplasia, squamous			5 (50%)	10 (100%)	10 (100%)	10 (100%)
Lung	(10)	(8)	(10)	(10)	(10)	(10)
Hemorrhage	10 (100%)	8 (100%)	10 (100%)	9 (90%)	10 (100%)	10 (100%)
Inflammation, chronic active		3 (38%)	1 (10%)	2 (20%)	1 (10%)	
Bronchus, metaplasia, squamous				1 (10%)	2 (20%)	5 (50%)
Nose (10)	(10)	(10)	(10)	(10)	(10)	
Olfactory epithelium, cytoplasmic alteration	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Respiratory epithelium, cytoplasmic alteration				8 (80%)	10 (100%)	10 (100%)
Special Senses System						
None						
Urinary System						
Kidney	(10)					(10)
Nephropathy	5 (50%)					5 (50%)

[1] Number of animals examined microscopically at site and number of animals with lesion.

TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Female F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
DISPOSITION SUMMARY						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(3)	(1)	(4)	(1)	(10)
Hepatodiaphragmatic nodule		2 (67%)	1 (100%)	4 (100%)	1 (100%)	1 (10%)
Inflammation, chronic		1 (33%)				
Inflammation, chronic active						1 (10%)
Necrosis						1 (10%)
Necrosis, focal	1 (10%)	1 (33%)				
Cardiovascular System						
Heart	(10)					(10)
Cardiomyopathy	4 (40%)					4 (40%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Ovary	(10)			(1)		(10)
Cyst	1 (10%)			1 (100%)		1 (10%)
Hematopoietic System						
Lymph node, mediastinal	(9)	(1)				(10)
Hemorrhage		1 (100%)				
Spleen	(10)		(1)			(10)
Fibrosis			1 (100%)			
Integumentary System						
Skin	(10)					(10)
Inflammation, chronic active	1 (10%)					
Musculoskeletal System						
None						
Nervous System						
None						

TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Female F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene (continued)

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
Respiratory System						
Larynx	(10)	(10)	(10)	(10)	(10)	(10)
Epiglottis, metaplasia, squamous		4 (40%)	4 (40%)	10 (100%)	10 (100%)	10 (100%)
Lung	(10)	(10)	(10)	(10)	(10)	(10)
Hemorrhage	10 (100%)	8 (80%)	6 (60%)	9 (90%)	7 (70%)	9 (90%)
Inflammation, chronic active		5 (50%)	2 (20%)		2 (20%)	2 (20%)
Alveolar epithelium, hyperplasia	1 (10%)					
Bronchus, metaplasia, squamous					2 (20%)	3 (30%)
Nose (10)	(10)	(10)	(10)	(10)	(10)	
Olfactory epithelium, cytoplasmic alteration	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Respiratory epithelium, cytoplasmic alteration				6 (60%)	10 (100%)	10 (100%)
Special Senses System						
None						
Urinary System						
Kidney	(10)					(10)
Nephropathy	1 (10%)					1 (10%)

[1] Number of animals examined microscopically at site and number of animals with lesion.

APPENDIX B

Organ Weights and Organ-Weight-to-Body-Weight Ratios

TABLE B Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
MALE						
n	10	10	10	10	10	10
Necropsy body wt	272 ± 5	269 ± 4	280 ± 6	273 ± 4	277 ± 5	262 ± 5
Heart						
Absolute	0.867 ± 0.013	0.852 ± 0.017	0.873 ± 0.018	0.862 ± 0.013	0.904 ± 0.013	0.851 ± 0.015
Relative	3.19 ± 0.02	3.17 ± 0.06	3.12 ± 0.06	3.17 ± 0.04	3.27 ± 0.03	3.25 ± 0.03
Right kidney						
Absolute	0.925 ± 0.014	0.947 ± 0.016	0.970 ± 0.016	0.966 ± 0.011	0.968 ± 0.019	0.920 ± 0.017
Relative	3.41 ± 0.04	3.52 ± 0.04	3.47 ± 0.05	3.55 ± 0.04	3.50 ± 0.04	3.51 ± 0.04
Liver						
Absolute	8.363 ± 0.142	8.773 ± 0.218	9.922 ± 0.233**	9.357 ± 0.142**	9.621 ± 0.233**	8.816 ± 0.221
Relative	30.80 ± 0.25	32.54 ± 0.50*	35.40 ± 0.40**	34.37 ± 0.49**	34.81 ± 0.72**	33.61 ± 0.42**
Lungs						
Absolute	1.295 ± 0.033	1.421 ± 0.046	1.525 ± 0.075**	1.424 ± 0.051	1.384 ± 0.045	1.354 ± 0.033
Relative	4.78 ± 0.13	5.28 ± 0.17	5.44 ± 0.24*	5.23 ± 0.20	5.00 ± 0.12	5.17 ± 0.12
Right testis						
Absolute	0.788 ± 0.061	0.732 ± 0.036	0.862 ± 0.059	0.830 ± 0.060	0.729 ± 0.063	0.722 ± 0.056
Relative	2.91 ± 0.22	2.72 ± 0.13	3.10 ± 0.23	3.07 ± 0.25	2.63 ± 0.21	2.77 ± 0.24
Thymus						
Absolute	0.236 ± 0.008	0.232 ± 0.011	0.242 ± 0.018	0.241 ± 0.008	0.245 ± 0.010	0.221 ± 0.010
Relative	0.87 ± 0.02	0.86 ± 0.04	0.87 ± 0.07	0.88 ± 0.02	0.89 ± 0.03	0.84 ± 0.03
FEMALE						
n	10	10	10	10	10	10
Necropsy body wt	170 ± 3	171 ± 3	169 ± 2	165 ± 2	168 ± 3	167 ± 3
Heart						
Absolute	0.597 ± 0.013	0.608 ± 0.013	0.604 ± 0.008	0.593 ± 0.009	0.582 ± 0.012	0.598 ± 0.011
Relative	3.51 ± 0.06	3.56 ± 0.04	3.59 ± 0.04	3.61 ± 0.06	3.47 ± 0.06	3.59 ± 0.02
Right kidney						
Absolute	0.608 ± 0.012	0.598 ± 0.016	0.595 ± 0.009	0.595 ± 0.011	0.605 ± 0.013	0.612 ± 0.011
Relative	3.58 ± 0.10	3.50 ± 0.08	3.53 ± 0.04	3.62 ± 0.07	3.61 ± 0.05	3.68 ± 0.06
Liver						
Absolute	5.296 ± 0.111	5.025 ± 0.102	5.062 ± 0.092	4.957 ± 0.134	5.149 ± 0.134	5.289 ± 0.165
Relative	31.14 ± 0.56	29.42 ± 0.34	30.02 ± 0.32	30.12 ± 0.71	30.68 ± 0.55	31.69 ± 0.68
Lungs						
Absolute	0.994 ± 0.032	1.015 ± 0.029	1.024 ± 0.022	0.938 ± 0.022	0.983 ± 0.040	1.011 ± 0.038
Relative	5.84 ± 0.14	5.94 ± 0.14	6.08 ± 0.15	5.71 ± 0.13	5.85 ± 0.17	6.05 ± 0.16
Thymus						
Absolute	0.196 ± 0.008	0.190 ± 0.007	0.181 ± 0.009	0.188 ± 0.004	0.191 ± 0.008	0.181 ± 0.008
Relative	1.15 ± 0.04	1.11 ± 0.04	1.07 ± 0.05	1.14 ± 0.02	1.13 ± 0.04	1.08 ± 0.04

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test.

APPENDIX C

Hematology and Clinical Chemistry Results

TABLE C1 Hematology Data for F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
MALE						
n	10	10	10	10	10	10
Manual hematocrit (%)	46.6 ± 0.4	46.1 ± 0.5	46.4 ± 0.3	46.5 ± 0.6	45.5 ± 0.3	45.4 ± 0.5
Packed cell volume (mL/dL)	44.9 ± 0.4	44.7 ± 0.4	44.2 ± 0.7	44.8 ± 0.6	44.4 ± 0.4	43.9 ± 0.4
Hemoglobin (g/dL)	15.4 ± 0.1	15.3 ± 0.1	15.2 ± 0.1	15.4 ± 0.1	15.1 ± 0.1	15.1 ± 0.1
Erythrocytes (10^6/µL)	8.91 ± 0.04	8.81 ± 0.07	8.83 ± 0.05	8.85 ± 0.07	8.71 ± 0.07	8.74 ± 0.06
Reticulocytes (10^6/µL)	0.16 ± 0.02	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.14 ± 0.01	0.12 ± 0.02
Mean cell volume (fL)	50.5 ± 0.4	50.8 ± 0.3	49.9 ± 0.8	50.7 ± 0.5	51.0 ± 0.3	50.2 ± 0.3
Mean cell hemoglobin (pg)	17.3 ± 0.0	17.3 ± 0.1	17.2 ± 0.1	17.4 ± 0.1	17.4 ± 0.1	17.2 ± 0.1
Mean cell hemoglobin concentration (g/dL)	34.4 ± 0.3	34.2 ± 0.1	34.4 ± 0.5	34.3 ± 0.3	34.1 ± 0.2	34.3 ± 0.2
Platelets (10^3/µL)	486.3 ± 7.3	462.5 ± 17.4	504.2 ± 22.1[2]	471.6 ± 15.0	485.4 ± 18.1	476.1 ± 6.2
Leukocytes (10^3/µL)	5.54 ± 0.18	5.83 ± 0.23	5.30 ± 0.18	5.83 ± 0.30	5.93 ± 0.43	5.22 ± 0.21
Segmented neutrophils (10^3/µL)	1.22 ± 0.04	1.23 ± 0.11	1.22 ± 0.17	1.31 ± 0.09	1.34 ± 0.25	1.21 ± 0.10
Lymphocytes (10^3/µL)	4.26 ± 0.18	4.52 ± 0.25	4.02 ± 0.10	4.42 ± 0.29	4.50 ± 0.30	3.95 ± 0.19
Monocytes (10^3/µL)	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.03	0.03 ± 0.01
Eosinophils (10^3/µL)	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.06 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
FEMALE						
n	10	10	10	8	10	9
Manual hematocrit (%)	46.4 ± 0.5	46.2 ± 0.7	47.3 ± 0.4	46.7 ± 0.3	47.8 ± 0.5	47.1 ± 0.8
Packed cell volume (mL/dL)	45.7 ± 0.6	45.3 ± 0.7	46.8 ± 0.6	46.5 ± 0.6	47.4 ± 0.7	46.2 ± 1.0
Hemoglobin (g/dL)	15.6 ± 0.2	15.5 ± 0.2	15.7 ± 0.2	15.9 ± 0.2	16.1 ± 0.1	15.7 ± 0.3
Erythrocytes (10^6/µL)	8.47 ± 0.08	8.51 ± 0.08	8.66 ± 0.07	8.71 ± 0.08	8.80 ± 0.08**	8.59 ± 0.13*
Reticulocytes (10^6/µL)	0.14 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.11 ± 0.03
Mean cell volume (fL)	54.0 ± 0.5	53.3 ± 0.6	54.0 ± 0.5	53.3 ± 0.6	53.9 ± 0.4	53.8 ± 0.5
Mean cell hemoglobin (pg)	18.4 ± 0.1	18.2 ± 0.1	18.1 ± 0.1	18.3 ± 0.1	18.3 ± 0.1	18.3 ± 0.0
Mean cell hemoglobin concentration (g/dL)	34.1 ± 0.3	34.2 ± 0.3	33.6 ± 0.3	34.2 ± 0.4	34.0 ± 0.3	34.1 ± 0.3
Platelets (10^3/µL)	725.5 ± 73.6	635.0 ± 27.2	697.1 ± 32.5	629.8 ± 39.3	647.7 ± 44.8	703.7 ± 69.8
Leukocytes (10^3/µL)	7.44 ± 0.46	6.60 ± 0.40	7.34 ± 0.34	7.60 ± 0.43	7.85 ± 0.55	7.39 ± 0.29
Segmented neutrophils (10^3/µL)	0.98 ± 0.10	0.88 ± 0.07	1.05 ± 0.08	1.33 ± 0.20	1.06 ± 0.17	1.17 ± 0.14
Lymphocytes (10^3/µL)	6.21 ± 0.43	5.61 ± 0.42	6.10 ± 0.32	6.17 ± 0.32	6.70 ± 0.43	6.08 ± 0.28
Monocytes (10^3/µL)	0.16 ± 0.05	0.06 ± 0.01	0.08 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.10 ± 0.02
Eosinophils (10^3/µL)	0.09 ± 0.02	0.05 ± 0.02	0.09 ± 0.03	0.06 ± 0.04	0.05 ± 0.02	0.05 ± 0.02

[1] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[2] n=9.
* Significantly different ($P \leq 0.05$) from the control group by Shirley's test.
** Significantly different ($P \leq 0.01$) from the control group by Shirley's test.

TABLE C2 Clinical Chemistry Data for F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

	0 mg/m^3	0.5 mg/m^3	2 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
MALE						
n	10	10	10	10	10	10
Urea nitrogen (mg/dL)	21.2 ± 0.3	21.4 ± 0.9	21.1 ± 0.5	21.9 ± 0.3	22.0 ± 0.8	22.0 ± 0.7
Creatinine (mg/dL)	0.77 ± 0.02	0.69 ± 0.03*	0.72 ± 0.03	0.69 ± 0.03*	0.67 ± 0.03*	0.65 ± 0.02**
Glucose (mg/dL)	134 ± 7	150 ± 11	136 ± 5	130 ± 4	135 ± 4	159 ± 11
Total protein (g/dL)	7.0 ± 0.1	6.8 ± 0.1	7.0 ± 0.1	6.9 ± 0.1	6.8 ± 0.1*	6.7 ± 0.1**
Albumin (g/dL)	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.0	4.2 ± 0.1	4.1 ± 0.1
Globulin (g/dL)	2.9 ± 0.1	2.7 ± 0.0	2.8 ± 0.0	2.7 ± 0.0*	2.6 ± 0.0**	2.5 ± 0.1**
Albumin/globulin ratio	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.6 ± 0.0**	1.6 ± 0.0*	1.7 ± 0.1**
Alanine aminotransferase (IU/L)	66 ± 3	57 ± 4*	57 ± 2*	55 ± 1*	55 ± 2*	52 ± 2**
Alkaline phosphatase (IU/L)	325 ± 10	305 ± 7	308 ± 8	323 ± 8	300 ± 8	291 ± 8*
Creatine kinase (IU/L)	191 ± 20	160 ± 20	145 ± 26	164 ± 30	148 ± 23	121 ± 17
Sorbitol dehydrogenase (IU/L)	17 ± 1	16 ± 1	15 ± 1	14 ± 1	15 ± 1	16 ± 1
Bile acids (µmol/L)	29.72 ± 2.40	30.99 ± 4.07	26.29 ± 2.16	31.04 ± 3.00	29.42 ± 1.74	36.64 ± 4.01
FEMALE						
n	10	10	10	10	10	10
Urea nitrogen (mg/dL)	21.1 ± 0.7	20.8 ± 0.6	21.8 ± 0.7	22.4 ± 1.0	21.6 ± 0.6	22.1 ± 1.3
Creatinine (mg/dL)	0.64 ± 0.02	0.60 ± 0.02	0.63 ± 0.02	0.64 ± 0.03	0.61 ± 0.01	0.61 ± 0.03
Glucose (mg/dL)	160 ± 12	174 ± 10	158 ± 11	174 ± 16	155 ± 8	165 ± 14
Total protein (g/dL)	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.4 ± 0.1	6.6 ± 0.1	6.4 ± 0.1
Albumin (g/dL)	4.0 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	4.0 ± 0.1
Globulin (g/dL)	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
Albumin/globulin ratio	1.6 ± 0.1	1.7 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	1.8 ± 0.2	1.7 ± 0.1
Alanine aminotransferase (IU/L)	47 ± 2	50 ± 2	44 ± 1	49 ± 1	53 ± 2	48 ± 3
Alkaline phosphatase (IU/L)	289 ± 7	273 ± 9	305 ± 17	287 ± 9	288 ± 9	283 ± 9
Creatine kinase (IU/L)	154 ± 17	174 ± 20	155 ± 26[2]	189 ± 19	166 ± 23	146 ± 18
Sorbitol dehydrogenase (IU/L)	14 ± 1	13 ± 1	13 ± 1	14 ± 1	13 ± 1	14 ± 1
Bile acids (µmol/L)	26.07 ± 3.40	40.41 ± 6.70	38.71 ± 6.95	38.53 ± 7.17	30.65 ± 6.07	21.88 ± 2.11

[1] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[2] n=9.
* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test.
** Significantly different ($P \leq 0.01$) from the control group by Shirley's test.

APPENDIX D

Reproductive Tissue Evaluations and Estrous Cycle Characterization

TABLE D1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

Study Parameters	0 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
n	10	10	10	10
Weights (g)				
Necropsy body weight	272 ± 5	273 ± 4	277 ± 5	262 ± 5
Left epididymis	0.263 ± 0.010	0.284 ± 0.011	0.265 ± 0.015	0.260 ± 0.011
Left cauda epididymis	0.078 ± 0.003	0.091 ± 0.002	0.081 ± 0.003	0.079 ± 0.006[2]
Left testis	0.772 ± 0.064	0.927 ± 0.067	0.735 ± 0.054	0.780 ± 0.064
Spermatid measurements				
Spermatid heads (10^7/g testis)	5.19 ± 0.86	7.03 ± 0.35	4.51 ± 0.53	5.91 ± 0.71
Spermatid heads (10^7/testis)	4.36 ± 0.91	6.56 ± 0.60	3.50 ± 0.65	4.89 ± 0.78
Spermatid count (mean/10^{-4} mL suspension)	36.30 ± 7.59	54.7 ± 5.01	29.13 ± 5.41	40.73 ± 6.54
Epididymal spermatozoal measurements				
Motility (%)	55.26 ± 12.53	75.89 ± 5.02	60.06 ± 9.58	63.12 ± 7.50[2]
Concentration (10^6/g cauda epididymal tissue)	183 ± 39	275 ± 61	219 ± 65	345 ± 77[2]

[1] Data presented as mean ± standard error. Differences from the control group for necropsy body and reproductive organ weights and spermatid and epididymal spermatozoal measurements are not significant by Dunn's test.
[2] n=9.

TABLE D2 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 13-Week Inhalation Study of 1-Nitropyrene[1]

Study Parameters	0 mg/m^3	8 mg/m^3	20 mg/m^3	50 mg/m^3
n	10	10	10	10
Necropsy body weight (g)	170 ± 3	165 ± 2	168 ± 3	167 ± 3
Estrous cycle length (days)	5.10 ± 0.10	5.10 ± 0.10	5.40 ± 0.22	5.20 ± 0.13
Estrous stages (% of cycle)				
Diestrus	41.7	41.7	43.3	43.3
Proestrus	18.3	19.2	19.2	19.2
Estrus	21.7	20.8	20.0	18.3
Metestrus	18.3	18.3	17.5	19.2

[1] Necropsy body weight and estrous cycle length presented as mean ± standard error. Differences from the control group for necropsy body weights are not significant by Dunn's test. By multivariate analysis of variance (MANOVA), exposed groups do not differ significantly from the control group in cycle length or in the relative length of time spent in the estrous stages.