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Synergistic Effects of Air Pollutants: Ozone Plus a Respirable Aerosol

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Includes the Commentary of the Institute's Health Review Committee

Research Report Number 38

HEALTH EFFECTS INSTITUTE

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INVESTIGATOR'S REPORT Jerold A. Last

HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

Synergistic Effects of Air Pollutants: Ozone Plus a Respirable Aerosol

Jerold A. Last[1]

ABSTRACT

Rats were concurrently exposed to mixtures of ozone or nitrogen dioxide and respirable-sized aerosols of sulfuric acid, ammonium sulfate, or sodium chloride, or to each pollutant individually. Their responses to such exposures were evaluated by various quantitative biochemical analyses of lung tissue or lavage fluids, or by morphometric analyses. Such studies were performed in the acute time frame, generally involving exposures of from one to nine days, depending on the assays used. Correlations between the biochemical and morphometric results were examined over a wide range of pollutant concentrations in the exposure chambers.

Good correlations were found between the most sensitive biochemical indicators of lung damage—the protein content of lung lavage fluid or whole lung tissue and the rate of lung collagen synthesis—and the morphometric estimation of volume density or volume percent of the centriacinar lung lesion characteristically observed in animals exposed to ozone.

Synergistic interaction between ozone and sulfuric acid aerosol was demonstrated to occur at environmentally relevant concentrations of both pollutants by several of the analytical methods used. Such interactions were demonstrated at concentrations of ozone as low as 0.12 parts per million (ppm)[2] and of sulfuric acid aerosol at concentrations as low as 5 to 20 $\mu g/m^3$. The acidity of the aerosol is a necessary (and apparently a sufficient) condition for such a synergistic interaction between an oxidant gas and a respirable aerosol to occur. A hitherto unexpected synergistic interaction between nitrogen dioxide and sodium chloride aerosol was found during these studies; it is hypothesized that this was due to formation of their acidic (anhydride) reaction product, nitrosyl chloride, in the chambers during exposure to the mixture.

Preliminary experiments treating exposed animals in vivo with various free-radical scavengers suggested that dimethylthiourea, a hydroxyl-radical scavenger, might be protective against effects of ozone on rat lungs. This observation might have mechanistic implications, but further studies will be necessary to determine the significance of these findings.

INTRODUCTION

Ozone (O_3) causes lung injury in laboratory animals exposed to high concentrations and in those continuously exposed to concentrations at or near the peak hourly values encountered during serious episodes of photochemical smog (National Research Council 1977; Watson et al. 1988). A broad range of disciplinary approaches including pathology, biochemistry, physiology, anatomy, and microbiology have been used to examine effects of O_3 in animal experiments, and it is clear that integration of findings across these different disciplines is not yet completely possible.

The current U.S. National Ambient Air Quality Standard (NAAQS) for O_3 is 0.12 ppm for the peak hourly value (U.S. Environmental Protection Agency 1986). Average peak hourly O_3 concentrations in urban atmospheres can vary widely, but in the South Coast Air Basin of California, peak values exceed the NAAQS about half the days of the year, and the worst observed value of about 0.4 ppm is not unusual during severe smog episodes (California Air Resources Board 1984). Outside of California, peak hourly concentrations in excess of 0.2 ppm are not unusual during severe summer smog episodes (Apling et al. 1977; National Research Council 1977). There is no current NAAQS for sulfuric acid aerosol; the threshold limit value (TLV) for occupational exposure is 1,000 $\mu g/m^3$ for one hour.

Among the most important potential threats to human and animal health in photochemical smog are those from the strong oxidant gases O_3 and nitrogen dioxide (NO_2). These pollutants arise in part as a consequence of oxidation of atmospheric nitrogen to nitrous oxide (NO) and NO_2 during high-temperature combustion of fossil fuels, such as occurs in automobile engines and in generation of electricity from coal, oil, or natural gas. Concentrations of O_3 and of NO_2 attained in photochemical smog are interdependent, with NO_2 being a precursor of most of the O_3 formed via a complex series of free-radical reactions occurring in the polluted atmosphere. A great deal of research has been done to determine the effects of controlled exposures to O_3 on human and animal lungs. Effects of controlled exposures to NO_2 have also been reported, but NO_2 has been much less extensively studied than has O_3. It seems that, in

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[2] A list of abbreviations appears at the end of this report for your reference.

general, NO_2, which is less soluble in water than O_3, must be present in higher concentrations in air than those required for O_3 to elicit equivalent biologic or toxicologic effects. My colleagues and I, using the elevated rate of lung collagen synthesis after seven days of exposure to the oxidant gas as a quantifiable index of lung injury, have previously determined that O_3 is about 18 times more toxic to rat lungs than is NO_2 at given exposure concentrations spanning the ranges of 5 to 25 ppm of NO_2 and 0.5 to 1.5 ppm of O_3 (Last et al. 1983). This toxicity ratio is similar to earlier estimates based on histologic comparisons (Freeman et al. 1974).

EFFECTS OF OZONE ON LUNGS OF LABORATORY ANIMALS

Acute exposure to very high concentrations of O_3, in excess of about 1 ppm, causes severe pulmonary edema and hemorrhage. Death of laboratory animals can result from exposure to O_3 concentrations in excess of 1.5 ppm for periods of several hours or days. However, with concentrations at or below approximately 1.0 ppm of O_3, effects of O_3 related to edema and cellular inflammation are more subtle and are reflected as increased lung weight, and as increased lung enzyme activities and lung content of a large variety of measurable indicators associated with cellular inflammation and edema (Chow and Tappel 1972, 1973; Werthamer et al. 1974; National Research Council 1977; Mustafa and Tierney 1978).

The major changes recorded in laboratory animals exposed to moderate concentrations of O_3 (below 1 ppm for hours or days) include damage to respiratory tract epithelium, especially loss of cilia from ciliated cells, and cell necrosis. The two major sites of damage appear to be the trachea and large bronchi, which undergo loss of cilia from epithelial cells, and the centriacinar region of the lung, which undergoes epithelial cell necrosis. The alveolar type I cells and the Clara cells seem to be particularly susceptible (National Research Council 1977). Other changes observed in animals exposed to moderate concentrations of O_3 include those typical of an inflammatory cell response, especially in the epithelial layer of small bronchioles in the centriacinar region (National Research Council 1977). Chronic exposure to O_3 is associated with continuing bronchiolitis and continuing inflammatory response of the centriacinar region (Eustis et al. 1981). Another chronic effect of prolonged exposure of animals to moderate levels of O_3 is pulmonary fibrosis; that is, the accumulation of collagen in the centriacinar region of the lung (Stokinger et al. 1957; Freeman et al. 1973; Last 1988). These results are consistent with the predicted sites of maximal deposition of O_3 in the respiratory tract on the basis of its relative solubility in water and from established models of O_3 dosimetry to the lung (see, for example, Watson et al. 1988).

Several laboratories have reported changes in the protein content of lung lavage fluid in animals exposed to O_3. Hu and associates (1982) reported increased accumulation of protein in lung lavage fluid from guinea pigs exposed for 72 hours to 0.26, 0.51, or 1 ppm of O_3. No effect was observed at 0.10 ppm. The increased protein in lavage fluid seemed to originate from serum, suggesting that the changes being measured arose from pulmonary edema. Costa and coworkers (1985) reported that rats exposed to O_3 had a higher alveolar permeability to serum albumin than did normal rats. Frank and colleagues (1979) also reported pulmonary edema in rabbits exposed to high concentrations of O_3 for short periods of time.

Bartlett and associates (1974) reported decreased lung tissue elasticity consistent with mild pulmonary fibrosis in young rats exposed to 0.2 ppm of O_3 for 30 days. Several groups have reported increased collagen synthesis by lungs of laboratory animals exposed to O_3 (Hussain et al. 1976; Last et al. 1979a; Bhatnagar et al. 1983; Filipowicz and McCauley 1986). The significance of these observations with regard to the etiology of pulmonary fibrosis is controversial. Filipowicz and McCauley (1986) have suggested that despite the increased rate of collagen synthesis observed in lungs of rats exposed to O_3, there is no net accumulation of total lung collagen, suggesting increased degradation of collagen accompanying (and offsetting) the increase in synthesis.

EFFECTS OF SULFURIC ACID AEROSOL ON LUNGS OF LABORATORY ANIMALS

Guinea pigs seem to be particularly susceptible to sulfuric acid aerosol, when compared with other laboratory animals. Guinea pigs show increased airway resistance after exposure for one hour to 100 to 1,000 $\mu g/m^3$ of the acid aerosol (Amdur et al. 1978). Interestingly, no long-term effects of exposure of guinea pigs to 100 $\mu g/m^3$ for 52 weeks were reported by Alarie and coworkers (1975).

Rats or monkeys are remarkably resistant to effects observed in the lung when acutely exposed to respirable aerosols of sulfuric acid (Alarie et al. 1973, 1975; Cavender et al. 1977; Schwartz et al. 1977), even at concentrations at or above 50,000 to 100,000 $\mu g/m^3$. On the other hand, guinea pigs and, to a lesser extent, mice respond to exposure to lower concentrations of sulfuric acid mist (Cavender et al. 1977; Schwartz et al. 1977; Amdur et al. 1978), and presumably represent susceptible species to this agent. Intermittent exposure of mice to 1,400 $\mu g/m^3$ sulfuric acid mist in

conjunction with carbon particles (1.5 mg/m^3; no group was exposed to acid alone) for 20 weeks caused decreased resistance to laboratory-induced pulmonary infections (Fenters et al. 1979). No effects of sulfuric acid aerosol exposure at 8,000 μg/m^3 in dogs and at 4,000 to 14,000 μg/m^3 in sheep were observed for various cardiopulmonary functions examined (Sackner et al. 1978). The relatively high resistance of laboratory animals to exposure to sulfuric acid aerosol is assumed to reflect its high absorption by the nasopharynx, thereby reducing to very low levels the dose reaching the distal lung. This is consistent with the high solubility of sulfuric acid in water and with established models of lung dosimetry.

Sulfuric acid aerosol exposure at relatively low concentrations has been associated with alterations in mucociliary clearance rates for various tracer substances introduced into respiratory airways of laboratory animals. Fairchild and associates (1975) reported that exposure of guinea pigs for one hour to concentrations of sulfuric acid between 30 and 3,000 μg/m^3 caused the sites of deposition of radioactively labeled streptococcus administered by aerosol to shift proximally (upward in the respiratory airways toward the nasopharynx and trachea). Rabbits exposed for one hour to 200 μg/m^3 of sulfuric acid aerosols showed altered rates of mucociliary clearance of radioactive tracer particles (Schlesinger et al. 1983), as did donkeys (Lippmann et al. 1987).

Chronic exposure of monkeys for up to 78 weeks to sulfuric acid aerosol at 1,000 to 5,000 μg/m^3 resulted in histologic changes to the bronchial mucosa and changes in epithelial cells of bronchi and respiratory bronchioles (Alarie et al. 1973, 1975). These results are consistent with the known sites of deposition for particles of about 0.5 to 5 μm, which are preferentially deposited in the deep lung (see, for example, Watson et al. 1988).

EFFECTS OF COMBINATIONS OF OZONE AND ACID AEROSOL ON LUNGS OF LABORATORY ANIMALS

Gardner and coworkers (1977) reported that mice exposed sequentially to 0.1 ppm of O_3 for three hours followed by 900 μg/m^3 of sulfuric acid aerosol for two hours showed significantly higher mortality when challenged with an aerosol of *Streptococcus pyogenes* than did mice challenged with either pollutant alone. Osebold and associates (1980) reported enhanced antigenicity of protein introduced into the respiratory tract in mice exposed to O_3 plus sulfuric acid as compared to those exposed to either agent alone. They interpreted their findings as indicative of increased epithelial permeability, which is an enhanced effect of O_3, in animals exposed to the mixture of gas and aerosol. Last and Cross (1978) reported synergism of O_3 effects on rat lungs by sulfuric acid aerosol during simultaneous exposure to concentrations of about 1,000 μg/m^3 of the aerosol and 0.4 ppm of O_3 for 3 to 14 days. Parameters evaluated included rate of secretion of mucous glycoproteins by tracheal explants, and lung content of water, DNA, RNA, and various lysosomal enzymes. This same laboratory later showed an enhanced effect of O_3 in lungs of rats simultaneously exposed to O_3 plus sulfuric acid aerosol as evaluated by elevated rates of collagen synthesis and by morphometric evaluation of lung and inflammatory cell populations at sites of lung lesions (Last et al. 1983, 1984). In all of these studies, sulfuric acid aerosol alone had little or no effect on the parameters being quantified; however, the acid aerosol significantly increased the effect of a given concentration of O_3, as if it were increasing the effective dose of O_3 delivered to the centriacinar region of the lung. Juhos and coworkers (1978) reported that histologic changes in lungs of small numbers of rats exposed to 0.9 ppm of O_3 were similar to, but less severe than, those observed in rats exposed to 0.9 ppm of O_3 plus 2,000 μg/m^3 of sulfuric acid aerosol, consistent with the observations of Last and coworkers described above.

There also have been reports of negative results of exposures to mixtures of O_3 and sulfuric acid aerosols. Cavender and colleagues (1977) reported no effects in rats or guinea pigs exposed for up to seven days to 2 ppm of O_3, or 10,000 μg/m^3 of sulfuric acid aerosol, or both, other than those ascribed to O_3 alone. It is, however, noteworthy that control animals lost weight during these exposures, suggestive of illness or other handling problems, and that 2 ppm of O_3 for seven days, which is well in excess of the median lethal dose (LD_{50}) for rats, provoked no mortality in this study. Human subjects exposed sequentially to 0.3 ppm of O_3 for two hours followed by 100 μg/m^3 of sulfuric acid aerosol for four hours showed no effects on pulmonary function tests (Kulle et al. 1982).

A hitherto unexpected synergism between the oxidant air pollutants O_3 or NO_2 and a respirable-sized aerosol of ammonium sulfate was observed during controlled exposures of rats to these substances (Last et al. 1983). Response of rat lungs to these pollutants was quantified by measuring in vitro the apparent collagen synthesis rates in minced lung tissue from exposed animals. Dose-response curves to either O_3 or NO_2 were altered in the presence of 5 mg/m^3 of ammonium sulfate aerosol. Morphometric and histologic observations of lungs from rats exposed to high levels of O_3, with and without concurrent exposure to the ammonium sulfate particles, confirmed such synergistic effects. In subsequent studies, cellular populations were examined at the sites of lesions in lungs of rats exposed for three or seven

days to 0.64 to 0.96 ppm of O_3 with or without 5 mg/m^3 of ammonium sulfate aerosol (Last et al. 1984).

Taken together, these studies gave rise to a hypothesis that the synergistic interaction between O_3 and acid aerosols may be mediated by changes in the pH of the lung lining fluid caused by local deposition of acid at or near sites of reaction of O_3 or NO_2 with molecules within lung cells or lining fluid. On the basis of these and earlier results, we proposed to examine further aspects of the O_3–acid aerosol interaction. We specifically proposed to relate our earlier findings at toxicologic concentrations of these pollutants to effects at concentrations of the pollutants that might occur in urban atmospheres. We also proposed to perform limited studies of the mechanisms underlying oxidant gas–acid aerosol interactions, so as to facilitate predictions of other such interactions that might occur during exposure to mixtures of pollutants.

SPECIFIC AIMS

This project examined the interaction between the gaseous oxidant pollutants O_3 or NO_2 and respirable-sized aerosols of ammonium sulfate, sulfuric acid, or sodium chloride. Individual agents or mixtures of oxidant and aerosol were breathed by rats in exposure chambers. The aim was to determine whether or not such interactions gave toxic effects on the lung that were additive or were greater than additive—that is, synergistic. The objectives of these experiments were to compare responses of the lung by biochemical, toxicologic, and morphologic criteria, to each pollutant, both individually and as a component of a controlled binary mixture. One hypothesis tested was that the response to binary mixtures of oxidant gases and respirable acid aerosols would exceed the sum of the responses to the individual pollutants.

A range of oxidant and aerosol concentrations was used during the study. An attempt was made to define the no-observable-effect level (NOEL) for oxidants and for acid aerosols by the most sensitive assays used, and to correlate the effects observed between various quantitative assays of lung composition and structure.

The first specific aim of this study was to determine whether or not synergism occurs between oxidant gases and respirable acid aerosols at ambient or near-ambient concentrations of O_3 or NO_2 and aerosol. To this end, we performed titration experiments, using various biochemical assays of whole lung tissue and of lung lavage fluid as response endpoints, to examine this question quantitatively. Morphometric analysis of the lung centriacinar region was used selectively to correlate structural and cellular changes in this region of the lung with the measured alterations in biochemical parameters.

The second specific aim was to determine what role aerosol acidity plays in the synergy with O_3 or NO_2. To this end, experiments were performed in which we attempted to correlate acidity of aerosol, as measured by the pH of eluates from sampling filters, with biologic response to the aerosol in combination with O_3. Such experiments also directly addressed the issue of whether or not measurable neutralization of acid aerosols occurs in the exposure chambers as a result of the reaction of acid droplets with volatile ammonia, arising from the animals themselves or from the actions of microorganisms upon their excreta.

The third specific aim was to determine the role of active oxygen species ("free radicals") in the mechanism of interaction between O_3 and respirable acid aerosols. To approach this objective, selected scavengers of free radicals were administered to rats before and during exposures to O_3 or acid aerosols or both in order to examine whether or not they were protective in vivo against the effects of O_3 or the combination of O_3 and aerosol. Experiments to pursue this aim were substantially reduced in scope and extent as a result of feedback from a Health Effects Institute site visit during the first year of this project; therefore, work toward this aim was limited to the development of methods to perform such experiments, and to preliminary experiments.

MATERIALS AND METHODS

GENERATING AND MONITORING AEROSOLS

Aerosols were generated as described in detail elsewhere (Last et al. 1979b). Solutions of sulfuric acid or ammonium sulfate in distilled water were nebulized with a Babington-type nebulizer (Solo-Sphere, McGaw Respiratory Therapy, Irvine, CA). The nebulizer was immersed in an ice-water bath during operation to reduce water evaporation from the solution. The aerosol was diluted with clean, dry air immediately downstream from the nebulizer to reduce the relative humidity. A ^{85}Kr discharger was used to reduce the aerosol electrostatic charge to Boltzmann equilibrium. The discharger was heated slightly with a heating tape to enhance droplet drying. The conditioned aerosol was then introduced into the inlet air stream of a 450-L exposure chamber (Schwartz et al. 1976). The chamber volumetric flow rate was 225 L/minute (30 air changes per hour). Chamber relative humidity was maintained at 70 to 80 percent at 22°C to preserve the integrity of the aerosol. Chamber aerosol mass concentrations were measured by collecting aerosol samples from known volumes of air on glass-fiber filters (Type

AE, Gelman Instrument Co., Ann Arbor, MI) and by weighing the filters before and after collection to determine their increase in mass. The aerodynamic size distributions were determined with a seven-stage cascade impactor (Model 04–002, Aries, Davis, CA) and by subsequent ion chromatographic analysis of the sulfate anion on each stage and on the backup filter. A log-normal distribution was fitted to each sample set of data and the values reported are the mass median aerodynamic diameter (MMAD) and geometric standard deviation (σ_g) for each fitted distribution.

Under our exposure conditions, stage 6 of the Aries B6 Cascade Impactor had an effective cutoff MMAD of about 0.78 μm, and stage 7 had an effective cutoff diameter of 0.29 μm at the air flow rates used.

The ammonium sulfate aerosols, generated with a Babington nebulizer at 5 mg/m^3, were 0.8 to 1.0 μm MMAD with σ_g of 1.8 to 2.0. Sulfuric acid aerosols generated from the Babington nebulizer were generally about 0.4 to 0.5 μm MMAD, with σ_g of 1.8 to 2.5. Specific monitoring details for each exposure are given in the tables and figure legends. Sodium chloride aerosols were also generated from the Babington nebulizer at about 1 mg/m^3, with 0.4 to 0.5 μm MMAD and σ_g of 2.5 to 2.6.

In some experiments samples of aerosolized sulfuric acid were collected for the measurement of pH. Collections were made on Versapor 800 (Gelman Instrument Co.) membrane filters (0.8 μm pore diameter) by sampling chamber aerosol mixtures under reduced pressure (approximately 1.2 pounds/sq in) for 60 minutes. Filters were then washed with a known volume of deionized water, and the pH (I 70 pH meter, Beckman, Palo Alto, CA) and the sulfate concentration (as determined by ion chromatography) of these aqueous solutions were measured.

GENERATING AND MONITORING OZONE AND NITROGEN DIOXIDE

Ozone was generated from medical-grade oxygen using silent arc discharge ozonizers (Model IV, Erwin Sander Co., Eltze, West Germany). Ozone concentrations are reported using the ultraviolet photometric standard. Each chamber was monitored every two minutes by an ultraviolet O_3 monitor (Model 1003-AH, Dasibi Environmental Corp., Glendale, CA) calibrated using an absolute O_3 photometer (Model 1008-PC, Dasibi Environmental Corp.). Monitoring was controlled and data were reported using a PDP LSI 11/23 computer (Digital Equipment Corp., Cambridge, MA). Final calculations were performed with a Burroughs (Unisys Corp., Detroit, MI) B7800 computer (Last et al. 1983; Warren and Last 1987).

Nitrogen dioxide was generated by dilution and metering of the dry tank gas (99 percent NO_2 in nitrogen), and NO_2 concentrations were measured by a chemiluminescence detector (Model 14T, Thermo-Electron Corp., Hopkinton, MA) controlled by the PDP LSI 11/23 computer as described above (Last and Warren 1987). The main thrust of the experiments in this study involved exposures to O_3 with or without acid aerosol; exposures to NO_2 were limited in scope. We used the relationship of relative toxicity of O_3 to NO_2 as being 18 to 1 (Last et al. 1983) to estimate the appropriate range of NO_2 concentrations to study, as judged from the results observed with rats exposed to O_3. For comparative purposes, 2, 5, and 10 ppm of NO_2 were, therefore, considered equivalent in biologic effect to 0.11, 0.28, and 0.56 ppm of O_3, respectively.

ANIMALS AND EXPOSURE CHAMBERS

Male Sprague-Dawley rats free of chronic respiratory disease and weighing 250 to 300 g (Banton-Kingman, Gilroy, CA) were used for most of the experiments. All animals were given an acclimatization period of two to seven days in the exposure chambers before use, and were maintained at the California Primate Research Center exposure facility (an animal facility certified by the American Association for the Accreditation of Laboratory Animal Care) on a 12-hour light and 12-hour dark schedule with free access to food (Purina rat chow pellets) and water. Rats were exposed to filtered air, to oxidant gases, to aerosols of sulfuric acid, ammonium sulfate, or sodium chloride, or to mixtures in glass and stainless-steel chambers of the Hinners type (Hinners et al. 1968) of 4.2 m^3. Air flow rates supplied 30 changes of chamber contents per hour. Rats were housed in pairs in stainless-steel mesh cages during exposures. Animal loading rates were less than 2 percent of chamber capacity. All animals were exposed continuously (23.5 hours/day) for the designated durations unless otherwise indicated.

In certain experiments, special care was taken to attempt to suppress the release of ammonia from microbial action upon animal excreta. In these exposures, the chambers were cleaned and rinsed as usual. The chamber drain valve was then securely closed and 10 gallons of a 0.02 percent solution of alkylbenzyl dimethylammonium chloride was placed in the bottom of the chamber, under the flooring grate. This fluid, which is bactericidal, should have helped to maintain any excretory products falling to the bottom of the chamber in an aseptic state (or at least have retarded the growth of microorganisms), and effectively suppressed (or diminished) a major component of the putative generation of ammonia from conversion of urea and other organic nitrogen compounds in urine and feces in the chambers.

RADICAL QUENCHING AGENTS AND TREATMENTS

Vitamin E acetate, β-carotene, and 1,3-dimethylthiourea were obtained from Corvitol (Chemical Division of Henkel Corp.), Sigma Chemical Co. (St. Louis, MO), and Aldrich Chemical Co. (Milwaukee, WI), respectively, and were used without further purification. Both vitamin E acetate and β-carotene were administered by intraperitoneal injection in up to 0.5-mL volumes at 0.1 g/kg doses in a diluent of 1 part Triton X-100 in 50 parts of sterile water. Controls were treated with an equivalent volume of diluent alone. Solutions for injection were homogenized by sonication immediately before use. A standard regimen was used for the treatment of rats with either diluent, vitamin E, or β-carotene: for one-day exposures, rats were injected once 48 hours before the start of exposure; and for seven-day exposures, rats were injected twice, two days before and three days after the start of exposure. Dimethylthiourea (DMTU) was administered by intraperitoneal injection in a solution of sterile 0.15 M sodium chloride solution, in doses ranging from 50 to 500 mg/kg per day, each day during exposure. Dosage regimens were chosen on the basis of preliminary experiments demonstrating elevated lung levels of the agent under the protocols used.

TISSUE ANALYSIS FOR QUENCHING AGENTS

Vitamin E was measured in blood and lung by the method of Zaspel and Csallany (1983). Plasma or whole lung homogenate was extracted with acetone. The resultant organic phase was analyzed by high-pressure liquid chromatography on a reversed-phase column. Fluorescence detection (Hitachi Model No. F-1000) (excitation at 295 nm, emission at 340 nm) gave a standard curve that was linear from 0.05 to 10 μg of vitamin E. Recovery of authentic α-tocopherol from lung tissue was greater than 90 percent.

β-Carotene was measured in blood or lung tissue by the method of Shapiro and associates (1984). Plasma or saponified lung homogenate was extracted with hexane. The organic phase was analyzed by high-pressure liquid chromatography on a reversed-phase column. Ultraviolet detection (Gilson [Middleton, WI] holochrome spectrophotometer using a wavelength of 470 nm) gave a standard curve that was linear from 1 to 60 ng of β-carotene. Recovery of authentic β-carotene from lung tissue was greater than 80 percent.

LAVAGEABLE PROTEIN AND ENZYME ACTIVITY

Immediately after exposure, animals were killed by pentobarbital overdose and prepared for lavage. The lavage procedure (Guth et al. 1986) consisted of slow washing of the lungs with six 5-mL volumes of normal saline via a tracheal cannula. Throughout this procedure care was taken to avoid visible blood contamination of the return lavage fluid. Recovery of lavage fluid was greater than 90 percent and total volume recovered was recorded for each animal. The six washings from each lung were pooled and centrifuged at 800 × *g* for 10 minutes to remove cells and debris, and the protein content was determined from a portion of the cell-free supernatant by the method of Lowry and associates (1951), which uses the Folin phenol reagent to form a colored reaction product with certain amino acid residues in the protein molecule. Extent of color formation was converted to milligrams of protein by use of a standard curve prepared with known concentrations of bovine serum albumin as a standard. Data were calculated and expressed as total milligrams of protein in the pooled total recovered lavage fluid. Portions of the cell-free supernatant were assayed for the activities of lactate dehydrogenase and of acid phosphatase (Guth and Mavis 1985; Guth et al. 1986), or of *N*-acetyl-β-D-glucosaminidase (Beaufay et al. 1974; Guth et al. 1986). Enzyme assays were performed on freshly prepared cell-free lavage fluid at 25°C. One unit of activity was defined as 1 μmol of product formed per minute. All lavageable protein content or enzyme activity data for each animal were expressed as milligrams of protein or units of activity per total recovered lavage fluid volume.

In some experiments, the cell pellet from centrifugation of pooled lavage fluid was resuspended in physiological saline (vortex mixed) and counted with an electronic cell counter (Coulter Instruments, Hialeah, FL). Data were expressed as total cells recovered per lavage. Gating (sizing) techniques were used to distinguish cells from acellular debris, and in some cases to speciate cell types in these experiments. Wright-Giemsa–stained slides of cell suspensions were also used to enumerate cell types in lavage fluid in some experiments.

TOTAL LUNG PROTEIN AND DNA CONTENT

The right apical and middle lung lobes were removed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in approximately 10 volumes of 0.15 M sodium chloride. A portion of the resulting homogenate was used for protein determination (Lowry et al. 1951). Calculations were based on values determined using four replicate 100-μL samples from 4 mL of total homogenate of the right middle plus right lower lobes of rat lung. Each 100-μL sample was precipitated with trichloroacetic acid (Cl_3CCOOH) and centrifuged, and the pellet was frozen. Protein was then solubilized in 0.5 mL of 0.5 N sodium hydroxide (NaOH) (37°C, 24 hours). Sodium hydroxide was neutralized with 0.5 mL of 0.5 N hydrochloric acid. Protein in each sample

was determined in duplicate. The protein contents for each homogenate were obtained by averaging the four sample protein values (eight determinations). Data for tissue protein content were expressed as milligrams per whole lung. This value was calculated for each animal as the product of the protein content per combined right apical and middle lobes times 4.29 (Walton and McGovern 1977). DNA content of lung tissue was quantified as described previously (Last and Cross 1978).

MOVEMENT OF LABELED TRACER FROM BLOOD TO LAVAGEABLE SPACE

For the assay of tracer movement from blood to lavage fluid, each rat was anesthetized with subcutaneously injected acepromazine (1 mg/kg of body weight) followed five minutes later by ketamine (50 mg/kg of body weight) injected intramuscularly. Approximately 10 μCi of ^{3}H-labeled albumin in a total volume of 0.10 mL of saline was injected via the tail vein. About 50 minutes after tracer injection, each rat was injected intraperitoneally with a lethal dose of sodium pentobarbital (200 mg/kg of body weight). The trachea was exposed and a cannula was inserted and tied into the trachea. Care was taken to avoid contamination with blood at this step. The thorax was then opened and a blood sample (approximately 3 to 5 mL) was drawn by cardiac puncture for preparation of serum. Exactly one hour after tracer injection the lungs were lavaged with six sequential 5-mL volumes of 0.15 M sodium chloride. The lavage samples were pooled and centrifuged at 700 × *g* for 10 minutes to remove free cells. The supernatant was recovered, an aliquot was counted for radioactivity, and the remainder was used for protein and enzyme assays. In some experiments ^{3}H-labeled albumin was instilled intratracheally in a total volume of 0.15 mL in order to quantify movement from airways to blood. Exactly one hour after albumin instillation the lungs were lavaged as described, and blood was collected for measurement of radioactivity.

Data were expressed as the total lavageable radioactivity divided by the radioactivity in 1 mL of serum (that is, disintegrations per minute [dpm] of tracer per milliliter of lavage fluid × milliliters of fluid recovered × 10^3/dpm of tracer per milliliter of serum). The factor of 10^3 was used to avoid awkward numbers of decimal places.

Clearance

To examine the rate of clearance of labeled albumin from blood, rats were injected intravenously as described with the tracer solution. Blood was collected from the orbital sinus under light carbon dioxide (CO_2) anesthesia at various times ranging from 15 minutes to four days after injection, and radioactivity remaining in serum was determined by liquid scintillation counting.

Preparation of Tracer

Bovine serum albumin (Fraction VI, Sigma Chemical Co.) was labeled using the conditions described by Gisslow and McBride (1975). Briefly, 20 μCi of ^{3}H-acetic anhydride (ICN, Irvine, CA) in benzene was added slowly to 3 g of albumin in 30 mL of 1 M potassium phosphate (K_2HPO_4) at pH 8.0 and 0°C with stirring. After one hour the product was dialyzed extensively against water and lyophilized. Just prior to use, the tracer was dissolved in 0.15 M sodium chloride at a final concentration of albumin of approximately 65 mg/mL, containing about 0.1 mCi/mL. More than 98 percent of the total radioactivity of the dissolved product was precipitable with 10 percent Cl_3CCOOH. The product was evaluated also by gel electrophoresis on 10 percent acrylamide followed by Coomassie blue staining and fluorography.

COLLAGEN SYNTHESIS RATE ASSAY

At the end of the experiment, each rat was weighed and killed with an overdose of sodium pentobarbital (injected intraperitoneally). The chest was opened, and the lungs were perfused with 0.15 M sodium chloride via the pulmonary artery to remove trapped blood. The lungs were then excised, trimmed free of extraneous tissue with scissors, and put into tissue culture medium (Dulbecco's modified Eagle's medium, GIBCO, Grand Island, NY).

Each lung was minced on a glass plate with two razor blades into pieces of approximately 1 mm^3. These pieces were divided randomly among three incubation vials (1.8 cm in diameter), each aliquot containing about 150 to 200 mg wet weight of lung tissue. Excess tissue was discarded or used for other analyses. The minced tissue was incubated for one hour in 1.8 mL of medium containing 100 μg/mL of ascorbate and 64 μg/mL of β-aminopropionitrile fumarate at 37°C, with vigorous shaking, under an atmosphere of O_2:CO_2 (95:5) in a Dubnoff metabolic shaker. This medium was then removed, fresh medium (0.5 mL), identical to that above but with the addition of 50 μCi of ^{3}H-proline (generally labeled, 490 Ci/mol), was added, and the incubation was continued. Routinely, vials were removed from the incubation chamber after one, two, and three hours of incubation to determine synthesis of hydroxyproline.

Medium was aspirated from the tissue with a Pasteur pipette and discarded because the large amount of ^{3}H-proline it contained would have interfered with the ^{3}H-hydroxyproline assay carried out later. Because more than

95 percent of the labeled collagen and more than 90 percent of the newly synthesized total protein remained with the tissue fraction, removing the medium did not significantly affect our results. The tissue was washed quickly with ice-cold phosphate-buffered saline, then suspended in 1.5 mL of 0.5 M acetic acid and homogenized (Polytron homogenizer, Brinkmann Instruments). Aliquots were taken from these crude homogenates for assay as follows: duplicate 100-μL aliquots for colorimetric analysis of DNA and protein content, 20 μL for Cl_3CCOOH-precipitable radioactivity (total protein), and 1.0 mL for determination of Cl_3CCOOH-insoluble ^3H-hydroxyproline and the specific activity of Cl_3CCOOH-soluble proline. Because the rate of synthesis of total protein or hydroxyproline (expressed as either counts per minute [cpm] per milliliter of homogenate or per milligram of protein) in minced lung tissue from rats administered a toxicant such as O_3 would be influenced by the actual proline pool size endogenous in the minced lung tissue, we routinely determined the actual specific activity of proline in the tissue homogenates. Specific activity was determined by adjusting the pH of the Cl_3CCOOH-soluble fraction with NaOH to fall between pH 1 and 7. Radioactivity other than ^3H-proline was removed with Permutit, and an aliquot of the supernatant fraction was counted by liquid scintillation. Another aliquot was chemically assayed for total proline content by a colorimetric method (Troll and Lindsley 1955). Specific activity, which tended to be lower in lungs of animals exposed to oxidants, was calculated by dividing the radioactivity (cpm of ^3H-proline) by the amount of proline present in a given volume of homogenate.

Under our incubation conditions, incorporation of ^3H-proline into total Cl_3CCOOH-insoluble protein and into ^3H-hydroxyproline was linear with respect to time for at least four hours in control rats. The best straight line was fitted by linear regression analysis to the data obtained at one, two, and three hours for each sample (plus a zero-hour value of hydroxyproline of 0 nmol/g of protein). The calculated collagen synthesis rate used for comparisons between groups of rats was determined by fitting regression lines to data points from each of the animals in the same group. The slopes of the group means for these regression lines (collagen synthesis rates) then were compared using Student's *t* test. Data were processed with a Burroughs Prime Minicomputer, using a published program written in Fortran for this purpose (Last 1989).

As a result of differences in the exact specific activity of the labeled proline added to the tissue culture medium and other experimental variables, the control values for collagen synthesis rate differed slightly from experiment to experiment (for a typical set of experiments, the mean value ± 1 SD for a group of 61 control rats analyzed from 16 different exposure groups was 14.1 ± 3.1 nmol of hydroxyproline synthesized per gram of protein per hour; that is, a coefficient of variation in this parameter of 22 percent). Therefore, the synthesis rate for each control animal within an individual experiment was normalized to a cumulative mean air-breathing control value (control values from all experiments) to increase the size of the control group, and thereby the statistical power of the analysis. Then, the rate from each exposed animal was multiplied by the ratio of the cumulative control value to the mean of the control values per individual experiment. The mean values ± 1 SD for each exposed group were then calculated.

MORPHOMETRIC EVALUATION OF LUNG DAMAGE

Histologic and morphometric analyses were performed on a different group of rats than those undergoing biochemical or toxicologic analysis, but both groups had been exposed at the same time to O_3, to O_3 plus acid aerosol, or to acid aerosol alone. After thoracotomy, the heart was ligated at its base to isolate the pulmonary vasculature. The trachea was cannulated and the lungs were fixed by airway perfusion with a cacodylate-buffered glutaraldehyde-paraformaldehyde fixative at 30 cm of fixative pressure. After at least two hours of fixation, the right cranial and caudal lung lobes were cut in a sagittal plane. A block 1 cm by 1 cm by 0.5 cm was cut at random from one of the sagittal slabs from each lobe. The blocks were dehydrated in a graded series of ethanol, embedded in glycol methacrylate, sectioned at 2 μm, and stained with hematoxylin and eosin, or they were embedded in Araldite 502, sectioned at 1 μm, and stained with methylene blue, azure II, and basic fuchsin.

Two slides for each of the (usually) six rats per group (that is, usually 48 slides per experiment) were used for morphometric analysis. For estimates of the volume ratio of lesion, all tissue present on each section was covered by stratified sampling by use of a double square lattice test system (D64) with 1,024 points and a rear-screen projection unit at a magnification of × 100. By use of a second cascade level of subsampling, the volumetric ratio of components constituting the lesion was estimated by systematic area-weighted quadrant sampling at a magnification of × 400. In this latter method of sampling, we used a simple square lattice system of 100 points in which one point was designated as the test point. The slide was scanned in a systematic manner until the test point fell on a lesion. Subsequently, a random number generator was used to select an additional point of the 100 possible points, which when selected was superimposed on the location originally marked by the test point. This method insures random location of the grid relative to

the lesion. Ten fields were used per slide for this subsampling method. Volume ratios were estimated by use of point count techniques. In the same manner the frequency of lesions per square micrometer was estimated. A region of interstitial fibrosis, of interstitial edema, of clusters of five or more inflammatory cells, or of intraluminal accumulations of five or more inflammatory cells within acini was defined as a lesion. This definition allowed classification of a region of an acinus as a lesion or nonlesion at a magnification of × 100 with a light microscope. Area-weighted periodic sampling was used as a second cascade level of subsampling for estimating volumetric densities (V_V) of interstitial cells and stainable (van Gieson's stain) collagen fibers within lesions, and for estimating numerical densities (N_V) of interstitial cell profiles per lesion (number per square millimeter).

Cells that were involved in the inflammatory response within the interstitium of the proximal acinus included macrophages, monocytes, fibroblasts, neutrophils, and lymphocytes. Interstitial cells were categorized by classic definitions of cell shape, nuclear shape, and heterochromatin nuclear:cytoplasmic ratio. When the ratio was less than 1:2, the cell was determined to be a monocyte. The N_V (calculated as the number of interstitial cells per cubic micrometer) for each cell category was estimated by dividing the profile tallies for each cell type by the appropriate mean nuclear caliper diameter ($\overline{D}$). We assumed $\overline{D}$ values of 5.13 μm for monocytes and macrophages and 4.47 μm for other interstitial cells, including fibroblasts. Numbers of cell profiles within the lesion (number per square millimeter) were estimated using the counting rule proposed by Gunderson (1977). Total cells within lesions of the total lung were calculated as the sum of the products of N_V, volume density of lung lesion ($V_{V\ lesion}$) per lung, and fixed lung volume for each cell type.

Sampling was sufficient to estimate the mean of all morphometric parameters tested within approximately 10 percent of the animal mean using a 95 percent confidence interval. All morphometric values were analyzed using analysis of variance (ANOVA) (P7D of BMDP program), with $p < 0.05$ being considered as the level of significance.

DATA ANALYSIS

Measurements of the collagen synthesis rate were computed by linear regression analysis of data (Last 1989). Slopes of the resultant plots were compared by a Student's *t* test as described in detail elsewhere (Last 1989). Data from other biochemical and toxicologic assays were generally compared by one-way ANOVA, using SAS software (SAS Institute, Cary, NC) or Minitab software (Pennsylvania State University, University Park, PA) on a Burroughs Prime Minicomputer. Morphometric analyses were compared with biochemical data by use of the BMDP statistical package on a PDP LSI 11/23 computer using various statistical tests, including Duncan's multiple range test and ANOVA (Welch, Brown-Forsythe, Levene, and Bonferroni tests). Generally, $p < 0.05$ was taken to indicate a significant change in all comparisons of data sets.

RESULTS

BIOCHEMICAL EFFECTS OF EXPOSURE TO OZONE OR NITROGEN DIOXIDE ON RAT LUNGS: EXPOSURE CONCENTRATION–RESPONSE RELATIONSHIPS (SPECIFIC AIM NO. 1)

Assays of Bronchoalveolar Lavage Fluid

We examined the exposure concentration–response behavior of several assays of whole lung lavage fluid from rats exposed for up to two days to nominal concentrations of 0 to 0.96 ppm of O_3. Such assays included the total protein content of lavage fluid and the activities of several enzymes including lactate dehydrogenase, acid phosphatase, and the lysosomal hydrolase *N*-acetyl-β-D-glucosaminidase. We also examined the movement of a radiolabeled tracer molecule, ^{3}H-albumin, from blood to lavage fluid (and vice versa) in these experiments (Guth et al. 1986).

Accumulation of lavageable protein in the lung has been used by others as a sensitive measure of lung damage by O_3 and other agents. Thus, we compared the tracer movement from blood to lavageable space with the total protein content of lavage fluid as two measures of lung damage after rats were exposed for six hours to a fairly wide range of O_3 concentrations, as shown in Figure 1. Both lavage protein content (Figure 1a) and tracer movement (Figure 1b) were significantly increased in rats after six hours of exposure to O_3 concentrations of 0.4 ppm or higher.

On the basis of these observations, we compared the assays of protein content of lavage fluid and tracer movement with the enzyme content of lung lavage fluid. After exposure of rats to 0.64 ppm of O_3 (nominal concentration) for six hours, the lavage protein was increased by about 80 percent (from 2.1 ± 0.2 to 3.7 ± 1.1 mg per lung), but the activities of lactate dehydrogenase, acid phosphatase, and *N*-acetyl-β-D-glucosaminidase in lavage fluid did not differ from control values. These data suggest that measurements of lavage fluid protein or of tracer movement from blood might be more sensitive to change after short exposures to O_3 than measurements of activity of lavageable enzymes. Ex-

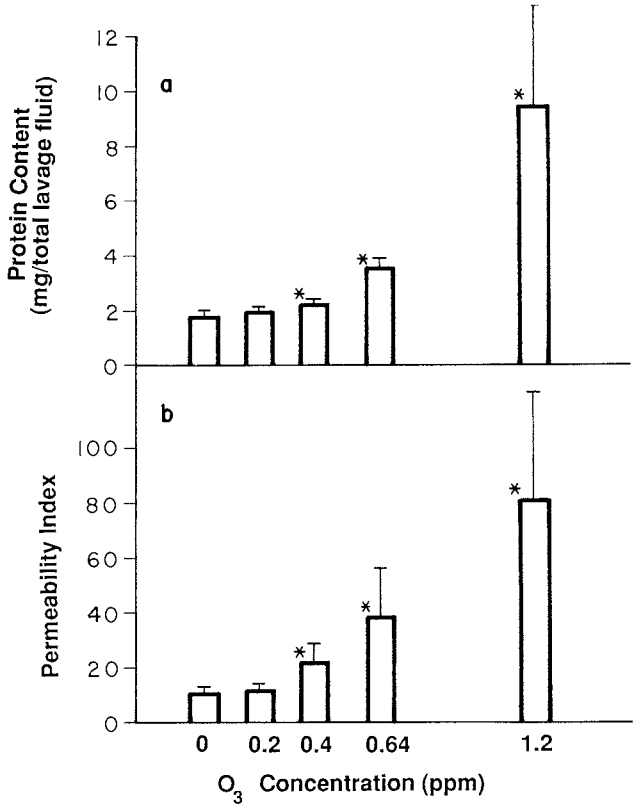

Figure 1. Effect of O_3 concentration, after exposure of rats for six hours, on (a) total protein content in lung lavage fluid and on (b) tracer movement from blood to lavage fluid (permeability index). Permeability index ([dpm recovered by lavage/dpm in 1 mL of serum] × 10^3) was determined one hour after intravenous injection of ^{3}H-labeled albumin. Values are means ± 1 SD for four animals per group. An asterisk indicates a significant difference ($p < 0.05$) from the control value. Actual O_3 concentrations were 0.20 ± 0.01, 0.40 ± 0.00, 0.63 ± 0.00, and 1.22 ± 0.02 ppm.

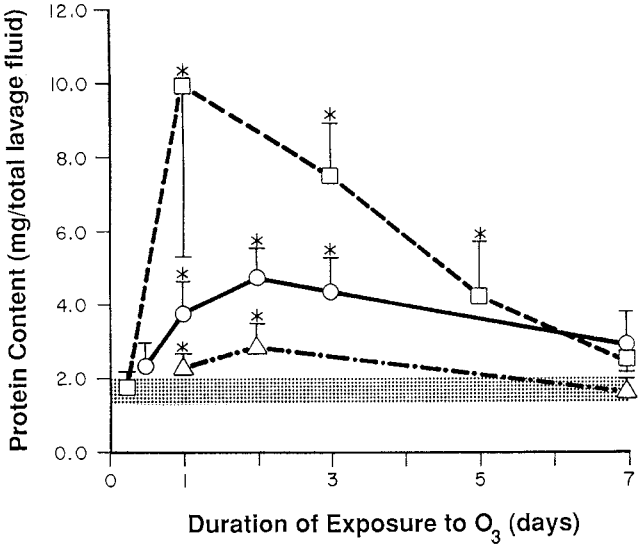

Figure 2. Total protein recovered by bronchoalveolar lavage from rats exposed to nominal concentrations of 0.12 (△), 0.20 (○), or 0.64 (□) ppm of O_3 for various durations. Data are presented as means ± 1 SD for each group. Shaded area indicates the mean ± 1 SD for all animals exposed to filtered air (n = 26; lavage fluid collected at various times). Asterisks indicate significant elevations ($p < 0.05$) as compared with values for animals exposed to filtered air. Actual concentrations of O_3 for the groups exposed to 0.12, 0.20, or 0.64 ppm were 0.12 ± 0.01, 0.20 ± 0.01, or 0.63 ± 0.03 ppm, respectively. n = 4, 4, and 3 for groups exposed to 0.12, 0.20, and 0.64 ppm of O_3, respectively.

posing rats (for six hours to seven days) to 0.64 ppm O_3 resulted in a maximal increase in total protein content of lavage fluid after one day, and then a return to control values by seven days of O_3 exposure (Figure 2). We therefore examined the responses of rats to a range of O_3 concentrations after one day of exposure, as shown in Figure 3. Total lavageable protein was significantly increased in animals exposed to concentrations of 0.12 ppm O_3 and higher (Figure 3a). Labeled albumin content of lavage fluid after intravenous injection was significantly elevated above values observed with control rats breathing filtered air after one day of exposure to concentrations of O_3 of 0.4 ppm and higher (Figure 3a). Acid phosphatase and *N*-acetyl-β-D-glucosaminidase activities were significantly elevated in lavage fluid of rats exposed to concentrations of 0.40 ppm or higher (Figure 3b). Lavageable lactate dehydrogenase activity was increased only in groups of rats exposed to 0.64 or 0.96 ppm of O_3 (Figure 3b).

We exposed other groups of rats to air (control), or 0.12 ppm, or 0.20 ppm of O_3 for two days and measured lavage fluid for protein content, labeled tracer content, and enzyme activities. Table 1 shows that increases in lavage fluid protein content were significant in rats exposed to 0.12 or 0.20 ppm of O_3, and changes in labeled tracer content were significant only in the rats exposed to 0.20 ppm of O_3. No significant effects on lavageable enzyme activities were detected at these low concentrations of O_3.

Protein content of lung lavage fluid was quantified after three days of exposure of rats to NO_2. Values observed were 225, 175, and 106 percent of control for 10, 5, and 2 ppm, respectively; the first two values (at 10 and 5 ppm) were significantly greater than the control values. Activities of the following enzymes were quantified in the lavage fluid from the rats exposed to 5 ppm of NO_2: lactate dehydrogenase, acid phosphatase, and *N*-acetyl-β-D-glucosaminidase, none of which differed significantly from control values.

Assays of Lung Tissue

The responses of rats exposed to concentrations of 0 to 0.96 ppm of O_3, in terms of lung protein content and apparent collagen synthesis rate by minced lung tissue, were determined prior to the outset of this study (Last et al. 1983, 1984). Briefly, a linear relationship was evident between exposure concentrations between 0.4 and 0.96 ppm of O_3 and

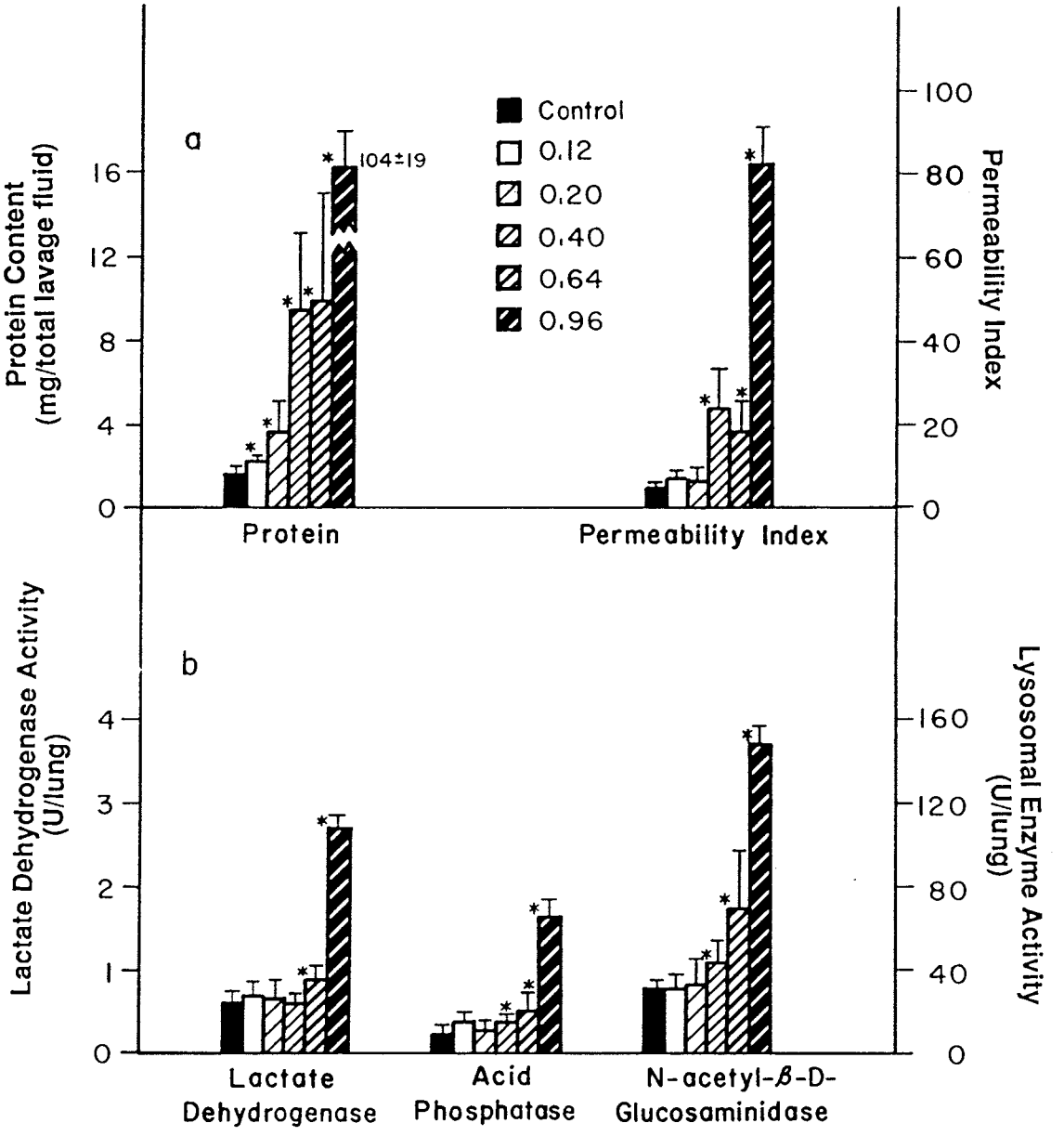

Figure 3. Concentration dependence of changes in tracer movement from blood to lavage fluid (permeability index) and in protein and enzyme content of lung lavage fluid after 24-hour exposures to various concentrations of O_3. A: total protein content (mg/total lavage fluid) and tracer movement ([total dpm recovered in lavage fluid/dpm in 1.0 mL of serum] × 10^3). B: lavage fluid content of lactate dehydrogenase and of the lysosomal enzymes acid phosphatase and *N*-acetyl-β-D-glucosaminidase (total units recovered in lavage fluid). Lung lavage was performed at the termination of exposure and cell-free lavage fluid was prepared for protein and enzyme assay as described in the Materials and Methods section. Data are expressed as means ± 1 SD with five animals per group. An asterisk indicates a significant difference ($p < 0.05$) from control. Actual O_3 concentrations were 0.11 ± 0.01, 0.21 ± 0.01, 0.40 ± 0.03, 0.66 ± 0.03, and 0.95 ± 0.03 ppm.

increase in collagen synthesis rate as evaluated after 7, 14, and 21 days of exposure, and in lung protein content after 7 or 9 days of exposure. Results obtained in studies performed under the aegis of this contract are presented below as a component of combined exposures including acid aerosols. Only preliminary data with regard to exposure to NO_2 were published prior to these studies (Last et al. 1983). Therefore, groups of rats were exposed for seven days to 10, 5, or 2 ppm of NO_2. Rates of lung collagen synthesis, as compared with those for control animals exposed to filtered air, were 210, 120, and 99 percent, respectively. The values at 5 and 10 ppm of NO_2 were significantly increased as

Table 1. Measurement of Labeled Tracer, Protein, and Enzymes in Lung Lavage Fluid of Rats Exposed for Two Days to 0.12 or 0.20 ppm Ozone[a]

Parameter Measured	Filtered Air Exposure	O_3 Exposure	
		0.12 ppm	0.20 ppm
Labeled tracer index	5.26 ± 1.13	5.39 ± 0.91	6.61 ± 0.62[b]
Protein (mg/total lavage fluid)	1.63 ± 0.32	2.80 ± 0.68[b]	4.18 ± 0.41[b]
Lactate dehydrogenase	0.44 ± 0.04	0.42 ± 0.08	0.45 ± 0.05
Acid phosphatase	19.6 ± 6.6	21.6 ± 4.8	24.5 ± 6.0
N-acetyl-β-D-glucosaminidase	28.4 ± 7.2	31.6 ± 9.7	30.1 ± 0.7

[a] Control rats were exposed to filtered air. Measurements were made on cell-free lavage fluid obtained at termination of exposure. Values are expressed as mean ± 1 SD for six animals per group. Enzyme activities are presented as total units recovered in lavage. Actual O_3 concentrations were 0.12 ± 0.00 and 0.19 ± 0.01 ppm.

[b] Significant ($p < 0.05$) difference from value for control group receiving filtered air.

compared with the control values. Thus, a reasonable concentration-response relationship was determined by this assay, with an apparent NOEL at 2 ppm of NO_2.

Protein content of lung tissue after seven days of exposure was significantly increased, to 122 percent of the control value, at 10 ppm of NO_2. Values observed at 5 and 2 ppm were, respectively, 98 and 109 percent of control values; neither value was significantly different from the control value. Thus, the apparent NOEL, as defined by this assay, was 5 ppm of NO_2.

EFFECTS OF EXPOSURE TO OZONE PLUS AMMONIUM SULFATE OR SULFURIC ACID AEROSOL ON RAT LUNGS: EXPOSURE CONCENTRATION–RESPONSE RELATIONSHIPS (SPECIFIC AIM NO. 1)

Assays of Bronchoalveolar Lavage Fluid

Exposure of rats for six hours to 0.64 ppm of O_3 caused significant increases in the protein content of lung lavage fluid (see Figure 1). No significant lung damage with respect to control values was observed in rats exposed to 1,000 μg/m^3 of sulfuric acid aerosol. When rats were exposed to 0.64 ppm of O_3 plus 1,000 μg/m^3 of sulfuric acid aerosol for six hours or one day, the increase in lavage fluid protein content was the same as that observed in rats exposed to the same amount of O_3 alone; that is, this assay gave no evidence that sulfuric acid aerosol potentiated the edema caused by this relatively high concentration of O_3 under these experimental conditions.

Exposure of rats to 0.20 ppm of O_3 for one or two days also significantly increased the protein content of their lung lavage fluid (Figure 3a, Figure 4). Exposure to the combination of 0.20 ppm of O_3 and 5 mg/m^3 of ammonium sulfate for two days resulted in a significantly greater increase in the protein content of lavage fluid as compared with results observed in rats exposed to O_3 alone (Figure 4); this assay revealed no response in rats to the ammonium sulfate aerosol alone (that is, results are indistinguishable from control values).

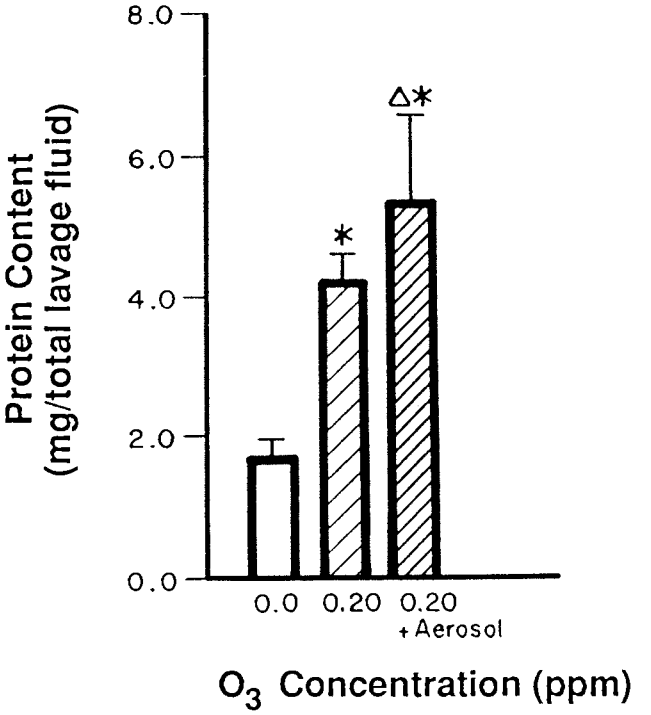

Figure 4. Total protein recovered by bronchoalveolar lavage from groups of rats exposed for two days to 0.20 ppm of O_3, or to 0.20 ppm of O_3 plus 5 mg/m^3 of ammonium sulfate aerosol. Control rats received filtered air. Data are presented as means ± 1 SD for each group. Asterisks indicate significant elevations ($p < 0.05$) as compared with values for control animals; triangle indicates a significant elevation as compared with the group exposed to 0.20 ppm of O_3 alone. The actual concentrations of O_3 for rats exposed to O_3 alone or to O_3 plus aerosol were 0.19 ± 0.01 ppm or 0.18 ± 0.02 ppm, respectively. The actual aerosol concentration was 5.44 ± 0.27 mg/m^3; MMAD = 0.52 μm; σ_g = 1.71. n = 6 for each group.

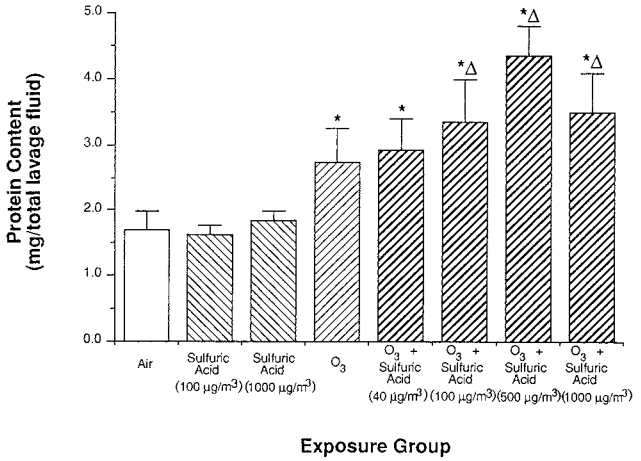

Figure 5. Total lavageable protein from groups of rats exposed for three days to sulfuric acid alone, 0.20 ppm of O_3 alone, or to combinations of 0.20 ppm of O_3 plus the indicated concentrations ($\mu g/m^3$) of sulfuric acid aerosol. Asterisks and triangles indicate significant differences ($p < 0.05$) from values for groups exposed to air alone or to 0.20 ppm of O_3 alone, respectively. Data for each exposure group are given in this form: Treatment group (target concentration) (n = group size): actual O_3 concentration, mean ± SD [number of samples taken]; actual aerosol concentration, mean ± SD [number of samples taken] (aerosol size in MMAD and σ_g [number of samples taken]).
Air-alone group (n = 14).
Sulfuric acid aerosol (100 $\mu g/m^3$)–alone group (n = 5): actual aerosol concentration, 80 ± 10 $\mu g/m^3$ [4] (MMAD = 0.43 µm; σ_g = 1.70 [1]).
Sulfuric acid aerosol (1,000 $\mu g/m^3$)–alone group (n = 5): actual aerosol concentration, 1,290 ± 380 $\mu g/m^3$ [4] (MMAD = 0.36 µm; σ_g = 1.83 [1]).
Ozone-alone group (n = 14): actual O_3 concentration, 0.19 ± 0.01 ppm [432].
Ozone plus sulfuric acid aerosol (40 $\mu g/m^3$) group (n = 5): actual O_3 concentration, 0.20 ± 0.01 ppm [425]; actual aerosol concentration, 42 ± 7 $\mu g/m^3$ [3] (MMAD = 0.42 µm; σ_g = 1.71 [1]).
Ozone plus sulfuric acid aerosol (100 $\mu g/m^3$) group (n = 14): actual O_3 concentration, 0.19 ± 0.01 ppm [423]; actual aerosol concentration, 100 ± 10 $\mu g/m^3$ [3] (MMAD = 0.49 µm; σ_g = 1.94 [1]).
Ozone plus sulfuric acid aerosol (500 $\mu g/m^3$) group (n = 6): actual O_3 concentration, 0.19 ± 0.01 ppm [427]; actual aerosol concentration, 340 ± 190 $\mu g/m^3$ [4] (MMAD = 0.45 µm; σ_g = 1.73 [1]).
Ozone plus sulfuric acid aerosol (1,000 $\mu g/m^3$) group (n = 10): actual O_3 concentration, 0.19 ± 0.02 ppm [434]; actual aerosol concentration, 740 ± 130 $\mu g/m^3$ [3] (MMAD = 0.41 µm; σ_g = 1.73 [2]).

Similarly, exposing rats to 0.20 ppm of O_3 plus 100 to 1,000 $\mu g/m^3$ of sulfuric acid aerosol for three days caused a significantly greater increase in protein content of lavage fluid than was observed in rats exposed to 0.20 ppm of O_3 alone (Figure 5). Exposure to sulfuric acid aerosol alone at 100 or 1,000 $\mu g/m^3$ elicited no response. Thus, at lower exposure concentrations of O_3, a synergistic response between O_3 and sulfuric acid aerosol was observed in this assay. We found an apparent no-observable-synergistic-effect level (NOSEL) (0.20 ppm of O_3) between 40 and 100 $\mu g/m^3$ of sulfuric acid aerosol (Figure 5), a result consistent with the lack of observable synergy, as determined by this assay, at 0.20 ppm of O_3 plus 5 or 20 $\mu g/m^3$ of sulfuric acid aerosol (data not shown).

In an attempt to examine further the exposure concentration–response relationship with respect to O_3 exposure, as determined by this assay, we also exposed rats for three days to 0.12 ppm of O_3 with or without 500 $\mu g/m^3$ of sulfuric acid aerosol. Protein content of lavage fluid was significantly greater in the rats exposed to O_3 alone than in control rats exposed to filtered air in this experiment, but results with sulfuric acid present were indistinguishable from those without sulfuric acid (Table 2). Thus, by the criterion of this assay, an apparent NOSEL was found between 0.12 and 0.20 ppm of O_3 (500 $\mu g/m^3$ of sulfuric acid aerosol), even though O_3 alone elicited significant response at both of these concentrations.

Table 2. Protein Content in Lung Lavage Fluid of Rats Exposed for Three Days to 0.12 ppm of Ozone with or without 500 $\mu g/m^3$ of Sulfuric Acid Aerosol[a]

Exposure Group	Protein Content (mg/total lavage fluid)
Filtered air	1.75 ± 0.23
O_3	2.79 ± 0.59[b]
O_3 + sulfuric acid aerosol	2.53 ± 0.93[b]

[a] Groups of rats were continuously exposed for three days to O_3 or O_3 plus sulfuric acid aerosol as described in the Materials and Methods section. Data are reported as means ± 1 SD for six animals per group. Chamber concentrations of O_3 and sulfuric acid aerosol were as follows (mean ± 1 SD [number of independent measurements]). O_3-alone group: 0.12 ± 0.0 ppm [414]. O_3 plus sulfuric acid group: O_3 concentration, 0.12 ± 0.0 ppm [320]; aerosol concentration, 610 ± 70 $\mu g/m^3$ [6] (MMAD = 0.42 µm; σ_g = 1.83).

[b] Significant ($p < 0.05$) difference from value for control group receiving filtered air.

Assays of Lung Tissue

Concentration-response relationships for rats exposed to O_3 with or without sulfuric acid aerosol, as determined by the key assays used, collagen synthesis rate and lung protein content, are presented only for experiments at or below 0.20 ppm of O_3 or 1,000 $\mu g/m^3$ of sulfuric acid aerosol or combinations of various doses of each (see also Appendix B). Data for responses to higher concentrations of O_3 and of sulfate aerosols are published and available (Last et al. 1983, 1984, 1986). Exposing rats for seven days to 0.20 ppm of O_3 caused significant increases in the apparent collagen synthesis rate evaluated with minced lung tissue (Figure 6), and exposure to sulfuric acid aerosol alone (at 1,000 $\mu g/m^3$) revealed no effect. Ozone plus sulfuric acid aerosol at concentrations of 200, 500, and 1,000 $\mu g/m^3$ showed a significantly greater response by this assay than did exposure to O_3 alone (Figure 6). When rats were exposed to 0.12 ppm of O_3 with or without 500 $\mu g/m^3$ of sulfuric acid aerosol, only those rats exposed to the mixture of pollutants showed a significant increase in their lung collagen synthesis rate as compared to control animals exposed to filtered air only

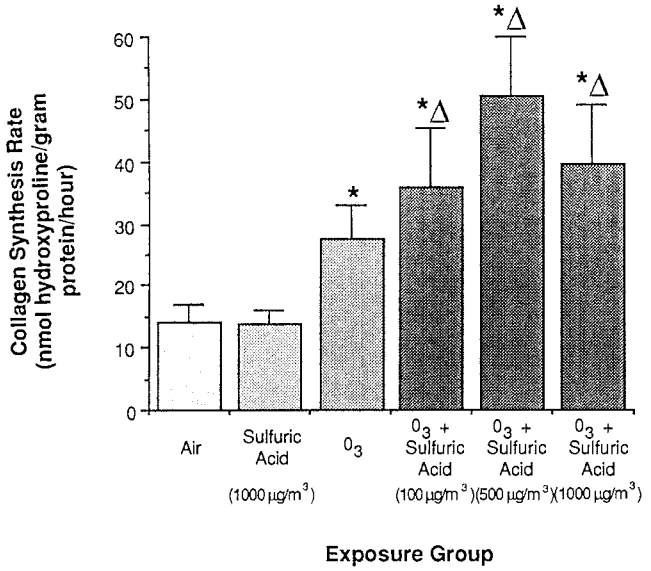

Figure 6. Apparent collagen synthesis rates from minced lung tissue of rats exposed for seven days to 0.20 ppm of O_3 alone or to 0.20 ppm of O_3 plus the indicated concentration ($\mu g/m^3$) of sulfuric acid aerosol. Values are presented as the means ± 1 SD for collagen synthesis rates (nanomoles of hydroxyproline incorporated per gram of protein per hour). Asterisks and triangles indicate significant differences ($p < 0.05$) from values for groups exposed to filtered air alone and to O_3 alone, respectively. Chamber pollutant concentrations were as described for Figure 5. Group sizes (n) were as follows: filtered air (41); sulfuric acid aerosol alone (12); O_3 alone (10); 0.20 ppm of O_3 plus aerosol at 100 $\mu g/m^3$ (9), or 500 $\mu g/m^3$ (4), or 1,000 $\mu g/m^3$ (6).

Table 3. Apparent Lung Collagen Synthesis Rates for Rats Exposed for Seven Days to 0.12 ppm of Ozone with or without Concurrent Exposure to 500 $\mu g/m^3$ of Sulfuric Acid Aerosol[a]

Exposure Group	n	Collagen Synthesis Rate (nmol hydroxyproline/g protein/hour)
Filtered air	8	14.0 ± 2.3
O_3	12	16.1 ± 2.9
O_3 + sulfuric acid aerosol	14	18.5 ± 3.4[b]

[a] Data are expressed as mean values ± 1 SD for normalized data from the indicated numbers of rats. Chamber concentrations for O_3 and sulfuric acid aerosol were as follows (mean ± 1 SD). O_3 alone: 0.12 ± 0.00 ppm. O_3 plus aerosol: O_3 concentration, 0.12 ± 0.00 ppm; aerosol concentration, 500 ± 120 $\mu g/m^3$ (MMAD = 0.45 ± 0.05 μm; σ_g = 2.15 ± 0.57).

[b] Significant ($p < 0.05$) difference (by ANOVA) from value for control rats exposed to filtered air.

(Table 3). Thus, the apparent NOSEL by the criterion of the lung collagen synthesis rate assay is below 0.12 ppm of O_3, and is apparently between 5 and 40 $\mu g/m^3$ of sulfuric acid aerosol (at 0.20 ppm of O_3) (data not shown).

Total lung protein content was evaluated in rats exposed to similar protocols. Synergistic interaction between O_3 and sulfuric acid aerosol was observed after seven days of exposure to 0.20 ppm of O_3 (which significantly increased lung protein content by itself) plus 100 or 500 $\mu g/m^3$ of sulfuric acid aerosol (data not shown; see Warren and Last 1987). Preliminary experiments suggest a NOSEL for O_3 at 0.12 ppm (with or without 500 $\mu g/m^3$ of sulfuric acid aerosol for seven days) by this measurement (data not shown). Analysis of response of rats to exposure for nine days to 0.20 ppm of O_3 with or without 5 or 20 $\mu g/m^3$ of sulfuric acid aerosol showed an apparent NOSEL for the acid aerosol at 5 $\mu g/m^3$, with a significantly enhanced response at 20 $\mu g/m^3$ of acid aerosol as compared to the response observed to O_3 alone (Figure 7).

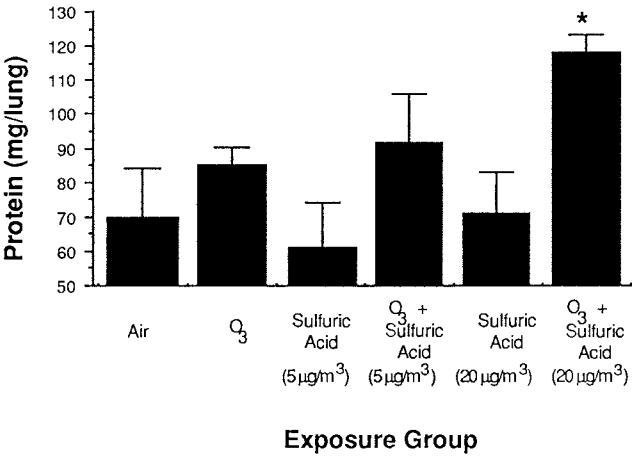

Figure 7. Protein content of lungs of rats exposed for nine days to air, or to 0.20 ppm of O_3 with or without 5 or 20 $\mu g/m^3$ of sulfuric acid aerosol, or to 5 or 20 $\mu g/m^3$ of aerosol alone. Data are presented as means ± 1 SD, with six animals in each experimental group. Asterisk indicates significant difference from control value.

RESPONSE TO NITROGEN DIOXIDE WITH OR WITHOUT SULFURIC ACID AEROSOL (SPECIFIC AIM NO. 1)

Limited experiments were performed, on the basis of observed results with O_3–sulfuric acid aerosol mixtures, to ascertain whether a similar synergistic interaction occurred when NO_2 was substituted for O_3. Rats were exposed for one or seven days to 5 ppm of NO_2 with or without 1,000 $\mu g/m^3$ of sulfuric acid aerosol. When rats were exposed to NO_2 plus acid aerosol for one day, protein content of lavage fluid was significantly increased to 215 percent of control values, a significantly higher value than that observed in rats exposed to NO_2 or sulfuric acid alone. Lung collagen synthesis rates, measured after seven days of exposure, were significantly increased in rats exposed to 5 ppm of NO_2 alone (120 percent of control values) but were not affected by acid aerosol alone (98 percent of control values); re-

sponse to 5 ppm of NO_2 plus 1,000 μg of sulfuric acid aerosol was 145 percent of control values, significantly greater than the response to NO_2 alone. Rats exposed to 2 ppm of NO_2 with or without 500 $\mu g/m^3$ of sulfuric acid aerosol for seven days showed the following results in the assay for lung collagen synthesis rate as compared to filtered air controls: NO_2 alone, 99 percent; sulfuric acid alone, 98 percent; NO_2 plus acid aerosol, 129 percent (a significant increase). We concluded that the NOSEL for NO_2 at 500 $\mu g/m^3$ of sulfuric acid aerosol by this assay is below 2 ppm.

MORPHOLOGICAL EFFECTS OF EXPOSURE TO OZONE OR NITROGEN DIOXIDE ON RAT LUNGS: EXPOSURE CONCENTRATION–RESPONSE RELATIONSHIPS (SPECIFIC AIM NO. 1)

Lungs from rats exposed to selected regimens were also analyzed by quantitative morphometry. Results are presented here only for exposures at or below 0.20 ppm of O_3 with or without sulfuric acid aerosol. Morphometric data from rats exposed to higher concentrations of O_3 with or without sulfuric acid aerosol have been published elsewhere (Last et al. 1983, 1984). When rats were exposed for seven days to 0.20 ppm of O_3 with or without 1,000 $\mu g/m^3$ of sulfuric acid aerosol, there was no observable difference in the total lung volume in either group when compared with control rats exposed only to filtered air. An apparent synergistic interaction was observed by quantifying the $V_{V\ lesion}$ (accumulation of inflammatory cells at the alveolar epithelial layer of the centriacinar region), which was $0.2 \pm 0.8 \times 10^3$ for filtered air, $1.8 \pm 0.7 \times 10^3$ for acid alone, $4.1 \pm 1.2 \times 10^3$ for O_3 alone, and $31.8 \pm 1.1 \times 10^3$ for O_3 plus acid aerosol; the effects of O_3 were significantly greater than those of air alone, and the effects of the combination were significantly greater than those of O_3 alone.

Rats were also exposed for seven days to 0.12 ppm of O_3 with or without 500 $\mu g/m^3$ of sulfuric acid aerosol. Several parameters evaluated, including $V_{V\ lesion}$, showed a trend toward higher values in the group exposed to O_3 plus acid aerosol than were observed in the group receiving O_3 alone (Table 4). Sulfuric acid aerosol alone gave results indistinguishable from the control values in several similar experiments performed; however, no matched group receiving acid aerosol alone was analyzed in this specific experiment. Thus, an apparent NOSEL by morphometry was observed at or near 0.12 ppm of O_3 (500 to 1,000 $\mu g/m^3$ of sulfuric acid aerosol) in this set of experiments.

Separate groups of rats were exposed for seven days to filtered air, 5 ppm of NO_2, 1,000 $\mu g/m^3$ of sulfuric acid aerosol, or NO_2 plus sulfuric acid or sodium chloride aerosol. For all parameters evaluated (Table 5), the results for acid aerosol alone were indistinguishable from those for control rats receiving filtered air so the data from these groups were combined; NO_2 alone significantly increased the observed lung inflammatory lesion. The combination of NO_2 and acid aerosol showed significantly higher values than those observed with exposure to NO_2 alone.

CORRELATION OF BIOCHEMICAL AND MORPHOMETRIC ANALYSES

At high exposure concentrations of O_3, all of the assays used in this study gave rise to results that were correlated with O_3 concentration. Thus, our problem here was to ask over the entire range of O_3 concentrations used in this study, without inappropriately weighting the higher concentrations, whether or not we could define the most sensitive assays for biochemical and morphological changes in the lung, and if so, which ones they were. If we could recognize such assays, especially across these disciplines, then

Table 4. Morphometric Analysis of Rat Lungs Exposed for Seven Days to 0.12 ppm of Ozone with or without 500 $\mu g/m^3$ of Sulfuric Acid Aerosol[a]

Exposure Group	Lung Volume (cm^3)	$V_{V\ lesion}$/Lung $\times 10^3$	$V_{V\ lesion}$/Parenchyma $\times 10^3$	V_{lesion} in Lung (mm^3)
Filtered air	13.0 ± 0.7	1.0 ± 0.5	1.2 ± 0.6	13.4 ± 6.0
0.12 ppm O_3	13.7 ± 1.0	1.5 ± 0.5	1.6 ± 0.5	20.1 ± 7.1
0.12 ppm O_3 + 500 $\mu g/m^3$ sulfuric acid aerosol	13.6 ± 0.8	1.9 ± 0.9[b]	2.1 ± 1.0[c]	25.8 ± 12.1[d]

[a] Data are expressed as mean values ± 1 SD (n = 6 for all groups). In this experiment the lesion as observed was primarily accumulation of alveolar macrophages in proximal acinar locations.

[b] Significant ($p < 0.05$) increase over value for control group exposed to filtered air.

[c] Trend toward increase over value for control group exposed to filtered air.

[d] Trend toward increase over value for control group exposed to filtered air ($p < 0.10$).

Table 5. Morphometric Analysis of Rat Lungs Exposed for Seven Days to Nitrogen Dioxide with or without Sulfuric Acid or Sodium Chloride Aerosol[a]

Exposure Group	*n*	V_{lesion} (mm^3)	$V_{V\ lesion}$ ($\times 10^3$)	P_{lesion}[b] ($\times 10^3$)
Air or 1,000 $\mu g/m^3$ sulfuric acid aerosol alone	6 (3 + 3)	20.4 ± 8.8	1.8 ± 0.7	100 ± 43
5 ppm of NO_2	6	155.9 ± 18.2[c]	13.0 ± 1.1[c]	765 ± 89[c]
NO_2 + 1,000 $\mu g/m^3$ sulfuric acid aerosol	6	174.8 ± 22.5[d]	15.2 ± 2.1[d]	858 ± 110[d]
NO_2 + 1 mg/m^3 sodium chloride aerosol	6	179.8 ± 17.4[d]	15.2 ± 1.0[d]	882 ± 85[d]

[a] Data are expressed as means ± 1 SD.

[b] P_{lesion} is points on lesion.

[c] Significantly greater ($p < 0.05$) than air- or sulfuric acid–alone groups by Duncan's multiple range test.

[d] Significantly greater ($p < 0.05$) than 0.5 ppm of NO_2- or air- or sulfuric acid–alone groups by Duncan's multiple range test.

we also wanted to know how closely correlated with one another were the responses, as quantified by these assays, both to O_3 alone and to O_3 plus acid aerosol. We approached this question by regression analysis of each assay against concentration of pollutants to define each of the assays used with regard to sensitivity and exposure concentration–response behavior. The most sensitive assays for biochemistry and morphometry were then compared with each other by linear regression analysis to determine their degree of correlation and the probability that a line of identity between the assays could occur by chance.

Obviously, with more than 10 years of experience performing these assays in this and similar animal models, one has a subjective sense of the most sensitive assays without any structured analysis of the data. For the biochemical analyses of effects of acute exposure regimens three candidates for the most sensitive methods exist: the collagen synthesis rate assay (day 7), whole lung protein content (day 7 and longer), and total lavageable protein (days 1 to 3). We examined each of these assays for their exposure concentration–response behavior by regression analysis, finding the following results for response versus O_3 concentration.

For the collagen synthesis rate assay, O_3 (n = 113 animals) gave a correlation coefficient r of 0.74 ($p < 0.0001$ for difference of slope from control values). This result may be compared with our earlier experiments (Last et al. 1979a) using different animals, different technicians, and higher O_3 concentrations, in which we found r = 0.79 for exposure concentration–response relationship, as quantified by this assay, with O_3 concentration. For O_3 in combination with acid (n = 120 animals), we found r = 0.73 ($p < 0.0001$ for difference of slope [concentration–response relationships] from control values; $p < 0.0001$ for difference of slope from O_3-alone values).

For the total lung protein content assay, comparisons of slopes of concentration-response plots between control and O_3-exposed rats were not significantly different (p = 0.06 for the pooled data from 116 animals).

For total lavageable protein, results (data from 54 animals were analyzed) showed significant differences between control and O_3-exposed animals ($p < 0.0001$), with r = 0.43. Data from 33 rats exposed to O_3 with or without acid aerosol also showed a significant ($p < 0.0001$) correlation with exposure concentration and a significant difference in slope from data for control animals breathing filtered air, but the slopes were not significantly different from those for rats exposed to O_3 alone. However, this lack of significance was due not to problems inherent in this assay, but to specific factors that need to be understood about the measurement of lavageable protein. The assay of lavageable protein is time sensitive, with optimal results observed between one and three days after initiation of exposure to O_3, depending on the O_3 concentration used. We pooled all available data for the correlations, not just data obtained at optimal times of exposure, thereby increasing the "noise" in this assay. The other biochemical assays, collagen synthesis rate and total lung protein, are less time sensitive, and they were routinely performed at seven days and at seven and nine days, respectively, from initiation of exposure, at or close to their optimal times for achieving a good ratio of signal to noise. Had we selected optimal data from the lavageable protein assay (that is, day 3 at 0.12 and 0.2 ppm of O_3, and day 1 at 0.64 ppm), there would have been a much higher correlation with the morphometric data or the

Table 6. Comparison of Biochemical and Morphometric Analyses of Lung Parameters in Rats Exposed to Ozone Plus Acid Aerosol

Morphometric Parameter	Correlation Coefficient (r) for Comparison with Biochemical Parameter	
	Collagen Synthesis Rate	Total Lung Protein
V_{lesion}, O_3	0.58	0.56
$V_{V\ lesion}$, O_3	0.57	0.58
V_{lesion}, O_3 + acid	0.64[a]	0.48
$V_{V\ lesion}$, O_3 + acid	0.66[a]	0.47

[a] Significantly different ($p < 0.05$) slope (by ANOVA) than for O_3 alone.

Table 7. Lung Protein Content of Rats Exposed for 15 or 30 Days to 0.20 ppm of Ozone with or without 1,000 $\mu g/m^3$ of Sulfuric Acid Aerosol[a]

Exposure Group	Lung Protein Content (mg/lung)	
	15 Days	30 Days
Filtered air	151.3 ± 5.0 (100)	173.0 ± 8.7 (100)
O_3	164.7 ± 1.2 (109)	188.0 ± 11.8 (109)
Sulfuric acid aerosol	153.6 ± 1.2 (102)	175.6 ± 11.7 (102)
O_3 + sulfuric acid aerosol	176.2 ± 1.2 (116)	194.7 ± 11.0 (112)

[a] Data are means ± 1 SD (n = 6 rats per group) for total lung protein content of rats for the various exposure regimens. Values in parentheses are percentages of values for control groups exposed to filtered air.

concentration-response plots as a function of O_3 or O_3 plus aerosol concentrations for this assay; however, the values of n in the various subgroups would have been small, therefore affecting our comparisons adversely.

We performed a similar analysis of the most sensitive morphometric measurements, volume of lung lesion (V_{lesion}) and $V_{V\ lesion}$. Ozone concentration–response analyses by linear regression gave the following results.

For V_{lesion}, O_3 (0.12 to 0.64 ppm; n = 28 animals) gave an r value of 0.84 and $p < 0.0001$ for the difference of the slope as compared to control values (filtered air). For O_3 plus sulfuric acid (n = 26 rats), the corresponding values were r = 0.84 and $p < 0.0001$ versus O_3 alone. When results with sulfuric acid and O_3 were combined with data from O_3 at 0.64 ppm plus 5 mg/m^3 of ammonium sulfate (n = 37 animals), we found r = 0.90 and $p < 0.0001$, as compared to values of slopes with O_3 alone.

For $V_{V\ lesion}$, very similar values were observed: r = 0.86 versus O_3 and r = 0.84 versus O_3 plus acid, both significant at $p < 0.0001$.

We next turned to a comparison of the biochemical and morphometric estimates, which, although performed on different animals, could be grouped by matched sets undergoing identical concurrent exposures in the same chambers. Choice of animals for biochemical or morphological workup was completely at random at the end of the exposure by the exposure facility technicians. The results of comparing the various possible combinations and permutations of assays are presented in Table 6. Given the strong existing correlation between all of the parameters chosen and concentration of O_3 to which animals were exposed, it is not surprising to find that there was a correlation between the biochemical and morphometric analyses. However, some useful generalizations do emerge from this analysis. First, whether comparisons were made with raw data or by transformation of all values to percentage of control, the regression analyses gave identical results. Second, collagen synthesis rate seemed to be much better correlated with morphometry than total lavageable protein or total lung protein content when O_3 plus acid aerosol exposures were examined.

SUBCHRONIC RESPONSE OF RAT LUNGS TO OZONE–SULFURIC ACID AEROSOL EXPOSURE

A single preliminary experiment was performed to examine the potential relevance of O_3–sulfuric acid interaction beyond the acute time frame (one to nine days) studied in these experiments. We examined the protein content of lungs from rats exposed for 15 and 30 days to 0.2 ppm of O_3 plus 1,000 $\mu g/m^3$ of sulfuric acid aerosol, to O_3 or sulfuric acid aerosol alone, or to filtered air. The results of this experiment are shown in Table 7. There was a small but significant increase in the protein content of the lungs of rats exposed to O_3 after 15 and 30 days of exposure. The relative response was similar at both time points both for O_3 alone and for O_3 plus sulfuric acid aerosol. The response to O_3 plus sulfuric acid aerosol was significantly greater than that to O_3 alone at 15 days; the difference between the animals exposed to O_3 plus sulfuric acid aerosol and O_3 alone was not significant at the 30-day time point. We concluded that total lung protein content may be a suitable measurement of lung alteration (damage or repair or both) during the subchronic time frame to use in the pursuit of answers to some of the remaining questions about O_3–sulfuric acid aerosol interaction in future studies. Much more work remains to be done, however, before any conclusions can be drawn regarding the occurrence of such an interaction in the subchronic time frame.

Table 8. Measurements of pH in Sulfuric Acid Aerosol Samples[a]

Nominal Sulfate Concentration ($\mu g/m^3$)	n	Actual Sulfate Concentration ($\mu g/m^3$)	pH
0 (filtered air)	5	–	4.80 ± 0.14
5	12	4.5 ± 0.8	4.44 ± 0.08
20	12	19.4 ± 3.0	4.35 ± 0.08
40	31	47 ± 10	4.27 ± 0.46
100	26	100 ± 30	3.73 ± 0.35
500	18	600 ± 80	3.15 ± 0.07
1,000	26	970 ± 220	2.93 ± 0.08

[a] The sulfate mass concentration and pH were measured from samples of sulfuric acid aerosol that were collected as described in the Materials and Methods section. Samples were collected from chambers containing groups of rats exposed to O_3 plus the indicated concentrations of sulfuric acid aerosol. Data are expressed as mean concentrations ± 1 SD of sulfate ion or as the pH of aerosol samples.

IMPORTANCE OF AEROSOL ACIDITY (SPECIFIC AIM NO. 2)

Studies of the effects of exposure of rats to various aerosols, alone and in combination with a relatively high concentration (0.64 ppm) of O_3, had shown synergistic interaction between O_3 and 5 mg/m^3 of ammonium sulfate (pH of stock solution = 5.1), with no interaction observed between O_3 and sodium chloride or sodium sulfate aerosols (pH of stock solutions = 7.0) (Last et al. 1986). These results suggested that the acidity of an aerosol might be the factor that determines whether or not the aerosol exhibited a synergistic interaction with O_3, and prompted a series of experiments to evaluate more precisely the role of aerosol acidity in predicting the response of rats exposed to mixtures of oxidant gases and aerosols.

We have examined the pH and sulfate concentration of aqueous extracts from filters used to trap sulfuric acid aerosols when sampling chamber atmospheres during the course of our experiments, including several of the exposure concentration–response experiments discussed above. The results of these measurements are shown in Table 8. The apparent pH of filters used to sample the filtered-air-only exposure chamber contents as measured using these procedures was 4.8. This pH value is similar to that for aqueous washes of unused filters (mean ± 1 SD was 4.62 ± 0.82; n = 5). As the chamber concentration of sulfuric acid was increased, the pH of the eluate from the filter extract was decreased. When the mean values for analytically determined sulfate mass concentration were plotted against the mean pH values for samples from atmospheres containing 100 to 1,000 $\mu g/m^3$ of sulfuric acid aerosol, a linear relationship was apparent (slope = −0.93; r = 0.98). Interestingly, the pH of the samples from chambers containing 40 $\mu g/m^3$ of sulfuric acid aerosol or less was slightly less acidic than the pH value calculated from linear extrapolation of the data from the higher concentrations of sulfuric acid aerosol (for example, expected and observed values for 40 $\mu g/m^3$ aerosol were 3.74 and 4.27, respectively).

These observations suggest two important conclusions. First, only moderately to strongly acidic aerosols result in a synergistic interaction of lung damage with O_3. As determined by the methods for measuring the aerosol pH used here, only those aerosols possessing a pH of less than approximately 4.4 have been shown to interact synergistically with O_3. Second, other workers have suggested that the pH of chamber atmospheres used for animal exposures might be altered by the presence of ammonia produced by animal metabolism or by breakdown of excreta by bacteria (Barrow and Dodd 1979; Barrow and Steinhagen 1980; Larson et al. 1982). The correlation coefficient of r = 0.98 between acid aerosol concentrations between 100 and 1,000 $\mu g/m^3$ and pH of filter eluates in our experiments suggests that any such neutralization of acid by ammonia in this range of sulfuric acid aerosol concentrations must have been negligible. However, the apparent loss of acidity from sulfuric acid aerosol at 40 $\mu g/m^3$ might have been due to partial neutralization by ammonia, although other explanations are also possible. Addition of a bactericidal detergent to the chambers (see the Materials and Methods section) had no effect on the pH of filter eluates at 40 or 100 $\mu g/m^3$ of acid aerosol.

Effect of Aerosol Size on Ozone–Acid Aerosol Interaction

Rats were exposed to 0.64 ppm of O_3 with or without 100 $\mu g/m^3$ of sulfuric acid aerosol. The aerosols were generated from either a Babington nebulizer (nominal 0.5 μm MMAD) or from a nuclei-mode generator (nominal 0.03 μm count median diameter [CMD] at the chamber sampling port) in these experiments, and the resultant lung response was quantified by various biochemical and morphological indices. As shown in Table 9, the response to the 0.5 μm aerosol was more pronounced by all the measurement parameters evaluated than was the response to the 0.03 μm aerosol, consistent with the known site of action of O_3 in the centriacinar region of the lungs and the known pattern of lung deposition for particles of these sizes.

Nitrogen Dioxide–Ozone Interaction

We have subjected rats to a single exposure to 0.2 ppm of O_3 with or without 5 ppm of NO_2 delivered in a mixture. Since NO_2 is the mixed anhydride of nitrous and nitric acids (HNO_2 and HNO_3), it is itself an acidogenic gas, and

Table 9. Effect of Sulfuric Acid Aerosol Size on Ozone-Aerosol Interaction: Changes in Lung Parameters After Rats Were Exposed for Three, Five, or Seven Days to 0.64 ppm of Ozone with or without 1,000 μg/m^3 of Aerosol[a]

Exposure Group	Protein Content (μg/lung)			Collagen Synthesis Rate (nmol hydroxyproline/g protein/hour)
	Three Days	Five Days	Seven Days	
Filtered air	–	23.2 ± 3.3 (15)[b]	–	24.6 ± 4.2 (4)
O_3[c]	24.3 ± 3.0 (4)	26.8 ± 2.8 (3)	31.0 ± 2.9 (8)	39.8 ± 3.6 (4)[d]
O_3 + 0.03 μm sulfuric acid[d]	26.0 ± 2.2 (4)	28.7 ± 1.9 (3)	32.0 ± 3.0 (8)	33.9 ± 5.1 (4)
O_3 + 0.5 μm sulfuric acid[d]	27.4 ± 4.3 (4)	ND[e]	33.3 ± 1.9 (8)	45.2 ± 4.7 (4)[d]

[a] Data are means ± 1 SD for protein content of washed right apical plus right middle lung lobes from rats exposed for 23.5 hours/day for the indicated number of days. The number of rats for each group is given in parentheses. Actual concentrations of inhaled agents (means ± 1 SD) were as follows. O_3 alone: 0.61 ± 0.02 ppm. O_3 plus sulfuric acid (nuclei mode): 0.64 ± 0.01 ppm; 980 ± 130 μg/m^3; 0.031 ± 0.006 μm CMD; σ_g = 1.70 ± 0.09. O_3 plus sulfuric acid (nebulizer): 0.62 ± 0.03 ppm; 940 ± 50 μg/m^3; 0.49 ± 0.01 μm MMAD; σ_g = 1.82 ± 0.06.

[b] Control values did not differ at days 3, 5, and 7, so the pooled mean ± 1 SD is reported. Lungs were not lavaged prior to analysis.

[c] Significantly different ($p < 0.05$) (by Wilcoxin paired-sample t test) from protein content value for control rats receiving filtered air.

[d] Significantly different ($p < 0.05$) (by ANOVA) from value for control rats breathing filtered air.

[e] ND = not determined.

we predicted that it would potentiate an oxidant gas such as O_3 during coexposure, were that to truly be the rule governing synergistic interactions in our experiments. Groups of six rats each were exposed to filtered air, to O_3 plus NO_2, or to NO_2 or O_3 alone. After nine days, the rats were killed and the total protein content of their lungs was quantified. Both the O_3-alone and the NO_2-alone exposure groups tended to have higher protein content than the air-breathing control rats (Table 10), but the small differences observed were not significant. The rats exposed to NO_2 plus O_3 had values that were significantly higher than values for the air-breathing control rats and that tended to be higher than the results from either of the groups exposed to the individual gases. These results suggest that there is indeed an interaction between O_3 and NO_2, but do not allow a determination of whether or not the observed effects are additive or synergistic.

Nitrogen Dioxide–Sodium Chloride Interaction

No interaction was observed between responses of lungs of rats exposed to O_3 (0.96 ppm) and to respirable aerosols of 5 mg/m^3 of sodium chloride under conditions identical to those that we demonstrated had elicited a synergistic interaction in our experiments with O_3 and ammonium sulfate aerosols (Last et al. 1986). Rats exposed to 5 ppm of NO_2 for seven days showed a significant increase in lung collagen synthesis rate (120 percent of control values). Groups of rats exposed to 5 ppm of NO_2 and 1,000 μg/m^3 of sulfuric acid aerosol had lung collagen synthesis rates of 145 percent of the control values (for rats exposed to filtered air), values significantly greater than those observed with the rats exposed to NO_2 alone, as reported above (exposure concentration–response experiments). Of interest here, however, is the response of rats exposed for seven days to 5 ppm of NO_2 plus 1 mg/m^3 of sodium chloride aerosol (Figure 8). These animals showed a lung collagen synthesis rate 165 percent of the values observed with control rats exposed to filtered air. In control experiments we found values of 98 and 95 percent of control values for rats exposed to sulfuric acid aerosol alone and sodium chloride aerosol alone, respectively, by this assay.

We also quantified the protein content of the lung lavage fluid from rats exposed for three days to 5 ppm of NO_2, with and without 1,000 μg/m^3 of sulfuric acid or 1 mg/m^3 of sodium chloride aerosol. As compared with control rats exposed to filtered air, we found values of 175 percent of control values in rats exposed to NO_2 alone, 180 percent of control values in rats exposed to NO_2 plus sulfuric acid

Table 10. Lung Protein Content of Rats Exposed for Nine Days to 0.20 ppm of Ozone with or without 5 ppm of Nitrogen Dioxide[a]

Exposure Group	Protein Content (mg/lung)
Filtered air	100.0 ± 6.1
O_3	106.6 ± 3.6
NO_2	106.4 ± 10.7
O_3 + NO_2	110.4 ± 6.9[b]

[a] Results are means ± 1 SD for groups of six animals.

[b] Significant difference ($p < 0.05$) (by ANOVA) from value for control rats receiving filtered air.

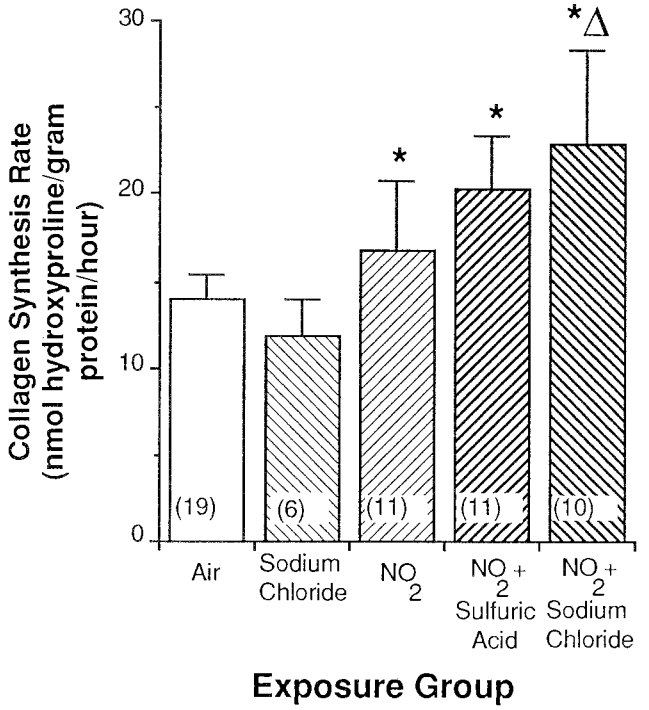

Figure 8. Apparent rates of lung collagen synthesis (nanomoles of hydroxyproline incorporated per gram of protein per hour) from groups of rats exposed for seven days to 1 mg/m^3 of sodium chloride aerosol, 5 ppm of NO_2, or 5 ppm of NO_2 plus either 1,000 μg/m^3 of sulfuric acid or 1 mg/m^3 of sodium chloride aerosol. Control rats were exposed to filtered air. Data are presented as means ± 1 SD for the number of rats per group (indicated in parentheses). Asterisks indicate a significant elevation ($p < 0.05$) from control values. The triangle indicates a significant elevation ($p < 0.05$) as compared with the group exposed to NO_2 alone. Actual concentrations of NO_2 or aerosols were as follows (means ± 1 SD). Sodium chloride group: 0.94 ± 0.04 mg/m^3 (MMAD = 0.38 μm; σ_g = 2.11). NO_2 group: 5.33 ± 0.66 ppm. NO_2 plus sulfuric acid group: NO_2 concentration, 4.90 ± 0.04 ppm; aerosol concentration, 890 ± 90 μg/m^3 (MMAD = 0.40 μm; σ_g = 2.16). NO_2 plus sodium chloride group: NO_2 concentration, 5.29 ± 0.74 ppm; aerosol concentration, 1.11 ± 0.19 mg/m^3 (MMAD = 0.47 μm; σ_g = 2.52).

aerosol, and 210 percent of control values in rats exposed to NO_2 plus sodium chloride aerosol. The increase in values for the group exposed to NO_2 plus sodium chloride was significant when compared to values observed in rats exposed to NO_2 alone (Last and Warren 1987).

We interpret these results as suggesting that a reaction product of NO_2 and sodium chloride is responsible for the interaction observed in these experiments, because no interaction was observed between sodium chloride and O_3, which do not react chemically. We hypothesize that the interaction between NO_2 and sodium chloride is due to their reaction to form nitrosyl chloride (NOCl), the mixed anhydride of hydrochloric, nitrous, and nitric acids, which could give rise to strong acids in the centriacinar region of the lung upon hydrolysis (Schroeder and Urone 1974; Finlayson-Pitts 1983; Last and Warren 1987). The possibility of additional acid aerosols (or acidogenic aerosols) arising in the atmosphere from sources other than sulfur dioxide (SO_2) or direct dissolution of NO_2 to give nitric and nitrous acids opens some interesting vistas toxicologically.

EFFECTS OF FREE-RADICAL SCAVENGERS ON OZONE-INDUCED LUNG DAMAGE (SPECIFIC AIM NO. 3)

Tests of Vitamin E or β-Carotene as Protective Agents

Rats were exposed for one or seven days to 0.64 ppm of O_3. The protein content of lung lavage fluid, the activities of enzymes from lavage fluid, and the whole lung protein content of control rats injected with diluent (2 percent weight/volume Triton X-100 detergent) alone were indistinguishable from prior data from untreated rats. These values also were similar to those from rats treated with vitamin E or β-carotene that were exposed to filtered air. Diluent-injected rats that were exposed to O_3 gave results for each of the aforementioned assays that were similar to prior data from rats exposed to 0.64 ppm of O_3. Furthermore, values from O_3-exposed rats treated with either vitamin E or β-carotene were indistinguishable from those of diluent-treated rats that were exposed to O_3 (data not shown). Thus, neither treatment with vitamin E nor with β-carotene had an observable effect on pulmonary biochemical responses of rats exposed to air or to 0.64 ppm of O_3 by the criteria used in these experiments. Morphometric estimation of cells (neutrophils, macrophages, monocytes, and lymphocytes) recovered in the bronchoalveolar lavage fluid after seven days of exposure of rats to O_3 similarly lacked any appearance of a protective effect of vitamin E. Rats treated with β-carotene showed a statistically significant decrease in neutrophils and monocytes recovered in their lavage fluid in these experiments, but no decrease in alveolar macrophages, the predominant cell type in the lavage. The biologic significance, if any, of this observation is not readily apparent.

We also exposed rats for three or seven days to 0.64 ppm of O_3 with or without 1 mg/m^3 of ammonium sulfate aerosol, with and without concurrent treatment with vitamin E as per the above protocol, and evaluated their lungs morphometrically. The results of these experiments are shown in Table 11. There was no effect of ammonium sulfate aerosol at this concentration; results were similar to those observed in rats exposed to O_3 alone. Ozone alone or with ammonium sulfate aerosol elicited lung lesions absent from control animals (exposed to filtered air), and from rats exposed to aerosol alone. Values in all assays tended to be lower in animals given vitamin E, the results being more pronounced at three days than at seven days after initiation

Table 11. Morphometric Analysis of Lungs of Rats Exposed for Three or Seven Days to 0.64 ppm of Ozone with or without 1 mg/m^3 of Ammonium Sulfate Aerosol and with or without Vitamin E[a]

	Three Days		Seven Days		
Exposure Group	V_V *lesion* ($\times 10^3$)	V *lesion* (mm^3)	V_V *lesion* ($\times 10^3$)	V *lesion* (mm^3)	No. of Macrophages per mm^3 of Lesion
Filtered air or aerosol alone	0	0	0	0	0
O_3	13.4 ± 5.6	165 ± 78	19.7 ± 3.2	271 ± 71	2,536 ± 771
O_3 + vitamin E	8.8 ± 2.1	97 ± 22	17.0 ± 2.6	233 ± 20	2,320 ± 213
O_3 + ammonium sulfate	11.1 ± 3.2	124 ± 28	14.6 ± 3.3	224 ± 66	2,353 ± 256
O_3 + ammonium sulfate + vitamin E	8.4 ± 3.9	95 ± 40	13.8 ± 4.9	190 ± 55	2,064 ± 106

[a] Data are means ± 1 SD, for groups of three to six rats for each experimental condition.

of exposure, but none of the observed decreases in vitamin E–treated animals was significant.

The content of vitamin E or of β-carotene was measured in lung lobes from groups of treated rats that were exposed to air or to 0.64 ppm of O_3 for one or seven days. The lungs were perfused in situ with 0.15 M sodium chloride via the vasculature before analysis to remove as much entrapped blood as possible, so as to reflect actual lung levels. A 1.5- to 2-fold increase in the lung content of vitamin E was observed from treated rats exposed either to air or to O_3, while the β-carotene content was increased approximately 1,000-fold in the lungs of all treated rats.

It is possible that lung damage caused by exposing rats to 0.64 ppm of O_3 was too great to have been altered by treatments with vitamin E or β-carotene. To test this hypothesis groups of rats were treated with Triton X-100 diluent, vitamin E, or β-carotene and were exposed either to 0.20 ppm of O_3 or to 0.20 ppm of O_3 plus 1,000 μg/m^3 of sulfuric acid aerosol for seven days. Control animals were exposed to filtered air. The apparent rate of lung collagen synthesis (Table 12) and the whole lung protein content were measured from these animals. Values from diluent-treated rats that were exposed to O_3 or to O_3 plus sulfuric acid aerosol were not significantly different from values for untreated exposed rats. No significant differences from diluent-treated animals were observed when exposed rats were treated with either vitamin E or β-carotene. The increase in lung protein content observed after exposure of rats to O_3 plus sulfuric acid aerosol was not affected by treatment with either vitamin E or β-carotene (data not shown). Thus, treatment with vitamin E or β-carotene, following the dosage schedules used in these experiments, failed to protect rats from lung damage induced by exposure to 0.20 ppm of O_3 with or without sulfuric acid aerosol.

Table 12. Apparent Lung Collagen Synthesis Rates for Rats Exposed for Seven Days to 0.20 ppm of Ozone, or to Ozone Plus 1,000 μg/m^3 of Sulfuric Acid Aerosol After Treatments with Diluent, Vitamin E, or β-Carotene[a]

	Collagen Synthesis Rate (nmol hydroxyproline/g protein/hour)		
Exposure Group	Diluent	Vitamin E	β-Carotene
Filtered air	14.6 ± 2.3 (6)	11.8 ± 1.3 (3)	14.0 ± 1.6 (3)
O_3	18.1 ± 4.6 (5)[b]	18.4 ± 2.0 (3)[b]	19.4 (2)
O_3 + sulfuric acid aerosol	21.7 ± 2.0 (6)[b,c]	23.0 ± 5.0 (3)[b,c]	24.8 ± 2.0 (3)[b]

[a] Rats were treated with either diluent or 0.1 g/kg of vitamin E acetate or β-carotene in diluent two days before and three days after initiation of exposure. Values represent the means ± 1 SD. Numbers of rats are given in parentheses. Chamber pollutant concentrations (means ± 1 SD) were as follows. Ozone alone: 0.20 ± 0.01 ppm. Ozone plus aerosol: ozone concentration, 0.19 ± 0.01 ppm; aerosol concentration, 1,030 ± 150 μg/m^3 (MMAD = 0.42 μm, σ_g = 1.75).

[b] Significant difference ($p < 0.05$) from value for filtered air–alone group.

[c] Significant difference ($p < 0.05$) from value for O_3-alone group.

Table 13. Measurements of Bronchoalveolar Lavage Fluid from Rats Exposed for One Day to 0.64 ppm of Ozone and Treated with Either Saline or 500 mg/kg of Dimethylthiourea[a]

Exposure Group	*n*	Total Protein Content (mg/total lavage fluid)	Acid Phosphatase (units)	*N*-Acetyl-β-D-glucosaminidase (units)
Air + saline	4	1.30 ± 0.61	12.4 ± 2.7	26.3 ± 9.1
O_3 + saline	5	5.89 ± 0.95	18.1 ± 3.2	74.7 ± 26.3
O_3 + DMTU	5	3.28 ± 0.79[b]	12.1 ± 2.6[b]	39.4 ± 7.3[b]

[a] Rats were treated immediately before exposure. Data represent means ± 1 SD for protein content or enzyme activities from lavage fluid for the indicated numbers of rats. The chamber O_3 concentration (mean ± 1 SD) was 0.63 ± 0.03 ppm.

[b] Significant differences ($p < 0.05$) from the group exposed to O_3 and treated with saline.

Dimethylthiourea as a Protective Agent

As shown in Table 13, we measured the protein content and the activities of acid phosphatase and *N*-acetyl-β-D-glucosaminidase in bronchoalveolar lavage fluid from rats treated with 500 mg/kg of DMTU immediately before exposure to 0.64 ppm of O_3 for one day. The protein content of lavage fluid was significantly elevated in groups of normal rats exposed to 0.64 ppm of O_3. A significant reduction from these elevated values was observed upon exposure of the DMTU-treated rats to 0.64 ppm of O_3. Moreover, similar findings were observed upon measurement of the lavageable activity of acid phosphatase and *N*-acetyl-β-D-glucosaminidase; activities from DMTU-treated rats exposed to O_3 were significantly lower than from saline-injected rats exposed to O_3 (Table 13). Lavageable activity of lactate dehydrogenase was also measured from DMTU-treated rats exposed to 0.64 ppm of O_3; values from treated and untreated rats were similar (data not shown).

Rats were treated once with 50 mg/kg of DMTU immediately before exposure for one day to 0.64 ppm of O_3 or to 0.64 ppm of O_3 plus 1,000 μg/m^3 of sulfuric acid aerosol. Lavageable protein content from DMTU-treated rats exposed to air was indistinguishable from that of lavage fluid from saline-treated control rats, as were values from groups exposed to O_3 plus sulfuric acid aerosol. However, protein content of lavage fluid from O_3-exposed rats treated with DMTU was significantly lower than that in lavage fluid from similarly exposed rats treated with saline (Warren et al. 1988). The lavageable activity of acid phosphatase or *N*-acetyl-β-D-glucosaminidase from DMTU-treated rats exposed to filtered air was similar to control values. Exposure of rats to 0.64 ppm of O_3, with or without accompanying aerosol, increased the lavageable activity of acid phosphatase or *N*-acetyl-β-D-glucosaminidase. However, the activity of these enzymes in lavage fluid of DMTU-treated rats exposed to O_3, with or without sulfuric acid aerosol, was significantly lower than values from similarly exposed rats treated with saline (Warren et al. 1988).

We also treated groups of rats with 50 mg/kg/day of DMTU during exposures for three days to either 0.20 ppm of O_3 or 0.20 ppm of O_3 plus 1,000 μg/m^3 of sulfuric acid aerosol. Lavage fluid from saline-treated rats exposed to O_3 alone or to O_3 plus sulfuric acid aerosol contained significantly more protein than did lavage fluid from control animals. Protein recovered by lavage from groups exposed to air, O_3, or O_3 plus sulfuric acid aerosol was as follows (mean ± 1 SD in mg): 1.50 ± 0.23 (n = 11), 2.97 ± 0.66 (n = 10), and 3.66 ± 0.56 (n = 10), respectively. Protein content of lavage fluid from DMTU-treated rats exposed to air, O_3, or O_3 plus sulfuric acid aerosol was indistinguishable from the content of similarly exposed groups treated with saline: 1.87 ± 0.23 (n = 5), 2.58 ± 0.41 (n = 5), and 3.63 ± 0.59 (n = 5), respectively.

Because the serum half-life of DMTU in rats is reported to be 34 hours (Fox et al. 1983), we expected substantial bodily accumulation of DMTU during a seven-day treatment schedule. Therefore, we used a lower daily dose of DMTU in conjunction with our measurements of the lung content of DNA and protein, of lung morphometry, or of lung collagen synthesis rate. Rats were treated with saline each day of exposure or with 500 mg/kg of DMTU just before the start of exposure and with 250 mg/kg of DMTU daily. Significant increases were observed in lung protein and DNA content of saline-treated rats exposed to 0.64 ppm of O_3 (Table 14). No differences were observed between air-exposed rats treated with saline and those treated with DMTU. However, DMTU-treated rats exposed to O_3 had significantly lower total lung protein and DNA content than O_3-exposed rats treated with saline; that is, DMTU prevented the O_3-induced increase in lung content of DNA and protein in these animals.

The rate of collagen synthesis assessed with minced lung tissue from rats treated with saline was significantly elevated from control values after exposure of the animals to 0.64 ppm of O_3 for seven days (Figure 9). Rats treated with 500 mg/kg of DMTU immediately prior to exposure to

Table 14. DNA or Protein Content of Lungs from Rats Treated with Either Saline or DMTU and Exposed for Seven Days to 0.64 ppm of Ozone[a]

Exposure Group	DNA Content (mg/lung)	Protein Content (mg/lung)
Air + saline	5.53 ± 0.69	106 ± 10
Air + DMTU	6.01 ± 0.69	111 ± 18
O_3 + saline	6.65 ± 0.43[b]	152 ± 9[b]
O_3 + DMTU	5.96 ± 0.47[c]	123 ± 11[c]

[a] Rats were treated with saline or DMTU (500 mg/kg immediately before exposure followed by 250 mg/kg/day thereafter) during exposure. Values represent means ± 1 SD for the total lung DNA or protein content for groups of six rats. The chamber O_3 concentration (mean value ± 1 SD) was 0.62 ± 0.03 ppm.

[b] Significant elevations ($p < 0.05$) from rats exposed to air and treated with saline.

[c] Significant differences ($p < 0.05$) from similarly exposed rats treated with saline.

0.64 ppm of O_3, followed by 250 mg/kg of DMTU each day thereafter, had significantly lower collagen synthesis rates than similarly exposed untreated rats. However, lung collagen synthesis rates in tissue from rats exposed to air and treated with DMTU were significantly lower than the corresponding values from control animals treated with saline.

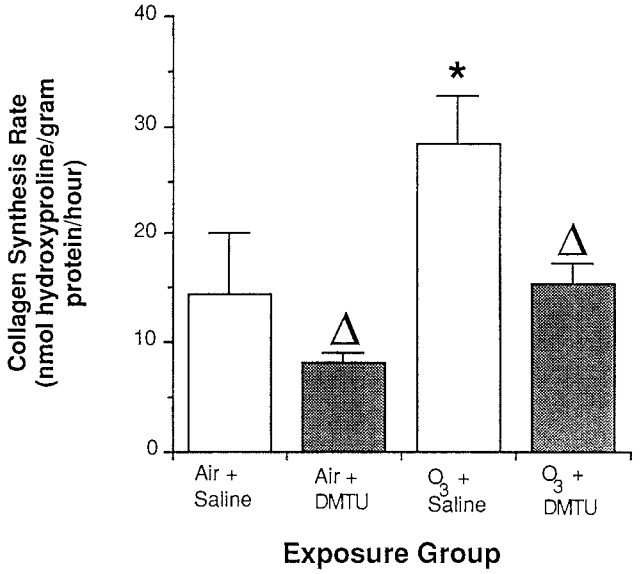

Figure 9. Apparent rates of lung collagen synthesis (nanomoles of hydroxyproline incorporated per gram of protein per hour) from rats exposed to filtered air or to 0.64 ppm of O_3. Groups in each chamber were treated immediately before exposure either with saline or with DMTU (500 mg/kg of body weight immediately before initiation of exposure and 250 mg/kg/day thereafter). Data are presented as means ± 1 SD for three rats per group. The asterisk indicates a significant elevation ($p < 0.05$) from values of saline-treated rats exposed to air; triangles indicate significant differences ($p < 0.05$) from values of similarly exposed rats treated with saline or DMTU. The chamber O_3 concentration (mean ± 1 SD) was 0.62 ± 0.03 ppm.

Thus, treatment with this high dose of DMTU for seven days apparently depressed the rate of collagen synthesis by lungs of rats exposed either to air or to 0.64 ppm of O_3.

The volume of proximal acinar lesions in lungs with rats exposed to 0.64 ppm of O_3, with or without 1,000 μg/m^3 of sulfuric acid aerosol, agreed well with the observed collagen synthesis rates (Figure 10). A trend toward a decreased volume of lesions resulted from O_3 (not significant) or O_3 plus sulfuric acid ($p < 0.05$) in rats treated with DMTU, indicating an amelioration of the inflammatory or fibrotic response. Histopathology confirmed this ameliorating effect of DMTU on the nature and extent of the lesions. Lungs of rats treated with DMTU showed a limited degree of interstitial inflammation (including decreased fibroblast accumulation and extracellular fibers as evaluated by point counting techniques) as compared with untreated rats.

Effect of Variation of Dose of Dimethylthiourea

In another experiment, the effects of different dosage schedules of DMTU on collagen synthesis rates were assessed. Rats were treated with 50, 120, or 250 mg/kg/day of DMTU for seven days. Treatment with 500 mg/kg on day 1 followed by 250 mg/kg/day of DMTU daily significantly reduced the apparent rate of collagen synthesis, as observed previously. A trend toward decreased rates of lung collagen synthesis was observed for rats treated for seven days with either 250 or 120 mg/kg/day of DMTU. Lung collagen synthesis rates from rats treated with 50 mg/kg/day of DMTU were indistinguishable from control rates in this experi-

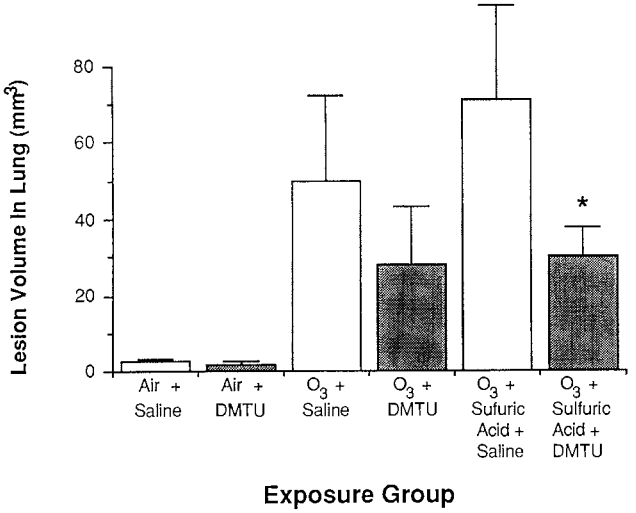

Figure 10. The volume (in mm^3) of the proximal alveolar lesion in the lungs of rats exposed for seven days to filtered air, or to 0.64 ppm of O_3, or to 0.64 ppm of O_3 with or without 1,000 μg/m^3 of sulfuric acid aerosol. Treatment with saline or DMTU was as described in Figure 9. Data presented are means ± 1 SD for two to six rats per group. The asterisk indicates a significant elevation from the value for saline-treated rats exposed to air.

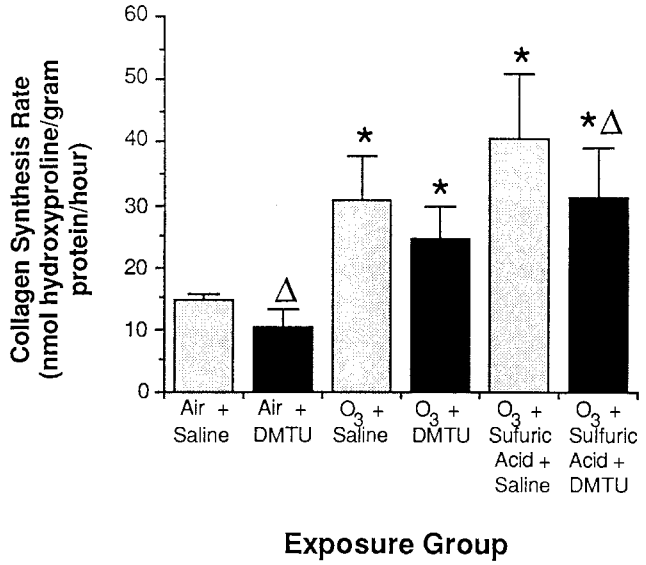

Figure 11. Apparent rates of lung collagen synthesis (nanomoles of hydroxyproline incorporated per gram of protein per hour) from rats exposed for seven days to filtered air, to 0.64 ppm of O_3, or to 0.64 ppm of O_3 plus 1,000 $\mu g/m^3$ of sulfuric acid aerosol. Groups in each chamber were treated immediately before exposure with either saline or 50 mg/kg/day of DMTU daily for 7 days. Data are presented as means ± 1 SD for groups of six rats. Asterisks indicate significant elevations ($p < 0.05$) from values observed with saline-treated rats exposed to air; triangles indicate significant differences ($p < 0.05$) from values for similarly exposed rats treated with saline or DMTU. Chamber pollutant concentrations were as follows (mean ± 1 SD). O_3-alone group: 0.63 ± 0.02 ppm. O_3 plus aerosol group: O_3 concentration, 0.64 ± 0.03 ppm; sulfuric acid aerosol concentration, 840 ± 10 $\mu g/m^3$ (MMAD = 0.52 μm; σ_g = 2.24).

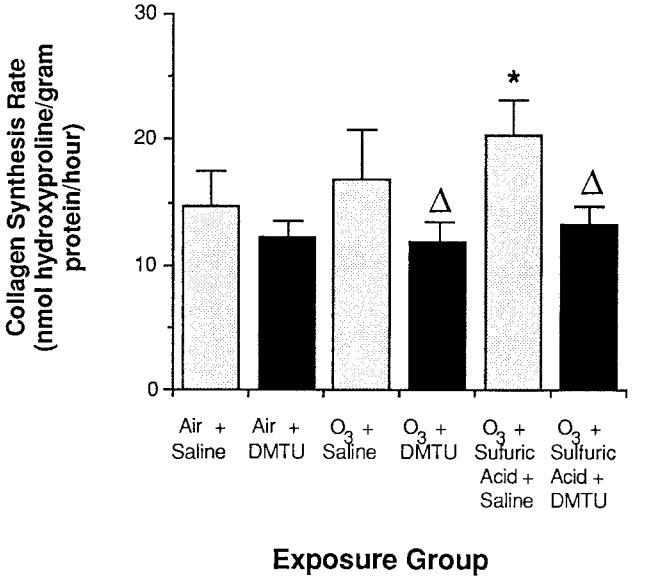

Figure 12. Apparent rates of lung collagen synthesis from rats exposed for seven days to filtered air, 0.20 ppm of O_3, or 0.20 ppm of O_3 with 1,000 $\mu g/m^3$ of sulfuric acid aerosol. Groups in each chamber were treated immediately before exposure with either saline or 50 mg/kg/day of DMTU daily for 7 days. Data are presented as means ± 1 SD. The asterisk indicates a significant elevation from values of saline-treated rats exposed to air; triangles indicate significant decreases ($p < 0.05$) from values of similarly exposed rats that were treated with saline or DMTU. Sizes of saline-treated and DMTU-treated groups exposed to air, O_3, or O_3 plus aerosol were 27 and 2, 8 and 3, or 10 and 4 rats, respectively. Chamber pollutant concentrations (mean ± 1 SD) were as follows. O_3-alone group: 0.20 ± 0.01 ppm. O_3 plus aerosol group: O_3 concentration, 0.19 ± 0.01 ppm; aerosol concentration, 970 ± 150 $\mu g/m^3$ (MMAD = 0.41 μm; σ_g = 1.73).

ment. Therefore, we repeated the previous experiment (seven-day exposure to 0.64 ppm of O_3) using this dose of DMTU; an additional group was exposed to O_3 plus 1,000 $\mu g/m^3$ of sulfuric acid aerosol.

Total lung protein content and the rate of collagen synthesis assessed with minced lung tissue from rats treated with 50 mg/kg/day of DMTU and exposed to 0.64 ppm of O_3 or to 0.64 ppm of O_3 plus 1,000 $\mu g/m^3$ of sulfuric acid aerosol for seven days were determined. Total lung protein content from rats exposed to air and treated with 50 mg/kg/day of DMTU for seven days was indistinguishable from the value for control rats. Treated rats exposed to 0.64 ppm of O_3 had significantly lower lung protein content than exposed rats treated with saline. The total lung protein content of rats treated with DMTU and exposed to O_3 plus sulfuric acid aerosol was indistinguishable from values of similarly exposed rats that were treated with saline.

The rate of collagen synthesis assessed with minced lung tissue from saline-treated rats exposed for seven days to 0.64 ppm of O_3, with or without 1,000 $\mu g/m^3$ of sulfuric acid aerosol, was significantly higher than control values (Figure 11). Treatment of rats with 50 mg/kg/day of DMTU did not significantly decrease the O_3-induced increase in lung collagen synthesis rate, although a trend was clearly apparent. Lung collagen synthesis rates from DMTU-treated rats exposed to O_3 plus aerosol were significantly lower than values from exposed rats treated with saline. No morphological differences were observed between the groups by the criteria of volume of lesion, the nature of the lesion upon histologic examination, or the total number of cells obtained by bronchoalveolar lavage. It should be noted that in this experiment DMTU-treated rats exposed to air had significantly lower rates of lung collagen synthesis than did saline-treated control rats.

The rate of collagen synthesis was also increased in rats exposed to 0.20 ppm of O_3 or to 0.20 ppm of O_3 plus 1,000 $\mu g/m^3$ of sulfuric acid aerosol for seven days (Figure 12). DMTU treatment (50 mg/kg/day) prevented the increase in lung collagen synthesis rate in both exposed groups. Values from DMTU-treated rats exposed to air tended to be lower than values from saline-treated control rats (Figure 12), although the decrease was not significant in this experiment. No differences were observed in the total number of cells obtained by bronchoalveolar lavage from the different groups in this experiment, or in the differential analysis of cell types in the lavage fluid.

Table 15. Summary of Results Examining Ozone–Sulfuric Acid Aerosol Interaction

Ozone Concentration (ppm)	Sulfuric Acid Aerosol ($\mu g/m^3$)	Interaction Determined by Assay[a]		
		Collagen Synthesis Rate	Protein Content of Lung Lavage Fluid	Lung Protein Content
0.64	1,000	+	–	+
	500	+	ND	–
	200	+	ND	–
	0[b]	+	+	+
0.20	1,000	+	+	±
	500	+	+	+
	100	+[c]	+	+
	40	+	–	+[c]
	20	ND	–	+
	5	ND	–	±
	0[b]	±	+	+
0.12	500	±	±	–
	0[b]	–	+	–

[a] Values for the O_3-alone group were compared to the values of the O^3-plus-aerosol group. + = synergistic interaction observed; – = interaction not observed; ± = trend with regard to interaction observed, but not statistically significant; ND = not done.

[b] Values with 0 acid aerosol (that is, O_3 alone) are indicated as + for significant increase, – for no increase, and ± for trend upon exposure to O_3, as compared with values for control rats exposed to filtered air.

[c] May be either additive or synergistic interaction.

DISCUSSION

The most important conclusions of this study are (1) that O_3–acid aerosol interaction occurs in rats at concentrations of each agent that approximate actually encountered ambient levels; (2) that several sensitive assays may be used to quantify the acute response of the lung to oxidants, alone and in combination with respirable acid aerosols; (3) that there is a reasonably good correlation between the most sensitive biochemical and morphometric indicators of the lung responses studied; (4) that acidity of an aerosol is apparently a necessary and sufficient condition for it to interact synergistically with an oxidant gas to cause increased lung damage; and (5) that neither vitamin E nor β-carotene is protective in vivo against lung damage caused by O_3, but an apparent protective effect of DMTU deserves further study.

Table 15 summarizes the results of the exposure concentration–response studies. The matrix of O_3 concentration, sulfuric acid aerosol concentration, and assays performed gives rise to several conclusions. At the highest O_3 concentration examined (0.64 ppm), all of the assays showed significant increases in effects on lungs of rats exposed to O_3 alone. A synergistic interaction between 0.64 ppm of O_3 and all three concentrations of acid aerosol tested was observed by the criterion of increased lung collagen synthesis rate. Only at the highest concentration of acid aerosol tested (1,000 $\mu g/m^3$) was such a synergistic interaction observed by assay of lung protein content. No significant interaction was observed by assay of protein content of lung lavage fluid at 1,000 $\mu g/m^3$ of acid aerosol. We interpret these observations as suggesting that the assays of total lavageable protein and of whole lung protein content cannot discriminate between the lung damage caused by O_3 and that caused by O_3 plus acid aerosol exposures at high concentrations of O_3.

Total lavageable protein and whole lung protein content were significantly elevated above control values after rats were exposed to 0.20 ppm of O_3. Lung collagen synthesis rates were significantly elevated above control values after rats were exposed to 0.20 ppm of O_3 in most of our experiments. A trend toward higher values was observed in the remainder of these experiments. We have previously reported significant increases in lung collagen synthesis rate when rats were exposed to 0.20 ppm of O_3 for seven days (Warren et al. 1986). A significant synergistic interaction was observed between 0.20 ppm of O_3 and all of the acid aerosol concentrations tested by essentially all of the assays performed, consistent with our interpretation that such interaction is most easily evaluated under conditions of minimal lung damage by O_3 alone.

Rats were exposed to 0.12 ppm of O_3, the current peak hourly NAAQS for O_3, with and without 500 $\mu g/m^3$ of sulfuric acid aerosol. When compared to the group exposed to filtered air, total lavageable protein was the only parameter

measured that was significantly elevated for the group exposed to 0.12 ppm of O_3 alone. Collagen synthesis rate was significantly elevated in lungs of rats exposed to 0.12 ppm of O_3 plus 500 $\mu g/m^3$ of sulfuric acid aerosol. Neither 0.12 ppm of O_3 alone nor 500 $\mu g/m^3$ of sulfuric acid alone evoked a significant increase by the criterion of this assay. Thus, concentrations of O_3 as low as 0.12 ppm may elicit responses from lungs of exposed rats and may be potentiated by concurrent exposures to acid aerosols.

It is not clear whether or not the small changes in total lavageable protein and in lung collagen synthesis rate observed in this experiment are necessarily predictive of long-term damage. In lungs of monkeys exposed to higher concentrations of O_3 (0.40 ppm), morphological lesions have been shown to persist during prolonged exposures and for a three-month period after termination of exposure. Measurements in lungs of rats exposed to 0.64 ppm of O_3 for one week showed increases in apparent collagen synthesis rate and in histologically observable collagen consistent with O_3-induced fibrosis. The connection (if any) between the early effects of exposure to 0.12 ppm O_3 shown here and long-term inflammatory and fibrotic changes has not yet been demonstrated. On the basis of results observed after exposing rats to higher levels of O_3 it might be prudent to examine further the possibility that these early changes are indeed predictive of long-term effects of O_3 on the lung at frequently encountered ambient concentrations.

We also conclude from these experiments that assays of enzyme activity in cell-free lavage fluid (Beck et al. 1983; Henderson 1984; Guth and Mavis 1985) are a relatively insensitive method of measuring O_3-induced lung damage. Acid phosphatase and *N*-acetyl-β-D-glucosaminidase enzyme activities failed to show significant changes at concentrations of O_3 below 0.40 ppm (24 hours); lactate dehydrogenase activity was an even less sensitive assay, with significant increases found only after exposure of rats to 0.64 ppm of O_3 for 24 hours. Preliminary experiments suggested that sialic acid content of lavage fluid was also an insensitive (and highly variable) measurement of O_3-induced lung damage, so this assay was not further examined. Increased movement of labeled tracer from blood to a lavageable compartment of the lung was a more sensitive index of lung damage than were any of the enzyme activity assays. The most sensitive indicator of O_3-induced lung response in lavage fluid was an increase in the total protein content (predominantly serum albumin) of the lung lavage fluid, which was significantly increased above control values after one or two days of exposure of rats to 0.12 ppm of O_3. Interestingly, the assay of movement of ^3H-labeled albumin from blood to lung lavage fluid does not show any synergistic interaction between O_3 and acid aerosols under conditions in which O_3 alone provokes a positive response and the assay of total lavageable protein content shows synergistic interaction between the mixture of pollutants. It would be of interest to see if this finding is also true with sequential exposure regimens, but such experiments were beyond the scope of these studies.

To examine further the range of concentrations of O_3 and acid aerosol exhibiting synergistic interactions, rats were exposed to 0.20 ppm of O_3 in conjunction with 40 $\mu g/m^3$ of sulfuric acid aerosol. A synergistic interaction between 0.20 ppm of O_3 and 40 $\mu g/m^3$ of sulfuric acid was demonstrated by assay of tissue protein content after seven or nine days of exposure and by assay of lung collagen synthesis rate. We also found interactions between 0.20 ppm of O_3 and 20 $\mu g/m^3$ of sulfuric acid, with an apparent NOSEL for sulfuric acid aerosol (plus 0.20 ppm of O_3) at or near 5 $\mu g/m^3$. The NOSEL for O_3 (plus 500 $\mu g/m^3$ of acid aerosol) is at or below 0.12 ppm.

Morphometric analyses done on replicate animals at some of the concentrations of O_3 and acid aerosol tested supported these conclusions, and were highly correlated with the observed biochemical lesions (Table 6).

The acidic aerosols most likely to occur in polluted urban air under conditions of photochemical smog generation are ammonium sulfate and bisulfate, although ambient concentrations of these aerosols are far lower than those used in most of these experiments. We have previously shown that aerosols of neutral salts (sodium chloride or sodium sulfate) do not interact synergistically with O_3 (Last et al. 1986). Furthermore, we have suggested that the acidity of an aerosol is the important determinant of whether the aerosol will synergistically interact with O_3, and we have proposed a mechanism that suggests that the primary determinant for the elicitation of synergy between O_3 and acid aerosols is the aerosol acidity (and not the mere presence of the aerosol) (Last et al. 1984). If this mechanism is correct, then weak acids, such as ammonium sulfate, should cause less of a synergistic response than should strong acids, such as sulfuric acid, in accord with our observations in these studies (compare NOSELs of 1 mg/m^3 or above for ammonium sulfate aerosol with about 5 $\mu g/m^3$ for sulfuric acid aerosol).

Treatment with DMTU protected rats from lung damage (as measured by some of our assays) resulting from exposure to oxidant gases, whereas no protection was observed after treatment of rats with either vitamin E or β-carotene under conditions where the total lung concentrations of these antioxidants were increased twofold and 1,000-fold, respectively. These findings suggest the inter-

esting possibility that the protective capability of DMTU might be related to its aqueous solubility. Being freely water soluble, DMTU might have access to compartments of the lung that are relatively unavailable to lipid-soluble compounds such as vitamin E or β-carotene. One such compartment of probable importance for interactions between oxidant gases and the lung is the fluid lining of the pulmonary epithelium. Alternatively, lipid peroxidation may not be of importance in lung injury (Risberg et al. 1987). In preliminary experiments, we have recovered substantial amounts of DMTU from lavage fluid of rats (670 μg in the total recovered lavage fluid from rats treated 24 hours previously with 500 mg/kg of DMTU, or 30 μg in the total recovered lavage fluid from rats treated with 50 mg/kg/day of DMTU for three days). The relationship of DMTU concentrations in lung lavage fluid to the capability of this compound to quench reactions involving reduced oxygen species in the intact lung remains to be proved.

Undoubtedly, many steps occur between oxidant insult to the lung and the manifestation of measurable damage. Currently the chemistry of reactions occurring in the lung at the initial stages of these processes is poorly understood. In vitro experiments have suggested the participation of hydrogen peroxide and superoxide radical in O_3-induced cytotoxicity to lung cells (Morgan and Wenzel 1985). That DMTU is able to protect rats from lung injury in our experiments may implicate the involvement of hydrogen peroxide in these processes. However, other reduced oxygen species, which may directly result from reaction between O_3 and the lung, may also form hydrogen peroxide in subsequent reactions in vivo, especially in the presence of ferrous ions and other catalytic species. A more complete understanding of the mechanism of oxidant insult to the lung is needed before we can interpret our observations of the partially protective effect of DMTU against O_3-induced lung damage. Similarly, a greater understanding of such mechanisms might also allow us to separate the actions of O_3 alone from those of O_3 in combination with sulfuric acid aerosol in the model system we have been studying.

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APPENDIX A. Validation of Methodology Used in This Study: ^{3}H-Albumin as a Tracer for Estimation of Altered Epithelial Permeability

Bovine serum albumin was labeled with ^{3}H by acetylation of ε-amino groups of lysine residues, as described in the Materials and Methods section. Labeled albumin gave a pattern after electrophoresis on 10 percent polyacrylamide gels that was identical to that of an albumin standard by protein staining and fluorography (data not shown). After injection, clearance of labeled albumin was examined over a four-day period. Figure A.1 shows the amount of radioactivity remaining in serum of rats at various times from 15 minutes until 96 hours after injection of albumin. The curve was fitted to a biphasic decay model by linear extrapolation of the slow component (40 to 96 hours) to an intercept on the Y axis. The fast component was then estimated by subtracting the slow component from the observed data at each time. The resultant curve describing the fast component of clearance is shown in the inset of Figure A.1. Half-times for serum clearance were estimated graphically to be 6.5 hours and 43 hours for the fast and slow components, respectively. On the basis of these results, we chose to measure tracer in lavage fluid one hour after injection in order to avoid large changes in serum concentration due to tracer clearance. As judged by the calculated half-times of clearance, less than 8 percent of the injected albumin would have been removed from circulating blood after one hour (Figure A.1).

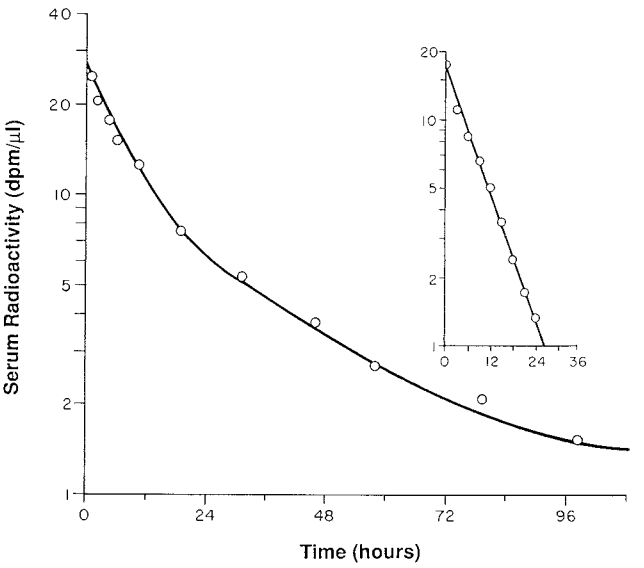

Figure A.1. Clearance of serum radioactivity after injection of ^{3}H-albumin. ^{3}H-albumin was injected via the tail vein at time zero, and serial blood samples were collected as described by Guth and associates (1986). The decay curve was fitted to a two-component model and the first component (inset) was obtained by graphical analysis of the residuals. Half-times for the fast and slow components were estimated as 6.5 hours and 43 hours, respectively.

To confirm that the tracer remained intact after injection, serum and lavage fluid were obtained from control rats and from rats exposed to 1.2 ppm of O_3 for six hours. Radioactivity in the lavage fluid was more than 80 percent precipitable by Cl_3CCOOH, and radioactivity in the serum was more than 95 percent precipitable by acid. Serum and lyophilized lavage fluid from control and O_3-exposed rats were examined by gel electrophoresis followed by fluorography (data not shown). The only bands detected by fluorography coelectrophoresed with authentic serum albumin, indicating that essentially all of the radioactivity recovered one hour after injection remained in the form of acetylated albumin.

Albumin tracer was also administered intratracheally to rats exposed for one day to 0.64 ppm O_3 in order to compare measurement of the movement of tracer from airways to blood with the movement from the blood to lavage fluid (compare with Figure 1). The radioactivity recovered in serum obtained one hour after instillation, expressed as 1,000 × percentage of instilled dose/milliliter of serum, increased from 2.01 ± 0.82 percent in control animals to 5.61 ± 2.06 percent in exposed rats, a smaller relative change than was observed for movement from blood to lavage (compare with Figure 1).

APPENDIX B. Interaction Between High Concentrations of Ozone and Ammonium Sulfate Aerosol

We examined several biochemical and morphometric parameters in lungs of rats exposed to 0.96 ppm of O_3 with or without 5 mg/m^3 of ammonium sulfate aerosol (0.5 to 0.6 μm MMAD). These animals showed significant increases in all of the parameters tested (Last et al. 1986) as compared to control rats breathing filtered air. In addition, groups of rats breathing O_3 plus ammonium sulfate aerosol had significant increases in these same parameters as compared with animals breathing O_3 alone. Ammonium sulfate aerosol alone produced no effects in these animals. Thus, O_3 and ammonium sulfate interacted synergistically. In addition to increases in apparent collagen synthesis rates and in various morphometric parameters, we found that the soluble proline content and the protein content of lungs from exposed animals were significantly increased (Last et al. 1986), with lungs from rats exposed to O_3 plus ammonium sulfate containing significantly more protein than those exposed to O_3 alone. Since the increased protein content in lungs from rats exposed to O_3 with or without ammonium sulfate was not accompanied by increased body weight (to the contrary, body weight was significantly decreased in both exposure groups), we interpreted the increased lung protein content as reflecting levels of pulmonary edema, or inflammatory cell infiltration, or both, with or without accompanying epithelial cell proliferation. There was no difference in blood content of lungs, as measured by the method of Cross and colleagues (1979), between control rats and any group of exposed rats in these studies (data not shown). Elevated apparent collagen synthesis rates and increased fibroblast content and extent of lung lesions in these rats are consistent with this conclusion. Because of the inherent limitations to interpretations of a negative result, we examined as many parameters as we could in these experiments to ensure that we would observe a synergistic interaction if it were actually occurring.

ABOUT THE AUTHOR

Jerold A. Last received his Ph.D. in biochemistry from the Ohio State University, Columbus, OH, in 1965. Postdoctoral work at New York University (on deciphering the genetic code) and the National Institutes of Health (on regulation of hemoglobin synthesis and purification of RNA ligase) continued an interest in protein biosynthesis as a research focus. After further studies of collagen biochemistry with Paul Doty at Harvard University, in 1976 he joined the faculty of the School of Medicine at the University of California, Davis, where he is now Professor of Pulmonary Medicine, Vice Chair of the Department of Medicine, and Director of the University of California Systemwide Toxic Substances Research and Teaching Program. His research interests have focused on lung collagen structure and synthesis in animal models of pulmonary fibrosis and in human tissue, and on the development of microanalytical techniques to study collagen composition and structure.

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ABBREVIATIONS

ANOVA	analysis of variance
Cl_3CCOOH	trichloroacetic acid
CMD	count median diameter
CO_2	carbon dioxide
cpm	counts per minute
$\overline{D}$	mean nuclear caliper diameter
DMTU	dimethylthiourea
dpm	disintegrations per minute
^3H	tritium
HCl	hydrochloric acid
HNO_2	nitrous acid
HNO_3	nitric acid
^{85}Kr	krypton-85
LD_{50}	median lethal dose
MMAD	mass median aerodynamic diameter
NAAQS	National Ambient Air Quality Standard
NaOH	sodium hydroxide
NO	nitrous oxide
NO_2	nitrogen dioxide
NOCl	nitrosyl chloride
NOEL	no-observable-effect level
NOSEL	no-observable-synergistic-effect level
N_V	numerical density
O_3	ozone
P_{lesion}	points on lesion
ppm	parts per million
r	correlation coefficient
σ_g	geometric standard deviation
SO_2	sulfur dioxide
TLV	threshold limit value
V_V	volumetric density
V_{lesion}	volume of lung lesion
$V_{V\ lesion}$	volume density of lung lesion

INTRODUCTION

A Request for Applications (RFA 84–3), which solicited proposals for "Mechanisms of Oxidant Toxicity," was issued by the Health Effects Institute (HEI) in the summer of 1984. In response to the RFA, Jerold A. Last, from the University of California at Davis, submitted a proposal entitled "Synergistic Effects of Air Pollutants: Ozone Plus a Respirable Aerosol." The HEI approved the three-year project, which began in April 1985. Total expenditures were $479,215. The Investigator's Report was received at the HEI in July 1988, and was accepted by the Health Review Committee in July 1989. During the review of the Investigator's Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigator's Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigator's Report in perspective as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1977. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and provide the EPA with limited discretion to modify those requirements. The Clean Air Act is currently undergoing revision, which may or may not affect regulatory policies.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current standards include those for ozone and nitrogen dioxide. The determination of the appropriate standards for emissions of oxidants depends, in part, on an assessment of the risks to health that they present. Because the ambient atmosphere contains pollutants in addition to oxidants, which may or may not influence oxidant-induced effects, understanding the impact of copollutants is important to informed regulatory decision making.

SCIENTIFIC BACKGROUND

OXIDANTS

Oxidants and their precursors are derived from mobile, stationary, and natural sources. Although nitrogen dioxide is produced from the combustion of fossil fuels and decomposition of vegetation, it is primarily derived from secondary atmospheric reactions. The NAAQS for nitrogen dioxide is 0.053 ppm averaged annually. Ozone is not directly emitted from motor vehicles but is formed as a result of complex photochemical reactions among oxides of nitrogen and volatile organic compounds. The NAAQS for ozone is 0.12 ppm averaged over one hour, not to be exceeded more than once a year. For ozone, the summertime peak hourly ambient levels range from 0.07 ppm in rural sections of the country to as high as 0.35 ppm in some urban areas (U.S. Environmental Protection Agency 1986b). Thus, ozone is one of the oxidants of primary concern because of the inability to maintain air quality within the NAAQS for an estimated 130 million persons who reside in nonattainment areas (U.S. Office of Technology Assessment 1988).

Data from animal, clinical, and epidemiological studies indicate that, even at concentrations near ambient levels, exposure to ozone exerts numerous effects on the respiratory tract (reviewed by the U.S. Environmental Protection Agency 1986a, 1988; Lippmann 1989). In animals, functional, biochemical, and structural changes have been documented. In humans, small reversible changes in pulmonary function and cellular alterations have been demonstrated. However, several issues, such as the relationship between acute reversible events and permanent structural alterations, need clarification before the significance of such reported changes can be interpreted.

Acute or short-term studies in laboratory animals have shown alterations in host defense mechanisms that include increased susceptibility to infection, impairment of alveolar macrophage function, and decreased mucociliary clearance. Inflammation is accompanied by increases in airway epithelial permeability. The long-term consequence of these functional impairments is unknown.

In animals exposed acutely or subchronically to ozone, structural and biochemical alterations, which suggest changes related to the development of pulmonary fibrosis, have been shown (reviewed by Last 1988). Cellular lesions in the centriacinar region of the lung include accumulation of macrophages in the proximal alveoli and damage to ciliated cells and type I epithelial cells, with subsequent proliferation of nonciliated bronchiolar (Clara) cells and

type II epithelial cells (Evans et al. 1976; Schwartz et al. 1976; Plopper et al. 1978; Boorman et al. 1980; Castleman et al. 1980). In subchronic studies, morphologic and ultrastructural morphometric analyses reveal significant changes in the centriacinar region of rats (Boorman et al. 1980; Moore and Schwartz 1981; Barry et al. 1985, 1988; Barr et al. 1988) and monkeys (Hyde et al. 1989). An important consequence of these cellular events is the thickening of the epithelium of the alveolar and respiratory bronchioles and an increase in the thickness and volume of the interstitium. Such structural changes at this critical intersection between the conductive and respiratory portions of the lung may have functional implications for gas exchange in this region and for gas delivery to more distal regions. Collagen content and synthesis rates increase, while intracellular collagen degradation decreases in animals exposed to moderate or high levels of ozone (Pickrell et al. 1987; Last 1988). The effects of exposure to low or ambient levels of ozone on collagen metabolism are less clear (Filipowicz and McCauley 1986; Wright et al. 1988).

In clinical studies (reviewed by the U.S. Environmental Protection Agency 1986a, 1988; Lippmann 1989), changes in forced expiratory volumes, forced expiratory rates, airway resistance, and airway compliance have been documented after an acute exposure to ambient levels of ozone. Although there is considerable variability among healthy individuals, these functional alterations are reproducible within an individual. It has not yet been possible to document a greater sensitivity among the elderly, persons with asthma, or persons with chronic obstructive lung disease. In addition to functional impairments, acute ozone exposure can cause cough, shortness of breath, and pain upon deep inspiration. Ozone exposure also increases airway reactivity and airway permeability. An increase in polymorphonuclear leukocytes and other mediators of inflammation can be measured in the bronchoalveolar lavage fluid of subjects eighteen hours after an acute exposure to ozone.

Results from epidemiological and field studies generally support the findings from clinical studies, with two notable exceptions. First, in field studies, decrements in lung function can be measured that are greater than those that occur at comparable ozone concentrations in chamber studies (Spektor et al. 1988). Second, it appears that people with asthma may be unusually sensitive to oxidant air pollution, a finding that cannot be demonstrated in chamber studies, with ozone (reviewed by Bromberg 1988; Bates 1989). Studies by Whittemore and Korn (1980), Bates and Sizto (1983), and Holquin and coworkers (1985) have documented increases in symptoms and in the use of health care facilities by people with asthma that correlated with increases in ambient ozone levels, as well as with other factors.

The effects of chronic exposure to ozone are less clear. Some experiments have evaluated acute effects after repeated exposures to ozone. In clinical studies, the extent of changes in pulmonary function diminish after repeated daily exposures (Hackney et al. 1977; Farrell et al. 1979; Folinsbee et al. 1980). This finding has led to the speculation that transient functional alterations may not represent important health effects. Experiments in animals show similar adaptation to functional responses after repetitive ozone exposures, but also reveal progressive epithelial injury (Tepper et al. 1987). With chronic exposures, numerous studies in rats and monkeys have demonstrated persistent proliferative responses and cellular remodeling in the centriacinar region (reviewed by Lippmann 1989). Several recent epidemiologic studies have shown progressive decrements in pulmonary function that could be interpreted as premature aging of the lung (Kilburn et al. 1985; Knudson et al. 1985; Detels et al. 1987). However, because of methodologic problems and coexposure to other pollutants, these effects cannot be ascribed conclusively and exclusively to ozone (U.S. Environmental Protection Agency 1988).

When trying to assess the health impact of chronic exposure to oxidant air pollution, several questions emerge. First, what is the health significance of the acute reversible effects that can be readily and repeatedly produced; that is, with chronic exposure, do repeated transient reversible responses lead eventually to irreversible chronic effects? Before this question can be adequately answered, additional research on the mechanisms of ozone-induced effects and on the pathogenesis of possibly related chronic diseases is needed.

Second, what are the reasons for the observed greater sensitivity to ozone shown in epidemiological and field studies that are not apparent in controlled clinical studies? Cumulative exposure to ozone, which occurs in ambient settings, is usually not evaluated in chamber studies. There is some evidence that exposure duration is just as important as concentration. Coexposure to other pollutants, which are usually not part of the test atmosphere in clinical studies, is also of primary concern. Copollutants under recent investigation include acid aerosols.

ACID AEROSOLS

Acid aerosols and gases are secondary pollutants that are formed from reactions of sulfur dioxide with nitrogen oxides. The predominant acidic sulfur oxide aerosols are sulfuric acid, ammonium sulfate, and ammonium bisulfate. Acidic nitrogen oxides exist primarily as gases, such as nitric acid and nitrous acid, and little information on their

ambient levels and their effects on the respiratory tract is available.

The data base on ambient levels of acid aerosols is limited because most studies are designed to characterize ambient air and not to assess human exposure (Lioy and Waldman 1989). Data are available on total sulfates and sulfuric acid levels. During the summer months, sulfate levels average around 8 $\mu g/m^3$, with peaks around 25 $\mu g/m^3$; sulfuric acid levels range from 2 to 10 $\mu g/m^3$, with peaks around 40 $\mu g/m^3$ (reviewed by Lioy and Waldman 1989). However, toxicologic data suggest that adverse effects are associated with the acidic, not the sulfate, ion (Amdur et al. 1978; Schlesinger 1985). Because measured sulfates include neutralized salts in addition to sulfuric acid, and measured sulfuric acid does not encompass all acidic compounds, these two measures are not true surrogates for acid aerosol levels. Because of the importance of assessing acid levels and the development of new analytical techniques, our knowledge of human exposure should improve in the future.

Acid sulfur oxides are known irritants of the respiratory tract, and numerous studies, in both animals and humans, have shown effects from these aerosols. At low concentrations, sulfuric acid accelerates tracheobronchial mucous transport and enhances alveolar clearance; at higher concentrations or under conditions of chronic exposure, the aerosol retards airway and alveolar clearance (reviewed by Schlesinger 1985; Wolff 1986). In rabbits, chronic exposure to sulfuric acid causes an increase in the density and acid content of secretory cells in the small airways as well as an increase in the percentage of small airways to total airways (Gearhart and Schlesinger 1989).

In human clinical studies, marked differences in responsiveness among different studies have been reported. Differences in exposure protocols can account for some of the discrepancies in the findings. Particle size, relative humidity, ventilation rates, mode of breathing, and potential for neutralization influence the acidity of the inhaled aerosol and the deposition site in the lung (Folinsbee 1989). In summary, normal individuals do not show functional changes when exposed for short periods to sulfates at concentrations less than 100 $\mu g/m^3$ (reviewed by Utell 1985). At much higher concentrations, changes in some pulmonary function measurements and bronchial reactivity have been reported. In contrast, people with asthma, and especially young people with extrinsic asthma, seem to be far more sensitive to sulfuric acid aerosols than healthy adults (Koenig et al. 1983; Utell et al. 1983b).

As with ozone, it has been difficult to attribute adverse health effects to ambient levels of acid aerosols based on epidemiologic studies (reviewed by Lippmann 1985). Studies by Kitagawa (1984), Schenker and colleagues (1984), Bates and Sizto (1987), and Wichmann and coworkers (1989) have demonstrated a correlation between respiratory disease and sulfates, sulfuric acid, sulfur dioxide, or particulate matter. However, without an actual measurement of acid aerosols, the causative agent could not be determined, only implied. In an effort to determine the contribution of acid aerosols to respiratory dysfunction, detailed acid measurements will be made as part of the ongoing Harvard School of Public Health Six-Cities study and a multicity cooperative study between the United States and Canada (reviewed by Speizer 1989).

It is also clear that additional attention must be given to the combined effects of acid aerosols and other inhaled pollutants. Studies, both animal and human, are limited, and findings are inconsistent. Animals exposed to high levels of ozone and sulfuric acid aerosols have shown increased susceptibility to respiratory infection (Gardner et al. 1977), increased pulmonary collagen synthesis rates, and inflammation of the respiratory tract (Last et al. 1983, 1984). In contrast, acid aerosols did not influence ozone-induced effects in human volunteers exposed to both pollutants either simultaneously (Stacy et al. 1983; Horvath et al. 1987) or sequentially (Kulle et al. 1982). Utell and coworkers (1983a) hypothesize, however, that a latent period may be necessary before the full impact of an acid aerosol exposure is apparent.

ANALYZING FOR SYNERGISM

Because humans are exposed more often to multiple, rather than single, pollutants, attempts to assess the health effects of complex mixtures are increasing. When there is coexposure, the question arises as to whether or not the exposure to one pollutant affects the outcome associated with another pollutant. Different possibilities can be considered. In one case, the first pollutant by itself has no effect, and in combination with the second pollutant, still has no effect. In the second case, the first pollutant has an effect, but when given in conjunction with the second pollutant, it has no effect on the measured outcome of the second pollutant, or vice versa; the two pollutants are said to be independent or additive in their effects. In the third case, the effects of exposure to the two pollutants together is greater or less than would be expected from the sum of their separate effects; the two pollutants are said to act synergistically. Statistical analysis of appropriately designed experiments can differentiate among these different kinds of combined effects.

The biological question of synergy is addressed directly by the statistical concept of interaction. A valid and efficient design for demonstrating synergy is the two-way fac-

torial experiment in which the response to two agents is measured under four possible conditions of treatment: either agent, neither agent, or both agents in combination. The appropriate analytical tool for this type of design is a two-factor analysis of variance (two-way ANOVA). In two-way ANOVA, each factor is independently assessed for its main effect. At the same time, the interaction between the two factors is assessed to determine if the response to both factors is equal to the sum of their effects alone. The three tests, two main effects and one interaction, are derived simultaneously from the combined body of data on all four experimental groups. This procedure guarantees more precision and statistical power than could be obtained from piecemeal comparisons.

The entire analysis may be focused on the single test for interaction. The test for interaction in a two-way analysis is not affected by the presence or absence of either main effect. This concept can be illustrated by describing the possible outcomes of the joint action of acid aerosols and ozone:

1. No effect of acid aerosol; that is, the combined response equals the ozone response.
2. Independent (additive) effect of acid aerosol; that is, the combined response equals the ozone response plus the aerosol response.
3. Interactive (synergistic) effect of acid aerosol; that is, the combined response is greater or lesser than the ozone response plus the aerosol response.

In addition to statistical hypothesis testing, dose-response surface analysis may also be used to assess synergistic effects. Dose-response surface analysis constructs a three-dimensional plot of the dose of each of two binary components versus a relative risk of the occurrence of a measurable health effect. For example, concentrations of ozone and acid aerosols would be plotted along two horizontal axes. The vertical axis represents an assay of lung damage. The surface can be described by simple mathematics and fitted to the data by bivariate regression. Thus, with the dose-response surface as an analytical tool, expressed mathematically and fitted statistically, several questions can be answered directly with a minimum of hypothesis testing:

1. Do successive cross-sections of the surface keep a constant shape (additive effects) or do they change shape (synergistic effects)? If synergy is present, what is its magnitude and what is the gradient of the surface (that is, the sensitivity of the assay)?
2. How does the shape of the dose-response surface vary among assays?
3. What are the effects of putative protective agents on the dose-response surface?

Several assays of lung damage are available that would be appropriate for assessing synergism between the effects of ozone and those of acid aerosols. As noted above, at relatively low concentrations, ozone induces changes in airway permeability and can be measured by the presence of serum proteins or tracer molecules in the bronchoalveolar lavage fluid. Inflammatory cells can also be recovered in lavage fluid and their numbers and functional activity assessed. Other mediators of inflammation or injury (for example, complement fragments, prostaglandins, proteinases, or fibronectin) can be measured. Because of the implications of ozone as a fibrogenic agent, collagen metabolism can be evaluated. Finally, structural changes in the centriacinar region of the lung can be described morphologically and quantified morphometrically.

JUSTIFICATION FOR THE STUDY

The HEI sought proposals that would improve our understanding of the relation between oxidant lung injury and lung disease. In particular, the Institute was interested in proposals that would evaluate mechanisms of injury resulting from exposure to oxidants at or near ambient levels. Studies that would integrate molecular and cellular events of injury and relate these changes to chronic lung disease processes were of interest. In addition, the Institute recognized the need to develop and evaluate sensitive methods for the detection of critical targets of oxidant exposure.

J.A. Last proposed to examine the effects of acid aerosols on the toxic effects of oxidants, an important issue of oxidant injury. Last hypothesized that ozone and sulfate aerosols act synergistically, and he sought to explore the interaction of these pollutants at concentrations near ambient levels. He also proposed to assess the mechanisms of any observed synergistic effects. Earlier work by the investigator and his coworkers had demonstrated an effect of acid aerosols on ozone-induced effects, but at ozone levels ranging from 0.64 to 1.2 ppm (Last et al. 1983, 1984). Thus, Last and his colleagues already had experience conducting studies similar to those proposed. The methods to assess injury were considered relevant and state-of-the-art. The multidisciplinary approach of evaluating biochemical and morphometric endpoints was supported by the expertise of the investigative team.

OBJECTIVES AND STUDY DESIGN

The objective of Last's proposal was to investigate the synergistic effects between inhaled oxidants and respirable-sized aerosols in rats. In contrast to previous studies by the

investigator, the proposed studies were conducted at concentrations closer to ambient levels. Biochemical and morphological assays were used to evaluate synergistic interactions. In addition, mechanistic studies were proposed to determine the cellular and biochemical basis for any synergism that might be observed. Specific aims of the proposal were:

1. To determine if synergism occurs between the effects of ozone or nitrogen dioxide and those of respirable acid aerosols;
2. To determine the role of aerosol acidity in any such synergistic interactions; and
3. To evaluate the role of free radicals in the mechanism of interaction between the effects of ozone and those of acid aerosols.

To pursue the first specific aim, Last conducted a series of experiments in which rats were exposed acutely to ozone alone, nitrogen dioxide alone, or either oxidant in combination with sulfuric acid aerosol or ammonium sulfate aerosol. Exposures were for 23.5 hours per day and ranged up to seven days; in one experiment, exposures were continued for 15 or 30 days. Ozone concentrations ranged from 0.12 to 1.2 ppm; nitrogen dioxide concentrations ranged from 2 to 10 ppm. To assay for effects, total protein content, enzyme activities, and radiolabeled albumin were measured in the bronchoalveolar lavage fluid; protein content and collagen synthesis rates were measured in lung tissues; and morphometric measurements were made of lung parenchyma. In addition to evaluating synergistic interactions, the investigator sought to determine the sensitivity of the various assays and to correlate biochemical and morphometric data.

To pursue the second specific aim, the investigator measured the pH and sulfate concentrations in eluates from filters used to sample the sulfuric acid aerosol. In addition to the acidity of the sulfuric acid aerosol, the investigator examined the effect of aerosol size on lung responses. Combinations of ozone and nitrogen dioxide or nitrogen dioxide and sodium chloride were also evaluated for the potential to generate other acidic species that might have synergistic effects.

To pursue the third specific aim, rats were treated with vitamin E, β-carotene, or dimethylthiourea prior to exposure to ozone alone, to ozone and ammonium sulfate aerosol, or to ozone and sulfuric acid aerosol.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

Although the investigator presented numerous results from a number of separate experiments, it is unclear, because of the methods used for data analyses, whether or not synergism occurred. With respect to the second specific aim, the investigator showed that acidity, not the sulfate, was necessary for acid aerosols to influence ozone-induced effects. The role of aerosol acidity, however, was not determined. With the third specific aim, the investigator tested the effects of various free radical scavengers. A protective effect was observed with dimethylthiourea, but not vitamin E or β-carotene; however, without a description of the rationale for the choice of scavengers or a discussion of how the results relate to a hypothesis, the mechanistic role of free radicals remains unclear.

ASSESSMENT OF METHODS AND STUDY DESIGN

The Methods section of the Investigator's Report does not include a clear description of the experimental design. Thus, what follows in the report appears as a collection of individual experiments rather than a coherent research plan. Furthermore, without an outline of the experimental design, it is difficult to determine the rationale for the choice of experiments. For example, although the investigator wanted to approximate ambient concentrations of the pollutants, it is not clear why he chose to conduct ozone exposures for 23.5 hours per day, which does not mimic environmental exposures.

Although one of the objectives was to compare responses to pollutants individually and in binary mixtures, few of the experiments showed data from a group exposed to the aerosol alone. The impact of this omission is discussed below (see Statistical Methods).

The assays chosen for assessment of ozone-induced effects were appropriate and state-of-the-art. The biochemical work performed to validate the assays was well conceived and conducted.

The number of samples selected for the morphometric studies was adequate; however, because of the importance of randomness in sample selection, a better explanation of how the blocks of lung tissue were "cut at random" for embedding in plastic is needed. Several problems with the calculations for the numbers of cells within the lesions also should be noted. Calculation of numerical density ($N_V = N_A/\overline{D}$) requires information about the number of profiles of the cells counted per unit area, N_A, and about the mean nuclear caliper diameter, $\overline{D}$, of the cell nuclei. The Investigator's Report states that values for the different interstitial cell types were assumed, but no reference for the source of the specific values was given. The investigator also did not state whether or not these values changed as a result of different exposure conditions. In addition, it is not appar-

ent if two correction factors for the $\overline{D}$ values, one for the section thickness and the other for missed cap sections, were included in the calculations.

STATISTICAL METHODS

The central goals of this study were to determine if synergy exists between the effects of oxidant gases and those of acid aerosols and to make comparisons among different assays of lung damage. The statistical analyses of data used in this project included linear regression, one-factor ANOVA, and a variety of multiple-comparison tests.

As noted in the Scientific Background, the evaluation of synergy depends on comparing the combined effects of the oxidant gas and acid aerosol with the sum of their individual effects; however, the investigator did not consistently present data on the effect of the aerosol alone, presuming it to have no effect. In many cases, the null effect of the aerosol was taken for granted as a historical control, so that no data for the aerosol alone were reported. This omission removes some weight from the right side of the equation (combined response is greater than the ozone response plus the aerosol response), thus exaggerating the evidence of synergy.

All results relating to synergy should have been supported by two-way ANOVA. Data on aerosol exposure alone should have been presented, so that both components of the binary mixture could have been analyzed. By using two-way ANOVA, the effect of either agent alone need not be assumed or demonstrated to be zero. To illustrate the advantage of a simple two-way ANOVA, consider the interpretation of Table 10. Exposure to ozone alone and nitrogen dioxide alone each produced a 6 to 7 percent increase in lung protein over the control value, each statistically insignificant. From combined exposure, the effect was 10 percent, significantly above the control value but not significantly above either agent alone and not above the sum of the two single effects. The investigator concluded that there was an "interaction" between the two oxidants, but he was unable to determine the nature, additive or synergistic, of their joint action. A two-way ANOVA would have ruled out a synergistic interaction.

Making use of the two-factorial design with the proper analysis is preferable to multiple-comparisons procedures. It produces fewer type I errors and more power, because the use of the full sample adds precision to the assessment of each main effect. Lack of power may explain the failure to detect synergy at low concentrations of ozone. If measuring a threshold level for synergy were the objective, it would have been important to use the most powerful analytical tool available. The investigator employed an informative group of study parameters, but by using an analytical approach of low power and by making multiple comparisons, he increased the risk of missing true differences (type II error) and observing false ones (type I error).

The conduct and reporting of data reduction and statistical testing deserve comment. Some procedures were applied without apparent aim, reported obscurely, and not interpreted. These include the Duncan tests and, particularly, the regressions and the correlations among assays. The stated aim was to define the most sensitive assays and to correlate them, but no inferential framework, either quantitative or probabilistic, was provided. No measurements of assay sensitivity were reported or compared. Some linear regressions were performed, but no graphical presentations were offered to support them.

An example of a typical problem with data reduction is the regression procedure used for calculating the collagen synthesis rate. To calculate this rate, a line was fitted to each animal's measurement of collagen at t = 1, 2, and 3 hours, "plus a 0-hour value of 0 nmol." The regression line should have been forced through the origin; instead, a false data point was added to pull the fitted line close to the origin. Both procedures yield an unbiased estimate of the collagen synthesis rate, but the variability of the estimate can be considerably greater if a false point is used rather than a zero-intercept regression (direct proportionality). High variability adversely affects statistical power. In this case, because of the false point, the variance of the estimate of collagen synthesis rate is higher by a factor of 3/2 than it would have been if the regression had been forced through the origin. Variance and sample size are inversely proportional at a given level of statistical power; thus, the investigator effectively reduced his sample size by a factor of 2/3.

The procedure used for normalization is unclear. For the collagen synthesis assays, it appears that values from control animals from all the experiments were combined to normalize the data from each experiment. Then it appears that these historical control values were used to test for significant changes in the rate of collagen synthesis in individual experiments. Because experimental conditions and animals vary appreciably from time to time, it is inappropriate to use historical control values.

In summary, the Investigator's Report contains several areas of misdirected statistical technique. The analysis rarely matched the experimental design. Where the biological question of synergy was addressed, the equivalent statistical question of interaction was not used. Linear regression was cited repeatedly without a clear definition or purpose. The descriptions of assays lacked descriptive statistics. Multiple comparison procedures and correlation coefficients were overused.

In addition to a more rigorous statistical approach, dose-response surface analysis could have been applied to the data obtained from these studies (see Scientific Background). By using such an analytical device the investigator could have differentiated between synergistic and additive effects, determined the relative sensitivity of the various assays, and better assessed the protective function of dimethylthiourea.

RESULTS AND INTERPRETATION

Based on his results, the investigator drew five conclusions:

1. At concentrations that approximate ambient levels, ozone and acid aerosols show synergistic effects in rats.
2. Several sensitive assays can be used to quantify the acute response of the lung to oxidants, either alone or in combination with acid aerosols.
3. The most sensitive biochemical and morphometric indicators correlate reasonably well.
4. The acidity of an aerosol apparently is a necessary and sufficient condition for the aerosol to interact synergistically with ozone.
5. Although dimethylthiourea in vivo appears to protect the lung against oxidant lung damage, neither vitamin E nor β-carotene offers the same protection.

The five conclusions are supported by the data to varying degrees. However, because of inadequate descriptions of experimental details and problems with statistical analysis, alternative interpretations of the data cannot be excluded.

With respect to the first conclusion, the combined effects of the acid aerosol and ozone exceeded those of ozone alone with regard to collagen synthesis rates, morphometric measures, and, occasionally, lavageable protein. The responses were usually small, and for ozone plus sulfuric acid, the degree of the response was not dependent on the amount of acid. Furthermore, because of the choice of the method of data analysis, it is not clear that synergism between the effects of acid aerosols and those of oxidants occurred. Additive effects were not considered as an alternative explanation. The drawback of using Table 15 as the basis for the conclusion that synergism occurred is that it lacks the necessary data; the reported results are probabilistic, not quantitative.

With regard to the second conclusion, a strong component of the study was the analysis of various endpoints to determine those most sensitive for detecting synergistic effects. For most of the experiments, collagen synthesis rates and lavageable protein appeared to be the most sensitive indicators of lung response. As the investigator states, the health consequences of the observed responses are not certain. More consideration could have been given to the meaning of the results relative to potential adverse health effects.

The morphometric data provided quantitative measures of lesions, but no qualitative information on the nature and sites of the lesions was provided. With such information, it would have been possible to determine if the lesions caused by the combined exposures differed from those caused by exposures to single pollutants.

With respect to the third conclusion, weaknesses in the analysis of the correlation among assays limit their interpretation. Descriptive statistics, such as estimates of sensitivity or specificity, were not provided. Correlation coefficients and p values were reported, but without explanation of their relevance. Given the physiology of the system under investigation, it would not have been surprising if these correlations among assays for lung damage had been strong; in fact, they were weak (r = 0.5 to 0.6).

For the fourth conclusion, the investigator demonstrated that acidity was necessary for a joint action between the effects of ozone and those of sulfuric acid aerosol. If more information on experimental details or additional discussion of data interpretation had been provided, more insight on the role or mechanism of action of an acid might have been gained.

The investigator states that the bactericidal fluids should have maintained any excretory products from the animals in an aseptic state. It is not clear, however, if this was only assumed or if the investigator tested for the presence of ammonia. Data from Table 8 indicate that a small amount of ammonia was present. The nonlinearity at the low end of the pH plot of the sampling filters was, conceivably, due to partial neutralization of the acid by ammonia in the exposure chamber. This amount of neutralization would not be detectable at the higher acid concentrations but would be detectable at the low end of the concentration curve.

Although the procedures for aerosol generation and for measurement of the nebulized acids were adequate, more information for the nuclei-mode aerosol could have been provided. Also, it is not clear why a particular aerosol size was selected. If the sizes were selected to differentiate between different respiratory tract deposition sites, it should be noted that an aerosol formed at 0.03 μm will rapidly grow in the chamber to approximately 0.1 to 1.0 μm and will deposit in regions similar to those of a 0.5 μm aerosol. Because the units of measurement of the smaller aerosol were given as count median diameter, it is not apparent whether or not the actual mass median aerodynamic diameter was measured; thus, it is difficult to compare the results between the two aerosol sizes.

The Investigator's Report does not provide enough details on the exposure chamber, gas distribution system, sampling system, and the composition of the medical grade breathing air. Without such information, the potential for the formation of other species cannot be evaluated. For example, in the presence of calcium chloride, sodium nitrate and hydrogen chloride would be formed, thus contributing to the acid burden of the exposure atmosphere. These reactions may explain the anomalous behavior observed in the presence of nitrogen dioxide and sodium chloride.

Although the conclusion that the addition of vitamin E and β-carotene did not protect against oxidant-induced lung damage was supported by the data, some of the protective effects of dimethylthiourea could have been discussed more thoroughly. For example, although dimethylthiourea protected against ozone-induced increases in lavageable lung protein, it did not prevent increases after ozone and acid aerosol exposure. In contrast, with the collagen synthesis response, the agent protected against the combination but not against the ozone alone. Without an explanation of what mechanisms the investigator was trying to elucidate, the significance of these observations is obscure. In addition, the interpretation of the results is further complicated because dimethylthiourea also lowered total lung protein and DNA content. Because vitamin E and β-carotene are dietary nutrients, the dietary regimen and food content of these compounds needs to be controlled by the use of a semipurified diet. The Investigator's Report does not indicate if this was done; thus, the interpretation of their protective function could be affected.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The need to determine if oxidants and acid aerosols act synergistically in the lung still remains. These two classes of pollutants coexist in the atmosphere, especially during the summer months when people are usually outdoors and physically active. Although this study presents data that are suggestive of a synergistic effect, further experiments are needed to confirm the findings.

The investigator demonstrated that certain biochemical parameters were sensitive indicators of oxidant-induced effects. The questions remain, however, regarding the importance and specificity of the changes and how these changes related to injury. If tissue or cell injury occurred, it should be possible to detect such injury by multiple related markers. Just one assay that shows changes while others remain unchanged may not signify injury. It was not an objective of this study to assess the relationship of these parameters to respiratory disease; their health significance remains to be determined in future studies.

It was an objective of this project to explore the mechanisms of any observed synergism. Although it appears that the acidity of the aerosol was an important factor, the mechanisms by which acid aerosols may enhance oxidant-induced damage remain unknown. This information has important implications for evaluating the potency of other acidic compounds in the atmosphere (for example, acid vapors such as nitric acid), assessing the sensitivity of subpopulations, and determining the nature and impact of ozone-induced effects.

CONCLUSIONS

The investigator addressed an important question in toxicology; that is, what are the combined effects of exposure to more than one pollutant? He hypothesized that the response to binary mixtures of oxidant gases and respirable acid aerosols would exceed the sum of the responses of the individual pollutants. The experiments involved exposures to different concentrations of pollutants for different lengths of time, with endpoints measured at varying times after exposure. Effects reported were both time- and endpoint-dependent.

The Investigator's Report contains the results of many interesting and useful experiments. The development of a coherent theme addressing the specific aims would have strengthened the study. The failure to apply dose combinations in a systematic way limited the interpretation of the data. The investigator demonstrated that several assays may be sensitive indicators of a response of the lung. Of the various biochemical and morphometric parameters investigated, only some showed measureable changes. Although these changes were used to assess "injury," "interaction," and "synergism," their biological significance remains unclear. Furthermore, although a joint action was observed at some time points, no overall pattern was apparent. A significant problem with the study was that the interpretations and conclusions were founded on statistical maneuvers that were not appropriate for the hypothesis being tested. Other approaches to data analysis could have more clearly supported his conclusions.

In summary, the strengths of this study were its ambitious scope and the large quantity of data that were gathered in one test system, the rat lung. The data included many strong hints that synergism may have occurred between ozone and acid aerosols, that some assays were more responsive than others, and that dimethylthiourea may have protected the lung against damage by mixtures of ozone and acid aero-

sols. However, the evidence for a synergistic effect was inconclusive. Detracting from the study was its piecemeal approach to experimental design and statistical analysis. The investigator did not capitalize on the strength of his experimental system and delineate clearly the effects of these environmental pollutants on the lung.

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LIST OF HEI PUBLICATIONS

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Special Reports

Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research (Supplement)	January 1988

Research Reports

Report No.	Title	Principal Investigator	Publication Date
1	Estimation of Risk of Glucose 6-Phosphate Dehydrogenase-Deficient Red Cells to Ozone and Nitrogen Dioxide	M. Amoruso	August 1985
2	Disposition and Metabolism of Free and Particle-Associated Nitropyrenes After Inhalation	J. Bond	February 1986
3	Transport of Macromolecules and Particles at Target Sites for Deposition of Air Pollutants	T. Crocker	February 1986
4	The Metabolic Activation and DNA Adducts of Dinitropyrenes	F.A. Beland	August 1986
5	An Investigation into the Effect of a Ceramic Particle Trap on the Chemical Mutagens in Diesel Exhaust	S.T. Bagley	January 1987
6	Effect of Nitrogen Dioxide, Ozone, and Peroxyacetyl Nitrate on Metabolic and Pulmonary Function	D.M. Drechsler-Parks	April 1987
7	DNA Adducts of Nitropyrene Detected by Specific Antibodies	J.D. Groopman	April 1987
8	Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung	J.L. Mauderly	May 1987
9	Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury	J.M. Patel	June 1987
10	Predictive Models for Deposition of Inhaled Diesel Exhaust Particles in Humans and Laboratory Species	C.P. Yu	July 1987
11	Effects of Ozone and Nitrogen Dioxide on Human Lung Proteinase Inhibitors	D.A. Johnson	August 1987
12	Neurotoxicity of Prenatal Carbon Monoxide Exposure	L.D. Fechter	September 1987
13	Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties	E.D. Crandall	October 1987
14	The Effects of Ozone and Nitrogen Dioxide on Lung Function in Healthy and Asthmatic Adolescents	J.Q. Koenig	January 1988
15	Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide	T.J. Kulle	January 1988
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Research Reports

Report No.	Title	Principal Investigator	Publication Date
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18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
19	Factors Affecting Possible Carcinogenecity of Inhaled Nitropyrene Aerosols	R.K. Wolff	August 1988
20	Modulation of Pulmonary Defense Mechanisms Against Viral and Bacterial Infections by Acute Exposures to Nitrogen Dioxide	G.J. Jakab	October 1988
21	Maximal Aerobic Capacity at Several Ambient Concentrations of Carbon Monoxide at Several Altitudes	S.M. Horvath	December 1988
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23	Responses of Susceptible Subpopulations to Nitrogen Dioxide	P.E. Morrow	February 1989
24	Altered Susceptibility to Viral Respiratory Infection During Short-Term Exposure to Nitrogen Dioxide	R.M. Rose	March 1989
25	Acute Effects of Carbon Monoxide Exposure on Individuals with Coronary Artery Disease	HEI Multicenter CO Study Team	November 1989
26	Investigation of a Potential Cotumorigenic Effect of the Dioxides of Nitrogen and Sulfur, and of Diesel-Engine Exhaust, on the Respiratory Tract of Syrian Golden Hamsters	U. Mohr (U. Heinrich)	May 1989
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