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Effects of Nitric Oxide and Nitrogen Dioxide on Bacterial Growth

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The effects of low concentrations of nitric oxide (NO) and nitrogen dioxide (NO_2) on actively dividing cultures of *Staphylococcus aureus, Micrococcus luteus, Micrococcus roseus, Serratia marcescens, Bacillus subtilis, Bacillus circulans, Bacillus megaterium*, and *Bacillus cereus* were studied. Fresh cultures of each organism were incubated for 24 h at 25°C on both nutrient agar and mineral salts glucose agar plates under atmospheres containing various low concentrations of NO in air (0 to 1.9 ppm [0 to 2.0 μg/g of air]), NO_2 in air (0 to 5.5 ppm [0 to 8.8 μg/g of air]), or NO and NO_2 in air. Bacteria grown under air only were used as controls. After incubation, the colonies that developed on the plates were counted. None of the bacteria tested was affected by NO or NO_2 at the indicated concentrations while growing on nutrient agar. *Serratia marcescens, B. circulans, B. subtilis, B. megaterium*, and *B. cereus* grown on mineral salts glucose agar were not significantly affected by NO or NO_2. Low concentrations (0 to 1.9 ppm) of NO were bacteriostatic to log-phase cultures of *M. roseus, M. luteus*, and *Staphylococcus aureus* grown on mineral salts glucose agar. Bacteriostatic activity over a 24-h interval was maximal at an initial NO concentration of 1 ppm. Appreciable amounts of NO_2 were produced in 24 h at initial NO concentrations greater than 1 ppm. These results suggest that NO_2 may reduce the bacteriostatic activity of NO. Low concentrations (0 to 5.5 ppm) of NO_2 in air did not affect any of the bacteria tested. At these low concentrations, NO affected bacterial growth, although NO_2, NO_2^-, and NO_3^- did not. In addition, it was determined that the bacteriostatic activity observed in this study was not due to an increase in the acidity of the medium.

The purpose of this investigation was to determine the effects of low concentrations of nitric oxide (NO) and nitrogen dioxide (NO_2) on actively dividing cultures of *Staphylococcus aureus, Micrococcus luteus, Micrococcus roseus, Serratia marcescens, Bacillus subtilis, Bacillus circulans, Bacillus megaterium*, and *Bacillus cereus*. These organisms were chosen because they are ubiquitous in nature. Because NO is an intermediate in denitrification and NO and NO_2 are becoming increasingly common forms of nitrogen in the environment, it is important to determine their effects on bacterial growth.

Numerous studies were undertaken to determine the interactions among the various nitrogen oxides and bacteria (1, 4, 16). Bancroft et al. (2) showed that heterotrophic bacterial populations in soil are inhibited by low nitrite concentrations and therefore may be affected by such compounds as NO_2. This fact may be important in soil microbial populations because NO_2 can be absorbed by soil constituents and converted to nitrate both abiotically and by nitrifying microorganisms (4, 8). Labeda and Alexander (12) found that NO_2 inhibited nitrification in certain soils.

The abiotic and biotic roles of nitrogen oxides in the environment have been established (4, 5, 16). They are major air pollutants (17), are a critical component of acid rain (14), and have a variety of effects on microorganisms. It has been shown that correlations exist between viable airborne bacterial density and NO and NO_2 (10, 13, 15, 22).

A statistically significant negative correlation was found between the number of viable bacteria isolated from urban air and the NO concentration of the air (13, 15). Shank et al. (18) found that NO had no effect on nondividing dessicated bacteria adsorbed onto a Millipore filter. Benbough (3) suggested that NO may protect dessicated organisms by reacting with free radicals formed after rehydration which might otherwise disrupt cellular lipids.

A statistically significant positive correlation was found between the number of viable airborne bacteria isolated from urban air and the NO_2 concentration of the air (15). Ehrlich and Miller (7) found that aerosolized spores of *B. subtilis* were not significantly affected by NO_2 at 10 ppm (16 μg/g of air). The bactericidal effects of NO_2 or the products formed from it in water increase as the pH decreases (20, 21). Gray (10) mixed high concentrations of NO and NO_2 and found that the toxicity of the mixture was approximately proportional to the amount of NO_2 present.

It is generally thought that the bactericidal effects of NO and NO_2 are due to their reaction with water to form nitrous and nitric acids (18), but this only appears to be true at high concentrations. The data presented here indicate that at low NO and NO_2 concentrations, acids are not present in high enough concentrations to act as toxic agents. Grant et al. (9) found that exposing acid forest soil to 1 ppm of NO_2 did not cause the soil pH to drop. The results of this study show that at low concentrations of NO and NO_2, the NO is bacteriostatic for some organisms and not for others, whereas NO_2 may protect some bacteria from the inhibitory effects of NO.

MATERIALS AND METHODS

Bacteria. *Staphylococcus aureus* ATCC 6538P, *M. luteus* ATCC 272, *M. roseus* ATCC 144, *Serattia marcescens* ATCC 60, *B. subtilis* ATCC 82, *B. circulans* ATCC 4513, *B. megaterium* ATCC 14581, and *B. cereus* ATCC 14579 were obtained from the American Type Culture Collection, Rockville, Md.

Media. Nutrient agar was prepared from distilled water (1 liter), Noble agar (25.0 g), and nutrient broth (8.0 g). Mineral salts glucose agar contained: distilled water, 1 liter; Noble agar, 25.0 g; glucose, 10 g; K_2HPO_4, 0.8 g; NH_4NO_3, 0.5 g; yeast extract, 0.5 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.1 g; and $FeCl_3$, 0.1 g. The pH was adjusted to 7.0, and the medium was autoclaved.

Gas exposure. Broth cultures were made for each organism and incubated at 25°C for 24 h. Serial 10-fold dilutions of 24-h log-phase cultures were made and surface plated onto nutrient and mineral salts glucose agars. The plates were placed into air-tight jars (standard Pyrex glass anaerobic jars, 23 by 13 cm, with aluminum tops fitted with a gas valve) for gassing. The jars were gassed to slight overpressure with various low concentrations of NO, NO_2, or both. The jars initially contained one of the following gas mixtures: NO (0 to 1.9 ppm) and air; NO_2 (0 to 5.5 ppm) and air; NO (0 to 1.9 ppm), NO_2 (0.5 ppm), and air; or air only. The plates in the jars gassed only with air were used as controls. All plates were incubated at 25°C for 24 h. After incubation, the colonies that developed on the plates were counted. This procedure was done at least in triplicate for each organism at each gas concentration.

Medium analysis. The pH of each medium was determined by mixing 1 g of the medium from each gas concentration and control jar with enough distilled deionized water to make a total volume of 10 ml. The various medium-water solutions were placed in a Sorvall Omni-mixer and mixed for 1 min. The pH of each sample was determined with a pH meter.

Effect of adding HNO_3 to media. Plates of mineral salts glucose agar were adjusted to pH 5.0, 5.5, 6.0, 6.5, or 7.0 by adding HNO_3 to the medium. Each organism in log-phase growth was plated at each pH and incubated at 25°C for 24 h. In addition, a set of plates at each pH was gassed with various amounts of NO (0 to 1.9 ppm) in air and incubated at 25°C for 24 h as described above. After 24 h the colonies that had developed on the plates were counted.

RESULTS

The bacteria grown on nutrient agar plates were not affected by low concentrations of NO or NO_2. *Serratia marcescens, B. circulans, B. subtilis, B. megaterium*, and *B. cereus* grown on mineral salts glucose agar were not significantly affected by NO or NO_2 at any of the concentrations or combinations tested. A monotonic decrease with increasing NO density was sometimes detected in the data for all of the *Bacillus* species, but it was nonsystematic. This variation could possibly be explained by spore formation and the resistance of spores to NO (10). Low concentrations (0.1 to 1.4 ppm) of NO were bacteriostatic to log-phase cultures of *M. roseus, M. luteus*, and *Staphylococcus aureus* grown on mineral salts glucose agar. The effects of NO seem to be bacteriostatic and not bactericidal at these concentrations (Fig. 1). If NO were bactericidal, higher initial concentrations would result in lower or equal numbers of surviving colonies instead of the increase that was seen. The bacteriostatic activity reached a maximum between initial NO concentrations of 0.8 and 1.0 ppm. Higher initial concentrations of NO were accompanied by an increase in bacterial survival. This relationship can be represented, based on consideration of chemical reactions, by the following equation:

$$Y = 100\% - \frac{A[NO_i]}{1 + \beta[NO_i]} + \frac{B\beta[NO_i]^2}{1 + \beta[NO_i]} \quad (1)$$

where Y represents percent survival, NO_i is the initial concentration of NO, and the last two terms in the equation represent the NO and NO_2 concentrations, respectively, after 24 h of incubation (see equations 2 and 3 below). The strength of the chemical reactions is characterized by parameter β, which equals 0.439 ppm^{-1}; its derivation is shown below. A and B are constants which were chosen by the nonlinear least-squares method. The characteristic time for sorption of NO_2 into the aqueous phase is directly proportional to the square of NO_2 density. At 1 ppm, the time constant for sorption of

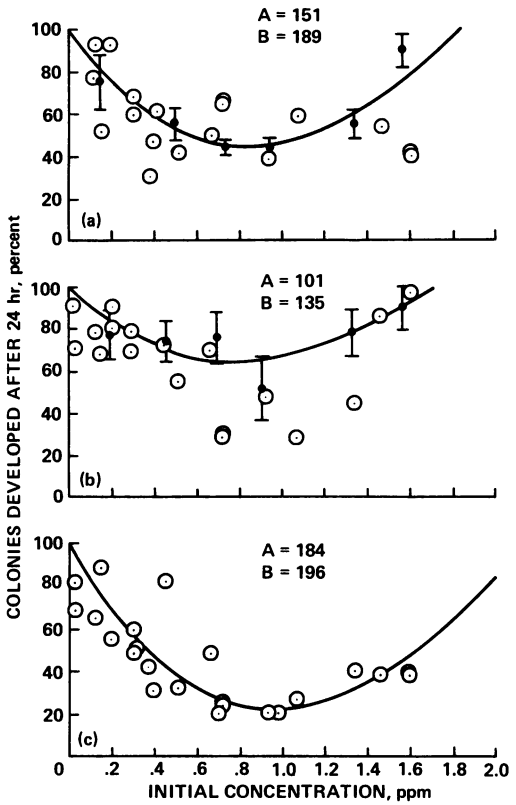

FIG. 1. Survival rates for colonies of (a) *M. roseus*, (b) *M. luteus*, and (c) *Staphylococcus aureus* after 24 h of incubation with various initial concentrations of NO in air. Error bars represent one standard deviation for those runs having eight plates at the same NO_i concentration; others had an average error of ±9%. Values for the constants *A* and *B* are shown in each panel (refer to equation 1).

NO_2 into the aqueous phase is 300 h (23). Therefore, the amount of NO_2 into the aqueous phase is 300 h (23). Therefore, the amount of NO_2 removed from the gaseous phase in 24 h is minute or essentially zero. The time constant for sorption of NO at 1 ppm is also several hundred hours (23). The ratio of the variance, the *F* test, was used to judge the significance of the fitted curve for both a random distribution and a simple exponential decay. In applying the *F* test, the data are assumed to have a normal distribution, and the residual variance of the data unexplained by the hypothesis is related to the residual variance unexplained by the alternative hypothesis as a ratio. The probability that the data support the hypothesis can then be determined (19). If both hypotheses are equally supported, the probability will be 50%. For *M. roseus* ($A = 151$, $B = 189$) the hypothesis of equation 1 is significant above the 99% confidence level for both a random distribution and a simple exponential. For *M. luteus* ($A = 101$, $B = 135$) the hypothesis of equation 1 is significant to the 90% level for both hypotheses. In both cases there was no statistical improvement (probability = 50%) with the simple exponential function over the random distribution. However, for *Staphylococcus aureus* ($A = 184$, $B = 196$) both the hypothesis of equation 1 and a simple exponential function fit the data well. The significance over a random distribution was 94 and 92%, respectively. This was expected because the trend toward higher survival rates at higher NO_i concentrations was not as strong for *Staphylococcus aureus* (Fig. 1c). Overall, the results indicated that the form of equation 1 is statistically valid.

NO_2 at 0.13 to 5.5 ppm did not have any effect on any of the bacteria tested. When various concentrations of NO (0 to 1.9 ppm) were mixed with 0.5 ppm of NO_2, the bacteriostatic effect of NO was still apparent. However, the average survival rate of the affected organisms was increased. This indicated that adding NO_2 to the system yielded higher colony counts at a given NO concentration, although they were still lower than those of the control. These data are consistent with the hypothesis that as the proportion of NO_2 to NO increases, more bacteria survive at high initial NO concentrations.

The pH of uninoculated plates of nutrient agar and mineral salts glucose agar after being gassed with NO and incubated for 24 h ranged from 6.5 for plates gassed with 1.4 ppm of NO to 6.8 for those gassed with 0.1 ppm of NO. The same pH results were obtained for inoculated plates.

Staphylococcus aureus, *Serratia marcescens*, *M. roseus*, and *M. luteus* organisms incubated on mineral salts glucose agar plates containing various amounts of HNO_3 were killed by increasing amounts of acid. When the same test was done under an atmosphere of NO, the bacteriostatic effect of NO for each organism was essentially the same as that shown in Fig. 1 at pH 7.0 and 6.5. At pH 5.0 and 5.5, the toxic effects of the acid obscured the NO effects. At pH 6.0 the bacteriostatic effect of NO could be detected, but the survival rate decreased an average of 20 to 35% for each organism.

DISCUSSION

The data presented in this paper indicate that NO at low concentrations affects bacterial growth, but NO_2, HNO_2, HNO_3, NO_2^-, and NO_3^- do not. No effect was observed on bacteria grown on nutrient agar, but certain bacteria grown on mineral salts glucose agar were affected. The mineral salts glucose medium was not as rich as the nutrient agar; therefore, the bacteria were more physically stressed in that medium.

These strict growth conditions should allow the bacteria to more readily exhibit signs of additional stress from the gases. Bacteria are physically stressed in an airborne environment and in some soil environments, the major conditions under which exposure to these gases may occur. The surface plating method used in this study approximated the way a growing organism would come in contact with NO while airborne or in some soils.

During the 24-h incubation period, the NO in the jars was oxidized to form NO_2 by the reaction $2NO + O_2 \rightarrow 2NO_2$. The rate of this reaction is dependent on the concentration of O_2 and the square of the NO concentration ($k = 2 \times 10^{-38}$ cm^{-6} s^{-1}) (11). Therefore, the duration of bacterial exposure to the full NO_i concentration was limited and sharply decreased with increasing NO concentration. In other words, although the absolute NO concentration at a given time after the start of incubation was always greater with a larger NO_i concentration, the fraction NO/NO_i is smaller, and therefore the fraction NO_2/NO is greater. The concentrations of NO and NO_2 after 24 h can be exactly expressed as a function of NO_i as follows:

$$NO = \frac{[NO_i]}{1 + \beta[NO_i]} \quad (2)$$

$$NO_2 = \frac{\beta[NO_i]^2}{1 + \beta[NO_i]} \quad (3)$$

where β, defined as the rate constant k times the oxygen concentration times the incubation period, has a numerical value of 0.439 ppm^{-1}.

The bacterial response to initial concentrations of NO was distinctly bimodal (Fig. 1). The survival curves for *M. roseus*, *M. luteus*, and *Staphylococcus aureus* all had the same shape. Each curve showed a decrease in survival with increasing NO concentrations up to an NO_i of ~1 ppm, followed by a marked increase in survival with increasing NO_i for an NO_i of >1 ppm. These data suggest that NO is bacteriostatic and that increasing NO_2 concentrations increasingly reduce this effect. The simplest equation that matches the data is $Y = 100\% - A\ NO + B\ NO_2$, where Y is the percent survival, NO and NO_2 are the concentrations (in cubic centimeters) of NO and NO_2, respectively, and A and B are constants determined in a nonlinear least-squares fit for each species (Fig. 1). Equations 2 and 3 were then substituted for NO and NO_2 to create equation 1.

When 0.8 ppm of NO_2 was added to 0 to 1.9 ppm of NO in air, the average survival rate of the exposed bacteria was somewhat increased. This is consistent with the hypothesis that NO_2 reduces the bacteriostatic effect of NO.

NO_2 combines with water in the medium to form nitrous and nitric acids, which are toxic to bacteria. However, at the low concentrations of NO_2 used in this study, the amount of acid formed in the medium was too small to substantially lower the pH and did not affect the growing bacteria. The data presented here indicate that at low concentrations NO is bacteriostatic for some bacteria but does not generate enough acids to be toxic. It is interesting to note that nonsporeforming gram-positive bacteria were affected by NO, but sporeforming and gram-negative bacteria were not affected. This effect may be due to differences between gram-negative and gram-positive cell wall structure and the resistance of the spores.

The results of this study agree with those of an earlier study that found that increases in atmospheric NO content were associated with a decrease in the number of viable airborne bacteria, whereas increased NO_2 concentrations were associated with an increase (15). The data presented here indicate that the effects of NO_2 depend on the effects of NO, such that NO_2 reduces the bacteriostatic effect of NO. Because it has been shown that bacteria can divide while airborne (6), the results of this study indicate that NO at the low concentrations found in the atmosphere can select for resistant bacteria in the air and affect the viable airborne bacterial population. In addition, low NO concentrations from denitrification (16) can select for or inhibit certain portions of the bacterial population in soil. This phenomenon appears to be self-regulating, because increases in NO concentrations lead to increases in NO_2 concentrations. The NO_2 in the atmosphere, from NO and other sources, in turn may reduce the selective effect of NO.

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