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Chemical Characterization and Toxicologic Evaluation of Airborne Mixtures

Inhalation Toxicology of Diesel Fuel Obscurant Aerosol In Sprague-Dawley Rats

FINAL REPORT,
PHASE 2, REPEATED EXPOSURES

Walden Dalbey, Ph.D.
Simon Lock, Ph.D.
Richard Schmoyer, Ph.D.

JULY, 1982

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A series of repeated exposures of rats to aerosolized diesel fuel was performed to help establish (1) indices of potential toxicity resulting from aerosol exposure and (2) the relative importance of duration of exposures, the frequence of exposures, and aerosol concentration in the induction of observed lesions. Twelve groups of animals (24 per sex in each group) were exposed to combinations of exposure frequency (1 or 3 exposures/week), exposure duration (2 or 6 hours) and aerosol concentration (expressed as the product of concentration × time for convenience, with values of 0, 8, or 12 mg h/L). Each group received

9 exposures. Body weight and food consumption were recorded on a weekly basis. Assays were performed on selected animals within 1-2 days after the last exposure or after 2 weeks without exposure. Endpoints included number and phagocytic activity of pulmonary free cells, pulmonary function tests, neurotoxicity assays, clinical chemistry, organ weights, and histopathology. Data were analyzed by analysis of variance.

After exposure, the primary target organ was the lungs. Focal accumulations of pulmonary free cells were observed in the lung parenchyma, associated with thickening and hypercellularity of alveolar walls. The number of lavaged pulmonary free cells correlated well with histologic observations, remaining elevated after two weeks without exposure. Lung volumes were altered by exposure, including increased FRC, decreased TLC, and decreased VC. Carbon monoxide diffusing capacity was decreased in several exposed groups also. None of the more systemic changes observed were considered to be of biologic significance, even though the exposure conditions were considered to result in a maximum tolerated dose. Frequency of exposure was the dominant variable over the range of parameters used in this study, 3 exposures/wk being more deleterious than 1/week. Variation in duration of exposure appeared to have very little effect and a "dose"-response was often not apparent with differences in concentration.

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INHALATION TOXICOLOGY OF DIESEL FUEL OBSCURANT AEROSOL IN SPRAGUE-DAWLEY RATS

FINAL REPORT, PHASE 2, REPEATED EXPOSURES

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EXECUTIVE SUMMARY

A series of repeated exposures of rats to aerosolized diesel fuel was performed to help establish (1) indices of potential toxicity resulting from aerosol exposure and (2) the relative importance of duration of exposure, frequency of exposure, and aerosol concentation in the induction of abnormalities. Twelve groups of rats (24 per sex in each group) were each exposed to a combination of exposure frequency (1 or 3 exposures/week), exposure duration (2 or 6 hours), and aerosol concentration (expressed as the product of concentration and time for convenience, with values of 0, 8, or 12 mg•hr/L). Each group received 9 exposures regardless of frequency of exposures.

Body weight and food consumption were measured over the course of exposures and a subsequent 2 week period without exposures. Assays performed within the first two days after the last exposure and two weeks later included number and phagocytic activity of pulmonary free cells, a series of pulmonary function and neurotoxicity tests, clinical chemistry, hematology, organ weights, and histopathology. Analysis of variance, with compensation for block effect resulting from three separate shipments of rats, was used to compare data derived from the various endpoints.

Some of the exposure conditions employed in this study were considered to result in a maximum tolerated dose, based on mortality among groups exposed to 12 mg•hr/L and loss of body weight with 3 exposures/week. Under these exposure conditions, the lung was the primary site of toxicity. Focal accumulations of pulmonary free cells were observed histologically in the lung parenchyma, with associated thickening and hypercellularity of nearby alveolar walls. The number of lavaged pulmonary free cells increased similarly, being elevated by 2 days postexposure and remaining above control values after 2 weeks without exposure. Pulmonary wet weight and dry weight were increased by exposure. The wet/dry ratio was constant, indicating that the weight increase was predominantly cellular rather than a result of fluid accumulation. Lung volumes were altered by exposure, including increased functional residual capacity and vital capacity. Carbon monoxide diffusing capacity was decreased in several exposed groups.

Systemic changes observed included a decreased liver weight, even though no abnormalities were observed histologically, and the number of circulating red blood cells decreased by about 13 percent among groups exposed 3 times per week, but not in groups exposed once per week. These changes were not considered to be of immediate biologic significance, although they will be investigated in the final, subchronic, phase of the inhalation toxicology study.

The second objective of this study was to examine the relative importance of concentration, duration of exposure, and frequency of exposure. It was found that frequency appeared to be the dominant variable over the range of parameters used in this study. Those biologic endpoints affected by exposure were generally more severely altered among groups exposed 3 times per week. Duration of exposure appeared to have very little effect

and a "dose"-response was often not evident with differences in concentration.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Uses of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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TABLE OF CONTENTS

	Page
EXECUTIVE SUMMARY	1
FOREWORD	3
LIST OF FIGURES	7
LIST OF TABLES	9
INTRODUCTION	13
MATERIALS AND METHODS	14
Experimental Design	14
Exposure Methods	16
Observations During Exposure	16
Pulmonary Free Cells	17
Clinical Chemistry	17
Pulmonary Function Tests	20
Organ Weight and Histopathology	23
Neurotoxicity Assays	24
RESULTS	26
Aerosol Concentration	26
Mortality	26
Body Weight	26
Food Consumption	30
Pulmonary Free Cells	30
Pulmonary Function Tests	33
Organ Weights	46
Histopathology	48
Neurotoxicity	48
Clinical Chemistry	51
DISCUSSION	53
LITERATURE CITED	57
APPENDIX A: STATISTICAL CONSIDERATIONS IN EXPERIMENTAL DESIGN AND ANALYSIS	59
Experimental Design	59
Analysis of Data	60
Weight Study	61
Feeding Study	62
Neurotoxicity Data	62
Clinical Chemistry Assays	63
Lung Lavages	63
Pulmonary Function	64
APPENDIX B: EXPOSURE AND ASSAY SCHEDULE	65

APPENDIX C: DEFINITIONS AND TABULATED DATA 67

APPENDIX D: HISTOPATHOLOGY 85

PERSONNEL 92

PUBLICATIONS 93

DISTRIBUTION LIST 95

LIST OF FIGURES

Page

1. Design of experimental matrix showing exposure groups (at intersections of lines) with varying combinations of frequency of exposure, exposure duration, and either (A) Ct product or (B) aerosol concentration 15

2. Body box used for pulmonary function tests. 18

3. A. Body box configuration during measurement of resistance. Note esophageal cannula connected to differential pressure transducer and similar transducer on box to measure air flow through pneumotachograph 19

   B. Body box configuration during multibreath nitrogen washout maneuver. Either air or oxygen flow past opening to tracheal cannula and N_2 analyzer. Lungs are inflated while solenoid (S) is closed. 19

   C. Plethysmograph configuration during measurement of FRC. Opening (A) is occluded at end-expiration. Changes in tracheal pressure and lung volume (plethysmograph pressure) are recorded as animal breathes against closure 19

   D. Plethysmograph configuration during quasistatic pressure-volume curve. Lung volume changes are recorded from pressure changes in plethysmograph as lungs are inflated and deflated by syringe. Transpulmonary pressure is difference between esophageal and tracheal pressures. Lungs are taken to -30 cm HOH by pressure reservoir on opposite side of solenoid (S) 22

   E. Body box configuration during maximal forced exhalation maneuver. Lungs are inflated by pressure reservoir at 30 cm HOH and then connected by solenoid (S) to subatmospheric reservoir for deflation. 22

   F. Body box configuration during measurement of single-breath CO diffusing capacity. 22

4. Schematic diagram of apparatus used for startle reflex assay. . . 25

5. Cumulative mortality over course of exposures among groups receiving 12 mg·hr/L. 27

6. Mean body weight of animals during exposure to aerosolized diesel fuel once per week. Exposures ended after the ninth week. 28

7. Mean body weight of animals during exposure to aerosolized diesel fuel three times per week. Exposures ended after the third week. 29

8. Mean weekly food consumption of animals during exposure to aerosolized diesel fuel once per week. Exposures ended after the ninth week. 31

9. Mean weekly food consumption of animals during exposure to aerosolized diesel fuel three times per week. Exposures ended after the third week. 32

10. Percent of macrophages associated with given number of yeast in phagocytosis assay. Arrow marks binding index or average number of yeast associated with a macrophage 34

11. Mean multibreath nitrogen washout curves for males of control group EA (○) and exposed group FA (●). End-tidal N_2 concentration is plotted against breath number. 36

12. Mean multibreath nitrogen washout curves for males of control group EA (○) and exposed group FA (●). End-tidal N_2 concentration is plotted against cumulative dilutions of FRC. . . 37

13. Quasistatic pressure-volume curves of lungs from males in control group HA (○) and exposed group (GA (●) at 2 weeks postexposure. Lung volumes shown are total lung capacity (TLC), vital capacity (VC), inspiratory capacity (IC), functional residual capacity (FRC), residual volume (RV), and expiratory reserve volume (ERV) 38

LIST OF TABLES

Page

1. Least squares means of total lung capacity (TLC) vs. Ct product, frequency, sex, and time postexposure 40

2. Least squares means of vital capacity (VC) vs. Ct product frequency, sex, and time postexposure 41

3. Interaction of vital capacity, Ct product, and time post-exposure 41

4. Least squares means of inspiratory capacity (IC) vs. Ct product, frequency, sex, and time postexposure 42

5. Least squares means of functional residual capacity (FRC) vs. Ct product, frequency, sex, and time postexposure 43

6. Least squares means of residual volume (RV) vs. Ct product, frequency, sex, and time postexposure 44

7. Least squares means of specific compliance (C_{sp}) vs. Ct product, frequency, sex, and time postexposure 45

8. Least squares means of diffusing capacity and diffusing capacity/TLC vs. Ct product, sex, and frequency 46

9. Least squares means of weight of right middle lung lobe vs. Ct product and recovery period 47

10. Least squares means of change from pre-exposure value of forelimb grip strength vs. Ct product and time postexposure . . 49

11. Least squares means of change from pre-exposure value of landing foot spread vs. Ct product and time postexposure 49

12. Least squares means of change from pre-exposure value of tail flick vs. Ct product and time postexposure 50

13. Pre-exposure means and postexposure differences in reaction time in the startle reflex assay (least squares means) 50

14. Pre-exposure means and postexposure differences in peak time in the startle reflex assay (least squares means) 51

15. Pre-exposure means and postexposure differences in peak height in the startle reflex assay (least squares means) 51

16. Least squares means of red blood cell count vs. Ct product and frequency 52

17. Least squares means of alkaline phosphatase activity vs. Ct product 52

18. Mean linear intercept and functional residual capacity 55

1B. Disposition of animals used in study 65

2B. Exposure and assay schedule for phase 2 diesel fuel aerosol exposure 66

1C. Mean aerosol concentration determined gravimetrically (Mean ± S.E.) and target concentration for each treatment group. Values are in mg/L. 68

2C. Least square mean values for body weight (g) differences during first 2 weeks of exposure for males (females) in relation to exposure time (grams). 68

3C. Least square mean values for body weight (g) differences during first 2 weeks of exposure for males (females) in relation to exposure frequency 68

4C. Least square mean values for food consumption (g/wk) during first 3 weeks of exposure for males (females) in relation to Ct product 69

5C. Least square mean values for food consumption (g/wk) during first 3 weeks of exposures for males (females) in relation to frequency of exposure. 69

6C. Millions of alveolar macrophages lavaged from lungs during phase 2 assays (Mean ± S.E.) 70

7C. Millions of pulmonary free cells other than alveolar macrophages lavaged from lungs during phase 2 assays (Mean ± S.E.). 70

8C. Binding index (mean number of yeast/macrophage) in phagocytosis assay of phase 2. (Mean ± S.E.) 71

9C. Pulmonary resistance (cm H_2O/mL/sec) of rats in phase 2 exposure to diesel fuel aerosol (Mean ± S.E.). 71

10C. Negative slopes of linear regression equations for multibreath nitrogen washout curves where y = log (Percent N_2) and x = cumulative dilutions of FRC (Mean ± S.E.) 72

11C. Total lung capacity (mL) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.) 72

12C. Vital capacity (mL) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 73

13C. Inspiratory capacity (mL) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.) 73

14C. Functional residual capacity (mL) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 74

15C. Specific compliance (mL/cm H_2O/mL) of groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 74

16C. Peak flow (mL/sec) in maximal forced expiration maneuver among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. . . . 75

17C. Expiratory flow (mL/sec) at 50 percent of vital capacity during maximal forced expiration maneuver among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 75

18C. Expiratory flow (mL/sec) after exhalation of 75 percent of vital capacity during maximal forced expiration maneuver among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 76

19C. Expiratory flow (mL/sec) at 50 percent of vital capacity during maximal forced expiration maneuver referenced to vital capacity (mL). Values are Mean ± S.E.. 76

20C. Single-breath carbon monoxide diffusing capacity [mL/(mmHg)(min)] among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 77

21C. Single breath carbon monoxide diffusing capacity referenced to total lung capacity (in liters) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. 77

22C. Liver weight (g) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 78

23C. Kidney weight (g) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 78

24C. Weight of adrenal glands (mg) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.). 79

25C. Wet weight of right middle lung lobe (mg) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. 79

26C. Wet/dry weight ratios of right middle lung lobes among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. . . . 80

27C. Severity of focal pneumonitis and associated histological changes among groups in phase 2 of diesel fuel exposures.. . . . 80

28C. Pre-exposure forelimb grip strength (kg) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. 81

29C. Pre-exposure landing foot spread (mm) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. 81

30C. Pre-exposure tail flick reflex (sec) among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. 82

31C. Pre-exposure peak height (gram weight) in startle reflex among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. . . . 82

32C. Pre-exposure peak time (msec) in startle reflex assay among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. . . . 83

33C. Pre-exposure reaction time (msec) in startle reflex assay among groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. . . . 83

34C. Least squares means of clinical chemistry parameters in sham groups in phase 2 of diesel fuel exposures (Mean ± S.E.).. . . . 84

INTRODUCTION

Battlefield smokes and obscurants are valuable tools of the armed forces for defending men, materiel, and installations against observation and bombardment. Because of their ability to degrade the performance of target acquisition and guidance devices, conceal friendly ground maneuver, deceive the enemy, and provide a means of signalling and marking, smokes and obscurants will be widely employed in the event of hostilities, and are increasingly being used in training in order to create realistic battlefield conditions. The U.S. Army Medical Bioengineering Research and Development Laboratory is actively investigating the toxic properties of various smoke/obscurant munitions and systems to estimate their potentials for adversely affecting the performance capabilities of soldiers in combat, for causing immediate or delayed health effects in troops exposed in training and for affecting the health, safety, and comfort of persons engaged in the manufacture of smoke munitions.

One material currently under study is diesel fuel aerosol. Aerosolized diesel fuel is a widely used visual obscurant. When injected into the exhaust manifold of a tactical vehicle, diesel fuel instantly vaporizes, is expelled with the vehicle exhaust, and upon exiting the exhaust system condenses to form a dense white "smoke" which rapidly provides a large and effective screen for the vehicle and supporting troops. Since there is a potentially large population at risk, and because little information is available on the potential health and performance effects of exposure to diesel fuel in this form, a number of studies have been designed to expand the available data base so that appropriate health protection decisions may be made. Inhalation exposures of rodents have been conducted to determine the biologic effects of exposure to variations in aerosol concentration, duration of each exposure, and frequency of exposures.

The first phase in these exposures was a series of acute, range-finding experiments to establish the maximum tolerated concentrations for a given exposure duration (2). The primary reason for conducting the acute exposures was to establish concentrations to be used in phase 2, repeated exposures, the subject of this report. The exposure durations to be used in the repeated exposures were 2 and 6 hours. A duration of 4 hours was also used in the acute exposures to provide more complete exposure-response information for statistical analysis. Information from the first two phases of this study was used to design the exposure regime for the third and final phase, subchronic (13-week) exposures, to be the subject of a future report.

Since concentration and duration of exposure were both variables, we also acquired whatever data were readily obtainable on the relationship of mortality to the Ct product. The multiplication product of concentration (C) of an airborne contaminant and time of exposure (t) has often been used as an index of the "dose" of material delivered to the body and therefore the exposure conditions required for a specific effect (1), although this relation is not always valid and must be used with caution. The applicability of the Ct product to mortality could serve as a guide for its usefulness later in the repeated-exposure studies.

Mortality was found to be highly correlated with the Ct product during single exposures ($p = 0.0001$) and 83 percent of the variation in mortality was explained by the Ct product (2). The Ct product was therefore used to estimate maximum tolerated exposure conditions to be used in repeated exposures. Eight mg•hr/L was the Ct product estimated to be two standard errors below the Ct value resulting in 1 percent mortality in acute exposures. Pilot work indicated that animals tolerated exposure to this level of aerosol well.

A matrix design of repeated exposures in Phase 2 was established using a Ct product of 8 mg•hr/L as a lower exposure regimen and 12 mg•hr/L as an upper level. Exposure variables were the Ct product, frequency of exposures (1 or 3/wk), duration of exposure (2 or 6 hr), sex, and time after last exposure (2 days or 2 weeks). All groups received a total of 9 exposures regardless of frequency of exposures. Thus, groups exposed once per week were treated for 9 weeks; those exposed 3 times per week were assayed after 3 weeks of exposure.

The assays performed on the animals were chosen on the basis of anticipated effects. They included pulmonary free cell number and phagocytic activity, pulmonary function, neurotoxicity, clinical chemistry, blood cell number, organ weight, and histopathology. Assays were performed within 2 days after the last exposure and after 2 subsequent weeks without exposure. This report summarizes observations made during these exposures and data obtained from the assays.

MATERIALS AND METHODS

Experimental Design

Figure 1 summarizes the groups in the experiment. Each of the 12 groups consisted of 48 Sprague-Dawley rats from Charles River (Wilmington, MA) (24 males and 24 females). The exposures were performed in 3 blocks of 4 groups. Statistical considerations in the design and in data analysis are given in Appendix A, with particular attention to compensation for block effects between shipments. A description of exposure schedules, multiple uses of animals, and ages at times of assay is given in Appendix B. Definitions of terms used in the analysis and presentation of data are given in Appendix C.

Once in the inhalation facility, rats were housed individually in hanging, stainless steel, wire mesh cages. Purina rat chow was provided *ad libitum* except during exposures. Water was provided using an automatic watering system. In order to control the possible presence of Pseudomonas aeruginosa the water supply was hyperchlorinated to 16 ppm as it entered the building. The actual chlorine concentration in the water the animals received was in the range of 3-5 ppm; a concentration range that is commonly used in animal facilities to prevent the spread of the bacteria. A 12 hr-on/12 hr-off light cycle was maintained.

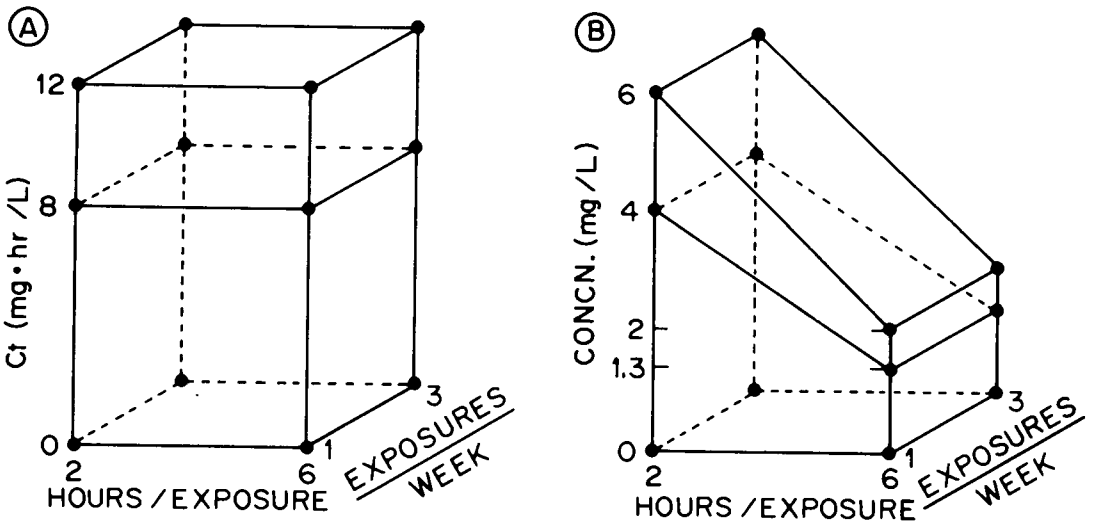

Figure 1

Design of experimental matrix showing exposure groups (at intersections of lines) with varying combinations of frequency of exposure, exposure duration, and either (A) Ct product or (B) aerosol concentration.

Exposure Methods

The exposure chambers and aerosol generation system have been previously described (3). The generator was designed to model the vehicle exhaust system used by the military to produce smoke from diesel fuel. It consisted essentially of a 1-in.-O.D. stainless steel tube about 1 m long with a Vycor heater fitted into one end. This heater was maintained at 600°C. The distal end of the generator was heated to 350°C by a heating tape. Nitrogen entered the end near the Vycor heater and exited at the opposite end of the tube. Diesel fuel was metered onto the tip of the Vycor heater where it was flash vaporized and carried by the hot nitrogen out of the generator and into the air entering the exposure chamber. Aerosol concentration in the chamber was controlled by the rate of flow of fuel into the generator at a constant flow rate of air through the chamber.

Exposures were whole-body and performed in 1.5 m^3 New York University chambers with rats housed individually in 6 tiers within the chamter. Aerosol concentration was monitored continuously by infrared backscatter probes at the top and bottom of the chamber. Particle size was determined routinely by cascade impaction. The mass median diameter was 0.9 - 1.1 μm with a geometric standard deviation of 1.4 - 1.5. Actual size varied slightly with aerosol concentration, as expected with a condensation aerosol at high concentrations. The percent of fuel in the vapor phase also varied with particle concentration but was on the order of 15-20 percent for most of the concentrations employed here. Aerosol distribution within the chamber was uniform (3), and there was no evidence of appreciable particle growth between the top and bottom of the chambers. Periodic filter samples were also taken for gravimetric determination of concentration during each exposure. These filter samples were also analyzed by high pressure liquid chromatography and gas chromatography as part of routine monitoring of stability of the fuel. All fuel was from one shipment of a standard blended fuel (Phillips Petroleum Co.) which has been extensively characterized at our laboratory.

Observations During Exposure

Individual records were kept for all animals. All animals were weighed once per week, on Monday. In addition, food consumption was determined for 12 animals (6 of each sex) in each treatment group. A large supply of food for each animal was kept in a large plastic jar, from which food was taken to supply a hopper on the side of the animal's cage. Once per week food in the hopper was returned to the jar and weighed. This method assumed that loss of food from the hopper other than by eating was uniform across all treatment groups.

Several assays were performed on animals at one to two days and at two weeks after the last exposure. The following sections describe the methods used in those assays.

Pulmonary Free Cells

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and killed by aortic bleeding. The abdomen was opened and the diaphagm cut to collapse the lungs. The trachea was exposed and cannulated with polyethylene tubing (PE205). Lungs were then lavaged with phosphate buffered saline at room temperature; volumes used were 40 percent of the estimated vital capacity based on body weight. The first lavage remained in the lung for 2 minutes before withdrawal; five subsequent washes were performed without waiting between injection and withdrawal.

Lavaged cells were kept on ice, centrifuged twice in refrigerated Hanks' balanced salt solution (HBSS) and resuspended in 6 mL of HBSS. Total alveolar cell and macrophage counts were performed on a hemocytometer. Cell viability was determined by trypan blue exclusion. Cells were diluted to obtain 2×10^5 cells/mL HBSS. In the assay of phagocytic activity, cells were incubated with yeast previously diluted to provide 10^7 individual yeast/mL HBSS. Incubations were done in a vial with a glass coverslip in its bottom and containing 2×10^6 yeast and 2×10^5 cells in 2.3 mL of HBSS. Vials were shaken gently for 60 min at 37°C, and then 30 min were allowed for cells to settle and attach onto the coverslip. The coverslips were gently washed with HBSS 3 times to remove excess yeast. Cells were then fixed with buffered formalin and stained with hematoxylin and eosin.

Coverslips were examined under 400× magnification to count the number of yeast associated with alveolar macrophages. Counts was made of the number of cells with yeast and of the number of yeast per individual macrophage. A limited number of differential counts was also made.

Clinical Chemistry

Before rats were used for lung lavages, blood was taken from them for clinical chemistry. Blood was taken by aortic puncture with a 21 g needle and drawn through a plastic 3-way valve with a 5 mL plastic syringe. The valve was then switched and additional blood was drawn into a heparinized 1 mL plastic syringe. The heparinized blood was used immediately for counts of red and white blood cells on a Fisher Autocytometer. Non-heparinized blood in the larger syringe was stored frozen in liquid nitrogen before being assayed for clinical chemistry parameters 3-5 days later. The following were measured routinely:

alkaline phosphatase	SGOT	cholesterol
triglycerides	uric acid	urea nitrogen
glucose	bilirubin	creatinine
sodium	potassium	

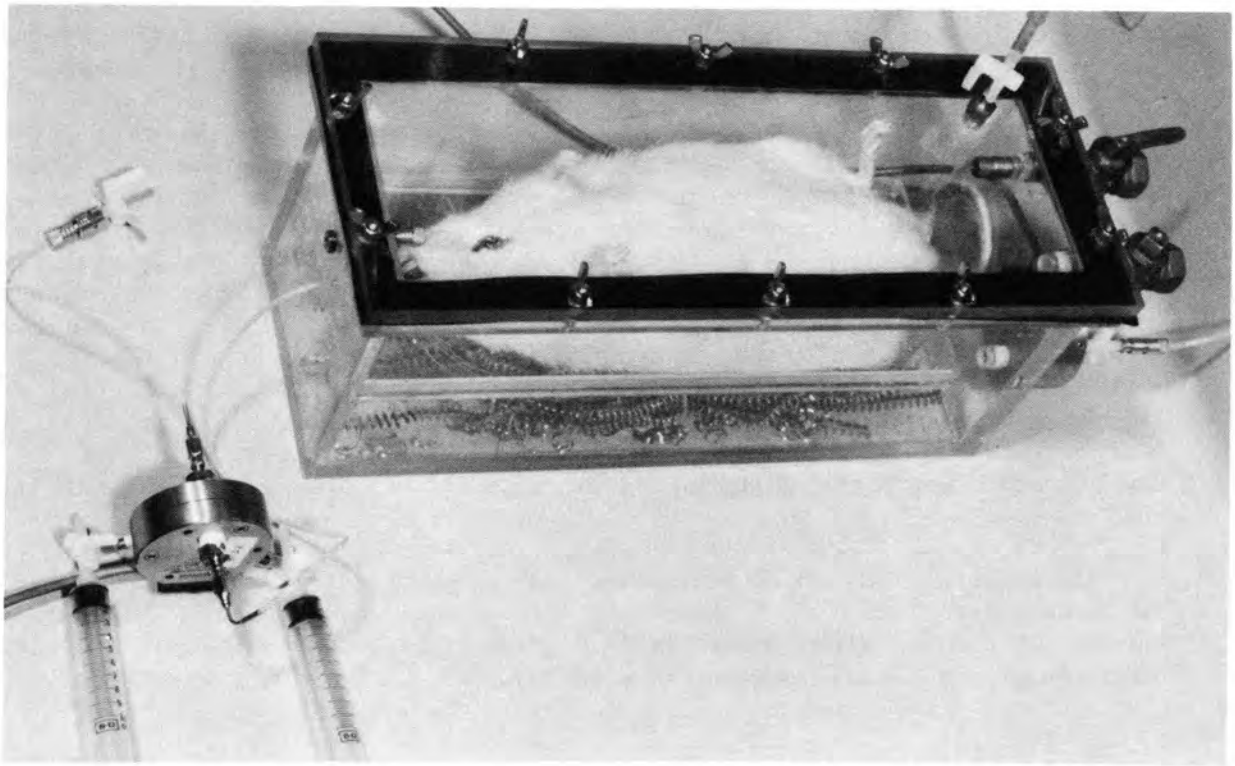

Figure 2

Body box used for pulmonary function tests.

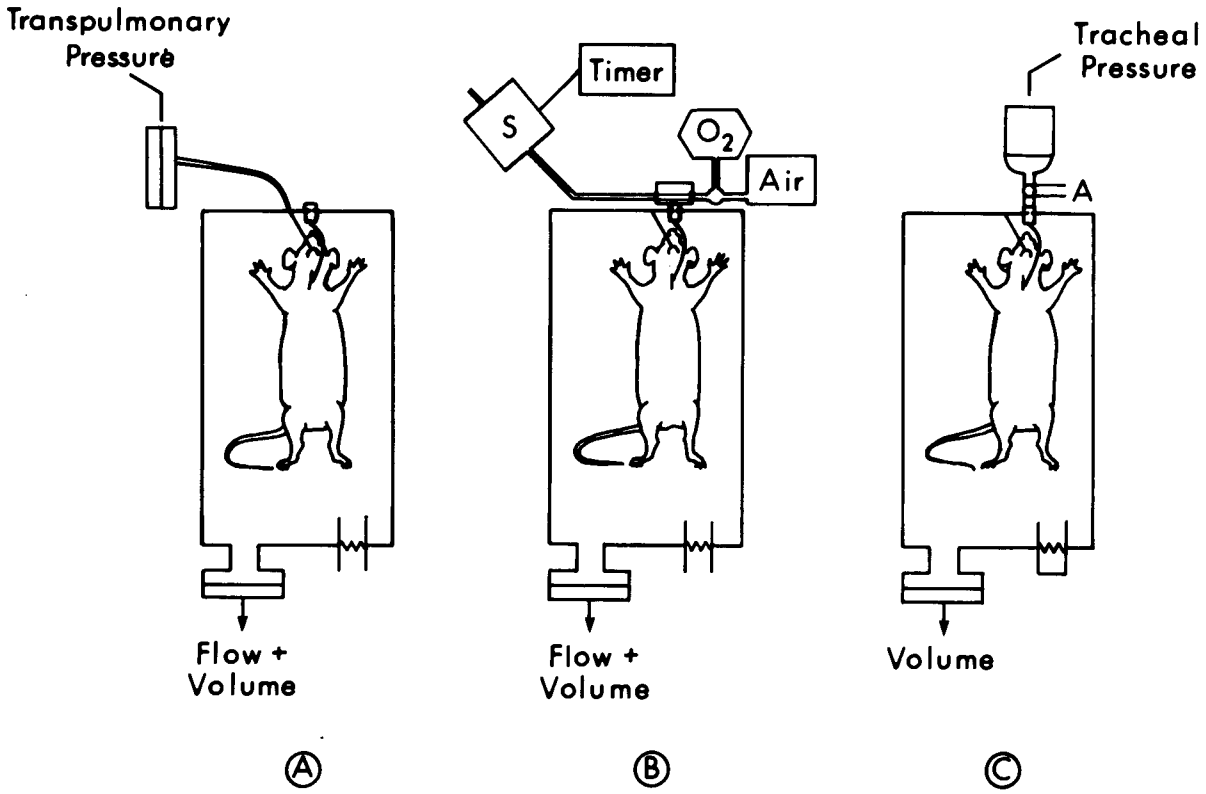

Figure 3

A. Body box configuration during measurement of resistance. Note esophageal cannula connected to differential pressure transducer and similar transducer on box to measure air flow through pneumotachograph.

B. Body box configuration during multibreath nitrogen washout maneuver. Either air or oxygen flow past opening to tracheal cannula and N_2 analyzer. Lungs are inflated while solenoid (S) is closed.

C. Plethysmograph configuration during measurement of FRC. Opening (A) is occluded at end-expiration. Changes in tracheal pressure and lung volume (plethysmograph pressure) are recorded as animal breathes against closure.

Pulmonary Function Tests

Terminal pulmonary function tests were performed on 12 animals (6 per sex) from each treatment group. All tests were performed in a plexiglass body box which could be sealed and used as a whole body plethysmograph (Fig. 2). Three pressure transducers were used for various tests; they were connected to amplifiers on a multichannel Electronics for Medicine electronic recorder. Tracings from these transducers, a nitrogen analyzer, and an electronic integrator were monitored on the oscilloscope of the recorder and recorded on light sensitive paper. All calibrations were performed by standard manipulations of the body box to mimic conditions during each test.

Rats were anesthetized with i.p. injection of 50 mg/kg of pentobarbital. The trachea was exposed and cannulated with a 4 cm length of polyethylene tubing (1.67 mm ID and 2.42 mm OD). The animal was placed on its back in the box. The tracheal cannula was directly connected to the outside of the box through a plastic tubing adapter. An open-ended, water-filled (1.3 mm ID and 2.0 mm OD) cannula was introduced into the esophagus and flushed with water to obtain maximal pressure deflections.

Respiratory flow was measured by a pneumotachograph and a Validyne MP45 differential pressure transducer, illustrated in Figure 3A. Flow signals were electronically integrated to provide a volume tracing. Changes in esophageal pressure were recorded from a water-filled Validyne MP45 differential pressure transducer, also illustrated in Figure 3A. Esophageal pressure, respiratory flow, and tidal volume were recorded during spontaneous breathing. Resistance was calculated from these recordings by the method of Amdur and Mead (4), with subtraction of resistance in the tracheal cannula and associated tubing.

The second lung function test was a multibreath nitrogen washout maneuver (5). Air (300 mL/min) flowed past a "T" connection to the tracheal cannula within a small plexiglas block (Figure 3B). A solenoid on the exit side of the block automatically cycled open and closed, alternately inflating the lungs for 0.5 sec and allowing 0.75 sec for deflation. A constant flow of air resulted in standardized positive-pressure ventilation of the lung. During the nitrogen washout maneuver, the air supply was changed to oxygen by turning a 3-way valve during exhalation. Thus the next inhalation was of 100 percent oxygen and there was no dead space in the oxygen delivery system. The probe for the nitrogen analyzer (Hewlett-Packard, Vertek Series) was on the tube between the "T" and the tracheal cannula and thus in position to detect end-tidal nitrogen concentration during several breaths until a concentration of 2 percent was reached.

Functional residual capacity (FRC) was the third lung function test and was measured by the Boyles law technique (6). The trachea was occluded at the end of exhalation (FRC), and changes in tracheal pressure and lung volume were recorded as the rat tried to breathe against the sealed tracheal cannula. The cannula was closed at FRC so that air pressure within the lungs was equivalent to atmospheric pressure. Thus, known values included

original pressure in the lungs, change in pressure during attempted inhalations, and change in lung volume as the animal's lungs expanded. Using Boyle's law the original volume or FRC can be calculated. Atmospheric pressure was recorded daily on a mercury manometer.

The schematic of the system used for FRC is shown in Figure 3C. A Statham P23ID pressure transducer was connected by a three-way fitting directly to the tracheal cannula so that the animal was breathing through the one open end, labeled A in Figure 3C. The body box was changed into a plethysmograph at this point by closing the valve on the pneumotachograph to increase the sensitivity of measurement of volume changes. At the end of an expiration, opening A was occluded with a finger. Three or more breaths were recorded and the entire procedure was repeated at least 3 times.

The basic intent of the quasistatic pressure volume maneuver, the next test, was to establish the pressure-volume relationships in the lung in situ. This was done in a semistatic or quasistatic manner. The lungs were inflated to a maximal lung volume and then deflated slowly (over 5-6 sec) so that there was adequate time for lung volume to essentially equilibriate with a continuous gradient of transpulmonary pressure. Maximal inflation was defined as lung volume at a transpulmonary pressure of 30 cm water. A schematic of the system is found in Figure 3D. One side of a differential pressure transducer was connected to the tracheal cannula by a water-filled tube while the other side was attached to the esophageal cannula. Transpulmonary pressure was taken as the difference between esophageal pressure and tracheal pressure. The trachea was connected to a 20-mL syringe by a three-way valve. The other limb of this valve was connected to a solenoid which in turn was connected to a pressure flask maintained at -30 cm of water pressure.

The first part of this maneuver was to establish the maximal inflation volume or inspiratory capacity by injecting air from the 20 mL syringe into the lungs beginning at the end of exhalation. Then the quasistatic maneuver was performed by injecting that volume of air slowly into the lungs, slowly withdrawing that volume of air over approximately 5-6 seconds, quickly switching the three-way valve to close off the syringe and connect the animal to the reservoir at a -30 cm water pressure. The body box was used as a plethysmograph during this maneuver. A 5-L flask was connected to the plethysmograph to prevent large pressure fluctuations. Lung volume changes were measured at increments in transpulmonary pressure of 5 cm water over inflation and deflation. Absolute lung volumes were calculated by combining FRC and the pressure-volume curves. Residual volume was defined as lung volume at a transpulmonary pressure of -30 cm water.

One of the primary means of detecting damage to the small airways is by maximal flow-volume curves or the flow during maximal forced exhalation (7). In this procedure, the animal was forced to inhale air to a maximal inspiratory pressure of 30 cm water and was then connected to a reservoir held at -30 cm water to achieve a maximal deflation rate. The pressure reservoirs were 5-gallon glass jugs so that connection to the animal did not decrease their pressure. The connection to the negative pressure reservoir was a three-way solenoid shown in Figure 3E. The system was

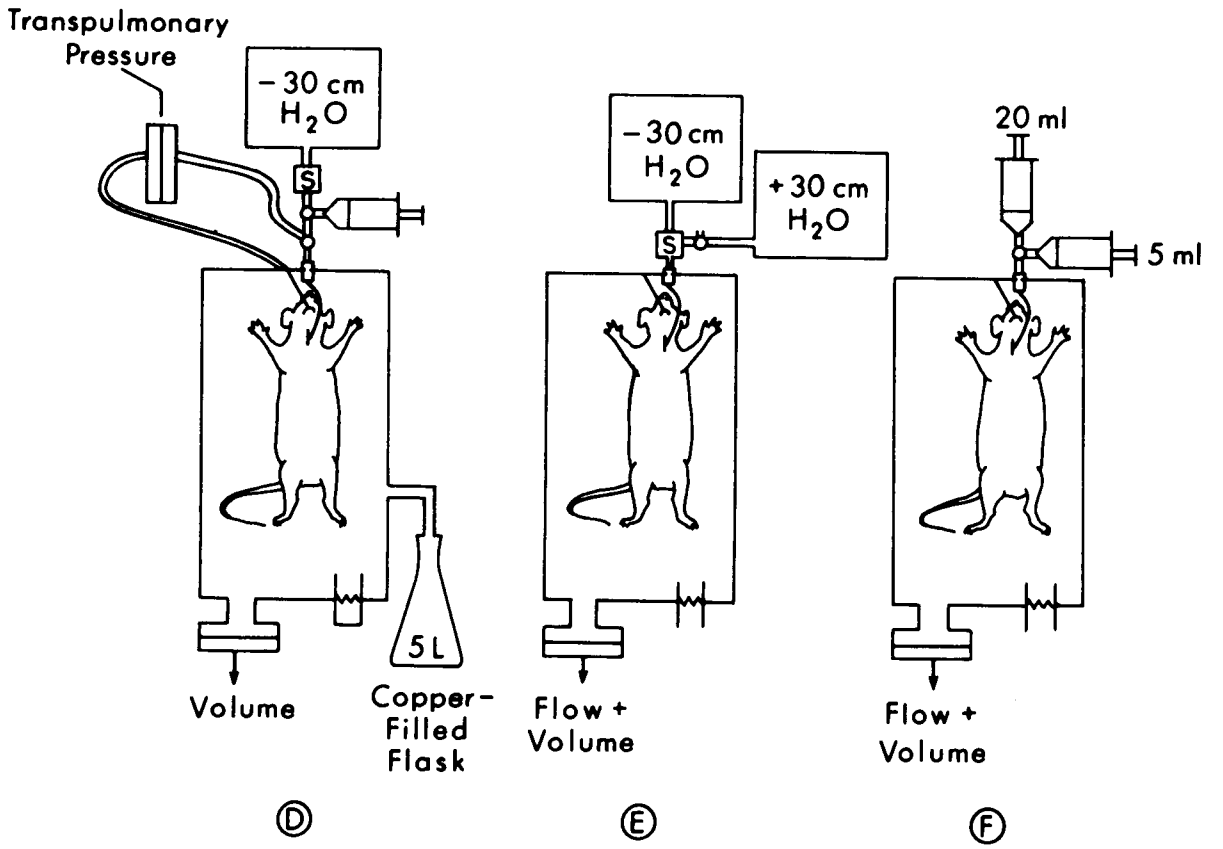

Figure 3 (continued)

D. Plethysmograph configuration during quasistatic pressure-volume curve. Lung volume changes are recorded from pressure changes in plethysmograph as lungs are inflated and deflated by syringe. Transpulmonary pressure is difference between esophageal and tracheal pressures. Lungs are taken to -30 cm HOH by pressure reservoir on opposite side of solenoid (S).

E. Body box configuration during maximal forced exhalation maneuver. Lungs are inflated by pressure reservoir at 30 cm HOH and then connected by solenoid (S) to subatmospheric reservoir for deflation.

F. Body box configuration during measurement of single-breath CO diffusing capacity.

designed and tested to assure that it was not limiting flow during forced exhalations.

A three-way valve, shown in the figure, was switched to connect the animal to the positive pressure reservoir. As soon as lungs were fully inflated, the solenoid was switched to connect the animal with the negative pressure reservoir, and flow and volume were recorded during deflation. The body box was used with a pneumotachograph. Volume changes were obtained by integration of the flow signal. At least two forced exhalations were performed and analyzed.

Single-breath carbon monoxide diffusing capacity was the last pulmonary function test performed. Diffusing capacity simply refers to the volume of gas which can be exchanged across the lungs in a given time. An air mixture containing neon, acetylene, and carbon monoxide was injected into the lungs and then rapidly withdrawn, the last portion of it being kept for analysis of neon and carbon monoxide concentrations. Neon is used as an insoluble tracer to help establish the volume of air in the lungs with which the injected gas mixed. Carbon monoxide was used to determine the rate of diffusion across the lung membranes, with the assumption that the blood was a sink for carbon monoxide.

A diagram of the procedure is shown in Figure 3F. The body box was used with an assembly of one three-way valve and two plastic syringes. The 20-mL syringe was filled with lung diffusion gas mixture obtained from Matheson Gas Co. (air with 0.4 percent carbon monoxide, 0.5 percent neon, 0.5 percent acetylene). The volume of gas injected into the lungs was the inspiratory capacity obtained during the quasistatic pressure-volume maneuver. While lung volume changes were being recorded, the gas mixture was rapidly injected into the lungs and immediately withdrawn until only 5 mL remain in the lungs. The three-way valve was quickly switched to connect the 5 mL syringe to the animal and the remaining 5 mL withdrawn. This 5 mL was immediately taken to a Carle Analytical Gas Chromatograph (Model 111) for analysis of neon and carbon monoxide concentrations. Diffusing capacity was calculated by a standard equation (8).

Organ Weight and Histopathology

The animals used in the pulmonary function tests were subsequently killed by aortic bleeding, and several tissues were taken for weighing and histopathology. The right middle lung lobe was tied off at the bronchus, removed, and weighed immediately. It was then dried at 95°C for 2 days and reweighed for dry weight. The remainder of the lung and trachea were fixed for 24 hours under a constant tracheal pressure of 25 cm of buffered formalin. The left adrenal gland, left kidney, and liver were also weighed. These organs were preserved in buffered formalin along with spleen, larynx, heart, brain, lumbar spinal cord, sciatic nerve, and nasal turbinates.

The following organs were routinely embedded in paraffin, sectioned and stained with hematoxylin and eosin: cross-sections of left lung lobe (just below entry of bronchus), larynx, trachea, nasal turbinates (standard

section in relation to grooves or palate), adrenal, cerebrum, spleen, and kidney. Longitudinal sections of the large lobe of the liver were also prepared.

Neurotoxicity Assays

Four neurotoxicity assays were performed on the same animals before any exposure, one day after the last exposure, and after two subsequent weeks without treatment. The assays included landing foot spread (9), tail flick (10), forelimb grip strength (11), and startle reflex (12). These assays were chosen because they are relatively simple to perform and interpret, are practical for use with rats, and are capable of measuring different neurological or neuromuscular functions. The modified procedures developed in our laboratory for use with rats are outlined below.

The tail flick is primarily a test of the "pain" reflex. Rats were held securely in a plastic holder from which their tail protruded. The tail was placed in a beaker of water maintained at 61°C by a heating coil and a YSI Thermistemp temperature controller. The time for the rat to remove its tail from the water was recorded. The mean of three trials was used as a value for each individual.

Landing foot spread is an index of peripheral neuropathy. It is a measure of the distance between the two hind feet when animals land after being dropped from 12 inches above a table. The palmar pads of their hind feet were inked to mark a paper on the table. The mean of three trials was used for data analysis.

Forelimb grip strength is the force required to pull an animal off a bar which it is grasping with its forefeet. The test centers on neuromuscular disturbances and requires some motivation of the animal. The apparatus used with our rats was modified from that used with mice. Basically, a Chatillion scale was placed horizontally with a horizontal grid on the end of a bar coming out from the scale. The grid extended into a narrow, high-walled plexiglas box without a top or end opposite the scale. Rats were held by the tail and placed so that they held the grid with their forefeet. They were gently pulled away from the scale, which recorded the maximum force required to make them let go of the grid.

The startle reflex assay tested the time to reaction and the force of the response when rats were startled by a sharp auditory stimulus. Rats were placed in a wire box within a larger sound-insulated box (Fig. 4). A constant white noise at 85 dB within the larger box helped eliminate outside noises. After an acclimation period of 10 min., rats received a series of five 10 msec pulses of noise at 13,000 Hz and 110 dB separated by 25 sec. Their response, or startle reflex, was monitored by a Gould load cell under the wire box. The entire procedure, including data acquisition and analysis, was controlled by an Apple microcomputer.

On the basis of pilot work using chlorpromazine and other drugs, it was decided that the neurotoxicity data could be analyzed best as

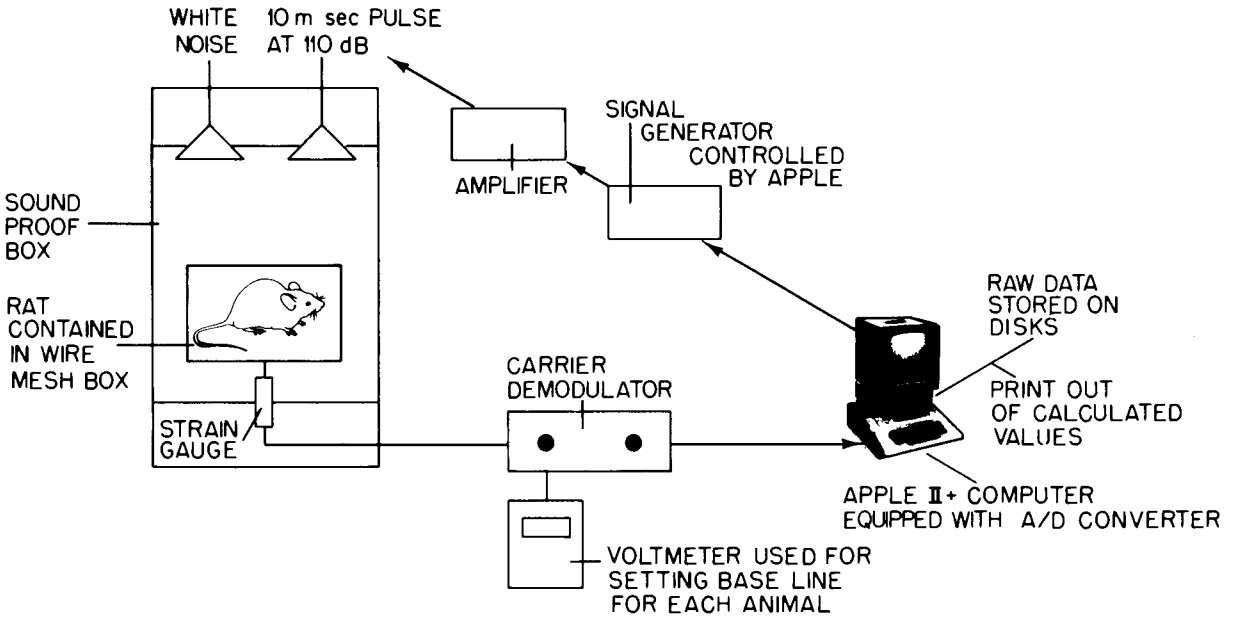

Figure 4

Schematic diagram of apparatus used for startle reflex assay.

differences between pre-exposure and postexposure values for each individual. Thus each animal served as its own control.

RESULTS

Aerosol Concentration

In addition to continuous monitoring of the aerosol concentration by infrared backscatter detectors, filter pad samples of chamber air were drawn during each exposure for gravimetric analysis of chamber concentration. A summary of the measured and target concentrations is in Table 1C (see Appendix C). Mean values for groups exposed to lower concentrations tended to be slightly more than target values while those for the two groups receiving 6 mg/L were slightly lower than the target. However, actual concentration was generally close to the target concentration; the study was therefore conducted in accordance with the experimental design.

Mortality

A Ct product of 8 mg·hr/L had been previously estimated to result in a dose of diesel fuel which approximated a maximum tolerated dose, in terms of mortality, and no deaths were seen in the repeated exposures with Ct products of 0 or 8 mg·hr/L. However, among the groups receiving 12 mg·hr/L there was 6.25 percent mortality overall. Eleven rats (10 females and only one male) died among the 4 groups receiving this higher exposure. Eight of these including the single male fatality were in the group exposed to 6 mg/L for 2 hr/exposure at 1 exposure/wk; one death was in each of the other 3 groups. The reason for this apparently unequal distribution of deaths is not known.

Deaths occurred within 48 hr after a given exposure, as in acute exposures used to define maximum tolerated exposure conditions. As seen in Figure 5, deaths occurred over the span of 9 exposures, although they were more frequent during the earlier exposures. At autopsy, lungs were enlarged and reddened. There occasionally was some fluid in the trachea. No other abnormalities were seen, again giving a picture similar to that after acute exposures. It appeared that there was no difference in the cause of death between single and repeated exposures and that the choice of a Ct product of 8 mg·hr/L for maximal exposure conditions resulting in no significant mortality was valid. With exposures to a Ct product 50 percent higher, significant mortality resulted.

Body Weight

Mean body weights during exposures given once per week or 3 times per week are shown in Figures 6 and 7 respectively. It is readily apparent from these figures that mean body weight was depressed in both sexes by exposure to the aerosol, especially with 3 exposures/week. Weight

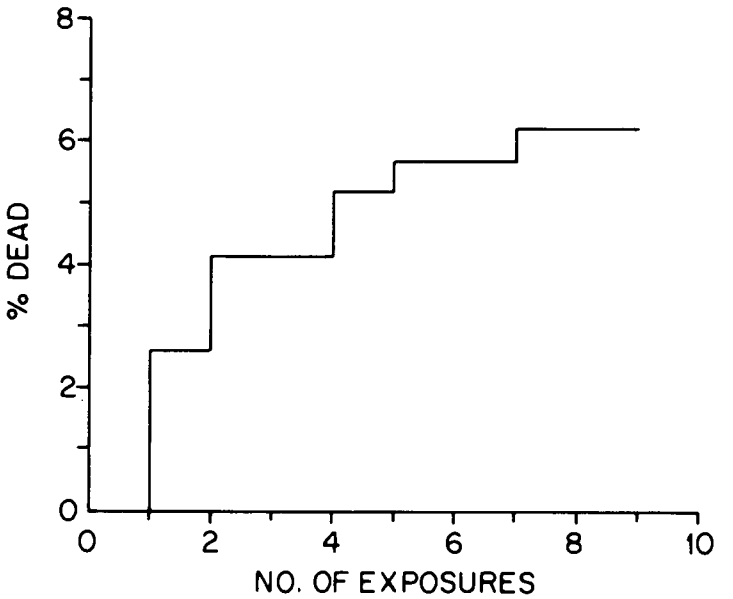

Figure 5

Cumulative mortality over course of exposures among groups receiving 12 mg·hr/L.

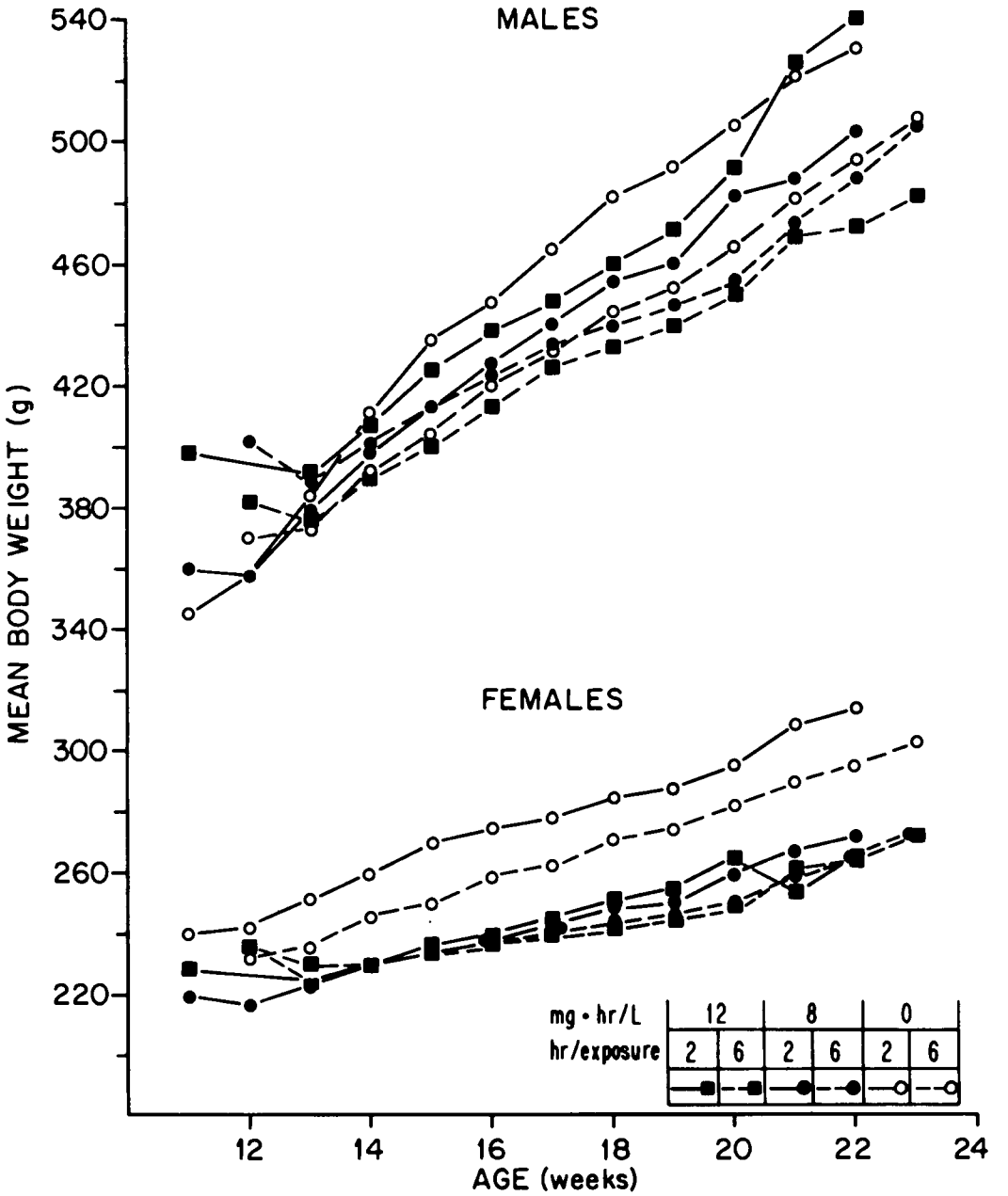

Figure 6

Mean body weight of animals during exposure to aerosolized diesel fuel once per week. Exposures ended after the ninth week.

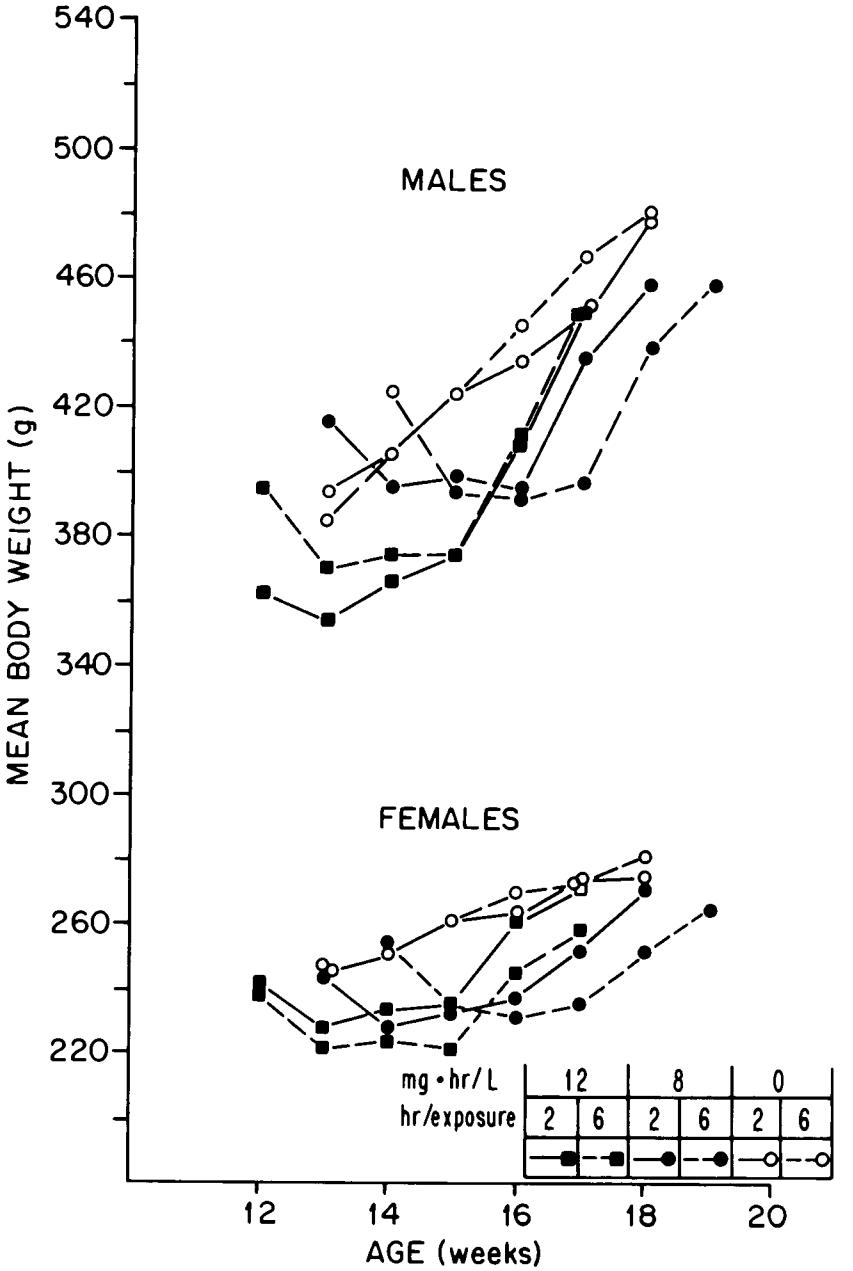

Figure 7

Mean body weight of animals during exposure to aerosolized diesel fuel three times per week. Exposures ended after the third week.

increased in the groups exposed once per week after an initial depression, the rate of gain being similar to that of sham-exposed controls. After an initial depression, weight in groups exposed 3 times per week was stable for the remainder of the exposure period and then rebounded toward control values during the 2 weeks post-exposure.

Weight data were treated statistically by analysis of variance of the weekly weight gains (or loss). Weight gain during the first few weeks of exposure was affected by all three exposure parameters (exposure, duration of exposure and frequency of exposure). During the first 2 weeks of exposure, body weight gain was significantly reduced in exposed animals compared to controls ($p = 0.0001$ for both sexes). However the extent of this difference was not related to Ct product. Frequency of exposure was also a significant factor; weight gain among groups exposed once per week was greater than it was in those exposed 3 times per week ($p = 0.0001$ for both sexes). Groups exposed for 6 hours gained less weight than those exposed for 2 hours ($p = 0.0001$ for both sexes). Least squares mean values reflecting these analyses are shown in Tables 2C and 3C. (See Appendix A for explanation of least squares means.)

Body weights rebounded toward control values after cessation of 3-times-per-week exposures (see Fig. 7). Among groups exposed once per week (Fig. 6), the effect of exposure on rate of weight gain lessened with time, until there was no significant rebound in weight when exposure was stopped after nine weeks.

Food Consumption

Weekly food consumption data were obtained for 6 males and 6 females in each group throughout the exposure and recovery periods. These values are summarized in Figs. 8 and 9. As with body weight, there was an obvious sex difference, and data were analyzed for each sex separately. No significant block effects were observed. And, as with body weight, the most significant effects were seen in the first few weeks of exposure. Food consumption generally decreased significantly with increasing Ct product or frequency during the first 3 weeks of exposure, as shown in Tables 4C and 5C. No significant relation of duration of exposure and depression of food consumption was observed.

Food consumption increased in the groups given 3 exposures per week after the third week, when those exposures were stopped. In the groups receiving 1 exposure per week, there was generally a significant depression in food consumption for the nine weeks of exposure with no discernable relation to the Ct product.

Pulmonary Free Cells

The number of alveolar macrophages lavaged from the lungs of all exposed groups at two days post exposure was significantly greater than that of controls ($p = 0.0001$). This increase is evident in the means shown

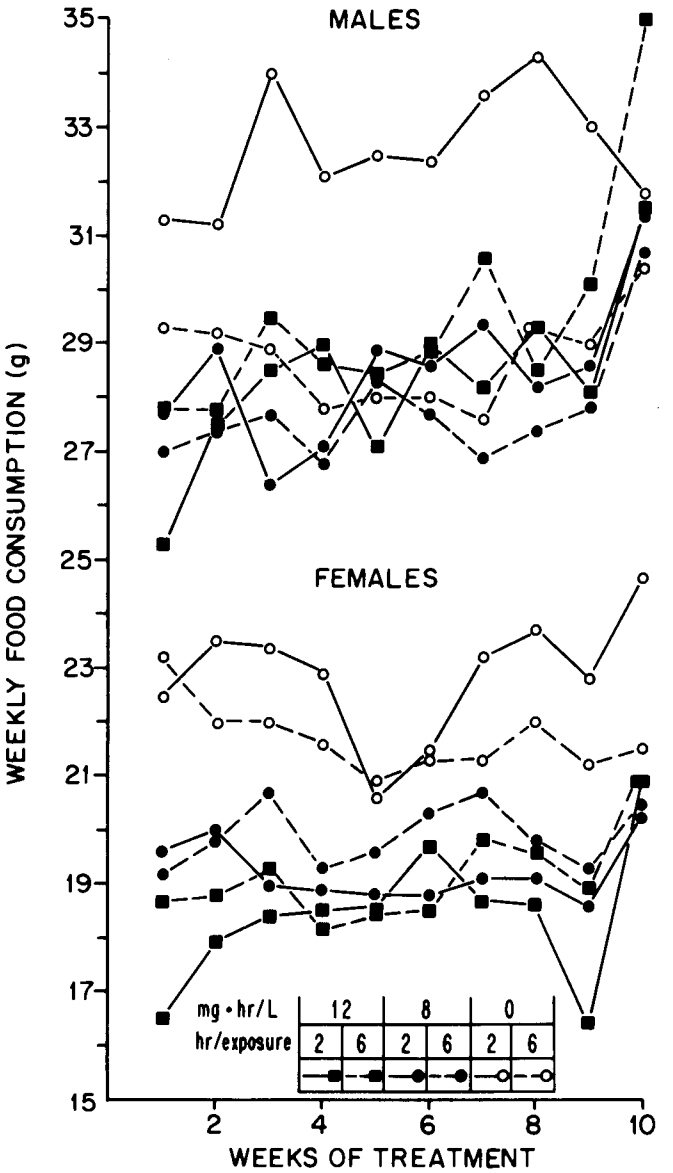

Figure 8

Mean weekly food consumption of animals during exposure to aerosolized diesel fuel once per week. Exposures ended after the ninth week.

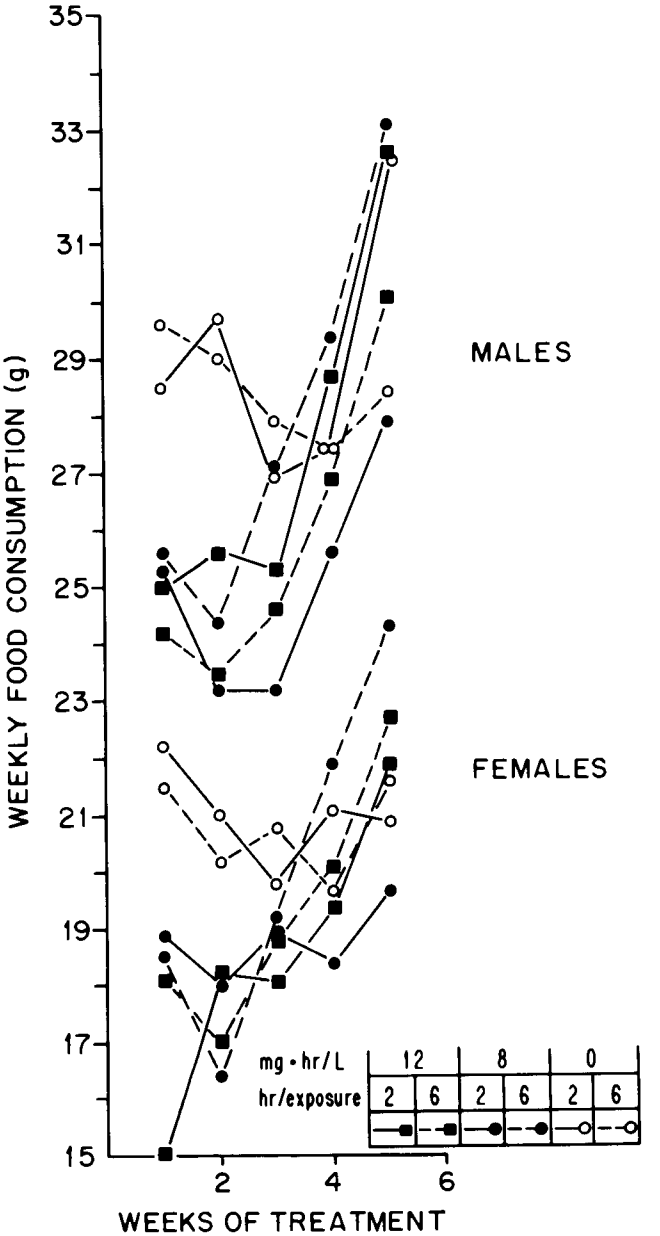

Figure 9

Mean weekly food consumption of animals during exposure to aerosolized diesel fuel three times per week. Exposures ended after the third week.

in Table 6C. There was also a clear sex difference ($p = 0.0001$) which was made essentially insignificant ($p = 0.012$) by referencing cell number to body weight. Control values were in the range of 5×10^6 cells/kg for both sexes. The increase in cell number was not related to Ct product. Interestingly, the two week period without exposure did not result in a significant change in the numbers of alveolar macrophages compared to values at 2 days post exposure ($p = 0.77$). This lack of a decrease to control values is also evident in the means in Table 6C. There was no significant effect of exposure on cell viability.

The number of lavaged cells other than alveolar macrophages increased very significantly in all groups of exposed animals ($p = 0.0001$), but again without a clear relation to the Ct product. The numbers among exposed groups were highly variable, as seen in Table 7C. These other cells consisted primarily of granulocytes. Differential counts of cells attached to the coverslips in the yeast assay of 3 groups showed that the percent of granulocytes among these "other cells" rose from 2 percent in control preparations to 87 percent at 2 days after exposure. Lymphocytes accounted for 7 percent of these cells and the remaining 6 percent were unidentified. The vast majority of granulocytes appeared to be neutrophils, as was previously observed after acute exposures. Unlike the number of macrophages, the number of "other cells" returned essentially to control values by 2 weeks without exposure, as seen in Table 7C.

Phagocytic activity of lavaged alveolar macrophages was expressed in terms of the number of yeast associated with the cells attached to the coverslip used in the assay. A typical example of the data from one animal is given in Figure 10. It was found that similar curves from all animals could be adequately described by a negative binomial distribution which could be completely described by its mean and variance. The mean of this frequency distribution is essentially the average number of yeast associated with all macrophages examined. This mean is referred to here as the binding index and is illustrated in Figure 10. Values for binding index among the treatment groups are summarized in Table 8C. There were no significant changes in binding index which could be related to exposure to the aerosol.

Pulmonary Function Tests

The results of each pulmonary function test are presented here in essentially the order in which the tests were done. Resistance was the first test, representing resistance of the respiratory tract below the tracheal cannula. Values are summarized in Table 9C. No differences in resistance were observed which were related to exposure to the aerosol. However, there was a significant sex-related difference ($p = 0.0001$). Least squares means were 0.290 ± 0.019 H_2O/mL/sec for males and 0.389 ± 0.019 cm H_2O/mL/sec for females.

Multibreath nitrogen washout curves were also performed. These can be analyzed in a variety of ways. All attempt to ascertain how efficiently N_2 present in the lungs is removed or washed out over a series of breaths

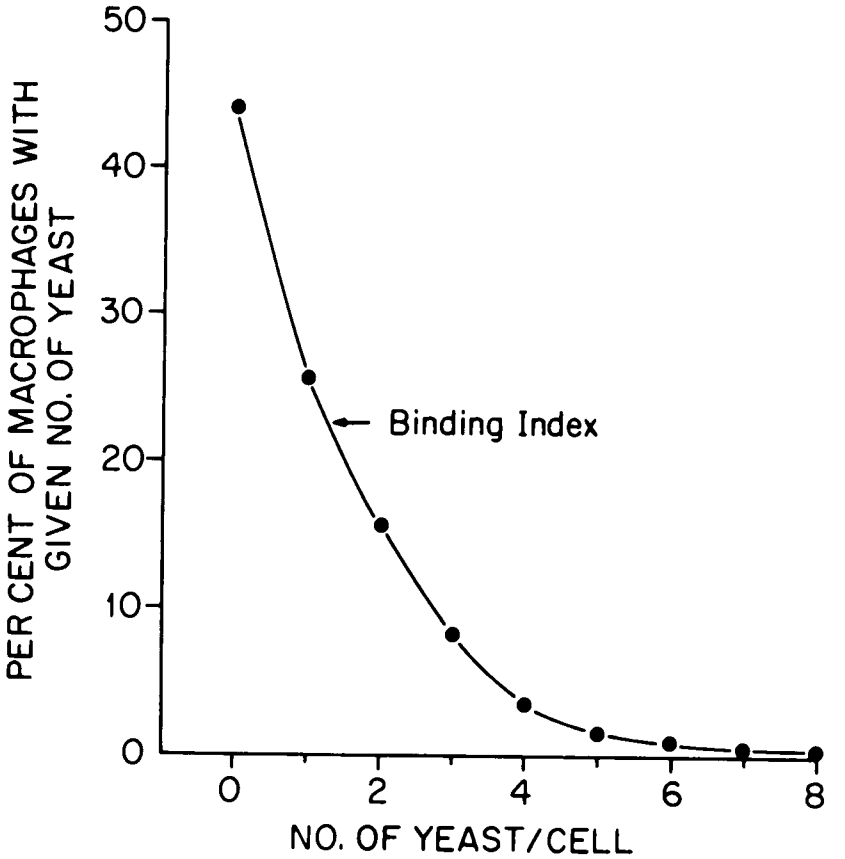

Figure 10

Percent of macrophages associated with given number of yeast in phagocytosis assay. Arrow marks binding index or average number of yeast associated with a macrophage.

of pure O_2. One simple method is to express end-tidal N_2 concentration in terms of breath number when tidal volume is constant, as illustrated in Figure 11 for males in a control and an exposed group at 2 weeks postexposure. The N_2 concentration at the end of exhalation was taken to best approximate alveolar concentration. In this case it appears that the lungs of the treated group were less well ventilated than those of the controls, requiring more breaths to expire the same amount of N_2. However, the resting volume of the lung (functional residual capacity or FRC) was 3.0 ± 0.2 mL for controls and 3.7 ± 0.2 mL for exposed animals in this example. This difference in lung volume must be compensated for in calculations on the washout curves if one is to separate changes in efficiency of ventillation from altered lung volume. In Figure 12 the end-tidal N_2 concentration is plotted against the cumulative number of times that FRC was diluted by successive breaths. After the elimination of the influence of FRC, it is apparent that the two groups do not differ from one another in this test. The apparent difference noted above was due to increased resting volume in exposed animals.

All calculations dealing with the nitrogen washout curves were made on the basis of cumulative dilutions of FRC. One of the analyses performed was a simple linear regression of the log (percent N_2) vs cumulative dilutions of FRC, on the assumption that the lungs washed out exponentially like a single compartment. This assumption proved to be nearly true, with correlation coefficients generally greater than 0.980. Thus the slope of the regression equation was taken as an index of the rate of clearance of N_2 from the lung. These slopes are summarized in Table 10C. The large number of standard errors equal to zero is an artifact of rounding. There was no exposure-related change in the slope. There was a significant sex difference, however ($p = 0.0001$); males had least squares mean slope of -0.188 ± 0.002 while females had a slope of -0.155 ± 0.003. Thus, females had relatively greater resistance to breathing and less rapid washout of N_2 from the lungs.

The number of dilutions required to reach end-tidal concentrations of 10 percent and 5 percent nitrogen were also calculated by extrapolation from the data points on either side of these nitrogen concentrations for each animal. Again no exposure-related changes were observed, but there was a sex difference ($p = 0.0001$). Least squares mean values for the dilutions to reach 10 percent N_2 were 5.19 ± 0.08 for females and 4.10 ± 0.08 for males; values for the number of dilutions to reach 5 percent N_2 were 7.23 ± 0.10 and 5.79 ± 0.10 for females and males respectively.

Lung volumes were obtained by combining data from two methods: FRC from the Boyle's Law method and a continuum of changes in volume (but not absolute volumes) ranging from total lung capacity (TLC) to residual volume (RV) from the quasistatic pressure-volume curve. These two assays were combined to produce pressure-volume curves of absolute lung volume. Examples of these curves are presented in Figure 13. The lung volumes derived from these curves are shown in the figure, including FRC, TLC, RV, vital capacity (VC), inspiratory capacity (IC), and expiratory reserve

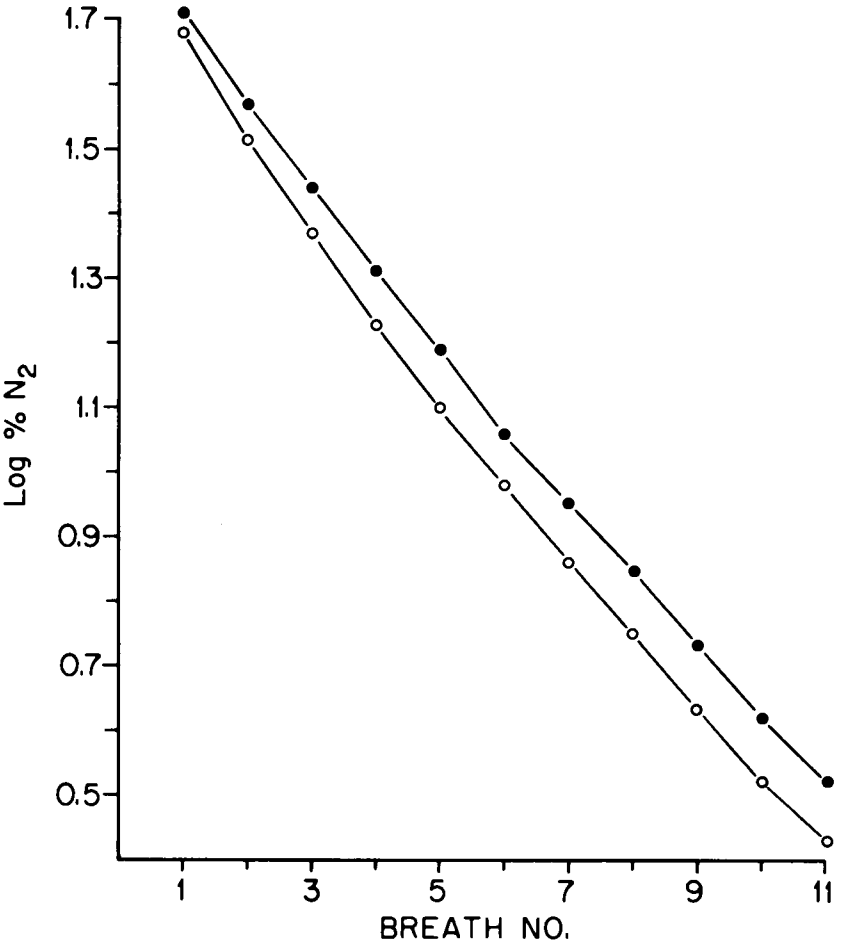

Figure 11

Mean multibreath nitrogen washout curves for males of control group EA (○) and exposed group FA (●). End-tidal N_2 concentration is plotted against breath number.

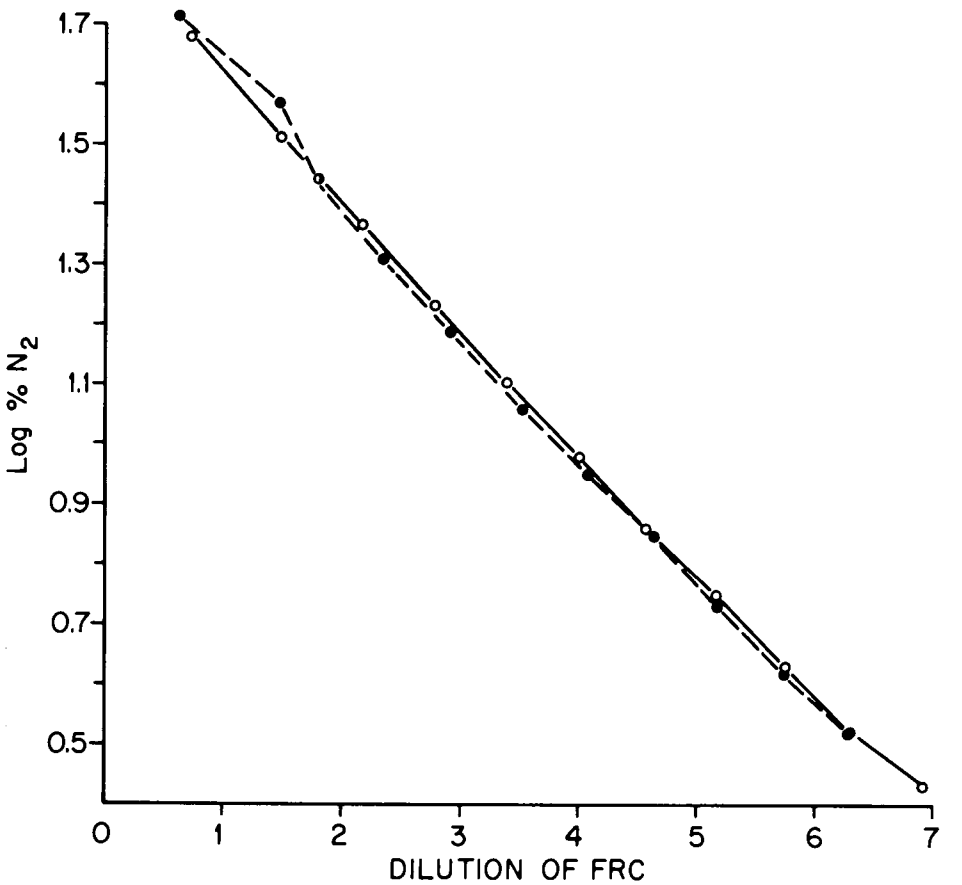

Figure 12

Mean multibreath nitrogen washout curves for males of control group EA (○) and exposed group FA (●). End-tidal N_2 concentration is plotted against cumulative dilutions of FRC.

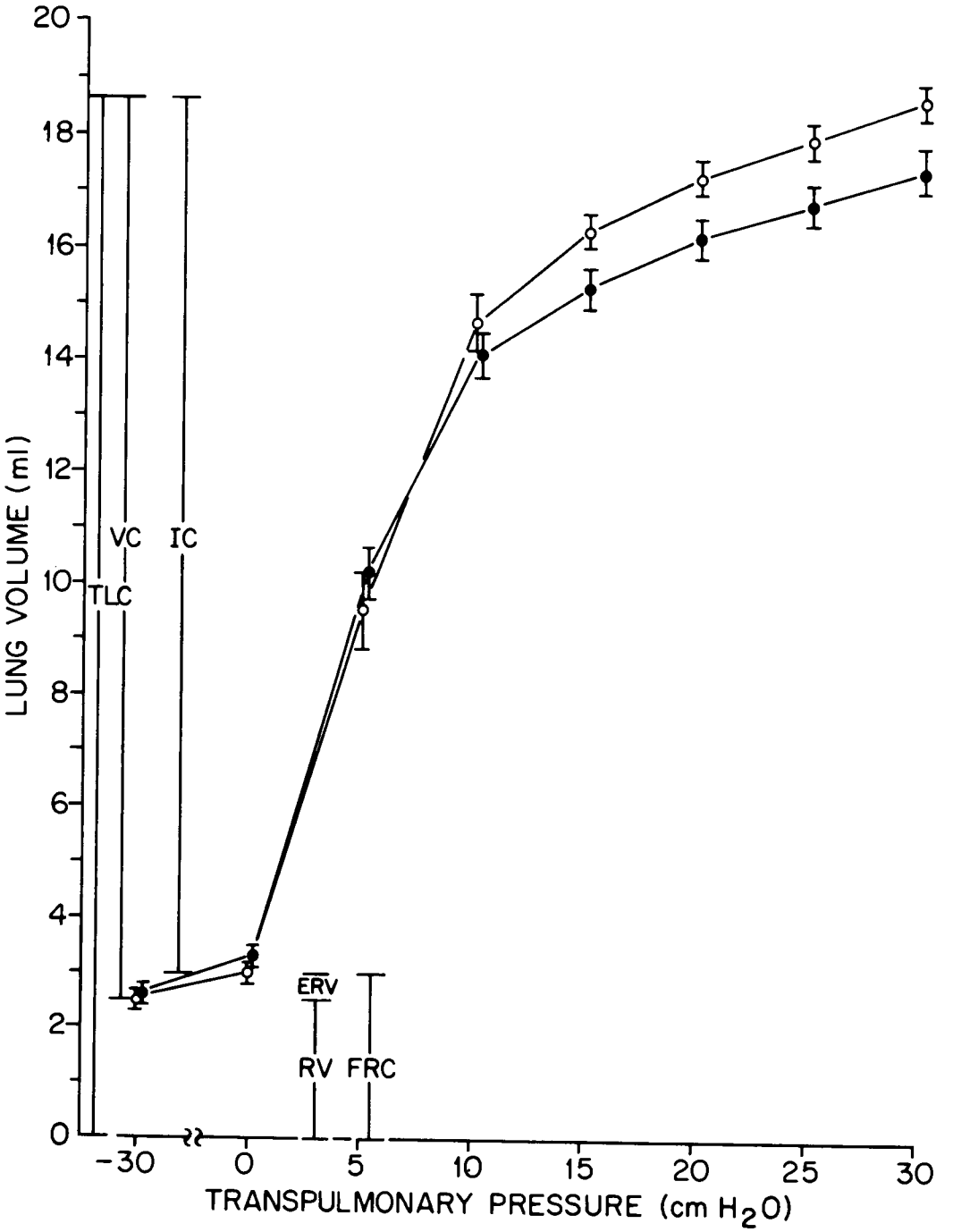

Figure 13

Quasistatic pressure-volume curves of lungs from males in control group HA (○) and exposed group (GA (●). Lung volumes shown are total lung capacity (TLC), vital capacity (VC), inspiratory capacity (IC), functional residual capacity (FRC), residual volume (RV), and expiratory reserve volume (ERV).

volume (ERV). Changes in these volumes could reflect alterations in architectural and/or elastic properties of the lung.

There was some concern that TLC and VC increased significantly with age over the course of exposures since these volumes appeared slightly lower in two control groups assayed at 16 weeks of age than in older controls. In a direct comparison, these two groups (HA and LA) had TLC's lower than the others by about 8 percent. If an age-related increase in lung volume were present, compensation for this change would have to be made. No such compensation was made for the following reasons:

1) A definite age-related change could not be described over the range of ages in this phase of the toxicologic evaluation of diesel fuel aerosols.

2) Groups of exposed animals were exposed over the same range of ages as controls and at approximately the same ages.

3) The youngest groups of exposed animals (aged 15 and 16 weeks) had TLC's in males which were about 14 percent below control groups of comparable age, an appreciably greater difference than the apparent 8 percent noted in controls above.

4) Among female controls in the 6 assays of older controls, there was as much variation in TLC as there was between those groups collectively and the two groups assayed at 16 weeks of age.

Values for several lung volumes are presented in Tables 11C-14C. Analysis of the values for TLC showed that it was affected by exposure ($p = 0.005$), frequency of exposure ($p = 0.0001$), time postexposure ($p = 0.0001$), and sex ($p = 0.0001$). There was also a significant interaction of exposure and duration of exposure ($p = 0.0005$). Table 1 summarizes the least squares means and standard errors for each of these variables. Definitions of terms used in this and subsequent tables are given in Appendix C.

TABLE 1. LEAST SQUARES MEANS OF TOTAL LUNG CAPACITY (TLC) VS. Ct PRODUCT, FREQUENCY, SEX, AND TIME POSTEXPOSURE

Ct Product	TLC (mL)
0	16.5 ± 0.2
8	15.9 ± 0.2
12	15.6 ± 0.2

Exposures Week	TLC (mL)
1	16.7 ± 0.1
3	15.3 ± 0.1

Sex	TLC (mL)
M	18.0 ± 0.1
F	14.0 ± 0.1

Time Postexposure	TLC (mL)
2 days	15.3 ± 0.1
2 weeks	16.7 ± 0.1

Ct Product	2 Hours Exposure	6 Hours Exposure
0	16.5 ± 0.3	16.5 ± 0.3
8	15.2 ± 0.3	16.7 ± 0.3
12	15.7 ± 0.3	15.4 ± 0.3

The larger TLC's in males were expected because of body size. It is interesting to note, however, that a highly significant ($p = 0.0001$) sex difference was present for TLC referenced to body weight. Females had 52.7 ± 0.4 μL/g while males had 38.8 ± 0.4 μL/g. Females therefore had larger lung volumes in proportion to their body weight.

Exposure to aerosol generally resulted in decreased TLC except among groups exposed for 6 hours/exposure to a daily Ct product of 8 mg·hr/L. The increase in TLC observed between 2 days after exposure and 2 weeks later occurred in most groups, exposed and control. The reason for this increase was not clear.

Vital capacity was significantly ($p = 0.0001$) affected by exposure, sex, frequency of exposure, and time postexposure. Least squares means and standard errors for these variables are summarized in Table 2.

TABLE 2. LEAST SQUARES MEANS OF VITAL CAPACITY (VC) VS. Ct PRODUCT FREQUENCY, SEX, AND TIME POSTEXPOSURE

Ct Product	VC (mL)
0	14.3 ± 0.2
8	13.6 ± 0.2
12	12.9 ± 0.2

Exposures Week	VC (mL)
1	14.2 ± 0.1
3	13.0 ± 0.1

Sex	VC (mL)
M	15.3 ± 0.1
F	11.9 ± 0.1

Time Postexposure	VC (mL)
2 days	13.1 ± 0.1
2 weeks	14.1 ± 0.1

As with TLC, females had proportionately higher VC's for their body weight than did males (44.7 ± 0.4 vs 32.9 ± 0.3 μL/g). Vital capacity changes followed those for TLC except that the exposure-time interaction was marginally significant ($p = 0.04$). Least squares means for this interaction are given in Table 3.

TABLE 3. INTERACTION OF VITAL CAPACITY Ct PRODUCT AND TIME POSTEXPOSURE

Ct Product	2 Hours Exposure	6 Hours Exposure
0	14.2 ± 0.2	14.4 ± 0.2
8	13.1 ± 0.2	14.0 ± 0.2
12	13.0 ± 0.2	12.9 ± 0.2

The pattern for inspiratory capacity was very similar, with significant ($p = 0.0001$) effects of exposure, sex, frequency of exposure, and time after last exposure. The exposure-duration interaction was marginal ($p = 0.016$). Again, least squares means are given in Table 4.

TABLE 4. LEAST SQUARES MEANS OF INSPIRATORY CAPACITY (IC) VS. Ct PRODUCT, FREQUENCY, SEX, AND TIME POSTEXPOSURE

Ct Product	IC (mL)
0	13.4 ± 0.2
8	12.5 ± 0.2
12	12.0 ± 0.2

Exposures Week	IC (mL)
1	13.1 ± 0.1
3	12.1 ± 0.1

Sex	IC (mL)
M	14.2 ± 0.1
F	11.1 ± 0.1

Time Postexposure	IC (mL)
2 days	12.1 ± 0.1
2 weeks	13.1 ± 0.1

Ct Product	2 Hours Exposure	6 Hours Exposure
0	13.3 ± 0.2	13.4 ± 0.2
8	12.0 ± 0.2	13.1 ± 0.2
12	12.0 ± 0.2	12.0 ± 0.2

Rather than decreasing with exposure, FRC significantly ($p = 0.0001$) increased by 9 percent among groups receiving 8 mg•hr/L and 15 percent after exposure to 12 mg•hr/L. There were also significant ($p = 0.0001$) effects of sex, frequency of exposure, time after exposure, and interaction of exposure and time. Animals exposed over a longer time (1/wk) had larger values (by 13 percent) for FRC which also increased during the 2 week postexposure period. In the exposure-time interaction, the effect was reversed from that of previous lung volumes; FRC increased among groups receiving 12 mg•hr/L and only in those having 6 hour exposures with 8 mg•hr/L. Least squares means and standard errors are given in Table 5.

TABLE 5. LEAST SQUARES MEANS OF FUNCTIONAL RESIDUAL CAPACITY (FRC) VS. Ct PRODUCT, FREQUENCY, SEX, AND TIME POSTEXPOSURE

Ct Product	FRC (mL)
0	3.13 ± 0.06
8	3.40 ± 0.06
12	3.59 ± 0.06

Exposures Week	FRC (mL)
1	3.58 ± 0.05
3	3.17 ± 0.05

Sex	FRC (mL)
M	3.82 ± 0.05
F	2.93 ± 0.05

Time Postexposure	FRC (mL)
2 days	3.19 ± 0.05
2 weeks	3.56 ± 0.05

Ct Product	2 Hours Exposure	6 Hours Exposure
0	3.12 ± 0.09	3.14 ± 0.09
8	3.15 ± 0.09	3.65 ± 0.09
12	3.41 ± 0.09	3.41 ± 0.09

Values for residual volume paralleled those for FRC in both the increase after exposure and levels of significant differences in the least squares means outlined in Table 6.

TABLE 6. LEAST SQUARES MEANS OF RESIDUAL VOLUME (RV) VS. Ct PROCUCT, FREQUENCY, SEX, AND TIME POSTEXPOSURE

Ct Product	RV (mL)
0	2.22 ± 0.06
8	2.39 ± 0.06
12	2.65 ± 0.06

Exposures Week	RV (mL)
1	2.54 ± 0.04
3	2.30 ± 0.04

Sex	RV (mL)
M	2.74 ± 0.04
F	2.10 ± 0.04

Time Postexposure	Rv (mL)
2 days	2.25 ± 0.04
2 weeks	2.59 ± 0.04

Ct Product	2 Hours Exposure	6 Hours Exposure
0	2.28 ± 0.07	2.16 ± 0.07
8	2.09 ± 0.07	2.69 ± 0.08
12	2.73 ± 0.08	2.57 ± 0.08

All lung volumes discussed thus far were derived from the combination of values for FRC and the quasistatic pressure-volume curves. Another way of analyzing the pressure-volume curves is in terms of compliance, the slope of the pressure-volume curve over a specified range of transpulmonary pressure. Compliance differs at various locations along the curve and with changes in lung site. A specific pressure range is used to define the location on the curve being examined. To help compensate for variations in lung volume, the compliance value may be divided by actual lung volume at the midrange of the chosen transpulmonary pressures to yield a number called specific compliance. The specific compliance values given in Table 15C are the changes in lung volume from transpulmonary pressures of 0 to 10 cm H_2O divided by lung volume at 5 cm H_2O.

Analysis of least squares means for specific compliance revealed significant effects of exposure ($p = 0.0001$), frequency of exposure ($p = 0.0001$), sex ($p = 0.007$), and interaction of exposure and duration of each exposure ($p = 0.005$). Least squares means (± S.E.) for these variables are in Table 7.

TABLE 7. LEAST SQUARES MEANS OF SPECIFIC COMPLIANCE (C_{sp}) VS. Ct PRODUCT, FREQUENCY, SEX, AND TIME POSTEXPOSURE

Ct Product mg·hr/L	C_{sp}
0	1.15 ± 0.01
8	1.04 ± 0.01
12	0.97 ± 0.01

Exposures Week	C_{sp}
1	1.02 ± 0.01
3	1.09 ± 0.01

Sex	C_{sp}
M	1.03 ± 0.01
F	1.08 ± 0.01

Ct product mg·hr/L	C_{sp}	
	2 hrs/exposure	6 hrs/exposure
0	1.16 ± 0.02	1.14 ± 0.02
8	1.08 ± 0.02	1.00 ± 0.02
12	0.95 ± 0.02	0.99 ± 0.02

As one would expect from the data on lung volumes, specific compliance decreased with exposure and was lower in animals exposed once per week. Values for females were higher than those for males.

The maximal forced exhalation maneuver was used to test for functional obstruction of the airways. In this assay, the animals were made to inhale to TLC and then forced to exhale under conditions such that their respiratory system limited the maximum flow achieved at any given lung volume. Expiratory flow was analyzed in terms of peak flow, flow at 50 percent of vital capacity, and flow when 25 percent of vital capacity remained. Means of the original flows at these specific lung volumes are in Tables 16C-18C. There appeared to be a decrease in maximal flow among the groups receiving the highest Ct product and more frequent exposures. However, vital capacity was also decreased among these groups. Since maximal flow is related to vital capacity, the question arose as to whether the apparent effect of exposure on maximal flows was a result of decreased VC. As seen in Table 19C, maximal flow at 50 percent of VC was not affected by exposure when the size of the lungs was accounted for by referencing flow to VC. A plot of peak flow against vital capacity led to the same conclusion. Therefore no evidence for functional obstruction of the airways was observed in this assay.

The rate of diffusion of carbon monoxide from the lung was used as an index of the efficiency of gas exchange across the lung. Values from the single-breath maneuver, in mL/(mmHg) (min), are summarized in Table 20C. Significant ($p = 0.0001$) effects were observed for sex, exposure, dose-time, and dose-frequency. However, the absolute value for diffusing capacity would be related to lung size; larger lungs should have larger diffusing capacities. Therefore diffusing capacity was referenced to total lung capacity (in liters), as summarized in Table 21C. Significant effects

(p = 0.0001) were again found for sex, exposure, dose-time, and dose-frequency. Least squares means are shown below in Table 8.

TABLE 8. LEAST SQUARES MEANS OF DIFFUSING CAPACITY AND DIFFUSING CAPACITY/TLC (IN LITERS) VS. Ct PRODUCT, SEX, AND FREQUENCY

Ct (mg·hr/L)	Diffusing Capacity	Diffusing Capacity/TLC
0	0.964 ± 0.018	57.6 ± 1.0
8	0.899	55.4
12	0.769	48.5

Sex	Diffusing Capacity	Diffusing Capacity/TLC
M	1.108 ± 0.014	61.6 ± 0.8
F	0.647	46.1

	Diffusing Capacity		Diffusing Capacity/TLC	
	Frequency		Frequency	
Ct	1/wk	3/wk	1/wk	3/wk
0	0.939	0.989 ± 0.025	54.7	60.5 ± 1.9
8	0.969	0.829	57.1	53.7
12	0.876	0.663	52.3	44.7

Although there was not a consistent exposure-related decrease in CO diffusion, the low values were among the groups receiving 3 exposures/week and especially those at the higher exposure level (See Tables 20C and 21C).

Organ Weights

Wet weights were taken on the right middle lung lobe, liver, left kidney, and left adrenal gland. Mean weights for the liver, kidney, and adrenal are in Tables 22C, 23C, and 24C, respectively. As expected, liver weight was greater in males than in females (p = 0.0001, 15.3 ± 0.1 and 9.0 ± 0.1, respectively) and weight tended to increase with age. Overall, there was a decrease in least squares means related to exposure (p = 0.0001).

Mean weight in controls (both sexes combined) was 12.74 ± 0.16 g, 11.74 ± 0.16 g in groups given 8 mg·hr/L, and 11.90 ± 0.16 g in groups given 12 mg·hr/L.

Kidney weight was also lower in females than in males ($p = 0.0001$, 0.96 ± 0.01 g vs 1.61 ± 0.01 g, respectively) and tended to increase with age. However, there was not an exposure-related change in mean weights. As is evident in Table 24C, adrenal weight was greater in females ($p = 0.0001$; 33.6 ± 0.4 mg in females and 28.0 ± 0.4 mg in males). Although exposure to diesel fuel itself did not influence adrenal weight, there was a small but significant increase ($p = 0.0001$) among groups exposed 3 times per week (31.9 ± 0.4 mg) over those exposed once per week (29.7 ± 0.4 mg). Adrenal weight did not change with age. This effect of frequency of exposures thus did not appear to be related to the diesel fuel but more to the stress of handling during exposure.

Wet weight of the right middle lobe also did not change with age in either sex; but there was an increase positively correlated with exposure to diesel fuel ($p = 0.0001$), as seen in Table 25C. Lobes from males had a higher ($p = 0.0001$) weight than those from females (202 ± 2 mg and 152 ± 2 mg, respectively). The mean weights from both sexes together increased with the Ct product when examined at both 2 days and 2 weeks postexposure. However, the weights of lobes taken from exposed animals 2 weeks postexposure were significantly lower than those of lobes taken 2 days postexposure, as seen in Table 25C of the raw data and the least squares means shown in Table 9.

TABLE 9. LEAST SQUARES MEANS OF WEIGHT OF RIGHT MIDDLE LUNG LOBE VS. Ct PRODUCT AND RECOVERY PERIOD

Time	mg·hr/L		
Postexposure	0	8	12
2 days	152 ± 3	188 ± 3	213 ± 3
2 weeks	156 ± 3	172 ± 3	181 ± 3

Even though wet weight was increased by exposure, the wet to dry ratio of lung weight was not significantly changed. As seen in Table 26C, this ratio remained constant at just over a value of 5 in most groups. This consistancy in the wet to dry ratio indicated that the wet weight increases probably were predominantly cellular rather than from edema.

Histopathology

The lung was the only organ examined histologically that consistently had lesions which could be related to diesel fuel exposure. There were no exposure-related lesions observed in the remainder of the respiratory tract or in any of the other organs examined. Control animals had occasional areas of focal pneumonitis, consisting of local accumulations of inflammatory cells within the alveoli, and peribronchial and perivascular inflammatory lesions. Since the lesions in exposed animals were quite similar in appearance to those in controls, care was taken to discriminate between them. Lesions observed after diesel fuel exposure were generally focal accumulations of alveolar macrophages and other free cells in the alveoli and associated thickening of the alveolar wall with hypercellularity (probably lymphocytes and histocytes). The presence of septal fibrosis could not be determined on the H&E slides routinely prepared.

A summary of individual observations is given in Appendix D. Group summaries of the severity of pulmonary lesions are shown in Table 27C. The scoring method was standardized by the pathologist and was based on the relative numbers of alveolar free cells and the hypercellularity of the alveolar walls. As seen in the table, there was some overlap between control and exposed groups with some exposed animals having essentially normal lungs. It should be emphasized, however, that only one section of the lung was taken. This section cannot be taken as being necessarily representative of the focal lesions potentially distributed throughout the lungs.

The animals exposed to a Ct product of 12 mg·hr/L had generally higher scores using the scoring scheme described above than those of groups exposed to 8 mg·hr/L. Those examined at 2 weeks after exposure had fewer alveolar free cells (as seen in Table 7C) but more cellularity of the alveolar walls, with a possible slight increase in the number of interstitial cells. Large numbers of free cells still remained in the alveoli. Rupture of alveolar walls was most common in groups GA (6 mg/L, 2 hr/exposure, 3 exposures/wk) and CA (2 mg/L, 6 hr/exposure, 3 exposures/wk), the two groups exposed to a CT product of 12 at the higher exposure frequency. Rupture was also noted in one animal each from groups DA (4 mg/L, 2 hr/exposure, 3 exposures/wk) and HA (control).

The control group HA also had a high incidence of liver and kidney alterations which were not observed among exposed animals (see Appendix D). These lesions were mild and were observed only among those animals killed at 2 days postexposure. They could not be related to housing conditions (or any other known factor) and appeared to be a random incidence of normal lesions observed in these animals. No histopathologic reason for the decrease in liver weight among exposed animals was observed.

Neurotoxicity

No significant alterations, which could be related to diesel fuel exposure, were observed in any of the four neurotoxicity assays. In each assay, pre-exposure values served as baseline data. Observations made at

one day or two weeks after the last exposure were related to these pretreatment values for each individual, and the changes in a given parameter for each individual animal were analyzed statistically. Thus, each animal served as its own control.

Pre-exposure means for forelimb grip strength appear in Table 28C. It is apparent from the table that males had higher values. And analysis of least squares means revealed a significant difference (0.97 ± 0.02 kg for males and 0.84 ± 0.02 kg for females, p = 0.0001). No exposure-related changes from the pre-treatment values were observed at 1 day or 2 weeks after the last exposure. The magnitudes of the observed decreases are summarized in Table 10.

TABLE 10. LEAST SQUARES MEANS OF CHANGE FROM PRE-EXPOSURE VALUE OF FORELIMB GRIP STRENGTH VS. Ct PRODUCT AND TIME POSTEXPOSURE

Ct Product mg•hr/L	1 Day Postexposure	2 Weeks Postexposure
0	-0.02 ± 0.03	-0.04 ± 0.04
8	-0.09 ± 0.03	-0.07 ± 0.04
12	-0.07 ± 0.03	-0.13 ± 0.04

Pre-exposure means for landing foot spread are shown in Table 29C. Males again had significantly greater values (114 ± 2 min vs 92 ± 2 min, p = 0.0001) than females and there were no significant changes related to exposure. Least squares means of the differences between pre-exposure and postexposure values are illustrated in Table 11.

TABLE 11. LEAST SQUARES MEANS OF CHANGE FROM PRE-EXPOSURE VALUE OF LANDING FOOT SPREAD VS. Ct PRODUCT AND TIME POSTEXPOSURE

Ct Product mg•hr/L	1 Day Postexposure	2 Weeks Postexposure
0	8.0 ± 3.3	6.0 ± 3.3
8	-1.2 ± 3.3	1.2 ± 3.3
12	3.9 ± 3.4	3.2 ± 3.3

Pre-exposure values for tail flick are in Table 30C. Males reacted more slowly than females with tail flick times of 1.73 ± 0.04 sec and 1.53 ± 0.04 sec respectively (p = 0.001). There was a significant exposure-related decrease at 1 day after the last exposure (p = 0.0003),

but this effect was not present two weeks later. If the effect was real, it was only transient in nature. The least squares means of the changes in tail flick time are shown in Table 12.

TABLE 12. LEAST SQUARES MEANS OF CHANGE FROM PRE-EXPOSURE VALUE OF TAIL FLICK VS. Ct PRODUCT AND TIME POSTEXPOSURE

Ct Product mg•hr/L	1 Day Postexposure	2 Weeks Postexposure
0	0.02 ± 0.07	-0.11 ± 0.08
8	-0.40 ± 0.07	-0.26 ± 0.08
12	-0.13 ± 0.07	-0.10 ± 0.08

Three types of data were derived from the startle reflex assay: reaction time (msec from the sound stimulus to start of response), peak time (msec from stimulus to maximal response), and peak height (magnitude of maximum reaction). Pre-exposure values for each are presented in Tables 31C-33C. There was a significant sex difference for each ($p = 0.0065$ for peak time and $p = 0.0001$ for reaction time and peak height). As could be expected, the peak height was greater in males than in females (least squares means of 357 ± 14 and 246 ± 15 g. wt., respectively). Reaction time was slightly longer in males (17.9 ± 0.1 vs 17.3 ± 0.1 msec), as was peak time (27.3 ± 0.3 vs 26.3 ± 0.3 msec). A significant block effect ($p = 0.001$) was observed only with peak height and this effect was compensated for in the statistical analysis.

At one day and at two weeks after the last exposure, reaction time and peak time were not significantly effected by exposure. The initial least squares means and differences between postexposure and pre-exposure values are given in Tables 13 and 14.

TABLE 13. PRE-EXPOSURE MEANS AND POSTEXPOSURE DIFFERENCES IN REACTION TIME IN THE STARTLE REFLEX ASSAY (LEAST SQUARES MEANS)

Ct Product mg•hr/L	Pre-exposure Mean (msec)	Postexposure Difference	
		1 Day	2 Weeks
0	17.7 ± 0.2	0.4 ± 0.3	-0.5 ± 0.2
8	17.5 ± 0.2	-0.2 ± 0.3	-0.2 ± 0.2
12	17.6 ± 0.2	0.1 ± 0.3	-0.2 ± 0.2

TABLE 14. PRE-EXPOSURE MEANS AND POSTEXPOSURE DIFFERENCES IN PEAK TIME IN THE STARTLE REFLEX ASSAY (LEAST SQUARES MEANS)

Ct Product mg•hr/L	Pre-exposure Mean (msec)	Postexposure Difference	
		1 Day	2 Weeks
0	26.7 ± 0.4	1.0 ± 0.5	0.6 ± 0.5
8	26.5 ± 0.4	0.1 ± 0.5	0.3 ± 0.5
12	27.1 ± 0.4	0.5 ± 0.5	0.6 ± 0.5

Unlike pre-exposure reaction time and peak time, pre-exposure values for peak height were slightly affected by dose group ($p = 0.014$) (see Table 15).

TABLE 15. PRE-EXPOSURE MEANS AND POSTEXPOSURE DIFFERENCES IN PEAK HEIGHT IN THE STARTLE REFLEX ASSAY (LEAST SQUARES MEANS)

Ct Product mg•hr/L	Pre-exposure Mean	Postexposure Differences	
		1 Day	2 Weeks
0	347 ± 20	61 ± 21	52 ± 22
8	263 ± 20	127 ± 21	114 ± 22
12	294 ± 20	90 ± 21	97 ± 22

Those groups with lower initial heights tended to have greater increases by one day postexposure, giving overall postexposure means within a relatively narrow range of 377 to 408 g. wt. Although there were not significant differences among the treatment groups in the height increases, it appeared as though initial variability among the groups decreased with age as the peak heights moved toward a common value of about 390 g. wt. regardless of aerosol exposure. Referencing values to body weight or age did not result in exposure-related differences for any of the neurotoxicity assays.

Clinical Chemistry

Both red and white blood cell counts were performed in addition to clinical chemistry on serum samples. There was a significant ($p = 0.0001$) block effect in RBC numbers. After compensation for the block effect, there still were significant effects of exposure and frequency of exposure ($p = 0.001$). The interaction of these two factors is demonstrated in Table 16.

TABLE 16. LEAST SQUARES MEANS OF RED BLOOD CELL COUNT VS. Ct PRODUCT AND FREQUENCY

Ct Product mg·hr/L	Exposures/Week 1	Exposures/Week 3
0	4.87 ± 0.10	5.09 ± 0.10
8	5.06 ± 0.10	4.32 ± 0.10
12	4.72 ± 0.13	4.30 ± 0.10

The number of RBC's was decreased among groups exposed 3 times/week but not among those exposed once weekly.

Changes in WBC number resulting from exposure were not as clear. Males had higher counts (3.79 ± 0.12 vs 1.78 ± 0.13 WBC × $10^3/mm^3$ in females, p = 0.0001). Those groups exposed 3 times/week had higher counts than those exposed once weekly (3.08 ± 0.12 vs 2.49 ± 0.13 WBC × $10^3/mm^3$, p = 0.0001). But the effect of exposure was marginal (p = 0.032). Least squares means were 3.13 ± 0.16 for controls, 2.54 ± 0.16 for a Ct product of 8 mg.hr/L, and 2.68 ± 0.19 for a Ct product of 12 mg.hr/L. Therefore, there was only a tendency for exposure to result in lower WBC counts.

A variety of clinical chemistry assays were performed on serum samples. These samples were frozen shortly after collection and assayed within 4 days. Dr. Carl Burtis of ORNL's clinical chemistry laboratory stated that only LDH values would be affected by this procedure (compared with fresh serum samples). Very little was observed which could be related to diesel fuel exposures. Therefore, only least square means for sham-exposed controls are presented in Table 34C to provide an idea of the data obtained. Alkaline phosphatase activity and concentrations of triglyceride and sodium were the only blood chemistry measurements that were affected significantly by diesel fuel exposure.

Alkaline phosphatase activity increased in exposed animals but only in females and with marginal statistical significance (p = 0.03), as illustrated in Table 17.

TABLE 17. LEAST SQUARES MEANS OF ALKALINE PHOSPHATASE ACTIVITY VS. Ct PRODUCT

Ct Product mg·hr/L	Males	Females
0	150 ± 7	108 ± 7
8	139 ± 7	135 ± 7
12	154 ± 7	148 ± 10

After consideration of the variability apparent from the standard errors, this increase can be considered slight. The increase in sodium concentration was also small (144.8 ± 0.3 in controls to 147.4 ± 0.3 in exposed groups) but significant ($p = 0.0001$). Triglyceride levels were also directly affected by exposure ($p = 0.005$), decreasing after exposure from 123.2 ± 5.1 for Ct = 0 to 80.0 ± 5.0 for Ct = 8 and 79.2 ± 6.0 for Ct = 12 mg•hr/L. The effect appeared to be transient; triglyceride rose significantly ($p = 0.0001$) from 82.0 ± 3.7 mg percent at 2 days after the last exposure to 106.3 ± 4.3 mg percent two weeks later. This pattern of exposure-related change in triglyceride was similar to those of body weight and food consumption and may be related to them.

DISCUSSION

The overall objective of this study, the second of three phases of which is reported here, was to determine the biologic effects of exposure to variations in aerosol concentration, duration of each exposure, and frequency of exposures. During a series of single acute exposures (the first phase of the study), mortality was observed to be highly related to the Ct product (2). Therefore, the Ct product was used in this study as a convenient parameter for setting exposure conditions. The primary objective of the study, however, was not to investigate the relation of the Ct product to given endpoints, but the relation of time, concentration, and frequency to those endpoints. Elucidation of a "dose"-response in terms of the Ct product was considered a secondary objective and no presumption was made on the linearity of a Ct-response curve.

The combinations of exposure durations and concentrations used in Phase 2 were based on mortality data from the acute exposures. Initially, a Ct-product of 8 mg•hr/L was estimated to be the lower confidence bound for LCt_{01} in acute exposures of Phase 1, and therefore, a maximum tolerated exposure level for repeated exposures in Phase 2. However, after a series of preliminary exposures with a Ct of 8 mg•hr/L as a maximal "dose", it was decided to start exposures again with 12 mg•hr/L as an upper limit, even though there was a significant likelihood of deaths as a result of the higher exposure level. This decision was based on the lack of highly significant effects from the initial series of exposures with 8 mg•hr/L. Mortality from the subsequent repeated exposures to 8 and 12 mg•hr/L approximated that expected on the basis of acute exposures. About 6 percent of the animals receiving the higher exposure level (12 mg•hr/L) died, while there were no deaths with 8 mg•hr/L.

Body weight was also affected by exposure to the aerosol, particularly among the groups exposed 3 times per week in which mean weights were depressed over the entire 3 weeks of exposure. Depressions in food consumption generally paralleled those in body weight. To help place the exposure conditions into perspective, exposures three times per week to Ct products of 8 or 12 mg•h/L caused a depression in body weight approximately 10 percent below that of controls. A maximum tolerated dose can be defined as a dose (Ct product) which produces less than a 10 percent depression in

body weight. In addition, approximately 6 percent of animals exposed to 12 mg•hr/L died; a maximum tolerated dose should result in no deaths. By either criteria, body weight or mortality, the exposure regimen used in Phase 2 resulted in effective doses of diesel fuel which could not have been increased without quite deleterious effects.

Of the organs examined, the lungs were most significantly affected by exposure. The small particle size (mass median diameter of about 1 μm) probably favored aerosol deposition in the lung rather than upper airways. Thus no effects were observed in the airways histologically or in pulmonary function tests associated with abnormalities in the airways. In the lung parenchyma, however, there was histologic evidence of generally focal accumulations of alveolar macrophages and other free cells in the alveoli along with associated thickening of the alveolar wall with hypercellularity. Those groups exposed to 12 mg•hr/L generally had the most severe lesions and, during the two-week postexposure period, the number of free alveolar cells decreased while the cellularity of the alveolar walls worsened.

The number of lavaged pulmonary free cells correlated well with histologic observations. The number of macrophages and other cells, predominantly neutrophils, was increased greatly in lavages at 2 days postexposure. After two subsequent weeks with no exposures, the number of macrophages remained elevated while the number of other free cells returned to control values. These observations appeared to reflect a persistent effect of the aerosol.

Additional evidence of changes in the lung parenchyma was seen in the pulmonary function tests. Carbon monoxide diffusing capacity was decreased in several exposed groups at both two days and two weeks after exposure, reflecting an impairment in gas diffusion from the lung to blood. Also, exposed groups usually showed changes in lung volumes including increased functional residual capacity (FRC) and decreased total lung capacity (TLC) and vital capacity (VC). These changes were accompanied by a decrease in specific lung compliance at low lung volumes.

FRC and TLC were most affected in groups CA and GA (12 mg•hr/L, 3 exposures/wk) and DA (8 mg•hr/L, 3 exposures/wk), as seen in the tables of results. Although the amount of histopathological examination of the lungs was limited to one slide per animal, it is interesting to note that alveolar rupture was noted most commonly in these groups, particuarly CA and GA. A decreased vital capacity, increased FRC and observable alveolar ruptures indicated the possibility of significant damage to the architecture of the lung parenchyma. Morphometric analysis of the lung could provide a quantitative correlation to the physiologic and more subjective histopathologic changes observed. However, it is very time consuming and tedious. Therefore, a limited amount of morphometry was performed on lungs of males from four groups, although it was not originally planned. The paraffin sections were examined at a magnification of 250X with a Weibel reticule and the mean linear intercept (Lm) in alveolar parenchyma determined. Results are given in Table 18.

TABLE 18. MEAN LINEAR INTERCEPT AND FUNCTIONAL RESIDUAL CAPACITY

Group	mg	Hours Exposure	Exposures Week	FRC (mL)	Lm (μm)
EA	0	2	1	3.0	105 ± 3
LA	0	2	3	3.7	102 ± 3
FA	2	6	1	3.7	190 ± 5
CA	2	6	3	4.9	116 ± 5

Although these data are extremely limited, it would seem that there is a relationship between alveolar rupture and mean linear intercept. Furthermore, although not reflected in this table, there appears to be a correlation between Ct, the amount of alveolar rupture observed, and FRC.

The increase in pulmonary free cell number was expected to occur after nine exposures. But the extent of changes in lung volumes was rather surprising. Whether these changes observed in the lung would resolve with additional time is not known; but there is the potential for essentially permanent damage to the lung.

There were more systemic changes observed in exposed animals other than those in the lung. Liver weight was decreased even though no abnormalities were observed histologically. The number of circulating red blood cells decreased by about 13 percent among groups exposed 3 times per week, but not in groups exposed once per week. The concentration of sodium in the serum of exposed groups increased by about 2 percent. And there was a transient decrease in serum triglyceride which seemed to parallel changes in body weight and food consumption. Of these observations, only the altered triglyceride levels could be related to other exposure-related changes.

None of these systemic changes can be considered of great biologic significance at this point. Liver weight, triglceride level and food consumption may be related to each other and reflect the stress of exposure. The change in serum sodium, a 2 percent increase, was statistically significant only because the variance for this parameter is normally very small. The decrease in circulating RBC's, although observed only with the higher frequency exposures, indicated a possibly significant impairment which should be monitored closely in future experiments.

In regard to the original objective of this study concerning the relative importance of concentration, duration of exposure, and frequency of exposures, it was frequency which appeared as the dominant variable over the range of parameters used in this study. Duration (2

or 6 hrs) appeared to have very little effect and a "dose"-response was often not apparent with differences in concentration.

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APPENDIX A: STATISTICAL CONSIDERATIONS IN EXPERIMENTAL DESIGN AND ANALYSIS

Experimental Design

The purpose of Phase II was to investigate the biological nature of the rat's response to diesel fuel aerosol, and in particular, to assess the relative importance of the factors C (concentration), t (time or duration of exposure), and F (frequency or number of exposures per week). Resource limitations on Phase II were the availability of only four chambers equipped for exposure to diesel fuel aerosol and which would each contain a maximum of 48 rats, and the manpower to run the various assays of interest on 4 × 48 = 192 rats during any period of time of length negligible to the growth and development of the rat.

The design of Phase II depended on two basic inferences from the acute exposures of Phase I. These were (1) that mortality could be modeled roughly as a function of the Ct product, and (2) that a Ct product of about 8 mg·hr/liter would, with reasonable certainty, lead to less than 1 percent mortality, at least for acute exposures. These conclusions were based on a statistical analysis of the Phase I data (2) which involved fitting and testing various models in C, t, and Ct, and extrapolating to a lower confidence bound for the LD_{01} (one-percent-mortality dose).

Because of the multiplicative nature of C and t in determining mortality, interesting ranges of C and t lead jointly to a Ct range surpassing 8 hr-mg/L and extending to Ct for which there would likely be no discernible effect at all. Therefore if C and t were design factors it would not be reasonable to employ a factorial design, that is one for which observations are taken at all possible combinations of specified factor levels. Factorial designs are desirable because they are generally much more efficient at estimating effects of interest, than are other designs. Thus, in order to achieve a factorial design, treatments were defined jointly by Ct, t, and F, instead of C, t, and F. An additional advantage of the design is that by comparing results for different t, the Ct-model for all observed responses could be assessed.

Two exposure durations were selected, t = 2 and t = 6 hours. Frequency regimes of 1 time per week for 9 weeks and 3 times per week for 3 weeks were also chosen. Ct levels of 0 (control), 8/3 and 8 hr-mg/liter were chosen, but these were later changed to 0, 8, and 12. (In view of the results of an initial aborted attempt at Phase II, it was decided that the Ct levels 8/3 and 8 were too low to give a significant response.) Thus 12 Ct-t-F treatment groups were needed. With interest in both sexes, in effects immediately post treatment as well as after a recovery period of two weeks, and with various assays some of which are invasive, it was necessary to assign 48 rats in all to each of the Ct-t-F groups. These twelve groups were to be run in three blocks of four. A good design for this experiment would permit estimating effects of interest without block bias, and with minimal variability.

The natural approach to designing the above constrained experiment was to try to equate the block effects with the Ct-t-F interaction. (There are two degrees of freedom for each.) Then all other effects could be estimated without bias. It was not possible to do this. Some bias in estimating other effects was therefore unavoidable, and it was necessary to select a design on the basis of what could be estimated without bias, as well as variability.

Another approach to designing Phase II was to assume that certain effects are negligible and to choose a design which estimates all other effects most efficiently. Attention must also be paid to the consequence of the negligible-effect assumption being false. Designs were found both for the assumption of negligible Ct-t-F interaction and for the assumption that the effect of block itself is negligible. (An assumption in both designs is that there are no interactions involving Ct, t, or F with block.) The former assumption (i.e. no Ct-t-F interaction) was considered to be the more tenable of the two, since if responses could be modeled as functions of Ct, then given Ct, t would be a factor of no importance at all. Therefore the design finally selected (described in Appendix B) is the one based on the former assumption. Under this assumption, all effects (including block effects) could be estimated without bias. A non-negligible Ct-t-F interaction, on the other hand, would bias both Ct and block effect estimates (cf, proposed design for Phase II).

Analysis of Data

The basic statistical method used to analyze the Phase II data is analysis of variance. The analysis of variance models, which were fitted by least squares, include terms in Ct, t, F, sex, and block. Certain groups of endpoints, the lung lavage, pulmonary function, and clinical chemistry assays were invasive. For these groups individual rats could not serve as their own controls (pre- vs post-treatment), and so an additional factor, TRT, with levels immediately post-treatment, and two weeks post-treatment, was included. For noninvasive end points, the weight and feeding studies and the neurotoxicity studies, pre- and post-treatment measurements were on the same rat and hence not independent, and so TRT could not be included in the model.

For noninvasive endpoints the analysis of variance model was

Endpoint = Intercept + Ct + t + F + sex + block
+ Ct*t + Ct*F + Ct* sex + T*F + T*sex
+ F*sex + Ct*t*sex + Ct*F*sex
+ T*F*sex + error

For invasive endpoints the model includes all terms listed above plus TRT and interactions involving TRT and all terms above except block, intercept, and, of course, error.

Interactions involving block were assumed negligible. The design precluded estimation of interactions involving block and Ct, t or F (inclusive) anyway. Since block was included as a factor, estimates of

other effects were adjusted for blocks. If, as is assumed in the above model, block effects are truly additive, the other effects are estimated without block bias. Fitting this model makes possible estimating the response of factor combinations other than those explicitly observed in the experiment. For example, data were recorded for Ct = 12, t = 6, F = 3, and block = 1, but not for Ct = 12, t = 6, F = 3, and block = 2 or 3. However the response at these latter, unobserved, treatment combinations is still estimated — as is clear from the model. Thus by fitting the model we can predict the outcome of a larger, ideal experiment in which all Ct-t-F treatment combinations are run in each of blocks 1, 2, and 3. By comparing all of these predictions or cell means, we can estimate treatment effects without block bias.

Simple averages of cell means are called least squares means and are an essential part of the analysis of Phase II. Least squares means take the place of usual means in situations in which block effects bias comparisons of usual means. Comparisons of least squares means are unbiased because they take all blocks into account equally. Comparisons of least squares means can be shown to produce the test statistics that we have used to test the significance of the various factors.

All the linear model analyses discussed here were performed using SAS GLM, and all hypothesis tests were "TYPE IV" (Goodnight, 1978).* The following discussion is intended to supplement that included in the main text.

Weight Study

Weekly body weights were recorded for all rats for the first five weeks of the study and for eleven weeks for rats exposed once/week. Initially, the data appeared to be free of outliers, though after weekly weight gains were computed, several obviously fallacious recordings were detected. These were amended after examining the records more closely.

Various transformations such as weight-divided-by-pretreatment-weight as well as weight gains and the untransformed data were analyzed. The untransformed data exhibited very strong block effects. It is particularly important to eliminate block effects after week 5. This is because data from both frequency classes are necessary to estimate the effect of block, but no F = 3 weight data are available after week 5. Weekly weight gains showed block effects which died out after the third week (males) or fourth week (females). Since, as is evident from graphing group mean weights over time, growth is approximately linear, block effects due to different starting weights are essentially eliminated by transforming to weight gains. (Different starting weights accounted for almost all of the observed differences between blocks.)

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*Goodnight, J. H. (1978) "Tests of Hypotheses in Fixed Effects Linear Models," SAS Technical Report R-101, Cary, NC: SAS Institute, Inc.

Feeding Study

The feeding study data consists of food consumption measurements (in grams) for up to ten weekly or nearly weekly intervals. Routine examination revealed no outliers. As with the weight study the issue of block bias is particularly critical to the analysis of data from weeks for which only F = 1 recordings were made. For intervals 1-5 all block-effect tests were nonsignificant except for males in interval 5 ($p = 0.03$). We assumed therefore that the effect of blocks is negligible in intervals 6-10. Hence Ct, t, and F effects can be estimated for these intervals as well.

Neurotoxicity Data

Neurotoxicity assays included startle reflex, grip strength, splay, and tail flick assays. The latter three assays were analyzed by analysis of variance in a straightforward manner. However, the startle reflex parallels that for the neurotoxicity data and therefore will not be discussed further here.

Each observation in the startle reflex data set consists of several measured characteristics of the response of a rat to a startling sound. Measurements were made on each rat prior to treatment (PRT), immediately post treatment (IPT), and two weeks post treatment (TWPT). At these particular times a series of five trials was made, and up to five measurements on each end point were recorded, depending on how many trials resulted in responses.

Out of a total of 2140 trials (PRT, PIT, and TWPT), 81 trials resulted in no response. An additional 7 responses yielded reaction times that were absurdly low (less than 5 msec — the rat probably decided to move for some other reason before he heard the sound), and 8 responses had absurdly high reaction times (more than 40 msec — the rat didn't respond but he started to move for some other reason). These latter 15 observations were also taken to be non-responses for a total of 96 non-responses in 2140 trials (4.5 percent). After discarding these 15 observations the data seemed generally free of outliers for all variables.

Most rats responded at least four times in any particular set of five trials, and no rat failed to respond in all five trials. Rats from every exposure group failed to respond and there was no clear connection between exposure and non-response. For this reason, and because there were so few non-responders anyway, the frequency of response was not analyzed further, at least in terms of exposure. Frequency of response nevertheless did affect the analysis as will be described. To analyze the data, means were computed for the up-to-five trials for each rat at PRT, IPT, and TWPT. Data for each rat were summarized as a mean of up-to-five observations. These means were in turn converted to differences IPT-PRT and TWPT-PRT (PRT values alone were also analyzed as a check).

Analyses of the differences would have been by straightforward analysis of variance except for the fact that the unequal numbers of responses used

to compute the means implies that the variances of the differences are heterogeneous. Thus a weighted analysis must be used. Proper weighting also involves estimating the correlation among observations on a given rat at a given time (PRT, IPT, and TWPT), - above and beyond that correlation associated with features of the rat which are stable in time and therefore cancel out when differences are computed. This (former) correlation was estimated and found to be quite small (0 - 0.15 for the various endpoints). Because there were very few non-responses, adjustment for a correlation this small has negligible effect on the analysis of variance and was assumed to be zero in all cases. The differences were weighted accordingly.

We considered adjusting maximum force and tensing rate by weight since it seems clear that these endpoints could depend on weight. However, this was not observed from the data as plots of peak height vs weight showed no discernible trend. Since we knew already that weight was affected by exposure, we chose not to adjust for it.

In addition to analyzing differences of means (post-treatment minus pretreatment), analyses were performed on pretreatment means, means at 2 days post-exposure, means at 2 weeks post-exposure, and the differences divided by pretreatment — 4 values. The results seemed clearest when expressed in terms of the differences.

Clinical Chemistry Assays

Clinical chemistry measurements were made (on different rats) both immediately post treatment (IPT) and two weeks post treatment (TWPT). Thus for the clinical chemistry data, we have the additional factor of treatment (TRT) with levels IPT and TWPT. The data set is relatively free of outliers, though there were a few unusual responses. Statistical analyses were made both with and without these unusual parts and inferences were generally the same in both cases (results are reported for the "with" case). The analysis of variance is straightforward.

Lung Lavages

Variables in this data set fall into two groups — lavaged cells and phagocytosis data. The phagocytosis data are the numbers of individual macrophages having attached yeast cells out of about 1000 macrophages scored for each rat. Scoring was 0,1...7, and >7 attached yeast cells/ macrophage. Analysis of the lavaged cell data parallels the analysis of variance of the clinical chemistry data. Again we have the factor TRT.

Several of the endpoints in the lavaged cell data may have distributions which are not Gaussian. For example the IPT values for total lavaged cells, group B females are:

6.87
6.54
12.12
4.23
6.90
7.71.

Overall, however, 12.12 is not unusual, and neither is this sort of pattern. Nevertheless, the standard analyses of these endpoints, which follow, generally make good sense. While the p-values quoted may only be approximate we did not feel that it was worth pursuing a robust analysis.

Weight adjustment was also investigated, but as with the startle reflex data there were no clear trends evident in plots of lavaged cells, lavaged macrophages, etc. against weight. Still in all some of the weight-adjusted analyses are a little more clear. Thus we included weight-adjusted endpoints in the analysis.

The phagocytosis data are extensive enough that condensing them even for each rat is advantageous. To this end, we fit the observed attached-yeast cell counts to two distributions, the Poisson and the negative binomial. Chi-square goodness of fit (GOF) tests revealed that the Poisson distribution fit the data quite poorly. GOF tests showed that the yeast counts sometimes departed significantly from the negative binomial distribution, but to a much lesser degree than from the Poisson. Since about 1000 cells were scored for each rat, even small departures can be quite significant. We feel that for our purposes the negative binomial distribution provides adequate fit to the phagocytosis data.

The negative binomial distribution can be completely characterized by its mean and variance. Accepting that this distribution provides adequate fit to the data then enables us to consider only sample means and sample variances. The mean here is the binding index. The variance does not have any clear interpretation and so we did not further analyze sample variances.

Pulmonary Function

This data set was remarkably complete and free of outliers. Again TRT is a factor and the analysis parallels that for the clinical chemistry data.

APPENDIX B: EXPOSURE AND ASSAY SCHEDULE

Animals were exposed either once per week or three times per week for a total of 9 exposures. Following exposure, half of each group was used in assays designated as "immediately post-exposure". The other half of the group was held in our animal facilities for a further two weeks, without additional exposure, before assay. These animals are refered to as "two weeks postexposure" in the text.

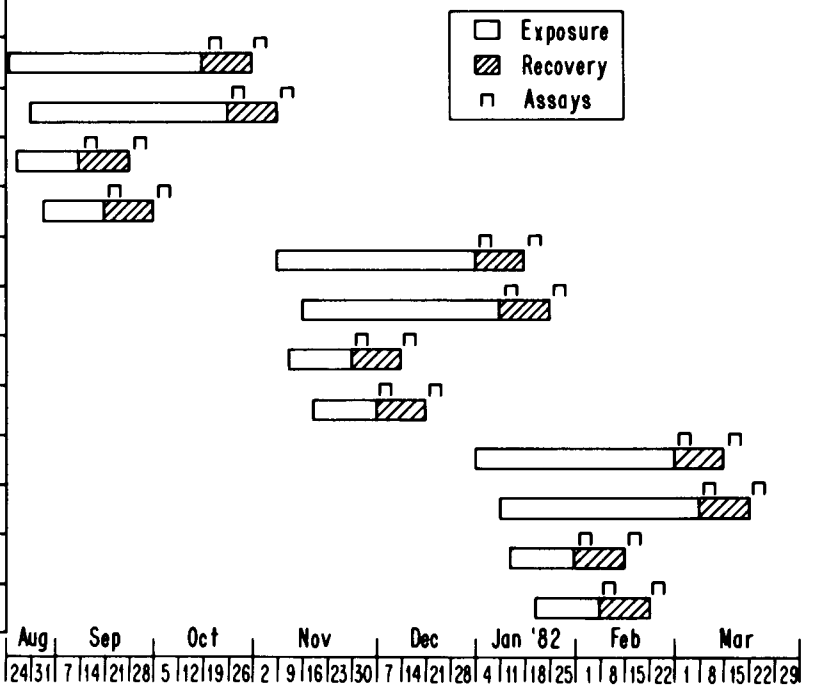
In the following tables (1B and 2B) the exposure and assay schedule and the utilization of animals are illustrated.

TABLE 1B. DISPOSITION OF ANIMALS USED IN STUDY

	Assay	Animal Numbers
Immediately Postexposure	Alveolar Macrophage Blood Urine	1 - 6 M 1 - 6 F
	Pulmonary Function Histology	13 - 18 M 13 - 18 F
	Neurotoxicology Food Consumption	19 - 24 M 19 - 24 F
Two Week Postexposure	Alveolar Macrophage Blood Urine	7 - 12 M 7 - 12 F
	Neurotoxicology Pulmonary Function Histology	19 - 24 M 19 - 24 F

TABLE 2B. EXPOSURE AND ASSAY SCHEDULE FOR PHASE 2 DIESEL FUEL AEROSOL EXPOSURE

Group Designation	Exposure Regime Ct	mg/L	hr	Times/week
AA	12	6	2	1
BA	8	1.3	6	1
CA	12	2	6	3
DA	8	4	2	3
EA	0	0	2	1
FA	12	2	6	1
GA	12	6	2	3
HA	0	0	6	3
JA	8	4	2	1
KA	0	0	6	1
LA	0	0	2	3
MA	8	1.3	6	3



APPENDIX C: DEFINITIONS AND TABULATED DATA

Exposure:	The condition of being exposed to diesel fuel aerosol, regardless of concentration, frequency and duration, as opposed to the sham exposures of controls where diesel fuel aerosol was not introduced into the chamber.
Frequency:	Either 1 or 3 exposures per week. All animals were exposed nine times, so that those exposed once per week were exposed for 9 weeks and those exposed 3 times a week were exposed for 3 weeks.
Concentration:	The mass concentration of aerosol in the chamber during exposure as measured by a mass monitor, calibrated by gravimetric samples (see Table 1C).
Time postexposure:	Half of the animals were assayed in the first two days post exposure. The remainder were kept for two weeks after the last exposure before being assayed in order to allow either recovery or progression of biological changes due to exposure.
Duration:	All animals were exposed for either 2 or 6 hours per exposure.
Sex:	Equal numbers of male and female rats were used in each exposure group and for determination of each biological end point except when this was prevented by mortality.
Ct Product:	The multiplication product of concentration (mg/L) and exposure duration (hrs).

TABLE 1C. MEAN AEROSOL CONCENTRATION DETERMINED GRAVIMETRICALLY (MEAN ± S.E.) AND TARGET CONCENTRATION FOR EACH TREATMENT GROUP. VALUES ARE IN mg/L.

Hours of Exposure	Target Concentration	Measured Concentration	Exposure Group
6	1.33	1.40 ± 0.04	B
	1.33	1.5 ± 0.05	M
	2	2.18 ± 0.03	F
	2	2.20 ± 0.07	C
2	4	4.24 ± 0.03	J
	4	3.88 ± 0.21	D
	6	5.73 ± 0.30	A
	6	5.75 ± 0.09	G

TABLE 2C. LEAST SQUARE MEAN VALUES FOR BODY WEIGHT (g) DIFFERENCES DURING FIRST 2 WEEKS OF EXPOSURE FOR MALES (FEMALES) IN RELATION TO EXPOSURE TIME (GRAMS)

Ct Product mg•hr/L	Hours/Exposure	
	2	6
0	30.7 (14.4)	27.4 (13.9)
8	6.4 (-2.8)	-11.1 (-14.3)
12	-2.4 (-5.1)	-8.9 (-9.9) ± 1.7 (1.1)

TABLE 3C. LEAST SQUARE MEAN VALUES FOR BODY WEIGHT (g) DIFFERENCES DURING FIRST 2 WEEKS OF EXPOSURE FOR MALES (FEMALES) IN RELATION TO EXPOSURE FREQUENCY

Ct Product mg•hr/L	Exposures/Week	
	1	3
0	27.1 (13.3)	31.1 (14.9)
8	14.5 (-0.8)	-19.3 (-16.3)
12	-1.1 (-4.6)	-10.2 (-10.3) ± 1.7 (1.1)

TABLE 4C. LEAST SQUARE MEAN VALUES FOR FOOD CONSUMPTION (g/day) DURING FIRST 3 WEEKS OF EXPOSURE FOR MALES (FEMALES) IN RELATION TO Ct PRODUCT

Ct Product mg•hr/L	Exposure Week		
	First	Second	Third
0	29.3 (22.5)	29.3 (21.2)	29.1 (21.6)
8	27.0 (18.8)	26.3 (19.2)	26.8 (19.7)
12	25.3 (17.0)	26.2 (19.1)	26.7 (18.3)
Standard Error	0.6 (0.5)	0.7 (0.9)	0.7 (0.4)
P-Value	0.0002 (0.0001)	(0.004) (0.19)	0.03 (0.0001)

TABLE 5C. LEAST SQUARE MEAN VALUES FOR FOOD CONSUMPTION (g/day) DURING FIRST 3 WEEKS OF EXPOSURES FOR MALES (FEMALES) IN RELATION TO FREQUENCY OF EXPOSURE

Exposures/wk	Exposure Week		
	First	Second	Third
1	28.1 (21.0)	28.6 (20.3)	29.2 (20.5)
3	26.4 (18.9)	25.9 (19.3)	25.8 (19.3)
Standard Error	0.4 (0.4)	0.5 (0.6)	0.5 (0.3)
P-value	0.01 (0.05)	0.0004 (0.19)	0.0001 (0.01)

TABLE 6C. MILLIONS OF ALVEOLAR MACROPHAGES LAVAGED FROM LUNGS DURING PHASE 2 ASSAYS (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	2.99±0.54	2.68±0.32	2.67±0.59	2.25±0.48
0	6	1	1.89±0.23	1.73±0.37	3.95±0.74	2.54±0.37
0	2	3	2.26±0.52	1.82±0.20	2.48±0.34	1.37±0.19
0	6	3	2.22±0.20	1.71±0.19	2.48±0.29	1.96±0.41
4	2	1	4.18±1.03	2.27±0.57	7.28±1.04	4.22±0.59
4/3	6	1	4.94±0.83	3.14±0.71	6.67±1.01	3.16±0.48
4	2	3	5.83±2.15	5.10±0.73	4.06±0.83	3.54±0.47
4/3	6	3	4.07±0.81	4.33±0.68	4.58±0.63	3.45±0.46
6	2	1	4.74±0.50	3.08±0.68	5.29±0.75	-
2	6	1	6.18±1.17	2.64±0.46	6.18±1.15	3.13±0.49
6	2	3	4.83±0.66	4.48±0.61	3.48±0.46	3.20±0.46
2	6	3	6.69±0.70	2.99±0.55	9.01±1.91	4.82±1.07

TABLE 7C. MILLIONS OF PULMONARY FREE CELLS OTHER THAN ALVEOLAR MACROPHAGES LAVAGED FROM LUNGS DURING PHASE 2 ASSAYS (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	2.05±1.20*	1.64±0.90	0.22±0.08	0.19±0.04
0	6	1	0.15±0.04	0.26±0.15	1.40±0.78	0.15±0.03
0	2	3	0.20±0.04	0.09±0.02	0.24±0.09	0.09±0.02
0	6	3	0.30±0.08	0.12±0.02	0.26±0.12	0.06±0.02
4	2	1	6.99±1.22	4.88±0.58	0.78±0.15	0.43±0.10
4/3	6	1	10.45±2.14	4.26±0.53	0.32±0.07	0.14±0.02
4	2	3	3.75±1.59	2.54±0.63	0.40±0.10	0.25±0.07
4/3	6	3	3.26±0.55	1.49±0.49	0.11±0.03	0.09±0.02
6	2	1	9.56±2.33	8.21±2.57	0.18±0.06	-
2	6	1	4.92±2.29	3.21±1.24	0.20±0.06	0.11±0.06
6	2	3	3.83±0.72	3.74±0.81	0.61±0.14	0.17±0.05
2	6	3	6.16±2.17	3.85±1.11	0.94±0.27	0.31±0.07

*One outlier was deleted.

TABLE 8C. BINDING INDEX (MEAN NUMBER OF YEAST/MACROPHAGE) IN PHAGOCYTOSIS ASSAY OF PHASE 2. (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	1.69±0.17	1.26±0.10	0.73±0.12	0.66±0.08
0	6	1	1.14±0.12	1.01±0.15	1.38±0.10	1.44±0.25
0	2	3	1.65±0.20	1.60±0.16	1.54±0.13	1.44±0.17
0	6	3	1.47±0.06	1.39±0.14	1.25±0.15	1.00±0.04
4	2	1	0.94±0.11	0.72±0.10	1.22±0.19	1.06±0.17
4/3	6	1	1.16±0.07	0.89±0.09	0.86±0.11	0.87±0.08
4	2	3	1.15±0.12	1.03±0.11	1.06±0.26	1.20±0.27
4/3	6	3	1.63±0.17	1.45±0.19	1.70±0.20	2.06±0.22
6	2	1	1.13±0.18	1.17±0.09	1.13±0.06	-
2	6	1	1.42±0.18	1.33±0.11	0.95±0.11	0.97±0.13
6	2	3	1.00±0.12	1.18±0.15	1.26±0.11	1.20±0.09
2	6	3	0.63±0.06	0.63±0.10	1.28±0.12	1.35±0.26

TABLE 9C. PULMONARY RESISTANCE (cm H_2O/mL/sec) OF RATS IN PHASE 2 EXPOSURE TO DIESEL FUEL AEROSOL (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	0.22±0.03	0.45±0.10	0.13±0.02	0.12±0.03
0	6	1	0.27±0.04	0.36±0.07	0.28±0.03	0.42±0.08
0	2	3	0.11±0.01	0.21±0.08	0.30±0.06	0.23±0.01
0	6	3	0.50±0.06	0.81±0.12	0.37±0.08	0.46±0.09
4	2	1	0.25±0.04	0.32±0.08	0.22±0.04	0.49±0.11
4/3	6	1	0.19±0.05	0.36±0.09	0.18±0.03	0.25±0.01
4	2	3	0.51±0.19	0.52±0.14	0.48±0.03	0.37±0.04
4/3	6	3	0.34±0.03	0.31±0.05	0.17±0.02	0.28±0.02
6	2	1	0.28±0.03	0.22±0.02	0.26±0.02	0.24±0.03
2	6	1	0.20±0.01	0.53±0.16	0.12±0.03	0.13±0.01
6	2	3	0.60±0.08	0.65±0.11	0.31±0.04	0.37±0.11
2	6	3	0.38±0.11	0.79±0.37	0.30±0.06	0.47±0.10

TABLE 10C. NEGATIVE SLOPES OF LINEAR REGRESSION EQUATIONS FOR MULTIBREATH NITROGEN WASHOUT CURVES WHERE y = log (Percent N2) AND x = CUMULATIVE DILUTIONS OF FRC (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	0.16±0.00	0.11±0.00	0.20±0.01	0.16±0.01
0	6	1	0.18±0.00	0.16±0.00	0.19±0.01	0.16±0.00
0	2	3	0.21±0.02	0.16±0.00	0.19±0.00	0.17±0.01
0	6	3	0.17±0.00	0.14±0.01	0.15±0.00	0.11±0.00
4	2	1	0.21±0.01	0.17±0.00	0.19±0.01	0.17±0.01
4/3	6	1	0.22±0.01	0.19±0.01	0.23±0.00	0.19±0.00
4	2	3	0.10±0.00	0.09±0.00	0.21±0.01	0.18±0.00
4/3	6	3	0.19±0.00	0.17±0.00	0.20±0.00	0.15±0.00
6	2	1	0.23±0.00	0.21±0.01	0.22±0.02	0.19±0.00
2	6	1	0.17±0.00	0.12±0.01	0.21±0.00	0.19±0.01
6	2	3	0.17±0.00	0.17±0.03	0.15±0.00	0.11±0.00
2	6	3	0.12±0.00	0.11±0.00	0.23±0.02	0.21±0.02

TABLE 11C. TOTAL LUNG CAPACITY (mL) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	18.7±0.6	14.0±0.6	19.1±0.4	15.4±0.6
0	6	1	19.7±0.8	14.0±0.7	18.7±0.5	15.3±0.6
0	2	3	16.7±0.5	13.1±0.5	18.8±1.1	15.9±0.6
0	6	3	17.8±0.6	13.5±0.4	18.7±0.3	14.2±0.5
4	2	1	18.4±1.3	14.0±0.5	18.5±0.4	13.8±0.4
4/3	6	1	19.0±0.7	14.0±0.4	22.2±1.5	14.8±0.4
4	2	3	15.0±0.7	12.1±0.8	16.3±0.3	13.8±0.5
4/3	6	3	16.0±0.9	14.0±0.9	19.3±0.8	15.0±0.4
6	2	1	17.5±0.7	14.7±0.6	20.0±1.4	14.8±0.6
2	6	1	18.6±1.0	12.6±0.6	17.8±0.9	15.6±0.6
6	2	3	15.1±0.6	12.6±0.4	17.4±0.4	13.3±0.5
2	6	3	14.9±0.6	11.8±0.5	18.1±0.6	13.7±0.2

TABLE 12C. VITAL CAPACITY (mL) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	16.4±0.6	12.4±0.6	16.4±0.3	13.3±0.5
0	6	1	17.1±0.7	12.2±0.7	16.2±0.5	13.4±0.6
0	2	3	14.4±0.6	11.3±0.5	15.7±0.7	13.6±0.6
0	6	3	15.7±0.5	11.8±0.2	16.2±0.3	12.5±0.4
4	2	1	15.9±1.1	12.1±0.5	15.9±0.3	11.7±0.4
4/3	6	1	16.0±0.7	11.5±0.3	18.5±1.4	12.5±0.4
4	2	3	13.5±0.6	10.9±0.6	13.4±0.3	11.4±0.4
4/3	6	3	13.2±0.8	11.8±0.8	16.1±0.7	12.7±0.4
6	2	1	14.3±0.6	11.9±0.5	16.5±1.1	12.3±0.6
2	6	1	15.2±0.9	10.3±0.5	14.9±0.7	13.2±0.6
6	2	3	12.5±0.5	10.2±0.4	14.8±0.2	11.3±0.4
2	6	3	13.3±0.6	10.0±0.7	14.6±0.7	11.2±0.2

TABLE 13C. INSPIRATORY CAPACITY (mL) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	15.3±0.5	11.6±0.4	15.8±0.3	12.6±0.6
0	6	1	16.1±0.7	11.5±0.7	15.2±0.5	12.4±0.5
0	2	3	13.5±0.5	10.8±0.4	15.1±0.8	13.0±0.4
0	6	3	14.6±0.4	11.0±0.2	15.4±0.4	11.7±0.4
4	2	1	14.1±1.1	11.0±0.4	14.7±0.4	10.9±0.3
4/3	6	1	14.7±0.6	10.5±0.2	16.8±1.1	11.4±0.4
1	2	3	12.4±0.6	10.1±0.5	12.3±0.3	10.5±0.3
4/3	6	3	12.5±0.7	11.0±0.8	15.2±0.7	12.1±0.4
6	2	1	12.9±0.5	10.8±0.5	14.9±0.9	11.2±0.5
2	6	1	14.4±0.8	9.8±0.5	14.1±0.7	12.5±0.5
6	2	3	11.5±0.5	9.6±0.4	13.7±0.2	10.4±0.4
2	6	3	12.2±0.5	9.4±0.4	13.2±0.6	10.1±0.2

TABLE 14C. FUNCTIONAL RESIDUAL CAPACITY (mL) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	3.1±0.1	2.2±0.2	3.0±0.2	2.5±0.1
0	6	1	3.7±0.2	2.6±0.1	3.5±0.1	3.0±0.2
0	2	3	3.2±0.2	2.3±0.1	3.7±0.4	2.9±0.2
0	6	3	3.1±0.2	2.4±0.2	3.0±0.2	2.3±0.2
4	2	1	4.2±0.4	3.1±0.1	3.8±0.2	2.9±0.2
4/3	6	1	4.3±0.2	3.4±0.2	5.4±0.5	3.3±0.1
4	2	3	2.7±0.2	2.0±0.3	4.0±0.3	3.3±0.2
4/3	6	3	3.6±0.3	3.0±0.2	4.1±0.1	3.0±0.1
6	2	1	4.5±0.3	3.8±0.3	5.1±0.5	3.6±0.2
2	6	1	3.9±0.2	2.5±0.2	3.7±0.2	3.0±0.1
6	2	3	3.4±0.1	2.9±0.5	3.3±0.2	2.6±0.1
2	6	3	2.8±0.2	2.4±0.2	4.9±0.2	3.5±0.1

TABLE 15C. SPECIFIC COMPLIANCE (mL/cm H_2O/mL) OF GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	1.10±0.03	1.23±0.06	1.20±0.04	1.23±0.08
0	6	1	1.06±0.03	1.10±0.04	1.11±0.06	1.12±0.05
0	2	3	1.12±0.07	1.23±0.04	1.14±0.11	1.17±0.08
0	6	3	1.12±0.05	1.21±0.08	1.24±0.07	1.33±0.09
4	2	1	0.92±0.04	0.99±0.05	1.03±0.04	1.08±0.05
4/3	6	1	0.96±0.02	0.94±0.03	0.95±0.03	1.00±0.05
4	2	3	1.11±0.05	1.20±0.09	1.06±0.06	1.16±0.06
4/3	6	3	0.94±0.02	0.97±0.04	1.02±0.03	1.10±0.06
6	2	1	0.93±0.02	0.92±0.04	0.91±0.05	0.92±0.03
2	6	1	0.99±0.02	1.07±0.06	1.03±0.03	1.02±0.03
6	2	3	1.01±0.05	1.02±0.03	1.06±0.03	1.08±0.04
2	63	3	1.08±0.06	1.03±0.03	0.94±0.04	0.95±0.03

TABLE 16C. PEAK FLOW (mL/sec) IN MAXIMAL FORCED EXPIRATION MANEUVER AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	47.4 ± 1.6	35.6 ± 1.5	47.5 ± 0.9	42.0 ± 2.3
0	6	1	51.2 ± 2.0	39.3 ± 1.9	49.1 ± 1.5	40.4 ± 1.1
0	2	3	45.2 ± 1.8	36.4 ± 2.0	47.4 ± 1.4	41.2 ± 1.1
0	6	3	38.4 ± 1.8	31.1 ± 1.3	47.4 ± 1.3	37.5 ± 1.5
4	2	1	44.7 ± 2.2	33.4 ± 2.6	46.3 ± 1.2	34.7 ± 2.5
4/3	6	1	48.9 ± 1.5	38.5 ± 0.6	52.3 ± 2.7	40.5 ± 1.2
4	2	3	37.2 ± 2.0	32.9 ± 2.0	42.9 ± 1.3	37.1 ± 1.0
4/3	6	3	39.4 ± 1.9	37.6 ± 2.2	46.7 ± 1.7	37.4 ± 1.4
6	2	1	44.2 ± 1.0	34.2 ± 1.6	49.9 ± 1.9	38.6 ± 2.6
2	6	1	44.5 ± 1.5	29.7 ± 1.7	42.3 ± 2.2	39.4 ± 2.8
6	2	3	29.5 ± 2.4	26.3 ± 1.4	42.8 ± 0.7	35.3 ± 0.9
2	6	3	35.0 ± 2.3	28.1 ± 2.0	42.6 ± 2.3	37.4 ± 0.5

TABLE 17C. EXPIRATORY FLOW (mL/sec) AT 50 PERCENT OF VITAL CAPACITY DURING MAXIMAL FORCED EXPIRATION MANEUVER AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	43.4 ± 2.0	30.2 ± 1.6	42.8 ± 0.9	36.5 ± 2.6
0	6	1	46.8 ± 2.7	34.0 ± 2.2	45.8 ± 1.6	36.7 ± 1.1
0	2	3	40.7 ± 2.2	31.7 ± 1.9	43.7 ± 1.5	36.7 ± 1.1
0	6	3	34.8 ± 2.4	25.9 ± 1.5	44.1 ± 1.6	32.1 ± 1.9
4	2	1	39.7 ± 2.3	29.8 ± 2.0	43.9 ± 9.9	30.5 ± 2.2
4/3	6	1	44.5 ± 1.9	33.2 ± 0.9	48.7 ± 3.5	35.2 ± 1.3
4	2	3	33.1 ± 2.5	28.9 ± 2.1	38.6 ± 1.3	32.0 ± 1.2
4/3	6	3	34.8 ± 1.9	33.8 ± 2.0	42.7 ± 1.4	32.7 ± 1.3
6	2	1	40.4 ± 1.2	31.0 ± 1.5	45.2 ± 2.4	33.4 ± 2.9
2	6	1	39.9 ± 1.8	25.1 ± 1.8	37.5 ± 2.3	34.5 ± 2.5
6	2	3	24.8 ± 2.1	21.6 ± 1.5	38.5 ± 0.8	30.0 ± 1.1
2	6	3	33.3 ± 2.6	25.3 ± 1.9	39.9 ± 2.7	32.6 ± 0.7

TABLE 18C. EXPIRATORY FLOW (mL/sec) AFTER EXHALATION OF 75 PERCENT OF VITAL CAPACITY DURING MAXIMAL FORCED EXPIRATION MANEUVER AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	25.8 ± 1.3	16.9 ± 0.9	23.8 ± 1.3	20.4 ± 1.3
0	6	1	26.4 ± 1.4	20.2 ± 1.1	26.1 ± 0.9	21.9 ± 0.7
0	2	3	22.8 ± 1.4	18.4 ± 1.2	25.2 ± 1.1	21.3 ± 0.7
0	6	3	19.1 ± 1.9	13.9 ± 1.0	26.3 ± 0.8	18.1 ± 1.4
4	2	1	22.8 ± 1.0	17.6 ± 1.1	25.6 ± 0.4	27.6 ± 1.2
4/3	6	1	25.9 ± 1.2	19.0 ± 0.9	28.3 ± 2.0	20.6 ± 0.8
4	2	3	19.8 ± 2.0	16.6 ± 1.1	22.2 ± 0.7	18.3 ± 1.0
4/3	6	3	19.9 ± 1.1	19.7 ± 1.1	24.1 ± 0.8	19.2 ± 0.6
6	2	1	23.1 ± 0.7	19.3 ± 0.7	25.7 ± 1.3	19.7 ± 1.9
2	6	1	21.7 ± 1.1	14.0 ± 1.1	21.3 ± 1.3	20.0 ± 1.8
6	2	3	13.9 ± 0.9	11.4 ± 0.9	21.6 ± 0.8	17.1 ± 0.8
2	6	3	20.4 ± 2.5	17.0 ± 1.4	23.1 ± 1.4	18.6 ± 0.6

TABLE 19C. EXPIRATORY FLOW (mL/sec) AT 50 PERCENT OF VITAL CAPACITY DURING MAXIMAL FORCED EXPIRATION MANEUVER REFERENCED TO VITAL CAPACITY (mL). VALUES ARE MEAN ± S.E.

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	2.65 ± 0.05	2.45 ± 0.09	2.62 ± 0.08	2.74 ± 0.12
0	6	1	2.74 ± 0.11	2.80 ± 0.08	2.83 ± 0.06	2.75 ± 0.07
0	2	3	2.82 ± 0.07	2.79 ± 0.07	2.80 ± 0.08	2.71 ± 0.10
0	6	3	2.22 ± 0.14	2.19 ± 0.09	2.73 ± 0.08	2.58 ± 0.17
4	2	1	2.52 ± 0.10	2.48 ± 0.15	2.71 ± 0.05	2.58 ± 0.12
4/3	6	1	2.78 ± 0.06	2.89 ± 0.03	2.63 ± 0.08	2.81 ± 0.07
4	2	3	2.47 ± 0.13	2.64 ± 0.11	2.88 ± 0.10	2.80 ± 0.08
4/3	6	3	2.65 ± 0.08	2.87 ± 0.04	2.66 ± 0.05	2.57 ± 0.05
6	2	1	2.84 ± 0.09	2.62 ± 0.14	2.77 ± 0.10	2.71 ± 0.16
2	6	1	2.63 ± 0.07	2.43 ± 0.11	2.52 ± 0.08	2.60 ± 0.13
6	2	3	1.99 ± 0.16	2.12 ± 0.13	2.61 ± 0.07	2.66 ± 0.08
2	6	3	2.51 ± 0.16	2.46 ± 0.14	2.72 ± 0.07	2.91 ± 0.06

TABLE 20C. SINGLE-BREATH CARBON MONOXIDE DIFFUSING CAPACITY [mL/(mmHg) (min)] AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	1.16 ± 0.07	0.61 ± 0.04	1.22 ± 0.04	0.75 ± 0.09
0	6	1	1.18 ± 0.07	0.74 ± 0.08	1.15 ± 0.07	0.79 ± 0.06
0	2	3	1.13 ± 0.06	0.67 ± 0.08	1.34 ± 0.09	0.88 ± 0.06
0	6	3	1.18 ± 0.05	0.73 ± 0.03	1.28 ± 0.07	0.81 ± 0.04
4	2	1	1.07 ± 0.07	0.69 ± 0.04	1.18 ± 0.03	0.68 ± 0.04
4/3	6	1	1.47 ± 0.15	0.77 ± 0.04	1.25 ± 0.11	0.62 ± 0.06
4	2	3	0.73 ± 0.03	0.40 ± 0.03	1.12 ± 0.05	0.67 ± 0.03
4/3	6	3	1.07 ± 0.09	0.72 ± 0.08	1.20 ± 0.11	0.70 ± 0.04
6	2	1	1.06 ± 0.07	0.66 ± 0.03	1.26 ± 0.08	0.63 ± 0.04
2	6	1	1.06 ± 0.13	0.59 ± 0.03	1.02 ± 0.10	0.65 ± 0.03
6	2	3	0.76 ± 0.03	0.50 ± 0.05	1.10 ± 0.04	0.57 ± 0.08
2	6	3	0.97 ± 0.07	0.45 ± 0.02	0.64 ± 0.06	0.25 ± 0.03

TABLE 21C. SINGLE BREATH CARBON MONOXIDE DIFFUSING CAPACITY REFERENCED TO TOTAL LUNG CAPACITY (IN LITERS) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	71.5 ± 4.8	49.4 ± 2.2	74.6 ± 1.9	56.1 ± 5.3
0	6	1	69.5 ± 4.7	61.3 ± 6.4	71.1 ± 3.7	59.6 ± 5.3
0	2	3	78.7 ± 4.1	58.2 ± 5.0	85.5 ± 4.5	64.5 ± 3.0
0	6	3	75.8 ± 3.2	62.0 ± 2.9	79.0 ± 3.2	65.1 ± 4.3
4	2	1	69.0 ± 6.6	57.8 ± 3.1	74.2 ± 1.4	57.3 ± 4.1
4/3	6	1	93.1 ± 10.9	67.3 ± 3.5	67.4 ± 3.9	49.6 ± 4.0
4	2	3	54.2 ± 1.8	36.9 ± 2.9	84.0 ± 4.4	58.4 ± 1.9
4/3	6	3	81.1 ± 6.0	60.7 ± 4.1	73.5 ± 4.5	55.6 ± 4.4
6	2	1	73.5 ± 2.1	55.6 ± 2.5	76.5 ± 1.6	51.1 ± 3.1
2	6	1	68.4 ± 6.7	57.6 ± 2.7	67.9 ± 5.5	49.7 ± 2.5
6	2	3	60.9 ± 2.8	48.6 ± 2.9	74.8 ± 2.3	50.0 ± 6.8
2	6	3	73.1 ± 5.1	47.1 ± 5.1	44.7 ± 5.2	22.2 ± 2.0

TABLE 22C. LIVER WEIGHT (g) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	15.6±0.9	9.3±0.3	18.1±0.7	11.1±0.8
0	6	1	15.5±0.5	10.2±0.8	18.1±0.9	9.8±0.2
0	2	3	14.8±0.5	9.1±0.4	15.4±0.9	9.7±0.5
0	6	3	14.3±0.3	10.0±0.5	14.3±0.8	9.6±0.5
4	2	1	14.8±0.6	8.7±0.3	15.7±0.2	8.3±0.3
4/3	6	1	14.6±0.6	8.9±0.2	14.8±0.7	8.2±0.4
4	2	3	14.4±0.2	8.4±0.3	14.7±0.9	8.4±0.2
4/3	6	3	14.4±0.4	8.8±0.3	15.8±0.8	9.6±0.3
6	2	1	14.6±0.7	8.3±0.3	17.1±0.6	8.4±0.5
2	6	1	15.7±0.9	8.0±0.5	15.7±0.6	8.9±0.3
2	2	3	14.1±0.9	8.3±0.3	14.4±0.6	9.1±0.2
6	6	3	14.2±0.7	8.2±0.2	15.2±0.8	8.6±0.3

TABLE 23C. KIDNEY WEIGHT (g) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	1.66±0.05	0.98±0.03	1.83±0.08	1.13±0.04
0	6	1	1.65±0.04	1.04±0.07	1.78±0.09	1.12±0.04
0	2	3	1.44±0.03	0.93±0.14	1.57±0.11	0.98±0.04
0	6	3	1.47±0.05	0.96±0.04	1.54±0.02	1.04±0.05
4	2	1	1.62±0.06	0.85±0.02	1.70±0.06	0.94±0.04
4/3	6	1	1.59±0.08	0.95±0.04	1.71±0.09	0.95±0.02
4	2	3	1.48±0.03	0.91±0.01	1.61±0.07	0.99±0.04
4/3	6	3	1.49±0.07	0.93±0.04	1.65±0.04	0.97±0.04
6	2	1	1.52±0.07	0.84±0.03	1.81±0.08	0.86±0.06
2	6	1	1.67±0.09	0.89±0.04	1.60±0.06	1.00±0.06
6	2	3	1.47±0.07	0.85±0.05	1.60±0.08	1.00±0.03
2	6	3	1.54±0.06	0.87±0.02	1.58±0.10	1.01±0.04

TABLE 24C. WEIGHT OF ADRENAL GLANDS (mg) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	26 ± 1	36 ± 2	27 ± 1	34 ± 2
0	6	1	24 ± 2	30 ± 2	26 ± 2	34 ± 3
0	2	3	29 ± 1	33 ± 1	28 ± 3	35 ± 2
0	6	3	28 ± 2	35 ± 2	33 ± 2	34 ± 2
4	2	1	26 ± 3	32 ± 1	25 ± 3	29 ± 2
4/3	6	1	28 ± 1	33 ± 3	29 ± 2	33 ± 2
4	2	3	29 ± 2	32 ± 2	25 ± 2	37 ± 2
4/3	6	3	28 ± 1	32 ± 1	28 ± 2	29 ± 4
6	2	1	26 ± 2	32 ± 3	29 ± 2	32 ± 4
2	6	1	30 ± 2	30 ± 3	26 ± 1	34 ± 2
6	2	3	27 ± 1	36 ± 1	32 ± 1	42 ± 1
2	6	3	30 ± 1	31 ± 2	32 ± 2	37 ± 1

TABLE 25C. WET WEIGHT OF RIGHT MIDDLE LUNG LOBE (mg) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	189 ± 9	134 ± 8	179 ± 7	141 ± 7
0	6	1	170 ±16	133 ± 8	170 ±10	143 ± 3
0	2	3	159 ± 6	120 ± 4	160 ± 8	121 ± 4
0	6	3	177 ±12	136 ± 9	180 ± 7	150 ± 6
4	2	1	216 ±10	156 ± 5	202 ± 8	141 ± 6
4/3	6	1	214 ±11	149 ± 5	211 ±12	148 ± 3
4	2	3	213 ± 8	157 ± 6	190 ± 7	145 ± 8
4/3	6	3	219 ± 9	154 ± 5	176 ± 6	145 ± 5
6	2	1	236 ±13	180 ± 9	227 ± 6	163 ±15
2	6	1	257 ±13	161 ± 5	198 ±15	145 ± 6
2	2	3	248 ± 9	196 ±11	220 ±12	174 ± 4
6	6	3	246 ±13	196 ± 9	196 ± 4	145 ± 7

TABLE 26C. WET/DRY WEIGHT RATIOS OF RIGHT MIDDLE LUNG LOBES AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure		2 Weeks Postexposure	
			Males	Females	Males	Females
0	2	1	5.04±0.03	5.04±0.05	5.12±0.03	5.01±0.03
0	6	1	5.20±0.04	5.11±0.05	5.19±0.06	5.23±0.05
0	2	3	5.33±0.03	5.38±0.04	5.17±0.06	5.20±0.05
0	6	3	5.04±0.03	5.09±0.05	5.35±0.06	5.26±0.07
4	2	1	5.01±0.04	5.18±0.05	5.17±0.01	5.20±0.05
4/3	6	1	5.10±0.03	5.01±0.04	5.21±0.05	5.18±0.05
4	2	3	5.17±0.05	5.10±0.05	5.04±0.05	5.07±0.08
4/3	6	3	5.19±0.04	5.32±0.05	5.19±0.02	5.04±0.06
6	2	1	4.73±0.17	4.89±0.17	4.96±0.05	4.92±0.04
2	6	1	5.14±0.05	5.22±0.05	5.09±0.03	5.17±0.06
2	2	3	5.24±0.06	5.20±0.02	5.08±0.04	4.92±0.12
6	6	3	5.11±0.04	5.21±0.04	5.11±0.03	5.03±0.09

TABLE 27C. SEVERITY OF FOCAL PNEUMONITIS AND ASSOCIATED HISTOLOGICAL CHANGES AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES

mg/L	Hours per Exposure	Exposures per Week	2 Days Postexposure	2 weeks Postexposure
0	2	1	-*	-
0	6	1	-	-
0	2	3	-	-
0	6	3	-	-
4	2	1	±	±
4/3	6	1	±	±/+
4	2	3	+/++	++
4/3	6	3	++/+++	±/+
6	2	1	+	±
2	6	1	++	+
2	2	3	+++	+/++
6	6	3	++/+++	++

* -, no observed focal pneumonitis,
±, equivocal change,
+, slight,
++, moderate,
+++, most severe observed.

TABLE 28C. PRE-EXPOSURE FORELIMB GRIP STRENGTH (kg) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	Males	Females
0	2	1	0.91 ± 0.04	0.78 ± 0.05
0	6	1	1.02 ± 0.04	0.74 ± 0.04
0	2	3	0.98 ± 0.02	0.76 ± 0.04
0	6	3	0.78 ± 0.07	0.85 ± 0.04
4	2	1	0.94 ± 0.06	0.77 ± 0.04
4/3	6	1	1.09 ± 0.06	0.92 ± 0.03
4	2	3	1.00 ± 0.09	0.88 ± 0.06
4/3	6	3	0.82 ± 0.07	0.90 ± 0.03
6	2	1	1.03 ± 0.05	0.93 ± 0.03
2	6	1	0.97 ± 0.08	0.71 ± 0.07
6	2	3	1.03 ± 0.04	0.81 ± 0.06
2	6	3	1.07 ± 0.04	0.96 ± 0.04

TABLE 29C. PRE-EXPOSURE LANDING FOOT SPREAD (mm) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	Males	Females
0	2	1	110 ± 10	98 ± 1
0	6	1	108 ± 8	80 ± 5
0	2	3	102 ± 8	96 ± 6
0	6	3	113 ± 8	102 ±10
4	2	1	106 ± 5	81 ± 4
4/3	6	1	134 ± 13	88 ± 4
4	2	3	132 ± 4	88 ± 4
4/3	6	3	127 ± 5	94 ± 8
6	2	1	112 ± 9	95 ± 6
2	6	1	110 ± 8	97 ±11
6	2	3	107 ± 1	95 ±11
2	6	3	111 ± 8	95 ± 8

TABLE 30C. PRE-EXPOSURE TAIL FLICK REFLEX (sec) AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (Mean ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	Males	Females
0	2	1	1.82 ± 0.17	1.45 ± 0.08
0	6	1	1.54 ± 0.16	1.09 ± 0.08
0	2	3	1.35 ± 0.13	1.27 ± 0.17
0	6	3	1.80 ± 0.15	1.63 ± 0.10
4	2	1	1.50 ± 0.25	1.35 ± 0.14
4/3	6	1	1.76 ± 0.13	1.71 ± 0.15
4	2	3	2.09 ± 0.31	1.59 ± 0.09
4/3	6	3	2.14 ± 0.29	1.87 ± 0.12
6	2	1	1.76 ± 0.11	1.54 ± 0.12
2	6	1	1.82 ± 0.09	1.78 ± 1.0
6	2	3	1.56 ± 0.17	1.49 ± 0.04
2	6	3	1.62 ± 0.18	1.51 ± 0.10

TABLE 31C. PRE-EXPOSURE PEAK HEIGHT (gram weight) IN STARTLE REFLEX AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	Males	Females
0	2	1	359 ± 64	166 ± 25
0	6	1	433 ± 39	330 ± 80
0	2	3	432 ± 72	388 ± 59
0	6	3	298 ± 51	252 ± 47
4	2	1	227 ± 43	135 ± 20
4/3	6	1	382 ± 70	135 ± 35
4	2	3	313 ± 49	261 ± 53
4/3	6	3	463 ± 81	346 ± 46
6	2	1	339 ± 35	212 ± 19
2	6	1	286 ± 34	172 ± 32
6	2	3	272 ± 35	229 ± 35
2	6	3	475 ± 48	215 ± 49

TABLE 32C. PRE-EXPOSURE PEAK TIME (msec) IN STARTLE REFLEX ASSAY AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	Males	Females
0	2	1	26.8 ± 0.5	26.1 ± 0.3
0	6	1	26.8 ± 0.4	27.2 ± 2.1
0	2	3	27.6 ± 0.8	25.3 ± 0.3
0	6	3	27.1 ± 0.9	27.5 ± 1.2
4	2	1	27.6 ± 0.9	24.7 ± 0.4
4/3	6	1	27.2 ± 0.7	25.6 ± 0.5
4	2	3	26.4 ± 0.4	27.4 ± 1.5
4/3	6	3	26.5 ± 0.7	27.4 ± 0.8
6	2	1	27.1 ± 1.0	26.3 ± 0.6
2	6	1	26.1 ± 0.5	29.1 ± 2.7
6	2	3	27.3 ± 1.0	26.4 ± 0.9
2	6	3	31.2 ± 2.1	25.7 ± 0.7

TABLE 33C. PRE-EXPOSURE REACTION TIME (msec) IN STARTLE REFLEX ASSAY AMONG GROUPS IN PHASE 2 OF DIESEL FUEL EXPOSURES (MEAN ± S.E.)

mg/L	Hours per Exposure	Exposures per Week	Males	Females
0	2	1	18.0 ± 0.4	18.0 ± 0.3
0	6	1	17.4 ± 0.3	18.8 ± 2.0
0	2	3	18.3 ± 0.5	17.3 ± 0.5
0	6	3	18.3 ± 0.3	16.9 ± 0.6
4	2	1	18.3 ± 0.4	16.7 ± 0.3
4/3	6	1	18.2 ± 0.5	19.0 ± 0.5
4	2	3	17.1 ± 0.4	18.0 ± 1.2
4/3	6	3	16.9 ± 0.4	16.9 ± 0.3
6	2	1	17.8 ± 0.4	17.4 ± 0.3
2	6	1	17.4 ± 0.6	18.6 ± 3.5
6	2	3	18.1 ± 0.2	17.7 ± 0.6
2	6	3	19.6 ± 0.9	16.6 ± 1.0

TABLE 34C. LEAST SQUARES MEANS OF CLINICAL CHEMISTRY PARAMETERS IN SHAM-EXPOSED GROUPS (± S.E.)

	Males	Females
Alkaline phosphatase (mU/mL)	150 ± 7	108 ± 7
SGOT (mU/mL)	155 ± 5	134 ± 5
Cholesterol (mg percent)	54.8 ± 2.3	70.7 ± 2.3
Triglycerides (mg percent)	119 ± 7	128 ± 7
Uric acid (mg percent)	2.15 ± 0.08	1.74 ± 0.07
Glucose (mg percent)	174 ± 5	153 ± 5
Urea nitrogen (mg percent)	23.4 ± 0.5	24.0 ± 0.5
Total bilirubin (mg percent)	0.59 ± 0.04	0.65 ± 0.04
Creatinine (mg percent)	0.79 ± 0.04	0.82 ± 0.03
Sodium (mEq percent)	145 ± 0.4	145 ± 0.4
Potassium (mEq percent)	4.86 ± 0.12	3.96 ± 0.11

APPENDIX D:

HISTOPATHOLOGY

The following pages contain notes on histopathological evaluation of individual animals at 2 days after the last exposure. All slides were coded by number so that the pathologist could not identify groups. The term "SD pneumonitis" is used for the pulmonary lesions observed in unexposed Sprague-Dawley rats, consisting primarily of peribronchial and perivascular inflammation with some focally increased numbers of pulmonary free cells in the alveoli. The term "alveolitis" refers to chronic infiltrate on the walls of the alveoli but not the lumen. The notation of "macrophages" refers to alveolar macrophages and other free cells within the alevoli. Animals are coded by a number and M for males or F for females. Individual data are not presented for animals examined at two weeks post exposure. There were no exposure-related changes observed in tissues other than the lung among these animals. The primary question, therefore was whether pulmonary lesions observed at 2 days postexposure were altered by the two week interim without exposure. This question was answered by blind scoring of the lesions on the basis of groups rather than individuals, as shown in Table 27C.

The following scoring criteria were used in the histopathological evaluations:

±, equivocal change,
+, slight,
++, moderate,
+++, most severe observed.

GROUP EA (0 mg/L, 2 hr/exposure, 1 exposure/wk)
2 days Postexposure

Animal	
13 M	Some SD pneumonitis
14 M	Some SD pneumonitis, macrophages (+)
15 M	normal
16 M	Some SD pneumonitis, macrophages (+)
17 M	Some SD pneumonitis
18 M	SD pneumonitis, alveolitis, macrophages (+), sinusitis
13 F	Some SD pneumonitis
14 F	normal
15 F	Some SD pneumonitis
16 F	Some SD pneumonitis, macrophages (+)
17 F	normal
18 F	normal

GROUP LA (0 mg/L, 2 hr/exposure, 3 exposures/wk)
2 Days Postexposure

Animal	
13 M	few macrophages
14 M	few macrophages
15 M	few macrophages
16 M	few macrophages
17 M	same with sinusitis
18 M	more cellularity and macrophages, slight squamous metaplasia in larynx
13 F	few macrophages
14 F	few macrophages
15 F	few macrophages
16 F	more cellularity and macrophages, slight squamous metaplasia in larynx
17 F	few macrophages
18 F	few macrophages

GROUP KA (0 mg/L, 6 hr/exposure, 1 exposure/wk)
2 Days Postexposure

Animal

13 M almost normal, some SD pneumonitis foci
All other animals were the same with some atrophic epithelium in the trachea.

GROUP HA (0 mg/L, 6 hr/exposure, 3 exposures/wk)
2 Days Postexposure

Animal

13 M	normal
14 M	glomerular hypercellularity
15 M	glomerular changes, chronic sinusitis and rhinitis (infiltration and squamous metaplasia)
16 M	renal tubular cloudy swelling, lung congestion, some alveolar macrophages
17 M	SD infiltrate in lung (++), renal tubular swelling
18 M	liver congestion, very slight centrilobular swelling, lymphocytic infiltrate, hepatocellular swelling
13 F	glomerular changes, slight hepatic infiltrate, slight alveolar rupture, sinusitis and slight rhinitis
14 F	SD infiltrate in lung (+)
15 F	centrilobular congestion and disintegration in liver (cloudy swelling and necrosis), sinusitis and rhinitis
16 F	Some alveolar macrophages, ruptured alveoli, liver congestion and hepatocellular swelling, slight interstitial medullary nephritis, slight laryngitis
17 F	normal
18 F	slight laryngitis

GROUP JA (4 mg/L, 2 hr/exposure, 1 exposure/wk)
2 Days Postexposure

Animal	
13 M	lung almost normal, some focal pneumonitis
14 M	lung almost normal, some focal pneumonitis, rhinitis
15 M	lung almost normal, some focal pneumonitis
16 M	lung almost normal, some focal pneumonitis
17 M	lung almost normal, some focal pneumonitis
18 M	lung almost normal, some focal pneumonitis
13 F	lung almost normal, some facal pneumonitis with more macrophages
14 F	lung almost normal, some focal pneumonitis
15 F	lung almost normal, some focal pneumonitis
16 F	lung almost normal, some focal pneumonitis
17 F	lung almost normal, some focal pneumonitis
18 F	lung almost normal, some focal pneumonitis

GROUP DA (4 mg/L, 2 hr/exposure, 3 exposures/wk)
2 Days Postexposure

Animal	
13 M	macrophages (++), ruptured walls
14 M	macrophages (+), SD pneumonitis, sinusitis
15 M	lung congestion, few macrophages
16 M	normal
17 M	normal
18 M	normal, sinusitis
13 F	normal
14 F	almost normal, SD pneumonitis, sinusitis
15 F	almost normal, SD pneumonitis, sinusitis
16 F	normal
17 F	normal, sinusitis
18 F	normal, sinusitis

GROUP BA (1-1/3 mg/L, 6 hr/exposure, 1 exposure/wk)
2 Days Postexposure

Animal	
13 M	Some hypercellular alveolar walls, ruptured walls, few macrophages
14 M	Some hypercellular alveolar walls, ruptured walls, few macrophages, SD pneumonitis
15 M	Some hypercellular alveolar walls, ruptured walls, few macrophages
16 M	Some hypercellular alveolar walls, ruptured walls, few macrophages, focus acute colonjitis
17 M	Some hypercellular alveolar walls, ruptured walls, few macrophages
18 M	Some hypercellular alveolar walls, ruptured walls, few macrophages, SD pneumonitis
13 F	Some hypercellular alveolar walls, ruptured walls, few macrophages but less severe
14 F	Some hypercellular alveolar walls, ruptured walls, few macrophages but less severe
15 F	Some hypercellular alveolar walls, ruptured walls, few macrophages, SD pneumonitis
16 F	SD pneumonitis, almost normal
17 F	Some hypercellular alveolar walls, ruptured walls, few macrophages, focus of interstitial nephritis
18 F	SD pneumonitis, almost normal

GROUP MA (1 1/3 mg/L, 6 hr/exposure, 3 exposures/wk)
2 Days Postexposure

Animal	
13 M	alveolar cellularity, few macrophages, sinusitis
14 M	alveolar cellularity, atrophic tracheal epithelium
15 M	alveolar cellularity, atrophic tracheal epithelium
16 M	alveolar cellularity, macrophages, atrophic tracheal epithelium
17 M	alveolar cellularity, macrophages, atrophic tracheal epithelium
18 M	alveolar cellularity, macrophages, atrophic tracheal epithelium
13 F	almost normal
14 F	alveolar cellularity, macrophages, atrophic tracheal epithelium
15 F	alveolar cellularity, macrophages, atrophic tracheal epithelium
16 F	alveolar cellularity, macrophages, atrophic tracheal epithelium
17 F	almost normal
18 F	alveolar cellularity, macrophages, atrophic tracheal epithelium

GROUP AA (6 mg/L, 2 hr/exposure, 1 exposure/wk)
2 Days Postexposure

Animal	
13 M	SD pneumonitis, rhinitis
14 M	SD pneumonitis, rhinitis
15 M	macrophages (+)
16 M	SD pneumonitis, rhinitis
17 M	small focus alveolitis
18 M	macrophages (+)
13 F	few macrophages
14 F	few macrophages, ruptured alveolar walls, rhinitis
15 F	few macrophages, ruptured alveolar walls, rhinitis
10 F	SD pneumonitis, rhinitis
16 F	SD pneumonitis, rhinitis
18 F	SD pneumonitis, rhinitis

GROUP GA (6 mg/L, 2 hr/exposure, 3 exposure/wk)
2 Days Postexposure

Animal	
13 M	macrophages (++), thickened alveolar wall, slight rhinitis
14 M	macrophages (++), thickened alveolar wall, slight rhinitis
15 M	macrophages (+/++), thickened alveolar wall, slight rhinitis
16 M	macrophages (+/++), thickened alveolar wall, slight rhinitis
17 M	macrophages (+/++), thickened alveolar wall, slight rhinitis
18 M	macrophages (+), thickened alveolar wall, slight rhinitis focus of SD pneumonitis
13 F	macrophages (±), lymphoid hyperplasia of nasal epithelium
14 F	macrophages (±), lymphoid hyperplasia of nasal epithelium
15 F	macrophages (++), subacute pneumonitis, some ruptured alveoli
16 F	few macrophages, ruptured alveoli
17 F	few macrophages, ruptured alveoli
18 F	few macrophages, laryngitis

GROUP FA (2 mg/L, 6 hr/exposure, 1 exposure/wk)
2 Days Postexposure

Animal	
13 M	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis
14 M	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis
15 M	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis
16 M	macrophages (++), alveolitis, sinusitis
17 M	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis
18 M	macrophages (++), alveolitis, sinusitis
13 F	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis, sinusitis
14 F	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis, chronic alveolitis
15 F	macrophages (+), thickened alveolar walls, alveolar rupture, foci of subacute pneumonitis, chronic alveolitis
16 F	macrophages (++), alveolitis, sinusitis
17 F	macrophages (++), alveolitis, sinusitis, slight laryngitis
18 F	macrophages (++), alveolitis, sinusitis

GROUP CA (2 mg/L, 6 hr/exposure, 3 exposures/wk)
2 Days Postexposure

Animal	
13 M	macrophages (+)
14 M	macrophages (+), SD pneumonitis
15 M	macrophages (++), some alveolitis, minimal glomerular lesion, sinusitis
16 M	macrophages (+)
17 M	macrophages (+)
18 M	macrophages (+), slight alveolitis, glomerular hypercellularity and swelling
13 F	ruptured alveolar walls, slight alveolitis, micronephrolithiasis
14 F	ruptured alveolar walls, slight alveolitis
15 F	macrophages (+), slight alveolitis, ruptured alveolar walls
16 F	macrophages (+), alveolitis, sinusitis
17 F	macrophages (+), alveolitis, sinusitis
18 F	macrophages (±), ruptured alveoli

PERSONNEL

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Principal Investigator	:	Walden Dalbey, PhD.
Research Scientist	:	Simon Lock, PhD.
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Statistician	:	Richard Schmoyer, PhD.
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Michael Guerin, PhD.
Robert Holmberg, PhD.
Roger Jenkins, PhD.
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PUBLICATIONS

Dalbey, W., S. Lock, S. Garfinkel, R. Jenkins, R. Holmberg and M. Guerin. Effects of repeated exposures to aerosolized diesel fuel. The Toxicologist 2: 50, 1982.

Dalbey, W., S. Lock, J. Moneyhun, R. Holmberg, and M. Guerin. Inhalation exposures of rats to aerosolized diesel fuel. Proceedings of the Symposium on the Toxicology of Petroleum Hydrocarbons. Held in Washington, D.C., May, 1982. (Ed. H. N. MacFarland, C. E. Holdsworth, J. A. MacGregor, R. W. Call, and M. L. Kane), pp. 13-25, API, Washington, D.C., 1982.

Lock, S., W. Dalbey, R. Rice, S. Garfinkel and L. Balogh. Toxicologic effects of aerosolized diesel fuel. Presented at American Industrial Hygiene Conference, Cincinnati, June, 1982.

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