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Early Markers of Lung Injury

John N. Evans, David R. Hemenway, and Jason Kelley
University of Vermont, Burlington, VT

Includes the Commentary of the Institute's Health Review Committee

Research Report Number 29

HEALTH EFFECTS INSTITUTE

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HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

Early Markers of Lung Injury

John N. Evans[1], David R. Hemenway, and Jason Kelley

ABSTRACT

The goal of this study was to develop an early marker of lung injury that might change in response to exposure to a mobile source emission. Nitrogen dioxide (NO_2)[2] was chosen as an example of an atmospheric pollutant that is related to automobile emissions. Since reorganization of the connective tissue matrix of the lung occurs in response to injury, markers of connective tissue metabolism were selected as targets. Hydroxylysine became the marker of choice. It is an amino acid that is virtually exclusive to collagen, although it does occur in minimal amounts in other proteins. Furthermore, it is excreted in the urine, which makes it readily available for analysis using noninvasive techniques. Other markers evaluated as part of the study included angiotensin-converting enzyme as a marker of lung injury, desmosine as a marker of elastin degradation, and hydroxyproline as another marker of collagen metabolism.

Male Fischer-344 rats were exposed in whole-body chambers to controlled concentrations of NO_2 for various doses and periods of time. The concentrations of NO_2 ranged from 0.5 to 30 parts per million (ppm); the rats were exposed for six hours per day for periods of two days to four weeks. Urine and bronchoalveolar lavage samples were collected and analyzed for the appropriate marker. In addition, pulmonary function studies and histologic examinations of the lungs were completed at selected time points.

Urinary hydroxylysine concentration increased as a function of NO_2 concentration during six-hour-per-day exposures for two days. This short-term exposure required relatively high doses to achieve significant changes in the hydroxylysine output. During one-week exposures to either 25 or 30 ppm NO_2, there was an increase in urinary hydroxylysine associated with changes in lavage concentrations of angiotensin-converting enzyme and hydroxylysine. The lungs of these animals demonstrated histologic changes typical of oxidant injury.

Four-week exposure protocols using 0.5 and 1 ppm NO_2 were most interesting in terms of the sensitivity of the marker. There was minimal damage revealed by the histology and function studies, yet there were significant increases in the excretion of hydroxylysine. It appears that hydroxylysine can be indicative of exposure when other parameters are normal. It will require long-term follow-up of exposed rats to determine whether or not the change in marker concentration is predictive of damage.

Hydroxylysine may be an excellent marker of exposure to oxidants in the human population. Controlled studies to establish base-line values are needed, followed by carefully controlled studies in individuals with connective tissue abnormalities of the lung.

INTRODUCTION

The overall goal of this study was to identify a biological marker, or markers, of lung injury that is induced by exposure to mobile source emissions. A basic operating premise was that the marker had to be available in a body fluid that could be monitored in a relatively noninvasive manner in humans. The marker should vary with the level of exposure and should reflect the extent of injury to the lung. The fluids deemed acceptable for analysis included bronchoalveolar lavage fluid, blood, and urine. Nitrogen dioxide was selected as an example of a pollutant associated with mobile source emissions. Indices of connective tissue metabolism were chosen as possible markers, for the reasons cited below.

Environmental pollutants that result from mobile source emissions, such as NO_2, have been implicated in the development of pulmonary injury. Evidence in humans and animals exists to demonstrate altered broncho-reactivity and lung compliance associated with metabolic and biochemical changes (for reviews see Mustafa and Tierney 1978; Dawson and Schenker 1979). Nitrogen dioxide is produced by oxidation of atmospheric nitrogen and is a ubiquitous pollutant of the environment. Urban concentrations can range from 0.08 to 0.2 ppm, with spikes to 1 ppm. In addition to exposure due to combustion engines, occupational exposures occur in arc welding, in copper dip tanks, and in agricultural settings. It is clear that a large number of individuals are exposed to a wide range of NO_2 levels derived from a variety of sources.

The response of the lung to NO_2 is complicated and depends on species, level of exposure, duration of exposure, and the time at which the lung is examined relative to exposure. There appear to be acute effects, including bron-

[1] Correspondence may be addressed to Dr. John N. Evans, College of Medicine, Department of Physiology and Biophysics, Given Building, University of Vermont, Burlington, VT 05405.

[2] A list of abbreviations appears at the end of this report for your reference.

chospasm, and long-term effects involving reorganization of the connective tissue matrix of the lung. Reported changes in pulmonary connective tissue range from the development of pulmonary fibrosis to emphysema. Hattori and Takemura (1974) reported the development of fibrotic lesions in mice after exposure to 0.5 ppm NO_2 for six months. Dróždž and coworkers (1977) exposed guinea pigs to 1 ppm NO_2 for six months and reported a significant decrease in lung collagen. Kleinerman and colleagues (1982) exposed hamsters to 30 ppm NO_2 for three weeks and reported a decrease in elastin and collagen during exposure, which returned toward normal after exposure. On the basis of light microscopic examination, Freeman and associates (1969) reported changes in collagen and elastin in alveolar septa when rats were exposed to 15 ppm NO_2 for one week. These studies are only a representative sampling of the literature on the effects of NO_2 on pulmonary connective tissue. It is clear that exposure induces changes in connective metabolism, with subsequent development of significant changes in lung architecture; the actual outcome is dependent on the level and duration of exposure and the species exposed.

The present study is based on the evidence, cited above, that alterations in the connective tissue do occur in NO_2 exposure. The two major components of this matrix are collagen and elastin. These proteins are synthesized and degraded in the lung as part of an ongoing turnover of connective tissue components. In the development of fibrosis or emphysema, the relationship of synthesis and degradation is altered, which results in a net change of collagen or elastin content in the lung.

We selected hydroxylysine as a potential marker of lung injury. It is a modified amino acid found in relatively few proteins. It is essentially unique to collagen and to proteins containing collagen-like sequences. It has greater specificity for these proteins than does hydroxyproline, another modified amino acid that has been used extensively in previous metabolic and lung injury studies. When peptides are degraded, the constituent hydroxylysine is not reutilized in the body, but rather is released in the urine, presumably in the form of small peptides.

Hydroxylysine has an advantage over hydroxyproline, the more commonly studied collagen amino acid, in that it is not found in appreciable amounts in the procollagen telopeptides. These are relatively long peptides that are cleaved from the collagen molecule as it is synthesized. Although most of the length of these peptides consists of globular, noncollagen domains, one of the procollagen propeptides contains an internal collagen-like sequence that is rich in hydroxyproline. With this knowledge in hand, it is no longer possible to think of hydroxyproline as a strict marker of degradative processes that attack mature tissue collagen. Because the procollagen peptides do not contain hydroxylysine residues, the presence of hydroxylysine in tissues more closely reflects tissue collagen levels, and its appearance in body fluids can be assumed to more accurately reflect degradative processes.

Desmosine is a complex cross-linked amino acid derivative that is specific for the connective tissue protein elastin. Previous work has shown that during elastin turnover in the body, desmosine is not reused, and is not taken up in the intestines. Desmosine released in elastin degradation is excreted in the urine. Goldstein and Starcher (1978) have demonstrated that urinary desmosine excretion is markedly increased after intratracheal instillation of elastase. Harel and coworkers (1980) have shown that urinary excretion of desmosine is elevated in patients with chronic obstructive pulmonary disease when compared with control subjects.

Angiotensin-converting enzyme is found in a number of tissues, including the capillary bed of the lung. Appearance of angiotensin-converting enzyme in bronchoalveolar lavage fluid serves as a sensitive and early marker of lung injury induced by pneumotoxic drugs (Newman et al. 1980) or by oxidant gases (Rowe et al. 1981). The enzyme in lavage fluid appears to be derived from endothelial cells and is not a transient exudate from the serum.

SPECIFIC AIMS

The specific aims of this project were (1) to identify markers of lung injury that could be measured in lavage fluid or urine; and (2) to determine if levels of these markers are altered when rats are exposed to controlled concentrations of NO_2.

METHODS

ANIMALS

Rats were selected as the experimental species for these studies because of their extensive use in previous studies of lung injury in this laboratory and others. Male Fischer-344 rats were obtained from Charles River Laboratory (Kingston, NY) breeders. These rats were born via Caesarean section and were maintained, upon delivery to the University of Vermont, in an isolated facility used only for exposure studies.

EXPOSURE

The rats were exposed in horizontal laminar flow cham-

bers (previously described in Hemenway et al. 1983). The rats were individually caged within the chamber, and, when appropriate, metabolic cages were employed to collect urine samples during the exposures.

As described previously (Hemenway and Jakab 1987), significant effort was expended to develop reliable and accurate methods of generating and monitoring NO_2 in the chamber. Briefly, in a fume hood, a 50-liter Teflon bag was filled with pure NO_2 and dry pure nitrogen. The concentration of NO_2 in this source bag was varied depending on the desired final concentration in the chamber. The bag was connected to a stainless-steel Teflon diaphragm pump, and flow from the bag was varied, relative to the main chamber flow, in order to achieve the desired chamber concentration. This system is highly accurate and repeatable. We have consistently achieved levels within 10 percent of target concentration in these studies. Nitrogen dioxide levels were monitored on-line in the chamber using a high-temperature converter, chemiluminescent NO_2 analyzer.

HYDROXYLYSINE

Total hydroxylysine, including both the free and glycosylated forms, was measured using a method developed as part of this project (Kelley and Chrin 1984). Briefly, we adapted a thin-layer chromatographic procedure capable of analyzing picomolar quantities of individual amino acids. The procedure uses ^{14}C-dansyl chloride to form ^{14}C-didansyl hydroxylysine, which can be readily separated from other amino acids by thin-layer chromatography on polyamide plates (Airhart et al. 1979). Quantification of nonradiolabeled hydroxylysine is carried out directly on acid hydrolysates of crude tissue and body fluid samples by mixing these with a precise amount of ^{3}H-hydroxylysine ("probe"). The degree to which the specific radioactivity of the latter isotope is lowered allows indirect, but very specific, quantification of picomolar quantities of hydroxylysine.

To quantify hydroxylysine content of lavage, urine, and crude tissue, aliquots were hydrolyzed overnight in redistilled hydrochloric acid (HCl) at 110°C under vacuum, evaporated to dryness, resuspended twice in redistilled water, and redried. The dried sample was resuspended in deionized water to the volume needed. An aliquot of hydrolysate was mixed with 10 µl of ^{3}H-hydroxylysine (300 pmol, unless otherwise stated), dried under vacuum, and solubilized in 20 µl of 1 M sodium-carbonate, sodium-bicarbonate buffer (pH 9). The pH of the resulting sample was tested, and 10 µl additional buffer was added if the pH remained less than 9.

An equal volume of ^{14}C-dansyl chloride solution in acetone was added to the sample and was reacted at 37°C in a humidified atmosphere for 30 minutes, or until the yellow color disappeared. The reacted sample was dried and then extracted three times in 100 µl of water-saturated ethyl acetate. The pooled samples in ethyl acetate were dried, and the sample was reconstituted in 10 µl deionized water.

Chromatography was performed, as previously described. In brief, the reaction mixture was applied near the corner of a 7.5 × 7.5-cm^2 micropolyamide plate by repeated spotting of individual microdrops from a capillary tube. The plates were developed first by ascending chromatography in covered glass tanks in formic acid (88 percent):water (1:50 v/v) and then dried. The plates were then turned 90 degrees and rechromatographed in benzene:glacial acetic acid (9:1 v/v). For optimal separation of hydroxylysine from other dansylated derivatives, chromatography was repeated in a second dimension with the same solvent system. Development in each tank was continued until the solvent front ran all the way to the top of the plate. Under the conditions of chromatography chosen, most of the amino acids were allowed to migrate, in both dimensions, further from the origin in order to provide optimal separation of didansyl-hydroxylysine from dansyl-hydroxide and other closely neighboring derivatives. Dansyl-amino acids were visualized under short-wave ultraviolet light through protective lenses, and were identified on the basis of patterns published in the literature (Kelley and Chrin 1984).

Individual dansylated derivatives were cut from the plate and placed in 7-ml scintillation vials, to which were added 0.15 ml fresh NCS tissue solubilizer (Amersham, Arlington Heights, IL) and 3 ml Econofluor. Radioassay was performed in a Packard Tri-Carb liquid scintillation spectrometer with average counting efficiencies of 0.35 for ^{3}H and 0.70 for ^{14}C. The ^{3}H-specific radioactivity of hydroxylysine (dpm/pmol) was then calculated as:

$$\frac{^{3}\text{H dpm} \times 2}{^{14}\text{C dpm}/7.2 \text{ dpm/pmol}}$$

where 7.2 represents the specific radioactivity of ^{14}C-dansyl chloride. The twofold correction is made because of the didansylated nature of the hydroxylysine species isolated.

Hydroxylysine in nonradioactive biologic samples was quantified by mixture with the ^{3}H-hydroxylysine probe, as described above. The expected reduction of specific radioactivity as increasing amounts of nonlabeled hydroxylysine were added can be expressed by the equation:

$$\text{pmol (unknown)} = \text{pmol (probe)} \times \left[\frac{\text{SA (probe)}}{\text{SA (mix)}} - 1 \right]$$

where *SA* stands for the specific activity, and *mix* represents the mixed probe and unknown standard (Airhart

et al. 1979). We set up the assay in such a way that unknown samples containing 300 pmol of hydroxylysine fell in the middle of the nonlinear dilution curve.

The hydroxylysine contents of the unknown samples were expressed directly or were normalized to volume or to total amino acid content (for urine and lavage samples), to dried weight or to protein content (for tissue homogenates), or to creatinine (for urine samples).

To quantify hydroxylysine in lavage fluid, a 1-ml aliquot of thawed fluid was precipitated overnight at 4°C by the addition of 10 ml of ice-cold 100 percent ethanol. The protein pellet was collected by centrifugation at 2,500 × g for 15 minutes at 4°C, and was dissolved in 1 ml of redistilled HCl. The sample was hydrolyzed overnight under vacuum, evaporated to dryness, resuspended twice in deionized water, and redried. The dried sample was then reconstituted in 10 µl of deionized water, mixed with 300 pmol of ^{3}H-hydroxylysine having a specific radioactivity of 32.0 dpm/pmol, dried under vacuum, and solubilized in 20 µl of 1 M sodium bicarbonate buffer (pH 10) for reaction with ^{14}C-dansyl chloride (see above). An equal volume of dansyl chloride solution (11.42 dpm/pmol) in acetone was added to the sample and was reacted at 37°C for 30 minutes. The final dansyl chloride concentration in the reaction mixture was 4.2 mM. The reacted sample was then dried down and extracted in 100 µl of water-saturated ethyl acetate. The ethyl acetate extract was evaporated to dryness, and then reconstituted in 10 µl of acetone for spotting on a micropolyamide plate (see above).

DESMOSINE

Desmosine was measured in urine samples by Dr. Shiu Yeh Yu, at the V.A. Medical Center in St. Louis, who employed a highly sensitive radioimmunoassay, capable of detecting as little as 200 pg of desmosine (Harel et al. 1980). Urine samples were hydrolyzed with 6 M HCl at 110°C for 48 hours. After hydrolysis, the sample was filtered and excess HCl was removed by evaporation in a rotary-vacuum evaporator. The dry residue was resuspended and desmosine was determined by radioimmunoassay.

CREATININE AND PROTEIN

Creatinine content of unprocessed urine was determined by the picric acid method, using a standard kit obtained from Sigma Chemical Co. Total amino acid content of urine or lavage fluid was measured using a ninhydrin-based reaction. Total protein was determined by the method of Lowry (1951).

ANGIOTENSIN-CONVERTING ENZYME

Angiotensin-converting enzyme activity was measured using the radioassay devised by Ryan and colleagues (1978) using the synthetic tritiated benzoyl-phe-ala-proline as the substrate. This method is capable of measuring angiotensin-converting enzyme in less than 10^{6} endothelial cells. All samples were assayed in triplicate, with and without the angiotensin-converting enzyme inhibitor captopril (5 µmol) present. The difference between the inhibited and uninhibited values was used as the basis for quantification of the enzyme activity. Unless otherwise indicated, inhibited samples were no different from base-line (no enzyme) samples, indicating that no other enzymatic activities in crude samples were capable of cleaving the synthetic substrate employed. One unit of enzyme activity was defined as the amount of enzyme digesting 1 percent of available substrate per minute. The sensitivity of the assay was such that a change in activity of less than 0.12 percent could be detected. All reactions were shown to be linear with time and amount of enzyme added. Incubation time was adjusted such that 5 to 20 percent of available substrate was hydrolyzed.

Electrophoretically pure rat lung angiotensin-converting enzyme, isolated by affinity chromatography (Bull et al. 1985), was used as a positive control in the enzyme assay. For this purpose, the enzyme was purified by single-step affinity chromatography on a lisinopril-sepharose column, as described by Bull and colleagues (1985). The column allowed a single-step 600-fold purification of angiotensin-converting enzyme from lung homogenates to a single peptide of 172 kDa as determined by polyacrylamide gel electrophoresis.

To standardize the enzyme assay in studies carried out on different days, samples of unknowns were run on more than one occasion and chromatographically purified rat lung angiotensin-converting enzyme was used as a standard.

HYDROXYPROLINE

Hydroxyproline was measured using the same derivatization thin-layer chromatography assay that was used for hydroxylysine (Airhart et al. 1979); however, the probe used was ^{3}H-hydroxyproline. This method allows detection of less than 1 pmol.

URINE COLLECTION

Urine was collected for biochemical studies by placing rats in individual metabolic cages specially constructed to fit within the NO_2 exposure chambers. This allowed full 24-hour urine collections to be carried out without inter-

ruption during the exposure periods. The urine was immediately acidified after collection to avoid the formation of precipitates. One drop of analytical grade 6 N HCl was added to each sample.

LAVAGE COLLECTION

Alveolar lavage was carried out by the sequential instillation of four boluses (7 ml each) of phosphate-buffered saline (PBS), through a polyethylene catheter that was ligated in place in the trachea after excision of the thoracic contents from the chest. Fluid recovery exceeded 24 ml in all animal groups studied. Prior to lavage, all lungs were perfused with PBS through the right ventricle at low pressure, to remove intravascular blood. Each lavage sample was centrifuged at 500 rpm in a clinical centrifuge for 10 minutes, to remove cells and debris, and then stored at −20°C until analyzed.

PULMONARY FUNCTION

Pulmonary function studies were completed using previously published methods from our laboratories (Kelley et al. 1980). Briefly, plethysmographic methods were used to determine lung volumes and capacities in spontaneously breathing rats that had been anesthetized. Quasistatic deflation pressure-volume curves were completed to determine lung compliance.

HISTOLOGY

Histologic evaluation was completed using previously described techniques (Adler et al. 1986). The rat was anesthetized and tracheostomized. The abdomen was opened and both hemidiaphragms were ruptured to allow the lungs to collapse. Fixative (10% neutral buffered formalin) was then instilled through a low-resistance system, and the lung held at the pressure of 20 cm H_2O for at least 1 hour. The lungs were then immersed in fixative at this pressure for 24 hours. Step sections were cut through the right lobe of each animal. Three sections were randomly selected and three areas were analyzed in each section. A general histologic evaluation was completed by a pathologist who was not informed about which tissues were experimental and which were control. In each of the areas, the mean linear intercept was determined as an index of alveolar size.

STATISTICS

Data analysis was performed using Statistical Analysis System (SAS) statistical software (SAS Institute Inc. 1985). Analyses of variance (ANOVAs) were computed for a variety of experimental designs to determine the effect of dose (or time) on the response variable. Contrasts were constructed to decompose the dose (or time) effect into linear and quadratic components. If the original ANOVA resulted in a significant treatment effect, controls were compared with each experimental treatment level via contrasts using the Bonferroni adjustment for the number of comparisons performed.

EXPOSURE PROTOCOL

Several experiments were conducted using different concentrations of NO_2 for various lengths of time. Table 1 summarizes the different experiments and analyses performed.

RESULTS

EFFECT OF DIET ON URINARY HYDROXYLYSINE

When standard rat chow, which contains 6 percent fish meal protein, was consumed, the rats had a urinary hydroxylysine output of 16.7 ± 5.6 μmol/24 hours, compared to 6.2 ± 3.1 μmol/24 hours in rats maintained on a diet free of meat protein. In order to minimize the effect of diet on hydroxylysine excretion in urine, all experiments were completed with rats maintained on meat-protein-free food. This provided a uniform and stable base-line level of hydroxylysine excretion throughout the course of these studies.

EFFECT OF OXYGEN INJURY ON URINARY HYDROXYLYSINE

Inhalation of high fractions of oxygen is well known as a method of inducing injury to the lung. Since the injury was predominantly limited to the lung, a preliminary study was completed to determine the effect of 85 percent oxygen on the urinary output of hydroxylysine in rats. The results are shown in Figure 1. This level of oxygen exposure is sublethal in the rat, yet there is a substantial increase of hydroxylysine during the exposure period that begins after approximately four days, with a maximum value after seven days. The effect is transient, with the hydroxylysine urinary output returning to control levels by nine days of exposure.

TWO-DAY EXPOSURES, MULTIPLE CONCENTRATIONS

In order to determine the effect of short-term exposure, a series of NO_2 levels were evaluated using a time sequence of six hours per day for two days. The following six exposure levels of NO_2 were achieved: 1.05 ± 0.01; 3.0 ± 0.02; 7.72 ± 0.17; 14.69 ± 0.30; 25.0 ± 1.1; 28.2 ± 1.5 (mean

Table 1. Description of Experiments

Overall Design	Concentration of NO_2 (ppm)[a]	Length of Exposure at Time of Sampling (days)	Number of Animals Analyzed						
			Hydroxylysine in Urine	Hydroxylysine in Lavage	Angiotensin-Converting Enzyme in Lavage	Desmosine in Urine	Hydroxyproline in Urine	Histology	Pulmonary Function Tests
6 hours/day for 2 days	unexposed	–	16	6	5				
	1	2	6	6	6				
	3	2	6	6	6				
	7.5	2	6	6	6				
	15	2	5	6					
	25	2	4						
	30	2	6	6	6				
6 hours/day for 5 days	unexposed	–	10		3			4	
	7.5	1	7		3				
	7.5	2	7		3				
	7.5	3	7		3				
	7.5	4	7		3				
	7.5	5	4		3			6	4
	unexposed	–	14		3			4	3
	30	1	6		3				
	30	2	7		3				
	30	3	6		3				
	30	4	3		3				
	30	5	7		3			4	4
6 hours/day, 5 days/week for 2 weeks; plus 2-week recovery period	unexposed	–	10		9	10	10	12	13
	25	2 (week 1)	4			6	6		
	25	4 (week 1)	6			5	5		
	25	5 (week 1)			6			4	4
	25	2 (week 2)	6			6	6		
	25	4 (week 2)	6			6	6		
	25	5 (week 2)			6			4	5
	25	3 (week 1 of recovery)	5			5	5		
	25	2 (week 2 of recovery)	5			5	5		
	25	5 (week 2 of recovery)						4	5
6 hours/day, 5 days/week for 4 weeks; plus 4-week recovery period	unexposed	–	7	22	12			8	8
	1	3 (week 1)	3						
	1	3 (week 2)	4						
	1	3 (week 3)	8						
	1	3 (week 4)	6						
	1	5 (week 4)		8	9			4	4
	1	3 (week 1 of recovery)	8						
	1	3 (week 3 of recovery)	7						
	1	3 (week 4 of recovery)	5						
	1	5 (week 4 of recovery)		12	12			4	4
6 hours/day, 5 days/week for 4 weeks; plus 4-week recovery period	unexposed	–	15	22	21			10	
	0.5	3 (week 1)	7						
	0.5	3 (week 2)	7						
	0.5	3 (week 3)	5						
	0.5	3 (week 4)	7						
	0.5	5 (week 4)		10	8			4	4
	0.5	3 (week 1 of recovery)	7						
	0.5	3 (week 2 of recovery)	8						
	0.5	3 (week 3 of recovery)							
	0.5	5 (week 4 of recovery)		11	12			5	5

[a] Target concentration; actual concentration slightly different (see text).

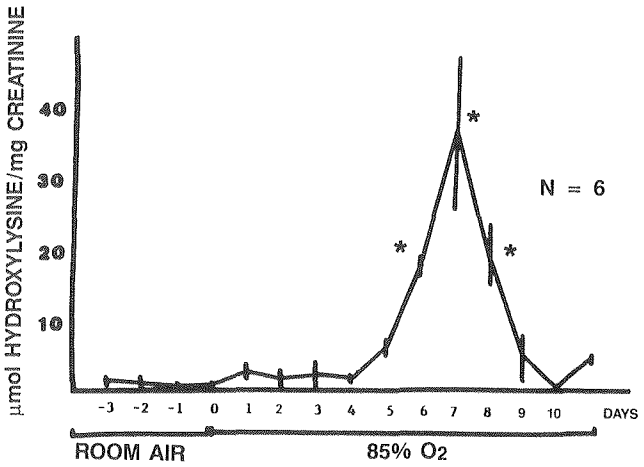

Figure 1. Urinary concentration of hydroxylysine per milligram of creatinine, based on 24-hour urine collections. During the four-day control period rats inspired room air, which was followed by 10 days of continuous exposure to 85 percent oxygen. Values expressed are means ± 1 SEM for six rats per group. * = significant ($p < 0.05$) difference from control.

± SD). Six male adult (300 to 320 g) rats were exposed to each concentration, and six rats served as unexposed control animals. There was no significant difference in the body weights between the exposed and control animals prior to or after the two-day exposure. In this series, the rats were killed within 18 hours of the termination of the experiment.

The ratio of the amount of hydroxylysine in the urine of exposed rats to the amount of the hydroxylysine in the urine of control rats is expressed in Figure 2. There were three separate groups of control animals for these experiments, and their hydroxylysine content in urine differed (0.30 to 0.60 µmol/ml); this necessitated the use of the experiment-to-control ratio in order to compare these six experiments. The increase in urinary hydroxylysine ratio was a significant ($p < 0.01$) linear function of exposure level, demonstrating a dose-dependent relationship. This result demonstrates that a two-day exposure, for six hours per day, results in an increase in urinary output of hydroxylysine. The urine volume and creatinine levels were not different in the experimental versus control groups.

When bronchoalveolar lavage was completed at the end of the two days of NO_2 exposure, the lavage concentration of hydroxylysine increased ($p < 0.05$) as a linear function of exposure level (Figure 3). Analyses of variance between experimental and control groups demonstrated significant changes at 7.5, 15, and 30 ppm. Angiotensin-converting enzyme levels in lavage fluid also increased ($p < 0.05$) as a linear function of NO_2 concentration, primarily as a result of the response seen at 30 ppm (Figure 4). Based on the ANOVA, there was a significant ($p < 0.05$) increase, compared to control animals, in the rats exposed to 30 ppm.

Although there were significant linear components to the relationship between lavage protein concentration and level of exposure, a significant ($p < 0.05$) increase was observed only at 30 ppm NO_2, when compared to the control levels (Figure 5). If the angiotensin-converting enzyme activity was normalized to the protein concentration, no significant changes were observed at any of the concentrations tested.

This series of experiments demonstrates that hydroxylysine levels in both the urine and the bronchoalveolar lavage fluid will increase when rats are exposed for relatively short times to moderate-to-high levels of NO_2. The angiotensin-converting enzyme level and the protein concentration are

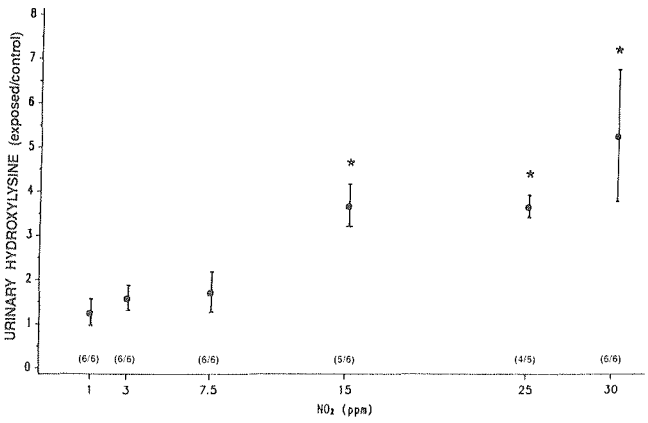

Figure 2. Urinary concentration of hydroxylysine, expressed as exposed value relative to control value, based on 24-hour urine collection during the second day of exposure to NO_2 (two days, six hours per day). The ratio of experimental to control urinary hydroxylysine has a significant ($p < 0.05$) linear dependence on dose. Values are expressed as means ± 1 SEM. The number of exposed/control rats is shown in parentheses. * = significant ($p < 0.01$) difference from control.

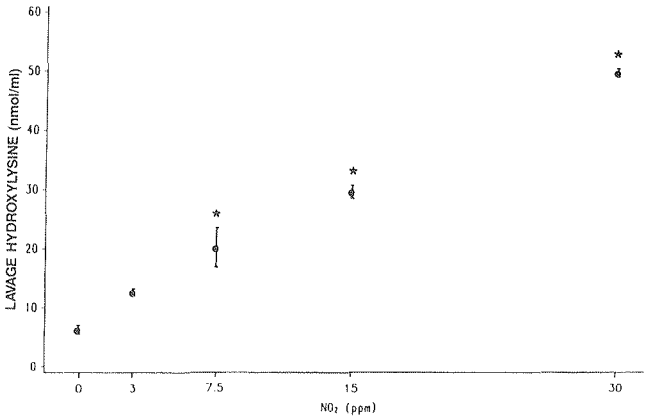

Figure 3. Concentration of hydroxylysine in bronchoalveolar lavage fluid after two days of exposure to NO_2 for six hours per day. Values are expressed as means ± 1 SEM for six rats per group. * = significant ($p < 0.05$) difference from control.

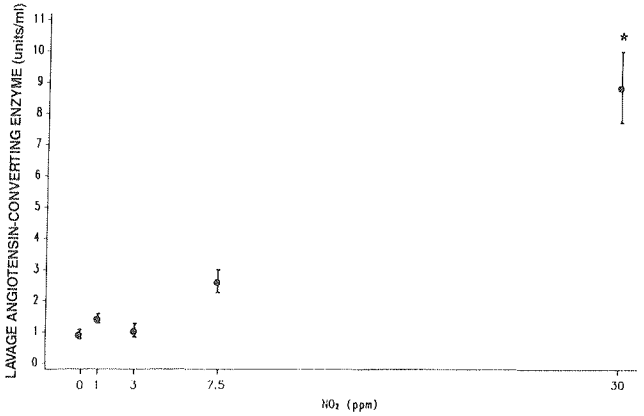

Figure 4. Activity of angiotensin-converting enzyme per milliliter of bronchoalveolar lavage fluid after two days of exposure to NO_2 for six hours per day. Values are expressed as means ± 1 SEM for six exposed rats and five control rats per group. * = significant ($p < 0.05$) difference from control.

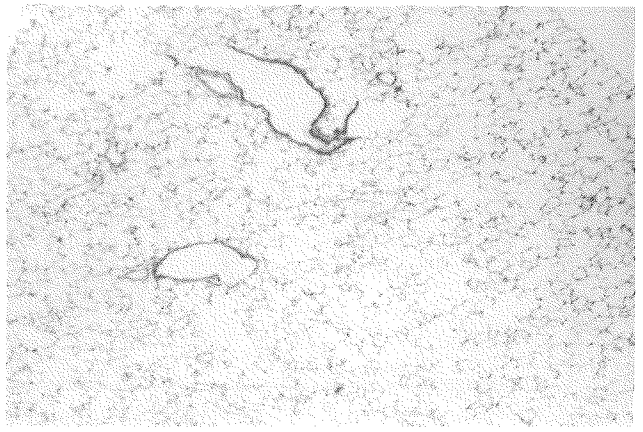

Figure 6. Histologic section of lung after three days of exposure to 7.5 ppm NO_2 for six hours per day. Magnification = × 40.

significantly increased only at the highest levels of exposure.

FIVE-DAY EXPOSURES, MODERATE AND HIGH CONCENTRATIONS

A moderate level of NO_2 (7.2 ± 0.1 ppm) was used in a six-hours-per-day, five-day exposure protocol. Thirty-two male Fischer-344 rats (200 to 230 g) and 32 nonexposed control rats of the same weight range were studied. There were no demonstrable effects on body weight as a function of exposure. Urine was collected during the exposure. Lavage analysis, histologic evaluation, and pulmonary function testing were completed at the end of the five days of exposure.

Histologic examination revealed that the exposure to 7.5 ppm NO_2 for six hours per day had only minor effects on the lung. After three days of exposure, there were no gross lesions and only slight evidence of epithelial thickening in alveoli near terminal bronchioles (Figure 6). Although there was some evidence of macrophage accumulation, this was also seen in the sections from control animals (Figure 7). After five days of exposure to 7.5 ppm NO_2, a slight thickening of the epithelium was observed again, and a minor loss of cilia was seen in both large and small airways (Figure 8). Control rats demonstrated no evidence of disease. The mean linear intercept was not different in the control (45.1 ± 5.3) and experimental (45.8 ± 4.02) groups. Evaluation of pulmonary function tests revealed a significant ($p < 0.02$) increase in lung compliance after five days of exposure (0.60 ± 0.03 vs. 0.78 ± 0.12 ml/cm water).

During the first day of exposure to 7.5 ppm NO_2, the urinary concentration of hydroxylysine rose significantly ($p < 0.05$) to three times that observed in the unexposed control

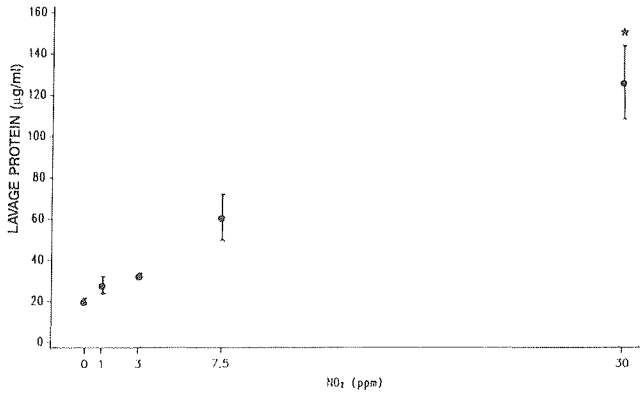

Figure 5. Concentration of protein in bronchoalveolar lavage fluid after two days of exposure to NO_2 for six hours per day. Values are expressed as means ± 1 SEM for six rats per group. * = significant ($p < 0.05$) difference from control.

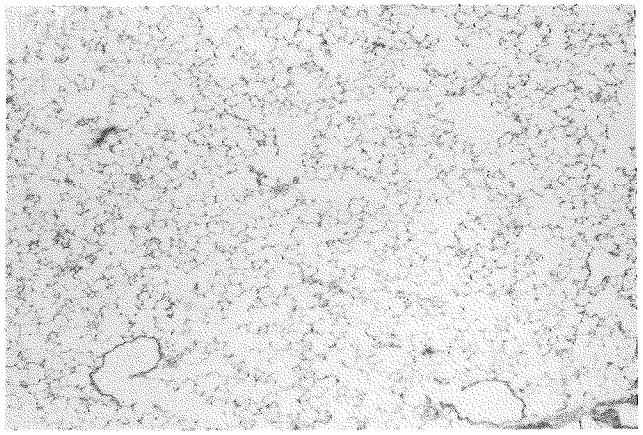

Figure 7. Histologic section of lung from a control animal. Magnification = × 40.

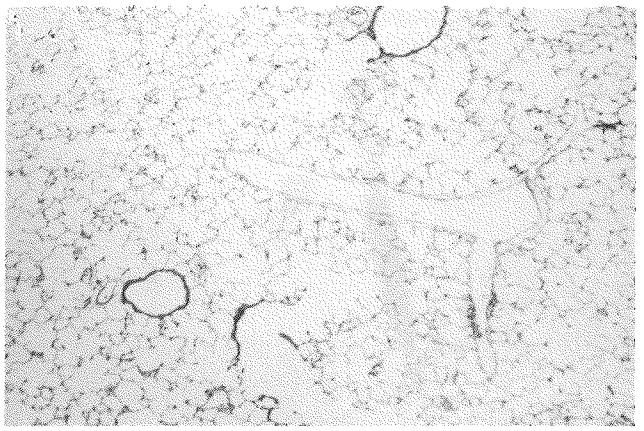

Figure 8. Histologic section of lung after five days exposure to 7.5 ppm NO_2 for six hours per day. Magnification = × 40.

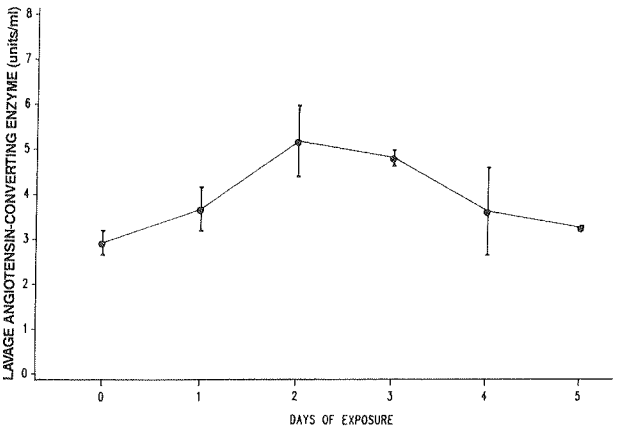

Figure 10. Activity of angiotensin-converting enzyme per milliliter of bronchoalveolar lavage fluid after exposure to 7.5 ppm NO_2 for six hours per day. Values are means ± 1 SEM for three rats per group. No significant change.

animals (Figure 9). This value decreased to levels not significantly different from the control levels over the next four days. Angiotensin-converting enzyme activity in lavage fluid was not significantly different when exposed rats were compared with unexposed control rats (Figure 10); however, the angiotensin-converting enzyme activity demonstrated a significant ($p < 0.05$) quadratic trend as a function of days of exposure. Lavage protein concentration had a tendency to rise, but no statistically significant (quadratic, $p < 0.7$) changes were detected (Figure 11).

This experiment demonstrates that a moderate level of exposure can result in significant increases in the urinary excretion of hydroxylysine at a time when bronchoalveolar lavage angiotensin-converting enzyme activity is not significantly changed. This transient increase occurs despite the fact that the histologic evidence of injury to the lung is minimal.

To test the effect of the five-day exposure to a higher (29.5 ± 0.3 ppm) level of NO_2, 64 male rats (body weight 175 to 200 g) were studied. Thirty-two rats were exposed for six hours per day for five consecutive days, and 32 served as control animals. Urine and bronchoalveolar lavage samples were collected over the course of the exposure from exposed and unexposed rats. In addition, lungs were fixed at the termination of the exposure for histologic examination and the determination of mean linear intercept. Pulmonary function was also compared in the exposed and control animals.

Exposure to 30 ppm NO_2 for six hours per day had overt effects on the lung as revealed by histologic examination. After three days of exposure, there was thickening of alveo-

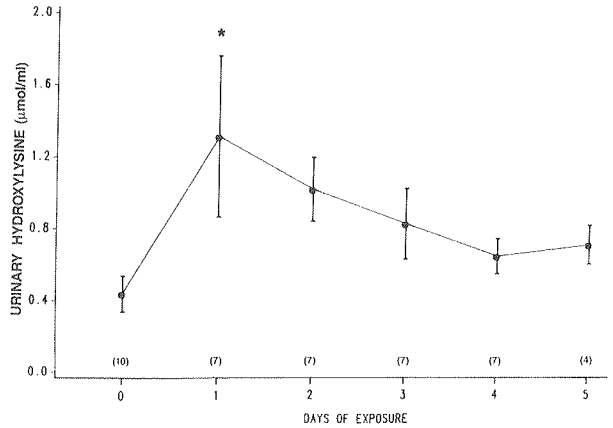

Figure 9. Urinary hydroxylysine concentration based on a 24-hour urine collection during exposure to 7.5 ppm NO_2 for six hours per day. Values are means ± 1 SEM. The number of determinations is shown in parentheses. * = significant ($p < 0.05$) difference from control.

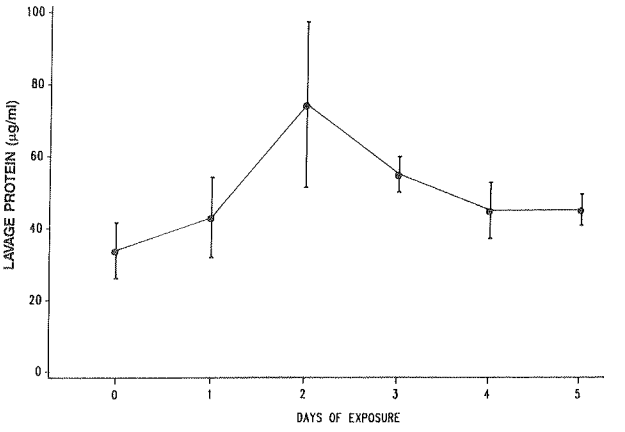

Figure 11. Concentration of protein in bronchoalveolar lavage fluid after exposure to 7.5 ppm NO_2 for six hours per day. Values are means ± 1 SEM for three rats per group. No significant change.

lar walls and alveolar ducts, with some sloughing of ciliated cells. The lungs of control animals were normal, with intact cilia and thin alveolar membranes. After five days of exposure, there was increased thickening of the epithelium in alveoli near terminal bronchioles and alveolar ducts. This thickened area was made up of cuboidal-like cells, with a large influx of macrophages. There was significant loss of cilia and sloughing of the airway epithelium. Control animals at this time appeared normal. The mean linear intercept was significantly ($p < 0.05$) reduced in the exposed (37.6 ± 2.71), compared with control (47.73 ± 3.45), samples, demonstrating a reduction in alveolar size. Pulmonary function analysis revealed a decrease in lung compliance, as well as in vital capacity and total lung capacity.

The concentration of hydroxylysine in the urine varied as a quadratic function of time ($p < 0.05$). The concentration was significantly ($p < 0.05$) different from the control level during the first day of exposure to 30 ppm NO_2, reaching three times the control value (Figure 12). During the remaining four days, the concentration diminished, reaching a level approximately twice that of the unexposed control. Analysis of the bronchoalveolar lavage fluid revealed a significant ($p < 0.05$) increase in angiotensin-converting enzyme activity per milliliter of lavage fluid on each day, relative to the control value (Figure 13). The change in angiotensin-converting enzyme activity took on a significant ($p < 0.05$) quadratic pattern over time. The maximum elevation, which was 10 times the control value, occurred on the third day of exposure, slightly decreasing on the fourth and fifth days. There was also a rapid increase in total protein in the lavage fluid during the exposure, demonstrating significant ($p < 0.05$) differences between experimental and control values on days 1, 2, and 3, and returning

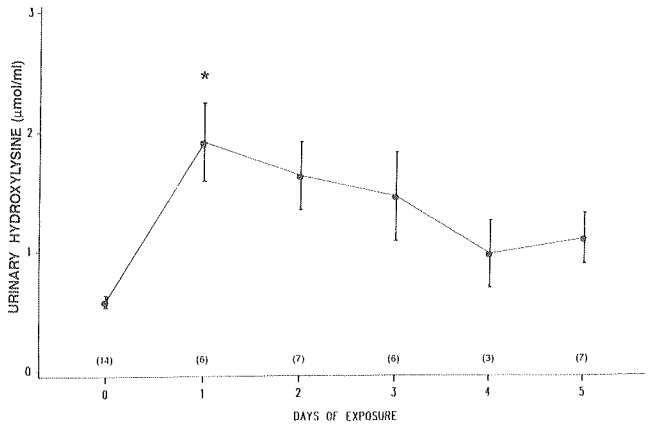

Figure 12. Urinary hydroxylysine concentration based on 24-hour urine collection during exposure to 30 ppm NO_2 for six hours per day. Values are means ± 1 SEM. The number of rats at each time point is given in parentheses. * = significant ($p < 0.05$) difference from control.

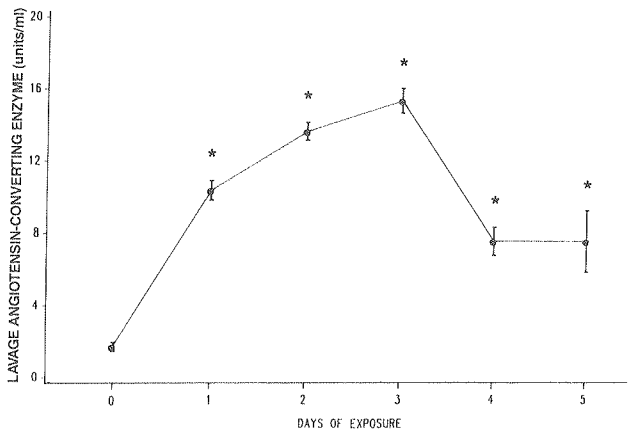

Figure 13. Activity of angiotensin-converting enzyme per milliliter of bronchoalveolar lavage fluid after exposure to 30 ppm NO_2 for six hours per day. Data represent means ± 1 SEM for three rats per group. * = significant ($p < 0.05$) difference from control.

to base line by days 4 and 5. When the angiotensin-converting enzyme activity was expressed as a percentage of total protein, that is, as the specific activity, there were no significant changes in the exposed rats.

These results demonstrate that exposure of rats to high concentrations of NO_2 for five days results in histologically demonstrable injury to the lung. This injury is associated with significant elevations of both the urinary concentration of hydroxylysine and the activity of angiotensin-converting enzyme in lavage fluid. The rise in hydroxylysine occurred early and demonstrated a return toward normal as exposure continued.

TWO-WEEK EXPOSURES, HIGH CONCENTRATIONS

Rats were also exposed to 25 ppm of NO_2 for six hours per day, five days per week, for two weeks. This is a total of 60 hours of exposure to a relatively high concentration of NO_2. Thirty-two male rats (225 to 275 g) were exposed, and 32 unexposed control animals of the same weight range were maintained in NO_2-free conditions. Urine samples were collected during exposure, and rats were killed at the end of each week of exposure, and two weeks after the termination of the exposure.

After five days of exposure to 25 ppm NO_2 for six hours per day focal accumulations of macrophages were observed in interstitial areas of the lung. There appeared to be areas of increased connective tissue accumulation and increased numbers of fibroblasts (Figure 14). The lungs of control animals at this time appeared normal. After two weeks of exposure, the lesions appeared to be worse, with increased areas of alveolar thickening, hypercellularity, and further evidence of connective tissue deposition in the interstitium

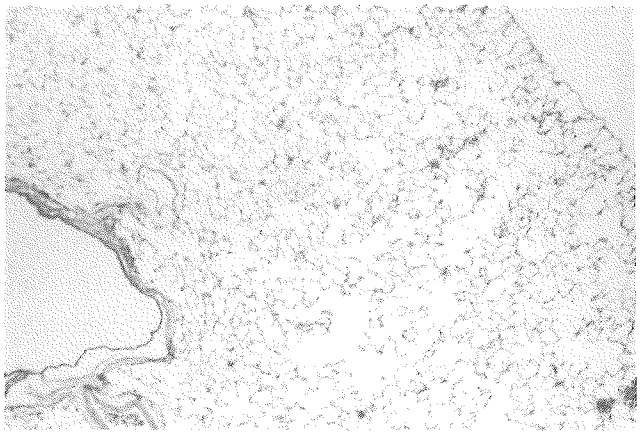

Figure 14. Histologic section of lung from rat exposed to 25 ppm NO_2 for six hours per day for five days. Magnification = × 40.

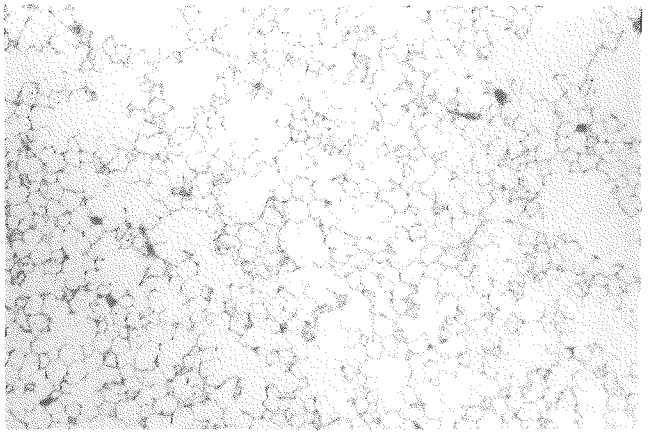

Figure 16. Histologic section of lung from rat two weeks after termination of exposure to 25 ppm NO_2 for six hours per day, five days per week, for two weeks. Magnification = × 40.

(Figure 15). When the lungs were examined two weeks after the termination of exposure, they appeared normal, with only a few areas of alveolar thickening (Figure 16), and they were not distinguishable from the lungs of control rats. After one and two weeks of exposure, the mean linear intercept was decreased in the exposed group, compared to the control group (40.1 ± 4.5 vs. 49.4 ± 4.1; $p < 0.05$). After two weeks of recovery, the mean linear intercept was not different in the experimental and control groups. No significant alterations in pulmonary function were detected at any of the time points.

Urinary hydroxylysine determinations were completed twice per week, and demonstrated a significant ($p < 0.05$) linear rise during the exposure period (Figure 17). The maximal value was observed at the end of the second week of exposure, and was 10 times that of the unexposed control group. At the end of the first week of recovery, the urinary levels had fallen substantially, but were still greater than those of the control group; this held true even after two weeks of recovery. The lavage angiotensin-converting enzyme activity, expressed per milliliter of recovered lavage fluid, was unchanged during the exposure. Lavage protein concentration was significantly ($p < 0.05$) elevated at the end of each week of exposure, and returned to control levels after two weeks of recovery.

The effects of NO_2 exposure on elastin degradation, as measured by urinary desmosine level, were monitored as part of this protocol (Figure 18). The urine samples collected from the rats exposed to 25 ppm NO_2 for two weeks were prepared for desmosine analysis.

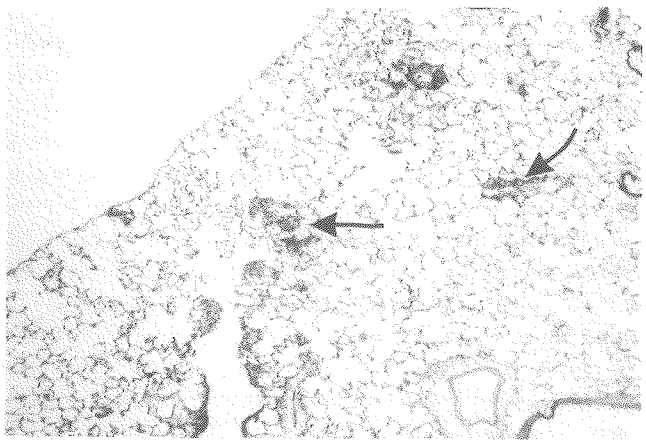

Figure 15. Histologic section of lung from rat exposed to 25 ppm NO_2 for six hours per day, five days per week, for two weeks. Arrows indicate alveolar thickening, hypercellularity, and beginning of collagen deposition. Magnification = × 40.

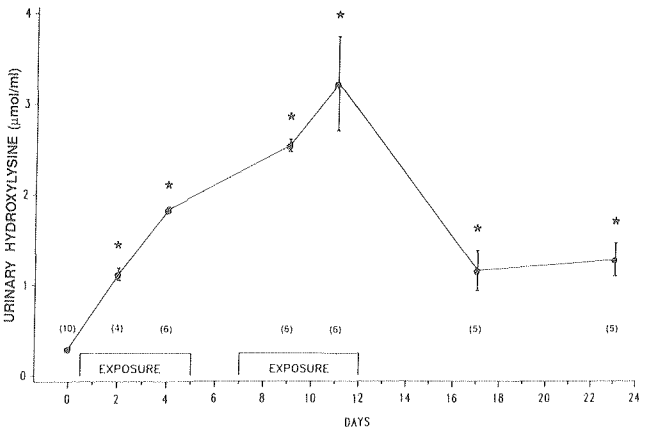

Figure 17. Urinary concentration of hydroxylysine based on 24-hour urine collection during exposure to 25 ppm NO_2 for six hours per day. Days on which exposure occurred are labeled. Values are means ± 1 SEM. The number of rats at each time point is given in parentheses. * = significant ($p < 0.05$) difference from control.

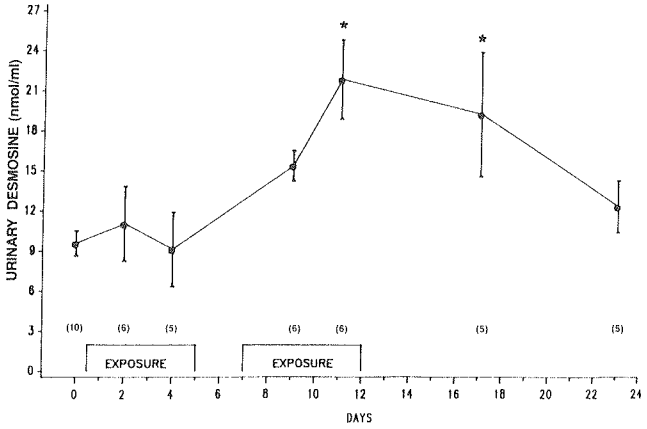

Figure 18. Urinary concentration of desmosine based on 24-hour urine collection during exposure to 25 ppm NO_2 for six hours per day. Days on which exposure occurred are labeled. Values are means ± 1 SEM. The number of rats assayed at each time point is shown in parentheses. * = significant ($p < 0.05$) difference from control.

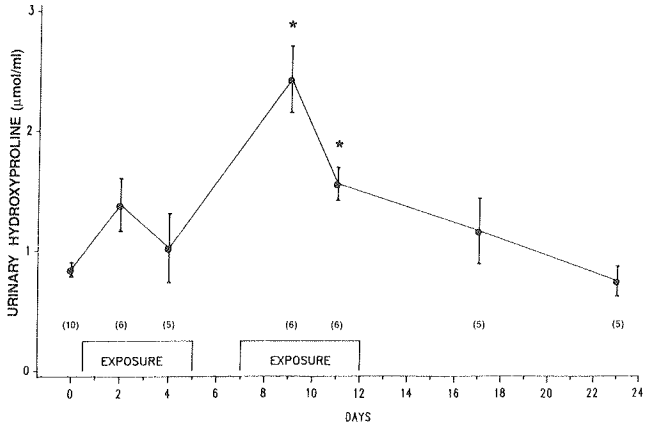

Figure 19. Urinary concentration of hydroxyproline based on 24-hour urine collection during exposure to 25 ppm NO_2 for six hours per day. Days on which exposure occurred are labeled. Values are means ± 1 SEM. The number of rats per group is shown in parentheses. * = significant ($p < 0.05$) difference from control.

Desmosine analyses of urine hydrolysates were expressed as a concentration and also were normalized to urinary creatinine concentration. The base-line value for urinary desmosine in control animals was 10.31 ± 2.92 nmol/mg creatinine (mean ± SD), or 9.61 ± 3.37 nmol/ml of urine. The urinary desmosine level demonstrated a significant ($p < 0.05$) linear increase during the exposure period. Based on ANOVA, the values on days 11 and 17 were significantly ($p < 0.05$) increased when compared to the control value. During the subsequent recovery period, during which the animals breathed only ambient air without further NO_2 exposure, the urinary desmosine levels remained elevated for one week, and then returned toward normal levels. Because NO_2 exposure had little effect on the urinary creatinine excretion, there were no differences in the data plotted for concentration versus concentration normalized to creatinine concentration.

Thus, elastin breakdown products can also be detected in statistically significantly elevated levels after high-dose exposure to NO_2 for two weeks. It is of interest that, compared with the immediate release of collagen-breakdown products (hydroxylysine) at this exposure level, there is an apparent delay in elastin degradation. The different enzymatic pathways involved may have differential latencies for activation.

The benefits of using hydroxylysine as a marker of collagen turnover have been discussed elsewhere (Krane et al. 1977). In this same exposure, we investigated whether the more abundant, but less specific, modified amino acid hydroxyproline also could serve as a marker of NO_2 exposure of rats. For this purpose, urinary samples were also analyzed for hydroxyproline concentration. The exposure protocol was as indicated above: two weeks of NO_2 at 25 ppm (five days per week, six hours per day), followed by a two-week recovery period.

The base-line value for urinary hydroxyproline in control animals that breathed room air was 1.2 ± 0.1 µmol/mg creatinine (mean ± SD), or 0.85 ± 0.1 µmol/ml of urine. With exposure to high levels of NO_2, the urinary hydroxyproline level rose slowly to a significantly ($p < 0.05$) elevated level during the second week of exposure (Figure 19). The maximum concentration occurred in the middle of the second week of exposure, and was three times above the control level. During the subsequent week of exposure, there was a decrease in the urinary hydroxyproline level, and during the recovery period, the urinary hydroxyproline concentration returned to normal levels. Because NO_2 exposure had little effect on the urinary creatinine excretion, there were no important differences in the data plotted for concentration versus concentration normalized to creatinine concentration.

These results contrast with the data on hydroxylysine excretion from the same experiment, in which there was an immediate, but more sustained, elevation for the full two weeks of exposure, followed by return toward normal levels. More noteworthy is the relative magnitude of the response to NO_2: In contrast to the 3-fold rise in hydroxyproline concentration, hydroxylysine rose 10-fold. The extremely low background would be expected based on the relative proportions of hydroxylysine and hydroxyproline in collagen. The lesser rise of hydroxyproline points to greater degradation of this amino acid in preference to excretion via the urine. On an absolute scale, the base-line hydroxylysine excretion (0.30 µmol/ml) is one-third the level of the

basal hydroxyproline, but it rises to somewhat higher levels in response to NO_2. This head-to-head comparison validates our hypothesis that hydroxylysine measurements should be more sensitive than hydroxyproline measurements to environmental perturbations.

FOUR-WEEK EXPOSURES, LOW CONCENTRATIONS

In order to study a low dose over a longer period, 32 male adult rats (200 to 275 g) were exposed to 0.95 ± 0.12 ppm NO_2 for six hours daily, five days per week, for four consecutive weeks. This is a total of 120 hours of exposure to NO_2. Urine samples were collected periodically during the experiment, and rats were killed after four weeks of exposure or after four weeks of recovery from the exposure. The control group consisted of 32 rats of the same weight range (200 to 275 g).

Exposure of rats to 1 ppm NO_2 for six hours per day, five days per week, for four weeks did not have any major effects on the lung, based on histologic examination. There were only subtle thickenings at alveolar ducts, which were limited to focal regions. The lungs of control animals were normal at this time point. Four weeks after the termination of the exposure, it was not possible to detect any differences between the exposed and the control lungs. After four weeks of exposure, the mean linear intercept was unchanged in the experimental, compared with the control, group (48.1 ± 8.1 vs. 46.7 ± 0.8). When compared four weeks after the termination of the exposure, no significant difference was detectable between the experimental and control mean linear intercepts (52.8 ± 5.2 vs. 50.2 ± 6.7). No significant differences in pulmonary function between exposed and control rats were detectable after four weeks of exposure or after the four-week recovery period.

As shown in Figure 20, urinary hydroxylysine demonstrated a gradual linear increase ($p < 0.05$) during the four-week exposure period. The concentration of urinary hydroxylysine during the control period was 0.62 ± 0.24 μmol/ml prior to exposure, a value that is typical for control rats. The hydroxylysine concentration in urine was at its maximum during the week after exposure, and was more than 300 percent of the control value. The urinary hydroxylysine concentration decreased during the recovery period, and was only 113 percent of the control value in the middle of the fourth week of recovery.

Three groups of rats were lavaged as part of this experiment: unexposed control animals; a group exposed for four weeks; and a group exposed for four weeks and then allowed to recover for four weeks. The lavage total protein was 32.1 ± 23.6 μg/ml in the control group. After four weeks of exposure the protein concentration was 50.5 ± 32.3 μg/ml,

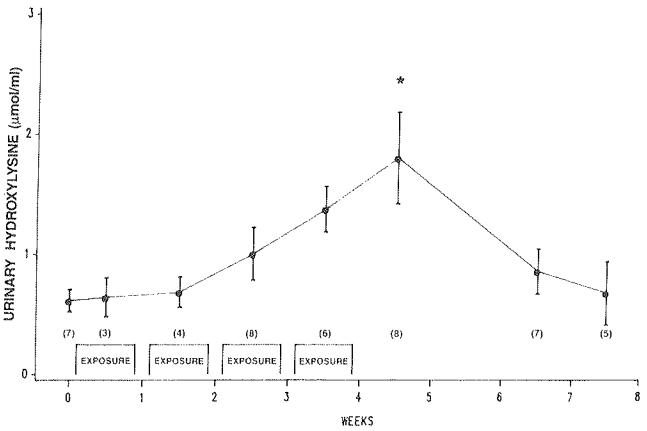

Figure 20. Urinary concentration of hydroxylysine based on 24-hour urine collection during exposure to 1.0 ppm NO_2 for six hours per day. Days on which exposure occurred are labeled. Values are means ± 1 SEM. The number of rats studied at each time point is shown in parentheses. * = significant ($p < 0.05$) difference from control.

and after the recovery period this value was 16.2 ± 5.9 μg/ml. These differences were not statistically significant because of a high level of variability. Angiotensin-converting enzyme activity was also determined in these lavage fluid samples. Lavage angiotensin-converting enzyme activity, expressed per milliliter of recovered lavage fluid, was 1.78 ± 0.42 in 12 control rats. In rats exposed for four weeks, the angiotensin-converting enzyme activity significantly ($p < 0.05$) increased to 2.50 ± 0.75, which is 140 percent of the activity in control animals. After four weeks of recovery, the angiotensin-converting enzyme activity had returned to control levels, at 1.31 ± 0.48.

The concentration of hydroxylysine in the lavage fluid of 22 unexposed control rats was 6.2 ± 2.1 nmol/ml, and was significantly lower (2.7 ± 1.0 nmol/ml) in 8 rats studied after four weeks of exposure. In 12 rats studied after four weeks of recovery, the lavage hydroxylysine concentration was greater (3.1 ± 0.95 nmol/ml), but was still significantly reduced compared to the control animals.

This experiment demonstrates that exposure to a relatively low concentration of NO_2 (1 ppm) for longer time periods than in earlier experiments causes increases in hydroxylysine excretion. The excretion decreased when the rats recovered from this level of exposure. The histologic changes were minimal at this level of exposure.

Another long-term exposure was conducted in which 32 male adult rats (175 to 225 g) were exposed to 0.5 ± 0.05 ppm NO_2 for six hours per day, five days per week, for four consecutive weeks. This results in a total exposure of 120 hours for the entire period. This level of exposure is the lowest reported in these studies. Urine samples were collected at frequent intervals throughout the protocol, and

lavage fluid was collected for analysis after four weeks of exposure, and after four weeks of recovery. Thirty-two unexposed rats of the same body weight served as control animals.

Exposure of rats to 0.5 ppm NO_2 for six hours per day, five days per week, for four weeks did not result in any pulmonary effects that were demonstrable histologically. It was not possible to detect differences between the lung sections from the exposed (Figure 21) and the control animals. When evaluated four weeks after the termination of the exposure, it was still not possible to detect differences in the two groups of tissues. At the end of the exposure period, no significant difference was observed in the mean linear intercept between the control (50.2 ± 0.71) and exposed (47.9 ± 4.39) groups. After the four-week recovery period, there was a significant increase in the mean linear intercept in the rats that had been exposed to NO_2. This change in mean linear intercept was associated with a significant increase in the vital capacity at the end of the four weeks of exposure, as well as with an increase in compliance after the four-week recovery period.

As shown in Figure 22, urinary concentration of hydroxylysine measured in rats exposed at weekly intervals rose sharply during the first two weeks of exposure, then decreased slightly during the next two weeks and during one week of recovery. The control value for the concentration of hydroxylysine in the urine was 0.37 ± 0.12 μmol/ml. The maximal mean value (1.55 ± 0.69 μmol/ml) observed in the urine occurred during the second week, and was 418 percent of the control value. During the remaining two weeks of exposure, the urinary hydroxylysine concentrations remained elevated (1.2 ± 0.3 μmol/ml), but gave no further evidence of an increase in response to continued exposure. During the recovery period, the concentration fell to approximately 0.72 to 0.75 μmol/ml, which was not significantly different from control levels. Unlike the urinary concentrations of hydroxylysine, the lavage concentrations did not change during the course of exposure or recovery.

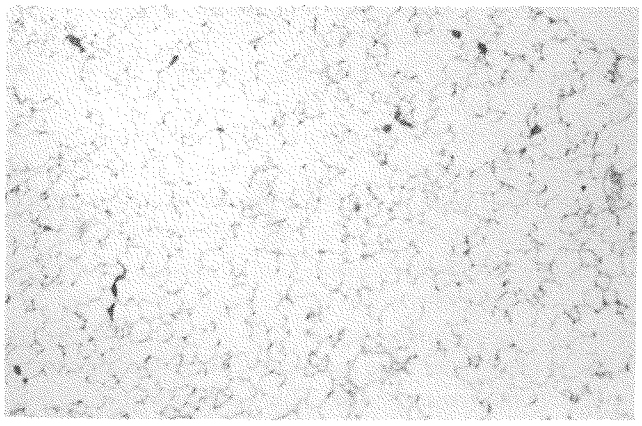

Figure 21. Histologic section of lung after four weeks of exposure to 0.5 ppm NO_2 for six hours per day, five days per week. Magnification = × 40.

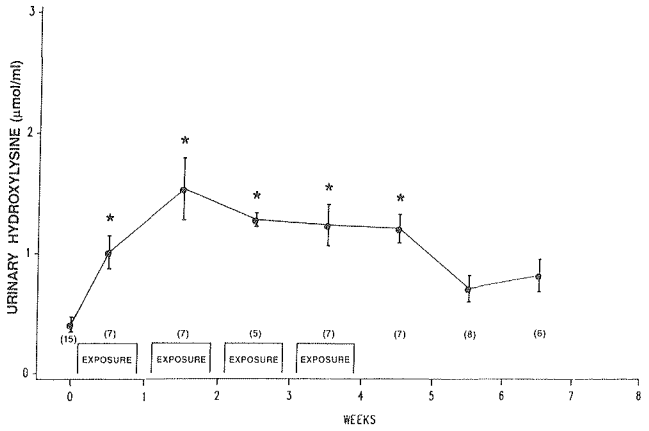

Figure 22. Urinary concentration of hydroxylysine, based on 24-hour urine collection during exposure to 0.5 ppm NO_2 for six hours per day. Days on which exposure occurred are labeled. Values are means ± 1 SEM. The number of rats studied at each time point is shown in parentheses. * = significant ($p < 0.05$) difference from control.

Lavage total protein concentration was determined in 21 control rats, 10 rats studied after four weeks of exposure, and 12 rats allowed to recover from the exposure for four weeks. The control lavage total protein concentration was 17.3 ± 7.8 μg/ml, which was unchanged after exposure (17.8 ± 7.8), or during the recovery period (19.1 ± 7.2). Unlike in the 1-ppm four-week exposure study, no changes were observed in lavage total protein.

Similarly, the lavage angiotensin-converting enzyme activity was unchanged in this exposure protocol. The value was 1.85 ± 0.60 units/ml in 21 control animals, 1.63 ± 0.85 units/ml in 8 rats studied after four weeks of exposure, and 2.27 ± 0.65 units/ml in 12 rats allowed to recover for four weeks. When angiotensin-converting-enzyme-specific activity was determined, no significant changes were observed over time.

Again, a low dose (0.5 ppm) of NO_2 exposure for longer time periods resulted in increased urinary output of hydroxylysine. It was not possible to detect changes in these lungs by histologic examination. There was a small increase in mean linear intercept, with an associated increase in vital capacity.

DISCUSSION

These studies indicate that hydroxylysine has potential as a marker of pulmonary changes associated with NO_2 exposure. Hydroxylysine concentration in the urine definitely

rises in response to NO_2. However, the rise is not as great as the rise seen with exposure to high concentrations of oxygen. An interesting question is whether or not the rise in urinary hydroxylysine is a more specific marker than other indices of exposure, such as lavage angiotensin-converting enzyme or protein levels. In this regard, it is most interesting that at the lowest levels of NO_2 used in these studies (0.5 ppm for four weeks), the urinary hydroxylysine rose significantly, an absolute fourfold elevation. In contrast, at the same dose, there was no elevation in lavage protein level. This suggests that hydroxylysine is superior to total protein as a marker, and that there is a selective alteration in the metabolism of hydroxylysine-containing proteins upon low-level exposure to NO_2.

Another index of specificity of hydroxylysine-containing peptides built into these studies was the use of creatinine to normalize urinary hydroxylysine concentration data. We were concerned that with exposure to high levels of NO_2, there might be alterations in animal behavior, fluid or food intake, and a generalized catabolic state, with release of elevated levels of urinary amines. Alternatively, there might be altered concentrations of urinary amino acids as a consequence of decreased fluid intake. Indeed, there was a rise in urinary creatinine levels during the early response to the highest levels of NO_2; however, this rise did not reach statistical significance in any given experiment. Under no circumstances did the minor changes in urinary creatinine levels explain changes in urinary hydroxylysine concentration. As a consequence of this observation, in most cases hydroxylysine data were not normalized to the measured creatinine levels. Furthermore, the changes in creatinine at high levels of exposure to NO_2 were paralleled by rises in urinary total nitrogen, measured early in this series of experiments.

Our understanding of the interactions between oxidant gases, such as NO_2, and connective tissue components has been advanced during the past several years since the present series of studies was first proposed (Riley and Kerr 1985; Riley et al. 1987). Connective tissue components can be directly degraded by oxidants in vitro. Given the vulnerability and first-line exposure of the lung to oxidants, pulmonary connective tissue might be expected to be particularly vulnerable to such an untoward oxidant effect. Indeed, it is not clear whether or not even the low levels of oxygen found in ambient air are adequate to promote connective tissue breakdown. If such were the case, it might be further hypothesized that any effects of low levels of NO_2 might act in concert with ambient oxygen to promote connective tissue breakdown. It is well recognized that exposure of animals to toxic levels of oxidant gases leads to emphysematous lesions within several weeks.

Oxidant gases can be expected to alter connective tissue metabolism at several points. They can interact with proteolytic enzymes, induce release of such enzymes from inflammatory cells, and directly interact with collagen and other matrix components in ways that make the latter more vulnerable to enzymatically mediated proteolysis. Riley and colleagues (1987) have documented increased levels of hydroxyproline-containing peptides in lavage fluid after exposure to oxygen. As in the present studies, they found that the content of such material rose fourfold to fivefold after a sublethal exposure to oxygen. In contrast to our studies with NO_2, there was no evidence of elastin breakdown after hyperoxic injury. When collagen cross-linking was prevented by pretreating animals with the lathyrogen, beta-aminopropionitrile, histologic changes of emphysema were significantly worse (Riley et al. 1987). This suggests that replacement of connective tissue is a normal part of the attempted repair process after severe hyperoxia.

All of the present observations are consistent with the notion that hydroxylysine found in urine samples of NO_2-exposed animals comes from the lung rather than from other tissues. The best data upon which to base this assertion come from the lavage studies, in which there is a high degree of correlation between hydroxylysine levels in lavage and urine. Moreover, the hydroxylysine levels in both fluids rise in direct proportion to each other. There is a clear exception to this observation at the lowest dose tested (0.5 ppm NO_2), where the lavage concentration dropped during exposure. We do not have an explanation for this result.

We had originally hypothesized that hydroxylysine would prove to be a more sensitive and specific marker than hydroxyproline. This indeed turned out to be the case. This comes about partly because of a lower initial background level of hydroxylysine. Although both modified amino acids rise to approximately the same absolute levels during high-level NO_2 exposure, the fact that hydroxylysine starts from a lower background level in unexposed animals has the effect of heightening the difference between control and exposed animals. In a similar fashion, hydroxylysine appears more sensitive than desmosine. Differences in the magnitude and time courses for these three markers could be important clues to our understanding of the connective tissue response.

In this study, we found that the absolute increases in hydroxylysine are significant at exposure levels as low as 0.5 ppm NO_2 after one week of exposure. Because monitoring at earlier time points at this exposure level was not carried out in this study, it is not clear whether or not urinary hydroxylysine rises even earlier at these low levels. It is interesting to contrast this observation with published studies of the effects of lethal levels of hyperoxia on the lung (Riley

et al. 1987). The magnitude of the rise of hydroxyproline in the study by Riley and coworkers is of the same magnitude as that seen with 0.5 ppm NO_2. Moreover, there were no accompanying changes in histology after exposure to this level of NO_2, making hydroxylysine measurements the most sensitive index of impact on the lung under the conditions of this study.

With regard to the sensitivity of indirect monitoring of lung connective tissue breakdown by urinary measurements, it is of interest that in the two studies using low levels of NO_2 exposure (0.5 and 1 ppm), the urinary levels were elevated, while other biochemical indices of direct lung changes were negative or not significantly elevated. For instance, during exposures to both 0.5 ppm and 1 ppm NO_2, urine hydroxylysine levels were significantly elevated. However, at neither of these exposures did lavage protein levels rise significantly. This is one of the most cogent arguments for the specificity of urinary hydroxylysine measurements in these studies. From a practical viewpoint, it suggests that in future studies involving low-level NO_2 exposure, investigators might best "normalize" hydroxylysine data to simultaneously measured total protein levels. As importantly, hydroxylysine levels changed when the histology was judged normal and few functional changes were observed. This leads us to recommend long-term follow-up experiments to assess whether lesions would develop over time.

When one compares the results from the studies presented in this report, there appear to be both acute and chronic responses to NO_2. This observation has direct implications for the design of potential studies in human populations, indicating the desirability of a study design with continuous rather than spot testing in human populations. Only with extreme exposure to NO_2 or other oxidant gases would human subjects show evidence of altered connective tissue metabolism and excretion of matrix peptides (Hatton et al. 1977).

On the basis of the biochemical and physiologic arguments put forward above, hydroxylysine and desmosine appear to be markers of degradation derived from connective tissue, and are, therefore, specific for degradation of matrix proteins. In contrast, excess levels of hydroxyproline in urine may reflect both synthetic as well as degradative processes occurring within the connective tissue compartment of the lung. It would seem valuable to study base-line hydroxylysine levels in a healthy volunteer population to determine human variability. Once this value is established, specific populations could be targeted in which one would expect abnormal connective tissue degradation. If such experiments demonstrate a reasonable base line for hydroxylysine, with changes of magnitude in disease states that are measurable, then a controlled study of people exposed to mobile source emissions could be undertaken.

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ABOUT THE AUTHORS

John N. Evans is an Associate Professor of Physiology and Biophysics at the University of Vermont College of Medicine. He earned his Ph.D. in physiology at the University of Florida College of Medicine in 1976. His major research interest is the cell biology of the lung, with particular emphasis on smooth muscle and the response of the lung to injury.

David R. Hemenway is Professor of Civil and Mechanical Engineering at the University of Vermont. Dr. Hemenway earned his M.S. and Ph.D. degrees in industrial hygiene at the University of North Carolina. His interests are in the effects of inhaled pollutants on the lung and the application of engineering principles to the design of aerosol exposures.

Jason Kelley is a Professor in the Department of Medicine at the University of Vermont College of Medicine. He received his M.D. degree from the University of Texas Southwestern Medical Center in Dallas and completed postdoctoral training at the College of Physicians and Surgeons of Columbia University as well as at the University of Vermont. Dr. Kelley has major research interests in connective tissue matrix and cytokine biology of the lung.

ABBREVIATIONS

ANOVA	analysis of variance
^{14}C	carbon-14
^{3}H	tritium
HCl	hydrochloric acid
NO_2	nitrogen dioxide
PBS	phosphate-buffered saline

INTRODUCTION

A Request for Applications (RFA 82–2), which solicited proposals for "Cellular and Biochemical Markers Related to Nonneoplastic Chronic Lung Diseases," was issued by the Health Effects Institute (HEI) in the summer of 1982. In response to the RFA, Drs. J. Evans and J. Kelley, from the University of Vermont, submitted a proposal entitled "Early Markers of Lung Injury." The HEI approved the three-year project, which began in July 1983. Total expenditures were $495,765. The Investigators' Report was received at the HEI in August 1987 and was accepted by the Health Review Committee in April 1988. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of the HEI and to the public.

THE CLEAN AIR ACT

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1977. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and provide the EPA with limited discretion to modify those requirements.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards to protect the public health. The current standards include those for ozone and nitrogen dioxide. The determination of the appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the risks to health that they present. It is recognized that, in animals, repetitive exposure to oxidant gases may lead to chronic loss of lung function; however, in humans, more information is available on the acute effects of exposure. Thus, identification of early markers of lung injury can contribute to the understanding of both acute and chronic effects caused by oxidants and, therefore, to informed regulatory decision making.

BACKGROUND

Oxidants represent a class of reactive pollutants derived primarily from atmospheric reactions between the combustion products of fossil fuels and oxygen, catalyzed by sunlight. In acute animal experiments, the inhalation of high concentrations of oxidants results in pulmonary edema, inflammation, and hyperplasia of constituent cells (U.S. Environmental Protection Agency 1986). The contribution of oxidants to the development of chronic lung disease, such as fibrosis or emphysema, has received considerable attention. However, because the early stages of these diseases are not well understood, it has been difficult to relate pollutant exposure to such delayed, chronic effects. Thus, the identification of biochemical or cellular markers that are associated with the onset of chronic pulmonary disease could aid in determining the role of oxidant exposure.

The biological basis for either emphysema or fibrosis resides in the connective tissue matrix of the lung parenchyma. The major components of lung connective tissue are collagen, elastin, glycosaminoglycans, and glycoproteins, such as fibronectin and laminin (Hance and Crystal 1975; Turino 1985). These components provide structural support and dictate the mechanical properties of the lung. Collagen, which exists as several types, and elastin have received the most study because they constitute more than 90 percent of the matrix components.

The formation of collagen fibrils requires intracellular synthesis of procollagen molecules and extracellular conversion of the procollagen to collagen (reviewed by Prockop et al. 1979a,b). Procollagen is a triple-helical, rigid molecule composed of three polypeptide chains, called α chains. Combinations of different subtypes of these chains determine the final collagen type. During synthesis of the α chains, some of the proline and lysine amino acids are hydroxylated. Subsequent to hydroxylation, sugar residues, specifically galactose and glucose, are attached to the hydroxylysine. These, as well as other modifications, are important in the assembly of the helical chains to form the procollagen molecule. After secretion, procollagen is processed to yield collagen, which self-associates to form collagen fibrils. The enzyme, lysyl oxidase, mediates the cross-linking of the collagen molecules. A number of factors have been identified that regulate the amount, as well as the characteristics, of the collagen fibrils.

Elastin is the protein component of the elastic fiber, and is derived from a soluble form, tropoelastin (reviewed by Sandberg et al. 1981). Tropoelastin is synthesized, undergoes posttranslational hydroxylation of some proline amino acids, and is secreted. Once in the extracellular space, tropoelastin is crosslinked to form the insoluble elastin fiber. Lysyl oxidase participates in the formation of the crosslinking amino acids, desmosine and isodesmosine, which are unique to elastin. In contrast to collagen, little is known about what regulates elastin metabolism.

Collagen and elastin are susceptible to degradation by specific proteases, which have either collagenase or elastase activity. The sources of these enzymes are the blood and inflammatory cells, such as polymorphonuclear leukocytes and alveolar macrophages. The activity of these proteolytic enzymes is regulated by inhibitor molecules called antiproteases or protease inhibitors.

Emphysema is characterized by permanent enlargement of the alveolar airspaces, destruction of alveolar walls, and an absence of fibrosis (Snider et al. 1985). Morphometric analysis of emphysematous tissues shows airspace enlargement (as measured by mean linear intercept, an estimator of average airspace size) and a decrease in alveolar surface area. Animal models of the disease are considered appropriate if enlargement of the airspaces distal to the terminal bronchioles can be demonstrated (Snider et al. 1986).

The loss of structural integrity of the lung parenchyma has led to research focusing on the role of the connective tissue components in the pathogenesis of emphysema. The most popular hypothesis for the development of this disease suggests that an imbalance between proteolytic enzymes and their inhibitors results in the destruction of the pulmonary interstitium (reviewed by Janoff 1985). Early-onset, familial emphysema occurs in individuals with an inheritable deficiency of the alpha-1-proteinase inhibitor. These individuals develop panlobular emphysema, in which all components of the acinus are destroyed. In contrast, with centrilobular emphysema, which is associated with cigarette smoking, the alveoli adjacent to the respiratory bronchioles are selectively destroyed, but the more distal alveolar tissue is spared.

Intratracheal instillation of elastase into animals produces lesions similar in appearance to human panlobular emphysema (Kaplan et al. 1973) and is used as a model to study the biochemical events of connective tissue turnover. After initial degradation, elastin, which normally exhibits little turnover, is rapidly synthesized (Kuhn et al. 1976; Yu et al. 1978; Karlinsky et al. 1983). In addition, collagen content decreases but is also replenished (Kuhn et al. 1976; Yu and Keller 1978; Karlinsky et al. 1983). The restoration of these two components in experimental systems may explain why the measurement of total elastin and collagen content in emphysematous lungs from humans reveals normal levels (reviewed by Hance and Crystal 1975). What may be of more importance are the organization of the elastic fiber network (Kuhn et al. 1976) and the relative proportions of elastin, collagen, and glycosaminoglycans (Karlinsky et al. 1983).

Pulmonary fibrosis is characterized primarily by excessive collagen, which results in a stiffer, less compliant lung (Karlinsky and Goldstein 1980). Histological examination of fibrotic lungs reveals excess collagen, deposited in an inhomogenous pattern, in the alveolar interstitium, alveolar ducts, and respiratory bronchioles. Increased levels of total lung collagen have been measured biochemically. The accumulation of collagen has been ascribed to an increase in collagen synthesis (Clark et al. 1980). In addition to being present in excess amounts, the type as well as the crosslinking pattern of collagen is altered (reviewed by Last 1988). Although less well described, there is also evidence for increases in elastin (reviewed by Karlinsky and Goldstein 1980) and glycosaminoglycans (reviewed by Turino 1985). Several fibrogenic agents have been identified, some of which have been used experimentally to study the pathogenesis of the disease. Experimental evidence suggests that if repair of the alveolar epithelium is delayed, there is an associated proliferation of interstitial fibroblasts and, presumably, altered collagen metabolism (Bowden 1985; Last 1988).

Although different models exist for the induction of either emphysema or fibrosis, it is apparent that the two diseases are closely related (Niewoehner and Hoidal 1982). Using an experimental system that usually produces interstitial fibrosis, Niewoehner and Hoidal (1982) showed that when hamsters were fed an inhibitor of lysyl oxidase (β-aminoproprionitrile), bullus emphysema was present in addition to the fibrotic lesion. The authors suggest that prevention of postinjury repair by β-aminoproprionitrile prolongs alveolar disruption and promotes subsequent bullus formation. These findings further emphasize the importance of the repair process in the pathogenesis of disease.

Oxidant injury to components of the connective tissue has been demonstrated in experimental systems; but how these forms of injury relate to human disease is not known. Furthermore, although ozone and nitrogen dioxide are both oxidants, experimental exposure to either of the two gases produces different alterations in the pulmonary connective tissue. Experimental evidence from animals exposed to ozone suggests that the gas may induce pulmonary fibrosis (reviewed by Last 1988). Increases in lung collagen content and collagen synthesis rates have been measured (Last 1988). In addition, morphometric analysis of parenchymal tissues reveals histological changes indicative of interstitial fibrosis (Boorman et al. 1980; Fujinaka et al. 1985).

Nitrogen dioxide exposure causes airspace enlargement with some alveolar septal fibrosis. Exposure of rats to 30 parts per million (ppm) of nitrogen dioxide continuously for three to six weeks results in mild emphysema (Freeman et al. 1968). This model has been adopted as a way of studying the morphological and connective tissue biochemistry during the early stages of the emphysematous lesions. During initial inflammation, large numbers of elastase-rich polymorphonuclear leukocytes are recruited from the blood to the alveolar airspace (Glasgow et al. 1987). Both pulmonary elastin and collagen content decrease during the first week of exposure. Collagen content returns to normal levels during oxidant exposure, whereas elastin content does not return to normal until exposure is terminated (Kleinerman and Ip 1979). In contrast to the instilled-elastase model, the nitrogen dioxide model results in centriacinar emphysema that progresses little after termination of exposure.

Since disruption of the balance between connective tissue synthesis and degradation is implicated in the pathogenesis of chronic lung disease, a better understanding of the dynamics of collagen, elastin, and, possibly, glycosaminoglycan (Karlinsky et al. 1983; Turino 1985) turnover is needed. Assays that measure components of metabolism are available and have been used primarily to analyze events in the lung tissue from animal models of either fibrosis or emphysema. There has been little application of these assays to sources that can be sampled relatively easily (urine, blood, bronchoalveolar lavage). Goldstein and Starcher (1978) demonstrated the usefulness of correlating levels of desmosine in the urine with pulmonary elastin content and turnover after elastase administration. With respect to the effects of oxidants, desmosine levels in hamster urine increased after exposure to 30 ppm nitrogen dioxide (Ip et al. 1984). In humans accidently exposed to very high levels of nitrogen dioxide, urinary levels of hydroxylysine glycosides rose (Hatton et al. 1977). A systematic evaluation has not been conducted in which markers of pulmonary connective tissue metabolism have been measured in accessible fluids after oxidant exposure. Ideally, it should be possible to relate such marker measurements to either exposure levels or the potential for disease.

JUSTIFICATION FOR THE STUDY

The HEI sought proposals that would improve our understanding of the relation between oxidant injury and lung disease. Of particular interest to the Institute was the identification of sensitive indicators of lung injury and disease as measured in human body fluids. A related goal was to develop objective measures of exposure or damage to the lung. In addition, the HEI sought to support research that could demonstrate predictive power from short-term bioassays for the evaluation of human risk to oxidant exposure.

Drs. Evans and Kelley hypothesized that alterations in connective tissue that are induced by exposure to atmospheric pollutants can be monitored by measuring markers of connective tissue metabolism. Using nitrogen dioxide as the test insult, the investigators proposed to measure markers of connective tissue synthesis (procollagen I terminal peptide, lysyl oxidase) and degradation (desmosine, hydroxylysine, hydroxyproline) in the serum, urine, or lavage fluids from rats. Other biochemical markers, lung physiology, and histology were to be used to assess lung injury. The ultimate goal of the proposal was to apply the relatively noninvasive means of measuring connective tissue metabolism to the human population.

The proposal was responsive to the RFA. The study was well planned, the specific aims logical, and the methods appropriate. The choice of markers was considered reasonable, based on the current knowledge of connective tissue metabolism.

OBJECTIVES OF THE STUDY

The primary goal of this project was to demonstrate that markers of connective tissue turnover, available from samples obtained relatively noninvasively, can be used to predict the occurrence of lung injury after exposure to oxidant gases. Specific aims of the original proposal were:

1. To demonstrate that selected markers (procollagen I terminal peptide, hydroxylysine, hydroxyproline, desmosine, lysyl oxidase, and angiotensin-converting enzyme) from serum, urine, or bronchoalveolar lavage fluid predict the time course and severity of lung injury after exposure of rats to nitrogen dioxide;
2. To correlate these biochemical markers with whole-lung physiology, lung histopathology, and biochemical measures of lung collagen metabolism;
3. To determine the sensitivity of these assays to nitrogen dioxide exposure as well as to determine the minimal level of exposure that affects marker levels; and
4. To develop a rationale for future application of these results to human populations.

In order to accomplish these aims, the investigators had to develop, as part of their project, some of the assays used to measure the markers of interest. In addition, the investigators had to set up and calibrate the nitrogen dioxide exposure facility. Thus, their project entailed considerable developmental work before actual experiments could be conducted. During the course of the project, changes in per-

sonnel necessitated the elimination of the lysyl oxidase measurements.

STUDY DESIGN

Adult male rats (Fischer-344) were exposed to various concentrations of nitrogen dioxide (ranging from 0.5 ppm to 30 ppm) for various lengths of time (ranging from two days to four weeks) in a series of separate experiments. Urine and bronchoalveolar lavage fluid were collected and analyzed for markers of lung injury: hydroxylysine, hydroxyproline, desmosine, and angiotensin-converting enzyme. In some experiments, lavage and urine volumes, dried lung weight, urinary creatinine, lavage and urine amino acid content, and lung tissue protein content also were measured and used to normalize some of the marker values. Pulmonary function studies and histologic examinations by light microscopy also were used to assess the extent of oxidant-induced lung injury.

Animals exposed to clean air were used as negative controls. For positive controls, rats were exposed to 85 percent oxygen, and urinary hydroxylysine was measured.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

Rather than using a predefined protocol to address a specific hypothesis, the investigators accomplished the specific aims with a series of exploratory experiments that addressed a sequence of questions.

METHODS AND STUDY DESIGN

Although the design and conduct of the study were appropriate in most respects, it is not clear whether or not the control animals were exposed to clean air in the same manner as other animals were exposed to nitrogen dioxide. Also, the two-day, short-term exposures used in the exposure concentration response curve (Figure 2) were not optimal, due to the small numbers of animals in each group and the limited range of data points between the apparent no-effect level and the highest concentration tested.

The choices of angiotensin-converting enzyme, desmosine, and hydroxylysine were appropriate, and the assay developed to measure hydroxylysine was elegant. However, the measures used to correlate lung injury (pulmonary function, light microscopy) with changes in marker levels were probably too insensitive at low levels of nitrogen dioxide exposure. Thus, at low oxidant levels, it might not have been possible with the methods used to determine confidently whether or not injury had occurred.

DATA PRESENTATION AND STATISTICAL ANALYSIS

This was a well-designed study, with an appropriate set of statistical analyses directed at improving the clarity of presentation and at making the statistics contribute more directly to the aims of the study. Significance tests were conducted by simple one-way analysis of variance (ANOVA) and were followed, in cases of significance, by multiple comparisons made appropriately stringent by the Bonferroni rule. Tests for linear and quadratic trend were also applied, but it is not clear how the evidence of these trends was weighed against the significance tests. One-way ANOVA and multiple comparisons analysis are likely to be statistically underpowered for a study like this, since they do not take into account the order of doses or times. Yet, the investigators downplay a significant linear trend (Figure 5) on the ground that ANOVA revealed only one significant elevation in lavage protein. The test for linear or quadratic trend is more convincing than ANOVA, because ANOVA is insensitive to the order of doses, while linear and quadratic contrasts are not.

Since this study is more descriptive than inferential, it would have been acceptable to place less emphasis on significance testing. The study aims to measure and compare the sensitivity of various markers to nitrogen dioxide exposure. Biological sensitivity does not always have to be quantified on a yes-or-no scale (significant or nonsignificant increase), but also can be expressed on a continuous scale, preferably logarithmic. The text frequently expresses results in terms of a threefold increase or a tenfold increase over control, which is an appropriate way to summarize the results. However, the report would have been strengthened if the results had been tabulated or compared in a comprehensive way. The investigators were systematic in their design, covering a range of acute and chronic exposure patterns. A summary table listing the response of each marker to each exposure, expressed as ratio of mean response to control, plus or minus standard error, would have been helpful.

In addition to facilitating comparisons, the inclusion of means and standard deviations would permit further analyses, such as the computation of coefficients of variation. The usefulness of additional analyses can be illustrated by considering the investigators' claim that as an index of lung injury, hydroxylysine is more sensitive than hydroxyproline because of its tenfold increase over control compared to the threefold increase seen for hydroxyproline. If, however,

hydroxylysine were ten times more variable than hydroxyproline, it would be no more useful as an index of injury.

The authors vacillate some between multiplicative and arithmetic scales of response. The text describes three- or tenfold increases, while the graphic presentation is made on an arithmetic response scale. To corroborate the text, the reader must "eyeball" the control and response levels and divide them mentally. The ANOVAs were probably done using arithmetic responses, despite the greater variability that is apparent at higher response levels in most of the figures (ANOVA assumes uniform variance). The investigators could have considered using log dose in their statistics and a logarithmic scale in their graphics. Using such an approach, controls would always be at 1.0, and multiples of response could be read directly. This could be more informative than the sole reliance on asterisks to indicate statistical significance.

RESULTS AND INTERPRETATION

The investigators have shown that urinary hydroxylysine is a sensitive indicator of nitrogen dioxide exposure over a range of concentrations from 0.5 to 30 ppm. Angiotensin-converting enzyme from the lavage fluid increased only at 30 ppm nitrogen dioxide and urinary desmosine responded significantly only at 25 ppm, while urinary hydroxylysine showed significantly measurable changes after exposure to oxidant concentrations as low as 0.5 to 1 ppm, levels at which quantitative histopathology showed no effect. The data are particularly convincing in those experiments in which animals were exposed to 0.5 ppm or 1 ppm nitrogen dioxide for four weeks and then allowed to recover for four weeks; hydroxylysine levels rose during the exposure period but then fell during the recovery period. The clear superiority of hydroxylysine over hydroxyproline as a marker is well illustrated by the data in Figure 17 as compared with those in Figure 19.

There is, however, no clear dependence of the urinary hydroxylysine levels on the nitrogen dioxide exposure dose. After three days of exposure, urinary hydroxylysine levels were similar (0.5 to 1.5 μmol/ml) at 0.5 or 1 ppm as at 30 ppm of nitrogen dioxide (see Figures 12, 20, and 22). In addition, at 7.5 ppm (Figure 9) and 30 ppm (Figure 12) nitrogen dioxide, the greatest response was observed at one day of exposure. At 0.5 ppm (Figure 22), 1.0 ppm (Figure 20), and 25 ppm (Figure 17) nitrogen dioxide, however, urinary hydroxylysine levels increased with increasing time of exposure. Clarification or discussion of some of these observations in the Investigators' Report would have been helpful. If the hydroxylysine arises from the interaction of nitrogen dioxide with collagen, as the investigators propose, it is not clear why this effect does not worsen with additional exposure, rather than being transient. Exposure protocols differed among experiments, which probably contributed to the lack of a dose dependence in the overall results. Furthermore, since connective tissue turnover is influenced by age (Last 1988), the use of rats with different weight ranges (and thus, presumably, different ages) among experiments may have affected a dose-dependent relationship; body weights ranged from a low of 175 to 200 g to a high of 300 to 320 g. It is also conceivable that the observed hydroxylysine production represents a nonspecific response, possibly related to the physical stress of exposure.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Data from these studies suggest, but do not prove, that urinary hydroxylysine can be used as a marker for lung injury from oxidant air pollution. Additional studies should be conducted to determine the source of the marker as well as its specificity to nitrogen dioxide exposure. A definitive set of experiments could be conducted, focusing on the relation between low-dose nitrogen dioxide exposure, metabolic or ultrastructural changes in the lung, and the threshold level of hydroxylysine excretion in the urine. These studies should be done at dose levels that are relevant to ambient exposures to nitrogen dioxide. Isotopic tracers could be used to ascertain whether or not the hydroxylysine originates in the lung or represents a metabolic product affected by nonspecific stresses.

The results do not change the perception that nitrogen dioxide is a potential risk factor for the development of fibrosis and emphysema, but do suggest a new approach for exposure assessment. The data shown in Figure 22 are especially noteworthy because they were observed after exposure at 0.5 ppm for six hours/day for four weeks; this level of exposure is high but approaches a realistic concentration for a possible worst-case human exposure. Potentially, the methodology developed by these investigators could be applied to the measurement of urinary hydroxylysine in humans. Such studies with humans exposed experimentally to low-dose concentrations of nitrogen dioxide or to ambient levels of pollutants in communities, occupational settings, or indoor environments could explore the potential of this assay for the assessment of oxidant pollution exposure.

CONCLUSIONS

This study showed that urinary hydroxylysine, a marker of collagen turnover, increased in rats after exposure to con-

centrations of nitrogen dioxide as low as 0.5 ppm; other related markers, such as angiotensin-converting enzyme, desmosine, and hydroxyproline, did not show this level of sensitivity to oxidant exposure. The evidence is not conclusive, however, that urinary hydroxylysine is a sensitive, specific, or quantitative marker for nitrogen dioxide injury in the lung. At low levels of nitrogen dioxide, the existence or extent of lung injury is inadequately defined both biochemically and histologically. Whether or not the hydroxylysine detected in the urine originates from the lung remains to be determined. Finally, the marker may not be sufficiently discriminating at a quantitative level for use in setting standards, since the urinary levels of hydroxylysine are the same at 0.5 ppm or 1 ppm nitrogen dioxide as at 30 ppm. However, because of the specificity of hydroxylysine for collagen degradation and the apparent sensitivity of this marker relative to other markers of connective tissue metabolism, the resolution of these uncertainties should be pursued.

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Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
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