Factors Affecting Sensitivity of Staphylococcus aureus 196E to Polyphosphates

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Received 7 March 1986/Accepted 18 June 1986

The effect of polyphosphates (eight compounds) on growth of Staphylococcus aureus 196E in brain heart infusion broth was studied. The organism was sensitive (in decreasing order) to chain polyphosphates with 21, 3, 13, and 15 PO₄ groups, and bactericidal effects were observed with 0.5% of these compounds. No inhibition was effected by PP₆ or a metaphosphate. The inhibitory effects were pH dependent, and bacterial sensitivity was highest at pH > 7.4. Initial populations affected the number of survivors. No growth was observed after 24 h at 35°C when the initial cell population was less than 10⁶ CFU/ml, and a 100- to 1,000-fold decline in cell numbers occurred when initial populations were higher than 10⁸ CFU/ml. Sodium tripolyphosphate produced less inhibition after heat sterilization (15 min, 121°C) than after filter sterilization, whereas sodium hexametaphosphate (n = 21) retained most of its antimicrobial activity after heat sterilization. Supplementation of broth with Mg²⁺ was effective in overcoming inhibition by 0.5% sodium tripolyphosphate, and an addition of 0.25 to 1.0 mM cation restored most of the growth. Inhibition was partially eliminated by Ca²⁺ and Fe²⁺, but not by Zn²⁺ or Mn²⁺.

Various phosphates are added to food products primarily for the purpose of retaining tenderness, retaining moisture, reducing shrinkage during cooking, emulsifying, buffering, and sequestering of cations (2). Because of their highly charged anionic nature, the polyphosphates form complexes with multivalent cations, binding cobalt and copper very strongly and magnesium, calcium, iron, zinc, and manganese to a lesser extent (16, 17). Chelation is influenced by pH, temperature, ionic strength, metal ion content, and composition of the medium. Formation of stable complexes with cations which are essential to normal growth of microorganisms may render them unavailable for metabolic functions and thereby cause growth inhibition.

Despite the potential antimicrobial activities of polyphosphates, there has been relatively little research on the antimicrobial effects and their mode of action. Polyphosphates inhibit pseudomonads in poultry meat (3), retard fungal growth on fresh cherries (11), and inhibit growth of Moraxella and Acinetobacter species in culture media (4). Effective concentrations were within a broad range, depending on compounds and bacterial species. A review of antimicrobial effects of phosphates in foods and culture media was published by Tompkin (15). More recently, antimicrobial effects against five bacterial cultures in agar media were reported (9). In a study with Clostridium botulinum, a delayed toxicity was observed in peptone-yeast extract-glucose broth containing 0.4% sodium acid PP₆ (19).

This study was undertaken to examine and compare the effectiveness of ortho- and polyphosphates as growth inhibitors of Staphylococcus aureus in liquid media. It was further directed at the investigation of the effects of pH, cell populations, heat, and metal ions on inhibition with selected effective compounds.

MATERIALS AND METHODS

Test microorganism. S. aureus 196E, an enterotoxin A and D producer, was maintained on brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) slants. Inocula of the organism used in the tests were from an 18-h culture grown in BHI broth at 35°C.

Test chemicals. Compounds used in this study consisted of sodium salts of chain phosphates in which PO₄ groups are held together through P-O-P linkages, and one ring phosphate. The compounds and their average chain length are given in Table 1. CaCl₂·2H₂O, FeSO₄·7H₂O, MgCl₂·6H₂O, and MnCl₂·4H₂O were from Spectrum Chemical Manufacturing Corp., Redondo Beach, Calif., and ZnCl₂ was from Fisher Scientific Co., Fair Lawn, N.J.

Screening tests of phosphates in culture media. Each of the phosphates was added to BHI broth in concentrations of 0.1, 0.3, and 0.5% (wt/vol). Flasks containing 20 ml of BHI broth and the phosphate were sterilized at 121°C for 15 min, inoculated with the test organism, and placed in a 35°C water bath shaker (60 rpm) (American Optical Corp., Buffalo, N.Y.). Viable counts were determined after 6, 24, 48, and 72 h by plating serial dilutions of broth samples on preprepared plate count agar (Difco Laboratories). Plates were incubated at 35°C, and colonies were counted after 48 h of incubation using a Quebec colony counter (American Optical Corp.).

Effect of pH. Samples of BHI broth (pH 7.4), alone and with 0.5% sodium tripolyphosphate (STPP), were adjusted to pH values of 4, 5, 6, 7, 8, and 9 before sterilization, by using solutions of 0.1 M HCl or 0.1 M NaOH and a digital pH meter (model 601A; Orion Research, Inc., Cambridge, Mass.). Inocula were added, samples were incubated at 35°C, and cell numbers were determined after 24 and 48 h of incubation as described above.

Effects of initial cell populations. To study the relationship between the inhibitory effect of the polyphosphate and initial bacterial populations, sterile BHI broth portions containing 0 and 0.5% STPP or sodium hexametaphosphate (SHMP) (n = 21) were inoculated with populations of an 18-h culture of

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**TABLE 1. Phosphate compounds screened**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>M.W.</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium Phosphate (DSP, J.T. Baker)</td>
<td>( \text{Na}_2HPO_4 )</td>
<td>142</td>
<td>( \text{O} \quad \text{Na}-\text{O}-\text{P}-\text{O}-\text{Na} )</td>
</tr>
<tr>
<td>Sodium Acid Pyrophosphate (SAPP, FMC)</td>
<td>( \text{Na}_2H_2P_2O_7 )</td>
<td>222</td>
<td>( \text{O} \quad \text{O} \quad \text{Na}-\text{O}-\text{P}-\text{O}-\text{P}-\text{O}-\text{Na} )</td>
</tr>
<tr>
<td>Tetrasodium Pyrophosphate (TSPP, Fisher)</td>
<td>( \text{Na}_4P_2O_7 )</td>
<td>266</td>
<td>( \text{O} \quad \text{O} \quad \text{Na}-\text{O}-\text{P}-\text{O}-\text{P}-\text{O}-\text{Na} )</td>
</tr>
<tr>
<td>Sodium Tripolyphosphate (STPP, Monsanto)</td>
<td>( \text{Na}<em>5P_3O</em>{10} )</td>
<td>368</td>
<td>( \text{O} \quad \text{O} \quad \text{O} \quad \text{Na}-\text{O}-\text{P}-\text{O}-\text{P}-\text{O}-\text{P}-\text{O}-\text{Na} )</td>
</tr>
<tr>
<td>Sodium Tetrametaphosphate (STMP, FMC)</td>
<td>( \text{m(NaPO}_3\text{)}_4 )</td>
<td>408</td>
<td>( \text{ONa} \quad \text{ONa} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{Na} \quad \text{Na} \quad \text{Na} \quad \text{Na} )</td>
</tr>
<tr>
<td>Sodium Hexametaphosphate (SHMP, FMC)</td>
<td>( \text{(NaPO}_3\text{)}_n )</td>
<td>1326 ( \text{n(n=13)} )</td>
<td>( \text{O} \quad \text{P} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{Na} \quad \text{Na} \quad \text{N} \quad \text{a} \quad \text{n} )</td>
</tr>
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</table>

S. aureus ranging from ca. 10^1 to 10^6 cells per ml. Samples were incubated, and viable counts were determined after 24 and 48 h as described above.  

**Effect of heat.** Flasks containing BHI broth with 0.25, 0.5, and 1% (wt/vol) STPP or SHMP were prepared either by (i) sterilizing at 121°C for 15 min or by (ii) preparing 10% (wt/vol) solutions of the polyphosphates, filter sterilizing them by using 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.), and adding appropriate volumes to presterilized broth to obtain the desired phosphate concentrations. The flasks were inoculated, and viable cells were determined after 24 and 48 h at 35°C as described above.  

Tests in culture media with added cations. The content of iron, magnesium, calcium, and zinc in BHI broth was measured by atomic absorption spectrophotometry (model 5000; The Perkin-Elmer Corp., Norwalk, Conn.). The effect of these cations on inhibition by the polyphosphates was studied by adding increments (to 1 mM) of FeSO₄·7H₂O, MgCl₂·6H₂O, CaCl₂·2H₂O, ZnCl₂, or MnCl₂·4H₂O to BHI broth alone, and to broth containing 0.5% STPP or SHMP. Broth samples containing the mineral alone, or in combination with the polyphosphates, were inoculated with the test organism, and viable counts were determined after 4, 8, and 24 h of incubation at 35°C as described above. Each test was repeated twice, in duplicate, and reported data represent mean values of these measurements.  

**RESULTS**  

**Screening tests.** Eight phosphate compounds were tested in culture media to identify compounds which inhibit the growth of S. aureus 196E (Table 1). Results showed no inhibitory effects of any of the phosphates in a concentration of 0.1% (wt/vol) after 24 h of incubation. At 0.3%, only SHMP, with approximately 21 PO₄ groups, inhibited growth of the organism, while at 0.5%, STPP and SHMP (n = 13, 15, and 21) showed inhibitory effects. Growth curves of S. aureus in BHI broth alone and in broth containing 0.5% of each of these compounds are shown in Fig. 1. At this concentration, SHMP (n = 21) was most effective, followed very closely by STPP and SHMP (n = 13). While cell numbers in broth increased from ca. 10^6 to 10^9 after 24 h of incubation, they declined 100-fold in the presence of 0.5% of any of these compounds. Further studies were conducted with STPP and SHMP (n = 21).  

**Effect of pH.** The addition of 0.5% STPP to BHI broth raised its pH from 7.4 to 7.7, but no change in broth pH was produced by 0.5% SHMP (n = 21). After heat sterilization
the respective pH values were 7.4 and 6.9. The effect of various pH levels on growth of *S. aureus* 196E in BHI broth alone and in broth containing 0.5% STPP is shown in Fig. 2. In broth alone the organism grew over the pH range of 6 to 9 in 24 h, with an optimal growth between pH 7.4 and 9.0. These findings are in agreement with previously reported values (12, 14). Growth was also observed at pH 5.0 after 48 h of incubation. In broth containing 0.5% STPP, the organism was least sensitive to the phosphate at pH 6 and displayed enhanced sensitivity at pH 7 and above. The number of survivors decreased from an initial population of $5.7 \times 10^6$ to $1 \times 10^3$ CFU/ml after 48 h of incubation at broth pH $> 7.4$.

**Effect of initial cell populations.** Incubation of initial bacterial populations ranging from $10^2$ to $10^6$ CFU/ml in broth alone resulted in viable numbers of ca. $10^9$ cells per ml after 24 h of incubation. Data comparing the effects of 0.5% STPP and SHMP ($n = 21$) are summarized in Table 2. No viable cells were recovered from broth samples containing either 0.5% STPP or SHMP and initial populations of less than $10^4$ cells per ml. Growth inhibition persisted even as inoculum sizes increased. At each inoculum level, the viable numbers

<table>
<thead>
<tr>
<th>Initial population (log CFU/ml)</th>
<th>0.5% STPP</th>
<th>0.5% SHMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.6</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>5.4</td>
<td>2.8</td>
<td>2.0</td>
</tr>
<tr>
<td>6.4</td>
<td>3.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* No growth in undiluted broth samples.
after 24 h declined 100- to 1,000-fold, and an additional 10-fold decline was evident after 48 h in most of the experiments with these compounds (Table 2).

Effect of heat. Growth inhibition in broth containing heat-sterilized polyphosphates was compared with inhibition in broth to which filter-sterilized phosphates were added. Three levels of STPP or SHMP were used: 0.25, 0.5, and 1% (wt/vol); results are summarized in Table 3.

No appreciable differences in growth inhibition were discerned between treatments at phosphate levels of 0.5 or 1%. In either sterilization procedure, these levels were bactericidal to the organism, causing approximately 1,000-fold reductions in cell numbers after 48 h of incubation. Pronounced differences by the sterilization treatments were observed at lower concentration of STPP (0.25%) with two levels of initial populations (3.8 x 10^4 and 3.8 x 10^6 CFU/ml). No growth was evident in the presence of the filter-sterilized STPP, and cell numbers declined during incubation. In each test, growth after 48 h of incubation was lower than the initial numbers, whereas the heat-sterilized STPP displayed little inhibitory effect. In contrast, the inhibitory effects of the heat- and filter-sterilized SHMP were comparable.

Effect of metals. Measurements of the metal content in BHI broth by atomic absorption spectrophotometry indicated the following concentrations: 0.35 mM Mg, 0.0425 mM Ca, 0.023 mM Fe, and 0.025 mM Zn. The effect of supplementation of these cations on growth inhibition by 0.5% STPP (13.6 mM) was examined with concentrations ranging from 0 to 1.0 mM and two levels of cell populations (10^4 and 10^6 CFU/ml).

Preliminary tests were done to determine the effect on growth of each of these cations in STPP-free broth. While some initial delay in growth was observed by the addition of the highest level of each cation to the broth (1.0 mM), no difference was seen in viable counts after 24 h of incubation.

The effects of four levels of each cation in combination with 0.5% STPP on growth of S. aureus after 24 h of incubation are summarized in Table 4. Mg^{2+} was the most effective cation, and inhibition was nearly totally eliminated by ca. 0.25 mM. The addition of 0.125 mM MgCl_2 . 6H_2O resulted in viable counts after 24 h of incubation which were only 1 log cycle lower than those in STPP-free media. The effectiveness of the minerals in overcoming inhibition by STPP was followed by Ca^{2+} and Fe^{3+}. The addition of 1 mM Fe^{2+} in combination with STPP caused the formation of a precipitate and a rapid decline in growth. Inhibition persisted with the addition of Zn^{2+}, in contrast to the effects of the other cations (Table 4).

Similar experiments with a higher initial cell population (5.7 x 10^6 CFU/ml) and two levels of cation supplementation (0.34 and 0.68 mM) including Mn^{2+} confirmed the results shown in Table 4. Mg^{2+} was the most effective in restoring growth, followed closely by Ca^{2+} and Fe^{2+}; viable numbers declined during incubation in the presence of added Zn^{2+} or Mn^{2+} (data not shown).

**DISCUSSION**

The straight-chain polyphosphates with chain length of 3, 13, 15, and 21 were inhibitory to growth of S. aureus 196E, whereas the PP₅ (sodium acid PP₅) and tetrasodium PP₅ or the cyclic phosphate (sodium tetrametaphosphate) had no effect on the growth of the organism. The binding sites of the condensed phosphates favor metal chelation and hence growth inhibition. In contrast, formation of a chelate ring may be hampered sterically in metaphosphates, and since the stability of complexes is generally influenced by the chain length of the polyphosphates, PP₅ metal complexes are less stable than the longer-chain phosphate (17).

No viable counts were recovered from broth containing a concentration of 0.5% STPP or SHMP and initial populations of less than 10^6 CFU/ml after incubation for 24 or 48 h. At higher inocula, numbers of surviving cells after 24 h at 35°C were 100- to 1,000-fold lower than the initial populations. Hence, this concentration of the polyphosphates resulted in a 99.9% reduction in viable cells. Such reduction may be particularly significant in food products which are expected to contain relatively low initial cell populations.

Temperature and pH are known to influence the rate of hydrolysis of polyphosphates (1). When STPP hydrolyzes, both Pi and PP₅ are formed, and these compounds have shown no antimicrobial activity against S. aureus 196E in the present study. Hydrolysis of SHMP is partly to Pi, and partly to triopolyphosphate (1). Based on results summarized in Table 3, hydrolysis to compounds lacking antibacterial activity appears to have occurred during heat sterilization of STPP. In contrast, SHMP retained most of its antibacterial activity after the sterilization treatment. The increased stability of SHMP is apparently associated with its increased chain length as compared with STPP (6).

Inhibition by the polyphosphates was pH dependent, and a reduction in bactericidal activity showed when the pH was lowered. In general, chelators become increasingly dissociated at elevated pH values, and the quantity of complexations increases (6). As a result, essential minerals become unavailable for bacterial growth and, in turn, inhibition is

<table>
<thead>
<tr>
<th>Phosphate</th>
<th>Level (%)</th>
<th>Log CFU/ml</th>
<th>Initial</th>
<th>Heat sterilized</th>
<th>Filter sterilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>STPP</td>
<td>0.25</td>
<td>4.58</td>
<td>2.69</td>
<td>6.82</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>6.58</td>
<td>7.32</td>
<td>8.54</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>6.40</td>
<td>4.23</td>
<td>3.34</td>
<td>4.50</td>
</tr>
<tr>
<td>SHMP</td>
<td>0.25</td>
<td>4.58</td>
<td>2.93</td>
<td>2.34</td>
<td>0^a</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>6.58</td>
<td>4.61</td>
<td>3.82</td>
<td>4.20</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>6.40</td>
<td>4.95</td>
<td>3.82</td>
<td>5.11</td>
</tr>
</tbody>
</table>

^a No growth in undiluted samples.
enhanced. This may explain the increased inhibition observed at elevated pH values in the present study, despite the fact that pH 7 or slightly higher is normally more favorable for growth of *S. aureus* than the low acid range.

Growth recovery by supplemented cations can be explained by the formation of stable soluble complexes between metals and the polyphosphates, which renders essential ions unavailable to the cells. The growth requirement for magnesium is absolute; it is associated with bacterial cell wall and membrane, is important in maintaining bacterial permeability barrier, and activates many enzymes. Sequestration of magnesium has been shown to affect cell metabolism and cause failure in cell division or loss of cell wall integrity (8, 13, 20). Deprivation of magnesium diminished repair of *S. aureus* from sublethal heat injury (5), and an effect on enterotoxin by magnesium was also reported (10). In the latter study, a maximum toxicity was produced in casein hydrolyzate medium containing 0.059 mM MgCl₂ · 6H₂O by adding 0.207 mM salt and 0.009 mM FeSO₄ · 7H₂O (10).

In the present study, the addition of 0.25 to 0.35 mM Mg²⁺ to BHI broth containing 0.5% STPP exerted the highest effect on growth restoration. Since BHI broth contains 0.35 mM Mg²⁺, most of it apparently became unavailable to the organism as a result of a stable chelate which formed with the polyphosphate and caused magnesium deficiency. In other studies conducted in our laboratory, Mg²⁺ addition was effective in restoring growth, inhibited by polyphosphates, of *Streptococcus mutans* (P. S. Kirk, M.S. thesis, Wayne State University, Detroit, Mich., 1984) and *Bacillus cereus* spores (S. Bernal, M.S. thesis, Wayne State University, Detroit, Mich., 1985). In studies with *B. cereus*, vegetative growth, but not spore germination, was inhibited. Vishniac (18) showed inhibition of yeast hexokinase by STPP and its restored activity by Mg²⁺, and Webb (20) reported restoration of normal morphology of gram-positive rods grown in magnesium-deficient medium after magnesium supplementation, although supplementation with other metals did not eliminate the effects.

Partial growth of *S. aureus* was restored by supplemented Ca²⁺ or Fe²⁺, but inhibition was not eliminated by either Zn²⁺ or Mn²⁺. The addition of manganese was reported to reduce magnesium requirements in *Bacillus megaterium* and *B. subtilis* (21). In the present study, it was not effective in overcoming the inhibition of *S. aureus* by STPP.

We speculate that the superior ability of Mg²⁺ to restore growth is not entirely caused by nutritional needs of the organisms for this specific mineral, but may also be related to a higher stability constant of the polyphosphate with Mg²⁺ than with other cations (6, 7). The addition to the medium of a cation with a high stability constant may cause the release of other essential chelated cations (or other vital soluble substances) with lower stability constants and make them available for bacterial utilization. Alkaline earth metals (e.g., Mg²⁺ and Ca²⁺) were reported to form more stable complexes with phosphates than other metals (16, 17). From data on dissociation constants of complexes formed between various metals and a polyphosphate (*n* = 5), reported by Van Wazer and Campanella (17), the complex stabilities for the cations investigated in the present study are Mg²⁺ > Ca²⁺ > Fe²⁺ > Zn²⁺ > Mn²⁺. Additional studies support the high effectiveness of complexes of polyphosphates with magnesium and calcium (6). If magnesium forms a more stable chelate with the polyphosphate molecules than other cations which are essential to microbial growth, an excess of magnesium in the medium may block any interaction between polyphosphate and other critical components. Additional studies are in progress in an attempt to confirm the mechanism of action of polyphosphates.

**LITERATURE CITED**