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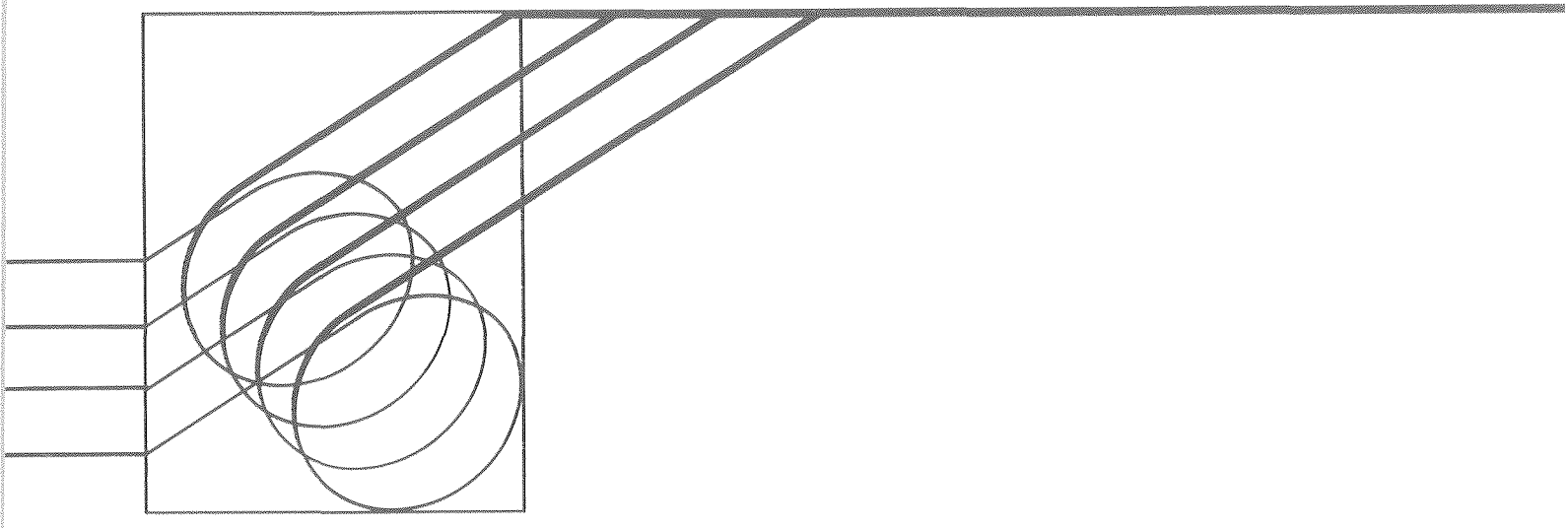

Transport of Macromolecules and Particles at Target Sites For Deposition of Air Pollutants

T. T. Crocker, M. D., and D. K. Bhalla, Ph.D.

University of California, Irvine
Department of Community and Environmental Medicine
College of Medicine
Irvine, CA

Includes the Report of the Institute's Health Review Committee

HEALTH EFFECTS INSTITUTE

The Health Effects Institute (HEI) is a non-profit corporation founded in 1980 to assure that objective, credible, high-quality scientific studies are conducted on the potential human health effects of motor vehicle emissions.

Funded equally by the U.S. Environmental Protection Agency (EPA) and 24 automotive manufacturers or marketers in the United States, HEI is independently governed. Its research projects are selected, conducted, and evaluated according to a careful public process, including a rigorous peer review process to assure both credibility and high scientific standards.

HEI makes no recommendations on regulatory and social policy. Its goal, as stated by former EPA Administrator William D. Ruckelshaus, is "simply to gain acceptance by all parties of the data that may be necessary for future regulations."

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Preface

THE HEALTH EFFECTS INSTITUTE AND ITS RESEARCH PROCESS

The Health Effects Institute (HEI) is an independent non-profit corporation that, according to its charter, is "organized and operated...specifically to conduct or support the conduct of, and to evaluate, research and testing relating to the health effects of emissions from motor vehicles."

It is organized in the following ways to pursue this purpose:

Independence in Governance

HEI is governed by a four-member board of directors whose members are William O. Baker, Chairman Emeritus of Bell Laboratories and Chairman of the Board of Rockefeller University; Archibald Cox, Carl M. Loeb University Professor (Emeritus) at Harvard University; Donald Kennedy, President of Stanford University; and Charles Powers, President, Clean Sites, Incorporated. Professor Cox chairs the Board. These individuals, who select their own successors, initially were chosen, after consultation with industry representatives and other individuals, by then Environmental Protection Agency Administrator Douglas M. Costle.

Two-Sector Financial Support

The Institute receives half of its funds from the United States government through the Environmental Protection Agency and half from the automotive industry. Twenty-four leading manufacturers of vehicles or engines that are certified for use on U.S. highways contribute to the Institute's budget, in shares proportionate to the number of vehicles or engines that they sell.

Research Planning and Project Evaluation

HEI is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is devised by the Health Research Committee, a multi-disciplinary group in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee defines an area of inquiry, the Institute solicits from the scientific community research proposals on a specific topic. The resultant applications are initially reviewed for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to the mission-oriented research program. Studies recommended by the Committee undergo a final evaluation by the Board of Directors, which also reviews the procedures, independence, and quality of the selection process.

When a study is completed, a draft final report is reviewed by a separate HEI Committee, the Health Review Committee, whose members are expert scientists representing a broad range of experience in environmental health sciences. The Review Committee has no role in the review of applications or in the selection of projects and investigators for funding. This Committee assesses the scientific quality of each study and evaluates each study's contribution to unresolved scientific questions.

Each funded proposal is assigned in advance of completion to a member of the Review Committee, who acts as "primary reviewer." When the draft report is received, the primary reviewer directs a peer review that involves: (1) a referral of the report to the appropriate technical experts and, when appropriate, (2) the involvement of the Review Committee's biostatistician who determines the appropriateness of the statistical methods used to evaluate the data. After the investigator comments on the technical evaluations, the primary reviewer drafts a review, which is examined by the full Review Committee and revised as necessary. After an evaluation by the HEI Board of Directors, the investigator's final report and the Review Committee's report are made available to the sponsors and to the public.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing and nature of the HEI report releases are tailored to ensure that the Review Committee's report does not interfere with the journal publication process.

INTRODUCTION

In the summer of 1982, the Health Effects Institute (HEI) issued a Request for Applications (RFA 82-5) soliciting proposals on "Dose to Target Tissues." In the fall of 1982, Drs. T. Timothy Crocker and Deepak K. Bhalla of the College of Medicine, Department of Community and Environmental Medicine, University of California, Irvine, proposed a project, entitled "Transport of Macromolecules and Particles at Target Sites for Deposition of Air Pollutants." HEI approved expenditures of $300,000 during a two-year period for this project. The investigators began their study in August, 1983, and the final report was accepted by the Health Review Committee in December, 1985. The following report by the Health Review Committee is intended to place the investigators' final report in perspective as an aid to the sponsors of HEI and to the public.

THE CLEAN AIR ACT

Under the Clean Air Act, as amended (1), EPA has broad authority to adopt emission standards for, *inter alia*, new motor vehicles (2), and any source of a "hazardous" air pollutant (one that may cause "an increase in mortality or an increase in serious irreversible, or incapacitating reversible, illness") (3). It may regulate the contents of motor vehicle fuels (4). In an emergency, in the absence of adequate state or local action, EPA may sue to enjoin any emissions contributing to "an imminent and substantial endangerment to the health of persons"(5). In making public health assessments and judgements in connection with the exercise of these powers, EPA considers, *inter alia*, the exposure of human beings to the substance at issue and its toxicity to exposed individuals. Research bearing on such exposure or toxicity is, thus, relevant to the exercise of the EPA's authorities to regulate emissions from motor vehicles.

BACKGROUND

The pulmonary epithelium is the first point of contact between the lung and inhaled air pollutants. It is important to add to our understanding of the epithelium because it is the first line of defense against injurious air contaminants. Changes in the pulmonary epithelium might provide early indications of exposure or signify potential lung damage.

Pulmonary Epithelium

The epithelium of the pulmonary system is a cellular, avascular layer of tissue that covers the surfaces of other tissues that face the lumen† and forms a barrier between the organism and the outside environment. The epithelium regulates the traffic of substances to and from its luminal or mucosal† surface to the underlying serosal† or submucosal† tissues. It therefore serves protective and transport functions. In order for the epithelium to maintain its integrity as a barrier, individual epithelial cells must maintain continuous lateral attachments with one another. An anastomosing network of intramembraneous proteins

† Technical terms marked with a dagger are explained in the glossary, page 14.

arranged in rows or fibrils fuses the neighboring cells at their lateral surfaces. This network, termed the "zonula occludens", or tight junction, forms a belt around each cell and limits the passage of material. Substances that do penetrate the epithelium must take a route either between (paracellular) or through (transcellular) cells. The transcellular route consists of two pathways: diffusion across the cell membrane or, alternatively, uptake by membrane-bound vesicles.†

Pulmonary Epithelial Permeability

Considerable effort has been focused both on investigating the movement of material across the pulmonary epithelium and on characterizing and understanding epithelial permeability. Experimental studies on epithelial permeability have tried to determine:

- the permeability to specific substances, such as ions, small nonelectrolytes, water, macromolecules, particles;
- the route and/or mechanisms of transport; and
- the permeability alterations by physiologic, pharmacologic,† or toxic agents.

Effects of Air Pollutants

Relatively few studies have attempted to assess pulmonary injury at air pollutant levels typically encountered in ambient environments. One difficulty in doing such experiments is identifying an endpoint that could serve as an early indicator of a more serious or debilitating injury. Edema,† altered mucous secretions, and enhanced airway reactivity† are associated with alterations in permeability. Because of the important protective and regulatory roles of the pulmonary epithelium, alterations in permeability may have significant consequences. Some fibrotic† diseases or lung carcinomas may originate from the retention of toxic or carcinogenic particles. Since exposure to air pollutants such as ozone (O_3) and nitrogen dioxide (NO_2) may cause pulmonary edema, increases in airway resistance,† and damage to epithelial cells, it seems reasonable that altered epithelial permeability may be an early sign of injury. Few studies, however, have attempted to relate alterations in permeability to air pollution exposure. Studies that have been done are summarized in Table 1.1 (ozone) and Table 1.2 (nitrogen dioxide). The studies have not been evaluated by HEI for technical quality.

HEI OBJECTIVES

Background

The Health Effects Institute has sponsored several projects designed to determine the dose-to-target tissues produced by different types of pollutant exposure. Research that examines the respiratory effects of gases that originate directly or indirectly from automotive exhaust, such as nitrogen oxides, ozone, and formaldehyde, is of special interest.

Table 1.1. Effects of ozone on pulmonary tissues associated with changes in permeability

Species	Concentration (ppm)	Time (Hour)	Effects	Reference
Guinea pig	8.0	0.5	Increased permeability to egg albumin	Matsumura (6)
Guinea pig	2.0, 5.0	0.5	Increased severity of dyspneic attacks and decreased survival after antigen aerosol	Matsumura (7)
Rats	0.25, 0.5 1.0, 2.5	6	Increased permeability to albumin	Albert (8)
Guinea pig	1.5, 2.0, 3.0, 3.5	0.5	Increased severity of dyspneic attacks and decreased survival after acetylcholine aerosol	Matsumura (9)
Dog	0.7-1.0	2	Increased airway resistance after histamine aerosol	Lee (10)
Human	0.6	2	Increased airway resistance after histamine aerosol	Golden (11)
Guinea pig	4.0	3	Increased permeability to mannitol, dextran, horseradish peroxidase	Davis (12)
	1.0	3	Increased permeability to mannitol, dextran	
Guinea pig	0.26, 0.51, 1.0	72	Increased protein levels in lavage fluid	Hu (13)
	0.51	3	Increased protein levels in lavage fluid 10 hours post-exposure	

Table 1.2. Effects of nitrogen dioxide on pulmonary tissues associated with changes in permeability

Species	Concentration (ppm)	Time	Effects	Reference
Guinea pig	40, 80	0.5 hr.	Increased severity of dyspneic attacks and decreased survival after antigen aerosol	Matsumura (7)
Guinea pig	30, 40 45, 50	0.5 hr.	Increased severity of dyspneic attacks and decreased survival after acetylcholine aerosol	Matsumura (9)
Human (asthmatics)	0.1	1 hr.	Increased airway resistance after carbacol aerosol	Orehek (14)
Guinea pig	5, 15	2 d, 14 d	Increased permeability of horseradish peroxidase	Ranga (15)
Guinea pig	28	6, 24, 48 hr.	Increased fragmentation of tight junctions with increasing dose	Case (16)

Justification for Study by HEI

HEI attempts to foster research that will lead to an improved understanding of the dose-to-respiratory tract tissues from inhaled vehicle emissions. Quantifying the cellular doses of specific emission constituents, or of their metabolites, is vital to our understanding of the health effects of any substance. Information in this area will help provide a basis for making two key extrapolations. First, it will aid in the extrapolation of dose-response information obtained in cell and tissue studies to the whole animal. Second, such information will be useful in the extrapolation of dose-response from one species to another.

There is some evidence to indicate that altered epithelial permeability may be an early marker of subsequent lung damage. Investigations of the relationship between air pollutant exposure, pulmonary epithelial permeability, and morphological lesions may provide valuable information about air pollution health effects. Research of this kind will aid HEI in improving our understanding of the dose delivered to target tissues for important vehicle-related air pollutants.

OBJECTIVES OF THE PROPOSED STUDY

The objectives of the investigators were to study the sites of injury in rat airways following inhalation of formaldehyde (HCHO), O_3, and NO_2: (a) alone or combined; (b) during rest or during exercise; and (c) under conditions of repeated exposure which might lead to progressive injury or adaptation.

Electron microscopy would be used to study the route and possible mechanism of increased permeability. Histologic findings were to be correlated with thymidine incorporation into the respiratory tract epithelial cells as reflective of possible cell killing or replacement. Changes in permeability were to be measured by determining the transfer from the respiratory tract lumen to the blood and from the blood to the lumen of a low molecular-weight substance, ^{99m}Tc-diethylenetriamine-pentaacetate (DTPA, mol. wt. 469), and a high molecular-weight substance, ^{125}I-bovine serum albumin (^{125}I-BSA, mol. wt. 69,000).

The purpose of these studies was to compare the permeability changes in three areas of the respiratory tract: the nose, the trachea, and the bronchoalveolar area. These comparisons made it possible to measure the loss or reduction of the normal barrier function of the respiratory epithelia, which might increase the susceptibility of each target zone to penetration by foreign molecules and particles.

Anticipated Results and Significance

The investigators hoped to localize sites of pollutant injury by identifying regions of 1) altered macromolecule uptake and transport in lung epithelial and lymphoid tissue and 2) altered vascular permeability to injected radiolabeled macromolecules. They planned to

alter the sites of injury by varying the factors expected to influence the deposition of toxic pollutants in the airways. Findings from the study were expected to be an important step toward confirming hypotheses related to factors that influence pollutant doses to respiratory system target tissues.

SUMMARY OF INVESTIGATORS' CONCLUSIONS

1. In control rats, the transfer of the isotopically-labeled test molecules from the respiratory tree lumen to the blood was lowest in the nasal area, highest in the trachea, and intermediate in the bronchoalveolar region.

2. Exposure for 2 hours to 0.8 and 2.0 ppm of O_3 did not alter nasal permeability, but it did increase the tracheal and bronchoalveolar permeability of labeled compounds. This effect was greatly enhanced and prolonged by a level of exercise that doubled oxygen consumption. In rats exposed to O_3 during exercise, the surface area of the lungs that was involved in inflammatory changes was also increased by four- to seven-fold. Ultrastructural analysis of the tracheal tissues from resting rats, which had been exposed to O_3, showed increases in endocytotic vesicles, as well as the presence of instilled horseradish perioxdase (HRP) in intercellular spaces. No HRP was found in the intercellular spaces in control rats.

3. Exposure of resting rats to 6 or 12 ppm NO_2 for 2 hours did not alter respiratory tract epithelial permeability, and no focal lesions in the lung were produced under these conditions. Exposure of rats to 12 ppm NO_2 during exercise, however, significantly increased tracheal and bronchoalveolar permeability to diethylenetriamine-pentaacetate (DTPA) and bovine serum albumin (BSA).

4. Exposure to 10 ppm formaldehyde for 4 hours increased nasal mucosal permeability and, after repeated exposure, increased nasal epithelial cell turnover.

5. Exposure to 0.6 ppm O_3 plus 2.5 ppm NO_2 for 2 hours produced a significant increase in bronchoalveolar permeability immediately after exposure, whereas neither of these gases alone at these concentrations produced a significant change in permeability. In rats exposed during exercise to 0.35 ppm O_3 plus 0.6 ppm NO_2, there were two- to three-fold more focal lesions than in exercising rats exposed to 0.35 ppm O_3 alone.

TECHNICAL EVALUATION

Assessment of Methods and Study Design

In general, the methods and study design were appropriate for the specific objectives of the study. The study design included replicate experiments on control (air-exposed) and experimental (irritant gas-exposed) rats. Typically, three animals were used at each exposure

concentration. The use of replicate experiments offered an opportunity for the verification of results and the collection of adequate numbers of the animals for analysis of differences between the control and experimental groups. In addition, the effects observed after exposure during exercise demonstrated an enhanced possibility to detect effects under physiologically-realistic stress conditions.

The methods used in this investigation appear to represent state-of-the-art technology for *in vivo* studies of permeability. Nevertheless, some questions remain unanswered, such as: (a) whether tissue sampling for electron microscopy and autoradiography was sufficiently selective to assure that samples were from the most affected areas, (b) whether correlative measures of ventilatory patterns are needed to maximize both dosage estimates and interpretation of the functional significance of epithelial permeability changes, (c) whether the method of instillation of the isotopically-labeled test substances assured adequate separation of nasal, tracheal, and bronchoalveolar areas of the lung, and (d) whether sufficient dose-response data were available on individual gases and their mixtures to reach conclusions about synergistic actions.

These concerns primarily relate to whether the primary data on epithelial transport can be used to extend interpretations with regard to mechanisms, interactive effects, and specificity of site of action. To some extent, these issues were addressed in the investigators' final report and do not seriously detract from the study's contribution. They do, however, affect our ability to extrapolate the results to *in vivo* studies in humans.

Interpretation of Results

The authors' conclusion that the rats' exposure to O_3 and NO_2, particularly during their exercise, enhances tracheal and bronchoalveolar permeability is well-supported by the data. The proposed pathway of increased transfer also seems reasonable, in view of the histological findings and electronmicrographs. The authors are cautious in drawing conclusions about the combined effects of O_3 and NO_2, although a synergistic phenomenon is implied. Clearly, the study design does not permit a definitive conclusion of synergism. Such a conclusion receives some support, however, from reports recently published by others (17, 18). The question of synergism between O_3 and NO_2 needs further investigation.

The research results demonstrate that pulmonary irritants affect the pulmonary epithelium in different ways and at different sites. The predominant effect of formaldehyde appears to be in the upper airways, while the predominant effects of ozone and nitrogen dioxide seem to be in lower airways. Previous studies using different endpoints have reached the same conclusions. This research does, however,

demonstrate that one can use the experimental approach of measuring epithelial permeability to study isolated areas of the respiratory tree. The study also establishes the relative permeability *in vivo* of the three areas of the pulmonary tree that were studied. This project also demonstrates the enhancement of exercise on the biological action of inhaled irritants.

With the exception of the histopathology results from the combined O_3 and NO_2 exposures during exercise, the concentrations of irritants that were used in this study are higher than those likely to be encountered by people in normal daily activities. In resting animals, permeability changes were not demonstrated with single acute exposures below concentrations at which other kinds of health effects were demonstrated. Bronchoconstriction with 10 ppm of formaldehyde previously was demonstrated in both lab animals and humans; studies in animals and humans on the mechanics of breathing demonstrated effects with levels of ozone at or below the biologically-active levels demonstrated in this study. Enhancement of bacterial infectivity also was demonstrated with nitrogen dioxide, as well as ozone, below the levels studied here.

Attainment of Study Objectives

The research findings make a contribution toward HEI's broad goal of defining the factors that influence the dose of pollutants to target tissues. It only partially meets the investigators' stated objectives, however. Specifically, no dose-response data or respiratory tract regional comparisons for formaldehyde are reported in the final report. Furthermore, the experiments on combination exposures to O_3 and NO_2, although very interesting, are not definitive enough to conclude that there is a synergistic effect.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Three areas of the study's results are broadly important and could stimulate considerable additional scientific effort:

- observation of an apparent correlation between the increased surface area of the vesicles and the increased permeability to marker molecules;
- evidence suggesting that there is a synergistic effect of O_3 and NO_2 on permeability; and
- apparent enhanced effects on permeability due to exercise.

Additional studies to confirm and extend these observations would be worthwhile. Furthermore, the issues of development of tolerance to the permeability enhancement, and of possible increased effects with repeated exposures were not tested in this study. The authors raise the issue of prostaglandins and their possible effects on hydrostatic

pressure, etc. Their discussion primarily is speculation, and however, suggests the need for further research on this question.

Specific research recommendations are summarized below.

1. Further studies of the possible interactive effects of O_3 and NO_2 on permeability and reversible morphologic changes are needed. The design should include tests over a range of concentrations and exposure durations that would delineate the conditions for synergism if it occurs. More dose-response data is needed on individual constituents as well as in combinations. In order to confirm that synergism occurs, it is necessary to know that the response to a mixture occurs at a combined molar dose, which is below the dose of a single agent that produces the same response.

2. More focused studies on the mechanism of permeability increases should include morphology/function correlation studies over a range of dose and time variables. The present report suggests that conditions that result in altered permeability also result in altered morphology of lung epithelium. However, this conclusion largely is based on the effects of both types observed in experiments done at a single time and concentration. To confirm the possible correlations and the order of cellular injury, one needs to have data over time and at different concentrations. For example, if morphologic changes in cells occurred earlier and/or at lower concentrations than permeability changes, one would have reasonable (though still not definitive) grounds for concluding that the permeability changes were the result of altered morphology. Such information would help in the selection of the most sensitive measurement for monitoring further experimental or clinical studies of interactions, etc.

3. It would be of interest to conduct studies to see whether lung-to-blood transport and effects of other pollutants are enhanced under conditions that enhanced the DTPA and BSA transfer observed in this study. Thus far, the significance of the reported altered permeability rests on the speculation that inhaled antigens, carcinogens, or other gases and vapors would be enabled to more readily reach the target sites. Some experimental work to confirm this hypothesis would be worthwhile.

IMPLICATIONS FOR PUBLIC POLICY AND RISK ASSESSMENT

As noted above, apparently harmful effects of the pollutants used in this study have been observed with other methods and at lower concentrations. In addition, it is critically important to combine the consideration of function with structural change if we are to effectively evaluate the effects of air pollutants. Nevertheless, the research findings reported in this project do have potential implications for risk assessment. If scientific agreement can be reached that synergism occurs between ozone and nitrogen dioxide, then the potential of

interaction between these two gases would have to be taken into consideration when assessing risks and setting air quality standards.

Because of the relatively high concentrations used in this study, it is important to determine the significance of the measured increases in pulmonary epithelial permeability. For example, do these changes occur at or near levels typically encountered in the urban atmosphere? The biological significance of the increased permeability effect also should be considered. The health significance of increased permeability has not been well established, but could include: a) enhanced sensitization to macromolecular antigens inhaled simultaneously with airborne irritants; b) enhanced retention of carcinogenic or other toxic macromolecules in the lung, which might initiate localized injury; or c) enhanced penetration of macromolecules into the blood stream, which might result in systemic effects.

A potential increased risk of lung injury in exercising individuals is suggested by this study. With the emphasis on exercise in current health promotion activities, exposure to ambient air pollutants, which are irritants, is likely to be experienced by many exercising individuals. People who exercise regularly outdoors may therefore be at greater risk of lung injury. If this is indeed the case, then regulators ought to consider this population subgroup when setting air pollution standards. In order to answer this question, the findings of this study should be considered in conjunction with other studies in humans and animals exposed to oxidants.

Notes

1. 42 U.S.C. Section 7401-7642 (1982).

2. Section 202 of the Act, 42 U.S.C. Section 7521 (1982).

3. Section 112(a)(1) of the Act, 42 U.S.C. Section 7412(a)(1)(1982).

4. Section 211 of the Act, 42 U.S.C. Section 7545 (1982).

5. Section 303 of the Act, 42 U.S.C. Section 7603 (1982).

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Glossary

Edema	An accumulation of excess fluid in cells, tissues or spaces.
Fibrotic	Inappropriate connective tissue.
Lumen	The space inside a tubular structure
Mucosal	Epithelial, mucous membrane.
Pharmacologic	Relating to the use of drugs.
Reactivity	Response of muscle to a stimulus.
Resistance	A passive force exerted in opposition to another force — in this case, the flow of gas out of the lungs during expiration.
Serosal	Outermost layer.
Submucosal	Tissue layers below the mucosal layer.
Vesicle	Small circumscribed compartment.

Transport of Macromolecules and Particles at Target Sites For Deposition of Air Pollutants

by T. T. Crocker and D. K. Bhalla

ABSTRACT

This study analyzed rats' nasal, tracheal and bronchoalveolar epithelial permeability to macromolecules after they were exposed, in 2- or 4-hour periods of rest or exercise, to ozone (O_3)(0.6, 0.8 or 2 ppm), nitrogen dioxide (NO_2) (2.5, 6 or 12 ppm) or formaldehyde (10 ppm). Exercise was performed on a treadmill operated at a speed that led to a 2-fold increase in oxygen consumption. Histopathologic and electron microscopic cytochemical and autoradiographic studies were performed to identify the structural aspects of mucosal response.

In rats not exposed to pollutants, the quantity of macromolecular tracers (^{99m}Tc-DTPA, ^{125}I-BSA) in blood sampled 6, 7, 8, 9 and 10 minutes after a slow 5-minute instillation of comparable quantities of tracer molecules in the lumen of each zone, was lowest in nasal, highest in tracheal, and intermediate in the bronchoalveolar region. Exposure of resting rats to O_3 did not affect nasal permeability, but tracheal and bronchoalveolar permeabilities increased by 2-fold 1 hour after the exposure. In rats exposed at rest to O_3, tracheal permeability was no longer elevated 24 hours after exposure, but bronchoalveolar permeability remained elevated at 24 hours after exposure and was normal at 48 hours. Exposure during exercise increased the effect of O_3 in the trachea and in the bronchoalveolar zone. However, exercise also prolonged the duration of the O_3 effect on the tracheal zone from 1 hour to 24 hours and, in the bronchoalveolar zone, from 24 hours to 48 hours.

Histologically, focal inflammatory lesions in the alveolar zone were maximal at 48 hours after a 4-hour resting exposure to O_3. After exposure during exercise, the area of lung involved by lesions increased 4- to 7-fold above the lesion-bearing area in rats exposed while resting. By electron microscopy, horseradish peroxidase (HRP) was localized in epithelial intercellular spaces, but not in the apical tight junctions, of tracheal epithelial cells from O_3-exposed rats; no HRP was found in intercellular spaces in controls. The number of HRP-containing endocytic vesicles in tracheal epithelial cells was 2-fold greater in rats exposed to O_3 than in control rats. This 2-fold increase in vesicles presumed to be transporting HRP matches the 2-fold increase in transfer of DTPA from the tracheal lumen to the blood. Electron microscopic autoradiography revealed ^{125}I-BSA accumulation in subepithelial connective tissue, and electron microscopic cytochemistry identified accumulation of HRP not only between cells but also at the basal

lamina. These accumulations suggest that exposure led to an overload of HRP and BSA at these sites, or to impaired removal of tracers from connective tissue into blood.

Formaldehyde (HCHO, 10 ppm) exposure increased nasal mucosal permeability immediately after a single 4-hour exposure, but permeability was normal 24 hours and 48 hours later. Autoradiographs of ^{3}H-thymidine-labeled nasal epithelia showed increased cell turnover following the above exposure, but not after a 4-hour exposure to 0.8 ppm O_3. Tracheal epithelial cell turnover was not increased by one exposure to either gas. Three daily 4-hour exposures to 10 ppm of HCHO increased nasal epithelial cell turnover, reduced the total number of nasal epithelial cells, and induced inflammatory changes in nasal mucosa. When 0.8 ppm O_3 was administered in combination with 10 ppm HCHO, effects on nasal epithelium were similar to the effects of HCHO alone. Ozone alone did not have any effect on nasal epithelial cell turnover after 3 daily 4-hour exposures.

Permeability in the tracheal and bronchoalveolar zones of rats exposed at rest or exercise to 6 ppm or 12 ppm NO_2 did not differ from controls. In rats exposed to 12 ppm NO_2 for 2 hours during exercise, there was a significant increase in tracheal and bronchoalveolar permeability to DTPA and BSA at 1 hour after exposure. Exposure at rest to 0.6 ppm O_3 plus 2.5 ppm NO_2 produced a significant increase in bronchoalveolar permeability immediately after exposure. This exposure had no effect on nasal permeability.

Histologically, focal lesions involved a small proportion of the alveolar zone of the lung 48 hours after a 4-hour exposure at rest to 0.35 ppm O_3. Exposure of resting rats to 6 ppm and 12 ppm of NO_2 produced no focal lesions. In rats exposed during exercise to 0.35 ppm O_3 plus 0.6 ppm NO_2, 2- to 3-fold more focal lesions occurred than were observed in exercising rats exposed to 0.35 ppm O_3 alone.

INTRODUCTION

Airway epithelial functions have been studied in recent years using morphologic characterization of the macromolecular transport system, through isotope tracer techniques for evaluating mucosal permeability characteristics and using measurements of transmucosal ionic flows. Under normal conditions, airway epithelium serves as a barrier by restricting the transfer of exogenous antigens, macromolecules, and particles from the airway lumen to the blood. Structural integrity and organization of the sealing fibrils of tight junctions have been recognized as one major basis for this barrier function (1, 2, 3). Interruption of this barrier leads to the "paracellular" permeation of electrolytes and macromolecules.

In addition, topically-applied macromolecules or particles may be taken up by endocytic vesicles of the epithelial cells and transported across the mucosa (3, 4). This is presumed to be a normal process subject to modulation by homeostatic requirements or pathologic events, and the mode of permeation could be regarded as paracellular or transcellular, as in vesicular transport by capillary endothelial cells.

Increased airway-to-blood transfer of macromolecules may occur following epithelial perturbations that result from mechanical insult (5); antigenic challenge (6); chemical modulation (7, 8); or inhalation exposure to pollutants such as cigarette smoke (9, 10), nitrogen dioxide (4) and ozone (11). Localization of horseradish peroxidase (HRP) in intercellular spaces was interpreted as an indication of tight junction impairment following insult.

Most studies of transport of molecules across respiratory mucosa in animals have dealt with trachea only, although studies in man, using inhaled ^{99m}Tc-diethylenetriaminepentaacetate (DTPA) aerosols, have involved the whole lung. Permeability changes in the airway mucosa after exposure to noxious agents, such as cigarette smoke or NO_2, have been observed both in laboratory animals and humans. By instilling HRP into the airway of guinea pigs and measuring HRP appearance in blood, Boucher *et al.* (9) found that increased permeability resulting from cigarette smoke was detectable after 5, 20 and 100 puffs of whole cigarette smoke, but morphologically, HRP was seen in intercellular spaces only after 100 puffs. Hulbert *et al.* (10) found that acute exposure of guinea pigs to 100 puffs of cigarette smoke resulted in an increase in airway permeability to HRP, with maximal increase 30 minutes after the exposure. A similar increase in airway epithelial permeability was observed by Ranga *et al.* (4) after exposure of guinea pigs to 5 or 15 ppm NO_2.

Jones *et al.* (12) used a ^{99m}Tc-DTPA aerosol to demonstrate a greater lung-to-blood transfer of DTPA in symptomless cigarette smokers than in nonsmokers. Minty *et al.* (13) and Mason *et al.* (14) found a similar increase in the clearance of aerosolized DTPA from the alveolar region, with a decreased clearance after discontinuation of smoking.

The increased permeability may result in exposure of irritant receptors to inhaled agents in guinea pigs as suggested by Boucher *et al.* (9) and in monkeys as suggested by Hogg *et al.* (6). However, reports on the relation between permeability and hyperreactivity are not in complete agreement. Kennedy *et al.* (15) reported increased permeability without increased hyperreactivity in cigarette smokers, but the study conflicts with earlier suggestions linking hyperreactivity in cigarette smokers with increased permeability (16).

Although direct instillation procedures are used to detect permeability changes across trachea and major airways (7), and radioaerosol procedures allow evaluation of smaller airways and alveoli (13, 17, 18), information on molecular transport across specifically isolated airway regions remains scanty. The present study investigates the effects of acute O_3, NO_2 or HCHO exposures, alone or combined, on permeability changes in localized regions of the respiratory system.

AIMS

Hypotheses To Be Tested

1. Epithelia of the nasal, tracheal and bronchoalveolar zones of the respiratory tract are target sites for deposition of air pollutants.
2. The effects of acute low-dose exposure to ozone (O_3), formaldehyde (HCHO) or nitrogen dioxide (NO_2), singly or combined, in rats exposed during rest or exercise will include changes in permeability of mucosal surfaces in these target sites. Increased permeability is expected and will be indicated by more rapid transport of non-ionic tracer molecules from the lumen of each site to the blood. Increased permeability of respiratory epithelia represents the loss, or reduction, of a normal barrier function of epithelia, with possible increased susceptibility of each target zone to penetration of foreign molecules and particles.

Specific Aims

1. Use three non-ionic molecular or macromolecular probes of different molecular weights.
2. Use different isotopes or cytochemical methods to trace each molecule as a means to study permeability changes in the nasal, tracheal and bronchoalveolar zones.
3. Examine permeability at 1, 24 and 48 hours after one or several exposures to O_3, NO_2 or HCHO, singly or combined. Measure transport of radiolabeled DTPA and ^{125}I-bovine serum albumin (BSA) from lumen-to-blood in each target site after exposure to gases. Measure blood-to-lumen transport of tracers by lung lavage after exposure.
4. Analyze, by electron microscopy, the route of molecular transfer and the role of fine structural components of the epithelial cells, such as the tight junctions, endocytic vesicles and degenerating cells, in the transport of tracers from the lumen to the blood.
5. Correlate histologic findings in each of the three zones of the respiratory tract with permeability, using tritiated thymidine incorporation into DNA of nasal and tracheal epithelial cells as a measure of cell-killing and -replacement.

METHODS

Animals

Male Sprague Dawley rats (Hilltop Laboratory, Scottdale, PA) that weighed 180 to 220 g and were about 49 to 53 days of age were delivered in filtered containers, maintained on a standard diet, and held in a laminar air barrier caging system for at least one week prior to experimentation. Ten percent of each batch was autopsied upon their arrival to assure their freedom from lung disease.

Gas Exposure of Resting Rats

Groups of 3 rats were exposed at rest to 0.8 ppm O_3 or 10 ppm HCHO, using a staggered schedule throughout a day to allow 1 of the 3 animals to be tested within 1 hour following completion of a 2-hour exposure. At 24 and 48 hours after exposure, the second and third animals were used to determine the duration of the effect of O_3 or HCHO on the permeability of respiratory epithelia. Control rats were sham-exposed to clean air in comparable chambers on the same schedule, so that one control and one exposed rat would be examined within 1 hour, and at 24 and 48 hours, following a 2-hour exposure. Three groups of 3 control and 3 O_3-exposed rats could be studied on a single day, providing only 3 control and 3 exposed rats for observation at 1, 24 or 48 hours for each zone of the respiratory tract. Each exposure experiment was performed at least twice, and the data were pooled for analysis. Exposure was conducted in rectangular stainless steel chambers (19) with rats held on a single level in individual wire cages.

Each of the exposure chambers was supplied with purified air from a high pressure air purification system. Outside air was first filtered and then compressed to about 100 psig, using a liquid ring compressor (Nash Engineering Company, Norwalk, CT). The high pressure air subsequently passed through a fixed Purafil ($KMnO_4$ on Alumina; HE Burroughs, Inc.) bed in order to remove gaseous contaminants such as O_3, SO_2, NO_2, and some hydrocarbons. Carbon monoxide was removed as it passed through a Hopcalite catalyst; a heatless dryer located just upstream of the catalyst bed prevented catalyst inactivation by moisture. Following filtration and thermal equilibration to laboratory temperature, the high-pressure purified air was throttled down to ambient pressure and humidified to 40 $\pm$ 2% RH. Delivery of purified air to the control chamber followed a final HEPA filtration. In the O_3 exposure chamber, following the HEPA filtration, O_3 was injected into the airstream.

Ozone was generated by passing medical-grade oxygen through an electrical ozone generator (Sander Ozonizer, Type III, Osterberg, West Germany) for addition to purified air. The O_3 concentration in the rat breathing zone was monitored continuously through Teflon sampling lines using a calibrated ultraviolet monitor (Dasibi Environmental Corporation, Glendale, CA). Ozone concentrations for this study were

0.8 and 2.0 ppm, with relative standard deviations of less than 5% in each case. In the control chamber the average ozone concentration was about 0.01 ppm.

Formaldehyde (HCHO) vapors were generated by passing dry, purified nitrogen through purified grade paraformaldehyde. Monomeric HCHO vapors were introduced into the chamber inlet stream. HCHO concentration was characterized using a CEA 555 monitor and chromotropic acid analysis. Rats were exposed to either 10 ppm HCHO or to clean air in stainless steel exposure chambers for 4 hours. Exposures were not staggered, as in the case of O_3, but all the animals exposed to clean air or HCHO were examined within 4 hours after completion of the exposures.

O_3 and NO_2 Exposure of Exercising Rats

Rats were trained on a 10-animal treadmill (Quinton Model 42-15, Quinton Instruments, Seattle, WA) for two days prior to the experimental exposure. They ran at 15 m/minute at 20% grade for 1 hour on the first day and for 2 hours on the second day. All rats underwent identical training and were then randomly assigned to exposure during rest or exercise. Rats exposed at rest were held in restraining plastic tubes and placed in a modified Quinton exposure treadmill in order to maintain the exposure conditions as close as possible to those for exercising rats. The exposure treadmill (20) was used to determine the mean metabolic rate of 10 rats at rest or at various running speeds and grades; the exposure regimen produced a 1.3- to 2-fold increase in oxygen consumption in exercising rats as compared to resting rats. O_3 concentrations were measured every 4 minutes in each of the 10 channels during a 2-hour exposure.

NO_2 was obtained from a cylinder of research-quality gas (1% NO_2 in zero air) and monitored continuously at the breathing zone of the animals through teflon tubing using a nitrogen oxides analyser (Monitor Labs, model 8840).

Permeability from Lumen to Blood

Animals were anesthetized intraperitoneally with sodium pentobarbital (Nembutal sodium, Abbott Laboratories, North Chicago, IL), using 5 mg per 100 g body weight. A polyethylene tube (PE-90 was placed in the trachea and a polyethylene catheter (PE-10, Clay Adams, Passippany, NJ) was placed in the femoral artery. The radiolabeled tracer inoculum contained ^{99m}Tc-diethylenetriaminepentaactetate (^{99m}Tc-DTPA, mol wt 492) and ^{125}I-bovine serum albumin (^{125}I-BSA, mol wt 69,000). For permeability testing in the nose, we performed a tracheostomy and filled the oropharynx with dental impression cream to block the posterior nares and prevent passage of label into esophagus or larynx. We instilled either 0.1 ml in each nostril or a volume of tracer solution sufficient to fill the nasal cavities, and recorded the volume for comparison with transport of the label to blood.

For tracheal instillations, 0.1 ml of the inoculum was delivered into the trachea using a PE-10 tube attached to a 1-ml syringe; inoculum was introduced through the tracheostomy tube while the rat was held supine in a nearly horizontal position. The tracer solution was delivered into the middle zone of the trachea about 0.5 cm beyond the tracheostomy tube over a 5-minute period.

For bronchoalveolar instillation, rats were held in a semi-vertical position. Inocula were delivered directly to a main stem bronchus using a longer PE-10 tube inserted through the tracheostomy tube. Heparin solution (20 units in 0.2 ml saline) was injected into rats through carotid or femoral artery catheters prior to blood sampling. Blood samples of 0.15 ml were drawn 6, 7, 8, 9 and 10 minutes after the start of instillation. In one experiment, blood samples were also drawn during the instillation period, i.e., at 1, 2, 3, 4 and 5 minutes after the start of instillation. No attempt was made to replace blood with plasma expanders. Each blood sample (0.15 ml) was added to 0.15 ml of the above heparin solution in glass microtubes for immediate counting of ^{99m}Tc in a NaI (Canberra Industries, Meriden, CT, Series 8100). Counting samples were held for 1 week and counted for ^{125}I radioactivity after ^{99m}Tc radioactivity had decayed.

Data Analysis

Isotope counts in 0.15 ml blood samples at the 6-, 7-, 8-, 9- and 10-minute time points for the two molecules were expressed as the percent of the inoculum transferred from the site of application to the entire blood volume of each rat. Blood volume was calculated as 6.4% of the body weight (21).

The label in blood samples was analyzed by obtaining the regression curve extrapolated to the intercept at the mid time point, T_i, for the 5 samples. Differences in the fractions of each labeled molecule transferred to the blood in both the control and the O_3-exposed groups were analyzed by analysis of variance, using a significance level of 0.05.

Permeability from Blood to Lumen

To determine whether radiolabeled tracers were transferred from blood into the lumen of the airways in a fashion comparable to the transfer from the bronchoalveolar lumen to blood, 0.1 ml of the labeled tracer solution was inoculated over 5 seconds into the femoral vein and a blood sample taken at 5 minutes. The animals were then exsanguinated, before their lungs were lavaged with 8 ml of phosphate buffered saline, at 6 minutes after inoculation. In these studies, rats were exposed to 0.8 or 2 ppm O_3 for 2 hours and the lungs were lavaged 1, 24 or 48 hours after the exposure. The total recovered label in lavage fluid was expressed either as the fraction of all label present in the entire blood volume of the rat at 5 minutes after injection, or as the fraction of the radiolabel inoculated.

Electron Microscopy

For cytochemistry, 0.5 mg of unlabeled horseradish peroxidase (HRP, mol. wt. 40,000 d) was added to 0.1 ml inoculum that contained the radiolabeled tracer molecules described above. Following instillation, portions of trachea and bronchi distal to the site of instillation were excised and fixed by immersion in 2.5% glutaraldehyde and 0.8% formaldehyde in 0.1 M phosphate buffer. HRP was detected by treatment with diaminobenzidine (DAB), and tissues were processed for electron microscopy.

For autoradiographic detection of ^{125}I-BSA, thin sections collected on cleaned carbon-coated grids were coated with Ilford L-4 emulsion and stored in the dark at 4°C. Sections were developed after 2 to 3 months, stained and observed in a Jeol JEM 100C electron microscope.

Histopathology

Tissue Preparation

Rats were killed 18 to 48 hours after exposure by exsanguination after deep anesthesia with sodium pentobarbital I.P. The thoracic cavity was opened after puncturing the diaphragm and the lungs and trachea carefully exposed. The trachea was transected 5 mm above the bifurcation and the distal portion cannulated. Lung surfaces were examined for abnormalities before and after removal from the thoracic cavity. The cannulated trachea with attached lungs and thoracic viscera were fixed for 3 days by airway perfusion with buffered 10% formalin at 30 cm fluid pressure for 72 hours (22). The remaining trachea with attached larynx was fixed in buffered 10% formalin.

The rat was decapitated, and the integument, cartilaginous snout, and lower jaw were removed. That portion of the head containing the intact nasal cavity was immersed for 3 days in buffered formalin under slight vacuum to remove trapped air and ensure that all surfaces were in contact with fixative.

Lung tissues embedded in paraffin were sectioned at 6 microns; tissues embedded in glycolmethacrylate were sectioned at 2 microns. A complete paraffin section of the left lung was taken close to, and parallel with, the midline of the main bronchus. The section was stained with hematoxylin and eosin and used for the overall microscopic survey and histologic analysis of the portion of the alveolar zone of the section that bears focal lesions characteristic of O_3 or NO_2 toxicity in rodent lungs (23). The right cranial and caudal lung lobes were cut in a saggital plane. One 1 cm x 1 cm x 0.5 cm block was cut at random from a saggital slab from each lobe. The blocks were embedded in glycol methacrylate, sectioned at 2 microns, and stained with hematoxylin and eosin for the cascade level I morphometric method of Elias and Hyde (24).

The trachea was split longitudinally, embedded in glycol methacrylate, sectioned at 2 microns and stained with toluidine blue. Fixed, dissected rat heads were decalcified for 3 weeks in TRIS buffered EDTA. Specimens of nasal cavity were prepared by cutting a 2-3 mm slice, freehand, in the dorso-ventral plane through the hard palate, using the incisive papillae as a reference point. These slices were perpendicular to both the plane of the hard palate and of the nasal septum. The slices were embedded, anterior face down, in glycol methacrylate, sectioned at 2 microns and stained with toluidine blue.

Histologic Analysis

The area of alveolar ducts and alveoli in a 6-micron paraffin section of the left lung was first scanned systematically with a one-hundred square ocular grid calibrated with a stage micrometer using a dissecting microscope at 12.5x. This procedure selected and recorded the number of grid spaces of the section occupied by terminal bronchioles, alveolar ducts and alveoli; bronchi and major vessels were excluded. A second scan was then performed with a similar grid in the compound microscope at 100x. Any grid space (10^4 microns^2) in the alveolar zone that contained a lesion was scored. The magnificiation factor for the two grid counting systems is 1:8. The area of the alveolar zone of the section, as measured in the first scan, was multiplied by 8 to provide the denominator in the same grid units as were used in the second scan. The number of the alveolar zone grids that bore lesions in the second scan was expressed as the percent of the total number of grid areas representing the entire alveolar zone in a section of the lung lobe.

Since the severity of lesions varies, a system was established to consider these variations. The lesion types were defined as:

Type 1: cells of any type were free in alveolar spaces within a grid area of 10^4 microns^2. This histologic state occurred in 1 to 3% of the grid spaces of the alveolar zone of normal rat lungs, but occurred with increased frequency in exposed lungs.

Type 2: increase in cellularity of alveolar duct walls and septae, with thickening of these structures whether or not there were free cells in air spaces. Type 2 lesions were never present in normal rat lungs. However, Type 2 lesions increased in proportion to O_3 concentration in exposed rats.

The percent area of a lung section bearing focal lesions was expressed either as the total of both Type 1 and 2 lesions observed or as the percent of the area bearing Type 2 lesions only.

Autoradiography

Rats were injected intraperitoneally with 1 μCi/g body weight of ^{3}H-methylthymidine (^{3}H-T, 50 Ci/mM, Schwartz/Mann, Spring Valley, NY) one hour before killing. After exposure, animals were killed at intervals to coincide with maximum cell death, with the time of appearance of inflammatory tissue effects of inhaled materials, and/or with the time when the maximum rate of DNA synthesis was expected to occur.

Slides with sections of nasal or tracheal epithelia were dipped in Kodak NTB2 emulsion, air-dried, and stored in light-tight boxes at refrigerator temperature for 30 days. After photographic development, slides were stained lightly with toluidine blue. The percentage of labeled cells per total epithelial cells was determined by cell counts of complete sections for each animal. A 50-micron separation between serial sections eliminated the possibility of re-counting portions of the same cell population.

Statistical Analyses of Lung Lesions

Unless otherwise stated above for morphometric data, statistical analysis to determine significant differences between animals within an experimental group, and between experimental groups, consisted of a two-tailed "t" test or analysis of variance, including multiple comparison methods.

RESULTS

Trachea-to-Blood Transfer

Independent Transfer of Molecules

Presence of more than one molecule in the inoculum raises the possibility of molecular interactions, which in turn may influence the independent transepithelial transfer of molecules. To determine the effect of the presence of one molecule on the transfer of another, 8 normal, unexposed rats received DTPA alone or DTPA plus BSA. Blood samples were collected as described, and the fraction of the instilled DTPA that was transferred from the tracheal lumen to the blood was calculated for each rat receiving DTPA alone and for each rat receiving DTPA in combination with BSA. No significant difference ($p < 0.05$) in the DTPA transfer from the trachea to the blood was observed in the two groups. This observation does not test whether DTPA might interfere with transfer of BSA; it was taken as a working hypothesis that the larger molecule (BSA) would be more likely to interfere with transfer of the smaller one (DTPA).

Table 1. Tracheal Mucosal Permeability to DTPA (a) at 0.5 Hour, 1.5 Hour, 2.0 Hour, 3.0 Hour and 4.5 Hour After a 2-Hour Exposure to 0.8 ppm Ozone

Labeled Molecule	Mol. Wt., [d]	Percent of Inoculum Present in Entire Blood Volume at Time T_i After Tracheal Instillation of Labeled Molecules					
		Clean Air	Ozone (Hours)				
			0.5	1.5	2.0	3.0	4.5
DTPA (a)	492	1.54 ±0.12 (b)	4.84 (c) ±1.93	2.48 (c) ±0.01	2.96 (c) ±0.22	2.77 (c) ±0.97	3.57 (c) ±0.97
Sample size [n]		4	4	2	2	4	4

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate

(b) Mean ± 1 standard deviation

(c) $p < 0.05$

Rats exposed to O_3 at rest (Wire cages, stainless steel chamber)

To determine the length of time that alterations in permeability produced by exposure to O_3 could be regarded as constant, DTPA was instilled at 0.5, 1.5, 2.0, 3.0 and 4.5 hours after a 2-hour exposure. Table 1 shows that 1.54% of the inoculum was present in the blood at T_i in control rats studied at one time; repeated studies were not done in controls exposed to clean air only. Fractions of 4.84% to 2.48% of inoculum were found at T_i, in rats studied at the above time intervals after the end of a 2-hour exposure to 0.8 ppm O_3. All of the values in O_3-exposed rats were higher than in the single group of sham-exposed controls, but none of the values in O_3-exposed rats were significantly different from one another. There was variability in the amount of transport scored by this method, but a statistically significant increase in permeability persisted for at least a 4.5-hour period following exposure.

In control rats exposed to clean air, the fraction of DTPA transferred from the tracheal lumen to the blood was greater than the fraction of the instilled dose of BSA (Table 2). One hour after a 2-hour exposure to O_3, both DTPA and BSA fractions appearing in blood were higher than in control rats. The difference between the two groups was significant and was maintained at all blood-sampling time points (Figure 1).

Table 2. Tracheal Mucosal Permeability to DTPA and BSA at 1, 24 or 48 Hours After a 2-Hour Exposure to 0.8 ppm Ozone

Labeled Molecule	Mol. Wt., [d]	Percent of Inoculum Present in Entire Blood Volume at Time T_i After Tracheal Instillation of Labeled Molecules			
		Clean Air	Ozone (Hours)		
			1	24	48
DTPA (a)	492	1.50 ±0.43 (b)	3.07 (c) ±0.58	1.85 ±0.42	1.29 ±0.28
BSA (d)	69,000	0.51 ±0.09	0.65 (c) ±0.14	0.49 ±0.11	0.54 ±0.13
Sample size [n]		15	6	6	6

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

The rapidly rising concentration of labeled molecules in the blood (Figure 1) reflected not only the progressive deposition of the label but also the rate of transfer of tracer from the tracheal lumen to the blood. Concurrent removal of label from blood caused the curve expressing blood concentrations of the labeled compounds to approach a plateau after 10 minutes from the start of instillation. Rates of transfer are not presented for purposes of this report because rates were not as useful in statistical analyses as the fraction of inoculum that had been transferred to blood at the intercept, T_i. (See Methods: Data Analysis).

Table 2 compares tracheal mucosal permeability to both DTPA and BSA, demonstrating that the proportion of inoculum present in the blood at time T_i was highest in animals examined 1 hour after a 2-hour exposure to ozone and was not significantly elevated at 24 and 48 hours after exposure.

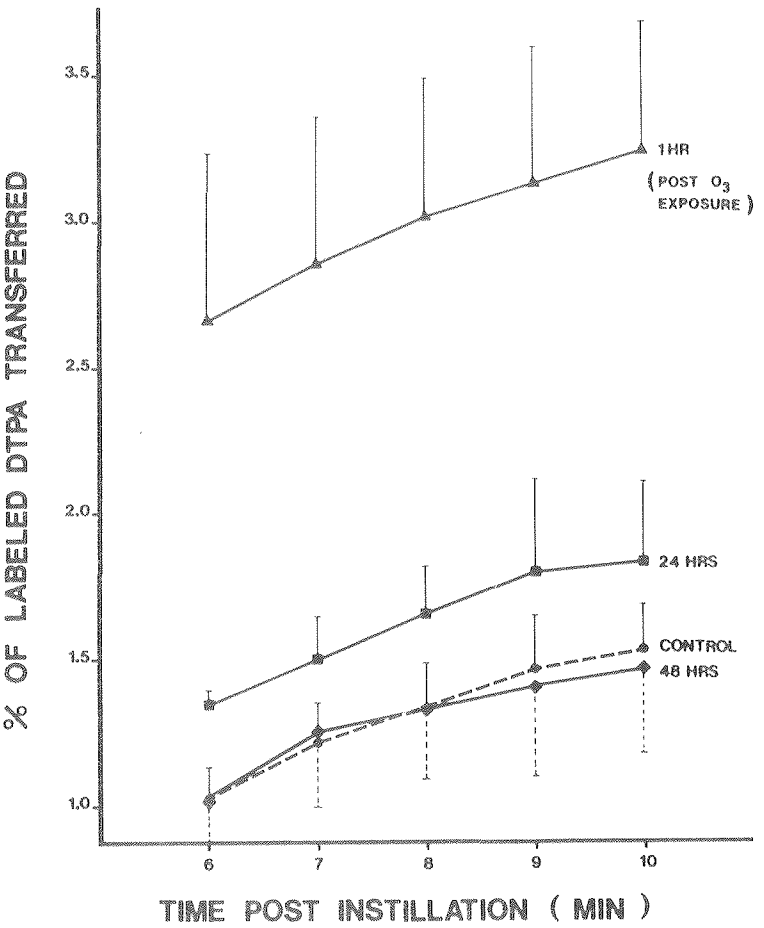

Figure 1. ^{99m}Tc-DTPA in 0.1 ml saline was instilled into the tracheas of anesthetized rats over a 5-minute period. Labeled tracer was measured in blood samples collected at 6,7,8,9 and 10 minutes after the start of tracer instillation, and the data were expressed as the percent of inoculum in blood at these time points. Percent transfer at all these time points was greater in tracheas from rats exposed to 0.8 ppm O_3 for 2 hours than in sham-exposed controls. x 4,200

Rats exposed to O_3 while at rest or exercise (Treadmill exposure system)

Exercise during sham-exposure to clean air did not alter tracheal mucosal permeability to DTPA or BSA. In rats exposed to O_3 while at rest, permeability at 1 hour was greater than in control rats but permeability was normal 24 hours after exposure. In rats exposed to O_3 during exercise, the transport of both DTPA and BSA was greater at 1 hour than in rats exposed to O_3 while at rest or to clean air while at rest or during exercise (Tables 2, 3). The increase in permeability persisted at 24 hours after exposure to O_3 during exercise.

Table 3. Tracheal Mucosal Permeability to DTPA and BSA at 1, 24 and 48 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 0.8 ppm O_3

Labeled Molecule	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Tracheal Instillation of Labeled Molecules									
	Clean Air Rest	Clean Air Exercise (Hours)			O_3 Rest (Hours)			O_3 Exercise (Hours)		
		1	24	48	1	24	48	1	24	48
DTPA (a)	1.35	0.95	0.96	1.02	3.31 (c)	1.91	1.36	4.25 (c)	4.05 (c)	1.54
	±0.45 (b)	±0.23	±0.13	±0.20	±2.10	±0.80	±0.56	±1.73	±2.38	±0.82
BSA (d)	0.70	0.95	0.54	0.48	1.08	0.10	0.37	1.80 (c)	1.50 (c)	0.49
	±0.30	±0.60	±0.20	±0.12	±0.73	±0.64	±0.21	±1.11	±1.31	±0.16
Sample size [n]	16	6	6	3	6	6	2	7	7	3

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Rats exposed to NO_2 while at rest or exercise

Exposure of rats trained to run, but exposed at rest while restrained in cylinders located in the treadmill, may be different from exposure of rats while at rest in wire cages in the Rochester-type chamber. The use of restraint in cylinders in the treadmill was undertaken to assure that the breathing zone of each resting rat was located at the same point of entry of O_3 to the treadmill as the breathing zone of rats exposed while running.

Tracheal permeability in rats exposed to 12 ppm NO_2 while at rest, or during exercise in the treadmill, has been studied twice. In rats exposed at rest to NO_2, the fractions of DTP and BSA in blood were not consistently increased either immediately after or 24 hours after the exposure (Table 4). In rats exposed during exercise, there was a significant increase in transfer of DTPA and BSA immediately after the exposure, but only DTPA transfer was significantly elevated 24 hours later.

Table 4. Tracheal Permeability to DTPA and BSA at 1 and 24 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 12 ppm NO_2

Labeled Molecule	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Tracheal Instillation of Labeled Molecules				
	Clean Air Exercise	12 ppm NO_2 Rest (Hours)		12 ppm NO_2 Exercise (Hours)	
		1	24	1	24
DTPA (a)	1.25 ±0.57 (b)	1.20 ±0.18	2.09 (c) ±0.80	2.53 (c) ±0.72	2.56 (c) ±0.89
BSA (d)	0.45 ±0.05	0.64 ±0.07	0.59 ±0.30	0.88 (c) ±0.35	0.50 ±0.17
Sample size [n]	6	3	3	3	4

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Transfer of Tracers from the Bronchoalveolar Zone to the Blood

Rats exposed to O_3 while at rest

As in the trachea-to-blood study, O_3 exposure increased the transfer of DTPA and BSA to blood, and the percent transfer was greater for DTPA than for BSA (Table 5). Unlike the trachea-to-blood study, the permeability induced by O_3 was greater 24 hours after exposure than at 1 hour after O_3 exposure.

Rats exposed to O_3 during exercise

As in the case of trachea-to-blood transfer of molecules, exercise alone did not produce any consistent, significant change in permeability from the bronchoalveolar zone to the blood, but exercise during exposure to O_3 resulted in increased transmural transfer of both DTPA and BSA (Table 6). Bronchoalveolar permeability in rats exposed during exercise to O_3 was greatest at 1 hour after exposure and remained high at 24 hours and 48 hours after the exposure, as compared to sham-exposed rats or rats exposed at rest to O_3.

Table 5. Broncho-Alveolar Mucosal Permeability to DTPA and BSA at 1, 24 or 48 Hours After a 2-Hour Exposure of Resting Rats to 0.8 ppm Ozone

Labeled Molecule	Mol. Wt., [d]	Percent of Inoculum Present in Entire Blood Volume at Time T_i After Broncho-Alveolar Instillation of Labeled Molecules			
		Clean Air	Ozone (Hours)		
			1	24	48
DTPA (a)	492	0.72 ±0.22 (b)	1.23 (c) ±0.33	1.51 (c) ±1.09	0.83 ±0.17
BSA (d)	69,000	0.44 ±0.10	0.56 ±0.15	0.80 (c) ±0.48	0.52 ±0.09
Sample size [n]		17	6	6	7

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Rats Exposed at Rest or Exercise to NO_2

Rats exposed while at rest or during exercise to 6 ppm or 12 ppm NO_2 did not differ from controls, but in rats exposed at exercise to 12 ppm, the bronchoalveolar zone showed significantly increased permeability to DTPA and BSA at 1 hour after a 2-hour exposure; the increased permeability was absent at 24 and 48 hours (Table 7).

Rats Exposed to O_3 + NO_2 While at Rest or Exercise

Bronchoalveolar permeability in rats exposed at rest to 0.6 ppm O_3 + 2.5 ppm NO_2 for 2 hours was significantly greater than in the sham-exposed controls immediately after the exposure, but there was no significant increase at 24 hours or 48 hours after the exposure (Table 8). Exposure of resting rats to 0.6 ppm O_3 alone did not produce a significant increase in permeability.

In rats exposed during exercise to 0.6 ppm O_3, there was a significant increase in permeability that persisted at 24 hours after the exposure. The increase in permeability was greatest immediately after exposure in the rats exposed during exercise to 0.6 ppm O_3 + 2.5 ppm NO_2. The increased permeability persisted at 24 hours after the exposure.

Table 6. Broncho-Alveolar Mucosal Permeability to DTPA and BSA at 1, 24 and 48 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 0.8 ppm O_3

Labeled Molecule	Clean Air Rest	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Broncho-Alveolar Instillation of Labeled Molecules								
		Clean Air Exercise (Hours)			O_3 Rest (Hours)			O_3 Exercise (Hours)		
		1	24	48	1	24	48	1	24	48
DTPA (a)	0.72	0.69	0.86	0.82	2.04 (c)	1.14 (c)	0.60	3.27 (c)	2.59 (c)	1.08
	±0.23 (b)	±0.11	±0.24	±0.15	±0.67	±0.31	±0.12	±0.78	±0.37	±0.28
BSA (d)	0.70	0.70	0.71	0.79	1.46 (c)	0.84	0.81	2.15 (c)	1.50 (c)	1.19 (c)
	±0.19	±0.14	±0.09	±0.14	±0.45	±0.15	±0.21	±0.62	±0.17	±0.20
Sample size [n]	16	6	6	6	7	8	5	7	6	5

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Table 7. Broncho-Alveolar Mucosal Permeability to DTPA and BSA at 1, 24, and 48 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 6 ppm and 12 ppm NO_2

Labeled Molecule	Clean Air Exercise	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Broncho-Alveolar Instillation of Labeled Molecules											
		6 ppm NO_2 Rest (Hours)			6 ppm NO_2 Exercise (Hours)			12 ppm NO_2 Rest (Hours)			12 ppm NO_2 Exercise (Hours)		
		1	24	48	1	24	48	1	24	48	1	24	48
DTPA (a)	0.64	0.51	0.67	0.53	0.66	0.75	0.52	0.82	0.88	—	1.58 (c)	0.82	0.48
	±0.28 (b)	±0.05	±0.07	±0.21	±0.29	±0.31	±0.15	±0.18	±0.40	—	±0.43	±0.41	±0.05
BSA (d)	0.43	0.46	0.51	0.46	0.43	0.53	0.41	0.37	0.41	—	0.73 (c)	0.46	0.45
	±0.08	±0.11	±0.09	±0.08	±0.11	±0.19	±0.05	±0.04	±0.03	—	±0.21	±0.11	±0.01
Sample size [n]	16	3	3	3	6	6	3	3	3		6	6	3

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Table 8. Broncho-Alveolar Mucosal Permeability to DTPA and BSA at 1, 24, and 48 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 0.6 ppm O_3 + 2.5 ppm NO_2, and 0.6 ppm O_3 Alone

Labeled Molecule	Clean Air	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Broncho-Alveolar Instillation of Labeled Molecules											
		0.6 ppm O_3 Rest (Hours)			0.6 ppm O_3 Exericise (Hours)			2.5 ppm NO_2 + 0.6 ppm O_3 Rest (Hours)			2.5 ppm NO_2 + 0.6 ppm O_3 Exercise (Hours)		
		1	24	48	1	24	48	1	24	48	1	24	48
DTPA (a)	0.90 ±0.47 (b)	1.41 ±0.18	1.03 ±0.10	0.77 ±0.07	2.02 (c) ±0.40	2.00 (c) ±0.49	— —	1.54 (c) ±0.56	1.28 ±0.23	0.91 ±0.39	2.82 (c) ±0.40	1.81 (c) ±0.54	— —
Sample size [n]	15	3	3	2	3	3		3	3	3	3	3	

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate

(b) Mean ±1 standard deviation

(c) $p < 0.05$

Blood-to-Lung Transfer

In control rats, transfer of the large molecule (BSA) to lavage fluid, expressed as a percent of the intravenous inoculum, was greater than that of the small molecule (DTPA) (Table 9). In contrast, the amount of DTPA in the lavage fluid exceeded that of BSA when expressed as a fraction of the isotope counts in the rats' whole blood volumes at 5 minutes after inoculation, i.e., immediately prior to lavage (Table 10). Exposure to O_3 increased the fractions of both DTPA and BSA transferred from blood to alveolar space in rats studied within 1 hour after exposure. In pilot experiments, the increased transfer was noticed as early as 1 minute after intravenous inoculation. At 6 or 10 minutes after intravenous inoculation, the fractions of tracers appearing in lavage fluid of O_3-exposed rats were still considerably higher than in control rats. Lavage samples taken 6 minutes after intravenous inoculation provided the greatest sensitivity in the detection of permeability changes occurring within 1-hour after a 2-hour exposure to 0.8 ppm O_3.

The definitive experiments were done with lavage of the lung at 6 minutes after intravenous inoculation of DTPA and BSA (Table 9). In rats exposed for 2 hours to 0.8 ppm O_3 while at rest in wire cages, DTPA but not BSA transfer increased significantly. Neither DTPA nor BSA transfer was elevated significantly at 24 or 48 hours after exposure to 0.8 ppm O_3. In contrast, after exposure to 2 ppm the permeability to both DTPA and BSA was increased significantly at 1, 24 and 48 hours after exposure.

Table 9. Percent of Each Molecular Species Transferred from Blood to Lavage Fluid 1, 24 and 48 Hours After a 2-Hour Exposure of Resting Rats to 0.8 or 2 ppm O_3

Molecule	Mol. Wt., [d]	Percent of Inoculum Transferred to 8 ml Lavage Fluid at 6 Minutes After I.V. Inoculation						
		Air	O_3 0.8 ppm (Hours)			O_3 2 ppm (Hours)		
			1	24	48	1	24	48
DTPA (a)	492	0.03 ±0.01 (b)	0.05 (c) ±0.02	0.04 ±0.02	0.03 ±0.01	0.12 (c) ±0.05	0.09 (c) ±0.02	0.05 (c) ±0.02
BSA (d)	69,000	0.06 ±0.04	0.07 ±0.02	0.07 0.02	0.05 0.02	0.13 (c) 0.05	0.14 (c) 0.11	0.10 (c) 0.05
Sample size [n]		15	10	8	7	10	8	8

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Table 10. Percent of Each Molecular Species Transferred from Blood to Lavage Fluid 1, 24 and 48 Hours After a 2-Hour Exposure of Resting Rats to 0.8 or 2 ppm O_3

Molecule	Mol. Wt., [d]	Percent of 5-Minute Blood Counts Transferred to 8 ml Lavage Fluid at 6 Minutes After I.V. Inoculation						
		Air	O_3 0.8 ppm (Hours)			O_3 2 ppm (Hours)		
			1	24	48	1	24	48
DTPA (a)	492	0.26 ±0.09 (b)	0.51 (c) ±0.27	0.22 ±0.09	0.18 ±0.05	0.97 (c) ±0.57	0.51 (c) ±0.03	0.19 ±0.08
BSA (d)	69,000	0.06 ±0.02	0.08 ±0.03	0.03 ±0.01	0.07 ±0.04	0.13 (c) ±0.05	0.07 ±0.03	0.07 ±0.05
Sample size [n]		8	8	4	3	8	4	4

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ± standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

When the transfer of labeled molecules from blood to lavage fluid was calculated as a percent of total ^{99m}Tc or ^{125}I radioactivity in the blood 5 minutes after intravenous injection of tracers, i.e., 1 minute prior to lavage (Table 10), the percent transfer was over 8 times higher for DTPA than that calculated as the percent of total injected radioactivity (Table 9). The transfer of BSA was similar whether the transferred fractions were calculated as the percent of the injected dose, or as the percent of the radioactivity in the total blood volume 5 minutes after intravenous injection. These results are compatible with more rapid removal of DTPA than of BSA from blood into body compartments other than the lung.

Transport of labeled molecules from the trachea and from the bronchoalveolar lumen to the blood correlated with transport from the blood to lung lavage fluid: transport in both directions was observed at 1 hour after the end of a 2-hour exposure to ozone. In both directions, more DTPA than BSA was transported and exposure to ozone increased the transport of DTPA to a greater degree than the transport of BSA.

There may be inconsistency between the measurement of transport *from* airway lumen as compared with transport *to* airway lumen at 24 hours after exposure: blood-to-lumen transport of DTPA was significantly elevated at 1 hour but not at 24 hours after exposure to 0.8 ppm ozone, while transport from lumen to blood was significantly increased at both 1 hour and 24 hours after exposure. The blood-to-lung procedure appears to detect permeability changes with lesser efficiency than the lumen-to-blood procedure.

Nose-to-Blood Transfer

Ozone exposure

Nasal mucosal permeability to DTPA and BSA in control rats sham-exposed to clean air was much lower than the tracheal or bronchoalveolar permeability (Table 11). The transfer of label in control animals was only 0.14% of the DTPA contained in the inoculum. While a statistically significant increase in transfer of DTPA was observed at 48 hours after exposure to 0.8 ppm O_3, the increase was so small that we regard the change in permeability to be 0 throughout the period of observation following exposure to ozone. A comparable lack of increased permeability to BSA was also observed.

Table 11. Nasal Mucosal Permeability to DTPA and BSA at 1, 24 or 48 Hours After a 2-Hour Exposure of Resting Rats to 0.8 ppm Ozone

Labeled Molecule	Mol. Wt., [d]	Percent of Inoculum Present in Entire Blood Volume at Time T_i After Nasal Instillation of Labeled Molecules			
		Clean Air	Ozone (Hours)		
			1	24	48
DTPA (a)	492	0.14 ±0.05 (b)	0.15 ±0.02	0.14 ±0.16	0.22 (c) ±0.03
BSA (d)	69,000	0.15 ±0.03	0.14 ±0.03	0.15 ±0.02	0.17 ±0.04
Sample size [n]		11	6	3	4

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Formaldehyde (HCHO) exposure in resting rats

Exposure to HCHO, 10 ppm for 4 hours, caused a significant increase in nasal mucosal permeability to DTPA (Table 12) and an increase in permeability to BSA which was not significant 1 hour after exposure. Nasal mucosal permeability to either molecule at 24 or 48 hours after exposure to HCHO was not significantly greater than that in the control rats.

No_2 and $NO_2 + O_3$ exposure at rest or exercise

Exposure of resting or exercising rats to 12 ppm NO_2 had no effect on the nasal permeability to DTPA immediately after the exposure or 24 hours later (Table 13). No significant increase in nasal permeability was observed in rats exposed to 0.6 ppm O_3 or to 0.6 ppm O_3 + 2.5 ppm NO_2 at rest or during exercise (Table 14).

Table 12. Nasal Mucosal Permeability to DTPA at 1, 24 or 48 Hours After a 4-Hour Exposure of Resting Rats to 10 ppm HCHO

Labeled Molecule	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Nasal Instillation of Labeled Molecules			
	Clean Air	HCHO (Hours)		
		1	24	48
DTPA (a)	0.31 ±0.36 (b)	0.83 (c) ±0.54	0.27 ±0.18	0.28 ±0.18
Sample size [n]	18	6	6	7
BSA (d)	0.41 ±0.07	0.51 ±0.03	0.42 ±0.08	0.52 ±0.24
Sample size [n]	9	3	3	4

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation
(c) $p < 0.05$
(d) BSA: ^{125}I-Bovine Serum Albumin

Route of Permeation of HRP and ^{125}I-BSA

Electron microscopic cytochemistry and autoradiography

The dense reaction product of HRP-DAB was present in the intercellular spaces of tracheal epithelia of all rats exposed to O_3 (Figure 2), but not in any control rats (Figure 3). The reaction layer stretched along the majority of the length of the junction between adjacent epithelial cells, but it was absent in the intact tight junctions near the luminal surface. The number of intercellular spaces containing HRP was considerably reduced at 24 hours after O_3 exposure; none contained HRP after 48 hours. HRP was localized to a variable extent over the luminal surfaces of epithelial cells and in endocytic vesicles, which were found in the apical and basal regions of the cytoplasm, and near to or attached to the lateral cell membranes (Figure 4).

In double labeling experiments involving simultaneous localization of HRP by cytochemistry and ^{125}I-BSA by autoradiography, the two tracer molecules were found at common sites: in endocytic vesicles, and in intercellular spaces. In addition, ^{125}I-BSA grains, unlike HRP, were not ony present in the subepithelial connective tissue but their concentration was greatest in this region (Figure 5). Autoradiographic grains produced by ^{125}I-BSA were not present in the cartilagenous zone.

Table 13. Nasal Permeability to DTPA at 1 and 24 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 12 ppm NO_2

Labeled Molecule	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Start of Instillation of Labeled Molecules				
	Clean Air Exercise	12 ppm NO_2 Rest (Hours)		12 ppm NO_2 Exercise (Hours)	
		1	24	1	24
DTPA (a)	0.16 ±0.09 (b)	0.11 ±0.02	0.15 ±0.14	0.21 ±0.02	0.10 ±0.04
Sample size [n]	8	4	4	4	4

(a) DTPA: ^{111}In-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation

Table 14. Nasal Mucosal Permeability to DTPA at 1 and 24 Hours After a 2-Hour Exposure of Resting or Exercising Rats to 0.6 ppm O_3 or 2.5 ppm NO_2 + 0.6 ppm O_3

Labeled Molecule	Clean Air Rest	Percent of Inoculum Transferred to Entire Blood Volume at Time T_i After Nasal Instillation of Labeled Molecules					
		2.5 ppm NO_2 + 0.6 ppm O_3 Exercise (Hours)		2.5 ppm NO_2 + 0.6 ppm O_3 Rest (Hours)		O_3 Exercise (Hours)	
		1	24	1	24	1	24
DTPA (a)	0.25 ±0.20 (b)	0.13 ±0.05	0.10 ±0.07	0.11 ±0.03	0.12 ±0.02	0.16 ±0.02	0.10 ±0.003
Sample size [n]	6	3	3	3	3	3	3

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate
(b) Mean ±1 standard deviation

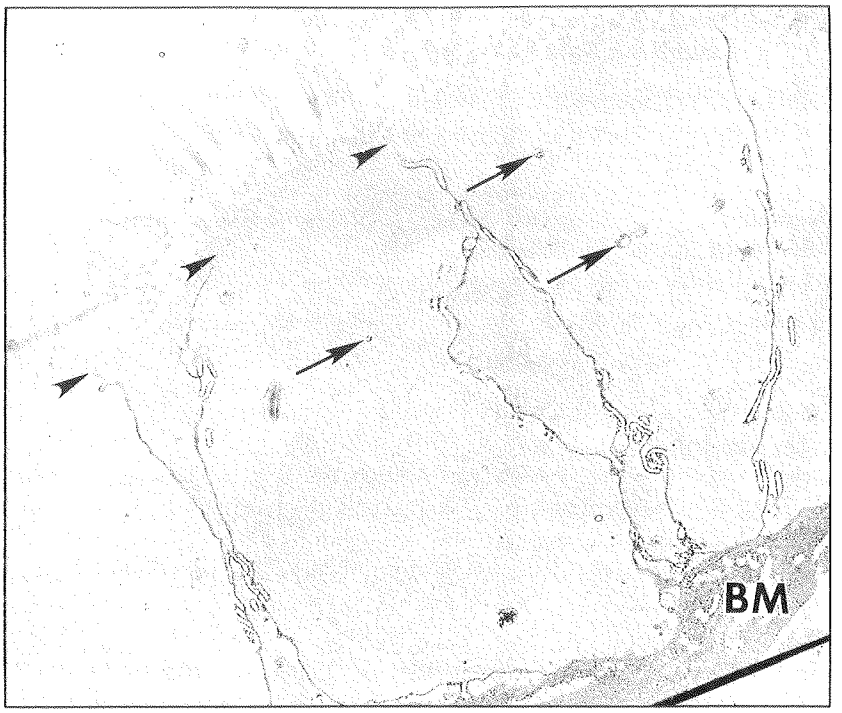

Figure 2. In tracheal epithelia of rats exposed to 0.8 ppm O_3 for 2 hours, intraluminally instilled HRP can be seen in endocytic vesicles (arrows) and intercellular spaces but there is no HRP reaction in the apical tight junction areas (arrowheads). Substantial accumulation of HRP is seen over the basement membrane (BM). x 7,000

To determine the contribution of endocytic transport of HRP from lumen to basolateral intercellular spaces of tracheal epithelial cells, thin sections were stained with lead citrate only. Sections were scanned for the presence of HRP at the luminal surface of cells to assure that all cells sampled had had the opportunity to incorporate HRP into endocytic vesicles. Portions of cells in these zones were photographed and printed at a final magnification of 20,000x. A complete montage of each cell was then assembled. Each cell was identified as a nonciliated cell with or without secretory inclusions, or as a ciliated cell. The number of endocytic vesicles containing HRP was counted in the cytoplasm. Care was taken to avoid identifying a vesicle as an invagination of basolateral cell membrane, and to avoid counting vesicles attached to the cell membrane. However, the presence of these forms was taken as evidence for endocytosis at the lumen and exocytosis at the basolateral surfaces of cells.

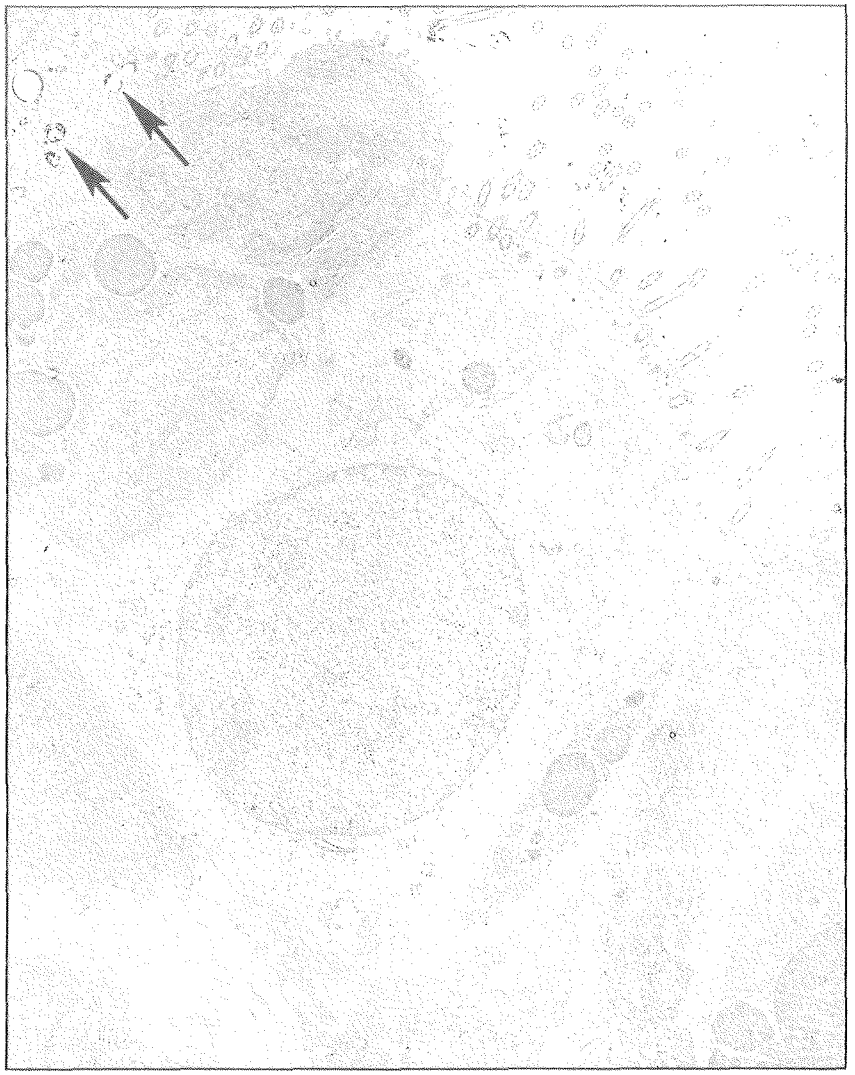

Figure 3. A section of trachea from a rat exposed to purified air. HRP instilled in the tracheal lumen is seen in the endocytic vesicles (arrows) in a ciliated cell but the intercellular spaces are devoid of HRP. x 5,000

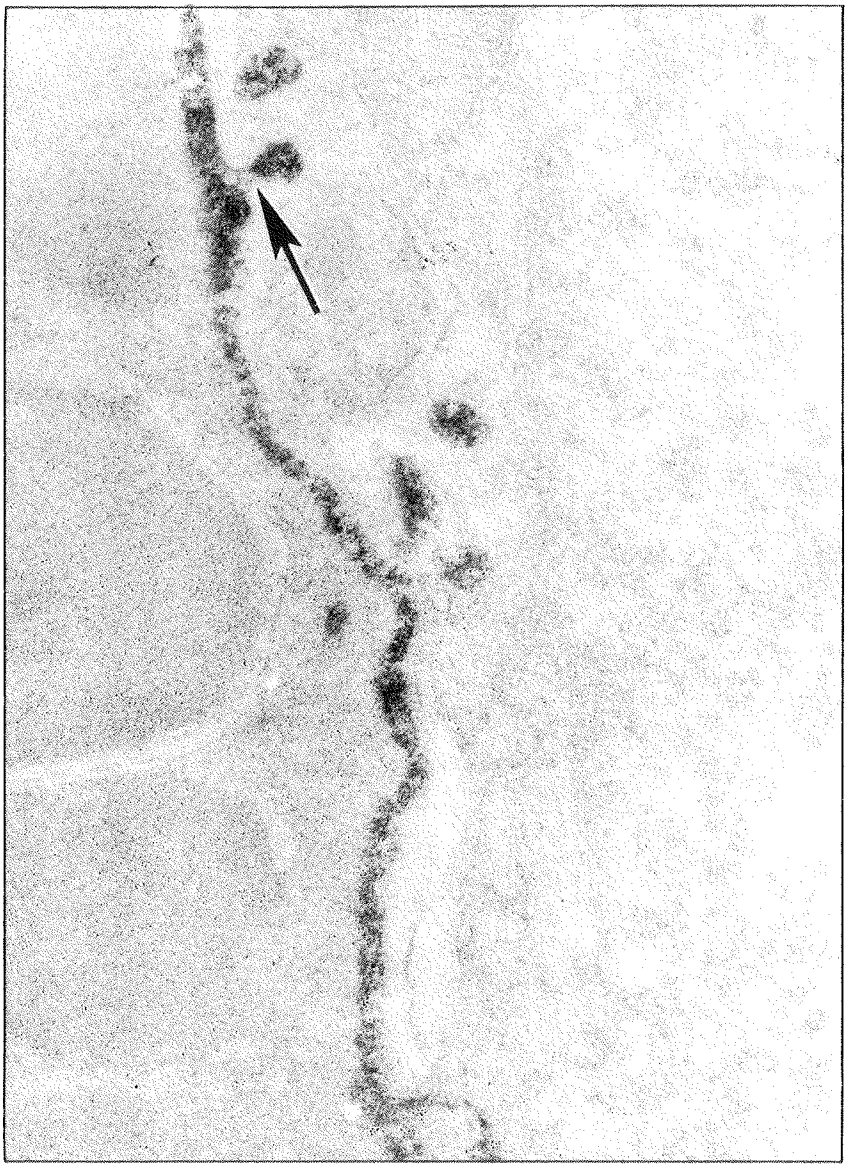

Figure 4. A portion of the tracheal epithelium from a rat exposed to 0.8 ppm O_3 for 2 hours. HRP-filled endocytic vesicles are seen in the cytoplasm and attached to the lateral cell membrane (arrow). HRP is seen in the intercellular space. x 50,000

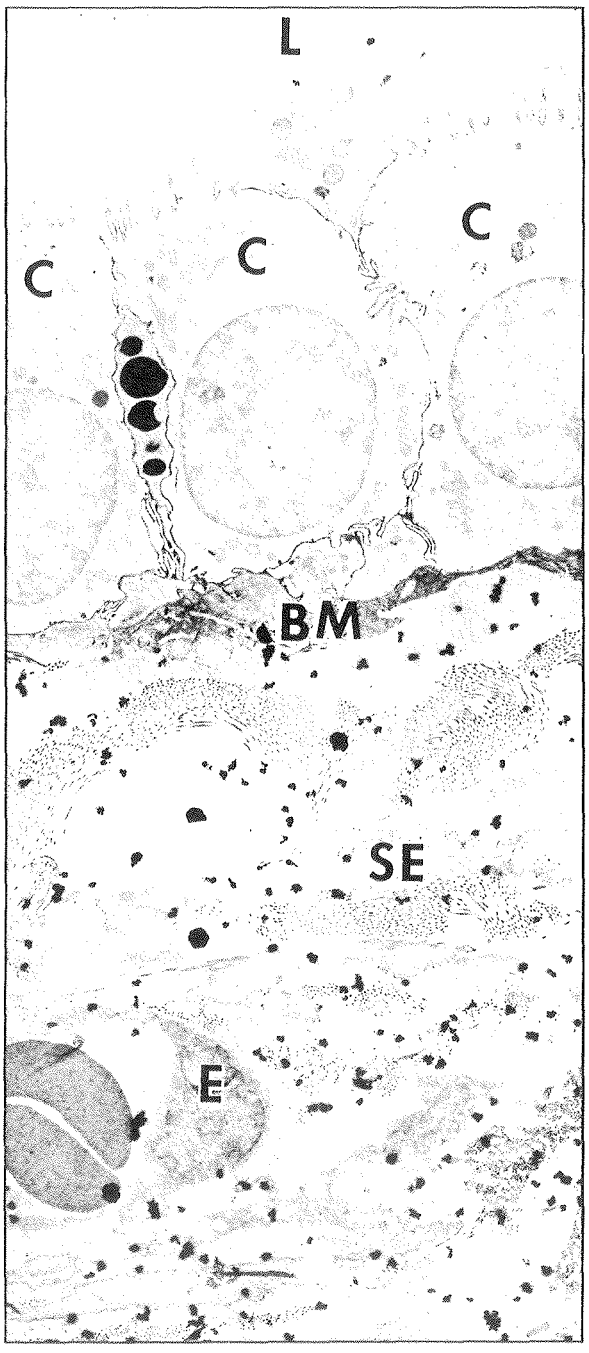

Figure 5. A section of trachea from a rat exposed to 0.8 ppm O_3 for 2 hours. Dense HRP reaction is seen in intercellular spaces and at the basement membrane (BM). Autoradiographic grains produced by ^{125}I BSA are accumulated in the subepithelial connective tissue (SE). L - lumen, C- ciliated cell, E - endothelial cell of a capillary. x 4,200.

The contribution of endocytic transport of HRP from the lumen to the basolateral intercellular spaces of tracheal epithelial cells was determined in thin sections stained with lead citrate only. In rats exposed to clean air, a total of 63 cells (42 nonciliated and 21 ciliated) were evaluated (Table 15). One cell of each type was excluded due to the excessive numbers of labeled vesicles; this unusual finding led to exclusion of these two cells. The composite mean number of vesicles per cell in the 63 columnar epithelial cells of control rat tracheas was 2.03. In O_3-exposed rats, the composite mean number of vesicles per cell of 4.39 in 57 cells was significantly greater than in the control rats ($p < 0.01$). The means were similar in the 35 nonciliated cells in tracheas of exposed rats and in the 42 nonciliated cells from control rats. However, the mean number of labeled vesicles in the 22 ciliated cells from tracheas of exposed rats (7.68 vesicles/cell) was significantly greater than the mean of 2.14 in the 21 ciliated cells from control rats ($p < 0.01$) The composite mean of vesicles per cells of all types was increased above controls by 2.16-fold in the combined tracheal cell population of O_3-exposed rats, and by 3.59-fold in the ciliated cells of tracheas from O_3-exposed rats; both increases were statistically significant.

The surface area of HRP-positive vesicles, expressed as a percent of the cytoplasmic area per cell (Table 16), showed a pattern of increase similiar to that seen in vesicle counts. The percent of cell surface area occupied by vesicles containing HRP was greatest for ciliated cells and was 2.85-fold greater in O_3-exposed rat tracheas than in the tracheas of clean air-exposed control rats. Although the increased vesicle count in the 25 nonciliated nonsecretory cells in O_3-exposed rats was not significantly greater than in the 33 similar cells in the controls (Table 15), the percent of the total cell surface area occupied by HRP-containing vesicles for this class of cells was 2.24-fold greater in tracheas of O_3-exposed rats than in controls ($p < 0.05$, Table 16). The percentage change in surface area in other cell types matched closely with the change in vesicle counts. The 2-fold increase in total mean surface area of vesicles and the 2-fold mean increase in the number of vesicles in tracheal epithelial cells of O_3-exposed rats, as compared to control rats are similar to the 2-fold increase in the transfer of DTPA from trachea to blood (Table 17).

Table 15. HRP-Positive Endocytic Vesicle Number in Tracheal Epithelia of Rats Exposed to Clean Air or 0.8 ppm Ozone for 2 Hours

Cell Type	Mean Number of Vesicles Per Cell		Change in Number of Vesicles
	Clean Air	Ozone	(Ozone/Clean Air)
Ciliated Cells	2.14 n = 21 (a)	7.68 n = 22 $p < 0.01$	3.59
Nonciliated Cells With Secretory Granules	3.33 n = 9	2.20 n = 10 p = 0.26	0.66
Nonciliated Cells Without Secretory Granules	1.61 n = 33	2.36 n = 25 p = 0.13	1.47
Total	2.03 n = 63	4.39 n = 57 $p < 0.01$	2.16

(a) n: Number of cells counted

Electron microscopic autoradiography of alveolar epithelia

In the blood-to-lavage-fluid study, autoradiographic grains produced by ^{125}I-BSA injected in blood were localized (1) in cytoplasmic vesicles in epithelial and endothelial cells of the alveolar septa, (2) in intercellular spaces, (3) in degenerating epithelial cells with swollen cytoplasm, and (4) in damaged cell membranes of rats exposed to 0.8 ppm O_3 for 2 hours and fixed within 30 minutes after exposure. Near the alveolar surface, grains were often associated with pits and deep invaginations in the epithelial cell membranes, suggesting a vesicular mode of transport of BSA from the blood to the alveolar space. Although disturbances in cellular contracts were noticed, most intercellular tight junctions appeared intact.

Table 16. HRP-Positive Endocytic Vesicle Area in Tracheal Epithelia of Rats Exposed to Clean Air or 0.8 ppm Ozone for 2 Hours

Cell Type	Percent of Cytoplasmic Area Occupied by HRP-Vesicles Per Cell		Change in Surface Area of Vesicles
	Clean Air	Ozone	(Ozone/Clean Air)
Ciliated Cells	0.109 ±0.140 n = 21 (a)	0.311 ±0.215 n = 22 p < 0.01	2.85
Nonciliated Cells With Secretory Granules	0.225 ±0.211 n = 9	0.213 ±0.205 n = 10 p = 0.90	0.95
Nonciliated Cells Without Secretory Granules	0.131 ±0.186 n = 33	0.293 ±0.372 n = 25 p < 0.05	2.24
Total Cells	0.139 ±0.177 n = 63	0.286 ±0.291 n = 57 p < 0.01	2.06

(a) n: Number of cells counted

Histopathology

Autoradiographic analysis of ^{3}H-T-labeled nasal and tracheal epithelia showed an increased percent of labeled nuclei in nasal epithelia after HCHO, but not O_3 or NO_2, exposure. Permeability changes appeared before the change in the percent of labeled epithelial cell nuclei (the Labeling Index) in the nasal mucosa after HCHO exposure. After exposure to NO_2 or O_3, however, the Labeling Index in the nasal or tracheal epithelia did not change.

A 4-hour exposure to 10 ppm HCHO resulted in an increased nasal epithelial Labeling Index in rats labeled at 17 hours and killed at 18 hours after exposure. There was a 4-fold increase in the Labeling Index of the columnar cells lining the maxillary fossa and the tips of maxillary and nasal turbinates, but not of the high columnar cells lining the medial surfaces of the turbinates, the nasal septum, or the olfactory epithelia. Repeated HCHO exposures for 3 days caused a substantial increase in cell turnover in the respiratory epithelia of the nose, but repeated exposure had little effect on tracheal cell turnover.

Table 17. Tracheal Mucosal Permeability to DTPA (a) and BSA (b) Immediately After a 2-Hour Exposure of Resting Rats to 0.8 ppm O_3

Experiment	Amount in 0.1 ml Inoculum (μg)		Fold Increase in Percent of Inoculum Transferred to Entire Blood Volume at Time T_i	
	DTPA (a)	BSA (b)	DTPA (a)	BSA (b)
1	75	2	1.91	1.35
2	75	12	2.26	1.20
3	75	35.6	2.92	1.68
4	75	29.8	1.66	1.06
Mean			2.19	1.32

(a) DTPA: ^{99m}Tc-diethylenetriaminepentaacetate, mol wt 492 d

(b) BSA: ^{125}I-bovine serum albumin, mol wt 69,000 d

In one experiment involving 0.8 ppm O_3 exposure for 4 hours and ^{3}H-T labeling at 0-, 1-, 2-, 4-, and 8-day time intervals, we did not find a consistent pattern of increased cell turnover in tracheal or nasal epithelia. Quantitative histologic evaluation of the alveolar zone after one 4-hour O_3 exposure followed by fixation 48 hours later, revealed increases of both Type 1 lesions (two or more free cells in the alveolar space but no septal thickening) and Type 2 lesions (increased cellularity and thickening of alveolar septae with or without free cells in the alveolar space).

Exposure to NO_2 was performed once in exercising rats during a 3-hour exposure at 0.6 ppm. In addition, NO_2, 0.6 ppm was combined with O_3, 0.35 ppm for comparison with O_3, 0.35 ppm alone. Histologic data for the bronchoalveolar zone only are available: O_3 produced a low level of focal lesions in the alveolar zone of the lung, NO_2 produced no focal lesions, but O_3 plus NO_2 produced 2- to 3-fold more focal lesions than O_3 alone.

DISCUSSION

Permeability Changes

The appearance of tracer molecules in the blood between 6 and 10 minutes after starting a 5-minute period of instillation of 0.1 ml of radiolabeled tracers into the trachea or a main stem bronchus, provides data for the rate of permeation across epithelial cells. Tracer concentrations in the blood progressively approach a plateau as the rate of "input" from lumen to blood is matched by "output" from blood via renal excretion of DTPA and sequestration of BSA in extravascular sites. At the 10-minute point, both control and O_3-exposed rats have similar slopes representing input-output rates that are becoming nearly parallel, but blood concentrations are different, owing to more rapid transfer of tracers across O_3-exposed rat tracheas during the initial 5-minute period of instillation of tracers. Although input rates from the tracheal lumen to blood can be differentiated for exposed and control rats by curve-fitting of data for 6 to 10 mm., for this report, differences in the fraction of the inoculum found in the whole blood volume at the time point $T_i$were used for comparison, rather than the differences in rates of permeation.

Molecular species recovered in the blood of control rats were related in a reverse order to their molecular weights. This observation is consistent with the suggestion that molecules are transferred through size-selective pores (11). Ozone increased the transfer of DTPA and BSA from the trachea to the blood at 1-hour after a 2-hour exposure. The proportional increase was greater for the smaller (DPTA) than for the larger molecule (BSA). Davis *et al.* (11), in their studies with O_3 exposure, found greater proportional change in transfer for larger molecules than for smaller ones. Since their changes in permeability were produced by longer exposure than used in this study and to O_3 concentrations five times higher than the concentrations used here, it is possible that the brief exposures and lower concentrations of O_3 used in the present experiments were sufficient only to increase the number of small pores for the passage of DTPA and were not sufficient to increase, to an equal degree, the number of larger pores presumed to be required for increased BSA permeation.

The fold increase in the transfer of label from lumen to blood for nasal, tracheal and bronchoalveolar zones in control rats and in rats exposed to O_3 or to NO_2 demonstrated marked variability in mucosal permeation. A relatively small fraction of the inoculum was transported across nasal mucosa (Tables 18 and 19), while more than 10-fold greater quantities of DTPA and more than 3-fold greater quantities of BSA were transported across the trachea, and an intermediate quantity of the labels was transported from the bronchoalveolar lumen to the blood. No correction has been made for the relative surface areas available for transfer of the tracers.

Table 18. Nasal, Tracheal and Bronchoalveolar Mucosal Permeability to DTPA at 1, 24 and 48 Hours After a 2-Hour Exposure to 0.8 ppm Ozone

Site of Transfer	Percent Transfer in Clean Air	Increase in O_3 Exposed Rats Over Clean Air (Hours)		
		1	24	48
Nose to Blood	0.15	1.1	0.9	1.5
Nose to Blood	0.12	1.2		
Trachea to Blood	1.65	1.9 (a)	1.3	0.8
Trachea to Blood	1.32	2.3 (a)	1.2	1.0
Bronchoalveolar Zone to Blood	0.7	2.0 (a)	2.8 (a)	1.0
Bronchoalveolar Zone to Blood	0.75	1.4	1.4 (a)	1.3

(a) $p = < 0.05$

Table 19. Nasal, Tracheal and Bronchoalveolar Mucosal Permeability to BSA at 1, 24 and 48 Hours After a 2-Hour Exposure to 0.8 ppm Ozone

Site of Transfer	Percent Transfer Clean Air	Increase in O_3 Exposed Rats Over Clean Air (Hours)		
		1	24	48
Nose to Blood	0.16	0.8	0.9	1.1
Nose to Blood	0.15	1.1		
Trachea to Blood	0.57	1.4 (a)	1.0	1.1
Trachea to Blood	0.44	1.2	1.0	1.1
Bronchoalveolar Zone to Blood	0.50	1.3	2.0 (a)	1.1
Bronchoalveolar Zone to Blood	0.39	1.2	1.5 (a)	1.3 (a)

(a) $p = < 0.05$

These results make several major points:

- There was a significant difference in the rate of transport in the three zones of the respiratory tract studied here.

- There was a significant difference in the effect of O_3 and NO_2 in each of these locations, with nasal mucosa being insensitive to oxidant gases when compared to the tracheal mucosa and the bronchoalveolar zone. Exposure to HCHO increased permeability and Labeling Index in the nasal mucosa. Study of the effect of HCHO on permeability in tracheal and bronchoalveolar zones was deferred because of lack of increased cell turnover or lesion formation in histologic studies.

- A time pattern of change in permeability after exposure to ozone was observed in tracheal and bronchoalveolar zones. The increase in mucosal permeability in trachea was virtually gone by 24 hours and had returned to normal by 48 hours, but increased permeability in the bronchoalveolar zone was greater in the exposed animals both at 1 hour and 24 hours after exposure.

- Exercise increased the effect of O_3 and NO_2, both in the tracheal and the bronchoalveolar zones. In addition, increased permeability induced by exercise plus O_3 lasted longer than that induced by O_3 at rest.

No structural damage or increase in Labeling Index appeared in the tracheas after O_3 or NO_2 exposure; hence increased permeability did not coincide with cell killing or obvious injury. Major airway constriction has been described in man within 1 to 2 hours after the onset of exposure to O_3 or NO_2. In dogs, airway constriction following O_3 exposure is modulated by reflex vagal pathways (25), and probably by local releases of thromboxane (26). Histamine and methacholine cause large airway constriction and, according to Boucher *et al.* (7) cause increased permeability of HRP from the tracheal lumen to the blood. The increased tracheal permeability observed here and by others may, therefore, be an effect of oxidant gases on permeability of epithelia that is causally related to neural reflex events. The causal link may be local release of cyclooxygenases, parasympathomimetic agonists, or other epithelial cell products. In any event, the permeability changes caused by O_3 or NO_2 in the rat trachea coincide in time with the early occurrence of airway constriction in dogs and man upon exposure to these gases.

A more prolonged effect of O_3 in the bronchoalveolar zone is consistent with the known pathogenicity of O_3 for the junction of the terminal bronchiole, alveolar duct, and proximal alveoli. At the last

site, type I cell death, type II cell replication, and inflammatory changes occur. Early death of type I cells may increase permeability at 1 hour, while inflammatory lesions which appear at 24 hours and peak at 48 hours after O_3 exposure may release bradykinin or other agonists, which could account for persistent permeability even if epithelia were replaced. Thus, increased immediate permeability, as compared to persistence of high permeability at 24 hours after O_3 exposure may constitute overlapping events with different causal mechanisms.

Exposure to O_3 is known to cause not only an increase in airway permeability (11, 27, 28) but also airway inflammation (29, 30). The presence of granulocytes in tracheal epithelia of dogs exposed to O_3 has been correlated with increased tracheobronchial constriction upon challenge with acetylcholine by Holtzman *et al.* (31). A role for granulocytes in endothelial permeability has been suggested by Shasby *et al.* (32). There may, therefore, be a function for granulocytes in epithelial permeability. The *in vitro* studies of Shasby *et al.* (32) show that PMA-stimulated granulocytes cause an increase in endothelial permeability, and that this effort is dependent upon a close association of granulocytes with target cells.

Both the greater resistance of nasal mucosa to the permeation of tracer molecules and to O_3 exposure, and the quick restoration of normal permeability in the tracheal mucosa after O_3 exposure, correlate with the absence of cell-killing by O_3. The lack of response of nasal epithelium to O_3 was observed in spite of maximum exposure of the nasal as compared to the other respiratory epithelia. This low degree of response to O_3 in nasal mucosa, and the low permeability of nasal mucosa in the controls, may be due to poor penetration of both O_3 and tracer molecules through mucus to cells. In addition, properties of the cell types in the nasal mucosa, and blood flow in this site, may differ from these characteristics of tracheal and bronchoalveolar zones. Factors controlling the penetration of tracers may also control the penetration of O_3 to each zone. For example, the solution of ozone may be different in the fluid phase over the epithelium in each zone. As a further example, the transport of BSA to the blood was slower than the transport of DTPA in the tracheal and bronchoalveolar zones. In contrast, BSA was transported across the nasal mucosa at about the same low rate as DTPA. This suggests that nasal permeability may have been low because of a failure of both tracer molecules to reach nasal epithelial cell surfaces.

The pattern of transfer of tracers from the blood to the airway lumens reached by lung lavage resembled transfer from the tracheal and bronchoalveolar zones to the blood in several aspects: the change in permeability could be detected immediately after O_3 exposure; and increased permeability was detected better within 1 to 6 minutes

following instillation or injection of tracers than after 10 or more minutes following instillation or injection. Further, in rats exposed to clean air, the fractional transfer for DTPA was greater than that for BSA, when expressed as the percentage of counts in the total rat blood volume 1 minute before lavage. Although the change in permeability was greater after exposure to 2.0 ppm than to 0.8 ppm of O_3 and persisted at 48 hours after the exposure, the increase in transfer of DTPA as compared to BSA was comparable to the transfer from bronchoalveolar lumen to blood, suggesting that pore size was not altered by the higher O_3 concentration.

Mode of molecular transfer

Intercellular spaces were the major site for localization of HRP in its transit from the airway to the blood, although tight junctions were intact and free of HRP. Absence of HRP from the spaces within tight junctions was unexpected in view of the hypothesis that O_3 impairs tight junctions as proposed by Simani *et al.* (33), Hogg *et al.* (6) and Boucher *et al.* (9). Alternatively, the number of HRP molecules that might be retained in junctions may be too small for detection by cytochemistry, even if the junctions opened after O_3 exposure. It is also possible that only a limited number of tight junctions were permeabilized by O_3, and that those containing detectable HRP were missed in sampling. HRP passing through this small number of impaired junctions might become evenly distributed in the intercellular spaces of all adjacent cells. Walker *et al.* (34) have suggested that permeability is not related to junctional organization along the lateral surfaces of all epithelial cells, but that leaks develop at the corners of the epithelia where three cells meet. Studies of Gordon *et al.* (35) suggest the added alternative that NO_2 exposure disrupts tight junctional strands only at certain sites around a cell. The perturbed junctions may be randomly distributed and may not be located at the point of juncture of three epithelial cells.

Endocytic vesicles may transport tracers from the luminal surface of epithelial cells to the lateral cell membranes. This process may contribute to the accumulation of molecules in the intercellular spaces (36). In these experiments, HRP was applied to the luminal surface of tracheal epithelia at approximately 20 minutes after completion of exposure, and the tracheas were fixed about 20 minutes later. The total elapsed time between introduction of HRP and fixation of tissures was not greater than 20 minutes. Transfer of HRP from the lumen to the basolateral intercellular space would therefore be fairly rapid by this criterion, and must have been even more rapid to account for transport of tracers from the lumen to the blood at increased rates within 1 to 10 minutes after instillation of tracers in permeability studies.

The question arises as to whether the intercellular spaces filled because of some prior relaxation of tight junctions or because of an increased rate of transport by way of endocytic vesicles. The number of vesicles in cells from O_3-treated animals was about 2.16-fold greater than in the controls and the increase in label found in the blood was about 2-fold greater. It is possible that the increased transmucosal transport of tracers involves principally an endocytic process. If this mechanism can be confirmed, the hypothesis that toxic gases cause increased transport by relaxation of tight junctions may be supplemented or replaced by the hypothesis that a greater rate of vesicular transport is a major mechanism of increased transmucosal permeability.

A mechanism is suggested by which increased vesicular transport might occur. Apical cell membrane invagination to form endocytic vesicles is a normal transmucosal transport process. Damage to apical membranes by ozone may lead to autophagocytosis of damaged membranes with increased formation of endocytic vesicles and increased transport of tracers from the lumen to the blood.

Accumulation of Tracers at Basal Lamina and Mucosal Connective Tissues

In O_3-exposed rats, HRP is clearly visualized in intercellular spaces and at the basal lamina. After diffusion into a larger subepithelial compartment, however, HRP was not discerned cytochemically. While ^{125}I-BSA was not localized with the same intensity as HRP over intercellular spaces, the amount of ^{125}I-BSA in the subepithelial region was great enough to produce extensive autoradiographic grains in mucosal connective tissues. It is possible that HRP is retained in intercellular spaces due to its nonspecific binding to cell membranes and to the components of basal lamina, following a 2-hour exposure to 0.8 ppm O_3. It is also possible that BSA passes through the epithelial layer relatively rapidly but becomes bound to surfaces in the subepithelial compartments, with the accumulation of BSA in the subepithelial connective tissue. In spite of the electron microscopic evidence for retention of BSA and HRP noted above, transport of BSA and of DTPA from the tracheal lumen to blood was increased by O_3. There may, therefore, be several concurrent processes that have the net effect of increasing lumen-to-blood transport while at the same time leading to the accumulation of tracers in sites between the epithelial tight junctions and the capillaries.

The first interpretation of the accumulation of tracers between and beneath O_3-exposed tracheal epithelial cells is that an overload had occurred. Delivery of tracers to the intercellular and subepithelial zones exceeded normal removal processes. An alternative interpretation is that the removal process was blocked. Removal processes could be delayed by increased pulmonary vascular pressure, with or without increased capillary permeability.

Endothelial cell damage in lung capillaries has been reported after exposure of mice, rats, and cats to O_3 (37, 29, 38). The close approximation of the capillaries to the air space would favor endothelial injury in alveoli with the probable consequence of increased capillary permeability. However, in the trachea the capillaries are located deep in the connective tissue, where O_3 might not penetrate to cause increased capillary permeability by direct endothelial cell injury.

Increased pulmonary vascular pressure could lead to increased interstitial pressure with or without increased capillary permeability. Release of thromboxane as a basis for constriction of bronchial smooth muscle has been described after O_3 exposure (26). Thromboxane and related prostaglandins increase pulmonary vascular pressure. Hence, a basis for increased interstitial hydrostatic pressure exists. Experimentally increased pulmonary microvascular pressure retarded clearance of liquid from lungs in lambs (39). In order for this mechanism to be invoked as a basis for delayed removal of tracer molecules in the tracheal walls after exposure to O_3, it would be necessary to establish that vasoactive substances are released shortly after O_3 exposure and that they induce increased pressure in the vascular distribution of the bronchial artery.

The entrapment of BSA in the subepithelial connective tissue may explain the lesser increase of BSA (1.32-fold), as opposed to the about 2-fold increase of DTPA in the blood of O_3-exposed rats, as compared to clean air controls (28) (see Table 17).

In spite of their differences in size, two markers used in this study (HRP, molecular weight 40,000d and BSA, molecular weight 69,000d), were transferred through the same epithelial routes, i.e., endocytic vesicles and intercellular spaces. Although the two markers followed a common transport pathway, they may have been complementary in their use as molecular probes because of different modes of binding to surface of cells or to extracellular structures in the pathway from the lumen to the blood.

The autoradiographic detection of intravenously injected ^{125}I-BSA in the alveolar epithelial and endothelial cells suggested multiple routes of molecular transfer. The transfer occurred through endocytosis (a relatively active process in endothelial cells), through degenerated alveolar epithelial cells, and through intercellular spaces. Junctional impairment did not appear concurrently.

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About the Authors

T. Timothy Crocker (M.D. U. Calif. San Francisco, 1944) is a Professor of Community and Environmental Medicine and founded that Department at U. Calif., Irvine, College of Medicine in 1971. He is the author of reports on epidemiology, virology, cell biology, chemical carcinogenesis and lung toxicity of air pollutants.

Deepak K. Bhalla (Ph.D., Howard University, Washington, DC, 1976) is an Adjunct Assistant Professor of Community and Environmental Medicine, College of Medicine, University of California, Irvine. After graduation in 1976, he held a postdoctoral position at Harvard Medical School and a research position at U.C. San Francisco before joining U.C. Irvine. He has published in the areas of inhalation toxicology, cell biology and the lymphoid system.

FOR FURTHER INFORMATION CONTACT:

The HEALTH EFFECTS INSTITUTE
215 First Street
Cambridge, MA 02142
(617) 491-2926

HEALTH EFFECTS INSTITUTE

215 First Street

Cambridge, MA 02142

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