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Polyphosphate Inhibition of Growth of Pseudomonads From Poultry Meat

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ABSTRACT

ELLIOTT, R. PAUL (Western Regional Research Laboratory, Albany, Calif.), ROBERT P. STRAKA, AND JOHN A. GARIBALDI. Polyphosphate inhibition of growth of pseudomonads from poultry meat. Appl. Microbiol. **12**:517-522. 1964.—Both commercial polyphosphates and equivalent mixtures of chemically pure polyphosphates inhibited the growth of nonfluorescent pseudomonads in a synthetic medium. Fluorescent strains grew after a short lag. Inhibition was not caused by high pH, but rather by chelation of metal ions essential to the growth of the bacteria. Mg^{++} and the natural competitive chelators, pyoverdine and bacteriological peptone, reversed the inhibition. Chilling chicken carcasses overnight in slush ice containing 3 and 8% polyphosphates lengthened subsequent shelf-life 17 and 25%, respectively. Chickens held in continuous contact with 3 and 8% solutions of polyphosphates during storage at 2.2 C kept 17 and 67% longer, respectively. Only fluorescent strains developed in the presence of 3 and 8% polyphosphates. Chickens held in antiseptic ice containing 8% polyphosphates kept 60% longer than did those in water ice.

Polyphosphates are widely used in cured meat where they contribute to flavor, water retention, color, tenderness, and juiciness, and decrease cooking shrinkage (Swift and Ellis, 1956, 1957; Klose, Campbell, and Hanson, 1963). Only recently have they been suggested for chilled or frozen products. Fish dipped in polyphosphates before freezing had less drip and better flavor (Anonymous, 1962; Tanikawa, Akiba, and Shitamori, 1963). Polyphosphates increased the water-binding capacity of meats (Hellendoorn, 1962) and of chicken (Mahon, 1962; Mountney and Arganosa, 1963; Schermerhorn and Stadelman, 1963; Thomson, Kotula, and Novotny, 1963), and decreased the cooking losses in chickens (Mountney and Arganosa, 1963; Klose et al., 1963; Schermerhorn and Stadelman, 1963; Monk, Mountney, and Prudent, 1964). Polyphosphates also improved tenderness and juiciness (Spencer and Smith, 1962; May, Helmer, and Saffle 1963), and delayed oxidative rancidity in poultry meat (Marion and Forsythe, 1962).

Spencer and Smith (1962) reported that chilling in a polyphosphate solution increased the shelf-life of chickens by 1 to 2 days. Post, Krishnamurty, and Flanagan (1963) described antibacterial action of sodium hexametaphosphate used in calcium alginate swabs.

Polyphosphates are well known as chelating or sequestering agents for metallic ions. The chain phosphates chelate strongly, the ring phosphates weakly, and the orthophosphates not at all (Van Wazer and Callis, 1958; Van Wazer and Campanella, 1950; Martell and Calvin, 1952).

The longer chains of phosphates form the most stable complexes (Martell and Calvin, 1952; Van Wezer and Callis, 1958). These compounds chelate the following metals (in order of decreasing stability of the complex): (i) Fe^{+++}, Pb^{++}; (ii) Co^{++}, Cu^{++}, UO_2^{++}; (iii) Ni^{++}, Zn^{++}; (iv) Fe^{++}, Mn^{++}, Ca^{++}, Mg^{++}; and (v) Sr^{++}, Ba^{++}, Ag^{+}; they chelate Li^{+}, NH_3^{+}, Na^{+}, and K^{+} only weakly (Van Wazer and Campanella, 1950).

Chelating agents may either enhance or hinder growth of microorganisms. Metals essential for growth may be more available after chelation by assimilable compounds (Lankford, Kustoff, and Sergeant, 1957). Chelators may remove toxic metals from effective concentration (Hutner et al., 1950; Mayer and Traxler, 1962; Jones, 1964). On the other hand, compounds such as 8-hydroxyquinoline or dithizone always inhibit growth, because they form highly stable chelates with metals essential to the growth of the microorganisms (Zentmyer, 1943, 1944; Albert et al., 1947; Lankford et al., 1957). Several workers (Mayer and Traxler, 1962; Newton, 1953; Weinberg, 1954; Bernheim, 1954; Saz and Slie, 1954; Albert et al., 1947; Plocke and Vallee, 1962; Garibaldi and Bayne, 1962) reversed the antibacterial action of chelating agents by adding metal ions.

Weinberg (1957), in a comprehensive review, related chelation of metal ions to the effectiveness of antibiotics. Chlortetracycline may owe its antibacterial action in beef to chelation of trace metals required by the microorganisms for growth (Jay, Weiser, and Deatherage, 1957). The magnesium of seawater reverses the inhibition by tetracycline of fish spoilage bacteria (Southcott and Tarr, 1961).

The pseudomonads predominate in chicken spoilage (Ayres, Ogilvy, and Stewart, 1950). Two investigations (Burton, Campbell, and Eagles, 1948; King, Campbell, and Eagles, 1948) showed the importance of trace metals in pigment production by these organisms; Katznelson

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and White (1950) reported that Na^+, K^+, Mg^{++}, S, P, and Cl^- or Br^- were necessary for growth of *Pseudomonas nigrifaciens*.

The objects of the present investigation were (i) to determine the effect of a commercial polyphosphate mixture (Kena; Calgon Corp., Pittsburgh, Pa.) on growth of pure cultures from poultry meat, (ii) to ascertain the mode of action of the inhibition of growth, and (iii) to confirm both in practical tests on chilled chickens.

MATERIALS AND METHODS

Basal medium. The medium used for all pure culture work was citric acid, 1 g; K_2SO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.8 g; $(NH_4)_2HPO_4$, 4 g; dissolved in 1 liter of demineralized water from a Barnstead Bantam demineralizer fitted with a standard cartridge. We added $ZnSO_4$, $MnSO_4$, $FeCl_3$, and $Cu(CH_3COO)_2$ solutions to give 1 ppm of each metal ion. The medium was autoclaved at 121 C for 15 min. Final pH was 6.8.

Preparation of polyphosphates. We analyzed the commercial polyphosphate mixture by the method of Van Wazer, Griffith, and McCullough (1954), and estimated it to contain 75% $Na_5P_3O_{10}$ and 25% $Na_4P_2O_7$. Orthophosphate constituted less than 1% of the total. To minimize hydrolysis to orthophosphate, solutions of the polyphosphate mixture were filter-sterilized and were added aseptically to the basal medium.

Cultures and inocula. Six strains of pseudomonads isolated from spoiled chicken were tested for their ability to grow in the basal medium in the presence of 0.1 to 5% polyphosphates. Four fluorescent strains grew after a somewhat increased lag, and two nonfluorescent strains failed to grow in the basal medium containing 1% polyphosphates in 95 hr at 15 C. One fluorescent strain, tentatively identified as *P. ovalis* (37F), and one nonfluorescent, tentatively identified as *P. fragi* (40N1), were chosen for further study. Both grew well at 0 C, and had maximal growth temperatures of 32.0 and 31.2 C, respectively, as determined with a temperature-gradient incubator (Elliott, 1963). Loopfuls of culture grown on a metal-deficient medium were used as inoculum. This medium was Na citrate, 1 g; K_2SO_4, 1 g; and $(NH_4)_2HPO_4$, 4 g; in 1 liter of demineralized water. The medium was treated by the method of Donald, Passey, and Swaby (1952) to remove trace metals, and then was autoclaved. Growth on this medium was barely visible after incubation for 24 to 48 hr at 28 C.

Measurement of growth. Growth measurements were made with the use of a Klett-Summerson photoelectric colorimeter with green filter no. 54 against the sterile basal medium as blank. Light transmitted by the violet no. 42 filter excited fluorescence from pyoverdine, thus giving high results from the fluorescent bacteria.

Effect of pH. Preliminary tests showed that the high pH of polyphosphates did not cause the inhibition. However, for constancy of conditions, the polyphosphate solutions were all adjusted to pH 6.8 with H_2SO_4.

Effect of ionic strength. Ionic strength was calculated by the formula of White et al. (1959). Ionization constants for the phosphoric, pyrophosphoric, and tripolyphosphoric acids were obtained from Van Wazer (1958). We assumed that citric acid and the sulfate salts were fully ionized. The ionic strength of the basal medium was then 0.1, and that of the 1% polyphosphate mixture was 0.31 at pH 6.8.

In a preliminary experiment, additional K_2SO_4 in the basal medium brought the ionic strength to that of the medium containing 1% polyphosphates, i.e., 0.41 (final concentration of K_2SO_4 was 0.13 M). The growth of both test organisms was nearly as rapid in the presence of this extra K_2SO_4 as in the basal medium. We concluded that high ionic strength could not explain the powerful inhibitory action of the polyphosphates.

Reversal of inhibition with added Mg^{++}. Sterile $MgSO_4$ solution was added to sterile, double-strength basal medium containing 2% of the polyphosphates. When 7.1 ml of a 1 M $MgSO_4$ solution had been added to 100 ml of the double-strength mixture, $MgNH_4PO_4$ began to precipitate. This concentration represented the sequestering or chelating capacity of the polyphosphate-medium mixture for

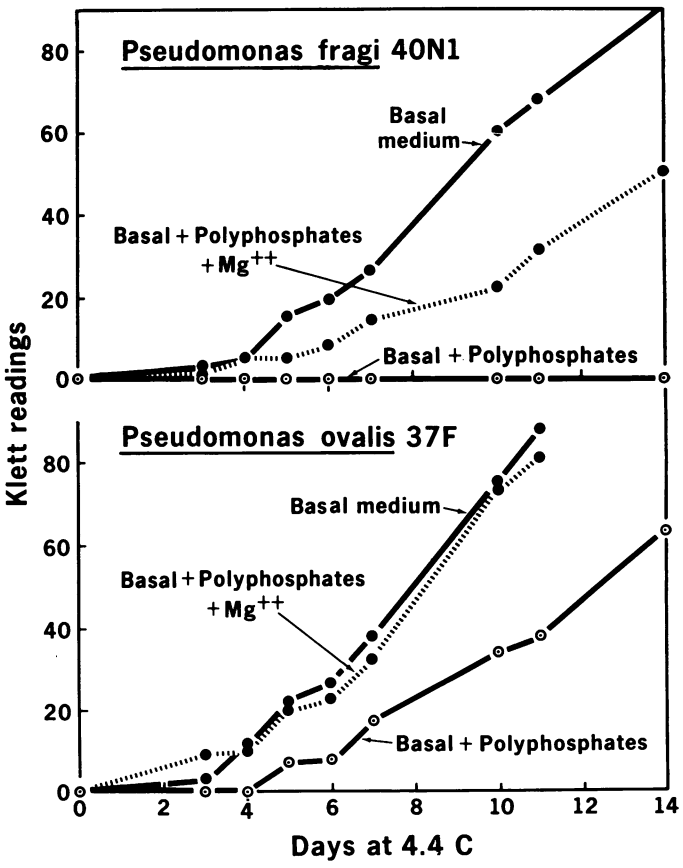

FIG. 1. *Inhibition of growth of two psychrophilic pseudomonads by 1% commercial polyphosphates in the basal synthetic medium, and reversal of inhibition by 0.035 M Mg^{++}.*

Mg^{++}. When the double-strength basal medium was diluted with sterile water to single strength, the concentration of the polyphosphates thus became 1%, and that of the Mg^{++}, 0.035 M. After 2 days, the clear supernatant fluid was poured aseptically to sterile Klett tubes, and was inoculated with loopfuls of the test organisms in parallel with suitable controls. Mg^{++} reversed the inhibiting effect of polyphosphates on both organisms (Fig. 1). A duplicate set of tubes gave similar results.

Confirmation with pure phosphates. A repeat experiment was conducted with purified phosphates of known composition. A mixture of 75% sodium tripolyphosphate (Fisher, purified) and 25% sodium pyrophosphate (Baker, reagent) was dissolved in water, adjusted to pH 6.8 with H_2SO_4, sterilized by filtration, and added aseptically to the basal medium. Results were essentially the same as those reported in Fig. 1. That is, *P. fragi* 40N1 was unable to grow in the presence of 1% polyphosphates, and *P. ovalis* 37F grew after a short lag. Mg^{++} reversed the inhibition.

Reversal of inhibition by natural chelators. To simulate conditions on the surface of poultry meat during spoilage, bacteriological peptone (Difco), creatine, and glutamic acid were added to basal media at 0.1 and 1% levels with and without 1% commercial polyphophates. The inoculated media were incubated at 14 C with continuous shaking in flasks with test-tube side arms calibrated for use on a Klett-Summerson photoelectric colorimeter. Of these additives, only bacteriological peptone permitted prompt growth of *P. fragi* 40N1 in the presence of the polyphosphates (Fig. 2, bottom), although glutamic acid permitted growth after a 4-day lag.

Because the fluorescent pseudomonads were less sensitive to the chelating power of polyphosphates than were the nonfluorescent pseudomonads, we felt that the pigment complex, pyoverdine, might be competing as a chelator against polyphosphates, much as it does against conalbumin in egg spoilage, as shown by one of us (Garibaldi, *unpublished data*). A crude preparation of the pigment complex was dissolved in water, filter-sterilized, and was then added at a 0.005% level to basal media both with and without 1% commercial polyphosphates. From its fluorescence, this level of pyoverdine was equivalent to 5 μg/ml (as riboflavine), when measured according to a method previously described (Elliott, 1962). The inoculated media were incubated in Klett tubes at 2.2 C, and were shaken each time before they were examined for turbidity. Pyoverdine at this level permitted growth of the nonfluorescent *P. fragi* 40N1 in the presence of 1% polyphosphates (Fig. 2, top). A duplicate set of tubes gave similar results, and a later experiment confirmed the effect. Pyoverdine also partially reversed the slight inhibition of *P. ovalis* 37F which was previously described.

In another experiment, 0.005% pyoverdine permitted *P. fragi* 40N1 to grow in the presence of 1% polyphosphates and 0.1% glucose. The experiment was conducted at 14 C in Erlenmeyer flasks with side arms calibrated for a Klett colorimeter. Results were similar to those described above, but with a greater growth due to the additional carbon source (Fig. 3). Pyoverdine also shortened the lag as compared with the control, but the reason for this is not clear.

Slush-ice chill tank experiments. Slush-ice chill tanks were prepared by prechilling water to 1 C, and adding appropriate chemicals and then enough ice to chill the chickens just to 1 C. Chicken fryers were killed and eviscerated, and were then immersed in the chill tanks, where they remained 20 to 24 hr. They drained for 2 to 3 min, and then were packaged in loosely closed polyethylene bags and were stored at 2.2 C. Each second day, duplicate sets of birds were removed, and were smelled by a panel of six to eight persons; entire chickens were weighed into 1 liter of 0.1% peptone. After being shaken for 2 min by hand, appropriate dilutions of this wash water were plated on Trypticase Soy Agar (BBL). Plates were incubated at 4.4 C for 2 weeks.

In the first experiment, chickens were killed and eviscerated in the laboratory processing plant. Parallel chill tanks contained (i) polyphosphates to give 3% in the final solution after all ice had melted, (ii) 3% polyphosphates and $MgSO_4$ to give 0.11 M Mg^{++}, and (iii) a control without additives. The solution containing $MgSO_4$ was somewhat cloudy, due to precipitation of magnesium orthophosphate. The solubilities of the magnesium complexes of the polyphosphates are temperature-dependent (Irani, 1961), and

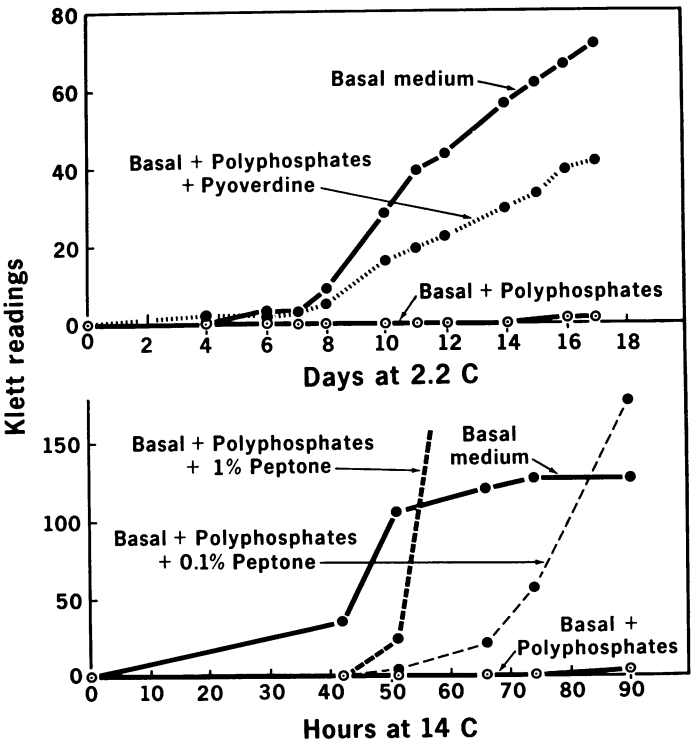

FIG. 2. *Inhibition of growth of Pseudomonas fragi 40N1 by 1% commercial polyphosphates in the basal synthetic medium, and reversal of inhibition by 0.005% pyoverdine and by bacteriological peptone.*

this amount of Mg^{++} was somewhat in excess of that which could be sequestered by the polyphosphate mixture. The magnesium orthophosphate formed only after the polyphosphate was saturated with Mg^{++}.

The control chickens chilled in slush-ice without additives spoiled, with off odors and counts of $10^{7.4}$ or higher per gram in about 12 days. The chickens chilled with 3% polyphosphates in the slush-ice reached this condition in about 14 days. The birds chilled with both polyphosphates and Mg^{++} spoiled slightly more quickly than did the controls (Fig. 4).

The chickens in this first experiment had a very low initial level of psychrophilic bacteria. In the second experiment, freshly killed and eviscerated fryers were obtained from a commercial source, and were chilled in the same way as were those in the first experiment, except that ice that had been used for shipping chickens to California from the East Coast was added. These chickens had a psychrophilic bacterial count of $10^{5.5}$ per gram at the time that they were packaged for storage. The birds were otherwise treated and examined as in the first experiment. The control birds spoiled in 4 days, as determined by odor and bacterial count. Those that had the polyphosphate chill treatment spoiled after 5 days. Mg^{++} at the 0.11 M level reversed the inhibiting effect of the polyphosphates.

The third experiment was designed to determine the effect of polyphosphates as a chill-tank additive at an 8% level. The control chickens chilled in ordinary ice slush spoiled with off odors and counts of 10^8 per gram in about 8 days, whereas those chilled in ice slush containing 8% polyphosphates reached this condition in about 10 days (Fig. 5).

Antiseptic ice experiments. A preliminary experiment was conducted to determine the effect of continuous contact between polyphosphates and chicken meat during spoilage. Single chickens, freshly killed and eviscerated in a commercial plant, were immersed in 2 liters of tap water with 3 and 8% commercial polyphosphates, and a control was immersed in tap water. The chickens remained in these solutions at 2.2 C until they spoiled. At intervals, they were shaken vigorously, and were then examined under ultraviolet light and were smelled; samples of the solutions were taken for 2.2 C plate count on Trypticase Soy Agar (BBL) and for streak plates on Pseudomonas Agar F (Difco) for estimation of fluorescent pseudomonads (Silliker, Shank, and Andrews, 1958).

Results (Fig. 6) show the marked inhibition of growth of psychrophilic bacteria by continuous contact with 8% polyphosphates. For simplicity of presentation, the data

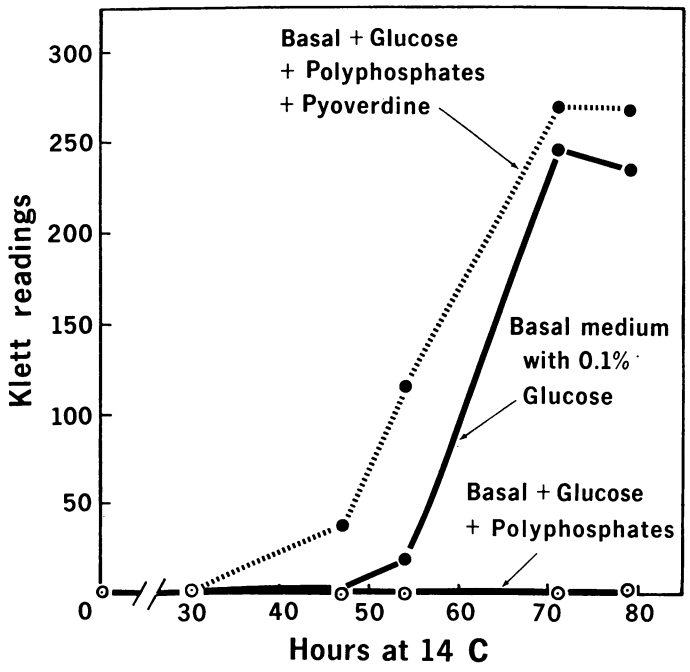

FIG. 3. *Inhibition of growth of Pseudomonas fragi 40N1 by 1% commercial polyphosphates in the basal medium with 0.1% glucose, and reversal of inhibition by 0.005% pyoverdine.*

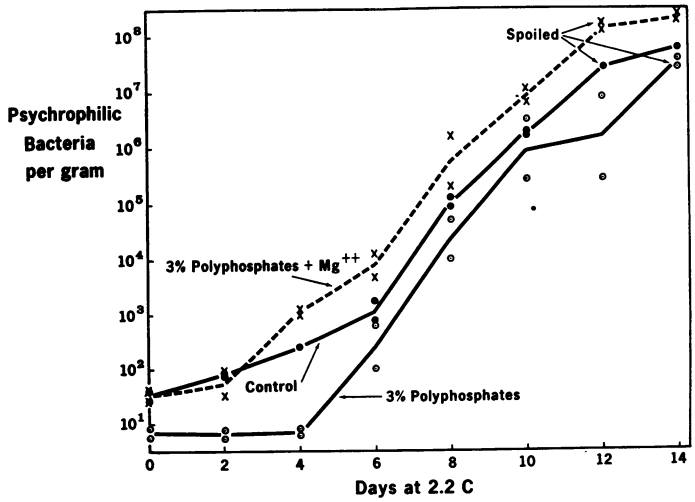

FIG. 4. *Inhibition of subsequent spoilage of chicken meat at 2.2 C by 3% commercial polyphosphates in a 24-hr ice-slush chill, and reversal of inhibition by 0.11 M Mg^{++}.*

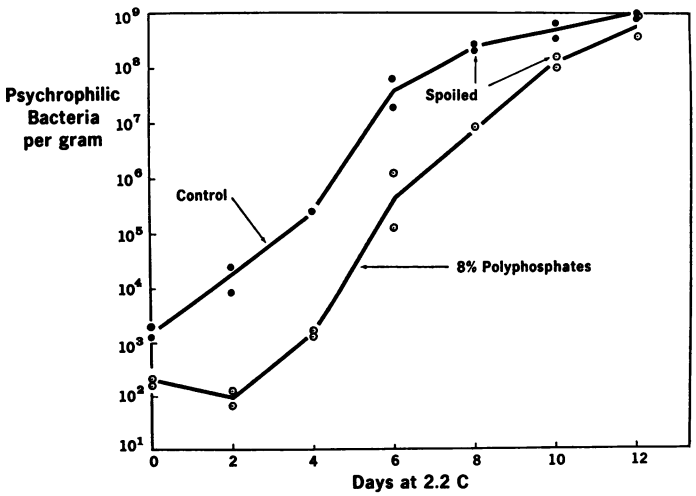

FIG. 5. *Inhibition of subsequent spoilage of chicken meat at 2.2 C by 8% commercial polyphosphates in a 24-hr ice-slush chill.*

for the 3% polyphosphates were omitted; they fell between the curves shown. The 3% solution enhanced keeping time 17%, and the 8% solution, 67%. In the presence of polyphosphates, the nonfluorescing strains failed to grow and the fluorescing strains grew only after a lag. These results fully confirmed the observations made on fluorescent and nonfluorescent pure cultures of *Pseudomonas*.

The next experiment was designed to determine how an ice containing 8% polyphosphates would affect the spoilage rate of chickens stored in it. Water containing 8% commercial polyphosphates was frozen and crushed in a commercial establishment, and was then stored at −11 C until used. Freshly killed and eviscerated fryers from a commercial source were air-chilled for 2 hr, and were then divided into two lots. One lot was stored in ordinary tapwater ice as controls, and the other was stored in the 8% polyphosphate ice, both in a room refrigerated at 2.2 C. Each day, additional ice was added to each lot; each second day, sample birds were taken for odor and bacterial counts as previously described.

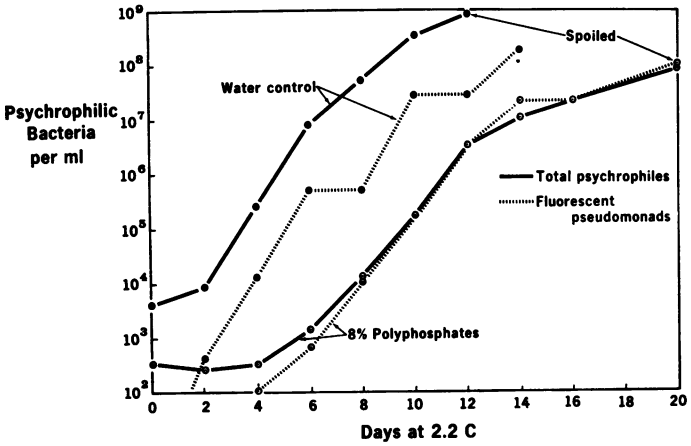

FIG. 6. *Inhibition of bacterial growth at 2.2 C in water and in a solution of 8% commercial polyphosphates containing whole eviscerated chickens. At 0 days, the chickens had been immersed about 20 min when the solutions were sampled.*

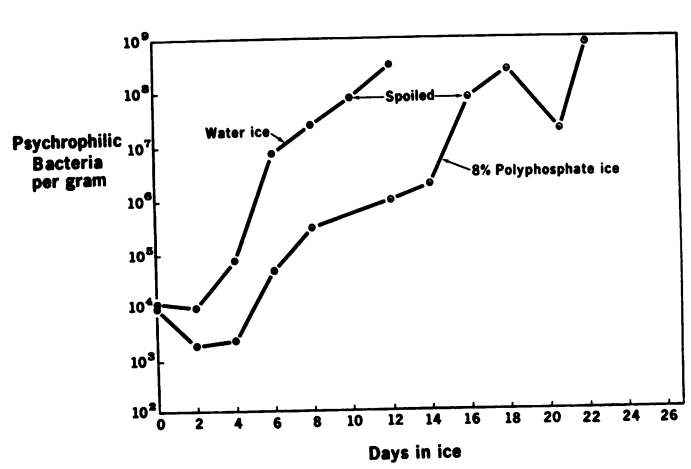

FIG. 7. *Inhibition of bacterial growth on chickens stored in ice containing 8% commercial polyphosphates.*

Results (Fig. 7) show a 60% enhancement of shelf-life in the ice with 8% polyphosphate. The water ice, of course, was at 0 C at all times during its melting. However, because solutes lower the melting point of ice, the pieces with the higher concentration of polyphosphates melted first. Thus, the polyphosphate ice was at −2 C shortly after each re-icing, and gradually approached 0 C as the ice melted, leaving nearly pure water ice. The longer keeping time in the polyphosphate ice can be attributed, in part, to this lower average temperature.

DISCUSSION

In chicken spoilage, polyphosphates inhibit or destroy a portion of the flora, and increase the lag for the organisms that eventually reproduce. They completely inhibit the growth of nonfluorescent strains in pure culture, but not in the mixed culture of chicken spoilage. Only when the polyphosphates were present in overwhelming amounts in intimate and continuous contact with the spoiling surfaces did they prevent the nonfluorescent organisms from growing on chickens (Fig. 6). Natural chelators such as pyoverdine or peptones reverse the effect of the polyphosphates (Fig. 2 and 3); one must assume that these and other natural chelators develop on chicken meat surfaces, permitting growth of both fluorescent and nonfluorescent bacteria after an increased lag.

The level of pyoverdine used in our experiments appears higher per gram than that ordinarily found in poultry meat spoilage (Elliott, 1962). However, in the microenvironment on the surface of poultry meat, one would expect at least as high a concentration as 5 μg/ml (calculated as riboflavine) during growth of fluorescent pseudomonads; therefore, the concentration we used in liquid culture is realistic.

We have shown the reversal of inhibition by Mg^{++}. Although more Mg^{++} than other metal ions is required by most microorganisms, it may not be the only important metal ion. Sufficient Mg^{++} to overwhelm the chelating power of polyphosphates may release Fe^{++}, Mn^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Li^{+}, Na^{+}, and K^{+} from chelation (Van Wazer and Campanella, 1950). The available data do not show the relative importance of the various metal ions to the organisms in question.

When the polyphosphates were in continuous contact with the spoiling surfaces, chickens kept much longer than did controls. However, the use of polyphosphates in antiseptic ice for shipping chilled birds might prove a greater expense than the enhancement of keeping time warrants. Furthermore, as the polyphosphate ice melts, the concentration of solubles diminishes progressively in the melting ice. To furnish to the spoilage surfaces a constantly fresh source of polyphosphates, frequent re-icing with polyphosphate ice is necessary. From the economic standpoint, an overnight chill in slush-ice containing polyphosphates might be better.

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