CONTRACT NO. A033-089
FINAL REPORT
AUGUST 1995

# Studies to Determine Long-Term Health Effects of Acidic Atmospheres

## Part 1

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
AIR RESOURCES BOARD
Research Division

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Final Report

Contract No. A033-089

Part 1

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August 1995

## ABSTRACT

Inhaled acids are associated with adverse health effects, a conclusion based largely on studies with particulate-associated acid sulfates. The acidic component of ambient air in some regions, e.g., California, contains nitric acid ($HNO_3$) vapor, but there is a limited database concerning its biological effects. Furthermore, effects of nitric acid may be modulated by co-exposure to other pollutants, such as ozone. Rabbits were exposed for 4 hr/d, 3 d/wk for 4 wk to nitric acid vapor at three concentrations, 50, 150 and 450 $\mu g/m^3$; 0.15 ppm ozone alone; or to a mixture of 50 $\mu g/m^3$ nitric acid plus 0.15 ppm ozone. Additional animals were exposed for 12 or 40 wk to the lowest level of nitric acid with and without ozone. Peak ambient concentrations of $HNO_3$ may reach 50 $\mu g/m^3$ and the two higher acid concentrations used then represent 3 and 9 times this peak level, reasonable values to develop an exposure concentration-response evaluation and to determine the significance of any biological responses to the acid. Exposure was followed by assays of biochemical markers in lavage fluid, pulmonary macrophage function, and *in vitro* bronchial responsivity to smooth muscle constrictor challenge. While 4 wk of exposure to acid resulted in reduced basal levels and stimulated production of superoxide anion by macrophages and release/activity of tumor necrosis factor, these effects resolved by 12 wk. Bronchi from rabbits exposed to $\geq 150$ $\mu g/m^3$ nitric acid exhibited reduced smooth muscle responsivity *in vitro* compared to control and this was also found in the lowest concentration group by 40 wk of exposure. Exposures to the mixture for 4wk resulted in no interaction for most endpoints, but antagonism was noted for stimulated superoxide production, while synergism was noted for spontaneous superoxide production and bronchial responsivity. These results indicate that short term repeated exposure to $HNO_3$ may adversely impact upon pulmonary health by affecting target sites throughout the lungs, and that inhalation of an $HNO_3/O_3$ mixture can produce synergistic interaction in affecting some biological parameters. Furthermore, it is likely that inhaled vapor phase $HNO_3$ reaches its target sites in the lungs in the particulate state.

ACKNOWLEDGMENTS

This project was performed with the assistance of Drs. Lung Chi Chen and Christine Nadziejko, Ms. Kathy Baker, Mr. James Monahan and Mr. John Gorczynski.

This report was submitted in fulfillment of Contract No. A033-089, "Studies to Determine Long Term Health Effects of Acidic Atmospheres", by New York University, Department of Environmental Medicine under the sponsorship of the California Air Resources Board. The work was completed as of December 31, 1994.

DISCLAIMER

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## SUMMARY AND CONCLUSIONS

The acidic component of ambient air in California often contains nitric acid ($HNO_3$) vapor, but there was a limited database concerning its biological effects. Furthermore, effects of $HNO_3$ may be modulated by co-exposure to other pollutants, such as ozone ($O_3$). In order to provide additional information as to potential health effects of exposure to nitric acid, rabbits were exposed for 4 hr/d, 3 d/wk for 4 wk to $HNO_3$ vapor at 0, 50, 150 and 450 $\mu g/m^3$ alone; 0.15 ppm $O_3$ alone; or to a mixture of 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$. Additional animals were exposed for 12 or 40 wk to 50 $\mu g/m^3$ $HNO_3$, 0.15 ppm $O_3$ or a mixture of 50 $\mu g/m^3$ $HNO_3$ + 0.15 ppm $O_3$. Exposures were followed by assays of biochemical markers in lavage fluid, pulmonary macrophage function, and *in vitro* bronchial responsivity to smooth muscle constrictor challenge. Nitric acid had no effect on viability or numbers of cells recovered, nor on lactate dehydrogenase or total protein in lavage. Following the 4 wk exposures, all acid concentrations reduced both basal levels and stimulated production of superoxide anion by macrophages, while the release/activity of tumor necrosis factor by stimulated macrophages was reduced following exposure to ≥150 $\mu g/m^3$ $HNO_3$. Bronchi from rabbits exposed to ≥150 $\mu g/m^3$ $HNO_3$ exhibited reduced smooth muscle responsivity *in vitro* compared to control. While 4 wk exposure to the $HNO_3/O_3$ mixture resulted in no interaction for most endpoints, antagonism was noted for stimulated superoxide production, while synergism was noted for spontaneous superoxide production and bronchial responsivity. Exposure to the mixture resulted in a total abrogation of response to spasmogens in most bronchi examined and a marked attenuation in others. Following 12 or 40 wk of exposure, the only effect was airway hyporesponsiveness following exposure to the $HNO_3$. These results indicate that short term repeated exposure to $HNO_3$, when inhaled in vapor phase, may adversely impact upon pulmonary health by affecting target sites throughout the lungs, and that inhalation of an $HNO_3/O_3$ mixture can produce synergistic interaction in affecting some biological parameters. A consistent effect on airway responsiveness was noted, but the biological significance of this is not clear at this time. Furthermore, it is likely that inhaled vapor phase $HNO_3$ is transformed into particles following inhalation, and reaches its target sites in the lungs in the particulate state, either as pure acid droplets or adsorbed onto

other particles already present, or formed in the respiratory tract by reaction with respiratory ammonia

## RECOMMENDATIONS

The results of this study suggest that further assessment of short-term repeated exposures to nitric acid, with and without ozone, may be needed to better assess the potential risk to human health.

## BACKGROUND

Exposures to airborne acids have been associated with adverse health effects in both epidemiological and experimental studies (USEPA, 1989). However, such relationships are based largely on particulate-associated acid, the predominate form of ambient acidity in the eastern United States. The acidic component of the atmosphere in many western regions is more often dominated by the vapor phase, of which nitric acid ($HNO_3$) is one common constituent (Pierson and Brachaczek, 1988; Munger et al., 1990). This nitric acid is formed within the photo-oxidation cycle of polluted air (Durham and Brock, 1986), deriving from primary emissions released by mobile sources. The major production pathway involves reaction between hydroxyl radicals ($OH\cdot$) and nitrogen dioxide ($NO_2$).

The available database concerning health effects of $HNO_3$ vapor is limited, since most studies examined the histological response to instilled or nebulized $HNO_3$, a procedure used in developing models of bronchiolitis obliterans (Totten and Moran, 1961; Greenberg et al, 1971; Mink et al, 1984; Peters and Hyatt, 1986). A few toxicological and controlled clinical studies of $HNO_3$ vapor have been reported (Abraham et al, 1982; Aris et al, 1991; Nadziejko et al, 1992; Mautz et al, 1993). Exposure concentrations ranged from 50-4120 $\mu g/m^3$, and exposure regimes varied from single to short-term repeated.

The purpose of the current investigation was twofold. First, it aimed to provide a more definitive assessment of the potential health significance from inhaled $HNO_3$ vapor by conducting a broad survey of the exposure concentration-dependence of pulmonary effects induced by short-term episodic exposures. This was achieved by examining, within one study, potential targets encompassing both the conducting and respiratory regions of the rabbit lung. A range of target sites was selected since the dosimetry of inhaled $HNO_3$ is unknown. Second, the study evaluated responses of these same pulmonary targets following exposure to a mixture of ozone ($O_3$) and $HNO_3$. While it is clearly important to assess potential health effects from single pollutants, ambient atmospheres generally consist of pollutant mixtures, and the biological effects of exposure may reflect responses to combinations of toxic substances (Goldstein, 1979; Schlesinger et al, 1992). Levels of atmospheric $HNO_3$ vapor generally peak during daytime hours

(Ellestad and Knapp, 1988), and a common co-pollutant within photochemical atmospheres having a similar temporal pattern is $O_3$. Although a number of studies have examined interactions between $O_3$ and particulate-associated acids (e.g., Schlesinger et al, 1992; U.S.E.P.A., 1989), there are only two reports that evaluated interactions between $O_3$ and $HNO_3$ vapor (Aris et al., 1991; Nadziejko et al, 1992).

## APPROACH

This project involved long-term repeated exposures of rabbits to nitric acid and ozone, alone and in combination, for assessment of biological responses. It consisted of four phases: 1) design, construction and testing of exposure systems; 2) development of a real-time nitric acid monitor for on-line measurements during exposure; 3) actual exposures of rabbits to pollutant atmospheres; and 4) assessment of physicochemical factors affecting nitric acid dosimetry in the respiratory tract.

## RATIONALE FOR EXPOSURE CONCENTRATIONS

The few available data for ambient $HNO_3$ suggest much variability. Concentrations ranged from 0.5-4.8 $\mu g/m^3$ in one southern California city, and 0.8-56 $\mu g/m^3$ in another (Munger, et al, 1990). Nitric acid levels (24 hr avg) in a rural area of North Carolina ranged from 0.8-2.1 $\mu g/m^3$ (Shaw et al., 1982). It appears that peak concentrations of $HNO_3$ may reach 50 $\mu g/m^3$, the lowest level used in this study. The two higher acid concentrations used then represent 3 and 9 times this peak level, reasonable values to develop an exposure concentration-response evaluation and to determine the significance of any biological responses to the acid. In addition, data from southern California (California Air Resources Board, personal communication) indicate annual 24 hr avg and maximum 24 hr avg. levels of total vapor phase acidity ranging from 27-45 $\mu g/m^3$, and 68-105 $\mu g/m^3$, respectively. If $HNO_3$ is considered to be a model for vapor phase acidity, then the exposure concentrations currently used also range down to relevant levels using this justification approach. The exposure concentration of $O_3$ used, namely 0.15 ppm, is only slightly above the Federal air quality standard (0.12 ppm) and is frequently exceeded in southern California (USEPA, 1988).

EXPOSURE SYSTEM

The animal exposure facility used in this study consisted of three units, each capable of holding up to 10 rabbits. One unit was used for exposure to nitric acid, another for ozone and the third for the acid/ozone mixture. A dedicated sham exposure unit was already available. Figure 1 shows a photograph of one of these units. All exposure atmospheres were equilibrated to 21°C, 55% RH in a Teflon (PFA) mixing chamber, and then delivered to individual exposure ports via a manifold system similar in design to that used in this laboratory for particulate acid exposures (Schlesinger et al, 1983; Schlesinger, 1987). All components of the system that contacted nitric acid or ozone were constructed from Teflon (PFA).

As shown in Figure 2, all incoming air was passed through a cleaning system which included a series of annular denuders to remove ambient ammonia and also contained HEPA filters, activated charcoal, and Purafil ($KMnO_4$-coated alumina) to remove ambient particles, sulfur dioxide, nitrogen oxides, and ozone.

During exposure, the rabbits were restrained in commercially available whole body restrainers, and exposures were nose-only using Teflon film masks. Samples for atmospheric analysis were drawn from a dedicated port on the exposure apparatus during each exposure.

Following construction, one of the exposure systems was used to assess any effect of humidity on nitric acid concentrations in the exposure system as well as the uniformity of nitric acid penetration into each of the individual nose-only ports. Nitric acid was generated into the unit and initially the acid concentration exiting into one of the exposure ports was measured using the real-time monitor. Testing of the system was performed at both a low and high concentration of nitric acid (159 and 591 $\mu g/m^3$) and at ambient relative humidities (RH) ranging from 30 - 65% (at 24.4°C.). The upper RH limit was considered as a worst case, since RH measurements at an actual exposure port with a rabbit on-line indicated that levels only up to 25 - 30% would be obtained during actual exposures. At the lowest RH, 87 - 93% of the expected concentration values were obtained at the exposure port after a 2 hr equilibration time during which time nitric acid was allowed to flow into the system, while at the highest RH penetration was 81%. In addition, loss of acid did not increase with time beyond the 2 hr equilibration period.

These results indicated that worst case loss of nitric acid between introduction into the exposure unit and the exposure port would be about 20%, and that this appeared to hold over a wide nitric acid concentration range. Thus, it was possible to increase delivery from the nitric acid generation system so as to make up for any loss. This was actually tested, and with proper adjustment of flow rate with the generation system, levels of nitric acid recovered to >90% of the expected values. Thus, with use of the real time monitor, it was possible to maintain the desired nitric acid levels in the exposure system with a high degree of precision. In addition, as part of routine protocol, prior to each daily exposure, nitric acid generation into the system was begun at least 2 hr prior to introduction of the rabbits into the system

The final test of each exposure unit was to determine uniformity of delivery of nitric acid to each individual exposure port. For this, a known concentration of nitric acid was generated into each exposure unit and the concentration at each nose-only port measured with the real time monitor. It was found that values for each port all were within 5% of each other, and within 5% of the generated concentration.

## REAL-TIME NITRIC ACID MONITOR

The concentration of nitric acid during exposure was routinely measured using a real-time $HNO_3$ monitor which was designed and constructed especially for this project by modification of an NOx chemiluminescence analyzer (Monitor Labs Model 8840). Filter samples were also routinely obtained during exposures for comparison with measurements using the monitor; filters were analyzed for nitrate ion ($NO_3^-$) using a Dionex 4000i ion chromatography system. During initial testing of the nitric acid monitor, it was found that various reported methods for extraction of nitric acid from filters were lacking, in that extraction efficiencies were quite variable, and ranged as low as 34%. Accordingly, modified procedures for extraction of nitric acid from nylon filters were developed. These are described in the Appendix of this Report.

A schematic diagram of the $HNO_3$ monitor is shown in Figure 3. Generated $HNO_3$ enters the monitor through a 37 mm Teflon filter, which removes any particulate nitrates, or other particles, from the incoming air. The sample stream is then divided into two channels. One of these

incorporates a 47 mm nylon (Nylasorb) filter, which collects vapor phase $HNO_3$. The second channel remains unchanged from the original $NO_x$ monitor. Each sample then passes into separate Molycon converters, which transform any $HNO_3$ vapor present in the stream into nitric oxide (NO). The concentration of $HNO_3$ is determined by the response difference between these two channels. To assure accurate measurement of nitric acid by the monitor, the Nylasorb filter must be 99+% efficient for removal of nitric acid, and the Molycon converter must have a consistent efficiency for converting nitric acid to NO. The actual efficiency is >96%.

In order to develop the $HNO_3$ monitor, a $HNO_3$ calibrator was required. A commercially available nitrogen dioxide ($NO_2$) calibrator (VICI Metronics) was modified to incorporate a NO dilution channel, an integrated "zero air" generation system, and denuders for removal of ambient ammonia. The NO dilution channel was designed in this laboratory, and then installed by the manufacturer (VICI Metronics). The zero air system was designed after a Monitor Labs calibrator, and incorporated an "OZATROG" clean air system and a three way solenoid valve to deliver zero air to the permeation oven and for dilution. This custom zero air system was installed into the calibrator in this laboratory.

The $HNO_3$ monitor was initially calibrated using the NO dilution channel of the calibrator. Certified NO gas was delivered to the monitor, and the relationship between NO concentration and monitor output voltage was assessed. To validate the accuracy of the monitor, certified $HNO_3$ permeation devices were used to generate acid at known concentrations, and the response of the monitor was examined. Results indicated a mean ratio of 0.98 (SD = 0.09) for $HNO_3$ mass concentration indicated by the monitor to that actually generated by the $HNO_3$ permeation device, for ten concentrations within the range used in this study.

To determine the response time for the real-time monitor, a known nitric acid concentration (350 $\mu g/m^3$) was generated into the monitor and the response of the monitor at various elapsed times from the start of sampling was determined. Results are shown in Figure 4. The concentration of nitric acid as measured by the monitor was consistently within 5% of the actual concentration generated.

## EXPERIMENTAL ANIMAL

Male, specific pathogen free (Pasteurella multocida) New Zealand white rabbits (3.5 - 4.5 kg; Hazelton Research Products, Denver, PA) were used. This species has served as a model for acid toxicity for a number of years in this laboratory. Animals were housed in stainless steel cages in temperature (20°C ) and humidity [50% relative humidity (RH)] controlled rooms, and had food (Purina Rabbit Chow-HF) and water provided *ad libitum*. They were quarantined for a 2 wk period prior to introduction into the exposure protocol. Nasal swabs and blood samples were routinely taken for clinical screening and sentinel animals were routinely used as colony health controls.

## EXPERIMENTAL EXPOSURE DESIGN

This project consisted of two exposure regimes, a 4 week subchronic screening exposure using three concentrations of nitric acid, followed by 12 and 40 wk chronic exposures using one concentration of nitric acid. All exposures for all regimes were 4 hr/d, 3 d/wk.

For the subchronic protocol, 42 rabbits were randomly assigned into seven groups of six each for exposure as follows: clean air I (sham control I); clean air II (sham control II); $HNO_3$ vapor at 50, 150, or 450 $\mu g/m^3$; $O_3$ at 0.15 ppm (290 $\mu g/m^3$); or a mixture of 50 $\mu g/m^3$ $HNO_3$ with 0.15 ppm $O_3$. The two control groups were exposed under temperature and humidity conditions identical to those used with the pollutants, and the two control exposures were separated by a period of 8 wk.

For each of the two chronic exposure protocols, 32 rabbits were randomly assigned into four groups of eight each for exposure as follows: clean air (sham control); $HNO_3$ vapor at 50 $\mu g/m^3$; $O_3$ at 0.15 ppm, or a mixture of 50$\mu g/m^3$ with 0.15 ppm $O_3$.

## BIOLOGICAL ASSAYS

Immediately following the final exposure after either 4, 12 or 40 wk, rabbits were euthanized (sodium pentobarbitol, 75 mg/kg *iv*) and the trachea and upper lung exposed. The right post-caudal lobe was clamped, and the remaining lung lavaged. Acellular lavage fluid was analyzed for lactate dehydrogenase (LDH) and total soluble protein. The former, a cytoplasmic enzyme, is a general marker for cytotoxicity, while the latter is an index of altered epithelial permeability (Henderson and Belinksy, 1993).

Specific characteristics of cells recovered by lavage were assessed. This included enumeration of cell types; cell viability; phagocytic activity of pulmonary macrophages; production of superoxide anion ($O_2^{\cdot -}$) by zymosan-stimulated and unstimulated macrophages; and release/activity of tumor necrosis factor (TNFα) elicited from lipopolysaccharide-stimulated macrophages. These macrophage functional and secretory parameters were selected as indices of the ability of pollutant exposure to modulate various aspects of host pulmonary defense. Phagocytosis is critical to the clearance of viable and nonviable particles from the lungs, as well as for the induction of immune responses (Crystal, 1991). TNFα is involved in tumoricidal activity as well as the modulation of fibroblast growth and the inflammatory response, while $O_2^{\cdot -}$ is one component of macrophage cytolytic defense (Wewers and Gadek, 1991).

Following the lavage procedure, the lungs were removed from the thorax, and sections of trachea and second generation bronchi were obtained for examination of airway responsivity to pharmacological agents, using an *in vitro* preparation (El-Fawal and Schlesinger, 1992). This assay was performed to determine the effect of pollutant exposure on conducting airway function. The post-caudal lobe was then unclamped and fixed by airway instillation of 2% glutaraldehyde in cacodylate buffer at a pressure of 30 cm $H_2O$. Tissue slabs were embedded in paraffin, sectioned at a thickness of 3μm and stained with hematoxylin and eosin for general pathological analysis by light microscopy. Alveolar septal thickness and alveolar airspace diameter were measured as morphometric indices of response.

**Bronchopulmonary Lavage:** Lavage was performed using a procedure described previously (Schlesinger et al, 1990). This involved *in situ* infusion six times with calcium- and magnesium-free phosphate buffered saline (PBS). The withdrawn fluid from each wash was centrifuged, and the cell pellets resuspended in Eagle's minimum essential medium with Hank's salts [EMEM(H)]. The supernatant from the first lavage only was used for LDH and total soluble protein analyses. The cells were pooled, respun and suspended in the appropriate medium for the assays described below.

All cell culture reagents were routinely tested for bacterial and fungal contamination. Endotoxin levels were assessed in lavage fluid and culture reagents using the Limulus amebocyte lysate assay (Whittaker M.A.

Bioproducts, Walkerville, MD); levels were routinely <0.5 ng. All glassware was autoclaved and heat sterilized (170 °C, 4 hr) prior to use.

**Lavage Fluid Analysis:** LDH in lavage fluid was measured using a commercially available kit (Sigma Chemical Co., St. Louis, MO), and the results expressed as BB units/ml. Total soluble protein content was also measured with a commercially available kit (Bio-Rad Laboratories, Richmond, CA) and results expressed as μg protein/ml lavage fluid.

**Lavaged Cell Characterization:** Total recovered cell count and cell viability were determined by hemocytometer counting and trypan blue exclusion, respectively. Differential cell counts were performed to assess any influx of polymorphonuclear leukocytes, an indication of an inflammatory response. This was done following fixation of cells with methanol and staining with Diff-Quik™; two hundred cells per animal were examined.

**Macrophage Phagocytosis:** Phagocytic activity was evaluated using a suspension assay, previously described (Schlesinger, 1987), in which macrophages were incubated for 1.5 hr with opsonized polystyrene latex microspheres (3 μm diam., Duke Scientific, Palo Alto, CA) and the phagocytic index, i.e., the percentage of the total number of viable macrophages that ingested latex particle(s), was quantitated by light microscopy.

**Tumor Necrosis Factor Activity:** The release/activity of TNFα elicited by lipopolysaccharide (LPS)-stimulated macrophages was assessed using a bioassay, described previously (Zelikoff et al., 1991), which measures cytotoxicity towards tumorigenic L-M cells (a clone of L-929 mouse fibroblasts; American Type Cell Culture Collection, Rockville, MD). TNFα release/activity was expressed as the inverse of the dilution which produced 50% cytotoxicity.

**Superoxide Anion Radical Production:** Production of $O_2^{\cdot -}$ by resting (spontaneous) and zymosan-stimulated macrophages was assessed using a microassay described previously (Zelikoff and Schlesinger, 1992). The assay is based upon the reduction of ferricytochrome c, and specificity of the reduction was controlled by its inhibition by superoxide dismutase (SOD). Results were expressed as nmol $O_2^{\cdot -}/3 \times 10^5$ macrophages.

***In Vitro* Nonspecific Airway Responsivity.** The response of airway smooth muscle to spasmogenic agents as a test of nonspecific airway responsivity was assessed using an *in vitro* assay (El-Fawal and Schlesinger, 1994). A section of distal trachea immediately proximal to the bifurcation and a section of a

second generation bronchus from the right lung were removed by blunt dissection. Each section was trimmed of excess connective tissue, and a tracheal or bronchial ring prepared. Each ring was mounted with clips in a 10 ml organ bath containing Krebs-Henseleit solution at 37°C and gassed with a mixture of 95%$O_2$/5%$CO_2$. Tissues were allowed to equilibrate for 1hr, under a resting tension of approximately 2g for trachea or 1g for bronchus. During this time, the bathing fluid was changed at intervals of 10-15min.

Tracheal and bronchial smooth muscle response to pharmacological challenge was measured using isometric transducers (Model FT03, Grass Medical Instruments, Quincy, MA) connected to a polygraph (Grass Model 7). To assess bronchial responsivity, cumulative concentration-response curves (CRC) to acetylcholine (ACh) and to histamine (Hist) were established. These two agonists were used since cholinergic drugs and histamine are the most common pharmacological agents employed in clinical bronchoprovocation challenge testing to assess the state of airway responsivity (Townley and Agrawal, 1990). The concentration range for both agonists, which began at the lowest effective concentration for each, extended from $10^{-8}$ to $10^{-3}$M. Tracheal responsivity was assessed using only acetylcholine.

Two parameters were used to relate changes in airway tension to dose of agonist as an index of the state of airway responsiveness. These were reactivity, defined as the slope of the agonist dose-response relationship for each airway and determined by linear regression analysis of tension vs. $\log_{10}$[agonist]; and $T_{max}$, the tension generated at the highest dose of agonist used ($10^{-3}$M).

Following each pharmacological assessment, the tracheal and bronchial rings were fixed in 15% formalin for histological evaluation. The rings were embedded in paraffin and 5µm serial sections were cut and stained with hematoxylin and eosin and evaluated for the integrity of the epithelial lining.

## STATISTICAL ANALYSIS

Effects of the individual pollutants upon lavage fluid and cellular parameters and airway $T_{max}$ were assessed by analysis of variance, followed by Dunnett's test to determine significance of changes from sham control. Data in the form of percentages were subjected to angular transformation prior to this analysis. The results of the two sham control tests for the subchronic study were pooled since these were not significantly different

from each other (t-test, $p>0.05$). In the graphs and tables, values for sham exposures represent these combined groups.

Comparison of airway reactivity between exposure cohorts was performed with analysis of covariance, followed as above by Dunnett's test. In order for airways of a particular pollutant exposure cohort to be considered as having responsivity different from control, a statistically significant difference in either reactivity or $T_{max}$ must have been found.

Statistical evaluation of interaction between $HNO_3$ and $O_3$ was performed using one way analysis of variance with multiple linear contrasts (Schlesinger et al., 1992). In this analysis, the absence of interaction is defined as the case in which the effect of exposure to the mixture is additive, i.e., not different from the sum of the effects from the individual pollutants. Thus, synergism is the case where the mixture produced an effect which was greater than additive, while antagonism is the case where response to the mixture was less than additive. All of the "effects" noted above are in relation to differences from sham control.

In all cases, statistical significance was accepted at $p<0.05$.

## GENERATION AND CHARACTERIZATION OF EXPOSURE ATMOSPHERES

Nitric acid vapor was generated using certified $HNO_3$ permeation devices (VICI Metronics, Santa Clara, CA). This represented a novel approach to vapor generation, inasmuch as other studies produced nitric acid atmospheres by nebulization of $HNO_3$ solutions and subsequent vaporization of the droplets in dry air. A schematic diagram of the $HNO_3$ generation system is shown in Figure 4.

Prior to use of each new tube, the output from these permeation devices was analyzed for any contaminant nitrogen oxides ($NO_X$), i.e. $NO_2$ or NO. A Monitor Labs $NO_2$ monitor, which incorporated a Nylasorb filter after the Teflon filter to remove nitric acid, was used to assess any $NO_X$ release by the permeation tubes. The sampling stream was divided into a total $NO_X$ and an NO channel. There was found to be no detectable NOx (minimum detectable level for $NO_2$ or NO is 0.004 ppm) released by any of the tubes used in this study.

Ozone was generated by passing oxygen (in argon) through an ultraviolet $O_3$ generator (OREC Model 03V1-0, Phoenix, AZ). The $O_3$

concentration during exposures was measured with an ultraviolet photometer (Dasibi Model 1008-PC, Glendale, CA).

Mixed pollutant atmospheres were obtained by generating both $HNO_3$ and $O_3$ into the Teflon mixing chamber prior to delivery to the rabbits. The $O_3$ was injected just prior to the mixing chamber, followed by the downstream injection of $HNO_3$. To assure adequate mixing of the two pollutants, a turbulence-inducing disc was incorporated into the airstream (Stairmand, 1951). In order to assess whether there was any chemical interaction prior to inhalation, the concentrations of $HNO_3$ and $O_3$ were first measured at their respective points of generation with the appropriate monitor, and then measured again after the pollutants were mixed. The results indicated no measurable loss of either pollutant prior to inhalation. This was confirmed by collection, from the mixed atmosphere, of $HNO_3$ on nylon filters, followed by extraction and analysis for $NO_3^-$ using ion chromatography.

Table 1 provides the actual concentrations of nitric acid vapor and ozone obtained during the 4, 12 and 40 week exposure series.

## RESULTS OF SUBCHRONIC EXPOSURE SERIES

### Responses to $HNO_3$

Table 2 presents results of 4wk $HNO_3$ exposure on cell characterization, lavage biochemistry and macrophage phagocytosis. No significant acid-exposure related effects were noted in total numbers of cells recovered by lavage, differential count, cell viability, nor in LDH or total soluble protein in lavage fluid. The phagocytic index of macrophages obtained from animals exposed to the highest acid concentration was somewhat reduced from control, but the difference was not statistically significant ($p>0.29$).

Figure 5 shows effects of $HNO_3$ exposure on the production of superoxide anion by pulmonary macrophages. Exposure to all concentrations of acid resulted in a significant reduction of superoxide levels.

Figure 6 shows effects of $HNO_3$ on TNF$\alpha$ release/activity. There was an apparent exposure concentration related trend towards a reduction in TNF$\alpha$ activity, with the change at $\geq 150\ \mu g/m^3$ becoming statistically significant from control.

Nitric acid exposure produced significant effects on bronchial responsivity, as determined using the *in vitro* assay of smooth muscle

response to pharmacological agents. Figure 7 shows the CRCs of bronchi to ACh and Hist, while Table 3 shows values for bronchial reactivity and $T_{max}$ derived from these data. [The coefficients of determination ($r^2$) for the individual regression analyses used to obtain reactivity were all highly statistically significant, and ranged from 0.85 to 0.97.] Exposure to 150 and 450 $\mu g/m^3$ $HNO_3$ produced a reduction in bronchial responsivity to both acetylcholine and histamine, indicating airway hyporesponsivity compared to sham (air) control. There was also some indication that effects at 450 $\mu g/m^3$ were somewhat less than those at 150 $\mu g/m^3$.

Tracheal reactivity and $T_{max}$ are also shown in Table 3. There were no statistically significant differences from sham control in these parameters for any of the exposures, indicating that $HNO_3$ did not affect nonspecific responsivity of this airway.

**Responses to $O_3$ and $HNO_3/O_3$ Mixture**

Table 4 presents results of exposures to $O_3$ and the $HNO_3/O_3$ mixture. There were no significant effects on numbers of recovered cells, cell differentials, cell viability, macrophage phagocytic activity nor on lavage fluid LDH or total soluble protein following exposure to $O_3$ alone or to the mixture.

Exposure to the mixture did result in a significant increase, compared to sham (air) control, in spontaneous superoxide production which was not seen with $O_3$ alone, and which contrasted with the reduction in superoxide production seen following exposure to 50 $\mu g/m^3$ $HNO_3$. The mixture produced a significant reduction in stimulated superoxide production compared to sham control, while $O_3$ alone showed no effect and 50 $\mu g/m^3$ $HNO_3$ decreased production. TNF$\alpha$ release/activity was significantly reduced, compared to sham control, following exposure to the mixture, while neither $O_3$ nor $HNO_3$ alone had any effect.

Perhaps the most dramatic response to the mixed atmosphere was noted for nonspecific bronchial responsivity. While neither $O_3$ nor 50 $\mu g/m^3$ $HNO_3$ alone had any significant effect on responsivity, bronchi obtained from four of the six rabbits exposed to the mixture showed no measurable response to either acetylcholine or histamine over the entire dose range of these spasmogens. While bronchi from the other two animals exposed to the mixture showed some measurable response, a severe attenuation compared

to sham control was observed. This is reflected in mean values for $T_{max}$ noted in Table 4.

The effects of the mixture on tracheal responsivity are also shown in Table 4. No significant differences from control were noted following exposure to the mixture, nor to ozone alone.

The results of the statistical interaction analysis are shown in Table 5. In most cases, there was no interaction between $O_3$ and $HNO_3$. However, the effect of the mixture on spontaneous superoxide production was synergistic, while antagonism was noted for stimulated production. In the latter case, addition of ozone to the exposure atmosphere lessened the effect from acid alone. Different qualitative responses for spontaneous or stimulated production of reactive oxygen species following pollutant exposure is not necessarily unexpected, since different mechanisms are involved in these two processes.

Interaction for bronchial responsivity, namely the induction of hyporesponsiveness, was synergistic, inasmuch as no change in reactivity was noted with $HNO_3$ or $O_3$ alone; the almost total abrogation of response to the spasmogens indicates that the acid/ozone mixture had a profound effect on airway physiology. No interaction was noted for TNF$\alpha$, since the response to the mixture, while different from control, was not different from that due to $O_3$ alone.

## RESULTS OF CHRONIC EXPOSURE SERIES

Results of the 12 and 40 week exposures are shown in Tables 6 and 7. It is clear from Table 6 that there are generally no significant pollutant exposure related effects on lavage or macrophage indices. Changes in superoxide production and TNF production/activity noted following the 4 wk exposure series resolved by 12 weeks of continued exposure.

On the other hand, airway reactivity was altered with the longer-term exposures. The 4 week exposure series resulted in bronchial hyporeactivity at the two highest levels of nitric acid used, namely 150 and 450 $\mu g/m^3$. Following 40 weeks of exposure at 50 $\mu g/m^3$, bronchi were found to be hyporeactive at this concentration as well (Table 7). No such effect was noted in the trachea. Figure 8 shows the progression of bronchial responsiveness to ACh from 4 to 40 weeks of exposure to $HNO_3$.

## HISTOPATHOLOGY

Histological analysis of conducting airway sections from all exposure groups indicated no overt pathology and normal airway epithelium.

Morphometric assessment of the tissues was performed to assess alveolar septal thickness and alveolar size. Originally, analysis of sections from the 4 wk exposures had indicated there to be statistically significant increases in alveolar wall thickness in animals exposed to ozone, and to 150 and 450 $\mu g/m^3$ nitric acid. However, there were internal inconsistencies in the dataset, in that: 1) the ozone exposure resulted in an apparent increase in septal thickness, yet the acid/ozone mixture did not and there was no basis to assume that the acid would "reverse" the effect of the ozone, so the original findings were illogical; and 2) there were no apparent visual differences between the exposure groups even though there was an apparent increase in alveolar size with the initial assessment. Thus, the original sections were re-examined, and the revised data for the 1 month exposures are presented in Table 8 alone with results of analyses of the 12 and 40 week exposures. No morphometric changes are now reportable in the 4 wk exposure group. It is likely that any changes were so subtle that they fell within the observational error of the readers and, thus, are of little biological significance. The statistically significant increase in alveolar size in the 12 wk exposure group is not considered to be biologically significant. Thus, the overall data set indicates there to be no biologically significant changes in alveolar morphometric indices following exposure to ozone, nitric acid, or their combination for up to 40 wk.

## ASPECTS OF NITRIC ACID DOSIMETRY

Results of the 4 wk exposures to nitric acid indicated that short term repeated exposures affected biological targets throughout the respiratory tract, including the alveolar region; this latter was an unexpected finding, in that nitric acid is both a highly soluble and reactive vapor and was expected to have its major impact in the nasal passages and upper bronchial tree.

We hypothesized that nitric acid reaches the deep lung via two mechanisms. One is formation of pure nitric acid droplets within the humid respiratory tract. The other involves adsorption of nitric acid vapor onto the surfaces of ammonium nitrate particles, which would be formed by reaction of inhaled nitric acid with endogenous respiratory tract ammonia ($NH_3$).

Understanding the dosimetry of inhaled nitric acid is important not only to explain the observed biological findings, but also to assist in extrapolation modeling to humans.

In order to examine dosimetric factors underlying the observed regional pattern of biological responses, a series of experiments was performed to provide information on the actual extent of penetration of nitric acid through the upper respiratory airways by examining the ability of the acid vapor to become pure acid particles and/or to be converted into ammonium nitrate particles within the lungs. This will allow determination of whether the biological effects seen in the deep lung were due to pure nitric acid vapor reaching this area and/or to particulate acidic nitrate species.

The experiments examined, under strictly controlled conditions, the formation of nitric acid droplets from acid vapor under conditions of increasing relative humidity and then examined the production of ammonium nitrate particles when nitric acid vapor was mixed with ammonia under conditions simulating those which would occur in the upper respiratory tract of the rabbit.

To these ends, a physical model system to allow for conditioning of generated nitric acid, i.e. exposing acid to various humidity levels, and to allow assessment of particle formation and of reaction products with ammonia was constructed, based upon a similar system used in this laboratory to study neutralization of acid sulfate aerosols. The system was made completely of nonreactive Teflon. Air entering the system was passed through HEPA filters, silica gel, citric acid coated glass wool, activated charcoal and citric acid coated annular denuders, this last assuring removal of any ambient ammonia. Relative humidity (RH) was controlled by use of a loop consisting of a distilled water-filled flask placed within a heating mantle, while temperature was controlled by heating tape and fiberglass insulation. Both were monitored using sensors placed at the beginning and end of the sampling portion of the system. A schematic diagram of this generation/conditioning system is shown in Figure 9.

Nitric acid, generated using permeation devices, was transported into the conditioning system. Two sampling ports (SP) were integrated into the system, one at the point of nitric acid injection (SP1) and another at a certain distance downstream, depending upon the experimental conditions desired (SP2). Thus, measurements taken at SP1 provided information on initial

injection chemistry, while those obtained at SP2 showed any alterations following transit of the acid through the system.

In the first phase of the study, nitric acid vapor was introduced into the conditioning system at the concentrations used in the 4 wk study, namely 50, 150 and 450 $\mu g/m^3$. The RH within the system was incrementally increased (from an initial value of 5%) in order to determine the relationship between RH and particle formation. The number concentration and size distribution of any aerosols produced under these experimental conditions were determined with a fast response real-time aerosol spectrometer in conjunction with a differential mobility analyzer (DMA) using samples obtained at SP2, while any remaining nitric acid vapor was monitored at SP2 with the $HNO_3$ monitor. The aerosol measurement system was interfaced with a microcomputer to control DMA voltage and for data acquisition. Size distribution data were transformed using a special program for calculation of number concentration and number median diameter (NMD), from which mass median diameter (MMD) of any aerosols produced was calculated.

The second phase of the study examined neutralization. Ammonia was generated at nominal concentrations of 20, 200, and 400 ppb from certified permeation devices and injected into the system at a point downstream from the acid injection site. The ammonia concentrations used were within the range reported to be found in the upper respiratory tracts of various species, including humans (Vollmuth and Schlesinger, 1984). A mixing baffle was incorporated into the system to ensure adequate mixing of ammonia with the nitric acid vapor. For this experiment, the RH within the conditioning system was maintained at 95% to simulate that occurring in the upper respiratory tract. Nitric acid generated at 50, 150 and 450 $\mu g/m^3$ was mixed with each ammonia concentration. Measurements were made of the number and size of any particles which formed using real time monitoring instrumentation described above. Particle samples for chemical analysis were also collected on tandem Teflon/nylon filters through Teflon tubing placed within the center of the airstream at SP2. Filter samples were analyzed for hydrogen ion as well as for ammonium and nitrate ions. These sampling procedures allowed determination of whether any free nitric acid was adsorbed onto the particles, i.e., whether there is excess acid that may be delivered to the deep lung. In addition, the conditioning tube was washed with distilled water and the recovered fluid chemically analyzed to account

for any wall loss. The transport time of aerosol between the point of ammonia injection and SP2 was set at 0.21 sec, which is approximately the residence time of the aerosol in the rabbit upper respiratory tract under the *in vivo* exposure conditions used in this project.

Figure 10 shows the loss of nitric acid vapor in the conditioning tube as RH is increased. The loss of acid vapor was accompanied by an increase in particle formation. For the specific experiment shown, nitric acid entered the tube at a concentration of 1320 $\mu g/m^3$. At this concentration, there appeared to be an RH threshold below which there was no measurable loss of vapor phase acid.

Figure 11 shows the loss of nitric acid vapor initially injected into the conditioning tube at various concentrations when ammonia at one concentration was added. It can be seen that as the acid concentration increases, the percentage of particulate-associated nitrate decreases. This is because the $NH_3$ is being consumed in the neutralization reaction by the excess acid.

In the last phase of this study, we examined the ability of other particles to act as vectors for adsorbed nitric acid, which could then be carried into the deep lung. We used sodium chloride aerosol as a model for aqueous particles which may exist in the humid atmosphere within the airways.

Sodium chloride aerosols were produced by nebulization of 0.5% solutions. The aerosols were mixed with humidified air and nitric acid vapor in a stainless steel tube. A laminar flow pattern was maintained within the tube. The aerosols passing through the center of the tube were reacted with water vapor and nitric acid vapor via diffusion processes. The length of the tube was such to have minimal loss of particles formed. At the tube exit, particles and vapors were collected on Teflon and Nylon filters, respectively. Filters were analyzed for nitrate and chloride using ion chromatography. Particle size was determined with a Mercer impactor.

The particle size of the sodium chloride with adsorbed nitric acid was 0.82 μm. From Figure 12 it can be seen that there is significant adsorption of acid onto the salt particles. Thus, any aerosol in the respiratory tract, whether water or ammonium nitrate, can act as a vector for inhaled nitric acid vapor.

Another issue addressed was the extent of particle production. Figure 12 also shows the relative amount of nitrate which was associated with particles following introduction of nitric acid into the experimental

apparatus, both in the presence and absence of ammonia. There is greater production of particles when ammonia is present at both levels of RH, but even in the absence of ammonia, almost 25% of the nitric acid forms particles at the higher RH. This confirms the hypothesis that nitric acid vapor, once inhaled into a humid atmosphere and in the presence of ammonia, will form a substantial number of particles.

Regarding the data for nitric acid only presented in Figure 12, it should be noted that in the experimental system used, an equilibrium is established between nitric acid vapor, nitric acid dissolved in water droplets, and water vapor in the surrounding air. If there were no equilibrium, then nitric acid could not remain in the water droplets. Since the system is maintained at a constant RH and vapor phase nitric acid concentration (the amount of nitric acid in water droplets is low compared to the concentration of vapor phase acid), then this equilibrium is maintained when the acid droplets are collected on the Teflon filters. This phenomenon differs from that occurring in ambient air. During ambient sampling for acid droplets, equilibrium is not achieved or maintained for a substantial time and, thus, nitric acid particles collected on filters are likely to evaporate and escape capture.

One of the other parameters of interest was the size of the particles produced when nitric acid was introduced into humid atmospheres. It was found that nitric acid in an atmosphere without ammonia at a relative humidity (RH) of 77% produced ultrafine particles having a diameter of 0.003 μm. When ammonia was added (at 400 ppb), the size of the particles increased to 0.15 μm at the same RH. Figure 13 shows the effect of increasing RH on particle size in an atmosphere containing ammonia.

## DISCUSSION

The main goal of this project was to assess the effects of inhaled $HNO_3$ vapor in both the conducting and respiratory airways. The results indicate that $HNO_3$ deposits in sufficient amounts on conducting airways so as to alter pharmacological responses, yet also reaches the respiratory airways where it affects macrophage functionality, this latter at least with short-term (4 wk) repeated exposures.

Nitric acid-induced alterations in both conducting and respiratory airways were also noted by Mautz et al (1993a,b), who observed changes in breathing pattern, alveolar macrophage receptor binding capacity, and

alveolar morphometry in rats exposed to 50, 170 and 470 $\mu g/m^3$ $HNO_3$ for 4 hr/d, 3 d/wk for 4 wk, similar concentrations and the same exposure regime as used herein. Further evidence for penetration of $HNO_3$ into the deep lung was provided by Nadziejko et al (1992), who noted reduced production of superoxide anion by macrophages harvested from rats exposed to 250 $\mu g/m^3$ for 4 hr/d for 4 d. In the current study, however, a similar effect was seen with longer duration exposures to a lower acid concentration, i.e., 50 $\mu g/m^3$. As in the current study, Nadziejko et al (1992) found no effects on total numbers of cells recovered by lavage, differential counts, nor in total soluble protein in lavage fluid.

The observations of $HNO_3$-induced effects throughout the lungs was initially somewhat surprising. Because of its high water solubility and reactivity, vapor phase $HNO_3$ would be anticipated to undergo significant removal within the upper respiratory tract, e.g., in the nasal passages. A water soluble gaseous pollutant with known dosimetry that may be compared to $HNO_3$ is sulfur dioxide ($SO_2$), and it has been suggested (Amdur, 1966) that at low concentrations pertinent to ambient air pollution, $SO_2$ is not as efficiently removed by the upper respiratory tract as might be anticipated and a significant fraction of the amount inhaled penetrates into the lungs. It is clear from the results presented here that this may also be true for $HNO_3$.

While both $SO_2$ and $HNO_3$ penetrate beyond the upper respiratory tract, a major difference is that the biological effects of the latter occur in much more distal regions that those observed for the former, which are found generally only in the larger conducting airways (N.A.S., 1978). A likely explanation is that gaseous $HNO_3$ is transformed into particulate form at some point following inhalation. The humidity within the airways is very high, and because of its solubility, some of the inhaled $HNO_3$ vapor within such an environment combines with water that had condensed into small droplets, and which can serve as vectors for acid delivery to the deep lung. In addition, endogenous ammonia within the airways may react with inhaled gaseous $HNO_3$, producing particulate ammonium nitrate. Once formed, these particles may then also serve as absorption surfaces, and additional vectors, for $HNO_3$. Such particle formation from nitric acid vapor is evident from the results presented herein.

Exposure to $HNO_3$ for 4 wk reduced the production and/or function of some important macrophage secretory products, namely superoxide and

TNFα. Similar results have been observed following exposures to particulate acid sulfates (Zelikoff and Schlesinger, 1992). The biological effects of acid sulfates are due to the hydrogen ion ($H^+$) rather than the associated anion (Schlesinger et al, 1990; Schlesinger, 1989), and the similarity of effects on macrophages from both nitric and sulfuric acids suggest this may also be true for $HNO_3$. On the other hand, while possible effects due to deposited nitrate ions cannot be ruled out, there is an insufficient database to allow any conclusion as to their role in altered macrophage function induced by $HNO_3$. In any case, TNF mediates a variety of biological responses associated with host defense as well as various non-immunological parameters (Kunkel et al, 1989), and alterations in its efficacy may have implications to overall pulmonary immunocompetence. Macrophage-mediated cytotoxicity depends upon target cell injury induced by various factors, of which superoxide is one (Nathan et al, 1981), and reduced production may also compromise host defense, perhaps by reducing pulmonary antibacterial activity (Amoruso et al., 1981).

Perhaps the most biologically significant and consistent effect occurred in the conducting airways, where exposure to $HNO_3$ lowered bronchial reactivity to acetylcholine and histamine *in vitro*, i.e., induced nonspecific airway hyporesponsivity. Most previously reported studies of $HNO_3$ effects on airway function used human asthmatics or animal models of asthma, and responses may not be indicative of those occurring in healthy individuals. Abraham et al. (1982) exposed normal sheep for 4 hr to 4,120 $\mu g/m^3$ $HNO_3$ and noted a decrease in specific pulmonary flow resistance, compared to preexposure control values, but no significant change in responsiveness to bronchoconstrictor challenge. Aris et al (1991) exposed normal human subjects for 4 hr to 500 $\mu g/m^3$ $HNO_3$ vapor and found no statistically significant change in airway resistance. Thus, while effects of $HNO_3$ on airway physiology may appear to be somewhat equivocal, perhaps the *in vitro* assay used herein can detect changes in airway responses prior to those measurable *in vivo*. In any case, the pathological significance of bronchial hyporesponsivity is not known.

It is interesting that exposure to the lowest acid concentration did not produce any change in responsiveness until 40 wk of exposure, yet the mixture of this acid concentration with ozone produced hyporesponsiveness at 4 wk and not for the longer exposure durations. These observations

highlight the complexity in attempting to predict effects of mixtures from effects due to exposure to the individual compounds and should not be interpreted as contradictory. The mechanisms underlying these differences in response are unknown.

The hyporesponsivity noted with $HNO_3$ differs from the hyperreactivity observed following exposure to particulate acid sulfates (El-Fawal and Schlesinger, 1994). The reasons for this difference are unclear. One possibility is an $HNO_3$-induced production of nitric oxide (NO), which has been demonstrated both *in vivo* and *in vitro* to be a bronchodilator (Dupuy et al., 1992), or some NO-containing chemical species. As an oxidizing agent, $HNO_3$ may react with airway mucus constituents containing sulfhydryl bonds, such as the amino acid cysteine, a component of bronchial mucin (Miller et al, 1993). This could result in formation of S-nitrosothiol, a class of chemicals that is much more stable than NO within the cellular milieu, and is also a potent relaxant for airway smooth muscle (Jansen et al, 1992).

It is interesting that tracheal responsivity was unaffected by exposure to $HNO_3$, while acid sulfates were found to alter both bronchial and tracheal response to spasmogens (El-Fawal and Schlesinger, 1994). This suggests differences in dosimetry, and a lack of significant deposition in the trachea for inhaled $HNO_3$ vapor, or its existence in particles much smaller than the acid sulfate droplets.

While, as mentioned above, the responses to acid sulfates are due to the $H^+$, the effects of nitrate ion on bronchial function should be considered in assessing results observed herein. However, limited data on inhaled nitrates suggest that they have minimal effects on airway physiology. For example, Sackner et al. (1976) exposed dogs to sodium nitrate at 740 or 4,000 $\mu g/m^3$ for 7.5 minutes, and found no effects on pulmonary function, while controlled clinical studies using nitrate aerosols at concentrations ranging from 200-7,000 $\mu g/m^3$ found no effects on airway resistance in normal subjects (Stacy et al., 1983; Utell et al, 1979).

In addition to examining responses to inhaled $HNO_3$, another goal of this study was to assess responses to an atmosphere containing a mixture of $O_3$ and $HNO_3$, since photochemical smog atmospheres likely contain both pollutants. For the most part, exposure to the mixture resulted in no toxicological interaction, but some synergism or antagonism were noted for some endpoints at 4 wk. It should be mentioned that in terms of effects on

macrophages, it is possible that different subpopulations may be affected by exposure to nitric acid and ozone, which would be reflected in responses to the mixture. However, this was not evaluated in this project.

There are only two previously reported studies of interactions between $HNO_3$ vapor and $O_3$. Nadziejko et al (1992) exposed rats for 4 hr to 0.6 ppm $O_3$ plus 1000 $\mu g/m^3$ $HNO_3$, or for 4 hr/d for 4 d to 0.15 ppm $O_3$ plus 250 $\mu g/m^3$ $HNO_3$. While most responses were additive, antagonism was noted following the single exposure regime for neutrophil counts and protein in lavage fluid. In the other study, Aris et al (1991) exposed normal humans to 500 $\mu g/m^3$ $HNO_3$ with 0.2 ppm $O_3$, and concluded that there was no interaction between the two pollutants in regards to airway resistance and various indices related to an inflammatory response.

Exposure to the acid/ozone mixture resulted in a complete loss of bronchial smooth muscle response to acetylcholine and histamine in 4 of 6 animals, a response which was not seen in the trachea, nor in the bronchi obtained from animals exposed to $O_3$ or $HNO_3$ alone. The cell membrane is the site of $O_3$ toxicity (Menzel, 1984), which likely results from reaction with various membrane components, including amino acids and fatty acids (Pryor et al, 1983; Pryor, 1992). Exposure to another oxidant, namely $NO_2$, was associated with alterations in membrane lipids, and a resultant change in membrane fluidity and interference with such membrane-dependent functions as receptor-ligand interaction (Patel et al, 1988). It is likely that $O_3$ would have similar effects, since there is some commonality in their toxic mechanisms related to membrane damage (Rietjens et al, 1986). Furthermore, it is possible that the extent of membrane lipid peroxidation due to exposure to the $O_3/HNO_3$ mixture may be synergistic compared to that from exposure to each pollutant alone (Ichinose and Sagai, 1989, 1991).

In conclusion, this results of this project add new information to the sparse database concerning the toxicology of inhaled $HNO_3$ vapor, and demonstrates that $HNO_3$ impacts upon both the conducting and respiratory airways, resulting in reduced immunocompetence of lung macrophages and apparent changes in bronchial response to pharmacological agents. Exposure to a mixture of $HNO_3$ and $O_3$ resulted in some synergistic or antagonistic interaction depending upon the biological endpoint; the dependence of type of interaction upon the endpoint was also noted in a recent study examining effects of ozone and acid sulfates (Schlesinger et al, 1992). The results of the

current study suggest that $HNO_3$ under ambient conditions may adversely impact pulmonary health by affecting target sites throughout the lungs, and that inhalation of $O_3/HNO_3$ mixtures common in photochemical smog atmospheres can produce interactions that may affect macrophage and bronchial airway function. The biological significance of the bronchial hyporesponsiveness noted with acid exposure is not clear at this time.

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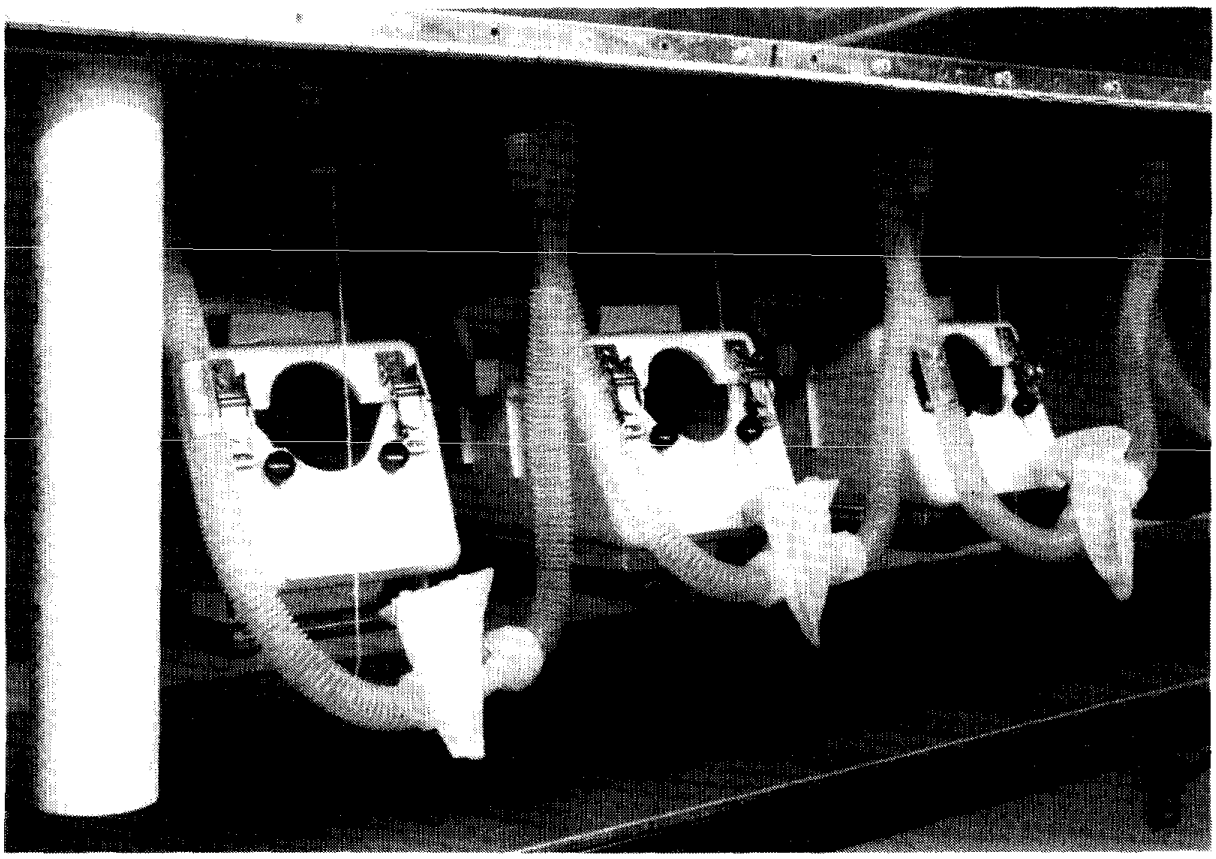


FIGURE 1. Photograph of rabbit exposure units.

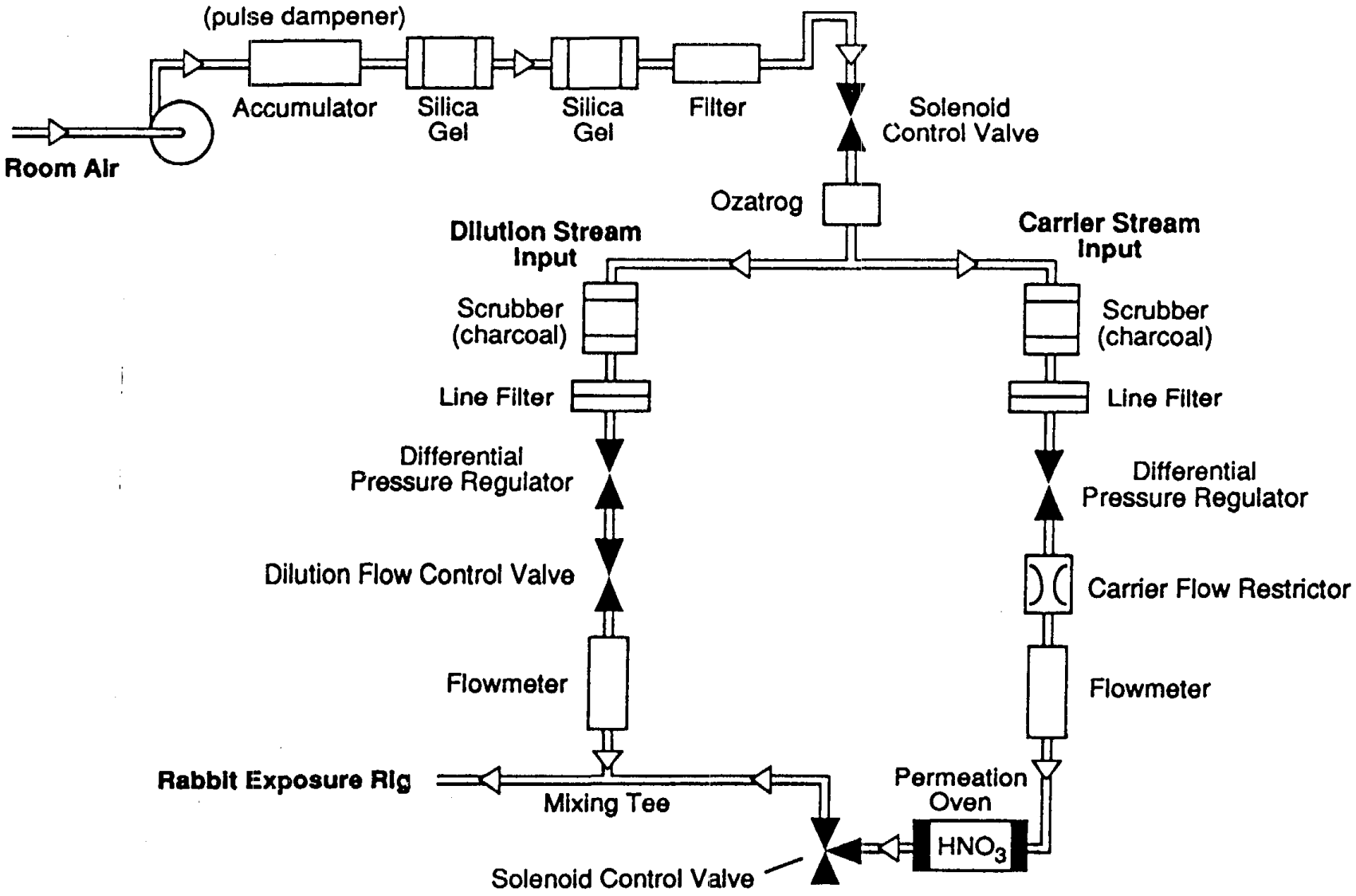



FIGURE 2. Schematic diagram of nitric acid generation system.

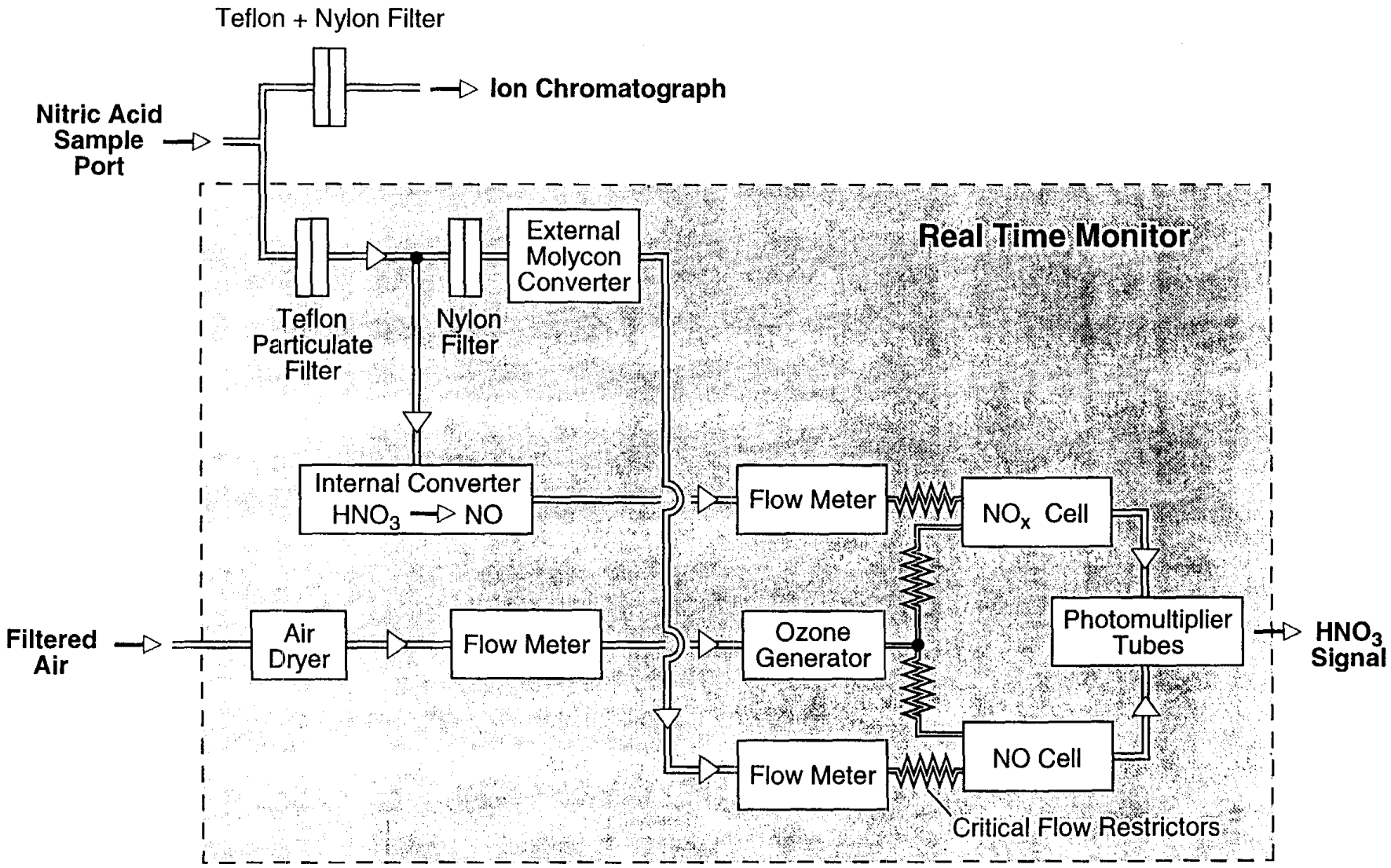
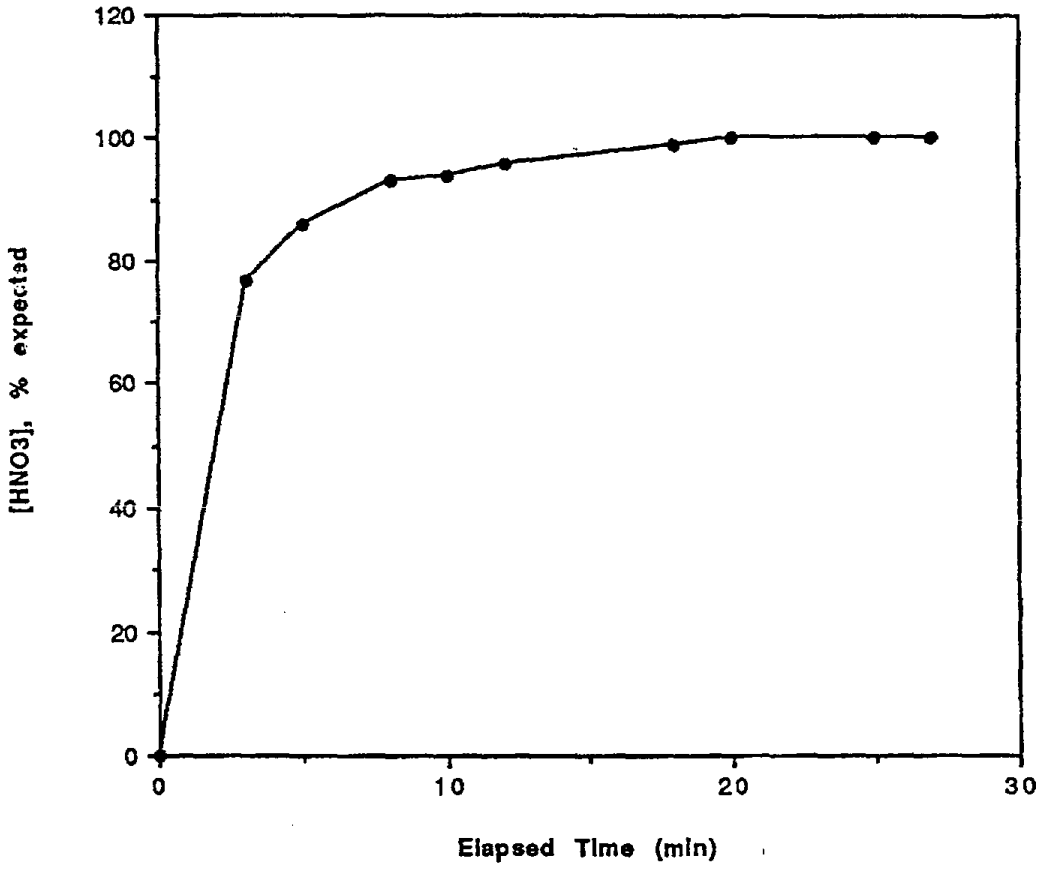



FIGURE 3. Schematic diagram of nitric acid monitor.

FIGURE 4 . RESPONSE OF REAL TIME MONITOR



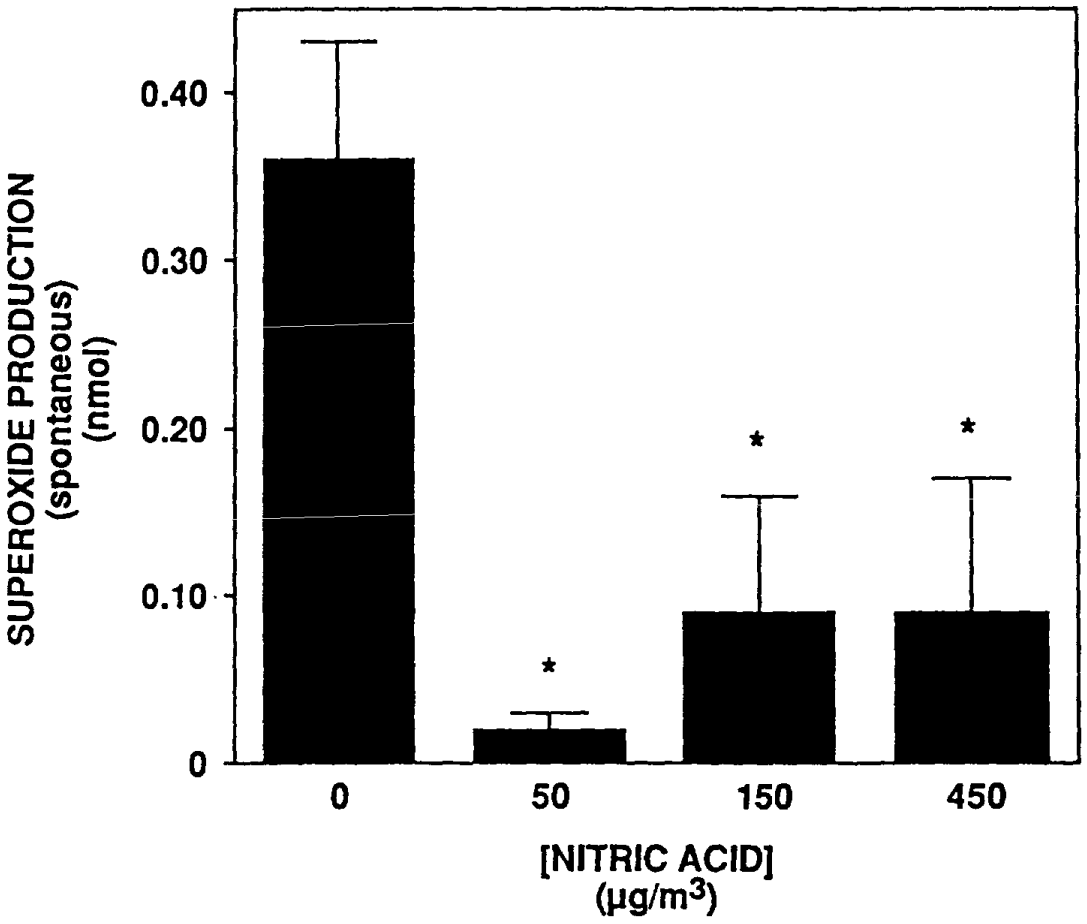



FIGURE 5 (A) Spontaneous production of superoxide by macrophages following exposure to nitric acid.

* significantly different from control

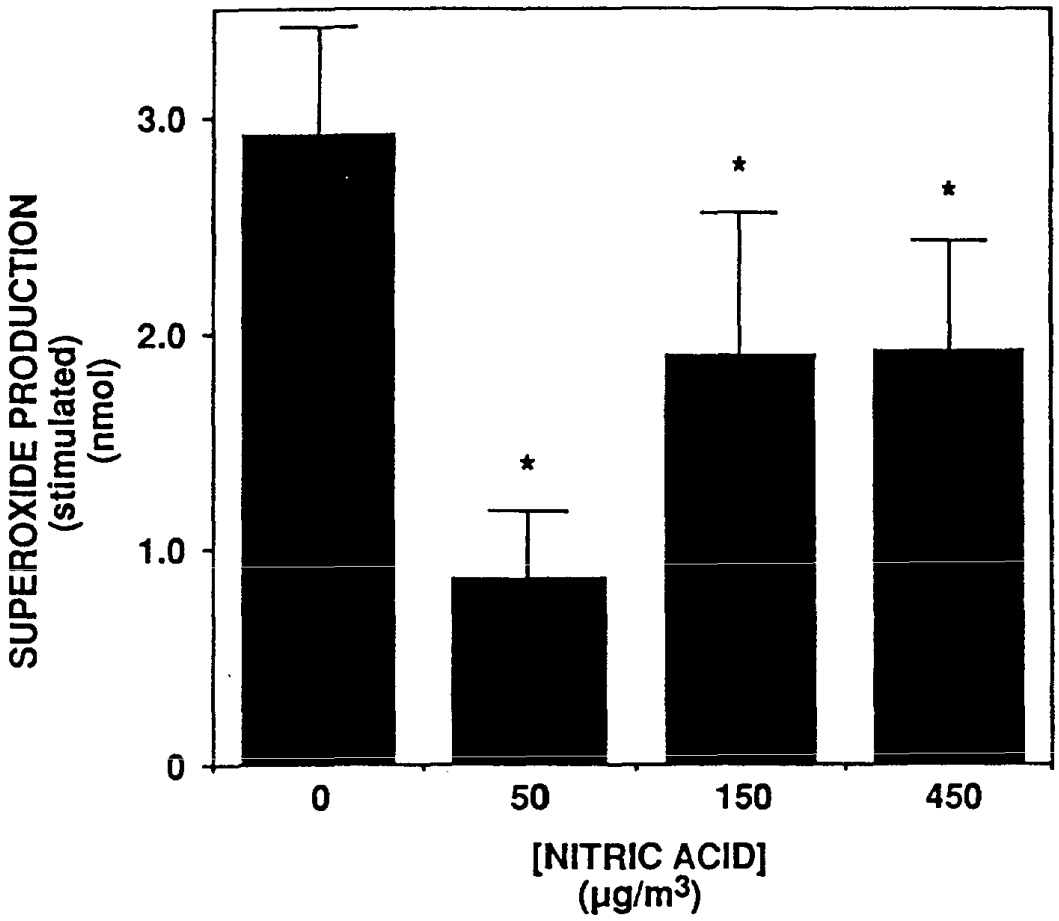



FIGURE 5 (B) Production of superoxide by stimulated macrophages following exposure to nitric acid.

* significantly different from control

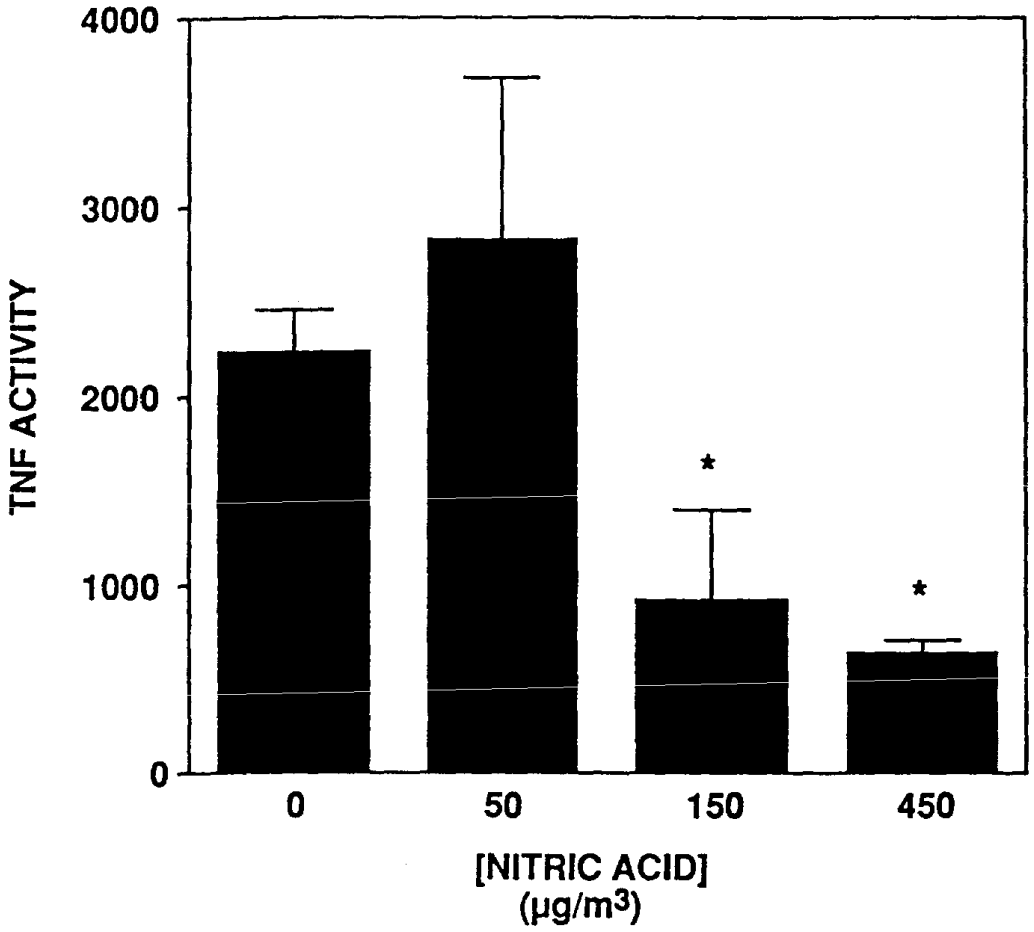



FIGURE 6. Production of TNF by macrophages following exposure to nitric acid.

* significantly different from control

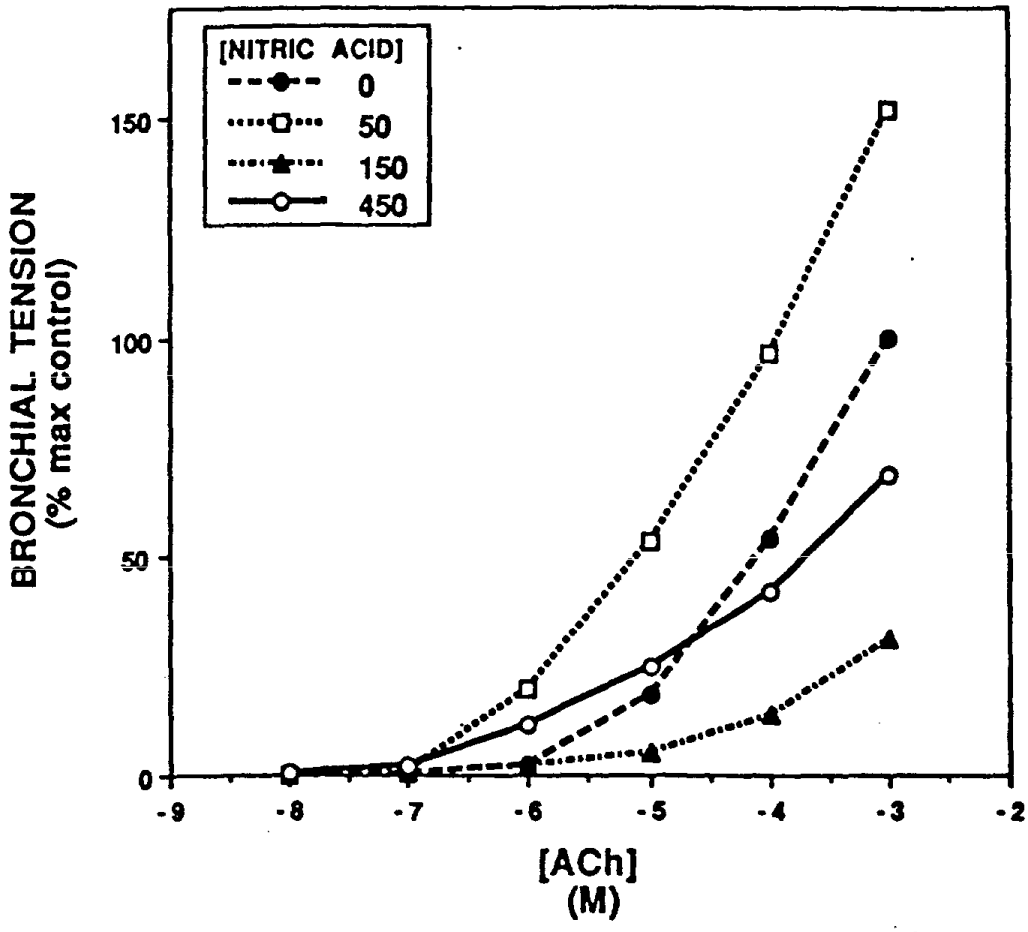



FIGURE 7 (A). Response of bronchial sections to ACh. Sections were obtained from animals exposed to nitric acid.

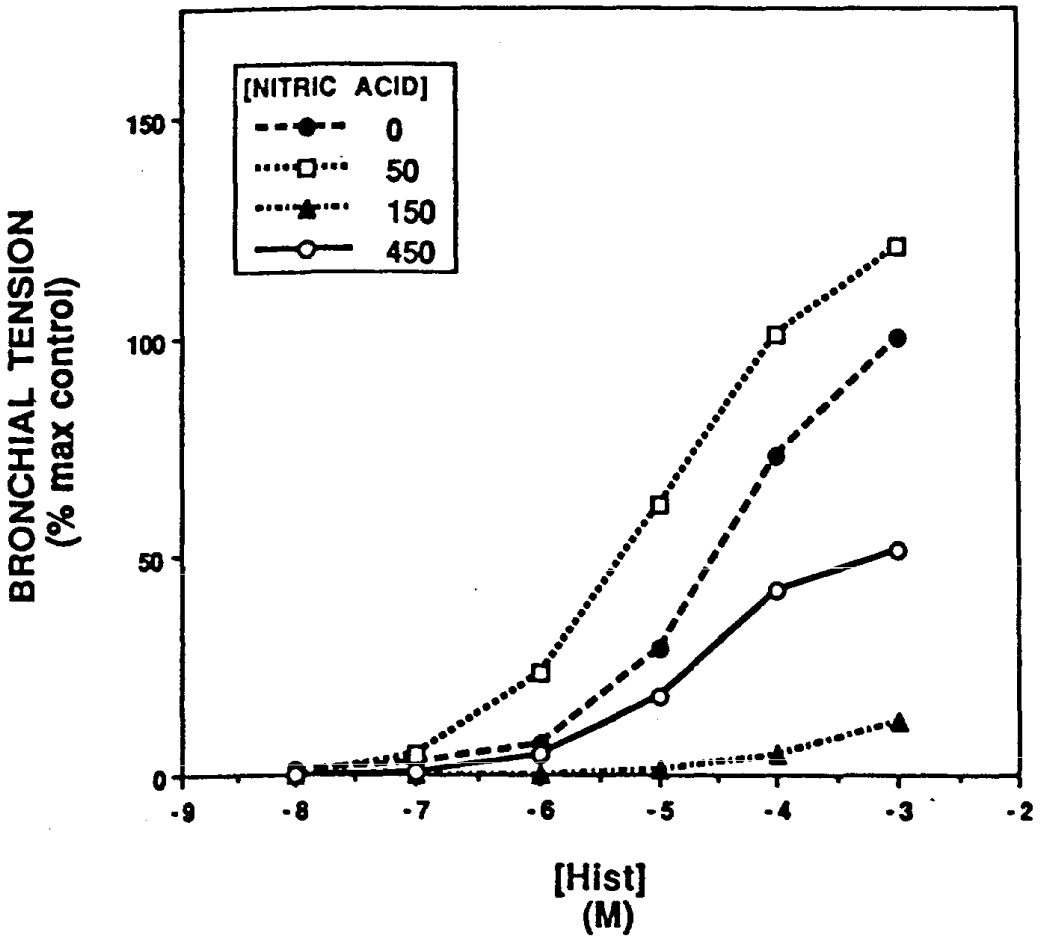



FIGURE 7 (B). Response of bronchial sections to Histamine. Sections were obtained from animals exposed to nitric acid.

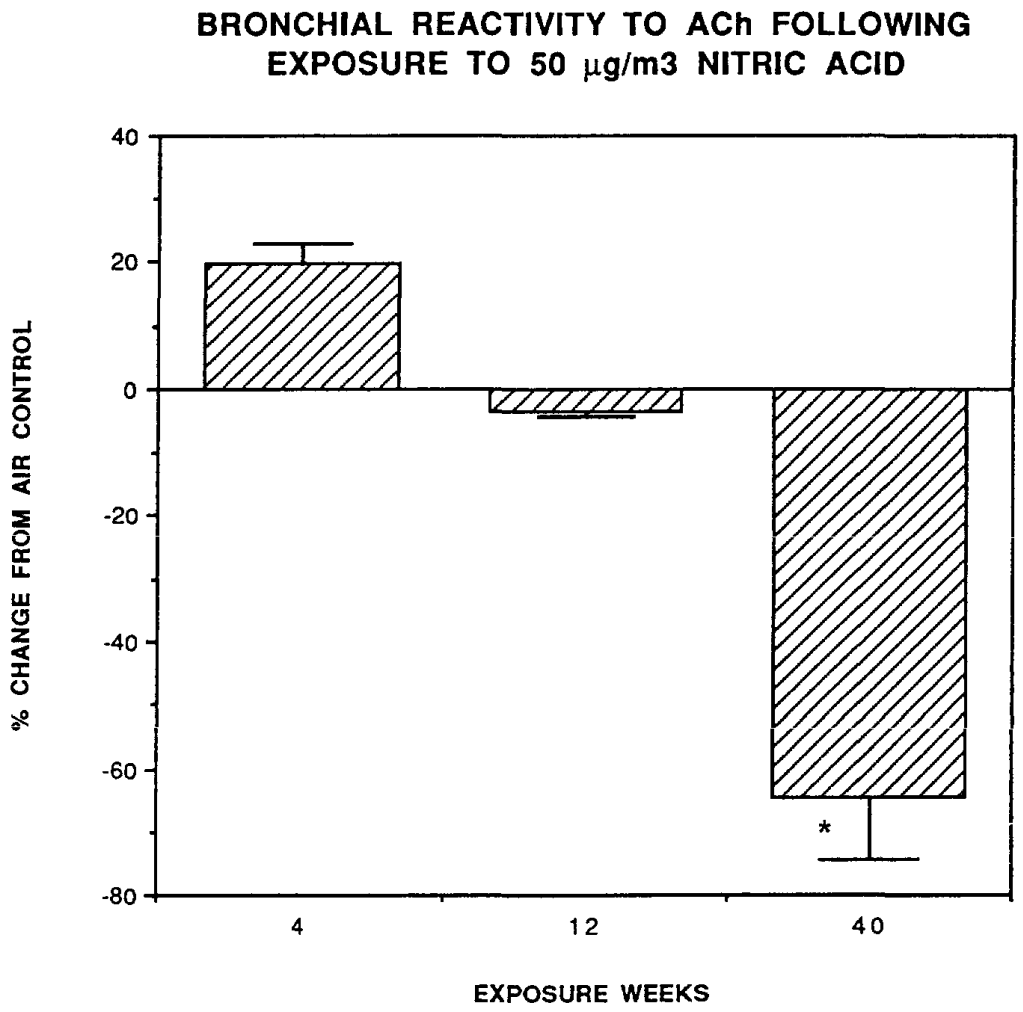



FIGURE 8. Changes in bronchial reactivity with continued exposure to nitric acid.

* significant change

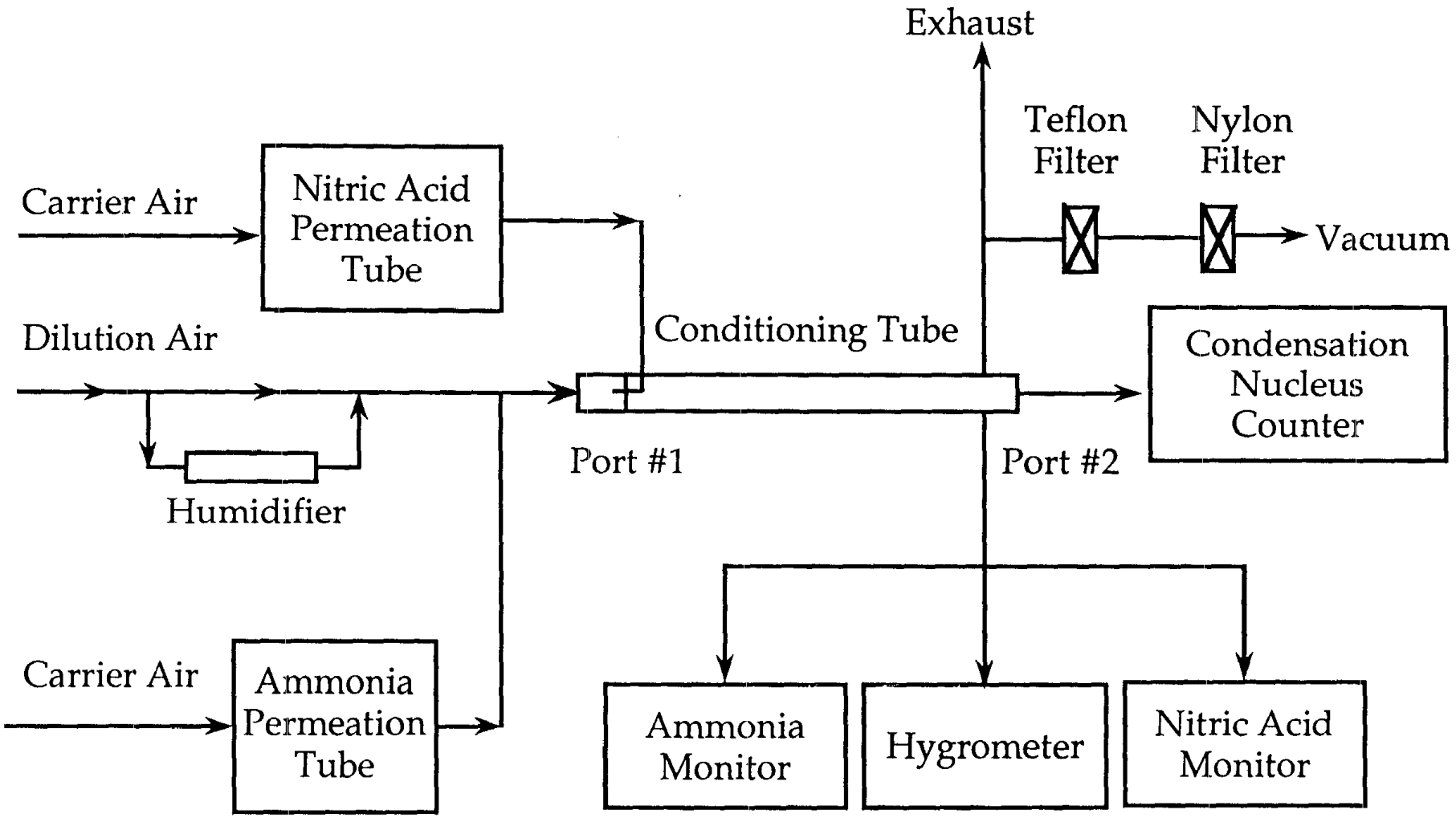



FIGURE 9. **The schematic diagram of the $NHO_3$ conditioning system.**

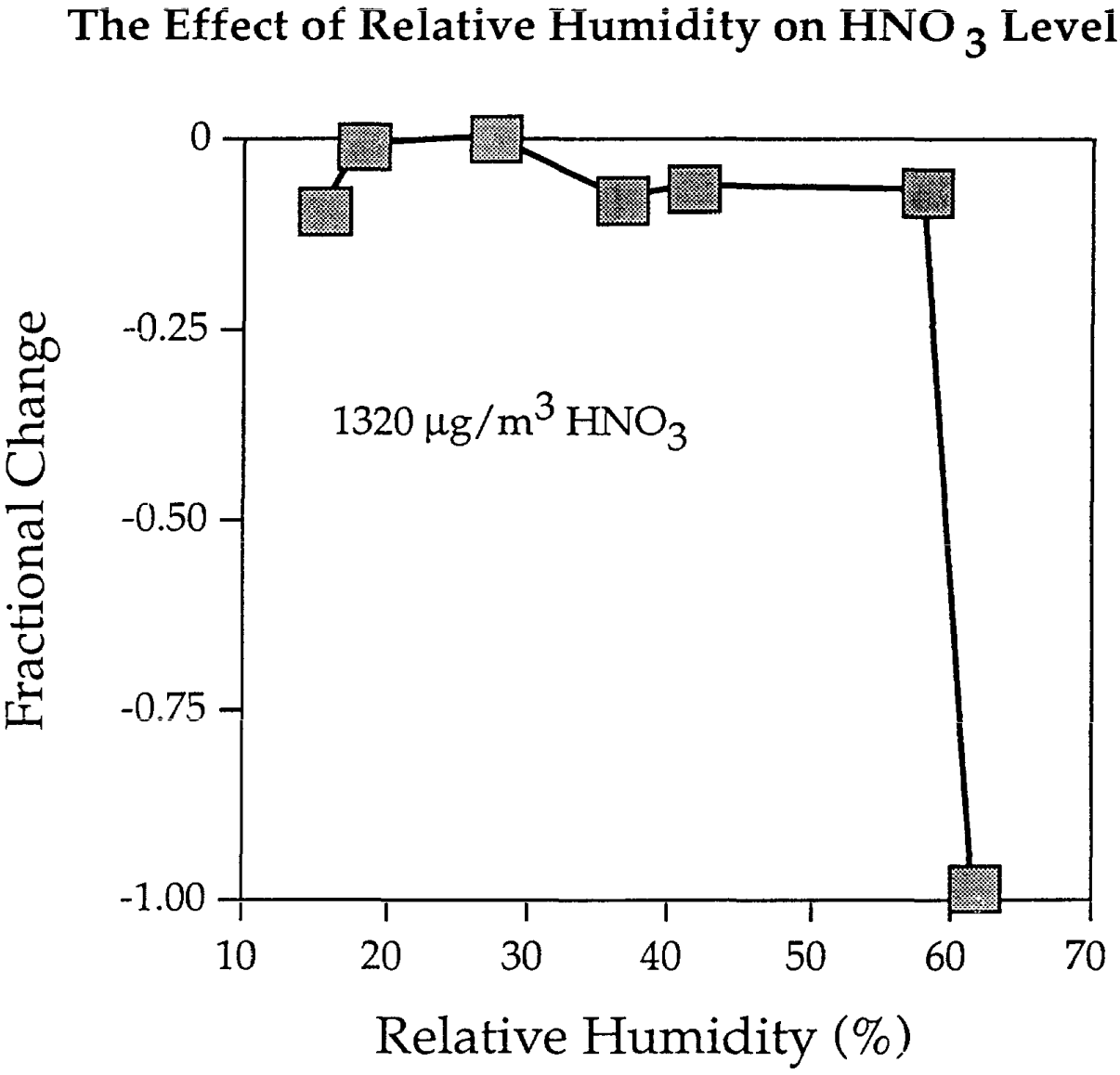



FIGURE 10. This shows the difference in nitric acid concentration between SP1 and SP2 when acid was introduced into the conditioning system maintained at different RH levels.

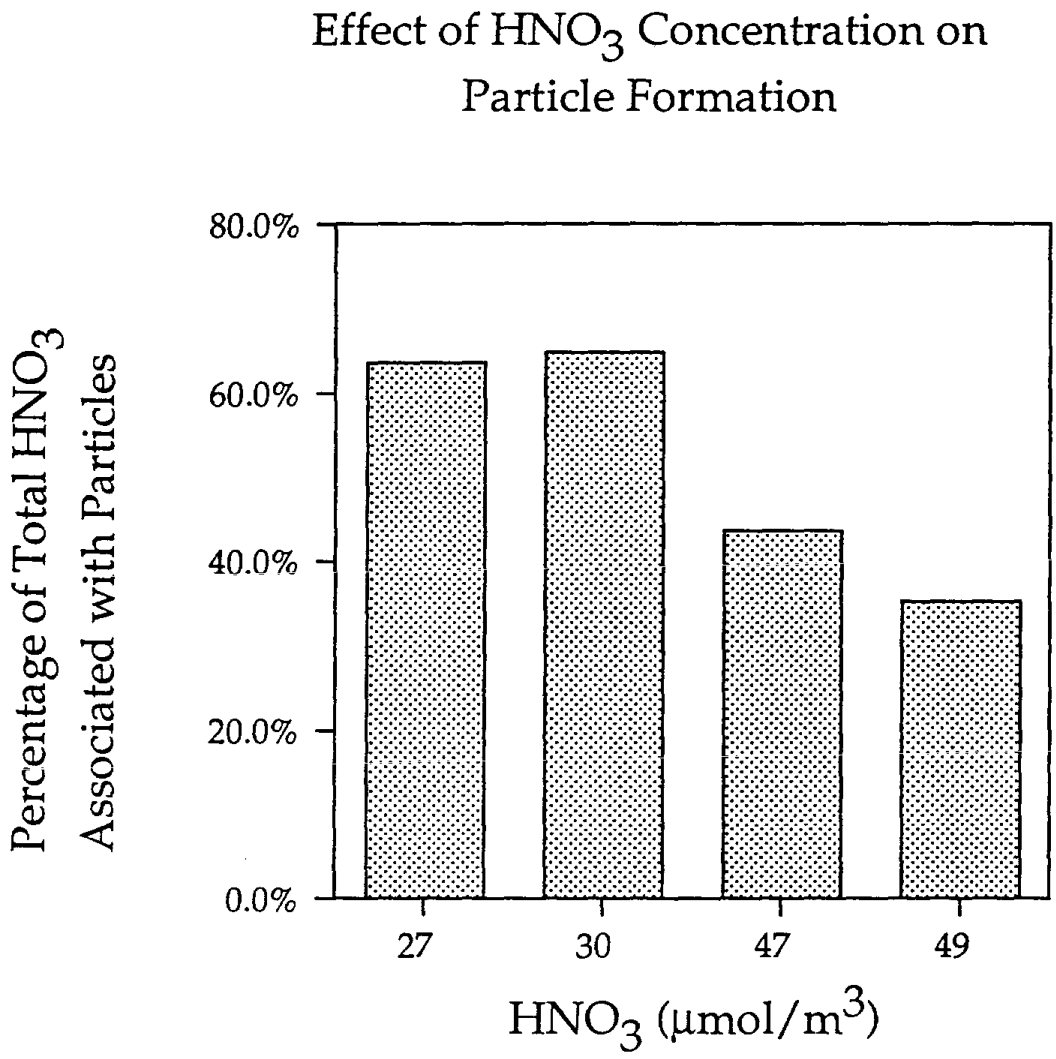



FIGURE 11. Production of particles by mixing nitric acid at various concentrations with ammonia at 460 ug/m3. Percentage of total nitric acid associated with particles was determined by the amount of nitrate collected on Teflon filters.

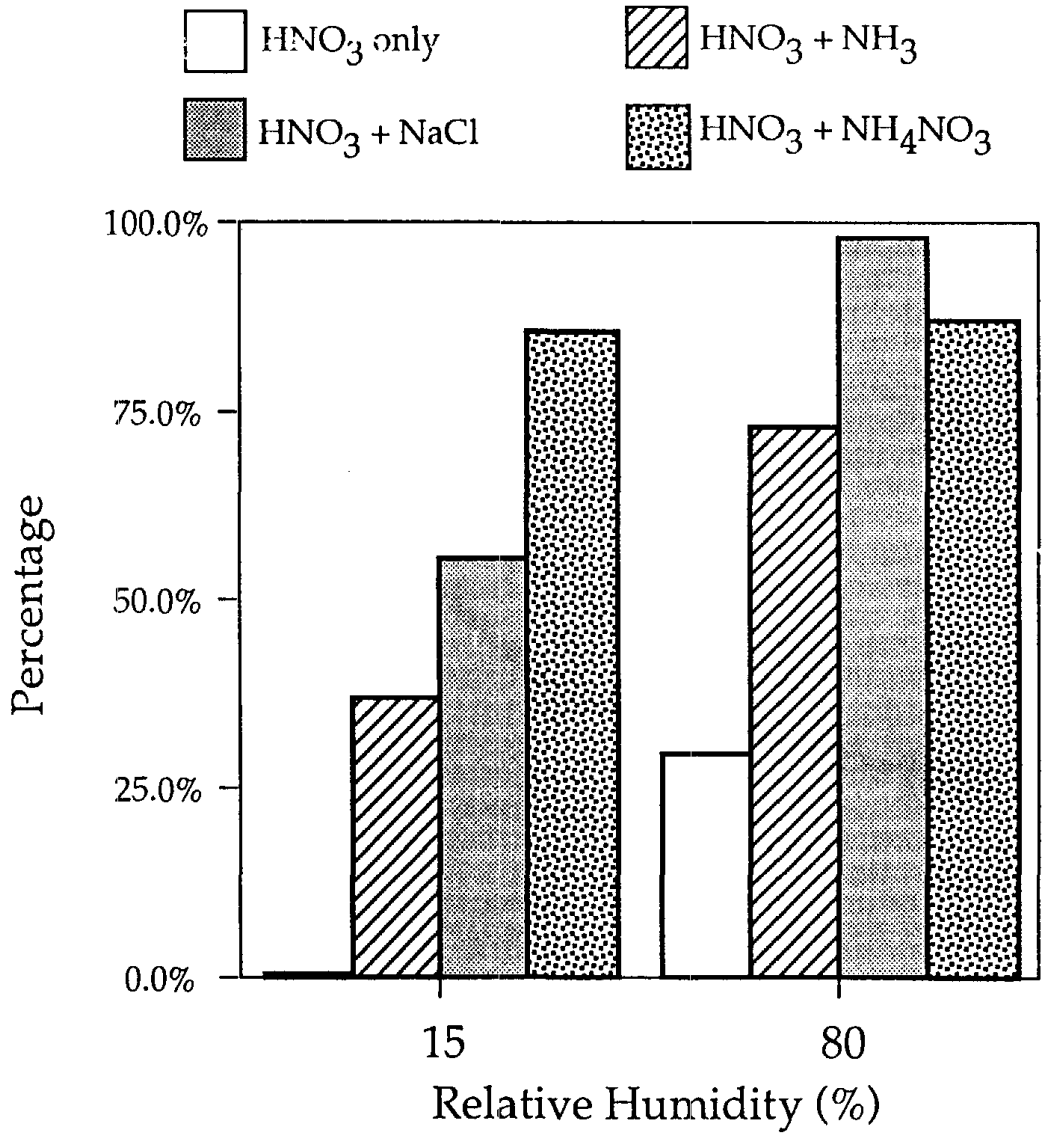



FIGURE 12. Results of mixing nitric acid (1947 ug/m3) with either sodium chloride or ammonium nitrate particles. Sampling was performed at SP2 and nitrate concentration was measured by IC.

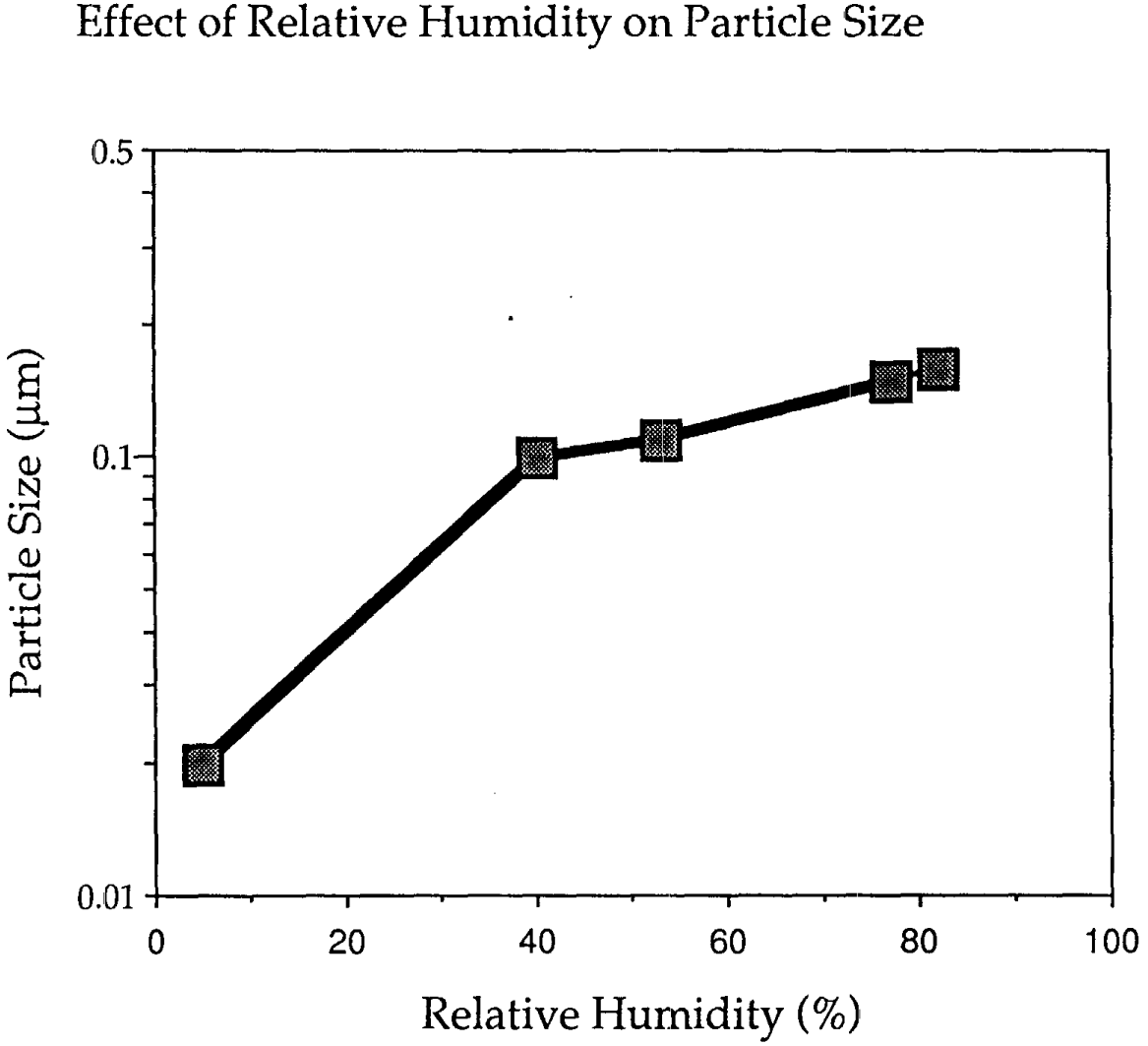



FIGURE 13. Nitric acid (1947 ug/m3) was mixed with ammonia (460 ug/m3) and the distribution of resulting particles was measured at SP2.

**TABLE 1**

**Exposure Concentrations**

| Exposure Group[a] | $[HNO_3]$[b] $\mu g/m^3$ | $[O_3]$[b] ppm |
|---|---|---|
| Control | O[c] | O[d] |
| Acid 50/4 | 50.8 (0.7) | - |
| Acid 150/4 | 153.0 (0.9) | - |
| Acid 450/4 | 457.0 (1.4) | - |
| Ozone/4 | - | 0.15 (0.001) |
| Mixture/4 | 55.3 (1.3) | 0.15 (0.001) |
| Acid 50/12 | 50.9 (0.6) | - |
| Ozone/12 | - | 0.15 (0.001) |
| Mixture/12 | 49.8 (0.7) | 0.15 (0.001) |
| Acid 50/40 | 50.5 (0.3) | - |
| Ozone/40 | - | 0.15 (0.001) |
| Mixture/40 | 50.9 (0.4) | 0.15 (0.001) |

[a] 4, 12 and 40 - denotes the 3 exposure durations.

[b] Grand means (± SE) of means of daily measures, each of which was based upon 12 readings from appropriate monitors over the course of each 4 hr daily exposure.

[c] $< 5 \mu g/m^3$

[d] < 0.002 ppm

**TABLE 2**

**Responses to $HNO_3$ Exposures**
(4 wk)

| | Lavaged Cell Characterization[a] | | | | Lavage Fluid Biochemistry[a] | |
|---|---|---|---|---|---|---|
| [$HNO_3$] ($\mu g/m^3$) | Total Cells Recovered (per kg BW x $10^6$) | Differential Count[b] (% MØ) | Cell Viability (%) | MØ Phagocytic Index (%) | LDH (BB units/ml) | Protein ($\mu g$/ml) |
| 0 | 10.7 (4.5) | 99.2 (0.7) | 97.3 (1.3) | 43.7 (12.4) | 20.3 (6.9) | 87.0 (12.6) |
| 50 | 9.0 (3.7) | 99.5 (0.8) | 98.3 (0.7) | 49.2 (14.3) | 17.3 (6.1) | 75.3 (12.1) |
| 150 | 7.7 (3.8) | 99.7 (0.5) | 97.7 (0.9) | 44.9 (10.2) | 18.5 (7.2) | 82.7 (14.6) |
| 450 | 10.3 (6.5) | 99.4 (0.4) | 95.8 (1.1) | 34.8 (10.5) | 16.1 (8.7) | 66.0 (17.4) |

[a] Mean (± SD).
[b] MØ = pulmonary macrophage.

**TABLE 3**

**Effects of $HNO_3$ on Airway Responsivity**
(4 wk)

| | Bronchus [a] | | | | Trachea [a] | |
|---|---|---|---|---|---|---|
| | ACh | | Hist | | ACh | |
| $[HNO_3]$ ($\mu g/m^3$) | Reactivity ($\Delta g/M$) | $T_{max}$ (g) | Reactivity ($\Delta g/M$) | $T_{max}$ (g) | Reactivity ($\Delta g/M$) | $T_{max}$ (g) |
| 0 | 0.50 (0.14) | 1.67 (0.56) | 0.46 (0.15) | 1.89 (0.56) | 0.31 (0.12) | 1.26 (0.68) |
| 50 | 0.60 (0.14) | 2.28 (0.25) | 0.62 (0.07) | 2.23 (0.25) | 0.27 (0.08) | 1.29 (0.39) |
| 150 | 0.15 (0.05)* | 0.52 (0.12)* | 0.09 (0.06)* | 0.23 (0.12)* | 0.27 (0.08) | 1.09 (0.48) |
| 450 | 0.25 (0.09)* | 1.14 (0.51) | 0.27 (0.13)* | 0.97 (0.51)* | 0.48 (0.22) | 1.91 (0.80) |

[a] Mean (± SD).
* Significant difference from control.

## TABLE 4

### Responses to $O_3$ and $HNO_3/O_3$
(4 wk)

| Endpoint | Exposure Atmosphere [a] | | | |
|---|---|---|---|---|
| | Sham Control | $HNO_3$ [b] | $O_3$ [c] | $HNO_3 + O_3$ |
| Cells Recovered (per kg BWx$10^6$) | 10.7 (4.5) | 9.0 (3.7) | 9.1 (6.1) | 6.5 (1.7) |
| % Macrophage (MØ) | 99.2 (0.7) | 99.5 (0.8) | 98.8 (0.8) | 99.5 (0.8) |
| Cell Viability (%) | 97.3 (1.3) | 98.3 (0.7) | 98.3 (0.9) | 96.8 (1.3) |
| MØ Phagocytic Index (%) | 43.9 (12.4) | 49.2 (14.3) | 42.0 (10.1) | 45.0 (8.7) |
| LDH (units/ml) | 20.3 (6.9) | 17.3 (6.1) | 19.5 (4.6) | 17.6 (3.8) |
| Soluble Protein (units/ml) | 87.0 (12.6) | 75.3 (12.1) | 59.8 (15.7) | 61.2 (20.1) |
| $O_2^{\cdot-}$ (spont)(nmol/3x$10^5$ MØ) | 0.36 (0.07) | 0.02 (0.01)* | 0.69 (0.12) | 1.55 (0.35)* |
| $O_2^{\cdot-}$ (stim)(nmol/3x$10^5$ MØ) | 2.92 (0.50) | 0.86 (0.31)* | 2.18 (0.21) | 1.81 (0.19)* |
| $TNF_\alpha$ (1/dilution for 50% toxicity) | 2237 (223) | 2828 (856) | 1245 (881) | 815 (362)* |
| Bronchial responsivity (ACh) | | | | |
| reactivity (Δg/M) | 0.50 (0.14) | 0.60 (0.14) | 0.46 (0.05) | - [d] |
| $T_{max}$ (g) | 1.67 (0.36) | 2.28 (0.62) | 1.83 (0.34) | 0.35 (0.76)*[e] |
| Bronchial responsivity (Hist) | | | | |
| reactivity (Δg/M) | 0.46 (0.15) | 0.62 (0.07) | 0.52 (0.15) | - [d] |
| $T_{max}$ (g) | 1.89 (0.56) | 2.23 (0.25) | 2.20 (0.50) | 0.05 (0.1)*[e] |
| Tracheal responsivity (ACh) | | | | |
| reactivity (Δg/M) | 0.31 (0.12) | 0.27 (0.08) | 0.29 (0.10) | 0.23 (0.03) |
| $T_{max}$ (g) | 1.21 (0.31) | 1.25 (0.29) | 1.22 (0.46) | 1.27 (0.23) |

[a] Mean (± SD)
[b] $HNO_3$ = 50 μg/$m^3$
[c] $O_3$ = 0.15 ppm.
[d] No measurable response from 4/6 animals.
[e] Based upon n=2 responding animals.
* Significant difference from control.

## TABLE 5

### Summary of Interactions

(4 wk)

| Endpoint | Response [a] | | | Interaction Analysis | |
|---|---|---|---|---|---|
| | $HNO_3$ [b] | $O_3$ | $HNO_3 + O_3$ | p[c] | Type of Interaction [d] |
| Cells recovered | ns | ns | ns | 0.97 | 0 |
| % Macrophage (MØ) | ns | ns | ns | 0.29 | 0 |
| Cell viability | ns | ns | ns | 0.42 | 0 |
| MØ Phagocytic Index | ns | ns | ns | 0.46 | 0 |
| LDH | ns | ns | ns | 0.52 | 0 |
| Soluble protein | ns | ns | ns | 0.87 | 0 |
| $O_2^{\cdot -}$ (spont) | ↓ | ns | ↑ | 0.01 | + |
| $O_2^{\cdot -}$ (stim) | ↓ | ns | ↓ | 0.001 | - |
| $TNF_\alpha$ | ns | ns | ↓ | 0.143 | 0 |
| Bronchial responsivity (ACh) | | | | | |
| reactivity | ns | ns | (e) | – | + |
| $T_{max}$ | ns | ns | ↓ | 0.001 | + |
| Bronchial responsivity (Hist) | | | | | |
| reactivity | ns | ns | (e) | – | + |
| $T_{max}$ | ns | ns | ↓ | 0.001 | + |
| Tracheal responsivity (ACh) | | | | | |
| reactivity | ns | ns | ns | 0.56 | 0 |
| $T_{max}$ | ns | ns | ns | 0.96 | 0 |

[a] ns = no significant difference from control.
↓ = significant decrease from control.
↑ = significant increase from control.

[b] $HNO_3$ = 50 $\mu g/m^3$; $O_3$ = 0.15 ppm.

[c] p values of multiple linear contrast.

[d] 0 = no interaction.
+ = synergism.
- = antagonism.

[e] 4 of 6 animals showed no response to spasmogen while two showed marked attenuation.

Table 6. Results of 12 and 40 week Exposures[a]

| | 12 week | | | | 40 week | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Acid | Ozone | Mixture | Control | Acid | Ozone | Mixture |
| Lavage LDH (units/ml) | 23 (2.6) | 21 (2.8) | 17 (1.8) | 19 (2.1) | 11 (2.2) | 16 (4.5) | 22 (3.3) | 6 (1.4) |
| Lavage Protein (μg/ml) | 61 (4.8) | 59 (7.3) | 61 (4.2) | 59 (4.0) | 71 (11) | 50 (9) | 57 (9) | 62 (3) |
| Hydrogen Peroxide (S)[b] (nmole/3x$10^5$ MØ) | 1.59 (0.13) | 1.27 (.23) | 1.45 (.13) | 1.70 (.19) | 1.69 (.19) | 1.84 (.3) | 1.53 (.22) | 1.56 (.21) |
| Hydrogen Peroxide (U)[c] | 1.25 (.12) | 0.92 (.11) | 1.04 (.14) | 1.18 (.11) | 1.09 (.16) | 0.96 (.2) | 0.89 (.14) | 0.80 (.13) |
| Superoxide (S)[b] | 1.22 (.07) | 1.32 (.23) | 1.17 (.12) | 1.39 (.18) | 2.42 (.24) | 2.13 (.19) | 2.56 (.28) | 2.18 (.20) |
| Superoxide (U)[c] | .34 (0.06) | .53 (.13) | .47 (.12) | .59 (.11) | .53 (.15) | .59 (.16) | .64 (.13) | .51 (.08) |
| Phagocytic Capacity[d] | 13 (1.7) | 6 (1.4) | 6 (1.8) | 13 (3.5) | Not Yet Available | | | |
| Phagocytic Index[e] | 47 (3.8) | 28 (1.8)* | 46 (3.1) | 46 (2.8) | | | | |
| Recovered Cells/BW (x$10^6$) | 8.2 (1.4) | 5.5 (0.7) | 6.4 (0.6) | 6.3 (.9) | 5.8 (.9) | 5.1 (.8) | 4.9 (.5) | 4.0 (.4) |
| Cell Viability (%) | 98 (.3) | 98 (.3) | 97 (.3) | 95 (1) | 9.5 (1.1) | 98 (0.8) | 95 (1.3) | 97 (.8) |

(a) Values are mean ± SE
(b) S = stimulated macrophages
(c) U = unstimulated macrophages
(d) Phagocytic capacity = % of cells containing ≥ 4 particles
(e) Phagocytic index = % of total cells which contain particles
* $p < 0.05$ compared to control

**Table 7. Airway Responsiveness**
(12 and 40 wk)

| | 12 week | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bronchi | | | | Trachea | | | |
| | Control | Acid | Ozone | Mixture | Control | Acid | Ozone | Mixture |
| Reactivity (A)[a] | .27 (.05) | .26 (.04) | .19 (.03) | .29 (.05) | .45 (.08) | .44 (0.8) | .35 (.06) | .27 (.06) |
| $T_{max}$ (A)[a] | 1.12 (0.18) | 1.18 (0.19) | 0.83 (0.12) | 1.14 (0.15) | 1.95 (.40) | 2.11 (.41) | 2.01 (.11) | 1.42 (.31) |
| Reactivity (H)[a] | .39 (.04) | .34 (.02) | .24 (.05) | .34 (.03) | | | | |
| $T_{max}$ (H)[a] | 1.60 (.11) | 1.32 (.13) | 1.05 (.19) | 1.44 (.15) | | | | |
| | **40 week** | | | | | | | |
| | Bronchi | | | | Trachea | | | |
| | Control | Acid | Ozone | Mixture | Control | Acid | Ozone | Mixture |
| Reactivity (A) | 0.31 (0.04) | 0.11 (0.03)* | 0.28 (0.02) | 0.26 (0.02) | 0.63 (0.23) | 0.42 (0.05) | 0.49 (0.05) | 0.36 (0.05) |
| $T_{max}$ (A) | 1.49 (0.22) | 0.40 (0.12)* | 1.20 (0.16) | 1.10 (0.16) | 2.74 (0.84) | 1.89 (0.24) | 2.08 (0.24) | 1.66 (0.30) |
| Reactivity (H) | 0.29 (0.03) | 0.14 (0.03)* | 0.31 (0.04) | 0.36 (0.04) | | | | |
| $T_{max}$ (H) | 1.81 (0.24) | 0.64 (0.14)* | 1.59 (0.16) | 1.51 (0.26) | | | | |

(a) A = ACh
H = Histamine
* $p < 0.05$ compared to control
(all values are mean ± SE)

TABLE 8

PULMONARY MORPHOMETRY

ALVEOLAR SEPTAL THICKNESS

| Exposure | 4 wk | 4 wk repeat | 12 wk | 40 wk |
|---|---|---|---|---|
| Control | 4.8 (0.4) | 4.6 (1.2) | 5.7 (1.4) | 3.8 (0.4) |
| 50 $HNO_3$ | 5.2 (0.5) | 3.8 (0.3) | 5.4 (0.7) | 4.3 (0.4) |
| 0.15 $O_3$ | 5.9 (0.8)* | - | 5.8 (1.6) | 4.1 (0.7) |
| $HNO_3$+$O_3$ | 5.6 (1.2) | - | 6.0 (1.3) | 4.4 (0.7) |
| 150 $HNO_3$ | 6.0 (0.5)* | 4.2 (0.6) | - | - |
| 450 $HNO_3$ | 7.1 (0.3)* | 4.3 (0.2) | - | - |

ALVEOLAR AIRSPACE SIZE

| Exposure | 4 wk | 4 wk repeat | 12 wk | 40 wk |
|---|---|---|---|---|
| Control | 32.4 (2.8) | 62.8 (5.3) | 47.8 (6.6) | 60.3 (9.1) |
| 50 $HNO_3$ | 31.8 (4.9) | 64.8 (2.8) | 47.5 (3.1) | 58.0 (3.9) |
| 0.15 $O_3$ | 48.0 (3.0)* | - | 56.2 (6.5)* | 53.2 (3.8) |
| $HNO_3$+$O_3$ | 41.2 (4.4)* | - | 61.2 (8.5)* | 56.2 (2.5) |
| 150 $HNO_3$ | 29.3 (1.4) | 67.1 (5.1) | - | - |
| 450 $HNO_3$ | 26.6 (4.7) | 57.8 (2.0) | - | - |

Values are mean (SD) in μm

* = significant difference from control ($p<0.05$)

# APPENDIX

# STANDARD PROCEDURES FOR THE EXTRACTION OF NITRIC ACID FROM NYLON FILTERS AND DENUDERS

SOP 91-101 ARB

September, 1991

**NOTE:** The following extraction protocols will result in an accurate measurement of collected nitric acid only if proper procedures are used to ensure removal of any free ammonia and/or particulate nitrates from the sampling system.

## I. REAGENTS AND MATERIALS USED

| | |
|---|---|
| Sodium Carbonate: | Fisher S-263-500 Certified |
| Sodium Bicarbonate: | Fisher S-233-500 Certified |
| Nitric Acid: | Fisher SA95 1N Certified |
| Glycerin: | Fisher 6153-1 Spectroanalyzed Certified |
| Alcohol: | Pharmco Ethyl Alcohol 200 Proof |
| Nylon Filter: | Gelman 47 μm Nylasorb (#66509) 1 μm |
| Caps: | Fisher Stopper Plug 14-959-17 |
| Annular Denuder: | University Research Glassware, NC |
| Extraction tubes: | VWR 3310-800 Polystyrene Tubes Round Bottom (12x75 mm) |
| Large Denuder Extraction Tube: | Sarstedt 62-554-002 (120X 17 mm) conical tubes & screw cap |

## II. EXTRACTION OF NITRIC ACID FROM NYLON FILTERS

### A. Preparation of Extraction Solution

1. Add 1 liter of 18 Ω water into a 2 L volumetric flask.
2. Weigh 0.3396 g sodium carbonate, and 0.5047 g sodium bicarbonate.
3. Add the above to the 2 L flask, and mix well until dissolved.
4. Bring contents to 2 L mark with 18 Ω water and mix again.

### B. Extraction Procedure

1. Place extraction tubes and caps in a rack inside $NH_3$ free hood.
2. Place all collected filter samples in petri dishes and place these and all denuders inside the hood.
3. Place clean forceps and a beaker of 100% ethanol in the hood.

4. Remove each filter from the petri dish using alcohol-washed forceps and place the filter into the extraction tube. Add 4 ml of extraction solution, and cap immediately.

5. Rinse forceps in 18 Ω water, then in alcohol between each use.

6. Remove the rack from the hood and vortex each tube (high level) for 1 minute .

7. These solutions will be used for ion chromatographic analyses. If saved for analysis at a future time, they must be refrigerated.

## III. EXTRACTION OF NITRIC ACID FROM CARBONATE/BICARBONATE DENUDERS

1. Place all labeled denuders and extraction solutions inside the $NH_3$ free hood.

2. Remove the cap of of one end of each denuder, and add 10 mL of 18Ω water and then cap it again.

3. Vortex for 1 minute, rotating the denuder at the same time.

4. Pour the contents into a 15 mL labelled tube and cap.

5. Note the volume recovered of the solution. This is extraction # I.

6. Repeat steps 2-4 for extraction # II.

7. Note the volume recovered each time.

8. These solutions will be used for ion chromatographic analyses. If saved, they must be refrigerated.