Effect of NO₂ on Airborne Venezuelan Equine Encephalomyelitis Virus

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Received for publication 19 November 1971

Studies were conducted to determine the effect of nitrogen dioxide (NO₂) on aerosol survival and biological decay rate of Venezuelan equine encephalomyelitis (VEE) virus and spores of Bacillus subtilis var. niger. The NO₂ concentrations used in the experiments were 0.5, 5, and 10 ppm at 24 C and 85% RH. The survival of airborne VEE virus disseminated as particles 1 to 5 µm in diameter was significantly influenced by the presence of 5 ppm of NO₂. At this concentration, the biological decay rate increased threefold and the aerosol recovery and aerosol survival of the VEE virus were significantly lower than at 0.5 ppm or in the absence of NO₂. Airborne spores of B. subtilis were not significantly affected by as much as 10 ppm of NO₂.

Of the six commonly found oxides of nitrogen, nitric oxide (NO) and nitrogen dioxide (NO₂) are considered of greatest importance from the standpoint of urban air pollution. NO represents the major product of the combustion of fossil fuels, and all oxides of nitrogen react in the air to produce NO₂ as the principal end product.

In urban air, concentrations of NO₂ display distinct diurnal variations related to the intensity of sunlight, the amount of atmospheric mixing, and the extent of vehicular traffic. Thus, a major peak concentration occurs between midmorning and noon, and a secondary, lesser peak occurs in the late afternoon. NO also shows seasonal variations, whereby higher concentrations are recorded in the late fall and winter months; this is usually not reported for NO₂.

Results of continuous monitoring of pollutants indicate that peak concentrations of NO exceeding 1 ppm are common; however, NO₂ concentrations rarely exceed 0.5 ppm. According to Tabershaw et al. (6), the highest concentration of NO₂ recorded in the Los Angeles area in 1964 was 1.3 ppm, and during the same year hourly averages of NO₂ exceeded 0.25 ppm about 9% of the days. Over a 3-year period from 1966 to 1968 the daily maxima of 0.3 ppm of NO₂ were exceeded on 10% of the days in the same area (7).

Although a considerable amount of information is available pertaining to the effects of NO₂ in experimental animals and to a lesser degree in man, data pertaining to the effects of this pollutant on airborne microorganisms are limited. In this respect, aerosol studies were carried out with Venezuelan equine encephalomyelitis (VEE) virus and spores of Bacillus subtilis var. niger serving as the physical decay tracer. The experiments were designed to determine the effects of NO₂ in concentrations ranging from 0.5 to 10 ppm on the aerosol survival of VEE virus, disseminated in a static aerosol chamber, as particles ranging in size from 1 to 5 µm.

MATERIALS AND METHODS

Virus. Frozen chick embryo suspensions of Trinidad strain of VEE virus and a concentrated slurry of spores of Bacillus subtilis var. niger (batch no. 91), provided by the Fort Detrick laboratories, were stored in individual vials at dry-ice temperature until used.

Inasmuch as information was not available on the effects of NO₂ on airborne spores of B. subtilis, the applicability of this organism as a physical decay tracer for these studies was initially determined. For these preliminary studies, 0.5 g of the frozen B. subtilis spores was mixed with 0.4 g of sodium fluorescein in 100 ml of phenolated gelatin phosphate diluent. After blending in a Waring Blender and screening through an 80-mesh stainless-steel screen, 1.0 ml of the mixture was used for dissemination.

For studies of NO₂ effects on the VEE virus, spores of B. subtilis were used as the physical decay tracer. The frozen microorganisms were thawed at 24 C and mixed to provide an approximate 10:1 ratio of VEE virus assay units to B. subtilis spores. A 1.0-ml sample of the blended mixture was disseminated...
by means of a two-fluid atomizer (FK-8 aerosol gun). This atomizer produced aerosol clouds having the majority of particles in the 1- to 5-μm size range.

**Aerosol chamber.** The desired environmental conditions of 24°C and 85% RH were established in the 2,500-liter stainless-steel aerosol chamber (9–5) before the introduction of the air pollutant or the dissemination of the biological suspensions. A measured amount of NO₂ was injected into the aerosol chamber first by using a syringe, followed by dissemination of the biological agent mixture. To maintain a uniform distribution of the agent cloud and the gas, a small fan was continuously operated in the chamber throughout the aerosol trials.

A modified Mast ozone meter was used to record the NO₂ concentration before and throughout each aerosol trial. The continuous recording permitted the estimation of the decay of NO₂ in the aerosol chamber during each 64-min aerosol trial. Based on the analytical data, the decay of NO₂ ranged from 0.3 to 0.5% per min. To minimize the potential effect of residual NO₂ in the aerosol chamber, the first aerosol experiment on a given day was a control trial without NO₂, and the concentration of NO₂ was increased in each subsequent aerosol trial.

**Aerosol sampling and assay.** Duplicate samples of the aerosol were collected for 1 min at 4, 16, 32, and 64 min cloud ages with all-glass impingers (AgI-30) operating at a sampling rate of 12.5 liters/min (2). A single-stage impactor (6) designed to remove particles larger than 5 μm replaced the conventional curved stem of the sampler. The AgI-30 samplers contained 20 ml of heart infusion broth and distilled water serving as collecting fluids for the VEE virus and *B. subtilis*, respectively. To prevent foaming during the sampling, the collecting fluids contained 0.15% Antifoam A (Dow-Corning) emulsion.

The duplicate samples were pooled and *B. subtilis* was assayed conventionally on Tryptose-agar (4). The VEE virus was assayed by the plaque technique in primary chick monolayer tissue culture (3). The plates were incubated at 37 ± 1°C for 72 hr, and the plaques were counted.

**Data analysis.** Mean estimates of aerosol recovery, survival, and decay rate based on six replicate aerosol experiments were calculated. The means were compared by standard analysis of variance technique, and the significance of the observed differences is reported at the 0.05 probability level.

**RESULTS AND DISCUSSION**

The decay of the aerosol was measured by quantitation of the airborne VEE virus, *B. subtilis* spores, and sodium fluorescein, respectively, at 4, 16, 32, and 64 min after dissemination. The slope of the curve, expressed in percent per minute units (%/min), defined the total decay rate of the cloud, which included the biological and physical losses of the aerosol. The biological decay rate which defined the losses due to inactivation or death of the microorganisms represented the difference between the total decay rate and the rate of decay of the respective material used as the physical tracer.

The results of preliminary studies indicated that the survival of airborne *B. subtilis* spores was not significantly influenced by the presence of NO₂ in the aerosol chamber. The death rate of *B. subtilis* was 0.15, 0.13, and 0.31%/min at NO₂ concentrations of 0.5, 5, and 10 ppm, respectively, whereas in NO₂-free atmosphere it was 0.10%/min. The aerosol recovery of *B. subtilis* at various cloud ages is shown in Fig. 1. Somewhat lower, although not significantly different, aerosol recoveries of viable spores were obtained at 10 ppm of NO₂ as compared to the recoveries at the two lower NO₂ concentrations or the control environment. Estimates of *B. subtilis* survival in aerosol were computed by dividing the ratio of *B. subtilis* to sodium fluorescein in the aerosol samples by the ratio of the two products in the dissemination mixture. These results were multiplied by 100 in order to provide percentage expressions. The survival properties of airborne *B. subtilis* spores were not influenced by the presence of as much as 10 ppm of NO₂ (Fig. 1). Therefore, when disseminated as an intimate mixture, the *B. subtilis* spores were acceptable as a physical tracer in aerosol studies designed to determine the effects of NO₂ on airborne VEE virus.

Recoveries of VEE virus at the four aerosol sampling periods and the death rates of the virus in the presence of various NO₂ concentrations are shown in Table 1. In general, the relationship between the aerosol death rate of the virus and the NO₂ concentration was linear. At 0.5 ppm of NO₂, deleterious effects of the pollutant were absent and the aerosol

![Fig. 1. Aerosol recovery and survival of B. subtilis spores at various cloud ages and NO₂ concentrations.](image-url)
recovery and the death rate of VEE virus were essentially identical to those observed in the control environment. At 5 ppm of NO₂, the death rate of the VEE virus was approximately threefold that in the control groups ($P \leq 0.05$). At this NO₂ concentration, aerosol recovery of the virus at a 4-min cloud age was only slightly lower than that of the control. However, during the remaining three sampling periods, the per cent of viable virus recovered was less than one-half of the control. Increasing the NO₂ concentration to 10 ppm produced a more than 10-fold increase in the biological decay rate ($P \leq 0.001$) and significantly reduced aerosol recoveries of viable VEE virus. The deleterious effect of NO₂ on aerosol recovery was already apparent during the early (4 min) sampling period and continued throughout the life of the aerosol cloud.

The effect of NO₂ can be further illustrated by plotting the aerosol survival of the VEE virus as a function of cloud age. The slopes of the best-fitted curves shown in Fig. 2 confirm the NO₂ effects of reduced aerosol survival and increased biological death rate.

Studies on the effects of NO₂ on airborne bacteria were reported by Won and Ross (9), who exposed mixtures of *Rhizobium meliloti* and *B. subtilis* spores to NO₂, sulfur dioxide, and vapors of Formalin. In their experiments, NO₂ was introduced into the chamber air after the bacteria were airborne. Thus, the effects of the air pollutants were observed within a microbial population prestressed by being airborne for 1 hr. *R. meliloti* was more sensitive to NO₂ than to Formalin, but it was relatively insensitive to sulfur dioxide. Combination of the gases did not produce any significant synergistic effects. The viability of *R. meliloti* decreased approximately one log over a 60-min cloud life in the presence of 3 ppm of NO₂ at 20 C and 50% relative humidity (RH). The authors also reported that the bactericidal activity of NO₂ was somewhat higher at 95% RH.

Data previously reported from our laboratories (3) indicated that the survival of airborne VEE virus did not vary significantly within a humidity range of 18 to 90% relative humidity at 24 C. However, it can be expected that increased water content in the air would promote the formation from NO₂ of oxidizing agents, such as nitric and nitrous acids. Such agents are known to actively react with cell proteins. More specifically, nitrous acid deamidates proteins as well as purines and pyrimidines in both single- and double-stranded nucleic acids. The presence of these agents in the air could be expected to increase the inactivation rate of the airborne virus by virtue of modification of the viral protein.

Another environmental factor which could enhance the inhibitory effect of NO₂ on VEE virus is the presence and intensity of solar radiation. Berendt and Dorsey (1) recently reported that after 1 hr of exposure to simulated solar radiation only 0.02 and 0.006% of aerosolized VEE virus survived at 30 and 60% RH, respectively. The inactivation rate was further intensified when sodium fluorescein was added to the viral suspension before dissemination. The threshold NO₂ concentration (< 5 ppm) at which the inhibitory effects on the airborne VEE virus were first apparent in our studies was markedly higher than that usually found.

### Table 1. Aerosol recovery and death rate of VEE virus at various NO₂ concentrations

| NO₂ (ppm) | Cloud age (min) | Death rate (%/min) |
|-----------|----------------|-------------------|
|           | 4   | 16  | 32  | 64  |
| 0         | 28.1* | 19.3 | 13.1 | 6.50 | 0.53 |
| 0.5       | 28.5 | 20.0 | 12.0 | 6.79 | 0.53 |
| 5         | 26.8 | 14.4 | 4.96 | 2.72 | 1.66 |
| 10        | 20.0 | 8.37 | 1.77 | 0.178 | 6.12 |

* Per cent viable recovery.
in polluted urban atmospheres. Nevertheless, this threshold level could be expected to be significantly lowered as a result of the combined effects of temperature, humidity, solar radiation, and other pollutants.

ACKNOWLEDGMENTS

We thank E. W. Larson and R. L. Walker, Aerobiology and Evaluation Laboratory, Fort Detrick, for providing the VEE virus and B. subtilis cultures and for many helpful suggestions.

This study was supported by funds provided by the Department of the Army, Fort Detrick, Frederick, Md.

LITERATURE CITED

1. Berendt, R. F., and E. L. Dorsey. 1971. Effect of simulated solar radiation and sodium fluorescein on the recovery of Venezuelan equine encephalomyelitis virus from aerosol. Appl. Microbiol. 21:447-450.

2. Brachman, P. S., R. Ehrlich, H. F. Eichenwald, V. J., Cabelli, T. W. Kethley, S. H. Madin, J. R. Maltman, G. Middlebrook, J. D. Morton, J. H. Silver, and E. K. Wolfe. 1964. Standard sampler for assay of airborne microorganisms. Science 144:1296.

3. Ehrlich, R., and S. Miller. 1971. Effect of relative humidity and temperature on airborne Venezuelan equine encephalomyelitis virus. Appl. Microbiol. 22:194-199.

4. Ehrlich, R., S. Miller, and R. L. Walker. 1970. Relationship between atmospheric temperature and survival of airborne bacteria. Appl. Microbiol. 19:245-249.

5. Ehrlich, R., S. Miller, and R. L. Walker. 1970. Effects of atmospheric humidity and temperature on the survival of airborne Flavobacterium. Appl. Microbiol. 20:884-887.

6. Malligo, J. E., and L. S. Idoine. 1964. Single-stage impact device for particle sizing biological aerosols. Appl. Microbiol. 12:32-36.

7. Mosher, J. C., W. G. MacBeth, M. J. Leonard, T. P. Mullins, and M. F. Brunelle. 1970. The distribution of contaminants in the Los Angeles basin resulting from atmospheric reactions and transport. J. Air Pollut. Contr. Ass. 20:35-42.

8. Tabershaw, I. R., F. Ottoboni, and W. C. Cooper. 1968. Oxidants: air quality criteria based on health effects. J. Occup. Med. 10:32-48.

9. Won, W. D., and H. Ross. 1969. Reaction of airborne Rhizobium meliloti to some environmental factors. Appl. Microbiol. 18:555-557.