# Adaptations to Ozone in Reference to Mucociliary Clearance

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ABSTRACT

Adaptation to ozone in respiratory tract mucociliary clearance was investigated in this study. Eighty rats inhaled insoluble radioactively labeled particles in order to permit monitoring of clearance. The respiratory tract and the feces were counted for radioactivity at fixed intervals to determine clearance rates. A brief challenge to 1.2 ppm of ozone following particle deposition caused a substantial delay in rapid (mucociliary) clearance. This delay (or "ozone effect") however, was eliminated by brief pre-exposure to 0.8 ppm of ozone 3 days prior to deposition of particles. When a 13-day period intervened between the ozone pre-exposure and challenge, a substantial delay (or "ozone effect") was again seen. Thus, the pre-exposure to ozone appeared to afford essentially complete protection at 3 days, and no protection by 13 days.

CLEARANCE EFFICIENCY of particles deposited in the respiratory system is of major interest in the field of inhalation toxicology. Any type of delay in clearance will increase the period for which inhaled particles remain in the respiratory system. If these particles are toxic, a greater retention time can cause greater susceptibility to attack. This makes vital the evaluation of exposure effects to pollutant atmospheres on particle clearance.

Upon inhalation, airborne particles are either deposited in the respiratory system or subsequently exhaled. Those deposited beyond the nasal region may be cleared out of the respiratory system by any of several mechanisms, depending on their size, shape, rate of dissolution, and region of deposition. However, for particles in the diameter size range of about one $\mu$m and above, three major mechanisms may be assumed primarily responsible for clearance.

1) Particles deposited on ciliated airways are primarily cleared by mucociliary action.
2) Particles deposited in the deeper non-ciliated portion of the lung may be cleared by macrophage cells which engulf and subsequently carry the particles from the respiratory system.
3) Particles with rapid rates of dissolution can dissolve through the respiratory surfaces into the blood stream, lymphatics, and other tissues.

One method of evaluating these pulmonary clearance mechanisms involves depositing radioactively labeled particles by inhalation and monitoring their clearance with external radiation detectors. The respiratory tract is counted for radioactivity at fixed intervals in order to determine the clearance rates of the tracer particles. When relatively insoluble tracer particles are used, very little dissolution through lung tissue occurs, therefore, clearance primarily involves the physical removal of these deposited particles from the respiratory tract. They are subsequently swallowed and eventually excreted. An alternative method of following clearance involves radioactive counting of fecal samples collected at fixed intervals.[1] Both methods of monitoring clearance were utilized in this investigation.

Figure 1 shows sample clearance curves for both monitoring methods using rats which have inhaled insoluble radioactively labeled particles. Two distinct rates of clearance are observed for each curve. Initially, a rapid clearance rate occurs which may be primarily attributed to mucociliary action in the nasopharyngeal and tracheobronchial regions. A slow clearance rate is seen about 12 to 18 hr after deposition. This phase continues for several days and is the result of deep lung clearance, presumably involving macrophage action and particle dissolution. Respiratory clearance data may be used to derive an excretory clearance curve. When this is done, good agreement is observed between the derived and experimental excretory clearance curves, indicating that each radiation monitoring system is independently capable of determining a clearance pattern.

A significant change in the clearance rates may result, if, after deposition of the radioactively labeled particles, the rat is placed into an exposure chamber containing a pollutant gas such as ozone. A delay is observed in the rapid (mucociliary) clearance portion of both curves as a result of a sufficiently high-level ozone challenge. This delay is more exemplified in the excretory clearance curve, where the counting errors in the curve are much less. Redistribution of particles in the respiratory tract causes

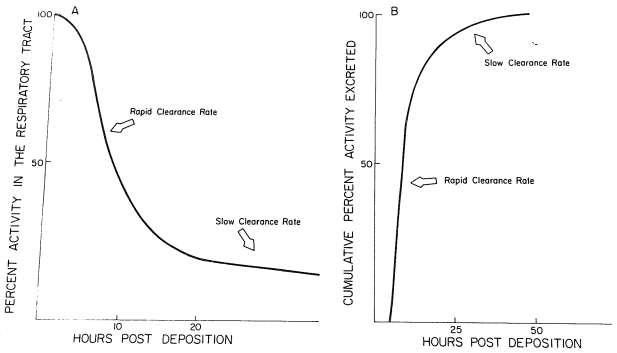


**Fig. 1. Clearance curves of rats which have inhaled insoluble tracer particles show both rapid and slow rates of clearance. A) Respiratory clearance curve determined by external radioactive counting of the respiratory tract at fixed intervals. B) Excretory clearance curve determined by radioactive counting of fecal samples collected at fixed intervals.**

greater counting errors in the respiratory clearance curve. The respiratory clearance curve is included only to show trends which were quantitated using the excretory clearance curve. In order to quantitate this delay, a $T_{50}$ parameter is introduced. This is defined as the time required for a 50% excretion of the radioactive particles with the feces. Comparing $T_{50}$ of a group of rats challenged to high levels of ozone after deposition of particles with $T_{50}$ of a group of rats without ozone challenge, a clearance blockage time is determined.

*Adaptation to Ozone*

Upon this basis, the possibility of adaptation to ozone in mucociliary clearance can be investigated. If adaptation to ozone is induced in mucociliary clearance, then subsequent exposure to ozone should not greatly hamper clearance. Thus, pre-exposure to ozone at a level sufficient to induce this adaptive effect should substantially reduce the blockage time associated with a future ozone challenge.

It has been shown that after low-level ozone pre-exposure, rats are able to tolerate what would otherwise be lethal doses of ozone.[2-5] Other pulmonary parameters, for which similar tolerance has been exhibited, weeks after ozone pre-exposure, include edema formation[4,5] and biochemical response.[6,7] Any protection in mucociliary clearance, however, has yet to be reported.

The goal of this investigation was to demonstrate the presence or absence of adaptation to ozone in mucociliary clearance. The procedure involved comparing the blockage times of rat groups challenged to ozone after particle deposition, with and without pre-exposure to ozone. If adaptation to ozone was observed, the duration of protection would be established by varying the period between pre-exposure and ozone challenge.

## Methods

Eighty white, male specific pathogen-free Sprague Dawley rats (Hilltop Lab Animals, Inc., Chatsowrth, Calif.) were used in this study. The animals were delivered in filtered boxes in order to minimize pre-experimental exposure to air pollutants. The rats' weights ranged from 175 to 224 g. The rats were randomly divided into eight groups of ten animals. Each rat group was treated according to the experimental protocol shown in Figure 2.

The radioactively labeled particles had to meet several requirements to allow unambiguous interpretation of the experiment's results. The particles had to be: essentially biologically inert; in the size range for significant deposition in the ciliated airways; relatively monodisperse; and possess a tightly bound radioactive label. Aerosol development over a period of 18 months prior to this experiment had yielded such an aerosol. The results of this development will be briefly mentioned here.

Monodisperse polystyrene latex (PSL) microspheres (Dow Chemical, Midland, Michigan) were selected for radioactive labeling with $^{51}Cr$ isotope (28-day half-life, 0.32 MeV gamma emission). The starting material—radioactive chromium chloride—was converted into a chromium acetylacetonate complex and bonded to the particles by repolymerization in the presence of a chelate. Bonding was enhanced by exposure to a high-level gamma ray field and labeled particles were finally purified by successive centrifugations.

Loss of label in the animals' lungs may occur by several processes, including leaching of the $^{51}Cr$, dissolution of the PSL particles, and breakup of the PSL surface. No attempt was made to separately evaluate each process, but the total $^{51}Cr$ loss rate was evaluated several ways. In vitro loss-rate studies were performed by placing PSL particles between two membrane filters using a filter holder designed for this purpose (Sandia Research and Development Corporation, Albuquerque, New Mexico), and submerging the filters in an aqueous sodium chloride solution containing bovine serum albumin. Aliquots taken from the solution at timed intervals were analyzed for $^{51}Cr$. In vivo studies included radioactive counting of various rat tissues at intervals after inhalation of the labeled PSL. Loss of label was very slow; about 0.1%/day in vitro and less than 0.3%/day in vivo.

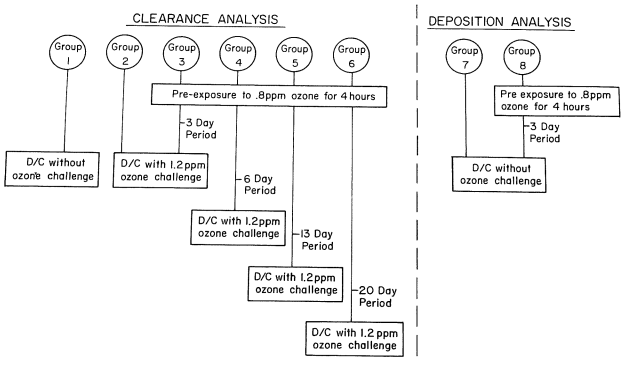


**Fig. 2. Experimental protocol for investigating ozone adaptation in mucociliary clearance. Eight groups of rats were evaluated with a deposition and clearance (D/C) test using insoluble radioactively labeled particles.**

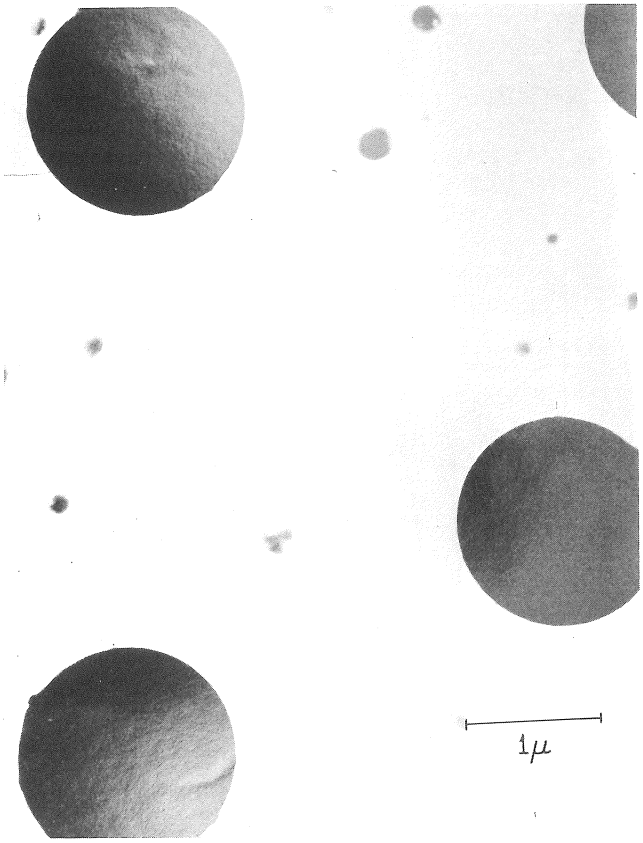


**Fig. 3. Electron micrograph of the polystyrene latex particles show rough-surfaced, monodisperse microspheres with a uniform count median diameter of 1.6 μm. The small fragments seen in the micrograph, formed by evaporation of nebulized droplets that did not carry a microsphere, contain less than 2% of the total radioactivity as determined by counting of cascade impactor samples.**

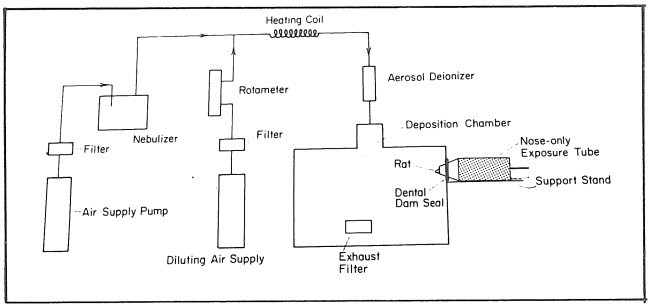


**Fig. 4. A schematic of the generation system used to deposit the tracer particles in the respiratory tract of rats. The deposition chamber has multiple ports, thereby allowing ten rats to be simultaneously exposed to the tracer particles. Exposure tubes are of perforated metal to prevent stress to the rats from temperature buildup.**

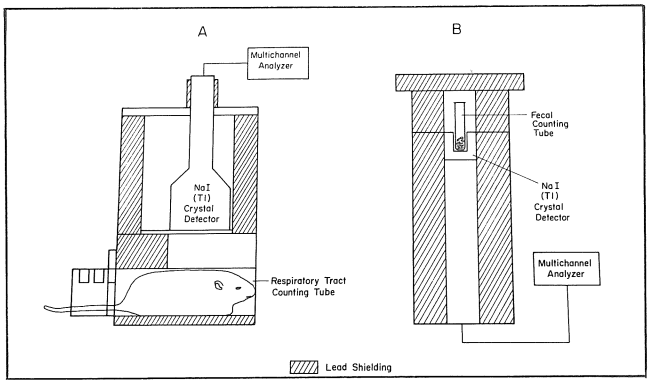


**Fig. 5. Diagrams of the counting systems used to monitor the tracer particles. A) System used for external radioactive counting of the respiratory tract, to determine the respiratory clearance curve. B) System used for radioactive counting of the feces, to determine the excretory clearance curve.**

Labeled PSL was aerosolized from a 0.1% aqueous suspension using a Lovelace-type compressed air nebulizer (ARIES, Inc., Albuquerque, New Mexico),[8] dried by heating and dilution with clean air, and passed through a 2-mCi $^{85}$K4 deionizer. The resultant aerosol had a mass median aerodynamic diameter of 2.3 μm and an associated geometric standard deviation (SD) of 1.2. Sizing was performed with a seven-stage Mercer-type cascade impactor with a back-up filter (ARIES, Inc., Albuquerque, New Mexico).[9] Electron microscopy of an electrostatic precipitor sample[10] of the particles as inhaled by the rats, showed slightly rough-surfaced monodisperse particles (Fig. 3).

The aerosol was nebulized into the deposition chamber, a 40-l aluminum-foil-lined cylinder with ports around the circumference for nose-only exposure of rats (Fig. 4). The rats were exposed to PSL particles for approximately 20 min , with aerosol being intermittently generated into the deposition chamber throughout this period. Following deposition of the radioactive aerosol, the nose-only exposure tubes were disconnected from the deposition chamber, the rats removed from the tubes, and wiped to remove particles which may have contaminated their heads. The rats were then put into individual counting restrainers which were placed beneath a collimated NaI (T1) gamma ray detector (Fig. 5*a*) to determine the initial (100%) amount of respiratory-tract radioactivity. Such counts were repeated at fixed intervals after exposure (to either clean air or ozone) to determine the respiratory clearance curves.

One hr after PSL-particle deposition the rats were placed in individual cage sections of open-mesh wire cages and challenged to 1.2 ppm ozone for 4 hr inside a one-m$^3$ Rochester-type exposure chamber.[11] This ozone concentration was chosen due to the substantial blockage time it produces in clearance, as observed in preliminary studies. Ozone was generated into the chamber by passing medical-grade oxygen through an electrical ozone generator (Sander, Osterberg, W. Germany). The flow rate through the chamber was maintained between 10 and 20 ft$^3$/min. The concentration was determined by a Dasibi ozone monitor by way of an inert sampling line from the chamber at the same height as the rat cages. The group of rats used to determine a normal clearance pattern was placed into a chamber with flowing, clean, filtered air for the 4-hr period, thus, establishing blockage times for the ozone-exposed groups.

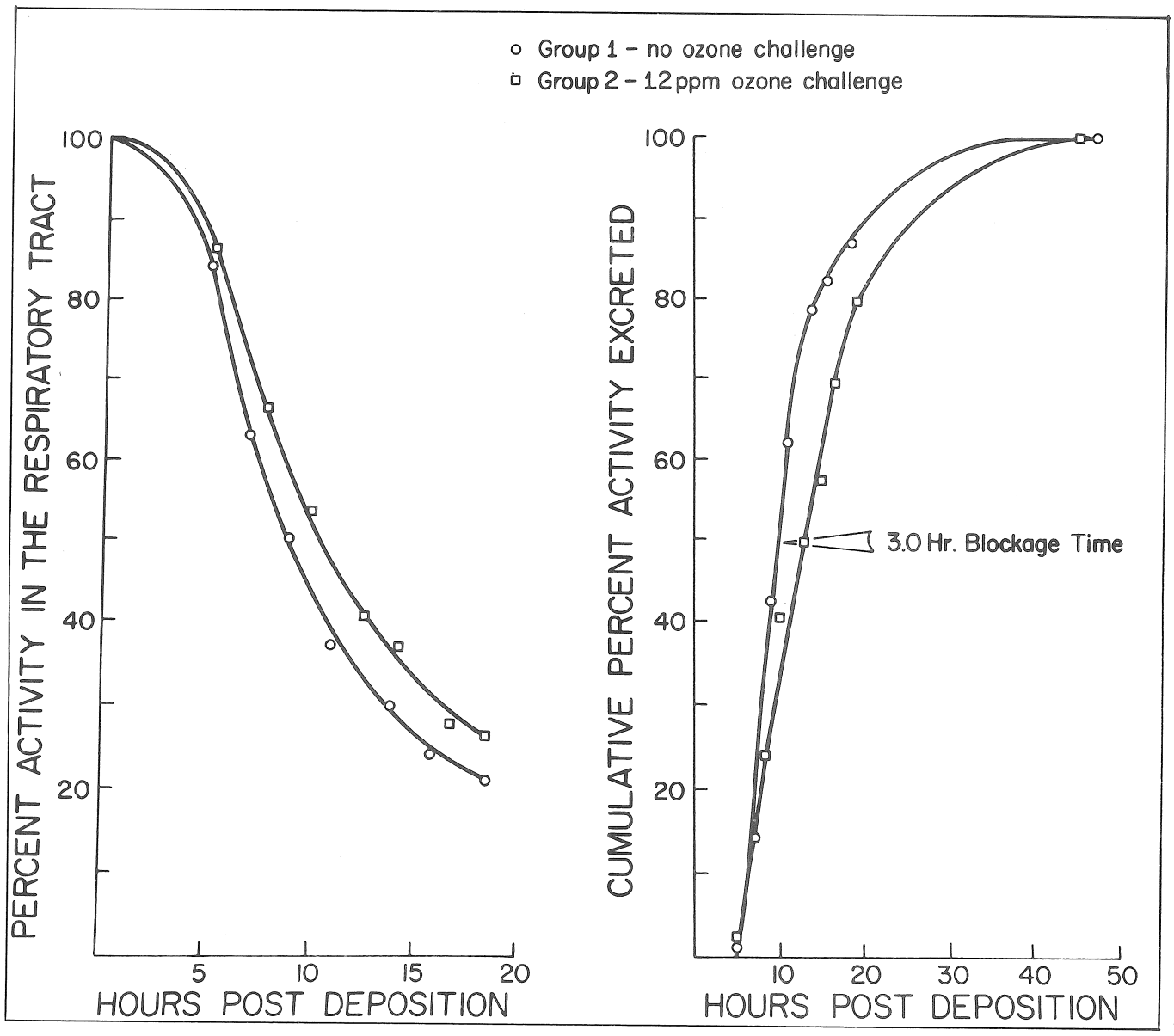


**Fig. 6. Respiratory and excretory clearance curves for inhaled insoluble particles both exhibited a substantial delay in rapid (mucociliary) clearance as a result of 1.2 ppm ozone challenge after deposition of PSL particles. The blockage time associated with this delay, determined by the excretory clearance curve, was 3.0 hr. Radioactivity in feces was essentially at background level after 50 hr, therefore, the amount excreted during the first 50 hr was taken as 100%.**

Fecal samples were collected from the cage sections of the individual rats at the termination of ozone challenge. These samples were counted for radioactivity in a NaI (T1) gamma ray detector well counter (Figure 5*b*). The feces of each rat were then collected at fixed intervals to determine the excretory clearance curve. Rat placement into individual counting restrainers stimulated fecal output, thereby making fecal collections at fixed intervals realistic. The total radioactivity excreted over the ensuing 50 hr was considered to be 100% since by that time, rats, whether exposed to ozone or clean air, had cleared about the same amount of radioactivity as observed from respiratory clearance curves in preliminary studies. Also, additional clearance of radioactive particles after 50 hr is negligible.

Rats, when pre-exposed to ozone (0.8 ppm for 4 hr) were similarly placed in individual cage sections in the Rochester chamber. Following pre-exposure these rats were returned to their housing cages until the day of particle deposition and subsequent ozone challenge.

## Results

Group 1 rats, after deposition of PSL particles, were allowed to clear without ozone challenge in order to provide a normal clearance curve for rats. Their $T_{50}$ was 9.6 ± 6 hr, which was used to establish the blockage times for other groups of rats.

Group 2 and Group 3 rats had PSL-particle deposition simultaneously to insure identical particle characteristics

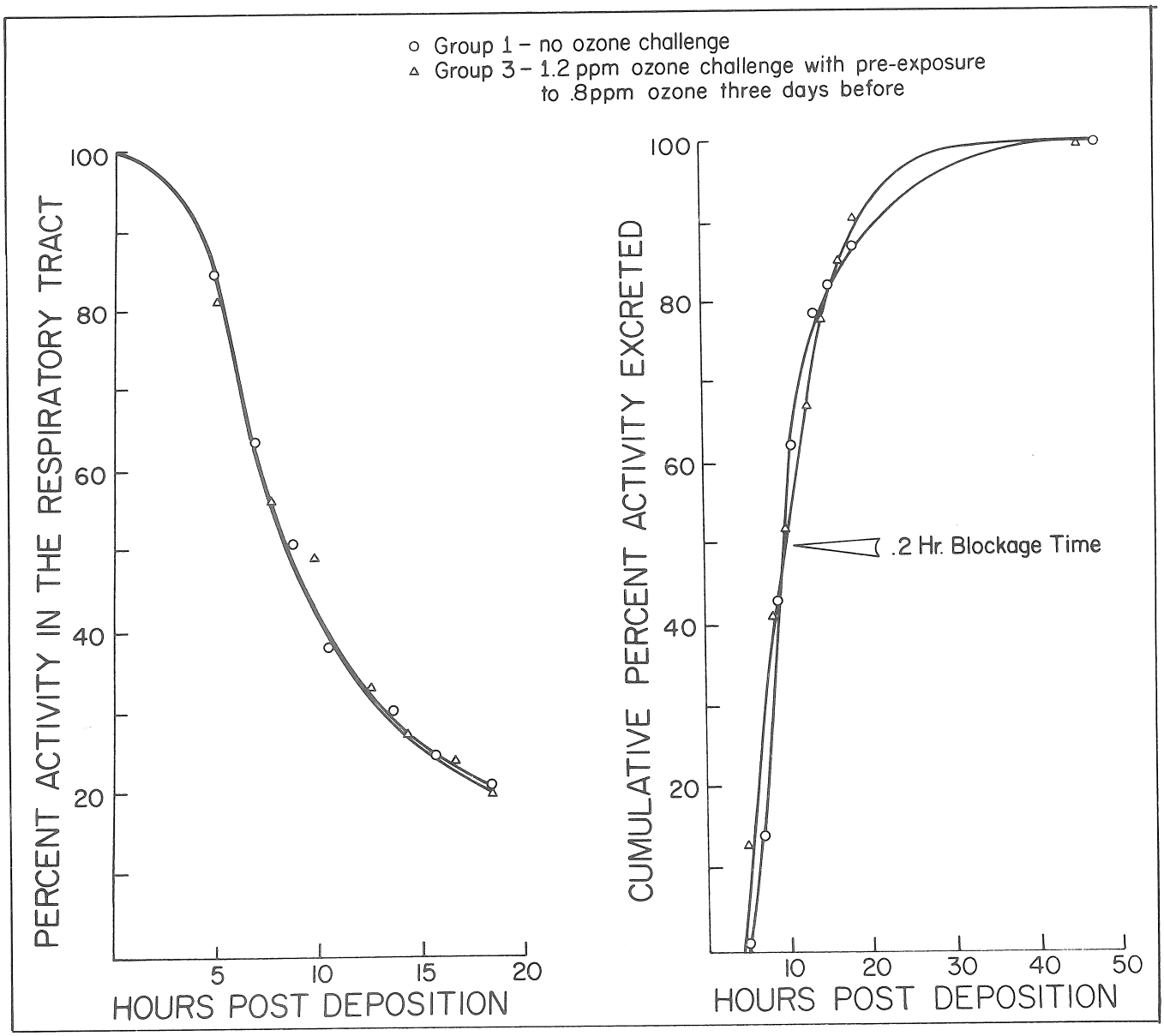


**Fig. 7. Respiratory and excretory clearance curves both showed the delay, resulting from 1.2 ppm ozone challenge, essentially eliminated as a result of pre-exposure to 0.8 ppm ozone 3 days before. The blockage time, determined by the excretory clearance curve, was reduced to an insignificant 0.2 ± 1.0 hr.**

for these two critical groups. Upon ozone challenge, Group 2 rats with no pre-exposure to ozone exhibited a substantial delay in clearance. This delay appeared in the rapid (mucociliary) clearance portion of both their respiratory and excretory clearance curves (Fig. 6). The $T_{50}$ for this group was 12.6 ± 1.2 hr, therefore, a blockage time of 3.0 ± 1.3 hr resulted from the ozone challenge.

Group 3 rats had been pre-exposed to ozone 3 days prior to ozone challenge. The delay from normal clearance was almost negligible in both the respiratory and excretory clearance curves (Fig. 7). The $T_{50}$ was 9.8 ± .8 hr, indicating an insignificant blockage time of only .2 ± 1.0 hr. Therefore, the pre-exposure 3 days before to ozone, had essentially eliminated the effect of a subsequent ozone challenge.

This result suggested an adaptation to ozone developed in Group 3. However, before this could be ascertained, it was necessary to show that both Groups 2 and 3 had essentially the same deposition pattern before clearance. If the airway of Group 3 rats were somewhat constricted as a result of previous exposure to ozone, greater deposition in the upper respiratory tract might result. Therefore, the clearance rate in these rats could be increased (with a reduced blockage time) due to differing deposition pattern and not to adaptation development.

Groups 7 and 8 had PSL-particle deposition simultaneously and were allowed to clear without ozone challenge, in order to insure that the deposition patterns with and without ozone pre-exposure were identical. Group 7

Table 1.—Summary of Results Showing Ozone Adaptation Occurrence in Mucociliary Clearance

| Group No. | Ozone Pre-Exposure Concentration (4 hr) (ppm) | Ozone Challenge Concentration (4 hr) (ppm) | Time Period (Days) between Ozone Pre-Exposure and Challenge | Time (hr) to Clear 50% of Tracer Particles ($T_{50}$) | Blockage Time (hr) |
|---|---|---|---|---|---|
| 1 | No pre-Exposure | No challenge | ----- | 9.6 ± .6 | 0, by definition |
| 2 | No pre-exposure | 1.21 ± .01 (.08) | ----- | 12.6*±1.2 | 3.0 ± 1.3 |
| 3 | .81 ± .00 (.02) | 1.21 ± .01 (.08) | 3 | 9.8*± .8 | .2 ± 1.0 |
| 4 | .81 ± .00 (.02) | 1.22 ± .01 (.03) | 6 | 10.7 ± .8 | 1.1 ± 1.0 |
| 5 | .81 ± .00 (.02) | 1.20 ± .02 (.09) | 13 | 12.7 ± .8 | 3.1 ± 1.0 |
| 6 | .81 ± .00 (.02) | 1.20 ± .01 (.04) | 20 | 12.4 ± .7 | 2.8 ± .9 |

NOTE: Values are accompanied by standard errors and standard deviations (in parentheses).

*A *t* test performed on the $T_{50}$ values of Groups 2 and 3 indicated that the $T_{50}$ of Group 3 was significantly lower, with greater than 95% certainty.

experienced no pre-exposure to ozone, while Group 8 had been pre-exposed to ozone 3 days before. The $T_{50}$ values were 9.6 ± .6 hr for Group 7 and 9.3 ± .4 hr for Group 8. The similarity of these values indicates that the pre-exposure to ozone 3 days before had no significant effect on the deposition pattern. The $T_{50}$ of Group 7 rats was identical to that of Group 1 rats, both clearing normally, which indicates the reproducibility of the data.

Adaptation to ozone in mucociliary clearance had thus been established. Pre-exposure to ozone at 0.8 ppm had rendered a 1.2 ppm ozone level 3 days later, which was almost ineffectual in producing delayed mucociliary clearance. The next step was to establish the duration of this protection.

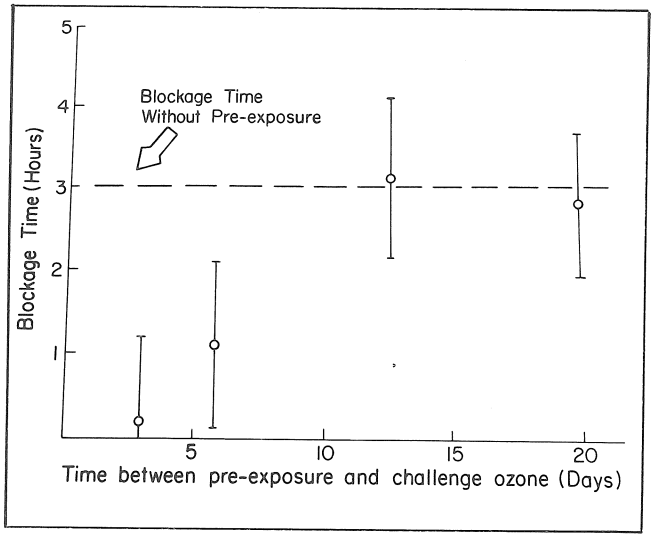


**Fig. 8. Protection afforded mucociliary clearance against future ozone exposure as a result of pre-exposure to ozone. There appeared to be essentially complete protection at 13 days, which decreased at 6 days and was completely lost by 13 days after the adaptation-inducing dose. The standard error bars indicate the significance of this response.**

Group 4 rats had been pre-exposed to ozone 6 days before deposition of PSL particles and ozone challenge. The $T_{50}$ of this group was 10.7 ± .8 hr, indicating a blockage time of 1.1 ± 1.0 hr. This would indicate that the degree of protection against ozone was lessening with duration after the adaptation-inducing dose.

Groups 5 and 6 had been pre-exposed to ozone 13 and 20 days, respectively, before ozone challenge. The $T_{50}$ values were 12.7 ± .8 hr for Group 7 and 12.4 ± .7 hr for Group 8, showing blockage times of 3.1 ± 1.0 and 2.8 ± .9 hr, respectively. These blockage times were almost identical to Group 2, without pre-exposure, indicating a loss of protection by 13 days after the adaptation-inducing dose. The results are summarized in Table 1 and the degree and duration of protection is shown in Figure 8.

## Conclusions

A 1.2 ppm ozone challenge for 4 hr causes a substantial delay in mucociliary clearance in rats. This delay is probably due to a slower rate of mucus transport, as is indicated by the reduction in the slope of the rapid clearance portion of the clearance curves. Slowing of mucus transport in both the rat and rabbit trachea, as a result of ozone exposure, has been previously reported.[12,13] This slower rate of clearance from the lung can be caused by alterations in mucus secretion, and/or changes in ciliary activity.

Morphologic observations of ozone exposure effects on the airways of the tracheobronchial tree by others have shown occurrence of extensive epithelial damage. Boatman[14] observed damage ranging from the largest bronchi to the respiratory bronchiole at 0.5 and 1.0 ppm ozone levels for 4.5 to 5.6 hr in the cat. His studies indicated that > 33% reduction in the number of ciliated cells occurred in the large- and medium-sized airways. Some desquamation of goblet cells also occurred in these airways. Large vacuoles in the ciliated epithelium and smaller vacuoles in the mucus gland cells were also reported. The 0.8 ppm ozone pre-exposure for 4 hr in this investigation

should have similarly caused extensive epithelial damage throughout the airways of the tracheobronchial tree.

A period of epithelial regeneration has been observed by Boatman[14,15] to follow this damage. He reported almost complete recovery of the ciliated epithelium in rabbits one wk post-exposure to 1.0 ppm ozone for 3 hr. He also reported that the goblet cells appeared intact, however, they had increased in size and had "filled in" areas previously occupied by the ciliated cells. Thus the period of regeneration correlates with the period of protection afforded by the 0.8 ppm ozone pre-exposure observed in this study. That is, during the period of epithelial regeneration there is protection against the effects of ozone exposure. The mechanism(s) involved in this protection is not clear.

A possible mechanism for this protection involves a thickening of the mucus layer. Boatman[14,15] reported the formation of larger goblet cells. This temporary structural change may lead to additional mucus secretion. A thicker mucus layer would offer the epithelium an extra physical barrier against ozone. As mucus secretion returns to normal, protection would be lost.

Another possible mechanism for this protection involves the ciliated cells and their cilia. Either the formation of intermediate cilia,[16] or the occurrence of some other temporary change in the regenerating ciliated cell could account for such protection. The protection would then be lost when the ciliated cell had essentially fully recovered.

It is interesting to note that the ozone challenge dose—1.2 ppm—is probably edemagenic. The occurrence of alveolar edema would not be expected to directly influence clearance from the rat's ciliated airways. It is possible that the reported alterations in mucus secretion, and/or ciliary activity are associated with the edema response.

Whatever the cause of this adaptation to ozone, its development has been shown in another function: mucociliary clearance. In this case, a brief exposure to a relatively low level of ozone afforded protection against a higher ozone level for about one wk. That the lung possesses significant adaptive potential to inhaled pollutants is intriguing. Description of both the limits of adaptive capability and the mechanisms of such capabilities appears to be a fruitful area for future investigation.

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