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Nitrite-induced Germination of Putrefactive Anaerobe 3679h Spores[1]

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Sodium nitrite alone has been shown to stimulate germination of PA 3679h spores. The process was accelerated by using increased concentrations of sodium nitrite, a low *p*H, and a high temperature of incubation. At low concentrations of nitrite (0.01 to 0.2%), the delay of 36 to 48 hr occurred before germination commenced at 37 C. However, with 3.45% nitrite at 45 C and *p*H 6.0, most of the spores germinated within 1 hr. At *p*H 7.0, the germination rate decreased markedly, and at *p*H 8.0 it was nil. The greatest acceleration in germination rate occurred near 60 C. Hydroxylamine was completely inhibitory to nitrite-induced germination. Sodium nitrite, in turn, inhibited germination by L-alanine, the degree of inhibition being influenced by nitrite concentration and *p*H.

Several ways have been found to induce germination of bacterial spores. In addition to the "physiological germinants," such as amino acids and carbohydrates, there have also been reports of "ionic germination" (5, 8). Hyatt and Levinson (5) were able to induce germination of *Bacillus megaterium* spores with either KNO_3 or KNO_2, but only if the spores were first subjected to a sublethal heat treatment of 10 min at 60 C. Likewise, Black (Bacteriol. Proc., p. 36, 1964) observed germination of *B. cereus* strain *terminalis* spores in 0.5 to 2.0 M $NaNO_2$ or KNO_2.

Studies of microcultures by Duncan and Foster (3) indicated that sodium nitrite, even in high concentration, would not prevent loss of refractility and swelling of putrefactive anaerobe 3679h (PA 3679h) spores, although very small amounts of the compound were able to inhibit division of newly emerged cells. These observations prompted an investigation of the effect of sodium nitrite on germination of PA 3679h spores.

MATERIALS AND METHODS

Spores of PA 3679h were prepared as described elsewhere (2). Reaction mixtures in new screw-capped tubes (16 × 125 mm) were adjusted to the proper temperature in a thermostatically controlled water bath; then 1.2×10^8 to 1.5×10^8 spores were added per ml. Germination was followed by measuring the decrease in optical density (expressed as change in OD) at 600 mμ with a Bausch & Lomb Spectronic-20 colorimeter. Germination was also validated by phase contrast microscopy and by staining with dilute methylene blue.

All reagents were prepared as concentrated stock solutions in deionized water and diluted as necessary to give a final reaction mixture volume of 6 ml. Fresh solutions of sodium nitrite were prepared before each experiment.

For some experiments, the germination rate of the spores was expressed as the change in OD per minute × 100, as measured over a 10-min period during the first 10 to 25 min of germination.

RESULTS

Increasing the concentration of sodium nitrite from 0.03 to 0.2% dramatically accelerated germination at *p*H 6.0 (Fig. 1). Essentially no germination occurred at *p*H 6.0 in 1 week without nitrite or at *p*H 7.0 with as much as 0.2% nitrite.

The 36-hr lag before germination commenced was eliminated by increasing the nitrite concentration to 0.5 M (3.45%) and the temperature to 45 C. Figure 2 shows the rapid germination at *p*H 6.0 under these conditions in contrast to a much slower rate at *p*H 7.0 and practically none at *p*H 8.0.

Figure 3 shows the effect of temperature on nitrite-induced germination. The greatest acceleration in germination rate occurred in the vicinity of 60 C. These results are in sharp contrast to the 45 C optimum reported by Uehara

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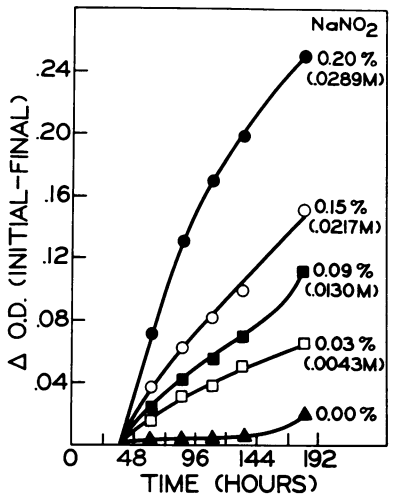

FIG. 1. *Effect of sodium nitrite on germination of PA 3679h spores. Spores were incubated at 37 C in 0.01 M phosphate buffer, pH 6.0, plus added nitrite. No germination was observed at pH 7.0 under the same conditions.*

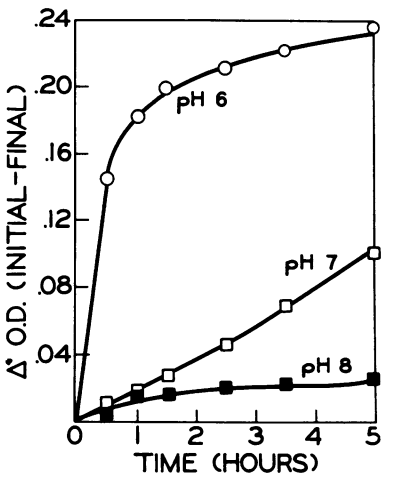

FIG. 2. *Effect of pH on germination of PA 3679h spores by sodium nitrite. Spores were incubated at 45 C in 0.01 M phosphate buffer plus 0.5 M (3.45%) sodium nitrite at the indicated pH values.*

and Frank (10) for the L-alanine germination system with spores of this same organism.

A sublethal "heat shock" is necessary to induce optimal germination in some bacterial spores. To see whether the temperature effect in Fig. 3 was the result of "heat activation," spores and sodium nitrite were heated separately and together, as shown in Table 1. Heating the spores alone (treatment 1), the nitrite alone (treatment 2), or both separately (treatment 3) gave about the same germination rates as no heating (treatment 4). Only when the spores and nitrite were heated together (treatment 5) was the germination rate increased appreciably. Thus, it must be concluded that the effect of increased temperature shown in Fig. 3 was not the result of "heat activation" of the spores or of heat-induced alteration of the nitrite ion, but rather was the result of greater interaction between nitrite and the spores.

Various compounds that have been shown to inhibit germination of bacterial spores were tested for their effect on nitrite-induced germination of PA 3679h (Table 2). Hydroxylamine was markedly inhibitory, as were tris(hydroxymethyl)aminomethane buffer (a known sequestering agent of metal ions), sodium azide, potassium cyanide, and hydrogen peroxide. All other com-

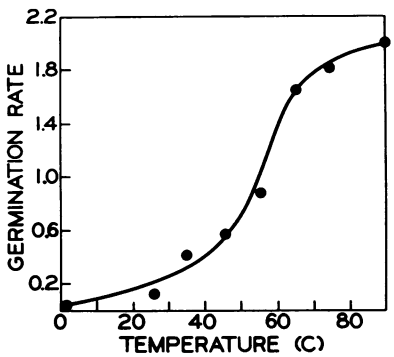

FIG. 3. *Effect of temperature on rate of PA 3679h spore germination by sodium nitrite. Final concentration of sodium nitrite was 0.5 M in 0.01 M phosphate buffer, pH 6.0.*

TABLE 1. *Effect of heating components of germination mixture on the germination rate of PA 3679h spores*[a]

Treatment	Germination rate
1. Spores heated at 65 C for 30 min in 0.01 M phosphate buffer; then removed and suspended in germination mixture at 35 C	0.33
2. Germination mixture heated at 65 C for 30 min; then cooled to 35 C and spores added	0.32
3. Spores and germination mixture heated separately at 65 C for 30 min; then cooled to 35 C and mixed	0.34
4. Unheated spores in unheated germination mixture at 35 C	0.34
5. Spores incubated in germination mixture at 65 C	1.65

[a] Germination mixture: 0.5 M sodium nitrite in 0.01 M phosphate buffer, pH 6.0.

TABLE 2. *Effect of various compounds on the initial rate of sodium nitrite-induced germination of PA 3679h spores*[a]

Test compound	Final concn	Germination rate	Change in germination rate[b]
			%
Control (phosphate buffered, 0.01 M)	—	.56	
Control (Tris-buffered, 0.01 M)	—	.04	−93
Hydroxylamine-HCl	500.0 mM	.00	−100
NaN_3	10.0 mM	.01	−98
KCN	10.0 mM	.17	−70
H_2O_2	2.0%	.23	−59
Glycine	10.0 mM	.56	0
$MnCl_2$	1.0 mM	.83	+48
8-Hydroxyquinoline	1.0 mM	.63	+13
$HgCl_2$	1.0 mM	.62	+11
2,4-Dinitrophenol	5.0 mM	.70	+25
$NaNO_3$	58.8 mM	.59	+5
$NaNO_3$	117.6 mM	.59	+5
NaCl	85.6 mM	.69	+23
NaCl	342.4 mM	.70	+25

[a] Germination mixture: 0.5 M sodium nitrite in 0.01 M phosphate buffer, *p*H 6.0, 45 C.
[b] Based on phosphate-buffered control.

pounds tested either had no effect or were slightly stimulatory. Except with hydroxylamine, the decreased germination rates in Table 2 were eventually overcome, and the final levels of germination were equivalent to those of the phosphate-buffered control. With hydroxylamine, however, there was no germination at all within 10 hr.

"Competitive" inhibition caused by increasing concentrations of hydroxylamine is shown in Fig. 4. This compound is known to inhibit bacterial growth (6). To see whether it prevented germination by reacting with some component of the spore, PA 3679h spores were suspended in 0.5 M hydroxylamine in 0.01 M phosphate buffer, *p*H 6.0, and incubated for 20 hr at 30 C. Then the spores were washed five times with cold deionized water and suspended in 0.5 M sodium nitrite in 0.01 M phosphate buffer, *p*H 6.0. The germination rate at 45 C was approximately the same as that with spores which had not been treated with hydroxylamine. Therefore, it would appear that the mechanism of inhibition by hydroxylamine is not that of a prior combination with some component of the spores.

Uehara and Frank (10) described a germination system for spores of PA 3679h consisting of L-alanine and sodium pyrophosphate. Germination was most rapid at *p*H 8.5 and 45 C; it was practically nil at *p*H 6.5 and below, or at temperatures above 55 C. Figure 5 shows the alkaline *p*H optimum of the L-alanine germination system in contrast to the acidic optimum of the nitrite system (Fig. 2).

Nitrite inhibited germination by L-alanine (Fig. 6). At *p*H 8.0, which is near the optimum

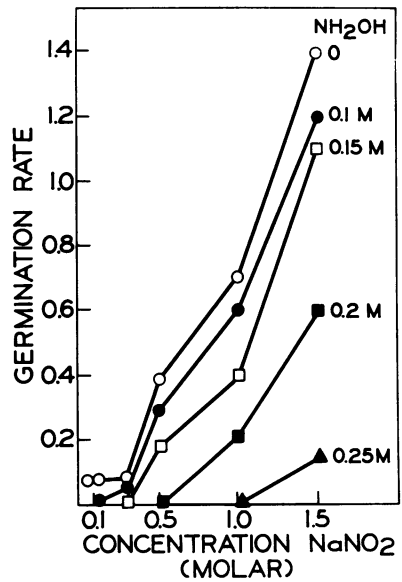

FIG. 4. *Effect of hydroxylamine on germination of PA 3679h spores by sodium nitrite. Spores were incubated at 45 C in 0.01 M phosphate buffer, pH 6.0, with added sodium nitrite and hydroxylamine hydrochloride.*

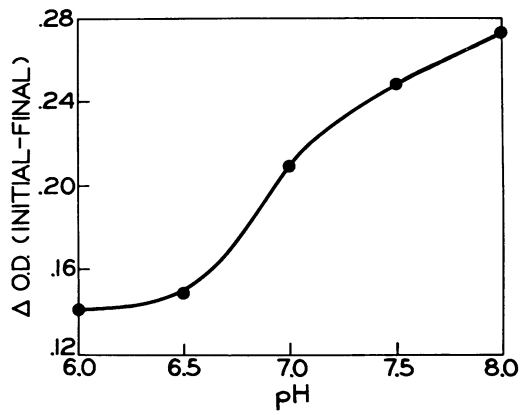

FIG. 5. *Effect of pH on germination of PA 3679h spores by L-alanine and sodium pyrophosphate. The germination mixture contained: L-alanine, 60 mM; sodium pyrophosphate, 35 mM; sodium thioglycolate, 8.8 mM; and phosphate buffer, 0.01 M; adjusted to the appropriate pH. The total change in optical density was measured after 9 hr at 45 C.*

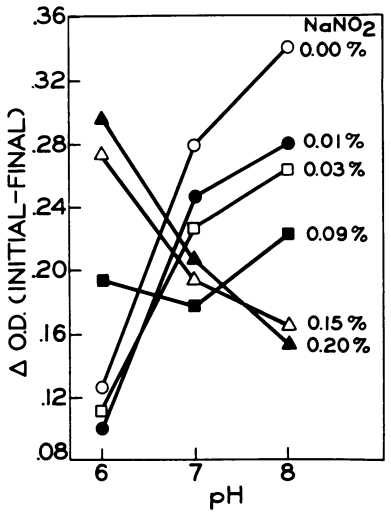

FIG. 6. *Effect of sodium nitrite on* L-*alanine-sodium pyrophosphate induced germination of PA 3679h spores. The germination mixture contained:* L-*alanine, 60 mM; sodium pyrophosphate, 35 mM; sodium thioglycolate, 8.8 mM; and phosphate buffer, 0.01 M; adjusted to pH 6, 7, or 8. Sodium nitrite was added to give the final concentrations shown. The total change in optical density was measured after 36 hr at 45 C. There was no change in optical density of control spore suspensions in 0.01 M phosphate buffer at the respective pH values.*

for the L-alanine system, increasing additions of nitrite decreased germination. However, at *p*H 6.0 which favors nitrite-induced germination, additional nitrite stimulated germination.

Inhibition of the L-alanine system by small amounts of nitrite is contrary to expectation. Fleming and Ordal (4) reported a synergistic effect of L-alanine and inorganic ions on germination of *B. subtilis* spores. They observed negligible germination in L-alanine at low ionic strength, but the rate accelerated as the ionic strength was increased up to about 0.1 M. This was true with a variety of salts, especially monovalent ones. However, with the system reported here, even as little as 0.01% (0.0014 M) sodium nitrite caused inhibition of L-alanine-induced germination.

DISCUSSION

The role of ions in germination of bacterial spores has been given little consideration, and most of the work reported previously has been done with aerobic sporeformers. The results reported here have shown that sodium nitrite is able to induce germination of spores of PA 3679h. This process was affected by the concentration of nitrite, the *p*H, and the temperature of incubation. The relatively low *p*H necessary for optimal activity indicates that undissociated nitrous acid probably is the effective compound.

The lag period of 36 to 48 hr at 37 C before commencement of germination in low concentrations of nitrite could be a reflection of the temperature of incubation. Increasing the temperature from 4 to 90 C resulted in progressively greater rates of germination (Fig. 3), with most rapid acceleration between 50 and 70 C. Black (Bacteriol. Proc., p. 36, 1964) also found nitrite germination of *B. cereus* spores to be accelerated with increasing temperatures above 43 C.

In contrast to our results, Black observed no germination within 60 min; then virtually all of the spores germinated in the next 0.5 hr. Other workers have found a heat shock to be necessary for ionic stimulation of germination, but, in our experience with nitrite, germination commenced immediately and without the necessity of prior heat shock.

It is possible that previous workers' failure to observe germination with ions alone was due to the incubation temperatures employed, usually 30 to 45 C which is well within the growth ranges of the organisms. In the nitrite system reported here, germination was fastest at temperatures far above the growth range of the organism.

Of the substances tested, only hydroxylamine was completely inhibitory to nitrite-induced germination. Results indicated that this inhibition was not caused by a prior reaction of hydroxylamine with some specific component of the spore, since it was effective only when present in conjunction with nitrite. The possibility of a chemical reaction between hydroxylamine hydrochloride (the actual compound used) and sodium nitrite should not be overlooked. It is known (6) that these two compounds may react to yield N_2O, NaCl, and water. However, this reaction is stoichiometric, and yet only 0.25 M hydroxylamine was required to effect complete inhibition of germination by 1.0 M sodium nitrite. It would appear that another reaction, presumably involving some spore structure, does occur. The effect of hydroxylamine may be exerted only after the initial reaction of nitrite with the spore.

The fact that sodium nitrite, even in low concentrations, inhibits germination by L-alanine is not in agreement with reports of a synergistic action between various ions and alanine. The lack of enhancement could be explained by the differences in *p*H optima of the two systems: at *p*H 8.0 nitrite is not an effective germinant, but at *p*H 6.0 it is active. However, this does not explain why very small amounts of nitrite inhibited the L-alanine germination system.

Various suggestions have been put forth to

explain the mechanism by which ions stimulate germination of spores. Alderton and Snell (1) suggested an indirect effect of ions on metabolic activity which might involve a volume change in the spore structure to a point of core hydration. Metabolic activity could begin after hydration.

In view of the variety of ions reported to be effective in germination, any role as an enzyme activator would seem to be nonspecific in nature. Also, the concentration of ions involved in germination is normally higher than that usually considered necessary for enzyme activation.

Rode and Foster (9) suggested that a number of germinants, viz., inorganic and organic ions, chelating agents, surfacants, and hydrogen ions, induce germination by altering the conformational structure of proteins and perhaps other macromolecules responsible for the dormant spore state.

Riemann (7) assumed a spore structure-Ca-dipicolinic acid or Enzyme-Ca-dipicolinic acid complex to be associated with dormancy in spores. He suggested that this complex could be disrupted by the chelation of calcium with agents such as ethylenediaminetetraacetic acid, dipicolinic acid, and amino acids, thus leading to germination.

Fleming and Ordal (4) suggested that Riemann's mechanism for germination could be extended to include germination with both monovalent and multivalent ions, since Fleming (Ph.D. Thesis, Univ. of Illinois, Urbana, 1963) had previously shown that increased ionic strength could favor dissociation of the chelated structure.

Black (Bacteriol. Proc., p. 36, 1964) suggested that germination of *B. cereus* spores by sodium nitrite was effected by reduction, with nitrite serving as an electron donor, and possibly mediated by the activity of spore catalase. If this is correct, it would appear that the action of nitrite is indeed nonspecific, since it also induced germination of PA 3679h spores, which presumably are devoid of catalase.

If nitrite does act by reduction, it could conceivably result in the alteration of the tertiary structure of a spore protein, which in turn may be involved in the calcium-dipicolinic acid complex. The increase in germination rate with increasing temperature and increasing nitrite concentration may be a reflection of such structural alteration.

ACKNOWLEDGMENT

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