ARB Project 7-077-1 Final Report

Development of a Biological Test System for Quantitating the Respiratory Hazard of Ambient Concentrations of Air Pollutants and Evaluation of Vitamin E in the Prevention of Oxidant Induced Impairment.

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TO: Air Resources Board

RE: ARB Project 7-077-1 entitled

"Development of a Biological Test System for Quantitating the Respiratory Hazard of Ambient Concentrations of Air Pollutants and Evaluation of Vitamin E in the Prevention of Oxidants Induced Impairment."

The specific tasks to be accomplished in this contract were as follows:

(1) To determine the effect of exposure to nitrogen dioxide upon murine intrapulmonary bactericidal function.

(2) To determine the effect of combined atmospheres of nitrogen dioxide and ozone on murine intrapulmonary bactericidal function.

(3) To evaluate the role of vitamin E as an *in vivo* protector against oxidant-induced inhibition of pulmonary bactericidal activity.

INTRODUCTION

Atmospheric concentrations of ozone, nitrogen dioxide, carbon monoxide, etc., considerably above ambient have been shown to represent a significant health hazard (1-5). Unfortunately, at the present time we do not have a laboratory test system to assess the biological significance of ambient concentrations of these pollutants. This inability to demonstrate biological consequences with relevant pollutant exposures necessitates that air pollution control standards be arbitrary and insufficiently objective. Recent studies in our laboratory (funded by the Clean Air for California Program Project S-5) have demonstrated in mice and rats that ozone exposure significantly impairs intrapulmonary bactericidal function (6-8). These experiments showed a concentration dependent inhibition in murine ability to kill inhaled bacteria following exposures of four hours or more to ozone concentrations of 0.62 ppm and greater.

Previous investigations have shown that intrapulmonary bactericidal activity is due to phagocytosis by pulmonary macrophages and that this cell system is the primary host defense against inhaled bacteria (9). Since the anatomic position of the pulmonary macophage virtually obligates intimate exposure to any inhaled pollutant, this key element in defense might well suffer when any potentially toxic substance is inhaled. Therefore, although the mechanisms responsible for many pollutant induced impairments in pulmonary function are not well understood, it is likely that abnormalities in bactericidal function will occur and correlate with increasing concentrations of the pollutant.

The aforementioned finding that atmospheric concentrations of ozone, at 0.62 ppm or greater for four hours or more, caused a measurable decrease in this important host defense system suggested that this system is sufficiently sensitive to assess the biological hazard of other pollutants as well as atmospheres containing combinations of pollutants. We extended the previously described experiments with ozone to include murine exposures to nitrogen dioxide alone, and in combination with ozone. In this manner a biological model was developed which can be used to establish air quality control standards for combinations of these two pollutants.

In addition to the above studies, the murine model was used to evaluate the potential role of vitamin E in oxidant induced respiratory disease. The role of vitamin E, the major antioxidant defense system of the body in preventing oxidant induced pulmonary damage was evaluated (10, 11). Both hypervitaminosis E and vitamin E deficiency were produced in our animals and these changes were correlated with oxidant induced inhibition of pulmonary bactericidal activity. Vitamin E did protect against oxidant injury, and therefore, it is conceivable that human studies with this vitamin will indicate therapeutic value.

METHODS OF PROCEDURE

Animals - Male, Swiss, albino mice weighing 25 to 30 gm, and Sprague-Dawley CRD-free rats weighing 60-70 grams were used in all experiments. The animals were housed in plastic cages and given food and water ad libitum.

Pollutant Exposures

Nitrogen Dioxide - Nitrogen dioxide was carried into the exposure chambers in a stream of nitrogen gas. Pure nitrogen dioxide was maintained in the liquid state at 4 C in an ice bath. Nitrogen, flowing at rates less than 1.5 ml/min,

was sparged in the liquid nitrogen dioxide by means of a fritted glass bubbler. The concentration of nitrogen dioxide in the gas stream was measured continuously with a nitrogen dioxide analyzer. At random intervals, samples of air were obtained from the chamber for determination of the concentration of nitrogen dioxide according to the chemical method suggested by Saltzman (12).

Ozone Exposure - Ozone was generated from oxygen by silent electrical discharge. The concentration of ozone was measured by microcoulomb ozone sensors attached to a multiple point recorder. Control animals were exposed to identical air flows containing 21 percent oxygen.

Nitrogen Dioxide and Ozone Exposures - The air pollution system was designed to minimize reactions between ozone and nitrogen dioxide. Pure nitrogen dioxide was maintained in a liquid state at 4°C in an ice bath. Nitrogen, flowing at rates of less than 1.5 ml/min was sparged in the liquid nitrogen dioxide by means of a fritted glass bubbler. Gaseous nitrogen dioxide and carrier nitrogen were diluted with air in a mixing chamber and then conducted into a second mixing chamber that contained ozone which had been generated from oxygen by silent electrical discharge. The ozone had been previously diluted with air in a separate mixing chamber prior to its addition to nitrogen dioxide. The air containing nitrogen dioxide and ozone flowed from the second chamber into the exposure chamber. The flow rate for the exposure chamber was set at 30 volume changes per hour in order to maintain high gas turnover and thereby reduce the time in which ozone and nitrogen dioxide could react to form nitrogen pentoxide. Because the conversion of ozone to nitrogen pentoxide is stoichiometric, estimates of this reaciton were obtained by measuring amperometrically the increase

in ozone concentration after terminating the flow of nitrogen dioxide (13). The error of amperometric measurement of ozone caused by nitrogen dioxide was corrected by use of a calibration curve. Our observations confirm those of others (14)--approximately 10 percent of the nitrogen dioxide that is present is misinterpreted as ozone by the amperometric meter. The concentration of nitrogen dioxide within the exposure chamber was measured continuously using a colorimetric method that is not influenced by low levels of ozone (15). Samples of air were also obtained at random intervals from the exposure chamber for verification of these metered concentrations by means of the chemical methods suggested by Saltzman (12).

Animal Exposure - 1) Evaluation of the effects of nitrogen dioxide. The methods used for the *in vivo* assessment of the bactericidal capacity of the murine lung have been published (16). Groups of 30 mice were infected with aerosols of *Staphylococcus aureus* labeled with radioactive phosphorus (^{32}P) in an aerosol apparatus designed to produce uniform inhalation dosages of infecting bacteria. Immediately after infection, ten mice were killed with ether (0 hour) and the radioisotope concentrations of their lungs were measured. Half of the remaining mice were exposed for four hours to concentrations of nitrogen dioxide between 1.9 and 14.8 ppm. The ten control mice were exposed to similar flows of room air. Transfer of animals from the infection chamber to the nitrogen dioxide chambers took 30 to 45 minutes. At the end of the experimental period, the treated and control mice were killed and pulmonary transport and bactericidal activity rates were computed from measurements of pulmonary radioactive phosphorus

and bacterial concentrations (16). These rates were computed from the following formulas:

$$\text{Percent bactericidal activity} = 1 - \left[\frac{\text{bact } ct_{t\ hr}}{^{32}P_{\ t\ hr}\ XK} \right] \times 100$$

$$\text{Percent bacterial removal} = \left[\frac{^{32}P_{0\ hr} - ^{32}P_{t\ hr}}{^{32}P_{0\ hr}} \right] \times 100$$

The percent bactericidal activity is the rate at which bacteria are killed within the lungs, where bact $ct_{t\ hr}$ equals bacterial count at t hours, and K is the aerosol labeling constant. In the formula to compute percent bacterial removal, $^{32}P_{0\ hr}$ equals the radioactive phosphorus counts of the lungs of mice killed immediately after aerosolization. The experiments performed at the 1.9, the 3.8, and the 7.0 ppm level of nitrogen dioxide were repeated once.

In a second series of experiments, mice were exposed for 17 hours to nitrogen dioxide in concentrations of 1.0, of 2.3, and of 6.6 ppm. These animals were then infected with radiolabeled staphylococci and pulmonary bactericidal function was determined four hours later. The experiments performed at the 1.0 and 2.3 ppm level were repeated once.

Pulmonary specimens were obtained from the various treatment groups for histological examination in each series of experiments.

2) The effect of exposure to combinations of ozone and nitrogen dioxide on murine pulmonary defense mechanisms was studied with the aforementioned methods with the exception that combinations of ozone and nitrogen dioxide were investigated instead of nitrogen dioxide by itself.

3) The experimental design that was used to study the influence of vitamin E on ozone induced bactericidal dysfunction consisted of feeding rats the test diet until a severe degree of vitamin E deficiency was present as measured by the dialuric acid hemolysis test (hemolysis more than 80 percent).

Experimental Design - Rats were fed the test diets until a severe degree of vitamin E deficiency was present as measured by the dialuric acid hemolysis test (hemolysis of more than 80 percent). The animals that were deficient in vitamin E and appropriate controls were infected with aerosols of radio-labelled staphylococci, and then transferred to the pollution chambers (30-45 minutes) where they were exposed for four hours either to ozone at concentrations of 0.5 to 1.0 ppm or to air containing 21 percent oxygen. Pulmonary bactericidal activity was measured immediately after these exposures (16). In a second series of experiments, rats with vitamin E deficiencies and control animals were exposed continuously for 7 days to 0.7 ppm of ozone following which their pulmonary bactericidal capacities were determined. During the exposure period the rats were maintained on their appropriate diet. In each series of experiments, pulmonary specimens were obtained from the various treatment groups for histological examination.

Dietary Treatment - According to the literature, diets containing 10 mg/kg or more of vitamin E are sufficient to meet murine nutritional requirements (16a). Five diets were used in these studies. One set of commercially

prepared diets consisted of pellets in which vitamin E was either absent or present at the concentration of 110 mg/kg. Such diets have been used in previous studies relating the vitamin E status of the host to susceptibility to ozone-induced abnormalities (10). Their composition corresponds to the formula developed by Draper and colleagues with the exception that lard was substituted for corn oil (16b). A second set of diets that were deficient or supplemented with vitamin E were prepared in our laboratory according to the directions of Draper and colleagues, with the exception that 15 percent of tocopherol stripped corn oil was substituted for 10 percent tocopherol stripped corn oil (16b). The composition of these diests is shown in Table 1. Vitamin E was absent in the deficient diet and 45 mg/kg of the vitamin was present in the supplemented diet. A standard laboratory diet (Purina Lab Chow) which contained at least 60 mg/kg of vitamin E was used as a control (17). The vitamin E status of the animals was determined by measuring the serum concentration of the vitamin with the dialuric acid hemolysis test (18). Murine weight was determined weekly.

Edema Formation - The presence of edema fluid was evaluated by calculating a wet to dry weight ratio of the lungs (19). The rats were sacrificed, their lungs removed, and the surfaces blotted with filter paper. After weighing these lungs, they were dried in an oven at 60°C for 24 hours and reweighed. In order to insure that a stable weight had been achieved after the initial 24 hours of drying, the lungs were replaced in the oven and weighed again at 48 hours. Weight changes were not noted in the 24 to 48 hour interval in these experiments.

RESULTS

1) Exposure to nitrogen dioxide.

The data for the experiments in which mice were infected with radiolabeled staphylococci and then exposed for four hours to concentrations of nitrogen dioxide ranging from 1.9 to 14.8 ppm are shown in Table 2. The mean values for the pulmonary radioactive phosphorus and bacterial concentrations, aerosol labeling ratio (K), and percentage decline in radioisotope count and bactericidal activity are also shown. According to these data, physical removal of bacteria was not affected by exposure to nitrogen dioxide. Similar amounts of radioisotope were present within the lungs of control and nitrogen dioxide-treated mice for each of the exposure levels studied. The decline in radioisotope concentration for the five-hour experimental period was small and tended to be less than 17%. Pulmonary bactericidal activity decreased progressively with exposure to increasing concentrations of nitrogen dioxide. These differences were significant ($P<.05$) for the comparison of controls and mice treated with 7.0 ppm of nitrogen dioxide or greater.

The effect of a 17-hour exposure to different concentrations of nitrogen dioxide on murine pulmonary bactericidal activity is shown in Table 3. The radioisotope concentrations of the lungs of control and treated mice were similar for each of the levels of nitrogen dioxide that were studied, i.e., there were no differences in inhalation as a result of exposure to the pollutant. Pulmonary bactericidal activity was reduced in mice that were exposed to either 2.3 or 6.6 ppm of nitrogen dioxide. The impairment in pulmonary bactericidal activity was small following exposure to 2.3 ppm of nitrogen dioxide. A much larger defect was noted in mice exposed for 17 hours to 6.6 ppm of nitrogen dioxide.

Histological examination of pulmonary specimens from mice exposed for four hours to 7.0 ppm or less of nitrogen dioxide did not show changes from control specimens. Sections from mice exposed to 9.2 and 14.8 ppm of nitrogen dioxide demonstrated vascular hyperemia. Neither intra-alveolar edema nor acute inflammation was found. The lungs of mice exposed for 17 hours to 2.3 or 6.6 ppm showed minor changes of vascular hyperemia and interstitial edema, and a rare focus of intra-alveolar edema. Foci of nonspecific inflammation were equally present in treated and control mice.

Effect of Exposure to Ozone and Nitrogen Dioxide - The experiments which were performed to estimate the reaction of ozone with nitrogen dioxide to form nitrogen pentoxide indicated that approximately 50 percent (range 25-70%) of the available ozone may have undergone a chemical reaction. Since each molecule of ozone would form only one molecule of nitrogen pentoxide (20), the concentration of nitrogen pentoxide approximated 0.15 ppm for most experiments and never exceeded 0.25 ppm.

It should be noted that ozone is a highly reactive compound which enters into other chemical reactions besides the formation of nitrogen pentoxide. Hence, the aforementioned estimates are intended only as a means for assessing maximal concentrations of this potentially toxic by-product.

Mice that were exposed to the polluted atmospheres were lethargic during and immediately following the exposure periods. In 7 of 9 experiments in which the exposures were of 17 hours duration, the treated mice lost significant amounts of weight (4-12%) despite the availability of ample quantities of food and water. In the two experiments which were performed

at low oxidant concentrations, neither weight loss nor bactericidal dysfunction were noted. Weight loss was 2 percent or less in the exposures of four hours duration.

The data for the experiments in which mice were infected with radiolabeled staphylococci and then exposed for four hours to concentrations of ozone of 0.11 to 0.39 ppm in combination with 2.04 to 7.27 ppm of nitrogen dioxide are shown in Table 4. The Table contains the mean values for ozone and for nitrogen dioxide, the pulmonary radioactive phosphorus and bacterial concentrations, the aerosol labeling (K), percentage decline in radioisotope count, bactericidal activity, and the wet to dry weight ratios. According to these data, physical removal of bacteria was not affected by exposure to combinations of the pollutants. The decline in radioisotope concentration for the experimental period tended to be less than 20 percent and was similar for treated and control mice. Pulmonary bactericidal activity was not significantly affected by exposure to atmospheres containing subthreshold levels of both pollutants (<0.4 ppm ozone; <7.0 ppm nitrogen dioxide). Exposure to threshold levels of ozone 0.36 and 0.39 ppm in combination with 4.06 or 6.84 ppm of nitrogen dioxide did result in significant decreases of pulmonary bactericidal activity ($P<0.05$).

The effect of 17 hour exposures to different concentrations of ozone and nitrogen dioxide are shown in Table 5. Exposure to 0.11 to 0.27 ppm of ozone in combination with 1.49 to 4.18 ppm of nitrogen dioxide resulted in severe decreases of 10 to 60 percent in the numbers of inhaled bacteria. Although the deficits in bacterial inhalation did not exactly parallel increases in pollutant concentration, the larger deficits coincided with

exposure to the higher levels of oxidant. Similarly, pulmonary bactericidal activity was impaired most severely in mice that were maintained in the more polluted atmospheres. Pulmonary bactericidal activity was not impaired by prolonged exposure to the lower concentrations 0.11 and 0.20 ppm of ozone and 1.74 and 1.49 ppm of nitrogen dioxide. It is noteworthy that for each pollutant combination, the deficit in bactericidal function was comparatively less than the deficit in ventilation as judged by the numbers of inhaled bacteria. Table 5 also shows that the bacterial clearance rates corresponded to the pulmonary bactericidal activity rates. The concordance of these two measurements demonstrates that the rate of radiophosphorus detachment and loss from the staphylococci was not specifically altered in mice that had been exposed to oxidants for 17 hours. The rates of bacterial removal, as measured by the decrease in radiophosphorus concentrations, were similar for control and treated mice in all experiments except one. In the experiment in which mice were exposed to 0.21 ppm of ozone and 4.18 ppm of nitrogen dioxide, the rate of radiophosphorus removal was significantly decreased in the treated mice ($P<0.05$).

Histological examination of specimens from mice that were exposed for four hours to combinations of ozone and nitrogen dioxide showed mild vascular hyperemia and occasional foci of hemorrhage. The lungs of mice exposed for 17 hours to pollutants did not show changes from control specimens. Small areas of non-specific peribronchial inflammation were infrequently present in treated and control mice.

Similar wet to dry weight ratios for murine lungs were found for each experimental group indicating that gross fluid retention was not a factor in these experiments (Tables 4, 5). The small increases in the ratios which were noted in mice that were exposed to the pollutants for four hours were not significant ($P>0.05$).

Influence of commercially prepared vitamin E test diets on susceptibility of murine pulmonary bactericidal activity to ozone-induced impairment - Figure 1 shows that rats fed the commercial test diets did not gain weight. The failure of these diets to support normal growth seemed to be related to the palatability of the diet as the animals did not consume normal quantities of pellets. This failure in growth was not related to the abrupt change from the post-weaning Lab Chow diet, since preconditioning for three weeks with the diet containing 110 mg/kg of vitamin E did not alter the abnormal growth pattern (Fig. 2). According to the results obtained with the dialuric acid hemolysis test, the animals maintained on diets that were severely deficient in vitamin E had hemolysis of 80 percent or more of their red blood cells after 2-5 weeks of dietary treatment; rats maintained on diets containing 110 mg/kg of vitamin E had an average percentage of hemolysis of 30 percent. Blood from animals fed Purina Lab Chow manifested an average of 38 percent hemolysis.

The data for the experiments in which the effect of a four hour exposure to ozone on pulmonary bactericidal activity of rats fed these diets are shown in Table 6. The mean values for ozone concentration, pulmonary radiophosphorus and bacterial concentrations, and percentage decline of

viable staphylococci in the lungs are shown. According to these data, smaller concentrations of radiophosphorus were present within the lungs of rats fed the two commercially prepared vitamin E test diets than were present within the lungs of rats fed Lab Chow. Similar numbers of bacteria were recovered from the lungs of rats fed the test diets following exposure to 21 percent oxygen or to ozone. Fewer bacteria were recovered from the lungs of rats fed diets of Lab Chow following the control exposure. Pulmonary bactericidal activity was decreased significantly and equivalently, in rats fed either test diet after exposure to atmospheres of 21 percent oxygen when compared with animals fed diets of Lab Chow. Exposure to 0.5 ppm of ozone resulted in a further decrease in bactericidal activity for the groups than the reduction in bacterial killing, although not statistically significant, appeared to be greater in animals that were deficient in vitamin E.

Similar decreases in pulmonary bactericidal activity occurred following 4 hour exposures to control atmospheres in animals that were preconditioned with the commercially prepared diet containing 110 mg/kg of vitamin E prior to the induction of vitamin E deficiency with the diet that did not contain vitamin E. Since the significance, if any, of the deficiency in vitamin E was obscured by the impairment in bactericidal function that occurred independently of the vitamin E status, these diets were not studied further.

Influence of laboratory prepared vitamin E test diets on susceptibility of murine pulmonary bactericidal activity to ozone-induced impairment - Figure 3 shows that rats fed diets that were deficient or supplemented with vitamin E in accordance with the modified Draper formula, manifested substantial

growth. This rate of growth was only slightly less than that observed for rats that were fed Lab Chow. According to the data in Table 7, lower concentrations of radiophosphorus and of staphylococci were recovered from the lungs of rats fed these test diets than from the lungs of rats fed control diets of Lab Chow. Since each of these measurements declined in an equivalent fashion, pulmonary bactericidal activity was not altered by the changes and similar rates of bacterial inactivation were observed for each experimental group. Exposure to 0.5 or 1.0 ppm of ozone caused equivalently significant decreases in pulmonary bactericidal activity in rats fed the supplemented diets as in rats fed the diets deficient in vitamin E (Table 7, Figure 4). Hence, the presence of vitamin E deficiency did not enhance the defect due to ozone and supplementation with vitamin E did not prevent the abnormality.

The data obtained for the experiments in which rats were fed the laboratory prepared vitamin E test diets, and then exposed to 0.7 ppm of ozone for seven days before measurements of pulmonary bactericidal activity were obtained are shown in Table 8. Because the previous experiments demonstrated that pulmonary bactericidal activity was not altered in rats maintained on these diets, animals fed Lab Chow were not included as controls. Murine weight increased normally prior to the exposure period. Exposure to ozone resulted in 7.0 percent weight loss for the rats without vitamin deficiency, and a 16.7 percent weight loss in rats with deficiencies. The difference in weight loss between the two groups is significant ($P<0.05$) demonstrating that a deficiency in vitamin E increases the rate at which weight is lost during exposure to ozone.

The vitamin E status of the animals did not affect pulmonary bactericidal activity as rats deficient in vitamin E killed inhaled staphylococci as rapidly as did rats without deficiencies. When infection occurred after exposure to ozone, a significant decrease in pulmonary radiophosphorus concentration was found in rats fed either test diet indicating an ozone-induced reduction in bacterial inhalation. Exposure to ozone also caused a decrease in pulmonary bactericidal activity for each treatment group. This decrease was significantly larger in rats with vitamin E deficiencies demonstrating that a deficiency in vitamin E enhances the degree of ozone-induced impairment.

The results of experiments in which the fluid content of the lungs was measured for animals that had been exposed to 0.7 ppm of ozone for 7 days, and for control animals are shown in Table 9. Calculation of the dry weight to wet weight ratio of the lungs revealed an equivalent content of 77 to 79 percent of water. It is noteworthy, that pulmonary wet and dry weight measurements were significantly higher in rats exposed for a 7 day period to ozone than in comparable controls ($P<0.001$). This ozone-induced increase in tissue mass occurred in all groups of rats independent of dietary therapy. Although not statistically significant ($P>0.05$) rats with vitamin E deficiencies appeared to manifest larger increases in pulmonary weight after exposure to ozone than did non-deficient controls.

The lung wet-dry-weight data for experiments in which rats were exposed to 0.5 or 1.0 ppm of ozone for 4 hours are shown in Table 10. Animals exposed to 0.5 ppm of ozone had lung wet weights, dry weights, and fluid contents

equivalent to the control animals. The finding of a significant increase in lung wet weights ($P<0.01$) and dry weights ($P<0.001$) of animals fed vitamin E supplemented diets and exposed to 1.0 ppm of ozone, and no increase in lung weights of the deficient-ozone exposed rats is surprising, and we do not have an explanation for these results.

Histological examination of the lungs of rats exposed to 0.5 or 1.0 of ozone for four hours showed increased vascular congestion when compared to controls. These levels of exposure to ozone did not cause abnormalities within the bronchial or alveolar regions. Infrequent foci of chronic inflammation were present to an equivalent extent in all groups. Acute inflammation was not found. Histological differences were not found in pulmonary specimens from rats fed diets that were deficient or supplemented with vitamin E. The lungs of animals exposed for 7 days to 0.7 ppm of ozone did not show vascular congestion. These specimens did demonstrate a moderate loss of bronchial epithelium as well as occasional intrabronchial exudates. Areas of fibrosis and thickening of interstitial tissues were found in these specimens (Fig. 5). A few pulmonary sections from the ozone treated rats contained an increased number of eosinophiles. Again, no distinction could be made histologically between lungs from rats fed diets that were supplemented or deficient in vitamin E.

DISCUSSION

These experiments demonstrate that exposure to nitrogen dioxide adversely affects the intrapulmonary defense mechanisms of the murine lung. This defect is present in animals exposed for brief periods (four hours) to levels of nitrogen dioxide of 7.0 ppm or greater. With more prolonged

exposures (17 hours) bactericidal dysfunction occurs at concentrations of 2.3 ppm or greater of nitrogen dioxide. It is noteworthy that these findings are quantitatively similar to data obtained from experiments in which pathogenic microorganisms (*Klebsiella pneumonia*) were used to assess the effect of nitrogen dioxide on the overall pulmonary bacterial defense systems (21).

Two mechanisms of host defense against inhaled bacteria are presently known: mechanical removal by the mucociliary stream and bactericidal destruction in situ by the intrapulmonary defense system (9). From the initial experiments, the defect in bactericidal activity is consequent on inhibition of in situ defense systems, whereas physical removal rates are not altered by exposure to nitrogen dioxide. Although there may be several mechanisms responsible for intrapulmonary killing, from previous experiments pulmonary alveolar macrophages are of primary importance (9). The pathogenesis of defective phagocytosis involves either or both hampered macrophage mobility (e.g., from intra-alveolar or interstitial edema, or a toxic effect on the cell itself.

Respiratory tissue damage and edema formation are known consequences of exposure to gaseous pollutants (1, 22, 23), changes that might prevent macrophages from trapping or killing inhaled microorganisms. However, in our experiments there were minimal abnormalities in the lungs of mice exposed to threshold levels of nitrogen dioxide. These results are consistent with reports by other investigators of only minor histological and ultrastructural change in the lungs of rodents subjected to low levels of nitrogen dioxide (24, 25). It is, therefore, unlikely that the defects in bactericidal

function which were found following 17-hour exposures to 2.3 ppm or 4-hour exposures to 7.0 ppm of nitrogen dioxide were due to anatomic abnormalities. Alternatively, nitrogen dioxide may have diminished macrophage function by peroxidation of lung lipids (26, 27), inactivation of enzyme systems (28), or denaturation of proteins (29). Such biochemical abnormalities could be important for they have been observed to follow exposures to nitrogen dioxide that were similar in concentration and duration to those used by us (26, 27).

The pattern of injury observed following nitrogen dioxide exposure is similar to that previously reported for ozone (6). Brief exposure to either pollutant results in progressive impairment in intrapulmonary bactericidal activity without significantly altering mucociliary function. Both are oxidants; however, ozone is approximately ten times more toxic than nitrogen dioxide: the threshold level for ozone-induced dysfunction is 0.66 ppm and that for nitrogen dioxide is 7.0 ppm.

An important physiological difference between ozone and nitrogen dioxide concerns the effect of each gas on ventilation. A 17-hour exposure to nitrogen dioxide failed to cause a reduction in the number of inhaled bacteria, whereas similar exposure to ozone profoundly decreased the number of inhaled bacteria. Since ozone causes severe bronchoconstriction (6), the previously reported reduction in inhaled bacteria probably represented an increase in airway resistance such that fewer bacteria were inhaled and those that were inhaled were deposited higher in the bronchial tree. This response to ozone can be considered protective since it diminishes the pulmonry bacterial burdern and redistributes the inhaled microorganisms to the upper airways. It is

noteworthy that prolonged exposure to nitrogen dioxide does not evoke this protective mechanism and nitrogen dioxide at levels of 2.3 ppm or greater cause significant decreases in pulmonary bactericidal activity.

Before discussing the data from the experiments in which mice were exposed to both ozone and to nitrogen dioxide, certain aspects of oxidant chemistry and of the measurement of bactericidal function merit consideration. When the ozone concentration is high, nitrogen dioxide is readily oxidized to nitrogen pentoxide (20). It has been estimated that at a nitrogen dioxide concentration of 1.0 ppm the half life of ozone is 8 minutes (20). At higher nitrogen dioxide concentrations the conversion rate may be even faster. In order to retard this chemical reaction, the nitrogen dioxide and ozone were diluted prior to mixing, and this mixture was flowed rapidly through the exposure chamber so that the time available for reaction and building up of reaction products would be reduced. These measures were partially successful, in that they kept the estimated concentration of nitrogen pentoxide below 0.25 ppm and enabled the maintenance of predetermined ozone and nitrogen dioxide levels. Although data concerning the toxicity of nitrogen pentoxide are few, the experiments that have been performed indicate that low levels of nitrogen pentoxide are unlikely to be important (22, 30).

Measurement of pulmonary bactericidal capacity is a precise means for quantitating the antibacterial properties of the lung (14). Previous investigations have shown that changes in bactericidal function correlate

with the severity of experimentally induced pathophysiologic abnormalities (31-33). Exposure to ozone or nitrogen dioxide results in a pattern of a concentration-related inhibition in pulmonary bactericidal function (6-8, 31, 34). Because of differences in producing and measuring oxidant concentrations, as well as differences in determining bactericidal activity, the threshold values for this dose-effect relationship may vary for individual experiments. However, the reproducibility of the threshold value is sufficiently reliable to allow its use in a comparative manner (7, 31). The cited references demonstrate that by studying a range of oxidant concentrations one can predict with considerable accuracy at which level bactericidal function will be diminished and at which level function will be unimpaired (6-8, 31).

The initial experiments were designed to determine if brief (4 hour) exposures to different combinations of ozone and nitrogen dioxide would result in a synergistic, antagonistic, or an indifferent kind of bactericidal injury. The data were interpreted by using the criteria mentioned by Jawetz (35). Synergy was present if the effect produced by the combination was greater than the sum of effects produced by each of the components. Antagonism was present if the effect of the combination was less than that produced by the sum of the effects of the components. Indifference occurred when the effect of the combination equaled the effect of the single more active component or the sum of the effects of the two individual gases. To take into account errors in determining bactericidal thresholds, a greater than twofold difference in concentration from the theoretical

additive effect was considered necessary in order to demonstrate a synergistic or antagonistic interaction. Previous studies had shown that for a four hour exposure the threshold level for an ozone-induced defect was approximately 0.4 ppm (31), and that the level for nitrogen dioxide was approximately 7.0 ppm (36). According to the data obtained in the present experiments, combinations of ozone and nitrogen dioxide act differently in that they cause bactericidal dysfunction at concentrations which approximate the injury thresholds of the individual gases. A significant bactericidal defect was present at ozone and nitrogen dioxide concentrations (ppm) of 0.29 and 7.16; 0.36 and 4.06; and 0.39 and 6.84. Because bactericidal abnormalities were not present when mice were exposed to lesser concentrations of each pollutant, synergy was not a factor in these experiments. The absence of a bactericidal abnormality in the experiment in which 0.4 ppm of ozone was combined with 2.17 ppm of nitrogen dioxide and in the experiments in which 6.33 and 7.27 ppm of nitrogen dioxide were combined with 0.16 and 0.11 ppm of ozone can be attributed to the experimental variation which occurs near the threshold level. It is unlikely that the two oxidants interacted antagonistically, because increasing concentrations of the pollutnats (0.36 and 0.39 ppm of ozone and 4.06 and 6.84 ppm of nitrogen dioxide) caused progressively severe defects in bactericidal function.

These investigations also demonstrate that the impairment in murine defenses which is induced by a brief exposure to ozone and nitrogen dioxide results from abnormalities in intrapulmonary bacterial killing rather than

defects in bacterial transport. In these experiments, as well as the previously cited studies of individual oxidant effects, bacterial removal mechanisms were not inhibited by exposure to polluted atmospheres (6, 7, 31, 36).

The experiments in which mice were exposed for 17 hours to combinations of ozone and nitrogen dioxide demonstrated that ventilatory disturbances as well as bactericidal defects are important pathophysiologic consequences of exposure to oxidants. In fact, according to these data, ventilatory dysfunction as measured by bacterial inhalation is a more sensitive parameter of oxidant-induced injury than is bactericidal function. Reductions in the numbers of inhaled bacteria occur at concentrations of pollutants that are below the threshold levels for bactericidal dysfunction. Furthermore, when both abnormalities are present, the physiological defect is usually more severe. Because low levels of ozone, but not of nitrogen dioxide, inhibit ventilatory function, this physiologic abnormality is primarily an ozone induced defect. The pathogenesis of the physiologic disturbance involves tracheobronchial irritation, increases in airway resistance, and the development of a rapid and shallow respiratory pattern (22, 23, 37, 38).

Unlike the ventilatory defect which can be attributed solely to ozone, the impairment in bacterial defense is probably a combined effect. The aforementioned investigations in which pollutants were studied individually have shown that significant reductions in bactericidal activity occur following 17 hour exposures to concentrations of ozone above 0.57 ppm (6),

and of nitrogen dioxide of 2.3 ppm. In the present experiments, impairments in bacterial defense appeared at or about the threshold level of nitrogen dioxide (set 3 and 4, Table 5) indicating that the defect was in part caused by nitrogen dioxide. The finding that the addition of 0.27 ppm of ozone to a subthreshold level of 1.78 ppm of nitrogen dioxide results in bactericidal impairment, whereas the addition of 0.11 or 0.20 ppm of ozone to slightly lower, subthreshold levels of nitrogen dioxide fails to induce impairment, is consistent with addition of the injurious effects of each gas--an indifferent, not synergistic effect. Had synergy occurred, combinations of the lower, subthreshold concentrations of the two oxidants should have depressed bactericidal activity.

In addition to a direct toxic action on the macrophage, these gases may have affected cellular function indirectly, by reducing the nutritional intake of food and water. Animals that are starved have impaired pulmonary antibacterial activity, presumably as a result of metabolically induced abnormalities in phagocytic function (3, 9, 40). Since mice that were exposed to polluted atmospheres for 17 hours lost weight, this nutritional effect may have accounted in part for the bactericidal defects. Also, in accordance with a nutritional effect is the finding that bactericidal function was unimpaired in the two experiments in which weight loss was absent. Weight loss was minimal in the four hour exposures, and hence, nutritional factors were of lesser importance in these experiments.

Although ozone and nitrogen dioxide are edemagenic (1), it is unlikely that sufficient edema was provoked to hamper macrophage mobility. The threshold level for edema formation in mice following a 20 hour exposure to

ozone is about 1.0 ppm (23), and that for nitrogen dioxide is above 10.0 ppm (41). Deficits in antibacterial function were found in many experiments following exposure to much lower pollutant concentrations. The postulated absence of edema in these subthreshold exposures is corroborated by the failure to demonstrate excess pulmonary fluid by histological examination and pulmonary wet weight and dry weight measurements.

With one exception the transport rates for bacteria were not affected by exposure to these levels of ozone and nitrogen dioxide. This observation is consistent with previous studies indicating that at these concentrations mucociliary transport is not altered by exposure to either ozone (6) or nitrogen dioxide (36). The decreased rate of bacterial removal which was noted after the 17 hour exposure to 0.21 ppm of ozone and 4.18 ppm of nitrogen dioxide is difficult to evaluate. These levels were among the highest that were studied and ciliary function may have been impaired by prolonged exposure to these high concentrations. Because the difference in transport rates was in part due to a negative radiophosphorus removal rate in the treated mice, future experimentation is necessary to determine the reproducibility of this result.

Deficiency of vitamin E augmented the adverse effect of prior, low-level exposure to ozone (0.7 ppm for 7 days) on murine pulmonary bactericidal capacity. On the other hand, identical hypovitaminosis failed to influence murine pulmonary killing of staphylococci inhaled prior to exposure to 0.5 or 1.0 ppm of ozone for 4 hours. These experiments will be discussed separately.

The additive effect of the vitamin deficiency plus the pollutant in the 7 day experiment probably reflects: 1) the interaction between the vitamin, the pollutant, and the alveolar macrophage system, and 2) an indirect nutritional effect which resulted in a more rapid rate of weight loss in rats that were deficient in vitamin E. Previous studies in this laboratory with the murine:inhaled *S. aureus* model have shown that ozone inhibits pulmonary defenses by reducing the intrapulmonary bacterial killing rate (6, 7). Pulmonary phagocytes play a major role in bacterial killing (9). Because ozone inflicts injury that is evident both morphologically (34, 42), and functionally (43), it is likely that toxicity can be attributed to damage to this cellular defense system. Consistent with this hypothesis, fibrosis was a prominent feature in the histological specimens that were obtained from rats exposed to ozone. Although the exact biochemical mechanisms by which ozone injures cells has not been determined, the effect is probably related to the production of free radicals and lipid peroxides (44-46). Vitamin E is a scavenger of free radicals, and it has been suggested that the vitamin prevents ozone-induced damage by inactivating such highly injurious compounds (11, 47, 48). In the present experiments, rats that were deficient in vitamin E should have lacked the antioxidant protection normally afforded by vitamin E; therefore, intrapulmonary defenses were inordinately vulnerable to the toxic action of ozone.

Vitamin E deficiency may also have altered murine susceptibility to the ozone in a non-specific way. Rats deficient in vitamin E lost more weight during the 7 day exposure period than did control animals. Previous experiments have shown that the nutritional status of the host is an important determinant

of pulmonary bactericidal activity. Animals that are starved or lose weight due to nutritional deprivation are unable to kill inhaled bacteria normally (39, 40). It is difficult to evaluate fully the nutritional status from measurements of weight alone. Nevertheless, a non-specific, nutritional effect may have accounted, in part, for the augmented reduction in pulmonary bactericidal activity that was noted in the deficient-ozone exposed rats.

Unlike the findings with prolonged exposures, deficiency in vitamin E did not further compromise pulmonary bactericidal capability as reduced by exposure to ozone for 4 hours. Two concentrations of ozone, 0.5 and 1.0 ppm, were studied. A 4 hour exposure to ozone at 0.5 ppm is the minimum that will cause measurable impairment of pulmonary bactericidal function (6, 31); deficiency of vitamin E did not enhance this minimal defect. Similarly, the defect caused by 4 hours exposure to 1.0 ppm was not made worse by pre-existing deficiency in vitamin E. Although it was not reflected in the wet-dry weight measurements, vascular congestion was present in histological sections from rats exposed to 0.5 or 1.0 ppm of ozone. This difference in sensitivity for the two measurements of pulmonary fluid has been observed previously by others (49). There are several possible explanations for the differences in results with the acute and the long term exposures.

Firstly, rats that were made deficient in vitamin E, as documented by assay of serum, may have had extravascular stores of the vitamin which allowed some protection from brief exposures to ozone. Rats that were exposed chronically, suffered protracted insults and such postulated stores of vitamin E would be insufficient to afford protection. In support of this concept, it should be noted that the dialuric acid test is performed on serum and indicates deficiencies rather than absences of vitamin E (16);

tissue levels of the vitamin may be present even when serum levels are inordinately low (15). It is difficult to correlate the percent hemolysis observed in the dialuric acid test with the exact concentrations of vitamin E in plasma because this relationship depends in part on the fat composition of the diet (50). Comparison of our data with data obtained in animals fed a similar diet indicates that values of less than 10 percent hemolysis correspond to concentrations of 421 μg/100 ml, or more, of vitamin E and values of 80 percent hemolysis correspond to concentrations of less than 135 μg/100 ml of vitamin E (50). Secondly, animals that were deficient in vitamin E had continued nutritional losses in the course of the prolonged exposure to ozone, unlike similarly exposed controls. Because nutritional status affects pulmonary bactericidal activity, this difference--which occurred only in chronically exposed rats--might account for the discrepancy in vitamin E effect.

Lastly, the abnormalities induced by an acute exposure to ozone differ from those that occur after chronic exposures. Brief exposures to sublethal levels of ozone cause vascular congestion, pulmonary edema, and decreases in certain sulfhydryl enzyme concentrations (23, 46). With continued exposure, tolerance develops to each of these initial abnormalities. This pharmacological action results in improvement in the initial defects despite continued exposure (1, 23, 51). At the same time, new abnormalities of pulmonary fibrosis and emphysema develop in chronically exposed animals (23, 52). The interrelationships between these pathophysiologic abnormalities, vitamin E, and pulmonary bactericidal activity are not known, but it seems likely that

vitamin E may provide varying degrees of protection for pulmonary defenses depending on the kind of ozone-induced abnormality.

The marked reductions in bacterial deposition that were observed are in accordance with the decreases in vital capacity and minute ventilation that have been shown to occur following exposure to ozone (23). These changes are consequences of ozone-induced increases in airway resistance (38). As a result, there is redistribution of inhaled bacteria within the pulmonary tree; however, recent studies in our laboratory indicate that neither bacterial transport rates nor bactericidal activity are altered (36).

The importance of the diet used to produce a deficiency in vitamin E is also shown in these experiments. The commercial vitamin E diets failed to support normal rates of murine growth. When pulmonary bactericidal activity was tested in rats fed such diets, it was impaired in controls as well as in animals with severe deficiency. In contrast, rats fed test diets that were prepared in our laboratory, according to the directions of Draper and colleagues, displayed nearly normal growth rates and pulmonary bactericidal activity. We are unsure of the factor(s) responsible for the cessation of growth that occurred when animals were fed the commercial diets. The primary difference(s) between the commercial and the laboratory prepared test diets is the substitution of lard for corn oil in the commercial product. This change may have decreased the palatability of these diets, an explanation which is consistent with the observation that fewer pellets were consumed by rats maintained on commercial diets.

SUMMARY

The following paragraphs summarize each of the three series of experiments that were performed in fulfillment of ARB Project 7-077-1.

The effect of nitrogen dioxide on murine resistance to infection was investigated by simultaneously determining physical removal and bactericidal activity rates of murine lung. Mice were exposed to various concentrations of nitrogen dioxide for 17 hours prior to or 4 hours after infection with aerosols of *Staphylococcus aureus* labeled with radioactive phosphorus (^{32}P). Animals infected and then exposed to levels of nitrogen dioxide above 7.0 ppm showed a progressive decrease in percent pulmonary bactericidal activity which could not be accounted for by physical removal of bacteria. Exposure to levels of 2.3 ppm or greater for 17 hours prior to staphylococcal infection caused decreases in bactericidal activity.

Since ozone also impairs pulmonary bactericidal activity, the effect of combinations of these gases was investigated in order to ascertain whether they interacted biologically in a synergistic, indifferent or an antagonistic manner. Mice were exposed to atmospheres of ozone and nitrogen dioxide for 17 hours prior to, or 4 hours after infection with aerosols of radiolabeled *S. aureus*. Bacterial transport and inactivation rates were measured at 4 hours. Animals infected and then exposed to a range of oxidant combinations manifested bactericidal dysfunction when the level of one of the pollutants approximated its individual threshold value (O_3 - NO_2 in ppm 0.29 - 7.16; 0.36 - 4.06; 0.39 - 6.84). Exposures to the pollutants for 17 hours prior to infection caused bactericidal dysfunction when a subthreshold

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TABLE 1. Composition of diets prepared in our laboratory according to the directions of Draper et al.

15.8%	Tocopherol stripped corn oil*
58.3%	Glucose
21.1%	Vitamin Free Casein
4.2%	Salt Mixture 4164†
0.6%	Vitamin Mix

*The diet described by Draper et al contained 10% tocopherol stripped corn oil.

†Draper et al, J. Nutrition 84:395, 1964.

TABLE 2 Effect of Exposure to Different Concentrations of Nitrogen Dioxide on the Defense Mechanisms of the Murine Lung.

Experimental Group[1]	Nitrogen Dioxide (ppm) Mean ± SD	Bacterial Count/ml Lung Homogenate[2]	^{32}P Count/ml Lung Homogenate[3]	Bactericidal Activity (%)[3,4]	^{32}P Removal (%)[3]
Control 0 hr (19)			803 ± 94		
Control 5 hr (19)	1.9 ± 0.3	41.7 ± 11.8	824 ± 57	78.0 ± 4.8	- 2.6 ± 14.0
NO_2 5 hr (18)		55.8 ± 14.3	857 ± 72	76.3 ± 4.7	- 6.7 ± 15.4
Control 0 hr (20)			1489 ± 147		
Control 5 hr (20)	3.8 ± 0.5	86.0 ± 16.1	1313 ± 122	87.3 ± 1.7	11.8 ± 12.0
NO_2 5 hr (20)		104.0 ± 12.1	1240 ± 127	83.6 ± 1.7	16.7 ± 11.8
Control 0 hr (19)			996 ± 95		
Control 5 hr (18)	7.0 ± 0.3	31.0 ± 4.7	873 ± 85	88.1 ± 2.2*	12.3 ± 11.9
NO_2 5 hr (19)		52.3 ± 3.3	921 ± 92	81.0 ± 1.9	7.5 ± 12.8
Control 0 hr (10)			1487 ± 114		
Control 5 hr (9)		60.2 ± 3.4	1471 ± 88	91.3 ± .4**	1.1 ± 9.6
NO_2 5 hr (9)	9.2 ± 0.6	158.0 ± 19.1	1507 ± 58	77.7 ± 2.9	1.3 ± 8.7
Control 0 hr (10)			425 ± 28		
Control 5 hr (10)	14.8 ± 0.3	30.0 ± 7.3	503 ± 33	81.6 ± 3.6**	-18.4 ± 11.0
NO_2 5 hr (10)		94.6 ± 13.2	464 ± 25	31.5 ± 12.5	- 9.2 ± 9.3

TABLE 2 continued

(1) Number in brackets is the number of animals in each group.

(2) Mean ± SE X 10^2

(3) Mean ± SE

(4) K values: 1.91 - K = 25.1

3.75 - K = 51.4

6.98 - K = 29.9

9.23 - K = 47.6

14.79 - K = 31.4

*$P<0.05$

**$P<0.01$

TABLE 3 Effect of a 17-Hour Exposure to Different Concentrations of Nitrogen Dioxide on the Bactericidal Activity Rate of the Murine Lung.

Set	Experimental Group[1]	Nitrogen Dioxide (ppm) Mean ± SD	Bacterial Count/ml Lung Homogenate[2]	^{32}P Count/ml Lung Homogenate[3]	Bactericidal Activity (%)[3,4]
1	Control (20)	1.0 ± 0.1	39.0 ± 7.2	930 ± 87	84.2 ± 2.8
	NO_2 (20)		45.9 ± 7.5	1000 ± 64	83.6 ± 2.3
2	Control (20)	2.3 ± 0.2	24.2 ± 5.4	1184 ± 95	92.0 ± 1.6*
	NO_2 (20)		38.1 ± 4.3	1061 ± 86	85.7 ± 1.7
3	Control (10)	6.6 ± 0.6	25.8 ± 1.7	172 ± 10	92.0 ± 0.6**
	NO_2 (10)		98.6 ±13.7	130 ± 12	57.3 ± 7.8

(1) Number in brackets is the number of animals in each group.

(2) Mean ± SE X 10^2

(3) Mean ± SE

(4) K values: Set 1 = 28.5; Set 2 = 27.7; Set 3 = 192.0.

* $P<0.05$

** $P<0.01$

TABLE 4. Effect of a 4 hour exposure to nitrogen dioxide and ozone on the defense mechanisms of murine lung

Set	Experimental Group	Pollutant (ppm ± SD) NO_2	Pollutant (ppm ± SD) Ozone	Bacterial Count per Milliliter Lung Homogenate[a]	^{32}P Count per Milliliter Lung Homogenate[a]	Bactericidal Activity %[a, b]	^{32}P Removal %[a]	$\left[1 - \frac{\text{dry weight}}{\text{wet weight}}\right] \times 100$ Percent ±SE
	Control 0 hr				775 ± 77			
1	Control 4 hr			6355 ± 1894	764 ± 77	88.6 ± 2.5	1.4 ± 13.9	73.5[e]
	NO_2—O_3 4 hr	2.04 ± .18	0.30 ± .04	8124 ± 2031	684 ± 58	82.8 ± 3.2	11.7 ± 11.5	73.9
	Control 0 hr				567 ± 40			
2	Control 4 hr			1867 ± 387	512 ± 44	88.1 ± 2.2	9.7 ± 10.0	72.3
	NO_2—O_3 4 hr	2.17 ± .13	0.40 ± .03	1215 ± 158	461 ± 32	91.7 ± 0.9	18.7 ± 8.0	74.5
	Control 0 hr				1667 ± 123			
3	Control 4 hr			5142 ± 1030	1365 ± 75	84.9 ± 2.9	18.1 ± 7.5	74.5
	NO_2—O_3 4 hr	4.08 ± .40	0.18 ± .04	4059 ± 714	1261 ± 65	86.9 ± 1.7	24.4 ± 6.8	72.3
	Control 0 hr				579 ± 76			
4	Control 4 hr			6112 ± 1055	498 ± 62	89.6 ± 1.3	14.0 ± 15.6	76.4
	NO_2—O_3 4 hr	4.04 ± .24	0.27 ± .04	8856 ± 1443	500 ± 60	86.3 ± 1.6	13.6 ± 15.4	77.0
	Control 0 hr				1351 ± 79			
5	Control 4 hr			2630 ± 314[d]	1062 ± 99	72.9 ± 2.1	21.4 ± 8.7	76.4
	NO_2—O_3 4 hr	4.06 ± .22	0.36 ± .03	5910 ± 1508	1038 ± 75	36.0 ± 14.7[c]	23.2 ± 7.1	77.2

TABLE 4 continued

Set	Experimental Group	Pollutant (ppm ± SD) NO_2	Pollutant (ppm ± SD) Ozone	Bacterial Count per Milliliter Lung Homogenate[a]	^{32}P Count per Milliliter Lung Homogenate[a]	Bactericidal Activity %[a, b]	^{32}P Removal %[a]	$\left[1-\frac{\text{dry weight}}{\text{wet weight}}\right] \times 100$ Percent ± SE
	Control 0 hr				586 ± 84			
6	Control 4 hr			3033 ± 815	510 ± 40	86.8 ± 2.9	13.0 ± 14.2	74.6
	NO_2—O_3 4 hr	6.33 ± .37	0.16 ± .07	2678 ± 383	738 ± 102	90.6 ± 1.9	-25.9 ± 25.1	76.3
	Control 0 hr				825 ± 64			
7	Control 4 hr			1886 ± 230	780 ± 67	90.8 ± 1.2	.5.5 ± 10.9	74.9
	NO_2—O_3 4 hr	7.27 ± .25	0.11 ± .02	2458 ± 259	716 ± 23	87.3 ± 1.3	13.2 ± 7.3	75.0
	Control 0 hr				991 ± 127			
8	Control 4 hr			1456 ± 321	929 ± 108	92.8 ± 1.0[d]	6.3 ± 16.2	75.2
	NO_2—O_3 4 hr	7.16 ± .42	0.29 ± .06	2012 ± 355	800 ± 114	86.5 ± 1.4	19.3 ± 15.5	77.5
	Control 0 hr				830 ± 42			
9	Control 4 hr			1555 ± 114	797 ± 69	82.5 ± 1.9	4.0 ± 9.6	74.8
	NO_2—O_3 4 hr	6.84 ± .23	0.39 ± .08	13659 ± 2170	879 ± 66	-28.8 ± 17.7[d]	-5.9 ± 9.6	75.4

(a) Mean ± SE

(b) K values: set 1 = 54; set 2 = 31; set 3 = 23; set 4 = 130; set 5 = 9.2; set 6 = 44; set 7 = 27; set 8 = 19.0; set 9 = 12.0.

(c) $P < 0.05$

(d) $P < 0.01$

(e) Standard deviations < 0.87 for each value; p values > 0.05 for all comparisons.

TABLE 5 Effect of a 17 hour exposure to nitrogen dioxide and ozone on the defense mechanisms of the murine lung.

mental up	Pollutant (ppm) Mean ± S.D. NO_2	Ozone	Bacterial Count per Milliliter Lung Homogenate[a] 0 hr	4 hr	Radiophosphorus Count per Milliliter Lung Homogenate[b] 0 hr	4 hr	Bactericidal Activity % [b]	Bacterial Clearance % [b]	^{32}P Removal % [b]	$\left[1 - \frac{\text{dry weight}}{\text{wet weight}}\right] \times 100$ % ± SE	Weight Change %
trol			296 ± 34	51 ± 17	924 ± 93[d]	768 ± 104	83.6 ± 3.9	82.7 ± 6.2	21.1 ± 13.1	79.4[f]	0
lutant	1.74 ± .26	0.11 ± .02	207 ± 24	45 ± 7	706 ± 65	572 ± 65	79.8 ± 2.3	77.9 ± 4.4	19.0 ± 11.8	80.2	+ 4
trol			363 ± 28	70 ± 11	1372 ± 128	1210 ± 156	81.5 ± 3.6	80.7 ± 3.6	11.8 ± 14.0	78.2	+ 2
lutant	1.49 ± .30	0.20 ± .05	324 ± 33	51 ± 7	1098 ± 122	1005 ± 132	84.2 ± 2.4	84.1 ± 2.7	8.5 ± 15.7	78.8	0
trol			346 ± 17	50 ± 64	916 ± 48[e]	756 ± 39	84.2 ± 2.0[e]	85.5 ± 2.0[c]	17.5 ± 6.0	77.5	0
lutant	2.30 ± .30	0.20 ± .03	196 ± 13	44 ± 47	559 ± 49	403 ± 43	70.9 ± 3.1	77.4 ± 2.8	27.9 ± 9.9	77.9	-13
trol			351 ± 38[e]	55 ± 47	1001 ± 89[e]	769 ± 70	83.9 ± 2.5[e]	84.2 ± 2.2[e]	23.1 ± 9.8	78.1	- 1
lutant	1.78 ± .20	0.27 ± .01	191 ± 25	71 ± 84	525 ± 47	416 ± 32	69.6 ± 4.7	62.7 ± 6.7	20.8 ± 9.4	78.3	- 5
trol			405 ± 37	73 ± 71	1341 ± 113[e]	1067 ± 71	80.5 ± 2.8[e]	81.9 ± 2.4[e]	20.4 ± 8.5[c]	78.0	0
lutant	4.18 ± .39	0.21 ± .04	108 ± 69	63 ± 82	423 ± 22	523 ± 49	68.1 ± 3.0	41.7 ± 8.5	-23.6 ± 13.2	78.6	- 5

Mean ± SE X 10^2

Mean ± SE

Average K values: Set 1 = 40.4; Set 2 = 35.2; Set 3 = 41.2; Set 4 = 55.5; Set 5 = 38.8

(d) $P<0.05$

(e) $P<0.01$

(f) Standard deviations for each value <1.13; P values >0.05 for all comparis

TABLE 6. Effect of a 4 hr exposure to 0.53 ppm ozone on the pulmonary bactericidal activity of rats fed vitamin E test diets formulated with tocopherol stripped lard.

Experimental Group No. of animals	Exposure	Bacterial Count per milliliter of lung*	^{32}P Count per milliliter of lung‖	Bactericidal Activity %†
b Chow, 8	Control	101 ± 17	2760 ± 324	78.7 ± 2.5
tamin E Test Diet 110 mg/kg, 17	Control	348 ± 71	2081 ± 87	53.6 ± 5.5
tamin E Test Diet 0 mg/kg, 15	Control	311 ± 44	2282 ± 192	56.5 ± 3.7
tamin E Test Diet 110 mg/kg, 15	.53 ± 0.05**	358 ± 78	1772 ± 114	39.8 ± 7.3
tamin E Test Diet 0 mg/kg, 17	.53 ± 0.05	411 ± 84	1903 ± 230	27.8 ± 10.3

*Mean ± S.E. X 10^2

†Mean ± S.E.

‖Mean ± S.E.

**Mean ppm ozone ± SD

Comparisons: Lab Chow, Control vs 110 mg/kg Vitamin E, Control $p < .02$

Lab Chow, Control vs 0 mg/kg Vitamin E, Control $p < .001$

All other comparisons, $p > .05$

TABLE 7. Effect of a 4 hr exposure to 0.50 ppm of ozone on the pulmonary bactericidal activity of rats fed vitamin E supplemented or vitamin E deficient diets formulated with tocopherol stripped corn oil.

Experimental Group No. of Animals	Exposure	Bacterial Count per Milliliter of Lung*	^{32}P Count per Milliliter of Lung‖	Bactericidal Activity %†
Lab Chow, 16	Control	309 ± 37	2823 ± 432	75.4 ± 2.0
Vitamin E Test Diet 45 mg/kg, 15	Control	174 ± 13	2102 ± 259	80.4 ± 1.9
Vitamin E Test Diet 0 mg/kg, 15	Control	245 ± 33	2357 ± 404	76.0 ± 2.6
Vitamin E Test Diet 45 mg/kg, 16	.50 ± .07**	247 ± 24	2060 ± 258	69.0 ± 3.7
Vitamin E Test Diet 0 mg/kg, 12	.50 ± .07	323 ± 53	2015 ± 277	67.1 ± 3.1

*Mean ± S.E. X 10^2

‖Mean ± S.E.

†Mean ± S.E.

**Mean ppm ozone ± SD

Comparisons: Vitamin E 45 mg/kg, Control vs Vitamin E 45 mg/kg, Ozone $p < .02$

Vitamin E 0 mg/kg, Control vs Vitamin E 0 mg/kg, Ozone $p < .05$

All other comparisons, $p > .05$

TABLE 8 The influence of a 7 day exposure to 0.70 ppm ozone on the pulmonary bactericidal activity of rats fed vitamin E test diets formulated with tocopherol stripped corn oil.

Experimental Group No. of Animals	Exposure	Bacterial Count per Milliliter of Lung*	^{32}P Count per Milliliter of Lung‖	Bactericidal Activity %†	Weight Before 7 Day Exposure	Weight After 7 Day Exposure	% Weight Change
min E Test Diet /kg 35	Control	321 ± 44	2561 ± 170	81.3 ± 1.5	283.4 ± 5.3	286.3 ± 4.8	+ 1.0
min E Test Diet /kg 25	Control	353 ± 39	2425 ± 150	80.4 ± 1.8	277.1 ± 6.0	287.8 ± 5.2	+ 3.9
min E Test Diet /kg 25	.70 ± .21**	162 ± 17	1189 ± 108	73.4 ± 2.3	278.2 ± 6.0	258.8 ± 6.8	- 7.0
min E Test Diet /kg 27	.70 ± .21	344 ± 45	1369 ± 140	50.3 ± 4.9	276.3 ± 3.6	230.2 ± 7.5	-16.7

*Mean ± S.E. X 10^2

†Mean ± S.E.

‖Mean ± S.E.

**Mean ppm ozone ± SD

Comparisons of bactericidal activity:

Vitamin E 45 mg/kg, Control vs Vitamin E 45 mg/kg, Ozone $p < .01$

Vitamin E 0 mg/kg, Control vs Vitamin E 0 mg/kg, Ozone $p < .001$

Vitamin E 45 mg/kg, Control vs Vitamin E 0 mg/kg, Control $p > .05$

Vitamin E 45 mg/kg, Ozone vs Vitamin E 0 mg/kg, Ozone $p < .001$

for 7 days to 0.7 ppm of ozone or 21 percent oxygen.

$$\left[100 - \frac{\text{dry weight}}{\text{wet weight}}\right] \times 100$$

Experimental Group No. of Animals	Wet Weight	Dry Weight	Percent ± S.E.
Vitamin E Test Diet, 45 mg/kg Control (16)	1.156 ± .063	0.256 ± 0.019	78.1 ± 0.4
Vitamin E Test Diet, 0 mg/kg Control (16)	1.145 ± .031	0.265 ± 0.014	77.1 ± 0.6
Vitamin E Test Diet, 45 mg/kg Ozone (21)	1.464 ± .044	0.332 ± 0.015	77.4 ± 0.4
Vitamin E Test Diet, 0 mg/kg Ozone (17)	1.569 ± .063	0.337 ± 0.018	78.6 ± 0.3

Wet Weight — Vitamin E Test Diet, 45 mg/kg, control vs Wet Weight — Vitamin E Test Diet, 45 mg/kg, ozone $p<.001$

Wet Weight — Vitamin E Test Diet, 0 mg/kg, control vs Wet Weight — Vitamin E Test Diet, 0 mg/kg, ozone $p<.001$

*Mean ± S.E.

exposed to 0.5 or 1.0 ppm of ozone for 4 hrs.

$$\left[100 - \frac{\text{dry weight}}{\text{wet weight}}\right] \times 100$$

Experimental Group No. of Animals	Wet Weight	Dry Weight	Percent ± S.E.
0.5 ppm Ozone Experiment			
Vitamin E Test Diet, 45 mg/kg Control (10)	1.275 ± .030	0.286 ± .007	77.5 ± 0.5
Vitamin E Test Diet, 0 mg/kg Control (10)	1.184 ± .033	0.267 ± .009	77.5 ± 0.2
Vitamin E Test Diet, 45 mg/kg 0.5 ppm Ozone (10)	1.287 ± .034	0.285 ± .006	77.8 ± 0.1
Vitamin E Test Diet, 0 mg/kg 0.5 ppm Ozone (10)	1.274 ± .042	0.281 ± .009	78.0 ± 0.1
1.0 ppm Ozone Experiment			
Vitamin E Test Diet, 45 mg/kg Control (10)	1.350 ± .050	0.287 ± .009	78.7 ± 0.3
Vitamin E Test Diet, 0 mg/kg Control (10)	1.410 ± .042	0.303 ± .010	78.5 ± 0.2
Vitamin E Test Diet, 45 mg/kg 1.0 ppm Ozone (10)	1.577 ± .054	0.330 ± .010	79.0 ± 0.1
Vitamin E Test Diet, 0 mg/kg 1.0 ppm Ozone (10)	1.333 ± .044	0.287 ± .008	78.4 ± 0.4

Comparisons:

Wet Weight — Vitamin E, 45 mg/kg, control vs Wet Weight — Vitamin E, 45 mg/kg, 1.0 ppm Ozone $p < .01$

Dry Weight — Vitamin E, 45 mg/kg, control vs Dry Weight — Vitamin E, 45 mg/kg, 1.0 ppm Ozone $p < .001$

All other comparisons: $p > .05$

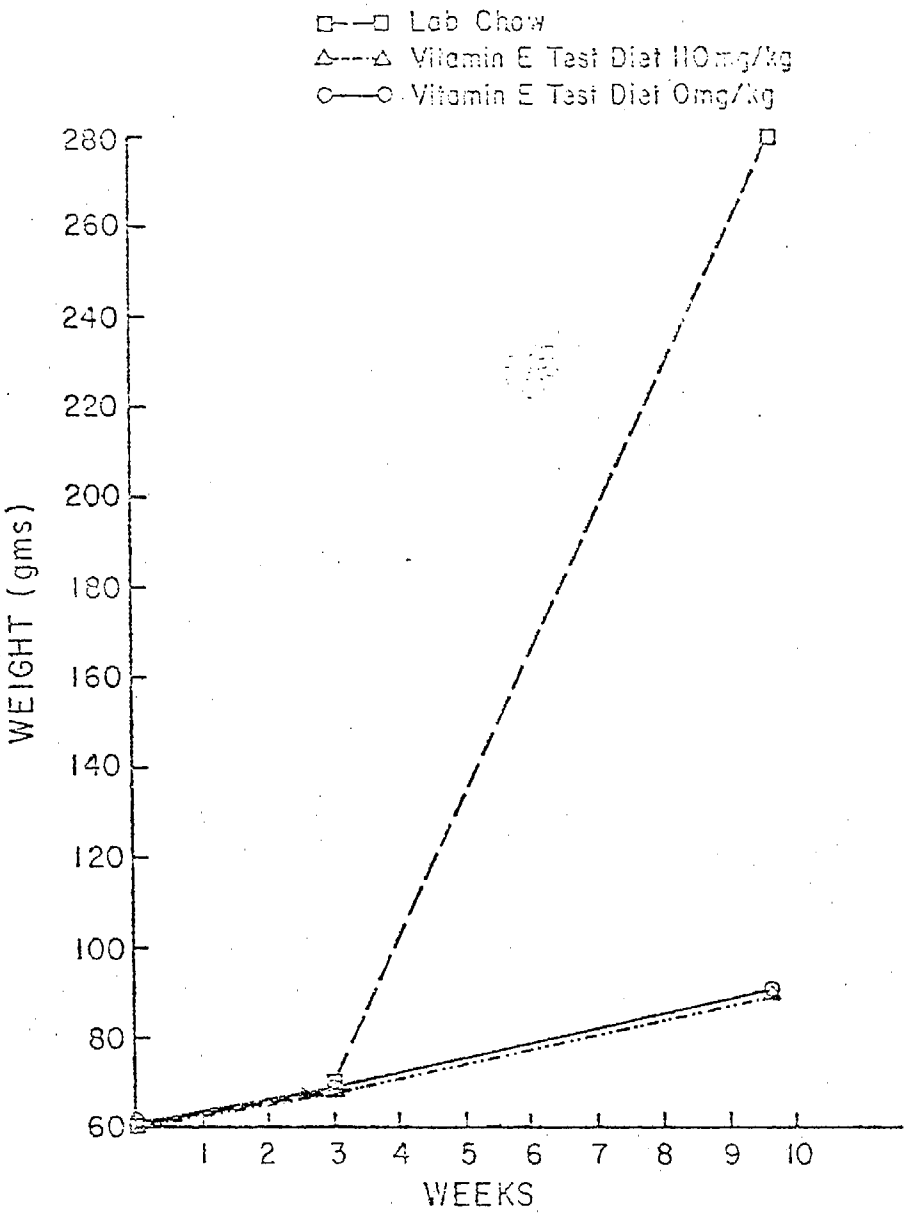

Figure 2. Comparison of weight gain for rats preconditioned with vitamin E test diets formulated with tocopherol stripped lard and control diets of Lab Chow.

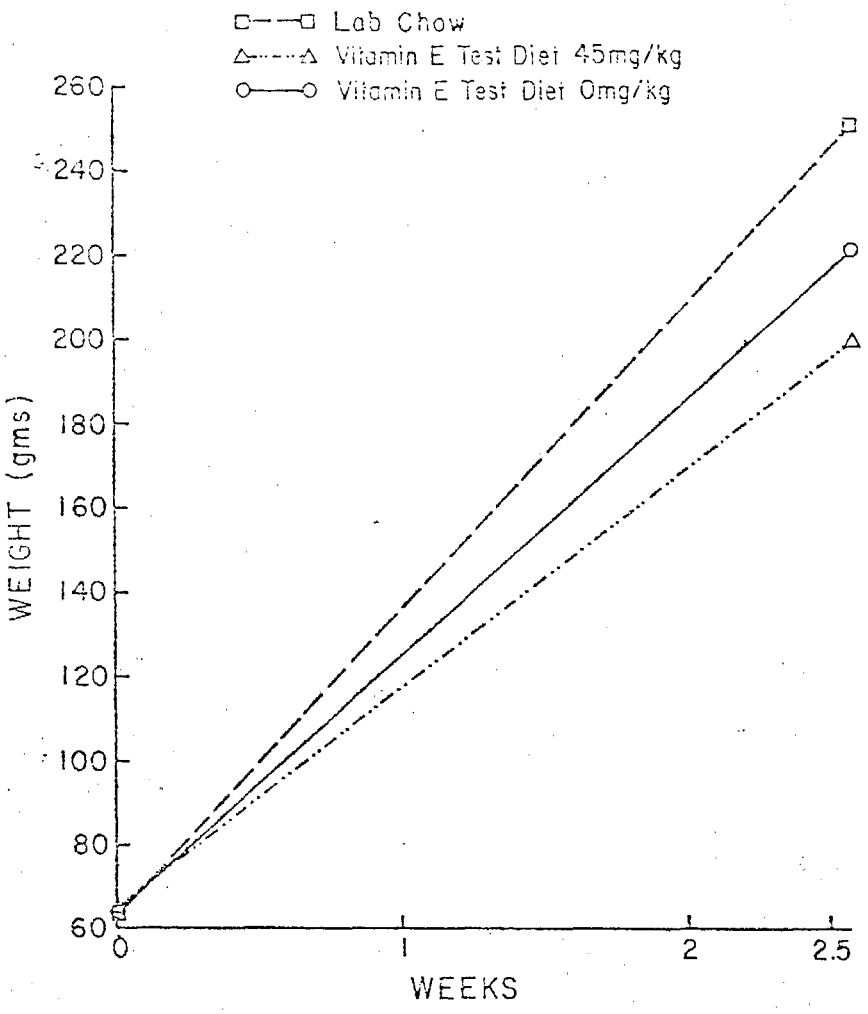

Figure 3. Comparison of weight gain for rats fed vitamin E test diets formulated with tocopherol stripped corn oil and control diets of Lab Chow.

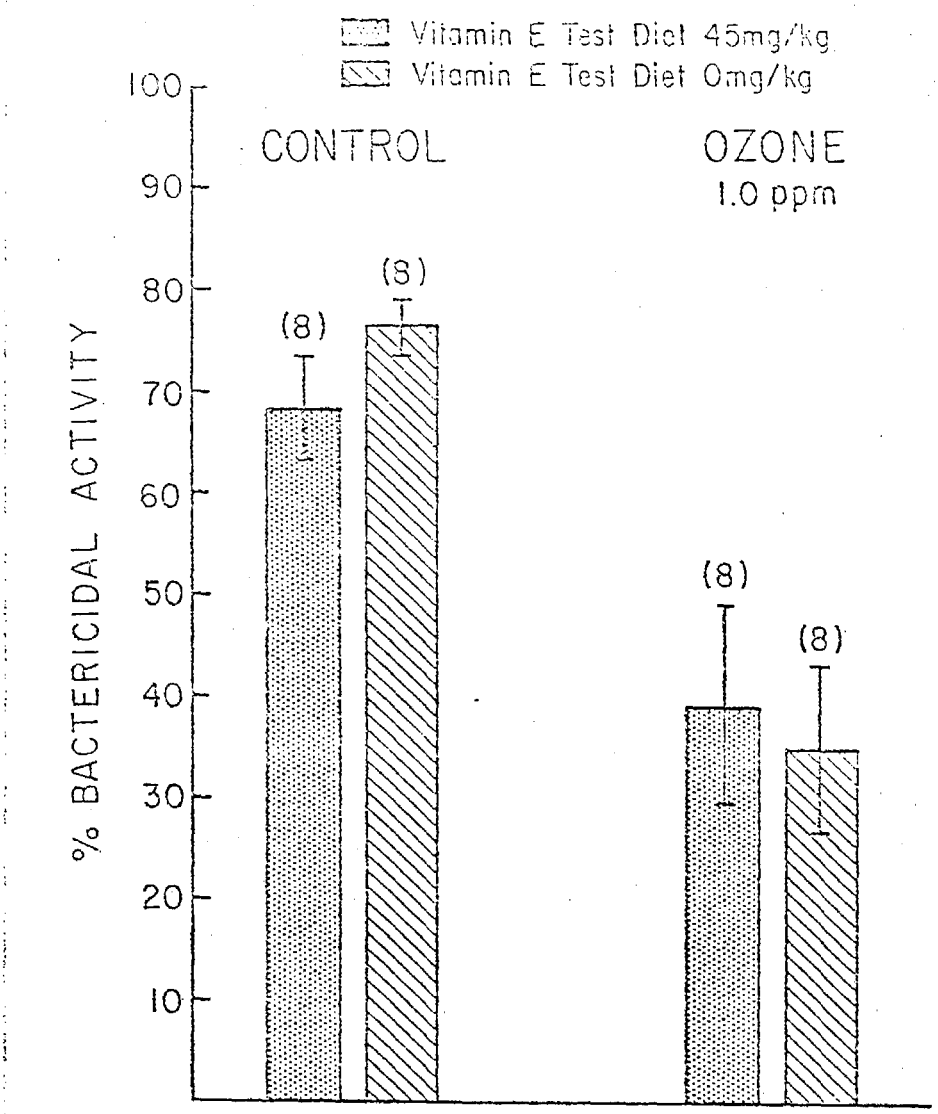

Figure 4. Effect of a 4 hour exposure to 1 ppm of ozone on the pulmonary bactericidal activity of rats fed vitamin E test diets formulated with tocopherol stripped corn oil.

Comparisons:

Vitamin E test diet 45 mg/kg, control vs ozone $p<0.05$

Vitamin E test diet 0 mg/kg, control vs ozone $p<0.01$

All other comparisons $p>0.05$

Figure 5. Photomicrograph of a section of lung from a rat exposed for 7 days to 0.7 ppm of ozone showing fibrosis and interstitial thickening.

HE stained, X 130