HEI HEALTH EFFECTS INSTITUTE

# Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

## Part V: Effects on Pulmonary Function

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**Includes the Commentary of the Institute's Health Review Committee**

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# HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the U.S. However, the Institute exercises complete autonomy in setting its research priorities and in disbursing its funds. An independent Board of Directors governs the Institute. The Research Committee and the Review Committee serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigator's Report and the Review Committee's evaluation of the work's scientific and regulatory relevance.

# HEI Statement

**Synopsis of Research Report Number 65 Part V**

# Pulmonary Function Alterations in Rats After Chronic Ozone Inhalation

## BACKGROUND

Ozone is the major pollutant in smog. It is formed by complex photochemical reactions between nitrogen oxides and volatile organic compounds in the presence of sunlight. Motor vehicle and industrial emissions are prominent sources of these compounds. Peak atmospheric ozone concentrations generally occur during the summer months because the photochemical reactions that produce ozone are enhanced by sunlight and high temperature. Ozone exposure is a major health concern because it is a highly reactive gas that, at sufficiently high concentrations, can injure cells and tissues. Because ozone has the capacity to damage cells, exposure to ozone as a risk factor for lung cancer is an important public health issue. Therefore, the National Toxicology Program (NTP) conducted a series of tests to evaluate ozone's carcinogenicity in rats and mice chronically exposed to this pollutant. This presented a unique opportunity to study ozone's noncancerous effects as well; therefore, the NTP and the HEI entered into a collaborative agreement. HEI-funded investigators studied whether long-term ozone exposure causes or enhances alterations that are characteristic of chronic lung diseases, such as fibrosis or emphysema, in the lungs of laboratory animals.

The current National Ambient Air Quality Standard for ozone is 0.12 parts per million (ppm), a level that is not to be exceeded for more than one hour once a year. This standard was established largely on the basis of results of studies of acute exposure in human subjects. In exercising young adults, exposure to elevated levels of ozone for relatively short time periods causes lung function to be temporarily reduced and markers of pulmonary inflammation to appear in the fluid used to wash cells and other materials from the lungs. Whether repeated inhalation of ozone produces long-term effects on lung function, potentially causing or aggravating chronic lung disease, is unknown.

## APPROACH

Drs. Harkema and Mauderly exposed 65 male and female F344/N rats to either 0.12 ppm, 0.5 ppm, or 1.0 ppm ozone for 7 hours/day, 5 days/week, for 20 months, and investigated the effects of this exposure on lung function. Within one to six days after completing the 20-month exposure, they performed a battery of pulmonary function tests on anesthetized rats. Rats exposed to filtered air free of ozone served as a control group. The investigators' goal was to characterize the nature and magnitude of pulmonary impairment that may be associated with chronic exposure to ozone.

Testing pulmonary function in laboratory animals is a sensitive procedure for detecting lung injury and physiological changes related to disease. In humans, pulmonary function testing is critical to evaluating lung abnormalities in a variety of clinical and subclinical disease states. In fact, alterations in pulmonary function are commonly seen in humans who have been exposed to ozone. Therefore, the availability of benchmark data on pulmonary function in this rat cohort was considered to be essential to the overall interpretation of the other seven NTP/HEI studies.

## RESULTS AND IMPLICATIONS

The investigators found that a 20-month exposure to ozone produced minimal changes in the pulmonary function of rats. The only statistically significant effects were observed in female rats exposed to 0.5 ppm ozone, a finding that does not have an obvious biological explanation. These female rats showed an average decrease of 40% in the measurements of lung residual volume, which is the amount of gas or air remaining in the lungs after a subject has exhaled completely. Although this reduction is small in absolute terms, when it is extrapolated from rats and considered over the course of a human lifetime, this decrease in residual volume could have important human health consequences.

The investigators appropriately note that the alterations in pulmonary function observed in the rats exposed to ozone may not accurately reflect what occurs in humans because of differences between the species. The investigators also properly caution that the effects of ozone exposure in humans may not be as benign as those observed in the rats because the duration of tissue exposure is much longer in the course of a human lifetime. In addition, human exposures to ozone occur in association with other insults to lungs, such as microorganisms or other air pollutants, and in the presence of existing lung disease. These factors could be important contributors to the extent and severity of the lung responses to ozone exposure.

This Statement, prepared by the Health Effects Institute (HEI) and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1991 to 1993. The inhalation component of this project was supported by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone. This study was conducted by Drs. Jack R. Harkema and Joe L. Mauderly of the Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute in Albuquerque, NM. The following Research Report contains an Introduction to the NTP/HEI Collaborative Ozone Project, the detailed Investigators' Report, and a Commentary on the study prepared by the Institute's Health Review Committee.

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When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in the selection or management of the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee, and, if necessary, revise the report.

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# INTRODUCTION

## The National Toxicology Program and Health Effects Institute Collaborative Ozone Project

The NTP/HEI Collaborative Ozone Project was a four-year project with many investigators that was organized to evaluate the effects of prolonged ozone exposure on lung injury in animals. The ozone exposures were conducted by the National Toxicology Program (NTP) at Battelle Pacific Northwest Laboratories. The individual investigators' studies, which addressed the pathologic and physiologic consequences of prolonged ozone exposure, were supported by the Health Effects Institute (HEI). A full description of the NTP/HEI Collaborative Ozone Project and the exposure protocol can be found in the Introduction and Supplement to Research Report Number 65 Part I. This information also will be published in Part VI of Research Report Number 65 that describes the exposure and distribution of the animals.

Briefly, in 1987, the Health Effects Institute entered into a partnership with the National Toxicology Program to evaluate the effects of chronic ozone exposure in rats. The NTP, consisting of four agencies of the U.S. Department of Health and Human Services, coordinates the nation's testing of potentially toxic and hazardous chemicals. The Health Effects Institute, an independent research organization supported by both government and industry, provides unbiased information on the health effects of motor vehicle emissions.

Because of the widespread exposure to ozone and concerns about its potential health effects, HEI and the California Department of Health and Human Services nominated ozone for carcinogenicity and toxicity testing by the NTP. The NTP, recognizing that cancer was only one of the chronic diseases of concern, included additional animals for HEI-supported studies of the pathologic and physiologic consequences of prolonged ozone exposures. The HEI animals were housed in cages that would otherwise have been empty. By developing a partnership, the HEI and NTP were able to leverage their funds to develop a comprehensive research program that extended beyond carcinogenicity endpoints; the HEI-sponsored research focused on the relation between long-term ozone exposure and the pathogenesis of chronic lung diseases, such as asthma, emphysema, and fibrosis. The Health Effects Institute would not have been able to undertake such an expensive project, which requires special facilities and trained personnel, without the NTP's support of the inhalation component and the cooperation of the NTP's contractor, Battelle Pacific Northwest Laboratories.

For the HEI component of the Project, eight studies were selected for funding from proposals submitted in response to the Request for Applications (RFA) 90-1, Health Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program–Health Effects Institute Studies, Part A: Respiratory Function Studies, and Part B: Structural, Biochemical, and Other Alterations. Because of the complexity of a project with many investigators and many endpoints, the HEI Health Research Committee also funded a Biostatistical Advisory Group to provide assistance with experimental design, animal allocation, and data analyses. Figure 1 presents a diagram of the studies in the NTP/HEI Collaborative Ozone Project and their relations to each other. They include those studies that were part of the NTP bioassay, the eight HEI-funded studies, and the biostatistical study. In addition, HEI engaged Battelle Pacific Northwest Laboratories to provide support services for the HEI-sponsored investigators.

Starting at six to seven weeks of age, male and female F344/N rats were exposed to 0, 0.12, 0.5, or 1.0 parts per million (ppm) ozone, six hours per day, five days per week. These concentrations were selected to include the maximum concentration the animals would tolerate (1.0 ppm), the current National Ambient Air Quality Standard (NAAQS) for ozone (0.12 ppm), and an intermediate concentration. The NTP's carcinogenicity bioassay consisted of a two-year study and a lifetime study in rats and mice, and a study of male rats exposed to 0.5 ppm ozone and two levels of a human pulmonary carcinogen, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The design of the HEI studies was directed, to some extent, by the constraints of the NTP protocol. These included ozone exposure concentrations that were set by the NTP, a limit on the sample size (164 rats) to the number of available exposure chambers, and quarantine restrictions that did not allow reentry of animals into the exposure chambers once they had been removed, thus eliminating the possibility of conducting serial tests.

The Biostatistical Advisory Group developed a sample allocation scheme that allowed several researchers to obtain measurements on tissue samples from the same subset of study animals, providing the maximum overlap of animals and tissues among the eight studies while ensuring balance with respect to dose, gender, and time of death. When the ozone exposure of the HEI animals ended (at 20

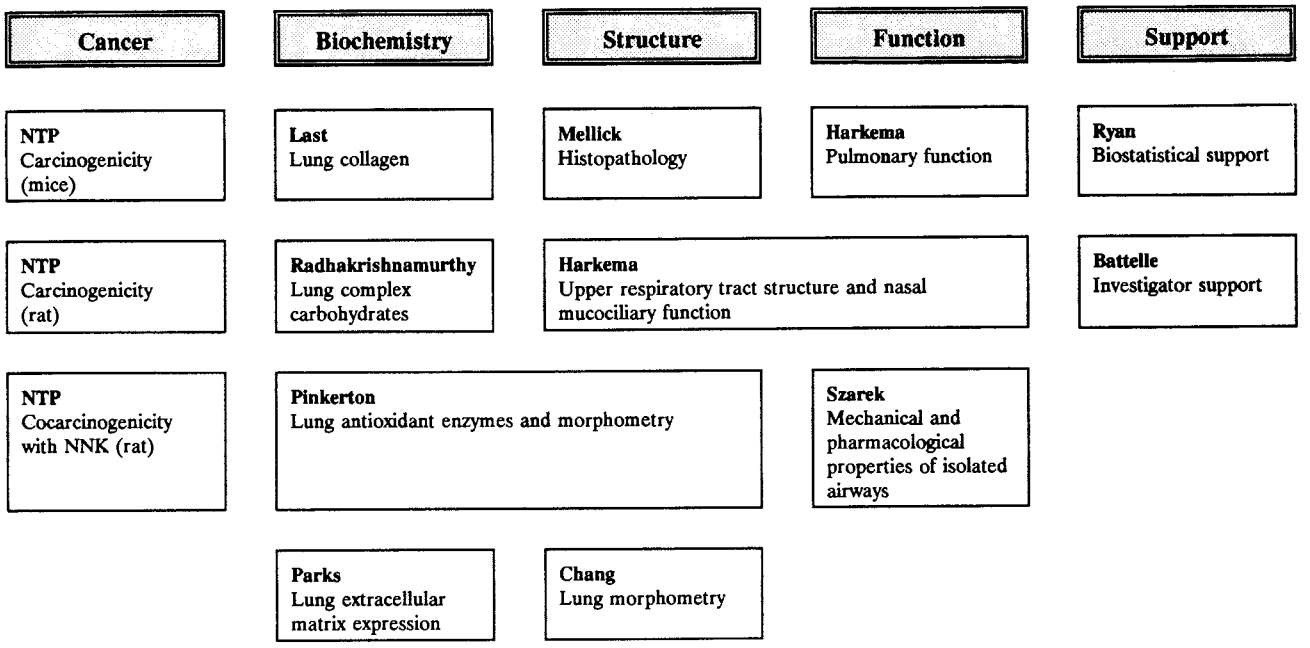


**Figure 1.** The NTP/HEI Collaborative Ozone Project: individual studies.

months), several investigators traveled to Battelle Pacific Northwest Laboratories to conduct their assays or to obtain samples on site. Battelle personnel prepared the tissues for off-site investigators and shipped them directly to their laboratories.

Because the studies varied in duration from six months to two years, HEI is publishing the reports for each individual study after the Institute's review process for each study is complete. Each Investigator's Report and a forthcoming Integrative Summary Report will be Parts of Report Number 65 of the HEI Research Report series. The present study by Drs. Jack Harkema and Joe Mauderly of the effects of long-term ozone exposure on pulmonary function is Part V. Other investigators in the Collaborative Ozone Project examined the effects of ozone on airway reactivity (Szarek), structural (Pinkerton, Chang), or biochemical (Last, Radhakrishnamurthy, and Parks) alterations.

Although some conclusions can be drawn on the basis of the results from each individual study, the interpretation of Dr. Harkema's findings will be strengthened when those data are correlated with the outcomes of the other investigators.

The importance of the collaborative NTP and HEI chronic ozone exposure studies is that they provide an unparalleled opportunity to examine the effects of prolonged ozone exposure using a variety of scientific approaches. The interaction of a number of methods to analyze the pathologic and physiologic consequences of chronic ozone exposure is one of this project's unique features. The results of these studies will provide new information about the threshold effects of ozone exposure on lung injury and the type and extent of damage in a well-established animal model. These results may be helpful for evaluating current standards of ozone exposure as they apply to human health and for designing future animal and human studies.

# Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

## Part V: Effects on Pulmonary Function

Jack R. Harkema and Joe L. Mauderly


## ABSTRACT

The impact of a 20-month exposure to ozone on the pulmonary function of rats was assessed from a single series of measurements made after exposures were completed. Four to ten male and female F344/N rats per group were exposed six hours per day, five days per week, for 20 months to ozone at 0.12, 0.5, or 1.0 parts per million (ppm)*, or to clean air as controls. One to three days after the last exposure, the rats were anesthetized using halothane, fitted with oral endotracheal and esophageal catheters, and measured using plethysmographic techniques. The differences between mean values for control and treated rats were tested for significance by multiple comparisons. The values and intersubject variability for more than 30 measured and calculated parameters were similar to those reported previously for rats of similar age. The only consistent exposure-related effect was a small reduction of residual volume measured during slow lung deflation. This trend was observed in most exposure groups, but was most significant in females exposed to ozone at the 0.5 ppm level. Fibrosis and epithelial changes were observed in the terminal bronchiole–alveolar duct region in parallel studies of different rats from the same exposure groups. We hypothesized that these changes stiffened airspace walls and acted to maintain the patency of the air pathway at a lower than normal lung volume during deflation. Overall, the exposures had little impact on the integrated pulmonary function of the lung as measured in anesthetized rats.


## INTRODUCTION

Ozone is a reactive, toxic oxidant gas produced by the reaction of nitrogen oxides and hydrocarbons in oxygen and sunlight, which occurs with other photochemical oxidants, nonoxidant gases and vapors, and fine particles in urban smog. The National Ambient Air Quality Standard for ozone is 0.12 ppm averaged for one hour, a level that is not to be exceeded more than once per year. In 1990, approximately 25% of the U.S. population was estimated to live in areas exceeding this standard (U.S. Environmental Protection Agency 1991).

Exposures to ozone, its toxic characteristics, and the present information base on health effects from epidemiological, clinical, and laboratory animal studies were reviewed by Lippmann in 1992. Ozone is readily absorbed when inhaled; Gerrity and associates (1988) demonstrated that humans at rest absorb approximately 90% of inhaled ozone. Ozone exposure reaches all conducting airways; however, the highest local dose in the lung is received at the junction between the small conducting airways and proximal alveoli (Miller et al. 1978; Hatch et al. 1989). Models of the dosimetry of ozone based on species-specific airway structure indicate that the estimated dose of ozone to the centriacinar region of the lung is two times greater in humans than in rats (Gerrity and Wiester 1987).

There is a substantial base of information on the transient effects of acute exposures of humans and animals to inhaled ozone; however, the health effects of long-term exposures of humans are uncertain. Ozone is known to have irritant and cytotoxic properties in humans and animals, and to have fibrogenic and genotoxic properties in animals (Lippmann 1992). However, the extent to which ozone might contribute to chronic lung diseases, including asthma, chronic obstructive pulmonary disease, interstitial pulmonary fibrosis, and cancer, among the human population is unknown. Our present understanding of potential long-term effects is based primarily on studies of animals exposed repeatedly, yet there have been no studies involving lifetime exposure.

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one section of Part V of Health Effects Institute Research Report Number 65, which also includes an Introduction to the NTP/HEI Collaborative Ozone Project, a Commentary by the Health Review Committee on the Investigators' Report, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Jack Harkema, Room A47, VMC, Department of Pathology, Michigan State University, East Lansing, MI 48824-1314.

This study was supported by HEI funds from the U.S. Environmental Protection Agency and the motor vehicle industry. The inhalation component of this project was sponsored by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred

Studies of monkeys exposed to various concentrations of ozone for up to 18 months have demonstrated bronchiolitis (Tyler et al. 1988), epithelial hyperplasia (Hyde et al. 1989), and the accumulation of structurally abnormal collagen (difunctional cross-links) consistent with progressive fibrosis (Reiser et al. 1987); however, exposures of this length represent only a small portion of the life span of monkeys.

Gross and White (1987) exposed male F344/N rats 20 hours per day, seven days per week, for 12 months to 0.5 ppm ozone. A 12-week recovery period followed. Histopathology at the end of the exposure consisted of mild inflammation centered on alveolar ducts. Significant differences in pulmonary function values from control values consisted of 7% to 11% increases in functional residual capacity (FRC) and residual volume (RV) and a 7% decrease in the lung's carbon monoxide diffusing capacity. These slight differences were reduced to nonsignificant levels during the recovery period.

In a study at the U.S. Environmental Protection Agency (EPA), male F344/N rats were exposed to a simulated urban ozone profile for up to 18 months (Grose et al. 1989; Tepper et al. 1991). The rats were exposed 13 hours per day, seven days per week to 0.06 ppm ozone. On Monday through Friday, a nine-hour ramped spike with a peak concentration of 0.25 ppm ozone and an integrated concentration of 0.19 ppm ozone was added to the 0.06 ppm ozone baseline exposure. After 12 months of exposure, there was a slight, but significant decrease in total lung capacity (TLC) compared with that of control rats, and a slight, near-significant decrease in RV. The slope of the values obtained in a multibreath nitrogen washout was steeper in exposed rats, but the difference disappeared when values were normalized by lung volume. These differences were reduced during a subsequent six-month recovery period. Although minor inflammations were observed in airways early during the exposure, the rats were described as having "no lesions of significance" at 18 months (Tepper et al. 1991). At that time, there was a slight, but significant decrease in the ventilatory (tidal volume) response of exposed rats to carbon dioxide and an increase in expiratory resistance.

Primarily on the basis of earlier inconclusive data from mice suggesting that ozone might act as a carcinogen or cocarcinogen (Witschi 1988), the National Toxicology Program (NTP) sponsored a bioassay of chronic ozone inhalation in rats and mice. The Health Effects Institute (HEI) worked with NTP to complement the standard bioassay by adding collaborative studies focused on various health effects endpoints. These studies made multiple use of a limited number of rats exposed for 20 months.

The study described in this report was included in the joint NTP/HEI effort to determine the impacts on pulmonary function of 20 months of exposure to ozone at three concentrations, including the current ambient air quality standard of 0.12 ppm. The ability to assess the pulmonary function of rats comprehensively by plethysmography was well developed, the assays had proved sensitive to numerous forms of lung injury, and substantial correlations between the functional responses of rats and those of humans to similar histopathologies had been established (Mauderly 1988, 1989). The purpose of this study was to determine whether or not the exposures caused measurable changes in function. If so, the study would provide data useful for examining structure-function correlations in conjunction with parallel studies of histopathology.

## SPECIFIC AIMS

This research was conducted in response to the HEI's Request for Applications No. 90-1, "Health Effects of Chronic Ozone Inhalation: Collaborative NTP/HEI Studies. Full Proposals." This request solicited collaborative studies of the health effects of 20-month exposures of rats to ozone, aimed at complementing the standard evaluations included in an NTP-sponsored cancer bioassay.

The principal aim of this study was to determine the existence, nature, magnitude, exposure-response relations, and gender specificity of pulmonary function impairments in rats due to chronic ozone exposure. The objective was to obtain in vivo measurements of pulmonary function, through multiple assays of breathing patterns, lung volumes, pressure-volume characteristics, airflow limitation, gas distribution uniformity, and alveolar capillary gas exchange, that simulated similar measurements in humans. The secondary aim of this study was to provide pulmonary function data useful, in conjunction with parallel studies, for determining structure-function correlations.

## METHODS

### EXPERIMENTAL DESIGN

Male and female F344/N rats were exposed by inhalation six hours per day, five days per week, for 20 months to ozone at 0.12, 0.5, or 1.0 ppm, or to air as controls. At one to six days after their last exposure, a comprehensive series of in vivo pulmonary function measurements were made by plethysmography, including assays of breathing pattern, lung mechanics, airflow limitation, lung volumes, gas distribution, and gas exchange. The rats recovered after the measurements and were later killed for other studies. The

number of rats available for this study was limited to 4 to 10 per gender per exposure group, because mortality prevented measurement of the planned 10 males and 10 females per group. Table 1 lists the sizes of the groups measured.

## PROCEDURES

### Subjects, Maintenance, and Exposures

Male and female F344/N rats (Simonsen Laboratories, Gilroy, CA) were randomly assigned at four to five weeks of age to control or ozone-exposed groups after a 10 to 14 day quarantine period. (Appendix A gives the NTP identification numbers for the rats used in this study.) Until the function tests the rats were housed in individual wire cages in 2.0-$m^3$ inhalation exposure chambers (H2000, Hazleton Systems, Aberdeen, MD). The animal maintenance and observation procedures were standard for an NTP-sponsored inhalation bioassay. In brief, the chambers were maintained at approximately 24°C, 59% relative humidity, with a flow rate providing 10 air changes per hour. The exposure rooms were lighted on a 12-hour cycle (from 0600 to 1800). Untreated paper cage board beneath the cages was changed twice daily, and chambers were washed weekly. The rats were provided with a pelleted ration (NIH-07, Zeigler Bros., Gardner, PA) ad libitum outside exposure hours and with water ad libitum at all times.

Groups of rats were exposed six hours per day (beginning at approximately 0730), five days per week (Monday through Friday), for 20 months to ozone at 0.12, 0.5, or 1.0 ppm, or to filtered air as unexposed controls. Charcoal and high-efficiency particulate air filters were used to purify ambient air, and ambient ozone was removed using potassium permanganate filters. Ozone was generated from 100% oxygen by corona discharge (OREC Model 03V5-0, Ozone Research and Equipment Corp., Phoenix, AZ). Ozone concentrations were measured using multiplexed ultraviolet spectrophotometric analyzers (Model 1003-AH, Dasibi Environmental Corp., Glendale, CA) calibrated by a chemical (neutral buffered potassium iodide) method. Ozone in the control atmosphere was below the limit of detection (0.002 ppm).

**Table 1.** Number of Rats Measured in Each Exposure Group

| Ozone Exposure (ppm) | Males | Females | Total |
|---|---|---|---|
| 0 (control group) | 9 | 9 | 18 |
| 0.12 | 4 | 4 | 8 |
| 0.5 | 8 | 10 | 18 |
| 1.0 | 7 | 10 | 17 |

### Pulmonary Function Measurements

Pulmonary function was measured using methods modified only slightly from previously reported methods (Harkema et al. 1982). Each rat was placed in a 1.0-L induction chamber, and anesthesia was induced with 5% halothane (Halothane USP, Halocarbon Laboratories, North Augusta, SC) vaporized in air (Vapor 19, North American Drager, Telford, PA). When the rat became immobile, it was removed from the chamber, a wire speculum (Mauderly 1977) was placed in its mouth, it was returned to the chamber, and anesthesia was continued to a light surgical plane. The rat was then removed from the chamber and placed on an intubation platform (Mauderly 1977), an endotracheal catheter was inserted, and the anesthetic plane was stabilized. The endotracheal catheter was 5.5 cm long, with an internal diameter of 1.78 mm, and was fabricated from a thin-wall, 14-gauge, silicon intravenous catheter (Cathlon IV, Jelco, Raritan, NJ) as described previously (Mauderly 1977).

After stabilization, the intubated rat was placed prone in a 1.4-L combination volume-displacement (flow) and constant-volume (pressure) plethysmograph. The plethysmograph, heated by a resistance element placed under the floor, was initially adjusted to approximately 37°C. A rectal thermistor was inserted, body temperature was monitored, and the heating element was adjusted as necessary to prevent hypothermia. The luer hub of the endotracheal catheter was attached to a breathing port at the front of the plethysmograph, which consisted of a luer fitting (No. 6161, Popper, New York, NY) drilled to an internal diameter of 2.5 mm. Between test procedures and during measurement of spontaneous respiratory variables, the breathing port was attached to a flow-through anesthetic circuit. With the rat positioned with its upper incisors resting on the luer breathing port, the total external dead space of the breathing pathway was approximately 0.4 mL.

A liquid-filled, open-tipped esophageal catheter of 2.2-mm outside diameter fabricated from a 6.5-French suction catheter (Digi-Trol, Seamless Hospital Products, Wallingford, CT) was inserted into the esophagus. The catheter was attached to a differential pressure transducer (MPX11DP, Motorola, Phoenix, AZ) with the other port open to ambient air. The depth of the catheter tip was adjusted to maximize the esophageal pressure signal. The signal conditioning and computational hardware and software required that the change in esophageal pressure be used as an estimate of the change in transpulmonary pressure ($P_{tp}$). This was deemed acceptable because the calculations required measurement of only $P_{tp}$ changes,

rather than absolute $P_{tp}$, the airway pressure fluctuations were negligible during tidal breathing, and the difference between esophageal and airway pressure was negligible during slow, induced inflations and deflations. A second liquid-filled catheter and identical transducer were connected to a Y-arm on the plethysmograph breathing port to measure airway pressure.

Respiratory flow was measured using a wire screen pneumotachograph and a differential pressure transducer (MP-45 2.0 cm $H_2O$, Validyne, Northridge, CA) open directly to the plethysmograph wall. The pneumotachograph consisted of six layers of 400-mesh wire cloth covering a 1.3-cm hole in the plethysmograph wall. When used in the mode for rapid events, the frequency response of the plethysmograph has been shown to be adequate for characterizing the details of these fast, forced expiratory events (Harkema et al. 1982). To use the plethysmograph in the pressure mode, the pneumotachograph hole was plugged, and the same transducer was used to measure box pressure.

The esophageal and airway pressure signals and plethysmograph flow (or pressure) signals were conditioned by preamplifiers and routed to a software-based data logger–respiratory mechanics analyzer (LS-14, Buxco Electronics, Sharon, CT). The logger-analyzer provided analog output to a strip-chart recorder, performed computations of parameters measured during spontaneous breathing, and provided digital data output to a monitor and data collection system based in a personal computer. Values for all parameters except respiratory frequency, tidal volume, minute volume, dynamic lung compliance ($C_{dyn}$), and total pulmonary resistance were calculated manually from strip-chart recordings, because comparisons between manual and electronic computations demonstrated the latter to be unreliable for all measurements except during spontaneous breathing. Calibrations were performed before the measurement of each rat, using a water manometer for pressure and a glass syringe for volume. This procedure also calibrated flow, from which volume was integrated. Periodic cross-calibrations of flow using a rotameter calibrated by timed volume collections ensured the accuracy and linearity of flow within the range of measurement.

Positive and negative airway pressures were used to induce lung inflations and both quasistatic (slow) and forced deflations simulating voluntary single-breath movements performed by humans. Reservoirs maintained at +40 and −50 cm $H_2O$ were connected to the airway by solenoid valves, which could be controlled manually or by the logger-analyzer system using programs specific to each test procedure. Inspiratory and quasistatic expiratory flows were limited by needle valves to 5 and 3 mL/second, respectively. Forced exhalation was induced by a valve with 9.5-mm internal diameter via a pathway in which flow impedance between the endotracheal catheter and the vacuum reservoir had been minimized.

After the rat was positioned in the plethysmograph with catheters in place, the halothane concentration was adjusted with the goal of maintaining a baseline respiratory frequency between 50 and 60 breaths/minute (typically 1.5% to 2.0% halothane). Anesthetic depth and respiratory pattern were allowed to stabilize at the baseline levels between each subsequent test procedure.

The breathing pattern and dynamic lung mechanics were measured, mean values were logged at 10-second intervals, and logged values were monitored until stable for a minimum of six time intervals (one minute). The stable values for respiratory frequency, tidal volume, minute volume, $C_{dyn}$, and total pulmonary resistance were then recorded. Subsequent measurements were made during temporary apnea induced by inflation of the lung using a syringe, which also standardized the lung volume history before each test.

The diffusing capacity for carbon monoxide ($D_{CO}$) was measured by a single-breath method (Ogilvie et al. 1957). The inflation volume required to raise $P_{tp}$ from apnea to +20 cm $H_2O$ was measured, and that volume of test gas containing 0.4% carbon monoxide and 0.5% neon in air was injected from a syringe during apnea. After six seconds, half of the injected volume was withdrawn, and the remainder, representing an "alveolar" sample, was withdrawn into a second syringe. The concentrations of carbon monoxide and neon in the alveolar sample were measured by gas chromatography (Model 111, Carle Instruments, Anaheim, CA). Diffusing capacity was calculated using the gas concentrations, breath-holding time as measured from a strip-chart recording of $P_{tp}$, and individual values for body weight and temperature. The alveolar volume during breath-holding was calculated from neon dilution.

Functional residual capacity was measured using a barometric (Boyle's law) technique (DuBois et al. 1956). The plethysmograph was switched to the pressure mode to enhance sensitivity, apnea was induced, the airway was plugged, and fluctuations in lung volume and airway pressure were measured as the rat resumed breathing attempts. Measured barometric pressure and body temperature were used for calculations.

A quasistatic deflation from TLC to RV was performed during apnea by inflating the lung slowly to +30 cm $H_2O$, then deflating it slowly until cessation of flow. The logger-analyzer program controlled inflation and deflation valves, and analog signals for volume and $P_{tp}$ were recorded. The inflation volume was measured as inspiratory capacity, the

deflation volume was measured as vital capacity (VC), the difference was calculated as expiratory reserve volume (ERV), and RV was calculated as the difference between FRC and ERV. Quasistatic chord compliance was calculated from the recorded $P_{tp}$ and volume differences between FRC and FRC at +10 cm $H_2O$ $P_{tp}$.

A forced exhalation from TLC was induced during apnea by inflating the lung to +30 cm $H_2O$, then deflating it without intentional flow limitation until cessation of flow. Recorded analog signals for flow and volume were analyzed to calculate forced vital capacity (FVC), peak expiratory flow rate (PEFR), percentage of FVC exhaled in 0.1 second, mean midexpiratory flow rate (MMEF) (25% to 75% of FVC), and the expiratory flows at 10%, 25%, and 50% of FVC.

A single-breath nitrogen washout was performed during apnea by deflating the lung to RV, inflating it with oxygen to +30 cm $H_2O$ $P_{tp}$, and then deflating it at the quasistatic rate (3 mL/second) to RV while recording the lung volume and exhaled nitrogen concentration (Nitralyzer 505, Med Science Electronics, St. Louis, MO). The slope of phase III ("alveolar plateau") of the washout, in units of percentage of nitrogen per millimeter, was measured from recorded lung volume and nitrogen traces.

After completing the above test procedures, which required approximately 30 minutes, the halothane vaporizer was turned off, and the rat was allowed to regain consciousness. At the first sign of movement or eye blink reflex, the esophageal and endotracheal catheters were removed, and the rat was removed from the plethysmograph, weighed, and returned to its holding cage.

All recorded traces and logged data were identified with the animal number and date and were retained. Parameters were calculated from the traces using calibration factors confirmed for each rat at the time of measurement, and calculated and logged values were entered into a computer database for manipulation, retention, and analysis.

### Statistical Analyses

The goal of the statistical analysis was to determine if pulmonary function values from ozone-exposed rats differed significantly from those from air-exposed control rats. The significance of exposure effects was analyzed by multiple pairwise comparisons using BMDP software (BMDP7D, "Description of Groups [Strata] with Histograms and Analysis of Variance," Edition 1983, Version 1987, BMDP Statistical Software, Los Angeles, CA). Three contrasts to control were tested using pooled variances and the Bonferroni adjustment for multiple comparisons. The criterion for statistical significance was set at a value of $p < 0.05$ for all comparisons. Values for the separate genders and for combined genders were analyzed for all parameters.

To evaluate the possibility of a dose response to ozone for the respiratory function values, linear regressions were performed using the concentration of ozone and gender as explanatory variables. Gender was treated as a categorical variable and ozone concentration as a continuous variable, so that there was a single slope for ozone with separate intercepts for males and females. A retrospective (posterior) calculation of power was performed for ozone using the actual estimate of the SE of the regression model. The results of this calculation were expressed as the slope for ozone concentration necessary to have 95% power (Neter et al. 1985).

## RESULTS

The tests were performed as planned, without difficulty. The window of tolerance to halothane was narrow for several rats, as is common for older subjects. These rats were stabilized at a slightly higher respiratory frequency than is typical for younger rats, because they became apneic if the halothane concentration was increased to lower the respiratory frequency further. All rats, however, could be stabilized at a respiratory frequency of 60 breaths/minute or lower, and there was no significant difference among control and exposed groups in the mean values for respiratory frequency.

Few parameters exhibited significant exposure-related differences from control values. For the combined genders (Table 2), only four parameters of the group exposed to 0.5 ppm ozone had mean values significantly different from control values. Insignificant differences consisting of a 4% lower TLC and a 2% higher VC for the group exposed to 0.5 ppm ozone combined to produce a significant difference of a 6% higher VC/TLC. Both the RV and the RV/TLC of that group were significantly lower than the control values. The magnitude of the difference in RV was 0.8 mL, or 38% of the control mean. Because values for the FRC of the control group and the group exposed to 0.5 ppm ozone were similar and the RV of the exposed group was smaller, the ERV of the exposed group was significantly higher (33%).

Only one value for exposed males differed significantly from that for control rats (Table 3). The mean forced expiratory flow rate at 10% of FVC for the males exposed to 1.0 ppm ozone was significantly lower (30%) than that for control rats when normalized by FVC, but was not significantly lower (27%) when expressed in milliliters per sec-

**Table 2.** Summary of Results for Male and Female Rats Combined

| Parameter | Units | Ozone Exposure[a] 0.0 ppm ($n$ = 18) | 0.12 ppm ($n$ = 8) | 0.5 ppm ($n$ = 18) | 1.0 ppm ($n$ = 17) |
|---|---|---|---|---|---|
| Body weight | g | 407 ± 24 | 411 ± 23 | 399 ± 17 | 389 ± 20 |
| Respiratory frequency | breaths/min | 57 ± 1 | 57 ± 1 | 57 ± 1 | 57 ± 1 |
| Tidal volume | mL | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.6 ± 0.1 | 1.6 ± 0.1 |
| Minute volume | mL/min | 97 ± 5 | 98 ± 5 | 89 ± 4 | 90 ± 5 |
| Dynamic lung compliance | mL/cm $H_2O$ | 0.43 ± 0.03 | 0.42 ± 0.06 | 0.48 ± 0.04 | 0.45 ± 0.04 |
| $C_{dyn}$/functional residual capacity | mL/cm $H_2O$/mL | 0.11 ± 0.01 | 0.12 ± 0.01 | 0.14 ± 0.01 | 0.12 ± 0.01 |
| Total pulmonary resistance | cm $H_2O$/mL/sec | 0.17 ± 0.01 | 0.21 ± 0.01 | 0.19 ± 0.01 | 0.19 ± 0.01 |
| Quasistatic chord compliance | mL/cm $H_2O$ | 0.85 ± 0.04 | 0.80 ± 0.04 | 0.86 ± 0.03 | 0.88 ± 0.04 |
| Total lung capacity | mL | 15.3 ± 0.6 | 14.2 ± 0.8 | 14.7 ± 0.5 | 15.4 ± 0.6 |
| TLC/body weight | mL/kg | 38 ± 2 | 35 ± 1 | 37 ± 1 | 40 ± 1 |
| Vital capacity | mL | 13.2 ± 0.5 | 12.7 ± 0.7 | 13.4 ± 0.5 | 13.6 ± 0.5 |
| VC/TLC | % | 86 ± 1 | 90 ± 1 | 91 ± 1[b] | 88 ± 1 |
| Functional residual capacity | mL | 3.9 ± 0.2 | 3.6 ± 0.2 | 3.6 ± 0.2 | 3.8 ± 0.1 |
| FRC/TLC | % | 26 ± 1 | 26 ± 2 | 25 ± 1 | 25 ± 1 |
| Residual volume | mL | 2.1 ± 0.1 | 1.5 ± 0.2 | 1.3 ± 0.2[b] | 1.6 ± 0.2 |
| RV/TLC | % | 14 ± 1 | 11 ± 1 | 9 ± 1[b] | 11 ± 1 |
| Expiratory reserve volume | mL | 1.8 ± 0.2 | 2.1 ± 0.2 | 2.3 ± 0.2 | 2.2 ± 0.1 |
| ERV/TLC | % | 12 ± 1 | 15 ± 2 | 16 ± 1[b] | 14 ± 1 |
| Carbon monoxide diffusing capacity ($D_{CO}$) | mL/min/mm Hg | 0.22 ± 0.01 | 0.20 ± 0.02 | 0.22 ± 0.01 | 0.24 ± 0.01 |
| $D_{CO}$/alveolar lung volume | mL/min/mm Hg/mL | 0.015 ± 0.001 | 0.014 ± 0.001 | 0.015 ± 0.001 | 0.016 ± 0.001 |
| Slope of phase III of nitrogen washout | % nitrogen/mL | 38 ± 3 | 40 ± 4 | 41 ± 4 | 35 ± 3 |
| Forced vital capacity | mL | 12.8 ± 0.5 | 12.5 ± 0.6 | 12.8 ± 0.5 | 13.2 ± 0.5 |
| % FVC exhaled in 0.1 sec | % | 64 ± 2 | 68 ± 2 | 63 ± 2 | 65 ± 1 |
| Peak expiratory flow rate | mL/sec | 112 ± 3 | 115 ± 3 | 112 ± 2 | 117 ± 2 |
| PEFR/FVC | mL/sec/mL | 9.0 ± 0.4 | 9.4 ± 0.4 | 9.0 ± 0.3 | 9.0 ± 0.3 |
| % FVC at PEFR | % | 67 ± 1 | 66 ± 1 | 66 ± 1 | 64 ± 1 |
| Mean midexpiratory flow | mL/sec | 84 ± 2 | 80 ± 2 | 84 ± 2 | 82 ± 2 |
| MMEF/FVC | mL/sec/mL | 6.8 ± 0.3 | 6.5 ± 0.3 | 6.7 ± 0.3 | 6.3 ± 0.2 |
| Expiratory flow at 50% FVC ($F_{50}$) | mL/sec | 97 ± 3 | 99 ± 4 | 102 ± 3 | 105 ± 4 |
| $F_{50}$/FVC | mL/sec/mL | 7.8 ± 0.3 | 8.1 ± 0.4 | 8.1 ± 0.3 | 8.1 ± 0.3 |
| Expiratory flow at 25% FVC ($F_{25}$) | mL/sec | 48 ± 2 | 45 ± 3 | 47 ± 2 | 49 ± 2 |
| $F_{25}$/FVC | mL/sec/mL | 3.9 ± 0.2 | 3.6 ± 0.3 | 3.8 ± 0.2 | 3.8 ± 0.2 |
| Expiratory flow at 10% FVC ($F_{10}$) | mL/sec | 15 ± 1 | 12 ± 1 | 13 ± 1 | 13 ± 1 |
| $F_{10}$/FVC | mL/sec/mL | 1.2 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 |

[a] Values are expressed as means ± SE.

[b] Difference from control mean value significant at $p < 0.05$; the Bonferroni adjustment was used for multiple comparisons.

**Table 3.** Summary of Results for Male Rats

| Parameter | Units | Ozone Exposure[a] 0.0 ppm (n = 9) | 0.12 ppm (n = 4) | 0.5 ppm (n = 8) | 1.0 ppm (n = 7) |
|---|---|---|---|---|---|
| Body weight | g | 474 ± 30 | 459 ± 29 | 452 ± 27 | 473 ± 15 |
| Respiratory frequency | breaths/min | 56 ± 1 | 56 ± 2 | 55 ± 1 | 57 ± 1 |
| Tidal volume | mL | 2.0 ± 0.1 | 2.0 ± 0.1 | 1.9 ± 0.1 | 1.9 ± 0.1 |
| Minute volume | mL/min | 112 ± 5 | 109 ± 6 | 103 ± 6 | 107 ± 7 |
| Dynamic lung compliance | mL/cm $H_2O$ | 0.46 ± 0.03 | 0.51 ± 0.09 | 0.53 ± 0.06 | 0.47 ± 0.05 |
| $C_{dyn}$/functional residual capacity | mL/cm $H_2O$/mL | 0.11 ± 0.01 | 0.12 ± 0.02 | 0.13 ± 0.02 | 0.12 ± 0.01 |
| Total pulmonary resistance | cm $H_2O$/mL/sec | 0.17 ± 0.02 | 0.19 ± 0.02 | 0.20 ± 0.01 | 0.20 ± 0.02 |
| Quasistatic chord compliance | mL/cm $H_2O$ | 0.96 ± 0.05 | 0.86 ± 0.06 | 0.97 ± 0.04 | 1.00 ± 0.06 |
| Total lung capacity | mL | 17.2 ± 0.6 | 15.8 ± 1.0 | 16.9 ± 0.5 | 18.0 ± 0.7 |
| TLC/body weight | mL/kg | 37 ± 2 | 35 ± 3 | 39 ± 3 | 38 ± 1 |
| Vital capacity | mL | 14.9 ± 0.6 | 14.0 ± 0.9 | 15.4 ± 0.5 | 15.9 ± 0.6 |
| VC/TLC | % | 87 ± 1 | 88 ± 2 | 91 ± 1 | 88 ± 2 |
| Functional residual capacity | mL | 4.2 ± 0.3 | 4.2 ± 0.1 | 4.0 ± 0.2 | 4.0 ± 0.2 |
| FRC/TLC | % | 25 ± 2 | 27 ± 2 | 24 ± 1 | 23 ± 1 |
| Residual volume | mL | 2.2 ± 0.2 | 1.8 ± 0.3 | 1.5 ± 0.2 | 1.8 ± 0.3 |
| RV/TLC | % | 13 ± 1 | 12 ± 2 | 9 ± 1 | 10 ± 2 |
| Expiratory reserve volume | mL | 2.0 ± 0.3 | 2.3 ± 0.3 | 2.6 ± 0.3 | 2.2 ± 0.3 |
| ERV/TLC | % | 11 ± 2 | 15 ± 3 | 15 ± 2 | 13 ± 2 |
| Carbon monoxide diffusing capacity ($D_{CO}$) | mL/min/mm Hg | 0.24 ± 0.02 | 0.21 ± 0.04 | 0.23 ± 0.02 | 0.28 ± 0.03 |
| $D_{CO}$/alveolar lung volume | mL/min/mm Hg/mL | 0.014 ± 0.001 | 0.013 ± 0.002 | 0.013 ± 0.001 | 0.016 ± 0.001 |
| Slope of phase III of nitrogen washout | % nitrogen/mL | 37 ± 3 | 43 ± 9 | 36 ± 3 | 33 ± 3 |
| Forced vital capacity | mL | 14.6 ± 0.6 | 13.8 ± 0.9 | 14.9 ± 0.5 | 15.3 ± 0.6 |
| % FVC exhaled in 0.1 sec | % | 59 ± 2 | 64 ± 2 | 56 ± 2 | 62 ± 1 |
| Peak expiratory flow rate | mL/sec | 112 ± 4 | 120 ± 2 | 113 ± 3 | 117 ± 4 |
| PEFR/FVC | mL/sec/mL | 7.7 ± 0.3 | 8.9 ± 0.8 | 7.7 ± 0.3 | 7.7 ± 0.2 |
| % FVC at PEFR | % | 69 ± 1 | 69 ± 1 | 68 ± 2 | 69 ± 1 |
| Mean midexpiratory flow | mL/sec | 83 ± 2 | 83 ± 3 | 83 ± 5 | 82 ± 4 |
| MMEF/FVC | mL/sec/mL | 5.8 ± 0.3 | 6.1 ± 0.4 | 5.6 ± 0.3 | 5.4 ± 0.2 |
| Expiratory flow at 50% FVC ($F_{50}$) | mL/sec | 99 ± 4 | 100 ± 6 | 104 ± 4 | 110 ± 5 |
| $F_{50}$/FVC | mL/sec/mL | 6.8 ± 0.2 | 7.3 ± 0.4 | 7.0 ± 0.2 | 7.3 ± 0.2 |
| Expiratory flow at 25% FVC ($F_{25}$) | mL/sec | 48 ± 1 | 47 ± 5 | 44 ± 5 | 49 ± 4 |
| $F_{25}$/FVC | mL/sec/mL | 3.4 ± 0.2 | 3.5 ± 0.3 | 3.0 ± 0.3 | 3.3 ± 0.2 |
| Expiratory flow at 10% FVC ($F_{10}$) | mL/sec | 15 ± 1 | 12 ± 1 | 12 ± 1 | 11 ± 1 |
| $F_{10}$/FVC | mL/sec/mL | 1.0 ± 0.1 | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.1[b] |

[a] Values are expressed as means ± SE.

[b] Difference from control mean value significant at $p < 0.05$; the Bonferroni adjustment was used for multiple comparisons.

ond. Values of RV and RV/TLC for exposed males were all lower than those for control rats (8% to 32%), but no differences were significant.

The significant exposure-related differences described above for the combined genders were largely driven by differences among the females exposed to 0.5 ppm ozone (Table 4). A slightly, but insignificantly, smaller TLC (3%) and larger VC (4%) resulted in a significantly higher VC/TLC (7%). The RV and RV/TLC values for the females exposed to 0.5 ppm ozone were significantly (40%) lower than those for control rats. Nearly identical differences for the group exposed to 0.12 ppm ozone gave $p$ values just above the criterion for significance.

As described above, with the exception of a single mean value of normalized flow rate at low lung volume for the males exposed to 1.0 ppm ozone, all consistent trends and significant differences from control values were related to a lower RV in exposed rats. This trend was consistent among all exposed groups, but was most significant in the females exposed to 0.5 ppm ozone. Group differences in

**Table 4.** Summary of Results for Female Rats

| Parameter | Units | Ozone Exposure[a] 0.0 ppm ($n$ = 9) | 0.12 ppm ($n$ = 4) | 0.5 ppm ($n$ = 8) | 1.0 ppm ($n$ = 7) |
|---|---|---|---|---|---|
| Respiratory frequency | breaths/min | 57 ± 1 | 58 ± 2 | 58 ± 1 | 56 ± 1 |
| Tidal volume | mL | 1.4 ± 0.1 | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 |
| Minute volume | mL/min | 81 ± 3 | 86 ± 1 | 78 ± 4 | 79 ± 3 |
| Dynamic lung compliance | mL/cm $H_2O$ | 0.40 ± 0.05 | 0.32 ± 0.03 | 0.45 ± 0.05 | 0.43 ± 0.07 |
| $C_{dyn}$/functional residual capacity | mL/cm $H_2O$/mL | 0.11 ± 0.01 | 0.11 ± 0.01 | 0.14 ± 0.01 | 0.12 ± 0.02 |
| Total pulmonary resistance | cm $H_2O$/mL/sec | 0.17 ± 0.02 | 0.23 ± 0.01 | 0.18 ± 0.01 | 0.18 ± 0.02 |
| Quasistatic chord compliance | mL/cm $H_2O$ | 0.73 ± 0.02 | 0.74 ± 0.02 | 0.78 ± 0.03 | 0.77 ± 0.01 |
| Total lung capacity | mL | 13.4 ± 0.2 | 12.6 ± 0.6 | 13.0 ± 0.4 | 13.6 ± 0.2 |
| TLC/body weight | mL/kg | 41 ± 1 | 35 ± 2 | 37 ± 1 | 42 ± 2 |
| Vital capacity | mL | 11.4 ± 0.2 | 11.5 ± 0.2 | 11.8 ± 0.4 | 12.0 ± 0.2 |
| VC/TLC | % | 85 ± 1 | 91 ± 2 | 91 ± 2[b] | 89 ± 1 |
| Functional residual capacity | mL | 3.6 ± 0.1 | 3.1 ± 0.2 | 3.3 ± 0.2 | 3.7 ± 0.1 |
| FRC/TLC | % | 27 ± 1 | 24 ± 2 | 25 ± 1 | 27 ± 1 |
| Residual volume | mL | 2.0 ± 0.2 | 1.2 ± 0.2 | 1.2 ± 0.2[b] | 1.6 ± 0.2 |
| RV/TLC | % | 15 ± 1 | 10 ± 2 | 9 ± 2[b] | 12 ± 1 |
| Expiratory reserve volume | mL | 1.6 ± 0.2 | 1.9 ± 0.3 | 2.1 ± 0.2 | 2.1 ± 0.1 |
| ERV/TLC | % | 12 ± 1 | 15 ± 3 | 16 ± 1 | 16 ± 1 |
| Carbon monoxide diffusing capacity ($D_{CO}$) | mL/min/mm Hg | 0.20 ± 0.01 | 0.19 ± 0.02 | 0.21 ± 0.01 | 0.22 ± 0.01 |
| $D_{CO}$/alveolar lung volume | mL/min/mm Hg/mL | 0.015 ± 0.001 | 0.015 ± 0.001 | 0.016 ± 0.001 | 0.016 ± 0.001 |
| Slope of phase III of nitrogen washout | % nitrogen/mL | 38 ± 6 | 37 ± 1 | 45 ± 6 | 36 ± 5 |
| Forced vital capacity | mL | 11.0 ± 0.2 | 11.2 ± 0.3 | 11.1 ± 0.3 | 11.7 ± 0.2 |
| % FVC exhaled in 0.1 sec | % | 68 ± 1 | 71 ± 1 | 68 ± 1 | 68 ± 2 |
| Peak expiratory flow rate | mL/sec | 112 ± 3 | 110 ± 4 | 111 ± 3 | 117 ± 3 |
| PEFR/FVC | mL/sec/mL | 10.3 ± 0.4 | 9.9 ± 0.3 | 10.0 ± 0.3 | 10.0 ± 0.2 |
| % FVC at PEFR | % | 64 ± 1 | 64 ± 2 | 65 ± 2 | 61 ± 2 |
| Mean midexpiratory flow | mL/sec | 85 ± 3 | 77 ± 1 | 84 ± 2 | 82 ± 2 |
| MMEF/FVC | mL/sec/mL | 7.7 ± 0.2 | 6.9 ± 0.2 | 7.6 ± 0.3 | 7.0 ± 0.2 |
| Expiratory flow at 50% FVC ($F_{50}$) | mL/sec | 95 ± 4 | 99 ± 6 | 100 ± 4 | 101 ± 5 |
| $F_{50}$/FVC | mL/sec/mL | 8.7 ± 0.3 | 8.9 ± 0.5 | 9.0 ± 0.3 | 8.7 ± 0.4 |
| Expiratory flow at 25% FVC ($F_{25}$) | mL/sec | 49 ± 3 | 42 ± 4 | 49 ± 2 | 49 ± 2 |
| $F_{25}$/FVC | mL/sec/mL | 4.4 ± 0.3 | 3.8 ± 0.4 | 4.5 ± 0.2 | 4.2 ± 0.2 |
| Expiratory flow at 10% FVC ($F_{10}$) | mL/sec | 16 ± 1 | 12 ± 1 | 13 ± 1 | 14 ± 1 |
| $F_{10}$/FVC | mL/sec/mL | 1.5 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 | 1.2 ± 0.1 |

[a] Values are expressed as means ± SE.

[b] Difference from control mean value significant at $p < 0.05$; the Bonferroni adjustment was used for multiple comparisons.

the subdivisions of lung volume for males and females are illustrated by bar graphs in Figure 1, which shows the nonsignificant trend toward smaller lungs (therefore lower values for TLC) in the males exposed to 0.12 ppm ozone and a smaller RV in the males exposed to 0.5 and 1.0 ppm ozone. The TLC of the females exposed to 0.12 ppm ozone was also slightly lower than that of control rats, and the trend toward lower values for RV is evident for all groups. These findings appear to indicate a true tendency for ozone exposure to reduce RV, with the greatest effect occurring at the 0.5 ppm level.

The single significant difference from control values related to the forced expiratory flow of the males exposed to 1.0 ppm ozone did not reflect a consistent trend among exposed rats of either gender. A slightly larger FVC and lower flow at 10% of FVC combined to produce this isolated significant difference. The fact that this difference did not reflect flow limitation can be seen in Figure 2,

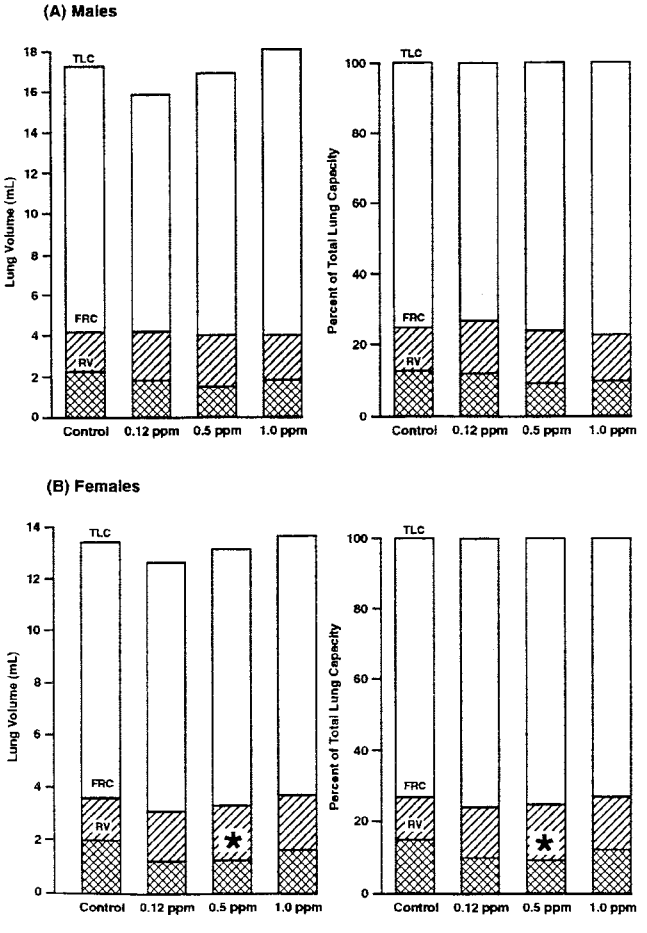


**Figure 1. The subdivisions of the lung volume of (A) the male and (B) the female rats.** The bar graphs were constructed from mean values listed in Tables 3 and 4. Volumes are plotted in milliliters on the left, and as percentages of TLC on the right. Asterisks indicate mean values significantly lower than those of controls.

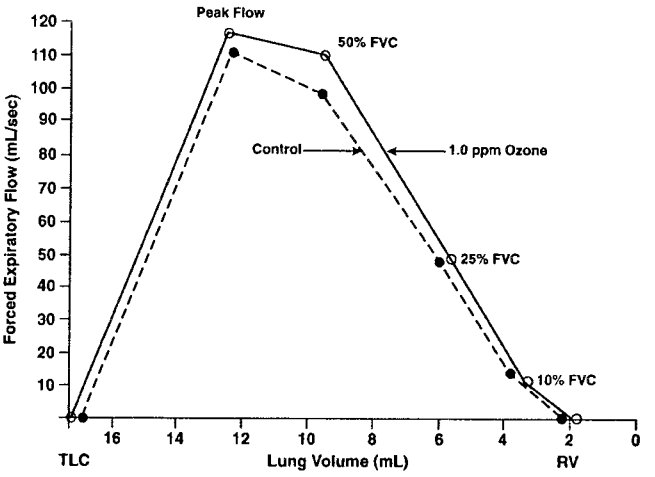


**Figure 2. Forced expiratory flow–volume curves of the data for the control group and male rats exposed to 1.0 ppm ozone.** The curves were reconstructed from mean values from Table 3 for TLC, PEFR, the percentage of TLC at peak flow, flow rates at 50%, 25%, and 10% of FVC, and RV. Closed circles indicate data for control rats, and open circles indicate data for rats exposed to 1.0 ppm ozone. Flow rates of the exposed rats exceeded those of control rats throughout the exhalation.

which gives the mean expiratory flow-volume curves, reconstructed from a few point measurements, of the control groups and the groups exposed to 1.0 ppm ozone. Forced flow is volume-dependent; thus, a stringent comparison involves determining whether or not flows are similar at similar lung volumes. As seen in Figure 2, although the flow of the males exposed to 1.0 ppm ozone was lower at 10% of FVC than that of the control rats, the flows of the exposed group were actually slightly higher than those of the control rats throughout the curve at equal lung volumes. Figure 2 illustrates that there is no suggestion of actual flow limitation among the exposed group at any point during the exhalation.

As mentioned above, linear regressions were performed, using the concentration of ozone and gender as explanatory variables, to evaluate the possibility of a dose response for the various parameters measured in this study. No interactions between gender and ozone were found for any of the parameters; however, six parameters (listed in Table 5) had marginally significant slopes for the ozone concentrations. All the $p$ values for the slopes of the regression lines for these parameters ranged from 0.011 to 0.048, and partial correlation coefficients for the slopes ($r$) were low, ranging from 0.26 to 0.31. This suggested only a weak relation between these parameters and the ozone exposures. In Table 5, the SE of the regression model is an estimate of the variability of an individual in the population. The SE and the change in slope for 95% power were both expressed as

**Table 5.** Linear Regression Analyses for Changes in Selected Respiratory Function Parameters in Response to Ozone Exposures[a]

| Parameter | Units | $p$ | Slope[b] | Standard Error of the Regression Model | $r$ | Fractional Standard Error of the Regression Model[b] | Fractional Slope for 95% Power[c] |
|---|---|---|---|---|---|---|---|
| Vital capacity | mL | 0.041 | 1.300 | 1.300 | 0.27 | 0.078 | 0.093 |
| Residual volume/ total lung capacity | % | 0.048 | −0.028 | 0.043 | 0.26 | 0.370 | 0.440 |
| Carbon monoxide diffusing capacity | mL/min/mm/Hg | 0.042 | 0.033 | 0.049 | 0.26 | 0.200 | 0.240 |
| Functional residual capacity | mL | 0.047 | 0.800 | 1.200 | 0.26 | 0.075 | 0.089 |
| Mean midexpiratory flow/ forced vital capacity | mL/sec | 0.031 | −0.520 | 0.850 | 0.28 | 0.140 | 0.170 |
| Expiratory flow at 10% FVC | mL/sec | 0.011 | −0.230 | 0.320 | 0.33 | 0.340 | 0.410 |

[a] A separate intercept was estimated for males and females.

[b] The fractional slope was calculated for 95% power as a test of the slope for ozone concentration being significant from 0 at the 5% level.

[c] The standard error of the regression model and the slope for 95% power were expressed as a fraction of the mean value of a control animal (average of a male and a female control rat).

a fraction of the mean value of a control animal (average of a male and a female). The change in slope for 95% power was only slightly larger (20%) than the SE of the regression model, indicating that the study had good power to detect changes in the slope that were only somewhat larger than the variability among the animals. All of the regression lines for the six respiratory function parameters had slopes smaller than the SE of the regression model, suggesting a very weak dose response for the concentrations of ozone used in this study.

## DISCUSSION AND CONCLUSIONS

### INTERPRETATION OF THE FINDINGS

By the measurements used in this study, exposure to ozone at 0.12 to 1.0 ppm for 20 months was shown to cause little decrement in the pulmonary function of male or female rats. The only consistent indication of an exposure effect was the reduction in RV. In anesthetized rats, RV is thought to be determined primarily by airway collapse; thus, the gas remaining in the lung at that point would primarily represent gas trapped distal to the location of airway closure (Leith 1976; Gillespie 1983). Because cartilage and wall thickness act to prevent collapse in larger conducting airways, it is assumed that collapse would occur in the terminal bronchiole–alveolar duct region of rats, where cartilage is absent and the airway wall is very thin. If this is true, a structural change acting to stiffen this region might allow the lung to deflate to a lower than normal volume. This hypothesis is supported in general terms by the observation that the lungs of certain diving mammals, in which cartilage extends more distally than in other mammals with flexible chest walls such as rats, can deflate to extremely small RVs (Leith 1976).

Quantitative structure-function correlates were not examined because detailed histopathologic or morphometric analysis was not performed on lungs of rats used in this study. An indirect comparison is possible, however, because rats from the same exposure groups were examined in parallel studies. Pinkerton and colleagues (1993) evaluated rats from the same exposures as those in this study. They observed that in exposed rats epithelial cell types and thicknesses more typical of bronchioles were located on alveolar septal tips more distal into the alveolar duct regions than in control rats. Last and coworkers (1993) also examined rats from the same exposure and observed an excess of stainable collagen and increased hydroxylysine-derived cross-linking of collagen in the centriacinar region. Although not conclusive, these findings support the hypothesis that an exposure-related stiffening of the terminal bronchiole–alveolar duct region might have allowed the lungs to deflate to the slightly greater degree observed in this study.

The interpretation of the finding that RV was affected more at the 0.5 ppm ozone exposure level than at 1.0 ppm ozone is uncertain. The RV difference between these exposed groups was small, but was consistent between genders. It might be conjectured that the effect of stiffening the terminal bronchiole–alveolar duct region would be offset in part by any narrowing of the air pathway. Although Pinkerton and colleagues (1993) did not report data for airway caliber and

thickness, one might suspect that epithelial thickening, in combination with surrounding fibrosis, could have acted to narrow the air pathways in this region. Regardless, this conjecture most likely exceeds the interpretive value of the differences measured in this study.

It is reasonable to question why the measures of lung compliance used in this study gave no indication of increased lung stiffness, if stiffening of the lung parenchyma affected deflation. Both $C_{dyn}$ measured during spontaneous breathing and quasistatic chord compliance measured during slow deflation target the elastic recoil of the lung in the volume region of normal breathing. This volume is well above the level at which RV is set. Both measures of compliance have been shown to reflect widespread or marked local changes in lung elasticity (Mauderly 1988, 1989). A comparison of different methods of characterizing the deflation pressure-volume curve of rats with ozone- or endotoxin-induced bronchiolitis or interstitial inflammation (Mauderly et al. 1988) demonstrated that the chord compliance used in this study had a sensitivity greater than that of dynamic compliance, maximum quasistatic compliance, or a pressure-volume curve shape factor. Most likely the focal tissue stiffening induced by ozone exposure in this study was not sufficiently severe or was not sufficiently widespread to influence total lung elastic recoil measurably, or the effect was manifested at a lung volume that was not measured by the assays used.

## SIMILARITY OF FINDINGS TO PREVIOUS RESULTS

The only previous assessments of the pulmonary function of rats exposed to ozone for 12 months or longer were those conducted at General Motors (Gross and White 1987) and at the EPA (Grose et al. 1989; Tepper et al. 1991), which are described in the Introduction section above. Because the exposure patterns, concentrations, and times used in these studies differed from those of the present study, neither of the previous studies provides a good direct comparison.

The study by Gross and White included rats exposed to 0.5 ppm ozone, but the rats were exposed for 140 hours per week in contrast to the exposure of 30 hours per week in the present study. Inflammation and fibrosis of alveolar duct walls, resulting in approximately a doubling of wall thickness, were observed after exposure. The increase in FRC observed after 12 months of exposure in that study may have resulted from a resetting of the relaxed lung position to a larger volume due to the pathology observed, as has been proposed for similar effects from fibrotic lung disease in rats (Mauderly 1988). The FRC of rats and other mammals with flexible chest walls is thought to be larger during consciousness than during anesthesia (Gillespie 1983). It is possible that proliferative and fibrotic changes, if sufficiently extensive, act to "fix" the lung at the larger relaxed volume during consciousness, and that such a lung does not deflate to a normally lower relaxed volume during anesthesia. In the present study, the less intense exposures probably resulted in less structural change than was observed in the Gross and White study; thus, no effect on FRC was manifested.

The description of the pathology observed in the Gross and White study does not allow more than a conjectural comparison with that in the present study. If pathology was more severe in the previous study than in the present study, or involved a greater epithelial and lumenal component and less interstitial change, it could have increased the RV by promoting earlier than normal airway closure during deflation, rather than supporting the patency of airways as hypothesized for the present effects. The reduction of diffusing capacity in the White and Gross study was also suggestive of more extensive pathology than in the present study, in which diffusing capacity was not significantly affected.

To compare exposures between the present study and that conducted by the EPA is even more difficult because of the variable exposure pattern used in the former. In contrast to the slight, but significant reduction of TLC observed by Grose and associates (1989), no consistent tendency toward reduction of TLC was observed in the present study. The trend toward reduction of RV was common to both studies. Ventilatory responses to carbon dioxide were not assessed in the present study, but forced expiratory parameters did not indicate an increase in expiratory flow resistance, as observed during exaggerated tidal breathing in the study of Tepper and coworkers (1991). Whether or not these subtle differences between the results of the studies are meaningful is unknown.

## ADEQUACY OF EVALUATION OF FUNCTIONAL CHANGES

The measurements used in this study have been applied in numerous studies of rats with experimentally induced lung abnormalities. The measurement procedures, their sensitivity and interpretive value in assessing different forms of lung injury, and the relevance of disease-related physiological changes in animals to those in humans have been discussed (Costa 1985; Mauderly 1988, 1989; Mauderly et al. 1988). There are numerous differences between the test procedures applied to rats and those applied to humans, but taking these and interspecies differences in normal lung structure and

function into account, the tests of animals are generally accepted as producing results that parallel those expected in humans with similar pathologies.

The sensitivity of the tests applied to rats is manifested in part by the finding of statistically significant differences between control and treated rats when absolute differences in mean values are in the range of 10% and group sizes are on the order of 10 rats. The variability of rat pulmonary function data from subject to subject or day to day is not typically less than that of human data. Without baseline or concurrent control data for humans, it is common to use a difference of approximately 20% between measured and predicted values as a criterion for "abnormality."

The intersubject variability of values for parameters measured in this study was typical of that reported for rats of similar age in previous studies. In an earlier study at this Institute using the same techniques (Mauderly and Gillett 1992), the pulmonary function of 20 male and 20 female unexposed control F344/N rats was measured at 22 months of age. The variability of values for selected parameters, expressed as coefficients of variation, is compared between these historic data and the present data from the nine male and nine female control rats in Table 6. Although some coefficients of variation for the present study were larger and some were smaller than the historic values, overall intersubject variability tended to be less in the present study.

Ozone exposure had little effect on the intersubject variability of the values obtained in this study. The statistical program used in this study included Levene's test of significance of differences in variance among the exposure groups. Of the 34 parameters listed in Tables 2, 3, and 4, variances differed significantly among groups for only 2 parameters, total pulmonary resistance and FRC. Indeed, it was because variances differed little among groups that the pooled variance was used to test the significance of differences between mean values.

The likelihood of calculating a significant difference by chance among the large number of statistical comparisons in this study must be considered. In total, the three contrasts to control for 34 parameters of two genders resulted in 204 individual comparisons. For this reason, it is important to examine the significant differences for patterns of consistency among related parameters. As described above, the trend toward a smaller RV was corroborated by trends in related parameters from several exposure groups of both genders. This supports the conclusion that the trend was not a manifestation of statistical chance, but was probably a true exposure effect. Conversely, there was little support among other parameters or other groups for concluding that the single significant difference in forced expiratory flow in the male rats exposed to 1.0 ppm ozone represented a true exposure effect.

## CONCLUSIONS

The results of this study demonstrated that exposures of F344/N rats six hours per day, five days per week, for 20

**Table 6.** Intersubject Variability, Expressed as Coefficients of Variation[a], of Selected Pulmonary Function Data from Control Rats in This Study Compared with That of Data from Control Rats of Similar Age in a Previous Study[b]

| | Males | | Females | |
|---|---|---|---|---|
| Parameter | This Study ($n$ = 9) | Previous Study ($n$ = 20) | This Study ($n$ = 9) | Previous Study ($n$ = 20) |
| Minute volume | 14 | 23 | 11 | 30 |
| Dynamic lung compliance | 19 | 36 | 37 | 32 |
| Quasistatic chord compliance | 16 | 14 | 7 | 15 |
| Total lung capacity | 10 | 12 | 6 | 13 |
| Vital capacity | 13 | 11 | 7 | 11 |
| Functional residual capacity | 19 | 21 | 8 | 23 |
| Residual volume | 24 | 26 | 31 | 24 |
| Carbon monoxide diffusing capacity | 21 | 17 | 18 | 12 |
| Slope of phase III of nitrogen washout | 24 | 40 | 45 | 39 |
| % Forced vital capacity exhaled in 0.1 sec | 9 | 8 | 6 | 10 |
| Mean midexpiratory flow | 8 | 9 | 12 | 22 |
| Expiratory flow at 10% FVC | 25 | 15 | 24 | 25 |

[a] Coefficient of variation = standard deviation divided by the mean, multiplied by 100.

[b] Values for 22-month-old, unexposed control rats reported by Mauderly and Gillett (1992).

months to ozone at 0.12, 0.5, or 1.0 ppm caused little decrement in the pulmonary function of intact, anesthetized subjects as measured by plethysmography. Parallel studies of rats from the same exposure groups have revealed subtle, dose-related histopathological alterations in the terminal bronchiole–alveolar duct region. Other than a reduction of RV, these lesions have little measurable impact on the integrated function of the lung as measured by techniques thought to produce results similar to those obtained by clinical assessments of pulmonary function in humans.

It might be conjectured from the present results, but was not proved by this study, that similar types, magnitudes, and distributions of structural changes in the lungs of humans would have similarly little impact on the measured pulmonary function. This study did not address the issue of whether or not similar structural changes would, in fact, occur in humans exposed as were the rats in this study. The interspecies differences in the dosimetry of ozone in the lower respiratory tract (Gerrity and Wiester 1987), and the fact that rats are obligatory nose breathers while humans are not, make it difficult to predict structural or functional changes in humans from the changes observed in rats. It seems likely that humans who undergo long-term exposure to ozone, in combination with other pollutants, and incur a spectrum of lung insults during their life spans would probably have a complex of lung structural changes that would not precisely parallel the changes observed in the short-lived, infection-free, singly exposed rats evaluated in this study.

It is difficult to infer potential adverse health effects in humans exposed chronically to ozone from the reduction in RV observed in the rats in this study. Concern for changes in the RV of humans with obstructive lung disease usually centers on increases in RV, rather than decreases. Reductions in the RV of humans usually accompany reductions in other lung volumes in restrictive lung disease. There is no known clinical precedent for a reduction of RV as an isolated finding. If the RV of the rats was reduced because of a slight stiffening of the terminal bronchiole–alveolar duct junction, as speculated, one might also speculate that chronic ozone exposure could cause deep lung fibrosis in humans. Because of differences in structure between the terminal airways of humans and rats, it is not clear that a reduction of RV would occur at some point in the development of fibrosis in humans, as it apparently did in rats. If, at some stage, a modest reduction of RV did occur as an isolated functional change in humans, it is not clear that the impact on ventilation or gas exchange would be adverse, nor that it would be detected in population studies.

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## APPENDIX A. Identification of Specific Animals in the Exposure Groups

**Table A.1.** Specific Animals Studied

| Ozone Exposure (ppm) | Gender | Identification Numbers for Specific Animals |
|---|---|---|
| 0 | Male | H1, H7, H16, H53, H61, H85, H133, H149, H161 |
| 0.12 | Male | H54, H62, H134, H150 |
| 0.5 | Male | H2, H8, H17, H23, H55, H63, H87, H163 |
| 1.0 | Male | H3, H9, H18, H24, H56, H88, H136 |
| 0 | Female | H4, H10, H13, H19, H57, H65, H89, H137, H157 |
| 0.12 | Female | H58, H66, H138, H154 |
| 0.5 | Female | H5, H11, H14, H20, H59, H67, H91, H139, H155 |
| 1.0 | Female | H6, H12, H15, H21, H60, H68, H92, H140, H156, H160 |

## ABOUT THE AUTHORS

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**Joe L. Mauderly,** D.V.M., received a doctorate in veterinary medicine from Kansas State University in 1967, and served as a laboratory animal veterinarian and physiologist for the U.S. Air Force at the U.S. Army Natick Laboratories, Natick, MA. He joined the Inhalation Toxicology Research Institute in 1969 and held a succession of research and management positions before becoming Director in 1989. He is currently president of the Lovelace Biomedical and Environmental Research Institute and Director of the Inhalation Toxicology Institute. Dr. Mauderly's primary research interests are the effects of inhaled toxic materials on the structure and function of the lung, relationships between lung lesions and respiratory functional changes, pulmonary carcinogenesis from inhaled particles, and the extrapolation of toxicological findings in laboratory animals to estimates of health risk for humans.

## PUBLICATIONS RESULTING FROM THIS RESEARCH

Harkema JR, Mauderly JL. 1995. Pulmonary function alterations in F344 rats following chronic ozone inhalation. In: Tropospheric Ozone: Critical Issues in the Regulatory Process. Air & Waste Management Association, Pittsburgh, PA (Proceedings of the Meeting of the Association, May 11–13, 1994, Orlando, FL). In press.

## ABBREVIATIONS

| | |
|---|---|
| $C_{dyn}$ | dynamic lung compliance |
| $D_{CO}$ | carbon monoxide diffusing capacity of the lung |
| ERV | expiratory reserve volume |
| EPA | U.S. Environmental Protection Agency |
| FRC | functional residual capacity |
| FVC | forced vital capacity |
| HEI | Health Effects Institute |
| MMEF | mean midexpiratory flow |
| NTP | National Toxicology Program |
| PEFR | peak expiratory flow rate |
| ppm | parts per million |
| $P_{tp}$ | transpulmonary pressure |
| $r$ | correlation coefficient |
| RV | residual volume |
| TLC | total lung capacity |
| VC | vital capacity |

## INTRODUCTION

Since the midnineteenth century, we have known that ozone is a powerful lung irritant (reviewed by Bates 1989). However, only in recent decades have the health effects of exposure to atmospheric oxidants been systematically measured (reviewed by the U.S. Environmental Protection Agency 1986, 1988, 1993; Lippmann 1989, 1992, 1993). When young adults are exposed to elevated levels of ozone for relatively short time periods while they exercise, lung function is transiently reduced and markers of inflammation appear in bronchoalveolar lavage fluid (Spektor et al. 1988a; Lippmann 1989). Whether or not repeated inhalation of ozone produces long-term effects on lung function, which may cause or aggravate chronic lung disease, is unknown.

In light of widespread exposure to ozone and the great uncertainty regarding the possible health risks of chronic exposure to this pollutant, the Health Effects Institute (HEI)* and the National Toxicology Program (NTP) undertook a collaborative project with laboratory animals to evaluate the effects of prolonged exposure to low, medium, and high concentrations of ozone. The Health Effects Institute funded several related studies that evaluated the biochemical, structural, and functional changes caused by prolonged ozone exposure in the respiratory tracts of rats. The data from the pulmonary function testing described in this report by Drs. Harkema and Mauderly constitute one facet of this integrative, multiinvestigator approach designed to improve our understanding of the effects of chronic exposure to ozone in humans.

In February 1990, the HEI issued a Request for Applications (RFA 901) that solicited proposals for studies on the "Health Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program–Health Effects Institute Studies, Part A: Respiratory Function Studies." Proposals submitted in response to this RFA were reviewed by an ad hoc review panel for scientific merit and by the HEI Research Committee for their contribution to a well-balanced program. A companion RFA (Part B), was subsequently issued in 1990 to seek applications for studies of structural, biochemical, and other changes in the animals from the NTP/HEI collaboration.

In response to RFA 90-1, Part A, Dr. Jack R. Harkema and colleagues of the Lovelace Biomedical and Environmental Research Institute submitted a proposal, entitled "Respiratory Function Alterations Following Chronic Ozone Inhalation in Rats." The proposed experiments were designed to measure ozone exposure in anesthetized rats and the effects on pulmonary function that are dependent on concentration. The one-year project began in September 1991 and total expenditures were $82,036. The Health Effects Institute received the Investigators' Report for review in August 1993. A revised report was received in April 1994 and was accepted by the Health Review Committee in July 1994.

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 following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective. A forthcoming Integrative Summary Report (Part X of Research Report Number 65) will summarize the biochemical, structural, and functional data, and discuss their implications for human health.

## REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission 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." Sections 202(a), (b)(1), (g), and (h), and Sections 207(c)(4), (5), and (6) impose specific requirements for reduction in motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide EPA with limited discretion to modify those requirements.

Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current NAAQS for ozone is 0.12 parts per million (ppm). This standard is exceeded when more than one day per year has a maximum hourly average concentration of ozone above 0.12 ppm. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

Section 109 of the Clean Air Act also requires periodic review and, if appropriate, revision of the NAAQS and of the air quality criteria on which they are based. The EPA completed its last formal review of the air quality criteria for ozone in 1989. Based on that review, the EPA announced a final decision on March 9, 1993, not to revise the existing ozone NAAQS. However, since 1989, a substantial number of new studies on the health and environmental effects of ozone have appeared in the peer-reviewed literature. As a result, on February 3, 1994, the EPA announced its intention to review the Air Quality Criteria for Ozone and Other Photochemical Oxidants as rapidly as possible. The EPA will then complete its scientific review of the NAAQS for ozone, consistent with assuring a sound, scientifically supportable decision concerning revision of the standard. Research into the health effects of ozone, like that described in this report, forms the basis of and is essential to the informed regulatory decisionmaking required by the Clean Air Act.

## SCIENTIFIC BACKGROUND

Ozone is the major pollutant in photochemical smog. It is formed by complex photochemical reactions between nitrogen oxides and volatile organic compounds in the presence of sunlight. Motor vehicle and industrial emissions are prominent sources of these compounds (U.S. Environmental Protection Agency 1991b). Peak atmospheric ozone concentrations generally occur during the summer months because the photochemical reactions that produce ozone are enhanced by sunlight and high temperature. Ozone exposure is a major health concern because it is a highly reactive gas that, at sufficiently high concentrations, can injure cells and tissues (U.S. Environmental Protection Agency 1986, 1991b).

The current NAAQS for ozone (0.12 ppm) was established largely on the basis of the results of studies of short-term or acute exposure in human subjects. In 1990, daily one-hour maximum ozone levels ranged from 0.06 ppm in less polluted areas of the United States to 0.3 ppm or higher in summer in the Los Angeles basin (U.S. Environmental Protection Agency 1991a). Peak ozone concentrations of 0.1 ppm and higher, lasting for 8 to 12 hours, have been reported in both the United States and Europe. This exposure pattern can continue for several days during a summer air pollution episode (Rombout et al. 1986; Van Bree et al. 1990). Therefore, in the United States, approximately 50 million residents live in areas in which the current ozone standard is exceeded (U.S. Environmental Protection Agency 1993).

The remaining sections of this scientific background summarize what is known about the effects of ozone on pulmonary function because this was the focus of the research described in this report. The first section is a brief discussion of the effects of ozone on human pulmonary function as measured in studies of controlled exposure to ambient air levels, and from field and epidemiologic studies. The second section is a review of the effects of ozone on pulmonary function in laboratory animals from both short-term and long-term exposures.

### HUMAN STUDIES

The available data about the adverse effects of ozone in humans are based on controlled exposures to ambient air levels, and on epidemiologic and field studies. Studies of controlled exposures typically use fixed concentrations of ozone under carefully regulated environmental conditions; field and epidemiologic studies typically examine variable concentrations of ozone under realistic exposure conditions that include copollutants such as fine particles, acid aerosols, nitrogen dioxide, and allergens.

In studies of controlled exposures to ambient air ozone levels, short-term exposures have been shown to produce a variety of pulmonary responses, including decreased inspiratory capacity, increased breathing frequency, nose and throat irritation, mild bronchoconstriction, airway hyperresponsiveness, cough, and subjective symptoms of pain on deep inspiration and shortness of breath (U.S. Environmental Protection Agency 1986; Lippmann 1989, 1992, 1993). Specifically, tests of pulmonary function in exposed individuals show a reduction in measurements of forced vital capacity (the amount of air that a subject can forcibly exhale in a single effort, after fully inflating the lungs), total lung capacity, and forced expiratory volume in one second ($FEV_1$; the volume of air that a subject can forcibly exhale during the first second of exhalation after fully inflating the lungs). The decrease in $FEV_1$ may be related to a reduced ability of subjects to inhale deeply due to a neural response to the inhaled pollutant (Bates 1989).

It is interesting to note that most subjects exposed to ozone for brief periods (one to six hours) on several consecutive days show a reduced response to ozone after three to five days of exposure (Hackney et al. 1977; U.S. Environmental Protection Agency 1986). The mechanisms by which the lungs adapt are unknown.

An extensive data base on pulmonary function responses to ozone exposure comes from field and epidemiologic studies of children who were exposed to elevated levels of ambient ozone while attending summer camps (Spektor et al. 1988a; Raizenne et al. 1989; Higgins et al. 1990; Avol et

al. 1991; Spektor et al. 1991). In these studies, $FEV_1$ decreased in response to ozone concentrations of up to 0.25 ppm. However, because other pollutants in addition to ozone were present in the ambient atmosphere, these results must be interpreted with caution. Decreases in peak expiratory flow rate (the highest rate at which a subject exhales gas or air) also have been reported to occur with higher concentrations of ambient ozone, especially in children with asthma (Lebowitz et al. 1991; Thurston et al. 1992). Two studies in which lung function was measured in adults before and after 30 minutes of exercise in the presence of up to 0.14 ppm ozone also have shown a decrease in $FEV_1$ (Selwyn et al. 1985; Spektor et al. 1988b).

For ethical and practical reasons, experimentally exposing humans to ozone for prolonged periods is not possible. Therefore, epidemiologists are faced with the problem of distinguishing the effects of ozone from the effects of other air pollutants. For this reason, animals have been used to investigate the pathophysiological mechanisms associated with prolonged ozone inhalation.

## ANIMAL STUDIES

### Short-Term Exposures

Most animal species exhibit rapid and shallow breathing in response to ozone challenges of less than one day (reviewed by U.S. Environmental Protection Agency 1986). For example, Amdur and colleagues (1978) showed that breathing frequency increased in guinea pigs exposed to ozone (0.2, 0.4, or 0.8 ppm) for two hours. Mautz and Bufalino (1989) measured breathing frequency and tidal volume, a lung volume related to the level of breathing, in unanesthetized rats exposed to ozone (0.2, 0.4, 0.6, or 0.8 ppm) for three hours. Increases in breathing frequency were correlated to the concentration of ozone, and were significantly different from control animals exposed to 0.4 ppm, and a maximal response was observed in animals exposed to 0.6 ppm. Values from the volume of air expired per minute decreased only at 0.6 and 0.8 ppm. Tepper and colleagues (1990) exposed unanesthetized rats to ozone (0.12, 0.25, 0.5, or 1.0 ppm) for 2.25 hours with a breathing regimen stimulated by carbon dioxide to mimic the use of an exercise regimen that is often used in human studies. Again, breathing frequency increased and tidal volume decreased between 0.25 and 1.0 ppm ozone, but volume of air expired per minute did not decrease. A similar pattern has been observed in dogs trained to run on a treadmill, exposed to 1.0 ppm ozone for two hours, and evaluated either 1 or 24 hours after exposure (Lee et al. 1979, 1980; Sasaki et al. 1987).

A few investigators have tested the effects of exposing laboratory animals to ozone repeatedly over a period of three to five days (U.S. Environmental Protection Agency 1986). Tepper and colleagues (1989) showed that the initial increased breathing frequency was attenuated after five consecutive days of ozone exposure in rats treated with an exposure protocol similar to that used in the repeated exposure studies of human subjects. Exposures were for 2.25 hours and included the challenge with carbon dioxide to mimic exercise. Functional changes were largest on days 1 and 2 with attenuation after two days; however, functional attenuation did not occur in the 1.0-ppm group. In a study conducted at the General Motors Research Laboratories in rats exposed to 0.7 ppm ozone for 20 hours/day for 28 days, Gross and White (1986) showed a significant reduction in forced expiratory volume, lung volume, and carbon monoxide diffusing capacity, as well as a significant increase in functional residual capacity, the amount of air remaining in the lung at the end of a tidal breath). These investigators interpreted their findings as indicating the presence of an obstructivetype lung lesion in ozonetreated animals. However, these effects were largely reversed after a nine-week recovery period in clean air.

### Prolonged Exposures

Several one- to two-year studies of prolonged ozone exposure using rodents have been conducted to address the issue of cumulative exposure over long time periods. The results of pulmonary function measurements from these studies are summarized in Table 1.

Gross and White (1987) demonstrated increases of approximately 10% in residual volume and functional residual capacity and a 7% decrease in the lung's carbon monoxide diffusing capacity in F344/N rats exposed to 0.5 ppm ozone for 20 hours/day, 7 days/week for one year. These measurements returned to normal after three months of breathing clean air. A second set of studies of prolonged ozone exposure, conducted at the U.S. EPA Health Effects Research Laboratories, was designed to mimic daily urban exposure to ozone (Grose et al. 1989; Tepper et al. 1991). F344/N rats were exposed for 12 or 18 months to a base ozone concentration of 0.06 ppm for 13 hours/day, 7 days/week, upon which was superimposed an ozone spike that reached 0.25 ppm for 9 hours/day, 5 days/week (resulting in a 9-hour time-weighted average of 0.19 ppm for 5 days/week). When pulmonary function measurements were made on anesthetized animals, the investigators noted a decrease in residual volume and total lung capacity (Grose et al. 1989). A cohort of unanesthetized animals exposed by Tepper and colleagues (1991) showed decreased breathing frequency and increased expiratory resistance.

**Table 1.** Effects of Prolonged Ozone Exposure on Pulmonary Function in F344/N Rats

| Ozone Concentration (ppm) | Exposure Duration | Anesthesia | Number of Measured Parameters | Statistically Significant Observations | Reference |
|---|---|---|---|---|---|
| 0.5 | 20 hrs/day, 7 days/wk for 12 months | Yes | 10 | Residual volume increased 11%<br>Functional residual capacity increased 7%<br>Carbon monoxide diffusing capacity decreased 7% | Gross and White 1987 |
| 0.06 base with 0.25 spike | 13 hrs/day, 7 days/wk; 9 hrs/day, 5 days/wk for 12 months | Unknown | 8 | Total lung capacity decreased 4%<br>Residual volume showed trend toward decrease (approximately 9%) | Grose et al. 1989 |
| 0.06 base with 0.25 spike | 13 hrs/day, 7 days/wk; 9 hrs/day, 5 days/wk for 18 months | No | 9 | Expiratory resistance increased 100%<br>Breathing frequency decreased 10% | Tepper et al. 1991 |
| 0.06 base with 0.25 spike | 13 hrs/day, 7 days/wk; 9 hrs/day, 5 days/wk for 18 months | Yes | 17 | Residual volume decreased 19%<br>Total lung capacity decreased 2% | Costa et al. 1994 |

In summary, studies on pulmonary function have produced somewhat conflicting results. Thus, it is still not known whether or not repetitive daily or intermittent exposures to ozone over the course of an animal's lifetime produce cumulative damage and lasting deficits in lung function, or if they alter airway structure; nor are dose-response data available. In the study presented in this Research Report, Drs. Harkema and Mauderly tested the hypothesis that prolonged ozone exposure affects pulmonary function in F344/N rats. The availability of animals from the NTP/HEI Collaborative Ozone Project allowed the investigators to examine these events in animal lungs exposed to ozone at levels equivalent to the current NAAQS and higher, and ultimately to correlate their results with the findings of other investigators in the NTP/HEI Collaborative Ozone Project.

## JUSTIFICATION FOR THE STUDY

The primary objectives for RFA 90-1, as issued by HEI, were to support biochemical, structural, and functional studies to determine whether or not prolonged inhalation of ozone caused changes in the respiratory system of rats that might potentially be related to chronic lung disease in humans. Drs. Harkema and Mauderly had extensive experience in measuring pulmonary function in animals, and proposed to examine pulmonary function in anesthetized rats.

Pulmonary function testing in laboratory animals is a sensitive procedure for detecting lung injury and physiologic changes related to disease. In humans, carefully executed pulmonary function testing is critical to the correlation of lung changes in a variety of clinical and subclinical disease states. In fact, alterations in pulmonary function are commonly seen in humans who have been exposed to ozone. Therefore, the availability of benchmark data on pulmonary function in this rat cohort was considered to be essential to the overall interpretation of the NTP/HEI studies.

## OBJECTIVES AND STUDY DESIGN

Drs. Harkema and Mauderly proposed to investigate the effects of a 20-month exposure to ozone on the pulmonary function of 65 male and female F344/N rats that had been exposed to ozone (0.12 ppm, 0.5 ppm, or 1.0 ppm) for 7

hours/day for 5 days/week. Their goal was to provide data on the nature and magnitude of pulmonary impairment that may be associated with chronic exposure to ozone. They performed a battery of pulmonary function tests on groups of 8 to 17 rats anesthetized with halothane within one to six days after the 20-month exposure ceased. Another group of 18 rats that had been exposed to filtered air free of ozone served as the control group. Each of the tested groups was nearly evenly divided between males and females.

The inhalation component of this project was conducted in compliance with the NTP health and safety regulations, and with the Food and Drug Administration Good Laboratory Practice Regulations. Animal care and use were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

## TECHNICAL EVALUATION

### ATTAINMENT OF STUDY OBJECTIVES

The investigators successfully accomplished the primary objective of their study, which was to complete a comprehensive battery of pulmonary function tests on rats exposed to air and the three concentrations of ozone in the NTP/HEI Collaborative Ozone Project. To accomplish this objective, the investigators transported their equipment for measuring pulmonary function to Battelle Pacific Northwest Laboratories in Richland, WA, where the ozone exposures had taken place.

### ASSESSMENT OF METHODS AND STUDY DESIGN

Within one to six days after the ozone exposure ceased, the animals were anesthetized with halothane so that lung function could be measured. The investigators used methods with which they have extensive experience, including inserting endotracheal and intraesophageal catheters into the anesthetized rats. They also used pneumotachographs, a body plethysmograph, and mechanical systems to inflate and deflate the lungs to known pressures, and to inflate the lungs with oxygen or other test gases in order to obtain data for other functional measurements.

This experienced team of investigators expertly performed the pulmonary function tests on these small animals. The study yielded data on respiratory rate, tidal volume, lung volume, maximal expiratory flow, dynamic compliance, respiratory resistance, single-breath carbon monoxide diffusing capacity, and the slope of phase three of the single-breath nitrogen washout. As noted by the investigators, general anesthesia can modify ventilatory responses; however, only the measurements of respiratory frequency and tidal volume were likely to have been affected by the anesthesia (Costa et al. 1992).

### STATISTICAL METHODS

Multiple pairwise comparisons were used to determine the significance of exposure effects. Data from exposed animals were compared with data from control animals for males and females separately and for both genders combined. Pooled variances and the Bonferroni adjustment for multiple comparisons were used. Dunnett's test was not used because the size of the exposed group was sometimes larger than that of the control group.

Linear regression analyses used gender and the concentration of ozone as explanatory variables to test for a response to ozone that correlated with the administered dose. Gender was the categorical variable and ozone concentration was the continuous variable. No significant interaction between gender and ozone concentration was found. A retrospective power calculation used the estimate of the standard error of the regression model.

### RESULTS AND INTERPRETATION

The investigators found that the 20-month exposures to ozone produced minimal alterations in lung function. At the level of statistical significance defined by the investigators ($p = 0.05$), the only statistically significant findings were 40% decreases in residual volume and in residual volume/total lung capacity in female rats exposed to 0.5 ppm; these percentages reflect a change from 2.0 mL to 1.2 mL in residual volume. Although this reduction is small in absolute terms, it could have important human health consequences, when extrapolated from rats and considered over the course of a human lifetime.

The investigators hypothesized that the observed decrease in residual volume might be attributable to fibrotic changes in the terminal airways resulting in increased stiffness, which allowed more air to be expelled from the gas exchange region during exhalation before these small airways closed. However, this finding was limited to female rats exposed to 0.5 ppm, and measurements of lung compliance gave no indication of increased lung stiffness. The investigators suggested that the focal tissue stiffening induced by ozone exposure was not sufficiently severe or widespread to influence total lung elastic recoil by a measurable amount. Furthermore, they suggested that no changes in residual volume were noted in the animals exposed to 1.0 ppm ozone because the increased stiffness that maintained the patency of the walls of the terminal airways was offset by a narrowing of the lumens caused by wall thickeningin these same airways.

Although quantitative correlations of structure and function were not examined in this study, Drs. Harkema and Mauderly noted that the observations of other NTP/HEI investigators provide some support for their interpretations. Using morphometric techniques, Pinkerton and colleagues (1993) found that the alveolar ductal region showed a bronchiolized epithelium that extended further into the alveolar septal tips. Also, rats exposed to 1.0 ppm ozone had moderate to marked levels of stainable centriacinar collagen when compared with control rats, but rats exposed to 0.5 ppm ozone varied in the amount of stainable centriacinar collagen measured (Last et al. 1994). Although these findings support the hypothesis of an exposure-related stiffening of the terminal bronchiole–alveolar duct region, they still do not explain why the residual volume values were unchanged in the group exposed to 1.0 ppm ozone.

The investigators appropriately note in their report that the alterations in pulmonary function observed in the rats exposed to ozone may not reflect what occurs in humans who are similarly exposed. Such disparities are based on confounding factors that frequently arise in extrapolating toxicologic data from animals to humans, such as differences in susceptibility to the toxic agent, breathing patterns, and respiratory tract geometry. For example, rats are obligate nose-breathers, whereas many humans are mouth-breathers. This difference can affect the dose of ozone delivered to the lower airways and gas exchange region and the extent of lung responses.

The investigators also properly caution that the effects of ozone exposure in humans may not be as benign as those observed in the rats because the duration of tissue exposure is much longer in the course of a human lifetime. In addition, human exposures to ozone occur in association with other insults to the lungs, including other air pollutants and tobacco smoke, and in the presence of existing lung disease. These factors could be important contributors to the extent and severity of the responses of the lungs to ozone exposure.

## REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

This was a carefully conducted study that generated a large volume of pulmonary function data, the great majority of which showed no change in animals exposed to ozone. In isolation, the change observed in residual volume is difficult to interpret because it did not vary as dose varied, and because it was limited to female rats at a dose of 0.5 ppm ozone. However, our understanding of the change in residual volume is enhanced by data from the other NTP/HEI studies that show some structural changes that correlate with these functional changes.

The results of the NTP/HEI Collaborative Ozone Project, and of this study in particular, suggest several avenues of future research. Because the rats were evaluated at only one point in time at the end of 20 months of ozone exposure, a study of the response to ozone over time would probably contribute greatly to our understanding of the physiologic and pathologic responses to ozone, especially given the data that show an adaptationlike response to ozone exposure over time. Also, our understanding of species differences in response to ozone remains incomplete, yet it is critically important for interpreting results and evaluating the implications for human health effects. For example, more ozone uptake occurs in the nose in rats than in humans. Future studies could address this and other potentially important species differences between humans and this laboratory animal. Finally, the NTP/HEI studies deal only with exposure to ozone; they do not address the issue of coincident insults from cigarette smoke and other pollutants. Experiments could be designed to study the effects of prolonged exposure to multiple pollutants.

## CONCLUSIONS

The investigators found that 20month exposures to 0.12, 0.5 or 1.0 ppm ozone produced minimal changes in the pulmonary function of F344/N rats. The only statistically significant effects were observed in female rats exposed to 0.5 ppm; these animals exhibited a decrease in residual volume and residual volume / TLC. The fact that this finding was limited to female rats does not have an obvious biological explanation.

The pathophysiologic and biological significance of these findings is unclear when the findings are viewed in isolation. However, the NTP/HEI Collaborative Ozone Project was designed to measure a variety of endpoints in order to form some generalized, comprehensive conclusions about ozone exposure in rats. As such, the data from this functional study are essential for subsequent correlations with data from structural and biochemical studies conducted by other investigators from the NTP/HEI Collaborative Ozone Project. Integration and careful synthesis of all of these data will provide a clearer and more comprehensive understanding of the effects of chronic ozone exposure on the pulmonary system and its potential implications for lifetime exposures of humans.

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