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Time–Dose Response for Nitrogen Dioxide Exposure in an Infectivity Model System

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The concentration of NO_2 in polluted atmosphere is subject to wide variation, according to peak traffic load, industrial productivity, intensity of sunlight and meteorological conditions. Normally NO_2 has a low basal concentration with superimposed spikes when the above conditions are optimal for its production. Thus, it is important to determine the relative importance of a short-term, relatively high concentration of NO_2 versus exposure for longer periods of minimal dose levels. This problem was approached experimentally by measuring the effect of NO_2 on an animal's resistance to the induction of bacterial pneumonia. The data collected indicate that: (1) in short-term dose-response studies using the same *Ct* (concentration x time) product of 7, the actual concentration exerts a greater influence on NO_2 effect than does the duration of exposure; (2) when concentration is held constant and the time increased, the average difference in mortality from controls can be seen after only 1 hr exposure to 3.5 ppm and after 3 weeks of exposure to 0.5 ppm; and (3) the relative mean survival time at 3.5 ppm for 1 hr was 18–36 hr less than that of the control animals.

Introduction

A number of atmospheric chemicals are characterized by very uneven concentrations from day to day or even hour by hour, as a result of the rate of their production and meteorological influences. This is particularly amplified in the case of chemicals formed secondarily through interactions occurring in the atmosphere. For instance, the level of atmospheric NO_2 is governed not only by the rate of production of NO, but also by factors existing independently in the environment, favoring its conversion to NO_2. This generally results in a low basal atmospheric concentration on which are superimposed peaks of higher concentrations, usually of very short duration and irregular occurrence. Thus, it is of great value to determine the relative importance of the more uniform low basal concentration (as might be modeled from a weekly, monthly, or yearly average) in comparison to the high, short peaks or vice versa. Furthermore, it is of importance to know what biological interactions may occur in both systems if applied in the same regimen of exposure.

The problem defined above was approached experimentally by conducting inhalation experiments in animals at various exposure regimens designed to elucidate the relative toxicologic importance of these factors, i.e., continuous basal dose versus short periods of exposure at higher levels. There is only a little information available in the literature concerning time–dose studies of NO_2. Gray et al. (*1*) found a sharp gradient in LC_{50} at exceedingly high doses in favor of concentration from exposure ranging from 2 to 240 minutes; for instance, 1445 ppm for 2 min and 88 ppm for 240 min. Thus, computed on the basis of the product of concentration and time (ppm × min) the values are approximately 2,890 and 21,120 respectively, a difference of 8-fold in the product *Ct*.

Wagner et al. (*2*) noted no pulmonary emphysema after exposure of rats to 25 ppm NO_2

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for 8 hr/day, 5 days per week. Similar negative results were reported by Hine et al. (*3*) and Boren (*4*) after intermittent exposure of rats to 25 ppm NO_2 for a duration of 4 months. On the contrary, pulmonary emphysema was noted by Freeman et al. (*5*) after continuous exposure of rats to comparable concentrations. Slower repair was observed by Kleinerman and Wright (*6*) after cessation of exposure of animals to NO_2 on a continuous basis as compared to similar experiments carried out on an intermittent schedule. Ehrlich and Henry (*7*) found emphysema in mice after both continuous and intermittent exposure at 0.94 mg/m^3 (0.5 ppm) NO_2. They also observed increased susceptibility to bacterial pneumonia when mice were subjected to the same exposure conditions.

Ehrlich et al. (*8*) reported that fluctuating concentrations of NO_2 represent a more significant factor in influencing immune response than exposure to a constant but higher concentration of NO_2: Thus, continuous exposure to 3.8 mg/m^3 (2 ppm) NO_2 did not influence the formation of antibodies or levels of immunoglobulins, while exposure to 0.94 mg/m^3 (0.5 ppm) with a daily 1-hr peak of 3.8 mg/m^3 (2.0 ppm) NO_2 depressed the ability to form serum neutralizing antibodies and significantly alter the levels of immunoglobulins IgM, IgG_1, and IgG_2.

Due to the ambiguities in the literature concerning dose response for NO_2, more definitive work remains to be done to determine the relative importance of various dose regimens and the possible influence of threshold, tolerance, healing, and adaptation in biological reactions to NO_2. Because of the interest in dose-response and the possibility that several mechanisms might be operating concurrently in NO_2 toxicity, this problem was incorporated in the U.S.–U.S.S.R. Health Exchange cooperative agreement.

In this program the following studies were to be undertaken: short acute exposure versus the same *Ct* over a longer period; continuous dose versus the intermittent application; superimposition of spike on a low basal dose versus continuous and intermittent at a single concentration. Preliminary data from some of these experiments are presented here.

The model that has been employed in this work involves primarily resistance to the induction of bacterial pneumonia. This parameter was selected because of its demonstrated effects at ambient concentrations of air pollutants (7). This model also probably reflects a summation of the total toxic assaults on the deep lung such as edema, inflammation, cellular necrosis, reduced macrophage function, and upper airway effects, such as ciliostasis (*9*). Briefly, this model consists of the superimposition of a pathogenic bacterial aerosol (*Streptococcus pyogenes,* Group C, isolated from a pharyngeal abscess of a guinea pig) following exposure to the gaseous toxicant. Control animals receive bacteria aerosol only.

This system has been efficiently utilized in environmental toxicologic studies of ozone (*9, 10*), nitrogen dioxide (*11*), irradiated auto smog (*12*), and trace metals, such as NiO and MnO_2 (*13, 14*). This model has been successfully employed to enhance the pulmonary infectivity in mice (*10, 11, 13, 14*), rats (*15*), hamsters (11), and squirrel monkeys (*16*). Among the infectious agents eliciting this reaction in the above animals are *Streptococcus pyogenes, Klebsiella pneumoniae, Diplococcus pneumoniae,* and influenza PR-8 virus. Thus, this model is an exquisitely sensitive indicator of biological effects *in vivo*. In addition, the model is useful in studying the mechanism of action of the pollutants (*17, 18*).

Results

In answer to the question raised in the introduction, the following results have been obtained.

Dose–response studies were conducted utilizing a concentration × time (*Ct*) product of 7, which was based on the published threshold for NO_2 using the infectivity model (3.5 ppm × 2 hr) (*11*). Figure 1 shows enhancement of mortality from pulmonary infection resulting from the same *Ct* exposure to NO_2 over different time periods. It will be noted that the actual concentration exerted more influence than the duration of exposure. If no interaction existed between concentration and time, (i.e., if they had equal importance), all the test groups should demonstrate an increase in mortality of about 15%, whereas the trend of the combined data indicate a gradient response from a high mortality enhancement of approximately 50% to a low equalling controls.

The interaction of a constant concentration with time over a longer period was also examined. It is the overall plan to construct a family of curves at various concentrations from 0.5 to 14 ppm. At the present moment, three concentrations of NO_2 have been studied. A level of 3.5 ppm was used for comparison with the study mentioned above. A lower value of 0.5 ppm was selected, since this concentration represented the lowest figure in the literature yielding positive results for chronic exposure in the infective system (*11*). Preliminary data at 1.5 ppm

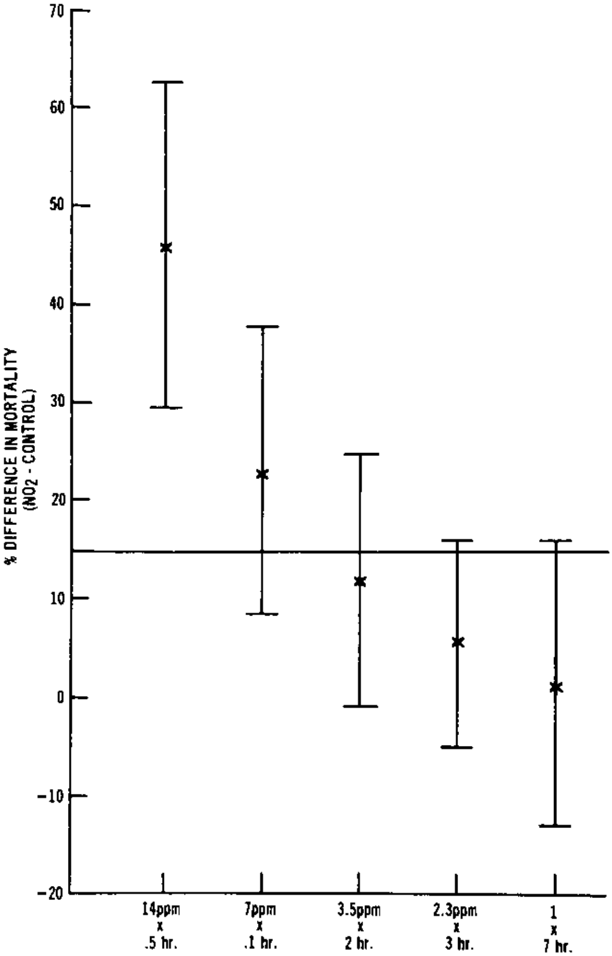

FIGURE 1. Enhancement of mortality from pulmonary infection resulting from the same concentration × time exposure of NO_2 over different exposure periods. Average per cent mortality differences are shown along with their 95% confidence intervals. The horizontal line indicates the predicted effect (observed data) if there were a direct relationship between time and concentration.

of NO_2 are also included. The data shown in Figure 2 represent 60 individual observations for the 3.5 ppm level, eight for the 0.5 ppm, and six for the 1.5 ppm level. The simple linear regression for the 3.5 ppm and 0.5 ppm of NO_2 shows that the mortality increased with time and concentration and was statistically significant at the 0.01 probability level. Estimates which can be generated from these regressions with respect to the true average differences in mortality show that statistical significant change from controls ($p < 0.05$) can be predicted after 1 hr exposure to 3.5 ppm, and after 3 weeks at the 0.5 ppm concentration. However, the regression for 1.5 ppm was significant only at the

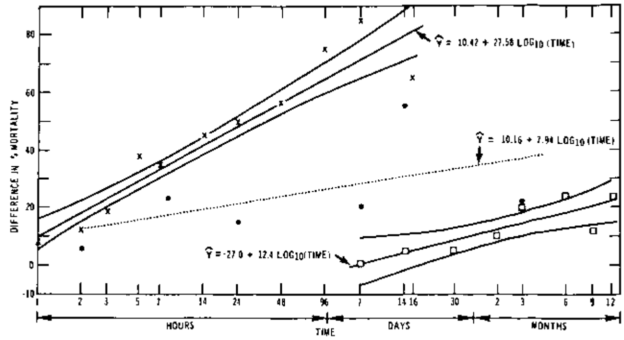

FIGURE 2. Difference in percent mortality vs. length of exposure to various concentrations of NO_2: (×) 3.5 ppm NO_2; (○) 1.5 ppm NO_2 (□) 0.5 ppm NO_2. The average per cent mortality difference (NO_2-control) represents 65, 6, and 8 observations for 3.5 ppm, 1.5 ppm, and 0.5 ppm NO_2 respectively. The 3.5 ppm and 0.5 ppm regression analyses were statistically significant at the 0.05 probability level. The simple linear regression equations are shown for these concentrations along with their 95% confidence limits. However, the regression for 1.5 ppm was significant only at the 0.25 probability level.

0.25 probability level and is represented by a dashed line. (Additional data will be collected in order to better define the 1.5 ppm response curve.) While it was noted in the first series of experiments (Fig. 1) that there was a strong interaction in favor of concentration over time, the latter study still illustrates the importance of obtaining toxicological information from long-term exposures to NO_2.

The data from the same series of experiments were analyzed for influences of exposure time to 3.5 ppm on mean survival period, as calculated according to the equation:

$$\mathrm{MST} = \left[\Sigma(AB) + (DL)\right]/n$$

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; and n is the initial number of mice in the experimental group. A statistically significant ($p <$ 0.05) regression with a negative slope was observed for mean survival of mice challenged with *Streptococci pyogenes* versus length of exposure to 3.5 ppm for various periods prior to the challenge. From Figure 3, it is evident that there is an inverse relationship between survival and length of exposure to a constant concentration of 3.5 ppm NO_2. With this parameter, after 1 hr of exposure to 3.5 ppm, one may be 95% confident that the NO_2-exposed mice, on the average, lived between 18 and 36 hr less than do control mice.

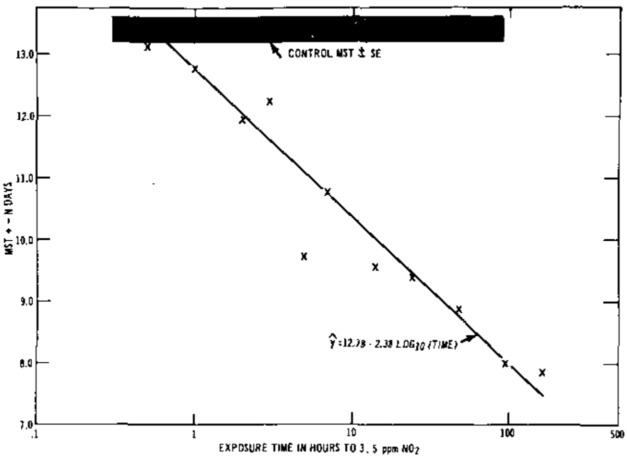

FIGURE 3. Mean survival time of mice following challenge vs. length of exposure to 3.5 ppm NO_2 before challenge. A statistically significant ($p < 0.05$) linear regression with a negative slope was observed for mean survival time in days of mice challenged with *Streptococcus* following different lengths of exposure to 3.5 ppm of NO_2.

For the same model system (fixed concentration versus time) other concentrations are now being tested for the purpose of developing information which can be used to develop regression curves at other concentrations which then can be used for predicting purposes.

Figure 4 presents preliminary data from experiments which were designed to test the effect of concentration and time as above, but utilizing an intermittent exposure model in place of a continuous exposure model. In these experiments, animals were exposed for 7 hr/days, 7 days/week to 3.5 ppm NO_2. At various times, animals were removed and given the bacterial aerosol. Figure 4 illustrates that NO_2 shows a significant increasing linear relationship with duration of exposure.

Conclusion

In short-term exposure to NO_2, the concentration employed has a much greater influence than duration of exposure in terms of the same *Ct*.

There is a significant increase in toxicity with time during a continuous exposure to a low concentration.

Intermittent response at a constant concentration also shows an increased effect with duration of exposure, although the exact slope at this time is still unclear.

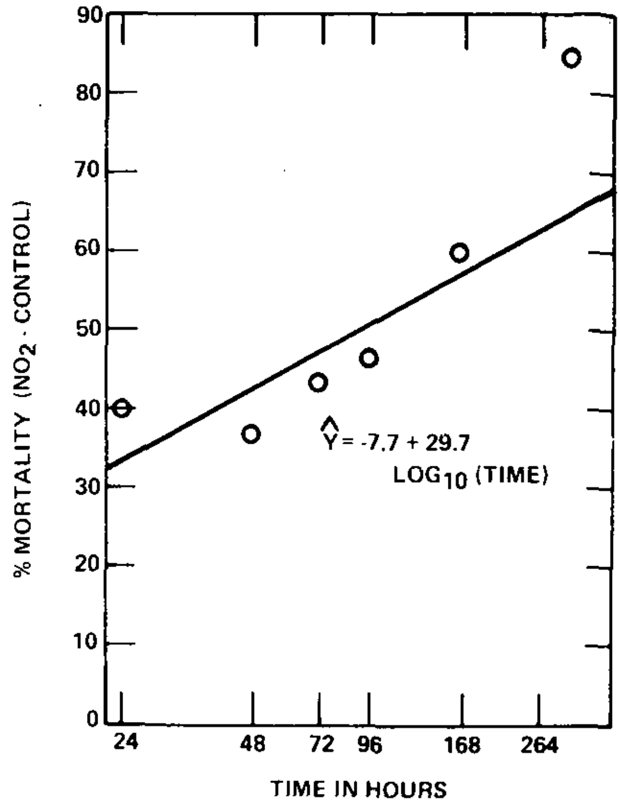

FIGURE 4. Linear regression of per cent mortality increase (NO_2-control) upon the logarithm of time for mice exposed to 3.5 ppm NO_2 7 hours/day for various numbers of days prior to challenge with *Streptococcus*. Statistically significant at the 0.05 probability level. The individual data points plotted represent four replications of the experimental regimen.

The question arises as to what is the cause of this increase in pulmonary infectivity which we enhance with NO_2

It has been demonstrated in previous work at our laboratory that acute exposure to NO_2 and O_3 have a destructive action on pulmonary alveolar macrophages (*17–19*), i.e., reduction in number, phagocytic competence, viability, and enzymatic function. These cells are postulated to be the chief pulmonary defense against infectious agents introduced via the airway (*20*). Thus, this effect on the macrophage could explain the effect seen after a single exposure to the pollutant. However, it is also known that the alveolar macrophages are renewable resources and that these cells are probably derived from the bone marrow and transported to the lung in the blood (*21*). Previous work (*17*) has indicated that the effect of a single dose of an oxidant on the alveolar macrophage lasts for approximately 24 hr. Thus, if the action of NO_2 on the infectivity parameter were solely to be a direct effect on the pulmonary macrophages, and that these cells were to be continually renewed, then no increase of toxic action with time would probably occur, particularly with the intermittent model. Therefore, the following hypotheses may be suggested to explain the enhancement of pulmonary infectivity following long term or repeated exposures.

Other damages which are known to occur in the lung, that is, various anatomic and chemical alterations, might have an influence on the role of the infection, and these lesions might increase with time (*22, 23*).

There is an effect on the alveolar macrophage prior to their emergence into the lung which could be mediated through free radicals, nitroxides, or peroxides (*24–26*).

A combination of the above two responses could also contribute to the alteration of the host's natural defenses against the inhaled microorganism.

These mechanisms will have to be tested through specific experiments designed to uncover the mode of action of NO_2. An amplification of the toxic effects of NO_2 may be examined through a combination of acute, chronic, and intermittent NO_2 exposures similar to the ones discussed in this paper.

REFERENCES

1. Grey, E. LeB., et al. Toxicity of the oxide of nitrogen. Arch. Ind. Hyg. Occup. Med. 10: 423 (1954).
2. Wagner, W. D., et al. Experimental study of threshold limit of NO_2. Arch. Env. Health 10: 455 (1965).
3. Hine, C. H., Cavalli, R. D., and Wright, R. R., unpublished results; cited by H. E. Stokinger and D. L. Coffin, in Air Pollution, 2nd ed. A. C. Stern, Ed. Academic Press, New York, 1968, pp. 446-546.
4. Boren, H. G. Carbon as a carrier mechanism for irritant gases. Arch. Env. Health 8: 119 (1964).
5. Freeman, G., et al. The subacute nitrogen dioxide-induced lesion of the rat lung. Arch. Environ. Health 18: 609 (1969).
6. Kleinerman, J., and Wright, G. W. The effects of prolonged and repeated nitrogen dioxide inhalation on the lungs of rabbits and guinea pigs. Paper presented at 5th Annual Air Pollution Medical Research Confer. Calif. State Dept. of Public Health, Los Angeles, Dec. 1961.
7. Ehrlich, R., and Henry, M. C. Chronic toxicity of nitrogen dioxide. I Effects on resistance to bacterial pneumonia. Arch. Env. Health 17: 860 (1968).
8. Ehrlich, R., et al. Immunologic response in vaccinated mice during long-term exposure to nitrogen dioxide. Environ. Res., 10: 217 (1975).
9. Coffin, D. L., and Gardner, D. E. Interaction of biological agents and chemical air pollutants. Ann. Occup. Hyg. 15: 219 (1972).
10. Coffin, D. L., et al. Effect of air pollution on alteration of susceptibility to pulmonary infection. In: Proceedings of 3rd Annual Conference on Atmospheric Contamination in Confined Space, Aerospace Medical Research Laboratory, 1968, pp. 71–80.
11. Ehrlich, R. Effects of nitrogen dioxide on resistance to respiratory infection. Bacteriol. Rev. 30: 604 (1966).
12. Coffin, D. L., and Blommer, E. J. Acute toxicity of irradiated auto exhaust. Its indication by enhancement of mortality from *Streptococcal pneumonia*. Arch. Environ. Health 15: 36 (1967).
13. Port, C. D. Interaction of nickel oxide and influenza infection in the hamster. Paper presented at Conference on Heavy Metals in the Environment; Environ. Health Perspect. 10:268 (1975).
14. Maigetter, R. Z., et al. Effect of manganese dioxide on resistance to respiratory infections. Abstracts, American Society of Microbiology, Chicago, Ill., 1974.
15. Blommer, E. J. Unpublished data.
16. Henry, M. C., Ehrlich, R., and Blair, W. H. Effect of nitrogen dioxide on resistance of squirrel monkeys to *K. pneumoniae* infection. Arch. Environ. Health 18: 580 (1969).
17. Coffin, D. L., Gardner, D. E., and Holzman, R. S. Influence of ozone on pulmonary cells. Arch. Environ. Health 16: 633 (1968).
18. Gardner, D. E., Holzman, R. S., and Coffin, D. L. Effects of nitrogen dioxide on pulmonary cell population. J. Bacteriol. 98: 1041 (1969).
19. Gardner, D. E., et al. Loss of protective factor for alveolar macrophages when exposed to ozone. Arch. Int. Med. 127: 1078 (1971).
20. Green, G. M., and Kass, E. H. The role of the alveolar macrophage in the clearance of bacteria from the lung. J. Exptl. Med. 119: 167 (1964).
21. Godleski, J. J., and Brain, J. D. The origin of alveolar macrophage in mouse radiation Chimeras. J. Exp. Med. 136: 630 (1972).
22. Freeman, G., et al. Pathology of pulmonary disease from exposure to ambient gases (nitrogen dioxide and ozone). Arch. Environ. Health 29: 203 (1974).
23. Buel, G. C., Tokiwa, Y., and Mueller, P. K. Lung collagen and elastin denaturation *in vivo* following inhalation of NO_2. Paper presented at Air Pollution Control Assn. Meeting, San Francisco, June 1966, APCA Paper 66–7.
24. Stokinger, H. E., and Coffin, D. L. Biological effect of air pollutants. In: Air Pollution 2nd ed. A. C. Stern, Ed. Academic Press, New York, 1968, pp. 446–546.
25. Menzel, D. B., Roehm, J. N., and Lee, S. D. Vitamin E: the biological and environmental antioxidant. Agr. Food Chem. 20: 481 (1972).
26. Chow, C. K., and Tappel, A. L. An enzymatic protective mechanism against lipid peroxidation damage to lung of ozone exposed rats. Lipids, 7: 518 (1972).