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Respiratory Carcinogenesis of Nitroaromatics

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IIT Research Institute, Chicago, IL

Includes the Commentary by the Institute's Health Review Committee

Research Report Number 32

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HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

Respiratory Carcinogenesis of Nitroaromatics

Richard C. Moon, Kandala V.N. Rao, Carol J. Detrisac[1]

ABSTRACT

The carcinogenic potential of 1-nitropyrene, a major mutagenic constituent of diesel-exhaust particles, was investigated using a hamster respiratory-carcinogenesis model. The specific aims of the investigation were to assess the activity of 1-nitropyrene as a complete carcinogen (Study 1) and as a cocarcinogen (Study 2) when administered in combination with the known environmental carcinogen benzo[*a*]pyrene. Preparations of 1-nitropyrene and benzo[*a*]pyrene adsorbed onto an equal mass of carbon carrier particles (Stokes diameter 2 to 5 μm, greater than 70 percent) were used for the intratracheal administration.

Evaluation of 1-nitropyrene as a complete carcinogen involved exposing male Syrian golden hamsters to 1 or 2 mg of 1-nitropyrene either once or twice each week. A once-per-week instillation of 2 mg of benzo[*a*]pyrene served as a positive control. Two groups of animals received sterile saline only (saline controls) or carbon particles suspended in saline (particle controls). In addition, a group of untreated hamsters served as shelf controls. Evaluation of 1-nitropyrene as a cocarcinogen involved treating animals once each week with either 1 or 2 mg of 1-nitropyrene with or without concomitant exposure to 0.25 mg of benzo[*a*]pyrene. The studies were terminated after 92 weeks of treatment.

In both studies, hamsters receiving 1-nitropyrene showed a dose-related decrease in survival and body-weight gain. In general, animals in Study 2 showed better survival than those in Study 1. A high intercurrent mortality was observed in the control groups of Study 1. In order to adjust for intercurrent mortality, tumor incidences were analyzed after modeling (using Cox regression) the effect of treatment in all animals for the period they were alive. A broad spectrum of neoplastic and nonneoplastic lesions was observed in the lungs and tracheas of all hamsters except shelf controls. Because of the histologic complexity of these lesions, the slides were coded, and the tissues were evaluated by an unbiased pathologist. The tumor types included papillomas, adenomas, adenocarcinomas, and squamous cell carcinomas, the latter being the most prevalent type in benzo[*a*]pyrene-treated animals. In view of the low incidence of tumors in 1-nitropyrene-treated hamsters, the tumor incidences in various experimental groups were compared regardless of the tumor type, location, and multiplicity. A small, but significant, increase in tumor incidence, with a dose-response trend, was observed only in Study 2 hamsters receiving 1-nitropyrene once weekly. In contrast, treatment with benzo[*a*]pyrene adsorbed onto carbon particles induced benign and malignant tumors virtually in all animals that survived more than 50 weeks of treatment. The lack of a significant effect in Study 1 may be due to various confounding factors, such as high mortality, toxicity of carbon particles alone at the high amount administered, and underlying virus infection. Evaluation of the cocarcinogenic effect of sequential exposure to 1-nitropyrene and benzo[*a*]pyrene revealed that the combined effect, although enhanced, was statistically no greater than could be expected from the addition of individual effects. However, there was an increase in the number of animals bearing multiple tumors in the combination groups, indicating a potential synergistic effect. The overall data from the current studies in the hamster model suggest that 1-nitropyrene in association with carbon particles is a weak respiratory carcinogen.

INTRODUCTION

The respiratory carcinogenicity of diesel-exhaust particles is of special concern in view of their mutagenic and genotoxic activities and the prospect of increased use of diesel-powered engines (Rosenkranz and Mermelstein 1983; Fraser 1986; McClellan 1986). While the contribution of diesel-exhaust exposure to lung cancer in exposed populations remains to be determined (Wynder and Higgins 1986), a recent case-control epidemiologic investigation indicated increased risk of lung cancer to railroad workers in high-exposure occupations (Garshick et al. 1987). Furthermore, a broad variety of biologically active compounds, including known carcinogens like benzo[*a*]pyrene (B*a*P)[2], have been isolated from the particle extracts (Pitts 1983). Much of the direct-acting mutagenicity of diesel-exhaust extracts is now accounted for by the presence of nitrated polynuclear aromatic hydrocarbons, which seem to be ubiquitous in the urban environment because of their facile formation from pyrene and nitrogen oxides generated in

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[2] A list of abbreviations appears at the end of this report for your reference.

various combustion processes (Tokiwa et al. 1987). Since very little is known about their respiratory carcinogenic effects, the present studies investigated the carcinogenic potential of 1-nitropyrene (1-NP), a major mutagenic constituent of diesel particles (Salmeen et al. 1982).

In addition to diesel exhaust, 1-NP has been detected in other combustion products, such as coal fly ash (Mumford et al. 1986), carbon black toners (Rosenkranz et al. 1980), and gasoline station waste (Manabe et al. 1984). Low levels of the compound were found in (yakitori) grilled chicken (Kinouchi et al. 1986) and in tea leaves (Dennis et al. 1984). Although 1-NP was a potent direct-acting mutagen in the Ames test (Pitts et al. 1982; Salmeen et al. 1982), it showed weak-to-moderate genotoxic activity in mammalian cell systems (Nachtman and Wolff 1982; Heflich et al. 1986; Patton et al. 1986; Lambert and Weinstein 1987). The potential genotoxicity of 1-NP in mammalian respiratory organs was indicated by the formation of DNA adducts, such as *N*-(deoxyguanosin-8-yl)-1-aminopyrene, in the lung and tracheal tissues of laboratory animals (Jackson et al. 1985; King et al. 1986; Mitchell 1988), and by the induction of unscheduled DNA synthesis in the tracheal epithelial cells of rabbits and humans (Sugimura and Takayama 1983; Haugen et al. 1986). Since nitropyrenes were active in the human tracheal cell system without the addition of the activating microsomal S9 mix, while B*a*P required the S9 mix for activation, Sugimura and Takayama (1983) suggested that nitropyrenes could be more important than B*a*P as a cause of human cancer.

The limited carcinogenicity bioassays with 1-NP in mice and rats produced inconsistent, and sometimes conflicting, results. El-Bayoumy and colleagues (1984) reported an increased incidence of lung adenomas in male and female A/J mice after intraperitoneal injection of 1-NP, but other investigators (Kouros and Dehnen 1985; Wislocki et al. 1986; Busby et al. 1989) did not find a significant effect when the compound was injected subcutaneously or intraperitoneally in newborn mice of other strains. However, Wislocki and coworkers (1986) observed a significant increase in the incidence of liver tumors in male mice. Subcutaneous injection of 1-NP induced injection-site sarcomas (malignant fibrous histiocytomas) in male and female rats and mammary adenocarcinomas in female rats (Ohgaki et al. 1982; Sugimura and Takayama 1983; Hirose et al. 1984; El-Bayoumy et al. 1987). These findings, however, were not substantiated by subsequent studies (Ohgaki et al. 1985; de Serres and Matsushima 1986). The tumor response observed in earlier studies might have been influenced by the age of the animals at the time of exposure, the possible impurity of 1-NP used, and the duration of the experiment. Although rats are now known to develop lung tumors after prolonged inhalation of high concentrations of diesel-exhaust particles (Brightwell et al. 1986; Heinrich et al. 1986; Mauderly et al. 1987), none of the previous studies with 1-NP in rats reported the induction of respiratory tumors. Maeda and associates (1986) found squamous cell carcinomas (75 percent of animals), undifferentiated carcinomas (7 percent), and squamous cell metaplasia (7 percent) in the lungs of Fischer-344 rats that had been injected with 0.15 mg of 1,6-dinitropyrene in a beeswax-tricaprylin vehicle directly into the lungs; no such lesions were found after similar administration of 1.5 mg of 1-NP in the same vehicle. The role of 1-NP as a complete carcinogen is yet to be established, and its effect on different phases of the carcinogenic process has not been fully investigated. It is interesting to note that 1-NP did not show tumor-initiating activity in mouse skin (El-Bayoumy et al. 1982; Nesnow et al. 1984), a model system that had consistently responded to the carcinogenic polynuclear aromatic hydrocarbons. Thus, additional studies are needed to assess the carcinogenic potential of 1-NP in general, and in the respiratory organs in particular. Since the anticipated human exposure to 1-NP is mainly through the respiratory route, a model system utilizing direct application of 1-NP to respiratory tissues is appropriate for assessing the carcinogenic potential of the compound alone or in environmental mixtures.

AIMS

The main objective of the studies was to assess the respiratory carcinogenic potential of 1-NP in a hamster model using the intratracheal route of exposure. The bioassays were designed to provide qualitative and quantitative information on two major aspects of 1-NP carcinogenesis: (1) the carcinogenicity of 1-NP when administered together with carbon particles for the life span of the animal (Study 1); and (2) the cocarcinogenic activity of the 1-NP and carbon mixture when given in combination with B*a*P, a known carcinogen present in diesel exhaust (Study 2).

MATERIALS AND METHODS

GENERAL CONSIDERATIONS

During the past two decades, several models have been introduced for investigations on respiratory tract carcinogenesis in laboratory animals (Nettesheim and Griesemer 1978; Pepelko 1984). The Syrian golden hamster model was selected for the present studies because it has been widely used for carcinogenicity bioassays of a variety of environ-

mental pollutants, especially those containing polynuclear aromatic hydrocarbons. Also, extensive data are available on the dose-tumor relation and the histogenesis of induced tumors.

The respiratory tract of the Syrian golden hamster is similar to that of humans, except for the sparsity of bronchial glands in the lower part of the tract and the absence of supporting structures, such as cartilage, in the bronchi (Becci et al. 1978). The incidence of spontaneous lung tumors in hamsters is very low, and consists of peripheral proliferative lesions with questionable neoplastic significance; malignant epithelial tumors are virtually unknown (Nettesheim and Griesemer 1978). Bronchogenic squamous cell carcinomas (Stenback 1977) and small-cell carcinomas (Schuller et al. 1988), resembling those found in human lung cancer, have been induced in the hamster by a variety of environmental chemical carcinogens.

Previous studies at the IIT Research Institute (IITRI) defined the relation between tumor response and dose of known carcinogens in the hamster respiratory tract, using either the Saffiotti or the Schreiber method of intratracheal administration (Henry et al. 1974; Grubbs et al. 1979; Becci et al. 1980). During the first 10-month period of the project reported here, efforts were made to administer 1-NP using the Schreiber method, which was developed and modified at IITRI for studying the carcinogenic, cocarcinogenic, and tumor-promoting activities of potential respiratory carcinogens. The intratracheal catheter and the ancillary equipment used for administering solutions of test agents are described by Grubbs and colleagues (1979). A major advantage of this method is that a well-defined area of the respiratory tract is preselected for the test-agent exposure, and the specified target area is exposed to a known amount of the agent for a predetermined period. The latency period for the development is usually short, and the tumors are easily quantified because they are localized in the selected area of exposure. Neither inhalation nor other intratracheal techniques can offer these advantages. The model is both cost- and time-effective. For this project, however, this approach was not successful because of the poor solubility of 1-NP in common solvents (dimethylsulfoxide, propylene glycol) that could be used without producing toxic effects. Consequently, the Saffiotti method (Saffiotti et al. 1968) was adopted for intratracheal administration of 1-NP. This method, which was already being used at IITRI for investigating the respiratory carcinogenic effects of diesel-fuel emissions in hamsters (Shefner et al. 1982, 1985), offered an opportunity to expose the animals to 1-NP attached to carrier carbon particles, thus simulating human exposure conditions.

Because diesel particulate pollutants contain a variety of organic compounds, including carcinogens such as B*a*P (Pitts 1983), the potential cocarcinogenic effects of 1-NP and B*a*P were investigated in Study 2. Study 2 was originally planned for one year's duration, but, at the recommendation of the HEI Research Committee, it was extended for the life span of the hamsters (100 weeks; 92 weeks of treatment). This change was made to match the duration of treatment of 1-NP groups common to Study 1.

In both studies, 1-NP was administered at two dose levels, 2 mg (high) and 1 mg (low), each with an equal amount of carrier carbon particles. The high dose of 1-NP (2 mg) was selected for the following reasons. Previous studies by Henry and coworkers (1974) at IITRI, and by Stenback and colleagues (1973), utilized 2 mg of the same carbon particles for intratracheal administration to hamsters. The maximum quantity of 1-NP that could be adsorbed onto this amount (2 mg) of carbon was found to be 2 mg. With greater amounts (3 mg or more of 1-NP per 2 mg of carbon), visible separation of the compound occurred when suspensions for intratracheal instillation were made in saline.

EXPERIMENTAL PROTOCOLS

The experimental designs for the two studies are shown in Tables 1 and 2.

Study 1: Carcinogenic Effect of 1-Nitropyrene

Study 1 was conducted to provide data concerning the carcinogenic effect of 1-NP after repeated intratracheal exposure for the life span of the hamsters. Groups of seven- to nine-week-old hamsters were exposed by intratracheal administration to a 2-mg (high) or 1-mg (low) dose of 1-NP, either once a week (on Mondays) or twice a week (on Mondays and Thursdays). A different group of hamsters was given 2 mg of B*a*P once a week and served as the positive control. Three other groups of hamsters received saline twice weekly (saline controls), or carbon particles twice weekly (particle controls), or were left untreated (shelf controls). The experiment was terminated after 92 weeks of treatment.

Study 2: Cocarcinogenic Effect of 1-Nitropyrene

Study 2 was conducted to assess the potential cocarcinogenic effects resulting from administration of 1-NP in combination with a low dose of B*a*P (0.25 mg). The 1-NP was given once each week at the same two dose levels used in Study 1, with or without additional treatment with B*a*P. Two other groups received B*a*P in combination with either saline or carbon particles. The multiple-treatment groups received one treatment on Tuesdays and the other treatment on Fridays. Control animals either were given carbon parti-

Table 1. Experimental Design for Assessing Carcinogenicity of 1-Nitropyrene by Intratracheal Administration to Hamsters, Study 1

Group	Initial No. of Hamsters	Treatment[a,b]	
		1-Nitropyrene	Other
1 (Shelf control)	20	0	0
2 (Saline control)	50	0	Saline, 0.2 mL 2/wk
3 (Particle control)	50	0	Carbon particles, 2 mg 2/wk
4	55	2 mg 1/wk[c]	0
5	55	1 mg 1/wk[c]	0
6	58	2 mg 2/wk[c]	0
7	60	1 mg 2/wk[c]	0
8	50	0	Benzo[*a*]pyrene, 2 mg 1/wk[c]

[a] All treatments were given in 0.2 mL of saline using the Saffiotti technique (1968).

[b] Duration of the experiment: 92 weeks.

[c] At each dose, the compound was adsorbed onto an equal mass of carbon carrier particles.

cles, or were not treated so that they could serve as shelf controls.

TEST COMPOUNDS

1-Nitropyrene (99.9 percent pure) was synthesized and supplied by the Midwest Research Institute (Kansas City, MO). Benzo[*a*]pyrene (95 to 99 percent pure) was procured from the National Cancer Institute Carcinogen Registry at IITRI. The compounds were stored desiccated in sealed amber-glass bottles at −70°C.

CARRIER PARTICLES

Carbon particles (Stokes diameter, 2 to 5 μm) were prepared at the Particle Chemistry Laboratories, IITRI, from carbon powder supplied by Barnebey-Cheney (Columbus, OH). The source of the carbon was charred nut shells. This material was chosen for the solid structure of the particles (as opposed to the agglomerate and partially fused aggregates found in carbon blacks), high surface area (due to particle porosity), high purity, and absence of extractable organic compounds. Emission spectroscopic analysis of the size-fractionated powders indicated the 2- to 5-μm powder used in this study was 99.0 percent pure. Major contaminants identified included aluminum (0.25 percent), calcium (0.04 percent), iron (0.01 percent), potassium (0.06 percent), and silicon (0.68 percent). The material was solvent-washed and vacuum-dried by the manufacturer. Initial studies at IITRI indicated that the concentration of benzene-soluble organic compounds was less than 500 parts per million (ppm). A lower concentration of organic

Table 2. Experimental Design for Assessing Cocarcinogenicity of 1-Nitropyrene by Intratracheal Administration to Hamsters, Study 2

Group	Initial No. of Hamsters	Treatment[a,b]		
		1-Nitropyrene	Benzo[*a*]pyrene	Other
1 (Shelf control)	20	0	0	0
2 (Saline control)	50	0	0.25 mg 1/wk[c]	Saline, 0.2 mL 1/wk
3 (Particle control)	50	0	0	Carbon particles, 2 mg 1/wk
4	50	1 mg 1/wk[c]	0	0
5	50	2 mg 1/wk[c]	0	0
6	50	0	0.25 mg 1/wk[c]	Carbon particles, 2 mg 1/wk
7	50	1 mg 1/wk[c]	0.25 mg 1/wk[c]	0
8	50	2 mg 1/wk[c]	0.25 mg 1/wk[c]	0

[a] All treatments were given in 0.2 mL of saline using the Saffiotti technique (1968).

[b] Duration of the experiment: 92 weeks.

[c] At each dose, the compound was adsorbed onto an equal mass of carbon carrier particles.

compounds can be expected in the preparation used because the carbon particles were washed with isopropanol during the size-fractionation process.

Particle-size analysis was performed by a sedimentation technique (Andreason pipette method; see Siebert 1979). This sizing technique provides mass-size distribution data based on particle Stokes diameter. All of the particle-size distribution data are shown in Table 3.

TEST PREPARATIONS

Carbon particles carrying the test compounds (1-NP or B*a*P) were prepared by Mrs. Jean Graf-Teterycz, Chemistry Division, IITRI, using the melt method. The test compound (6 g) was first dissolved in hot acetone (300 mL) and mixed thoroughly with an equal amount (6 g) of carbon particles. The solvent was allowed to evaporate overnight at room temperature, and a slurry was prepared from the residue by the addition of acetone. After careful removal of the solvent from the slurry by gentle heating on a thermostatically controlled hot plate, the compound was melted into the porous carbon particles by increasing the temperature of the hot plate without causing sublimation of 1-NP from overheating. The mixture was thoroughly stirred during melting. After cooling, the product was collected into actinic containers, sealed, and stored at −10°C until use. The actual preparation of the melted product was conducted in a laboratory hood equipped with a high-efficiency particulate air (HEPA) filter. Pure compounds and the test preparations were handled in a HEPA-filtered glove box, thus avoiding exposure to light and atmospheric oxygen.

ANALYTICAL METHODS

Purity of 1-NP and B*a*P was checked by the suppliers before shipment. Subsequent analyses of the compounds and their test preparations by high-pressure liquid chromatography (HPLC) were carried out by Dr. Walter Eisenberg, Chemistry Division, IITRI. For the assays, 10-mg samples of test preparations, containing the compounds attached to carbon particles, were individually extracted in a Soxhlet apparatus for six hours with 200 mL of methylene chloride ($MeCl_2$). The $MeCl_2$ extract was evaporated to 1 mL, and then the solvent was exchanged to acetonitrile. The extracts were analyzed by reverse-phase HPLC using the conditions for individual compounds listed in Table 4.

Recoveries of 1-NP and B*a*P from fresh batches of test preparations ranged from 87 to 99 percent. The losses were minimal (less than 10 percent) when the preparations were stored at −10°C for six to eight weeks. Therefore, all test preparations were used within six weeks after preparation. Storage for longer periods resulted in a 20 percent loss of the 1-NP preparation. The loss could be due to some separation of 1-NP from the carbon particles, possibly resulting from conversion to a crystalline state. Analysis by HPLC showed no degradation products in these samples.

ANIMALS AND ANIMAL CARE

Outbred male Syrian golden hamsters, five to seven weeks old, were obtained from Harlan Sprague Dawley (Indianapolis, IN) for Study 1, and from the Mammalian Genetics and Animal Production Section, National Cancer Institute (Bethesda, MD) for Study 2. The hamsters were

Table 3. Particle-Size Distribution as Determined by the Andreason Pipette Method[a]

Size[b] (μm)	Cumulative Mass (% less than size)
9.0	99.5
7.0	97.0
5.0	90.2
3.0	48.8
2.0	20.5
1.0	0.8

[a] See Siebert 1979.

[b] Stokes diameter. Particle morphology of the carbon powder was examined by both optical and scanning electron microscopy. In general, particles were slightly elongated fragments with sharp angular edges.

Table 4. High-Pressure Liquid Chromatography Conditions

	Compound	
	Benzo[*a*]pyrene	1-Nitropyrene
Detector	Liquid chromatography fluorometer	Fluorescence spectrophotometer
	Range 1.0 Sensitivity 532 λ setting = 280 nm Cutoff filter 389 nm	Range 10.0 Sensitivity 532 Excitation λ = 360 nm Emission λ = 430 nm
Column	Zorbax ODS 4.6 mm × 25 cm P.N.	Zorbax ODS 4.6 mm × 15 cm P.N.[a]
Solvent	82% Methylcyanide 18% Water Isocratic	75% Methanol 25% Water pH 8.0
Flow rate	1.0 mL/min	1.0 mL/min

[a] Catalytic column packed with law 1–100 HN 3179–01 (ground and sieved < 125 μm, > 45 μm).

housed, in groups of five per cage, in polycarbonate cages provided with hardwood-chip laboratory animal bedding (Sani-chips, P.J. Murphy Forest Products Corporation, Rochelle Park, NJ). The bedding material was heat-treated and checked by the manufacturer for the presence of *Pseudomonas*, *Salmonella*, and *Shigella* (all negative), and for pesticide residues (less than 0.01 ppm), aflatoxins (less than 20 parts per billion), and heavy metals (less than 0.5 ppm). Racks carrying the animal cages were kept in presterilized, windowless, air-conditioned rooms (23° ± 1°C) that were allotted exclusively for the study animals. The cages and bedding were changed twice weekly, or more frequently if necessary. Used cages and water bottles were cleaned, washed, and sterilized in an automatic washing machine (180°F) or autoclave. Throughout the study, hamsters were maintained on a pelleted stock diet (Wayne Laboratory Blox, Wayne Pet Food Division, Continental Grain Co., Chicago, IL) and given tap water ad libitum. They were observed daily for symptoms of illness, particularly of respiratory tract disease, morbidity, and mortality. Body weights of all animals were recorded weekly until termination of the study. After two weeks of quarantine, the hamsters were stratified by body weight and were randomly allocated to the various treatment and control groups so that all groups had uniform average body weight. Each animal was ear-punched for individual identification. Each cage carried a color-coded tag, unique for the group, showing the treatment, group number, and number of hamsters in the cage.

INTRATRACHEAL INSTILLATION

Suspensions of test and control preparations were made in sterile saline just before use. Appropriate amounts of the material were carefully mixed with the required volume of the saline in actinic flasks, so that the required dose could be delivered in 0.2 mL of the final suspension. The suspensions were prepared immediately before dosing and kept homogenous by frequent vortexing.

Intratracheal instillations were carried out according to the procedure described by Saffiotti and coworkers (1968), with the modification that halothane was used instead of methohexital (brevital) as the anesthetic. The hamsters were immobilized in an anesthetic apparatus similar to that described by Smith and associates (1973). A mixture of halothane and oxygen was kept circulating through the anesthetic chambers by means of a Heidbrink model 960 veterinary anesthesia machine (Ohio Chemical & Surgical Equipment Co., Madison, WI), connected to a Harvard model 607 variable-speed dog ventilator (Harvard Apparatus, Natick, MA). Under these conditions, the induction and the recovery periods were short (one to two minutes) and no adverse reactions (for example, laryngospasm) were encountered during the instillation procedure.

NECROPSY AND COLLECTION OF TISSUES

Hamsters that died during the study or were killed when found moribund were promptly necropsied to collect fresh tissues. At the termination of the studies, all animals were killed by carbon dioxide asphyxiation. The larynx of each hamster was severed, and the trachea and lung tissues were removed en bloc and slowly infused with 10 percent neutral buffered formalin. The most anterior portion of the trachea was then ligated, and the tissue placed in formalin. Other tissues, showing gross abnormalities, were also taken for histopathologic examination. In addition, blood samples were collected when animals were killed at the end of each study for determination of serum antiviral antibody titers.

HISTOPATHOLOGY

Tissues were trimmed by first removing the trachea below the bifurcation of the main bronchi. The trachea was evenly divided into anterior and posterior pieces, and lung lobes were trimmed to include major airways and then embedded. All grossly visible lesions were also embedded. Step sections (every 125 μm) were taken from both regions of the trachea. Replicate sections were obtained from all lung specimens. All 5-μm paraffin sections were stained with hematoxylin and eosin for microscopic examination.

Tissues from Study 1 were examined first, and then those from Study 2. The slides were randomized for microscopic examination so that all slides from a single animal were viewed together, but the pathologist was aware neither of the treatment received by the animal nor of the duration of treatment. Slides from each hamster were examined in the sequence of anterior trachea, posterior trachea, and lung. Diagnoses were recorded immediately after each slide was examined. No data were collected to indicate the number of lesions per animal.

All hyperplastic, metaplastic, and dysplastic lesions, and all neoplasms observed in the epithelium of the trachea and lungs were recorded. Inflammatory or reactive changes that occurred in epithelial or nonepithelial tissues were not evaluated because they were seen in all tissues bearing carbon particles. Although accumulations of intraalveolar macrophages were occasionally seen, these cells were considered inflammatory and, therefore, did not constitute a proliferative lesion.

No effort was made to discriminate the site of origin of lesions within the lung (bronchus, pulmonary bronchiole, respiratory bronchiole, alveolar duct, or alveolus) for two rea-

sons. First, many of the lesions obliterated the site of origin, and second, the presence of large amounts of carbon often obscured the site of origin (all of the lung was opaque except for the proliferative lesions, which characteristically contained fewer carbon particles).

STATISTICAL ANALYSIS

Body-weight data were analyzed by analysis of variance (Gad and Weil 1982). Because of the high mortality rate, the tumor-incidence distributions in all groups were compared after modeling the effect of treatment in all animals for the period they were alive. Because of the low incidence of tumors in groups treated with 1-NP, no distinction was made as to the number, location (trachea or lung), type, and malignancy of the tumors. The tumor-incidence data were analyzed as to the main effects and interactions in 2-by-2 or 3-by-2 designs, as specified by the HEI Review Committee. The designs were evaluated for effects on time-to-tumor data using a Cox regression model, and in terms of simple tumor incidence using a log-linear model. In the presence of a significant main effect or interaction, pairwise comparisons were made using the Mantel-Cox statistic for time-to-tumor data and a chi-squared statistic for tumor-incidence data. The methodology, results, and a general interpretation of the findings are presented in the Statistical Report (Appendix A).

RESULTS

SURVIVAL AND BODY-WEIGHT CHANGES

Study 1

The survival rates and body-weight gains of hamsters receiving the various treatment regimens are shown in Figures 1 and 2, respectively. After five weeks of treatment, 75 percent of the hamsters in the saline control group died, apparently from pneumonia. The causative factor or factors, however, are not known. After 20 to 30 weeks of treatment, increased mortality rates were observed in hamsters treated twice weekly with high- and low-dose levels of 1-NP, as well as in the particle control and the B*a*P-treated animals. The mortality rate was particularly high in hamsters receiving the highest dose of 1-NP (2 mg) and in the particle control group, both treated on a twice-weekly schedule. Since both had 2 mg of carbon particles, the twice-weekly administration of this amount of carbon particles seems to be a major factor contributing to the observed toxicity in these groups. At 52 weeks, the survival rates of animals in the 1-NP groups were as follows: low dose 1/wk, 76 percent; high dose 1/wk, 69 percent; low dose 2/wk, 53 percent; high dose 2/wk, 38 percent. The particle control and the B*a*P-treated animals had a survival rate of 18 percent and 54 percent, respectively. Thereafter, a steady decline in the survival rate of all groups, including the shelf control group, was observed.

The effect of intratracheal instillation per se on the body-weight gain was apparent after the first few weeks of treatments. Irrespective of the treatment, the mean body weights were consistently lower than those of the shelf control animals. This effect was greatest ($p < 0.05$) for hamsters treated with the highest dose of 1-NP (2 mg 2/wk) and for those receiving carbon particles only (2/wk). The large increases and fluctuations observed toward the end of the study in all groups were due to the deaths of animals that had lost substantial body weight and to the relatively small group sizes resulting from the loss of animals. The increased body weight of some animals from pathological conditions, such as ascites and generalized edema, may have also contributed to the fluctuations. The body-weight losses were most apparent among the hamsters that received 2 mg of 1-NP twice weekly (group 6), and were least apparent in those receiving 1 mg of 1-NP once a week (group 5), a trend similar to that observed with the survival rate.

Study 2

Survival rates and mean body weights of animals in all treatment groups are shown in Figures 3 and 4. Compared with Study 1, all groups in this study showed good survival rates; at 36 weeks, the survival rate ranged from 80 percent for particle control animals (2 mg 1/wk) to 98 percent for animals treated with the high dose of 1-NP (2 mg 1/wk). Thereafter, the mortality rate increased in all groups, particularly those treated with particulate material.

In general, the body-weight gains of all treated groups were similar, and were consistently lower than those of the shelf control group. After 36 weeks of treatment, the mean body weights of all treated groups (range 160 to 172 g) were not significantly different ($p > 0.05$) from the mean body weight of the shelf control group (172 g). As in Study 1, toward the end of the experiment, the mean body weights fluctuated because of the pathological conditions of individual animals and the associated mortality. Records of the survival and body-weight changes of animals in Studies 1 and 2 are given in Appendix B[3].

[3] Appendix B is available on request from the Health Effects Institute.

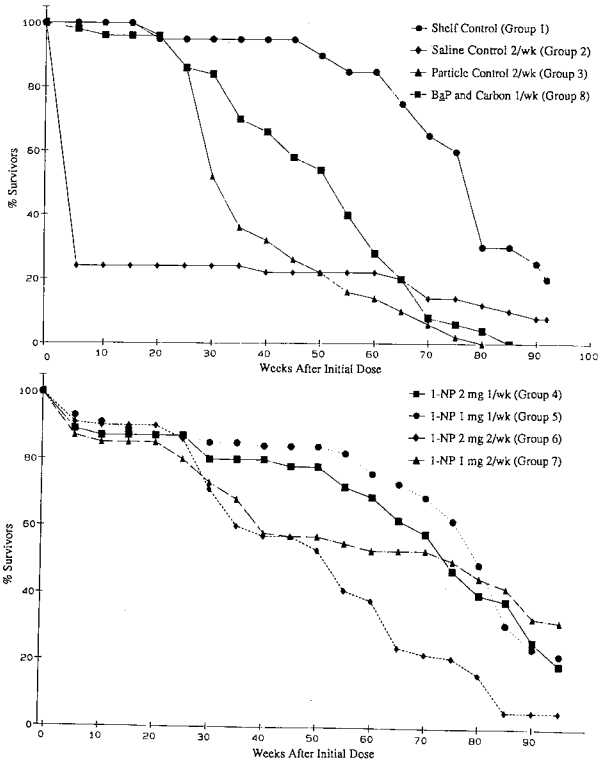

Figure 1. Study 1: Percentage of survivors among hamsters treated with 1-NP, BaP, and control preparations.

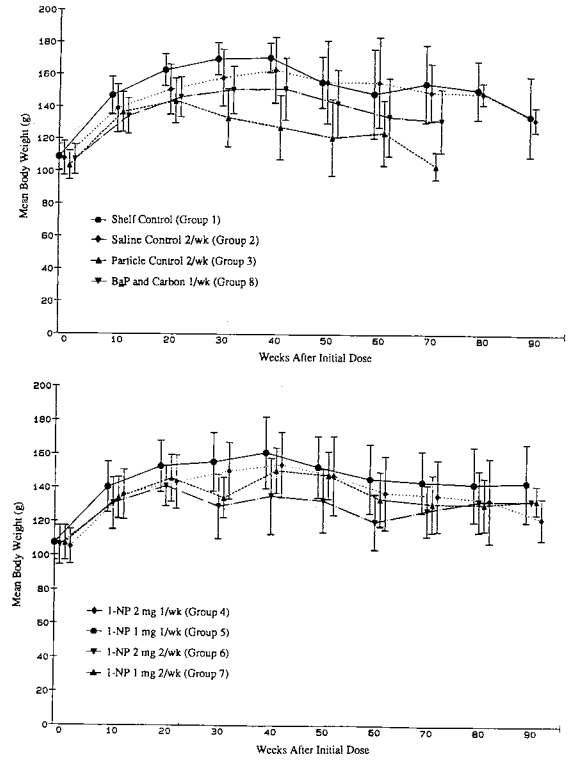

Figure 2. Study 1: Mean body weights ± SD of hamsters treated with 1-NP, BaP, and control preparations.

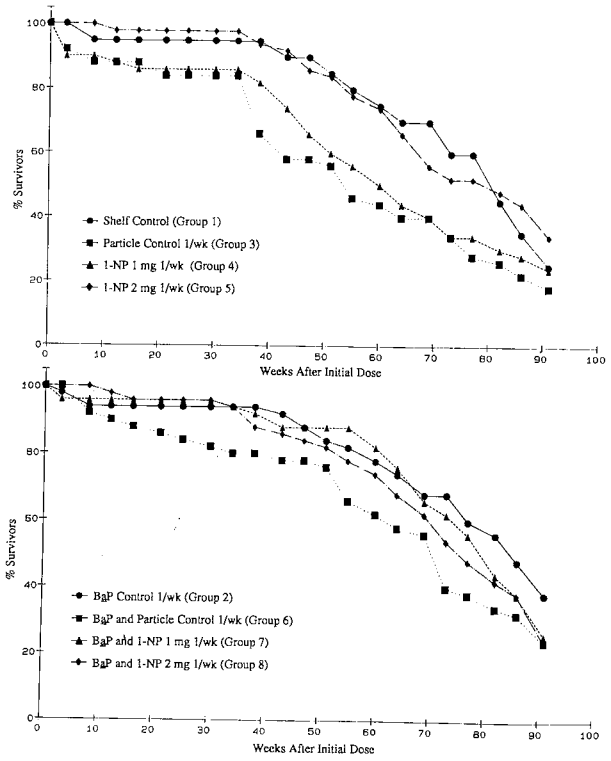

Figure 3. Study 2: Percentage of survivors among hamsters treated with 1-NP, BaP, and control preparations.

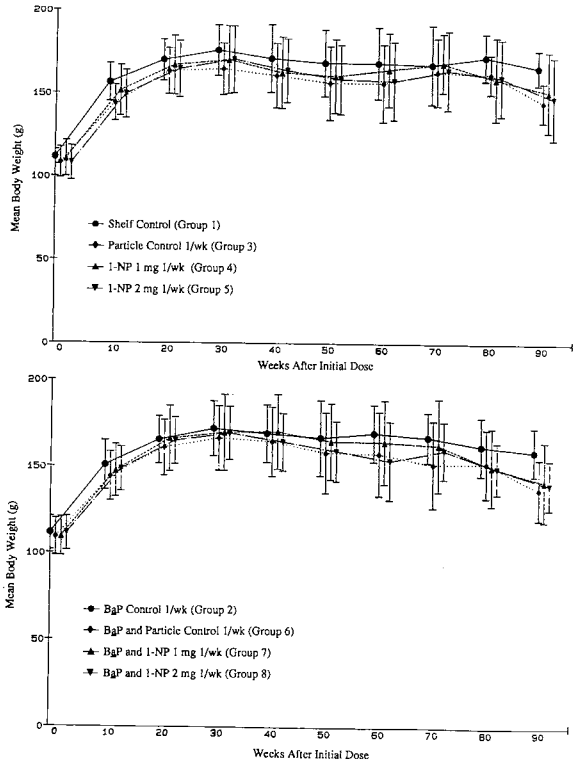

Figure 4. Study 2: Mean body weights ± SD of hamsters treated with 1-NP, BaP, and control preparations.

Table 5. Study 1: Yield of Tissues for Histopathologic Examination to Determine Carcinogenicity of 1-Nitropyrene

Group	Initial No. of Hamsters	No. of Hamsters with Tracheas Examined	No. of Hamsters with Lungs Examined
1	20	20	20
2	50	49	50
3	50	48	48
4	55	54	55
5	55	53	54
6	58	53	56
7	60	58	58
8	50	48	49

Table 6. Study 2: Yield of Tissues for Histopathologic Examination to Determine Cocarcinogenicity of 1-Nitropyrene

Group	Initial No. of Hamsters	No. of Hamsters with Tracheas Examined	No. of Hamsters with Lungs Examined
1	20	19	19
2	50	49	50
3	50	46	47
4	50	48	48
5	50	46	46
6	50	48	49
7	50	48	48
8	50	45	46

HISTOPATHOLOGY

An individual histopathology report was prepared for each animal. The lesions were recorded by site (trachea or lung) and length of treatment. This was done to determine the age of the animals at diagnosis of the lesions. Not all hamsters in the study had trachea and lung tissue examined microscopically. Losses of tissue were due to autolysis or cannibalization, or both. Hamsters routinely cannibalize the cheek pouches and cervical tissues of dead cagemates. Understandably, tracheal tissue was lost to cannibalization more often than was lung tissue. If any part of the trachea was acceptable for microscopic examination, the tissue was counted as examined for calculation of incidence. Table 5 lists the yield of tissue by group for Study 1; Table 6 lists the yield for Study 2. Incidences of lesions are shown in Ta-

Table 7. Study 1: Incidence of Tracheal Lesions in Hamsters After Exposure to 1-Nitropyrene by Treatment Group

	None (Shelf Controls)	Saline	Carbon Particles	1-Nitropyrene				Benzo[a]-pyrene
Experiment Parameters								
Dose	0	0.2 mL	2 mg	2 mg	1 mg	2 mg	1 mg	2 mg
Dosing schedule	0	2/wk	2/wk	1/wk	1/wk	2/wk	2/wk	1/wk
Group	1	2	3	4	5	6	7	8
No. of hamsters with tracheas examined	20	49	48	54	53	53	58	48
Nonneoplastic[a]								
Hyperplasia	2 (10.0)	17 (34.7)	34 (70.8)	13 (24.1)	15 (28.3)	33 (62.3)	36 (62.1)	34 (70.8)
Squamous metaplasia		4 (8.2)	2 (4.2)	5 (9.3)	3 (5.7)	12 (22.6)	3 (5.2)	19 (39.6)
Dysplasia		20 (40.8)	18 (37.5)	4 (7.4)	4 (7.5)	19 (35.8)	15 (25.9)	19 (39.6)
Micropapillomatosis	1 (5.0)		9 (18.8)	3 (5.6)	3 (5.7)	9 (17.0)	12 (20.7)	13 (27.1)
Neoplastic[a]								
Papilloma		1 (2.0)				1 (1.9)		10 (20.8)
Polyps			1 (2.1)		1 (1.9)	1 (1.9)	3 (5.2)	10 (20.8)[b]
Carcinoma in situ								1 (2.1)
Squamous cell carcinoma								6 (12.5)
Anaplastic carcinoma								1 (2.1)
Basal cell carcinoma								1 (2.1)
Unspecified carcinoma								1 (2.1)
Total animals with tracheal tumors	0 (0)	1 (2.0)	1 (2.1)	0 (0)	1 (1.9)	2 (3.8)	3 (5.2)	24 (50.0)

[a] Values indicate numbers of lesions. Numbers in parentheses are the percentages of hamsters with tracheas examined that had tumors.

[b] One animal had two morphologically distinct polyps.

Table 8. Study 1: Incidence of Lung Lesions in Hamsters After Exposure to 1-Nitropyrene by Treatment Group

	None (Shelf Controls)	Saline	Carbon Particles	1-Nitropyrene				Benzo[a]-pyrene
Experiment Parameters								
Dose	0	0.2 mL	2 mg	2 mg	1 mg	2 mg	1 mg	2 mg
Dosing schedule	0	2/wk	2/wk	1/wk	1/wk	2/wk	2/wk	1/wk
Group	1	2	3	4	5	6	7	8
No. of hamsters with tracheas examined	20	50	48	55	54	56	58	49
Nonneoplastic[a]								
Hyperplasia		8 (16.0)	23 (47.9)	29 (52.7)	27 (50.0)	36 (64.3)	27 (46.6)	21 (42.9)
Bronchiolar hyperplasia	1 (5.0)	4 (8.0)	16 (33.3)	31 (56.4)	30 (55.6)	32 (57.1)	28 (48.3)	28 (57.1)
Squamous metaplasia			6 (12.5)	11 (20.0)	8 (14.8)	15 (26.8)	8 (13.8)	23 (46.9)
Mucous metaplasia				2 (3.6)			3 (5.2)	2 (4.1)
Adenomatosis		9 (18.0)	15 (31.3)	38 (69.1)	42 (77.8)	35 (62.5)	33 (56.9)	19 (38.8)
Neoplastic[a]								
Adenomas				1 (1.8)		2 (3.6)	3 (5.2)[b]	18 (37.5)[c]
Mastocytoma		1 (2.0)					1 (1.7)	
Bronchial carcinoma								1 (2.0)
Papillary carcinoma								1 (2.0)
Adenocarcinoma								1 (2.0)
Squamous cell carcinoma			1 (2.1)		1 (1.9)			38 (77.6)
Mixed squamous adenomatous carcinoma								1 (2.0)
Alveolar bronchiolar carcinoma					1 (1.9)		1 (1.7)	13 (26.5)
Anaplastic carcinoma								2 (4.1)
Total animals with lung tumors	0 (0)	1 (2.0)	1 (2.1)	1 (1.8)	2 (3.8)	2 (3.6)	4 (6.9)	39 (79.6)

[a] Values indicate numbers of lesions. Numbers in parentheses are the percentages of hamsters with lung tissue examined that had tumors.

[b] One animal had two morphologically distinct adenomas.

[c] Four animals had two morphologically distinct adenomas.

bles 7 and 8 for Study 1, and in Tables 9 and 10 for Study 2. Incidences of animals with multiple tumors are shown for Study 1 in Table 11, and for Study 2 in Table 12. A synopsis of neoplastic and nonneoplastic lesions seen in Studies 1 and 2, with a narrative description of the general microscopic appearance of each type of lesion, is included in Appendix C.

There were two apparent concurrences of a nonneoplastic lesion with a neoplastic lesion, one comprising squamous metaplasia and squamous cell carcinoma in the lung, and the other being adenomatosis in the lung that was possibly a preneoplastic lesion. The Syrian golden hamster has a very low incidence of naturally occurring respiratory neoplasms (Nettesheim and Griesemer 1978). Shelf control hamsters from both studies were free of respiratory tumors. All treatment groups receiving intratracheal instillations contained some animals with respiratory tumors, except for the particle control group in Study 2.

In Study 1, a number of hamsters died early of acute severe pneumonia. Although autolytic changes were seen in the tracheal and lung tissues of many of these animals, the determination of neoplasia in these tissues was seldom a problem. On the other hand, classification of nonneoplastic lesions often was difficult. The infusion of formalin into the autolytic lungs tended to displace cuboidal bronchiolar epithelium into the junction of the respiratory bronchioles. This occasionally mimicked hyperplasia of bronchiolar epithelium (bronchiolization), which was a common finding in Study 1. Infusion of formalin into the lungs, followed by ligation of the proximal trachea, tends to smooth the fixed mucosa of the trachea. If cannibalization or autolysis precluded tying off the trachea, the mucosa was often thrown up into microscopic folds that tended to mimic micropapillomatosis (a proliferative change).

There was considerable reactive change in the lungs that contained carbon, characterized by granulomatous pneumonitis with giant cells and peribronchiolar fibrosis. The fibrosis was often profuse, and it occasionally contained

Table 9. Study 2: Incidence of Tracheal Lesions in Hamsters After Exposure to 1-Nitropyrene and Benzo[*a*]pyrene by Treatment Group

	None (Shelf Controls)	Benzo[*a*]-pyrene	Carbon Particles	1-Nitropyrene		Benzo[*a*]pyrene + Carbon Particles	1-Nitropyrene + Benzo[*a*]pyrene	
Experiment Parameters								
Dose	0	0.25 mg	2 mg	1 mg	2 mg	0.25 mg + 2 mg	1 mg + 0.25 mg	2 mg + 0.25 mg
Dosing schedule	0	1/wk	1/wk	1/wk	1/wk	1/wk	1/wk	1/wk
Group	1	2	3	4	5	6	7	8
No. of hamsters with tracheas examined	19	49	46	48	46	48	48	45
Nonneoplastic[a]								
Hyperplasia		14 (28.6)	2 (4.3)	6 (12.5)	10 (21.7)	23 (47.9)	21 (43.8)	29 (64.4)
Squamous metaplasia		1 (2.0)		1 (2.1)		5 (10.4)	10 (20.8)	13 (28.9)
Dysplasia		13 (26.5)	3 (6.5)		5 (10.9)	17 (35.4)	19 (39.6)	20 (44.4)
Micropapillomatosis		3 (6.1)	1 (2.2)		2 (4.3)	1 (2.1)	5 (10.4)	7 (15.6)
Neoplastic[a]								
Papilloma		1 (2.0)				5 (10.4)	4 (8.3)	8 (17.8)
Polyps		6 (12.2)[b]			1 (2.2)	5 (10.4)	8 (16.7)[b]	17 (37.8)
Carcinoma in situ				1 (2.1)				
Squamous cell carcinoma						1 (2.1)		
Anaplastic carcinoma							1 (2.1)	
Fibrosarcoma		1 (2.0)						2 (4.4)
Total animals with tracheal tumors	0 (0)	8 (16.3)	0 (0)	1 (2.1)	1 (2.2)	10 (20.8)	12 (25.0)	22 (48.9)

[a] Values indicate numbers of lesions. Numbers in parentheses are the percentages of hamsters with tracheas examined that had tumors.

[b] One animal had two morphologically distinct polyps.

metaplastic transformation to bone. The physical presence of large amounts of carbon pigment in the bronchial tree and alveoli obscured fine detail in the pulmonary tissue.

Adenomatosis and hyperplasia of alveolar epithelium frequently occurred in the same focus. The diagnostic discrimination was based on the predominance of one condition over the other. Hyperplasia was usually present at the periphery of an adenomatosis lesion.

The differentiation of malignant tumors from benign tumors and proliferative changes in the trachea was not difficult. Malignant tracheal tumors had submucosal infiltration, whereas benign tumors were almost invariably elevated well above the mucosal surface, with a variable ratio of epithelial to stromal elements. Proliferative nonneoplastic changes in the trachea were diffuse or, if local, were flat or plaque-like.

Differentiation between focal proliferative nonneoplastic lesions and benign tumors in the lungs was difficult. Benign lung tumors tended to be larger and to have more disorganized microarchitecture than was seen in adenomatoses or hyperplasias. The differentiation of malignant from benign alveolar/bronchiolar tumors was based on the presence of aggressive tissue at the margins of the tumor or a more disorganized microarchitecture, or both. Though the majority of the carcinomas were well differentiated, they were locally invasive and often quite large.

Study 1: Carcinogenicity of 1-Nitropyrene

The incidences of nonneoplastic and neoplastic lesions seen in the trachea and lungs of various experimental and control hamsters are shown in Tables 7, 8, and 11. Photomicrographs of representative nonneoplastic lesions are presented in Figures 5 through 19.

Nonneoplastic Findings. The shelf control hamsters were largely free of nonneoplastic lesions in either the trachea or the lung. The incidence of lesions of the trachea in group 2 (saline control) showed the same spectrum of lesions (with the noticeable absence of squamous metaplasia) as groups 3 through 8, but with lower incidence. Less bronchiolar hyperplasia was seen in the particle control animals (group 3) than was seen in groups 4 through 8. Group 8 (B*a*P, 2 mg 1/wk) had a higher incidence of squamous metaplasia than did any other group. A high incidence of adenomatosis occurred in hamsters after approximately one year of treatment, regardless of group.

Neoplastic Findings. The incidences of individual types of neoplasms in the lung or trachea were not markedly higher

Table 10. Study 2: Incidence of Lung Lesions in Hamsters After Exposure to 1-Nitropyrene and Benzo[*a*]pyrene by Treatment Group

	None (Shelf Controls)	Benzo[*a*]-pyrene	Carbon Particles	1-Nitropyrene		Benzo[*a*]pyrene + Carbon Particles	1-Nitropyrene + Benzo[*a*]pyrene	
Experiment Parameters								
Dose	0	0.25 mg	2 mg	1 mg	2 mg	0.25 mg + 2 mg	1 mg + 0.25 mg	2 mg + 0.25 mg
Dosing schedule	0	1/wk	1/wk	1/wk	1/wk	1/wk	1/wk	1/wk
Group	1	2	3	4	5	6	7	8
No. of hamsters with lung tissue examined	19	50	47	48	46	49	48	46
Nonneoplastic[a]								
Hyperplasia	1 (5.3)	10 (20.0)	3 (6.4)	4 (8.3)	8 (17.4)	17 (34.7)	14 (29.2)	14 (30.4)
Bronchiolar hyperplasia	6 (31.6)	41 (82.0)	35 (74.5)	39 (81.3)	41 (89.1)	44 (89.9)	40 (83.3)	46 (100)
Squamous metaplasia		2 (4.0)			1 (2.2)	3 (6.1)	6 (12.5)	5 (10.9)
Mucous metaplasia		7 (14.0)			1 (2.2)		2 (4.2)	2 (4.3)
Adenomatosis		12 (24.0)	17 (36.2)	14 (29.2)	30 (65.2)	29 (59.2)	26 (54.2)	32 (69.6)
Neoplastic[a]								
Adenomas		11 (22.0)		1 (2.1)	4 (8.7)	5 (10.2)	9 (18.8)[b]	12 (26.1)
Mastocytoma			1 (2.1)					
Papillary carcinoma								2 (4.3)
Squamous cell carcinoma		3 (6.0)			1 (2.2)	6 (12.2)	6 (12.5)	11 (23.9)
Alveolar/bronchiolar carcinoma		3 (6.0)				2 (4.1)	2 (4.2)	2 (4.3)
Anaplastic carcinoma							2 (4.2)	
Basal cell carcinoma							1 (2.1)	
Sarcoma					1 (2.2)			1 (2.2)
Total animals with lung tumors	0 (0)	16 (32.0)	1 (2.1)	1 (2.1)	6 (13.0)	11 (22.4)	12 (25.0)	24 (52.2)

[a] Values indicate numbers of lesions. Numbers in parentheses are the percentages of hamsters with lung tissue examined that had tumors.

[b] One animal had two morphologically distinct adenomas.

in the 1-NP-treated groups than in the appropriate control groups. A wide spectrum of tumor types, occurring from low to high incidence, was seen in both trachea and lung tissue in group 8 hamsters (B*a*P, 2 mg 1/wk). Incidences of microscopic findings, shown in Tables 7 and 8, are summarized as follows:

Group 1, Shelf Control. No tumors were seen in the shelf control hamsters.

Group 2, Saline Control. Two tumors, a tracheal papilloma and a lung mastocytoma, were diagnosed in different animals.

Group 3, Particle Control, 2/wk. A tracheal adenomatous polyp was seen in one animal, and another animal had squamous cell carcinoma of the lung.

Group 4, 1-Nitropyrene, 2 mg 1/wk. Only one tumor, a solid adenoma of the lung, was diagnosed in this group.

Group 5, 1-Nitropyrene, 1 mg 1/wk. One adenomatous polyp was noted in the trachea of one hamster. Two lung tumors, a squamous cell carcinoma and an alveolar/bronchiolar carcinoma, were detected in two other hamsters.

Group 6, 1-Nitropyrene, 2 mg 2/wk. Four animals were diagnosed with tumors in this group: one had a myxomatous polyp of the trachea, one had a tracheal papilloma, one had a lung acinar adenoma, and one had an alveolar/bronchiolar adenoma.

Group 7, 1-Nitropyrene, 1 mg 2/wk. Seven hamsters in this group bore tumors. Two animals were diagnosed with tracheal adenomatous polyps. Four other hamsters were noted with single tumors: one with a lung acinar adenoma, one with an alveolar/bronchiolar adenoma, one with an alveolar/bronchiolar carcinoma, and one with a lung mastocytoma. The seventh animal had three tumors—an adenomatous tracheal polyp, a cuboidal lung adenoma, and an alveolar/bronchiolar adenoma.

Group 8, Positive Control, Benzo[*a*]pyrene, 2 mg 1/wk. A wide variety of tumors were diagnosed in both the trachea and lung tissues of these animals.

A total of 42 hamsters were diagnosed with at least one neoplasm. Fourteen animals were found with only one tumor: 3 had tracheal polyps (one each of mixed, adenoma-

Table 11. Study 1: Number of Hamsters Exhibiting Single or Multiple Respiratory Tumors After Exposure to 1-Nitropyrene

Group	Treatment	No. of Animals with						Total No. of Tumor-Bearing Hamsters
		1 Tumor	2 Tumors	3 Tumors	4 Tumors	5 Tumors	6 Tumors	
1	None (shelf controls)	0	0	0	0	0	0	0
2	Saline, 0.2 mL 2/wk	2[a]	0	0	0	0	0	2
3	Carbon particles, 2 mg 2/wk	2[b]	0	0	0	0	0	2
4	1-Nitropyrene, 2 mg 1/wk	1[c]	0	0	0	0	0	1
5	1-Nitropyrene, 1 mg 1/wk	3[d]	0	0	0	0	0	3
6	1-Nitropyrene, 2 mg 2/wk	4[e]	0	0	0	0	0	4
7	1-Nitropyrene, 1 mg 2/wk	6[f]	0	1[g]	0	0	0	7
8	Benzo[*a*]pyrene, 2 mg 1/wk	14[h]	6[i]	10[j]	7[k]	4[l]	1[m]	42

[a] First animal diagnosed with tumor (lung mastocytoma) at 539 days of treatment.

[b] First animal diagnosed with tumor (tracheal adenomatous polyp) at 210 days of treatment.

[c] Animal diagnosed with tumor (lung adenoma) at 644 days of treatment.

[d] First animal diagnosed with tumor (tracheal adenomatous polyp) at 392 days of treatment.

[e] First animal diagnosed with tumor (tracheal mixed polyp) at 272 days of treatment.

[f] First animal diagnosed with tumor (tracheal adenomatous polyp) at 203 days of treatment.

[g] Animal diagnosed with three tumors (tracheal adenomatous polyp, lung adenoma, and alveolar/bronchiolar adenoma) at 644 days of treatment.

[h] First animal diagnosed with tumor (tracheal adenomatous polyp) at 161 days of treatment.

[i] First animal diagnosed with two tumors (lung squamous cell carcinoma and alveolar/bronchiolar carcinoma) at 218 days of treatment.

[j] First animal diagnosed with three tumors (tracheal adenomatous polyp, lung squamous cell carcinoma, and alveolar/bronchiolar carcinoma) at 298 days of treatment.

[k] First animal diagnosed with four tumors (tracheal papilloma, lung papillary adenoma, alveolar/bronchiolar adenoma, and alveolar/bronchiolar carcinoma) at 377 days of treatment.

[l] First animal diagnosed with five tumors (tracheal and lung anaplastic carcinomas, lung squamous cell carcinoma, alveolar/bronchiolar adenoma, and alveolar/bronchiolar carcinoma) at 370 days of treatment.

[m] One animal diagnosed with six tumors (tracheal papilloma, polyp, and basal cell carcinoma; lung squamous cell carcinoma, alveolar/bronchiolar adenoma, and alveolar/bronchiolar carcinoma) at 374 days of treatment.

tous, and myxomatous) and 11 had lung squamous cell carcinoma.

Six hamsters were noted with two morphologically distinct lesions. One animal was diagnosed with squamous cell carcinomas in the lung and trachea. Two hamsters had an adenoma in the lung (one papillary and one acinar) combined with lung squamous cell carcinoma. Another animal was found with an alveolar/bronchiolar adenoma and a squamous cell carcinoma of the lung. Two other hamsters had alveolar/bronchiolar carcinoma and lung squamous cell carcinoma.

Ten animals in this group bore three tumor types. One hamster was noted with a tracheal papilloma, an alveolar/bronchiolar adenoma, and a lung squamous cell carcinoma. Two animals had a tracheal papilloma combined with an alveolar/bronchiolar carcinoma and a lung squamous cell carcinoma. One hamster was diagnosed with a tracheal papilloma, alveolar/bronchiolar adenoma, and squamous cell carcinoma in the lung. Two other hamsters were noted to have an adenomatous tracheal polyp, an alveolar/bronchiolar carcinoma, and a lung squamous cell carcinoma. Another animal was found with three carcinomas: two in the trachea (unspecified and squamous cell carcinoma) and a lung squamous cell carcinoma. One other hamster had a carcinoma in situ in the trachea, a bronchial carcinoma, and a squamous cell carcinoma in the lung. Two animals were found with three lung lesions: one with an alveolar/bronchiolar adenoma, an alveolar/bronchiolar carcinoma, and a squamous cell carcinoma; the other with an alveolar/bronchiolar adenoma, an alveolar/bronchiolar carcinoma, and an anaplastic carcinoma.

Seven hamsters were found with four distinct tumor types. One animal had a tracheal papilloma combined with an alveolar/bronchiolar adenoma, an alveolar/bronchiolar carcinoma, and a lung squamous cell carcinoma. Another two hamsters were noted with a tracheal papilloma, an alveolar/bronchiolar adenoma, a lung squamous cell carcinoma, and a lung adenoma (one solid and one papillary). One animal was found with a tracheal papilloma and a squamous cell carcinoma in the trachea, and an adenocar-

Table 12. Study 2: Number of Hamsters Exhibiting Single or Multiple Respiratory Tumors After Exposure to 1-Nitropyrene and Benzo[*a*]pyrene

Group	Treatment	No. of Animals with						Total No. of Tumor-Bearing Hamsters
		1 Tumor	2 Tumors	3 Tumors	4 Tumors	5 Tumors	6 Tumors	
1	None (shelf controls)	0	0	0	0	0	0	0
2	Benzo[*a*]pyrene, 0.25 mg 1/wk	12[a]	7[b]	0	0	0	0	19
3	Carbon particles, 2 mg 1/wk	1[c]	0	0	0	0	0	1
4	1-Nitropyrene, 1 mg 1/wk	0	1[d]	0	0	0	0	1
5	1-Nitropyrene, 2 mg 1/wk	7[e]	0	0	0	0	0	7
6	Benzo[*a*]pyrene, 0.25 mg 1/wk + particles, 2 mg 1/wk	13[f]	4[g]	1[h]	0	0	0	18
7	Benzo[*a*]pyrene, 0.25 mg 1/wk + 1-nitropyrene, 1 mg 1/wk	12[i]	2[j]	3[k]	1[l]	0	1[m]	19
8	Benzo[*a*]pyrene, 0.25 mg 1/wk + 1-nitropyrene, 2 mg 1/wk	10[n]	13[o]	5[p]	1[q]	0	0	29

[a] First animal diagnosed with tumor (tracheal fibrosarcoma) at 360 days of treatment.

[b] First animal diagnosed with two tumors (tracheal papilloma and alveolar/bronchiolar adenoma) at 570 days of treatment.

[c] Animal diagnosed with tumor (lung mastocytoma) at 643 days of treatment.

[d] Animal diagnosed with two tumors (tracheal carcinoma in situ and alveolar/bronchiolar adenoma) at 643 days of treatment.

[e] First animal diagnosed with tumor (lung sarcoma) at 422 days of treatment.

[f] First animal diagnosed with tumor (lung squamous cell carcinoma) at 367 days of treatment.

[g] First animal diagnosed with two tumors (tracheal papilloma and lung squamous cell carcinoma) at 489 days of treatment.

[h] Animal diagnosed with three tumors (alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, and lung squamous cell carcinoma) at 646 days of treatment.

[i] First animal diagnosed with tumor (lung squamous cell carcinoma) at 245 days of treatment.

[j] First animal diagnosed with two tumors (anaplastic carcinoma in trachea and lung) at 623 days of treatment.

[k] First animal diagnosed with three tumors (lung adenoma, alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma) at 630 days of treatment.

[l] Animal diagnosed with four tumors (tracheal papilloma and polyp, alveolar/bronchiolar adenoma, and lung squamous cell carcinoma) at 646 days of treatment.

[m] Animal diagnosed with six tumors (tracheal papilloma, alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, lung squamous cell carcinoma, lung anaplastic carcinoma, and lung basal cell carcinoma) at 583 days of treatment.

[n] First animal diagnosed with tumor (tracheal fibrosarcoma) at 388 days of treatment.

[o] First animal diagnosed with two tumors (tracheal papilloma, and lung squamous cell carcinoma) at 422 days of treatment.

[p] First animal diagnosed with three tumors (tracheal polyp, tracheal fibrosarcoma, and alveolar/bronchiolar adenoma) at 492 days of treatment.

[q] One animal diagnosed with four tumors (tracheal papilloma, tracheal polyp, alveolar/bronchiolar adenoma, and lung squamous cell carcinoma) at 597 days of treatment.

cinoma and squamous cell carcinoma in the lung. A single hamster was diagnosed with an adenomatous tracheal polyp, an alveolar/bronchiolar adenoma, an alveolar/bronchiolar carcinoma, and a lung squamous cell carcinoma. One other hamster was noted with anaplastic carcinoma in the trachea as well as three lung tumors: an alveolar/bronchiolar adenoma, a mucinous adenoma, and a squamous cell carcinoma. Another animal was found with a tracheal adenomatous polyp, a papillary lung adenoma, an alveolar/bronchiolar adenoma, and a lung squamous cell carcinoma.

Four animals were diagnosed with five neoplasms. One hamster was found with two tracheal polyps (adenomatous and mixed), a tracheal and lung squamous cell carcinoma, and an alveolar/bronchiolar adenoma. Another animal had an adenomatous tracheal polyp, a papillary lung adenoma, and papillary, mixed cell, and squamous cell carcinomas in the lung. One hamster was found with a squamous cell carcinoma in both the trachea and lung, a tracheal papilloma, an alveolar/bronchiolar adenoma, and an alveolar/bronchiolar carcinoma. Another hamster was noted with tracheal and lung squamous cell carcinomas, an alveolar/bronchiolar adenoma, an alveolar/bronchiolar carcinoma, and an anaplastic lung carcinoma.

A single hamster was diagnosed with the following six

Figure 5. Normal tracheal tissue from hamster in shelf control group at 644 days. Magnification is × 100.

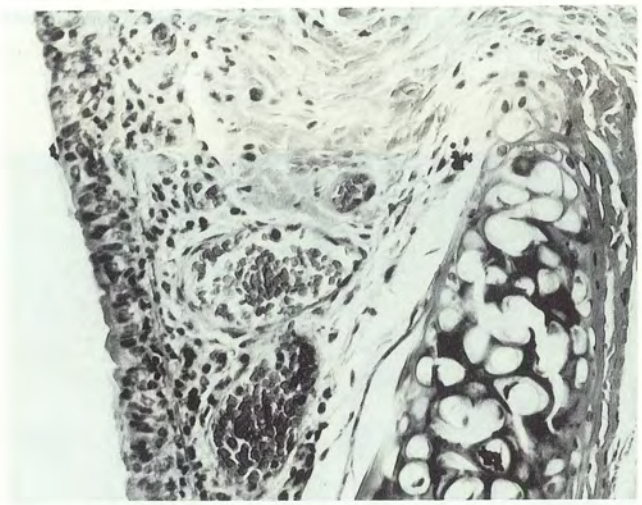

Figure 6. Tracheal tissue from hamster exposed to 1-NP, 2 mg 2/wk (at 246 days) showing hyperplasia. Thickening of the tracheal epithelium with non-squamous epithelial cells is apparent. Magnification is × 200.

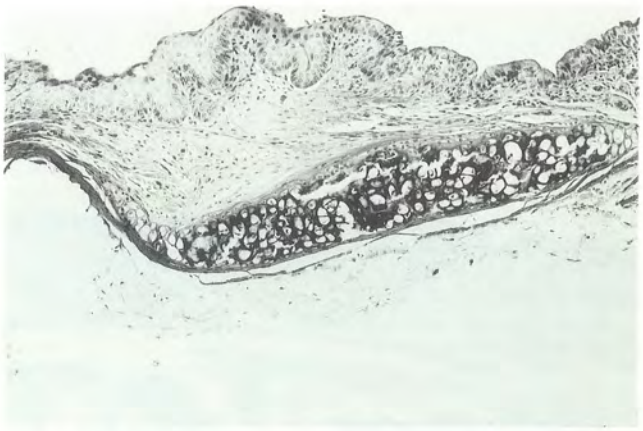

Figure 7. Tracheal tissue from hamster exposed to 1-NP, 2 mg 2/wk (at 258 days) showing squamous metaplasia. Change of focal areas of the tracheal mucosa into stratified squamous epithelium can be seen. Magnification is × 100.

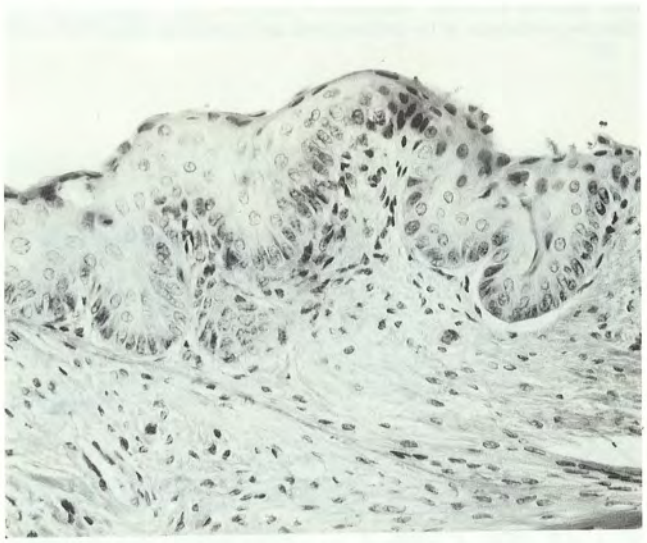

Figure 8. Same as Figure 7 at higher magnification (× 250).

tumor types: tracheal adenomatous polyp, papilloma and basal cell carcinoma, alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, and lung squamous cell carcinoma.

Study 2: Cocarcinogenicity of 1-Nitropyrene Administered with Benzo[a]pyrene

The incidences of nonneoplastic and neoplastic lesions observed in the study are presented in Tables 9, 10, and 12.

Nonneoplastic Findings. The incidence of nonneoplastic lesions in the trachea was higher in hamsters exposed to B*a*P than in those not exposed to B*a*P. The incidence of non-neoplastic lesions in the lung tended to be uniform in all groups that received carbon particles alone or in concert with B*a*P, either with or without 1-NP exposure. A high incidence of adenomatosis was seen in hamsters after approximately one year in the study, a finding similar to that in Study 1.

Neoplastic Findings. The incidence of tumors in the trachea and lungs of hamsters in groups given B*a*P was higher than for those not receiving B*a*P. The incidence of tracheal and lung tumors in hamsters in group 5 (1-NP, 2 mg 1/wk) was higher than that seen for group 4 (1-NP, 1 mg 1/wk). Correspondingly, the tumor incidence of group 8 (B*a*P,

Figure 9. Tracheal tissue from hamster exposed to 1-NP, 2 mg 2/wk (at 246 days) showing dysplasia. Alteration of normal ciliated pseudostratified columnar epithelium of the tracheal mucosa is apparent. Magnification is × 200.

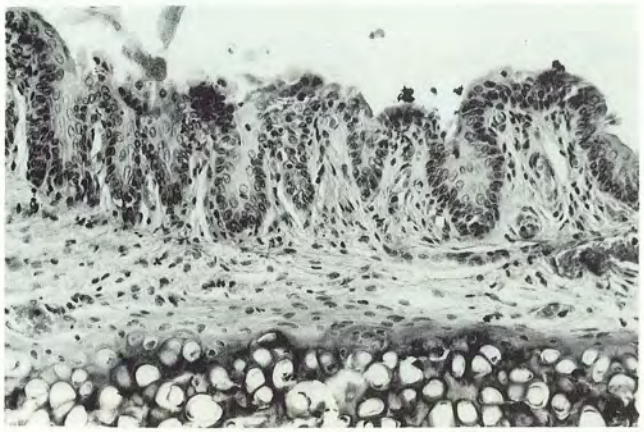

Figure 10. Tracheal tissue from hamster exposed to 1-NP, 2 mg 2/wk (at 260 days) showing micropapillomatosis. Proliferation of tracheal mucosa into microscopic serpentine folds can be seen. Magnification is × 200.

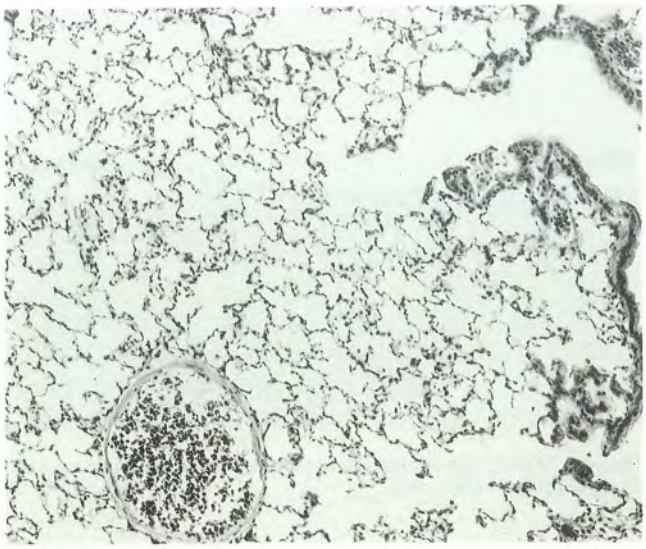

Figure 11. Normal lung tissue from hamster in shelf control group (at 644 days). Magnification is × 40.

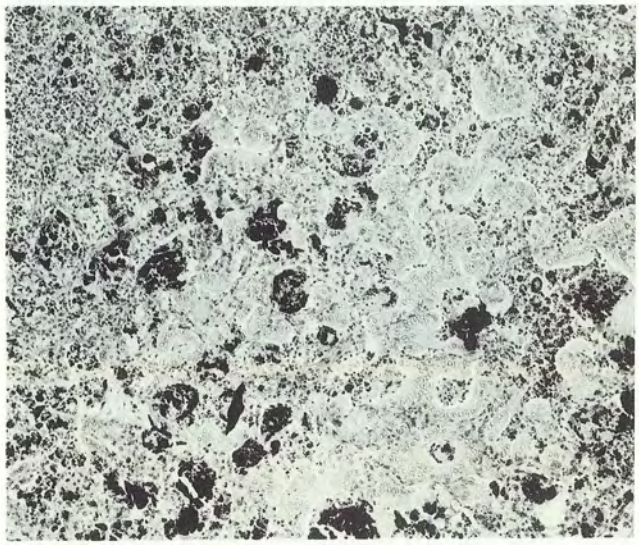

Figure 12. Lung tissue from hamster exposed to 1-NP, 2 mg 2/wk (at 241 days) showing hyperplasia. Proliferating alveolar epithelium, several layers thick, can be seen. Magnification is × 100.

1/week, and 1-NP, 2 mg 1/wk) is higher than that of group 7 (B*a*P, 1/week, and 1-NP, 1 mg 1/week). The incidence of histopathologic findings is shown in Tables 9 and 10. Histopathology findings by group are as follows:

Group 1, Shelf Control. All animals were free of neoplastic lesions.

Group 2, Benzo[*a*]pyrene, 1/wk. Nineteen hamsters were diagnosed with tumors in this group. Twelve had only one tumor type: one had a tracheal adenomatous polyp, one had a tracheal fibrosarcoma, one had a papillary lung adenoma, six had alveolar/bronchiolar adenomas, one had an alveolar/bronchiolar carcinoma, and two had lung squamous cell carcinomas.

Seven animals were diagnosed with morphologically distinct tumors. One hamster had two tracheal polyps (adenomatous and myxomatous). Two animals had an adenomatous tracheal polyp combined with a lung ade-

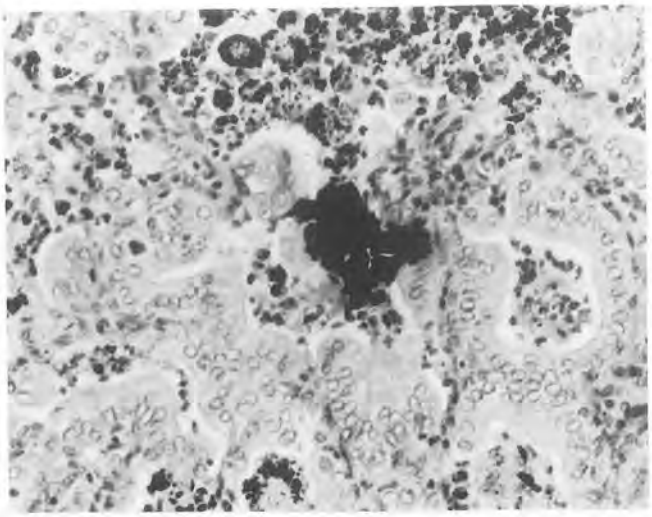

Figure 13. Same as Figure 12 at higher magnification (× 200).

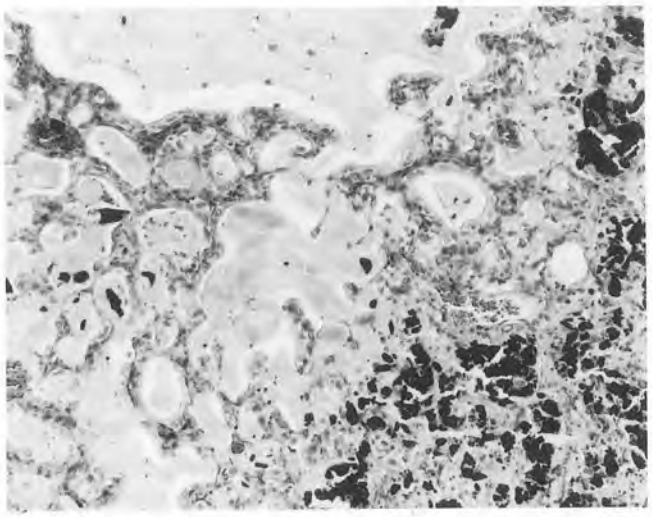

Figure 14. Lung tissue from hamster in particle control group (at 529 days) showing adenomatosis. Collections of pink-staining colloid material in the lumina of alveoli are apparent. The alveolar epithelium is thickened and irregular and the alveoli are distended. Magnification is × 80.

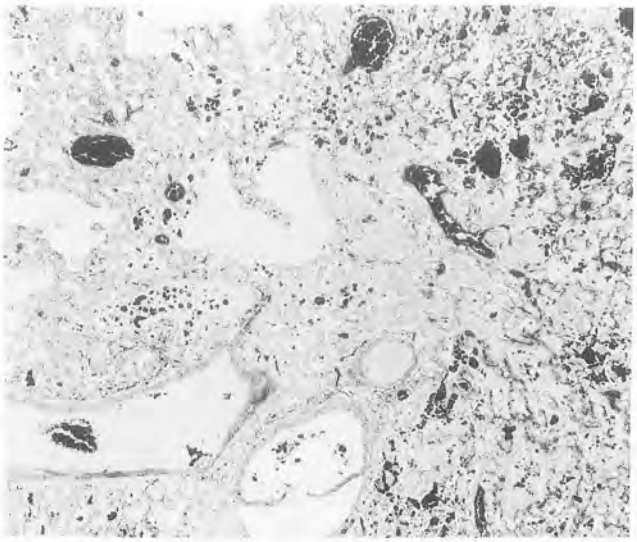

Figure 15. Lung tissue from hamster in particle control group (at 529 days) showing adenomatosis (center) and hyperplasia of the bronchial epithelium (right bottom). Magnification is × 40.

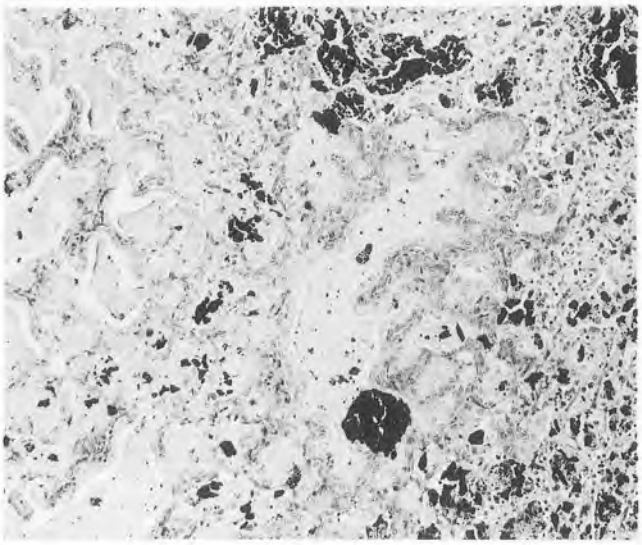

Figure 16. Lung tissue from hamster exposed to 1-NP, 2 mg 2/wk (at 242 days) showing squamous metaplasia. Magnification is × 120.

noma (one acinar and one papillary). Another hamster with a tracheal adenomatous polyp also had a lung squamous cell carcinoma. One other hamster was noted with a mixed-cell polyp of the trachea and an alveolar/bronchiolar carcinoma. One animal was diagnosed with a tracheal papilloma and an alveolar/bronchiolar adenoma, and another animal with an alveolar/bronchiolar adenoma and an alveolar/bronchiolar carcinoma.

Group 3, Particle Control, 1/wk. One mastocytoma of the lung was observed in this group.

Group 4, 1-Nitropyrene, 1 mg 1/wk. One hamster was diagnosed with two lesions, a tracheal carcinoma in situ and an alveolar/bronchiolar adenoma.

Group 5, 1-Nitropyrene, 2 mg 1/wk. Seven animals in this group had one tumor each. The tumor types were an adenomatous polyp in the trachea, three alveolar/bronchiolar adenomas, a papillary lung adenoma, a lung squamous cell carcinoma, and a lung sarcoma.

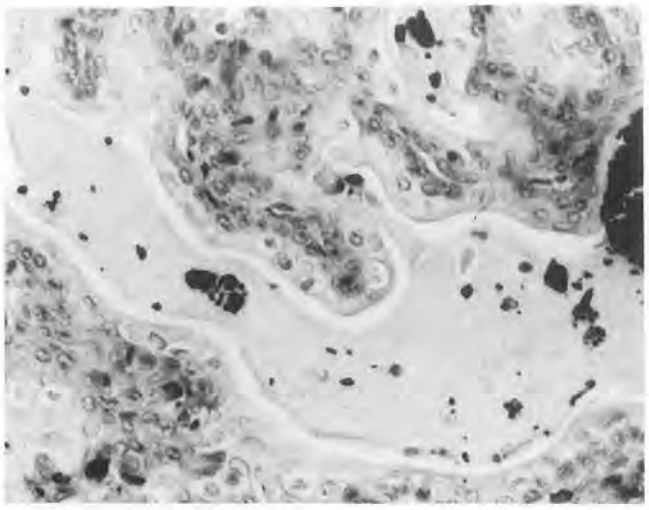

Figure 17. Same as Figure 16 at higher magnification (× 240).

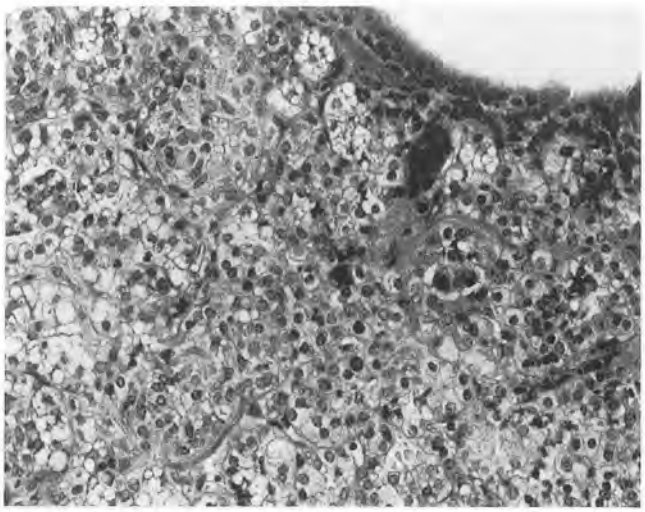

Figure 19. Same as Figure 18 at higher magnification (× 160).

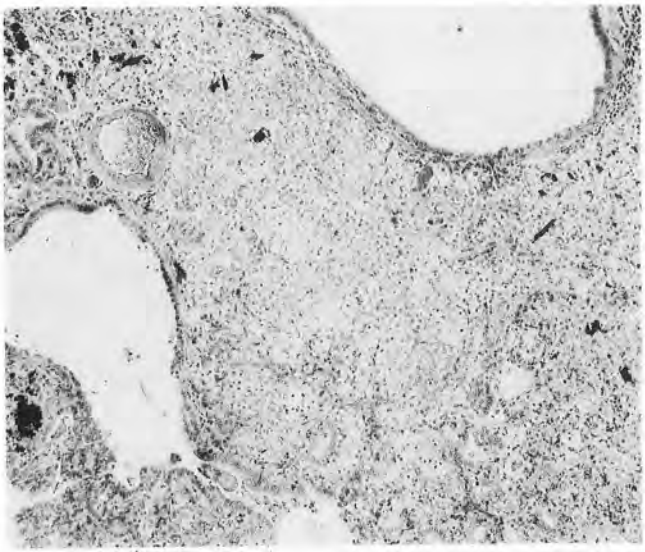

Figure 18. Lung tissue from hamster exposed to 1-NP, 1 mg 1/wk (at 299 days) showing mucous metaplasia. Magnification is × 80.

Group 6, Benzo[*a*]pyrene, 1/wk, and Particles, 1/wk. Eighteen animals were diagnosed with neoplastic lesions. The following were noted to have only one lesion: two hamsters with tracheal papillomas, four with tracheal polyps (two myxomatous, one adenomatous, and one mixed), three with lung adenomas (two alveolar/bronchiolar and one solid), one with an alveolar/bronchiolar carcinoma, and three with lung squamous cell carcinomas. One hamster had two tracheal tumors, a papilloma and a squamous cell carcinoma. Another animal was diagnosed with an adenomatous polyp in the trachea and a papillary adenoma in the lung. Two hamsters had both a tracheal papilloma and a lung squamous cell carcinoma. The tissues from a single animal in this group were found to contain three neoplasms: an alveolar/bronchiolar adenoma, an alveolar/bronchiolar carcinoma, and a lung squamous cell carcinoma.

Group 7, Benzo[*a*]pyrene, 1/wk, and 1-Nitropyrene, 1 mg 1/wk. A total of 19 hamsters were diagnosed with at least one neoplastic lesion. Of the animals with only one tumor, the following were found: one with a tracheal papilloma, six with tracheal polyps (three adenomatous, two chondromatous, and one mixed), three with alveolar/bronchiolar adenomas, and two with lung squamous cell carcinomas.

One hamster was noted to have an anaplastic carcinoma in both the trachea and deep lung, while another had an alveolar/bronchiolar adenoma and a squamous cell carcinoma in the lung.

Three hamsters were found to have three neoplasms each: one had a tracheal papilloma, a papillary adenoma in the lung, and a lung squamous cell carcinoma; one had two tracheal polyps (adenomatous and myxomatous) and an alveolar/bronchiolar adenoma; and one had two lung adenomas (solid and alveolar/bronchiolar) plus an alveolar/bronchiolar carcinoma.

One hamster had four distinct lesions: a papilloma and a chondromatous polyp in the trachea, an alveolar/bronchiolar adenoma, and a squamous cell carcinoma in the lung.

A single animal in this group was noted to have six distinct neoplasms: a tracheal papilloma and five lung lesions (alveolar/bronchiolar adenoma, alveolar/bronchiolar carci-

noma, squamous cell carcinoma, basal cell carcinoma, and anaplastic carcinoma).

Group 8, Benzo[*a*]pyrene, 1/wk, and 1-Nitropyrene, 2 mg 1/wk. In this group, 29 animals were diagnosed with neoplasms. Ten animals had only one lesion: three had tracheal polyps (one mixed, one chondromatous, and one adenomatous), one had a tracheal fibrosarcoma, two had lung adenomas (alveolar/bronchiolar and acinar), three had lung squamous cell carcinomas, and one had a lung sarcoma.

Thirteen animals had two distinct lesions. One hamster had a papilloma and an adenomatous polyp in the trachea. Two animals were noted with the combination of a tracheal papilloma and a lung adenoma (one alveolar/bronchiolar, one acinar). Two hamsters had tracheal chondromatous polyps and alveolar/bronchiolar adenomas. One animal was found with a mixed-cell polyp in the trachea and a papillary adenoma in the lung. Another two animals had a tracheal polyp (one mixed, one adenomatous) combined with a lung squamous cell carcinoma. Two hamsters were diagnosed with a tracheal polyp (one mixed, one adenomatous) and an alveolar/bronchiolar carcinoma. A single animal was noted with a tracheal papilloma and a papillary carcinoma of the lung. Tissues from another hamster contained a tracheal papilloma and a lung squamous cell carcinoma, and one hamster was diagnosed with two lung lesions—a solid adenoma and a squamous cell carcinoma.

Five animals were found with three distinct tumor types. One hamster was noted with a papilloma and a mixed-cell polyp in the trachea, combined with an alveolar/bronchiolar adenoma. Another animal had a papilloma and a mixed-cell polyp in the trachea, plus a lung squamous cell carcinoma. Another hamster was diagnosed with an adenomatous tracheal polyp and two lung lesions (squamous cell carcinoma and papillary carcinoma). One other animal was found with a mixed-cell tracheal polyp, a papillary lung adenoma, and a lung squamous cell carcinoma. The fifth hamster had two tracheal lesions (adenomatous polyp and fibrosarcoma) and an alveolar/bronchiolar adenoma.

Only one hamster in this group was diagnosed with four different neoplasms, two tracheal (papilloma and myxomatous polyp) and two lung (alveolar/bronchiolar adenoma and squamous cell carcinoma).

A number of hamsters in both Study 1 and 2 had lung parenchyma containing both acute and chronic inflammatory cells. The repeated deposition of carbon particles in the alveolar sacs acted to inhibit the resolution of the inflammatory response. At the end of the 92-week experimental period, blood samples were collected randomly for serum antiviral antibody determinations (Microbiological Associates, Bethesda, MD). Positive titers for mouse pneumonia virus were obtained from all 30 serum samples from Study 1, and from only 1 (from the particle control group) of the 20 serum samples collected from Study 2. None of the sera yielded positive titers for Sendai virus.

Some of the hamsters that died late in the study showed gross lesions consistent with amyloidosis, a common finding in aging Syrian golden hamsters (Renshaw et al. 1975). These lesions included subcutaneous edema, ascites and pneumothorax, a pitted renal cortical surface, and slightly enlarged lymph nodes and spleen. The majority of animals displayed more than one of these changes.

STATISTICAL ANALYSIS

The results of the statistical analysis of the main effects and interactions are described in the Statistical Report (Appendix A). They are summarized in the following paragraphs.

Study 1

Increased exposure frequency to 1-NP led to significantly decreased tumor latency. The absence of a significant interaction between exposure frequency and 1-NP dose level indicates that the effect of exposure frequency was consistent for both low and high doses of 1-NP. In terms of simple tumor incidence, the main effect of exposure frequency approached statistical significance ($p = 0.07$).

Study 2

Exposure to both B*a*P and 1-NP significantly decreased tumor latency and significantly increased tumor incidence. When B*a*P and 1-NP were given in combination, no interaction was observed; their joint effect did not exceed what would be expected by the addition of their individual effects.

The between-study comparison of the 1-NP low-dose and high-dose groups revealed a significant difference between the two studies for tumor incidence, but not for tumor latency. In the first study, 1-NP low-dose animals exhibited an increased tumor incidence compared with high-dose animals, but in the second study, low-dose animals had decreased tumor incidence compared with high-dose animals.

DISCUSSION AND CONCLUSIONS

The overall data from the two studies demonstrated that prolonged exposure to carbon particulate preparations containing 2 mg of 1-NP had a weak carcinogenic effect on the respiratory tissues of the hamster. In contrast, the high

potency of B*a*P was readily revealed in this model by the development of a variety of benign and malignant tumors of the respiratory tract in virtually all hamsters that survived more than 50 weeks of treatment.

The results seem to parallel closely other reports of weak, or no, genotoxic activity of 1-NP in mammalian cell systems (Nakayasu et al. 1982; Edwards et al. 1986; Haugen et al. 1986; Heflich et al. 1986), and of lack of significant respiratory carcinogenic effects in other rodent models (Maeda et al. 1986; Busby et al. 1989). Furthermore, they are consistent with the predicted low carcinogenicity of 1-NP by the carcinogenicity prediction and battery selection method (Rosenkranz and Howard 1986). Although Study 1 failed to show a significant carcinogenic effect of 1-NP, a weak carcinogenic effect was apparent from the increased incidence of tumors and the dose-response trend observed in Study 2. The absence of a dose-response relation in Study 1 could be due to the low tumor incidence in all 1-NP treatment groups, possibly from high mortality during the early phase of the study. The highest dose regimen used in the study (1-NP, 2 mg 2/wk) was toxic, as demonstrated by consistently low mean body weights and poor survival rate. The toxic effects could not be attributed exclusively to 1-NP exposure, because they were also seen in particle control animals, albeit to a lesser degree. Previous studies at IITRI, reported by Henry and associates (Henry and Port 1973; Henry et al. 1974), showed body-weight loss in hamsters exposed once a week for 30 weeks by intratracheal instillation to carbon particles (2 mg) of the same size (2 to 5 μm). Carbon particles alone had no tumorigenic effect on the respiratory organs, nor did they enhance the respiratory carcinogenesis by diethylnitrosamine (Henry and Port 1973; Stenback et al. 1973). In the present Study 1, two hamsters in the particle control group had tumors, but their relation to treatment with carbon particles alone is doubtful, because a similar tumor incidence was seen in the saline control group. However, the influence of loading the lungs with particulate matter on histologic changes and tumor development in the respiratory tract needs to be investigated further (Morrow 1988; Burger et al. 1989).

A variety of nonneoplastic lesions were seen in the lungs and tracheas of hamsters treated with the various test preparations (Figures 5 through 19). Some of them possibly were preneoplastic, and others might have played a role in neoplastic development and ultimate tumor response.

Tracheal hyperplasia was noted in nearly all groups, with the highest incidences (70.8 percent) in the particle control group and the B*a*P group (Study 1, groups 3 and 8). From a comparison of the particle control groups of Study 1 (group 3, 2/wk) and Study 2 (group 3, 1/wk), it appears that hamsters treated twice per week had a much higher incidence of this nonneoplastic proliferation. A similar pattern is seen with 1-NP at either concentration. Tracheal squamous metaplasia was associated primarily with exposure to B*a*P, but when animals were treated with B*a*P plus 1-NP (1 mg or 2 mg), the incidence of metaplasia was increased. This suggests that 1-NP may enhance the squamous metaplastic response to B*a*P.

The incidence of adenomatosis of the lung was highest in animals treated with 1-NP. Although this alteration was also seen in B*a*P-treated hamsters (Study 1, group 8), there was no significant change in incidence when hamsters were exposed to both B*a*P and 1-NP. Although adenomatosis has not been considered a proliferation that is definitely preneoplastic, this possibility could explain some of the data obtained in these studies. In Study 1, we observed a much higher incidence of adenomatosis in animals treated with 1-NP (groups 4 through 7, 57 to 78 percent) than in animals treated with B*a*P (group 8, 39 percent).

Concurrently, there were many more hamsters that were diagnosed with lung tumors in the B*a*P-treated group (group 8, 80 percent) than in the 1-NP-treated groups (groups 4 through 7, 2 to 7 percent). If adenomatosis is a preneoplastic change, then its increased incidence caused by 1-NP reflects an increase in lesions that have not yet progressed to tumors; the decreased incidence of adenomatosis in the B*a*P-treated hamsters would indicate that the lesions already had progressed to tumors. If this were the case, the incidence of adenomatosis in the B*a*P-treated animals would be lower in later deaths. After 52 weeks, all hamsters in group 8 had tumors, but only 52 percent (14 of 27) were diagnosed with adenomatosis. Those hamsters treated with 1-NP had a much higher incidence of adenomatosis in deaths after 52 weeks (group 4, 95 percent [36 of 38]; group 5, 88 percent [38 of 43]; group 6, 84 percent [32 of 38]; group 7, 91 percent [29 of 32]). This suggests that adenomatosis in the present context may be a premalignant alteration that is elevated in 1-NP-exposed hamsters.

The inflammatory reaction, cell damage, and cell proliferation observed in the present studies could have modulated the tumor development by different mechanisms. Upon exposure of animals to 1-NP-coated coal fly ash, Mumford and coworkers (1986) reported a direct relationship between an increased number of alveolar macrophages in cell culture and a decreased mutagenic activity and 1-NP recovery. It is possible that the macrophage infiltrates seen in the lungs of 1-NP-treated animals reduced the bioavailability of 1-NP at the target tissues and contributed to the low incidence of tumors, especially in Study 1. Also, the influence of other factors, such as the presence of viral infections, is not known. Serum samples collected from all Study 1 hamsters killed at termination of the experiment showed signifi-

cant antiviral antibody titers for mouse pneumonia virus. In the past, few investigators addressed the question of the effect of respiratory infections on lung carcinogenesis in laboratory animals (Nettesheim et al. 1974). Studies in Strain A mice enzootically infected with Sendai virus show both enhancement and suppression of chemically induced pulmonary adenomas, depending on the carcinogen employed (Peck et al. 1983). A recent analysis by Rao and associates (1989) using a large data base from several carcinogenesis bioassays in mice revealed no significant effect of virus infections on tumor prevalence. A modulating role of the virus infection in the development of tumors in Study 1, however, cannot be excluded.

The uptake, bioavailability, and lung retention of 1-NP associated with diesel particles and coal fly ash were reported by others (Bond et al. 1986; Mumford et al. 1986; Wolff et al. 1989), but no such data are available for the 1-NP preparation used in our studies. However, it is reasonable to expect that 1-NP adsorbed onto the carbon particles would behave similarly. At the high concentrations (50 percent) used, a very efficient release can be expected. This is supported by the high tumor response observed in B*a*P-treated animals. However, studies with pure 1-NP suspensions are needed to provide base-line information regarding the carcinogenicity of 1-NP without the complicating effects of carrier particles. Takayama and associates (1985) have demonstrated the respiratory carcinogenicity of 1,6-dinitropyrene in hamsters by intratracheal administration of suspensions of the pure compound. Use of pure compound may also be advantageous for studying cocarcinogenic and tumor initiation and promotion effects.

An interesting finding in this study is a possible cocarcinogenic effect from combined exposure to 1-NP and B*a*P (Study 2). Although the tumor incidence from the combined treatment did not exceed what would be expected by the addition of individual effects, a substantial increase in the number of animals bearing multiple tumors was observed in the high-dose combination group (Table 12). A cocarcinogenic effect is consistent with the findings of Mitchell (1985) and of Howard and coworkers (1986) that pretreatment with B*a*P increases the binding of 1-NP metabolites to the DNA of lung tissues in mice. Because a variety of environmental chemicals are strong microsomal enzyme inducers, enhanced carcinogenic effects may result from the combined exposure to such inducers and 1-NP. This aspect, as well as the potential tumor-promoting activity of 1-NP, must be further evaluated to assess the carcinogenic risk of 1-NP present in environmental pollutant mixtures.

Recent epidemiologic (Garshick et al. 1987) and experimental (Brightwell et al. 1986; Heinrich et al. 1986; Mauderly et al. 1987) investigations showed increased carcinogenic risk from prolonged exposures to relatively high concentrations of diesel-exhaust particles. Among the laboratory animals investigated, a consistent positive response was observed only in rats. Studies of mice and hamsters either gave conflicting results or did not show a significant positive response. It is not known whether or not a species-specific effect is involved. For example, the pattern of 1-NP metabolism varied among different species (Rosenkranz and Howard 1986). Furthermore, inhalation of large amounts of a variety of apparently nongenotoxic particulate matter, such as quartz, induced lung tumors in rats (Vostal 1986). The reason for the absence of a carcinogenic, or a tumor-promoting, effect of diesel exhaust in hamsters (Shefner et al. 1985; Brightwell et al. 1986; Heinrich et al. 1986) is not known. However, several factors might have played a role in this respect. A majority of the tumors in rats developed after a long latent period (more than 130 weeks). The relatively low longevity of hamsters (about 100 weeks) might not have permitted the development of some of the proliferative lesions into benign and malignant tumors (Pepelko 1984). Large group sizes (200 animals), such as those employed in studies with rats, might be necessary for demonstrating a significant increase in tumor incidence. This is a general problem with weak carcinogens, and their evaluation is complicated further by the confounding effects when they are present in mixtures. On the other hand, the present studies with B*a*P, and those of Takayama and colleagues (1985) with 1,6-dinitropyrene, demonstrate that the hamster does respond to potent carcinogens. In conclusion, the present studies suggest that the carcinogenic risk from the low levels of 1-NP present in diesel-exhaust particles and environmental pollutants is very small, and other potent carcinogens, such as B*a*P and dinitropyrenes, may be contributing to the observed carcinogenic effects of diesel-exhaust particles.

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APPENDIX A. Statistical Report[4]

INTRODUCTION

The purpose of these analyses was to test for main effects and interactions in the 2-by-2 and 3-by-2 designs shown in Tables A.1 through A.3. In the tables in this Appendix, "0" and "1" are used to denote treatment without or with the specific agent; "+" and "–" are used to denote animals with or without tumors. The raw data on tumor latency distribution and survival distribution are available on request from Health Effects Institute.

STATISTICAL METHODS

These subdesigns were evaluated for effects on time-to-tumor data using a Cox (1972) regression model, and in terms of simple tumor incidence using a loglinear model (Bishop et al. 1975). In the presence of a significant main effect or interaction, pairwise comparisons were performed using the Mantel-Cox statistic (Mantel 1966) for time-to-tumor data and a chi-squared statistic for tumor-incidence data.

RESULTS

Analysis of the Study 1 subdesign revealed a significant main effect of exposure frequency ($p < 0.02$) on the time to tumor (that is, once per week versus twice per week), but no significant main effect of dose or exposure by dosage interaction (see Table A.4). In terms of tumor incidence, the log-linear model revealed no significant main effects or interaction (see Table A.5); however, the exposure main effect did approach significance ($p < 0.07$), which is consistent with the time-to-tumor result at $p < 0.02$.

In terms of the Study 2 data (animals not exposed to 1-NP), the Cox regression model revealed that the B*a*P-by-carbon interaction was not significant; however, both main effects of B*a*P and carbon were significant at the $p < 0.01$ level (see Table A.6).

[4] This report was prepared by Dr. Robert D. Gibbons, Associate Professor of Biostatistics, University of Illinois at Chicago.

Table A.1. Study 1: Animals Exposed to 1-Nitropyrene

Exposure Frequency	1-Nitropyrene	
	High Dose	Low Dose
1/wk	Group 4	Group 5
2/wk	Group 6	Group 7

Table A.2. Study 2: Animals Not Exposed to 1-Nitropyrene

Carbon Particles	Benzo[*a*]pyrene	
	0	1
0	Group 1	Group 2
1	Group 3	Group 6

Table A.3. Study 2: Animals Not Exposed to Carbon Particles

1-Nitropyrene	Benzo[*a*]pyrene	
	0	1
0	Group 1	Group 2
Low	Group 4	Group 7
High	Group 5	Group 8

Table A.4. Study 1 Data: Estimated Mean Tumor Latency[a]

Exposure Frequency	1-Nitropyrene	
	High Dose	Low Dose
1/wk	644.0	629.7 ± 9.7
2/wk	591.7[b] ± 25.8	613.4 ± 15.0

[a] Values are means ± SE given in days. Where no SE is given, all animals were killed on the day indicated. The Kaplan-Meier estimate was used.
[b] Significantly different from high dose, 1/wk ($p < 0.01$).

Table A.5. Study 1 Data: Tumor Incidence

Exposure Frequency	1-Nitropyrene: High Dose −	High Dose +	Low Dose −	Low Dose +
1/wk	54	1	51	3
2/wk	52	4	51	7

Table A.6. Study 2 Data for Animals Not Exposed to 1-Nitropyrene: Estimated Mean Tumor Latency[a]

Carbon Particles	Benzo[*a*]pyrene 0	Benzo[*a*]pyrene 1
0	647.0	622.6[b] ± 10.2
1	643.0	595.3[b,c] ± 14.4

[a] Values are means ± SE given in days. Where no SE is given, all animals were killed on the day indicated. The Kaplan-Meier estimate was used.

[b] Significantly different from no B*a*P ($p < 0.01$).

[c] Significantly different from no carbon ($p < 0.01$).

In terms of simple tumor incidence (see Table A.7), the carbon-by-B*a*P interaction was not significant; however, the main effect of B*a*P was highly significant ($p < 0.0001$). In contrast to the results for time to tumor, the main effect of carbon was not significant for simple tumor incidence.

In terms of the Study 2 data for animals not exposed to carbon particles, the Cox regression analysis revealed a nonsignificant 1-NP-by-B*a*P interaction. In contrast, the main effects of B*a*P and of 1-NP were both highly significant ($p < 0.0001$ for both; see Table A.8).

With respect to simple tumor incidence, the results were identical. The 1-NP-by-B*a*P interaction was not significant, but both main effects were highly significant (for 1-NP, $p < 0.0004$, and for B*a*P, $p < 0.0001$; see Table A.9).

Finally, in terms of between-study comparisons—that is, the consistency of the difference between 1-NP (low) and 1-NP (high) across Study 1 and Study 2—we found nonsignificant interactions for time-to-tumor data (see Table A.10), but a significant interaction ($p < 0.02$) for simple tumor incidence (see Table A.11). The interaction for tumor incidence was due to increased incidence in the low-dose 1-NP group relative to the high-dose 1-NP group in Study 1, and the reverse in Study 2.

Comparing these two studies in terms of actual survival, a significant interaction was observed for the time-to-mortality data ($p < 0.01$; see Table A.12) and a marginally sig-

Table A.7. Study 2 Data for Animals Not Exposed to 1-Nitropyrene: Tumor Incidence

Carbon Particles	Benzo[*a*]pyrene 0 −	Benzo[*a*]pyrene 0 +	Benzo[*a*]pyrene 1 −	Benzo[*a*]pyrene 1 +
0	19	0	31	19
1	46	1	31	18

Table A.8. Study 2 Data for Animals Not Exposed to Carbon Particles: Estimated Mean Tumor Latency[a]

1-Nitropyrene	Benzo[*a*]pyrene 0	Benzo[*a*]pyrene 1
0	647.0	622.6[b] ± 10.2
Low	643.9 ± 0.1	613.5[b,c] ± 12.0
High	633.9 ± 8.7	580.2[b,c] ± 13.5

[a] Values are means ± SE given in days. Where no SE is given, all animals were killed on the day indicated. The Kaplan-Meier estimate was used.

[b] Significantly different from no B*a*P ($p < 0.01$).

[c] Significantly different from no 1-NP ($p < 0.01$).

Table A.9. Study 2 Data for Animals Not Exposed to Carbon Particles: Tumor Incidence

1-Nitropyrene	Benzo[*a*]pyrene 0 −	Benzo[*a*]pyrene 0 +	Benzo[*a*]pyrene 1 −	Benzo[*a*]pyrene 1 +
0	19	0	31	19
Low	47	1	29	19
High	40	7[a,b]	17	29[a,c]

[a] Significantly different from particle control ($p < 0.05$).

[b] Difference from no 1-NP approaches significance ($p < 0.09$).

[c] Significantly different from no 1-NP ($p < 0.05$).

Table A.10. Comparison of Studies 1 and 2: Estimated Mean Tumor Latency[a]

1-Nitropyrene	Study 1	Study 2
Low	629.7 ± 9.7	643.9 ± 0.1
High	644.0	633.9 ± 8.7

[a] Values are means ± SE given in days. Where no SE is given, all animals were killed on the day indicated. The Kaplan-Meier estimate was used.

Table A.11. Comparison of Studies 1 and 2: Tumor Incidence

	Study 1		Study 2	
1-Nitropyrene	–	+	–	+
Low	51	3	47	1
High	54	1	40	7

Table A.12. Comparison of Studies 1 and 2 for Time to Mortality: Estimated Mean Survival Time[a]

1-Nitropyrene	Study 1	Study 2
Low	435.2 ± 25.2	405.7 ± 29.5
High	400.0 ± 27.8	525.6 ± 19.1

[a] Values are means ± SE given in days. The Kaplan-Meier estimate was used.

nificant interaction was observed for the mortality incidence data ($p < 0.055$; see Table A.13). This interaction for actual survival was due to increased survival in the high-dose 1-NP group relative to the low-dose 1-NP group in Study 2 and to the lack of significant difference in survival between the two groups in Study 1.

SUMMARY

The results of these analyses revealed the following. First, with respect to Study 1, increased exposure frequency led to significantly decreased tumor latency. The absence of a significant exposure frequency by 1-NP dosage interaction indicated that the effect of exposure frequency was consistent for both low and high dosages of 1-NP. In terms of simple tumor incidence, the main effect of exposure frequency approached statistical significance ($p < 0.07$).

Second, Study 2 revealed that both B*a*P and carbon exposure significantly decreased tumor latency; however, their joint action was no greater than or less than would be expected by the addition of their individual effects. In terms of simple tumor incidence, the effect of B*a*P was retained, but carbon did not increase the incidence of tumors.

Table A.13. Comparison of Studies 1 and 2: Mortality Incidence

	Study 1		Study 2	
1-Nitropyrene	Alive	Dead	Alive	Dead
Low	5	49	6	42
High	4	51	17	30

Third, Study 2 also revealed that both 1-NP and B*a*P significantly decreased tumor latency and significantly increased tumor incidence. Again, there was no interaction indicating that their joint effect did not exceed what we would expect from the addition of their individual effects.

Fourth, the between-study comparison of the 1-NP low-dosage and high-dosage groups revealed a significant difference between the two studies for tumor incidence but not tumor latency. In Study 1, 1-NP low-dose animals exhibited an increased tumor incidence relative to high-dose animals, whereas in Study 2, low-dose animals had decreased tumor incidence relative to high-dose animals. Although not statistically significant, low-dose animals in Study 1 did have a decreased tumor latency (630 days) relative to high-dose animals (644 days); whereas in Study 2, the tumor latency of low-dose animals (644 days) was increased relative to the high-dose animals (634 days). In terms of actual survival, a significant interaction for time to mortality and a marginally significant interaction for mortality incidence indicated decreased mortality for the high-dose animals of Study 2 relative to the low-dose animals, and no significant difference between the two dosage groups of Study 1.

APPENDIX B. Survival, Body Weight, and Body Weight Gain of Hamsters in Studies 1 and 2

This appendix is available on request from the Health Effects Institute.

APPENDIX C. Synopsis and Narrative Description of Histopathologic Lesions Found in Studies 1 and 2

TRACHEA

Neoplastic

Papilloma. A benign neoplasm of tracheal mucosa that is predominantly epithelial tissue, usually stratified squamous. A small amount of stroma may be present. The tumor is elevated above the normal surface of the mucosa.

Polyp. A benign neoplasm of tracheal mucosa that is usually attached by a small base and has one or more bulbous tips. There is usually a generous stroma.

Adenomatous. Covered with single or multiple cell layers of nonsquamous epithelial cells.

Mixed. Covered with a mixture of epithelial types, areas of stratified squamous epithelium, and areas of multiple cell layers of nonsquamous epithelium.

Myxomatous. A stroma composed of generous amounts of

a loose network of stellate connective tissue cells mixed with amorphous intercellular material.

Chondrous. A stroma composed of cartilage.

Unspecified.

Squamous Cell Carcinoma. A malignant neoplasm of stratified squamous epithelium that infiltrates below the basement membrane of the mucosa.

Basal Cell Carcinoma. A malignant neoplasm of tracheal mucosa, composed of tumor cells derived from the stratum germinitivum. They tend to form rosettes, multiple serpentine folds, and linear arrays of cells.

Anaplastic Carcinoma. A malignant neoplasm of tracheal mucosa, composed of undifferentiated primitive cells that lack an organoid structure.

Unspecified Carcinomas. A malignant neoplasm of tracheal mucosa not otherwise specified.

Fibrosarcoma. A malignant tumor of fibroblasts, characterized by highly cellular lobules and very little collagen production.

Nonneoplastic

Hyperplasia. Thickening of the tracheal epithelium with nonsquamous epithelial cells.

Squamous Metaplasia. Change of focal areas of the tracheal mucosa into stratified squamous epithelium.

Micropapillomatosis. Proliferation of tracheal mucosa such that the epithelium is thrown up into microscopic serpentine folds.

Dysplasia. Alteration of the normal ciliated pseudostratified columnar epithelium of the tracheal mucosa to an epithelium that has lost its normal microscopic appearance.

LUNG

Neoplastic

Adenoma. A focal benign neoplasm of lung epithelium.

Acinar. One composed of tumor cells that tend to be arranged into spherical secretory units.

Papillary. One composed of cuboidal to columnar epithelium that tends to be thrown into complex folds and fronds inside the tumor.

Solid. One composed of tightly packed tumor cells that lack epithelial orientation.

Cuboidal. One composed of rows of cuboidal cells, often with a serpentine configuration.

Mucinous Cell. One composed of epithelial cells that resemble goblet cells and contain mucinous cytoplasmic contents.

Mastocytoma. A focal benign interstitial collection of neoplastic cells with the characteristics of mast cells.

Alveolar/Bronchiolar Tumors. A focal neoplasm composed of sinuous folds of cuboidal to columnar epithelium that, more or less, conforms in places to the alveolar microarchitecture.

Adenoma. A benign neoplasm composed of uniform cells, usually one layer thick, with tumor margins that are smooth.

Carcinoma. A malignant tumor composed of uniform cells that are, in places, piled up. At some sites, the tumor cells may be anaplastic. There may be infiltration of tumor cells at the margins of the tumors.

Adenocarcinoma. A malignant tumor of the epithelium in which there is a resemblance to the secretory endpiece microarchitecture.

Papillary Carcinoma. A malignant lung tumor composed of bronchiolar epithelium that is arranged in multiple infoldings and frond-like structures. The cells are often piled up and anaplastic.

Basal Cell Carcinoma. A malignant epithelial tumor of the stratum germinitivum of stratified squamous epithelium that forms rosettes, folds, and linear arrays of low cuboidal cells.

Squamous Cell Carcinoma. A malignant tumor composed of stratified squamous epithelium. Often large amounts of keratin are seen within the tumor. These tumors tend to be well differentiated. They were the largest tumors seen in these studies, and they tended to be multiple when they occurred.

Mixed Carcinoma. A malignant tumor composed of a mixture of adenomatous and squamous epithelial areas.

Anaplastic Carcinoma. A malignant epithelial tumor composed of cells that are primitive and lack normal polarity to each other and to the basement membrane.

Sarcoma. A malignant tumor composed of connective tissue cells too anaplastic to identify as to tissue cell type.

Nonneoplastic

Hyperplasia. Proliferating alveolar epithelium several layers thick, seen with focal distribution usually at the margins of areas of adenomatosis.

Hyperplasia, Bronchiolar Epithelium. Usually multifocal, excessive numbers of cuboidal respiratory epithelial cells at the distal terminal bronchiole. There is often transformation of neighboring simple squamous alveolar respiratory epithelium to cuboidal cells (bronchiolization).

Adenomatosis. Collections of pink-staining colloid material in the lumina of alveoli about the terminal bronchioles. The alveolar epithelium is thickened and irregular. The alveoli are often distended.

Squamous Metaplasia. The focal transformation of bronchiolar or alveolar epithelium into stratified squamous epithelium.

Mucous Metaplasia. Focal alteration of alveolar/bronchiolar epithelium to nodules of large cells stuffed with intracytoplasmic collections of mucus.

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ABBREVIATIONS

B*a*P	benzo[*a*]pyrene
HEPA	high-efficiency particulate air (filter)
HPLC	high-pressure liquid chromatography
IITRI	IIT Research Institute
$MeCl_2$	methylene chloride
1-NP	1-nitropyrene
ppm	parts per million

INTRODUCTION

A Request for Applications (RFA 82–1), which solicited proposals for "Studies on the Metabolism and Biologic Effects of Nitropyrene and Related Nitro-Polycyclic Aromatic Compounds," was issued by the Health Effects Institute (HEI) in the summer of 1982. In response to the RFA, Dr. Richard Moon from the IIT Research Institute in Chicago, IL, submitted a proposal entitled "Respiratory Carcinogenesis of Nitroaromatics." The HEI approved the three-year project, which began in December 1983. Total expenditures were $348,011. The Investigators' Report was received at the HEI in September 1987 and accepted by the Health Review Committee in July 1988. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of the HEI and to the public.

THE CLEAN AIR ACT

The Environmental Protection Agency (EPA) sets standards for diesel (and other) emissions under Section 202 of the Clean Air Act, as amended in 1977. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(A)(i) specifically directs the Administrator to "prescribe regulations . . . applicable to emissions of carbon monoxide, hydrocarbons, and oxides of nitrogen from classes . . . of heavy-duty vehicles or engines." Section 202(a)(3)(A)(iii) similarly requires regulations applicable to emissions of particulate matter from classes or categories of vehicles.

Under these provisions, the EPA has taken regulatory actions with respect to diesel engines. In 1980, the EPA set light-duty diesel particulate matter standards and, in 1984, granted a two-year delay in their effective date. The EPA established emissions-averaging in 1983, and it set nitrogen oxides standards in 1985. For heavy-duty diesel engines, the EPA set hydrocarbon and carbon monoxide standards in 1983, and nitrogen oxides and particulate matter standards in 1985. In 1988, the agency established revised particulate matter standards for certain light-duty diesel trucks. In addition, under Section 109 of the Act, the EPA established National Ambient Air Quality Standards for particulate matter. Those standards were most recently revised in July 1987.

Research on the carcinogenic effect of 1-nitropyrene and other nitrated polycyclic aromatic hydrocarbons can contribute to an increased understanding of the risks to humans from exposure to diesel-engine exhaust particulates, and can contribute, thereby, to informed decision-making with respect to standards under the Clean Air Act.

BACKGROUND

Exhaust from diesel engines contains a variety of compounds adsorbed to the soot particles as well as gases that are produced by the combustion process. During the oil crisis of the mid-1970s, there was concern that increased use of vehicles equipped with the more fuel-efficient diesel engines would increase the levels of airborne particulates and gases produced by such engines. During this same time, several investigations showed that organic solvent extracts of diesel-exhaust particles, particularly the nitroaromatic fraction containing nitropyrenes, were mutagenic in bacteria (Lewtas 1983). Subsequent studies indicated that these extracts also produced mutagenicity and chromosomal damage in mammalian cells (Claxton 1983; Lewtas 1983). More recent studies have shown that chronic inhalation of high levels of diesel-engine emissions induced tumors in the lungs of laboratory rodents (Brightwell et al. 1986; Heinrich et al. 1986; Ishinishi et al. 1986; Iwai et al. 1986; Mauderly et al. 1986). Several epidemiological studies also have suggested an association between chronic exposure to diesel-engine exhaust and an increased risk of lung cancer and bladder cancer in humans (Silverman et al. 1986; Steenland 1986; Garshick et al. 1987, 1988; International Agency for Research on Cancer 1989). These findings have prompted research to seek a better understanding of the potential mechanisms of toxicity of components in diesel-engine exhaust and how these mechanisms may contribute to the induction of cancer.

Nitropyrenes are polycyclic aromatic hydrocarbons that contain one or more nitro ($-NO_2$) groups. During fuel combustion, aromatic hydrocarbons and nitrogen oxides that are produced react to form nitrated polycyclic aromatic hydrocarbons, including the nitropyrenes. The number and positions of the nitro groups determine the type of nitropyrene. Although they can exist in crystalline form in diesel-engine exhaust, nitropyrenes are predominantly adsorbed to the soot particles. 1-Nitropyrene is the most abundant nitropyrene (approximately 100 μg/g of particles), while

dinitropyrenes account for only 1 μg/g of particles. These soot particles range in size from 0.1 to 0.3 μm (Tokiwa and Ohnishi 1986), and it is estimated that after inhalation, 25 to 35 percent of the particles deposit in the deep alveolar regions of the lungs (McClellan 1987). Thus, nitropyrenes, which are genotoxic in cell culture, can be in contact with respiratory tract tissues; however, the role of these compounds in the increased incidence of pulmonary tumors in animals after chronic exposure to diesel-engine exhaust is unknown.

Results from animal studies using pure compounds suggest that nitropyrenes are mammalian carcinogens (reviewed by Hecht 1988; International Agency for Research on Cancer 1989). Dinitropyrenes, particularly the 1,6- and 1,8-isomers, have been shown to be tumorigenic in several model systems (Ohgaki et al. 1984; Tokiwa et al. 1984; Rosenkranz and Mermelstein 1985; Takayama et al. 1985; King 1988). In contrast, as summarized in the Investigators' Report, the results of 1-nitropyrene carcinogenicity studies have been contradictory and inconclusive. One of the reasons for the inconsistent results may be that it is difficult to separate completely 1-nitropyrene from the dinitropyrene isomers during purification. Contamination of 1-nitropyrene preparations by only trace amounts of the more potent dinitropyrenes could dramatically influence the observed carcinogenic potential (Ohgaki et al. 1985). The inconsistent results regarding 1-nitropyrene carcinogenicity also relate to differences in the model system used. King (1988) has reported that the route of administration, and the age as well as the strain of laboratory animal used, influence the results. (It should be noted that when Dr. Moon proposed his study, some reports indicated that 1-nitropyrene was mutagenic, and preliminary data suggested a carcinogenic potential.)

Most animal studies attempt to associate excess tumors with a given exposure; this approach, however, does not provide insight into the mechanisms of carcinogenesis (Kaufman 1988). Because diesel-engine exhaust is a complex mixture, it may contain, in addition to potential carcinogens, cocarcinogens, initiators, promoters, and inhibitors of carcinogenesis. Collectively, these constituents determine the ultimate carcinogenic response, and they do so through different mechanisms of action. Some components, such as polycyclic aromatic hydrocarbons, may alter genetic material, whereas others may stimulate a proliferative response of the pulmonary epithelium, thus increasing the susceptibility of the tissue to cancer induction. One methodology that is employed to assess the cocarcinogenic, promotional, or inhibitory potential of a compound is to test the compound in conjunction with a known carcinogen. The premise is that the known carcinogen will initiate a specific tumor incidence rate, which will be altered in an additive, synergistic, or inhibitory manner, depending on the nature of the test compound. For studies of respiratory carcinogenesis, various protocols have been used, the most popular involving the intratracheal instillation of a polycyclic aromatic hydrocarbon or the injection of a nitrosamine.

During the 1970s, the Syrian golden hamster became one of the most widely used species for studies of respiratory carcinogenesis (reviewed in Nettesheim 1972). In this species, the histology of the respiratory tract is similar to that in humans; pulmonary infections are infrequent; spontaneous lung tumors are rare; and carcinomas similar to the type found in humans (bronchogenic squamous cell) can be induced. Using the hamster, a model of respiratory carcinogenesis was developed (Saffiotti et al. 1968) whereby carcinomas in the trachea and bronchi could be induced by the intratracheal instillation of polycyclic aromatic hydrocarbons adsorbed onto iron oxide particles.

More recent studies, however, suggest that the hamster may not be suitable for the testing of the carcinogenicity of diesel-engine exhaust. In chronic inhalation studies, rats developed respiratory tract tumors (Brightwell et al. 1986: Heinrich et al. 1986; Ishinishi et al. 1986; Iwai et al. 1986; Mauderly et al. 1986), but hamsters did not (Wehner et al. 1979; Heinrich et al. 1982; Brightwell et al. 1986; Heinrich et al. 1986). Furthermore, it appears that the induction of pulmonary and bronchial tumors in hamsters has occurred only by intratracheal instillation of carcinogens or by systemic administration of nitrosamines (Homburger 1972). Recent studies by Keenan and coworkers have shown that intratracheal instillation produces tracheal lesions, which affect the proliferative response of the airways (Keenan et al. 1989a); carcinogen administration by intralaryngeal instillation does not produce respiratory tract tumors (Keenen et al. 1989b). Thus, the sensitivity of the hamster, as well as the appropriateness of the intratracheal instillation technique for assessing respiratory tract carcinogenicity, needs to be considered. (It is important to note that when Dr. Moon proposed his study, these problems with the model system were not recognized.)

JUSTIFICATION FOR THE STUDY

The HEI solicited proposals that would provide information on the metabolism, biochemistry, and biologic effects of 1-nitropyrene and related nitroaromatics. Of particular interest to the Institute were studies, in rodents, on the potential carcinogenicity of these compounds, either alone or in combination with other relevant materials.

Dr. Moon proposed to obtain dose-response data on the activity of 1-nitropyrene in the induction of respiratory tract cancer in the Syrian golden hamster. Using a specialized catheter system, the investigators proposed to instill 1-nitropyrene, alone or with other agents, and assess the carcinogenic, tumor-initiating, tumor-promoting, and cocarcinogenic potential of the nitropyrene. Dr. Moon had considerable experience conducting similar studies, and it appeared that the proposed study would provide useful information on the mode of action of 1-nitropyrene.

OBJECTIVES AND STUDY DESIGN

The primary objective of the investigation proposed by Dr. Moon was to assess the carcinogenic potential of 1-nitropyrene to the respiratory tract. To achieve this goal, the investigators conducted two studies that were designed to answer two specific aims: Study 1 focused on whether or not 1-nitropyrene could act as a complete carcinogen, and Study 2 addressed whether or not 1-nitropyrene could act as a cocarcinogen. Using the intratracheal route of exposure, Syrian golden hamsters were exposed to 1-nitropyrene alone or in combination with a known carcinogen.

During the first 10 months of the investigation, efforts were made to administer the 1-nitropyrene by the modified Schreiber method (Grubbs et al. 1979), in which a solution of the pure compounds is administered to a limited region of the trachea. This method has the advantages that dose can be quantified and the effect is highly localized. However, because of the poor solubility of the 1-nitropyrene in solvents that did not produce inflammation in the respiratory tract, the method was replaced by the Saffiotti intratracheal instillation technique (Saffiotti et al. 1968). Thus, the final study design was as described below.

In Study 1, Syrian golden hamsters were exposed by intratracheal instillation to 1-nitropyrene adsorbed onto carbon particles. Either once or twice a week, animals received 1 mg or 2 mg of the compound adsorbed onto equal amounts of carbon (Stokes diameter, 2 to 5 μm). Treatments were carried out over 92 weeks. For negative controls, animals received the saline alone, particles alone, or no treatment. For positive controls, animals received benzo[*a*]pyrene adsorbed onto carbon particles.

In Study 2, Syrian golden hamsters were exposed by intratracheal instillation to 1-nitropyrene adsorbed onto carbon particles in combination with benzo[*a*]pyrene, also adsorbed onto carbon. Once a week, animals received 1 mg or 2 mg of 1-nitropyrene along with 0.25 mg of benzo[*a*]pyrene. The duration of the study was 92 weeks. Control groups consisted of those receiving no treatment, carbon particles alone, particle-bound 1-nitropyrene alone, particle-bound benzo[*a*]pyrene alone, and particle-bound benzo[*a*]pyrene with carbon.

Animals were killed for histopathological assay at the termination of either study or at any time during the study if found in a moribund condition. Hamsters that died during the course of the study also were examined. The trachea and lungs were evaluated by light microscopy for any pathologic lesions.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The first aim was achieved to a limited degree; however, methodological problems and intercurrent mortality of laboratory animals prevented thorough comparisons of the carcinogenic responses among the different treatment groups. The second aim was achieved to the extent that a cocarcinogenic effect was observed in one of the treatment groups (0.25 mg benzo[*a*]pyrene plus 2 mg 1-nitropyrene); however, it was not observed in the other treatment groups and no dose-response effect was apparent.

METHODS, STUDY DESIGN, AND DATA ANALYSIS

The study design and methodology were adequate in most respects, based on the data available at the outset of the investigation; however, it has since become apparent that the hamster may not be a satisfactory test system (see the Background section). Furthermore, the use of intratracheal instillation may have given misleading results in that the long period of treatment (92 weeks) and the epithelial tissue damage incurred could have influenced carcinogen localization and tissue responses (see the Background section).

Because histopathology was the primary endpoint of interest, it might have been helpful to have used more sensitive histologic methods, which are routinely available. The investigators preserved tracheal and lung tissues in 10 percent formalin, embedded them in paraffin, and then took 5 μm sections for histopathology. These methods are standard techniques for routine tumor analysis. However, fixatives made from paraformaldehyde or containing glutaraldehyde are preferable to formalin because of their superior fixation (Hayat 1981; Fox et al. 1985). In addition, the use of a water-soluble plastic-embedding material would have permitted the cutting of thinner (1 to 2 μm) sections (Ruddell 1967). Although more expensive and labor intensive, these alternative methods produce histologic samples that

can be analyzed with greater ease and less ambiguity. With improved resolution, it might have been possible to obtain more data on the nonneoplastic and preneoplastic lesions as well as better differentiation of the anatomic sites of the lesions.

RESULTS AND INTERPRETATION

In Study 1, no significant increase in the final cumulative incidence of respiratory tract tumors was observed in animals treated with 1-nitropyrene, whereas a significant increase was observed in those animals treated with the positive control, benzo[*a*]pyrene. In Study 2, a suggestive increase in the cumulative incidence of respiratory tract tumors was observed in animals treated once weekly with 1-nitropyrene. In the group of animals treated with both 1-nitropyrene and benzo[*a*]pyrene, the increase in respiratory tract tumor incidence appeared to be significantly greater than that attributable to benzo[*a*]pyrene alone. This result suggests that 1-nitropyrene acted as a cocarcinogen in combination with benzo[*a*]pyrene.

Adequate interpretation of the results from the project, however, is hampered by several problems. In Study 1, a high rate of intercurrent mortality occurred in the animals. When the animals were killed at the end of the study, serum was collected to determine viral antibody titers, and high levels of antibody to mouse pneumonia virus were found. However, in those animals that died early, it is not clear if serum was collected or if the animals were free of known pathogenic viruses at the time of the unscheduled deaths. Thus, the reason for the intercurrent mortality is unknown. It is unclear how infection modulates the carcinogenic response. Schreiber and associates (1972) reported an increase in respiratory tumors in infected rats, whereas Rao and colleagues (1989) saw no effect in mice. The health status of laboratory animals remains an important issue in the interpretation of the results.

Limitations in the necropsy and histopathology procedures used by the investigators compound the difficulties in evaluating the mortality and carcinogenicity data. At the time of death, the tracheas and lungs were removed and prepared for histopathology. All neoplasms, but not inflammatory or reactive changes, were recorded. Because of the difficulties in discriminating the sites of origin of the lung lesions, this information also was not recorded. As stated by the investigators, differentiation between malignant and benign tumors was easy, but distinguishing between benign tumors and focal proliferative nonneoplastic lesions was more difficult. Several comments are in order. First, with respect to the sampling of the tissues for histopathology, ligation of the trachea after severing the larynx may have damaged a significant area of the trachea. Second, because the larynx is a known target of carcinogens in this experimental system (Keenan et al. 1989b), it is unfortunate that the larynx was not included in the tissues taken for histologic examination. Third, it would have been preferable to score the tumors and other lesions for each segment of the respiratory tract separately. The failure to include the larynx routinely among the organs examined and the combining of the bronchi and the peripheral airways as "lungs" detract from the value of the observations. Fourth, the failure to collect data on the numbers of lesions per animal was also unfortunate, since without these data, the extent of tumorigenesis cannot be adequately assessed. As already noted, improved resolution of the sectioned material might have alleviated some of the difficulties in differentiating pulmonary substructure.

Mastocytomas were observed in animals from a saline-control and a 1-nitropyrene-treated (1 mg 2/wk) group from Study 1, and in one animal from the particle-control group from Study 2. However, the observed mastocytomas were not confirmed histochemically. Mastocytomas are unusual tumors, especially in hamsters, which have few mast cells in the lung. Because this type of tumor was observed in control groups, its occurrence in hamsters exposed to 1-nitropyrene may have been fortuitous.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Several questions that remain unresolved include the reproducibility of the enhancing effect of 1-nitropyrene on the carcinogenicity of benzo[*a*]pyrene, and the roles that tissue injury and intercurrent infection play in determining this response. Since both 1-nitropyrene and benzo[*a*]pyrene coexist in automotive emissions, it would be of interest to explore their combined effects in an appropriately sensitive species. From what we now know, benzo[*a*]pyrene may have an effect on the metabolism, and therefore the carcinogenicity, of 1-nitropyrene. By interfering with the cytochrome-P450-mediated oxidation of 1-nitropyrene, a detoxification mechanism, benzo[*a*]pyrene may allow nitroreduction to predominate, thus permitting the activation of 1-nitropyrene to a DNA-reactive species. It also appears that tissue injury and inflammation, which were present in the current project, may have modulated the carcinogenic response in ways that need clarification.

On the basis of this study and other recent investigations, it appears that the hamster intratracheal instillation model may not be an appropriate experimental system to evaluate the cocarcinogenic potential of 1-nitropyrene. The rat,

which is known to develop lung tumors from the inhalation of diesel-engine exhaust, may be more appropriate for future studies. However, even with this apparently sensitive species, attention should be paid to strain differences; King (1988) has shown that CD rats, but not Fischer-344 rats, developed mammary tumors after administration of 1-nitropyrene. Finally, the influence of the intratracheal installation procedure on tumorigenicity should be assessed further.

CONCLUSIONS

Although the hamster may not be the most appropriate test species for diesel-engine exhaust, the results of this project are in agreement with those of other recent carcinogenicity studies, which have shown 1-nitropyrene to be only weakly carcinogenic or noncarcinogenic (El-Bayoumy et al. 1982; Nesnow et al. 1984; Tokiwa et al. 1984; Ohgaki et al. 1985; King 1988). Weak or borderline carcinogenic potential is consistent with the absence of detectable DNA adducts in tissues from rats injected with 1-nitropyrene (Beland 1989). Although the results of this study suggest that 1-nitropyrene may act as a cocarcinogen with benzo[*a*]pyrene, such an interpretation must await further data. Unfortunately, for reasons that are unclear, this project was compromised by poor survival of the animals, which was unrelated to neoplasia and was not limited to the saline-control group. Although extensive data were collected on pulmonary pathology, abridged necropsy and histopathologic procedures limit the evaluation of the full pathogenic potential of the compounds administered.

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LIST OF HEI PUBLICATIONS

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Special Reports

Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research (Supplement)	January 1988

Research Reports

Report No.	Title	Principal Investigator	Publication Date
1	Estimation of Risk of Glucose 6-Phosphate Dehydrogenase-Deficient Red Cells to Ozone and Nitrogen Dioxide	M. Amoruso	August 1985
2	Disposition and Metabolism of Free and Particle-Associated Nitropyrenes After Inhalation	J. Bond	February 1986
3	Transport of Macromolecules and Particles at Target Sites for Deposition of Air Pollutants	T. Crocker	February 1986
4	The Metabolic Activation and DNA Adducts of Dinitropyrenes	F.A. Beland	August 1986
5	An Investigation into the Effect of a Ceramic Particle Trap on the Chemical Mutagens in Diesel Exhaust	S.T. Bagley	January 1987
6	Effect of Nitrogen Dioxide, Ozone, and Peroxyacetyl Nitrate on Metabolic and Pulmonary Function	D.M. Drechsler-Parks	April 1987
7	DNA Adducts of Nitropyrene Detected by Specific Antibodies	J.D. Groopman	April 1987
8	Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung	J.L. Mauderly	May 1987
9	Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury	J.M. Patel	June 1987
10	Predictive Models for Deposition of Inhaled Diesel Exhaust Particles in Humans and Laboratory Species	C.P. Yu	July 1987
11	Effects of Ozone and Nitrogen Dioxide on Human Lung Proteinase Inhibitors	D.A. Johnson	August 1987
12	Neurotoxicity of Prenatal Carbon Monoxide Exposure	L.D. Fechter	September 1987
13	Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties	E.D. Crandall	October 1987
14	The Effects of Ozone and Nitrogen Dioxide on Lung Function in Healthy and Asthmatic Adolescents	J.Q. Koenig	January 1988
15	Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide	T.J. Kulle	January 1988
16	Metabolism and Biological Effects of Nitropyrene and Related Compounds	C.M. King	February 1988

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Research Reports

Report No.	Title	Principal Investigator	Publication Date
17	Studies on the Metabolism and Biological Effects of Nitropyrene and Related Nitro-polycyclic Aromatic Compounds in Diploid Human Fibroblasts	V.M. Maher	March 1988
18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
19	Factors Affecting Possible Carcinogenecity of Inhaled Nitropyrene Aerosols	R.K. Wolff	August 1988
20	Modulation of Pulmonary Defense Mechanisms Against Viral and Bacterial Infections by Acute Exposures to Nitrogen Dioxide	G.J. Jakab	October 1988
21	Maximal Aerobic Capacity at Several Ambient Concentrations of Carbon Monoxide at Several Altitudes	S.M. Horvath	December 1988
22	Detection of Paracrine Factors in Oxidant Lung Injury	A.K. Tanswell	February 1989
23	Responses of Susceptible Subpopulations to Nitrogen Dioxide	P.E. Morrow	February 1989
24	Altered Susceptibility to Viral Respiratory Infection During Short-Term Exposure to Nitrogen Dioxide	R.M. Rose	March 1989
25	Acute Effects of Carbon Monoxide Exposure on Individuals with Coronary Artery Disease	HEI Multicenter CO Study Team	November 1989
26	Investigation of a Potential Cotumorigenic Effect of the Dioxides of Nitrogen and Sulfur, and of Diesel-Engine Exhaust, on the Respiratory Tract of Syrian Golden Hamsters	U. Mohr (U. Heinrich)	May 1989
27	Cardiovascular Effects of Chronic Carbon Monoxide and High-Altitude Exposure	J.J. McGrath	July 1989
28	Nitrogen Dioxide and Respiratory Infection: Pilot Investigations	J.M. Samet	September 1989
29	Early Markers of Lung Injury	J.N. Evans	September 1989
30	Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust	J.L. Mauderly	October 1989
31	DNA Binding by 1-Nitropyrene and Dinitropyrenes in Vitro and in Vivo: Effects of Nitroreductase Induction	F.A. Beland	October 1989

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The Health Effects Institute (HEI) is an independent nonprofit corporation that is "organized and operated . . . to conduct, or support the conduct of, and to evaluate research and testing relating to the health effects of emissions from motor vehicles." It is organized in the following ways to pursue this purpose.

INDEPENDENCE IN GOVERNANCE

The Institute is governed by a four-member Board of Directors whose members are Archibald Cox (Chairman of the Board), Carl M. Loeb University Professor (Emeritus) at Harvard University; William O. Baker, Chairman (Emeritus) of Bell Laboratories and Chairman of the Board of Rockefeller University; Donald Kennedy, President of Stanford University; and Walter A. Rosenblith, Institute Professor (Emeritus), Massachusetts Institute of Technology.

TWO-SECTOR FINANCIAL SUPPORT

The Institute receives half of its funds from the United States government through the Environmental Protection Agency, and half from the automotive industry. Twenty-eight domestic and foreign manufacturers of vehicles or engines contribute to the Institute's budget in shares proportionate to the number of vehicles or engines that they sell in the United States.

THE HEI RESEARCH PROCESS

The Institute is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is developed by the Health Research Committee, a multidisciplinary group of scientists knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific community that research proposals are being solicited on a specific topic. Applications are reviewed first for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to HEI's mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, who review the merits of the study as well as the procedures, independence, and quality of the selection process.

THE HEI REVIEW PROCESS

When a study is completed, a final report authored by the investigator(s) is reviewed by the Health Review Committee. The Health Review Committee has no role either in the review of applications or in the selection of projects and investigators for funding. Members are also expert scientists representing a broad range of experience in environmental health sciences. The Committee assesses the scientific quality of each study and evaluates its contribution to unresolved scientific questions.

Each Investigator's Report is peer-reviewed, generally by a biostatistician and three outside, independent reviewers chosen by the Review Committee. At one of its regularly scheduled meetings, the Review Committee discusses the Investigator's Report. The comments of the Committee and the peer reviewers are sent to the investigator, and he or she is asked to respond to those comments and, if necessary, revise the report. The Review Committee then prepares its Commentary, which includes a general background on the study, a technical evaluation of the work, a discussion of the remaining uncertainties and areas for future research, and implications of the findings for public health. After evaluation by the HEI Board of Directors, the HEI Research Report, which includes the Investigator's Report and the Review Committee's Commentary, is published in monograph form. The Research Reports are made available to the sponsors, the public, and many scientific and medical libraries, and are registered with NTIS and MEDLINE.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing of the release of an HEI Research Report is tailored to ensure that it does not interfere with the journal publication process.

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