Air Pollution Health Effects Laboratory
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SULFATE, NITRATE INHALATION TOXICITY

FIRST ANNUAL REPORT

November 16, 1974 to December 31, 1975

for the

State of California Air Resources Board

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edited by

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I. Summary of Progress to December 31, 1975

This first year-end report describes progress made between November 16, 1974 and December 31, 1975. Two major categories of effort are described: a) the start-up of the new health-effects facility (Section II of this report); and b) scientific progress (Sections III, IV and V). Sections on Personnel, status of budget, Results and Discussion and Appendices are also included.

The research is conducted in a 2,200 square foot laboratory building on the North Campus of the University of California, Irvine. The facility, consecutively called SNIF for "Sulfate, Nitrate Inhalation Facility" and IRF for "Inhalation Research Facility" was re-named "Air Pollution Health Effects Laboratory (APHEL). The new name, selected in consultation with University administrative officials, more clearly reflects the current research programs and is hopefully more meaningful to the University community and to the public. The official opening under this name, held May 2, 1975, was attended by University administrators and representatives of the California Air Resources Board. Additional "open-house" ceremonies were held for University personnel and for the general public.

The completed laboratory contains a central exposure room with four main exposure chambers (University of Rochester type, each one cubic meter in volume, Reference: Leach et al., 1958) which are suitable for handling gaseous and particulate atmospheres of varying toxicity including urban and industrial air pollutants, corrosive mists, pathogens and radioactive materials. Small laboratory animals are exposed inside the chambers in any of several modes including: free in wire cages, inside "nose-only" exposure tubes or in body plethysmographs. Larger animals, including human subjects, dogs and pigs can be exposed outside of the chambers to pollutant atmospheres through flexible stainless steel hoses leading to masks. Supporting facilities in the building include a large air purification system, a very complete aerosol laboratory, a gas laboratory, physiology and histopathology laboratories, an animal housing wing, a PDP-11 mini-computer, radiation detection equipment, a small shop, office space, storage space, a small photographic darkroom, and a cage washing area. Basic equipping and staffing are complete and a relatively well-coordinated research program is in progress.

Several problems identified in previous quarterly progress reports have seen satisfactory resolution. A license for use of radioisotopes was obtained in April, 1975 (Appendix A), modifications to the animal housing wing (sloped and drained flooring, impermeable wall coating, area caging, and additional air-cleaning equipment) have been made that not only meet the recommendations of the American Association for Accreditation of Laboratory Animal Care but also anticipate future requirements for provision of exercise and animal-to-animal contact.

Scientific progress has been made in three areas: 1) development of animal methods; 2) development of pollutant atmospheres; and 3) animal exposures to pollutant atmospheres. The laboratory presently works with two species, rat and dog, in order to avoid drawing conclusions that may be limited to a single species.

Experimental Animals

The rat is used because it is relatively inexpensive, is known to respond to several common air pollutants, and because of the large available data base with respect to behavior, housing, handling and biologic parameters. Rats are primarily used in sacrifice studies where tissues must be examined to determine effects.

The dog as used in our laboratory is a trained, cooperative subject, each individual known by name, and having an always-growing laboratory record of her own normal biologic parameters and individual responses to air pollutants. The dogs are not used in terminal studies. Dogs make good subjects for several reasons: 1) they appear to have small airways (a critical site for several natural and environmentally induced diseases) that are quite similar anatomically to those of man; 2) they have a range of variability in response similar to man; 3) a large physiological data base exists for the dog; 4) they are exceptionally cooperative so that fairly difficult "clinical-type" tests may be performed; 5) the young, developing dog may eventually be used as a laboratory model for the young developing child; 6) the dog is a convenient size, near enough to man so that similar, often identical, equipment can be used for both; and 7) the dog is a reasonably economical subject with respect to purchase, care and housing.

Experimental Methods

Methods developed for these two species were aimed at detection of changes within the respiratory system that are relevant to pathogenesis of chronic respiratory disease, to aggravation of disability in subjects with bronchitis, asthma or emphysema and to short-term discomfort or disability in normal subjects exposed to a 1 or 2 day episode of moderate to severe air pollution.

Abnormal distribution of gases and impaired entry or release of gases in the lung induced by bronchial constriction or interstitial edema are tested in dogs by nitrogen washout and in rats by radioxenon wash-in and washout. Abnormalities of this type would be expected to contribute to disability in subjects with pre-existing disorders of ventilation during brief exposure as in a one or two day air pollution episode. Altered clearance of inhaled particles may occur during brief exposures and would enhance the risk of infection in the lung in normal as well as in previously impaired subjects.

In this first year we have: 1) finalized and repeatedly used an exposure system for dogs that allows us to expose calm, unanesthetized animals, via a comfortable mask for periods in excess of 4 hours; 2) adapted pulmonary function tests to the dog including measurements of FRC, rate, tidal volume and multi-breath nitrogen washout, a sensitive indicator of small airways status; 3) established normal values of pulmonary function as measured by this method in dogs; 4) discovered a range of individual responses of our dogs to a brief exposure to 0.6 ppm ozone; 5) generated initial data for the effects on dogs of other levels of ozone and of some sulfate aerosols; 6) designed, built, tested and used a radioactive-gas wash-in/washout manifold for examining lung function in rats; 7) performed initial tests of deposition and clearance of radiolabeled, monodisperse, near micron-sized aerosols in rats; 8) obtained initial data on the effect of ozone and salt aerosols in rats; 9) evaluated the miniature pig, using ozone, as a laboratory subject in air pollution studies; and 10) set up and used a procedure for precise, controlled fixation of rats' lungs for pathologic study.

Experimental Atmospheres

In establishing methods for generation of aerosol/gas pollutant atmospheres the critical requirements include: 1) elimination of unwanted contaminants; 2) precise control of amounts of materials in the exposure atmosphere; 3) control of aerosol particle size in the 0.1 to 1.0 (mass median) micrometer diameter

range; 4) ability to measure exposure levels at the point of inhalation, i.e., in the breathing zone; and 5) ability to control environmental temperature and humidity during exposure. Our first-year accomplishments in this phase include: 1) ability to generate, characterize and control aerosols of sodium chloride, ferric sulfate, ammonium sulfate and sulfuric acid mist in the micrometer and submicrometer range at maximum concentrations that do not lead to aggregation of particles with resultant loss of control over particle size, i.e., concentrations of up to 7 mg/m^3; 2) establishing routine capability for aerodynamic and electron microscopic sizing of aerosols; 3) ability to control exposure levels at 40% to 80% relative humidity at 65-72°F; 4) virtual elimination of unwanted contaminants from exposure atmospheres; 5) ability to precisely monitor gases and aerosols in the breathing zone of animals; and 6) capability to repeat exposures under identical exposure conditions.

Experimental Observations

This past year, we have exposed rats and/or dogs to the following atmospheres: clean filtered air, ozone, sodium chloride, ferric sulfate, ammonium sulfate, and some gas/aerosol combinations. Results, though mostly preliminary, are beginning to emerge. Tentative conclusions that are possible at this time are subject to confirmation but include: 1) ozone at 0.6 ppm for 2 hours does produce a temporary effect in the dog that can be interpreted as a ventilatory impairment; 2) at the high mass concentration studied (3-4 mg/m^3), while submicron salts of NaCl and ferric nitrate do not appear to impair ventilation, ammonium nitrate may; 3) the range of response from individual to individual is large and a subject animal that has a large response to ozone may or may not have a large response to particulates; and 4) the only combination aerosol and gas for which data analysis is complete (ozone + NaCl in rat) indicates no potentiating effect due to the presence of an inert aerosol.

Comment

In brief, we are on schedule in overall progress and revisions to the project flow diagram (Figure 1) have been made only on the basis of negotiated changes in the order of some tests. Our successes are due to intense efforts by APHEL personnel, the high level of support from University administrators, faculty and staff and the close cooperation and support of individuals in the State of California Air Resources Board. We have, over the year, held weekly meetings with all project staff, many of which were also attended by ARB personnel. We feel that the present atmosphere for our research is highly

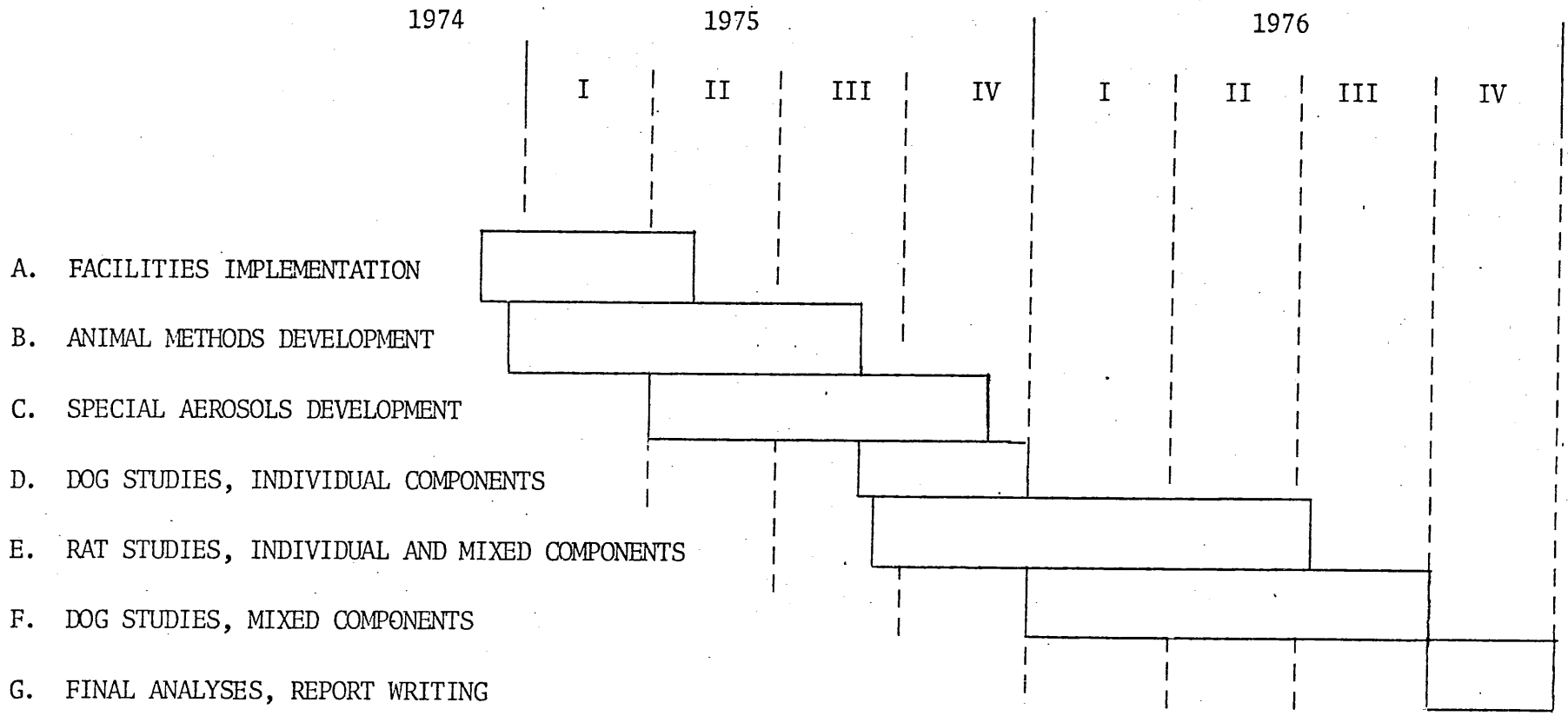


Figure 1. Flow sheet of research for California Air Resources Board, being performed at the University of California, Irvine Air Pollution Health Effects Laboratory

productive and look forward to providing major input to the methodology of laboratory investigation and to decisions on abatement of air pollutants.

As researchers, we are obligated to maintain contacts within and outside of the scientific community. A summary of professional activities is included as Appendix B. During the past year we have seen several visitors from the toxicology discipline at our laboratory including: Andrea Bianco, M.D. (research toxicologist from the Italian Atomic Energy Agency in Rome); Gunter Oberdoerster, D.V.M. (inhalation toxicologist from the Institute for Aerobiology, Germany); Walter Tyler, Ph.D., D.V.M. (Director, California Primate Research Center, UC Davis); Donald Dungworth, Ph.D., D.V.M. (Chairman, Veterinary Pathology, UC Davis); Douglas MacEwen, Ph.D. (Director of the Toxic Hazards Research Unit, Dayton, Ohio); Donald Hausknecht, Ph.D. (Manager of Environmental Systems for Scientific Associates, Inc., El Segundo, Ca.): Richard Ziskind, Ph.D. (in environmental health modeling at Scientific Associates, Inc.); Seung Han Lee, M.D. (Catholic Industrial Accident Hospital, Seoul, Korea); and researchers from inhalation groups at San Diego State University, Rancho Los Amigos Hospital in Downey, and from the Inhalation Toxicology Research Institute in Albuquerque, New Mexico.

II. Facilities

A. Furnishing of the Facility

Furnishing of the 2200 square foot facility has been completed with the installation of chambers, flooring, a mini-computer, a fume hood, a burglar alarm and laboratory and office furniture. The floor plan, shown in Figure 2, indicates the present space assignments.

Chambers

Though chambers of the University of Rochester design have already proven their utility and reliability in animal inhalation studies, any new exposure system must be tested with respect to its operating characteristics before meaningful studies can proceed. Accordingly, initial tests were performed to insure suitability of the chambers for our purposes. Criteria established for evaluation of chamber system performance included:

1. Proper air-flow rates with simultaneous proper operating pressures in the Rochester chambers. Animal exposures to gas/particle air pollutant atmospheres require operation of the chambers at a pressure of minus 0.1 to 0.5" of water over a range of about 2 to 25 cubic feet per minute air flow. Negative pressure in the chambers is required to prevent leakage of exposure atmospheres into the laboratory proper.

2. Stability of flows and pressures during operation of the chamber system. Proper air flow and chamber pressure must be maintained over the time period of animal exposure (up to 8 hrs per run). Few, if any, adjustments in flow-controlling equipment should be required during a chamber run.

3. Negligible leaks in the system. Leakage of exposure atmosphere out of the chambers is prevented by negative pressure within the system. Leakage of room air into the chamber must also be minimized to prevent alteration of exposure environments.

4. Characterization of gas velocity profiles in Rochester chambers. The velocity of air should be relatively uniform throughout the chambers to insure uniform pollutant exposure and proper removal of heat, CO_2 and ammonia from the vicinity of the animals.

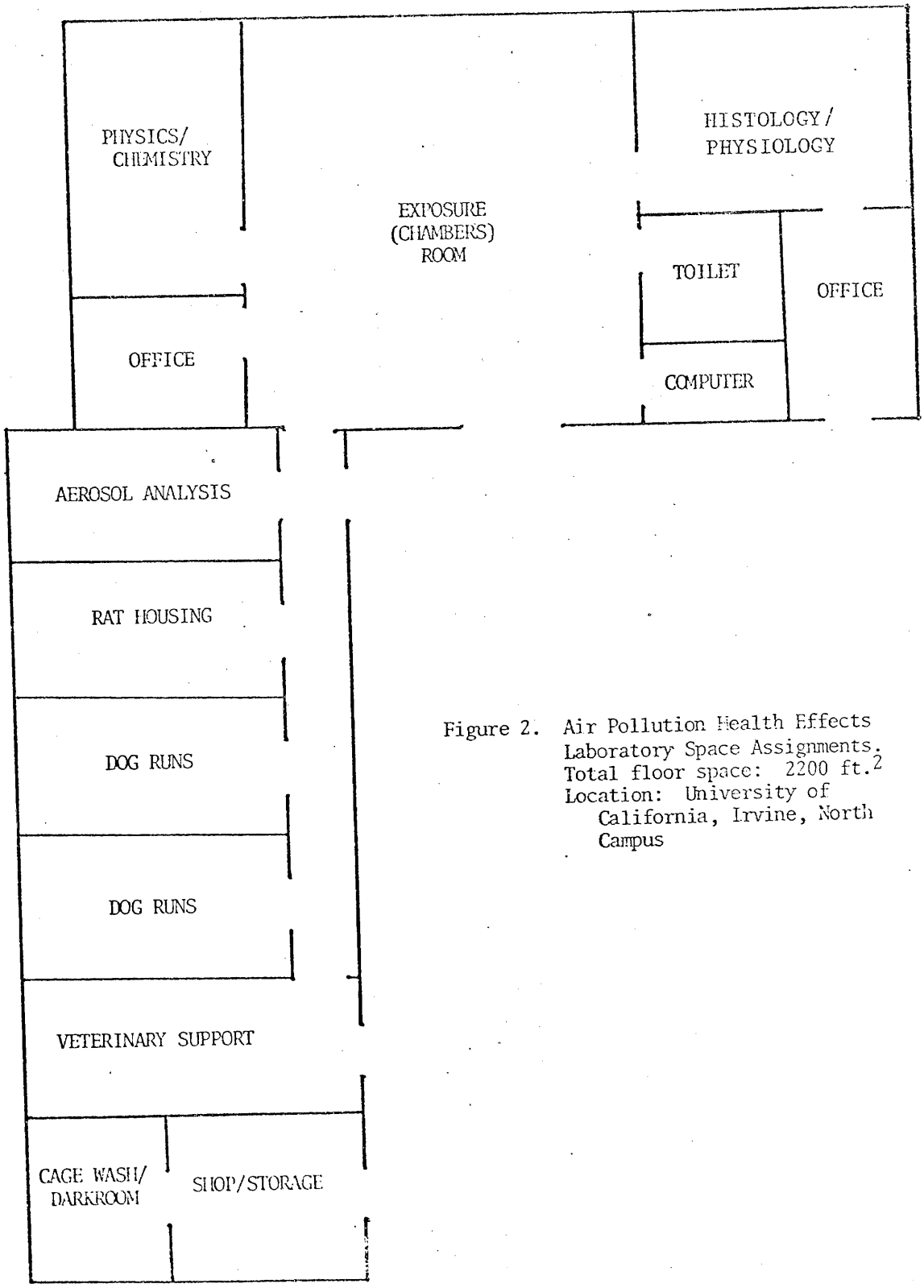


Figure 2. Air Pollution Health Effects Laboratory Space Assignments. Total floor space: 2200 ft.^{2} Location: University of California, Irvine, North Campus

5. Specification of losses of aerosols to walls and surfaces of chambers. Electrostatic, gravitational, inertial and diffusional forces cause losses of particles to chamber and cage surfaces. The rate of loss of particles during chamber operation should be known.

6. Negligible background level of aerosol particles and contaminant gases in chambers under operating conditions. The air-purification system must reduce unwanted contaminant airborne pollutants to levels that will not interfere in any way with the controlled exposure of animal subjects.

7. Homogeneous distribution of exposure aerosols and gases in chambers. Each animal within a chamber should be exposed to the same level of pollutant gases and aerosols. Uniform distribution of aerosols is more difficult to achieve than uniform distribution of gases due to the lower diffusional velocities of aerosol particles. This factor justifies the use of the Rochester-type chamber design, which has an upper mixing section.

8. Significant efficiency of aerosol deposition in animals in chambers. Each animal, inside a cage within a chamber, must have significant deposition of aerosol particles in the respiratory tract. Effective deposition of aerosols in animals during chamber operation must be demonstrated.

Each of the above items requires a specific test. Table I summarizes the current status of such tests, including the testing method and results.

Floors

Floors in the chamber room, washroom, and entire animal housing wing have been covered with Crossfield Products' "Dexotex-Neotex" industrial floor, which has a non-skid, sand-entrained surface. The walls received a 6" covering of the same material. This flooring, covered with epoxy paint, is disinfectable, waterproof, non-skid when wet and resistant to attack by common solvents and corrosive chemicals. Remaining floors have been covered with 1/8" thick vinyl-asbestos tile.

Table I

Current Status of Exposure Chamber Tests

Criterion	Method	Results or Comments
1. Proper flows and pressures in chambers	Observe pressure manometer and flow gauge readings under operating condition	Chambers operate at proper flows and pressures
2. Stability of proper flows and pressures	Observe gauges during simulated 8-hour chamber run	Stability within proper limits maintained without need for adjustments for 8 hour operation
3. No leaks in system	Compare airflow rates through intake manifold and exhaust lines	Leaks are negligible
4. Uniform gas velocity profile in chambers	Read with anemometer probe	Test postponed, acceptable distribution of gas seen in Test 7, below
5. Low background levels		
a) contaminant aerosols	Weigh absolute fiber filters before and after sample during simulated chamber run with and without absolute filters in place	Contaminant aerosols are negligible with chamber filters in place
b) contaminant gases	Analysis of gas samples during simulated chamber run	Performed for O_3, NO, NO_2 and SO_2: level for each below 10 ppb
7. Good mixing of exposure atmosphere in chambers		
a) aerosols b) gases	Measure three age distribution functions, $E(\theta)$, $I(\theta)$ and $\Lambda(\theta)$ for test aerosol and gas	Good mixing of gases and aerosols
8. Known deposition efficiency of test aerosol in rats inside chamber	Run chambers with test aerosol with caged rats present, dissect animals and assay respiratory tract and fur for aerosol material	Material found on fur, in nose and in lung in the following ratios 37:1:1.7

Computer

The mini-computer, a Digital Equipment Corp. PDP 11/10, arrived in early July. A Teletype Corp. Model 33 terminal was rented from on-campus for use with the PDP-11. Since the teletype terminal is equipped with a paper tape reader/punch, we have the capability to generate, store and execute programs for data analysis. The capability is now being utilized in programs dealing with particle analysis and physiological data. A Digital Equipment Corp. LA 30 Decwriter arrived in September and is presently interfaced for high-speed data output. The computer is interfaced to several pieces of pulmonary function equipment and serves to directly receive and process data.

Burglar Alarm

An Intruder Alarm System was completed on July 11. The system, a Dynaplex-2000, delivers a silent alarm directly to UCI campus police upon unauthorized entry outside of normal working hours.

B. Housing of Animals

The goals of the project necessitate housing of dogs for longer than one year and of rats for up to two months. The original design and subsequent modifications to the animal holding wing have been aimed at achieving healthy, comfortable living conditions for the animals as well as assuring full accreditation by the American Association for Accreditation of Laboratory Animal Care.

Original modifications to the animal holding wing necessary to assure conformance with recommendations for the housing of animals set forth in Guide for the Care and Use of Laboratory Animals (DHEW, 1972) were identified. Drains were installed in all animal holding rooms and adequate waterproofing of the existing wall structures in the animal rooms was accomplished by the application of Cross-Gard Wallcote, a two-component resin wall surfacing. Hot and cold water hose bibs were installed in the animal rooms. Further modifications for beagle housing (2 rooms) included uniform sloping floors of ¼" per foot to the drain located in the rear of each room and addition of kennel runs.

The dog runs were designed with both AALAS regulations and the comfort and safety of the dogs in mind. The kennel runs manufactured by the Bob Long Environmental Systems (Gambrills, Md.) (Fig. 3) were selected for the housing

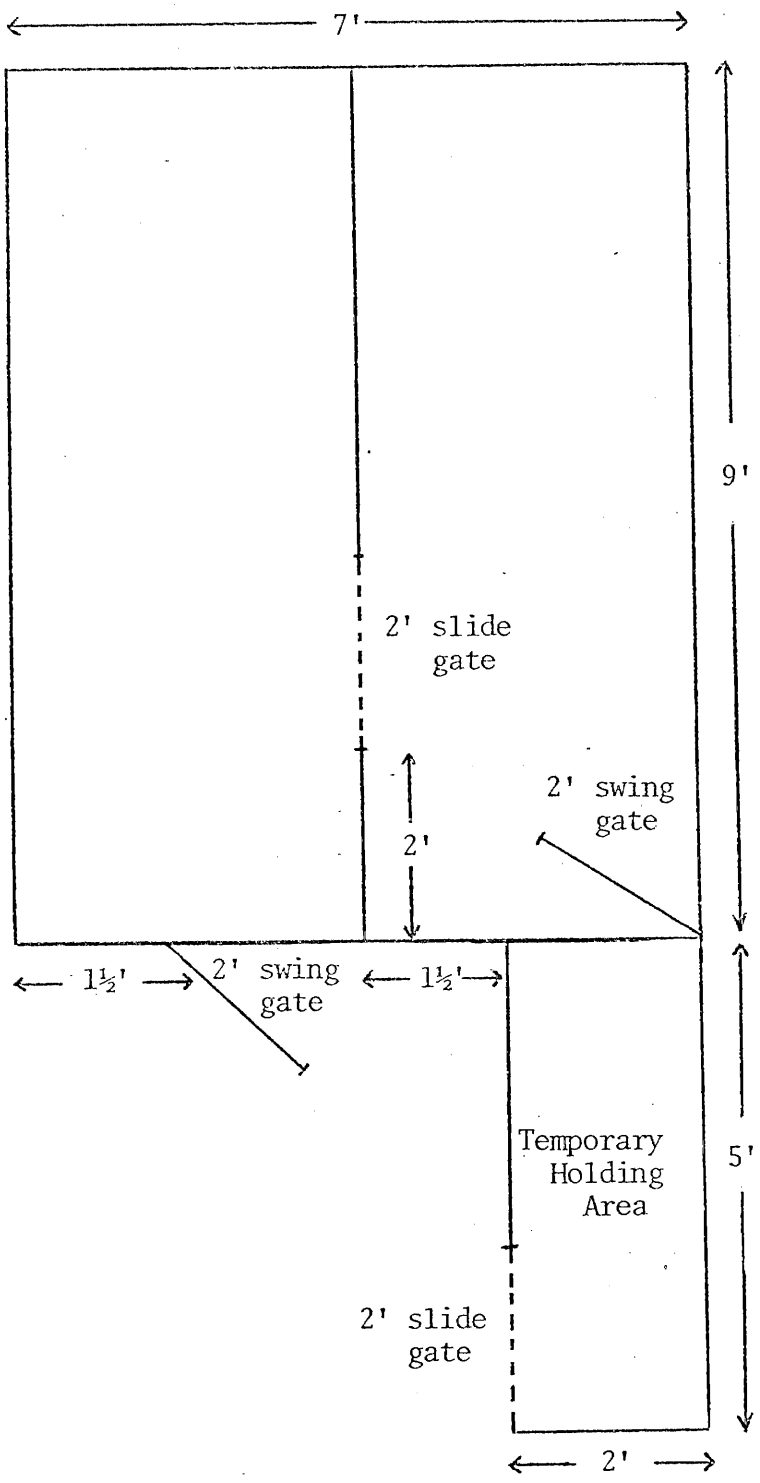


Figure 3. APHEL indoor caging system installed for dogs. The runs are designed to allow room for exercize and to permit animal to animal contact.

of dogs. The caging is constructed of flexible 1¼" wire mesh panels 5' tall and of varying lengths to fit laboratory needs. All wire ends are folded at both top and bottom to provide a safer environment for the animals, and self-locking latches are provided on the swing and slide gates, adding to the efficiency of the system. The panels rest on 1" extensions (legs) to provide easy cleaning access. The dogs may be moved at any time from the main area of the run to the smaller extension of the run (shown in the lower right of the diagram) to facilitate cleaning or other procedures.

Laboratory rats are housed at the facility in stainless steel hanging cages fitted with wire-mesh bottoms (20 cages per rack). Previous concern for the respiratory health of these animals appears to have been alleviated by the arrival of recirculating air filtration units (Dexon, Inc., Minneapolis, Minn.), which remove essentially all particulates and most vapors from the animal room. Rock salt has also been substituted for conventional wood shavings bedding as a deterrent to bacterial or fungal growth. To our knowledge the use of rock salt is unprecedented and we are attempting to define any adverse aspects of this procedure. Presently, the facility has the capability of housing 200 adult rats.

C. Purified Air System

The purified air system was designed to chemically and physically scrub pollutants from air supplied to the four Rochester chambers. The system creates a clean reference air into which known quantities of pollutants can be injected and their biologic effect monitored without interfering effects from unwanted contaminants in the experimental atmosphere.

The design parameters for reference air are:

Air flow	0-100 standard cubic feet per minute (scfm)
Air temperature	72°F
Humidity	40-80% RH, variable and controllable (± 5%)

Contaminant	Probable maximum Inlet Conditions	Outlet (to Chambers)
nitric oxide	1 ppm	10 ppb
nitrogen dioxide	1 ppm	10 ppb
hydrocarbons (as C_2H_4)	100 ppm	500 ppb
carbon monoxide	100 ppm	2 ppm
sulfur dioxide	1 ppm	50 ppb
particulates (DOP)		.003%/.3 um
ozone	500 ppb	10 ppb
ammonia	10 ppm	2 ppb

Component Status (refer to Fig. 4)

1. Humidity and Temperature Control. Humidity and temperature control have been accomplished by a BEMCO PTHS Air Servo (Pacoima, Ca.). This unit has a refrigerator-drier and deionized water injection for humidity control plus heaters for temperature control. The Air Servo was designed with the capability of handling either inside or outside air. Incorporated into the design of the Air Servo is a Barneby-Cheney (Columbus, Ohio) activated charcoal filter which catalytically reduces ozone and adsorbs hydrocarbons remaining after treatment in previous components. Presently, preliminary calibration has been performed on the unit. The unit allows control of temperature in the Rochester chambers between 50° and 100°F and relative humidity between 40 and 80%.

2. Purafil Module. The module has arrived and has been filled with Purafil medium. The medium consists of activated alumina molecular sieve pellets impregnated with potassium permanganate oxidant (Purafil Inc., Chamblee, Ga.). It removes NO, NO_2, SO_2, most hydrocarbons, NH_3, and some CO by adsorption, absorption and oxidation.

The system itself consists of two parallel filter beds. Each bed has greater than one effective bed length when operated solely, but dual operation insures prolonged operation in the desired efficiency range and diminishes the probability of pollutant "breakthrough" during a chamber run. The unit has been temporarily connected to the intake manifold of the Bemco Air Servo until the catalytic oxidation unit arrives. At that time, the oxidation unit will be installed between the Purafil module and the Air Servo.

3. Catalytic Oxidation Unit. Catalytic oxidation is desirable for efficient removal of carbon monoxide as the oxidation reaction between the potassium permanganate in Purafil and CO is relatively slow. Englehard Corporation successfully bid on the catalytic oxidation unit last February. Although a disagreement between Englehard and the University regarding the terms of purchase has been resolved, further delay in arrival of the unit has resulted from a misunderstanding relating to the physical placement of the unit, necessitating a slight revision in the construction plans. Revised drawings for the unit have been approved by this laboratory and returned to Englehard Corporation. As of this date, delivery is expected within the next two months.

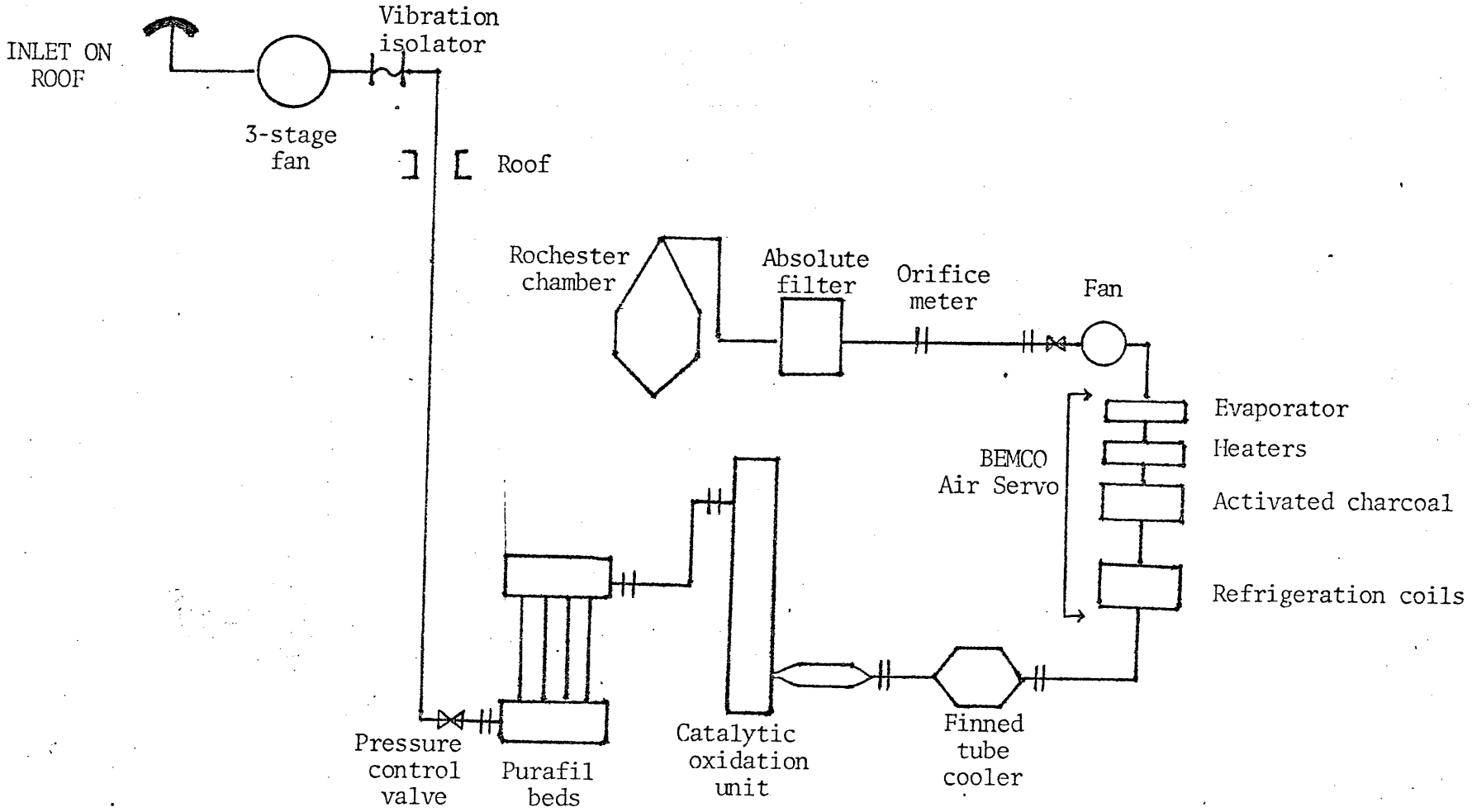


Figure 4. Pure Air System for Air Pollution Health Effects Laboratory

Currently, permanent stainless steel pipe has been installed from the Bemco Air Servo outlet to the Rochester chamber inlets. The system is complete at this time for all practical purposes except for absolute removal of CO from the ambient air. Since ambient levels of CO are not generally great in the Irvine area, animal exposures to controlled experimental atmospheres have proceeded.

D. Animal Wing Air Conditioning

Presently, air quality is maintained in the animal housing wing by individual Whirlpool room air conditioners, portable fans and recirculation of air through air-cleaning modules fitted with absolute particle filters and chemical filtering media. To eliminate the large time commitment and labor associated with this present system and to improve the constancy of the environment we are in the process of procuring a single, central air-conditioning system. The new system, scheduled for completion in May, 1976, has chemical and particulate filters incorporated into its design. The criteria for design of the system, developed in consultation with UC Irvine and UC Davis Veterinary School personnel, are as follows:

Temperature	68-74°F (controllable)
Humidity	greater than 20%, year-round
Particulates	removal efficiency at 99.97% or greater at 0.3 um diameter and above
Gases controlled	CO, NH_3, NO, amines, NO_2, hydrocarbons, SO_2, ozone

The main component of the system will be a commercially-available heating and cooling unit (Fig. 5). The cooling coils will operate continuously while the heaters will be used only when the thermostat calls for heat. The air passing over the cooling coils will be lowered to a temperature of 55°F and this will be the maximum dewpoint experienced.

Air purification will be achieved by a chemical filter composed of Purafil medium. Purafil consists of buffered potassium permangenate impregnated on activated alumina pellets. It is an effective "odor oxidant" which can remove a wide range of polar and organic molecules through absorption, adsorption, and oxidation. The purification unit will be downstream of the air conditioning unit so that outside air will be cleaned before entering the cage rooms.

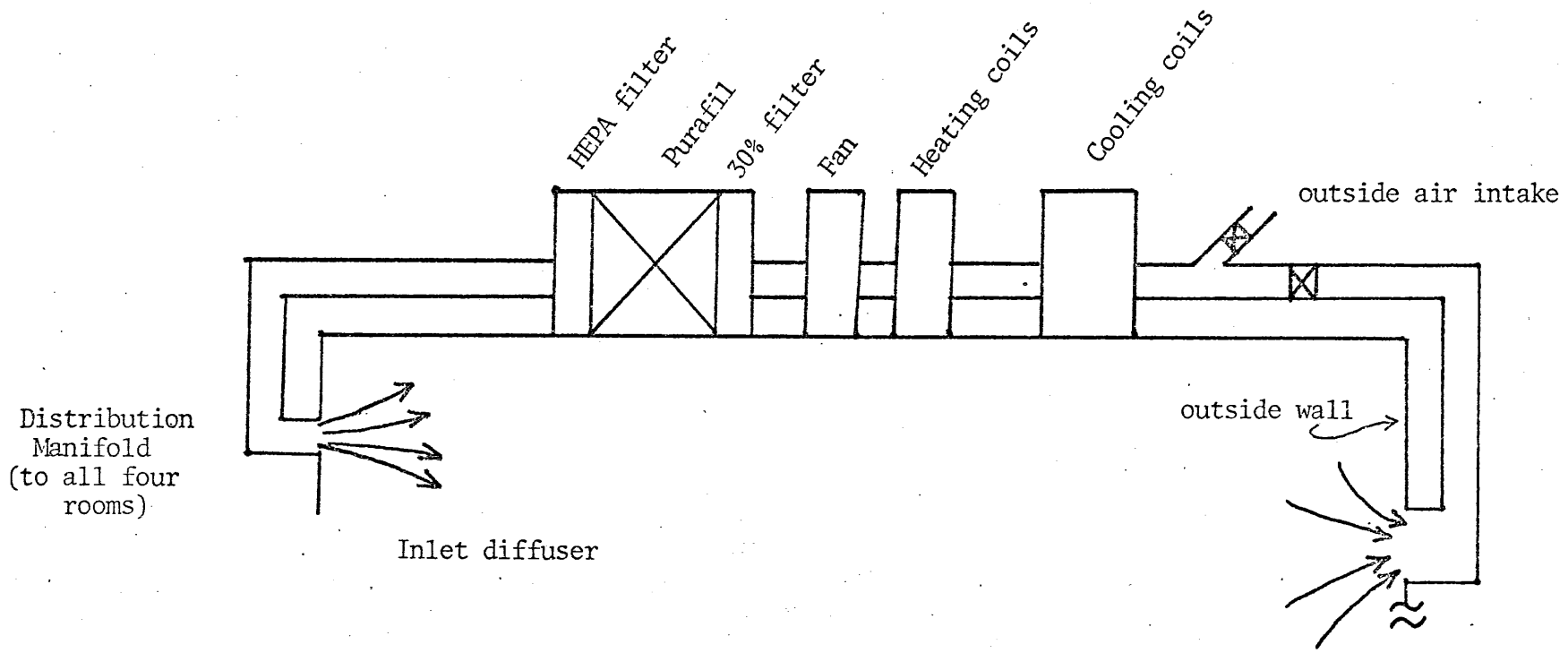


Figure 5. Side View of Upgraded Air-Conditioning in Animal Housing Wing.

Particulate matter will be removed in two stages. First, there will be a "30% gross filter" which will remove large particulates and protect the Purafil from blinding. The second stage will follow the chemical Purafil filter. This filter, a HEPA type, will operate at nearly absolute efficiency for particulates 0.3 μm diameter and above.

III. Animal Methods

A. Animal Handling

The importance of animal quality in establishing reliability and credibility of the project cannot be overlooked. It appears that the quality of the supplier is a critical factor in acquisition of acceptable animals.

Laboratory rats have been obtained from two major sources in an attempt to locate an animal fit for pulmonary research. The original source selected was Blue Spruce Farms, Inc. (Altamont, N.Y.), which provided male BLU-Long Evans hooded rats. More recently, specific-pathogen free Sprague Dawley rats have been procured from Hilltop Lab Animals, Inc. (Chatsworth, Calif.). Male rats weighing between 175 and 195 grams are brought directly to the facility in specially filtered shipping containers and housed in a rat room provided with a laminar flow air purification system containing an absolute particulate filter and a pelletized chemical absorbant filter (Purafil).

Newly arrived animals are assigned a unique, sequential number and are toe-clipped by standard methods. The procedure allows quick identification of each individual. The rats are then housed in stainless steel hanging cages and randomly placed in an experimental protocol. Each animal's weight is recorded and subsequent growth is followed.

Laboratory rats are rigorously maintained to diminish the possibility of respiratory infection. Besides the resident pure-air system, the animals' caging is completely changed and the room disinfected once per week with a rotational change to sterilized caging once every three weeks. Conventional bedding in the rat trays has been replaced by rock salt, which mixes with excreted urine to create an environment undesirable to bacterial growth. Routine mopping also aids in the prevention of particulate contaminants.

Criteria for acceptable dogs were established early in the program and appear to have been met satisfactorily by Laboratory Research Enterprises, Inc., Kalamazoo, Michigan. The animals were to be purebred female Beagle dogs, relatively matched in weight and size with no physical defects and a clean respiratory history. Complete medical histories on each dog accompanied the animals to insure appropriate cognizance of each individual and her individual care schedule. Animals arrived already tattooed for identification purposes and with a temporary debarking performed by the supplier at the age of five months.

New dogs are quarantined for seven days to allow acclimation to the new environment as well as confirmation of the animal's health. Weight and body temperature of each animal are recorded and are measured every other day thereafter. The animals are housed in kennel runs which are cleaned three times daily; the caging is scrubbed and disinfected once per week. The caging system permits exercise and animal-to-animal contact.

The cleanliness of the individual dogs is difficult to maintain in a laboratory atmosphere, but appears to be in control with shampooing all animals and cleaning each individual's ears once per week.

Dogs are maintained on a commercial dry diet which is supplemented by canned-meat wet food once per week. Each animal receives vitamins daily (Norden Laboratories, Lincoln, Neb.) to insure a properly balanced nutritional program.

Routine mopping and air-purification modules also aid in maintaining a healthy environment for the dogs.

B. Exposure System for Dogs

1. Mask for Dogs

The exposure mask used for dogs is made by us using a liquid latex molding compound. The basic design of the mask was taken from a successful dog mask loaned to us by Joseph Mauderly, D.V.M. (Inhalation Toxicology Research Institute, Lovelace Foundation, Albuquerque, New Mexico). The latex portion of the mask is placed around a section of a 500 ml polyethylene bottle. The mask is secured to the dog by cloth straps with plastic fasteners that fasten behind the head (Fig. 6). Originally, the mask rubber was made about 1 mm thick for durability and to create an effective seal around the dog's muzzle. It was found, however, that the mask interfered with venous blood return flow and caused noticeable swelling of the dog's muzzle when worn for several hours. Thinner masks (about ¼ mm) were then made. The mask currently being used is thinner, does not cause swelling of the dog's muzzle, and with the aid of water-soluble sealing gel is as effective as the original mask. The dogs appear to accept the thinner masks & have worn them for several hours without any indication of discomfort and without interference with physiologic functions.

2. Restraint for Dogs

When conducting lengthy exposures (several hours) on unanesthetized Beagle dogs, it is necessary to partially restrain the dogs in a natural standing

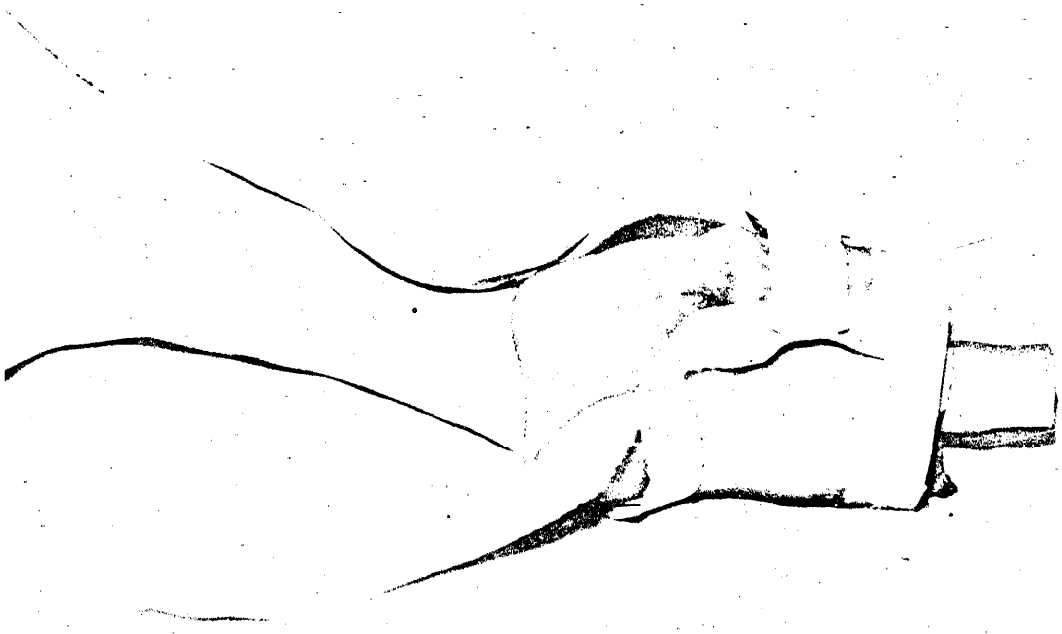


Figure 6. Mask for unanesthetized dog used in exposure to air pollutants and in pulmonary function testing. The latex mask, built around a 500 ml polyethylene bottle, is secured to the dog by straps.

position with no restriction on breathing. Two methods have been developed to accomplish this. The first method is the use of padded supports that enclose the legs and gently restrain the dogs (Fig. 7). The second method of restraint developed is an adjustable cotton sling mounted on a mobile platform (Fig. 8). The sling is equipped with adjustable restraining straps for the dog's front legs and across the dog's back. These straps are only necessary during a training period of approximately 1-2 weeks. After this acclimation the dogs accept the sling well enough so that the restraints are not necessary. The height of the sling is adjusted so that each dog is in a natural standing position with a minimum amount of restraint. After the dog has become accustomed to standing quietly in the sling, it is lowered somewhat so that the dog's weight is supported by its legs and not resting on the chest.

Experience with the sling and stocks indicates that the sling is the better of the two methods. While in the sling, the dogs appear to be more comfortable and are less difficult to handle. The sling, at this time, is the only restraining device used during exposures.

C. Pulmonary Function Testing in Dogs

Pulmonary function testing as a method of ascertaining exposure effects is being performed on all dogs at the Facility. The tests include nitrogen washout , tidal volume , and functional residual capacity (FRC). All tests are conducted on unanesthetized animals.

Basic equipment utilized in pulmonary function testing include spirometer, x-y plotter, nitrogen analyzer, and vacuum pump (see Figure 9). The dog is gently restrained in a sling during the procedure, with the animal's feet touching the floor to prevent hampered breathing. A flexible, counterweighted gas delivery and monitoring system is attached to the dog via a latex mask. The system consists of flexible, 1-inch polyethylene tubing connected from an oxygen tank to a Rudolph valve and from the nitrogen analyzer head to the spirometer. Appropriate counter-weighting of the system via a rope and pulley allows freedom of head movement for the masked dog.

A more efficient mask was recently developed for use during pulmonary function testing. This mask conforms more closely to the dog's muzzle to drastically reduce dead space inside the mask. A further refinement proved to be the use of a water-based lubricating jelly (K-Y) to obtain a firmer seal of the latex mask to the dog's muzzle. Data recorded during implementation of these new materials indicate greater precision and reproducibility.

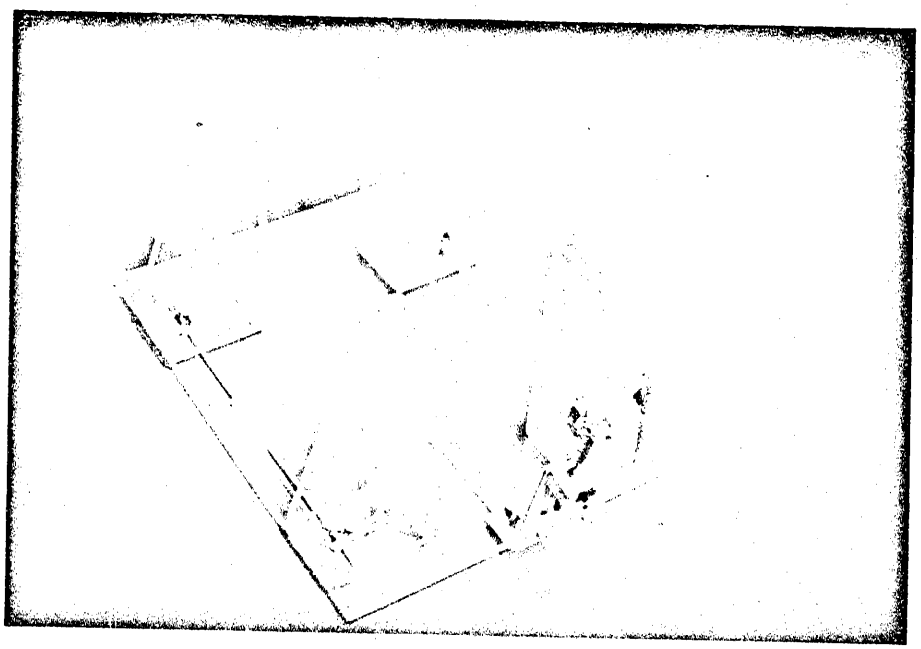


Figure 7. Plexiglass stocks for gently restraining laboratory dogs. Cushions are placed between legs and vertical posts and legs are secured to posts.

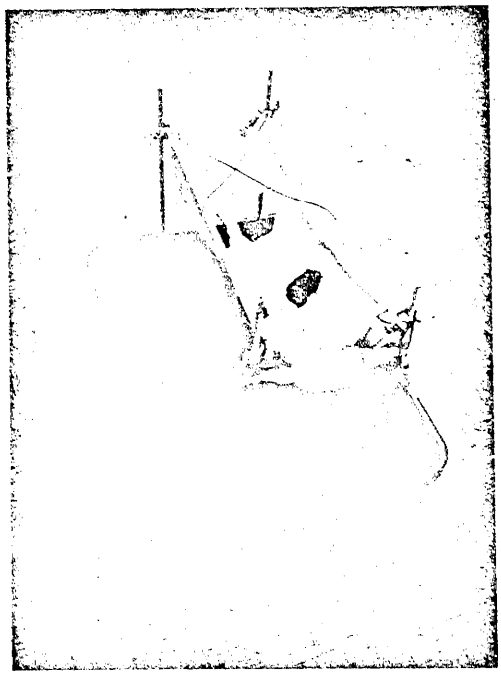


Figure 8. Cloth sling used to gently restrain dogs during inhalation exposures and pulmonary function testing. After an initial training period the sling is lowered so that the dog's weight rests on its legs.

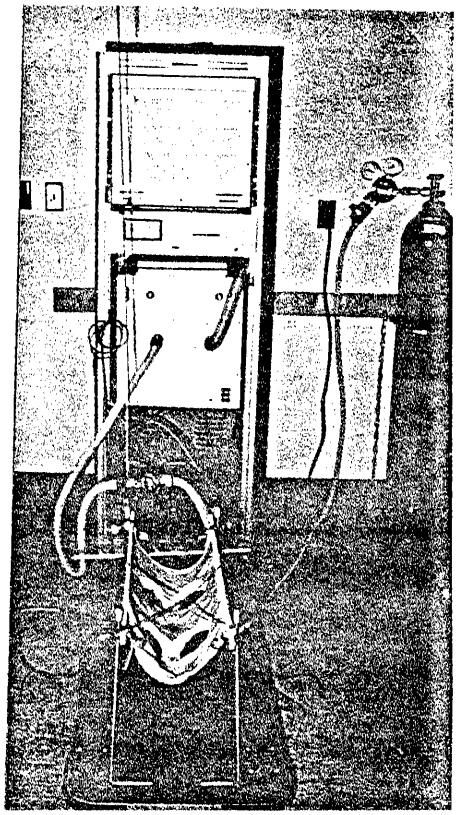


Figure 9. Rack-mounted equipment and sling used for pulmonary-function testing in dogs.

Analysis of data obtained through pulmonary testing has been refined with the use of the PDP-11 minicomputer. Individual nitrogen washout curves are entered into the computer to be normalized by the division of the animal's cumulative expired volume (CEV) by its functional residual capacity. The resulting normalized curves, expressed as the decrease in nitrogen concentration versus the dilution volume of oxygen as the number of FRCs, provide data somewhat independent of breathing frequency and tidal volume.

Comparative studies of nitrogen washout curves taken both before and after exposure to a pollutant atmosphere are conducted after normalization. The least squares method of best fit is employed to yield a polynomial equation best representing the normalized data; that expression is then examined using moment analysis. Application of this technique to multibreath nitrogen washouts has been described in the literature. The relationship of the normalized first moment (M_1/M_0) plotted against the normalized second moment (M_2/M_0) has been found to be an index of pulmonary ventilation (Saidel, et al., 1975). Computer analysis of nitrogen washouts at APHEL similarly utilize these moments where M_0 is the area under the curve, and with increasing moments (M_1, $M_2 \ldots M_n$), greater weight is given to the tail region of the curve. This type of analysis appears to be a very sensitive measure of lung function. A schematic representation of data handling is shown in Figure 10.

Pulmonary function testing of dogs at the facility thus far has provided reliable and reproducible data. It is anticipated that the described methods will be further refined as the project continues.

D. ^{133}Xenon Wash-in/Washout in Rats

Abnormalities in the rates with which a test gas fills or empties the lung can indicate significant health impairment. In man, the "nitrogen washout test" is used clinically to assess damage in small airways. Radioactive gases can also be used to quantitate wash-in/washout dynamics in breathing subjects. In order to assess these phenomena in rodents we have devised equipment and procedures using radioactive xenon gas. The apparatus, protocol and data handling are described in this section.

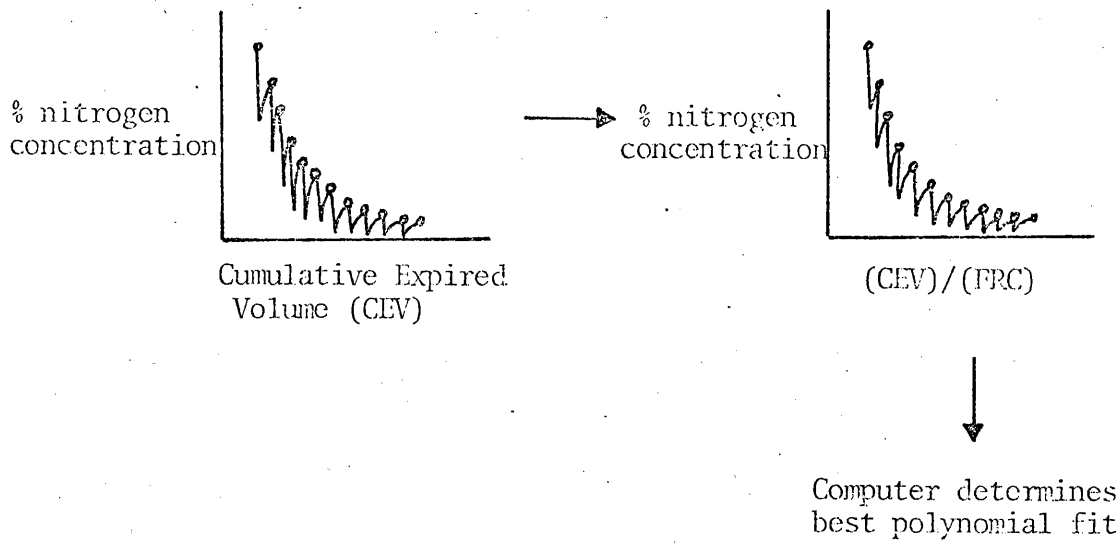
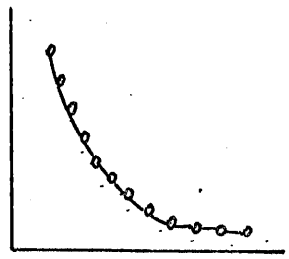
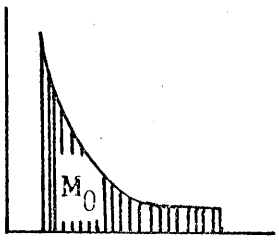


Figure 10. Schematic representation of nitrogen washout data analysis performed by the PDP 11 minicomputer.



Computer performs moment analysis on this curve



Computer output
M_0 , M_1, M_2
M_1/M_0, M_2/M_0

Statistical tests performed to detect pulmonary impairment

Equipment

The xenon method utilizes four pieces of equipment: a manifold with attached plethysmographs; a VR-6 amplifier and oscilloscopic recorder (Electronics for Medicine, White Plains, N.Y.); a radiation counting system; and a PDP-11/10 minicomputer (Figure 11).

The manifold from which rats breathe (Fig. 12) consists of a machined brass chamber having an internal volume of about 200 cc. Attached to this are electric valves and a fan that can both move air out of the manifold or recirculate air within the manifold. The attached plethysmographs are made of aluminum so that body heat of the animal is efficiently conducted away. They are plugged into the manifold so that the entire manifold-plethysmograph interior may be sealed by closing the solenoid valves. The internal volume of the closed system, including manifold and fan, is about 300 cc. A plexiglas window on the upper side of the plethysmograph allows observation of the animal and counting of radioactivity from the chest. Sodium iodide crystal radiation detectors are placed over each plethysmograph window (centered above the rat lung); collimation is used to reduce counts from areas other than the thorax. The crystal detector is connected to an amplifier and count-rate meter to convert count rate to a DC electrical signal which is approximately proportional to the amount of radioactive gas in the lung. This signal is amplified, passed to an oscilloscopic recorder, recorded, properly buffered and passed to a PDP-11 computer. The DC analog signal is converted to digital values and these values are stored as a matrix in the computer. This digital information is later stored on paper tape, used to generate tables, and analyzed for statistically significant changes.

Pressure transducers monitor the pressure changes in the plethysmograph that are caused by the rat's breathing. The transducer signal is passed to the VR-6 amplifier and the signal is recorded along with the radioactivity count-rate signal. From the pressure signal, breathing rate and tidal volume are determined.

Procedure

The basic procedure involves following radioactivity in the chest while two rats breathe xenon from the chamber (2 minutes) and then 100% fresh air (5 minutes). Details will now be described.

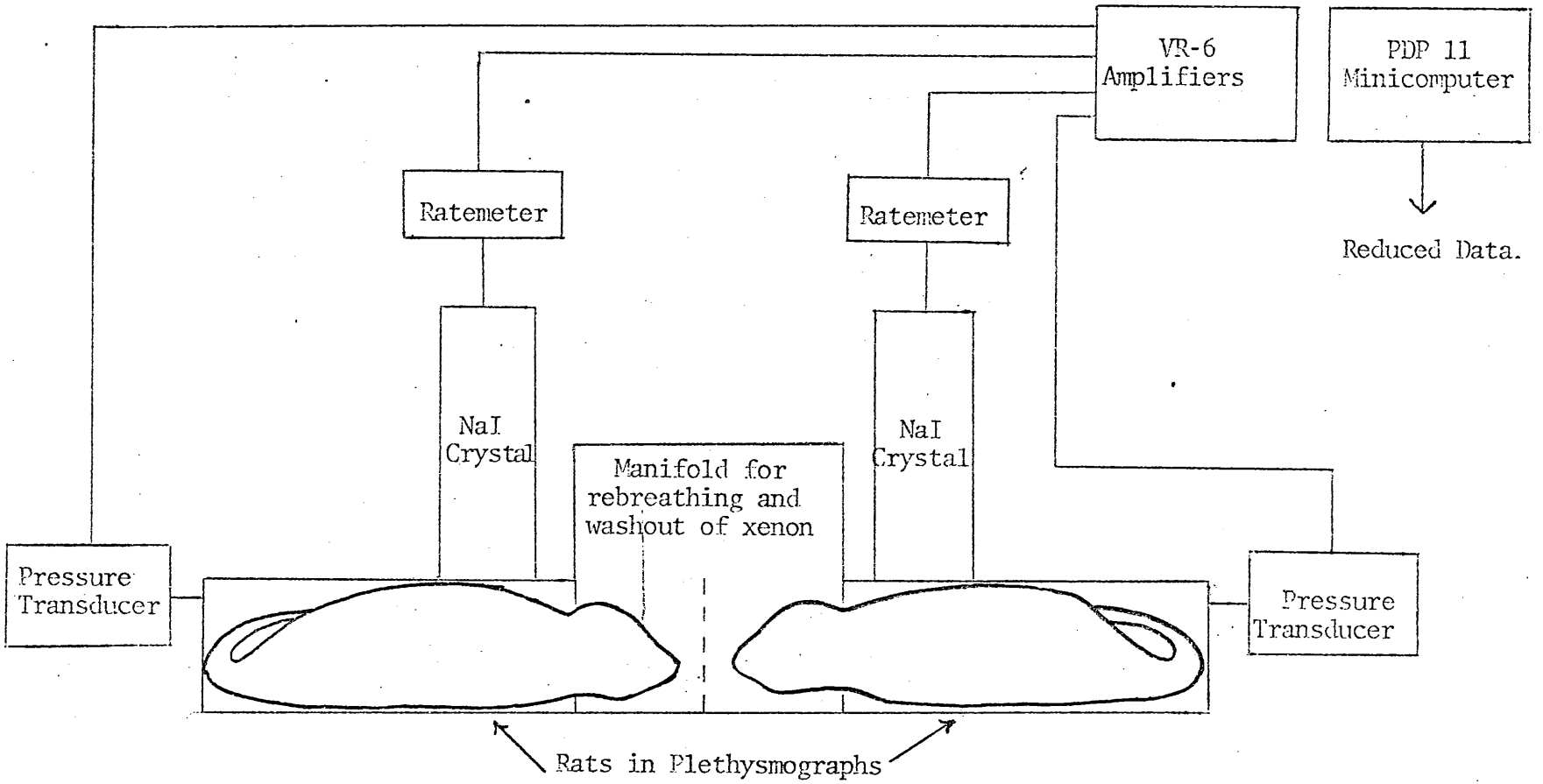


Figure 11. Schematic of current data acquisition and analysis system for xenon washout tests in rats..

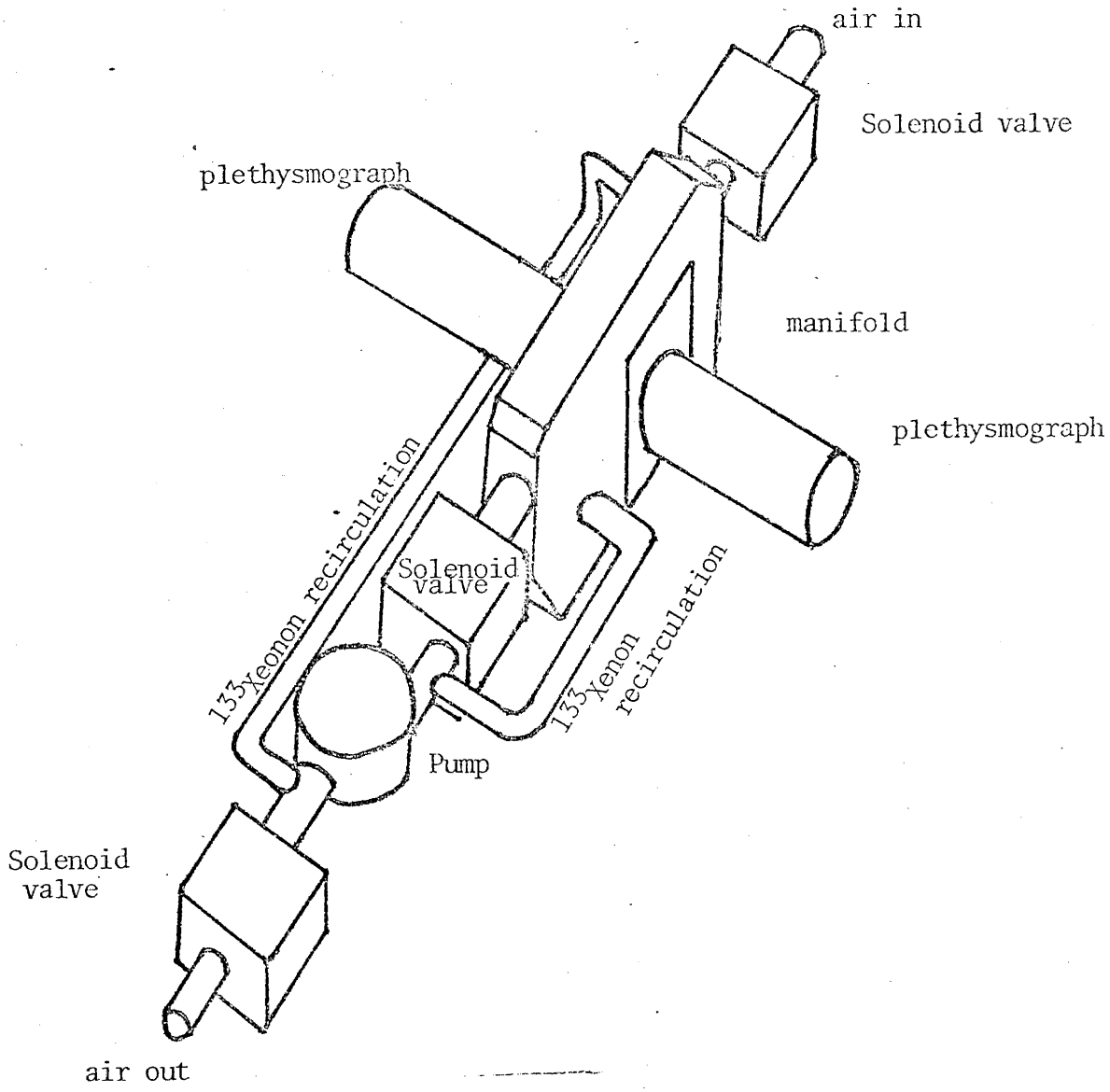


Figure 12. Device designed and assembled at APHEL for obtaining ^{133}Xe washout curves in rodents. Construction is mainly of brass and aluminum. Device is about 18" in length. (not drawn to scale).

Before being placed in a plethysmograph, the animals are lightly anesthetized by intraperitoneal injection with veterinary grade sodium pentobarbitol. Twenty-six to 30 mg/kg of body weight has been determined to be the optimum dosage level. In practice this has been accomplished by injecting a standard 0.1 cc of anesthetic solution into each animal. This provides a light level of anesthesia that keeps the animal quiet for the test; recovery begins within about 15 minutes after the testing is completed. K-Y water soluble jelly (Johnson & Johnson) is used liberally around the neck of the anesthetized animal to assure a good seal to the thin rubber gasket of the plethysmograph. The rubber neck seal is slipped over the neck of the rat, the animal placed in the plethysmograph body and attached to the manifold so that the head protrudes into the the manifold's interior. The neck seal is tested for leaks after the pressure transducer is attached to the plethysmograph body. At this time, a small volume of air (3-5 cc) is introduced into the plethysmograph. If no leak is occurring around the neck seal, a deflection of the transducer trace displayed will be observed and the stability of this deflection is a measure of the quality of the seal. A second connecter at the rear of the plethysmograph is used for connecting a vacuum line to circulate fresh air around the body after the test.

The vaccum line is plugged into the plethysmograph via a three-way stopcock and the stopcock is initially positioned so that the plethysmograph is sealed. The pressure trace generated by the rat breathing is then displayed on the oscilloscope of the VR-6. The manifold internal recirculation is turned on so that the rat is breathing circulated room air from the manifold. The operator assures that the breathing trace is strong when the rat is connected, to make sure that the head and neck of the rat are resting unobstructed. Two rats are attached to the manifold and wash-in/washout curves are taken for both rats at once.

With the animal in place, "RUN" is typed on the teletype and all questions are then answered. RETURN is then pressed to collect background counting data. When background is taken, the computer will then be ready to accept washout data. All valves are closed (a green panel light indicates this condition) and a sample of xenon gas is drawn into a syringe. The activity should be between 15,000 & 20,000 counts/min in contact with a low-efficiency portable G.M. detector, and the total volume should not exceed 2 ml. This xenon sample is injected into the injection

point of the manifold, located behind the left plethysmograph, and the empty syringe withdrawn. At the time the sample is injected, a stopwatch is started and "RETURN" is pressed on the teletype. Count rate data from the lung will then be taken. The visual trace on the VR-6 should be checked occasionally to make sure that the count rate is increasing during wash-in and that both rats have a good breathing signal. At two minutes, all valves are opened (a red light is turned on) and washout continues for 5 minutes.

At the end of the washout period, the computer signals ready for flushing of the plethysmograph with room air. The operator removes the transducer connections so that the back of the plethysmograph is open to room air and turns the second stopcock to apply suction to the back of the the plethysmograph. This allows a flow of air to pass through the plethysmographs and flushes out any xenon that has leaked inside. When flush begins, "RETURN" is pressed on the teletype. The entire washout procedure is completed when the teletype answers "MORE ANIMALS?" If more animals are to be run, "YES" should be typed; if not, "NO" should be typed.

When washouts are completed for all animals the computer is ready to output. Questions are asked by the computer and the operator can chose any or all modes. The choices, in order, are: 1) paper tape punch?; 2) data table?; 3) moment analysis?; and 4) plot of mean and confidence interval?

For calibration of the breathing signal of the plethysmograph an inflatable object of a known volume similar to that of the rat is placed inside and a seal is placed over the plethysmograph's neck opening. An oscillating known volume signal is supplied by a syringe attached to the inflatable object inside the plethysmograph. This produces a cyclic deflection on the recorder chart. A family of these deflections are then generated for each plethysmograph. Thus, one obtains a calibrated curve from which measurement of tidal volume of the rat may be calculated.

Data

The program for assessing and analyzing xenon wash-in/washout data is written in Dartmouth BASIC. During these tests the computer is dedicated to the acquisition and processing of xenon data and cannot accomplish any other tasks until the procedure is completed. Upon completion of a test, the operator may select as output a punched paper tape of all data taken, a table of all radioactivity

values as percent of highest count, a table of the moments for each rat during both wash-in and washout, and a plot of the wash-in and washout for a group of rats showing the mean percentage of radioactivity $\pm$ one standard deviation plotted versus time.

After the program has been read in to the computer from paper tape, "RUN" is typed on the teletype. The computer will answer that the program has been loaded and is ready. Questions are then asked of the operator as to date, exposure material, concentration and duration of exposure.

The program has four sections or modules. The first module initializes all computer registers and accepts data as to date, exposure concentration and time. Module 2 has a large loop containing three sub-loops. The number of the animal being tested is typed in. Background radioactivity (before xenon is used) is then established by the first subloop. The ratemeter signal is accessed through an analog to digital converter for each rate ten times during a 1 min. period, and these ten values are averaged for the background count. The background values are printed and the program waits for a command to continue. At injection of the xenon-laden air into the manifold, the ready signal is passed to the computer. This starts a timing loop which accesses data from the ratemeter every four seconds. This loop continues throughout wash-in (2 mins.) and washout (5 mins.) and stores 100 data points of amount of radioactivity for each rat. The computer then communicates that washout is complete and the program is ready to receive information during flushing of the plethysmograph with room air. The plethysmograph is opened to the vacuum line and the program is again called. This accesses 2 data points, one-minute apart, to determine if xenon that escaped around the neck seal was present in the body of the plethysmograph (if this is the case, a drop in radioactivity is seen during flushing). After flush is complete, the program asks if more animals are to be run and, if so, recycles to the animal-number sequence and continues on command to acquire new data.

If no more animals are to be run, the program will, on command, punch a paper tape (Module 3 of program) of all data taken so that a hard copy of the data is available for future use or storage. Punched information is formatted so that each count rate is recorded in sequence, starting at background, continuing through wash-in, washout, and flush, and ending with the rat number. The total punched tape for a group of animals ends with the date, pollutant exposure parameters, and time after exposure.

The Fourth Module provides different types of data output. Choices are: 1) all data as percent of the highest radioactive count in columns headed by rat numbers; 2) moment analysis of the wash-in-washout of each rat; and 3) a plot of the combined group showing variability around each point. Any or all of these options may be chosen on command. Options are selected by answering questions posed by the computer.

Breathing rate is counted from the VR-6 trace and tidal volume measured. Using this data, an average tidal volume, average respiratory rate, and average minute ventilation is calculated and recorded.

Moments of both the wash-in and washout curves are calculated as described by Saidel, et al. (1975) by the computer. The parameters we have been most interested in are the ratios of moments (M_1/M_0 and M_2/M_0). These moments are plotted on linear graph paper with M_1/M_0 plotted on the y axis and M_2/M_0 plotted on the x axis. The differences of each moment ratio from their pre-exposure values are calculated and statistical tests for significance performed. More sophisticated analysis will include normalization of data to a mean of zero and standard deviation of 1 and performing multivariant clustering procedures. Figures 13, 14 and 15 demonstrate the outputs presently obtained from the computer.

E. Labelling of Polystyrene Test Spheres

1. Objectives

The primary objective of this experimental phase was to develop very stable, micron-range monodispersed radioactive aerosol particles suitable for use in tests of deposition and clearance competency in animals. A parallel interest was to develop high yield procedures which would allow the experimentor to work with lower quantities of radioactive material. The criteria established for the particles in order to be useful in diagnostic tests for respiratory-tract injury are summarized below. All the requirements were successfully accomplished or exceeded in this initial year of research.

1) To insure reproducible aerosol behavior and adequate penetration within the lung, the particle aerodynamic median diameter should have a fixed value between 0.5 and 2 microns, reproducible within 10% for successive preparations. Also, the geometric standard deviation of mass distribution should be similarly reproducible and 1.4 or less (preferably less than 1.2). These criteria insure adequate penetration into the entire respiratory system when inhaled, and reproducibility of the deposition pattern.

```
6 DEC 1975
0.8 OZONE + NACL
MED POST

ANIMAL NUMBER
 81    82    83    84    85    86    87    88

 43    46    44   172    76    32    56    60    42    STD. TO 50%

MOMENT ANALYSIS

WASH IN
M0              M1              M2              M1/M0           M2/M0

 1719           29146           579760          16.9552         337.266
 1660           27872           554214          16.7663         333.864
 1552           26680           578266          17.1987         346.821
 1823           30810           592876          16.4619         325.22
 1695           28743           572355          16.8553         337.677
 1767           29869           572961          16.4884         324.992
 1687           28726           572992          17.0279         339.651
 1797           29922           592308          16.6882         330.045
PUNCH OUT
 1516 DATA 16.9552 , 16.7663 , 17.1987 , 16.4619
 1517 DATA 16.9553 , 16.4884 , 17.0279 , 16.6882
 1518 DATA 337.266 , 333.864 , 346.821 , 325.22
 1519 DATA 337.677 , 324.992 , 339.651 , 330.045

WASHOUT

 2259           61302           2.69003E+06     27.1368         1193.81
 2384           62143           2.61265E+06     26.0667         1095.37
 2501           69264           3.02687E+06     27.6945         1210.25
 3865           123650          5.69607E+06     31.9922         1473.76
 2256           59923           2.58733E+06     26.5616         1146.86
 2371           65989           2.89785E+06     27.8171         1222.32
 2206           56606           2.49176E+06     25.6768         1087.21
 3461           108675          4.97858E+06     31.2195         1437.21
PUNCH OUT
 1520 DATA 27.1368 , 26.0667 , 27.6945 , 31.9922
 1521 DATA 26.5616 , 27.8171 , 25.6768 , 31.2195
 1522 DATA 1193.81 , 1095.37 , 1210.25 , 1473.76
 1523 DATA 1146.86 , 1222.32 , 1087.21 , 1437.21
```

Figure 13. Computer printout of wash-in/washout data in reduced form. These data were acquired immediately after a group of 8 rats were exposed to an atmosphere containing 0.8 ppm ozone and NaCl. Listed are animal numbers, the time (in seconds) for the radioactivity in the lung to reach 50% of the highest value during the washout and the moment analysis for each rat.

0.8PPM OZONE +NACL

N	PRE	IMED	3HR	24HR
W/I - M1/M0				
81	16.8648	.536021	-4.56691	-2.16308
82	15.64	7.2014	4.12468	8.53004
83	17.0331	.92526	-2.69886	-.97574
84	17.0207	-3.28306	6.67364	-.294349
85	16.7575	1.19349	3.44084	5.3206
86	16.544	-.336069	-.11122	3.23682
87	16.6584	2.2181	-.154286	1.67183
88	16.836	-.877884	-2.97754	-1.6334
SX		7.57725	3.73034	13.6927
SX2		71.01	110.432	122.728
N		8	8	8
MEAN		.947157	.466293	1.71159
STD.DEV.		2.82474	3.686	3.52299
STD.ERR.		.998696	1.3032	1.24557
T		.948393	.357806	1.37415
W/I - M2/M0				
81	334.954	.690248	-6.91348	-3.12102
82	300.759	11.0072	6.18802	11.872
83	341.112	1.67364	-4.31911	-1.37082
84	341.357	-4.72732	7.84076	-.752585
85	333.257	1.3251	4.67087	7.52302
86	326.252	-.386208	-.260228	4.77606
87	330.612	2.73401	-.325767	1.75946
88	335.941	-1.66577	-5.36463	-3.31248
SX		10.6509	1.51643	17.3736
SX2		158.937	216.99	246.605
N		8	8	8
MEAN		1.33136	.189554	2.1717
STD.DEV.		4.25378	5.2046	5.10973
STD.ERR.		1.50394	1.8401	1.80656
T		.885248	.103013	1.20212

Figure 14. Computer printout summarizing the moment analyses performed on wash-in data obtained for a group of 8 rats exposed to 0.8 ppm ozone + NaCl. Listed are M_1/M_0 and M_2/M_0 for the pre-exposure wash-in data and the % change in those values for the wash-ins performed immediately, 3 hours and 24 hours after the exposure. Also included are statistical values including the mean, standard deviation, standard error, and T-test value. The same type summarization is output by the computer for washout data.

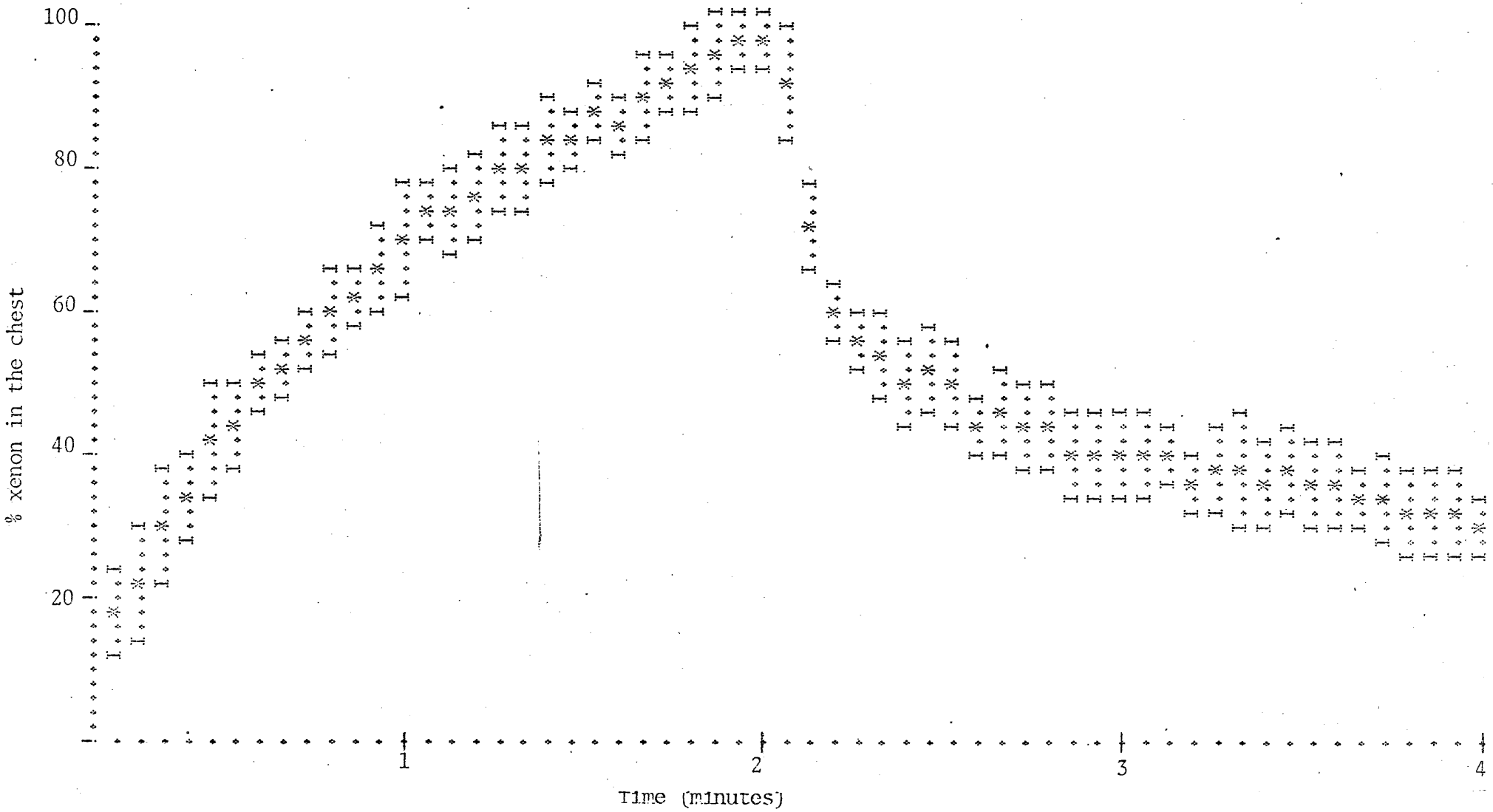


Figure 15. Computer printout of wash-in and the first two minutes of washout of radioactive xenon from the lungs of a group of rats. The wash-in lasts two minutes and washout is observed for 5 minutes. Error bars are ± one standard deviation about mean values.

2) The aerosol should be labeled with a radioactive tag which will decay by gamma emission with sufficient energy for external detection with sodium iodide detectors. Preferably, the isotope should be a pure gamma emitter (as in electron capture decay modes) to reduce the potential physiologic effects associated with beta ionization in tissue. The half-life of decay should be greater than 1 day, and the specific activity should be such that microcurie amounts could be deposited in the lungs of animals by inhalation.

3) The radioactive tag should have a stability such that less than a few per cent per day is removable from the particles in water , in tissue-fluid simulant, and in the lung itself.

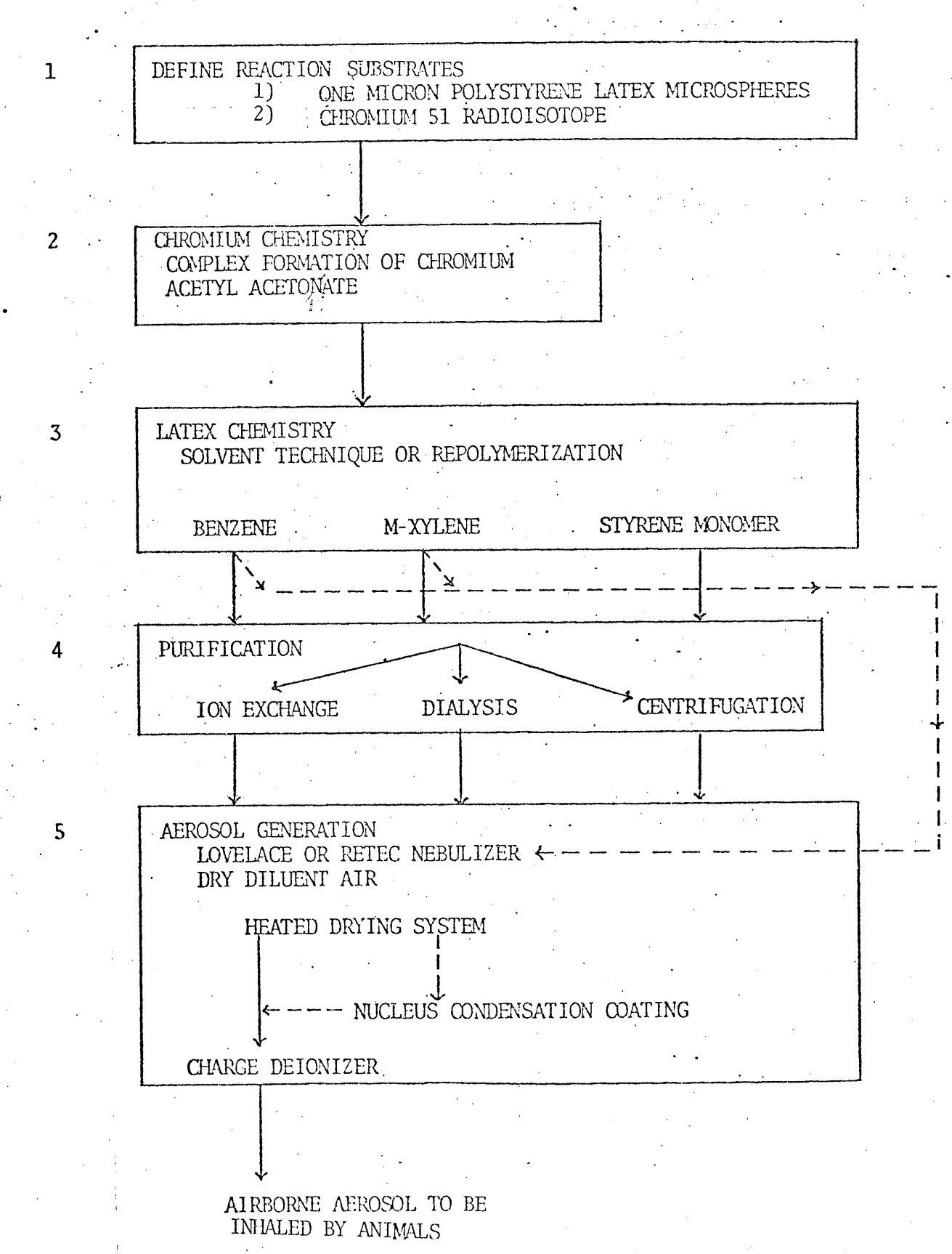
2. Materials and Methods

Five stages of procedure were used to satisfy the overall final objectives (summarized in Figure 16).

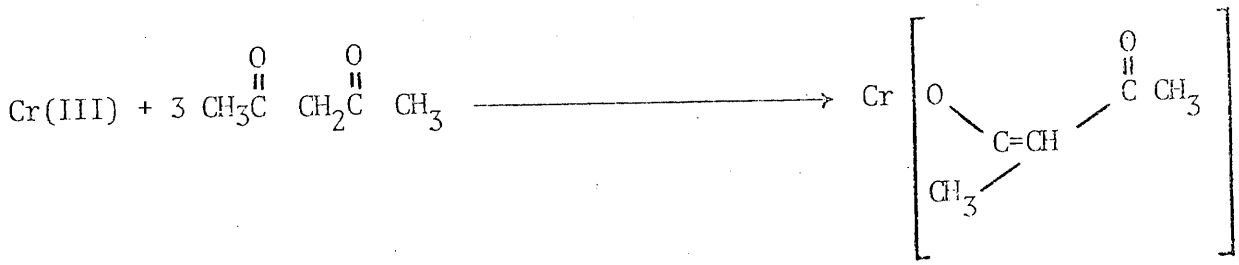
The first stage was to identify appropriate reaction materials which could meet the stringent requirements proposed. In order to satisfy these criteria, the decision was made to purchase monodisperse microspheres and to attach a radioactive label. Commercially-available polystyrene-latex particles from Dow Chemical, often used for calibration of aerosol instruments, were well characterized in the literature (Stöber and Flachsbart, 1971), and were apparently suitable for our purposes. Polystyrene microspheres of 1.099 micron diameter were chosen as the initial starting material. The radioactive isotope selected had to exhibit the additional properties of chelation into an appropriate organometallic complex which is stable at a neutral pH, biologically non-toxic, and easily eliminated from the organism. For these reasons, chromium-51 radioactive isotope (used in several diversified human studies) was selected. The chromium (III) state of this element is readily converted to chromium acetylacetonate. Its half-life is 27.8 days and it decays by electron capture, emitting only gamma radiation.

Stage Two, an initial part of the reaction chemistry, was the development of exact experimental procedures which would convert the Cr(III) state, with high reproducible yields, into a chromium acetylacetonate complex having the required properties (Szende and Udvarheti, 1975; Albert, et al., 1964). The basic procedure was obtained from Szende and Udvarheli (1975) and modified to increase chelation yields.

FIGURE 16. Radiochemistry of Polystyrene Particles



Several noteworthy pitfalls in the procedure were encountered. The pH of the reaction medium with free Cr(III) in 0.1 N HCl aqueous solution was adjusted with sodium hydroxide. It was discovered that if the hydroxide is added too rapidly, a grey-colored solution resulted which did not form the chelate desired. This is probably the result of chromium(III) oxide and/or hydroxide formation which blocks the chelating action. This problem is prevented by slow addition of the neutralizing base. Also, one must be sure that the acetylacetone chelating reagent is pure. The solution should be clear to slighly yellow and have the physical properties listed in Table II Room temperature reaction was associated with poor yields. A modification to the reaction was to operate at an elevated temperature (65°C) in order to increase the chelation-step yield (see Table III). The chromium chelation reaction is the following.



Specifically, carrier-free radioisotope (30 m Ci) was injected into a 25 x 200 mm test tube and diluted to 8-10 ml with deionized water. A small magnetic stirring bar was used for light agitation. A pH electrode fitted with a capillary injection tube (Figure 17) was inserted into the solution for slow neutralization (one drop per second) of the solution to a pH of 5.0 with 0.2 N sodium hydroxide by injection through the capillary tube. Two-tenths ml of acetylacetone (2,4 pentanedione) was added and agitated by shaking until thorough homogeniety was attained (1-2 mins). The solution was further neutralized until a steady pH of 6.5-7.0 was obtained. The pH electrode was withdrawn and rinsed lightly into the reaction solution with deionized water. The solution was placed in a constant temperature bath at 65-70°C without agitation for one hour. After cooling for 1-½ hours the solution was extracted with 10 ml of benzene which had been thoroughly shaken with the reaction medium. A modified 25 ml pipette was used to facilitate this separation (Fig.18). The extraction was repeated with 5 ml of benzene and the two extracts passed through a Gelman absolute glass filter and evaporated to dryness at 65°C under a clean filtered air stream. A small amount of radioactivity may or may not become airborne with the solvent vapor during

TABLE II

PHYSICAL PROPERTIES OF REACTANT MATERIALS

Material	Appearance	Molecular Weight	Density gm/ml	Refractive Index	Melting Point °C	Boiling Point °C
Acetyl acetone	clear to yellow liquid	100.13	0.9565	1.4014	---	108
Chromium-acetyl acetonate	Red crystals	349.33	---	---	216	340
Polystyrene Microspheres	milky white suspension	---	1.05	1.5905	---	---

References: Dow Chemical Technical Bulletin on Latex Microspheres, and Handbook of Chemistry and Physics, Edition 54, Chemical Rubber Publishing Company, 1973.

TABLE III

EFFECT OF REACTION TIME & TEMPERATURE ON PERCENT BINDING OF CHROMIUM TO ACETYLACETONE

Reaction time (mins.)	Temperature (°C)	Yield (%)
10	25	32.9
5	65	71.3
10	65	70.6
15	65	72.0
30	65	82.6
60	65	93.0
90	65	82.8
60 (repeat)	65	93.1
90 (repeat)	65	83.1
150	25	70

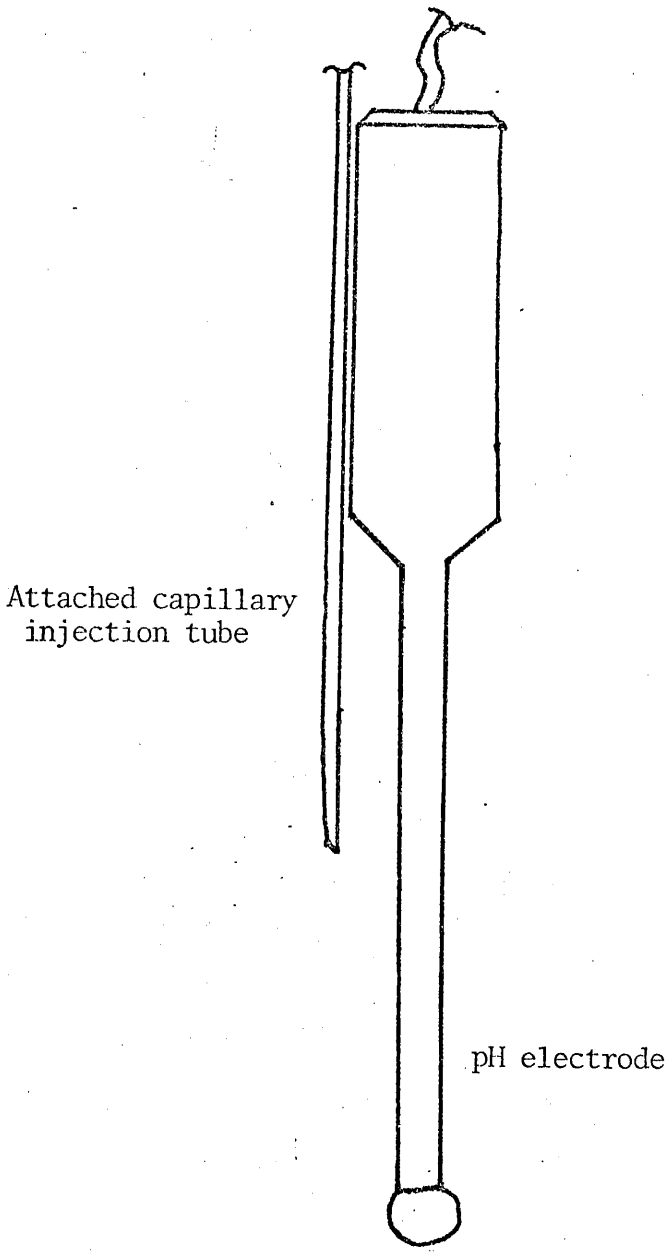


Figure 17. pH electrode with injection tube fastened to its side for simultaneous measurement of solution pH while adding reagents

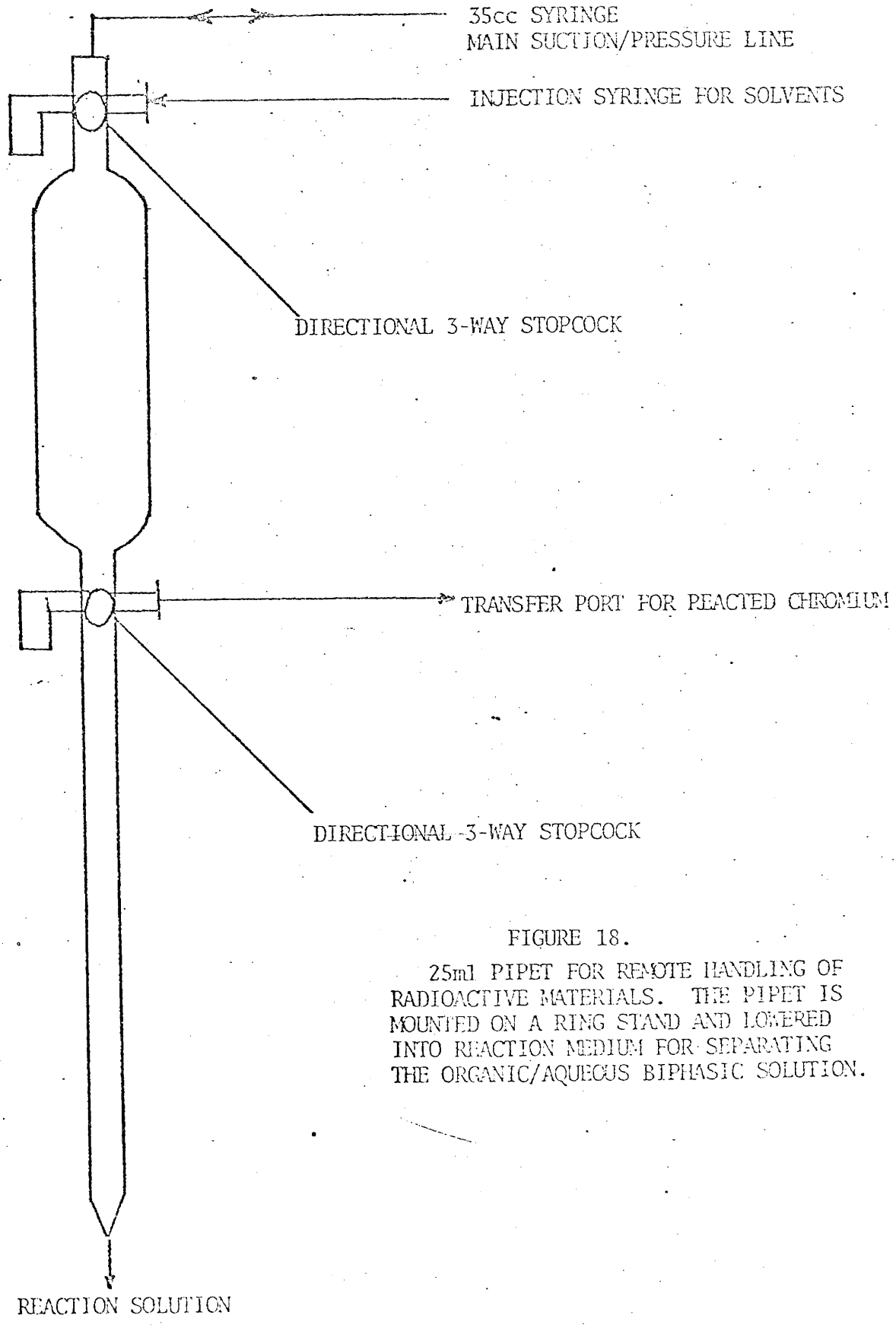


FIGURE 18.

25ml PIPET FOR REMOTE HANDLING OF RADIOACTIVE MATERIALS. THE PIPET IS MOUNTED ON A RING STAND AND LOWERED INTO REACTION MEDIUM FOR SEPARATING THE ORGANIC/AQUEOUS BIPHASIC SOLUTION.

drying. Yield measurements were made by radioactive counting of the aqueous residual and extraction solutions under identical counting geometry and volume conditions and comparing the measured activities. Results are summarized in Table IV. Reproducible yields averaging 90% have been obtained.

Stage Three involves the attachment of radioactive label in chelated form to the latex particles. Several different techniques were evaluated before the final procedure was realized. The scientific literature contains several methods for attaching iodine or bromine to polystyrene microspheres (McCann, 1975; Black and Walsh, 1970; Bogen, 1969; and Singer et al., 1969). These references were useful for describing a variety or reactions that the particles may undergo. Further, Dr. Leigh Bangs of Dow Chemical Central Research, Plastics Department (Midland, Michigan) supplied additional literature and information regarding stability and reactivity of the microspheres. Selected physical data on the particles is given in Table II. They possess temperature stability up to their glass transition temperatures of $\sim 80^{o}C$. They are supplied suspended in water solution which can be redispersed upon settling by agitation until $\sim 50\%$ w/v solution concentration is obtained. There are many reactive sulfate groups on the particles.

Chromium chelate bonding to the particles was attempted through two main routes. First was a solvent evaporation technique that has been used in the past for dying latex particles (Vanderhoff, 1974). Basically, the method is to solvate the organometallic complex in benzene or m-xylene and let adsorption occur with the microspheres. The apolar organometallic complex and solvent is presumably entrapped in the hydrophobic interior of the spheres. The solvent is then evaporated under reduced pressure leaving the radioactive complex behind in the particles interior. The yield and leaching characteristics of this method were such than only approximately 28% of the activity remained with the particles after precipitating on 0.45 um pore size filter paper and rinsing with deionized water. To better entrap the activity, a second system was employed to coat the external surface of these particles. This combined system of solvent entrapment and coating, called nucleus condensation coating (N.C.C.), has been used by Drs. Prodi and Spurny (1972) to encapsulate particles in carnauba wax. This method improved the yield up to 40-50% but routine use would involve considerable experimental design changes. This method holds promise for future work but the excellent results from parallel work with repolymerization techniques has taken priority at this time.

TABLE IV

CHROMIUM CHELATE YIELD RESULTS

Reaction Number	% Yield
Cr - 13	93.1
Cr - 14	83.1
Cr - 15	87.7
Cr - 16	97.5
Cr - 17	90.7
Cr - 18	86.3
Cr - 19	93.2
Average	90.2

The use of seed emulsion repolymerization of particles is not uncommon, A good specific reference for chromium work being Szende et al. (1975). The procedure developed by us is derived from this reference and from descriptions by Bogen (1970). The method of repolymerizing the particles in the presence of a chelate and using radiation-induced reaction chemistry to enhance incorporation (Huh, 1974) was also applied to this study. Special care is required as the monomer:particle:surfactant ratios (used to stabilize the particulate suspension) must be carefully controlled during the reaction or polydispersed particles will be obtained.

The dried chromium complex was dissolved in 0.2 ml of freshly distilled styrene monomer in a 25 x 200 mm test tube. Two ml of 1.099 micron polystyrene microspheres (10% w/v) were pipetted into the test tube and an additional 3 ml of deionized water injected into the solution. The mixture was agitated gently with a wrist shaker for 5 hours at room temperature. Five ml of deionized water was added and the solution placed in a chamber without agitation for 19 hours with an 11,000-curie gamma ray source (^{137}Cs). Approximately 10 mg of potassium persulfate radical initiator was added and the reaction shaken in a constant temperature bath at 65-70°C for 4-6 hours. The solution was then diluted to 80 ml with deionized water and passed through a filter paper. The solution was then ready for purification and aerosolization. A "transfer" yield is measured at this point to determine the amount of activity not lost to walls or residual particles. This was measured by counting the total vial activity before and after dilution transfer. The vial was re-diluted after transfer to exactly the same volume and geometry as was used in the first count. The average transfer yield was greater than 90%.

The purification stage of this experiment consisted of evaluating three possible methods which could remove labile or non-bound chromium from the particles. The first involved use of ion exchange resin columns as proposed by Van den Hul and Vanderhoff (1968) and by McCann (1975). This technique is effective in removing ionic components but did not effectively remove unbound organometallic complex. It was also a time-consuming process to properly prepare the resins. A second "clean-up" process was to dialyze the particles as described by Szende (1975) and by Bogen (1970). The main difficulty here was the inconveniently long time required for complete dialysis (3-5 days). The third method proved to be both effective and fast (3 hours). We developed a centrifuge procedure which would separate out the particles in the size

range of interest without compacting all of the solids in the bottom of the centrifuge tube. The time and speed of centrifugation, experimentally determined for a centrifuge with a 6" radial arm, was 2000 rpm for 20 minutes using a 20 ml sample at 0.12% w/v concentration. This would settle the majority of the particles to the tube bottom and leave a light particle cloud in the bottom 5 ml of the supernatant. The upper 15 ml of the supernatant was removed, particles diluted and resuspended, and centrifugation repeated. This supernatant contained the non-bound activity plus some latex particles. By counting a standard sample of supernatant and plotting its activity versus wash cycle the removal of non-bound radioactive material can be followed (Fig. 19). About 4 wash cycles are sufficient but six were used routinely. After the last resuspensation with fresh water, three drops of a 5% sodium lauryl sulfate surfactant was added to help stabilize the solution. We also used brief ultrasonic vibration to fully disperse settled particles. The activity yield (% radiolabel attached to washed particles) after completion of this stage averaged 86.8% (Table V).

The final stage, Stage Five, was to aerosolize monodispersed aerosol for the deposition and clearance inhalation studies and to test the particles against the requirements established in Stage One. Two methods have been used thus far. A nucleus condensation coating technique described by Prodi (1972) in which the radioactive aerosol is passed through a vapor of carnauba wax was employed, as previously mentioned, to generate high yields without a purification step. This method has not been fully developed at this time due to the successful completion of a simpler procedure. The second system was designed to use high solution concentrations of 0.1% w/v of latex particles and still produce an aerosol with greater than 95% airborne singlets using a Lovelace-type nebulizer. The nebulizer output is coupled to a diluting dry air flow of 8:1 which acts to dry the particles and diminish the formation of agglomerates. The dilution air and particles pass through a heated copper tube into a krypton electrical charge neutralizer to remove static charge and is finally passed into an inhalation chamber (Fig.20).

Results

The aerosol was evaluated for size distribution and radioactive stability using electron microscopy, cascade impactors, and in-vivo intraperitoneal injection, intra-tracheal injection, and aerosol inhalation. Electron microscopic examination of the aerosol particles was of samples obtained by electrostatic precipitation. These were examined and photographed, final magnifications being determined by measurement of photographs of precision

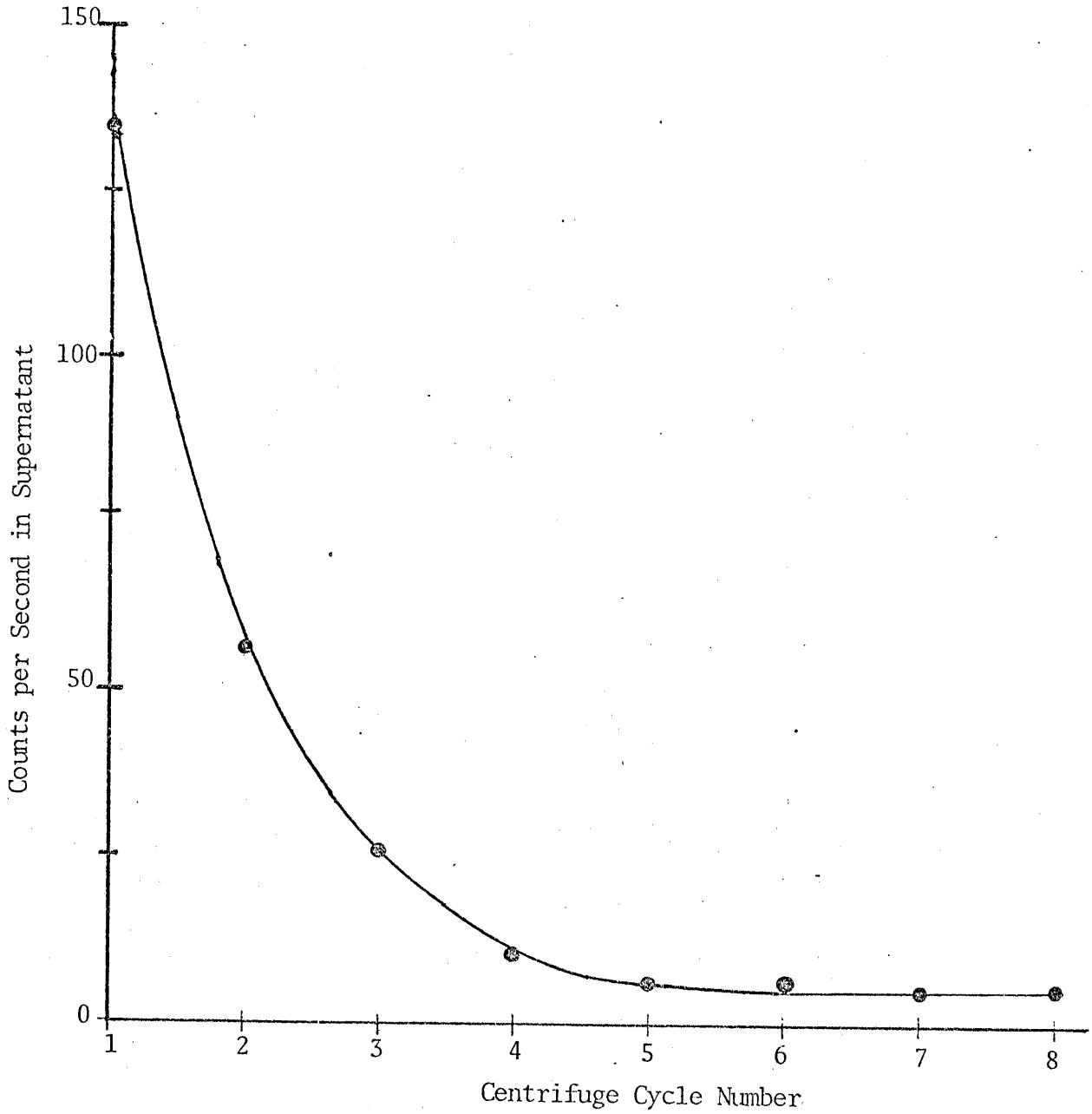


Figure 19. Purification of labelled latex particles by sequential centrifugation. A 20-ml test solution was spun on a 6" arm at 2000 rpm for 20 minutes for each cycle. Fifteen mls of supernatant was removed during each cycle after the first centrifugation and freshly filtered deionized water was added to resuspend the particles for the next cycle. The curve shows radioactivity in counts/min recovered in supernatant after each cycle.

TABLE V

RADIOACTIVE YIELD DETERMINATIONS ON SAMPLES TAKEN FROM TWO SEPARATE BATCHES OF LABELED POLYSTYRENE MICROSPHERES

Batch Number	Sample Number	% Yield*
PX - 14	1	92.1
	2	95.1
	3	95.4
PX - 15	1	73.1
	2	78.4

*Yield is based on activity bound to the particles versus the total activity of the sample aliquot at end of reaction chemistry. Average yield - 86.8%

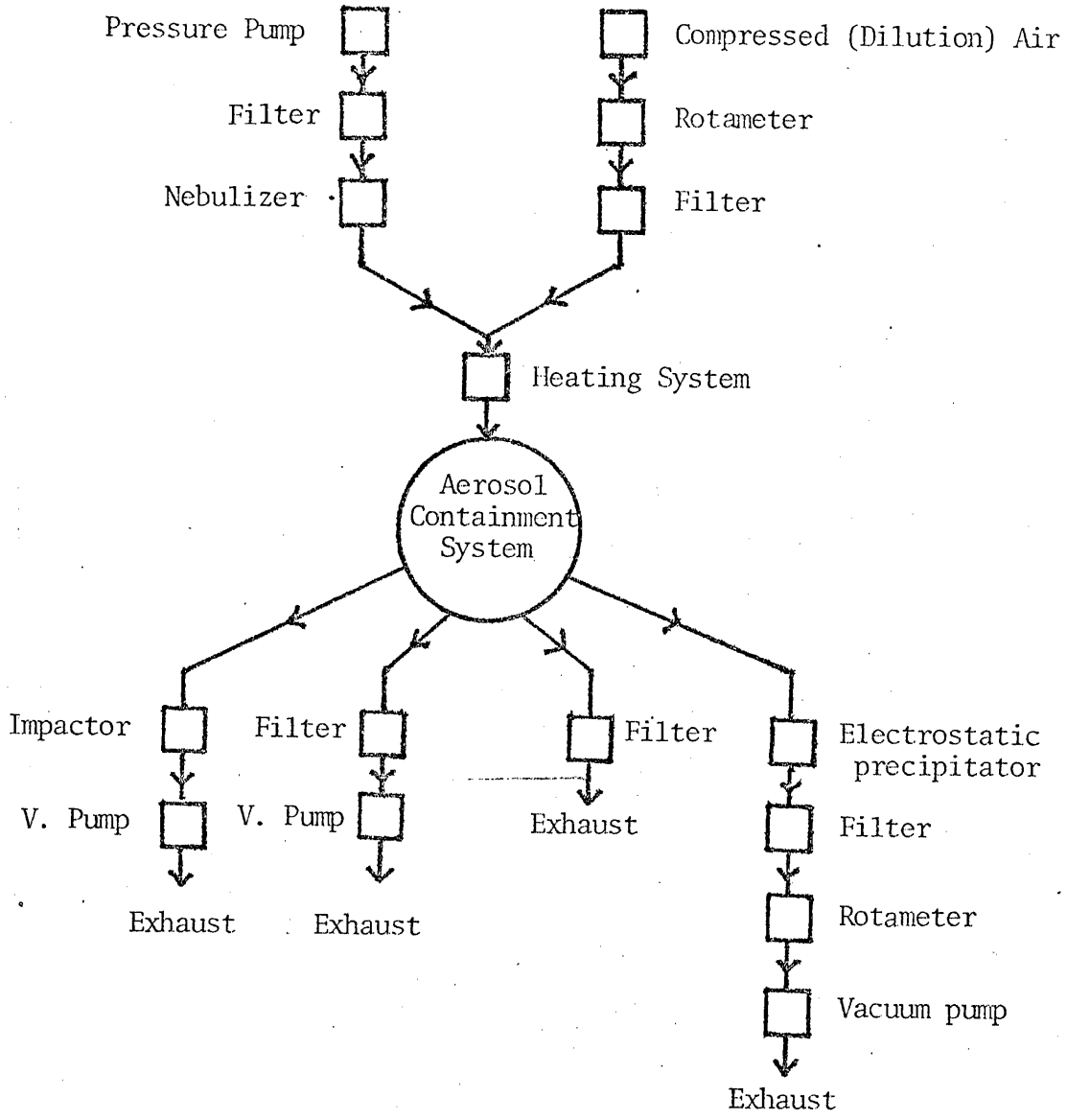


Figure 20. Aerosol generation and monitoring of ^{51}Cr-labeled latex spheres.

replicas of diffraction gratings. The results (Fig . 21) show a monodispersed aerosol with a count median diameter of 1.43 microns. Small particles, less than 0.3 microns in diameter, representing less than 2% of the total particle radioactivity (cascade impactor measurement) are also seen in the background of the photograph. They probably originate from normal airborne particulates and from dried residuals of the reaction chemistry. The cascade impactor data (Table VI) was used to obtain aerodynamic diameter and geometric standard deviation of the aerosol size distribution. The average aerodynamic diameter was 1.75 um with an average geometric standard deviation of 1.26.

The radioactive label stability, or the ability of the particles to retain their radioactive tag, was tested in rats using several methods. The first involved intraperitoneal injection. Intraperitoneal injection of polystyrene latex microspheres has been used by others to test for leaching of radioactivity (Szende, 1975 and Albert, 1964). The particles themselves are expected to be retained in the abdomen while any chromium acetylacetonate complex is readily eliminated from the organism. To further test for destruction of the complex, which should liberate free chromium within the organism, the animal was sacrificed and various organs counted for chromium activity. Further, feces and urine were collected over a period of 7 days post injection and also measured for radioactivity. The excreted activity was compared to a standard sample of the material injected into the animal diluted to the same volume and geometry. Results (Tables VII & VIII) show that less than 1% of the retained activity was found in organs other than the abdominal wall after 7 days and the total amount excreted was less than 2%.

Another test of label stability was to inject a small quantity of the particles into the lung via the trachea to determine leaching characteristics under lung-tissue conditions. The animal was sacrificed after 24 hours and the organs counted for activity. Ideally, only the lungs and gastrointestinal tract should contain any activity. Less than 0.2% of the activity was observed in the combined activity of the other organs (Table IX). Finally, aerosol inhalation tests were used to confirm the tracheal injection data. After sacrifice at 24 hours post-inhalation exposure no significant activity (above background) was found in any organ except the lungs and gastrointestinal tract. All of these tests are indicative that the activity is strongly bound and does not separate from the particles when exposed to normal metabolic processes within the organism (Table X).

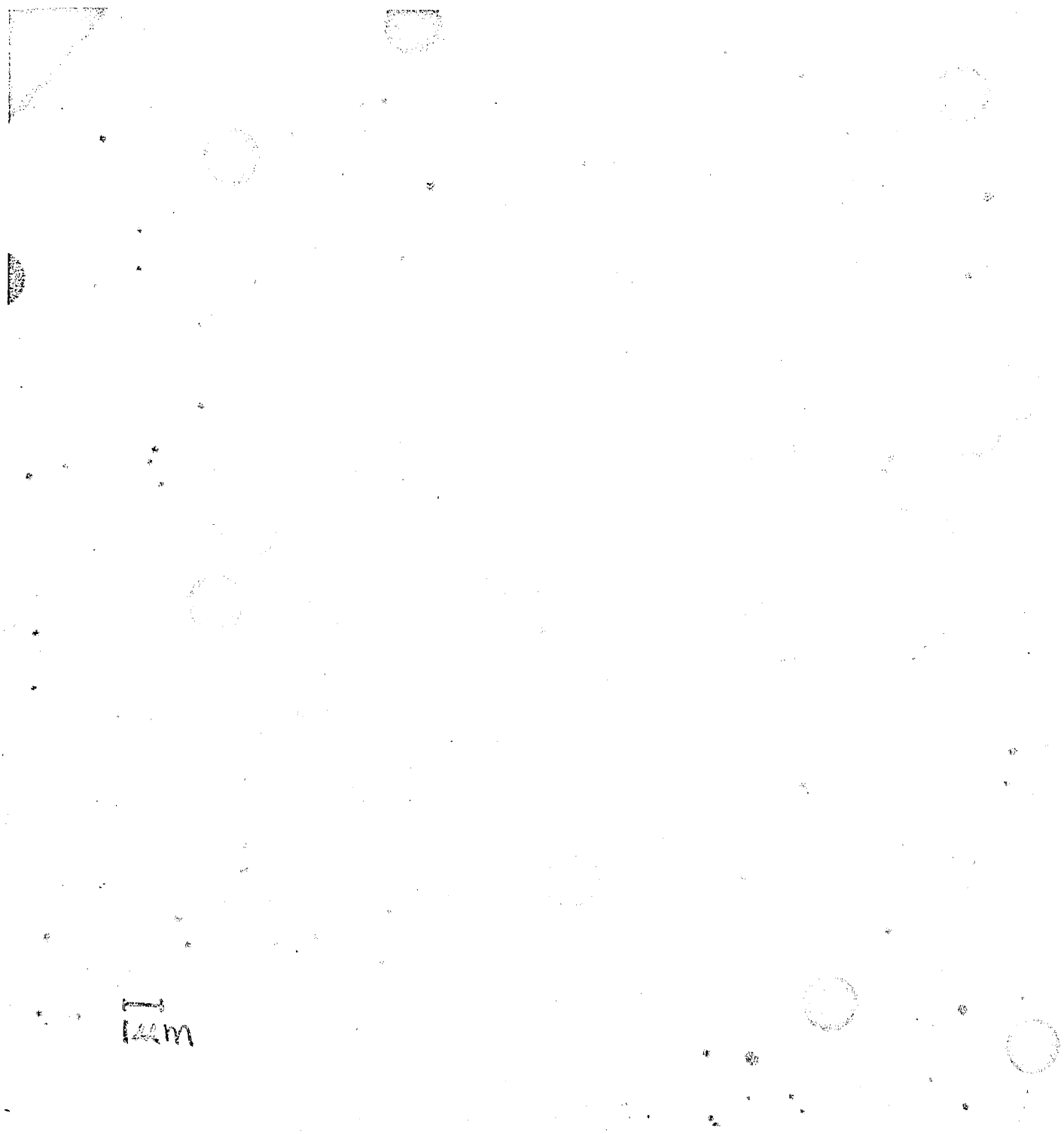


Figure 21. Electron micrograph of polystyrene latex microspheres labeled with ^{51}Cr. The count median diameter of the larger particles is 1.43 microns. The smaller particles seen in the background contain less than 2% of the total particle radioactivity. The origin of the smaller particles is probably from dried residues of the reaction chemistry and dissolved materials in the nebulized suspension.

TABLE VI

CASCADE IMPACTOR RESULTS

Material	Aerodynamic Median Diameter	Geometric Standard Deviation	% Activity < 0.3 microns
PX - 14 Impactor #1	1.7	1.18	< 2%
PX - 15 Impactor #1	1.75	1.35	< 2%
PX - 15 Impactor #2	1.81	1.24	< 2%

TABLE VII

INTRAPERITONEAL INJECTION EXCRETION DATA

Animal	1 day	2 days	4 days	5 days	7 days	Total after 7 days
Rat #26	0.68%		0.43%		0.21%	1.32%
Rat #35	0.10%	0.08%		0.09%	0.04%	0.31%
Rat #36	0.15%	0.10%		0.10%	0.04%	0.39%

% activity found in the excretion compared to the quantity injected. The numbers represent to total activity excreted from each previous measurement with the total shown in the last column.

TABLE VIII

INTRAPERITONEAL INJECTION, % ORGAN ACTIVITY* AFTER 7 DAYS POST INJECTION

Animal	Abdominal Wall	Intes-tines	Lungs & Heart	Liver	Spleen	Kidney	Total % not on abdominal wall
Rat #25	99.49%	0.05%	0.25%	0.06%	0.02%	0.13%	0.51%
Rat #26	99.8%	0.03%	0.01%	0.02%	0.01%	0.04%	0.11%
Rat #36	99.9%	0.01%	0.01%	0.01%	0.01%	0.01%	<0.1%

*% activity represents the activity of each organ compared to the sum total of the activity of all the organs with each animal.

TABLE IX

TRACHEAL INJECTION ORGAN ACTIVITY 24 HOURS POST INJECTION

Lungs	Stomach & Intestines	Heart	Liver	Kidney	% Total* Leached
58.1%	41.3%	0.02%	0.02%	0.02%	$<$0.1%

*Total leached refers to total activity not found in the lungs or gastro-intestinal tract.

TABLE X

AEROSOL INHALATION ORGAN ACTIVITY 24 HOURS POST EXPOSURE

Lungs	Stomach & Intestines	Heart	Liver	Kidney	Ribs & Thoracic Wall	% Total Leached
63.8%	36.1%	BK	BK	BK	BK	$<$0.1%

BK = activity within background limits of detection.

Finally, the labeled particles are being monitored to determine their stability under refrigerated storage. Thus far, after two months no degradation of the particles or loss of the radioactive tag has been observed. The overall radioactive yield from free chromium to labeled particles averaged 70.5%, a value that is considered desirably high for this type of chemistry.

F. Deposition and Clearance Challenge in Rats

The purpose of these tests is to evaluate the efficiency of mechanisms by which the respiratory tract clears itself of inhaled, deposited particles. A radiolabeled aerosol was developed (Section III-E) that has an aerodynamic diameter and real size similar to those of bacteria and near the size of some atmospheric inorganics. The aerosol is then inhaled and followed as a sham infectious or injurious particle. The rationale, prior use, and scientific considerations for such deposition and clearance tests are discussed in a paper (Appendix E) that will be published in the Proceedings of the Sixth Annual Conference on Environmental Toxicology.

The aerosol generation and monitoring system used in the particle deposition and clearance experiments is illustrated in Figure 20. Latex microspheres labeled with ^{51}Cr are aerosolized with a Lovelace nebulizer. Diluting air is mixed with the output of the nebulizer to prevent agglomeration of the particles. The ratio of the amount of diluting air to the amount of air from the nebulizer is maintained at approximately 8:1. The mixture is sent through a heating system which increases the temperature to above 40°C for the purpose of drying the aerosol. The radioactive particles are nebulized into a five gallon, sealed can which is lined with aluminum foil to assist in the radioactive cleanup. Four holes are positioned around the periphery of the can so that the nose-only exposure tubes containing the rats may be plugged into the aerosol containment system. The rats are allowed to breathe the radioactive particles, nose-only, after the volume of the can has been filled with aerosol and the generator turned off. The exposure tubes consist of 8" sections of 2" diameter galvanized pipe, narrowed at one end in a funnel shape. The rats are placed in these tubes and gently but firmly secured so that the tip of their noses protrude through a hole in the tip of the funnel. The exposures of these animals to the microspheres are up to about 20 minutes in length depending on the specific activity of the aerosol. During animal exposure, cascade impactor and electron micrograph samples can be taken for aerosol characterization.

After the animals have inhaled a sufficient amount of radioactive aerosol, the exposure tubes are removed from the containment system, the rats are removed from the tubes and wiped to remove particles which may have contaminated their heads, noses, or fur during the exposure. The rats are then placed in individual counting restrainers and placed beneath a collimated NaI (Tl) gamma-ray detection system (Figure 22) to determine the initial amount of radioactivity in the respiratory tract. These counts are repeated at given intervals in order to observe the kinetics of clearance of particles from the respiratory system. Counts of unexposed rats are also obtained to establish the radiation background of the experimental set-up.

Additional information on the fate of inhaled particles can be obtained by analysis of excreta for particles. Excreta are collected at regular intervals and characterized by determination of time parameters such as time of first appearance, time required to excrete half of the total excreted, etc.

Preliminary studies involving 2-hour exposures to 2 ppm ozone indicate that deposition and clearance are altered (Figure 23).

G. Pulmonary Function/Response to Ozone Using Miniature Swine

1. Rationale

The following discussion, prepared by R. Fairshter, establishes the rationale for considering alternative large animals to supplement the dog in our research program .

Recent studies in man (McFadden, 1974; Abbour and Morton, 1975; Begin, et al. 1975) have established that detection of small airway obstruction (SAO) may require several different methods of testing. Specifically, some cigarette smokers can be shown to be normal by one method and abnormal by another testing procedure. Preliminary observations in our laboratory indicate that these same principles probably also apply to non-smoking human subjects with subclinical airway disease. Therefore, in order to detect effects of air pollutants in human lungs, multiple testing procedures probably should be applied.

The problem is somewhat more complex in animals. Humans may be easily taught to perform a variety of procedures which measure various aspects of pulmonary function. The number of procedures applicable to unanesthetized animals is limited. Therefore, more than one species might be considered as a test animal.

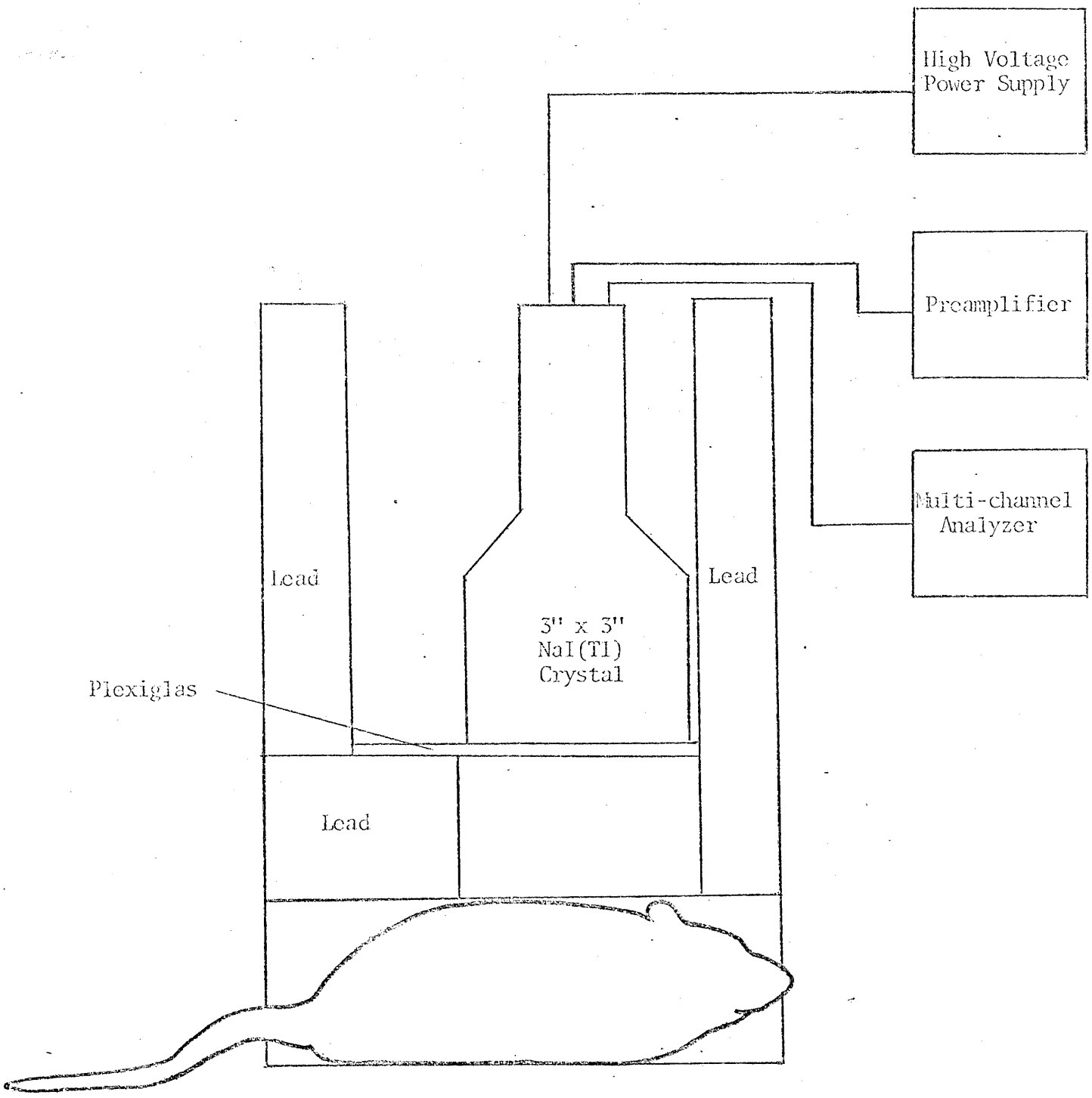


Figure 22. Schematic of radiation detection system used to observe the amount of radioactivity in the thoracic and head region of a rat after deposition of latex microspheres tagged with ^{51}Cr.

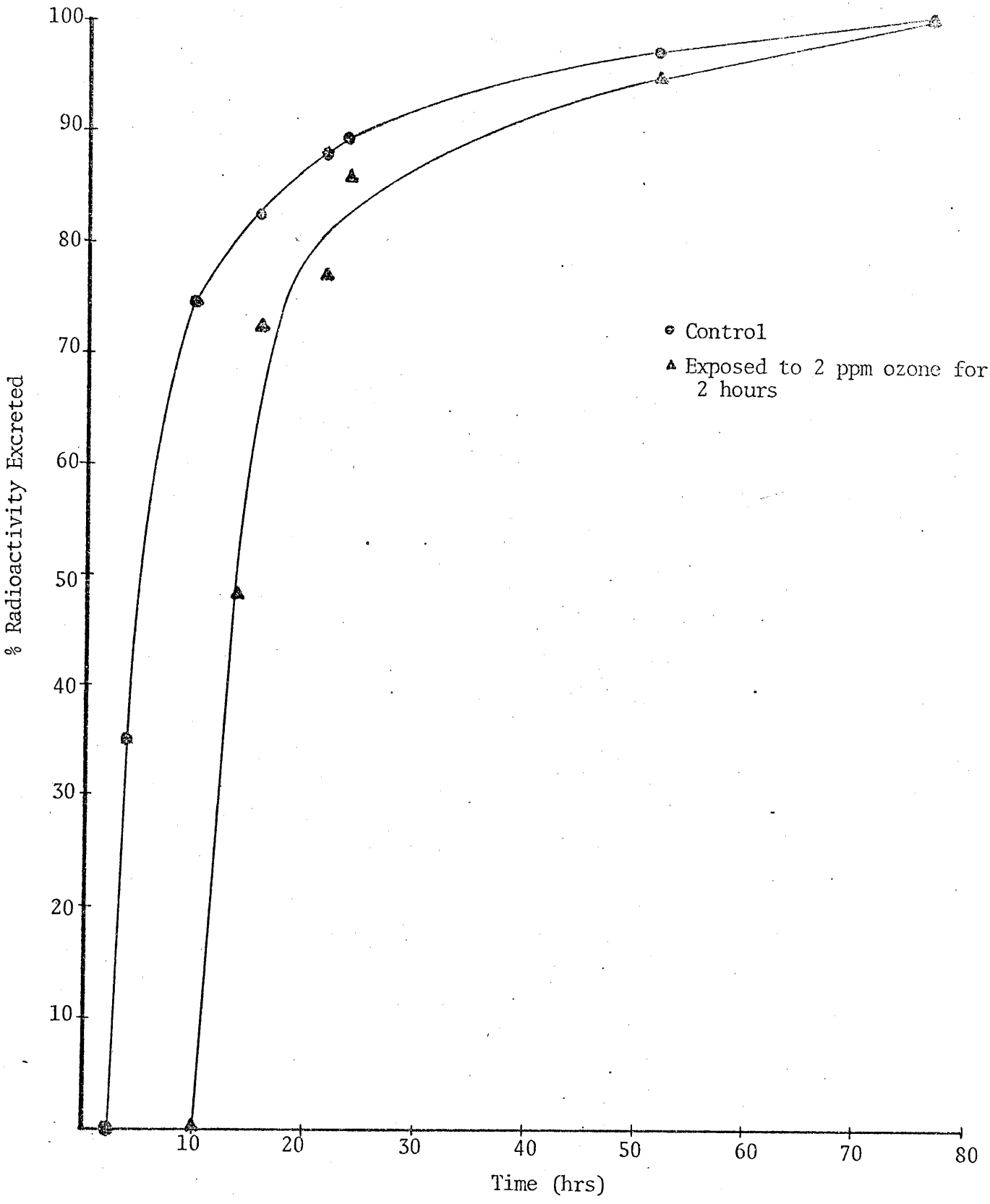


Figure 23. Fecal data from deposition/clearance study with rats. Results clearly show a delayed clearance of particles in rats that were exposed to 2 ppm ozone for 2 hours.

Specifically, multibreath nitrogen washout depends upon a difference in time constants (product of resistance x compliance) of different parenchymal units. In their classic paper, Otis et al. (1956), analyzed the lung in terms of an electrical analogue consisting of multiple parallel units with variable resistances in series leading to separate compliant parenchymal (capacitance) units. They demonstrated that the distribution of ventilation depended upon the time constants of the separate resistance-compliance units. Later, Chiang (1971), Ingram and Schilder (1967) and Wanner (1974) related distribution of ventilation as measured by multibreath nitrogen washout to frequency dependence of lung compliance. These authors demonstrated that abnormal nitrogen washout correlates very well with frequency dependence of compliance and the presence of two or more lung compartments with varying time constants. A four-fold difference in time constants must exist between different units in order to obtain frequency dependence of compliance and, presumably, abnormal nitrogen washout.

The lungs of dogs are well-known to have a highly developed system of collateral ventilation. Therefore, even in the presence of airway disease, parenchymal units may be well ventilated through collateral units (inter-alveolar pores, intra-bronchial communications). This high capacity for collateral ventilation might be expected to decrease the sensitivity of the nitrogen washout test since lung units with prolonged time constants might be bypassed by collateral channels.

Therefore, for this project, the future use of other large animals should be considered. The mini-pig has a poorly developed system of collateral lung ventilation. Early lesions which are not easily detectable by physiologic techniques in dogs may be detectable in the mini-pig. Since we should attempt to define the threshold levels of pollutants capable of causing lung damage, even if non-detectable physiologically in man (the collateral lung ventilation in man is intermediate between that of dog and the pig), use of the mini-pig as a test animal should be carefully considered. We do not suggest abandoning the dog as a test animal. We simply suggest that the use of more than one experimental animal might be advantageous to this project. To this end, a summer-student project has been devised to start development of a nitrogen-washout technique in the pig.

2. Objectives

The objectives in this study were twofold: to develop the nitrogen-washout technique in the miniature swine, and to evaluate him as a model for humans with respect to pulmonary function changes upon exposure to ozone.

3. Methods

Two female miniature swine, 6 months old and weighing approximately 30 kilograms, purchased from the Sinclair Comparative Medicine Research Farm associated with the University of Missouri, arrived at the lab in late June, 1975.

In order to obtain respiratory measurements on these animals, two specific experimental devices were developed: a restraint-transportation apparatus and an airtight mask. The restraint-transportation device developed consisted of a sling built into a heavy wooden frame, 2' x 2' x 3', mounted on four 8" rubber wheels. A 20" x 36" heavy sheet of canvas with 4 leg holes serves as a sling. Three automobile seat belts provided ventral restraint. The leg holes as well as the neck areas of the sling were padded with thick foam to assure comfort for the animal (Figure 24). The pig mask was modeled after the handmade latex masks which are presently being used on dogs in this lab. A cylindrical latex sleeve, 6" in length with a diameter of 2½", was the basic design for the mask. In order to assure an airtight seal, the animal's snout was first shaved, then a thin piece of closed-pore weather stripping foam was wrapped twice around the snout at the end of the mouth. Water-soluble jelly was applied to the outside of this foam, the mask was slipped over the mouth and snout, and held tightly by 3 rubber bands.

4. Procedures

Three mask-exposures to 1 ppm and 2 ppm ozone concentrations were conducted. Pig 116 was exposed to 2 ppm ozone for 4 hours. Pig 117 was exposed to 1 ppm ozone for 4 hours, and after a two-week recovery period was also exposed to 2 ppm ozone for 4 hours. The mini-pigs proved to be both noisy and a bit obstinate. They are relatively strong animals and hence the restraint necessary to conduct a 4-hour expousre was somewhat difficult to maintain. N_2 washout curves were obtained prior to, immediately after and 24 hours after the ozone exposures. Pre-exposure curves allowed each animal to serve as its own control with regard to analyzing the post-exposure data. Washout curves were obtained by plotting per cent N_2 in expired air vs. volume exhaled (in liters). The

Figure 24. Sling-type restrainer for pig, used during pulmonary function testing.

procedure involved was similar to that used for obtaining dog washouts. These data were analyzed using a modified student's T statistical test.

The washout data can be classified as follows, where n = number of washout curves used in data analysis:

Pig 116	Pre-exposure	Immediate post-exposure (2 ppm for 4 hrs)	24 hours post-exposure (2 ppm for 4 hrs)
	n = 8	n = 3	n = 3

Pig 117	Pre-exposure	Immediate post-expos. (1 ppm for 4 hrs)	17 hrs. post-expos. (1 ppm for 4 hrs)	Immediate post-expos. 2 ppm/4 hrs.
	n = 6	n = 3	n = 4	n = 4

5. Results

Nitrogen washout curves before and after exposure to 2 ppm ozone are shown in Figure 25. Confidence limits were set at 95%. At graduations of 5 liters, points taken from the post-exposure washout curves were analyzed by comparing them to the pre-exposure (control) points. In Tables XI-XV a "+" denotes a significant difference in the curves being compared whereas a "-" denotes no significant difference.

6. Conclusions

Data and observations obtained in this study seem to indicate that:

a. Miniature swine are more difficult to work with than are Beagle dogs.
b. At 1 ppm ozone, lung functions do not appear to be significantly impaired (Pig 116 had no response, Pig 117 a small response).
c. At 2 ppm ozone, lung functions do appear to be significantly impaired.

These preliminary data indicate that the pig is less sensitive to ozone than is the dog, therefore less suited as a model for humans with respect to pulmonary function changes upon exposure to ozone. As a result we have no immediate plans to replace the dog as an experimental subject in this project. (Note: both tidal volume and respiratory rate were measured before and after exposures to ozone. However, the variability in the values of these parameters was large and no significant changes were detected.)

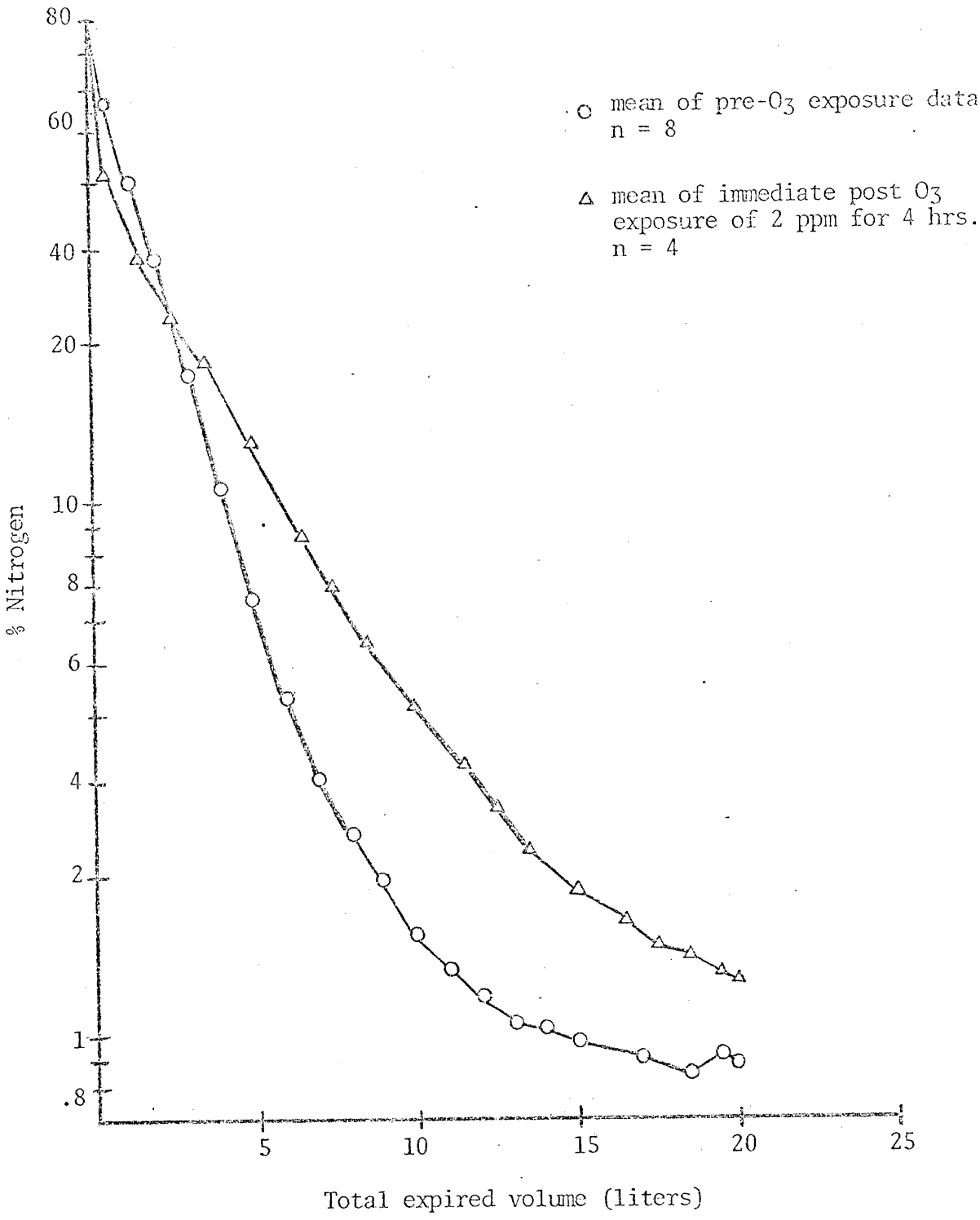


Figure 25. These N_2 washout curves illustrate the most marked effect seen in the mini-pigs following an ozone exposure. (Data from Pig 116)

TABLE XI. Comparison of Pre-Exposure with Immediate Post-exposure to 1 ppm O_3 for 4 hours on Pig 117. V_E is expired volume, U is number of degrees of freedom, and t is the value of the t statistic.

V_E (liters)	U	t	Significance
5	4	.80	-
10	3	1.37	-
15	7	2.96	+
20	7	3.93	+

TABLE XII. Comparison of Pre-exposure with 17 hours Post-Exposure to 1 ppm O_3 for 4 hours. Pig 117

V_E (liters	U	t	Significance
5	7	2.97	+
10	6	.12	-
15	6	1.95	+/-
20	3	.54	-

TABLE XIII. Comparison of pre-exposure with Immediate Post-exposure to 2 ppm O_3 for 4 hours. Pig 117

V_E (liters	U	t	Significance
5	7	4.45	+
10	4	4.13	+
15	4	2.28	+
20	6	2.28	+

TABLE XIV. Comparison of Pre-exposure with Immediate Post-exposure to 2 ppm O_3 for 4 hours. Pig 116

V_E (liters)	U	t	Significance
5	3	3.20	+
10	3	4.40	+
15	3	4.61	+
20	5	5.44	+

TABLE XV. Comparison of Pre-Exposure with 24 hrs Post-Exposure to 2 ppm O_3 for 4 hours. Pig 116

V_E (liters)	U	t	Significance
5	3	5.31	+
10	2	2.94	+
15	2	2.15	-
20	2	1.23	-

II. Pathology

1. Preparation of Tissue

The lung, an easily-deformed, easily-damaged organ, requires the utmost care when it is prepared for pathologic examination. Endpoints of pathology, such as alveolar and bronchiolar size, and thickness of membranes and septa, are all dependent on state of inflation. Identification of numbers and types of cells present, degree of ciliation, and the separation of membranes can be impossible if fixation is not rapid. We have, therefore, looked toward rigorous methods for dissection and rapid fixation at a constant pressure. The procedures described below, and discussed more generally in the attached pre-print (Appendix E) are adapted from methods used in preparing lungs for detailed morphometric examination.

2. Dissection

The rats sacrificed for histologic examination of the lung are killed by intraperitoneal injection of sodium pentobarbital. The procedure for the dissection is:

a) the abdominal cavity is opened and the abdominal aorta and/or the posterior vena cava cut in order to bleed the animal.
b) the trachea is then exposed, tied off at the upper end, and cut.
c) the thoracic cavity is opened, exposing the lungs and heart.
d) beginning with the trachea, tissue is cut or blunt-dissected away, partially freeing the lungs and heart.
e) the inferior vena cava, aorta, and esophagus are cut next to the diaphragm, totally freeing the lungs, heart and trachea.
f) the lung/heart unit is ready for fixation.

During the procedure of excising the lungs, "Dissection and Lung Catalog-Record" form is filled out and signed (Figure 26). The lower right corner of this form is detached and placed inside the specimen bottle with the excised lungs. The rest of the form is kept in a laboratory notebook. This notebook contains pertinent information on all of the dissected rats.

3. Lung Fixation

Objective: to develop a method for fixing the lung that uses a simple apparatus, that is rapid, and that is reproducible.

Figure 26

CATALOG # _____

DISSECTION AND LUNG CATALOG-RECORD

RAT # _____ DATE LOT RECIEVED _____ DISSECTION DATE _____

EXPOSURE HISTORY-

PREVIOUSLY EXPOSED? YES ____ NO ____ EXPOSURE DATE _____

ATMOSPHERE _____ CONCENTRATION _____ DURATION _____

TIME FROM EXPOSURE _____

ANIMAL CONDITION-

BREED _____ SEX M F WEIGHT _____

APPEARANCE? SHINY _____ ROUGH _____

PHYSICAL CONDITION? GOOD _____ POOR _____

EXPLAIN _____

_____

DISSECTION RECORD-

ABDOMINAL VISCERA? NORMAL _____ ABNORMAL _____

EXPLAIN _____

_____

APPEARANCE OF LUNGS-

TRACHEA? NORMAL _____ ABNORMAL _____

EXPLAIN _____

	COLOR	EXPLAIN-
RIGHT UPPER LOBE	_____	_____
RIGHT MIDDLE LOBE	_____	_____
RIGHT LOWER LOBE	_____	_____
LEFT UPPER LOBE	_____	_____
LEFT LOWER LOBE	_____	_____

CAUSE OF DEATH- NEMBUTAL _____

OTHER _____

DISSECTED BY _____

CATALOG # _____

RAT # _____ REC. _____ DISS. _____

PREV. EXP. _____ DATE _____

ATMOS. _____ CONC. _____

DURATION _____

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Criteria for suitable fixation are:

a) production of minimal artifacts

b) fixation at normal state of lung inflation

c) economical with respect to time and cost

Modern methods of lung fixation range from simple immersion in liquid fixative, through tracheal and vascular perfusion, to the complex formalin steam method at constant inflation volume described by Weibel and Vidone (1961). We have constructed a simple apparatus, suggested by Fawell and Lewis (1971), (Figure 27) for fixation by intratracheal instillation of fixative at a constant pressure.

The apparatus used routinely with the lungs of rats consists of a tracheal cannula connected to a reservoir of fixative. The solution presently used for the fixation of rat lungs is 10% neutral buffered formalin. The recipe for preparation of one liter is:

Formaldehyde	100 ml
Monobasic Sodium Phosphate	4.0 gm
Dibasic Sodium Phosphate	6.5 gm
Distilled H_2O	900 ml

Another solution to be used in the future is a modification of Karnovsky's formaldehyde/glutraldehyde fixative with added calcium chloride. The creation of an air-lock in the pipette bulb maintains a constant pressure head of fixative fluid, the inflation pressure being the distance from the meniscus in the lower bulb to the level of formalin in the bath. In our work, we use 30 cm of pressure as suggested by Dungworth (1976). On the average, during 30 minutes fixation at 30 cm pressure head, a lung weighing 5 gm increases to 19 gm in weight. After immersion in fixative for one month, the weight decreases to 16 gm because of shrinkage. For proper and adequate fixation, 3 hours-18 hours at 30 cm pressure is used before disconnecting and placing the lung in a jar of fixative. To date, about 50 lungs have been fixed by this method.

4. Histopathologic Examination

A local arrangement for histopathologic examination of rat lungs has been developed by the voluntary participation of Hubert Pirkle, M.D., Associate Professor of Pathology, College of Medicine, UC Irvine. Dr. Pirkle's aid will include supervision of tissue processing (fixing, blocking, sectioning and mounting of tissues on microscope slides for reading by a histopathologist).

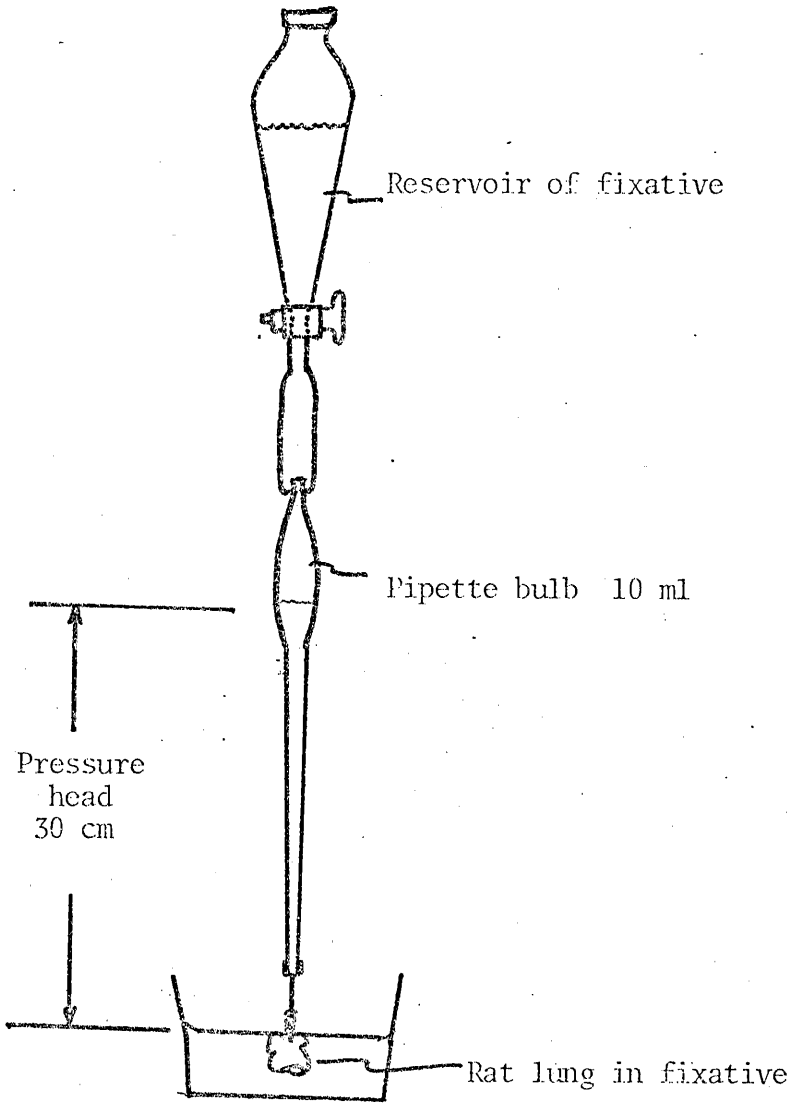

Figure 27. Device for instillation-fixation of rat lung at constant pressure. This method of fixation, often used in rigorous morphometric studies, assures reproducible, rapid fixation with near-minimal artifacts.

Processing costs will be covered by the histopathology service funds in the present budget. The slides will be read by Dr. Pirkle as an interested collaborator. If time and effort factors reach significant proportion, Dr. Pirkle will be paid for his participation.

This service arrangement is informal but has the advantages of being local, rather than remote as originally planned. The use of a consulting service at UCLA will be more seriously considered as workload increases.

Present need for pathology service has been related to quality control in laboratory animals, especially rats, before exposing the animal or conducting baseline physiological studies. This scope will increase as experiments require histologic study of all experimental animals to assess normality of lung. Development of local pathology service is desirable and will be continued if compatible with workload.

IV. Monitoring of Pollutant Aerosols

A. General

Illustrated in Figure 28 is some of the aerosol monitoring and characterizing equipment at the inhalation facility. Each of these instruments has limitations and the complete battery is often necessary to fully characterize the size of the constituents of an aerosol cloud.

The Royco Model 225 aerosol particle analyzer is capable of sizing particles which range from 0.5 μm to greater than 3.0 μm in diameter; its mode of analysis is that of light scattering. This apparatus has been used to measure the geometric size of sulfuric acid mist particles. Its performance has been consistent and the acid mist itself has not been detrimental to the Royco instrument.

The Thermo Systems Model 3030 Electrical Aerosol Size Analyzer is capable of sizing aerosol particles which range from 0.0032 μm to 1 μm in diameter. This instrument is based on a diffusion-charging, mobility-analysis principle. It is being utilized to measure the geometric size distribution of salt aerosol particles generated with the Environmental Research Corporation fluid atomizer. The performance of this equipment has also been consistent. Table XVI shows electrometer current readings for various salt aerosols and clean Rochester chamber air. Differences between adjacent current readings are used to calculate number of particles in each size interval. Though the instrument does not appear to provide an unbiased size distribution, it is useful for monitoring the stability of an aerosol during an exposure.

A Zeiss TGZ-3 particle size analyzer is presently being used to determine the size distribution of salt aerosols from electron micrographs. A particle population may be separated into a maximum of 48 size intervals so that the distribution can be well defined. This equipment is easily and efficiently operated; it is now essentially interfaced with the PDP-11 minicomputer.

Seven-stage cascade impactors from ARIES, Inc. (Albuquerque, New Mexico) are presently in use at this facility. This equipment can reliably measure the aerodynamic size distribution of aerosol particles which range from 0.3 μm to 5 μm in diameter. The total mass concentration of an aerosol can also be determined with this instrument. The impactors are being used in sulfuric acid mist and sodium chloride experiments. They are also used in the labeling

TABLE XV. ELECTROMETER CURRENT READINGS FOR SALT AEROSOLS

Channel	μm	Sodium Chloride 10%			Ammonium Sulfate 10%			Ferric Sulfate 10%			Clean Air		
1	.0032	/	/	/	/	/	/	/	/	/	/	/	/
2	.0056	/	/	/	/	/	/	/	/	/	/	/	/
3	.0100	9.23	9.01	9.06	11.05	10.91	10.72	12.58	12.38	12.43	.110	.105	.101
4	.0178	9.01	8.89	8.77	11.00	10.88	10.72	12.51	12.26	12.44	.110	.104	.101
5	.0316	8.65	8.42	8.44	10.35	10.46	10.17	12.05	11.68	11.98	.104	.099	.097
6	.0562	7.84	7.57	7.63	9.67	9.59	9.41	11.16	11.01	11.15	.100	.096	.090
7	.100	4.39	4.25	4.32	5.88	5.89	5.85	6.80	6.62	6.87	.074	.070	.068
8	.178	.88	.85	.85	1.46	1.52	1.42	1.76	1.69	1.56	.027	.026	.025
9	.316	.11	.11	.11	.23	.26	.27	.26	.23	.26	.007	.006	.007
10	.562	.04	.04	.04	.08	.08	.08	.08	.08	.08	.004	.005	.004
11	1.000	.03	.03	.03	.04	.04	.04	.04	.04	.04	.004	.004	.003

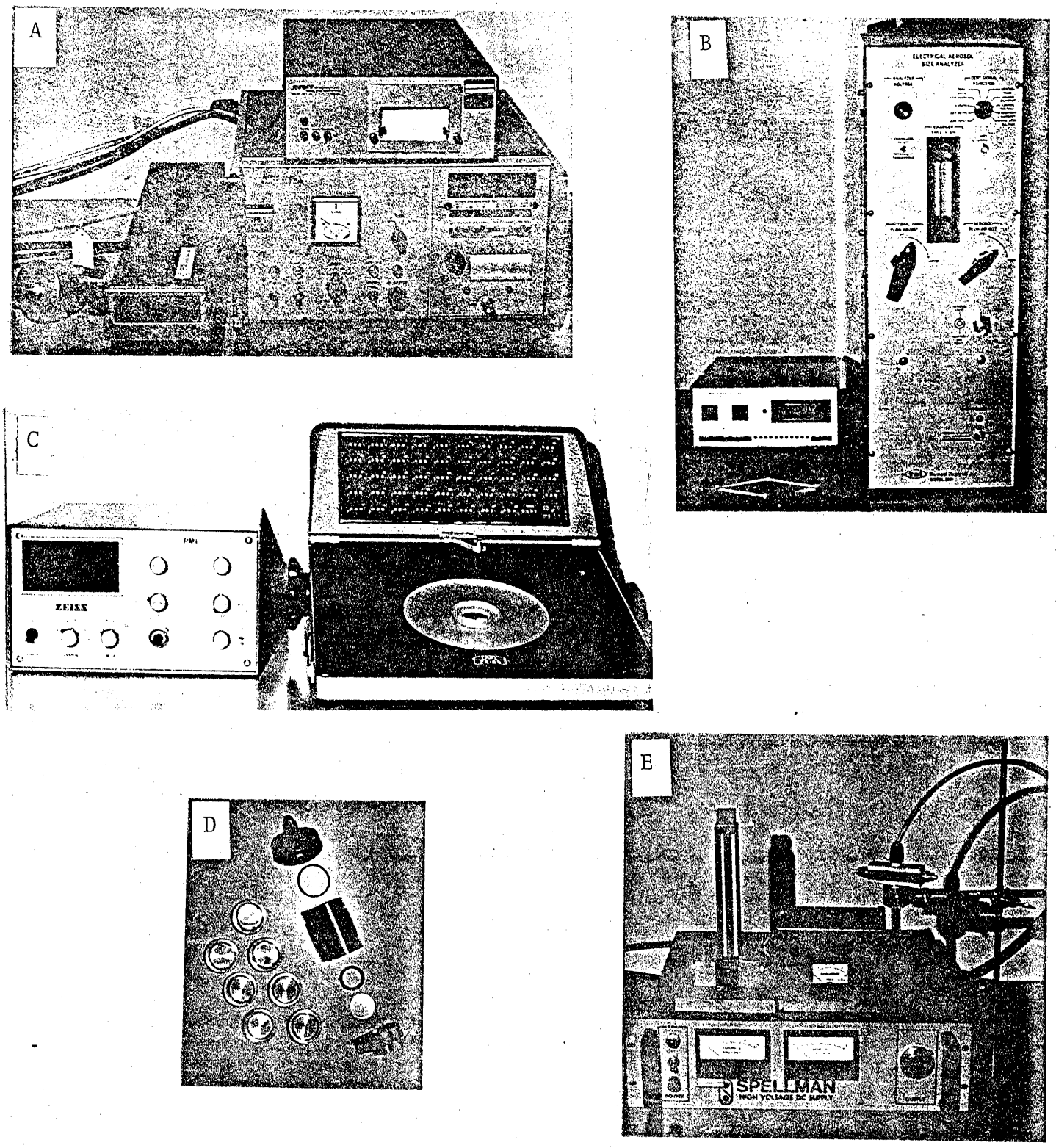

Figure 28. Some of the aerosol monitoring and characterizing equipment at APHEL. A. Royco 225 optical size analyzer. Sizes airborne aerosols in the range of 0.5 to greater than 5.0 μm. B. Whitby-type electrical size analyzer. Sizes in ten classifications, all below 1.0 μm particle diameter. C. Zeiss TGZ3 particle size analyzer. Has 48 size-interval channels for precisely determining size distribution data from electron micrographs. D. ARIES seven-stage cascade impactor. Provides aerodynamic size distribution between 0.3 and 5.0 μm. E. ARIES electrostatic precipitator. Obtains sample of aerosol for electron microscopy.

experiments of polystyrene latex with Cr-51 to determine how much of the radioactivity has been transferred to small, secondary particles and the extent to which particle agglomeration has occurred.

Electrostatic precipitators, also from ARIES, Inc., are being employed to obtain samples for electron microscopy in the salt aerosol exposures and the Cr-51 labeling study. From the electron micrographs obtained using this equipment, not only a size distribution is determined, but also visual information on the aerosol is obtained. Particle shape, state of agglomeration, presence of foreign materials and state of dryness are ascertained from such photographs. Without electron microscopy, background information such as this will go undetected and factors influencing exposure may not be observed.

B. Generation and Characterization of Sulfuric Acid Mist Aerosols

The biological effects of sulfuric acid mist undoubtedly depend on particle size, mass concentration in the air and concentration of the acid. The following ultimate criteria were selected for the generation and monitoring of sulfuric acid aerosol in order to insure well-characterized animal exposures.

1. A size distribution and mass concentration that is stable over a period of hours.
2. A selectable mass concentration from 0.3- 15 mg/m^3
3. A unimodal mass distribution, with greater than 95% of the total mass below 3 microns in diameter.
4. A means to vary the humidity of the exposure chamber.
5. A means to measure the mass concentration of the aerosol, with verification by an independent method.
6. A means to measure the pH or molarity of the aerosol, with verification by an independent method.
7. Determination of whether or not the sulfuric acid aerosol is in aqueous equilibrium (i.e., neither evaporating nor taking up water) with the water vapor present in the chamber
8. Determination of the particle size distribution (by count and by mass) of the aerosol with verification by an independent method.

It is desirable to initially determine such parameters as mass concentration, pH and particle size distribution by two independent techniques in order that possible errors in one or both of the techniques be detected. If both techniques are sound, they should both yield essentially the same result. The simplest technique may then be adopted for routine measurements.

Scientific progress relating to the development of sulfuric acid aerosols for future inhalation studies may be conveniently divided into three categories: aerosol generation, aerosol containment, and aerosol characterization. Progress made in each area may be summarized as follows:

1. Aerosol generation

Several published papers refer to generation of sulfuric-acid aerosols (Pattle, 1956: Alarie, 1973: Treon, 1950; Fairchild, 1975). To meet all of our criteria the decision was made to use compressed air nebulization. In the search for a suitable nebulizer for the generation of sulfuric acid aerosol, four nebulizers were considered: Lovelace-type, Retec, Puratin-Bennet, and a custom glass nebulizer constructed in this laboratory. Nebulizers were compared on the basis of output rate, maximum unattended operating time, size of particles generated, resistance to corrosion, and other characteristics. The custom glass nebulizer with a large-particle trap (Figure 29) was found to be most compatible with the aerosol containment system and research objectives.

At present, the following generation capability exists with respect to sulfuric acid aerosols:

a. Capability to produce any mass median aerodynamic diameter from .3 um to 2.3 μm.

b. At 1.5 μm or 2.3 μm, capability of varying relative humidity while holding mass concentration and particle size constant.

c. Capability of producing mass concentrations as high as 120 mg/m^3.

2. Aerosol containment

One of the laboratory Rochester chambers was selected as the containment system for sulfuric acid aerosol early in this research. Three important

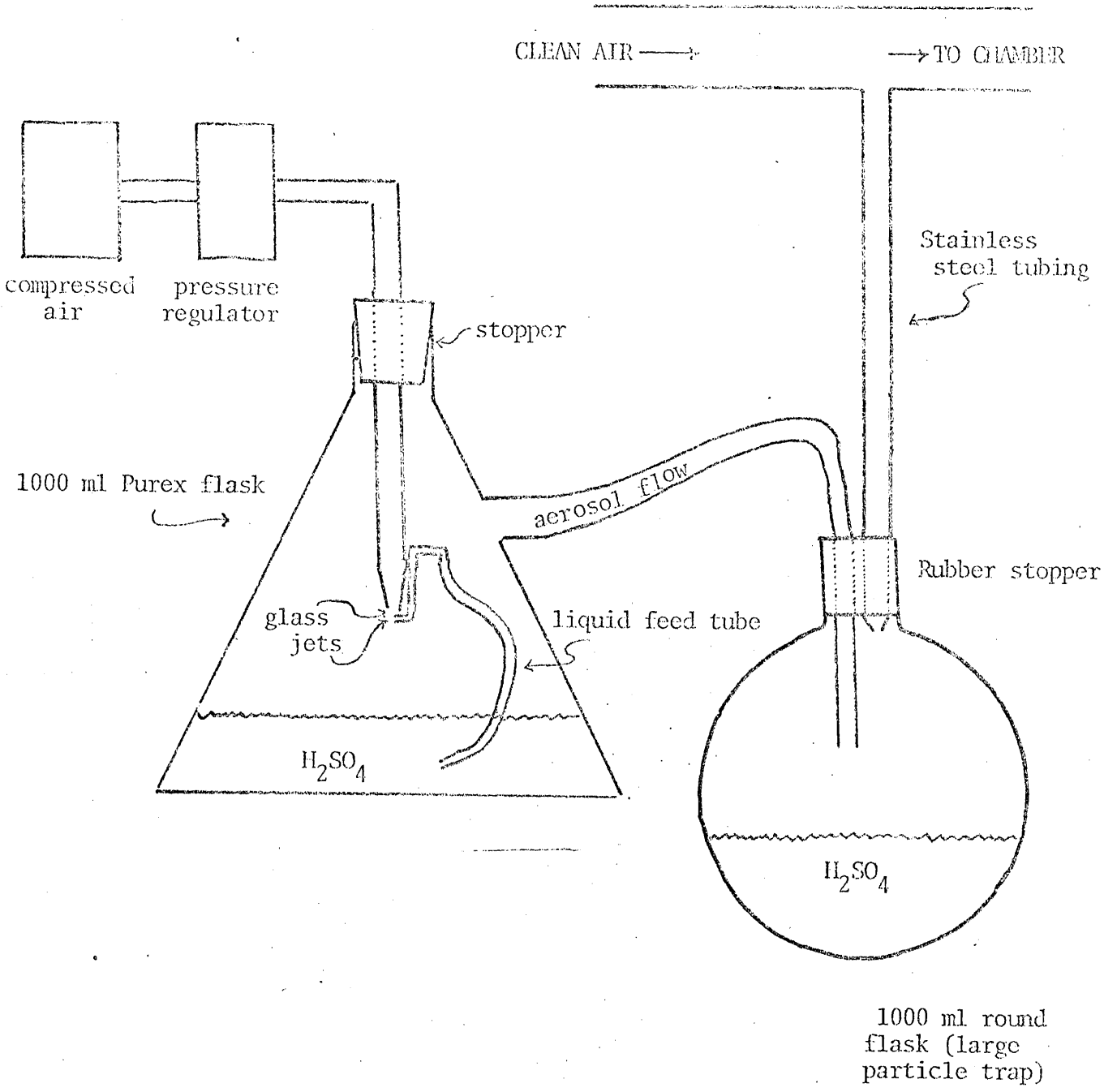

Figure 29. Sulfuric acid mist generator, developed at APHEL for animal exposure studies. The compressed air nebulizer can generate sub-micron aerosols at high mass concentrations for several hours at a time.

advantages of the Rochester chambers over less sophisticated aerosol containment systems are as follows:

a. Good mixing. Age distribution studies using aerosol and gaseous tracers have been performed which confirm the assumption of good mixing in the Rochester chambers. Chamber mixing appears to be especially good for gaseous pollutants, and acceptable for particulates.

b. Temperature and humidity control. With the recent installation of stainless steel pipe on the downstream side of the Bemco Air Servo, full capability for temperature and humidity control has been achieved. Temperature is controllable between 50° and 100°F and relative humidity between 40 and 80%.

c. Air purification. The purafil module, presently located directly upstream of the Bemco unit, removes laboratory and atmospheric pollutants (such as ammonia) that could interfere with the sulfuric acid aerosol research.

3. Aerosol characterization

Methods have been developed to measure: a) aerosol particle size and distribution; b) mass concentration of aerosol; c) average acid concentration inside aerosol particles; and d) time stability of aerosol. A brief description of each technique is as follows:

a. Aerosol particle size and distribution. A cascade impaction method is used to measure particle size and mass distribution. After collection of a sulfuric acid aerosol sample, the fraction of total mass on each stage is estimated by acid-base titration. A log normal mass distribution is assumed, allowing determination of mass median aerodynamic diameter and geometric standard deviation (Fig. 30). Good agreement has been obtained between this method and the electrostatic precipitation/electron microscopy method used to estimate sodium chloride aerosol size.

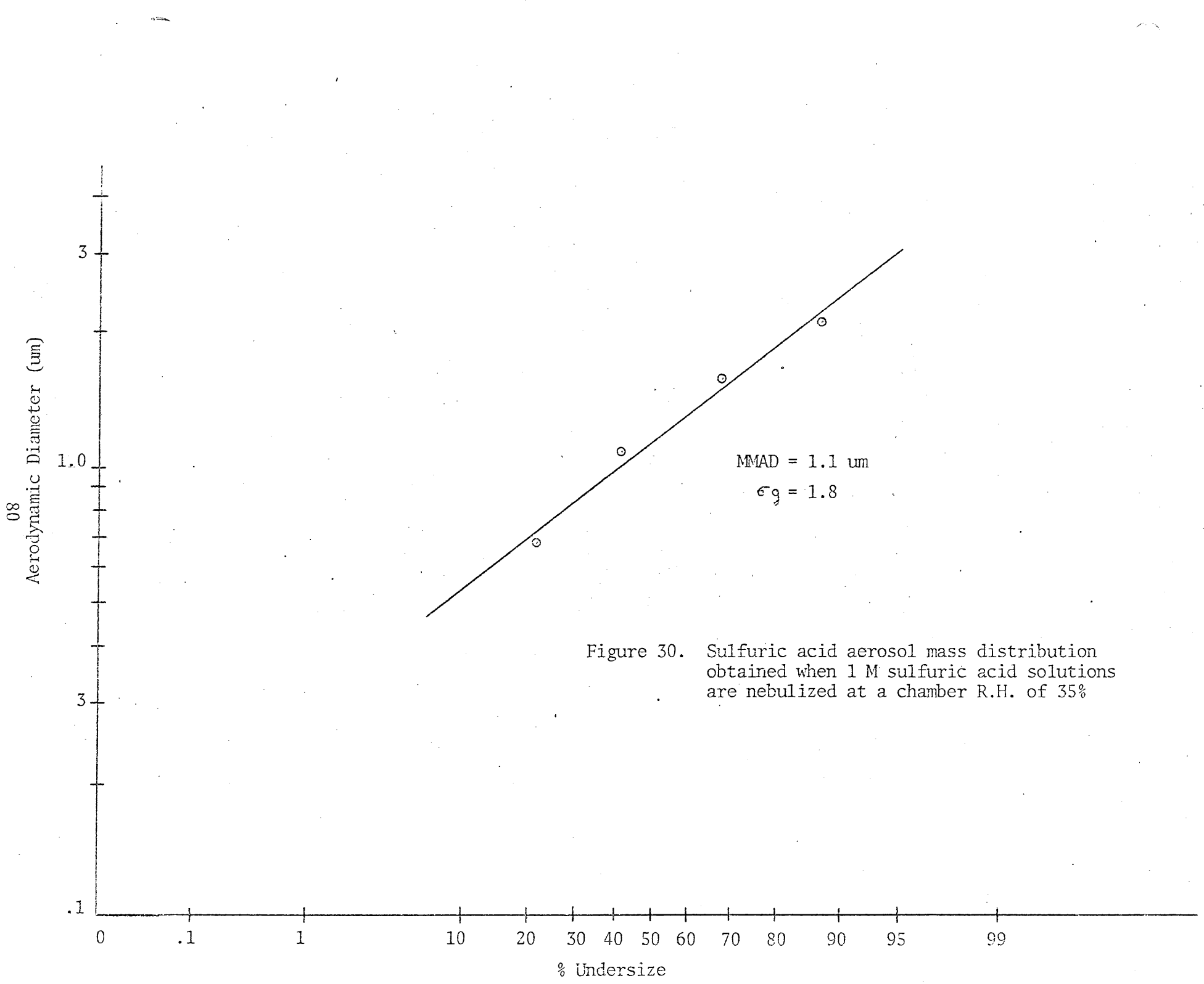

Figure 30. Sulfuric acid aerosol mass distribution obtained when 1 M sulfuric acid solutions are nebulized at a chamber R.H. of 35%

b. Mass concentration of aerosol. To measure aerosol mass concentration, a known volume of aerosol is collected upon specially treated fiberglass filters and the quantity of acid caught is determined by titration.

c. Average acid concentration inside aerosol particles. A known weight of aerosol is collected by filtration and titrated with standard alkali. Corrections are made for non-acid particulates, hygroscopicity of sulfuric acid, and humidity effects.

d. Time stability. The Royco Model 225 aerosol particle counter is used to provide a continuous record of aerosol time stability. Particle number concentration measurements are made in five size ranges from .5 to 3 μm at periodic intervals during the exposure period. The custom glass nebulizer has been shown to provide good time stability for periods exceeding 5 hours.

As of this report date a preliminary two-hour exposure of a rat to sulfuric acid mist aerosol has been made using the generation equipment described above. The sulfuric acid aerosol generation and characterization systems are thus ready for full scale use in animal exposures.

C. Generation and Monitoring of Pollutant Salt Aerosols

Sodium chloride, ferric sulfate, sulfuric acid mist and ammonium sulfate are the pollutant aerosols inhaled by animals in this project. The basic objective is to reproducibly generate aerosol particles with mass median diameters below 1 μm, having geometric standard deviations of less than 2, and mass concentrations up to a few mg/m^3 of air. We have generated stable, controllable salt aerosols with mass median diameters between 0.2 and 0.4 μm, having geometric standard deviations between 1.4 and 1.8 and at mass concentrations of up to 3 or 4 mg/m^3 of air. This mass concentration is near the maximum mass concentrations that we can practically generate and maintain singlet particles for both rat and dog exposures. At much higher number concentrations particle agglomeration causes changes in particle size. The overall set-up

for generation and monitoring of aerosols used in animal studies in shown in Fig. 31.

Generation

Aerosols have been generated from 10% sodium chloride, ferric sulfate, and ammonium sulfate solutions using a compressed air atomizer made by Environmental Research Corporation (Fig. 32). The procedure is as follows:

Protocol for generation of salt aerosols

1. Prepare salt solution (10% by weight). Example: 270 g distilled water
   30 g sodium chloride
2. Fill liquid reservoir with solution to a level between indicator marks.
3. Install liquid reservoir and the atomizer assembly in the generator housing.
4. Connect air supply line to generator. The supply air pressure must be at least 45 psig (set at 50 psig). Make sure the aerosol line is secured to the Rochester chamber inlet and that no leaks are present.
5. Adjust the pressure regulator until the gauge on the aerosol generator reads 35 psig.
6. Read atomizer flowmeter. A reading of 75 to 90 indicates the atomizer is operating properly.
7. Set the dilution air valve close to zero.
8. Check the exposure chamber flow rate. 0.03" to 0.04" water pressure on chamber flow gauge will give 3 to 4 mg/m^3 mass concentration.
9. Check the negative pressure in the exposure chamber. For dog exposures, make sure that there is flow through the one-way valve to the exhaust chamber; however, pressure difference should not exceed 1.5 inches.
10. Check chamber humidity and temperature and record them.
11. Determine the mass concentration in the exposure chamber by sampling through a pre-weighed fiberglass filter at a flowrate of 16.6 liters/min. for 1 hour. This can be performed before the exposure begins.
12. During the dog exposure, sample aerosol from chamber as before or in front of the dog mask with a fiberglass filter at a flowrate of 2 liters/min for 2 hours and compare the result with filter sampled in the chamber.

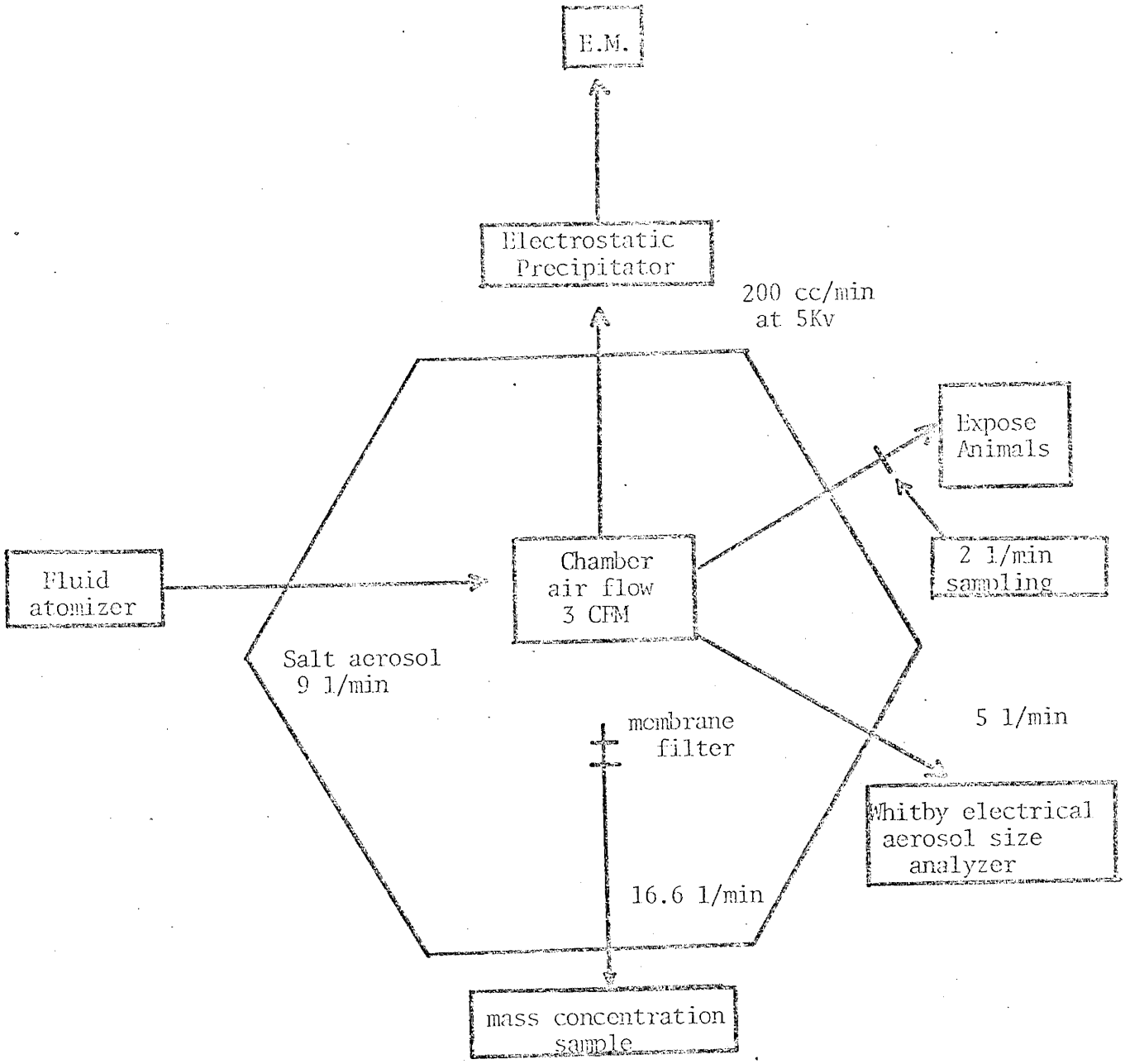

Figure 31 Schematic diagram of aerosol system for animal exposure to salt aerosols.

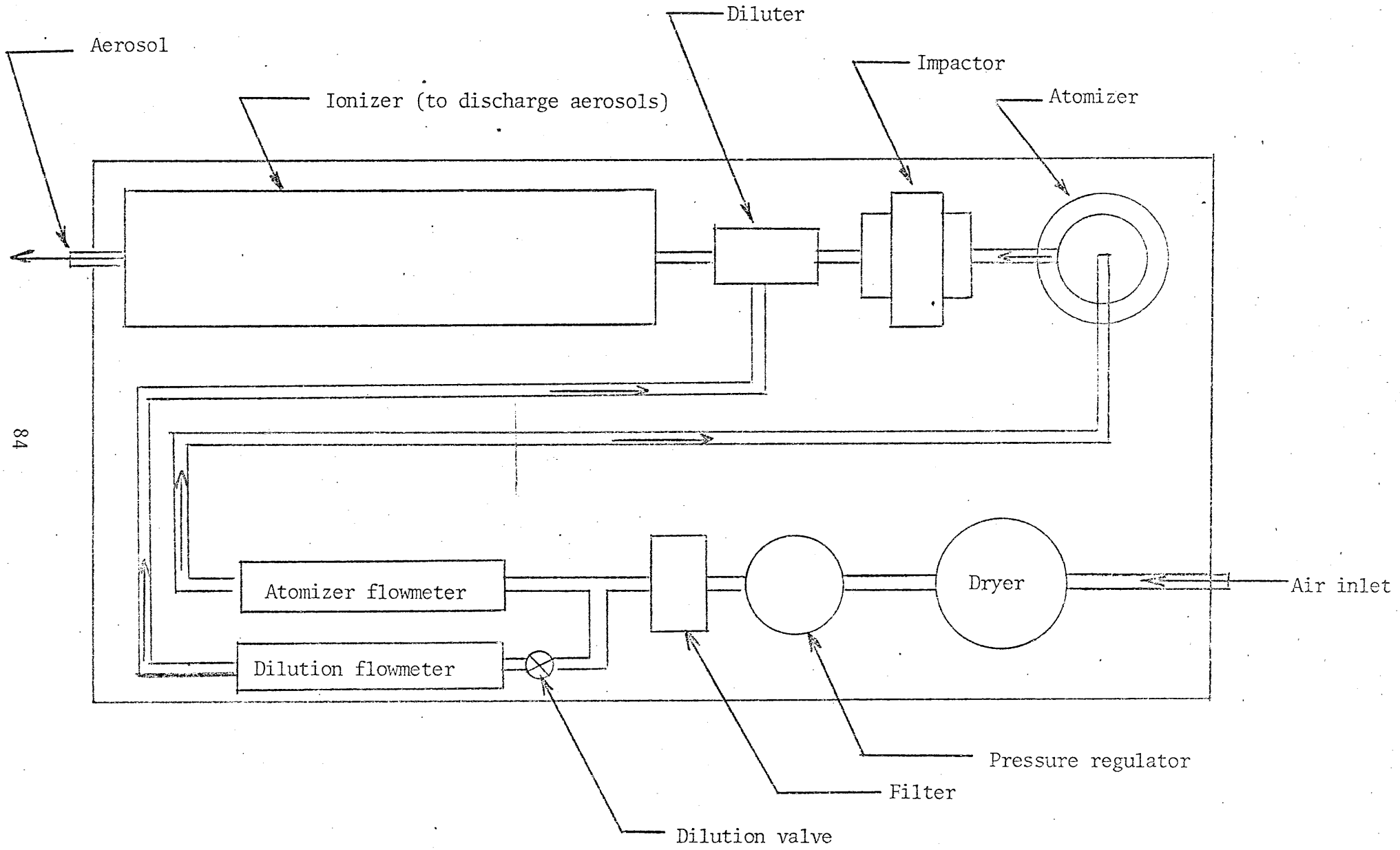

Fig. 32. Nebulizer, manufactured by Environmental Research, used in generation of salt aerosols.

13. Take electron microscope samples on grids before, during and immediately after the exposure.

14. Check liquid reservoir in generator periodically; refill the solution at least once every hour.

15. Disconnect compressed air supply when exposure is finished.

16. Wash and clean liquid reservoir, impactor and atomizer in the ultrasonic cleaner.

Whitby Electrical Size Analyzer

Size distributions of salt aerosols are monitored using the Whitby-type Electrical Size Analyzer. Five out of the eleven size-interval channels are in the 0.1 to 1.0 um diameter range in which we are interested. Readouts from the electrometer currents of the device also yield information on the mass concentration of aerosols inside the Rochester chamber. Though this instrument is not used to determine reliable size estimates, it does permit one to see any drift in particle size.

Electron Microscopy

Sodium chloride, ferric sulfate and ammonium sulfate particles are also collected on an electron microscope grid using the electrostatic precipitator made by ARIES, Inc. (Albuquerque, New Mexico) (Fig. 28). The accurate size distribution, count median diameter, mass median diameter and geometric standard deviation are then determined by analysis of photographs (Figs. 33, 34 and 35) using a Zeiss TGZ-3 particle size analyzer. Computer output from sizing of salt aerosols is found in Appendix D. Median projected area diameter, mass median diameter and geometric standard deviation of aerosols from 10% sodium chloride, ferric sulfate and ammonium sulfate solutions are shown in Table XVII. Figure 36 shows the cumulative size distributions of salt aerosols as plotted on log-probability paper; log-normally distributed data plot as straight lines on such paper. Differential size distributions of the same aerosols are shown in Figure 37.

Airborne mass concentrations are determined by putting two fiberglass filters in series inside the chamber and sampling with 16.6 liters/min for 1 hour. The first filter captures all the mass and the second filter gives the change of filter weight due to humidity and acts as a control. During

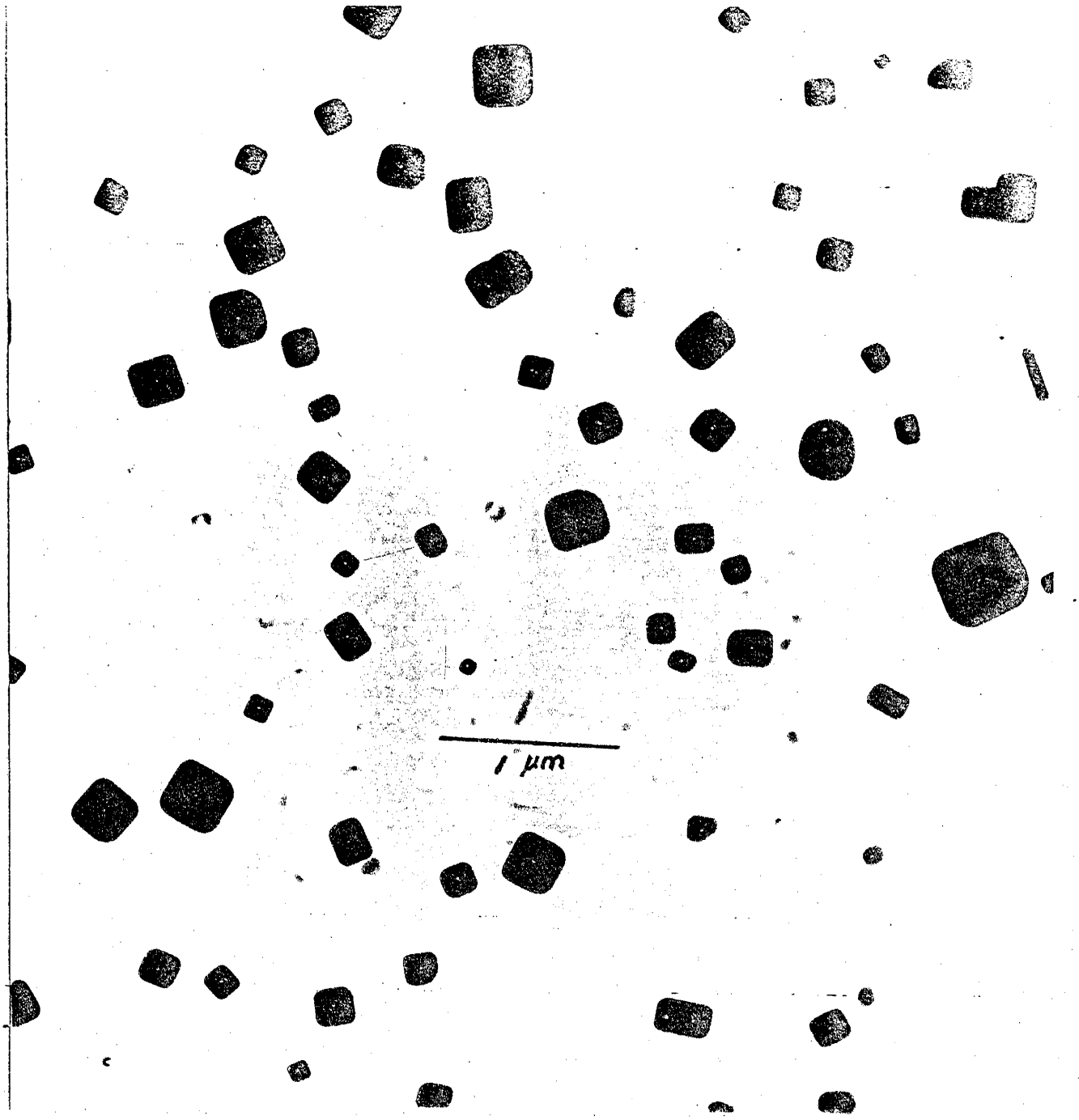

Figure 33. Electron micrograph of sodium chloride aerosol sampled from Rochester chamber. Aerosol was prepared by drying a nebulized solution (10% w/vol) of the salt. Bar on photograph represents 1.0 µm.

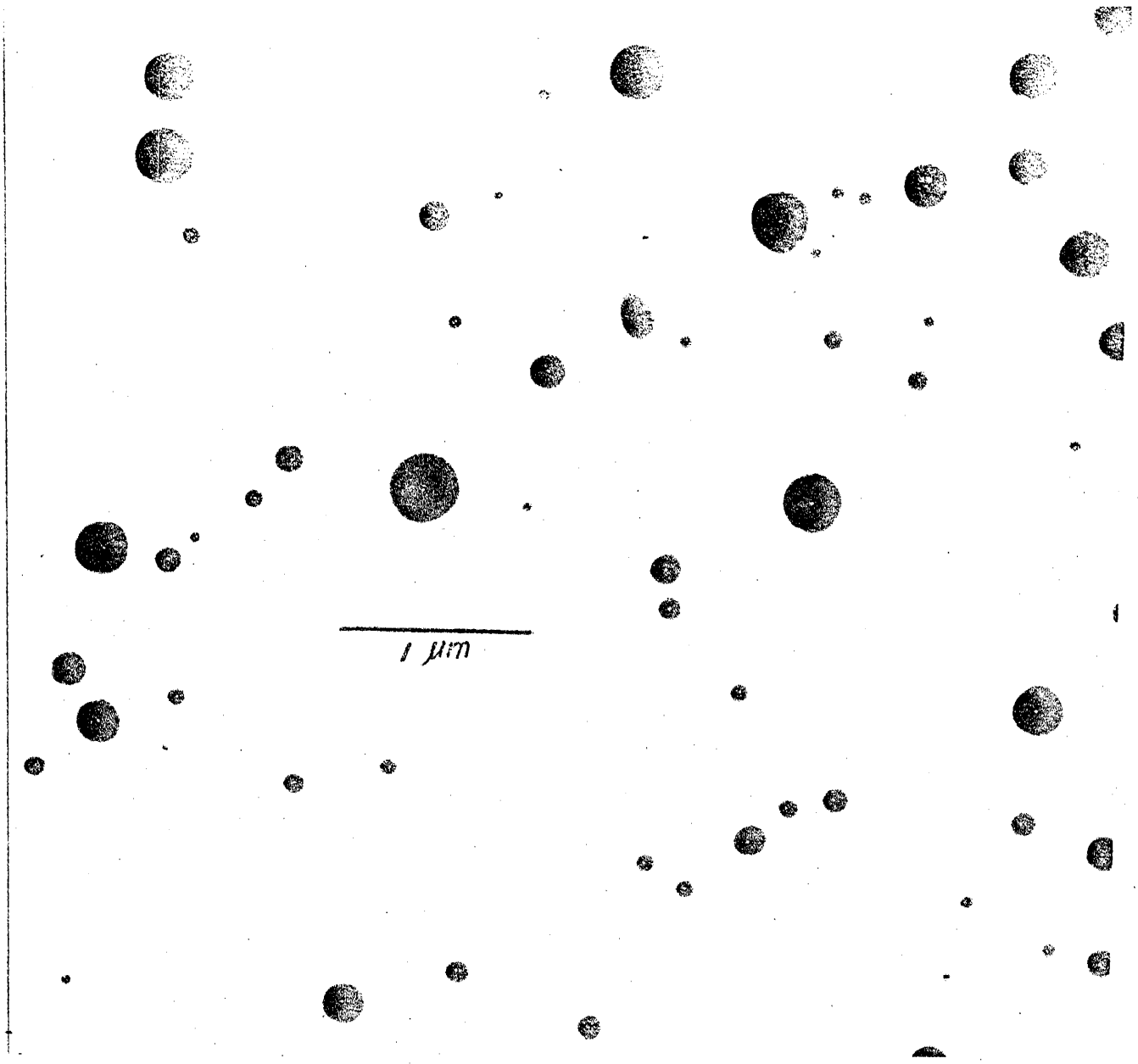

Figure 34. Electron micrograph of ferric sulfate aerosol sampled from Rochester chamber. Aerosol was prepared by drying a nebulized solution (10% w/vol) of the salt. Bar on photograph represents 1.0 μm.

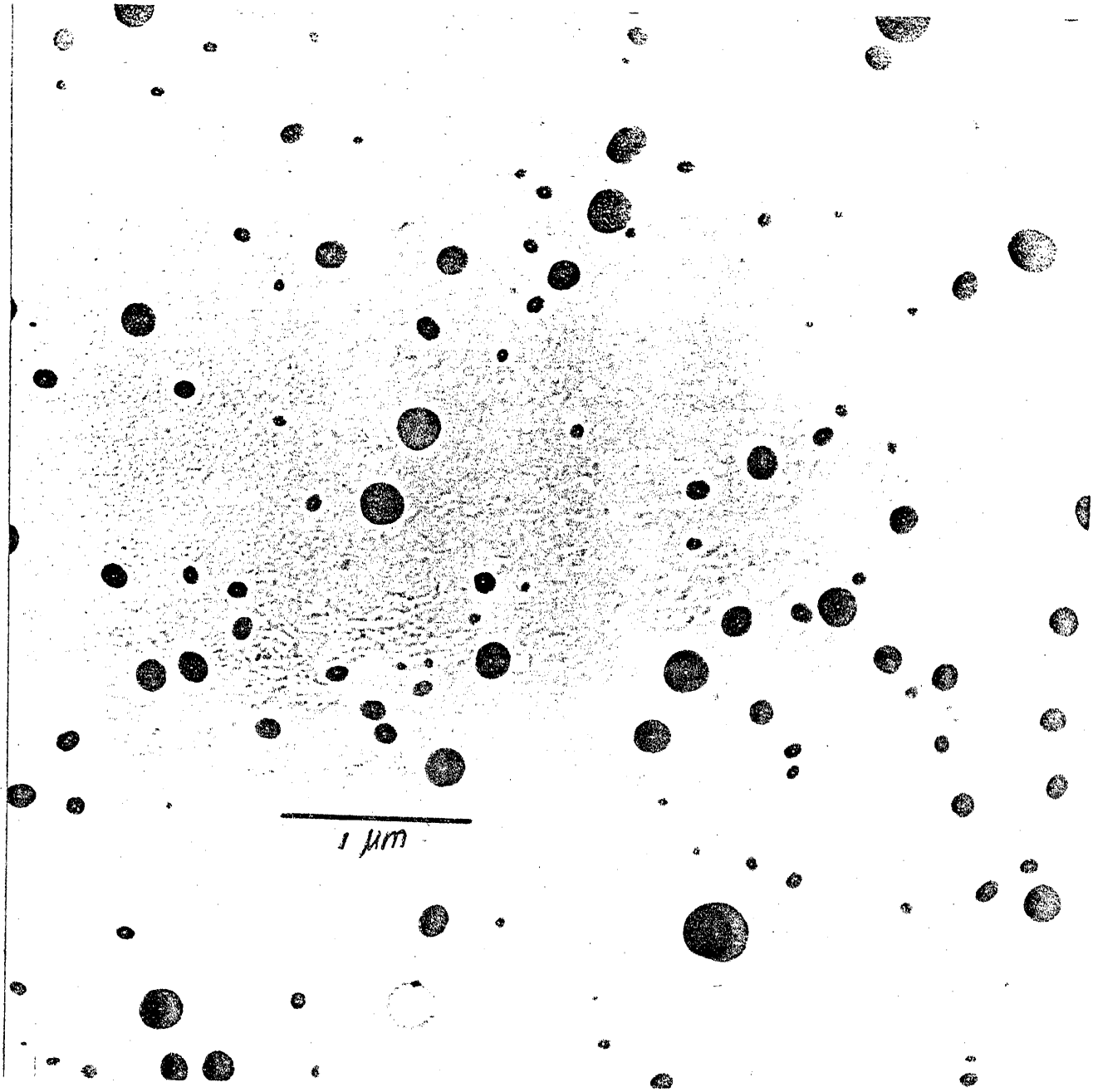

Figure 35. Electron micrograph of ammonium sulfate aerosol sampled from Rochester chamber. Aerosol was prepared by drying a nebulized solution (10% w/vol) of the salt. Bar on photograph represents 1.0 μm.

TABLE XVII

Aerosol	Density of Solid	Method	Number of Particles Counted	Count Median Diameter	Mass Median Diameter	Aerodynamic Diameter*	Geometric Standard Deviation
Sodium chloride	2.2	EM	202	.16 μm	.28 μm	.42	1.52
Ferric sulfate	2.1	EM	409	.11 μm	.22 μm	.32	1.67
Ammonium sulfate	1.8	EM	297	.16 μm	.33 μm	.44	1.65

*calculated by MMD x $(\text{density})^{\frac{1}{2}}$

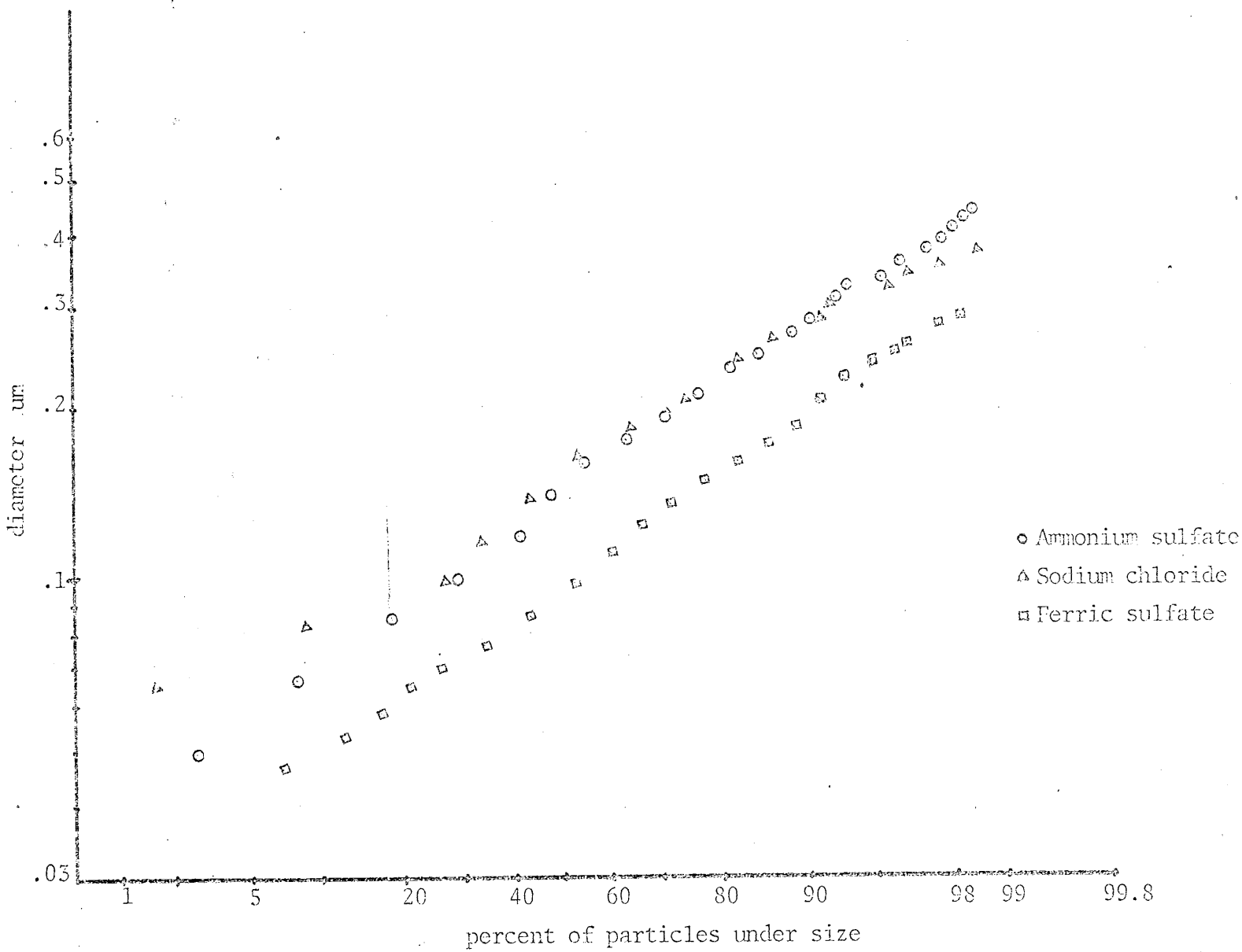

Figure 36. Cumulative size distribution of sodium chloride, ferric sulfate, and ammonium sulfate generated from 10% solutions. Sizing was performed on electron micrographs.

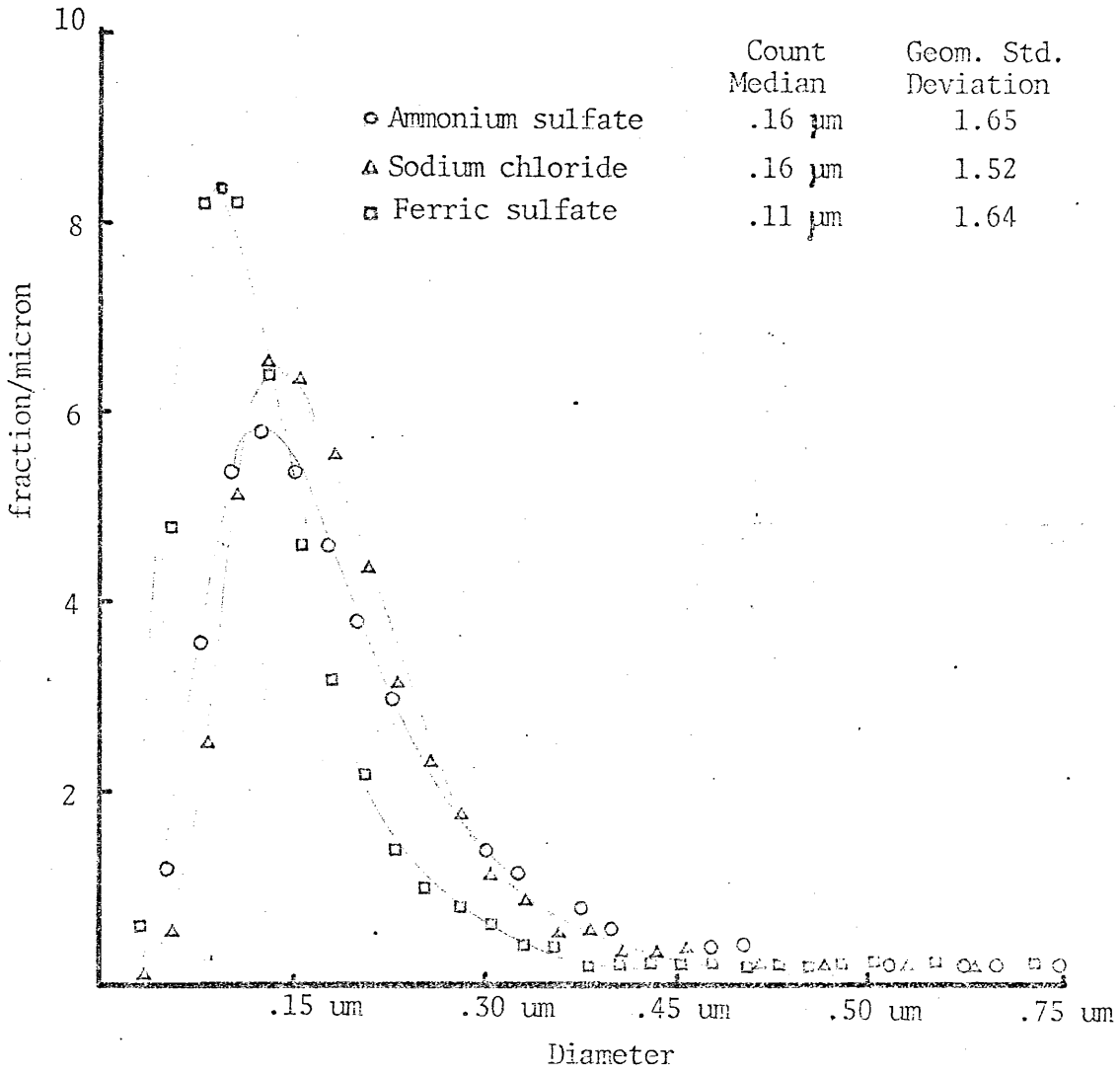

Figure 37. Log normal distribution curve of ammonium sulfate, ferric sulfate and sodium chloride

the dog exposure, sample aerosol in front of the dog mask with a membrane filter at a flow rate of 2 liters/min for 2 hours and compare the result with filters sampled in the chamber. Table XVIII summarizes the mass concentrations of three aerosols inside a chamber and at the dog mask. During these studies, chamber temperature and relative humidity are controlled by a Bemco humidity and temperature control unit to give an average temperature and relative humidity of 23°C and 40%, respectively.

D. Generation and Monitoring of Pollutant Gases

Animal exposures have been performed in which constant levels of ozone have been maintained for several hours. Ozone, produced by passing medical grade oxygen through an electrical ozone generator (Scientific Industries of California, Inc. or Ozonizer, Type III from Sander), is introduced into a Rochester chamber which is run at constant flow rate and negative pressure. The flowrate through the chamber is maintained between 10 and 20 cubic feet per minute, which is the region for best stability of the atmosphere. During rat exposures, the animals are inside all-wire cages, located on a shelf inside a Rochester chamber and the ozone concentration is monitored through an inert line whose inlet is at the same height as the cages. For dog exposures (Figure 38), sampling and exhaust lines, respectively, connect to one Rochester chamber used for mixing the gas and to a second one used for exhaust. The second chamber is run at a more negative pressure than the mixing chamber; therefore; there is a constant flow of ozone past the dog masks and through the flexible hose connecting the two chambers. The concentration of ozone is determined by the Dasibi ozone monitor which samples from the line joining the chambers at a point just in front of the dog masks; when located at this position the monitor measures the ozone concentration presented to the animal, not the concentrations in the mixing chamber or in the exhaled breath of the animal. The tubing from the first Rochester chamber to the animal position is constructed of flexible stainless steel. The lines from the exposure mask to the exhaust chamber are constructed of polyethylene and are also flexible. Flexible lines permit the dogs to move their heads and to find comfortable positions during exposure. The sampling and exhaust lines for the ozone monitor are ¼" teflon tubing which we have found to be relatively inert to ozone. Once the animals are in position and are breathing through the exposure mask, the desired ozone concentration can be obtained and maintained constant by using methods described

TABLE XVIII

MASS CONCENTRATION

	In the center of Rochester chamber	In front of dog mask
Sodium chloride	3.51 mg/m^3	2.95 mg/m^3
Ferric sulfate	4.8 mg/m^3	3.8 mg/m^3
Ammonium sulfate	3.73 mg/m^3	3.54 mg/m^3

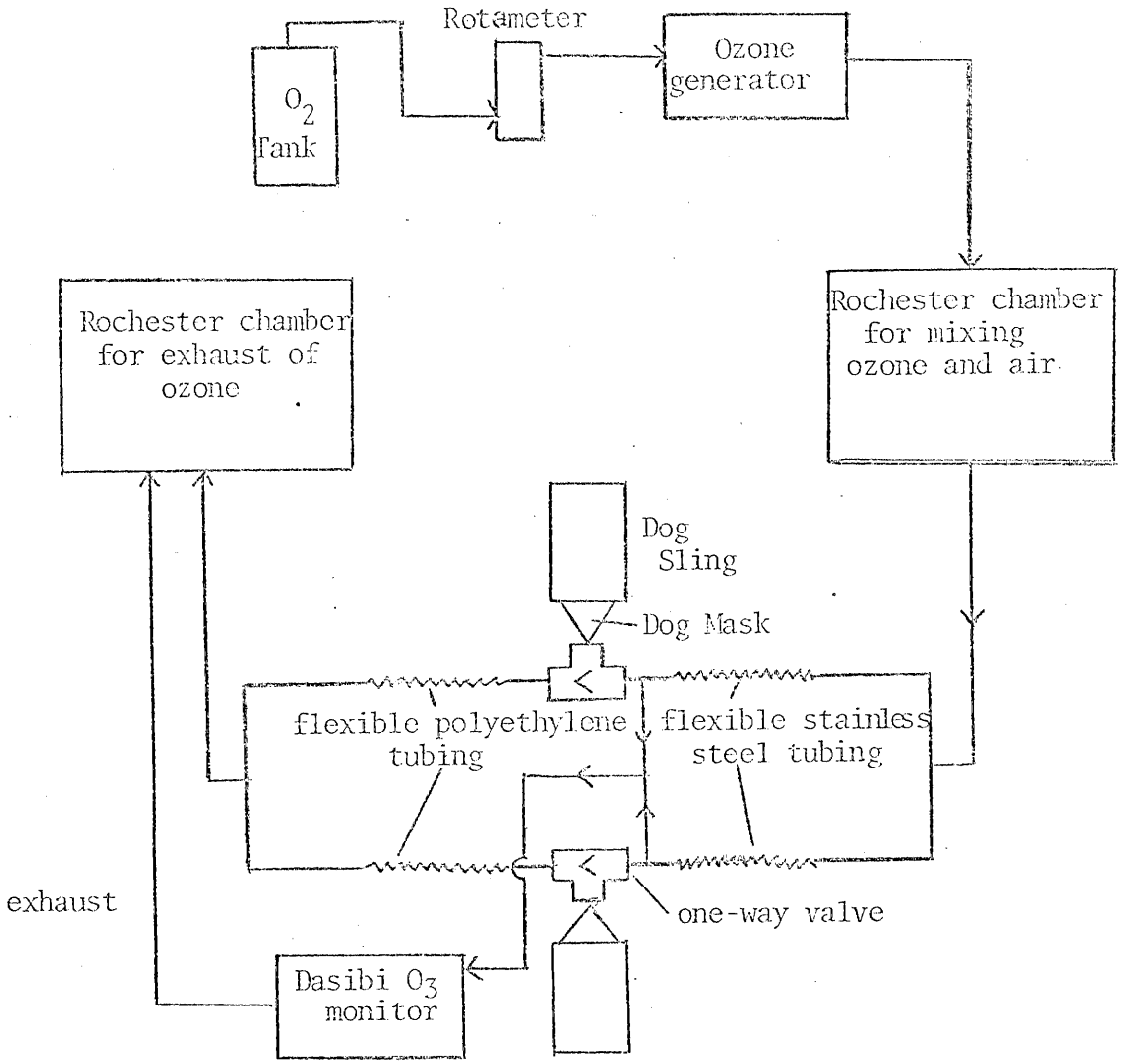

Figure 38. Schematic of ozone generation and monitoring system for dogs. Two animals may be exposed simultaneously. The arrows indicate the direction of the flow of gas. Note that the ozone concentration is monitored near the point of animal exposure

in the following protocol. This system has been used to expose unanesthetized dogs, nose only, to ozone for continuous periods of up to four hours. Details are as follows:

Protocol for Generation/Monitoring of Ozone

General

1. Close all chamber doors and make sure each chamber is sealed.
2. Turn on the blowers to establish air flow for all of the chambers that will be in use.
3. Turn on the first three switches to the BEMCO unit ("fan and control", "cool", "heat").
4. Pressure conditions: a) keep the line pressure ~0.1" of water; b) the pressure difference between the dog exposure and the dog exhaust chambers should be about 1" of water; and c) the exposure chambers should all have negative pressures.

Ozone Generation and Monitoring

1. Make sure that the teflon connectors from the ozone generator are not leaking and are attached to the mixing chamber.
2. Plug in the ozone generator and open the needle valve to the oxygen tank.
3. Dasibi calibration:
   a) connect the calibration unit to the Dasibi ozone monitor and set the control to provide a calibration level near the desired exposure concentration.
   b) turn the Dasibi and the calibration unit on and allow a minimum of 15 minutes warm-up.
   c) check the Dasibi sample frequency and the control frequency to see if they are at the appropriate levels: 450,000-480,000 Hz. and 230,000-260,000 Hz., respectively. If they are not, adjust the position of the ultraviolet source until they are at the correct levels.
4. In order to achieve the desired concentration of ozone in the chamber, three variables may be changed: the flowrate of air through the mixing chamber, the flowrate of oxygen entering the ozone generator, and the voltage of the ozone generator. For an increase of ozone concentration in the chamber either decrease the flowrate of air through the chamber, increase the amount of oxygen to the ozone generator, or increase the ozone generator voltage. To decrease the ozone concentration in the chamber, perform opposite adjustments.

Shutdown

1. Close the main valve to the oxygen tank and disconnect the ozone generator from the chamber.
2. Disconnect the Dasibi from the sampling line and re-check the Dasibi calibration to make sure it has not changed during the exposure.
3. Turn off switches to the appropriate chambers when they are not in use but make sure that another experiment or exposure in another chamber is not affected; that is, the flowrate and/or pressures are not changed.

E. Generation and Monitoring of Aerosol/Gas Combinations

The generation of an atmosphere containing both ozone and aerosol is accomplished by operating both generation systems at the same time and introducing the gas and particles into the inlet of the same chamber. The basic requirements for each system are the same as when they are used together as when they are used separately.

The method of monitoring the mass concentration of the aerosol in the exposure atmosphere (determination of mass collected on a filter) is not changed when ozone and particles are mixed. The method of measuring the ozone concentration in the combined atmosphere, however, is different than for ozone alone. The Dasibi ozone monitor is not capable of quantitating the concentration of ozone when a significant amount of aerosol is also present in the atmosphere. The particles interfere with the operation of this type of gas monitor and a higher level of ozone is observed than is actually present. The readings of the aerosol and the ozone recorded by the Dasibi separately seem to be additive when they are combined, but only for a short period of time. After approximately one hour the readings are no longer stable. An in-line fiberglass filter is not suitable to use in this instance; even though it prevents the particles from entering the sampling chamber, the ozone reacts with the filter and a lower concentration than the true level is observed. After a period of time, a build-up of particles on the filter will also change the flowrate through the Dasibi, and hence its calibration. The possibility of using a teflon filter has been recognized and one is presently on order.

The Model SM400 second-derivative spectrometer, manufactured by Lear Siegler, is used to monitor the concentration of ozone in the pollutant atmosphere when particles in high mass concentrations are also present. This instrument outputs

an electrical signal which is proportional to the amount of gas present in a dynamic collection chamber. This signal is derived from absorption of light at a specific wavelength for each gas and is not affected by the presence of other materials or fluctuations in intensity of the light source with time. The measurement of the ozone concentration by this apparatus is not changed when an aerosol is generated into the same atmosphere. A second derivative spectrum of ozone as measured during an exposure is represented in Figure 39. It is also possible to monitor the concentrations of many other types of gases with this equipment. Presently, the Lear Siegler is calibrated by comparing its output voltage with the concentration detected by the Dasibi ozone monitor after the Dasibi has been calibrated in the absence of aerosol. An independent calibration for the Lear Siegler is being developed at this time. For all exposures involving the combination of ozone and aerosols, the Lear Siegler Derivative Spectrometer will be used to analyze the level of ozone present. Its performance thus far has been quite acceptable and the concentrations observed with it are reliable. The detailed protocol for generation and monitoring ozone plus aerosol is:

General Requirements

The same general requirements as stated for the ozone and aerosol exposure systems separately are applicable for this system.

Ozone and Aerosol Generation and Monitoring

1. Procedures are the same for the generation of both the ozone and the aerosol as when each is generated alone.
2. The flowrate of the chamber must be low (less than 2cfm) if a high mass concentration is desired.
3. Lear-Siegler calibration
   a. turn the power and lamp switches to the "on" position and allow a minimum of 15 minutes for warm-up time
   b. generate ozone into the Rochester chamber and monitor the concentration with the Dasibi ozone monitor after it has been calibrated.
   c. set the following controls to the appropriate settings:

Time constant	3
Attenuation	0.33
Light source	UV
Blower	on
Wavelength drive	STOP
Wavelength (millimicrons)	253

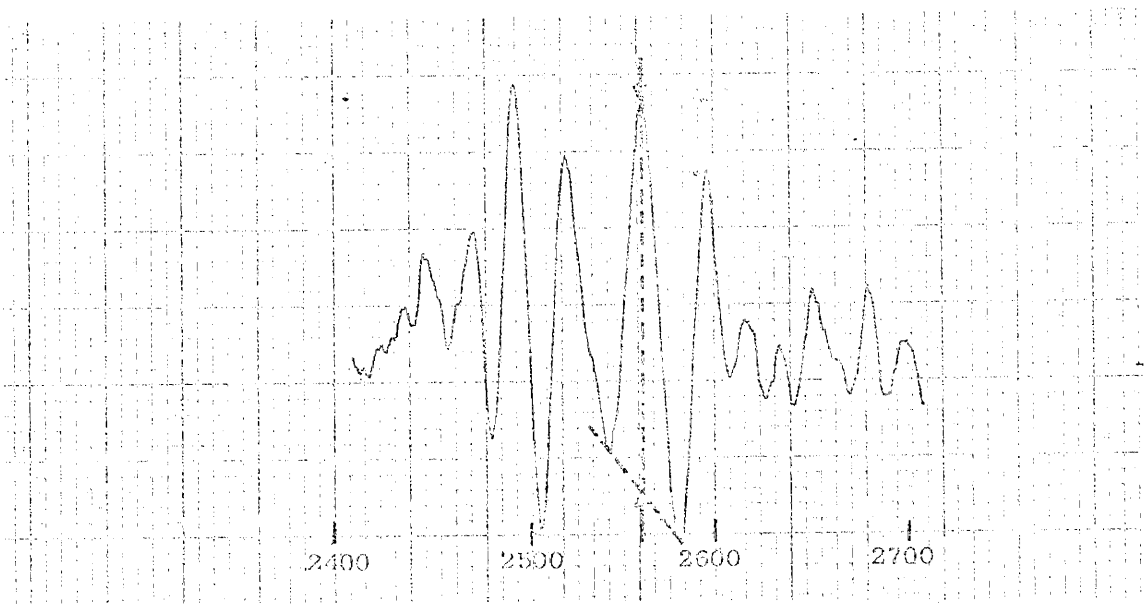

Figure 39. A second derivative spectrum of ozone as monitored by the Lear Siegler SM400 Derivative Spectrometer. The peak at 2570 A^{o} (marked by upper arrow) is the one analyzed because it is affected minimally by spectra of other gases. The distance between arrows (a voltage) is proportional to ozone concentration.

d. connect the output to the strip chart recorder (20 volts full scale) and turn on

e. set the wavelength drive to FAST FORWARD and scan until the wavelength indicator reaches 259 millimicrons

f. set the wavelength drive to STOP and turn off the recorder. Reset to 253 millimicrons.

g. measure the 257 millimicron peak height from the chart paper. This voltage represents the ozone concentration that is present in the exposure chamber.

h. change the ozone concentration in the Rochester chamber and allow it to stabilize

i. repeat steps (d) through (g) until a calibration curve is completed for the output voltage versus the ozone concentration. It is best if most of the data points are taken with concentrations close to the one that is to be used for the exposure.

4. Disconnect the Dasibi ozone monitor from the exposure chamber and turn it off.
5. Begin the generation of aerosol into the chamber and allow a minimum of 15 minutes for it to stabilize.
6. Adjust the level of ozone until the desired concentration is reached.
7. After all the rats are in the chamber or the dog exposure is under way and the ozone concentration has stabilized, it is necessary to check the concentration of ozone only every 10-15 minutes.
8. The monitoring of the mass concentration of the aerosol should be performed before and during the exposure.

Shut-Down Procedures

Shut-down procedures are the same as used for each system separately except that the Lear Siegler's calibration has to be checked with the Dasibi.

V. Animal Studies

A. Ozone in Dogs

The effect of ozone on pulmonary function in the dog is currently under investigation. Concentration is on status of delicate small airways, known to be the site of injury for many inhaled toxins. In order to facilitate proper interpretation of animal response, nine animals were screened by an initial exposure to 0.6 ppm ozone to identify individual differences early in the study. It was thus established whether or not a particular animal tended to fall into a category of " tolerant " or " sensitive " to air pollutants. Each dog was then able to be rated with respect to her individual sensitivity using the nitrogen washout technique.

The methodology of the exposure system has previously been described in Section III-B. Each dog was exposed via mask to 0.6 ppm ozone from a chamber for 2 hours through a flexible stainless steel tube and Rudolph low dead-space pulmonary valve. Three nitrogen washout tests were conducted immediately prior to and after exposure for each dog. Washouts, not yet analyzed, were also performed at 3, 24, 48 and 72 hours post exposure.

Moment analysis of the washout curves was carried out as described in Section III-C, "Pulmonary Function Testing in Dogs." The derived moments for both pre- and post-exposure washouts were subjected to a Student's t test of significance employing the method of Behrens and Fisher for calculating degrees of freedom, necessitated by the different variances of the pre- and post-moments (Behrens, 1929; Fisher and Yates, 1957). The results are shown in Table XIX. Data indicate that an exposure to a 0.6 ppm ozone for two hours has a significant detrimental effect (95% confidence level) on lung function.

The dogs in Table XIX are listed in order of increasing sensitivity to ozone. A wide variation of response was elicited, ranging from no apparent response to a large response in which the animal had outward signs of ill health. Symptoms exhibited by the large-response individual included coughing up of mucus, lethargy and apparent disorientation. Graphic illustration of the Beagles' nitrogen-washout response to 0.6 ppm ozone is shown in Figure 40.

B. Ozone and Ozone Plus Aerosols in Rats

Xenon wash-in/washout tests have been performed in rats before and after exposure to ozone and ozone in the presence of NaCl aerosol. Exposures

Dog	Pre M_1/M_0	Post M_1/M_0	% Δ M_1/M_0	Pre M_2/M_0	Post M_2/M_0	% Δ M_2/M_0
Daphne	11.32	8.93	-21.10	320.7	214.3	-33.20
Addy	8.53	8.65	1.41	151.6	139.1	-8.25
Cindy	9.34	9.47	1.39	180.3	174.7	-3.11
Irene	8.82	9.51	7.82	185.6	197.4	6.36
Klingon	10.55	11.65	10.40	218.7	256.2	17.15
Frankie	8.22	11.81	43.70	129.2	234.3	81.4
Heidi	9.35	13.66	46.12	212.2	394.9	86.1
Bonnie	7.31	12.85	75.85	110.9	310.1	179.6
Jonnie	7.83	15.03	91.95	145.3	450.5	210.1
$\bar{X}$	9.03	11.28	28.61	183.8	263.5	59.6
S.D.	1.28	2.28	37.90	62.9	103.3	86.61
S.E.	0.43	0.76	12.63	20.9	34.5	28.87
	t = 2.59			t = 1.78		
	d.f. = 13			d.f. = 13		
	sig. = 95%			sig. = 95%		

Increasing sensitivity to ozone

TABLE XIX. Summary of moment analysis performed on normalized nitrogen washout curves before and after 2-hour exposures to 0.6 ppm ozone. Dogs are listed in order of increasing sensitivity to ozone. The Student T-test was performed to test for differences between pre- and post-exposure values of moments. The Behrens-Fisher method of calculating degrees of freedom was used due to probable unequal variances. The ozone exposure had a significant detrimental effect on ventilation for the group of dogs.

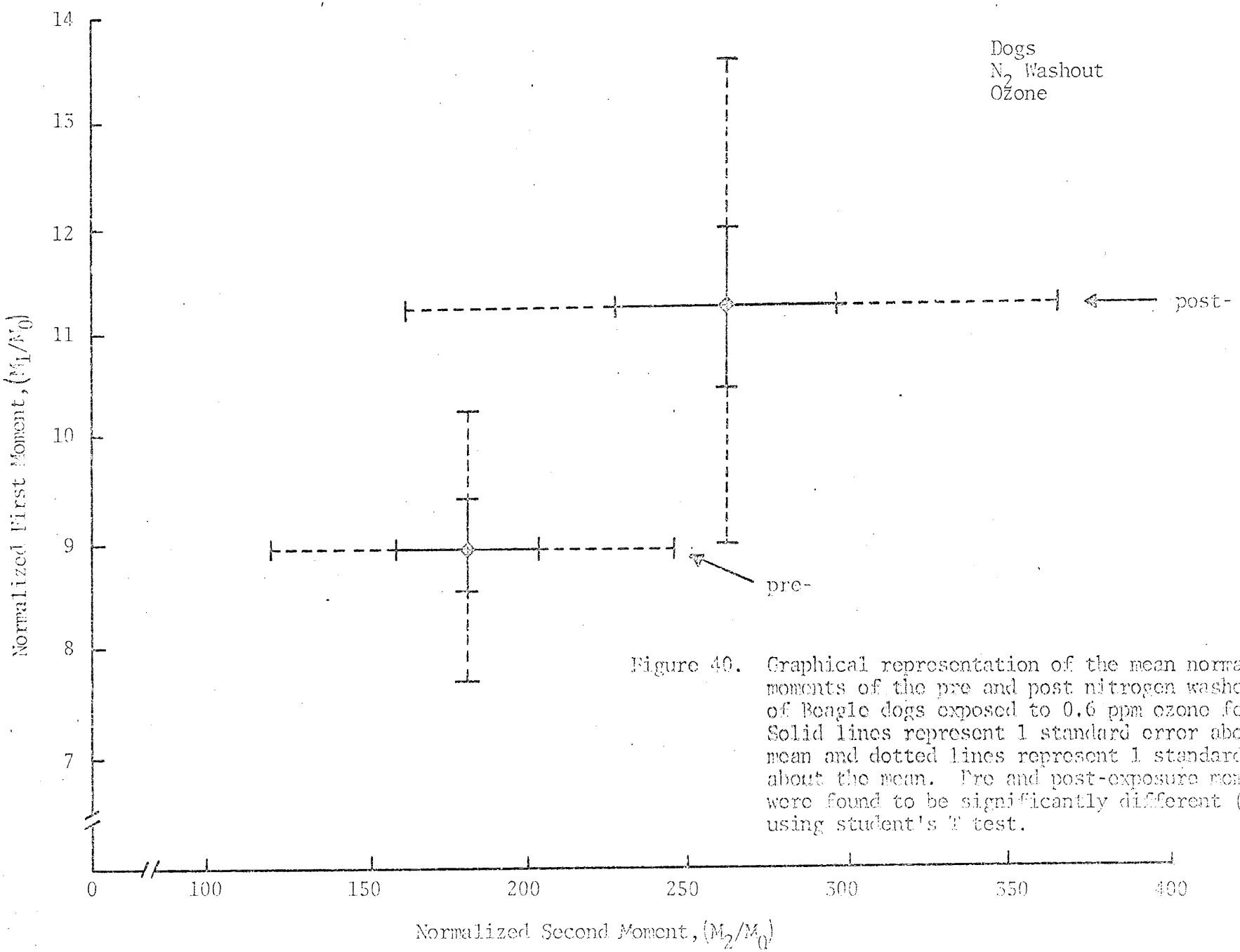

Figure 40. Graphical representation of the mean normalized moments of the pre and post nitrogen washout data of Beagle dogs exposed to 0.6 ppm ozone for 2 hours. Solid lines represent 1 standard error about the mean and dotted lines represent 1 standard deviation about the mean. Pre and post-exposure moment values were found to be significantly different ($\alpha = .025$) using student's T test.

were for 4 hours inside a Rochester-type chamber at levels of ozone ranging from 0.2 ppm to 1.0 ppm and at about 3-7 mg/m^3 (.5 μm MMAD) aerosol. Data were analyzed by analysis of moments of wash-in and washout curves and comparison of changes in moments after exposure. At the time of this report the data have only been partially analyzed. For those data that have been analyzed (Table XX) the following observations can be made:

The data are highly variable and detailed analysis to identify the major sources of variability are being started. Major contributions to the variability possibly include:

a) variable level of sedation of animals;
b) changes in tidal volume and breathing rate due to pollutant exposure (in our present data analysis these effects were not compensated for), and
c) inclusion of female rats in some groups (the experiments were to be performed on all males, which have more stable physiology, but the supplier could not supply the required number of male animals.)

Tentative conclusions being considered at this time include:

1. NaCl aerosol, even at the high mass concentrations used, did not enhance the physiologic effects of ozone,
2. Ozone, at all levels studied, appeared to influence the dynamics of wash-in/washout of ^{133}Xe, and

During these wash-in/washout tests it appeared visually that animals exposed to lower levels of ozone sometimes reacted more severely than animals exposed to higher levels. Calibration tests before and after exposures lend confidence to the exposure levels involved, and the moment analysis data are not inconsistent with this observation. The possibility that an undiscovered artifact is responsible, however, remains. One might speculate that a protective response, perhaps in the pattern of breathing, is responsible for decreasing the actual dose to deep lung when the exposure level is high enough to stimulate neuronal receptors.

C. Exposure of Dogs to Salt Aerosols

Nine unanesthetized Beagle dogs at the facility were exposed via a latex mask to aerosols of ferric sulfate, sodium chloride and ammonium sulfate for a standard period of four hours per exposure. Nitrogen washouts were performed on

TABLE XX

SUMMARY OF POLLUTANT EXPOSURES USING RATS

Date	# animals	Pollutant Levels O_3 (ppm)	Pollutant Levels NaCl (mg/m^3)	% Change in Moments ($M_1/M_0 + M_2/M_0$) From Controls (std. error of mean) Wash-in	Washout
11-25-75	8	0.2	---	*	*
11-26-75	8	0.6	---	*	*
12-1-75	8	0.8	---	*	*
12-2-75	8	0.0	---	0 ± 1	0 ± 11
12-2-75	8	0.2	---	*	*
12-4-75	8	0.6	3.5	*	*
12-7-75	8	0.4	6.2	7 ± 2	20 ± 4
12-8-75	8	0.4	---	*	*
12-10-75	8	0.8	---	12 ± 1	38 ± 3
12-11-75	8	0.2	---	25 ± 2	43 ± 7
12-15-75	8	0.6	6.5	*	*
12-16-75	8	0.4	6.6	9.7 ± 1.0	37 ± 1
12-16-75	8	0.2	6.4	9.9 ± 2	35 ± 1

*data generated but not yet analyzed

each dog prior to and after exposure in an attempt to measure effect on pulmonary function. Three dogs were exposed for four hours each to an atmosphere of submicron ferric sulfate aerosol ranging in concentration from 3.8 mg/m^3 to 4.2 mg/m^3. Summarization of the results of moment analysis on the pre- and post-nitrogen washouts is shown in Figure 41 and Table XXI. The results of these exposures indicate that ferric sulfate at these concentrations has no apparent effect on pulmonary function. However, because of the small sample size further study should be implemented before any firm conclusions are drawn.

An additional three dogs at the facility were exposed for four hours each to sodium chloride aerosols varying in concentration from 2.97 mg/m^3 to 3.5 mg/m^3. Results for this series of exposures are shown in Figure 42 and Table XXII. Though the data is inconclusive due to the small sample size, no measurable response is indicated.

Three more dogs at the facility were exposed for four hours to ammonium sulfate aerosols ranging in concentration from 3.54 mg/m^3 to 3.92 mg/m^3. Figure 43 and Table XXIII summarize the results for these experiments. No quantitative conclusions can be drawn at this time due to small sample size, but these preliminary data suggest a response of the lung to the ammonium sulfate aerosol with little or not response to sodium chloride or ferric sulfate aerosol. More exposures are planned to quantitate this possible response.

For a graphic representation of a comparison of ozone and various aerosols on pulmonary function refer to Figure 44. The mean moments ± 1 standard error for all pre-exposure values are represented by the dotted lines. The post-exposure values are represented by solid lines.

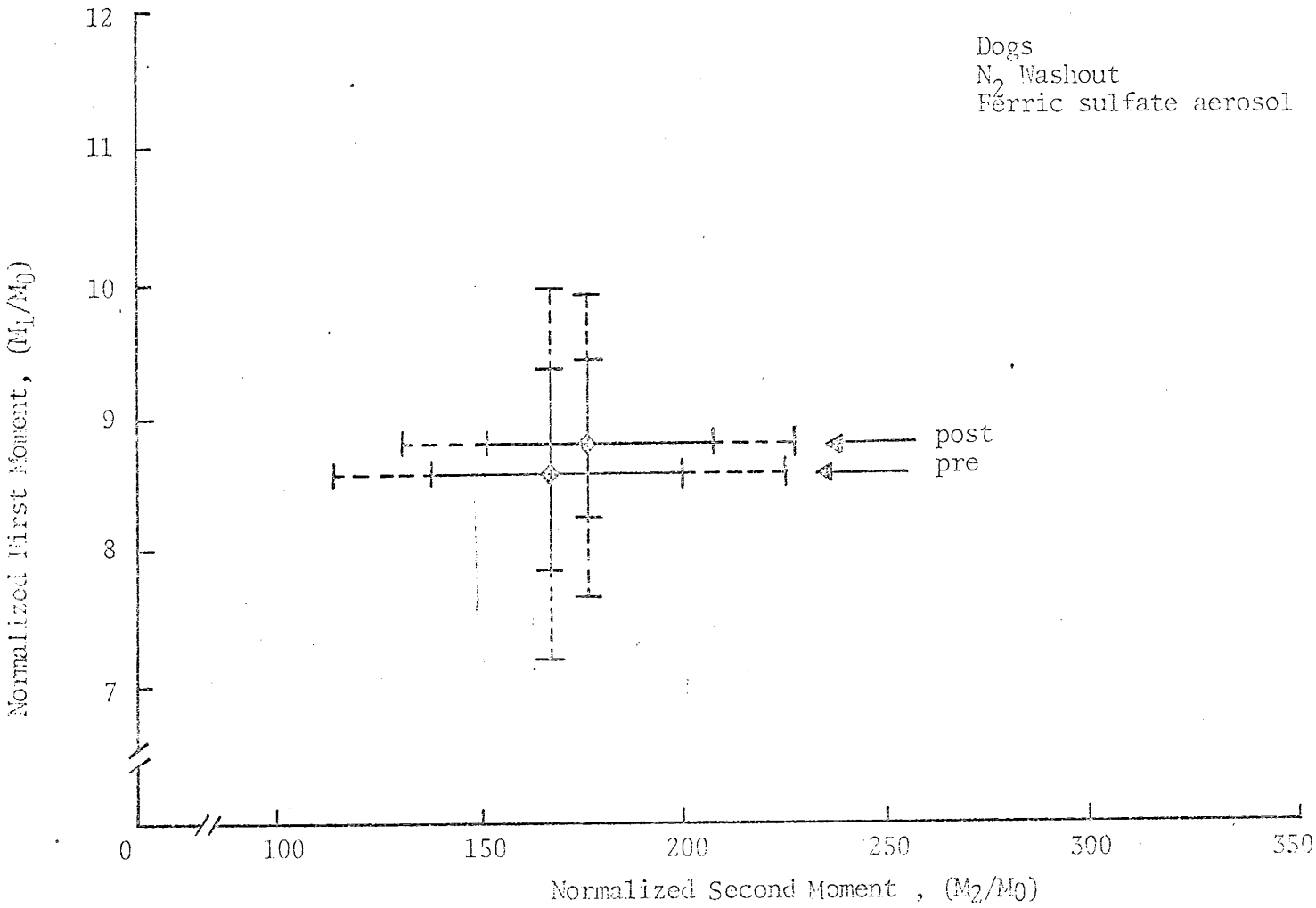

Figure 41. Graphical representation of the mean normalized moments of the pre and post exposure nitrogen washout data of Beagle dogs exposed to ferric sulfate aerosol. Solid lines represent 1 standard error about the mean and dotted lines represent 1 standard deviation about the mean.

Dog	Pre M_1/M_0	Post M_1/M_0	% Δ M_1/M_0	Pre M_2/M_0	Post M_2/M_0	% Δ M_2/M_0
Cindy	9.95	9.95	0	228.3	227.6	0.3
Irene	8.59	8.7	1.28	164.5	183.3	11.43
Heidi	7.24	7.69	6.22	116.3	131.9	13.41
$\bar{X}$	8.59	8.78	2.50	169.7	180.9	8.18
S.D.	1.36	1.13	3.28	56.18	47.89	7.07
S.E.	.79	.66	1.90	32.66	27.84	4.09

TABLE XXI. Summary of moment analysis performed on normalized nitrogen washout curves before and after 4-hour exposures to ferric sulfate aerosols. Dogs are listed in order of increasing sensitivity to the aerosol.

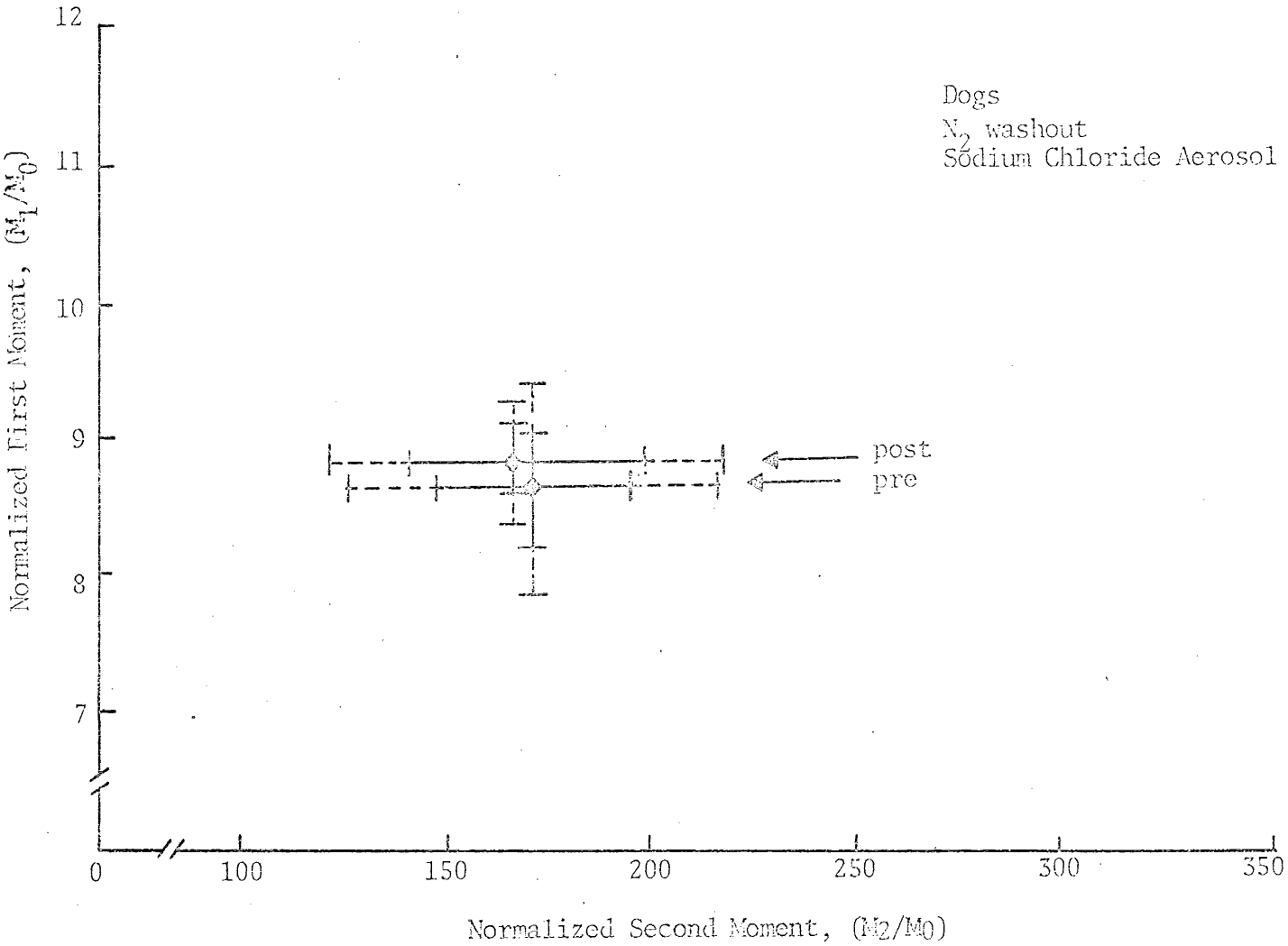

Figure 42. Graphical representation of the mean normalized moments of the pre-and post-exposure nitrogen washout data of Beagle dog exposed to sodium chloride aerosol. Solid lines represent 1 standard error about the mean and dotted lines represent 1 standard deviation about the mean.

Dog	Pre M_1/M_0	Post M_1/M_0	% Δ M_1/M_0	Pre M_2/M_0	Post M_2/M_0	% Δ M_2/M_0
Bonnie	8.05	8.52	5.84	140.0	127.9	-12.4
Daphne	9.56	9.39	-1.78	223.3	220.4	-1.3
Addy	8.51	8.81	3.53	146.5	153.9	5.05
$\bar{X}$	8.71	8.91	2.5	171.9	167.4	-2.88
S.D.	.77	.44	3.9	45.5	47.7	8.83
S.E.	.45	.26	2.3	25.7	27.6	5.10

TABLE XXII. Summary of moment analysis performed on normalized nitrogen washout curves before and after 4-hour exposures to NaCl aerosol. Dogs are listed in order of increasing sensitivity to the aerosol.

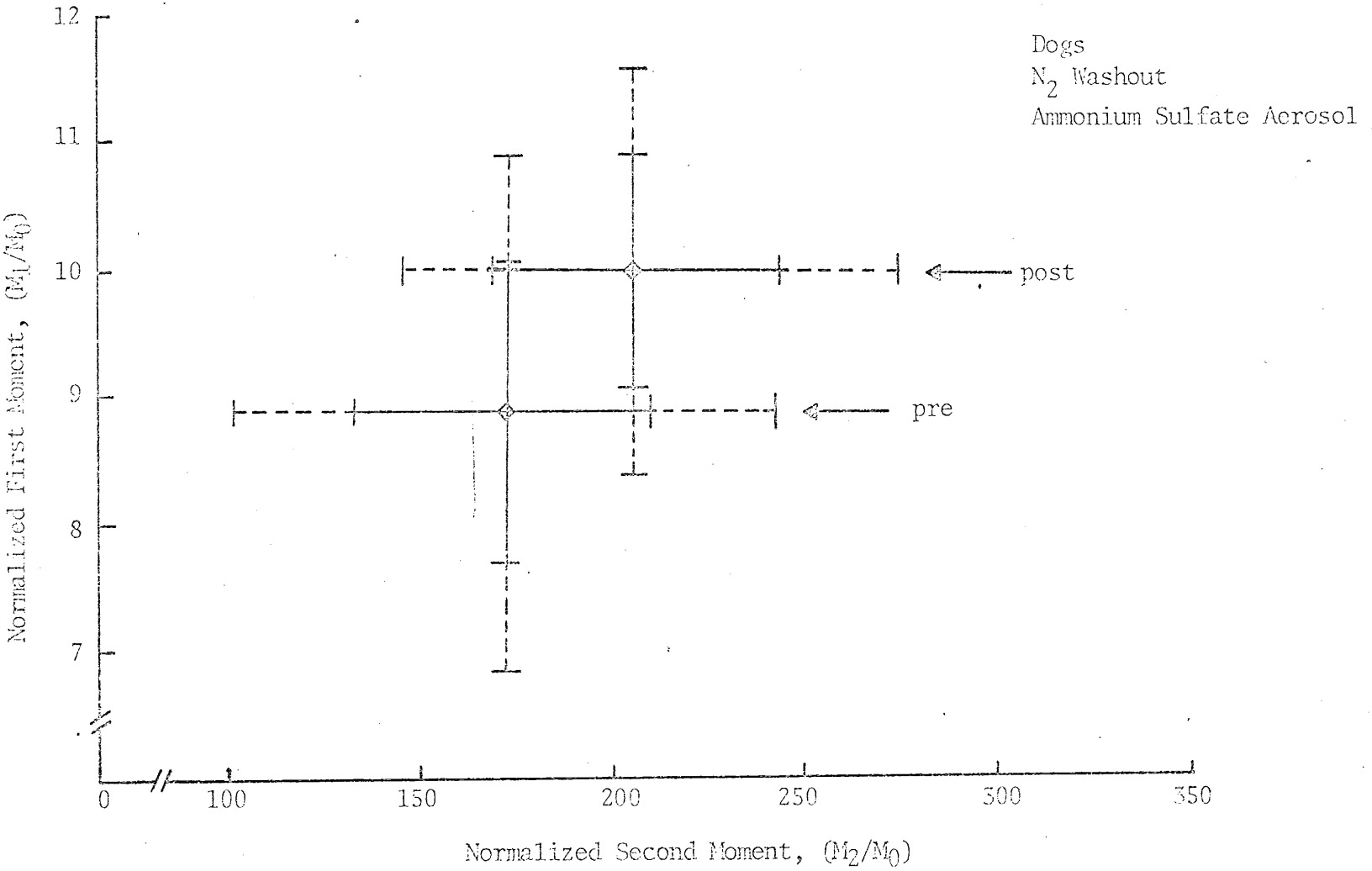

Figure 43. Graphical representation of the mean normalized moments of the pre and post-exposure nitrogen washout data of Beagle dogs exposed to ammonium sulfate aerosol. Solid lines represent 1 standard error about the mean and dotted lines represent 1 standard deviation about the mean.

Dog	Pre M_1/M_0	Post M_1/M_0	% Δ M_1/M_0	Pre M_2/M_0	Post M_2/M_0	% Δ M_2/M_0
Klingon	11.22	11.75	4.72	252.4	274.4	8.72
Bonnie	7.89	8.64	9.51	139.8	148.7	6.37
Daphne	7.54	9.62	27.59	126.3	197.0	55.98
$\bar{X}$	8.88	10.0	13.94	172.8	206.7	23.69
S.D.	2.03	1.59	12.06	69.2	63.4	27.99
S.E.	1.17	0.9	6.97	40.0	36.7	16.18

TABLE XXIII. Summary of moment analysis performed on normalized nitrogen washout curves before and after 4-hour exposures to ammonium sulfate aerosol. Dogs are listed in order of increasing sensitivity to the aerosol.

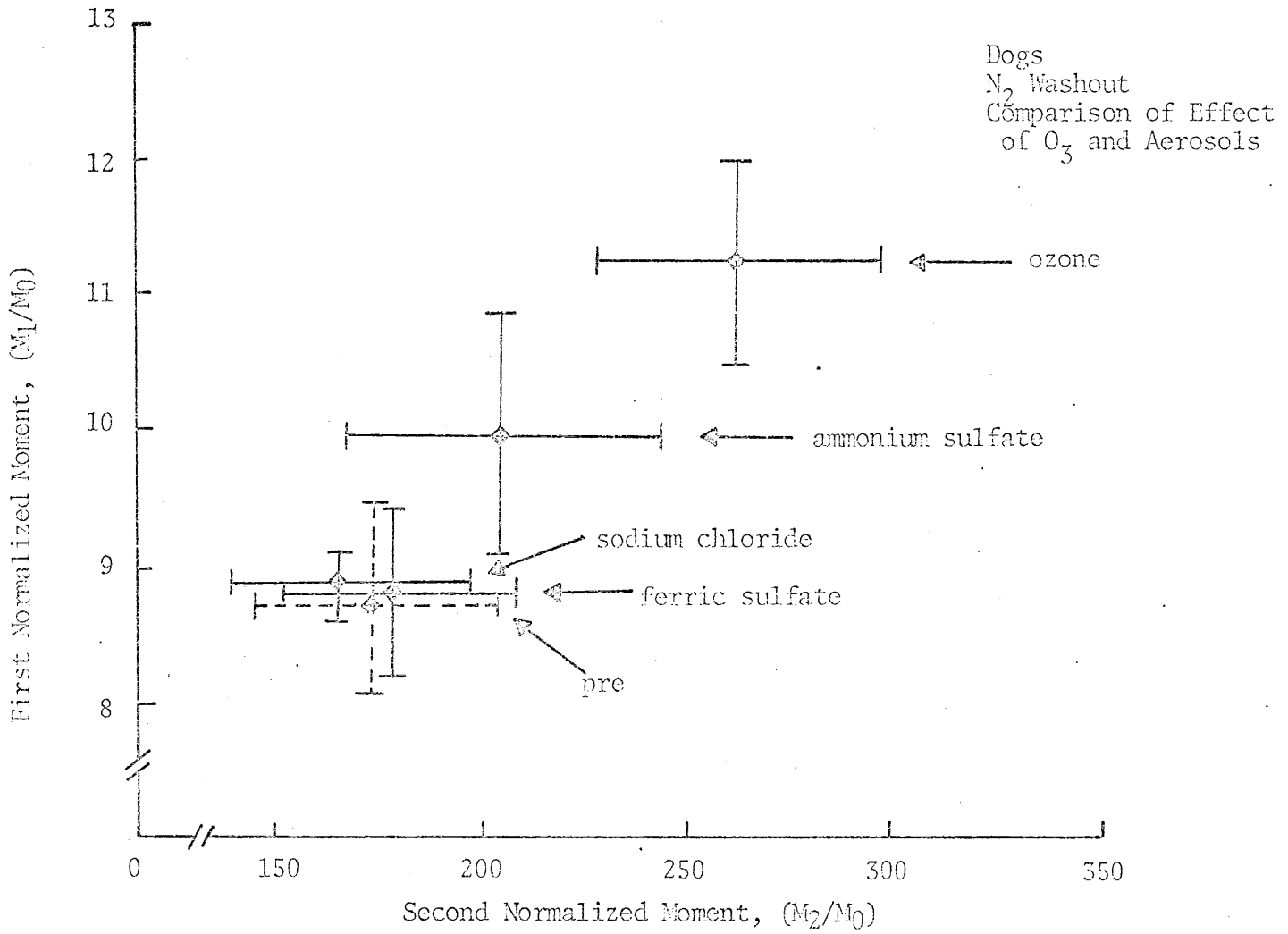

Figure 44. A summary of the mean normalized moments of the pre and post-exposure nitrogen washout data of Beagle dogs exposed to ozone, ammonium sulfate aerosol, ferric sulfate aerosol, and sodium chloride aerosol. The dotted lines represent the mean normalized moments of all dogs before exposure. Solid lines represent the mean post normalized moments for any given gas or aerosol. All values are represented as 1 standard error about the mean.