VI. Personnel

Staffing has been completed. Below are descriptions of scientific personnel and their involvement in the research effort. Included in this list are laboratory personnel whose salaries are derived from sources other than the California Air Resources Board.

Crocker, T.T., M.D. — Principal Investigator

Involved in scientific planning, experimental design, and in interpretation of results in all phases of project.

Samuelsen, G.S., Ph.D. — Co-Principal Investigator

Involved in facilities planning and implementation, and in directing generation and characterization of pollutant atmospheres (Involvement terminated July, 1975)

Wilson, A.F., M.D., Ph.D. — Co-Principal Investigator

Involved in supervision of development of animal methods, design of pulmonary testing protocols and in interpretation of animal response data

Phalen, R.F., Ph.D. — Project Director

Involved in project coordination, development of animal methods, generation and characterization of aerosol and gaseous pollutants, conduct of animal exposures and animal testing, and handling and interpretation of data.

Fairshter, R.D., M.D. — Pulmonary Specialist

Involved in animal methods development and interpretation of pulmonary function data.

Davis, J., B.S. (biologist) — Staff Research Associate

Involved in animal testing, development of masks, plethysmographs, animal restraint devices, exposure of animals, dissections, preparation of histologic samples, calibration and maintenance of physiologic equipment, computer interfacing and data handling.

Dennison, W., M.S. (engineer) — Engineering Aide

Involved in general facilities implementation, equipment fabrication, design fabrication & checkout of pure air system, and animal & environmental systems

Kenoyer, J.L., M.S. (physicist) — Staff Research Associate

Involved in aerosol and gas generation and characterization, radiation safety, radiolabeling, calibration & maintenance of exposure chambers and equipment, computer interfacing and data handling.

Truglio, N., B.S. (biologist) — Animal Caretaker

Involved in care, handling and training of animals and support of animal methods development.

Hinrichs, R.J., B.S. (chemist) — Research Assistant

A.E.C. licensed senior operator for the UC Irvine nuclear facility. Involved in radionuclear chemistry of polystyrene-latex microspheres.

Walters, R., B.S. (chemist-engineer) — Engineering Aide

Involved in generation and characterization of sulfuric acid aerosols, preparation of Rochester chambers, design and equipment selection for cleaning air in animal chambers and animal housing areas, and general facilities.

Katz, A. (pre-medical student) — Summer Student

Involved in developing nitrogen washout technique in the mini-pig and performing exposures with ozone.

Ho, A.T., M.S. (engineer) — Staff Research Associate

Involved in aerosol generation and characterization, computer interfacing, software development, implementation of pure air system, and development of fixation methods for rodent lungs.

Murdock, L.M., M.A. (physiologist) — Staff Research Associate

Involved in pulmonary function studies, animal care and training, general animal methods development and supervision of animal housing wing.

*Stavert, D.M., B.S. (biologist) — Laboratory Helper

Involved in care, handling and training of laboratory animals and in animal methods development

Moreshead, M. — Animal Caretaker

Involved in animal handling, training and care, maintenance and fabrication of masks, slings and miscellaneous equipment. Performs rodent dissections.

*Salary wholly derived from other than State of California funds.

VII. Status of the Budget

In this draft of the final report on Contract ARB 4-611, budget figures are not complete because our University accounting procedures are such that there is a delay in providing each project with the detailed computer runs. This problem has been discussed with Kathy Whorley at ARB, and it is understood that the final budget figures will be incorporated in the final report. The official billing and accounting of expenses of Contract ARB 4-611, however, will be forthcoming from the University Accounting Office.

During the period of the Contract, adjustments have been made within expenditure categories, but we have not exceeded our total dollar allotment. The Quarterly Progress Reports have listed details of some of the early transfers, and the final report will outline a compilation of these adjustments.

All equipment purchases over $500 required approval from ARB. Necessary approval was obtained for all such equipment purchases and there follows a detailed accounting of equipment purchases made during the contract period. Included in this listing is the amount originally budgeted for the item of equipment in the proposal and the subsequent savings or overrun for each item. Equipment purchases were within the amount proposed in the original budget.

P.O. Number	Item	ARB #	Property #	Budgeted	Actual Price	Savings	Overrun
	Pure Air System						
75812	1 # A-17956 Deoxo Catalytic Oxidation System		7590-00233	$ 16,000.00	$ 6,436.08	$ 6,891.46	
76698	1 Halstead-Mittchel cooling tower	03407	7590-01396		392.20		
75824	1 Air Purification Unit	03394	7590-00281		1,781.00		
76699	1 Cincinnati 3 stage blower	03401	7590-00464		499.26		
	Ozone Monitor, NO_2/NO and SO_2			$ 17,685.00		$ 2,500.00	
46136	1 used SM4 Derivatire Spectrometer		7590-01802				
	1 used Ultraviolet source						
	1 used SO_2 sealed standard cell				$15,185.00		
	1 used NO sealed standard cell						
	1 used NO_2 sealed standard cell						
	Strip Recorder						
75886	1 Multipoint Recorder, Esterline Angus Model E 1124E	03399	7590-00358	$ 2,500.00	$ 2,635.00		$ 135.00
	Integrating Nephelometer	03397					
75598	1 Royco Model 225		7590-00259	$ 3,500.00	$ 8,056.00		
	1 Model 127 Digital Printer	03398	7590-00260		1,134.20		
	1 MSA Absorber Cartridge				106.00		$ 5,796.20
	Electrostatic Aerosol Sampler						
75621	1 Regulated High Voltage D.C. Power Supply	03138	7590-00135	$ 2,300.00	$ 561.98	$ 1,738.02	
	Brady Array			$ 1,000.00		$ 1,000.00	
	Seven Stage Cascade Impactor						
75562	1 Seven Stage Cascade Impactor 1/2-1 liter with calibration curves for lea level	03139	7590-00157	$ 225.00	$ 397.44		$ 172.44
	Seven State Cascade Impactor						
76545	1 Seven Stage Cascade Impactor 120 cc/min with calibration curves for sea level	03395	7590-00284	$ 225.00	$ 397.45		$ 172.45
	Programmable Calculator, Interface & Output Typewriter						
76091	1 PDP 11/10-SC CPU with 16 K core	03403	7590-00490	$ 5,000.00	$ 7,495.00		
	1 AR11 16 channels ADC, 10-Bit, scope control and real time clock			600.00 2,000.00	1,712.00		
	1 BM873 Multi-device bootstrap loader				400.00		

P.O. Number	Item	ARB #	Property #	Budgeted	Actual Price	Savings	Overrun
	1 DD11-B mounting panel H9 cabinet				250.00		
	1 QJ900-AB PTS/Basic Software License				500.00		
	1 H970-CA cabinet with power distribution, fans & end panels				800.00		
	1 teletype(ASR) -33 kit				130.00		
	Less 15%				-1,693.05		2,569.58
	Tax				575.63		
	Aerosol Neutralizer			$ 615.00			
	Ozonotor						
75561	1 Ozonator, Model 1012	03140	7590-00187	$ 2,000.00	$ 514.10	$ 1,485.90	
	Spirometer, Nitrogen analyzer, Nitrogen residual volume calculator, Recorder, Recorder interface, CO_2 absorber and valving, Respiratory mechanics unit and Table Top Console			$ 17,100.00			
75573	1 Linear Nitrogen Analyzer	03145	7590-00204		$ 3,047.50		
75575	2 DC Amplifiers	03141	7590-00211				
		03142	7590-00212		770.00		
	1 Pulmonary Function Module	03143	7590-00213		825.00		
	2 Pressure Differential Transducers				650.00		
	1 Twelve liter rolling seal spirometer with electrical flow & volume signals & dumping option	03144	7590-00214		2,320.00		
	Tax				273.90		
75576	1 Six-channel Oscilloscopic Recorder	03147	7590-00205		5,200.00		
	4 Pressure amplifiers for Statham transducers	03148	7590-00206				
		03149	7590-00207				
		03382	7590-00208				
		03383	7590-00209		3,520.00		
	Tax				583.20		1,189.60
	Small low energy 1-1/2" probe, holder for probe, dual channel analyzer, dual channel rectilinear			$ 1,500.00 225.00 1,575.00			
75565	2 802-3 Scintillation Dectector Nal	03384	7590-00262	1,245.00			
		03385	7590-00263		$ 560.00		

P.O. Number	Item	ARB #	Property #	Budgeted	Actual Price	Savings	Overrun
	1 3002-3KU power supply	03386	7590-00264		535.00		
	2 each 802-9 tube base with preamplifier	03387 03388	7590-00265 7590-00266		420.00		
	2 each 816A/830A amplifier/ single channel analyzer	03389 03390	7590-00267 7590-00268		1,050.00		
	1 1771/1772 scaler/scaler timer	03391	7590-00269		1,045.00		
	1 1400 AEC standard bin/ power supply	03392	7590-00270		600.00		
	1 1401-L lin/log ratemeter	03393	7590-00271		575.00		
	Accessory Systems Cables and Terminal				100.00		
	Less 5%				-244.25		
	Less 1/4%				- 12.42		
	Tax and shipping				328.44		411.77
	Radiometer			$ 5,400.00		$ 5,400.00	
	Spirometer			$ 2,500.00		$ 2,500.00	
	Wilhemy Balance			$ 1,750.00		$ 1,750.00	
79118	Operating Table #1110-K-15 Animal Operating Table	03408	7590-01391	$ 550.00	$ 300.00	$ 250.00	
77180	Metabolic cages 2 stacking custom stainless metabolism cages		7590-00997 7590-00998	$ 3,600.00	$ 1,510.60	$ 2,089.40	
	Kennel runs			$ 600.00		$ 600.00	
	Animal racks and cages			$ 2,400.00		$ 2,400.00	
	Benches			$ 3,000.00		$ 3,000.00	
75563	Radiation Survey Meter 1 Beta-Gama Survey Meter	03146	7590-00215	$ 238.00	$ 203.58	$ 34.42	
46150	2 Dexon 2 X 6 Laminar Flow units	03411 03412	7590-01791 7590-01792		$ 1,484.00		$ 1,484.00

P.O. Number	Item	ARB #	Property #	Budgeted	Actual Price	Savings	Overrun
46173	1 Maco bench scale, model # 25		7590-01800		$ 233.30		$ 233.30
76641	1 Gas flow calibrator	03400	7590-00365		$ 393.26		$ 393.26
76136	1 Hewlett-Packard (HP-45) hand held calculator	03137	7590-00149		$ 220.74		$ 220.74
76520	1 PH meter	03402	7590-00483		$ 269.77		$ 269.77

VIII. Discussion

General Aims

Appropriate advice as to management of ambient air may be achieved if an adequate scientific data base on the characteristics, sources and long- and short-term health effects is compiled. A solid foundation of pertinent information is still being compiled. Only when the relevant pollutants, their detailed characteristics and interactions, and their important biologic actions are elucidated, can decision makers be secure in their strategies and actions. Science has been utilized effectively in this country in the past in the solution of major problems. Key factors in this success have been: 1) identification of relevant questions that require answers; 2) development and validation of methodologies powerful enough to find valid answers; 3) application of these methods to the pertinent questions. Success is inevitably due to the parallel and cohesive efforts of many persons -- some directly in the heart of the problem area, others working on peripheral problems. The Air Pollution Health Effects Laboratory has been developed and is currently engaged with the description of toxicity of inhaled solids, liquid droplets and gases, considered singly and in combinations. An attempt has been consciously made to derive methods, data, and questions that are important to understanding and elucidating current pertinent problems relating to atmospheric air standards.

As scientists collaborating toward developing information on the health effects of air pollutants, we have established a laboratory whose major concern is inhalation toxicology and which has the necessary depth and breadth to provide valid answers in this area. We have equipped and staffed for concentration of effort in the areas of: a) respiratory physiology and pathology using relevant animal models including the use of humans at a later time ; b) pollutant properties that are pertinent to health effects, especially for aerosols and aerosols in the presence of various gases; and c) the conduct of precisely controlled inhalation experiments. This first annual report sets forth important methodologies developed specifically to serve scientific investigators, points out those characteristics of aerosols that are important to their toxicology and provides preliminary toxicologic data on important basic components of the polluted atmosphere. This and subsequent research at this laboratory can be expected to contribute materially to the proper

management of our air resource.

Specific Aims

It is possible to identify needs for research toward answering several relatively specific questions that bear on the evaluation of health effects of air pollutants. Examples of such questions that we plan to take a leading role in answering include:

1. Of the large number of possible aerosol/gas air-pollutant combinations which are the most potent, and therefore worthy of more detailed study?
2. What is the role of relative humidity as a modifying factor?
3. What is (are) the site(s) and nature of injury to lung from mixed air pollutants?
4. How are important defense mechanisms of the lung affected by exposure to air pollutants?
5. What is the time course of recovery after an acute air-pollutant episode?
6. In particulate salt toxicity, what are the relative toxicities of various anions and cations, e.g. Na vs. Fe and nitrate vs. sulfate?
7. What is the role of pH of acid droplets in degree of injury to the respiratory tract?
8. What forms of adaptation of sensitization can one expect upon repeated exposure to various air-pollutants?
9. What are those most critical health effects upon which exposure limits for general populations depend?
10. What are the most sensitive sub-populations, the very young, the old, the debilitated, etc.?
11. What are the delayed effects after exposure of the young, developing respiratory system?
12. What aerosol properties other than size, e.g., surface area and rate of dissolution in lung fluids, might be important to toxic response?
13. How do chronic exposure effects differ from those of acute exposure?
14. What are the best animal species for air-pollution research?
15. How does constant living in a relatively clean or relatively polluted environment influence the response to an episode?

16. What should the general public learn about health effects of air pollutants and what should be taught in public schools?
17. What sort of education on air pollutants should be included in medical schools, colleges and universities?
18. How should future researchers in air pollution be trained?
19. What should the short and long-range objectives be with respect to health effects research?
20. What can be expected with respect to future airborne toxins in our changing society?

Initial Results/Relevance

Perhaps the most important result of this first year's effort is that the laboratory has been installed, staffed and engaged in research. The smoothness of the development of methods and initial gathering of data verifies that the planning and implementation have been sound. The laboratory now stands as an important resource for identifying and solving relevant problems.

A more specific accomplishment is in the controlled generation of particulate pollutants, with sizes, compositions and concentrations that mimic actual ambient aerosols. These aerosols can be generated into very clean air within a broad, controlled range of relative humidities and under precise temperature control. In the past, much potentially valuable toxicologic information has been compromised by gross uncertainties, or in appropriate values in the exposure environment. We feel that our methods for purification of carrier air, control of humidity and temperature, generation and mixing of aerosols, and careful characterizations constitute a valuable contribution to the science of inhalation toxicology.

Another contribution lies in the successful adaptation of inhaled gas wash-in/washout methods to animals. Such tests, of recent use in human evaluation, shed light on the previously "silent" zone of the lung: the small delicate airways. Performance of these tests in animals permits screening of many atmospheric combinations at various levels, acidities, humidities, etc., that would be prohibitive in human subjects.

Of the three aerosols tested in dogs, sodium chloride and ferric sulfate appear to have little acute effect on small airways patency while ammonium nitrate does interfere with pulmonary function. The level of ammonium nitrate initially studied, 3-4 mg/m^3 of air, is much higher than that in ambient air,

so it is now necessary to drop the level to determine a "no-effect" threshold for the healthy subject.

Initial results of an aerosol/gas combination, sodium chloride plus ozone, indicate that an inert salt does not potentiate the acute physiologic response to ozone.

The hint that, under some circumstances, a lower level of a pollutant may be more toxic than a higher one is an important lead to be followed as time permits. It is possible, as previously stated, that a low enough level does not trigger the protective breathing response seen at a slightly higher, more irritating level. When the protective breathing response is absent, deeper-lying tissues might be more heavily exposed.

Recommendations

Recommendations for a long-term research program have been made in a research proposal, "Sulfate, Nitrate Inhalation Toxicity", submitted to the California Air Resources Board in August, 1975. At that time, program expansion into one or more of 5 possible areas was discussed and two of these, short-term exposures of normal and abnormal human subjects, and long-term exposures beginning in the young, pre-adolescent animal were recommended as having high priority. We believe that program expansion is desirable on behalf of the public good and policy questions for control of air pollution. To these ends, discussions of long-term program planning between the staff of the Air Pollution Health Effects Laboratory and the staff of the California Air Resources Board should be instituted. No new recommendations of a short-term nature are made at this time. The research program is in an interesting and productive phase and, we feel, should proceed as planned.

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APPENDIX A

RADIATION USE AUTHORIZATION NO. IRS-7509 Rev.

50,000,000

Class ..... EOY 3,000,000

User Robert F. Phalen, Ph.D. Dept. Comm & Env Medicine Expires 04-76

No. Form No.

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RADIATION SAFETY MANUAL #93 WORK AUTHORIZED

Locations SNIF/IRF facility, North Campus

Personnel R. Phalen, J. Davis, J. Kenoyer, R. Hinrichs, N. Truglio, T. Crocker, G. Samuelsen, R. Fairshter, A. Wilson, B. Dennison, R. Walters

Description Isotopes will be used in research animals for diagnostic purposes and as tracers with inhaled aerosols. A sealed Kr-85 source will be used to remove static charge from aerosol particles. Labeled aerosols will be injected into a double confinement apparatus described in the application. Large animals will be exposed using a mask over the breathing openings.

PRECAUTIONS REQUIRED

Isotope Limits

Isotope	Chem. Form	Phys. Form	mCi/order	mCi/total
^{85}Kr	gas	sealed source	2	by EH & S approval only
^{133}Xe 1)	saline solution	liquid	1500	25000
2)	gas	gas	1500	25000
^{99}Tc	ammonium hydroxide	liquid	1	5
^{51}Cr	chloride or chromate	liquid	1	5
^{59}Fe	chloride	liquid	1	5
Other ^{131}I	sodium iodide	liquid	1	5

1. All persons involved shall be familiar with and comply with provisions of the Irvine Campus Radiation Safety Manual.
2. All counter surfaces for radioisotope work will first be covered with a plastic-backed absorbent paper, available at the storeroom.
3. All radioactive waste will be delivered to EH&S except short half lives.
4. All liquid radioactive waste will be collected in 1 gal. bottles protected from breakage or lab grade nalgene containers of 1 gal. max.
5. All dry radioactive waste will be collected in EH&S-supplied cardboard boxes of 2 or 4.5 cu. ft., lined with a plastic bag.
6. A Geiger-Mueller counter or other suitable detector must be immediately available when working with the isotopes listed.
7. Film badges will be worn when working with the isotopes listed.
8. Radioactive gases evolved during work with Xe-133 shall not be released in concentrations greater than 3×10^{-7} μCi per ml air at the point of release
9. Iodine handling must be performed using veterinarian's gloves fit to the shoulder and in a hood with 100 linear feet per minute(lfpm)face velocity.
10. Live animals or cages will be labeled or posted with "radioactive materials" signs after injection or ingestion. Plastic bags CONT ON NEXT PAGE

Personal Prot. Eqpt. Required.

- ☒ Film Badge
- ☐ Pocket Dos.
- ☐ .....
- ☐ .....
- ☒ Lab Apron or
- ☒ Lab Coat
- ☐ Coveralls
- ☐ .....
- ☐ .....
- ☒ Imperv. Gloves
- ☒ veterinarian gloves
- ☐ Shoe Covers
- ☐ .....
- ☐ .....
- ☐ Half Mask
- ☐ Face Mask
- ☐ .....
- ☐ .....
- ☐ .....
- ☐ .....

Bioassay

- ☐ Urinalysis
- ☐ Thyroid Scan
- ☐ Whole Body Ct.

AUTHORIZATION

We certify that all work will be as described and will be performed in accordance with the precautions listed.

Authorized User Robert F. Phalen 4/24/75 Date

Dept. Chairman J. Jeffrey Carter Date

We approve the radiation use as described subject to the precautions listed.

Rad. Safety Officer [signature] 4-25-75 Date

Rad. Safety Subcommittee ..... Date

RADIATION USE AUTHORIZATION NO. JRS-7509 Rev. PAGE 2

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Class..... HGV 3,000,000

User Robert E. Phalen, Ph.D. Dept. Comm. & Env. Medicine Expires 04-76

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IATION SAFETY MANUAL #93 WORK AUTHORIZED

Locations

Personnel

Description

PRECAUTIONS REQUIRED

Isotope Limits

Isotope	Chem. Form	Phys. Form	mCi/order	mCi/total

Other

1. All persons involved shall be familiar with and comply with provisions of the Irvine Campus Radiation Safety Manual.

10. cont.
containing sacrificed animals will be labeled before disposal.
11. Isotopes transferred between UCI, OCMC leased space, or OCMC must be accompanied by a proper transfer form, a copy of which must be sent to UCI EH&S Office.
12. Animal disposal will be in accordance with the Medical Vivarium Committee Policy, radioisotope laden or not.

Personal Prot. Eqpt. Required.

- ☐ Film Badge
- ☐ Pocket Dos.
- ☐ .....
- ☐ .....
- ☐ Lab Apron
- ☐ Lab Coat
- ☐ Coveralls
- ☐ .....
- ☐ .....
- ☐ Imperv. Gloves
- ☐ .....
- ☐ .....
- ☐ Shoe Covers
- ☐ .....
- ☐ .....
- ☐ Half Mask
- ☐ Face Mask
- ☐ .....
- ☐ .....
- ☐ .....
- ☐ .....

Bioassay

- ☐ Urinalysis
- ☐ Thyroid Scan
- ☐ Whole Body Ct.

AUTHORIZATION

We certify that all work will be as described and will be performed in accordance with the precautions listed.

Authorized User Robert E Phalen 4/24/75
Date

Dept. Chairman J. Frantz Crosbie
Date

We approve the radiation use as described subject to the precautions listed.

Rad. Safety Officer.....
Date

Rad. Safety Subcommittee.....
Date

REVIEW

Robert F. Phalen, Ph.D.
Department of Community and Environmental Medicine

IRS-7509

Dr. Phalen is with the Community and Environmental Medicine Program officed in Medical Surge II with research facilities being completed on North Campus and referred to as SNIF or IRF. The license is a new request and the following information has been obtained and three inspections and reviews of those facilities and license desires.

The facility is adequately designed for the use of radioactive materials, as well as for the inhalation studies designed to be conducted within the chamber containment of the facility. The chambers are Rochester chambers and inspected by me to insure that radioactivity would not escape into the room while injecting into the chamber volume the atmosphere to be breathed by the animals.

The protocol set up for air pollution studies and dogs has been submitted, and there are no areas where there is a concern with the regard to the methods and design of the particular research where isotopes may be used. The facility is adequate to safely house the particular experiment.

Although the primary function of the facility is to study inhalation research, there will be occasions when tagged aerosolized particles will be used in order to determine particle size parameters with regard to lung clearing capabilities of several animals. The animal facility will be complete with dog runs rat cages, and presently under consideration is the use of even larger animals, such as sheep or donkies.

The project director will be Robert Phalen and his background is certainly adequate to indicate that he has an impressive amount of experience in this area both from Rochester and Lovelace. His total experience with radiation and radioactive materials dates back to his being a Ph.D. student in the University of Rochester; and he has served as an Assistant Radiological Safety Officer at San Diego State University from 1964 to 1966. He has over eleven years experience with the use of radioisotopes and his experience with the procedures and techniques involved here are extensive and adequate for direction of such a program, as well as training personnel within his supervision. Dr. Phalen has several personnel coming onboard who will be individually approved as independent users of radioisotopes. Experience and training forms on all independent users and the chief researcher are adequate.

No medical examination will be required at this time unless the strangeness of the medical aspects and the toxic properties of the materials to be used require it. Various types of radiations are being utilized owing to the nature of the tagging that needs to be done. The facility has adequate storage space, has good confinement for radiation exposure control and is quite compatible with adjacent projects in the area. The minimum laboratory safety criteria have been met. The facility would be a little difficult to decontaminate with the new roughed floor covering that has been added, but there is no anticipation of a problem owing to the radioactive work.

The radioisotope handling procedures, as given to me verbally and in the initial description of the project submitted December, 1974, are adequate to control the release of radioactivity to the environment. Procedures for prevention of ingestion, inhalation or absorption by the workers and the selected protective equipment should further enhance the safety aspects of the program. There will be on-hand monitoring

equipment which is being bid out now. Film badges will be required where gamma emitters are going to be used.

There will be a radioactive waste management program to be considered. There will probably be liquid and dry waste collection for long life radioisotopes. Short life radioisotopes will merely be disposed of through permitting 8+ half-life decay in order to achieve the levels not to exceed the table II quantities of schedule A of the title 17, section 30355. Where waste is required to be removed by this department there will be a recharge.

There are suitable warning signs, labels and locked cabinets in order to control radioactive materials. Waste materials will be properly contained in EH & S-issued vessels. No request for radioactive materials will be on the facility door. Cages, refrigerator and other equipment, glassware and devices should be labeled when radiation or radioactivity is present. No x-rays are anticipated for use at this time. Notice to the employees sign has not been posted in the facility in that the bulletin boards have not been installed at this time.

Both inhouse as well as pre-trained personnel will be utilized. No bio-assay requirements will be specified at this time. EH & S will monitor the facility on a quarterly basis. The user is asked to monitor after each major use. Dosimetry requirements will be x-ray, beta, gamma badges. Ring badges may be considered for specialized procedures but not as a routine monitoring device. The fumehood is just now being completed and the survey should be made of that device in order to establish its flow criteria. The license itself will describe the radioisotope complement desired in order to conduct this research project. Instrumentation will have to be calibrated once every six months and such information kept in a log book. All log books should also be maintained for the radioisotope inventory. The review was completed on December 16, 1974.

December 16, 1974

William W. Wadman III
Radiation Safety Officer

WWW:ckk

APPENDIX B

Professional Activities of the Air Pollution Health Effects Laboratory Staff (1974-1975)

Publications

*Crocker, T.T. (ed.). Conference on Health Effects of Atmospheric Salts and Gases of Sulfur and Nitrogen in Association with Photochemical Oxidant, Volumes I and II, prepared for California Air Resources Board, January, 1974.

*Crocker, T.T. Effects of Sulfur Oxides in Animals and Man, Conference on Health Effects of Atmospheric Salts and Gases of Sulfur and Nitrogen in Association with Photochemical Oxidant, Volume II, California Air Resources Board, January 1974.

Davis, J. and B.H. Brattstrom. Vocalization of the California Newt, Taricha Eorosa, J. of Herp. (accepted for publication)

Dennison, William J. Design of High Flow Ambient Air Purification System. School of Engineering Report - UCI-ARTR-75-1, University of California, Irvine

*Dungworth, D.C., R.F. Phalen and W.S. Tyler. Systematic approach to methods for evaluation of pulmonary toxicity in animals. Proc. of the Sixth Annual Conf. on Environmental Toxicology, Dayton, Ohio, October 21-23, 1975 (in press)

Fairshter, R.D. and A.F. Wilson. Paraquat poisoning - manifestations and therapy. Am. J. Medicine (in press)

Glauser, F., A. Wilson, L. Carothers, J. Higi, D. White and J. Davis. Pulmonary parenchymal tissue volume (V_t) measurements in graded degrees of pulmonary edema in dogs. Circ. Res. 36:229, 1975.

Ho, A. The evaluation of V_{max} as contractility index of myocardial muscle. M.S. Thesis, School of Mechanical Engineering, California State College at Sacramento, June, 1974.

Kenoyer, J.L., Dissolution of ^{169}Yb Fused Clay Particles, M.S. Thesis, San Diego State University, Department of Physics, 1975.

Murdock, L. The physiology and bioenergetics of the American coot, Fulica Americana. M.A. Thesis, California State University at Fullerton, 1975.

Phalen, R.F. Respiratory Tract Morphology: Summary of a Conference. Bio.Sci. 24:612, 1974.

Phalen, R.F. and O.G. Raabe. Aerosol particle size as a factor in pulmonary toxicity. Proc. of the Fifth Ann. Conf. on Environmental Toxicology, AMRL-TR-74-125, pp.353-366, 1974.

*Acknowledgement given to California Air Resources Board for support of research

Phalen, R.F., W.L. Cannon and D. Esparza. Comparison of impaction, centrifugal separation and electron microscopy for sizing cigarette smoke. Proc. of the Symposium on Fine Particles, Environmental Protection Agency, 1975.

*Phalen, R.F., J.D. Hallford and J.L. Kenoyer. Particle deposition and clearance as a test of toxic effect. Proc. of the Sixth Ann. Conf. on Environmental Toxicology (in press)

Phalen, R.F. Inhalation exposure of animals. Environmental Health Perspectives (in press)

Raabe, O.G., H.C. Yeh, G.J. Newton, R.F. Phalen and D.J. Velasquez. Deposition of inhaled monodisperse aerosols in small rodents. (to appear in Inhaled Particles IV, Pergamon Press, 1976)

Shepherd, A.F., J.C. Sutherland and A.F. Wilson. Continuous spectrophotometric measurements of arteriovenous oxygen difference. J. Appl. Physiol. 39:152-155, 1975.

Wilson, A.F., R. Honsberger, J.T. Chiu and H.S. Novey. Transcendental meditation and asthma. Respiration 32:74, 1975.

Yeh, H.C., A.J. Hulbert, R.F. Phalen, D.J. Velasquez and T.D. Harris. A stereo-radiographic technique and its application to the evaluation of lung casts. J. Invest. Radiol. 10:351-357, 1975.

*Acknowledgement given to California Air Resources Board for support of research.

Presentations

Ho, A. V_{max} as the Index of Myocardial Contractility. Conference on Engineering in Biology and Medicine, New Orleans, Sept. 1975.

Kenoyer, J.L. Radiation Physics and Safety in the Laboratory. Seminar, Air Pollution Health Effects Laboratory, University of California, Irvine, June 17, 1975.

Phalen, R.F. Inhalation Program at University of California, Irvine. Conference on Health Effects of Air Pollution, University of California, Los Angeles, November 26, 1975.

Phalen, R.F. Health Effects of Air Pollution. Presented to Orange County Pharmaceutical Association, Newport Beach, California, October 15, 1975.

Phalen, R.F. The Human Tracheobronchial Tree. Annual American Industrial Hygiene Association Conference, Minneapolis, Minnesota, June 5, 1975.

Phalen, R.F. Inhalation Exposure of Animals. Target Organ Toxicity and Lung Conference sponsored by Society of Toxicology, Environmental Protection Agency and National Institute of Environmental Health and Safety, Cincinnati, Ohio, September 16, 1975.

Phalen, R.F., J.D. Hallford and J.L. Kenoyer. Particle Deposition and Clearance-Relationship to Recognition of Toxic Effects. Sixth Ann. Conf. on Environmental Toxicology, Dayton, Ohio, October 21, 1975.

Davis, J. Health Consequences of Air Pollution, Lecture, Corona del Mar High School, Newport Beach, California, December, 1975.

Ho, A. Sources and Mechanics of Air Pollution, Lecture, Corona del Mar High School, Newport Beach, California, December, 1975.

Other Professional Activities

Davis, J. Attendance of 55th Ann. Meeting of the American Society of Mammalogists, Missoula, Montana, June, 1975.

Davis, J. and R. Phalen. Attendance of Sixth Ann. Conference on Environmental Toxicology, Dayton, Ohio, October, 1975.

Davis, J. Visit to NIOSH laboratories, Cincinnati, Ohio, October, 1975.

Davis, J. Visit to Pulmonary Research Unit, University of Cincinnati, Cincinnati, Ohio, October, 1975.

Davis, J. Visit to Inhalation Toxicology Research Institute, Lovelace Foundation, Albuquerque, New Mexico, October, 1974.

Kenoyer, J., T. Crocker, J. Davis and R. Phalen. Visit to Inhalation Research Group, University of California, Davis, June 1975.

Murdock, L. Attendance of American Institute of Biological Scientists Conference, Corvalis, Oregon, June, 1975.

Walters, R. Visit to Air Pollution Project, University of California, Riverside, August 25, 1975.

Kenoyer, J., J. Davis and R. Phalen. Visit to laboratory of Dr. Jack Hackney at Rancho Los Amigos Hospital, Inc., Downey, California, December 23, 1975.

Kenoyer, J., J. Davis and R. Phalen. Visit to U.C.L.A. School of Medicine, Pulmonary Division, Los Angeles, California, December 23, 1975.

APPENDIX C. DESCRIPTION OF UCI NUCLEAR REACTOR FACILITY

The Chemistry Department at the University of California, Irvine, has developed a special facility in support of radionuclear chemistry. The focal point is a Triga Mark I class nuclear research reactor. The reactor operates at 250 kw (1.8×10^{12} neutrons/cm^2 sec) steady state power and is capable of pulsing to 100 Mw peak power. This supplies a suitable neutron source for most activation studies or isotope production. Other equipment in the facility includes a 15 mev neutron generator for inducing reactions requiring fast neutrons, a 11,000 Ci cessium gamma-ray source for photon chemistry, specialized detection equipment including a germanium-lithium high resolution crystal, a 4096 pulse height multichannel analyser, NaI (Tl) detection systems, and a high precision (0.1%) oxygen analysis system. This facility is available to support project areas of radioisotope generation and particle tracer chemistry of the type required at the Air Pollution Health Effects Laboratory.

APPENDIX D

Computer Output of Size Distribution of Salt Aerosols, as obtained from analysis of electron microscopic photographs

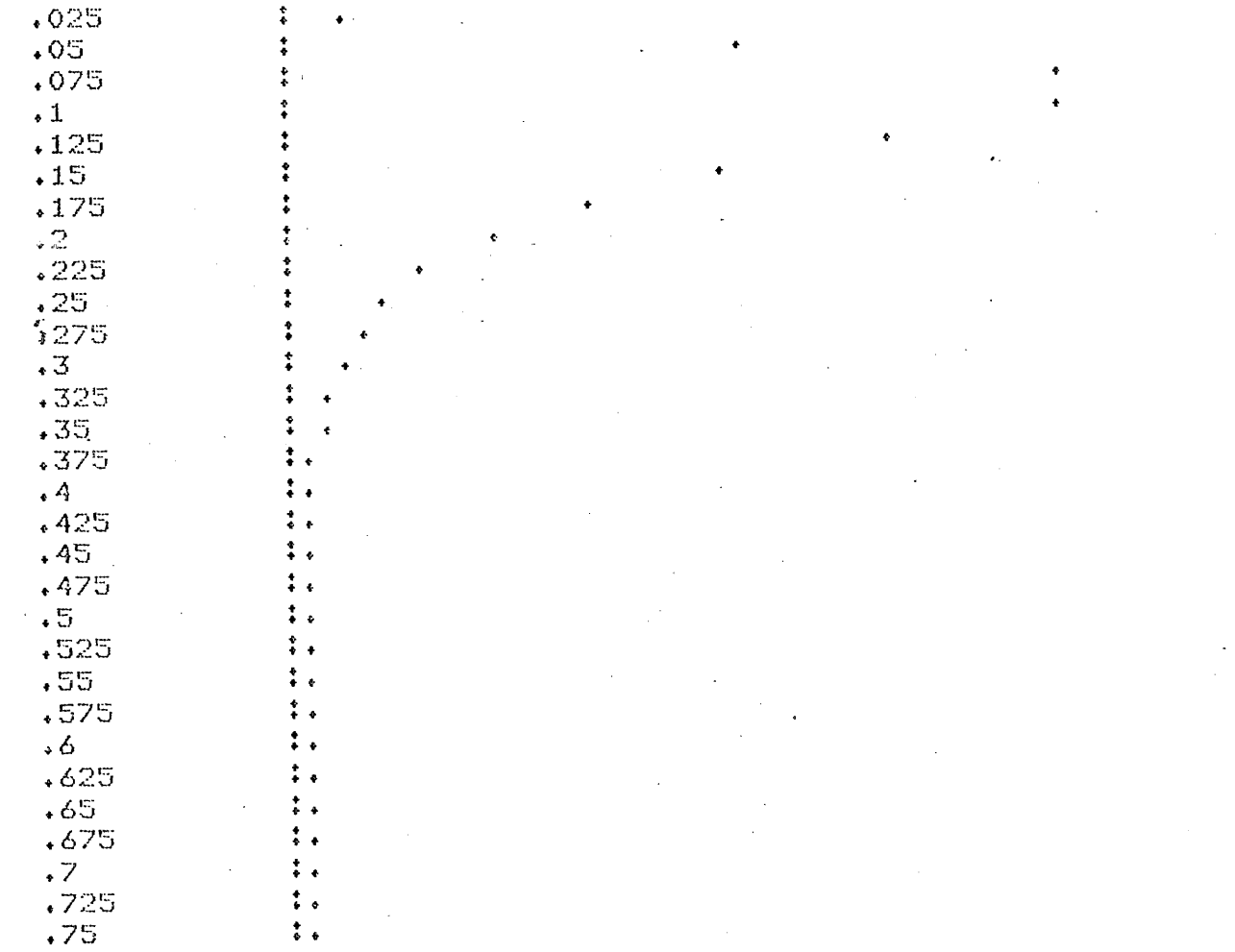
FERRIC SULFATE

TODAY IS 11 / 24 / 75
THE CONCENTRATION IS 10 PERCENT
THE CHAMBER TEMPURATURE IS 23 C
THE CHAMBER FLOW RATE IS 3 CUBIC FEET PER MINUTE
THE RELATIVE HUMIDITY IS 40 %
THE ZEISS MAGNIFICATION IS 30140

DIAMETER	COUNT	LESS THAN	LESS THAN PERCENT
.0165893	0	0	0
.0225614	0	0	0
.0285335	0	0	0
.0348374	3	3	7.33496E-03
.0408096	6	9	.0220049
.0471135	16	25	.0611247
.0530856	22	47	.114914
.0590577	18	65	.158924
.0653616	17	82	.200489
.0713338	23	105	.256724
.0776377	31	136	.332518
.0836098	18	154	.376528
.0895819	19	173	.422983
.0958859	22	195	.476773
.101858	17	212	.518337
.108162	12	224	.547677
.114134	14	238	.581907
.120106	14	252	.616137
.12641	11	263	.643032
.132382	9	272	.665037
.138686	10	282	.689487
.144658	17	299	.731051
.15063	12	311	.760391
.156934	14	325	.794621
.162906	7	332	.811736
.16921	6	338	.826406
.175182	8	346	.845966
.181155	7	353	.863081
.187459	5	358	.875306
.193431	4	362	.885086
.199735	3	365	.892421
.205707	3	368	.899755
.211679	2	370	.904646
.217983	1	371	.90709
.223955	3	374	.914425
.230259	4	378	.924205
.236231	4	382	.933985
.242203	3	385	.94132
.248507	2	387	.94621
.254479	3	390	.953545
.260783	1	391	.95599
.266755	0	391	.95599
.272727	8	399	.97555
.279031	0	399	.97555
.285003	2	401	.98044
.291307	2	403	.98533
.297279	1	404	.987775
.303251	5	409	1

COUNT MEDIAN DIAMETER IS .11
STANDARD DEVIATION IS 1.64

Y-AXIS: FROM 0 TO 10 IN INTERVALS OF .2



TYPE GO TO 410 IF YOU WANT THE AREA UNDER THE CURVE

STOP
READY

AMMONIUM SULFATE

TODAY IS 12 / 1 / 75
THE CONCENTRATION IS 10 PERCENT
THE CHAMBER TEMPURATURE IS 23 C
THE CHAMBER FLOW RATE IS 3 CUBIC FEET PER MINUTE
THE RELATIVE HUMIDITY IS 40 %
THE ZEISS MAGNIFICATION IS 30140

DIAMETER	COUNT	LESS THAN	LESS THAN PERCENT
.049436	8	8	

%SYN AT LINE 1650

READY

1650 PRINT D(I),N(I),Q(I),Q(I)/Q(V)
GO TO 1640

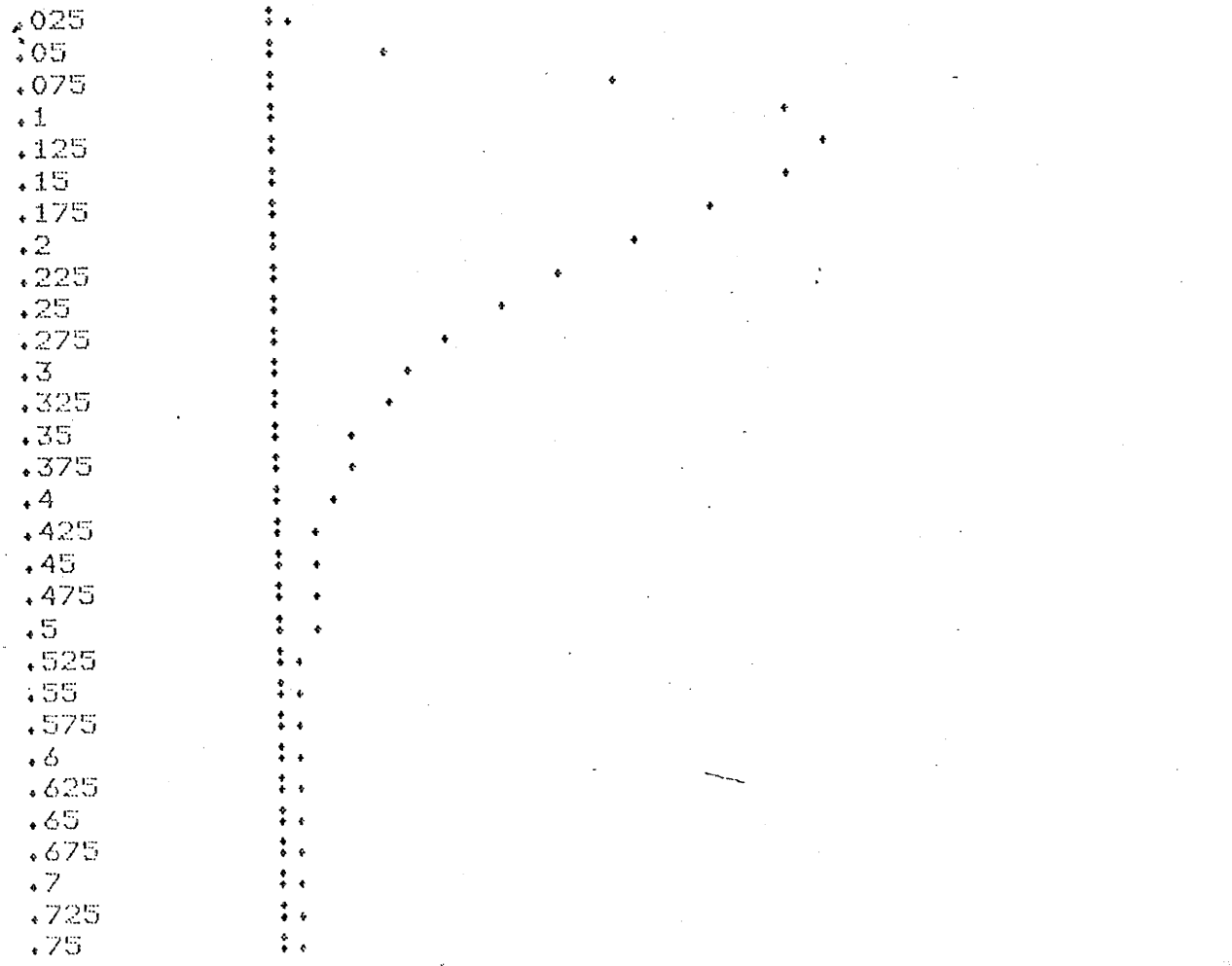
DIAMETER	COUNT	LESS THAN	LESS THAN PERCENT
.049436	8	8	.026936
.0676841	15	23	.0774411
.0859323	29	52	.175084
.10418	34	86	.289562
.12276	32	118	.397306
.141009	22	140	.47138
.159257	17	157	.52862
.177505	27	184	.619529
.195753	21	205	.690236
.214333	19	224	.754209
.235567	16	240	.808081
.250829	9	249	.838384
.269078	14	263	.885522
.287326	5	268	.902357
.305906	6	274	.922559
.324154	1	275	.925926
.342402	7	282	.949495
.36065	4	286	.962963
.378898	3	289	.973064
.397478	0	289	.973064
.415727	2	291	.979798
.433975	1	292	.983165
.452223	3	295	.993266
.470471	1	296	.996633
.489051	1	297	1

CNUNT MEDIAN DIAMETER IS .155099
MASS MEDIAN DIAMETER IS .331029
STANDARD DEVIATION IS 1.65319

READY

COUNT MEDIAN DIAMETER IS .16
STANDARD DEVIATION IS 1.65

Y-AXIS: FROM 0 TO 10 IN INTERVALS OF .2



SODIUM CHLORIDE

TODAY IS 12 / 5 / 75
THE CONCENTRATION IS 10 PERCENT
THE CHAMBER TEMPURATURE IS 23 C
THE CHAMBER FLOW RATE IS 3 CUBIC FEET PER MINUTE
THE RELATIVE HUMIDITY IS 40 %
THE ZEISS MAGNIFICATION IS 30825

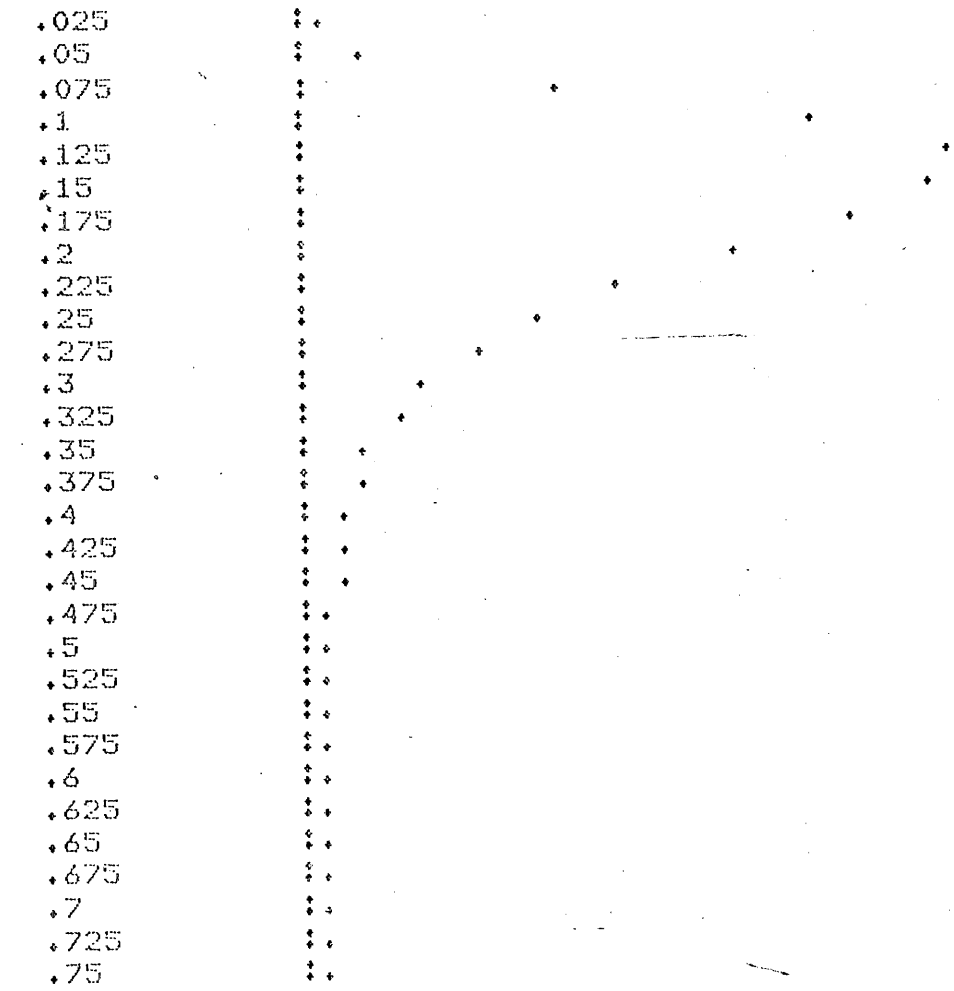
DIAMETER	COUNT	LESS THAN	LESS THAN PERCENT
.0483374	0	0	0
.0661801	3	3	.0148515
.0840227	14	17	.0841584
.101865	25	42	.207921
.120032	23	65	.321782
.137875	19	84	.415842
.155718	22	106	.524753
.17356	22	128	.633663
.191403	13	141	.69802
.20957	8	149	.737624
.230333	5	154	.762376
.245255	10	164	.811881
.263098	10	174	.861386
.280941	10	184	.910891
.299108	3	187	.925743
.316951	6	193	.955446
.334793	2	195	.965347
.352636	2	197	.975248
.370478	2	199	.985148
.388646	1	200	.990099
.406488	1	201	.995049
.424331	1	202	1

COUNT MEDIAN DIAMETER IS .162793
MASS MEDIAN DIAMETER IS .275016
STANDARD DEVIATION IS 1.51903

READY

COUNT MEDIAN DIAMETER IS .16
STANDARD DEVIATION IS 1.52

Y-AXIS: FROM 0 TO 10 IN INTERVALS OF .2



TYPE GO TO 410 IF YOU WANT THE AREA UNDER THE CURVE

STOP

APPENDIX E

Particle Deposition and Clearance as a Test of Toxic Effect*

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*supported in part by the Air Resources Board of the State of California under Contract Number 4-611

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The objective of tests of aerosol deposition and clearance in toxicology is to assess the effect of exposure to toxic materials on an important defense mechanism, the ability of the lung to handle inhaled particles. Tests of deposition and clearance patterns have been performed by various investigators using human as well as large and small animal subjects. Several agents do alter deposition and/or clearance phenomena in the lung. The effect of cigarette smoke exposure on the clearance of inhaled aerosols in the rat is given as an example of use of deposition and clearance tests in the laboratory.

I. Introduction

The field of inhalation toxicology is currently in an intriguing active phase characterized by two features: 1) compilation, description and quantitation of effects other than mortality that include physiological, morphological and biochemical parameters, and 2) development of an understanding of biologic responses in terms of the physical and chemical properties of inhaled materials. Further development in both of these areas seems necessary for inhalation toxicology to become a predictive discipline as opposed to merely a descriptive one. The phenomena associated with deposition and clearance of inhaled particles have recently been recognized as important aspects of the defensive mechanisms of the lung. The purpose of this paper is to justify the use of deposition and clearance tests in the inhalation toxicology laboratory.

Particles, or aerosols as they are called when airborne, can be broadly classified with respect to their origin. Naturally occurring aerosols include spores, pollens, microorganisms, inorganic dusts, ash, degradation products of various plants, liquid condensation droplets and various solids formed from naturally occurring volatile materials. In and about areas populated by humans additional aerosols include those produced from combustion of fuels, a multitude of industrial processes, erosion of machinery and building and household materials, spraying devices, and again, reactions of gaseous materials. Aerosols in and about localized workplaces form another practically innumerable sub-category. A common belief, probably justifiable, is that virtually all aerosols are capable of producing toxic responses in humans if inhaled in sufficient concentrations over a sufficient time (R.V. Christie, 1967).

For purposes of scientific elucidation, the fate of many inhaled aerosols can be analyzed into two phases: 1) deposition on surfaces of the respiratory

tract, and 2) clearance (or lack of) after deposition has occurred.

The patterns of deposition of inhaled aerosols are becoming understood in terms of forces that act on airborne particles, the air-flow characteristics of breathing, and the geometric properties (morphology) of the respiratory tract. Mammalian respiratory systems have geometric and air-flow properties such that particles within a given range of size and shape tend to deposit preferentially in characteristic locations. For example, the human nose is known to be highly efficient in collecting particles with aerodynamic diameters greater than a few micrometers. The deep lung can only collect particles that have eluded the nose (or mouth) and the tracheobronchial tree; that is, particles with aerodynamic diameters below a few microns.

It appears that clearance mechanisms at various levels in the respiratory tract are efficient in handling particles in the size ranges that preferentially deposit at a given site. For example, alveolar macrophages seem to exhibit efficient engulfment for particles in the one micron diameter size range; this is just in the size range of high deposition probability in alveoli. Infectious organisms are often in the size range for deposition in the deep lung, where conditions are usually favorable for their rapid reproduction. Fortunately, macrophages can inactivate many infectious organisms. Similarly, the nose effectively clears the largest inhalable particles via sneezing, blowing and mucus movement, and the moving mucus of the tracheobronchial tree is efficient in transporting large quantities of solid or liquid particles of various sizes, shapes and densities. The point is that deposition and clearance phenomena appear to be somewhat matched or balanced with respect to particle size characteristics. This being the case, one might suspect that alterations in either the deposition or clearance patterns could predispose one toward future injury from inhaled aerosols. In some instances, of course, shifts in deposition or clearance patterns might act to afford increased protection.

Fortunately, despite differences in size and morphology, most mammals appear to have clearance phenomena that are remarkably similar with respect to rates and mechanism; for example, they commonly have mucociliary clearance in the nose and tracheobronchial tree and a macrophage response in the alveolar spaces. Also, though correspondence is not as close here, basic similarities exist in aerosol deposition characteristics and in responses to toxic materials. Thus, it is reasonable to consider tests of aerosol deposition and clearance in animals in toxicologic evaluations of injury.

II. Inhaled Agents that Alter Deposition or Clearance Patterns

Several materials are known to alter deposition or clearance (including killing or inactivation of microorganisms) of inhaled particles. A few examples will serve to illustrate. Cigarette smoke, an almost ever-present co-insult in human inhalation exposure situations, has understandably been well studied. The controlled studies of Albert, et al. (1969, 1970, 1974) show effects in humans and donkeys that depend on dose and exposure time. Low single doses or early effects of repeated exposure to smoke were associated with acceleration of clearance rates in the tracheobronchial tree of both species. Heavier doses and long-term repeated exposures were associated with sporadic clearance, intervals of clearance stasis, and even retrograde movement of deposited particles (again in both species). Cigarette smoke exposures (220 mg/m^3) have been shown to increase deposition and delay clearance in rats (Garver, 1968) and to increase the survival of inhaled viable bacteria in hamsters (Henry, Spangler, Findlay, and Ehrlich, 1970). In the hamster study, excess deaths due to bacterial infections were seen in animals exposed to cigarette smoke for 2 hours ("3% v/v").

Pre-existing influenza infection has been shown to impair both upper and lower respiratory tract clearance. Studies by Green (1965) with P-8 virus infected mice that were exposed to viable staphylococcus bacteria showed that

infected animals did not effectively kill the bacteria. Similarly, Creasia, Nettesheim, and Hammons (1973) found that P-8 virus-infected mice had drastically impaired clearance of radioactive "insoluble" particles. In humans, Camner (1973) found that influenza infection could impair tracheobronchial clearance for up to one month after disappearance of the familiar clinical symptoms.

Elliot Goldstein and co-workers (1971, 1974) reported work in which mice were challenged with radiolabeled viable staphyloccocus both before and after exposures to relatively low levels of ozone and nitrogen dioxide. Prior exposure to ozone (.6-2 ppm, 17 hours) or ozone plus nitrogen dioxide (.1-.3 and 1.5-4.2 ppm, 17 hours) lead to: 1) decreased overall deposition of bacteria, and 2) impaired killing of deposited bacteria. In the same series of studies, exposures of ozone plus nitrogen dioxide (.4 and 4-6.8 ppm, 4 hours) after inhalation of bacteria, caused increased survival of the bacteria. Ozone alone at 2 ppm (4 hours) was observed to cause increased survival and increased clearance of the inhaled bacteria.

Sulfuric acid mist exposures by Fairchild, Stultz and Coffin (1975) at 3 mg/m^3 (1.8 μm CMD) in guinea pigs caused increased total deposition of inhaled streptoccocus.

Sulfur dioxide (1 ppm, 7 hours, 5 days to 25 days) has been shown to diminish the clearance of inert particles in both the lung and tracheobronchial tree in rat by Ferin and Leach (1973). A similar effect was seen in donkeys after brief exposure (300 ppm SO_2, 30 min) by Spiegelman et al. (1968).

Many other agents have been reported to change deposition and/or clearance patterns in various species.

III. Experimental Design for Deposition and Clearance Testing

A successful test of deposition and clearance implies that certain criteria have been met. These criteria apply to the test aerosol, animal subjects,

experimental plan and analysis of data. Recommendations can be made in each of these areas.

The aerosol

For deposition and clearance studies, the aerosol should be inhaled by the subjects and have an aerodynamic size that permits significant deposition beyond the nose. For most studies an aerosol smaller than about 5 μm in diameter is appropriate. Monodisperse aerosols, or at least those that have diameters distributed with geometric standard deviations less than about 1.3, should be used. Aerosol characteristics should be reproducible from one run to the next. The aerosol should be detectable in the lung, either by external radiation detection or chemical/biological assay in excised lung. Radioactive aerosols should have a tag that does not have excessive leaching; a few percent per day in the lung environment is sufficient. Initial activity of labeled aerosol should be on the order of one microcurie to allow for precise external counting. An aerosol that fits the above criteria is radiolabeled polystyrene-latex. The basic particles are available commercially (Dow Chemical Company, Midland, Michigan) in several sizes, and methods for labelling with radioisotpes are available (Szende, et al., 1975 and Black and Walsh, 1970).

The animal.

Unanesthetized animals should be used when possible in order to avoid variable and often uncertain effects associated with anesthesia. In most studies two species should be used, especially when one is either the guinea pig or the rat. Rats tend to have respiratory infections and guinea pigs appear to have unusually reactive airway musculature. Individual subjects should serve as their own controls to reduce variability in the data. Healthy individuals usually have relatively repeatable deposition and clearance phenomena, but variation within a group may be large.

Experimental Plan

Exposure to the aerosol should be to the nose or mouth to avoid deposition of large amounts on fur or skin. Deposition of material on fur of laboratory animals can interfere with subsequent assay of amounts in lung and can lead to ingestion of large quantities of particles. A determination of the initial deposited amount should be made immediately after exposure (within minutes), and the inhalation exposure should not last more than about 20-30 minutes. Long inhalation exposures are complicated by concurrent clearance occurring during deposition.

Amount in the lung should be determined with sufficient frequency to define the clearance curve. Ideally, the amoung in the lung should be quantitated hourly for the first few hours and daily for several days. It is important that clearance be followed for long enough to properly define the clearance curve. The tracheobronchial tree is usually cleared by about 1-2 days but deep lung clearance can require several days, even months or years, for highly insoluble materials.

Data Handling

To determine the effect of toxic agents on clearance, statistical tests should be employed. This necessitates reducing clearance curves to numerical values; the fewer parameters used to define the curve, the simpler the tests will be. Figure 1 depicts three out of the many ways of quantitating clearance curves: 1) analysis into exponential components; 2) fitting with a polynomial function, and 3) calculation of moments. Each method yields numerical values that can be given mean values and standard deviations for a group of observations. Statistical tests for significance can then be performed on these values, permitting one to demonstrate significant changes in clearance patterns.

VI. Effect of Cigarette Smoke Exposure on Particle Clearance in the Rat

Data resulting from a modest study will be presented to demonstrate the effect of a toxic agent on tracheobronchial clearance. Rats were used as they were inexpensive and easy to handle. Eight Sprague Dawley rats were briefly exposed, nose only, to a radioactive silver aerosol. The animals were then divided into two groups: one group was exposed to cigarette smoke for four hours, the other group breathed ordinary air and served as a clearance control. Clearance curves were determined for each animal by measuring the radioactivity in the thorax every 100 minutes for the first day, every 200 minutes the second day and less frequently for about eight days. These measurements were made by using a collimated gamma-ray detector placed above an opening in a lead shield. The animals were placed beneath this collimator shield such that only gamma rays emitted from the thoracic region were measured. Feces were collected every time a thoracic count was made, and the radioactivity in each sample was determined.

Aerosol Exposure

An exploding wire aerosol generator (a 4 microfarad capacitor charged to 6.5 kilovolts) of the type described by Karioris and Fish (1962) was used to aerosolize 20 cm of 0.005 inch diameter silver wire. The wire had previously been neutron irradiated and had an induced activity of about 0.75 microcuries of ^{110m}Ag (beta and gamma, 250 day half-life) per cm of wire. The aerosol had spherical primary particles that were distributed approximately log-normally with a count median diameter of 0.07 microns and a geometric standard deviation of 1.6. Electron micrographs indicated that most of the aerosol was in the form of agglomerates of primary particles with an aerodynamic median diameter of about 1 um when inhaled by the animals. The radioactive wire was exploded inside a 22 liter exposure chamber, five minutes were allowed for settling of large particulates, and the animals were exposed nose-only to the aerosol through

ports in the chamber walls (Fig. 3). During the actual explosion, rats were held in a separate room to isolate them from the loud noise. The exposure time was 15 minutes and the mean initial lung deposition was estimated (using wheat-filled phantoms) to be about 0.1 microcuries, or about 0.2 milligrams of silver. No anesthetics were used as the animals were docile and cooperative during both the aerosol exposure and the thoracic counting procedures.

Smoke Exposure

The experimental group was exposed to fresh tobacco smoke generated by a machine which drew air continuously through several lit unfiltered cigarettes and gently blew the smoke into a large box (one cubic meter volume). The animals were placed inside cages within this box 30 minutes after exposure to the silver aerosol. They remained in the smoke for four consecutive hours, being removed only twice for two minutes each time, for thoracic activity measurements. The smoke concentration was maintained at a level such that taking a breath inside the smoke chamber (smoke exposure box) gave the experimentors the same subjective experience as inhaling during normal cigarette smoking.

Clearance Measurement

The animals were placed in plastic restrainers beneath a NaI(Tl) crystal (3" diameter) for gamma activity determinations of their thoracic regions. Two inches of lead were used to shield the head and gastrointestinal tract from the gamma detector. The shield, with a 2½" wide opening above the thoracic area, had been designed using roentgenograms of all of the rats so that ^{110m}Ag in either the head, stomach or intestines was shielded from the detector.

Results

Longitudinal body scans of radioactivity indicated high initial activities in the head region, and it was feared that the fur on the head had been contaminated

with significant amounts of ^{110m}Ag. However, this activity declined rapidly indicating a fairly clean nose-only exposure, and indicating that the initial activity was probably due to high deposition inside the nose and throat.

When compared to the air breathing group, the animals in the smoke-filled chamber were less active when in their cages, preferring to sit quietly, but they resisted handling by struggling considerably more than the control animals.

Clearance curves for control and smoke-exposed groups (Figs. 4, 5) were arrived at by converting the raw data (counts per minute) into percent of initial value and then averaging the values of all animals in each group. Cumulative activity excreted in the feces (Fig. 6) is shown for both groups and is in terms of percent of total activity excreted during the data collection period.

The thoracic clearance curves appear linear (on a semi-log plot) after about 40 hours. This linear portion has a half-life of about 285 hours, with no significant difference between the two groups. Extrapolating this linear curve toward zero time and subtracting it from the original clearance curve produces a so-called "short-term" clearance curve. For rats, this curve is usually also linear on a semi-log plot and is often assumed to represent mucociliary clearance of particles deposited on the ciliated portions of the respiratory tract. The control group's short-term curve is linear over its entire range and has a half-life of 6½ hours. The smoke-exposed group's short-term curve does not appear linear until 12 hours after the animals were removed from the smoke chamber. The linear portion of this curve has a half-life of 6 hours which is not significantly different from the control value.

The time at which cumulative fecal excretion of ^{110m}Ag reached 50% of the total excreted was 8 hours for the control group and 21 hours for the smoke-exposed group. The difference, 12 hours, is almost identical to the period of time during which thoracic clearance was depressed in the smoke group. This lag

in excretion was not due to fecal retention by the smoke group since both groups produced fecal pellets at the same rate throughout the experimental period. It is therefore concluded that the brief exposure to cigarette smoke blocked movement of silver from the respiratory tract to the gastrointestinal tract, and that this block was effective for 12 hours after the smoke exposure terminated.

V. Conclusion

The status of deposition and clearance phenomena is an important consideration in inhalation toxicology. The techniques of aerosol challenge have been recently developed to a sufficient degree that routine testing of deposition and clearance of inhaled particles is now feasible in the toxicology laboratory.

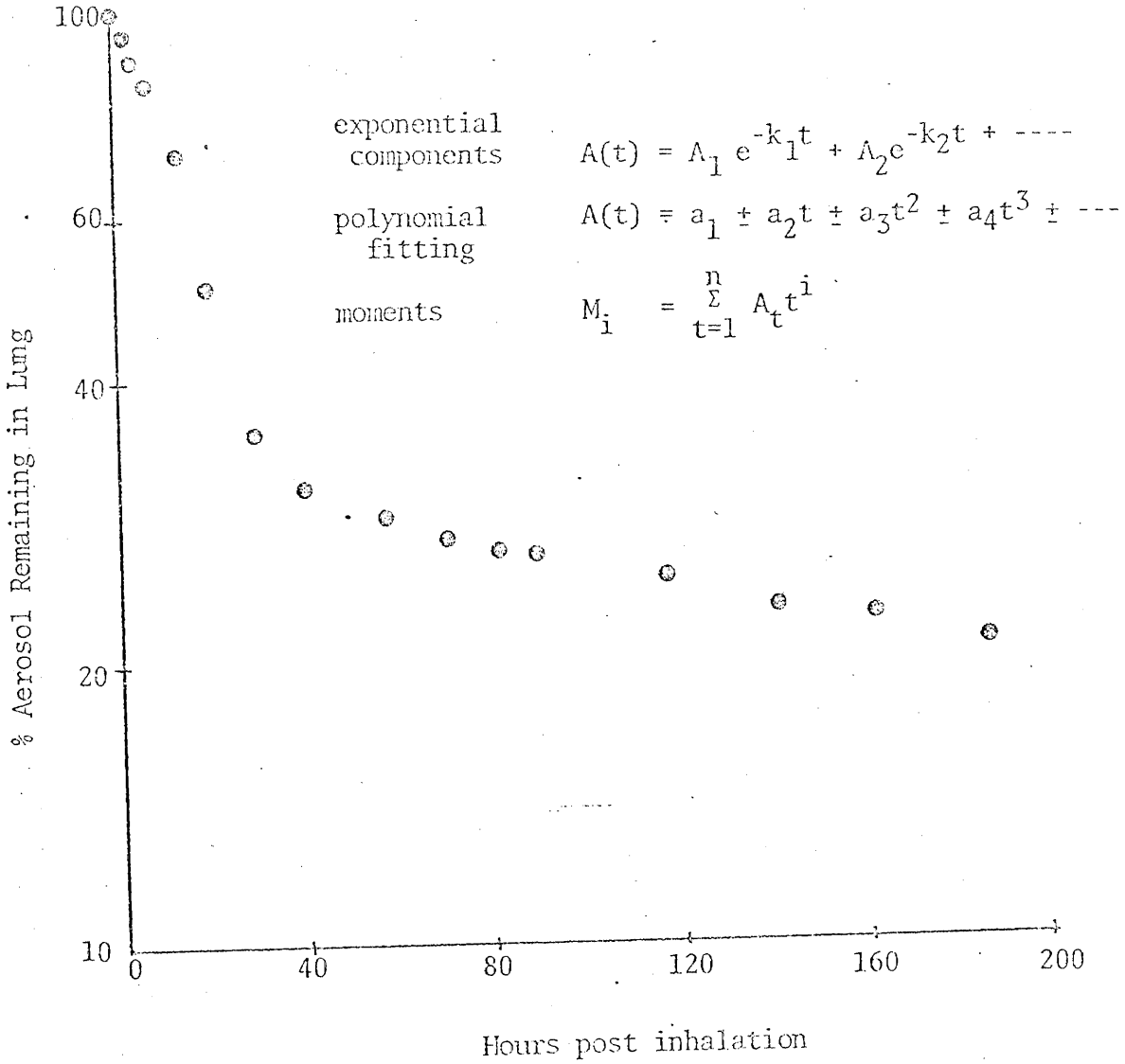
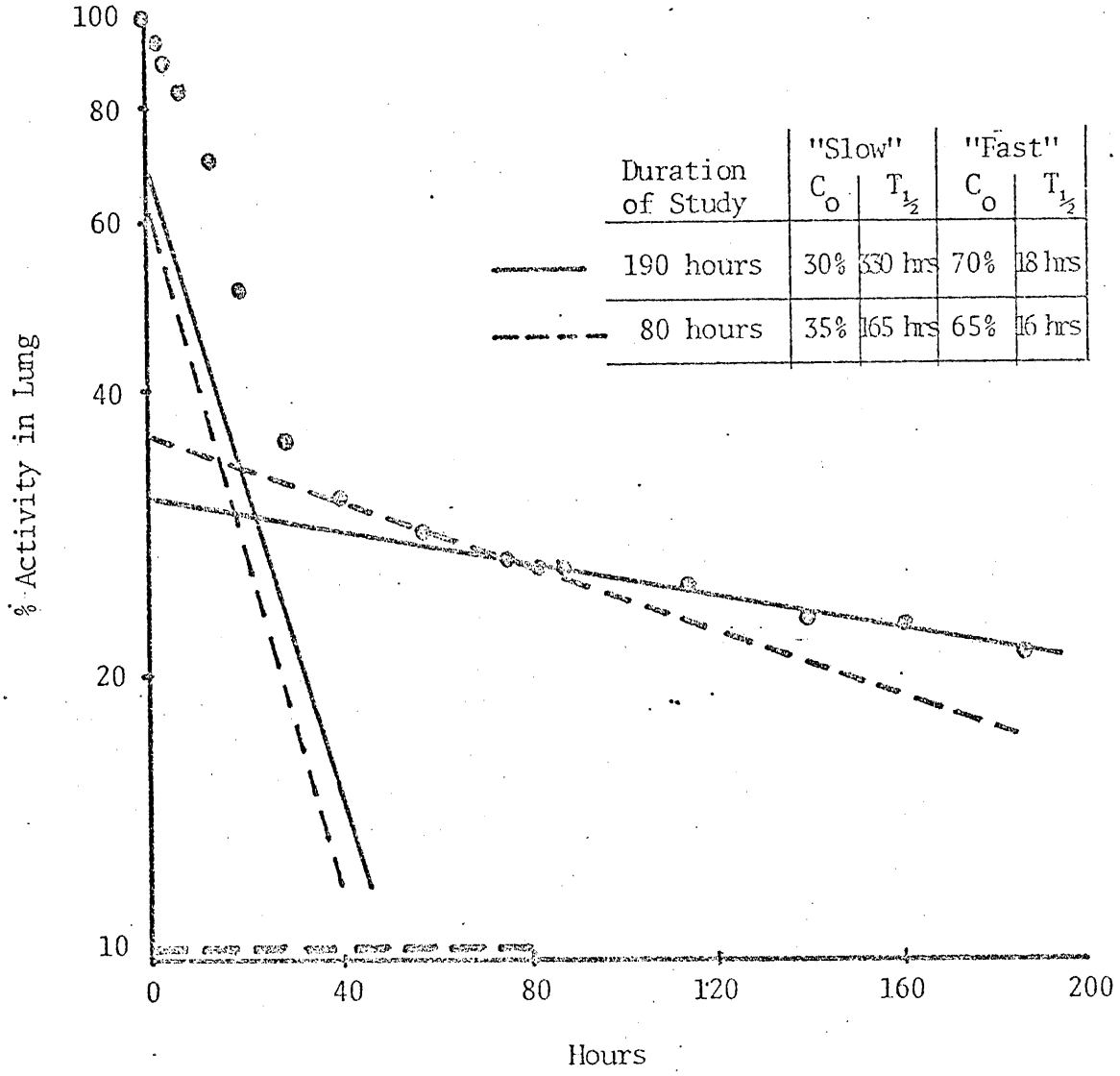


Figure 1. Three methods of reducing a clearance curve to quantitate parameters that can be used in statistical testing. The method that gives fewer parameters for testing would in general be more sensitive for detecting differences between groups.



Duration of Study	"Slow" C_o	"Slow" $T_{1/2}$	"Fast" C_o	"Fast" $T_{1/2}$
190 hours	30%	330 hrs	70%	18 hrs
80 hours	35%	165 hrs	65%	16 hrs

Figure 2. Demonstration that the duration of a clearance study has an effect on the exponential components of a clearance curve. Had this study been stopped at 80 hours different component curves would have resulted.

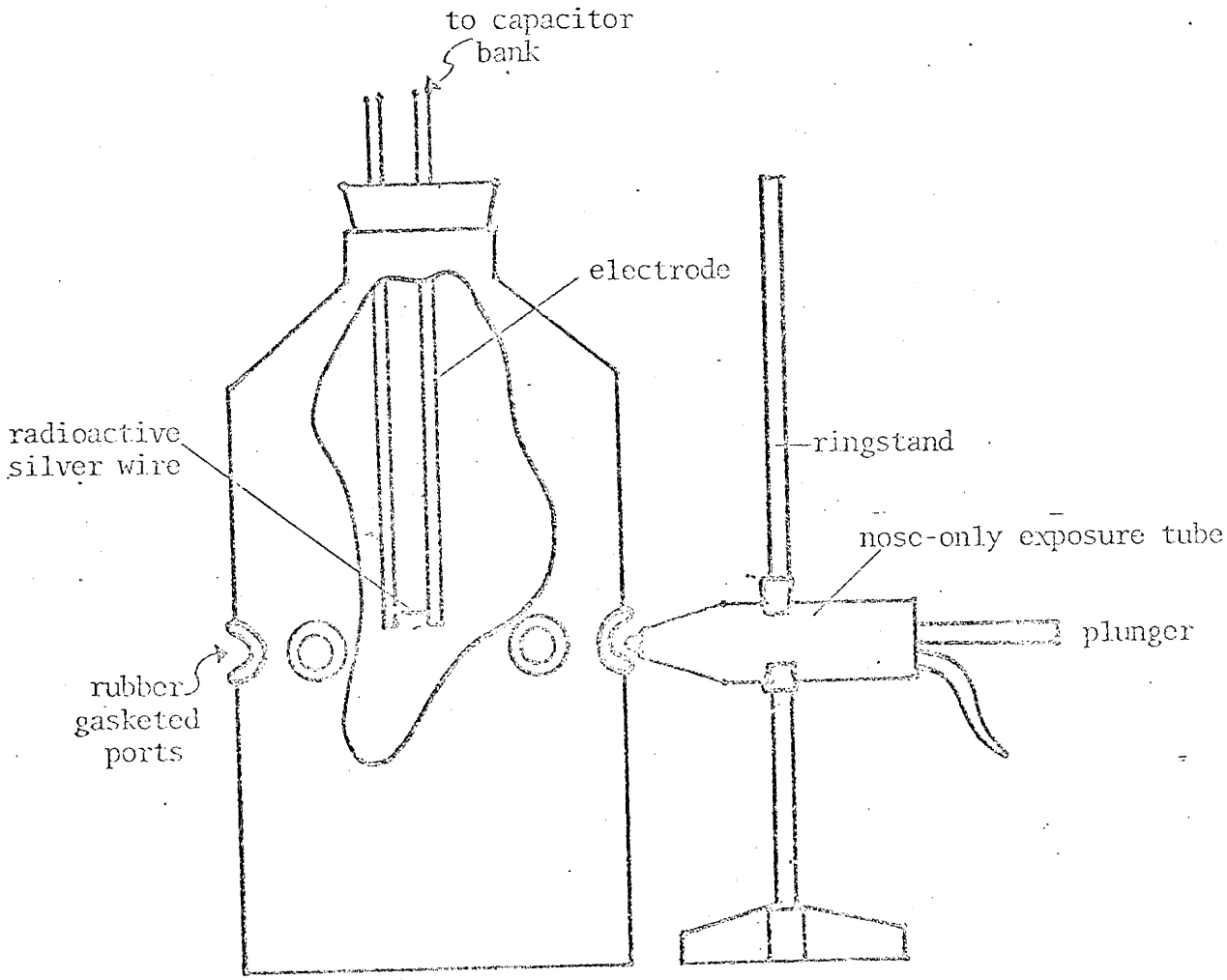


Figure 3. Aerosol exposure set-up for simultaneous nose-only exposure of 8 rats to radioactive silver particles

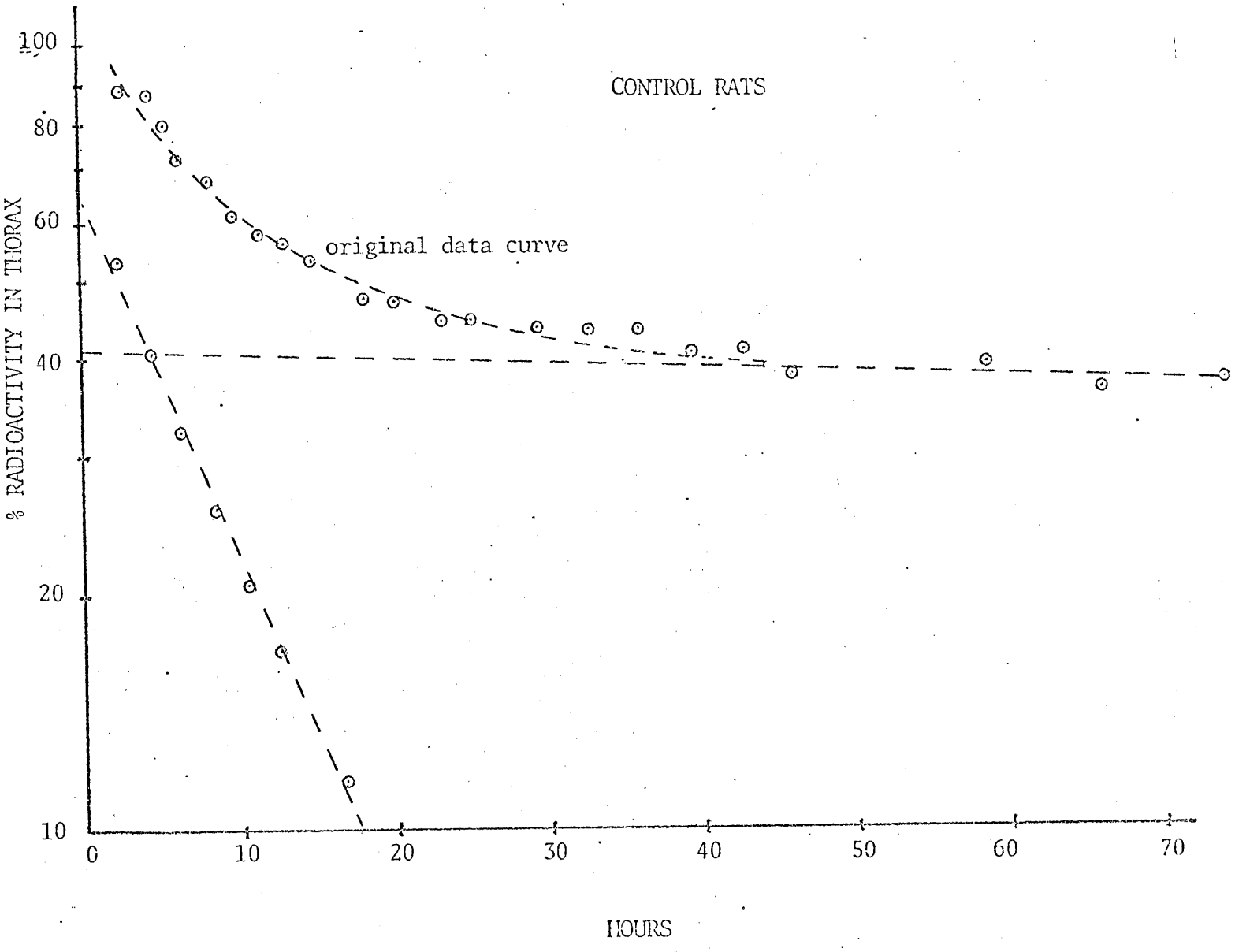


Figure 4. Thoracic clearance curves for control rats. Exponential components, "short" and "long" term, are shown.

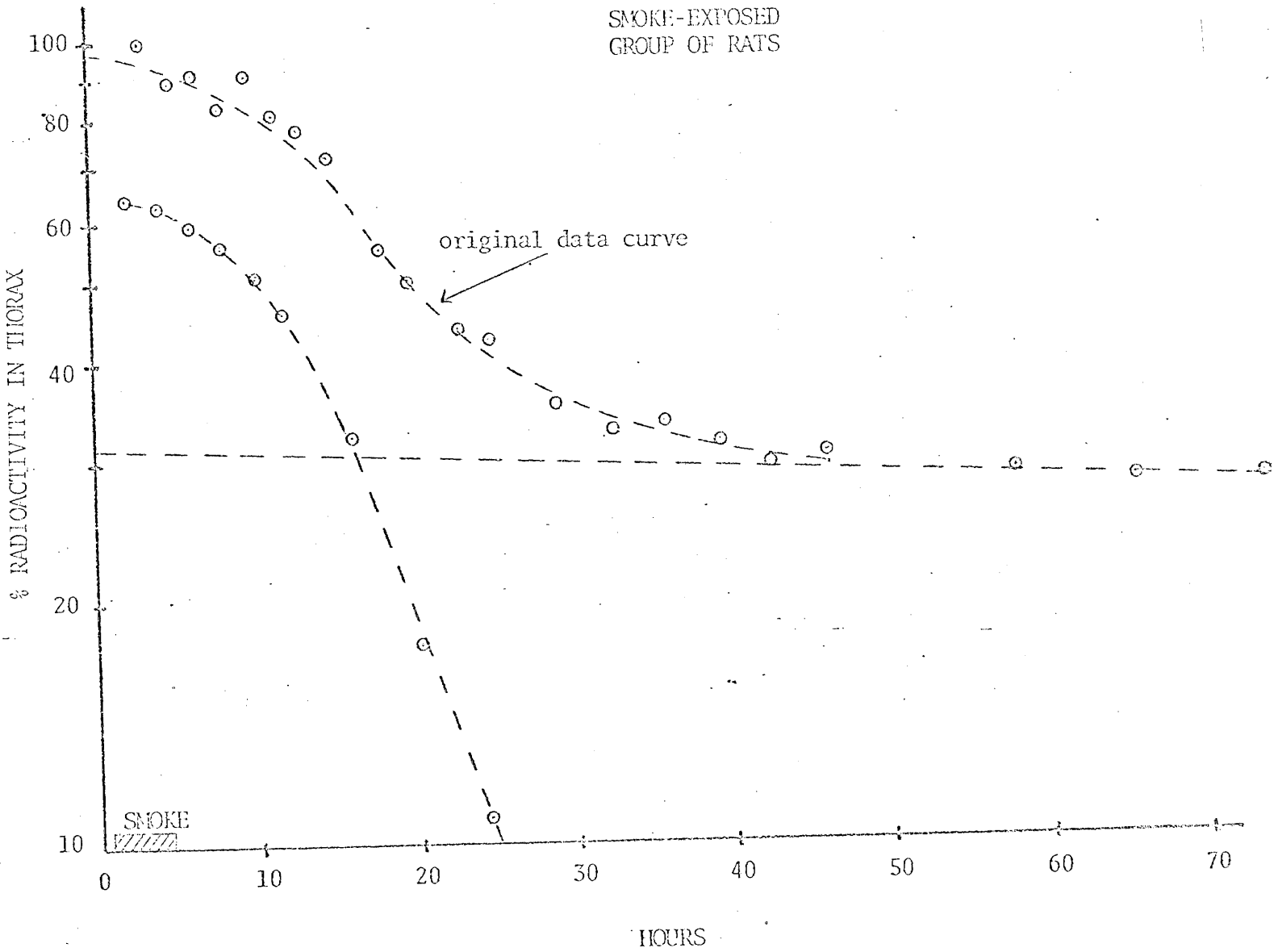


Figure 5. Thoracic clearance curves for rats exposed to cigarette smoke. Exponential components, "short" and "long" term, are shown.

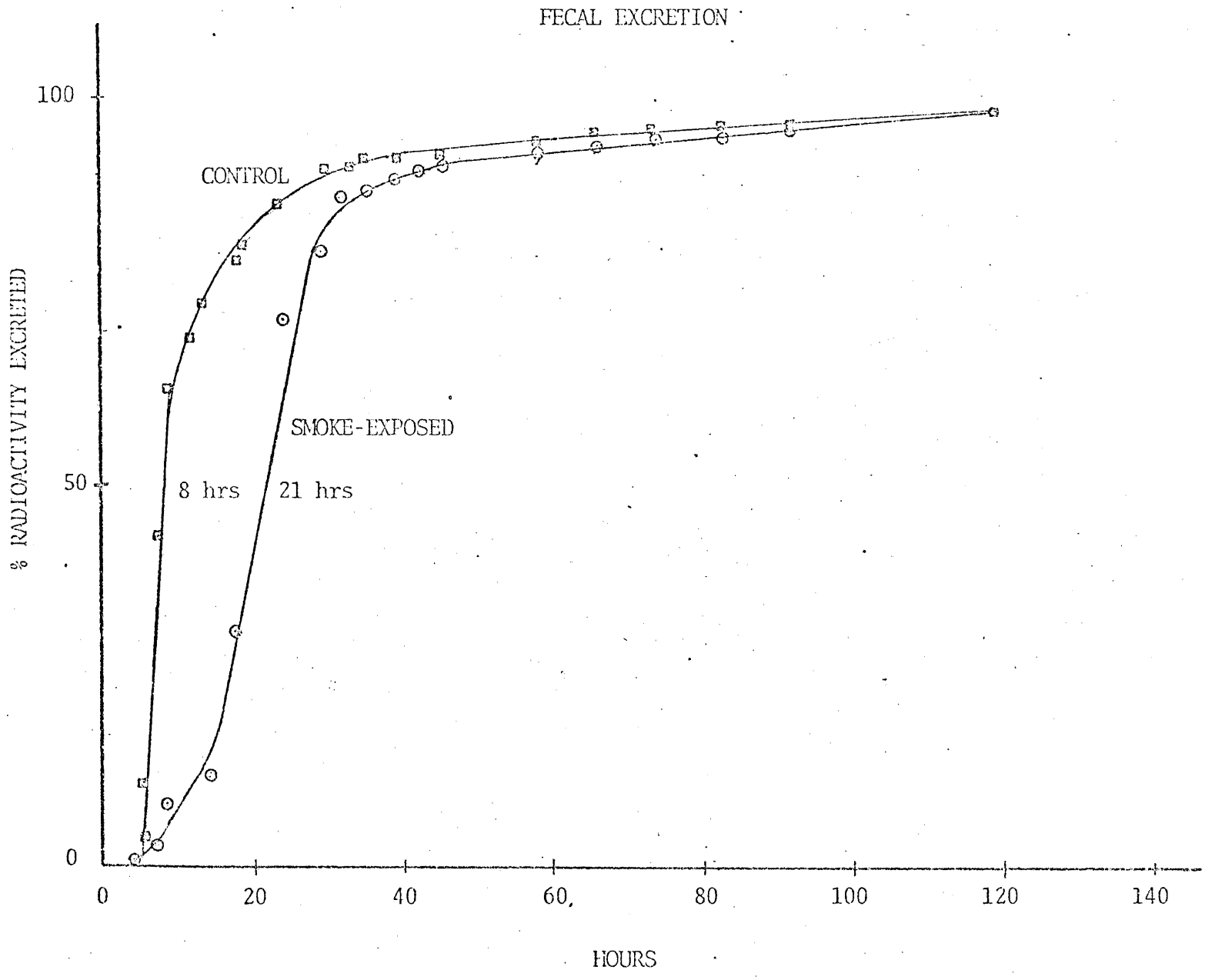


Figure 6. Cumulative fecal excretion of inhaled radioactivity for control and cigarette smoke-exposed rats. Half of the activity was excreted in the first 8 hours by the control group and in the first 21 hours by the smoke-exposed group.

Acknowledgments

Dr. L. L. Skolil of the Physics Department, San Diego State University, encouraged and aided the original experimental work reported in Section VI.

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Morphological Methods for Evaluation of Pulmonary Toxicity in Animals

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Running head--Morphological Evaluation of Lung

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INTRODUCTION

The mammalian respiratory system has a variety of important functions in addition to the primary one of gaseous exchange (1,2). The corresponding diversity of structural components of the respiratory tract, compounded by the inhomogeneity of morphologic responses of the lung to damaging agents, necessitates extremely careful selection and implementation of the several morphological methods required for its examination. Methods must be sensitive enough to reveal the presence and nature of subtle effects, and also provide information on which useful hypotheses of pathogenesis can be based.

This review is designed to present the important considerations in the choice of methods and is a guide to references describing them in more detail. It is not intended to be a detailed critique of methods or a complete laboratory protocol. The majority of the review will deal with the routine necessary for the satisfactory search for and documentation of toxic effects. Emphasis will be on the *sine qua non* for detecting subtle effects, which provide the most discriminating information relevant to pulmonary toxicity. The remainder of the review briefly addresses special methods for investigating various aspects of the pathogenesis of pulmonary lesions likely to be encountered and which are necessary for furthering the understanding of pulmonary pathobiology.

ROUTINE EVALUATION

Gross Examination

The methods to be described in this and subsequent sections are post-mortem procedures, although most are applicable to surgical specimens. Radiographic studies, therefore, will not be discussed. They can provide indications of gross and subgross morphologic changes *in vivo*, however, and are particularly pertinent to chronic studies involving the larger species of experimental animals.

TRACHEOBRONCHIAL TREE AND PARENCHYMA The animal is deeply anesthetized by sodium pentobarbital and killed by exsanguination. The trachea and lungs are carefully exposed after the diaphragm is punctured, and search is made for abnormalities of the pleural cavity and its parietal and visceral surfaces (e.g., excessive fluid, adhesions). The trachea is transected 3-5 rings distal to the larynx, and the distal portion with attached lungs and other thoracic viscera removed. The surfaces of the trachea and lungs are examined for signs of abnormalities (e.g., indications of edema, hemorrhage, consolidation, emphysema, scarring, possible tumor nodules). These can be documented photographically or schematically in outline drawings. The partially collapsed state of the normal regions of the excised lung results in exaggerated appearance of the abnormalities and enables detection of small lesions that sometimes cannot be discerned in the inflated state. The extent to which the major airways and pulmonary parenchyma need be opened depends on the amount of gross damage. If there is no sign of edema or an exudative lesion, the examination of airways and parenchyma is left until after fixation. Even where major airways are opened, samples of lungs should be retained for perfusing fixation by the airways. The weight and fluid-displacement volume of the lungs can be obtained after tying off the major vessels and dissecting away the heart and mediastinum, if the degree and nature of the abnormalities observed indicates these would be useful quantitative paremeters. The volume of fresh unfixed lungs is better measured from radiographs, however, as recommended by Dunnill et al. (3).

NASOPHARYNX AND LARYNX These structures should be surveyed for damage and the need for more extensive examination determined. In laboratory rodents, the nasal sinuses and turbinates can be examined by removing the overlying nasal bone with forceps or by sagittal section. In larger animals such as the dog, a sagittal section is made. Excepting in cases of tumors or severe upper respiratory

irritation by inhaled materials, microscopic methods are usually necessary for detection of changes in these regions.

Fixation

CHOICE OF FIXATIVE AND METHOD OF FIXATION Criteria for suitable fixation are:

a) production of least artifact

b) reproducibility

c) simplicity and cost

a) The major aim with respect to production of least artifact is to retain as close as possible the in vivo appearance of the lung immediately preceding death. With pulmonary tissue, in addition to the usual fixation artifacts which have to be considered (e.g., shrinkage, mechanical distortion, changes in cellular organelles) there is the need to prepare pulmonary parenchyma for microscopic examination such that the correct configurations and relationships of airspaces are retained. Fixation by immersing small pieces of lung in various fluids is a common routine procedure. With the exception of severe exudative processes or where there are solid lesions such as tumors, however, immersion-filled lungs do not provide proper definition of either normal or abnormal components. The preferred method of distending the lungs with perfusion of fixative through the airways eliminates these disadvantages by returning the lung to a state similar to that in vivo.

The work of Heard and colleagues (4,5) is the basis for most of the methods of perfusion via the airways used today. After the lungs have been examined grossly, they are inflated with fixative via the trachea at 30 cm of fluid pressure measured from the surface of the fixative bath in which the lungs are immersed. We have used pumps to provide the necessary height of fixative in the reservoir for large animals (e.g., horses) but have found the marriott bottle to be the most suitable device for lungs from animals the size of dogs or monkeys down to

mice. We routinely use 30 cm of water pressure since this is clearly on the plateau of the pressure-volume curve for all of these species and does not result in tearing or rupture of any tissues. Fixation of dog lungs at 25 cm of water pressure has resulted in incompletely filled or distended alveoli. This is characterized by folds in the interalveolar septum which at total lung capacity should be straight. Specimens prepared at pressures which result in incomplete distension of the alveoli and airways are not suitable for morphometric analysis using stereological procedures, and are less suitable for scanning electron microscopy due to local variations in the degree of distension and therefore interrelationships of the component parts. The airway perfusion method can be applied equally well to one lung or, as is sometimes necessary in large animals, to one lobe or bronchopulmonary segment. A more extensive discussion of general methods of fixation can be found in the report by Dunnill et al. (3).

The perfusion method of fixation by the airways not only maintains the dimensions and configurations of the tissues at total lung capacity, but also provides the large volume of fixative in intimate contact with the various surfaces which is essential to rapid fixation. The distance the fixative must diffuse for complete penetration is minimal. This method has for general studies the additional advantage of providing a relatively unobstructed view of cell surfaces for scanning electron microscopy by flushing off mucous coat and alveolar lining material. It has the disadvantage of causing some translocation of exudates and particles and providing a specific artifact of increased tissue spaces around pulmonary vessels, the so-called edema artifact.

The choice of fixative is also a major consideration in view of the large numbers of fixatives which have been used on the respiratory system. The main components of these fixatives are usually one or more aldehydes, buffer, and various salts with high purity water so that the fixative has a constant pH and

osmolality. Many investigators today use a mixture of glutaraldehyde and formaldehyde made from paraformaldehyde which results in rapid penetration and thorough fixation. Cacodylic acid is generally preferred as the buffer because it results in resilient lungs; that is, blocks of lung compressed by cutting rapidly resume their original fixed volume when placed in fresh fixative. A small amount of calcium is commonly added to the fixative to preserve phospholipids associated with pulmonary surfactant as well as those which are components of the various cell membranes. Although iso-osmotic fixatives are used, we prefer a hypertonic fixative (approximately 550 milliosmoles). [All of the above desirable characteristics are achieved using a modification of Karnovsky's formaldehyde/glutaraldehyde fixative with added calcium chloride ((paraformaldehyde-40 g/liter; glutaraldehyde 100 ml of 50% solution/liter; calcium chloride--0.5 g/liter cacodylic acid--12.8 g/liter)) which is diluted 1 to 4.5 before use with cacodylic acid (32 g/liter) and the pH adjusted to 7.2 with 1.0 N HCl (6).] The fixative is relatively simple to prepare and can be stored in the refrigerator for several months. It has the advantage of being a good room temperature storage fluid for fixed tissues. Using this fixative at 30 cm of pressure, fixation is rapid and complete. Fixation times of 2 and 4 hours are acceptable, but we prefer to maintain the 30 cm of pressure overnight or for 18 hours. Samples cut from these lungs are placed in fresh room temperature fixative where they may be stored without damage or deterioration for more than one year.

b) Fixation of lungs at a standard pressure of 30 cm of the fluid provides the most reproducible appearance for general purposes. Considerations of reproducibility and least artifact become more critical relative to morphometry. Here again, for purposes of pathology we find perfusion of the excised lung to be the method of choice. The alternative approach used for morphometry of normal lungs is perfusion via the trachea with the lungs in situ within the thoracic cavity (7).

c) Perfusion of excised lungs by trachea or major bronchus is a relatively simple procedure for rodents, once a series of delivery tubes leading from marriott bottle reservoirs is provided. Larger reservoirs are needed for lungs of larger species. Although the perfusion method cannot be performed as rapidly as immersion of samples in fixative, the greater effectiveness in enabling detection and evaluation of subtle or mild lesions more than outweighs the greater cost in time taken. Where large numbers of animals per treatment group are involved, at least a significant proportion of lungs should be fixed by airway perfusion.

FIXATION OF LUNG BY PERFUSION THROUGH AIRWAYS As will be evident from the foregoing discussion, our preferred routine method of fixation is perfusion by the airways with modified Karnovsky's fixative at 30 cm of fluid pressure (6). We find that after partial collapse of the lungs has occurred on excision, no degassing is necessary to obtain complete distribution of the perfusate. Degassing is in fact contraindicated for most purposes because it increases the cumbersomeness of the technique, lessens the degree of reproducibility of reinflation and makes redistribution of components of any lesion more likely.

FIXATION OF LUNG BY IMMERSION Massively consolidated or edematous parenchyma, or large solid lesions such as tumors, have to be fixed by immersion in fixative fluid. For subsequent study by light microscopy Zenker-formol is preferable to formalin because it heightens the contrast of hematoxylin and eosin staining, especially the eosinophilia of proteinaceous transudates or exudates. The shrinkage caused by immersion in fixative is used to advantage in enumeration of tumor nodules in lungs of strain A mice which is the basis of a carcinogenesis bioassay system (8). Tissue to be examined by electron microscopy is immersed in the modified Karnovsky's fixative described previously.

Immersion fixation is also used when the redistribution of intraluminal particles, cells or exudates might interfere with the objectives of the study, as in determining the fate of inhaled particles (9,10).

FIXATION OF NASOPHARYNX AND LARYNX After gross examination, these structures in small animals (i.e., rodents) can be fixed in toto in the modified Karnovsky's fixative after flushing surfaces with fixative to remove trapped air bubbles and mucous coat. Samples of tissues from recognized lesions and representative portions of the nasoturbinate region, pharynx and larynx need to be dissected out in large animals.

Sampling for Microscopic Examination

The size and diversity of components of the respiratory tract pose a considerable sampling problem in the thorough search for lesions. This is compounded by the inhomogeneity of morphologic responses of the tract to irritants as was mentioned in the introduction. These two features together require sampling be both wide in distribution and specific in anatomic localization. The number of large blocks taken for examination by light microscopy and scanning electron microscopy will be determined by the compromise between thoroughness and the practical limit in terms of cost of preparation and examination. But there is a minimum below which the risk of spurious conclusions due to serious sampling errors becomes unacceptable. Any sampling of parenchyma must take into account vertical (gravitational) gradients affecting the distribution patterns of certain lesions and the difference between hilar and peripheral regions of lobes.

TRACHEOBRONCHIAL TREE AND PARENCHYMA The sampling procedure varies according to the size of the lung. In the case of rodents such as rats and hamsters, sampling of the trachea presents few problems other than to be aware of possible differences in the mucosa over the cartilagenous and intercartilaginous membrane portions of the trachea as has been found in the rat (11). A block containing

a longitudinal section of distal trachea and the bifurcation into bronchi suffices for nonparenchymal regions. The preferred planes of section for rodents' lungs are illustrated in Fig. 1. These are vertical sections in the sagittal plane for the left lung and from the hilus along the axis of major airways for the cranial, middle and caudal lobes of the right lung. All of these blocks can be sectioned whole for histologic examination. Although the sagittal section of the left lung is a common section for major attention, we prefer the sections from the right middle and caudal lobes. One reason is that unless the section of the left lung is cut very close to the mid-line, most of the airways are cut transversely. The longitudinal sections of airways present in blocks from the right middle and caudal lobes reveal bronchial and acinar orientations of lesions much more readily, especially by scanning electron microscopy (SEM). A second reason is that the blocks from the right lung are a more convenient size to mount whole for light microscopy.

More care in sampling is required for lungs of larger animals such as dogs and monkeys because of the bulk of tissue to be surveyed and the increased likelihood of regional variations in response being manifested. To minimize sampling errors, standard parenchymal sampling sites covering both dorsoventral and hilar-peripheral axes should be chosen. The 9 sampling sites we take from parenchyma of the 4 lobes of the right lung of the dog are illustrated in Fig. 2. Because we frequently use one lung of dogs and monkeys for biochemical studies or those requiring special fixation, such as freezing, we derive most morphologic information on the basis of one lung. If the two lungs are available, samples can be taken from both. Samples of major airways typically consist of proximal trachea, bifurcation of trachea, and lobar bronchus.

Evaluation of pulmonary toxicity invariably involves the comparison of lungs from two or more groups of animals. For this specific comparison, by qualitative

or quantitative (morphometric) means, we use the same sampling sites for tissue blocks in all animals (12) rather than the method of stratified random sampling using a random number table together with a numbered sampling grid (13). The latter method relates to statistical confidence with which the sample represents the lung from which the sample is taken rather than comparison among lungs where the lesion can be affected by specific anatomic location.

When detailed comparisons are required within or among groups of animals, a useful approach is to select a specific bronchopulmonary segment of the lung for more specific study. Sections of segmental bronchus, terminal bronchiole, and more distal lobular tissue can be sliced out of the desired bronchopulmonary segment under a dissecting microscope. These sections, as well as those of trachea and lobar bronchus, can then be closely compared.

Any gross lesions not represented in the samples described previously must also be selected.

NASOPHARYNX AND LARYNX Blocks are taken representing proximal and distal regions of nasal sinuses and turbinates, and the pharynx and larynx. Again, more are required for larger animals. For some studies it is desirable to dissect mucosa from the nasal septum or turbinates and prepare it as a whole mount for morphological examination. Further details of the use of whole mounts and sections of nasal regions can be found elsewhere (14,15).

Microscopic Examination

The need for examination of a wide sampling of pulmonary tissue has already been stressed. Requirements for cost effectiveness in the evaluation of lungs from large numbers of animals in toxicity trials means that the microscopic methods most useful are those that provide for examination of large samples, that is light microscopy (LM) and scanning electron microscopy (SEM). For initial detection and analysis of lesions we use correlated LM and SEM. The best

way to do this generally is to take complementary blocks of tissue from the same sampling site, embed one in plastic suitable for large 1μ sections and process the other for SEM. The surface and sectioned views can then be compared for interpretation. The advantage of the large 1μ section is that it not only provides the best resolution for LM, but also enables precise selection of anatomic locations for thin sections to be examined by transmission electron microscopy (TEM).

This is a discussion of routine microscopic methods and we recognize that the word can take on shades of difference in meaning according to the objectives of the investigations. Often, most microscopic screening is by LM alone because of the bulk of specimens. Equally so, it must be realized that in the search for subtle effects or in the description of damage once it is found, at least a significant number of lungs from animals in the critical experimental groups should be examined by correlated LM, SEM and TEM.

LIGHT MICROSCOPY (LM) Survey by LM of sections carefully prepared from vacuum-embedded paraffin blocks and stained by hematoxylin and eosin provide the basis for other modes of microscopic investigation (Fig. 3). More definitive study of cellular components of lesions is made on the 1μ sections cut from large plastic-embedded blocks and these provide the essential link between LM and TEM (see segment on TEM below). The paraffin sections also provide the basis for a large variety of special staining methods (16).

SCANNING ELECTRON MICROSCOPY (SEM) The large, approximately 12x10x4 mm samples of tissue selected for SEM are the complementary halves of blocks used for LM and are cut so as to include longitudinal sections of airways in the surface to be examined (Figs. 4-6). The tissue blocks are dehydrated in graded ethanol and then dried by the critical point procedure using CO_2 (6,17). The dried tissue is attached to standard SEM stubs put in a high vacuum coating device on a tilting

and rotating stage and coated first with carbon then with gold palladium (18). Such tissues can be stored in a dessicator for prolonged periods and still be useful for SEM.

Although not a routine procedure, to enable precise correlation between surface features seen by SEM and cross sectional features of selected areas, blocks can be removed from the SEM stub after evaluation and prepared for LM and TEM. They are placed in 100% ethanol, which is then substituted by propylene oxide, and are then embedded in an Epon-Araldite mixture. The tissue is examined by LM of 1μ sections and specific regions can be selected for TEM (18). Information on interior aspects of tissues and cells can be obtained by SEM after the tissue has been fractured either before (19) or after (20) drying. It can also be obtained from plastic-embedded tissue after iodine and acetone surface etching (21)

TRANSMISSION ELECTRON MICROSCOPY Because lesions in the lung are frequently focal and have a specific orientation relative to the acinar structure of the pulmonary parenchyma it is essential to know precisely the anatomic location in the small airways or acinus from which the TEM blocks are taken. This precise location can be learned by several routes. The oldest is a modification of the procedure used by Grimley et al. (22) wherein large, 2x2 cm blocks of tissue are embedded as for TEM and alternate 30μ and 10μ sections cut on a large microtome commonly used for metal or bone. The 10μ sections are evaluated using light microscopy and the precise lesion area is dissected from the adjacent 30μ section, cemented on a block from a beam capsule and ultrathin sections cut (23). It has the disadvantage of relatively low resolution for LM due to the thickness of the section. This can be avoided by embedding slightly smaller sections (i.e., 12x10 mm) and cutting 1μ sections on a Sorval JB-4 microtome using glass knives. These thin sections can be stained using various dyes and provide high resolution for evaluation of

the tissue by LM (Figs. 7A, B). The areas of interest are selected in the one-micron section, identified in the block, and the surrounding tissue removed leaving a plastic mesa containing the required region (24). This mesa is sectioned in the usual manner and examined by TEM (Fig. 7C).

SPECIAL METHODS FOR GROSS AND SUBGROSS EVALUATION

Whole Lung Sections

The technique of preparing whole sections from human lungs was first described by Gough and Wentworth (25) and was used in their studies of emphysema in man. The sections can be useful as permanent records or illustrations of whole lung involvement in certain types of disease processes. Subsequent developments of the technique and their use in the measurement or grading of emphysema in human lungs is briefly discussed in a report by Dunnill et al. (3). Preparation of lung macrosections and their permanent mounting by their lamination between sheets of transparent plastic film has also been described (26,27).

Vascular Injection Technique

A technique using thin slices of lungs in which the vessels have been injected with multicolored latex, has been used in studies of the comparative subgross pulmonary anatomy of a variety of mammals (28,29). A major focus of attention in these studies was the comparative anatomy of the vascular tree. Vascular injection and casting has been used in investigations into the vascular changes accompanying emphysema in man (30).

Airway Casting

This is useful for development of mathematical models for behavior of inspired gases and particles (31) and for the study of airway disease (32) and the pathogenesis of emphysema (33).

Replica casts of airways down to and including alveoli can be prepared in situ for large and small animals (31). The method involves replacement of air by cyclic

ventilation with CO_2, filling with degassed saline and slowly injecting silicone rubber through the trachea while allowing saline to drain from the thorax via slits between ribs. After curing (2-20 hours) the organ is removed from the thorax and the tissue digested away. Morphometric measurements that may be made on such casts include branching angles and dimensions of airways and alveoli. In some cases alveolar pores can be seen and their relative sizes determined via the scanning electron microscope.

The major limitation in using airway casts is that all but the simplest measurements made on them may require considerable time, effort and skill. On the other hand, a replica cast captures and preserves the entire airway structure, allowing precise determination of the spatial and structural distribution of lesions.

SPECIAL METHODS OF MICROSCOPIC EVALUATION

A variety of investigative methods is needed in the search for pathogenetic mechanisms underlying disease processes in the lung, as it is for any organ. These methods are relevant to studies of both cellular biology and pathobiology, and unavoidably investigations into the one have considerable impact on the other. The techniques in question have for the most part either been in use for a relatively short time, or are in the process of being explored. Only an introduction to these topics will therefore be provided.

Histochemistry

Histochemistry and cytochemistry are essential for the full elucidation of the pathogenesis of toxic changes in inhomogeneous organs such as the lung because it is necessary to localize biochemical changes to the specific cells or cell populations involved.

Many specialized methods of tissue preparation and incubation are required for the broad spectrum of histochemistry. Enzyme histochemistry and histochemistry for certain cellular components is best done on cryostat sections. Like all

sections of the respiratory system, distended cryostat sections are much easier to evaluate and provide more useful information. Usually the tissue is distended with a cryostat embedding material, commonly 4% gelatin, as originally described by Tyler and Pearse (34). This embedment has the double advantage of distending the lung and also providing a medium or embedment which permits much more complete sections than can be obtained if the lung is handled like other organs or tissues. Without such embedding, only fragments of sections are obtainable. These are extremely difficult to evaluate in terms of total distal airway and parenchymal morphology. Freezing of the gelatin infiltrated section is generally accomplished using freon-22 cooled to near its freezing point of -160°C. Freezing directly in liquid nitrogen is considerably slower and frequently distorts the tissue blocks. While most cryostat sections tend to be thicker and therefore provide less resolution that paraffin sections, with appropriate equipment it is possible to serially cut frozen sections at 5 or 6 microns. Such sections are suitable for a wide variety of histochemical procedures which are commonly applied to the serial section in order to obtain correlated biochemical and morphological information at the cellular level.

Histochemical procedures have been diversified significantly in recent years (35) and include methods for many enzymes as well as cell inclusions and intercellular material. Many of these procedures can be applied at both the light and electron microscopic levels of observation of the lung (36-40) and some are suitable for automated image analysis (41). The studies of Spicer et al. (42) and Lamb and Reid (43,44) concerning toxic effects of inhaled gasses on respiratory mucopolysaccharides is especially noteworthy. Another example is the fluorescent amine technique of Falck that has been used in studies of serotonin-producing cells of neuroepithelial bodies present in respiratory mucosa (45).

A promising new area of chemical analysis that can be applied to the respiratory system is that of analyzing X-rays, cathodoluminescence or back-scattered electrons generated by the interaction of the electron beam of an SEM or TEM with the atoms of cellular components, inclusions, or histochemical final reaction products *in situ*, thus providing elemental analysis of endogenous or foreign materials in cells and tissues (46-49).

Autoradiography

Autoradiography has been used to determine the cytokinetics of pulmonary cells responding to damage caused by toxic environments, such as in the demonstrations that alveolar type 2 epithelial cells are the precursors of type 1 epithelial cells (50,51). The second major use of autoradiographic techniques is for tracing the intracellular pathways traversed by radiolabeled precursors of known or hypothesized cell products (52-54). A third use is in studying the deposition and fate of inhaled particles (55).

Morphometry

Morphometry is necessary for precise correlation of structure and function in both normal and diseased organs. It can provide accurate measurement of the severity of damage in diseased organs and it is the only means of confirming, by statistical methods, the existence of significant subtle lesions in a particular treatment group of experimental animals.

A systematic approach to a quantitative morphologic analysis of the architecture of the pulmonary system using manual methods has been provided by Dunnill (56), Weibel (57) and Thurlbeck (58). Those authors established the formulae and methods necessary to obtain statistically reliable quantitative values for the pulmonary system. Recently quantitation of the pulmonary system has been automated by use of computed pattern recognition techniques (59) and automated measuring microscopes (60). The greatest application of automation has been with

automated measuring microscopes. They have been used to quantitate selected features of conducting airways in normal and experimental bronchitis (61) and of distal airspaces in normal (62), emphysematous (63), and experimental, pollutant-damaged lung (12,64). Pattern recognition techniques have also recently been used to classify and measure the distal airways on an automated measuring microscope (65).

Freeze-fracture

Freeze-fracture is a method of looking at replicas of fractured surfaces at very high resolution using TEM. Like the SEM, it provides a view of surfaces rather than cross sections. Thus for low magnification and low resolution of natural or fractured surfaces, the SEM is the most appropriate instrument, whereas for high magnification, high resolution freeze-fracture or freeze-etch is the most appropriate technique.

Freeze-fracture procedures avoid the necessity for including chemical interactions, which may cause artifacts in the preparation of tissue, and reveal an en face view of membranous surfaces. In the pulmonary system, the method has been used for study of cell organelles, particularly during secretion and phagocytosis (66), for visualization of the alveolar lining layer (67) and in the examination of endothelial cells relative to their capability for metabolizing circulating vasoactive agents (68). The method is also necessary for the study of normal and abnormal cell junctions (69).

Tracer Techniques

These have been used in studies of the permeability of the pulmonary vasculature in both normal and edematous lungs. Horseradish peroxidase, hemoglobin, microperoxidase, ferritin and colloidal particles have been used (70-74). The investigations on pathways of clearance of inhaled iron oxide aerosols (10) or

intratracheally instilled ferritin or colloidal carbon (9) referred to earlier also involved the use of tracers.

Thick Histologic Sections

These were principally used in the study of human emphysema (75). To some extent they have been superceded by SEM, but they still have an important role in documenting the pattern of collagenous and elastic fibers in interalveolar septa and determining their abnormalities during the pathogenesis of diseases such as emphysema.

ADDITIONAL SPECIAL METHODS OF FIXATION

No one fixation procedure is appropriate for all investigative purposes. To the extent that considerations of methods of fixation are intimately related to the techniques of evaluation for which they are to be employed, common fixation techniques have already been discussed. There remain several, however, that have special indications to be matched with the specific aims of the investigator.

Vapor Fixation

Methods have been developed for the use of formalin vapor (76,77) or formalin steam (7) but have little to offer in the way of advantages and nothing at all in convenience. Air fixation likewise has no usefulness other than to provide a convenient gross anatomical reference. A recent method of vapor fixation using osmium tetroxide suspended in cooled fluorocarbon has been briefly reported by Kilburn and McKenzie (79). The mixture was injected intratracheally into breathing hamsters to fix the lungs while inflated and to lessen the chance of translocation of cells and particles on luminal surfaces of airways.

Vascular Perfusion

The primary use of this method has been in the demonstration of extracellular lining layers of alveoli and bronchioles by electron microscopy (80,81). Translocation of cells and particles should be less than by intratracheal perfusion,

which may make this method useful for localization of these components.

Rapid freeze method

This method was developed by Staub and Storey (82). It provides an accurate representation of the morphologic state of the lung "frozen" at a point in time in its cycle of dynamic events. The animal's lungs are frozen while it is alive, at the desired phase of the respiratory cycle. The procedure does require thoracotomy with good exposure of the lungs. Carefully controlled ventilation is required to maintain physiological state with the ability to momentarily hold the lung at the desired degree of inflation or vascular perfusion. Freon 22 cooled to near its freezing point of -160°C or propane cooled to -175°C is used as the cryogenic agent for rapid freezing as each of them absorbs significantly more heat per unit volume of weight than liquid nitrogen which rapidly absorbs heat then boils, forming an air interface which effectively reduces the transmission of additional heat from the specimen to the cryogenic agent. Only the first few millimeters of tissue under the pleura are extremely rapidly frozen; deeper tissue is frozen considerably more slowly. Tissues frozen in this manner may be freeze-dried or freeze-substituted for subsequent critical point drying followed by evaluation in the scanning electron microscope (6) or followed by embedding in paraffin or plastic for light microscopy (23) or TEM. In the SEM, the general architecture of the pulmonary tissues are well preserved and available for evaluation, but the surface detail of the cells is obscured by the mucous coat or alveolar lining layer in the airways or alveoli respectively.

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LEGENDS FOR FIGURES

Fig. 1--Schematic outline of the dorsal view of a rat's lung illustrating the vertical planes of section for sampling tissue. The contour of the accessory lobe is indicated by the narrow broken line. LL--left lung; RCr--right cranial lobe; RM--right middle lobe; RCa--right caudal lobe.

Fig. 2--Schematic outline of the lateral view of the four lobes of a dog's right lung illustrating nine sampling sites.

Fig. 3--Light microscopy of properly prepared paraffin sections provides a relatively simple and rapid means of screening all levels of the tracheobronchial tree and parenchyma. Normal rat lung, H&E stain, 12x.

Fig. 4--SEM enables evaluation of surfaces and is intermediate in resolution between LM and TEM. It allows the examination of relatively large areas and a great depth of field as illustrated in this micrograph of normal rat lung. Tb--Terminal bronchiole; Ad--Alveolar duct, 30x.

Fig. 5A--The transition region from terminal bronchiole to alveolar duct is an area frequently damaged by inhaled irritants. Occasional macrophages (arrow) can be observed within proximal alveoli of this alveolar duct from a normal rat, 170x.

B--Compared to the normal, in the rat following exposure to ozone (0.8 ppm for 7 days) the terminal bronchiole has a flattened surface appearance (Tb) and proximal alveoli contain clusters of infiltrating inflammatory cells and debris (arrows), 160x.

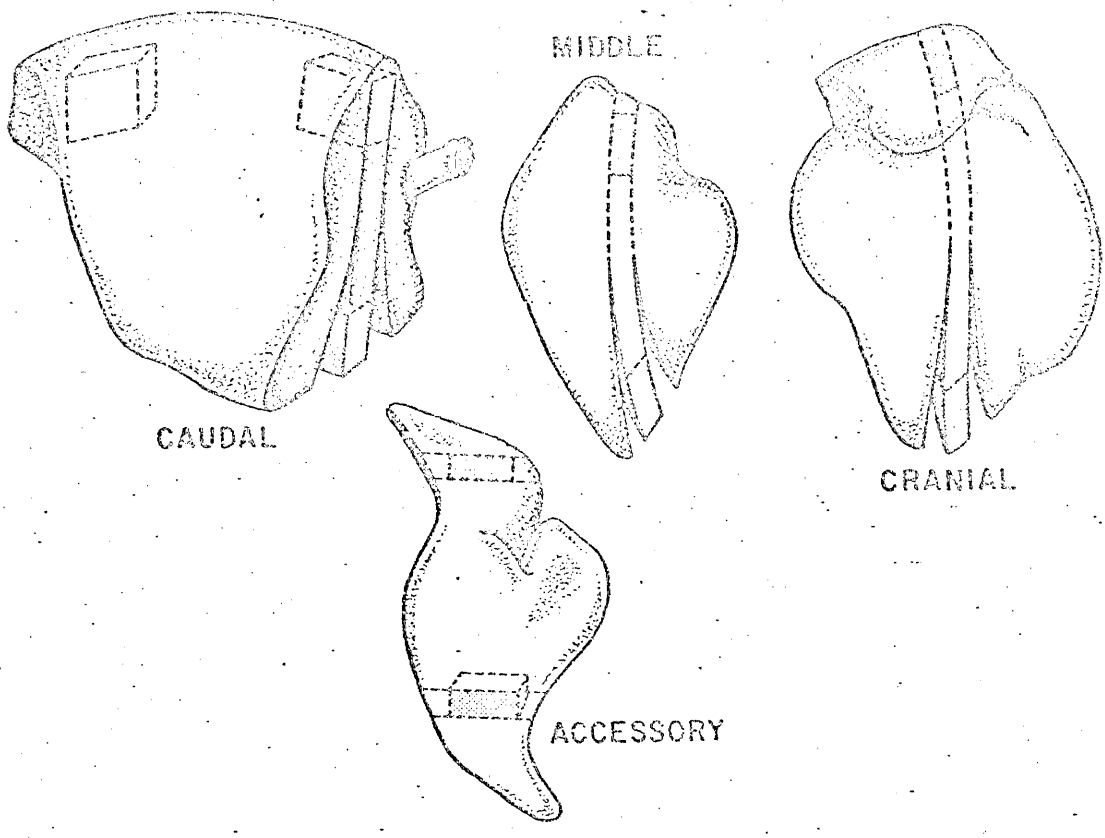
Fig. 6A--Highly magnified SEM view of normal terminal bronchiolar epithelium in the rat, 2800x.

B--In contrast, note the loss of surface projections of nonciliated bronchiolar (Clara) cells and the shortening and reduced density of cilia in a rat exposed to 0.8 ppm ozone for 7 days, 2300x.

Fig. 7A--One micron section from plastic-embedded lung of a rat exposed to 0.8 ppm ozone for 2 days provides good resolution of cellular detail by LM and localization of specific region of interest (circled). Richardson trichrome, 111x.

B--Higher magnification of area circled in A, 500x.

C--Using the mesa technique, a portion of the lesion, such as circled in 7B, can be selected and thin sections from the same region of the block examined by TEM. Uranyl acetate and lead citrate, 3750x.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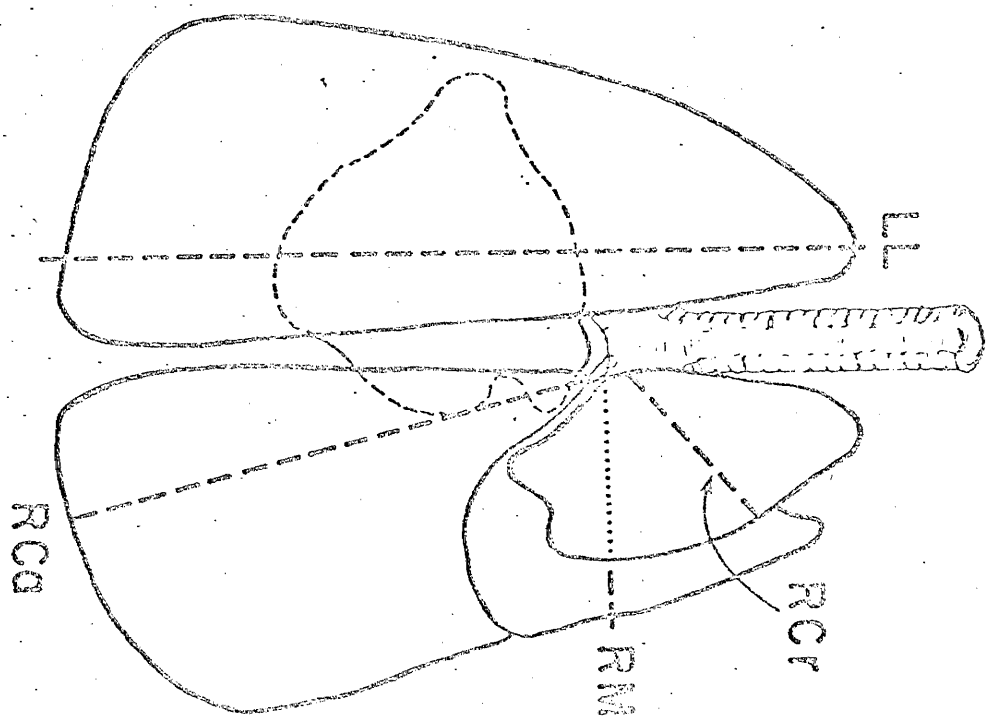
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