Growth from Spores of Clostridium perfringens in the Presence of Sodium Nitrite

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The method by which sodium nitrite may act to prevent germination or outgrowth, or both, of heat-injured spores in canned cured meats was investigated by using Clostridium perfringens spores. Four possible mechanisms were tested: (i) prevention of germination of the heat-injured spores, (ii) prior combination with a component in a complex medium to prevent germination of heat-injured spores, (iii) inhibition of outgrowth of heat-injured spores, and (iv) induction of germination (which would render the spore susceptible to thermal inactivation). Only the third mechanism was effective with the entire spore population when levels of sodium nitrite commercially acceptable in canned cured meats were used. Concentrations of 0.02 and 0.01% prevented outgrowth of heat-sensitive and heat-resistant spores, respectively. Nitrite-induced germination occurred with higher sodium nitrite concentrations.

Although sodium nitrite plays an important role in the preservation of canned cured meats, the actual mechanism by which it prevents spoilage is obscure. It is believed that the stability of these meat products is due to the ability of the curing salts, sodium nitrite and sodium chloride, to block some stage in the outgrowth or germination, or both, of the indigenous bacterial spores which have been heat-injured by the brief thermal processing that these canned meats undergo. The mild thermal processing presumably renders the spores more sensitive to the curing salts; however, the transitional stages of spore germination or outgrowth, or both, at which sensitization occurs remain unclear.

Sodium nitrite, when heated in a laboratory medium, forms a bacterial inhibitor which has been found to be highly effective in preventing the growth of several clostridial species (11, 12). However, recent evidence indicates that such an inhibitory product may not be active in cured meat products (9).

It is known that commercially acceptable concentrations of sodium nitrite may interfere with the growth of bacterial cells (2). Another postulated role of sodium nitrite in the preservative system of cured meats may be its ability to induce spore germination, thus making the spores susceptible to subsequent heat processing (4). Indeed, nitrite-induced germination has been shown to occur with putrefactive anaerobe 3679h (5), Clostridium butyricum (1), and Bacillus cereus (S. H. Black, Bacterial Proc., p. 36, 1964).

The present study was initiated to determine whether the inhibition of microbial growth from spores by commercially acceptable levels of nitrite (0.02% maximum residual nitrite) occurs at both the level of germination and outgrowth.

MATERIALS AND METHODS
Preparation of spore suspensions. Spores of C. perfringens strain FD1, obtained from the Food and Drug Administration, and C. perfringens NCTC 8798 (Hobbs type 9) were used. Strain FD1 produces heat-sensitive spores that require no prior heat shock for germination, and strain NCTC 8798 produces heat-resistant spores that require heat shock for optimal germination (Labbe and Duncan, unpublished data).

The organisms initially were grown in 15 ml of Fluid Thioglycollate Medium (BBL); successive 10% inocula were made at 16-hr intervals until a 1,500-ml culture was obtained. The latter culture was inoculated into 15 liters of D-S sporulation medium (6). All incubations were at 37 C. When the number of free spores reached a maximum (about 15 hr for FD1 and 20 hr for NCTC 8798), the culture was harvested by continuous-flow centrifugation. The spores were suspended in distilled deionized water and were subjected to ultrasonic treatment to remove spores still in their sporangia. Subsequently, the spores were washed several times with cold deionized water until a suspension of clean spores was obtained. During

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cleaning and storage in deionized water, about 3 to 4% of the spores became phase dark. A stock spore suspension adjusted to an optical density (OD) of about 325 Klett units was prepared, and the remainder of the spores was lyophilized.

Assessment of initiation of germination and outgrowth. Initiation of germination was measured as a decrease in OD at 660 nm by using a Bausch & Lomb Spectronic-20 colorimeter; initiation was confirmed by determining loss of spore-phase brightness by using a Zeiss phase-contrast microscope.

Some of the data are presented as per cent germination with the decrease in OD of the positive control taken as 100% germination.

Spore outgrowth was determined by the increase in OD that occurred subsequent to an initial decrease in OD due to initiation of germination. Outgrowth was confirmed by phase-contrast microscopy.

In studying the inhibitory effect of sodium nitrite upon germination and outgrowth, a complex medium was used which contained a final concentration of 2.5% Brain Heart Infusion (Difco) plus 0.27% added yeast extract. The pH was adjusted to give a final pH of 7.0 or 6.0. In this medium, outgrowth of non-heat-injured spores began after 15 to 20 min. Reagents for the nitrite-induced germination experiments were prepared as concentrated stock solutions in deionized water and diluted as necessary. A final reaction mixture volume of 6 ml was used. Fresh stock solutions of sodium nitrite were prepared before each experiment.

Strain FD1 spores initiated germination and grew without prior heat shock; strain NCTC 8798 spores were heated at 70°C for 15 min to initiate germination. In all germination and outgrowth studies, the reaction mixtures, contained in screw-cap tubes (16 by 125 mm), were preheated to the appropriate temperature in a thermostatically controlled water bath; spores were then added to a final concentration of about 3 × 10⁷/ml.

Preparation of heat-injured spores. Heat-injured spores were prepared by heating 5 ml of a stock spore suspension containing about 2 × 10⁷ spores/ml in a screw-cap tube (16 by 125 mm); the spores were heated either at 90°C for 5 min in the case of FD1 or at 100°C for 18 min in the case of strain NCTC 8798. These times and temperatures resulted in an approximately 0.33 D value (time in minutes for inactivation of 90% of the spores) for spores of both strains.

Viable counts of heat-injured spores were made by means of pour plates by using TSN agar (10). Incubation was at 37°C for 24 hr (strain FD1) or 48 hr (strain NCTC 8798) under a gas mixture of 90% nitrogen and 10% carbon dioxide.

RESULTS

Four mechanisms for possible inhibition of microbial growth from spores by sodium nitrite were investigated. These included (i) prevention of germination of heat-injured spores in a complex medium, (ii) prevention of germination of heat-injured spores in a complex medium by an inhibitory product formed by prior combination of sodium nitrite with a component in the medium during autoclaving, (iii) inhibition of outgrowth of heat-injured spores, and (iv) induction of germination (which would render the spore susceptible to thermal inactivation).

Inhibition of germination. The effect of adding sodium nitrite to a complex medium before or after autoclaving (15 min at 121°C) on the germination of heat-injured or non-heat-injured FD1 spores is shown in Fig. 1. At pH 7 and at sodium nitrite concentrations less than about 0.3%, heat-injured spores were more susceptible to inhibition of germination by nitrite regardless of whether nitrite was added before or after autoclaving. Addition of sodium nitrite before autoclaving the medium did not reduce the per cent germination of heat-injured spores, as compared to the germination obtained when nitrite was added after autoclaving. However, germination of non-heat-injured spores was reduced when nitrite was added before autoclaving. At pH 6, the results were nearly the reverse of those obtained at pH 7.0. At
Effects of sodium nitrite on germination are probable due primarily to species differences. Considering the maximum residual sodium nitrite concentration of 0.02% that is actually permissible in practice, it is unlikely that either sodium nitrite or an inhibitory product formed by heating nitrite with a complex medium contributes to the preservation of canned cured meat by blocking germination of all contaminating spores. The possibility that nitrite may block germination of a fraction of the heat-injured spore population is discussed later.

Inhibition of outgrowth. The effect of nitrite upon outgrowth of unheated *C. perfringens* spores is shown in Fig. 2. At pH 6, 0.04% sodium nitrite was required to inhibit outgrowth of unheated strain FDI spores. Ten times that amount was required at pH 7. Also at pH 7, a concentration of 0.4% nitrite decreased the rate of germination.

Heat-injured FDI spores were much more sensitive to nitrite than non-heat-injured spores, with a concentration of 0.02% preventing outgrowth at pH 6 and 0.1% preventing outgrowth at pH 7 (Fig. 3). Small amounts of sodium nitrite apparently stimulated outgrowth of heated spores.

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**Fig. 2. Effect of sodium nitrite on outgrowth of non-heat-injured *Clostridium perfringens* strain FDI spores.** Spores were incubated at 45°C in medium consisting of 2.5% Brain Heart Infusion and 0.27% yeast extract. Sodium nitrite was added to the sterile medium to give the concentrations indicated in the figure.

Sodium nitrite concentrations of 0.2% or more, the percent germination of heat-injured spores was greater than that of non-heat-injured spores regardless of whether nitrite was added before or after autoclaving. The addition of sodium nitrite to the medium before autoclaving markedly reduced the germination of non-heat-injured spores at pH 6.0 as compared to the germination obtained when nitrite was added after autoclaving the medium. At concentrations greater than 0.3%, the germination of heat-injured spores was only slightly reduced when nitrite was added before autoclaving. Heat-injured or non-heat-injured NCTC 8798 spores were not prevented from germinating when sodium nitrite concentrations as high as 2% were added to the complex media after autoclaving.

Duncan and Foster (3) observed that even as much as 4% sodium nitrite does not prevent loss of refractivity of PA 3679b spores in liver-veal-agar. Gould (7), on the other hand, noted inhibition of several *Bacillus* species in yeast-glucose-agar by 0.075 to 0.25% nitrite. The various effects of sodium nitrite on germination are probably due primarily to species differences. Considering the maximum residual sodium nitrite concentration of 0.02% that is actually permissible in practice, it is unlikely that either sodium nitrite or an inhibitory product formed by heating nitrite with a complex medium contributes to the preservation of canned cured meat by blocking germination of all contaminating spores. The possibility that nitrite may block germination of a fraction of the heat-injured spore population is discussed later.

**Fig. 3. Effect of sodium nitrite on the outgrowth of heat-injured *Clostridium perfringens* strain FDI spores.** Incubation conditions were the same as those described in Fig. 2, except for the concentrations of sodium nitrite used.
at pH 7, since the OD at concentrations of 0.01% was greater after 150 min than that of the control.

Similar experiments were conducted with NCTC 8798, a heat-resistant strain. This strain was more sensitive to nitrite than was FD1; concentrations of 0.02 and 0.3% were required for inhibition of outgrowth of non-heat-injured spores at pH 6 and 7, respectively (Fig. 4). As with strain FD1, heat-injured spores were more susceptible to inhibition of outgrowth by sodium nitrite both at pH 6 (0.01%) and pH 7 (0.04%; Fig. 5).

Although commercial heat treatments employed in the processing of canned cured meats may destroy heat-sensitive spores, it is unlikely that all spores of resistant strains are so affected. That the latter may be more sensitive to inhibition of outgrowth by sodium nitrite is therefore of added importance.

Sodium nitrite-induced Germination. There have been several reports on induction of spore germination by sodium nitrite (1, 5; S. H. Black, Bacterial. Proc., p. 36, 1964). Generally, the temperatures of incubation in these studies were below that to which spores are subjected during the thermal processing of canned cured meats. We have confirmed that nitrite induction of germination does occur with C. perfringens and have investigated the possibility that this may be a factor in preservation of canned cured meats. If commercially acceptable concentrations of nitrite induced germination of the spores in a meat product during processing, the spores would become heat-labile and would therefore be inactivated by the thermal process.

The effect of nitrite concentration on the induction of germination of strain FD1 spores is shown in Fig. 6. There is a limiting concentration (about 1.5%) above which the level of initiation of germination will not increase under the stated conditions. The effects of temperature and pH are shown in Table 1. As the temperature increased, the per cent initiation of germination increased at pH 6. Complete germination occurred in 90 min at low temperatures (25 C) and at pH 6 only if the nitrite concentration was sufficiently high (6%). Essentially no germination occurred at pH 8. Nitrous acid appears to be the effective agent, since the extent of initiation of germination was greatest at pH 6. No germination occurred after 2 hr at pH 6 when the spores were incubated at temperatures of 25, 37, 45, 60, 75, or 90 C with as much as 0.02% sodium nitrite, the maximum
with a phase-dark periphery but a refractile core. It is unlikely that most of these spores remained viable since this strain, FD1, is itself heat-sensitive with a D0 equal to about 15 min.

It may be concluded that, although sodium nitrite may induce bacterial spore germination, concentrations that are commercially acceptable will not.

**DISCUSSION**

The method by which sodium nitrite may act to prevent microbial growth of heat-injured spores in canned cured meats was investigated by using *C. perfringens*. Four possible mechanisms were tested: (i) prevention of germination of heat-injured spores, (ii) prior combination with a component in a complex medium to prevent germination of heat-injured spores, (iii) inhibition of outgrowth of heat-injured spores, (iv) induction of germination (which would render the spore susceptible to subsequent heat treatments).

When sodium nitrite is heated in a complex medium, it combines with some component in the medium to yield an unknown but extremely potent inhibitory agent; e.g., autoclaving 3.5 μg/ml (0.00035%) in a medium for 20 min at 109 C produces enough inhibitor to inhibit 8 x 10⁶ vegetative cells in 50% of the trials (12). This product has been shown to be effective against vegetative cells of several *Clostridium* species (11). However, the present results indicate that, even when using concentrations of sodium nitrite up to 10 times greater than commercially acceptable levels, the inhibitory agent, if indeed produced, is not effective in preventing initiation of germination of most of the intact or heat-injured *C. perfringens* spores. For instance, at pH 6, about 0.5% nitrite was required to reduce the per cent germination of heat-injured strain FD1 spores to 50% of the control, whether sodium nitrite was added before or after autoclaving.

It has been found (9) that the inhibitory agent formed by heating nitrite in a complex medium may have little effect in the preservation of canned cured meats. A meat suspension heated with 150 μg (0.015%) of sodium nitrite per ml was inhibitory to *C. botulinum*; however, the inhibition was thought to be due to residual inorganic nitrite since dialysis removed the inhibitory activity. Furthermore, the inhibitory factor produced by heating nitrite in a complex medium could be inactivated by nonfat meat solids.

The present results show that sodium nitrite at greater than commercially acceptable concentrations does not prevent initiation of germination of the majority of non-heat-injured or heat-injured *C. perfringens* spores, regardless of whether it is added before or after autoclaving the complex

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**Table 1. Effects of temperature and pH on nitrite-induced germination of Clostridium perfringens FD1 spores**

<table>
<thead>
<tr>
<th>Temp and pH</th>
<th>Germination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 C, pH 8</td>
<td>4</td>
</tr>
<tr>
<td>45 C, pH 7</td>
<td>7</td>
</tr>
<tr>
<td>45 C, pH 6</td>
<td>18</td>
</tr>
<tr>
<td>60 C, pH 8</td>
<td>4</td>
</tr>
<tr>
<td>60 C, pH 7</td>
<td>11</td>
</tr>
<tr>
<td>60 C, pH 6</td>
<td>46</td>
</tr>
<tr>
<td>75 C, pH 8</td>
<td>0</td>
</tr>
<tr>
<td>75 C, pH 7</td>
<td>4</td>
</tr>
<tr>
<td>75 C, pH 6</td>
<td>96</td>
</tr>
</tbody>
</table>

*Germination mixtures contained 1% NaNO₃ and 33 mm phosphate buffer. Germination was determined after 1 hr of incubation.*
germination medium. However, a small number of the spore population may actually have been blocked from initiating germination. These blocked spores would not have been detected by the technique used for assessment of spore germination, since extinction measurements are of little value in estimating germination above about 90% (8). It would be difficult to distinguish a low number of nitrite-blocked spores from the "superdormant" spores (8) that may be present in a normal spore population.

The fact that a small number of spores may be found by postprocessing analysis of canned cured meats (13) indicates that either inhibition of the initiation of germination of the heat-injured spores has occurred or that the spores represent a superdormant fraction. Indeed, superdormancy may have been enhanced by heat injury.

Although sodium nitrite alone did not prevent germination of the majority of the spore population, it may have inhibited germination of small numbers of spores. This inhibition should be greater in combination with the curing salt sodium chloride.

Sodium nitrite and sodium chloride are not solely responsible for the preservation of canned cured meats. Heat plays an important role, although it is not sufficient to destroy all bacterial spores. The thermal treatment is thought to injure the spores, making them more sensitive to curing salts in the outgrowth medium (3, 14). This phenomenon was observed in the present studies with heat-damaged C. perfringens spores. At pH 6.0, concentrations of 0.02 and 0.01% sodium nitrite inhibited outgrowth of heat-damaged spores of a heat-sensitive and a heat-resistant strain, respectively. Unheated spores required 0.04 and 0.02%, respectively. It should be pointed out that the incubation temperature was 45°C for 3 hr. It is conceivable that, with additional incubation, inhibition might have been overcome. A temperature of 45°C was used since it is near the optimum for growth of C. perfringens. Roberts and Ingram (14) pointed out that the injured spore may be affected by the temperature prevailing while it is struggling against the inhibitory salts. Undoubtedly, inhibition of outgrowth of germinated spores by sodium nitrite is an important aspect of canned meat preservation, since commercially acceptable concentrations were effective.

Nitrite-induced germination has been observed with several organisms. We confirmed its occurrence with C. perfringens. High temperature, low pH, and high sodium nitrite concentrations yielded optimal germination. Undissociated nitrous acid presumably was the effective agent. Duncan and Foster (4) postulated that the phenomenon of nitrite-induced germination may be involved in the stability of the preservative system. Once germinated by sodium nitrite, the spores would be destroyed by the heat treatment rendered the particular product. This seems unlikely, since, in the present experiments, germination was not obtained after 1 hr at 90°C with as much as 0.02% sodium nitrite, the maximum concentration allowable in commercial use. Whether spores of other organisms would be similarly affected is not known.

An important factor in the safety and stability of canned cured meats which must be mentioned is that of cell numbers (13). In the present experiments, concentrations of about 10^9 spores per ml were used; this is much greater than the actual spore load in meats. Concentrations of sodium nitrite lower than those used here may have been equally effective against germination and outgrowth had we employed a smaller number of spores.

In these experiments no attempt was made to investigate the additive effect that a combination of sodium chloride plus sodium nitrite might have in inhibiting growth from spores. Conceivably, the combined inhibitory effect would be greater than that reported for nitrite alone.

ACKNOWLEDGMENTS

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LITERATURE CITED