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Influence of Exposure Mode on the Toxicity of NO_2

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Pollutant gases are subject to a variety of physical and chemical interactions within the atmosphere due to cyclic production and various meteorological influences. In consequence there is generally a diurnal concentration profile for NO_2 which consists of peaks of short duration and irregular occurrence superimposed on a low background. Since this variation could play an important role in the toxic effect of NO_2, the influences of various exposure modes was studied. Continuous and intermittent exposure studies were used to determine the relationship between biological response and length of exposure to various concentrations of NO_2. As the concentration decreased, the slope of the regression line decreased. After adjusting for total differences in the product concentration × time, the response for the two exposure modes was essentially the same. When a constant concentration × time level was employed, a short-term exposure to a high concentration produced a greater effect than exposure to a lower concentration administered over a longer period. Using these curves, the relationship between level of effect, concentration, and time can be determined. Results of these studies indicated that the frequency and amplitude of short-term peaks are of significance even though the exposure is interrupted with periods of zero concentration of NO_2.

Introduction

Nitrogen oxides formed in combustion processes are due to either the thermal fixation of atmospheric nitrogen in the combustion air or the conversion of chemically bound nitrogen in the fuel. In the United States, about half of the atmospheric nitrogen oxides is derived from products of automobile exhaust emissions and the remaining half is derived from stationary source emissions of various types. Concentration profiles for man-made oxides of nitrogen vary according to population density and combustion activity; therefore, significant elevations above the background level often occur.

Since atmospheric nitrogen dioxide (NO_2) is derived from nitric oxide, principally through the photochemical process, its concentration varies with the rate of combustion, the presence of other atmospheric pollutants, and various meteorological conditions-intensity of light, wind speed and direction, height of inversion layer, and temperature.

As a consequence of these variables, there are often low background levels of NO_2 on which higher diurnal peaks are superimposed. These peaks are usually of short duration and of irregular occurrence. Aerometric sampling devices provide air quality data expressed in terms of instantaneous hourly or daily integrated values. However, this data base is reduced to a simple annual arithmetic average and compared to the National Air Quality Standard for NO_2, which is set at an annual average of 100 $\mu g/m^3$ (0.05 ppm). This average greatly minimizes the sporadic pollutant peaks which could be of toxicological importance. When such averages are used as indices of air pollution, the implication of the particular exposure profile on the health of the population at risk may not be immediately obvious.

Current literature contains few toxicological data that systematically compare the influence of mode of dose on the health effects of NO_2. Therefore, experiments were designed to examine and compare several different exposure regimens, by using a single sensitive parameter—host resistance to respiratory infections. This model probably best reflects a summation of all the possible responses to the pollutant assault on the lung, such as edema, inflammation, and subtle immunological and cellular alterations (*1*).

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Several species of animals have been employed in this model system to demonstrate the adverse effects of other environmental pollutants, such as irradiated automobile exhaust (*2*), O_3 (*3*), NiO (*4*), $CdCl_2$ (*5*), and MnO_2 (*6*). Influenza PR-8 virus, *Klebsiella pneumoniae*, *Diplococcus pneumoniae*, and *Streptococcus pyogenes* are examples of the types of microorganisms that have been used in these types of investigations. This model appears to be a sensitive biological indicator for toxicological studies.

Experimental

Pathogen-free Swiss Albino female mice, strain CD-1 (Charles River Laboratory) weighing 20-25 g, were exposed in a stainless steel chamber to various exposure regimes of NO_2. Each mouse was in an individual compartment, and was provided food and water *ad libitum* whenever exposures were for longer than 3 hr. Control animals were treated similarly. The NO_2 concentration within the chamber was monitored continuously by the standard chemiluminescence method (*7*). In addition, the chamber concentration was periodically (three times/day) determined manually by the Saltzman method (*8*).

At various times during the exposure studies, groups of 20 mice were removed from the treatment chamber, combined with 20 control mice which had breathed only clean filtered air, and immediately exposed for 15 min to an aerosol of viable microorganisms (*Streptococcus pyogenes*, Group C). The organisms were grown in brain heart infusion broth (Difco) for 24 hr prior to use. Prior to aerosolization, the organisms were washed three times and resuspended to a final concentration of approximately 10^6 organisms/cm^3. A 5.0 ml aliquot of this suspension was aerosolized, and the microbes were delivered to the test animals immediately following the NO_2 exposure. The controls were again separated from the NO_2-exposed animals, and both groups were observed for 15 days in order to determine mortality rate. The data are reported as the difference in percent mortality between the NO_2 test group and control.

In addition to measuring the differences in mortality rates, a second parameter—the relative mean survival time—was also analyzed in order to determine the influence of exposure time and concentration on the mean survival period of the exposed animals. The relative mean survival time (RMST) reflects the average number of days the test animals lived during the experimental period.

It is calculated according to the equation:

$$\text{RMST} = [(D\ L) + \Sigma\ (A\ B)]/n$$

where A is the last day on which any individual mouse was alive; B is the number of mice surviving A days; D is the last day of the experiment (in this case 15); L is the number of mice which were alive on day D; n is the initial number of mice in the experimental group; and Σ represents the summation over the appropriate terms. The data are represented as the difference in relative mean survival time between the NO_2-exposed group and the controls.

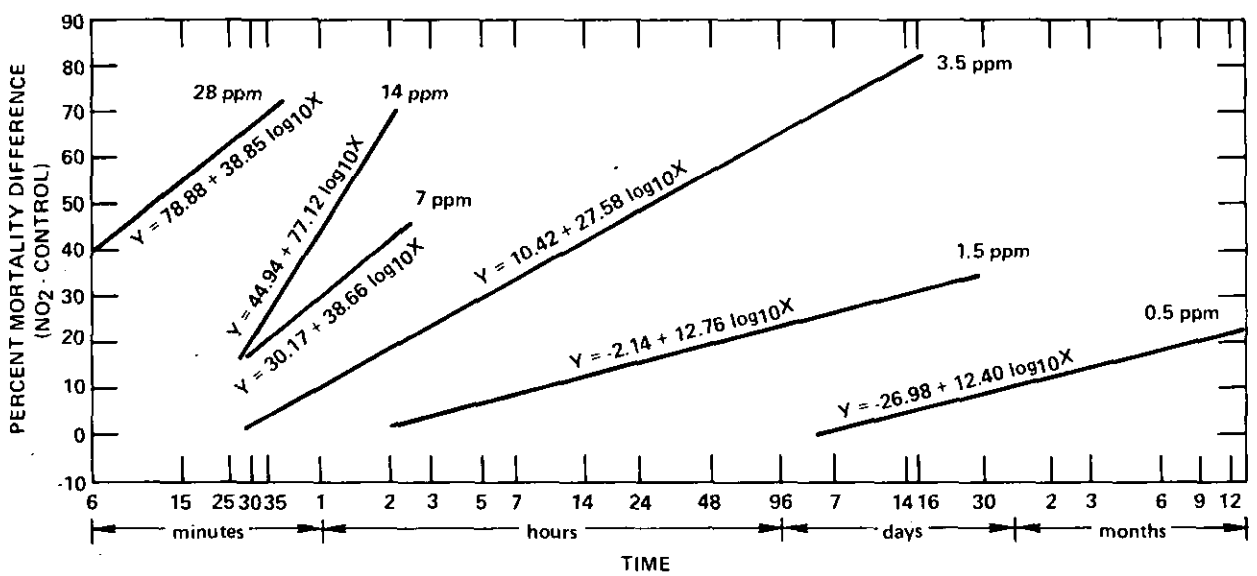

FIGURE 1. Percent mortality of mice versus length of continuous exposure to various NO_2 concentrations prior to challenge with streptococci.

Results and Discussion

Using the enhancement of mortality as a biological endpoint for measuring the toxic effects of NO_2, studies were conducted in which the length of continuous exposure varied from a few minutes to several months. To date, six different concentrations have been studied, ranging from 0.94 mg/m³ (0.5 ppm) NO_2 to 526.7 mg/m³ (28 ppm) NO_2. Regression analysis was used for examining the interrelationship between percent mortality and the length of exposure to NO_2. Figure 1 presents the regression equations for each of the concentrations studied. All of the regression lines were statistically significant at the 0.05 probability level. The experimental data base used to develop these curves is given in Table 1. When comparisons are made of the various lines, it is evident that with increasing concentrations, the slope of the regression line becomes steeper; that is to say, with increasing concentrations of NO_2, the rate of increase in mortality also increases. From these curves various estimates can be derived for combinations of concentrations, lengths of exposure, and specific mortality responses. For example, the predicted length of exposure needed to produce a 20% enhancement in mortality varies from approximately 6150 hr for 0.94 mg/m³ (0.5 ppm) NO_2 to 0.5 hr for 26.3 mg/m³ (14 ppm) NO_2.

A common method for comparing the relationship of concentration and time to a specific toxic effect is on a concentration × time ($C \times T$) basis. If no interaction occurred between concentration and time, then no statistical difference in response should be noted when either factor is varied, providing that the product remains a constant value.

Table 2 indicates that in the infectivity model the concentration has a greater influence on the observed effect than does the length of exposure. For each of the given $C \times T$, there is a gradient response in mortality which would not be expected if the effect of NO_2 were directly related to concentration and time. To illustrate this point, at a $C \times T$ of 21, the overall expected average mortality increase could be 45.1%. However, the predicted mortality value from Figure 1 varied from a low of 12.5%

Table 1. Experimental data from studies designed to determine the effects of continuous exposure to various NO_2 concentrations with time.[a]

NO_2 level, ppm	Exposure length, hr	Mortality, % (NO_2 control)
0.5	168	0.8
	336	5.1
	720	5.0
	1440	10.0
	2160	19.9
	4320	23.7
	6480	12.3
	8760	23.4
1.5	2	6.7
	5	0.0
	8	24.4
	18	25.0
	24	15.6
	96 (6)	10.8
	126	35.0
	168 (4)	25.0
	222	25.0
	336 (4)	31.3
	504 (3)	45.0
3.5	0.5 (5)	10.0
	1.0 (10)	8.0
	2.0 (2)	12.5
	3.0 (10)	19.0
	5.0 (2)	37.5
	7.0 (14)	34.3
	14.0 (4)	45.0
	24.0 (7)	49.3
	48.0 (8)	56.3
	96.0	75.0
	168.0	85.0
	384.0	65.0
7.0	0.5 (6)	20.8
	1.0 (6)	29.2
	1.5 (6)	28.3
	2.0 (6)	49.2
14.5	0.5 (3)	23.3
	1.0 (3)	38.3
	1.5 (3)	66.7
	2.0 (3)	65.0
28	0.10 (5)	41.6
	0.25 (5)	53.6
	0.42 (5)	60.6
	0.58 (5)	73.6

[a] The number of replicate experiments is indicated in parentheses.

Table 2. Influence of concentration and time on enhancement of mortality resulting from various NO_2 concentrations.[a]

Concentration, ppm	$C \times T$	Time, hr	Mortality, %
1.5	7	4.7	6.4
3.5		2.0	18.7
7.0		1.0	30.2
14.0		0.5	21.7
28.0		0.25	55.5
1.5	14	9.3	10.2
3.5		4.0	27.0
7.0		2.0	41.8
14.0		1.0	44.9
28.0		0.5	67.2
1.5	21	14.00	12.5
3.5		6.00	31.9
7.0		3.00	48.6
14.0		1.50	58.5
28.0		0.75	74.0

[a] These are predicted values obtained from Figure 1.

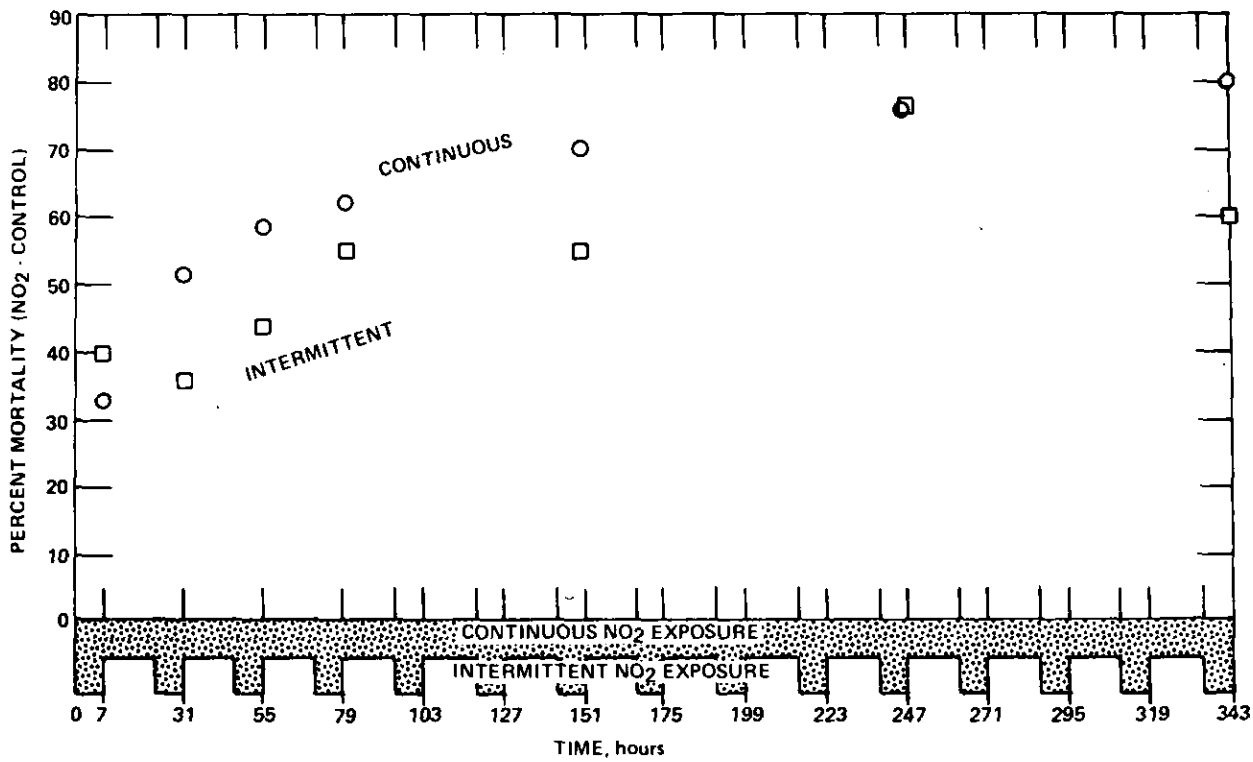

FIGURE 2. Percent mortality of mice versus the length of either continuous or intermittent exposure to 3.5 ppm NO_2 prior to challenge with streptococci.

Table 3. Comparison of the effects of $C \times T$ and mode of exposure to 3.5 ppm NO_2 on percent mortality.

Concentration × time ($C \times T$)	Mortality, %	
	Intermittent exposure	Continuous exposure
49.0	37	42
73.5	43	47
98.0	55	50
171.5	55	57
269.5	75	62
367.5	60	66

obtained with 2.8 mg/m³ (1.5 ppm) × 14 hr to a high of 74% obtained with 52.7 mg/m³ (28 ppm) × 0.75 hr. This indicates that in this system concentration is a more important factor in eliciting the toxicological response than is time.

Thus, in order to examine the adverse effects of this pollutant, it becomes necessary to clearly define the exact exposure pattern. The experiments described thus far provide information on what might be expected if the exposure regimen were of a continuous, nonvarying pattern. The $C \times T$ data indicated that substantially different effects can result from varying the exposure scheme. Also, since ambient concentrations of NO_2 are irregular, sporadic, and follow a diurnal mode, it was of interest to include in the study an intermittent exposure regimen and to compare those responses to those from the continuous exposure.

In order to test the effect of concentration and time, mice were intermittently exposed for 7 hr/day, 7 days/wk to either 2.8 mg/m³ (1.5 ppm) NO_2 or 6.6 mg/m³ (3.5 ppm) NO_2. At various times animals were removed and given the bacterial challenge, and their response was compared to the animals exposed continuously.

Figure 2 illustrates the results from continuous and intermittent exposure to 6.6 mg/m³ (3.5 ppm) NO_2 for periods up to 15 days. There was a noticeable increase in percent mortality for each experimental group with increasing length of exposure. But for each given length of exposure, there was no statistical difference ($p = 0.05$) between the continuous and the intermittent exposure groups. After adjusting the data for actual exposure time, and thus for total difference in $C \times T$, the percent mortality rate in the two exposure modes was essentially the same (See Table 3).

Similar studies conducted at a lower concentration of NO_2 (2.8 mg/m³, 1.5 ppm) produced a different response (Fig. 3). Again, there was a significant increasing linear relationship with duration of exposure. However, initially the mortality rate was significantly higher in mice exposed to the pollutant

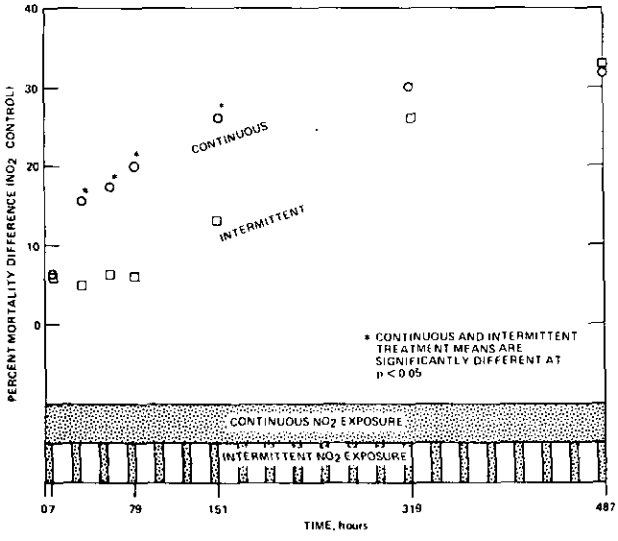

FIGURE 3. Percent mortality of mice versus length of either continuous or intermittent exposure to 1.5 ppm NO_2 prior to challenge with streptococci.

Table 4. Effects of 7, 14, and 28 ppm of NO_2 on relative mean survival time in mice exposed for various lengths of exposure.[a]

$C \times T$	NO_2 Concentration, ppm	Time, hr	Difference in RMST, days	Standard error
2.8	28	0.10	−2.99	0.77
3.5	7	0.50	−1.51	0.72
7.0	7	1.00	−2.28	0.43
7.0	14	0.50	−1.43	0.84
7.0	28	0.25	−4.61	0.47
10.5	7	1.50	−1.93	0.50
11.7	28	0.42	−4.76	0.74
14.0	7	2.00	−4.03	0.84
14.0	14	1.00	−2.65	0.10
16.3	28	0.58	−6.56	0.64
21.0	14	1.50	−5.60	0.33
28.0	14	2.00	−5.97	0.10

[a] The number of replicate experiments for each $C \times T$ level was 6, 3, and 5 for 7, 14, and 28 ppm NO_2, respectively.

continuously as compared to the intermittent treatment. This difference became nondistinguishable following 14 days of exposure.

The question arises as to the cause of the variation in the early response between the continuous and intermittent exposure at 2.8 mg/m³ (1.5 ppm). From the continuous exposure curves (see Fig. 1), a statistically significant increase in mortality of approximately 15% would not be expected earlier than 24 hr at this low level of NO_2. Thus, during the first four initial periods of intermittent exposure, the accumulated $C \times T$ is below this critical threshold, whereas the continuous treatment exceeds this 24 hr criteria and hence causes a significant increase in mortality. After the seventh intermittent exposure to 2.8 mg/m³ (1.5 ppm) NO_2, the pollutant produces a significant increase in mortality, and the effect begins to approach that of the continuous treatment group.

In conjunction with measuring the enhancement in mortality, a second endpoint was used to illustrate the relationship between concentration and length of exposure. The relative mean survival times for mice continually exposed to the three higher concentrations of NO_2 (13.2, 26.3 and 52.7 mg/m³) are given in Table 4. The data demonstrate that at the concentrations and exposure periods studied, the survival time of the NO_2-exposed mice was significantly less than those in the control group. The relative mean survival time decreased with increasing concentration and correlated with the mortality enhancement presented in Table 2.

The relative mean survival times at lower concentrations of NO_2 (2.8 and 6.6 mg/m³) are seen in Table 5 where a comparison is made between continuous and intermittent exposure. Consistent statistical differences in the rate of survival were seen after four or more intermittent exposures to 6.6 mg/m³ (3.5 ppm) NO_2. All continuous exposures at this level of NO_2 produced statistically different relative mean survival times, as compared to control. However, the pattern of statistical difference for the 2.8 mg/m³ (1.5 ppm) exposure mode was somewhat ambiguous. This may reflect a decrease in sensitivity of this parameter as compared to the mortality model system, or it may reflect simply a lesser response to this lower level of NO_2.

Summary

This study has described variations in response with different concentrations, modes, and durations of NO_2 exposure. Continuous exposure to different levels of NO_2 resulted in a family of linear regression lines which related enhancement of mortality with duration of exposure. As the concentration of NO_2 increased, the slope of the resulting linear regression also increased.

Sidorenko and Pinigin (*9*) demonstrated that in continuous inhalation exposures to organic compounds the concentration-time relationship is linear on a log-log scale. Utilizing the family of curves presented in Figure 1, a concentration–time curve was prepared using the endpoint of 20% mortality (Fig. 4). The regression thus obtained shows that the concentration-time relationship for mortality resulting from exposure to NO_2 can also be represented by a straight line on a log-log scale.

The relationship between concentration and time produced significantly different mortality responses, although $C \times T$ was held constant. The

Table 5. Effects of intermittent and continuous exposure to 1.5 and 3.5 ppm NO_2 on relative mean survival time in mice exposed for various periods.[a]

Consecutive exposures	NO_2 level, ppm	$C \times T$ difference in RMST					
		Intermittent regimen (7 hr/day)			Continuous regimen (24 hr/day)		
2	1.5	21.0	0.46	(4, 0.64)	72.0	1.08	(4, 0.83)
3		31.5	−0.55	(4, 0.20)	108.0	−0.58	(4, 0.75)
4		42.0	−0.79	(5, 0.16)	144.0	−0.78	(6, 0.46)
7		73.5	−1.34	(8, 0.50)	252.0	−1.95	(5, 0.97)
14		147.0	−2.16	(6, 0.52)	504.0	−2.42	(3, 0.58)
21		220.5	−2.88	(2, 1.03)	864.0	−4.05	(3, 1.13)
2	3.5	49.0	−2.83	(3, 1.12)	168.0	−4.67	(7, 0.56)
3		73.5	−0.93	(4, 1.65)		No data	
4		98.0	−3.59	(4, 0.97)	336.0	−6.95	(1, —)
7		171.5	−3.48	(3, 0.71)	588.0	−7.10	(1, —)
11		269.5	−4.90	(3, 1.85)		No data	
15		367.5	−5.55	(2, 0.05)	1260.0	−5.85	(1, —)

[a] Numbers in parentheses represent sample size and standard error, respectively.

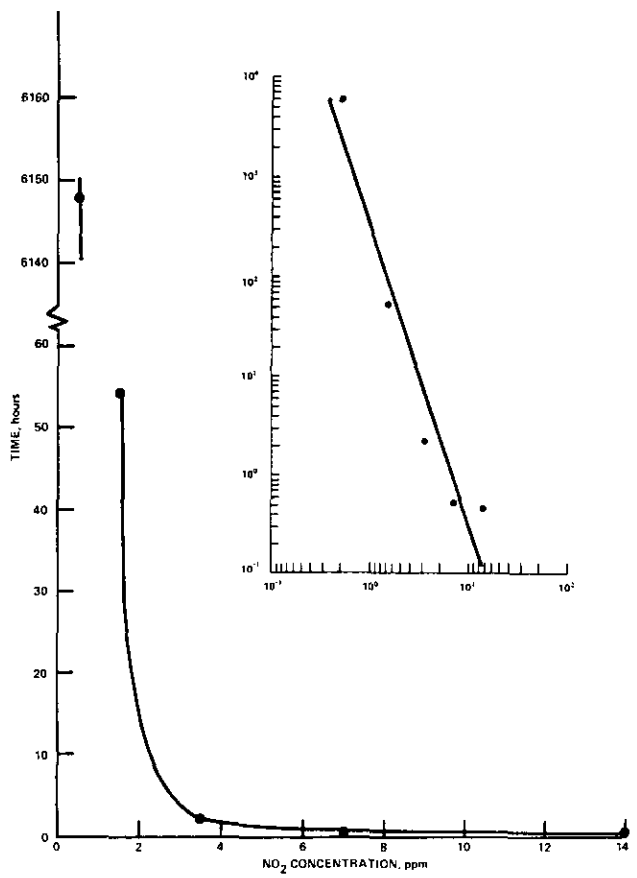

FIGURE 4. Time necessary to elicit a 20% mortality in mice versus concentration of NO_2.

ranking of the effects in the infectivity model suggests that concentration is the more important factor for a fixed $C \times T$ level. However, deleterious effects can also result from chronic exposure to low levels of NO_2, as evidenced from significantly increasing mortality with long-term exposure to 0.94 mg/m³ (0.5 ppm) NO_2.

This seemingly contradictory role that time plays with respect to host responses to bacterial infections may indicate that there is more than one mechanism of NO_2 injury. At higher levels of NO_2 and for shorter time periods, the destructive action of this pollutant may be primarily on the pulmonary alveolar macrophage (*10*). These cells are postulated to be the chief pulmonary defense against inhaled infectious agents, and a variety of environmental pollutants have been shown to alter the functioning of these cells (*10–13*). However, after long-term, low-level exposures, certain anatomical and biochemical changes do occur, such as desquamation of type 1 epithelial cells (*14*), loss of lung recoil (*15*), and pulmonary emphysema (*16, 17*). Therefore the observed increase in mortality at 0.94 mg/m³ (0.5 ppm) NO_2 may indicate that the effects of this pollutant can be mediated through numerous subtle alterations in several host defense mechanisms.

Of particular importance were the results obtained when comparisons were made between intermittent and continuous exposure to 2.8 and 6.6 mg/m³ (1.5 and 3.5 ppm) NO_2. Differences in mortality responses between the two exposure modes may be resolved on the basis of $C \times T$. These data indicate the importance that short-term peaks may have upon responses to environmental pollutants. Consequently, air quality standards which do not account for the frequency and amplitude of such spikes may allow excess risk. At the present time, research is being conducted to investigate the effects resulting from the superimposition of spikes on lower basal concentrations of NO_2.

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