FINAL REPORT

ARB CONTRACT NO. A6-218-30

CORRELATIVE AND SENSITIVE DISCRIMINANTS FOR AIR QUALITY CONTROL

Period: July 26, 1977 - October 31, 1978

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1. ABSTRACT

This is the final report of a first year contract: July 26, 1977 to July 25, 1978, with a three-month extension to and including October of 1978. This project is the first quantitative measurement of the influence of an ambient level of NO_2 exposure (intermittent 0.34 ppm for six weeks) on the alveolar cell populations of the mouse lung. In one of four major studies, the lungs of 120 Swiss-Webster male mice, equally divided into control and exposed groups, were quantitated by computer assisted image analysis for eight variables which include numbers of Type 2 cells, areas of Type 2 cells, alveolar wall areas, internal surface area of the lung, and linear intercepts. The main finding was an increase in Type 2 cells which was statistically significant ($.01 < p < .025$) by Chi Square upper quartile analysis. In addition, a two-factor analysis of variance with slide as the repeated measure (an analysis of specific lung areas) showed significant differences between the control and exposed groups of animals, with the numbers of Type 2 cells in the lungs of exposed animals apparently changing from the patchy variation of the control group to a putative widespread hyperplasia. Similar trends were found in the earlier study. Other data collation studies are in progress.

One major part of the study, a molecular probe quantitation for protein leakage in the lung, has provided data from three independent experiments that show significant increases in the amount of horseradish peroxidase (HRP) in the lungs of mice exposed to ambient levels of NO_2. Of six test periods, five showed an increase in lung HRP for the exposed animals. Three of the five periods were statistically significant by analysis of all data, and all five were significant by upper quartile statistical testing. Ultrastructural studies established HRP retention and distribution in the lung 5½ hours after injection; to date, differences

between groups are not clear cut.

Data was also obtained in peripheral areas: 1) a suggestive correlation between an increase in urinary protein excretion of students and periods of high NO_2 ambient air exposure, 2) a statistically significant decrease in spleen weights and lymphoid nodules of the spleen in mice exposed to intermittent 0.34 ppm NO_2; and 3) the finding of a destructive human lung disease related to the inhalation of silicate dust and presumably an as yet unidentified air pollutant.

The image analysis and HRP tests point to a noxious effect on the lung by an ambient level of NO_2. Further studies are needed to confirm the findings, achieve greater levels of sensitivity and statistical significance, and expand the data with particular attention to the degree of reversibility. The ultimate goal is a census of the alveolar cell populations and their unit structure, the alveoli. In effect, a census will define the status of the cellular reserves of the alveoli, and will measure the influence of air pollutants on the rate of depletion of both the alveolar cell populations and the alveolar units themselves. A reduction in lung reserve that may be caused or exacerbated by air pollutants is a major concern for air quality control. Covert lung destruction and loss of variable magnitude appears to be a common occurrence in the well population. It undoubtedly facilitates lung disease in general, is a part of the pathogenesis of the emphysematous diseases specifically, and predisposes to systemic disease.

2. EXECUTIVE SUMMARY

The major goal of the study is an evaluation of adverse health effects of ambient levels of nitrogen dioxide (NO_2), with special attention to serious but subclinical (covert) types of lung damage. The experimental model is the Swiss-Webster male mouse, and in all studies NO_2 was applied six hours per day and five days per week, with levels of 0.8± .04 ppm (Experiment M113), 0.34± .02 ppm (Experiment M114), 0.27± .03 (Experiment M115), and 0.26± .03 ppm(for Experiment M116 from 6/20 to 8/19 and 0.24± .03 from 8/21 to 9/18). Experiments M115 and M116 afford data for the effects of NO_2 on the developing mouse lung.

Three major kinds of lung damage have been investigated; 1) the loss of the Type 1 cell of the alveolus as measured by Type 2 cell replacement; 2) ultrastructural abnormalities of the Type 2 cell quantitated by image analysis measurements of mitochondria and lamellar bodies within the cell; and 3) the leakage of protein into the alveolus as measured by quantitation of a molecular probe, horseradish peroxidase (HRP). In the latter respect, electron microscopy was also done to evaluate abnormalities of cell junctions and structural components of the protein transport system.

The three investigative approaches were chosen for the following reasons: 1) the ultrathin Type 1 cell is primarily responsible for gas exchange in the lung, and it is also the cell type primarily responsible for preventing the leakage of protein into the air spaces of the alveoli; 2) Type 1 cell loss is a common finding in a great variety of human lung diseases, and it is a very early structural alteration as judged by the very early occurrence in lung disease of increased numbers of Type 2 cells (hyperplasia or population shift); 3) the weeping lung, as with weeping eyes, is also a very early and highly sensitive indicator of a noxious effect on sensitive membranes. In

the lung, this is seen as the leakage of protein into the air spaces and the structures in the supporting tissues of the lung; 4) the replacement of the Type 1 cell by a cell (Type 2) that is more than 50 times thicker,and leakage of protein rich fluid into the air spaces are very,serious impediments to gas exchange between the red blood cells in lung capillaries and the air spaces; 5) damage to the Type 2 cell itself, especially if irreversible, is a particularly serious alteration since the Type 2 cell is believed to be the progenitor of the Type 1 cell, i.e., the loss of both Type 1 and Type 2 cells of the alveolus is tantamount to loss of the alveolus itself from a functional standpoint; 6) both clinical and pathologic studies of the human lung indicate that Type 1 cells, Type 2 cells, and entire alveoli are being progressively lost to some extent in everyone and the loss increases progressively with time. There is great variation in the amount of loss between age groups but factors other than "normal" aging are involved.

With the foregoing in mind, it is clear that the interaction of many environmental and other noxious agents is causing some destruction of lung cells and tissue each day. However, the magnitude of the destruction in the well population has not yet been established nor has the degree to which the diverse noxious agents are individually responsible for this covert loss. Thus, it is the ultimate goal of the studies initiated to determine the increment of lung tissue structural and functional reserves consumed by specific air pollutants, singly and in combination, and thereby provide assistance in the establishment of air quality standards for the various pollutants. It should be emphasized that the loss of reserve tissue not only leads to disease in itself, e.g., the emphysematous diseases of the lung, but places the individual at great risk with respect to all diseases where increased burdens are placed on the lung, as for example, pneumonias and cardiovascular

disease. A lung with poor reserves also makes "well people" susceptible at one time or another to infections, diseases in general, and adverse effects of stress in particular.

To accomplish the goals specified above, three interrelated and major methodologies have been developed in this laboratory and are as follows: 1) image analyzer quantitation of Type 1 cell loss. After the mice have been exposed to NO_2 (the details of each experiment are summarized below), microscopic sections of the lung are prepared which bring out the Type 2 cell selectively. Since millions of Type 2 cells are present in the lung sections to be quantitated, computer assisted image analyzer is necessary for an appropriate quantitation. The image analysis study included eight measurements; numbers of Type 2 cells according to three different diameters (cells larger than 8 micra, 10 micra, and 12 micra), Type 2 cell size or area, the amount or area of the lung supporting tissues (connective tissues), the internal surface area of the air spaces or alveoli, and a mathematical measurement for either numbers of alveoli or wall area (mean linear intercept). With this approach, the preliminary data have shown: 1) a highly significant increase in the numbers of Type 2 cells in the lungs of 64 young adult mice as compared to the lungs of 64 control animals in a study where the animals had been exposed to 0.34 ppm NO_2 for six hours/day, five days/week and for six weeks (M114); 2) the increase in Type 2 cells (or loss of Type 1 cells) in the NO_2 exposed animals was highly significant statistically when the data were analyzed according to an upper quartile testing of the combined control and exposed animal groups (Type 2 cell count adjusted to alveolar wall baseline). With the upper quartile test, the NO_2 exposed animals outnumbered the control animals by a ratio of two to one ($p < .025$). This type of data analysis is particularly pertinent to the human experience since it most

likely reflects a subpopulation of animals especially susceptible to NO_2 exposure. Also, air pollutants are just one of many noxious environmental agents that adversely affect the lungs of the animals. In other words, the state of health of the animal colonies has a broad "normal" variation, as is also true for the state of health of human "well populations"; 3) no difference was found in the amount of lung supporting tissue between control and exposed animals. This finding indicates that the increase in Type 2 cells, based on the ratio of Type 2 cells to wall area, is real and not a decrease in wall area; 4) statistically significant differences between the lungs of control and NO_2 exposed animals were found with respect to the location of the lung section examined, i.e., the eight lung sections used for quantitation encompassed different levels of the lung, running from medial to lateral, and also (with four fields per section) apical anterior, apical posterior, basal anterior, and basilar posterior. The findings suggest, as would be anticipated from the human experience, that some portions of the lung are especially susceptible to noxious influences, e.g., human tuberculosis is almost invariably found in the upper lobe. Moreover, the exposed group of animals showed a greater uniformity of Type 2 cell numbers throughout the different lung areas. This suggests that NO_2 exposure has converted amny of the more protected or more resistant alveoli to the high Type 2 cell populations found in more vulnerable locations, or found in alveoli that bear a heavier burden of "ordinary" noxious agents; 5) the M116 experiment testing the effects of NO_2 on the developing lung are incomplete but the preliminary data from image analysis quantitation after four and 12 weeks of exposure again indicate an increase in the numbers of Type 2 cells for the NO_2 exposed animals and also more uniformity in Type 2 cell populations. Included in the study is an electron microscopic-image analyzer quantitation of mitochondria of the Type 2

cells and their lamellar bodies. The data are presently being analyzed for evidence of damage to subcellular organelles by NO_2 exposure. If present, it would imply an increased burden of repair for a cell that not only fulfills important biochemical functions but is believed to be the progenitor of the Type 1 cell. This study is the first of its kind to be carried out. Amongst the biochemical activities is the secretion of a substance (surfactant) that acts as a naturally occurring detergent in the air spaces and prevents the alveoli from collapsing on expiration. In the latter respect, alveolar space collapse (or atelectasis) is a serious complication that can follow surgical operations or simple immobilization in bed; 6) protein leakage, polyacrylamide gel (PAGE) quantitations of a molecular probe (HRP) were carried out in several experiments, including three earlier studies where missing data have been supplied by the present investigation. In the three studies, a greater retention of HRP (or essentially an increased retention of plasma protein) was found in the lungs of mice exposed to 0.5 ppm continuous NO_2, intermittent 0.6 ppm NO_2, and intermittent 0.8 ppm NO_2. In all experiments, the lungs were tested after two periods of NO_2 exposure, three weeks and six weeks. Thus, there were six test periods, i.e., three experiments with two tests per each experiment. Of the six test periods, five showed a greater HRP content for the exposed animals and three of these were statistically significant. Using an upper quartile (upper 25%) statistical analysis as a means of separating out animals believed to be more susceptible to the NO_2, five of the six periods showed statistically significant increases in HRP for NO_2 exposed animals; 7) the electron microscopic studies of the horseradish peroxidase protein leakage tests showed conclusively that HRP was in fact retained in the lungs 5-1/2 hours after injection and this served to lend support to PAGE HRP quantitation studies, i.e., after 5-1/2 hours there was still HRP to be cleared from the

cell and tissue structures involved in protein transport. In addition, the distribution of HRP in the various compartments of the lung was demonstrated, and this is of assistance in identifying those aspects of the protein transport mechanisms that may be altered by the NO_2 exposure; 8) investigations not in the formal proposal but carried out as opportunities to provide new kinds of useful data are also a part of the work accomplished in this period. One of the studies showed a highly significant decrease in the weights of spleens from animals (the M116 sutdy) exposed to 0.34 ppm NO_2. In addition, there was a decrease in the lymphoid tissue content of the spleens from NO_2 exposed animals, plus a decrease in spleen/body weight ratios and body weights themselves.

A second peripheral study was an extension of the PAGE testing, but for the quantitative measurement of urine protein content rather than HRP. During a four-week summer period, urines were obtained every other day from six medical students and the total urinary protein content measured by a dye-binding assay method. Twice a week, the urines were also analyzed for comparisons between low molecular and high molecular weight proteins, using the PAGE methodology. The main purpose of the testing was to establish the feasibility of large volume protein measurements and protein separations. Carrying out both measurements in a well population had not been done previously, and this is also the first search for air pollution correlation. No conclusions could be drawn from the medical school students but there were suggestive trends of increased urine excretion following NO_2 peak episodes of 0.2 ppm and higher. The total protein content also indicates that the well population will be found to have individuals with variable levels of protein excretion and that this may in part be a sign of subpopulations with increased susceptibility to urinary protein leakage. One of our earlier studies with guinea pigs, using PAGE tests, demonstrated increased protein leakage in the urine of the animals

exposed to NO_2.

A third and final peripheral study is that we reported a new human lung disease, silicate pneumoconiosis, as part of our continuing study of human lung pathology, and with special emphasis on covert types of air pollution effects. The silicate particle that was identified in the lung sections is believed to cause relatively little harm ordinarily but produces severe destructive lung disease when the particle is inhaled at a time when some other toxic substance is adsorbed to it. To date, the adsorbed toxic substance has not been identified but the evidence available strongly implicates a pesticide as one of the major noxious agents. Whether or not NO_2 and other gaseous air pollutants are adsorbed to silicates and in this way play a role in destructive lung disease is clearly an important question to answer.

3. ACKNOWLEDGMENTS

The following key personnel participated in the work of the first year:

1. Valda Richters, Ph.D.: Dr. Richters was in charge of the histopathology-histochemistry workload. This included supervision and assistance with animal surgery, gelatin inflation of lungs, and the histochemical tests (lactate dehydrogenase). In addition, the general processing of lungs for frozen section and accessioning of the material were under her supervision.

   Dr. Richters also worked on the development of the Quantimet image analysis procedure. The analysis of M113 was done by Dr. Richters, with the assistance of the principal investigator.

2. Dorothy Mundy was in charge of the biochemical aspects of the study, in particular the horseradish peroxidase molecular probe tracer studies.

3. The Medical Image Science Group of the University of Southern California provided two key personnel for assistance in the project, specifically Dr. Werner Frei and Robert Erbe. This group was responsible for computer assistance, in particular conversion of cassette data to tape and the setting up of the data in the format requested by the statistical group.

4. U.S.C. Biomedical Statistical Group. This group, headed by Dr. Stanley P. Azen (consultant for statistics on this project) provided with computer and statistical analysis services. Dr. Azen was assisted by Michael Jones who was directly responsible for data application to the statistical program packages (available to us through the University Computer Center of the University of Southern California).

5. Exposure Chamber Monitoring. The Air Resources Board provided a Beckman Liquid Saltzman monitor for our continuous monitoring needs. In the earlier part of the program, chemiluminescent units were used with the assistance (for the initial months) of our part-time consultant, Mr. Jack Littman.

   From the onset of the program, and continuing throughout the program, fritted bubbler analysis of nitrogen dioxide has been carried out. The monitoring device includes a bubble meter as an integral part of the unit.

   The workload for the fritted bubbler measurements was carried by Dr. Arnis Richters, Dorothy Mundy and Kestutis Kuraitis.

6. Histopathologic sectioning was done by Donna Smith.

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4. DISCLAIMER

The statements and conclusions in this report are those of the contractor and are not necessarily those of the California Air Resources Board. The material reported herein is not to be construed as either an actual or implied endorsement of such products.

5. TABLE OF CONTENTS

		Pages
1.	Abstract	2,3
2.	Executive Summary	4-10
3.	Acknowledgments	11
4.	Disclaimer	12
5.	Table of Contents	13-14
6.	Legends	15-16
7.	List of Tables	17-18
8.	Materials and Methods	19-24
9.	Summary and Conclusions	25-35
10.	Recommendations	36-37
11.	Introduction to Body of Report	38-40
	a) Scope and purpose of the project	41-43
	b) The design of the experiment and the materials of of instrumentation required	44-45
	c) Operational and experimental phase	45-53
	d) Discussion	54-58
12.	Text References	59-60
13.	List of inventions reported and publications	61
14.	Abbreviations	62
15.	Figures 1-3	63-64
16.	Tables 1-22	65-104
17.	Appendix	13-14

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Preprint: Barr, S.A., and Sherwin, R.P.: A correlation of urinary protein content and air pollution exposure of medical students.

Reprint: Sherwin, R.P., Barman, M.L., and Abraham, J.L.: Silicate pneumoconiosis of farm workers. Lab. Invest. 40:576-582, 1979

6. LEGENDS

Figure 1. Two-Way Nested Design on Data from Experiment M114 (animals listed within group; slide and field are considered replicates); internal surface area ÷ wall area (an exploratory measurement for degree of lung inflation and compartmentalization).

The mean ratios for each slide show relatively little variation for the control group animals as compared to those of the exposed group. The slide numbers represent sections running from the hilar portion of the lung to the periphery in the sagittal plane. This group-slide difference was found to be significant in terms of the interaction ($p < .02$). However, there was no group alone difference (between the mean values for the exposed and control animals). cf Table 15

Figure 2. Two-Way Nested Design on Data from Experiment M114 (animals listed within group; slide and field are considered replicates); numbers of Type 2 cells (expressed as wall area ÷ numbers of Type 2 cells 8μ and larger.

Different parts of the lungs of the exposed animals showed a remarkably constant number of Type 2 cells, whereas there was great variability for the control animals. cf Table 15

Figure 3. Two-Factor Analysis of Variance of Data from Experiment M114 with Slide as a Repeated Measure.

The three figures shown (a-c) are plots of the group-slide interactions for three measurements: a) wall area ÷ 10,000; b) wall area with a 8μ sizing factor ÷ 10,000, and c) wall area - Type 2 cell (8μ sizing factor) area ÷ 10,000. Each measurement is a reflection of the numbers of alveoli per field, and generally varies according to the degree of lung inflation (or atelectasis). The three different measurements of the same parameter

show relatively little difference. In principle, Figure 3c is optimal since it eliminates the possible complication of Type 2 cell hyperplasia causing an improper increase in the wall area measurement. The interactions shown in all three Figures are statistically significant ($p < .02$ or $.03$), and again show a tendency for slide uniformity in the exposed group in contrast to considerable variation in the control group. The meaning of this requires further study; a working hypothesis is that the lungs of the exposed group may be exhibiting peripheral atelectasis and central overinflation (? emphysema). Conversely, the control lungs show a variable degree of inflation with no special pattern. Speculatively, the control lungs may be exhibiting a primarily functional variation whereas the exposed lungs are largely affected by pathologic variations. The mean values of each group are not significantly different apparently because of broad variation in the control group.

7. LIST OF TABLES

		Pages
Table 1 - M113:	Experimental Design	65-66
Table 2 - M114:	Experimental Design	67-68
Table 3 :	Key to Image Analysis Study	69
Table 4a- M114:	Image Analysis of Mouse Lungs I. Variable Measurements	70
4b- M114:	II. Variable Ratios	71
4c- M114:	Percentages of Exposed and Control Groups Comprising the Upper 5% Tails	72
4d- M114:	Percentages of Exposed and Control Groups Comprising the Lower 5% Tails	73
Table 5 - M115:	Experimental Design	74-75
Table 6 - M116:	Experimental Design	76-77
Table 7 - M116:	Ratio of Wall Area to Type 2 Cell Number (Desk Top Calculator)	78
Table 8 :	Mouse Body Weights (Gms)	79
Table 9 :	Mouse Spleen/Body Weight Ratios	80
Table 10- M113:	0.8 ppm NO_2, 6 hrs/day x 5 days/week Number Type 2 Cell/Wall Area	81
Table 11- M114:	Statistical Analysis (60 control and 60 NO_2 exposed mice)	82
Table 12 -M114:	Means and Standard Deviations for Control and Exposed Animals According to Slide Number	83-84
Table 13 -M114:	Two-Factor Analysis of Variance Results with Slide as a Repeated Measure	85
Table 14a-M114:	Chi-Square Analyses of Contingency Tables. Animals are Classified as being Part of the Upper Quartile (U) or the Remainder (L)	86-87
14b-M114:	Two-Way Nested Design.. Animal Nested within Group; Slide and Field are Considered Replicates	88-89
Table 15 -M114:	Means ± Standard Deviations for Exposed and Control Groups by Slide. Only Animals with Complete Data Used	90-92
Table 16 -M114:	Two-Factor Analysis of Variance Results with Slide as a Repeated Measure	93-94

List of Tables - Contd.

		Pages
Table 17 -M114:	Chi-Square Analyses of Contingency Tables. Animals are Classified as being Part of the Upper Quartile (U) or the Remainder (L)	95
Table 18	: HRP Tests; Upper Quartile Statistical Analysis	96-97
Table 19	: Workload of Horseradish Peroxidase - Protein Leakage Testing and HRP Sensitivity (RHZ)	98
Table 20 -M114:	Data Collection Format	99
Table 21 -M116:	Quantitative Ultastructural Study of Type II Cells and their Organelles	100-101
Table 22	: Computer Assisted Statistical Analysis - Work Listing	102-104

8. MATERIALS AND METHODS

1. NO_2 Exposure Methodology

For each experiment, colonies of 150 mice were generally used, equally divided into control and exposed groups and housed in identical exposure chambers. Details of the chambers and the NO_2 method have been reported earlier (18). In brief, room air was filtered by a combination of particle and NO_2 adsorbing (Purafil) filters, and NO_2 applied to the air intake mixing unit of the exposure chamber through a silicone drip method. The silicone used is a high viscosity (500 centistokes) medical grade having an essentially negligible vapor pressure. The delivery of the silicone is dependent on the caliber of the teflon delivery tube, the height of the column, and the air flow. A voltage regulator is used to maintain a constant fan rpm. The following means of NO_2 monitoring were employed: 1) fritted bubbler readings of approximately 4 liter air samples; 2) Beckman liquid analyzer continuous flow recording; 30 chemiluminescent continuous recordings (Teco). Measurements were obtained of control chamber, NO_2 exposure chamber, and ambient room atmosphere. The Purafil filter was changed according to periodic checks of a color change of the pellets. There were also daily recordings of room temperature, chamber temperatures, and humidity. A rheostat controlled air conditioner maintained room temperature at $70^{o}F \pm 2$. The cages were generally cleaned two to three times per week, according to animal census. Periodic calibration of the monitoring equipment was carried out through the assistance of the Air Resources Board, El Monte division.

The animals were fed a standard pellet chow and received water ad libitum. Animal weights were recorded at the time of shipment and at the time of testing. The separation of the animals was according to animal weights, with generally five animals per cage. There were two environmental chambers, the one most recently in use having laminar air flow and stainless steel fabrication.

2. Quantimet Image Analysis. Quantitation of Type 2 pneumocytes.

The lungs are inflated with 6% gelatin through a 22 gauge cannula inserted into the trachea, and with the lungs in a plastic Petri dish cooled to $4^{o}C$. The lungs are inflated to approximate thoracic age volume using loss of the sharp lobar edge as a discriminant. The gelatin inflated lung is placed in a refrigerator at $4^{o}C$ to solidify the gelatin. The lobes of the lung are then separated, placed on a Mylar sheet with each lobe identified, and wrapped in aluminum foil for deep freezing at $-75^{o}C$. Sections 15 micra thick are cut on a Cryostat, with 12 sections from each of three lobes from each animal. Staining is carried out in groups of generally eight slides, each from six pairs of control and NO_2 exposed animals. The details of the LDH enzyme reaction can be found in an earlier report (2). In brief, a tetrazolium salt is used as an electron acceptor and is reduced to an insoluble formazan in the prsence of the LDH enzyme, with lactate as the substrate. The formazan reaction product is essentially selective for the Type 2 pneumocyte since this cell is the only one in the alveolus with a heavy perinuclear cytoplasmic desposit. Other alveolar cells (Type 1 pneumocytes, endothelial cells, and macrophages) show finely granular, diffuse reaction products in their cytoplasm which the Image Analyzer will not detect when the appropriate gray scales are set. Cells of the bronchial mucosa which stain with LDH are usually absent in the alveolar field selected or, when present, are excluded by the image editor. Both bronchial and bronchiolar lining cells are easily identified in the televised lung image. The LDH response that occurs in cells of the alveolar wall other than the Type 2 cell serves to delineate the walls for measurement by a second detector gray value setting of the image analyzer.

The image analysis is carried out by a single technician who analyzes four fields per each slide and obtains measurements of lung sections from control and exposed animals in an alternating sequence. Standardization of the measurements is achieved through periodic display of a standardization grid containing squares and circles with established measurements. The gray values are set to a point where a positive detected image corresponds to the displayed image, with the maximum setting at a point just below the first recognition of detection noise.

With the two detector settings set for Type 2 cells and wall area, an automatic program is used for measurement recordings. The format for the measurements is provided in Table 3. The eight measurements obtained through the automatic programmer are recorded on a cassette tape. The cassette data are transcribed by a telephone adaptor to the Medical Image Science Group where the following is accomplished: 1) conversion of the cassette data from ASCII to EBCDIC; 2) editing of the tape data; 3) parsing and computer format. Statistical programs at the USC Computer Center are used for the statistical analysis.

2. Capillary permeability-protein leakage studies of the lung with the molecular probe horseradish peroxidase (HRP)

Following each test period (includes NO_2 exposure studies, HRP linearity studies, and HRP clearance studies), the mice to be tested were placed in an infrared lamp heated chamber at 37°C for a period of 30 minutes for each individual mouse as a means of dilating the tail vein. Horseradish peroxidase (50 mg HRP Sigma Type 2/ml basal salt solution) was injected intravenously at a dosage of 0.5 mg (0.01 ml) per Gm of body weight using a 1 ml syringe, a 27 gauge needle and an approximately 30 second infusion time. In the later tests, the HRP dosage was reduced to 0.25 mg per Gm animal body weight. Intraperitoneal 0.5 ml sodium pentobarbital was used to kill each

animal at the designated time period, from immediately after injection to 5-1/2 hours after HRP injection. On opening the thoracic cavity, blood was collected from the severed jugular veins and spun down in a microfuge (Beckman) for five minutes. The serum obtained was immediately placed in a deep freeze at -85°C. The right ventricle of the heart was perfused with basal salt solution (BSS; 22°C) until maximal blanching of the lung was achieved. Generally no more than 20 ml BSS was used in a one minute period. The lungs were removed enbloc, placed on a BSS moistened gauze sponge, compressed gently two to three times by fingertip pressure to express fluid and residual blood, placed in a tared vial, weighed, and then frozen and stored in a deepfreeze at -85°C. In the later studies, a Sage pump was used to effect a timed volume control of the intravenous injection. For the HRP assay, frozen lung tissue was homogenized at 5°X in 2.0 ml of BSS containing 10% isopropanol and 10^{-4} phenylmethasulfonyl fluoride. The homogenate was spun down in a Beckman microfuge for 5 minutes. Duplicate 25 µL and 50 µL samples of the supernate were loaded on polyacrylamide disc gels (PAGE). In some studies the lung homogenate was adjusted to produce a constant 100 mg of lung tissue per 100 ml of BSS, as a means of providing a fixed protein load for the gel electrophoresis. A portion of the supernate was used to determine protein content. The electrophoresis apparatus was cooled by cold (4°C) water and the run carried out at 2-3 mA per gel for three hours. Protein was measured by the Lowry method or dye binding.

The gels from the 25 µL samples were incubated in a benzidine dihydrochloride-guiacol (DAB) stain for 30 minutes in the dark at 25°C and were then fixed in 7% acetic acid. Each gel was scanned with a Beckman Acta 3 spectrophotometer at 525 nM with a scanning speed of 1.5 cm/minute. The tracings were measured through the use of an integrating recorder with settings at 10 mV at 12,000 cpm, a chart speed of 4 cm/

minute and a span of 2. In some studies, an iron heme stain was substituted for the benzidine guiacol or the two stains were used in parallel for comparison purposes.

Electronmicroscopy

The electron microscopy procedure was carried out in two major directions. ultrastructural abnormalities (including image analysis of Type 2 pneumocyte organelles) and the distribution and clearance of HRP in the lung of control and exposed animals. The latter study included 0 minute to 5-1/2 hour intervals after HRP injection as a means of evaluating HRP clearance. For the 10 minute postinjection study each mouse received 6 mg of HRP in BSS intravenously. Ten minutes later, the right ventricle of the pentobarbital killed animal was perfused with 3 ml of BSS and this was followed by 3 ml of 2% of glutaraldehyde in 0.1 M cacodylate buffer (GTA) (right ventricle). With an allowance of 5 minutes for postperfusion fixation, 5 ml of diaminobenzidine (DAB) was perfused through the right ventricle and the entire lung suspended in the DAB solution for 30 minutes. The lung was then sliced into 1 mm cubes and immersed in a DAB-peroxide solution. This was followed by routine processing for electron microscopy, including osmium fixation, which produces the final HRP-DAB-osmium complex.

The electron microscopic processing in brief includes, as mentioned earlier, postfixation of the section in osmium, washing in 0.1 M cacodylate buffer, dehydration in graded alcohols, clearing in hydroxypropylmethacrylate and embedment in epon. Thin sections were obtained with and without uranyl acetate and lead citrate staining, and grids were examined with a Philips 301 electron microscope.

Electron micrographs were obtained in accordance with the two major directions, i.e., Image Analysis Quantitation, 32 electron micrographs of Type 2 pneumocytes of 5700 x 3 from each, and HRP distribution in the lung

parenchyma and pleura. For the distribution studies, the dosage of HRP was identical to the methodology used for the PAGE studies in order to provide direct correlative data. There were also additional fixation testing studies involving tying of the trachea, suspending the lungs in 2% GTA solution for two hours, and then processing the thinly sliced sections for comparative electron microscopic studies.

9. SUMMARY AND CONCLUSIONS

The experiments, results, and discussions are presented in chronological order in two parts; Part I, computer assisted image analysis; Part II, electronmicroscopy and protein leakage studies, with a concluding section on peripheral investigations.

This study involved an image analysis quantitation of the numbers of Type 2 cells in the lungs of young adult mice, half of which had been exposed to intermittent 0.8 ppm NO_2 (6 hrs/day x 5 days/week). The lungs from 70 control and 70 NO_2 exposed animals were analyzed at two periods, i.e., six and ten weeks of NO_2 exposure and 35 animal pairs for each period. This study was not completed for the following reasons: a) the initial attempt to change from a desk top computer analysis to an on-line analysis by the University Computer Center resulted in an excessively cumbersome data format: b) it was deemed more profitable to apply data in its new format from Experiment M114 to the analysis effort because of the lower level of NO_2 exposure (0.34 NO_2 for M114 vs. 0.8 ppm for M113). A limited desk top computer analysis was carried out for the first ten control and ten exposed animals and the analysis showed a trend for greater numbers of Type 2 pneumocytes in the lungs of the NO_2 exposed animals (Table II). This is in accord with an expected loss of Type I cells from NO_2 exposure.

2. Experiment M114. Image analysis.

Half of a colony of 128 mice were exposed to 0.34 ppm NO_2 for 6 hrs/day, 5 days/week and for 6 weeks. The data obtained from the image analysis study of the left lung were subjected to three different computer analyses: t-test evaluations, two factor analysis of variance, and upper quartile analysis. The results are as follows;

a) <u>t-test for independent means</u>:

1) a highly significant ($p \rightarrow 0$) increase, for NO_2 exposed mice, in Type 2 cells by absolute numbers and by wall/cell ratio (Table 13, Addendum pp 10, 11).

2) no difference between control and exposed animals in wall measurement; this finding is evidence in support of Type 2 cell hyperplasia.

3) exposed animals had an increase in perimeter, or internal surface area ($p < .003$) this finding may reflect irregularity and disruption of the structure of the alveolar walls for the exposed animals. Elaboration of these findings is presently in progress

4) in the upper 5 percentile study there was a very significant difference in the wall to cell ratio between control and exposed groups ($p < .0001$), 103 vs. 57 respectively (cf T2-10μ Ratio, Table 4c). Note: the smaller value for the exposed group indicates a greater number of Type 2 cells, i.e., wall area/cell number, with wall areas of C and X equal. Significance at the same level ($p < .0001$) was also noted, but in the opposite direction, for the lower 5 percentile (cf Table 4d, 10μ T2-Ratio).

5) an increase in mean cell area (Total area T2/T2 cell number) was noted for the exposed animals, but the difference was just short of significance by 2-tail test ($p < .088$), but $p < .044$ on 1-tail test (Table 13, Addendum page 11).

b) A two-way nested analysis of variance (cf Page 16) and Table 13 also showed an increased number of Type 2 cells for the exposed animals, but great variations and skewing within groups apparently precluded statistical significance.

c) A non-parametric test, upper quartile analysis according to animals, showed a high statistical significance, i.e., $.01 < p < .025$ (Table 19b, data item "new 4/7").

d) There also appeared to be statistically significant differences according to the location of the lung section, i.e., the 8 slides analyzed represented medial and lateral portions of the lung in a sequential order. Examples of the differences in patterns can be found in Figures 1, 2, 3a, 3b, and 3c. The patterns generally show that the exposed animals had less variation in Type 2 numbers, a pattern that also seems to be the case with lung protein and horseradish peroxidase content as will be described later. The pattern differences suggest that the numbers of Type 2 cells per alveolus varies according to anatomical location, i.e., Type 1 cells in some areas of the lung are more susceptible to noxious agents and these areas will have a Type 1 to Type 2 population shift. Thus our working hypothesis is that the exposed animals show less variation because NO_2 exposure damages "intermediate" resistant Type 1 cells and results in a more uniform Type 2-Type 1 cell distribution in alveoli throughout the lung.

The different statistical analysis approaches have been employed to cover special aspects of the analytical problem. All animals show some indigenous infectious, parasitic, or degenerative lung disease. The t-test for independent means attempts to resolve this background "noise" by creating a single (composite) control lung and a single (composite) exposed lung, i.e., each slide is treated as an anatomically independent lung site. In the latter respect, it should be noted that complete independence is not obtained even with data based on animal rather than lung slide since animal itself is not completely independent, e.g., 5 mice/cage and 12 cages/chamber. In view of the question just raised of the independence of lung slide, the extremely high statistical significance found by t-test analysis may not be entirely valid. The more orthodox statistical approach based on animals,

a 2-factor analysis of variance, showed a trend for greater numbers of Type 2 cells in the lungs of the NO_2 exposed animals, but the analysis fell short of significance undoubtedly due to the great variation found between animals and the relatively small numbers of animals (60 pairs). The addition of new data found on analysis of the right lung, an incomplete part of the M114 study, will double the volume of data for the 2-factor analysis of variance study and may thus reduce animal variation. The right lung study will also provide comparison data between left and right lungs.

The third approach to statistical analysis takes advantage of the putative amplifying effect of NO_2 exposure, i.e., a subpopulation of animals is expected to be particularly susceptible to NO_2 exposure, and this should be reflected in especially high Type 2 cell counts for the susceptible subpopulation. Thus, the highly significant statistical finding with the uppr quartile analysis ($.01 < p < .025$) is perhaps the single most outstanding finding of the M114 experiment.

The significance of Type 2 cell increase is as follows: 1) an increase in Type 2 cells occurs at the expense of a loss of the Type 1 cell; 2) the Type 2 cell is over 12 micra in thickness whereas the Type 1 cell is less than 0.1 micron in thickness. Replacement of the alveolar lining with a cell over 100 x the thickness of the normal lining is a serious impairment to gas exchange; 3) damage to and loss of Type 1 cells places a heavy burden on the protein transport system of the lung since it is the Type 1 cell that is the principal barrier to the leakage of protein from capillaries into the alveolar lumina; 4) an increase in Type 2 cells is very likely associated with damage to the Type 2 cell itself, i.e., the greater resistance of the Type 2 cell to NO_2 and other noxious agents is merely relative to that of the Type 1 cell. Lethal damage to the Type 2 cell as well as to the Type 1 commonly occurs in the human lung in everybody to some

degree, and this sets the stage for the complete loss of alveoli, or emphysema. In part, Type 2 hyperplasia can be considered a prelesion of emphysema, with progress to emphysema depending upon the irreversibility of the Type 2 hyperplasia and subsequent progression to Type 2 cell death itself.

3. Experiment M115. Image Analysis.

This is the first study to involve the developing mouse lung. There were 20 control and 20 NO_2 pregnant exposed females, with NO_2 exposure at 0.27 ppm for 6 hrs/day and 5 days/week. Image analysis studies of the mice born in the chambers were carried out after five weeks of NO_2 exposure. This experiment was deemed inappropriate for analysis in view of two complications: 1) of 54 presumably pregnant mice, only 26 actually delivered; and 2) a large number of immature Type 2 cells were noted at 5 weeks of age, and this required modification of the image analysis methodology for their detection. Attention was therefore turned to a present experiment, M116.

4. Experiment M116. Image Analysis.

This repeat experiment of M115 was carried out with NO_2 exposure at 0.3 ppm for 6 hrs/day and 5 days/week. A total of 160 newborn mice were obtained and studied in this experiment, equally divided into control and exposed groups. Image analysis was carried out after four weeks of exposure and after 12 weeks of exposure. At the time of this report, image analysis has been completed for 20 pairs of males at four weeks and 20 pairs of males at 12 weeks, and with respect to the left lung. Computer analysis of the data is pending completion of the studies. A preliminary analysis of the data that is presently available was accomplished through the use of a desk top computer, and the results have been summarized in Table 8. The findings are considered inconclusive, but NO_2 exposed animals had, as expected from Experiment M114, a higher number of Type 2 pneumocytes.

A second part of the M116 image analysis study involved quantitative measurements of subcellular structures of the Type 2 cells, specifically mitochondria and lamellar bodies. This analysis, using electron micrographs and a macroviewer, is presently under way. The data are intended to search for alterations of Type 2 cell organelles, i.e., Type 2 cell damage secondary to in vivo NO_2 exposure. The quantitative data obtained will help to establish the extent of the NO_2 damage and in particular the potential for irreversibility of both Type 1 and Type 2 cell damage since the Type 2 cell is believed to be the progenitor of the Type 1 cell. More specifically, 20 male mice (ten control and ten exposed to 0.3 ppm NO_2) have been processed for macroviewer image analysis measurements of the Type 2 cell, including its subcellular organelles. Half were studied at 4 weeks of exposure, and half at 12 weeks. A 5700X magnification was used for all photographs, and a total of 320, or 32 per animal are on hand for quantitation; analysis has been delayed pending arrival of an adaptor. Thus the two interrelated studies involve a total quantitation of 320 electron micrographs, with generally one Type 2 cell and its multiple organelles per photograph (Table 23).

5. Computer analysis program accomplishments

Mention should be made of accomplishments in computer analysis. The several parts of the analytical chain have now been successfully established and linked together, namely: 1) an optimal data format for recording at our terminal (casette); 2) semiautomated Quantimet programmer added; 3) ASCII to EBCDIC conversion, casette to tape conversion, special data processing format conversion, and parsing, all by the U.S.C. Image Science Group; and 4) tape to disc conversion and statistical analysis through the Medical Campus terminal (Dr. Stanley Azen) of the University Computer Center (U.C.C.) using packaged programs of the U.C.C. (also, cf Table 12. Computer Data Copy).

Part II: Electron microscopy, protein leakage, and peripheral studies

6. Experiments M113 and M114. Electron Microscopy

A need of the first year study was to demonstrate structurally that a molecular probe for protein leakage, horseradish peroxidase (HRP) was in fact retained in lung tissue 5-1/2 hours after intravenous HRP injection. The demonstration was accomplished and provided proof that a biochemical measurement of HRP content of the lung is in part a measure of protein transport and clearance. As mentioned earlier, the Type 1 cell is the major barrier to protein leakage into alveoli. Damage or loss of the Type 1 cell therefore results in a protein transport burden for the lung, i.e., an increased protein reabsorption workload. The ultrastructural studies also provided data on HRP distribution and clearance on the basis of a series of timed studies from 10 minutes to 5-1/2 hours (M113, M114), and also established sites of HRP retention (see Section 7).

Ultrastructural studies have also been carried out in a search for parenchymal abnormalities that can be attributed to NO_2 exposure. From the subjective evaluation carried out, no differences were found between control and exposed groups, including a search of bronchioloalveolar structures as well as subcellular organelles, e.g., mitochondria, lamellar bodies, and endoplasmic reticulum.

7. Protein Leakage Studies

The HRP studies involved three major directions: a) the electron microscopic demonstration of HRP in lung tissues as just discussed above; b) completion of earlier PAGE studies dealing with the quantitation of HRP retention in the lung of control versus NO_2 exposed animals; c) the uptake of HRP by macrophages as a means of achieving a quantitative, image analyzer measurement of macrophage populations in the lung. The HRP work accomplished included the following: 1) New data to complete HRP experiments initiated prior to the Contract Award, including: a) linearity determinations

of HRP measurements; b) adjustments of HRP quantitations to a common baseline for three independent experiments; and c) reproducibility tests relating to HRP shelf life and stability within the acrylamide gels; 2) tests for HRP lung and serum clearance at multiple test periods from 10 minutes to 5-1/2 hours; 3) the development of a methodology for controlling pressure and volume (time) of intravenous HRP injection; 4) the testing of different doses of intravenous HRP with respect to a) optimal lung retention versus plasma clearance, b) optimal polyacrylamide gel separation versus HRP concentration in lung homogenate, and c) optimal HRP stain densities for accurate photometric (gel scan) quantitation; 5) correlative electron microscopic studies to demonstrate the distribution, transportation vehicles, and clearance of HRP in the lung.

The principal findings of the HRP molecular probe studies are as follows: a) Three independent studies have shown a greater retention of HRP in the lungs of mice exposed to ambient levels of NO_2 (ranging from intermittent or continuous 0.5 to 0.8 ppm). Of the six testing periods (i.e., 3 week and 6 week tests per each three experiments), five showed a greater content of HRP in the lungs of animals exposed to NO_2, and three of the five were statistically significant (Tables 20a & b). With a top quartile statistical analysis, five of the six test periods were significantly different; see Table 20a; b) Both light and electron microscopic studies showed that HRP remained in the lung at 4-1/2 hours postinjection and could be found in basement membranes, subpleural lymphatics, macrophages, and pinocytotic vesicles of the endothelium and mesothelium. On a subjective and non-quantitative appraisal, no differences in distribution of HRP could be demonstrated between control and exposed groups. A methodology for quantitative measurements of HRP distribution are presently being developed through the application of the macroviewer of the image analyzer; c) Formulae for HRP

linearity were established and will be employed in the ongoing studies where the same lot number of HRP will be used.

The reason for special attention to HRP and protein transport is that abnormalities of protein transport represent one of the earliest and most important findings in a great number of important lung diseases. Moreover, protein leakage can be expected to be a very early effect of the inhalation of noxious substances such as NO_2, ozone, SO_2, and other such irritating agents. Fluid leakage in the lung is a very serious matter since it indicates damage or total loss of the Type 1 cell, can become a serious barrier to gas exchange, and predisposes to the emergence of opportunistic organisms. Moreover, protein leakage is undoubtedly associated with cellular metabolic abnormalities including an altered production of surfactant by the Type 2 cell. In the latter respect, the quantitative measurement by image analysis of Type 2 cell lamellar bodies and mitochondria will provide important information to supplement the data obtained concerning protein leakage.

Peripheral investigations

To make use of the tissues that would ordinarily be discarded, and to take advantage of the participation offered by a predoctoral student, a peripheral study was carried out. This data has been presented at a Scientific meeting and the abstract accompanies this report. In brief, the data showed that mice exposed to 0.34 ppm nitrogen dioxide (animals from Experiment 116) demonstrated a decrease in body weight ($p < .05$), a decrease in spleen weight ($p = .0025$), a decrease in spleen/body weight ratio ($p < .005$), a decrease in average nodule size ($p < .005$), a decrease in total nodule

area ($p = .05$), and a decrease in average spleen area around each nodule ($p < .0005$). These studies included the use of the Quantimet Image Analysis for measuring total lymphoid nodule area, nodule number, and spleen area. The published abstract credits the California Air Resources Board Contract 86-218-30 for partial support of this study.

A second peripheral study was SDS-PAGE separations of human urinary proteins and dye binding assays of urinary protein involving six medical students during a four-week summer period (July-August 1978). The data are too limited for conclusions to be drawn but there is a suggestion that a rise in urinary protein content followed elevations of NO_2 above 0.2 ppm one hour maximum levels at the nearby monitoring station (1). This is the first study where a feasible methodology has been worked out for a definitive investigation. It is an important direction for NO_2 health effects studies since it bears on key health areas, i.e., a systemic (renal) effect and an early abnormality (protein loss). From the spleen study findings just mentioned, from an earlier study of proteinuria in guinea pigs exposed to NO_2, and other studies, it is apparent that NO_2 has systemic effects. The loss of protein in the urine is a health concern similar and in part related to the problem of elevated blood pressure. There is no sharp cut-off between normal and abnormal blood pressures, but a rise is clearly undesirable and at a more or less arbitrary level warrants treatment in the absence of symptoms.

A third peripheral study involved human lungs exhibiting a newly recognized disease, silicate pneumoconiosis. The study was completed with partial support from the ARB, and a report recently published in Laboratory Investigation, 40:576-582, 1979, with partial support from the ARB acknowledged. The pathology of the lung disease is especially pertinent to the general problem of air pollution in that the occurrence of a presumably inert or relatively harmless silicate particle in the lung has resulted in severe

destructive disease with fibrosis. The reason for this is presently believed to be in the presence of some toxic substance adsorbed to the silicate, with the combination having a synergistic, severely destructive effect. The identity of the putative toxic substance is unknown but may be a pesticide as judged by correlations recently made with an ongoing study. There is also the implication that particulates in the air through the adsorption of noxious agents such as NO_2 may play important roles in facilitating destructive lung diseases.

10. RECOMMENDATIONS

The major recommendations for the ongoing project are as follows:

1. With respect to the needs for statistical analysis, our consultants have recommended that we increase the number of animals in the study and analyze the data by several statistical approaches, with emphasis on non-parametric methods.

2. Additional work is needed in the area of ultrastural correlation with the image analysis measurements of Type 2 cells. While damage to the Type 1 cell has been shown to be correlated with an increase in the Type 2 cells, little attention has been given to damage to the Type 2 cell itself. Our ongoing proposal includes quantitative measurements of subcellular structures of the Type 2 cells as seen in electron micrographs. The material for this study is already on hand and awaits an adapter for interfacing the macro lens with the Vidicon camera of the image analyzer.

3. Our attempt to quantitate Type 2 cells in the newborn could not be carried out because of immaturity of the Type 2 cell, i.e., a remarkably different lactate dehydrogenase response from the young adult. We propose to establish a new marker for the Type 2 cell of the immature lung, and to exploit the finding as a means of quantifying adverse effects of NO_2 and other pollutants on the developing lung.

4. Reversibility is a critical consideration in the hypoplasia of Type 2 cells and our ongoing proposal includes an experimental design that will allow us to test reversibility after varying periods following the cessation of NO_2 exposure.

5. The linearity studies we have carried out with HRP indicate that greater sensitivity and accuracy can be achieved in testing through the use of appropriate mathematical formulae derived from the linearity determination.

Recommendations - Contd.

We have in the past purchased HRP in large quantities to avoid variation between lots.

6. The use of the HRP molecular probe in combination with other probes appears to have great potential for providing new and useful information.

II. INTRODUCTION TO BODY OF REPORT

This is the final report for the first year of the Contract Award. The specific aim of the first year was to apply a new methodology to the evaluation of air quality, beginning with studies involving the exposure of mice to ambient levels of nitrogen dioxide (NO_2). The new methodology, developed in this laboratory, has three principal parts: a) the selective staining of the Type 2 lung cell, one of the two major epithelial cells found in the alveolus of the lung; b) the counting of Type 2 cells by an image analyzer, i.e., large volume quantitation of cell numbers and cell sizes; and c) the measurement of lung wall area by the image analyzer to provide a baseline for Type 2 cell measurements and new kinds of data (linear intercepts, and internal surface area). The importance of the image analysis study is that it can provide new discriminants for evaluating air quality that are highly sensitive and especially relevant to the problem of air pollution as concerns the human population. Our working hypothesis, with supporting data from our studies of both "healthy" and diseased human lungs, is as follows: lung reserve cells and tissue are being consumed at variable rates in the human population at large, and air pollution is one of many important factors that modulates the expenditure of pulmonary reserves. Of paramount importance, the expenditure of reserves can be entirely covert and can progress to the point where the complete loss of lung cell reserves becomes a serious and irreversible clinical disease, emphysema being a classical example. Thus, one of the major goals of the studies we have proposed and done is concerned with the problem, specifically, an <u>inventory</u> of the major epithelial cell populations of the alveoli where gas exchange takes place. The measurement of Type 2 cell populations that we achieve are indirect measures of damage to and loss of Type 1 cells. Further details on the rationale are provided in other sections that follow.

There are two other major specific goals of the program, an electron microscopic study which is attempting to identify the type of Type 1 cell damage that leads to Type 2 cell replacement and a molecular probe quantitation of protein leakage in the lung. The data being derived gives information on the functional status of the alveolar wall and is especially significant for correlation with the Type 2 cell-Type 1 cell population shift since it is the integrity of the Type 1 cell that is principally responsible for the prevention of intraalveolar protein leakage (or pulmonary edema). Within the period of this final report, work has been accomplished in all three major goal areas. In addition, two new methodologies have been developed to provide important supplementary data, namely the quantitation of macrophages in the lung and the quantitative measurement of subcellular structures of the Type 2 pneumocyte.

The work was accomplished through the carrying out of four definitive experiments, M113, M114, and M115 and M116 and a number of pilot studies limited to small groups of animals. The details of the Experimental Designs and Results are given in the text that follows. All of the experiments were concerned with nitrogen dioxide exposures. In the initial proposal, ozone studies were included. However, events taking place as the program developed indicated that the NO_2 studies should be continued and expanded. Particularly influenctial in the decision were the following: 1) the preliminary finding that a level of intermittent NO_2 as low as 0.34 ppm was by itself causing an increase in Type 2 cells, i.e., Type 2 cell hyperplasia putatively related inversely to Type 1 cell damage and loss; 2) protein leakage was found following intermittent 0.5 ppm NO_2; 3) NO_2 exposure has been receiving increasing attention with respect to health effects and its occurrence in the atmosphere. Moreover, some modification of the Experimen-

tal Design for studying NO_2 was called for, in particular the use of increased numbers of animals and the development of an optimal statistical approach to data evaluation. Further, a thorough evaluation of NO_2 exposure was needed to provide a foundation for expansion into two important directions, the influence of NO_2 exposure on the developing lung and an evaluation of reversibility of structural-functional alterations.

One other aspect of the program carried out during the period of this report is the commitment of a small amount of ARB support to several small pilot studies. The latter include: 1) a quantitative measurement of lymphoid nodules of the spleen by the image analysis methodology; 2) a study of urine protein content of six students on the Medical Campus during the summer smog period of 1978; 3) the investigation and subsequent establishment of a new lung disease. Further details concerning these pilot studies are given in the text that follows. The publications that resulted from each of the studies are included in the Appendix of this report. A preprint concerning protein leakage is also appended; it deals with the quantitation of horseradish peroxidase (HRP) and its increase in lung tissues of NO_2 exposed animals. The HRP tests, by polyacrylamide gel electrophoresis (PAGE) and enzyme kinetic assays, are measurements of the bidirectional transport of protein in the lung. The distribution of HRP in lung tissues and cells was demonstrated by electron microscopy. Linearity and clearance tests were also run. A definitive manuscript on the Type 2 cell-Type 1 cell population shift established by image analysis is in preparation.

a. Scope and purpose of the project:

This research project is primarily concerned with the effect of ambient air pollutants on the cellular ecology of the lung. The purpose was to determine the lowest level of NO_2 that caused shift in lung cell populations of mice. The initial proposal to study Ozone as well was changed to NO_2 only in view of the importance of adverse health effects of NO_2. The change was with approval of the project officer. Special attention was given to Type 2/Type 2 cell population shifts, and to a pathophysiologic lung abnormality (protein leakage). The specific aim was to achieve quantitative measurements of the consumption of structural and functional reserves of the lung secondary to air pollutant exposure in order to assist with the establishment of air quality standards.

General background of the proposal: The research work is founded on prior studies showing that a cell population shift can be demonstrated following exposure of guinea pigs to 2 ppm NO_2, and that protein leakage follows exposure of mice to 0.4 ppm NO_2. The cell measurement studies included hand count and image analysis methods (1,2,3). The close correlation that was found between the two latter methods provided the foundation for a large volume, semiautomated quantitation of Type 2 cell populations in the lung. An earlier study was the first to demonstrate that Type 2 cell populations could be selectively stained (for lactate dehydrogenase - LDH) for the sharp contrast demanded by hand and image analysis quantitation methods (4). With respect to the functional studies of protein leakage, this laboratory developed a variety of approaches to its measurement including tracheobronchial lavage where the protein content was quantitatively measured through liquid scintillation counting of tritium labeled albumin (5) and, later, protein characterization and quantitation through polyacrylamide gel electrophoresis (6). Subsequently, we showed that the lung tissue content of plasma proteins was also increased in response to an ambient level of NO_2 exposure in vivo

using either a fluorescent label for plasma proteins (7) or a molecular probe (8,9; also see Preprint).

Theoretical approach to the solution of the problem: The most overlooked aspect of the problem of adverse health effects of air pollution is the cost of poor air quality in terms of the consumption of structural and functional reserves of the lung and other organs. A first step in the irreversible loss of lung tissue is a shift in cell populations. It is now generally accepted that an increase in Type 2 pneumocytes of the lung is largely a reflection of loss of Type 1 cells. This particular shift in a cell population is one of the earliest of the pathologic lesions we now recognize as a sequella of the inhalation of many noxious substances. A prototype experimental example of this is the complete replacement of alveolar lining cells by Type 2 pneumocytes following the inhalation of 100 percent oxygen by primates. The lesion is early in the sense that there is no overt loss of lung architecture and is presumed to be mainly reversible following the gradual reduction of the oxygen level. Considerable functional alteration can be expected prior to and concomitant with the loss of Type 1 cells, and paramount in this respect are two major considerations: altered gas exchange and altered permeability of capillaries to plasma protein. Moreover, an increase in Type 2 cells is a common denominator and a very early event in a large number of diverse human lung diseases. Thus, measurements of cell population shifts and altered capillary permeability can be expected to be very sensitive and very meaningful methods for evaluating air quality. One other important consideration is that human lung pathology studies indicate that some lung tissue is irreversibly lost by everyone continuously, i.e., on a very roughly chronological or "aging" basis. In effect, there can be little doubt that environmental exposures of all kinds are responsible for incremental increases in the rate and total consumption of lung and other reserve tissues. The actual

rate of consumption, and in particular the influence of specific environmental noxious agents on the rate, are just beginning to be recognized as cardinal questions in the evaluation of air quality. From this concept, it can be seen that qualifications such as "margins of safety" and "threshold levels" are not strictly applicable to the problem, i.e., progressively decreasing levels of a noxious agent can be expected to have progressively decreasing (but not necessarily proportional) influences on the consumption of structural and functional reserves. The consumption of great amounts of reserves is often subclinical.

Limitations of the work: The initial proposal included both ozone (O_3) and nitrogen dioxide. However, the focus of the work has been entirely on NO_2 because of two main considerations: 1) the methodology originally proposed has had to be reworked due to a major change in the research protocol, specifically conversion from desk top computer recording and analysis to a University computer tie-line. This recommendation was made by the ARB and we are in full agreement that we will in the long run obtain far better results in terms of the breadth and sensitivity afforded by computer assistance; 2) in depth exploration of nitrogen dioxide now seems to be of greater importance than previously recognized, both with respect to its control in the environment and its adverse health effects. Moreover, we are particularly concerned about its influence on the developing lung, and have introduced newborn mouse lung studies. It is also pertinent to point out that ongoing studies are concerned with the necessity for testing reversibility at varying postexposure periods. Most importantly, the principles established with the NO_2 model [i.e., the experimental design for extent of lung quantitation (cell, lobe, and animal numbers), extent of supporting ultrastructural and protein leakage studies, and computer analysis of programming] will be readily applicable to the studies of other pollutants planned, and will in this way greatly expedite the continuing investigation.

b. The design of the experiment and the materials and instrumention required were in large part established by prior work. The two major new needs were: 1) the lease or purchase of an image analyzer (Quantimet 720); and 2) computer interfacing with the image analyzer. With respect to the Quantimet, a decision on purchase as opposed to leasing was made October 6, 1977. However, the Quantimet did not arrive until January of 1978 and it did not become operative until February 15, 1978 (following the completion of several service calls required to make the instrument operative. A further complication to the operation of the Quantimet stemmed from a change in the research protocol recommended by the ARB, namely, as mentioned earlier, interfacing with the University computer rather than the IBM desk top computer originally specified. For the contract submitted, the IBM desk top computer served three functions, programming of the Quantimet for image analysis, recording of data, and statistical analysis. With later revision to University computer use, no programmer for the Quantimet was provided. With the start of the analysis work, a completely manual programming proved to be a serious obstacle to the work output. A request was submitted to obtain either a desk top programmer, a computer interface, or an automated programmer module as an accessory for the Quantimet. A decision in favor of the latter was made following an offer to us by the Company of a demonstrator model at half price. This unit arrived in March of 1978. A new programming was initiated subsequently, with the definitive image analysis studies beginning anew as of April 1978. Several weeks were required to effect a working program, with collaborative efforts by specialists in the areas of statistical and computer assistance. In the latter respect, several options afforded by the University were investigated and a decision made to effect a liaison with the USC Medical Imaging Science Group (MISG). The facilities they offered were: 1) a telephone link to their computer; 2) conversion of our cassette tapes in

ASCII to their tape reels, and in EBSDIC; 3) parsing of the tape with data printout proof; 4) setting up of a tape format according to that specified by the statistician; 5) general consultant assistance with the ongoing program and for new considerations.

With respect to the statistical analysis, Dr. Stanley Azen, Consult Statistician for the program, assigned an associate (Michael Jones, a statistician-computer programmer) to assist us. He concurred with the use of the services of the MISG, and the jointteam then consisted of Michael Jones, Robert Erbe of MISG) and the supervisory assistance of Drs. Azen (USC) and Werner Frei (MISG). Tapes prepared by the MISG were converted to discs at the University Computer Center of USC (UCC) by Michael Jones, and data analysis was obtained through package statistical programs and the computer facilities of the UCC.

c. Operational and experimental phase:

1. Details concerning procedural and instrumentation progress with the Image Analyzer.

In the initial runs with the image analyzer, it was found that the program worked out by the programmer-statistician was unsatisfactory in that an inordinate amount of time was required by the computer to carry out the statistical analysis. The specific problem was excessive identifying data preceding the experimental findings. The identification data also consumed an inordinate amount of time for the operator carrying out the image analysis, i.e., three hours per 32 lung fields for each animal. Following the analysis of 20 animals (10 pairs of control and exposed mice; Experiment M113, 0.8 ppm NO_2 exposure), the image analysis was stopped and efforts made to develop a new format. The working group developed a format appropriate to the needs of both efficient analysis and efficient handling of large volumes of data by the computer-statistician group. This format was found to be highly

satisfactory in the studies that followed and is currently in use. In particular, the three-hour image analysis time was reduced to one hour and computer use greatly facilitated.

2. Operational details, data, and results.

As mentioned, initial data recording and computer analysis began with Experiment M113. This data proved too unwieldy for computer analysis and was analyzed by data entry into a desk top computer, the Statistician (Compucorp). The data entry format for this first program (set up on March 15, 1978) is presented in Table 22a. The data analysis was carried out on 20 animals (10 pairs) and the preliminary data obtained are provided in Table 1. The results of the analysis showed a trend towards an increase in Type 2 pneumocytes for the exposed animals , but the findings are inconclusive due to limitation of the study.

The new data recording and computer analysis format was established on July 7, 1978 and was immediately applied to Experiment M114 since the lung tissues had been completely processed by this time and the lower NO_2 level applied in M114 was deemed a more important priority (preliminary analysis of data from Experiment M113 had already pointed to a Type 2 pneumocyte hyperplasia trend). The specific details of the data analysis are provided in Tables 4,5,12-19 & 24. The findings of the study were presented as a preliminary report: Type 2 pneumocyte hyperplasia and hypertrophy in response to 0.34 ppm nitrogen dioxide; an image analyzer computer quantitation, and the abstract (10) published in Fed. Proc. (38:1352, 1979). The Air Resources Board was acknowledged for partial support of the preceding publication.

The operational and experimental plans of the project also included three other areas of work: 1) polyacrylamide gel electrophoresis (for the quantitative measurement of a molecular probe tracer, horseradish peroxidase); 2) electron microscopy (for subcellular lung alterations; and 3) macroviewer

image analysis (for subcellular measurements using electron micrographs).

The biochemical study effort was targeted for the completion of earlier studies, in particular linearity testing, determining absolute levels of HRP in the lung tissues and blood, remeasurements of HRP gel scan areas (with adjustments to a common baseline for the three independent studies and comparison testing with a benzidine-iron as opposed to guiacol enzyme staining. It should be noted that the quantitative HRP molecular probe tracing methodology originated in this laboratory as a means of assaying lung tissue protein. The test was developed in order to gain greater sensitivity for measurement of protein leakage in the lung, i.e., whatever excess amount of protein is found in lung lavage fluid represents a small fraction of the actual leakage since most of the protein leaked into alveoli is reabsorbed by the lung (i.e., there is a bidirectional lung transport of plasma proteins). We had earlier developed the first methodology to test for an increase in lung tissue protein following pollutant exposure, namely fluorometric quantitation of fluorescamine labeled plasma protein (7). Our development of the HRP method was prompted by the greater ease and accuracy of measurement by gel electrophoresis of this highly appropriate molecular probe (40,000 MW). Germanely, there is a recent report which adds strong support to the rationale of this approach, specifically the demonstration of increased lung tissue albumin by immunologic assay following a virus infection of the lung (11).

The results of our HRP studies indicate that intermittent exposure of mice to 0.5 ppm NO_2 results in increased capillary permeability and an increased burden of plasma protein transport by the lung. The specific details are provided in the accompanying preprint "Serum and lung clearance of horseradish peroxidase; influence of low levels of nitrogen dioxide." Other results not covered in the published report, and presently incomplete, include tests carried out to refine the methodology, including tests for optimal HRP

dosage, clearance rate distribution curves, and optimal protein concentrations for gel loading.

The ultrastructural studies were carried out primarily for two purposes: a) to determine the subcellular distribution of horseradish peroxidase in the lung, as part of the HRP capillary permeability studies; and b) to detect structural alterations. Since all mice have some degree of endemic pneumonitis (there are no "germ-free" mice in this regard), the target of the search for ultrastructural abnormalities has been quantitative measurements of organelles and other structures by image analysis.

More specifically with respect to capillary permeability, special attention was given to the distribution of HRP in epithelial and endothelial pore systems, and the basement membranes in general. The majority of the effort has been exploratory. The latter included: 1) studies of HRP distribution according to time, e.g., 0 hours to 5-1/2 hours (see Table 20 for the workload details); 2) distribution of HRP according to intravenous dosage used; 3) the testing of different fixation procedures, including perfusion of the pulmonary arteries through the right ventricle, tracheobronchial tree perfusion, tissue mincing, and combined methodologies; 3) microwave fixation of the lung was also tested, but the preliminary results indicate that with the microwave energy used air within the tissues and air spaces apparently results in undesirable cell vacuolization. At the time of this report, definitive results have not been obtained that serve to distinguish the NO_2 exposed animals from the controls.

More specifically, with respect to the ultrastructural studies, a major direction is, as mentioned earlier, quantitative measurements by image analysis of subcellular structures, e.g., nuclei, mitochondria, lamellar bodies, and pinocytotic vesicles. For these studies, a macroviewer was ordered as part of the original equipment requested. However, the demonstrator on loan as of

May 18, 1978 and the new unit (received December 1978) were defective in that the camera lens mount that interfaced with the Vidicon camera of the image analyzer had an incompatible threading. As of the period of this report, the proper lens mount had not been received (on hand as of March 1979). Tissue processing for these studies was, however, on schedule and the workload that has been carried out is presented in Table 7 . All photomicrographs have been taken at a single magnification and 32 technically suitable Type 2 pneumocytes within each grid section have been photographed, and the 3X enlarged prints are awaiting macroviewer analysis.

In addition to the main study, a relatively small part of the research effort was directed to peripherally related projects. Some ultrastructural and manuscript support was given to the publication of a report on a newly recognized lung disease "Silicate pneumoconiosis in the farm worker (a reprint accompanies this report); partial support by the ARB was acknowledged[12]. Credit was also given to the ARB for the following two preliminary reports: "Decrease in spleen weights and spleen lymphoid nodules following exposure to 0.34 ppm nitrogen dioxide (NO_2)," Kuraitis,K.V., Richters,A., and Sherwin, R.P; and 2) "Urinary protein excretion of students; possible influence of air pollution," Barr, S., and Sherwin, R.P.[13,14]. Further discussion of the foregoing reports is presented in Section 11 D of this report.

The subject matter that follows summarizes the workload carried out during the period of this report.

1. See page 44
2. Experiment M114. This experiment involved 128 mice, half exposed to 0.34 ppm NO_2 (6 hours/day x 5 days/week x 6 weeks) studied by lung image analysis and an additional four pairs (eight) of mice studied by electron microscopy for the distribution of horseradish peroxidase (HRP) in lung tissue (10-minute postinjection interval) as part of a lung permeability study. The exposure period was from 12/21/77 to 2/7/78. The workload for

both the image analysis study and the ultrastructural invesigation is given in Table 2.

The Quantimet image analysis studies were carried out between August 15, 1978 through October 15, 1978. The left lung of each animal was analyzed (120 animals x eight slides/animal, or 960 total slides and 3840 microscopic fields). The specific details of the image analysis are given in Tables 3,21,22b. As pointed out in Table 3, there were four parts to the analysis, a computer study of the top 5 percentile, and a study of the bottom 5 percentile. The calculations involved statistical evaluations of nine variable measurements, five variable ratios, and five variable factors concerning experiment, duration, lobe, animal, and group (Table 3). The findings with respect to the various group measurements are given in Table 4, and a summary of the significant findings is provided in Table 5. Also, a portion of the data was collated earlier for a preliminary report, for presentation at the Federation meetings, April 9, 1979 (Fed. Proc., page 1352, #5945). A definitive report is in preparation (cf addendum to preprint: "Type 2 cell hyperplasia of lung following 0.4 ppm nitrogen dioxide exposure; an image analysis quantitation"). The analysis of the data has shown a significant increase in Type 2 pneumocytes based on a study of the left lungs alone and upper quartile statistical analysis of animal comparisons (and also by total data if one assumes that each lung field is an independent subset of the lung cell populations). The conclusion being drawn from the analysis is that the trend found in M114 and also M113 can best be documented by doubling animal numbers in order to satisfy the conservative statistical approach. The statistical approach is covered in more detail later on under Discussion. At the time of this report, a more intensive evaluation of the data is under way by our statistical consultants, and the data presently on hand will be supplemented by a new image analysis quantitation

of the right lower lobe. The latter will serve to guide the experimental design for the ongoing studies with expanded numbers of animals and will provide us with new information on comparisons between right and left lungs. With respect to the right lower lobe analysis of the 60 pairs of animals approximately half have been quantitated.

With respect to the ultrastructural study, the workload is presented in Table 2. A portion of this work has been published in a preliminary report (9) presented March 6, 1979(15) and is reported in detail in the definitive report (8; cf Preprint). The purpose of the study was to establish the distribution of HRP in the lung shortly after injection (10-minute interval) for correlation with the 4-1/2 hour postinjection findings, where the greater part of the HRP would have been cleared from blood and tissues. The high concentration of HRP at this early injection period affords certain data on the permeability of intercellular spaces, as discussed in detail in the preprint (8) submitted with this application. Most importantly, from the standpoint of a new and recent contribution, the 10-minute and the 4-1/2 hr HRP studies provide structural evidence in support of the conclusions derived from the biochemical investigation.

3) M115 is an experiment carried out between 4/28/78 and 6/9/78. The specific details concerning exposure and workload are given in Table 6. The workload includes studies of pregnant mice and their litters. The microscopic slide preparation was completed but the material has not been analyzed in view of the low newborn yield (only 26 of 54 presumably pregnant mice delivered) and technical difficulties with the experimental design, i.e., the experiment demonstrated that the Type 2 pneumocyte of the newborn does not have the perinuclear distribution of LDH that permits appropriately fast and accurate image analysis until the animals are from four to six weeks' old. An attempt to develop a means of achieving the contrast needed

for image analysis beginning at birth may be made in the future depending upon the outcome of the revised experimental design, Experiment M116, where the study of newborn mice begins at four weeks.

4) M116: This M116 study is the first image analysis application to the lungs of newborns, i.e., the developing lung, and was carried out between 6/20/78 and 9/28/78. It involved image analysis and electron microscopy following four and 12 weeks of NO_2 exposure. A third part of the study involved frozen section-light microscopy of lungs from animals previously injected with HRP, a new type of HRP study designed for the quantitation of macrophages by image analysis. Another new study introduced with Experiment 116 is the measurement of Type 2 cells using macroviewer image analysis of electron micrographs, specifically measurements of the sizes of Type 2 cells, nuclei, mitochondria, and lamellar bodies. The technical workload for this study has been completed, namely 32 Type 2 cells per animal and ten animals for each of the two exposure periods, i.e., a total of 20 animals and 640 Type 2 cells with several organelles per cell. The analysis part of this study has been held up due to faulty machining of the lens mount for the macroviewer. The specific details of the experiment and the workload accomplished are given in Table 7. All of the slide preparations from the three lobes of the lung have been completed. Image analysis has been completed for 20 pairs of males at four weeks and 20 pairs of males at 12 weeks, and with respect to the left lung. Computer analysis of the data is now in progress; the results are not yet available. A preliminary analysis of the data, based on the use of a desk top computer, has been summarized in Table 8, and includes significant differences for animal weights (Table 9).

A third <u>new</u> part of the study will be a comparison of the influence of NO_2 on female newborn animals in comparison to males. The latter analysis of the slide material on hand, is the next priority in our program.

With respect to the HRP-frozen section-light microscopy quantitation of macrophages in the lungs of these animals, our preliminary work indicates that the slides will have to be stained in order to provide sufficient background contrast for ratio determinations of macrophages to wall area. To accomplish this, a small pilot study is now in progress to select an optimal stain for the alveolar walls.

1. Experiment M113. 180 young adult mice, half of which were exposed intermittently to .8 ppm NO_2 (6 hrs/day x 5 days/week), were used in this study with an exposure period from 6/24/77 - 11/10/77. The experimental design (Table 1) included three parameters: 1) Type 2 study by image analysis using two exposure periods, six and 10 weeks with 35 pairs per each period; 2) Macrophage population study, employing HRP as markers, by frozen section and image analysis, using 10 pairs of mice following six and 10 weeks NO_2 exposure, and 3) Ultrastructural study of HRP distribution in lung using 10 pairs of mice after six weeks exposure to NO_2.

The frozen sectioning for LDH and HRP was completed 1/17/78. The Quantimet studies of LDH stained lungs of 10 pairs was started 3/15/78 and data analyzed by desk computer in June, 1978. The statistical data are summarized in Table 11.

d - Discussion:

The progress that has been made can be divided into two categories, the data analysis obtained in the 15-month period and the work done (M113, M114, M115, and M116) to get the program under way. With respect to data analysis, the single most important finding concerns the major direction of the proposal, namely that an intermittent 0.34 ppm level of NO_2 has resulted in a population shift. The increase in Type 2 cells found ($.01 < p < .025$) is believed to have occurred at the expense of damage to and loss of Type 1 cells. Most importantly, this damage and loss have occurred in the absence of pathologic evidence from light microscopy studies. Recent evidence indicates that electron microscopy semiquantitative studies can demonstrate Type 1 cell damage of this subtle nature (16). The latter type confirmation is planned for a later date, and in particular for image analysis rather than a subjective (0-4+) appraisal of damage. It should be noted that a semiquantitative EM study of Type 1 cells, and especially image analysis quantitation using EM photos, are extremely time consuming and cannot substitute for large volume image analyzer quantitation of Type 2 cells.

The finding of significant Type 2 cell hyperplasia requires confirmation and this is planned in ongoing studies where the numbers of animals will be doubled, from 60 pairs of control and exposed animals to 120 pairs (total - 240). The primary need for an expanded study is the presence of a variety of indigenous lung diseases in the mouse colony. Murine pneumonitis is especially important since it produces lesions independently of pollutant exposure and is very likely modulated by the pollutant. The murine pneumonitis complication does have an important usefulness; one of the most serious aspects of community air pollution is that it exacerbates a great variety of human lung diseases, non-infectious as well as

infectious. Thus, the mouse model is in this respect very appropriate to the human problem with air pollution.

In support of the principal finding of Type 2 cell hyperplasia are findings from three independent studies where a molecular probe (HRP) has provided evidence for increased protein leakage following exposure to ambient levels of NO_2. Pulmonary edema is a common denominator for almost all human lung responses to the inhalation of noxious substances, and protein leakage is a very early and sensitive measurement of impending or actual edema. Five of six test periods showed significant differences between the animal pairs by upper quartile analysis. The improved statistics with the non-parametric test again show that the complication of indigenous disease results in a control population that is not normally distributed. These new protein leakage studies have the support of our early work with lung lavage. There is also the support of the electron microscopic studies; they have clearly demonstrated that HRP is in fact retained in the lung tissue and that we are obtaining a measurement that does reflect the bi-directional transport of protein in the lung. Work carried out in the period of this report has substantially advanced the methodology so that we can expect greater sensitivity and accuracy in the measurements. Other accomplishments are demonstrations of a mesothelial cell transport system showing that the measurement of protein loss into the pleural cavity may be a very useful means of monitoring capillary permeability.

While the data obtained with respect to image analysis have been studied definitively only with Experiment M114, the other three experiments M113, M115, and M116 have advanced our methodology. M116 is also expected to provide data for a second definitive study; at the time of this report image analysis was just getting started. We have set aside the material from M115 since it was the prototype for M116; we learned from it that

the developing Type 2 cell in the mouse lung has a distribution pattern for lactate dehydrogenase that will require a modified staining reaction for the purpose of image analysis. This finding affords an opportunity to study altered maturation of the developing Type 2 cell secondary to pollution exposure. Both the M115 and M116 experiments are new directions, i.e., not mentioned in the original proposal. They are important directions since an influence of an air pollutant on the developing lung of an animal would potentially have very important meaning. Further discussion of M115, M116, and also M113 can be found in the earlier part of this section and in the text in general.

Other aspects of accomplishments relating to getting the program underway and developing the methodolcgy are the following: 1) the work has produced a definitive computer assisted image analysis and statistical analysis program; 2) improvements have been made in the HRP molecular probe methodology, including the derivation of formulae from linearity curves and advances in sensitivity of the HRP detection; 3) two new methodologies have been introduced, image analysis of the lung macrophage population, and quantitative measurements by macroviewer image analysis of subcellular structures of the Type 2 cell.

A final consideration is information gained from peripheral studies: 1) Alterations in spleen weight and lymphoid nodule sizes were detected using the image analyzer and material from M116. The details are provided in the accompanying report of the abstract presentation (13). If confirmed, the findings indicate not only a systemic influence of NO_2 at an ambient level but also an effect on a key part of the defense system. For orientation, it is useful to consider that most of the human deaths that have occurred with Babesiosis have been in people who have had their spleens

removed before they acquired the infection. There is of course the more general question as to the effects on the host defense system throughout the body; 2) A study of urinary protein excretion in six students on this Medical School campus indicates that a definitive study of this type should be set up and carried out in a broad sampling of the well population. No conclusions could be drawn but a combined methodology has been developed which appears to be very suitable for such a study (3); 3) The lungs from some of the animals of M116 were subjected to a pilot study for the quantitation of retrovirus present in the cells of lung, i.e., a molecular hybridization test which is an extremely sensitive means of detecting virus quantitatively. A 2.4x increase was found in the lungs of the exposed animals, in line with our belief that NO_2 may facilitate the expression of virus, in part or in whole, in the cells of the lung. Our earlier work with macrophages and the phenomenon of congregation provided a foundation for this hypothesis. Separate support for this investigation is being sought; 4) A small amount of ARB support was applied to two pathologic studies, one of which has resulted in a publication of a new kind of dust disease in the lungs of farm workers (12). The two most important aspects of this disease are: a) it appears to be related to the inhalation of an unidentified noxious pollutant that combines with clay silicates in the soil and/or in commercial products, and b) both clinical and pathologic features of the disease have masqueraded as a variety of lung abnormalities.

One other pathologic study is still in progress and concerns the light microscopy findings in the lungs of the well population as reflected in Medical Examiner cases. The preliminary indications are that subclinical disease is surprisingly common even in the young age group. Since the study is concerned with overt chronic inflammatory and other lesions, it

can be expected that image analyzer quantitation would greatly amplify the amount of lung damage sustained and in particular would largely answer the question of what cellular and tissue reserves remain. In the latter respect there have been no studies, human or animal, that have quantitated the Type 2 cell populations, especially in terms of the rate of depletion of Type 1 cell reserves. The studies being done under this proposal are the first step in this direction. The early findings, which we emphasize are preliminary, are consistent with the working hypothesis that the single most destructive effect of air pollutants is on the cellular ecology. Thus, we are seeking to measure the "normal" rate of cell and tissue depletion and then to determine the direct or modulating influence of air pollutants, singly and in combination, on the acceleration of the rate beyond the normal. Further, the ongoing studies will be concerned with the very critical question of reversibility of Type 1 cell damage and loss, and also the question of damage and loss of the Type 2 cell itself. With the loss of both cell types, bronchiolar cells may migrate into the alveoli, or the alveoli may be destroyed. Thus our ultimate goal is an appropriate inventory of the alveolar epithelial cells (Type 1 and 2) and the alveoli themselves.

A final consideration is the interaction we have shown between structural and functional alterations of mouse lung, serum, and spleen. In particular the putative shift in Type 2 cell population occurred at a level where increased protein leakage in the lung was also detected. This is in accord with the fact that the Type 1 cell is the critical barrier for alveolar protein leakage, and HRP retention and Type 2 increase reflect damage to and loss of this cell type. The spleen findings point to a systemic (immune) influence of NO_2. In addition, human studies add support to a systemic effect (urine protein increase suggested at ambient smog levels) and emphasize the potentiation for lung disease that can result when dusts and noxious substances combine (silicate pneumoconiosis).

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13. LIST OF INVENTIONS REPORTED AND PUBLICATIONS

No inventions have been produced through this work. The presentations and published abstracts that have resulted are as follows:

1) Sherwin, R.P., Okimoto, D.T., and Bernett, J.T.: Serum and lung clearance of exogenous horseradish peroxidase: influence of low levels of nitrogen dioxide (cf Preprint)

2) Sherwin, R.P., Okimoto, D., Mundy, D., and Bernett, J.: Clearance of horseradish peroxidase in the lungs of mice exposed to an ambient level of nitrogen dioxide. Lab. Invest. 40:49, 1979 (cf Addendum)

3) Sherwin, R.P., Kuraitis, K.V., and Richters, V.: Type 2 pneumocyte hyperplasia and hypertrophy in response to 0.34 ppm nitrogen dioxide; an image analyzer computer quantitation. Fed. Proc. 38#5945):1352, 1979 (cf Addendum)

4) Kuraitis, K.V., Richters, A., and Sherwin, R.P.: Decrease in spleen lymphoid nodules following exposure to 0.34 ppm nitrogen dioxide (NO_2). Fed. Proc. 38#5944:1352, 1979 (cf Addendum)

5) Sherwin, R.P., Barman, M.L., and Abraham, J.L.: Silicate pneumoconiosis of farm workers. Lab. Invest. 40:576-582, 1979 (Reprints will be submitted when received)

6) Barr, S.A., and Sherwin, R.P.: A correlation of urinary protein content and air pollution exposure of medical students. Submitted to Archives of Internal Medicine (cf Preprint)

Publications in preparation:

1) Sherwin, R.P., Kuraitis, K.V., and Richters, V.: Type 2 pneumocyte hyperplasia and hypertrophy in tesponse to 0.34 ppm nitrogen dioxide; an image analyzer computer quantitation. Definitive manuscript.

2) Sherwin, R.P., Lindal, R.G., and Sobin, S.S.: The lung of the well population; a Medical Examiner Case Study.

14. ABBREVIATIONS

BSS - basal salt solution

EM - electron microscopy

HRP - horseradish peroxidase

LDH - lactate dehydrogenase

MISG - Medical Imaging Science Group of University of Southern California

NO_2 - nitrogen dioxide

PAGE - polyacrylamide gel electrophoresis

ppm - part per million

SW(m) - Swiss-Webster mice (males)

T1 cell - Type 1 pneumocyte of the lung

T2 cell - Type 2 pneumocyte of the lung

3H-TdR - Tritiated thymidine

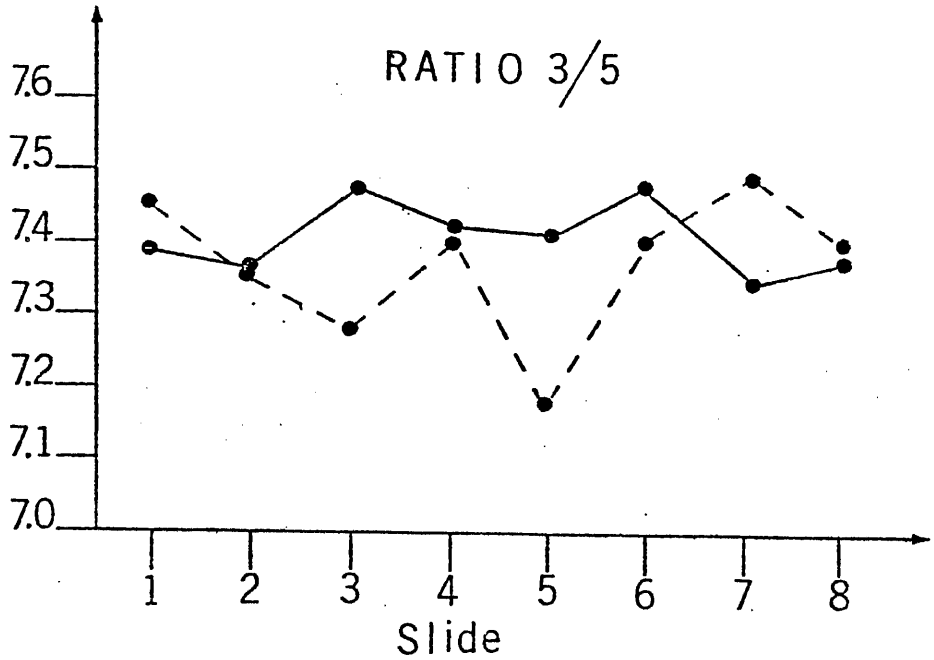
MISG - Medical Imaging Science Group, University of Southern California

UCC - University Computer Center, University of Southern California

ASCII - American Standard Code for Information Interchange

EBCDIC - Extended Binary Coded Decimal Interchange Code

Fig. 1. Two-way Nested Design on Data from Experiment M114
Internal Surface ÷ Wall Area (Legend page 8)



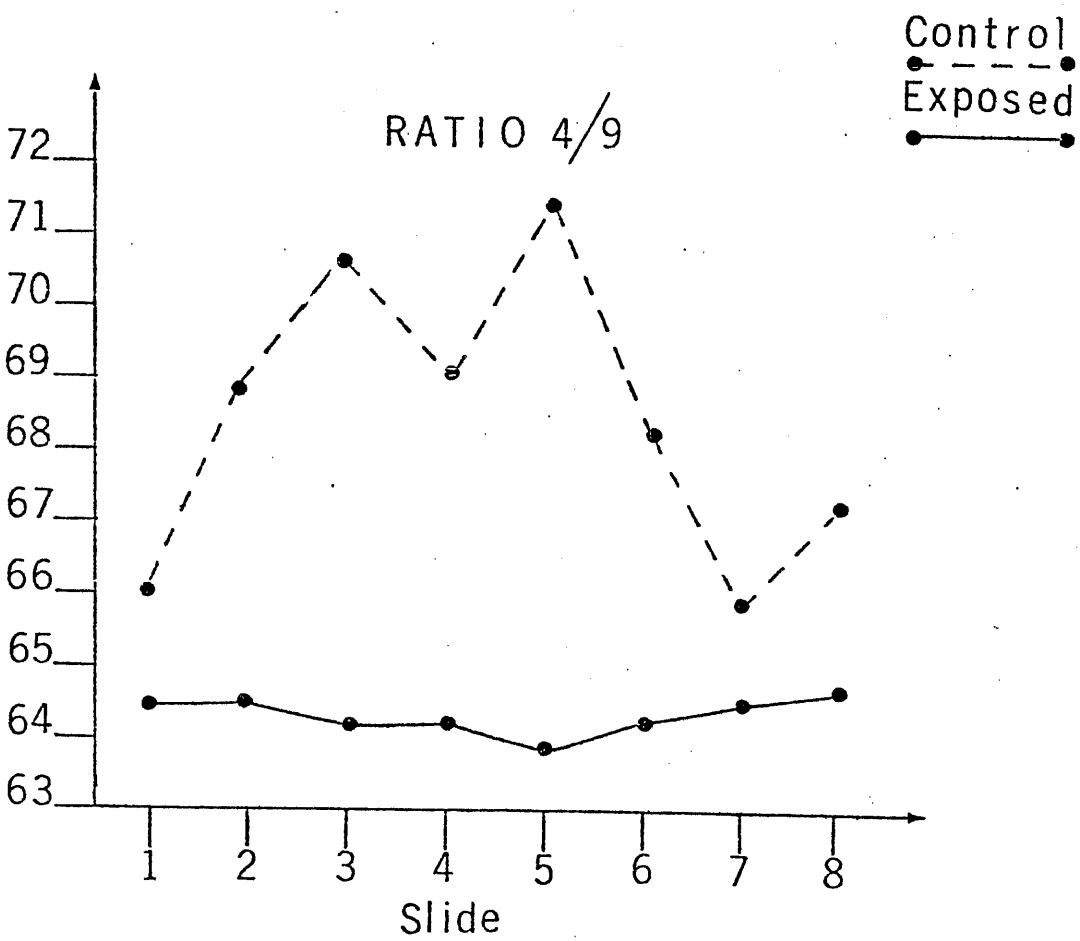


Fig. 2. Two-way Nested Design on Data from Experiment M114
Numbers of Type 2 Cells (Legend page 8)

Fig. 3. Two Factor Analysis of Variance of Data from Experiment M114 with Slide as a Repeated Measure (Legend page 8)

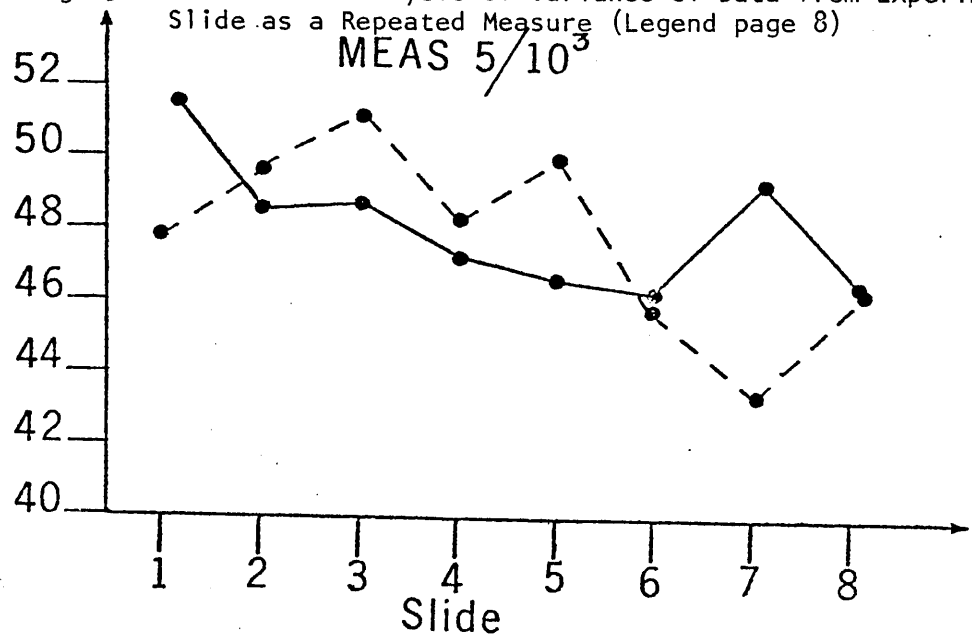


Fig. 3a

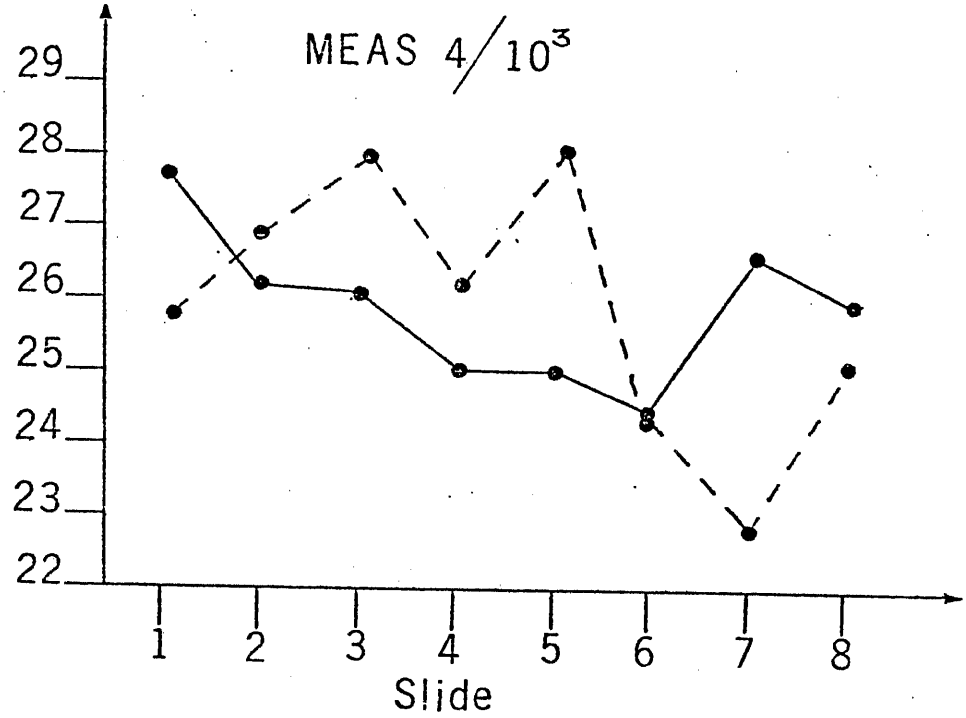


Fig. 3b

Control

Exposed

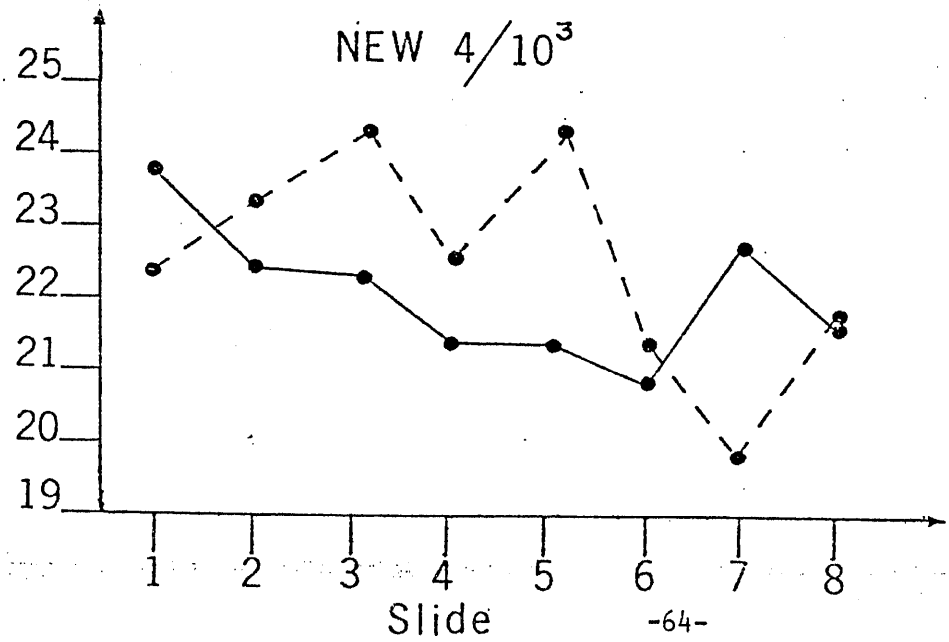


Fig. 3c

TABLE 1

M113
6/24/77 - 11/10/77

NO_2 : .8 ppm 6 hrs/day x 5 days/week
Duration : 1) 6 weeks
2) 10 weeks

Animals : S/W mice, ♂ young adults
90 controls
90 experimental

Experimental Design

1) Frozen sections : LDH, Safranin O, other

	6 week exposure					10 week exposure		
	No. animals	No. slides LDH	Saf. O	Other		No. animals	No. slides LDH	Saf. O	Other
RUL	35C+35X	560	140	140		35C+35X	560	140	140
RLL	35C+35X	560	140	140		35C+35X	560	140	140
LL	35C+35X	560	140	140		35C+35X	560	140	140
		1680	420	420			1680	420	420

Total No. Slides : LDH - 3360
Saf. O 840
Other 840

2) Frozen sections : HRP ; 4½ hrs post-injection ; .25 mg/gm

	6 week exposure					10 week exposure		
	No. animals	No. slides HRP				No. animals	No. slides HRP	
RLL	10C+10X	200				10C+10X	200	
LL	10C+10X	200				10C+10X	200	
		400					400	

Total No. Slides : 800 Grand Total: 5,840

3) EM : HRP - 4½ hrs. post-injection ; .25 mg/gm

		No. animals available	No. animals examined	blocks	No. grids examined	prints
RUL	6 weeks exposure	10C	5C	50	~25	81
		10X	5X	50	~25	89
	10 weeks exposure	10C	5C	50	~25	102
		10X	5X	50	~25	69

4) Quantimet measurements

	animals	No. slides	No. fields	No. Measurements Obtained: Type II count	Type II area	Alveolar wall area	Alveolar P	Linear intercept
LL 6wks. exposure	10C	77	385	2304	2310	2304	2310	2310
	10X	78	390	2340	2340	2334	2340	2340

TABLE 2

M114

12/21/77 - 2/7/78

NO_2 : .34 ppm ± .02 ; 6 hrs/day x 5 days/week

Duration : 6 weeks

Animals : S/W mice, ♂ young adults

64 control

64 experimental

Experimental Design

1) Frozen sections : LDH and Safranin O

	No. animals	No. slides LDH	No. slides Saf. O
RUL	60C+60X	960	240
RLL	60C+60X	960	240
LL	60C+60X	960	240
	Total :	2880	720

Grand Total: 3,600

2) EM : HRP - 10 min. post-injection ; 6 mg/animal

No. animals available	No. animals examined	blocks	No. grids examined	prints
4C	4C	40	~ 20	42
4X	4X	40	~ 20	85

3) Quantimet - Computer Study of LDH preparations

	Left lung								
					Measurements Obtained				
		No.			Type II		Alveolar wall		Linear
	animals	slides	fields		count	area	area	P	intercept
6 weeks	60C	480	1920		See Table 3				
	60X	480	1920						

4) Mouse weight comparisons

See Table 9

Table 3

KEY TO IMAGE ANALYSIS STUDY

I. Measurements by Image Analyzer

9 = Numbers of Type 2 cells, $\geq$ 8μ in diameter

8 = " " " " " , $\geq$ 12μ " "

7 = " " " " " , $\geq$ 10μ " "

6 = Area of Type 2 cells, $\geq$ 10μ " "

5 = Total Wall Area ÷ 10*

4 = Total Wall Area, $\geq$ 10μ sizing and minus Type 2 cell area

3 = Perimeter of Walls**

2 = Intercepts of Walls†

Missing Slide: AB AB AB AB

Missing Fields: AB

II. Ratio Measurements analyzed by computer

1) 4/7: Inverse of Numbers of Type 2 cells ($\geq$ 10μ)

2) 4/8: Inverse of Numbers of Type 2 cells ($\geq$ 12μ)

3) 6/7: Cell Area (Type 2 $\geq$ 10μ)

4) 5/8: Cell Area (Type 2 $\geq$ 12μ)

5) 3/5: Internal Surface Area/Wall area

6) 3/7: Internal Surface Area/number of Type 2 cells ($\geq$ 10 μ)††

Note: Type 2 cells and linear intercepts expressed in numbers per field. All other values in units (1 unit = $3.2\mu^2$)

A circle with a 103 micra diameter has a diameter of 20 mm projected on the screen (a magnification of 190x)
A triangle with base 500 micra=95mm on screen and 34,240 units; with an altitude of 435 micra, 108,750 square micra is equal to 34,240 Quantimet units, and 1 unit = 3.176 square micra.

*Computed by: multiplying by 10 and subtracting Type 2 cell area
**Perimeter is in effect a measure of internal surface area
†Linear intercept is a sampling type approach to wall-alveoli measurement
††A linear rather than area baseline

Table 4a. IMAGE ANALYSIS OF MOUSE LUNGS*
I. Variable Measurements
Experiment M114

	Mean	S.D.	S.E.	T Value	D.F.	Prob. 2-Tail
Numbers of Type 2 Cells (8μ+)						
Exposed	314.35	145.46	3.58	5.39	3211	p → 0
Control	288.05	130.04	3.30			
Numbers of Type 2 Cells (12μ+)						
Exposed	193.21	105.23	2.60	4.60	3185	p → 0
Control	177.11	91.48	2.33			
Numbers of Type 2 Cells (10μ+)						
Exposed	250.87	123.15	3.03	5.29	3208	p → 0
Control	229.18	108.20	2.74			
Area of T2 Cells (10μ+)						
Exposed	3129.09	2885.15	71.01	4.42	3210	p → 0
Control	2722.58	2265.38	57.34			
Alveolar Wall Area†						
Exposed	2120.70	1394.16	34.30	0.77	3211	0.441
Control	2082.02	1453.28	36.78			
Internal Surface Area†						
Exposed	2747.92	1231.38	30.30	2.95	3211	0.003
Control	2622.36	1180.57	29.88			
Linear Intercepts						
Exposed	8229.40	3749.41	92.25	3.45	3210	0.001
Control	7786.04	3526.56	89.29			

*Selected Data:††64 pairs of mice, half exposed to .34± .02 ppm NO_2, 6 hours/day x 5 days/week x 6 weeks.

† ÷10

Note: Type 2 cells and linear intercepts = Numbers/Field. All other values in area units (1 unit = $3.2\mu^2$) cf Table 3.

The initial use of a t test for independent means is discussed on pages 15-16.

†† Other data, variants of the above, gave similar results. (See Tables 15 and 18 for an expanded data analysis)

Table 4b: IMAGE ANALYSIS OF MOUSE LUNGS*
II. Variable Ratios
Experiment M114

	Mean	S.D.	S.E.	T Value	D.F.	Prob. 2 Tail
Wall Area/T2 Cells						
Exposed	82.84	28.82	0.71	-5.16	3207	$p \to 0$
Control	88.61	34.43	2.87			
T2 Size/T2 Cell Number						
Exposed	10.91	4.94	0.12	1.70	3206	0.09
Control	10.64	3.99	0.10			
Internal Surface Area/Wall Area						
Exposed	6.88	0.96	0.02	0.54	3210	0.59
Control	6.86	1.04	0.03			

*Selected Data: 64 pairs of mice, half exposed to .34± .02 ppm NO_2, 6 hours/day x 5 days/week x 6 weeks.

Note: a) Wall area ÷ Type 2 cells = inverse of numbers of Type 2 cells per lung field adjusted to amount of lung tissue (or numbers of alveoli)
b) Type 2 size ÷ Type 2 number = Type 2 cell area (1 unit = $3.2\mu^2$)

The initial use of a t test for independent means is discussed on pages 15-16.

Table 4c. PERCENTAGES OF EXPOSED AND CONTROL GROUPS COMPRISING THE UPPER 5% TAILS
Experiment M114

	Exposed			Control			p
	FO	FI	%	FO	FI	%	
T2 Cells (8μ+)	1545	108	6.53	1509	51	3.27	.0001ψ
T2 Cells (12μ+)	1530	111	6.76	1498	48	3.10	.0001ψ
T2 Cells (10μ+)	1543	109	6.60	1508	50	3.21	.0001ψ
T2 Cell Area	1540	111	6.72	1512	49	3.14	.0001ψ
Wall Area (÷10)	1579	74	4.48	1474	86	5.51	.1772
Wall Area†	1580	72	4.36	1473	88	5.64	.0957
ISA ††	1565	87	5.27	1488	73	4.68	.4424
Intercepts	1562	90	5.45	1490	70	4.49	.2110
T2 (10μ+) Ratio*	1594	57	3.45	1455	103	6.61	.0001ψ
T2 (10μ) Ratio* Wall (Sizing)	1578	62	3.78	1449	97	6.27	.0012ψ
T2 (12μ+) Ratio*	1574	67	4.08	1453	92	5.95	.0153ψ
T2 Cell Size	1540	110	6.67	1508	50	3.21	.0001ψ
ISA/Wall	1575	77	4.66	1477	83	5.32	.3905
ISA/T2	1573	78	4.72	1476	82	5.26	.4834

FO: # Fields 95%

FI: # Fields 5%

* Inverse

† 10μ sizing

†† ISA = Internal Surface Area (Perimeter)

ψ Highly significant

Note: See Table 3 for key, and Table 19b for Upper Quartile testing by Chi-Square analysis.

Table 4d. PERCENTAGES OF EXPOSED AND CONTROL GROUPS COMPRISING THE LOWER 5% TAILS
Experiment M114

	Exposed			Control			p
	FO	FI	%	FO	FI	%	
T2 Cells (8μ+)	1574	79	4.78	1479	81	5.19	.5906
T2 Cells (12μ+)	1554	87	5.30	1465	81	5.24	.9373
T2 Cells (10μ+)	1576	76	4.60	1475	83	5.33	.3428
T Cell Area	1571	80	4.85	1481	80	5.12	.7161
Wall Area (÷10)	1569	84	5.08	1484	76	4.87	.7846
Wall Area †	1565	87	5.27	1488	73	4.68	.4424
ISA ††	1571	81	4.90	1482	79	5.06	.8373
Intercepts	1570	82	4.96	1482	78	5.00	.9623
T2 (10μ+) Ratio*	1536	115	6.97	1513	45	2.89	.0001ψ
T2 Cell Size	1567	83	5.03	1481	77	4.92	.9088
T2 (12μ+) Ratio*	1533	108	6.58	1493	52	3.37	.0001ψ
ISA/Wall	1578	74	4.48	1474	86	5.51	.1785
ISA/T2	1536	115	6.97	1513	45	2.89	.0001ψ
T2 Ratio* (10μ wall)	1533	107	6.52	1493	53	3.43	.0001ψ

FO: # Fields 95%

FI: # Fields 5%

* Inverse

† 10μ sizing

†† ISA = Internal Surface Area (Perimeter)

ψ Highly significant

Note: See Table 3 for key, and Table 19b for Upper Quartile testing by Chi Square analysis.

Table 5

M115

4/28/78 - 6/13/78

NO_2 : .27 ppm ± .03

6 hrs/day x 5 days/week

Length of experiment: a) Newborns - 5 weeks ; LDH

6 weeks ; HRP

b) Adult ♀ - 6 weeks ; HRP

Animals used : S/W mice - a) adult ♀ , 20C and 20X

- b) newborns ♀ and ♂ , 48C and 48X

Experimental design:

1) LDH study ; frozen sections

Safranin O / frozen sections

		No. animals			No. slides cut and stained			
		available	cut		LDH	Saf. O		
Newborns NO_2 exposure *								
< 1 day	RUL, RLL, LL	3C + 3X	3C + 3X		90	0	No T2 ;	walls LDH +++ **
10 days	" " "	1C + 1X	1C + 1X		30	0	T2 + ;	walls LDH +++ **
14 days	" " "	1C + 1X	1C + 1X		30	0	T2 + ;	walls LDH +++ **
21 days	" " "	2C + 2X	2C + 2X		30	0	T2 + ;	walls LDH +++ **
26 days	" " "	1C + 1X	1C + 1X		30	0	T2 + ;	walls LDH + but can be used now to detect T2's on Quantimet
	* Length of exposure equals age of newborn animals							
	** Background too heavy for Quantimet work							
Newborns 5 week exposure	RUL	30C+30X	30C+30X		480	120		
	RLL				480	120		
	LL				480	120		
					1440	360		

Table 5 (Contd.)

2) HRP - macrophage study ; frozen sections ; 4½ hrs post-injection

a) Newborns - HRP given IP .35 mg / gm body wt.

b) Adult ♀ - " " " .5 mg / gm body wt.

		No. animals		No. slides cut and stained
		available	cut	HRP
Newborns 6 weeks exposure	RUL	10C+10X	5C+5X	100
	RLL			100
	LL			100

		No. animals		No. slides cut and stained
		available	cut	HRP
Newborns 6 week exposure	RUL	10C+10X	C+ X	100
	RLL			100
	LL			100
Postpartem ♀ 6 week exposure	RUL	10C+10X	0	
	RLL			
	LL			
~~F~~5 litters 6 week exposure	RUL	10C+10X	0	
	RLL			
	LL			

3) Mouse weight comparisons

see Table 9

4) Quantimet - Computer Study of LDH slides

not done

Table 6

M116

6/20/78 – 9/28/78

NO_2 : .3 ppm ± .05

6 hrs/day x 5 days/week

Length of experiment : Newborns – a) 4 weeks ; LDH , spleen

b) 5 weeks ; HRP , spleen

c) 12 weeks ; LDH , spleen

d) 13 weeks ; HRP , spleen

Animals used : S/W mice – a) adult ♀ , pregnant , 20C and 20X

b) newborns , ♂ – 87C and 91X

♀ – 116C and 97X

Experimental Design :

1) LDH study ; frozen sections :

	4 week exposure				12 week exposure			
	No. animals	No. slides LDH	No. slides Other		No. animals	No. slides LDH	No. slides Other	
♂ RUL	20C+20X	320	80		20C+20X	320	80	
♂ RLL		320	80			320	80	
♂ LL		320	80			320	80	
		960	240			960	240	
			Total: 1200					Total : 1200
♀ RUL	20C+20X	320	80		20C+20X	320	80	
♀ RLL		320	80			320	80	
♀ LL		320	80			320	80	
		960	240			960	240	
			Total: 1200					Total : 1200
						Grand total: 4800 slides		

Table 6 Contd. (M116)

2) HRP- macrophage study ; frozen sections ; 4½ hrs postinjection HRP given IP ; .5 mg/gm body wt.

	5 week exposure				13 week exposure		
	No. animals	No. slides HRP	No. slides Other		No. animals	No. slides HRP	No. slides Other
♂ RUL	not done				10C+10X	160	40
♂ RLL						160	40
♂ LL						160	40
						480	120
							Total : 600
♀ RUL	10C+10X	160	40		10C+10X	160	40
♀ RLL		160	40			160	40
♀ LL		160	40			160	40
		480	120			480	120
			Total: 600				Total : 600
						Grand total : 1800 slides	

3) Ultrastructural study - Type II pneumocytes

NO_2 Exposure	No. animals			No. blocks	No. grids	No. prints
4 weeks	5C	5X		100	40	320
12 weeks	5C	5X		100	40	320

4) Quantimet - Computer study

a) Type 2 cell - lung quantitation ; LDH

	Left lung animals	No. slides	fields		Type count	No. Measurements Obtained II area	Alveolar wall area	Alveolar wall P	Linear intercept
4 weeks	20C	160	640						
4 weeks	20X	160	640						
12 weeks	20C	160	640			Now in progress			
12 weeks	20X	160	640						

RUL
RLL } to be done

Table 7

RATIO OF WALL AREA TO TYPE 2 CELL NUMBER*

(M116, Desk Top Calculator)

Duration NO_2	Exp. Grp.**	N	$\bar{M}$	S.D.	Var.	S.E.	Confidence Limits 95%			
4 Weeks †	C	472	95.52	28.88	834.28	1.32	98.13	92.92	$\bar{M}_x - \bar{M}_c$	4.39
	X	482	99.91	29.42	865.86	1.34	102.54	97.29	D.F.	952
									t	2.32
									p	=.01
12 Weeks	C	474	86.05	20.65	426.69	0.94	87.91	84.19	$\bar{M}_x - \bar{M}_c$	-4.83
	X	520	81.21	14.64	214.35	0.64	82.47	79.96	D.F.	992
									t	4.28
									p	→0

* Numbers of Type 2 cells (+/or altered wall; wall measurements pending computer analysis); expressed as inverse ratio, i.e., higher ratio = smaller number.

** 20 pairs male mice @ 4 weeks (954 of 1280 Fields)
20 pairs male mice @ 12 weeks (994 of 1280 Fields)

† NOTE: The mouse lung at 4 weeks of age does not show the heavy perinuclear distribution of LDH in Type 2 cells that is characteristic of the young adult and adult mouse. Also, maturation of the alveolar walls is incomplete at the 4 week period. For these reasons, the 4 week quantitation was technically unsatisfactory, and will be repeated using a new approach now in a development stage. Of interest, it was the lungs of exposed animals primarily that proved to be most difficult to analyze (? slower development under the NO2 exposure and greater disruption of walls). See Table 10 for spleen data reversal 6vs.12 wks.

The 12 week animal groups were readily analyzed and again showed a highly significant difference in Type 2 cell numbers($p \rightarrow 0$);computer study pending.

Table 8

Mouse Body Weights (Gms)

Exper. No.	Age	Sex	Exposure Group	NO_2 Exposure (Weeks)	No.	Wt. ($\bar{M}$)	S.D.	p Value
M114	Adults	M	C	--	64	36.94	±2.91	N.S.
	"	M	X	6	64	36.65	±2.83	
M115	5 wks	M	C	--	30	16.39	±4.37	N.S.
	"	M	X	5	30	16.28	±2.43	
M116	3 wks	M	C	--	81	9.19	±2.14	<.005
	"	M	X	3	60	8.27	±2.10	
	"	F	C	--	88	8.61	±2.06	<.0005
	"	F	X	3	74	7.64	±2.15	
	4 wks	M	C	--	20	14.18	±2.72	N.S.
	"	M	X	4	20	13.87	±3.40	
	"	F	C	--	30	17.48	±4.18	<.025
	"	F	X	4	30	15.62	±3.67	
	12 wks	M	C	---	37	34.95	±1.94	<.01
	"	M	X	12	41	33.31	±3.49	
	"	F	C	--	56	28.86	±2.29	N.S.
	"	F	X	12	37	28.36	±2.40	

Table 9

Mouse Spleen/Body Weight Ratios *

Exper. No.	Age Weeks	Sex	Group	NO_2 Exposure (Weeks)	No.	Spleen/Body Wt. Ratios	S.D.	p Value
M116	12	M	C		37	.47295	±.09387	<.01
	12	M	X	12	41	.41787	±.09906	
	12	F	C		56	.54590	±.23014	N.S.
	12	F	X	12	37	.55129	±.08794	

*cf Ref. 13, and copy of published abstract

Table 10

Experiment M113, 0.8 ppm NO_2, 6 hrs/day x 5 days/week
Number Type 2 Cell/Wall Area*

A) Data Analyzed

1) Left lung from 90 control and 90 exposed animals processed

2) Desk top computer analysis of 20 animals (10 pairs); on-line computer program could not be carried out.

B) Statistical analysis by independent T-Test

1) Exposed animals

389.0000 Number of fields
,0486 Mean ratio
.0074 S.D.
.0000 Variance
.0003 Standard error of mean
.0493) 95% confidence limits
.0478)

2) Control animals

382.0000 Number of fields
.0469 Mean ratio
.0060 S.D.
.0000 Variance
.0003 Standard error of mean
.0475) 95% confidence limits
.0463)

*Expressed as fraction (subsequent analyes by inverse, i.e., wall area ÷ Type 2, which results in a whole number rather than these small ratio fractions).

Table 11: Statistical Analysis, Experiment M114
(60 control and 60 NO_2 exposed mice)*

Ratio		DF	$\bar{M}$	F	p value
4/7	A(G)	112	22,978.22	120.67	$p \to 0$
	G	1	22,970.69	1.00	NS
	Residual	3068	190.42		
4/8	A(G)	112	45,948.70	82.03	$p \to 0$
	G	1	47,489.00	1.03	NS
	Residual	3068	560.16		
6/7	A(G)	112	317.22	34.31	$p \to 0$
	G	1	66.87	0.21	NS
	Residual	3068	9.24		
5/8	A(G)	112	140,653.19	45.67	$p \to 0$
	G	1	110,129.00	0.78	NS
	Residual	3068	3,079.71		
3/5	A(G)	112	0.2143	68.50	$p \to 0$
	G	1	0.0392	0.18	NS
	Residual	3068	.0031		
3/7	A(G)	112	17,468.36	33.20	$p \to 0$
	G	1	9,374.00	0.54	NS
	Residual	3068	526.19		
4/9	A(G)	112	13,826.88	130.86	$p \to 0$
	G	1	12,087.19	0.87	NS
	Residual	3068	105.66		
5/7	A(G)	112	60,080.36	82.44	$p \to 0$
	G	1	52,348.00	0.87	NS
	Residual	3068	728.76		

*Two way nested design (animals nested within group; fields are considered replicates)

A(G): Control-exposed animal differences
G : Control vs exposed group differences

DF = Degree of Freedom
F = F Statistic

Residual: From total of 8 slides x 4 fields x 112 animals (complete data only)

For a statistical study where slide is the repeated measure, and for the definition of this analysis, see Tables 17 (mean values per slide) and Table 18 (two factor analysis of variance).

Table 12a: Experiment M114
Means and Standard Deviations for Control and Exposed Animals According to Slide Number*

Ratio 4/7

Slide	Exposed (N=50)	Control (N=44)
1	81.71±29.60	84.55±30.50
2	81.81±26.40	87.63±30.11
3	81.97±26.88	89.54±32.31
4	82.03±28.93	88.30±33.26
5	81.21±26.74	89.87±31.45
6	81.41±26.37	87.45±30.46
7	81.48±27.50	83.84±28.42
8	31.84±27.69	85.34±27.29
	Overall Mean 81.68	Overall Mean 87.064

Ratio 4/8

Slide	Exposed (N=50)	Control (N=44)
1	109.47±40.68	115.61±47.18
2	110.07±36.69	118.25±43.41
3	110.91±36.71	119.89±44.53
4	111.02±39.16	118.55±47.19
5	109.95±35.99	119.03±40.93
6	109.92±37.04	119.95±46.80
7	109.42±36.87	113.00±38.65
8	110.79±37.07	114.02± 37.82
	Overall Mean 110.192	Overall Mean 117.285

Ratio 6/7

Slide	Exposed (N=50)	Control (N=44)
1	10.79±3.70	10.37±3.43
2	11.10±3.74	10.76±3.18
3	10.68±4.74	10.96±3.40
4	10.68±4.12	10.87±3.97
5	10.83±4.43	11.28±4.21
6	10.87±4.03	10.37±3.12
7	11.25±4.59	10.23±2.91
8	11.31±5.81	10.72±3.18
	Overall Mean 10.938	Overall Mean 10.696

Ratio 5/8

Slide	Exposed (N=50)	Control (N=44)
1	211.96±72.54	226.94±87.88
2	213.11±68.74	227.45±80.38
3	218.08±70.25	231.76±85.27
4	217.92±68.16	231.04±93.88
5	214.63±62.96	223.59±67.29
6	215.95±66.64	235.81±92.21
7	211.55±66.26	221.80±68.70
8	214.87±66.85	221.30±68.64
	Overall Mean 214.750	Overall Mean 227.454

Ratio 3/5

Slide	Exposed (N=50)	Control (N=44)
1	0.74±0.10	0.75±0.11
2	0.74±0.09	0.74±0.10
3	0.75±0.10	0.73±0.11
4	0.74±0.09	0.74±0.10
5	0.74±0.08	0.72±0.10
6	0.75±0.09	0.74±0.09
7	0.74±0.09	0.75±0.09
8	0.74±0.10	0.74±0.87
	Overall Mean 0.741	Overall Mean 0.737

Ratio 3/7

Slide	Exposed (N=50)	Control (N=44)
1	113.34±27.72	118.99±28.71
2	113.28±28.24	120.51±32.83
3	117.60±31.411	121.03±32.95
4	116.02±27.07	123.65±40.66
5	113.84±23.91	116.56±24.88
6	116.34±24.39	122.91±33.62
7	111.96±24.78	119.44±27.09
8	113.30±27.44	119.45±26.19
	Overall Mean 114.457	Overall Mean 120.32

*Only animals with complete data used
See next page for key to ratios, or Table 3

Table 12b: Experiment M114
Means and Standard Deviations for Control and Exposed Animals According to Slide Number*

Ratio 4/9			Ratio 5/7		
Slide	Exposed (N=50)	Control (N=44)	Slide	Exposed (N=50)	Control (N=44)
1	64.48±23.80	60.05±23.59	1	157.39±48.48	163.79±50.08
2	64.57±21.17	68.84±23.14	2	156.75±43.41	167.33±50.65
3	64.20±21.55	70.70±24.96	3	160.28±46.48	170.29±53.36
4	64.31±23.13	69.04±24.97	4	160.06±46.79	170.40±58.80
5	63.92±21.58	71.47±25.66	5	157.13±43.33	167.15±46.04
6	64.24±21.03	68.19±22.64	6	158.72±43.53	169.83±52.57
7	64.53±22.12	65.89±22.30	7	155.79±43.22	163.24±46.65
8	64.72±22.58	67.22±21.06	8	156.75±44.10	164.37±45.18
	Overall Mean 64.37	Overall Mean 68.424		Overall Mean 157.86	Overall Mean 167.05

Ratio Abbreviations:

1. 4/7: Inverse of Type 2 cell number, 10μ+, wall area 4
2. 4/8: Inverse of Type 2 cell number, 10μ+, wall area 4
3. 6/7: Area of Type 2 cell
4. 5/8: Inverse of Type 2 cell number, 12μ+, wall area 5
5. 3/5: Internal surface area ± wall area 5
6. 3/7: Internal surface area ± Type 2 cell number, 10μ+
7. 5/7: Inverse Type 2 cell number, 10μ+, wall area 5

M/B. See also Table 3

Table 13. Experiment M114

Two-Factor Analysis of Variance Results with Slide as a Repeated Measure

	p- Values*			
Ratios	G	S	SG	
4/7	.347	.243	.249	
4/8	.368	.471	.680	
6/7	.733	.779	.306	
5/8	.362	.461	.937	
3/5	.839	.316	.023**	See Figure 1
3/7	.260	.223	.944	
4/9	.367	.248	.065**	See Figure 2
5/7	.322	.183	.980	

* The p-values under G indicate the significance of testing Exposed vs. Control animals: S for differences among the eight slides; and SG for interactions between factors S and G.

At the 0.5 significance level, only two interactions appear significant or close to it. There are no group differences and no slide differences.

See Table 3 for ratio key, and also Table 18 for definition as well as other ratio determinations, e.g., "4/7" vs "New 4/7." "New 4/7" represents a change in wall area calculation, specifically wall area minus Type 2 cell area.

Table 14a: Experiment M114

Chi-Square* Analyses of Contingency Tables. Animals are Classified as being Part of the Upper Quartile (U) or the Remainder (L)

Measure 9

	L	U	Total
X	38	19	57
C	47	10	57
Total	85	29	114

X_1^2=3.75 (.05<p<.1)

Measure 8

	L	U	Total
X	38	19	57
C	47	10	57
Total	85	29	114

X_1^2=3.75 (.05<p<.1)

Measure 7

	L	U	Total
X	38	19	57
C	47	10	57
Total	85	29	114

X_1^2=3.75 (.05<p<.1)

Measure 6

	L	U	Total
X	37	18	57
C	46	11	57
Total	85	29	114

X_1^2=2.63 (NS)

Measure 5

	L	U	Total
X	44	13	57
C	41	16	57
Total	85	29	114

X_1^2=0.42 (NS)

Measure 4

	L	U	Total
X	44	13	57
C	41	16	57
Total	85	29	114

X_1^2=0.42 (NS)

Measure 3

	L	U	Total
X	41	16	57
C	44	13	57
Total	85	29	114

X_1^2=0.42 (NS)

Measure 2

	L	U	Total
X	40	17	57
C	45	12	57
Total	85	29	114

X_1^2=1.16 (NS)

*Significant X_1^2 indicates an association between beingin a particular group (X or C) and belong to the upper quartile. p is reported if p < .1, otherwise non-significant (NS)

See Table 3 for ratio key

Table 14a: Experiment M114 (Contd.)

Chi-Square* Analyses of Contingency Tables.
Animals are classified as being part of the upper quartile (U) or the lower (L).

Ratio 4/7

	L	U	Total
X	48	9	57
C	37	20	57
Total	85	29	114

X_1^2=5.60 (.01 < p < 025)

Ratio 4/8

	L	U	Total
X	46	11	57
C	39	18	57
Total	85	29	114

X_1^2= 2.27 (NS)

Ratio 6/7

	L	U	Total
X	43	14	57
C	42	15	57
Total	85	29	114

X_1^2=0.05 (NS)

Ratio 5/8

	L	U	Total
X	45	12	57
C	40	17	57
Total	85	29	114

X_1^2=1.16 (NS)

Ratio 3/5

	L	U	Total
X	42	15	57
C	43	14	57
Total	85	29	114

X_1^2=0.05 (NS)

Ratio 3/7

	L	U	Total
X	44	13	57
C	41	16	57
Total	85	29	114

X_1^2- 0.42 (NS)

Ratio 4/9

	L	U	Total
X	47	10	57
C	38	19	57
Total	85	29	114

X_1^2=3.75 (.05 < p < .1)

Ratio 5/7

	L	U	Total
X	46	11	57
C	39	18	57
Total	85	29	114

X_1^2=2.27 (NS)

Note: 29/114 $\cong$ 25%

* Significant X_1^2 indicate an association between being in a particular group and belonging to the upper quartile. p reported if p<.1, otherwise nonsignificant (NS)

See Table 3 for ratio key

Table 14b

Two-Way Nested Design on Experiment M114. Animal Nested within Group; Slide and Field are Considered Replicates

Measure		DF	Mean Square	F Ratio	p Value
9	A(G)	112	327,573	32.96	p → 0
	G	1	323,856	0.99	NS
	Residual	3067	9,939		
8	A(G)	112	169,835	32.99	p → 0
	G	1	122,024	0.72	NS
	Residual	3067	5,148		
7	A(G	112	230,859	32.51	p → 0
	G	1	217,392	0.94	NS
	Residual	3067	7,101		
6	A(G)	112	107,837,392	33.25	p → 0
	G	1	91,516,928	0.85	NS
	Residual	3067	3,242,773		
5	A(G)	112	10,420,183,040	51.50	p → 0
	G	1	58,261,504	0.06	NS
	Residual	3067	202,329,168		
4	A(G)	112	4,300,115,968	48.17	p → 0
	G	1	26,804,224	0.01	NS
	Residual	3067	89,261,632		
3	A(G)	112	3,270,255,616	47.24	p → 0
	G	1	675,282,944	0.21	NS
	Residual	3067	69,220,512		

See Table 3 for ratio key

Table 14b Contd.

Two-Way Nested Design on Experiment M114. Animal Nested within Group; Fields are Considered Replicates*

Measure		DF	Mean Square	F Ratio	p Value
2	A(G)	112	289,475,840	45.99	p → 0
	G	1	78,778,368	0.27	NS
	Residual	3067	6,294,144		
New 4	A(G)	112	3,493,660,160	54.38	p → 0
	G	1	217,186,304	0.06	NS
	Residual	3067	64,243,968		
New 4/7	A(G)	112	22,468	129.69	p → 0
	G	1	25,392	1.13	NS
	Residual	3069	173		
New 4/8	A(G)	112	46,313	81.41	p → 0
	G	1	51,724	1.12	NS
	Residual	3069	569		
New 4/9	A(G)	112	13,263	159.04	p → 0
	G	1	13,609	1.03	NS
	Residual	3069	83		
Ratio 5/9	A(G)	112	33,445	131.58	p → 0
	G	1	26,747	0.80	NS
	Residual	3069	254		

* See Table 3 for ratio key

Note: For a statistical study where slide is the repeated measure, and for the definition of this analysis, see Tables 17 (mean values per slide) and Table 18 (two factor analysis of variance). A different weighting of the data results from mean values based on fields as opposed to slides.

Table 15a: Experiment M114

Means ± Standard Deviations for Exposed and Control Groups by Slide.
Only Animals with Complete Data Used

Measure 9			Measure 8		
Slide	Exposed (N=50)	Control (N=44)	Slide	Exposed (N=50)	Control (N=44)
1	413.21±130.68	373.95±136.80	1	251.94± 89.44	226.14± 97.11
2	396.83±124.03	381.24±122.92	2	241.57± 90.96	233.82± 89.13
3	396.24±152.46	386.21±135.83	3	239.94±105.51	240.40± 96.34
4	381.38±146.46	368.42±130.55	4	230.05±105.04	228.37± 95.57
5	381.46±129.49	380.45±120.08	5	229.61± 94.57	234.74± 88.41
6	370.63±133.03	350.02±117.47	6	225.72± 94.64	210.66± 82.16
7	396.43±146.23	340.69±104.91	7	243.50±106.75	206.61± 74.75
8	384.75±141.29	369.32±106.83	8	234.20±104.75	227.52± 75.93
	Overall Mean 390.10	Overall Mean 368.77		Overall Mean 237.06	Overall Mean 226.03

Measure 7			Measure 6		
Slide	Exposed (N=50)	Control (N=44)	Slide	Exposed (N=50)	Control (N=44)
1	329.24±108.24	296.31±113.89	1	3853.49±2452.66	3393.09±2077.54
2	316.53±106.16	304.12±103.47	2	3785.18±2227.57	3540.70±1997.93
3	315.57±127.83	311.14±114.11	3	3759.75±2777.51	3701.06±2109.66
4	302.89±122.46	293.22±109.43	4	3601.19±2625.77	3511.50±2556.87
5	303.20±110.06	304.40±101.45	5	3597.05±2501.33	3726.19±2662.62
6	295.03±111.70	278.01± 98.24	6	3519.60±2284.69	3115.80±1758.52
7	317.17±123.83	271.13± 88.61	7	3962.49±2840.90	2916.04±1443.11
8	306.70±119.36	295.23± 90.67	8	3851.02±3147.98	3355.50±1841.32
	Overall Mean 310.78	Overall Mean 294.19		Overall Mean 3741.03	Overall Mean 3407.42

Measure 5			Measure 4		
Slide	Exposed (N=50)	Control (N44)	Slide	Exposed (N=50)	Control (N=44)
1	51534.58±23896.55	47804.88±24714.48	1	27720.70±15166.38	25834.84±15911.93
2	48689.28±20465.65	49582.86±21443.20	2	26291.10±13443.72	26910.00±14118.00
3	48878.42±22209.38	51256.34±24431.98	3	26124.70±13885.51	28122.52±16084.82
4	47244.30±22666.21	48117.29±22279.39	4	25066.70±14285.25	26124.20±15391.56
5	46938.86±20658.17	50498.16±21997.11	5	25008.20±12815.68	28089.02±15790.05
6	46167.70±20438.79	45933.38±21214.42	6	24442.40±12164.77	24457.02±13658.00
7	49228.74±24124.14	43420.57±17959.96	7	26831.36±15927.55	22914.99±11277.74
8	46964.06±21000.24	46944.45±17734.92	8	26008.32±15330.95	25192.70±11880.47
	Overall Mean 48204.20	Overall Mean 47944.54		Overall Mean 25936.39	Overall Mean 25955.32

Table 15 b: Experiment M114

Means ± Standard Deviations for Exposed and Control Groups by Slide.
Only Animals with Complete Data Used

Measure 3			Measure 2		
Slide	Exposed (N=50)	Control (N=44)	Slide	Exposed (N=50)	Control (N=44)
1	35914.22±13278.02	33170.27±13184.34	1	10699.17±4038.73	9876.23±3960.93
2	34208.68±11296.10	34752.84±11852.98	2	10216.65±3402.25	10254.38±3437.13
3	34779.48±13680.29	35210.27±13430.14	3	10386.85±4080.94	10477.30±3970.70
4	33397.00±13133.94	33770.66±12176.30	4	9993.61±3925.63	9982.64±3526.98
5	33399.80±12456.32	34276.25±10845.37	5	9980.56±3716.16	10164.63±3239.11
6	33135.34±12844.41	32466.86±11852.53	6	9888.08±3856.75	9641.91±3504.31
7	34183.86±13473.37	31103.98±10285.22	7	10271.52±4121.86	9186.82±3048.05
8	33292.78±12541.91	33327.02± 9729.99	8	9980.73±3758.31	9834.14±2851.65
	Overall Mean 34038.70	Overall Mean 33509.54		Overall Mean 10176.82	Overall Mean 9926.95

New 4			New 4/7		
Slide	Exposed (N=50)	Control (N=44)	Slide	Exposed (N=50)	Control (N=44)
1	23867.22±13628.31	22441.77±14295.30	1	70.92±29.20	74.18±29.90
2	22505.94±11925.92	23369.35±12611.73	2	70.70±26.00	76.87±29.60
3	22365.00±12157.20	24421.48±14396.50	3	71.28±26.45	78.58±31.42
4	21484.02±12346.54	22612.75±13300.87	4	71.38±28.23	77.43±32.22
5	21411.26±11333.39	24362.86±13687.20	5	70.38±26.43	78.59±29.91
6	20922.90±10750.40	21341.21±12312.46	6	70.54±26.23	77.07±29.84
7	22868.92±13830.71	19998.93±10288.16	7	70.24±26.68	73.61±27.84
8	21701.38±12142.96	21837.22±10562.86	8	70.45±26.33	74.62±26.68
	Overall Mean 22140.49	Overall Mean 22547.82		Overall Mean 70.73	Overall Mean 76.37

New 4/8			New 4/9		
Slide	Exposed (N=50)	Control (N=44)	Slide	Exposed (N=50)	Control (N=44)
1	95.31±40.72	101.87±47.13	1	55.84±23.24	57.70±22.49
2	95.55±36.99	104.14±43.31	2	55.66±20.43	60.22±22.46
3	96.77±37.13	105.59±44.07	3	55.67±20.77	61.87±23.98
4	96.92±39.09	104.36±46.83	4	55.83±22.27	60.37±23.78
5	95.68±36.46	104.47±40.05	5	55.28±21.00	62.40±24.10
6	95.66±37.64	106.28±46.82	6	55.54±20.60	59.90±21.81
7	94.77±36.93	99.52±38.52	7	55.48±21.06	57.73±21.59
8	95.93±36.74	99.99±37.61	8	55.58±21.03	58.64±20.37
	Overall Mean 95.82	Overall Mean 103.28		Overall Mean 55.61	Overall Mean 59.85

Table 15c: Experiment M114

Means ± Standard Deviations for Exposed and Control Groups by Slide. Only Animals with Complete Data Used

Ratio 5/9		
Slide	Exposed(N=50)	Control(N=44
1	413.21±130.68	373.95±136.80
2	396.83±124.03	381.24±122.92
3	396.24±152.46	386.21±135.83
4	381.38±146.46	368.42±130.55
5	381.46±129.49	380.45±120.08
6	370.63±133.03	350.02±117.47
7	396.43±146.23	340.69±104.91
8	384.75±141.29	369.32±106.83
	Overall Mean 390.10	Overall Mean 368.77

See Table 3 for ratio key

Table 16. Experiment M114

Two-Factor Analysis of Variance Results with Slide as a Repeated Measure

	p-Values* Group (G)	p-Values* Slide (S)	p-Values* Group-Slide Interaction (GS)	
Measure 9	.371	.008	.134	
Measure 8	.514	.041	.070	
Measure 7	.406	.014	.109	
Measure 6	.431	.457	.101	
Measure 5	.949	.036	.034	See Figure 3a
Measure 4	.994	.088	.021	See Figure 3b
Measure 3	.819	.041	.100	
Measure 2	.717	.050	.110	
New 4	.862	.052	.022	See Figure 3c
New 4/7	.315	.216	.451	
New 4/8	.344	.481	.819	
New 4/9	.330	.179	.110	
Ratio 5/9	.327	.120	.693	

* The p-values under G indicate whether there exist significant differences between Exposed and Control animals, under S for differences among the eight slides, and under GS for interactions between factors S and G.

At the .05 significance level, there were six significant slide differences and three significant interactions. Two slide differences and and one additional interaction approached significance.

See Table 3 for ratio key

Table 16 (Contd.)

Note: In this Table 18, SLIDE is the repeated measure, i.e., the lung has been sectioned in a sagittal plane from hilum to the lung periphery and the analysis is concerned with a comparison of the eight different mediolateral plane areas with each other. This tests the concept that cells in the medial plane may have a different "normal" baseline and different susceptibilities than those in the mid and lateral portions. Moreover, it is also possible that Type 2 cell hyperplasia in some parts of the lung may by averaging of lung data be "neutralized" by damage so severe in other parts as to destroy Type 2 cells as well as Type 1 cells, i.e., the "early" lesion of Type 1 cell loss later followed by Type 2 cell loss is undoubtedly one of the pathogenic pathways to emphysema.

As an example, Measure "4/9" is a quantitation of numbers of Type 2 cells per field, adjusted to wall area. The mean values for each of the eight slides is given in Table 14b and plotted in Fig. 3c (Page 95). Interaction between the slides of the control and exposed gorups is significant, meaning that the responses interact rather than run parallel. Also, variability for the exposed group is much less, a finding commonly noted in the other analyses. Note that the lower the number, the greater is Type 1 cell loss and Type 2 cell increase. The variability of the control group (vs the exposed animals) is consistent with the concept that the patchy loss of lung reserves in the control animals (due to "ordinary" assaults on the lung) has become widespread and almost uniform in the exposed group. However, the data analysis is of a preliminary nature, and the experimental study itself at a beginning stage.

Table 17: Experiment M114

Chi-Square* Analyses of Contingency Tables. Animals are Classified as being Part of the Upper Quartile (U) or the Remainder (L)

New 4

	L	U	Total
X	44	13	57
C	41	16	57
Total	85	29	114

X_1^2=0.42 (NS)

New 4/7**

	L	U	Total
X	48	9	57
C	37	20	57
Total	85	29	114

X_1^2=5.60 (

New 4/8

	L	U	Total
X	45	12	57
C	40	17	57
Total	85	29	114

X_1^2=1.16 (NS)

New 4/9

	L	U	Total
X	47	10	57
C	38	19	57
Total	85	29	114

X_1^2=3.75 (.1>p>.05)

Ratio 5/9

	L	U	Total
X	46	11	57
C	39	18	57
Total	85	29	114

X_1^2=2.27 (NS)

*Significant X_1^2 indicates an association being in a particular group (X or C) and belong in the upper quartile. p is reported if p < .1, otherwise non-significant (NS)

**Considered optimal measurement since derived from Type 2 cells 10μ or greater, with the same wall sizing and Type 2 cell area subtracted from the wall area

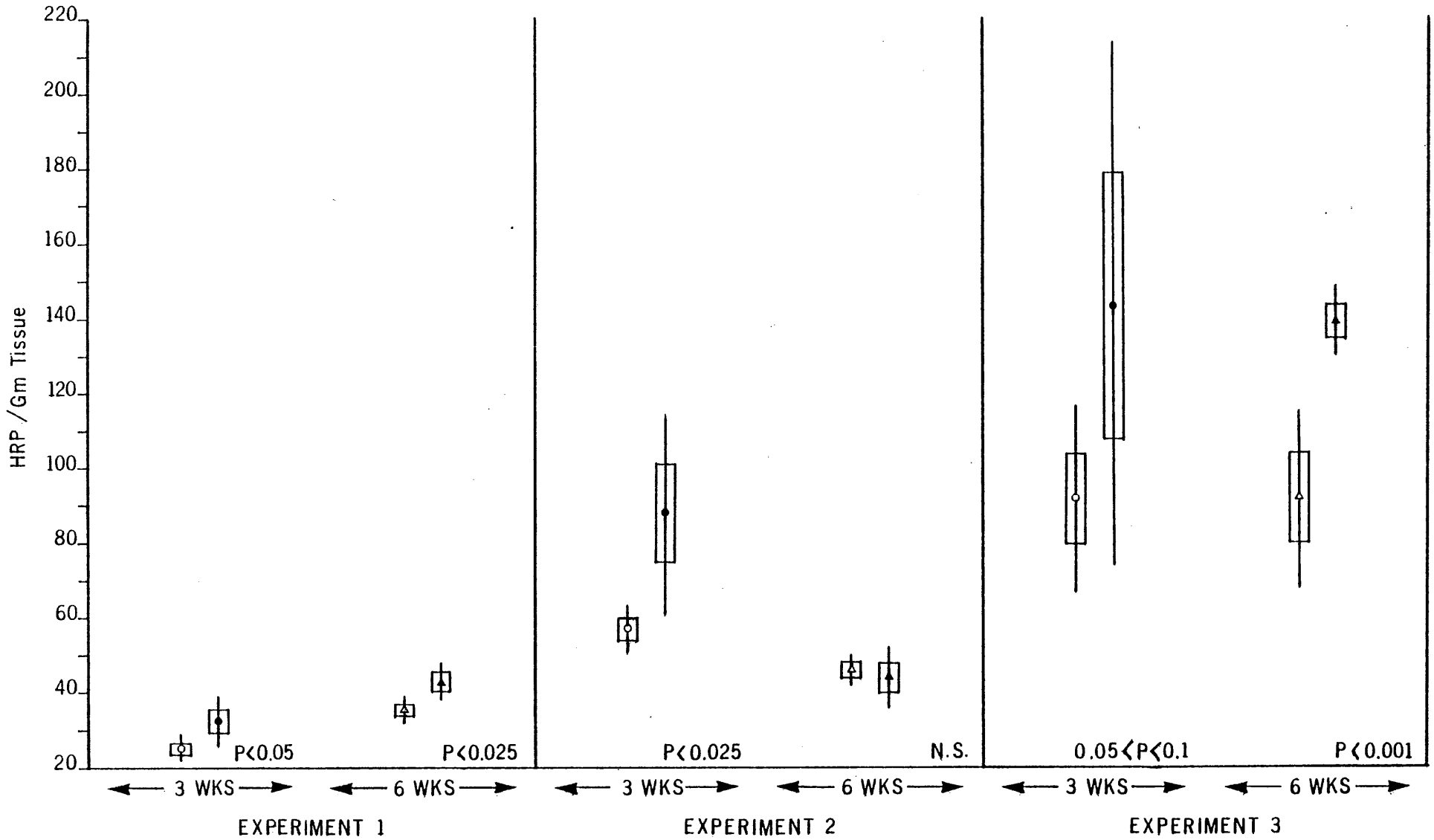


Table 18a: HRP Tests; Upper Quartile Statistical Analysis
cf Next page

Table 18a (Contd.)

Legend:

Center open circles: Control animals at three weeks post NO_2 exposure; open triangle at six weeks.

Center solid circles: Exposed animals at three weeks; solid triangle at six weeks

Rectangles: Standard error

Vertical lines: 95% Confidence limits

(see Preprint)

Table 19 : Workload of Horseradish Peroxidase - Protein Leakage Testing and HRP Sensitivity (RHZ)

I. Baseline Studies

1. Linearity testing: a) Schrauen vs. Hemè-iron
   b) Intra- and inter-experiment testing
   c) HRP lot numbers

2. Clearance rate: lungs, serum, kidneys

3. Optimal dosage determination: a) Organ concentration
   b) Blood loading
   c) Loading gel (PAGE)

4. Storage effects on degradation: a) Frozen intact lungs and other organs: serum
   b) Frozen organ homogenates
   c) Frozen gels
   d) Stained gels (next day reading; light exclusion)

5. Controlled injection rate and pressure (sage pump)

6. Gel staining: Schrauen vs. heme-iron

II. Definitive Experiments

1. Independent tests (3) of concentration of HRP in lung, kidney, and serum:
   a) Integrated chart analysis
   b) Statistical analysis

2. Tests for dosage vs. lung and serum concentrations

3. Brain concentration studies

4. Electron microscopic studies: a) Clearance in lung
   b) Distribution in lung
   c) Optimal fixation tests
   d) Optimal HRP staining tests
   e) Optimal tissue staining
   f) Blood HRP concentration vs. distribution

5. Macrophage identification and quantitation:
   a) Light microscopy and EM
   b) Image analysis of electron micrographs

TABLE 20: DATA COLLECTION FORMAT FOR M114

Good slide - good architecture a LDH
↑ bronchi/ bronchioles/ vessels
∴ ↑ # editing.

"43/55" 022 1 AB

00597	00409	00490	07746	11726	68946	59247	18252	AB
00578	00405	00489	09533	11447	71803	52454	15832	AB
00590	00407	00490	08116	10746	65461	51721	15576	AB
00503	00332	00411	05502	10010	56097	53247	16259	AB
00728	00500	00604	12911	12724	81453	52355	16652	AB
00554	00397	00472	08602	10893	67732	49970	15210	AB
00524	00370	00430	07405	10680	62967	53140	16294	AB
00730	00523	00602	12509	14317	90960	62520	19354	AB
00746	00507	00602	12162	12917	81170	55878	17287	AB
00724	00486	00602	09913	13052	81793	60356	17976	AB
00506	00355	00418	06928	10235	60474	50817	15520	AB
00677	00482	00562	11692	13363	86512	57552	17602	AB
00700	00507	00591	11874	12806	81737	55947	17157	AB
00443	00299	00358	05555	07511	44974	36034	11048	AB
00515	00343	00424	06024	09635	57069	47751	14803	AB
00612	00414	00506	10407	11923	77570	50636	15670	AB
00561	00386	00463	06819	09799	56820	49785	15414	AB
00558	00361	00466	04978	09821	55581	53028	16424	AB
00419	00275	00336	03848	07655	42364	42974	13152	AB
00398	00277	00337	06027	07461	46365	35010	10568	AB
00714	00490	00588	10517	12289	75046	55812	17621	AB
00469	00303	00380	04830	08135	45637	44273	13423	AB
00393	00270	00323	05967	07988	47729	37958	12063	AB
00490	00335	00407	08038	08183	50052	38896	11896	AB
00578	00380	00473	06353	10051	57066	52243	16225	AB
00571	00387	00465	06764	10118	57539	52946	16357	AB
00404	00279	00342	04720	08043	44103	45232	13833	AB
00544	00365	00448	08136	10210	60343	51837	15819	AB
00586	00357	00454	05119	10025	55894	54415	16803	AB
00487	00315	00395	04825	08450	46308	46098	14222	AB
00457	00285	00367	04211	08712	47800	50279	15150	AB
00515	00328	00416	05209	09517	52614	53653	16290	AB

* See Table 3 for key, and compare with format for M113(Table 22a); Also see Table 22b

Table 21: M116: Quantitative Ultrastructural Study of Type II Cells and their Organelles

Animal No.	Sex	Age	Duration of NO_2 Exposure	Tissue Used	Block #	Sections Made: 1μ Epoxy	Sections Made: EM Grids	No. Photos Taken
C14	M	4 Weeks	4 Weeks	Cardiac Lobe	C8574 C8581	X	X	32
X14	"	"	"	"	C8586 C8588	X	X	32
C15	"	"	"	"	C8600 C8595	X	X	32
X15	"	"	"	"	C8003 C8012	X	X	32
C16	"	"	"	"	C8617 C8614	X	X	32
X16	"	"	"	"	C8629 C8623	X	X	32
C17	"	"	"	"	C8633 C8624	X	X	32
X17	"	"	"	"	C8645 C8652	X	X	32
C18	"	"	"	"	C8654 C8659	X	X	32
X18	"	"	"	"	C8666 C8663	X	X	32

Note: This study will measure nuclei, mitochondria and lamellar bodies of Type 2 cells, using 32 electron micrographs per animal and the image analyses with microviewer.

X = Generally one grid and one epoxy section for each lung tissue block; 16 electron micrographs from each grid and 32/animal.

Table 21: Contd. M116: Quantitative Ultrastructural Study of Type II Cells and their Organelles

Animal No.	Sex	Age	Duration of NO_2 Exposure	Tissue Used	Block #	Sections Made 1μ Epoxy	Sections Made EM Grids	No. Photos Taken
C65	M	12 Weeks	12 Weeks	Cardiac Lobe	C8842 C8845	X	X	32
X65	"	"	"	"	C8851 C8852	X	X	32
C66	"	"	"	"	C8867 C8869	X	X	32
X66	"	"	"	"	C8873 C8879	X	X	32
C67	"	"	"	"	C8894 C8897	X	X	32
X67	"	"	"	"	C8886 C8890	X	X	32
C68	"	"	"	"	C8902 C8909	X	X	32
X68	"	"	"	"	C8920 C8915	X	X	32
C69	"	"	"	"	C8928 C8929	X	X	32
X69	"	"	"	"	C8833 C8938	X	X	32

X = Generally one grid and one epoxy section for each block; 16 photos from each grid. This listing is example of format for workload inventory.

Table 22: Computer Assisted Statistical Analysis - Work Listing

Job	00052, Robert Erbe, 11/27/78	M114 Data Set 1: Statistical analysis
	00053, Robert Erbe, 11/28/78)	) M114 Data Set 1
	3252, Michael Jones, 11/29/78)	
	3440, Michael Jones, 11/29/78	Descriptive statistics M114
	00124, Robert Erbe, 12/28/78	M114 Data Set 2
	00126, Erbe/Jones, 12/28/78	M114 Data Set 2 Print-out
	975, Michael Jones, 1/3/79)	) M114 -1&2 Statistical analysis
	2115, Michael Jones, 1/3/79)	
	1956, Michael Jones, 1/3/79	Combined T tests - Sets 1&2 M114
	5609, Michael Jones, 1/17/79	Descriptive statistics - Set 2 M114 (M2 &M3)
	5506, Michael Jones, 1/17/79	Descriptive statics - Set 2 M114 (M9 &M4)
	5700, Michael Jones, 1/17/79	Descriptive statistics - Set 1 (M2 & M3)
	5701, Michael Jones, 1/17/79	Descriptive statistics - Set 1 (M9 & M4)
	4130, Michael Jones, 1/31/79	T tests M114 Set 2
	3916, Michael Jones, 1/31/79	
	102, Michael Jones, 1/31/79	Descriptive statistics Set 1 (M2 & M3) corrected data (c)
	4131, Michael Jones, 1/31/79	Combined T tests M114 (Sets 1&2)(b)
	4127, Michael Jones, 1/31/79	M114 data 1 T tests (c)
	3852, Michael Jones, 1/31/79	M114 Data 2 Descriptive statistics (c)
	127, Michael Jones, 1/31/79	M114 Descriptive statistics: Set 2 (c)
	101, Michael Jones, 1/31/79	M114 Descriptive statistics: Set 1 c c)
	4734, Michael Jones, 1/31/79	M114 data analysis 2 - upper 5% test
	7316, Michael Jones, 2/2/79	M114 data analysis 1 &2 - upper 5% test
	2293, Michael Jones, 2/28/79	
	7205, Michael Jones, 2/2/79	M114 data set 1 analysis upper 5%
	366, Erbe/Jones, 2/8/79	M116 - distribution of data
	715, Michael Jones, 2/27/79	M114 data analysis 1 lower 5%
	2293, Michael Jones, 2/28/79	M114 data analysis 1&2 lower 5%

Table 22: Computer Assisted Statistical Analysis - Work Listing - Contd.

Job:	4419, Michael Jones, 4/25/79	M114 data listing
	4386, Sarah Schoentgen, 4/25/79	M114 ratio tests: A
	3785, Sarah Schoentgen, 4/25/79	M114 ratio tests: B
	5508, Sarah Schoentgen, 4/26/79	M114 ranking of ratios: A
	5504, Sarah Schoentgen, 5/8/79	M114 ranking of ratios: B
	4552, Michael Jones, 5/2/79	M114 creation of data
	4417, Michael Jones, 5/2/79	M114 M9-M2 and new M4
	4722, Sarah Schoentgen, 5/2/79	M114 data listing
	4790, Sarah Schoentgen, 5/2/79	M114 programming
	7079, Michael Jones, 5/4/79	M114 upper 25%; M9-M6
	7081, Michael Jones, 5/4/79	M114 upper 25%; M5-M2
	6960, Michael Jones, 5/4/79	M114 M2-M4; 4/7,4/8,4/9
	7000, Michael Jones, 5/4/79	M114 ratio 5/9
	7083, Michael Jones, 5/4/79	M114 rankings: 4/1,4/7,4/8,4/9,5/9
	6931, Michael Jones, 5/4/79	M114 data listing
	6915, Michael Jones, 5/4/79	M114 new 4/7,4/8,4/9,5/9

Table 22: Computer Assisted Statistical Analysis - Work Listing - Contd.

Job:	2286, Michael Jones, 2/28/79	M114 data analysis 2, lower 5%
	354, Pete Siemsen, 3/29/79	Edit instructions
	353, Erbe/Jones, 3/29/79	M116 data listing
	35, Erbe/Jones, 3/29/79	M116 data printout
	6754, Michael Jones, 3/30/79	
	6883, Michael Jones, 3/30/79	M114 revised (new M5) data T-Tests (Set 1)
	6837, Michael Jones, 3/30/79	M114 revised data T-Tests (Set 2)
	6754, Michael Jones, 3/30/79	M114 revised data T-Tests (sets 1&2)
	5889, Michael Jones, 4/5/79	M116 data distribution
	5821, Michael Jones, 4/5/79	M116 F rtran listing & created disk data set
	5675, Michael Jones, 4/5/79	Tape third & tape dump (EBCDIC)
	528, Erbe/Jones, 4/9/79	M116 data
	529, Erbe/Jones, 4/9/79)	M116 Set 2 data listing
	2431, Michael Jones, 4/10/79)	
	2317, Michael Jones, 4/10/79	M116 set 2 data listing (fourth)
	2439, Michael Jones, 4/10/79	M116 distribution of data
	2483, Michael Jones, 4/10/79	M116 T-Tests set 1 (corrected)
	6070, Michael Jones, 4/11/79	M114 data sets 1&2 (new M5) lower 5%
	3354, Michael Jones, 4/11/79	M116 descriptive statistics set 1
	3349, Michael Jones, 4/11/79	M116 descriptive statistics set 1
	3421, Michael Jones, 4/11/79	M116 descriptive statistics set 2
	900, Michael Jones, 4/11/79	M114 - 1,2,3,&4 parts T-tests
	883, Michael Jones, 4/11/79	M114 - 1,2,3,&4 parts T-tests (exposed only)
	3419, Michael Jones, 4/11/79	M116 descriptive statistics set 2 (corrections indicated)
	5940, Michael Jones, 4/12/79	M114 -1,2,3,&4 parts T-tests (control only)
	3788, Michael Jones, 4/18/79)	M114 - T-tests upper 5% - sets 1&2
	3980, Michael Jones, 4/18/79)	
	4300, Sarah Schoentgen, 4/25/79)	M114 analysis of variance (data distribution)
	3871, Sarah Schoentgen, 4/25/79)	

Presentation choice: Platform ☒ Poster Session ☐ **SCIENTIFIC PAPER**

Title:
Clearance of Horseradish Peroxidase in the Lungs of Mice Exposed to an Ambient Level of Nitrogen Dioxide

Name(s) of authors, institutional affiliations and addresses:
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Department of Pathology
2025 Zonal Ave., Los Angeles, Calif. 90033

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Abstract — Double space.

Horseradish peroxidase (HRP) was injected intravenously into control mice and those exposed to intermittent and continuous low levels (0.5 ppm to 0.8 ppm) of nitrogen dioxide (NO_2) to study permeability and alveolar reabsorption properties of the lung. In three independent studies (264 mice) polyacrylamide gel electrophoresis (PAGE) of lung homogenates and serum for HRP quantitative analyses were carried out, with postinjection intervals of 0 to 5-1/2 hours and with major emphasis at 4-1/2 hours. The PAGE studies were supplemented by enzyme kinetic assays and ultrastructural studies. The major findings were a greater HRP content of lung tissue from the exposed animals in all three experiments (49 ± 18 vs. 39 ± 14 ($p < .05$); 89 ± 49 vs. 69 ± 26 ($p < 0.05$) and 84 ± 40 vs. 54 ± 17 ($p < .025$); in one experiment, the increase was noted at six weeks postexposure instead of at three weeks. The levels of HRP in lung and serum peaked at one hour and at 2-1/2 hours. Ultrastructurally, HRP was found to be quickly distributed in both the large and small pore systems and had ready access to endothelial cell junctions, interstitial tissues, and basal lamina. In part, the greater 4-1/2 hour residual content of HRP in the lung is believed to be related to an increased number and/or increased HRP content of macrophages. Slower clearance and/or greater amounts of HRP in lung cells and tissues was also believed to contribute to the differences found.

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TYPE 2 PNEUMOCYTE HYPERPLASIA AND HYPERTROPHY IN RESPONSE TO 0.34 PPM NITROGEN DIOXIDE; AN IMAGE ANALYZER COMPUTER QUANTITATION.

Russell P. Sherwin, Kestutis V. Kuraitis* and Valda Richters, *University of Southern Calif., Los Angeles 90033

Of 120 Swiss-Webster male mice, half were exposed to 0.34 ppm NO_2, 6hrs/day, 5days/week, for 6 weeks. Lactate dehydrogenase positive Type 2 cells were quantitated by an image analyzer (Quantimet 720) using 30 pairs (60 animals) of control and exposed animals, the left lobe of lung, 8 slides/lobe and 4 fields/slide. For each of 8 measurements, there were 1920 data bits, a total of 15,360. Computation of the data provided: 1) the inverse of number of Type 2 cells ÷total wall area, M_c: 91.5±33.7, M_x: 83.7±29 ($p \to 0$); 2) size of Type 2 cells, cell area ÷cell number, M_c: 11.8±4.7, M_x: 12.6±6.1 ($p<.007$); 3) inverse of number of cells ÷internal surface area, M_c: 121.0±40.8, M_x: 114.7±37.6 ($p<0.002$); 4) internal surface area ÷wall area, M_c: 6.70±1.1, M_x: 6.77±1.1 (NS); 5) absolute cell counts per field, cells larger than 9μ and 11μ respectively: (a) M_c: 360.7±127.8, M_x: 397.0 ±143.4 ($p<.005$); (b) M_c: 222.7±97.6, M_x: 248.9±111.5 ($p<.0001$); 6) absolute cell area per field, M_c: 3782±2738, M_x: 4537±3505 ($p<.0001$); 7) internal surface area per field, M_c: 32609±11097, M_x: 34118±11724 ($p<.01$); 8) linear intercepts per field, M_c: 9708±3339, M_x: 10231±3576 ($p<.004$). The findings indicate a shift in cell populations following Type 1 cell loss from short term exposure to an ambient level of NO_2. The significance of Type 2 cell hypertrophy is unknown. (A6-218-30, Calif. Air Resources Board)

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DECREASE IN SPLEEN WEIGHTS AND SPLEEN LYMPHOID NODULES FOLLOWING EXPOSURE TO 0.34 PPM NITROGEN DIOXIDE (NO_2).

Kestutis V. Kuraitis*, Arnis Richters, Russell P. Sherwin.
University of Southern California, Los Angeles, CA. 90033

Effects of ambient level NO_2 exposure on the spleen were studied. Spleen weights, expressed as percent of body weight, were recorded from 37 control and 41 S/W male mice following exposure to 0.34 ppm NO_2, 6 hrs/day, 5 days/week, for 12 weeks. The exposed animals demonstrated a decrease in body weight ($p<0.05$), spleen weight ($p=0.0025$), and spleen/body weight ($p<0.005$). Another experiment was conducted to quantitatively determine what spleen tissues were responsible for the lower weight. Computed image analysis (Quantimet 720) was performed on hematoxylin-stained spleen sections, 4 sections/spleen at 4 different levels, 2 fields/section. Measurements of total lymphoid nodule area, nodule number, and spleen area were expressed as the following 3 ratios: 1) total nodule area÷spleen area, 2) total nodule area÷nodule number, and 3) spleen area÷nodule number. The data demonstrate that the NO_2-exposed animals had a decrease in the average nodule size ($p<0.0005$), a decrease in the total nodular area occupying a given area of spleen ($p=0.05$), and a decrease in the average spleen area found around each nodule ($p<0.0005$). This is the first report relating ambient level NO_2 exposure to a quantitative depletion of splenic lymphoid tissue. This may compromise immune reactivity of NO_2-exposed animals.
(Supported in part by Calif. Air Resources Board, A6-218-30)

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