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RESPIRATORY EFFECTS OF ACID CONTAINING MULTICOMPONENT POLLUTANT ATMOSPHERES

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RESPIRATORY EFFECTS OF ACID-CONTAINING MULTICOMPONENT ATMOSPHERES

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ABSTRACT

The purpose of this study was to assess the possible adverse effects on the respiratory system of acid air pollutants inhaled in mixtures with other pollutant compounds commonly present in California urban air. A 7-component mixture of acid-forming oxidant pollutants representing the principal components of California urban air pollution was generated which was composed of 0.35 ppm ozone, 1.3 ppm nitrogen dioxide, 2.5 ppm sulfur dioxide, 1.1 mg/m^3 manganese, ferric and ammonium sulfite, and 0.5 mg/m^3 carbon soot at 85% relative humidity. Respiratory effects were compared to those produced by acid components (1 mg/m^3 nitric acid + 0.5 mg/m^3 sulfuric acid) and oxidant components (0.35 ppm O_3 + 1.3 ppm NO_2) alone at 85% RH. The O_3 and NO_2 had time to react to form HNO_3, nitrate radical (NO_3), and N_2O_5. HNO_3 formed in these reactions was measured at test exposure points. Laboratory rats were exposed 4 h to the pollutant atmospheres and a variety of respiratory effects were examined among different sets of atmospheres including 1) reflex changes in breathing pattern, 2) histopathological analysis of tissue damage to the lung and nasal epithelium, 3) particle clearance of the respiratory tract, and 4) analysis of structural changes in pulmonary surfactant using infrared and ultraviolet spectroscopy, gas chromatography, and surface pressure measurements. At the concentrations tested, the acid components alone had no significant effects on the respiratory parameters examined. Mixtures containing 0.35 ppm ozone caused lung tissue damage, end-exposure breathing pattern changes, and pulmonary surfactant effects similar to effects of 0.35 ppm O_3 alone. The mixture of 0.35 ppm O_3 + 1.3 ppm NO_2 resulted in an early-exposure breathing pattern change consistent with an upper airway irritant effect of HNO_3 formed in reactions between the oxidants. No evidence of synergism between oxidants, O_3 and NO_2, or modifying effects of acids on O_3 responses was found at these low concentrations in contrast to earlier studies using higher concentrations or exercise exposure. No significant effect on particle clearance of the 7-component atmosphere was observed although the trend of change was in the same direction as expected for an O_3 effect. The chemical composition of pulmonary surfactant from rats exposed to 0.35 ppm O_3 alone or in combination with 1.3 ppm NO_2 was shown to change 24-48 h after the exposure and methodology for isolating surfactant was shown to be critical for detecting the changes. The changes included shifts in the fatty acid composition and the formation of oxidized compounds in surfactant. The time course for the changes was consistent with the course of pulmonary inflammatory response to oxidant damage.

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Laboratory rats were exposed 4 h to acid-containing and acid-forming air pollutant mixtures to assess the possible adverse effects on the respiratory system of acid air pollution. A 7-component mixture of pollutants representing the principal components of California air pollution was generated in the laboratory and its effects were compared to the acid and oxidant components to test the hypotheses that acid and oxidant compounds act synergistically to enhance respiratory tract injury. The 7-component mixture was composed of 0.35 ppm O_3, 1.3 ppm NO_2, 2.5 ppm SO_2, 0.01 mg/m^3 $MnSO_4$, 0.6 mg/m^3 Fe_2SO_4, 0.5 mg/m^3 $(NH_4)_2SO_4$, and 0.5 mg/m^{3}carbon soot aerosol. When generated in the laboratory, this mixture was expected to form primarily HNO_3 vapor and some acid sulfate and bisulfate particles. The formed acid was almost entirely HNO_3 vapor at about 0.1 mg/m^3. The subsets of components for comparison were 0.35 ppm O_3 + 1.3 ppm NO_2 (forming about 0.5 mg/m^3 HNO_3 vapor), 0.35 ppm O_3, and 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4. In addition, the effects of concentration of the 7-component mixture and of the O_3 + NO_2 mixture were studied in exposures reduced from the above concentrations by factors of 0.5 and 0.25.

The respiratory effects examined among selected sets of pollutant atmospheres included 1) reflex breathing pattern changes in response to inhaled irritants, 2) histopathological analyses of tissue damage to the lung parenchyma and nasal epithelium, 3) effects on particle clearance rates of the respiratory tract, and 4) analysis of structural changes in pulmonary surfactant using infrared and ultraviolet spectroscopy, gas chromatography, and surface pressure measurements. Breathing pattern changes were measured during exposures from rats in nose-only flow plethysmographs. Tissue damage was measured in the lung by light microscopy and quantitative analysis of oxidant lesion areas in lung parenchyma. Tissue damage in nasal epithelium was measured by cell proliferation response to injury using incorporation of radiolabeled thymidine into replicating DNA. Pulmonary surfactant was recovered by lavage, and chemically isolated for spectroscopic, gas chromatographic, and surface pressure analysis.

At the concentrations tested, acid components had no significant effects on the respiratory parameters examined. While earlier studies in this laboratory had shown synergistic interactions on lung tissue damage between 0.6 ppm O_3 and 2.5 ppm NO_2 (forming HNO_3) in resting exposures or 0.35 ppm O_3 and 0.6 ppm NO_2 in exercising exposures, at the lower concentrations and resting exposures studied here, no detectable synergistic effects on lung tissue injury

were found. Multi-component mixtures containing 0.35 ppm O_3 in the present study showed lung tissue damage, and end-exposure breathing pattern changes, similar to effects of 0.35 ppm O_3 alone. These consisted of slight development of a rapid and shallow breathing pattern response and a slight induction of lung parenchymal lesions. There was no demonstrable effect of the acids HNO_3 + H_2SO_4 or mixed acid and oxidants on nasal tissues. The combination of 0.35 ppm O_3 and 1.3 ppm NO_2 resulted in an early exposure breathing pattern change toward slow-deep respiration which was indicative of an upper airway irritant response that may have resulted from inhalation of HNO_3 formed by reactions between the oxidants. The slight rapid-shallow breathing response characteristic of O_3 and observed at the end of O_3 exposures was suppressed when O_3 was combined with NO_2. Despite these alterations in breathing pattern which can affect inhaled dose-distribution of pollutants, no evidence of synergism between the oxidants O_3 and NO_2 was observed for lung tissue injury at the lower concentrations studied. There was also no significant effect of the 7-component atmosphere observed on particle clearance rates.

Changes in the chemical composition of pulmonary surfactant on exposure to O_3, O_3 + NO_2, and a mixture of HNO_3 and H_2SO_4 were compared to clean air controls using Fourier transform infrared (FTIR) and ultraviolet (UV) spectroscopy, as well as gas chromatographic analysis of the fatty acid methyl esters. The surface pressure-surface area isotherms were also measured. In addition, the change in composition with sacrifice time after exposure to the O_3 + NO_2 combination was measured. Exposure to the O_3 + NO_2 combination resulted in a change in the fatty acid composition of surfactant 24 hours after the exposure; infrared and ultraviolet spectroscopy indicate an accumulation of oxidation products up to 48 hours after the exposure. The plateau observed in the surface pressure-area isotherms of the clean air controls disappears in the exposed samples. In addition, the chemical composition and surface pressure-area isotherms of the surfactant from rats exposed to a mixture of sulfuric and nitric acids were similar to that of the clean air controls; however, the composition and surface pressure-area isotherms of surfactant from rats exposed to O_3 or to O_3 + NO_2 mixtures and sacrificed 44 hours after the exposure differed significantly from the clean air controls, but were similar to each other.

Finally, isolation techniques for surfactant recommended in the literature were compared in the O_3 + NO_2exposure. It was shown that sucrose density gradient centrifugation must be used in isolation of pulmonary surfactant. An "abbreviated" method used in many exposure studies reported in the literature cannot be used if studying surfactant is the goal.

In summary, in single resting 4 h exposures of rats to 0.35 ppm O_3, addition of 1.3 ppm NO_2 and the principal acid reaction product of this addition (0.1-0.5 mg/m^3 HNO_3) or the addition of 2.5 ppm SO_2, particle sulfates (1 mg/m^3), and carbon (0.5 mg/m^3) can modify the reflex breathing pattern response to O_3 but do not significantly modify O_3-induced effects on tissue injury, or significantly affect particle clearance. Although other pollutant compounds may profoundly affect O_3 damage at higher concentrations or during exercise exposure, at the low concentrations examined in this contract, O_3 accounted for the toxic effects of the inhaled pollutant mixtures.

RECOMMENDATIONS

The results of the present investigation indicated no strong interactions between acid air pollutants and oxidants at low concentrations in single 4 h exposures. However the earlier studies working at higher concentrations and showing enhancement of O_3 effects on lung tissue damage by presence of acid air pollutants, and the strong interaction between O_3 and NO_2 (with reaction products HNO_3, NO_3 radical, and N_2O_5) are still cause for attention to the importance of air pollution mixtures. It is not known if interactive effects may be revealed at lower concentrations with repeated exposures. It is known that exercise exposure resulted in a strong interactive effect between O_3 and NO_2 at low concentrations: 0.35 and 0.6 ppm respectively. Thus exercise exposure continues to be a very important variable to be considered in assessing the potential adverse health effects of air pollutant exposure. It is not clear what the mechanism for the observed synergistic interactions between O_3 and NO_2 and between O_3 and acids at the higher concentrations might be. For example it may be inorganic chemical reactions between pollutants producing potent toxicants such as NO_3 and N_2O_5 as products, it may be biochemical interactions between pollutant compounds and tissues, or it may be physiological interactions affecting dose-distribution of inhaled pollutant compounds. Elucidating the mechanisms for such interactions requires working at high concentrations or exposure conditions (such as exercise) that give strong positive effects in order to compare different experimental manipulations. A better understanding of the mechanisms of toxic interactions

among pollutants then permits a more efficient assessment of the potential for important interactions to be occurring among the complex human exposure situations of the urban environment.

I. INTRODUCTION

The importance of acidic compounds in causing adverse health effects associated with air pollution exposure is of increasing concern because of high levels of acidity observed in fogs not only in the South Coast Air Basin (Brewer et al., 1983; Waldman et al., 1982; Richard et al., 1983; Munger et al., 1983, 1984; Jacob et al., 1985) but in less populated portions of California as well (Munger et al., 1986; Jacob et al., 1986). Acid compounds are formed as atmospheric oxidation products of oxides of sulfur and nitrogen, hence exposure of the public to these acids often occurs simultaneously with exposure to other pollutants such as O_3, NO_2, SO_2, etc. It has become apparent that toxicological studies of individual components of airborne pollutants do not adequately predict effects on health. The chemistry, dosimetry, and toxicity of these materials are profoundly affected by the other constituents of the complex mixture of gases and aerosols comprising the polluted urban atmosphere. Recent studies have implicated acids in synergistic interactions between ozone and aerosols that enhance lung tissue damage (Last et al., 1983, 1984, 1986; Last and Cross, 1978). ARB supported research in our laboratory has documented a synergistic interaction between inhaled O_3 and NO_2, a mixture which forms secondary compounds including nitric acid, N_2O_5, and the nitrate-radical (Mautz, 1984). It is important to examine the physical and chemical interactions that occur among air pollutants in mixtures and to investigate how these interactions affect toxicity of mixed pollutants because 1) combinations may be potentially more toxic than their individual components, and 2) combinations are a more realistic representation of the conditions of human exposure in urban settings.

The purpose of this project was to assess the possible adverse effects of inhaled acidic air pollutant mixtures on the respiratory system. Mixtures of acid-forming pollutants and oxidants representing the principal components of California urban air pollution were generated and respiratory effects were compared between those produced by the acid components and the oxidant components alone. The exposure atmosphere used was composed of 7 compounds: 0.35 ppm O_3; 1.3 ppm NO_2; 2.5 ppm SO_2; 0.01 mg/m^3 $MnSO_4$; 0.5 mg/m^3 $Fe_2(SO_4)_3$; 0.5 mg/m^3 $(NH_4)_2SO_4$; and 0.5 mg/m^3 carbon aerosol. When mixed together these compounds engage in chemical reactions that form acids. Fe^{+3} and Mn^{+2} were included to catalyze formation of acid sulfates (Kleinman et al., 1985). O_3 and NO_2 mixtures with water vapor form HNO_3 vapor via reactions 1-3.

(1) $O_3 + NO_2 \rightleftharpoons NO_3 + O_2$

(2) $NO_3 + NO_2 \rightleftharpoons N_2O_5$

(3) $N_2O_5 + H_2O \rightleftharpoons 2\ HNO_3$

Thus the reactive species NO_3 and N_2O_5 were present in addition to the oxidants and acids. From the change in the O_3 concentration when the NO_2 was turned off, the measured gas phase HNO_3, and the known kinetics of these reactions (Finlayson-Pitts and Pitts, 1986), we estimate that typical NO_3 and N_2O_5 concentrations at the exposure ports were 100 ppt and 100 ppb respectively. These can be compared to ambient peak concentrations observed in California of 430 ppt for NO_3 (Platt et al., 1984) and calculated N_2O_5 peak levels of approximately 15 ppb (Atkinson et al., 1986). When generated in the laboratory at 85% relative humidity, the 7-component mixture was expected to acidify the ammonium sulfate particles (ammonium bisulfate) and form HNO_3 vapor. Acid formation was one of the bases for selecting mixture components for separate tests. The subsets of components to be compared to the full mixture were 0.35 ppm O_3 alone, and the combinations 0.35 ppm O_3 + 1.3 ppm NO_2, and 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4. Dose-response tests of two of the mixed atmospheres were performed at lower concentrations to extend toxicity assessment to include more common urban ambient concentrations for comparison to concentrations likely to produce detectable effects. Because the combination of O_3 and NO_2 has been demonstrated to have synergistic effects on lung tissue damage and to form NO_3, N_2O_5, and HNO_3 vapor, a dose-response test was performed with this mixture using the concentrations of the 7-component mixture (0.35 ppm O_3 + 1.3 ppm NO_2) compared to levels reduced by factors of 0.5 and 0.25. A dose-response test was also performed for the full 7-component mixture at concentration reduction factors of 1.0, 0.5, and 0.25.

Exposure subjects were laboratory rats. This animal exposure model is commonly used as a means of discovering potential human health hazards from inhaled pollutants. This standard model can be maintained in good lung health in the laboratory, thus reducing the possible confounding effects of infection. The rat model permits us to test higher doses than would be ethically possible with human subjects in order to discern important interactions between component pollutants, and to provide assessment of toxicity using a variety of biological endpoints. These include invasive measurements of tissue and biochemical changes which are not possible to perform with human volunteers. There is some question as to whether the rat is

more or less sensitive than other laboratory animals with respect to inhaled acids. This must be considered in the context of the endpoints being used. For breathing pattern changes, Alarie (1973) and others have demonstrated for a wide variety of compounds that rat responses were predictive of responses observed in other mammals, including humans. Acids in the presence of oxidant gases have been shown to cause histopathological and morphological changes in nasal and pulmonary airways consistent with those observed in rabbits. We do not know whether the rat or the rabbit has inherently more disruptable clearance mechanisms. Studies of acids with rabbits differ in experimental design than those we have done in rats.

This project used several biological endpoints to evaluate respiratory effects of selected pollutant atmospheres. These included 1) reflex changes in breathing pattern (frequency and tidal volume), minute ventilation, and metabolic gas exchange, 2) autoradiographic measurement of epithelial cell turnover in the nose, 3) histopathological analysis of deep lung tissue damage, 4) measurement of respiratory tract clearance of radiolabeled insoluble tracer particles, and 5) an analysis of chemical changes in pulmonary surfactant measured by Fourier transform infrared spectroscopy (FTIR), ultra violet spectroscopy, and gas chromatography of the fatty acid methyl esters and by surface tension measurements of pulmonary surfactant.

Respiratory reflex responses to inhaled irritants are complex, and different irritants can induce opposing reflexes. For example, ozone is a pulmonary irritant and induces a rapid-shallow breathing response while upper airway irritants like formaldehyde or airborne acids induce a slow-deep breathing response (Alarie, 1973). Reflex irritant responses may defend the respiratory system against inhalation injury or they may be simply a pathologic response. Thus, there is strong potential for important interactions between reflex responses to single and combined pollutants and lung tissue damage. These interactions could affect dose distribution of inhaled pollutants and may explain the enhancing effects of exercise exposure on toxicant inhalation injury and synergistic interactions observed between pollutant compounds such as O_3 and airborne acids.

Histopathological endpoints provide quantitative information on the direct effects of pollutants on epithelial tissues of the respiratory tract. Strong oxidants like O_3 primarily affect the deep lung tissues and produce focal lesions in terminal bronchioles and proximal alveoli (Schwartz et al., 1976; Last et al., 1983; Crapo et al., 1984; Evans, 1984; Mautz et al., 1985a).

There is evidence that O_3 acts synergistically with acid aerosols (Last and Cross, 1978; Last et al., 1983, 1984, 1986; Kleinman et al., 1985) and with NO_2 (Mustafa et al., 1984; Mautz, 1984) to produce lung tissue damage. Tissue injury from inhalation of airborne acids occurs in the nasal epithelium, and there is evidence that O_3 present as a co-pollutant can enhance nasal epithelial injury from acid particles (Kleinman et al., 1985).

Particle clearance from the respiratory tract is one of the critical pulmonary defense mechanisms. The function of respiratory clearance is to efficiently remove particles deposited by inhalation, and the consequences of clearance failure include increased retention of infectious and toxic agents potentially accompanied by increased risks of acute and chronic diseases (Phalen et al., 1980, 1984). Respiratory clearance is a sensitive index of toxic inhalation exposures and is affected by a variety of air pollutants. Inhalation of sulfuric acid aerosol has been shown to induce changes in particle clearance rates (Schlesinger et al., 1978, 1979, 1983), and clearance of staphyloccus bacteria was inhibited by exposure to O_3 or NO_2 (Goldstein et al., 1971).

The alveolar region of the lung where gas exchange occurs is lined with a thin layer of fluid containing a surfactant (Clements, 1956, 1961; Clements et al., 1958, 1961, 1976, 1984; Pattle, 1955, 1958; Pattle and Thomas, 1961). This surfactant layer plays a vital role in maintaining the integrity of the alveolar spaces by lowering the surface tension of the fluid at the air-water interface (Scarpelli, 1968). Alveolar collapse (atelectasis) is therefore prevented in the expiration phase of breathing, and pulmonary edema associated with high alveolar surface tension is also prevented (Avery and Mead, 1959; Nieman and Sredenberg, 1984). Abnormalities in the surfactant system are responsible for diseases such as respiratory distress syndrome. The lipids are believed to lower alveolar surface tension by organizing themselves at the air-fluid interface in a parallel fashion, with their polar, hydrophilic ends projecting into the water and their relatively non-polar, hydrophilic ends projecting into the air.

Air pollutants entering the alveoli first contact the pulmonary surfactant lipids, which thus present a potential reaction site for air pollutants prior to their reaching the cells. Alternatively, exposure to the pollutants may induce a biological inflammatory response which may alter the composition of the surfactant. However, despite the variety of <u>in vivo</u> as well as <u>in vitro</u> studies, little is known about whether air pollutants actually react with the

lipids of pulmonary surfactant *in vivo* as they cross the alveolar liquid lining (Haagsman and van Golde 1985). In addition, very little is known about possible changes in the surfactant due to inflammatory responses induced in the animal by the exposure.

The respiratory effects studied in this project include sensitive indications of respiratory responses (reflex changes studied in breathing pattern and changes in mucociliary clearance) and histopathologic measurements of more serious injury in nasal and deep lung tissue. Chemical changes in pulmonary surfactant are indicative of possible direct chemical alterations and biological responses induced by oxidant and acid inhalation. This study provides a multi-endpoint set of toxicological data on pollutant combinations which are environmentally relevant, have not been studied previously, and have a strong potential for synergistic interactions leading to enhanced adverse health effects on the respiratory system.

II. MATERIALS AND METHODS

A. Generation and Characterization of Exposure Atmospheres

Components of mixed pollutant atmospheres were metered into purified air at rates sufficient to yield desired concentrations at the breathing zone of exposed animals. Nitrogen dioxide and sulfur dioxide were metered from tanks of compressed gas. Ozone was generated by metering medical grade oxygen through a Sander Ozonizer. Solutions containing ammonium, manganese, and ferric sulfates were aerosolized using a Collison nebulizer; the resulting aerosol was dried with dilution air and passed through a ^{85}Kr discharger to reduce static charge to Boltzman equilibrium levels and brought to equilibrium at 85% relative humidity. Carbon aerosols were produced by combustion of propane under controlled conditions (Lee and Mulholland, 1980). Nitric acid vapor and sulfuric acid aerosol were generated by metering nitric acid vapor and sulfur trioxide gas into humidified, purified air.

Atmospheric aerosols were continuously monitored during exposures using an electrical mobility classifier (TSI Model 3030) and an optical particle counter (Climet Model 208). These devices provide particle size and concentration data on a real-time basis as a check on atmosphere stability during experiments. Integrated samples were collected using an 8-stage Sierra-type cascade impactor. A point-to-plane electrostatic precipitator was used to collect samples on carbon-coated electron microscope grids for transmission electron microscope particle size analysis. Electron microscopy was used to confirm the calibration of the impactor and the real-time particle counting systems for carbon aerosol sizing.

Nitrogen oxide gases were continually monitored by chemiluminescence (Monitor Labs 8840); sulfur dioxide was monitored using a pulsed fluorescence detector (Thermo Electron Corp., Model 43); and ozone was monitored using UV absorption (Dasibi Model 1003 AH).

Atmospheric acidity consisted of nitric and sulfuric acids, and most, if not all, of the nitric acid was present in the vapor phase. A 2-stage filter collection system was used for HNO_3 analysis. A prefilter (Zeflour 0.5 um pore size) collected carbon and acid aerosols. Nitric acid vapor passed through this filter and was trapped by a nylon backup filter (Appel et al., 1980). Zeflour filters were extracted and aliquots analyzed for sulfate and nitrate

ion concentrations by ion chromatography. The nylon filters were extracted and analyzed for nitrate by ion chromatography, and vapor phase HNO3 was computed.

Total carbon content of aerosol mixtures was analyzed by combustion conversion of carbon to CO_2. The method was specially adapted at this laboratory for this contract. Particle samples, collected on Gelman microquartz filters, were placed in a quartz tube and combusted at 1000° C in a steam of pure oxygen. A downstream MnO_2 catalyst, also at 1000° C, ensured quantitative oxidation of all carbonaceous species to CO_2. The CO_2 formed in the combustion section was swept by the O_2 carrier gas into the sample cell of a Dasibi Model 3003 CO analyzer, modified for the measurement of CO_2. Sample carbon content (elemental plus organic carbon) was proportional to the quantity of CO_2 evolved, or area under the CO_2 peak produced by the sample.

Overall precision of the carbon analysis method was evaluated by performing replicate analysis of standards of known carbon content. The coefficient of variation was approximately 4%. The minimum detectable airborne carbon concentration was limited primarily by the carbon blank of the filter media and the air sampling volume. 47 mm dia. Gelman microquartz filters, if used as received, typically had a carbon blank of about 100 ± 15 μg (x ± SD). Using an air sampling volume of 1 m^3 the resulting carbon detection limit was roughly 60 $\mu g/m^3$. Detection limits were reduced about 50% by prefiring the quartz filters to reduce this carbon blank. Standards for method calibration may be either organic or elemental carbon. Since the propane soot aerosol was almost entirely elemental carbon, this form of carbon was chosen for calibration. Commercial activated carbon, heated at 300° C for 30 min to remove organic impurities (Ohta and Okita, 1984; Ohta et al., 1984), was used. Since good linearity (r=.997) between CO_2 peak height and mass of elemental carbon combusted was observed in the 0-.5 mg carbon range, this peak parameter (rather than peak area) was used to quantify the carbon content of samples.

Acidification of the aerosol phase was assessed by direct potentionetric measurements and also computed by mass balance methods. Filter samples were extracted in distilled water, the pH was measured and the hydrogen ion content calculated. This H^+ content was corrected for background acidity resulting from hydrolysis of Fe^{+3} (a component of the 7-component mixture) which occurs after filter extraction. The correction factor (μ eq. H^+ released per μ Mole Fe^{+3} in the extract) was determined empirically as a function of sulfate concentration in sample extract solution using dilutions of a solution similar in concentration to the nebulizing solution used for aerosol generation. The

mass balance method used was to calculate total sulfate aerosol content from the Fe^{+3} content in filter extracts as determined by atomic absorption spectrophotometry. The difference between total measured sulfate and calculated sulfate (assuming no significant fractionation in the aerosol phase) which is termed excess sulfate is assumed to represent the amount attributable to oxidation of So_2 to SO_4^{-2} as sulfuric acid. In addition, particulate phase nitrate is assumed to represent adsorption of nitric acid vapor by the particulate phase. Particulate phase acidity is thus reported as the μ eq. of H^+/m^3 due to adsorbed HNO_3 and H_2SO_4 from conversion of SO_2 and NO_2 in the atmosphere mixture.

B. Animals

Exposure subjects were male, barrier reared Sprague-Dawley rats, (240-280 g.) from Hilltop Labs (Scottdale, PA). The animals were shipped in air-filtered cartons and trucked to us from the airport by special carrier to avoid contact with less healthy rats. Upon arrival the rats were checked for appearance, (shiny coat, nasal discharge, activity, etc.). Animals were held in our facility at least one week before experimental exposure. A sample 10% of the animals was taken at random for quality control examination. Quality control animals were killed by pentabarbital overdose, their thoracic and abdominal viscera inspected for gross abnormalities, and the lungs removed and fixed by airway perfusion. Lung sections were examined histologically and graded according to morphological guidelines for lab rats. Acceptance and use of the animals was based on this examination. Quarterly necropsy and viral serological testing reports were sent to Hilltop Labs at our request for our records. These tests were routinely done by Microbiological Associates, Bethesda, MD. Rats were housed in front of custom-designed horizontal laminar airflow isolation racks (Burleson-Airtech, Orange, CA), and the animals' bedding was changed three times per week with a rotational change to sanitized caging once every two weeks. Conventional litter in the rat trays beneath the wire cage floors has been replaced by rock salt which dries excreted urine and feces to suppress ammonia production due to bacterial growth.

C. Plethysmography

Breathing pattern changes of rats exposed to the test atmospheres were measured with a body plethysmograph. The plethysmograph provided measurements of tidal volume, breathing frequency, and minute ventilation of 8 rats during exposure. Rats were held in 57 mm inside diameter acrylic plastic tubes with

aluminum nose cones similar to nose-only inhalation exposure tubes described by Raabe et al. (1973) and modified to function as flow plethysmographs during exposure to O_3. A truncated cone of latex dental dam was clamped between the latex cone and subtended an angle 4° smaller than the metal nose cone. The latex fit snugly around the anterior angle of the rat's head and the nose and mouth protruded from the tip of the cone thus separating respiratory orifices from the body. Thoracic displacement of air during respiration was measured with a #0000 pneumothachograph (Dyna Sciences, Bluebell, PA) and differential pressure transducer (Validyne MP-45, Northridge, CA) connected between the body tube and a port in the metal nose cone opening at the rat's nose. The signal was electrically integrated and counted to display tidal volume and breath frequency on a chart recorder (Gould Model 2800S, Cleveland, OH). Eight plethysmograph exposure tubes were plugged into ports of a Rochester exposure chamber and exposure atmospheres were drawn past the aluminum nose cones to provide a nose-only exposure of the rats. Metabolic gas exchange of rats in the plethysmograph was measured by collecting expired air into the stream of air drawn past the rat's nose and mouth at 3 L min^{-1}. The air passed to a 2 L mixing chamber and through a flow controlling rotameter. The diluted, mixed, expiratory air was sampled for O_2 and CO_2 fractions and water vapor content using a dew point sensor (EG&G, Model 911, Waltham, MA) and a mass spectrometer (Perkin-Elmer model 1100, Pomona, CA). Measurements were made by switching instruments to sample between upstream and downstream gases and successively sampling from 8 rats exposed simultaneously. Metabolic rate was calculated from standard equations for open flow respirometers (Mautz et al., 1985d) and expressed at STPD conditions. The system enabled us to measure metabolic gas exchange and ventilation equivalents for oxygen in addition to the more standard plethysmographic measures of frequency, tidal volume and minute ventilation.

During an exposure, the animals breathed clean air for 0.5 hour while recovering from the excitement of handling and loading, followed by a 4 hour exposure to the test atmosphere. The 8 animals exposed in the plethysmograph were included as the majority of the complete sample (n = 10) exposed for histopathologic analyses. Respiratory rate, tidal volume, minute ventilation, oxygen consumption, and ventilation equivalents for O_2 and CO_2 were measured at 20 min intervals and averaged over hourly intervals throughout the exposure. Responses to exposure groups were then analyzed by comparison to clean air control exposed rats using analysis of variance and Tukey multiple comparisons of group effects (Zar, 1984).

D. Histopathology

Tissue injury was quantified in the alveolar region of the lung in a procedure similar to cascade level 1 stratified sampling analysis (Last et al., 1983). Rats were deeply anesthetized by sodium pentabarbital and killed by exsanguination. The thoracic cavity was opened after puncturing the diaphragm and the lungs and trachea carefully exposed. The trachea was transected 5 mm above the bifurcation and the distal portion cannulated. Lung surfaces were examined for abnormalities before and after removal from thoracic cavity. The cannulated trachea with attached lungs and thoracic viscera were fixed by airway perfusion with 10% neutral buffered formalin at 30 cm fluid pressure for 72 hours (McClure, 1982).

After two days the lungs were removed from the perfusion apparatus and the caudal lobe of the right lung was separated. Appropriate portions of this lobe were prepared for embedding on an automatic tissue processor under a constant vacuum for rapid and complete infiltration of the tissue. Lung tissues were embedded in paraffin and sectioned at 6 μm on a rotary microtome. Complete lobal sections, cut close to the midline of the main bronchus, were used for microscopic examination and grid area determinations. These selected slides were stained with hematoxylin and eosin.

Lung grid area measurements were made by light microscopy using a one-hundred square ocular grid calibrated with a stage micrometer. Total lung parenchymal area was first measured using a dissecting microscope. Lung lesion area measurements were then made using a compound microscope. The magnification factor for the two grid counting systems was 1:8, and all values were corrected accordingly for computation of percent lesion area. We have defined 2 levels of lesion severity in our analyses:

Type 1 Lesion

Two or more cells lie free in the lumen of an alveolus within a single grid area of 10,000 sq. μm. This feature also occurs at small frequency in lungs of rats exposed to clean air, but is properly categorized as a lesion because the proportion of the alveoli bearing free cells increases markedly after exposure to oxidant pollutants.

Type 2 Lesion

Similar to Type 1 except that there is an increase in cellularity of alveolar septae and thickening of the septal wall.

Autoradiography was used to identify sites of cell killing and number of cells killed in nasal epithelial tissues. Thymidine labeled with tritium was incorporated into DNA of dividing cells and subsequently quantified using autoradiographic techniques. Rats were injected intraperitoneally with tritiated thymidine, (^{3}HT, 1 μCi/g body wt.) 18 hrs after exposure. After sacrificing the animals at 48 h post-exposure as described above, the rats were decapitated, and the integument, cartilaginous snout, and lower jaw removed from the heads. The portion of the heads containing intact nasal cavities was immersed in formalin fixative. The head segments were put under slight vacuum during fixation to ensure that all surfaces were in contact with solution. After fixation the heads were decalcified for 3 weeks in TRIS buffered EDTA. Specimens of nasal cavity were prepared by free hand cutting a 2-3 mm slice through the hard palate at the incisive papillae. This slice was perpendicular to the plane of the hard palate and nasal septa producing a block with dorsal-ventral orientation. This slice was embedded anterior face down in glycol-methacrylate, sectioned at 2 μm, dipped in Kodak NTB 2 emulsion, air dried and stored in light tight boxes at refrigerator temperature for 30 days.

After photographic development, slides were stained lightly with Toluidine Blue. The percentage of labeled cells per epithelial cell population in nasal cavity was determined by cell counts of complete sections (20-30 grid fields; 2 mm each) for each animal. The 50 μm separation between sections counted eliminated the possibility of counting portions of the same cell population.

E. Particle Clearance.

Particle clearance from the respiratory tract was measured by depositing radiolabeled polystyrene particles in the rats prior to pollutant exposure (Frager et al., 1979; Phalen et al., 1980; Kenoyer et al., 1981; Mannix et al., 1982; Mannix et al., 1983). The tracer particles were labeled at our laboratory (Hinrichs et al., 1978) with tightly bound ^{51}Cr. These particles were produced from commercially available monodisperse polystyrene latex microspheres (Duke Scientific Corp., Palo Alto, CA). Aerosols were generated from a 0.1% (by volume) aqueous suspension of the particles using a Lovelace compressed air nebulizer (Raabe, 1972). The aerosolized particles were dried

by heating and dilution with filtered air and passed through a ^{85}Kr discharger before entering the nose-only exposure chamber (Raabe et al., 1973). Twenty rats were exposed simultaneously to the radioactive aerosol in this system for 20 min. The aerosol, sampled from the breathing zone of the rats using a calibrated seven-stage impactor, had an activity median aerodynamic diameter of about 1.8 μm and a geometric standard deviation of about 1.2.

After the deposition was completed the rats were removed from the nose-only system and their noses washed to reduce externally deposited radiolabeled particles. The animals were then placed in individual plastic counting tubes and inserted into a collimated 3 in NAI(T1) gamma ray counting apparatus. Upper and lower pulse height discrimination was used to detect the ^{51}Cr gamma rays (320 keV). All of the rats were counted for 100 seconds in this system before they were placed into nose-only exposure tubes and plugged into the chambers for the 4 hr exposures to purified air or the 7-component mixture. After the rats were removed from the exposure chambers their feces were collected at fixed times during the first 50 hr after the deposition of the tracer particles. Early clearance was characterized by the resulting fecal activity excretion curves. During the first 400 hr post deposition of the tracer particles thoracic counts were performed on each animal in order to characterize late clearance. In addition, the rats were sacrificed at 30 days post deposition and their lungs were counted in order to provide another measure of late clearance (termed the A_{30}). Early 50% clearance times ($T_{50\%}$), late clearance biological half-times (T_L), and A_{30} values were determined for each rat. Means and standard deviations were calculated for the purified air and 7-component mixture exposed groups of rats, and the group $T_{50\%}$, T_L, and A_{30} values were compared statistically using two-tailed t-tests.

F. Chemical Characterization of Pulmonary Surfactant.

In the exposure experiments to the combination of ozone and nitrogen dioxide, three variations of the isolation technique for pulmonary surfactant were tested. The first was what we refer to as the "extended" isolation procedure; this is the method recommended by Steim et al. (1969) and involves sucrose density gradient centrifugation (SDGC) to separate mucus as well as dialysis at several stages to remove metals used in the lavage solution, sucrose from the SDGC, etc. The second method, which we refer to as the "full" procedure includes the critical SDGC steps, but omits the dialysis steps. The thrid method, the "abbreviated" procedure, omits both dialysis and SDGC. These methods are described in more detail below.

In all methods, pulmonary surfactant (PS) was obtained using lavage of the lung. After exposure, rats were deeply anesthetized with sodium pentabarbitol IP and the abdominal aorta was cut to drain blood from the heart and lungs. The trachea was cut perpendicular to its length, and a short piece of tygon tubing inserted into the trachea and tied in position. Through this tubing two 7.0 ml volumes of chilled (on ice) 0.15 M saline and containing 0.003 M $MgCl_2$ and 0.003 M $CaCl_2$ were injected and then withdrawn sequentially to obtain the surfactant.

In independent studies, the use of two sequential 7.0 ml lavages was shown not to cause significant cellular disruption; the activity of the enzyme lactate dehydrogenase, a marker for cellular disruption, was measured in each of 10 lavages carried out sequentially on one rat (Bergmeyer et al., 1974). It was only at the 5th-6th lavage that the level of this enzyme rose significantly, indicating that at least 5 sequential lavages were required to produce significant cellular disruption.

The lavage fluid from each group of 4-6 rats was pooled and was stored on ice during the trip from U.C. Irvine to California State University Fullerton (~30 minutes), where the isolation and characterization of PS were carried out. For the "extended" isolation procedure, the method of Steim et al. (1969) was chosen to isolate PS, based on the review of various isolation techniques (Frosolono et al., 1970; Frosolono, 1982). Briefly, the lavage fluid was centrifuged at 600 g for 25 minutes to sediment cellular debris; the supernatant was then recentrifuged at 16,000 g for one hour to sediment the surfactant pellet. The pellet was resuspended in saline and dialyzed overnight against 0.02 M Tris-EDTA at pH 8.0, then against distilled water for 24 hours. This dialyzed pellet was layered over a sucrose solution of density 1.01 and centrifuged at 1.04×10^5 g in a TI50 rotor for one hour. The pellet obtained from this step was resuspended in water, layered on a sucrose solution of density 1.055 and centrifuged for one hour at 8.72×10^4 g in the TI50 rotor. After this step, the surfactant appeared as an opalescent band at the water-sucrose solution interface, and a yellow mucoid material sedimented. The top layer containing the surfactant was drawn off, pelleted out in distilled water with another centrifugation and then relayered on the 1.055 density sucrose solution. After ultracentrifugation for one hour, no mucoid sediment was observed; the surfactant was pelleted out and dialyzed against distilled water. It was then extracted in a 2:1 chloroform-methanol mixture for analysis. All steps in the isolation were carried out at $4^{\circ}C$. Because mucus

was not observed in the second ultracentrifugation on the more dense sucrose solution, this second step was omitted after the first few experiments.

Although Steim et al. (1969) state that 98% of the protein remains in the supernatant when the surfactant is first pelleted out, some protein does pellet out with the surfactant, particularly in the case of the animals exposed to the combination of O_3 and NO_2, where protein levels in the lavage fluid are elevated. This causes an emulsion of denatured protein to form during the last extraction step. This emulsion was allowed to settle at the solvent-water interface and was not drawn off with the surfactant.

The "full" isolation procedure involved the same approach as that in the "extended" procedure, except that the dialysis steps were omitted. The reason for trying to avoid dialysis is twofold; first the possibility that it could introduce spurious oxidation during the isolation procedure, and second, the possibility that it could reduce the yield by adsorption of surfactant on the dialysis tubing. While these points were not addressed by Steim et al. (1969), and discussions with biochemists confirmed that the use of dialysis is an accepted method which they do not expect to introduce artifacts, we felt that if it were not necessary, it should be avoided since small amounts of oxidation products could be formed in the exposure studies.

In the method of Steim et al. (1969), the first dialysis steps against EDTA and then distilled water were apparently intended to remove divalent cations such as Mg^{2+} and Ca^{2+}. However, it was not clear that this was necessary, since these cations are expected to remain in the supernatant after the surfactant is first pelleted out by centrifuging at 16,000 g. To test this, we measured the concentrations of both Mg^{2+} and Ca^{2+} using atomic absorption emission spectrometry in the supernatant as well as in the surfactant which had pelleted out and then been resuspended in water. As expected, essentially all of the Mg^{2+} and Ca^{2+} in the original saline used for lavage was in the supernatant, and undetectable levels were present in the resuspended surfactant. Hence this dialysis step proved unnecessary.

The second dialysis step in the method of Steim et al. (1969) was of the top layer and interface containing the surfactant from the density 1.055 sucrose density gradient centrifugation. This step presumably removed any sucrose which was removed with the interface. However, this dialysis was replaced by adding another ultracentrifugation step in which the surfactant was pelleted out in distilled water. This separated essentially all of the sucrose

which remained dissolved in the supernatant. In addition, since sucrose is not soluble in chloroform/methanol solvent, the final extraction step ensures that sucrose is not carried along with the surfactant. However, this was also checked by examining the infrared spectrum for sucrose bands; as expected, none were observed.

The third method, the "abbreviated" procedure, involves simply removing the cellular debris and pelleting out the surfactant at 16,000 g as in the first two steps of the "extended" and "full" procedures as described above. The material is then extracted into chloroform/methanol as in the final stages of the first two methods. This technique, which has been used quite extensively in the past by researchers in the field of air pollutant exposures, has the advantage that it is simpler, and hence saves time and reduces the risk of sample contamination; it also yields approximately an order of magnitude more material. However, as discussed in more detail below, our results indicate that if examining pulmonary surfactant is the goal, this method is not satisfactory. Thus it appears that extraneous material, likely mucus, accounts for the increased mass yield, and hence any effects observed do not reflect the much smaller amount of surfactant present.

After isolation of the surfactant, the FTIR spectrum was obtained by evaporating an aliquot of the surfactant, dissolved in chloroform/methanol, onto a germanium crystal (25 mm x 10 mm x 2 mm, 45°); a Mattson Sirius 100 spectrometer with 2 cm^{-1} resolution was used, and 64 scans were co-added to improve the signal-to-noise ratio. The UV spectrum was obtained by evaporating the solvent from an aliquot of PS and redissolving it in methanol; the spectrum was scanned using a Varian Model 2200 UV/visible spectrometer. The fatty acid composition was determined by gas chromatographic analysis of the methyl esters formed using the method of Mason and Waller (1964), with calibrations using authentic samples of the methyl esters. In one run, combined GC-MS was also carried out to confirm the identification of the fatty acids.

Finally, surface pressure-surface area isotherms were determined using a Lauda film balance at 21°C on a 0.15 M NaCl subphase. Surface pressure (P) is related to surface tension (t) by the equation:

$$P = t_o - t$$

where t_o is the surface tension of the subphase and t is the surface tension when the monolayer is spread on the subphase.

G. Exposure Design and Schedule

Table 1 shows the exposure experiment plan for this contract. Each exposure experiment has a list of the target test atmospheres to which groups of rats were exposed and the kinds of experimental responses (endpoints) examined. The first year of the contract was devoted to construction and development of the rodent plethysmograph for breathing pattern analyses and the development of the pulmonary surfactant analysis techniques. During the second year, we performed the exposures.

Table 1.

Exposure Experiments for ARB Contract A4-112-32: "Respiratory Effects of Acid Containing Multicomponent Atmospheres"

1. Preliminary oxidant exposure to determine effective sacrifice time for analysis of lung surfactant changes.

Atmospheres: a) Control; b) 0.35 ppm O_3 + 1.3 ppm NO_2.

Endpoints: pulmonary surfactant analysis and breathing pattern analysis.

2. Comparison of acid atmosphere and acid forming oxidant atmospheres

Atmospheres: a) Control; b) 0.35 ppm O_3; c) 0.35 ppm O_3 + 1.3 ppm NO_2; d) 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4.

Endpoints: pulmonary surfactant analysis, breathing pattern analysis, and histopathology.

3. Dose-response of acid forming O3 + NO2 mixture.

Atmospheres: a) Control; b) 0.35 ppm O_3 + 1.3 ppm NO_2; c) Atmosphere b components at 1/2 concentration; d) Atmosphere b components at 1/4 concentration.

Endpoints: breathing pattern analysis.

4. Comparison of 7-component mixture to subsets of components.

Atmospheres: a) Control; b) 7-component mixture: 0.35 ppm O_3, 1.3 ppm NO_2, 2.5 ppm SO_2, 0.01 mg/m^3 $MnSO_4$, 0.5 mg/m^3 $Fe_2(SO)_3$, 0.5 mg/m^3 $(NH_4)_2SO_4$, and 0.5 mg/m^3 carbon aerosol; c) 0.35 ppm O_3; d) 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4.

Endpoints: Breathing pattern analysis and histopathology

5. Dose-response of 7-component mixture.

Atmospheres: a) Control; b) 7-component mixture at concentrations listed in exposure experiment 4, atmosphere b; c) 7-component mixture at 1/2 concentration; d) 7-component mixture at 1/4 concentration.

Endpoints: Breathing pattern analysis and histopathology.

6. Effects of 7-component mixture on mucociliary clearance.

Atmospheres: a) Control; b) 7-component atmosphere.

Endpoints: clearance

III. RESULTS

A. Exposure Atmospheres

Concentrations of pollutant compounds used in the exposures for this contract are given in Tables 2 through 5. The gases O_3, NO_2, and SO_2 were measured in real time and were continuously adjusted to control concentrations at target values in the breathing zone of the rats. Analytical procedures for the vapor acid, HNO_3, and aerosol compounds were more protracted, and many of these compounds were involved in chemical reactions and losses to the surface of the exposure system. Thus, their concentrations were not subject to such tight control and exhibited more variability. Generally pollutant concentrations were close to target values with one important exception. No significant excess acidity could be detected in the particle phase in excess of the background attributable to iron. The small amount of particle acidity, as determined by mass balance analysis was all attributable to nitric acid adsorbed on the particles from the vapor phase. No sulfate in excess of that originally added was detected. The acid component of the 7-component mixtures was primarily in the form of HNO_3 vapor, and being a reaction product of the mixture, HNO_3 was variable between different exposure experiments. The consequence of these results for the interpretation of the experiments was most important in experiment 4. Here the acid mixture HNO_3 + H_2SO_4 selected for comparison to the 7-component mixture had acid concentrations that exceeded acids formed in the 7-component mixture. However because we observed no tissue damage and only minor alterations in breathing pattern in response to this acid mixture, the increased concentrations used do not lead to erroneous conclusions about effects of acids in the 7-component mixture.

B. Plethysmography

The first experiment for the contract was a test of the effects of 0.35 ppm O_3 + 1.3 ppm NO_2. This was a preliminary experiment to test the performance of the rodent plethysmograph and to determine the most effective post-exposure sacrifice time for detecting changes in pulmonary surfactant. Table 6 shows breathing pattern and minute ventilation of rats during the first and fourth hour of the exposure. The effects of upper airway irritants (such as HNO_3) were expected to be immediate while the effects of the lower airway irritants (the oxidants) were expected to appear as rapid and shallow breathing toward the end of the exposure. There was a trend in the exposure group toward slow and deep respiration in the beginning of the exposure with a shift towards

Table 2. Pollutant Concentrations (Mean ± SD) in Exposure Atmospheres for Experiments 1-4.

Exposure Experiment	Group	RH (%)	O_3 (ppm)	NO_2 (ppm)	HNO_3 µg	H_2SO_4 µg	Aerosol Size µm MMAD	GSD
1.	Clean Air	83.9±0.7						
	O_3+NO_2	83.9±0.7	0.36±0.03	1.3±0.1	800±130			
2.	Clean Air	82.6±1.1						
	O_3	84.8±2.1	0.36±0.01					
	HNO_3+ H_2SO_4	84.6±1.4			790±60	500±10	0.15	3.2
	O_3+NO_2	84.5±1.0	0.36±0.01	1.3±0.1	600			
3.	Clean Air	84.6±0.6						
	O_3+NO_2	85.4±1.3	0.34±0.02	1.3±0.1	400±110			
	O_3+NO_2	84.5±0.4	0.19±0.01	0.70±0.01	100±10			
	O_3+NO_2	85.0±1.3	0.09±0.01	0.30±0.01	20±10			
4.	Clean Air	83.6±0.8						
	O_3	84.3±0.4	0.34±0.01					
	HNO_3+ H_2SO_4	83.5±0.7			670±100	390±30	0.17	3.6
	7-Component Mixture	See Table 3.						

Table 3. Pollutant Concentration (Mean ± SD) in 7-Component Atmosphere in Exposure Experiment 4.

Relative Humidity		72.5±0.7
O_3	(ppm)	0.36±0.01
NO_2	(ppm)	1.2±0.01
SO_2	(ppm)	2.5±0.1
Mn^{+2}	($\mu g/m^3$)	3.5
Fe^{+3}	($\mu g/m^3$)	77
Carbon	($\mu g/m^3$)	648
	Compounds Formed or Modified in Reactions	
SO_4^{-2}	($\mu g/m^3$)	600±39
NO_3^{-1}	($\mu g/m^3$)	23.9±8.5
H^+	(μ Eq/m^3)*	0.38±0.13
HNO_3(vapor)	($\mu g/m^3$)	106±18
	Aerosol Characteristics	
Size	(μm MMAD)	0.48
Geometric SD (μm)		3.02
Mass Concentration	($\mu g/m^3$)	1373±99

*H^+ computed by mass balance - all acidity attributable to adsorption of HNO_3 by the particulate phase.

Table 4. Pollutant Concentrations (Mean ± SD) in 7-Component Atmospheres for Experiment 5 (Effects of Concentration).

Relative Humidity (%)	Full Concentration	Half Concentration	Quarter Concentration
Relative Humidity (%)	82.7±0.9	83.1±1.0	82.7±1.1
O_3 (ppm)	0.35±0.02	0.18±0.01	0.09±0.01
NO_2 (ppm)	1.3±0.1	0.7±0.01	0.37±0.04
SO_2 (ppm)	2.5±0.1	1.3±0.01	0.60±0.01
Mn^{+2} ($\mu g/m^3$)	6.4	3.7	0.4
Fe^{+3} ($\mu g/m^3$)	258	152	9.3
Carbon ($\mu g/m^3$)	680±85	515±49	165±35
Compounds Formed or Modified by Reactions			
SO_4^{-2} ($\mu g/m^3$)	574±36	339±31	132±64
NO_3^{-1} ($\mu g/m^3$)	40±9	26±4	17±5
H^+ (μ Eq/m^3)	0.63±0.14	0.41±0.06	0.26±0.08
HNO_3(vapor) ($\mu g/m^3$)	77±9	29±5	12±2
Aerosol Characteristics			
Size (MMAD) (μmMMAD)	0.3	0.3	Not detectable
Geometric SD	3.2	4.6	Not detectable
Mass Concentration ($\mu g/m^3$)	1552±221	788±144	395±93

Table 5. Pollutant Concentrations (Mean ± SD) in 7-Component Atmosphere for Experiment 6 (Effects on Respiratory Tract Clearance).

Relative Humidity (%)		83.3±1.0
O_3	(ppm)	0.36±0.01
NO_2	(ppm)	1.3±0.1
SO_2	(ppm)	2.5±0.1
Mn^{+2}	($\mu g/m^3$)	2.1
Fe^{+3}	($\mu g/m^3$)	90
Carbon	($\mu g/m^3$)	615±120
	Compounds Formed or Modified in Reactions	
SO_4^{-2}	($\mu g/m^3$)	525±33
NO_3^{-1}	($\mu g/m^3$)	8±7
H^+	(μ Eq/m^3)	0.13±0.11
HNO_3(vapor)	($\mu g/m^3$)	96±21
	Aerosol Characteristics	
Size	(μm MMAD)	0.36±0.3
Geometric DS (μm)		2.6±0.2
Mass Concentration ($\mu g/m^3$)		1348±208

Table 6. Breathing Pattern and Ventilatory Responses of Rats Exposed to 0.35 ppm O_3 + 1.3 ppm NO_2 (Experiment 1).

	Hour 1 of Exposure			Hour 4 of Exposure		
	Mean	SE	n	Mean	SE	n
Breath Frequency (min^{-1})						
Clean Air	161	4.4	8	161	9.4	8
0.35 ppm O_3 + 1.3 ppm NO_2	152	4.0	7	173	10.1	7
Tidal Volume (ml)						
Clean Air	0.96	0.05	7	0.99	0.05	7
0.35 ppm O_3 + 1.3 ppm NO_2	1.08	0.09	7	0.90	0.10	7
Minute Ventilation ($ml \cdot min^{-1}$)						
Clean Air	157	13	7	166	13	7
0.35 ppm O_3 + 1.3 ppm NO_2	163	11	7	153	12	7

rapid and shallow respiration toward the end of the exposure, but none of the respiratory variables were significantly different from control clean air exposure (t-tests, $p < 0.05$).

Tables 7 and 8 show breathing pattern, ventilation, and metabolic rates of rats exposed in experiment 2 to oxidant and acid subcomponents of the 7-component mixture. There were significant differences in breathing pattern responses to these atmospheres (Table 8). During the first hour of exposure the acid mixture and the $O_3 + NO_2$ mixture induced a shift toward slow and deep breathing which was significant for a tidal volume increase in the $O_3 + NO_2$ exposure. The O_3 exposure group showed significantly increased breath frequency at the end of exposure, but tidal volume was unchanged. Changes in breathing pattern resulted in changes in minute ventilation, and while these were not statistically significant changes, ventilation equivalent for oxygen consumption (V_E/V_{O2}) did show significant changes in the final hour of exposure. $O_3 + NO_2$ exposure resulted in depression of V_E/V_{O2} while exposure to O_3 alone or $HNO_3 + H_2SO_4$ resulted in an increased V_E/V_{O2}.

Exposure to the series of $O_3 + NO_2$ mixtures differing in concentration by successive factors of 2 (experiment 3) gave no significant differences in breathing pattern, metabolic rate or ventilation equivalent for oxygen (Table 9 and 10). At the time of this exposure, measurement of core body (rectal) temperature was added to the battery of physiological variables recorded, and no effects of the oxidants on body temperature were observed in this exposure. The highest exposure concentration, which was equivalent to concentrations of O_3 and NO_2 used in experiments 1 and 2, showed a trend similar to the earlier exposures, with depressed frequency in hour 1, and depressed frequency, tidal volume, minute ventilation, and ventilation equivalent for O_2 in hour 4. However, these trends were not statistically significant and this emphasizes the fact that effects observed in $O_3 + NO_2$ exposures in experiments 1 and 2, although significant, were small.

Exposure to the full 7-component mixture and selected subcomponents was performed in Experiment 4 (Tables 11 and 12). There were no significant effects of these atmospheres during the first hour of exposure. However, significant effects were present by the fourth hour. These included increased breathing frequency with greatest development for the 7-component mixture, a trend (but not significant shift) toward decreased tidal volume in the 7-component group, significant increase in minute ventilation in the O_3 exposure group, depression in oxygen consumption in the 7-component exposure group, and

Table 7. Breathing Pattern, Ventilatory, and Metabolic Responses of Rats Exposed to Mixed Acid and Oxidant Pollutants (Experiment 2).

	Hour 1 of Exposure			Hour 4 of Exposure		
	Mean	SE	n	Mean	SE	n
Breath Frequency (min^{-1})						
Clean Air	148	7.3	8	145	7.2	8
0.35 ppm O_3	156	10.8	8	175	8.5	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	138	12.4	7	139	6.0	7
0.35 ppm O_3 + 1.3 ppm NO_2	133	4.5	7	140	4.9	7
Tidal Volume (ml)						
Clean Air	1.00	0.08	8	0.82	0.07	8
0.35 ppm O_3	1.06	0.06	8	0.82	0.06	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	1.13	0.06	7	0.95	0.04	7
0.35 ppm O_3 + 1.3 ppm NO_2	1.23	0.06	7	0.79	0.11	7
$\dot{V}_E$ (ml/min)						
Clean Air	143	8.1	8	117	10.5	8
0.35 ppm O_3	162	11.2	8	142	8.1	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	155	13.6	7	131	3.6	7
0.35 ppm O_3 + 1.3 ppm NO_2	162	4.9	7	109	12.9	7
$\dot{V}_{O2}$ (ml/kg/min)						
Clean Air	28.6	1.3	8	26.9	1.0	8
0.35 ppm O_3	27.9	1.1	8	26.2	0.7	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	27.4	0.8	7	26.0	0.9	7
0.35 ppm O_3 + 1.3 ppm NO_2	28.5	0.7	7	27.0	0.7	7
$\dot{V}_E/\dot{V}_{O2}$ (ml/ml)						
Clean Air	20.8	1.8	8	18.5	2.2	8
0.35 ppm O_3	22.6	1.6	8	21.8	1.3	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	23.0	2.1	7	21.8	0.6	7
0.35 ppm O_3 + 1.3 ppm NO_2	22.0	0.9	7	15.9	1.5	7

Table 8. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Responses in Experiment 2 ($p < 0.05$).[1]

Variable	Hour 1 of Exposure	Hour 4 of Exposure
Breath Frequency	NS	Clean Air; O_3+NO_2; $HNO_3+H_2SO_4$; O_3
Tidal Volume	Clean Air; O_3; $HNO_3+H_2SO_4$; O_3+NO_2	NS
Minute Ventilation	NS	NS
$\dot{V}_{O2}$	NS	NS
$\dot{V}_E/\dot{V}_{O2}$	NS	O_3+NO_2; Clean Air; $HNO_3+H_2SO_4$; O_3

[1]Overhead lines cluster groups not significantly different from each other.
NS is not significant.

Table 9. Breathing Pattern, Ventilatory, and Metabolic Responses of Rats Exposed to Different Concentrations of Combined O_3 + NO_2 (Experiment 3).

	Hour 1 of Exposure			Hour 4 of Exposure		
	Mean	SE	n	Mean	SE	n
Breath Frequency (min^{-1})						
Clean Air	156	9	8	148	8	8
0.35 ppm O_3 + 1.3 ppm NO_2	130	6	8	139	14	8
0.18 ppm O_3 + 0.7 ppm NO_2	151	10	8	155	8	8
0.09 ppm O_3 + 0.3 ppm NO_2	156	6	8	142	8	8
Tidal Volume (ml)						
Clean Air	1.10	0.08	8	0.98	0.07	8
0.35 ppm O_3 + 1.3 ppm NO_2	1.10	0.10	8	0.94	0.08	8
0.18 ppm O_3 + 0.7 ppm NO_2	1.07	0.06	8	1.04	0.10	8
0.09 ppm O_3 + 0.3 ppm NO_2	1.01	0.05	8	0.98	0.06	8
$\dot{V}_E$ (ml/min)						
Clean Air	167	12	8	143	9	8
0.35 ppm O_3 + 1.3 ppm NO_2	143	16	8	126	13	8
0.18 ppm O_3 + 0.7 ppm NO_2	156	10	8	156	8	8
0.09 ppm O_3 + 0.3 ppm NO_2	158	8	8	135	7	8
$\dot{V}_{O2}$ (ml/kg/min)						
Clean Air	29.4	0.7	8	28.3	1.0	8
0.35 ppm O_3 + 1.3 ppm NO_2	27.6	1.2	8	26.0	1.3	8
0.18 ppm O_3 + 0.7 ppm NO_2	28.8	1.0	8	26.6	0.8	8
0.09 ppm O_3 + 0.3 ppm NO_2	27.2	1.0	8	26.3	1.1	8
$\dot{V}_E/\dot{V}_{O2}$ (ml/ml)						
Clean Air	22.5	1.6	8	20.3	1.0	8
0.35 ppm O_3 + 1.3 ppm NO_2	20.4	1.7	8	18.7	1.2	8
0.18 ppm O_3 + 0.7 ppm NO_2	22.1	1.5	8	21.7	0.9	8
0.09 ppm O_3 + 0.3 ppm NO_2	21.7	0.8	8	19.7	1.4	8
Body Temperature ^oC						
Clean Air	38.7	0.4	7	38.3	0.2	7
0.35 ppm O_3 + 1.3 ppm NO_2	38.2	0.2	4	37.7	0.4	4
0.18 ppm O_3 + 0.7 ppm NO_2	38.0	0.9	7	38.1	0.2	7
0.09 ppm O_3 + 0.3 ppm NO_2	38.7	0.3	5	38.1	0.1	5

Table 10. ANOVA Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Responses in Experiment 3 ($p < .05$). NS is not significant.

Variable	Hour 1 of Exposure	Hour 4 of Exposure
Breath Frequency	NS	NS
Tidal Volume	NS	NS
Minute Ventilation	NS	NS
$\dot{V}_{O2}$	NS	NS
$\dot{V}_E/\dot{V}_{O2}$	NS	NS
Body Temperature	NS	NS

Table 11. Breathing Pattern, Ventilatory, and Metabolic Responses of Rats Exposed to 7-Component Mixed Acid & Oxidant Pollutants & Subsets of the Mixture (Experiment 4).

	Hour 1 of Exposure			Hour 4 of Exposure		
	Mean	SE	n	Mean	SE	n
Breath Frequency (min^{-1})						
Clean Air	138	5.7	8	134	5.1	8
0.35 ppm O_3	145	8.5	8	160	13.1	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	136	3.3	8	147	3.5	8
7-Component Mixture	137	5.4	8	166	9.1	8
Tidal Volume (ml)						
Clean Air	0.99	0.05	7	0.93	0.05	7
0.35 ppm O_3	1.06	0.06	8	0.94	0.08	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	1.02	0.03	8	0.89	0.05	8
7-Component Mixture	1.08	0.19	8	0.79	0.05	8
$\dot{V}_E$ (ml/min)						
Clean Air	139	11.6	7	121	7.4	7
0.35 ppm O_3	151	7.7	8	144	5.9	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	139	5.2	8	130	5.4	8
7-Component Mixture	146	6.2	8	128	4.8	8
$\dot{V}_{O2}$ (ml/kg/min)						
Clean Air	32.3	1.2	8	31.3	1.2	8
0.35 ppm O_3	31.3	0.9	8	29.7	0.7	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	30.6	1.2	8	29.1	0.5	8
7-Component Mixture	28.8	0.6	8	24.5	0.7	8
$\dot{V}_E/\dot{V}_{O2}$ (ml/ml)						
Clean Air	16.8	1.2	7	15.3	1.0	7
0.35 ppm O_3	18.2	0.8	8	18.0	0.6	8
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	16.4	0.6	8	16.4	0.6	8
7-Component Mixture	19.0	0.8	8	19.0	0.8	8
Body Temperature ^oC						
Clean Air	38.2	0.1	6	37.9	0.1	6
0.35 ppm O_3	38.0	0.1	6	37.4	0.2	6
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	38.3	0.1	8	38.0	0.1	8
7-Component Mixture	38.0	0.2	8	37.6	0.1	8

Table 12. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Responses in Experiment 4 ($p < 0.05$).[1]

Variable	Hour 1 of Exposure	Hour 4 of Exposure			
Breath Frequency	NS	Clean Air	HNO_3+ H_2SO_4	O_3	7-Comp.
Tidal Volume	NS		NS		
Minute Ventilation	NS	Clean Air	HNO_3+ H_2SO_4	7-Comp.	O_3
$\dot{V}_{O2}$	NS	Clean Air	O_3	HNO_3+ H_2SO_4	7-Comp.
$\dot{V}_E/\dot{V}_{O2}$	NS	Clean Air	HNO_3+ H_2SO_4	O_3	7-Comp.
Body Temperature	NS		NS		

[1]Overhead lines cluster groups not significantly different from each other.
NS is not significant.

elevation of ventilation equivalent for oxygen in the O_3 and 7-component mixture groups.

The final exposure involving breathing pattern analysis (experiment 5) was a comparison of the 7-component mixture at different concentrations varying by factors of 2 (Tables 13 and 14). Breathing pattern showed a shift to rapid and shallow respiration in the fourth hour of exposure that was significantly different from control for the full concentration mixture. Minute ventilation was significantly elevated during both time periods for the half concentration group.

C. Histopathology

Lung tissue injury from pollutant exposures was analyzed in experiments 2, 3, and 5. The comparison of oxidant and acid components (experiment 2) is shown in Table 15. There were no significant differences in Type 1 lesion areas due to exposure to any of the pollutants, however the oxidant pollutants, 0.35 ppm O_3 and 0.35 ppm O_3 + 1.3 ppm NO_2 induced a small percentage of Type 2 lesions. There was no significant difference between lesion areas induced by the two oxidant exposures ($p < 0.05$).

Table 16 shows a comparison of the two component acids and O_3 to the full 7-component mixture (experiment 3). In parallel to the results of the previous experiment, there were no significant differences in Type 1 lesion areas, and the two atmospheres containing O_3 (O_3 alone and 7-component mixture) induced Type 2 lesions, but there was no significant difference between the effects of the two atmospheres.

A comparison of the 7-component mixture at different concentrations experiment 5, Table 17), showed significant induction of both types of parenchymal lesions from exposure to the full concentration mixture (ANOVA and Tukey multiple comparisons, $p < 0.05$), but lower concentrations did not induce significant lung tissue lesion areas.

Analysis of nasal epithelial injury was performed in exposures to acid mixtures (Experiments 2, 4, and 5). Epithelial injury was measured by cell turnover rate as indexed by tritiated thymidine incorporation (Table 18). There were no significant differences in epithelial labeling index among exposure groups of these experiments.

Table 13. Breathing Pattern, Ventilatory, and Metabolic Response of Rats Exposed to Different Concentrations of the 7-Component Mixture (Experiment 5).

	Hour 1 of Exposure			Hour 4 of Exposure		
	Mean	SE	n	Mean	SE	n
Breath Frequency (min^{-1})						
Clean Air	125	4.2	8	131	7.7	8
Full Concentration	130	5.9	8	161	6.9	8
½ Concentration	144	3.4	8	149	6.8	8
¼ Concentration	143	6.5	8	145	4.9	8
Tidal Volume (ml)						
Clean Air	1.04	0.04	8	0.94	0.04	8
Full Concentration	1.08	0.05	8	0.73	0.08	8
½ Concentration	1.08	0.05	8	0.97	0.04	8
¼ Concentration	0.99	0.08	8	0.96	0.06	8
$\dot{V}_E$ (ml/min)						
Clean Air	129	3.1	8	121	5.9	8
Full Concentration	139	5.6	8	114	8.6	8
½ Concentration	154	7.2	8	144	7.6	8
¼ Concentration	138	5.4	8	139	7.7	8
$\dot{V}_{O2}$ (ml/kg/min)						
Clean Air	26.5	0.9	8	27.5	1.3	8
Full Concentration	27.6	0.8	8	25.9	1.0	8
½ Concentration	29.3	1.1	8	29.3	1.6	8
¼ Concentration	27.9	0.8	8	28.8	0.9	8
$\dot{V}_E/\dot{V}_{O2}$ (ml/ml)						
Clean Air	19.0	0.5	8	17.4	0.4	8
Full Concentration	19.1	0.6	8	16.4	0.7	8
½ Concentration	14.2	0.8	8	17.9	0.7	8
¼ Concentration	17.6	0.8	8	16.8	0.6	8
Body Temperature (°C)						
Clean Air	38.6	0.1	7	38.1	0.1	7
Full Concentration	38.5	0.1	7	37.9	0.1	7
½ Concentration	38.4	0.1	8	38.1	0.1	8
¼ Concentration	38.4	0.1	8	38.1	0.1	8

Table 14. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Responses in Experiment 5 ($p < 0.05$).[1]

Variable	Hour 1 of Exposure	Hour 4 of Exposure
Breath Frequency	NS	Clean ¼ ½ Full
Tidal Volume	NS	Clean ¼ ½ Full
Minute Ventilation	Clean ¼ Full ½	Clean ¼ Full ½
$\dot{V}_{O2}$	NS	NS
$\dot{V}_E/\dot{V}_{O2}$	NS	NS
Body Temperature	NS	NS

[1]Overhead lines cluster groups not significantly different from each other. NS is not significant.

Table 15. Lung Parenchymal Lesion Injury Resulting from Exposure to O_3, HNO_3 + H_2SO_4, and O_3 + NO_2 (Experiment 2). Rats were exposed nose-only for 4 h.

Rat Exposure Group		Type 1 Lesion (Area %)		Type 2 Lesion (Area %)	
	n	Mean	SE	Mean	SE
Clean Air	10	2.13	0.30	0.0	0.0
1.0 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	9	2.45	0.22	0.0	0.0
0.35 ppm O_3	10	2.55	0.31	0.60	0.29
0.35 ppm O_3 + 1.3 ppm NO_2	10	2.56	0.25	0.22	0.09

Table 16. Lung Parenchymal Lesions resulting from Exposure to O_3, HNO_3 + H_2SO_4, and 7-Component Mixture (Experiment 4).

Rat Exposure Group		Type 1 Lesion (Area %)		Type 2 Lesion (Area %)	
	n	Mean	SE	Mean	SE
Clean Air	9	2.19	0.23	0.0	0.0
1.0 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	10	2.64	0.29	0.0	0.0
0.35 ppm O_3	9	2.48	0.31	1.24	0.41
7-Component Mixture	9	3.17	0.45	0.93	0.43

Table 17. Lung Parenchymal Lesions Resulting from Exposure to 7-Component Mixture at Varying Concentrations (Experiment 5).

Rat Exposure Group		Type 1 Lesion		Type 2 Lesion	
	n	Mean	SE	Mean	SE
Clean Air	9	1.68	0.16	0.0	0.0
7-Component, Full Concentration	10	3.71	0.28	0.63	0.19
7-Component, ½ Concentration	9	2.53	0.26	0.05	0.04
7-Component, ¼ Concentration	9	2.18	0.22	0.0	0.0

Table 18. Nasal Epithelial Injury from 7-Component and Subcomponent Mixture Exposures (Experiments 2, 4, and 5).

		Nasal Respiratory Epithelium	
		Label Index (% of Labeled Cells)	
	n	Mean	SE
Experiment 2			
Clean Air	8	1.02	0.19
0.35 ppm O_3	5	1.71	0.28
1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	6	1.11	0.18
0.35 ppm O_3 + 1.3 ppm NO_2	4	1.02	0.23
Experiment 4			
Clean Air	7	0.78	0.14
1.0 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	9	0.72	0.11
7-Component	7	0.56	0.13
Experiment 5			
Clean Air		0.10	0.10
7-Component, Full Concentration		0.25	0.21
7-Component, ½ Concentration		0.10	0.08
7-Component, ¼ Concentration		0.06	0.08

D. Particle Clearance

The results of the experiment performed to evaluate the effects of the 7-component mixture on early and late clearance in the rat are shown in Table 19. The data from the fecal analysis indicate that the rats exposed to the 7-component mixture did not exhibit a statistically significant ($p < 0.1$) alteration in early (upper respiratory tract) clearance as compared to the purified air exposed rats. The mean early 50% clearance time ($T_{50\%}$) for the 7-component mixture exposed rats was nearly the same as that of the purified air exposed rats. The two measures of late (deep lung) clearance (from decline in thoracic counts and 30 day postdeposition lung counts) showed a trend toward accelerated clearance in the 7-component mixture exposure but neither change was statistically significant.

E. Chemical Characterization of Pulmonary Surfactant

In initial studies, the "extended" isolation procedure of Steim et al. (1969) was shown to give material which had the necessary characteristics of surfactant (Goerke, 1974; Kotas, 1982) in terms of surface tension reducing properties, fatty acid composition, and UV spectrum. While the infrared spectrum had not been reported in the literature, it was found to be very similar to that of phosphatidylcholines, the major component of surfactant. Thus this method was used in most of the exposure studies.

In later work, the use of the "abbreviated" versus the "full" isolation procedures was tested on the lavage fluids from rats exposed to the O_3 + NO_2 combination at 85% relative humidity. This is also described below.

1. Experiment 1. Exposure to O_3 + NO_2: Effect of Post-Exposure Sacrifice Time

Exposure to air pollutants may alter the composition of pulmonary surfactant either by reacting directly with it, or by inducing a biological response which then alters the surfactant composition. It is expected that a direct chemical reaction would be observed immediately after the exposure, while an inflammatory response might be delayed and peak some hours after the exposure.

TABLE 19

EFFECTS OF THE 7-COMPONENT MIXTURE ON EARLY AND LATE CLEARANCE OF RADIOLABELED TRACER PARTICLES

Early Clearance	Number*	$\overline{T}_{50\%}$ ± SD(hr)	$\Delta T_{50\%}$ ± SE(hr)	p†
Purified Air, 85% RH	29	10.0±1.5	----	----
7-Component Mixture	29	10.2±1.8	0.2±0.4	0.64
Late Clearance	**Number**	$\overline{T}_L$ ± SD(hr)	ΔT_L ± SE(hr)	p†
Purified Air, 85% RH	30	617±187	----	----
7-Component Mixture	30	553±178	-64±47	0.18
A_{30} (Index of Late Clearance)	Number	$\overline{A}_{30}$ ± SD	ΔA_{30} ± SE	p†
Purified Air, 85% RH	30	21.3±3.8	----	----
7-Component Mixture	30	20.8±3.6	-0.5±1.0	0.65

* Two rats were excluded from the early clearance data analysis for failure to meet established defecation and water consumption criteria.

† Two-tailed t-test.

To test whether direct reaction or a biological response, or both, occurred on exposure to O_3 + NO_2 at high relative humidities, rats exposed to the atmosphere in Experiment 1 (Table 20) were sacrificed at three different times, 0, 24, and 48 hours, after the exposure. The 24 and 48 hour groups were kept in clean air after the exposure. The O_3 and NO_2 had mixed for approximately four minutes prior to reaching the exposure ports, where their concentrations were measured, and hence had reacted to form the NO_3 radical and N_2O_5 at calculated concentrations of 1.1×10^2 ppt and 1.7×10^2 ppb respectively, based on the measured change in the ozone concentration when the NO_2 was turned off at the end of the experiment, and the measured HNO_3 concentration of 0.56 ± 0.47 mg/m^3 (220 ± 20 ppb).

Because of the extremely small yields obtained from the pooled lavage fluids of 4-6 rats, typically 0.1 - 0.4 mg, it was not possible in one experiment to examine the surfactant from individual rats or from two or more groups in order to assess individual variability in the data. In order to address this variability, we have relied as much as possible on duplication of experiments. Since the goals of the studies focused on a qualitative assessment of changes in the surfactant, this was a satisfactory first approach.

a. FTIR and UV Spectroscopy

In a variety of both in vivo and in vitro (i.e., model compound) studies, we have found FTIR and UV spectroscopy to be very useful and sensitive techniques for detecting oxidation products. However, because of their sensitivity and the very small yields of surfactant available for analysis, great care must be taken to avoid contamination and/or oxidation during sample isolation. Hence reproducibility from one set of experiments to another must be established in order to confirm any observed changes. We summarize here the changes we have observed in the infrared and ultraviolet spectra which have been consistent from one experiment to another. In particular, we find that the appearance of certain infrared bands seems to be associated with UV absorptions which have been reported in the literature to be indicative of the formation of lipid oxidation products. Lung surfactant is a complex biological material of variable molecular composition. Many of the components are not well characterized. A quantitative spectral analysis of surfactants obtained in this study would have been beyond the scope of the proposed work because this would have required establishing identities and extinction coefficients for all of the relevant absorbing species. The qualitative spectral analyses,

Table 20. Summary of Conditions of In Vivo Exposures. Exposures for Pulmonary Surfactant Experiments.

Exposure Experiment No.	Atmospheres	Conditions
1.	a) Clean Air b) 0.35 ppm O_3 + 1.3 ppm NO_2	Three groups of 4-6 rats exposed to (O_3 + NO_2) were sacrificed at 0, 24 and 48 hours respectively after the exposure; pure air exposed rats were sacrificed at 0 hours. Surfactant was isolated using"extended"procedure.
2.	a) Clean Air b) 0.35 ppm O_3 + 1.3 ppm NO_2 c) 0.35 ppm O_3 d) 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4	Clean air group, (O_3 + NO_2), O_3 only and acid exposed groups sacrificed 44 h after exposure; an additional (O_3 + NO_2) exposed group was sacrificed at 0 hr. Surfactant was isolated using "extended"procedure.
3.	a) Clean Air b) 0.35 ppm O_3 + 1.3 ppm NO_2	Same conditions as experiment #1; surfactant was isolated using "abbreviated" and the "full" procedures.

when coupled to the detailed chemical analyses, were able to provide the data required for the evaluation of the changes of interest in surfactants caused by oxidant/acid exposures.

The region of the infrared spectrum which has proven to be most useful is the 2000 cm^{-1} to 1200 cm^{-1} region. Figure 1a shows the spectrum from 2000 cm^{-1} to 800 cm^{-1} of a typical surfactant isolated from rats exposed to clean air; this can be compared to the spectrum of a commercially available sample of dipalmitoyl phosphatidylcholine (DPPC) shown in Figure 1b where the functional groups responsible for the major absorption bands are identified. Clearly, the spectrum of surfactant is very similar to that of DPPC, as expected.

Figure 1c shows the spectrum of surfactant from rats which have been exposed to O_3 + NO_2 and sacrificed 48 hours after the exposure. The arrows mark the regions where new bands have appeared or changes in existing bands are noted.

Figure 2 shows the UV absorption spectra of the surfactant obtained from the clean air controls as well as the exposed animals in Experiment 1 (Table 20). For comparison, the spectrum of a commercial sample of DPPC is shown in Figure 3. The spectrum of surfactant from the clean air control looks similar to that of the DPPC, as expected. However, the surfactant from the exposed rats shows a continuous increase in absorption at approximately 235 nm and 275 nm. The same qualitative trend was observed, although not as pronounced, when the exposure was repeated but a modified isolation procedure (the "full" procedure) was used (Experiment 3, Table 20).

b. Fatty Acid Composition

Table 21 shows the composition of the surfactant as a ratio to palmitic acid, the major fatty acid present. There was a consistent drop in the amounts of stearic, oleic, and linoleic acids on exposure to the air pollutants, with the maximum effect occurring 24 hours after the exposure. When the exposure was repeated, but the "full" isolation procedure was used in place of the "extended" procedure, the same qualitative trend was observed, but it was not as pronounced as in Experiment 1.

FIGURE 1 : FTIR SPECTRA OF PULMONARY SURFACTANT FROM RATS AND OF L-α-DIPALMITOL PHOSPHATIDYLCHOLINE(DPPC)

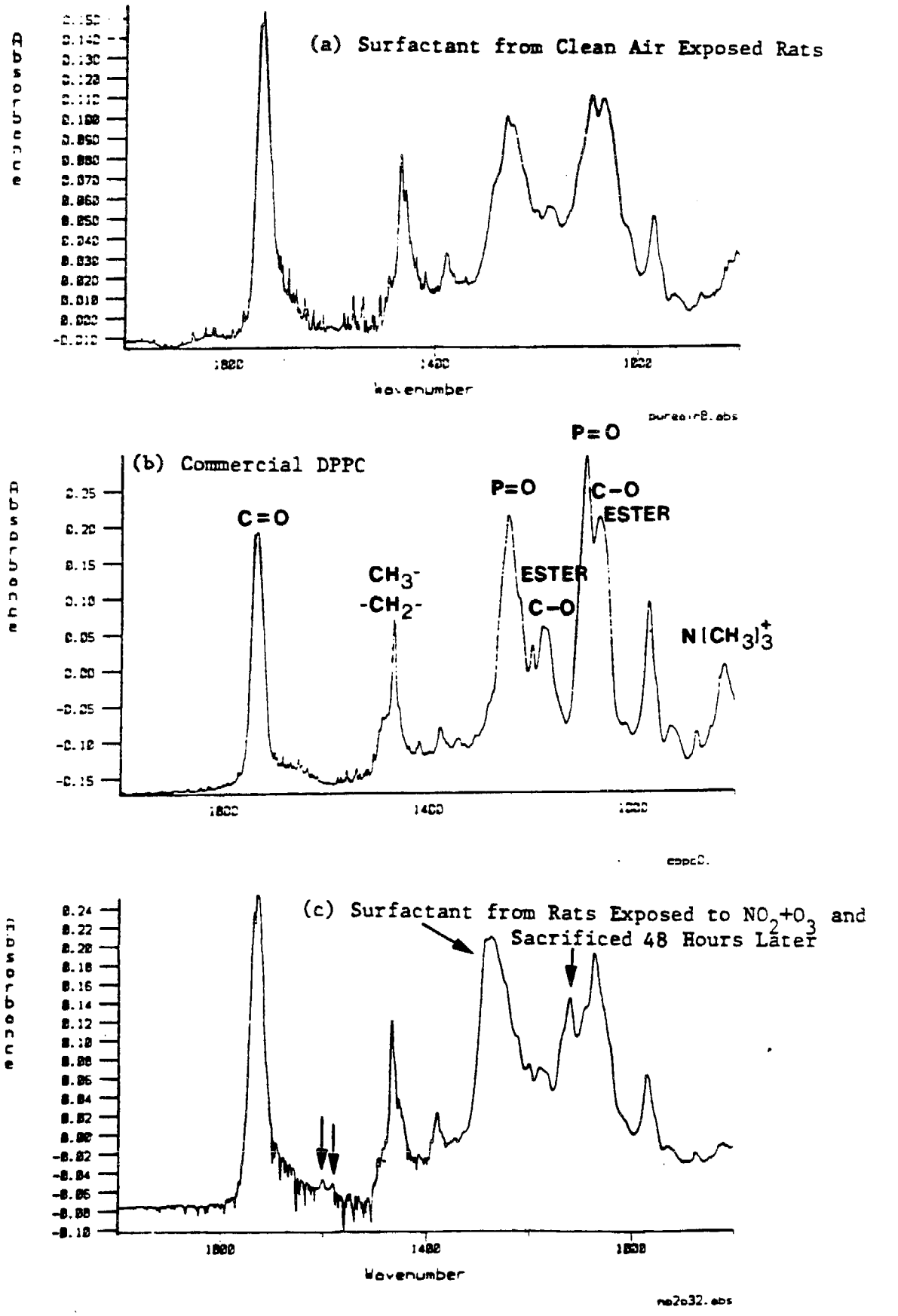
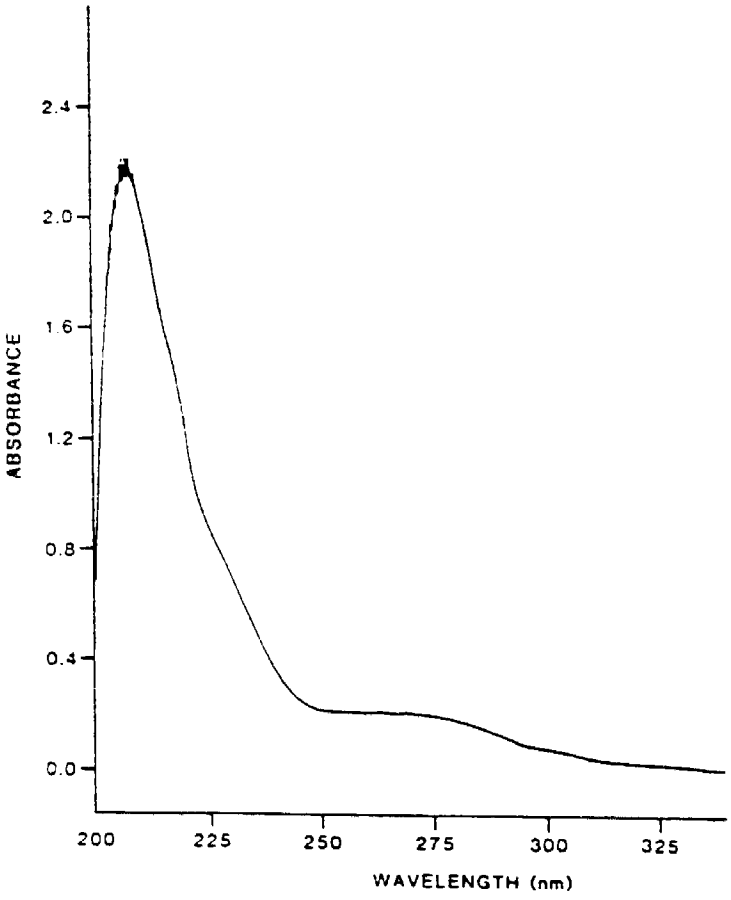
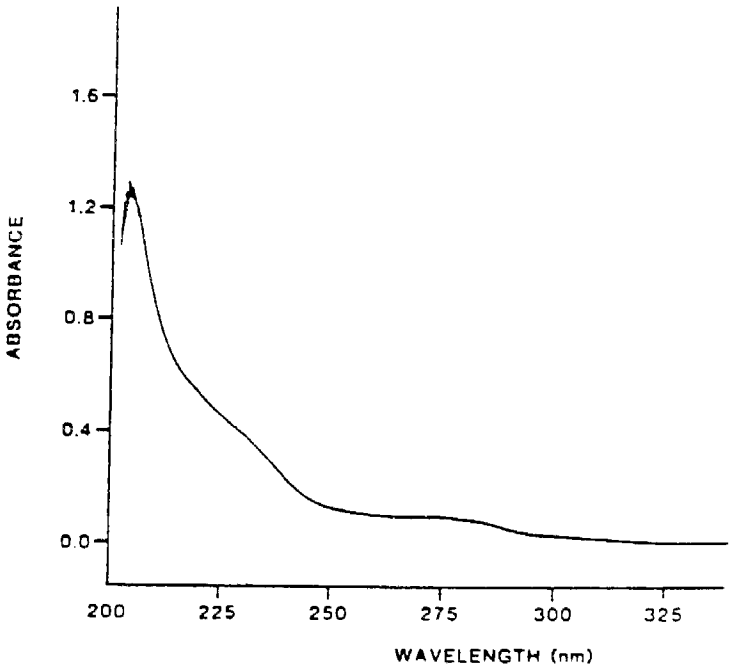


FIGURE 2: ULTRAVIOLET SPECTRA OF SURFACTANT FROM RATS EXPOSED TO CLEAN AIR OR TO NO_2+O_3 AND SACRIFICED AT 0,24 OR 48 HOURS LATER

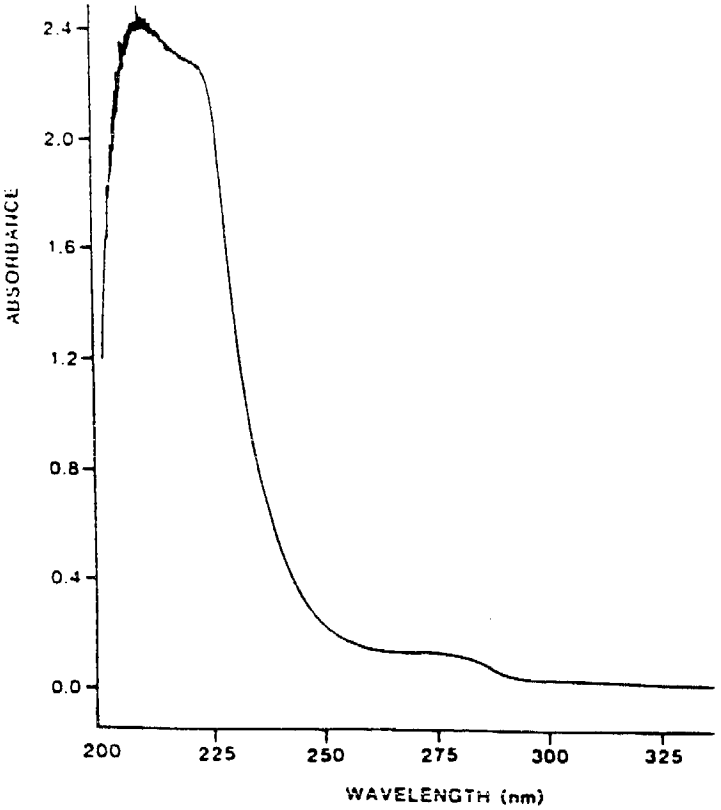
UV Spectra of Surfactant from Pure Air Exposed Rats
(3.5 mg/ml in MeOH)



UV Spectra of Surfactant from NO_2/O_3 Exposed Rat
0 Hours (0.35 mg/ml in MeOH)



UV Spectra of Surfactant from NO_2/O_3 Exposed Rats,
24 Hours (0.35 mg/ml in MeOH)



UV Spectra of Surfactant from NO_2/O_3 Exposed Rats,
48 Hours (0.35 mg/ml in MeOH)

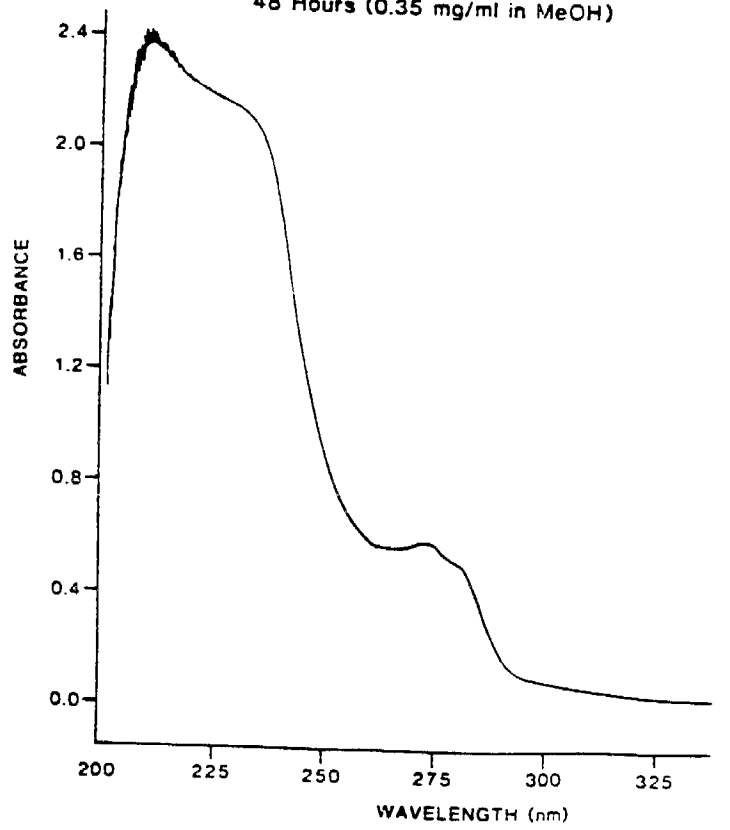


FIGURE 3: Ultraviolet Absorption Spectrum of L-α-Dipalmitoyl Phosphatidylcholine (0.35 mg/ml)

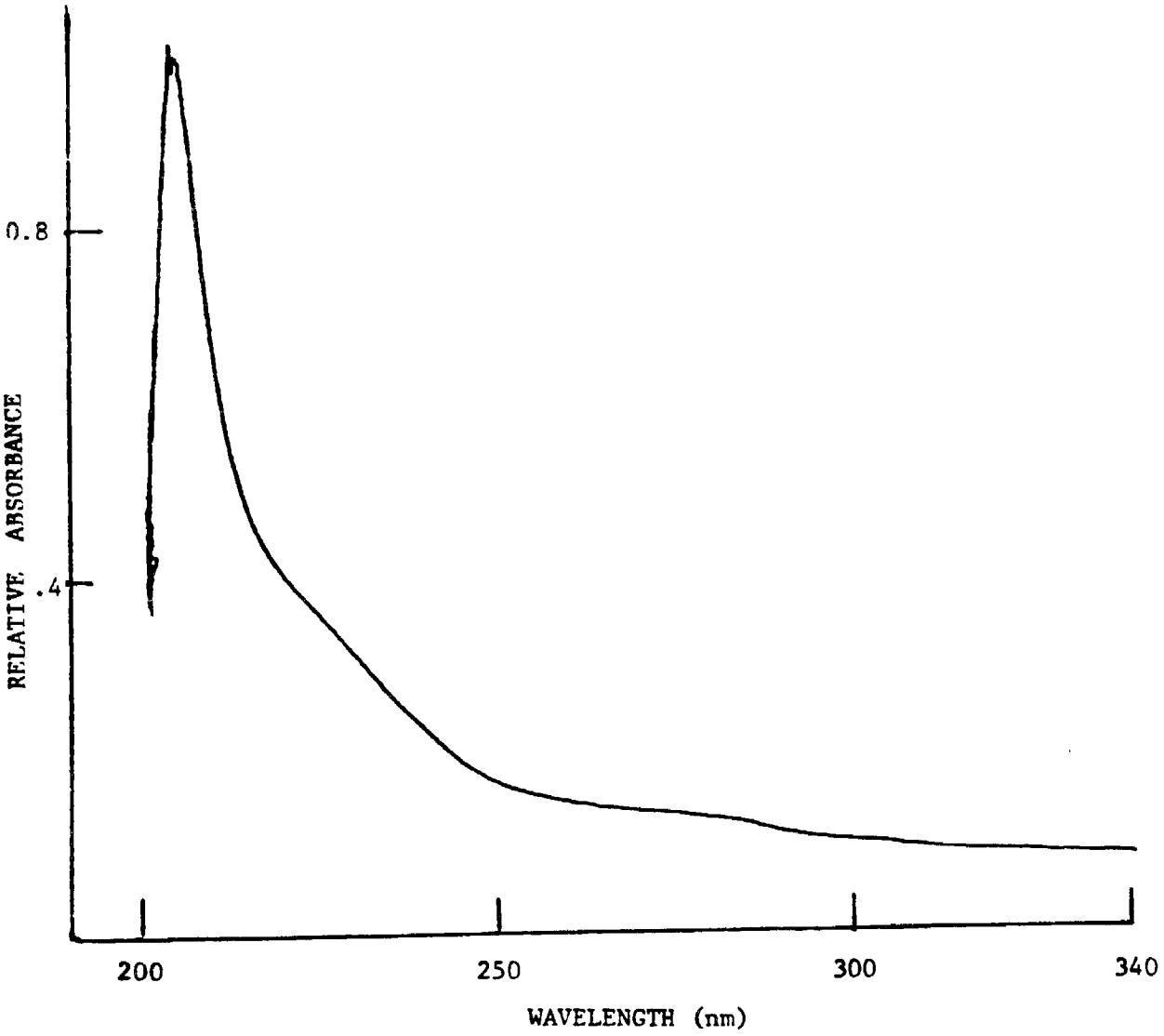


Table 21. Fatty Acid Composition of Pulmonary Surfactant of Rats Exposed to Clean Air or to a Combination of O_3 (0.35 ppm) and NO_2 (1.3 ppm) at 85% Relative Humidity for 4 Hours and Sacrificed at 0, 24 and 48 Hours After the Exposure, Respectively.

Experiment Number (Table 20)	Time of Sacrifice and Atmosphere	10^2 x Molar Ratio[a] Stearic / Palmitic	Oleic / Palmitic	Linoleic / Palmitic
1.	0 Hrs; Clean Air	12	14	36
2.		19	14	27
3.		26	8.3	8.8
1.	0 Hrs; O_3 + NO_2 Exposed	6.3	2.9	2.2
2.		9.9	9.8	13
3.		26	9.9	12
1.	24 Hrs; O_3 + NO_2 Exposed	3.3	0.7	<0.3
3.		17	6.3	6.9
1.	48 Hrs; O_3 + NO_2 Exposed	3.9	2.6	<1
2.		6.0	5.7	13
3.		40	12	13

[a]Typical analytical errors in these measurements are ±15%.

c. Surface Pressure-Area Isotherms

Figure 4 shows typical surface pressure-surface area isotherms of surfactant from rats exposed to clean air or to the O_3 + NO_2 combination and sacrificed at 0 or 44 hours after the exposure (Experiment 2, Table 20). While no decrease in the peak surface pressure was observed in the exposed animals, the plateau around 15-20 dynes/cm which is typical both of surfactant from clean air exposed animals as well as samples of commercially available DPPC (Figure 5), disappeared in the exposed samples. This loss of the plateau was most prominent at 48 hours.

2. Comparison of Exposure to O_3, O_3 + NO_2, or HNO_3 + H_2SO_4

The results of Experiment 1 (Table 20) suggested that recovery of the animals from the exposure was not complete 44-48 hours after the exposure. In addition, the UV and FTIR bands observed at that time (Figures 1 and 2) indicated that maximum oxidation products might be observed at the 44-48 hour point. Thus, the intercomparison of the effects of exposure to O_3 alone, the O_3 + NO_2 combination and the acid mixture was carried out by sacrificing the animals 44 hours after the exposure.

a. FTIR and UV Spectroscopy

Figure 6 shows the UV spectra of each of the samples. For unknown reasons, the clean air controls showed strong UV absorptions at 235 and 275 nm, characteristic of the presence of oxidized products. The UV spectra of the acid and ozone only exposed samples also showed evidence of oxidation products, while surprisingly, the O_3 + NO_2 exposed sample showed less.

The FTIR spectra (Figure 7) appear to parallel the UV spectra, in that the samples showing strong UV bands also show the new IR bands at 1600 and 1580 cm^{-1}, which also appear to be characteristic of the presence of oxidation products.

b. Fatty Acid Composition

Table 22 gives the fatty acid composition of the clean air controls as well as of the surfactant from rats exposed to each of the atmospheres. Given the approximately 15% estimated analytical error, the effects of O_3 alone and O_3 + NO_2 are considered to be within experimental error of each other. Both

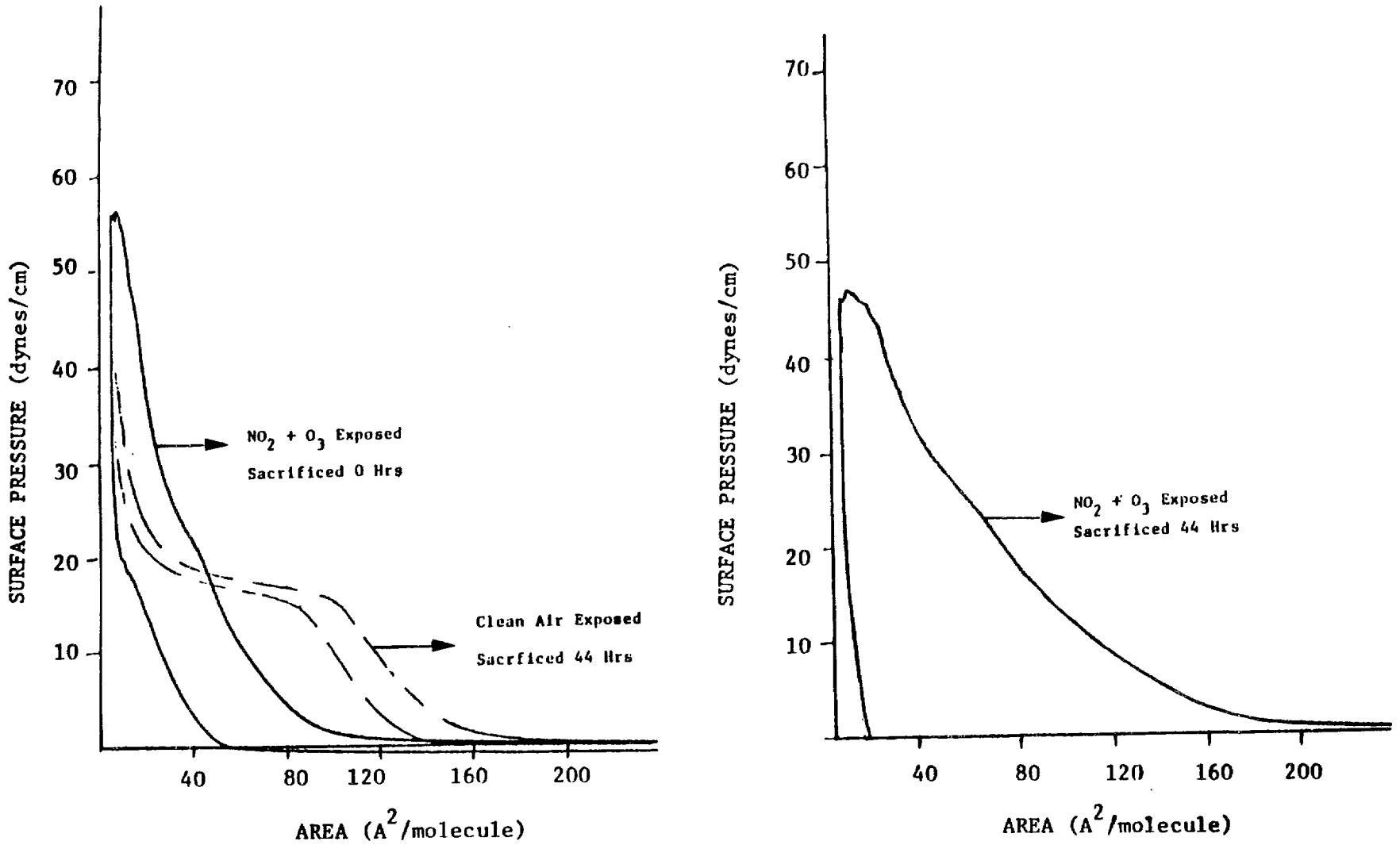


FIGURE 4: SURFACE PRESSURE-AREA ISOTHERMS OF SURFACTANT FROM RATS EXPOSED TO CLEAN AIR OR NO_2+ O_3

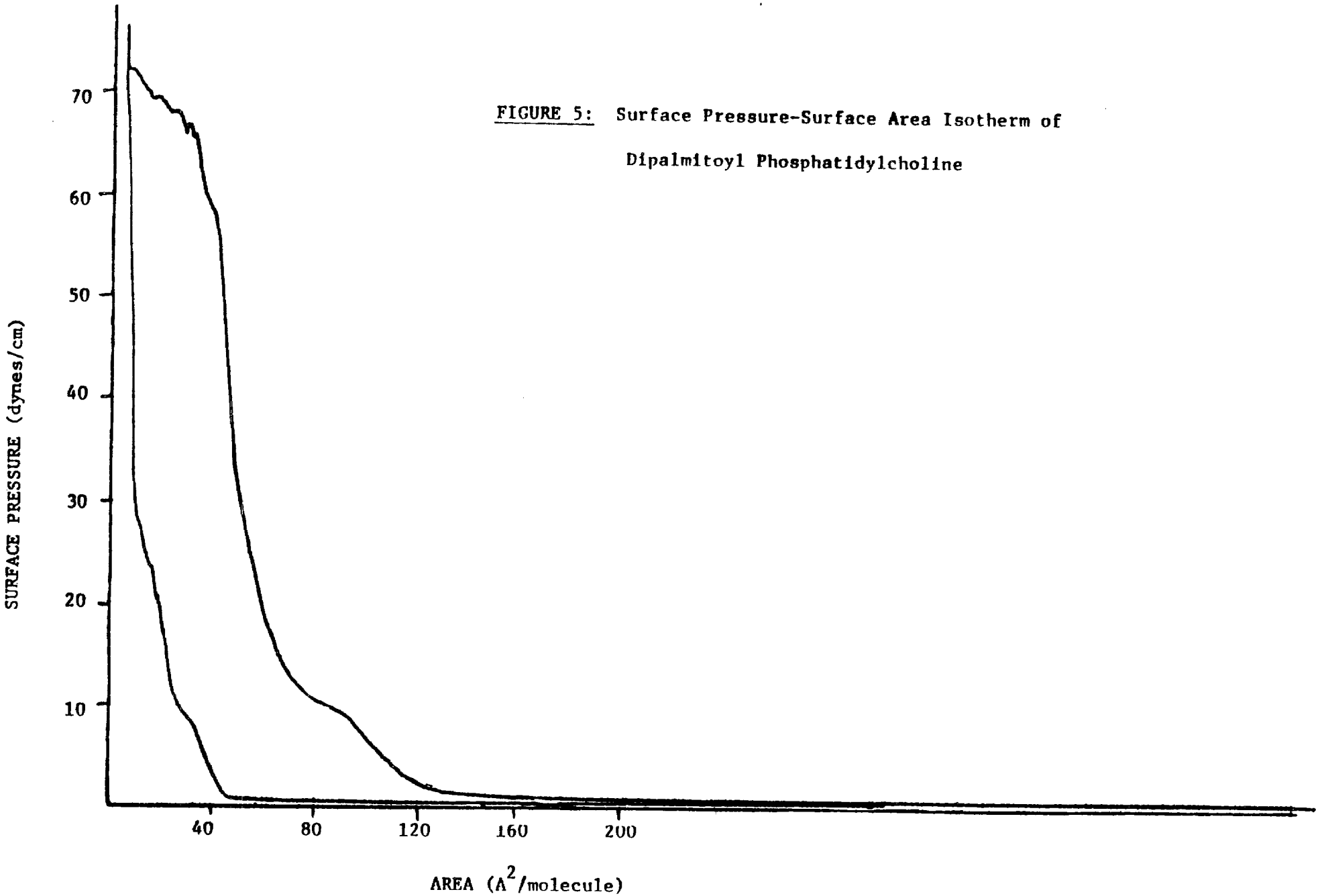


FIGURE 5: Surface Pressure-Surface Area Isotherm of Dipalmitoyl Phosphatidylcholine

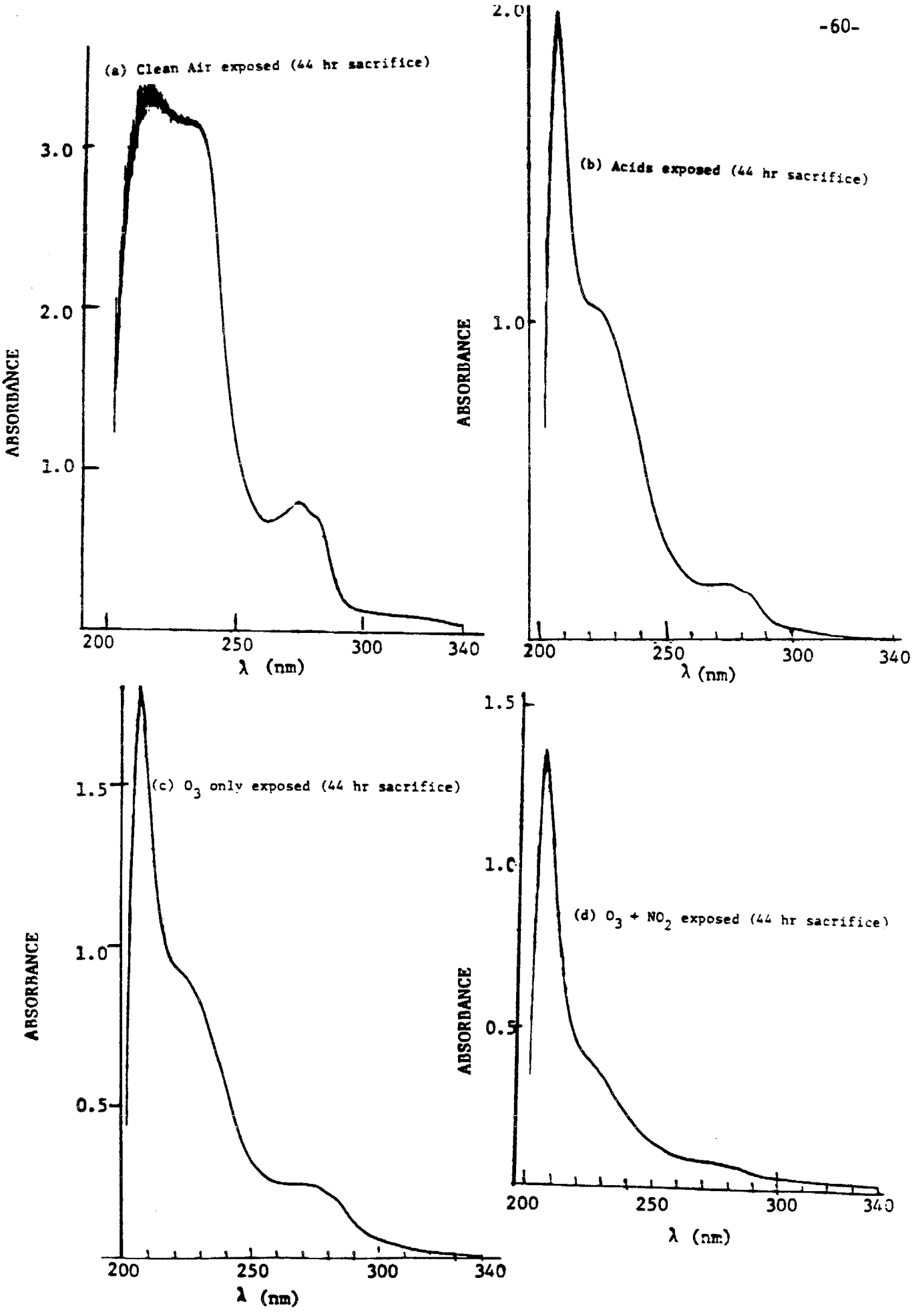


FIGURE 6: UV Absorption Spectra for Pulmonary Surfactant of Rats Exposed to (a) Clean Air, (b) Acids, (c) O_3, (d) NO_2 + O_3

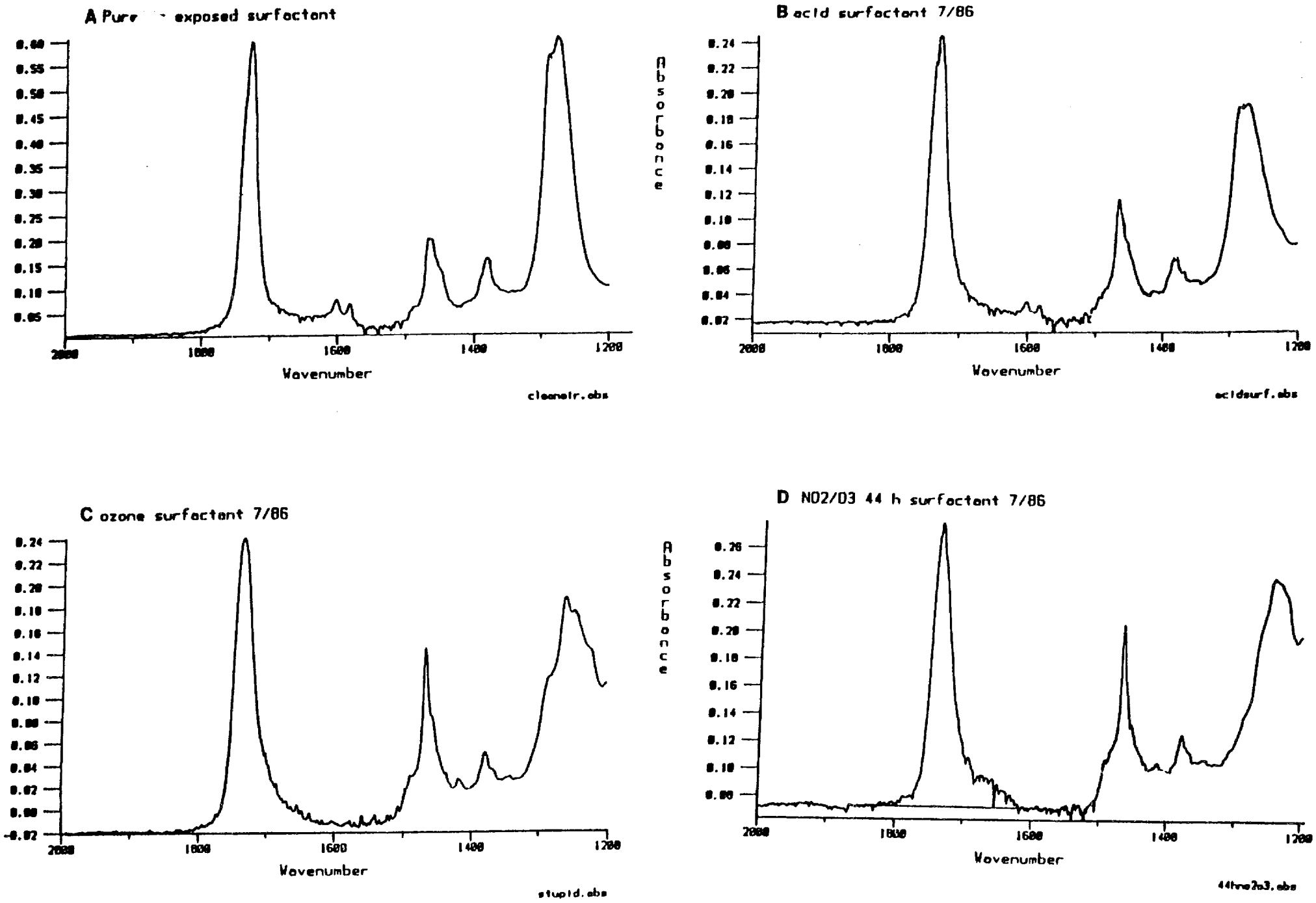


FIGURE 7: FTIR Spectra for Pulmonary Surfactant of Rats Exposed to Clean Air, Acids, O_3 only, and $NO_2 + O_3$

Table 22. Fatty Acid Composition of Pulmonary Surfactant from Rats Exposed to Clean Air, 0.35 ppm O_3, 0.35 ppm O_3 + 1.3 ppm NO_2 or an Acid Mixture, 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4[a] and Sacrificed 44 Hours After the Exposure.

Exposure Atmosphere	10^2 x Molar Ratio Stearic / Palmitic	Oleic / Palmitic	Linoleic / Palmitic
Clean Air	19	14	27
O_3 Only	7.3	7.3	10
O_3 + NO_2	6.0	5.7	13
HNO_3 + H_2SO_4	16	20	39

[a] Experiment 2, Table 20.

exposures resulted in a significant decrease in the stearic, oleic, and linoleic acid components compared to the clean air controls. The rats exposed to the acid mixture showed smaller effects, if any, and in the opposite direction.

c. Surface Pressure-Surface Area Isotherms

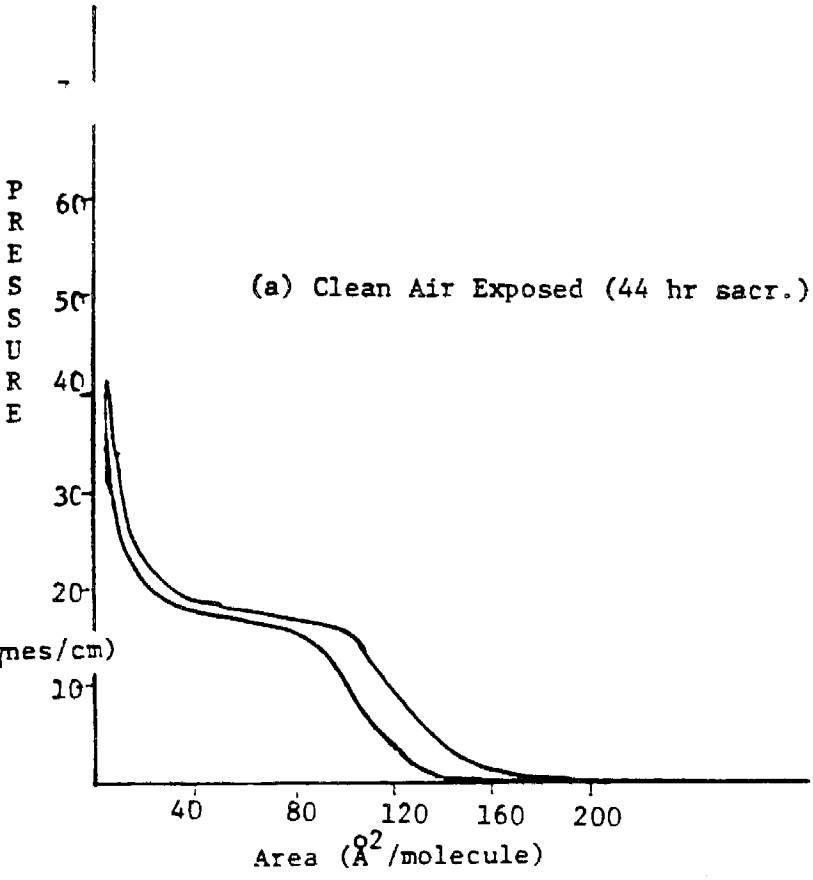
Figure 8 shows the surface pressure-surface area isotherms. The acid-exposed sample was similar to the clean air control, showing a plateau in the 15-20 dynes/cm region. However, the plateau was non-existent in the O_3 and O_3 + NO_2 exposed samples. This similarity in the clean air controls and the acid exposed rats on the one hand, and the O_3 and O_3 + NO_2 exposed animals on the other, may be related to the similarity in their respective fatty acid compositions (Table 22).

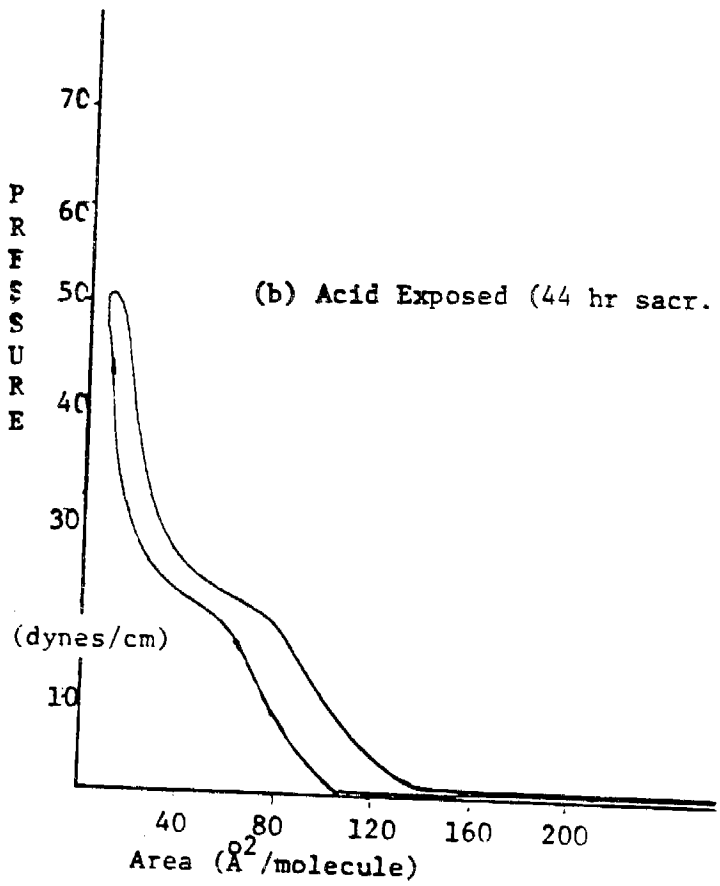
3. Intercomparison of Isolation Techniques

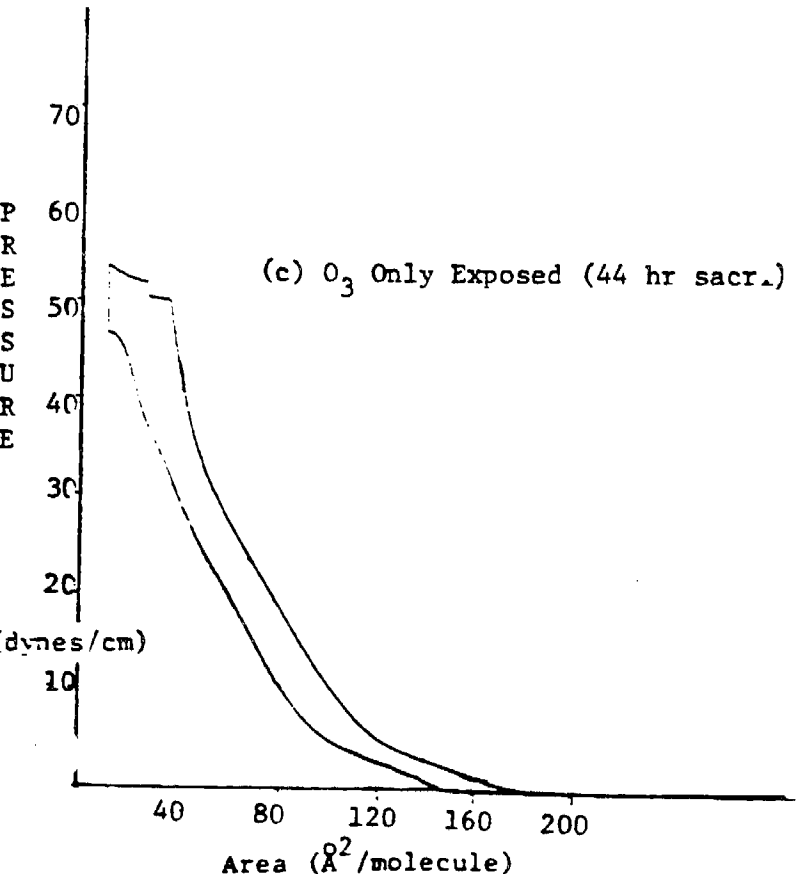
The "extended" isolation procedure is a thorough process recommended in literature studies of the physiological role of pulmonary surfactant, and it was adopted in most of these studies. However, a recent detailed review of the literature on the effects of inhalation of air pollutants revealed that the vast majority of these studies used a much simpler isolation method for surfactant. This consisted of removing the cellular debris, pelleting out the surfactant and extracting it into an appropriate solvent, a method we refer to as the "abbreviated' isolation technique.

Use of this method in future studies would have significant advantages over the "extended" method, including increased mass yield, decreased sample handling and hence less chance of accidental sample contamination, and less analysis time per experiment, permitting more experiments and/or samples to be studied. However, in order to test whether this method gave comparable results to the "extended" technique, Experiment 1 of Table 20, i.e., examination of the effect of sacrifice time on the surfactant after exposure to O_3 + NO_2 mixtures, was repeated. This is denoted Experiment 3 in Table 20.

The details of this experiment are described in the draft of the paper in Appendix 1, which is being submitted to Fundamental and Applied Toxicology for publication. Briefly, the fatty acid composition and UV spectra of the samples isolated using the "abbreviated" isolation technique were obtained and the remainder of the sample then resuspended in water and subjected to sucrose







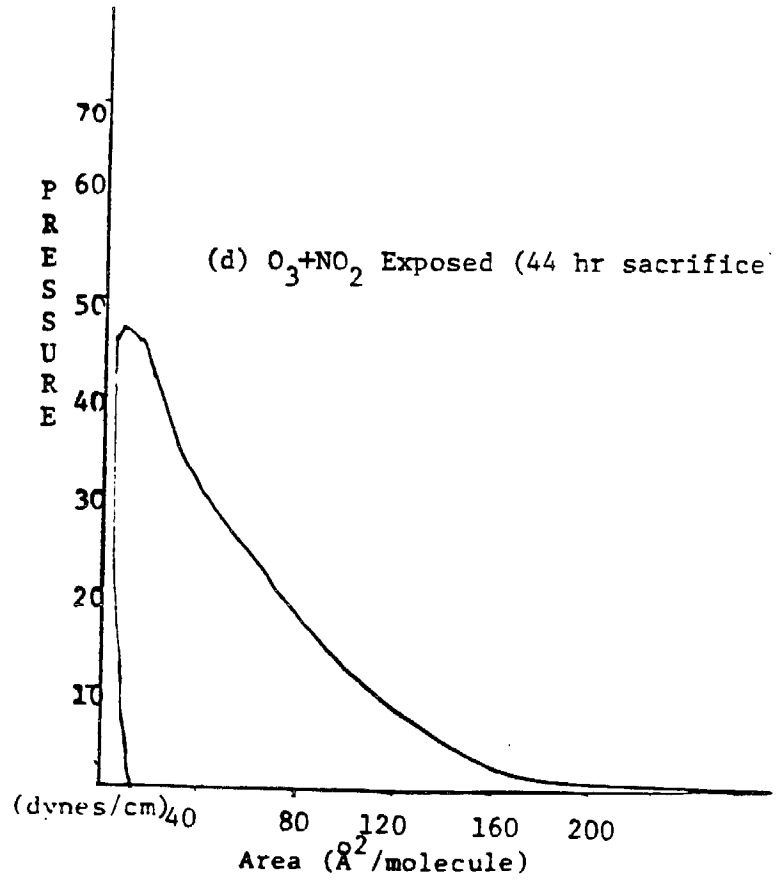


Figure 8: Surface Pressure-Area Isotherms for Pulmonary Surfactant of Rats Exposed to Clean Air, Acids, O_3, and O_3 + NO_2

density gradient centrifugation (SDGC). The samples isolated using the "abbreviated" method showed a significantly different fatty acid composition, and more importantly, did not show the change in composition with sacrifice time which had been observed in other experiments (Table 21). In addition, the UV spectra showed no evidence of oxidation products in any of the exposed samples. However, when the second portion of these samples was subjected to SDGC, the trend in fatty acid composition with sacrifice time was observed, and the UV showed evidence of oxidation products, especially in the 48 hour sample.

Because the yield of material was much greater with the "abbreviated" technique, we concluded that this material was a combination of surfactant and larger amounts of other substances, most likely mucus. If the mucus does not change on exposure of the animal, any change in the smaller quantities of surfactant present will be masked by the mucus.

IV. DISCUSSION

Evaluation of the health effects of acid air pollution is a complex problem for scientific investigation because of the complex mixture in which acidic air pollutants are formed. Earlier investigations of air pollution health effects focused on the direct physiological effects of prominant gaseous air pollutants such as SO_2, O_3, and NO_2. Acid air pollutants are present as a variety compounds in both vapor and particle phases and act in chemical environments with varying buffering capacities. The possible health effects of inhaled airborne acids are similarly complex. There is evidence that acid compounds may enhance the adverse health effects of oxidants (Last et al., 1983, 1984, 1986; Last and Cross, 1978), implying that evaluation of the health effects of acids should consider the effects of exposure to pollutant mixtures. There is an element of controversy about the importance of buffering capacity of the respiratory tract in the form of endogenous ammonia production, and how this might neutralize inhaled acids and mitigate health effects of exposure (Larson et al., 1977, 1982; Barrow and Steinhagen, 1980).

In consideration of these uncertainties about the important health effects of acid inhalation, the principal goals of this contract were:

1) To examine the toxicological effects of inhaled acid compounts in acute (4 h) exposures of laboratory rats.

2) To compare the effects of a multicomponent mixture of pollutants to selected subcomponents.

3) To investigate a variety of biological responses possibly affected by acid inhalation.

The choice of mixture components was governed by the need to represent pollutant agents that are prominant in the South Coast Air Basin and by our earlier striking observations that O_3 and NO_2 behave synergistically, inducing enhanced levels of lung tissue injury when inhaled in a mixture (Mautz et al., 1984, 1985c). Thus, a major thrust of this contract was to look for synergistic interactions among component pollutants of more complex mixtures and at lower concentrations.

A. Breathing Pattern Changes and Histopathology

These two biological endpoints are considered together here because of the interest in determining whether exposures that produced tissue injury could be related to the effects on the non-invasive measure of breathing pattern changes. The principal effect of the exposures on these endpoints was due to O_3. Parenchymal lung lesions were detected only in atmospheres that contained O_3 at 0.35 ppm, the highest concentration tested (Tables 15, 16, 17). O_3 alone and in the 7-component mixture at 0.35 ppm induced a small degree of rapid and shallow breathing at the end of these exposures, and in some cases the response was present in frequency but not significantly in tidal volume (Tables 7, 8, 11, 12). Acid components alone did not result in lung tissue injury or significant nasal epithelial injury (Tables 15, 16, 18) and at the concentrations studied, did not appear to modify the effects of O_3 on the lung. With only a marginal appearance of lung tissue damage from exposure to atmospheres containing O_3 at 0.35 ppm, it is difficult to detect any modifying effects of other pollutant compounds. These results are in contrast to earlier work in our laboratory with O_3 at 0.6 ppm in resting exposures or O_3 at 0.35 ppm in exercising exposures (Mautz et al., 1984, 1985c). In resting exposures of rats to O_3 at 0.6 ppm combined with NO_2 at 2.5 ppm there was strong enhancement (approximately 2 fold increase) of the O_3 effects on parenchymal lung lesions, and this enhancement was also apparent for O_3 at 0.35 ppm combined with NO_2 at 0.6 ppm when the exposure was performed during exercise. In the present study, however, 0.35 ppm O_3 at rest had such a small effect on the lung that any enhancing effect of 1.3 ppm NO_2 or reaction products of O_3 + NO_2 mixtures on lung lesions was not detectable.

A distinct effect of the O_3-NO_2 combination was apparent in breathing pattern responses. While 0.35 ppm O_3 alone induced a small degree of rapid and shallow respiration (Tables 7, 8, 11, 12), the combination with 1.3 ppm NO_2 suppressed this response (Tables 7, 8). Combinations of O_3 + NO_2 induced no significant breathing pattern changes at the end of exposures (Tables 6-10), and the only change observed in any of the O_3 + NO_2 exposures was an initial trend (non-significant) toward slow respiration and significant shift to deep respiration in one of the exposures (Tables 7, 8). It appears that the combination of O_3 + NO_2 with the formation of reaction products HNO_3, N_2O_5, and nitrate radical is capable of altering breathing pattern responses to O_3 at the concentrations tested. While such alterations could be responsible for the synergistic interactions on lung damage observed at higher concentrations of the pollutants or during exercise (Mautz et al., 1984), there was no observed

interaction on parenchymal lesions in the lower concentration exposures studied here. It is noteworthy that the 7-component mixture, which contained both O_3 and NO_2, resulted in an O_3 breathing pattern response (Tables 12, 13) and did not show the suppression of breathing pattern response to O_3 that occurred in O_3 + NO_2 mixtures (Tables 6-10). Clearly, the effects of mixed pollutants on breathing patterns are profoundly influenced by the complex chemistry of the mixtures and cannot be simply predicted as additive effects of the components. The consequences of these differences are expected to have major effects on dose-distribution of inhaled pollutant compounds, however at the relatively low concentrations examined in this project, these were not revealed in any significant patterns of lung parenchymal lesions or nasal epithelial injury.

Breathing patterns in the rat are sensitive to the effects of inhaled upper and lower respiratory tract irritants and responses in rats can be related to those of humans. In humans, rats, dogs, guinea pigs, rabbits, and probably in mammals in general, lower airway irritants such as O_3 and NO_2 induce a rapid-shallow breathing pattern, which is the probable consequence of tissue injury leading to stimulation of lung C fibers (Alarie, 1973; Coleridge and Coleridge, 1984). Rapid-shallow breathing is one of the prominant indicators of O_3-induced pulmonary function changes in humans and is accompanied by reductions in forced vital capacity (FVC) and forced expiratory volume at one second (FEV 1.0). While the magnitude of the breathing pattern response (e.g., a 10% shift) may differ between species in O_3 exposures, the approximate threshold for inducing a response (3-4 h in 0.35-0.4 ppm O_3 at rest) is similar for rats and humans. Concentrations of ozone that induced rapid-shallow breathing in rats (Tables 7, 11, and 13) also induced significant lung tissue injury (Tables 15, 16, and 17). It is, however, notable that the combinations of 0.35 ppm O_3 and 1.3 ppm NO_2 did not induce significant rapid-shallow breathing (Tables 7 and 9). Lesions to lung parenchyma were less severe (although not significantly so) than exposure to 0.35 ppm O_3 alone (Table 15). An exposure of human subjects to the combination 0.5 ppm O_3 + 0.5 ppm NO_2 also failed to induce a rapid-shallow breathing response (Folinsbee et al., 1981). The combination of O_3 and NO_2 appears to possess some special characteristics, as we observed synergistic interactions on lung tissue injury in exercising exposure of rats to 0.35 ppm O_3 and 0.6 ppm NO_2 (Mautz et al., 1983c). Nevertheless, the lack of breathing pattern responses observed in the present study of rats parallels observations in humans. Our current research on the presence of oxidant breathing pattern responses and lung histopathology in resting and exercising exposures of rats will clarify these relationships

and further establish the parallel responses of rats and humans to low concentrations of lower airway irritants.

The finding that the effects of pollutant mixtures are not generally predictable from the responses to individual components has been shown to be true for histopathology, permeability, and now breathing pattern changes. Changes in breathing pattern are likely to alter lung dose distribution and may well be an important protective function of lung defenses which can be disrupted by antagonistic, or competing toxicants in pollutant mixtures.

B. Particle Clearance

Ozone-containing multicomponent atmospheres generally produce effects on early and late particle clearance which are in the same direction as the effects produced following exposure to the ozone alone (Phalen et al., 1980; Kenoyer et al., 1981). The presence of ozone in a pollutant atmosphere tends to cause a delay in early clearance and an acceleration in late clearance. The magnitude of these effects may be greater or lesser than those produced by the ozone alone, depending upon the composition of the atmosphere and the exposure conditions (resting or exercising exposure, % RH, etc.). The results of 7-component exposure experiment indicate that the effects were in the same direction as those which would normally be observed following an exposure to ozone alone, but these effects were not statistically significant. Ozone alone at a concentration of 0.35 ppm does not significantly alter clearance rates (Kenoyer et al., 1981), and while the effect of including other components in the atmosphere was unknown, addition of nitrogen dioxide to an ozone-containing atmosphere often enhances the effects produced (effects in the same direction but of greater magnitude). In a previous experiment propane soot, in combination with sulfuric and nitric acids, caused effects which were in the opposite direction (Mannix and Phalen, 1986). Therefore, possible reasons why the 7-component mixture did not greatly affect clearance are 1) the concentrations of the pollutants were too low, and 2) the various components produced antagonistic effects.

C. Chemical Characterization of Pulmonary Surfactant

There have been numerous studies of the chemical composition of the alveolar surfactant layer in various animal species since initial work of Clements and Pattle and their co-workers. (For reviews, see Clements and King, 1976; Goerke, 1974; Sanders, 1982). Phospholipids are major surface-active components with phosphatidylcholine (lecithin) being predominant in a variety of species, including humans and rats. The fatty acid residues (R_1, R_2) in phosphatidylcholine are mainly the saturated residue palmitate (~55-80% of the total fatty acids present), and smaller amounts of stearate with the unsaturated carbon-carbon double bond containing fatty acids, such as oleic acid, forming the next major components (~5-15% each in both humans and rats). Fatty acids with 2 or more double bonds are also present to a smaller extent. Neutral lipids, especially cholesterol, comprise approximately 10-15% of the total lipids. In addition, surfactant contains proteins with molecular weights from 11,000 up to possibly 250,000.

Analysis of pulmonary surfactant effects focused on O_3 + NO_2 exposures and on a comparison of oxidant to acid atmospheres because of the possible reactions of O_3 and the reactive intermediates, NO_3 and N_2O_5, directly with pulmonary surfactant.

1. O_3 + NO_2 exposures. The increase in UV absorption observed at 235 and 275 nm is believed to be indicative of the formation or conjugated dienes and trienes, and possibly carbonyl groups, due to the accumulation of lipid oxidation products (Klein, 1970). These data indicate that such oxidation products are accumulating with time after the exposure, up to 48 hours.

Regardless of the particular exposure conditions (see below), the presence of these strong UV absorptions seems to be correlated with the appearance of the new FTIR bands at approximately 1600 and 1580 cm^{-1} and the changes in the bands around 1285 and 1130 cm^{-1} (Figure 1c). It is possible that these new infrared bands are due to 1, 3-dienes which according to Colthup et al. (1975) have absorption bands in the 1600 cm^{-1} region, and possibly to hydroperoxides which absorb around 1100 and 1300 cm^{-1}. However, studies of model compound oxidation under controlled laboratory conditions will be required to test these tentative assignments.

The reason for the change in the shape of the surface pressure-surface rea isotherms upon exposure to the air pollutants is not clear. The plateau in the clean air exposed sample and in DPPC is due to the fluid to solid phase transition (Goerke, 1974; Weiss and McConnell, 1985). The loss of the plateau reflects a change in the chemical composition of the surfactant, preventing formation of the solid phase. Such a change can be mimicked, for example, by increasing the percentage of oleic acid residues in a commercial sample of dipalmitoyl phosphatidylcholine (Figure 9).

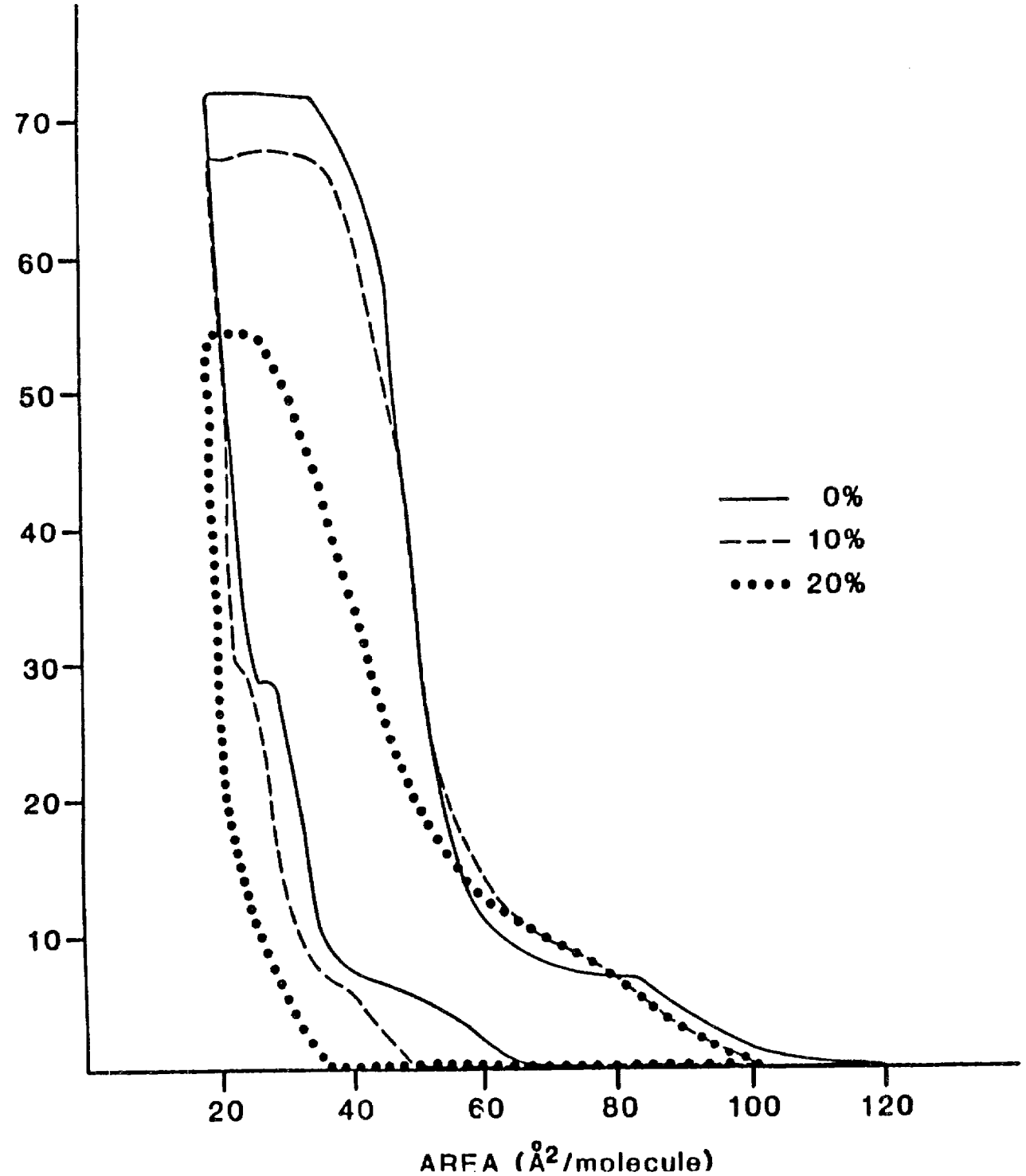
The combination of the fatty acid data, the FTIR and UV spectra, and the surface pressure-surface area isotherms all suggest that exposure to the combination of O_3 and NO_2 induces a biological response in the animal which changes the chemical composition of the liquid layer lining the alveoli. One hypothesis suggested by the data is that an oxidation is initiated in this liquid layer and that this oxidation peaks approximately 24 hours after the exposure. This would explain the decrease in the oleic and linoleic fatty acids whose changes were greatest 24 hours after the exposure. If the products of this oxidation could not be reutilized by the Type II cells to resynthesize surfactant, as has been shown to be the case for some DPPC analogs (Jacobs et al., 1984), they would build up in the liquid layer and be most evident 48 hours after the exposure. Thus the UV and FTIR spectra, as well as the surface pressure-surface area isotherms, likely reflect this accumulation of oxidation products.

A change in the biosynthesis of surfactant may also be occurring. Thus one would not expect stearic acid to decrease significantly relative to palmitic acid due to oxidation processes, since both are saturated, i.e., contain no reactive C=C bonds. However, as seen in Table 21, stearic acid decreased significantly in the exposed samples. This may reflect a change in the synthesis induced by the exposure. One note of caution must be sounded with respect to the stearic acid data, in that in our experience, stearic acid tends to show much more variability from experiment to experiment than any of the other fatty acids.

2. Exposure to O_3, $O_3 + NO_2$, or $HNO_3 + H_2SO_4$. The reason for the presence of oxidation products in the clean air controls as seen by both FTIR and UV, is not clear. All samples were treated identically and care was taken to avoid introducing air or contaminants into the samples. It may reflect an unusual set of animals, perhaps due to handling prior to reaching the laboratory; for example, this experiment was carried out in late June/early

FIGURE 9:

SURFACE PRESSURE OF DIPALMITOYL PHOSPHATIDYLCHOLINE WITH X% β-OLEOYL-γ-PALMITOYL PHOSPHATIDYLCHOLINE



July and hence the animals were shipped during a hot season, whereas the other experiments were carried out in January. Another possibility is oxidation introduced during the dialysis step of the isolation procedure; however, since all samples were treated identically, this would not explain why some of the samples show evidence of oxidation, and others do not.

While the strong UV and FTIR absorptions in the clean air controls makes interpretation of these data in the exposed animals difficult, it does serve to confirm the relationship between the UV and IR spectra suggested by the other studies, namely that the oxidation products responsible for the strong UV absorptions at 235 and 275 nm also account for, or are associated with, the new IR bands at 1600 and 1580 cm^{-1}. Since this is the first application of FTIR in such air pollution studies, this in itself is useful and suggests that future studies should concentrate on the laboratory oxidation of model compounds in order to identify the species responsible for these UV and IR bands.

Overall, the surfactant from animals exposed to the acid mixture was very similar to that from the clean air controls, whereas that from the animals exposed to O_3 alone was similar to that from rats exposed to the $O_3 + NO_2$ combination. From the standpoint of changes in surfactant, the effects of oxidants appear to be far more significant than those of the acid mixture, which are difficult to distinguish from clean air with our current techniques.

3. Intercomparison of Isolation Techniques. It is clear from the work on intercomparison of the "extended," "full" and "abbreviated" isolation techniques that if studying surfactant is the goal of the investigation, it is imperative to carry out sucrose density gradient centrifugation to separate surfactant from mucus. This is an important finding, especially given that most studies of air pollutant exposure in the past have used the "abbreviated" method and thus likely examined a combination of mucus and surfactant.

However, while SDGC is clearly essential, it is also important to minimize sample handling as much as possible, especially given the very small amounts of surfactant even from a pooled sample from 4-6 rats. Based on our experience with surfactant isolation over the last three years, we have developed a method, described earlier as the "full" isolation method, which includes SDGC but does not require dialysis. As described in the Methods section, this avoids loss of sample on the walls of the dialysis tubing, and rules out any spurious oxidation during dialysis. This method should be used in all future studies.

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Appendix: Manuscript submitted to Fundamental and Applied Toxicology

INHALED OXIDANT AIR POLLUTANTS: EFFECTS OF ISOLATION PROCEDURES ON OBSERVED PULMONARY SURFACTANT COMPOSITION

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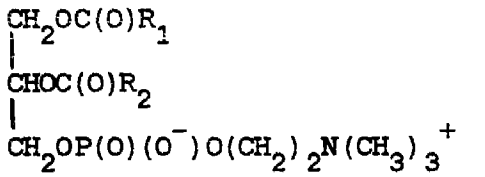
ABSTRACT

Inhaled Oxidant Air Pollutants: Effects of Isolation Procedures on Observed Pulmonary Surfactant Composition. Finlayson-Pitts, B. J., Sweetman, L.L., and Mautz, W. J. (1987). Fundam. Appl. Toxicol.

The goals of this study were: (1) to determine whether addition of sucrose density gradient centrifugation (SDGC) in the isolation of pulmonary surfactant from rats exposed to combined O_3 and NO_2 yields material with the same composition as that obtained without this step, and (2) to examine the effects of these air pollutants on the chemical composition of lavage fluids. Albino male Sprague-Dawley rats were exposed for four hours, nose only at rest, to a combination of O_3 (0.35 $\pm$ 0.02 ppm) and NO_2 (1.3 $\pm$ 0.1 ppm) at 84 $\pm$ 1% relative humidity and 22 $\pm$ 0.4 ^{o}C. The clean air controls and one group of exposed rats were sacrificed immediately after exposure; two exposed groups were sacrificed 24 and 48 hours later, respectively. The lavage fluids from half of each group were pooled to provide two replicate samples. These fluids were subjected to low speed centrifugation to remove cells and then pelleted out and extracted into chloroform/methanol, an "abbreviated" method often used in air pollutant exposures. A portion of these extracts was resuspended in water and further purified using SDGC, referred to here as the "full procedure". The fatty acid composition of the material from the abbreviated and full isolation procedures was found to differ significantly; in addition, a trend of change in composition with sacrifice time after the pollutant exposure was observed only when the full procedure was followed. UV studies provided evidence of the formation of oxidation products only in the samples from the full procedure 48 hours after the exposure. These results establish that both qualitative and quantitative observations of the chemical composition of lung lavage fluids can depend critically on the isolation procedures used, and suggest that a postexposure inflammatory response of the lung occurs in rats exposed to combined O_3 and NO_2.

Introduction

The alveolar region of the lung is lined with pulmonary surfactant which lowers the surface tension of the liquid layer lining the alveolar surface (Pattle, 1955; Clements, 1956); this surfactant prevents alveolar collapse (Avery and Mead, 1959) as well as pulmonary edema (Clements, 1961) which would result if the surface tension of water was not reduced. One of the major classes of compounds responsible for this surfactant action is phosphatidylcholine (PC):



The major fatty acid component (R_1,R_2) is the saturated palmitic acid [$R = CH_3(CH_2)_{14}-$], designated 16:0 to indicate 16 carbon atoms in the acid which contains no double C=C bonds; however, smaller amounts of other fatty acids, both saturated, e.g. stearic (18:0), and unsaturated, e.g. palmitoleic (16:1), oleic (18:1), and linoleic (18:2), are also present (Sanders, 1982). Because some of these fatty acids contain double bonds, inhalation of reactive air pollutants might change the chemical composition of the surfactant by direct reaction. Also, by inducing a biological inflammatory response, altered cellular processes might lead to changes in surfactant composition.

Samples of pulmonary surfactant are usually obtained via tracheostomy using lavage with a saline solution, followed by low speed centrifugation to sediment cellular debris. However, since mucus lines the surfaces of the respiratory tract to the end of the bronchi, the material thus obtained contains in addition to surfactant, "variable amounts of ...bronchial mucus and plasma proteins" (Goerke, 1974). The proteins remain in the supernatant when the decanted surfactant is pelleted out by high speed centrifugation.

Studies of the physiological function of surfactant generally have used subsequent isolation steps such as sucrose density gradient centrifugation on the cell-free pelleted material (Goerke, 1974; Frosolono, 1982; King, 1982; Kotas, 1982) in order to remove mucoid material. However, many studies of the effects of the inhalation of air pollutants on animals have used the material remaining after only the cells and protein have been removed (e.g. see Huber et al., 1971; Roehm et al, 1971; Menzel et al, 1972; Kyei-Aboagye et al, 1973; Arner and Rhoades, 1973; Shimasaki et al, 1976; Sevanian et al, 1979).

The purpose of the present study was to determine whether the use of sucrose density gradient centrifugation (hereafter referred to as

"SDGC") altered the observed fatty acid composition of the material obtained from rat lungs by lavage and pelleted out after spinning down the cells. We refer throughout the paper to material from which only the cellular debris has been removed as that from the "abbreviated" procedure; the material obtained after the SDGC steps is referred to as that from the "full" procedure.

A second purpose of the study was to examine whether the observed effects of exposure to a combination of two air pollutants, ozone and nitrogen dioxide, were the same using the abbreviated and full isolation procedures respectively. The combination of O_3 and NO_2 is of interest because they react in urban air to form the reactive nitrate radical (NO_3)

$$O_3 + NO_2 \longrightarrow NO_3 + O_2 \qquad (1)$$

as well as dinitrogen pentoxide

$$NO_3 + NO_2 \longrightarrow N_2O_5 \qquad (2)$$

In the presence of water, N_2O_5 reacts to form nitric acid, a reaction which is catalyzed by surfaces (Finlayson-Pitts and Pitts, 1986):

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

The nitrate radical has been measured in air at concentrations up to 430 ppt (v:v) (Platt et al, 1984) and the N_2O_5 concentration estimated to be as high as 15 ppb (Atkinson et al, 1986). Thus studies of the effects of combinations of O_3 and NO_2 which have had sufficient time to react to form NO_3 and N_2O_5 are of interest.

Methods

Experimental animals were seven week old male, barrier-reared Sprague-Dawley rats (Hilltop Laboratories, Scottsdale, PA) whose average weight was 265 $\pm$ 14 g. (All errors given are $\pm$ 1 S.D.). Rats were shipped in filter-equipped containers and housed at the laboratory in a laminar flow filtered air caging system. Ten percent of rats in each shipment were autopsied to verify the animals were free of lung disease. Eighteen rats were exposed at rest, nose only, for four hours to a combination of O_3 (0.35 $\pm$ 0.02 ppm) and NO_2 (1.3 $\pm$ 0.1 ppm) at 84 $\pm$ 1% relative humidity at 22 $\pm$ 0.4 °C. The two pollutants had several minutes to react prior to the exposure ports, so that their reaction products NO_3 and N_2O_5, as well as HNO_3, were also present. Target concentrations of O_3 and NO_2 were controlled at the exposure ports. From the known reaction kinetics of reactions (1) and (2) (Finlayson-Pitts and Pitts, 1986), the measured change in the ozone concentration when the NO_2 was turned off, and

the measured gas phase HNO_3 concentration of 2.2×10^2 ppb, the concentrations of NO_3 and N_2O_5 at the exposure ports were estimated to be 1.1×10^3 ppt and 1.2×10^2 ppb respectively. Simultaneously, a group of six rats were exposed to clean air. The exposure chambers are described in detail elsewhere (Mannix et al, 1982).

After the exposure, the oxidant exposed rats were divided into three groups. One group of six was sacrificed immediately after the exposure, the second group of six after 24 hours, and the third group of six after 48 hours. The clean air controls were sacrificed immediately after the exposure. The rats were deeply anesthetized with sodium pentobarbitol IP (36 mg/rat) and the abdominal aorta was cut to drain blood from the heart and lungs. Each lung was lavaged through a cut in the upper trachea with two 7 ml volumes of 0.15 M NaCl containing 0.003 M $CaCl_2$ and 0.003 M $MgCl_2$, added to maintain tight epithelial junctions (Henderson, 1984). The lavage fluid from three rats in each group was pooled in order to give two samples for each exposure to test for lavage sample variability as well as analytical precision.

A modification of the procedure recommended by Steim et al (1969) was used for isolating pulmonary surfactant. Briefly, the lavage fluid was centrifuged at 600 xg for 20 minutes to sediment the cellular debris, and the supernatant from this step then centrifuged for one hour at 16,000 xg. The pellet obtained was resuspended in deionized, distilled water and extracted into a chloroform/methanol (2:1 v/v) solvent. This is referred to as the "abbreviated procedure". Typical yields of material were 1-2 mg for the pooled sample from three rats.

Each sample was divided into 3 portions; 25% was esterified to obtain the fatty acid composition, 25% was used to obtain the ultraviolet absorption spectrum, and 50% was stored in the solvent under N_2 at $-15^{\circ}C$ until the subsequent isolation steps involving SDGC could be carried out. Formation of the fatty acid methyl esters was carried out by the method of Mason and Waller (1964) and they were analyzed by gas chromatography using a Hewlett Packard Model 5750 GC equipped with a wide-bore capillary column (Supelcowax 10) and flame ionization detector. The GC was calibrated using mixtures of commercially available fatty acid methyl esters. The UV spectra were obtained by evaporating the solvent and redissolving the samples in methanol; spectra were obtained on a Varian 2200 UV/visible spectrophotometer.

To examine the effects of subsequent SDGC, the solvent was evaporated from the stored samples which were resuspended in water. In order to obtain sufficient material for analysis after the SDGC steps, the replicate samples from each group of rats were combined, resulting in one, rather than two, samples for each exposure/sacrifice condition. These resuspended samples were layered on a sucrose solution of density 1.010 and centrifuged at 1.04×10^5 xg in a TI50 rotor for one hour; the

pelleted material was resuspended, layered on a sucrose solution of density 1.055 and centrifuged for one hour at 8.72×10^4 xg in the TI50 rotor. The opalescent material floating at the solution interface was collected and pelleted out by centrifuging at 1.04×10^5 xg for one hour. All steps were carried out at 4°C. The isolation method including SDGC is referred to as the "full" procedure.

The resuspended pellet was extracted into chloroform/methanol (2:1 v/v) and its fatty acid composition and UV spectrum obtained as described above.

Results

Figure 1 shows the major fatty acids in the material from the abbreviated and full isolation procedures, respectively. The composition is expressed as a ratio of the stearic, oleic and linoleic acids respectively, to palmitic acid which is the major fatty acid present; the ratios rather than absolute yields are used because of the difficulty in accurately weighing sub-microgram yields of total surfactant from the full procedure, and the presence of some smaller amounts of fatty acids which were not identified in these studies. The data in Figure 1 fall within the typical ranges reported in the literature (Sanders, 1982).

The fatty acid composition of the replicate samples from the abbreviated method, each comprised of the pooled lavage fluids from three rats from which only the cellular material and protein has been removed, are in excellent agreement. In addition, no clear effect of the exposure of the rats to the O_3 + NO_2 combination on the fatty acid composition is observed. However, in the material isolated using the full procedure, a distinct trend in the fatty acid composition is evident with time of sacrifice after the exposure, with a decrease in the relative amounts of stearic, oleic and linoleic acids 24 hours after the exposure, followed by a recovery from 24 to 48 hours.

Clearly, there are significant differences in the composition of the material isolated by the two methods. As seen in Figure 1, there is relatively more stearic and less linoleic acid in the material isolated using the full procedure. In other experiments, significant differences were also seen between the composition of the material isolated from the lavage fluids of clean air controls prior to SDGC compared to that afterwards. The concentration of linoleic acid was consistently lower, by a factor of 2-3, after SDGC, while the oleic acid was not changed by more than 50%. However, the change in the stearic acid component was much more variable, both quantitatively and with respect to the direction of the change.

The UV spectra of the material from the abbreviated procedure showed little increase in absorption at 233 and 275 nm; increased absorbance at these wavelengths are both indicators of lipid oxidation

(Klein, 1970). However, the sample from rats sacrificed 48 hours after the exposure isolated using the full procedure did show increased absorptions both at 233 and 275 nm, suggesting the presence of lipid oxidation products.

Discussion

These studies establish that differences in the techniques used for isolating pulmonary surfactant from lavage fluids can greatly influence the observed chemical composition with respect to the fatty acids as well as the presence of lipid oxidation products.

The agreement between the fatty acid composition of the two samples isolated using the abbreviated procedure in each exposure group shows that the sample variability in this one experiment is relatively small and the analytical precision is good.

The significant decrease observed in the relative amounts of stearic, oleic and linoleic acid components 24 hours after the exposure in the material isolated using the full procedure but not in that using the abbreviated procedure can be explained if the material isolated from the lavage fluid prior to SDGC is a mixture of mucus and a much smaller quantity of surfactant. Thus the fatty acid composition and UV analysis should reflect primarily the mucus whose composition may not change significantly on exposure to the air pollutants. However, the SDGC steps are recommended by Steim et al (1969) to remove mucoid material; in this case the material remaining after SDGC should be primarily surfactant. Supporting this interpretation is the much smaller yield of material obtained after SDGC. While it was difficult to accurately weigh such small amounts of material, yields were typically approximately 10% of the mass prior to SDGC.

The change in the fatty acid composition 24 hours after the exposure in the surfactant isolated using the full procedure indicates a postexposure inflammatory response of the lung to the inhalation of the $O_3 + NO_2$ combination. The change in the relative amounts of the major fatty acids may reflect a change in the biosynthesis of pulmonary surfactant and/or oxidation processes in the lung initiated by the pollutant exposure. The increase in the UV absorbance at 233 and 275 nm 48 hours after the exposure is characteristic of conjugated dienes and trienes and/or carbonyl groups respectively, which are formed by the oxidation of unsaturated fatty acids (Klein, 1970). This indicates that oxidative processes contribute at least in part to the observed changes. Thus the exposure may induce the release of oxidants into the liquid layer lining the alveoli, altering the fatty acid composition of the surfactant, and simultaneously forming oxidized products; if these products cannot be reutilized by the Type II cells for the synthesis of surfactant, as has been shown to be the case for a number of analogs of phosphatidylcholines (Jacobs et al, 1984), they will remain in the liquid layer and hence be expected to accumulate, giving observed

concentrations which are highest at 48 hours. However, the simultaneous change in saturated stearic acid as well as in the unsaturated oleic and linoleic acids suggests that a change in the biosynthesis may also be induced by the exposure.

This postexposure period in comparable histologic studies of the response to O_3 alone coincides with the influx of alveolar macrophages and edema of alveolar septa (Mautz et al, 1985; Evans, 1984). While permeability is increased in the air-to-blood (Bhalla et al, 1986) and blood-to-air (Bhalla and Crocker, 1987) transfer of albumin or smaller trace molecules, more marked histologic changes (Kleinman et al, 1985) and increase in air-to-blood permeability (Bhalla et al, unpublished data) occur after exposure to $O_3 + NO_2$. Changes in surfactant fatty acids observed at 24 hours after exposure are therefore associated with death of alveolar epithelial (Type I) cells; this presumably results from direct cell injury either by oxidative reactions between gases and cell membranes, or alternatively as a result of reactions between gases and surfactant lipids with formation of toxic species associated with oxidant-surfactant interactions.

Summary

The isolation techniques applied to the lavage fluids from rats exposed to clean air as well as to a combination of the criteria air pollutants O_3 and NO_2 is critical both to the quantitative results obtained on the chemical composition as well as to the trend observed in the composition with sacrifice time after the exposure. The results presented here suggest that if studying pulmonary surfactant is the goal, it is essential to carry out the full isolation procedure including sucrose density gradient centrifugation. Application of this procedure to the lavage fluids from rats exposed to the O_3-NO_2 combination shows that the fatty acid composition of the surfactant changes 24-48 hours after the exposure, with evidence of the formation of lipid oxidation products; this time lag indicates that that the exposure triggers a postexposure inflammatory response.

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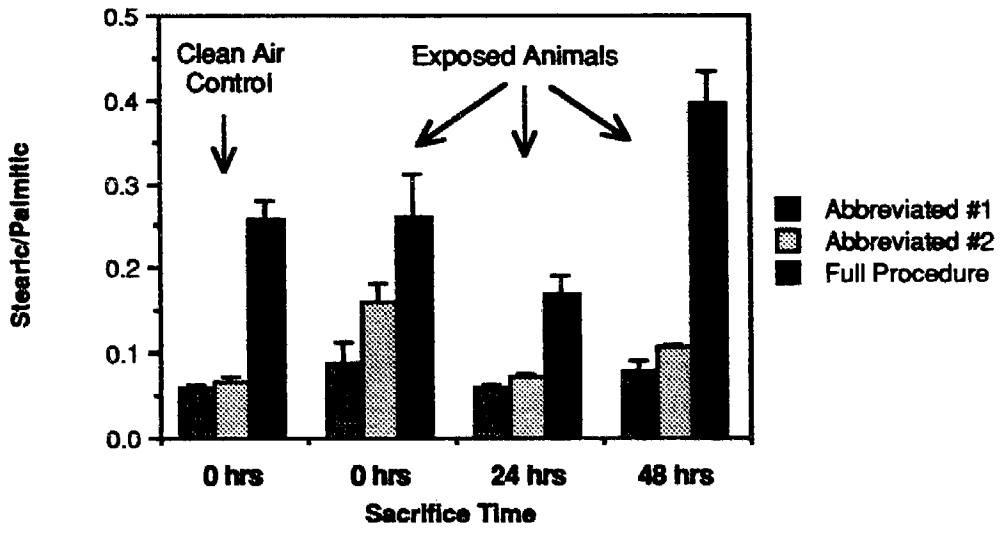
FIGURE CAPTION

Figure 1: Fatty acid composition of material isolated from lavage fluids of rats exposed to a combination of O_3 (0.35 ppm) + NO_2 (1.3 ppm) at 83.5% R.H. at 22 ^{o}C for four hours and sacrificed at 0, 24 or 48 hours after the exposure respectively. The two samples marked "Abbreviated-1" and "Abbreviated-2" represent pooled lavage fluids from three rats each, from which only the cellular debris and protein has been removed. These samples from replicate experiments were pooled and sucrose density gradient centrifugation carried out to obtain the bars designated as "Full Procedure". The error bars represent two standard deviations of the analytical error associated with the mean of three GC injections.


Stearic/Palmitic
0.5
0.4
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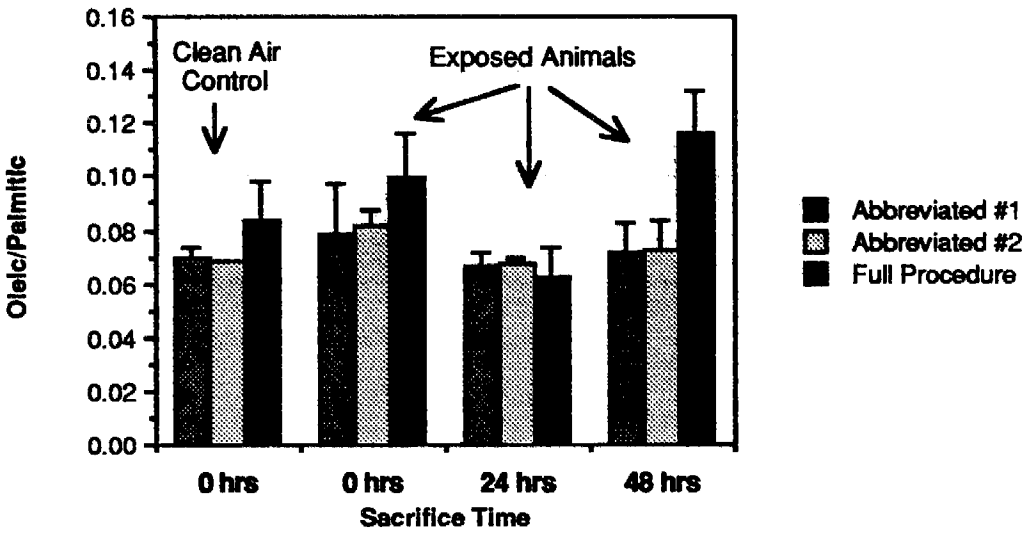
0.2
0.1
0.0
Clean Air Control
Exposed Animals
0 hrs
0 hrs
24 hrs
48 hrs
Sacrifice Time
Abbreviated #1
Abbreviated #2
Full Procedure


Oleic/Palmitic
0.16
0.14
0.12
0.10
0.08
0.06
0.04
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Clean Air Control
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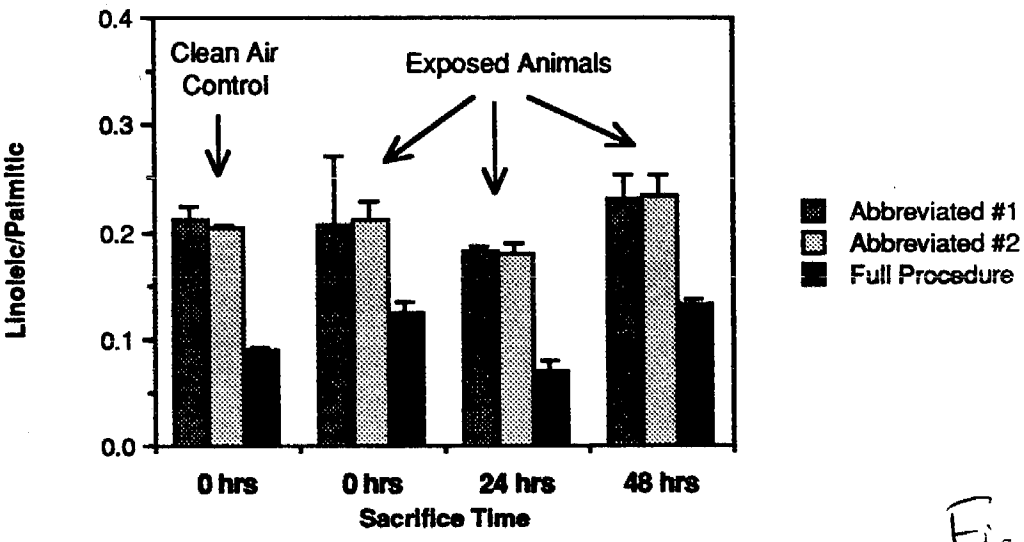
Full Procedure


Linoleic/Palmitic
0.4
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0.1
0.0
Clean Air Control
Exposed Animals
0 hrs
0 hrs
24 hrs
48 hrs
Sacrifice Time
Abbreviated #1
Abbreviated #2
Full Procedure

Fig 1.

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