Contract No. A6-148-33

ACID AIR POLLUTANT MIXTURES: RESPIRATORY SYSTEM RESPONSES AND EFFECTS OF EXERCISE

Final Report

Prepared for the California Air Resources Board

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Draft Final Report

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I. ABSTRACT

A toxicology study was performed to evaluate the effects on the respiratory system of inhalation of airborne acids and ozone (O_3). Rats were exposed to O_3 alone and in combination with a mixture of nitric acid (HNO_3) and sulfuric acid (H_2SO_4). Exposures were also performed with hydroxymethanesulfonic acid (HMSA) alone and in combination with O_3. Test atmospheres at two concentration levels were generated into stainless steel exposure chambers and healthy rats were exposed 4 h nose-only at rest or while walking on a treadmill at 10 m/min and 30% grade. This exercise level increased metabolic rate by a factor of about 1.5 over resting rates. Target concentrations for the lower concentration levels were 0.2 ppm O_3, 0.5 mg/m^3 HNO_3, 0.25 mg/m^3 H_2SO_4, and 0.25 mg/m^3 HMSA and for the higher concentration levels were 0.4 ppm O_3, 1.0 mg/m^3 HNO_3, 0.5 mg/m^3 H_2SO_4, and 0.5 mg/m^3 HMSA. In each exposure a matched group of rats exposed to purified air served as controls. The evaluation of respiratory effects consisted of (1) measures of respiratory irritancy including breath frequency, tidal volume, minute ventilation, oxygen consumption, ventilation equivalent for O_2, and rectal temperature; (2) analyses of tissue injury to the lung and nasal epithelium; and (3) biochemical analyses of pulmonary surfactant (PS) including Fourier transform infrared spectrometry (FTIR) and ultraviolet spectrometry (UV) of PS, gas chromatographic analysis of fatty acid composition of PS, and total protein content of lung lavage fluid. Exposure to 0.4 ppm O_3 at rest induced changes in breathing pattern and metabolic rate indicative of pulmonary irritation, induced lung parenchymal tissue injury, and changed the fatty acid composition of PS. Exposure to 0.2 ppm O_3 at rest did not result in significant effects on irritancy, tissue injury, or PS chemistry. Exercise exposure enhanced the effects of O_3 on irritancy and tissue injury. Resting exposure to 0.5 mg/m^3 HMSA alone had mild irritant effects but did not result in tissue injury or changes in PS chemistry. Addition

of HNO_3 + H_2SO_4 or HMSA to O_3 in resting exposures altered some pulmonary function measures of irritancy but tissue injury effects were similar to those observed from exposure to O_3 alone. Exposure to the O_3 + HNO_3 + H_2SO_4 combination gave evidence by FTIR for formation of some new compounds which are as yet unidentified. Exercise exposure to O_3 + HNO_3 + H_2SO_4 at the higher concentration level reduced the post-exposure breathing pattern responses and lung tissue injury observed for exercise exposure to O_3 alone possibly as a result of the influence of HNO_3 on exercise breathing pattern and dose distribution. Exercise exposure to O_3 + HMSA at the higher concentration level produced nasal epithelial injury that was not observed in O_3 alone or in resting exposures to O_3 + HMSA. The results of this investigation showed that the respiratory effects of single 4 h exposures at rest and exercise to ozone and mixtures were dominated by the effects of O_3. While the presence of acids in some instances significantly modified the effects of O_3, at the concentration tested, there was not a clear pattern of consistent interactions between these air pollutants. Such interactions may require higher concentrations or long term repeated exposures to be observed and understood.

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V. SUMMARY AND CONCLUSIONS

Laboratory rats were exposed 4 hours to mixtures of ozone (O_3) and airborne acids. The purpose of the project was to determine if acute inhalation exposures to oxidant and acid pollutants characteristic of air pollution in the South Coast Air Basin of Southern California showed evidence of synergistic interactions that enhanced injury to the respiratory tract. The effects of O_3 alone were compared to a combination of O_3 + nitric acid vapor (HNO_3) + sulfuric acid aerosol (H_2SO_4) and to a combination of O_3 + hydroxymethanesulfonic acid aerosol (HMSA). The comparisons were made at 2 concentration levels. Target concentrations for the lower levels were 0.2 ppm O_3, 0.5 mg/m^3 HNO_3, 0.25 mg/m^3 H_2SO_4, and 0.25 mg/m^3 HMSA and target concentrations for the higher levels were 0.4 ppm O_3, 1.0 mg/m^3 HNO_3, 0.5 mg/m^3 H_2SO_4, and 0.5 mg/m^3 HMSA. Rats were exposed under both resting and exercising conditions to test the possibility that important interactions between the pollutants may be present in exercise exposure that are not detected in resting exposures.

Analyses of biological effects included measures of respiratory irritation, analyses of respiratory tract tissue injury, and evaluation of alterations of pulmonary surfactant (PS). Irritancy was measured during and shortly after exposure (resting exposures) or shortly after exposure (exercising exposures) as changes in breathing pattern and metabolic rate. The parameters measured included breath frequency, tidal volume, minute ventilation, oxygen consumption, ventilation equivalent for oxygen, and rectal temperature. Histopathological analyses of tissue injury were performed including morphometric measurements of lesion formation in the lung parenchyma and autoradiographic measurements of cell turnover in nasal epithelium. The effects of exposures on PS were determined using Fourier transform infrared (FTIR) spectrometry and ultraviolet (UV) absorption spectrometry to characterize PS isolated from lavage fluid, gas chromatography to measure specific

fatty acid methyl esters in PS, and total protein content in lavage fluid to measure possible exudates due to tissue injury. PS was analyzed in the higher concentration exposures.

At the relatively low concentrations tested in these exposures, interactions between the acids and O_3 in their effects on irritancy, respiratory tract tissue injury, and changes in PS chemistry were complex and there was not a consistent pattern of synergistic interaction. Exposure to O_3 at 0.4 ppm at rest induced irritant responses (a rapid-shallow breathing pattern and depression in metabolic rate), induced lung parenchymal tissue injury, and changed the fatty acid composition of PS. At 24 hours post exposure, the concentrations of the minor fatty acids were decreased relative to the major fatty acid component, palmitic acid, with linoleic acid, the most unsaturated acid studied, showed the greatest change. Exposure to 0.2 ppm O_3 at rest did not result in significant irritancy responses or tissue injury. Exercise exposure enhanced the effects of O_3 on irritancy and tissue injury. Irritant effects on breathing pattern and metabolic rate were present in the 2nd hour after exercise exposures to both concentrations of O_3 while recovery occurred in the rest exposures to 0.4 ppm O_3 by hour 2 post-exposure. Tissue injury to the nose and lung was also increased by exercise exposure. Exercise exposure to O_3 + HNO_3 + H_2SO_4 at the higher concentrations showed a reduction in the hour 2 post-exposure breathing pattern response to O_3 alone and reduced parenchymal lung injury compared to the effects of O_3 alone. Exposure to HMSA at rest resulted in a small irritant effect (depression of metabolic rate) but no detectable tissue injury or effects on PS. HMSA combined with O_3 in resting exposures produced effects similar to the effects of O_3 alone on irritancy, tissue injury, or PS, however body temperature depression was greater in the combination exposure than in O_3 alone. Exercise exposure to 0.4 ppm O_3 + 0.5 mg/m^3 HMSA produced highly significant nasal epithelial injury that was not observed in O_3 alone or in resting exposures to O_3 + HMSA. The results of

this project showed that the respiratory effects of acute exposures to O_3 and acids were dominated by the effects of O_3 in inducing inflammatory injury to the lung which was revealed in measures of irritancy, tissue histopathology, and changes in pulmonary surfactant chemistry. Exercise exposure enhanced the effects of O_3 on the lung and the effects of HMSA on the nose. The presence of acids in combination with O_3 in some instances modified the pulmonary effects of O_3, but at the concentrations tested, there was not a clear pattern of consistent interactions between these air pollutants to permit an interpretation of the mechanism of toxic interactions. Such interactions may require study at higher concentrations in acute exposures or long term repeated exposures to gain clear understanding of the basis for the interplay of toxic effects.

VI. RECOMMENDATIONS

1. Toxicology studies with longer term periods of exposure should be performed to evaluate the effect of repeated inhalation exposure to acid and oxidant air pollutant mixtures. Observations of synergistic interactions between acids and ozone have been observed in multiple daily exposures (Last and Cross, 1978; Last et al. 1983, 1984, 1986) and in single acute exposures to higher O_3 concentrations than used in this study (Kleinman et al., 1989). The present research using single acute exposures at relatively low O_3 concentrations did not reveal a clear pattern of enhancement of O_3 toxicity by presence of HMSA or HNO_3 + H_2SO_4 in a mixture, and the synergistic phenomenon between acids and oxidants may be effected by repeated exposures. Acute exposure studies at higher concentrations are important for cost-efficient identification of important interactions and understanding biological mechanisms for the interactions. These should be advanced as a guide for choosing pollutant combinations to be studied in longer term exposures at urban ambient concentrations.

2. Investigators should continue to seek an explanation for the synergistic interactions between O_3 and NO_2 and to determine how HNO_3 may influence O_3 toxicity. Our earlier studies (ARB A2-129-33 Final Report 1984; ARB A4-112-32 Final Report 1988; Mautz et al., 1988) demonstrated strong synergistic interactions between O_3 and NO_2, and in other studies (Kleinman et al., 1989) we found that H_2SO_4 enhanced the O_3-induced lung injury. However in the present study, HNO_3 combined with H_2SO_4 and O_3 in an exercising exposure had a smaller effect on the lung parenchyma than O_3 alone. This new, contrasting result should be confirmed, and the role of HNO_3 in particular vs. vapor phase acids or upper airway irritants in general in this response needs to be determined.

3. Chemical analysis of pulmonary surfactant using FTIR and UV spectroscopy and gas chromatographic analysis of fatty acid esters was a sensitive indicator of oxidant-induced pulmonary inflammation. Research on PS should be continued to establish the time course of changes in these indicators of injury and inflammation. The effects of oxidant exposure on model compounds for PS should be carried out to identify the as yet unknown infrared absorption bands formed from the exposure of rats to the $O_3 + HNO_3 + H_2SO_4$ combination and to identify the important chemical changes that occur.

VII. INTRODUCTION

A. Purpose

People who live in polluted urban environments are exposed to mixtures of many different gases and particles. Oxidant air pollutants, particularly ozone (O_3), have long been recognized as major toxic components of polluted air in the South Coast Air Basin of Southern California. Acid compounds are of increasing concern because of the high levels of acidity measured in ambient air (Solomon et al., 1988) and in fogs in the South Coast Air Basin (Waldman et al., 1982; Brewer et al., 1983; Richard et al., 1983; Munger et al., 1983, 1984; Jacob et al., 1985), and because acids were reported to enhance the toxic effects of inhaled oxidants (Last and Cross, 1978; Warren and Last, 1987).

The purpose of this project was to evaluate the possible toxic effects to the respiratory system of inhalation of airborne acids. Because acids and oxidants occur together in the South Coast Air Basin and because the effects of acids in enhancing oxidant injury may be more important than the direct effects of acids alone, this study examined the effects of mixtures of O_3 and acids. Acid compounds were selected that are important components of urban air pollution in Southern California including sulfuric acid (H_2SO_4), nitric acid (HNO_3), and hydroxymethanesulfonic acid (HMSA) for inhalation exposure experiments with rats. This project followed our earlier study of acid air pollutant mixtures (ARB Contract A4-112-32, Final Report, 1988), and focused on several questions that were raised by the earlier research. In our earlier ARB contract and in other studies (Mautz et al., 1988), we found strong interactions between O_3 and NO_2, oxidants that in a mixture form HNO_3. It was possible that HNO_3 was responsible for this interaction, so in the present study, we included HNO3 in combination with O3 and H_2SO_4 to test whether the acids could modify the effects observed in rats exposed to O_3 alone. At the relatively low component concentrations of the multicomponent

mixtures we studied previously (0.35 ppm O_3, 1.3 ppm NO_2, 2.5 ppm SO_2, 1.0 mg/m^3 HNO_3, and 0.5 mg/m^3 H_2SO_4) the effects of O_3 dominated the respiratory responses and the exposures were all performed with resting rats. Exercise exposure, however, can greatly enhance the pulmonary effects of O_3 and the interaction between O_3 and NO_2 (Mautz et al. 1985a, 1988). In the present study we therefore used exercising exposures of rats to determine whether important effects were present under exercise conditions that were not detected in resting exposures. Finally, because HMSA has been reported to be a significant acidic component of fog water in Southern California, (Munger et al., 1986), we performed exposures to HMSA aerosol alone and in combination with O_3.

B. Previous Studies of Biological Effects of Oxidants and Acids

Studies of the health effects of O_3 exposure have shown that O_3 induces acute changes in lung function in humans and corresponding injury to epithelia of the respiratory systems of animals (Folinsbee et al., 1978; Adams et al., 1981; McDonnell et al., 1983; Mautz et al., 1985a, 1988). Human exposure studies have demonstrated significant toxic effects of O_3 at concentrations as low as 0.12 ppm, a level commonly recorded in heavily polluted urban areas, and ozone remains an important focus of attention for toxicological research and for environmental regulation. Studies of the health effects of inhaled acids have for the most part been confined to sulfuric acid aerosols. Sulfuric acid has been shown to induce changes in the lung's ability to clear foreign particles, to produce injury to respiratory system epithelia and to change pulmonary function (Schlesinger, 1985; Schlesinger et al., 1978, 1979, 1983; Schiff et al., 1979; Leikauf et al., 1981; Kerr et al., 1981; Chen and Schlesinger, 1983; Utell et al., 1983, 1985). Epidemiological evidence has been assembled that implicates acid sulfates as important air pollutants (Ozkaynak and Spengler, 1985). The correlation of hospital admissions for respiratory disease in Southern Ontario, Canada, with SO_2 and temperature by Bates and

Sizto (1983) and the further correlation between asthma admissions and SO_4-2 (Bates and Sizto, 1985, cited by Lippmann, 1985) is interpreted by Lippmann (1985) as evidence that sulfate serves as a surrogate for the more acidic sulfates (H_2SO_4 and NH_4HSO_4). Another observation made by Bates and Sizto (1985) is cited by Lippmann as follows: "elevated O_3 appeared to contribute to the effect of sulfate (or H^+) on respiratory morbidity."

While the toxicology of H_2SO_4 has been intensively studied, there has been little work on other acidic pollutant compounds such as HNO_3 or organic acids. Many kinds of acids may be present in polluted air and most are formed in oxidation reactions of sulfur oxides, nitrogen oxides and organic compounds. There is evidence from exposures of laboratory rats that synergistic interactions between acids and O_3 enhance the toxicity of the oxidant (Last et al., 1983, 1984, 1986; Last and Cross, 1978), and epidemiological reports of Bates and Sizto (1983, 1985) suggest that O_3 is an important co-pollutant with potentially acidic sulfates. A study of sodium hydroxymethanesulfonate (5 mg/m^3) exposure of rats for 3 days (Last et al., 1980) did not show significant effects on tracheal glycoprotein secretion rate, lung DNA, RNA and protein content, and lung wet to dry weight ratio.

C. Acid Air Pollution in Southern California

In photochemically polluted air in Southern California, the dominant acid species is often HNO_3 produced by the daytime oxidation of NO_2 by OH radicle or by nighttime reactions of the nitrate radical (NO_3) (Finlayson-Pitts and Pitts, 1986). Lioy (1983) summarized ambient data for acid sulfates and has reported sulfuric acid concentrations ranging up to 28 $\mu g/m^3$. Ambient concentrations of HNO_3 have not been well characterized, however Spicer et al. (1982) report typical peak hourly concentrations of 30 $\mu g/m^3$ (12 ppb) in Los Angeles and gas phase concentrations as high as 130 $\mu g/m^3$ (50 ppb) have been reported in Los Angeles (Tuazon et al., 1981). Maximum 24 hr averages measured in cities

within the South Coast Air Basin ranged from 5 to 20 $\mu g/m^{-3}$ in 1986 (Solomon et al., 1988). Nitric acid has a high vapor pressure, and most nitric acid in ambient air will be in the vapor phase in contrast to sulfuric acid which exists as an aerosol.

A number of recent studies of the chemical composition of fog water (Munger et al., 1983, 1984; Boyce and Hoffman, 1984) have established that sulfur with valence IV, S (IV), is often present in the aqueous phase in concentrations far in excess of those expected based solely on the well known equilibria (1-3) below:

$$SO_2(g) + H_2O \rightleftharpoons SO_2 \cdot H_2O \qquad (1)$$

$$SO_2 \cdot H_2O \rightleftharpoons HSO_3^- + H^+ \qquad (2)$$

$$HSO_3^- \rightleftharpoons SO_3^{-2} + H^+ \qquad (3)$$

The case for formaldehyde (HCHO) is the same. HCHO concentration in aerosols has been observed to be greater than expected from its gas phase concentration and the Henry's law constant (Klippel and Warneck, 1980). The reaction of HCHO and HSO_3^- yields the hydroxymethanesulfonate ion, $H_2COHSO_3^-$, which has been observed in fog water samples in California at significant concentrations (Munger et al., 1986). Indeed, 50% or more of the S (IV) in fog water samples has been shown to exist in the form of HMSA. As the HMSA complex forms, more S (IV) is drawn into solution acidifying it via reaction (2). Interestingly, HMSA is relatively stable towards oxidation so that the acidification of the droplet can occur without accompanying S (VI) formation.

D. Exercise as a dose factor in the health effects of air pollution

In inhalation studies exercise has a direct effect on ventilation and dose rate. Furthermore, exercise affects the dose distribution of inhaled compounds and numerous studies have shown

that exercise exposure can have a much greater impact on the effects of inhaled toxicants than expected from simple inhaled dose increases. Our previous ARB supported research on oxidant toxicity and exercise has demonstrated in acute exposure studies strong enhancement of ozone-induced lung tissue injury when exposures were performed during exercise (Mautz et al., 1985a), and exercise enhancement of the toxic interaction between O_3 and NO_2 (Mautz et al., 1988). The conditions of exposure of the public to air pollution may involve heavy exercise, and exercise is thus an important exposure variable. We therefore examined how exercise exposure altered the toxicity of acid-oxidant mixtures.

E. Biological Effects Assessment

The respiratory system responses we measured ranged from the whole animal to tissue and to molecular levels of organization. Reflex breathing responses provided a sensitive and comparative measure of irritancy and we developed measurements of breathing pattern of rats during exposures in our earlier study (ARB A4-112-32, Final Report, 1988). Respiratory reflex response to inhaling ozone or acidic pulmonary irritants consist of shifts to slow-deep breathing and depression of minute ventilation with acids, or to rapid-shallow breathing with ozone, (Alarie, 1973; Kane and Alarie, 1977; Kane et al., 1979; Chang et al., 1981; Coggins et al., 1981, 1982; Landrey et al., 1983).

Tissue injury resulting from the exposures was assessed in the parenchyma of the lung and in the epithelium of the nose. Oxidant exposure typically results in lesion injury of the lung parenchyma, and this injury was quantified by morphometric techniques (Mautz et al., 1985a, 1988). Acids on the other hand, can injure the nasal epithelium (Kleinman et al., 1985; Mautz et al., 1988), and nasal tissue injury was analyzed by measuring increased rates of epithelial cell turnover as the incorporation of radiolabeled thymidine into the DNA of proliferating epithelial cells.

Exposure-related changes in the chemical characteristics of pulmonary surfactant was also assessed in this study. The gas exchange portion of the respiratory tract consists of alveolar sacs and alveoli. Type I and Type II alveolar cells line the surface, and a thin layer of fluid known as pulmonary surfactant (PS) covers these cells, forming an interface between the airspace and the alveoli (Scarpelli, 1968). Pattle and co-workers (1955, 1958) and Clements and co-workers (1956, 1958, 1961a,b) first demonstrated that this surfactant layer plays a key role in lung function by lowering the surface tension of the fluid at the air-water interface, maintaining the integrity of the alveolar spaces. Thus PS prevents alveolar collapse (atelectasis) in the expiration phase of breathing and prevents pulmonary edema which would occur if the surface tension in the alveoli was high (Avery and Mead, 1959; Clements et al., 1961b). Abnormalities in the surfactant system are associated with such diseases as hyaline membrane disease in newborns and respiratory distress syndrome in adults (Haagsman and van Golde, 1985). Pollutant exposure that modifies the chemistry of PS could alter its surfactant properties and thus aggravate or possibly irritate lung injury.

Numerous studies of the chemical composition of PS have established that phospholipids are the major surface-active components, with phosphatidylcholine being predominant in a variety of species, including humans and rats. The fatty acid residues are mainly palmitate (55-80% of the total fatty acids present) with smaller amounts of other fatty acids, both saturated (e.g. stearic) and unsaturated (e.g. palmitoleic, oleic, linoleic) also present (Sanders, 1982). 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, hydrophobic ends projecting into the air.

It should be noted that the definition of what constitutes PS is not universally agreed upon, and is sensitive to the isolation

technique used to obtain the alveolar material. PS is normally obtained by rinsing the lung with a saline solution, followed by various centrifugation steps to remove cellular debris and mucus collected into the lavage solution from the airways (Goerke, 1974; King, 1982; Kotas, 1982; van Golde et al., 1988).

Oxidant air pollutants, especially O_3, have been observed to lead to changes in a variety of biochemical and physiological parameters in the lung, including changes in material isolated from lavage fluids. For example, while Huber et al. (1971) found no change in the fatty acid composition of lavage fluids from rabbits exposed to 5 ppm O_3 for three hours, Menzel et al. (1972) showed that linoleic and linolenic acids in the lavage fluids of rats exposed to 1.6 ppm O_3 continuously decreased after several days. Similarly, Roehm et al. (1972) found that exposure to 0.5 ppm O_3 for 6 weeks gave a statistically significant change only in vitamin E-depleted rats; arachadonic acid increased and palmitoleic acid decreased in the exposed animals. Shimasaki et al. (1976) exposed rats to 1.1 ppm O_3 for 5-7 days and showed that the fatty acids in the phosphatidylcholine component of the lavage fluid changed significantly; palmitic acid decreased, while stearic, oleic, linoleic, and arachadonic acids increased. While a number of studies have examined the effects of exposure to O_3 alone on the composition of lavage fluids, there appear to be no studies of the effects of O_3 combined with nitric and sulfuric acids on PS.

It should be noted that in all of these studies of the effects of air pollutants on the material lining the lung, only a low speed centrifugation to remove the cellular debris was used. Thus the changes in fatty acid composition may reflect changes in a combination of PS and mucus. Recent studies (Finlayson-Pitts et al., 1989) have shown that the omission of time-consuming ultracentrifugation steps recommended for the isolation of PS from lavage fluids may give a different fatty acid composition and also may lead to a significant underestimate of the effects of oxidants on PS.

The goals of the PS portion of this project were threefold: 1) To assess whether a combination of nitric and sulfuric acids, which in our earlier study (ARB contract A4-112-32, Final Report, May 1988) did not significantly alter PS themselves, enhanced the effects due to O_3 alone; 2) To determine whether exposure to HMSA alone induces an inflammatory response in the animal which alters the chemical composition of PS 24 hours after the exposure, as we observed for oxidant. air pollutants in our earlier study; 3) To investigate whether HMSA interacts with O_3 in a synergistic manner with respect to changes in the PS composition, i.e., whether the changes induced by the combination of HMSA + O_3 are greater than the sum of the changes induced by these two pollutants individually.

VIII. MATERIALS AND METHODS

A. Exposure Experiments

Table 1 shows the exposure experiment plan for this contract. Each exposure experiment shows the set of exposure groups and target concentrations for the pollutant atmospheres. The endpoints are the set of biological responses that were measured in each experiment.

B. Generation and Characterization of Exposure Atmospheres

Single and mixed components of test pollutant atmospheres were metered into purified air at rates sufficient to yield target concentrations at the breathing zone of exposed animals. Ozone was generated by metering medical grade oxygen through a Sander Ozonizer. 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 provided 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. HMSA was generated from acidified sodium hydroxymethanesulfonate (Kok et al., 1986). Sodium hydroxymethanesulfonate, purchased as Sodium Formaldehyde Bisulfate (Eastman), was dissolved in deionized, distilled water, and recrystallized according to the procedure described by Dasgupta et al. (1980), to ensure purity. A 10% solution of the recrystallized salt was adjusted to pH 4.0 with sulfuric acid. The adjusted solution was aerosolized with a Collison nebulizer, diluted, and equilibrated, with purified air at 85% relative humidity, and passed through a ^{85}Kr discharger to reduce the electrostatic charge on the particles to the Boltzmann equilibrium level.

TABLE 1. Exposure Experiments for ARB Contract A6-148-33: "Acid Air Pollutant Mixtures: Respiratory System Responses and Effects of Exercise"

1. Resting exposure to ozone and acids.

   Atmospheres: a) control; b) 0.4 ppm O_3; c) 0.2 ppm O_3; d) 0.4 ppm O_3 + 1.0 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4; e) 0.2 ppm O_3 + 0.5 mg/m^3 HNO_3 + 0.25 mg/m^3 H_2SO_4.
   Endpoints: breathing pattern analysis, histopathology, and pulmonary surfactant analysis (for high concentrations).

2. Exercising exposure to ozone and acids.

   Atmospheres: a) control; b) 0.4 ppm O_3; c) 0.2 ppm O_3; d) 0.4 ppm O_3 + 1.0 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4; e) 0.2 ppm O_3 + 0.5 mg/m^3 HNO_3 + 0.25 mg/m^3 H_2SO_4.
   Endpoints: breathing pattern analysis and histopathology.

3. Pilot exposure at rest to hydroxymethanesulfonic acid.

   Atmospheres: a) control; b) 0.5 mg/m^3 HMSA.
   Endpoints: breathing pattern analysis, histopathology,and pulmonary surfactant analysis.

4. Resting exposure to ozone and hydroxymethanesulfonic acid.

   Atmospheres: a) control; b) 0.4 ppm O_3; c) 0.5 mg/m^3 HMSA;d) 0.4 ppm O_3 + 0.5 mg/m^3 HMSA; e) 0.2 ppm O_3 + 0.25 mg/m^3 HMSA.
   Endpoints: breathing pattern analysis, histopathology, and pulmonary surfactant analysis.

5. Exercising exposure to ozone and hydroxymethanesulfonic acid.

   Atmospheres: a) control; b) 0.4 ppm O_3; c) 0.2 ppm O_3; d) 0.4 ppm O_3 + 0.5 mg/m^3 HMSA; e) 0.2 ppm O_3 + 0.25 mg/m^3 HMSA.
   Endpoints: breathing pattern analysis, and histopathology.

Ozone was monitored using a UV absorption O_3 analyzer (Dasibi Model 1003 AH). In mixtures of nitric and sulfuric acids 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 (Zefluor 0.5 μm pore size) collected acid aerosols. Nitric acid vapor passed through this filter and was trapped by a nylon backup filter (Appel et al., 1980). Zefluor 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 HNO_3 was computed. In HMSA exposures, aerosol samples were collected from the rat's breathing zone, on acid-washed, teflon-coated glass fiber filters (Pallflex TX40H120WW). The filters were weighed and extracted in aqueous solution. A fraction of that solution was added to concentrated sulfuric acid to decompose the HMSA. The formaldehyde released by the decomposition of the HMSA was reacted with chromotropic acid and analyzed spectrophotometrically, against standard curves prepared using purified HMSA salt as a reference.

All exposures of rats at rest were performed in Rochester-type exposure chambers modified to give the rats a nose-only exposure to the pollutant compounds (Bhalla et al., 1987). Nose-only exposure ensured that test acidic compounds were not neutralized by any ammonia produced from the rats' excreta. Exercising exposures were performed in a Quinton 42-15 rodent treadmill modified to contain the exposure atmosphere delivered from the Rochester chambers (Mautz, 1985a, b). Pollutant concentrations in the treadmill were monitored and controlled based on samples from the breathing zone of the rats
in the treadmill runways.

C. 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. These animals were killed by pentobarbital 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 laboratory rats. Acceptance and use of the animals was based on this examination. Quarterly necropsy and viral serological testing reports were provided by 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 two times per week. A rotational change of the rats to sanitized caging was performed once every two weeks. Conventional litter in the rat trays beneath the wire cage floors was replaced by rock salt which dries excreted urine and feces and suppresses ammonia production due to bacterial growth.

In experiments involving exercise exposure, rats were trained to achieve an exposure exercise capability of running at 10 $m \cdot min^{-1}$ at 30% grade for 4 h. Exercise protocols in earlier exposures were 15 $m \cdot min^{-1}$ at 20% grade for 3 h and increased metabolic rate by a factor of 2 over resting rates. In order to increase the time duration of exercise performance to 4 h, a different protocol was used with the lower running speed and steeper slope. Training on day 1 was continuous running for 3 h at 8 $m \cdot min^{-1}$, 20% grade; day 2 was continuous running at 10 $m \cdot min^{-1}$ 20% grade for 4 h and day 3 was continuous running at 10 $m \cdot min^{-1}$, 30% grade for 4 h. Training was designed to acquaint the rats with the treadmill and to ensure that they could complete the exposure protocol. This exposure

exercise level increased metabolic rate of the rats by a factor of about 1.5.

D. Pulmonary Function

Breathing pattern changes of rats exposed to the test atmospheres were measured with body plethysmographs. The plethysmographs 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). The tubes were modified to function as flow plethysmographs during exposure to O_3. To separate a rats' respiratory orifices from the body, a truncated cone of latex dental dam was clamped between the metal nose cone and body tube and subtended an angle 4° smaller than the metal nose cone. The latex fit snugly around the anterior angle of the head, and the nose and mouth protruded from the tip of the cone. Thoracic displacement of air during respiration was measured with a #0000 pneumotachograph (Dynasciences, 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 nose. The signal from the transducer was electrically integrated and counted to provide tidal volume and breath frequency displayed 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 at 3 L min^{-1} 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. 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 for water vapor content using a mass spectrometer (Perkin-Elmer model 1100, Pomona, CA) and a dew point sensor (EG&G, Model 911, Waltham, MA).

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., 1985b) and expressed at standard temperature, pressure, dry 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 h while recovering from the excitement of handling and loading, followed by a 4 h exposure to the test atmosphere. Following the exposure, rats were transferred to a clean air chamber and breathing pattern and metabolic rate measurements were made between 1 and 2 h post-exposure. Breathing pattern measurements could not be made from animals while they were running during exercise exposures, so exercising rats were removed from the treadmill following exposure, loaded into plethysmographs and exposed to clean air. Breathing pattern and metabolic rate measurements were made between 1 and 2 h post-exposure for comparison to post-exposure measurements from rats exposed at rest. 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 during 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 (pairwise) of group effects (Zar, 1984).

E. Histopathology

The 8 animals exposed in the plethysmographs were included as the majority of the complete sample (n = 10) exposed for histopathologic analyses. 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 pentobarbital and killed by exsanguination. The thoracic cavity was opened after puncturing the diaphragm and the lungs and trachea carefully isolated. 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 et al., 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 um 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 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. Two levels of lesion severity were defined:

Type 1 Lesion: Two or more cells lie free in the lumen of an alveolus within a single grid area of 10,000 sq. micrometers. This feature also occurs at small frequency in lungs of rats exposed to clean air, but is 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 microcurie/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 and placed under slight vacuum 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 glycolmethacrylate. Sections (2 μm) separated by 50 μm were 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.

F. Chemical Characterization of Pulmonary Surfactant

1. Isolation of Pulmonary Surfactant.

Pulmonary surfactant (PS) was obtained by lavage. The rats were deeply anesthetized with sodium pentobarbital IP 24 hours after the exposure, and the abdominal aorta cut to drain the blood from the heart and lungs. A short piece of tygon tubing inserted into a cut in the trachea was used to introduce and withdraw sequentially two 7.0 ml volumes of chilled 0.15 M NaCl containing 0.003 M $MgCl_2$ and 0.003 M $CaCl_2$ respectively. This saline solution containing the surfactant was stored on ice for approximately one half hour during the trip from U.C. Irvine to C. S. U. Fullerton. Twelve rats were used for each exposure condition for the surfactant analysis. Because only microgram amounts of isolated surfactant can be obtained from one rat, the surfactant from six rats was pooled to obtain sufficient sample for analysis. Thus for each exposure, there were two replicate samples representing pooled samples from six rats each.

The pooled PS containing saline solution was centrifuged at 600 g for 20 minutes to sediment cellular debris. The supernatant was then centrifuged at 16,000 g for one hour to sediment the surfactant pellet, along with other contaminants such as mucus. The pellet was resuspended in distilled, deionized water and stored at -15°C overnight; the supernatant was stored at 4°C and analyzed for total protein content using the Folin protein assay (Clark and Switzer, 1977).

The thawed surfactant solution was layered on a sucrose solution of density 1.01 (3.26 % w/v) and centrifuged at 1.04 x 105 g in a TI50 rotor for one hour. The pellet obtained was resuspended in water, layered on a second sucrose solution of density 1.055 (14.44% w/v), and centrifuged for one hour at 8.72 x 104 g in the TI50 rotor. The surfactant appeared as an opalescent band at the water-sucrose solution interface; in some samples, a pale yellow mucoid material appeared as a sediment. The surfactant was removed with a pipette and recentrifuged at 1.04

x 105 g to sediment the surfactant and separate it from any remaining sucrose. All centrifugation steps were carried out at 4°C.

The pellet from the final centrifugation was resuspended in water and then extracted sequentially with three 10 ml volumes of $CHCl_3/CH_3OH$ (2:1 v/v). The sample was then split into four equal parts for analysis. All samples were stored under N_2 at -15°C.

2. Chemical Analysis

The chemical composition of isolated PS was characterized by Fourier transform infrared spectroscopy (FTIR), ultraviolet spectroscopy (UV) and by gas chromatography (GC) of the fatty acid methyl esters formed from the surfactant. Protein analyses was carried out on the supernatants from the first centrifugation step.

a. FTIR. An aliquot of the isolated surfactant dissolved in $CHCl_3/CH_3OH$ was layered onto a germanium ATR (attenuated total reflectance) crystal (25 mm x 10 mm x 2 mm, 45°C) one drop at a time; the solvent generally rapidly evaporated, but when necessary, it was evaporated off with a stream of dry N_2. The ATR crystal was placed in the purged sample compartment of a Mattson Sirius 100 FTIR and the spectrum recorded at 2 cm^{-1} resolution, with 64 scans added to improve the signal-to-noise ratio.

b. UV. The solvent was evaporated off a second aliquot and CH_3OH added to redissolve the sample. Because PS is not as soluble in CH_3OH as in the $CHCl_3/CH_3OH$ mixture, the sample was allowed to stand under N_2 for approximately 30 minutes at room temperature, with periodic swirling of the ampule during this time. The spectra were scanned using a Varian Model 2200 UV/visible spectrometer with CH_3OH as the reference. After the spectra were obtained, the samples were stored in CH_3OH under N_2 at -15°C.

Subsequently, all UV spectra were recorded again using a Perkin Elmer Lambda 3B UV/visible spectrometer which had just become

available in the California State University Chemistry Department. These second set of spectra for all experiments using the Perkin Elmer reported here were recorded the day after the Varian spectra were obtained for the O_3 vs HMSA vs (O_3 + HMSA) exposure (Experiment 4). While artifactual formation of UV absorbing products would not have occurred in the samples from this last experiment, it is possible that it could have taken place during storage of the samples for two to three months from the first two experiments. Thus the spectra from these first two experiments, obtained using the Varian immediately after isolation of the PS, were carefully compared to those recorded subsequently using the Perkin Elmer spectrometer. No evidence of change in these samples during storage was found. Thus the spectra presented here are those from the Perkin Elmer instrument, since the computer interface and software allow more quantitative data manipulation and comparisons.

c. Gas Chromatography (GC) of Fatty Acid Methyl Esters. The fatty acid composition of the PS was obtained using GC of the methyl esters formed using a modification of the method of Mason and Waller (1964). Briefly, the solvent was evaporated from an aliquot of the PS sample, and 0.5 ml dry CH_3OH acidified with HCl, 0.1 ml dimethoxypropane and 1.4 ml $CHCl_3$ were added to the sample. It was left overnight under N_2 to react to completion, and then neutralized with 0.25 g of a mixture of $NaHCO_3$, Na_2CO_3 and Na_2SO_4 (2:1:2 w/w/w) which had been dried overnight at 110°C. The liquid/salt mixture was filtered using Acrodisc filters which had been previously cleaned with $CHCl_3/CH_3OH$. The solvent was evaporated to concentrate the sample to 100-200 microliters, which gave a sample of suitable concentration for analysis.

The major fatty acids (palmitic, palmitoleic, stearic, oleic, and linoleic) were identified by comparison of their retention times to those of authentic, commercially available samples of the methyl esters, and their relative yields obtained by calibrating the GC with these known standard methyl esters. The GC was a

Hewlett Packard Model 5750 equipped with a flame ionization detector and a Supelcowax 10 capillary column (Supelco Inc).

d. Protein Analysis. Although total protein analysis was not included in the original proposal to The California Air Resources Board, an undergraduate student at C. S. U. Fullerton, Ms. Marybeth Norgren, was available to carry out these analyses at no cost to the project while fulfilling her senior research requirement for graduation. She had experience with this assay in earlier research studies with Dr. Finlayson-Pitts, and hence carried out the Folin assay (Clark and Switzer, 1977) on the lavage fluids obtained in the experiments described here. To provide a more complete picture of the effects of O_3, HMSA and acids on the lung, the results of these studies were included in this report.

e. Statistical Analysis. Although the FTIR and UV data are qualitative in nature, the quantitative data on the fatty acid composition data are suitable for statistical analysis, which was performed at no cost to the project by Dr. Karen Messer of the Department of Mathematics at C. S. U. Fullerton.

The data for each fatty acid are analyzed separately. As stated above, two independent pooled samples for fatty acid determination were obtained for each exposure condition. The statistical model accounts for two types of random variability whose magnitude can be estimated from the data: (1) the variability between the mean GC readings of the two independent pooled samples, denoted by τ_{ij}, and (2) the variability between the GC readings on the same pooled sample, denoted by ϵ_{ijk}. In these symbols, the subscript i = 1,2,..,5 denotes exposure condition, the subscript j = 1,2 denotes the two pooled samples for each exposure, and the subscript k = 1,2,..,"n sub ij" denotes the GC measurements on each pooled sample.

The two types of random variability in the model both combine multiple sources of variation which cannot be separated. Term (1)

combines the natural variation among rats and the variation in the chemical isolation technique. Term (2) combines variation among the subsamples from a pooled sample and variation in the performace of the gas chromatograph.

The statistical model for each fatty acid is then

$$Y_{ijk} = \beta_i = \tau_{ij} = \epsilon_{ijk}$$

where Bi is the mean fatty acid concentration for the exposure condition and τ_{ij} and ϵ_{ijk} are assumed to be statistically independent random effects. These random effects are modeled as normal random variables with zero means and respective variances $\sigma_{\tau i}2$ and $\sigma_{\epsilon}2$. Thus the model allows the magnitude of variability between mean GC readings of the two pooled samples to be different for each exposure condition, while the variability $\sigma_{\epsilon}2$ is assumed to be the same for all exposure conditions.

The model was estimated by a maximum likelihood algorithm programmed in MATLAB on an AST/286. An EM algorithm was used and is available from Dr. Messer. A Wald test of significance ("t"-like ratios) was performed (Rao, 1965). Those τ_i which were not significantly different from zero or were negative were constrained to be zero. A small Monte Carlo study was run prior to the data analysis to check that the test gives reasonable results on the given sample sizes.

IX. RESULTS

A. Exposure Atmospheres

Concentrations of pollutant compounds used in the exposures for this contract are shown in Tables 2 and 3. O_3 was measured continuously during the exposure and O_3 generation rates were easily adjusted with a short time lag to control concentrations at target values in the breathing zone of the rats. The analyses of the vapor acid, HNO_3 and aerosol acids, H_2SO_4 and HMSA, involved lengthy sample acquisition times and HNO_3 in particular participated in wall surface reactions and possibly physical adsorption losses to the exposure system. Thus, concentrations of these compounds were not subject to the same close control as O_3, and they exhibited more variability. Generally, the mean pollutant concentrations were close to target values. Air temperature and relative humidity during exposures were controlled at 22.7 ± 0.3°C and 83.2 ± 1.2%.

B. Pulmonary Function

Breathing pattern, ventilatory and metabolic rate responses to ozone alone and in combination with HNO_3 and H_2SO_4 in resting exposures are shown in Table 4. Analysis of variance was used to determine if there were statistically significant differences between atmosphere groups. If differences were present, Tukey multiple comparison tests were performed to determine which atmosphere groups were significantly different from one another. The most concise way to express these results is to show (as in Table 5a) the groups in rank order by group mean and clustered by overhead lines that show sets of groups that are not significantly different from one another (Rohlf, 1969; Zar, 1984). Any pair of groups that are not directly connected by a single overhead line were sufficiently different to be significant at the $p<0.05$ level. In addition to this presentation, we have provided in part b of this and other multiple comparison tables, a listing of the

TABLE 2. Test Pollutant Concentrations (Mean ± SD) in Exposure Experiments 1 and 2.

Experiment and Exposure Groups	O_3 (ppm)	HNO_3 Vapor mg/m^3	Particulate Nitrate mg/m^3	H_2SO_4 (mg/m^3)	Aerosol (umMMAD)	Size (GSD)
1. Rest						
A. Clean Air						
B. O_3	0.20±0.01					
C. O_3	0.40±0.01					
D. O_3+HNO3 +H_2SO_4	0.21±0.01	0.64±0.08	0.024±0.007	0.25±0.03	0.27	2.0
E. O_3+HNO_3 +H_2SO_4	0.41±0.01	0.67±0.12	0.011±0.011	0.61±0.06	0.32	2.3
2. Exercise						
A. Clean Air						
B. O_3	0.21±0.01					
C. O_3	0.40±0.03					
D. O_3+HNO_3 +H_2SO_4	0.21±0.01	0.48±0.04		0.25±0.03	0.27	2.93
E. O_3+HNO_3 +H_2SO_4	0.40±0.03	0.91±0.16		0.51±0.03	0.21	3.05

TABLE 3. Test Pollutant Concentrations (Mean±SD) in Exposure Experiments 3, 4, and 5.

Experiment and Exposure Groups	O_3 (ppm)	HMSA (mg/m^3)	Aerosol Size (umMMAD)	(GSD)
3. Rest				
A. Clean Air				
B. HMSA		0.56±0.01	0.32	2.13
4. Rest				
A. Clean Air				
B. O_3	0.40±0.01			
C. HMSA		0.47±0.03	0.15	3.1
D. HMSA+O_3	0.20±0.1	0.23±0.01	0.34	2.9
E. HMSA+O_3	0.40±0.01	0.38±0.06	0.32	3.6
5. Exercise				
A. Clean Air				
B. O_3	0.20±0.01			
C. O_3	0.40±0.02			
D. O_3+HMSA	0.20±0.01	0.19±0.05	0.85	1.96
E. O_3+HMSA	0.40±0.02	0.46±0.06	0.79	2.66

TABLE 4. Breathing Pattern, Ventilatory, and Metabolic Rates of Rats Exposed at Rest to Ozone and Acids (Experiment 1). Data are mean (standard error, n).

Variable Exposure Group	Hour 1 of Exposure	Hour of Exposure	Hour 2 Post-exposure
Breath Frequency (min-1)			
Clean Air	150 (12.1, 8)	166 (12.3, 8)	166 (12.0, 8)
0.2 ppm O_3	150 (8.7, 8)	149 (6.4, 8)	162 (7.8, 8)
0.4 ppm O_3	150 (5.6, 8)	184 (4.3, 8)	166 (7.5, 8)
0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	146 (9.2, 8)	151 (7.6, 8)	152 (5.5, 8)
0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6 mg/m^3 H_2SO_4	138 (9.0, 8)	186 (8.2, 8)	180 (10, 8)
Tidal Volume (ml)			
Clean Air	0.90 (0.10, 8)	0.79 (0.08, 8)	0.75 (0.06, 8)
0.2 ppm O_3	0.95 (0.07, 8)	0.89 (0.07, 8)	0.86 (0.07, 8)
0.4 ppm O_3	0.98 (0.05, 8)	0.72 (0.03, 8)	0.72 (0.03, 8)
0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	0.96 (0.04, 8)	0.95 (0.02, 8)	0.84 (0.04, 8)
0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6 mg/m^3 H_2SO_4	1.08 (0.06, 8)	0.72 (0.04, 8)	0.78 (0.05, 8)
$\dot{V}_E$ (ml/min)			
Clean Air	122 (9.6, 8)	125 (7.7, 8)	122 (8.8, 8)
0.2 ppm O3	139 (7.7, 8)	130 (8.7, 8)	137 (8.1, 8)
0.4 ppm O_3	146 (8.9, 8)	131 (5.8, 8)	123 (6.8, 8)
0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	137 (5.0, 8)	142 (5.8, 8)	128 (5.3, 8)
0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6 mg/m^3 H_2SO_4	146 (9.0, 8)	130 (5.0, 8)	137 (5.0, 8)
$\dot{V}_{O2}$ (ml/kg/min)			
Clean Air	30.6 (1.15, 8)	31.6 (1.10, 8)	28.7 (1.21, 8)
0.2 ppm O_3	29.9 (0.97, 8)	29.3 (0.84, 8)	30.1 (1.20, 8)
0.4 ppm O_3	29.4 (0.93, 8)	28.2 (0.87, 8)	27.7 (1.52, 8)
0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	28.0 (0.71, 8)	29.1 (0.46, 8)	29.4 (0.83, 8)
0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6mg/m^3 H_2SO_4	28.4 (0.81, 8)	26.8 (0.70, 8)	29.7 (0.75, 8)
$\dot{V}_E/\dot{V}_{O2}$ (L/mmol)			
Clean Air	0.337(0.018, 8)	0.337(0.024, 8)	0.362(0.026, 8)
0.2 ppm O_3	0.377(0.023, 8)	0.360(0.027, 8)	0.372(0.029, 8)
0.4 ppm O_3	0.404(0.017, 8)	0.379(0.018, 8)	0.364(0.020, 8)
0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	0.385(0.011, 8)	0.379(0.009, 8)	0.340(0.015, 8)
0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6 mg/m^3 H_2SO_4	0.438(0.033, 8)	0.411(0.024, 8)	0.388(0.018, 8)
T_R (°C)			
Clean Air	38.4 (0.09, 8)	37.9 (0.11, 8)	37.7 (0.14, 8)
0.2 ppm O3	38.0 (0.09, 8)	37.9 (0.16, 8)	37.9 (0.14, 8)
0.4 ppm O_3	38.4 (0.18, 8)	38.0 (0.20, 8)	37.5 (0.26, 8)
0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	38.4 (0.19, 8)	38.0 (0.20, 8)	38.1 (0.19, 8)
0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6 mg/m^3 H_2SO_4	38.5 (0.13, 8)	37.9 (0.13, 8)	37.7 (0.11, 8)

TABLE 5a. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Rate Responses in Experiment 1 ($p<0.05$). Overhead lines cluster groups not significantly different from each other. NS is not significant. "High" and "Low" refer to relative concentrations of pollutants (see table 4). SE is the standard error for Tukey pairwise multiple comparisons = square root of the mean square error term from the ANOVA.

Variable	Exposure Time	SE	Exposure Group Ranking				
Frequency							
	Hr 1 of Expos.	9.16			NS		
	Hr 4 of Expos.	8.17	O_3 Low	O_3 +Acids Low	Clean Air	O_3 High	O_3 +Acids High
	Hr 2 Post-expos.	8.85			NS		
Tidal Volume							
	Hr 1 of Expos.	0.065			NS		
	Hr 4 of Expos.	0.056	O_3 +Acids Low	O_3 Low	Clean Air	O_3 High	O_3 +Acids High
	Hr 2 Post-expos.	0.050			NS		
$\dot{V}_E$							
	Hr 1 of Expos.	8.21			NS		
	Hr 4 of Expos.	6.73			NS		
	Hr 2 Post-expos.	6.96			NS		
$\dot{V}_{O2}$							
	Hr 1 of Expos.	0.92			NS		
	Hr 4 of Expos.	0.82	Clean Air	O_3 Low	O_3 +Acids Low	O_3 High	O_3 +Acids High
	Hr 2 Post-expos.	1.14			NS		
$\dot{V}_E/\dot{V}_{O2}$							
	Hr 1 of Expos.	0.022	Clean Air	O_3 Low	O_3 +Acids Low	O_3 High	O_3 +Acids High
	Hr 4 of Expos.				NS		
	Hr 2 Post-expos.				NS		
T_R							
	Hr 1 of Expos.				NS		
	Hr 4 of Expos.				NS		
	Hr 2 Post-expos.				NS		

TABLE 5b. Significant differences ($p<0.05$) from multiple comparison tests among exposure groups in Experiment 1. Any of the exposure groups on the left is significantly different from any of the groups on the right of the vs. symbol.

Variable	Exposure Time	Significant Group Differences				
Frequency	Hr 4 of Expos.	[O_3 Low, O_3 +Acids Low]	vs.	[O_3 High, O_3 +Acids High]		
Tidal Volume	Hr 4 of Expos.	[O_3 Low, O_3 +Acids Low]	vs.	[O_3 High, O_3 +Acids High]		
$\dot{V}_{O2}$	Hr 4 of Expos.	Clean Air	vs.	[O_3 +Acids Low, O_3 High, O_3 +Acids High]		
		[O_3 Low, O_3 +Acids Low, O_3 High]		vs.	O_3 +Acids High	
$\dot{V}_E/\dot{V}_{O2}$	Hr 1 of Expos.	Clean Air	vs.	[O_3 High, O_3 +Acids High]		

significant differences. It is possible to obtain data in which a group with a mean intermediate between two other groups is not significantly different from either extreme group, yet the extreme groups are significantly different from each other. Overhead lines clustering groups with no significant differences will overlap under these circumstances. This does not mean that there are no differences between the groups. . It means that due to the variability of the data the intermediate group cannot be uniquely clustered with either extreme group. That the extreme groups are significantly different is expressed by the fact that they are not connected by a single overhead line. Ozone at 0.4 ppm with and without acids present induced a trend toward rapid shallow breathing that was evident by the fourth hour of exposure, while O_3 at 0.2 ppm combined with acid induced a trend toward slow and deep breathing. None of the frequency changes were large enough to be significantly different from clean air control (Table 5 a,b), however the opposing directions of the breathing pattern shift significantly distinguished O_3 at higher concentration from O_3 at lower concentrations. Tidal volume changes showed a similar pattern, however the group exposed to the combination of 0.2 ppm O_3 with lower concentrations of acids had tidal volume significantly greater than the clean air control group. During the second hour post-exposure, breathing patterns showed recovery from the irritant effects. Differences in frequency and tidal volumes remained in the direction of the earlier irritant effects, but no patterns were statistically significantly different from one another. Minute ventilation (V_E) was not significantly affected at any time during the exposures, but oxygen consumption (VO_2) during the fourth hour of exposure was significantly depressed in O_3 at 0.4 ppm and in both combination exposures (Tables 4 and 5 a,b). Presence of the acids at high concentration significantly reduced VO_2 compared to O_3 alone. Ventilation equivalent for oxygen consumption (V_E/VO_2) was significantly elevated during the first hour of exposure for the higher concentration exposures to O_3 alone

and O_3 combined with acids. Body temperature was not significantly affected by the exposures.

Exercise exposure to these combinations of pollutants produced greater breathing pattern and metabolic rate effects and these persisted after the exposure. It was not possible to measure these variables from the rats while they were running, so the comparison between experiments 1 (rest) and 2 (exercise) is restricted to the time between 1 and 2 hours after exposure (hour 2 post-exposure). While exposures at rest (Experiment 1) did not induce effects persisting into the second post-exposure hour, exercise exposure in Experiment 2 induced changes that did not show such rapid recovery (Tables 6 and 7). Exposure to 0.4 ppm O_3 induced rapid-shallow breathing that continued into the second hour after exposure, and all exposure groups had significantly depressed oxygen consumption and body temperature. Although differences in V_E were not significant, the trends were in the same direction as VO_2, and the ratio of these variables, ventilation equivalent for O_2, was not significantly different among the groups.

Table 8 shows the effects of HMSA exposure on pulmonary function of rats exposed at rest (Experiment 3). There were no significant changes in breathing pattern or V_E, and the only significant effects observed were a small decline in VO_2 during the fourth hour of exposure that persisted 2 hours post exposure and an increase in ventilation equivalent for O_2 during the fourth hour of exposure. Tables 9 and 10 show the results of HMSA and O_3 exposure alone and in combination (Experiment 4). O_3 alone and in combination with HMSA at the higher concentrations induced rapid-shallow breathing by the fourth hour of exposure that persisted through 2 hours post-exposure. HMSA alone at 0.5 mg/m^3 did not induce changes in breathing pattern as was also observed in Experiment 3 (Table 8). VO_2 was significantly depressed in all exposure groups during hour 1 and in the high concentration HMSA and O_3 + HMSA groups during hour 4 of exposure. Observation of significant differences in VO_2 between groups during the first hour

TABLE 6. Breathing Pattern, Ventilatory, and Metabolic Rates of Rats Exposed During Exercise to Ozone and Acids (Experiment 2). Data are Mean (Standard Error, n).

	Hour 2 Post-exposure
Breath Frequency (min-1)	
Clean Air	130 (3.1, 8)
0.2 ppm O_3	128 (6.2, 8)
0.4 ppm O_3	150 (8.8, 8)
0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	121 (5.0, 8)
0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	123 (8.0, 8)
Tidal Volume (ml)	
Clean Air	0.86 (0.04, 8)
0.2 ppm O_3	0.79 (0.04, 8)
0.4 ppm O_3	0.63 (0.04, 8)
0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	0.84 (0.02, 8)
0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	0.89 (0.06, 8)
$\dot{V}_E$ (ml/min)	
Clean Air	111 (4.6, 8)
0.2 ppm O3	100 (7.2, 8)
0.4 ppm O_3	89 (8.4, 8)
0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	101 (6.1, 8)
0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	107 (5.2, 8)
$\dot{V}_{O2}$ (ml/kg/min)	
Clean Air	30.6 (1.07, 8)
0.2 ppm O_3	25.8 (0.78, 8)
0.4 ppm O_3	24.3 (1.10, 8)
0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	23.6 (1.73, 8)
0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	24.7 (0.51, 8)
$\dot{V}_E/\dot{V}_{O2}$ (L/mmol)	
Clean Air	0.344 (0.018, 8)
0.2 ppm O_3	0.337 (0.015, 8)
0.4 ppm O_3	0.328 (0.021, 8)
0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	0.372 (0.017, 8)
0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	0.378 (0.021, 8)
T_R (°C)	
Clean Air	38.2 (0.12, 8)
0.2 ppm O_3	37.4 (0.40, 8)
0.4 ppm O_3	37.3 (0.22, 8)
0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	37.4 (0.17, 7)
0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	37.1 (0.32, 8)

TABLE 7a. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Rate Responses in Experiment 2 ($p<0.05$). Overhead lines cluster groups not significantly different from each other. NS is not significant. "High" and "Low" refer to relative concentrations of pollutants (see table 6). Se is the standard error for Tukey pairwise multiple comparisons = square root of themean square error term for the ANOVA.

Variable	SE	Hour 2 Post-exposure				
Frequency	6.57	O_3 +Acids Low	O_3 +Acids High	O_3 Low	Clean Air	O_3 High
Tidal Volume	0.040	O_3 +Acids High	Clean Air	O_3 +Acids Low	O_3 Low	O_3 High
$\dot{V}_E$	5.93			NS		
$\dot{V}_{O2}$	0.715	Clean Air	O_3 Low	O_3 High	O_3 +Acids High	O_3 +Acids Low
$\dot{V}_E/\dot{V}_{O2}$	0.018			NS		
T_R	0.195	Clean Air	O_3 +Acids Low	O_3 Low	O_3 High	O_3 +Acids High

TABLE 7b. Significant differences ($p<0.05$) from multiple comparison tests among exposure groups in Experiment 2. Any of the exposure groups on the left is significantly different from any of the groups on the right of the vs. symbol.

Variable	Hour 2 Post-exposure					
Frequency	Clean Air	O_3 Low	O_3 +Acids Low	O_3 +Acids High	vs.	O_3 High
Tidal Volume	Clean Air	O_3 Low	O_3 +Acids Low	O_3 +Acids High	vs.	O_3 High
$\dot{V}_{O2}$	Clean Air	vs.	O_3 Low	O_3 High	O_3 +Acids High	O_3 +Acids Low
	O_3 Low	vs.	O_3 +Acids Low			
T_R	Clean Air	vs.	O_3 +Acids Low	O_3 Low	O_3 High	O_3 +Acids High

TABLE 8. Breathing Pattern, Ventilatory, and Metabolic Rates of Rats Exposed at Rest to Hydroxymethanesulfonic Acid (Experiment 3). Data are Mean (Standard Error, n). NS is not significantly different from clean air control at p=0.05 level.

	Hour 1 of Exposure	Hour 4 of Exposure	Hour 2 of Post-exposure
Breath Frequency (min-1)			
Clean Air	145 (6.2,8)	150 (7.0,8)	160 (8.6,8)
0.56 mg/m^3 HMSA	153 (5.6, 8)	162 (8.5,8)	168 (8.1,8)
	NS	NS	NS
Tidal Volume (ml)			
Clean Air	1.00 (0.04,8)	0.96 (0.04,8)	0.84 (0.06,8)
0.56 mg/m^3 HMSA	0.93 (0.05,8)	0.86 (0.03,8)	0.80 (0.03,8)
	NS	NS	NS
$\dot{V}_E$ (ml/min)			
Clean Air	143 (4.9,8)	142 (6.1,8)	134 (11.7,8)
0.56 mg/m^3 HMSA	141 (7.2,8)	139 (7.3,8)	133 (6.6,8)
	NS	NS	NS
$\dot{V}_{O2}$ (ml/kg/min)			
Clean Air	33.0 (0.93,8)	33.4 (0.83,8)	32.2 (1.07,8)
0.56 mg/m^3 HMSA	30.5 (1.29,8)	29.7 (1.36,8)	27.8 (1.36,8)
	NS	t=2.3, p<0.04	t=2.5, p<0.03
$\dot{V}_E/\dot{V}_{O2}$ (L/mmol)			
Clean Air	0.352 (0.012,8)	0.344 (0.007,8)	0.334 (0.225,8)
0.56 mg/m^3 HMSA	0.371 (0.005,8)	0.376 (0.071,8)	0.389 (0.021,8)
	NS	t=2.5, p<0.03	NS
T_R (°C)			
Clean Air	38.1 (0.10,8)	37.9 (0.15,8)	37.8 (0.13,8)
0.56 mg/m^3 HMSA	38.1 (0.25,8)	37.9 (0.45,8)	37.9 (0.38,8)
	NS	NS	NS

TABLE 9. Breathing Pattern, Ventilatory, and Metabolic Rates of Rats Exposed at Rest to Ozone and Hydroxymethanesulfonic Acid (Experiment 4). Data are Mean (Standard Error, n).

	Hour 1 of Exposure	Hour 4 of Exposure	Hour 2 Post-exposure
Breath Frequency (min-1)			
Clean Air	144 (6.7,8)	146 (4.9,8)	151 (6.8,8)
0.4 ppm O_3	151 (6.3,8)	190 (8.5,8)	187 (9.3,8)
0.47 mg/m^3 HMSA	138 (5.8,8)	154 (6.6,8)	162 (8.9,8)
0.2 ppm O_3+0.23 mg/m^3 HMSA	143 (5.1,8)	146 (6.9 8)	159 (6.1,8)
0.4 ppm O_3+0.38 mg/m^3 HMSA	150 (4.3,8)	202 (6.1,8)	188 (6.7,8)
Tidal Volume (ml)			
Clean Air	0.93 (0.05,8)	0.89 (0.06,8)	0.86 (0.05,8)
0.4 ppm O_3	0.96 (0.02,8)	0.68 (0.05,8)	0.68 (0.03,8)
0.47 mg/m^3 HMSA	1.02 (0.05,8)	0.94 (0.06,8)	0.85 (0.05,8)
0.2 ppm O_3+0.23 mg/m^3 HMSA	0.96 (0.05,8)	1.05 (0.08,8)	0.93 (0.10,7)
0.4 ppm O_3+0.38 mg/m^3 HMSA	0.90 (0.06,8)	0.64 (0.03,8)	0.69 (0.04,8)
$\dot{V}_E$ (ml/min)			
Clean Air	132 (5.7,8)	128 (6.8,8)	129 (7.5,8)
0.4 ppm O_3	144 (6.4,8)	126 (6.3,8)	125 (5.3,8)
0.47 mg/m^3 HMSA	140 (7.5,8)	143 (6.8,8)	136 (6.1,8)
0.2 ppm O_3+0.23 mg/m^3 HMSA	136 (7.5,8)	152 (11.6 8)	145 (15.5,7)
0.4 ppm O_3+0.38 mg/m^3 HMSA	135 (6.9,8)	128 (3.5,8)	128 (5.5,8)
$\dot{V}_{O2}$ (ml/kg/min)			
Clean Air	33.2 (1.36,8)	33.6 (1.27,8)	33.6 (0.72,8)
0.4 ppm O_3	29.9 (0.95,8)	30.8 (1.15,8)	30.4 (0.81,8)
0.47 mg/m^3 HMSA	28.4 (0.81,8)	28.8 (0.90,8)	29.9 (1.42,8)
0.2 ppm O_3+0.23 mg/m^3 HMSA	27.9 (1.52,8)	30.8 (1.09,8)	30.5 (0.74,8)
0.4 ppm O_3+0.38 mg/m^3 HMSA	29.4 (0.81,8)	28.1 (0.53,8)	30.8 (0.60,8)
$\dot{V}_E/\dot{V}_{O2}$ (L/mmol)			
Clean Air	0.346 (0.013,8)	0.337 (0.022,8)	0.344 (0.027,8)
0.4 ppm O_3	0.400 (0.014,8)	0.343 (0.015,8)	0.343 (0.013,8)
0.47 mg/m^3 HMSA	0.385 (0.026,8)	0.387 (0.021,8)	0.357 (0.021,8)
0.2 ppm O_3+0.23 mg/m^3 HMSA	0.389 (0.027,8)	0.393 (0.031,8)	0.323 (0.057,8)
0.4 ppm O_3+0.38 mg/m^3 HMSA	0.361 (0.020,8)	0.360 (0.013,8)	0.325 (0.016,8)
T_R (°C)			
Clean Air	38.4 (0.06,8)	38.0 (0.11,8)	38.0 (0.17,8)
0.4 ppm O_3	38.4 (0.08,8)	37.9 (0.15,8)	37.9 (0.06,8)
0.47 mg/m^3 HMSA	38.3 (0.26,8)	38.1 (0.21,8)	38.0 (0.17,8)
0.2 ppm O_3+0.23 mg/m^3 HMSA	38.2 (0.17,8)	38.0 (0.13,8)	38.0 (0.18,8)
0.4 ppm O_3+0.38 mg/m^3 HMSA	38.5 (0.09,8)	37.4 (0.11,8)	37.6 (0.14,8)

TABLE 10a. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Rate Responses in Experiment 4 ($p<0.05$). Overhead lines cluster groups not significantly different from each other. NS is not significant. "High" and "Low" refer to relative concentrations of pollutants (see table 9). SE is the standard error for Tukey pairwise multiple comprisons = square root of the mean square error term from the ANOVA.

Variable	Exposure Time	SE	Exposure Group Ranking				
Frequency							
	Hr 1 of Expos.	5.27			NS		
	Hr 4 of Expos.	6.70	Clean Air	O_3 +HMSA Low	HMSA High	O_3 High	O_3 +HMSA High
	Hr 2 Post-expos.	7.67	Clean Air	O_3 +HMSA Low	HMSA High	O_3 High	O_3 +HMSA High
Tidal Volume							
	Hr 1 of Expos.	0.048			NS		
	Hr 4 of Expos.	0.056	O_3 +HMSA Low	HMSA High	Clean Air	O_3 High	O_3 +HMSA High
	Hr 2 Post-expos.	0.054	O_3 +HMSA Low	Clean Air	HMSA High	O_3 +HMSA High	O_3 High
$\dot{V}_E$							
	Hr 1 of Expos.	6.83			NS		
	Hr 4 of Expos.	7.47			NS		
	Hr 2 Post-expos.	8.27			NS		
$\dot{V}_{O2}$							
	Hr 1 of Expos.	1.13	Clean Air	O_3 High	O_3 +HMSA High	HMSA High	O_3 +HMSA Low
	Hr 4 of Expos.	1.02	Clean Air	O_3 High	O_3 +HMSA Low	HMSA High	O_3 +HMSA High
	Hr 2 Post-expos.	0.91			NS		
$\dot{V}_E/\dot{V}_{O2}$							
	Hr 1 of Expos.	0.021			NS		
	Hr 4 of Expos.	0.021			NS		
	Hr 2 Post-expos.	0.031			NS		
T_R							
	Hr 1 of Expos.	0.15			NS		
	Hr 4 of Expos.	0.15	HMSA High	Clean Air	O_3 +HMSA Low	O_3 High	O_3 +HMSA High
	Hr 2 Post-expos.	0.15			NS		

TABLE 10b. Significant differences ($p<0.05$) from multiple comparison tests among exposure groups in Experiment 4. Any of the exposure groups on the left is significantly different from any of the groups on the right of the vs. symbol.

Variable	Exposure Time	Significant Group Differences					
Frequency							
	Hr 4 of Expos.	Clean Air	O_3 +HMSA Low	HMSA High	vs.	O_3 High	O_3 +HMSA High
	Hr 2 Post-expos.	Clean Air	O_3 +HMSA Low	HMSA High	vs.	O_3 High	O_3 +HMSA High
Tidal Volume							
	Hr 4 of Expos.	Clean Air	O_3 +HMSA Low	HMSA High	vs.	O_3 High	O_3 +HMSA High
	Hr 2 Post-expos.	Clean Air	O_3 +HMSA Low	HMSA High	vs.	O_3 High	O_3 +HMSA High
$\dot{V}_{O2}$							
	Hr 1 of Expos.	Clean Air	vs.	O_3 High	O_3 +HMSA High	HMSA High	O_3 +HMSA Low
	Hr 4 of Expos.	Clean Air	vs.	HMSA High	O_3 +HMSA High		
T_R							
	Hr 4 of Expos.	Clean Air	O_3 +HMSA Low	HMSA High	O_3 High	vs.	O_3 +HMSA High

of exposure is unusual in our experience with O_3 exposures and inspection of the raw data revealed a single rat in the clean air group with an unusually high metabolic rate (41.1 $ml \cdot kg^{-1} \cdot min^{-1}$) during the first hour of exposure and mean 38.2 ml $kg^{-1} \cdot min^{-1}$ over the entire hour 1, hour 4, and 2 h post-exposure period). Dropping this outlier animal from the entire analysis resulted in no significant difference in VO_2 among groups during hour 1 and did not affect the pattern of significant differences among groups at other times. The only other significant change was a depression in body temperature observed in exposure to the mixture O_3 + HMSA at higher concentration during the fourth hour of exposure.

Exercise exposure to O_3 and O_3 + HMSA induced significant shifts to high frequency breathing observed during the second hour after exposure to the higher concentrations (Table 11 and 12). VO_2 also showed significant changes, although curiously, it was the lower concentration of combined O_3 + HMSA group that was significantly lower than the control clean air group. Because measurements of breathing pattern and metabolic rate responses from exercising exposures occurred 1-2 hours post-exposure, they combine the effects of exposure and recovery processes and may be the result of more complex relationships between exposure groups.

C. Histopathology

Tissue injury to the respiratory system from the exposures was evaluated in the lung parenchyma and in the respiratory epithelium of the nose. Tables 13 and 14 show quantitative measurements of injury from exposures at rest and during exercise to O_3 alone and combined with HNO_3 + H_2SO_4 (Experiments 1 and 2). Resting exposure to these compounds did not significantly affect the R-1 nasal epithelium. Exposure to 0.4 ppm O_3 alone and in combination with acids had a small effect on lung parenchyma. The elevations of the indices for Type 1 injury were not statistically significant, and although the Type 2 lesion index was also not significant in ANOVA,

TABLE 11. Breathing Pattern, Ventilatory, and Metabolic Rates of Rats Exposed during Exercise to Ozone and Hydroxymethanesulfonic Acid (Experiment 5). Data are Mean (Standard Error, n).

Variable Exposure Group	Hour 2 Post-exposure
Breath Frequency (min-1)	
Clean Air	123 (4.1, 8)
0.2 ppm O_3	126 (10.5, 8)
0.4 ppm O_3	161 (6.7, 8)
0.2 ppm O_3+0.19 mg/m^3 HMSA	128 (6.5, 8)
0.4 ppm O_3+0.46 mg/m^3 HMSA	155 (6.4, 8)
Tidal Volume (ml)	
Clean Air	0.86 (0.05, 8)
0.2 ppm O_3	0.78 (0.08, 8)
0.4 ppm O_3	0.73 (0.10, 8)
0.2 ppm O_3+0.19 mg/m^3 HMSA	0.80 (0.06, 8)
0.4 ppm O_3+0.46 mg/m^3 HMSA	0.63 (0.06, 8)
$\dot{V}_E$ (ml/min)	
Clean Air	105 (5.2, 8)
0.2 ppm O_3	93 (4.3, 8)
0.4 ppm O_3	117 (4.5, 8)
0.2 ppm O_3+0.19 mg/m^3 HMSA	101 (7.1, 8)
0.4 ppm O_3+0.46 mg/m^3 HMSA	98 (10.9, 8)
$\dot{V}_{O2}$ (ml/kg/min)	
Clean Air	26.0 (1.03, 8)
0.2 ppm O_3	23.3 (1.28, 8)
0.4 ppm O_3	26.8 (0.80, 8)
0.2 ppm O_3+0.19 mg/m^3 HMSA	22.5 (0.93, 8)
0.4 ppm O_3+0.46 mg/m^3 HMSA	25.6 (1.27, 8)
$\dot{V}_E/\dot{V}_{O2}$ (L/mmol)	
Clean Air	0.379 (0.019, 8)
0.2 ppm O_3	0.344 (0.013, 8)
0.4 ppm O_3	0.390 (0.012, 8)
0.2 ppm O_3+0.19 mg/m^3 HMSA	0.383 (0.019, 8)
0.4 ppm O_3+0.46 mg/m^3 HMSA	0.334 (0.023, 8)
T_R (°C)	
Clean Air	37.3 (0.16, 8)
0.2 ppm O_3	37.2 (0.11, 8)
0.4 ppm O_3	37.1 (0.14, 8)
0.2 ppm O_3+0.19 mg/m^3 HMSA	37.5 (0.14, 8)
0.4 ppm O_3+0.46 mg/m^3 HMSA	37.0 (0.14, 8)

TABLE 12a. ANOVA and Tukey Multiple Comparison Significance Tests for Breathing Pattern, Ventilatory, and Metabolic Rate Responses in Experiment 5 ($p<0.05$). Overhead lines cluster groups not significantly different from each other. NS is not significant. "High" and "Low" refer to relative concentrations of pollutants (see table 11). SE is the standard error for Tukey multiple comparisons = square root of the mean square error term from the ANOVA.

Variable	SE	Exposure Group Ranking				
Frequency	7.16	Clean Air	O_3 Low	O_3 +HMSA Low	O_3 +HMSA High	O_3 High
Tidal Volume	0.056			NS		
$\dot{V}_E$	6.85			NS		
$\dot{V}_{O2}$	1.08	O_3 High	Clean Air	O_3 +HMSA High	O_3 Low	O_3 +HMSA Low
$\dot{V}_E/\dot{V}_{O2}$	0.017			NS		
T_R	0.141			NS		

TABLE 12b. Significant differences ($p<0.05$) from multiple comparison tests among exposure groups in Experiment 5. Any of the exposure groups on the left is significantly different from any of the groups on the right of the vs. symbol.

Variable	Hour 2 Post-exposure					
Frequency	Clean Air	O_3 Low	O_3 +HMSA Low	vs.	O_3 +HMSA High	O_3 High
$\dot{V}_{O2}$	Clean Air	O_3 High	vs.	O_3 +HMSA Low		
	O_3 High	vs.	O_3 Low			

TABLE 13. Nasal Epithelial (R-1 region) Injury and Lung Parenchymal Lesion Injury Resulting from Exposure of Rats to O_3 and O_3 + HNO_3 + H_2SO_4. Data are Mean (standard error, n).

Experiment Exposure Group	R-1 Nasal Epithelium (% labeled cells)	Lung Parenchymal Lesions Type 1 (Area %)	Type 2 (Area %)
1. Rest			
A. Clean Air	1.14 (0.43,7)	1.95 (0.12,9)	0.0
B. 0.2 ppm O_3	1.10 (0.15,6)	2.33 (0.34,8)	0.0
C. 0.4 ppm O_3	1.07 (0.18,6)	2.74 (0.46,9)	0.11 (0.06,9)
D. 0.2 ppm O_3+0.6 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	1.37 (0.31,6)	2.24 (0.28,8)	0.0
E. 0.4 ppm O_3+0.7 mg/m^3 HNO_3+0.6 mg/m^3 H_2SO_4	1.57 (0.38,7)	2.46 (0.24,9)	0.31 (0.17,9)
2. Exercise			
A. Clean Air	0.83 (0.16,8)	2.20 (0.24,9)	0.0
B. 0.2 ppm O_3	2.03 (0.43,7)	2.70 (0.29,9)	0.35 (0.14,9)
C. 0.4 ppm O_3	2.37 (0.49,7)	5.67 (0.47,9)	5.57 (0.95,9)
D. 0.2 ppm O_3+0.5 mg/m^3 HNO_3+0.25 mg/m^3 H_2SO_4	0.66 (0.16,7)	2.61 (0.29,10)	0.26(0.11,10)
E. 0.4 ppm O_3+0.9 mg/m^3 HNO_3+0.5 mg/m^3 H_2SO_4	1.31 (0.19,7)	4.08 (0.54,9)	3.22 (0.68,9)

TABLE 14a. ANOVA and Tukey Multiple Comparison Significance Tests for Nasal Epithelial and Lung Parenchymal Injury Resulting from Exposure of Rats to O_3 and $O_3 + HNO_3 + H_2SO_4$ at Rest and Exercise. Overhead lines cluster groups not significantly different from each other. NS is not significantly different from control. "High" and "Low" refer to relative concentrations of pollutants (see table 13). SE is the standard error for Tukey pairwise multiple comparisons = square root of the mean square error term from the ANOVA.

Experiment	SE	Rank Order of Group Means				
1. Rest						
Nasal Epithelium (R-1), n=6,6.	0.345			NS		
Lung Parenchymal Lesion						
Type 1, n=8,8.	0.326			NS		
Type 2, n=8,8.	0.091			NS		
2. Exercise						
Nasal Epithelium (R-1), n=8,8.	0.299	O_3 +Acids Low	Clean Air	O_3 +Acids High	O_3 Low	O_3 High
Lung Parenchymal Lesion						
Type 1, n=9,9.	0.385	Clean Air	O_3 +Acids Low	O_3 Low	O_3 +Acids High	O_3 High
Type 2, n=9,9.	0.522	Clean Air	O_3 +Acids Low	O_3 Low	O_3 +Acids High	O_3 High

TABLE 14b. Significant differences ($p<0.05$) from multiple comparison tests among exposure groups for nasal epithelial and lung parenchymal injury in exposures of rats to O_3 and $O_3 + HNO_3 + H_2SO_4$ at rest and exercise (see Tables 13 and 14a). Any of the exposure groups on the left is significantly different from any of the groups on the right of the vs. symbol.

Experiment	Exposure Groups						
2. Exercise							
Nasal Epithelium (R-1)	Clean Air	vs.	[O_3 Low	O_3 High]			
Lung Parenchymal Lesion							
Type 1	[Clean Air	O_3 +Acids Low	O_3 Low]	vs.	[O_3 +Acids High	O_3 High]	
	O_3 +Acids High	vs.	O_3 High				
Type 2	[Clean Air	O_3 +Acids Low	O_3 Low]	vs.	[O_3 +Acids High	O_3 High]	
	O_3 +Acids High	vs.	O_3 High				

Type 2 lesions are not observed at all in the absence of oxidant exposure, and qualitative presence of Type 2 lesion is indicative of an oxidant effect in the 0.4 ppm O_3 exposures. Exercise exposure induced significant injury to the R-1 nasal epithelium in 0.2 and 0.4 ppm O_3 exposures but there was no significant effect when acids were present with O_3. Significant injury to the lung parenchyma occurred when O_3 was present at the higher concentrations, and 0.4 ppm O_3 alone produced a greater parenchymal injury effect than 0.4 ppm O_3 combined with acids.

Tables 15 and 16 show tissue injury resulting from exposures to O_3 and HMSA alone and in combination at rest and during exercise (Experiments 3, 4, and 5). HMSA exposure at rest in Experiment 3 had no significant effect on nasal epithelium or on the lung. In Experiment 4 resting exposures there were no significant effects of any of the exposures on the nasal epithelium, but 0.4 ppm O_3 exposure alone and in combination with acids induced significant Type 2 lung tissue injury. In exercising exposures (Experiment 5), O_3 exposures resulted in increases in the nasal epithelium index of tissue injury, but the increases were not significantly different from clean air control. However, 0.5 mg/m^3 HMSA combined with 0.4 ppm O_3 induced a highly significant increase in cell turnover that was indicative of nasal epithelial tissue injury. Exposures to O_3 at both concentrations and in combination with acids induced Type 2 parenchymal lesions. Lesion areas were significantly elevated for the exposures to the higher concentrations, and there was no apparent modification of the effect of O_3 by the presence of HMSA.

D. Chemical Characterization of Pulmonary Surfactant

1. Exposure to O_3 or to an O_3 and Acid Combination.

a. Fourier Transform Infrared FTIR Spectroscopy. Appendix I contains the FTIR spectra in the 2000 - 1300 cm^{-1} range for the PS from rats exposed to clean air (controls), 0.40 ppm O_3 or to 0.41 ppm O_3 combined with 0.67 mg/m^3 HNO_3 and 0.61 mg/m^3 H_2SO_4 (Experiment 1). This region of the infrared has proven to be the most

TABLE 15. Nasal Epithelial (R-1 region) Injury and Lung Parenchymal Lesion Injury Resulting from Exposure of Rats to Hydroxymethanesulfonic Acid Alone and in Combination with O_3. Data are Mean (standard error,n).

Experiment Exposure Group	R-1 Nasal Epithelium (% labeled cells)	Lung Parenchymal Lesions Type 1 (Area %)	Type 2 (Area %)
3. Rest			
A. Clean Air	0.48 (0.10,5)	1.92 (0.16,8)	0.0
B. 0.56 mg/m^3 HMSA	0.62 (0.09,5)	1.98 (0.13,9)	0.0
4. Rest			
A. Clean Air	1.34 (0.32,9)	1.67 (0.18,10)	0.0
B. 0.4 ppm O_3	(no data)	2.03 (0.15,9)	0.40(0.12,9)
C. 0.47 mg/m^3 HMSA	1.00 (0.23,9)	1.46 (0.12,9)	0.0
D. 0.2 ppm O_3+0.23 mg/m^3 HMSA	1.06 (0.17,10)	1.62 (0.20,10)	0.0
E. 0.4 ppm O_3+0.38 mg/m^3 HMSA	1.10 (0.21,10)	1.92 (0.25,10)	0.54(0.18,10)
5. Exercise			
A. Clean Air	0.69+0.10,7	1.79 +0.22,9)	0.0
B. 0.2 ppm O_3	0.91+0.18,7	2.20 +0.16,9)	0.66(0.19,9)
C. 0.4 ppm O_3	0.94+0.50,7	3.27 +0.42,10	3.07(0.73,10)
D. 0.2 ppm O_3+0.19 mg/m^3 HMSA	0.93+0.19,7	2.04 +0.18,9	0.26 +0.09,9
E. 0.4 ppm O_3+0.46 mg/m^3 HMSA	3.12+0.24,6	3.29 +0.47,8)	3.58 +0.85,8

TABLE 16a. ANOVA and Tukey Multiple Comparison Significance Tests for Nasal Epithelial and Lung Parenchymal Injury Resulting from Exposure of Rats to O_3 and O_3 + HMSA at Rest and Exercise. Overhead lines cluster groups not significantly different from each other. NS is not significantly different from control. "High" and "Low" refer to relative concentrations of pollutants (see table 15). SE is the standard error for Tukey pairwise multiple comparisons = square root of the mean square error term from the ANOVA.

Experiment	SE	Rank Order of Group Means				
4. Rest						
Nasal Epithelium (R-1), n=9,9,	0.240			NS		
Lung Parenchymal Lesion						
Type 1, n=9,9.	0.196			NS		
Type 2, n=9,9.	0.102	Clean Air	HMSA High	O_3 +HMSA Low	O_3 High	O_3 +HMSA High
5. Exercise						
Nasal Epithelium (R-1), n=7,7.	0.178	Clean Air	O_3 Low	O_3 +HMSA Low	O_3 High	O_3 +HMSA High
Lung Parenchymal Lesion						
Type 1, n=9,9.	0.316	Clean Air	O_3 +HMSA Low	O_3 Low	O_3 High	O_3 +HMSA High
Type 2, n=9,9.	0.504	Clean Air	O_3 +HMSA Low	O_3 Low	O_3 High	O_3 +HMSA High

TABLE 16b. Significant differences ($p<0.05$) from multiple comparison tests among exposure groups for nasal epithelial and lung parenchymal injury in exposures of rats to O_3 and O_3 + HMSA at rest and exercise (see Tables 15 and 16a). Any of the exposure groups on the left is significantly different from any of the groups on the right of the vs. symbol.

Experiment	Exposure Groups					
4. Rest						
Lung Parenchymal Lesion						
Type 2	Clean Air	HMSA High	O_3 +HMSA Low	vs.	O_3 High	O_3 +HMSA High
5. Exercise						
Nasal Epithelium (R-1)	Clean Air	O_3 Low	O_3 +HMSA Low	O_3 High	vs.	O_3 +HMSA High
Lung Parenchymal Lesion						
Type 1	Clean Air	O_3 +HMSA Low	O_3 Low	vs.	O_3 High	O_3 +HMSA High
Type 2	Clean Air	O_3 +HMSA Low	O_3 Low	vs.	O_3 High	O_3 +HMSA Low

informative and hence is used throughout these studies. Replicate samples for each exposure represented the pooled PS from six rats. Overall, the spectra from the O_3 exposed rats are not different from those of the clean air controls. However, as seen in Figures 1a,b and 2a,b, in the rats exposed to the O_3-acid combination, there is a strong absorption band around 1706 cm-1 which is not seen in the clean air controls.

b. Ultraviolet (UV) Spectroscopy. Appendix II contains the UV spectra of the PS from rats exposed to clean air (controls), O_3 or to the O_3-acid combination. (The spectra near the baseline showing no peaks are the methanol (MeOH) solvent blanks recorded before each sample spectrum). Figures 3 and 4 give the UV data in a form more suitable for comparison. The spectra for the two groups #1 and #2 for each exposure condition have been averaged to give one representative spectrum for each exposure. In Figure 3, the average clean air control spectrum is overlaid with the average spectrum from the ozone exposed rats. Figure 4 compares the average clean air control spectrum to the averaged spectra of PS from rats exposed to the O_3-acid combination. (The averaged spectra have been multiplied by a constant factor to adjust the peak at 205 nm to approximately the same value to simplify the comparison). A comparison of these averaged spectra shows no obvious differences in the UV spectra. Furthermore, the spectra are virtually identical to that of an authentic (analytical reagent) sample of dipalmitoyl phosphatidylcholine, the major component of PS (Sanders, 1982).

c. Fatty Acid Composition. Table 17 shows the composition of the major fatty acids in the PS from the clean air controls as well as from the exposed rats, expressed relative to the major fatty acid component, palmitate. It is seen that all of the minor fatty acids are lower in the exposed animals than in the clean air controls, with stearic acid showing the least reduction and linoleic the greatest. The statistical analysis given in more

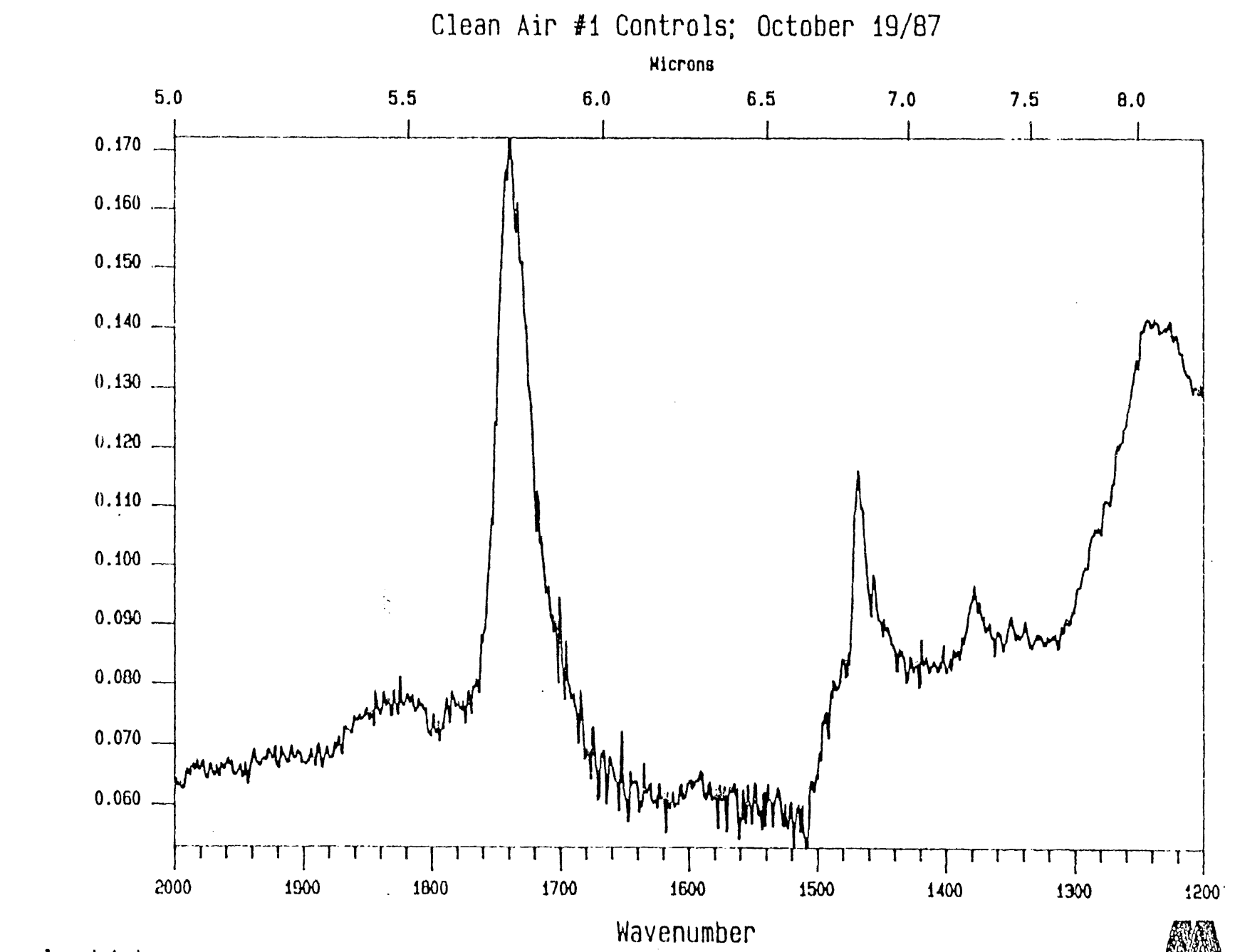


FIGURE 1a. FTIR SPECTRUM OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS, GROUP #1

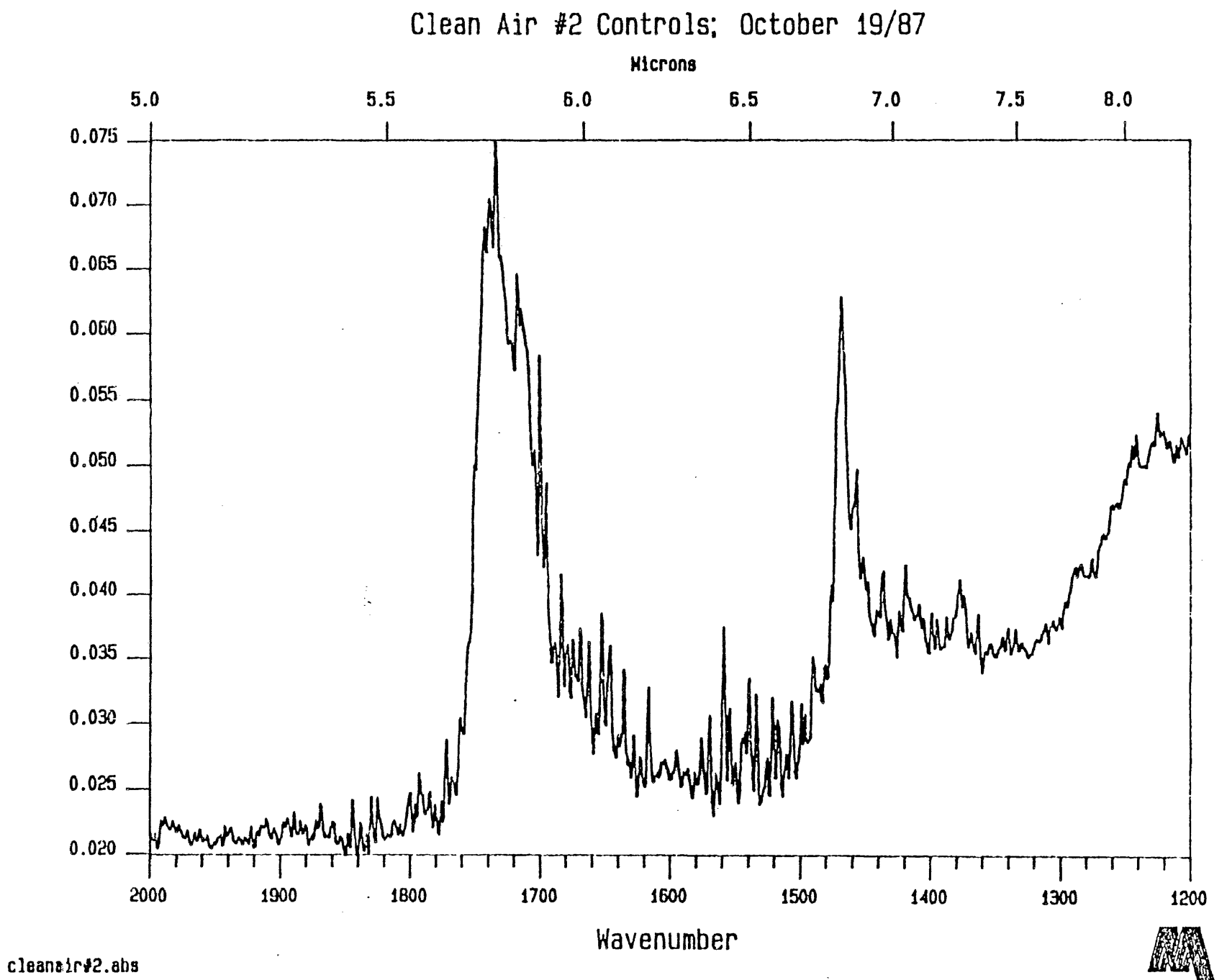


FIGURE 1b. FTIR SPECTRUM OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS, GROUP #2

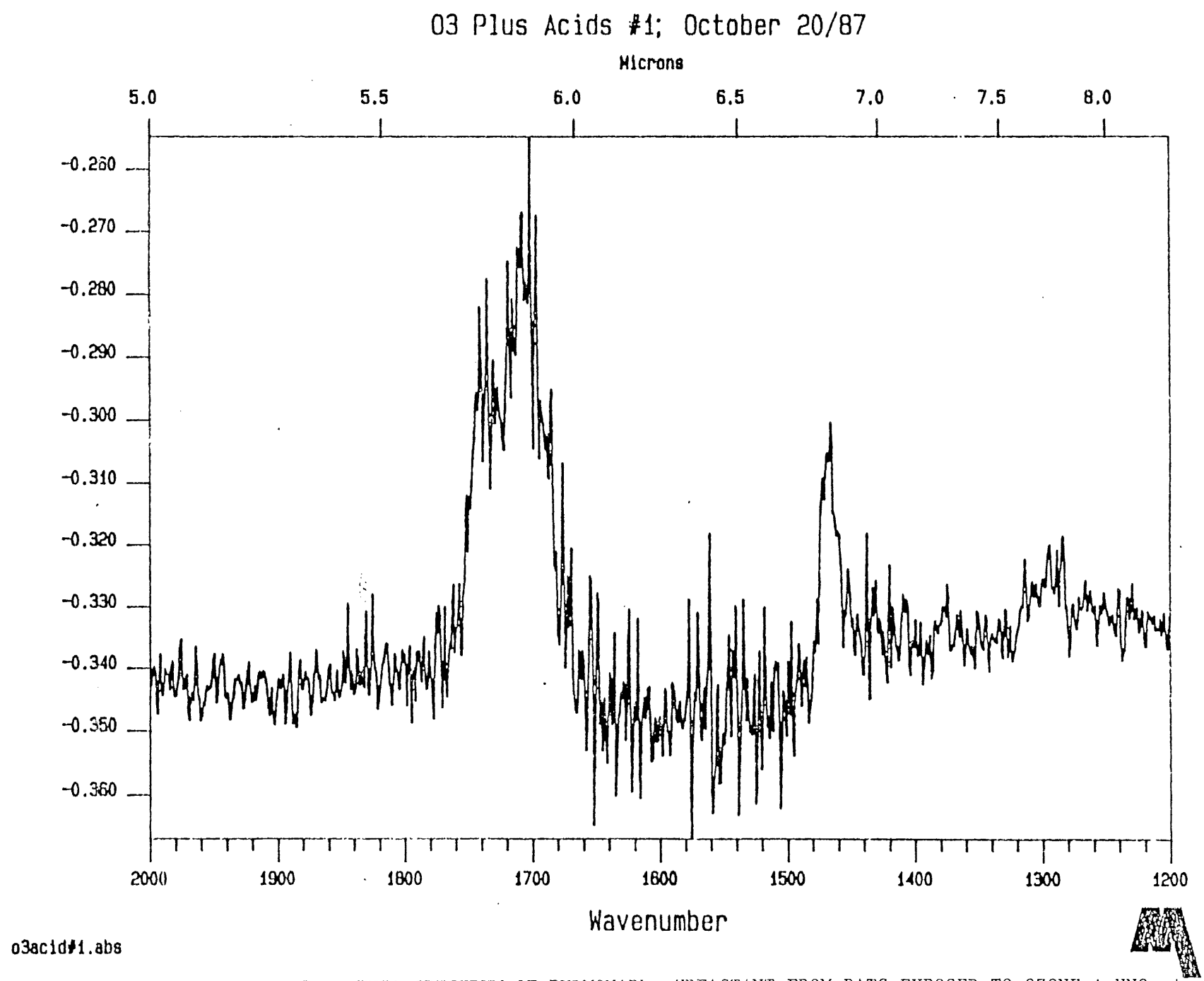


FIGURE 2a. FTIR SPECTRUM OF PULMONARY SURFACTANT FROM RATS EXPOSED TO OZONE + HNO_3 + H_2SO_4, GROUP#1

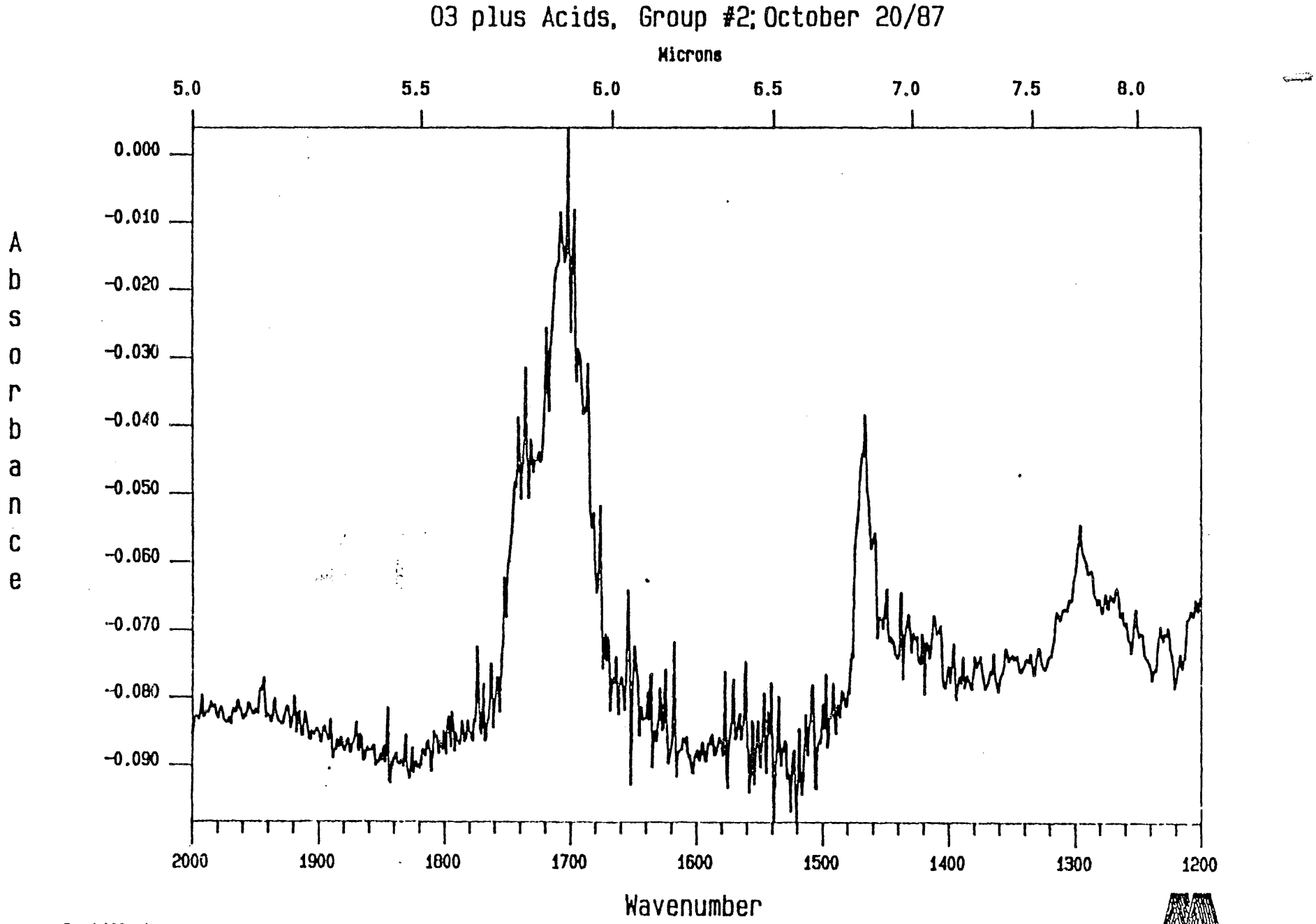


FIGURE 2b. FTIR SPECTRUM OF PULMONARY SURFACTANT FROM RATS EXPOSED TO O_3 + HNO_3 + H_2SO_4, GROUP #2

FIGURE 3. COMPARISON OF AVERAGE ULTRAVIOLET SPECTRUM OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS TO THAT FROM RATS EXPOSED TO 0.40 ppm OZONE FOR FOUR HOURS

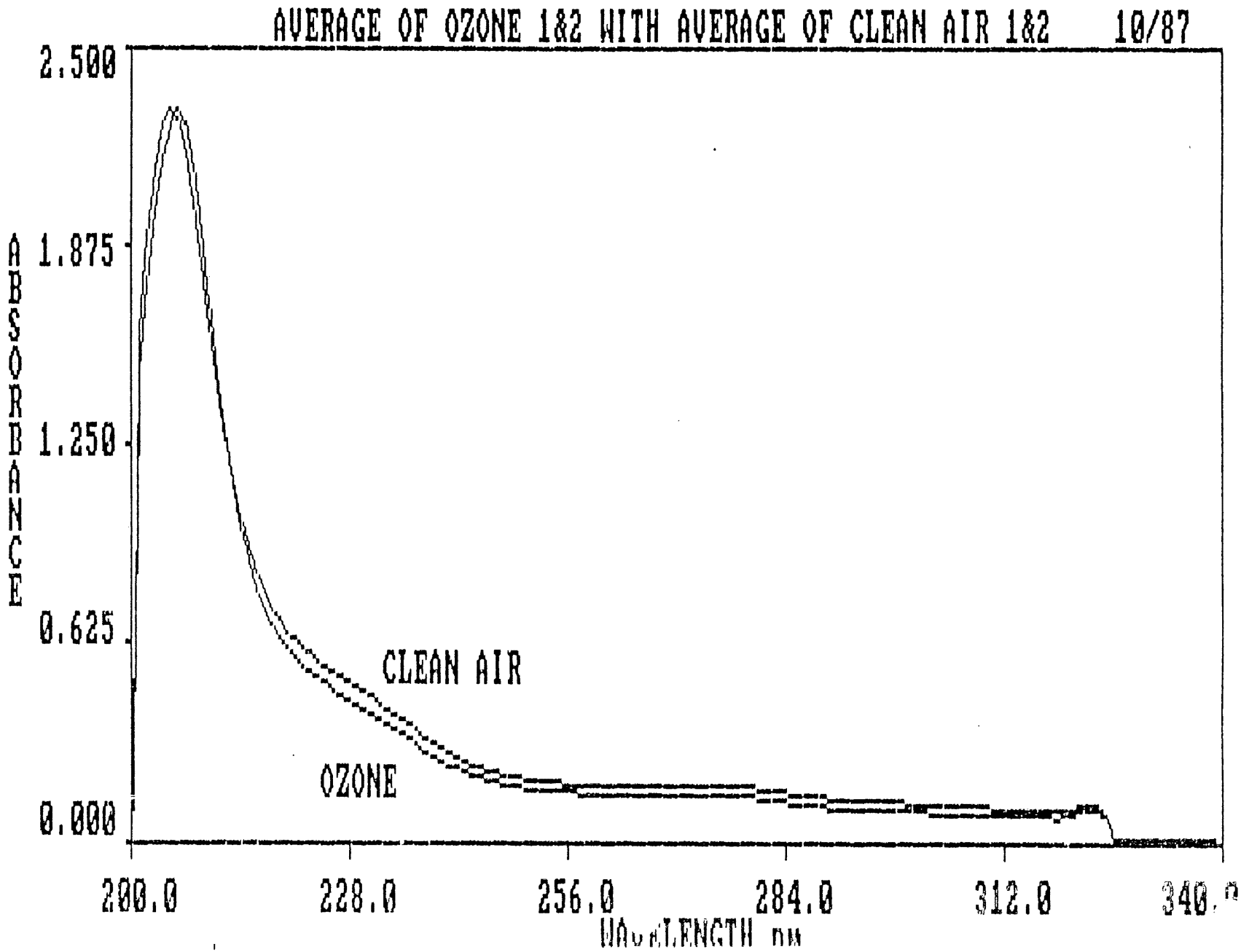


FIGURE 4. COMPARISON OF AVERAGE ULTRAVIOLET SPECTRUM OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS TO THAT FROM RATS EXPOSED TO 0.41 ppm O_3 + 0.67 mg/m^3 HNO_3 + 0.61 mg/m^3 H_2SO_4 FOR 4 HOURS

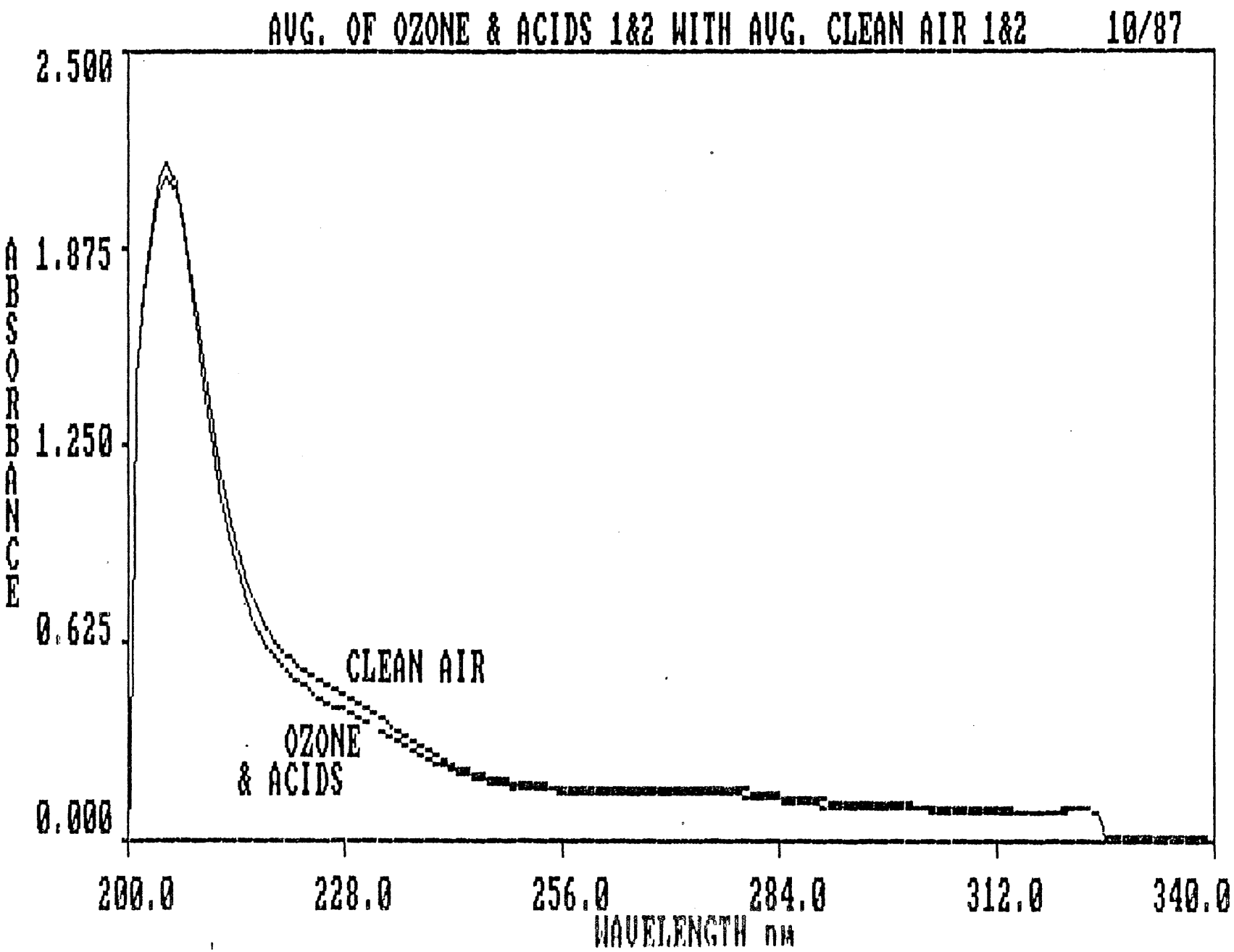


TABLE 17. Fatty Acid Composition of Pulmonary Surfactant from Rats Exposed at Rest to Ozone and Acids (Experiment 1). The O_3 alone exposure was 0.40 ± 0.01 ppm; the O_3 + Acids exposure was with 0.41 ± 0.01 ppm O_3, 0.67 ± 0.12 mg/m^3 HNO_3 and 0.61 ± 0.06 mg/m^3 H_2SO_4 at a relative humidity of 81.5 - 83%. MLE = maximum likelihood estimate, which is equivalent to the mean of the two groups, ± 2 standard deviations (SD) as computed by the statistical model.

Exposure	Molar Ratio of Fatty Acid to Palmitic Acid			
	Palmitoleic	Stearic	Oleic	Linoleic
CLEAN AIR				
Group #1	0.098	0.092	0.097	0.370
Group #2	0.068	0.079	0.074	0.233
MLE ±2SD	0.083 ±0.008	0.086±0.006	0.086±0.006	0.301±0.023
O_3 ONLY				
Group #1	0.047	0.064	0.050	0.151
Group #2	0.072	0.062	0.069	0.233
MLE ±2SD:	0.060±0.008*	0.063±0.005*	0.059±0.006*	0.192±0.023*
O_3 + ACIDS				
Group #1	0.059	0.070	0.060	0.197
Group #2	0.050	0.064	0.051	0.171
MLE ±2SD:	0.054±0.006*	0.067±0.004*	0.056±0.004*	0.184±0.016*

* Statistically different from the clean air controls; see Table20

detail below shows that all of these reductions are statistically significant with p values less than 0.001.

d. Protein Analysis. Table 18 gives the results of the Folin protein assay performed on the supernatant after pelleting out the surfactant plus mucus and other possible contaminants in the second centrifugation step. The lavage fluid from O_3 exposed animals showed a small, but statistically significant increase in total protein while the O_3 + acids exposure was within experimental error of the clean air controls.

2. Exposure to HMSA and O_3 Alone and in Combination (Experiments 3 and 4). Experiment 3 was an exposure to HMSA compared to clean air controls, while Experiment 4 assessed the effects of the higher concentration of HMSA alone, O_3 alone, or the combination of HMSA and O_3 compared to clean air controls. The data from both of these experiments are combined for analysis.

a. FTIR. Appendix III contains the FTIR spectra in the 2000 - 1300 cm^{-1} range for the four different exposure conditions (clean air, HMSA, O_3, or HMSA + O_3). As in the first experiment, the FTIR spectra for the O_3 exposed rats were essentially the same as those for the clean air controls. The spectra from the HMSA exposed rats, and from the O_3 + HMSA exposed animals were also the same as the clean air controls.

b. Ultraviolet Spectroscopy. Appendix IV contains the UV spectra of the PS from rats exposed to the four different atmospheres. Figures 5 - 8 show the spectra obtained by averaging those from groups #1 and #2 for each exposure, compared to the clean air control average spectrum. Three conclusions can be drawn from these comparisons. 1) The UV spectra from the HMSA exposed animals are the same as the clean air controls. 2) The UV spectra from the O_3 exposed animals show a slight increase in absorbance at

TABLE 18. Protein Content of Lavage Fluids from Rats Exposed at Rest to Ozone and Acids (Experiment 1). The O_3 only exposure was at 0.40 ± 0.01 ppm; the O_3 + Acids exposure was with 0.41 ± 0.01 ppm O_3, 0.67 ± 0.12 mg/m^3 HNO_3 and 0.61 ± 0.06 mg/m^3 H_2SO_4 at RH's of 81.5 - 83%.

	Total Protein (X 10^{-2} mg/ml)		
Exposure	Group #1	Group #2	Average ± 1 SD
Clean Air	3.91	3.58	3.75 ± 0.23
O_3 Only	4.61	4.91	4.76 ± 0.21*
O_3 + Acids	3.25	3.92	3.59 ± 0.47

*Statistically different from clean air controls ($p<0.03$).

FIGURE 5. UV SPECTRA OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS AND FROM RATS EXPOSED TO HM (EXPERIMENT #2); SPECTRA FROM REPLICATE GROUPS HAVE BEEN AVERAGED IN EACH CASE

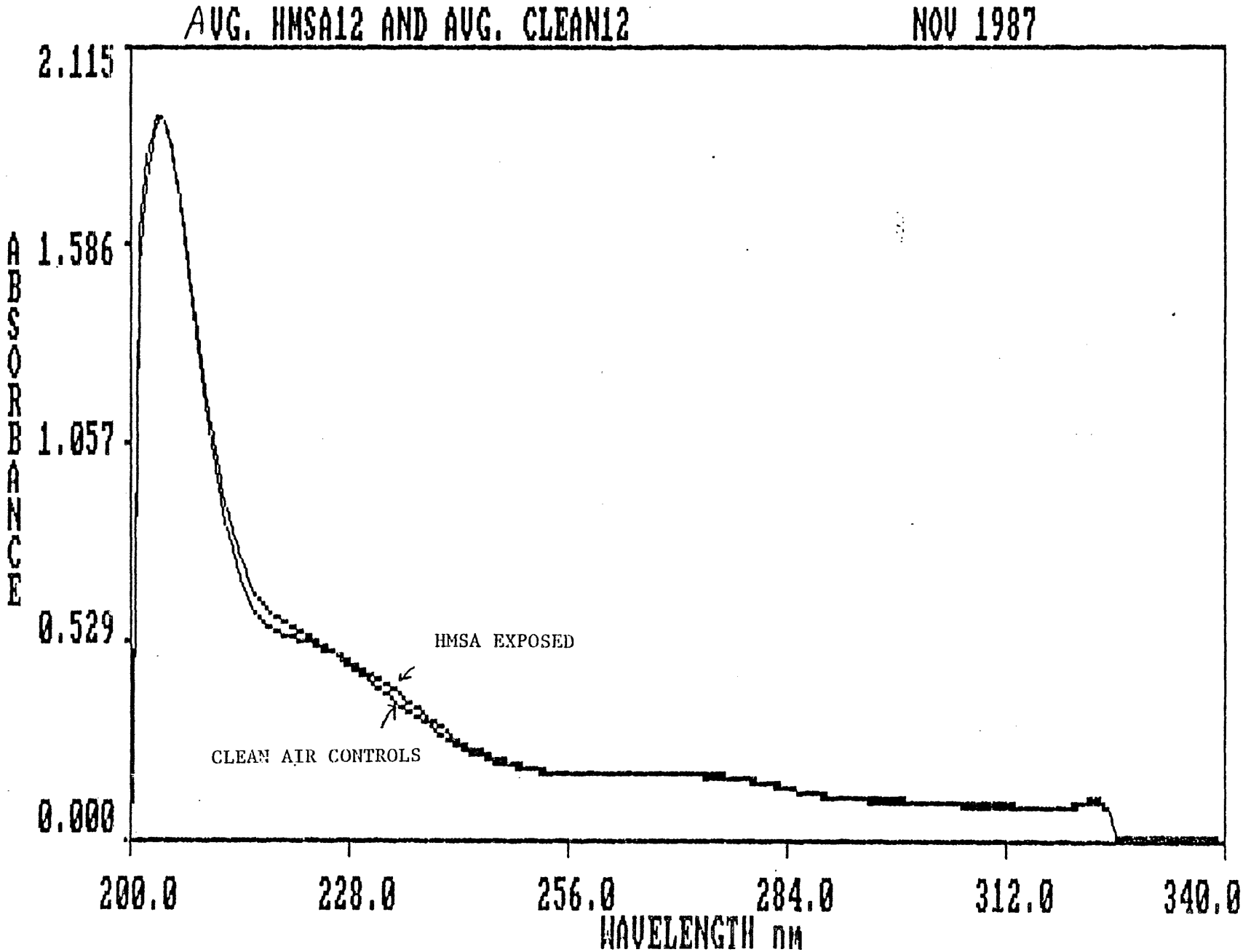


FIGURE 6. COMPARISON OF AVERAGED UV SPECTRA OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS AND FROM RATS EXPOSED TO 0.40 ppm O_3 FOR FOUR HOURS AT REST

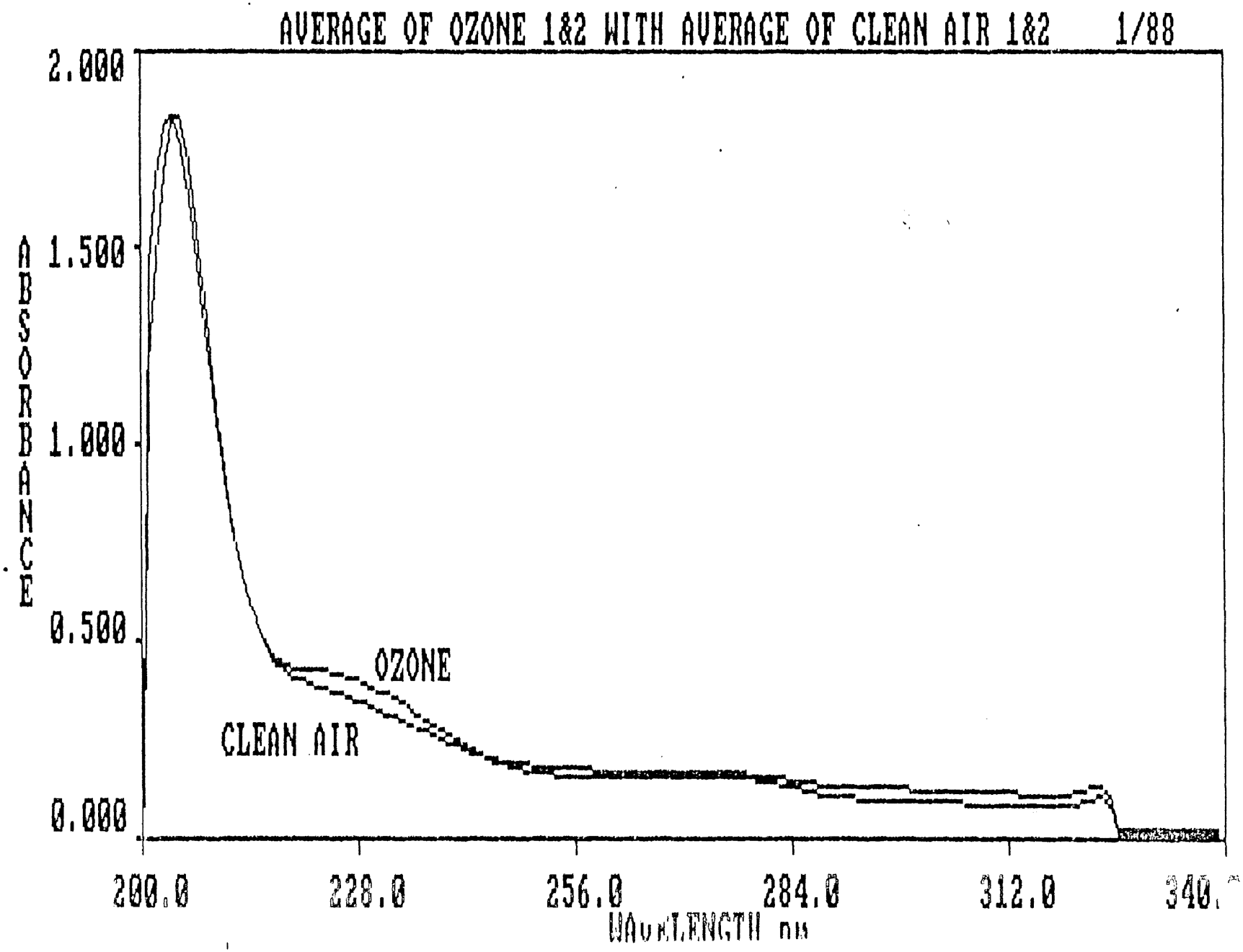


FIGURE 7. AVERAGE UV SPECTRA OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS AND FROM RATS EXPOSED TO 0.47 mg/m^3 HMSA FOR FOUR HOURS

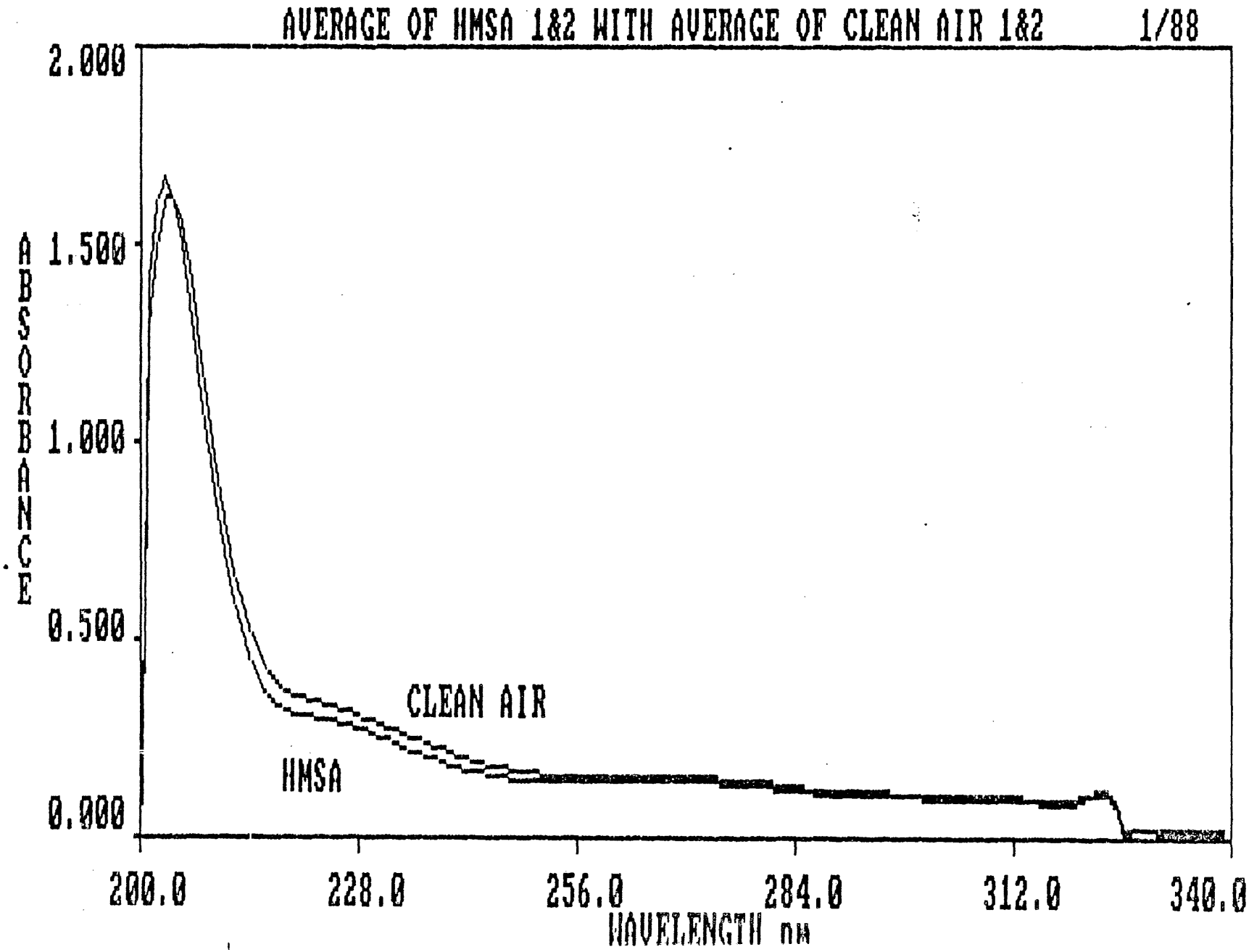
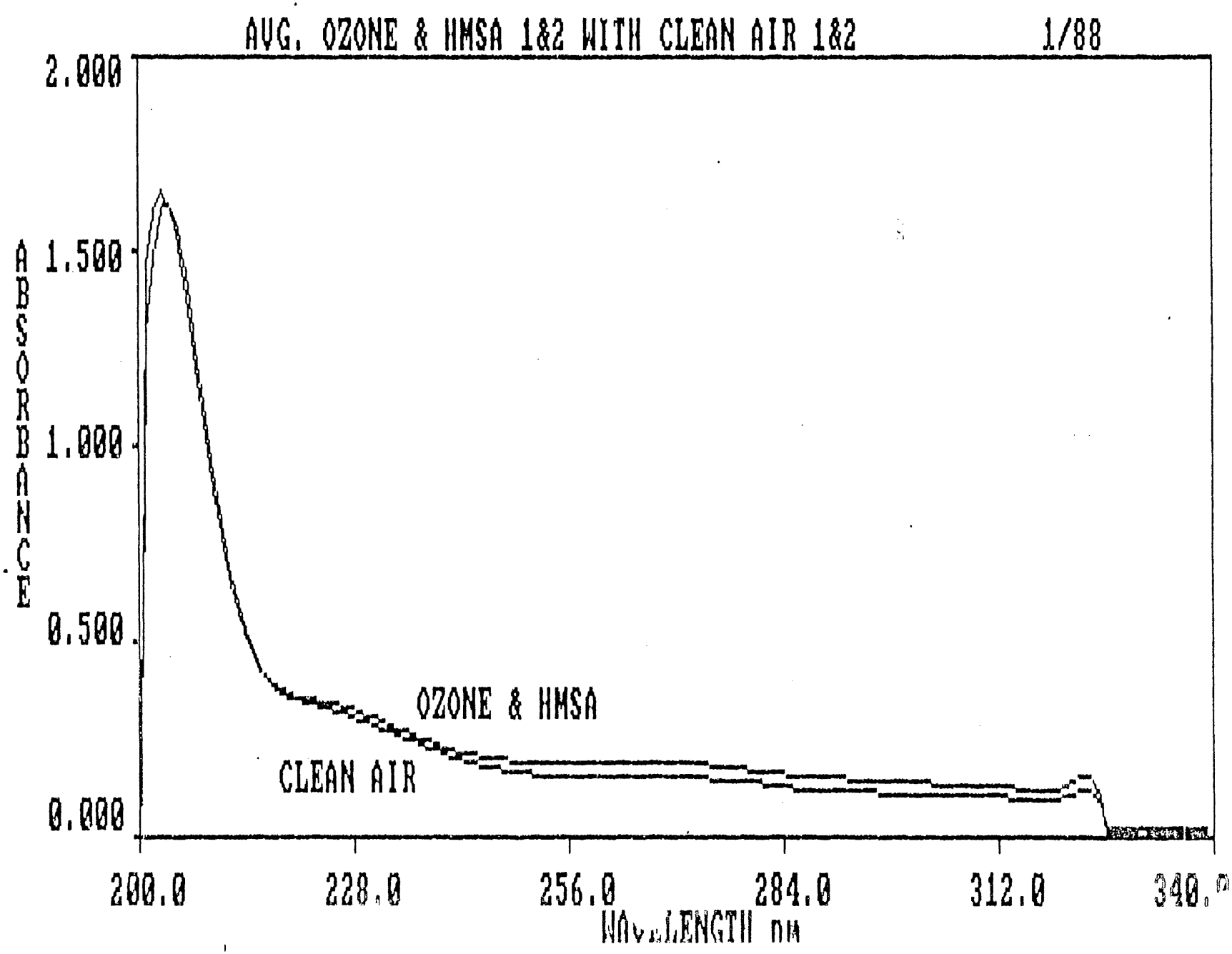


FIGURE 8. AVERAGE UV SPECTRA OF PULMONARY SURFACTANT FROM CLEAN AIR CONTROLS AND FROM RATS EXPOSED TO A COMBINATION OF 0.40 ppm O_3 + 0.38 mg/m^3 HMSA FOR FOUR HOURS



approximately 230 nm, characteristic of conjugated dienes and trienes from oxidation of PS (Klein, 1970). 3) The UV spectra of the PS from the animals exposed to the O_3 + HMSA combination are very similar to those from clean air controls.

c. Fatty Acid Composition. Table 19 shows the fatty acid composition of the PS from the four exposures and Table 20 summarizes the statistical analyses of the GC data. Taking these data as a whole, three conclusions can be drawn. 1) The ratio of the minor fatty acids to palmitic acid in the O_3 exposed animals again decreased significantly relative to the clean air controls, in agreement with the results of Experiment #1. 2) The fatty acid composition for the HMSA exposed rats was not significantly different from those of the clean air controls. 3) The statistical analysis shows that in comparing the results of the O_3 + HMSA exposure to the clean air controls, stearic and linoleic acids differ significantly while palmitoleic and oleic do not. However, this should be interpreted with caution due to outliers in the data. Thus Group #1 in the O_3 + HMSA exposed rats shows much higher stearic acid than the clean air controls, which is in the opposite direction to the effects expected for oxidant exposures. Group # 2, however, is similar to the clean air controls. For linoleic acid, Group #1 of the exposed animals is lower than the clean air controls while Group #2 is within experimental error of the clean air controls. This wide variability between the replicate groups in the O_3 + HMSA exposed animals suggests that any difference between these and the clean air controls should be viewed with caution. As discussed below, the statistical analysis shows that the O_3 exposure does not differ significantly from the O_3 + HMSA exposure, except for the stearic acid outlier. This suggests that the effects observed in the exposure are due to O_3 alone.

TABLE 19. Fatty Acid Composition of PS from Rats Exposed at Rest to O_3 and HMSA Alone or in Combination (Experiments 3 and 4). Exposure conditions were as follows: Experiment 3, HMSA 0.56 ± 0.01 mg/m^3; Experiment 4, O_3 0.40 ± 0.01 ppm, HMSA 0.47 ± 0.03 mg/m^3, and O_3 0.40 ± 0.01 ppm combined with 0.38 ± 0.06 mg/m^3 HMSA. MLE = maximum likelihood estimate, which is equivalent to the mean of the two groups, ± 2 standard deviations (SD) as computed by the statistical model.

Exposure	Molar Ratio of Fatty Acids to Palmitic Acid			
	Palmitoleic	Stearic	Oleic	Linoleic
CLEAN AIR				
Experiment 3				
Group #1	0.054	0.078	0.049	0.139
Group #2	0.053	0.049	0.052	0.153
MLE±2SD:	0.053±0.002	0.064±0.007	0.050±0.003	0.146±0.009
Experiment 4				
Group #1	0.086	0.072	0.071	0.217
Group #2	0.088	0.143	0.068	0.227
MLE±2SD:	0.087±0.005	0.107±0.005	0.070±0.002	0.222±0.009
O_3 ONLY				
Experiment 4				
Group #1	0.067	0.083	0.053	0.155
Group #2	0.082	0.091	0.056	0.170
MLE±2SD:	0.075±0.009*	0.087±0.016*	0.054±0.003*	0.162±0.009*
HMSA ONLY				
Experiment 3				
Group #1	0.074	0.056	0.064	0.221
Group #2	0.056	0.059	0.056	0.154
MLE±2SD:	0.065±0.007	0.057±0.002	0.060±0.002	0.187±0.010
Experiment 4				
Group #1	0.073	0.088	0.067	0.211
Group #2	0.081	0.107	0.064	0.188
MLE±2SD:	0.077±0.006	0.097±0.008	0.066±0.003	0.199±0.011
O_3 + HMSA				
Experiment 4				
Group #1	0.068	0.282	0.056	0.102
Group #2	0.094	0.092	0.066	0.223
MLE±2SD:	0.081±0.003	0.187±0.013*	0.061±0.006	0.162±0.023*

*Statistically different from clean air controls

TABLE 20. Summary of Statistical Analysis of Gas Chromatograph Data. Entries are p values for the given comparison. NS is not significant ($p > 0.1$).

Group Comparison	Molar Ratio of Fatty Acid To Palmitic Acid			
	Palmitoleic	Stearic	Oleic	Linoleic
Air vs O_3	<0.001	0.001	<0.001	<0.001
Air vs (O_3 + Acids)	<0.001	<0.001	<0.001	<0.001
Air vs HMSA	NS	0.02	0.021	0.06
Air vs (O_3 + HMSA)	0.047	<0.001	0.024	<0.001
O_3 vs (O_3 + Acids)	NS	NS	NS	NS
O_3 vs (O_3 + HMSA)	NS	<0.001	0.06	NS
HMSA vs (O_3 + HMSA)	NS	<0.001	NS	NS

d. Protein Analysis. Table 21 gives the results of the Folin protein assay for Experiments #3 and #4. Exposure to O_3 alone or to the O_3 + HMSA combination gave slightly increased protein levels in the lavage fluid (p values were 0.03 and 0.07 respectively).

E. Summary of Results of Exposure Experiments

Table 22 is a summary of statistically significant differences observed in this study. The combined results for effects of the exposures on pulmonary function, histopathology, and pulmonary surfactant showed a consistent pattern of pulmonary irritation induced by O_3 and a variable influence of airborne acids in modifying the O_3 effects. Resting exposures to 0.4 ppm O_3 produced significant changes in breathing pattern, metabolic rate, and pulmonary surfactant and induced lung parenchymal lesions, all indicative of a tissue injury and inflammation response. The effect of exercise exposure was to induce enhanced effects at 0.4 ppm O_3 and some significant effects and some non-significant trends in the respiratory variables at 0.2 ppm O_3. The effect of adding HNO_3 and H_2SO_4 to O_3 induced some significant modifications of O_3 effects on breathing pattern, metabolic rate, and histopathology but not on pulmonary surfactant chemistry. For some variables and concentrations of pollutants, the modification was in the direction of enhancing O_3 effects while in other cases, the direction of modification was the opposite; the effects of the acids on responses to O_3 were complex at the concentrations studied. Resting exposure to 0.5 mg/m^3 HMSA induced some significant pulmonary function effects indicative of respiratory irritation but did not produce significant tissue injury in the nose or lung or significant alteration in pulmonary surfactant chemistry. However, while exercise exposure to 0.4 ppm O_3 did not produce significant nasal epithelial injury, the combination of 0.4 ppm O_3 and 0.5 mg/m^3 HMSA produced a highly significant injury to the nasal epithelium.

TABLE 21. Protein Content of Lavage Fluids from Rats Exposed at Rest to O_3 and HMSA alone and in Combination (Experiments 3 and 4). Exposure conditions were as follows: Experiment 3, HMSA 0.56 ± 0.01 mg/m^3; Experiment 4, O_3 0.40 ± 0.01 ppm, HMSA 0.47 ± 0.03 mg/m^3, and O_3 0.40 ± 0.01 ppm combined with 0.38 ± 0.06 mg/m^3 HMSA.

Exposure	Total Protein (X 10^{-2} mg/ml)		
	Group #1	Group #2	Average + 1SD
CLEAN AIR			
Expt 3	4.39	3.83	4.11 ± 0.40
Expt 4	4.00	4.16	4.08 ± 0.11
O_3 ONLY			
Expt 4	4.43	5.12	4.78 ±0.49*
HMSA			
Expt 3	4.41	3.95	4.18 ±0.33
Expt 4	3.62	3.62	3.62
O_3 + HMSA			
Expt 4	4.31	5.88	5.10 ± 1.11*

*Exposures significantly different from clean air controls; p=0.03 for O_3 exposure and p=0.07 for O_3 + HMSA exposure.

TABLE 22. Summary of Statistically Significant Differences Observed in Exposure Experiments. Where two concentration levels were examined L=lower concentration and H=higher concentration (see Tables 1,2, and 3 for levels).

Experiment 1. Comparison of effects of exposure at rest to O_3 alone and O_3 + HNO_3 + H_2SO_4 at 2 concentration levels.

Pulmonary Function

Frequency	[O_3 L], [O_3+acids L] vs. [O_3 H], [O_3+acids H]
Tidal Volume	[O_3 L], [O_3+acids L] vs. [O_3 H], [O_3+acids H] [O_3+acids L] vs. [clean air]
$\dot{V}_E$	[O_3+acids L], [O_3 H], [O_3+acids H] vs. [clean air] [O_3+acids H] vs. [all other groups]
V_{O2}	[O_3 H], [O_3+acids H] vs. [clean air]

Tissue Injury

Lung	[O_3 H] induced a qualitative change compared to clean air.

Pulmonary Surfactant

Fatty Acid Composition	[O_3 H], [O_3+acids H] vs. [clean air]
Lavage Protein Content	[O_3 H] vs. [clean air]

Experiment 2. Comparison of effects of exposure during exercise to O_3 alone and O_3 + HNO_3 + H_2SO_4 at 2 concentration levels.

Pulmonary Function

Frequency	[O_3 H] vs. [all other groups]
Tidal Volume	[O_3 H] vs. [all other groups]
V_{O2}	[all exposure groups] vs. [clean air] [O_3+acids L] vs. [O_3+acids H]
TR	[all exposure groups] vs. [clean air]

Tissue Injury

Nasal Epithelium	[O_3 H] vs. [O_3+acids L], [O_3+acids H], [clean air] [O_3 L], [O_3 H] vs. [clean air], [O_3+acids L] [O_3 L] vs. [O_3+acids L]
Lung	[O_3 H] vs. [all other groups] [O_3+acids H] vs. [clean air], [O_3 L], [O_3+acids L]

Experiment 3. Exposure at rest to HMSA.

Pulmonary Function

$\dot{V}_{O2}$	[HMSA] vs. [clean air]
$\dot{V}_E/\dot{V}_{O2}$	[HMSA] vs. [clean air]

Experiment 4. Comparison of effects of exposure at rest to O_3 and HMSA alone and in combination. Combination exposures were at 2 concentration levels.

Pulmonary Function

Frequency	[O_3 H], [O_3+HMSA H] vs. [clean air], [O_3+HMSA L], [HMSA H]
Tidal Volume	[O_3 H], [O_3+HMSA H] vs. [clean air], [O_3+HMSA L], [HMSA H]
$\dot{V}_{O2}$	[clean air] vs. [all other groups]
TR	[O_3+HMSA H] vs. [all other groups]

Tissue Injury

Lung	[O_3 H], [O_3+HMSA H] vs. [clean air], [HMSA H], [O_3+HMSA L]

Pulmonary Surfactant

Fatty Acid Composition	[O_3 H], [O_3+HMSA H] vs. [clean air]
Lavage Protein Content	[O_3 H], [O_3+HMSA H] vs. [clean air]

Experiment 5. Comparison of effects of exposure during exercise to O_3 alone and in combination with HMSA at 2 concentration levels.

Pulmonary Function

Frequency	[O_3 H], [O_3+HMSA H] vs. [clean air], [O_3 L], [O_3+HMSA L]
$\dot{V}_{O2}$	[O_3 H], [clean air] vs. [O_3+HMSA L] [O_3 H] vs. [O_3 L]

Tissue Injury

Nasal Epithelium	[O_3+HMSA H] vs. [all other groups]
Lung	[O_3 H], [O_3+HMSA H] vs. [clean air], [O_3+HMSA L], [O_3 L]

X. DISCUSSION

This study has investigated the toxic effects of inhaled acids using a set of acid species found in the air pollution mixture of the South Coast Air Basin and using exposure conditions. HNO_3 and H_2SO_4 are the dominant inorganic acids in California air pollution (Solomon et al., 1988), and toxicologically these acids are interesting because they are differently partitioned to predominate in the vapor (HNO_3) and liquid particle (H_2SO_4) phases respectively. HMSA is an organic acid compound which is particularly interesting because its formation increases the capacity of droplets to load acidic sulfur compounds and thus to subsequently unload acid compounds on deposition in the respiratory tract. Other investigations of the toxicity of acids and oxidants have indicated that acid particles can act synergistically in enhancing the effects of O_3 on the lungs (Last and Cross, 1978; Last et al., 1983, 1984, 1986), consequently we examined the effects of mixtures of these acids with O_3. Exercise was used to study the toxicology of airborne acids and O_3 because exercise has been shown to enhance the effects of O_3 exposure and to enhance the interaction between mixtures of air pollutants (Mautz et al., 1985a, 1988).

A. Breathing Pattern Changes and Histopathology

Rats exposed at rest to 0.4 ppm O_3 alone or in combination with the acids (Tables 4 and 5) showed a trend of development of rapid and shallow breathing in the fourth hour of exposure and a significant depression of metabolic rate. This is the expected irritant response to O_3 at this concentration (Mautz and Bufalino, 1989). Rapid-shallow breathing pattern responses were not observed at 0.2 ppm. The presence of acids did not significantly modify the O_3 effect, and all breathing pattern responses showed recovery by 2 h post-exposure (Tables 4 and 5). However during hour 4 of exposure, 0.2 ppm O_3 combined with the lower concentrations of

acids induced a tidal volume significantly larger than clean air control (Table 5). Upper airway irritants, such as HNO_3, generally induce a slow-deep breathing pattern while lower airway irritants, such as O_3, induce a rapid-shallow breathing pattern (Alarie, 1973). In exposures to mixtures, the conflicting influences of the two irritants on breathing pattern could produce complex interactions. It is possible that the effects of the upper airway irritant acid dominate at low concentrations of the mixture while the lower airway irritant oxidant dominates breathing pattern changes at the higher concentrations. In our earlier ARB contract A4-112-32 (Final Report May, 1988), we observed significant interactions between the effects of 0.35 ppm O_3 alone and in combination with 1.3 ppm NO_2. The combination suppressed the small rapid-shallow breathing pattern responses observed in exposure to 0.35 ppm O_3 alone and had a small effect on shifting initial breathing pattern toward slow and deep respiration. We hypothesized that HNO_3 (about 0.05 mg/m^3) formed in the reaction of O_3 and NO_2 could be responsible for the interaction. However, in the present study, O_3 at 0.4 ppm induced a more extreme rapid-shallow breathing pattern and HNO_3 at a much higher concentration of 0.67 mg/m^3 (Table 2) in combination with H_2SO_4 did not significantly alter the O_3 effect. Although this experiment involved H_2SO_4 in combination with HNO_3 and thus was not an entirely unambiguous test of the effect of HNO_3 on O_3 breathing pattern reflex responses, the results showed that acid and oxidant breathing pattern interactions can be profoundly affected by concentration of the components.

Tissue injury from the resting exposures was not detected in the nose (Tables 13 and 14) and injury in the lung was detected only in 0.4 ppm O_3 exposures both alone and in combination with acids. Although the statistical test did not indicate significant differences in lung injury (Table 14), the presence of Type 2 lesions is qualitatively indicative of oxidant injury, and our observations Type 2 lesions have always been associated with O_3

exposure. Overall, in both breathing pattern reflex responses and in tissue injury, we observed a small effect of O_3 at 0.4 ppm which was not significantly augmented by the presence of the acids, HNO_3 and H_2SO_4, in a mixture with O_3.

Exercise enhanced the breathing pattern and tissue injury effects of ozone exposure. In contrast to the pattern of recovery observed 2 h following the resting exposure, exercise effects were still present at 2 h post exposure, and showed the expected O_3 effect of rapid-shallow breathing for O_3 at 0.4 ppm and depression of VO_2 and rectal temperature in all exposures (Tables 6 and 7). There were indications in this exposure that the presence of acids modified the O_3 effect. The rapid shallow breathing response observed following exposure to O_3 alone was not present following exposure to the mixture. This difference could represent a more rapid recovery from the effects of exposure to the mixture than from O_3 alone or the result of conflicting irritant responses to O_3 vs. acids. Metabolic rate depression 2 h following exercise exposure was strongest for the mixed O_3 and acid exposures which suggests that recovery from the irritant effects was not more rapid in the mixture. Tissue injury was present in the nose and was substantial in the lungs as a result of exercise exposure to O_3, but the pattern among different atmosphere groups was difficult to interpret based on our previous studies of O_3 and H_2SO_4. Lung tissue injury was present in 0.4 ppm O_3 exercising exposures, however the injury was significantly lower in the exposure to 0.4 ppm O_3 combined with acids compared to 0.4 ppm O_3 alone (Tables 13 and 14). In our earlier investigations using 0.6 ppm O_3 and 1.0 mg/m^3 H_2SO_4, exercise exposure to the mixture produced greater parenchymal lesion than exposure to O_3 alone (Kleinman et al., 1989). In the present exposures, O_3 alone injured the nasal epithelium but O_3 in combination with acids did not produce significant injury. However, in earlier exercising exposures to 0.6 ppm O_3 and 1.0 mg/m^3 H_2SO_4, O_3 alone did not result in significant nasal injury, but the acid alone and in combination

with O_3 resulted in similar levels of injury. There were several important differences in design between the present and former exposures which may explain these results. Exposure duration was shorter (3 h), exercise level was higher (2 times resting metabolic rate), and H_2SO_4 and O_3 concentrations were higher in the earlier study. The most important difference, however, was the presence of HNO_3 in the present experiment which may have altered breathing pattern and the relative distribution of inhaled air between the mouth and nose. At present, the results of this experiment stand in contrast to our expectations of acid enhancement of O_3 effects on lung parenchyma, and cannot be readily interpreted without more information on the effects of resting vs. exercising exposure to O_3 alone and in combination with single acid compounds. The results of exposure experiment 2 did not support the hypothesis that airborne acids H_2SO_4 + HNO_3 will interact synergistically with O_3 in their effects on lung tissue injury and indicate again that at low concentrations, the interactions between effects of O_3 and acids can be complex.

HMSA had some interesting toxicological effects in single and mixed exposures. Although breathing patterns were not significantly changed during resting exposure to 0.5 mg/m^3 HMSA, there was a trend toward rapid-shallow breathing and VO_2 was significantly depressed during the 4th h of exposure and 2 h post-exposure (Table 8) suggesting the presence of mild respiratory tract irritation. Exposures to HMSA in combination with 0.4 ppm O_3 showed significant rapid-shallow breathing patterns in response to O_3 with no significant modification when 0.5 mg/m^3 HMSA was present. However, VO_2 was again significantly depressed in HMSA alone as well as in combination with O_3 and body temperature showed a greater depression in the combination exposure than in either single component (Table 10). In its effects on VO_2 HMSA shows irritant properties that we have not observed in exposures to 1.0 mg/m^3 H_2SO_4 (unpublished data) and suggests that it may enhance the pulmonary function effect of 0.4 ppm O_3 as revealed by a trend

toward a more extreme rapid-shallow breathing response and significantly greater body temperature depression in combination exposures to O_3 and HMSA (Table 10). Exercise exposure showed at 2 h post exposure a similar high breath frequency response for 0.4 ppm O_3 alone and in combination with 0.5 mg/m^3 HMSA (Table 12). However while O_3 alone did not show significant nasal epithelial injury, HMSA combined with O_3 clearly injured the nasal epithelium (Table 15 and 16). Because the primary purpose of the experiment was to test for a modification of an O_3 effect on the lung by presence of HMSA, there was no direct comparison of an exercising exposure to HMSA alone. However, the level of nasal epithelial injury observed in this exposure to combined O_3 + HMSA was similar to nasal injury observed in exercising exposures to 1.0 mg/m^3 H_2SO_4 alone and in combination with 0.6 ppm O_3 (Kleinman et al., 1989). This strong effect of exercise exposure to acid aerosol on the nasal epithelium again contrasts with the unexpected result of Experiment 2 that HNO_3 combined with H_2SO_4 and O_3 did not produce a strong nasal epithelial injury (Table 14).

B. Chemical Characterization of Pulmonary Surfactant

The four parameters used to assess changes in the chemical composition of PS (FTIR, UV, GC, and total protein) examined different effects of the air pollutant exposures. Both of the spectroscopic methods assessed the formation of new IR or UV-absorbing species in PS due to the exposure, while GC measured the change in the original fatty acid composition. Total protein was indicative of transudation across the alveolar-capillary barrier (Henderson, 1984).

Our previous studies (ARB Final Report, 1988; Finlayson Pitts et al., 1989) established that exposures to the combination of the oxidant air pollutants, O_3, and NO_2, induced inflammatory responses in the lung. PS responses were characterized by a decrease in the amounts of the minor fatty acids relative to the major component, palmitic acid, with the more unsaturated fatty acids showing the

greatest relative changes. These changes in the fatty acid composition were maximal approximately 24 hours after the exposure while the formation of IR and UV absorbing products was greatest at the longest delay time studied, 48 hours.

Thus the oxidant inflammatory response in the alveolar region of the lung is characterized by the oxidation of fatty acids, especially unsaturated ones, and the build-up of oxidation products in the liquid layer lining the alveoli. Because the lung continuously recycles surfactant with turnover times in adult rabbits, for example, of approximately 3 - 11 hours (Clements et al., 1984; van Golde et al., 1988) the fatty acid composition returns to the clean air control values 48 hours after the exposure. However, the oxidation products may not be reutilized in making new surfactant as some analogs of phosphatidylcholines are (Jacobs et al., 1984), so they continue to build up in this liquid layer. Thus the change in the fatty acid composition precedes the observation of IR and UV absorbing products.

The measurement of either the change in the fatty acid composition or the formation of oxidation products was optimized at different post-exposure sacrifice times. In these studies we chose a sacrifice time of approximately 24 hours, optimizing the observed changes in the fatty acids. The rationale for this was that these data have proven to be the most sensitive and reproducible markers of effects on PS. Furthermore, the data were quantitative in nature so that a statistical analysis could be carried out. It should be noted, however, that by choosing the 24 hour sacrifice time, observation of the buildup of oxidation products by FTIR and UV was not optimized.

In the exposures involving O_3 in the studies presented here, the fatty acid composition of PS 24 hours after the exposure was significantly different than that of the clean air controls. The trend in the individual fatty acids was what one would expect from oxidation processes, i.e., saturated stearic acid generally showed the least change and the most unsaturated fatty acid studied,

linoleic acid, showed the greatest.

Exposure to HMSA alone had no significant effect on the fatty acid composition 24 hours after the exposure; thus it does not induce an inflammatory response as does O_3. Interestingly, the addition of either HMSA or the combination of nitric and sulfuric acids to O_3 during the exposure did not alter the effects compared to O_3 alone.

The FTIR spectra showed clear evidence of a change in PS only in the case of exposure to a combination of O_3 and nitric and sulfuric acids. The fact that this change was observed in both groups #1 and #2 in the studies reported here suggests it is not an artifact. However, the change in chemical composition which is responsible for this is not clear. Current studies under NIEHS support are directed to developing HPLC techniques for separation of the products from the PS; once these techniques are established, it will be applied to this system to identify this new IR absorbing species.

The only evidence for the buildup of oxidation products was in the 0.4 ppm O_3 exposure in Experiment 4. Failure to observe this response in other 0.4 ppm O_3 exposures was probably due to the choice of a 24 hour sacrifice time; as discussed above, in past studies of oxidant exposures the UV-absorbing products peaked at the 48 hour mark. Thus it was likely that in the present experiments the samples were collected too early in the course of the inflammatory response to show this effect. In this particular 0.4 ppm O_3 exposure (Experiment 4) in which UV-absorbing products appeared, the fatty acid composition was closer to the clean air controls than in Experiment 1 where UV absorbing products were not observed. For example, in Experiment 4 linoleic acid was 73% of the clean air controls, whereas in Experiment 1, it was 64%. This is consistent with the course of the inflammatory response being further advanced along in Experiment 4; thus the change in the fatty acid composition has already peaked and started to recover, and the oxidation products have built up to a greater extent. Why

the inflammatory response is further developed in this case is not clear.

There was a slight, statistically significant, increase in total protein in the exposures to O_3 alone and in the exposure to combined O_3 and HMSA. Changes in total protein in lavage fluids due to exposure to O_3 have been observed in some other studies. For example, Hu et al. (1982) exposed guinea pigs to 0.26 - 1.00 ppm O_3 for 3 h and followed the change in total protein in the lavage fluids as a function of time up to 24 h post-exposure. Total protein increased by approximately a factor of two 10 - 15 h after the exposure, but was indistinguishable from the controls at 24 h. In other studies, a significant increase in total protein was observed immediately after the exposure, but these experiments generally involved exposure to much higher oxidant concentrations and/or longer exposure times. For example Van Bree et al. (1988) observed a significant increase in protein in the lavage fluids from rats exposed to 2 ppm O_3 for 4 hours, while Nachtman et al. (1986) observed an increase in protein of 360% after exposure of rats to 0.8 ppm O_3 for 18 hours. On the other hand, Guth et al. (1986) observed a small, but statistically significant, increase in lavageable protein in rats exposed to 0.4 ppm O_3 for 6 h and sacrificed immediately after the exposure; after a 24 h exposure, the increase was much larger, approximately a factor of five compared to the clean air controls.

There are several factors which may explain why the changes in total protein in the lavage fluids we observed on exposure to O_3 tend to be less than those reported in the literature: (1) The dosage of O_3 was much lower in our studies; (2) In our work the rats were sacrificed 24 hours after the exposure, whereas many of the studies in the literature which report large increases in total protein sacrifice the animals immediately after the exposure. If the change in protein was maximal immediately after the exposure, we might not have observed it; (3) In our lavage saline solution we included small amounts of Ca^{2+} and Mg^{2+}, which are recommended

to help seal epithelial junctions (Henderson, 1984). This may also help to prevent leakage of protein into the liquid alveolar lining. Most studies of lavage fluids have not incorporated these cations in the lavage solution, and hence the observations may differ. However, we believe that for our purposes, i.e., studying only what is in the alveolar liquid lining, this lavage solution was the most appropriate.

In summary, O_3 induced an inflammatory response in rats which changed the fatty acid composition of PS 24 hours after the exposure. Exposure to HMSA alone produced no discernible effect on PS. The addition of either HMSA or a combination of HNO_3 and H_2SO_4 to O_3 did not significantly change the effects due to exposure to O_3 alone; the only exception was the possible formation of new products seen in the infrared spectra of PS from the O_3 + HNO_3 + H_2SO_4 exposure.

The results of our studies of acid and oxidant effects on the respiratory system revealed a strong correspondence between the different biological endpoints in identifying exposures producing significant responses. Table 23 summarizes the results of experiments using the variety of biological endpoints for exposures to acids and oxidants at moderate concentrations. Exposures that induced an oxidant effect produced significant effects in all of the endpoint categories: breathing pattern change, lung tissue injury, and change in PS composition. When O_3 concentrations were 0.2 ppm, neither breathing pattern nor tissue injury endpoint categories showed significant effects of the exposure. This pattern of responses supports the idea that these different endpoint categories are different measures of an overall injury-inflammation process and suggests that the non-invasive breathing pattern measurements are indicators of the other respiratory system effects of oxidant air pollutants.

While the pattern of oxidant induced injury and inflammation was generalized among the biological endpoints examined in this study, the effects of acids both directly on the respiratory tract

TABLE 23. Summary of Changes in Endpoints Observed in Acid and Oxidant Mixture Studies (ARB contracts A4-112-32 and A6-148-33). Exposures were at rest for 3 or 4 h. A "+" means that a significant change in this endpoint was observed during exposure to the atmosphere shown; a "-" means no significant effects were observed.

Endpoint	Exposure Atmosphere					
	O_3[1]	O_3+NO_2[1]	Acids[2]	HMSA[3]	O_3+Acids	O_3+HMSA
Reflex Change to Rapid-shallow Breathing Pattern	+	+	-	-	+	+
Respiratory Tract Tissue Injury						
-Lung	+	+	-	-	+	+
-Nasal Epithelium	+/-	-	-	-	+	+
Change in Fatty Acid Composition of Pulmonary Surfactant	+	+	-	-	+	+

[1]At concentrations of 0.35 or 0.4 ppm O_3 and 1.3 ppm NO_2.
[2]Acids = combination of 1 mg/m^3 HNO_3 + 0.5 mg/m^3 H_2SO_4.
[3]HMSA = hydroxymethanesulfonic acid at 0.5 mg/m^3.

and in modifying O_3 responses were complex. In some cases, the acid air pollutants significantly enhanced the irritant effects of O_3. Examples included the irritant effects on VO_2 of HNO_3 + H_2SO_4 + O_3 compared to O_3 alone (Tables 4 and 5) and the effects of HMSA + O_3 in depressing TB compared to O_3 alone (Tables 9 and 10). In other cases, presence of acids in a mixture with O_3 did not significantly modify the effects of O_3 and in one experiment (exercise exposure to O_3 alone and in combination with HNO_3 + H_2SO_4), O_3-induced lung tissue injury was significantly lower when acids were present with O_3 (Tables 13 and 14). It is clear from this and other studies (Last et al., 1983, 1984, 1986; Kleinman et al., 1989) that acid air pollutants can have important effects on the respiratory tract responses to O_3. At the relatively low concentrations of O_3 examined in acute exposures for this study, mixtures of O_3 and acids did not produce a clear pattern of interaction among the multiple biological effects examined that can permit a strong interpretation of the mechanisms for the interaction. There were suggestions that the magnitude and direction of the interactions depend on concentrations of O_3 and acids and that HNO_3 and H_2SO_4 may differently influence the interaction with O_3. Exercise exposure was hypothesized to enhance the respiratory effects of the pollutants and enhance their interactions at low concentrations as we have observed previously in studies of combinations of O_3 and NO_2 (Mautz et al., 1984, 1988). Exercise exposure demonstrated a strong effect of 0.5 mg/m^3 HMSA + 0.4 ppm O_3 in injuring the nasal epithelium, however the interactions between O_3 and this and other acids during exercise exposure remained complex. Repeated exposures to oxidants and acids may demonstrate a more clear pattern of interactions at urban ambient concentrations. Because such studies are much more expensive than acute exposure studies, the toxicological investigation of acid-oxidant interactions should use single acute studies at higher concentrations to determine mechanisms of interactions which can then be used to guide more extensive studies incorporating lower concentrations and repeated exposures.

XI. References

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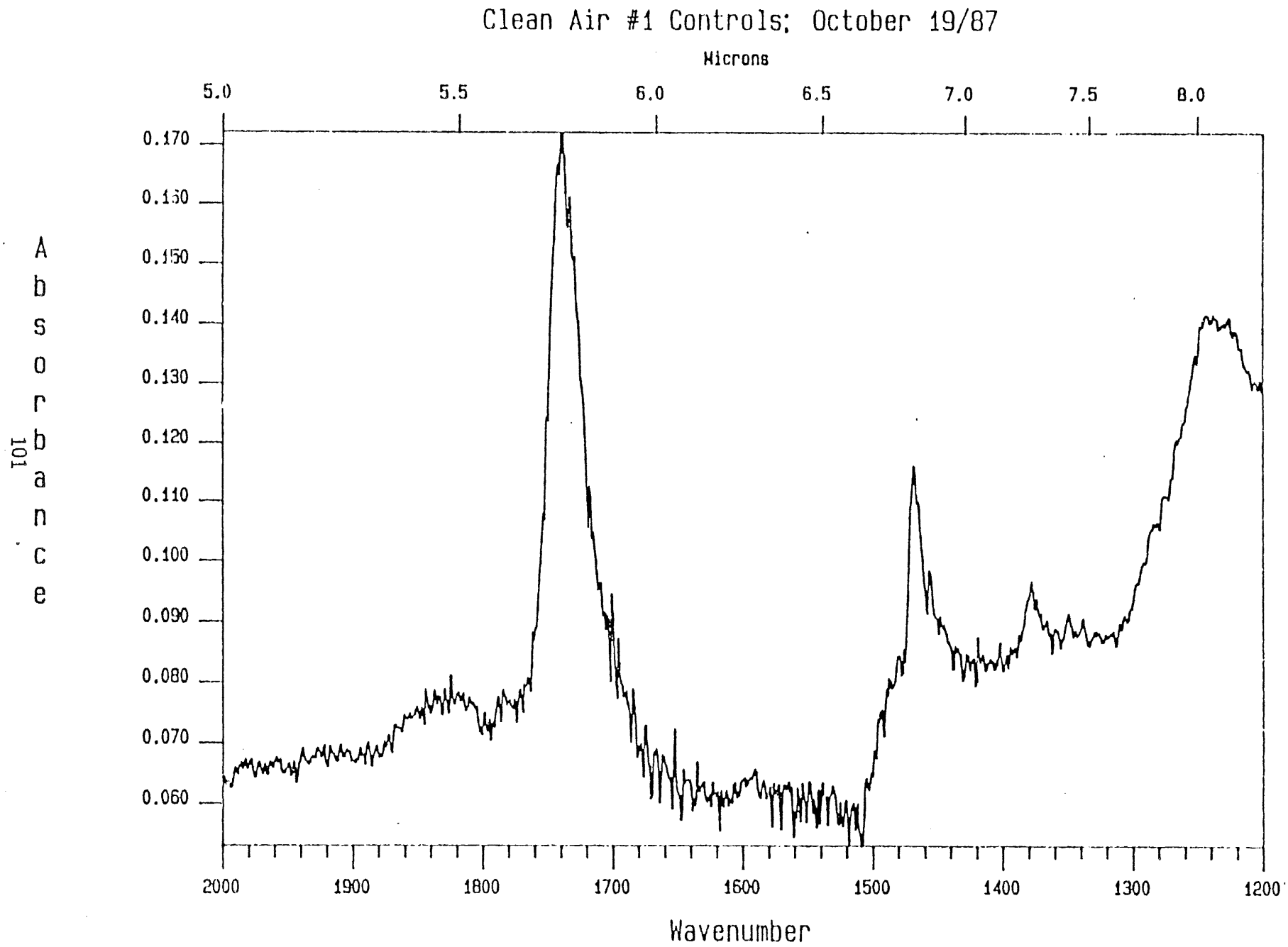
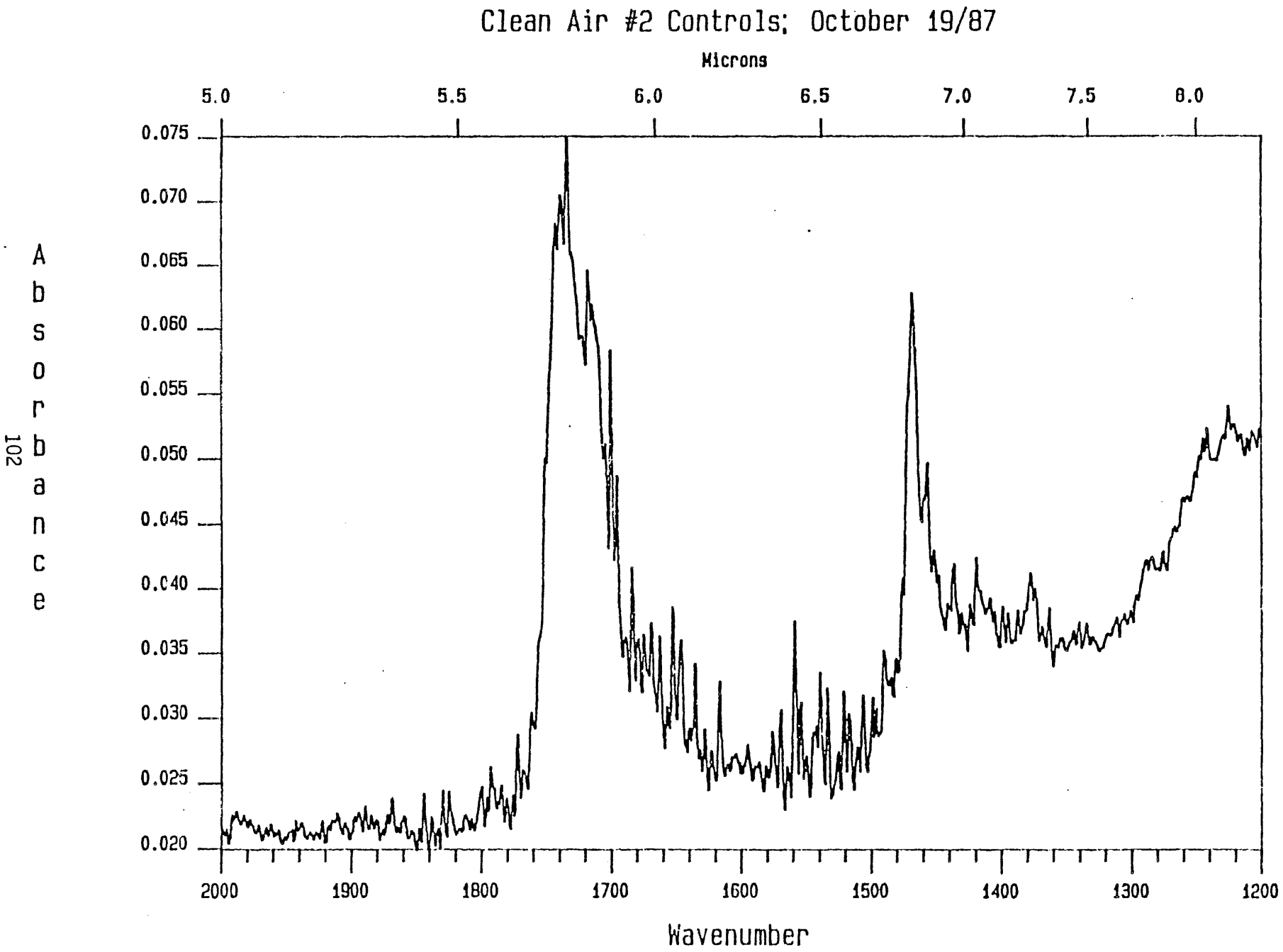
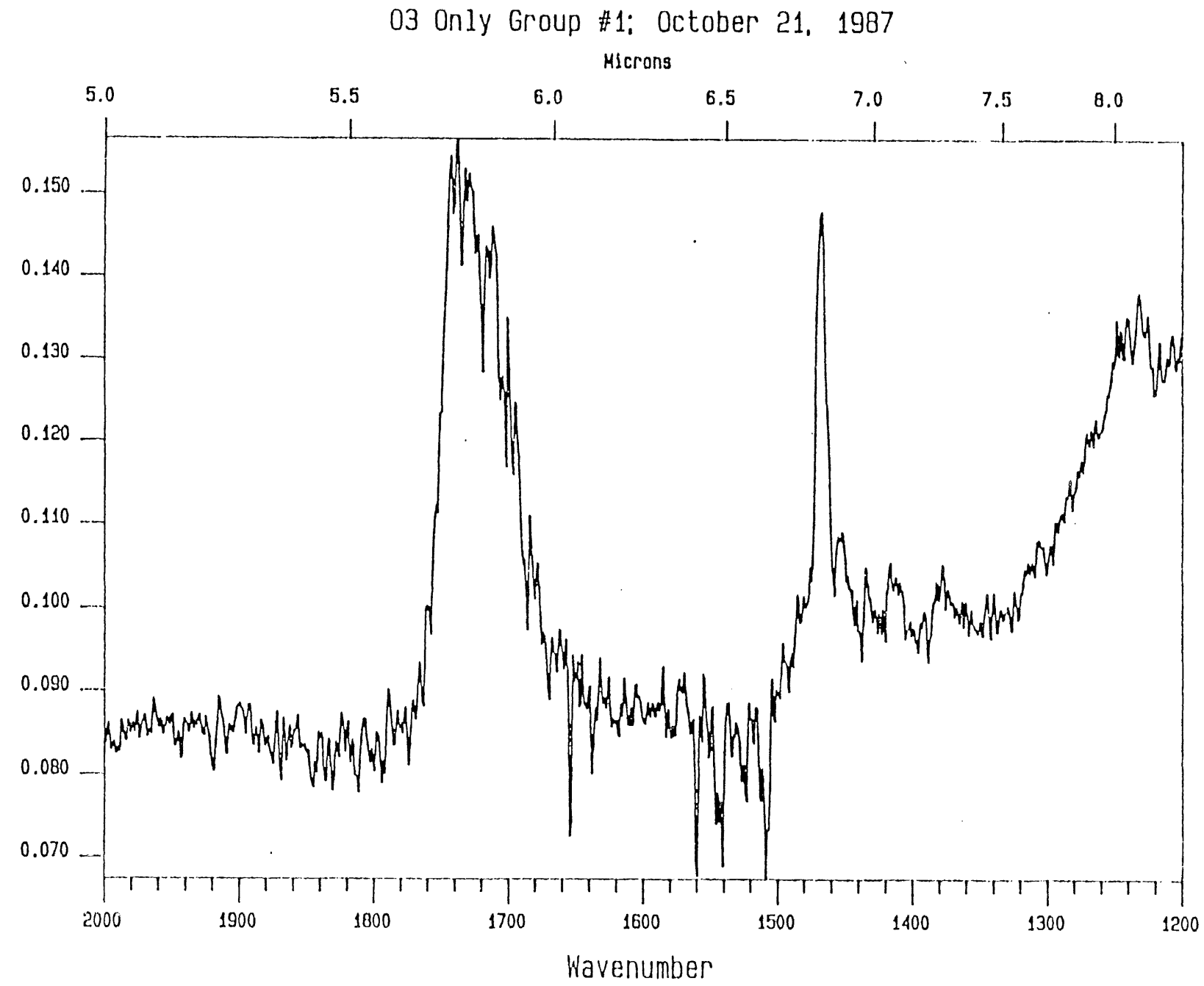
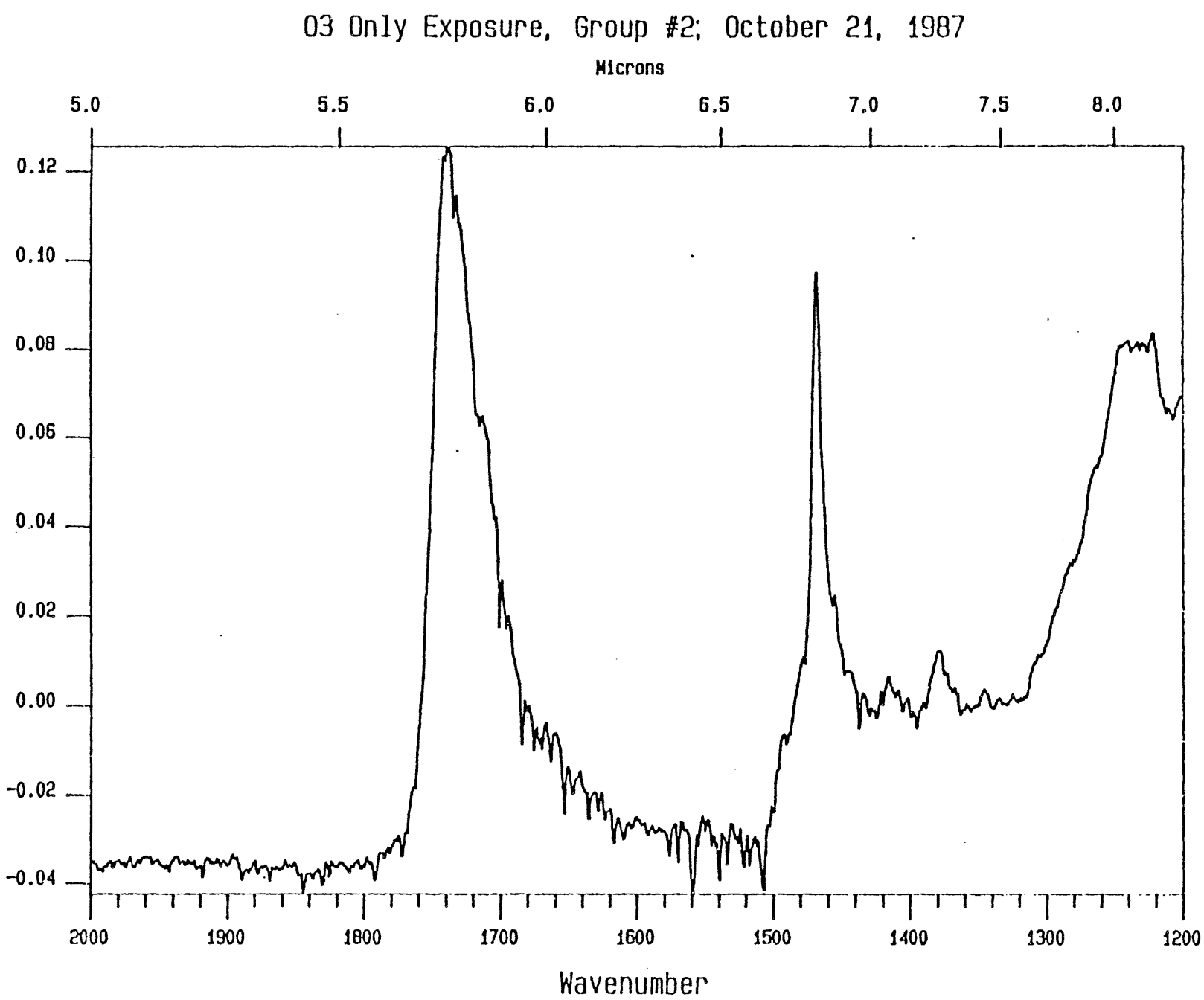
XII. APPENDIX I. FTIR SPECTRA (2000-1300 cm^{-1}) OF PULMONARY SURFACTANT FROM RATS EXPOSED TO CLEAN AIR, 0.40 ppm O_3, OR 0.41 ppm O_3 COMBINED WITH 0.67 mg/m^3 HNO_3 AND 0.61 mg/m^3 H_2SO_4 at RH = 81.5-83%.


Clean Air #1 Controls; October 19/87
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.170
0.160
0.150
0.140
0.130
0.120
0.110
0.100
0.090
0.080
0.070
0.060
Absorbance
101
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


Clean Air #2 Controls; October 19/87
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.075
0.070
0.065
0.060
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
Absorbance
102
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


03 Only Group #1; October 21, 1987
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.150
0.140
0.130
0.120
0.110
0.100
0.090
0.080
0.070
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


03 Only Exposure, Group #2; October 21, 1987
Microns
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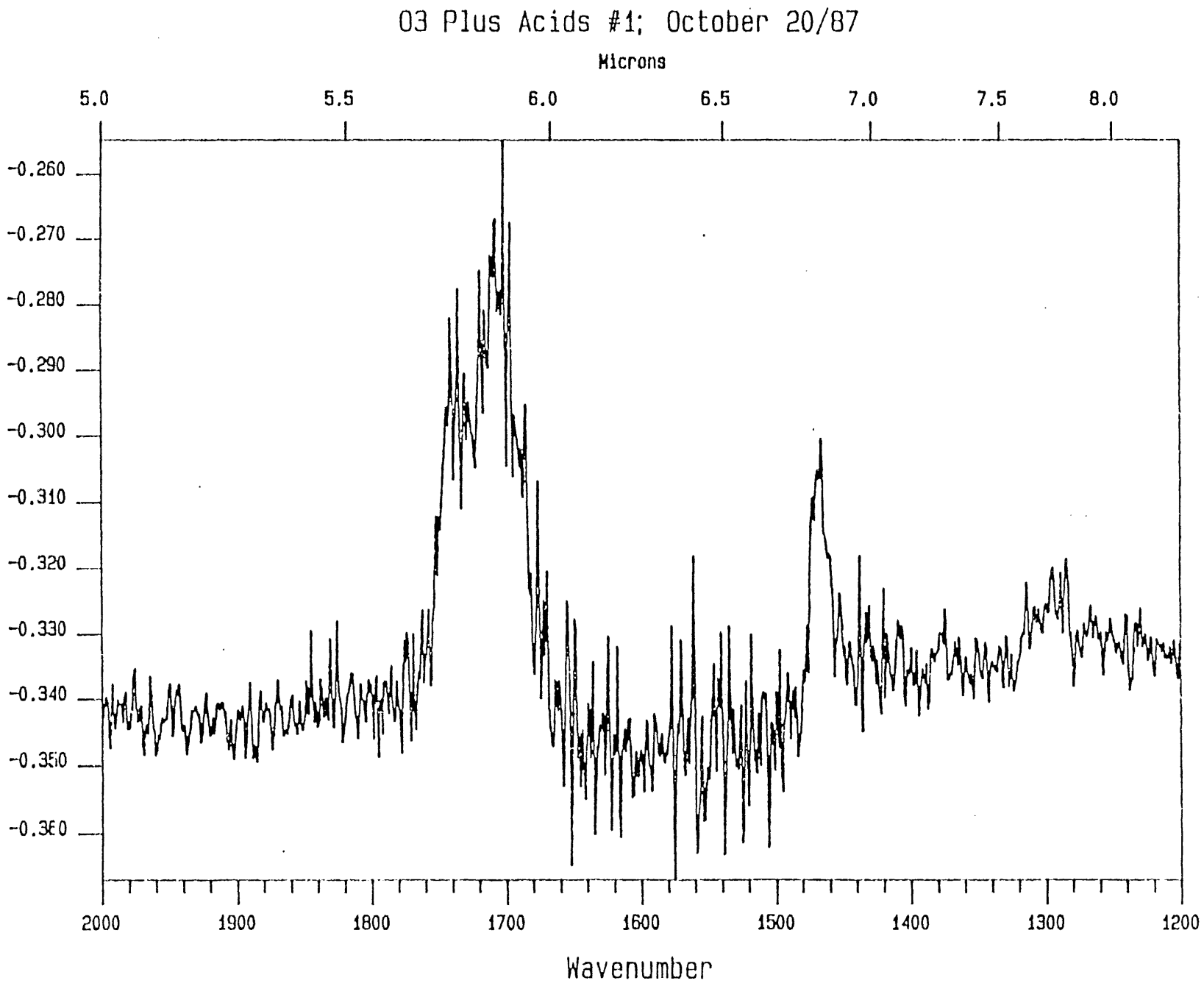
5.5
6.0
6.5
7.0
7.5
8.0
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


03 Plus Acids #1; October 20/87
Microns
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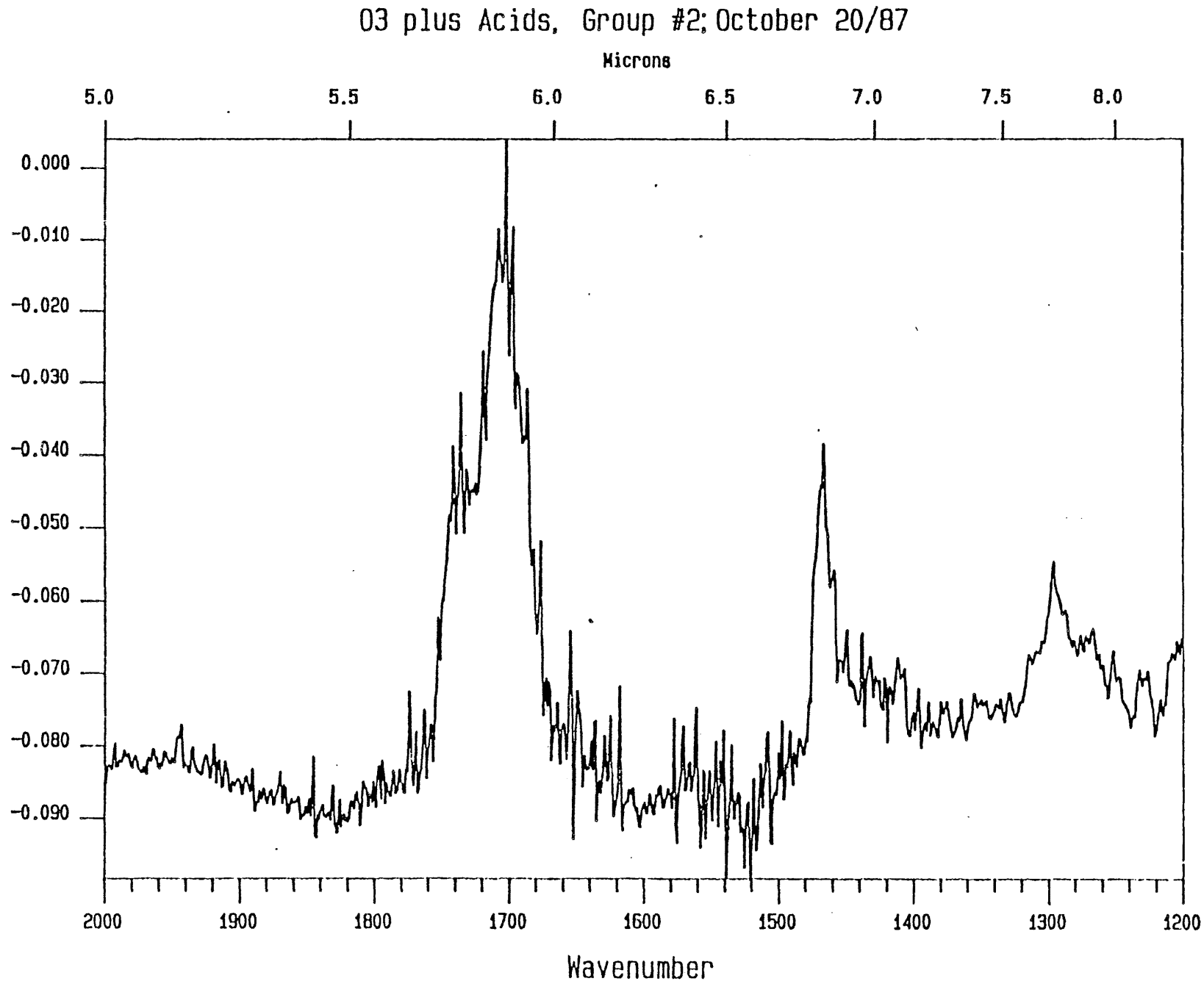
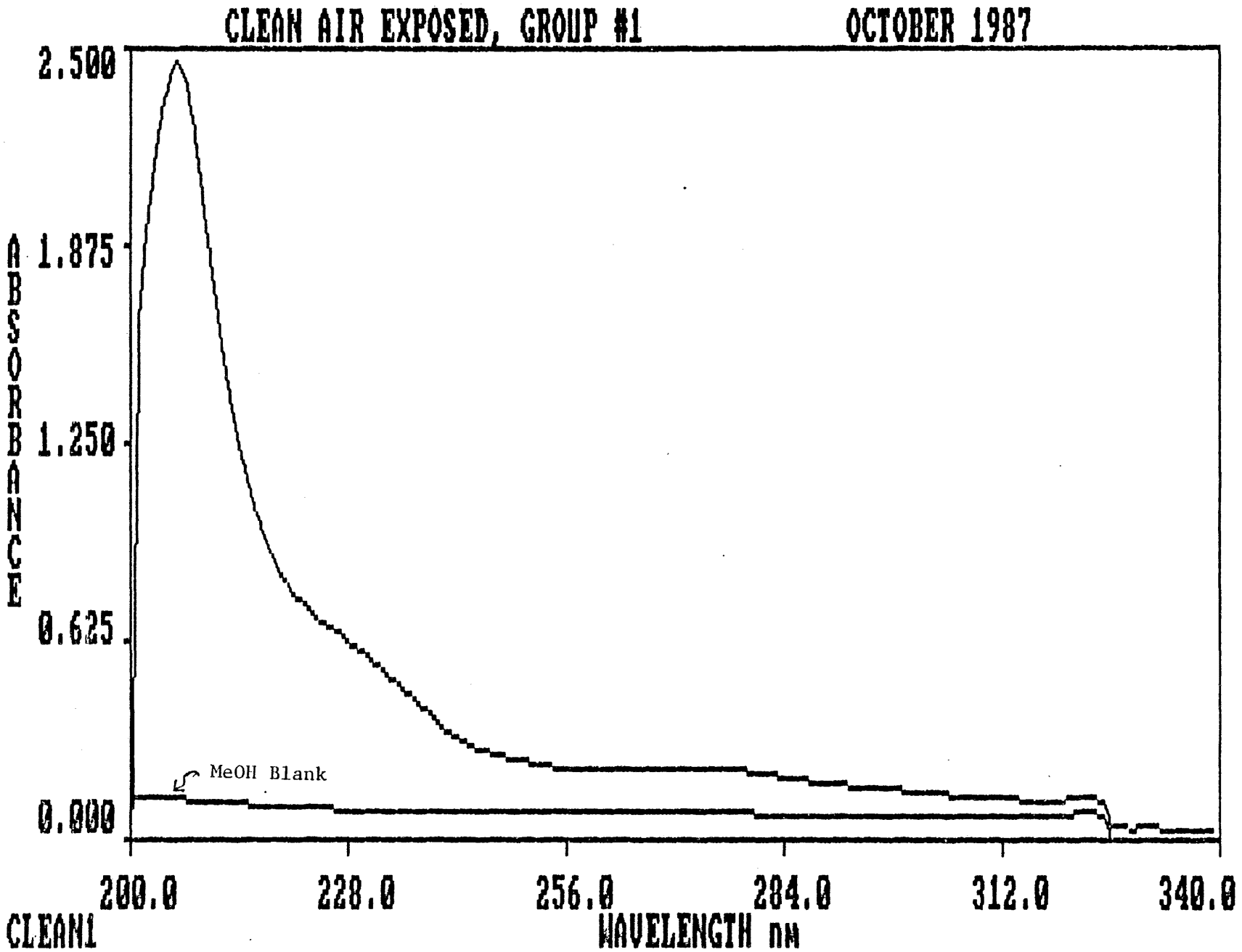
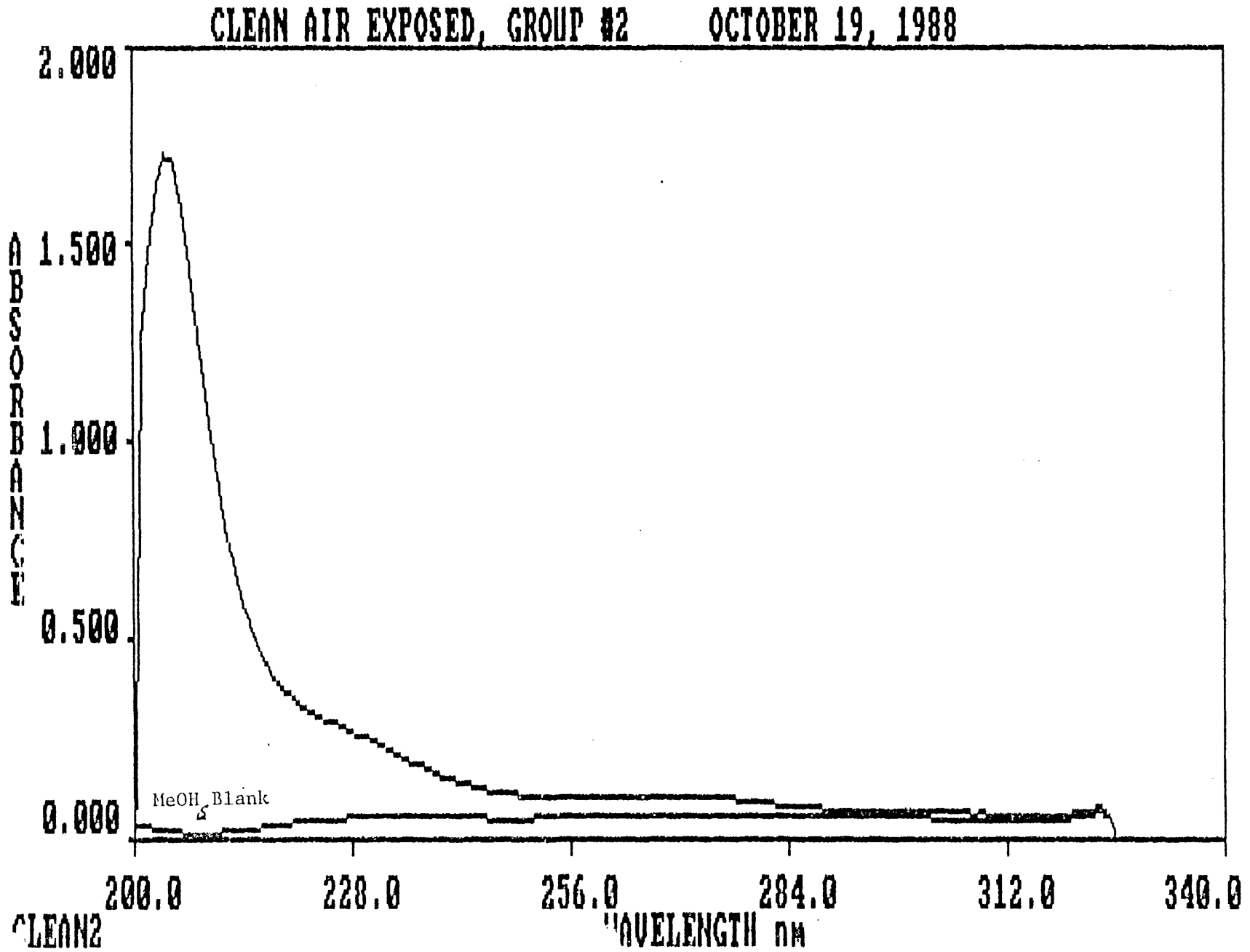
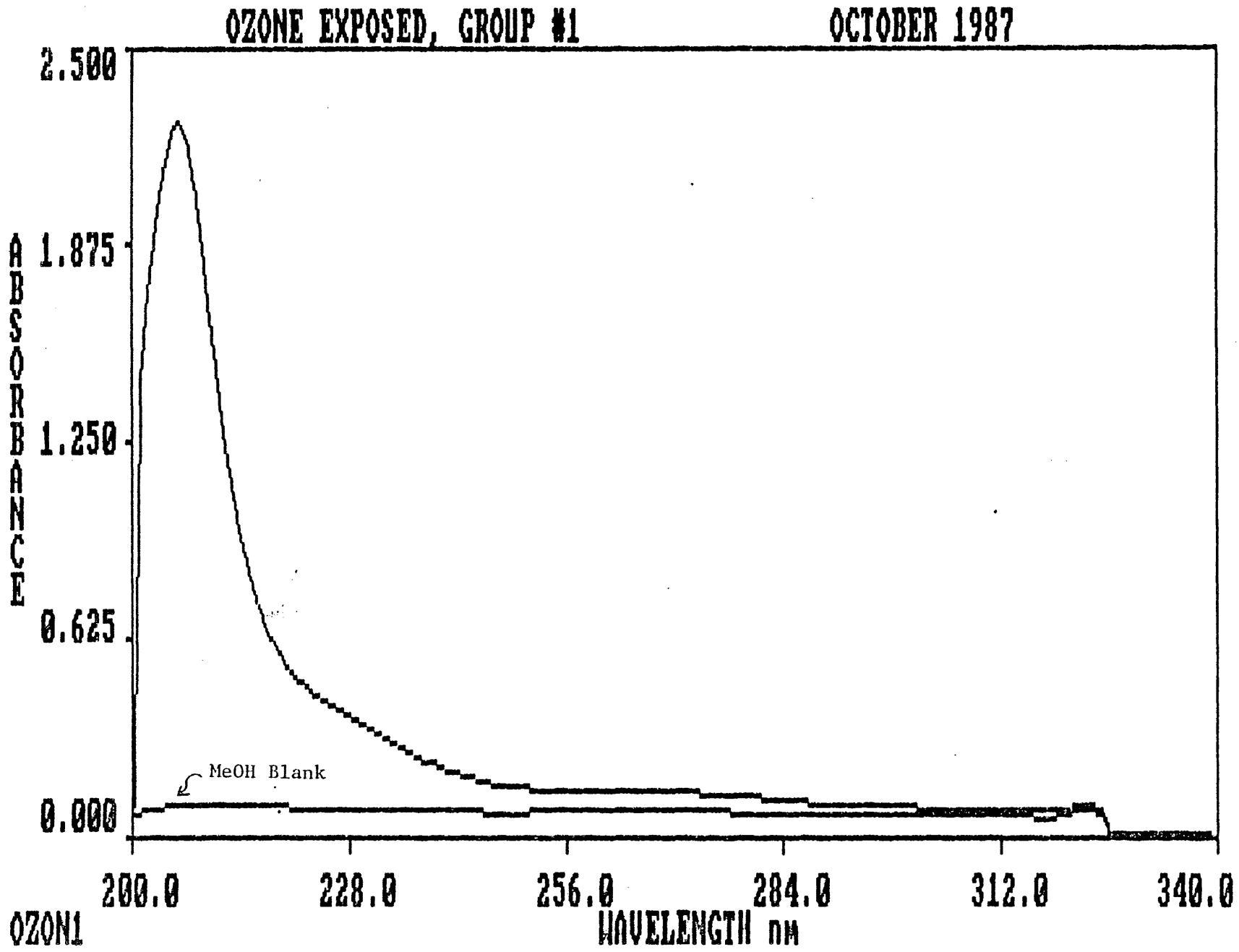
5.5
6.0
6.5
7.0
7.5
8.0
-0.260
-0.270
-0.280
-0.290
-0.300
-0.310
-0.320
-0.330
-0.340
-0.350
-0.360
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


03 plus Acids, Group #2; October 20/87
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.000
-0.010
-0.020
-0.030
-0.040
-0.050
-0.060
-0.070
-0.080
-0.090
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

XIII. APPENDIX II. ULTRAVIOLET SPECTRA OF PULMONARY SURFACTANT FROM RATS EXPOSED TO CLEAN AIR, 0.40 ppm O_3, OR 0.41 ppm O_3 COMBINED WITH 0.67 mg/m^3 HNO_3 AND 0.61 mg/m^3 H_2SO_4 AT RH = 81.5 - 83%.


CLEAN AIR EXPOSED, GROUP #1
OCTOBER 1987
2.500
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
CLEAN1
WAVELENGTH nm


CLEAN AIR EXPOSED, GROUP #2 OCTOBER 19, 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
CLEAN2
WAVELENGTH nm


OZONE EXPOSED, GROUP #1
OCTOBER 1987
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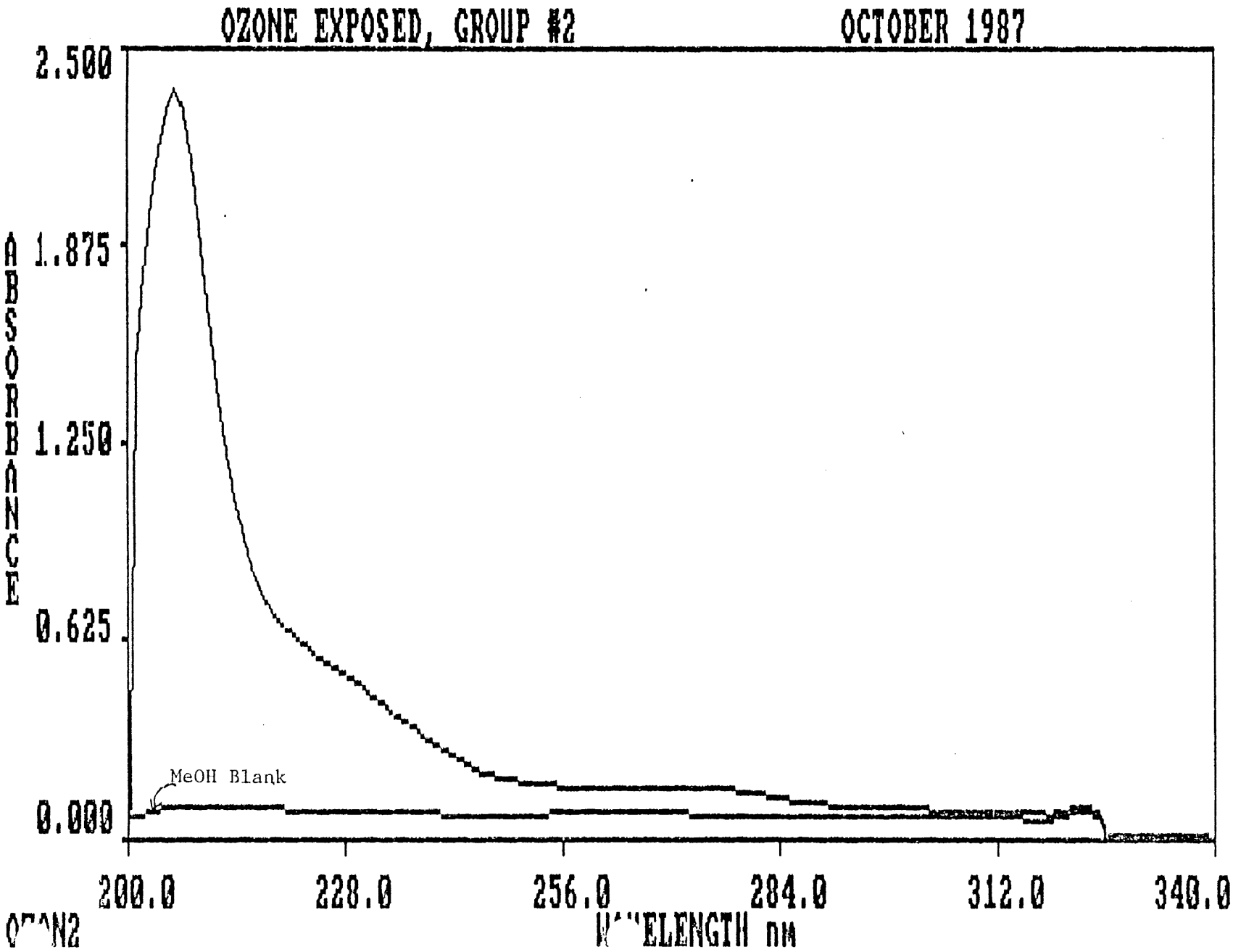
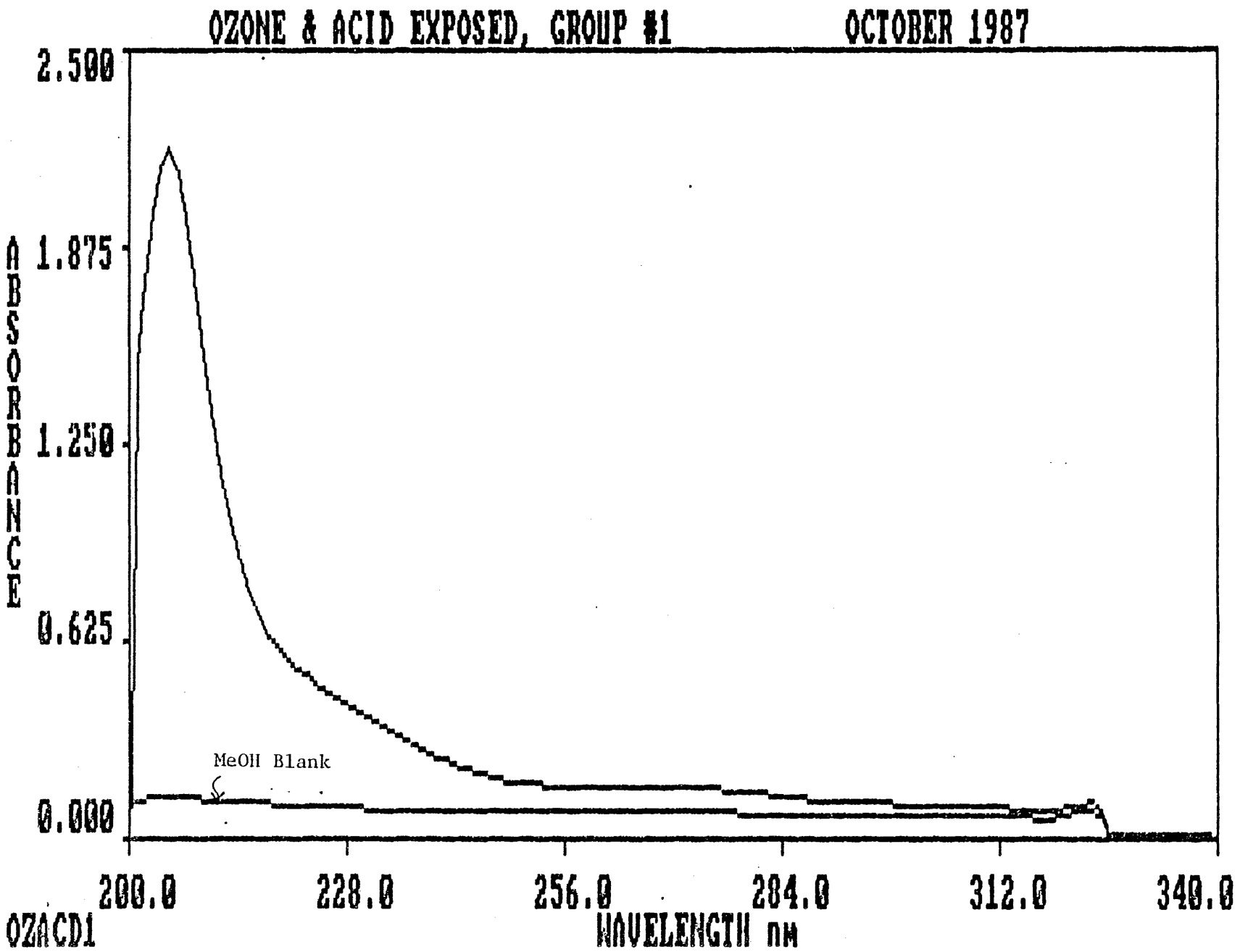
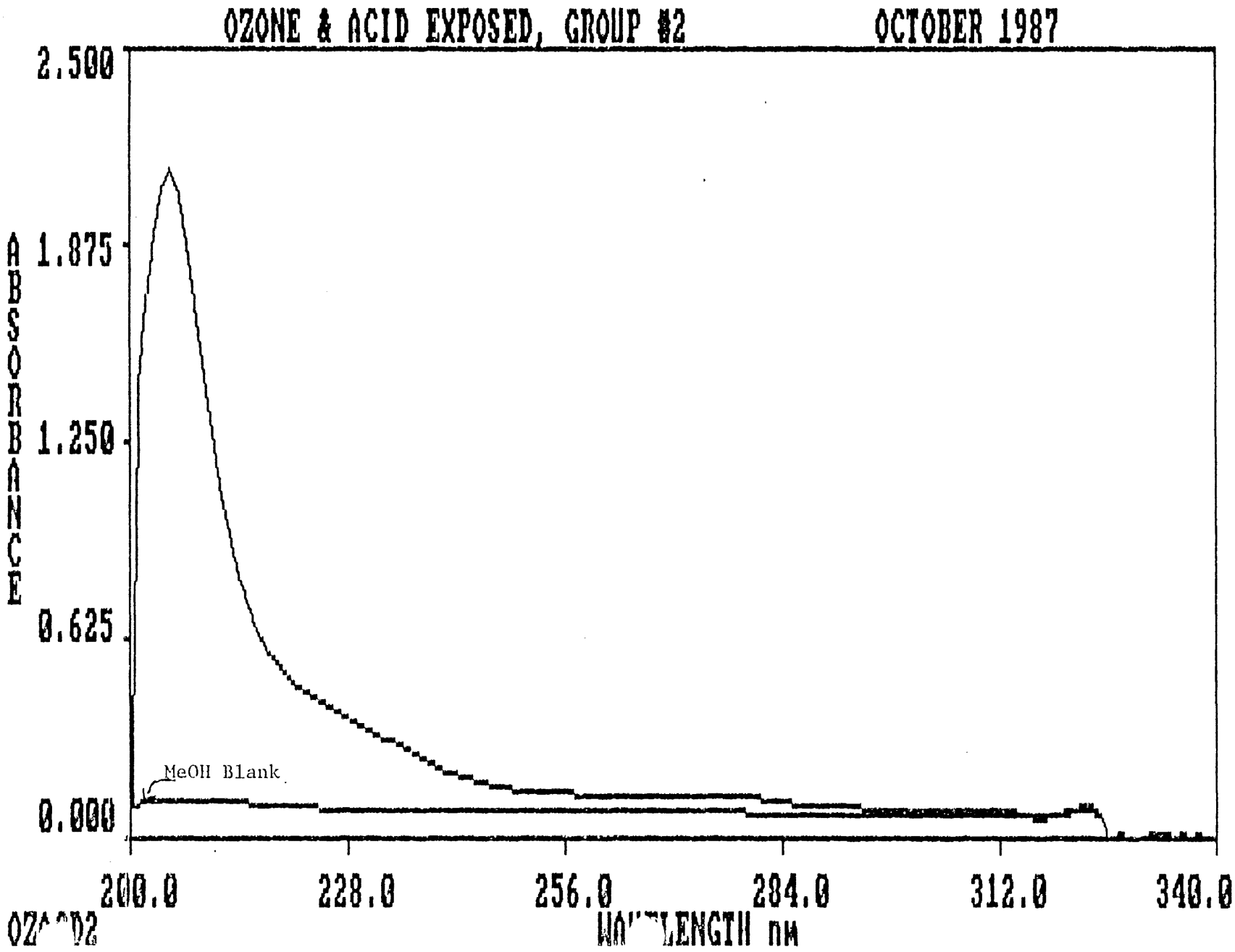
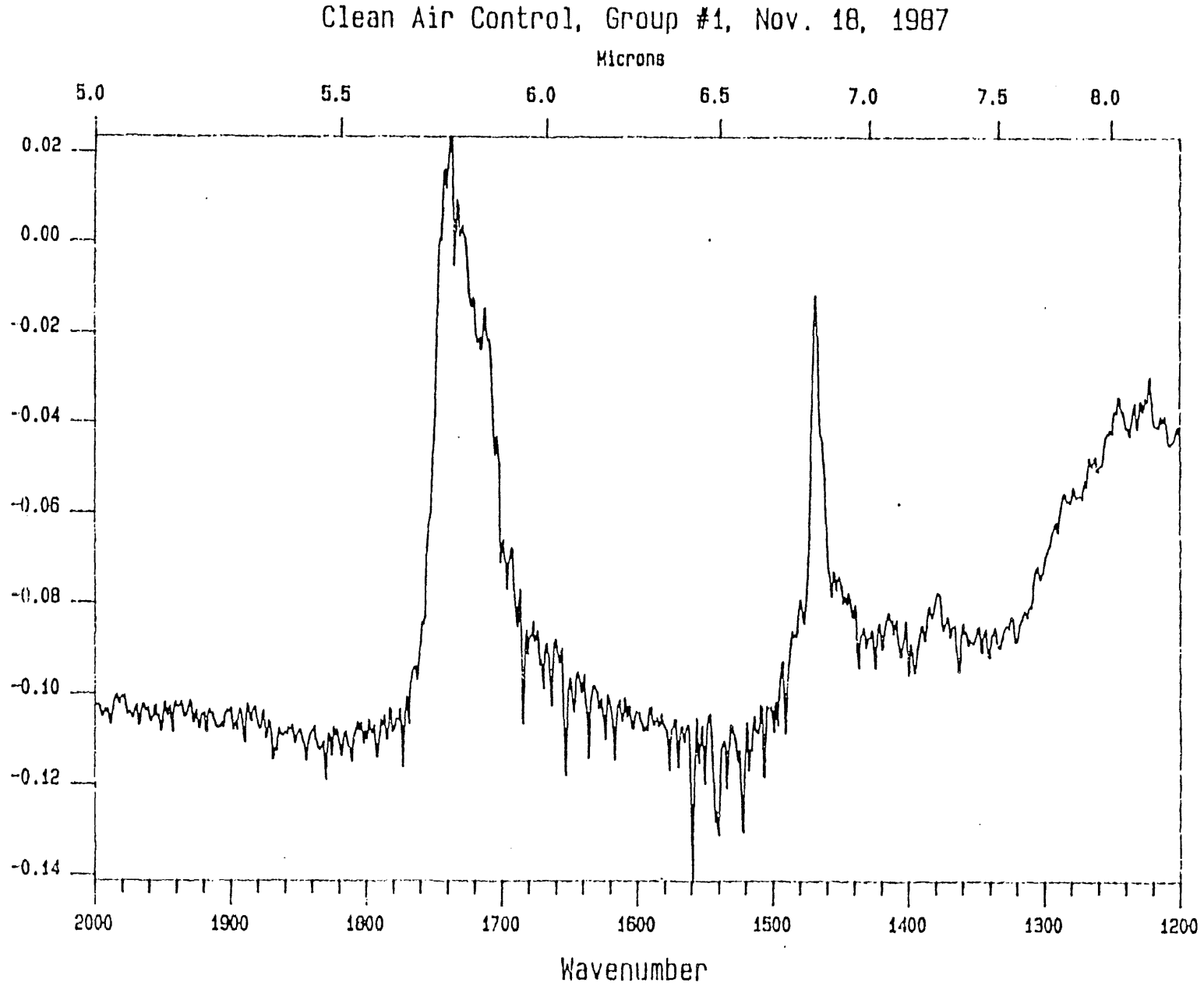
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
OZON1
WAVELENGTH nm

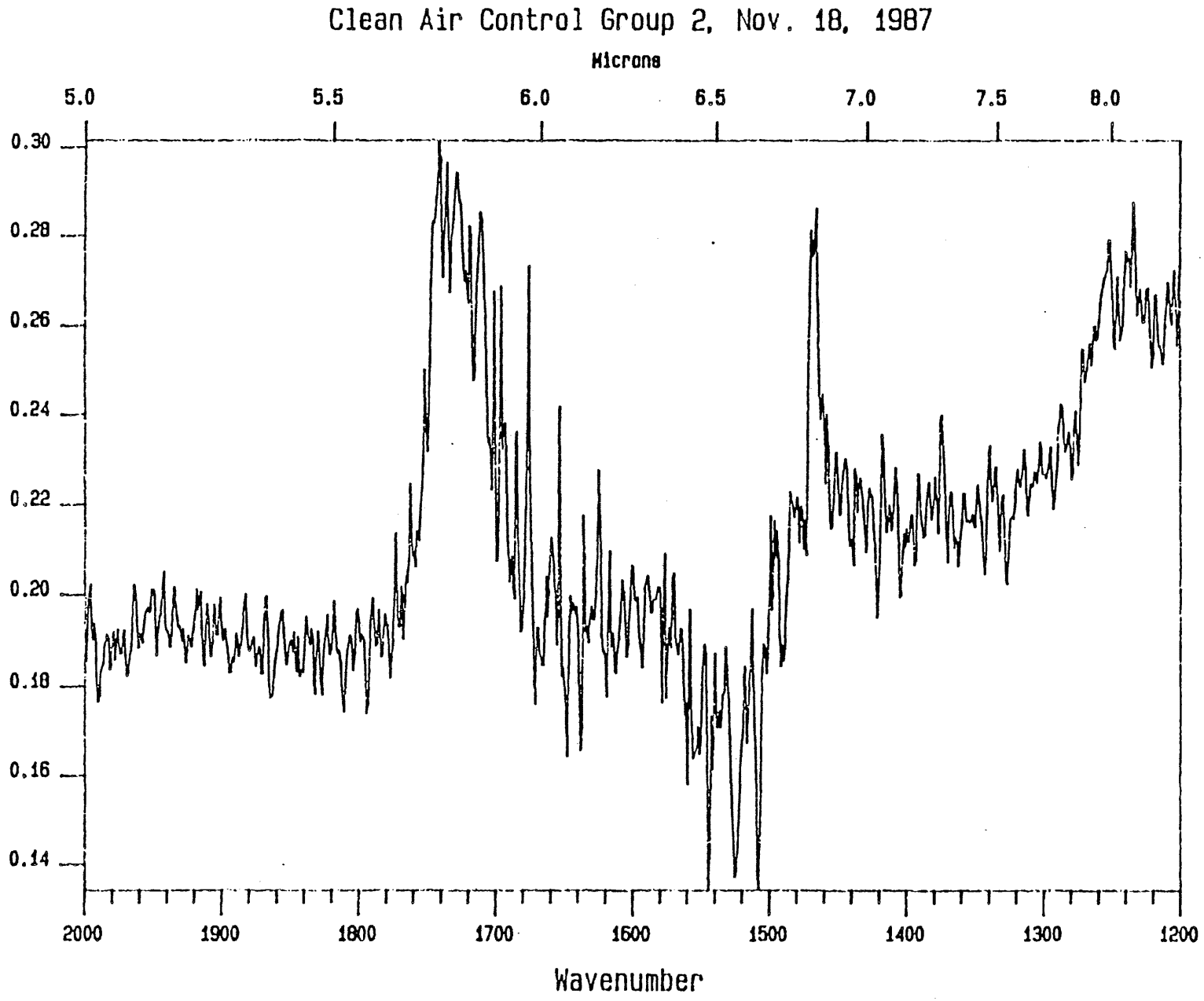

OZONE EXPOSED, GROUP #2
OCTOBER 1987
2.500
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
WAVELENGTH nm

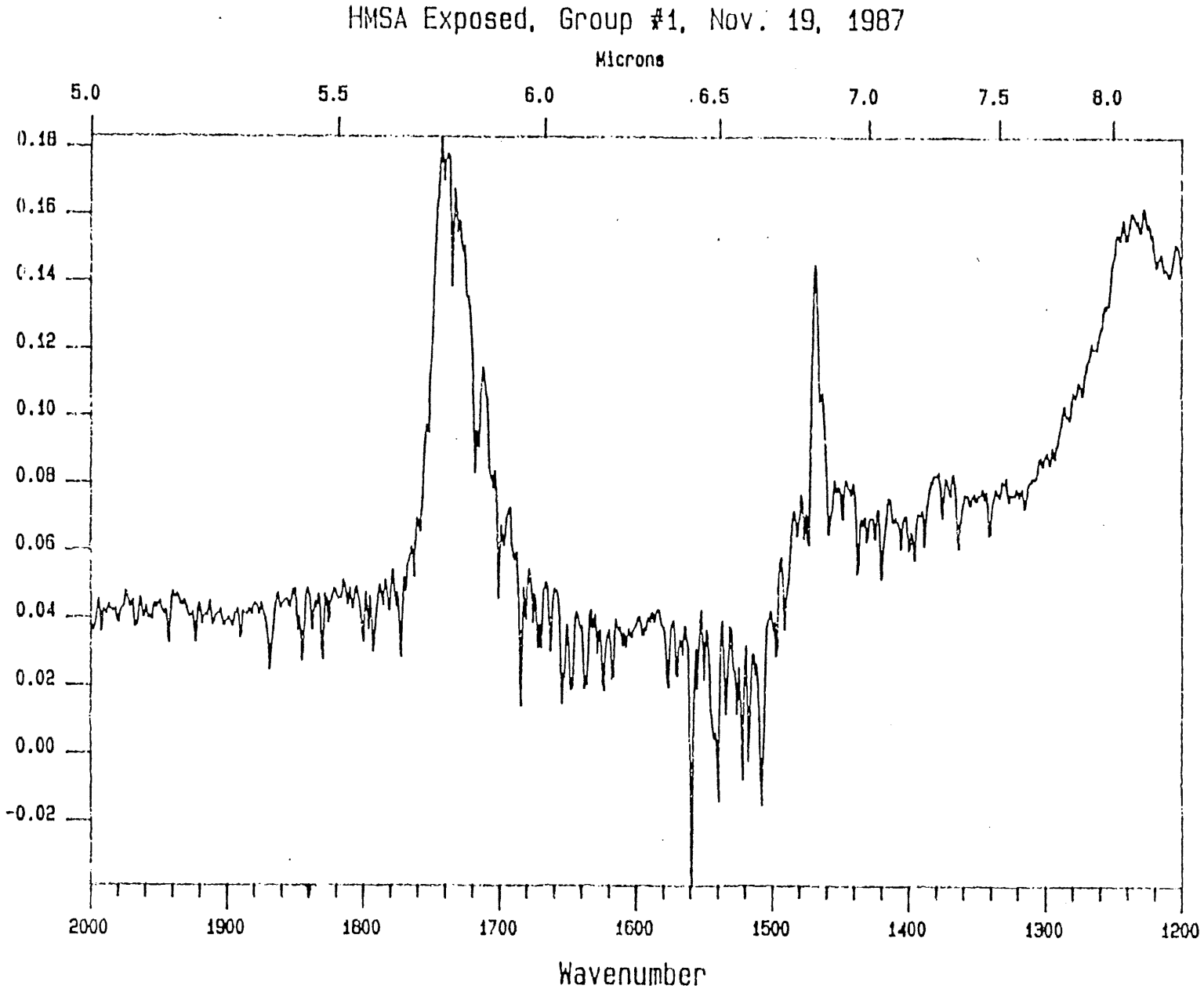

OZONE & ACID EXPOSED, GROUP #1
OCTOBER 1987
2.500
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
OZACD1
WAVELENGTH nm

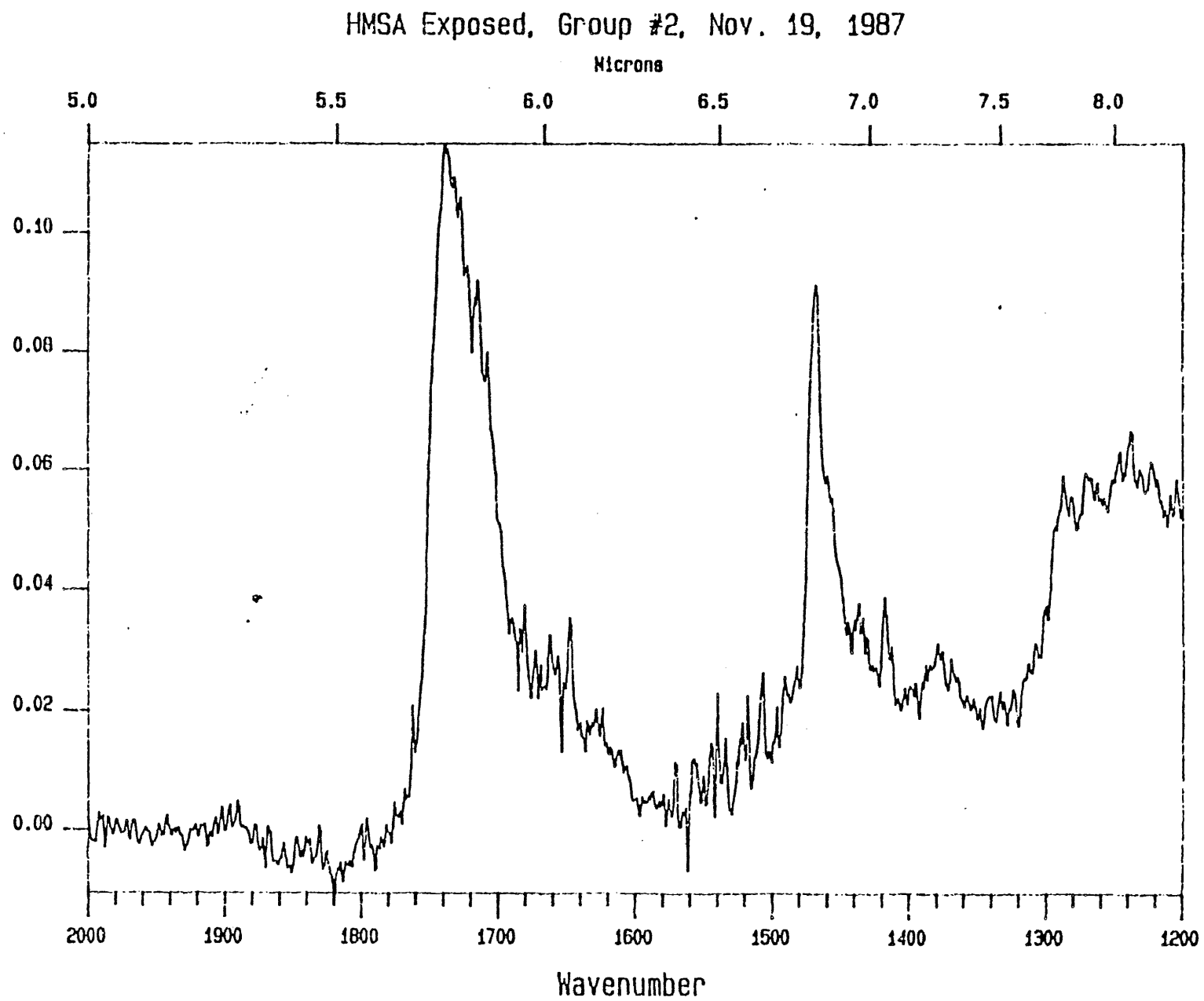

OZONE & ACID EXPOSED, GROUP #2
OCTOBER 1987
2.500
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
WAVELENGTH nm

XIV. APPENDIX III. FTIR SPECTRA (2000- 1300 cm^{-1}) OF PULMONARY SURFACTANT FROM RATS EXPOSED TO CLEAN AIR, HMSA (0.47-0.56 mg/m^3), 0.40 ppm O_3 OR TO 0.40 ppm O_3 COMBINED WITH 0.38 mg/m^3 HMSA, RH = 82-83%.


Clean Air Control, Group #1, Nov. 18, 1987
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


Clean Air Control Group 2, Nov. 18, 1987
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


HMSA Exposed, Group #1, Nov. 19, 1987
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


HMSA Exposed, Group #2, Nov. 19, 1987
Microns
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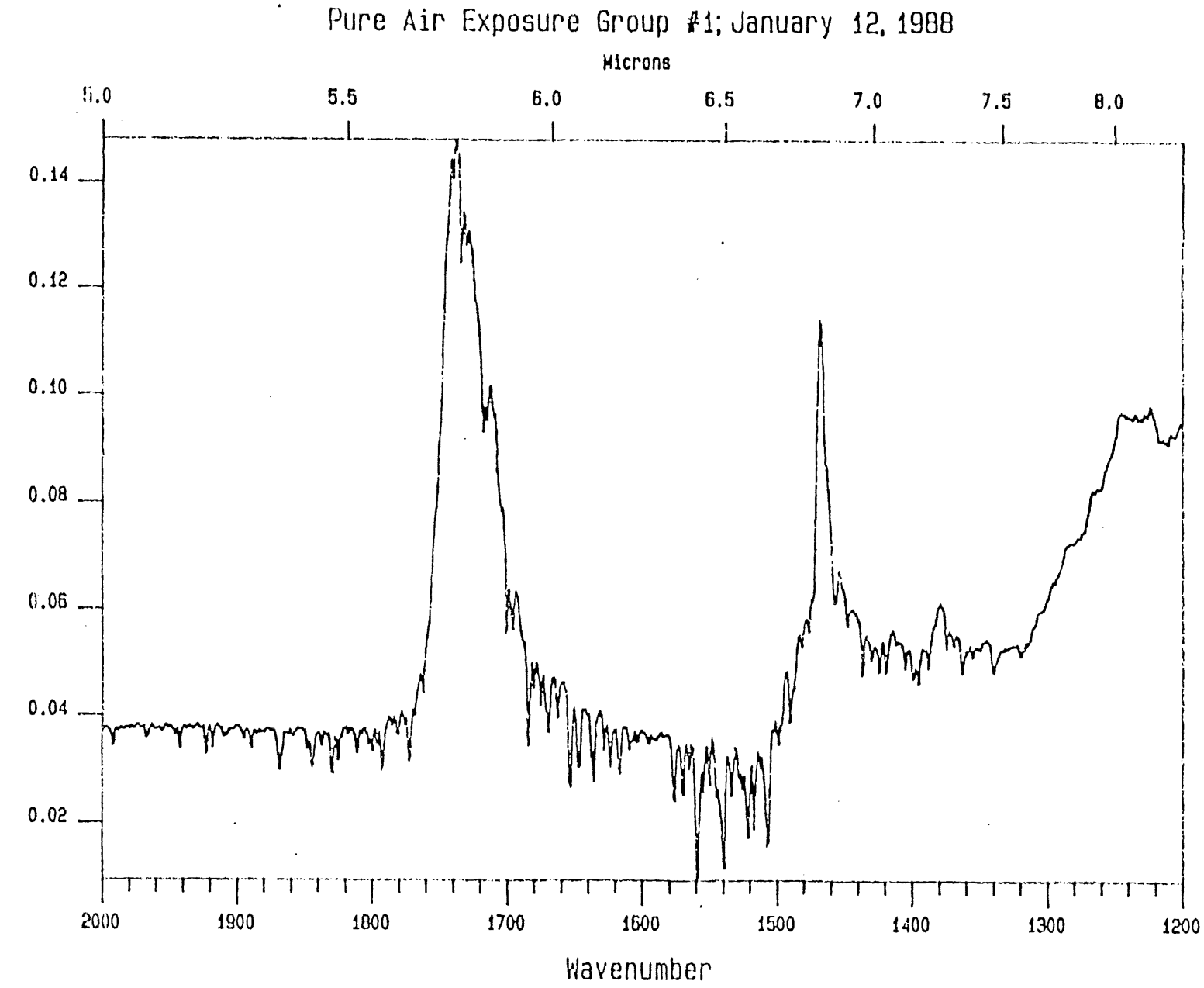
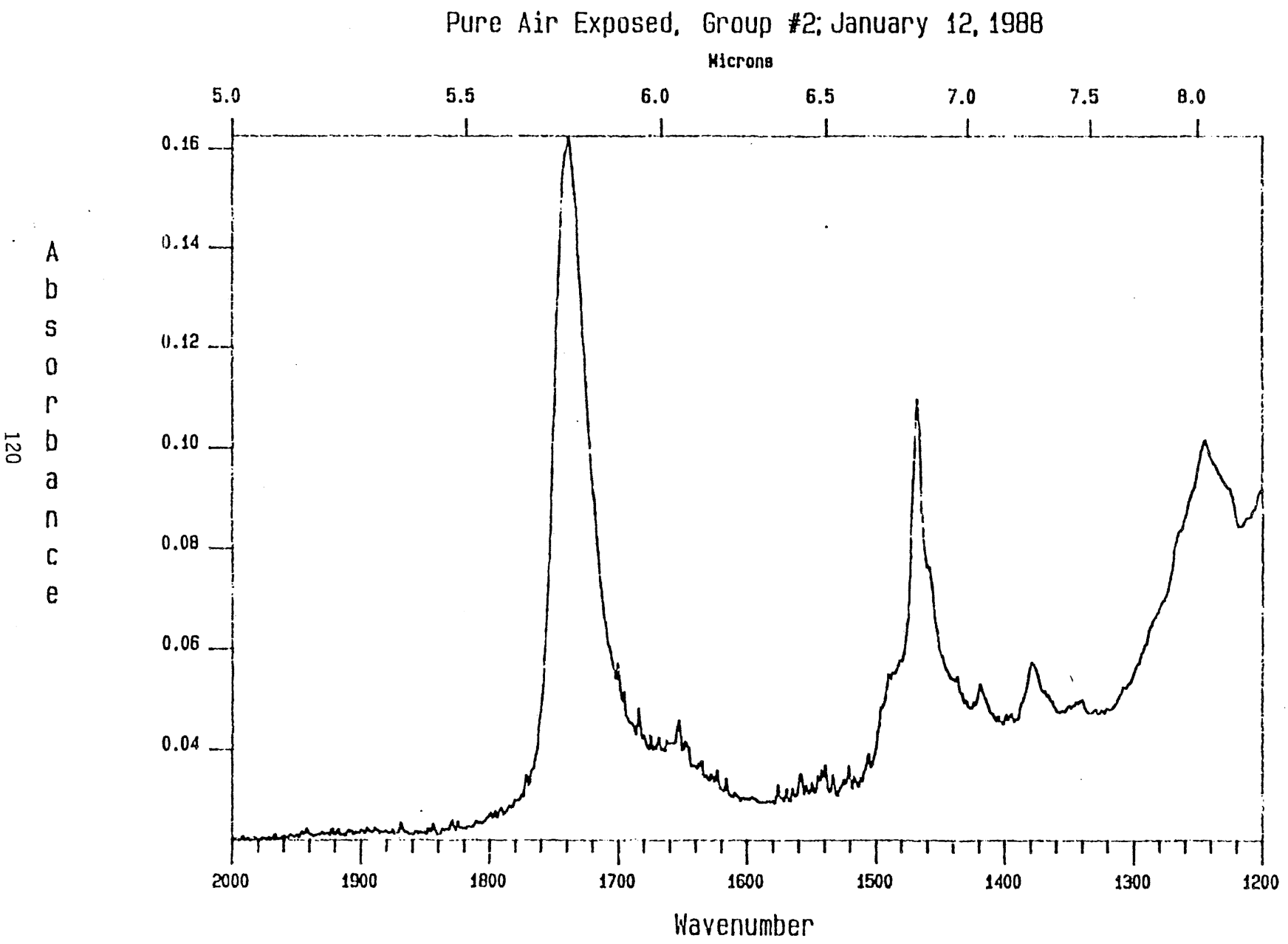
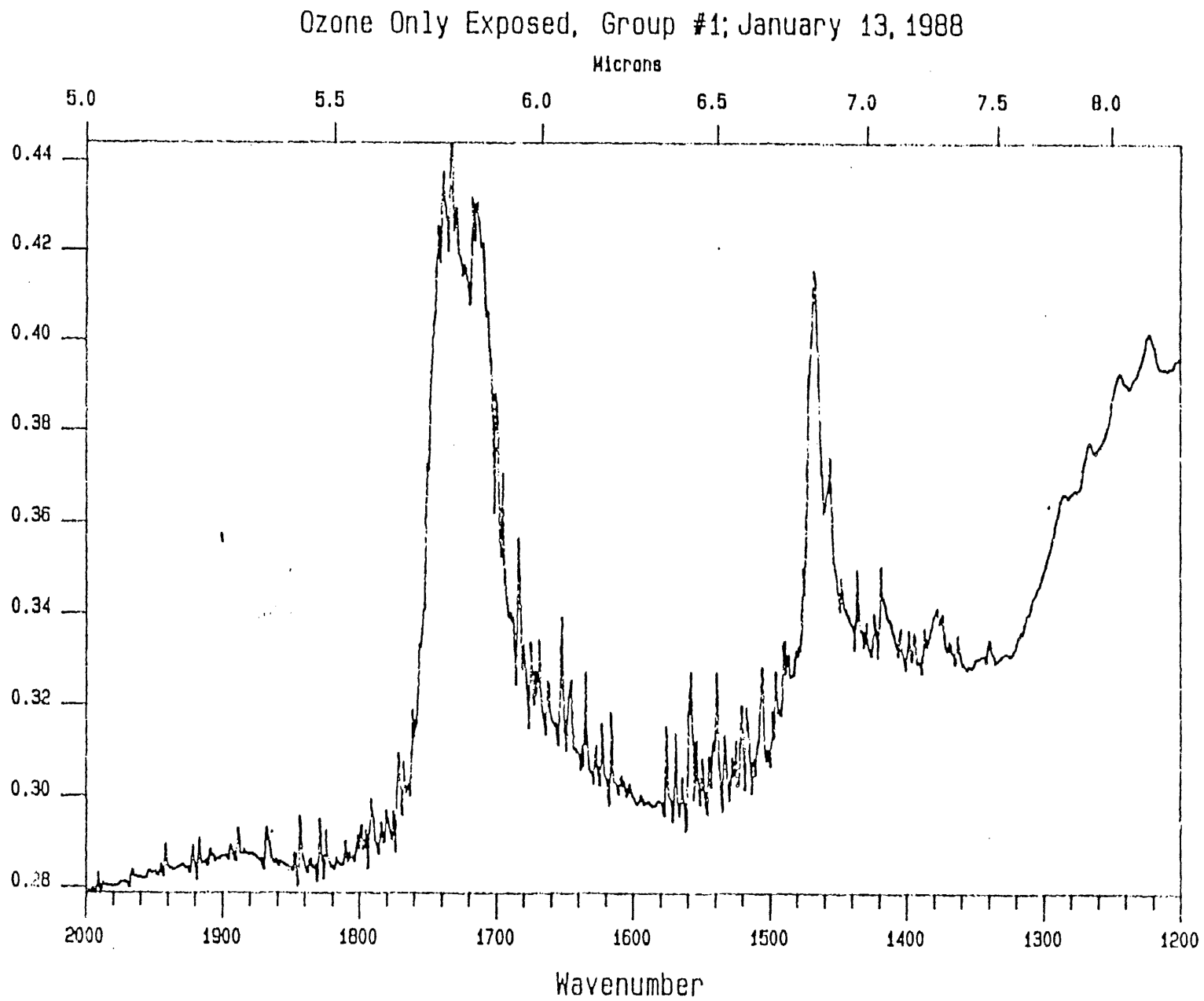
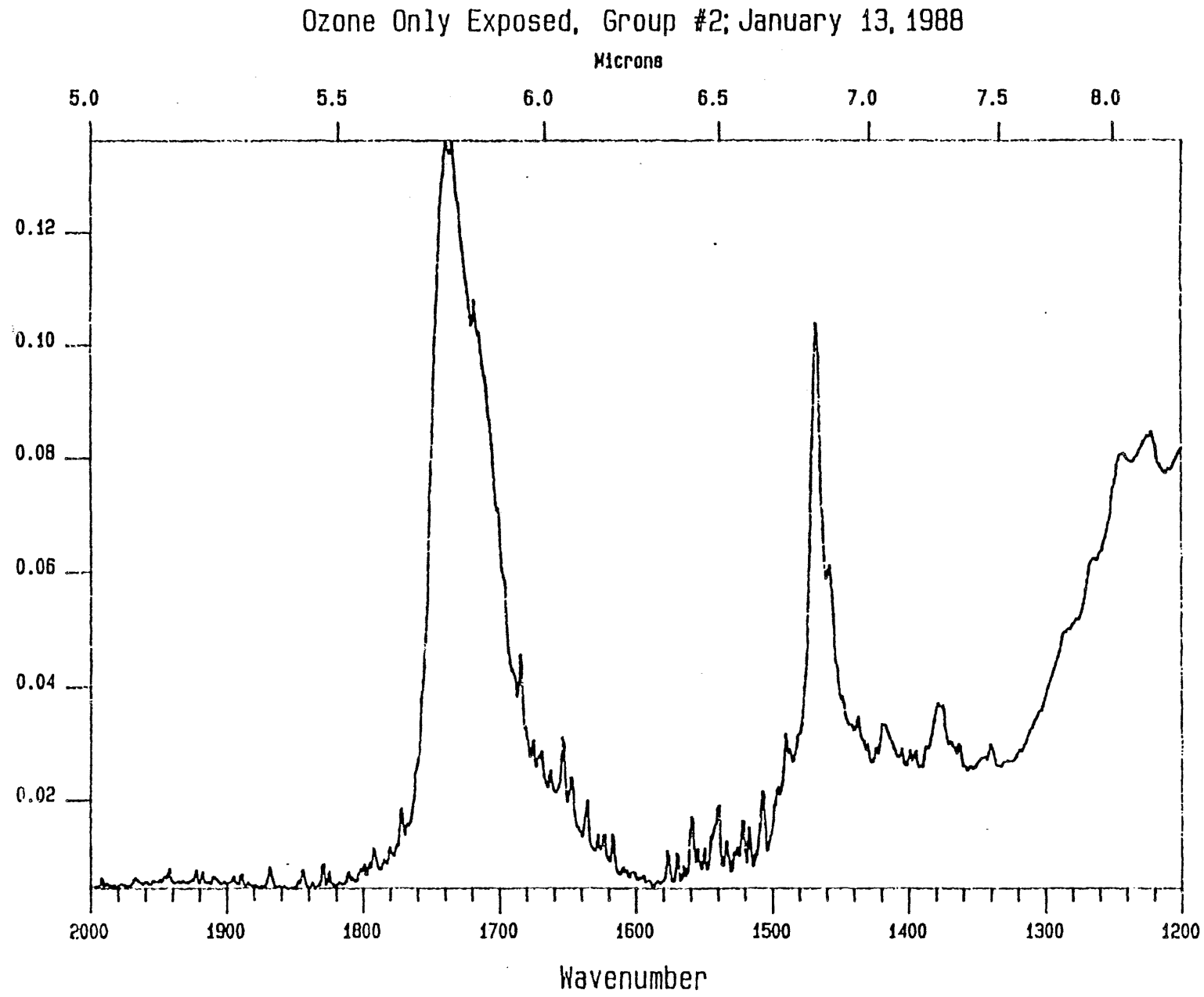
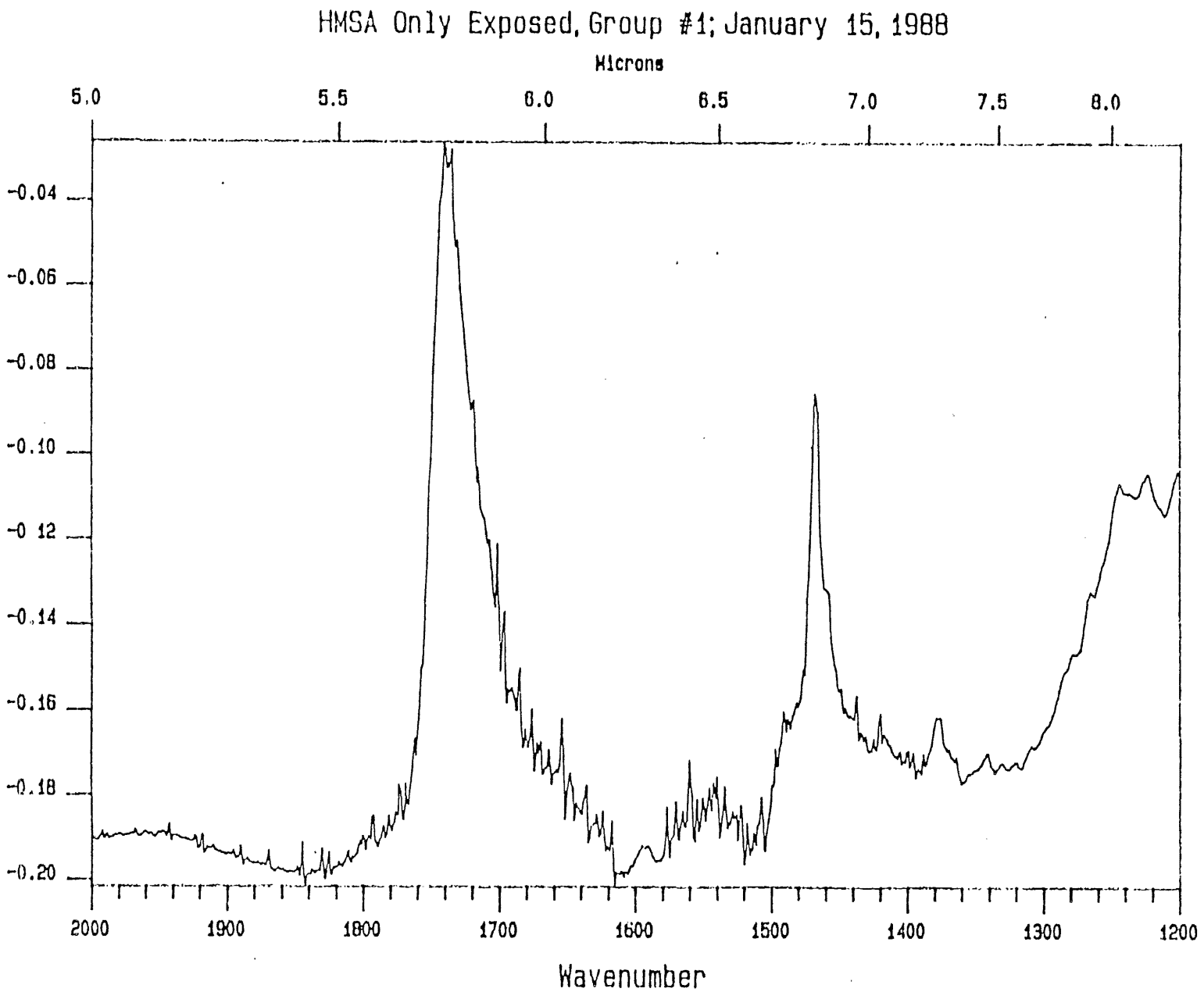
5.5
6.0
6.5
7.0
7.5
8.0
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

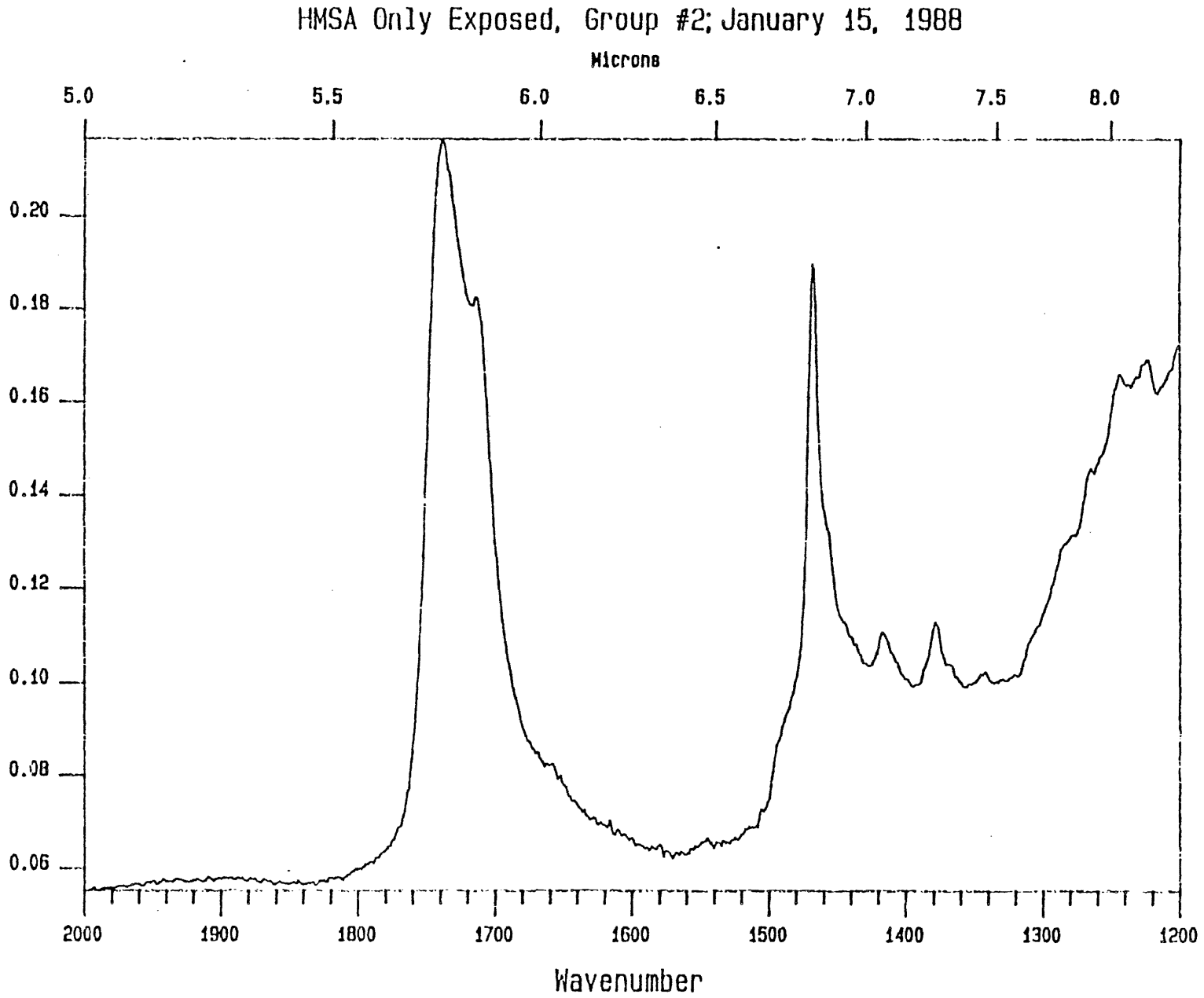

Pure Air Exposure Group #1; January 12, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.14
0.12
0.10
0.08
0.06
0.04
0.02
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

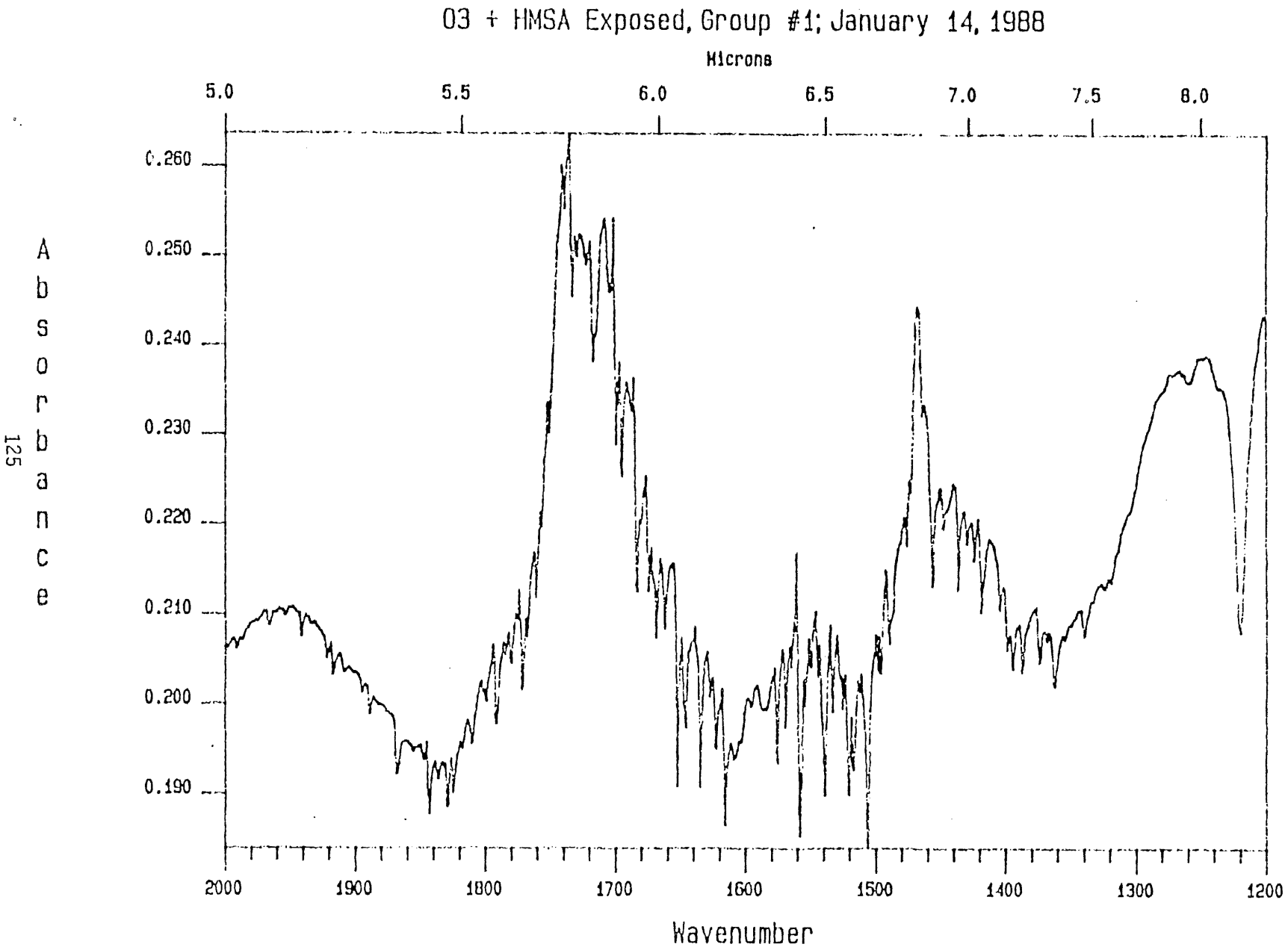

Pure Air Exposed, Group #2; January 12, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.16
0.14
0.12
0.10
0.08
0.06
0.04
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

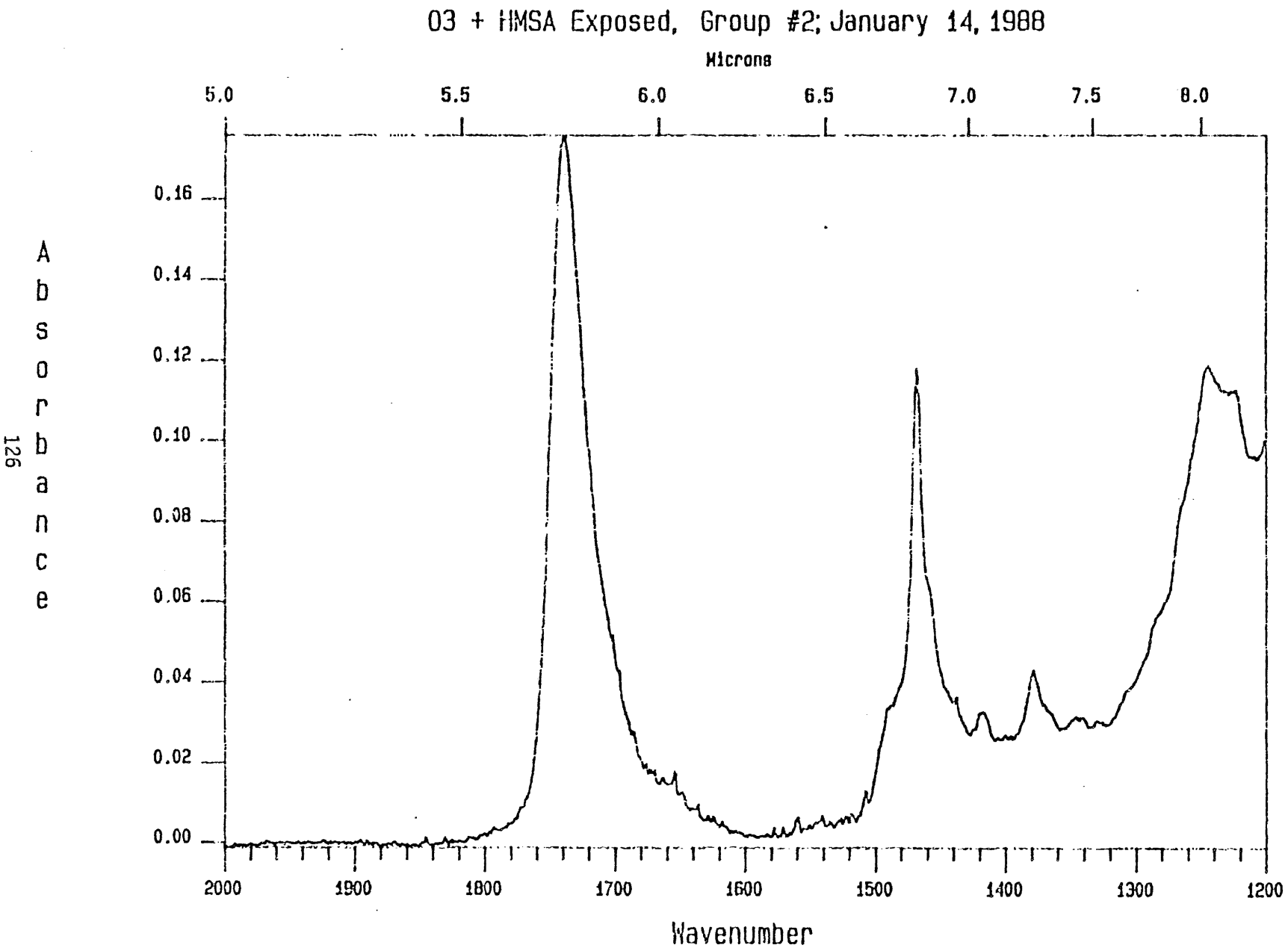

Ozone Only Exposed, Group #1; January 13, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.30
0.28
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber


Ozone Only Exposed, Group #2; January 13, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.12
0.10
0.08
0.06
0.04
0.02
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

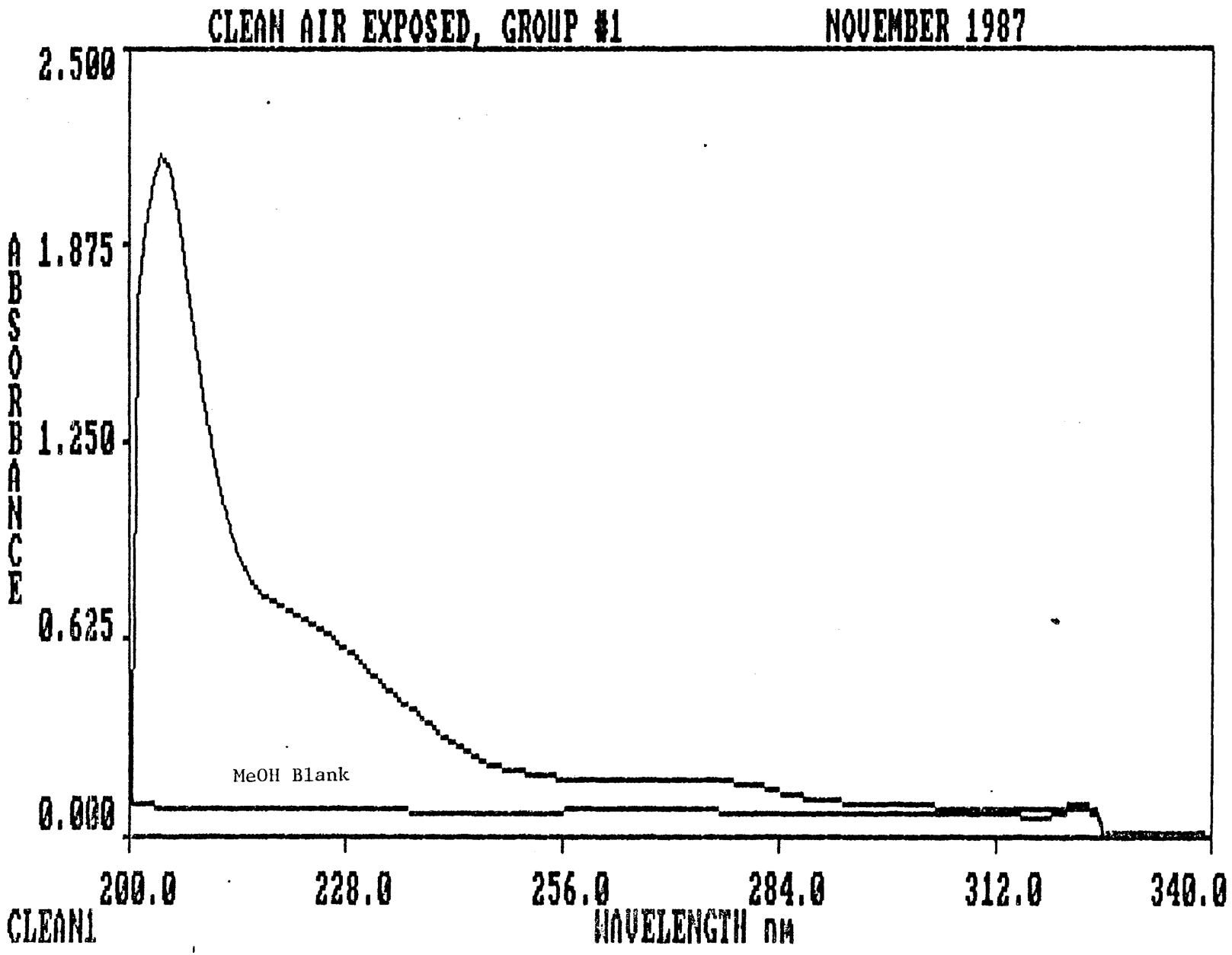

HMSA Only Exposed, Group #1; January 15, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
-0.04
-0.06
-0.08
-0.10
-0 12
-0.14
-0.16
-0.18
-0.20
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

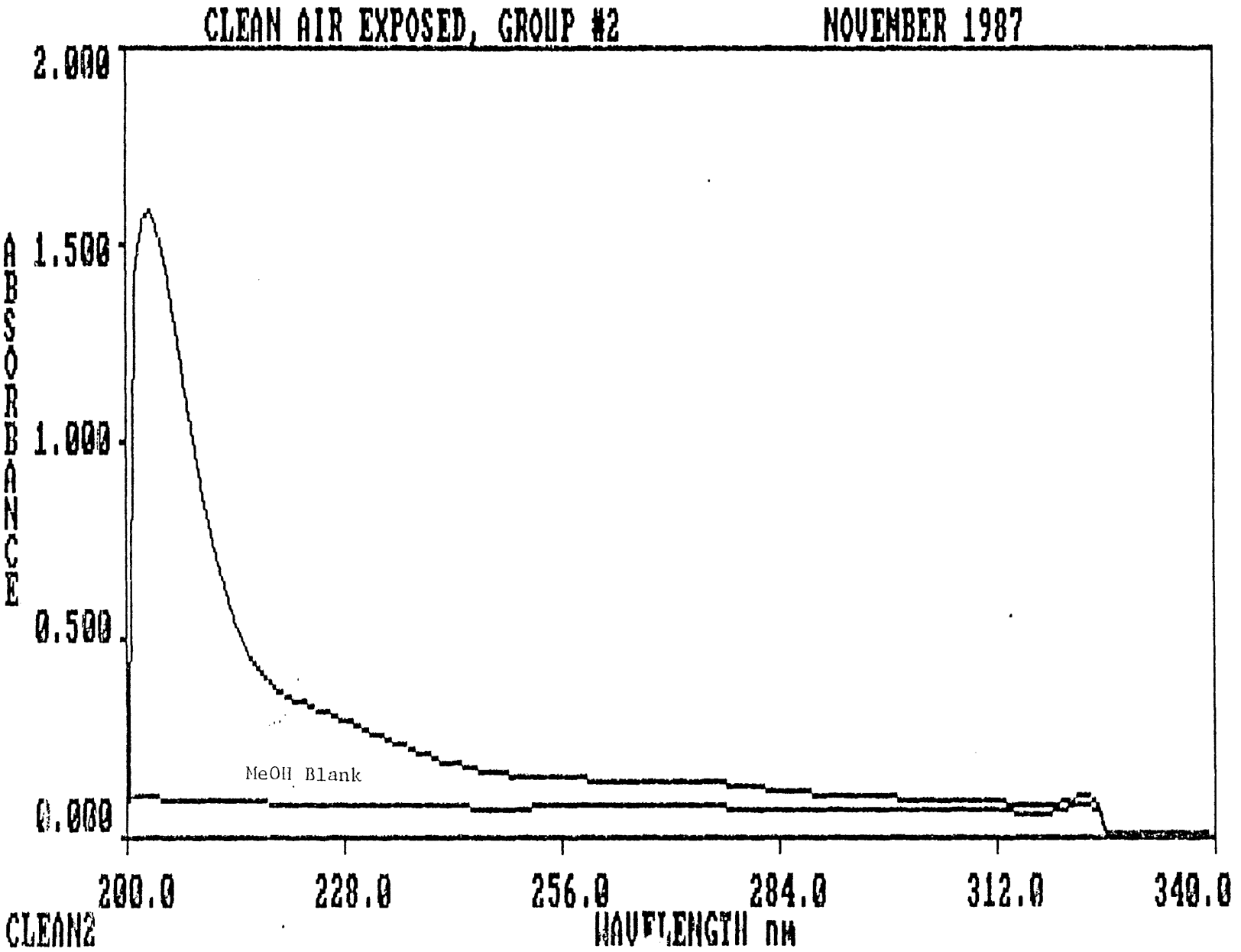

HMSA Only Exposed, Group #2; January 15, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

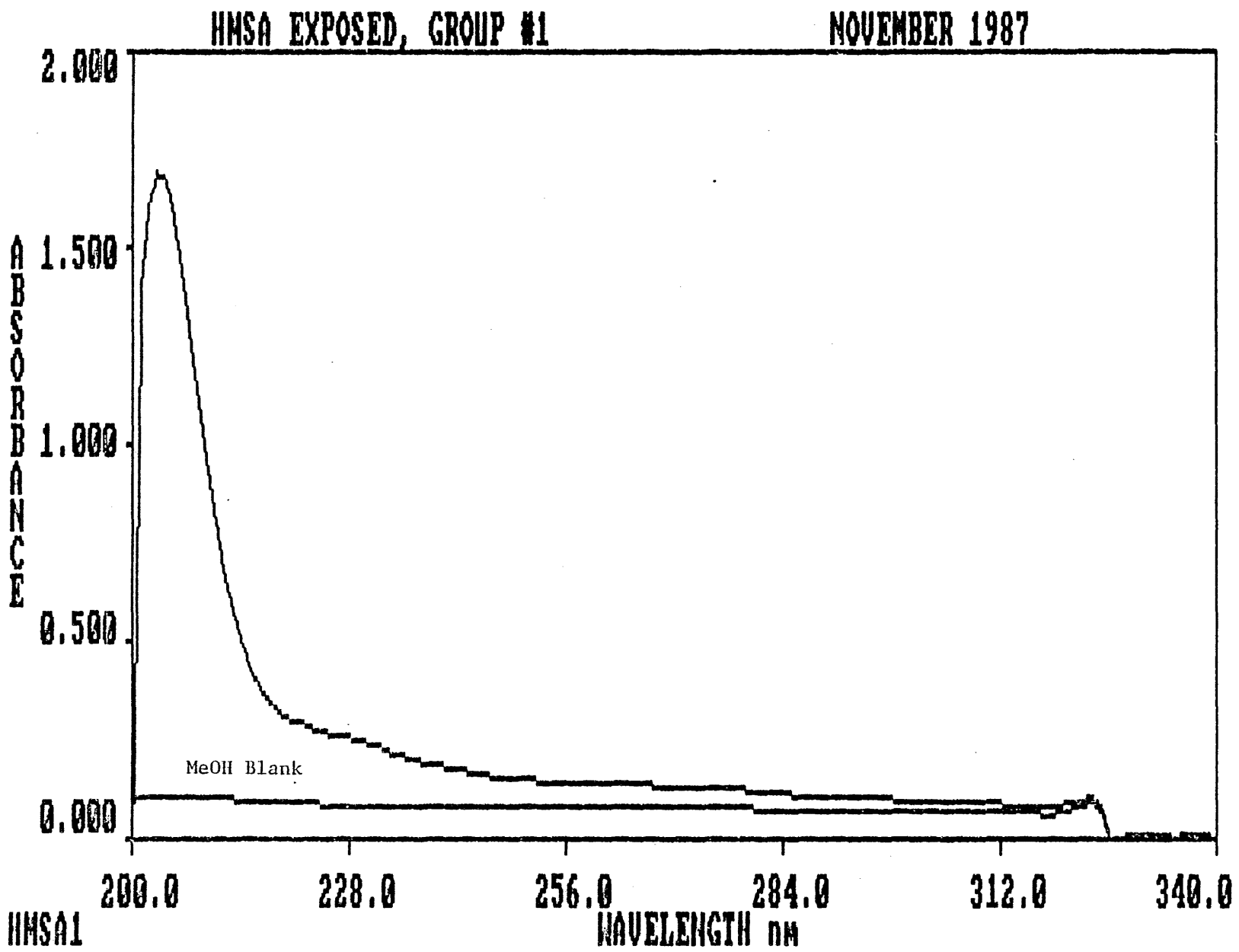

O3 + HMSA Exposed, Group #1; January 14, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.260
0.250
0.240
0.230
0.220
0.210
0.200
0.190
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber

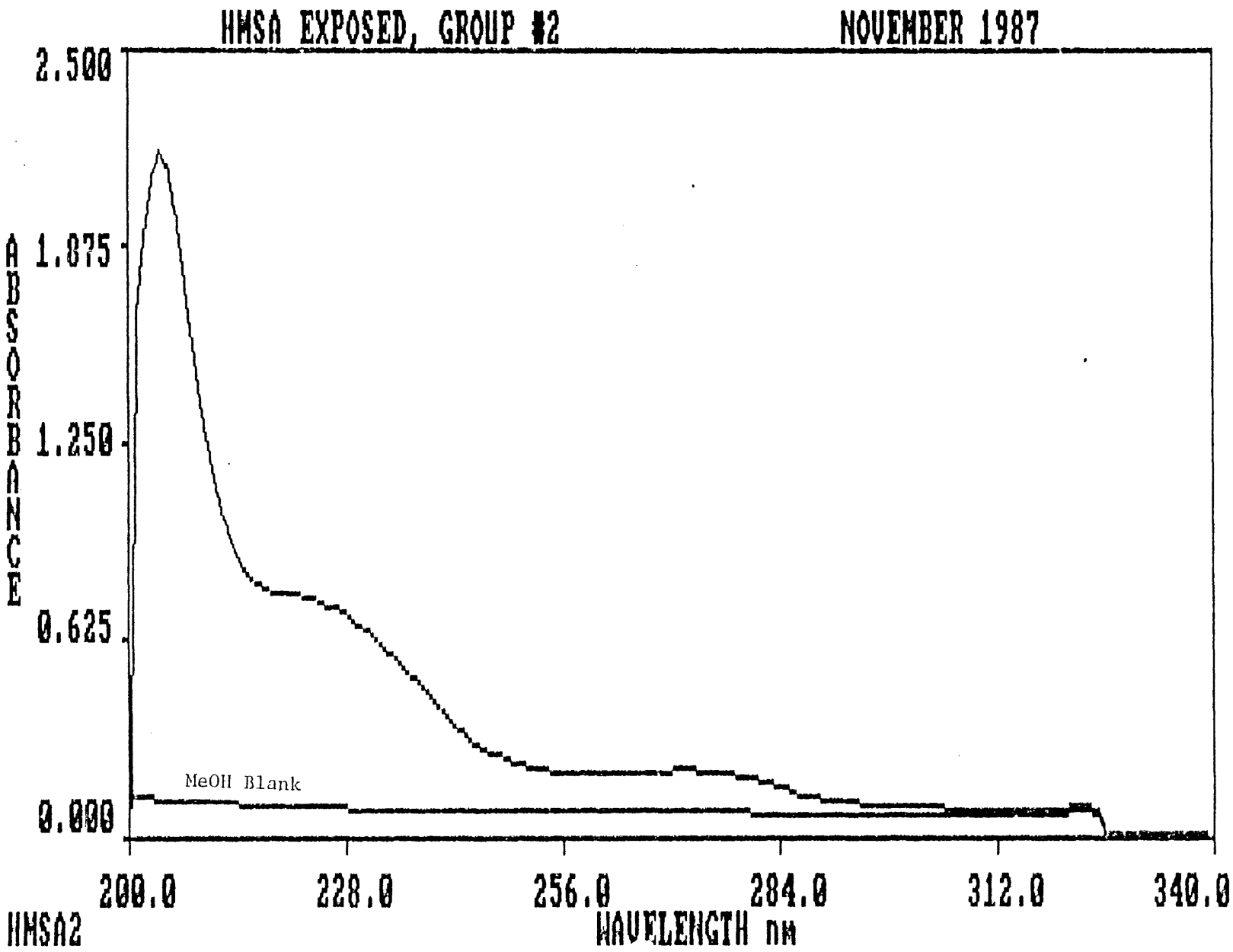

O3 + HMSA Exposed, Group #2; January 14, 1988
Microns
5.0
5.5
6.0
6.5
7.0
7.5
8.0
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance
2000
1900
1800
1700
1600
1500
1400
1300
1200
Wavenumber
o3hmsa2.abs

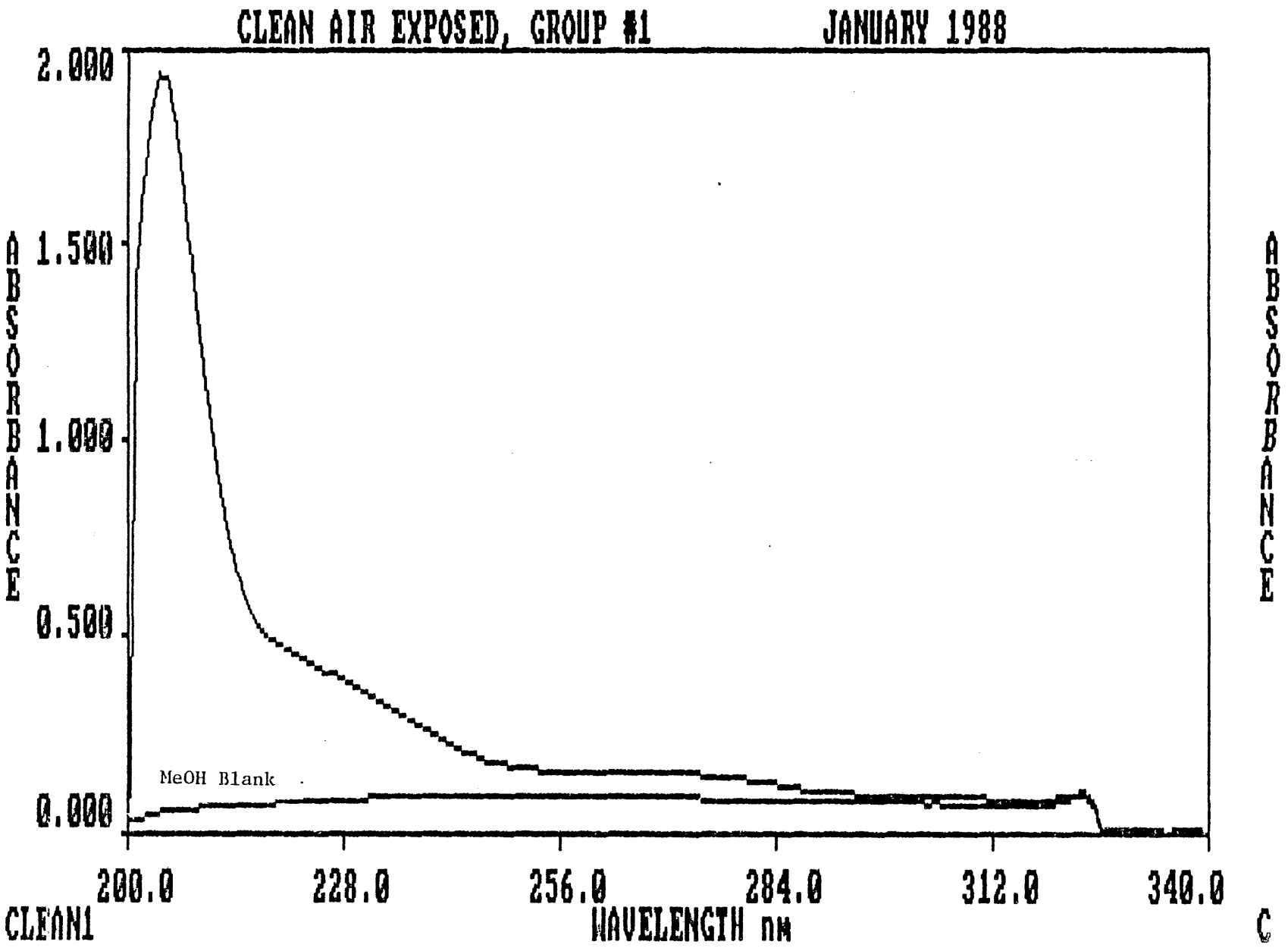
XV. APPENDIX IV. ULTRAVIOLET SPECTRA OF PULMONARY SURFACTANT FROM RATS EXPOSED TO CLEAN AIR, HMSA (0.47-0.56 mg/m^3), 0.40 ppm O_3 OR TO 0.40 ppm O_3 COMBINED WITH 0.38 mg/m^3 HMSA, RH = 82-83%.

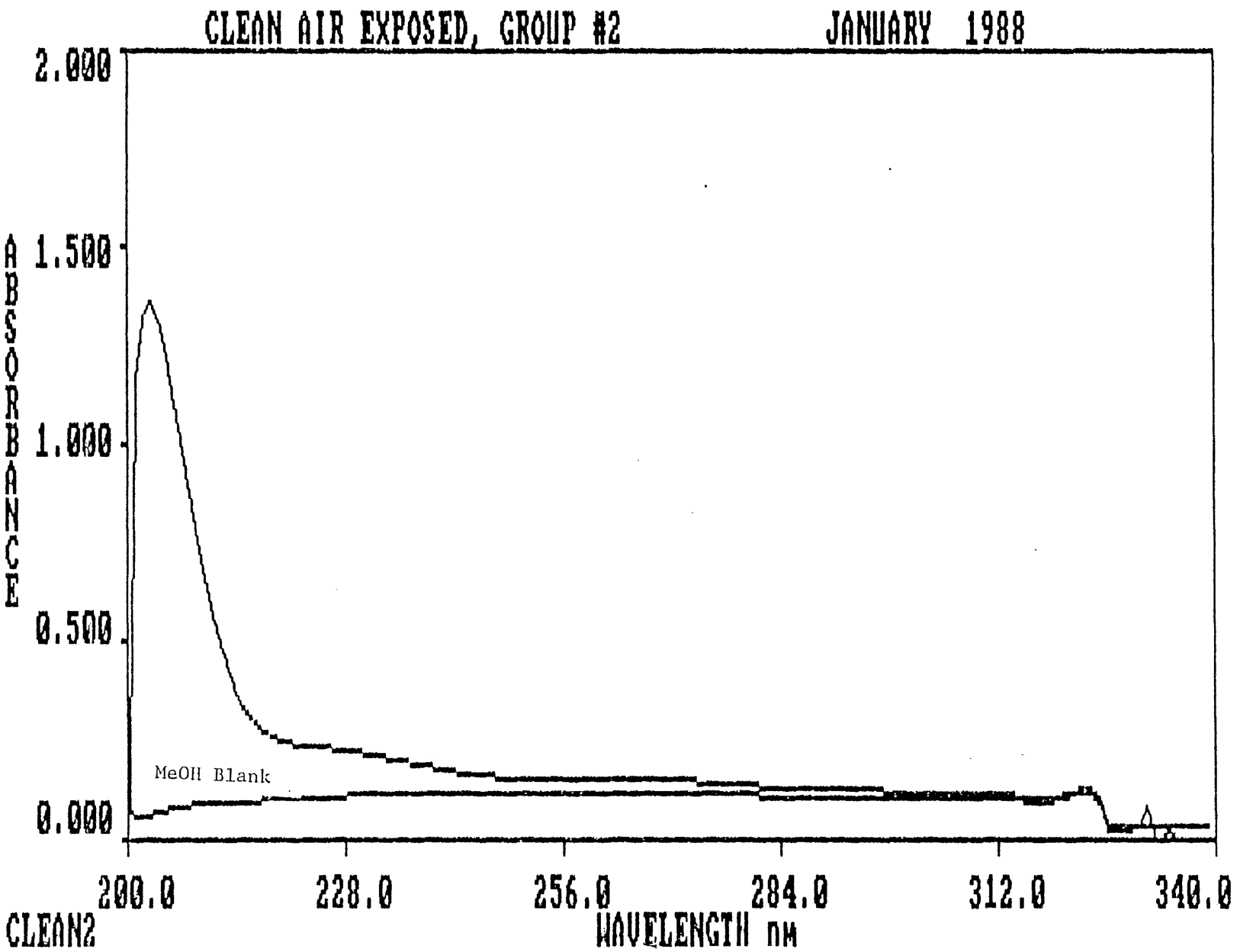

CLEAN AIR EXPOSED, GROUP #1
NOVEMBER 1987
2.500
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
CLEAN1
WAVELENGTH nm

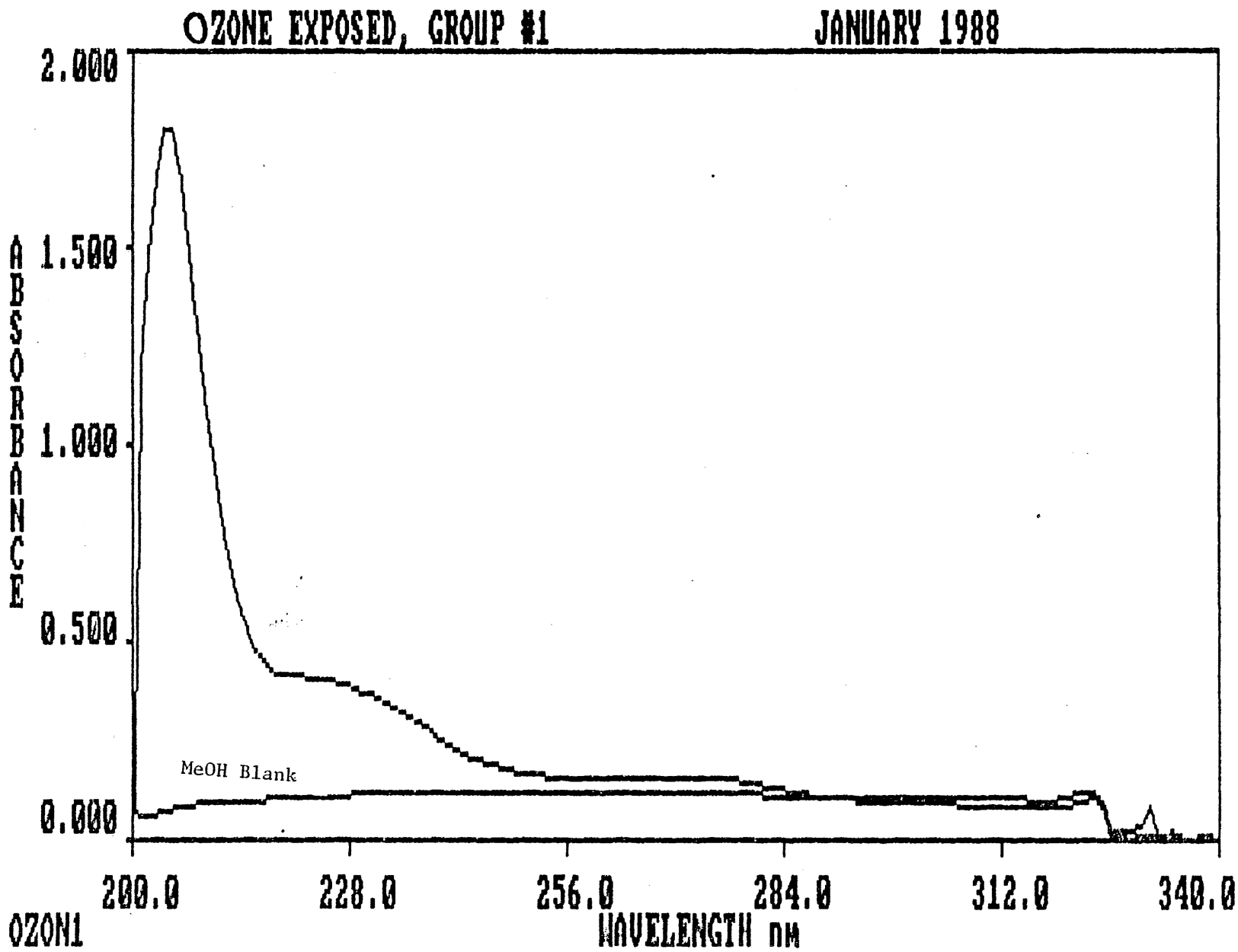

CLEAN AIR EXPOSED, GROUP #2
NOVEMBER 1987
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
CLEAN2
WAVELENGTH nm

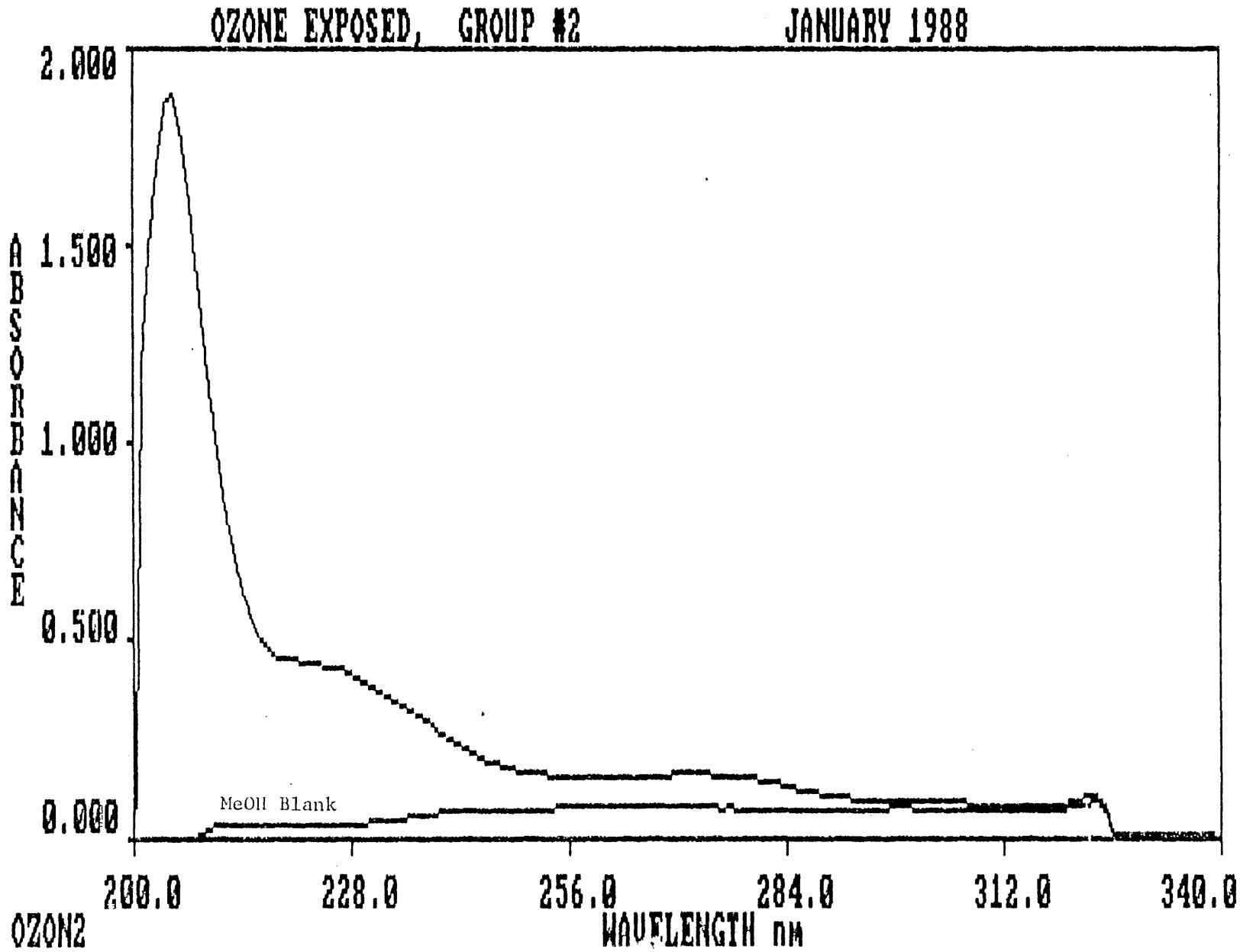

HMSA EXPOSED, GROUP #1
NOVEMBER 1987
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
HMSA1
WAVELENGTH nm

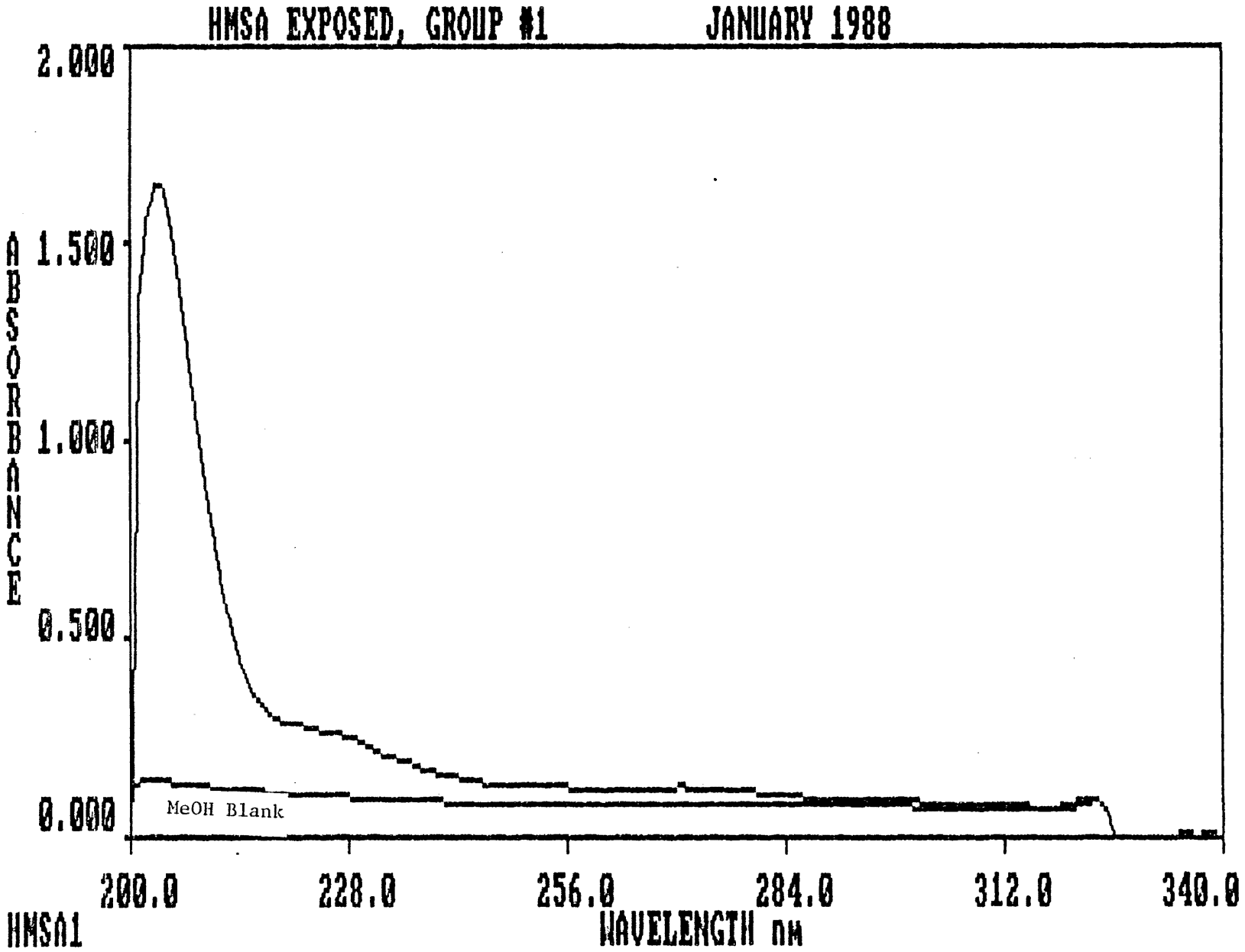

HMSA EXPOSED, GROUP #2
NOVEMBER 1987
2.500
1.875
1.250
0.625
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
HMSA2
WAVELENGTH nm

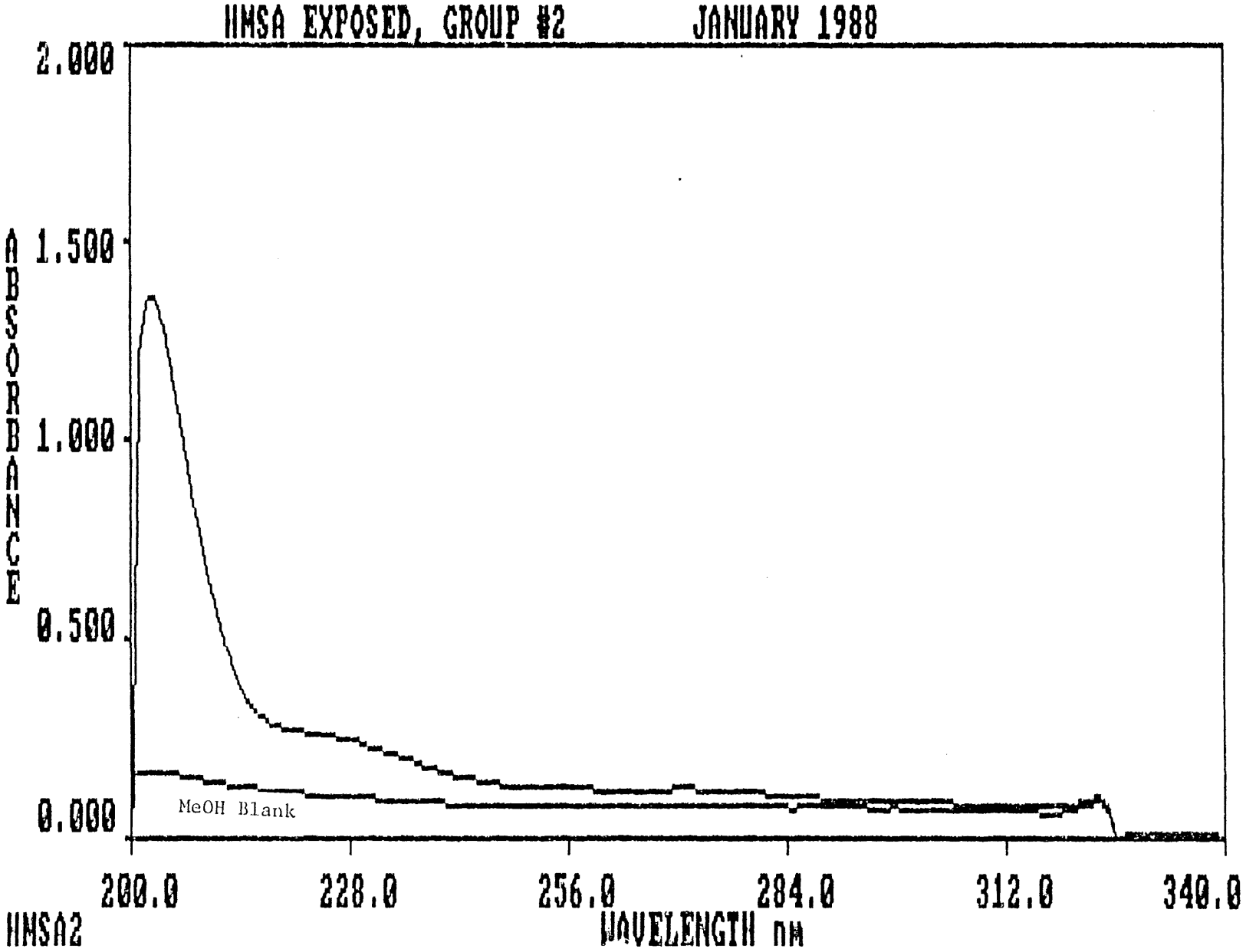

CLEAN AIR EXPOSED, GROUP #1
JANUARY 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
CLEAN1
WAVELENGTH nm
C

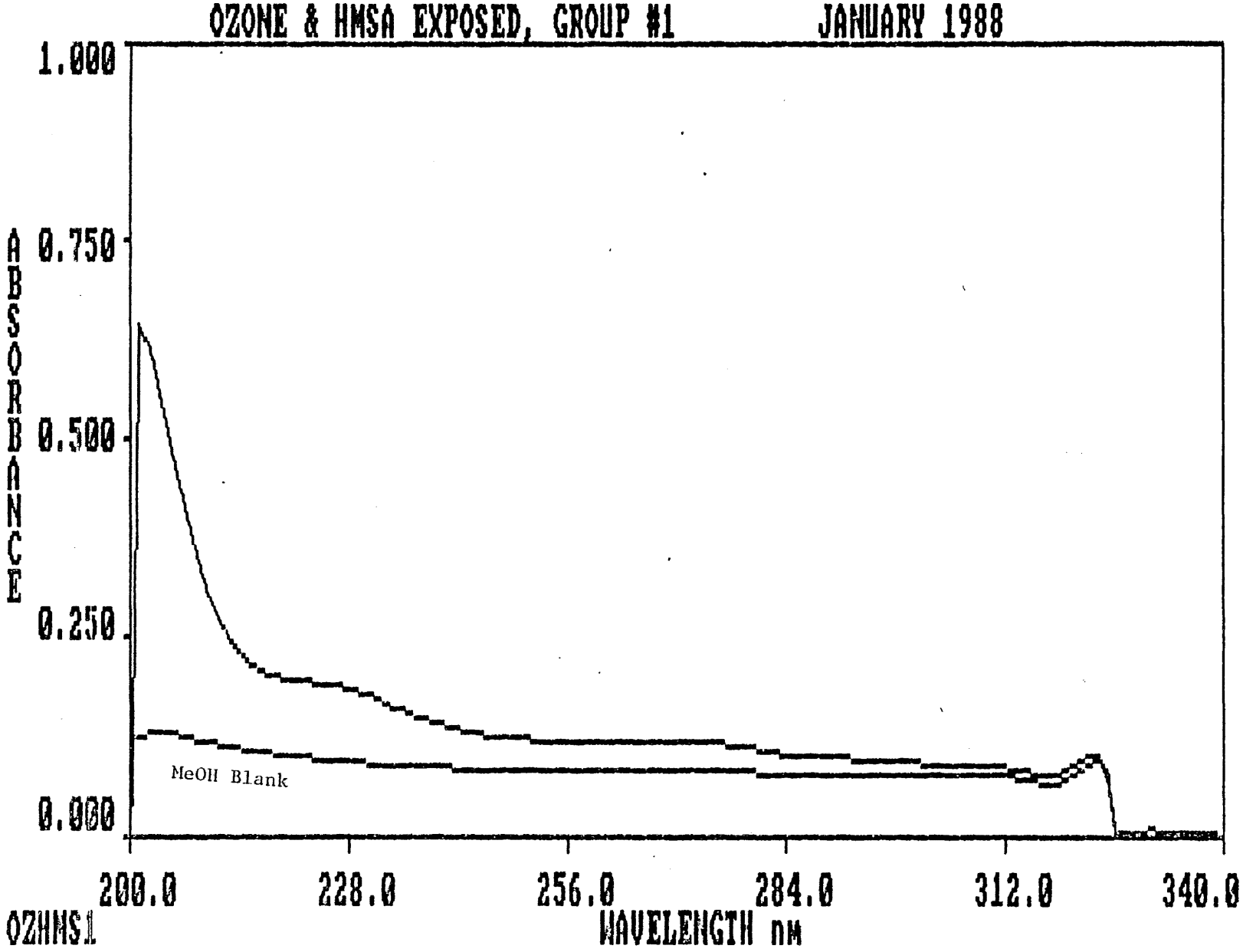

CLEAN AIR EXPOSED, GROUP #2
JANUARY 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
CLEAN2
WAVELENGTH nm

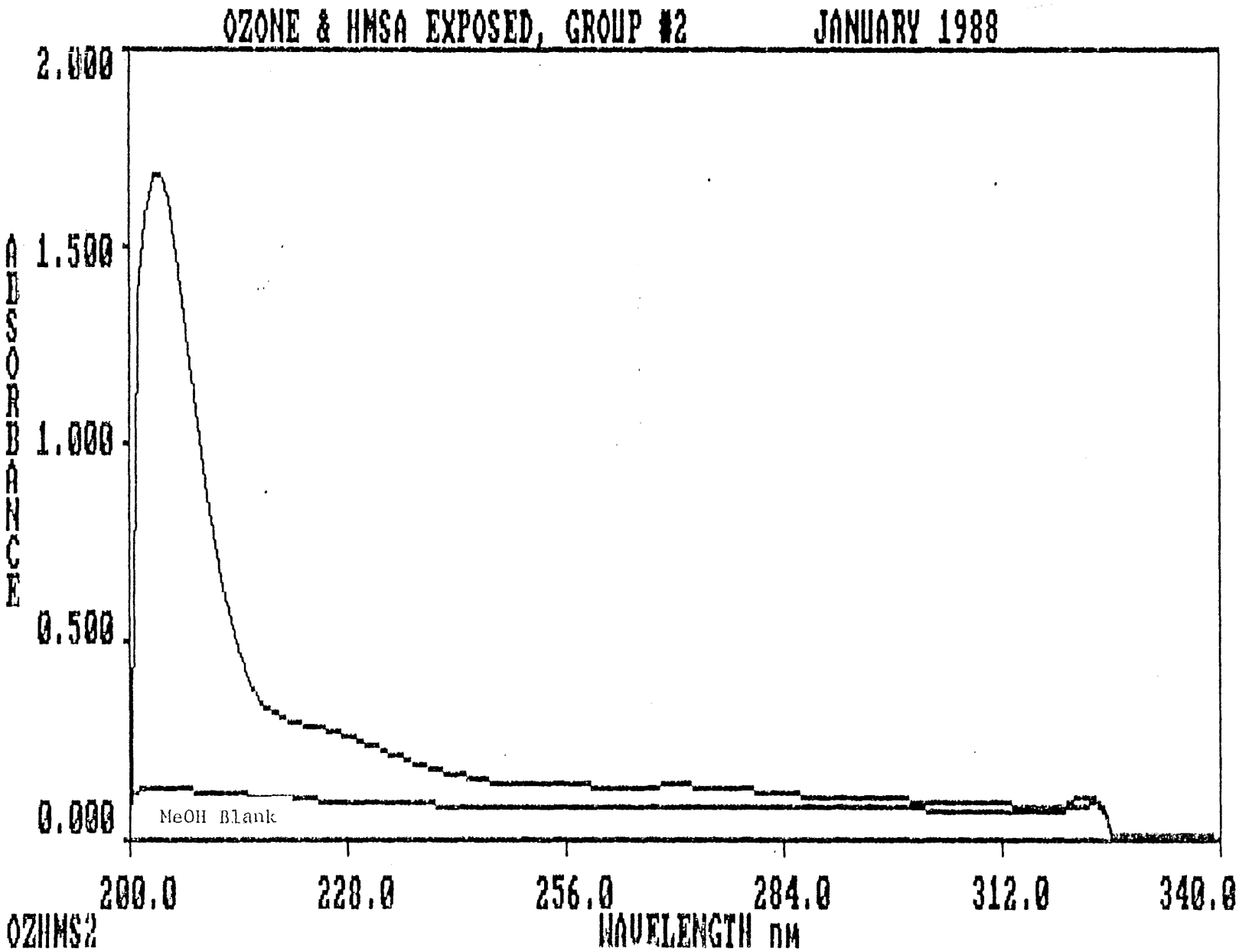

OZONE EXPOSED, GROUP #1
JANUARY 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
OZON1
WAVELENGTH nm


OZONE EXPOSED, GROUP #2
JANUARY 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
OZON2
WAVELENGTH nm


HMSA EXPOSED, GROUP #1
JANUARY 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
HMSA1
WAVELENGTH nm




OZONE & HMSA EXPOSED, GROUP #1 JANUARY 1988
1.000
0.750
0.500
0.250
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
OZHMS1
WAVELENGTH nm


OZONE & HMSA EXPOSED, GROUP #2 JANUARY 1988
2.000
1.500
1.000
0.500
0.000
ABSORBANCE
MeOH Blank
200.0
228.0
256.0
284.0
312.0
340.0
OZHMS2
WAVELENGTH nm