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FOREWORD

This final report summarizes investigations conducted from FY85-FY93 in the project entitled ""Evaluation of $NO_x$-induced Toxicity". During the course of the studies, investigations were undertaken to address numerous objectives. Specific information provided in this report, accordingly, is formatted in the context of the objective(s) to which it pertains. Each Section is prefaced by a statement of the objective(s) it concerns, a brief summary of accomplishments and findings that are detailed in the section, and citations of reports and publications that have emanmated in total or in part from research performed during the accomplishment of the objective(s). Each Section begins with its own Introduction, which is then followed by a Materials and Methods section, a Results section, and a Summary or Discussion section.

**A summary of the project's overall objectives and the Sections in which they are addressed in this Final Report are as follows:**

## SECTION A

Objective 1: To design and fabricate an exposure system for multiple-animal (rats) exposures to stable, pure, and reproducible atmospheres of $NO_2$ and NO.

Objective 2: To characterize the kinetic course of development and severity of $NO_2$-induced lung injury following the inhalation of 25-250 ppm $NO_2$ for durations ranging from 5 to 30 min.

Objective 3: To assess $NO_2$ exposure concentration and exposure duration in terms of which of these two variables are of greater importance in determining the severity of $NO_2$-induced lung injury.

Objective 4: To identify cellular changes in response to $NO_2$ by electron microscopic analyses.

## SECTION B

Objective 5: To determine the consequences of post-exposure exercise on the severity of $NO_2$-induced lung injury.

## SECTION C

Objective 6: To develop testing procedures, using an exercising rat model, that relate metabolic parameters to endurance in order to predict work performance capability during acute high level and also longer term, lower level exercise.

Objective 7: To characterize work performance degradation following acute inhalation of high concentrations of $NO_2$.

## SECTION D

Objective 8: To examine the toxicologic effects of NO in the lung.

Objective 9: To obtain information on the relative pulmonary toxicities of NO and $NO_2$ when inhaled at relatively high mass concentrations.

## SECTION E

Objective 10: To characterize methemoglobin formation and dissociation kinetics in the rat durring and following the inhalation of NO.

## SECTION F

Objective 11: To examine the relationship between blood methemoglobin and work performance capacity .

## SECTION G

Objective 12: To develop measurement techniques using partial body plethysmography and multi-animal measurement data acquisition systems to record and calculate ventilatory parameters during exposure to toxic substances.

Objective 13: To characterize the ventilatory response to inhaled $NO_2$ and enhanced ventilation during $NO_2$ + $CO_2$ inhalation in order to relate $NO_2$-induced injury with an estimate of inhaled dose.

## SECTION H

Objective 14: To develop a means by which pulmonary extracellular fluid lining constituents and blood compartment biochemical constituents can be resolved and quantified in order to characterize the hyperpermeability response to $NO_2$.

Objective 15: To assess the relationship between changes in the numbers and types of lung free cells following $NO_2$ exposure and the hyperpermeability response.

## SECTION I

Objective 16: To characterize the injurious response following brief, "high burst" exposures of rats to high mass concentrations of $NO_2$.

Objective 17: To investigate the effects of post-exposure exercise on the severity of lung injury following brief, "high burst" exposures to $NO_2$.

## SECTION J

Objective 18: To evaluate $NO_2$ and NO as being additive or synergistic in causing decrements in work performance capacity following their concurrent inhalation.

Objective 19: To determine whether or not the co-inhalation of high concentrations of $NO_2$ and NO result in more severe lung injury than that which occurs following exposure to $NO_2$ only.

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# Reports that have emanated from the project (most recent first):

*Lehnert, B.E., Archuleta, A.C., Ellis, T., Session, W.S., Lehnert, N.L., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environmental and Nutritional Interactions (in press), 1993.*

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*Oberdörster, G., Ferin, J., Finkelstein, J., Baggs, R., Stavert, D.M., Lehnert, B.E.: Potential health Hazards from thermal degradation events: Particulate vs. gas phase effects. SAE Technical Paper Series, The Engineering Society for Advancing Mobility Land Sea Air and Space, pp.1-15, 1992.*

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*Lehnert, B.E.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Walter Reed Army Institute of Research, Department of Respiratory Research, Washington, D.C., October 23, 1992.*

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*Stavert, D.M., Lehnert, B.E.: Potentiation of the expression of nitrogen-dioxide induced lung injury by post exposure exercise. Environ. Res. 48:87-99, 1989.*

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*Wilson, J.S., Stavert D.M., Archuleta R.F., Lehnert, B.E.: A novel inhalation chamber for exposure of rats to oxidant gases. 1987 Annual Meeting of the Society of Toxicology, February 24-27, 1987, Washington, DC, The Toxicologist 7:813, 1987.*

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*Stavert, D.M., Archuleta, D.C., Holland, L.M., Lehnert, B.E.: Subchronic exposure of rats to 35 ppm $NO_2$ does not produce emphysema or cause a progression of pre-existing emphysema. Mountain States Chapter of the Society of Toxicology, Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM, October 3-4, 1985.*

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# SECTION A

## Objective 1: To design and fabricate an exposure system for multiple-animal (rats) exposures to stable, pure, and reproducible atmospheres of nitrogen dioxide ($NO_2$) and nitric oxide (NO).

**Results:** A state-of-the-art quartz crystal exposure system for $NO_2$ and NO was designed and fabricated with the following attributes: 1) no surface reactivity, 2) fast response for burst atmosphere exposures, and 3) non-constraining, non-avoidance animal exposure capability.

**Reports:**

*Stavert, D.M., Lehnert, B.E.: Nitric oxide and nitrogen dioxide as inducers of acute pulmonary injury when inhaled at relatively high concentrations for brief periods. Inhalation Toxicol. 2:53-67, 1990.*

*Wilson, J.S., Stavert, D.M., Archuleta, R.F., Lehnert, B.E.: A novel inhalation chamber for the exposure of rats to oxide gases. 1987 Annual Meeting of the Society of Toxicology, February 24-27, 1987, Washington, D.C., The Toxicologist 7:a813, 1987.*

## Objective 2: To characterize the kinetic course of development and severity of $NO_2$-induced lung injury following the inhalation of 25-250 ppm $NO_2$ for durations ranging from 5 to 30 min.

**Results:** Baseline lung gravimetric studies were performed and lung weight changes as a function of animal growth and age were characterized. Lung gravimetric and histopathologic alterations resulting from the various $NO_2$-exposure regimens were used as toxicologic endpoints for quantitatively characterizing the kinetic development and magnitude of pulmonary injury over a 48 hr post-exposure period. For a given duration of $NO_2$ exposure, the resulting severity of lung injury in the rat lung, as indexed by lung gravimetric and histopathologic criteria, increases with increasing exposure concentration.

**Reports:**

*Lehnert, B.E., Archuleta, D.C., Ellis, T., Session, W.S., Lehnert, N.M., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environmental & Nutritional Interactions (in press), 1993.*

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*Lehnert, B.E., Stavert, D.M., Wilson, J.S., Kusewitt, D.F., Behr, M.J.: Acute and subacute pathologic changes caused by high levels of nitrogen dioxide in the lungs of Fischer-344 rats. 38th Annual Meeting of the American College of Veterinary Pathologists, Monterey, CA, November 9-13, 1987.*

*Lehnert, B.E., Wilson, J.S., Stavert, D.M.: Early lung changes following acute exposures to relatively high concentrations of nitrogen dioxide. 1987 Annual Meeting of the Society of Toxicology, February 24-27, 1987, Washington, D.C. The Toxicologist 7:A45, 1987.*

*Wilson, J.S., Tillery, S.I., Lehnert, B.E.: Lung weight studies involving aging Fischer-344 rats and different shipment groups of age-matched rats. 1986 Society of Toxicology Annual Meeting, New Orleans, LA. The Toxicologist 6:A204, 1986.*

*Tillery, S.I., Lehnert, B.E.: Age-body weight relationships to lung growth in the Fischer-344 rat as indexed by lung weight measurements. Lab. Animal Science 20:189-194, 1986.*

*Stavert, D.M., Archuleta, D.C., Holland, L.M., Lehnert, B.E.: Exposure to 35 ppm $NO_2$ for 25 days does not produce emphysema of cause a progression of pre-existing emphysema in the rat lung. J. Toxicol. Environ. Health 17:249-267, 1986.*

*Stavert, D.M., Archuleta, D.C., Holland, L.M., Lehnert, B.E.: Subchronic exposure of rats to 35 ppm $NO_2$ does not produce emphysema of cause a progression of pre-existing emphysema. Mountain States Chapter of the Society of Toxicology, Lovelace Inhalation Toxicology Institute, Albuquerque, NM, October 3-4, 1985.*

## Objective 3: To assess $NO_2$ exposure concentration and exposure duration in terms of which of these two variables are of greater importance in determining the severity of $NO_2$-induced lung injury.

**Results:** Lung gravimetric and histopathologic evidence indicated that $NO_2$ exposure concentration is of greater importance in inducing lung injury than exposure time when the product of $NO_2$ concentration and exposure time is kept constant.

**Reports:**

*Lehnert, B.E., Archuleta, D.C., Ellis, T., Session, W.S., Lehnert, N.M., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass*

*concentrations of nitrogen dioxide. Environmental & Nutritional Interactions (in press), 1993.*

*Stavert, D.M., Lehnert, B.E.: Concentration versus time is the more important exposure variable in nitrogen dioxide-induced acute lung injury. 27th Annual Meeting of the Society of Toxicology. Dallas, TX, February 1988. The Toxicologist 8:A556, 1988.*

## Objective 4: To identify cellular changes in response to $NO_2$ by electron microscopic analyses.

**Results:** Cuboidal cell hyperplasia that occurs following $NO_2$ exposure was confirmed to represent Type II cell hyperplasia. Brief exposures to high concentrations of $NO_2$ may injure or otherwise compromise the resident population of type II pneumocytes and thereby limit their ability to proliferate as an early stage of lung repair.

**Reports:**

*Lehnert, B.E., Archuleta, D.C., Ellis, T., Session, W.S., Lehnert, N.M., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environmental & Nutritional Interactions (in press), 1993.*

*Kusewitt, D.F., Stavert, D.M., Behr, M.J., Lehnert, B.E.: Delayed nitrogen dioxide ($NO_2$)-associated type II cell hyperplasia with increasing concentrations of inhaled $NO_2$. 27th Annual Meeting o the Society of Toxicology. Dallas, TX, February 1988. The Toxicologist 8:A555, 1988.*

# INTRODUCTION

Several studies have shown that acute exposures to nitrogen dioxide ($NO_2$) can result in an array of effects including airway and alveolar epithelial cell damage (Gordon et al., 1986; Evans et al., 1986; Cabral-Anderson et al., 1977; Kleinerman, 1970; Stephens et al., 1972; Evans et al., 1973a, 1973b, 1977), pulmonary edema (Carson et al., 1962; Brown et al., 1983; Rossano, 1945), enhanced susceptibility to pulmonary infections (Goldstein and Haeprich, 1973; Ehrlich and Findlay, 1979; Illing et al., 1980; Gardner et al., 1979), enhancements in the tumoricidal and phagocytic characteristics of alveolar macrophages (Sone et al., 1983), changes in lung clearance (Vollmuth et al., 1986), bronchiolitis obliterans (Milne, 1969), pneumonia (Adley 1946), and death (Hine et al., 1970). Aside from those few investigations in which mortality has been studied as a toxicological endpoint (Hine et al., 1970), most laboratory studies involving acute exposures to $NO_2$ have employed near-peak ambient or supra-ambient concentrations $\leq$ 50 ppm $NO_2$ as test atmospheres primarily to induce pulmonary dysfunction and pathology for detailed investigations of specific phenomena at play in oxidant gas-induced injury (e.g., Guidotti, 1978; Johnson et al., 1982; Crapo, et al., 1978a, b; Sagai et al., 1982; Williams et al., 1971; Sevanian et al., 1979; Wright and Mavis, 1981; Mustafa and Tierney, 1978; Menzel, 1976; Evans et al., 1977, 1973a,b, 1975, 1976, 1977, 1978a,b; 1986; Freeman et al., 1969; Hackett, 1979; Elsayed and Mustafa, 1982; Csallany and Ayaz, 1978; Kim, 1978; Gordon et al., 1986; Vollmuth et al., 1986). Thus, although the effects of inhaling $NO_2$ and the processes underlying such effects have received considerable experimental attention, an information gap continues to exist regarding the induction and expression of pulmonary injury *per se* as a function of $NO_2$ exposure concentration and exposure time when $NO_2$ is acutely breathed. Such information is of essential importance in the setting of standards and predicting the severity of lung injury arising from brief exposures to high levels of $NO_2$, given the various occupational scenarios in which acute human exposures to high mass concentrations of $NO_2$ may occur e.g., firefighters, miners, welders, silo-fillers, military personnel, *inter alios.*

The primary objective of this study, accordingly, was to assess the

severity of lung injury induced in the laboratory rat as a mammalian model following brief exposures (2 to 30 min) to relatively high concentrations of $NO_2$ ranging from 25 ppm $NO_2$ to 250 ppm $NO_2$. Lung gravimetric and histopathologic alterations resulting from the various $NO_2$-exposure regimens have been used as toxicologic endpoints for quantitatively characterizing the kinetic development and magnitude of pulmonary injury over a 48 hr post-exposure period. Additionally, the data base generated in this study has been used to examine the relative importance of exposure concentration of $NO_2$ versus exposure duration in producing pulmonary injury.

## MATERIALS AND METHODS

### Animals, Animal Exposures, Sacrifice Schedules

Adult, male Fischer-344 rats (specific-pathogen-free, Sasco, Omaha, Neb; specific-pathogen-free, virus-free, Harlan Sprague Dawley, Indianapolis, Inc.) were delivered to Los Alamos National Laboratory and acclimated for a minimum of ten days prior to experimental use in a facility accredited by the American Association of Laboratory Animal Care. During this period and following the test gas exposures, the rats were housed in rooms that received HEPA-filtered air. The animals were maintained in suspension cages (two per cage) covered with spun polyester filters (DuPont number 22 Spinbound Polyester Filter, E.E. DuPont Co., Wilmington, Del.) Standard diet and water were provided ad libitum. Serum from several representative animals from each rat shipment group was collected upon arrival as well as from animals maintained in the animal facility over the course of each exposure study for serologic analyses (Microbiological Associates, Inc., Bethesda, MD). Consistently, all animals were found to be negative for Reovirus Type 3, Kilham's rat virus, Pneumonia virus of mice, Sendai virus, Mycoplasma pulmonis, rat coronavirus, Sialodacryoadenitus virus, and cilia-associated respiratory bacillus. At the time of the actual exposures, all the rats weighed between 210-295 g with the vast majority of the animals weighing 240-260 g. As previously reported (Tillery and Lehnert, 1986), the lung weights of healthy rats in the above weight range are closely similar.

Separate groups of 8-12 animals were exposed to 25, 50, 75, 100, 150, 200, or 250 ppm of $NO_2$ (see Table 1 for mass concentration equivalents) for durations of 2, 5, 15, or 30 min; exposure equivalents ([$NO_2$ in ppm] x exposure duration in minutes) administered by these various exposure regimens are given in Table 2 for reference purposes.

Table 1: Concentration (ppm)/Mass Concentration ($mg/m^3$) Equivalents*

| ppm | $mg/m^3$ |
|---|---|
| 25 | 46.9 |
| 50 | 93.9 |
| 75 | 140.8 |
| 100 | 187.8 |
| 150 | 281.6 |
| 200 | 375.5 |
| 250 | 469.4 |

* $mg/m^3 = ppm \frac{(M.W.\ NO_2 : 46)}{24.5}$

Table 2: $NO_2$ Exposure Equivalents Administered During the Various Exposure Regimens

| Exposure Condition | Exposure Equivalents (ppm) | Exposure Condition | Exposure Equivalents (ppm) |
|---|---|---|---|
| 25 ppm x 5 min | 125 | 150 ppm x 2 min | 300 |
| 25 ppm x 15 min | 375 | 150 ppm x 5 min | 750 |
| 25 ppm x 30 min | 750 | 150 ppm x 15 min | 2250 |
| | | 150 ppm x 30 min | 4500 |
| 50 ppm x 2 min | 100 | | |
| 50 ppm x 5 min | 250 | 200 ppm x 2 min | 400 |
| 50 ppm x 15 min | 750 | 200 ppm x 5 min | 1000 |
| 50 ppm x 30 min | 1500 | 200 ppm x 15 min | 3000 |
| | | 200 ppm x 30 min | 6000 |
| 75 ppm x 2 min | 150 | | |
| 75 ppm x 5 min | 375 | 250 ppm x 2 min | 500 |
| 75 ppm x 15 min | 1125 | 250 ppm x 5 min | 1250 |
| 75 ppm x 30 min | 2250 | 250 ppm x 15 min | 3750 |
| 100 ppm x 2 min | 200 | | |
| 100 ppm x 5 min | 500 | | |
| 100 ppm x 15 min | 1500 | | |
| 100 ppm x 30 min | 3000 | | |

After each exposure, the animals were returned to their cages. Animal sacrifices (N = 8-12) were performed at 4, 8, 24, and 48 hr following the exposures. After some of the earliest exposure studies, some animal groups were sacrificed for subsequent study at 18 hr post-exposure instead of the 8 hr sacrifice time. Corresponding control groups of animals consisted of rats that were exposed to anhydrous filtered air only for a period of 30 min. Members of the sham-exposed groups were also sacrificed (N = 8-12) for subsequent lung studies at 4, 8 or 18, 24, and 48 hr following the air-only exposures. A separate shipment group of animals was used for each $NO_2$ exposure concentration x exposure time regimen and corresponding sham-air controls. Accordingly, results obtained at each post-exposure sacrifice time following a particular $NO_2$ exposure regimen could be directly compared with data obtained with sham-air treated animals from the same shipment group of rats. It should be noted that preliminary as well as parallel studies conducted with those described herein have demonstrated that 30 min exposures to air-only do not bring about alterations in lung histology or lung wet or dry weights (data now shown).

All studies described in this report were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (1985).

Generation of Nitrogen Dioxide and Exposure System

Nitrogen dioxide was generated from dinitrogen tetroxide ($N_2O_4$) (Freeman and Haydon, 1964) contained within a stainless steel chamber and maintained at 24°C by a circulating water bath. The $NO_2$ was mixed with anhydrous and HEPA-filtered air in a mixing chamber, Figure 1.

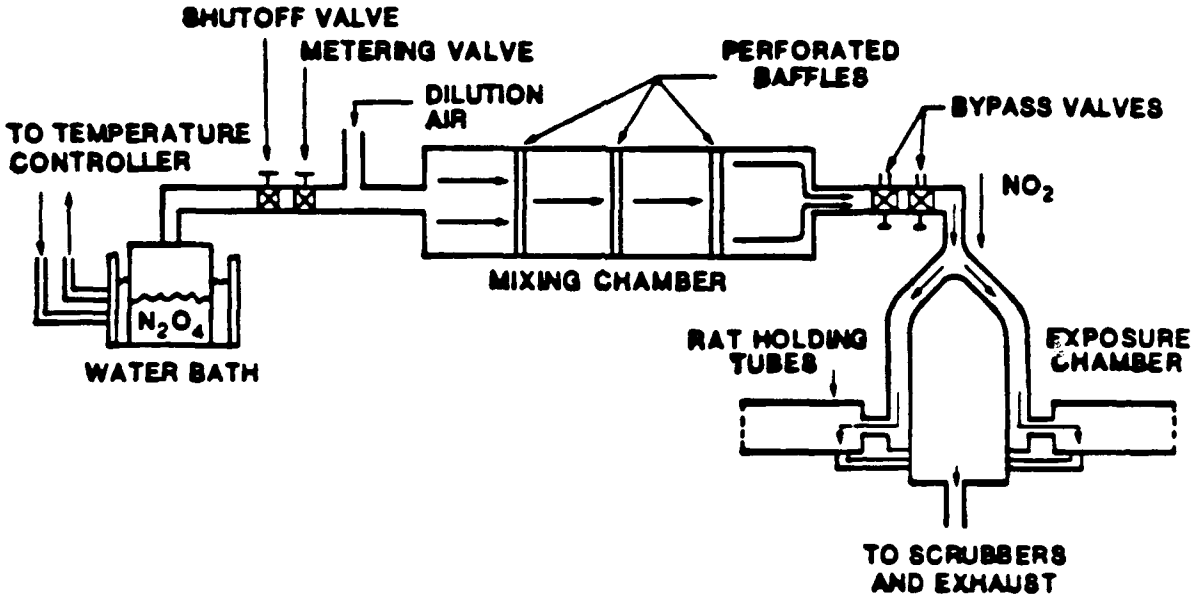



Figure 1: Diagram of the exposure system and associated components used to administer the test atmospheres.

Internal baffles inside the mixing chamber assured mixing and stabilization of the exposure atmospheres. $NO_2$ concentrations were regulated with a needle valve that controlled the amount of concentrated $NO_2$ added to the airstream entering the mixing chamber. From the mixing chamber, airflow was directed to a low internal volume, 12 port inhalation chamber, Figure 2.

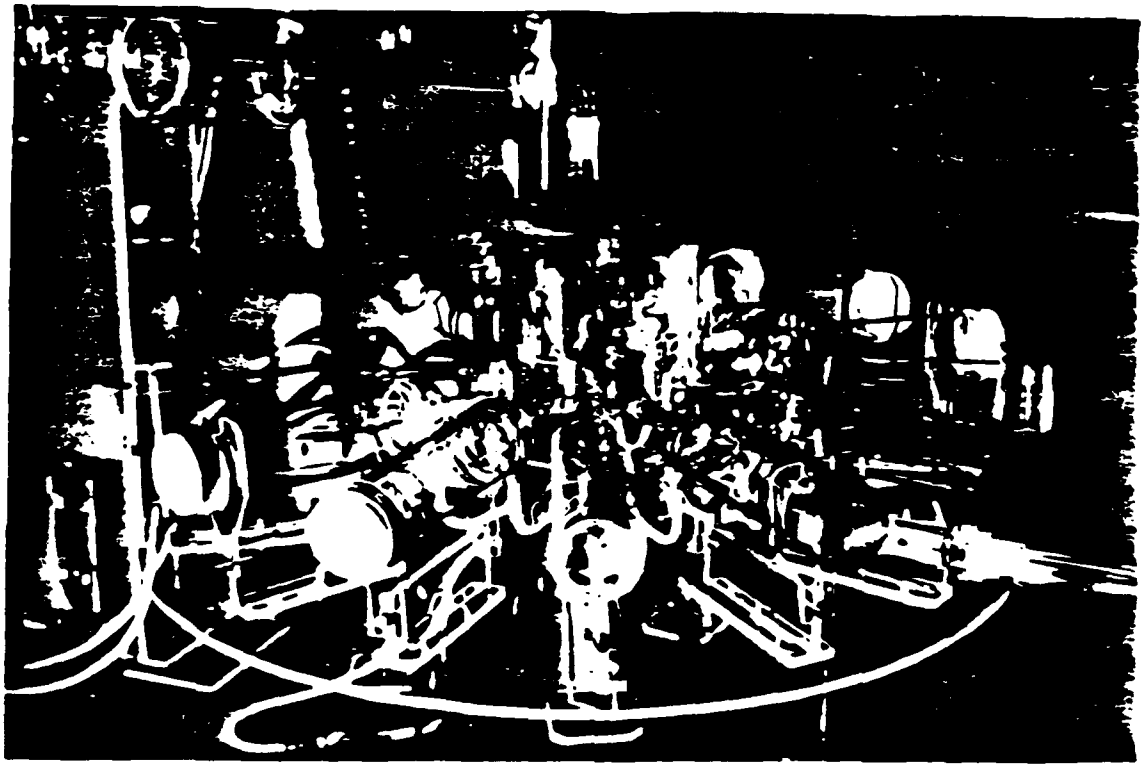


Figure 2: Radial quartz exposure chamber used to administer the $NO_2$ atmospheres.

Non-avoidance, non-constraining animal exposure tubes were attached to each port of the inhalation chamber and airflow was directed through each exposure tube as illustrated in Figure 3.

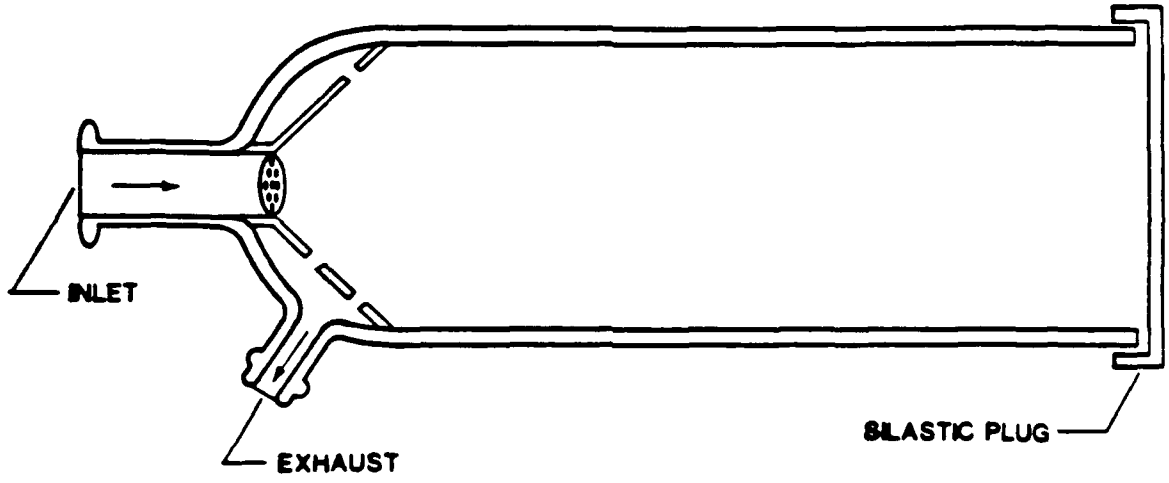



Figure 3: Diagram of a non-avoidance, non-constraining animal exposure tube. The tubes have been designed to provide the continual passage of an exposure atmosphere over the facial region of the rat.

Quartz glass valves located at the inhalation chamber inlet (see Figure 1) were used to temporarily direct the $NO_2$ airstream during the attachment of the exposure tubes to the chamber; fresh air only was delivered to the chamber during this time. The mixing chamber, inhalation chamber, and animal exposure tubes are fabricated from quartz glass (Quartz International, Albuq., NM) in order to minimize surface reactivity and maximize the purity of the exposure atmospheres. During the exposures, gas flow to the chamber was maintained at 12 liters $\cdot$ $min^{-1}$; over the course of the exposures, the inhalation chamber was maintained at a negative pressure of 0.2 inches of $H_2O$. After exiting the inhalation system, the exhaust stream of the test atmospheres was bubbled through a concentrated solution of sodium hydroxide prior to venting from the laboratory. A preliminary report on the exposure system has been presented elsewhere (Wilson et al., 1987).

Exposure atmosphere concentrations were continuously monitored with a

dual channel IR-UV spectrophotometer (Binos Inficon, Leyhod-Heraeus, Fed. Rep. of Germany) calibrated with primary gas standards (N.B.S.). Exposure concentrations of 25 ppm to 250 ppm $NO_2$ have been generated with rise times to equilibrium in the chamber of less than 15 sec, Table 3. As well, nominal gas concentrations within the chamber have been found to be stable over the course of prolonged exposure periods. For example, exposures to 150 ppm $NO_2$ deviate by less than 4 ppm from the nominal gas concentration over a 30 min exposure period. Nitric oxide (NO) concentrations generated during the exposures were always less than 30 ppm, Figure 4. Recent studies in our laboratory have indicated that NO concentrations well above those generated during the $NO_2$ exposures do not bring about histopathologic alterations or gravimetric changes in the lungs of rats (Lehnert and Stavert, 1987).

Table 3

| Nominal Concentration (ppm) | Measured $NO_2$ Concentration (ppm ± SD) | Range (ppm) | Rise Time - t 1/2 (sec) | Decay Time - t 1/2 (sec) |
|---|---|---|---|---|
| 25 | 26.1±1.0 | 23-27 | 5.2±0.9 | 8.2±2.9 |
| 50 | 49.2±2.0 | 47-52 | 4.6±0.9 | 8.4±2.5 |
| 150 | 152±3.4 | 145-158 | 5.3±0.7 | 9.6±1.9 |
| 200 | 203±4.2 | 194-211 | 6.2±1.6 | 8.6±2.1 |

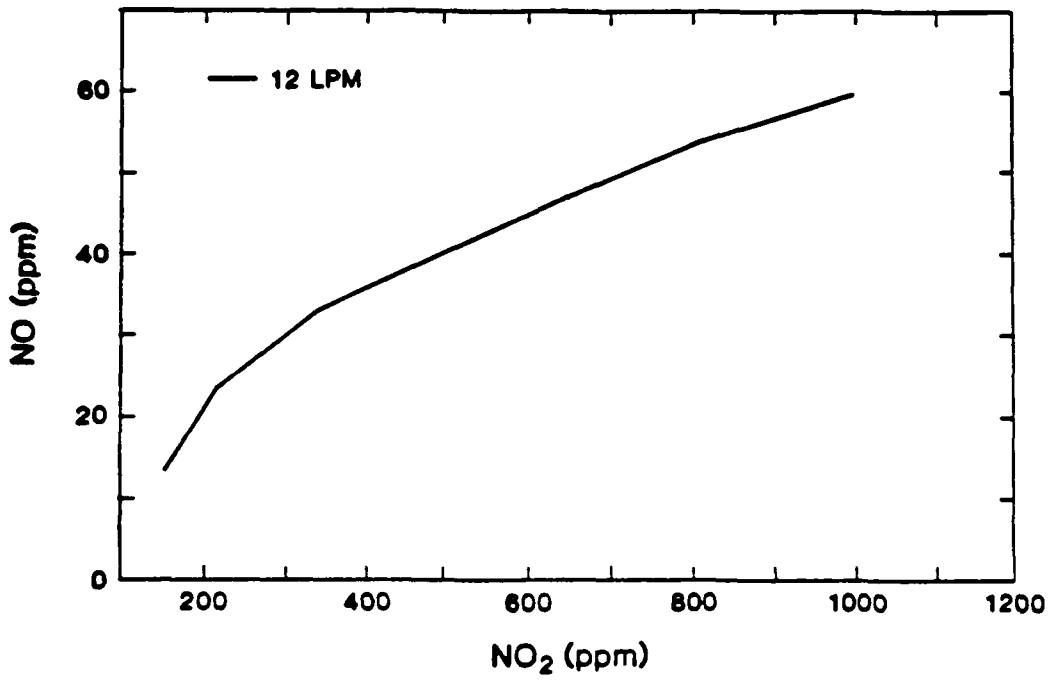



Figure 4: Background NO contamination in the exposure chamber as a function of $NO_2$ concentration delivered to the experimental animals.

Animal Sacrifices, Lung Gravimetric Measurements, and Tissue Processing

Rat sacrifices at the designated post-exposure times were initiated by intraperitoneal injections of 50 mg pentobarbital sodium. Upon complete apnea, the tracheae were cannulated with 18-gauge rods that were secured with ligature. The thoracic cages were surgically opened immediately thereafter, and each animal was exsanguinated via cardiac puncture and the aspiration of blood from the right ventricle. The trachea and lungs were then excised, and after removal of the heart, extra-pulmonary mediastinal tissue, and the esophagus, the volume of the lung preparation, or excised lung volume (ELV), was measured by saline displacement (Lai and Hildebrant 1978, Stavert et al., 1986). The lungs were then blotted dry and the tracheal cannulae removed. The bronchus leading to the right cranial lobe (RCL) was ligated with fine suture and the RCL was removed. The remaining lung preparation and the RCL were then collectively weighed (Lung Wet Weight, LWW), and the RCL were weighed separately (Right Cranial Lobe Wet Weight, RCLWW). Following the gravimetric measurements, the trachea and lungs, minus the RCL, was cannulated with an 18-gauge needle secured with ligature and subsequently infused and fixed at a constant pressure of 30 cm $H_2O$ with 10% formalin in phosphate-buffered saline for 48 hr. Thereafter, the post-fixation volume ($TLC_{30\ cm\ H_2O\text{-}Fixed}$) was measured by saline displacement (Stavert et al., 1986; Lai and Hildebrandt, 1978). The RCL were oven dried to a constant weight at 35°C for 36 hr and reweighed (Right Cranial Lobe Dry Weight, RCLDW). Inasmuch as the ELV and $TLC_{30\ cm\ H_2O\text{-}Fixed}$ measurements were found not to usefully serve as indicators of lung changes brought about by the $NO_2$ exposures (Lehnert et al., 1987), these data will not be presented in this report.

For the histologic analyses, the fixed left lungs were uniformly sliced on the same plane as the main-stem bronchi from the apex to the base along a line between the most posterior to the most anterior aspects to expose the maximal planar surface area for sectioning (Stavert et al., 1986). The blocks of tissue were embedded in paraffin, and 4 $\mu$m sections (two per lung) were prepared and stained with hematoxylin and eosin by conventional methods.

## Histopathology

$NO_2$-induced lung injury in the rat predominantly occurs in the terminal bronchioles, alveolar ducts, and proximal alveoli (e.g., Rombout et al., Hackett, 1979; Cabral-Anderson et al., 1977; Evans et al., 1977; Stephens et al., 1972; Evans et al., 1972). Interms of histopathologic alterations caused by the various exposure conditions used in this study, attention is especially focused on changes that occur in the alveolar duct-alveolar region of the lung where gas exchange is most prominent.

Hallmarks of $NO_2$ injury in the alveolar region of the lung that are detectable by light microscopy include the appearance of fibrin in the alveolar spaces, accumulations of polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM) in the alveolar spaces, the extravasation of erythrocytes into the alveolar space compartment, and, hyperplasia of type II pneumocytes. Fibrin, abnormal accumulations of PMN and AM, and the extravasation of erythrocytes are indicative of acute lung injury and the associated inflammatory response (Dradin et al., 1986; Warnock, 1982). Inasmuch as type II cell hyperplasia occurs in response to type I cell death (Rombout et al., 1986; Evans et al., 1980; Evans et al., 1977), the magnitude of the former likely serves as an index of the prevalence of the latter. In order to characterize the occurrence of these oxidant gas-induced abnormalities for comparative purposes, a grading scale has been used to quantitatively describe the relative severity of each of the above pathologic features in terms of their intensity of expression as well as their distribution in the lung. The intensity index scale for a given feature, e.g., fibrin accumulation, ranges from 0-4 with 0=not detected, 1=trace to mild in appearance, only few proximal alveoli contained fibrin; 2=present in moderate degree, several proximal alveoli contained fibrin; 3=present in high degree, many proximal alveoli and some more distal alveoli contained fibrin; and 4=present in highest intensity, essentially all proximal and more distal alveoli contained fibrin. The relative distribution index for a given pathologic feature ranges from 0-3 with 0=not present, 1=focal, i.e., present in occasional alveolar duct and proximal alveolar structures, 2=multifocal, i.e., present in several alveolar duct and proximal alveolar structures=, and 3=diffuse, i.e., present in virtually all alveolar duct and proximal alveolar structures.

For further comparative purposes involving analyses of fibrin and type II cell hyperplasia, the intensities and distributions of these $NO_2$-induced lung lesions were expressed as "generalized" indices. The "generalized" indices were formulated by multiplying the original intensity index value by 10, multiplying the corresponding distribution index by 13.33, and then multiplying these products; the 13.33 factor was used to normalize the distribution data to the same scale as that used for the intensity data. Accordingly, both the intensity and distribution component of the fibrin and type II cell hyperplasia data were given equivalent importance in the development of the "generalized" indices.

Statistical Analyses

The lung gravimetric data were analyzed by one way analysis of variance (Snedecor and Cochran, 1969a). When a significant value for the F ratio was obtained, data from each $NO_2$-exposed group was compared to data obtained from the corresponding sham-air exposed (Control) group using Dunnett's t-test (Gad and Weil, 1982; Dunnett, 1955; Dunnett, 1964). Regression lines were calculated by least squares fit analyses (Snedecor and Cochran, 1969b). Pearson's correlation coefficient, or the r value, was determined and its significance was evaluated by the r-test (Snedecor and Cochran, 1969c). Probability values (p) less than 0.05 were considered significant. Unless otherwise indicated, values presented in figures represent group averages; this manner of data presentation was selected for the sake of clarity. Actual means and standard deviations of these data are presented in the Addendum.

## RESULTS

Lung Wet Weights (LWW): Relative to control values, the average percent changes in the LWW of animals exposed to the various $NO_2$ regimens and subsequently sacrificed at 4, 8 or 18, 24, and 48 hr thereafter are graphically summarized in Figures 5A-5D. The absolute lung weights of the different animal groups are expressed as means and corresponding standard deviations in the Addendum, Table A1.

As indicted in Figure 5A, two minute exposures to the various

concentrations of $NO_2$ did not significantly affect the LWW with the exception of the 200 ppm exposure group in which the LWW parameter was slightly but significantly elevated. Following the 5 min exposures, the LWW of animals exposed to 150, 200, and 250 ppm $NO_2$ were significantly increased at virtually all post-exposure times with the greatest increases occurring after exposure to 250 ppm $NO_2$ (~ 128% increase). Increases in the LWW of animals exposed to 150, 200, and 250 ppm became more markedly pronounced following the 15 min exposures, Figure 5C. With the 250 ppm exposure, for example, the LWW had increased about two-fold as early as 4 hr after exposure. Unlike the 5 min exposure, the 15 min exposure to 100 ppm $NO_2$ resulted in significant elevations in LWW as of the 24 and 48 hr sacrifice times. As shown in Figure 5C, the $NO_2$-induced increases in LWW at the various post-exposure times were dependent upon exposure concentration with the highest exposure concentration studied giving the greatest effect. No trends were observed for elevations in the LWW of rats exposed for 15 min to 25, 50, or 75 ppm $NO_2$. Further increases in LWW were observed with the animals exposed to 100,150, and 200 ppm $NO_2$ for 30 min, Figure 5D. No exposure studies were conducted for this duration using a 250 ppm $NO_2$ concentration inasmuch as all the animals exposed to 200 ppm for 30 min died before the 8 hr sacrifice time; at the 4 hr sacrifice time, the LWW of rats that breathed 200 ppm $NO_2$ for 30 min had increased over three-fold relative to control LWW values. Also of notable importance, increases in LWW became detectably significant at the 4 hr time point after the animals breathed 75 ppm $NO_2$ for a 30 min duration, Figure 5D.

A summary of the 24 hr post-exposure changes in LWW caused by the various $NO_2$ concentrations administered for 2, 5, 15, and 30 min is illustrated in Figure 6. For concentrations $\geq$ 75 ppm $NO_2$, increases in the gravimetric parameter increased as a function of exposure time. A generalization that increasing concentrations of inhaled $NO_2$ produces corresponding increases in LWW when inhaled over the same exposure period was especially supported by the LWW data obtained after the 15 min exposure, Figure 7.

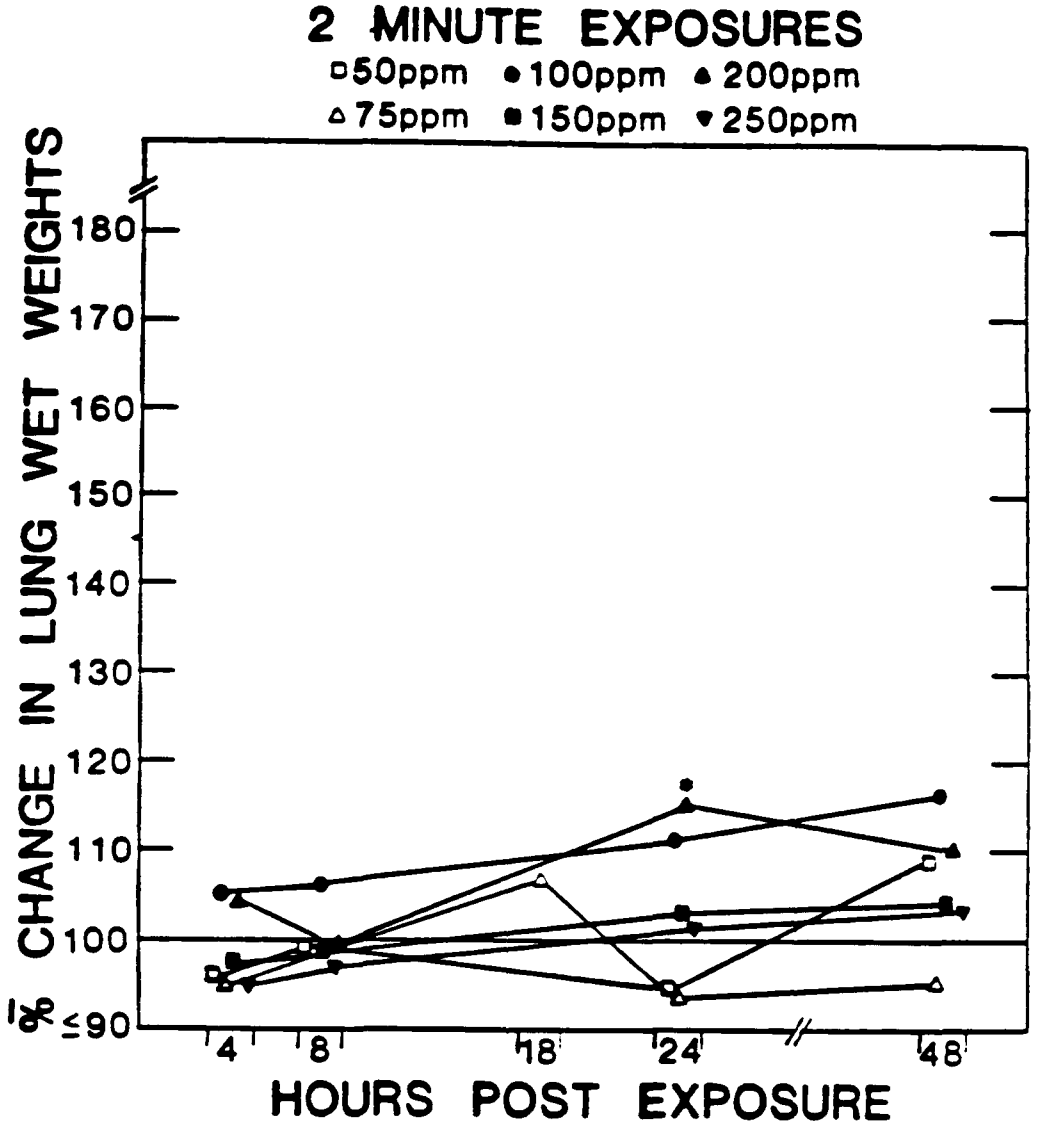



Figure 5A: Average percent change in the lung wet weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 2 minutes. Marked values (*) are significantly higher than control values, $p < 0.05$.

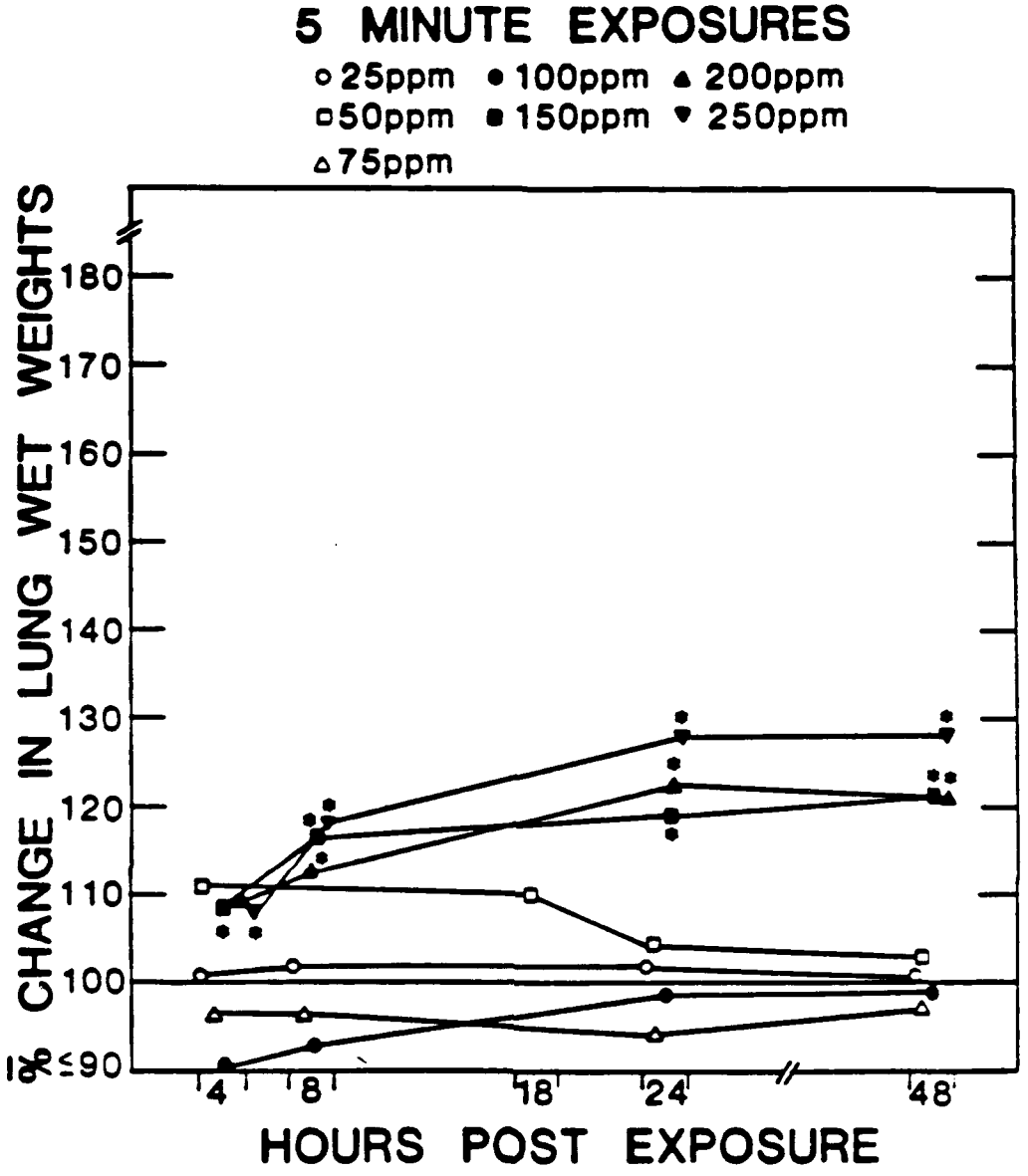



Figure 5B: Average percent change in the lung wet weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 5 minutes. Marked values (*) are significantly higher than control values, $p < 0.05$.

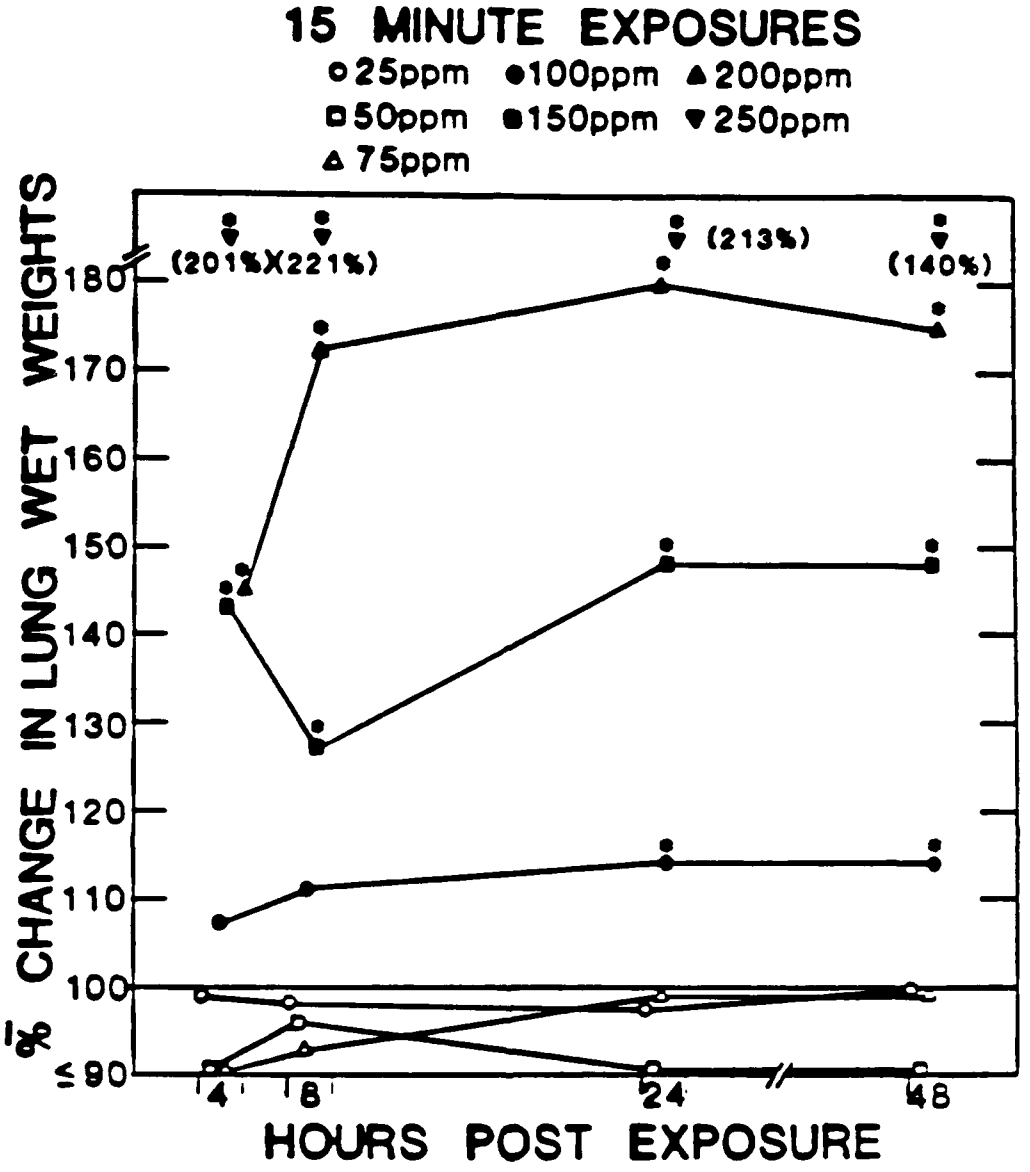



Figure 5C: Average percent change in the lung wet weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 15 minutes. Marked values (*) are significantly higher than control values, $p < 0.05$.

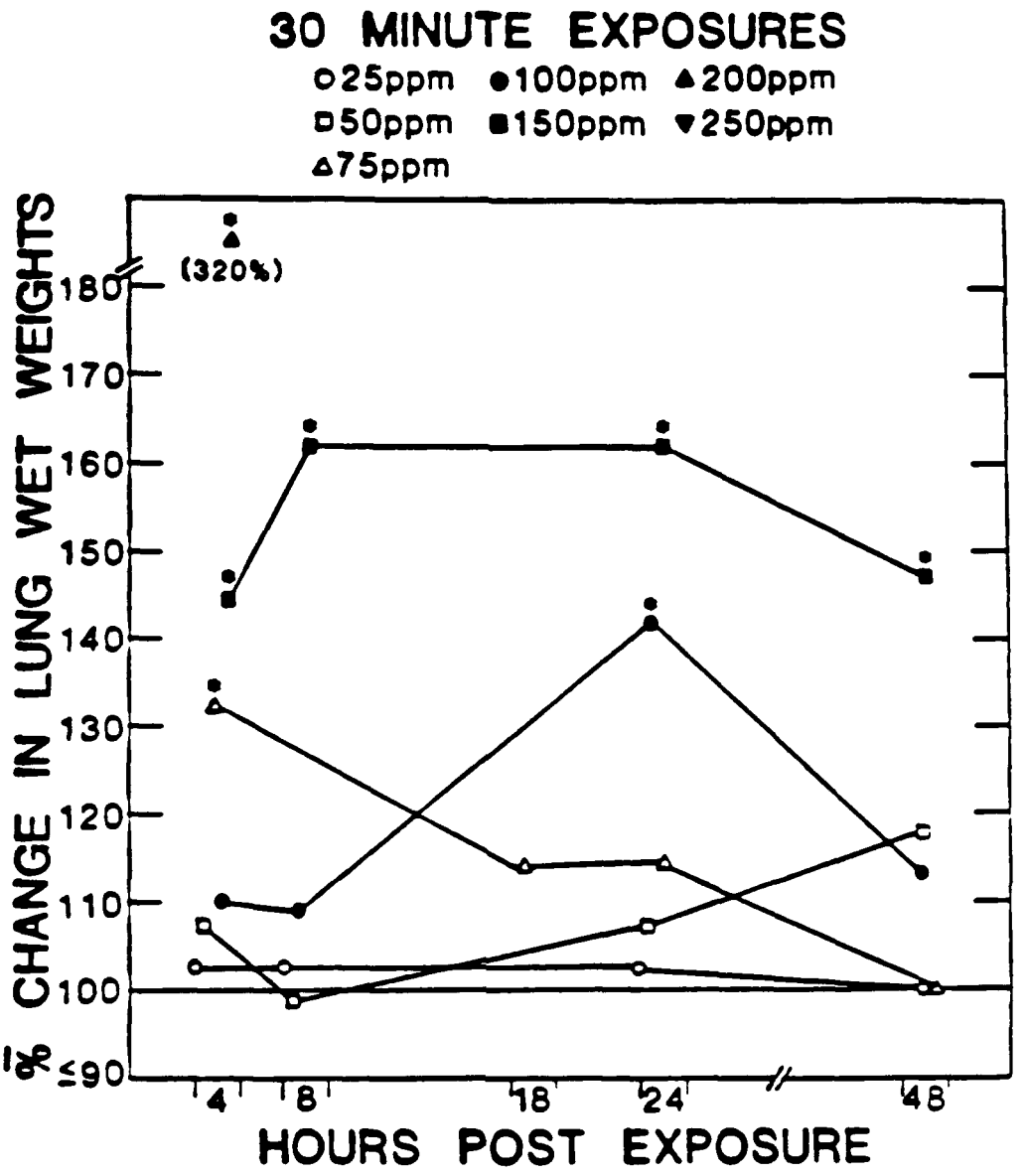



Figure 5D: Average percent change in the lung wet weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 30 minutes. Marked values (*) are significantly higher than control values, $p<0.05$.

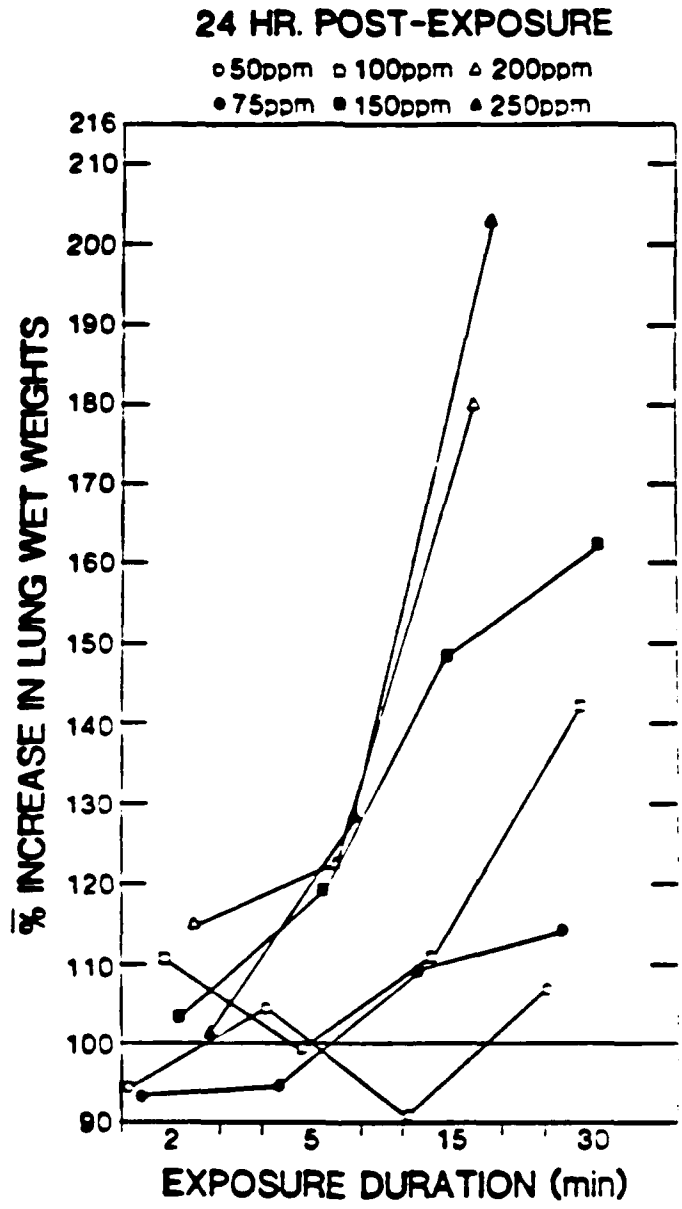



Figure 6: Changes in lung wet weights following exposures to the various $NO_2$ concentrations administered for 2, 5, 15, and 30 min.

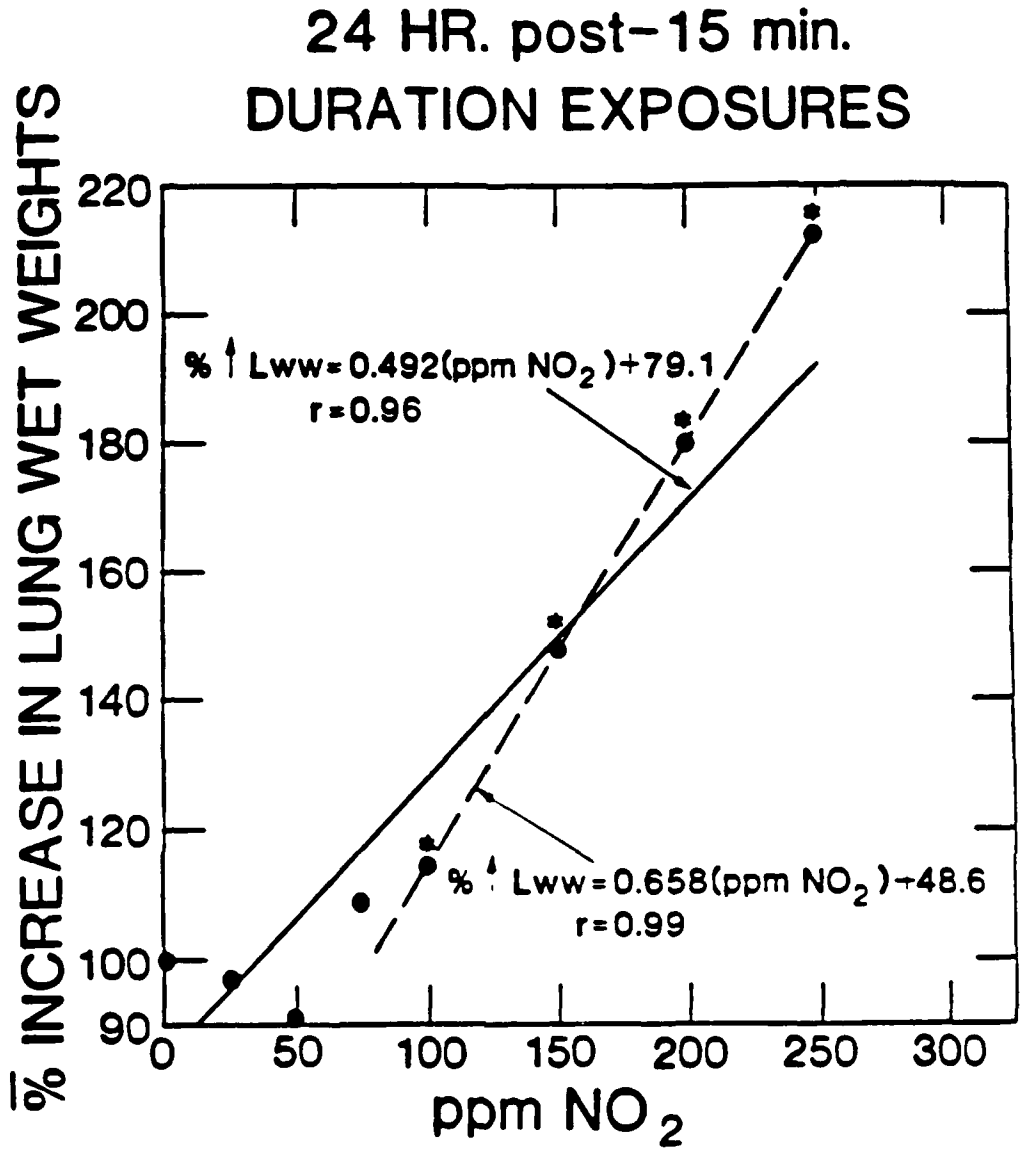



Figure 7: Increases in lung wet weights as a function of exposure concentration when the $NO_2$ was delivered to the animals for a 15 min duration. The solid line represents all data, regardless of significance. The dashed line was obtained by linear regression analysis of data points that were found to be significantly increased: Marked values (*) are significantly greater than control values. Both correlation coefficients are significant, $p < 0.01$.

Right Cranial Lobe Dry Weights (RCLDW): The average percent changes in the RCLDW of rats exposed to the various $NO_2$ regimens and subsequently sacrificed at 4, 8, or 18, 24, and 48 hr thereafter are graphically summarized in Figures 8A-8D. The absolute weights of the dried right cranial lobes are expresssed as means and correseponding standard deviations in the Addendum, Table A1.

Whereas some significant increases in RCLDW values were found following the 2 min exposures to 50,100,200, and 250 ppm $NO_2$, Figure 8A, these statistically significant alterations did not appear to follow a uniform pattern over the post-exposure sacrifice times; some of the RCLDW values determined to be elevated by the statistical tests may have been due to happenstance sampling. For example, the statistical analyses indicated significant elevations in RCLDW as of 8 hr after exposure to 150 ppm, even though the average percent increase was only ~ 105%. Generally, such low percentage differences are within the range of variation of the gravimetric assay. Regardless, more definitively distinct alterations in the RCLDW values were evident following the exposures to 150, 200, and 250 ppm $NO_2$ for 5 min, Figure 8B. As early as 8 hr after these exposures, the RCLDW had increased about 120% above control values. No significant increases were found in RCLDW at any of the sacrifice times after the 5 min exposures to 50,75, or 100 ppm $NO_2$, Figure 8B. Similar to the LWW results, exposure concentration-related increases in RCLDW were evident following the 15 min exposures to $NO_2$ with the greatest increases in RCLDW following the exposure to 250 ppm $NO_2$, Figure 8C. Notably, the 15 min exposures to 25,50, and 75 ppm did not produce elevations in the RCLDW of the experimental animals. With the 30 min exposures to $NO_2$, Figure 8D, those exposure concentrations that produced increases in RCLDW following the 15 min exposures brought about substantially greater elevations in this gravimetric parameter at virtually all post-exposure times as the exposure duration was prolonged. Although the RCLDW values tended to be elevated following the 75 ppm exposures (post-exposure hours 4,24, and 48), and at 48 hr after the 30 min exposure to 50 ppm, the RCLDW values obtained with the rats exposed to 25,50, and 75 ppm $NO_2$ were not significantly different from the RCLDW values obtained with the sham exposed, or control rats, Figure 8D.

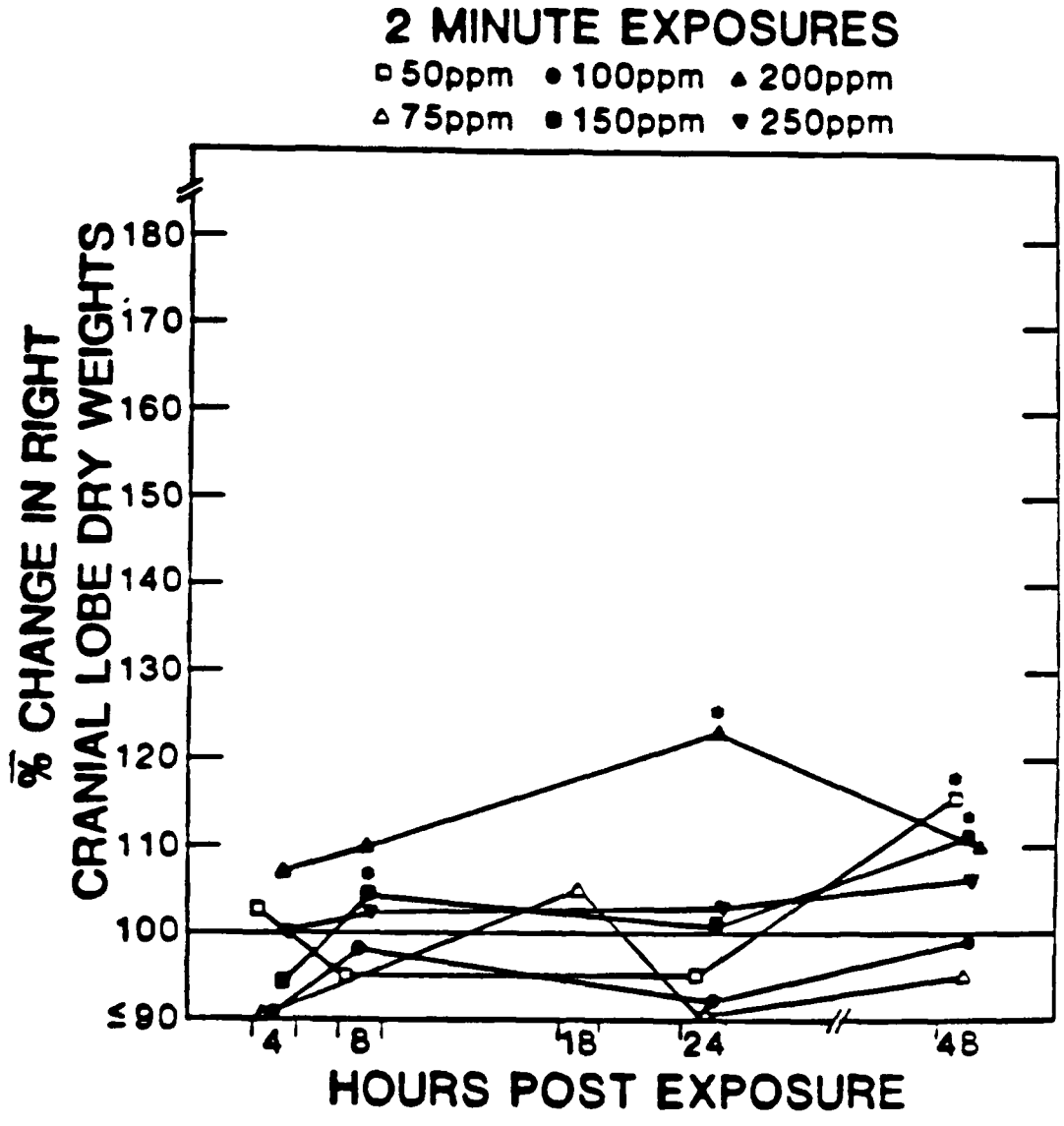



Figure 8A: Average percent change in the right cranial lobe dry weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 2 minutes. Marked values (*) are significantly higher than control values, $p < 0.05$.

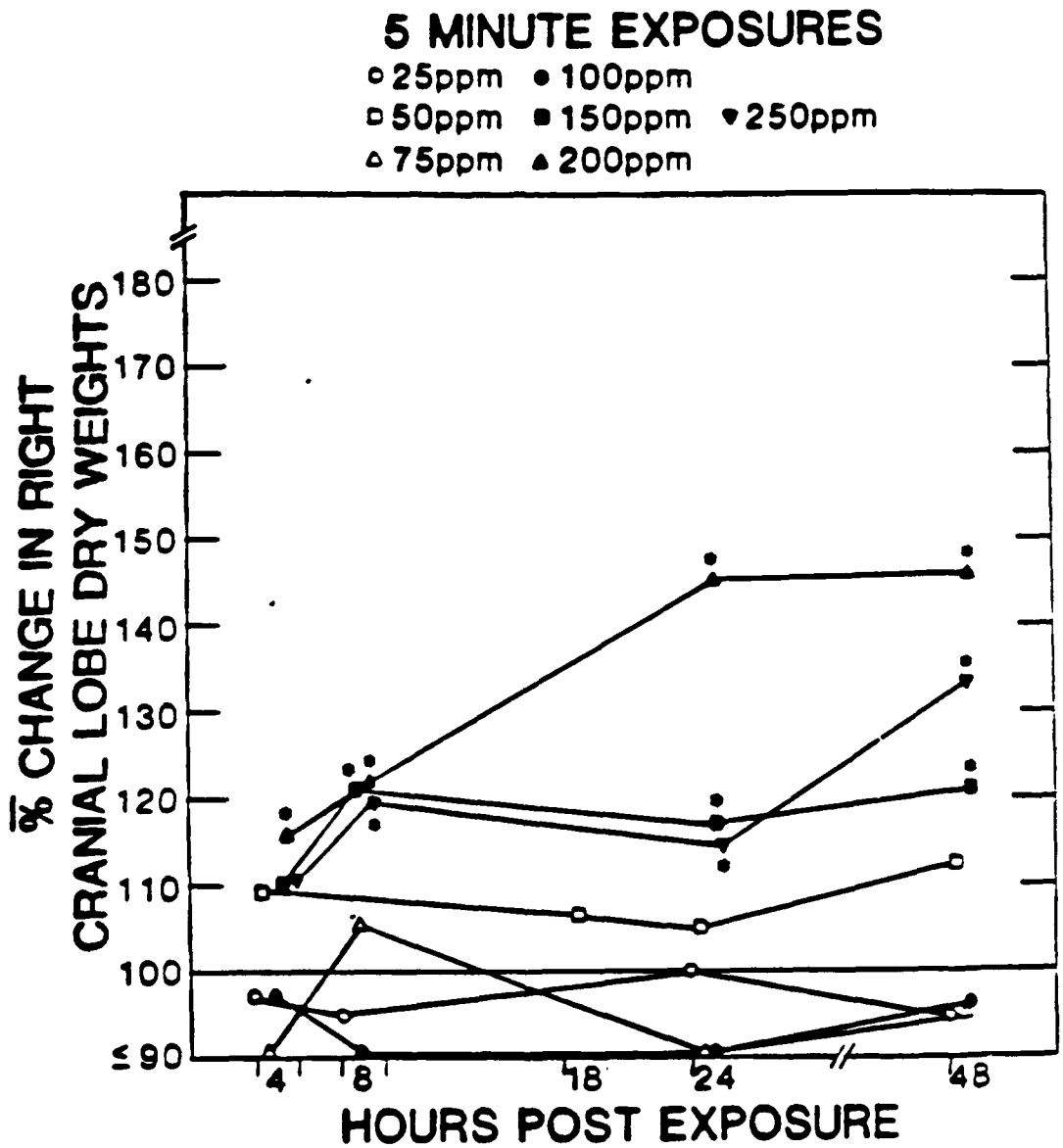



Figure 8B: Average percent change in the right cranial lobe dry weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 5 minutes. Marked values (*) are significantly higher than control values, $p < 0.05$.

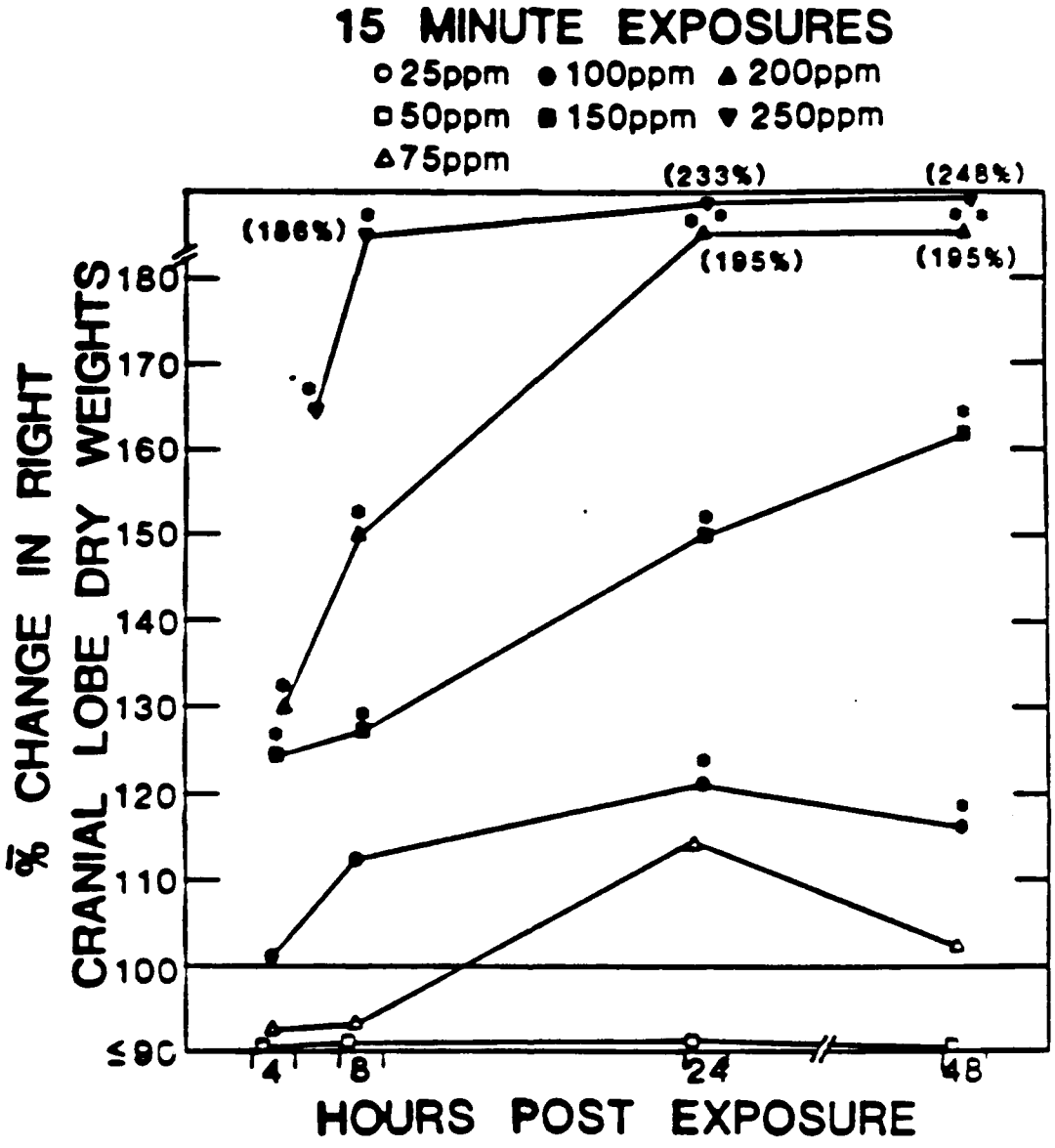



Figure 8C: Average percent change in the right cranial lobe dry weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 15 minutes. Marked values (*) are significantly higher than control values, $p < 0.05$.

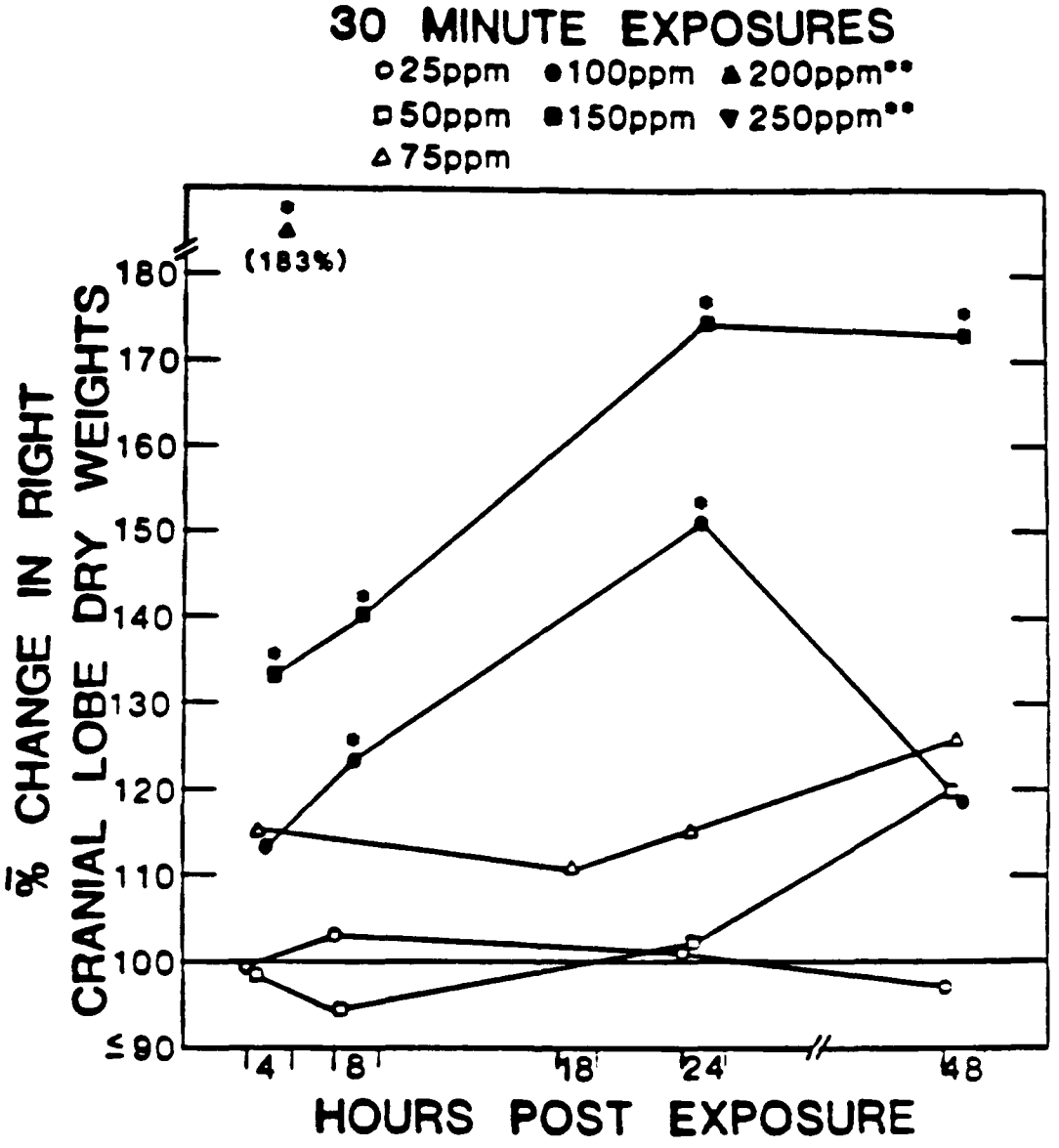



Figure 8D: Average percent change in the right cranial lobe dry weights of animals exposed to the various $NO_2$-containing atmospheres for a duration of 30 minutes. Marked values (*) are significantly higher than control values, $p<0.05$.

## Histopathology

General Observations: As reported by other investigators (e.g., Stephens, 1972; Rombout et al., 1986; Evans, 1973a) the pulmonary lesion produced by $NO_2$ in the present studies centered around the terminal bronchiole-alveolar duct-proximal alveolar regions in the lungs. Actual changes in the epithelial linings of the conducting airways, including the terminal bronchioles, were difficult to assess quantitatively at the light microscopic level. At the higher $NO_2$ concentrations and more prolonged exposure durations, however, some alterations in the epithelium of the terminal bronchioles was discernible. These changes included the sloughing of epithelial cells, and the appearance of aggregates of clumped, presumably exfoliated cells on the luminal surface. The most prominent lesions produced by the inhalation of $NO_2$, however, were found in the alveoli immediately surrounding alveolar ducts and, in instances of more pronounced injury, in the alveolar ducts themselves. The main immediate effect of $NO_2$ appeared to be damage to the local pulmonary vasculature and resulting changes in the permeability of the capillary-alveolar epithelial barrier inasmuch as fibrin and extravasated erythrocytes were early hallmarks of $NO_2$-induced injury. An acute recruitment of polymorphonuclear leukocytes usually accompanied this early response, especially when vascular leakage was prominent. These acute changes appeared as early as 4 hr post-exposure, but usually not latter than 8 hr after exposure if they were to be expressed at all. Moreover, the acute disorders were generally maximally severe at the 8 or 18 and/or 24 hr post-exposure times; the intensities and distributions of fibrin at these sacrifice times, for example, were usually closely similar and often times virtually identical. The most consistent component of the acute lesion was the presence of fibrin strands in the alveoli surrounding the terminal bronchioles. The numbers of alveoli containing fibrin and the numbers of terminal bronchioles and alveolar ducts surrounded by fibrin-containing alveoli generally increased with increasing exposure concentrations of $NO_2$ administered over a given exposure duration, or when exposure durations at a given $NO_2$ exposure concentration were more prolonged. In the lower $NO_2$ concentration range and in some of the shorter duration exposure studies in which lung injury was mild, fibrin disappeared from the alveoli by 48 or

even 24 hr after exposure. At higher $NO_2$ concentrations and more prolonged exposures, however, the appearance of fibrin persisted in the alveoli for up to 48 hr post-exposure. The appearance of erythocytes in the alveoli usually paralleled the presence of fibrin and increased in abundance with increasing exposure concentrations and exposure times. In general, the numbers of alveoli containing erythrocytes peaked at 4-8 hr post exposure; at high exposure concentrations and longer exposure times, however, red blood cells continued to be present in the alveoli for longer periods of time. Despite the evidence for extravasation of blood constituents and pulmonary edema provided by the presence of fibrin and red blood cells in the alveoli, little evidence of peribronchiolar vascular congestion, perivascular edema, or septal edema was seen, especially with the lower concentrations of $NO_2$ and shorter exposure times. At higher concentrations and more prolonged exposure times, a suggestion of peribronchiolar congestion and perivascular edema was observed, but these disorders were difficult to quantitate. It remains possible that these lesions were more prominent very soon after the $NO_2$ exposures and may have largely resolved as of 4 hr post-exposure. Regardless, no evidence of septal edema per se was seen at the light microscopic level following any of the exposures. As to the recruitment of neutrophils to the involved sites, their numbers were usually maximal at 24 hr after exposure. When the neutrophil response was not intense, it usually subsided by 48 hr post-exposure, but it was longer lived after the exposures to the higher $NO_2$ concentrations and longer duration exposures.

Resolution of the acute lesion consisted of partial resorption, phagocytosis, dissolution, or alveolar clearance of fibrin, red blood cells, and polymorphonuclear leukocytes. In most exposed groups, the resolution phase was accompanied by the appearance of alveolar macrophages in abnormal abundance in alveoli containing fibrin; often times these cells appeared to be enmeshed in the alveolar fibrin. The alveolar macrophages appeared as early as 4 hr after some exposure studies and as late as 24 hr after exposure in others. Generally, the appearance of alveolar macrophages was more rapid as the $NO_2$ exposure concentrations and exposure times were increased. Regardless, their numbers tended to be greater as of 48 hr after exposure.

A prominent later-phase change arising from the $NO_2$ exposures was the

development of type II epithelial cell hyperplasia in the peri-terminal bronchiolar alveoli, Figure 9. Type II cell hyperplasia was seen in all groups except those in which only slight or the mildest acute lesions were observed. Evidence of this hyperplastic change was seen in some groups as early as 24 hr after exposure, but the appearance of type II cell hyperplasia was more pronounced as of 48 hr after the exposures. In general, the intensities and distributions of type II cell hyperplasia paralleled those of the acute lesion.

Figure 9: Electron micrograph of the alveolar region demonstrating type II cell hyperplasia 48 hr after exposure to 150 ppm $NO_2$ for a 30 min duration.

Quantitative Histopathology: The average values for the intensity and distribution indices for fibrin, extravasated erythrocytes, polymorphonuclear leukocytes (PMN), alveolar macrophages (AM), and type II cell hyperplasia following the various $NO_2$ exposure regimens are graphically summarized in Figures 10A-10R. The means and standard deviations of the intensity and distribution indices for each of these histologic features are given in the Addendum, Table A2. For the sake of clarity, the histologic effects of inhaling $NO_2$ will be described on the basis of exposure duration. Because the appearance of fibrin and type II cell hyperplasia appeared to be the most consistent histopathologic indicators of $NO_2$-induced injury, histologic comparison of the lung toxicities of the various $NO_2$ exposure concentrations will primarily focus on these pathologic changes.

Figure 10A: Intensities and distributions of fibrin at 4hr after exposure to the various $NO_2$ exposure regimens.

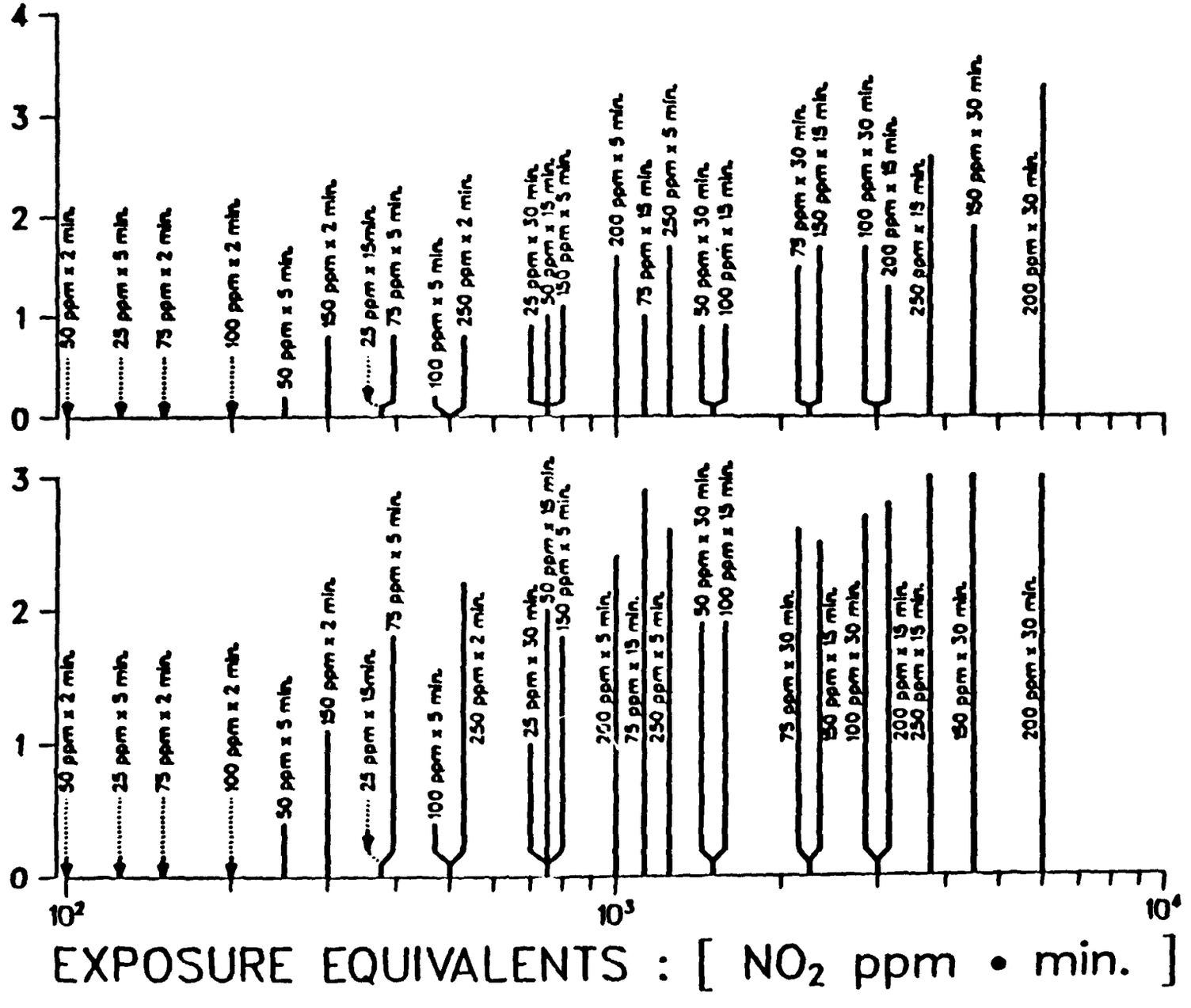


Figure 10B: Intensities and distributions of fibrin at 8hr after exposure to the various $NO_2$ exposure regimens.

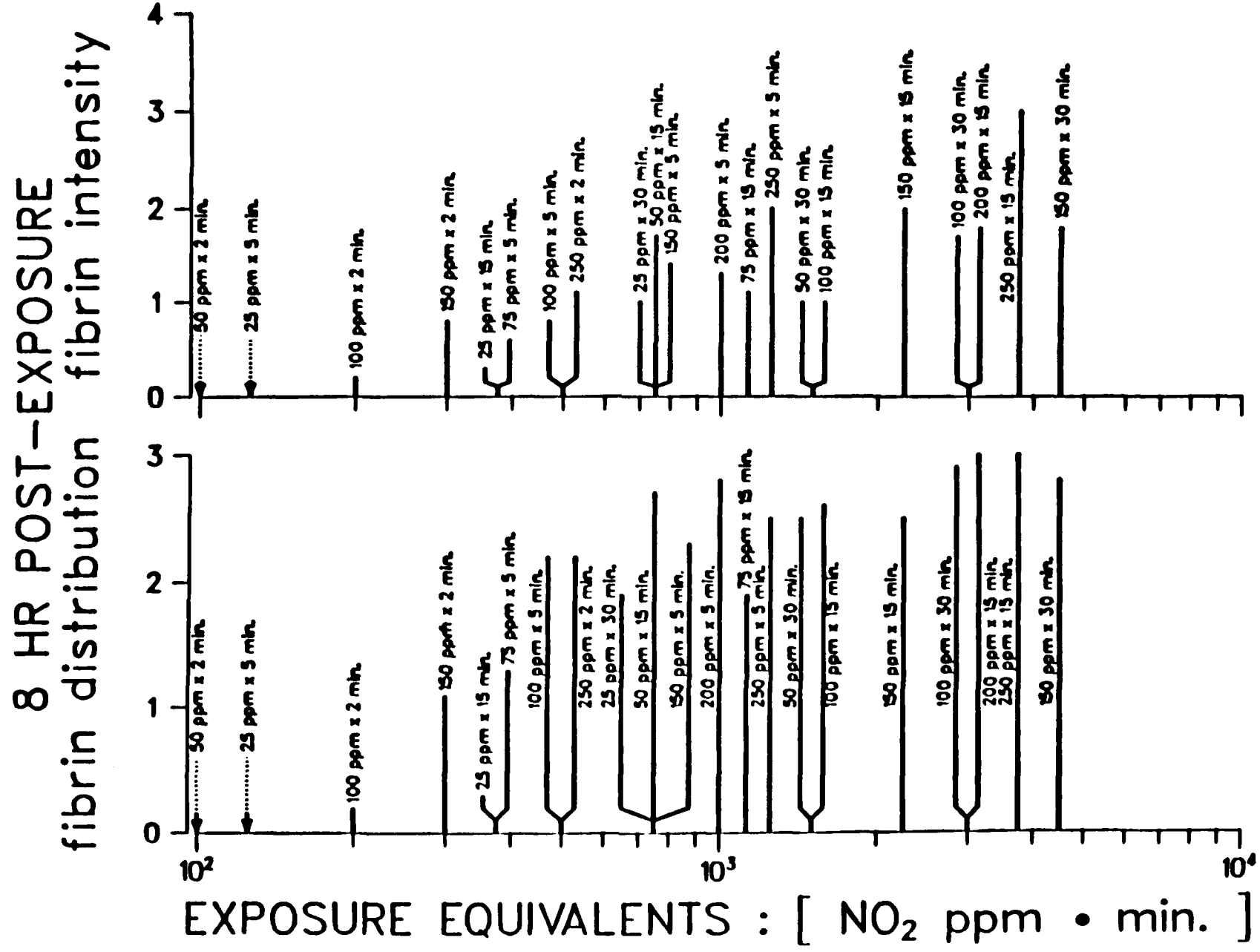


Figure 10C: Intensities and distributions of fibrin at 24hr after exposure to the various $NO_2$ exposure regimens.

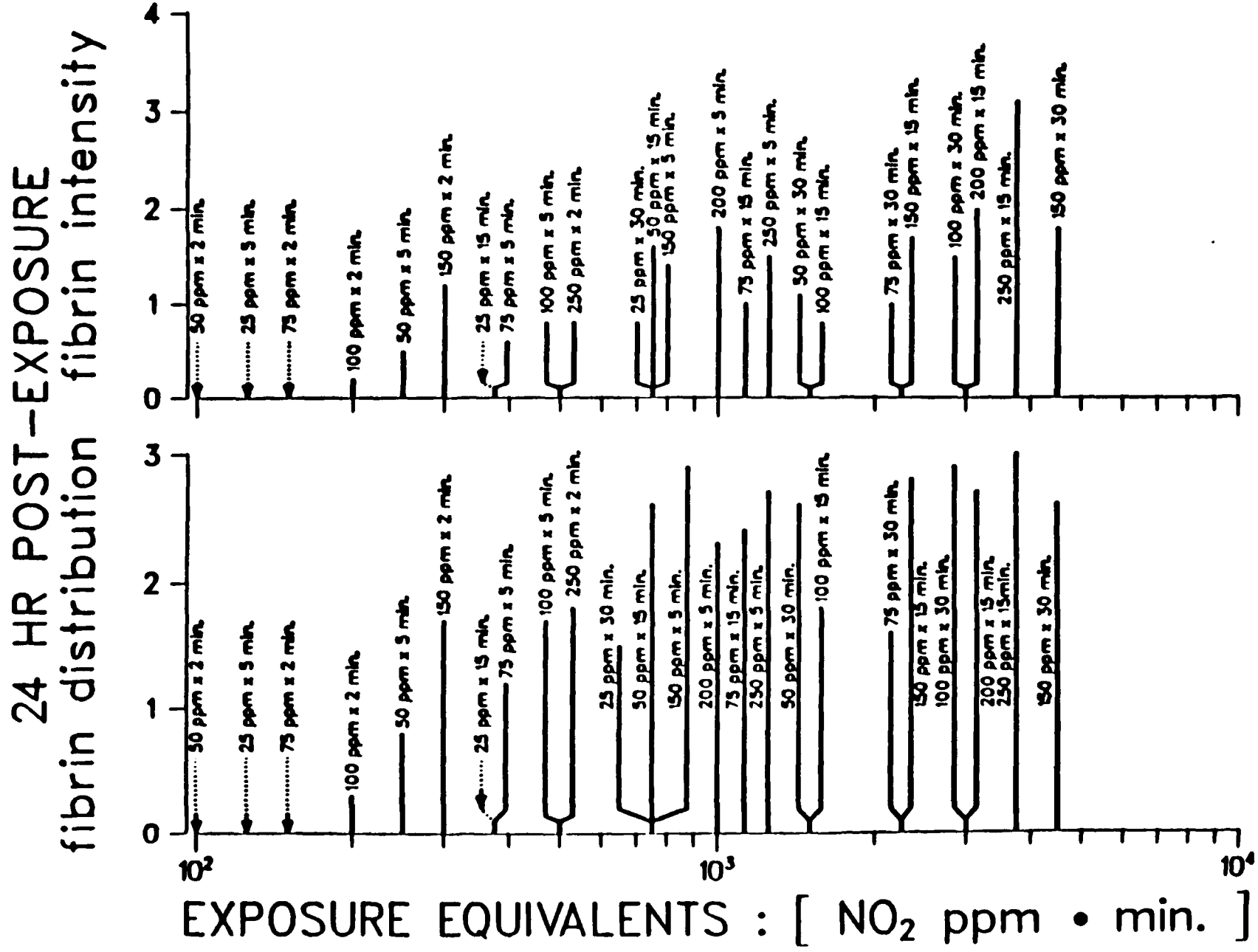


Figure 10D: Intensities and distributions of fibrin at 48hr after exposure to the various $NO_2$ exposure regimens.

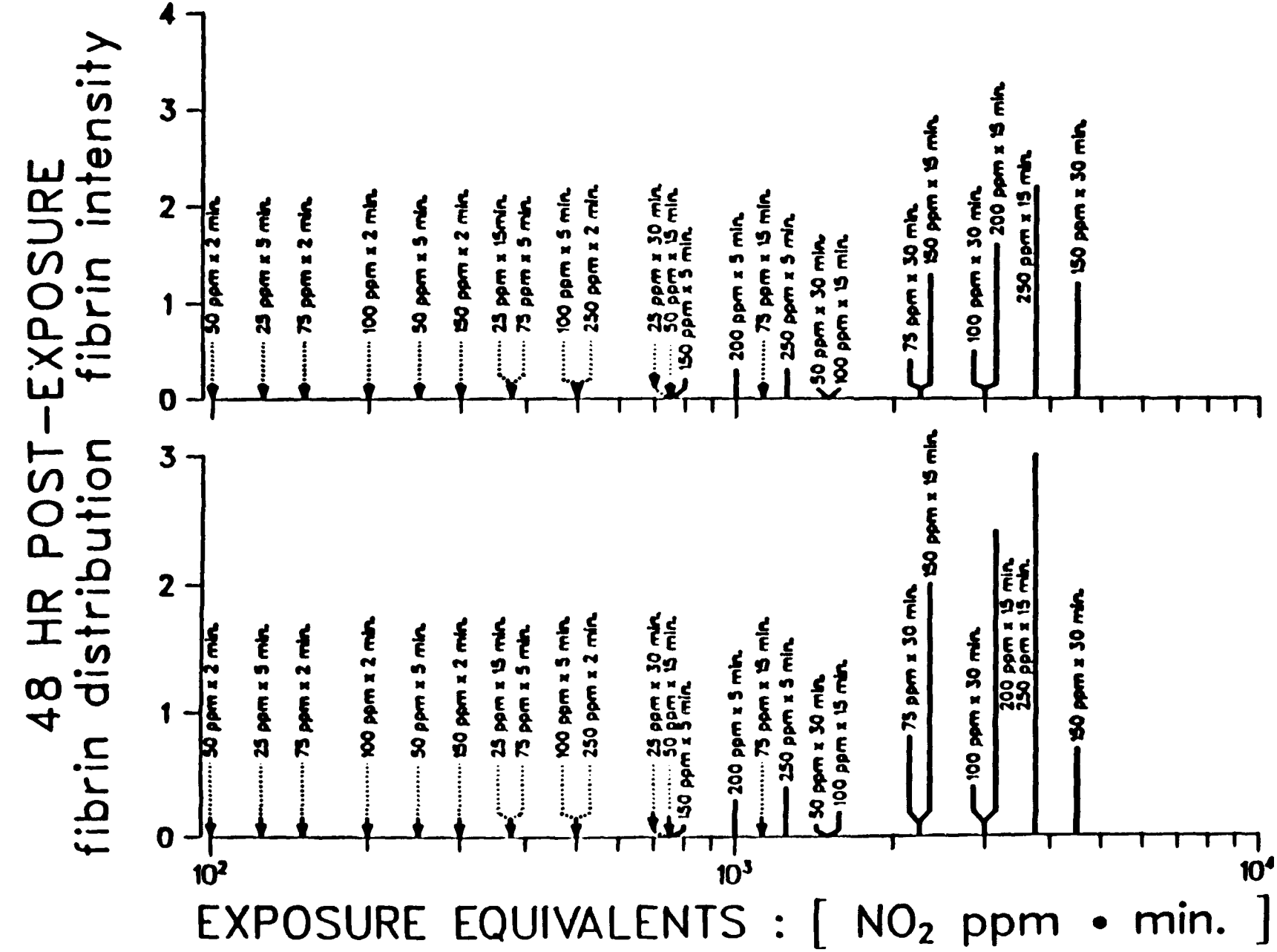


Figure 10E: Intensities and distributions of extravasated erythrocytes at 4hr after exposure to the various $NO_2$ exposure regimens.

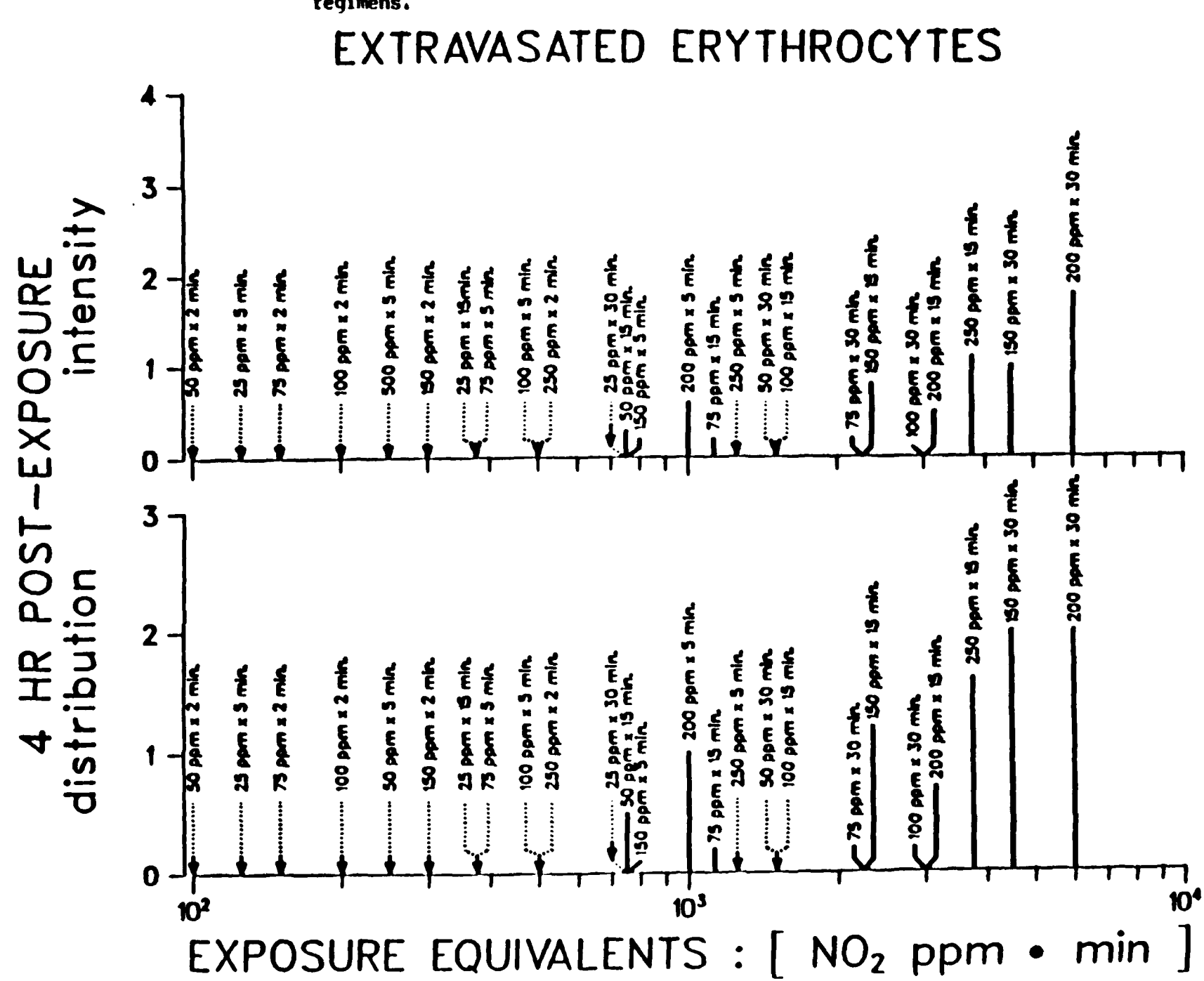


Figure 10F: Intensities and distributions of extravasated erythrocytes at 8hr after exposure to the various $NO_2$ exposure regimens.

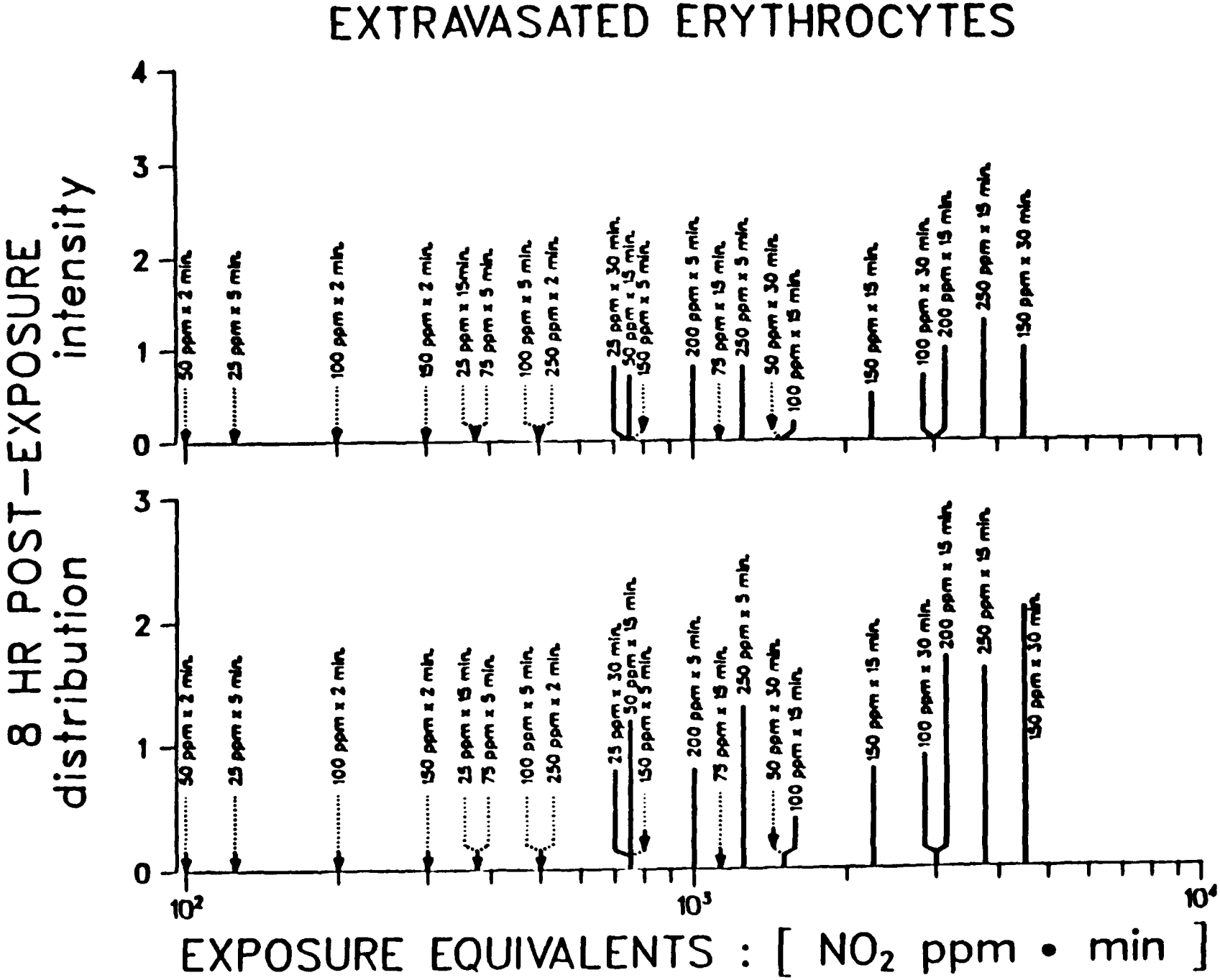


Figure 10G: Intensities and distributions of extravasated erythrocytes at 24hr after exposure to the various $NO_2$ exposure regimens.

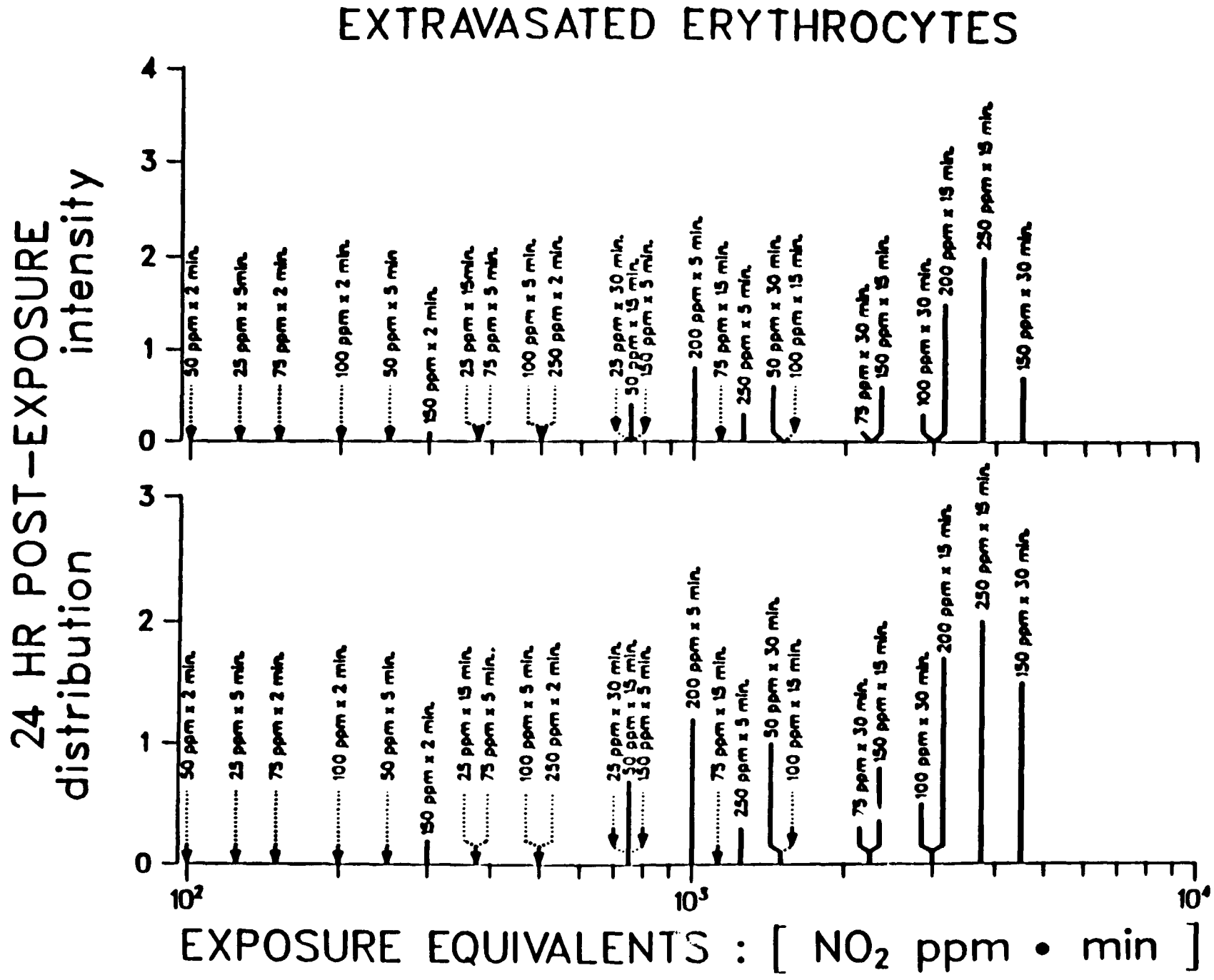


Figure 10H: Intensities and distributions of extravasated erythrocytes at 48hr after exposure to the various $NO_2$ exposure regimens.

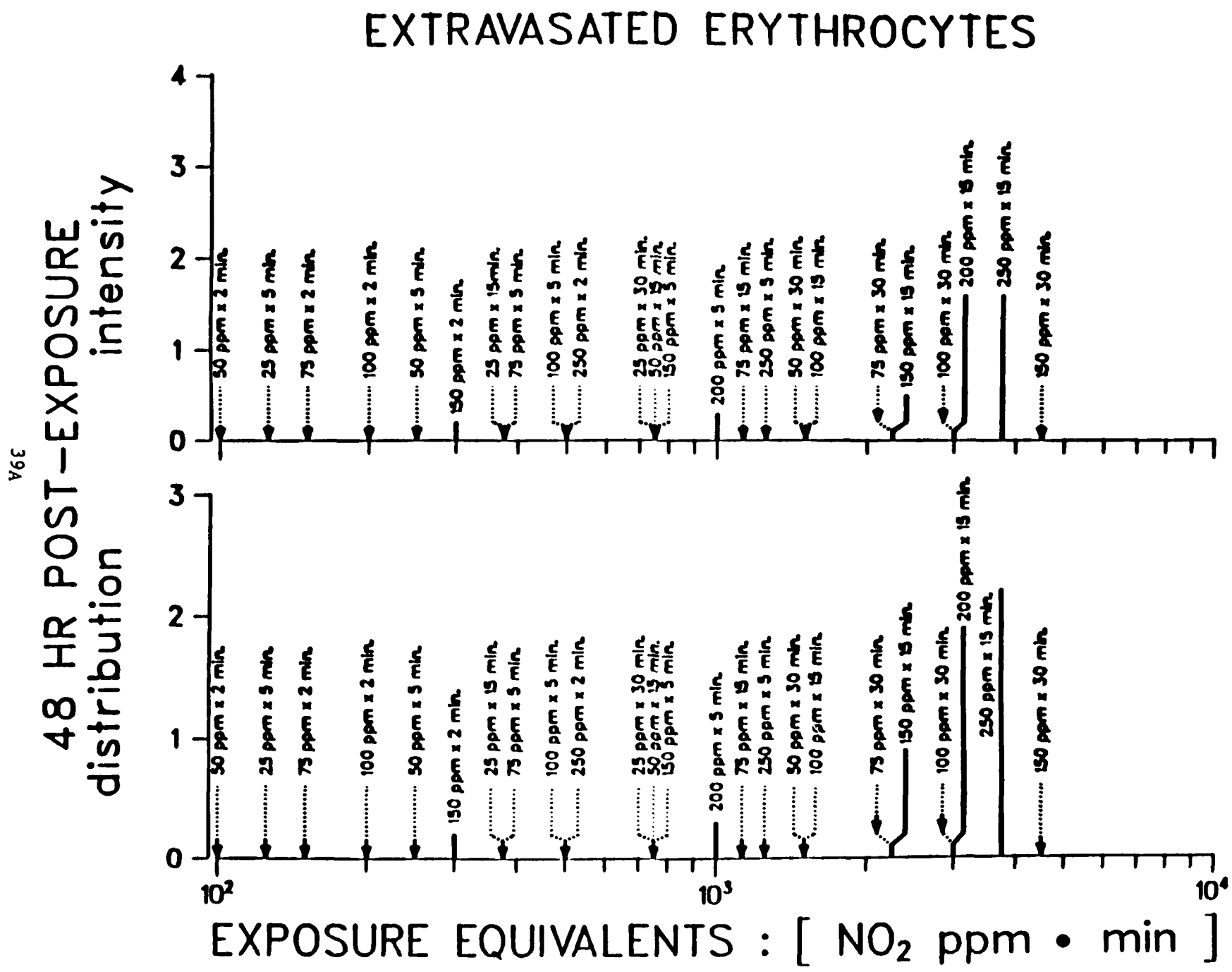


Figure 101: Intensities and distributions of polymorphonuclear leukocytes at 4hr after exposure to the various $NO_2$ regimens.

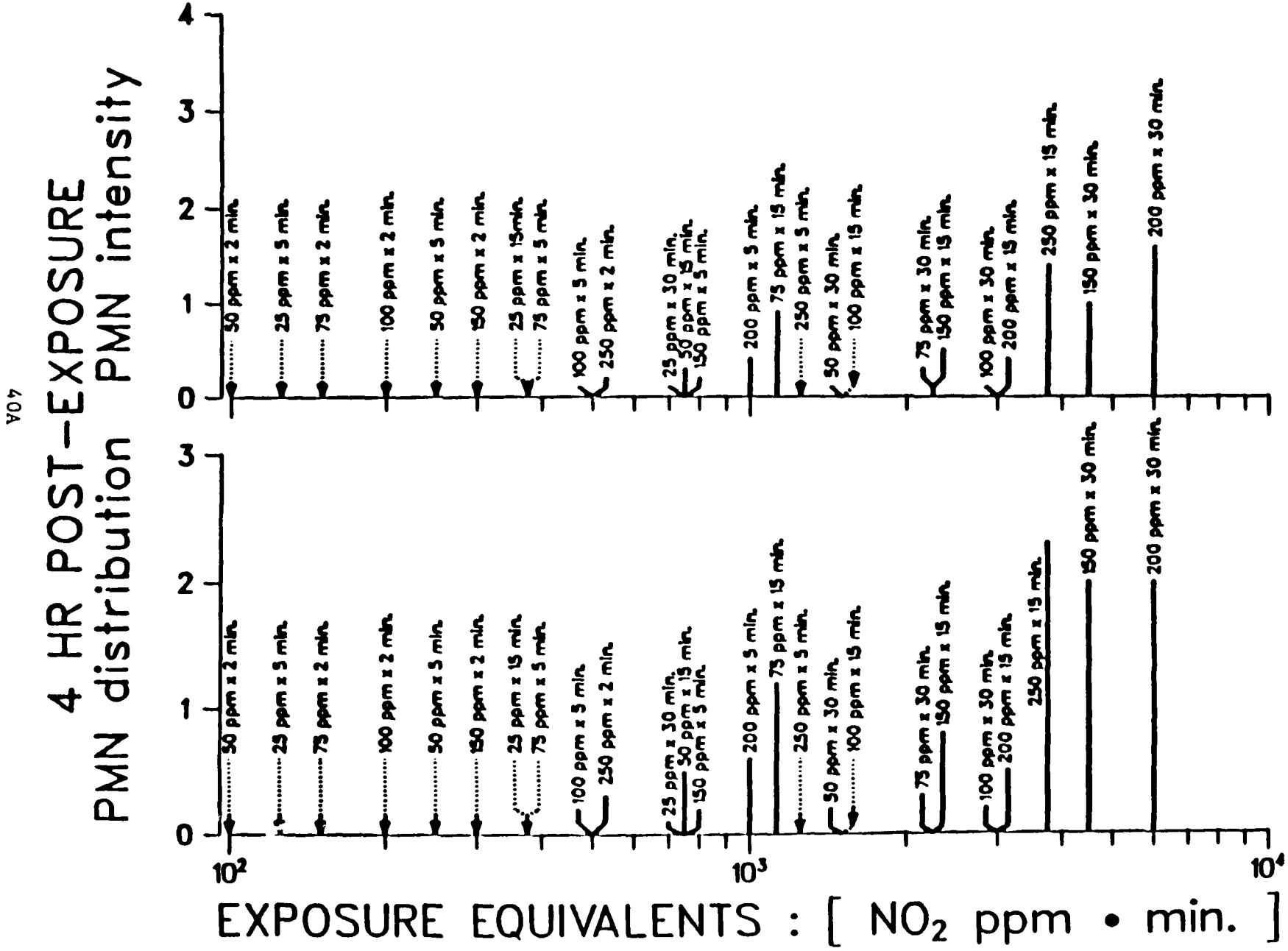


Figure 10J: Intensities and distributions of polymorphonuclear leukocytes at 8hr after exposure to the various $NO_2$ regimens.

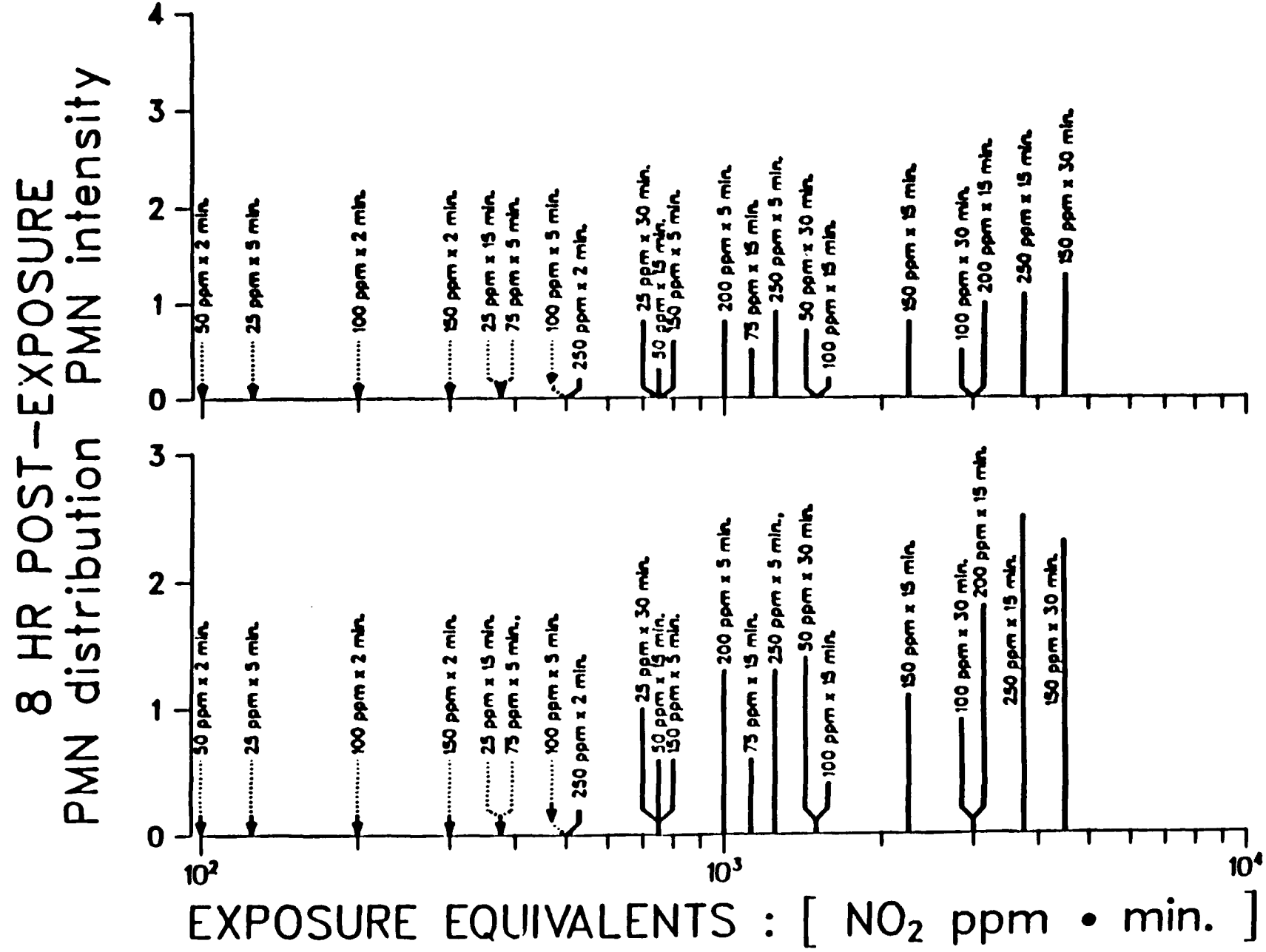


**Figure 10K: Intensities and distributions of polymorphonuclear leukocytes at 24hr after exposure to the various $NO_2$ regimens.**

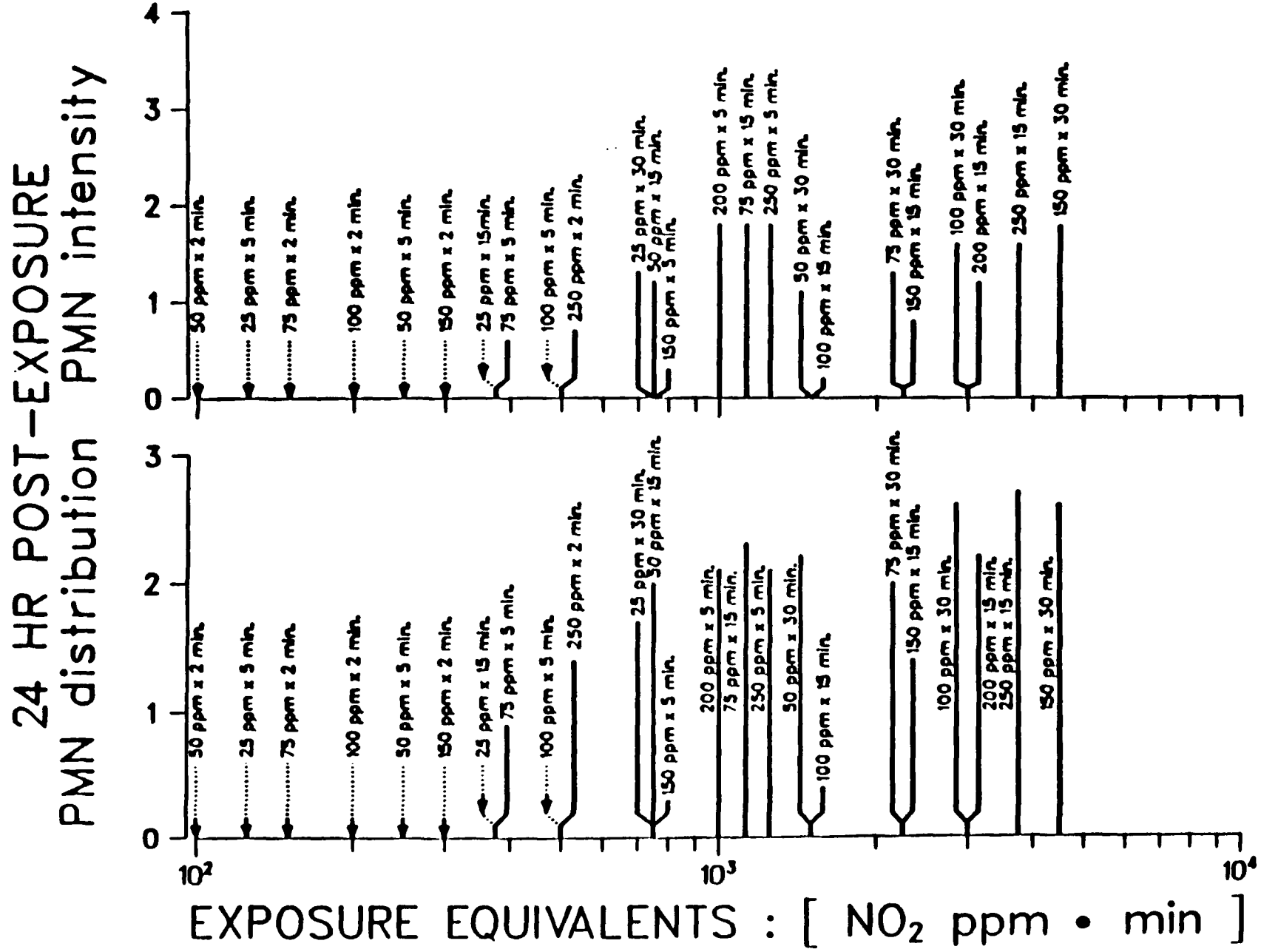


Figure 10L: Intensities and distributions of polymorphonuclear leukocytes at 48hr after exposure to the various $NO_2$ regimens.

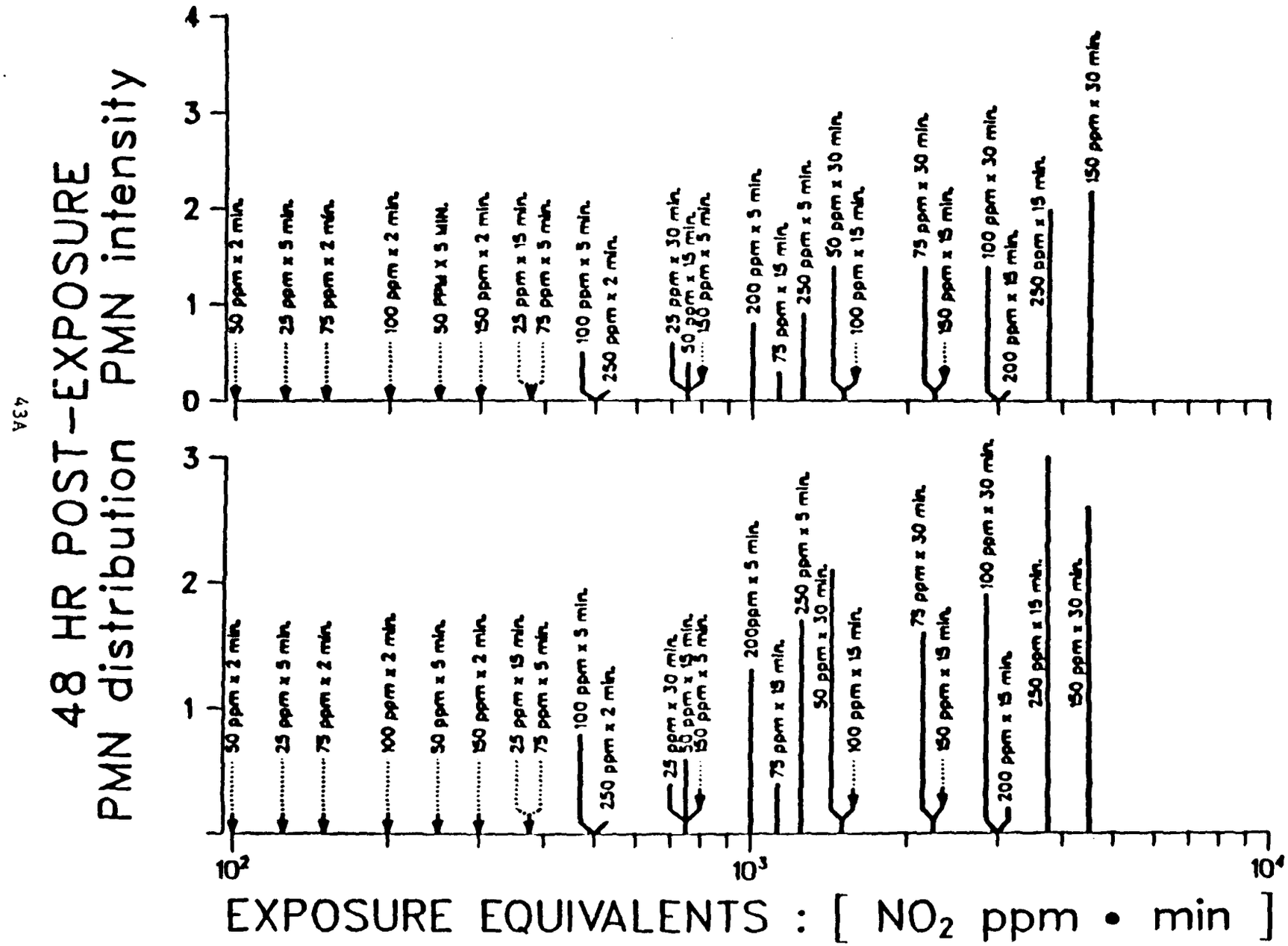


Figure 10M: Intensities and distributions of alveolar macrophages at 4hr after exposure to the various $NO_2$ regimens.

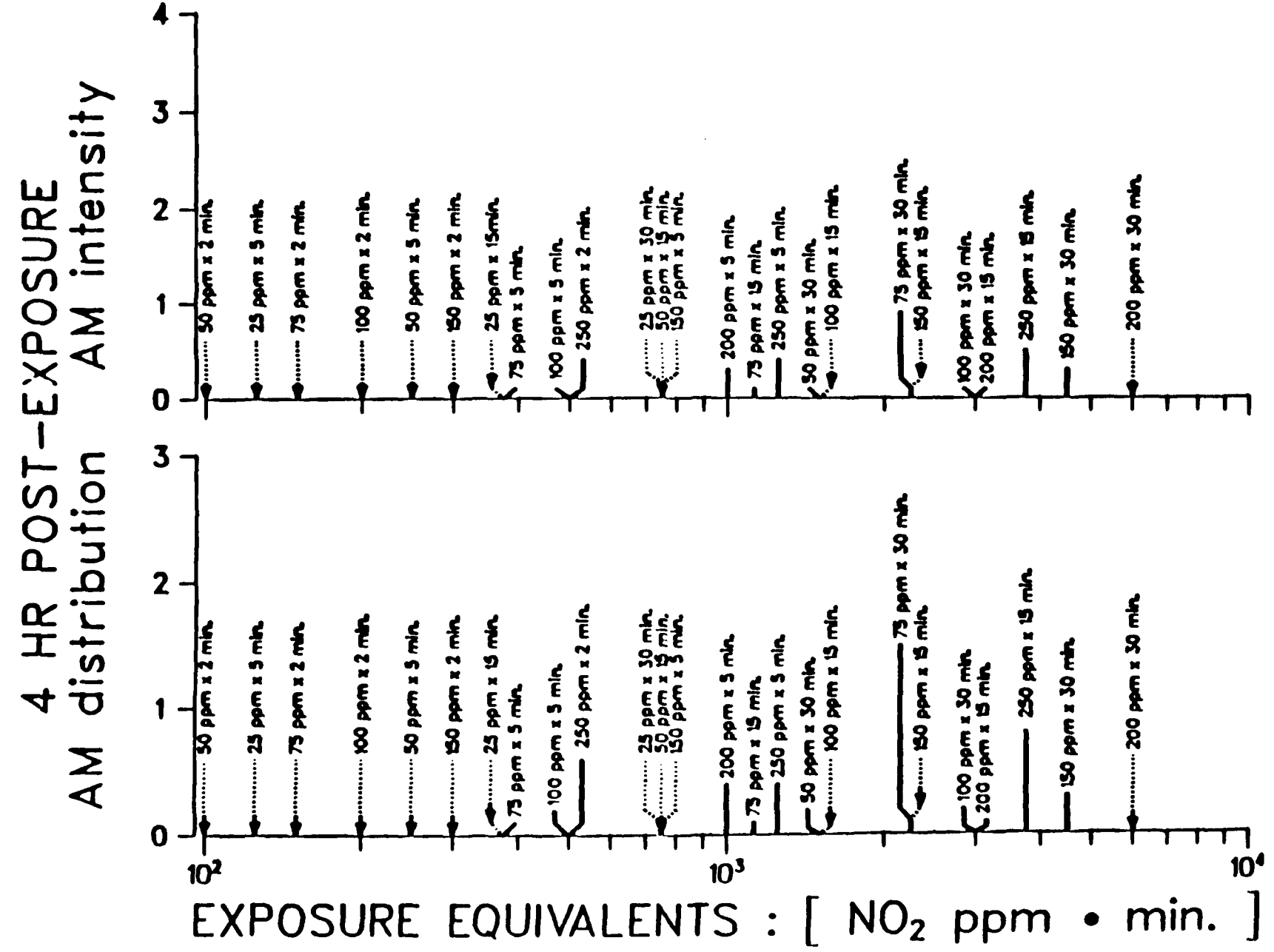


Figure 10N: Intensities and distributions of alveolar macrophages at 8hr after exposure to the various $NO_2$ regimens.

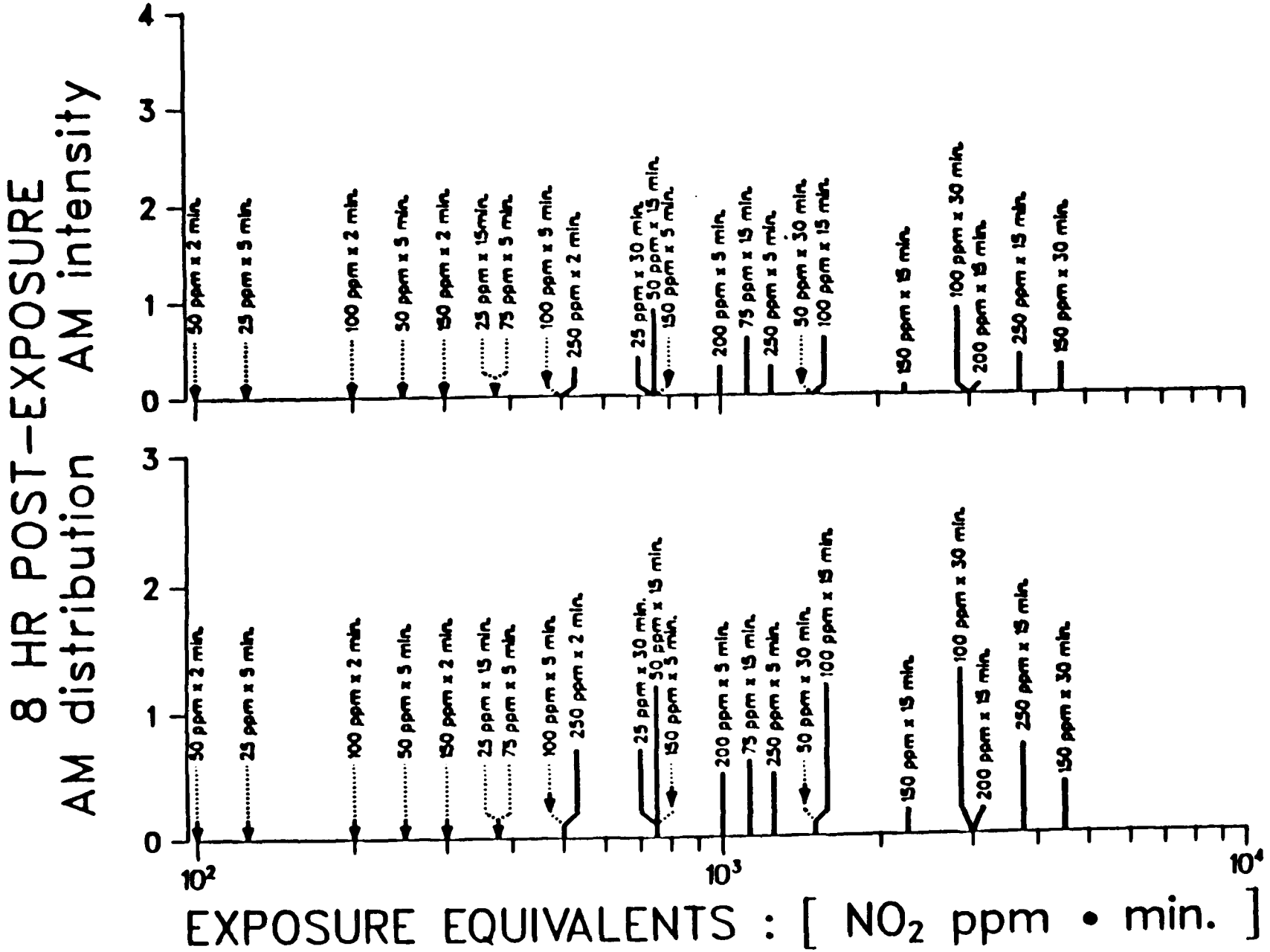


Figure 100: Intensities and distributions of alveolar macrophages at 24hr after exposure to the various $NO_2$ regimens.

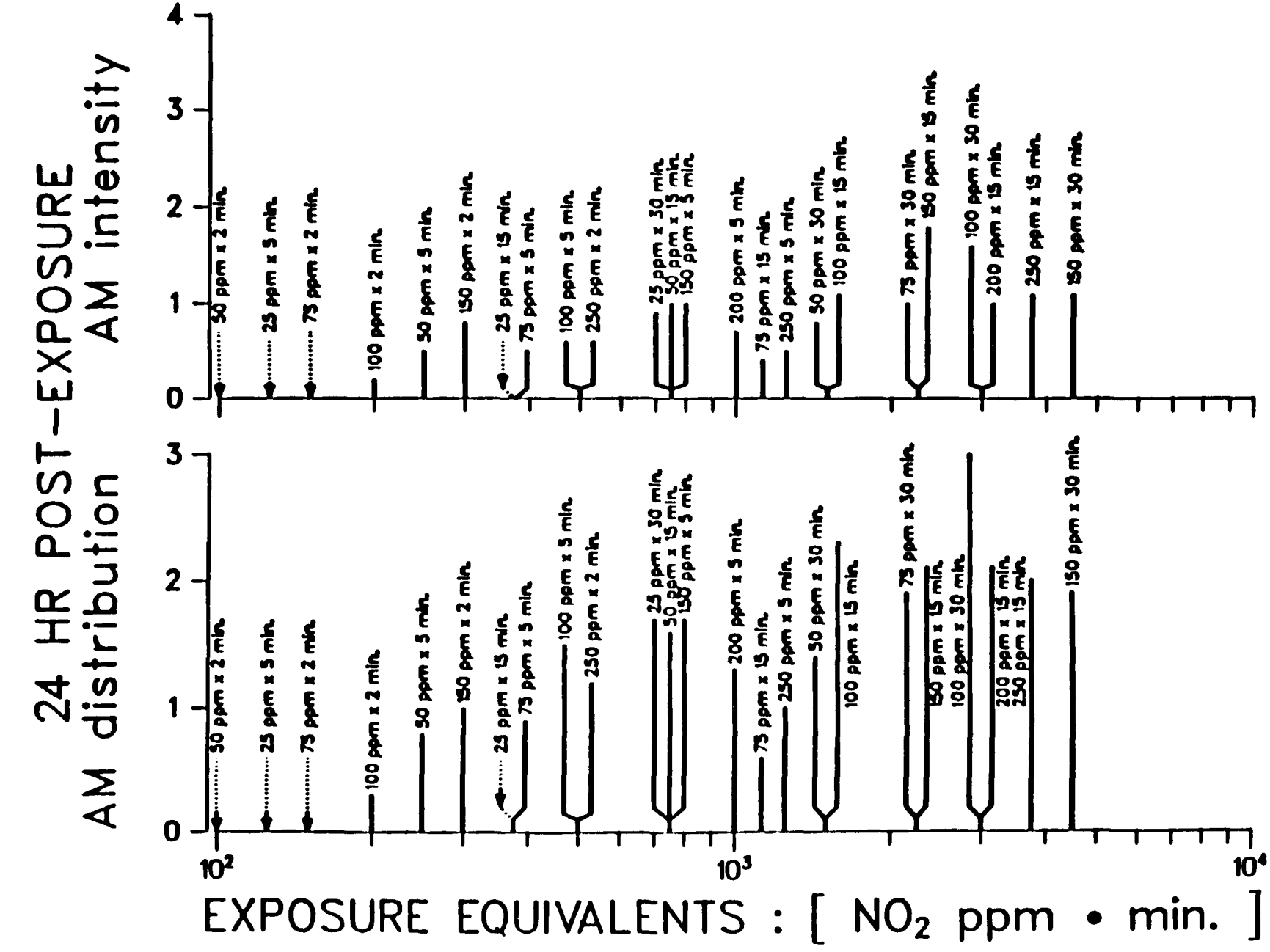


Figure 10P: Intensities and distributions of alveolar macrophages at 48hr after exposure to the various $NO_2$ regimens.

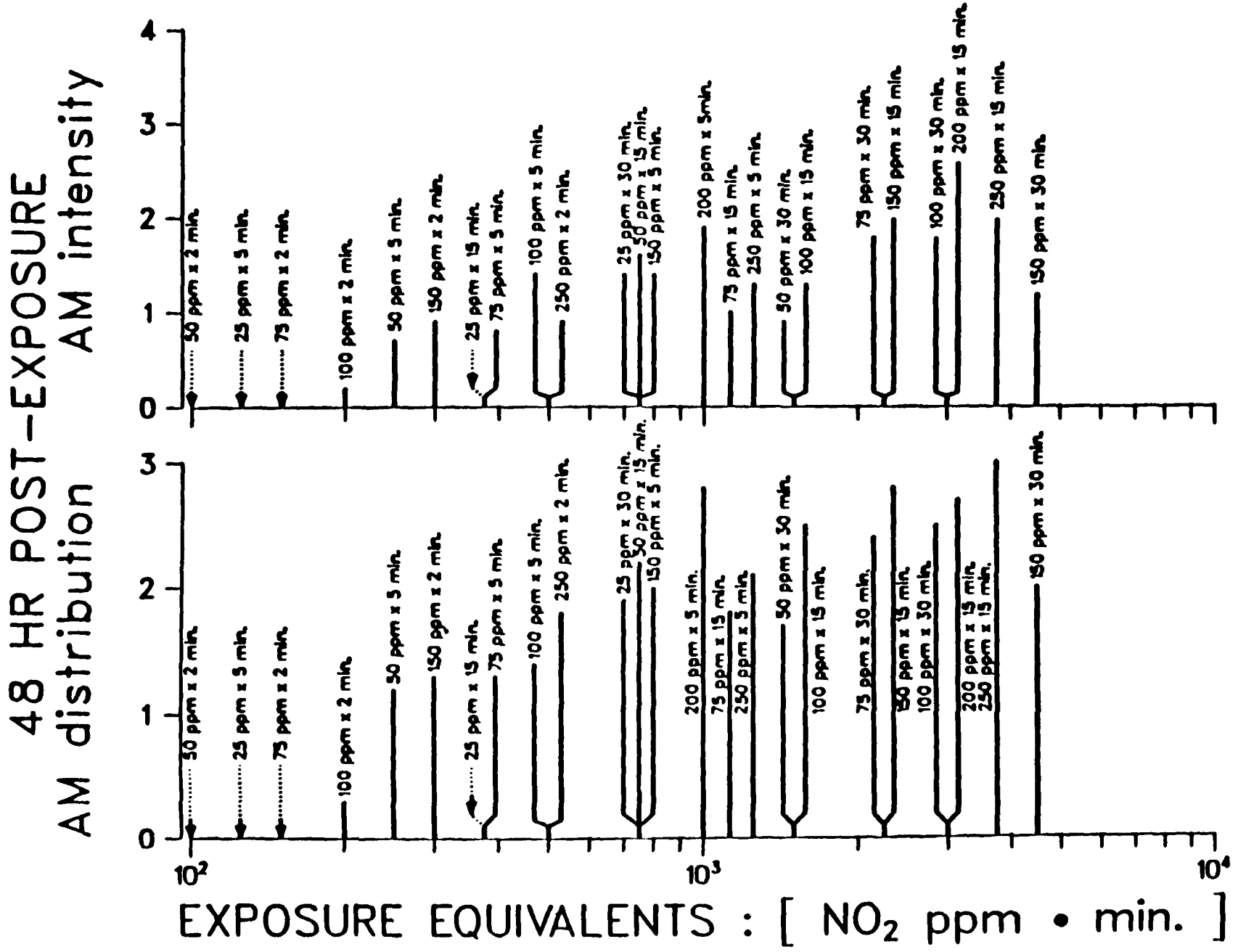


Figure 10Q: Intensities and distribution of type II cell Hyperplasia at 24hr after exposure to the various $NO_2$ regimens.

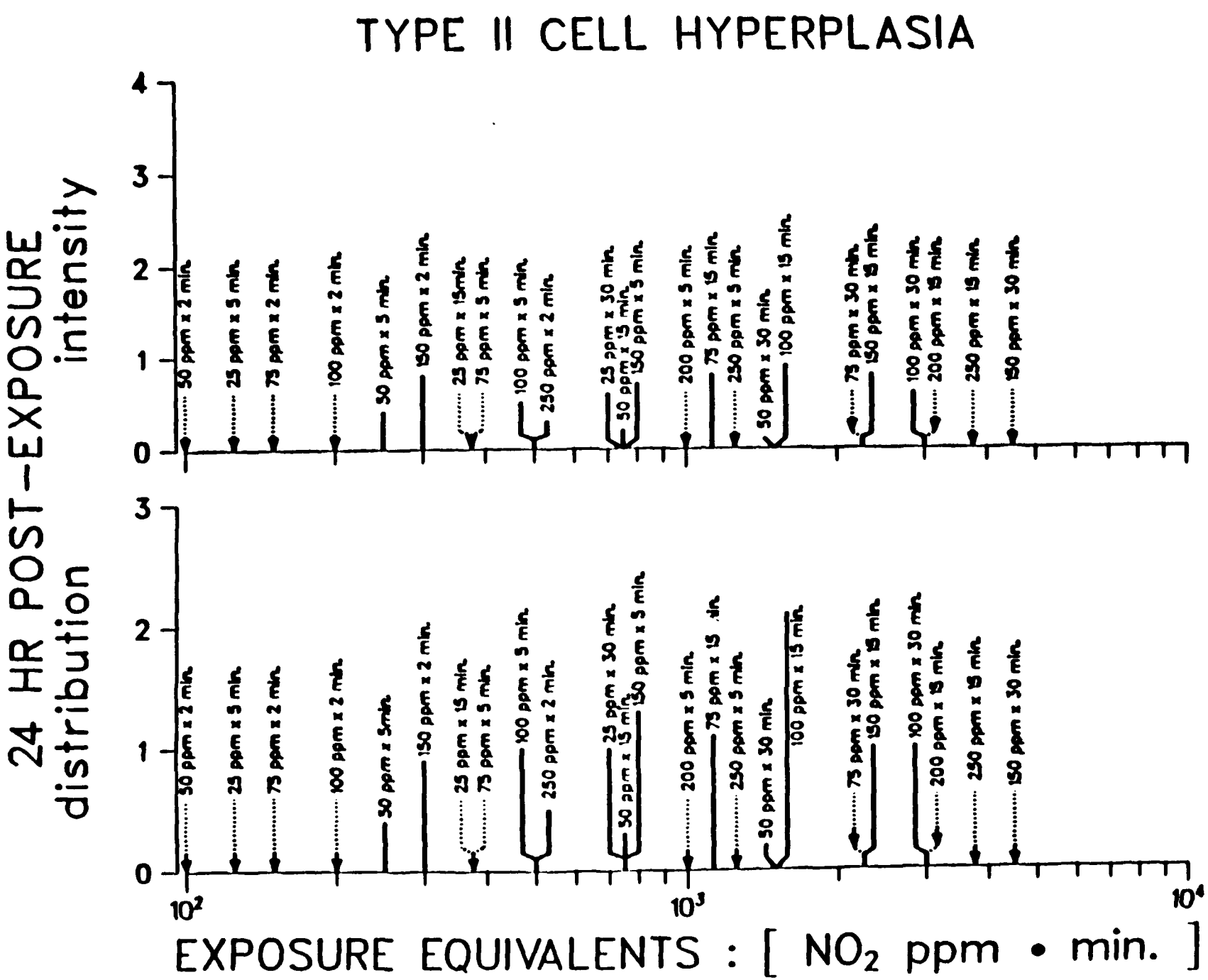
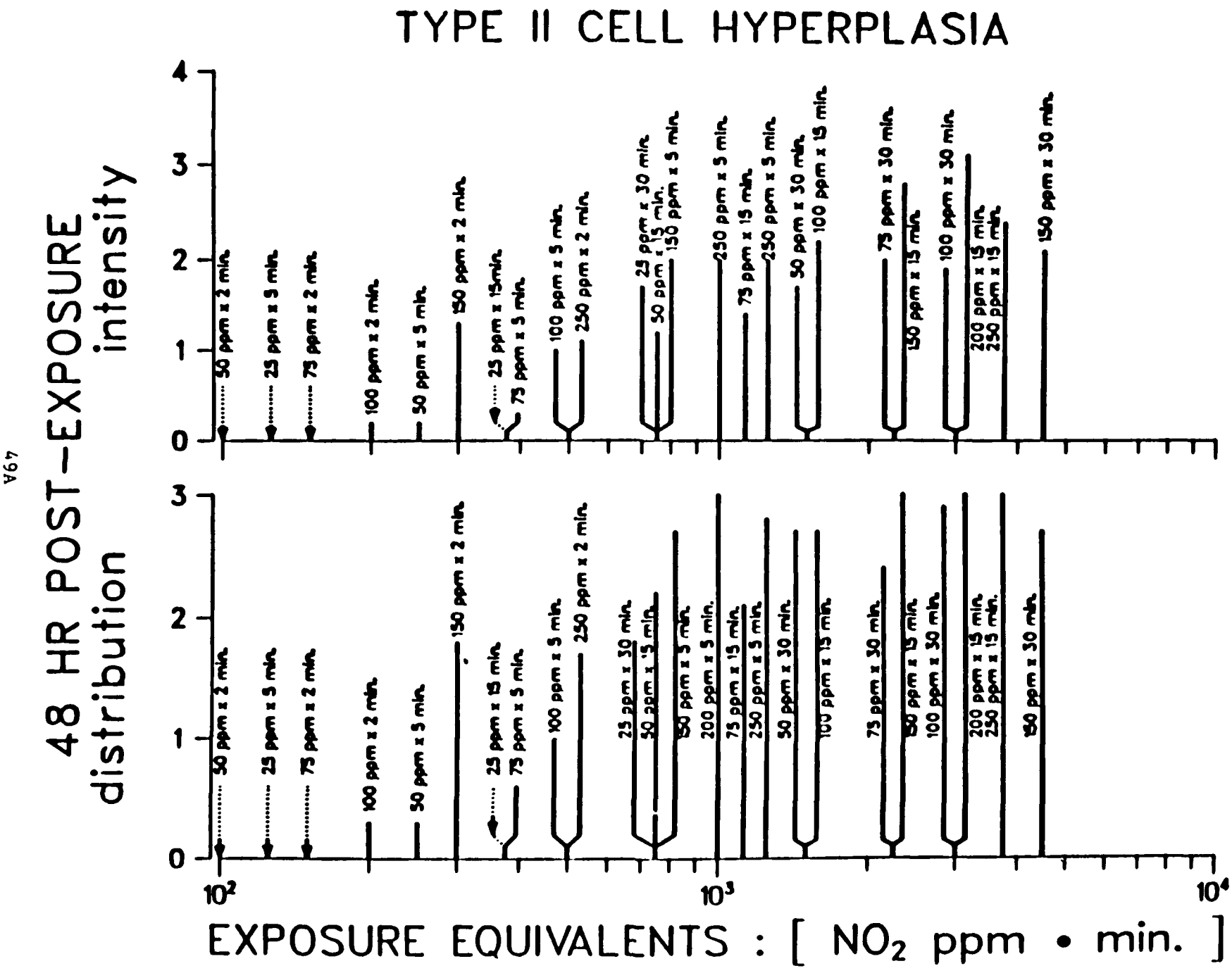


Figure 10R: Intensities and distribution of type II cell hyperplasia at 48hr after exposure to the various $NO_2$ regimens.



Two Minute Exposures: No evidence of injury was seen in animals exposed for 2 min to 50 or 75 ppm $NO_2$; two minute exposures to 25 ppm $NO_2$ were not conducted based on the 50 ppm $NO_2$ results. Fibrin was detectable in some alveoli as of 8 hrs after the exposure to 100 ppm $NO_2$, Figure 10B, but it was no longer detectable as of 48 hrs post-exposure, Figure 10D. It should be noted that when the mean intensity and distribution values are less than 1, some of the lungs in an exposure group did not express a given lesion. The low level injury produced by the 100 ppm exposure resulted in only slight, but detectable type II cell hyperplasia at the 48 hr time point, Figure 10R. At higher concentrations of $NO_2$, fibrin accumulations appeared earlier (by 4 hr) and their intensities and distribution were slightly higher than those found after the 100 ppm $NO_2$ exposures, Figure 10A. Type II cell hyperplasia at 48 hr following exposures to 150 and 250 ppm $NO_2$ was also more marked than that following the 100 ppm $NO_2$ exposure, Figure 10R; histologic analyses of lungs from animals exposed to 200 ppm $NO_2$ for 2 min are presently ongoing. Overall, these results indicate that the threshold concentration of $NO_2$ for a histopathologic effect in the rat's lung following a 2 min exposure is >75ppm and <100 ppm $NO_2$. Also, 2 min exposures to up to 250 ppm produces only mild lung injury.

Five Minute Exposures: Exposure of the rats to 25 ppm $NO_2$ did not produce observable lung injury at any time after the exposure. Exposures to 50,75, and 100 ppm $NO_2$ all produced trace to mild levels of lung injury, which was detectable by the appearance of fibrin as early as 4 hr after the exposure, Figure 10A. The levels of injury produced by these exposure concentrations were virtually identical in terms of the resulting fibrin intensities and fibrin distributions. With these exposures, however, the fibrin was no longer evident in the alveoli as of 48 hrs after exposure, Figure 10D. As well the 50,75, and 100 ppm $NO_2$ exposures for 5 min appeared to produce comparably modest type II cell hyperplasia as of 48 hr after exposure, Figure 10R. All of the above lesions were focal in nature. With the higher $NO_2$ concentrations (150,200,250 ppm $NO_2$), fibrin accumulations appeared to increase with increasing $NO_2$ concentrations. Both fibrin intensities increased and the distribution of the fibrin component became multifocal as early as 4 hr after the exposures, Figure 10A. The appearance of fibrin progressed towards a diffuse state as of 24 hr after

the exposures, Figure 10C. Extravasated erythrocytes were usually only apparent focally in low intensity following the 5 min exposures to 200 and 250 ppm $NO_2$, but they were not observed with the lower $NO_2$ concentrations, Figures 10E-10H. Generally the 150,200, and 250 ppm $NO_2$ exposures resulted in moderate type II cell hyperplasia that was multifocal in appearance as of 48 hr after exposure, Figure 10R. The intensities and distributions of the type II cell component of the responses to these high gas concentrations were closely similar.

Fifteen Minute Exposures: All exposure concentrations resulted in the appearance of fibrin, Figures 10A-10D. For the 25 ppm exposure, however, fibrin was only observed at the 8 hr time point, and, at that time, it was only evident in trace amounts in the lungs of some of the animals, Figure 10B. The fibrin response with the other exposures was greater as the $NO_2$ exposure concentration was increased, Figure 11. With the generalized index, 1-133 can be considered to represent trace to mild injury, 134-533 represents mild to moderate lung injury, 534-1197 represents moderate to severe lung injury, and 1198-1599 represents severe to very severe lung injury. Thus, the 15 min exposures to 50,75, and 100 ppm $NO_2$ produced a mild to moderate degree of lung damage as indexed by the appearance of fibrin, whereas the injury levels produced by the 150,200, and 250 ppm exposures may be categorized as severe. Extravasated erythrocytes were also a hallmark of the lung injuries arising from the 15 min exposures with the response being generally greater and longer lived following exposures to 200 and 250 ppm $NO_2$, Figures 10E-10H. Below 150 ppm $NO_2$, erythrocytes were not observed as of 48 hr after exposure. As to type II cell hyperplasia, this response was not observed in the lungs of the animals exposed to 25 ppm $NO_2$ for 15 min at either 24 or 48 hr post exposure, Figures 10Q and 10R. All other exposure concentrations did produce type II cell hyperplasia, and the level of expression of type II cell hyperplasia tended to correlate with exposure concentration, Figure 12. It is interesting to note that the level of type II cell hyperplasia produced by the 15 min exposure to $NO_2$ was less than that observed following equivalent time exposures to 150 and 200 ppm $NO_2$. Conceivably the 250 ppm $NO_2$ injured or otherwise compromised the resident population of type II pneumocytes during the exposure and thereby limited their ability to proliferate. Upon

exclusion of the 250 ppm $NO_2$ data point in Figure 12, the level of type II cell hyperplasia clearly was linearly related to $NO_2$ exposure concentration.

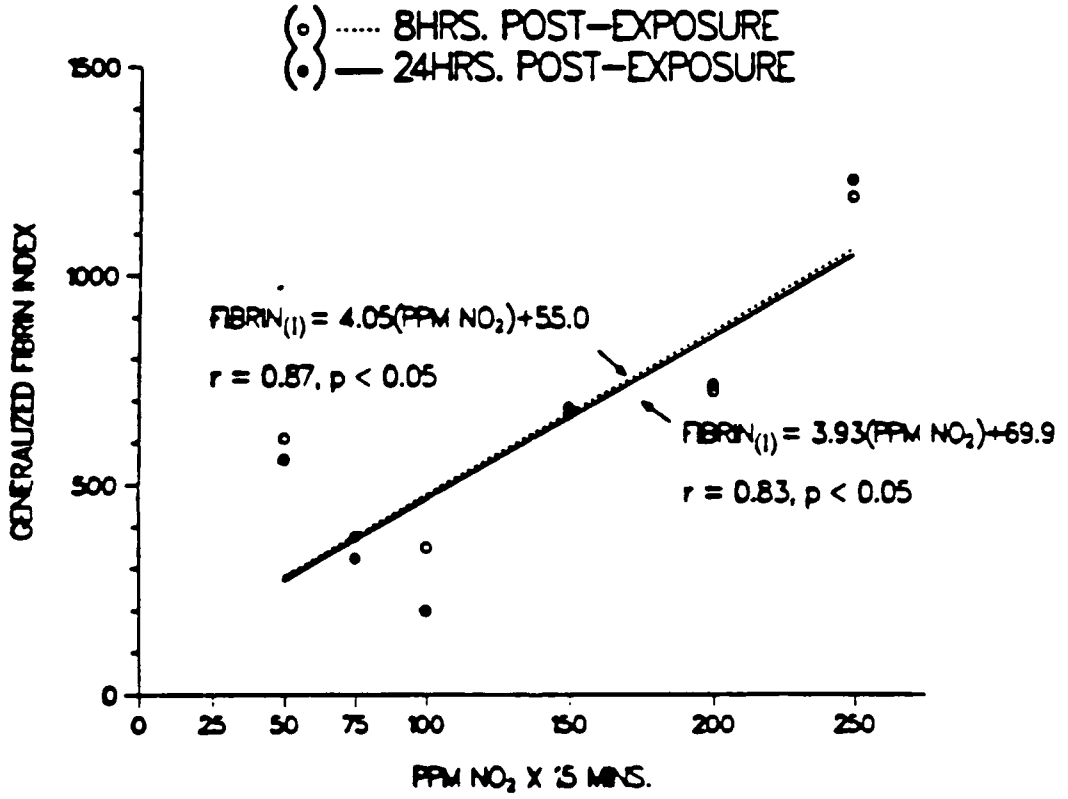



**Figure 11:** Generalized fibrin indices as of 8 hr and 24 hr after the 15 min exposures.

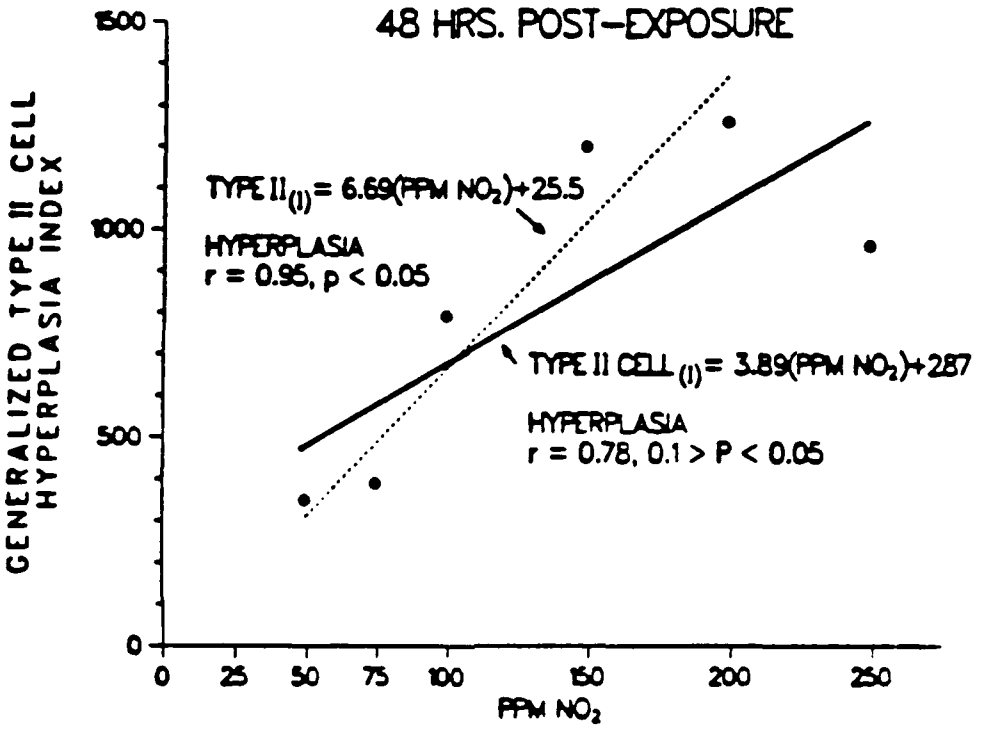



Figure 12: Generalized Type II cell hyperplasia indices as of 48 hr after the 15 min $NO_2$ exposures. The solid line resulted from least square fit analysis of all data whereas the dotted line includes data from the 50-200 ppm $NO_2$ exposures.

Over-all, the levels of lung injury following the 15 min exposures to the various $NO_2$ concentrations may be summarized as follows according to the histopathologic findings: 25 ppm, trace level of injury; 50-100 ppm $NO_2$, mild to moderate degrees of lung injury; 150-200 ppm $NO_2$, severe lung injury; 250 ppm, very severe lung injury.

Thirty Minute Exposures: Thirty minute exposures to all $NO_2$ concentrations studied resulted in histopathologic evidence of significant lung injury. The severity of injury, based on the appearance of fibrin (Figures 10A-10D), increased as a function of increasing exposure concentration, Figure 13. Lung damage apparently was most pronounced following the 200 ppm $NO_2$ exposure; no animals exposed to this concentration survived to the 8 hr sacrifice time. Accordingly, an exposure to 250 ppm $NO_2$ for 30 min was not conducted, as previously indicated. Extravasated erythrocytes were generally observed after all of the exposures but the abnormal appearance of these was no longer a major feature by the 48 hr sacrifice times, Figures 10E-10H. All of the 30 min exposures produced type II cell hyperplasia as of 48 hr after exposure. The degree of type II cell hyperplasia appeared to increase with increasing exposure concentrations, Figure 14. Overall, the severity of lung injury arising from the various 30 min $NO_2$ exposures can be classified as follows: 25 ppm, mild lung injury; 50 ppm, moderate lung injury; 75 ppm, moderate lung injury; 100 ppm, moderate to severe lung injury; 150 ppm, moderate to severe lung injury; $\geq$ 200 ppm, very severe lung injury and resulting death.

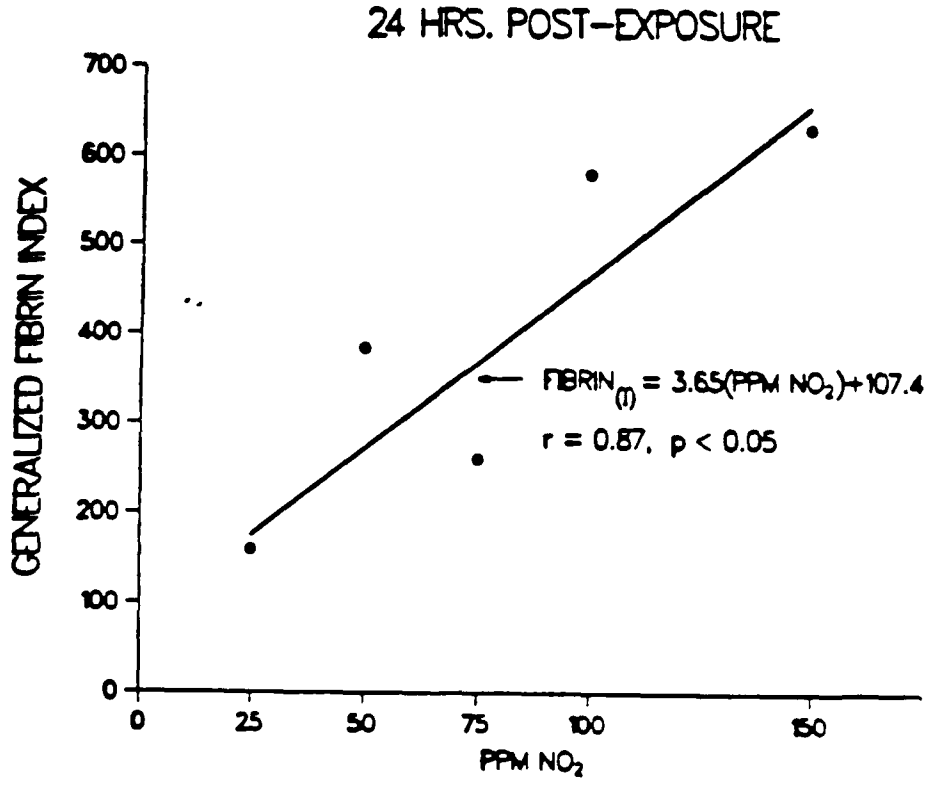



**Figure 13:** Generalized fibrin indices as of 24 hr after 30 min exposures to 25–150 ppm $NO_2$.

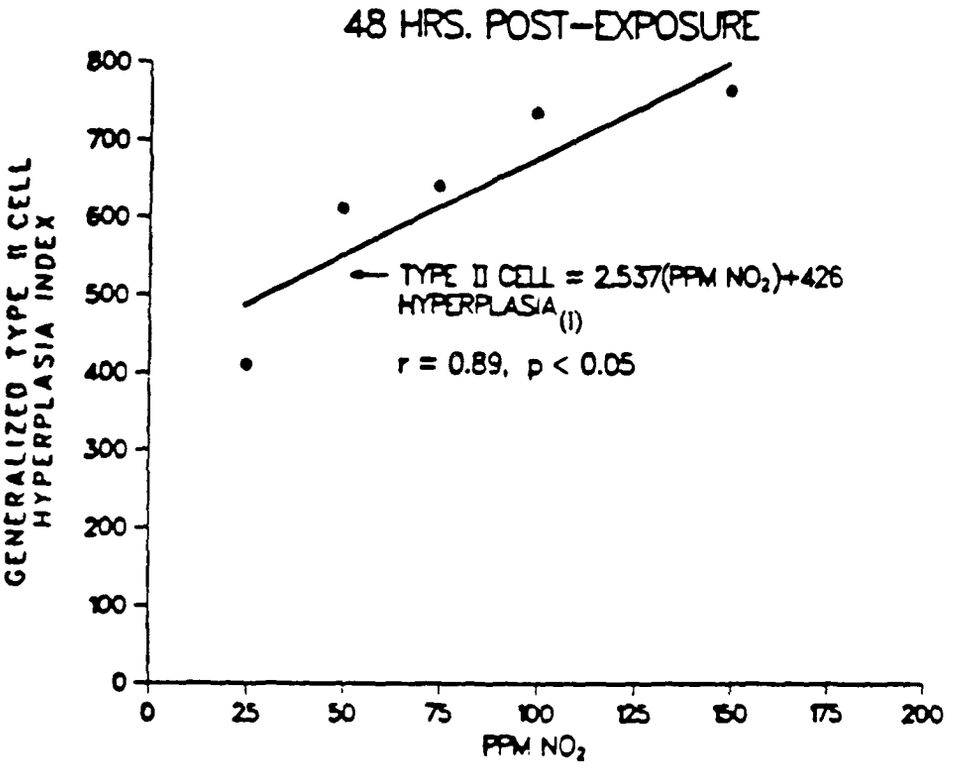



Figure 14: Generalized Type II cell hyperplasia indices as of 48 hr following 30 min exposures to 25-150 ppm $NO_2$.

Importance of Exposure Concentration Versus Exposure Duration

Various lines of evidence obtained in this study indicate exposure concentration plays a more important role in inducing lung alterations than exposure time when $NO_2$ is acutely inhaled. This generalization is illustrated in Figures 15 and 16, which show the average percent changes in LWW and RCLDW values, respectively, obtained as of 24 hr following exposure protocols in which the product of $NO_2$ concentration and time were kept constant; the post-exposure 24 hr time point was selected for these analyses inasmuch as $NO_2$-induced gravimetric changes were usually maximal at that time (see Figures 5A-5D, 8A-8D, and Addendum Table A1). In all instances where significant increases in the above gravimetric parameters were found, the most pronounced increases in LWW and RCLDW were produced by the shorter duration exposures involving the higher or highest concentration of $NO_2$ administered at each of the exposure equivalents studied. With few exceptions, the predominant importance of exposure concentration versus exposure time was also demonstrated by quantitative histopathologic assessments of the $NO_2$ exposed lungs. Figures 17 and 18 give the 24 hr post-exposure fibrin and 48 hr post exposure type II cell hyperplasia data, respectively, in the form of "generalized" indices. Again, these post-exposure times were selected for such presentation because the above histologic features almost always were found to have near maximal or maximal intensities and distributions at the above times. Aside from the fibrin values found for the two exposures giving 1500 ppm $NO_2$·min equivalents, the expression of fibrin was highest at the other exposure equivalents when the $NO_2$ concentrations were highest, Figure 17. Generally, type II cell hyperplasia, a proliferative response to type I pneumocyte destruction (Rombout et al., 1986; Evans et al., 1980; Evans et al., 1977; Evans et al., 1978a), also followed this pattern, Figure 18.

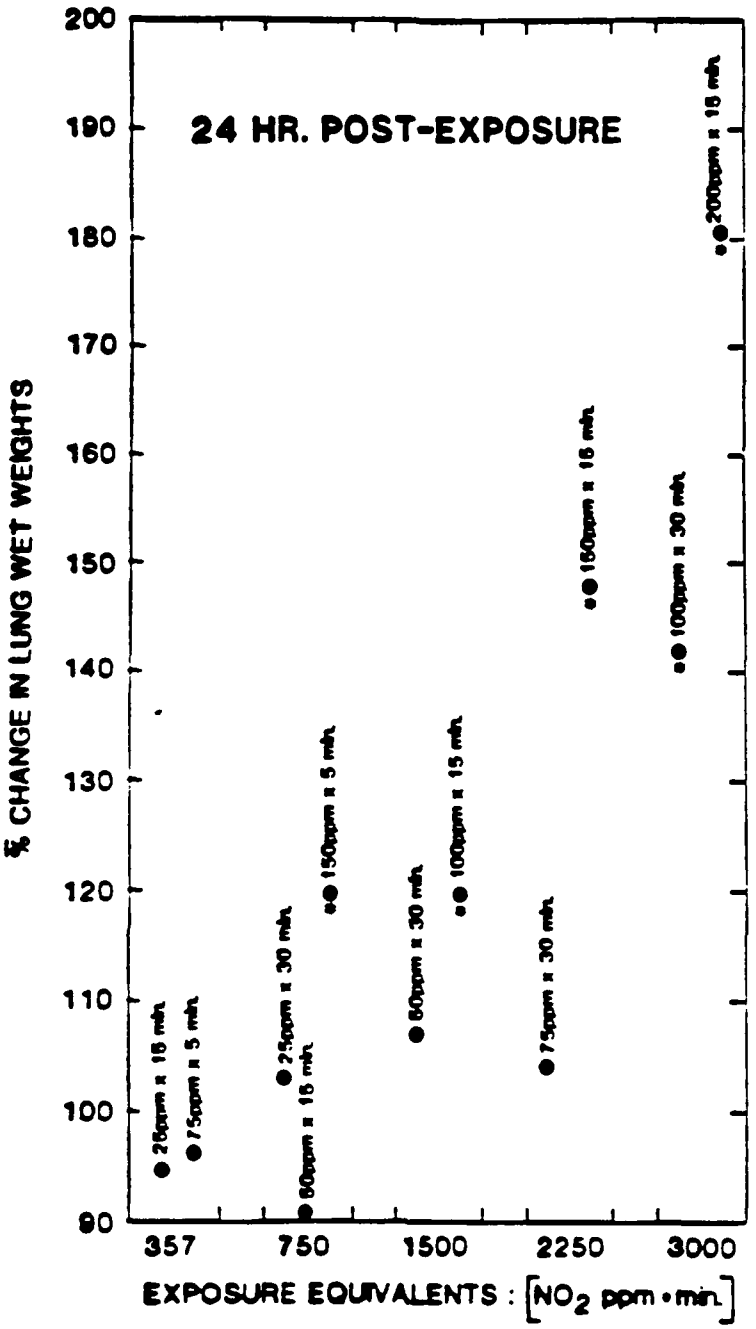



Figure 15: Average percent change in lung wet weights (LWW) from control values as of 24 hrs. after exposure to various exposure equivalents of $NO_2$. Marked values (*) are significantly higher than control values $p < 0.05$.

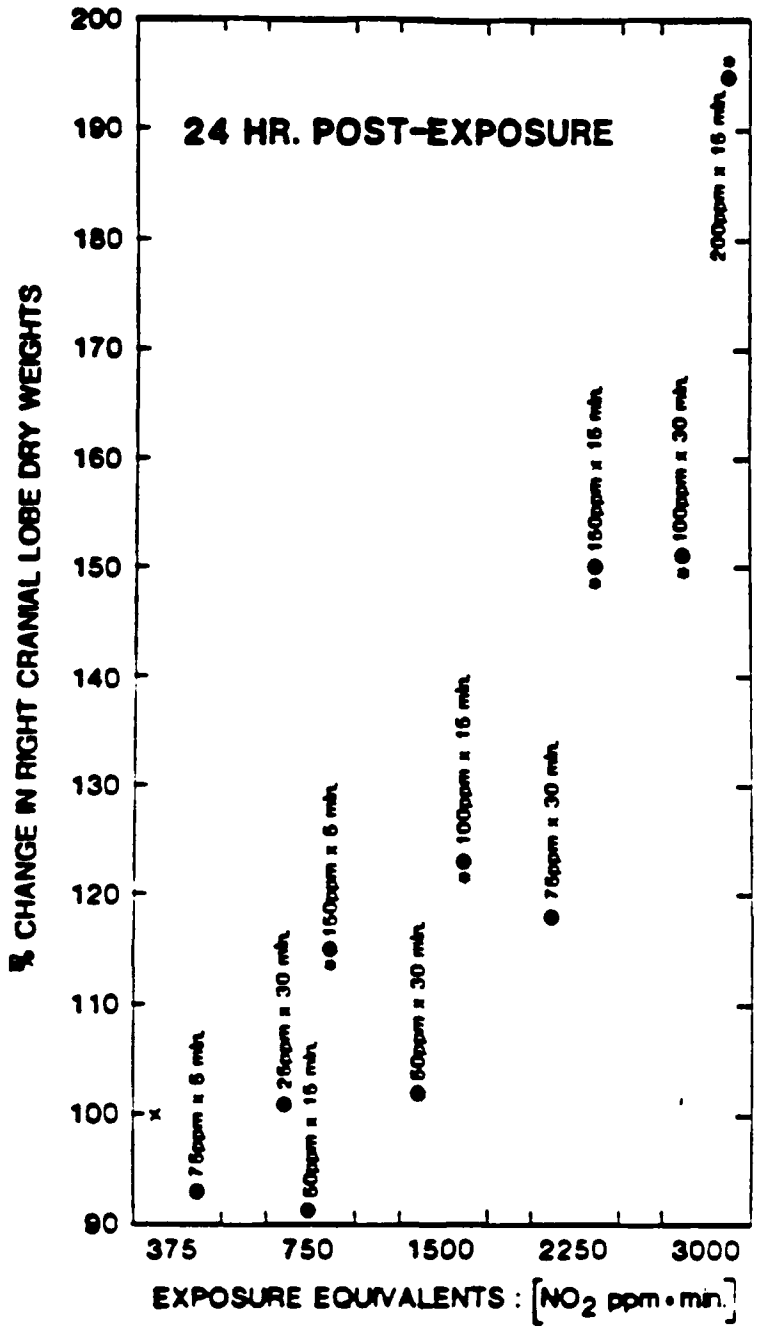



Figure 16: Average percent change in right cranial lobe dry weights (RCLDW) from control values as of 24 hr after exposure to various exposure equivalents of $NO_2$. Marked values (*) are significantly higher than control values $p < 0.05$. X: actual value was not obtained due to a technical over-sight.

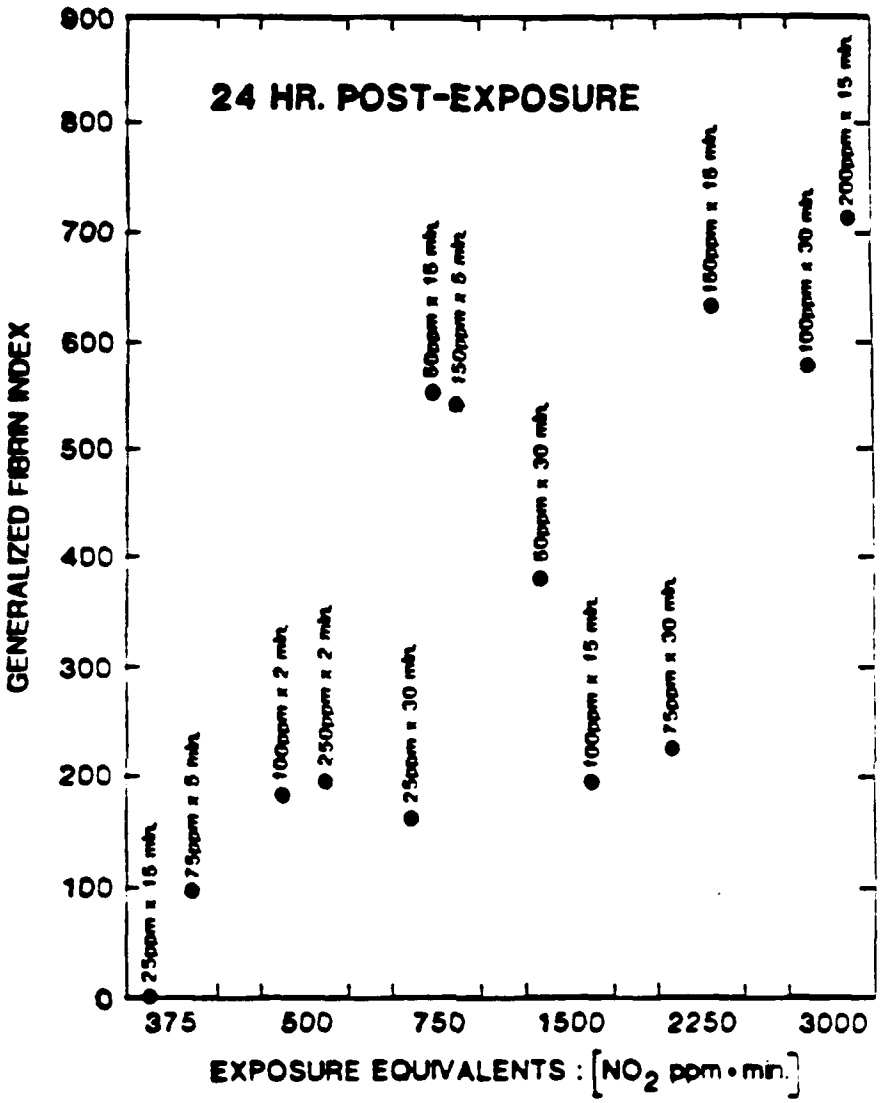



Figure 17: Generalized fibrin indices as of 24 hr after exposure to the various equivalents of $NO_2$.

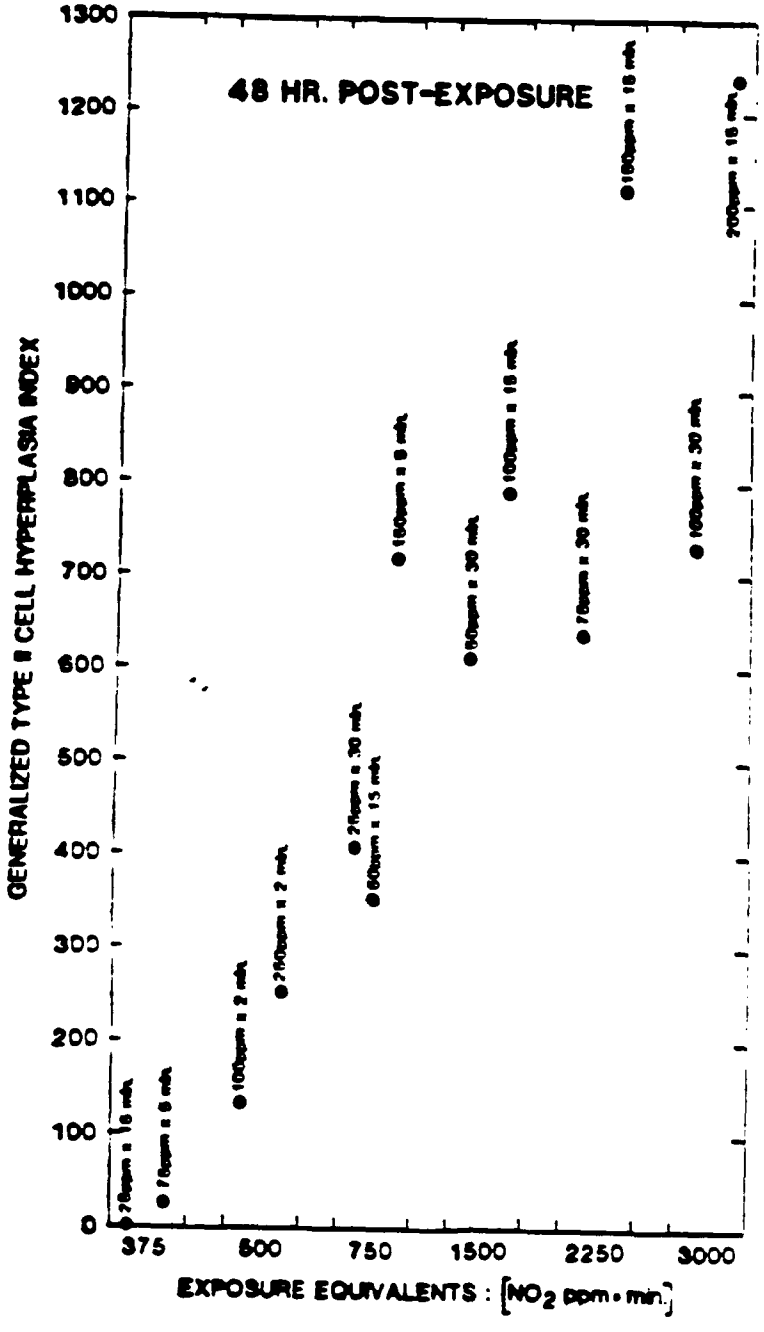



Figure 18: Generalized Type II cell hyperplasia indices 48 hr after exposure to the various exposure equivalents of $NO_2$.

## Summary

The present study was undertaken in order to assess the severity of acute lung injury induced in the laboratory rat by brief exposures to relatively high concentrations of $NO_2$. Two major endpoints used to evaluate lung toxicity were lung wet weight and lung dry weight measurements using a representative lung lobe for the latter (Greenburg et al., 1978; Cross et al., 1982). The lung wet weight measurements were made in order to index increases in lung water, a hallmark feature of pulmonary edema arising from alterations in lung permeability, and the lung dry weight measurements were made to index infiltrating blood-derived protein, potential changes in lung interstitial components such as connective tissue alterations and inflammatory cell influxes, influxes of inflammatory cells into the alveoli, and to index cell proliferation (Cross et al., 1982) e.g., type II cell hyperplasia. As well, lung injury resulting from the exposures was characterized by performing quantitative histologic analyses with emphasis on the peri-terminal bronchiolar-alveolar duct-alveolar region of the lung. The following summary on the severity of lung injury produced by the various $NO_2$ exposure regimens used in this study is based upon considering the collective results obtained using the above toxicologic endpoints.

Lung Injury Levels Following the Two Minute Exposures:

25 ppm $NO_2$: Not assessed
50 ppm $NO_2$: None unequivically detected
75 ppm $NO_2$: None unequivically detected
100 ppm $NO_2$: Trace
150 ppm $NO_2$: Trace to mild
250 ppm $NO_2$: Mild

Lung Injury Levels Following the Five Minute Exposures:

25 ppm $NO_2$: None detected
50 ppm $NO_2$: Trace
75 ppm $NO_2$: Trace
100 ppm $NO_2$: Trace to mild
150 ppm $NO_2$: Mild to moderate
200 ppm $NO_2$: Moderate
250 ppm $NO_2$: Moderate

Lung Injury Levels Following the Fifteen Minute Exposures:

25 ppm $NO_2$: Trace
50 ppm $NO_2$: Mild to moderate
75 ppm $NO_2$: Mild to moderate
100 ppm $NO_2$: Mild to moderate
150 ppm $NO_2$: Severe
200 ppm $NO_2$: Severe
250 ppm $NO_2$: Very Severe

Lung Injury Levels Following the Thirty Minute Exposures:

25 ppm $NO_2$: Mild
50 ppm $NO_2$: Moderate
75 ppm $NO_2$: Moderate
100 ppm $NO_2$: Moderate to severe
150 ppm $NO_2$: Moderate to severe
200 ppm $NO_2$: Very severe, acutely lethal

In addition to the above component of the study, the $NO_2$ toxicity data base was used to examine the relative importance of exposure concentation of $NO_2$ versus exposure duration in producing pulmonary injury. Similar to findings recently reported for subacute exposures to $NO_2$, (Rombout et al.,

1986), analyses described herein indicate $NO_2$ concentration plays a more important role in inducing pulmonary lesions than dose exposure time when the product of concentration and time is kept constant.

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# ADDENDUM TO SECTION A

Mean and standard deviations of the lung gravimetric data and the quantitative histopathology data obtained from analyses of the alveolar duct-alveolar region.

Table A1: Lung Gravimetric Values

| Exposure Condition | Post-Exposure Time (hr) | Lung Wet Weight (g) | Lung Wet Weight as % of Control | Right Cranial Lobe Dry Weight (g) | Right Cranial Lobe Dry Weight as % of Control | N |
|---|---|---|---|---|---|---|
| 25 ppm x 5 min | 4 | 1.265±0.067 | 101 | 0.023±0.002 | 97 | 12 |
| | 8 | 1.280±0.094 | 102 | 0.023±0.002 | 95 | 12 |
| | 24 | 1.276±0.082 | 102 | 0.024±0.002 | 100 | 12 |
| | 48 | 1.270±0.045 | 101 | 0.023±0.002 | 95 | 11 |
| Air | 24 | 1.252±0.061 | — | 0.024±0.001 | — | 10 |
| 25 ppm x 15 min | 4 | 1.238±0.049 | 99 | 0.021±0.002 | — | 12 |
| | 8 | 1.227±0.071 | 98 | 0.023±0.002 | — | 12 |
| | 24 | 1.218±0.079 | 97 | 0.022±0.002 | — | 12 |
| | 48 | 1.250±0.068 | 100 | N.D. | — | 12 |
| Air | 24 | 1.255±0.054 | — | N.D. | — | 12 |
| 25 ppm x 30 min | 4 | 1.270±0.165 | 103 | 0.022±0.002 | 99 | 12 |
| | 8 | 1.266±0.093 | 103 | 0.023±0.002 | 103 | 12 |
| | 24 | 1.266±0.101 | 103 | 0.023±0.002 | 101 | 12 |
| | 48 | 1.226±0.038 | 100 | 0.022±0.001 | 97 | 12 |
| Air | 24 | 1.230±0.059 | — | 0.023±0.002 | — | 12 |
| 50 ppm x 2 min | 4 | 1.290±0.137 | 96 | 0.026±0.004 | 103 | 9 |
| | 8 | 1.327±0.157 | 99 | 0.024±0.002 | 95 | 6 |
| | 24 | 1.262±0.081 | 94 | 0.024±0.003 | 95 | 10 |
| | 48 | 1.468±0.155 | 109 | 0.029±0.004 | 116 | 8 |
| Air | 24 | 1.343±0.120 | — | 0.025±0.002 | — | 7 |
| 50 ppm x 5 min | 4 | 1.417±0.204 | 111 | 0.028±0.017 | 109 | 10 |
| | 18 | 1.407±0.181 | 110 | 0.028±0.004 | 107 | 10 |
| | 24 | 1.321±0.046 | 104 | 0.027±0.002 | 105 | 11 |
| | 48 | 1.318±0.214 | 103 | 0.029±0.006 | 113 | 9 |
| Air | 24 | 1.276±0.202 | — | 0.026±0.004 | — | 12 |

Lung Gravimetric Values

| Exposure Condition | Post-Exposure Time (hr) | Lung Wet Weight (g) | Lung Wet Weight as % of Control | Right Cranial Lobe Dry Weight (g) | Right Cranial Lobe Dry Weight as % of Control | N |
|---|---|---|---|---|---|---|
| 50 ppm x 15 min | 4 | 1.273±0.066 | 79 | 0.025±0.001 | 80 | 6 |
| | 8 | 1.546±0.121 | 96 | 0.029±0.004 | 91 | 9 |
| | 24 | 1.444±0.131 | 90 | 0.029±0.003 | 91 | 9 |
| | 48 | 1.442±0.153 | 90 | 0.028±0.004 | 89 | 5 |
| Air | 24 | 1.600±0.262 | — | 0.031±0.008 | — | 9 |
| 50 ppm x 30 min | 4 | 1.621±0.206 | 107 | 0.029±0.008 | 98 | 8 |
| | 8 | 1.483±0.238 | 98 | 0.027±0.004 | 94 | 8 |
| | 24 | 1.609±0.101 | 107 | 0.030±0.002 | 102 | 5 |
| | 48 | 1.780±0.304 | 118 | 0.035±0.008 | 120 | 6 |
| Air | 24 | 1.510±0.232 | — | 0.029±0.006 | — | 10 |
| 75 ppm x 2 min | 4 | 1.244±0.112 | 94 | 0.023±0.002 | 88 | 9 |
| | 18 | 1.417±0.147 | 107 | 0.028±0.004 | 105 | 8 |
| | 24 | 1.232±0.110 | 93 | 0.023±0.002 | 85 | 8 |
| | 48 | 1.257±0.101 | 95 | 0.025±0.003 | 95 | 5 |
| Air | 24 | 1.322±0.166 | — | 0.027±0.005 | — | 11 |
| 75 ppm x 5 min | 4 | 1.270±0.166 | 96 | 0.025±0.004 | 98 | 12 |
| | 8 | 1.268±0.166 | 96 | 0.025±0.003 | 96 | 8 |
| | 24 | 1.240±0.168 | 94 | 0.024±0.003 | 93 | 10 |
| | 48 | 1.291±0.157 | 97 | 0.025±0.003 | 96 | 9 |
| Air | 24 | 1.326±0.168 | — | 0.026±0.003 | — | 10 |

Lung Gravimetric Values

| Exposure Condition | Post-Exposure Time (hr) | Lung Wet Weight (g) | Lung Wet Weight as % of Control | Right Cranial Lobe Dry Weight (g) | Right Cranial Lobe Dry Weight as % of Control | N |
|---|---|---|---|---|---|---|
| 75 ppm x 15 min | 4 | 1.195±0.067 | 87 | 0.023±0.002 | 92 | 9 |
| | 8 | 1.254±0.167 | 109 | 0.023±0.003 | 93 | 10 |
| | 24 | 1.511±0.206 | 109 | 0.029±0.004 | 114 | 10 |
| | 48 | 1.215±0.082 | 88 | 0.026±0.004 | 102 | 10 |
| Air | 24 | 1.380±0.163 | — | 0.025±0.003 | — | 7 |
| 75 ppm x 30 min | 4 | 1.838±0.245 | 132 | 0.031±0.003 | 115 | 6 |
| | 18 | 1.585±0.191 | 113 | 0.030±0.004 | 111 | 8 |
| | 24 | 1.602±0.241 | 114 | 0.031±0.004 | 115 | 8 |
| | 48 | 1.400±0.221 | 100 | 0.034±0.007 | 126 | 8 |
| Air | 24 | 1.400±0.245 | — | 0.027±0.007 | — | 6 |
| 100 ppm x 2 min | 4 | 1.437±0.371 | 105 | 0.0023±0.004 | 85 | 6 |
| | 8 | 1.453±0.175 | 106 | 0.026±0.002 | 98 | 6 |
| | 24 | 1.522±0.144 | 111 | 0.024±0.004 | 92 | 7 |
| | 48 | 1.589±0.088 | 116 | 0.026±0.003 | 99 | 10 |
| Air | 24 | 1.374±0.161 | — | 0.027±0.003 | — | 7 |
| 100 ppm x 5 min | 4 | 1.359±0.249 | 88 | 0.028±0.006 | 97 | 9 |
| | 8 | 1.428±0.167 | 93 | 0.026±0.002 | 90 | 10 |
| | 24 | 1.523±0.191 | 99 | 0.026±0.003 | 90 | 10 |
| | 48 | 1.527±0.168 | 99 | 0.027±0.004 | 96 | 10 |
| Air | 24 | 1.538±0.170 | — | 0.029±0.005 | — | 11 |

Lung Gravimetric Values

| Exposure Condition | Post-Exposure Time (hr) | Lung Wet Weight (g) | Lung Wet Weight as % of Control | Right Cranial Lobe Dry Weight (g) | Right Cranial Lobe Dry Weight as % of Control | N |
|---|---|---|---|---|---|---|
| 100 ppm x 15 min | 4 | 1.428±0.148 | 107 | 0.026±0.002 | 101 | 7 |
| | 8 | 1.482±0.033 | 111 | 0.029±0.002 | 112 | 3 |
| | 24 | 1.523±0.072 | 114 | 0.032±0.003 | 121 | 10 |
| | 48 | 1.525±0.117 | 114 | 0.030±0.004 | 116 | 10 |
| Air | 24 | 1.340±0.136 | — | 0.026±0.003 | — | 7 |
| 100 ppm x 30 min | 4 | 1.774±0.260 | 110 | 0.033±0.005 | 113 | 8 |
| | 8 | 1.769±0.285 | 109 | 0.035±0.006 | 123 | 9 |
| | 24 | 2.288±0.192 | 142 | 0.044±0.006 | 151 | 8 |
| | 48 | 1.831±0.177 | 113 | 0.034±0.003 | 118 | 6 |
| Air | 24 | 1.616±0.089 | — | 0.029±0.003 | — | 6 |
| 150 ppm x 2 min | 4 | 1.317±0.083 | 97 | 0.021±0.002 | 94 | 12 |
| | 8 | 1.322±0.070 | 98 | 0.023±0.001 | 104 | 12 |
| | 24 | 1.391±0.072 | 103 | 0.023±0.002 | 101 | 12 |
| | 48 | 1.401±0.063 | 104 | 0.025±0.001 | 111 | 12 |
| Air | 24 | 1.352±0.098 | — | 0.023±0.002 | — | 12 |
| 150 ppm x 5 min | 4 | 1.317±0.089 | 108 | 0.024±0.003 | 110 | 11 |
| | 8 | 1.414±0.098 | 116 | 0.026±0.002 | 121 | 12 |
| | 24 | 1.454±0.120 | 119 | 0.025±0.003 | 117 | 12 |
| | 48 | 1.477±0.073 | 121 | 0.026±0.002 | 121 | 12 |
| | 24 | 1.221±0.094 | — | 0.022±0.001 | — | 12 |

Lung Gravimetric Values

| Exposure Condition | Post-Exposure Time (hr) | Lung Wet Weight (g) | Lung Wet Weight as % of Control | Right Cranial Lobe Dry Weight (g) | Right Cranial Lobe Dry Weight as % of Control | N |
|---|---|---|---|---|---|---|
| 150 ppm x 15 min | 4 | 1.750±0.131 | 143 | 0.029±0.005 | 124 | 7 |
| | 8 | 1.556±0.102 | 127 | 0.029±0.002 | 127 | 6 |
| | 24 | 1.804±0.174 | 148 | 0.035±0.007 | 150 | 8 |
| | 48 | 1.804±0.205 | 148 | 0.037±0.007 | 159 | 12 |
| Air | 24 | 1.223±0.048 | — | 0.023±0.003 | — | 10 |
| 150 ppm x 30 min | 4 | 2.056±0.194 | 144 | 0.035±0.006 | 133 | 8 |
| | 8 | 2.301±0.795 | 162 | 0.037±0.008 | 140 | 12 |
| | 24 | 2.302±0.373 | 162 | 0.046±0.010 | 174 | 11 |
| | 48 | 2.095±0.337 | 147 | 0.046±0.007 | 173 | 8 |
| Air | 24 | 1.424±0.171 | — | 0.027±0.005 | — | 10 |
| 200 ppm x 2 min | 4 | 1.260±0.101 | 104 | 0.024±0.002 | 107 | 12 |
| | 8 | 1.200±0.353 | 99 | 0.025±0.003 | 110 | 12 |
| | 24 | 1.391±0.115 | 115 | 0.028±0.004 | 123 | 12 |
| | 48 | 1.325±0.079 | 110 | 0.025±0.003 | 110 | 12 |
| Air | 24 | 1.208±0.054 | — | 0.023±0.002 | — | 12 |
| 200 ppm x 5 min | 4 | 1.333±0.106 | 109 | 0.024±0.003 | 116 | 12 |
| | 8 | 1.376±0.062 | 112 | 0.025±0.002 | 121 | 12 |
| | 24 | 1.492±0.135 | 122 | 0.030±0.004 | 145 | 12 |
| | 48 | 1.477±0.132 | 121 | 0.030±0.005 | 146 | 12 |
| Air | 24 | 1.225±0.060 | — | 0.020±0.001 | — | 6 |

Lung Gravimetric Values

| Exposure Condition | Post-Exposure Time (hr) | Lung Wet Weight (g) | Lung Wet Weight as % of Control | Right Cranial Lobe Dry Weight (g) | Right Cranial Lobe Dry Weight as % of Control | N |
|---|---|---|---|---|---|---|
| 200 ppm x 15 min | 4 | 1.715±0.222 | 145 | 0.030±0.003 | 130 | 12 |
| | 8 | 2.038±0.877 | 172 | 0.034±0.004 | 150 | 11 |
| | 24 | 2.135±0.279 | 180 | 0.045±0.005 | 195 | 9 |
| | 48 | 2.075±0.207 | 175 | 0.045±0.007 | 195 | 11 |
| Air | 24 | 1.183±0.108 | — | 0.023±0.003 | — | 10 |
| 200 ppm x 30 min | 4 | 4.243±1.021 | 320 | 0.044±0.005 | 183 | 10 |
| | 24 | 1.325±0.073 | — | 0.024±0.001 | — | 11 |
| 250 ppm x 2 min | 4 | 1.132±0.063 | 94 | 0.023±0.003 | 100 | 12 |
| | 8 | 1.167±0.084 | 97 | 0.023±0.002 | 103 | 12 |
| | 24 | 1.215±0.087 | 101 | 0.024±0.002 | 103 | 12 |
| | 48 | 1.246±0.118 | 103 | 0.024±0.003 | 106 | 12 |
| Air | 24 | 1.207±0.173 | — | 0.023±0.003 | — | 11 |
| 250 ppm x 5 min | 4 | 1.393±0.087 | 108 | 0.026±0.002 | 110 | 12 |
| | 8 | 1.530±0.147 | 119 | 0.028±0.004 | 119 | 12 |
| | 24 | 1.642±0.075 | 128 | 0.027±0.006 | 114 | 12 |
| | 48 | 1.650±0.187 | 128 | 0.031±0.006 | 133 | 12 |
| Air | 24 | 1.284±0.067 | — | 0.023±0.003 | — | 11 |
| 250 ppm x 15 min | 4 | 2.527±0.649 | 201 | 0.035±0.005 | 164 | 11 |
| | 8 | 2.779±0.800 | 221 | 0.040±0.009 | 186 | 12 |
| | 24 | 2.687±0.537 | 213 | 0.050±0.008 | 233 | 12 |
| | 48 | 2.395±0.147 | 190 | 0.054±0.005 | 248 | 12 |
| Air | 24 | 1.259±0.048 | — | 0.022±0.002 | — | 12 |

Table A2: Alveolar Duct-Alveolar Histopathology

25 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| 25 ppm x 5 min | | | | | |
| 4 hr | 0 | 0 | 0 | 0 | 0 |
| 8 hr | 0 | 0 | 0 | 0 | 0 |
| 24 hr | 0 | 0 | 0 | 0 | 0 |
| 48 hr | 0 | 0 | 0 | 0 | 0 |
| 25 ppm x 15 min | | | | | |
| 4 hr | 0 | 0 | 0 | 0 | 0 |
| 8 hr | (0.3±0.5)[0.3±0.5] | 0 | 0 | 0 | 0 |
| 24 hr | 0 | 0 | 0 | 0 | 0 |
| 48 hr | 0 | 0 | 0 | 0 | 0 |
| 25 ppm x 30 min | | | | | |
| 4 hr | (0.9±0.5)[1.0±0.6] | 0 | (0.1±0.3)[0.1±0.3] | 0 | 0 |
| 8 hr | (1.0±0.0)[1.9±0.8] | (0.4±0.5)[0.7±0.9] | (0.8±0.5)[1.0±0.7] | 0 | (0.8±0.3)[0.8±0.3] |
| 24 hr | (0.8±0.4)[1.5±0.8] | (0.9±0.7)[1.7±1.1] | (1.3±0.9)[1.7±1.1] | (0.6±0.7)[1.0±1.1] | 0 |
| 48 hr | 0 | (1.4±0.5)[1.9±0.3] | (0.6±0.8)[0.4±0.5] | (1.7±0.5)[1.8±0.4] | 0 |

(Intensity): 0 = no lesion; 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5–12 animals.

## 50 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| 50 ppm x 2 min | | | | | |
| 4 hr | 0 | 0 | 0 | 0 | 0 |
| 8 hr | 0 | 0 | 0 | 0 | 0 |
| 24 hr | 0 | 0 | 0 | 0 | 0 |
| 48 hr | 0 | 0 | 0 | 0 | 0 |
| 50 ppm x 5 min | | | | | |
| 4 hr | (0.2±0.4)[0.4±0.8] | 0 | 0 | 0 | 0 |
| 18 hr | (0.8±0.4)[1.2±0.8] | (0.1±0.3)[0.2±0.6] | 0 | 0 | 0 |
| 24 hr | (0.5±0.7)[0.8±1.0] | (0.5±0.7)[0.8±1.0] | 0 | (0.4±0.7)[0.4±0.7] | 0 |
| 48 hr | 0 | (0.7±0.5)[1.2±1.0] | 0 | (0.2±0.4)[0.3±0.7] | 0 |
| 50 ppm x 15 min | | | | | |
| 4 hr | (1.0±0.0)[2.0±0.0] | 0 | (0.3±0.5)[0.5±0.8] | 0 | (0.3±0.5)[0.5±0.8] |
| 8 hr | (1.7±0.5)[2.7±0.5] | (0.9±0.6)[1.2±0.8] | (0.3±0.5)[0.6±1.0] | 0 | (0.7±0.5)[1.2±1.0] |
| 24 hr | (1.6±0.5)[2.6±0.5] | (1.0±0.7)[1.6±1.0] | (1.2±0.7)[2.0±1.0] | (0.2±0.7)[0.3±1.0] | (0.4±0.5)[0.7±0.9] |
| 48 hr | 0 | (1.6±0.5)[2.2±0.4] | (0.4±0.9)[0.6±1.3] | (1.2±0.4)[2.2±0.4] | 0 |
| 50 ppm x 30 min | | | | | |
| 4 hr | (0.9±0.3)[1.9±1.0] | (0.1±0.3)[0.2±0.6] | (0.1±0.3)[0.2±0.6] | 0 | 0 |
| 8 hr | (1.0±0.0)[2.5±0.5] | 0 | (0.7±0.5)[1.4±1.0] | 0 | 0 |
| 24 hr | (1.1±0.3)[2.6±0.5] | (0.8±0.4)[1.4±1.2] | (1.1±0.6)[2.2±1.0] | (0.1±0.3)[0.2±0.7] | (0.6±0.5)[1.0±1.0] |
| 48 hr | (0.1±0.3)[0.1±0.3] | (0.9±0.3)[1.7±0.7] | (1.4±0.5)[2.1±0.3] | (1.7±0.5)[2.7±0.5] | 0 |

(Intensity): 0 = no lesion, 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5–12 animals.

## 75 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| 75 ppm x 2 min | | | | | |
| 4 hr | 0 | 0 | 0 | 0 | 0 |
| 18 hr | 0 | 0 | 0 | 0 | 0 |
| 24 hr | 0 | 0 | 0 | 0 | 0 |
| 48 hr | 0 | 0 | 0 | 0 | 0 |
| 75 ppm x 5 min | | | | | |
| 4 hr | (0.8±0.3)[1.8±1.0] | (0.1±0.3)[0.1±0.3] | 0 | 0 | 0 |
| 8 hr | (0.6±0.5)[1.3±1.2] | 0 | 0 | 0 | 0 |
| 24 hr | (0.6±0.5)[1.2±1.0 ] | (0.5±0.5)[0.9±1.0] | (0.6±0.7)[0.9±1.0] | 0 | 0 |
| 48 hr | 0 | (0.8±0.4)[1.3±0.9] | 0 | (0.3±0.5)[0.6±0.9] | 0 |
| 75 ppm x 15 min | | | | | |
| 4 hr | (1.0±0.0)[2.9±0.3] | (0.1±0.3)[0.1±0.3] | (0.9±0.3)[1.2±0.6] | 0 | (0.2±0.4)[0.2±0.4] |
| 8 hr | (1.1±0.5)[1.9±0.8] | (0.6±0.5)[0.6±0.5] | (0.5±0.5)[0.6±0.8] | 0 | 0 |
| 24 hr | (1.0±0.0)[2.4±0.7] | (0.4±0.5)[0.6±0.9] | (1.8±0.6)[2.3±1.0] | (0.8±0.9)[1.1±1.2] | 0 |
| 48 hr | 0 | (1.0±0.5)[1.8±0.6] | (0.3±0.7)[0.4±0.8] | (1.4±0.5)[2.1±0.6] | 0 |
| 75 ppm x 30 min | | | | | |
| 4 hr | (1.5±0.5)[2.6±0.5] | (0.9±0.7)[1.5±1.0] | (0.3±0.5)[0.3±0.5] | 0 | (0.2±0.4)[0.2±0.4] |
| 18 hr | (1.7±0.5)[2.8±0.4] | (1.8±0.4)[2.3±0.5] | (1.3±0.8)[1.7±0.8] | 0 | (0.3±0.5)[0.7±1.0] |
| 24 hr | (1.0±0.8)[1.6±1.1] | (1.0±0.6)[1.9±0.9] | (1.3±1.0)[2.0±1.0] | 0 | (0.1±0.4)[0.3±0.8] |
| 48 hr | (0.4±0.5)[0.8±1.1] | (1.8±0.5)[2.4±0.5] | (1.4±1.3)[1.6±1.5] | (2.0±0.0)[2.4±0.5] | 0 |

(Intensity): 0 = no lesion; 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5-12 animals.

100 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| **100 ppm x 2 min** | | | | | |
| 4 hr | 0 | 0 | 0 | 0 | 0 |
| 8 hr | (0.2±0.4)[0.2±0.4] | 0 | 0 | 0 | 0 |
| 24 hr | (0.2±0.4)[0.3±0.6] | (0.2±0.4)[0.3±0.6] | 0 | 0 | 0 |
| 48 hr | 0 | (0.2±0.4)[0.3±0.6] | 0 | (0.2±0.4)[0.3±0.6] | 0 |
| **100 ppm x 5 min** | | | | | |
| 4 hr | (0.2±0.4)[0.4±0.8] | (0.1±0.3)[0.2±0.6] | (0.1±0.3)[0.2±0.6] | 0 | 0 |
| 8 hr | (0.8±0.4)[2.2±1.2] | 0 | 0 | 0 | 0 |
| 24 hr | (0.8±0.6)[1.7±1.2] | (0.6±0.5)[1.5±1.2] | 0 | (0.5±0.5)[1.0±1.0] | 0 |
| 48 hr | 0 | (1.4±1.3)[1.4±1.3] | (0.5±0.9)[0.8±1.3] | (1.0±1.3)[1.0±1.3] | 0 |
| **100 ppm x 15 min** | | | | | |
| 4 hr | (0.9±0.4)[1.9±0.9] | 0 | 0 | 0 | 0 |
| 8 hr | (1.0±0.0)[2.6±0.5] | (0.6±0.5)[1.2±1.1] | (0.2±0.4)[0.4±0.9] | 0 | (0.2±0.4)[0.4±0.9] |
| 24 hr | (0.8±0.4)[1.8±1.0] | (1.1±0.3)[2.3±0.5] | (0.2±0.4)[0.4±0.8] | (0.9±0.3)[2.1±0.8] | 0 |
| 48 hr | (0.1±0.3)[0.2±0.6] | (1.3±0.5)[2.5±0.5] | 0 | (2.2±0.4)[2.7±0.5] | 0 |
| **100 ppm x 30 min** | | | | | |
| 4 hr | (1.7±0.5)[2.7±0.5] | (0.1±0.3)[0.2±0.7] | (0.1±0.3)[0.2±0.7] | 0 | (0.1±0.3)[0.2±0.7] |
| 8 hr | (1.7±0.5)[2.9±0.3] | (0.9±0.8)[1.3±1.0] | (0.5±0.7)[0.9±1.1] | 0 | (0.7±0.8)[0.9±1.0] |
| 24 hr | (1.5±0.5)[2.9±0.3] | (1.6±0.5)[3.0±0.0] | (1.6±0.7)[2.6±0.7] | (0.6±0.5)[1.0±0.9] | (0.3±0.6)[0.5±1.0] |
| 48 hr | (0.5±0.8)[0.4±0.5] | (1.8±0.5)[2.5±0.5] | (1.4±0.7)[1.9±1.0] | (1.9±0.6)[2.9±0.4] | 0 |

(Intensity): 0 = no lesion; 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5–12 animals.

150 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| 150 ppm x 2 min | | | | | |
| 4 hr | (0.8±0.6)[1.1±0.9] | 0 | 0 | 0 | 0 |
| 8 hr | (0.8±0.5)[1.1±0.8] | 0 | 0 | 0 | 0 |
| 24 hr | (1.2±0.4)[1.7±0.5] | (0.8±0.4)[1.0±0.6] | 0 | (0.8±0.4)[0.8±0.4] | (0.1±0.3)[0.1±0.3] |
| 48 hr | 0 | (0.9±0.3)[1.3±0.7] | 0 | (1.3±0.5)[1.8±0.5] | (0.2±0.6)[0.2±0.6] |
| 150 ppm x 5 min | | | | | |
| 4 hr | (1.1±0.3)[1.8±0.5] | 0 | (0.2±0.4)[0.2±0.4] | 0 | (0.2±0.6)[0.1±0.3] |
| 8 hr | (1.4±0.5)[2.3±0.5] | 0 | (0.6±0.5)[0.6±0.5] | 0 | 0 |
| 24 hr | (1.4±0.5)[2.9±0.3] | (1.0±0.4)[1.7±0.8] | (0.3±0.5)[0.3±0.5] | (0.7±0.5)[1.3±1.0] | 0 |
| 48 hr | (0.2±0.6)[0.1±0.3] | (1.4±0.5)[2.0±0.4] | 0 | (2.0±0.4)[2.7±0.5] | 0 |
| 150 ppm x 15 min | | | | | |
| 4 hr | (1.7±0.7)[2.5±0.9] | 0 | (0.5±0.5)[0.8±0.9] | 0 | (0.8±0.5)[1.2±0.8] |
| 8 hr | (2.0±0.0)[2.5±0.5] | (0.1±0.3)[0.2±0.6] | (0.8±0.4)[1.1±0.7] | 0 | (0.5±0.5)[0.8±0.9] |
| 24 hr | (1.7±0.5)[2.8±0.4] | (1.8±0.4)[2.1±0.3] | (0.8±0.4)[1.4±0.8] | (0.8±0.5)[1.0±0.7] | (0.6±0.7)[0.8±0.9] |
| 48 hr | (1.3±0.5)[2.0±0.0] | (2.0±0.6)[2.8±0.4] | 0 | (2.8±0.4)[3.0±0.0] | (0.5±0.5)[0.9±1.0] |
| 150 ppm x 30 min | | | | | |
| 4 hr | (1.9±0.7)[3.0±0.0] | (0.3±0.5)[0.3±0.5] | (1.0±0.0)[2.0±0.0] | 0 | (1.0±0.6)[2.0±1.0] |
| 8 hr | (1.8±0.9)[2.8±0.9] | (0.3±0.7)[0.4±0.8] | (1.3±0.7)[2.3±0.9] | 0 | (1.0±0.4)[2.1±0.8] |
| 24 hr | (1.8±0.6)[2.6±0.5] | (1.1±0.3)[1.9±0.6] | (1.8±0.4)[2.6±0.5] | 0 | (0.7±0.5)[1.5±1.2] |
| 48 hr | (1.2±1.0)[0.7±0.7] | (1.2±0.6)[2.0±0.8] | (2.2±0.6)[2.7±0.5] | (2.1±0.6)[2.7±0.5] | 0 |

(Intensity): 0 = no lesion; 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5–12 animals.

200 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| 200 ppm x 5 min | | | | | |
| 4 hr | (1.6±0.5)[2.4±0.5] | (0.3±0.5)[0.4±0.7] | (0.4±0.5)[0.6±0.8] | 0 | (0.6±0.5)[1.0±1.0] |
| 8 hr | (1.3±0.5)[2.8±0.5] | (0.3±0.5)[0.5±0.8] | (0.8±0.6)[1.3±0.9] | 0 | (0.8±0.3)[0.8±0.3] |
| 24 hr | (1.8±0.4)[2.3±0.5] | (0.7±0.5)[1.3±1.0] | (1.8±0.5)[2.1±0.3] | 0 | (0.8±0.5)[1.2±0.8] |
| 48 hr | (0.3±0.5)[0.3±0.5] | (1.9±0.5)[2.8±0.5] | (0.8±0.6)[1.3±1.1] | (2.0±0.6)[3.0±0.0] | (0.3±0.5)[0.3±0.5] |
| 200 ppm x 15 min | | | | | |
| 4 hr | (1.3±0.5)[2.8±0.4] | (0.1±0.3)[0.1±0.3] | (0.4±0.5)[0.5±0.7] | 0 | (0.5±0.5)[0.7±0.8] |
| 8 hr | (1.8±0.5)[3.0±0.0] | (0.1±0.3)[0.2±0.6] | (1.0±0.5)[1.8±0.9] | 0 | (1.0±0.0)[1.7±0.5] |
| 24 hr | (2.0±1.0)[2.7±0.9] | (1.0±0.4)[2.1±0.8] | (1.2±0.6)[2.2±0.9] | 0 | (1.5±0.7)[1.7±0.6] |
| 48 hr | (1.6±0.7)[2.4±0.9] | (2.6±0.5)[2.7±0.5] | (0.1±0.3)[0.2±0.6] | (3.1±0.7)[3.0±0.0] | (1.6±0.5)[1.9±0.3] |
| 200 ppm x 30 min | | | | | |
| 4 hr | (3.3±0.7)[3.0±0.0] | 0 | (1.6±0.7)[2.0±0.0] | 0 | (1.8±0.5)[2.0±0.0] |

(Intensity): 0 = no lesion; 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5-12 animals.

250 ppm $NO_2$ Exposures

| EXPOSURE CONDITIONS/ SACRIFICE TIMES | FIBRIN | MACROPHAGES | NEUTROPHILS | TYPE II CELL HYPERPLASIA | EXTRAVASATED ERYTHROCYTES |
|---|---|---|---|---|---|
| 250 ppm x 2 min | | | | | |
| 4 hr | (0.8±0.4)[2.2±1.2] | (0.4±0.5)[0.6±0.8] | (0.2±0.4)[0.3±0.6] | 0 | 0 |
| 8 hr | (1.1±0.5)[2.2±0.8] | (0.3±0.5)[0.7±1.0] | (0.2±0.4)[0.2±0.4] | 0 | 0 |
| 24 hr | (0.8±0.5)[1.8±1.2] | (0.6±0.5)[1.2±1.0] | (0.7±0.5)[1.4±1.1] | (0.3±0.5)[0.5±0.8] | 0 |
| 48 hr | 0 | (0.9±0.7)[1.8±1.3] | (0.1±0.3)[0.1±0.3] | (1.1±0.8)[1.7±1.2] | 0 |
| 250 ppm x 5 min | | | | | |
| 4 hr | (1.7±0.5)[2.6±0.5] | (0.4±0.5)[0.4±0.5] | 0 | 0 | 0 |
| 8 hr | (2.0±0.7)[2.5±0.5] | (0.3±0.5)[0.5±0.9] | (0.9±0.7)[1.3±0.9] | 0 | (0.8±0.4)[1.3±0.8] |
| 24 hr | (1.5±0.5)[2.7±0.5] | (0.5±0.5)[1.0±1.0] | (1.8±0.6)[2.1±0.3] | 0 | (0.3±0.5)[0.3±0.7] |
| 48 hr | (0.3±0.5)[0.4±0.8] | (1.3±0.5)[2.1±0.7] | (0.9±0.5)[1.7±0.8] | (2.0±0.0)[2.8±0.5] | 0 |
| 250 ppm x 15 min | | | | | |
| 4 hr | (2.6±0.7)[3.0±0.0] | (0.5±0.7)[0.8±1.0] | (1.4±0.5)[2.3±0.5] | 0 | (1.1±0.7)[1.6±0.8] |
| 8 hr | (3.0±0.6)[3.0±0.0] | (0.4±0.5)[0.7±1.0] | (1.1±0.3)[2.5±0.5] | 0 | (1.3±0.9)[1.6±0.8] |
| 24 hr | (3.1±0.7)[3.0±0.0] | (1.1±0.3)[2.0±0.0] | (1.6±0.5)[2.7±0.5] | 0 | (2.0±0.0)[2.0±0.0] |
| 48 hr | (2.2±0.6)[3.0±0.0] | (2.0±0.0)[3.0±0.0] | (2.0±0.0)[3.0±0.0] | (2.4±0.7)[3.0±0.0] | (1.6±0.5)[2.2±0.4] |

(Intensity): 0 = no lesion; 1 = trace to mild; 2 = moderate; 3 = severe; 4 = very severe.

[Distribution]: 0 = not observed; 1 = focal; 2 = multifocal; 3 = diffuse.

The above values represent the mean ± S.D. of data obtained with 5–12 animals.

# SECTION B

## Objective 5: To determine the consequences of post-exposure exercise on the severity of $NO_2$-induced lung injury.

**Results:** The results of our studies: 1) demonstrate exercise following exposure to $NO_2$ can markedly enhance the expression of lung injury, 2) the "windowm of susceptibility" to the potentiation effects of post-exposure exercise under the conditions studied is > 8 hrs. but normally < 24 hrs., 3) two exercise bouts performed during the "windowm of susceptibility" results in a higher level of expression of lung injury than a single bout alone, and 4) an early post-$NO_2$ exercise bout can extend the period of time during which susceptibility to the potentiating effects of exercise can be demonstrated .

**Reports:**

*Lehnert, B.E., Archuleta, D.C., Ellis, T., Session, W.S., Lehnert, N.M., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environ. & Nutritional Interact. (in press), 1993.*

*"Post-Exposure Potentiation of Lung Injury by Exercise and Work Performance Degradation Following the Inhalation of Pyrolysis Products", National Aeronautics and Space Administration, Johnson Space Center, Houston, TX, November 13, 1992.*

*"Lung Injury Following Exposure of Rats to Relatively High Mass Concentrations of Nitrogen Dioxide". Walter Reed Army Institute of Research, Department of Respiratory Research, Washington, D.C., October 23, 1992.*

*Lehnert, B.E.: Toxicology of nitric oxide and nitrogen dioxide. In: Handbook of Hazardous Materials. (M. Corn, ed.) Academic press, San Diego, CA (in press), 1992.*

*Lehnert, B.E., Stavert, D.M.: Potentiation of lung injury by exercise following the inhalation of toxic gases: Evidence consistent with a postulated mechanism. 1991 Society of Toxicology Annual Meeting, Dallas,TX, February 25-March 1, 1991. The Toxicologist 11(1):A336, 1991*

*Lehnert, B.E., Stavert, D.M.: Relationship between pre-existing pulmonary edema induced by the inhalation of toxic gases and potentiation of lung injury by post-exposure exercise. Am. Rev. Respir. Dis. 143:A697, 1991.*

*Stavert, D.M., Lehnert, B.E.: Potentiation of expression of nitrogen dioxide-induced lung injury by post exposure exercise. Environ. Res. 48:87-99, 1989.*

*Stavert, D.M., Lehnert,B.E.: Window of susceptibility for post-exposure exercise potentiation of nitrogen dioxide-induced lung injury. 1988 Annual Meeting of the American Thoracic Society, Las Vegas, NV, May 8-11, 1988. Am. Rev. Resp. Dis. 137:A354, 1988.*

*Lehnert, B.E., Wilson, J.S., Stavert, D.M.: Exercise potentiates nitrogen dioxide ($NO_2$) toxicity. 1987 Annual Meeting of the Society of Toxicology, February 24-27, 1987, Washington, D.C. The Toxicologist 7:A46, 1987.*

*Stavert, D.M., Wilson, J.S., Archuleta, D.C., Lehnert, B.E.: The effects of nitrogen dioxide inhalation are potentiated by post-exposure exercise. American Thoracic Society Annual Meeting, May 10-13, 1987, New Orleans, LA., Am. Rev. Resp. Dis. 135:A281, 1987.*

## INTRODUCTION

Exercise (E) during the inhalation of oxidant gases such as nitrogen dioxide (NO2) and ozone results in more pronounced pulmonary disturbances than when these agents are breathed during rest (e.g., Mautz *et al.*, 1985; Illing *et al.*, 1980; McDonnell *et al.*, 1983; Adams and Schelegal, 1983; Fukase *et al.*, 1978). While E may effectively increase the dose of an oxidant gas delivered to the lung (Silverman *et al.*, 1976; Delucia and Adams, 1977; Mautz *et al.*, 1985), how E-associated events per se may contribute to the expression of oxidant gas-induced pulmonary injury such as pulmonary edema have not received experimental evaluation. Conceivably, events common to E, including increases in cardiac output, pulmonary vascular pressures, and pulmonary vascular perfusion (Wagner *et al.*, 1986; Hammond *et al.*, 1986; Harf *et al.*, 1978), in addition to increases in the mechanical movement of lung tissue and associate pressure alterations that occur during E (Rodarte and Fung, 1986; Linnarsson, 1974), could enhance the level of expression of pulmonary edema following oxidant gas-induced permeability changes.

We observed an increase mortality in rats if they were exercised after acute exposure to high concentrations of $NO_2$ (Stavert *et al.*, 1987a, b). This and earlier observations (Hine *et al.*, 1970) point to exercise as a post-exposure factor that can impact on the expression of acute $NO_2$-induced lung injury. In this Section, we describe our investigations of the potentiating effects of E on $NO_2$-induced lung injury with the objectives being: 1) to characterize the potentiation of expression of $NO_2$-induced pulmonary injury in the rat using lung gravimetric and histologic criteria, 2) to characterize the post-exposure period during which the expression of $NO_2$-induced lung injury can be potentiated by E, i.e., the "window of susceptibility", 3) to determine whether two E bouts performed during the window of susceptibility can cause even more marked increases in the expression of injury; and 4) to determine if early post-exposure E can extend the window of susceptibility.

## MATERIALS AND METHODS

*Animals and exercise protocol.* Male Fischer-344 rats (Specific-Pathogen-Free, Sasco, Inc., Omaha, NE) weighing between 250 and 300 g were used in this study. Before use, the animals completed a 19-day training program designed to condition them to perform on a treadmill (Stavert *et al.*, 1987a). The work intensities and durations of E were increased daily until the rats were capable of performing a "ramp" E protocol. The ramp protocol started at a treadmill velocity of 10 M/min. Every 30 sec thereafter, the treadmill speed was increased by 5 M/min up to 60 M/min (15% grade). Prior to the ramp runs, the rats performed a

"familiarization run" consisting of two short runs (20 M/min for 3 min, 15 % grade) separated by a 3-min rest period and finally followed by a 10-min rest period before initiation of the actual ramp protocol. Subsequent reference to E hereafter includes the familiarization run.

*Overview of objectives and approaches.* Rats were exposed at rest to 100 ppm $NO_2$ or filtered air only for 15 min. The rats were then subdivided into groups to be sacrificed 1, ~8, or ~24 hrs after an exposure. To determine the immediate effect E has on $NO_2$-induced pulmonary injury, some groups were exercised 1 hr before sacrifice. The E protocol required ~30 min. Accordingly, animals that were exercised immediately after exposure experienced a 30-min "rest" period prior to the 1-hr sacrifice time. To characterize the course of progression of E-associated potentiation of $NO_2$-induced injury, some rats were exercised immediately after $NO_2$ exposure and sacrificed at 8 or 24 hrs. Still other groups of $NO_2$ exposed animals were exercised 8 or 24 hr after exposure and sacrificed 24 hr, or ~8.5 hr and ~24.5 hrs, respectively, after exposure to gain information on the potentiating effects of E when performed at later postexposure times. To elucidate potential additive or synergistic effects of two postexposure E bouts, some animals that exercised immediately after the $NO_2$ exposure were again exercised at 8 hrs and sacrificed either after the second run (~8.5 hrs) or 24 hrs later. To determine if early postexposure E can extend the window of time within which E potentiates injury, some rats that were exercised immediately after $NO_2$ exposure were again exercised 24 hrs post-exposure and subsequently sacrificed. Air exposed control animals were either rested after exposure or exercised and sacrificed 1, 8, or 24 hrs after exposure.

*$NO_2$ exposures.* Nitrogen dioxide was generated from dinitrogen tetraoxide (Stavert *et al.*, 1986) and delivered to the rats via a quartz glass exposure system (Wilson *et al.*, 1987). Exposure concentrations were monitored with a dual channel ir-uv spectrophotometer (Binos Inficon, FRG) calibrated with primary gas standards (N.B.S. grade).

*Animal sacrifices, lung gravimetric measurements, and tissue processing.* The rats were anesthetized with ip injections of 50 mg pentobarbital sodium. After exsanguination by cardiac puncture, the trachea and lungs were excised, and extrapulmonary tissues were removed. The bronchus leading to the right cranial lobe (RCL) was ligated with suture and the RCL was removed. The remaining lung preparation and the RCL were then collectively weighed (lung wet weight, LWW). The RCLs were dried in an oven (150°C) for 48 hrs and weighed (right cranial lobe dry weight, RCLDW). The lungs were infused and fixed at a constant pressure of 30 cm $H_2O$ with 10% formalin in phosphate-buffered saline for 48 hrs.

The left lung lobes were trimmed (Stavert *et al.*, 1986) and embedded in paraffin, and 4 μm sections were stained with hematoxylin and eosin.

*Semi-quantitative histopathology.* Histopathologic assessments focused on changes that occur in the periterminal bronchiolar-alveolar region of the lung. These included the appearance of fibrin, accumulations of polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM), the extravasation of erythrocytes, vascular congestion, and alveolar cuboidal cell hyperplasia, i.e., type II pneumocyte hyperplasia. With the exception of vascular congestion, a grading scale was used to describe each of these features in terms of their (1) distribution, i.e., relative number of terminal bronchioles showing a lesion in associated alveolar structures; (2) severity, or the relative number of periterminal bronchiolar alveolar structures affected; and (3) intensity, the relative amount of material or relative alterations of cells in the alveoli. The distribution index for a given feature, e.g., fibrin, ranged from 0 to 4 with 0 = not observed; 1 = single or focal in appearance; 2 = few but multifocal; 3 = moderate number to many involved terminal airways; and 4 = all or essentially all alveolar structures were affected, i.e., diffuse. The relative severity index for a given pathologic feature ranged from 0 to 4 with 0 = no abnormality; 1 = the focal appearance of the abnormality in the periterminal alveolar structures; 3 = several affected alveolar structures; and 4 = many to all periterminal bronchiolar alveolar structures demonstrated the disorder. The relative intensity index also ranged from 0 to 4 with 0 = no abnormality; 1 = trace but detectable alterations in amount of abnormal material or numbers of cells; 2 = mild amount or small changes in cell numbers; 3 = moderate amount of material or moderate numbers of cells; and 4 = large amounts of intraalveolar material or large changes in cell numbers. For grading vascular congestion, only distribution and severity indices were used. In this case, the distribution index refers to the relative numbers of periterminal bronchioles that showed capillary congestion in associated alveolar septal walls and the severity index references the relative numbers of alveoli involved. The above histopathologic examinations were performed statistically blind.

*Statistical analyses.* Statistical comparisons of the lung gravimetric data were performed using the least significant difference method of multiple comparisons (Christensen, 1987). When the overall $F$ tests were significant at the 0.05 level, these tests were followed by one-tailed $t$ tests for significant increases (Snedecor and Cochran, 1969). The histopathologic variables of the treatment groups were compared pairwise using a $\chi^2$ test of independence (Brownlee, 1965).

## RESULTS

*Air exposures and exercise. E* after the air exposures resulted in no significant changes in the lung gravimetric parameters at any of the postexposure times (Figures 1 and 2). Histologically, no abnormalities in the lungs of the air exposed rats whether rested or exercised after exposure, were observed.

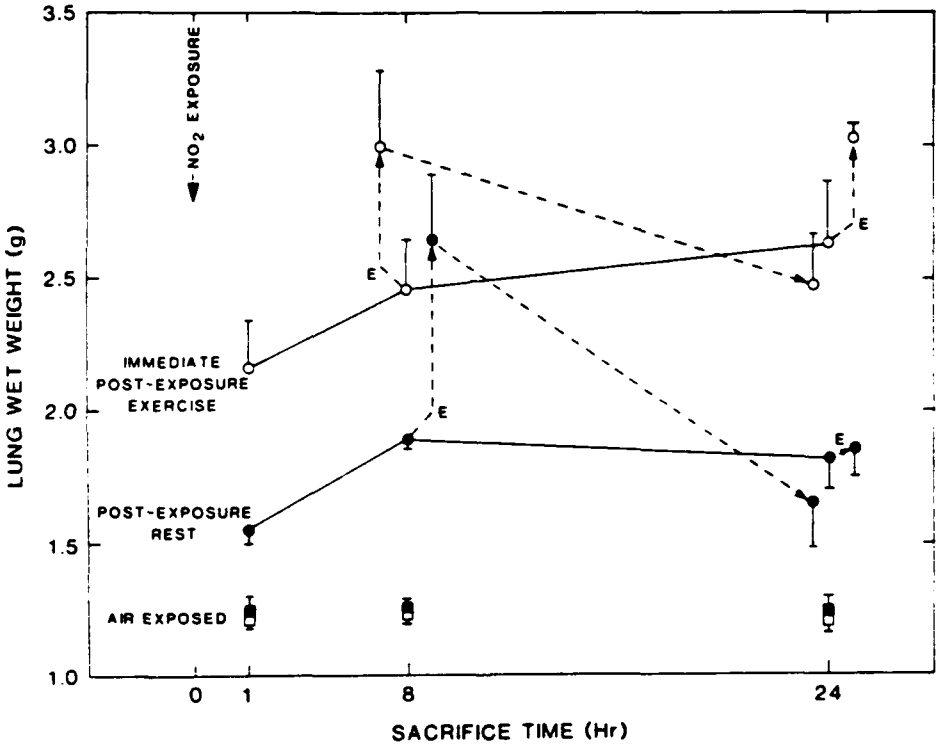


FIG. 1. Lung wet weights of the animals in the various exposure groups. (□) Air-exposed rats; (■) air-exposed and exercised rats; (●) $NO_2$-exposed and rested rats (associated dotted lines and arrows indicate postexposure values obtained when animals were exercised and/or sacrificed after an exercise bout); (○) $NO_2$-exposed and immediately exercised animals (associated dotted lines and arrows indicate postexposure values obtained when animals were subjected to a second exercise bout and/or sacrificed following the second exercise bout). Values in the figure represent means ± SE of measurements made on lungs of four to eight rats per group.

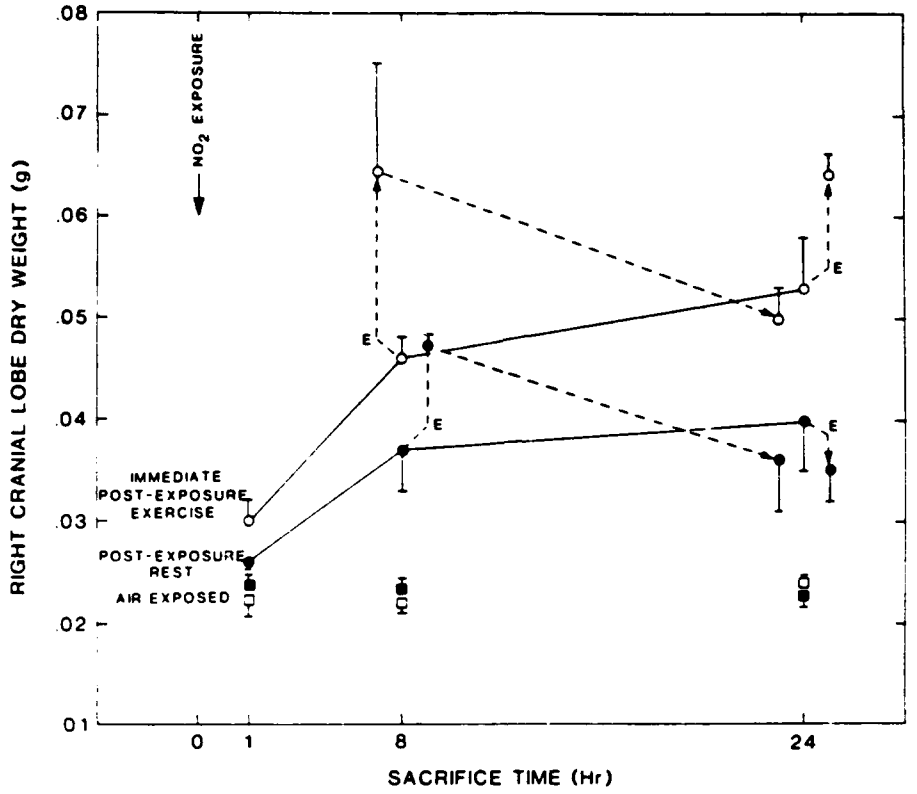


FIG. 2. Right cranial lobe dry weights of rats in the various exposure groups. (□) Air-exposed animals; (■) air-exposed and exercised rats; (●) $NO_2$-exposed and rested rats (associated dotted lines and arrows indicate postexposure values obtained when animals were exercised and/or sacrificed after an exercise bout); (○) $NO_2$-exposed and immediately exercised rats (associated dotted lines and arrows indicated postexposure values obtained when the animals were subjected to a second exercise bout and/or sacrificed following the second exercise bout). Values in the figure represent means ± SE of measurements made with the lungs of four to eight animals per group.

*$NO_2$ exposure followed by a single exercise bout immediately after exposure.* The LWW of rats exposed to the $NO_2$, exercised immediately thereafter, and sacrificed 30 min later was ~40% greater than the LWW of animals that were exposed to the $NO_2$ but rested prior to the I hr sacrifice time, Figure 1, P = 0.01. The RCLDW of the exercised rats also increased relative to the $NO_2$-exposed but rested condition (P = 0.03), but E-associated enhancements in this parameter, in terms of percentage changes, were less than that found for the LWW, i.e., ~ 17~ increases, Figure 2. Histologically, E immediately after $NO_2$-exposure resulted in increases in the distribution and severity of fibrin (P = 0.02, P = 0.02, respectively), and

tended to increase the intensity of fibrin, P = 0.06, Table 1. E immediately after $NO_2$ exposure also tended to facilitate the extravasation of erythrocytes (P = 0.07) and promote an earlier than normal influx of PMN into the alveoli, Tables I and 2.

The E-associated increases in the gravimetric parameters were persistent. For those animals exercised immediately after exposure and sacrificed 8 hrs thereafter, their LWW and RCLDW values were elevated ~30% and ~24%, respectively, over their equivalently exposed but rested counterparts (P = 0.01, P = 0.03), Figures 1 and 2. Histologic analyses at the 8-hr sacrifice time suggested E-associated enhancements in fibrin intensity (P = 0.07) and PMN intensity (P = 0.08). Also, vascular congestion at the 8-hr time appeared to be more widely distributed in the lungs of the exercised animals, although the indices for this disturbance were not significantly different from the $NO_2$ exposed and rested condition, Table 1. As of 24 hrs after the immediate postexposure E, the LWW and RCLDW were increased ~45% and ~40% over the LWW and RCLDW values obtained from the $NO_2$ exposed but rested rats, (P = 0.01, P = 0.03), Figures 1 and 2. The main histologic distinctions between the lungs of the rested and exercised animals were increases in fibrin severity (P < 0.01) and intensity (P = 0.06), and increases in the severity indices for extravasated erythrocytes (P = 0.01) and AM (P = 0.04) in the latter group of rats. No evidence was observed for an E-associated increase in type II cell hyperplasia, Table 2.

*$NO_2$ exposure and exercise at 8 or 24 hrs post- exposure.* When the rats were exercised 8 hrs post-exposure and sacrificed ~30 min later, their LWW and RCLDW increased to levels comparable to those found at the 8-hr sacrifice time point following $NO_2$ exposure and immediate E, Figures 1 and 2. Most of the histologic indices of lung injury for these latter two groups of animals were similar. When the rats were exercised 8 hrs post-exposure and examined 16 hrs later, their LWW and RCLDW values were similar to those obtained with rats exposed to the $NO_2$ and allowed to rest prior to sacrifice 24 hrs after the exposure, Figures 1 and 2. Thus, the E-associated increases observed for the gravimetric parameters when the animals were exercised 8 hrs post-$NO_2$ exposure and sacrificed shortly thereafter subsided by the 24-hr sacrifice time. Also, the histologic indices of lung injury for rats that were exercised 8 hr post-$NO_2$ exposure and for rats exposed to the $NO_2$ but allowed to rest were nearly all identical at the 24-hr sacrifice time. The major difference between the groups was an increase in fibrin severity (P = 0.04) in the lungs of the exercised animals, Table 1. Compared to the 24-hr sacrifice time following the immediate E post-$NO_2$ exposure condition, E at 8 hrs post-exposure tended to result in lesser fibrin severity and intensity and fewer extravasated erythrocytes, Table 1.

TABLE I

OCCURRENCE OF FIBRIN, EXTRAVASATED ERYTHROCYTES, AND VASCULAR CONGESTION FOLLOWING $NO_2$ EXPOSURES

$NO_2$-exposed animals*

| Postexposure condition(s) | Postexposure sacrifice time | Fibrin | | | Extravasated erythrocytes | | | Vascular congestion | |
|---|---|---|---|---|---|---|---|---|---|
| | | Distribution | Severity | Intensity | Distribution | Severity | Intensity | Distribution | Severity |
| Rest | 1 hr (5) | 2.5 ± 0.4 | 1.0 ± 0.0 | 1.0 ± 0.0 | 0 | 0 | 0 | 0 | 0 |
| Rest | 8 hr (6) | 4.0 ± 0.0 | 1.2 ± 0.2 | 1.8 ± 0.3 | 2.7 ± 0.2 | 1.0 ± 0.0 | 1.3 ± 0.2 | 0.5 ± 0.5 | 0.2 ± 0.2 |
| Rest | 24 hr (5) | 4.0 ± 0.0 | 1.0 ± 0.0 | 1.8 ± 0.4 | 3.0 ± 0.3 | 1.0 ± 0.7 | 2.0 ± 0.3 | 0 | 0 |
| Immediate exercise | 1 hr (4) | 4.0 ± 0.0 | 1.8 ± 0.3 | 1.5 ± 0.3 | 1.0 ± 0.6 | 0.5 ± 0.3 | 0.5 ± 0.3 | 0 | 0 |
| Immediate exercise | 8 hr (4) | 4.0 ± 0.0 | 1.8 ± 0.3 | 2.5 ± 0.3 | 3.0 ± 0.0 | 1.0 ± 0.0 | 2.3 ± 0.3 | 1.8 ± 0.8 | 0.5 ± 0.3 |
| Immediate exercise | 24 hr (5) | 4.0 ± 0.0 | 2.4 ± 0.3 | 3.6 ± 0.3 | 2.8 ± 0.2 | 1.8 ± 0.2 | 3.2 ± 0.4 | 0 | 0 |
| Rest, exercise at 8 hr | ~8.5 hr (4) | 3.8 ± 0.3 | 1.5 ± 0.3 | 2.3 ± 0.3 | 2.8 ± 0.3 | 1.0 ± 0.0 | 2.0 ± 0.4 | 0.8 ± 0.8 | 0.3 ± 0.3 |
| Rest, exercise at 8 hr | 24 hr (6) | 4.0 ± 0.0 | 1.6 ± 0.3 | 2.4 ± 0.4 | 1.4 ± 0.6 | 0.6 ± 0.2 | 1.4 ± 0.6 | 0 | 0 |
| Rest, exercise at 24 hr | ~24.5 hr (6) | 4.0 ± 0.0 | 1.0 ± 0.0 | 2.3 ± 0.2 | 2.8 ± 0.2 | 1.0 ± 0.0 | 2.0 ± 0.3 | 0 | 0 |
| Immediate and 8 hr exercise | ~8.5 hr (6) | 4.0 ± 0.0 | 2.8 ± 0.3 | 3.2 ± 0.2 | 3.3 ± 0.2 | 1.5 ± 0.2 | 2.8 ± 0.2 | 1.5 ± 0.7 | 0.7 ± 0.3 |
| Immediate and 8 hr exercise | 24 hr (6) | 4.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 | 3.5 ± 0.3 | 3.0 ± 0.0 | 3.3 ± 0.3 | 0 | 0 |
| Immediate and 24 hr exercise | ~24.5 hr (5) | 4.0 ± 0.0 | 3.2 ± 0.2 | 3.0 ± 0.0 | 3.4 ± 0.3 | 2.8 ± 0.2 | 2.8 ± 0.4 | 0 | 0 |

* No pathologic changes were observed in the lungs of rats exposed to filtered air only, $N$ = 5–6 animals per group.

( ) Numbers of animals studied per group.

Each of the above values represent the means ± SE.

TABLE 2

RELATIVE OCCURRENCE OF POLYMORPHONUCLEAR LEUKOCYTES AND ALVEOLAR MACROPHAGES, AND ALVEOLAR CUBOIDAL EPITHELIAL CELL HYPERPLASIA FOLLOWING THE $NO_2$ EXPOSURES

| $NO_2$-exposed animals* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Postexposure condition(s) | Post-exposure sacrifice time | Polymorphonuclear leukocytes | | | Alveolar macrophages | | | Cuboidal epithelial cell hyperplasia | | |
| | | Distribution | Severity | Intensity | Distribution | Severity | Intensity | Distribution | Severity | Intensity |
| Rest | 1 hr (5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rest | 8 hr (6) | 2.7 ± 0.2 | 1.0 ± 0.0 | 1.3 ± 0.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rest | 24 hr (5) | 1.2 ± 0.5 | 0.6 ± 0.2 | 0.8 ± 0.4 | 3.0 ± 0.0 | 1.4 ± 0.3 | 2.4 ± 0.4 | 2.4 ± 0.5 | 2.2 ± 0.2 | 1.0 ± 0.0 |
| Immediate exercise | 1 hr (4) | 0.5 ± 0.5 | 0.3 ± 0.3 | 0.3 ± 0.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| Immediate exercise | 8 hr (4) | 3.0 ± 0.0 | 1.0 ± 0.0 | 2.3 ± 0.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| Immediate exercise | 24 hr (5) | 2.0 ± 0.5 | 1.0 ± 0.3 | 1.2 ± 0.4 | 3.4 ± 0.3 | 2.0 ± 0.0 | 2.4 ± 0.3 | 3.2 ± 0.2 | 2.0 ± 0.0 | 1.2 ± 0.2 |
| Rest, exercise at 8 hr | ~8.5 hr (4) | 2.0 ± 0.7 | 0.8 ± 0.3 | 1.8 ± 0.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rest, exercise at 8 hr | 24 hr (6) | 1.2 ± 0.5 | 0.6 ± 0.3 | 1.0 ± 0.5 | 3.2 ± 0.4 | 1.4 ± 0.3 | 2.2 ± 0.2 | 3.0 ± 1.0 | 2.0 ± 0.0 | 1.0 ± 0.0 |
| Rest, exercise at 24 hr | ~24.5 hr (6) | 1.8 ± 0.4 | 0.8 ± 0.2 | 1.2 ± 0.3 | 2.7 ± 0.9 | 1.0 ± 0.0 | 1.7 ± 0.2 | 3.2 ± 0.2 | 2.0 ± 0.0 | 1.0 ± 0.0 |
| Immediate and 8 hr exercise | ~8.5 hr (6) | 3.3 ± 0.2 | 1.5 ± 0.2 | 2.3 ± 0.4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Immediate and 8 hr exercise | 24 hr (6) | 3.5 ± 0.3 | 2.5 ± 0.3 | 2.5 ± 0.3 | 4.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 | 2.3 ± 0.3 | 1.8 ± 0.3 | 1.0 ± 0.0 |
| Immediate and 24 hr exercise | ~24.5 hr (5) | 3.0 ± 0.3 | 2.2 ± 0.2 | 1.8 ± 0.2 | 3.8 ± 0.2 | 2.6 ± 0.3 | 2.2 ± 0.2 | 3.2 ± 0.2 | 2.0 ± 0.0 | 1.4 ± 0.5 |

* No pathologic abnormalities were observed in the lungs of rats exposed to filtered air only, $N$ = 5–6 rats per group.

( ) Numbers of animals studied per group.

Each of the above values represents the means ± SE.

The lung gravimetric values obtained from the animals that were exposed to the $NO_2$, exercised 24 hr later, and sacrificed 30 min thereafter were not significantly different from those obtained from the rats that were allowed to rest for 24 hrs after the $NO_2$-exposure, Figures 1 and 2. As well, no evidence of an E-associated potentiation in the severity of lung injury was observed, Tables 1 and 2.

*$NO_2$ exposure and immediate and subsequent exercise.* In this component of the study, rats were exposed to $NO_2$, exercised immediately after exposure, and again exercised at 8 or 24 hrs. The rats that were exercised immediately and 8 hrs post-$NO_2$ exposure were sacrificed either 30 min after the last exercise bout or 16 hrs later and those that were exercised immediately after exposure and again at 24 hrs post-exposure were sacrificed ~30 min after their last E bout. As shown in Figure 1, the average LWW of rats exercised 8 hrs following the immediate postexposure E further increased ~25% above that observed at the 8-hr postexposure time with rats that were exposed to the $NO_2$ and exercised immediately afterward, P = 0.09. No evidence for a persisting two-exercise bout-associated increase in LWW, however, was found when rats were exposed to the NO2, exercised immediately and 8 hr thereafter, and allowed to rest until sacrifice 24 hr after exposure, Figure 1. On average, the RCLDW of the rats that received two E bouts and were sacrificed ~8 hr postexposure appeared to be elevated ~37% above the RCLDW values obtained from rats administered only the immediate E bout, Figure 2, although the values for these two groups were not significantly different. Fibrin intensity for this group of animals, nevertheless, was greater than that for rats that were exercised immediately or 8 hrs post-exposure and sacrificed at the ~8-hr sacrifice time (P = 0.04, P = 0.04), Table 1. Regardless, like the LWW endpoint, the RCLDWs of rats that were exercised immediately and 8 hrs post-exposure and were then allowed to rest until the 24-hr sacrifice time were essentially identical to those of rats that were only exercised immediately after exposure, Figure 2. Relative to the lungs of rats that were only exercised immediately after exposure and sacrificed at the 24-hr time point, immediate and 8-hr E resulted in significant increases in the severity indices for extravasated erythrocytes and AM (P = 0.01, P = 0.003, respectively), and appeared to have caused increases in fibrin severity (P = 0.06) and intensity (P = 0.06). Compared to the histopathologic profile at 24 hr that followed E at 8 hr only, the immediate and 8-hr bouts resulted in further increases in fibrin severity (P < 0.01); marked enhancements in the distribution, severity, and intensity indices for extravasated erythrocytes (P < 0.01) and PMN (P = 0.01); and an increase in the severity of AM (P < 0.01), Tables 1 and 2. The LWW and RCLDW of animals exercised immediately and 24 hrs after exposure tended to be significantly increased over the LWW and RCLDW of rats that were only exercised immediately after the $NO_2$ exposure and sacrificed 24 hr later (P = 0.07, P

= 0.06, respectively), Figures 1 and 2. Also, the LWW and RCLDW of the rats exercised immediately and 24 hr postexposure were substantially greater than the LWW and RCLDW of animals that were only exercised at 24 hr postexposure ($P < 0.001$, $P < 0.001$), Figures 1 and 2. The second exercise bout at 24 hrs resulted in increases in fibrin intensity ($P = 0.04$), the severity of extravasated erythrocytes ($P = 0.03$), the severity of AM ($P = 0.04$), and a trend toward an increase in PMN severity ($P = 0.08$) relative to the expression of these abnormalities in rats that experienced immediate post-$NO_2$ exposure E only, Tables 1 and 2.

## DISCUSSION

On the basis of gravimetric and histopathologic criteria, postexposure E can significantly potentiate the expression of lung injury *after* brief exposure to the oxidant gas $NO_2$. Relative to the $NO_2$-exposed but rested condition, E performed immediately after exposure resulted in substantially greater increases in lung water as of 30 min thereafter, whereas increases in the lung dry weights and fibrin were less pronounced. As of 8 hrs after immediate postexposure E, the lung wet and dry gravimetric parameters further increased essentially proportionately above those measured for the resting condition. These effects of immediate postexposure E continued for at least 24 hr. By this latter time, the lung gravimetric values of the exercised rats were further increased ~40-45% over those obtained with the NO2-exposed but rested animals. Such E-associated enhancements in the expression of NO2-induced lung injury were accompanied by further increases beyond the resting condition in alveolar fibrin and erythrocytes. Importantly, type II cell hyperplasia, a well-documented response to type I pneumocyte injury (Rombout *et al.*, 1986; Evans *et al.*, 1978), was not detectably affected by E. This finding suggests that the potentiation in the expression of lung injury by E shortly after $NO_2$ exposure may not be due to furthering the initial level of cellular injury caused by the $NO_2$.

We have found that the animals remained susceptible to the potentiating effects of E for at least 8 hrs post-$NO_2$ exposure. A fundamental difference between the effects of the immediate and 8-hr E bouts, however, is noteworthy. Subsidences in the 8-hr E-associated elevations in the gravimetric parameters as of 24 hrs were not unlike those observed with rats exposed to the $NO_2$ but rested for the 24-hr postexposure period, whereas after immediate E, the LWW and RCLDW remained elevated above the $NO_2$-exposed but rested condition as of 24 hrs post-exposure time. Thus, the nature of the enhanced expression of lung injury caused by E immediately and 8 hr post-exposure differ qualitatively. Regardless, although we have not firmly established the precise postexposure duration of time during which E can potentiate the

expression of $NO_2$-induced lung injury, it appears that the window of susceptibility is closed as of 24 hrs post-$NO_2$ exposure.

In another component of the study, rats subjected to a second E bout 8 hrs after immediate post-exposure E were found to have lung gravimetric increases that were even higher shortly after the 8-hr E bout than they were when animals were exercised immediately after exposure and sacrificed 8 hrs thereafter. As well, the lung weights of the rats receiving two E bouts were also substantially higher than those of rats that were exposed to the $NO_2$, exercised 8 hrs later, and subsequently sacrificed after E. For the gravimetric parameters, further potentiation of the expression of lung injury by the second episode of E appeared to be merely additive. Of particular interest, further increases in the lung weight parameters, which did not appear to be simply due to increased vascular congestion, subsided by the 24-hr sacrifice time to levels found 24 hrs after immediate E only. In this regard, the second E bout-associated increase in the lung weights and their subsequent subsidence were qualitatively similar to those observed with the rats that were exposed to $NO_2$ and exercised 8 hrs later.

A second exercise bout performed 24 hrs after $NO_2$ exposure and immediate E was also found to further increase the expression of lung injury. Inasmuch as E performed 24 hrs after $NO_2$ exposure and rest did not have this effect, we conclude that E immediately after $NO_2$ exposure can prolong the window of susceptibility to the potentiating effects of E. This outcome of our study suggests that E after $NO_2$ exposure can either directly or indirectly worsen the initial $NO_2$-induced lung lesion or modify repair processes in a manner that extends the state of abnormal permeability. The histopathologic studies do suggest that E after $NO_2$ exposure can lead to an enhanced recruitment of inflammatory cells, and it remains possible that their more abundant presence may contribute, via a variety of mechanisms (Staub *et al.*, 1982; Eiermann *et al.*, 1983; Kreiger *et al.*, 1984; Steinberg *et al.*, 1986), to the observed increases in the expression of lung injury.

Aside from the effects E may have on cellular changes in $NO_2$-exposed lungs, the potentiated expression of injury may be due largely to physical mechanisms. Several investigators (Maron *et al.*, 1979; Buono *et al.*, 1982; Miles *et al.*, 1983, 1986; Miles and Durban, 1985) have found an apparent uncoupling between diffusion capacity and cardiac output after E that is consistent with an E-associated, transient, subclinical pulmonary edema. In the context of Starling's equation for fluid movement (Starling, 1896), when normal lung permeability is breached and pulmonary edema ensues, E-associated elevations in cardiac output and pulmonary vascular pressure would be expected to facilitate the translocation of water and blood solute constituents into extravascular compartments. Accordingly, the potentiation in expression of $NO_2$-induced lung injury by E may represent the imposition of forces involved in bringing about quasi-hydrodynamic pulmonary edema atop the complex

factors involved in permeability pulmonary edema (Crandall *et al.*, 1983; Bhattacharya, 1987; Staub, 1974). Another factor possibly underlying the potentiation of expression of lung injury is the increase in ventilation associated with E. Albelda and colleagues (1986) have shown that when ventilation was increased in sheep, lung lymph flow increased ~27% and the ratio of total protein concentration in the lymph relative to plasma fell ~10%. Such results are consistent with a hyperpnea-related increase in the pulmonary transvascular hydrostatic pressure gradient (Brigham, 1979; Parker *et al.*, 1981; Roselli, 1984). Of importance, pulmonary lymph flow has also been reported in increase (up to threefold) in exercising sheep (Coates *et al.*, 1983).

Although physiologic considerations may offer some explanation for various aspects of the potentiation of expression of $NO_2$-induced pulmonary edema, they do not provide direct insight as to the underlying bases of others. For example, it is unclear why the kinetics of subsidence of pulmonary edema following immediate postexposure E and immediate and 8 hr postexposure E differ. The findings made in this study seem to point to the translocation of water and blood solutes into at least two compartments that have differing rates of resolution of edema fluid. Immediate post-$NO_2$ exposure E appears to result in the further filling of one of these with edema fluid beyond the level produced by $NO_2$ exposure and rest only. This potential compartment evidently continues to fill for at least 24 hrs post-exposure. On the other hand, a second, more rapidly resolving compartment accumulates fluid during or shortly after a second E bout following the first. It is tempting to speculate that the immediate post-$NO_2$ exposure E bout resulted primarily in the further filling of the lung's interstitium with fluid, whereas the second E bout mainly promoted the translocation of edema fluid from the already well-filled interstitium into the more rapidly clearing alveoli (Havill and Gee, 1987). From a mechanistic perspective, the driving force(s) favoring such fluid movements could include E-associated increases in vascular distension with corresponding decreases in interstitial compliance, increased vascular filtration pressure, and an already well-hydrated interstitial compartment (Crandall *et al.*, 1983).

We note here that our studies involved a concentration of $NO_2$ that substantially exceeds levels found in most settings. However, we also noted that our observations were made following brief 15-min exposures to the $NO_2$, and that the high concentration of $NO_2$ used in this study can be encountered under some conditions (Guidotti, 1978). Whether our findings that postexposure E can potentiate the expression of lung injury following the inhalation of $NO_2$ at lower concentrations and longer durations requires further investigation.

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# SECTION C

## Objective 6: To develop a testing procedure, using an exercising rat model, to assess for degradations in work performance capacity.

## Objective 7: To characterize work performance degradation following acute inhalation of high concentrations of $NO_2$.

**Results:** A treadmill-metabolic chamber and stress testing protocol was developed to evaluate aerobic work performance on exercising rats. Using maximum oxygen consumption ($VO_{2max}$) as an index of work performance, several atmospheres were evaluated ranging from 25 ppm $NO_2$ to 2000 ppm $NO_2$. Definite decrements in work performance were observed. Work performance decrements were found to be linearly related to the severity of pulmonary edema, as indexed by lung wet weight increases after exposure to $NO_2$.

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## INTRODUCTION

Acute exposures to supra-ambient concentrations of nitrogen dioxide ($NO_2$) have been shown to produce histopathologic, biochemical, and functional alterations in the pulmonary system (e.g., Stavert and Lehnert, 1989; Evans, 1986; Elsayed and Mustafa, 1982; Evans et al., 1976; Johnson et al., 1982; Mustafa and Tierney, 1978). The major anatomical sites in the lung that are involved in $NO_2$-induced acute injury include the terminal bronchioles, the alveolar ducts, and the proximal alveoli with more distal alveolar involvement becoming evident with increasing $NO_2$ exposure concentrations (Stavert and Lehnert, 1989). Specific disturbances following the inhalation of $NO_2$ include endothelial cell swelling and cytoplasmic changes, type I pneumocyte destruction, increases in lung permeability and the development of pulmonary edema, and an acute inflammatory response, *inter alia* (Guidotti, 1978, 1980, 1985; Stephens, 1972; Dowell, 1971; Stavert and Lehnert, 1989; Gordon, 1983, Johnson, 1982; Evans et al., 1973a, 1973b, 1980; Goldstein, 1973; Hine, 1970; Mustafa and Tierney, 1978). Inasmuch as these pulmonary abnormalities are likely initiated immediately after the inhalation of high concentrations of $NO_2$ (Stavert and Lehnert, 1989) an early functional compromise minimally involving gas exchange in the lung would be expected. How such an impairment, as well as other pathophysiologic phenomena associated with $NO_2$-induced acute lung injury, may subsequently impact on work performance capacity or endurance has not been experimentally evaluated. A major objective of the present study, accordingly, was to obtain information as to how physical activity or work performance is affected by brief exposures to high and very high concentrations of $NO_2$. In order to do so, we ulitized an exercising rat model developed at Los Alamos National Laboratory and evaluated how maximum oxygen consumption, or $VO_{2max}$, is altered by the $NO_2$ exposures. $VO_{2max}$ was selected as the major parameter to index potential disturbances in work performance because of its close association with endurance (Astraud, 1970).

## MATERIALS AND METHODS

*Animals and Training Protocol:* Adult, male Fischer-344 rats (specific-pathogen-free, virus-free, Harlon Sprague Dawley, Indianapolis, Ind.) weighing between 250 and 300 grams were housed and cared for in facilities accredited by the American Association of Laboratory Animal Care; all studies described herein were conducted in accordance with the

"Guide for the Care and Use of Laboratory Animals," 1985. Before experimental use, the animals completed a 19 day training program (unless otherwise indicated) in order to train them behaviorally and to condition them physically to perform (run) on a treadmill. The progressive training program began on day 1 with 5 periods of intermittent exercise per day that consisted of 2 min durations of running on a treadmill at low work loads (10-15 M/min ~ 15% grade). Each running period was separated by 2 min rest periods. The work loads and durations of exercise were increased daily until the rats were running 40 M/min for 5 min, 4 times per day by day 13. From day 13 through day 19, rats were exercised twice per day using a "ramp" exercise protocol (to be described). During some of these training periods, oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) were measured and used to evaluate pre-exposure control values. To stabilize baseline resting oxygen consumption before the "ramp" protocol was initiated, a "familiarization run" was performed. This consisted of two short-duration, low-intensity runs on the treadmill (3 min, 20 M/min at a 15% grade) separated by a 3 min rest period and finally followed by a 10 min rest period. The "ramp" test was conducted as follows (see Figure 1): after an initial two min rest period during which resting $VO_2$ and $VCO_2$ values were measured, the actual ramp test was begun by turning on the treadmill to an initial velocity of 10 M/min. Every 30 sec thereafter, treadmill speed was incrementally increased by 5 M/min. The grade of the treadmill was held constant at 15% throughout the protocol. The test was continued until exhaustion or until an animal attained its maximum oxygen consumption ($VO_{2max}$), as described below. Work rate (Kg·M/min) at each incremental step was calculated as the product of the mean animal weight (280 gm, treadmill speed, and percent gradient over time (Donovan and Brooks, 1977), Figure 1.

*Experimental Protocol:* Upon completion of the training protocol, the rats entered the exposure phase of the study in which groups of animals were exposed to nitrogen dioxide ($NO_2$) or filtered air only. The exposure system and associated technique for exposing the animals to the test gas atmospheres have been described both earlier in this report as well as elsewhere (Wilson et al., 1987; Stavert and Lehnert, 1989). All the $NO_2$ and air exposures were conducted with resting animals. In the first study series, the $NO_2$ exposures were conducted with 10, 25, 50, or 100 ppm $NO_2$ delivered to the rats for a 15 min duration. In a second, more recent study series, rats were exposed to higher concentrations of $NO_2$, i.e., 200-2000 ppm before the work performance assessments.

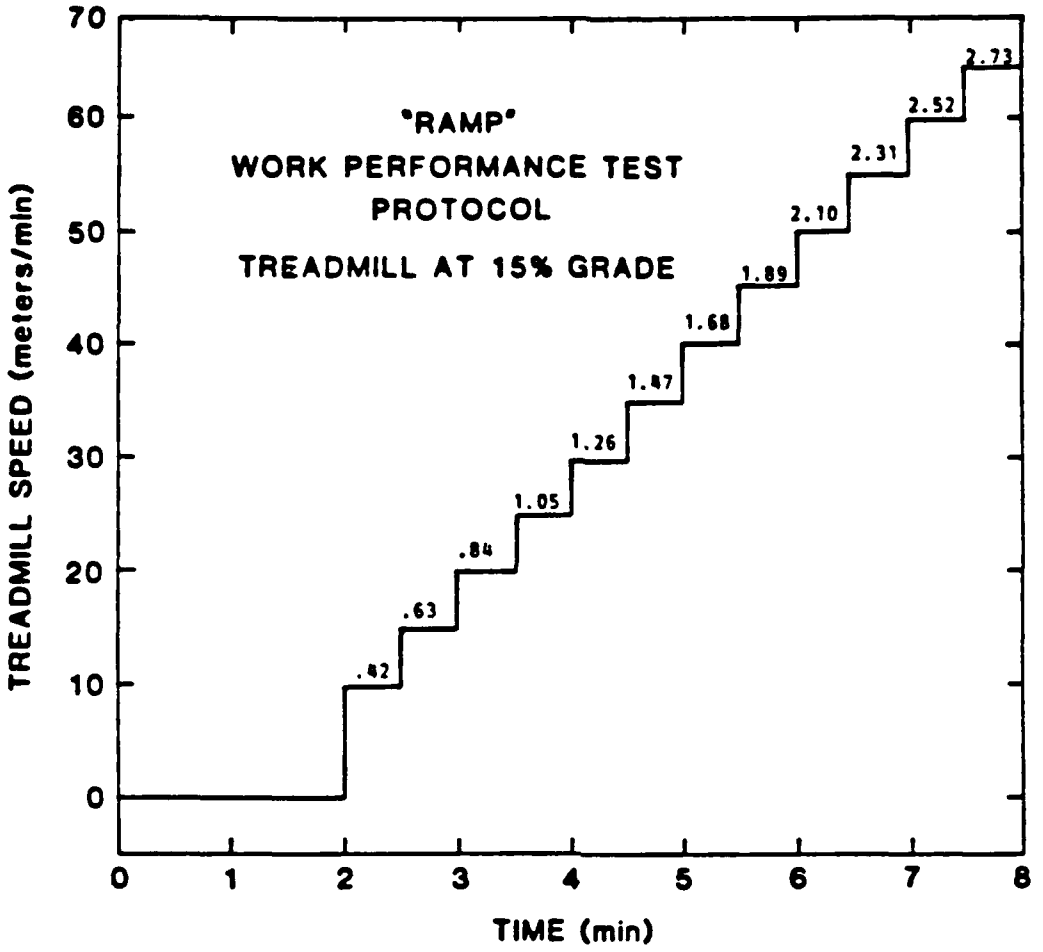


Figure 1: The "ramp" work performance test begins with two minutes of rest followed by a 10 M/min starting work load. Increases of 5 M/min in treadmill speed occur every 30 sec until the rat attains its maximum oxygen consumption. Work rates at each incremental step are in Kg·M/min.

*Measurements of $VO_{2max}$:* The treadmill used for the "ramp" protocol is contained in a metabolic chamber which provides the necessary means to measure $O_2$ consumption as a rat exercises, Figure 2. Airflow from the chamber (14 $L \cdot min^{-1}$) is dried (Silica Gel, J.T. Baker Chemical Co., Phillipsburg, NJ) and measured electronically using a pneumotachograph (Fleish No. 0, Gould Inc., Cleveland, OH) and transducer (Validyne Engineering, Northridge, CA) calibrated spirometrically. $O_2$ content in the effluent airstream is measured with a suitable analyzer (Ametek S-3A $O_2$ Analyzer, Ametek, Pittsburg, PA) that

is calibrated with a primary gas standard (National Bureau of Standards grade, Matheson Gas, LaPorte, TX). $O_2$ consumption is calculated every 5 sec via a data acquisition and computer system (HP-3497A, Hewlett-Packard, Corvallis, OR) using the equations of Mautz (1985). $VO_{2max}$, or the plateau of $O_2$ consumption achieved with increasing work loads, is expressed as ml $O_2$/kg body weight/min following correction for standard temperature and pressure. As illustrated in Figure 3, the $VO_{2max}$ values of animals performing the "ramp" protocol become satisfactorily stable and reproducible during the last several days of the training program. As also indicated in Figure 3, trained rats usually maximally consume $O_2$ ~5-6 min after the "ramp" protocol is initiated.

## RESULTS

*Work Performance After Exposure to the 10-100 ppm Concentrations of $NO_2$:* Almost all of rats were capable of completing the "ramp" exercise test after exposure to $NO_2$. No significant differences were found in the work rate at which maximum oxygen consumption was attained between the $NO_2$ exposed and air exposed animals, Table 1.

Work Rate at Which Maximum Oxygen Consumption Was Attained
(kg·M/min)

| | Pre-Exposure | Immediately Post-Exposure | 8 Hr Post-Exposure |
|---|---|---|---|
| Control Air | 1.92 ± 0.24 | 2.07 ± 0.31 | 2.07 ± 0.21 |
| 10 ppm $NO_2$ | 2.07 ± 0.25 | 1.72 ± 0.21 | 2.07 ± 0.25 |
| 25 ppm $NO_2$ | 1.81 ± 0.41 | 1.85 ± 0.38 | 1.64 ± 0.23 |
| 50 ppm $NO_2$ | 1.96 ± 0.29 | 1.93 ± 0.16 | 1.82 ± 0.29 |
| 100 ppm $NO_2$ | 1.93 ± 0.34 | 1.65 ± 0.34 | 1.64 ± 0.60 |

Table 1: Work rate at which maximum oxygen consumption was attained in rats exercising before, immediately, and 8 hr post-exposure to air or various concentrations of $NO_2$. The above values represent the mean ± S.D. of a minimum of 5-6 animals per exposure condition.

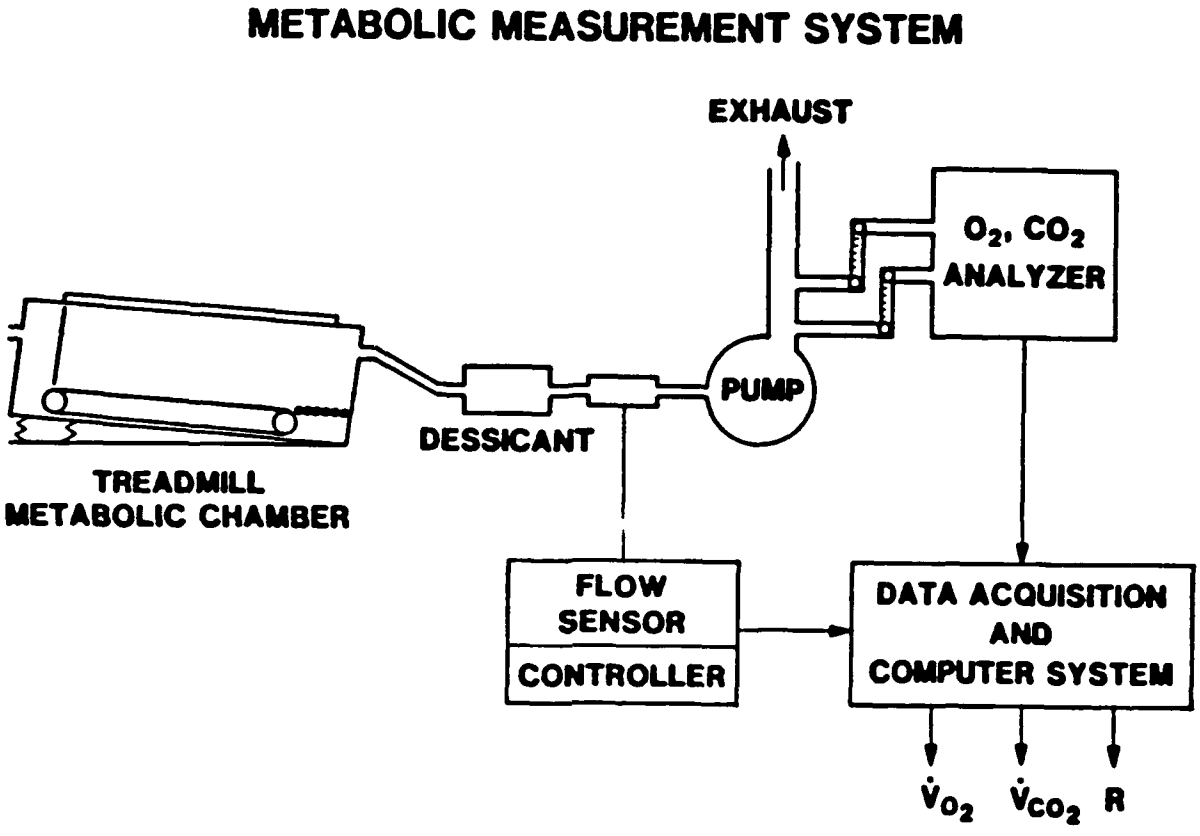


Figure 2: Schematic representation of the treadmill-metabolic chamber system used for measuring $VO_{2max}$.

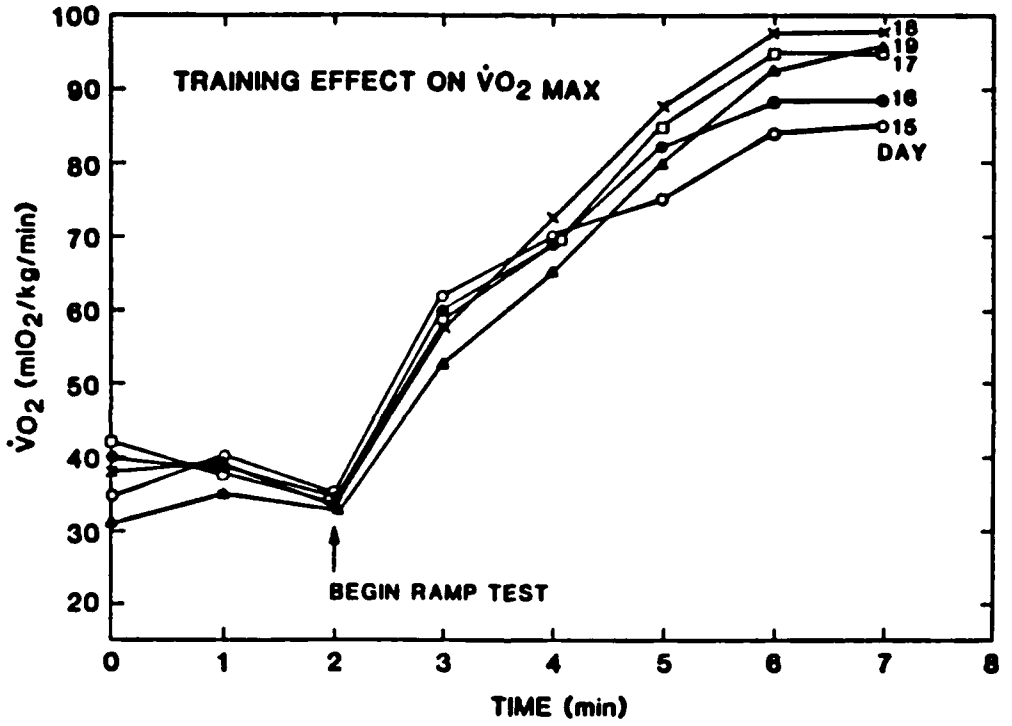


Figure 3: Maximum oxygen consumption by rats during the last several days of the 19 day training protocol.

It should be noted that one animal exposed to 100 ppm $NO_2$ for 15 min died within 1 hr after the post-exposure "ramp" test, whereas no animals died after an identical exposure followed by rest. Also, one animal exposed to 100 ppm $NO_2$ was incapable of completing the "ramp" test at the 8 hr time point. Data from this animal has been excluded from the above table. Even though most animals were capable of completing the "ramp" test protocol, significant reductions in $V0_{2max}$ were measured in rats after being exposed to greater than 25 ppm $NO_2$ relative to their pre-exposure control values obtained at the end of training period prior to the $NO_2$ exposures, Table 2. Reductions in $VO_{2max}$ were noted at 8 hrs post-exposure with animals exposed to 25 ppm $NO_2$ , and immediately and at 8 hrs post-exposure with animals exposed to 50 and 100 ppm $NO_2$.

## WORK PERFORMANCE EVALUATION

**Maximum Oxygen Consumption (ml $O_2$/Kg $min^{-1}$)**

| | Pre Exposure | Immediately Post Exposure | 8 Hours Post Exposure |
|---|---|---|---|
| SHAM AIR | 99.5 ± 3.8 (6) | 107.0 ± 7.2 (6) | 106.0 ± 7.7 (6) |
| 10 ppm $NO_2$ | 101.0 ± 2.3 (6) | 100.1 ± 2.7 (6) | 100.1 ± 1.3 (6) |
| 25 ppm $NO_2$ | 98.2 ± 5.6 (6) | 94.6 ± 8.3 (5) | 92.0 ± 5.0 (6)* |
| 50 ppm $NO_2$ | 94.5 ± 2.4 (6) | 81.6 ± 3.9 (6)* | 78.3 ± 8.5 (6)* |
| 100 ppm $NO_2$ | 102.0 ± 5.4 (6) | 88.5 ± 6.7 (6)* | 69.4 ± 19.0 (5)* |

Table 2: Maximum oxygen consumption of rats before, immediately, and 8 hrs after 15 min exposures to air or the various concentrations of $NO_2$, $p < 0.05$.

*Work Performance After Exposure to >100 ppm $NO_2$:* In a more recently performed series of studies, rats were exposed to higher concentrations of $NO_2$ and exercised immediately after the exposures for the work performance studies. Most of these exposures were very brief, "high burst" exposures ranging from 1-3 min in duration. The results from these studies are summarized in Figures 4 and 5.

Relative to the air-exposed control condition, 1 min exposures to $NO_2$ at concentrations of 500-2000 ppm resulted in significant reductions in work performance capacity, Figure 4. Such reductions in $VO_{2max}$ generally scaled with exposure concentration. Figure 5 summarized $VO_{2max}$ values obtained immediately after 2-15 min exposures to 200-1000 ppm $NO_2$. Comparisons of the these data with corresponding exposure concentration data in Figure 4 generally indicate that even short extensions of the 1 min exposures times to a given concentration of $NO_2$ can result in more marked reductions in work performance capacity. Comparisons of the 200 ppm $NO_2$ data in Figure 4 with the 100 ppm $NO_2$ data shown in Table 2 serves to illustrate that when these two concentrations are inhaled over a more extended period of time, i.e., 15 min, greater reductions in $VO_{2max}$ follow exposure to the higher of the two concentrations.

## DISCUSSION

### *Work Performance After Exposure to the 10-100 ppm Concentrations of $NO_2$*

With the exception of two animals that were exposed to 100 ppm $NO_2$ for 15 min, all of the other rats exposed to the $NO_2$ atmospheres used in this study were able to complete the "ramp" work performance test administered immediately and 8 hrs after the exposures. Moreover, those animals that did successfully complete the two "ramp" tests attained their maximum oxygen consumption ($VO_{2max}$) values at virtually the same times during the tests, regardless of their $NO_2$-exposure histories. Yet, the acute inhalation of $\geq$25 ppm $NO_2$ did result in demonstrable reductions in $VO_{2max}$, with increasing reductions occurring with increasing concentrations of inhaled $NO_2$. Such observations were particulary apparent as of the 8 hr post-exposure times when decreases in $VO_{2max}$ were most pronounced. The greater compromises seen during the 8 hr post-exposure exercise bout are not totally unexpected given the kinetics of expression of $NO_2$-induced lung injury (Lehnert et al., 1993; Stavert and Lehnert, 1989). By this time, the appearance of fibrin in the alveoli, at least, is maximally or

## Effects of High Concentrations of $NO_2$ on Work Performance

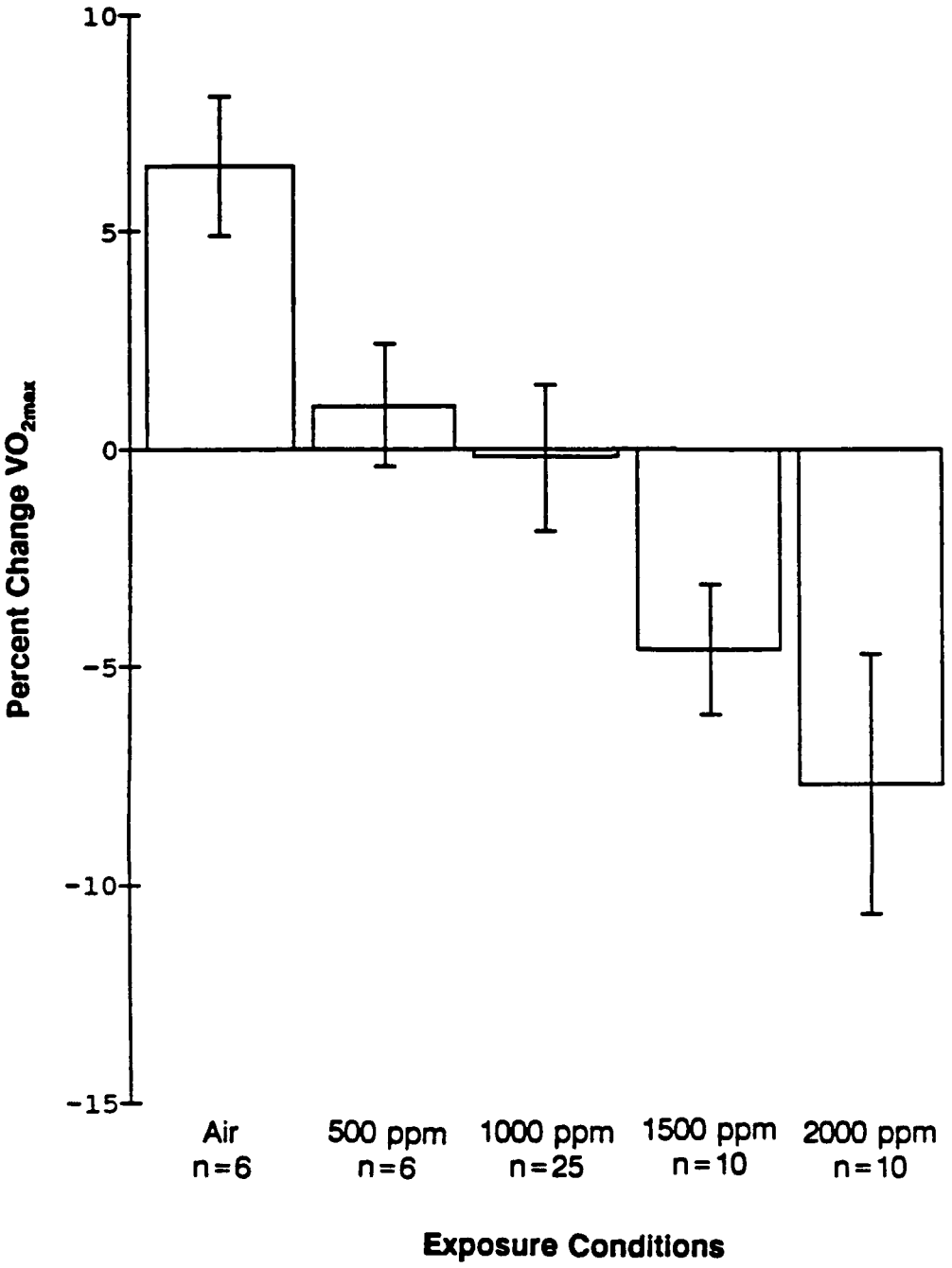


**Figure** 4 Percent change in maximum oxygen consumption ($VO_{2max}$) values representing reductions in work performance immediately following one minute exposures to various concentrations of $NO_2$. Values for all groups exposed to $NO_2$ were significantly lower than values of the air-exposed group, $p \leq 0.05$.

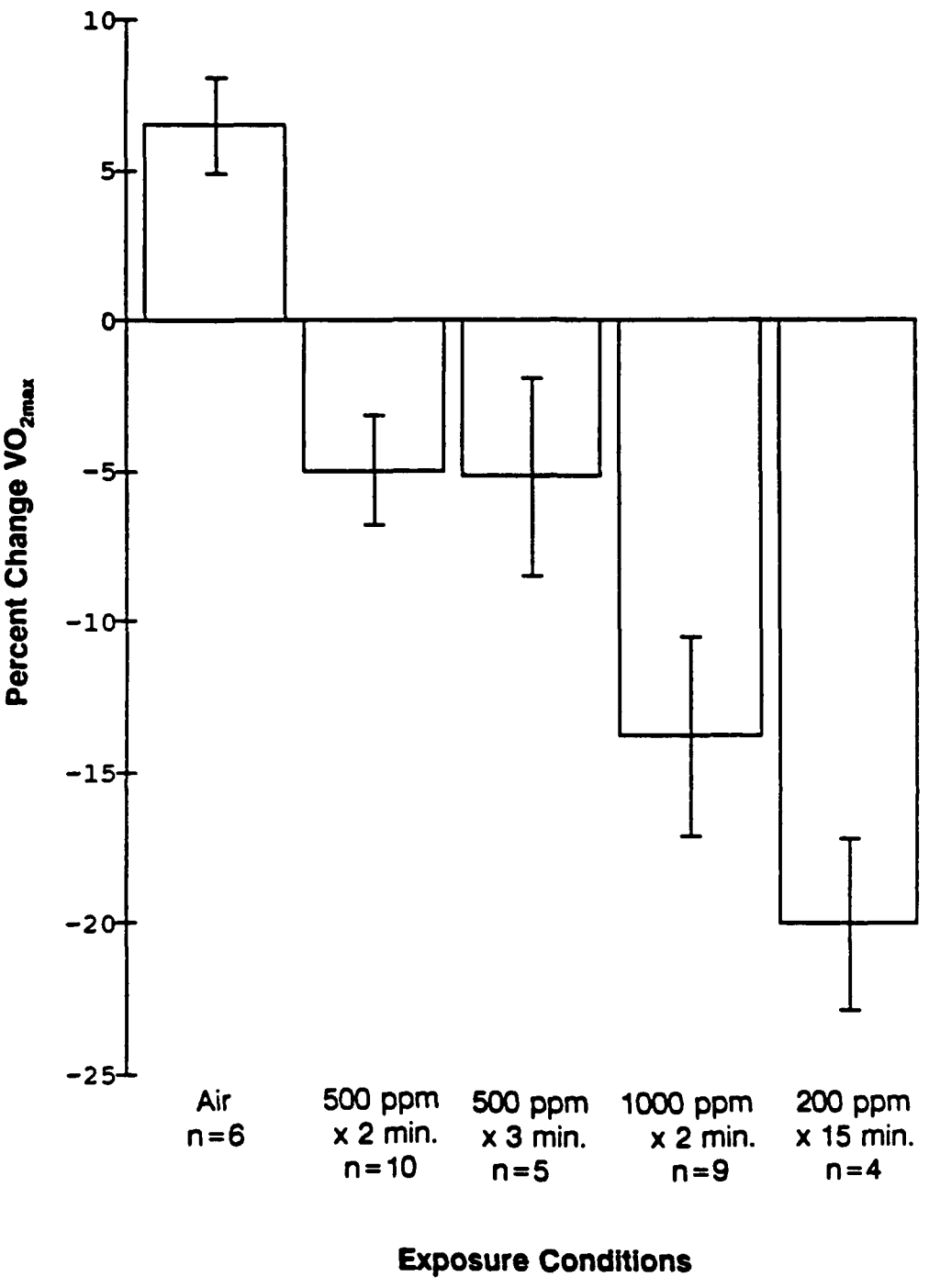


**Figure** 5 Percent change in maximum oxygen consumption ($VO_{2max}$) values representing reductions in work performance immediately following exposures to various concentrations of $NO_2$. Values for all groups exposed to $NO_2$ were significantly lower than values of the air-exposed group, $p \leq 0.05$.

near maximally expressed (Lehnert et al., 1993; Stavert and Lehnert, 1989). On the other hand, it remains possible that a potentiation in the expression of the severity of lung injury induced by the $NO_2$ exposures occurred as a result of the exercise involved in performing the "ramp" test immediately after the $NO_2$ exposures. Regardless, the reductions in $VO_{2max}$ values following the $NO_2$ exposures suggest that endurance can be significantly affected by acute $NO_2$ exposures like those used in the above study component.

*Work Performance After Brief Exposures to Higher Concentrations of $NO_2$*

The results from our studies in which rats were briefly exposed to high mass concentrations of $NO_2$ demonstrate that exposure durations of a little as 1 min can result in significant compromises in work performance capacity when exercise is performed immediately after exposure. However, for the sake of balance, we do note that in all of the above studies, work performance capacity was never reduced by more than ~20% as of the post-exposure times when $VO_{2max}$ was assessed.

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# SECTION D

## Objective 8: To examine the toxicological effects of NO in the lung.

## Objective 9: To obtain information on the relative pulmonary toxicities of NO and $NO_2$ when inhaled at relatively high mass concentrations.

**Results:** The results of our studies indicate that NO does not cause significant acute lung injury in the rat when inhaled at very high mass concentrations for durations up to 30 min. The toxicity of NO in the lung has been found to be at least 30 times less than that found after inhalation of $NO_2$.

**Reports:**

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*Lehnert, B.E.: Inhalation toxicology of nitric oxide and nitrogen dioxide. In: Handbook of Hazardous Materials. Academic Press, Inc. (In Press), 1992.*

# INTRODUCTION

Nitric oxide (NO) is the major oxide of nitrogen formed during a variety of high-temperature combustion processes and, accordingly, this gas represents a potentially hazardous air contaminant in settings where such processes occur. Although the pulmonary toxicity of NO is thought to be less than that of nitrogen dioxide ($NO_2$), little experimental information as to the toxicity of NO in the respiratory tract, or the relative pulmonary toxicities of inhaled NO and $NO_2$, is currently available. In most of the few studies in which the toxicity of NO has been evaluated or compared to inhaled $NO_2$, the actual compositions of the exposure atmospheres in terms of relative purity for a given nitrogen oxide species have been questionable, as have some of the experimental approaches used to assess the severity of resulting injury. Holt et al. (1979), for example, exposed mice for 2 h/d, 5 d/wk, to NO or $NO_2$ at nominal concentrations of 10 ppm for up to 30 wk. Exposures to either gas resulted in similar effects on leukocytosis, increased humoral immune responses to sheep red blood cells, decreased phytohemagglutinin responses with spleen cells, and decreases in tumor rejection. In that study, NO appeared to produce more advanced emphysematous changes in the lungs than those observed in the $NO_2$-exposed lungs. Such conclusions were subjectively drawn and not formulated from quantitative histologic analyses. Additionally, the actual concentrations of NO and $NO_2$ in the exposure system were not determined; gas turnover times in the animal exposure chamber used suggest the residency time of NO-containing atmospheres in the chamber would allow at least partial conversion of NO to $NO_2$ during exposures of the mice to the former. In another study, Hugod (1979a) examined the lungs of rabbits that were continuously exposed to 5 ppm NO for 14 d. Hugod reported the appearance of endothelial cell vacuolization as a result of the exposure. However, this abnormality, which may have been related to less than optimal tissue fixation, was also observed in lung samples from clean-air-exposed animals.

In a less recent series of studies (Greenbaum et al., 1967; Toothill, 1967; Shiel, 1967), dogs that were exposed to nominal nitric oxide at $6.2 \times 10^3$ mg/m$^3$ (5000 ppm) for 24 min or more succumbed to pulmonary edema after exposure. With shorter exposures, the dogs survived but exhibited respiratory distress. Physiologic disturbances attributed to the NO exposures included respiratory acidosis, decreased lung compliance, increased airway resistance, diminished cardiac output, and a marked reduction in heart rate. A major biochemical abnormality was the timerelated formation of methemoglobin. Given the known pathologic features of $NO_2$ toxicity (Evans et al., 1977; Evans and Freeman, 1980;

Rombout et al., 1986) and the observations made with these NO-exposed dogs (i.e., pulmonary edema, hyperinflation, hemorrhage, desquamation of mucosa, and bronchopneumonia), as well as the likely contamination of the NO atmospheres with nitrogen dioxide, it has been suggested (Committee on Medical and Biological Effects of Environmental Pollutants; 1977) that some, if not all, of the pathologic disturbances in the above dog study were caused by $NO_2$ and not NO. It should be pointed out that although NO has undoubtedly been present in instances of human poisoning due to oxides of nitrogen, poisoning from NO alone in breathed air has never been reported (Stokinger and Coffin, 1968).

The objectives of this component of the project were to (1) examine the acute pulmonary toxicologic effects of inhaling NO and (2) obtain information on the relative toxicologic effects of NO and $NO_2$ when breathed at relatively high mass concentrations during brief exposure periods. The first objective was undertaken to model exposure scenarios in which humans may encounter high concentrations of NO for brief durations. Inasmuch as typical NO-containing atmospheres also contain $NO_2$, the second objective was undertaken as one means to distinguish NO effects from those effects caused by the acute inhalation of $NO_2$ only. In order to achieve these objectives, we utilized an exposure system and exposure procedures that provided a means to expose rats to high concentrations of NO while minimizing background contaminations of the exposure atmospheres with $NO_2$.

## MATERIALS AND METHODS

*Animals:* Adult, male Fischer 344 rats (specific-pathogen free, virus-free, Harlan Sprague Dawley, Indianapolis, Ind.) were delivered to Los Alamos National Laboratory and acclimated in a facility accredited by the American Association of Laboratory Animal Care for a minimum of 10 d prior to experimental use. During this period and following the test gas exposures, the rats were housed in rooms that received HEPA-filtered air. The animals were maintained in suspension cages (two per cage) covered with spun polyester filters (DuPont no. 22 spinbound polyester filter, E. I. DuPont Co., Wilmington, Del.). Sterilized rat food [Teklad mouse/rat food (L-485), Harlan Sprague Dawley Inc., Madison, Wis.] and water were provided ad libitum. Serum from several representative animals were obtained upon arrival, as well as from animals maintained in the animal facility over the course of each exposure study, for serologic analyses (Microbiological Associates, Inc., Bethesda, Md.). Consistently, all animals were found to be negative for reovirus Type 3, Kilham's rat virus, pneumonia virus of mice, Sendai virus, Mycoplasma pulmonis, rat

coronavirus, sialodacryoadenitus virus, and cilia-associated respiratory bacillus. At the time of the actual exposures, all the rats weighed between 220 and 270 g with the vast majority of the animals weighing 240-260 g. Test gas-exposed groups of rats and their corresponding controls (sham-air exposed animals) were matched according to age and body weight at the time of the exposures.

*Experimental Design:* NO Studies In the initial exposure studies, groups of rats were exposed to 500, 1000, or 1500 ppm NO for a duration of 5 min. The animals were returned to their cages and sacrificed 24 h later for lung studies; previous studies in our laboratory have revealed that inflammatory responses following acute inhalations of $NO_2$, at least, are well developed as of 24 h after the exposures (Lehnert et al., 1987). Corresponding control groups of animals consisted of rats that were exposed to anhydrous filtered air only for a period of 5 min. Members of the sham-exposed groups were also sacrificed 24 h after the air-only exposures. In a subsequent series of studies, groups of rats were exposed to 1500 ppm NO for 15 min or 1000 ppm NO for 30 min periods in order to determine whether more prolonged breathing of this oxide of nitrogen results in more pronounced lung alterations. Sham-exposed rats in these studies were correspondingly exposed to anhydrous filtered air only, or anhydrous filtered air passed through soda lime, for durations of 15 or 30 min; the latter control groups were used in studies in which soda lime was added to the exposure system to reduce background $NO_2$ contaminations during the NO exposures (to be discussed).

*$NO_2$ Studies:* Groups of rats were exposed to 10, 25, 50, or 100 ppm $NO_2$ for durations of 5, 15, or 30 min. Corresponding control groups of animals consisted of rats that were exposed to anhydrous filtered air only for a period of 30 min. Following the $NO_2$ or air exposures, the animals were sacrificed 24 h later for lung gravimetric and histopathologic analyses. Results obtained following a particular $NO_2$ exposure regimen were directly compared with data obtained with sham air-treated control animals.

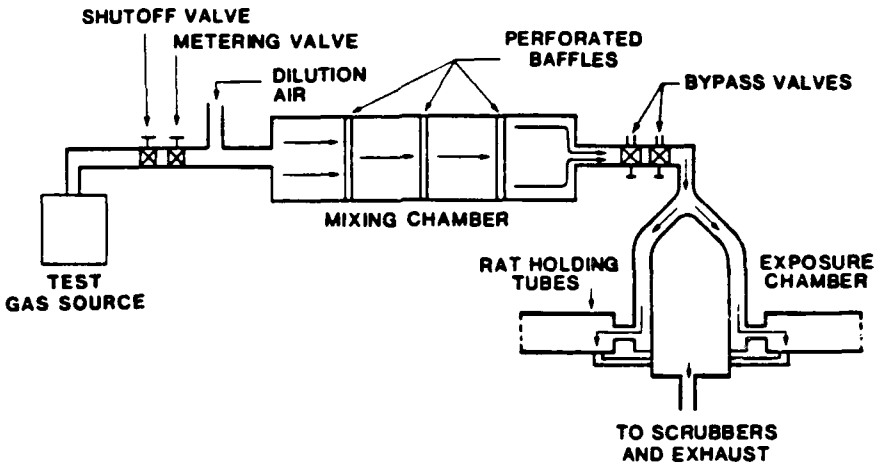
*Exposure Methods:* NO Exposures Pure nitric oxide (Matheson Gas Co., LaPorte, Tex.) was diluted with anhydrous HEPA-filtered air to produce the NO exposure atmospheres. The NO concentrations were regulated by adjustment of a needle valve that controlled the amount of concentrated NO added to a stream of anhydrous, filtered air directed into the exposure system. The NO was added to the airstream immediately before entering a mixing chamber, and delivered to the experimental animals contained in nonavoidance, nonconstraining exposure tubes attached to a radial, quartz glass exposure

chamber (Figures. 1a-c); the design, construction, and use of the exposure system have been described elsewhere (Wilson et al., 1987; Stavert and Lehnert, 1989). High flow rates (60 L/min) were used in order to reduce the residency time of the exposure atmospheres in the chamber system and thereby minimize the time for conversion of NO to $NO_2$. The mixing chamber was partially filled with soda lime (Baker Analyzed Reagent, 4-8 mesh size, J. T. Baker Chemical Co., Phillipsburg, N.J.) during all the exposures to 1500 ppm NO and in some 1000 ppm NO exposures in order to further reduce contaminating $NO_2$ (Oda et al., 1975); see Table 1. The ability of soda lime to reduce background $NO_2$ in the exposure system during the delivery of NO is illustrated in Figure. 2.

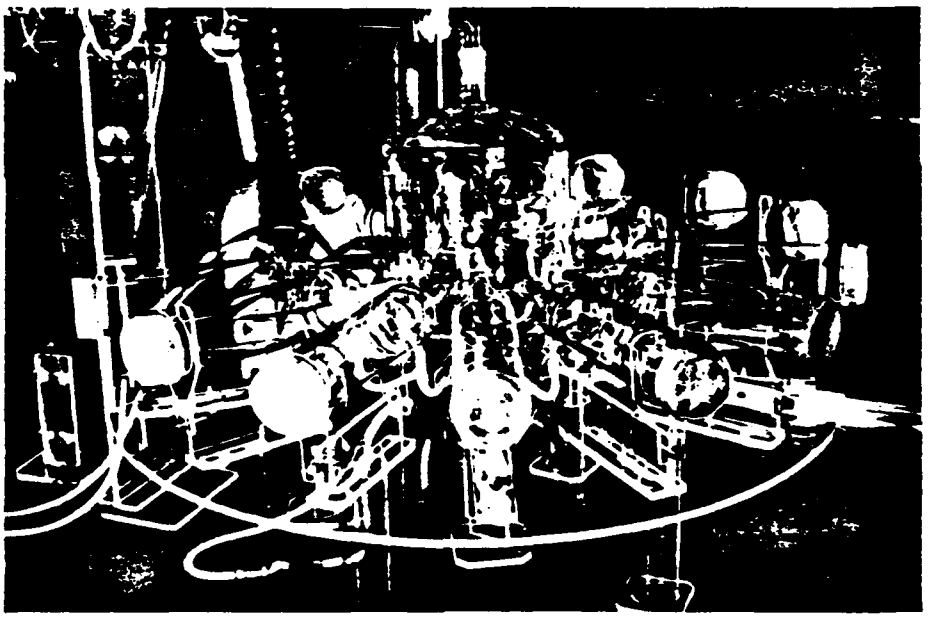
Exposure atmospheric concentrations of NO and $NO_2$ were continuously monitored with a dual-channel IR-UV spectrophotometer (Binos Inficon, Leyhold-Heraeus, Federal Republic of Germany) that was calibrated daily with primary gas standards (Matheson Gas Co., La Porte, Tex.); the primary gas standards were verified using Fourier transform infrared analyses and checked for impurities with mass spectrometry. Measured equilibration time ($t_{99}$) for the nominal concentrations of NO in the exposure system were ~34 s. The various NO exposure regimens and protocols, including sham-exposure conditions, are detailed in Table 1.

*$NO_2$ Exposures:* Nitrogen dioxide was generated from dinitrogen tetroxide ($N_2O_4$) (Freeman and Haydon, 1964; Stavert et al., 1986) contained within a stainless steel chamber and maintained at 24°C by a circulating water bath. The $NO_2$ was added to a stream of anhydrous and HEPAfiltered air and fed into a mixing chamber (see Figure 1a); $NO_2$ concentrations were regulated with a needle valve that controlled the amount of concentrated $NO_2$ added to the airstream entering the mixing chamber. From the mixing chamber, airflow was directed to the exposure chamber-animal holding tube assembly (Figure 1b) at a flow rate of 12 L/min. Exposure atmosphere concentrations were continuously monitored with the dual-channel IR-UV spectrophotometer calibrated with primary gas standards. As with the NO exposures, the $t_{99}$ equilibration values for the exposure concentrations of 10-100 ppm $NO_2$ in the chamber were ~34 s. Nominal gas concentrations within the chamber also have been found to be stable over the course of prolonged exposure periods (Table 2). Nitric oxide (NO) concentrations generated during the exposures were always less than 30 ppm (Figure 3).

*Animal Sacrifices, Lung Gravimetric Measurements, and Tissue Processing:* Rat sacrifices were initiated by intraperitoneal injections of 50 mg pentobarbital sodium.

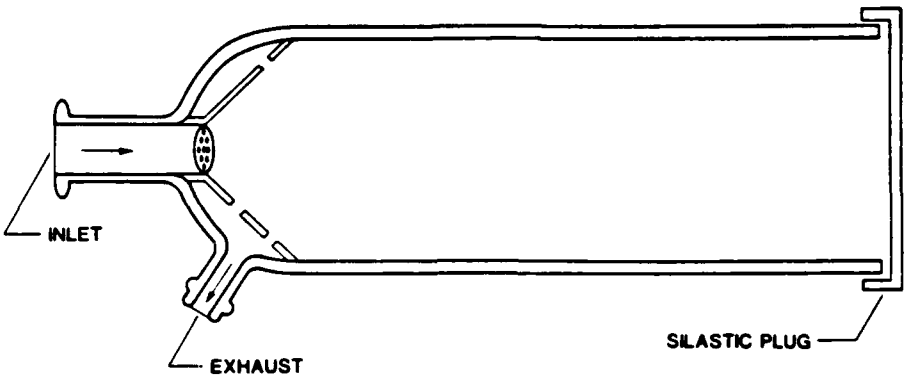



(*a*)



(*b*)

**FIGURE 1.** (*a*) Schematic representation of the gas delivery system and associated components used to administer the test atmosphere. (*b*) Quartz glass, 12-port radial exposure system used to deliver the test atmospheres to the experimental animals.




(c)

**FIGURE 1** (*Continued*). (c) Cross-sectional diagram of a nonavoidance, nonconstraining animal exposure tube. The tubes have been designed to provide continual passage of an exposure atmosphere over the facial region of a rat.

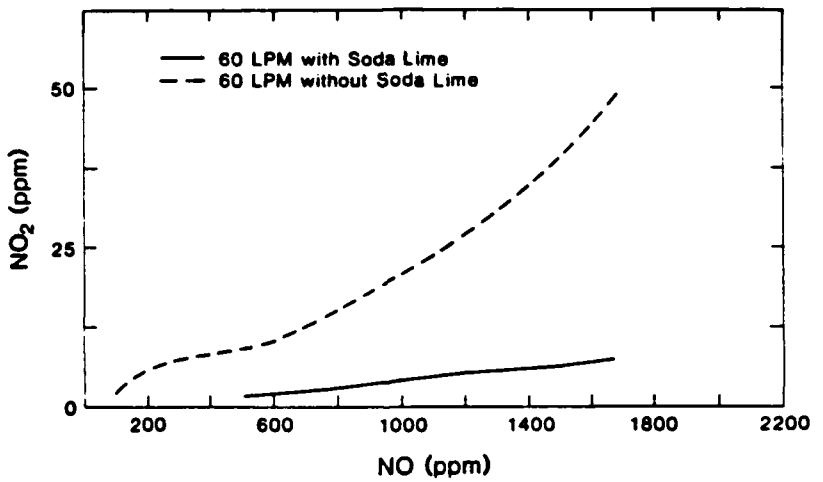



**FIGURE 2.** Diminution of contaminating $NO_2$ in the NO atmospheres by adding soda lime to the mixing chamber of the exposure system.

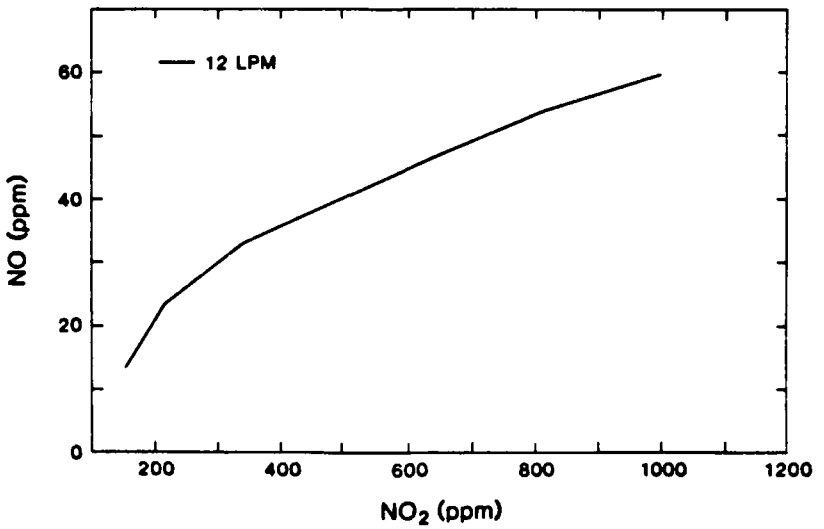



**FIGURE 3.** Background NO contamination in the exposure chamber as a function of $NO_2$ concentration delivered to the experimental animals.

**TABLE 1.** NO Exposure Regimens and Protocols

| Nominal exposure atmospheres | Exposure duration | Measured NO concentration (ppm)[a] | Measured $NO_2$ concentration (ppm)[a] | Soda lime |
|---|---|---|---|---|
| Air (sham controls) | 5 min | — | — | Not used |
| Air (sham controls) | 5 min | — | — | Used |
| 500 ppm NO | 5 min | 500 ± 5 | 4 ± 1 | Not used |
| 1000 ppm NO | 5 min | 1000 ± 5 | 23 ± 1 | Not used |
| 1500 ppm NO | 5 min | 1500 ± 10 | 17 ± 1 | Used |
| 1500 ppm NO | 15 min | 1500 ± 10 | 17 ± 1 | Used[b] |
| 1000 ppm NO | 30 min | 1000 ± 3 | 11 ± 1 | Used[b] |
| Air (sham control) | 15 min | — | — | Used[c] |
| Air (sham control) | 30 min | — | — | Used[c] |

[a]Values represent mean ± SD of multiple measurements made during the exposures.
[b]A greater mass of soda lime was used in this exposure in order to decrease the contaminating concentration of $NO_2$ below 25 ppm.
[c]The same mass of soda lime was used in this air exposure study as that used for the 30 min, 1000 ppm NO exposure.

**TABLE 2.** $NO_2$ Exposure Concentrations

| Nominal $NO_2$ concentration (ppm) | Measured $NO_2$ concentration (ppm) | Concentration range (ppm) |
|---|---|---|
| 10 | 10 ± 0.5 | 9.8–10.2 |
| 25 | 26 ± 1 | 23–27 |
| 50 | 49 ± 2 | 47–52 |
| 100 | 100 ± 3 | 97–104 |

Note. Values represent means ± SD of multiple measurements made during the exposures.

Upon complete apnea, the thoracic cages were opened and each animal was exsanguinated via cardiac puncture. The trachea and lungs were excised, and the heart, extrapulmonary mediastinal tissue, and esophagus were removed. The lungs were then blotted dry. The bronchus leading to the right cranial lobe (RCL) was ligated with fine suture and the RCL was removed. The remaining lung preparation and the RCL were then collectively weighed (lung wet weight, LWW). The trachea and lungs, minus the RCL, was cannulated with an 18-gauge needle secured with ligature and the lungs were infused and fixed at a constant pressure of 30 cm $H_2O$ with 10% formalin in phosphate-buffered saline for 48 h. The RCL were oven dried to a constant weight at 35°C for 36 h and reweighed (right cranial lobe dry weight, RCLDW); the LWW and RCLDW were used as end points for detecting $NO_2$-induced pulmonary edema (Cross et al., 1982; Stavert and Lehnert, 1989).
For the histologic analyses, the left lungs were sliced on the same plane as the main-stem bronchi from the apex to the base along a line between the most posterior to the most anterior aspects to expose the maximal planar surface area for sectioning (Stavert et al., 1986). The blocks of tissue were embedded in paraffin, and 4 μm sections were prepared and stained with hematoxylin and eosin by conventional methods.

*Histopathology:* Semiiquantitative histopathologic assessments focused on changes in the periterminal bronchiolar-alveolar duct-alveolar region of the lung (Stavert and Lehnert, 1989). Hallmarks of injury in this region that are detectable by light microscopy include the appearance of fibrin in the alveolar spaces, accumulations of polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM) in the alveolar spaces, the extravasation of erythrocytes into the alveolar space compartment, and hyperplasia of type II pneumocytes (Evans and Freeman, 1980; Rombout et al., 1986; Warnock, 1982; Kradin et al., 1986). A grading scale was used to describe quantitatively the relative severity of each of the above pathologic features in terms of intensity of expression as well as their distribution in the lung. The intensity index scale for a given feature, such as fibrin accumulation, ranged from 0 to 4 with 0 = not detected; 1 = trace to mild in appearance, only a few proximal alveoli contained small amounts of fibrin; 2 = present in moderate degree, several proximal alveoli associated with a given terminal bronchiole contained fibrin; 3 = present in high degree, most proximal and some distal alveoli associated with a given terminal bronchiole contained fibrin; and 4 = present in high intensity, essentially all alveoli associated with a given terminal bronchiole contained fibrin. A distribution index was used to quantitate the relative numbers of periterminal bronchiolar-alveolar structures showing an abnormality. The distribution index for a given pathologic

feature ranged from 0 to 3 with 0 = not present, 1 = focal, 2 = multifocal in occurrence, and 3 = diffuse in occurrence, that is, present in virtually all alveolar duct and proximal alveolar structures.

*Statistical Analyses:* The lung gravimetric data were analyzed by a two-tailed t test for unpaired data (Snedecor and Cochran, 1969). Probability (p) values less than .05 were considered significant.

## RESULTS

### NO Exposures

*Lung Gravimetric Analyses:* The acute, 5 min exposures to 500, 1000, or 1500 ppm NO produced no measurable increases in the LWW or the RCLDW of the exposed animals as of 24 h postexposure (Table 3). Likewise, more prolonged exposures to NO produced no demonstrable increases in these gravimetric parameters (Table 3). Accordingly, the wet and dry lung tissue gravimetric data collectively indicate that the NO exposures did not result in significant permeability changes in the lung, that is, no evidence of pulmonary edema.

*Histopathology:* No evidence of pathologic alterations was observed in the lungs of experimental animals acutely exposed to the various NO concentrations for a duration of 5 min. The inhalation of high concentrations of NO for 15 or 30 min durations also did not result in any detectable lung histopathologic disturbances in the periterminal bronchiolaralveolar duct-alveolar region. Subsequent electron microscopic assessments of this region after NO exposure (1000 ppm x 15 min) confirmed the absence of injury, for example, no evidence of cellular damage or fibrin extravasation was observed, as determined by light microscopy. In addition to these findings, no pathologic alterations were observed along the epithelial linings of the conducting airways, including the terminal bronchioles.

*Other Observations:* By the end of the 30 min exposure to 1000 ppm NO, the exposed rats were observably cyanotic. Eleven of 20 animals exposed at this concentration and time died within 1/2 h after the end of the exposure. This outcome was most likely due to the formation fo methemoglobin (to be discussed).

## $NO_2$ Exposures

*Lung Gravimetric Analyses:* Acute exposures of the rats to 10 ppm $NO_2$ for a duration of 30 min or to 25-50 ppm $NO_2$ for periods of up to 15 min produced no significant increases in LWW or RCLDW. Significant increases in these parameters were found, however, following exposure of the animals to 50 ppm $NO_2$ for 30 min and after exposures to 100 ppm $NO_2$ for periods of 5-15 min (Table 4). Such increases generally paralleled increasing exposure concentrations of the $NO_2$ and prolongation of exposure times.

*Histopathology:* Histologic disturbances caused by the various $NO_2$ exposure regimens are summarized in Table 5. No evidence of lung injury was observed following exposures of the rats to 10 ppm $NO_2$ for 30 min, or after the exposures to 25 ppm $NO_2$ for durations of 5 and 15 min. Detectable lung changes were observed after the animals breathed 25 ppm $NO_2$ for 30 min and following exposure to 50 ppm for only 5 min. Generally, the severity of $NO_2$-induced lung injury, expressed as the accumulation of fibrin, PMN, AM, extravasated erythrocytes, and type ll pneumocyte hyperplasia as of 24 h postexposure, became more pronounced with increasing $NO_2$ exposure concentrations and times.

## DISCUSSION

The results of this project component indicate that NO does not cause significant acute lung injury in the rat when inhaled at very high mass concentrations for durations up to 30 min. Whereas histologic disturbances were evident 24 h after rats were exposed to 50 ppm $NO_2$ for 15 min, exposure of rats to 1500 ppm NO for this same duration produced no such lesions. Additionally, exposures of rats to 25 ppm $NO_2$ for 30 min resulted in mild but demonstrable lung injury, while 30 min exposures to 1000 ppm NO, like the 30 min exposures to 10 ppm $NO_2$, caused no detectable injury by histologic or lung gravimetric criteria. Thus, it appears that the toxicity of NO in the lung is at least 30 times or so less than that of $NO_2$; it remains possible that NO per se is innocuous in the lung. A conclusion that NO has little to no toxicity in the lung is consistent with the light and electron microscopic study of Hugod (1979b) in which no pathologic disturbances were found in the lungs of rabbits continuously exposed to 40 ppm NO for 6 d. Although various lines of evidence suggest inhaled NO readily reaches deeper regions of the lung (Wagner, 1970; Yoshida and Kasama, 1987), and at least partial conversion of the NO to some level of $NO_2$ might be expected there, our results also suggest that $NO_2$

**TABLE 3.** Lung Gravimetric Values Obtained 24 Hours after the NO Exposures

| Exposure condition | Lung wet weights (g) | Right cranial lobe dry weights (g) | No. rats/group |
|---|---|---|---|
| Sham control (filtered air, 5 min) | 1.170 ± 0.066 | 0.024 ± 0.002 | 12 |
| Sham control (filtered air, 5 min; soda lime used) | 1.172 ± 0.065 | 0.024 ± 0.002 | 12 |
| 500 ppm NO, 5 min | 1.190 ± 0.054 | 0.024 ± 0.003 | 12 |
| 1000 ppm NO, 5 min | 1.238 ± 0.069 | 0.025 ± 0.002 | 12 |
| 1500 ppm NO, 5 min | 1.194 ± 0.064 | 0.025 ± 0.002 | 12 |
| Sham control (filtered air, 30 min) | 1.179 ± 0.037 | 0.024 ± 0.001 | 12 |
| 1000 ppm NO, 30 min | 1.176 ± 0.065 | 0.023 ± 0.002 | 9 |
| Sham control (filtered air, 15 min; soda lime used) | 1.175 ± 0.055 | 0.023 ± 0.002 | 12 |
| 1500 ppm NO, 15 min | 1.139 ± 0.034 | 0.023 ± 0.002 | 12 |

Note. Each of the values represents the mean ± SD. None of the above values obtained from the various NO exposed groups of animals are significantly different from those obtained from their *corresponding sham control counterparts.*

**TABLE 4.** Lung Gravimetric Values Obtained 24 Hours after the $NO_2$ Exposures[a]

| *Exposure* condition | *Lung wet* weights (g) | Right cranial lobe dry weights (g) | No. rats/group |
|---|---|---|---|
| 10 ppm $NO_2$, 30 min | 1.215 ± 0.004 | 0.023 ± 0.001 | 6 |
| Sham control | 1.180 ± 0.037 | 0.024 ± 0.001 | 12 |
| 25 ppm, 5 min | 1.276 ± 0.082 | 0.024 ± 0.002 | 12 |
| Sham control | 1.252 ± 0.061 | 0.024 ± 0.001 | 10 |
| 25 ppm, 15 min | 1.218 ± 0.079 | 0.022 ± 0.002 | 12 |
| Sham control | 1.255 ± 0.054 | 0.024 ± 0.001 | 12 |
| 25 ppm, 30 min | 1.266 ± 0.101 | 0.023 ± 0.002 | 12 |
| Sham control | 1.230 ± 0.059 | 0.023 ± 0.002 | 12 |
| 50 ppm, 5 min | 1.321 ± 0.046 | 0.027 ± 0.002 | 11 |
| Sham control | 1.276 ± 0.202 | 0.026 ± 0.004 | 12 |
| 50 ppm, 15 min | 1.350 ± 0.066 | 0.026 ± 0.002 | 6 |
| Sham control | 1.260 ± 0.048 | 0.023 ± 0.002 | 12 |
| 50 ppm, 30 min | 1.450 ± 0.121[b] | 0.029 ± 0.005[b] | 6 |
| Sham control | 1.183 ± 0.108 | 0.023 ± 0.003 | 10 |
| 100 ppm, 5 min | 1.426 ± 0.141[b] | 0.027 ± 0.002 | 6 |
| Sham control | 1.223 ± 0.048 | 0.023 ± 0.003 | 10 |
| 100 ppm, 15 min | 1.523 ± 0.072[b] | 0.032 ± 0.003[b] | 10 |
| Sham control | 1.340 ± 0.136 | 0.026 ± 0.003 | 7 |

[a]Each of the values represents the mean ± SD.
[b]Significantly higher than corresponding control values, $p < .05$.

**TABLE 5. Periterminal Bronchiolar-Alveolar Duct-Alveolar Histopathology Following the $NO_2$ Exposures**

| $NO_2$ exposure conditions | Fibrin | Polymorphonuclear leukocytes | Alveolar macrophages | Extravasated erythrocytes | Type II cell hyperplasia |
|---|---|---|---|---|---|
| 10 ppm, 30 min | 0 | 0 | 0 | 0 | 0 |
| 25 ppm, 5 min | 0 | 0 | 0 | 0 | 0 |
| 25 ppm, 15 min | 0 | 0 | 0 | 0 | 0 |
| 25 ppm, 30 min | (0.8 ± 0.4) [1.5 ± 0.8]<br>(1.0) [2.0] | (1.3 ± 0.9) [1.7 ± 1.1]<br>(2.0) [2.0] | (0.9 ± 0.7) [1.7 ± 1.1]<br>(1.0) [2.0] | 0 | (0.6 ± 0.7) [1.0 ± 1.1]<br>(0.5) [0.5] |
| 50 ppm, 5 min | (0.5 ± 0.7) [0.8 ± 1.0]<br>(0) [0] | 0 | (0.5 ± 0.7) [0.8 ± 1.0]<br>(0) [0] | 0 | (0.4 ± 0.7) [0.4 ± 0.7]<br>(0) [0] |
| 50 ppm, 15 min | (1.6 ± 0.5) [2.6 ± 0.5]<br>(2.0) [3.0] | (1.2 ± 0.7) [2.0 ± 1.0]<br>(1.0) [2.0] | (1.0 ± 0.7) [1.6 ± 1.0]<br>(1.0) [2.0] | (0.4 ± 0.5) [0.7 ± 0.9]<br>(0) [0] | (0.2 ± 0.7) [0.3 ± 1.0]<br>(0) [0] |
| 50 ppm, 30 min | (1.1 ± 0.3) [2.5 ± 0.5]<br>(1.0) [3.0] | (1.1 ± 0.6) [2.2 ± 1.0]<br>(1.0) [2.0] | (0.8 ± 0.4) [1.4 ± 1.2]<br>(1.0) [2.0] | (0.6 ± 0.5) [1.0 ± 1.0]<br>(1.0) [1.0] | (0.1 ± 0.3) [0.2 ± 0.7]<br>(0) [0] |
| 100 ppm, 5 min | (0.8 ± 0.6) [1.7 ± 1.2]<br>(1.0) [2.0] | 0 | (0.6 ± 0.5) [1.5 ± 1.2]<br>(1.0) [2.0] | 0 | (0.5 ± 0.5) [1.0 ± 1.0]<br>(0) [0] |
| 100 ppm, 15 min | (1.0 ± 0.0) [3.0 ± 0.0]<br>(1.0) [3.0] | (0.6 ± 0.2) [0.9 ± 0.4]<br>(0.5) [0.5] | (1.4 ± 0.3) [2.0 ± 0.0]<br>(1.0) [2.0] | (1.0 ± 0.7) [2.3 ± 0.2]<br>(1.0) [2.0] | (2.2 ± 0.2) [1.8 ± 0.4]<br>(2.0) [2.0] |

Note. (Intensity): 0 = no detectable abnormality, 1 = trace to mild in appearance, 2 = moderate, 3 = severe, 4 = very severe. [Distribution]: 0 = not observed, 1 = focal in appearance, 2 = multifocal, 3 = diffuse. Underlined numbers represent median values; otherwise each of the above values represents the mean ± SD of values obtained with 6–12 animals per exposure condition.

concentrations that may have developed in the deep lung upon the inhalation of high concentrations of NO (e.g., 1000 ppm) were less than 25 ppm.

Although no detectable pulmonary toxicity following the inhalation of NO was observed in our studies, evidence for extrapulmonary NO toxicity was obtained. As indicated in the Results section, rats exposed to 1000 ppm NO for a 30 min duration became overtly cyanotic and some animals so exposed died within 30 min after the exposure. Although NO-induced nervous system disturbances cannot be ruled out (Pflesser, 1935) as a contributing component to the cyanosis and animal death (e.g., respiratory drive depression), it is likely that formation of methemoglobin (Oda et al., 1975; Toothill, 1967; Kon et al., 1977; Maeda et al., 1987) as a result of the NO exposure was the main contributing factor. Typically, rats that are exposed to 1000 ppm for ~30 min develop methemoglobin concentrations >65% (Ripple et al., 1989). Methemoglobinemia is a well recognized cause of anemic hypoxia (Kon et al., 1977; Smith, 1986).

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# SECTION E

## Objective 10: To characterize methemoglobin formation and dissociation kinetics in the rat during and following the inhalation of NO.

**Results:** The formation and elimination of methemoglobin (MetHb) during and after exposure to nitric oxide (NO) has been characterized under a variety of conditions. A model has been developed to relate NO exposure concentrations to the rate of MetHb formation in the rat. The model indicates that the rate of MetHb formation is proportional to NO exposure concentration. Additionally, the rate of MetHb formation accumulation most simply models as a product of minute ventilation and exposure concentration; increasing minute ventilation during inhalation of a given concentration of NO results in a proportional increase in the rate of MetHb formation. MetHb removal approximately follows first order kinetics but the removal rate is dependent upon the initial post-exposure MetHb saturation. MetHb removal is not dependent on minute ventilation.

**Reports:**

*Ripple, G. Mundie, T., Stavert, D.M., Lehnert, B.E.: Kinetics of methemoglobin formation and elimination as a function of inhaled nitric oxide concentration and minute ventilation. 28th Annual Meeting of the Society of Toxicology. Atlanta, Ga, February 27-March 3, 1989. The Toxicologist 9:A745, 1989.*

*Lehnert, B.E.: Physiological and biochemical endpoints in inhalation toxicology", International Programme on Chemical Safety, World Health Organization, Hannover, FRG, October 6-October 9, 1992.*

## INTRODUCTION

Nitric oxide (NO) is a major oxide of nitrogen that can be produced during combustion processes. While recent studies have indicated that the acute inhalation of even high concentrations of this nitrogen oxide species does not cause an inflammatory response in the lung like that produced by nitrogen dioxide (Stavert and Lehnert, 1990), one toxicologic effect of the inhalation of NO is the formation of methemoglobin (MetHb). This occurs following the binding of NO to hemoglobin to form nitrosylhemoglobin (Hb-NO) and the subsequent, rapid oxidation of ferrous Hb-NO in the presence of oxygen to form ferric MetHb (Maeda et al, 1987). The overall outcomes of these reactions are decreases in both the oxygen carrying and delivery capacities of the erythrocytes due to a reduction in the availability of normal Hb to bind $O_2$ and to a decrease in the dissociation of $O_2$ from Hb (Kon et al, 1977; Kon et al, 1980; Maeda et al, 1983). This physiologic impairment is brought about by the initial binding of NO, which binds to hemoglobin ~3 x $10^5$ times more strongly than does $O_2$.

Little experimental information is currently available as to the rate(s) at which MetHb accumulates relative to inhaled NO concentration and minute ventilation. Additionally, surprisingly little data presently exists as to the rate by which MetHb is removed from the blood following NO exposure. Using the Fischer-344 rat model, two objectives of the present study, were: 1) to investigate the kinetics of MetHb accumulation during the inhalation of NO, and 2) to examine the kinetics of MetHb elimination following cessation of exposure to NO. The resulting data base from these studies was then used to develop an initial kinetic model for MetHb formation and elimination as a function of NO exposure concentration and minute ventilation.

## MATERIALS and METHODS

*NO Exposures:* Pure nitric oxide (Matheson Gas Co., LaPorte, TX) was diluted with anhydrous HEPA-filtered air to produce the NO atmospheres. The NO concentrations were regulated by adjusting a needle valve that controlled the amount of concentrated NO that was added to a stream of anhydrous, filtered air directed into the exposure system. The NO was added to the airstream immediately before gas entry into a mixing chamber and subsequent delivery to animals contained in nonavoidance, nonconstraining exposure tubes attached to a radial, quartz glass exposure chamber. Details about the design, construction, and use of the exposure system used in this study are described in Sections A

and D of this report and they have have been described in several previous reports (Wilson et al, 1987; Stavert and Lehnert, 1989; Stavert and Lehnert, 1990).

*MetHb Kinetic Studies*: Adult Fischer 344 rats were anesthetized with ethrane and catheters were placed into their middle caudal arteries. Approximately 30 min after the animals recovered from the anesthteic agent, 20 µl of arterial blood was sampled from each resting animal and analyzed for % MetHb saturation (Radiometer OSM-3). The rats were then exposed to 1000 ppm NO for 23 min, or to 500 ppm NO for 15 min, or to 200 ppm NO for 120 min. Blood samples were taken and analyzed for % MetHb at various times during and after the NO exposures. The total amount of blood cumulatively taken from each animal during any experiment was < 0.4 ml, and usually < 0.3 ml.

*MetHb Kinetic During Increased Minute Ventilation (VE)*: Prior to NO exposure, rats were placed into partial body flow plethysmographs. Pressure fluctuations within the plethysmographs were conditioned and amplified and analyzed with a Buxco pulmonary mechanics computer. The rats were exposed to 500 ppm NO for 15 min or to 500 ppm NO in combination with 5% $CO_2$ for 15 min. Blood samples were collected during the exposures and up to 1 hr thereafter for % MetHb saturation measurements.

## RESULTS

*Patterns of MetHb Formation and Elimination*: The patterns of MetHb accumulation and elimination obtained during and after the exposures to 1000 ppm NO x 23 min and exposures to 200 ppm NO x 120 min are illustrated in Figures 1 and 2. The 1000 ppm NO exposure resulted in the formation of ~60% MetHb whereas ~25% MetHb saturation resulted from the 200 ppm NO exposure.

The % MetHb saturations of arterial blood collected during and after exposure to 500 ppm NO for 15 min or to 500 ppm NO in combination with 5% $CO_2$ are illustrated in Figure 3. The inhalation of $CO_2$ brought about an approximately 1.6-fold increase in minute ventilation ($V_E$), Figure 4, which was attributable to increases in both tidal volume and breathing frequency, Figures 5 and 6. Importantly, the addition of $CO_2$ to the exposure atmosphere resulted in an ~1.6-fold increase in the % MetHb formed during the 500 ppm NO exposure relative to that formed during normal $V_E$ conditions (~25% MetHb vs ~15% MetHb), Figure 3.

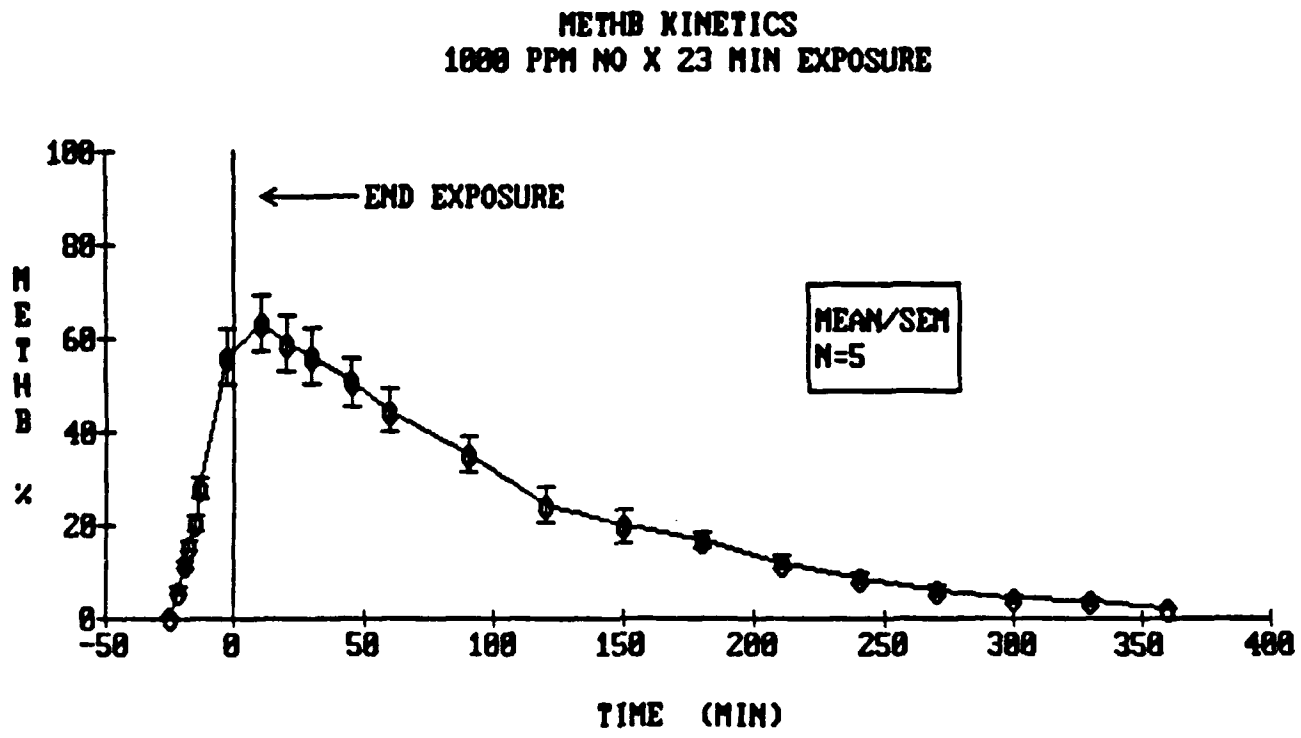



Figure 1: Methemoglobin saturation of arterial blood samples collected from rats during and after inhalation exposure to 1000 ppm NO for a 23 min period of time. Each point represents the mean and standard error of the mean for N = 5 animals.

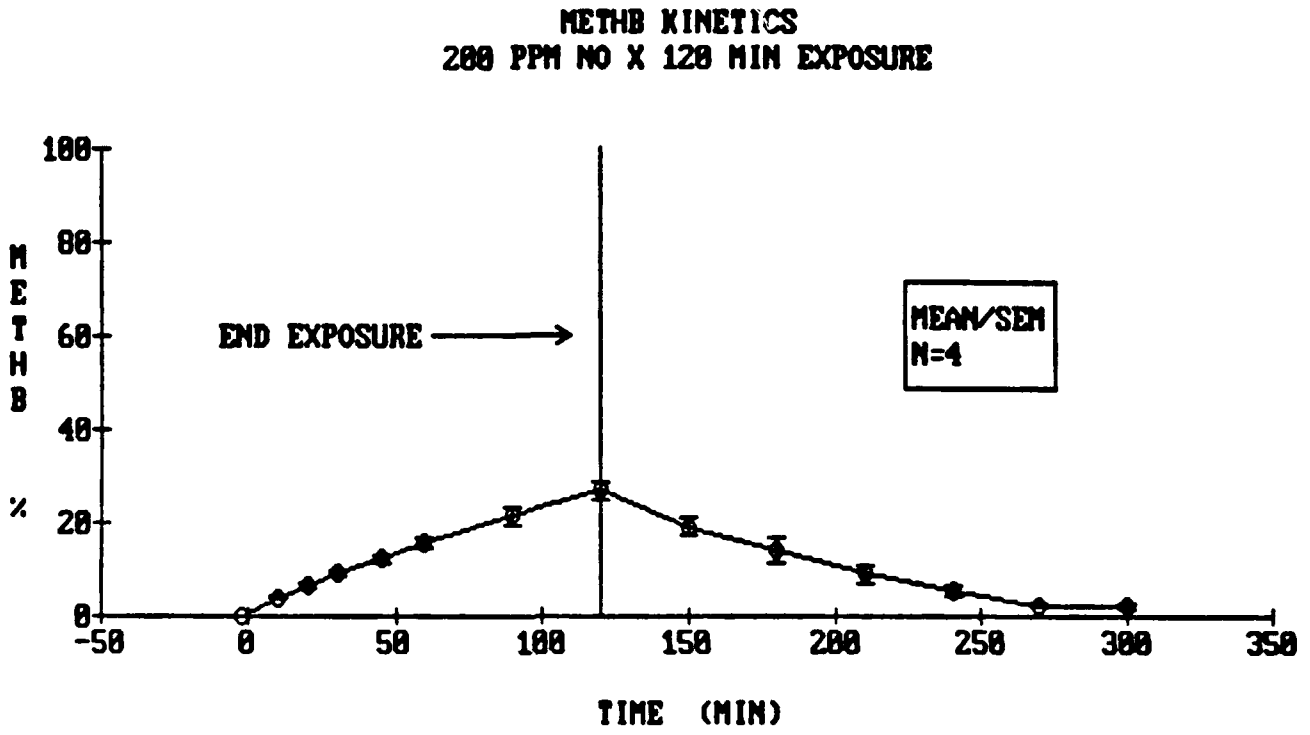



Figure 2: Methemoglobin saturation of arterial blood samples collected from rats during and after inhalation exposure to 200 ppm NO for a period of 120 min. Each point represents the mean and standard error of mean for N = 4 animals.

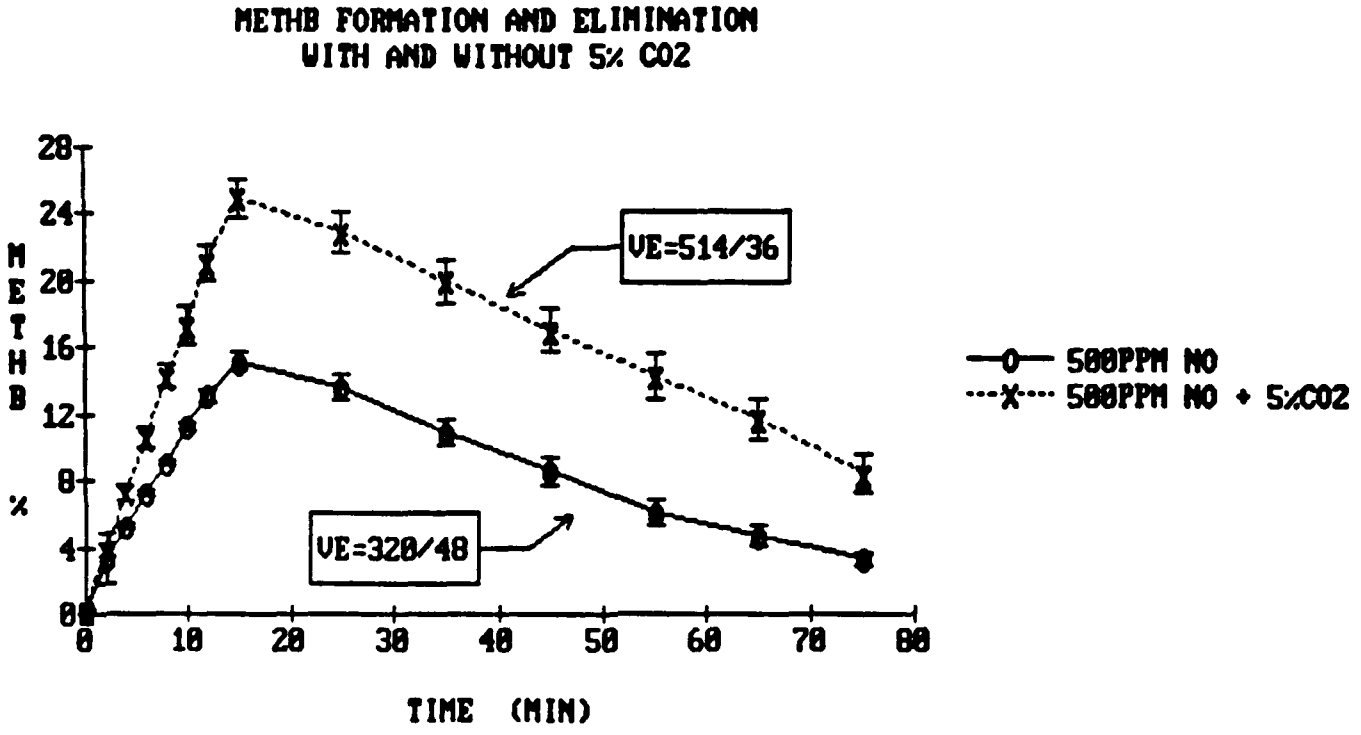



Figure 3: Methemoglobin saturation of arterial blood collected during and after exposure to 500 ppm NO for 15 min or to 500 ppm NO in combination with 5% $CO_2$ for 15 min. Each point represents the mean and standard error of the mean on 4 or 5 animals.

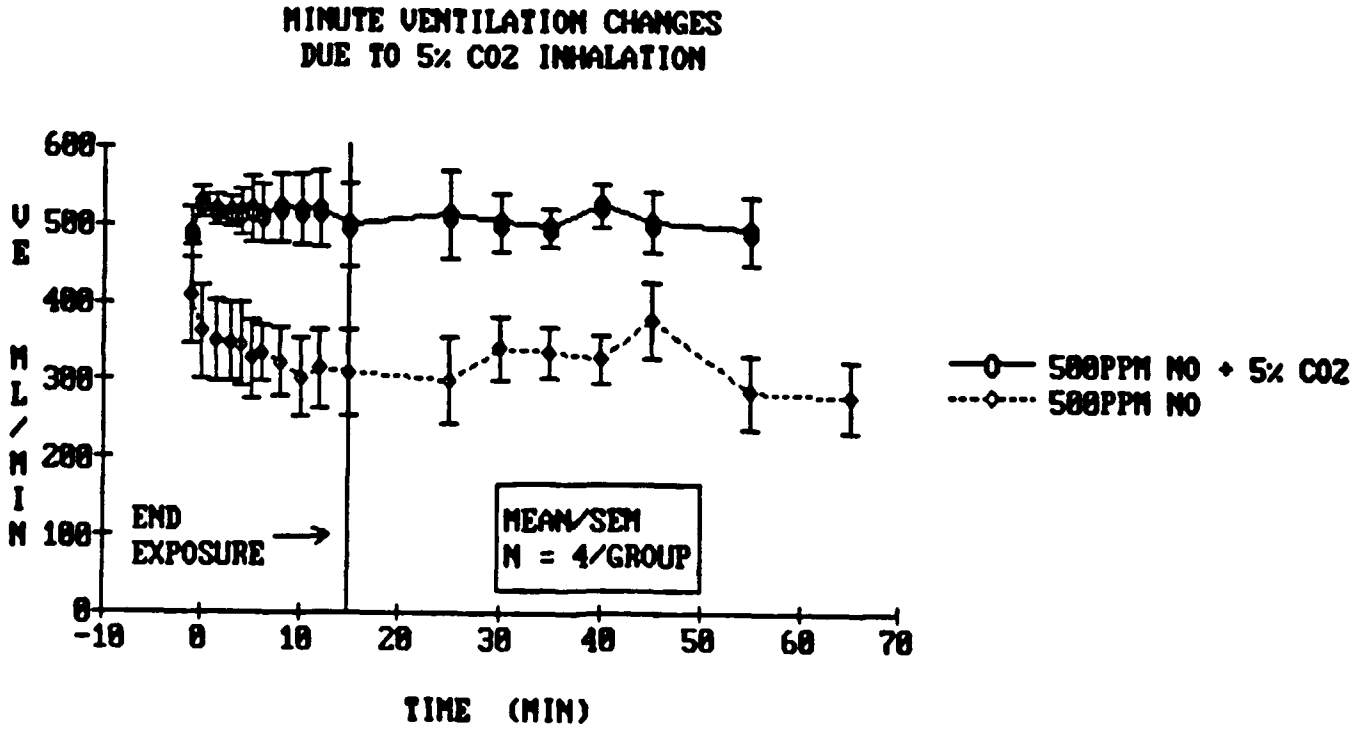



Figure 4: Minute ventilation ($\dot{V}E$) measured on rats during and after exposure to 500 ppm NO or 500 ppm NO in combination with 5% $CO_2$. Each point represents the mean and standard error of the mean of 4 or 5 animals.

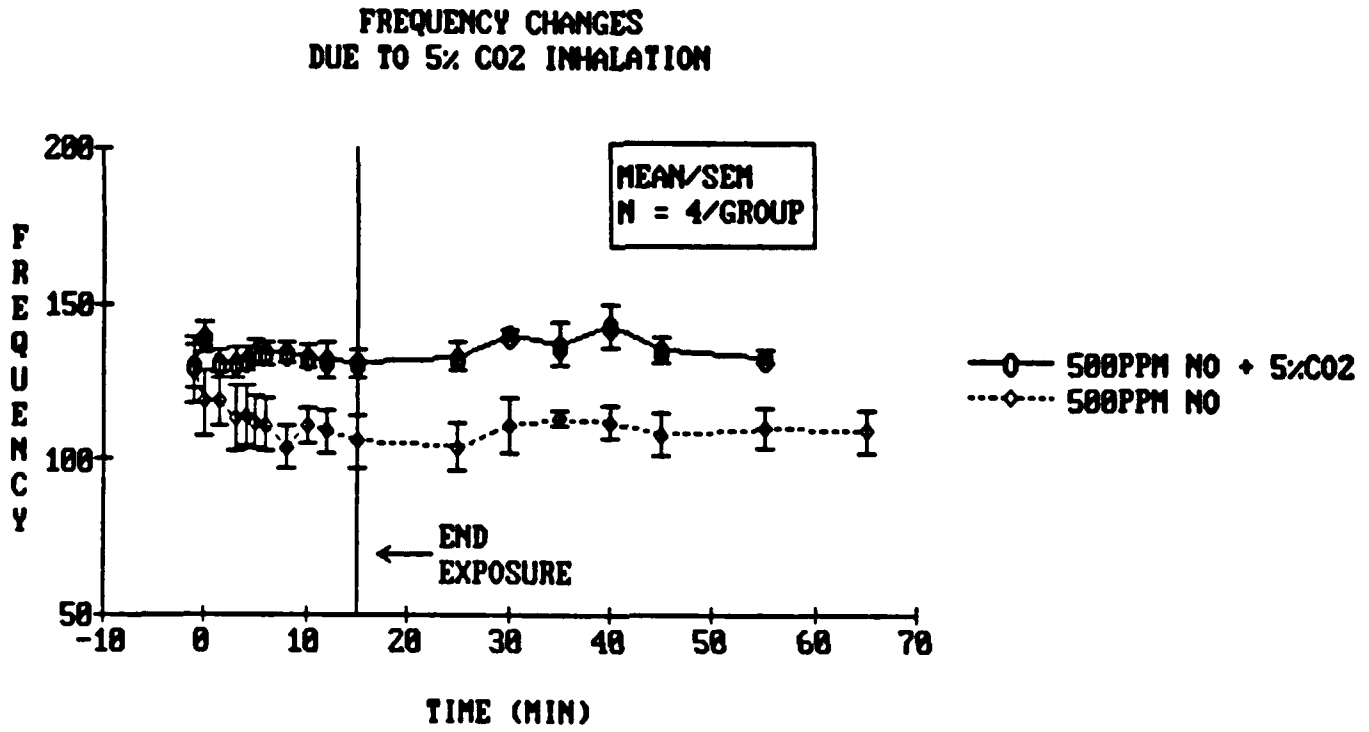



Figure 5: Breathing frequency (f) measured on rats duirng and after exposure to 500 ppm NO or 500 ppm NO in combination with 5% $CO_2$. Each point represents the mean and standard error of the mean of 4 or 5 rats.

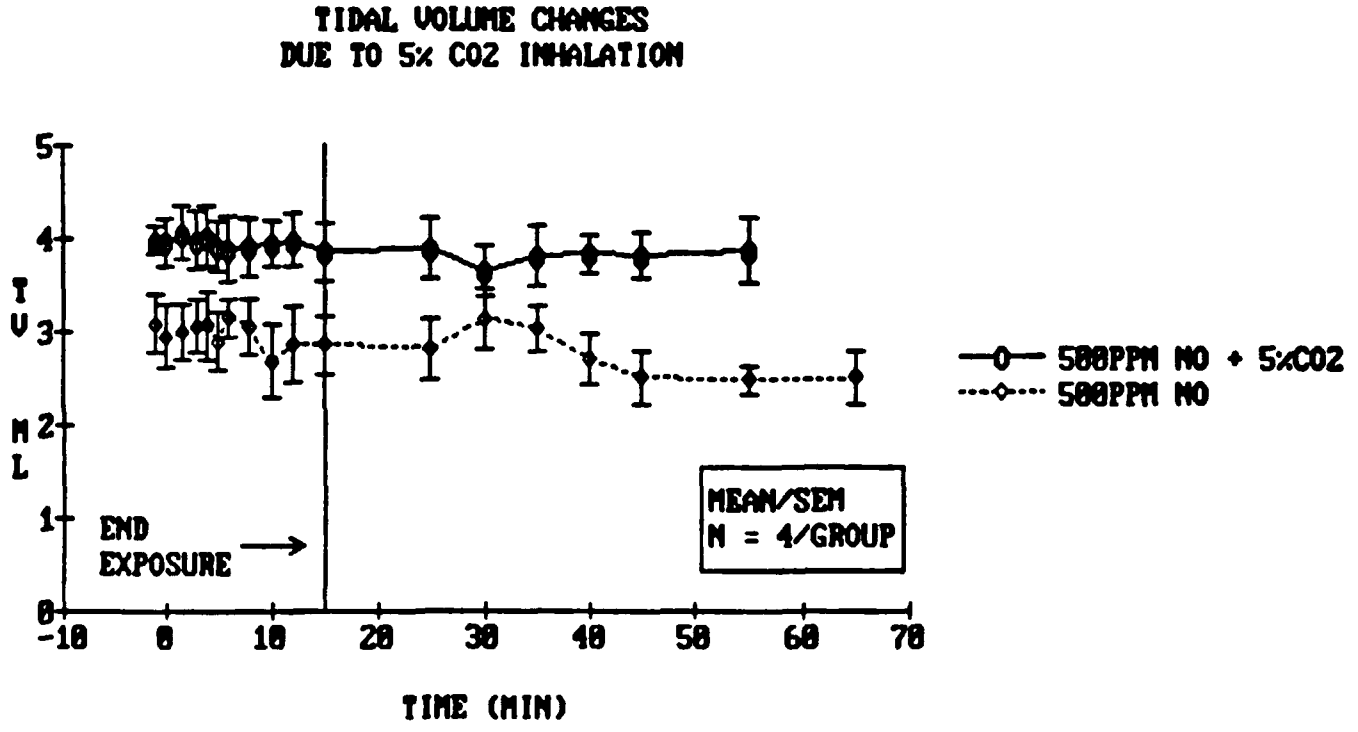
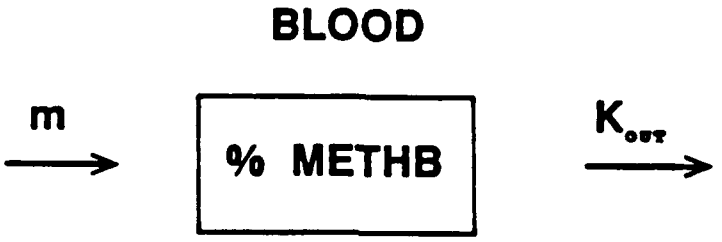



Figure 6: Tidal volume ($V_t$) measured on rats during and after exposure to 500 ppm NO or 500 ppm NO in combination with 5% $CO_2$. Each point represents the mean and standard error of the mean of 4 or 5 rats.

*Model for Methemoglobin Formation and Elimination in the Rat:* Assessments of the elimination kinetics of MetHb following cessation of the exposures to 1000, 500, and 200 ppm NO indicated that MetHB elimination followed a pattern typical of a simple first order process, Figure 7. Given the similar constants for the rate of MetHb elimination, or $K_{OUT}$, that were estimated from least squares analyses of the actual experimental data for each exposure, the data from all exposures (excluding those involving $CO_2$) were pooled and reanalyzed to better estimate the actual value for $K_{OUT}$, Figure 8. This value was found to be -0.00903 % MetHb • $min^{-1}$, r = 0.95 ($P < 0.01$).

In order to model the formation of MetHb during the exposures to NO, the following model was considered:




,where m is analogous to a constant rate of infusion of MetHb into the blood, or, in other words, the % increase in MetHb in the blood • $min^{-1}$. During exposure to NO, actual values for % MetHb are measured values and $K_{OUT}$ was estimated as previously described. m was considered to have a unique value for each exposure concentration considered; $V_E$ was assumed to be constant in all studies not involving concurrent $CO_2$ exposure.

The following equation was used to determine m for each exposure concentration:

$$\%\ \text{METHB}_{(t)} = \frac{m}{K_{out}} \left(1 - e^{-K_{out}(t)}\right)$$

, where % $MetHb_{(t)}$ is the percent blood saturation with MetHb at any given time during the NO exposures. It will be noted that the above equation concurrently accounts for the removal of some MetHb during the actual exposure period. (After cessation of the exposure, reductions in MetHb are simply described by % $MetHb_{(t)}$ = % MetHb

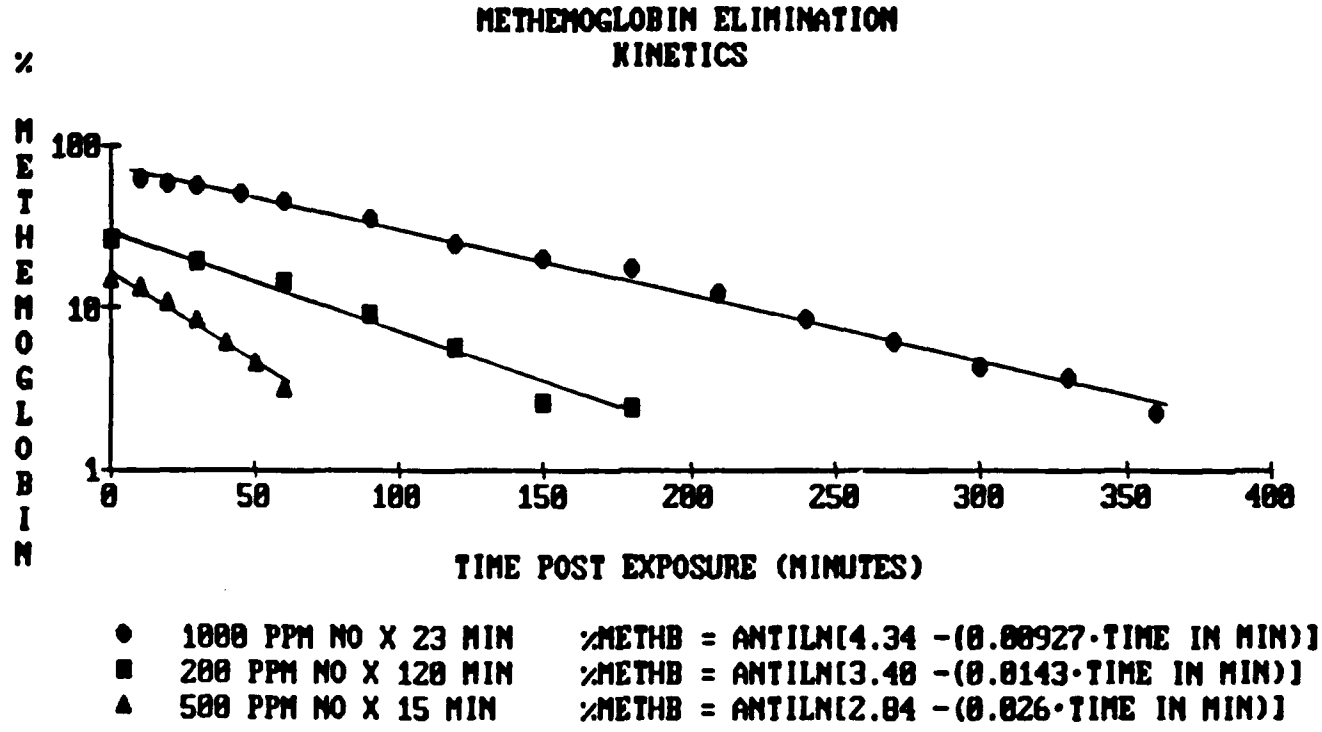



Figure 7: Log plot of % MetHb upon cessation of the 1000 ppm, 500 ppm, and 200 ppm NO exposures.

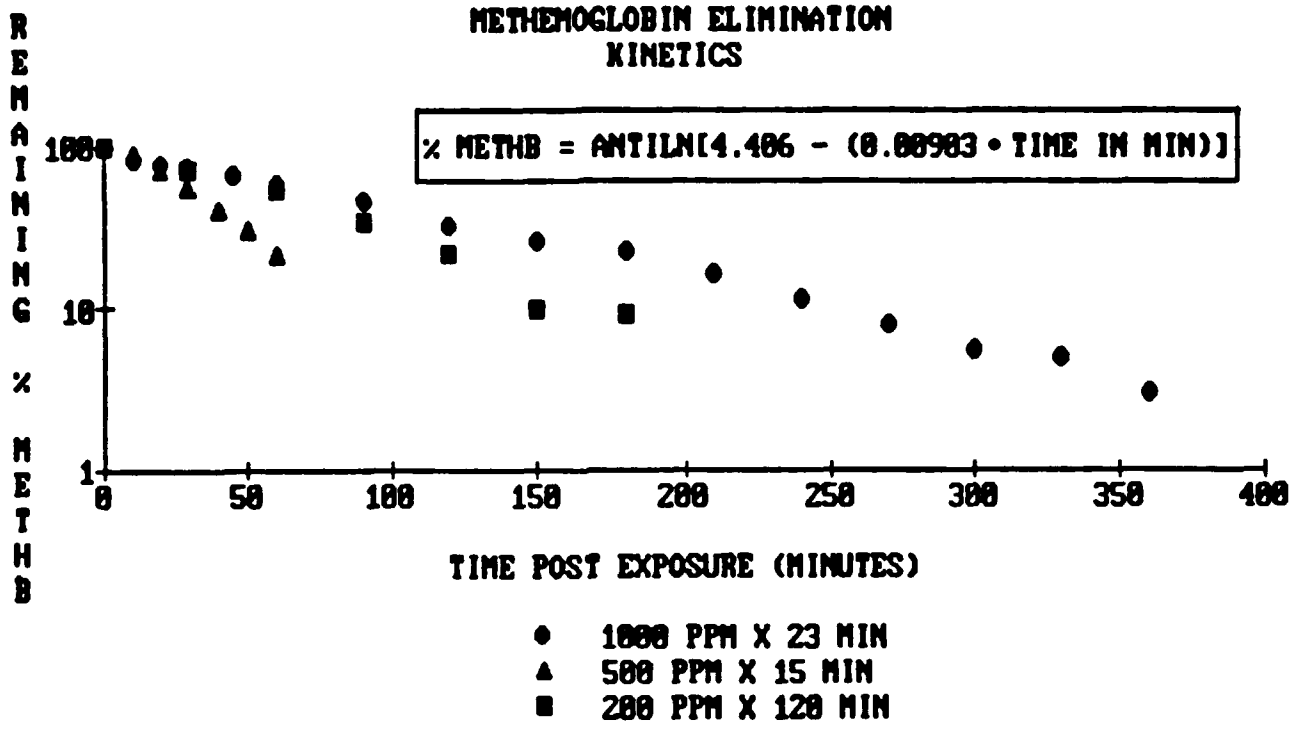



Figure 8: Pooled values plotted represent the percentages of the initial % MetHb at t = 0 post exposure that remained at subsequent post exposure times.

immediately post exposure • $e^{-K_{out}(t)}$. Values ascertained for m for each NO concentration are shown in Table 1 and Figure 9. These data could satisfactorily be described by the following equation that relates the rate of MetHb accumulation to NO exposure concentration under normal ventilating conditions: in % MetHb • $min^{-1}$ = -0.189 + [0.00313 x NO exposure concentration], r = 0.98, Figure 8. Thus the rate of MetHb accumulation can be predicted with foreknowledge of NO exposure concentration and the value for $K_{out}$.

Predicted values for % MetHb that were derived from the kinetic model are compared to measured values obtained from animals exposed to 1000, 500, 200 ppm NO in Tables II-IV.

TABLE 1

Estimated Values for "m"

| | |
|---|---|
| 1000 ppm NO: | m = 3.07684 % MetHb • $min^{-1}$ (t = 20 min) |
| 500 ppm NO: | m = 1.15188 % MetHb • $min^{-1}$ (t = 12 min) |
| 200 ppm NO: | m = 0.34324 % MetHb • $min^{-1}$ (t = 60 min) |
| 500 ppm NO with a 1.6 Fold in $V_E$: | m* = 1.8465 % MetHb • $min^{-1}$ (t = 12 min) |

( ): Exposure times at which m was estimated.

*: Estimated value for m is 1.6 times greater than that estimated for the inhalation of 500 ppm under resting conditions.

TABLE II

Predicted Values for % MetHb During and
After Exposure to 1000 ppm NO

| During Exposure | Predicted* | Measured % MetHb |
|---|---|---|
| t = 6 min | 17.9% | 16.0% |
| t = 8 min | 23.7% | 20.7% |
| t = 10 min | 29.4% | 28.4% |
| t = 20 min | 56.3% | 56.3% |
| **After Exposure** | | |
| t = 10 min | 70.1% | 58.8% |
| t = 50 min | 48.8% | 44.5% |
| t = 120 min | 26.0% | 24.3% |
| t = 200 min | 12.6% | 12.3% |
| t = 300 min | 5.1% | 3.8% |

* Predicted post-exposure values were determined using the zero time post exposure value for % MetHb extrapolated from least squares fit analyses of all post-exposure data.

TABLE III

Predicted Values for % MetHb During and
After Exposure to 500 ppm NO

| During Exposure | Predicted* | Measured % MetHb |
|---|---|---|
| t = 2 min | 2.3% | 3.4% |
| t = 6 min | 6.7% | 7.3% |
| t = 8 min | 8.9% | 9.1% |
| t = 12 min | 13.1% | 13.1% |
| t = 15 min | 16.2% | 15.1% |
| **After Exposure** | | |
| t = 10 min | 13.9% | 13.6% |
| t = 20 min | 12.7% | 10.9% |
| t = 40 min | 10.6% | 6.1% |
| t = 60 min | 8.8% | 3.3% |

* Predicted post-exposure values were determined using the % MetHb value measured immediately upon the cessation of the NO exposure.

TABLE IV

Predicted Values for % MetHb During and
After Exposure to 200 ppm NO

| During Exposure | Predicted* | Measured % MetHb |
|---|---|---|
| t = 10 min | 3.3% | 4.2% |
| t = 30 min | 9.0% | 9.2% |
| t = 90 min | 21.1% | 21.5% |
| t = 120 min | 25.1% | 26.9% |
| **After Exposure** | | |
| t = 30 min | 20.5% | 19.1% |
| t = 90 min | 11.9% | 9.1% |
| t = 120 min | 9.1% | 5.8% |
| t = 180 min | 5.2% | 2.5% |

* Predicted post-exposure values were determined using the % MetHb values measured immediately upon the cessation of the NO exposure.

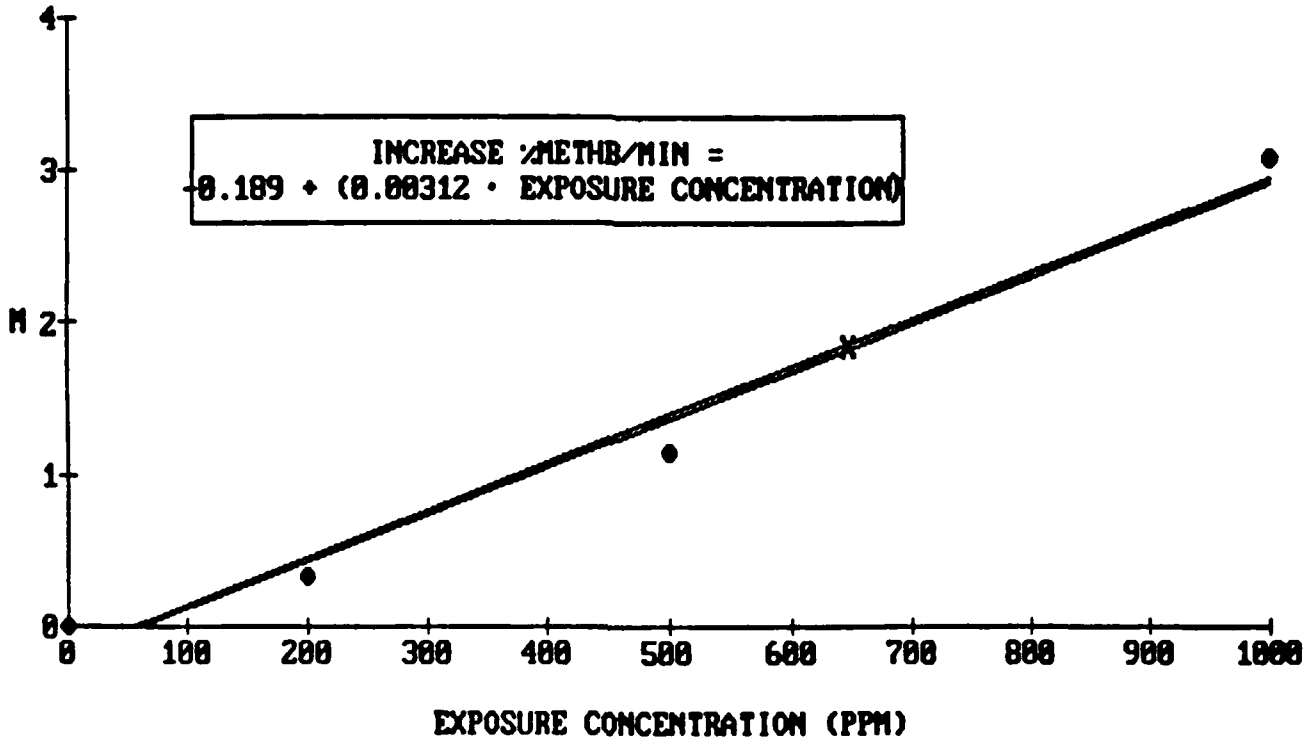



Figure 9: Values for m as related to exposure concentration. X: value for m when minute ventilation was increased 1.6 fold.

## DISCUSSION

In this component of the project, we characterized the formation of methemoglobin during exposures of rats to different NO concentrations and we characterized the kinetics of MetHb removal following cessation of the NO exposures. Included in these studies was an examination of the effects of $V_E$ on MetHb. A model has also been developed to relate NO exposure concentrations to the rate of MetHb formation in the rat. The emperical data and the model indicate that the rate of MetHb formation is proportional to NO exposure concentration. Additionally, the rate of MetHb accumulation most simply models as the product of minute ventilation and exposure concentration; increasing minute ventilation during the inhalation of a given concentration of NO results in a proportional increase in the rate of MetHb formation. The model also accounts for the removal of MetHb both during and after NO exposures. Future NO exposure studies with simultaneous ventilatory measurements could be performed to more closely refine estimates for m and $K_{out}$.

For a given exposure to NO, m can be related to the product of NO concentration and minute ventilation, which in turn, can be used to predict delivered dose. Accordingly, alterations in m should be proportional to $V_E$ at a fixed exposure concentration. The results of our studies support such a relationship. As shown in Figure 9 and Table I, a 1.6 fold increase in $V_E$ due to $CO_2$ inhalation appeared to have the same effect on m as would an increase in NO exposure concentration inhaled under normal conditions. In that NO Exposure Concentration ~= [m - (-0.189)]/0.00313, a 1.6 fold increase in $V_E$ is equivalent to exposing the animals to ~650 ppm NO, Figure 9, under resting conditions.

In the original model described herein, $K_{OUT}$ was estimated as a pooled value from all collected MetHb elimination data, without considering how the saturation level of MetHb achieved in a given exposure to NO may have impacted on this parameter. While the previously described model rather closely approximated the actual experimental data, the MetHB elimination data shown in Figure 10 has suggested to us that $K_{OUT}$ may actually be a function of the % saturation of MetHB achieved during an exposure to NO in that the rate of MetHb elimination appears to diminish as the maximal %MetHb attained increases. Such a relationship is further suggested by the data shown in Figure 11, where the various $K_{OUT}$s obtained after several different NO exposure regimens are plotted against the natural log of the maximal percentage of MetHb saturation achieved during each exposure. It would appear, accordingly, that $K_{OUT}$ may be more suitably modeled as a continuously changing variable during the accumulation of MetHb during NO exposure.

From the data shown in Figure 11, it was estimated that:

$$-K_{OUT} = (0.01) \ln \%MetHb + (-0.05), \text{ or}$$
$$K_{OUT} = (-0.01) \ln \%MetHb + (0.05).$$

We next solved for "m" during the NO exposure-MetHb accumulation phase, while considering $K_{OUT}$ as being a continuously changing parameter that varys with the %MetHb present at a given time. The accumulation of MetHb during three exposure conditions was modeled using Newton's method. The exposure conditions were: 1000 ppm NO x 23 min (3.035), 500 ppm x 15 min (1.221), and 200 ppm x 120 min (0.486). The values in the parentheses are for "m". The results of this change in the model relative to fitting the actual experimental data are summarized in Figure 12. The fits are nearly exact, and they are well within the error of the actual MetHb measurements.

Some problems with the model persist and require further investigation. For example, use of continuously changing $K_{OUT}$s for the MetHb elimination component of the model have as yet to be evaluated, but the inclusion of such a feature in the model may be useful. In the initial form of the model, $K_{OUT}$ was assumed to be a first order process, and this assumption resulted in reasonably good fits between the model's results and the empirically-derived data. However, if $K_{OUT}$ is an unvarying constant, one interpretation of the data summarized in Figure 10 is that the mechanism(s) underlying the removal of MetHb following cessation of NO exposure may be increasingly compromised with increasing maximal %MetHb achieved during an exposure. Given our original assumption that the rate of removal of MetHb is first order, which was based on our initial analyses of MetHb elimination data collected as of the time of development of our first model, a persistence in the "poisoning" of the MetHb elimination mechanism would be predicted. However, we have been unable to demonstrate such an effect. Figure 13 summarizes the results from a study in which rats were exposed to 1000 ppm NO to bring about an ~65% MetHb saturation level in their blood. After a post-exposure period during which MetHb was allowed to decrease to a saturation level of <5%, the rats were re-exposed to NO to raise their %MetHb to ~20%, and the rate of MetHb elimination thereafter was monitored. No evidence was found to support a persistent retardation in MetHb elimination due to the animals having had a recent history of a high percentage of MetHb saturation. We are reaching the conclusion that $K_{OUT}$ during the elimination of MetHb should not be modeled as a simple first order process. Indeed, more recent data, such as those summarized in Figure 14, strongly suggest $K_{OUT}$ cannot be simply viewed so simplistically.

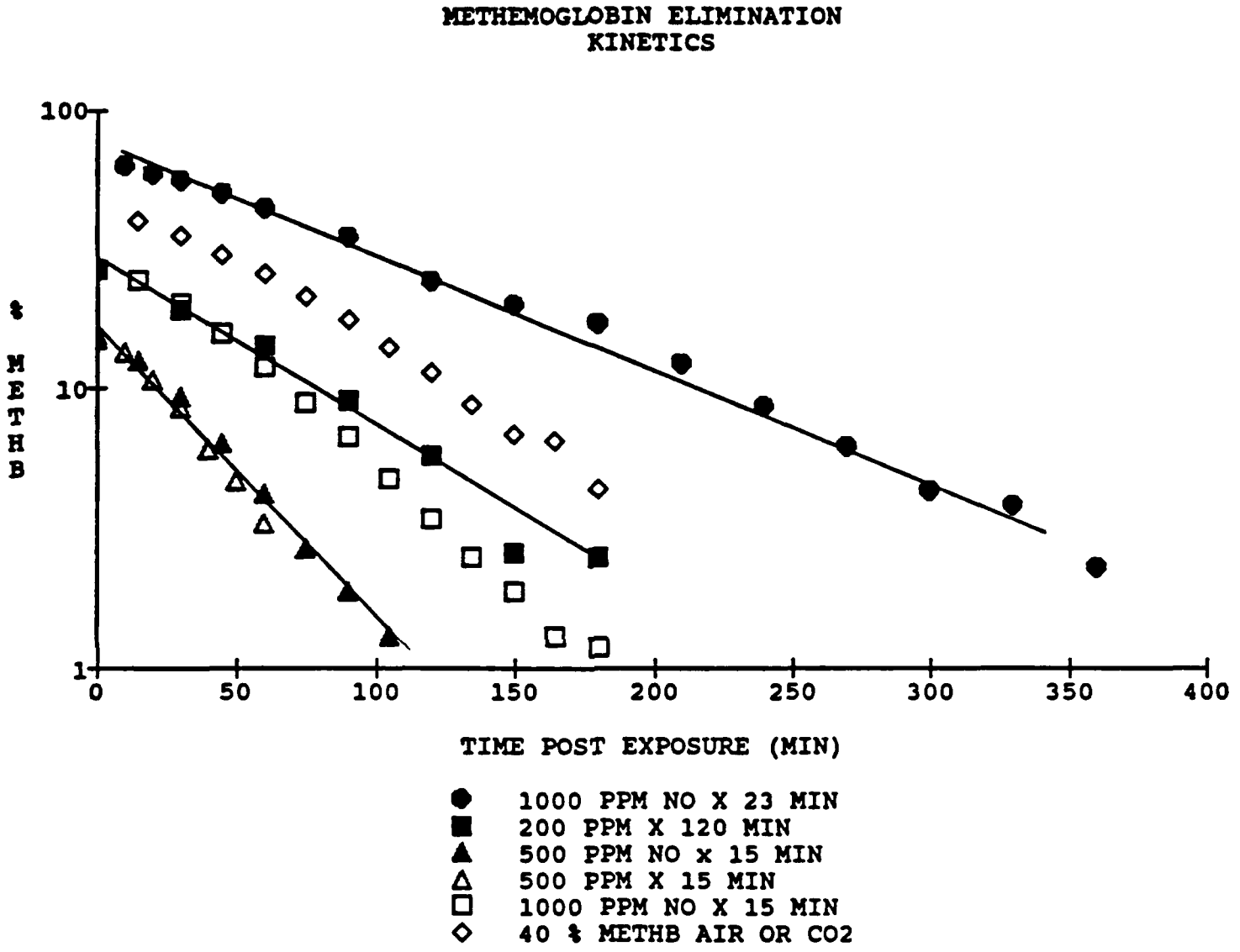
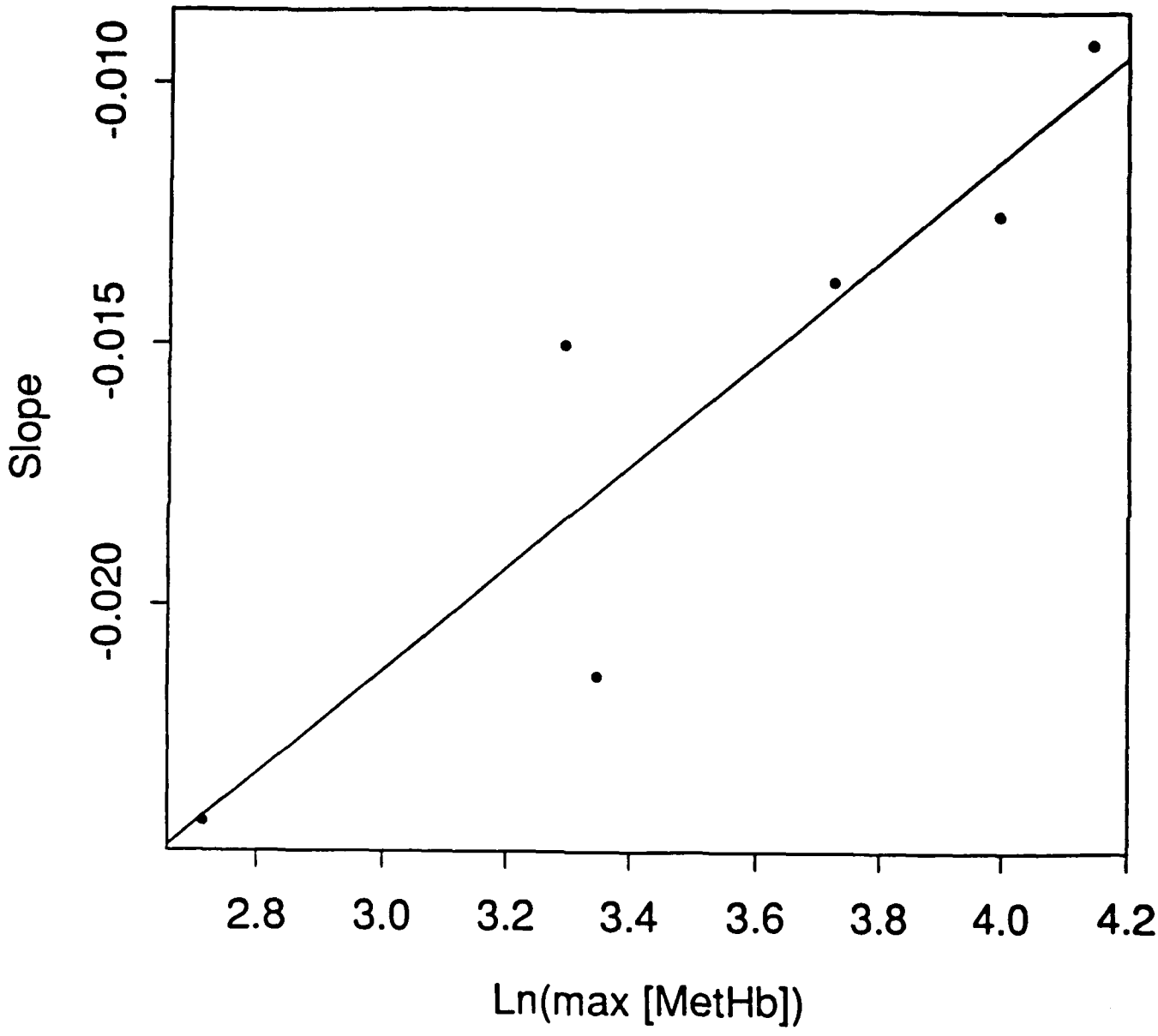



Figure 10: Elimination kinetics of MetHb modeled as a simple first order process.

**Figure 11: Plot of -$K_{OUT}$ (slopes) versus the natural log of the maximal MetHb concentrations achieved during several NO exposure studies. The line describing the data was determined by linear regression.**



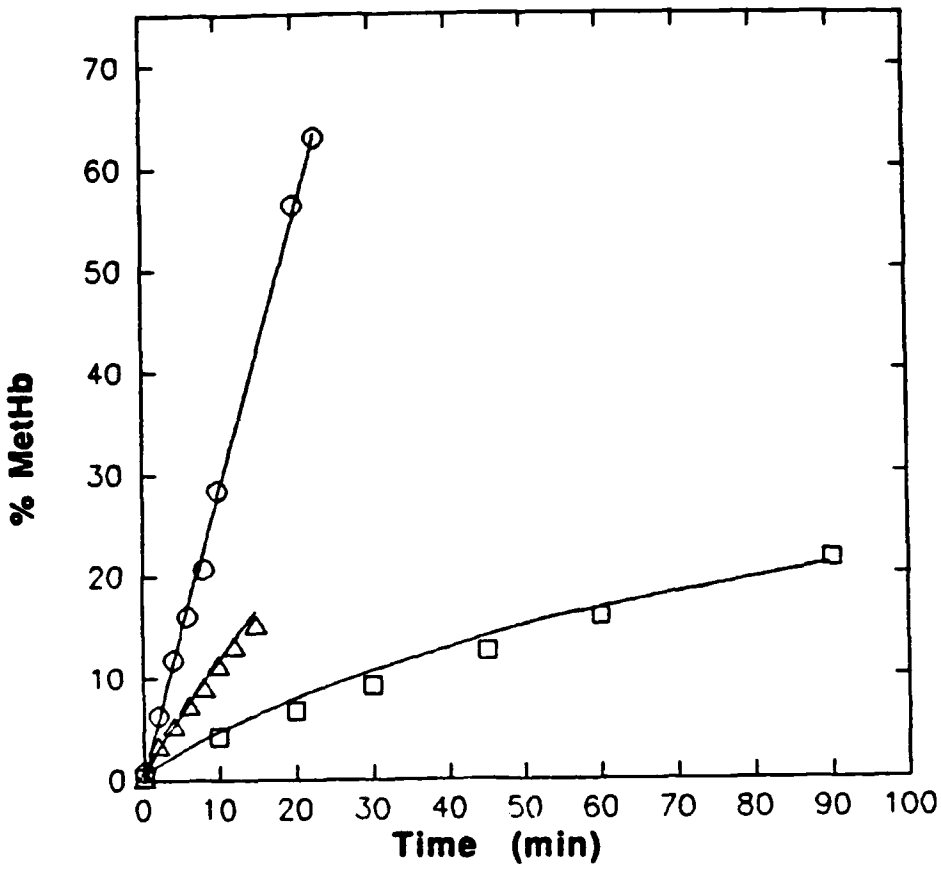



Figure 12: Model fits (lines) of the accumulation of MetHb (experimental data:symbols) during exposure to NO. Circles: 1000 ppm NO; Triangles: 500 ppm NO; Squares: 200 ppm NO.

Figure 13: Lack of an effect of an initially high saturation MetHb level on the rate of removal of MetHb achieved by as second NO exposure. If the initially high MetHb level persistently compromises MetHb elimination, the rate of MetHb elimination observed after the second exposure should be prolonged, e.g., ~ the same as that observed with the high MetHb level.

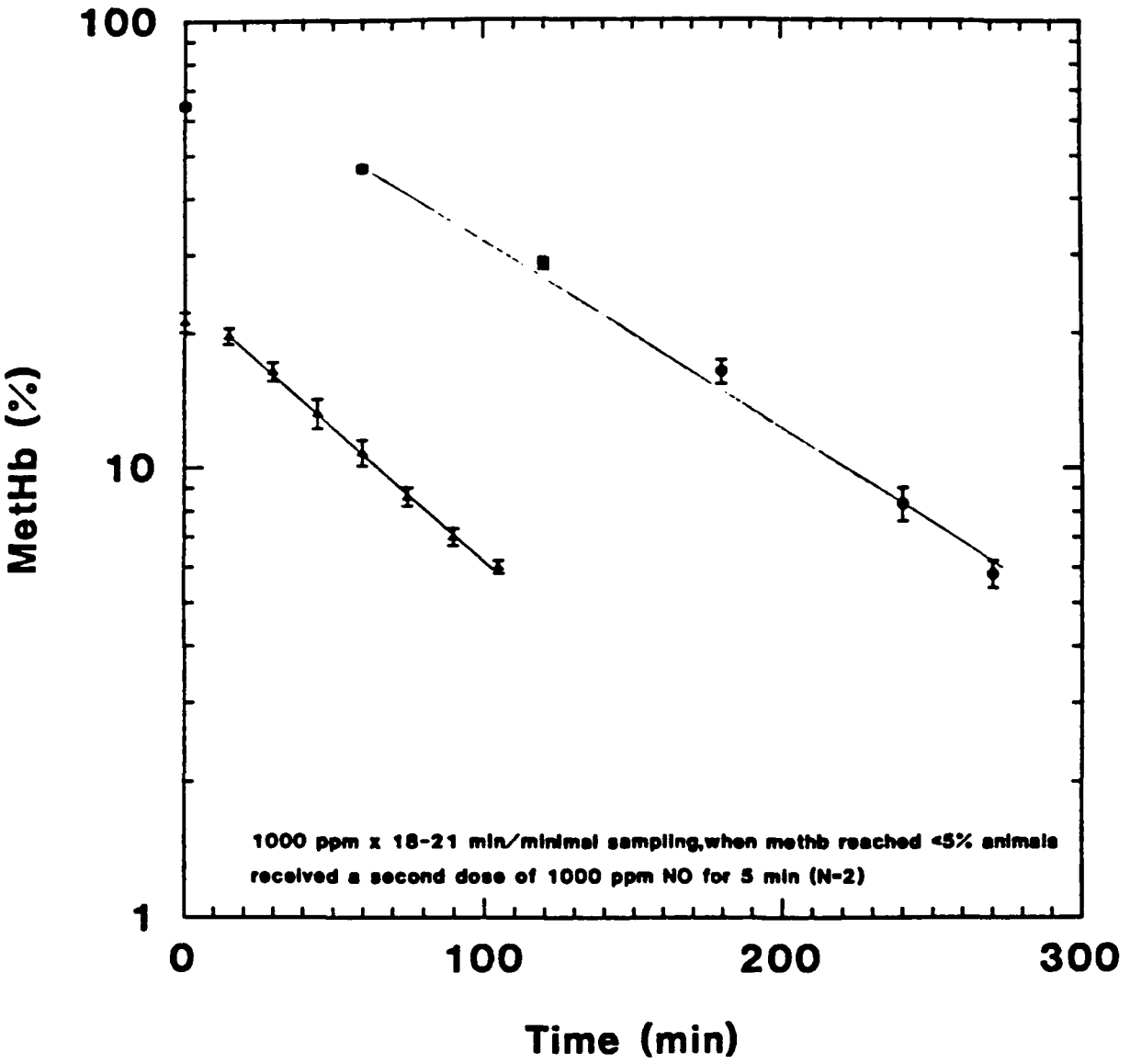
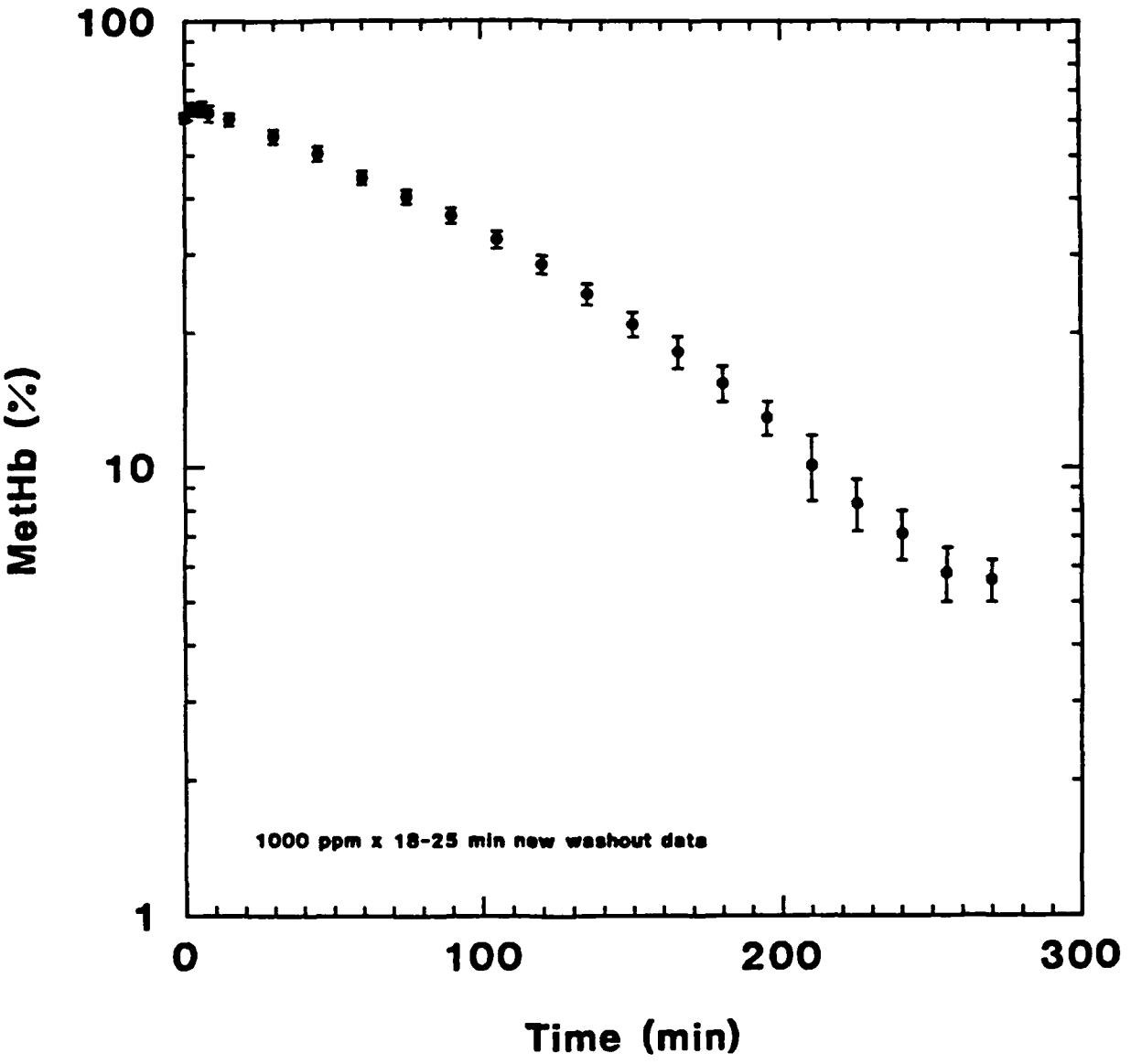


Figure 14: Complexity of the pattern of MetHb elimination when examined over a prolonged period after the exposure of rats to 1000 ppm NO for 18-25 min.



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# SECTION F

## Objective 11: To investigate the relationship between blood methemoglobin and work performance capacity.

**Results:** A direct relationship was found between reductions in $VO_{2max}$ and the level of methemoglobin present at the time of exercise.

**Reports:**

*Stavert, D.M., Ripple, G., Mundie, T.M., Lehnert, B.E.: $VO_{2max}$ decreases inversely with blood methemoglobin concentration following the inhalation of nitric oxide. 28the Annual Meeting of the Society of Toxicology. Atlanta, Ga, February 27-March 3, 1989. The Toxicologist 9:A744, 1989.*

*Lehnert, B.E.: "Physiological and Biochemical Endpoints in Inhalation Toxicology", International Programme on Chemical Safety, World Health Organization, Hanover, FRG, October 6-October 9, 1992.*

*"Post-Exposure Potentiation of Lung Injury by Exercise and Work Performance Degradation Following the Inhalation of Pyrolysis Products", National Aeronautics and Space Administration, Johnson Space Center, Houston, TX, November 13, 1992.*

*"Lung Injury Following Exposure of Rats to Relatively High Mass Concentrations of Nitrogen Dioxide". Walter Reed Army Institute of Research, Department of Respiratory Research, Washington, D.C., October 23, 1992.*

*Lehnert, B.E.: Toxicology of nitric oxide and nitrogen dioxide. In: Handbook of Hazardous Materials. (M. Corn, ed.) Academic press, San Diego, CA (in press), 1992.*

## INTRODUCTION

Nitric Oxide (NO) is the major oxide of nitrogen formed during a variety of high-temperature combustion processes and, accordingly, NO represents a potentially hazardous air contaminant in settings where such processes occur. Previously, we have shown that inhalation exposures to high concentrations of NO (1000 ppm) are innocuous in the rat's lung using histopathologic and gravimetric criteria (Stavert and Lehnert, 1990). However, evidence for extra-pulmonary toxicity was obtained in this previous study, due presumably to the rapid formation of methemoglobin (MetHb). MetHb, like carboxyhemoglobin (COHb) formed during the inhalation of carbon monoxide (CO), reduces the oxygen carrying capacity of the blood. As previously demonstrated by others (Weiser et al., 1978), acute exposure to CO impairs exercise performance as measured by maximum oxygen consumption ($VO_{2max}$) and the decrease in $VO_{2max}$ proportional to the HbCO saturation. Comparable information regarding relationship(s) between percent MetHb saturation and exercise performance, however, is currently non-existent. The purpose of this study, accordingly, was to evaluate work performance using $VO_{2max}$ as an indicator of work capacity at various MetHb saturation values in the rat. For comparative purposes, we also examine our results in the context of work performance degradation in the human as a function of COHb saturation.

## MATERIALS AND METHODS

*Metabolic Measurements and Exercise Protocol:* Measurements of work performance were performed while rats were exercising on a treadmill contained within a metabolic chamber, as described in Section C. Airflow through the chamber was measured and oxygen ($O_2$) and carbon dioxide ($CO_2$) in the effluent airstream were determined. Oxygen consumption ($VO_2$), carbon dioxide production ($VCO_2$), and respiratory quotient (R) were calculated every 5 seconds with a data acquisition and management system. Prior to the work performance studies, adult, male Fischer-344 rats were subjected to a 19 day training program in which they were behaviorally and physically conditioned to perform a "ramp" exercise test on a treadmill (15% grade), also as described in Section C. Maximum oxygen consumption was determined before and after the gas exposures with each conditioned animal serving as its own control.

*Nitric Oxide Exposures:* Atmospheres of 1000 ppm NO were generated by mixing pure NO with HEPA filtered anhydrous air. The atmospheres were passed through soda lime before

entering a quartz crystal exposure chamber , as described in Section D, to maintain $NO_2$ contamination below 25 ppm. The rats were exposed to NO for periods of 23 min. Thereafter, they were divided into groups that were exercised at 2, 60, 120, 240, and 480 min post-exposure for the work performance studies. Parallel studies conducted with separate groups of rats indicated % MetHb saturation values corresponding to these post-exposure times were ~65, 45, 24, 9, and 2 percent, respectively, Figure 1.

*Artifically Induced Anemia:* In another component of the study, we evaluated work performance degradation due to simple hemoglobin reduction. Blood was drawn in quantities of 2 and 4 ml aliquots from the middle caudual artery of rats while under local anesthesia. Equivalent volumes of plasma were reinjected into the animals. Control animals were identically prepared, but no blood was removed. 15 min after blood removal, the animals were evaluated for work performance capability. The percent changes in the hematocrit values of these animals were used as "MetHb-equivalent saturation" values in Figure 3.

## RESULTS AND DISCUSSION

The kinetics of MetHb formation and disappearance during and after the inhalation of 1000 ppm NO by unanesthetized rats have been described in Section D, see Figure 1. Maximum oxygen consumption ($VO_{2max}$) values at the indicated post-exposure % MetHb saturations are shown in Table I and Figure 2. A marked reduction in $VO_{2max}$ was observed shortly after the 23 min exposure to NO, Figure 6 (MetHb saturation = 63 percent), and decrements in $VO_{2max}$ at subsequent times proportionately scaled inversely with % MetHb saturation. Work performance capability increased to control values as MetHb saturation approached zero. Overall, the decriments in $VO_{2max}$ correlated ($r = 0.99$; $P < 0.001$) with % MetHb saturation ($VO_{2max} = 0.67 \times \%\ MetHb + 0.67$), with an intercept value close to zero % MetHb. $VO_{2max}$ values measured after hemoglobin reduction brought about by artificial anemia were similar to those measured in rats with comparable functional decreases in Hb due to MetHb, Figure 3.

The above findings are consistent with the possibility that the reduction of $VO_{2max}$ seen during heavy exercise after NO exposure was primarily due to the functional reduction of hemoglobin (via the formation of MetHb) and not importantly due to MetHb-associated changes in the $O_2$ dissociation curve (leftward shift) (Darling and Roughton, 1942).

By comparison, the magnitude of work performance degradation measured in this study with rats experiencing methemoglobinemia quantitatively differs from that measured with humans during COHb intoxication, Figure 4. In the human condition, reductions in $VO_{2max}$ more directly scale with COHb saturation, ($VO_{2max} = 1.08 \times COHb + 0.35$), than the $VO_{2max}$ reductions due

to MetHb saturation we measured in the rat model, see slope, Figure 4. Conceivably, this may be due to a more direct coupling of blood oxygen carrying capacity to maximal work performance in the human than in the rat.

## DISCUSSION

Nitric oxide (NO) can be inhaled at high mass concentrations in a variety of occupational settings where thermal combustion processes occur. Inhalation of this agent results in the formation of methemoglobin (MetHb), which decreases the oxygen carrying capacity of the blood. In this study, we examined the relationship of endurance, as indexed by $VO_{2max}$, and MetHb concentration [MetHb] following inhalation exposure to NO. Behaviorally and physically conditioned Fischer-344 rats were exposed to 1000 ppm NO for 23 min and exercised on a treadmill contained in a metabolic chamber at 2, 60, 120, and 240 min thereafter. [MetHb] at these post-exposure times were ~65%, 45, 25, and 10%, respectively. At a [MetHb] of ~65%, $VO_{2max}$ was only ~55 ml $O_2$/kg/min, but $VO_{2max}$ increased to within control limits (~95 ml $O_2$/kg/min) as [MetHb] approached zero %. Overall, $VO_{2max}$ closely scaled inversely with [MetHb], $r=-0.99$, $P<0.001$. This study demonstrates that: 1) brief exposure to a relatively high concentration of NO can result in marked compromises in endurance, but 2) recovery to normal endurance capacity predictably occurs as MetHb is eliminated. Thus, we summarize our findings as follows:

- Maximal work performance as indexed by $VO_{2max}$ was found to be inversely proportional to % MetHb saturation in rats after an acute inhalation of a high concentration NO.

- $VO_{2max}$ reductions measured in anemic rats were of the same magnitude as those seen with comparable reductions in functional Hb due to MetHb formation.

- These findings suggest leftward changes in the $O_2$ dissociation curve (Darling and Roughton, 1942) due to MetHb has a minor, if any, impact on $VO_{2max}$ in the rat.

- The reduction of $VO_{2max}$ due to MetHb in the rat was found to be much less than that reported for the human with comparable decrements in Hb availability due to the COHb formation (Weiser et al., 1978).

TABLE I

Maximum Oxygen Consumption ($\dot{V}O_{2max}$) Values at Various % MetHb Saturation Levels After a 23 Min Exposure to 1000 ppm NO.

| Time Post-Exposure (hr) | MetHb (%) | $O_2$ Ct (Vol %) | $\dot{V}O_{2max}$ (ml $O_2$/kg/min) Pre-Exposure | Post-Exposure | % Change $\dot{V}O_{2max}$ |
|---|---|---|---|---|---|
| 0.17 | 63.0 ± 5.8 | 8.0 ± 1.2 | 91.4 ± 0.7 | 54.3 ± 8.5 | -41.0 ± 9.3 |
| 1.0 | 44.5 ± 4.6 | 11.4 ± 0.9 | 93.7 ± 1.2 | 64.8 ± 1.5 | -30.8 ± 1.5 |
| 2.0 | 24.7 ± 3.8 | 15.2 ± 0.7 | 88.0 ± 1.4 | 76.3 ± 2.7 | -13.2 ± 2.0 |
| 4.0 | 8.6 ± 1.1 | 18.3 ± 1.2 | 94.4 ± 0.9 | 89.0 ± 1.8 | - 5.8 ± 1.0 |
| 8.0 | 0.5 ± 0.3 | 20.4 ± 0.6 | 91.4 ± 0.9 | 91.0 ± 1.0 | - 0.4 ± 1.0 |

Values Represent Means and SEM (N = 5-6 rats per post-exposure time point)

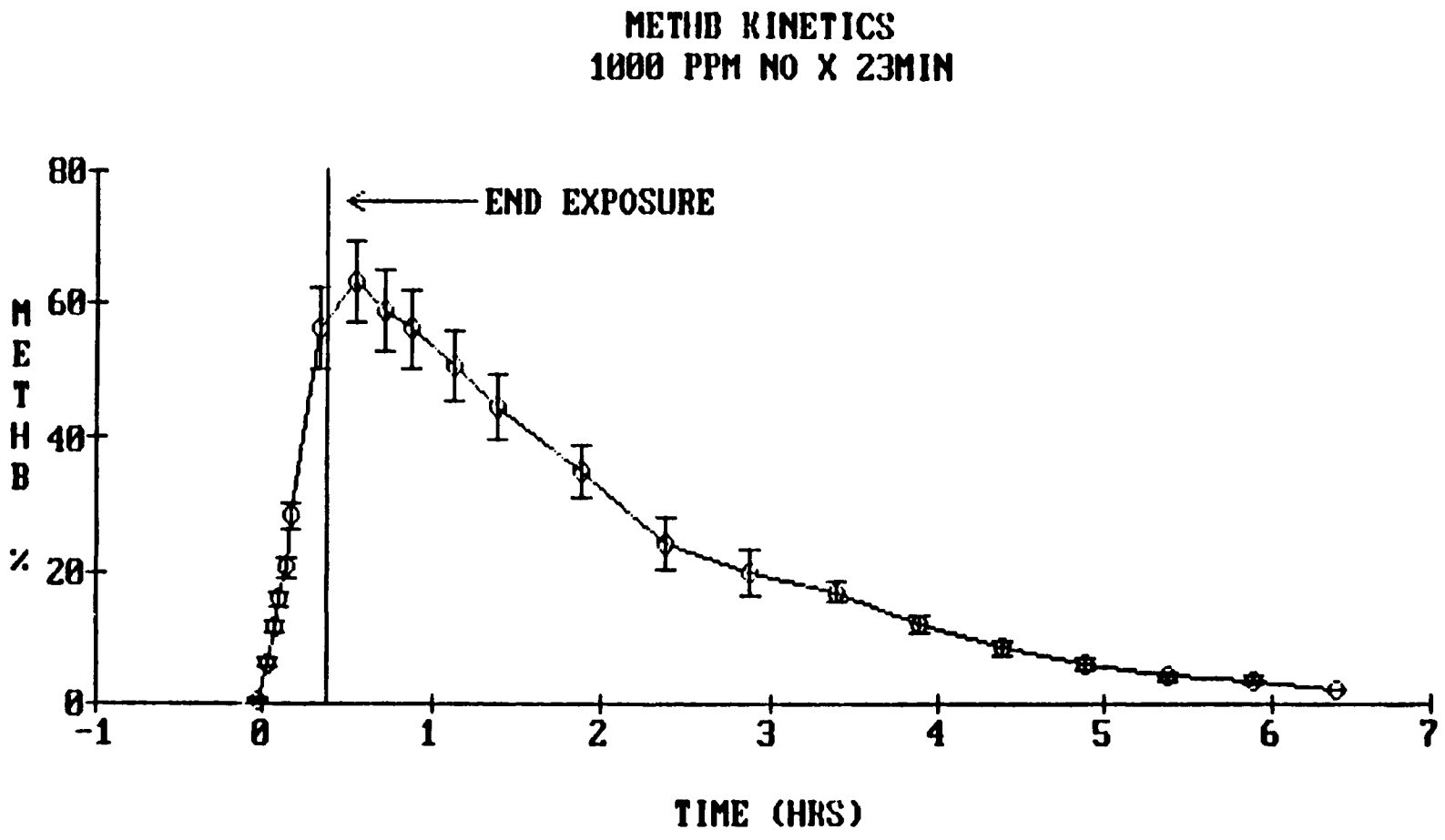


Figure 1: % MetHb saturation measured in blood samples during and after exposure to 1000 ppm NO.

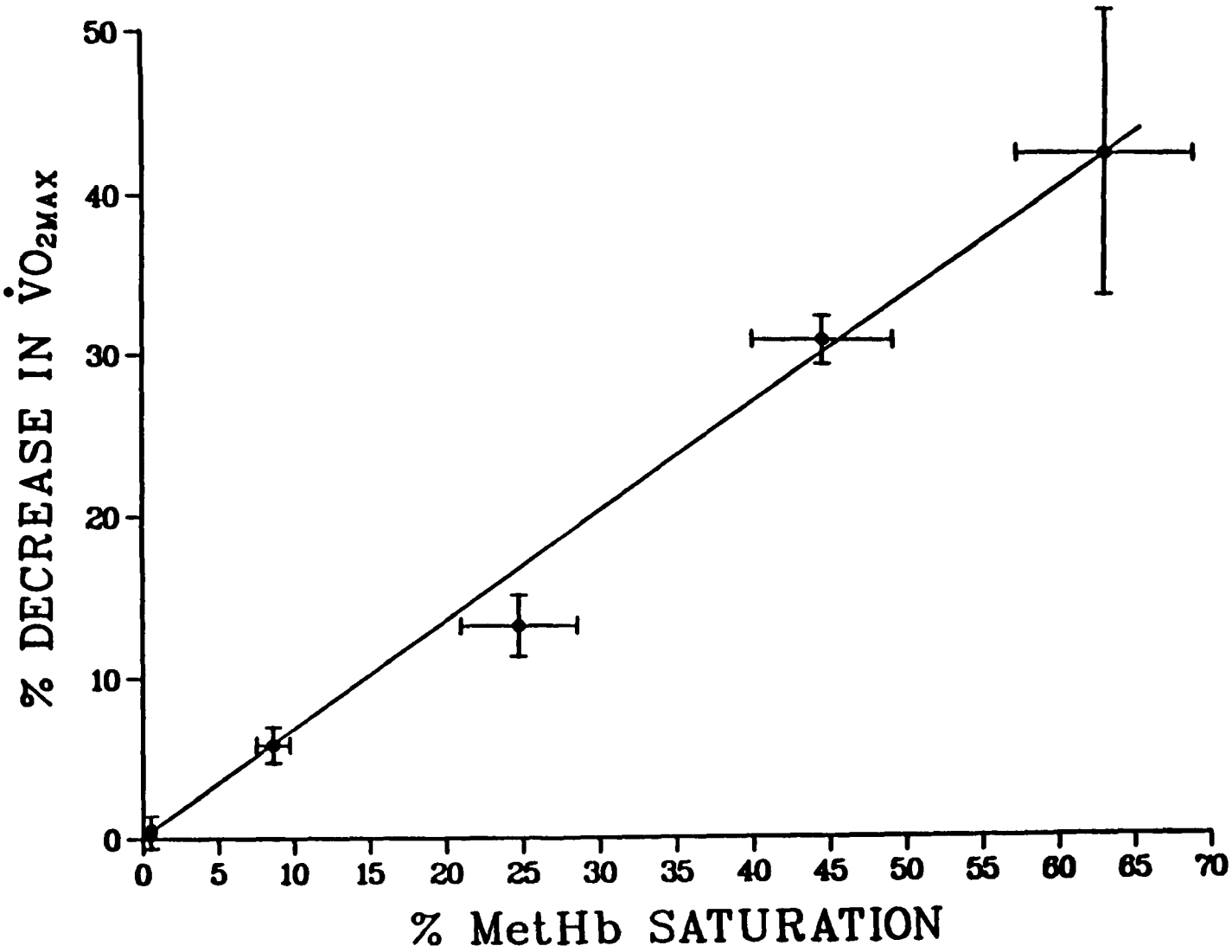


**Figure 2 :** Percentage decreases in maximal oxygen consumption ($\dot{V}O_{2max}$) as a function of % MetHb saturation during the elimination phase of MetHb.

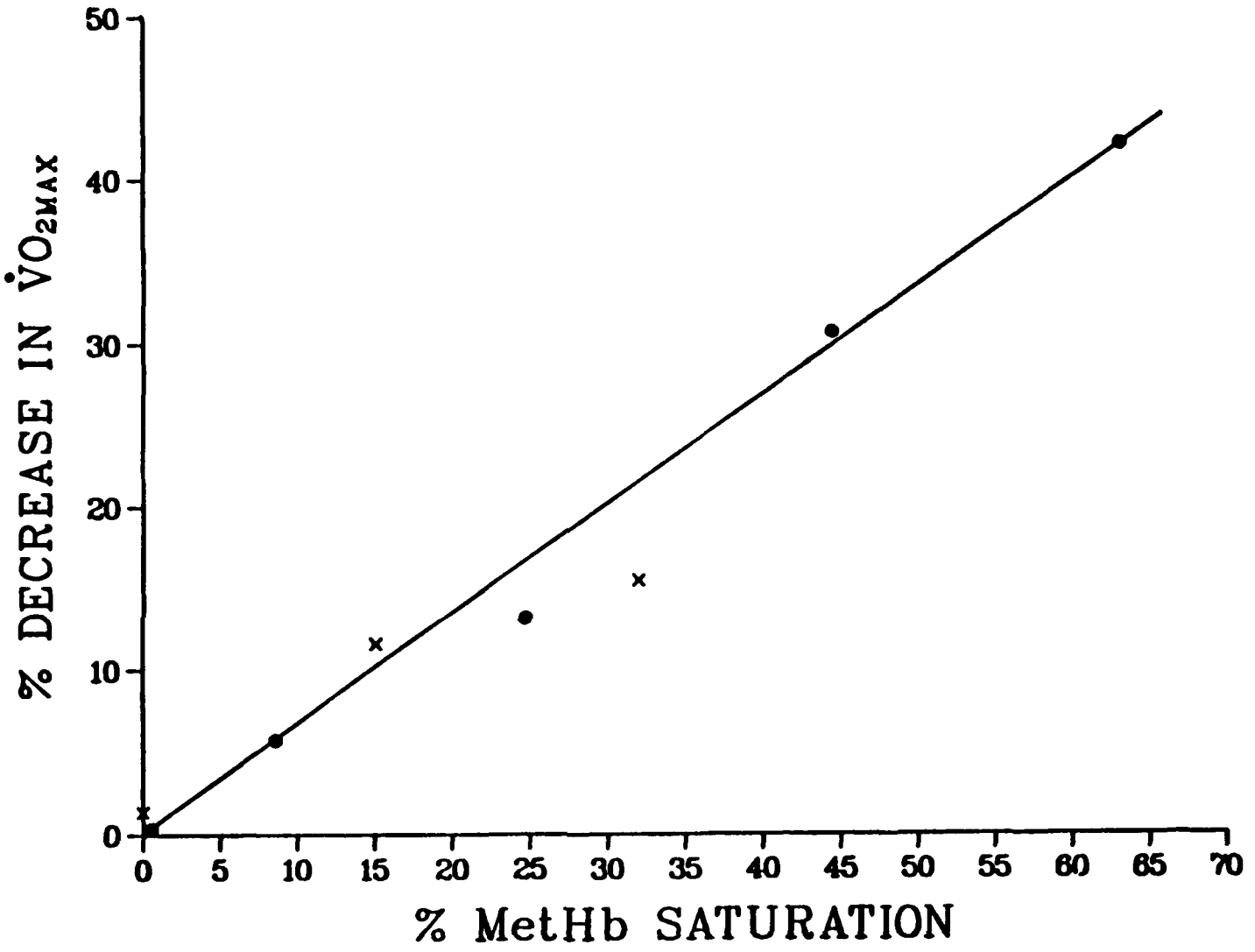


Figure 3: Percentage decreases in maximum oxygen consumption ($\dot{V}O_{2max}$) following artificially-induced anemia (X) compared to MetHb-associated reductions in maximum oxygen consumption (●).

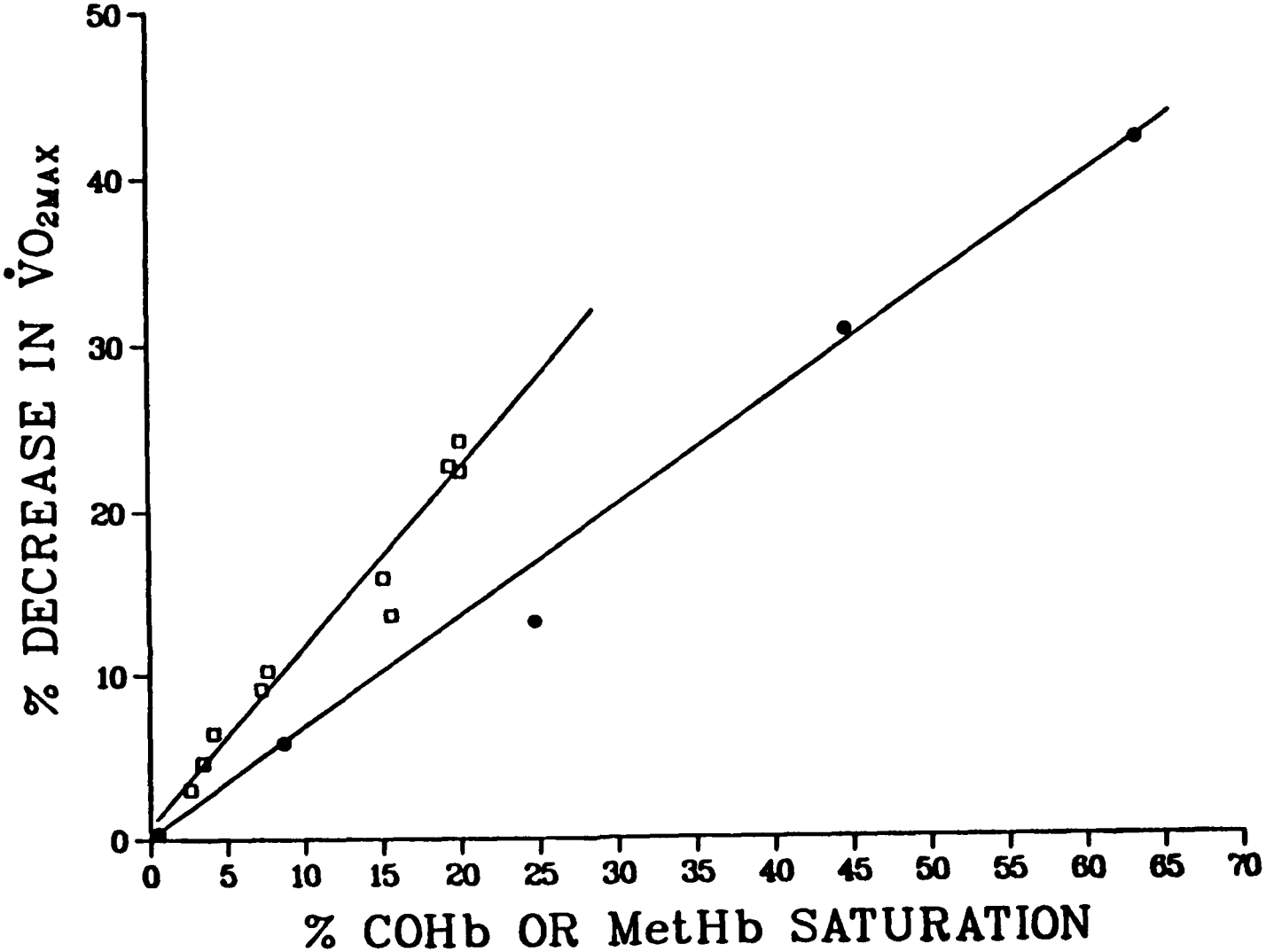


**Figure 4 :** Reported percentage reductions of maximum oxygen consumption in the human at varying % carboxyhemoglobin saturations (□) relative to the percentage reductions in maximum oxygen consumption observed in the rat experiencing differing levels of methemoglobinemia (●).

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Kon, K., Maeda, N., and Shiga, T.: Effect of nitric oxide on the oxygen transport of human erythrocytes. J. Toxicol. Environ. Health 2:1109-1113, 1977.

Stavert , D.M., Lehnert, B.E.: Nitric oxide and nitrogen dioxide as inducers of acute pulmonary injury when inhaled at relatively high concentrations for brief periods. Inhal. Toxicol. 2:53-67, 1990.

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# SECTION G

## Objective 12: To develop measurement techniques using partial body plethysmography and multi-animal measurement data acquisition systems to record and calculate ventilatory parameters during exposure to toxic substances.

## Objective 13: To characterize the ventilatory response to inhaled $NO_2$ and enhanced ventilation during $NO_2$ + $CO_2$ inhalation in order to relate $NO_2$-induced injury with an estimate of inhaled dose.

**Results:** In collaboration with the WRAIR Respiratory Branch, a system was successfully developed to measure ventilatory patterns of up to 6 animals simultaneously during periods of gas inhalation. Minute ventilation ($V_E$) is reduced with increasing $NO_2$ exposure concentrations. $V_E$ progressively falls as the duration of exposure to $NO_2$ increases, and this progressive reduction is exclusively related to a reduction in tidal volume ($V_t$). $CO_2$-induced increases in $V_E$ are attenuated by the concurrent inhalation of high concentration of $NO_2$ and they are essentially eliminated at very high concentration of $NO_2$. The diminished $V_E$ response to $CO_2$ during exposure to high concentrations of $NO_2$ are exclusively due to decreases in $V_t$. The severity of acute lung injury increases with increasing equivalents to $NO_2$. $V_E$ decreases as $NO_2$ concentration increases. Generally, the severity of acute lung injury in response to $NO_2$ inhalation is increased by an increase in $V_E$ during exposure. The severity of acute lung injury for a given concentration of $NO_2$ tends to scale proportionally with inhaled dose of $NO_2$. With or without $CO_2$, the highest $NO_2$ concentration studied (1000 ppm), caused a greater than expected injurious response.

**Reports:**

*Lehnert, B.E., Archuleta, A.C., Ellis, T., Session, W.S., Lehnert, N.L., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environmental and Nutritional Interactions (in press), 1992.*

*Stavert, D.M., Archuleta, D.C., Lehnert, B.E.: Relative acute toxicites of hydrogen fluoride, hydrogen chloride, and hydrogen bromide in nose- and pseudo-mouth-breathing rats. Fund. Appl. Toxicol. 16:636-655, 1991.*

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*Martinez, M., Archuleta, D., Stavert, D.M., Lehnert, B.E.: Nitrogen dioxide ($NO_2$) induced acute lung injury relative to increased minute ventilation during $NO_2$ exposure. The Toxicologist 11:A340, 1991.*

*Martinez, M., Archuleta D., Stavert, D.M., Lehnert, B.E.: Nitrogen dioxide ($NO_2$) induced acute lung injury relative to increased minute ventilation during $NO_2$ Exposure. 30th Annual Meeting of the Society of Toxicology, Dallas TX, The Toxicologist 11:A340, 1991.*

*Mundie, T., Ripple G., Stavert, D.M., Lehnert B.E.: Ventilatory changes upon the inhalation of high concentrations of nitrogen dioxide. 28the Annual Meeting of the Society of Toxicology, Atlanta, GA., 1989. The Toxicologist 9:A746, 1989.*

## INTRODUCTION

How much $NO_2$ is actually inhaled during an acute exposure to this oxidant gas is a major determinant of the resulting pathological response. Essentially no information is available about how minute ventilation ($V_E$) may be altered during the inhalation of high concentrations of $NO_2$. Additionally, little experimental attention has been given to characterizing the effects of increased ventilation during exposure to $NO_2$, such as may occur during the concurrent inhalation of $CO_2$, a major gas constituent in many typical combustion atmospheres where $NO_2$ may be present. The major objectives of this component of the project were to: 1) to further characterize the ventilatory response(s) to the inhalation of relatively high concentrations of $NO_2$, 2) to examine how the ventilatory response to $CO_2$ is altered by the concurrent inhalation of high concentrations of $NO_2$, 3) to investigate the relative severities of pulmonary injury caused by $NO_2$ when inhaled under "normal" $V_E$ and "$CO_2$-driven" $V_E$ conditions, 4) to examine the severity of $NO_2$-induced lung injury in the context of actual inhaled dose, and 5) to further assess the relationship of $NO_2$-induced lung injury in terms of mass concentration and exposure duration.

## MATERIALS AND METHODS

*Measurement of the Ventilatory Status of Rats:* In order to assess the pulmonary functional status of rats during and after exposure to test atmospheres, groups of nose breathing rats were placed in partial body-flow plethysmographs that were attached to an exposure chamber. Prior to the actual exposures, the rats were trained daily for 3 days to sit quietly within the plethysmographs.

The plethysmographs consist of a nose section, head section, body section, and a flow resistance plug, Figure 1. The nose section allows for the attachment of the plethysmograph to an exposure system manifold. The head section, made from Teflon®, contains a rubber dam diaphragm that effectively seals the body of the rat into the plethysmograph. The head portion also contains a neck brace to positionally maintain the head of a rat in the diaphragm. The body section of the plethysmograph, which contains the body of the rat, attaches to the nose section and the flow resistance plug via O-rings. The flow resistance plug consists of a port covered with four layers of 400-mesh SS cloth, which provides a resistance to flow created by the expansion and contraction of the thorax within the body section of the plethysmograph. Another port provides attachment to a differential pressure transducer (± 2.5 cm $H_20$, Validyne Engineering Corp., Northridge, CA) connected to an automated data acquisition and analysis system that consists of an IBM PC running ASYST (Adaptable Laboratory Software, Rochester, NY) software tools and custom software. The plethysmograph is linear to 100 ml/sec steady flow, and it gives a 1.54 cm

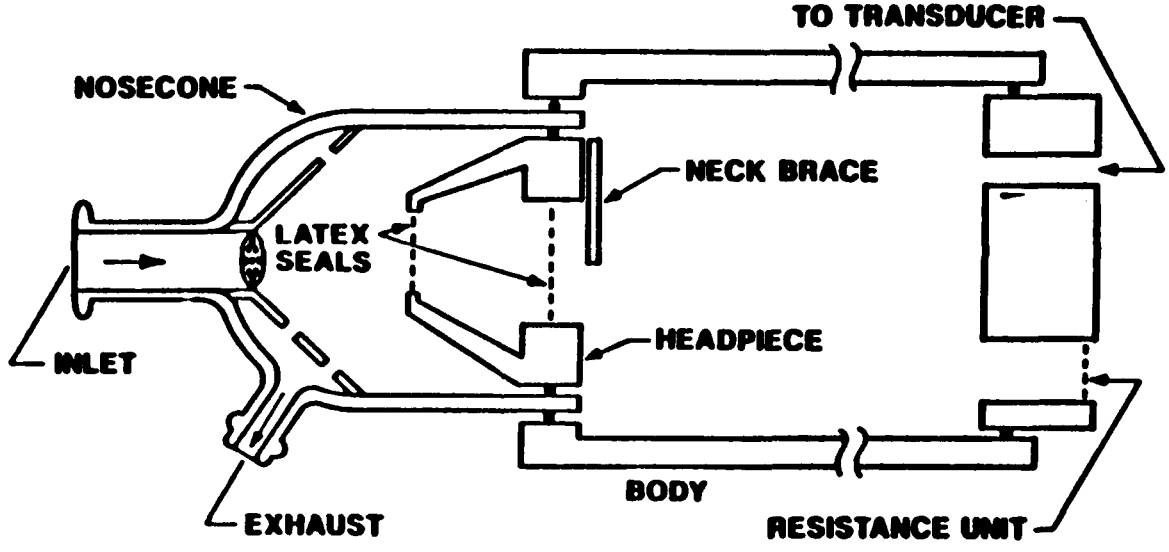


**Figure 1.** Illustration of a partial body flow plethysmograph used for the ventilatory measurements in this study. The nose cone is the front portion of a LANL quartz glass exposure tube. The headpiece is designed to hold the head of the rat in position during an exposure as well as to seal the animal within the body portion of the plethysmograph. The body portion and resistance unit allow for the measurement of pressure fluctuations within the plethysmograph, which are proportional to volume changes.

$H_2O$ response at this latter flow. The dynamic performance of the plethysmographic system has a uniform response up to 7 Hz (Stavert et al., 1986; Stavert and Lehnert, 1988). The system is able to continuously measure ventilatory patterns of up to six rats contained in separate plethysmographs and convert the flow signals to breath-by-breath measurements of minute ventilation ($V_E$), breathing frequency (f), tidal volume ($V_t$), inspiratory time, expiratory time, and inspiratory time/expiratory time ratios.

*Exposures:* On the day of exposure, the animals were placed into the plethysmographs and they were provided clean filtered air for 5 min while pre-exposure ventilatory parameters, i.e., $V_E$, tidal volumes ($V_t$), and breathing frequencies (f), were measured. Exposure atmospheres, consisting of either filtered air with or without 5% $CO_2$ (controls), or 100, 300, or 1000 ppm $NO_2$ with or without 5% $CO_2$, were then delivered to the rats for various durations ranging from 1 min to 20 min while the ventilatory parameters were measured. For 1000 ppm short-duration exposures, the animals were pre-exposed to 5% $CO_2$ before the $NO_2$ exposure to initiate an increase in $V_E$. After cessation of the exposures, clean air was again delivered to the animals for 5 to 15 min while post-exposure ventilatory parameters continued to be measured. After the exposures, the animals were returned to their cages until they were sacrificed 24 hrs later for lung gravimetric measurements.

$NO_2$ exposure atmospheres were generated by mixing pure $NO_2$ with anhydrous HEPA filtered air within the quartz glass mixing chamber. $CO_2$ was added and adjusted to 5% immediately upstream of the exposure chamber. As previously indicated, the animals were exposed to the atmospheres while in the flow plethysmographs. A Teflon head piece with 2 rubber dams effectively sealed the body of the animal within the plethysmograph, and, as previously indicated, a neck brace ( Figure 1) stabilized the animal during exposure.

*Lung Gravimetric Studies:* Lung gravimetric increases were used to index the severity of the pulmonary edematous response in these studies. Rat sacrifices were initiated by I.P injections of 50 mg pentobarbital sodium. The trachea and lungs were excised, and the heart, extra-pulmonary mediastinal tissue, and the esophagus were removed. The lungs were blotted and weighed (Lung Wet Weight, LWW). The bronchus leading to the right cranial lobe (RCL) was ligated with fine suture and the RCL was removed and weighed (Right Cranial Lobe Wet Weight, RCLWW). Following the gravimetric measurements, the trachea and lungs, minus the RCL, was cannulated with an 18-ga. needle that was secured with ligature and the lungs were subsequently infused and fixed at a constant pressure of 30 cm $H_2O$ with 10% formalin in phosphate buffered saline. The RCLs were oven dried to a constant weight at 100°C for 36 hrs and reweighed (Right

Cranial Lobe Dry Weight, RCLDW). Inasmuch as the RCLWW and RCLDW values generally scaled with the corresponding LWW values, only the LWW data will be summarized herein.

## RESULTS

*Ventilatory Responses to $NO_2$:* In general, $V_E$ was reduced upon the inhalation of most $NO_2$ atmospheres, Figure 2, compared to the $V_E$ measured from air exposed animals, and the $NO_2$-associated reductions in $V_E$ were greater with the higher concentrations of inhaled $NO_2$. Overall $V_E$ reductions of ~7% and ~15% were measured during the 100 ppm $NO_2$ x 15 min and 20 min exposures, respectively, while greater reductions in $V_E$ of ~20% and ~28% were measured during the 1000 ppm $NO_2$ x 1 and 2 min exposures. The above reductions of $V_E$ were primarily due to reductions in $V_t$, Figure 2. Exposures to 100 ppm $NO_2$ for 10 min, however, did not produce any major changes in $V_E$. While f increased slightly during most $NO_2$ exposures compared to f measurements on air exposed animals, Figure 2, $V_t$ decreased by as much as 40%, with the greatest reductions in $V_t$ occurring during the exposures to the highest $NO_2$ concentrations studied.

*Ventilatory Responses to $CO_2$ and Co-Inhaled $NO_2$ and $CO_2$:* The ventilatory response to $CO_2$ during air breathing was a $V_E$ increase of ~65% due to increases in both $V_t$ (~42%) and f (~21%), Figure 2. The ventilatory response to $CO_2$ during concurrent $NO_2$ inhalation were reduced, Figure 2. Some of these are graphically depicted in Figures 3 and 4. $CO_2$-associated increases in $V_E$ averaged only ~40% during the 100 to 300 ppm $NO_2$ + 5% $CO_2$ exposures, compared to only a ~25% increase in $V_E$ during the 1000 ppm $NO_2$ for 2 min exposure, Figure 2. No significant increase in $V_E$ occurred during the 1000 ppm $NO_2$ for 1 min exposure. The increased $V_E$ measured during $NO_2$ + $CO_2$ exposures was primarily related to increases in $V_t$ and to a lesser extent to increases in f. Breathing frequencies actually fell slightly from air +$CO_2$ control values during the 1000 ppm $NO_2$ + $CO_2$ exposure.

*Lung Gravimetric Changes:* The LWW, Figure 5, measured 24 hrs after the $NO_2$ exposure regimens were all significantly greater than the values obtained with animals exposed to air only. LWW tended to increase with increasing $NO_2$ exposure equivalents. The LWW of animals exposed to the 100 ppm and 300 ppm $NO_2$ + 5% $CO_2$ atmospheres were also increased above values measured from animals exposed to the various concentrations of $NO_2$ alone. LWW values from animals exposed to the 1000 ppm $NO_2$ + 5% $CO_2$ atmospheres, however, were not significantly higher than values obtained from the group of rats that were exposed to $NO_2$ only. This latter observation may be explained by the substantial attenuation of the ventilatory response

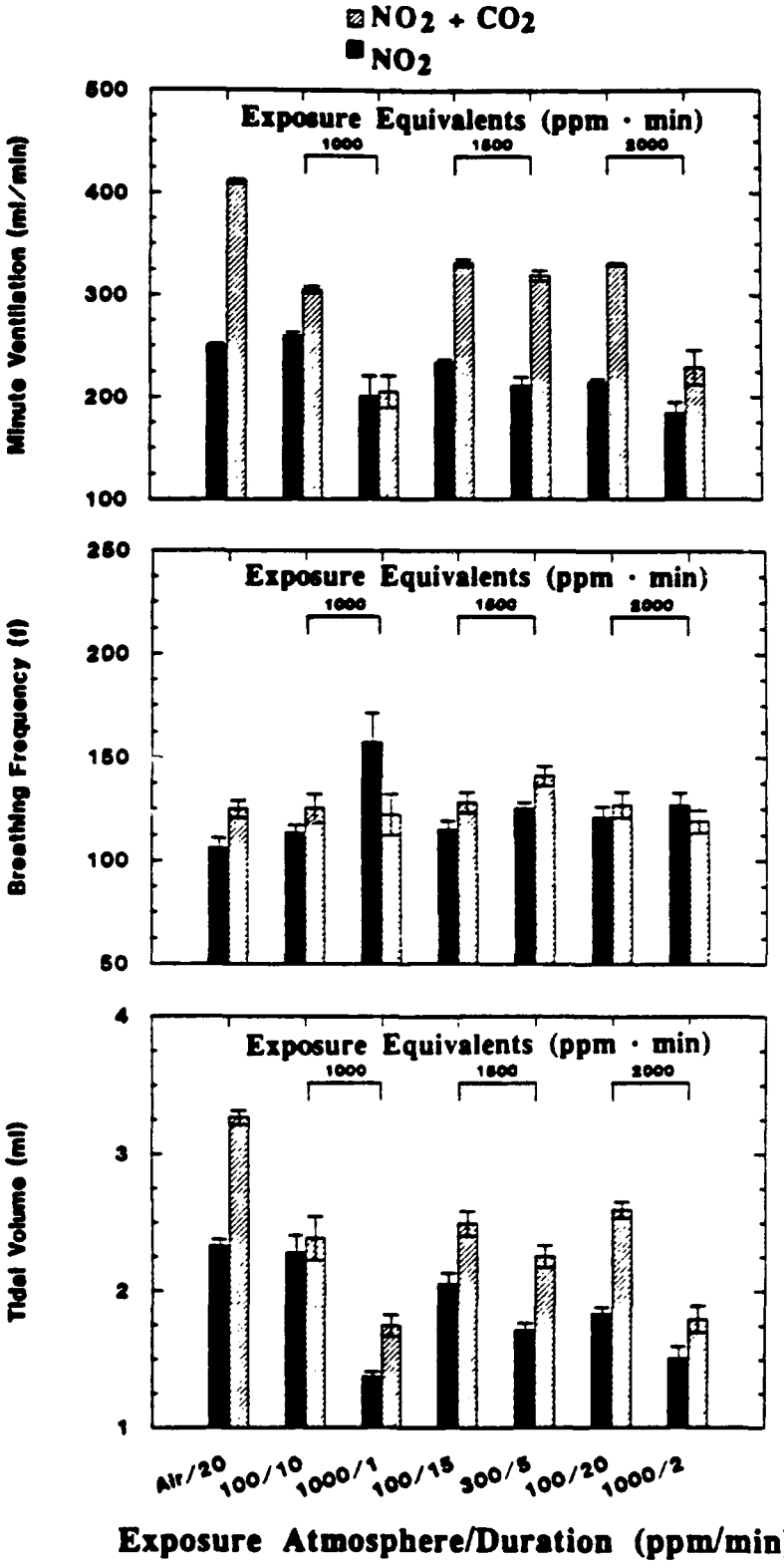


Figure 2: Minute ventilations ($V_E$), breathing frequencies (f), and tidal volumes ($V_t$) of rats during exposures to air or to $NO_2$ at different concentrations (ppm) and durations (min) in the presence or absence of 5% $CO_2$. Each bar represents the mean ± S.E. of average values measured over the course of exposure of 6-12 rats.

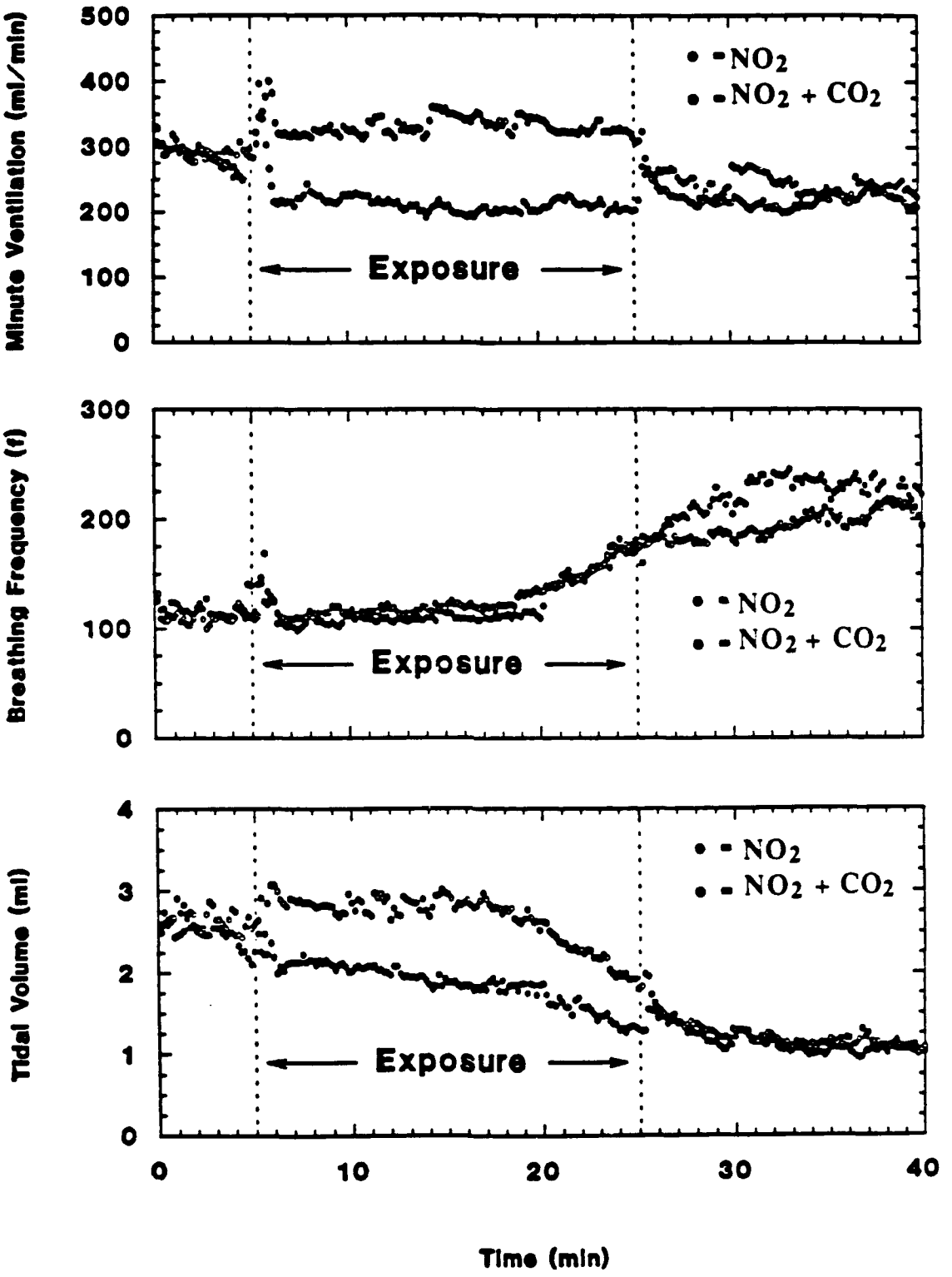


Figure 3: Minute ventilations ($V_E$), breathing frequencies (f), and tidal volumes ($V_t$) measured before, during, and after the exposure of rats to 100 ppm $NO_2$ for 20 min in the presence or absence of 5% $CO_2$. Each point represents the mean values of breath by breath ventilatory measurements made over a 10 sec period (N=12 animals).

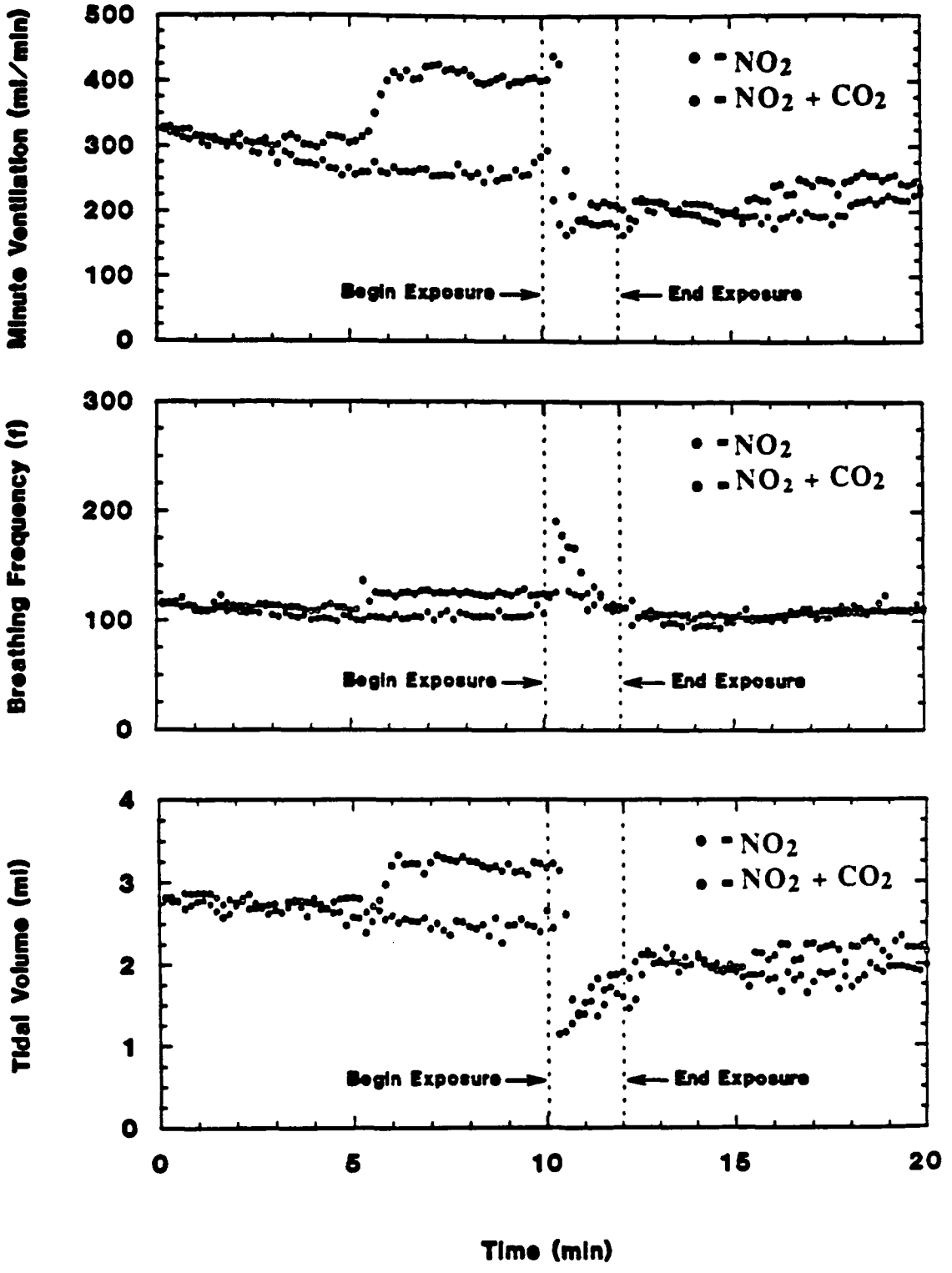


Figure 4: Minute ventilations ($V_E$), breathing frequencies (f), and tidal volumes ($V_t$) measured before, during, and after the exposure of rats to 1,000 ppm $NO_2$ for 2 min in the presence or absence of 5% $CO_2$. In this experiment, the $NO_2$ + $CO_2$ group of rats were "pre-exposed to 5% $CO_2$ during the last 5 min of the pre-$NO_2$ exposure period. Each point represents the mean values of breath by breath ventilatory measurements made over a 10 sec period (N=9-11 animals).

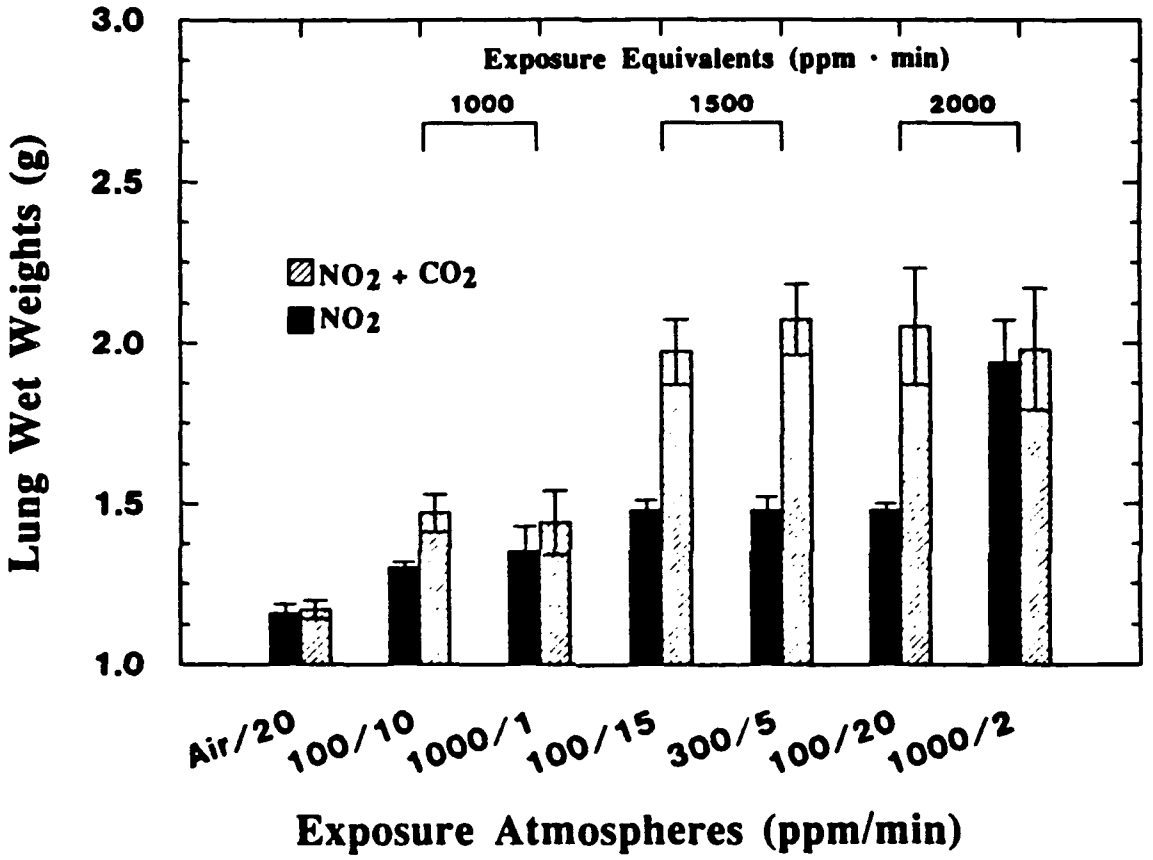


Figure 5: Lung wet weights (LWW) of rats 24 hrs after exposure to the various air or $NO_2$ test atmospheres with and without 5% $CO_2$. Each bar represents the mean ± S.E. of data obtained from 6-12 rats.

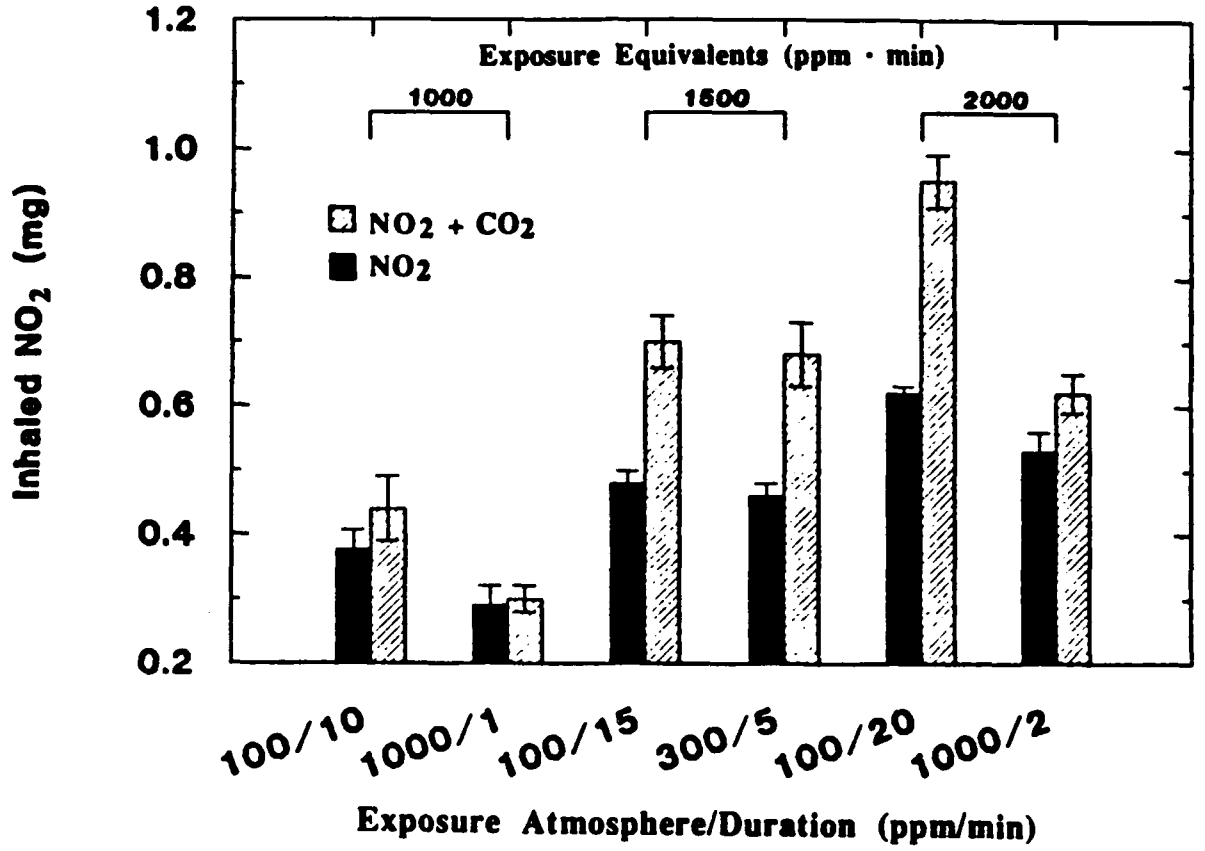


Figure 6: The inhaled doses of $NO_2$ in the $NO_2$ and $NO_2$ + $CO_2$ exposures were calculated as: (exposure atmosphere concentration ($mg \cdot L^{-1}$) · (sum of the mean minute ventilation over a given exposure duration). Nominal exposure concentrations were used in these calculations, but measured exposure concentrations did not vary from the nominal concentrations by more than 2%.

to $CO_2$ by the 1000 ppm $NO_2$, and, accordingly, by the closeness of the inhaled doses of $NO_2$ in the presence and absence of $CO_2$, Figure 6. Generally, the lung gravimetric parameters correlated with the calculated dose of inhaled $NO_2$. [The estimated inhaled dose of $NO_2$ was calculated as the sum of the $V_E$ (ml) times the exposure concentration of $NO_2$ (mg/L).] A notable exception was that the LWW values for animals exposed to the 1000 ppm $NO_2$ atmospheres for 2 min were greater than expected from the estimated inhaled dose, see Figures 5 and 6.

## SUMMARY

The results of this study component indicate: 1) $V_E$ is reduced with increasing $NO_2$ concentrations, 2) with the exception of exposures to very high mass concentrations of $NO_2$, i.e., 1000 ppm, $V_E$ progressively falls as the duration of exposure to $NO_2$ increases, and this progressive reduction is essentially exclusively related to a reduction in $V_t$, 3) $CO_2$-induced increases in $V_E$ are attenuated by the concurrent inhalation of high concentrations of $NO_2$, and they are nearly eliminated at very high concentrations of $NO_2$, 4) the diminished $V_E$ response to $CO_2$ during high concentration $NO_2$ exposure are exclusively due to decreases in $V_t$, 5) the severity of acute lung injury generally increases with increasing exposure equivalents to $NO_2$, even though $V_E$ decreases as $NO_2$ concentration increases, 6) generally, the severity of acute lung injury in response to $NO_2$ inhalation is increased by an increase in $V_E$ during exposure, 7) the severity of acute lung injury for a given concentration of $NO_2$ does not necessarily scale proportionally with inhaled dose of $NO_2$, and 8) with or without $CO_2$, a 1000 ppm $NO_2$ concentration caused a greater than expected injurious response.

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# SECTION H

**Objective 14: To develop a means by which pulmonary extracellular fluid lining constituents and blood compartment biochemical constituents can be resolved and quantified in order to characterize the hyperpermeability response to $NO_2$.**

**Objective 15: To assess the relationship between changes in the numbers and types of lung free cells following $NO_2$ exposure and the hyperpermeability response.**

**Results:** Development of an HPLC system that quantitatively resolves eleven lavage fluid constituents, including proteins and phospholipids, and the identification of three lavage fluid constituents that are common to the blood compartment. Development of a more rapid ion exchange HPLC technique for quantitating albumin, transferrin and immunoglobulins in lavage fluid. Demonstration of a lack of a relationship between $NO_2$-induced hyperpermeability and increases in polymorphonuclear leukocytes and alveolar macrophages in the lung's lavageable air space compartment.

**Reports:**

*Lehnert, B.E., Archuleta, A.C., Ellis, T., Session, W.S., Lehnert, N.L., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environmental and Nutritional Interactions (in press), 1992.*

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## INTRODUCTION

The kinetics of development of pulmonary edema and possible relationships between this response and excessive occurrences of polymorphonuclear leukocytes (PMN) and alveolar macrophages in the air spaces following exposure to high concentrations of $NO_2$ have received little experimental attention. Yet, such cells have been implicated in mediating hyperpermeability changes in the lung's microvasculature in a variety of conditions, probably via the elaboration of injurious reactive oxygen species, proinflammatory cytokines, granule proteins, and proteases (e.g., Johnson and Malik, 1980; van der Valk and Herman, 1987; Okrent et al., 1990; Sibille and Reynolds, 1990), and it remains possible that the recruitment of PMN and additional AM into the lung's air spaces may contribute to the pulmonary edematous response to $NO_2$ by causing injury to epithelial cells in addition to that caused by the direct action of $NO_2$. In this study component, accordingly, we exposed laboratory rats to a relatively high concentration of $NO_2$ (100 ppm for 15 min) in order: 1) to investigate the post-exposure pattern of development of pulmonary edema, as assessed by changes in protein constituents in bronchoalveolar lavage fluids, and 2) to assess how the pulmonary edematous response may be temporally related to $NO_2$-induced alterations in the numbers and types of lung free cells.

## Methods and Materials

*Animals:* Adult, male Fischer-344 rats (240-260 g, specific-pathogen-free, Sasco, Omaha, NB, specific-pathogen-free, virus-free, Harlon Sprague Dawley, Indianapolis, IN) were delivered to Los Alamos National Laboratory and acclimated in a facility accredited by the American Association of Laboratory Animal Care for a minimum of 10 days prior to experimental use. During this period and following the test gas exposures, the rats were housed in rooms that received HEPA-filtered air. The animals were maintained in suspension cages (two per cage) covered with spun polyester filters (DuPont no. 22 spinbound polyester filter, E.I. DuPont Co., Wilmington, DE). Sterilized rat food (Teklad mouse/rat food, L-485, Harlan Sprague Dawley Inc., Madison, WI) and water were provided *ad libitum*. Sera samples were obtained from representative animals upon arrival, as well as from animals maintained in the animal facility over the course of each exposure study, for serologic analyses (Microbiological Associates, Inc., Bethesda, MD). Consistently, all animals were found to be negative for reovirus Type 3, Kilham's rat virus, pneumonia virus of mice, Sendai virus, and cilia-associated respiratory bacillus.

$NO_2$-exposed groups of rats and their corresponding controls (sham-air) were closely matched according to age and body weight at the time of the exposures so that the sizes of their lungs, as indexed by mass, would be closely similar at the time of exposure (Tillery and Lehnert, 1986).

*$NO_2$ , Air Exposures, and Animal Sacrifice Times:* $NO_2$, generated from dinitrogen tetroxide (Stavert et al., 1986; Stavert and Lehnert, 1989; ), was added to anhydrous and HEPA-filtered air and fed into a mixing chamber. The test atmospheres containing 100 ppm $NO_2$ were routed at a flow rate of 12 $L \cdot min^{-1}$ to animals contained in nonavoidance, nonconstraining exposure tubes attached to a radial, quartz glass exposure system, as described elsewhere (Stavert and Lehnert, 1989; Stavert and Lehnert, 1990). Exposure concentrations of $NO_2$ were monitored with a dual channel ir-uv spectrometer (Binos Inficon, FRG) calibrated with a primary gas standard (N.B.S. grade). The $t_{99}$ equilibrium values for the nominal $NO_2$ concentrations used in this study were <34 sec. The rats were exposed to the 100 ppm concentration of $NO_2$ for 15 min. Control animals were exposed to filtered air only for 15 min.

Following $NO_2$-exposure, the rats were sacrificed at 8, 24, 48, 72, and 96 hrs for the lavage studies. Air exposed rats were sacrificed 24 hrs after exposure for the lavage studies. It should be noted that in several different studies, we have found no differences in the lavage fluid constituents of air exposed rats relative to cage control animals over extended post-exposure periods.

*Bronchoalveolar Lavage Studies:* Bronchoalveolar lavage was used in the study component in which the relationship between high concentration $NO_2$-induced hyperpermeability and the recruitment of lung phagocytes into the alveoli was examined. The animals from groups of $NO_2$- and air-exposed animals were anesthetized by I.P. injections of pentobarbital sodium (50 mg). Prior to complete apnea, the rats were exsanguinated via carotid artery transection, and each animal's trachea was cannulated with a blunt, 18 ga. needle secured with ligature. The lungs and trachea were removed *en bloc* from the thoracic cavity after perforation of the diaphragm, and the heart and esophagus were removed. The bronchoalveolar lavage procedure consisted of six sequential lung wash cycles with 8 ml of room temperature, divalent cation-free, phosphate-buffered saline (PBS, made with HPLC grade water) being used for each cycle (Lehnert et al., 1990). The lung washings were performed with gentle massage of the lungs during the infusion and aspiration of the PBS. The lavage fluid cumulatively retrieved from each animal's lung was pooled in a centrifuge tube maintained on ice. On average, >90% of the instilled

lavage fluid was recovered from the lungs of the air-exposed and $NO_2$-exposed rats. Small aliquots of the retrieved fluids were used to obtain hemocytometric counts of the lung free cells and to examine the viabilities (or actually cell membrane integrities) of the cells using trypan blue dye. The lavage fluids were then centrifuged at 300 g for 10 min at 4°C to sediment the cells, and the supernates above the cell pellets were removed and recentrifuged at 2300 g for 10 min at 4°C to remove remaining particulate, acellular material (Gurley et al., 1988). The supernates obtained after this last centrifugation cycle were stored in glass tubes at -70°C until further analyzed. These samples are referred to herein as bronchoalveolar lavage fluid (BALF).

Cytocentrifuged slide preparations of the cells recovered from the lung washings were made with a cytocentrifuge (Shandon Southern Cytospin, Shandon Southern Products, Ltd., Cheshire, UK). Cell differentials (light microscopy) were performed by examining 200-400 cells that were stained with Camco Quik Stain® (Cambridge Chemical Products, Inc., Ft. Lauderdale, FL).

As a general index of permeability pulmonary edema, aliquots of BALF were used to determine the amount of lavaged protein (Lehnert et al., 1991) using the method of Lowry et al. (1951) and bovine serum albumin as standards (Sigma Chemical Co.). Specific biochemical constituents in BALF were further resolved and quantitated via ion exchange high performance liquid chromatography (HPLC) (Lehnert et al., 1992). Briefly, the basic HPLC system consisted of a Waters Model 600E Controller (Waters Associates, Milford, MA) and an ion exchange column (IEC) (Protein-Pak DEAE-5PW column, 7.5 mm x 7.5 cm, Waters Associates). A 300 µl BALF sample was loaded onto the IEC, and constituents contained therein were eluted over a 30 min period at a flow rate of 1 $ml \cdot min^{-1}$ using a linear NaCl and pH gradient ranging from 20 mM Tris buffer (pH 8.5) to 20 mM Tris buffer containing 0.3 M NaCl (pH 7.0). Eluted fractions were detected by uv absorbance at 280 nm using a Waters 484 tunable absorbance detector (Waters Associates) that was connected to a Waters Model 745B Data Module (Walters Associates). The various fractions were quantified by measuring the areas under their resolved peaks. To identify blood-derived protein constituents in BALF, standards for the proteins of major interest, e.g., rat albumin (Organon Treknika Corp., West Chester, PA), were prepared in PBS for the HPLC analyses.

## RESULTS

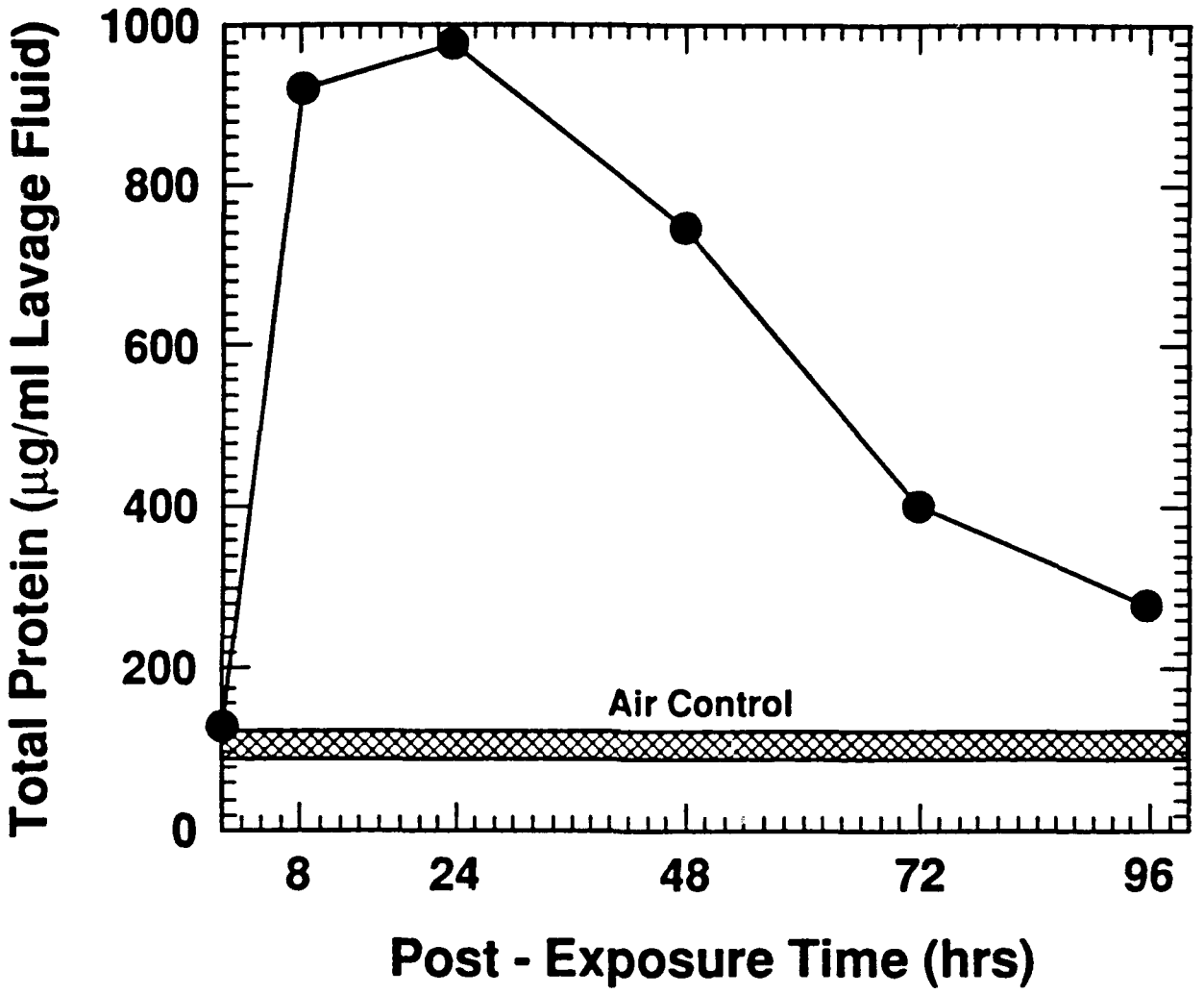
*$NO_2$-Induced Biochemical Changes in Lavage Fluids:* The temporal pattern of the hyperpermeability response to high $NO_2$, as indexed by increases in lavageable protein, is shown in Figure 1. A near-maximal increase in protein was well in place at the earliest 8

hr post-exposure time point, with the maximal increase in protein occurring 24 hrs after $NO_2$ exposure. Consistent with ongoing lung repair, lavageable protein levels progressively subsided thereafter. In that the Folin-Lowry assay for measuring protein is also sensitive to some non-protein biochemicals, e.g., hydrolytic products of nucleic acids (Shepard and Hopkins, 1963) that may occur in the lung's extracellular lining fluid upon cell death and disintegration, and because other sources of abnormal increases in protein in BALF may include the excessive production of airway mucus (glycoproteins) and the release of proteins from lysed cells, we further confirmed that the increases in lavaged protein were indeed associated with increases in BALF albumin, a protein derived from the blood compartment, Figure 2. Additionally, the pattern of increases in lavageable transferrin, another blood-derived protein, was virtually identical to that of albumin following the $NO_2$ exposure (data not shown), thus further confirming that the more general protein response summarized in Figure 1 reliably indexed changes in the lung's permeability status.

*$NO_2$-Induced Changes in Lung Free Cells:* The kinetic patterns of increases in lavageable PMN and AM are summarized in Figure 3. Although an increase in PMN was observed as of 8 hrs after $NO_2$ exposure, major increases in this cell type did not occur within the first 24 hr post-exposure period when the hyperpermeability response was well in place. In fact, the maximal increase in lavageable PMN numbers was observed 72 hrs after $NO_2$ exposure, when the hyperpermeability response had substantially subsided. As well, no evidence of an increase in AM numbers was obtained during the post-exposure period when the increase in lung permeability increase was maximal. The peak increase in this cell type occurred at the 48 hr post-exposure time point when the hyperpermeability response was in fact decreasing.

## DISCUSSION

Collectively, these findings suggest that no relationship exists between the hyperpermeability response to $NO_2$ and elevated numbers of PMN and AM in the alveoli. The above results, of course, do not prove that neutrophils and excessive macrophages have no influence on the epithelial injury caused by $NO_2$. If they do, however, their overall contribution to the acute hyperpermeability response would seem to be minor compared to the direct epithelial cytotoxicity of $NO_2$. This conclusion is similar to that recently derived from a study of the role PMN may play in mediating epithelial cell damage following exposure to ozone, another oxidant gas (Pino et al., 1992; Young and Bhalla, 1992).




Figures 1: Changes in lavageable protein over a 96 hr period following exposure to 100 ppm $NO_2$ for 15 min. N=4 rats per time point.

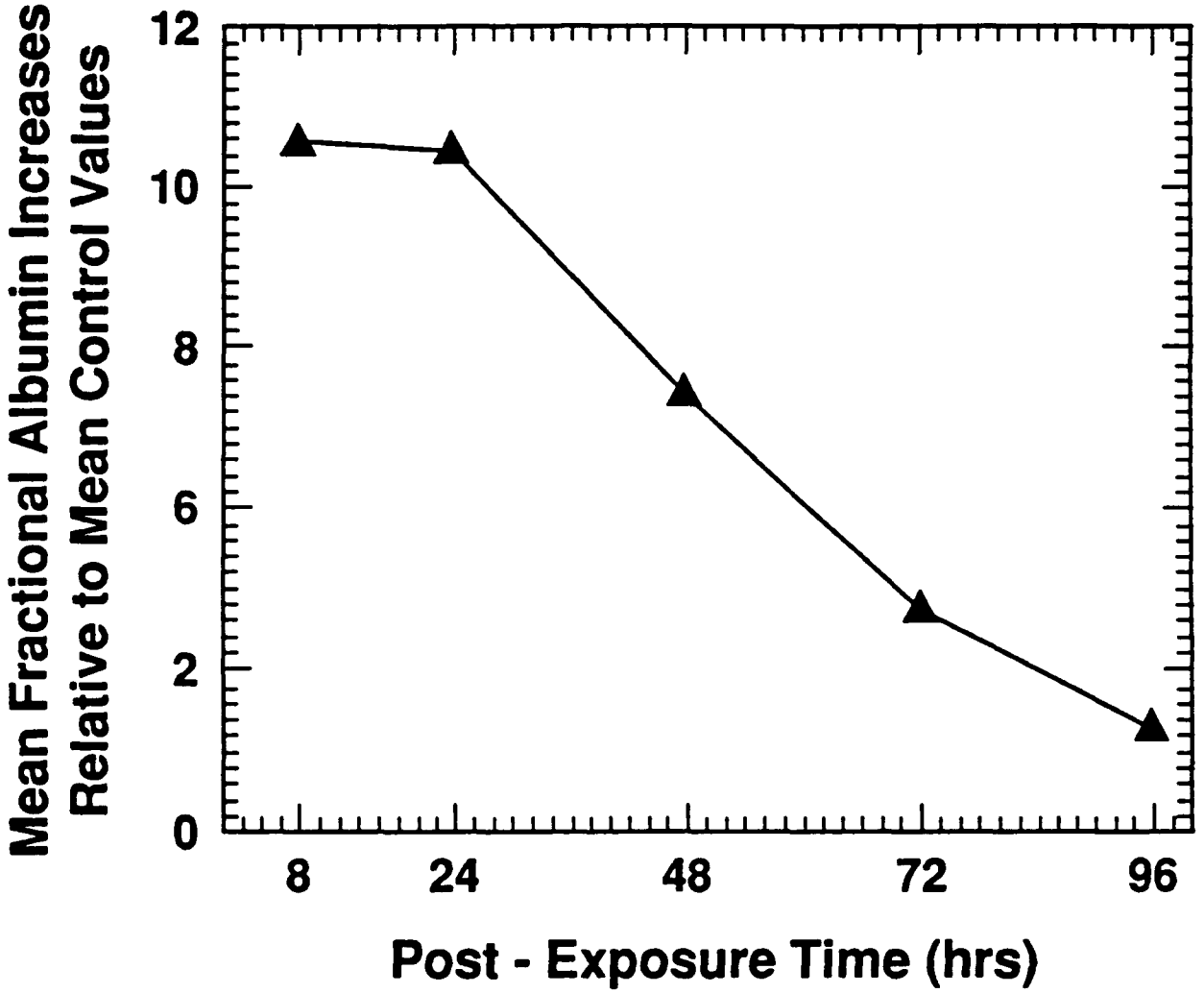



Figure 2: The post-exposure increases in BALF protein were associated with corresponding increases in lavaged albumin , as ascertained by ion exchange HPLC analysis (Lehnert et al., 1992). N=4 rats per time point. The hyperpermeable response was essentially totally in place as of 8 hrs after $NO_2$ exposure, whereas major increases in lavaged PMN occurred at much later post-exposure times, see Figure 3.

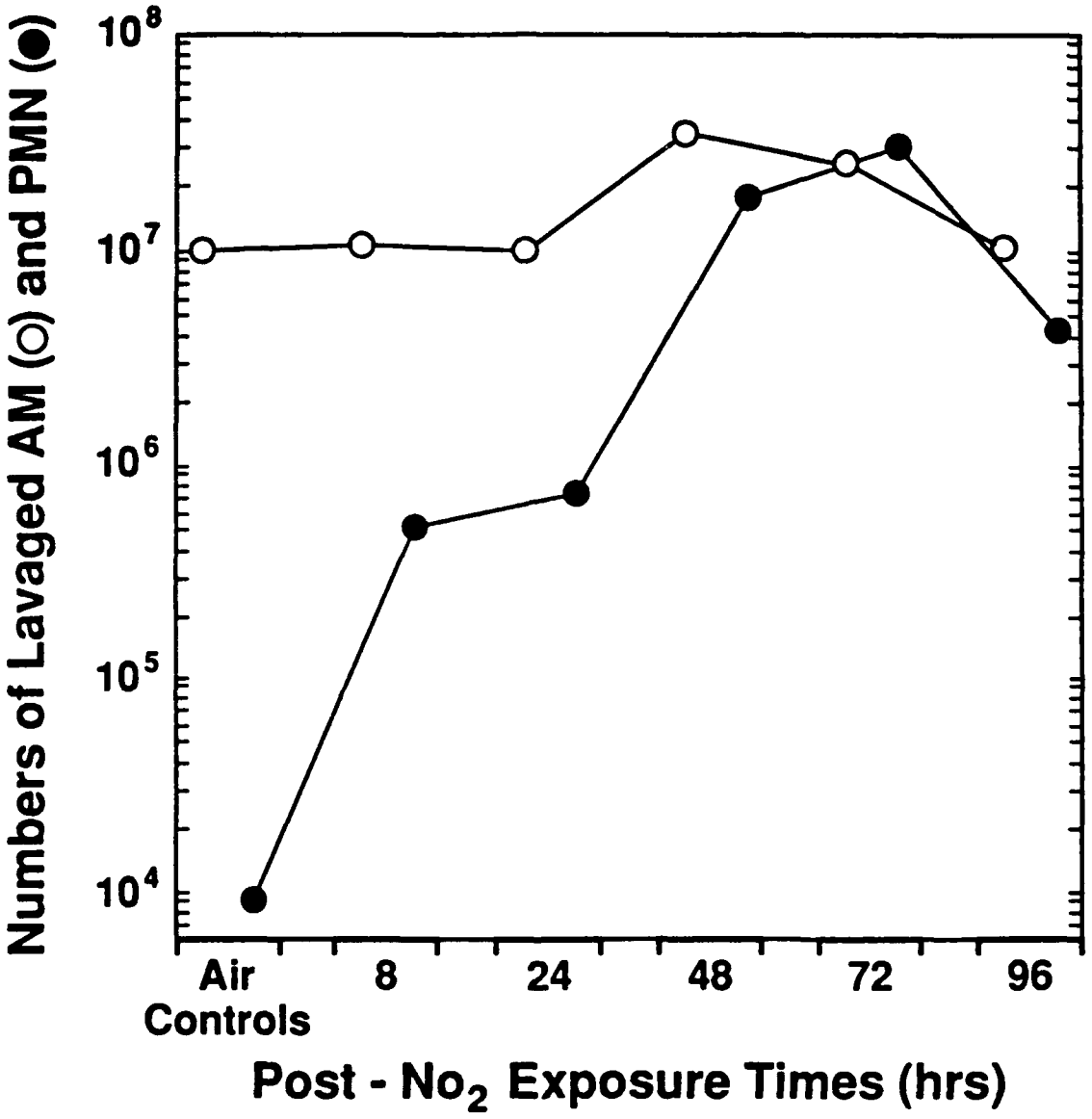



Figure 3: Lung "free cell response" following the exposure of rats to 100 ppm $NO_2$ for 15 min. No elevations in AM numbers occurred as of 24 hrs after the exposure. Although detectable increases in PMN were observed as early as 8 hrs after exposure, substantial increases in this cell type did not occur within the first 24 hr post-exposure time. N=4 rats per exposure condition (air or $NO_2$) and time point.

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# SECTION I

## Objective 16: To characterize the injurious response following brief, "high burst" exposures of rats to very high mass concentrations of $NO_2$.

## Objective 17: To investigate the effects of post-exposure exercise on the severity of lung injury following brief, "high burst" exposures to $NO_2$.

**Results:** Demonstration that very brief exposures to high mass concentrations can result in significant lung injury. Post-exposure exercise following brief exposures to high concentrations of $NO_2$ can substantially increase the pulmonary edematous response to the point of increasing mortality.

**Reports:**

*Lehnert, B.E., Archuleta, D.C., Ellis, T., Session, W.S., Lehnert, N.M., Gurley, L.R., Stavert, D.M.: Lung injury following exposure of rats to relatively high mass concentrations of nitrogen dioxide. Environmental & Nutritional Interactions (in press), 1993.*

*Lehnert, B.E.: Post-exposure potentiation of lung injury by exercise and work performance degradation following the inhalation of pyrolysis products, National Aeronautics and Space Administration, Johnson Space Center, Houston, TX, November 13, 1992.*

## INTRODUCTION

We have previously demonstrated that exercise following the inhalation of high concentrations of $NO_2$ for a duration of 15 can significantly increase the severity of $NO_2$-induced lung injury (Stavert and Lehnert, 1989; Stavert and Lehnert, 1988; Lehnert et al., 1987; Stavert et al., 1987). In that some human exposure conditions could entail brief exposures to very high mass concentrations of $NO_2$, groups of rats were exposed for short periods to "high bursts" of $NO_2$ delivered at mass concentrations ranging from 500-2,000 ppm before being sacrificed 24 hrs later for lung gravimetric assessments to index the severity of resulting pulmonary edema. Additionally, because human exposures to very high mass concentrations could be followed by exercise, such as during the escape from $NO_2$-contaminated settings, how the injurious responses to high burst exposures to the $NO_2$ are affected by post-exposure exercise were also investigated. [It should be noted here that no significant increases in lung wet weights were observed in previous studies conducted in our laboratory after rats were exposed to up to 150 ppm $NO_2$ for 2 min durations (see Section A; Lehnert et al., 1987).]

## METHODS AND MATERIALS

*Animals:* Adult, male Fischer-344 rats (240-260 g, specific-pathogen-free, Sasco, Omaha, NB, specific-pathogen-free, virus-free, Harlon Sprague Dawley, Indianapolis, IN) were delivered to Los Alamos National Laboratory and acclimated in a facility accredited by the American Association of Laboratory Animal Care for a minimum of 10 days prior to experimental use. During this period and following the test gas exposures, the rats were housed in rooms that received HEPA-filtered air. The animals were maintained in suspension cages (two per cage) covered with spun polyester filters (DuPont no. 22 spinbound polyester filter, E.I. DuPont Co., Wilmington, DE). Sterilized rat food (Teklad mouse/rat food, L-485, Harlan Sprague Dawley Inc., Madison, WI) and water were provided ad libitum. Sera samples were obtained from representative animals upon arrival, as well as from animals maintained in the animal facility over the course of each exposure study, for serologic analyses (Microbiological Associates, Inc., Bethesda, MD). Consistently, all animals were found to be negative for reovirus Type 3, Kilham's rat virus, pneumonia virus of mice, Sendai virus, and cilia-associated respiratory bacillus. $NO_2$-exposed groups of rats and their corresponding controls (sham-air) were closely matched according to age and body weight at the time of the exposures so that their baseline

lung gravimetric values would be closely similar at the time of exposure (Tillery and Lehnert, 1986). It should also be noted that the conduct of these studies was in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Council (DHEW Pub. No. [NIH] 85-23, 1985).

*$NO_2$ and Air Exposures:* $NO_2$, generated from dinitrogen tetroxide (Freeman and Haydon, 1964; Stavert et al., 1986), was added to anhydrous and HEPA-filtered air and fed into a mixing chamber. In these studies, the $NO_2$-containing atmospheres were delivered to the animal exposure system (see Sections A, B. and D; Stavert and Lehnert, 1989; Stavert and Lehnert, 1990) at a flow rate of 40 $L \cdot min^{-1}$ so that the nominal concentrations inhaled by the rats contained in nonavoidance, nonconstraining exposure tubes attached to the radial, quartz glass exposure system were achieved within 4 sec after the onset of the exposures. Exposure concentrations of $NO_2$ were monitored with a dual channel ir-uv spectrometer (Binos Inficon, FRG) calibrated with a primary gas standard (N.B.S. grade), as previously described. Control animals were exposed to filtered air only.

*Post-Exposure Exercise:* Rats were exercised or rested after being exposed to $NO_2$ or air. Before use, these animals were subjected to a 19-20 day training program designed to behaviorally and physically condition them to perform on a treadmill, as described elsewhere (see Section B; Stavert and Lehnert, 1989); rats that were observed to be "non-runners" during the training sessions were eliminated from further study. During training, the work intensities and durations of exercise were increased daily until the rats were capable of performing a "ramp" exercise protocol. As described in Section B, the "ramp" protocol started at a treadmill velocity of 10 $M \cdot min^{-1}$, and every 30 sec thereafter the treadmill velocity was increased by 5 $M \cdot min^{-1}$ up to 60 $m \cdot min^{-1}$ (15% grade). Maintenance of running speed was encouraged by electro-shock stimulation (40 V, 2 mA) delivered via a grid mounted behind the treadmill. Prior to the "ramp" runs following exposure to test atmospheres, the rats routinely were subjected to a "familiarization run" consisting of two short runs (20 $M \cdot min^{-1}$ for 3 min, 15% grade) separated by a 3 min rest period and finally followed by a 10 min rest period before initiation of the actual "ramp" protocol.

*Animal Sacrifices and Lung Gravimetric Studies:* Rat sacrifices were initiated by intraperitoneal injections of 50 mg pentobarbital sodium. Immediately upon the onset of

complete apnea, the thoracic cages were opened and each animal was exsanguinated via cardiac puncture. The trachea and lungs were excised, and the heart, extrapulmonary mediastinal tissue, and esophagus were removed. The lungs were then blotted dry. The bronchus leading to the right cranial lobe (RCL) was ligated with fine suture and the RCL was removed and weighed (right cranial lobe wet weight, RCLWW). The remaining lung preparation and the RCL were then collectively weighed (lung wet weight, LWW). The RCL were oven-dried to a constant weight and reweighed (right cranial lobe dry weight, RCLDW). Inasmuch as the post-exposure changes in the LWW, RCLWW, and RCLDW values after $NO_2$ exposure usually paralleled one another, only the LWW data will be reported herein as an endpoint for characterizing the severity of $NO_2$-induced pulmonary edema (Cross *et al.*, 1982; Stavert and Lehnert, 1989; Lehnert et al., 1993).

## RESULTS

*Short Burst, High $NO_2$ Concentration-Response Studies:* Figure 1 summarizes the average percentage increases in LWW values obtained with groups of animals that were subjected to various high concentration, short duration exposures and then rested after until they were sacrificed 24 hrs after exposure. It is evident from these studies that even very short exposures to very high concentrations of $NO_2$ can result in significant lung injury. As we have reported earlier (see Section A), pulmonary injury after exposure to a particular $NO_2$ exposure equivalent (concentration x time) is greater in animals exposed to higher concentrations of $NO_2$ for a shorter duration than that observed with animals exposed to lesser concentrations of $NO_2$ for longer durations. In other words, concentration rather than exposure duration is the more important exposure variable in $NO_2$-induced acute lung injury. The present studies with short burst $NO_2$ atmospheres, in conjunction with additional data obtained in previous studies performed in our laboratory, confirm these earlier findings. Figure 2 represents the percent changes in LWW 24 hrs after exposure to various concentration x time $NO_2$ exposure regimens. In this figure, we grouped the data from the experimental animals into exposure concentration families of low concentration, long duration (25 ppm to 50 ppm, 15 to 30 min), medium concentration, medium duration (75 ppm to 200 ppm, 5 to 15 min) and the short burst, high concentration group (500 ppm to 2000 ppm, 1 to 2 min). As illustrated in Figure 2, at any particular exposure equivalent, the higher the concentration and shorter duration the $NO_2$ exposure, the greater the resulting pulmonary edema.

*Post-Exposure Exercise Potentiation of Lung Injury Induced by High Concentration $NO_2$:* Exercise following 15 min exposures to relatively high concentrations of $NO_2$ can markedly increase the severity of the pulmonary injurious response beyond that which occurs when exposure is followed by rest. For example, performance of the previously described ramp exercise protocol immediately after a 15 min exposure to 100 ppm $NO_2$ dramatically increases the severity of the pulmonary edematous response in rats above that observed when $NO_2$ exposure is followed by rest, as described in Section B. Exercise after shorter duration, high burst exposures to $NO_2$ also results in a potentiation of the severity of resulting pulmonary edema. Figure 3 summarizes the percentage increases in LWW values obtained with groups of rats that were subjected to various high concentration, short duration exposures to $NO_2$ and then rested until they were sacrificed 24 hrs after the exposures (solid line), as well as the responses of groups of rats exposed in an identical manner but exercised within 1 hr after exposure and then sacrificed 24 hrs later. For any particular exposure equivalent, the animal group exposed to the lower $NO_2$ concentration (of this high $NO_2$ concentration family) for the longer duration had a much greater response to the potentiating effects of exercise. As rather dramatic examples, the group of animals exposed to 1000 ppm $NO_2$ for 1.5 min experienced 80% mortality after exercise compared to no exercise-associated mortalities after exposure to 1500 ppm for 1 min, and rats exposed to 1000 ppm $NO_2$ for 2 min experienced 100% mortality following exercise (data not shown), whereas rats exposed to 2000 ppm $NO_2$ for 1 min showed only 60% exercise-associated mortalities. Thus, in terms of the relative importance of exposure concentration versus exposure duration in the in context of the ability of exercise to potentiate lung injury following brief exposures to high $NO_2$ concentrations, exposure time appears to be the more important variable.

## DISCUSSION

The results from this component of the project demonstrate that very brief exposures (1-2 min) to very high concentrations of $NO_2$ can result in the development of profound pulmonary edema (Lehnert et al., 1993). This injurious response was observed to be proportional to the mass concentrations of $NO_2$ used in the exposure studies, i.e., a linear relationship was found between ppm $NO_2$ in exposure atmospheres and the severities of the resulting pulmonary edematous responses.

The results shown herein also show that exercise following very brief exposures to $NO_2$ can have profound effects on potentiating the injurious response to the $NO_2$. In previous studies (Stavert and Lehnert, 1989), we showed that such a potentiating effect can

occur after more extended $NO_2$ exposures, i.e., 15 min, but these more recent results indicate that exercise can exert a potentiating effect when exposure durations are as short as 1 min. Such findings are consistent with the hypothesis that $NO_2$ causes an essentially immediate change in the lung's permeability status (Lehnert and Stavert, 1991a; 1991b), and that the potentiating effect of post-exposure exercise is due to the imposition of cardiovascular events associated with exercise on an already "leaky" lung.

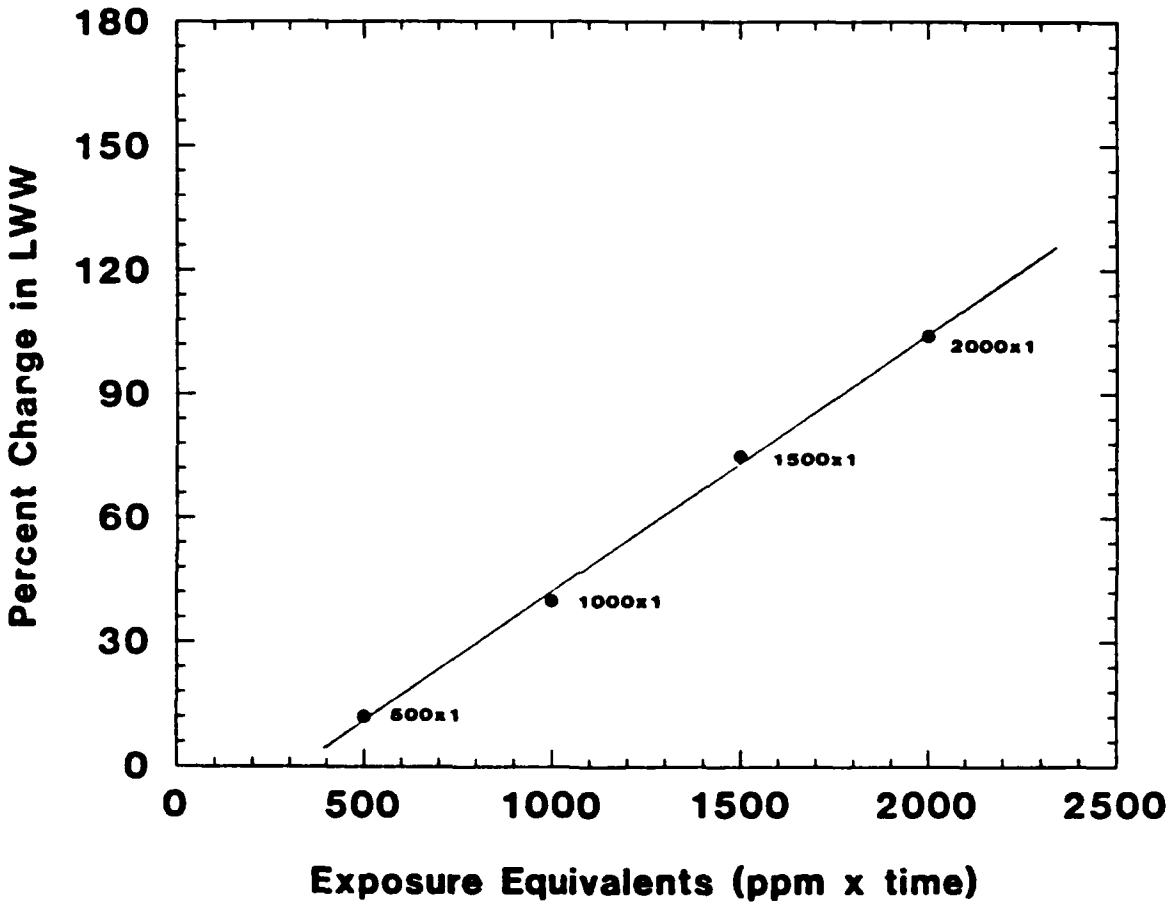


Figure 15: Percentage change in lung wet weights (LWW) as of 24 hrs after groups of rats were exposed to high concentrations of $NO_2$ (500-2,000 ppm) for a short duration of 1 min. Each point represents the mean obtained from 5-12 animals.

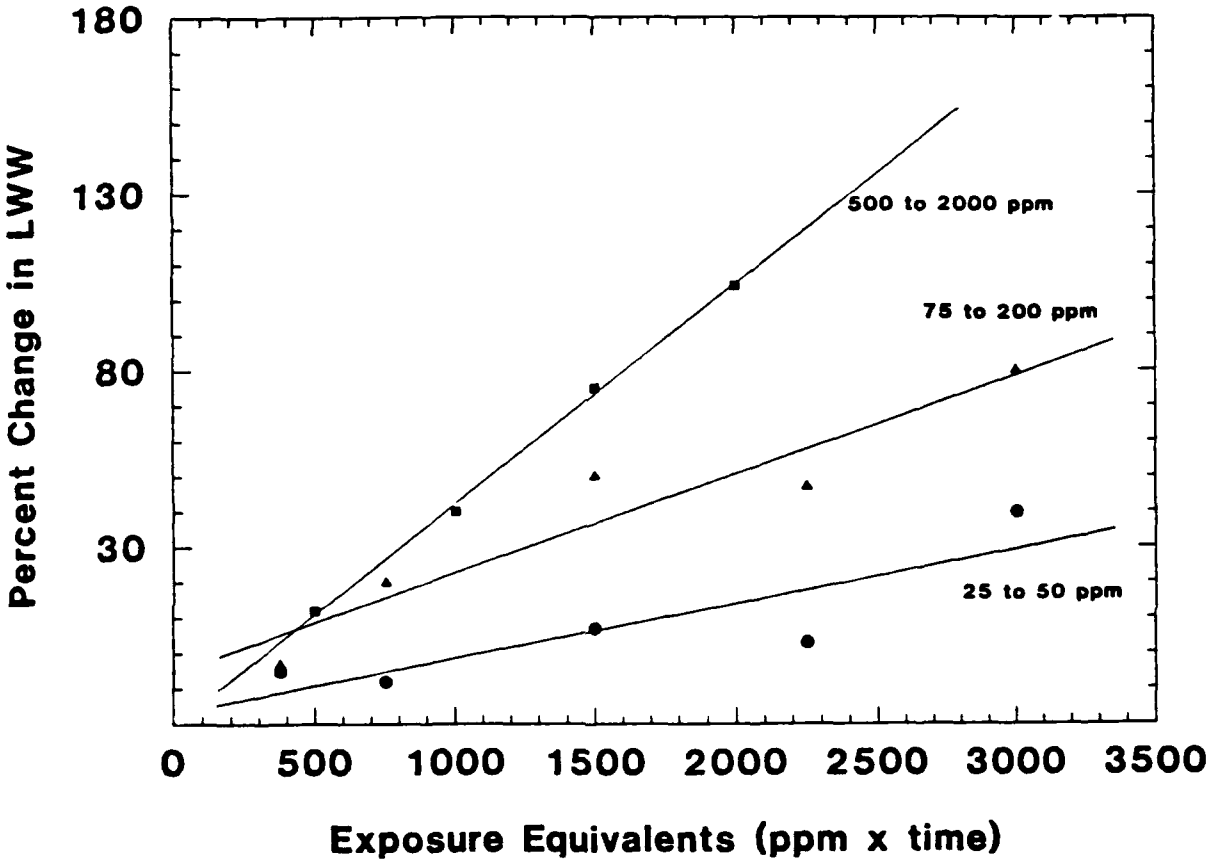


Figure 2: Percent changes in the lung wet weights (LWW) of groups of rats 24 hrs after being exposed to various concentrations of $NO_2$ for differing periods of time. The results have been grouped into $NO_2$ exposure concentration families of lower concentrations (circles, 25-50 ppm for 15-30 min), medium concentrations (triangles, 75-200 ppm for 5-15 min) and higher concentrations (squares, 500-1,000 ppm for 1-2 min). Each point represents mean values obtained from 6-18 rats. Lines were fitted to each $NO_2$ concentration family by linear regression.

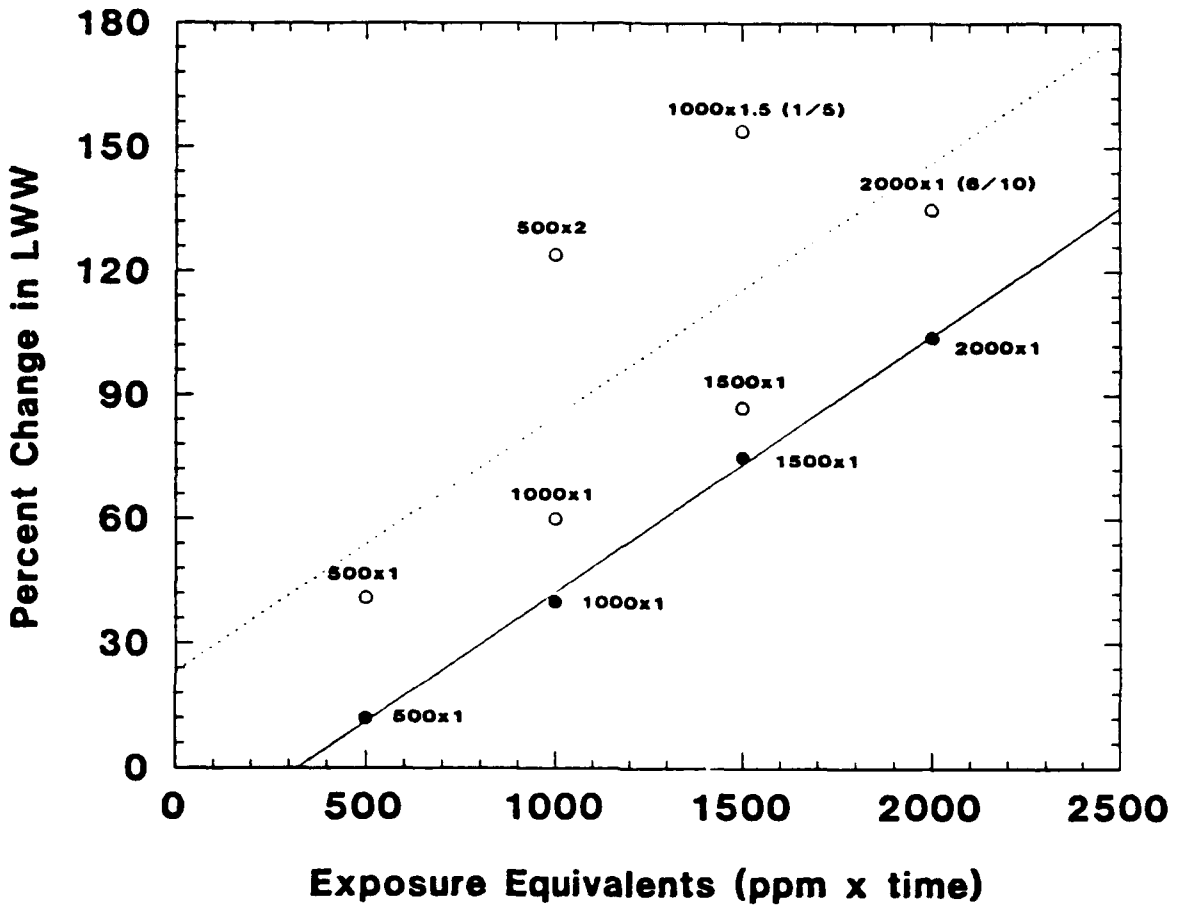


Figure 3: Percent changes in the lung wet weights (LWW) of groups of rats 24 hrs after that were exposed to high concentrations of $NO_2$ (500-2,000 ppm) for short durations (1-2 min) and either rested after exposure (solid circles, solid line) or exercised within 1 hr after exposure (open circle, dashed line). The exercised groups are further identified with concentration and time labels; when deaths occurred, the number of survivors from the group is given in parentheses. Each point represents mean values obtained from 5-12 rats.

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# SECTION J

**Objective 18: To evaluate $NO_2$ and NO as being additive or synergistic in causing decrements in work performance capacity following their concurrent inhalation.**

**Objective 19: To determine whether or not the co-inhalation of high concentrations of $NO_2$ and NO result in more severe lung injury following exercise than that which occurs following exposure to $NO_2$ only.**

**Results:** Demonstration that the combined effects of co-inhaled NO and $NO_2$ on work performance capacity are additive. Under some exposure and post-exposure conditions involving exercise, the pulmonary edematous response to co-inhaled NO and $NO_2$ can be more severe than when $NO_2$ is inhaled alone.

## INTRODUCTION

In previous studies, we found that work performance, as indexed by maximum oxygen consumption during exercise (or $VO_{2max}$) can be significantly compromised following exposure to $NO_2$ and NO when each of these gases are inhaled alone (see Sections C and F). With $NO_2$, this response evidently is related to the severity of acute lung injury present at the time of exercise, whereas with NO, reductions in $VO_{2max}$ directly scale with the level of blood methemoglobin saturation. It is currently unknown as to how $NO_2$ and NO may affect work performance capacity when these two gases are co-inhaled. Thus, this last component of the project was directed primarily to determine whether the combined effects of $NO_2$ and NO on $VO_{2max}$ are merely additive or synergistic. Additionally, we also set out to determine whether or not the co-inhalation of $NO_2$ and NO causes a greater level of acute lung injury following exercise, as indexed by lung gravimetric changes, than when $NO_2$ is inhaled alone.

## MATERIALS AND METHODS

The general techniques, e.g., generation and characterizations of exposure atmospheres, blood methemoglobin and work performance capacity ($VO_{2max}$) measurements, etc., that were used in this study component have been detailed previously in this report and elsewhere (e.g., Stavert and Lehnert, 1987; Lehnert, 1992). It should be noted, however, that previous studies of work performance capacity in our laboratory used rats that were conditioned over a 20 day period prior to exposures and $VO_{2max}$ measurements. Because our animal training has progressively become more aggressive over the last several years, we performed a comparative study to determine if animals trained over a 10 day period could be used in the $VO_{2max}$ studies. As shown in Figures 2A and 2B, we have repeatedly observed that the exercise time required to achieve $VO_{2max}$ and the actual $VO_{2max}$ values of rats trained for 10 days are virtually identical to those of rats trained over a 20 day period. Thus, in this study series, we assessed work performance capacity in rats that were trained over a 10 day period.

The following describes the experiments that were performed toward achieving the above objectives:

*Experiment 1:* Five rats were exposed simultaneously to 1000 ppm NO and 200 ppm $NO_2$ for a 15 minute exposure duration. This exposure regimen gave an exposure

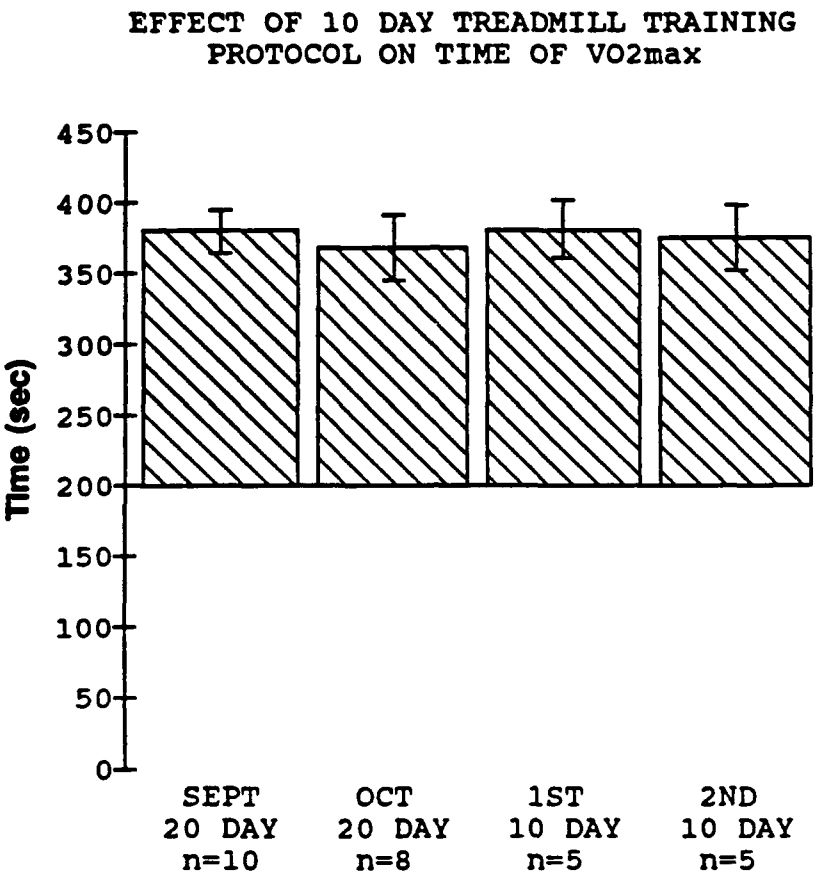


Figure 2A: Time period of exercise needed to reach $VO_{2max}$ with rats that were trained and conditioned for 10 or 20 days to perform the ramp protocol. SEPT, OCT: Groups of rats that were trained for 20 days; 1ST, 2ND: Groups of rats that were trained for 10 days. Time values represent the means ± S.E. of 5-10 rats. There are no significant differences among the groups.

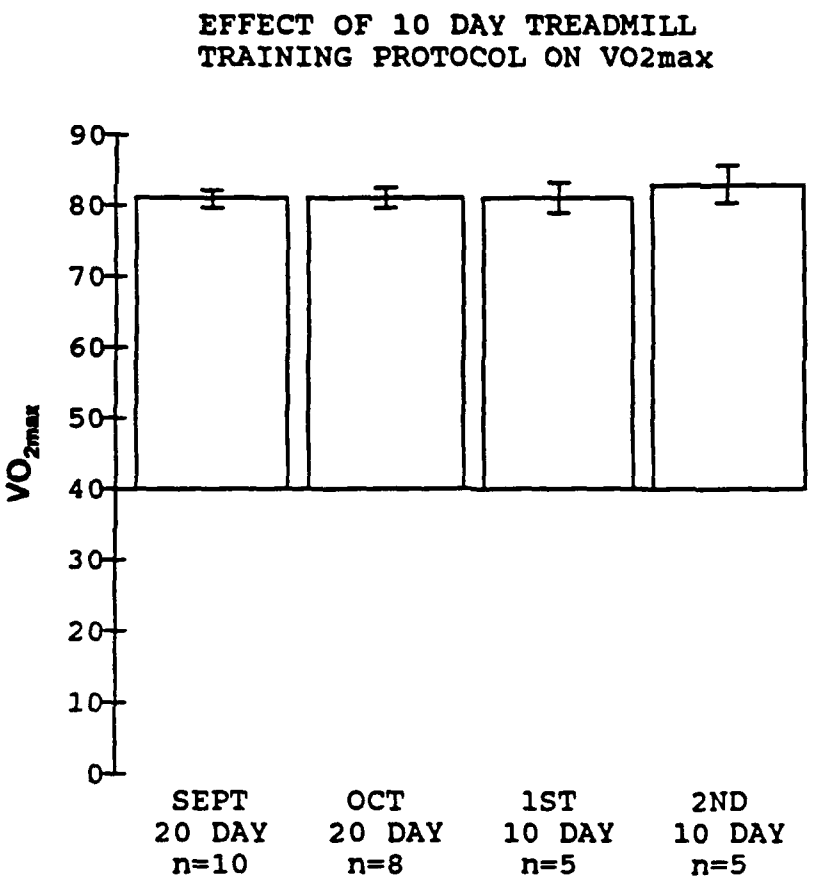


Figure 2B: Maximum oxygen consumption by 4 groups of rats that were trained and conditioned for 10 or 20 days to perform the ramp protocol. SEPT, OCT: Groups of rats that were trained for 20 days; 1ST, 2ND: Groups of rats that were trained for 10 days. $VO_{2max}$ values represent the means ± S.E. of 5-10 rats. There are no significant differences among the groups.

ratio of $NO_2$ : NO of 1: 5, or 3000 equivalents (ppm x min) of $NO_2$ to 15000 equivalents of NO. Immediately following the exposure, animals began the standard work performance protocol which consisted of 9 min of "warm-up" running (familiarization run) followed by 5 min of rest. Thereafter, the rats were subjected to the ramp exercise protocol, during which $VO_{2max}$ was measured as an index of work performance incapacitation (see Sections B and C). Blood samples for %methemoglobin (%MetHb) analyses were taken immediately preceding and immediately following the running of the "ramp" protocol. Blood %MetHb levels reported herein are from blood sampled immediately prior to the ramp protocol, which, on average occurred 18 min after the end of the exposure period. Following the work performance evaluations, the animals were returned to their cages until 1 hr post-exposure, at which time they were sacrificed by lethal pentobarbital sodium injection for lung gravimetric measurements. As a control for the above exposure conditions, a second group of five rats was exposed to 200 ppm $NO_2$ for 15 min. The remainder of their treatment was identical to that of the first exposure group described above. Blood %MetHb levels in this group were sampled at an average 15 min after the end of the exposure period.

*Experiment II*: Five rats were exposed to 1000 ppm NO for 15 min, and then exposed to 2000 ppm $NO_2$ for 1 min. This gave a collective exposure ratio of $NO_2$ : NO of 1: 7.5, or 2000 equivalents of $NO_2$ to 15000 equivalents of NO. The rationale behind this exposure regimen was to develop a pre-existing state of methemoglobinemia prior to the short burst exposure to the high 2000 ppm concentration of $NO_2$. Work performance was evaluated immediately following the exposure, and animals were sacrificed for lung gravimetric data at 1 hour post-exposure. Blood samples for %MetHb analyses were taken at an average 20 minutes after exposure, immediately preceding the running of the ramp protocol. As a control for the above exposure conditions, a second group of five animals was exposed to 2000 ppm $NO_2$ for 1 minute. The remainder of their treatment was identical to that of the first exposure group described above. Blood was sampled for %MetHb measurements in this group ~12 min after the end of the exposure period.

*Experiment III*: Five rats were exposed to 750 ppm NO for 6 min and then 200 ppm $NO_2$ for 15 min. This gave a collective exposure ratio of $NO_2$ : NO of 1: 1.5, or 3000 equivalents of $NO_2$ to 4500 equivalents of NO. Again, the rats were pre-exposed to the NO before the $NO_2$ exposure to produce a pre-existing state of methemoglobinemia in the animals. Work performance was evaluated immediately following the exposure, and the animals were sacrificed to obtain lung gravimetric data at 1 hr post-exposure.

Blood samples were taken for %MetHb analyses at ~33 min post-exposure, immediately preceding the running of the ramp protocol.

*Experiment IV*: Five rats were exposed simultaneously to 750 ppm NO and 200 ppm $NO_2$ for 15 min. This exposure regimen gave an exposure ratio of $NO_2$ : NO of 1: 3.75, or 3000 equivalents of $NO_2$ to 11250 equivalents of NO. Work performance was evaluated immediately following the exposure, and the animals were sacrificed for lung gravimetric data at 1 hr after the exposure. Blood samples were obtained for %MetHb determinations ~12 min after the exposure, immediately preceding the performance of the ramp protocol.

*NO and Methemoglobin:* In the current study, animals were exposed to NO in combination with $NO_2$ or to $NO_2$ only, as previously indicated. Expected blood %MetHb levels following exposure to NO only were predicted for comparison with data obtained in this study from previous data and a MetHb model obtained in our earlier investigations of the kinetics of formation and elimination of MetHb with NO exposure (Section E), as were estimates of expected changes in $VO_{2max}$ as a function of %MetHb (Section F). This approach was used because of our findings concerning the reproducibility of levels of %MetHb that occur in rats over time when that are exposed to closely-controlled concentrations of NO, and because of the linear relationship we previously found between %MetHb and reductions in $VO_{2max}$.

## RESULTS

### Work Performance Capacity

*Experiment I: Exposure to 1000 ppm NO and 200 ppm $NO_2$ for 15 min.*

Blood %MetHb levels at ~18 min post-exposure (immediately prior to the ramp exercise protocol) were 37.4 ± 3.7% (mean ± S.E.). Post-exposure $VO_{2max}$ averaged 48 ± 1.9% below that of the same animals' pre-exposure levels. Lung wet weight (LWW) averaged 1.48 ± 0.12 g.

Blood %MetHb levels after exposure to 200 ppm $NO_2$ only for 15 min were 1.5 ± 0.2% as of ~15 min post-exposure. Post-exposure $VO_{2max}$ averaged 20.3 ± 2.8% below

that of the same animals' pre-exposure $VO_{2max}$ values. The post-exposure LWW of these rats were 1.54 ± 0.13 g.

Based on our predictive model for MetHb accumulation and elimination (Section E), a 15 min exposure to 1000 ppm NO in the absence of high concentrations of $NO_2$ normally results in a %MetHb level of ~43% immediately after exposure and 36.7% as of 18 min after the exposure. This latter value is virtually identical to that obtained in this set of experiments. A 37% level of MetHb saturation, in turn, is associated with a 25% reduction in $VO_{2max}$ (Section F, Figure 2).

As previously indicated, $VO_{2max}$ was reduced by ~48% following exposure to both NO and $NO_2$. In that exposure to 200 ppm $NO_2$ alone causes an $VO_{2max}$ reduction of ~20%, and an exposure to 1000 ppm of NO alone causes a 25% reduction in $VO_{2max}$ under the above experimental conditions, the results from Experiment I suggest that the combined effects of NO + $NO_2$ exposure on work performance capacity are simply additive.

*Experiment II: Exposure to 1000 ppm NO for 15 min and then 2000 ppm $NO_2$ for 1 min.*

Blood %MetHb levels as of ~20 min after the exposures were 41.6 ± 3.1%. Post-exposure $VO_{2max}$ values were 36.6 ± 3.4% below that of the same animals' pre-exposure values. The post-exposure LWW of the rats were 1.61 ± 0.08 g.

Blood %MetHb levels present ~12 min after exposure to 2000 ppm $NO_2$ for 1 min were 1.1 ± 0.08%. Post-exposure $VO_{2max}$ values averaged 15.6 ± 1.7% below the pre-exposure values obtained with the same animals. The post-exposure LWW of these animals were 1.88 ± 0.18 g.

Based on our predictive model for MetHb accumulation and elimination (Section E), a 15 min exposure to 1000 ppm NO in the absence of high concentrations of $NO_2$ normally results in a %MetHb level of ~43% immediately after exposure and 36% as of 20 min after the exposure. This level of %MetHb saturation, in turn, is associated with a 24.8% reduction in $VO_{2max}$ (Section F, Figure 2).

Like in Experiment I, the reduction in $VO_{2max}$ that occurred after the rats were pre-exposed to NO and then subjected to a short burst exposure to the 2000 ppm $NO_2$ concentration was closely similar to sum of the $VO_{2max}$ reductions that follow exposure to the 1 min exposure to the high $NO_2$ only and the reduction in $VO_{2max}$ that follows exposure to NO only under the above experimental conditions (41.6% versus 40.4%).

*Experiment III: Exposure to 750 ppm NO for 6 min and then 200 ppm $NO_2$ for 15 min.*

Blood %MetHb levels present ~33 min after the exposures were 8.8 ± 1.2%. Post-exposure $VO_{2max}$ values were 32 ± 2.6% below the pre-exposure $VO_{2max}$ values obtained from the same rats. The post-exposure LWW of the rats were 2.16 ± 0.08 g.

From Experiment I, blood %MetHb levels after exposure to 200 ppm $NO_2$ only for 15 min were 1.5 ± 0.2% as of ~15 min post-exposure. Post-exposure $VO_{2max}$ averaged 20.3 ± 2.8% below that of the same animals' pre-exposure $VO_{2max}$ values. The post-exposure LWW of the Experiment I rats that were exposed to the 200 ppm $NO_2$ only were 1.54 ± 0.13 g.

Based on our predictive model for MetHb accumulation and elimination (Section E), a 6 min exposure to 750 ppm NO in the absence of high concentrations of $NO_2$ normally results in a MetHb level of ~12.3% immediately after exposure and 9.1% as of 33 min after the exposure. This level of %MetHb saturation, in turn, is associated with a 6.8% reduction in $VO_{2max}$ (Section F, Figure 2).

The expected reduction in $VO_{2max}$ in this experiment due collectively to the NO exposure and $NO_2$ exposure was 27.1%, which is similar to the value obtained experimentally.

*Experiment IV: Exposure to 750 ppm NO and 200 ppm $NO_2$ for 15 min.*

Blood %MetHb levels ~12 min after the exposure were 28.1 ± 1.7%. Post-exposure $VO_{2max}$ values of the animals were 44 ± 3.7% below their pre-exposure values. The post-exposure LWW of the rats were 2.45 ± 0.24 g.

From Experiment I, blood %MetHb levels after exposure to 200 ppm $NO_2$ only for 15 min were 1.5 ± 0.2% as of ~15 min post-exposure. Post-exposure $VO_{2max}$ averaged 20.3 ± 2.8% below that of the same animals' pre-exposure $VO_{2max}$ values. The post-exposure LWW of the Experiment I rats that were exposed to the 200 ppm $NO_2$ only were 1.54 ± 0.13 g.

Based on our predictive model for MetHb accumulation and elimination (Section E), a 15 min exposure to 750 ppm NO in the absence of high concentrations of $NO_2$ results in a 29.6% MetHb level immediately after exposure and ~26.6% MetHb as of 12 min after the exposure. This level of %MetHb saturation, in turn, is associated with an 18.5% reduction in $VO_{2max}$ (Section F, Figure 2).

As with the previous set of experiments, the data obtained in Experiment IV support the general conclusion that the combined effects of concurrent NO and $NO_2$ exposure on work performance capacity are merely additive. Specifically, a reduction in $VO_{2max}$ of ~44% was measured after the NO and $NO_2$ exposure, which is closely similar to the sum of the $VO_{2max}$ reductions that occur following exposures to NO alone and $NO_2$ alone under the conditions of Experiment IV, i.e., 38.8%.

## Lung Gravimetric Changes

As we have shown previously, short-term exposures to high concentrations of NO alone does not cause lung injury that is detectable by increases in lung wet weight (LWW) or histopathological changes that are discernible at the level of light microscopy (Section D). On the other hand, $NO_2$ is well recognized as a pneumoedematogenic gas. It might be expected, accordingly, that any increases in the LWW of animals exposed to a mixture of NO and $NO_2$ would be equivalent to that of animals exposed to $NO_2$ alone.

*Experiment I: Exposure to 1000 ppm NO and 200 ppm $NO_2$ for 15 min.*

The lung wet weight (LWW) of the rats that were exposed to both NO and $NO_2$ were 1.48 ± 0.12 g. The post-exposure LWW of rats exposed to 200 ppm $NO_2$ only for 15 min were 1.54 ± 0.13 g. The LWW values for these two exposure groups were not significantly different from one another.

*Experiment II: Exposure to 1000 ppm NO for 15 min and then 2000 ppm $NO_2$ for 1 min.*

The post-exposure LWW of the rats exposed to both the NO and $NO_2$ were 1.61 ± 0.08 g. The post-exposure LWW of the animals exposed to 2000 ppm $NO_2$ for 1 min were 1.88 ± 0.18 g. The LWW values for these two exposure groups were not significantly different from one another.

*Experiment III: Exposure to 750 ppm NO for 6 min and then 200 ppm $NO_2$ for 15 min.*

The post-exposure LWW of the rats exposed to both the NO and $NO_2$ were 2.16 ± 0.08 g. The post-exposure LWW of the Experiment I rats that were exposed to the 200 ppm $NO_2$ only were 1.54 ± 0.13 g. The LWW of the rats exposed to the NO and $NO_2$ were significantly higher than the LWW from rats exposed to the NO only, $P<0.05$.

*Experiment IV: Exposure to 750 ppm NO and 200 ppm $NO_2$ for 15 min.*

The post-exposure LWW of the rats that were exposed to both the NO and $NO_2$ were 2.45 ± 0.24 g. The post-exposure LWW of the Experiment I rats that were exposed to the 200 ppm $NO_2$ only were 1.54 ± 0.13 g. The LWW of the rats exposed to the NO and $NO_2$ were significantly higher than the LWW from rats exposed to the NO only, $P<0.05$.

## DISCUSSION

The results from previous study components have indicated that the inhalation of NO results in a predictable manner to the formation of methemoglobin (MetHb) (Ripple et al., 1989), and that reductions in work performance capacity, as indexed by decreases in $VO_{2max}$, are directly proportional to the percentage of blood hemoglobin that is in the form of MetHb (Stavert et al., 1989). Previous studies have also demonstrated that the inhalation of high concentrations of $NO_2$ also result in work performance degradations that appear to scale with the severity of pulmonary edema present at the time $VO_{2max}$ is measured . In the present study component, evidence has been obtained that strongly indicates that when high concentrations of NO and $NO_2$ are co-inhaled, the resulting degradations in work performance capacity are due to the additive effects of methemoglobinemia that result from NO and the acute injurious effects that are associated with $NO_2$.

In two of the experiments (Experiments I and II), we found no evidence that the co-inhalation of NO and $NO_2$ results in more severe pulmonary edema shortly after exercise than the level of injury that occurs following $NO_2$ only exposure. This finding was not unexpected, given our previous observations that high concentrations of NO alone are not pneumoedematogenic (Behr et al., 1988; Stavert and Lehnert, 1990). However, in two other sets of experiments (Experiments III and IV) some evidence was obtained that suggest that a greater than expected pulmonary edematous response may occur after co-exposure to NO and $NO_2$ under some exposure conditions. The

underlying explanation for the results from these latter experiments requires further investigation.

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