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Particle Clearance from the Respiratory Tract as a Test of Toxicity:

Effect of Ozone on Short and Long Term Clearance

Judson L. Kenoyer*, Robert F. Phalen, and James R. Davis

ABSTRACT: *The ability of the lung to mechanically remove inhaled deposited particles is an important mammalian defense mechanism that can be evaluated in the laboratory. Experiments that measure clearance kinetics have been performed by various investigators using human as well as large and small laboratory animals as subjects. Several agents have been shown to significantly alter clearance phenomena in the lung. This paper describes quantitative clearance experiments that used radioactive tracer particles to assess lung damage after exposure of rats to ozone, a photochemical air-pollutant gas. Radioactively labeled tracer microspheres were inhaled by groups of 30 rats prior to exposure to ozone. Ozone levels studied were 0.4, 0.8, and 1.0 ppm and all exposures were 4 hr in length. These exposures caused a delay in the early (0–50 hr postdeposition) clearance and an acceleration in the late (50–300 hr postdeposition) clearance rate of the tracer particles. Dose response curves show that clearance was affected more by the higher concentrations of ozone.*

INTRODUCTION

An area of inhalation toxicology that has undergone substantial evolution in recent years involves analysis of lung defense mechanisms [1]. The phenomena associated with clearance of inhaled insoluble particles have been recognized as important aspects of these defense mechanisms of the respiratory tract.

The patterns of deposition of inhaled aerosols are now becoming understood in terms of physical forces that act on airborne particles, the air flow patterns during breathing, and the geometric properties of the respiratory tract [2]. Mammalian respiratory systems have geometric and air flow properties such that particles within a given range of size and shape tend to deposit preferentially in characteristic anatomical locations. For example, the human nose is known to be an efficient impactor collecting many particles with aerodynamic diameters greater than a few micrometers. The aerodynamic diameter is defined as the geometric diameter of a unit density (1 g/cm^3) sphere with the same terminal settling velocity in still air as the particle under consideration. The deep lung can only collect particles that have eluded capture in the nose or mouth and passed through the tracheobronchial tree; that is, particles with aerodynamic diameters typically below a few micrometers.

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The nose effectively collects larger particles and efficiently clears them via sneezing, blowing, and ciliary-driven mucus movement. The moving mucus of the tracheobronchial tree is efficient in transporting large quantities of solid or liquid particles of various sizes, shapes, and densities upward to the region of the epiglottis where they can be swallowed. Alveolar macrophages that reside in the deep lung seem to exhibit efficient engulfment for particles in the micrometer diameter size range [3]; this is also in the size range of high deposition probability in alveoli. Viable microorganisms are often in this size range and deposit significantly in the deep lung, where conditions are favorable for their rapid reproduction. Fortunately, macrophages can inactivate many infectious organisms by engulfment and proteolytic enzyme action. Clearance from the pulmonary region is not yet completely understood, but the active mechanisms appear to include: (a) the dissolution of relatively soluble material with absorption into the systemic circulation, (b) direct passage of particles into the blood, (c) phagocytosis of particles by macrophages with translocation to the ciliated airways, and (d) transfer of particles via the lymphatic system to lymph nodes or blood. The fate of particles deposited in this region is probably strongly dependent on their mechanical stability, i.e., their rate of dissolution in lung fluids [4].

Despite great differences in body size and respiratory tract morphology, most mammals appear to have clearance phenomena that are remarkably similar with respect to mechanism; for example, mucociliary clearance in the nose and tracheobronchial tree and a macrophage response in the alveolar spaces. Also, though correspondence is not always close, basic similarities exist among mammalian species in regional patterns of aerosol deposition as well as in responses to individual toxic materials. Thus, it is not unreasonable to include measurements of aerosol deposition and clearance in laboratory animals in toxicologic evaluations that are eventually aimed at understanding human risks.

METHODS AND MATERIALS

Specific pathogen-free Sprague Dawley rats (Hilltop Lab Animals, Inc., Chatsworth, CA) were used in these experiments. Male rats weighing approximately 200 g were delivered to the laboratory in filtered shipping containers to minimize prior exposure to pollutants. All animals were housed in a laminar air barrier caging system in wire-bottom stainless steel cages over a nonstandard relatively dust-free sodium chloride litter for about 1 week prior to exposure to ozone.

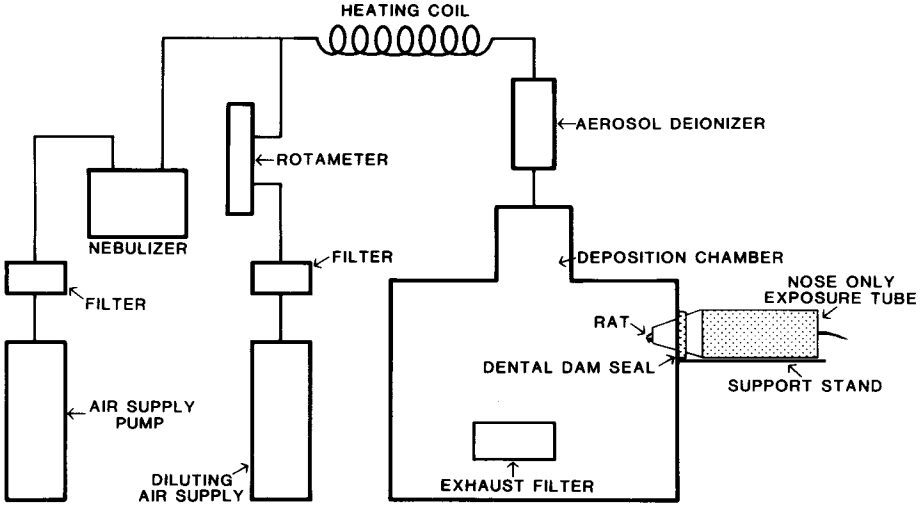
The tracer microspheres were labeled at this laboratory with ^{51}Cr [5]. The labeled particles employed for this study were produced from commercial monodisperse polystyrene latex microspheres (Dow Chemical Company, Midland, MI). Other methods of labeling monodisperse particles have been documented [6–8]. After aerosolization with a Lovelace-type compressed air nebulizer [10] (ARIES, Inc., Davis, CA) of a 0.1% (by volume) aqueous suspension of particles, the aerodynamic diameter of dried particles was measured using a calibrated seven-stage impactor [11] (ARIES, Inc.). The aerosol measured in the breathing zone of the rats had an activity median aerodynamic diameter of 1.6 μm with a geometric standard deviation of 1.2. Less than 1% of the radioactivity was in the fine fraction collected on the fiberglass backup filter in the impactor. Electron microscopy samples were collected from the inhalation exposure unit using a point-to-plane electrostatic precipitator [9] (ARIES, Inc.). Electron microscopy of the labeled particles gave a count median diameter of 1.4 μm with a geometric standard deviation of less than 1.1. The aerosol size distributions of the tracer

microspheres were well approximated by log-normal functions. In vitro leaching studies showed a leaching rate of ^{51}Cr from the particles of less than 0.1% per day [5]. The specific activity of the labeled microspheres was determined to be approximately 300 Ci/gm. This isotope of chromium emits a 0.32 MeV gamma ray. The estimated dose to a rat over the length of the experiment from the ^{51}Cr is less than 1 mrem. Counting times in the thoracic activity counting system ranged from 100 sec (initial activity) to 500 sec for the final count with low-activity animals; a minimum of 1000 gross counts were obtained for each activity determination.

The aerosolized particles had been dried by heating and dilution with clean air and passed through an ^{85}Kr deionizer (TSI Inc., St. Paul, MN) before being passed into a nose-only exposure chamber. This chamber was an aluminum-foil-lined cylinder (40-l volume) with circular exposure ports around the circumference. This nose-only exposure system has been described previously [12]. A maximum of 15 rats were exposed simultaneously to the radioactive aerosol in this system (Fig. 1) for approximately 20 min. The nose-only exposure tubes that held the unanesthetized animals were constructed of perforated metal to minimize thermal stress to the animals from body heat. The average amount of ^{51}Cr deposited per rat was less than 0.1 μCi and was contained in less than 1 μg of particles.

After the deposition episode was completed, the rats were removed from the system and their noses washed with wet paper towels to reduce the amount of externally deposited radioactivity. The animals were then placed in plastic counting tubes and positioned beneath a collimated 3-in. NaI(TI) gamma ray detection system (Fig. 2) shielded from background radiation with lead. Collimation was established with lead shielding such that the ^{51}Cr gamma rays emitted from the respiratory tract were favored for detection over those from the

Figure 1 A schematic of the generation system used to deposit the radioactive tracer particles in the respiratory tract of rats. The deposition chamber has multiple inhalation ports, thereby allowing 15 rats to be simultaneously exposed. The nose-only exposure tubes are constructed of perforated metal to prevent thermal stress to the rats from body heat.



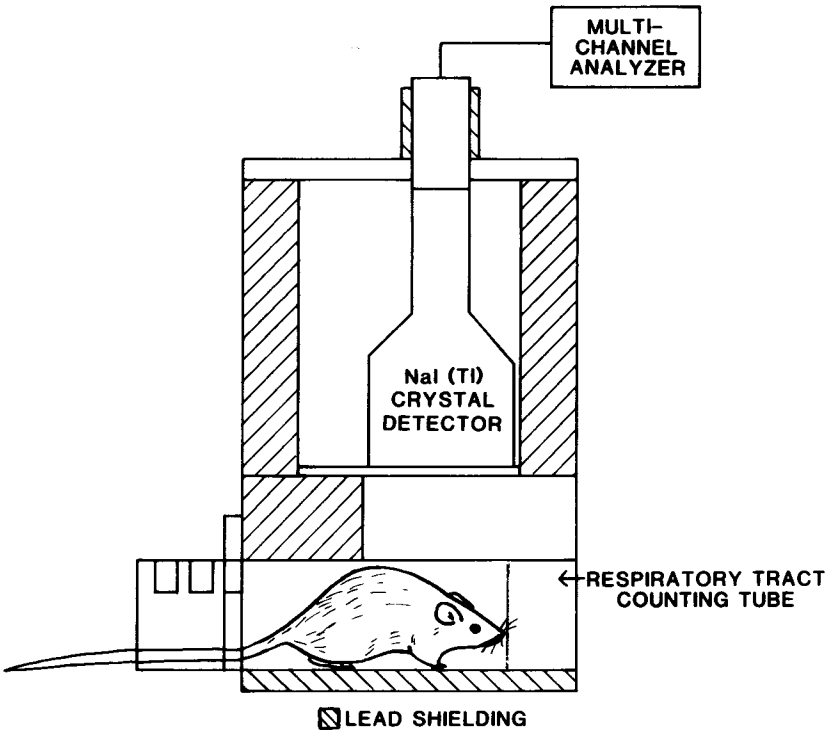


Figure 2 Diagram of the thoracic counting system used to determine the amount of radioactivity in the respiratory tract of the rat. The lead shielding in this external radiation detection system is such that radiation emitted from the respiratory tract is favored for detection over that from the stomach and intestines.

stomach and intestines; radiographs of rats were used to determine the dimensions of the collimation and shielding. If an animal moved forward or backward or twisted sideways during a counting interval, it was recounted. The initial amount of respiratory tract activity (defined as 100% for each rat) was determined before the animals went into either ozone-containing or clean-air atmospheres. The time between the end of particle exposure and counting the first and last animals ranged from 2–50 min, respectively. There is some particle clearance from the lung during this time but the effect on the subsequent data analysis is minimized by using the midpoint of the group counting interval in calculations.

One hour after the exposure to tracer particles, the animals were divided randomly into two groups and placed into individual compartments of open-mesh stainless-steel exposure cages. These cages were then positioned on a single level in a Rochester-type hexagonal cross section 1 m^3 volume stainless-steel chamber for a 4-hr exposure to either clean air or an ozone atmosphere. For each ozone exposure, clean-air-exposed animals from the same supply batch were used as controls.

Both of the exposure chambers were supplied with purified air which had been passed through filters, gas scrubbers, a cooler–drier, a humidifier, a heater, and a high-efficiency particulate filter. Temperature and humidity were controlled in the chambers during all exposures. The humidity was approximately 40% and chamber flow rates ranged from 0.3 – 0.6 m^3/min. Ozone was generated

by passing medical grade oxygen through an electrical ozone generator (Sander Ozonizer, Typer III, Osterberg, West Germany) and injected into the filtered chamber air. The ozone concentration was measured continuously at the breathing zone of the animals through inert sampling lines using a calibrated ultraviolet monitor (Dasibi Environmental Corporation, Glendale, CA). In the clean-air control chamber the average concentration of ozone was always less than 0.01 ppm.

After the animals were removed from the 4-hr exposure, collections of all of the feces were performed at fixed intervals selected to allow determination of the fecal excretion activity curve. Eleven fecal collections from each rat were made during the first 50 hr postdeposition of the tracer microspheres. After that time, feces were not collected because levels of excreted radioactivity were negligible. The total amount of radioactivity excreted in feces in the first 50 hr postdeposition corresponds closely to the amount of activity cleared from the respiratory system as measured with the thoracic counter during the same time interval. Fecal excretion curves were fitted to a log-normal function and the time at which 50% of the total activity was excreted determined for each rat; this time is referred to as the 50% clearance time ($T_{50\%}$). If the 50% clearance time for the ozone-exposed animals was greater than that for the control animals, then the difference between them was defined as a delay in early clearance. During the time interval of 50–300 hr postdeposition of the radiolabeled particles, four respiratory tract counts were performed on each animal using the plastic counting tubes. The percentages of these counts of the initial respiratory tract activity were calculated, plotted versus time, and fitted to a single exponential function. From this function a half-time for long-term clearance of the tracer microspheres was obtained for each rat.

Means and standard deviations were calculated for each group of rats and the early 50% clearance time and late clearance biological half-times were compared for the clean-air-exposed and the ozone-exposed groups using two-tailed *t*-tests.

Some animals were not included in the data analysis. Animals that did not eat food, drink water, or did not excrete during three consecutive fecal sampling intervals were excluded from the early clearance analysis. Animals with such a small amount of radioactivity in their lungs that a thoracic count could not be significantly distinguished from background were excluded from the long-term clearance analysis.

RESULTS

The effects of 4-hr exposures of rats to three concentrations of ozone (0.4 ppm, 0.8 ppm, and 1 ppm) on the clearance of radiolabeled tracer particles were measured (Table 1). At each of the three concentrations of ozone, a delay was observed in early (0–50 hr post deposition) clearance and an acceleration was seen in long-term (50–300 hr postdeposition) clearance in comparison to the matched control animals.

The effect of exposure to 1 ppm ozone on the short-term clearance of tracer particles is shown in Fig. 3 where the cumulative percentage of activity excreted in feces is plotted versus time for both the control and ozone-exposed groups. The data indicate that the clearance of the tracer particles in the animals exposed to the ozone was significantly delayed when compared to the early clearance in the control animals. The time required for the ozone-exposed animals to excrete 50% of the total amount of radioactivity excreted the first 50 hr postdeposition was approximately 5 hr longer than the time required by the control animals.

The effect of exposure to 1 ppm ozone on the retention of thoracic radioactiv-

Table 1 Effects of Three Concentrations of Ozone Exposure on the Early and Late Clearance of Radiolabeled Tracer Microspheres.

Exposures were 4 hours in length. $T_{50\%}$ = *Time required to excrete 50% of total activity excreted through 50 hr postdeposition.* T_L = *Long-term biological half-time.*

Early Clearance

Ozone concentration (ppm)	Exposed animals		Control animals		
	Number	$\bar{T}_{50\%}$ ± SD (hr)	Number	$\bar{T}_{50\%}$ ± SD (hr)	$\Delta T_{50\%}$ ± SE (hr)
0.4	8	11.5 ± 1.6	6	11.3 ± 1.0	0.2 ± 0.7
0.8	39	12.0 ± 2.4	40	11.1 ± 2.0	0.9 ± 0.5[a]
1.0	9	15.9 ± 2.1	10	11.1 ± 2.6	4.8 ± 1.1[b]

Late Clearance

Ozone concentration (ppm)	Exposed animals		Control animals		
	Number	$\bar{T}_L$ ± SD (hr)	Number	$\bar{T}_L$ ± SD (hr)	ΔT_L ± SE (hr)
0.4	8	554 ± 355	6	631 ± 238	−77 ± 151
0.8	40	346 ± 205	40	498 ± 360	−152 ± 66[b]
1.0	9	341 ± 177	10	667 ± 343	−326 ± 129[b]

[a] $P < 0.10$ (two-tailed t test)

[b] $P < 0.05$ (two-tailed t test)

[c] Negative sign (−) refers to acceleration in clearance

ity is shown in Fig. 4 where the radioactivity in the respiratory tract (as a percentage of the initial activity) is plotted versus time for both the control and ozone-exposed animals. These data show the delay in early clearance in the ozone-exposed animals as was seen with the excretion data. The respiratory tract clearance curves also indicate that the long-term (50−300 hr postdeposition) clearance of the tracer particles from the lungs was significantly accelerated in animals exposed to ozone when compared to that of control animals. The mean long-term biological clearance half-time for the ozone-exposed animals was over 300 hr faster than that of the control animals.

The data in Fig. 5 (listed in Table I) show the dose response relationship between ozone concentration and indices of both short and long-term clearance. The data indicate that as the concentration of ozone to which the animals were exposed was increased, both the length of delay of early clearance and the change in late clearance biological half-time increased as well. Ozone exposures also produced a significant change ($P < 0.05$) in long-term clearance in the animals exposed to 0.8 and 1.0 ppm ozone when compared to control animals. An acceleration of long-term clearance in the ozone-exposed animals was seen.

DISCUSSION

A test measuring the effect of a toxic agent on the clearance of tracer particles from the respiratory tract has been presented and shown to be of use in the laboratory. This experiment gives information on both the early and late clearance of radiolabeled tracer microspheres. Statistical tests can be performed that compare exposed with control animals. Dose response curves can be generated showing a greater effect for higher level atmospheres. The data are reproducible; four

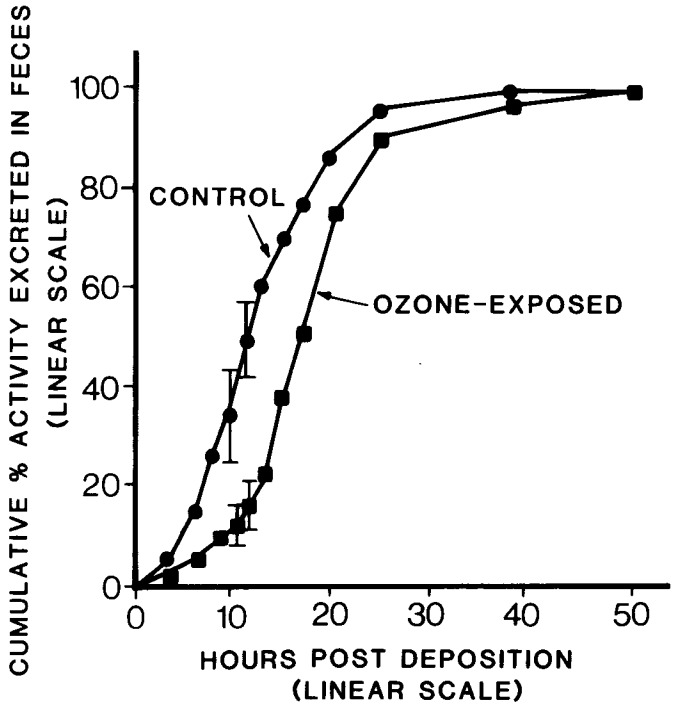


Figure 3 Activity excretion curves for control animals and animals exposed to 1 ppm ozone for 4 hr. When comparing the time required to excrete 50% of the total activity excreted during the first 50 hr for the two groups, a delay of approximately 5 hr in clearance of the radioactive tracer aerosol is seen in the ozone-exposed group. Ten animals were exposed in each group. Error bars represent ± 1 standard error of the mean.

groups of animals were exposed to 0.8 ppm ozone with similar results observed after each exposure. In all groups of animals exposed to ozone, a delay in early clearance and an acceleration in late clearance was seen.

A delay in early clearance could be caused by a decrease in mucus transport. Possible reasons for a decrease in transport velocity include a change in ciliary beat rate and changes in amount or properties of the mucus. Acceleration of clearance rate of particles observed in late clearance is most likely due to an increase in numbers or activity of deep-lung macrophages.

Other investigators have employed tests of clearance to determine the effects of ozone and other toxic agents. The effects of cigarette smoke on particle clearance have also been well studied. Albert et al. [13, 14] showed effects in humans and donkeys that depended on concentration of smoke and exposure duration. Low single doses or early effects of repeated exposure to smoke were associated with acceleration of clearance rates in the tracheobronchial tree of both species. Heavier doses and long-term repeated exposures were associated with sporadic clearance, intervals of clearance stasis, and even retrograde movement of deposited particles (again in both species). In a study of human smokers, Sanchis et al. [15] found slowed early clearance but faster late clearance.

Goldstein and coworkers [16, 17] reported work in which mice were challenged with radiolabeled viable staphylococcus both before and after exposures to relatively low levels of two environmental air pollutants, ozone and nitrogen

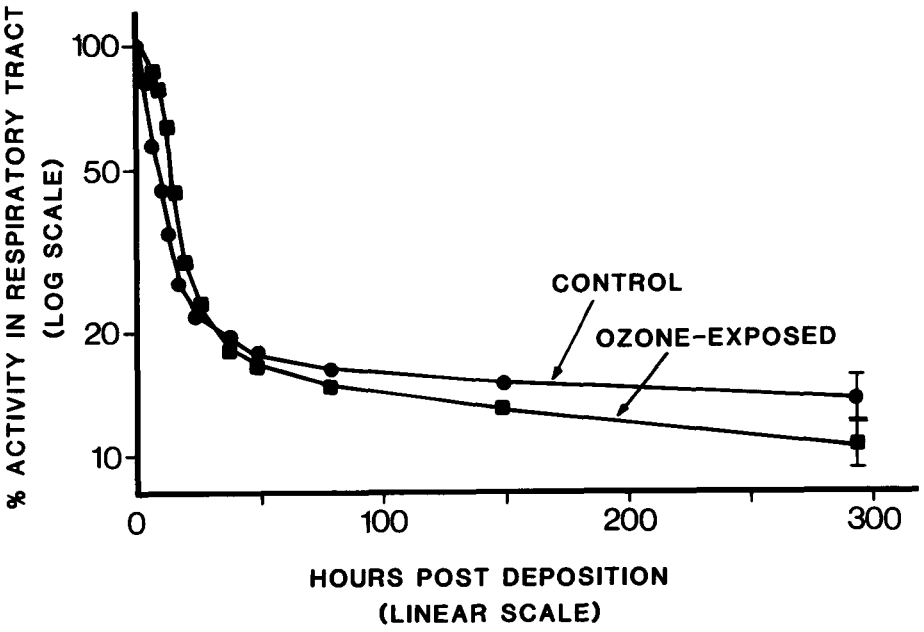


Figure 4 Clearance curves of ^{51}Cr-labeled tracer particles is measured by respiratory tract counting. Animals exposed to 1 ppm ozone for 4 hr show a delay in early clearance and an acceleration in late clearance when compared to control animals. Exposure to ozone or clean air was from hours 1–5 postdeposition of the tracer particles. Ten animals were exposed in each group. Error bars represent ± 1 standard error of the mean.

dioxide. Prior exposure to ozone (0.6–2 ppm, 17 h) or ozone plus nitrogen dioxide (0.1–0.1 and 1.5–4.2 ppm, 17 h) lead to impaired killing of deposited bacteria. In the same series of studies, exposures to ozone plus nitrogen dioxide (0.4 and 4–6.8 ppm, 4 hours) after inhalation of bacteria also led to increased survival of the bacteria. Ozone alone at 2 ppm (4 hr) was observed to cause both increased survival and increased mechanical clearance of the inhaled bacteria.

Ehrlich et al. [18] observed that exposures to various mixtures of nitrogen dioxide and ozone reduced the resistance of mice to streptococcal pneumonia. Daily 3-hr exposures (5 days/week) to a combination of 0.5 ppm NO_2 and 0.1 ppm O_3 for 2–6 months were very effective in reducing the host's resistance to this infectious organism. Schlesinger et al. [19] observed a short-term slowing of tracheobronchial mucociliary clearance of an inert test aerosol in donkeys after a 1-hr exposure to sulfuric acid mist at 194–1364 $\mu g/m^3$ of air (0.3–0.6-μm mass median aerodynamic diameter). Schlesinger et al. [20] also studied the effect of chronic inhalation exposures of donkeys to sulfuric acid mist upon mucociliary clearance. Exposures were 1 hr/day, 5 days/week, for 6 months to 102–106 $\mu g/m^3$ (0.5-μm mass median aerodynamic diameter) aerosol. Bronchial clearance became erratic within the first week of exposure and clearance rates of the exposed animals were significantly different, usually slower, than the control animals. Two of the exposed animals showed a sustained impairment of clearance toward the end of the 6-month exposure period.

Sulfur dioxide (1 ppm, 4 hr, up to 25 days) has been shown to diminish the clearance of inert particles in both the deep lung and tracheobronchial tree in the rat by Ferin and Leach [21]. A similar effect was seen in donkeys after a brief exposure (300 ppm SO_2, 30 min) by Spiegelman et al. [22].

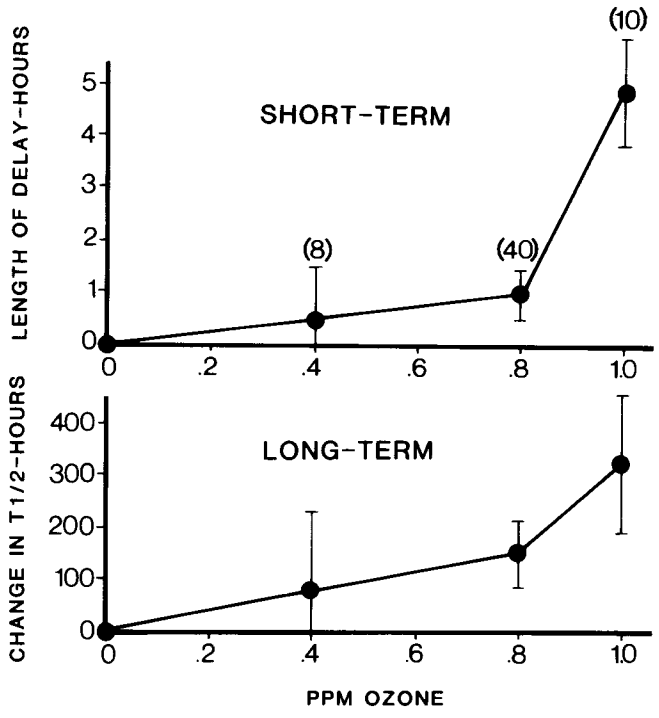


Figure 5 Dose response curves for both short- and long-term clearance of the tracer particles. Animals were exposed to 0, 0.4, 0.8, or 1.0 ppm ozone for 4 hr. The approximate number of animals in each exposure group is shown in parentheses; refer to Table 1 for numerical values. Error bars represent ± 1 standard error of the mean.

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