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Effects of Ozone on Airway Epithelial Permeability and Ion Transport

Philip A. Bromberg, Venkatachalam Ranga, and M. Jackson Stutts
Division of Pulmonary Diseases, Department of Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC

Includes the Commentary of the Institute's Health Review Committee

Research Report Number 48

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HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

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Philip A. Bromberg[1], Venkatachalam Ranga[2], and M. Jackson Stutts

ABSTRACT

Ozone is a highly reactive form of oxygen produced in the atmosphere by photochemical reactions involving substrates emitted from automobile engines. Outdoor air concentrations as high as 0.4 parts per million[3] (ppm) occur. The respiratory tract extracts about 90% of inhaled ozone. From the chemical reactivity of ozone, it is expected to attack organic molecules located on or near the respiratory surfaces. The airways are covered with a cohesive layer of epithelial cells that forms the boundary between the external environment and the respiratory tissues. One important role of this epithelial layer is its barrier function. Airborne particles that deposit (and dissolve) in the airway surface liquid are not readily absorbed, and soluble tissue components are excluded from the surface liquid. The epithelium also controls the volume and composition of the surface liquid. One important process in this regard is the absorption and secretion of ions and water.

We have studied the effects of inhalation of ozone on the barrier function (permeability to dissolved molecules) and the ion transport activity of epithelium using both in vivo and in vitro techniques. All our experiments were performed with male Hartley strain guinea pigs. Conscious, unrestrained animals were exposed to a concentration of ozone of 1 ppm for three hours in controlled environmental chambers in the Health Effects Research Laboratory, U.S. Environmental Protection Agency (EPA), Research Triangle Park, NC.

Such exposures caused a marked increase in the rate of appearance in blood of various water-soluble compounds instilled onto the surface of the trachea, indicating increased permeability of the airway epithelium. This interpretation was supported by electron microscopy, which showed that the tracer molecule horseradish peroxidase was present in the intercellular spaces of tracheal epithelium from ozone-exposed, but not air-exposed (control), animals. However, when the tracheas were excised after ozone exposure and mounted in a tissue bath before measurement of permeability, no increase was found. We suggest that the effect of ozone inhalation on airway permeability requires the action of mediators that are washed out in the in vitro situation.

When we exposed animals to 1 ppm for 3 hours daily, we found that the increased permeability in vivo was no longer demonstrable after the fourth exposure. The mechanisms for this type of "adaptation" are not known. The phenomenon recalls the response of human subjects to repeated daily ozone exposures.

Ozone exposure caused a sharp increase in active ion transport by tracheal epithelium. This is probably due to increased absorption of sodium ion (Na^+) because it was inhibited by 30 μM amiloride, a potent blocker of Na^+ channels in the apical (luminal) membrane of epithelial cells. The increased transport was observed in tracheal tissues bathed in physiologic salt solution and was present in tracheas removed from animals up to three days after a single exposure. It probably reflects a direct and specific effect of ozone on a component or components of the apical membrane.

These data indicate that ozone exposure produces major changes in airway epithelial permeability and ion transport in guinea pigs. The mechanisms of these effects and their importance in humans remain to be established.

INTRODUCTION

Ozone (O_3) is a powerful and reactive oxidant. In the ambient atmosphere it is generated by complex photochemical reactions that involve reactants found in automotive emissions. Although urban ambient air O_3 levels (one-hour averages) generally are less than the current National Ambient Air Quality Standard (NAAQS) of 0.12 ppm(v), concentrations higher than the NAAQS are commonly found during summer daylight hours, and levels of 0.3 ppm and even higher have been detected in the Los Angeles area.

In 1983 when the studies reported here began, it was known that some healthy adults exercising vigorously for one hour in atmospheres containing as little as 0.12 ppm O_3 during a two-hour exposure developed cough and substernal pain, as well as a decrease in vital capacity (McDon-

[1] Correspondence may be addressed to Dr. Philip A. Bromberg, Division of Pulmonary Diseases, Department of Medicine, School of Medicine, CB 7020, 724 Burnett-Womack Building, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7020.

[2] Dr. Ranga died in 1985, midway through this project.

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

nell et al. 1983). These changes regressed within a few hours after cessation of exposure. Despite decreased vital capacity, O_3 exposure provoked relatively little bronchoconstriction (McDonnell et al. 1983), and patients with asthma (Linn et al. 1978; Silverman 1979; Koenig et al. 1985) or atopy (Holtzman et al. 1979) or chronic obstructive pulmonary disease (Linn et al. 1982, 1983; Solic et al. 1982, Hackney et al. 1983; Kehrl et al. 1983, 1985) were not found to be unusually sensitive to controlled O_3 exposures. Two other O_3-related phenomena had been observed: (1) repetitive daily exposure of subjects to effective levels of O_3 resulted initially in enhancement of the vital capacity response, followed by progressive decrease and abolition of the response over a four- to five-day period (Hackney et al. 1977); (2) after a single exposure of human subjects to an effective level of O_3, there was a moderate increase in bronchial reactivity to challenge with inhaled aerosolized bronchoconstrictor agents like histamine and beta-methacholine (Golden et al. 1978; Holtzman et al. 1979).

The precise mechanisms of these effects remain obscure. The decrease in vital capacity that is characteristic of the respiratory effects of O_3 exposure in human subjects could not be directly duplicated in animal models because this change is not caused by an alteration in lung mechanics but, rather, by an involuntary inhibition of maximum inspiration (Hazucha et al. 1989). However, in several mammalian species, transient bronchial hyperreactivity was demonstrated after O_3 exposure (Easton and Murphy 1967; Lee et al. 1977; Abraham et al. 1980). In dogs this phenomenon appears to be related to the development of neutrophilic inflammation in the airways (Holtzman et al. 1983). Another important observation from animal toxicology is that exposure to O_3, even at concentrations less than 1 ppm, caused damage to ciliated epithelial cells in the airways and to type I alveolar epithelial cells (Boatman et al. 1974; Stephens et al. 1974; Mellick et al. 1977).

Airway epithelium has been recognized as having several functions:

1. It serves as a self-cleansing barrier between inhaled materials and particles (including microorganisms) deposited on the luminal side of the epithelium and the tissues of the airway wall.
2. It maintains the composition and volume of the airway surface liquid, which (among other things) is essential to the process of mucociliary clearance, in conjunction with the activity of the airway ciliated and secretory cells.
3. It produces and secretes mediators that may participate in inflammation or act on other cells in the airway wall, including smooth muscle.

Because inhalation of O_3 was known to be able to damage ciliated cells in airways, and because O_3 was a highly reactive substance, we proposed to investigate further the possibility that exposure to relatively low concentrations of O_3 would affect some of these functions of airway epithelium.

In 1980 we had reported that in guinea pigs exposed to 4 ppm O_3 for three hours the rate of uptake of polar probe molecules instilled in aqueous solution onto the tracheal surface was sharply increased (Davis et al. 1980). This was interpreted as showing an O_3-induced increase in permeability of the airway epithelium. The precise pathways by which these solute probes traversed the epithelium were not clearly established. We suggested increased permeability of the paracellular pathways with loss of molecular sieving, and demonstrated damage to the intercellular tight-junctional apparatus by freeze-fracture electron microscopy in these guinea pigs (Boucher 1981).

We were also interested in establishing whether or not O_3 exposure could alter ion transport by airway epithelium. Since 1975, a number of studies in mammals, including humans, have shown that airway epithelium actively absorbed Na^+. This process depends on a sodium-potassium (Na^+-K^+) pump in the basolateral cell membranes, which requires adenosine 5′-triphosphate (ATP) and is inhibitable by ouabain. In cells that incorporate a sodium-chloride (Na-Cl) or Na-K-2Cl cotransporter in the basolateral membrane and a Cl channel in the apical membrane, the Na^+-K^+ pump can drive Cl^- secretion as well as Na^+ absorption. The evidence supporting this model of ion transport in airway epithelium has been reviewed by Welsh (1987). Thus, airway epithelium can, under various circumstances, either absorb Na^+ from airway surface liquid (with Cl^- and water [H_2O] presumably following Na^+ passively), or can secrete Cl^- into airway surface liquid (with Na^+ and H_2O following Cl^- passively). Alterations in these epithelial ion transport processes could cause changes in the volume and composition of the airway surface liquid.

Because inhaled O_3 was known to cause epithelial cell changes and should react especially with apical cell membrane (i.e., surface) components, we thought that an O_3 effect on airway epithelial ion transport was not unlikely.

Alternatively, O_3 could react with other elements located in the superficial portion of the airway epithelium (e.g., sensory nerve endings), causing secondary changes in the function of airway epithelium.

SPECIFIC AIMS

To test the hypothesis that exposure to toxicologically relevant O_3 concentrations causes alteration of important

functions of airway epithelium, the specific aims of this study were as follows:

1. to characterize changes in the bioelectric properties of tracheal epithelium after in vivo exposure to O_3; and
2. to characterize changes in the permeability of tracheal epithelium to several polar molecules after in vivo exposure to O_3.

METHODS

ANIMALS AND EXPOSURES

We selected male Hartley strain guinea pigs as the laboratory animals for our studies because in our previous studies using these animals the effects of exposure to 1.0 ppm O_3 (unpublished observations) and 4 ppm O_3 (Davis et al. 1980) on in vivo permeability of airway epithelium to polar uncharged molecules were striking. Conscious, unrestrained animals were exposed in stainless-steel cages in the facilities of the Inhalation Toxicology Branch, Health Effects Research Laboratory. (We are grateful to Dr. Fred Miller and Dr. Judy Graham at the Health Effects Research Laboratory for the purchase, maintenance, and exposure of the animals.) The animals (Charles River Breeding Laboratories, Kingston, NY) were purchased by the EPA at six weeks of age, were housed two to a cage at 22°C with a 12-hour light, 12-hour dark cycle, and were fed with standard chow (Wayne guinea pig diet, Chicago, IL) and water ad libitum. They were used when body weights were 300 to 600 g, except for the Ussing chamber studies, which required 800- to 1,000-g animals (to obtain a sufficiently large tracheal caliber). At the required time after exposure, the animals were transferred to portable cages and brought by automobile (12 miles) to our laboratory in Chapel Hill, NC.

Clean air (sham) exposures were always interspersed with O_3 exposures to control for possible effects of season, supplier of animals, or other factors. All reported exposures were to 1.0 ppm O_3 or to filtered air (sham exposure control) for three hours. The O_3 was generated from oxygen by an electric spark generator (OREC Corp., Phoenix, AZ) and diluted with filtered air. The O_3 level in the exposure chamber was continuously monitored with a Bendix (Cincinnati, OH) Model 8003 O_3 analyzer periodically calibrated against a primary reference standard (RFOA-0176-007). During exposure, animals were placed in individual stainless-steel wire cages in a 0.3-m^3 Young and Bertke (Ronceverte, WV) exposure chamber with 9 to 10 changes of air per hour.

When animals were not used immediately after exposure, they were returned to their holding area and maintained on chow and ad libitum water until use. In some experiments, animals were exposed on four consecutive days before being studied immediately after the final exposure.

Neither O_3-exposed nor control animals appeared to be in distress at the time of study. The lungs showed no gross signs of pneumonia. Random histologic evaluation of airways and lungs of control animals did not show inflammation.

BIOELECTRIC AND PERMEABILITY MEASUREMENTS IN VITRO

Under deep nembutal anesthesia, and with supplementary local lidocaine injection, we excised the trachea and immediately placed it in mammalian Krebs-Ringer bicarbonate glucose solution (KRB) equilibrated with 5% carbon dioxide (CO_2) and 95% oxygen (O_2) at 37°C. The tracheal

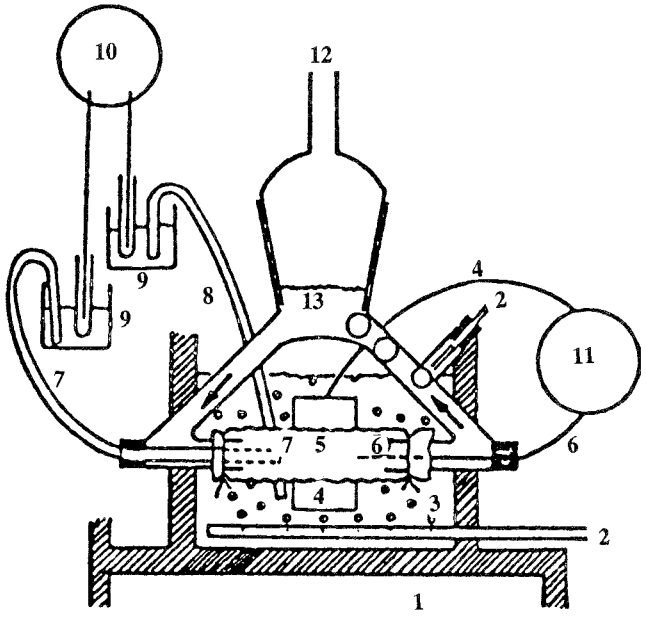

Figure 1. Clarkson chamber for studying transport in cylindrical tissues. The tracheal segment (5) is positioned horizontally, immersed in the bath (3), and secured to the arms of the inverted Y-shaped luminal perfusion chamber by ligatures. This isolates the luminal (13) from the serosal baths. A gas lift of 5% CO_2 and 95% O_2 (2) maintains circulation in the luminal bath. The serosal bath is gassed with 5% CO_2 and 95% O_2 via a horizontal perforated catheter (2). The *PD*-sensing circuit (left side) consists of conductive intraluminal (7) and external bath (8) polyethylene tubes filled with KRB-agar, each connected to calomel half-cells (9), which in turn are connected to a potentiometer (10). The current-passing circuit (right side) involves a Ag/AgCl needle electrode (6), which passes into the tracheal lumen through a plug in the right-sided arm. A cylindrical Ag electrode (4) in the serosal bath is positioned concentrically around the tracheal segment. The position of the luminal needle electrode is adjusted manually to obtain the highest value of I_{sc}. The circuit is connected to the voltage clamp (11) that passes sufficient current (I) across the tissue to clamp the transtracheal *PD* to any desired value. If *PD* is clamped at zero, the required current is the I_{sc}. The open-top plug (12) provides access to the intraluminal bath liquid for additions and sampling.

cylinder was trimmed of external adventitious tissue and mounted in the special apparatus shown in Figure 1.

The luminal and serosal surfaces were bathed in identical solutions of KRB, 5% CO_2, and 95% O_2. The luminal and serosal fluids were separated by the tracheal cylinder. Polyethylene bridges containing KRB-agar connected the luminal solution and the external bathing solution to identical calomel half-cells, and the potential difference (*PD*) between the cells was measured with a World Precision Instruments (New Haven, CT) voltage clamp apparatus. Direct current (DC) was passed from an automatic clamping circuit through a silver/silver chloride (Ag/AgCl) needle electrode carefully advanced into the lumen of the tissue. The current flowed to a cylindrical Ag/AgCl electrode plate positioned concentrically around the tissue cylinder. We adjusted the position of the internal electrode so as to minimize the current flow required to null the spontaneous electrical potential developed by the tissue to zero. This current is called the short-circuit current (I_{SC}).

After an initial one-hour period of equilibration at 36° to 37°C, the tissues were maintained open-circuited (i.e., their *PD* was continuously monitored). The I_{SC} was measured during a 45-second interval every 15 minutes. The ratio I_{SC}:*PD* was used to measure electrical conductance (G), assuming the tissue behaves like an ohmic resistor. We discarded tissues with $G > 18$ mS/cm^2. We added 1 mM dithiothreitol to the bath to preclude any toxic effects from liberation of Ag^+ from the electrode.

Isotopes were added to the luminal fluid or the external bath as required for solute flux measurements. Samples for counting radioactivity were withdrawn from the "source" side at the beginning and end of the experiment and were averaged. Samples were withdrawn from the "sink" side every 15 minutes, and the volume was replaced with KRB.

After counting samples in the appropriate spectrometer and correcting for dilutions on the sink side, we plotted the sink radioactivity against time and obtained a best linear fit by regression. The slope of this line was used to calculate the clearance of isotope from the known volume of source solution. At the end of the experiment we dissected the tracheal cylinder into a flat sheet and measured the surface area on a grid. Dividing the clearance (cm^3/sec) by the surface area (cm^2) provides a measure of permeability (P) in centimeters per second.

The permeability probes used included ^{14}C-mannitol, ^{3}H-inulin, ^{111}In-diethylenetriamine pentaacetic acid (^{111}In-DTPA), ^{125}I-albumin, and ^{3}H-dextran (molecular weight 10,000) and ^{14}C-dextran (molecular weight 70,000), in various combinations. The dextrans and inulin were gel-filtered to remove small molecular weight fragments just before use. When morphologic studies requiring horseradish peroxidase (HRP) were undertaken, the HRP was added to the appropriate bath 30 minutes before the termination of the experiment.

For measurements of bidirectional fluxes (and thus, flux ratios), we attempted to match the electrical conductances of the tissues used for the mucosal-to-serosal (m→s) and serosal-to-mucosal (s→m) flux measurements.

We used the Ferry-Faxen equation (Dowben 1969) to estimate the equivalent pore radius of the tissues using ratios of the permeability of the various probes to that of mannitol, and using molecular radii taken from the literature. We did not attempt to fit the data to more than one pore size. We followed the procedures used by Boucher (1980), Gatzy and Stutts (1980), and Gatzy (1982) to analyze permeability data for canine airways and for bullfrog lung. These concepts are derived from the work of Solomon (1968).

Because of our concerns about the validity of the I_{SC} measurements obtained with tracheal cylinders (the current flux across the tissue may not be homogeneous; I_{SC} was dependent on the exact positioning of the internal Ag electrode along the lumen of the tissue cylinder), we repeated the bioelectric measurements using conventional Ussing chambers. The tissue to be studied was mounted in planar fashion between two identical conically shaped half-chambers. The current-passing electrodes were positioned at the apices of the half-chambers, and we believed the current flux to be homogeneous.

The trachea was slit open longitudinally so as to spare the noncartilaginous portion. Even using larger (older) guinea pigs, we were barely able to cover a 0.26-cm^2 orifice. As many tissues as possible were mounted from each trachea without regard for region. Measurements of *PD*, I_{SC}, and G were made as described previously, except that the tissues were maintained short-circuited and *PD* was measured every 15 minutes. Tissues with $G > 18$ mS/cm^2 were discarded. We used only ^{14}C-mannitol (10 μCi) as a permeability marker in these studies. We used $^{36}Cl^-$ (3 μCi) and $^{22}Na^+$ (2 μCi) to measure bidirectional fluxes of these ions in tissue pairs matched for G. In some experiments, 30 μM amiloride was added to the mucosal media after the baseline bioelectric and flux data were obtained (75 minutes) and observations were continued for an additional 45 minutes (Stutts and Bromberg 1987).

In these Ussing chamber experiments, the investigators were blind as to the exposure condition of the animals available for study. An exposure log was maintained at the EPA exposure facility and the animals were coded. The investigators were provided animals in pairs and knew that one animal had been exposed to air (control) and the other had been exposed to O_3.

AIRWAY EPITHELIAL PERMEABILITY IN VIVO

We used the method of Boucher and associates (1978). Guinea pigs were anesthetized with nembutal (20 mg/kg of body weight) administered intraperitoneally. Using supplementary local lidocaine anesthesia, we placed a carotid artery catheter (PE 50) and tracheal cannula (PE 240) in the neck. The animal was placed supine on a board and tilted about 20° head down. Body temperature was monitored and maintained with a lamp. Phosphate-buffered saline, 0.2 mL (pH 7.40), containing probe molecules was instilled through the tracheal cannula onto the tracheal surface over a five- to six-minute period. This procedure is designed to minimize aspiration of the solution into the deep lung. The probes used were 2 μCi ^{14}C-mannitol, 10 μCi ^{111}In- DTPA, 5 μCi ^{3}H-inulin, and in 25% of animals, 0.2 mg of unlabeled HRP. In the latter animals, the lower tracheal surface was observed to have peroxidase reaction product after fixation.

We withdrew 1.0 mL of heparinized blood via the arterial catheter just before the tracheal instillation and at 5, 10, 15, 20, and 30 minutes after beginning the instillation. The blood was replaced by an equal volume of saline. Plasma, 0.45 mL, was immediately counted for ^{111}In ($t_{1/2}$ = 2.8 days) in a gamma spectrometer. The remaining isotopes were counted 28 days later and corrected for ^{14}C spill into ^{3}H (10%). The total quantity of probe-associated activity in plasma at any sampling time was calculated assuming a constant plasma volume of 3.75 mL/100 g of body weight and expressed as a fraction of the instilled dose of that probe-associated radioactivity.

To express the data in terms of uptake rates, we measured the slope of the best-fit line for the points at 0, 5, 10, and 15 minutes. The 20- and 30-minute data were excluded because they often exhibited a plateau, especially in animals that had been exposed to O_3. Although the probes all shared the same distribution in the airways, the exact surface area across which uptake occurred is not known, and the concentration of probe molecules in the source solution (the airway surface liquid) was not constant. Thus, we were unable to calculate the permeability constants from these data. Furthermore, the plasma levels were affected by the loss of probe molecules from the plasma (e.g., via renal excretion). Nevertheless, the data were quite reproducible among groups of animals, and the uptake rates were in qualitative agreement with our expectations for a diffusion-limited process.

We assumed that the ^{14}C label in mannitol is firmly incorporated into the probe molecule. Indium is very firmly bound to DTPA and remains bound even in oxidizing environments (Nolop et al. 1987). Inulin is relatively unstable. In addition to gel-filtering inulin immediately before use, we intermittently gel-filtered serum samples after probe instillation and always found more than 80% of the radioactivity associated with the high-molecular-weight fraction. Probe molecule solutions were instilled only after completion of exposure and were never directly exposed to O_3.

HISTOLOGY

Microscopy generally followed the procedures of Ranga and Kleinerman (1980, 1982). Tracheal tissue was fixed in 2.5% glutaraldehyde in 0.075 M cacodylate buffer (pH 7.4).

For transmission electron microscopy, the tissue was sliced in 2 × 2-mm blocks, further fixed for two hours at 4°C, and washed overnight at 4°C in 0.01 M cacodylate and 0.2 M sucrose (pH 7.4). Several blocks were treated with diaminobenzidine tetrahydrochloride, according to the method of Graham and Karnovsky (1966). Blocks were postfixed in 1% osmium tetroxide (OsO_4) with 1.5% potassium ferrocyanide ($K_4Fe(CN)_6$) for two hours on ice to enhance cell membrane visualization and cell localization in grids stained with uranyl acetate (Watson and Brain 1979). The blocks were then dehydrated in alcohol and embedded in Epon 812. Sections 1 μm thick were cut with a glass knife and counterstained with 0.05% toluidine blue (pH 5.0) to quantify "senescent" cells and to survey the tissue with light microscopy. Thin sections were cut with a diamond knife, mounted on copper grids (unstained or stained with uranyl acetate), and examined with a Zeiss EM-IOA electron microscope at 60 kV.

For other light microscopic studies, the fixed tissue was processed and embedded in paraffin. Sections 4 μm thick of the entire tracheal circumference were obtained and stained with hematoxylin-eosin or Alcian blue and periodic acid-Schiff (pH 2.6).

STATISTICS

Tables 1 and 2 present baseline data on bioelectric properties and permeability to several polar solutes of cephalad (upper) and caudad (lower) trachea when measured in vitro. These were evaluated using paired two-tailed t tests. However, the comparison of m→s and s→m fluxes for individual solutes in either the upper or lower trachea was evaluated using an unpaired t test.

Table 3 presents data on the effect of a single in vivo exposure to O_3 on the in vitro bioelectric properties of upper and lower trachea from two hours to seven days after exposure. For each parameter (PD, I_{sc}, G), a one-way analysis of variance (ANOVA) was performed for the data at all time points. Significant changes revealed by ANOVA were fur-

ther analyzed at individual times using Dunnett's two-tailed *t* test.

Tables 4 and 5 present data on the changes of in vitro bioelectric properties and permeability to polar solutes of lower guinea pig trachea obtained at various times (2 hours to 72 hours) after a single in vivo exposure to O_3. The data for all time points were analyzed by one-way ANOVA for each parameter. Significant changes revealed by ANOVA were further analyzed at individual times using Dunnett's two-tailed *t* test.

Table 6 compares the in vitro bioelectric properties of cylindrical preparations of the lower trachea with planar (0.26-cm^2) preparations. Comparisons of mean values for *PD*, I_{sc}, and *G* were made with unpaired *t* tests.

Table 7 compares the effects of in vivo O_3 exposure with air exposure on in vitro bioelectric properties of planar (0.26-cm^2) preparations of guinea pig trachea at a single time point. Unpaired *t* tests were used to evaluate differences between O_3- and air-exposed values for *PD*, I_{sc}, and *G*, and to evaluate the significance of differences between unidirectional ion fluxes.

Unpaired *t* tests were used to compare in vivo probe uptake rates in groups of guinea pigs after O_3 and air exposure (see Figures 4, 5, and 6).

RESULTS

NORMAL BIOELECTRIC PROPERTIES AND PERMEABILITY OF TRACHEA MEASURED IN VITRO

Because previous studies by Boucher and associates (1980) had shown that guinea pig airways (and mammalian airways in general) exhibited the highest *PD* values in the trachea, and lower values in the main bronchi and smaller bronchi, we first compared upper and lower trachea. The data are shown in Table 1 for 18 tracheas.

Regional Bioelectric and Permeability Characteristics

The upper trachea had a significantly higher *PD* than the lower trachea. The latter value (−7.9 ± 0.7 mV) is comparable to the in vivo mean value of −7.8 ± 1.1 mV reported by Boucher and colleagues (1980). Because the I_{sc} was similar in both regions, *G* was smaller in the upper trachea. The *G* value was paralleled by the smaller permeabilities (P_{solute}) observed in the upper trachea for each probe molecule, ranging from 40% to 60% of the permeabilities in the lower trachea. The monotonic decrease in solute permeability as a function of increasing molecular weight (and radius) was compatible with a model having a single population of cylindrical aqueous pores with an equivalent pore radius of 9 to 10 nm.

The data suggest that the relatively decreased electrical resistance of the lower trachea is due, in part at least, to increased passive ion movement through paracellular pathways. However, electron microscopic examination of tissues exposed in vitro to HRP, fixed at the conclusion of the experiment and stained for peroxidase activity, revealed very few intercellular spaces containing HRP in either the lower or the upper trachea. The lower trachea appeared to have more ciliated epithelial cells with diffuse non-membrane-bound uptake of HRP ("senescent" cells) than did the upper trachea. However, it is generally assumed that even mannitol (radius = 0.44 nm) is excluded from the cellular space. If polar probe molecule transit across this epithelium normally occurs largely through "senescent" ciliated epithelial cells, then there would be no obvious mechanism for molecular selectivity on the basis of molecular size.

Table 1. Bioelectric Properties of Normal Guinea Pig Trachea[a]

	PD[b] (mV)	I_{sc} (μA/cm^2)	*G* (mS/cm^2)	Permeability Coefficients of Solutes ($\times 10^{-7}$ cm/sec)			
				$P_{mannitol}$	P_{inulin}	$P_{dextran\text{-}10,000}$	$P_{dextran\text{-}70,000}$
Upper trachea (n = 18)[b]	−13.4 ± 0.9	70.3 ± 7.9	5.69 ± 0.65	2.29 ± 0.29	0.57 ± 0.13	0.18 ± 0.02	0.06 ± 0.02
Lower trachea (n = 18)	−7.9 ± 0.7	68.0 ± 10.4	9.59 ± 1.50	4.46 ± 0.39	0.94 ± 0.13	0.50 ± 0.08	0.14 ± 0.03
p Value, upper vs. lower trachea	< 0.001	NS[c]	< 0.013	< 0.001	< 0.05	< 0.005	< 0.05

[a] Data are given as mean ± SEM. Comparisons between parameter values for upper and lower trachea were analyzed by paired *t* tests.

[b] n = number of tissues studied.

[c] NS = not significant.

Table 2. Permeability Coefficients for Solute Movement Across Guinea Pig Trachea In Vitro Under Open Circuit Conditions

Solute	Molecular Radius (nm)	Molecular Weight	P_{solute} ($\times 10^{-7}$ cm/sec)[a] Upper Trachea	n[b]	Lower Trachea	n	p Value[c]
Mucosal→Serosal (m→s)							
^{14}C-mannitol	0.4	182	2.218 ± 0.256	23	4.529 ± 0.405	21	< 0.001
^{3}H-inulin	1.4	5,500	0.364 ± 0.079	19	0.782 ± 0.119	19	< 0.005
^{3}H-dextran	1.6	10,000	0.245 ± 0.039	6	0.597 ± 0.085	6	< 0.005
^{125}I-albumin	3.6	69,000	0.333 ± 0.039	6	0.433 ± 0.118	6	NS
^{14}C-dextran	3.8	70,000	0.072 ± 0.022	5	0.152 ± 0.046	5	< 0.05
Serosal→Mucosal (s→m)							
^{14}C-mannitol	0.4	182	2.733 ± 0.358	27	4.805 ± 0.664	28	< 0.01
^{3}H-inulin	1.4	5,500	0.479 ± 0.090	20	0.732 ± 0.141	21	< 0.05
^{3}H-dextran	1.6	10,000	0.450 ± 0.060	11	0.472 ± 0.062	12	NS
^{125}I-albumin	3.6	69,000	0.091 ± 0.025	7	0.089 ± 0.013	7	NS
^{14}C-dextran	3.8	70,000	0.062 ± 0.013	10	0.095 ± 0.016	11	NS

[a] Data are given as mean ± SEM.

[b] n = the number of tissues studied.

[c] Comparisons between upper and lower trachea were analyzed by paired t tests; NS = not significant.

Bidirectional Permeability of Probe Molecules

We then performed experiments in which permeabilities in both the m→s and s→m directions were measured. Individual tissues were used for m→s or for s→m fluxes. We attempted to match the electrical conductances of "pairs" of tissues (obtained from different guinea pigs) for these experiments to reduce the possible effect of different G values on the m→s:s→m flux ratios. In addition to the probes used previously, we also used ^{125}I-albumin in some experiments.

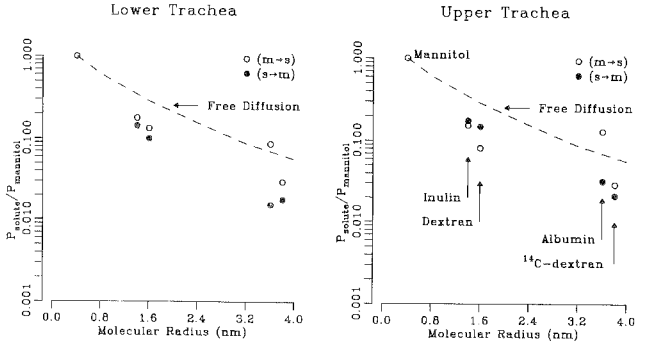

Figure 2. Bidirectional solute permeabilities of normal guinea pig trachea in vitro. Permeabilities of trachea to inulin, dextran-10,000, albumin, and dextran-70,000 (Table 2) normalized to mannitol are plotted on a logarithmic scale against their molecular radii. Data for lower and upper tracheal segments are in the left and right panels, respectively. The p values in the m→s and the s→m directions are shown in open and filled circles, respectively, for each solute. The identical dashed line in each panel represents the effect of molecular size on the unrestricted diffusion of solute in water.

The (unpaired) comparisons between m→s and s→m fluxes in upper and lower trachea for the various probes (Table 2) show that the differences are generally small except for albumin, whose absorption across airway epithelium is now known to represent an active process (Johnson et al. 1989). Thus, the difference (m→s vs. s→m) for upper trachea $P_{mannitol}$ is not significant ($p > 0.2$), nor are the differences for P_{inulin} ($p > 0.2$) and $P_{dextran\text{-}70,000}$. Only for $P_{dextran\text{-}10,000}$ is the difference significant ($p = 0.02$). We have no explanation for this finding. For the lower trachea, the m→s versus s→m differences are nonsignificant for all probes except albumin. In the case of albumin, however, the m→s permeability significantly exceeds s→m permeability ($p_{upper} = 0.01$; $p_{lower} = 0.02$).

Albumin is an important endogenous macromolecule. Its concentration in airway surface liquid in vivo has been reported to be of the order of 40 mg/dL, which is only about 1% of the serum albumin level (Boucher et al. 1981). It is therefore noteworthy that there is a marked asymmetry in the apparent unidirectional albumin permeabilities of guinea pig trachea (Table 2 and Figure 2). Flux ratios of 4.5:1 to 5:1 favoring albumin absorption were observed. In the s→m direction (Table 2), $P_{albumin}$ (0.9×10^{-7} cm/sec) was similar to the values for $P_{dextran\text{-}70,000}$ (0.8×10^{-7} cm/sec), and these molecules have a similar molecular radius. This suggests that albumin transport in the s→m direction may follow pathways similar to those used by polar probe molecules.

Table 3. Effect of Ozone on Bioelectric Properties of Excised Trachea from Two Hours to Seven Days After Exposure[a]

Exposure Group	n[b]	PD (mV)		I_{sc} (μA/cm²)		G (mS/cm²)	
		Upper Trachea	Lower Trachea	Upper Trachea	Lower Trachea	Upper Trachea	Lower Trachea
Air (control)	15	−10.2 ± 1.0	−6.4 ± 0.8	42.0 ± 3.6	31.8 ± 3.5	4.53 ± 0.50	6.08 ± 0.91
2 Hours after O_3	8	−15.6 ± 2.0	−11.6 ± 2.0	86.7 ± 14.4	89.1 ± 26.1	5.56 ± 0.53	7.94 ± 1.49
1 Day after O_3	8	−16.5 ± 2.9	−10.7 ± 0.8	50.3 ± 9.6	55.4 ± 6.4	3.82 ± 1.03	5.47 ± 0.80
2 Days after O_3	7	−12.0 ± 2.2	−12.0 ± 1.9	48.4 ± 9.7	47.0 ± 6.6	5.00 ± 1.54	4.70 ± 0.72
3 Days after O_3	7	−13.2 ± 3.3	−8.2 ± 1.4	43.0 ± 6.6	34.0 ± 8.2	4.44 ± 1.04	4.07 ± 0.72
7 Days after O_3	5	−10.8 ± 2.4	−6.7 ± 1.8	53.6 ± 21.0	30.9 ± 7.5	5.75 ± 2.08	6.08 ± 1.40

[a] Data are given as mean ± SEM.

[b] Number of tissues.

Table 4. Effect of Ozone on Bioelectric Properties and on Bidirectional Mannitol Permeability of Excised Lower Tracheal Segments from 2 to 72 Hours After Exposure[a]

Exposure Group	n[b]	PD (mV)	I_{sc} (μA/cm²)	G (mS/cm²)	$P_{mannitol}$ (× 10^{-7} cm/sec)	
					m→s	s→m
Air	16	−5.7 ± 0.6	20.7 ± 2.7	3.76 ± 0.36	2.88 ± 0.53	2.37 ± 0.52
2 Hours after O_3	16	−11.7 ± 1.5	51.1 ± 7.8	5.00 ± 0.79	1.55 ± 0.43	2.41 ± 0.41
24 Hours after O_3	12	−14.9 ± 1.2	42.6 ± 6.8	2.79 ± 0.33	1.26 ± 0.21	1.26 ± 0.16
48 Hours after O_3	11	−12.2 ± 1.0	39.7 ± 7.5	3.38 ± 0.63	1.42 ± 0.38	1.68 ± 0.39
72 Hours after O_3	11	−9.5 ± 1.5	34.9 ± 6.0	4.21 ± 0.72	2.30 ± 0.44	2.04 ± 0.40

[a] Data are given as mean ± SEM.

[b] n is the number of tissue pairs.

However, in the m→s direction, $P_{albumin}$ is anomalously high. (Johnson and coworkers [1989] have confirmed the sharp asymmetry of transepithelial albumin uptake in short-circuited canine bronchi in vitro at 37°C. They have shown that the transported albumin is extensively degraded [presumably by intracellular proteolysis] during the transport. At 4°C the flux ratio approaches 1, indicating a metabolic dependence for albumin absorption.)

The equivalent pore analysis for both lower and upper trachea is similar to that calculated for the previous set of experiments (Figure 2); that is, a single population of cylindrical aqueous pores with an equivalent pore radius of 9 to 10 nm. This is similar to what has been found for canine trachea in vitro (Boucher 1980).

EFFECT OF OZONE EXPOSURE ON BIOELECTRIC PROPERTIES OF TRACHEA MEASURED IN VITRO

The effects of a single in vivo three-hour exposure to 1 ppm O_3 on the bioelectric properties of excised guinea pig trachea measured from two hours to seven days after exposure are summarized in Table 3. A one-way ANOVA for these data shows the following significant changes after O_3 exposure: increased PD in the lower trachea ($p = 0.01$) and increased I_{sc} in both the lower trachea ($p = 0.001$) and the upper trachea ($p = 0.02$). Application of Dunnett's two-tailed t test showed that the PD increase (lower trachea) is driven by significant changes at two hours and at two days after exposure, and that the increased I_{sc} (both upper and lower trachea) is driven by significant changes at two hours after exposure. The changes in G were not significant.

Inspection of the mean control data for the bioelectric parameters reveals that the values are lower than in our earlier experiments. In particular, I_{sc} values (Table 3) are about half of those reported in Table 1. This may be due to the technical experience gained with this cylindrical tissue preparation between the time the earlier (Table 1) and later (Table 3) experiments were performed. As noted in the Methods section, the measurement of I_{sc} in a cylindrical tissue preparation is sensitive to the precise positioning of

Table 5. Effects of Ozone on Bidirectional Permeability of Excised Lower Tracheal Segments to Several Solutes from 2 to 72 Hours After Exposure[a]

Exposure Group	n[b]	$P_{mannitol}$		P_{DTPA}		P_{inulin}		$P_{albumin}$	
		m→s	s→m	m→s	s→m	m→s	s→m	m→s	s→m
Air	16	2.88 ± 0.53	2.37 ± 0.52	2.51 ± 0.50	2.20 ± 0.54	0.40 ± 0.12	0.50 ± 0.10	0.41 ± 0.13	0.094 ± 0.025
2 Hours after O_3	16	1.55 ± 0.43	2.41 ± 0.41	1.42 ± 0.40	1.61 ± 0.37	0.20 ± 0.07	0.44 ± 0.07	0.25 ± 0.10	0.078 ± 0.020
24 Hours after O_3	12	1.26 ± 0.21	1.26 ± 0.16	1.13 ± 0.19	1.01 ± 0.17	0.18 ± 0.04	0.33 ± 0.04	0.18 ± 0.03	0.058 ± 0.010
48 Hours after O_3	11	1.42 ± 0.38	1.68 ± 0.39	1.26 ± 0.31	1.37 ± 0.36	0.19 ± 0.07	0.35 ± 0.08	0.35 ± 0.11	0.054 ± 0.011
72 Hours after O_3	11	2.30 ± 0.44	2.04 ± 0.40	2.07 ± 0.43	1.64 ± 0.35	0.33 ± 0.08	0.45 ± 0.08	0.29 ± 0.09	0.069 ± 0.016

[a] Data are given as mean ± SEM × 10^{-7} cm/sec.

[b] n is the number of tissue pairs.

the intraluminal current-passing electrode. We may have become more adept at minimizing I_{sc} by careful positioning in the later experiments. Alternatively, there may have been some change over time in the lots of animals provided by the supplier. In cylindrical preparations, *PD* is a more reliable measure than is I_{sc}. The values in Table 3, though lower, are less divergent from those in Table 1. The value of G is calculated from *PD* and I_{sc} ($G = I_{sc}/PD$). The lower values of G in Table 3 would generally suggest a better tissue preparation and less edge damage.

To further evaluate the changes after O_3 exposure (1 ppm for three hours), we repeated this experiment with the addition of several permeability probes to either the mucosal or serosal sides of the bath. ^{14}C-mannitol and ^{3}H-inulin are typical uncharged aqueous probes that are thought to be excluded from the cellular compartment and therefore provide information about the paracellular pathway. ^{111}In-DTPA provided information about a molecule that is used for in vivo studies of respiratory epithelial permeability in humans (e.g., Kehrl et al. 1987). ^{125}I-albumin was used to confirm our finding of asymmetrical flux in control animals (absorption being four to five times more rapid than flux in the s→m direction) and to note the effect of O_3 exposure on this process.

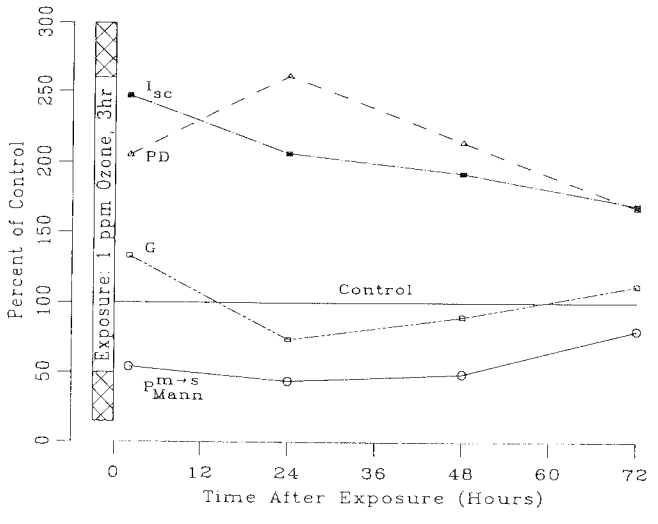

Figure 3. Time course of effects of O_3 exposure on bioelectric properties of guinea pig trachea. The data from Table 4 are presented graphically for each variable as a percentage of the control value: *PD* = −5.7 mV; I_{sc} = 20.7 μA/cm^2; G = 3.8 mS/cm^2; $P_{mannitol}$(m→s) = 2.9 × 10^{-7} cm/sec.

We killed the animals at 2 hours (n = 16), 24 hours (n = 12), 48 hours (n = 11), or 72 hours (n = 11) after termination of the O_3 exposure. Air-exposed animals killed 2 to 72 hours after exposure served as controls (n = 15). The control data showed no trends as a function of time after air exposure and were pooled.

The results for lower tracheal segments are shown in Table 4 (bioelectric properties and mannitol fluxes) and Table 5 (all permeability data). The results in Table 4 are also shown in Figure 3, in which the control values for I_{sc}, *PD*, G, and $P_{m\to s}$ of mannitol are normalized to 100% and the post-O_3 data at various times after O_3 exposure are represented as a percentage of this baseline.

Except for the period two hours after O_3 exposure, the bidirectional mannitol fluxes (last two columns of Table 4) have a ratio close to unity. We have no explanation for the deviation from unity at the two-hour point. (As shown in Table 5, the flux ratios for ^{111}In-DTPA, measured on the same tissues as mannitol, remained close to unity in all groups, including the animals evaluated two hours after O_3 exposure. Mannitol and ^{111}In-DTPA have very similar permeabilities).

An ANOVA for bioelectric parameters in Table 4 shows that the increase in *PD* after O_3 exposure was highly significant (p = 0.0001). This was driven by significant changes 2, 24, and 48 hours after exposure (Dunnett's two-tailed t test). The increase in I_{sc} was significant (p = 0.0092) due to the change at two hours after exposure. The changes in G had a p value of 0.11. ANOVA for the probe per-

meability data in Table 5 indicate that the decrease in m→s mannitol flux after O_3 exposure was significant ($p = 0.04$), and the decrease in m→s DTPA flux approached significance ($p = 0.08$). The change in $P_{m\rightarrow s}$ of mannitol was driven by the 24-hour postexposure point.

A possible explanation of the bioelectric changes (Table 4) is that apical membrane permeability to Cl^- and Na^+ is increased after O_3 exposure. Increased Na^+ entry across the apical membrane stimulates the basolateral Na^+-K^+ pump and causes an increase in Na^+ absorption that is electrogenic. Increased apical Cl^- permeability could also enhance electrogenic secretion of Cl^- delivered in neutral form into the cell by cotransport of Na-Cl (or of Na-K-2Cl) across the basolateral membrane. In addition, because passive ion conductances in airway epithelia are generally dominated by Cl^- (see Table 7: J_{Cl^-} m→s vs. J_{Na^+} s→m), such an increase in apical membrane permeability to Cl^- could also account for the trends toward increase in the mean value of G despite a decrease in the mean values for conductance through the paracellular pathway (i.e., probe permeabilities) at two hours after O_3 exposure. The trend toward a decrease in G at 24 hours after O_3 exposure, at a time when $P_{mannitol}$ has reached its nadir (and $P_{m\rightarrow s}$ mannitol is significantly reduced), would then require a decrease in transcellular Cl^- conductance. The persistence of a high PD value at this time might, therefore, suggest persistently elevated electrogenic Na^+ absorption.

In Table 5, the mannitol data are repeated from Table 4. The mannitol and DTPA permeabilities have already been discussed. The mean $P_{m\rightarrow s}$ values for inulin remained approximately 0.14 of the $P_{m\rightarrow s}$ values for mannitol at all times. This was somewhat smaller than our previously measured ratios for m→s fluxes of inulin and mannitol (0.17 to 0.18) in upper and lower tracheal segments from control animals. In those measurements, the m→s and s→m flux ratios for inulin and for mannitol were close to 1. However, in the data in Table 5, inulin flux was consistently larger in the s→m than the m→s direction. We have no explanation for this consistent inequality (which is not present in the simultaneously measured mannitol and DTPA fluxes).

The albumin fluxes again showed a highly asymmetrical flux ratio of 4.4, favoring absorption in the control group. After O_3 exposure, mean albumin m→s fluxes decreased, reaching a nadir of 44% of control at 24 hours after exposure. This trend did not, however, attain statistical significance. The s→m albumin fluxes also decreased (though not significantly), so that the flux ratio remains at least 3.0 at all times after O_3 exposure. Because albumin movement in the absorptive (m→s) direction is largely a transcellular process that depends on a specific apical membrane uptake mechanism (Johnson et al. 1989), the trend toward decrease in m→s flux after O_3 exposure could indicate a disruption of this process. If it occurred in vivo, it would favor higher albumin concentrations in airway surface liquid. Indeed, bronchoalveolar lavage in guinea pigs 15 hours after exposure to only 0.26 ppm O_3 for three hours (Hu et al. 1982) showed increased albumin levels. (Koren and coworkers [1989] also found a two-fold greater concentration of albumin in the bronchoalveolar lavage fluid from human subjects 18 hours after a two-hour exposure to 0.4 ppm O_3 when compared to the fluid from control subjects.) These findings were attributed by the authors to increased permeability, that is, increased s→m albumin flux. However, it is possible that impaired albumin resorption may also be responsible.

EFFECT OF OZONE EXPOSURE ON AIRWAY EPITHELIAL PERMEABILITY IN VIVO

As noted above, the paracellular permeability of guinea pig trachea, studied in vitro following in vivo exposure to O_3, tends to decrease, and is certainly not increased. We had reason to believe that this would not be the case in vivo (Davis et al. 1980). We therefore reinvestigated this question using a technique developed and applied by Dr. R. C. Boucher in Dr. J. C. Hogg's laboratory at McGill University (Boucher et al. 1978). The technique and its limitations are described in the Methods section.

We exposed the animals to 1 ppm O_3 for three hours, or to purified air (control) in a similar manner. In addition, we exposed animals to 1 ppm O_3 (three hours) or to purified air (control) on four consecutive days. After termination of the final exposure (either one exposure or four consecutive daily exposures), we anesthetized and prepared the animal for the in vivo permeability measurement.

In Figure 4, the single-exposure data are shown in the left-hand panels for each probe. The lowest panel (^{14}C-mannitol) shows that progressive uptake occurs in control animals. The process is greatly augmented after O_3 exposure. The plateau of plasma levels (20 and 30 minutes) probably is due to the progressive depletion of the source (airway lumen) and to the rapid exit of mannitol from the plasma compartment. The data for ^{111}In-DTPA are almost identical to those for mannitol. As expected, the inulin data show much lower uptakes for the control animals (the ordinate scale is reduced) but with a sharp increase in uptake after O_3 exposure. Significant but quantitatively smaller increases in airway permeability in vivo have been observed by us after a single exposure of guinea pigs to 0.3 ppm O_3 (Figure 5). These data confirm that the apparent effect of O_3 exposure on airway permeability to aqueous probe molecules depends on whether the measurements are carried

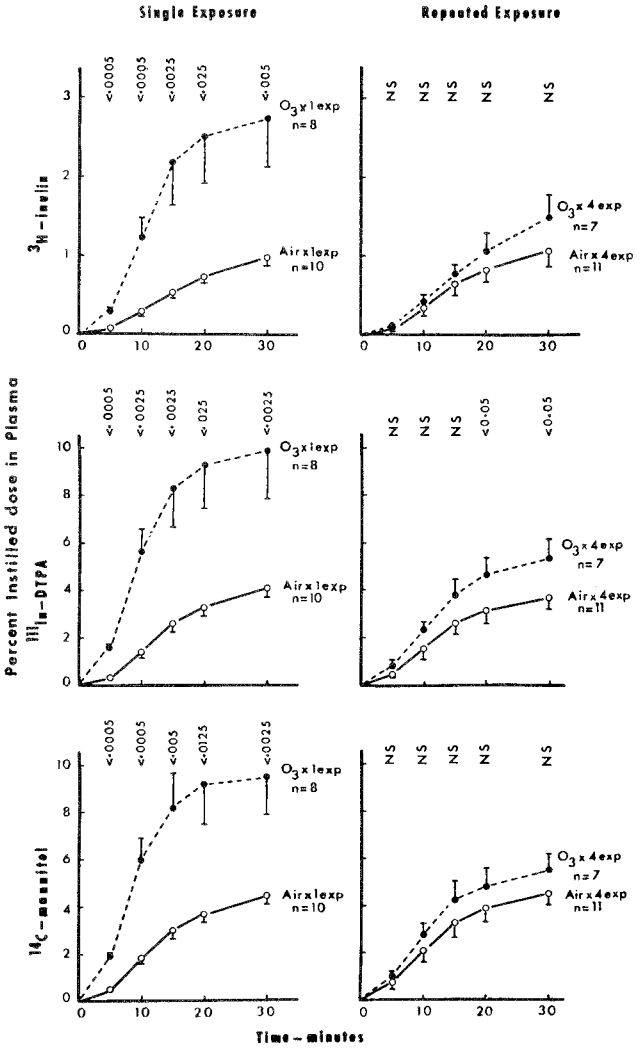

Figure 4. Effect of O_3 exposure on guinea pig solute permeability in vivo. In vivo appearance of solutes (^{14}C-mannitol, ^{111}In-DTPA, and ^{3}H- inulin) in blood plasma at 5 to 30 minutes after their simultaneous instillation onto the surface of the trachea in sham-exposed (open circles) and in O_3-exposed (1 ppm) (filled circles) guinea pigs. The plasma concentrations are expressed as percentage of instilled dose (see text). The three panels on the left depict mean data (± SEM) after a single exposure. The three panels on the right depict data following four consecutive daily exposures. The numbers above each panel represent the *p* values for the significance of the difference between the experimental and control groups. The number of animals in each experimental group is shown for each panel.

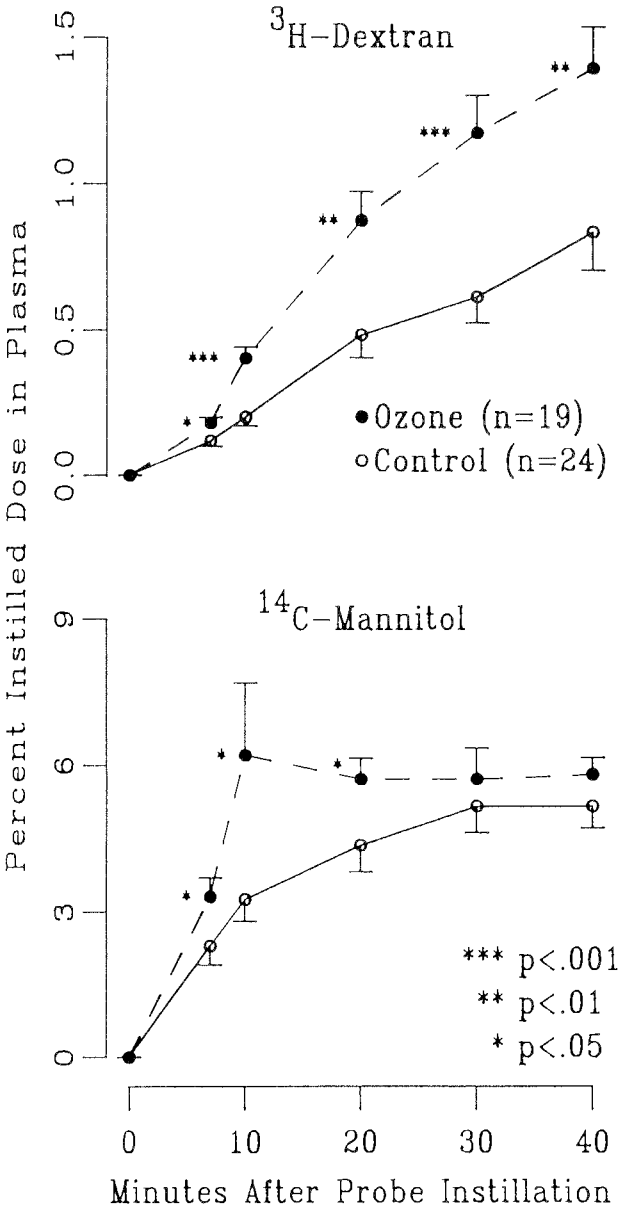

Figure 5. Effect of exposure to 0.3 ppm O_3 on guinea pig tracheal solute permeability in vivo. In vivo appearance of solutes (^{3}H-dextran-10,000, top; ^{14}C-mannitol, bottom) in blood plasma at 6 to 40 minutes after their simultaneous instillation onto the surface of the trachea in sham-exposed guinea pigs (open circles) and in animals exposed once to 0.3 ppm O_3 for three hours (filled circles). Asterisks indicate *p* values for the difference between the control and experimental groups at each time point.

out in vitro or in vivo. Possible reasons for this dependence are discussed below.

The data after four consecutive daily exposures are shown in the right-hand panels of Figure 4. The group of control animals for this protocol is indistinguishable from the control group for the single exposure (compare right with left panels). However, the effect of O_3 exposure has been greatly attenuated. Indeed, in comparing control exposure with O_3 exposure, only the 20- and 30-minute values for ^{111}In-DTPA were statistically significantly higher.

Figure 6 summarizes these in vivo permeability data. The ordinate represents the initial rate of plasma accumulation

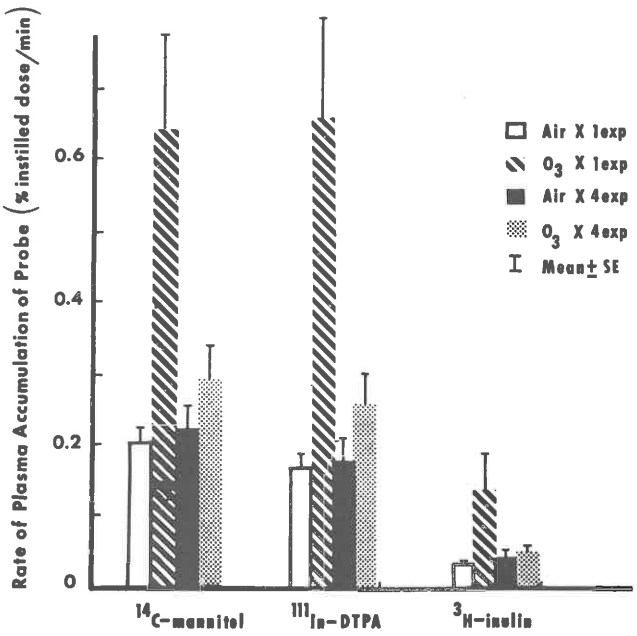

Figure 6. Effect of exposure to 1 ppm O_3 on guinea pig tracheal solute permeability in vivo. The heights of the bars represent mean uptake rates for solutes into plasma (percentage of instilled dose per minute) during the first 15 minutes after the start of instillation onto the trachea. Four experimental groups are depicted for each of three solutes (mannitol, DTPA, and inulin). Open bar indicates single sham exposure (n = 10); cross-hatched bar indicates single O_3 exposure (n = 8); solid bar indicates four consecutive daily sham exposures (n = 11); stippled bar indicates four consecutive daily O_3 exposures (n = 7).

for each probe computed from the slope of a linear fit to the points for 0, 5, 10, and 15 minutes. The two air controls are indistinguishable for each of the three probes. The single O_3 exposure increases the uptake rates for mannitol, DTPA, and inulin threefold to fourfold. However, after the fourth consecutive daily O_3 exposure, the mean probe molecule uptake rates are only 1.3- to 1.4-fold higher than those of the controls, and these increases did not attain statistical significance. The large differences between the mean probe molecule uptake rates in comparing a single exposure with four consecutive daily O_3 exposures are, however, highly significant.

Ozone-induced in vivo hyperpermeability therefore undergoes "adaptation" on repeated daily exposures, with a time course generally compatible with that described for "adaptation" of the effects of O_3 exposure on lung function in human subjects (Hackney et al. 1977). Because the latter effects are likely to involve toxicity to superficial airway structures (rather than lung parenchyma), an animal model in which adaptation of an O_3-induced airway effect is demonstrable may be of particular relevance.

MORPHOLOGIC CHANGES AND PATHWAYS OF TRANSEPITHELIAL SOLUTE MOVEMENT

From the work of Boucher and associates (1978), it is known that HRP (molecular radius 3.4 nm) instilled onto the airway surface of normal anesthetized guinea pigs in vivo slowly appears in the plasma. The precise anatomic pathway by which HRP uptake across airway epithelium occurs remains unclear. However, smaller probes (e.g., mannitol [molecular radius 0.44 nm], inulin [molecular radius

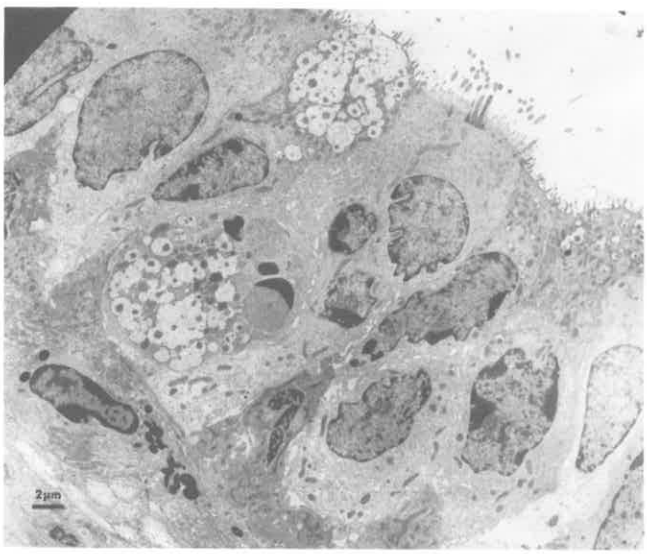

Figure 7. Electron micrograph of control trachea fixed in vivo in the presence of luminal HRP and stained for peroxidase activity. Several epithelial cells are seen, including secretory and ciliated cells. The lumen is to the upper right. No reaction product is seen in the intercellular spaces or in the secretory cells.

Figure 8. "Senescent" cells in control trachea fixed in vivo in the presence of luminal HRP and stained for peroxidase activity. The lumen is above. Three diffusely staining ciliated cells are present, indicating in vivo HRP uptake.

1.4 nm]) probably move largely through paracellular channels. The state of our understanding of these pathways for transepithelial movement of solutes in the airways has been reviewed and summarized by Boucher and Ranga (1988).

In our in vivo studies of guinea pig tracheal epithelium, we rarely saw peroxidase reaction product in an intercellular space in control animals (Figure 7). We did note that certain ciliated cells ("senescent" cells) stained profusely and diffusely for HRP (Figure 8). However, we were unable to define clearly any reaction product escaping into the submucosa from these potential sites of HRP passage.

After an acute three-hour exposure to 1 ppm O_3 (Figures 9 and 10), there was abundant reaction product in about two-thirds of the intercellular spaces in the epithelium, extending up to the region of the zonula occludens (tight junction). Although this does not prove that HRP penetrated through the tight junctions, it is quite suggestive. The number of "senescent" epithelial cells was decreased to 36% of their baseline number at 2 hours after O_3 exposure, and this decrease persisted for 72 hours. Another potential pathway for HRP penetration is via secretory cells that have been stimulated to secrete mucus into the lumen. Ozone exposure causes striking mucin secretion from the superficial epithelium (Figures 11a and 11b) as well as inflammatory cell exudation (Figure 11c). After O_3 exposure, HRP reaction product can be identified within vesicles in secretory cells, and HRP uptake may occur by this mechanism. It is difficult, however, to show convincing evidence of HRP secretion across the basolateral membranes of such cells and to quantify such phenomena.

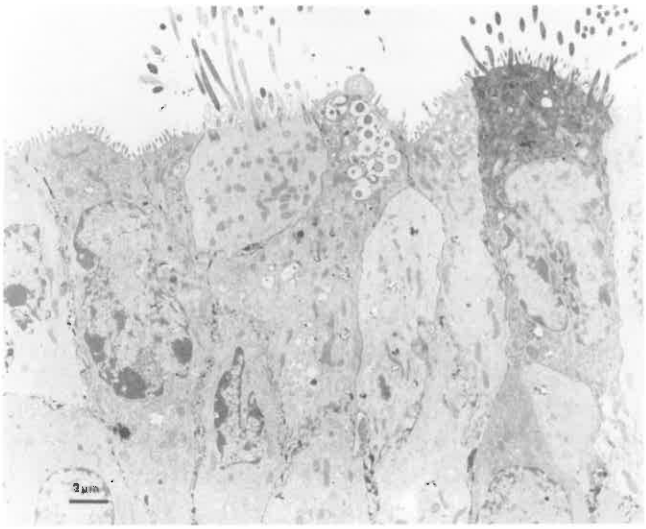

Figure 9. Effect of O_3 exposure on HRP penetration of guinea pig tracheal epithelium. Electron micrograph of trachea fixed in vivo in the presence of luminal HRP after O_3 exposure. Peroxidase reaction product is diffusely present in the intercellular spaces. In addition, vacuole-bounded reaction product is present in secretory cells.

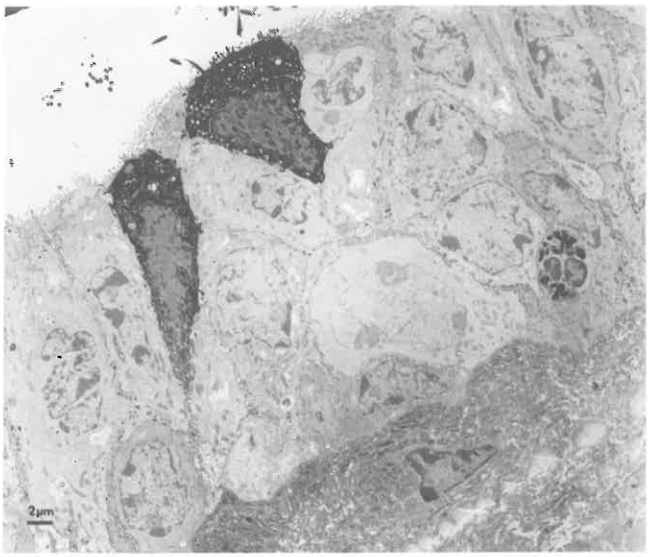

Figure 10. Effect of O_3 exposure on HRP penetration of guinea pig tracheal epithelium. Electron micrograph of trachea fixed in vivo in the presence of luminal HRP after O_3 exposure. In addition to peroxidase activity in the intercellular spaces, two ciliated cells are seen to be heavily and diffusely stained with reaction product.

Thus, we suspect that after O_3 exposure, HRP uptake occurs principally via the paracellular pathway following penetration across tight junctions. The smaller probe molecules probably follow the same pathway. However, in a blinded study, Dr. John Carson (Department of Pediatrics) in our institution examined freeze-fracture replicas of guinea pig tracheal epithelium and did not detect any change in tight-junction structure following exposure to 1 ppm O_3 for three hours.

When the O_3-exposed trachea was excised and mounted in a bath in vitro before addition of HRP to the luminal fluid, no reaction product was identified in the intercellular spaces. This is consistent with the absence of any permeability increase for mannitol and other aqueous probes (previously discussed) when the measurements are carried out in vitro.

ADDITIONAL STUDIES OF THE EFFECTS OF OZONE EXPOSURE ON BIOELECTRIC PROPERTIES

In contrast to our in vitro finding of a prolonged increase in transepithelial electrical *PD* in excised lower tracheal cylinders after O_3 exposure, measures of in vivo lower tracheal surface *PD* in anesthetized, tracheotomized guinea pigs, performed in collaboration with Dr. M. R. Knowles in

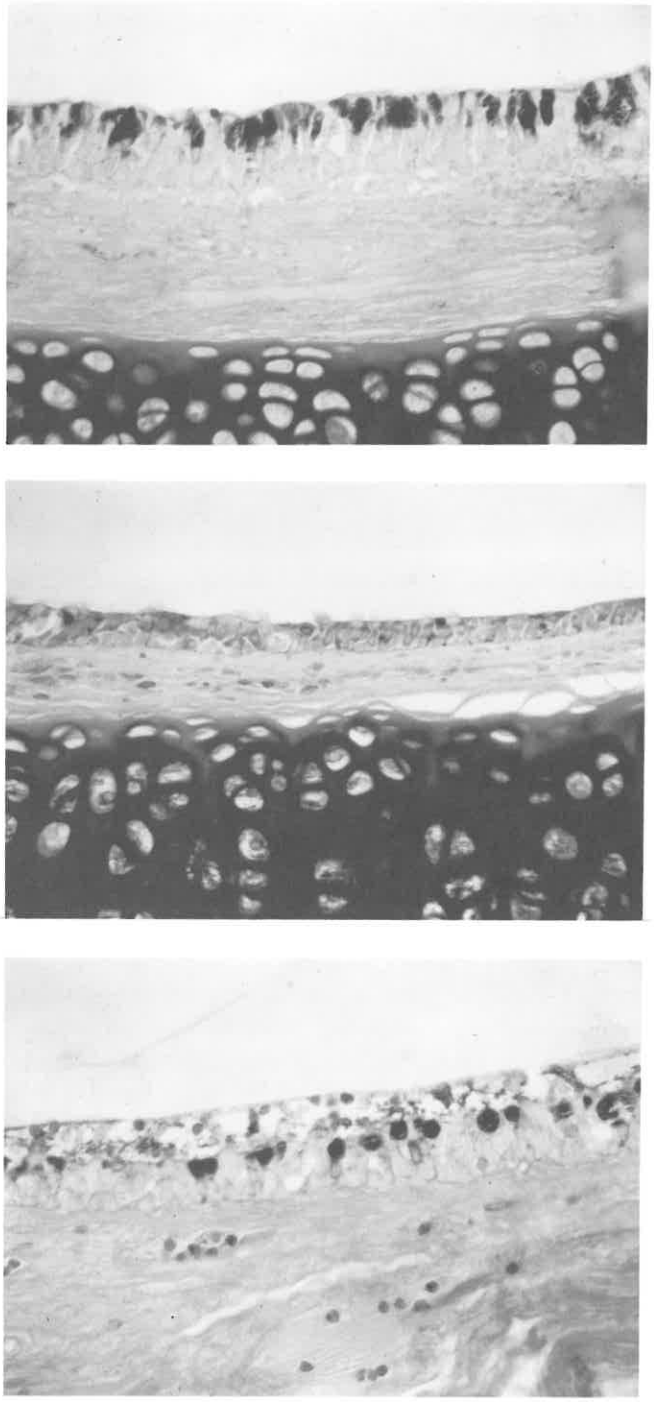

Figure 11. Inflammatory effects of O_3 exposure on guinea pig tracheal epithelium. Tracheal cross sections were stained for mucin with periodic acid-Schiff and Alcian blue. Top: Control animal showing many surface cells filled with mucin. Middle: After O_3 exposure, few stainable secretory cells are visible on the surface. Bottom: After O_3 exposure, exudate is visible on the luminal surface.

our Division, revealed no increase after similar exposure. The technique used was that described by Boucher and coworkers (1980). Normal guinea pig tracheal surface *PD* ranges from −6 to −10 mV (Boucher et al. 1980). This is quite consistent with the control data obtained from in vitro preparations. However, after O_3 exposure, no elevation of in vivo *PD* was found in six animals. (We did not attempt to study these tracheas in vitro.)

We therefore decided to remeasure the bioelectric properties of guinea pig trachea in vitro using the classical planar tissue configuration as the barrier between the two halves of an Ussing chamber. No effort was made to distinguish lower from upper trachea.

In Table 6, we compare the mean values for baseline (control) bioelectric properties of planar preparations with values for cylindrical preparations from the lower trachea taken from Table 4. Although the voltages are relatively similar, the planar preparation has a higher I_{sc} value and higher conductance. The mean mannitol permeability of the planar preparations was 23.8×10^{-7} cm/sec, as compared to our mean for cylindrical preparations of 2.9×10^{-7} cm/sec (Table 4). This eight-fold difference suggests that our planar preparations had significant edge damage, due to the very small chamber surface area (0.26 cm^2) and to the technical difficulties of mounting the tissue. Such artifact reduces the sensitivity of ion flux measurements to the effect of specific cellular mechanisms.

Nevertheless, we proceeded to study the effects of our three-hour, 1 ppm O_3 exposure protocol on these older, larger guinea pigs. The investigators were blind as to the exposures received by any particular animal (i.e., purified air or O_3; see the Methods section).

Table 7 shows that O_3 exposure was again found to increase I_{sc} significantly (by about 40%) without change in G (or in $P_{mannitol}$), resulting in a significant increase of about 45% in *PD*. As noted above, control $P_{mannitol}$ was relatively high, and control G was also higher than in the tracheal cylinder preparations.

The net sodium flux (J_{Na^+} net) accounted for 93% of control I_{sc}. However, the ion flux data did not clearly reveal the source of the 40% increase in I_{sc} following O_3 exposure. The expected difference in net ion flow for an increase in I_{sc} of 15 $\mu A/cm^2$ is only 0.5 $\mu Eq/cm^2/hr$, which is at the limit of resolution of these techniques. The post-O_3 changes in mean net Na^+ and Cl^- flows were in the right directions but were not statistically significant.

We added 30 μM amiloride, a relatively specific blocker of apical membrane Na^+ channels, to the luminal bath of control tissues and of O_3-exposed tissues. In control tissues, this resulted in a decrease in net Na^+ absorption to 0.4 ± 0.3 $\mu Eq/cm^2/hr$, a value not significantly different

Table 6. Comparison of Bioelectric Properties of Excised Cylindrical Segments of Lower Trachea with Planar Tracheal Preparations from Both Upper and Lower Trachea

	n[a]	PD (mV)	I_{SC} ($\mu A/cm^2$)	G (mS/cm^2)
Cylindrical (lower trachea)	16	− 5.7 ± 0.6	20.7 ± 2.7	3.8 ± 0.4
Planar (all parts of trachea)	28	− 4.7 ± 0.5	37.3 ± 2.9	8.6 ± 0.5

[a] n is number of tissues.

Table 7. Effect of Ozone Exposure on Bioelectric Properties, Bidirectional Sodium and Chloride Fluxes, and Mannitol Permeability in Planar Tracheal Preparations[a]

	Control (n = 14)[b]	Immediately (3 hr) After O_3 Exposure (n = 7)
PD (mV)	− 4.7 ± 0.5	− 6.8 ± 1.0[c]
I_{SC} ($\mu A/cm^2$)	37.3 ± 2.9	52.5 ± 6.5[c]
G (mS/cm^2)	8.6 ± 0.5	8.3 ± 0.6
J_{Na^+} ($\mu Eq/cm^2/hr$)		
m→s	4.3 ± 0.5	4.3 ± 0.8
s→m	3.0 ± 0.6	2.8 ± 0.6
Net	1.3 ± 0.4[d]	1.5 ± 0.3[d]
J_{Cl^-} ($\mu Eq/cm^2/hr$)		
m→s	6.1 ± 0.5	6.6 ± 0.5
s→m	6.7 ± 0.3	7.4 ± 0.7
Net	− 0.6 ± 0.6	− 0.8 ± 0.7
$P_{mannitol}$ ($\times 10^{-7}$ cm/sec)		
m→s	23.8 ± 3.6	19.5 ± 3.6
s→m	23.1 ± 3.0	24.2 ± 6.1

[a] Data are given as mean ± 1 SEM.

[b] n is number of tissue pairs.

[c] Different from sham exposure, $p < 0.05$.

[d] Net difference from zero, $p < 0.05$.

from zero. I_{SC} was, however, almost unchanged (decrease of 2 $\mu A/cm^2$). This is presumably due to increased secretion of Cl^- driven by hyperpolarization of the apical membrane. After O_3 exposure, amiloride caused an absolutely and proportionately larger decrease in I_{SC} (from 52 to 30 $\mu A/cm^2$). This is compatible with increased Na^+ entry across the apical membrane (leading to increased Na^+ absorption) as a mechanism underlying the response of the epithelium to O_3 exposure.

We also attempted to study trachea from animals killed 24 hours after O_3 exposure. Unfortunately, the electrical conductance values and mannitol permeabilities were markedly elevated and the data were deemed unacceptable.

The results of this blinded experiment using conventional methodology to assess bioelectric properties of guinea pig tracheal epithelium in vitro generally confirm our more extensive studies using cylindrical preparations. We are, therefore, confident that O_3 exposure of guinea pigs induces stimulation of active ion transport by airway epithelium. This change appears to be intrinsic to the epithelium and is not likely to depend on the presence of diffusible mediators produced by other cells. The persistence of the PD increase (in lower tracheal cylinders) also suggests that O_3 exposure causes a relatively irreversible, but specific, change in the epithelial cells themselves.

DISCUSSION

These studies in guinea pigs demonstrate that exposure to moderate concentrations of O_3 produces important effects on airway epithelial function. Permeability and ion transport are both affected, and the changes evolve over a period of days following a single exposure.

The permeability effects are easily detected in guinea pigs in vivo and appear to be present in human subjects as well (Kehrl et al. 1987). When the trachea is removed from the O_3-exposed animal, however, and then studied in vitro, no increase in permeability is seen. Similar observations have been made in O_3-exposed Sprague-Dawley rats (Bhalla and Crocker 1986; Bhalla et al. 1986; Rasmussen and Bhalla 1989), although the degree of in vivo permeability increase caused by O_3 may be less in this species than in guinea pigs. Bhalla and associates (1988) and Rasmussen and Bhalla (1989) have suggested that the transduction of the epithelial permeability response to O_3 in rat airways may involve destabilization of the microfilament component of the cytoskeleton.

The procedure used to evaluate airway epithelial permeability in vivo does not strictly limit instilled probe molecules to the trachea in spite of our efforts to minimize diffuse distal aspiration (see Methods section). Furthermore, it is known from histopathologic and mitotic index studies that O_3 exposure damages ciliated epithelial cells in small airways and type I alveolar epithelial cells. Therefore, it is possible that our in vivo permeability data could reflect O_3-enhanced probe molecule uptake from more distal airways without an effect on the trachea itself. The in vitro experiments with tracheal cylinders removed from O_3-exposed animals would be expected to give different results under this set of assumptions.

However, toxicologic studies show damage to ciliated cells in the trachea as well as distal airways following O_3 exposure, and our own histologic observations show that O_3 inhalation caused the appearance of inflammatory cells and marked mucin secretion in the tracheal epithelium (Figure 11), as well as more distally. Furthermore, HRP was abundantly present in the intercellular spaces of tracheal epithelium in O_3-exposed (but not control) animals when this probe molecule was instilled in vivo (Figures 9 and 10).

Thus, we believe the evidence supports the interpretation that the trachea participates in the genesis of increased solute permeability observed in vivo after O_3 exposure; the absence of these permeability increases when tracheas are removed from O_3-exposed animals and are studied in vitro requires further discussion.

We suggest that the post-O_3 increase in tracheal epithelial permeability in vivo may depend on soluble mediators that modulate epithelial function and are diluted or washed out in the in vitro situation. Because O_3 exposure resulted in an inflammatory response in our experiments, these putative mediators may be linked in some manner to the inflammatory response. (However, note the absence of neutrophilic infiltrate in the tracheas of Long-Evans rats following O_3 exposure [Evans et al. 1988].)

The demonstration of a post-O_3 increase in airway epithelial permeability to molecules as large as HRP raises the possibility that transepithelial penetration of inhaled allergens might be facilitated following O_3 exposure. Some evidence of enhanced response of experimentally sensitized rodents to challenge with antigen after exposure to high O_3 concentrations has been published (Matsumura 1970a,b,c; Osebold et al. 1980). Individuals with asthma do not seem to hyperreact to O_3 exposure (Linn et al. 1978; Silverman 1979; Koenig et al. 1985). However, experiments involving antigen challenge of lower airways after O_3 exposure have not been reported.

Our observation that the in vivo permeability response to O_3 exposure is abolished after four consecutive daily exposures offers an experimental approach to defining the mechanisms responsible for the increased permeability. This "adaptation" phenomenon also recalls the adaptation to repeated daily O_3 exposures observed in human subjects. Work is in progress that is designed to establish whether or not "adaptation" to O_3 in humans is accompanied by the disappearance of the inflammatory response that characterizes a single exposure (Seltzer et al. 1986; Koren et al. 1989).

Other approaches to studying the pathogenesis of increased permeability after O_3 exposure could follow the procedures adopted by investigators at the Cardiovascular Research Institute in San Francisco and by Murlas and his colleagues in their studies of the pathogenesis of airway hyperreactivity after O_3 exposure. These investigators used various pharmacologic inhibitors in dogs (O'Byrne et al. 1984a; Lee and Murlas 1985; Murlas and Lee 1985; Murlas et al. 1986) and neutrophil depletion protocols in guinea pigs (O'Byrne et al. 1984b; Murlas and Roum 1985b). Both exposed the animals to O_3 concentrations of at least 2 ppm. Pharmacologic inhibition of enzymes responsible for oxidative metabolism of arachidonate to active eicosanoids appeared to alter the hyperreactivity observed after O_3 exposure (O'Byrne et al. 1984a; Lee and Murlas 1985; Murlas and Lee 1985; Murlas et al. 1986). Interestingly, Schelegle and colleagues (1987) have reported that indomethacin pretreatment caused partial inhibition of the lung function decrement observed in healthy humans after O_3 exposure. They made no observations on the inflammatory response. Using ibuprofen to block cyclooxygenase, our group (M. J. Hazucha, personal communication, 1990) confirmed the observation of Schelegle and coworkers (1987) but found that the neutrophilia observed in bronchoalveolar lavage fluid after O_3 exposure was unaffected.

The chemotactic factors responsible for the O_3-induced neutrophilic inflammation of airways remain unclear. Bronchoalveolar lavage studies after O_3 exposure have failed

to reveal increased concentrations of known chemotactic agents like complement 5a (C5a), leukotriene B_4 (LTB_4), or 15-hydroxyeicosatetraenoic acid (15-HETE) (Seltzer et al. 1986; Koren et al. 1989). Platelet-activating factor (PAF) is chemotactic for neutrophils, and Drs. J. Samet and M. Friedman in our group have recently obtained evidence (unpublished) that PAF production by cultured alveolar macrophages is increased by O_3 exposure. They have not yet looked for in vivo correlations, however.

We have considered the possibility that O_3 directly or indirectly causes neurogenic inflammation in the airways. Neurogenic inflammation involves stimulation of the C-fiber system with consequent local and regional release of tachykinins. The intraepithelial location of some C-fiber branches makes them a potential target for O_3 effects (Lundberg et al. 1983a,b, 1984). We attempted to ablate the C-fiber system by pretreatment of male Hartley guinea pigs with capsaicin. Such "prepared" animals were later exposed to 1 ppm O_3 for two hours. Unexpectedly, however, capsaicin pretreatment appeared to potentiate rather than inhibit O_3-induced changes in respiratory frequency and tidal volume (Tepper et al. 1990).

The effects of O_3 exposure on airway epithelial ion transport assessed in vitro imply significant alterations in the complex mechanisms that regulate transcellular ion transport in this tissue. The high reactivity of O_3 and the presence of these post-O_3 changes in tissues bathed in KRB medium suggest a direct effect of O_3 on ion transport mechanisms in the apical membrane of the epithelial cells. The ability of amiloride to abolish these O_3-induced changes suggests further that increased Na^+ absorption is involved.

The disease cystic fibrosis is marked by airway hyperabsorption of Na^+ (as well as Cl^- impermeability). The recent identification of the gene that is mutated in most patients with cystic fibrosis and the inferred amino acid sequence of the complex membrane-spanning protein resulting from transcription and translation of this gene (Riordan et al. 1989) may also offer insights into possible modes of action of O_3 on ion transport across the apical membrane of respiratory epithelial cells.

We suspect that the effects of O_3 on airway epithelial ion transport in vivo are masked by the marked increase in paracellular permeability (previously discussed) associated with increased paracellular ionic conductance. Such changes would allow active ion transport to increase without a corresponding change in the transepithelial electrical potential difference.

Ozone exposure produces multiple effects on the lower airways. These include discharge of mucin from surface secretory cells (guinea pigs), neutrophilic inflammation (guinea pigs, dogs, and humans), increased in vivo permeability to polar molecules (guinea pigs, rats, and humans), and airway hyperreactivity to bronchoconstricting autocoids like histamine and acetylcholine (several mammalian species including humans). We have now also demonstrated an enhancement of active tracheal epithelial ion transport (guinea pigs). In addition, O_3 exposure causes altered respiratory regulation with decreased respiratory tidal volume and increased respiratory frequency (rats, dogs, and humans), probably via neural reflexes originating in the lower airways.

It seems unlikely that these multiple effects are attributable to a single locus of O_3 attack. Because of the high reactivity of this oxidant pollutant, however, it is likely that the most superficial tissues of the lower airways are the site of chemical attack. In addition to the epithelial cells themselves, sensory nerve endings are found intraepithelially. Macrophages are found on the surface of the deeper regions of the lung and may also be present more proximally. Mast cells and neurosecretory cells are also normally found close to the surface. In terms of sheer number the epithelial cells must constitute a major target, and the particular susceptibility of ciliated cells in the airways to O_3 damage (morphologic criteria) has been well established (Boatman et al. 1974; Stephens et al. 1974; Mellick et al. 1977).

Our data on bioelectric properties and ion transport support the proposition that O_3 directly attacks specific molecules associated with the apical membrane of airway epithelial cells. Epithelial cells subjected to various stimuli are currently under intense scrutiny as the possible source of a variety of mediators that may serve autocrine and paracrine functions or that may have chemotactic activity. Eicosanoids and other derivatives of phospholipid metabolism have received particular attention. Such mediators might then be responsible for a variety of O_3 effects including mucin discharge, sensory neural stimulation, hyperpermeability, neutrophilic inflammation, and bronchial hyperreactivity.

The precise relations among these multiple effects are unclear at present. Murlas and Roum (1985a) have been able to demonstrate bronchial hyperreactivity to parenteral acetylcholine immediately after very short exposures of guinea pigs to 3 ppm O_3, before demonstrable neutrophilic inflammation, or even in neutropenic animals (Murlas and Roum 1985b). Evans and coworkers (1988) found that O_3-induced (4 ppm for two hours) bronchial hyperreactivity was not accompanied either by neutrophil influx or by increased microvascular permeability in the trachea in Long-Evans rats. Yet, in dogs there is good evidence for an association between neutrophilic inflammation and bronchial hyperreactivity (Holtzman et al. 1983; Fabbri et al. 1984; O'Byrne et al. 1984b). In humans there appeared to be a

correlation between the increase of neutrophils in bronchoalveolar lavage fluid several hours after O_3 exposure (0.4 and 0.6 ppm) and the development of bronchial hyperreactivity (Seltzer et al. 1986).

Thus, there are likely to be important interspecies differences and even interstrain differences within a species relevant to these phenomena. The choice of animal models for toxicologic studies of O_3 therefore requires careful attention if the results are to be applied to humans.

Recently, immortalized human bronchial epithelial cell lines have been developed that produce epithelial monolayers in culture. These cultured immortalized epithelia retain a number of features of native epithelium and of primary cultures of disaggregated epithelial cells, although they are not normally differentiated morphologically. The availability of such cultured epithelia will help to overcome the problem of limited availability of normal human respiratory epithelial cells for experimental studies of toxicant effects.

IMPLICATION OF FINDINGS

The continuing widespread presence of tropospheric O_3 at levels that have been shown to cause decrements in human lung function, and the difficulties likely to be encountered in attempting to reduce these levels by regulation of various emissions, make it particularly important to understand in some detail the toxicology of repeated, as well as single, exposures to this oxidant, and particularly its potential for causing irreversible changes. Such information will be invaluable in regulatory decision-making, in enforcement, and, ultimately, in public acceptance of these decisions.

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ABOUT THE AUTHORS

Philip A. Bromberg received the M.D. from Harvard Medical School in Boston, MA, in 1953. He is currently the Bonner Professor of Pulmonary Medicine at the University of North Carolina School of Medicine in Chapel Hill, NC, where he is also director of the Center for Environmental Medicine and Lung Biology. His research interests focus on environmental toxicology.

Venkatachalam Ranga received the M.B.B.S. from the All India Institute of Medical Sciences in 1971. After clinical training in the United States, he was a research fellow with Professor J. C. Hogg at McGill University, Montreal, Quebec, Canada, and with Professor J. Kleinerman at Case Western Reserve University, Cleveland, OH. At the time of his death in 1985 he was an Assistant Professor of Medicine (Respiratory Diseases) and a staff member of the Center for Environmental Medicine and Lung Biology, University of North Carolina School of Medicine, Chapel Hill, NC.

M. Jackson Stutts received the Ph.D. in pharmacology and toxicology from the University of North Carolina at Chapel Hill in 1978. After postdoctoral work with Professor Richard Boucher in the Respiratory Diseases Division in the same institution, he joined the faculty and is currently a Research Associate Professor of Medicine. His research interests focus on respiratory epithelial physiology and toxicology.

PUBLICATIONS RESULTING FROM THIS RESEARCH

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Stutts MJ, Bromberg PA. 1987. Effects of ozone on airway epithelial permeability and ion transport. Toxicol Lett 35:315–319.

Boucher RC, Ranga V. 1988. Fate and handling of antigens by the lung. In: Immunology and Immunologic Diseases of the Lung (Daniel RP, ed.) pp. 55–78. Blackwell Scientific Publications, Boston, MA.

ABBREVIATIONS

Ag^+	silver ion
Ag/AgCl	silver/silver chloride
ANOVA	analysis of variance
ATP	adenosine 5′-triphosphate
^{14}C	carbon-14
C5a	complement 5a
Cl	chloride
Cl^-	chloride ion
CO_2	carbon dioxide
DTPA	diethylenetriamine pentaacetic acid
G	conductance
^3H	tritium
15-HETE	15-hydroxyeicosatetraenoic acid
HRP	horseradish peroxidase
^{125}I	iodine-125
I_{sc}	short-circuit current
^{111}In	indium-111
J_{Cl^-}	flux for chloride ion
J_{Na^+}	flux for sodium ion
KRB	Krebs-Ringer bicarbonate glucose solution
$K_4Fe(CN)_6$	potassium ferrocyanide
LTB_4	leukotriene B_4
m→s	mucosal-to-serosal (flux measurements)
MW	molecular weight
Na	sodium
Na^+	sodium ion

NAAQS	National Ambient Air Quality Standard
O_2	oxygen
O_3	ozone
OsO_4	osmium tetroxide
P	permeability coefficient
PAF	platelet-activating factor
PD	potential difference
ppm	parts per million
s→m	serosal-to-mucosal (flux measurements)
SEM	standard error of the mean
$t_{1/2}$	half-life

INTRODUCTION

A Request for Applications (RFA 82–2) that solicited proposals for "Cellular and Biochemical Markers Related to Nonneoplastic Chronic Lung Disease" was issued by the Health Effects Institute (HEI) in the summer of 1982. In response to the RFA, Dr. Philip A. Bromberg, from the University of North Carolina at Chapel Hill, submitted a proposal entitled, "Effects of Ozone on Airway Epithelial Permeability and Ion Transport." The three-and-a-half-year project began in August 1983, and total expenditures were $248,417. The Investigators' Report was received at the HEI for review in October 1989. The revised report was received in January 1991 and was accepted by the Health Review Committee in January 1991. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and Sections 207(c)(4), (5), and (6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide the EPA with limited discretion to modify those requirements.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current primary and secondary NAAQS for ozone is 0.12 parts per million[1] (ppm). This standard is met when no more than one day per year has a maximum hourly average concentrations of ozone above 0.12 ppm. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

Because determining appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the health risks that they present, research into the health effects of ozone in studies such as this one is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

Evidence from experiments with humans and with animals indicates that exposure to ozone affects the respiratory tract. The pulmonary epithelium, which lines the respiratory tract, protects the underlying tissues and regulates the transport of fluids and solutes. The mechanisms available to transport selectively or exclude materials from underlying tissues are complex and may be injured as a result of ozone exposure, thus compromising the functions of this important barrier. Various techniques have been used to assess the properties and integrity of the pulmonary epithelium. Understanding some of the principles of these techniques aids in the interpretation of results from studies on the pulmonary epithelium, such as the one conducted by Dr. Bromberg and his colleagues.

OZONE

Ozone is a highly reactive gas and a major constituent of photochemical smog. Although not emitted directly from motor vehicles and other combustion sources, it is formed as a result of complex photochemical reactions among oxides of nitrogen and volatile organic compounds. The NAAQS for ozone is defined as a daily maximum value of 0.12 ppm (averaged over one hour), and this maximum value should not be exceeded more than once a year. For the 10-year period from 1980 through 1989, the U.S. average of the second highest one-hour ozone concentration fluctuated slightly above the standard (0.12 to 0.14 ppm). In the Los Angeles basin, the summertime peak levels of ozone can exceed 0.30 ppm. Because of the inability to maintain air quality within the NAAQS for an estimated 67 million persons in the United States who reside in nonattainment

[1] A list of abbreviations appears at the end of the Investigators' Report for your reference.

areas, ozone is an oxidant of primary concern (U.S. Environmental Protection Agency 1991).

Data from human and animal studies indicate that, even at concentrations near ambient levels, exposure to ozone exerts numerous effects on the respiratory tract (reviewed by the U.S. Environmental Protection Agency 1986, 1988; Lippmann 1989). Small reversible changes in pulmonary function and cellular alterations have been observed in humans. After an acute exposure to ambient levels of ozone, changes in forced expiratory volumes, forced expiratory rates, airway resistance, and airway compliance have been documented in healthy adults during moderate to heavy exercise. Although there is considerable variability in ozone responsiveness among individuals, these functional alterations are reproducible within an individual. The extent of changes in pulmonary function diminishes after repeated daily exposures. A greater response to ozone among the elderly, persons with asthma, or persons with chronic obstructive lung disease has not been reported in exposed subjects. In addition to functional impairments, acute ozone exposure can cause cough, shortness of breath, and pain upon deep inspiration. Ozone exposure also increases airway reactivity in response to bronchoconstrictive agents. Increases in airway permeability also have been reported. Finally, increases in polymorphonuclear leukocytes and other mediators of inflammation have been measured in the bronchoalveolar lavage fluid of subjects after an acute exposure to ozone.

With animal studies, it has been possible to document structural and biochemical alterations after acute or subchronic exposures to ozone (reviewed by the U.S. Environmental Protection Agency 1986, 1988; Lippmann 1989). Cellular lesions in the centriacinar region of the lung include accumulation of macrophages in the proximal alveoli, and damage to ciliated cells and type I epithelial cells, with subsequent proliferation of nonciliated (Clara) cells and type II epithelial cells. In subchronic studies, morphologic and ultrastructural morphometric analyses reveal significant changes in the centriacinar region of the lungs of rats and monkeys. An important consequence of these cellular events is the thickening of the epithelium of the alveolar and respiratory bronchioles and an increase in the thickness and volume of the interstitium. In addition to these structural changes, other effects from short-term exposure to ozone have been reported (reviewed by the U.S. Environmental Protection Agency 1986, 1988; Lippmann 1989). Alterations in host defense mechanisms that include increased susceptibility to infection, impairment of alveolar macrophage function, and decreased mucociliary clearance have been shown. Inflammation has been accompanied by increases in airway epithelial permeability.

The relationship between the acute reversible events demonstrated in humans and the more permanent structural changes reported in animals requires better understanding. In both human and animal studies, the pulmonary epithelium is a target tissue for ozone exposure. The question has been raised as to whether or not changes in epithelial integrity can serve as a sensitive indicator of ozone-induced damage.

AIRWAY EPITHELIUM

Structure and Properties

An epithelium is a cellular, avascular layer of tissue that covers the free surfaces of other tissues and forms a barrier between the organism and the outside environment. The epithelium that lines the airways regulates the traffic of substances to and from its luminal or mucosal surface (the side facing the lumen) to the underlying serosal or submucosal tissues; thus, it serves both protective and transport functions. To maintain its integrity as a barrier, individual epithelial cells are attached to one another by a network of intramembraneous proteins. This network, termed the tight junction, forms a belt around each cell and limits the passage of material between cells.

Water and solutes (such as ions and nonelectrolytes) do permeate the pulmonary epithelium and must take a route either between (paracellular) or through (transcellular) cells (reviewed by Effros and Mason 1983; Wright 1983). For small nonelectrolytes, aqueous-filled pores have been proposed as a means by which these substances pass through or between cells. Based on experimental studies measuring the movement of different-sized molecules, models have been developed of pore systems with different-sized openings, termed equivalent pore radii. Regional differences in pore size exist throughout the respiratory tract, including the alveolar region (Cotton et al. 1983; Crandall and Kim 1991). Located in tight junctions, equivalent pore radii range from 3 to 10 nm. It has been suggested that pores of approximately 0.5 nm in radius reside in the cell membrane. The rate of diffusion (a process that does not require energy) of nonelectrolytes through aqueous-filled pores is inversely proportional to their molecular weight. Ions diffuse primarily through the junctional pores, but some diffusion across membrane pores occurs. It should be noted that pores have been described functionally, but have yet to be identified morphologically.

Ions also may be actively transported (a process that requires energy) across epithelial tissues. During active transport of one ionic species, the counter ion (an ion with the opposite electrical charge) will diffuse passively in order to

neutralize the electrochemical gradient created by the transport of the first ion. Proteins, which assist in the transport of ions, are located in the apical (surface of the cell facing the lumen) and basolateral (surface of the cell facing the adjacent epithelial cell) membranes of epithelial cells. The two major active transport systems in airway epithelium regulate the absorption of Na^+ from the lumen and secretion of Cl^- into the lumen (reviewed by Welsh 1987; reviewed by Widdicombe 1991). Species and regional differences exist with respect to which ion transport system predominates.

Assays of Epithelial Integrity

Efforts to characterize epithelial integrity have focused on developing techniques to measure bioelectric properties and solute transport. Originally developed to measure active transport in frog skin, Ussing chambers (Ussing and Zerhan 1951) have been used to measure bioelectric properties and ion fluxes in the airway epithelium. Sheets of epithelial tissues can be mounted in these chambers and test solutions added to either the luminal or serosal sides. Alternatively, excised, but intact, tracheal tubes can be mounted in cylindrical chambers.

Most assays of bioelectric properties of pulmonary tissues are conducted in vitro, although limited studies have been done in vivo. The airway epithelium generates a transmural potential difference, which can be measured with a voltmeter. This potential difference represents the sum of active and passive transport systems. The potential difference across pulmonary epithelia is always negative when referred to the submucosa, reflecting the fact that anions (primarily Cl^-) are being transported toward the lumen and cations (primarily Na^+) are moving toward the submucosa. By imposing an electric current from an external battery through the tissue, the potential difference can be reduced to zero. The current needed to achieve this is called the short-circuit current. Under short-circuit conditions, there is no difference in charge between the lumen and the submucosa; therefore, there is no electrochemical driving force for solute movement, and any flux of ions is due to active transport. Assuming that the epithelium acts as an ohmic resistor, the conductance (G) can be calculated from the potential difference (PD) and the short-circuit current (I_{sc}) with the equation $G = I_{sc}/PD$. The value for conductance reflects the passive ion movement. For example, large G values indicate a leaky epithelial barrier.

The movement, or flux, of solute can be measured by putting probes, radiolabeled ions or small nonelectrolytes, on either the submucosal or mucosal sides of the epithelium and measuring their rate of appearance on the opposite side. Net fluxes are calculated from unidirectional flux values. Under conditions in which passive diffusion dominates, the net flux is equal to one; that is, there is no difference in the directional flow of solute. The physical basis by which ions and nonelectrolytes diffuse across a barrier is given in Fick's equation:

$$\text{Flux} = DA/T\,(C_1 - C_2),$$

where D is the diffusion constant, A is the surface area of the barrier, T is the thickness of the barrier, and $(C_1 - C_2)$ is the concentration gradient of the solute across the barrier. The ratio of D to T is called the permeability coefficient (P). Studies can be done in vivo or in vitro using Ussing chambers. The advantage of in vitro experiments is that the surface area of the tissue in the chamber and the volume of the bath solutions, both of which are essential components of the Fick equation, are known. With in vivo studies, pulmonary surface areas and vascular volumes are estimated. The use of radioisotopes and radioimmunoassay allows detection of picogram quantities of the probe. Spurious results may occur, however, if the radioactive label becomes detached from the probe or if the molecule is metabolized during transport; thus, biochemical analysis of the probe, after transport, is essential in any study of permeability.

Microscopy is another method that has been used to assess epithelial permeability, especially of macromolecules and particles. The enzyme horseradish peroxidase is the most commonly used tracer. The product of the enzymatic reaction forms a precipitate that can be seen by both light and electron microscopy. Localization of the reaction product in the lateral spaces between epithelial cells is used as an indicator of paracellular transport pathways, and the presence of material inside cells is considered evidence for transcellular transport pathways. However, because no one has seen reaction product within tight junctions, it also is conceivable that the presence of material between cells is due to intracellular transport and discharge across lateral cell surfaces.

When used in combination, these techniques can provide information on the nature (active or passive) and pathways (paracellular or transcellular) of transport. Active transport systems exist on apical or basolateral cell membrane surfaces and therefore direct the passage of ions through cells. Passive diffusion of small solutes through aqueous-filled pores occurs primarily through tight junctions but can also occur, in the case of ions, across cell membranes. Injury to cells can affect the integrity of tight junctional complexes, cell membrane composition, or membrane ion pumps. Therefore, assays of epithelial integrity can provide useful information on whether or not the functions of protection and transport have been compromised.

Role of Airway Permeability in Disease

Changes in the integrity of the airway epithelium have several implications for pulmonary function. Two areas under active investigation include airway hyperreactivity, and ion transport and airway secretions. Persons with asthma exhibit exaggerated bronchoconstriction in response to toxic agents, histamine, or methacholine. Increases in mucosal permeability have been implicated as a possible mechanism by which antigens or mediators reach neural afferents, smooth muscle, or both. For example, the rate at which instilled tracer molecules appear in plasma was greater in sensitized animals exposed to an antigen aerosol than a saline aerosol (Boucher et al. 1979; Ranga et al. 1983; Taylor et al. 1983). Increased permeability of tracer molecules also occurred in nonsensitized animals exposed to aerosols of histamine or methacholine (Boucher et al. 1978). Although the above data indicate a possible association between hyperpermeability and bronchial hyperreactivity, results from other studies suggest that intrinsic permeability defects are not responsible for an enhanced bronchoconstrictive response. Comparisons of permeability to ^{99}Tc–diethylene triamine pentacetic acid (DTPA) aerosols in asymptomatic subjects with and without asthma revealed no differences (Elwood et al. 1983; O'Byrne et al. 1984). However, when subjects were challenged with histamine (O'Byrne et al. 1984) or methacholine (Elwood et al. 1983), differences in airway reactivity between the two groups were apparent. Thus, permeability defects associated with hyperreactivity seem to occur only during provoked bronchoconstriction.

Active transport and passive diffusion of ions, with the subsequent diffusion of water, affect both the volume and viscosity of epithelial surface fluid. The efficiency of mucociliary clearance of particles and infectious organisms is a function of the viscosity and thickness of the mucous layer. Persons with certain forms of asthma (Keal 1971; Charman and Reid 1972) or cystic fibrosis (Charman and Reid 1972; Wood et al. 1976; Boucher et al. 1983) exhibit alterations in the composition of their mucous secretions, which subsequently compromise pulmonary clearance. This situation, in turn, could make these persons more susceptible to respiratory tract infections.

EFFECTS OF OZONE ON AIRWAY EPITHELIAL INTEGRITY

Matsumura (1970) was one of the early investigators to evaluate the effects of ozone on epithelial permeability. In that study, guinea pigs were exposed to 8 ppm ozone for 30 minutes. Albumin radiolabelled with ^{131}I was instilled into the lungs, and then the levels of the tracer were measured in the plasma. Animals exposed to ozone exhibited a more rapid accumulation of the tracer in the plasma than did the nonexposed animals. Using concentrations that were 10 times lower, Bhalla and coworkers (1986) exposed rats to 0.8 ppm ozone for two hours and noted an increase in the transport of bovine serum albumin and DPTA across tracheal and bronchoalveolar regions into the plasma; the increase in transport was greater for the DPTA than for the albumin. The transport of albumin from the blood into the lungs also has been measured and noted to increase in rats exposed to 2 ppm ozone for five hours (Costa et al. 1985), to 0.5 ppm ozone for six hours (Albert et al. 1971), and to 0.4 ppm for six hours (Guth et al. 1986). Using tracers other than albumin, Davis and coworkers (1980) exposed guinea pigs to 4 ppm ozone for three hours, instilled different-sized probes (mannitol, molecular weight [MW] 182; dextran, MW 10,000; and horseradish peroxidase, MW 40,000), and then measured their rate of accumulation in the plasma. Significant differences in accumulation rates of all three probes occurred between animals exposed to ozone and those exposed to air immediately after exposure and 24 hours later. Hu and associates (1982) reported increases in protein concentration in lung lavage samples from guinea pigs exposed to 0.26, 0.51, or 1 ppm ozone for 72 hours, suggesting leakage from the blood into the airspaces. Increased protein, however, may not necessarily represent transport of proteins from the circulation, but could reflect other aspects of pollutant toxicity, such as increased secretion or cell death.

Abraham and colleagues (1984) evaluated both airway permeability and airway reactivity in sheep. Animals were exposed to 0.5 or 1 ppm ozone for two hours on two occasions separated by 24 hours. After the second exposure, animals inhaled aerosolized ^{3}H-histamine, and airway resistance and plasma levels of the tracer were measured. In most animals, ozone exposure caused an increase in both parameters.

The effects of ozone on permeability and pulmonary function have been investigated in humans, with contradictory results. Kehrl and coworkers (1987) measured increases in ^{99}Tc-DTPA transport to the plasma in human subjects exposed to 0.4 ppm ozone for two hours with intermittent exercise. Increases in airway resistance and decreases in forced vital capacity also were observed. In contrast, Utell and associates (1985) observed no differences in ^{99}Tc-DTPA transport in volunteers exposed to 0.5 ppm ozone for 30 minutes without exercise. In these subjects, no changes in pulmonary function were measured. Marked differences in protocols, which probably resulted in differences in total ozone doses to the lungs, may account for the different findings in the two studies.

In summary, ozone exposure increases airway permeability, and in some studies, this effect has been associated with increases in airway resistance. The effects of ozone on ion transport have received far less attention. In one study, Phipps and coworkers (1986) exposed sheep in vivo to 0.5 ppm ozone for four hours for two days ("acute") or for six weeks ("chronic"). Bioelectric properties, glycoprotein secretion, and ion and water fluxes were measured in vitro 24 hours after the termination of exposure. Another group of sheep was allowed to recover for seven days after a six-week exposure before being evaluated. Under acute conditions, the short-circuit current decreased, but it increased under chronic conditions; the conductance remained unchanged with both exposure protocols. These findings indicate that ozone affects active transport. After the acute exposure, secretion of sulfated glycoproteins increased, net Na^+ absorption decreased, and there was no effect on Cl^- secretion. After chronic exposure, net Cl^- secretion, net Na^+ absorption, and water secretion all increased. After seven days of recovery, the increased secretion of glycoproteins, Cl^-, and water persisted. The authors concluded that acute and chronic exposures to ozone cause airway mucous hypersecretion. This and other studies have shown histologic evidence of increases in mucin secretion and secretory cell hyperplasia (Hyde et al. 1989), but the ultimate effects of ozone exposure on airway surface fluid volume and viscosity are unknown.

JUSTIFICATION FOR THE STUDY

The HEI sought proposals that would improve our understanding of the relationship between oxidant lung injury and lung disease. Of particular interest to the Institute was the development of short-term tests that measure biological effects relevant to the pathogenesis of chronic lung disease. Bioassays that could be developed in animals and would have predictive power for assessing the human risk of lung disease were of major interest. It was considered important that the responses seen in animals could be correlated with responses observed in humans. Dr. Bromberg and his colleagues proposed to evaluate the kinetics of alterations in permeability produced by ozone exposure and to determine the time after exposure when the permeability of the airway epithelium to molecular probes is the greatest. The investigators proposed to expose guinea pigs acutely to ozone, and then measure permeability in vivo and in vitro using excised tracheas. In addition, they proposed to correlate permeability with bioelectric and morphologic parameters. Information gained from the proposed studies would be used to interpret further permeability alterations in humans.

The proposal was considered well focused, and it was believed that the information obtained would contribute to our understanding of the effects of ozone on airway permeability. The objectives were considered attainable because of the expertise and previous experience of the investigator and his colleagues. Previous observations by the investigators (Davis et al. 1980) would be extended, and the proposed work could provide a sound basis for subsequent work in humans.

SPECIFIC AIMS AND STUDY DESIGN

The investigators explored the hypothesis that exposure to ozone alters the integrity of the tracheal epithelium. The specific aims of the study were: (1) to characterize changes in bioelectric properties; and (2) to investigate changes in permeability to polar molecules.

In all the exposure studies, intact guinea pigs were exposed to ozone or filtered air. Exposures were conducted by Drs. F. Miller and J. Graham at the Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC. The effects of ozone were evaluated either in vitro, with excised tracheas, or in vivo.

First, the investigators characterized in vitro the bioelectric properties and permeability of excised tracheas of unexposed adult male guinea pigs. Regional differences between upper and lower sections of trachea were compared.

Second, the investigators determined the effect of a single exposure to 1 ppm ozone for three hours on bioelectric and permeability properties. Animals were killed at 2, 24, 48, or 72 hours after the termination of ozone exposures, and their tracheas were excised and evaluated in vitro. Potential difference (PD) and short-circuit current (I_{sc}) were measured, and conductance (G) was calculated to assess bioelectric properties. In addition, the ion fluxes of Cl^- and Na^+ across the epithelial tissues were measured. In some experiments, amiloride, an inhibitor of Na^+ transport across apical cell membranes, was added to the mucosal bath. Permeability of the tracheas was evaluated by adding several radiolabeled probes, including ^{14}C-mannitol (MW 182, molecular radius 0.4 nm), ^{111}In-DTPA (MW 492, molecular radius 1.0 nm), ^{3}H-inulin (MW 5,500, molecular radius 1.4 nm), ^{3}H-dextran (MW 10,000, molecular radius 1.6 nm), ^{131}I-albumin (MW 68,000, molecular radius 3.6 nm), and ^{14}C-dextran (MW 70,000, molecular radius 3.8 nm). Because of their small size, it was expected that mannitol, DTPA, and inulin would diffuse freely through aqueous-filled pores and would provide information on paracellular routes of transport. The investigators also used DTPA because this molecule is frequently used in human studies

and information from the proposed studies could be compared with results obtained in humans. Finally, albumin was chosen because there was some indication, which has recently been confirmed (Johnson et al. 1989), that this molecule's mode of transport varies, depending on the direction of movement. The majority of the in vitro experiments used tracheal cylindrical preparations, although a few experiments used planar sheets of tracheal tissues mounted in Ussing chambers.

Third, epithelial permeability was assessed in vivo. After a three-hour exposure to 1 ppm ozone, ^{14}C-mannitol, ^{111}In-DTPA, and ^{3}H-inulin were instilled onto airway surfaces, and their accumulation in the circulation was assayed for up to 30 minutes. Exposures to ozone were either for one day or for four consecutive days.

Electron microscopic studies with horseradish peroxidase (MW 40,000, molecular radius 3.4 nm) were included to confirm the morphologic pathway of transport. The tracer was added both in vitro and in vivo. The location of the peroxidase reaction product was used to determine whether macromolecules were transported by way of paracellular or transcellular routes.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators characterized the bioelectric properties and evaluated the permeability of the guinea pig trachea after acute exposures to 1 ppm ozone. Assays were conducted in vitro and in vivo after exposure of the intact animal. Thus, all the study objectives were attained.

STUDY DESIGN AND METHODS

This study represents a well-conducted series of experiments. The investigators used state-of-the art techniques, and the experiments were well controlled. Several approaches were used in an effort to characterize any effects of ozone exposure on tracheal permeability and ion transport.

STATISTICAL METHODS

Comparisons between mean values for the parameters measured (for example, potential difference, short-circuit current, probe permeabilities, and ion fluxes) were made using either paired or unpaired *t* tests, which, in most cases, were appropriate. However, the data in Table 2 show that the tissues were matched for electrical conductance; because the fluxes in the two directions were still paired, these data should have been analyzed by the paired *t* test. If matching the electrical conductance reduced the variability of the differences, the paired *t* test would have lowered the variance, thus making it more likely that a statistically significant result would have been detected. In addition, if the tissues presented in Table 2 were matched, it is unclear why the *n* values are different in panel A and panel B. If there are missing observations, analysis of matched data should have taken this into account.

Comparisons were made between regions (upper versus lower trachea), direction of flux movement (mucosal versus serosal), exposure conditions (air versus ozone), and preparations (cylindrical versus planar). When time was introduced as a third variable, the investigators correctly analyzed their data using a one-way analysis of variance. Significant results were further analyzed using Dunnett's two-tailed *t* test.

RESULTS AND INTERPRETATION

In unexposed guinea pigs, the upper trachea was less permeable than the lower trachea. The upper trachea was able to maintain a greater electrical difference between the luminal and serosal sides, as reflected by the greater potential difference and smaller conductance. The smaller value for solute permeability in the upper trachea and the absence of directional differences in solute movement support the conclusion that regional differences in permeability were due to differences in paracellular pathways. These findings are in agreement with other studies suggesting that permeability may increase as one moves distally down the conducting airways. In guinea pigs, the potential difference is lower in the bronchi than the trachea (Boucher et al. 1980). In sheep, the pore size is estimated to be greater in the bronchi than the trachea (Cotton et al. 1983). In rats, the tight junctional network between cells appears less complex as one moves distally out the airways from the trachea (Schneeberger 1980). Comparisons of regional differences with previous and future studies would have been more meaningful, however, had the investigators provided a definition of their classification of upper versus lower trachea. Finally, calculations of equivalent pore radii suggested a single population of aqueous-filled pores, with equivalent pore radii of 9 to 10 nm. This dimension also is consistent with other reports of pore radii in dogs (Boucher 1980) and in sheep (Cotton et al. 1983).

In the in vitro experiments with excised tissue from animals exposed to ozone, the potential difference increased and remained elevated after the exposure. Because the short-circuit current increased and the conductance remained unchanged, the data suggest an effect of ozone on active transport systems. The investigators attempted to perform ion flux measurements on tissues mounted in Ussing

chambers. However, because of the small size of guinea pig tracheal tissues relative to the chamber, substantial damage to the edge of the tissue was apparent. Measurements of Na^+ and Cl^- fluxes were unremarkable. However, adding amiloride to Ussing chamber preparations inhibited the increase in short-circuit current observed after ozone exposure, suggesting an effect on Na^+ absorption. The finding of an effect of ozone on active transport, and that the effect persisted after the termination of exposure, is in agreement with the study by Phipps and coworkers (1986).

When evaluated in vivo, the permeability to probe molecules increased markedly after a single ozone exposure. After four daily exposures, this response diminished and was not significantly different from the permeability measurements in the control animals. In contrast, in the in vitro studies, the permeability of mannitol decreased after ozone exposure. This discrepancy between the in vivo and in vitro results is an area of concern. The investigators suggest that the increase in permeability observed in vivo is due to the presence of mediators that were washed out in the in vitro preparations. The authors acknowledge the possibility that, with the in vivo model, the probe molecules had access to the bronchi and lower airways. As detailed above, several lines of evidence suggest that the bronchi may be more permeable than the trachea. Dosimetry models and histologic evidence indicate that ozone exerts its greatest impact in the lower airways. Thus, it is conceivable that in this study, the increases in permeability observed in vivo, but not in vitro, resulted from ozone-induced damage of the more permeable epithelium, which was excluded from the in vitro preparation. The investigators argue, however, that the trachea was the site of transport of the instilled probe molecules in the in vivo model. Furthermore, when using a similar instillation procedure, Bhalla and associates (1986) showed that the distribution of India ink in rats was limited to the trachea and mainstem bronchus. A similar demonstration in guinea pigs in this study would have been helpful. Thus, although it is more than likely that the observed transport of probe molecules occurred across tracheal tissues, it cannot be ruled out that the lower airways were also involved.

The finding of an attenuated response with the multiple ozone exposures is of interest because a similar adaptation response has been observed with pulmonary function measurements reported from other studies. Changes in ozone-induced pulmonary function in human subjects diminished after three to four days of exposure (Hackney et al. 1977; Farrell et al. 1979; Folinsbee et al. 1980). With repeated exposure, it is conceivable that the mucous layer increased in thickness; thus, less ozone would have reached the epithelial cells. Preliminary support for this hypothesis is derived from the findings of Phipps and coworkers (1986), which provided evidence suggesting an increase in mucous secretion after multiple exposures to ozone. Metabolic changes or activated antioxidant enzyme systems also could account for the adaptation (Grose et al. 1989).

The reaction product of the horseradish peroxidase was detectable in the intercellular spaces between epithelial cells and in membrane-bound vesicles within secretory cells in tissues from animals exposed to ozone. In tissues from animals exposed for four days, the lack of reaction product in the intercellular spaces paralleled other indications of adaptation. The route of transport, however, remains unresolved. The investigators postulated that the presence of the horseradish peroxidase in intercellular spaces was attributable to the passage of material through disrupted tight junctions, which is clearly possible. However, Bhalla and Crocker (1986) suggested that intracellular transport of horseradish peroxidase by endocytic vesicles is the predominant route. In their study of rats exposed to 0.8 ppm ozone for two hours, they noted an increase in the number and surface area of endocytic vesicles in the epithelial cells. It is not surprising that neither group of investigators was able to localize the horseradish peroxidase reaction product within tight junctions, which would confirm the hypothesis that increased permeability was due to decreased integrity of the junctional network. With the techniques currently available, it is not possible to "catch" tight junctions in the open position. As discussed in the Scientific Background section, distinguishing between paracellular and transcellular routes of transport of this macromolecule with this technique is difficult. Also, because horseradish peroxidase is a macromolecule, it may use a route of transport (that is, endocytic uptake) not routinely used by smaller molecules (Crandall and Kim 1991). Thus, although the use of horseradish peroxidase can provide information on epithelial transport of large molecules (such as antigens), it may not be an appropriate surrogate for small solutes.

Although not a stated objective of this study, it is important to note that the experiments did not elucidate any mechanisms for the observed changes in epithelial integrity after acute exposure to ozone. Interpretation of the data remains speculative, and additional research is needed to clarify the mechanisms of the observed effects of ozone on epithelial permeability and bioelectric properties.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

This and other studies have shown that ozone causes alterations in airway permeability (Matsumura 1970; Albert et

al. 1971; Davis et al. 1980; Hu et al. 1982; Abraham et al. 1984; Costa et al. 1985; Bhalla et al. 1986; Guth et al. 1986) and that these alterations, as reported by Kehrl and coworkers (1987), can be measured in humans. The current issues of interest are which mechanisms are involved and how do these mechanisms relate to other ozone-induced effects; future research could address these issues. Furthermore, elucidating these mechanisms may help to explain the adaptation response to ozone seen in this study and in other studies. It will be important to determine whether short-term adaptation is related to long-term lung injury.

The investigators postulated that mediators derived from inflammatory cells were responsible for the increased permeability to probe molecules seen in vivo. The authors referred to several recent studies that have explored the relationships among ozone, inflammation, and airway hyperreactivity; however, a coherent understanding of this relationship is not evident. Similar types of studies that would also relate defects in permeability to ozone exposure and inflammatory processes could be conducted. In addition, the influence of epithelial-derived mediators on epithelial permeability could be explored. Thus, the source, as well as the identity, of putative soluble mediators requires elucidation.

The significant discrepancies between the in vitro and in vivo results raise questions that require clarification before the in vitro model can be used in future studies assessing the effects of ozone on epithelial permeability. Whether the problem is the absence of appropriate mediators that are present in vivo, or differences in the airway distribution of test probes, it remains to be proven that the in vitro model is reliable for evaluating the effects of inhaled ozone on airway permeability.

The effect of ozone on ion transport by airway epithelia has received little attention. Because of the small size of guinea pig tracheal tissues relative to the size of the Ussing chambers, the investigators had to perform ion flux measurements on damaged tissues. Additional studies using tissues from larger animals with similar ion transport systems could be conducted to clarify the ion transport mechanisms affected by ozone. Additional pharmacological studies could confirm the effect of ozone on the apical transport of Na^+. The ultimate impact of oxidant exposure on airway surface fluid volume and viscosity remains unexplored.

The cellular site of ozone-induced injury remains unclear. The authors suggested that ozone probably has a direct effect on membrane active transport systems for ions and an indirect effect on paracellular transport for nonelectrolytes. Bhalla and colleagues (1988) suggested that ozone disrupts the microfilament component of the cell cytoskeleton, which, in turn, affects the intracellular endocytic and extracellular junctional transport systems. These possibilities need confirmation.

Finally, the concentration of ozone used in this study (1 ppm) is considerably higher than the levels found in ambient air. However, the study was designed to detect a biologic effect at a concentration lower than that used in previous studies (Davis et al. 1980), and mimicking human exposures was not an objective of this study. Because other researchers have detected effects on airway permeability (Albert et al. 1971; Hu et al. 1982; Abraham et al. 1984; Bhalla et al. 1986; Guth et al. 1986; Kehrl et al. 1987) and ion transport (Phipps et al. 1986) at lower ozone concentrations, future studies should use levels more relevant to ambient levels.

CONCLUSIONS

After exposing intact animals to 1 ppm ozone for three hours either for one day or for four successive days, the bioelectric and permeability properties of the guinea pig tracheal epithelium were studied in vitro and in vivo. Bioelectric properties of excised tracheas were measured in vitro using established techniques. Permeability of tracheas was measured by instilling water-soluble molecular probes onto either the mucosal or serosal side of the tracheal epithelium in vitro, or on the luminal side in vivo. Electron microscopic studies with horseradish peroxidase were included for morphologic confirmation of the transport pathways.

These experiments provide evidence that ozone exposure affects ion transport and the permeability of the tracheal epithelium. In vivo exposure to the oxidant caused an increase in active ion transport in the in vitro preparations; the increase in potential difference persisted for up to three days after a single exposure. The effect of ozone on active transport was inhibited by amiloride, suggesting that the oxidant affected the absorption of Na^+. However, additional studies are needed to confirm the ion species involved, as well as the ultimate effect of ozone exposure on airway surface fluid characteristics.

After ozone exposure, transport of probe molecules across the airway epithelium increased in the in vivo studies. After four successive ozone exposures, this defect in epithelial integrity was significantly attenuated. Histological examination of the tissues showed a postexposure infiltration of polymorphonuclear leukocytes into airway lumens. In contrast, the results from the in vitro studies showed a slight decrease in permeability to probe molecules after ozone exposure. The discrepancy between the in vivo and in vitro results of the transport of probe molecules may be due to the absence of soluble mediators in the in

vitro preparation, the distribution of the probe molecules in the in vivo studies, or both.

The electron microscopic studies revealed horseradish peroxidase in the intercellular space of the tracheal epithelium from animals exposed to ozone, but not animals exposed to air. The authors interpret this finding as indicating that increases in solute permeability are due to the disruption of junctional integrity and that transport occurred by paracellular pathways. Although the evidence for paracellular transport is suggestive, it is not conclusive because of the difficulties in distinguishing between the two pathways of transport with this technique. Furthermore, it is unclear if horseradish peroxidase can serve as an appropriate surrogate for solutes that normally traverse the epithelium by way of junctional pores.

The mechanisms responsible for the reported effects of ozone on the integrity of the tracheal epithelium remain unknown. Future studies to evaluate permeability should be conducted with in vivo model systems so that the role of soluble mediators can be investigated. Additional mechanistic studies could contribute to our understanding of the adaptation seen in this study and in pulmonary function measurements observed in other studies. The results from this study suggest that ozone may have a direct effect on epithelial cells, as evidenced by its effect on active transport; the oxidant also may have an indirect effect on epithelial cells, as evidenced by its effect on solute permeability. The design of future studies should consider what is known or what needs to be known about airway hyperreactivity and disorders in mucous secretion.

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Title	Publication Date
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Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
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