EPA-600/1-76-021
April 1976

TRACE SUBSTANCES AND TOBACCO SMOKE IN INTERACTION WITH NITROGEN OXIDES.

Biological Effects

By

Gustave Freeman and Laszlo T. Juhos
Stanford Research Institute
Menlo Park, California 94205

Contract No. 68-02-1243

Project Officer

David L. Coffin
Clinical Studies Division
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

LIBRARY
...TION

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
HEALTH EFFECTS RESEARCH LABORATORY
RESEARCH TRIANGLE PARK, N.C. 27711

DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

CONTENTS

LIST OF ILLUSTRATIONS AND TABLES iii

INTRODUCTION 1

SPECIALIZED FACILITIES AND EQUIPMENT 2

RESULTS AND DISCUSSION 3

Response of Newborn Rats to NO_2 3
Long-Term Exposure of Primates to Nitrogen Dioxide and Ozone 3
The Presence and Role of NO_2 in Tobacco Smoke and Its Relationship to Atmospheric NO_2 14
Initial Reaction Between NO_2 and Tissue Components 17

PUBLICATIONS RESULTING FROM THIS RESEARCH 23

APPENDICES

A DELAYED MATURATION OF RAT LUNG IN AN ENVIRONMENT CONTAINING NITROGEN DIOXIDE 24

B ADDRESS TO CDC/EPA/WHO INTERNATIONAL SYMPOSIUM 31

ILLUSTRATIONS

1 Mean Body Weights and Mean Respiration Rates of Monkeys on 0, 2, and 9 ppm NO_2 5

2 Weight Curves of Growing Stump-Tailed Macaques on 0, 2, and 9 ppm NO_2 6

3 Cigarette Smoking Apparatus for Rats 15

4 Modification of Smoking Apparatus 16

TABLES

1 Review of Repeated Functional Respiratory Studies on Monkeys Exposed to 0, 2, and 9 ppm NO_2 7

2 Observed Functional Residual Capacity of Control and 2 and 9 ppm NO_2-Exposed Macaques 8

3 Functional Residual Capacity and Related Respiratory Parameters for Control Monkey and for One Exposed to 9 ppm NO_2 10

4 Hematological Parameters for Control and 2 and 9 ppm NO_2-Exposed Monkeys 11

5 Hematologic Observations of Monkey Exposed Continuously to 0.91 ± 0.08 ppm Ozone 12

6 Hematologic Parameters for Control Monkey and for One Exposed to 9 ppm NO_2 13

7 Reported Values for the Oxides of Nitrogen from Tobacco Smoke 18

8 ^{15}N Content of Various Rat Lung Protein Samples Exposed to $^{15}NO_2$ 20

9 ^{15}N Content of Various Rat Lung Protein Samples Exposed to $^{15}NO_2$ 21

INTRODUCTION

During the past several years, we have been observing and reporting the effects of oxides of nitrogen on rats and on monkeys (Macaca speciosa). As a result of exposure to nitrogen dioxide (NO_2), rats developed a disease resembling human emphysema. In numerous publications, we have described morphological, physiological, and hematological changes accompanying this disease.*

During the past contract year (July 16, 1973, through September 15, 1974), emphasis was on determining the response of newborn animals living in an environment containing NO_2. Also, continuing ongoing observations, we examined:

- Both mature and newborn monkeys exposed continuously to NO_2 or to ozone.
- The relative effects of tobacco smoke compared with those of NO_2.
- The binding of NO_2 in tissue, based on the use of isotopically labeled, nonradioactive $^{15}NO_2$.
- The detailed hematologic effects of exposure to NO_2 or to ozone.

In addition to the usual parameters for detecting changes in the erythrocytic series, we conducted biochemical studies on blood of exposed animals.

This final report describes progress in these areas of investigation. This report is final only in an administrative sense, since we are continuing the work in particular unresolved areas. However, the published paper, "Delayed Maturation of Rat Lung in an Environment Containing Nitrogen Dioxide," does describe research completed during the contract year. Thus, this report is in its final form for Contract No. 68-02-1243, covering the period July 1973 to September 1974.

---

*Refer to "Publications Resulting from This Research" for the most recent papers.

SPECIALIZED FACILITIES AND EQUIPMENT

Approximately 3,000 square feet of laboratory space was divided into an exposure-chamber facility and into physiology, pathology, electron microscopy and cell biology, biochemistry, and tissue culture laboratories. A mass spectrometer, scanning electron microscope, and a variety of other high-precision instruments were available within the Institute for various phases of the project.

Five 80-cubic-foot exposure chambers, each capable of holding 60 full-grown rats or four adult monkeys, were used. Each chamber was equipped with the necessary control and safety devices. With an air-turnover rate of 40 cubic feet per minute, the gas concentrations were maintained within 10% of the nominal value so that feedback control techniques were not necessary. An alarm system signaled equipment failures, and necessary repairs could be made without materially upsetting exposure schedules.

Four NO_2 exposure chambers were equipped with hardware facilities to permit the addition and monitoring of ozone (O_3) concentrations in their atmospheres.

A Bendix Model 8101-BX NO_2 analyzer provided by EPA was installed for determining the concentrations of NO_2 alone and in mixtures with O_3. NO_2 values were confirmed by use of a modified Saltzman method on wet samples. Ozone determinations were made on a Bendix Model 8002 ozone analyzer, provided by SRI, and were confirmed with a Mast Meter.

The Bendix NO_2 analyzer has not worked satisfactorily. The manufacturer kept it for six weeks for repair and replacement of a failing solenoid valve, but the analyzer still performed unsatisfactorily after reinstallation. Our initial observations suggested a loss of analytical range and general instrumental instability. Also, ammonia from the excrement of animals in the chambers interfered with the determination of NO_2. Although we have been communicating with the Application Research Department of the Bendix Corporation in West Virginia to correct these problems, no solutions appear to be forthcoming. We are considering returning the analyzer to EPA. For the time being, we will make no further attempts to automate analysis of NO_2 concentrations.

Because proper automated control, monitoring, and recording of gas concentrations requires continuous surveillance, revision, and maintenance, hand sampling and flow rate control are being used at least twice daily to monitor desired concentrations. Although this system is satisfactory, it is time consuming and inefficient. A new analyzer, preferably a Meloy, is urgently needed.

RESULTS AND DISCUSSION

Response of Newborn Rats to NO_2

With pregnancy and birth occurring regularly in smoggy atmospheres, it is important to assess the relative vulnerability of newborn infants and their responses to the oxides of nitrogen, especially to NO_2. We already were observing monkeys born to mothers exposed continuously to either 2 or 9 ppm of NO_2. Offspring were born and reared in each of the atmospheres, and those born in NO_2-free chambers served as controls. The response of monkeys to the NO_2-containing environments is described in the following sections.

Meanwhile, we initiated a study of neonatal growth, using the rat because of its relatively shorter growth period and life span and because of our extensive experience with that species. Results of this study were just published in the American Review of Respiratory Disease (Appendix A).

Long-Term Exposure of Primates to Nitrogen Dioxide and Ozone

To evaluate the influence of the oxides of nitrogen on the increasing prevalence of chronic obstructive pulmonary diseases in man, an experiment was designed to simulate in an animal model the insidious, long-range pathogenesis of these diseases. In view of the 25 to 40 years required by man to develop overt emphysema, primates were chosen for study over rats because of their extended life span (only three years in the rat) and because of their closer biological resemblance to man. For more than seven years, we have clinically observed the macaques living continuously in either 2 or 9 ppm of NO_2. Their growth and respiratory rates have been monitored regularly, erythropoietic responses have been followed, and selected studies have been performed periodically. During the latter part of this contract year, we were developing techniques for the determination of specific functional states of ventilation of the exposed monkeys as compared with their controls.

For ten months, we conducted experiments involving exposure of a monkey to ozone and planned to add NO_2 to form a more realistic mixture. Toward the end of the contract year, we planned to sacrifice the monkeys and designed procedures for terminal evaluation of the effects of their chronic exposure to NO_2 and ozone.

Following is an account of the effort expended toward the assessment and monitoring of the influence of the atmospheric oxidants on the monkeys.

Growth and Respiratory Rates of Control and NO_2- and O_3-Exposed Monkeys

Monitoring of the growth and clinical conditions of control and of NO_2- and O_3-exposed monkeys continued monthly throughout the contract year. Body weights and respiratory rates of adult monkeys appear in Figure 1, and the body weights of juvenile monkeys are presented in Figure 2.

The adult control and exposed monkeys maintained relatively constant weights, although all groups appeared to show a moderate sinusoidal seasonal weight fluctuation.

At age five years, four juveniles (Be ♀, Ce ♀, In ♂, and Dy ♂) appear to have reached the range of mature body weights, and they are continuing to gain weight. Three of these animals were born in the NO_2-containing environment--two in 2 ppm and one in 9 ppm. In general, the juvenile monkeys continued to grow at equivalent rates, regardless of the NO_2 concentration (0, 2, or 9 ppm), although the weight of the one in the higher concentration was less for a considerable period.

The monkey exposed to 0.9 ppm ozone appears to have been losing weight since the beginning of exposure and had not regained preexposure body weight in ten months.

Functional Measurements of NO_2-Exposed Monkeys

Table 1 presents the functional respiratory parameters measured over time in control and NO_2-exposed monkeys. To improve instrumental and general hardware capabilities for measuring pulmonary residual volume (RFC and RV) and carbon monoxide diffusion capacity (D_{CO}), a new test gas mixture, gas analyzer, and spirometer were acquired at no cost to this contract.

We made functional measurements and studied the pulmonary wash-out pattern of control monkeys and of those exposed to 2 and 9 ppm NO_2. Monkeys were respired for about two minutes in a closed circuit, rebreathing system, starting with pure oxygen contained in a small animal spirometer (Warner L. Collins, Inc.) equipped with a kymograph. The gas volume change in the spirometer at the end of the breathing period was analyzed by a Carle Model 8000 gas chromatograph. From the data, the functional residual capacity (FRC) was calculated at expiratory end-tidal moments. Although increased FRCs were expected among the NO_2-exposed monkeys, the findings (Table 2) were to the contrary. Among the possible reasons for the apparently reduced functional residual volumes may be the existence of poorly ventilated portions of the NO_2-exposed lungs in which the nitrogen was not in equilibrium with gases in other portions of the lung and with the total test system at the termination of the experiment.

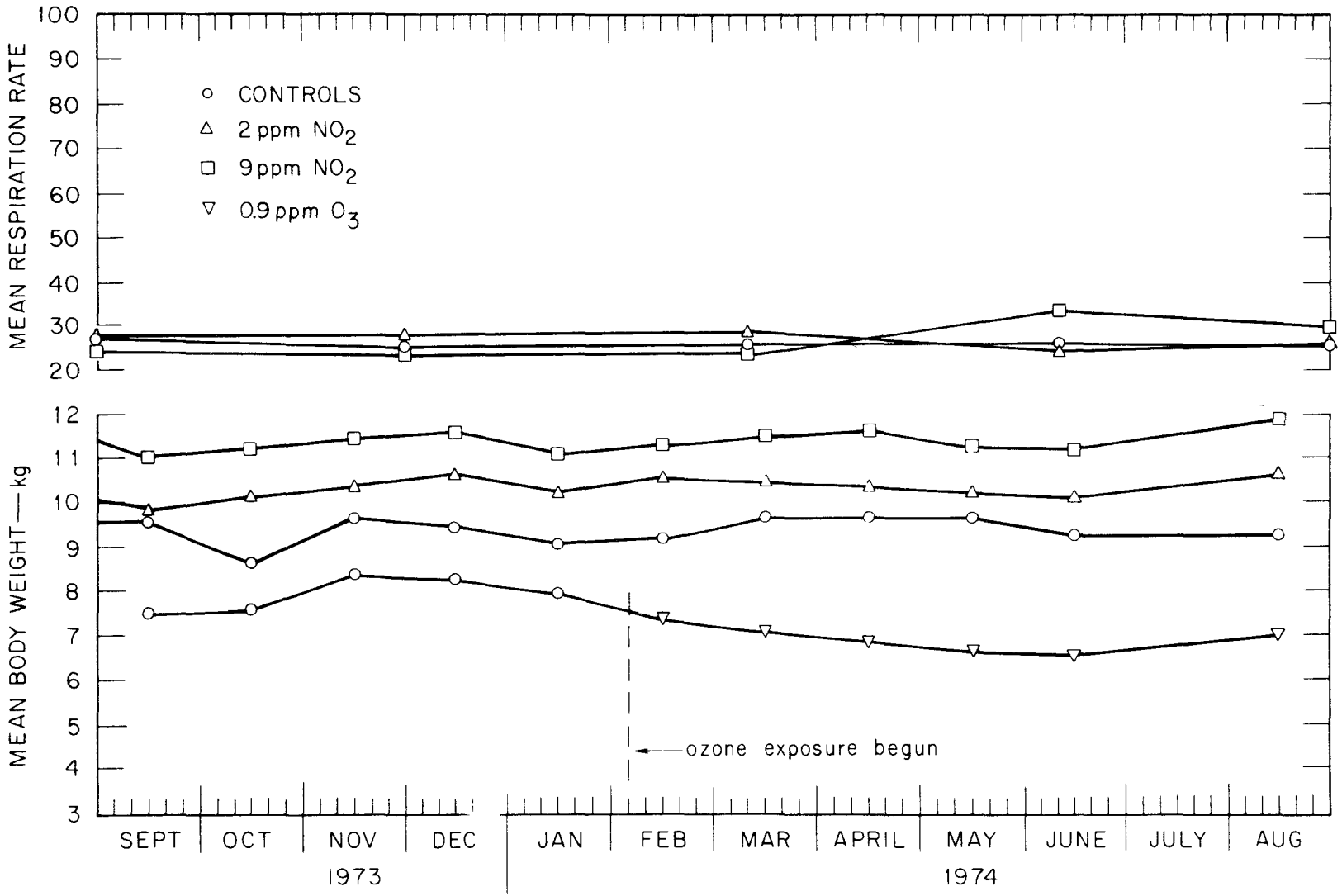

FIGURE 1 MEAN BODY WEIGHTS AND MEAN RESPIRATION RATES OF MONKEYS ON 0, 2, AND 9 ppm NO_2

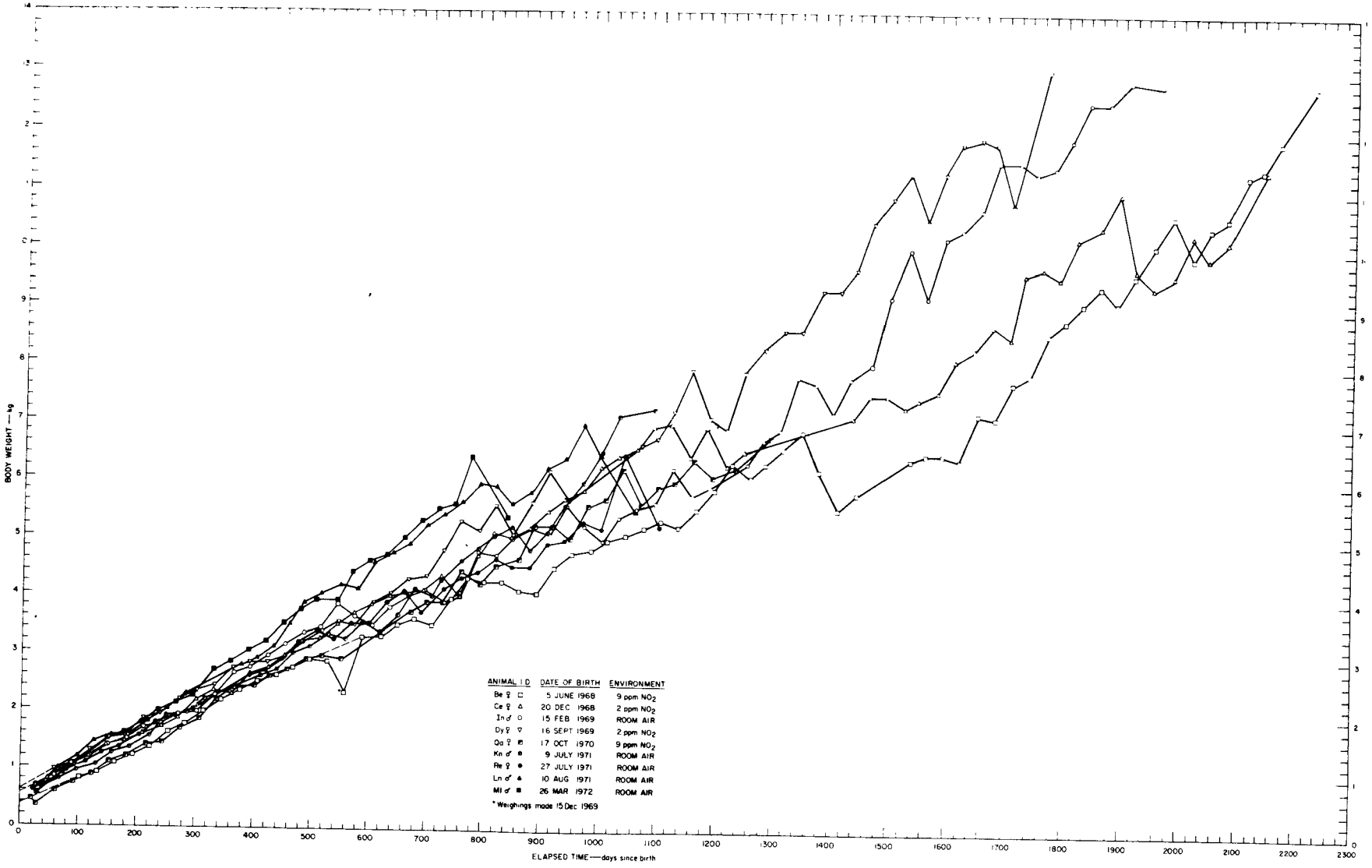

FIGURE 2 - WEIGHT CURVES OF GROWING STUMP-TAILED MACAQUES ON 0, 2 AND 9 ppm NO_2

Table 1

REVIEW OF REPEATED FUNCTIONAL RESPIRATORY STUDIES
ON MONKEYS EXPOSED TO 0, 2, and 9 ppm NO_2

Group	Quarterly Report (No./Page)	Body Weight (kg)	Respiratory Rate (Resp/min)	Minute Volume (ml/min)	ΔP_{max} (cm H_2O)	Compliance (cc/cm H_2O)	Compliance (cc/cm/H_2O/kg)	Resistance cm/H_2O ℓ/sec
Control								
An ♀	15/4	6.0	32	1280			2.92	1.23
	19/6	7.5	43	1715	1.5	43.5	5.80	0.64
	33/2	8.2	36	1650	9.1	13.9	1.70	1.25
Br ♂	15/4	9.1	28	1390			0.71	0.68
	33/2	16.6	24	432	21.0	0.9	0.05	4.76
Fy ♀	15/4	6.3	27	1050			0.42	1.78
	19/6	6.6	36	1285	1.1	39.2	5.90	0.64
	33/2	10.1	22	1012	9.4	6.9	0.68	5.00
Rh ♀	15/4	5.9	28	1285			3.07	0.18
	20/8	5.8	31	1540	5.1	11.6	2.00	0.84
	33/2	6.5	18	925	6.4	9.0	1.39	4.05
2 ppm NO_2								
Gy ♀	20/8	8.2	27	1600	2.4	26.5	3.20	0.34
	33/2	11.9	27	810	3.7	8.1	0.68	0.57
Jl ♀	15/4	5.6	28	910			7.79	0.26
	33/2	8.4	26	640	3.7	7.0	0.83	4.38
Ls ♀	15/4	5.5	23	525			0.54	7.17
	19/6	8.2	26	1500	2.7	22.3	2.70	0.54
	33/2	8.7	29	899	2.3	17.6	1.53	1.25
9 ppm NO_2								
Cy ♂	15/4	8.5	33	1040			0.48	2.57
	20/8	9.2	33	2345	1.9	43.1	4.70	0.39
	29/5	9.9	24	2120	2.1	10.0	1.00	2.70
	33/2	11.1	41	1025	17.0	1.7	0.15	17.17
Ee ♀	15/4	4.2	31	750			1.52	0.50
	19/6	6.0	32	1135		27.5	4.60	1.31
	20/8	5.6	23	1130	4.4	12.2	2.20	1.80
	33/2	7.2	24	1056	5.2	8.7	1.21	1.30
Gr ♂	19/6	14.4	23	2690	4.4	48.0	3.40	0.36
	20/8	14.5	19	3343	0.3	59.5	4.10	2.80
	33/2	16.0	17	451	13.3	2.1	0.13	15.56
Ml ♀	29/5	7.5	31	2700	2.7	45.7	6.10	0.54
	33/2	7.5	26	1378	9.5	6.7	0.89	3.95

Table 2

OBSERVED FUNCTIONAL RESIDUAL CAPACITY (FRC) OF CONTROL (UNEXPOSED) AND 2 AND 9 ppm NO_2-EXPOSED MACAQUES

I.D.		Weight (kg)	FRC (ml)	FRC/ kg
Juvenile macaques				
Control				
In ♂		11.4	246.6	21.6
Re ♀		4.9	84.6	23.9
Ln ♂		5.8	176.3	30.4
	Av	7.4	169.2	25.3
2 ppm NO_2-exposed				
Dy ♀		11.3	195.0	21.3
Ce ♀		9.4	116.5	12.4
	Av	10.4	155.8	16.9
9 ppm NO_2-exposed				
Be ♀		10.4	68.1	6.6
Qa ♀		5.2	214.8	37.1
	Av	7.8	141.5	21.9
Adult macaques				
Control				
Br ♂		15.5	376.1	24.3
Ay ♀		13.7	143.1	10.4
	Av	14.6	259.6	17.4
2 ppm NO_2-exposed				
Gy ♀		15.2	170.9	11.2
Ls ♀		11.9	154.6	13.0
	Av	13.6	162.7	12.1
9 ppm NO_2-exposed				
Gr ♂		17.6	173.7	9.9
Cy ♂		10.8	61.5	5.7
	Av	14.2	117.6	7.8

To examine further the FRCs of a monkey exposed to 9 ppm NO_2 and of a control, we made measurements repeatedly with a double-indicating system. The monkeys respired in a closed system containing neon and oxygen, with selective adsorption of carbon dioxide from the expired air. Neon and nitrogen are expected to exchange--neon from the spirometer to the lung and nitrogen from the lung to the spirometer--and attain equilibrium between the lungs and the spirometer. Although, theoretically, differences in the results of calculated FRC could be justified, the values should be close. Table 3 presents the results of such measurements.

Again, the FRC was not enlarged. The apparently greater oxygen consumption by the monkey exposed to 9 ppm NO_2, despite its lower minute volume and frequency, suggests a probable arterial-venous difference in partial pressures of oxygen.

Assessment of Small-Airway Obstruction

Small-airway disease is indicated when compliance of the lungs decreases as the frequency of breathing increases. Dr. William R. Powell* conducted preliminary experiments to measure frequency-dependent compliance for assessing small-airway obstruction in the rats developing NO_2-induced emphysema. Lungs of these rats readily demonstrated frequency dependence of compliance, whereas normal control lungs showed no decrease in compliance when respired at the same series of frequencies. This is strong functional support for the morphologic evidence of small-airway disease that occurs early in the pathogenesis of NO_2-induced emphysema in the rat (Freeman and Haydon, Arch. Environ. Health, 1964) and in man with developing emphysema.

Preparatory to measuring frequency-dependent compliance on excised lungs of monkeys, we are perfecting the procedure using excised rat lungs. The plethysmographic equipment was modified and recalibrated, and tidal excursions are now controlled by extrapulmonary pressure changes rather than by intratracheal positive pressure. Lungs of control and NO_2-exposed rats were measured for static compliance at a transpulmonary pressure ranging from ambient to -30 cm H_2O; such lungs also were measured for frequency-dependent dynamic compliance at each cm H_2O pressure decrement within the same transpulmonary pressure range decrement, i.e., at frequencies of 1 to 4 Hz. The analysis of data is in progress.

---

*Dr. Powell, a visiting scientist from the Engineering Department of the University of West Virginia, performed these tests at no cost to the contract.

Table 3

FUNCTIONAL RESIDUAL CAPACITY (FRC) AND RELATED RESPIRATORY PARAMETERS FOR CONTROL MONKEY AND FOR ONE EXPOSED TO 9 ppm NO_2*

	Control (Br ♂)	Exposed to 9 ppm of NO_2 (Gr ♂)
Body weight (kg)	16.2	15.5
Number of measurements	5	5
Duration of test (min, sec)	4, 15	4, 11
Volume of inspiration (ml)	122	102
Volume of expiration (ml)	119	97
Tidal volume (ml)	120	100
Respiratory frequency (respirations/min)	36	25
Minute volume (liter/min)	4.3	2.5
Oxygen consumption (ml/min/kg)	11.0	12.2
FRC (ml)		
Calculated on basis of nitrogen volume	658	430
Calculated on basis of neon volume	630	590
Average (nitrogen and neon)	644	510
Weight-normalized FRC† (ml/kg)	39.7	32.9

*Monkey has been exposed for eight years.

†Normal value, 35.7 ml/kg.

Hematologic Data on Monkeys Exposed to Environmental Oxidants of 2 and 9 ppm NO_2 and 0.9 ppm O_3

During the six years of this research, we have observed that exposure to both 2 and 9 ppm NO_2 results initially, within two to three weeks, in the development of a slightly microcytic polycythemia with normal mean corpuscular hemoglobin concentrations. This polycythemia later tended to return to normal levels as the monkeys became acclimated to the environment.

Hematologic studies were repeated on adult monkeys that have been exposed continuously to NO_2 for over six years. Circulating blood cells were counted and evaluated, and Table 4 compares the results of the two methods of cell enumeration and characterization. Compared with earlier hematologic studies in the same animals, the data indicate that the animals accommodated appreciably to the environment.

Table 4

HEMATOLOGICAL PARAMETERS FOR CONTROL AND 2 AND 9 ppm NO_2-EXPOSED MONKEYS
Numbers in Parentheses Determined by Coulter Counter, Model S

	Controls (5 monkeys)	Concentration of NO_2: 2 ppm (6 monkeys)	Concentration of NO_2: 9 ppm (4 monkeys)
RBC ($\times 10^6$)	5.6 (5.4)	5.5 (5.6)	6.0 (6.0)
Hbg (g %)	13.6* (14.1)†	13.3 (14.2)	14.7 (14.8)
Hct (%)	42.6 (41.3)‡	43.4 (41.4)	48.5 (44.4)
MCV (μ^3)	77‡ (76)	80 (74)	81 (77)
MCH ($\mu\mu$g)	24.3 (25.9)‡	24.2 (25.3)	24.5 (25.5)
MCHC (%)	-- (34.4)‡	-- (34.3)	-- (33.8)
WBC ($\times 10^3$)	12.7 (12.1)	16.1 (14.8)	14.0 (13.9)

*Wong iron method.

†Coulter colorimetric method.

‡Calculated.

One macaque (Rh ♀) was exposed to 0.91 ± 0.08 ppm O_3 for 28 days. This exposure level was at the upper limit of the analytical capability of the Bendix chemoluminescence ozone analyzer. This 7.4-lb adult monkey showed no evidence of difficulty in tolerating this concentration. Hematologic evaluation of the monkey was made just before exposure, and 24 and 72 hours and 4 weeks after the introduction of ozone. The available hematologic results, presented in Table 5, demonstrate early development of polycythemia. By the third day, accommodation is evident, although young cells--indicated by their reduced fragility--still predominate. Complete adaptation was achieved before day 28.

Table 5

HEMATOLOGIC OBSERVATIONS OF MONKEY (Rh ♀)
EXPOSED CONTINUOUSLY TO 0.91 ± 0.08 ppm OZONE

Sample	RBC ($\times 10^6/mm^3$)	Hct (%)	WBC ($\times 10^3/mm^3$)	Hb (gm/100 ml)	Osmotic Fragility*
Pre-exposure	4.8	42	14.4	13.21	0.42
Post-exposure					
24 hr	6.2	47	14.0	14.77	0.40
72 hr	5.5	46	13.5	13.41	0.38
28 days	4.9	40.5	12.5	12.06	0.42
8 months	6.7	42.0	13.2	-	0.42

*Arbitrary units.

Rh ♀ now has been exposed continuously to 0.9 ppm ozone for ten months. Its survival suggests that monkeys are more resistant than rats to ozone, since this exposure level resulted in an LD_{50} of about 19 days for rats. We plan to evaluate the ozone-caused changes after the sacrifice of the monkey.

Preparatory to sacrifice of the selected monkeys and assessment of the damage of atmospheric oxidants to the lungs of monkeys, we measured hematologic parameters for another monkey exposed to 9 ppm NO_2 and then compared them with those of an unexposed monkey of approximately the same age and weight. Table 6 presents the results.

Table 6

HEMATOLOGIC PARAMETERS FOR CONTROL MONKEY
AND FOR ONE EXPOSED TO 9 ppm NO_2

	Control (Br ♂)	Exposed (Gr ♂)
RBC ($10^6/mm^3$)	5.50	5.25
WBC ($10^3/mm^3$)	11.5	10.4
Hematocrit (%)	44.5	41.5
Hemoglobin (g/100 ml)	13.51	13.48

No significant differences exist within this pair of monkeys.

Plans for sacrificing NO_2-exposed monkeys (and appropriate controls) have been formulated for the final evaluation of the changes sustained by their lungs. Evaluation will include measurements of:

(1) Physiological functional parameters *in vivo*, mainly carbon monoxide-diffusing capacity and FRC.

(2) Hematological profile including hematocrit, total white and red cell counts, differential counts, mean corpuscular volume, and red cell fragility.

(3) Selected chemical parameters of blood reported to be influenced by exposure to atmospheric oxidants, such as erythrocytic cholinesterase, tocopherols, glucose-6-phosphate dehydrogenase, 2,3-diphosphoglycerate, and glutathione reductase.

(4) Pulmonary cell renewal as determined with tritiated thymidine.

(5) Selected chemical constituents of lung tissue such as glutathione reductase and tocopherols.

(6) Static and dynamic compliance and resistance to airflow of the excised lungs.

The whole monkey will undergo pathological examination, and the lungs will be studied by electron microscopy.

Sacrifice of the monkeys will commence when plans and procedures, as well as time tables of processing, are finalized.

The Presence and Role of NO_2 in Tobacco Smoke and Its Relationship to Atmospheric NO_2

These preliminary studies related to the content of NO_2 in cigarette smoke likely to be inhaled and likely to contact pulmonary tissue *in vivo*. Although we did examine using rats the apparatus we developed, the changing interests of the Environmental Protection Agency precluded our conducting detailed experiments. Progress made thus far is as follows.

During the 1972-73 contract year, we had constructed a cigarette smoking apparatus for rats (Figure 3). Initial tests of moisture retention of the original glass "trachea" showed that the unit could not be kept uniformly wet. An improved glass "trachea" was designed using a porous glass tube filter enclosed in a glass envelope (Figure 4). A special vessel for humidifying the air was built.

Initial testing of the smoking unit involved burning Camel cigarettes in the apparatus while simultaneously removing a sample of smoke from one of the rat "portholes." The sample was to be analyzed continuously with a Bendix Model 8101-B (chemoluminescent) oxides of nitrogen analyzer connected to a recorder. Further experiments were planned to determine the NO/NO_2 ratio produced by the machine at various dilutions.

The NO_2 content of the tobacco smoke reaching the lung, as simulated in our smoking machine, could not be analyzed by direct sampling with the Bendix gas analyzer. Removal of the aerosol by a millipore filter did not provide a sufficiently clean gas sample suitable for analysis by the chemoluminescence reaction. To solve this problem, we considered removing all particulate matter from the tobacco smoke by filtering and cryogenic techniques.

We found a filter that was potentially suitable for removing particulate material from fresh cigarette smoke and for producing useful samples for gas analysis by chemoluminescence. The Teflon filter material removed all microscopic particulate matter from smoke and retained 99% of the gaseous components that were reactive with Saltzman's reagent. However, the filter proved to have a finite capacity to hold back such components; once this limit was reached, gases were allowed to pass through.

The reactivity of the retained particulate matter with the Saltzman reagent could not be ascertained readily for its relationship to NO_2 or other reactive organic or inorganic nitrites.

The cryogenic fractionation of the fresh tobacco smoke, although potentially effective as a separation technique, would not produce a continuous stream of sample analyzable by either the Saltzman or the chemoluminescence methods, so we have not yet attempted using the technique.

Progress in these evaluations also has been hindered by the unsatisfactory performance of the Bendix NO_2 analyzer, a problem compounded by

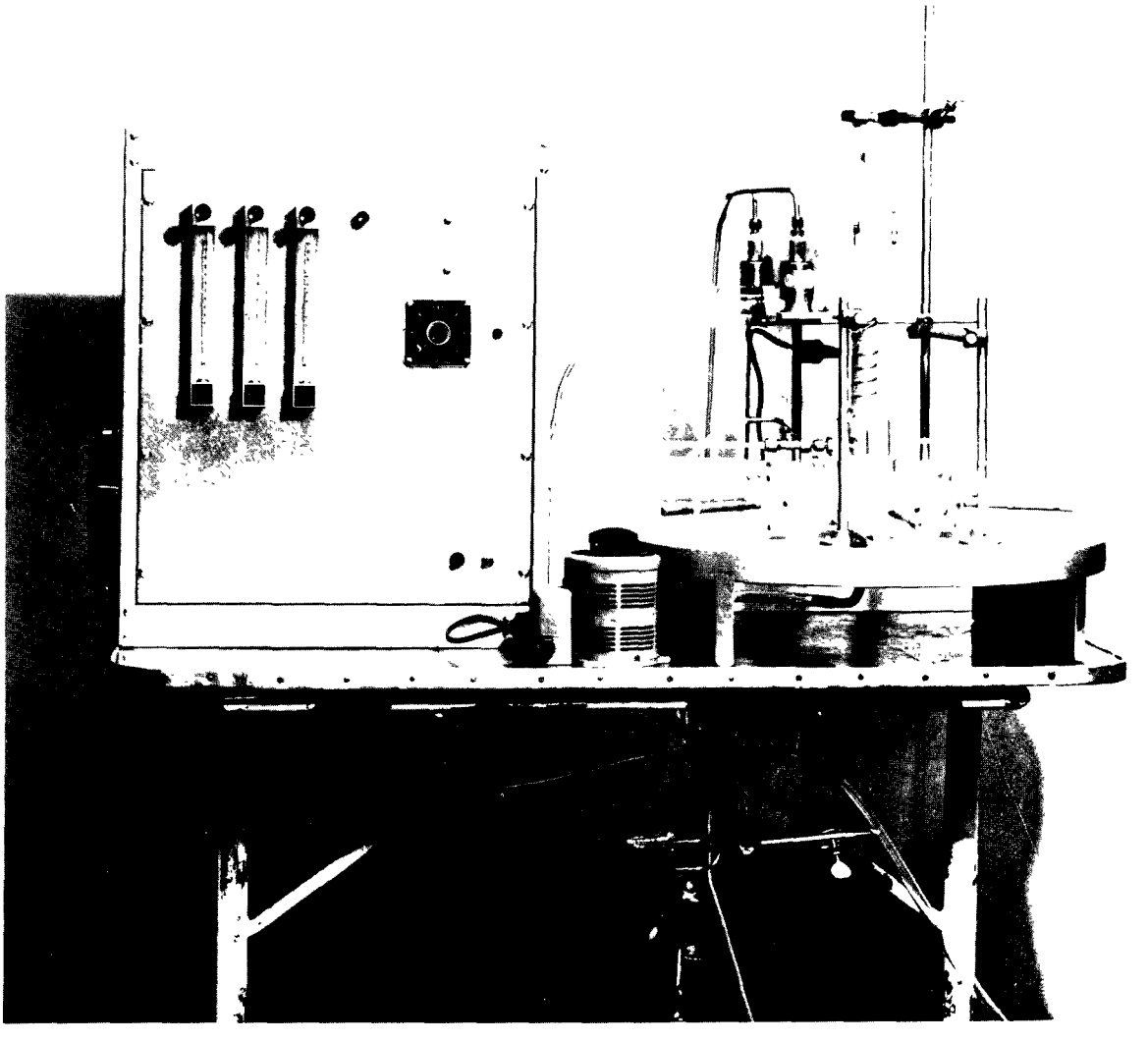

FIGURE 3 CIGARETTE SMOKING APPARATUS FOR RATS

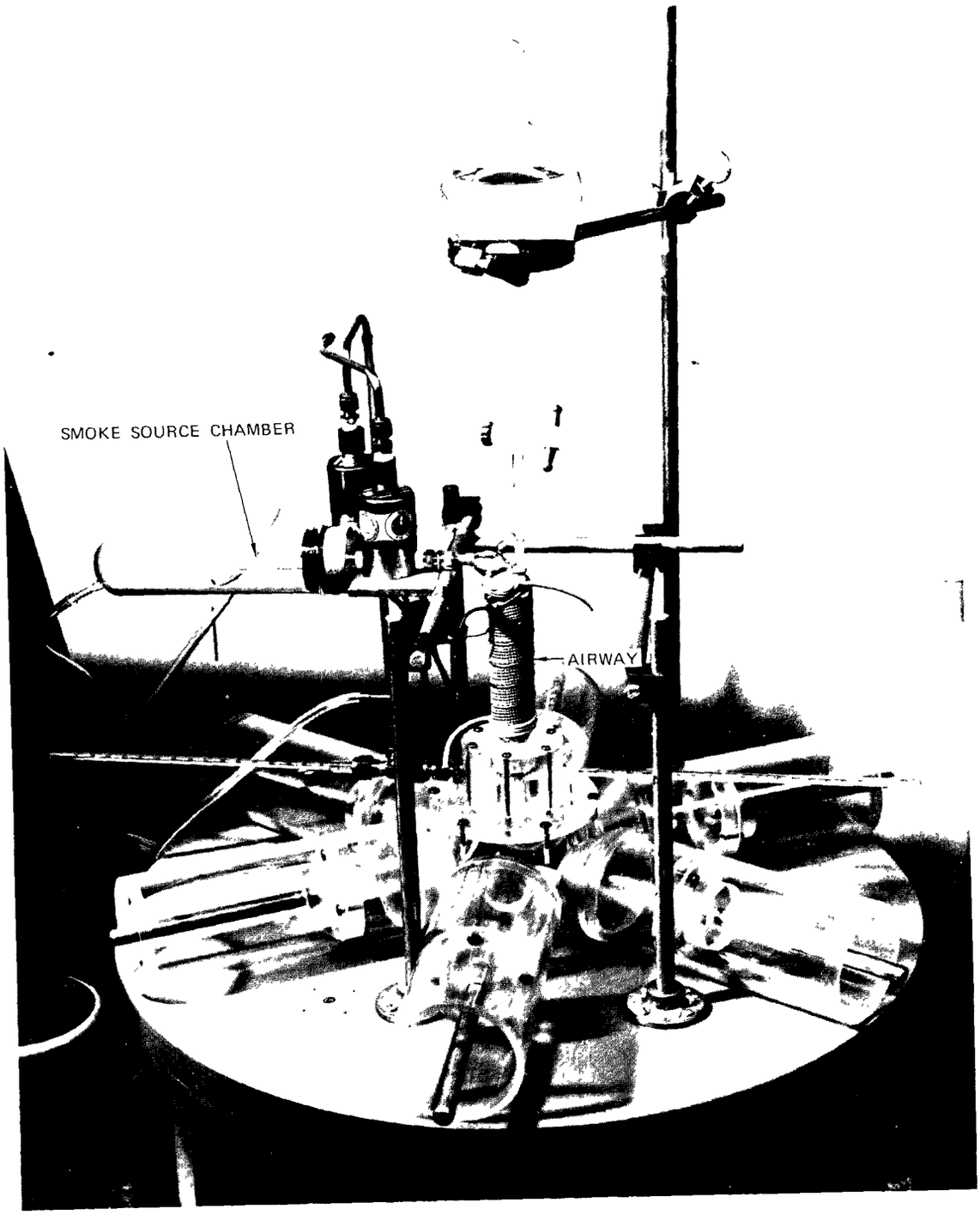

FIGURE 4 MODIFICATION OF SMOKING APPARATUS

the interference of ammonia with the chemoluminescence method of analysis that we discovered. This problem warrants further analytical work.

We reviewed the literature to gather reported values for the oxides of nitrogen contained in tobacco smoke. Table 7 lists these values together with references. Because the wide range of reported values renders the estimation of exposure of smokers rather difficult, the design of realistic model experiments is frustrating indeed.

Most of the analytical work on the oxides of nitrogen in cigarette smoke was reviewed by R. L. Stedman* and in a subsequent symposium on the chemical composition of tobacco and tobacco smoke held in Washington, D.C., in 1971, summarized by I. Schmeltz in The Chemistry of Tobacco and Tobacco Smoke. We found no publications after 1971 on the analytical problems involved in measuring the oxides of nitrogen in tobacco smoke.

Part of the difficulty in achieving accurate analysis in tobacco smoke is the lack of direct, specific analytical methods for the individual oxides and the continuous intermolecular chemical reactions among them.

Our theoretical calculations indicate that NO would oxidize to NO_2 at a rate of 0.001 ppm/sec, using the rate constant of $k = 7.5 \times 10^{-10} ppm^{-2} min^{-1}$ for the reaction of $2NO + O_2 \rightarrow 2\ NO_2$ applied to the average 50-ml puff of cigarette smoke, containing about 300 ppm of NO_x and diluted to 500 ml in the process of inhalation with 20% oxygen-containing air. Since these values are based on *in vitro* conditions, extrapolation to *in vivo* would be difficult.

Initial Reaction Between NO_2 and Tissue Components

Not only is the biochemical fate of NO_2 obscure, but its initial reactions with tissues also are unknown. As a result, our investigations were initiated along two lines. One was to observe reactions of thin films of representative model molecular species with gaseous NO_2 in chambers; the other was to identify reaction products between isotopically labeled ^{15}N in $^{15}NO_2$ and exposed tissue, both as a homogenate and as intact lungs of rats *in vivo*. The first approach revealed a striking reactivity of vitamin E (α-tocopherol) and also of vitamin A with NO_2. (This avenue is being pursued further under an NIH grant.) The other area of investigation is directed toward determining whether the nitrogen in NO_2 is covalently bound to tissue elements during exposure.

---

*R. L. Stedman, The chemical composition of tobacco and tobacco smoke, Chem. Rev. 68, 153-207 (1968).

Table 7

REPORTED VALUES FOR THE OXIDES OF NITROGEN FROM TOBACCO SMOKE

Reference	Method of Analysis	Concentration of NO_x
Haagen-Smit et al. Arch. Ind. Health 20, 53/399-54/400 (1959)	Saltzman*	Cigarette $NO + NO_2$ 145-655 ppm (v/v) Cigar $NO + NO_2$ 167-1250 ppm (v/v) Pipe tobacco $NO + NO_2$ 126-1154 ppm (v/v)
Bokhoven et al. Nature, p. 458, Nov. 4, 1961	"Normal analytical methods"	Cigarette $NO + NO_2$ 170-210 ppm NO_2 80-120 ppm
Kensler et al. New Engl. J. Med. 269, 1161 (1963)	Saltzman*	Cigarette 50 μg/puff of NO_2
Tada et al. Rep. Inst. Sci. Lab. Kurashiki 60, 7 (1962)		NO 72-271 ppm NO_2 19-118 ppm
Wescott et al. 18th Tobacco Chemist Research Conference, Raleigh, 1964		NO_x 96-1120 ppm
Norman et al. Nature, p. 915, Feb. 27, 1965	Saltzman*	Cigarette NO 698-1008 ppm NO_2 0-25 ppm
Newsome et al. Tobacco Science 9, 102 (1965)	Saltzman*	Cigarette NO 500-800 ppm NO_2 20-80 ppm
P. H. Abelson. Science, Dec. 22, 1967		Cigarette NO_2 250 ppm
G. Neurath. Experientia 23, 400-404, (1967)		No quantitative data; the path of NO and NO_2 to nitrosamines is described
Rowlands et al. Environ. Res. 2, 47-71 (1968)	Electron spin resonance	Implied qualitative presence of NO_x; no quantitative data

*Saltzman, Anal. Chem. 26, 1949 (1954).

The potential relevancy of these studies to lung cancer, as well as to emphysema, is considerable because nitrous acid, a product of NO_2 and water, forms nitrosamines in the presence of secondary amines, and certain of these are potent carcinogens.

Several other experiments were conducted, and typical data are compiled in Table 8. In the first type of experiment, frozen normal rat lung was powdered and exposed to 0.7 mmol of $^{15}NO_2$ for 30 minutes. Chloroform/methanol (1/1) was added to remove lipids. The lipid-free residue was suspended in an $NaNO_3$-$NaNO_2$ solution for 2 hours, removed by centrifugation, dialyzed overnight, and then recovered by centrifugation. A portion was removed for ^{15}N analysis, and the remainder was hydrolyzed in 6N HCl at 110°C for 18 hours. The acid was removed by evaporation in a vacuum desiccator containing KOH pellets. The residue was taken up in water and applied to a Dowex 50 (acid form) column, which was then washed with 50 column volumes of H_2O. The amino acids were removed with 5 M NH_4OH and recovered by evaporation. A portion was taken for ^{15}N assay, and the remainder was reacted with ninhydrin to release amino nitrogen, which also was assayed for ^{15}N.

Table 9 shows an increased level of ^{15}N in all fractions assayed. The ^{15}N content also was elevated when the lung homogenate was first treated with 10% TCA for 1 hour and then boiled for 2 hours before $^{15}NO_2$ exposure. When 1 ml of 0.05 M phosphate buffer (pH 7.4) is exposed to the equivalent amount of $^{15}NO_2$ for 30 minutes, flushed with nitrogen, and the powdered lung is added, significant amounts of ^{15}N appear in protein amino nitrogen. Thus, when rats were exposed to 18 ppm $^{15}NO_2$ for 6 hours, significant amounts of ^{15}N were found in lung protein, and lesser amounts were found in plasma and red blood cells. These experiments suggest the incorporation of the nitrogen of $^{15}NO_2$ into lung and blood protein of rats.

Further work was conducted to determine the location of ^{15}N in protein, lipid, and other cellular constituents, the mechanisms of incorporation, and the effect of incorporation on biochemical parameters. The data in Table 9 reflect incorporation of ^{15}N from $^{15}NO_2$ into the amino nitrogen of amino acids derived from lung protein of exposed lung homogenate (Experiments 3 through 8). When homogenate was exposed to $^{15}NO_3^-$ and $^{15}NO_2^-$ (prepared by reacting $^{15}NO_2$ with buffer), the amino nitrogen of amino acids became labeled also (Experiment 9), suggesting that one or both of the ions were intermediates in the reaction of NO_2 with lung homogenate. However, incorporation was equally effective when lung homogenate was first boiled and then treated with trichloroacetic acid (Experiment 10), indicating a nonmetabolic reaction. The presence of ^{15}N in amino acid nitrogen is surprising because conversion of NO_2 to R-NH_2 probably would be metabolic. Our tentative explanation, therefore, is that the reaction of $^{15}NO_2$ (and $^{15}NO_3^-$ + $^{15}NO_2^-$) with tissue was primarily chemical in nature, but that a portion of the initial product(s) was metabolized to ammonia during the 30-minute incubation period and was incorporated into amino acids at that time.

Table 8

^{15}N CONTENT OF VARIOUS RAT LUNG PROTEIN SAMPLES EXPOSED TO $^{15}NO_2$

Experiment	Sample	Nitrogen	$^{15}N/^{14}N$	^{15}N Atom Percent
1	Reagent ammonium sulfate	^{14}N	0.0028	0.28
2	Reagent ammonium sulfate	^{14}N	0.0025	0.25
3	Protein--unhydrolyzed	Total	0.00594	0.59
4	Protein--hydrolyzed	Amino acid	0.0516	4.91
5	Protein--0 to 60% ammonium sulfate	Total	0.0515	4.90
6	Protein amino acids	Amino	0.018	1.8
7	Protein amino acids	Amino	0.033	3.2
8	Protein amino acids	Amino	0.074	6.9
9	Protein amino acids--$^{15}NO_3^-$, $^{15}NO_2^-$-treated lung homogenate	Amino	0.039	3.8
10	Protein amino acids--boiled, TCA-treated lung homogenate	Amino	0.056	5.3
11	Reagent ammonium sulfate--95 atom percent ^{15}N	^{15}N	20.306	95.31
12	Reagent ammonium sulfate--95 atom percent ^{15}N	^{15}N	20.580	95.37
13	Reagent ammonium sulfate--45.88 atom percent ^{15}N	^{15}N	0.896	47.26

Table 9

^{15}N CONTENT OF VARIOUS RAT LUNG PROTEIN SAMPLES EXPOSED TO $^{15}NO_2$

Experiment	Fraction	Atom Percent ^{15}N
1 *In vitro*	Unhydrolyzed protein	0.50
	Amino acids	0.60
	Amino nitrogen	0.85
2 TCA control	Unhydrolyzed protein	0.53
	Amino acids	0.72
3 Ions	Unhydrolyzed protein	0.72
4 *In vivo*	RBC - unhydrolyzed protein	0.32, 0.67
	Plasma - unhydrolyzed protein	0.45, 0.64
	Lung - unhydrolyzed protein	0.72, 0.85
5 ^{14}N control	Lung - unhydrolyzed protein	0.34, 0.41
6 Natural abundance		
Literature		0.37
SRI		0.36, 0.38

Throughout the experimental work, we have followed the fate of the nonradioactively labeled NO_2 by mass spectrometry; these analyses have been supported by this contract, and the rest of the endeavor has been funded by an NIH grant. The results in Tables 8 and 9 depend on an as-yet-unresolved question about the relative validities of using masses 14 and 15 or 28 and 29 as ratios between the normal and isotopic nitrogens in the spectrometric analyses. The data presented were based on the former ratio. When N_2 masses are used as the baseline, there is no clear evidence of incorporation or reaction of the isotopic nitrogen with any tissue component. This impasse is to be resolved.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Published

Changes in Dogs' Lungs After Long-Term Exposure to Ozone. G. Freeman, R. Stephens, D. Coffin, and J. Stara. Arch. Environ. Health 26, 209 (1973).

The Effects of Mixed Sodium Chloride Aerosol and Nitrogen Dioxide in Air on Monkeys and Rats. N. J. Furiosi, S. C. Crane, and G. Freeman. Arch. Environ. Health 27, 405 (1973).

Cytological Changes in Dog Lung Induced by Chronic Exposure to Ozone. R. Stephens, G. Freeman, J. F. Stara, and D. L. Coffin. Am. J. Pathol. 73, 711 (1973).

Delayed Maturation of Rat Lung in NO_2-Containing Environment. G. Freeman, L. T. Juhos, N. J. Furiosi, R. Mussenden, and T. A. Weiss. Am. Rev. Respirat. Disease 110, 754 (1974). (See Appendix A)

In Preparation

The Effects of Long-Range NO_2 Exposure on Monkey Lungs.

Other

Dr. Freeman presented a paper, "Criteria from Animals Exposed to Known Concentrations of Nitrogen Dioxide and Ozone, With Potential Use in Epidemiology," at the CDC/EPA/WHO International Symposium in Paris (24-28 June 1974). A copy of the manuscript is presented in Appendix B. Also published was "Pathology of Pulmonary Disease From Exposure to Ambient Gases (Nitrogen Dioxide and Ozone)," G. Freeman, L. T. Juhos, N. J. Furiosi, R. Mussenden, R. J. Stephens, and M. J. Evans, Arch. Environ. Health 29, 203 (1974).

This research was performed principally with funds provided by the National Institutes of Health. However, the Environmental Protection Agency participated in having shared in the support of the central facility for controlled exposure of animals.

Delayed Maturation of Rat Lung in an Environment Containing Nitrogen Dioxide[1-3]

GUSTAVE FREEMAN, LASZLO T. JUHOS, NAZZARENO J. FURIOSI, ROWENA MUSSENDEN, and THOMAS A. WEISS

SUMMARY

The maturation of lungs was monitored by morphometric quantification of the number of alveoli in rats born and raised for 75 days in "clean" air and in air containing 15 ppm of nitrogen dioxide. Although numbers of alveoli in control lungs and lungs exposed to nitrogen dioxide were comparable at birth, there was a consistent delay in maturation in the exposed lungs from the ages of 3 to 60 days. At 75 days, i.e., at the termination of the experiment, however, the deficit had been made up. Thus, the delay in maturation is probably not an appreciable factor in the attrition of alveolar surface area apparent in mature rats exposed similarly for months or years.

Introduction

The number of parenchymal air spaces and the related gas-exchanging surface area were reduced in emphysematous rats that had been exposed continuously to nitrogen dioxide (NO_2) from weaning through old age (1–4). It was considered that this deficiency might have reflected a failure in early maturation, in part, and attrition of pulmonary tissue later, due to the continuous exposure. To test the concept of early retardation of growth in a preliminary experiment, the lungs of 90-day-old rats that had been exposed from birth to approximately 10 ppm of NO_2 were studied. No deficiency in the number of alveoli was revealed. Subsequently, the effects of 15 ppm of NO_2 on the lung were explored more definitively during growth from birth to 75 days. Evidence indicating a temporary lag in "septation" of air spaces during the first 6 weeks after birth is presented here.

(Received in original form June 28, 1974 and in revised form September 6, 1974)

[1] From the Department of Medical Sciences, Stanford Research Institute, Menlo Park, California 94025.

[2] This work was supported mainly by the Division of Health Effects Research, National Environmental Research Center, Environmental Protection Agency, Research Triangle Park, North Carolina, Contract No. 68-02-1243, and partly by the National Institute of Environmental Health Sciences, National Institutes of Health, Bethesda, Maryland, Grant No. ES-00842-02.

[3] Requests for reprints should be addressed to Gustave Freeman, M.D., Stanford Research Institute, 333 Ravenswood Avenue, Menlo Park, California 94025.

Materials and Methods

Experiment A: Ten rats, approximately 14 days' pregnant, were placed in chambers containing 10 ± 2 ppm of NO_2 in air, where they subsequently delivered their progeny. A comparable number delivered their offspring in a controlled environment of ambient air without added pollutants. Progeny were weighed at 62 days ± 1 day, and their skeletal length was measured from the tip of the nose to the end of the outstretched tail. At age 90 days, randomly selected male and female control rats and rats exposed to NO_2 were sacrificed, and their lungs were prepared for quantitative assessment of alveolation, as described earlier (1).

Experiment B: Hilltop rats, 18 days' pregnant, were placed either in exposure chambers containing approximately 15 ppm of NO_2 or in an environment without added contaminants. The concentration of NO_2 was monitored by frequent sampling and use of the Saltzman reaction. Three rats from each of 3 different litters born in environments

TABLE 1

RELATIVE ALVEOLAR MATURATION AT 90 DAYS OF AGE IN NEONATAL CONTROL RATS AND RATS EXPOSED TO 10 PPM OF NITROGEN DIOXIDE

Lungs	Mean ±SD Grid Count* *(alveoli/equivalent square)*	Mean ±SD Linear† *(points on equivalent length of line)*
Control	50.5 ± 11.5	22.1 ± 3.0
Exposed to NO_2	49.0 ± 14.2	23.5 ± 4.9

*Approximately 2,300 alveoli per lung were counted.
†Approximately 400 line intercepts per lung were counted.

with and without NO_2 were removed at 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, and 75 days.

After anesthesia was induced with sodium pentobarital, the lungs were exposed, and the trachea was cannulated with a shortened hypodermic needle. The lungs were left in the opened thorax of rats younger than 10 days, but were excised from older ones. An apparatus was constructed for fixing the lungs with formaldehyde steam, according to the method of Weibel and Vidone (5), and was modified to accept a hypodermic needle-cannula. The degree of distention of the lungs was controlled by holding the pressure of the fixing chamber at −15 cm H_2O and keeping the pressure of formaldehyde slightly higher than atmospheric, sufficient to overcome the resistance of the cannula, which was considerable in the small cannulas used in very young rats. This resulted in uncertain pressure control of the formaldehyde steam, as reported also by Weibel (6).

Fixed lung volume was determined by water displacement. Lungs of animals of adjacent ages (having similar volumes) were grouped, and an average age was assigned. Histologic slides were prepared by cutting 4-μ sections uniformly through the greatest diameter of the right lung from apex to base. Adjacent sections were stained with hematoxylin-eosin and with acid orcein. Counts on 10 randomly selected parenchymal fields of each lung were made at a magnification of 200×. The 10 fields covered extensive portions of the parenchyma at all levels of the relatively small neonatal lungs. Equivalent portions of all lobes were counted in all lungs.

The proportion of space to tissue in the parenchyma was determined by the point-counting method (7, 8), without differentiating between alveoli and ducts, and by using a 100-point integrating eyepiece (Zeiss). The transections of alveolar and ductal airspace units also were counted (7, 8). Transections extending beyond the upper horizontal and the righthand vertical limits of the integrating eyepiece field were included, and those that extended beyond the lower horizontal and lefthand vertical borders were excluded. The parenchymal portion of a lung was assumed to represent 90 per cent of the whole lung volume, as estimated by Weibel (8), Dunnill (9), and Angus and Thurlbeck (10).

Results

The relative number of alveoli in the lungs from Experiment A was determined as described earlier (1). The relative numbers of alveolar air spaces per unit volume of lung, adjusted for differences in distention, were proportionally equivalent to absolute alveolar counts in Experiment B (table 1). No significant difference was found between the degrees of alveolation in control lungs and lungs exposed to NO_2 at age 90 days.

TABLE 2

EFFECT OF EXPOSURE SINCE BIRTH TO APPROXIMATELY 10 PPM OF NITROGEN DIOXIDE ON WEIGHT AND LENGTH OF RATS DURING 62 DAYS OF GROWTH

NO_2 Exposure *(ppm)*	No.	Sex	Average Weight *(g)* Mean	SD	Average Height *(cm)* Mean	SD
0	12	M	313.8	28.1	40.9	1.5
10 ± 2	31	M	275.7	30.6	38.6	1.4
P value			< 0.001		< 0.001	
0	5	F	210.8	10.9	37.9	0.9
10 ± 2	10	F	190.8	15.4	35.5	1.2
P value			< 0.025		< 0.005	

The body weights and skeletal lengths for control rats and those exposed to 10 ppm of NO_2 at 62 days of age are presented in table 2.

In calculating the number of parenchymal air spaces for Experiment B, the method of Weibel (7, 8) was used, as expressed by the formula:

$$N_L = 0.9 \times V_{L_F} \times \frac{n^{3/2}}{\beta\sqrt{\delta}},$$

where N_L = total number of parenchymal (alveolar plus ductal) air spaces in the whole lung; 0.9 = the assumption that 90 per cent of the whole lung is parenchyma; V_{L_F} = volume of whole lung (in milliliters) after fixation; n = number of transectional units in 1 cm^2 of histologic preparation of parenchyma; $^3/_2$ = conversion factor from 2-dimensional planar to 3-dimensional volumetric units; $\beta = 1.92$, a shape coefficient (8) based on the assumption that two thirds of the parenchymal air space consists of spherical alveoli, and one-third consists of cylindrical alveolar ducts; δ = proportion of space to tissue in the parenchyma, determined by point counting.

Lung volumes and transectional and point counts are presented in table 3. At the termination of the study (75 days), lung volumes had not reached a plateau and presumably were continuing to increase in porportion to body weight until maturity; however, from approximately 25 days of age, the lung volumes of rats exposed to NO_2 were considerably larger than those of control rats. The proportion of space to tissue fluctuated with age more in the parenchyma of control lungs than in lungs exposed to NO_2, and the mean value was slightly higher in control lungs. The number of parenchymal air spaces per unit volume (1 cm^3) of parenchyma and per whole lung (figure 1) are presented in table 4. Also listed are the computed rates of formation of individual parenchymal airspace units between the sampling periods and the average volumes.

The significant difference ($P < 0.05$) between the over-all means of the transectional counts of

TABLE 3

VOLUMES AND TRANSECTIONAL AND POINT COUNTS OF PARENCHYMAL AIR SPACES IN MATURING CONTROL LUNGS AND LUNGS EXPOSED TO NITROGEN DIOXIDE (NO_2)

Age (days)	No.	Fixed Lung Volume (cm^3) Mean	SE	No. of Transections/Grid of 0.0016 cm^2 Mean	SE	Per cm^2 ($\times 10^3$)	Proportion of Space to Tissue in Parenchyma by Point Counts (%) Mean	SE
Rats exposed to air								
1	3	0.30	0.08	16.8	0.80	10.5	71.8	1.67
4	6	0.90	0.09	16.2	0.95	10.1	69.9	1.48
10	3	1.23	0.08	30.9	1.61	30.9	65.4	1.15
15	3	1.59	0.42	34.0	1.67	21.2	66.7	1.09
25	6	2.58	0.51	31.1	1.50	19.4	66.9	1.73
40	3	2.60	0.03	41.8	2.60	26.1	49.8	2.48
50	3	3.48	0.65	34.8	2.56	21.7	58.8	5.05
60	3	5.70	0.35	26.7	2.00	16.7	66.2	1.90
75	3	7.05	0.70	23.4	1.51	14.6	67.2	2.01
Mean				28.4		19.0		
Rats exposed to approximately 15 ppm of NO_2 in air								
1	2	0.35	0.15	19.5	1.52	12.2	61.4	9 79
3	3	0.60	0.10	18.9	1.20	11.8	69.4	3.73
10	9	1.02	0.16	21.6	0.92	13.5	69.2	0.90
20	3	2.55	0.00	23.0	1.99	14.4	69.9	3.00
35	5	3.90	0.68	19.8	1.59	12.4	73.9	1.41
55	4	7.64	1.53	20.6	1.32	12.9	71.1	3.14
75	3	7.95	2.30	21.7	2.35	13.6	69.9	1.80
Mean				20.7		13.0		
P value (control versus experimental)				< 0.05		< 0.05		

the lungs from control rats and rats exposed to NO_2 (table 3) generated the significant difference between the means of the number of parenchymal air spaces per unit volume (1 cm^3) in table 4. In lungs from both 1- and 75-day-old control rats and rats exposed to NO_2, the total numbers of parenchymal airspace units were approximately equal. Between the ages of 10 and 45 days, however, there was approximately a 36 per cent deficit in the number of parenchymal airspace units among the rats exposed to NO_2 and, presumably, in "over-all pulmonary maturation" (figure 1). Comparison of the slopes of the growth curves between these ages for both groups revealed a significantly reduced rate of "septation" for the rats exposed to NO_2 ($P < 0.05$).

The calculated formation rates of new parenchymal airspace units varied between lungs from control rats and rats exposed to NO_2. Between the ages of 4 and 10 days, the rate of approximately 7,400 units per day in the control group was 32 per cent higher than that in the group exposed to NO_2; however, during the latter part of the experimental period, the growth rate of new air spaces in the lungs exposed to NO_2 was somewhat higher than that in control lungs.

Variations in the average size of individual parenchymal airspace units in the control group indicate that normal growth of lungs involves periodic expanding and stretching of the parenchyma in addition to formation of new units. In contrast, the relative homogeneity in average size of individual parenchymal airspace units in the lungs exposed to NO_2 suggests persistent distention with increased volume of individual air spaces of approximately 30 per cent.

Discussion

The total alveolar count in lungs was used as a measure of pulmonary maturation by Dunnill (9) and Davies and Reid (11) in children from birth to 8 years of age, and by Angus and Thurlbeck (10) in adults from 12 through 85 years of age. In rats, Weibel (12) and Burri and associates (13) used parameters other than direct alveolar counts. Bartlett (14–16) studied the effects of altitude and endocrine constituents on pulmonary development in terms of numbers of alveoli.

It was apparent that Weibel's formula (8) for enumerating parenchymal air spaces in the normal lung might not be appropriate when applied wholly to lungs altered by disease. Diseased lungs with changes in cellularity, connective tissue, fluid content, types of cells, thickness of basement membranes, and alterations of alveolar septa and of distensibility under standard pressure (−15 cm H_2O) might not meet the criteria for shapes of air spaces (β) or proportions of space to tissue (δ) or both applied to normal lungs. Fortuitously, the over-all average ratio of space to tissue (table 3) revealed little or no difference between the control group and the group exposed to NO_2, although there was greater homogeneity of this ratio among the latter. During growth of diseased animals, therefore, maturation in terms of total number of parenchymal air spaces can only be expressed as an estimate.

Maximal growth rate of airspace units (table 4) occurred between 4 and 10 days of age in the control group. This is consistent with "Phase II" of pulmonary alveolation described by Burri and associates (13). The lack of statisti-

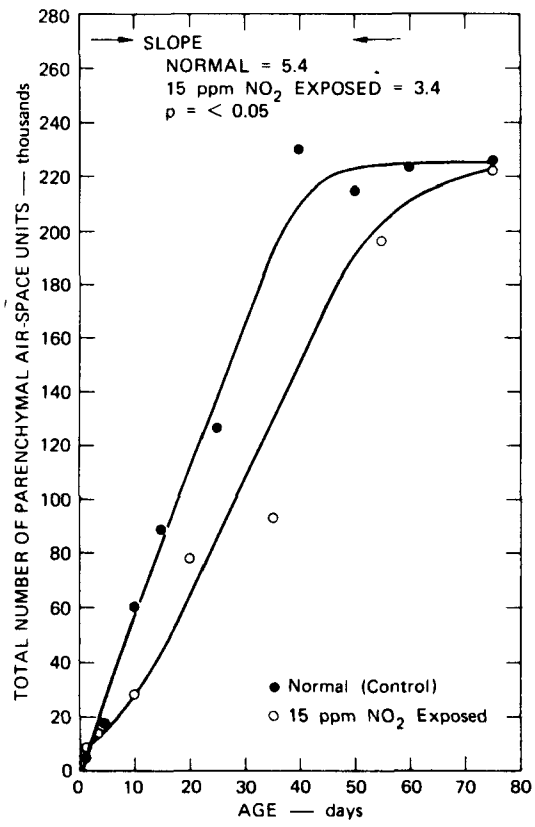

Fig. 1. Comparative growth of parenchymal airspace units in lungs from maturing control rats and rats exposed to 15 ppm of nitrogen dioxide (NO_2).

TABLE 4

NUMBER OF PARENCHYMAL AIRSPACE UNITS (ALVEOLAR AND DUCTAL), RATES OF FORMATION, AND INDIVIDUAL VOLUMES IN LUNGS OF MATURING CONTROL RATS AND RATS EXPOSED TO NITROGEN DIOXIDE (NO_2)

Age (days)	No.	No. of Parenchymal Airspace Units: Per Unit Volume ($\times 10^3$)	Per Whole Lung ($\times 10^3$)	Formed per Day ($\times 10^3$)	Volume of Individual Airspace Units ($ml \times 10^{-5}$)
Exposed to air					
1	3	20.9	5.6		4.8
				3.2	
4	6	20.0	16.2		5.0
				7.4	
10	3	54.6	60.4		1.8
				5.7	
15	3	62.2	89.1		1.6
				3.7	
25	6	54.4	126.3		1.8
40	3	98.3	230.0		1.0
50	3	68.3	214.8	1.9	1.5
60	3	43.7	224.0		2.3
75	3	35.3	224.9		2 8
Mean		50 9			
Exposed to approximately 15 ppm of NO_2 in air					
1	2	28.3	8.9		3.5
				2.4	
3	3	25.3	13.7		3.9
				2 1	
10	9	31.0	28.5		3.2
				5.0	
20	3	34.1	78.2		2.9
35	5	26.5	93.0		3.8
				2.6	
55	4	28.6	196.8		3.5
75	3	31.2	223.3		3.2
Mean		29.3			
P value (control versus experimental)		< 0.05			

cal distinction in number of air spaces beyond the age of 60 days supports the earlier observation in 90-day-old rats exposed to approximately 10 ppm of NO_2; however, the difference in slope of the two "alveolar growth" curves (figure 1) between 10 and 45 days of age indicates a temporary lag, or delay, in pulmonary maturation in rats exposed to NO_2. This effect may reflect an error in assuming identical coefficients for alveolar shape for both control lungs and lungs exposed to NO_2. Bignon and associates (17) also did not use an altered value in their detailed study of human chronic, obstructive, bronchopulmonary disease.

Some pregnant rats died from the NO_2 exposure before delivering their progeny, and litters generally were smaller than those of control rats. The mortality of the exposed progeny remained high for 15 days, indicating that animals used in our experiments were survivors of an environment that was only marginally compatible with life.

The extent of the observed lung enlargement in these experiments is similar to that reported by Bartlett (14) in studies of altitude-imposed hypoxia, but it is unlikely that the basic mechanisms are the same.

It was anticipated that continuous exposure of newborn animals to a high "subacute" concentration of NO_2 would result in injury and disease to their lungs, as it did in older animals (1–4). Although the pulmonary tissue fixed with volatilized formaldehyde did not provide optimal visualization of pathologic detail, mi-

croscopy revealed a lack of both fixed tissue reaction and macrophage aggregation in these immature lungs exposed to NO_2.

The objective of this study was to explore further the basis for the reduced number of parenchymal air spaces in the lungs of rats rendered emphysematous during long-term, intermittent exposure to approximately 15 ppm of NO_2 from the age of 1 month (1). The data support evidence for loss of parenchymal tissue essentially by a destructive process during the latter part of the animals' lives, although there also may well have been a transient neonatal delay in maturation. Such an effect was unlikely, however, because the emphysematous rats (1) had not been exposed to NO_2 until they were at least 1 month old.

References

1. Freeman, G., Crane, S. C., Furiosi, N. J., Stephens, R. J., Evans, M. J., and Moore, W. D.: Covert reduction in ventilatory surface in rats during prolonged exposure to subacute nitrogen dioxide, Am Rev Respir Dis, 1972, *106*, 563.
2. Haydon, G. B., Freeman, G., and Furiosi, N. J.: Covert pathogenesis of NO_2-induced emphysema in the rat, Arch Environ Health, 1965, *11*, 776.
3. Freeman, G., and Haydon, G. B.: Emphysema after low-level exposure to NO_2, Arch Environ Health, 1964, *8*, 125.
4. Freeman, G., Crane, S. C., Stephens, R. J., and Furiosi, N. J.: Pathogenesis of the nitrogen dioxide-induced lesion in the rat lung: A review and presentation of new observations, Am Rev Respir Dis, 1968, *98*, 429.
5. Weibel, E. R., and Vidone, R. A.: Fixation of the lung by formalin steam in a controlled state of air inflation, Am Rev Respir Dis, 1961, *84*, 856.
6. Weibel, E. R.: Morphological basis of alveolar gas exchange, Physiol Rev, 1973, *53*, 452.
7. Weibel, E. R., and Gomez, D. M.: A principle for counting tissue structures on random sections, J Appl Physiol, 1962, *17*, 343.
8. Weibel, E. R.: Morphometry of the Human Lung, Academic Press, Inc., New York, 1963.
9. Dunnill, M. S.: Postnatal growth of the lung, Thorax, 1962, *17*, 329.
10. Angus, G. E., and Thurlbeck, W. H.: Number of alveoli in the human lung, J Appl Physiol, 1972, *32*, 483.
11. Davies, G., and Reid, L.: Growth of the alveoli and pulmonary arteries in childhood, Thorax, 1970, *25*, 669.
12. Weibel, E. R.: Postnatal growth of the lung and pulmonary gas-exchange capacity, in *Development of the Lung*, A. V. S. de Reuck and R. Porter, ed., Churchill, London, 1967, p. 131.
13. Burri, P. H., Dbaly, J., and Weibel, E. R.: Postnatal growth in the rat lung, Experientia, 1972, *28*, 727.
14. Bartlett, D., Jr.: Postnatal growth of the mammalian lung: Influence of low and high oxygen tensions, Resp Physiol, 1970, *9*, 58.
15. Bartlett, D., Jr.: Postnatal growth of the mammalian lung: Influence of exercise and thyroid activity, Resp Physiol, 1970, *9*, 50.
16. Bartlett, D., Jr.: Postnatal growth of the mammalian lung: Influence of excess growth hormone, Resp Physiol, 1971, *12*, 297.
17. Bignon, J., Khoury, F., Even, P., Andre, J., and Brouet, G.: Morphometric study in chronic obstructive bronchopulmonary disease, Am Rev Respir Dis, 1969, *99*, 669.

CRITERIA FROM ANIMALS EXPOSED TO KNOWN CONCENTRATIONS OF NITROGEN DIOXIDE AND OZONE, WITH POTENTIAL USE IN EPIDEMIOLOGY

G. FREEMAN, L. T. JUHOS, N. J. FURIOSI,
W. R. POWELL, AND R. MUSSENDEN

Stanford Research Institute
Menlo Park, California, USA

DR. POCKIANELLA, LADIES AND GENTLEMEN:

Much has been learned about the responses of laboratory animals to atmospheres polluted by either nitrogen dioxide (NO_2) (Freeman et al. [1-4]) or ozone (O_3) (Stokinger et al. [5], Werthamer et al. [6], Freeman et al. [7]). But applying this knowledge to the epidemiology of disease is difficult because of the variability of man's activities, the complexity of photochemical reactions [8,9], and the difficulties in devising effective epidemiologic designs.

At least four criteria are required to group individuals for a meaningful epidemiologic study of the effects of oxides of nitrogen (NO_X) and oxidants. The first concerns residence in crowded, traffic-laden, and industrialized areas where the toxic, interdependent, photochemically induced atmospheric pollutants NO_2 and O_3 cycle simultaneously during half of each day in varying ratios and in similar average concentrations [8,9]. Thus, epidemiologic observations are related to the impact of mixtures. (Our experience with rats reveals that in short-term experiments, O_3 is about 10- to 20-fold more injurious to the lungs than NO_2 (Freeman et al. [10])).

Whereas one may limit atmospheric components to NO_2 and O_3 under laboratory conditions, in smoggy atmospheres peroxyacetyl nitrates also exist as photochemical products [8], and independent pollutants such as sulfur dioxide (SO_2) [11] appear frequently to complicate interpretation.

Cigarette smoking is a second factor [12]. Among the injurious components of tobacco smoke, oxides of nitrogen are found in hundreds of parts per million (Haagen-Smit et al. [13]), of which an undetermined portion is NO_2. Almost all the NO_X carried into the lungs with each bolus of inhaled smoke is retained (Freeman et al. [14]). The close correlation between the prevalence of human emphysema and chronic bronchitis and the amount of tobacco smoke inhaled over a period of

years [12] is especially noteworthy. Pulmonary disease is readily induced in rats by NO_2 in concentrations similar to those in tobacco smoke [4]. As a corollary, nonsmokers are largely spared from chronic obstructive pulmonary diseases, regardless of their environments.

A third factor is occupation. Hazardous conditions are produced by confined concentrations of NO_2 or O_3, or both, independent of photochemical activity. Moreover, certain activities create high concentrations of NO_2 or O_3 not achieved in photochemical reactions (Stokinger [15]). NO_X also is produced naturally by bacterial action on crops stored in silos and is the cause of silo-filler's disease in farmers. Industrial processes such as welding and the use of explosives in mines also can be hazardous when high temperatures accelerate the oxidation of nitrogen to form toxic oxides [9].

The fourth factor is individual susceptibility, which includes age at initial exposure. Generally, the very young are poorly defended against exogenously induced disease compared with more mature individuals, but this may not be a universal truth, as we shall see. The elderly also are vulnerable because of their susceptibility to cardiopulmonary disease and possibly because of aging. Individuals prone to respiratory allergy are particularly responsive to atmospheric irritants. With regard to chronologic age, the type or response to exogenous insults may depend on whether an individual was born in a polluted environment or moved into it later in life.

What is the evidence for this? We exposed four-week-old rats continuously to NO_2. Their rates of red cell formation increased rapidly, and the rats developed polycythemia [3]. [See Slide 1.] In contrast, animals exposed from birth generated red cells less rapidly than rats born in clean air. As a result, such exposed rats were relatively anemic at seven weeks of age and contrasted sharply with polycythemic rats of the same age that were exposed only during the last two of the seven weeks [14].

Also, in rats exposed from birth to high subacute concentrations of NO_2 (~15 ppm), the mechanical compliance of lungs was not affected by increasing the frequency of respiration. Lungs of animals of the same age, exposed after they were a month old, developed frequency dependence of compliance, because of changes in the small airways [14] [Slide 2]. Thus, the failure of animals exposed from birth to have developed small airway disease, as judged by frequency dependence of compliance, may have been determined by neonatal contact with NO_2. Both the hematologic and respiratory observations, then, suggest altered responsiveness. Incidentally, growth of the lung defined as the rate of formation of new alveoli--in animals exposed continuously to NO_2 from birth--was less than normal for the first ten weeks [14]. [See Slide 3.] The significance of very early exposure relative to subsequent pathogenesis appears to deserve further study.

Whereas smoking clearly is associated with chronic pulmonary disease, ambient ozone has not yet been so identified epidemiologically [8]. What is ozone's impact on the nonsmoking population? The odor of ozone is known to be detectable at a concentration of about 0.02 ppm; increased susceptibility to infection in animals begins at about 0.08 ppm; and eye irritation to peroxyacetyl nitrates is noticeable at about 0.1 ppm [8]. At the 0.1-ppm level, increased airway resistance also becomes evident in some individuals and is readily apparent in subjects breathing a concentration of about 0.5 ppm for three hours on five consecutive days. Exposure to about 1.0 ppm rapidly becomes intolerable to man [8].

In contrast, there is little clinical evidence of an adverse effect on the respiratory tract during experimental exposure to levels of NO_2 below 1.0 ppm, although the odor of 0.1 ppm is readily detectable. To simulate maximum human exposure to the mixture of NO_2 and ozone on a cyclic photochemical basis, we exposed rats four hours per day and observed pathologic effects in the lungs that could relate to chronic pulmonary disease [10]. At a concentration of about 0.9 ppm of each gas, a lesion developed at the narrow junction of the respiratory bronchiole and alveolar duct through which air is conducted to and from the alveoli. [Slide 4] Epithelial cells were injured and replaced (Evans et al. [17]), often by hypertrophic cells. The hypertrophic epithelium, together with fibroblasts and chronic inflammatory cells, proliferated at the level of the smallest airways to form denser tissue. The effects of these changes on lung function have not been explored. As with high subacute concentrations of NO_2 [1,4], continuous exposure of rats to a mixture of 0.9 ppm of ozone and of NO_2 led to the development of an emphysema-like disease. [Slide 5] Also, continuous exposure to a mixture of 2.5 ppm of NO_2 and 0.25 ppm of ozone resulted in disease of the alveolar ducts characteristic of the response to ozone rather than to NO_2. [Slides 6 and 7] Thus, ozone, clearly, is the primary cause of deep-seated pulmonary disease in the experimental animal when mixtures of the agents are present on a schedule approximating man's experience in photochemically smoggy atmospheres.

Let us shift our attention to animal studies with higher subacute concentrations of NO_2--about 14 to 17 ppm [3,4]. The relationship of smoking to the epidemiology of chronic obstructive pulmonary diseases [12], to which NO_2 may contribute, now comes into focus. Extrapolation to chronic human disease from the rat, which has a life span of about 4 percent that of man, is hazardous. Nevertheless, the fortuitous similarity in the relative time required for the development of emphysema in the rat compared with that in man warrants attention [1,3,4]. The clinical and morphological features associated with habitual smoking of cigarettes are consistent with the changes in the lungs of rats exposed to high subacute concentrations of NO_2 for long periods [1,3,4]. It is noteworthy, also, that ozone, which is absent from tobacco smoke, induces a similar disease in the rat at approximately one-twentieth the concentration of NO_2 when mixed with it in equal amounts [10].

In summary, it would not seem feasible to distinguish between the specific effects of NO_2 and O_3, epidemiologically, except in the cases of high occupational concentrations and of habitual smoking. As photochemically interdependent gases, they coexist and act almost simultaneously.

This research was supported by Contract NO. 68-02-1243 of the Environmental Protection Agency (USA), Division of Health Effects Research, Research Triangle Park, North Carolina, and by Grant ES00842-02 of the National Institutes of Health.

References

1. FREEMAN, G., HAYDON, G.B., "Emphysema after low-level exposure to NO_2," Arch. Environ. Health 8, 125 (1964).

2. FREEMAN, G., STEPHENS, R.J., CRANE, S.C., FURIOSI, N.J., "Lesion of the lung in rats continuously exposed to two parts per million of nitrogen dioxide," Arch. Environ. Health 17, 181 (1968).

3. FREEMAN, G., CRANE, S.C., STEPHENS, R.J., FURIOSI, N.J., "Pathogenesis of the nitrogen dioxide-induced lesion in the rat lung: A review and presentation of new observations," Am. Rev. Respirat. Disease 98, 429 (1968).

4. FREEMAN, G., CRANE, S.C., FURIOSI, N.J., STEPHENS, R.J., EVANS, M.J., MOORE, W.D., "Covert reduction of ventilatory surface in rats during prolonged exposure to subacute nitrogen dioxide," Am. Rev. Respirat. Disease 106, 536 (1972).

5. STOKINGER, H.E., WAGNER, W.D., DOBROGORSKI, O.J., "Ozone toxicity studies: III. Chronic injury to lungs of animals following exposure at low level," Arch. Industr. Health 16, 514 (1957).

6. WERTHAMER, S., et al., "Ozone-induced pulmonary lesions: Severe epithelial changes following sublethal doses," Arch. Environ. Health 20, 16 (1970).

7. FREEMAN, G., STEPHENS, R.J., COFFIN, D.L., STARA, J.F., "Changes in dogs' lungs after long-term exposure to ozone," Arch. Environ. Health 26, 209 (1973).

8. U.S. Public Health Service, Air Quality Criteria for Photochemical Oxidants, National Air Pollution Control Administration Publication No. AP-63, Washington, D.C. (1970).

9. U.S. Environmental Protection Agency, Air Quality Criteria for Nitrogen Oxides, Air Pollution Control Office Publication No. AP-84, Washington, D.C. (1971)

10. FREEMAN, G., JUHOS, L.T., FURIOSI, N.J., MUSSENDEN, R., STEPHENS, R.J., EVANS, M.J., "Pathology of pulmonary disease from exposure to ambient gases (nitrogen dioxide and ozone)," Arch. Environ. Health 29, 203 (1974).

11. U.S. Public Health Service, Air Quality Criteria for Sulfur Oxides, National Air Pollution Control Administration Publication No. AP-50, Washington, D.C. (1969).

12. U.S. Department of Health, Education and Welfare, The Health Consequences of Smoking, Chapter 2, Public Health Service, January 1973, pp. 31-62.

13. HAAGEN-SMIT, A.J., BRUNELLE, M.F., HARA, J., "Nitrogen oxide content of smoke from different types of tobacco," Arch. Industr. Health 20, 399 (1959).

14. FREEMAN, G., et al. Unpublished data.

15. STOKINGER, H.E., "Ozone toxicology. A review of research and industrial experience: 1954-1964," Arch. Environ. Health 10, 719 (1965).

16. SCHWARTZ, R., DAMESHEK, W., "Drug induced immunologic tolerance," Nature 183, 1682 (1959).

17. EVANS, M.J., STEPHENS, R.J., CABRAL, L.J., FREEMAN, G., "Cell renewal in the lungs of rats exposed to low levels of NO_2," Arch. Environ. Health 24, 180 (1972).

18. BUCKLEY, R.D., HACKNEY, J.D., CLARK, K., POSIN, C., "Some effects of ozone inhalation on human erythrocyte metabolism" (Abstract), Fed. Proc. Abstracts 33, 335 (1974).

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/1-76-021	2.	3. RECIPIENT'S ACCESSION NO.
4. TITLE AND SUBTITLE TRACE SUBSTANCES AND TOBACCO SMOKE IN INTERACTION WITH NITROGEN OXIDES. Biological Effects.		5. REPORT DATE April 1976
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Gustave Freeman and Laszlo T. Juhos		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Stanford Research Institute Menlo Park, California 94025		10. PROGRAM ELEMENT NO. 1AA601
		11. CONTRACT/GRANT NO. 68-02-1243
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711		13. TYPE OF REPORT AND PERIOD COVERED Final. July 1973/Sept. 1974
		14. SPONSORING AGENCY CODE EPA-ORD

15. SUPPLEMENTARY NOTES

16. ABSTRACT

The emphasis of this study is on determination of the response of newborn animals living in an environment containing nitrogen dioxide.

The following are also examined:

a. Both mature and newborn monkeys (Macaca speciosa) exposed continuously to NO_2 or to ozone.

b. The relative effects of tobacco smoke compared with those of NO_2.

c. The binding of NO_2 in tissue, based on the use of isotopically labeled, nonradioactibe NO_2.

d. The detailed hematologic effects of exposure to NO_2 or to ozone.

In addition to the usual parameters for detecting changes in the erythrocytic series, biochemical studies were conducted on the blood of exposed animals.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Nitrogen Oxide Nitrogen Dioxide Tobacco Ozone Laboratory animals Respiration Hematology	Tobacco smoke	06, F, T

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 41
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)