Contract No. A5-129-33

Research Contract Final Report

INHALATION TOXICOLOGY OF COMBINED ACID AND SOOT PARTICLES

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Submitted to

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by

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I. ABSTRACT

As a result of a request by the California Air Resources Board for proposals dealing with fine particles, an innovative inhalation toxicology study, using a reliable animal model, was designed and successfully conducted. The objective of the study was to evaluate the respiratory system toxicity of both diesel-engine-generated and propane-flame-generated fine carbon particles, especially when an acid mixture of the type observed in California's air was also present. Atmospheres were generated into stainless-steel exposure chambers and healthy rats breathed the pollutants nose-only for 5 hours per day for 5 consecutive days. The atmospheres studied included (a) dilute diesel exhaust soot + nitric acid + sulfuric acid, and (b) propane-flame-generated carbon soot + nitric acid + sulfuric acid. Groups of rats were also exposed to (a) the nitric and sulfuric acid mixture, (b) diesel soot, or (c) propane soot in order to examine the effects of the components of the soot + acids mixtures. Controls for exposure were rats exposed using the same schedule to purified air. When pollutants were present the concentrations of soot or of the combined acids were about 0.5 mg/m^3.

The evaluation of potential health effects in exposed rats included: (a) measurement of respiratory tract clearance of insoluble tracer particles; (b) histopathologic examination of respiratory tract tissues, including autoradiographic measures of cell turnover (an index of cell killing); (c) morphometric analysis of deep lung tissues; and (d) measurement of effects on pulmonary alveolar macrophages (immunological endpoints).

Biological effects were seen for each of the atmospheres studied. The propane soot + acids atmosphere produced a statistically significant ($p < 0.1$) delay in deep lung particle clearance; this effect was not seen with the diesel soot + acids atmosphere, however. Although none of the atmospheres produced lung cell killing, retained soot deposits were seen in the lung after exposure to atmospheres that contained soot. Diesel soot + acids, diesel soot alone and the acids alone all depressed pulmonary macrophage functions. Disturbances in deep-lung structure were associated with the inhalation of diesel soot alone and when it was combined with acids.

In summary, the agents studied were toxic in diverse ways. The effects of the combinations of soot plus acids appear to be attributable to the effects of the individual components. The interference with normal macrophage functions is likely to have the most significant implications to human health.

II. IMPORTANCE OF THE STUDY

This study provides important toxicologic information on previously unstudied combinations of California air pollutants. Assessments of lung injury cover structural and functional (including immunological) factors. Key features of this study include (a) sensitive and varied measures of biological effects, and (b) the application of a nose-only exposure system which prevents neutralization of airborne acids by animal-generated ammonia. The results are of relevance for several reasons: the mixture is chemically environmentally relevant from a human health point of view; the multiple repeated exposures are more representative of the exposure of human populations than either single acute exposures or continuous (24 hour/day) exposures; and the endpoints have been selected due to their link to health.

The epidemiological literature, and the past great air pollution disasters, indicate that mixtures of air pollutants can be injurious to people. Acute toxicology studies implicate certain individual pollutants, but have not addressed mixtures very well. Our study is among the few to bridge the gap by examining the toxicologic effects of complex mixtures of air pollutants in the laboratory.

III. SUMMARY AND CONCLUSIONS

Rats were exposed by inhalation for 5 hours per day for 5 successive days to atmospheres containing fine carbon particles and/or an acid mixture of the type observed in the South Coast Air Basin of southern California. The target concentrations of the multicomponent (soot + acid) atmospheres studied were (a) 0.5 mg/m^3 dilute diesel exhaust (diesel soot) + 0.35 mg/m^3 nitric acid + 0.15 mg/m^3 sulfuric acid, and (b) 0.5 mg/m^3 propane-flame-generated carbon (propane soot) + 0.35 mg/m^3 nitric acid + 0.15 mg/m^3 sulfuric acid. In addition, groups of rats were exposed to 0.5 mg/m^3 concentrations of (a) the acid mixture, (b) diesel soot, or (c) propane soot in order to examine the effects of the components of the multicomponent atmospheres. In each exposure a matched group of rats exposed to purified air served as controls. A total of 871 laboratory rats were involved in the study.

The biologic evaluations performed using the exposed animals were chosen in order to allow us to observe both structural (S) and functional (F) alterations in the upper respiratory tract (URT) and deep lung (DL) regions.

These evaluations included: (a) measurement of early (F, URT) and late (F,DL) respiratory tract clearance of insoluble tracer particles; (b) histopathologic examination of respiratory tract tissues (S, DL), including autoradiography of cell turnover (S, URT); morphometry of deep lung tissues (S, DL); and effects on pulmonary alveolar macrophages - the immunological endpoints (F, DL). In addition to these proposed endpoints, the accumulation of soot particles in the deep lung (F, DL) was also determined for rats exposed to soot-containing atmospheres. These evaluations provided major indicators of injury at critical sites in the respiratory tract that are commonly injured by air pollutants.

Positive effects were seen in at least one endpoint for each of the atmospheres studied. The particle clearance studies indicated that the propane soot + acids atmosphere produced a statistically significant ($p < 0.1$) delay in late (presumably deep lung) clearance; the diesel soot + acids atmosphere did not significantly affect particle clearance rates. None of the atmospheres produced significant cell killing (histopathology), but retained soot was present in the deep lung both immediately and seven days post-exposure when propane soot or diesel soot was inhaled. Diesel soot + acids, diesel soot alone and the acids alone were each found to produce effects - of varying degrees - on pulmonary macrophage functions. Morphometric analyses (by USC investigators) indicated that significant disturbances in deep-lung structure were associated strongly with exposures to diesel soot + acids and diesel soot alone.

From a regulatory issues viewpoint, this study was important for several reasons. First, a unique California-type mixture was studied. Previous studies by us and others had not addressed this mixture. Second, the exposure schedule used was realistic with respect to the exposure of human beings during a severe acid-smog episode. Third, the use of many endpoints proved to be more powerful in terms of assessing potential risks than single endpoints would have been. Finally, the use of the sensitive macrophage endpoints clearly pointed to the importance of including immunological measures in animal toxicology studies.

It is concluded that the studied atmospheres significantly affected the deep lung tissues rather than the upper airways. The major effects were seen in lung macrophage functions and in lung alveolar elastic tissue; both types of effects are likely to be indicators of the potential for the eventual development of lung disease. The most biologically active atmospheres were the combinations of soot and acids - with effects of the combinations apparently

attributable to the combined effects of the components. The dilute diesel exhaust, especially when combined with acids, was toxic, and we recommend that it be presumed to be capable of producing lung disease upon prolonged inhalation at elevated concentrations. The data from this study have already resulted in two peer-reviewed publications (see Appendicies), and a third scientific paper is now in preparation.

IV. RECOMMENDATIONS

There are several recommendations that we make as a result of this study:

1. Long-term (6 months or longer) inhalation studies at even lower concentrations are strongly recommended in order to provide toxicology information on the chronic effects of exposure to atmospheres containing acids and fine particles. The present study involving only 25 hours of exposure produced changes in respiratory system structure and various fuctions that could - with more prolonged exposures - lead to respiratory disease. Had the findings in this study been negative, such studies would not be indicated.

2. The extent to which human populations are exposed to airborne acids (including nitrogen-containing acids) needs to be determined and appropriate epidemiological studies (that include immunological assessments) should be conducted. The effects of (a) the activity (exercise) level of the exposed individuals, (b) age (lung development), and (c) the presence of co-pollutants in the air also need to be better defined. Eventually, after additional studies are completed, environmental air-contaminant criteria for airborne acids need to be considered.

3. In order to provide support for human risk estimates for the non-cancer-producing effects of fine particles, a dose-response study is needed. Such a study would provide detailed information on the exposure levels at which effects are observed and are not observed. We estimate that effects on macrophage functions would still be present at much lower doses than were studied in this project.

4. Our study alone does not challenge either the existing 50 microgram/m^3 24-hour averaging-time California air standard, or the 30 microgram/m^3 annual average PM_{10} standard. Additionally, our study does not support these existing standards because these low concentrations were not studied.

5. Investigators should be advised to use the nose-only exposure method and to continue the use of rats in air pollution studies. This animal has been observed to be sensitive to the effects of many pollutants, including mixtures of acids and fine carbon particles. The rat was sensitive to the atmospheres studied during this contract with respect to particle clearance, alveolar structure and macrophage function. Such effects are quite likely to lead to chronic respiratory health disturbances - especially following chronic exposures.

V. INTRODUCTION

A. Atmosphere Selection

Persons living in areas heavily impacted by vehicular and stationary source air emissions are exposed to a large variety of airborne particles and gases. Included in the particulate pollutants are various metals, minerals, water-insoluble inorganic compounds, water-soluble inorganic compounds including weak and strong acids, a great variety of organic compounds, elemental carbon, and water. This study focused on a particular combination - fine carbon particles plus acid - which has been associated with excess deaths and morbidity in past air pollution disasters (Goldsmith and Friberg, 1977), and which may represent a significant present and future airborne health hazard in California.

Elemental (graphitic) carbon in the fine particle size range (aerodynamic diameters below 10 micrometers) is emitted into the South Coast Air Basin at a rate of about 15 metric tons per day (Cass et al., 1982). The dominant source is internal combustion, with the diesel engine responsible for an estimated 60% of the total emission (Cass et al., 1982). Although elemental carbon represents only about 1/3 of the total particulate emission in that air basin, it is not only a substantial portion of the total fine particle inventory, but also is known to have significant retention times in the lung due to its resistance to normal particle clearance mechanisms (Nau et al., 1962; Wiester et al., 1980; Griffis et al., 1983; McClellan, 1987).

Acid mists and fogs in the South Coast Air Basin have been collected and found to have pH values as low as 2.2 (Brewer et al., 1983). In such samples ion analyses indicate that nitrate ion and sulfate ion are present in ratios that are consistent with the relative emission rates of nitrogen oxides and sulfur dioxide into the air (Waldman et al., 1982; Brewer et al., 1983). The

acid mist or acid fog droplets contain a variety of anionic and cationic species, but hydrogen, ammonium, nitrate and sulfate ions account for about 90% of the total ionic species on a chemical equivalency basis (Brewer et al., 1983).

In view of the preceding, inhalation toxicology studies are needed in which laboratory animals are exposed to combinations of carbon particles and sulfuric and nitric acids (H_2SO_4 and HNO_3, respectively). Such a combination has a counterpart in the South Coast Air Basin. Since diesel engine-generated carbon particles (diesel soot) may differ from other flame-generated graphitic carbon particles (such as propane soot) in their biological effects (especially when mixed with acid), both should be studied. Both types of carbon particles have been found in environmental air samples in the same size range (0.1-0.2 micrometers), but diesel particles have a higher percent of extractable organics than do the propane flame-generated carbon particles (Vostal, 1983).

B. Previous Studies

The forms of airborne carbon to which people are exposed are highly varied (natural graphite, coal dust and combustion-generated particles are examples). Graphite, in its natural form (which contains contaminant crystalline silica), is known to produce progressive and disabling pneumoconiosis, and there is evidence that pure (silica-free) graphite may have similar effects (Key et al., 1977). Similar deleterious effects on the lung have also been seen with coal dusts (Key et al., 1977). An important form of combustion-generated carbon is found in diesel exhaust, which has been used to represent ambient fine carbon particles in toxicological studies. Studies of its effects using laboratory animals show accumulation of particles in the deep lung, production of "nonspecific" cellular damage, and deterioration of lung defenses (Health Effects Panel of the Diesel Impacts Study Committee, 1981; McClellan, 1987). Toxicologic studies involving carbon particles also show changes in function and structure of various respiratory tract cells and biochemical changes in respiratory tract mucus. The cellular and mucus changes, except for those observed following long-term exposures or high concentration studies, appear to reverse over time. Repeated exposure, however, appears to lead to prolonged retention times for carbon in lung tissue over those seen after a single exposure (Griffis et al., 1983). In one set of long-term studies, diesel exhaust alone produced lung fibrosis and cancer in chronically exposed rats (McClellan, 1986). These effects occurred only at concentrations many times higher than that observed in ambient air, and the effect was greater with

higher particle concentrations. In the lungs of the groups of rats exposed to 3.5 milligrams/m^3 or above, clusters of macrophages filled with black pigment were observed in areas that did not yet show fibrotic or cancerous changes. In rats inhaling 0.35 milligrams/m^3, the carbon particle load did not continue to increase, even with extended times of exposure; hence, the rate of particle clearance matched the rate of particle deposition. Pigment storage in macrophages was dose-related; i.e., there was more pigment and more pathology in lungs of rats exposed to higher particle concentrations. Recently, McClellan (1987) estimated that the cancer risk to human populations (U.S. total population) was very low - less than 200 deaths/year. However, non-cancer risks are also possible, and our study was designed to predict the possibility of such risks.

Most of the available literature regarding the health effects of acid is concerned with sulfuric acid. There are suggestions that there are variations in responses of exposed subjects between species, within a given species (including humans), and across the biological endpoints used to measure effects. To some extent these variations may be due to the size of the particles used in exposures, the amount of ammonia neutralization occurring during exposure, and physical factors such as the temperature and relative humidity during exposure. The variability may also be attributable in part to host factors such as ammonia production rate, buffering capacity of mucus, and airway morphology.

Sulfuric acid droplets, when inhaled, have been associated with several effects. These include bronchoconstriction, alterations in ciliary beating, loss of cilia, sloughing of epithelial cells and alterations in clearance of particles from the lung (Amdur, 1958; Lippmann et al., 1987; Schlesinger et al., 1978; Schiff et al., 1979; Phalen et al., 1980; Wolff et al., 1981, 1986; Chen and Schlesinger, 1983). Very similar effects on particle clearance have been seen in rats, rabbits, dogs, donkeys and humans. At low concentrations of inhaled H_2SO_4 a stimulation of clearance is observed, but clearance is depressed at higher levels (Chen and Schlesinger, 1983). A single one hour exposure to 1 mg/m^3 of H_2SO_4 aerosol can cause effects on clearance that persist to 1 week post-exposure (Wolff et al., 1981). Exposures (up to 120 hours, cumulatively) of laboratory animals to H_2SO_4 aerosols at concentrations ranging from 0.3 to 5 mg/m^3, and sizes in the range of 0.3 to 1.0 micrometer mass median aerodynamic diameter (MMAD), have resulted in changes in respiratory tract morphology including concentration-dependent increases in the thickness of the tracheal mucus layer in guinea pigs (Wolff et al., 1986),

edema and cellular infiltration around bronchi and parenchymal blood vessels in guinea pigs (Bushtueva, 1957), and increased epithelial thickness and increased numbers of small airways and secretory cells in rabbits (Schlesinger et al., 1983). Exposures to H_2SO_4 for longer durations (cumulative exposure times between 120 and 700 hours) resulted in hypertrophy (excessive growth) of epithelial cells, mainly in the alveolar duct region in rats (Juhos et al., 1978), and increased density of secretory cells throughout the bronchial tree accompanied by decreased lumen sizes of small airways in rabbits (Gearhart and Schlesinger, 1988).

Despite the fact that nitric acid is widely used in industry, the toxicology data base is extremely limited. Abraham et al. (1982) exposed sheep to 1.6 ppm (5.5 mg/m^3) of nitric acid for 4 hours and found that airway reactivity (to aerosolized carbachol) was increased. Studies of the ozone + nitrogen dioxide combination at high relative humidity, in which nitric acid is readily formed, show that both airway permeability and lung pathology are altered (Bhalla et al., 1987; Mautz et al., 1988). In these studies effects were seen when the ozone and nitrogen dioxide concentrations were as low as 0.35 ppm and 0.6 ppm respectively, and the associated nitric acid formed was about 0.05 mg/m^3.

Recently, Koenig et al. (1989) at the University of Washington exposed allergy-prone human adolescents to sulfuric and nitric acids for 40 minutes (30 minutes at rest, followed by 10 minutes with moderate exercise). A preliminary analysis of data obtained using 9 subjects indicated that 0.068 mg/m^3 of H_2SO_4 decreased the FEV_1 by about 6% and increased total respiratory resistance by about 5% over clean air controls. Nitric acid exposure (0.05 ppm, or about 0.02 mg/m^3) was associated with a small decrease (4%) in FEV_1, but a substantial 23% increase in total respiratory resistance. The authors warn that this is a preliminary result, but if it holds up it indicated that nitric acid may be a toxicologically significant air pollutant.

In a study by Schiff et al. (1979) hamsters were exposed for 3 hours to carbon alone (1.5 mg/m^3, 0.3 micrometer diameter), H_2SO_4 alone (1.1 mg/m^3, 0.12 micrometer diameter), or to the mixture. Carbon alone did not change the tracheal cilia beating frequency, but it produced occasional rounded, swollen epithelial cells and focal loss of ciliated cells. Sulfuric acid alone was associated with statistically significant lowering of ciliary beating frequency, patchy uneven swollen epithelium, some loss of cilia, rounding and sloughing of epithelial cells, and alterations in the mucus which covers the

trachea. The carbon plus H_2SO_4 had effects that were the type seen with acid alone, but in general were more severe and of longer duration. In another study, mice exposed to acid-coated carbon particles (300 hours cumulative at 2.9 mg/m^3) showed greater mortality and pulmonary consolidation following a challenge exposure to viruses than did rats exposed to equivalent amounts of either carbon alone or acid alone (Fenters et al., 1979).

C. Biologic Endpoints Used

The selection of the biologic endpoints employed in this study was based on (a) the sensitivity of the endpoints in identifying effects at many levels of the respiratory tract, and (b) the fact that these endpoints can be used in assessing the effects of pollutant atmospheres on both respiratory tract structure and function (defense mechanisms). The biological endpoints included: (a) measurement of respiratory tract clearance of insoluble tracer particles, (b) histopathology of respiratory tract tissues, including autoradiography (a measure of cell killing), (c) morphometry of deep lung tissues, and (d) effects on pulmonary alveolar macrophages (immunological endpoints). Each of these will be separately discussed below.

1. Particle Clearance

The ability of the airways to rid themselves of insoluble debris is a vital respiratory tract defense. Without effective clearance the respiratory tract would quickly overload with chemical and microbiological contaminants and disease would be inevitable. In mammals the airways of the head, neck and bronchial tree are coated with mucus which is steadily driven by small hair-like cilia toward the throat for swallowing. The proper functioning of this clearance mechanism requires mucus-producing cells and glands and a large healthy population of mature ciliated cells. Deeper in the lung the clearance of foreign materials depends largely upon a population of self-mobile cells called macrophages. The macrophages actually engulf foreign material and bring it into contact with internally present digestive chemicals that inactivate microorganisms and also dissolve many inorganic contaminants. Macrophages transport material to the mucus, whereby it is subsequently swallowed, or to lymph nodes for long-term storage. In some cases macrophages with ingested debris remain in the deep lung for long periods.

Our study included assessments of these clearance phenomena during the early (mucociliary) and late (macrophage) clearance phases. The tracer particles used to follow clearance were radiolabeled by us (Hinrichs et al., 1978) so that they could be easily monitored by external detectors after inhalation. The monodisperse tracer particles were about 1.8 micrometers in aerodynamic diameter, and thus deposited throughout the entire respiratory tract.

2. Histopathology

Changes in the structure of respiratory tract tissues were investigated using histopathologic (light microscopy) techniques. Histopathologic evaluations were made of the respiratory tract from the nasal cavity to the lung alveolar region in order to directly measure any cellular injury or derangement induced by inhalation of the air pollutants. The mechanisms of respiratory system injury and repair inevitably involve interactions between epithelial (facing the air), endothelial (facing the blood) and mesenchymal (within tissue) cells. Conditions which delay the normal orderly process of cell regeneration and replacement (of dead cells) or disturb the essential interplay between the cells of the respiratory tract predispose the development of respiratory disease. Continued exposure to irritant gases or aerosols in sufficient concentrations can be expected to result in alteration of cellular structure in the nasal cavity, trachea, terminal bronchioles and alveoli.

In conjunction with histologic analyses of tissue structure, measurement of cell killing and subsequent stimulated cell division provide analysis of tissue pathologic effects of the exposures. Sites of injury in nasal, tracheal, and bronchial tissues are identified using an autoradiographic technique after labeling the whole animal with a radioactive DNA precursor.

In addition to the above endpoints, an additional assessment - quantitation of the number of lung cells containing soot particles - was also performed. The significance of soot accumulations following exposures is not presently known, but we were interested in the effects of acidity on the degree of accumulation of soot particles in the lung. This is important because excessive accumulation of soot has been implicated as a contributor to lung cancer (McClellan, 1987).

3. Morphometry

The lung morphometry endpoints were included to identify changes in deep lung structure following inhalation exposures. In these studies, important architectural features of the lung such as the elastic supporting matrix and the air-blood barrier were measured. These studies, performed by Drs. Sherwin and Richters of USC, required the use of very sophisticated computer-aided methods. Among the parameters measured were: lung volume; elastin fiber (flexible tissue elements) number, intercept (which gives the average distance between the fibers), and field area; alveolar wall area; and alveolar perimeter. Changes in these parameters are of interest because they are indicative of disruption of the alveoli in the lung and are potential indicators of eventual chronic respiratory illness. Lung volume is a coarse measure of gross organ damage that will indicate a significant change in tissue stiffness or tissue proliferation or destruction. Elastin fiber numbers, intercepts and areas are proportional to the amount of elastic tissue in the lung. Changes in elastin would indicate a disruption in the pliable architecture of the lung. One would expect such a disruption to occur in emphysema, for example. Alveolar wall areas and alveolar perimeters are measures that provide information on the available surface for gas exchange. Increases in area would indicate thicker walls, and thus imply hampered gas exchange. Decreases in perimeter would indicate less surface area for gas exchange.

4. Macrophage Studies

Pulmonary macrophages were evaluated to determine if this crucial element of pulmonary defense was compromised by the exposures. Pollutant exposure can result in increased rates of respiratory infections; this is borne out by studies with laboratory animals which demonstrate increased susceptibility to infection after exposure to acids and oxidant pollutants (Rose et al., 1988). Changes in pulmonary macrophages (a key part of the immune defense system) resulting from pollutant exposure are presumed to be good indicators of adverse effects of air pollutants.

Macrophages possess receptors on their surfaces for the Fc portion of immunoglobulin (IgG); these receptors help in the recognition of foreign particles (Gaafar et al., 1971). They also facilitate phagocytosis and destruction of foreign material by the macrophages (Boltz-Nitalescu et al., 1981). The binding of these receptors with immune complexes facilitates the

phagocytosis of IgG-coated particles (such as bacteria). The function of the macrophage Fc receptor in antigen-antibody response was assessed by measuring the capacity for binding sheep red blood cells in a rosette assay. The ability of macrophages to engulf foreign particles (polystyrene latex microspheres) by phagocytosis was measured using macrophages recovered from lungs of exposed and control rats. Using these techniques the effects of the pollutant atmospheres on two functions of macrophages which play major roles in host immunity were determined.

VI. MATERIALS AND METHODS

A flow diagram of the general procedures used in performing the study is shown in Figure 1. The details of the methods employed are described below.

A. Exposure Techniques

Barrier-reared Sprague-Dawley rats used in this study were purchased from Hilltop Lab Animals, Inc. Male rats were delivered to the laboratory in filter-equipped shipping boxes to minimize prior exposure of the animals to particulate pollutants. The rats were then housed in a laminar air-barrier caging system (with high-efficiency gas and particle filtration) for about one week before the start of the exposure. Microbiological assays, supplied by Hilltop, indicated that the rats were free of respiratory infection. This fact was confirmed by quality control histopathologic examinations at our laboratory.

Groups of rats were exposed to either purified air or the pollutant atmospheres using unique 1 m^3 stainless-steel Rochester chambers modified by us for nose-only exposure (Figure 2). The modification included a dander removal system that was necessary to preserve the air quality in the laboratory. Such modifications were necessary to permit exposure to acids and to protect laboratory personnel. Exposures were 5 hours per day for 5 consecutive days. Each rat was placed into a tube , designed by us, with an aluminum nosepiece having a conical interior cavity tapered toward the forward end so that only the tip of the rat's nose protruded into the atmosphere. The tubes were inserted and sealed (with double o-rings) into nose-only ports in the walls of chambers. Each port was supplied by a channel on the interior wall of the chamber which directed the atmosphere downward past the nose of the rat to prevent rebreathing of that atmosphere by the exposed rat and to prevent sharing of that sample of atmosphere with another rat. This exposure system

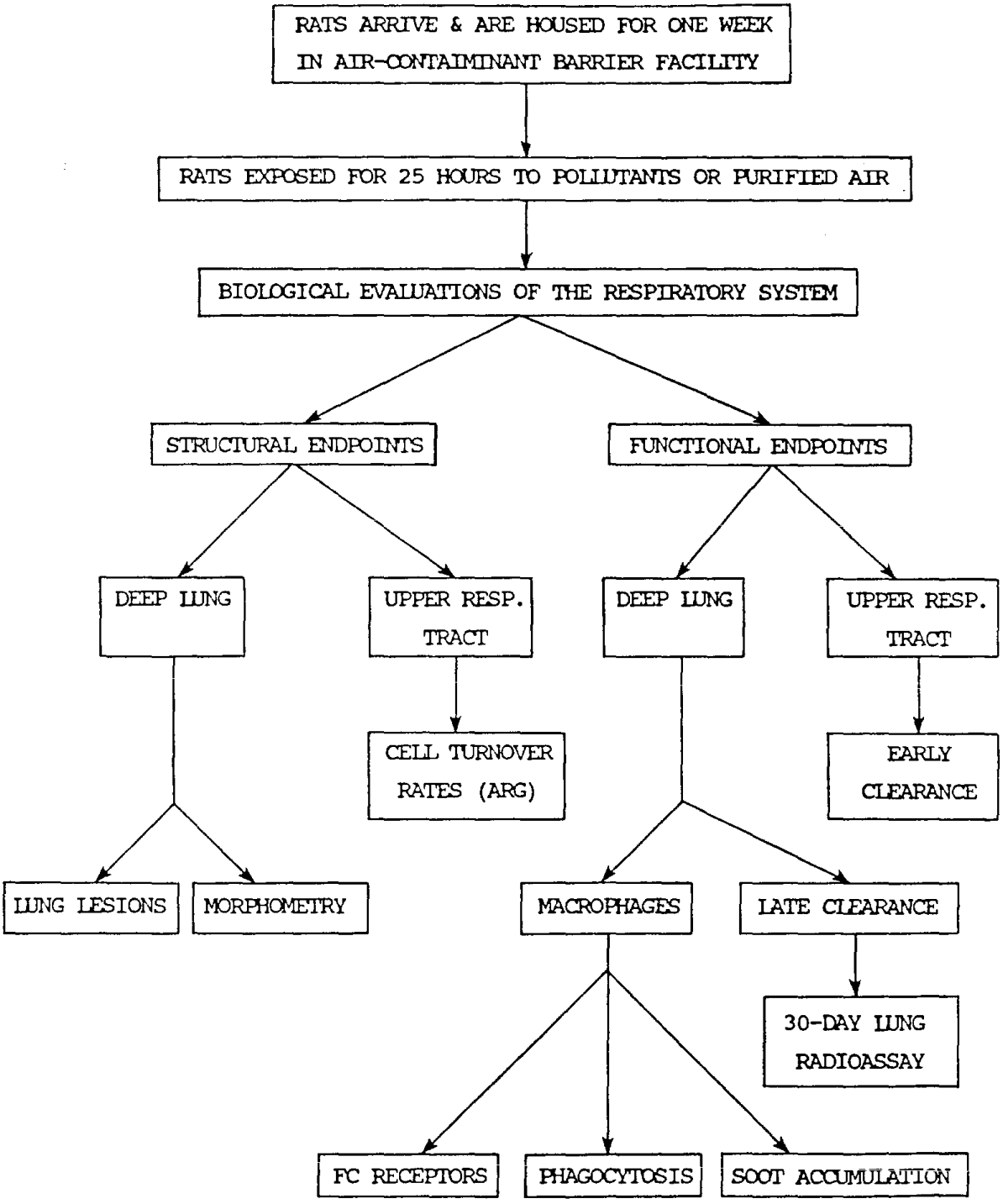
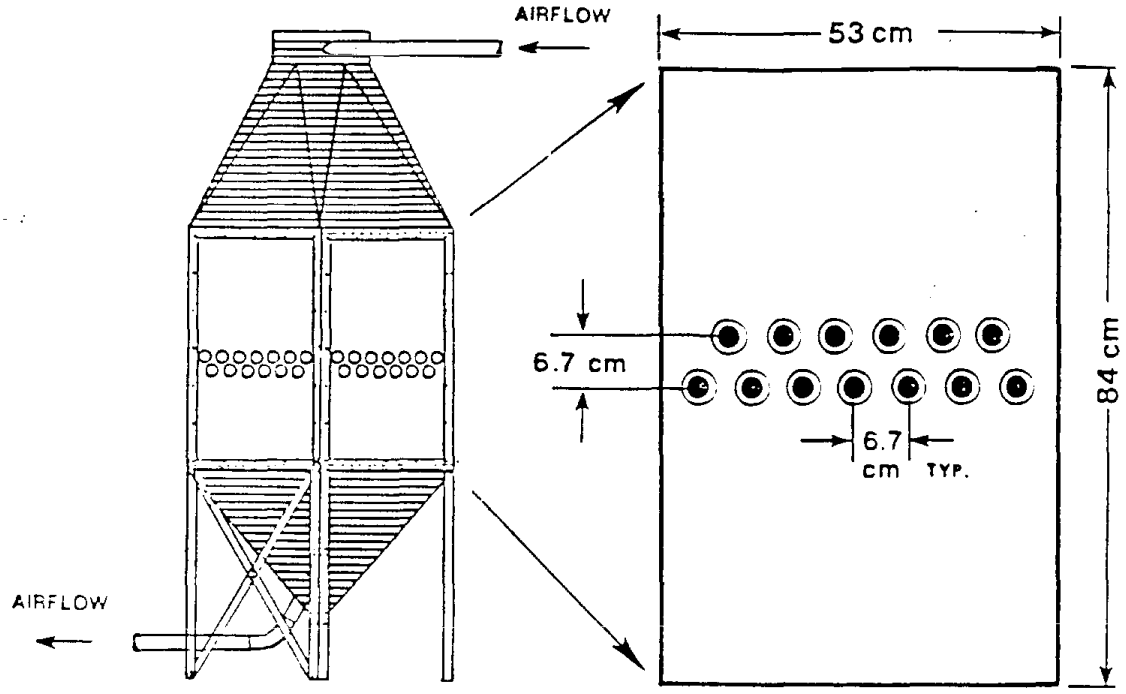


FIGURE 1. Flow diagram of the experimental procedures used in the study.

A. NOSE-ONLY MODIFICATION TO ROCHESTER CHAMBERS



TYPICAL ORIENTATION OF NOSE-ONLY PORTS

B. SECTIONAL VIEW

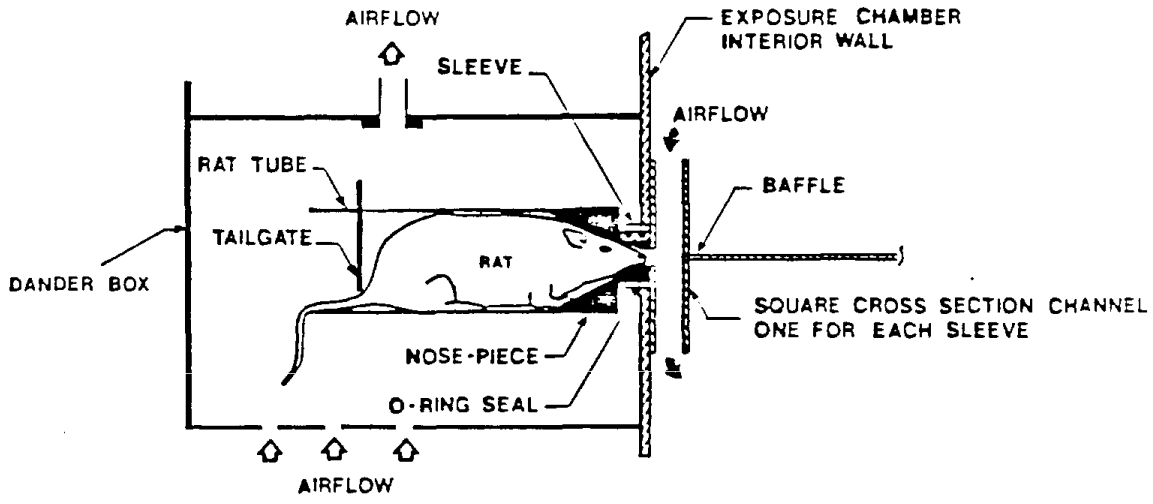


FIGURE 2. Rochester chambers modified for nose-only exposure.

was specifically designed to provide comfort to the rats (to minimize stress) and to prevent the neutralization of acids in the pollutant atmospheres by ammonia generated from the rats' excreta. The chambers were supplied with purified air (prior to the injection of pollutants) at a temperature of about 23 ± 1°C, and a relative humidity of 83 ± 2%.

Propane-flame-generated carbon aerosols were produced by a system developed and used by the National Bureau of Standards and the U.S. Environmental Protection Agency (EPA) for inhalation study purposes (EPA report published in February of 1980, #EPA-600/1-80-14). This generator, as modified for use in our studies (addition of acid generation capability), is shown in Figure 3. Diesel exhaust aerosols were obtained from an 11 horsepower, single-cylinder diesel engine operating at constant speed (1800 r.p.m.) and load (80% of maximum). The engine exhaust was rapidly diluted with purified air to prevent particle agglomeration before inhalation by the rats. (A schematic diagram of the diesel soot + acids generation system is presented in Figure 4.) The fuel used was Phillips #2 Diesel Control Fuel. (An interim report submitted by our laboratory to the California Air Resources Board in May 1986 describes in detail the specifications of the diesel engine and the characteristics of the diesel fuel. Copies of this document are available upon request.) The sulfuric acid coating on both the propane soot and diesel soot aerosols was produced by bubbling a stream of dry air through fuming sulfuric acid. The resultant sulfur trioxide vapor was mixed with the carbon aerosol - in the presence of water vapor - to produce the sulfuric acid coating on the surface of the particles (Walters et al., 1988, Appendix A). Electron micrographs of electrostatic precipitator-collected samples of the propane soot aerosol, the diesel soot aerosol, and the acid-coated diesel soot aerosol are shown in Figure 5. Nitric acid vapor was generated by bubbling purified air through concentrated nitric acid immersed in a constant temperature bath.

Cascade impactor samples were used to size-classify particles for chemical and gravimetric analyses. The size (mass median aerodynamic diameter) of the acid-coated propane soot and diesel soot aerosols was typically about 0.10 to 0.20 micrometers. The geometric standard deviation was approximately 2. Aerosols and vapors were collected on filters for gravimetric and chemical analyses. The analysis of filter samples collected during the studies indicated that the propane soot aerosols were composed of about 97% elemental carbon (and 3% extractable organic matter), and the diesel soot aerosols were composed of about 92% elemental carbon (and 8% extractable organic matter). The aerosol stability during exposures was determined using a real-time aerosol

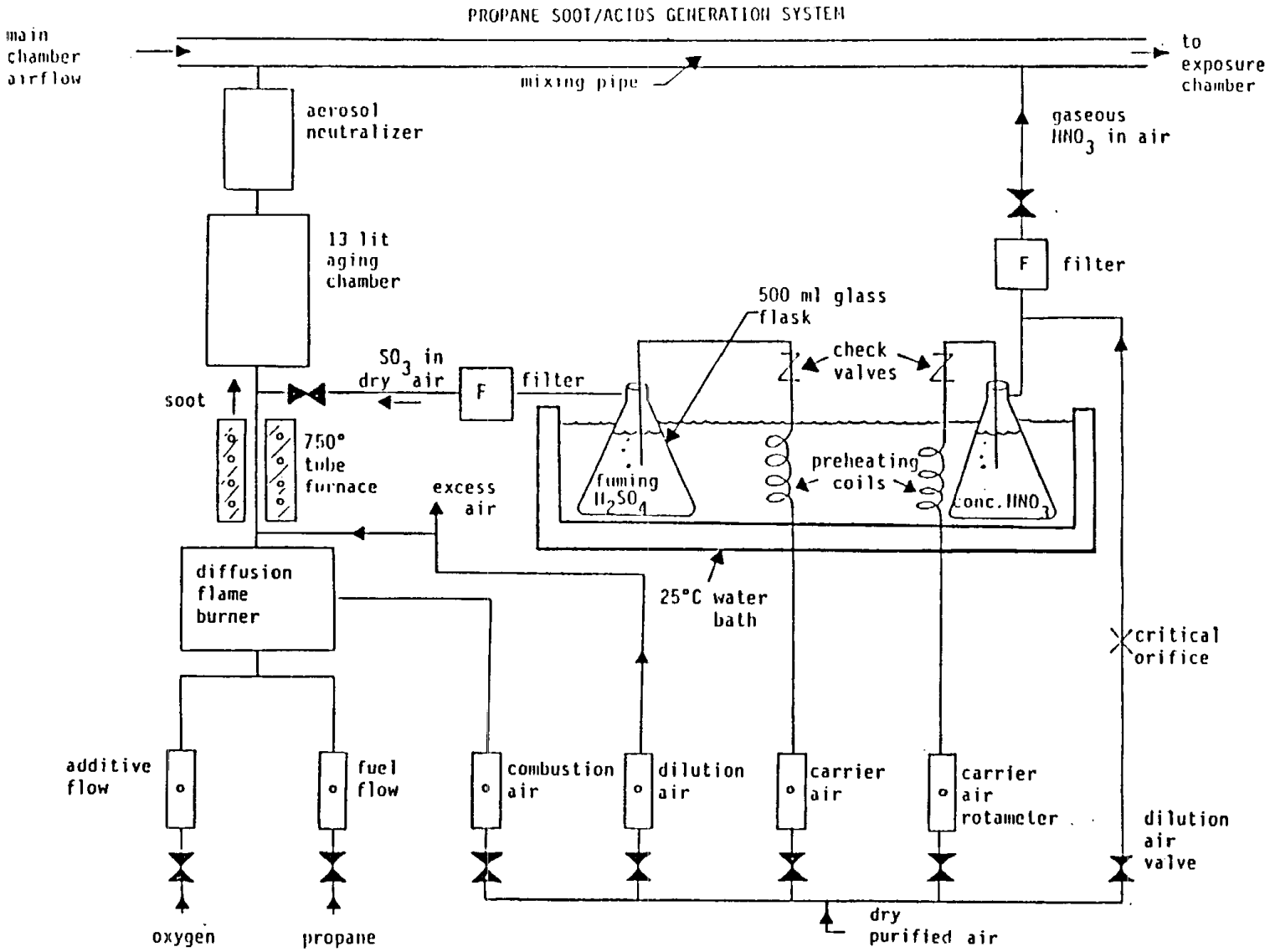


FIGURE 3. System used in generating propane soot + acids atmosphere.

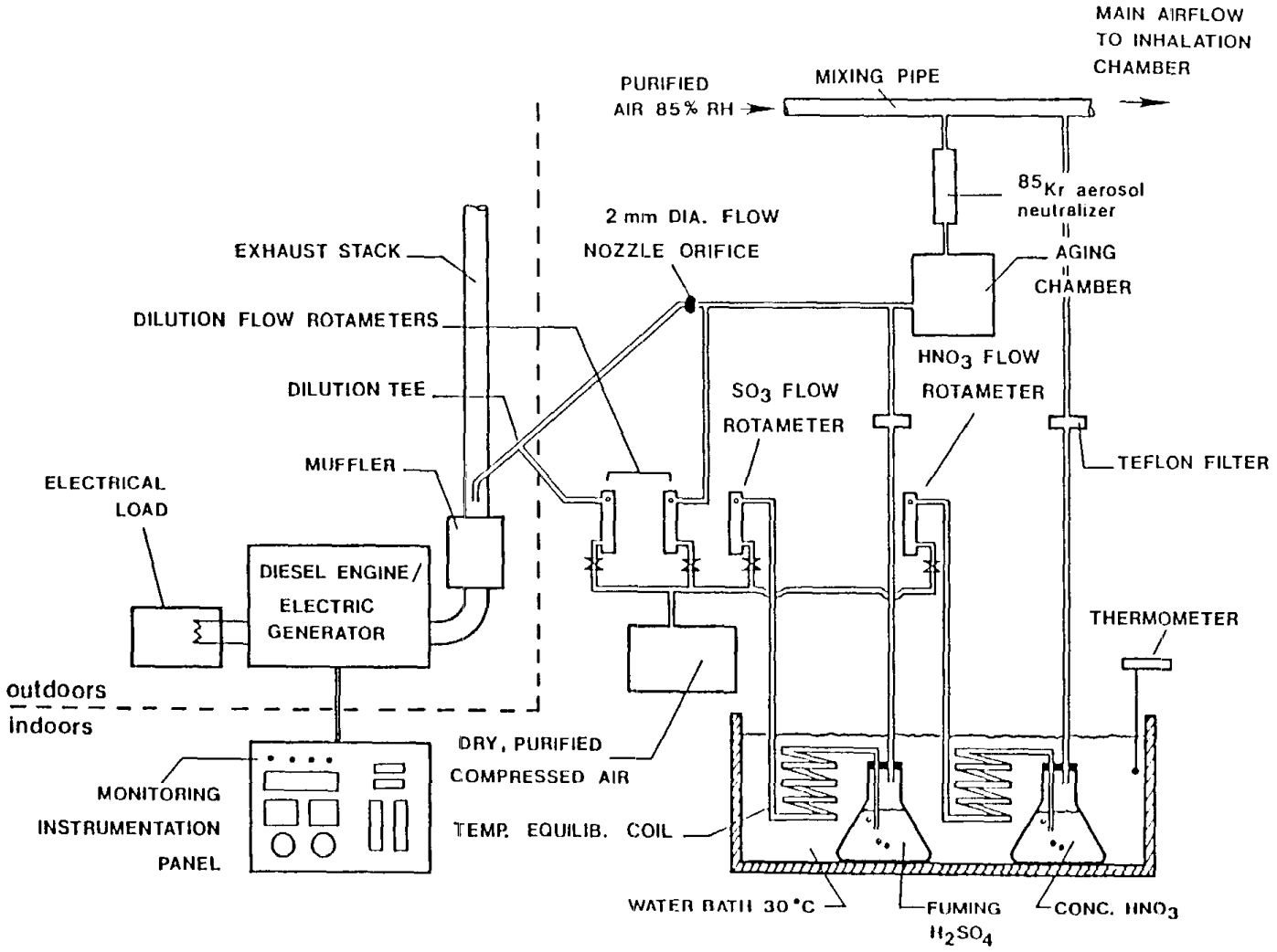


FIGURE 4. Schematic diagram of the diesel soot + acids generation system.

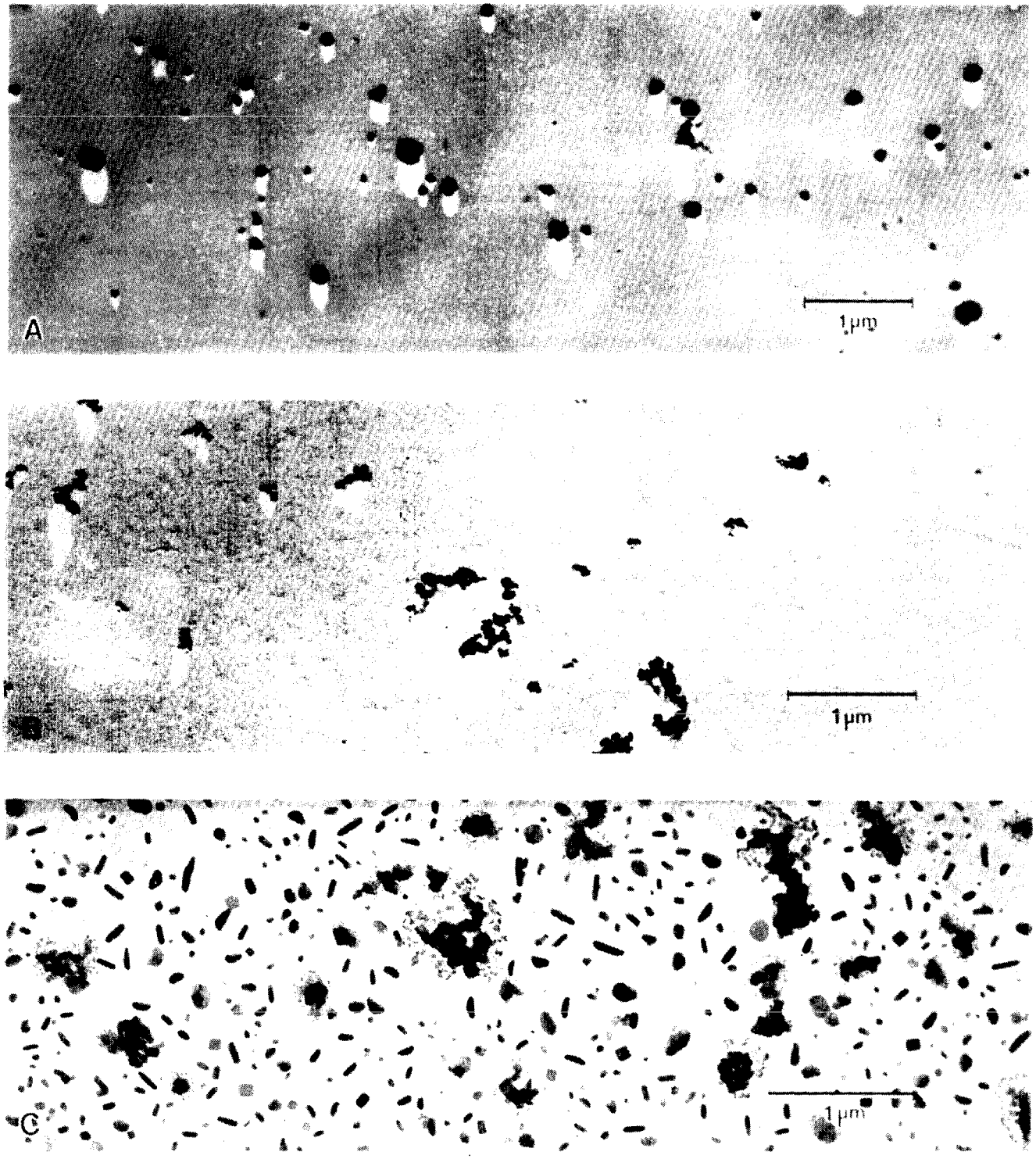


FIGURE 5. Electron micrographs of (A) the propane soot aerosol, (B) the diesel soot aerosol, and (C) the acid-coated diesel soot aerosol.

mass monitor. In the case of diesel exhaust-containing atmospheres, nitrogen oxides were monitored using a calibrated gas monitor. All sampling (for both aerosols and gases) was from the rat's breathing zone. (The atmosphere characteristics which were calculated from data obtained during the exposures performed for this contract are summarized in Table 1.)

B. Biologic Endpoints

1. Particle Clearance

The tracer particles were labeled at this laboratory (Hinrichs et al., 1978) with tightly bound ^{51}Cr. Aerosols were generated using a Lovelace compressed air nebulizer. The aerosolized particles were dried, diluted with filtered air and passed through a ^{85}Kr discharger before entering the nose-only exposure chamber. Rats were exposed to the radioactive aerosol in this system for 20 minutes. The aerosol, sampled from the breathing zone of the rats using a calibrated seven-stage impactor, had an activity median aerodynamic diameter of about 1.8 micrometers and a geometric standard deviation of about 1.2. After the deposition was completed the animals were placed in individual plastic counting tubes and inserted into a collimated 3-in NaI(Tl) gamma ray detector apparatus. All of the rats were counted for 100 seconds in this system before they were placed into the purified air or pollutant atmosphere chambers for their final 5 hour exposure (on the fifth exposure day). After the rats were removed from the exposure chambers their accumulated feces were collected at 10 fixed times during the first 50 hours after the deposition of the tracer particles. Early (upper respiratory tract, or mucociliary) clearance was characterized by the resulting fecal activity excretion curves. During the first 400 hours post-deposition of the tracer particles, 5 thoracic counts were performed on each animal in order to characterize late (deep lung, or macrophage-mediated) clearance. In addition, the rats were sacrificed at 30 days post-deposition and their lungs counted in order to provide another measure of late clearance (termed the A_{30}, for activity remaining at 30 days). Early clearance 50% clearance times ($T_{50\%}$), late clearance biological half-times (T_L), and A_{30} values were determined for each rat individually. Means and standard deviations were calculated for the purified air and pollutant exposed groups of rats, and the group $T_{50\%}$, T_L, and A_{30} values were compared statistically using two-tailed t-tests.

TABLE 1

Mean Values of Atmosphere Measurements During Exposures. Target Concentrations: Propane Soot, 0.5 mg/m^3; Diesel Soot, 0.5 mg/m^3; HNO_3, 0.35 mg/m^3; H_2SO_4, 0.15 mg/m^3.

	mg/m^3				ppm			
Exposure No. (Endpoint)	Propane Soot	Diesel Soot	HNO_3	H_2SO_4	NO	NO_2	%RH	T(^oC)
1 (M)	0.5	---	0.32	0.15	0.2	a	83.4	22.2
2 (H)	0.5	---	0.05	0.11	0.3	a	83.1	23.3
3 (M)	0.6	---	0.34	0.16	0.2	a	81.6	23.4
4 (H)	0.4	---	0.39	0.19	b	a	85.1	23.8
5 (H)[c]	0.3	---	0.20	0.08	b	a	84.6	22.8
6 (H)[d]	---	0.6	0.50	0.21	2.5	b	84.2	23.2
7 (C)	0.5	---	0.35	0.22	b	a	84.2	23.6
8 (C,I)	---	0.5	0.38	0.18	9.0	3.0	84.2	22.1
9 (M,H,I)	---	0.6	---	---	3.8	0.3	83.1	22.8
10 (M,H,I)	0.5	---	---	---	0.2	a	82.8	22.5
11 (M,H,I)	---	---	0.40	0.17	a	a	82.9	22.5
12 (C)	---	0.6	0.35	0.18	6.8	0.3	82.3	22.6
13 (M)	---	0.5	0.35	0.17	5.7	1.1	83.1	22.4

a - Less than 0.05 ppm.

b - Not measured.

c - Atmospheric component target concentrations for this study were one-half those of the other studies.

d - This 25 hour exposure was performed over 4 days rather than 5 days due to equipment failure.

Abbreviations for Endpoints: C - Clearance; H - Histology; I - Immunology; M - Morphometry.

A collimated radiation counting system which allowed separate detection of radiolabeled material in the nasopharyngeal and deep lung regions of the rat was designed and fabricated. This system is shown in Figure 6. The NaI(Tl) detector on the right was collimated such that radiation emitted from the tip of the rats' nose to approximately the middle of the trachea was detected. Radiation from the middle of the trachea through the lung region was shielded by lead from this detector, and was monitored by the other NaI(Tl) detector. The second detector was shielded by lead so that interfering radiation from the gastrointestinal region was minimized. The counting system was tested, and the results are shown in Figure 7. These tests indicate that the system performed adequately with respect to the separation of the nasopharyngeal and deep lung regions.

Due to several practical limitations, this system was not used during the particle clearance experiments performed for this contract. The system which had been used since 1980 was fully capable of providing data which could be used to determine the late clearance biological half-times. Our intention was to use the newly fabricated system to supply us with information concerning the rate of particle clearance from the nasal and upper tracheal regions - information which is normally obtained using the fecal activity excretion curves. There were three reasons that the usual method, rather than the new method, was used. Firstly, during the exposure of the rats to the tracer particles some radioactivity was deposited on the exterior of the noses and muzzles - thus interfering with nasal counts in the new system. Although some of this material was removable with cotton swabs, the radioactivity which remained confounded the nasal counts - especially since it seemed that the ratio of material deposited in the nose to that deposited on the outside of the nose varied greatly, even for rats with nearly identical deep lung particle depositions. Secondly, such a small percentage of the total deposited radioactivity was in the nose (about 5%) as opposed to that in the lungs (about 95%) that the nasopharyngeal region counting times were necessarily quite lengthy, and the counting statistics were poor (large relative standard deviations). Thirdly, it was observed during several test runs that the rats noses seemed to clear the deposited particles quite readily. In fact, almost all of the material was cleared within four hours of the deposition. Since our experiments have been designed such that the tracer particle deposition occurs just prior to the last exposure, most of the activity would have already been cleared before the end of the last five hour exposure. Use of the older method did not compromise the study in any way.

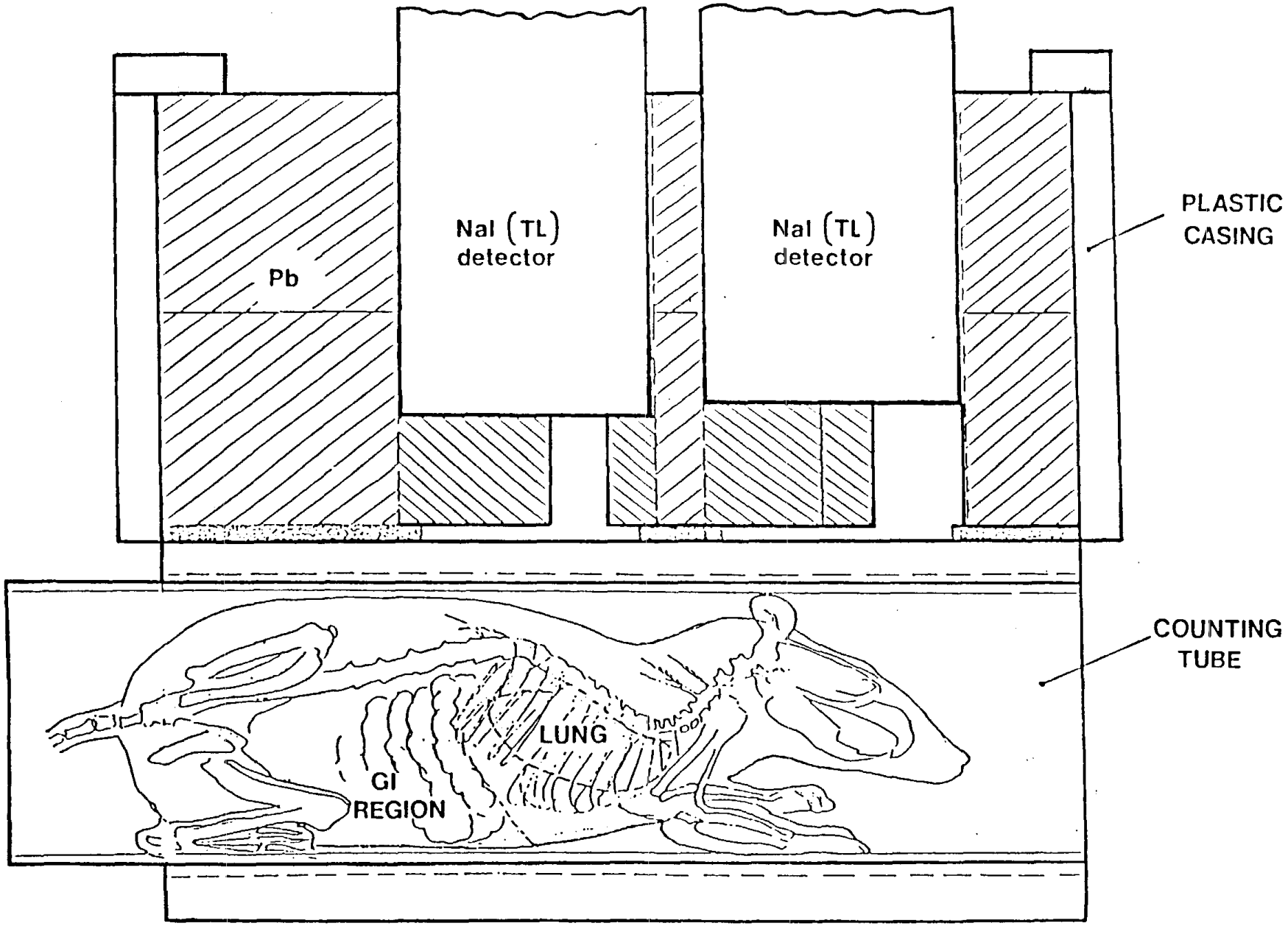
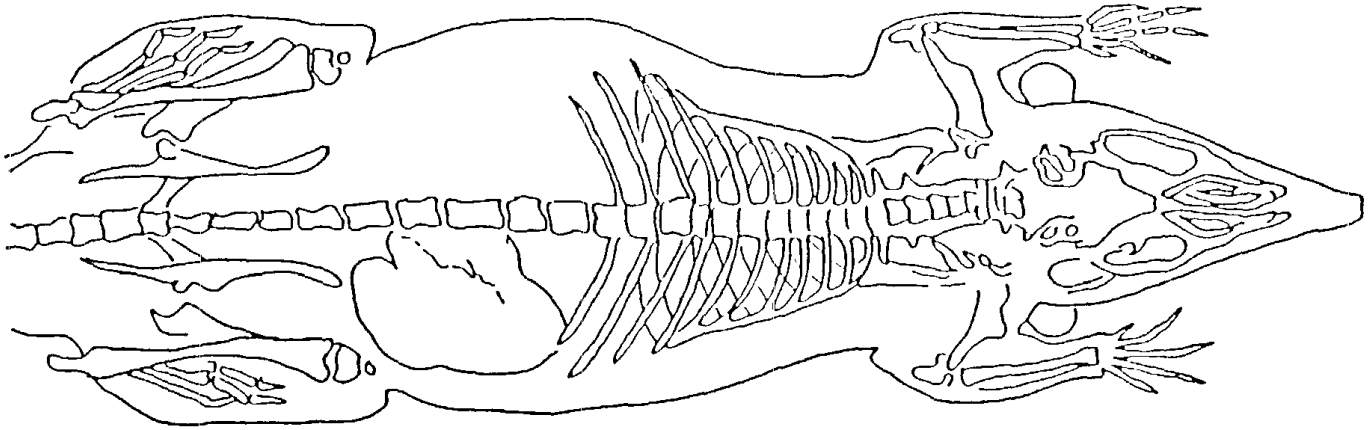


FIGURE 6. Diagram of collimated counting system designed to allow separate detection of radiolabeled material in the nasopharyngeal and deep lung regions of the rat.



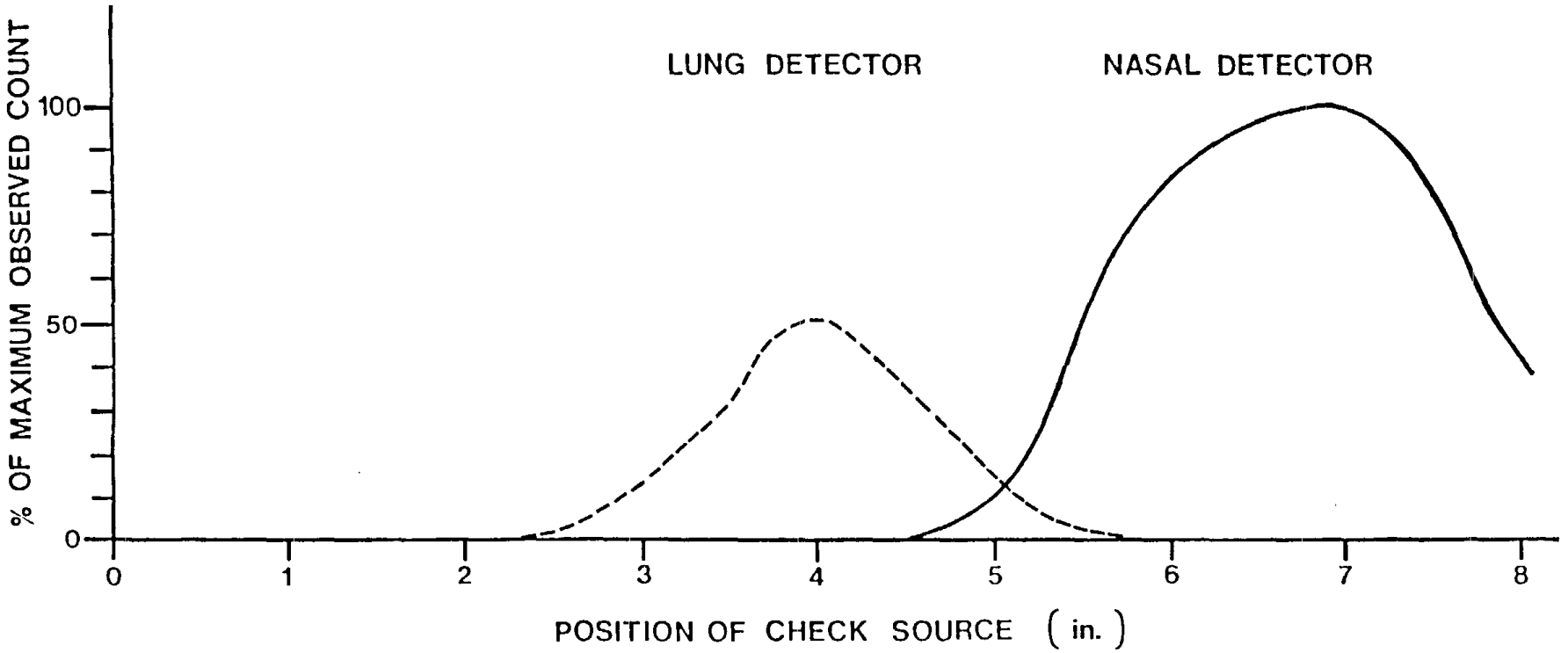


FIGURE 7. Response of nasopharyngeal and deep lung detectors as a function of radioactive "point source" position.

2. Histopathology

This endpoint was used to quantitate tissue injury in the respiratory system. A detailed description of the histological techniques used has been presented elsewhere (Mautz et al., 1985). A summary is provided here. Rats were deeply anesthetized and then humanely killed by exsanguination. The thoracic cavity was opened and the lungs and trachea carefully exposed. Lung surfaces were examined for abnormalities before and after their removal from the thoracic cavity. The distal portion of the trachea was cannulated and this segment with attached lungs was fixed by airway perfusion with 10% neutral-buffered formalin at 30 cm fluid pressure for 72 hours. The remaining trachea with attached larynx was removed and immersed in formalin fixative. In addition, the rat was decapitated, and that portion of the head containing the intact nasal cavity was immersed in formalin fixative.

The lungs were subsequently removed from the perfusion apparatus and lobes of the right lung were separated. Appropriate portions of the caudal lobe were prepared for embedding in an automatic tissue processor. Lung and tracheal tissues were embedded in paraffin and sectioned at 6 micrometers on a rotary microtome. Complete lobal sections of lung were cut close to the midline of the main bronchus, and were used for microscopic examination and grid area determinations. The trachea were split longitudinally and cell turnover rates were examined.

After fixation the heads were decalcified and 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 sectioned at 4 micrometers and stained.

Lung grid area determinations were made using an ocular grid calibrated with a stage micrometer. Total lung parenchyma determinations were similarly made at a lower magnification. Only lung parenchyma was counted. The magnification factor for the two grid counting systems was 1:8. Total lung parenchyma was computed and the percent lung lesion obtained. The linear grid scans of lung parenchyma gave a count of total lesion area per alveolar zone of lung section.

Autoradiographic techniques (Mautz et al., 1988) were used to identify sites of cell killing and numbers of cells killed in various regions in the respiratory tract. Rats were injected intraperitoneally with tritiated thymidine, a DNA precursor (H^3T, 1 µCi/gm body wt.), one hour before killing.

Tissue slides were dipped into sensitive photographic emulsion. After photographic development, slides were stained and the percentage of labeled cells per epithelial cell population in the trachea and nasal cavity was determined by cell counts for each animal.

As an additional measure of the effects of the soot-containing atmospheres on the deep lung, a quantitation of the lung cell population containing soot particles was also performed. Lung sections of rats exposed to either diesel soot or propane soot atmospheres were scanned with a light microscope at low power using an ocular grid in order to determine the total number of grid units contained in the sections. This number was divided by 40 to establish the interval between counting fields. The lung sections were then magnified to high power and each field was scanned for soot-containing cells. All of the cells in every fifth field in the section were then counted in order to determine the average total number of cells per field. The percentage of cells containing soot for the lung section was therefore the total number of soot-containing cells divided by the total number of cells.

3. Morphometry

With one exception (the group exposed to propane soot + acids and sacrificed one week post-exposure), all animals were shipped immediately to the USC laboratory following exposure. Coded labels were used to identify the animals, and uncoding was done only after the morphometric data had been received by the UCI investigators. The rats were killed by intraperitoneal injection of 2.0 ml of sodium pentobarbital (60 mg/ml). The lungs were removed en bloc from the chest cavity with the trachea intact. The right lung was inflated with 2% buffered glutaraldehyde at 25 cm water pressure until the volume of the lung approximated the volume of the right thoracic cavity. Lung volume was measured 24 hours after fixation and the lung was stored in phosphate buffer prior to processing. Processing was accomplished by dehydration of the lung in graded alcohols, xylene clearing, and embedding the tissue in paraffin. Whole right lower lobe sections of 6 micrometer thickness were obtained from the lung periphery (lateral plane) to exclude bronchovascular structures and thus minimize image analysis editing. An aldehyde-fuchsin stain for elastin was applied to four serial sections from each lung, and a Metanil Yellow stain was then added for delineating the alveolar walls. All lung sections from each test group were stained together to ensure uniform tissue staining.

Detection and quantitation were done with a Quantimet 720 image analyzer. The lung field viewed with the light microscopic was displayed on the monitor screen and two detector units were set manually to reproduce the image electronically. For accurate reproduction, comparisons were made between all three images-the light microscopic, the displayed, and the electronically detected. More specifically, one "gray level" was set to detect elastic fiber area (an electronic replica of the elastica seen in the microscopic field), and a second "gray level" was set for the detection of alveolar wall area (again matched to the microscopic field). We used four sections of the right lower lobe and analyzed three lung fields per section (apical, basilar, and central), for a total of 12 measurement fields for each animal. An automated programmer effected 15 measurements, including elastic tissue area, elastic fiber number, elastic fiber perimeter, elastic fiber linear intercepts, several sizings of elastic area (small to large fibers), alveolar wall area, alveolar wall perimeter, and alveolar wall linear intercept. Bronchovascular structures were excluded by image editing, and measurements were obtained with and without editing. (Only measurements made with editing were used in the final data analysis.) Elastic fiber field area for each lung field, divided by elastic fiber number in the same field, provided a measurement of mean elastic fiber area. Lung volume measurements were obtained by submerging the formalin-fixed right lower lobe in a previously tared beaker containing a phosphate buffer solution. The weight recorded was translated directly into lung volume. For each of the above measurements, the statistical analysis was performed using the two-tailed Student's t-test, with significance at the 95 percentile level.

4. Macrophage Studies

A flow diagram of the experimental procedures used in the macrophage studies is presented in Figure 8. After exposure to pollutants, each group of rats was sacrificed by sodium pentobarbital injection at pre-planned time intervals. The lungs were removed under sterile conditions, cut into small fragments and residential cells were obtained by thoroughly shaking the fragments in sterile tissue culture medium (Hanks' medium). The contaminating red cells were lysed using Boyle's solution. The intact cells were washed with medium, centrifuged at 300 x g for 10 minutes, and the concentration of the cells was determined and adjusted to 1×10^5 cells in 0.1 ml of RPMI (Roosevelt Park Memorial Institute) medium supplemented with 15% FCS (Fetal Calf Serum). The phagocytic activity was measured in Lab-Tek chambers (Rao et al., 1980). In brief, 0.1 ml of each cell suspension was placed into Lab-Tek chambers containing 0.5 ml of the medium and was incubated for 1 hour at 37°C. The

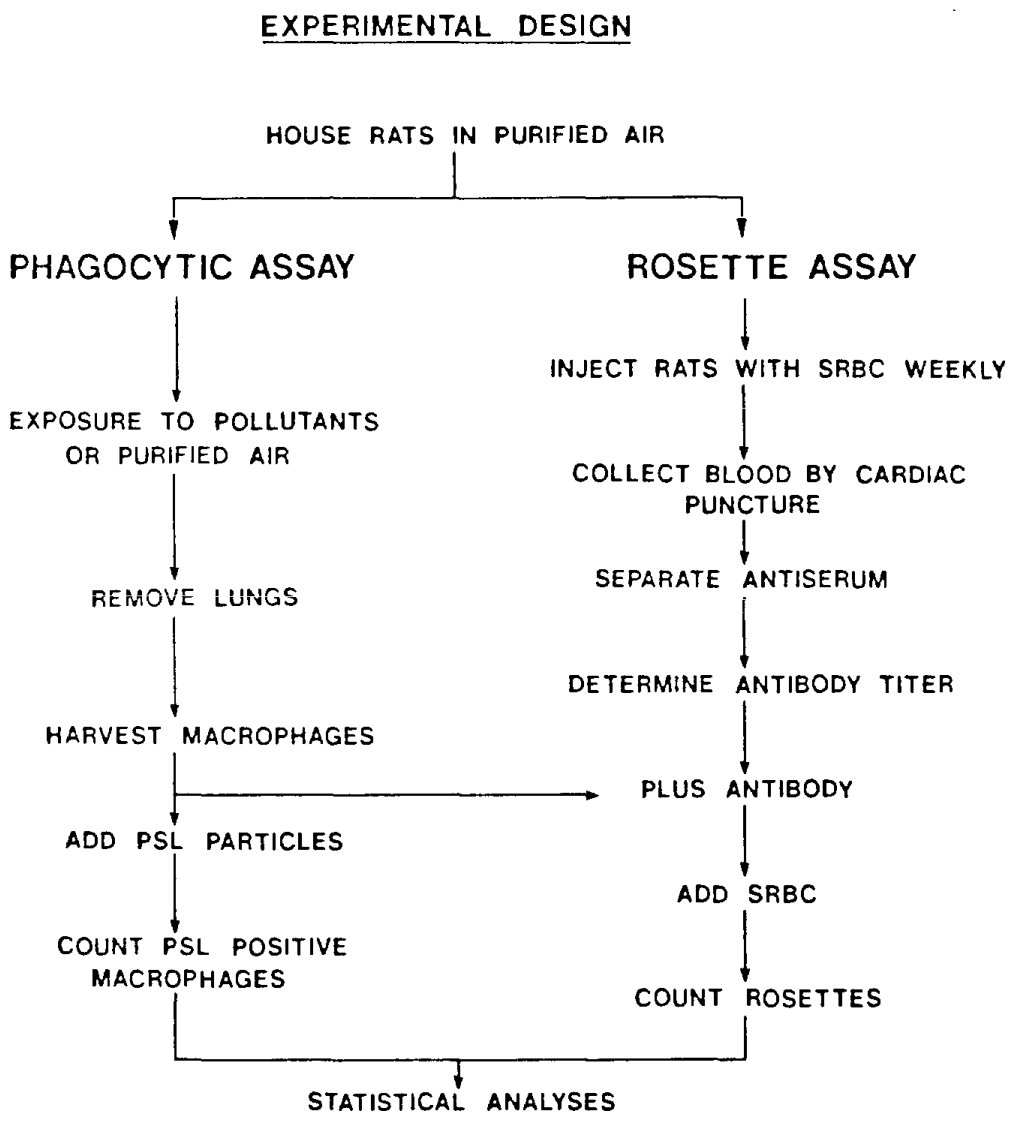


FIGURE 8. Flow diagram of procedures used in the macrophage studies.

chambers were then washed with the medium to remove the non-adherent cells. Then 0.1 ml of a suspension of spherical latex particles (diameter 1.1 micrometers) was added to each chamber and they were incubated for 60-90 minutes. The cells were then washed with calcium-free and magnesium-free PBS (Phosphate Buffered Saline, pH 7.2) to remove any non-engulfed latex particles. After dismantling the cell chambers from the Lab-Tek slides, the adherent cells (macrophages) were observed under an inverted-stage phase contrast microscope. The percentage of latex positive cells was determined. Matched groups of rats exposed to purified air were used as controls. The percent of the total macrophage population that had engulfed latex particles was calculated.

A rosette assay was used to determine the effect on surface Fc receptors which bind antigen to macrophages. Lab-Tek chambers, each containing 1×10^5 cells in 0.1 ml of RPMI medium supplemented with FCS and 0.59 ml of Hanks medium, were prepared as described earlier, for the individual rat lungs of each group. The Lab-Tek chambers were incubated for 1 hour at 37°C and later the non-adherent cells were removed by washing with the medium. The Fc receptors on macrophages were measured by rosette assay (Rao et al., 1980). In brief, 0.1 ml of anti-SRBC (SRBC = Sheep Red Blood Cell) serum - at a concentration determined by trials prior to the experiment - in RPMI medium was added to each of the chambers and incubated for 30 minutes at 37°C. After the incubation 0.1 ml of SRBCs (1×10^7 cells) was added to each of the chambers containing macrophages and was incubated for 30 minutes at room temperature. The unbound SRBCs were washed away gently using the medium. The number of cells forming rosette's with SRBCs were counted using a microscope. Cells with three or more SRBCs attached were counted as positive rosettes. One to two hundred counts were accumulated. The percentage of rosette-forming macrophages was then calculated.

Preparation of anti-SRBC serum: SRBCs obtained in Alsever's solution were washed with PBS (thrice) and the cell count was adjusted to 5×10^9 cells in 1.0 ml. Adult rats were injected IP with 0.2 ml of 5×10^8 SRBCs in PBS. Each received four such injections at weekly intervals. Ten days after the last injection the rats were bled and the serum was separated. The antiserum was inactivated at 57°C for 30 minutes and the titer of the antibody was detected by its ability to bind Fc receptors to macrophages as determined by the rosette assay.

Data for each rat, in the form of latex-positive macrophages or net rosettes, were assembled, group means and standard deviations calculated, and two-tailed t-tests (exposed vs. controls) and an analysis of variance were performed. A level of significance of 0.05 or less was considered statistically significant. (More specific information concerning the techniques used in performing the macrophage studies can be found in a publication by Prasad et al., 1988, Appendix B.)

VII. RESULTS

A. Particle Clearance

The results of the particle clearance experiments in which rats were exposed to the combined acid and soot atmospheres are shown in Tables 2 to 4. The data from the propane soot + acids experiment (Table 2) indicate that this combined pollutant atmosphere did not significantly affect the early clearance of the tracer particles. However, this atmosphere did produce a statistically significant delay in late clearance. The results of both the thoracic counting (T_L) and lung sacrifice (A_{30}) analyses are in agreement that the clearance of the radiolabeled tracer particles from the deep lung region of the rats was dramatically slowed as a result of the 25 hour propane soot + acids exposure. The results of the two diesel soot + acids experiments (Tables 3 and 4) indicate that this atmosphere did not produce significant effects on either early or late clearance. (Since the propane soot + acids atmosphere had such a pronounced effect on late clearance, the absence of effects following exposure to the diesel soot + acids atmosphere was somewhat surprising. The diesel experiment was repeated to verify the negative results from the previous study.)

B. Histopathology

The results of the histopathology studies are listed in Table 5. Despite the intense histopathological coverage and large numbers of careful measurements performed, no significant injury was observed. This is a clear negative with respect to tissue disruption at the light-microscopic level. None of the atmospheres studied (propane soot + acids, diesel soot + acids, or the component atmospheres) produced statistically significant quantities of deep lung focal lesions in pollutant exposed rats. The autoradiographic analyses of respiratory tissues indicated that none of these atmospheres

TABLE 2

Effects of Propane Soot + Combined Acid Atmosphere on Early and Late Clearance of Radiolabeled Tracer Microspheres

Early Clearance	Number	$\overline{T}_{50\%} \pm$ SD(hr)	$\Delta T_{50\%} \pm$ SE(hr)	p[b]
Purified Air	30	10.9±1.5	---	---
Propane Soot + Acids	28[a]	10.8±1.4	-0.1±0.4	0.79

Late Clearance	Number	$\overline{T}_L \pm$ SD(hr)	$\Delta T_L \pm$ SE(hr)	p[b]
Purified Air	30	651±202	---	---
Propane Soot + Acids	30	827±223	176±66	0.002

A_{30} (Index of Late Clearance)	Number	$\overline{A}_{30} \pm$ SD	$\Delta A_{30} \pm$ SE	p[b]
Purified Air	30	22.4±4.5	---	---
Propane Soot + Acids	30	25.7±3.2	3.3±1.0	0.001

$T_{50\%}$ = time required to excrete 50% of the total activity excreted through 50 hours postdeposition. T_L = late clearance biological half-time.

a - Two rats were excluded from the early clearance data analysis for failure to meet previously-established defecation criteria. Periodically in clean air or pollutant-exposed groups, some rats do not excrete sufficient feces to analyze.

b - Two-tailed t-test.

TABLE 3

Effects of Diesel Soot + Combined Acid Atmosphere on Early and Late Clearance of Radiolabeled Tracer Microspheres

Early Clearance[a]	Number	$\overline{T}_{50\%} \pm$ SD(hr)	$\Delta T_{50\%} \pm$ SE(hr)	p[b]
Purified Air	33	10.4±2.0	---	---
Diesel Soot + Acids	27	9.9±2.3	-0.5±0.6	0.36

Late Clearance	Number	$\overline{T}_L \pm$ SD(hr)	$\Delta T_L \pm$ SE(hr)	p[b]
Purified Air	33	609±181	---	---
Diesel Soot + Acids	27	647±218	38±52	0.46

A_{30} (Index of Late Clearance)	Number	$\overline{A}_{30} \pm$ SD	$\Delta A_{30} \pm$ SE	p[b]
Purified Air	33	20.7±3.7	---	---
Diesel Soot + Acids	27	21.3±4.0	0.6±1.0	0.55

$T_{50\%}$ = time required to excrete 50% of the total activity excreted through 50 hours postdeposition. T_L = late clearance biological half-time.

a - No rats were excluded from the early clearance data analysis for failure to meet established defecation criteria.

b - Two-tailed t-test.

TABLE 4

Effects of Diesel Soot + Combined Acid Atmosphere on Early and Late Clearance of Radiolabeled Tracer Microspheres

Early Clearance	Number	$\overline{T}_{50\%} \pm$ SD(hr)	$\Delta T_{50\%} \pm$ SE(hr)	p[b]
Purified Air	30	9.4±2.0	---	---
Diesel Soot + Acids	29[a]	10.0±2.1	0.6±0.5	0.31

Late Clearance	Number	$\overline{T}_L \pm$ SD(hr)	$\Delta T_L \pm$ SE(hr)	p[b]
Purified Air	30	641±253	---	---
Diesel Soot + Acids	30	646±196	5±58	0.93

A_{30} (Index of Late Clearance)	Number	$\overline{A}_{30} \pm$ SD(hr)	$\Delta A_{30} \pm$ SE	p[b]
Purified Air	30	20.6±4.1	---	---
Diesel Soot + Acids	30	20.6±3.8	0±1.0	0.95

$T_{50\%}$ = time required to excrete 50% of the total activity excreted through 50 hours postdeposition. T_L = late clearance biological half-time.

a - one rat was excluded from the early clearance data analysis for failure to meet established defecation criteria.

b - Two-tailed t-test.

TABLE 5

Histopathological Data

Atmosphere	Sac Time	Measurement	Ratio(Exp/Ctl)	P	n
Diesel Soot + Acids	Immed.	Type I	1.10(2.47/2.25)	0.43	8
		ARG(R)	2.43(0.34/0.14)	0.15	7
		ARG(R1)	1.19(0.37/0.31)	0.72	7
		ARG(T)	1.32(0.37/0.28)	0.56	7
		ARG(LB)	1.71(0.12/0.07)	0.64	7
Diesel Soot + Acids	7-Day	Type I	0.97(2.07/2.13)	0.86	5
		ARG(R)	2.0 (0.28/0.14)	0.32	5
		ARG(R1)	2.0 (0.62/0.31)	0.19	5
		ARG(T)	2.11(0.59/0.28)	0.19	5
		ARG(LB)	2.29(0.16/0.07)	0.46	5
Propane Soot + Acids	Immed.	Type I	1.16(2.31/2.00)	0.23	9
		ARG(R)	0.50(0.07/0.14)	0.45	8
		ARG(R1)	1.05(0.21/0.20)	0.93	8
		ARG(T)	0.76(0.41/0.54)	0.54	8
		ARG(LB)	1.29(0.09/0.20)	0.24	8
Propane Soot + Acids	7-Day	Type I	1.14(2.39/2.10)	0.44	10
		ARG(R)	1.20(0.18/0.15)	0.81	8
		ARG(R1)	0.47(0.18/0.38)	0.29	8
		ARG(T)	0.97(0.94/0.97)	0.93	8
		ARG(LB)	1.79(0.52/0.29)	0.23	8
Propane Soot + Acids*	Immed.	Type I	1.32(2.54/1.93)	0.09	8
		ARG(R)	0.36(0.14/0.39)	0.93	7
		ARG(R1)	1.33(0.16/0.12)	0.67	7
		ARG(T)	0.55(0.29/0/53)	0.08	7
		ARG(LB)	1.00(0.09/0.09)	1.00	7

TABLE 5 (cont'd)

Propane Soot + Acids*	7-Day	Type I	1.15(2.56/2.23)	0.25	9
		ARG(R)	0.64(0.25/0.39)	0.37	7
		ARG(R1)	2.25(0.27/0.12)	0.20	7
		ARG(T)	1.77(0.94/0.53)	0.27	7
		ARG(LB)	1.44(0.13/0.09)	0.70	7
Diesel Soot	Immed.	Type I	1.20(2.62/2.19)	0.10	9
		ARG(R)	1.18(0.58/0.49)	0.65	6
		ARG(R1)	1.62(0.73/0.45)	0.27	6
		ARG(T)	1.80(0.63/0.35)	0.06	6
		ARG(LB)	2.18(0.24/0.11)	0.35	6
Propane Soot	Immed.	Type I	1.21(2.66/2.19)	0.13	9
		ARG(R)	1.16(0.57/0.49)	0.73	6
		ARG(R1)	1.53(0.69/0.45)	0.36	6
		ARG(T)	1.63(0.57/0.35)	0.43	6
		ARG(LB)	1.82(0.20/0.11)	0.57	6
Acids	Immed.	Type I	1.12(2.39/2.14)	0.41	10
		ARG(R)	1.27(0.62/0.49)	0.57	7
		ARG(R1)	1.33(0.60/0.45)	0.55	7
		ARG(T)	1.40(0.49/0.35)	0.74	7
		ARG(LB)	3.00(0.33/0.11)	0.23	7

NOTE: *Atmospheric component target concentrations for this study were one-half those of the other studies.

Immed. = Immediate Sac; 7-Day = 7 Day Sac.

ARG = Autoradiography; Type I = Alveolar Lesions; R and R1 are regions of the nasal epithelium; T = Trachea; LB = Lobar Bronchi

produced a significant acceleration in cell turnover (as a result of cell death) in the nasal, tracheal or deep lung epithelia.

The data from the endpoint involving the quantitation of lung cell populations containing soot particles were more interesting (Table 6). The cells containing soot were mostly free cells (i.e., macrophages) which were found in alveolar spaces or against the alveolar walls. The distribution of these cells appeared to be random throughout the lung - although an occasional aggregate of soot-containing cells (between 3 and 8 cells) was found in proximal ducts. There did not appear to be a relationship between cell aggregates and the type of soot exposure (diesel or propane). In two separate experiments rats were exposed to the propane soot + acids atmosphere, and then were sacrificed either immediately or 7 days post-exposure. In both cases the percent of cells containing soot was higher in the group sacrificed right after the end of the exposure. The results of the diesel soot + acids study demonstrate that a considerably lower percent of cells containing soot was found than were seen in the propane soot + acids exposed animals. With regards to the study in which rats were exposed to propane soot alone or diesel soot alone, the lungs of rats receiving the diesel soot had a significantly higher ($p<0.01$, two-tailed t-test) percent of soot-containing cells per cell population than did the lungs of rats exposed to propane soot atmospheres (immediate sacrifice only in this case). (This study was performed using one batch of rats, making it possible to statistically compare the results obtained for the two groups of rats without the complication of a batch effect. All of the other studies were performed using different batches of rats.) We do not consider this observation to be of health significance, however. The acid did not appear to interfere with the clearance of soot particles in this study, but larger numbers of measurements would be required to adequately test this tentative conclusion.

C. Morphometry

Table 7 summarizes the results of the morphometric analyses performed by the USC investigators. The animals exposed to diesel soot alone had greater numbers of elastin fibers ($p<.02$) immediately following exposure than did the control rats, but they exhibited a smaller mean fiber area ($p<.02$). These results suggest that a fragmentation of the fibers has occurred (i.e., a significant increase in the number of elastin fibers without a significant rise in amount of elastin field area). Alveolar wall area, with or without elastin included, also increased significantly ($p<.02$) over the controls. Since the

TABLE 6

Comparison of Carbon Retention in the Deep Lung for Groups of Rats Exposed to Soot-containing Atmospheres.

4-Hour Sacrifice	NO.OF RATS N	CELLS WITH SOOT PER FIELD(SE)	% OF TOTAL CELLS WITH SOOT(SE)	% OF SOOT CELLS HEAVILY LADEN(SE)
Propane Soot + Acids	6	1.28(0.06)	1.20(0.06)	13(3.3)
Propane Soot + Acids[a]	7	1.11(0.11)	0.79(0.07)	10(1.2)
Propane Soot	7	0.72(0.08)	0.60(0.05)	5(1.9)
Diesel Soot + Acids	7	0.75(0.06)	0.51(0.04)	14(2.8)
Diesel Soot	6	1.15(0.03)	1.07(0.05)	17(1.6)
7-Day Sacrifice				
Propane Soot + Acids	7	0.82(0.04)	0.75(0.03)	12(2.7)
Propane Soot + Acids[a]	6	0.97(0.11)	0.77(0.07)	9(1.3)
Diesel Soot + Acids	7	0.63(0.11)	0.48(0.07)	10(1.4)

a - Concentrations of atmosphere components approximately one-half those used in the other studies.

TABLE 7

Summary of Selected Morphometry Data

(Percent Change in Parameter Above Mean Control)

	Evaluation Time	Rt Lung Volume	Elastin Fibers					Alveolar Wall	
			Number	Field Area	Mean Area	Inter-cepts	Peri-meter	Area	Peri-meter
Diesel Soot + Acids	Immediate	3.1	7.4	4.4	-0.6	3.6	5.2	9.0*	9.2
Diesel Soot + Acids	7-Day	4.5	0.7	-12.5*	-12.8*	-8.7*	-8.3*	0.5	2.0
Propane Soot + Acids	Immediate	-4.2	-0.7	2.6	2.5	-0.3	0.9	-3.3	-3.5
Propane Soot + Acids	7-Day	5.9	-8.4	-7.9**	0.1	-6.4	-6.8**	-9.4**	-2.4
Diesel Soot	Immediate	-6.4	21.7*	3.8	-14.8*	21.5*	11.4	17.6*	6.4*
Propane Soot	Immediate	-3.9	-9.5	6.9	15.8**	0.5	-0.4	21.0*	0.8
Acids	Immediate	-10.2*	-0.3	11.7	12.3	9.2	8.7	-1.3	-8.0

Immediate = Evaluation immediately after end of exposure

7-Day = Evaluation 7 days after end of exposure

\- = Decrease with respect to controls

Acids = Sulfuric and nitric

* = Significant at $p<0.05$, two-tailed t-test

** = $.1>p>.05$, two-tailed t-test

difference in lung volume was not significantly different from the control group, we cannot attribute the increase in alveolar wall area to atelectasis. Moreover, the ratio of elastin field area to alveolar wall area differed at a level approaching significance ($p<.07$), which adds support to some alteration of the alveolar wall instead of an elastin change. In addition, alveolar wall area with or without elastin areas included also significantly increased immediately following exposures to diesel soot + acids and to propane soot alone ($p<.001$ and $p<.002$, respectively), but with no other significant differences. For the propane soot alone group, alterations of elastin fell short of significance ($.1>p>.05$).

For the two groups evaluated 7 days post-exposure (diesel soot + acids, and propane soot + acids), the findings were reversed compared to the groups tested immediately after exposure. Specifically, the group exposed to propane soot + acids had decreases approaching significance in elastin field area ($p=.06$), alveolar wall area ($p=.07$), and elastin perimeter ($p=.07$). If one restricts the analysis to the smallest subset of elastin fibers, the decrease in elastin area becomes significant ($p<.04$), suggesting that the very delicate elastin fibers have been preferentially reduced in area. For the diesel soot + acids group there are significant decreases in elastin field area ($p<.001$), mean elastin fiber area ($p<.01$), and mean elastin fiber intercept ($p<.001$). In addition, there were decreases from the control values in the ratio of elastin fiber perimeter to alveolar wall perimeter ($p<.05$), and in the ratio of elastin field area to alveolar wall area, with and without exclusion of elastin area ($p<.001$). In view of the ratio differences, and the lack of statistically significant differences in alveolar wall area and lung volume, a decrease in lung elastin for the diesel soot + acids group is indicated. On turning to the data for the group exposed to diesel soot + acids and sacrificed immediately, only one significant change is found--an increase in alveolar wall area, with or without exclusion of elastin field area ($p<.01$ and $p<.02$, respectively).

D. Macrophage Studies

The results of the experiment performed to examine the effects of the diesel soot + acids atmosphere on the Fc receptors and on the phagocytic capability of rat alveolar macrophages are listed in Table 8. The data indicate that there was a significant reduction ($p < 0.05$) in the binding of sheep red blood cells to the macrophages obtained from the soot and acid exposed group of rats on days 0 (the final exposure day) and 2. This is evidenced by the reduction in the percentage of rosettes formed as compared to

TABLE 8

The Effects of the Diesel Soot + Acids Atmosphere on Pulmonary Macrophages

	Percent of Rosettes Formed on Day D_i After Pollutant Exposure[a]		
Purified Air Controls	D_0	D_2	D_3
28.6±5.9 n=8	16.8±4.2[b] n=5	16.1±1.8[b] n=5	19.1±1.3 n=5

	Percent of Macrophages With Polystyrene Latex Particles on Day D_i After Pollutant Exposure[a]		
	D_0	D_2	D_3
66.1±8.8 n=8	41.1±9.4[b] n=5	53.3±3.6[b] n=5	50.7±2.5[b] n=5

a - Values are the means ± S.D.

b - $p < 0.05$ vs control (two-tailed t-test).

the purified air controls. Consistent with these results, it was observed that there was a significant reduction in the percentage of macrophages from the diesel soot + acids group which engulfed polystyrene latex particles. This effect was also present immediately after exposure and persisted for at least 3 days.

The results from the study which involved exposures to the component parts of the soot + acids atmospheres (diesel soot alone, propane soot alone, and the acid combination alone) are presented in Tables 9 and 10. The acid combination produced a statistically significant reduction in the binding of the sheep red blood cells to the macrophages obtained from the pollutant-exposed group of rats on days 0, 2 and 4. A significant decrease in rosette formation was also noted on days 0 and 2 with rats exposed to diesel soot. By day 4 the percentage of rosettes had returned to normal. The propane soot atmosphere did not produce an effect on rosette formation on days 0 or 2. However, a significant reduction in rosette formation was observed in macrophages obtained from the rats sacrificed on day 4 - indicating the possible presence of a delayed effect. Regarding the effects of the atmospheres on phagocytosis, the acid combination significantly depressed phagocytic activity immediately after the exposure, and the effects persisted through day 4. A similar effect was observed with rats exposed to diesel soot, but in this case the effect only existed in rats sacrificed 2 days post-exposure. The propane soot atmosphere did not significantly affect phagocytic activity at any of the three time-points.

An analysis of variance was also used to compare the results (Fc receptors and phagocytosis) of the diesel soot + acids study to the results of diesel soot alone and acids alone studies. A statistically significant difference in the effects of the three atmospheres was noted. However, the analysis revealed that there was a significant "study" (or batch) effect - i.e., the control values varied greatly between the diesel + acids study and the other two studies. A Newman-Keuls test of multiple comparison was then used, and the results of this test indicated that any differences in the effects of the diesel soot + acids atmosphere and the other two atmospheres were marginal. In other words, both the diesel soot and the acids atmospheres produced effects which were not statistically different from the effects of the diesel soot + acids atmosphere.

TABLE 9

The Effect of Soot and Acid Atmospheres on the Fc Receptors of Pulmonary Macrophages

		Percent of Rosettes Formed on Day D_i After Pollutant Exposure[a]		
	Purified Air Controls	D_0	D_2	D_4
Diesel Soot	44.0±3.6 n=12	18.7[b]±2.8 n=6	35.8[b,c]±3.7 n=4	42.0[c]±3.2 n=4
Propane Soot	46.5±3.8 n=10	44.5±3.7 n=6	46.7±4.5 n=6	38.8[b,c]±3.4 n=4
Acids	42.1±3.7 n=12	12.8[b]±1.7 n=6	23.0[b,c]±2.2 n=4	35.5[b,c]±3.4 n=4

a - Values are the means ± SD.

b - $p < 0.05$ vs control (two-tailed t-test).

c - $p < 0.05$ vs D_0 (two-tailed t-test)

TABLE 10

The Effect of Soot and Acid Atmospheres on the Phagocytic Activity of the Macrophages

		Percent of Macrophages with Polystyrene Latex Particles on Day D_i After Pollutant Exposure[a]		
	Purified Air Controls	D_0	D_2	D_4
Diesel Soot	83.2±2.9 n=12	79.8±4.7 n=6	77.5[b]±3.1 n=4	80.5±2.4 n=4
Propane Soot	76.6±6.9 n=10	76.2±3.7 n=6	72.8[d]±4.3 n=6	69.8±5.9 n=4
Acids	77.0±4.2 n=12	65.0[b]±2.4 n=6	71.5[b,c]±3.9 n=4	71.5[b,c]±2.1 n=4

a - Values are means ± SD.

b - $p < 0.05$ vs control (two-tailed t-test).

c - $p < 0.05$ vs D_0 (two-tailed t-test).

d - Values were obtained on Day 1.

VIII. DISCUSSION

The study utilized over 850 healthy laboratory rats, and involved over a dozen detailed biological assessments. The findings are intriguing from a toxicological point of view. They indicate that the deep lung region was the most affected by the exposures. The fine particles were able to penetrate into the deep lung because of their low deposition efficiency in the nose. Diesel soot (actually dilute diesel engine exhaust), the acid combination (nitric plus sulfuric acids) and the combinations of soot plus acids all affected the deep lung. Most severely affected were lung macrophage cells and the elastic tissue of alveoli. The macrophage changes warn of the potential for immunologic disruption (which could lead to increased sensitivity to infection and to the effects of any of the several particulate air pollutants). The latter change (in elastin) could imply the possibility for development of fibrosis (scar formation) or emphysema (loss of alveolar tissue) upon longer-term exposure.

The results of the particle clearance experiments performed following exposures to the propane soot + acids atmosphere and the diesel soot + acids atmosphere present some interesting similarities and differences. Neither atmosphere significantly affected the early clearance of the tracer particles. However, the propane soot + acids atmosphere produced a large delay in late clearance which did not occur with the rats exposed to diesel soot + acids. One possible reason for this difference is that the propane flame-produced soot + acids atmosphere is considerably "cleaner" than the carbon atmosphere generated by the diesel engine. The diesel exhaust contains much higher levels of nitrogen oxides and carbon monoxide, in addition to various hydrocarbons (aldehydes, ketones, etc.) which are virtually absent in the propane soot atmosphere. The presence of these contaminants in the atmosphere may have produced an antagonistic effect on clearance, thereby counteracting the effect which would have been produced by the carbon and acids alone. Another possibility for the difference in the effects of the two atmospheres is the distinct nature of the carbon aerosol particles themselves. The carbon aerosol particles generated by the diesel engine are much more oily than those produced by the propane flame, and they have a higher fraction of extractable organics. Perhaps, due to this coating, the effects produced in the deep lung were reduced.

The morphometry findings do not permit conclusions about the significance of the changes in elastin fiber number and area. However, the finding of elastin changes, whether observed as increases or decreases, has several

implications. The lung scaffolding in general, and the elastin network in particular, play major roles in the recoil mechanism of the lung. Damage to elastin implies some impairment in respiratory function, at least in terms of functional reserves. Also, repair of damage to the overlying epithelium (alveolar lining cells, and Type 1 cells especially) is contingent on the integrity of the lung scaffolding. An altered elastin network tends to imply less than complete reversibility. With injury to the elastin, a loss of elastin first takes place, as has been demonstrated with both the papain and elastase models of emphysema. However, over the long term, the experimentally induced emphysemas and desctructive diseases of the human lung demonstrate an increase in elastin in association with fragmentation, thickening, and other changes. The reversal in differences between the immediately studied groups and those examined 7 days post-exposure may relate to the biphasic response of elastin to injury. These findings suggest the need for follow-up studies that investigate the physiologic and morphologic consequences of longer-term exposures to the atmospheres.

It is not simple to rank the studied atmospheres in order of their toxicity because each had unique effects. The major disruptions seen, an interference with macrophage surface receptors and phagocytic efficiency, were most severe following the acids and the diesel soot + acids exposures. Depressions were also seen after exposure to diesel soot alone. From a macrophage immune function point of view, the acids alone and the diesel soot alone produced deleterious effects. Several morphometric endpoints were statistically significantly altered. Acids alone and propane soot + acids were the least disruptive of deep lung structure. The most effective of our exposures, with respect to structural disruption, were diesel soot alone and diesel soot + acids.

Finally, due to the similarity of the human and rat respiratory tracts at the cellular level, we would expect similar effects to occur in human populations exposed to the air pollutants in many communities in California. These communities would be those simultaneously impacted by airborne acidity and fine particles (PM_{10}). As more information on the acid content of community air becomes available, the significantly-impacted areas will become more sharply defined.

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APPENDIX A: Cover page of paper by Walters et al.

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A GENERATOR FOR THE PRODUCTION OF SULFURIC ACID-COATED DIESEL SOOT AEROSOLS

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Abstract – Diluted diesel engine exhaust was mixed with sulfur trioxide and the resulting acid–soot aerosol characterized. The generation system, which is suitable for inhalation toxicology studies, was characterized at a soot concentration of approximately 0.5 mg m^{-3} and H_2SO_4 coating levels from 0 to 5 mg m^{-3}. Aerosol characterization included measurement of particle size (cascade impactor + electrical aerosol analyzer tandem sampler) and acid–soot association (electron microscopy of $BaCl_2$-coated sample grids).

The mass distribution of both acid-coated and uncoated diesel soot was unimodal and approximately lognormal. The acid coating increased both the size and monodispersity of the soot aerosol. The mass median Stokes equivalent diameter and geometric standard deviation were 0.12 μm and 2.4 for the uncoated soot, and 0.28 μm and 1.9 at 5.0 mg m^{-3} H_2SO_4. Approximately 95% of the soot particles were coated with H_2SO_4; the remainder appeared uncoated. Some H_2SO_4 droplets without a visible nucleus were also observed.

Key word index: Diesel soot, sulfuric acid, aerosols, air pollution.

INTRODUCTION

The diesel engine meets many needs in the transportation industry and in other areas. However, concern about the effects of emissions from the diesel has led in recent years to an increase in research and regulatory activity. The particulate emissions are of special concern since they play a role in urban area visibility degradation, and may have adverse health effects.

Under certain circumstances, diesel soot aerosols may be expected to be associated with H_2SO_4. In the atmospheric environment, SO_2 may be oxidized on the surface of solid soot particles (Novakov *et al.*, 1974) or in liquid droplets containing suspended soot particles (Benner *et al.*, 1982), with the resultant formation of H_2SO_4. Such particles may exist in urban areas which have both widespread use of diesel engines and significant SO_x emissions. Acidic soot aerosols are also expected to be present in some underground mining environments. About 60% of U.S. mine diesels are estimated to use exhaust oxidation catalysts to reduce CO and HC emissions. Unfortunately, use of the catalyst promotes oxidation of a significant fraction of the exhaust SO_2, resulting in mine H_2SO_4 levels of several mg m^{-3} (French and Mildon, 1984). Some of this H_2SO_4 is likely to be coated on the exhaust soot aerosols.

H_2SO_4-coated diesel soot particles have small aerodynamic sizes and an insoluble core. Therefore, inhalation of these particles may result in sustained contact of H_2SO_4 with deep lung tissues. Since there is the potential for substantial human exposure in the environments above, the inhalation toxicology of this pollutant combination is of concern.

The present work describes an H_2SO_4-coated diesel soot generation system and a limited physical characterization of the aerosol produced. The authors are not aware of any previous publications on the generation of acid-coated diesel aerosols, although other workers appear to have produced an acid-coated propane soot (Britton and Clarke, 1980; Brorström-Lundén and Lindskog, 1985) or acetylene soot (Thomas *et al.*, 1974) in the course of their experiments.

Although the present work is concerned only with the physical characterization of H_2SO_4-coated diesel soot aerosols, we note the increasing evidence for the reactivity of H_2SO_4 with some of the components of diesel exhaust, to produce products of unknown toxicity (Schuetzle, 1983; Wall and Hoekman, 1984).

METHODS AND MATERIALS

A diagram of the system used to generate the H_2SO_4-coated diesel soot aerosols is shown in Fig. 1. The engine (an 817 cm^3 displacement, single-cylinder, direct injection unit) was operated at constant speed (1800 r.p.m.) and load (78% of maximum). The loading method was a direct-coupled generator with a resistive electrical load. Monitoring instrumentation allowed the continuous measurement of intake and exhaust pressures and temperatures, engine r.p.m. and generator load. The fuel used was Phillips No. 2 Diesel Control Fuel, which was purged with N_2 upon receipt, and kept under positive N_2 pressure in a constant temperature environment.

The exhaust leaving the engine was allowed to age for a residence time typical of automotive exhaust systems (0.2 s),

APPENDIX B: Cover page of paper by Prasad et al.

EFFECTS OF POLLUTANT ATMOSPHERES ON SURFACE RECEPTORS OF PULMONARY MACROPHAGES

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The effects of two multicomponent pollutant atmospheres on the surface receptors (FcR) and phagocytic activity of rat pulmonary alveolar macrophages have been studied. FcR are crucial for the macrophages to become cytotoxic against target cells. The atmospheres were composed of pollutants that are prevalent in the South Coast Air Basin of southern California. Rats were exposed nose-only to a 7-component oxidant- and sulfate-containing atmosphere for 4 h/d for either 7 or 21 consecutive days. In another experiment rats were exposed 5 h/d for 5 consecutive days to another pollutant combination—acid droplets plus carbon-containing dilute diesel engine exhaust. In both experiments matched rats were exposed nose-only to purified air to be used as controls. Each of the atmospheres studied significantly reduced FcR activity for at least 3 d following the exposure, with the group of rats exposed to the 7-component atmosphere for 21 d exhibiting the most pronounced effect. Macrophages from rats exposed to the diesel exhaust plus acid atmosphere and the 7-component atmosphere for 7 d had significantly reduced phagocytic activity for at least 3 d postexposure, while the macrophages from rats exposed to the latter atmosphere for 21 d had phagocytic activity near control values. The decrease in phagocytosis and inhibition of FcR of macrophages suggests an impairment of macrophage function that probably renders the host vulnerable to bacterial and/or viral infections.

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13. Type of Report & Period Covered
Final Report

14.

15. Supplementary Notes

16. Abstract (Limit: 200 words)

Rats were exposed nose-only for 5 hours/day for five consecutive days to different atmospheres which included: 1)diesel exhaust, nitric acid, and sulfuric acid; 2) diesel exhaust; or 3) nitric and sulfuric acids in order to examine the effects of the mixtures and of the individual components. Matched control groups of rats were exposed to purified air following the same schedule. The target concentrations of pollutants were: 0.5mg/M^3 of soot in diesel exhaust; 0.35mg/M^3 of HNO_3; and 0.15mg/M^3 of H_2SO_4.

The evaluation of potential toxic effects on the respiratory tract included: a) measurement of clearance using tracer particles; b) microscopic examination for histopathology and soot accumulation; c) auto-radiography for increased cell turnover; and d) measurement of Fc receptor and phagocytic activity of pulmonary macrophages to assess functional changes.

None of the pollutant atmospheres altered particle clearance rates nor did they cause histopathologic changes or increased cell turnover. However, soot accumulation was increased in rats exposed to the diesel exhaust when compared to rats exposed to the mixture of diesel exhaust and acids. In addition, the mixture caused a significant inhibition of macrophage functions. We conclude that short-term exposures to diesel exhaust may elicit adverse effects in the respiratory tract and further studies are necessary to clearly understand the influence of acids on diesel exhaust effects.

17. Document Analysis a. Descriptors

Diesel Soot, Diesel Exhaust

b. Identifiers/Open-Ended Terms

Macrophages, Toxic Effect

c. COSATI Field/Group Toxicology/Health Effects

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