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Modification of Membrane Sulfhydryl Groups in Bacteriostatic Action of Nitrite

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The mechanism by which nitrite inhibits outgrowing spores of *Bacillus cereus* T was examined by using techniques developed earlier for nitrite analogs. The morphological stage of inhibition, cooperativity effects, effect of pH on inhibition, kinetics of protection against iodoacetate incorporation into membrane sulfhydryl groups, and protection against the bacteriocidal effect of carboxymethylation by iodoacetate indicate that nitrite acts as a membrane-directed sulfhydryl agent. The mechanism by which nitrite modifies the chemical reactivity of the sulfhydryl group could be either direct covalent modification or inactivation through communication with another modified membrane component. Profiles of pH effects suggest that the active agent is the protonated form of nitrite. The nitrite concentrations which modify membrane sulfhydryl activity coincide with those which have a bacteriostatic effect. These results are consistent with membrane sulfhydryl modification as a component of the mechanism of nitrite-induced bacteriostasis in this aerobic sporeformer.

Nitrite has been used since antiquity as a food preservative, first as a fortuitous component of curing salts and later as a recognized chemical agent with unique bacteriostatic properties (1, 19). Despite considerable effort to find alternative agents, none has been discovered (18). It seems probable that if the mechanism by which nitrite interferes with cell growth were understood, in terms of both the cellular target of nitrite action and the chemical events which lead to growth inhibition, other compounds which mimic the nitrite mechanism could be rationally designed or selected. Such compounds might be important as new food preservatives, antibiotics, or general bacteriostatic agents.

The mechanism of nitrite bacteriostatic action is elusive for several reasons, one being the tendency of nitrite to react so ubiquitously with nucleophilic groups (2) that determination of the reactions that cause bacteriostatic effects has not been possible. Moreover, the unavailability of radioactive nitrite has limited isotope tracer studies to those done with nonradioactive heavy isotopes, which require difficult and cumbersome mass spectrometric analysis (16). These limitations led to a great deal of work (reviewed by Reddy et al. [13]) in which nitrite interactions with bacterial cell components were observed; however, such interactions have never been linked to the chemical mechanism by which nitrite actually exerts its bacteriostatic effect in its role as a food preservative. In this paper, we present evidence that nitrite-induced bacteriostasis in an aerobe is associated with inactivation of membrane sulfhydryl groups. In ths respect, nitrite mimics the action of nisin, which is a natural peptide antibiotic (11), and *S*-nitrosothiols, which are analogs of nitrous acid (8) that have been implicated in the covalent modification of membrane sulfhydryl groups.

We previously explored the bacteriostatic mechanism of *S*-nitrosothiols (RSN=O), which are structural analogs of nitrous acid (HON=O), by examining their effects on the germination and outgrowth of spores of *Bacillus cereus* T (5, 8–11). Studying the effects on these bacterial spores is appropriate, since a primary value of nitrite as a food preservative is its ability to protect against the toxic effects caused by growth of bacterial sporeformers (1). Results obtained with these analogs indicated that the bacteriostatic mechanism involves covalent attachment of the analog to membrane sulfhydryl groups of germinated spores (5, 8–11). If *S*-nitrosothiols are a suitable model for the study of nitrite action, we should obtain similar results with nitrite.

MATERIALS AND METHODS

Materials. Tritiated iodoacetate (5 mCi) (New England Nuclear Corp.) was diluted to 5 ml with distilled water and filtered through a 0.45-μm-pore Millipore filter to remove impurities which bound nonspecifically to spores. We do not know the nature of these impurities. Unlabeled sodium iodoacetate was obtained from Sigma Chemical Corp.; reagent-grade sodium nitrite was obtained from J. T. Baker Chemical Co.; and tryptone was obtained from Difco Laboratories.

Spore preparation. Spores of *B. cereus* T were prepared and stored as previously described (20). Lyophilized spores (12.5 mg) were suspended in 20 ml of distilled water with a glass homogenizer. The spores were heat shocked by being placed in a 65°C water bath for 2 h. They were then centrifuged at 4°C for 10 min at 7,500 × *g*, and the spore pellet was suspended in 0.5 ml of water. Spores were added to the outgrowth medium to a final concentration as indicated for each experiment. There were approximately 10^9 CFU/mg of spores.

Measurement of inhibitor activity. The inhibitory effectiveness of nitrite was measured as described previously for nitrite analogs (3). Spores (0.37 mg) were added to 5 ml of 1% tryptone and incubated at 35°C in a drum shaker rotating at 20 rpm. After 18 min, phase-contrast microscopy showed that the spores were phase dark, establishing that germination had occurred. An aliquot of germinated spores was then added to 5 ml of 1% tryptone, which contained the inhibitor, to give a final spore concentration of 0.075 mg/ml, which corresponds to about 7.5×10^7 spores per ml. The tubes were then incubated on the rotary drum shaker for an additional 3 h and examined again. Under these conditions, spores in the absence of an inhibitor reach the vegetative state after about 100 min. In the presence of an inhibitor such as nitrite, interference with the outgrowth process occurs. The spores were defined as inhibited when at least 50% of

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the population was still in the phase-dark stage after the 3-h period. The amount of inhibition was the fraction of spores still in the phase-dark condition.

Reversibility of inhibition. Spores at a concentration of 0.075/ml were germinated as described above and added to tryptone that contained various levels of nitrite. After exposure to nitrite for 5 to 15 min, the spores were centrifuged, washed twice by centrifugation from 5 ml of 1% tryptone, and suspended in a final 5-ml volume of 1% tryptone. The spores were then incubated for 3 h and examined for signs of inhibition.

Effect of pH on inhibitory effectiveness of nitrite. Spores were germinated in 1% tryptone (pH 6.8). The spore suspension was centrifuged, and the pelleted spores were suspended at a spore concentration of 0.075 mg/ml in 5-ml volumes of 1% tryptone which had been adjusted to the desired pH with HCl. The inhibitory effectiveness was then determined as described above.

Measurement of iodoacetate incorporation into germinated spores. Spores were germinated as described above, except that they were at a concentration of 0.6 mg/ml. The spores were centrifuged and suspended at 1.2 mg/ml with 2% tryptone at the appropriate pH. Aliquots of the spore suspension were then each added to an equal volume of the appropriate concentrations of radiolabeled iodoacetate to give a final volume of 5 ml. Incorporation was allowed to proceed for 20 min during incubation at 35°C. Incorporation was halted by addition of a large excess of unlabeled iodoacetate (20 ml of 1 mM iodoacetate) that had been chilled to 0°C on ice. The labeled spores were centrifuged, suspended in 20 ml of 1 mM iodoacetate, and centrifuged again. This wash step was repeated. The radioactive spore pellet was then suspended in 0.5 ml of water and added to 5 ml of Aquafluor (100 g of naphthalene, 7 g of PPO [2,5-diphenyloxazole], 1.5 g of POPOP [1,4-bis(5-phenyloxazolyl) benzene] per liter of 1,4-dioxane), and the amount of radioactivity was determined with a scintillation counter. Nitrite protection against iodoacetate incorporation was effected by suspending the germinated spores in 2% tryptone which contained various concentrations of nitrite and incubating them for 3 min before adding the radioactive iodoacetate.

RESULTS

Figure 1 shows inhibition profiles in which nitrite was used to inhibit outgrowing spores under various pH conditions. The curves are highly sigmoid, with cooperativity indices of 5, 7, and 6 for pH 6.8, 6.2, and 5.6, respectively. The cooperativity index, which estimates sigmoidicity, was calculated as the $[S]_{0.9}/[S]_{0.1}$ ratio (11, 17), which in this case was the amount of inhibitor required to give 90% inhibition divided by the amount required to give 10% inhibition. Examination of the nitrite-inhibited spores by phase-contrast microscopy showed that the spores were inhibited (data not shown) at the early outgrowth stage. When the inhibited spores were centrifuged out of the medium and suspended in fresh medium with nitrite, they rapidly recovered, elongated, and proceeded through outgrowth into vegetative growth. Recovery was independent of nitrite exposure time in the 5- to 15-min range used. Calculation of the nitrous acid concentration (pK_a = 3.4) for the samples with 50% inhibition gave a constant value of 30 to 40 μM (0.002 to 0.003 mg/ml) (Fig. 1). Even at pH 5.6, the lowest pH tested, only 0.6% of the nitrite was in its protonated form. The pH-independent constant value implicates a protonated species in the inhibitory mechanism. This is consistent with nitrous acid as the active component (as was proposed many years ago [14]), with nitrite itself acting as a reservoir to replenish nitrous acid as it is consumed by various reactions. The cooperativity index, morphological stage at which inhibition occurs, and rapid reversibility were comparable to those observed with the *S*-nitrosothiols (5, 8–11).

We then determined whether nitrite inactivates membrane sulfhydryl groups in germinated spores, as seen by interference with the ability to incorporate radioactive iodoacetate. Figure 2 shows double-reciprocal plots of inhibition data obtained at different pH values. These plots are comparable to those obtained for nitrosothiols in that they are linear and the lines intersect at a point in the upper left quadrant (8, 11). Moreover, interference with iodoacetate incorporation at each pH occurred as if nitrous acid were the interfering component. Accordingly, nitrite at 7 mM and pH 5.6 showed the same slope as that at 26 mM and pH 6.2 and at 85 mM and pH 6.8. These were the concentrations that gave 50% inhibition at each pH (Fig. 1). Interference with iodoacetate incorporation therefore consistently reflected the amount of nitrous acid present and occurred at the same concentration of nitrous acid that inhibited outgrowth. Since our earlier work established that essentially all of the iodoacetate incorporation into outgrowing spores is directed to membrane groups (8, 10, 11), we conclude that nitrite interference with iodoacetate incorporation reflects its ability to protect membrane sulfhydryl groups against iodoacetate uptake.

An even better test of sulfhydryl group protection by nitrite is determination of its ability to protect against iodoacetate-induced viability loss. That iodoacetate should kill outgrowing bacterial spores is not surprising in view of its characteristics as a general agent for irreversible carboxymethylation of sulfhydryl groups (6). Sulfhydryl groups frequently occur as critical catalytic and structural residues in enzymes and other proteins. Despite this, we observed that reversibly bound nitrosothiols protect against the iodoacetate killing effect (11). Reversibly bound nitrite showed a

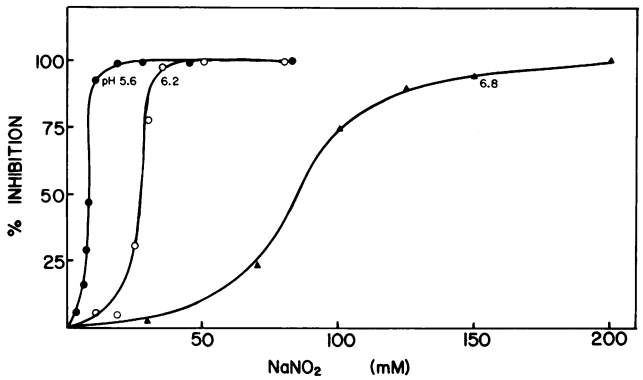

FIG. 1. Effect of pH on inhibitory effectiveness of $NaNO_2$ toward outgrowing *B. cereus* spores. Spores were prepared and heat shocked as described in the text. They were suspended in 5 ml of 1% tryptone to a final concentration of 0.075 mg/ml and allowed to germinate for 18 min (to phase-dark stage) at 35°C. They were then centrifuged and suspended in fresh 1% tryptone, which had been adjusted to pH 5.6 (●), 6.2 (○), or 6.8 (▲) with HCl or NaOH and to which the appropriate amount of $NaNO_2$ had been added. The final pH was checked. Outgrowth was allowed to continue at 35°C on a rotary drum shaker turning at about 20 rpm. The spores were incubated for 3 h, and inhibitory effects were evaluated by phase-contrast microscopy.

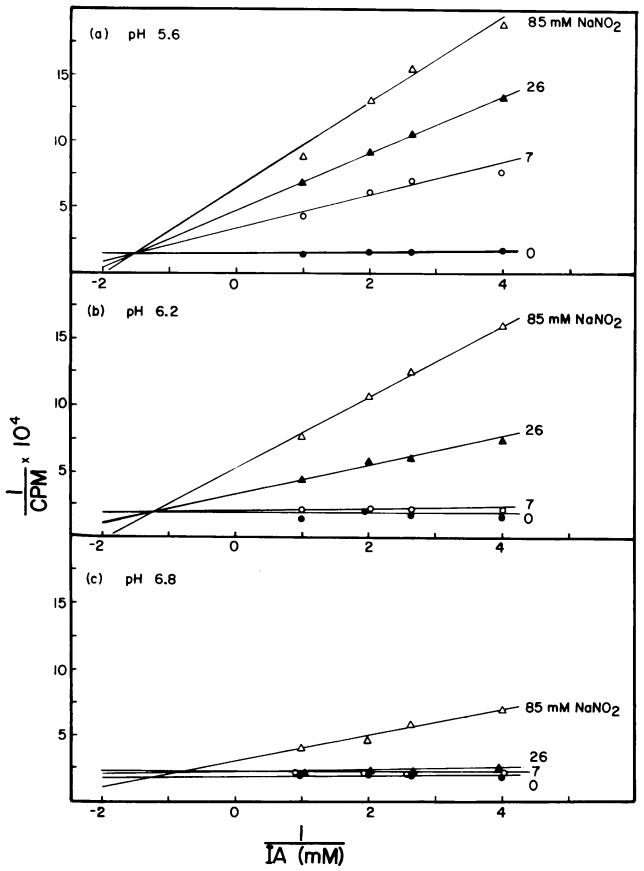

FIG. 2. Inhibition of incorporation of radioabeled iodoacetate (IA) into membrane sulfhydryl groups of germinated spores by $NaNO_2$ at pH 5.6 (a), 6.2 (b), and 6.8 (c). Spore samples were germinated, centrifuged, suspended in 2.5 ml of 2% tryptone to a final concentration of 0.6 mg/ml at the appropriate pH and nitrite concentration as described in the legend to Fig. 1, and incubated for 3 min at 35°C. Tritiated iodoacetate (specific activity, 15 mCi/mmol) was then added, and incubation was continued for 20 min. The reaction was quenched by addition of 20 ml of 1 mM unlabeled iodoacetate; the samples were washed by centrifugation, and the amount of label in the pellet was determined.

similar protective effect (Fig. 3). Addition of nitrite followed by iodoacetate (in excess of the amount to fully and irreversibly inhibit spore outgrowth in the absence of nitrite) to germinated spores fully protected them from the iodoacetate killing effect. Washing to remove nitrite and unbound iodoacetate allowed the entire population of spores to reach the vegetative state. Moreover, protection against iodoacetate killing occurred at the same nitrite concentration that inhibited spore outgrowth (cf. Fig. 1 and 3) and interfered with the uptake of radioactive iodoacetate (Fig. 2). This result provided a convincing link between the nitrite bacteriostatic effect and the ability of nitrite to modify the reactivity of critical membrane sulfhydryl groups.

DISCUSSION

The considerable reactivity of nitrite toward a wide variety of nucleophiles (2) complicates the problem of determining the mechanism of nitrite-induced bacteriostasis. Indeed, it is so reactive that its low toxicity and consequent suitability as a food preservative is surprising. Over a period of several decades, workers in many laboratories have proposed mechanisms of nitrite action based on observations of nitrite-induced cellular modifications. Nitrite can modify cytochromes and other heme proteins (15), inactivate certain enzymes associated with energy metabolism (12, 21), and modify iron-sulfur centers (13). It interferes with oxidative phosphorylation (3). It can affect membrane permeability, causing proton gradients to collapse (7). There is no question that nitrite is a reactive molecule which can modify many cellular components. It is less clear which modification(s) actually contributes to the preservative action of nitrite. Many observations were made with nitrite levels vastly greater than that required to exert a bacteriostatic effect. The results presented here clearly establish that membrane sulfhydryl modification accompanies nitrite inhibition and that these sulfhydryl groups are critical for cell viability. Most importantly, the results correlate sulfhydryl modification with the actual inhibitory event. The facile reversibility of nitrite inhibition argues against extensive modifications of critical intracellular proteins and enzyme systems, since this would be a burden that the cell could not rapidly discard or repair. Moreover, it was observed that nitrite-preserved foods contain viable outgrowth-inhibited spores (1) that can develop normally once they are removed from their inhibitory environment. This is not consistent with the occurrence of extensive intracellular damage, even over long periods.

Although the specific function of the membrane sulfhydryl groups has not been identified, our earlier studies with nitrite analogs and sulfhydryl-specific modifying agents suggested that their modification interferes with transport and that they are components of, or are in communication with, membrane permeases (11). Workers in several laboratories observed that nitrite interferes with active transport (22). Cohn et al. used membrane vesicles of *Escherichia coli* to demonstrate that sulfhydryl-directed agents inactivate active transport systems and that the reactivity of the sensitive sulfhydryl group depends on the conformation of the sulfhydryl environment (4). We observed that the sulfhydryl groups

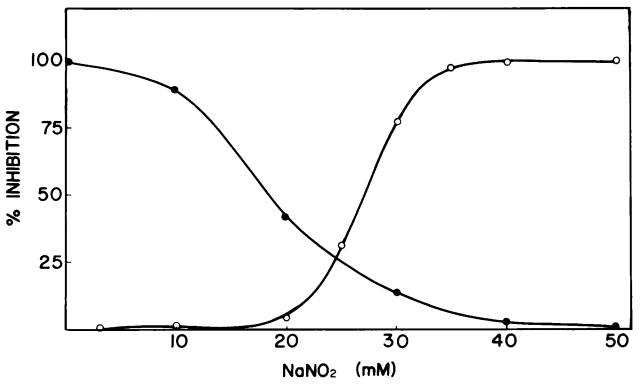

FIG. 3. Nitrite protection against the bacteriocidal effect of iodoacetate. Spore samples were germinated and centrifuged as in the legend to Fig. 1. They were suspended at a spore concentration of 0.075 mg/ml in tryptone (pH 6.2) that contained various concentrations of nitrite and incubated for 5 min at 35°C. This was followed by addition of 0.3 mM iodoacetate and incubation for an additional 15 min. The spores were then washed by centrifugation, suspended in fresh tryptone (pH 6.2), and outgrown for 3 h at 35°C. Symbols: ●, inhibition determined by phase-contrast microscopy as described in the legend to Fig. 1; ○, control experiment showing nitrite-induced inhibition in the absence of iodoacetate and without a wash to remove reversibly bound nitrite.

modified by nitrite are the specific target of nisin, a natural peptide antibiotic (11). Both the results presented in this paper and the successful use of nitrite as a food preservative suggest that the sulfhydryl groups of bacterial membranes constitute an antibiotic target about which a thorough understanding would be useful. We hope to exploit this understanding to aid in the rational design of new antibacterial agents. Whether these agents, which are directed toward membrane sulfhydryl targets in an aerobic *Bacillus* sporeformer, will prove to be effective against anaerobic sporeformers such as *Clostridium botulinum* is not yet known. It will depend upon whether the membrane sulfhydryl groups of aerobic and anaerobic sporeformers have similar functions and chemical environments.

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LITERATURE CITED

1. **Benedict, R. C** 1980. Biochemical basis for nitrite inhibition of *Clostridium botulinum* in cured meat. J. Food Prot. **43**:877–891.
2. **Cassens, R. G., M. L. Greaser, T. Ito, and M. Lee.** 1979. Reactions of nitrite in meat. Food Technol. **33**:46–57.
3. **Chea, K. S.** 1974. Effect of nitrite on respiration and oxidative phosphorylation. Int. J. Biochem. **5**:349–352.
4. **Cohn, D. E., G. J. Kaczorow, and H. R. Kaback.** 1981. Effect of the proton electrochemical gradient on maleimide inactivation of active transport in *Escherichia coli* membrane vesicles. Biochemistry **20**:3308–3313.
5. **Hansen, J. N., and R. A. Levin.** 1975. Effect of some inhibitors derived from nitrite on macromolecular synthesis in *Bacillus cereus*. Appl. Microbiol. **30**:862–869.
6. **Means, G. E., and R. E. Feeney.** 1971. Chemical modifications of proteins, p. 105–138. Holden-Day, Inc., San Francisco.
7. **Meijer, E. M., J. W. van der Zwan, R. Wever, and A. H. Stouthamer.** 1979. Anaerobic respiration and energy conversion in *Paracoccus denitrificans*. Eur. J. Biochem. **96**:69–76.
8. **Morris, S. L., and J. N. Hansen.** 1981. Inhibition of *Bacillus cereus* spore outgrowth by covalent modification of a sulfhydryl group by nitrosothiol and iodoacetate. J. Bacteriol. **148**:465–471.
9. **Morris, S. L., and J. N. Hansen.** 1981. Interaction of nitrosothiol and iodoacetate with membrane sulfhydryls of outgrowing *Bacillus cereus* spores, p. 269–272. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
10. **Morris, S. L., R. A. Levin, C. Wright-Wilson, and J. N. Hansen.** 1978. Effect of *S*-nitrosothiol structure on inhibition of germination and outgrowth of *Bacillus cereus* spores, p. 85–89. *In* G. Chambliss and J. C. Vary (ed.), Spores VII. American Society for Microbiology, Washington, D.C.
11. **Morris, S. L., R. C. Walsh, and J. N. Hansen.** 1984. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. J. Biol. Chem. **259**:13590–13594.
12. **O'Leary, V., and M. Solberg.** 1976. Effect of sodium nitrite inhibition on intracellular thiol groups and on the activity of certain glycolytic enzymes in *Clostridium perfringens*. Appl. Environ. Microbiol. **31**:208–212.
13. **Reddy, D., J. R. Lancaster, and D. P. Cornforth.** 1983. Nitrite inhibition of *Clostridium botulinum*: electron spin resonance detection of iron-nitric oxide complexes. Science **221**:769–770.
14. **Roberts, T. A., and M. Ingram.** 1966. The effect of sodium chloride, potassium nitrate and sodium nitrite on the recovery of heated bacterial spores. J. Food Technol. **1**:147–163.
15. **Rowe, J. J., J. M. Yarbrough, J. B. Rake, and R. G. Eagon.** 1979. Nitrite inhibition of aerobic bacteria. Curr. Microbiol. **2**:51–54.
16. **Sebranek, J. G., R. G. Cassens, W. G. Hoekstra, W. C. Winder, E. V. Podebradsky, and E. W. Kielsmeier.** 1973. N-15 tracer studies of nitrite added to a comminuted meat product. J. Food Sci. **38**:1220–1222.
17. **Segal, I. H.** 1975. Enzyme kinetics, p. 170–198. John Wiley & Sons, Inc., New York.
18. **Sofos, J. N., and F. F. Busta.** 1980. Alternatives to the use of nitrite as an anti-botulinal agent. Food Technol. **34**:244–251.
19. **Tarr, H. L. A.** 1941. Bacteriostatic action of nitrates. Nature (London) **147**:417–418.
20. **Vary, J. C., and H. O. Halvorson.** 1965. Kinetics of germination of *Bacillus* spores. J. Bacteriol. **89**:1340–1347.
21. **Woods, L. F. J., and J. M. Wood.** 1982. A note on the effect of nitrite inhibition on the metabolism of *Clostridium botulinum*. J. Appl. Bacteriol. **52**:109–110.
22. **Yarbrough, J. M., J. B. Rake, and R. G. Eagon.** 1980. Bacterial inhibitory effects of nitrite: inhibition of active transport, but not of group translocation, and of intracellular enzymes. Appl. Environ. Microbiol. **39**:831–834.