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FINAL REPORT

THE INFLUENCE OF EXERCISE ON LUNG INJURY INDUCED BY OZONE AND NITROGEN DIOXIDE

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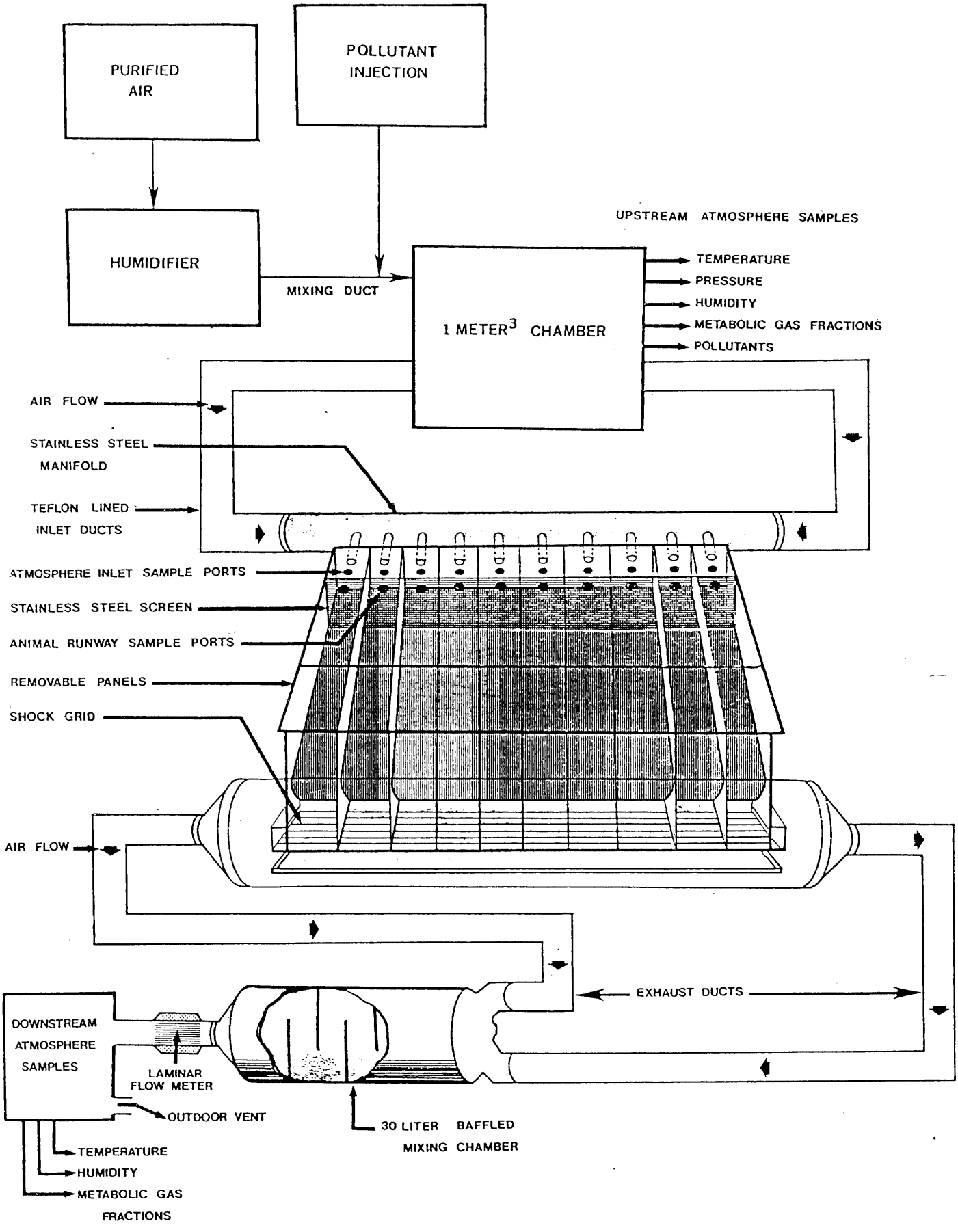
EXECUTIVE SUMMARY

The purpose of this research was to examine factors modifying lung lesions induced in laboratory rats by ozone inhalation. An earlier study (ARB Contract A0-129-32) demonstrated enhancement of lung damage by light exercise; in exposures with intermittent rest and exercise, ozone concentration and the proportion of time spent in exercise were important factors increasing abundance and severity of lung lesions. For the present study a rat exercise protocol was developed to utilize continuous exercise at higher workloads. In terms of the increase in metabolic gas exchange over resting rates, the moderate exercise levels in this study were analogous to human walking or light manual labor. Lung lesions were quantified as percent of parenchymal cross section area involved for two morphological types of lesions: Type I: free cells in alveolar spaces, and Type II: alveolar duct walls and alveolar septa thickened due to infiltrating cells. Three experiments were performed: 1) Effect of duration of exposure to 0.35 ppm O_3 during exercise, 2) Effect of increasing exercise workload at constant effective dose of 0.35 ppm O_3, and 3) Effect of 0.35 ppm O_3 and 0.6 ppm NO_2 inhaled alone and in combination during exercise. In the test of exposure duration, rats were exposed to 0.35 ppm O_3 while running 15 m/min at 20% grade for 0.5, 1.0, 2.0, or 3.0 hours. Type I lesion areas did not differ from control (1.8% of parenchymal section area) for 0.5 and 1.0 h exposures but increased by nearly a factor of 2 (3.4% and 3.6%) in 2.0 and 3.0 h exposures respectively. Type II lesions were first apparent in the 1.0 h exposure (0.18%) and increased to 2.6% and 3.2% in 1 and 3 h exposures.

In the second experiment, rats were again exposed to 0.35 ppm O_3. Minute ventilation could not be measured to accurately calculate effective dose of O_3, however oxygen consumption was measured and used as an index proportional to ventilation. Exposure duration was adjusted to hold the quantity (ppm O_3)·(duration)·(average $\dot{V}_{O_2}$) constant. Exercise levels and corresponding exposure durations were continuous rest (3.4 h), run 8 m/min at 0% grade (2.75 h), 15 m/min at 20% grade (2.33 h), and 30 m/min


PURIFIED AIR
POLLUTANT INJECTION
HUMIDIFIER
MIXING DUCT
UPSTREAM ATMOSPHERE SAMPLES
1 METER^3 CHAMBER
TEMPERATURE
PRESSURE
HUMIDITY
METABOLIC GAS FRACTIONS
POLLUTANTS
AIR FLOW
STAINLESS STEEL MANIFOLD
TEFLON LINED INLET DUCTS
ATMOSPHERE INLET SAMPLE PORTS
STAINLESS STEEL SCREEN
ANIMAL RUNWAY SAMPLE PORTS
REMOVABLE PANELS
SHOCK GRID
AIR FLOW
EXHAUST DUCTS
DOWNSTREAM ATMOSPHERE SAMPLES
LAMINAR FLOW METER
OUTDOOR VENT
30 LITER BAFFLED MIXING CHAMBER
TEMPERATURE
HUMIDITY
METABOLIC GAS FRACTIONS

at 20% grade with 2 rest periods of 7 min (1.75 h). Despite near equivalence of effective dose of O_3, lesion areas increased with increasing exercise intensity. Type I lesion areas for rest and 8 m/min, 0% grade groups were similar to control, but increased by factors of 1.5 and 3 (to 3.2% and 6.4% of parenchymal area) for exercise at 15 m/min, 20% grade and 30 m/min, 20% grade respectively. Type II lesions were present at low incidence (0.15% of parenchymal area) in the rest exposure and progressively increased by factors of 3 (to 0.5%), 16 (to 2.4%), and 33 (to 5.0%) for the 3 successive exercise workloads.

In the O_3 and NO_2 interaction experiment, rats ran at 15 m/min 20% grade for a 3 hour exposure. Exposure groups were 0.35 ppm O_3, 0.6 ppm NO_2, and combination 0.35 ppm O_3 and 0.6 ppm NO_2. Exposure to NO_2 alone resulted in no difference from clean air control, however NO_2 in combination with O_3 resulted in enhancement of lesion areas induced by O_3 alone. The experiment was repeated, and the results were similar. Combined results of the experiments showed Type I lesion areas induced by O_3 alone were increased above control by a factor of 1.6 (to 2.9% of parenchymal area), but in combined O_3 and NO_2, lesion areas increased by a factor of 2.7 over control (to 4.8% of parenchymal area). Type II lesion areas (not found in control clean air exposure) induced by exposure to O_3 alone (2.2% of parenchymal area) were increased by a factor of 2.3 (to 5.1%) when NO_2 was present as a co-pollutant.

Results of these experiments demonstrated the critical importance of common modifying factors such as exercise and co-pollutants to damage to the respiratory system. Although it is not likely that rats and humans develop the same quantitative extent of tissue damage from oxidant exposures, the qualitative effects and relative importance of the exposure variables tested (exercise and co-presence of NO_2 with O_3) are expected to be similar. Furthermore, sensitivity of both humans and animals is highly variable, and the range of human sensitivity may overlap that of the rat model.

INTRODUCTION

Inhalation of airborne pollutants during exercise is expected to increase risk of adverse health effects because exercise involves an increase in respiratory ventilation and thus increase in inhaled dose rate. Furthermore, the toxic effects of air pollutant inhalation may not be simply proportional to increased dose rate due to exercise ventilation. Changes in the rate and depth of ventilation with exercise may result in an altered distribution of inhaled dose, and protective breathing patterns operating at rest in response to irritant compounds may be overridden by the demands for increased gas exchange during exercise. Both these factors could result in greater penetration of pollutant compounds to the deep lung tissues and enhance the effect of increased dose. Thus risk during exercise exposure may be considerably greater than predicted by simple proportion to inhaled dose.

Numerous investigations have demonstrated pulmonary function decrements in humans exposed to ozone with enhanced effects evident in exercising exposures (Bates et al., 1972; Folinsbee et al., 1975, 1977; Silverman et al., 1976). Low concentrations of ozone (less than 0.3 ppm) that do not result in pulmonary function decrements in resting subjects can provoke responses under conditions of intermittent exercise (Hazucha et al., 1973; Silverman et al., 1976), and particularly sensitive subpopulations are responsive to ozone concentrations as low as 0.15 ppm (DeLucia and Adams, 1977). Recent efforts to study the relationship between ozone concentration, exercise level, and duration of exposure have focused on the effective dose, the product of concentration, ventilation, and duration (Silverman et al., 1976; Hackney et al., 1975; Adams et al., 1981).

Nitrogen dioxide is a less powerful oxidant than ozone, and its toxic effects are apparent at much higher concentrations and exposure durations (Hine et al., 1970; Morrow, 1975; Dawson and Schenker, 1979). In exercising exposures of humans to concentrations comparable to ambient levels, pulmonary function decrements are generally not observed (Hackney et al., 1978; Folinsbee et al., 1978), and pulmonary

function response to a combination of ozone and nitrogen dioxide was similar to the effect of exposure to ozone alone (Folinsbee et al., 1981). However, some evidence for a sensitive subpopulation among asthmatics was found in a study of bronchoconstrictor challenge following nitrogen dioxide exposure (Orehek et al., 1976), and ambient nitrogen dioxide has been implicated as an important factor in epidemiological studies of acute respiratory disease (Perlman et al., 1971; Love et al., 1982).

Laboratory animal studies permit measures of actual tissue damage following oxidant exposure. Because tissue damage in rats occurs at ozone concentrations similar to those which produce human functional decrements, animal studies are likely to be useful in estimating human tissue damage levels. Furthermore, animal studies permit an efficient way to examine the effects of several variables on toxic response. Such variables include concentration, exposure duration, level of exercise, and composition of pollutant mixtures. Ozone and nitrogen dioxide have similar effects on lung tissue morphology. Distal portions of the tracheobronchial tree and alveolar regions of the lung appear to be the primary targets. Effects of acute exposures to low concentrations of ozone (less than 1 ppm) or nitrogen dioxide (less than 5 ppm) include increased numbers of macrophages, loss of Type I alveolar cells, increased numbers of Type II alveolar cells, interstitial edema, and increased susceptibility to pulmonary infection challenges (Goldstein et al., 1973; Stephens et al., 1974a, 1974b; Morrow, 1975; Schwartz et al., 1976; Coffin and Stokinger, 1977; Ehrlich et al., 1977). A recent study documented a synergism between O_3 and NO_2 effects on lung collagen synthesis rates (Last et al., 1983).

Pulmonary irritants like ozone and nitrogen dioxide can induce a protective rapid-shallow breathing pattern in animals (Alarie, 1973), and it is important to consider the effect of exercise on toxic response. The demands for gas exchange in exercising animals may eliminate or at least diminish the rapid shallow breathing response leading to increased delivery of the pollutant to the deep lung. In an earlier ARB-supported

study at the Air Pollution Health Effects Laboratory, we examined the effect of exercise on ozone-induced lung damage in rats (Mautz et al., 1982, 1984). Rats exposed for 3.75 h to 0.20 and 0.38 ppm ozone developed focal lung parenchymal lesions which increased in abundance and severity when the animals were exposed during intermittent treadmill exercise. Increases in the proportion of time the rats spent running further increased alveolar zone areas involved in lesions. Exposure to 0.20 ppm O_3 during exercise produced lesions which were not observed in a similar exposure at rest, and in other exercise exposures the lesion response was greater than that predicted by a simple proportion to ventilation dose rate. The results indicated that exercise has a greater impact than can be explained by increased minute ventilation, and that the duration of exercise exposure has a large influence on ozone lung damage as well.

The purpose of this research project was to extend our earlier ARB study of lung tissue damage and ozone inhalation during exercise. Exposure experiments with 0.35 ppm O_3 and 0.6 ppm NO_2 were conducted to address the following questions:

1. What is the relationship between O_3 induced lung parenchymal lesions and duration of exercising exposure?
2. How do different intensities of exercise affect O_3 induced lung injuries?
3. Are the effects of O_3 and NO_2 inhaled during exercise as a mixture simply additive or is there significant interaction?

MATERIALS AND METHODS

Treadmill exposure system.

Rats were exposed to O_3 and NO_2 while exercising in a modified Quinton 42-15, variable speed and grade treadmill. The treadmill was enclosed, lined with stainless steel sheet to reduce pollutant losses to the structure, and instrumented to measure average metabolic gas exchange of 10 exercising rats. The exposure atmosphere was generated by mixing O_3 or NO_2 with purified air. O_3 was generated from medical grade O_3 by an

electrostatic discharge generator (Sander Ozonizer, Type III). NO_2 was admitted to the air stream from a tank of compressed gas. The treadmill and atmosphere exposure system have been described in detail in the Final Report for ARB contract AO-129-32 (Mautz, 1984) and in a manuscript submitted for publication which is appended to this report.

Oxidant gas concentrations in the treadmill were measured repeatedly from individual runways. Sample points were isolated from direct contact by rats with a coarse mesh (2 x 2) stainless steel screen. O_3 was measured with a Dasibi model 1003-AH ozone analyzer calibrated before and after exposure with a Dasibi calibrator. Periodically Dasibi instruments were calibrated using an ultraviolet photometric standard at the Dasibi factory and at the ARB El Monte Laboratory. NO_2 was measured with a chemilumeniscent detector (Monitor Laboratories, Model 8840). The instrument was calibrated with a Dynacal NO_2 permeation tube (VICI Metronics, Santa Clara, CA).

Tests were performed with mixed O_3 and NO_2 atmosphere to examine the possible formation of acid reaction products. Particles were sampled on a 47 mm membrane filter in tandem with a nylon filter which quantitatively collected ntiric acid vapor. Membrane and nylon filters were extracted with distilled water and analyzed for nitrate ion by ion chromatography.

Histopathology.

Tissue injury was measured in lung sections. Two days post exposure rats were deeply anesthetized with i.p. injections of sodium pentobarbital and killed by exsanguination through the abdominal aorta. The remote injection of anesthetic, immediate preparation and fixation of tissues, and identical treatment of control and pollutant exposed animals ensured that lung tissues were not damaged by the alkaline pentobarbital injection. The rib cage was opened after puncturing the diaphragm and allowing the lungs to collapse. The trachea was transsected 3-5 rings below the larynx, and the distal portion with attached lungs and thoracic viscera removed. Lung surfaces

were rinsed with saline and examined for gross abnormalities. The trachea was cannulated and the lungs fixed by airway perfusion with 10% neutral-buffered formalin at 30 cm fluid pressure for 72 hours (McClure et al., 1982). Lungs were then removed from the perfusion-fixation apparatus and apical, middle, and caudal lobes of the right lung separated and cut along the main lobar bronchus. A block containing a half lobe was prepared from the caudal lobe by cutting in a plane parallel to the center line of the lobar bronchus. The half lobe block was dehydrated and infiltrated with paraffin under vacuum on an automatic tissue processor. Paraffin imbedded tissues were sectioned at 6 microns and stained with hematoxylin and eosin. Total parenchymal cross section area of the lung section from each rat was measured using a dissecting microscope with a one-hundred square ocular grid calibrated with a stage micrometer. Large bronchi and vessels were excluded, and total parenchymal area was recorded for calculating percent of the area involved in lesions. The section was then examined systematically with a compound microscope to measure the lesion areas. An ocular grid at 100x covered a field of 1 x 1 mm. Each subdivision in the grid covered a region of 0.1 x 0.1 mm which included sections of about 3-5 alveoli. If any grid space contained part or all of a lesion, the whole space was recorded as a lesion. The sum of lesion areas divided by the total parenchymal area (corrected for the difference in magnification of the dissecting and compound microscopes) provided an estimate of percent of parenchyma occupied by lesions. Lung lesions had a focal distribution and varied in severity. The simplest lesions were foci of 3 to 5 alveoli in which 1 to 5 cells were found in alveolar spaces. Other lesions had similar cellular deposits associated with thickening of alveolar septa by increased numbers of nuclei and eosinophilic material denoting interstitial edema. Lesions types were defined based on these morphological differences as follows:

Type I. Free cells of any type in alveolar spaces with no apparent change in septal walls. Control animals exhibited these features to a small degree (1-3% cross section area), however the finding was categorized as a lesion because the incidence increased following exposure to ozone.

Type II. Alveolar duct walls and alveolar septa thickened due to infiltrating cells. Free cells may or may not be present.

In earlier analyses at this laboratory, the Type II lesion was subdivided into Type II and Type III. The Type II and Type III lesion areas were always considered together as a sum because they were intergrading. Here we have nominally lumped them into a single category, the Type II lesion. Histopathologic analyses were blind as the reader did not know exposure histories. Rats occasionally showed evidence of pulmonary infections consisting of peribronchial lymphocytic follicles or perivascular infiltrates of inflammatory cells. Lung sections were graded by infection criteria. Animals exhibiting perivascular cuffing associated with interstitial inflammatory disease were excluded. Approximately 10% of the animals had to be rejected, and rejection rate was not correlated with ozone exposure. In rare cases tissue preparations were rejected because of presence of a lung tumor or accidental puncture during dissection.

Animals, training, and exposure protocol.

Experimental animals were barrier-reared prague-Dawley rats (Hilltop Laboratory Animals, Inc., Scottdale, PA). The animals were shipped in filter barrier containers, and housed at the laboratory in isolation units supplied with Purafil scrubbed and HEPA filtered air. Rats were held in wire bottomed cages over beds of rock salt whose drying action on feces and urine suppressed ammonia production. Rats were received at age 6 weeks, and held at the laboratory for one week before exposure.

Development of training protocols for exercising rats at the laboratory was advanced to the point where we no longer need to rely on intermittent exercise. Training

and exposure protocols are shown in Table I. Rats were trained over 3 days with incremental exercise to achieve exposure runs at 15 m/min, 20% grade for 3 h. In the experiment testing effects of exercise intensity which included exercise at near maximal levels (30 m/min, 20% grade), two additional training days were added to the protocol to give the rats experience at this level. In the exposure runs of this experiment, duration of exposure was adjusted to hold constant our best estimate of effective dose based on metabolic gas exchange. The maximal exercise group (group 5) was run first. These rats ran at 30 m/min and 20% grade; they were given rest periods of about 7 min when they began to tire, and the experiment was terminated when they could no longer continue to run. The group completed 3 bouts of exercise (total time 1.5 h) with 2 rest periods (total time 0.25 h). Metabolic rate was measured during the exposure and used to calculate an index proportional to effective dose: (O_3 concentration)·(exposure duration)·(oxygen consumption rate). This value was used to estimate appropriate time durations for other exposure groups using measurements of metabolism at the lower exercise levels. By this procedure the effect of exercise ventilation on effective dose was compensated by the effect of time duration of exposure.

Table 1. Training and Exposure Protocols for Exercising Rats

	1. Effects of O_3 Exposure Duration	2. Effects of O_3 and Exercise Intensity	3. Effects of Combined O_3 and NO_2
Training			
Day 1	Continuous running, 3.0 h 8 m/min, 20% grade	Continuous running, 3.0 h 8 m/min, 20% grade	Continuous running, 3.0 h 8 m/min, 20% grade
Day 2	Intermittant running, 2.9 h Run 15 m/min, 20% gd, 0.5 h Rest 0.09 h	Intermittant running, 2.9 h Run 15 m/min, 20% gd, 0.5 h Rest 0.09 h	Intermittant running, 2.9 h Run 15 m/min, 20% gd, 0.5 h Rest 0.09 h
Day 3	Continuous running, 3.0 h 15 m/min, 20% grade	Intermittant running, 1.5 h 15 m/min, 20% gd, 0.25 h 30 m/min, 20% gd, 0.25 h	Continuous running, 3.0 h 15 m/min, 20% grade
Day 4		Intermittant running, 1.5 h 15 m/min, 20% gd, 0.25 h 30 m/min, 20% gd, 0.25 h	
Day 5		Continuous running, 2.0 h 30 m/min, 20% gd	
Exposure Rat Group			
1	Clean Air, Run 15 m/min, 20% gd, 3.0 h	Clean Air, Intermittant running, 1.13 h Rest 0.75 h Run 30 m/min, 20% gd, 0.83 h	Clean Air, Run 15 m/min, 20% gd, 3.0 h
2	O_3, Run 15 m/min, 20% gd, 0.5 h	O_3, Rest, 3.42 h	O_3, Run 15 m/min, 20% gd, 3.0 h
3	O_3, Run 15 m/min, 20% gd, 1.0 h	O_3, Run 8 m/min, 0% gd, 2.75 h	NO_2, Run 15 m/min, 20% gd, 3.0 h
4	O_3, Run 15 m/min, 20% gd, 2.0 h	O_3, Run 15 m/min, 20% gd, 2.33 h	O_3 and NO_2, Run 15 m/min, 20% gd, 3.0 h
5	O_3, Run 15 m/min, 20% gd, 3.0 h	O_3, Intermittant running, 1.75 h, Run 30 m/min, 20% gd, 1.50 h, Rest 0.25 h	

Statistical Analysis.

Data from the exposures were a measurement of Type I and Type II parenchymal lesion areas from each rat in an exposure group. In the first experiment, the effect of O_3 exposure duration on lesion areas was tested in stepwise regression (Dixon and Brown, 1979). Independent variables were exposure duration hours raised to powers 1, 2, and 3 in the successive steps. The dependent variable was lesion area, and separate analyses were done for Type I and Type II lesion areas. The second experiment, effects of exercise intensity at constant effective dose, was also analyzed by stepwise regression. Exercise intensity was measured by average oxygen consumption during the exposure, and independent variables were average $\dot{V}_{O_2}$ raised to the powers 1, 2, and 3 in the successive steps. The data set was confined to groups that received O_3 at constant effective dose (i.e., clean air control group with dose of 0.0 was excluded). Lesion areas were the dependent variables in separate analyses for Type I and Type II lesions. Effect of combined O_3 and NO_2 was tested in a 2-way analysis of variance (Dixon and Brown, 1979). The factors were O_3 (present or absent) and NO_2 (present or absent).

RESULTS

Ozone and nitrogen dioxide exposure concentrations.

Concentrations of the test oxidants measured in the treadmill are shown in Table 2. Target concentrations of O_3 (0.35 ppm) and NO_2 (0.6 ppm) were achieved in all exposures.

In combined O_3 and NO_2 exposures, initial tests showed that adding either gas to an atmosphere with an established concentration of the other gas resulted in a decline in concentration. This pattern is illustrated with O_3 addition to a 0.58 ppm NO_2 atmosphere in Figure 1. NO_2 fell to about 0.45 ppm as O_3 was raised to 0.35 ppm. When O_3 was turned off, the NO_2 concentration returned to the original value of 0.58 ppm. Particulate aerosol sampling with an electrical aerosol size analyzer (Thermo-Systems

Table 2. Ozone and nitrogen dioxide concentrations measured from the treadmill during exposures.

Group		Atmosphere	Relative Humidity (%)	Mean (ppm)	SD	n	Range
1. Effects of O_3 Exposure Duration							
	Duration (h)						
1	3.0	Clean Air	40				
2	0.5	O_3	40	0.351	0.016	90	0.289-0.391
3	1.0	O_3	41	0.350	0.021	80	0.310-0.397
4	2.0	O_3	40	0.356	0.016	168	0.318-0.394
5	3.0	O_3	40	0.354	0.018	216	0.208-0.399
2. Effect of O_3 and Exercise Intensity							
	Exercise Condition						
1	15 m/min, 20 grade	Clean Air	40				
2	Rest	O_3	40	0.349	0.015	344	0.311-0.381
3	8 m/min, 0% grade	O_3	40	0.354	0.014	214	0.279-0.386
4	15 m/min, 20% grade	O_3	41	0.351	0.019	212	0.300-0.393
5	30 m/min, 20% grade	O_3	40	0.355	0.019	147	0.261-0.395
3. Effects of Combined O_3 and NO_2							
1		Clean Air	40				
2		O_3	40	0.351	0.023	261	0.267-0.398
3		NO_2	40	0.60	0.02	320	0.55-0.69
4		O_3	40	0.352	0.033	243	0.224-0.425
		NO_2		0.59	0.03	162	0.51-0.07
4. Effects of Combined O_3 and NO_2							
1		Clean Air	46				
2		O_3	45	0.345	0.019	300	0.284-0.383
3		NO_2	45	0.60	0.04	72	0.53-0.65
4		O_3	45	0.341	0.020	279	0.281-0.384
		NO_2		0.58	0.04	166	0.47-0.66

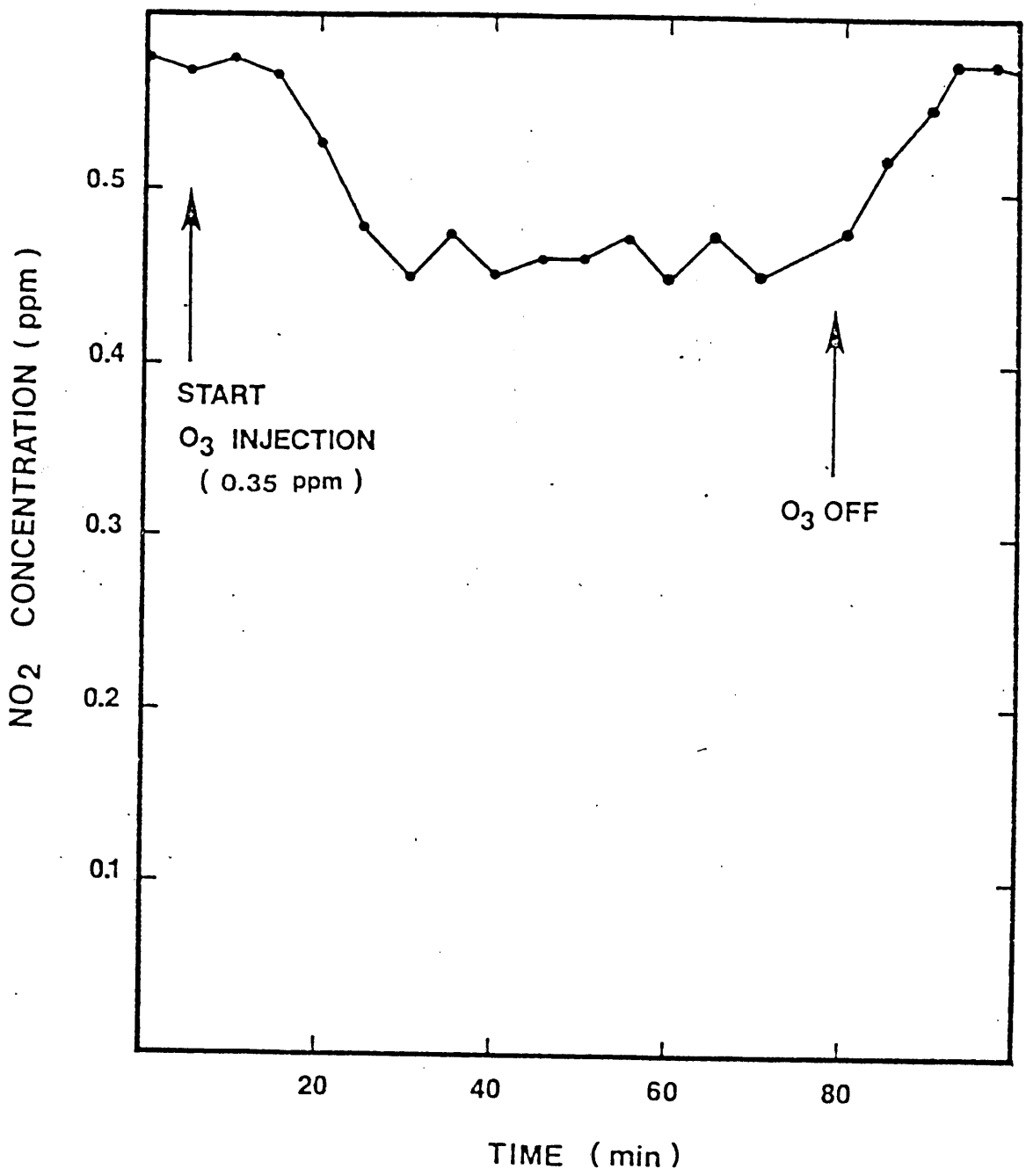


Figure 1. Concentrations of NO_2 are reduced when O_3 is added to exposure atmosphere.

Model 3030) and an optical particle counter (Climet Model 208) showed no significant formation of particles in the mixed oxidant atmosphere. A probable reaction product was nitric acid vapor, so during test runs and during one of the rodent exposures, we sampled acid products just upstream from the treadmill. The nylon backup filter method provided separate measurements of particle (Zeflour filter) and vapor phase (Nylasorb filter) acid. NO_2 and O_3 concentrations were adjusted so that the final treadmill concentrations during exposure were 0.6 ppm NO_2 and 0.35 ppm O_3. Concentrations of gases and ionic components based on filter sampling during the exposures are given in Table 3. Analysis of particle fractions showed very little acid products confirming the negative results of the earlier test for particulate aerosols with the optical particle counter and aerosol size analyzer. However, the vapor fraction showed significant formation of acid reaction product. Nitric acid concentrations were estimated by two independent methods. Sample extracts were analyzed for nitrate and for hydrogen ion concentrations and the corresponding HNO_3 levels were computed assuming that HNO_3 was the sole source of both ions. Close agreement was achieved within the limits of precision imposed by the sampling and analytical method. Conversion of all of the "missing" NO_2 to HNO_3 based on fractional losses of NO_2 in the earlier test (Figure 1) would have yielded concentrations of about 200 micrograms per cubic meter. Only 30% of the missing NO_2 was collected as HNO_3 (60 $\mu g/m^3$) on the filters, however it is quite possible that large fractions of vapor HNO_3 were scrubbed in passage through the atmosphere delivery system duct.

Effects of ozone exposure duration. In continuous exercise (5 m min^{-1}, 20% grade) exposures to 0.35 ppm O_3 for increasing durations, Type II lung lesions were first detected in exposures of 1 h (Table 4). Both lesion types showed large increases between 1 and 2 h exposure duration, however, stepwise regression analysis yielded relations with near terms (Type I = 1.59 + 0.67 (duration), $r = 0.71$, $F = 44.3$, $p<0.001$; Type II = -0.43 + 1.25 (duration, $r = 0.81$, $F = 82.6$, $p<0.001$). Average $\dot{V}_{O_2}$ during running at 15 m min^{-1}, 20% grade was elevated over resting rates by a factor of about 2.3.

Table 3. Characterization of mixed O_3 and NO_2 exposure atmospheres. Sample point was just upstream of treadmill.

Atmosphere	Relative Humidity (%)	O_3 (ppm)	NO_2 (ppm)	Filter	Particle Fraction Analysis: Effective H^+ (moles/m^3)	Particle Fraction Analysis: NO_3^- ($\mu g/m^3$)	Particle Fraction Analysis: Estimated HNO_3 ($\mu g/m^3$) a*	Particle Fraction Analysis: Estimated HNO_3 ($\mu g/m^3$) b†	Vapor Fraction Analysis: Effective H^+ (moles/m^3)	Vapor Fraction Analysis: NO_3^- ($\mu g/m^3$)	Vapor Fraction Analysis: Estimated HNO_3 ($\mu g/m^3$) a*	Vapor Fraction Analysis: Estimated HNO_3 ($\mu g/m^3$) b†
NO_2 (dry run)	45	0.0	0.63	Zeflour Nylasorb	1.4×10^{-8}	2.8	0.9	2.8				
$NO_2 + O_3$ (dry run)	45	0.35	0.53	Zeflour Nylasorb	4.1×10^{-8}	3.8	2.6	3.9	8.4×10^{-8}	11.3	5.3	11.5
$NO_2 + O_3$ (rat exposure)	45	0.34	0.58	Zeflour Nylasorb	2.0×10^{-8}	1.9	1.3	1.9	1.1×10^{-6}	58.3	70.1	59.2
									7.0×10^{-7}	40.4	44.1	41.1

*a: Based on hydrogen ion concentration
†b: Based on ion chromatographic determination of nitrate ion.

ABLE 4. Effects of exercise exposure duration on lung parenchymal lesions induced by 0.35 ppm ozone. Rats ran continuously at 15 m·min and 20% grade.

Rat Exposure Group	Duration (h)	Average $\dot{V}_{O_2}$ (ml/Kg/min)	Type I Lesion (Area %)			Type II Lesion (Area %)		
			Mean	SD	n	Mean	SD	n
1. Clean Air Control	3.0	51.6	1.82	0.14	10	0.0	0.0	10
2. Ozone	0.5	62.2	1.98	0.14	10	0.0	0.0	10
3. Ozone	1.0	55.5	1.66	0.14	9	0.18	0.09	9
4. Ozone	2.0	58.4	3.35	0.40	8	2.61	0.68	8
5. Ozone	3.0	59.7	3.56	0.23	9	3.22	0.33	9

Effect of exercise intensity at constant effective ozone dose. Table 5 shows lesion areas resulting from exposures to O_3 at different exercise levels. Following the highest intensity exercise exposure, each subsequent exposure was terminated based on O_3 concentration and estimated metabolic gas exchange to achieve equivalent index of effective dose $\left[(O_3 \text{ concentration}) \cdot (\dot{V}_{O_2}) \cdot (\text{duration})\right]$ (Table 6). The exercising O_3 exposures gave similar values of the index, however the resting exposure was slightly underestimated. Despite the similarity of effective doses of O_3, increasing levels of exercise during exposure had a large effect on O_3 lesion areas; by comparison to the lowest intensity exercise exposure, the highest intensity exercise increased Type I lesion areas by a factor of 2.7 and Type II areas by a factor of 10. Lesion areas of O_3 exposure groups regressed against average $\dot{V}_{O_2}$ yielded second order relationships. Type I = 0.87 + 0.0009 $(\dot{V}_{O_2})^2$, r = 0.78, F = 55.8, $p<0.001$; Type II = -1.13 + 0.0011 $(\dot{V}_{O_2})^2$, r = 0.77, F = 51.0, $p<0.001$.

Effects of combined ozone and nitrogen dioxide. Results of exposure experiments testing O_3 and NO_2 atmospheres alone and in combination are shown in Table 7. There was a pronounced interaction between these gases on the induction of parenchymal lung lesions. In the November 1983 exposure, NO_2 alone had no demonstrable effect on lesion areas. O_3 alone increased Type I lesion areas and induced Type II lesions. Exposure to the combination of gases, however, resulted in an approximate doubling of both Type I and Type II lesion areas compared to O_3 alone. The interaction was highly significant (Type I lesion interaction O_3-NO_2: F = 6.9, $p < 0.01$; Type II lesion interaction O_3-NO_2: F = 5.9, $p < 0.02$). The experiment was repeated in April, 1984, and the results were the same. Lesion areas induced by O_3 were doubled when NO_2 was also present (Type I lesion interaction: F = 18.9, $p < 0.0001$; Type II lesion interaction: F = 9.4, $p < 0.0004$).

Statistical Analysis.

A summary of the results of test statistics for these three experiments is given in Table 8.

TABLE 5. Effect of exercise on lung parenchymal lesions induced by 0.35 ppm ozone. Rats ran at different speeds and grades, and exposure duration was adjusted to give the same effective dose to each group. Similar effective doses were estimated by the proportional quantity (ppm O_3)·(exposure duration)·(oxygen consumption rate). See Table 6.

Rat Group	Duration (h)	Average $\dot{V}_{O_2}$ (ml/Kg/min)	Type I Lesion (Area %)			Type II Lesion (Area %)		
			Mean	SD	n	Mean	SD	n
1. Clean Air Control (rest; run 15 and 30 m/min, 20% grade)	1.58	60.4	2.13	0.54	9	0.0	0.0	9
2. Ozone (rest)	3.42	30.5	2.26	0.92	9	0.15	0.20	9
3. Ozone (run 8 m/min, 0% grade)	2.75	44.5	2.40	0.63	10	0.49	0.40	10
4. Ozone (run 15 m/min, 20% grade)	2.33	54.8	3.17	1.10	8	2.38	2.16	8
5. Ozone (rest and run 30 m/min, 20% grade)	1.75	75.4	6.43	2.14	10	4.96	2.51	10

Table 6. Effective dose of ozone in relative units based on oxygen consumption.

Rat Group	Exposure Condition	O_3 (ppm)	Time Duration (h)		$\dot{V}_{O_2}$ ml/Kg/min		$\dot{V}_{O_2} \cdot T \cdot (O_3)$ ppm·ml/kg
			Rest	Run	Rest	Run	
1.	30 m/min, 20% grade, with rest periods	0.0	0.75	0.83	34.7	83.7	0.0
2.	Continuous rest	0.349	3.42		30.5		36.5
3.	8 m/min, 20% grade	0.354		2.75		44.5	43.3
4.	15 m/min, 20% grade	0.351		2.33		54.8	44.9
5.	30 m/min, 20% grade, with rest periods	0.355	0.25	1.50	33.1	82.4	46.8

Table 7. Effect of 0.35 ppm ozone and 0.6 ppm nitrogen dioxide alone and in combination on lung parenchymal lesions. Rats were exposed during exercise at 15 m/min and 20% grade for 3 hours.

	Average $\dot{V}_{O_2}$ (ml/Kg/min)	Type I Lesion (Area %)			Type II Lesion (Area %)		
		Mean	SD	n	Mean	SD	n
Experiment 1, November 1983							
1. Clean Air Control	47.6	1.81	0.22	8	0.00	0.00	8
2. NO_2 alone	43.7	1.42	0.15	10	0.00	0.00	10
3. O_3 alone	51.1	2.64	0.33	9	1.52	0.38	9
4. NO_2 and O_3 combined	51.3	3.98	1.43	10	4.15	0.91	10
Experiment 2, April 1984							
1. Clean Air Control	56.2	1.79	0.54	10	0.00	0.00	10
2. NO_2 alone	49.6	1.87	0.17	9	0.00	0.00	9
3. O_3	50.7	3.15	0.41	8	2.92	0.63	8
3. NO_2 and O_3 combined	45.0	5.80	0.39	9	6.08	0.87	9

Table 8. Summary statistics for exposure experiments.

1. Effect of exposure duration tested in stepwise regression lesion areas vs. duration.

A. Type I Lesion Area

ANOVA

	df	Mean Square	F	p
Regression	1	23.727	44.25	< 0.001
Residual	43	0.536		

Regression	R	Std. Error of Coeff.
Type I = 1.594 + 0.674 (Duration)	0.712	0.101

B. Type II Lesion Area

ANOVA

	df	Mean Square	F	p
Regression	1	81.209	82.59	< 0.001
Residual	43	0.983		

Regression	R	Std. Error of Coeff.
Type II = -0.432 + 1.246 (Duration)	0.811	0.137

2. Effect of exercise intensity tested in stepwise regression, lesion areas vs. average oxyge consumption during O_3 exposures.

A. Type I Lesion Area

ANOVA

	df	Mean Square	F	p
Regression	1	105.606	55.78	< 0.001
Residual	35	1.893		

Regression	R	Std. Error of Coeff.
Type I = 0.867 + 0.00093 $(\dot{V}_{O_2})^2$	0.784	0.00013

B. Type II Lesion Area

ANOVA

	df	Mean Square	F	p
Regression	1	138.224	50.96	< 0.001
Residual	35	2.712		

Regression	R	Std. Error of Coeff.
Type II = -1.129 + 0.0011 $(\dot{V}_{O_2})^2$	0.770	0.00015

3. Effect of O_3 and NO_2 alone and in combination tested in 2 way ANOVA

	df	Mean Square	F	p
Experiment 1				
A. Type I Lesion Area				
O_3-NO_2 interaction	1	6.863	6.94	0.01
Error	3	0.989		
B. Type II Lesion Area				
O_3-NO_2 interaction	1	15.800	5.93	0.02
Error	33	2.666		
Experiment 2				
A. Type I Lesion Area				
O_3-NO_2 interaction	1	14.848	18.91	0.0001
Error	32	0.785		
B. Type II Lesion Area				
O_3-NO_2 interaction	1	22.379	9.44	0.004
Error	32	2.370		

DISCUSSION

Exercise poses an important challenge to normal respiratory defenses against toxic effects of pollutant atmospheres because of the increased rate of ventilation and altered distribution of ventilation within the deep lung. With the support of the California Air Resources Board, we have developed a rodent exercise inhalation model in conjunction with quantitative lung histopathology. These techniques have enabled us to assess the importance of exercise and other modifying factors on the toxicity of inhaled substances.

In this contract we extended the rat exercise model to include exposure conditions at high exercise levels, tested the effect of exercise exposure duration on O_3 induced lung damage, examined the effects of exercise workload during O_3 exposure, and measured the interactive effects of NO_2 as a co-pollutant with O_3 in producing lung tissue injury. Oxidant exposure concentrations selected for this study were within or near ambient levels associated with air pollution episodes. O_3 was 0.35 ppm and equivalent to a 2nd stage smog alert in the South Coast Air Basin. The test concentration of NO_2, 0.6 ppm, was at near ambient level. (0.4 ppm NO_2 is equivalent to a stage 3 pollution alert). In performing exercise exposures with the rat model, we expect to increase inhaled dose rate and to change dose distribution in a manner analogous to the conditions of human exercise exposure. In the absence of specific information on dose distribution changes and relative tissue sensitivity between species, the most reasonable approach to similarity of exercise conditions is equivalent factor increases in ventilation and metabolic gas exchange above resting rates. Comparisons of exercise metabolism to rest in rats were based on resting measurements made over extended periods in clean air to achieve a quiet resting state ($\dot{V}_{O_2}$ = 22-25 $ml \cdot Kg^{-1} \cdot min^{-1}$). Animals at rest briefly between bouts of exercise, or animals at rest in an irritating atmosphere could have higher metabolic rates. Exercise level for most of the exposures in this contract was constant running at 15 m/min, 20% grade except for the specific test of exercise level as a variable. 15 m/min, 20% grade was a level which the rats could

sustain continuously for 3 hours in O_3 atmospheres and involved a metabolic gas exchange about 2.5 times resting metabolic rate. This was mild exercise with a walking gait, and in terms of increment in metabolic gas exchange, it was analogous to mild exercise in humans. Table 9 lists metabolic rate increments of some common human activities for comparison. The most intense exercise level used in the rat exposures, 30 m min^{-1} and 20% grade, was about 3.5 times resting metabolic rate and still in the range of moderate states of exercise.

In our previous ARB contract research on O_3 toxicity and exercise (Mautz, 1981), we found that increases in the proportion of time rats spent running in intermittent rest-exercise exposures to O_3 had an important effect on lung parenchymal lesion areas. In exposures to 0.35 ppm O_3 for total durations of 3.75 h increasing exercise time from 1.3 to 2.5 h resulted in lesion area increases by a factor of about 2. The duration exposure test here (Table 4 and Figure 2) tested this directly and more simply, with continuous exercising exposures to 0.35 ppm O_3 for several fixed time durations. There was little effect in exposures up to 1 h, but large increases in Type I and Type II lesion areas resulted from 2 h exposures. Continued exposure up to 3 h resulted in a much smaller increase in lesion areas. It appears that 2 h exposure represents an important time duration for induction of parenchymal lesions.

Table 9. Metabolic cost of various activities, 80 Kg human.

	$\dot{V}_{O_2}$ (L/min)	Factor Increase Relative to Rest
Basal Metabolism	0.24	
Resting (sitting)	0.26	1.0
Walking (4 km/h)	0.82	3.2
Running (10 km/h)	2.9	11.0
Maximum $\dot{V}_{O_2}$	4.2	16.0
Light Manual Labor		
(carpentry, painting, raking)	0.7-0.9	3.0-3.5
Heavy Manual Labor		
(digging, woodcutting)	1.4-1.8	5.4-6.9
Recreational Exercise		
(bicycling, tennis, basketball, dancing)	0.8-2.0	3.1-7.8
(cross-country running)	2.1	8.0
Average Daily Metabolic Expenditure	0.45	1.7
Average Metabolic Expenditure		
During Waking Hours	0.50	2.1

Data based on Consolazio et al (1963) and Schoeller and van Santen (1982).

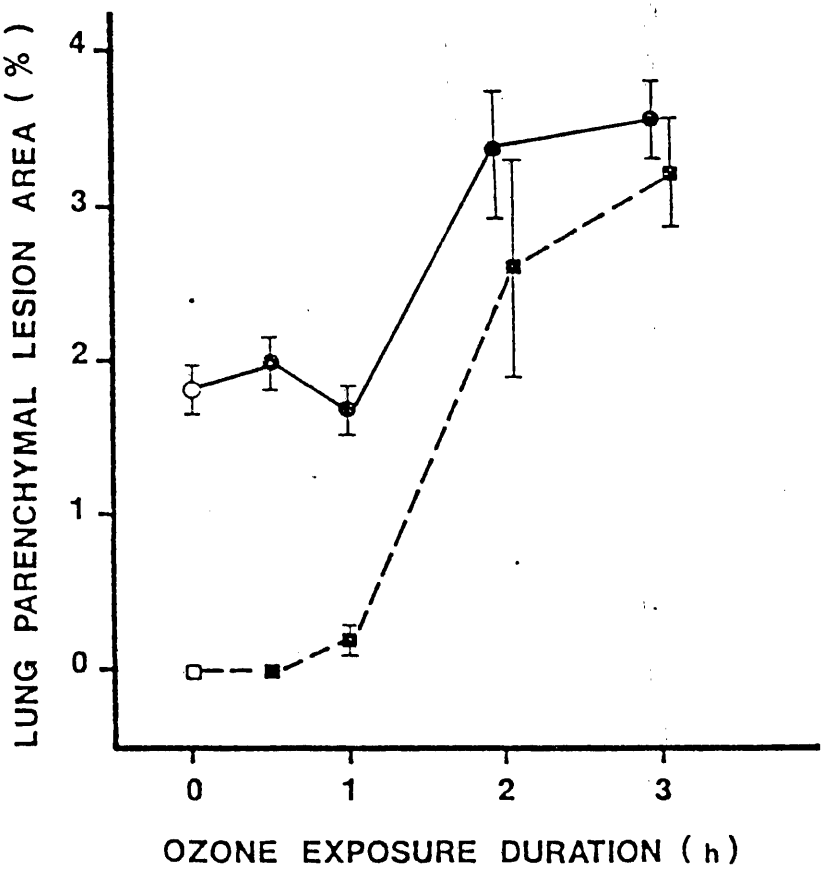


Figure 2. Effect of exercise exposure duration on lung parenchymal lesions induced by 0.35 ppm ozone. Rats ran at 15 m/min and 20% grade. Data are mean ± SE of Type I (circles) and Type II (squares) lesion areas. Open symbols are clean air control.

The test of exercise intensity showed that increasing exercise level had a large effect on lung lesion response which was not dependent on inhaled dose of O_3. The design of this experiment served to give rats the same equivalent dose of O_3 at different exercise workloads by appropriately shortening the time duration of the exposure to compensate for the increased dose rate at high exercise levels. Figure 3 illustrates the effect of exercise intensity with plots of lesion area against oxygen consumption rate of rats exercising at different speeds and grades. The exercise levels spanned a large portion of the aerobic capacity of laboratory rats. Maximal rates of oxygen consumption of Sprague-Dawley rats are about 110 ml/kg/min and sustainable for short time periods (Armstrong et al., 1983; Bedford et al., 1979; and Appendix). In the O_3 exposure experiment, we were able to get rats to perform at 82 ml/kg/min in 3 bouts with intervening rest periods. Average oxygen consumption for this exposure was 75 ml/kg/min, a level about 3.5 times resting metabolism and 70% of maximal metabolic rate. The rat group exposed at rest to 0.35 ppm O_3 had an unusually high metabolic rate (30.5 ml/kg/min), and this may have resulted from the irritating effect of the O_3 atmosphere. Resting rates measured from rats placed briefly in the treadmill or between bouts of intermittent exercise were usually about 28 ml/kg/min while rats permitted extended time periods of an hour or more to settle down have oxygen consumption rates about 22 ml/kg/min (Mautz, 1984 and Appendix). The effective dose concept does not provide a complete explanation for variation in toxic responses to O_3. Human pulmonary function changes have been found to have significant linear or second order polynomial relationships to effective O_3 dose, with ozone concentration often identified as exerting a primary influence (Silverman et al., 1976; Adams et al., 1981). In our study of tissue damage to the deep lung, we have shown that exercise ventilation is a very important variable in the lesion response, and at the low O_3 concentrations tested in this and our previous ARB study, exercise level was more important than O_3 concentration in the injury response.

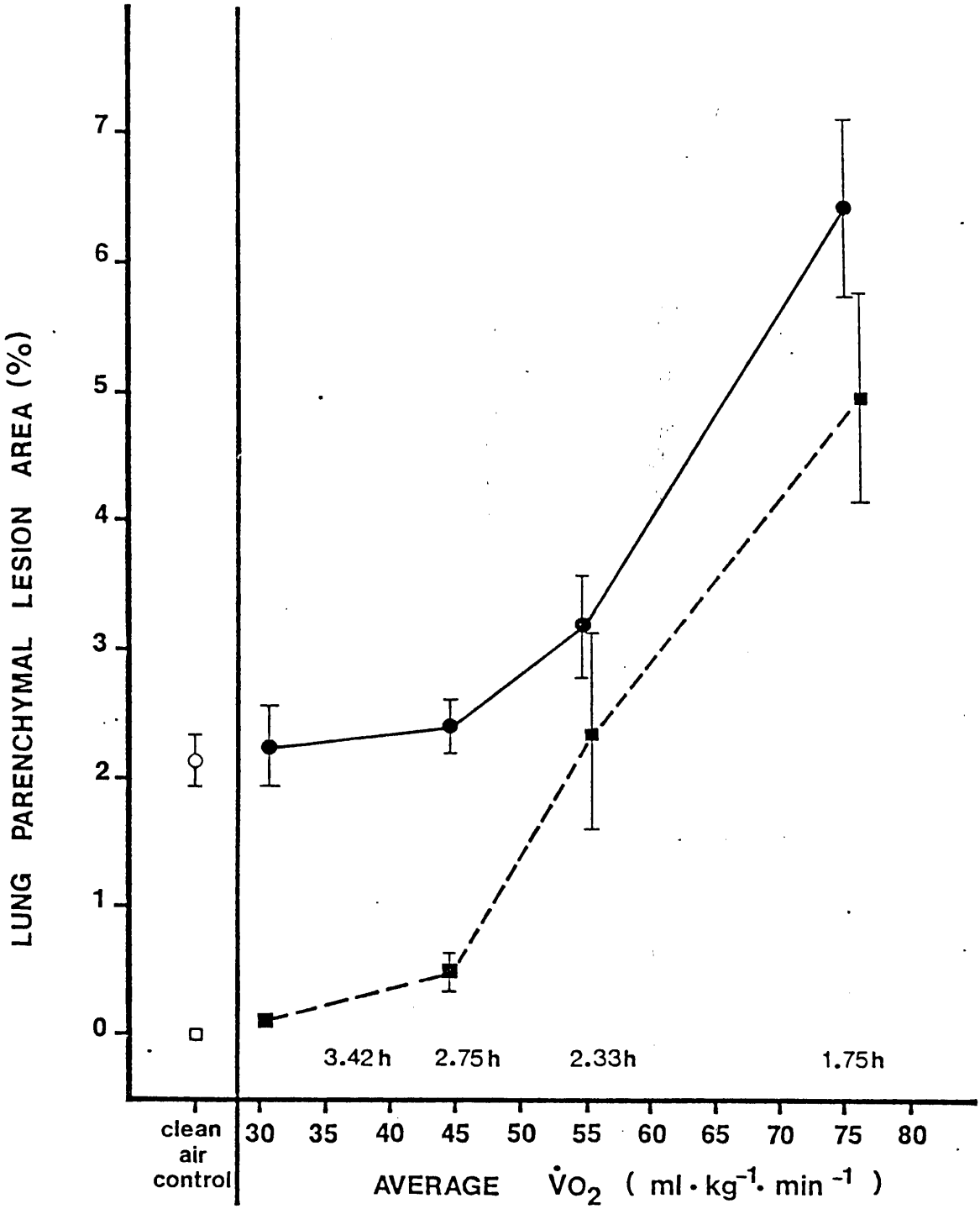


Figure 3. Effect of exercise on lung parenchymal lesions induced by 0.35 ppm ozone at constant effective dose. Data are plotted as a function of average oxygen consumption for different exercise intensities. Exposure durations are listed along the abcissa. Data are mean ± SE of Type I (circles) and Type II (squares) lesion areas.

The strong synergism observed between 0.35 ppm O_3 and 0.6 ppm NO_2 was impressive considering the low concentrations used in the experiment. The results of both replicate experiments combined gave the following mean values for Type I parenchymal lesion areas: Clean air control 1.80%, NO_2 alone 1.63%, O_3 alone 2.88%, and combined NO_2 and O_3 4.84%. Combined results for Type II lesion areas were Clean air control 0.0%, NO_2 alone 0.0%, O_3 alone 2.18%, combined NO_2 and O_3 5.08%. The interaction between these oxidant pollutants was highly significant in enhancing tissue damage to the deep lung; the presence of NO_2 with O_3 approximately doubled tissue damage induced by O_3 alone. Synergistic interactions between these oxidants affecting rat lung collagen synthesis rates have been observed (Last et al., 1983), however their exposure concentrations were much higher (O_3: 0.4-1.2 ppm; NO_2: 5-25 ppm), and exposure duration much longer (7 days) than in the present study. Extrapolation of dose response data (variable concentration) in the collagen synthesis study to the range of concentrations used in the present study would not yield a detectable interaction. The large effect we observed at low concentrations was probably due to the exercise exposure conditions, which may be expected to enhance the effects of the oxidants alone as well as in combination. It is noteworthy that in our experiments, NO_2 alone did not result in a detectable lesion response. The combined gases may react to yield several new compounds that could be toxic themselves or enhance O_3 toxicity. Dark reactions, such as could occur in the exposure experiment involve formation of nitrate radical:

$$NO_2 + O_3 \rightarrow NO_3^* + O_2 \qquad (1)$$

An equilibrium with N_2O_5 is rapidly established,

$$NO_2 + NO_3^* \rightleftarrows N_2O_5 \qquad (2)$$

and N_2O_5 can react with water to form nitric acid

$$N_2O_5 + H_2O \rightarrow 2\ HNO_3 \qquad (3)$$

These and other reactions have been reported in ambient air studies by Platt et al. (1984). The reactions are influenced by humidity with humidities of 40% or greater favoring the formation of HNO_3, and lower humidities favoring buildup of N_2O_5.

Vapor phase HNO_3 was detected during the exposure (Table 3), and synergism between O_3 and acid aerosols has been observed in other studies (Gardner et al., 1977; Last et al., 1983; Juhos et al., 1978). Proposed mechanisms for gas-aerosol synergistic effects often involve solution of gas into droplets and consequent changes in dose-distribution of the gas in the respiratory system. However, significant aerosol formation was not detected in the present study; HNO_3 was present in vapor phase. This leaves open the possibility that radicals or other reaction products produced the synergistic effects.

Conclusions.

The results of these experiments clearly demonstrated that acute lung tissue damage in laboratory rats exposed to O_3 can be extensively enhanced by exposure during exercise and by presence of NO_2 as a co-pollutant. Oxidant exposure concentrations were close to levels occurring in urban air pollution episodes (O_3: 0.35 ppm, NO_2: 0.6 ppm) and exposure durations were similar to periods of ambient episodes (3 h). Exercise levels were mild involving a 2.5-3.5 factorial increase in resting metabolic rate. Lung damage was near the lower limit of detection in 3.4 h resting exposures or in mild exercising exposures less than in 1 h duration, but increased markedly with moderate exercise exposures of more than 2 h duration. 0.6 ppm NO_2 had no detectable effect when inhaled

alone, but in combination with 0.35 ppm O_3 there was strong synergism enhancing lung tissue damage.

A critical question concerns the significance to human health of results obtained with the rat model. It is not possible to directly extrapolate tissue damage observed in rat lungs to firm conclusions about the quantitative extent of injury to humans. However, the qualitative effects and relative importance of the exposure variables tested may be expected to be similar. I feel there is a strong possibility that humans exercising at the oxidant pollutant levels tested in this study could receive similar increments in lung tissue damage compared to exposures at rest. Furthermore, while animal models may differ in absolute sensitivity to inhalation insults, sensitivity within a species is highly variable, and a conservative approach is to assume that the range of human sensitivity may overlap that of animal models (Dawson and Schenker, 1979).

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APPENDIX

A Rodent Treadmill for Inhalation Toxicologic Studies and Respirometry

by

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ABSTRACT

A 10 runway treadmill was enclosed for inhalation toxicologic studies of rodents under exercise exposure to environmental pollutants. The exposure system was lined with sheet stainless steel to minimize scrubbing of charged particles and reactive gases. Average metabolic gas exchange of exercising animals was derived from measurements of inlet or outlet air flow and data from an O_2 analyzer in conjunction with either a CO_2 or N_2 analyzer. An air flow rate of 400 $L \cdot min^{-1}$ ensured a response time of 1 min to reach 95% of a step change in metabolic rate and held scrubbing losses of an ozone test atmosphere to less than 2% of treadmill inlet concentration. Gas exchange averaged for 10 rats during incremental exercise up to their highest collective performance was similar to published data for rats tested individually.

INDEX TERMS

Inhalation exposure apparatus, air pollutant, metabolic gas exchange, rodent exercise, rodent treadmill.

INTRODUCTION

Although inhalation exposures of laboratory animals are commonly performed at rest, there are good reasons to conduct studies during exercise. In many human exposures, occupational and environmental, people working or playing have significantly elevated ventilation rates. In contrast, it is not uncommon to observe experimental animals maintaining minimal states of activity, in many cases apparently sleeping, during exposures in inhalation chambers. In studies using animals as models of human inhalation exposure the lack of similarity in such cases is striking.

Several events occur during exercise that are known or suspected to alter the biologic effects of a given concentration of an airborne toxin. Increased minute ventilation will lead to greater total internal exposure doses per unit time. For the average adult human, resting minute ventilation is about 7 $L \cdot min^{-1}$. During brief heavy exercise an increase to over 100 $L \cdot min^{-1}$ has been observed (3). Thus, exercise can increase the rate of delivery of an inhaled toxin by more than ten fold. Other events occurring during exercise that may modify response include greater depth of breathing, decreased contact times between inspired air and upper airways, an increase in the fraction of air drawn through the mouth (rather than the nose), and the potential overriding of reflex-mediated protective breathing patterns (for example shallow breathing) which are normally stimulated by irritants (2, 6, 28). In general, higher flow rates will change the deposition efficiencies for gases and particles in all regions of the respiratory tract, leading to a redistribution of the dose pattern and possibly a change in the total combined dose delivered to all regions. In many cases these factors would be expected to lead to increased responses to inhaled pollutants. Indeed, inhalation studies with both humans and laboratory animals have typically demonstrated greater responses during exposures at exercise than were seen at rest (9, 12, 22, 23, 24).

For the purpose of examining the effect of exercise as a modifier of the toxic response to air pollutant compounds, an enclosed treadmill exposure system was developed for use with rats as an exercising animal model. The treadmill was designed to simultaneously expose 10 running animals to test gases or aerosols and to permit simultaneous measurement of group respiratory gas exchange as an index of metabolic workload during exposure. The treadmill was used to determine the relationship between running speed and metabolic gas exchange at two elevation grades (0 and 20%). In addition, procedures were tested for measuring ozone concentration in the treadmill with rats present.

METHODS

Treadmill design. The treadmill exposure system was constructed from a Quinton 42-15 rodent treadmill (Figure 1). The treadmill contained 10 individual runways and controls for varying belt speed and inclination. An electrically charged grid at the rear of the belts motivated the animals to continue exercise. The treadmill was enclosed with polycarbonate plastic (6 mm), and sealed against significant leaks with neoprene gaskets. The drive motor was external to the enclosure, and a seal on the belt drive shaft was effected with a pair of semi-circular teflon bushings mounted in an aluminum panel. Inner surfaces of the runways were lined with stainless steel sheet to minimize both electrostatic charge effects and reaction with corrosive atmospheres. Inlet ports were centered at the front of each runway. 1 cm diameter stainless steel disk baffles mounted 1 cm behind the ports and stainless steel screens (2x2 mesh) positioned at 10 cm distributed the atmosphere uniformly in the runways and prevented rats from contacting inlet ports.

Removable panels above the charged grid provided access to individual runways. Another removable panel spanning the front of the treadmill below the inlet ports gave access to belt tension adjustment screws and to the enclosure floor beneath the belts. Excreta fell onto a removable tray on the enclosure floor and fecal masses clinging to the belts were brushed loose with wire wipers mounted to press lightly on the belts. During operation the belts became wet with urine and spotted with smeared fecal matter. This raised concern about the possible development of significant ammonia levels in the treadmill although continuous inflow of exposure atmosphere made this unlikely. NH_3 concentration was measured from the treadmill while rats performed intermittent exercise and rest. Atmosphere was sampled from a runway, and NH_3 was collected into 0.05 M H_2SO_4. The solution was adjusted to pH 13, and NH_3 concentration was measured with an ion selective electrode (HNU Systems, Newton, MA). Airborne NH_3 concentration was found to be less than 0.1 ppm.

A pair of exhaust lines at the rear of the treadmill conducted the atmosphere to a 30 L baffled mixing chamber, then through a flowmeter to sample ports for humidity and metabolic gas fraction measurement, and to an outdoor vent blower. Air inflow and outflow rates were adjusted to regulate the treadmill at ambient pressure.

Exposure atmospheres were produced by mixing test compounds or aerosols with purified air and delivering the mixture to each runway of the treadmill. Supply air was initially compressed and purified by passage through Purafil (Atlanta, GA) and Del-Monix (Deltech, Newcastle, DE) gas scrubbing filters, then decompressed, passed through a high efficiency particulate absolute (HEPA) filter, and humidified by controlled injection of water vapor. The mixed atmosphere was passed to a 1 m^3 stainless steel chamber containing a temperature and humidity sensor (Hygrodynamics, Silver Spring, MD), then through paired teflon-lined flexible ducts to a stainless steel manifold which distributed the flow to the 10 treadmill runways. A pair of atmosphere sample ports was located in

each runway at positions 3 cm upstream and 3 cm downstream of the stainless steel screen barriers. The first port withdrew samples of the inlet atmosphere and the second withdrew the atmosphere in the immediate vicinity of the rats.

Metabolic rate measurement. Average metabolic rate of 10 rats exercising in the treadmill was determined by analysis of input and output fractional gas composition, water vapor content, and flow rate (see Appendix for details.) Measurements were made by alternately sampling upstream and downstream air with a mass spectrometer (Perkin Elmer Model 1100, Pomona, CA) calibrated with gravimetric standard gases (Liquid Carbonics, Los Angeles, CA). Atmosphere flow rates were measured either with a single Fleisch No. 4 pneumotachograph (Dynascience, Blue Bell, PA) in the exhaust stream or paired Fleisch No. 1 pneumotachographs on the input manifold in conjunction with differential pressure transducers (Validyne MP-45, Northridge, CA). Humidity and temperature were measured with a dew point sensor (EG&G Model 911, Waltham, MA). Output of all instruments was displayed on a chart recorder (Gould Model 2800, Cleveland, OH) and digitized at 40 Hz for calculation of respiratory gas exchange with a PDP 11-10 computer system (Digital Equipment, Maynard, MA). To perform a measurement under steady state conditions, downstream and upstream gas fractions, along with atmosphere flow rate, temperature, and humidity. Were sampled 5 times over a 30 sec period. Gas exchange was then calculated by computer from mean values of the variables and expressed as the mean value per individual rat based on the number of animals in the treadmill.

Gas turnover kinetics of the treadmill atmosphere were evaluated by analyzing the response to step changes in a calibration gas input to treadmill runways. An exponential first order relation between outflow gas fraction and time provided an adequate fit to the treadmill response. Logarithmic transformation of gas fraction data yielded a straight line relation to time with slope $1/t$ and intercept $-d/t$, where t is the time constant and d is the delay time (19).

Correlation coefficients for repeated trials (n=8) ranged from 0.97 to 0.99 for atmosphere flow rates of 400 $L \cdot min^{-1}$ or 100 $L \cdot min^{-1}$. Time constants at these flow rates were 10 and 51 sec, respectively, and corresponding delay times were 29 sec and 81 sec. These values resulted in an expected time to reach 95% of steady-state gas fraction following a step change of 59 sec at 400 $L \cdot min^{-1}$ input flow and 233 sec at 100 $L \cdot min^{-1}$ input flow.

For gas exchange measurements, the system was tested before and after an experiment with gravimetric standard calibration gas elevated in CO_2 and depressed in O_2 composition. Calibration gas was bled into the treadmill runways and expected values of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were calculated from equations 5 and 6, or 7 and 8 (Appendix) using calibration gas composition as output fractions, treadmill inlet gas composition as input fractions, and metered flow rate (Bubble Meter, SKC West, Inc., Fullerton, CA) of calibration gas into the treadmill.

Animals, training, and exercise protocol. The relation between metabolic rate and running speed was determined for Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) with mean weight at testing of 272 ± 21 g. The animals were trained to run at level grade in the treadmill over a three day period commencing with 15 min of running at 8 $m \cdot min^{-1}$ alternating with 5 min of rest for 1.6 h. On the second day rats alternated 30 min of running with 10 min of rest for 3.2 h, and on the third day they ran continuously for 3 h. The purpose of the training protocol was to acquaint the rats with the treadmill and shock grid, and to identify individuals that would not readily run in the apparatus. About 3% of the animals could not be trained to run reliably and were excluded from the experiment. Metabolic rate was measured from groups of 10 rats at an air flow through the treadmill of 400 $L \cdot min^{-1}$. Following initial measurements at rest, treadmill speed was set at 10 $m \cdot min^{-1}$ at level grade and speed was increased in step changes of 5 $m \cdot min^{-1}$ at 6 min intervals. Metabolic rate was measured at each step after output flow

gas fractions stabilized, and the test was terminated when rats failed to maintain continuous exercise. Additional sets of measurements were made at 20% grade with 10 $m \cdot min^{-1}$ step increases over the lower range of speeds.

Pollutant atmosphere. An ozone atmosphere was used to test treadmill distribution of a pollutant compound and assess possible scrubbing by reactive components of the treadmill inhalation system. Ozone was produced by passing medical grade oxygen through an electrostatic discharge ozone generator (Sander Ozonizer, Type III, Osterberg, West Germany) and diluted into the humidified air stream. Samples of the exposure atmosphere were drawn in succession from three positions in the atmosphere stream: 1) in a 1 m^3 stainless steel chamber just before the atmosphere entered the treadmill manifold, 2) in the treadmill runways between the inlet ports and stainless steel screens, and 3) in the treadmill runways behind the stainless steel screen. Samples were drawn through teflon tubing and ozone concentration was measured by ultraviolet spectrophotometry in a Dasibi model 1003-AH ozone analyzer. The third position most closely approximated the rat breathing zone. However, because the rats had direct access to these sample tubes, samples could contain expired respiratory air or air aspirated through fur. Ozone distribution in the treadmill was measured with no animals present, animals present at rest, and animals exercising at 10.7 $m \cdot min^{-1}$.

RESULTS

The relation between running speed and metabolic gas exchange for groups of 10 rats running at level grade and 20% grade is shown in Figure 2. On level grade, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ increased linearly between 10 and 20 $m \cdot min^{-1}$. At higher speeds the response was attenuated as the rats approached exhaustion, and the experiment was terminated at 40 $m \cdot min^{-1}$ as animals became unable to continue running. Rats running at 20% grade

exhibited only slightly higher rates of metabolic gas exchange at speeds less than 30 $m \cdot min^{-1}$; however, the response was linear over the entire range of speeds at which they were able to perform.

Distributions of ozone concentrations (ppm) in the treadmill exposure system are shown in Table 1. Ozone losses between the 1 m^3 chamber and the first sample ports upstream of the stainless steel screens were 5-6%. When rats were not present in the treadmill, an additional 1-2% loss occurred in passage to the second sample port behind the screens. When rats were present in the treadmill losses measured in the runways were greater. With animals running, the measured loss between sample ports was 5% and when the animals were at rest in the treadmill the loss was 20%. However, mass spectrometer analysis of air samples from the second sample ports behind the screens revealed elevated and highly variable CO_2 fractions which indicated that these samples contained expired respiratory air. Variation in ozone concentration among runways was statistically significant ($F=7.3$, $p<0.001$) for samples upstream of screens when rats were present, however the runway differences were not large. Mean concentrations in individual runways measured at upstream sample ports with rats present ranged from 0.372 to 0.382 ppm with the treadmill running and from 0.371 to 0.379 ppm with the treadmill off.

DISCUSSION

Exercise is expected to be an important modifier of the toxic effects of inhaled pollutant compounds and a number of investigations have shown that exercise exposure to pollutant compounds exacerbates induced changes in pulmonary function of humans (1, 9, 22). With the development of exercising animal models, inhalation toxicologic studies may address questions which cannot be approached with human subjects, such as exercise

effects on histopathology and biochemistry. The enclosed multi-channel treadmill system described here provided an efficient means for exposing up to 10 exercising rats simultaneously and uniformly to a controlled atmosphere. A preliminary experiment with the apparatus (15) demonstrated exercise enhancement of ozone-induced lung lesions.

Fulfilling a basic requirement to contain exposure atmosphere in the treadmill serves in adapting the system for open flow respirometry and quantifying metabolic workload of the exercising animals. The choice of computational method for measuring metabolic gas exchange is governed by available instrumentation and practical considerations of exposure system design. The method presented (Appendix) is based on water vapor free gas fractions and correction of atmosphere flow to STPD. Techniques with single gas analyzers with and without water vapor and CO_2 absorption have been described (10, 11, 14, 27), and other sampling arrangements and equations appropriately derived from mixed and component gas flows (Eq. 1-4, Appendix) may be used. High flow rates in inhalation exposure systems make gas scrubbing of the total flow impractical; however, samples may be subjected to differential absorption prior to analysis. One may choose to measure input flow or output flow and apply different gas exchange equations for the two cases. If there are any leaks in the treadmill enclosure, an output flow measurement is preferable. The treadmill may then be operated under slightly negative pressure so that any leakage of air is directed inward, mixed with respired gas, and included in the measurements of total flow and output metabolic gas fractions. There will be no error in metabolic rate estimates unless respiratory gas fractions of the inlet exposure atmosphere differ from room air. If input flow is measured, any leakage (outward prior to complete mixing or inward) will alter output metabolic gas fractions and not be accounted in the flow measurement. Air flow rate through the treadmill must be set sufficiently high to ensure uniform exposure atmosphere distribution and an adequate response time to changes in metabolic rate; however, the flow must not be so great as to compromise resolution of metabolic gas fractions. We found a flow of 400

$L \cdot min^{-1}$ appropriate for exposing 10 rats and measuring gas exchange in the treadmill system.

The relation between running speed and metabolic gas exchange for rats running in the exposure system was in agreement with data reported in other studies of exercise metabolism of laboratory rats (4, 5, 7, 13, 18, 20, 21). There is a large degree of variation in results of these separate studies, and most investigators believe that regression slopes, presence of metabolic plateaus, and the values of $\dot{V}_{O_2}$ max and its corresponding running speed depend on animal training, exercise protocols, and selection criteria for performance (4, 13). Data for exercising Sprague-Dawley rats of mass similar to our animals (4, 5, 13, 21, 22) fall within the range of results reported here (Figure 2). Gleeson and Baldwin (13) reported a sharp plateau in $\dot{V}_{O_2}$ of untrained rats performing incremental exercise and others (5, 7, 18, 20, 21) have observed attenuation of metabolic response or plateaus with increasing running speed. In our experiments $\dot{V}_{O_2}$ continued to increase with increments of running speed, but while the response remained linear for running at 20% grade, an attenuated response and lower peak $\dot{V}_{O_2}$ were observed for level running. It is possible that attenuation would have been observed at 20% grade if additional measurements were made between 30 and 35 $m \cdot min^{-1}$. However, there was an important difference between level and 20% grade protocols; rats running uphill had larger speed increments and shorter total exercise duration to reach peak sustained speed. Thus rats running at 20% grade became exhausted after 4 speed steps (18-24 min of running) while rats on level grade attained 7 speed steps (36-42 min of running). Extended incremental exercise protocols may induce exhaustion and refusal to run at sub-maximal exercise levels. In another study using rats trained for several weeks, selected for superior running performance, and tested directly at each speed without progressive incremental increase (4), metabolic plateaus were not observed and the animals were capable of performing at speeds up to 50 $m \cdot min^{-1}$ at 17.8% grade. The diversity of metabolic responses and exhaustion behavior of rats under conditions of

maximal exercise has made it difficult to precisely and reproducibly characterize $\dot{V}_{O_2}$ max. The highest $\dot{V}_{O_2}$ measured in the present study (103 ± 14 $ml \cdot kg^{-1} \cdot min^{-1}$) fell within the range of $\dot{V}_{O_2}$max values reported in other studies using both incremental and single speed protocols (5, 7, 13, 20, 21), although our values were based on repeated measurements of the collective sustained performance of 10 animals and might be expected to underestimate an average of individual animal measurements. In view of the variability of $\dot{V}_{O_2}$ max determinations and frequent absence of definitive physiological indicators such as metabolic plateaus and continuous lactate accumulation (4, 5, 25), any investigation of the effects of toxic substances on maximal metabolic rate must use control groups strictly matched in age, size, and exercise experience and must consider the influence of running behavior on the endpoint.

Characterization of inhaled pollutant concentration is an important problem in inhalation toxicology, as particles and gases may be scrubbed from the test atmosphere along the delivery path. In addition, atmosphere sampling in the breathing zone of test animals introduces the possibility of contaminating the sample with expired respiratory gas. Ideally one would like samples of inhaled concentration at the mouth and nose such as might be obtained with a respiratory mask and valve. For exposures of animals in chambers, however, inhaled concentration is usually assumed to be equivalent to that of inlet air. With sufficient input flow, losses to the chamber structure can be minimized, but evasive maneuvers by test subjects such as tucking the head in the fur remain a potential problem. Rats exercising in the enclosed treadmill system were advantageously exposed head-on to a continuous flow of test atmosphere. Samples drawn directly from the runway in the breathing zone of the exercising animals were often spoiled by collection of expired respiratory gas, and this problem was greatest when the animals were at rest. Resting animals spent considerable time in close proximity to the sample line. The most representative atmosphere samples were obtained upstream, separated from the animals by coarse mesh stainless steel screens. Exhalant gas was not present in

these atmosphere samples and with no animals present in the treadmill, losses of ozone flowing down the runway were minimal (Table 1).

APPENDIX

Methods for measuring metabolic gas exchange in open flow respirometers have been developed for several particular cases (7, 8, 10, 11, 14, 15, 26, 27). All these methods can be related by a set of equations describing the balance flow of each component gas in question, and the total flow rate of mixed atmosphere into and out of the respirometer. By substitution, oxygen consumption and carbon dioxide production may be expressed in a minimum number of variables appropriate to available instruments. Symbol terminology follows earlier analyses (10, 11, 14, 27):

$\dot{V}_{O_2}$ = Rate of oxygen consumption of animals in the treadmill (STPD).

$\dot{V}_{CO_2}$ = Rate of carbon dioxide production of animals in the treadmill (STPD).

$\dot{V}_{N_2}$ = Rate of nitrogen exchange (STPD) assumed to be zero.

$\dot{V}_I$ = Rate of airflow into the treadmill (STPD).

$\dot{V}_E$ = Rate of airflow out of the treadmill (STPD).

F_{IO_2} = Dry volume fraction of oxygen in inlet air.

F_{EO_2} = Dry volume fraction of oxygen in output air.

F_{ICO_2} = Dry volume fraction of carbon dioxide in input air.

F_{ECO_2} = Dry volume fraction of carbon dioxide in output air.

F_{IN_2} = Dry volume fraction of nitrogen in input air.

F_{EN_2} = Dry volume fraction of nitrogen in output air.

The basic equations for volume flow rates of component gases are:

$$\dot{V}_{O_2} = \dot{V}_I F_{IO_2} - \dot{V}_E F_{EO_2} \quad (1)$$

$$\dot{V}_{CO_2} = \dot{V}_E F_{ECO_2} - \dot{V}_I F_{ICO_2} \quad (2)$$

$$\dot{V}_{N_2} = \dot{V}_E F_{EN_2} - \dot{V}_I F_{IN_2} = 0 \quad (3)$$

The relation between total input and output volume flow rates is:

$$\dot{V}_E = \dot{V}_I - \dot{V}_{O_2} + \dot{V}_{CO_2} \quad (4)$$

Equations 1 and 2 contain both input and output air flow terms, $\dot{V}_I$ and $\dot{V}_E$. By rearranging equation 4 and substituting for either $\dot{V}_I$ or $\dot{V}_E$ in equations 1 and 2, respiratory gas exchange may be expressed in terms of either the input or the output flow, whichever is more conveniently measured. Both terms $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are introduced into each of the equations by this substitution, but by simultaneous solution of the two equations for these two variables, expressions for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are obtained in terms of input and output fractional gas concentrations and a total air flow rate, be it either input or output flow. For the case in which $\dot{V}_E$ is measured, these expressions are:

$$\dot{V}_{O_2} = \dot{V}_E \frac{F_{IO_2}\,(1-F_{ECO_2}) - F_{EO_2}\,(1-F_{ICO_2})}{1-F_{IO_2} - F_{ICO_2}} \tag{5}$$

$$\dot{V}_{CO_2} = \dot{V}_E \frac{F_{ECO_2}\,(1-F_{IO_2}) - F_{ICO_2}\,(1-F_{EO_2})}{1-F_{IO_2} - F_{ICO_2}} \tag{6}$$

Alternatively, $\dot{V}_I$ or $\dot{V}_E$ may be eliminated from equations 1 and 2 by rearrangement and substitution of equation 3. This procedure yields expressions with the Haldane transformation using input and output nitrogen fractions (8). Again, the equations may be derived for measurements of either $\dot{V}_I$ or $\dot{V}_E$. The case for $\dot{V}_E$ measurement is:

$$\dot{V}_{O_2} = \dot{V}_E \left[F_{IO_2} \frac{F_{EN_2}}{F_{IN_2}} - F_{EO_2} \right] \tag{7}$$

$$\dot{V}_{CO_2} = \dot{V}_E \left[F_{ECO_2} - \frac{F_{EN_2}}{F_{IN_2}} F_{ICO_2} \right] \tag{8}$$

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Table 1. Ozone concentrations (ppm) achieved in the treadmill exposure system.

	Treadmill Running, 11 m·min^{-1}			Treadmill Off		
	Mean ± SD	n	Range	Mean ± SD	n	Range
No Rats Present						
1 m3 source chamber	0.359±0.011	20	0.335-0.375	0.355±0.014	20	0.330-0.375
Upstream of runway screens	0.341±0.009	20	0.326-0.351	0.333±0.010	20	0.315-0.348
Downstream of runway screens	0.336±0.010	20	0.314-0.348	0.326±0.012	20	0.311-0.347
Rats Present						
1 m3 source chamber	0.401±.009	5	0.391-0.413	0.398±.006	7	0.389-0.406
Upstream of runway screens	0.377±0.005	86	0.361-0.387	0.375±0.006	183	0.357-0.389
Downstream of runway screens	0.359±0.014	42	0.316-0.382	0.300±0.050	78	0.184-0.384

Data are combined for all 10 runways and when rats were present, sampling emphasized exposure region. In put flow rate was 400 L·min^{-1}.

Fig 1

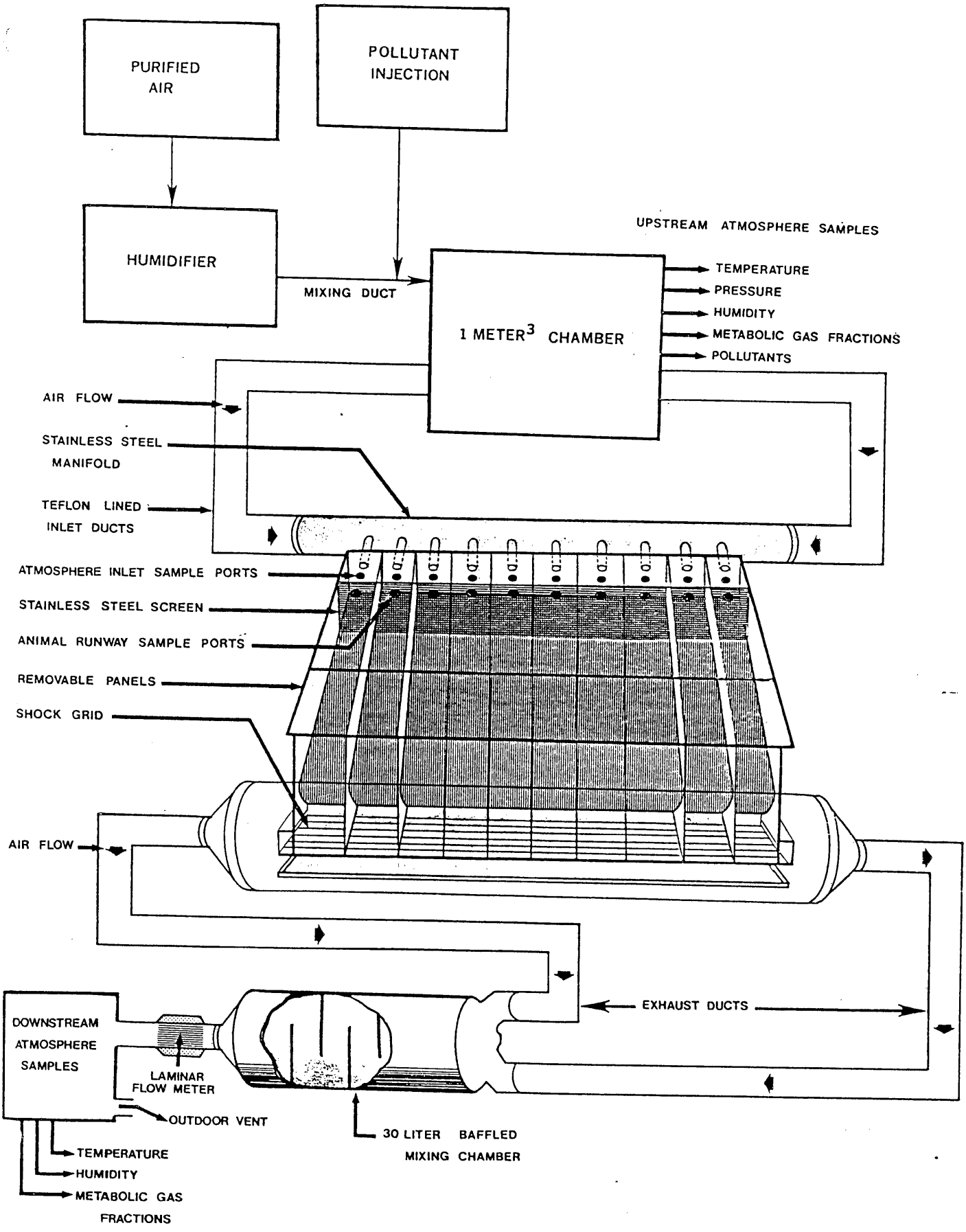
PURIFIED AIR
POLLUTANT INJECTION
HUMIDIFIER
MIXING DUCT
UPSTREAM ATMOSPHERE SAMPLES
1 METER^3 CHAMBER
TEMPERATURE
PRESSURE
HUMIDITY
METABOLIC GAS FRACTIONS
POLLUTANTS
AIR FLOW
STAINLESS STEEL MANIFOLD
TEFLON LINED INLET DUCTS
ATMOSPHERE INLET SAMPLE PORTS
STAINLESS STEEL SCREEN
ANIMAL RUNWAY SAMPLE PORTS
REMOVABLE PANELS
SHOCK GRID
AIR FLOW
EXHAUST DUCTS
DOWNSTREAM ATMOSPHERE SAMPLES
LAMINAR FLOW METER
OUTDOOR VENT
30 LITER BAFFLED MIXING CHAMBER
TEMPERATURE
HUMIDITY
METABOLIC GAS FRACTIONS

Figure 1: Rodent treadmill exposure system. Test atmosphere is individually delivered to 10 animals running in separate channels. Exposure atmosphere can be sampled at several positions and metabolic rate is measured as an average for the animal group. See Text for details.

Figure 2: Metabolic gas exchange (STPD) of groups of 10 rats as a function of running speed on level grade (circles) or 20% grade (squares). Data are means ± SD, and numbers are sample sizes of measurements from animal groups. Means have been offset to avoid overlap; measurements were made at the speeds listed.