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Antibacterial mechanism of phosphates in Staphylococcus aureus

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Antibacterial mechanism of phosphates in *Staphylococcus aureus*

by

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DEDICATION

This dissertation is submitted in humble gratitude to God
and with my earnest prayer

O Lord, I will praise You for I am fearfully and wonderfully made;
O Lord, You have taught me from my youth that
for as the heavens are high above the earth,
so great is Your mercy toward those who fear You;
the fear of the Lord is the beginning of wisdom;
a good understanding have all those who do Your commandments.

What shall I render to my Lord for all Your benefits toward me?
I will take up the cup of salvation, and call upon the name of my Lord.

May the words of my mouth and the meditation of my heart
be acceptable in Your sight;
May the beauty of my Lord be upon me, and establish the work of my hands;
May I grow in the grace and knowledge of my Lord and Savior Jesus Christ;

To You be all the glory both now and forever. Amen.

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Introduction

Meat products have been an important part of the human diet throughout history. Today, meats are still one of the most universally accepted foods. Among the factors that affect the selection of meat as a dietary constituent, palatability is the major factor. Palatability includes three major factors, namely: color, flavor and tenderness (Halliday, 1978). Therefore, the meat industry works in parallel with consumer preferences and concerns to maximize the palatability of meat products.

After the conversion of animal muscles into meat products, rapid microbiological and chemical degradations proceed. In order to preserve meat palatability, food additives and various preservation techniques are used. Apart from salt and sugar, phosphates are the most important food additives used in meat processing. During the 1970s, more than forty million pounds of phosphates were used annually by the meat industry (Molins, 1991).

Phosphates perform three basic functions, namely: sequestration of metal ions, control of pH by buffering and increase of ionic strength of solutions by providing polyvalent anions (Halliday, 1978; Shimp, 1983; Steinhauer, 1983). In meat systems, phosphates are used to improve product quality by increasing water binding and meat binding, enhancing emulsification, retarding oxidative rancidity and color deterioration, and enhancing cured-color development (Ellinger, 1972; Shimp, 1983). Recent applications of phosphates in muscle foods include their use in low-sodium sausage formulations, in muscle pigment protection, in the manufacture of restructured meat products, and as

antimicrobials in cooked meat products (Bolin and Connick, 1976; Brotsky and Everson, 1973; Merkenich, 1977; Steinhauer, 1983).

There is an increasing trend of phosphate utilization in meat products (Sofos, 1986) due to the following three reasons. First, three successive reports published by the National Academy of Sciences (NAS) in the early 1980s stated that there are excessive levels of sodium in the average American diet (NAS, 1980) and that carcinogenic nitrosamines could be formed from nitrite in cured meats (NAS, 1981; NAS, 1982). These reports raised consumer concerns about sodium-related hypertension and nitrite-related cancer. As a result of these concerns, the meat industry was prompted to use more phosphates to compensate for reduced sodium chloride and nitrite levels in meat processing. A second reason for increased phosphate utilization in meat products was the approval (USDA, 1982) of the use of certain food-grade phosphates (Table 1) as food additives in a wider variety of meat products, including fresh meats that will be frozen, cooked or further processed but excluding hamburger, ground beef, and fresh pork sausage. The third reason for increased phosphate utilization was the demand for more wholesome and safe meat products.

The changes in USDA regulations greatly stimulated research on the applications of phosphates in foods. Although phosphates have never been classified as antimicrobial agents (Molins, 1991), some phosphates in combination with other preservatives have exhibited antimicrobial properties in microbiological media and food products (Ivey and Robach, 1978; Nelson et al., 1983; Sofos, 1986; Tompkin, 1983; Wagner and Busta, 1983). Several investigators have shown that phosphates have direct antimicrobial activity (Kelch and Bühlmann, 1958; Post et al., 1963). In general, phosphates are effective

Table 1. A complete list of approved food-grade phosphates [a] for the meat, poultry and seafood industry by the Meat Inspection Division of the United States Department of Agriculture in 1982

Class	Common name	Abbreviation
Ortho-	Monosodium phosphate	MSP
	Monopotassium phosphate	MKP
	Disodium phosphate	DSP
	Dipotassium phosphate	DKP
Pyro-	Sodium acid pyrophosphate [b]	SAPP
	Tetrasodium pyrophosphate [b]	TSPP
	Tetrapotassium pyrophosphate	TKPP
Tripoly-	Sodium tripolyphosphate [bc]	STPP
	Potassium tripolyphosphate	KTPP
Poly-	Sodium polyphosphate glassy [b]	SPG
	Sodium ultraphosphate [bd]	UP

[a] The legal use limit of phosphates is up to 0.5% by weight of the final product in the United States.

[b] These phosphates were studied in this research project.

[c] STPP is the most popular phosphate used in meat products in the United States.

[d] UP is an acidic, extremely long-chained polyphosphate, glassy with 71% P_2O_5 content.

antibacterial agents in fresh poultry and cooked meats, but not in fresh meats (Molins, 1991). Phosphates enhance the activity of other chemical preservatives in fresh meats (Jarvis and Burke, 1976; Ueoka, 1981), but phosphates alone have no antibacterial activity (Molins et al., 1985, 1987), probably because of the presence in meats of hydrolytic enzymes that rapidly breakdown polyphosphates into orthophosphates. Unlike that in fresh red meats, phosphates are effective antibacterial agents in fresh poultry (Chen et al., 1973; Elliott et al., 1964; Foster and Mead, 1976; Synder and Maxcy, 1979) and cooked meats (Molins, 1991). In fresh poultry, phosphatases are membrane-bound and they are not released even upon extensive meat-processing operations, whereas phosphatases are denatured by heat in cooked meats.

Although considerable research has been devoted to phosphate utilization in meats, the antibacterial mechanism remains largely unknown (Hargreaves et al., 1972; Sofos, 1986; Tompkin, 1983). Current hypotheses of the antibacterial mechanisms of phosphates include chelation of structually essential metal ions (magnesium, calcium) in the cell walls (Gray and Wilkinson, 1965; Post et al., 1963), chelation of metal ions (magnesium, iron) in the cytochrome system (Firstenberg-Eden et al., 1981; Molins et al., 1984; Seward et al., 1982), repression of enzyme synthesis and inhibition of enzyme activity (Wagner and Busta, 1985; 1986; Wood and Clark, 1988), and change in the water activity of the media (Synder and Maxcy, 1979).

The hypothesis that phosphates inhibit by metal-ion chelation is supported by data showing that reversal of inhibition is achieved when excess metal ions are added to the media. This could be a result of several different mechanisms. Phosphates might inhibit by the removal of essential metal ions

from bacteria. Another possibility is that certain phosphate groups might be saturated by excess metal ions. A third possibility is that phosphates chelate metal ions such as magnesium and calcium and release metal ions such as iron, copper, or zinc (Molins, 1991).

Currently, phosphates are used to enhance the four major functional properties in meats described above, but not for antibacterial purposes. Considering that phosphates are used in practically all processed meat, poultry and fish products, and have great potential to be antimicrobial or antibotulinal agents in foods (Sofos and Busta, 1980; Tompkin, 1983), information about the antibacterial mechanism of phosphates is urgently needed. Such information could be applied to the microbiological safety of cooked meat products, such as modifying the ingredients or procedures in meat processing to achieve direct and maximum antibacterial effects.

The knowledge of the antibacterial mechanism(s) of SAPP and UP are especially important because SAPP is an effective anticlostridial agent in cooked meat products and its effects are additive to that of sodium nitrite (Barbut et al., 1986; Madril and Sofos, 1986; Molins et al., 1984; 1985; 1986; 1987; Nelson et al., 1983; Wagner and Busta, 1983). Furthermore, an acidic, extremely long-chained ultraphosphate having a P_2O_5 content of 74% (UP-74) has unique antimicrobial properties; it inhibits mold growth and toxin production (Lebron et al., 1989ab). UP-74 (74% P_2O_5) exceeds the limit (< 72% P_2O_5) for being classified as a hexametaphosphate approved by the USDA for use in meat products. However, similar very long-chain polyphosphates may also have antimicrobial properties. This speculation was examined by Kung (1991) and Marsh (1992). Kung (1991) studied the effects of polyphosphates in frankfurters and found that 0.4% UP

(71% P_2O_5) had the best antilisterial activity when compared to TSPP and STPP at the same level. Although UP did not significantly affect sensory attributes, acceptable product color, emulsion stability, purge and firmness characteristics were obtained from combinations of 0.1% UP and 0.3% TSPP, and 0.1% UP and 0.3% STPP. Marsh (1992) studied the effects of phosphates on the growth, protease production and protease activity of *Pseudomonas fragi*. UP significantly reduced the growth rate and levels of protease activity of this Gram-negative bacterium in bacteriological media.

Chemical Properties of Phosphates

Sequestration of metal ions

Sequestration, or soluble complex formation, is the result of a competitive reaction between a sequestrant anion and a precipitating anion for a metal ion (Ellinger, 1972). In general, polyphosphates have greater sequestering or chelating ability than orthophosphates (van Wazer and Callis, 1958). That is, tripolyphosphates form stronger complexes with metal ions than those of pyrophosphates, which in turn form stronger complexes than orthophosphates.

Polyphosphates are defined as unbranched structures of the elementary composition $M_{n+2}P_nO_{3n+1}$ where M is a metal or hydrogen (Harold, 1966). Linear polyphosphates form complexes with most cations due to the rotational flexibility of P-O-P linkages within the polyphosphate chain (Corbridge, 1974). Cations may be divided into three groups according to the strength of the complexes formed with polyphosphates : quaternary ammonium ions < alkali metals (K < Na) < alkaline earth metals (van Wazer and Campanella, 1950). The ability of polyphosphates to complex metal ions is proportional to the total

number of phosphorus atoms in the molecule, regardless of the chain length. This suggests that fragmentation of long phosphate chains would not affect metal ion chelation. In alkali metal-polyphosphate solutions, most metal cations form insoluble precipitates. However, those precipitates become soluble in the presence of high levels of polyphosphate due to complex ion formation.

Although pyrophosphates are best for sequestering heavy metal ions such as iron and copper (Steinhauer, 1983), they form weaker complexes than those of polyphosphates. In order to chelate one part of heavy metals, five to ten parts of TSPP are required (Greenfield and Clift, 1975). In general, TSPP forms insoluble precipitates with multiply charged metal ions.

Although orthophosphate complexes of the alkali and alkaline earth metals are weaker than those formed by polyphosphates, orthophosphates form relatively stable complexes with transition metals (van Wazer and Callis, 1958). Ferric ion complexes with orthophosphates are more stable than manganese ion complexes, which suggests that iron-oxygen covalent bonds are formed in ferric ion complexes with orthophosphates.

The chelating ability of phosphates is pH dependent (van Wazer and Callis, 1958). At pH values below 8, longer chain polyphosphates chelate calcium more effectively than pyrophosphates or tripolyphosphates. As pH increases from 8 to 11, chelation of calcium increases. A continuous decrease of calcium chelation is observed as pH increases beyond 11 or drops below 8. Unlike that of calcium, pyrophosphates chelate magnesium more effectively than tripolyphosphates, and in turn more effectively than the glassy phosphates. However, polyphosphates chelate five times more magnesium than calcium. Therefore, long chain polyphosphates are still the best sequestering agents for

calcium and magnesium (Steinhauer, 1983). Their sequestering efficiency increases as pH increases. Also, in contrast to calcium, chelation of ferrous and ferric ions by polyphosphates decreases with increases of pH and chain length (Irani and Morgenthaler, 1963). Likewise, the sequestering efficiency of short-chain phosphates, especially pyrophosphates, decreases as pH increases (Steinhauer, 1983).

The chelating ability of phosphates is temperature-dependent (Molins, 1991). Chelation increases with increasing temperature.

Acidification and pH buffering

pH buffers are mixtures of weak acids and their conjugate bases which are used to resist pH change in a system. In phosphoric acids, there is one strongly ionized hydrogen for every phosphorus atom and there is one weakly dissociated hydrogen at each end of the phosphate molecule, which in turn defines the ratio of strong to weak titratable acid functions (van Wazer and Holst, 1950a). It is this ratio that determines the buffering capacity of phosphate. The longer the chain length of a phosphate molecule, the greater the ratio of strong to weak titratable acid functions, and therefore the lower the buffering capacity of that phosphate molecule. This explains why polyphosphoric acids and their salts have limited buffering capacity, whereas ortho-, and pyrophosphoric acids and their salts are important, active acidifying and buffering agents (Molins, 1991). The active pH ranges of ortho-, and pyrophosphates are 2 to 3, 5.5 to 7.5, and 10 to 12 (van Wazer, 1971).

Polyanionic property

All phosphates behave like polyanions. Polyphosphates with lots of negatively charged ions exhibit more pronounced polyanionic effects than those with fewer negatively charged ions. If one end of their chain is attached to a positively charged site on a particle surface, the rest of the chain can attract water molecules from the solution. This stabilizes particles in suspension by separating them with water molecules (Steinhauer, 1983). In contrast, polyphosphates can precipitate particles by binding two or more positively charged sites (Steinhauer, 1983).

The polyanionic properties of phosphates are applied in both food and nonfood industries. In the food industry, phosphates are used to stabilize food emulsions and to disperse colloidal suspensions (Molins, 1991). In the dairy industry, phosphates are used to stabilize colloidal suspensions by increasing the negative charge of particles in aqueous suspension, and thereby enhancing repulsion between similarly charged particles (Knightly, 1969). In nonfood industries, this property is applied to processes such as deflocculation of clays or flocculation of paper pulp (Guarro, 1973). In water treatment, sodium polyphosphate glassy (SPG) is used to prevent clogging of pipes by blocking the formation of calcium carbonate crystals from hard water (Molins, 1990).

Phosphate hydrolysis

Phosphate hydrolysis is a problem in meat processing because of the possible loss of functional phosphate properties, including its antimicrobial activity. Studies of phosphate hydrolysis have shown that it occurs in

microbiological media and foods due to high temperatures, enzyme activity, and bacterial metabolism (Molins, 1991).

Linear polyphosphates are stable in aqueous solutions at 25°C, whereas tridimensional ultraphosphates are rapidly degraded in solution (van Wazer and Holst, 1950b). Although linear polyphosphates are relatively stable in alkaline or neutral solutions at 25°C, increasing temperatures or pH shifts away from neutral values accelerate their hydrolysis (Greenfield and Clift, 1975). Bell (1947), one of the pioneers of phosphate chemistry, found that hydrolytic susceptibilty at 100°C increased with increasing chain length: TSPP < STPP < SPG. This hydrolytic instability increases until a chain length of 10 is reached and then decreases afterwards (Corbridge, 1974). TSPP does not break down, even after 10 hours at 100°C (Bell, 1947). It has been shown that the heat-induced hydrolysis of phosphates reduces their antibacterial activity in culture media (Firstenberg-Eden et al., 1981), although TSPP is an exception (Molins et al., 1984; Zessin and Shelef, 1988). Moreover, SAPP that had been heated in an autoclave (121°C) was shown to enhance bacterial growth (Molins et al., 1984).

Phosphate hydrolysis is also affected by the presence of heavy metals such as calcium and magnesium, especially in alkaline solution (Dwyer, 1964). In the case of calcium and magnesium ion chelation, there is an increase in the positive charge of the phosphorus atom, which results in a higher susceptibility of the phosphorus atom to nucleophilic attack by water or by hydroxyl ions (Corbridge, 1986; van Wazer and Campanella, 1950). Thus, the presence of some heavy metals can result in increased rates of phosphate hydrolysis.

In general, the time between the addition of phosphates to raw meats and cooking allows meat phosphatases to hydrolyze the added phosphates. The

variations in this processing time by different personnel or companies may result in variations in the extent of phosphate hydrolysis, and thereby, lead to variations in product quality (Molins, 1991). It has been demonstrated that heat inactivation of meat phosphatases begins at about 40°C; all meat phosphatases are destroyed at 60°C during cooking (Awad, 1968). However, more research is needed to test the possibility of reactivation of those enzymes in cooked meat products during refrigerated storage.

The rate of phosphate hydrolysis also increases in the presence of microbial phosphatases (Molins, 1991) and, up to a point, increasing phosphate concentration in the solution (Bell, 1947).

Functional Properties of Phosphates in Meat Processing

Increase in water binding and meat binding

During meat processing and marketing from slaughter through porcessing, packaging and storage, muscle tissue is stressed which results in significant water loss. The undesirable effects of water loss from muscle tissue include decreased meat palatability from drier and tougher muscle, lower nutritional value from loss of soluble proteins, vitamins, minerals, and lipids, and enhanced meat spoilage.

Protein (18% w/w) is responsible for retaining water (75% w/w) in muscle tissue. The major protein component of muscle tissue is actomyosin. Certain phosphates (TSPP, STPP) have a unique ability to separate this protein into actin and myosin. When separated, the actin and myosin develop strong water-binding capacities, which reduces water loss (Knipe, 1982).

If the ionic strength of the meat fluids is high enough, myosin will solubilize and increase the viscosity of meat fluids. This helps to seal pores in the meat and physically prevents water, protein and mineral loss. In addition, it retards the diffusion of oxygen into the meat. Sodium chloride is used synergistically with phosphates to raise the ionic strength of meat fluids and, therefore, increase water binding (Knipe, 1982).

High pH also increases water binding. At the isoelectric point of muscle protein (pH 5.4), muscle protein is stable because of zero net internal charges. As the pH is raised, proteins unfold and bind ions and polar water molecules to maintain charge balance, and thereby to regain stability. Apart from separating actomyosin, alkaline phosphates (TSPP and STPP) also increase water binding by their alkaline pH. Trout (1984) reported that a pH 6.0 and a total ionic strength of 0.6 are needed for maximum binding and cooking yield.

Moreover, TSPP and STPP are capable of increasing meat binding by solubilizing myosin into viscous meat fluids (Siegel et al., 1978). When the meat product is heat cured, protein coagulates and this natural cement binds meat pieces into a cohesive whole for further processing or sale.

Therefore, phosphates (TSPP > STPP > SPG) increase water binding and meat binding in beef (Shults et al., 1972), chicken (Shults and Wierbicki, 1973), and frankfurters (Knipe et al., 1985), which in turn enhance meat palatability by preserving tenderness and flavor. In contrast, SAPP lowers the pH of meats, and thereby decreases water binding in beef, pork, chicken, and turkey muscle (Terrell et al., 1982).

Enhancement of emulsification

The emulsifying properties of proteins are important to maintain the stability of a three-phase (protein, fat, water) system in emulsified meat products. Proteins form a network or coating around the fat molecules to prevent the formation of large fat globules. Phosphates further enhance emulsification by blocking the formation of fat pockets. Barbut (1988) studied the effects of phosphates in reduced-salt poultry-meat batters and found that 0.4% SPG or SAPP greatly enhanced emulsion stability as observed under the scanning electron microscope. Of the two phosphate preparations, SPG was the most effective. Therefore, phosphates enhance meat palatablity by enhancing emulsification, which preserves tenderness and flavor.

Retardation of oxidative rancidity and color deterioration

Color deterioration in muscle foods is a primary factor affecting consumer acceptance. The major cause of color and flavor deterioration is oxidation. Oxidation of myoglobin in fresh and cured red meats leads to faded color. In white and light meats, the oxidation of lipids results in development of a yellow color. Lipid oxidation also is the cause of rancid off-flavors in all meat products.

The occurrence of oxidation in meats requires three elements : oxygen, an oxidizable substrate, and a catalyst. Molecular oxygen is almost everywhere, but oxidation initiated directly by oxygen is relatively insignificant in meats. Peroxides initiate oxidation in meats. Fortunately, the generation of peroxides and subsequent oxidation of meat constituents proceeds slowly unless a catalyst is present. Although heat and light are common, generally unavoidable catalysts, their effects are relatively minor when compared to metals such as iron and

copper (Shimp, 1983). Iron is the carrier of oxygen in most of the living animals. Copper is the carrier of oxygen in shellfish. These metal ions are regulated by proteins in living animals. After death, regulatory mechanisms are disrupted and destructive oxidation catalyzed by these metals results.

The use of an oxygen-scavenging chemical like erythorbate and the use of vacuum packaging retard the development of oxidative rancidity and faded color. In addition, it has been demonstrated that ascorbic acid acts synergistically with pyro- and polyphosphates in protecting cooked meat against oxidative rancidity (Tims and Watts, 1958). Therefore, nearly complete protection against oxidation can be achieved by the addition of phosphates: SAPP (St. Angello et al., 1988), TSPP and STPP (Shahidi et al., 1986; Tims and Watts, 1958), and SPG (Tims and Watts, 1958). It has been suggested that as the chain length of phosphates lengths, the ability to prevent lipid oxidation decreases (Shahidi et al., 1986). These phosphates sequester iron and copper effectively, thereby decreasing the ability of the catalysts to participate in oxidation reactions. They also protect the product during processing and prevent premature depletion of erythorbate. In 1987, the USDA approved a color-protecting composition that included phosphates for use in fresh meats (Molins, 1991).

Enhancement of cured-color development

The color of red meats is imparted by an iron-containing protein, myoglobin. The color of cured meats is attributed to a nitric oxide-myoglobin complex, which is formed by an interaction between nitrite and myoglobin. Although the cured color-fixing reaction is rapid, cured color may not be fully developed under some processing conditions. Therefore, a cure accelerator, such

as sodium erythorbate, is usually used, especially when rapid curing is required in some continuous production lines. The acidification of meat emulsions also results in acceleration of cured-color development (Molins, 1991). However, the lower pH brings meat proteins closer to their isoelectric points, which decreases water binding and emulsification. SAPP solves this problem by providing the lower pH for rapid color development as well as dissociating actomyosin to increase water binding to compensate for the loss of water from low pH. If sufficient curing time is available, alkaline phosphates (TSPP, STPP) should be used for cured-color development and gaining the beneficial effects of enhanced water binding and emulsification.

Current Hypotheses of Antibacterial Mechanism of Phosphates

Chelation of metal ions in the cell walls

Post et al. (1963) discovered that Gram-negative bacteria were phosphate-resistant and could survive 10% SPG. In contrast, Gram-positive bacteria were inhibited by 0.1% SPG and lysed almost immediately in 10% SPG. Because bacterial inhibition and lysis could be overcome by adding $MgSO_4.7H_2O$ and NaCl, Post et al. postulated that SPG interferes with divalent cation metabolism, particularly magnesium, which in turn blocks cell division and affects cell wall integrity.

Elliott et al. (1964) reported that 1% polyphosphates or 0.75% STPP + 0.25% TSPP were inhibitory to nonfluorescent *Pseudomonas* spp. in a synthetic medium, whereas fluorescent strains of pseudomonads produced a strong metal chelator called proverdine, which was able to overcome the inhibition. Moreover, the antibacterial activity of phosphates was overcome by magnesium

and bacteriological peptone, which moved Elliott et al. (1964) to support the hypothesis that phosphates acted primarily by metal-ion chelation, not by phosphate-induced pH changes.

Gray and Wilkinson (1965) further advanced the metal-ion chelation hypothesis. They discovered that the bactericidal activity of SPG was a result of chelation of structurally essential metal ions, particularly calcium, from the cell wall of *Pseudomonas aeruginosa*. The end effects were leakage of cytoplasmic material that absorbed at 260 nm, cell lysis, and loss of cell viability. Roller and Woods (1989) also found that polyphosphates affected cell viability, magnesium binding, and leakage of cell constituents. They concluded that the antibacterial mechanism of polyphosphates was damage to the bacterial envelope. Firstenberg-Eden et al. (1981) studied the inhibition of *Moraxella-Acinetobacter* by phosphates. They also supported the metal-ion chelation postulation. Blankenship and Craven (1985) found that leakage of cytoplasmic material from *Campylobacter jejuni* increased at higher pH values. The addition of divalent cations helped to stabilize the membranes and ribosomes.

A heat-sensitization study of salmonellae by polyphosphates was conducted by Seward et al. (1968). They concluded that, during heating, polyphosphates first adsorbed onto bacterial cells, followed by chelation and extraction of stabilizing cellular components.

Kulaev (1979) claimed that tripolyphosphate was the form of polyphosphate appropiate for transport through membranes of fungal cells and possibly cells of other organisms. This suggestion correlates with the finding that the uptake rate of tripolyphosphate from the media to cotton roots is the most rapid of several polyphosphates tested, including orthophosphate (Valikhanov

and Sagdullaev, 1979). In addition, tripolyphosphate is a primer for the biosynthesis of high molecular-weight polyphosphates in the cells.

Knabel et al. (1991) used a simple well-plate technique to determine the effects of various metal cations on the growth of microorganisms on media containing phosphates. As polyphosphates have higher cation-binding affinities (Irani, 1961; Irani and Callis, 1962; Irani and Morgenthaler, 1963) than peptidoglycan (Matthews et al., 1979), teichoic acids and teichuronic acids (Heckels et al., 1977; Lambert et al., 1975), the authors speculated that polyphosphates inhibited Gram-positive bacteria by removing essential metal cations from these cation-binding sites in the cell walls.

In general, the antibacterial property of phosphates is more effective in simple cultural media than in complex media (Zessin and Shelef, 1988). In order to achieve the same antibacterial effect, more phosphates will be needed in complex food systems than in culture media. Also, Gram-positive bacteria and young (3-hour-old) cultures are generally more sensitive than Gram-negative bacteria and older (24-hour-old) cultures (Chen et al., 1973; Firstenberg-Eden et al., 1981; Molins et al., 1984; Tutumi et al., 1976; Zessin and Shelef, 1988) to phosphates. It is still unclear why Gram-positive bacteria are more susceptible than Gram-negative bacteria to phosphates. Some researchers have suggested that phosphates induce the formation of inhibitory compounds in culture media. Carlsson et al. (1978) reported that autoclaved, anaerobic media containing phosphate and glucose tended to accumulate hydrogen peroxide. *Peptostreptococcus anerobius* was killed by superoxide from the autooxidation of accumulated hydrogen peroxide. In constrast, Gram-negative bacteria survived better than Gram-positive bacteria because of their enhanced manganese-

containing superoxide dismutase (MnSOD) and/or catalase synthesis under elevated oxygen pressure, resulting in the conversion of toxic superoxide and hydrogen peroxide to less harmful products (Schiavone and Hassan, 1985). On the other hand, Knabel et al. (1991) believed that Gram-negative bacteria were more resistant than Gram-positive bacteria to polyphosphates because the Gram-negatives possessed high-affinity systems to bind and transport essential metal cations.

Chelation of metal ions in the cytochrome system

Sculman and Dwyer (1964) reported that various chelating agents could be used to select petite colonies of some strains of *Staphylococcus aureus*. These petite colonies were virulent and antibiotic-resistant. They had slower growth rates, increased glucose requirements, and decreased capacity to use oxygen. These characteristics indicate the presence of an impaired cytochrome system and reduced ATP synthesis. Pin-point or petite colonies with defective cell division also were observed in culture media containing subinhibitory concentrations of phosphates (Firstenberg-Eden et al., 1981; Molins et al., 1984; Seward et al., 1982). The chelation of magnesium and iron, needed for the activity of the cytochrome system, also may be involved in the antimicrobial mechanism of phosphates (Molins, 1991).

Repression of enzyme synthesis and inhibition of enzyme activity

Cell walls, membranes, and cytoplasm are the target sites of many inhibitory compounds (Hugo, 1976). Many researchers have focused on the cytoplasm and have demonstrated that metal-ion chelation by phosphates results

in the inhibition of essential bacterial enzymes. Many bacterial phosphatases and other enzymes require specific metal ion cofactors, particularly magnesium or manganese, to function (Molins, 1991). For examples, polyphosphate kinase in *Escherichia coli* requires magnesium for the biosynthesis of long chain polyphosphate (Kornberg et al., 1956), polyphosphate-adenosinemonophosphate (AMP)-phosphotransferase in *Corynebacterium xerosis* requires magnesium for the degradation of polyphosphate (Dirheimer and Ebel, 1965), polyphosphate glucokinase in *Mycobacterium phlei* requires magnesium for polyphosphate degradation (Szymona and Ostrowski, 1964), and polyphosphatases in *Corynebacterium xerosis* (Muhammed et al., 1959) and *Aerobacter aerogenes* (Harold and Harold, 1965) require magnesium for polyphosphate degradation. Specific concentrations of these cofactors are needed for optimum activity (Foster et al., 1978). When these metal ion cofactors are chelated by phosphates, the enzymes cannot function properly, thereby affecting bacterial metabolism in many ways.

Akagi and Campbell (1963) reported that magnesium and manganese were required for optimum activity of an alkaline pyrophosphatase in *Desulfovibrio desulfuricans*. Further, the optimum ratio of enzyme to magnesium was 1:1 at pH 8.0. The alkaline pyrophosphatase was rapidly inactivated at 40^{o}C. Alkaline phosphatases are inhibited by orthophosphate, pyrophosphate, and other chelating agents, such as EDTA. Various alkaline phosphatases contain zinc, iron, manganese or magnesium (Whitaker, 1972).

In general, little is known about why phosphate blends composed of SPG and pyrophosphates (SAPP and/or TSPP) are more effective as antibacterial agents than individual phosphates. Additive or synergistic interactions may exist between pyro- and polyphosphates (Molins, 1991). Weimberg and Orton

(1963) studied two acid phosphatases in a yeast, *Saccharomyces mellis*. One of the acid phosphatases was a pyrophosphatase (pH optimum 7.5) that required magnesium for activity. If SAPP and SPG are added to the fresh, postrigor meats (pH 4.5-6.0), chelation of magnesium by SPG inactivates the pyrophosphatase, which in turn prevents the hydrolysis of SAPP. More studies on the optimum combination of pyro- and polyphosphates for maximum antibacterial properties, and on the characterisitics of microbial and meat phosphatases, should result in the ability to optimize the effectiveness of phosphates in meat processing.

Another possible antibacterial mechanism of phosphates, particularly SAPP, on enzyme inhibition can be proposed, based on the works of Kornberg (1957) and Harold and Harold (1965). Harold and Harold (1965) found that external orthophosphates repressed synthesis of intracellular polyphosphate kinase in *Aerobacter aerogenes*, thereby inhibiting internal polyphosphate formation. Kornberg (1957) reported that external orthophosphates repressed an intracellular polyphosphate kinase in *E. coli* that catalyzed ATP formation from intracellular polyphosphate and ADP. These results suggested that external orthophosphates are involved in the inhibition of enzyme synthesis and activity essential for nucleic acid synthesis (Molins, 1991). This supports the observation that an antagonistic relationship between polyphosphates and nucleic acid metabolism may exist in bacteria (Mudd et al., 1958; Smith et al., 1954; Wilkinson and Dugoid, 1960).

Further, McKellar and Cholette (1984, 1985) found that excess orthophosphates inhibited proteinase synthesis in *Pseudomonas fluorescens*. The partial reversal of inhibition of enzyme synthesis by calcium and manganese

suggests that metal-ion chelation may be involved in the the antibacterial mechanism.

Apart from serving as an energy source in several reactions in pro- and eucaryotes (Baltscheffsky, 1967), inorganic pyrophosphates regulate and inhibit enzymes (Kleppe, 1966; Pina et al., 1975; Sawhney and Nicholas, 1978). For example, Wagner and Busta (1985) reported that SAPP inhibited the toxin-activating protease of *Clostridium botulinum* when SAPP was added to culture media. SAPP may be a good antibotulinal agent in meats. Later, the same authors attempted to define the antibotulinal mechanism of SAPP on protease activity and found that [^{32}P]-SAPP was associated with the RNA fraction of *Clostridium botulinum* (Wagner and Busta, 1986). They proposed that the synthesis of proteases mediated by RNA may be affected by the association of SAPP with RNA. More study is needed to prove this hypothesis.

In Pacific cod fish muscle, Tarr et al. (1969) demonstrated that SAPP inhibited a 5'-nucleotidase which catalyzes the hydrolysis of adenosinemonophosphate (AMP) to adenosine and orthophosphate.

Change in water activity of the media

Radiation-resistant strains of *Moraxella-Acinetobacter* isolated from meats were inhibited in SPG-reduced water activity ($a_W < 0.99$) cuture medium (Synder and Maxcy, 1979). Normal bacterial growth resumed when the a_W was brought back to the original level. This phenomenon was observed in both culture media and in ground beef and pork, which led the authors to postulate that bacterial inhibition by SPG was caused by decreased a_W. However, Tompkin (1983) and

Sofos (1986) suggested that phosphates have little or no effect on a_W at the levels used in foods.

Staphylococcus aureus

A model bacterium

Staphylococcus aureus is a Gram-positive, nonmotile, nonsporulating, facultative anaerobic coccus. It is often present in raw meats and poultry. In addition to members of the genera *Micrococcus, Streptococcus,* and *Lactobacillus, Staphylococcus aureus* is a bacterium found frequently in cured and processed meats (Aspelund, 1984; Jay, 1986). There are four major types of food infections or intoxications associated with cured and processed meats: salmonellosis, staphylococcal intoxication, botulism, and perfringens intoxication. The enterotoxigenic strain of *Staphylococcus aureus* is one of the longest known and best understood bacterial foodborne pathogen that causes food intoxication. Moreover, it causes the second most costly foodborne disease in the United States (Todd, 1989).

Staphylococcus aureus survives environments that are high in protein, sugar, or salt (17%) content and that possess a low water activity (Aw 0.83-0.86) (Molins, 1986). It tolerates moderate levels of selective compounds like polymyxin, mercuric chloride, neomycin, sodium azide, and tellurite (Jay, 1986). *Staphylococcus aureus* also produces many extracellular products, such as thermostable nuclease, proteases, coagulase, and phosphatases (Jay, 1986). The bacteria are rapidly destroyed by moderate heat, but the enterotoxins are thermostable. In general, growth occurs over the range of 6.7-47^{o}C (Molins, 1986). The optimum growth temperature is 35^{o}C. Enterotoxins are produced

between 10-46°C during all phases of growth (Jay, 1986). The lowest pH of growth is pH 4.8 in the presence of molecular oxygen and pH 5.5 in the absence of oxygen (Molins, 1986). From the results of previous and present studies, Gram-positive bacteria are more sensitive to phosphates than are Gram-negative bacteria. Among all the tested Gram-positive bacteria, *Staphylococcus aureus* is the most sensitive to polyphosphates. In addition, it is also the species used to study the antibacterial mechanism of nitrite. Therefore, *Staphylococcus aureus* was chosen as a model bacterium in this study.

Cell wall structure

In Gram-positive eubacteria, the cell wall comprises 40-50% of the cellular dry weight in staphylococci, streptococci and bacilli (Doyle, 1989). The cell wall is about 25 nm thick and is comprised primarily of peptidoglycan (Beveridge, 1989). Peptidoglycan consists of repeating units of ß(1->4)-linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). It is a rigid backbone to which cell wall polysaccharides are attached. According to the structural characteristics, cell wall polysaccharides are classified into three groups: teichoic acids (glycerol teichoic acids, ribitol teichoic acids), teichuronic acids, and other polysaccharides (Munson and Glaser, 1981). In *Staphylococcus aureus,* the major cell wall polysaccharide is ribitol teichoic acid. These are known as group- and type-specific antigens in the serological classification of Gram-positive bacteria (Knox and Wicken, 1973). They also participate in cation assimilation (Beveridge and Murray, 1976b; Ellwood and Tempest, 1973; Hoover and Gray, 1977), serve as a phosphate reserve (Grand, 1979), are involved in bacteriophage attachment (Chatterjee, 1969; Coyette and Ghuysen, 1968), and

control autolytic activity (Höltji and Tomasz, 1975). However, their actual biological functions are not well defined (Lambert et al., 1975a). They are under physiological control and affected by the levels of phosphates and magnesium in the growth medium (Archibald, 1974).

Before the early 1970s, the linkage between peptidoglycan and cell wall polymers was believed to be a covalent acid-labile phosphodiester bond (Araki and Ito, 1989). Today, it is known that the linkage unit is (glycerol phosphate)3-N-acetylmannosamine-(ß1->4)N-acetylglucosamine. The linkage is: teichoic acid-(Gro-P)3-ManNAc-GlcNAc-P-peptidoglycan.

The phosphodiester groups of teichoic acids and the carboxyl groups of peptidoglycan are potent metal coordinators in Gram-positive bacteria (Beveridge and Murray, 1976; Lambert et al., 1975ab). It was demonstrated that *Staphylococcus aureus* cell walls have cation-binding capacities in the following descending order: trivalent > divalent (Mg=Ca) > monvalent (Archibald et al., 1973; Cutinelli and Galdiero, 1967). Galdiero et al. (1967) also reported that binding capacity decreased in the following order: $Ce^{3+} > Cu^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+} > K^{+}, Na^{+}$. Irving and Williams (1948) further defined the stability of divalent cations complexed to be Pd > Cu > Ni > Co > Zn > Cd > Fe > Mn > Mg. In addition, Galdiero (1967) suggested that different mechanisms of interaction existed between the cell wall of *Staphylococcus aureus* and mono- and divalent cations. For divalent and trivalent cations, their counterions are closely bound by electrostatic interaction. In contrast, the monovalent counterions form a very highly mobile monolayer on the surface of the polymer in the cell wall. In general, the association between metal ions and cell wall is quite low, on the

order of 10^3-10^4 M^{-1} (Doyle, 1989). There are more electronegative sites on the outer surfaces than on the inner surfaces of the wall (Umeda et al., 1987).

As mentioned before, ribitol teichoic acid in the *Staphylococcus aureus* cell wall participates in cation assimilation, particularly magnesium. Ribitol teichoic acid is the major component of the walls responsible for binding magnesium (Heptinstall et al., 1970) because the peptidoglycan of *Staphylococcus aureus* is highly cross-linked (Archibald, 1972). In *Bacillus licheniformis* cell walls, teichoic acid also is the major site of metal binding (Beveridge et al., 1982). It was shown that all of the magnesium and half of the calcium in the wall are bound to teichoic acid, whereas peptidoglycan is involved in less than 10% of the metal bound.

Magnesium is required for the assembly and stability of bacterial cell membranes (Fiil and Branton, 1969), for the integrity of the outer membrane of the cell wall of Gram-negative bacteria (Rogers et al., 1969), for the stabilization of intracellular structures, particularly RNA and DNA, and for the function of macromolecules involved in the production and use of ATP (Ahrland et al., 1958). Calcium is the major mineral in bacterial spores (Collins and Stotzky, 1989). Magnesium and calcium also are components of the membrane and are required for the correct packing order of the lipid components (Ferris and Beveridge, 1986). These metals are an integral "wall cement" for teichoic acids of bacilli and lipopolysaccharides of coliforms (Ferris and Beveridge, 1984; 1986; Lambert et al., 1975). Magnesium stabilizes cell walls by cross-linking wall polymers, whereas other multivalent cations help to withstand osmotic stress and plasmolysis by cross-linking membrane lipoteichoic acids to anionic wall polymers. Metal ions are required for the integrity of bacterial cell walls and for

the attachment of their superficial layers (Beveridge and Murray, 1976a). They also are required for the activity of many enzymes located at or near the cell surface. These include teichoic acid synthetases, phospholipid-synthesizing enzymes, peptidoglycan synthetases, and autolysins (Doyle, 1989).

Ribitol teichoic acid provides a reservoir of bound magnesium in the cell wall by selectively chelating magnesium from the surrounding medium (Hughes et al., 1973). Magnesium forms bridges across phosphate groups in adjacent ribitol teichoic acid chains in *Staphylococcus aureus* cell walls (Lambert et al., 1975a). Magnesium is then transferred to membrane teichoic acid and is involved in the activity of various enzyme systems, membrane stability, and transfer to the cytoplasm (Hughes et al., 1973). During the exponential growth of species of *Staphylococcus, Bacillus, Lactobacillus, Listeria, Escherichia, Salmonella, Neisseria,* and others, wall components are shed into the medium during each cell division (Doyle et al., 1988). These wall components are not reutilized or recycled. In *Bacillus subtilis,* more than 50% of the wall is lost in cell-wall turnover (Doyle and Koch, 1987). However, the mechanisms of the binding and transfer of metal ions remain largely unknown (Lambert et al., 1975a).

Analytical observations of these wall polymers show that much more metal is bound than can be accomodated on the available chemical groups in the wall (Beveridge and Murray, 1976b). Also, metal precipitates have been observed on and within the walls by using electron microscopy. These facts suggest that metal deposition occurs as a two-step process. The initial step involves reaction between the metal and the reactive chemical groups, which provides nucleation sites for the secondary deposition of more metal ions (Beveridge, 1978).

Before the 1970s, the bacterial cell wall was viewed as a rigid structure. Today, it is known that bacterial cell walls are flexible (Ou and Marquis, 1970). Metal ions can neutralize the repulsive negative charges in walls and cause the walls to contract. Lambert et al. (1975a) found that at 0.2 mM or higher concentrations of magnesium, alanyl esters in the*Staphylococcus aureus* cell wall control the amount of bound magnesium. On the other hand, the growth of cells in reduced levels of magnesium gave rise to cells with walls containing high amounts of phosphorus which binds more magnesium (Heptinstall et al., 1970). However, in the presence of sodium chloride, the amount of alanyl ester in *Staphylococcus aureus* cell walls is greatly decreased and the walls still bind more magnesium (Heptinstall et al., 1970). An increase of wall-bound teichoic acid is observed in high sodium chloride concentrations, which results in greater magnesium binding capacity (Ellwood and Tempest, 1972). Increasing concentrations of sodium chloride also produce a contraction of the cell walls of Gram-positive bacteria (Ou and Marquis, 1970). In contrast, more esterified D-alanine is present in walls at low pH, which decreases magnesium binding (Archibald et al., 1973). Metal-ion binding seems to be tightly coupled with high ratios of negative to positive charges (Doyle, 1989). The versatility of Gram-positive bacteria in response to changes in their growth environment is remarkable.

Research Objectives

The approval (USDA, 1982) of the use of certain food-grade phosphates (Table 1) as food additives in a wide variety of meat products greatly stimulated research on the applications of phosphates in foods. Although phosphates have

never been classified as antimicrobial agents (Molins, 1991), several studies have shown that phosphates have indirect (Ivey and Robach, 1978; Nelson et al., 1983; Sofos, 1986; Tompkin, 1983; Wagner and Busta, 1983) and direct (Kelch and Bühlmann, 1958; Post et al., 1963) antimicrobial activity. Considerable research has been devoted to phosphate utilization in meats, but the antibacterial mechanism remains largely unknown (Hargreaves et al., 1972; Sofos, 1986; Tompkin, 1983).

Currently, phosphates are used as additives to enhance the major four functional properties in meats, but not for antibacterial purposes. Considering that phosphates are used in practically all processed meat, poultry and fish products, and have great potential to be antimicrobial or antibotulinal agents in foods (Sofos and Busta, 1980; Tompkin, 1983), information about the antibacterial mechanism of phosphates is urgently needed.

The objectives of this study were to define the antibacterial mechanism(s) of phosphates (TSPP, STPP, SAPP, SPG, and UP) allowed for use in meat products. Attempts were made to elucidate their target site(s) in early-exponential phase cells of *Staphylococcus aureus* and to trace their subsequent effects on the cells. Another objective was to apply this knowledge to the development of recommended procedures for the use of phosphates, particularly ultraphosphates, in meat processing to achieve direct and maximum antibacterial effects.

MATERIALS AND METHODS

Bacterial Cultures

Staphylococcus aureus ISP40 8325 (Dr. Peter A. Pattee, Department of Microbiology, Immunology and Preventive Medicine, ISU, Ames, IA) was the principal organism used in this study. The following organisms were used in the minimum inhibitory concentration (MIC) study: *Staphylococcus aureus* (A-16) IDM Q3, *Escherichia coli* K 12 and *Pseudomonas fluorescens* EV-21 were obtained from the culture collection in the Department of Microbiology, Immunology and Preventive Medicine at ISU; *Escherichia coli* 15 TAU was obtained from Dr. Barbara J. Bachman, Department of Biology, Yale University; *Streptobacterium* sp. was isolated from extended-storage, refrigerated, vacuum-packaged cooked pork.

Stock cultures of bacteria were maintained by monthly subculture on brain heart infusion (BHI, Difco Laboratories, Detroit, MI) agar slants and stored at 4°C.

Media

All bacterial culture media, phosphate stock solutions, metal solutions, and 0.85%(w/v) NaCl suspending medium (saline) were prepared with distilled water that had passed through a 3-stage deionizer unit (Barnstead-Millipore Corp., Boston, MA). BHI broth, saline and metal solutions were adjusted to pH 6 with 5 N HCl or NaOH, followed by sterilization in an autoclave at 121°C for 15 minutes. Modified complete defined synthetic (mCDS) medium (Table 2, modified from Dr. Peter A. Pattee, Department of Microbiology, Immunology and Preventive Medicine, ISU) and phosphate stock solutions were adjusted to

Table 2. Composition of modified complete defined synthetic (mCDS) medium [a]

Ingredient	Amount/liter	Ingredient	Amount/liter
Amino Acids (100X) [b]		N-acetylglucosamine (100X)	20mg
L-alanine	60mg	Purines & pyrimidines (200X)	
L-arginine	10mg	Adenine	10mg
L-aspartic acid	90mg	Cytosine	10mg
L-cystine	20mg	Guanine	10mg
L-glutamic acid	100mg	Uracil	10mg
glycine	50mg	Thymine (50X)	10mg
L-histidine	20mg	Salts (5X)	
L-isoleucine	30mg	K_2HPO_4	7.000g
L-leucine	90mg	KH_2PO_4	7.565g
L-lysine	50mg	$MgSO_4$	0.050g
L-methionine	10mg	$Na_3citrate.2H_2O$	0.400g
L-phenylalanine	40mg	$(NH_4)_2SO_4$	1.000g
L-proline	80mg	Vitamins (100X)	
L-serine	30mg	Biotin	0.005mg
L-threonine	30mg	Ca pantothenate	0.250mg
L-trytophan	10mg	Niacin	1.200mg
L-tyrosine	50mg	Thiamine	1.000mg
L-valine	80mg	Distilled & deionized H_2O	1000ml
Glucose (100X)	5.0g		

[a] mCDS medium was prepared by mixing appropriate volumes of each stock (Sigma Chemical Co., St. Louis, MO), adjusting to pH 6 with 5 N HCl and sterilized by filtration.

[b] Stock concentration from which mCDS medium was prepared. All stocks were sterilized in an autoclave (except the vitamins were filter-sterilized) and stored at 4^oC.

pH 6 with 5 N HCl or NaOH, followed by filter sterilization through a 0.2-μm membrane filter (Supor-200, 47-mm, Gelman Sciences Inc., Ann Arbor, MI) or 0.22-μm syringe filter (Cameo IIS, Micron Separation Inc., Westborough, MA). All pH measurements were done by using a Fisher Accumet 950 pH meter connected to a pH electrode (Standard Ag/AgCl model 13-620-104, Fisher Scientific Co., Pittsburgh, PA). Bacteria were grown in sterile BHI (pH6) or mCDS (pH6) medium at 37°C in a reciprocating water bath (Model 129, Fisher Scientific Co.) at 120 strokes per minute. To simulate the pH of fresh postrigor meats and prerigor cooked processed meats, a pH of 6.0 was selected.

Phosphates

Because long-chain polyphosphates have high molecular weights and the molecular weight of sodium ultraphosphate is undetermined, percentages were used as the concentration units. Five percent (w/v) stock solutions of the following commercial-grade phosphates (BK Ladenburg Corp., Cresskill, NJ) were prepared at room temperature and used within 3 hours: tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium acid pyrophosphate (SAPP), sodium polyphosphate glassy (SPG, n~20), and sodium ultraphosphate (UP, sodium acid hexametaphosphate). Appropriate volumes of phosphate stock solutions were added to sterile media to achieve the final phosphate concentrations, followed by adjustment to pH 6 and sterilization by filtration. Selected physical and chemical properties of these phosphates are summarized in Table 3.

Table 3. Selected physical and chemical properties of phosphates [a]

Properties	TSPP	STPP	SAPP	SPG	UP [b]
Chemical formula	$Na_4P_2O_7$	$Na_5P_3O_{10}$	$Na_2H_2P_2O_7$	$(NaPO_3)_{20}.H_2O$	?
Molecular weight	266	368	222	2058	?
pH [c]	10.3	9.9	4.1	6.6	2.0
Solubility [d]	6	15	13	40	?

[a] Information supplied by BK Ladenburg Co., Cresskill, NJ

[b] UP is also known as Latal AC U and Bekaplus Ultra

[c] pH was measured at 1% aqueous solution

[d] Solubility was expressed as grams per 100 grams water at 25°C

Metals

One-tenth molar (w/v) stock solutions of the following reagent-grade metals (Fisher Scientific Co., Fair Lawn, NJ) were prepared and sterilized in an autoclave: anhydrous $CaCl_2$, $FeCl_3.6H_2O$, $MgCl_2.6H_2O$, and $MnCl_2.4H_2O$. Appropiate volumes of metal stock solutions were added to sterile media to achieve the final concentrations, followed by adjustment to pH 6 and filter sterilization.

Chemicals

Polymyxin B sulfate (Sigma Chemical Co.) was dissolved in water at 200 μg/ml, adjusted to pH 6, filter sterilized and used immediately. Disodium ethylenediamine tetraacetate (EDTA, Sigma Chemical Co.) was dissolved in water at 0.0034 M, adjusted to pH 6, filter sterilized and used immediately. Sucrose and NaCl for leakage studies were obtained from Fisher Scientific Co. DNase, RNase and proteinase K were used in microscopy studies. One milligram of pancreatic DNase (Amersham Corp., Arlington Heights, IL) was dissolved in 1 ml of a 50% (w/v) solution of glycerol containing 20 mM Tris.Cl, pH 7.5, plus 1 mM $MgCl_2$. DNase-free RNase was prepared by dissolving pancreatic RNase A (Sigma Chemical Co.) at 10 mg/ml in 10 mM Tris (pH 7.5) and 15 mM NaCl. After boiling for 15 minutes and cooling slowly (about 30 minutes) to room temperature, 0.5-ml aliquots were stored at -20°C. Proteinase K (fungal source, Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD) was dissolved in water at 20 mg/ml, filter sterilized and used immediately.

Minimum Inhibitory Concentration (MIC) Studies

MIC studies of phosphates were performed in BHI (pH6), mCDS (pH6) and meat. BHI (pH6) and mCDS (pH6) media containing serial concentrations of phosphates (TSPP: 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0%; STPP: same as TSPP plus 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0%; SAPP: same as STPP; SPG: same as SAPP plus 0.05, 10, 15, and 20%; UP: same as TSPP plus 0.05%) were prepared by adding appropriate volumes of phosphate stock solutions to sterile BHI and mCDS media, followed by adjustment to pH 6 and filter sterilization. Meat (pH6) medium containing serial concentrations of phosphates (TSPP: 1, 2, 3, 4, 5, 6, and 7%; STPP: same as TSPP plus 8 and 9%; SAPP: same as STPP; SPG: same as SAPP plus 0.5, 10, 15, and 20%; UP: same as TSPP plus 0.1 and 0.5%) were prepared by grinding the inner part of a large piece of vacuum-packaged beef (Meats Laboratories, ISU) in a sterile Oster food grinder inside a laminar flow hood. The metal parts of the food grinder were sterilized in an autoclave and the plastic parts were sanitized by soaking in 2% phosphate-free Liqui-Nox solution, Alconox Inc., New York, NY). Five-gram portions of the ground beef were distributed aseptically to sterile 6-oz (151-g) Whirl-Pak bags (catalog B1062WA, Nasco, Fort Atkinson, WI) embedded in ice. Appropriate volumes of phosphates were mixed with the meat samples to achieve serial phosphate concentrations, followed by pH adjustment.

Overnight bacterial cultures were inoculated into BHI (pH6) and mCDS (pH6) to obtain early-exponential phase cells. The cells were inoculated into phosphate-containing BHI (pH6) and mCDS (pH6) media, and incubated for 24 hours at optimum growth temperature of the corresponding bacteria. The MIC values of phosphates were determined as the lowest concentration of phosphates

with no growth either visually or spectrophotometrically (by Suprasil quartz semimicro UV cells and Kontron UVIKON 930 spectrophotometer, Kontron Instruments, Zurich, Switzerland) after 24 hours of incubation.

For meat media, 10^3 early-exponential phase cells were inoculated and the media were incubated for 24 hours at the optimum growth temperature of each corresponding bacterium. Viable counts were determined by using pour plates of tryptic soy agar (TSA, Difco Laboratories); the plates were incubated for 24 hours. The MIC values of phosphates were determined as the lowest concentration of phosphates that reduced viable colony counts by 99.9% or greater. The bacterial species that was the most sensitive to phosphates was selected as the principal organism used in subsequent studies.

Growth Studies

Growth curve of S. aureus ISP40

S. aureus ISP40 was inoculated on fresh BHI agar slants and incubated for 20 hours at 37°C. The culture was harvested by suspending the growth in 5 ml of 0.1% Difco peptone water. The bacterial suspension was agitated for 30 seconds at high speed on a vortex mixer to separate *S. aureus* cells, which tended to clump. A 0.5-ml volume of bacterial suspension was added to 100 ml of prewarmed mCDS (pH6) medium in a 250-ml Erlenmeyer flask containing a cotton plug and the flask was incubated for 20 hours at 37°C in a reciprocating water bath at 120 strokes per minute. Ten ml of culture were transferred to 100 ml of fresh, prewarmed mCDS (pH6) medium and incubated at 37°C. Three-ml samples were taken every hour until stationary phase, for absorbance (660 nm) and pH measurements. Also, cell suspensions were diluted with 0.1% peptone

water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log colony forming unit (CFU) per ml.

Effect of phosphates on growth

Growth studies were performed to determine the antibacterial effects of phosphates on *S. aureus* ISP40. *S. aureus* ISP40 was inoculated on fresh BHI agar slants and incubated for 20 hours at 37°C. The culture was harvested by suspending the growth on each slant in 5 ml of 0.1% peptone water. The bacterial suspensions were then agitated for 30 seconds at high speed on a vortex mixer to disrupt clumps of *S. aureus* cells. A 0.5-ml volume of bacterial suspension was inoculated to 100 ml of prewarmed mCDS (pH6) medium in a 250-ml Erlenmeyer flask containing a cotton plug and the flask was incubated for 20 hours at 37°C in a reciprocating water bath at 120 strokes per minute. Ten ml of culture were transferred to 100 ml of fresh, prewarmed mCDS (pH6) medium and the culture was incubated for 3 hours. Five ml of 3-hour *S. aureus* culture were then inoculated into 25 ml of mCDS (pH6) medium containing the following MIC levels of phosphates: 0.5% TSPP, 0.5% STPP, 0.5% SAPP, 0.1% SPG, and 0.1% UP. At 0, 1, 2, 3 hours of incubation, absorbance (660 nm) measurements were taken from 3-ml samples. Also, the cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log colony forming unit (CFU) per ml.

Leakage Studies

Preparation of final washed pellets

A 20-hour culture of *S. aureus* ISP40 on a BHI agar slant was harvested by suspending the growth in 5 ml of 0.1% peptone water. The bacterial suspension was agitated for 30 seconds on a vortex mixer. Two ml of bacterial suspension were inoculated into 100 ml of prewarmed BHI (pH6) medium and the culture was incubated for 3 hours at 37°C in a reciprocating water bath at 120 strokes per minute. Thirty ml of early-exponential phase cells were transferred aseptically to a sterile centrifuge tube and the cells were harvested by centrifugation (7700 x g, 5 minutes) in a Sorvall superspeed RC-2 refrigerated centrifuge. The supernatant was discarded and the pelleted cells were washed twice with saline. The washed pellets were used for subsequent experiments.

Selection of a suspending medium

To select a suitable suspending medium, washed pellets were resuspended in 30 ml of pH 6 NaCl (0.85%, 1.7%, 2.34%, 3.5%), sucrose (8.56%), and NaCl (0.59%)-sucrose (8.56%) solutions. After 0, 0.5, 1, and 1.5 hours of incubation at 25°C, 10 ml of cell suspension was removed and the cells were sedimented by centrifugation (20000 x g, 5 minutes). Three ml of cell-free supernatant were transferred to a Suprasil quartz semimicro UV cell and the absorbance (260 nm) was measured on a Kontron UVIKON 930 spectrophotometer. The absorbance at 260 nm of the supernatant, against distilled water, was used as an index of leakage of intracellular nucleotides.

Bacteriolytic effect of phosphates

To study the effect of phosphates on cell leakage, washed cell pellets described above were resuspended in 30 ml of pH 6 phosphate (0.5% TSPP, 0.5% STPP, 0.5% SAPP. 0.1% SPG, 0.1% UP), EDTA (0.0034 M), saline, and boiled saline (100°C, 15 minutes). The same procedure as described above was used to obtain cell-free supernantant for absorbance (260 nm) measurements.

Microscopy

The same procedure described above was used to obtain washed cell pellets. The cells were suspended in 30 ml of pH 6 saline, SPG (0.1%) and UP (0.1%). After 30 minutes of incubation at 25°C, 0.1 ml of cell suspension was smeared onto a microscope slide; the film was air-dried, heat-fixed, and stained with crystal violet for 3 minutes. To characterize the massive gelatinous aggregates that were observed microscopically, enzymes were used. The same procedure described above was used to obtain washed cell pellets. The pellets were suspended in 30 ml of pH 6 SPG (0.1%), SPG plus DNase (2 µl), SPG plus RNase (2 µl), SPG plus proteinase K (2 µl), and SPG plus DNase (2 µl), RNase (2 µl), and proteinase K (2 µl). After 1 hour of incubation at 25°C, 0.1 ml of cell suspension was smeared onto a microscope slide, and the preparation was air-dried, heat-fixed, and stained with crystal violet for 3 minutes. Photographs were taken by using Kodak Ektachrome 160 Tungsten film in an Olympus BH2 camera with an automatic exposure photomicrographic system.

Target site of phosphates

Polymyxin B sulfate and EDTA were used as positive controls. Polymyxin is a peptide antibiotic that causes cell membrane damage by combining with the ionized phosphate groups of the phospholipid components of the cell membrane (Newton, 1954; 1956). This results in a decrease in interfacial tension at the cell membrane, and therefore the disorganization of the cell membrane. EDTA is a metal chelator that removes metal cations in the cell wall (Gray and Wilkinson, 1965). This results in a loss of cell integrity. To define the target site of phosphates on the cell envelope of *S. aureus*, the washed cell pellets (described above) were suspended in 30 ml of pH 6 NaCl (0.4 M and 0.6 M), polymyxin B sulfate (200 μg/ml), polymyxin B sulfate (200 μg/ml) with NaCl (0.4 M and 0.6 M), EDTA (0.0034 M), EDTA (0.0034 M) with NaCl (0.4 M), and phosphates: TSPP (0.5%), TSPP (0.5%) with NaCl (0.4 M and 0.6 M), SPG (0.1%), SPG (0.1%) with NaCl (0.4 M), UP (0.1%), UP (0.1%) with NaCl (0.4 M and 0.6 M). The same procedure as described above was used to obtain cell-free supernantants for absorbance (260 nm) measurements.

pH study for metal chelation mechanism

The same procedure as described above was used to obtain the washed cell pellets. The cells were suspended in 30 ml of 0.85% NaCl (pH 6, pH 7, pH 8), and phosphates (0.5% TSPP, 0.1% SPG, 0.1% UP) at pH 6 and pH 8. The same procedure as described above was used to obtain cell-free supernantants for absorbance (260 nm) measurements.

Metal-Ion Studies

Reversal of growth inhibition by metal ions

One molar stock solutions of Ca^{2+}, Fe^{3+}, Mg^{2+}, and Mn^{2+} were prepared and sterilized in an autoclave. Serial dilutions of the metal solutions were made to obtain 0.1, 0.01 and 0.001 M final concentrations in mCDS (pH6) medium containing phosphates. A 3-hour *S. aureus* culture was prepared by using the procedure described in growth studies. An aliquot (0.5 ml) of this culture was inoculated into each 5-ml tube of mCDS (pH6)-metal-phosphate medium and the tubes were incubated at 37°C for 24 hours. Reversal of growth inhibition by a metal was indicated by the appearance of visible bacterial growth.

Reversal of UP inhibition by metal ions

Reversal of bacteriolytic effect of UP by metal ions

The same procedure described above was used to obtain the washed cell pellets. The cells were suspended in 30 ml of pH 6 UP (0.1%), UP plus Ca^{2+} (0.01 M), UP plus Mg^{2+} (0.01 M), and controls (saline, 0.01 M Ca^{2+}, and 0.01 M Mg^{2+}). The same procedure described above was used to obtain cell-free supernatants for absorbance (260 nm) measurements.

Reversal of bactericidal effects of UP by metal ions

The reversal of the bactericidal effects of UP by metal ions was performed to differentiate between metal-protection and metal-rescue studies, and to clarify each study. In the metal-protection study, cells were protected by metals one hour before exposure to UP. The same procedure as described above was used to obtain a 3-hour culture of *S. aureus*. Five ml of 3-hour culture were inoculated

into 25 ml of mCDS (pH6) medium containing Ca^{2+} (0.01 M) and Mg^{2+} (0.01 M). After 1 hour of incubation, UP (0.1%) was added to a flask of mCDS (pH6) medium containing Ca^{2+} (0.01 M) and Mg^{2+} (0.01 M). At 0, 1, 2, 3, 4 hours of incubation, cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log CFU per ml.

In the metal-rescue study, UP-treated cells were rescued by the addition of metals after one hour of incubation. The same procedure as described above was used to obtain a 3-hour culture of *S. aureus*. Five ml of 3-hour culture were inoculated into 25 ml of mCDS (pH6) medium containing UP (0.1%), UP (0.1%, overnight preparation), UP (0.2%, overnight preparation), and UP (0.3%, overnight preparation). After 1 hour of incubation, Ca^{2+} (0.01 M), Mg^{2+} (0.01 M) and Ca^{2+} (0.01 M) plus Mg^{2+} (0.01 M) were added (final concentration). At 0, 1, 2, 3, 4 hours of incubation, cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log CFU per ml.

Reversal of SPG inhibition by metal ions

Reversal of bacteriolytic effect of SPG by metal ions

The same procedure as described above was used to obtain cell pellets. The cells were suspended in 30 ml of pH 6 SPG (0.1%), SPG (0.1%) plus Mg^{2+} (0.01 M), SPG (0.1%) plus Ca^{2+} (0.01 M), and controls (saline, 0.01 M Ca^{2+}, 0.01

M Mg^{2+}). The same procedure described above was used to obtain cell-free supernatants for absorbance (260 nm) measurements.

Reversal of bactericidal effects of SPG by metal ions

The reversal of the bactericidal effects of SPG by metal ions was performed to differentiate between metal-protection and metal-rescue studies, and to clarify each study. In the metal-protection study, cells were protected by metals one hour before the exposure to SPG. The same procedure as described above was used to obtain a 3-hour culture of *S. aureus*. Five ml of 3-hour culture were inoculated into 25 ml of mCDS (pH6) medium containing Ca^{2+} (0.01 M) and Mg^{2+} (0.01 M). After 1 hour of incubation, SPG (0.1%) was added to a flask of mCDS (pH6) medium containing Ca^{2+} (0.01 M) and Mg^{2+} (0.01 M). At 0, 1, 2, 3, 4 hours of incubation, cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log CFU per ml.

In the metal-rescue study, SPG-treated cells were rescued by the addition of metals after one hour of incubation. The same procedure as described above was used to obtain a 3-hour culture of *S. aureus*. Five ml of 3-hour culture were inoculated into 25 ml of mCDS (pH6) medium containing SPG (0.1%). After 1 hour of incubation, Ca^{2+} (0.01 M), Mg^{2+} (0.01 M) and Ca^{2+} (0.01 M) plus Mg^{2+} (0.01 M) were added (final concentration). At 0, 1, 2, 3, 4 hours of incubation, cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log CFU per ml.

Reversal of TSPP inhibition by metal ions

The reversal of TSPP inhibition by metal ions was performed to differentiate between metal-protection and metal-rescue studies, and to clarify each study. In the metal-protection study, cells were protected by metals one hour before the exposure to TSPP. The same procedure as described above was used to obtain a 3-hour culture of *S. aureus*. Five ml of 3-hour culture were inoculated into 25 ml of mCDS (pH6) medium containing TSPP (0.5%). After 1 hour of incubation, Fe^{3+} (0.01 M) was added (final concentration). At 0, 1, 2, 3, 4 hours of incubation, cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log CFU per ml.

In the metal-rescue study, TSPP-treated cells were rescued by the addition of metals after one hour of incubation. The same procedure as described above was used to obtain a 3-hour culture of *S. aureus*. Five ml of 3-hour culture were inoculated into 25 ml of mCDS (pH6) medium containing Fe^{3+} (0.01 M). After 1 hour of incubation, TSPP (0.5%) was added to a flask of mCDS (pH6) medium containing Fe^{3+} (0.01 M). At 0, 1, 2, 3, 4 hours of incubation, cell suspensions were diluted with 0.1% peptone water and spread-plated on BHI agar plates in triplicate. Visible colonies of *S. aureus* were counted after 24 hours of incubation. The counts obtained were expressed as mean log CFU per ml.

Free-Metal Dialysis Study

The same procedure as described above was used to obtain the cell pellets. The cells were suspended in 30 ml of pH 6 saline, phosphates (0.1% UP and 0.1%

SPG), phosphate plus metal (UP plus 0.01 M Ca^{2+}, UP plus 0.01 M Mg^{2+}, SPG plus 0.01 M Ca^{2+}, SPG plus 0.01 M Mg^{2+}), and metal (0.01 M Mg^{2+}, 0.01 M Ca^{2+}). Ten-ml samples were transferred to a Spectra/Por sterile CE dialysis membrane (MWCO 100, Spectrum, Houston, TX) with 2 Spectra/Por sterile closures. One end was closed with a closure and the other end was closed with a stirring bar attached to a closure. The dialysis membranes were dialyzed in 2 L of saline with moderate stirring motion in a beaker at 25°C. At 0, 0.5, 1, 1.5 hour of incubation, a 20-ml sample was taken from each dialysis beaker and stored at 4°C in a 20-ml scintillation vial (Kimble Glass, Inc., Vineland, NJ). Free metals (Ca^{2+}, Mg^{2+}) were detected and quantified with an atomic absorption (AA) spectrophotometer (Instrumentation Laboratory, Inc., Massachusettes).

Metal Leakage Study

The same procedure as described above was used to obtain cell pellets. The cells were suspended in 30 ml of pH 6 phosphates (0.1% UP and 0.1% SPG), polymyxin (200 ug/ml), and saline. After 0, 0.5, and 1 hour of incubation, cell-free supernatants were obtained using the procedure described above. Metals (Ca^{2+}, Mg^{2+}) were detected and quantified by using an AA spectrophotometer.

Statistical Analysis

All experiments were replicated three times and the mean values were reported. Data were analysed for mean standard deviation and to determine statistical difference between means using t test in the StatView Student program (Abacus Concepts, 1991).

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration of Phosphates

Minimum Inhibitory Concentration (MIC) studies of phosphates were performed in brain heart infusion (BHI, pH6), modified complete defined synthetic (mCDS, pH6) and meat (pH6) for two purposes: first to compare the antibacterial effect of phosphates in culture media and in meat, second to select a phosphate-sensitive bacterium for this study.

The MIC values of phosphates in BHI (pH6), mCDS (pH6) and meat (pH6) media for different bacteria are shown in Table 4. The MIC values of phosphates in meat (pH6) media were generally higher than those in culture media because of the hydrolysis of phosphates by meat phosphatases. Also, Gram-positive bacteria were generally more sensitive to phosphates than Gram-negative bacteria. These results were consistent with other researchers' findings (Knabel et al., 1991; Molins et al., 1984). Among the three tested Gram-positive bacteria, *Staphylococcus aureus* ISP40 8325 was the most sensitive to phosphates. *S. aureus* ISP40 was totally inhibited by 0.5% pyrophosphates (SAPP: 0.0225 M, TSPP: 0.0188 M), 0.5% tripolyphosphate (STPP: 0.0136 M), and 0.1% long-chain polyphosphates (SPG: 0.0005 M, UP) in culture media. Therefore, *S. aureus* ISP40 8325 was selected as the principal organism for use in subsequent studies.

Table 4. Minimum inhibitory concentration (MIC, in percentage) when different bacteria were tested against five different phosphates in culture and meat media

Bacteria	TSPP		STPP		SAPP		SPG		UP	
	C[a]	M[b]	C	M	C	M	C	M	C	M
S. aureus (A-16) IDM Q3	2	>6	2	>6	2	>6	2	>6	0.2	>2
S. aureus ISP40 8325	0.5	>6	0.5	>6	0.5	>6	0.1	>4	0.1	>2
Streptobacterium (isolated from pork)	>4	6	>4	>6	4	>5	2	>5	6	>6
E. coli K 12	6	7	7	>9	9	>9	>20	>20	>6	>7
E. coli 15 TAU	6	6	>9	9	>9	>8	>15	>15	>6	6
Pseudomonas fluorescens EV-21	2.5	>4	3.5	>7	2.5	>6	5	>15	2.5	6

[a] MIC of phosphates in culture media: brain heart infusion (BHI, pH6) and modified complete defined synthetic (mCDS, pH6) media

[b] MIC of phosphates in meat (pH6) media

Growth Studies

Sampling time for early-exponential phase cells

Because young (early-exponential phase) cultures are generally more sensitive to phosphates than older (stationary phase) cultures (Molins et al., 1984), early-exponential phase cells were used to study the antibacterial mechanism of phosphates. Growth curves of *S. aureus* ISP40 in mCDS (pH6) medium were performed and expressed in absorbance 660 nm (Figure 1) and mean log CFU/ml (Figure 2). The comparison of these growth curves was shown in Figure 3. Three hours was chosen as the sampling time of early-exponential phase cells for subsequent experiments (Figures 1, 2 and 3).

Effect of phosphates on growth

The effect of phosphates on growth of *S. aureus* ISP40 in mCDS (pH6) medium at 37°C are shown in Figures 4-14: TSPP (Figures 4, 5), STPP (Figures 6, 7), SAPP (Figures 8, 9), SPG (Figures 10, 11), UP (Figures 12-14).

With the increase of TSPP concentrations (0.05%, 0.1%, 0.25%, and 0.5%), there was a decrease of *S. aureus* growth (Figures 4, 5). At 0.5% TSPP, *S. aureus* stopped growing after 1 hour of incubation (Figure 5). Moreover, a bactericidal effect of 0.5% TSPP (MIC) was observed after 2 hours of incubation. Bacterial count of 0.5% TSPP treatment was significantly ($P<0.05$) lower than that of the control by 3 hours of incubation.

A similar concentration effect was observed in STPP treatment (Figures 6, 7). When compared to the control, an initial increase of *S. aureus* growth by all concentrations of STPP (0.1%, 0.25% and 0.5%) was observed by 1 hour of incubation. After 2 hours of incubation, bactericidal effects of STPP (0.25%, 0.5%)

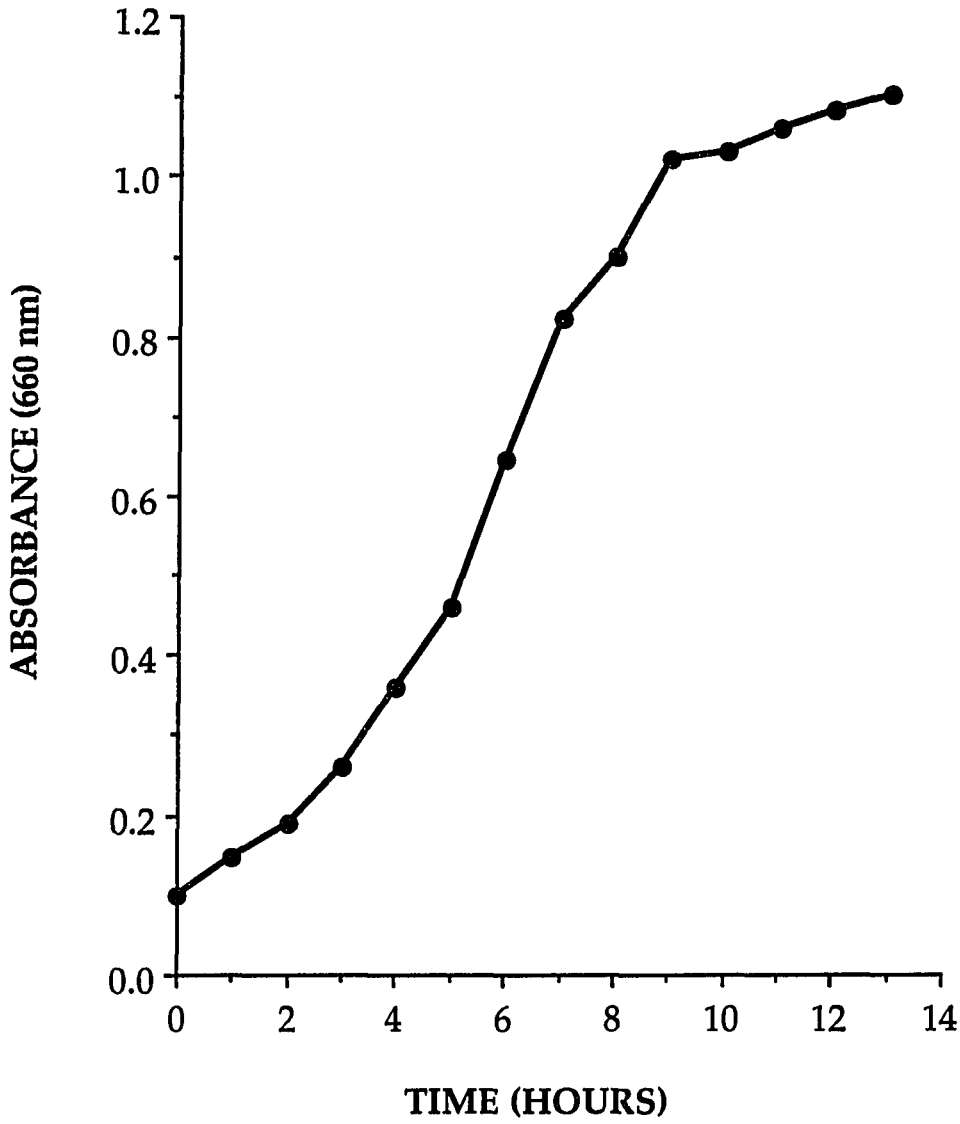


Figure 1. Growth curve (absorbance 660 nm) of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation of this figure was 0.01.

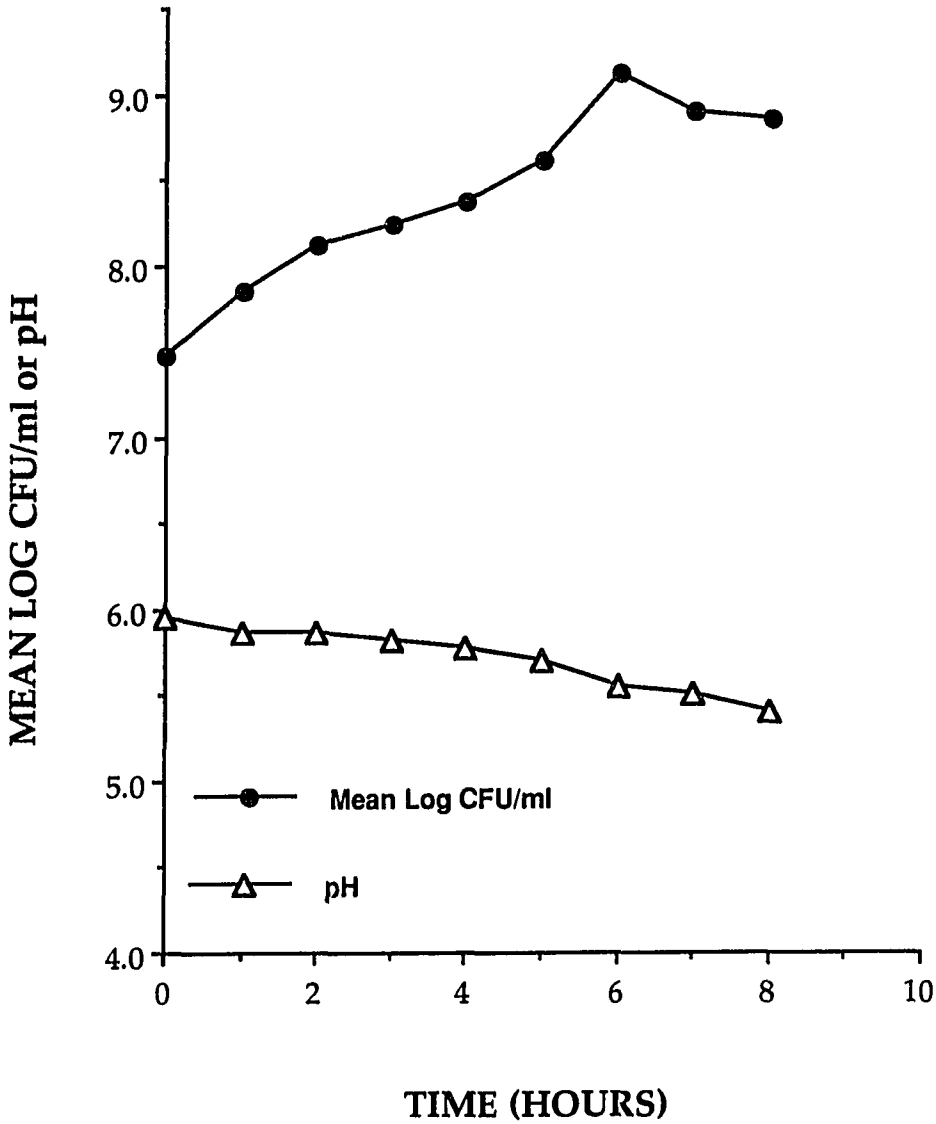


Figure 2. Growth curve (mean log CFU/ml) and pH of the medium of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.06.

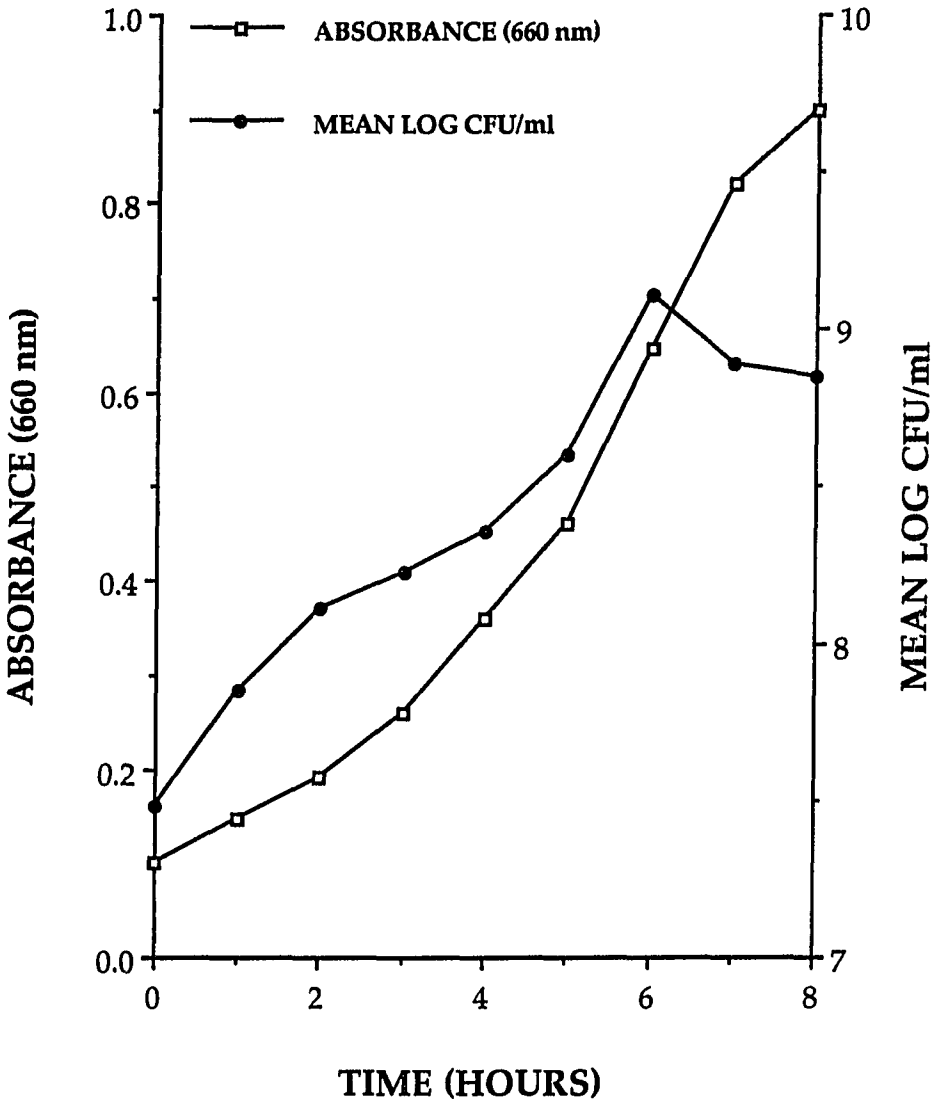


Figure 3. Comparison of growth curves (mean log CFU/ml and absorbance 660 nm) of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C.

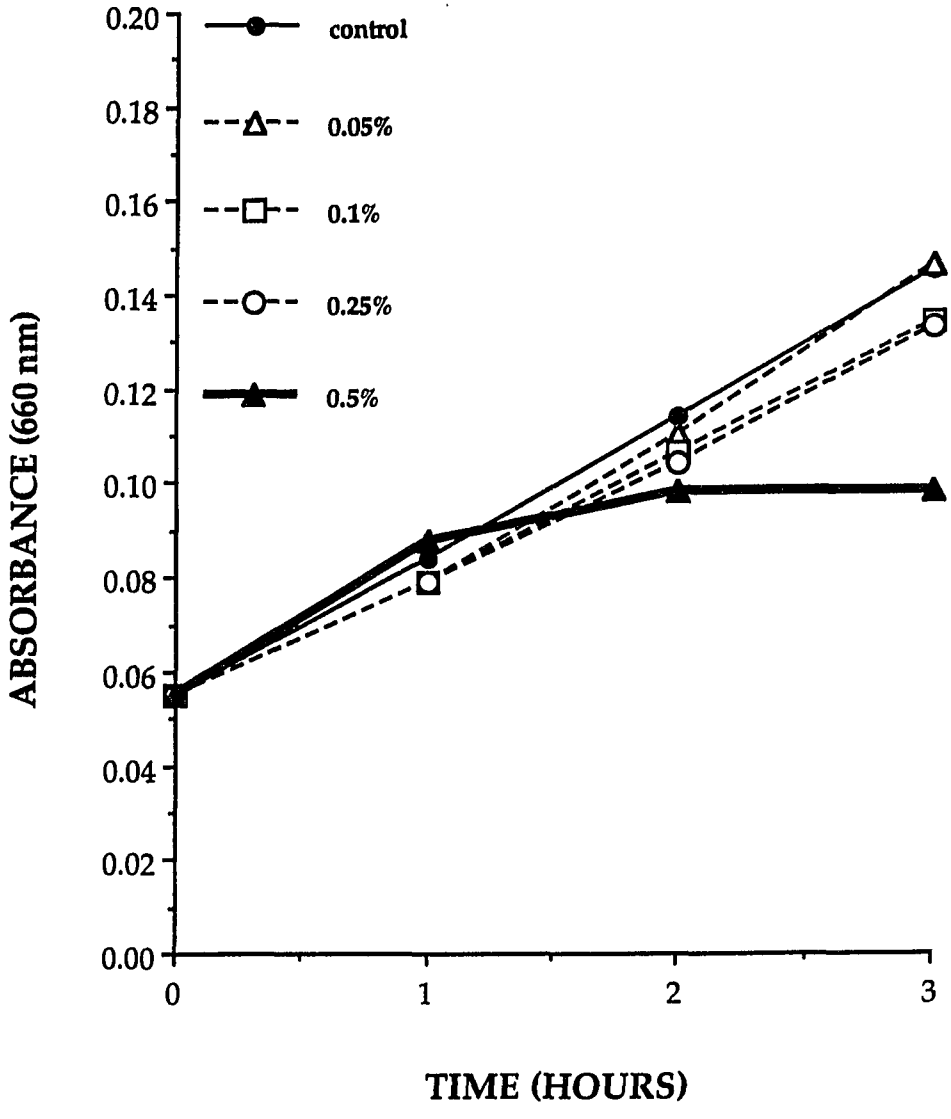


Figure 4. Effect of TSPP on growth (absorbance 660 nm) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.01.

which include bias correction are considered. Comparisons of coverage percentages and widths were made in all cases. Only results for 90% intervals are presented; results for the other levels are similar.

For the situations involving four parameters, results are generally sufficiently similar across the four parameters to provide a meaningful comparison among methods by averaging parameter estimates, bias values, MSEs or variances. Systematic differences among the parameters are discussed further as needed.

Although the data are generated from a continuous distribution, use of Breslow's approximation to the likelihood (2), as done in this study, can result in negatively-biased estimates of β (Farewell and Prentice, 1980) because both bootstraps impose ties on the resampled data sets. Since multiple selection of the same residual for different values of x results in distinct probability-scale failure times, the effect of ties is expected to be less severe in the residual bootstrap than in the vector bootstrap.

It is important to note that occurrence of monotone likelihood precludes the estimation of β in some samples. As detailed by Bryson and Johnson (1981), β is not estimible if any of the explanatory variables (or certain functions thereof) is monotone with respect to the ordered failure times. In the two-treatment problem, this occurs when the last observed failure for one treatment precedes the first observed failure for the other treatment. This structure will result in a likelihood that has no global maximum for $\beta \in \mathbb{R}^p$. As recommended by Bryson and Johnson, such samples are detected and replaced with new simulated samples in the Monte Carlo study. Hence, all estimates of bias and variance are made conditional on the existence of a finite $\hat{\beta}$.

The incidence of monotone likelihood in the Monte Carlo trials and bootstrap replicates is detailed in Table 4.1. As expected, the problem virtually disappears as n

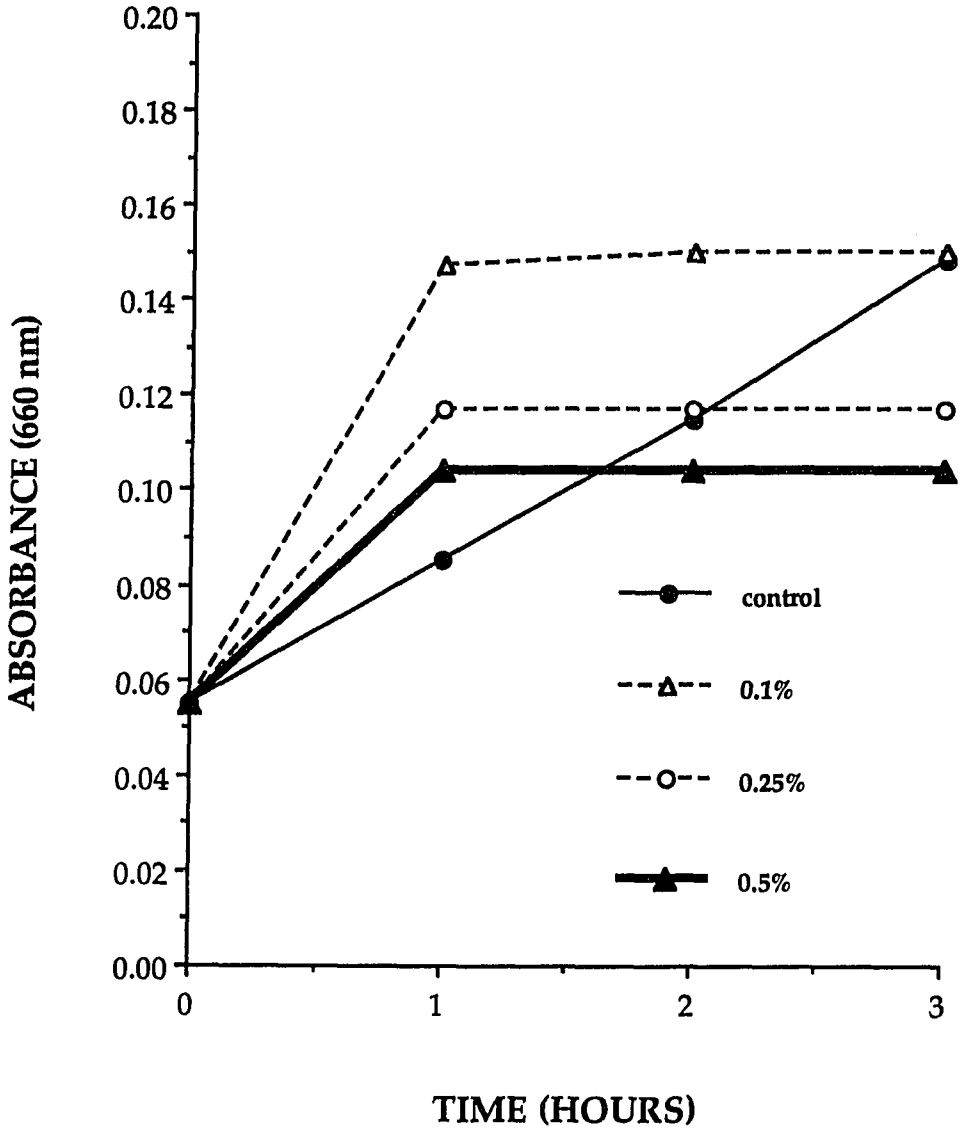


Figure 6. Effect of STPP on growth (absorbance 660 nm) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.01.

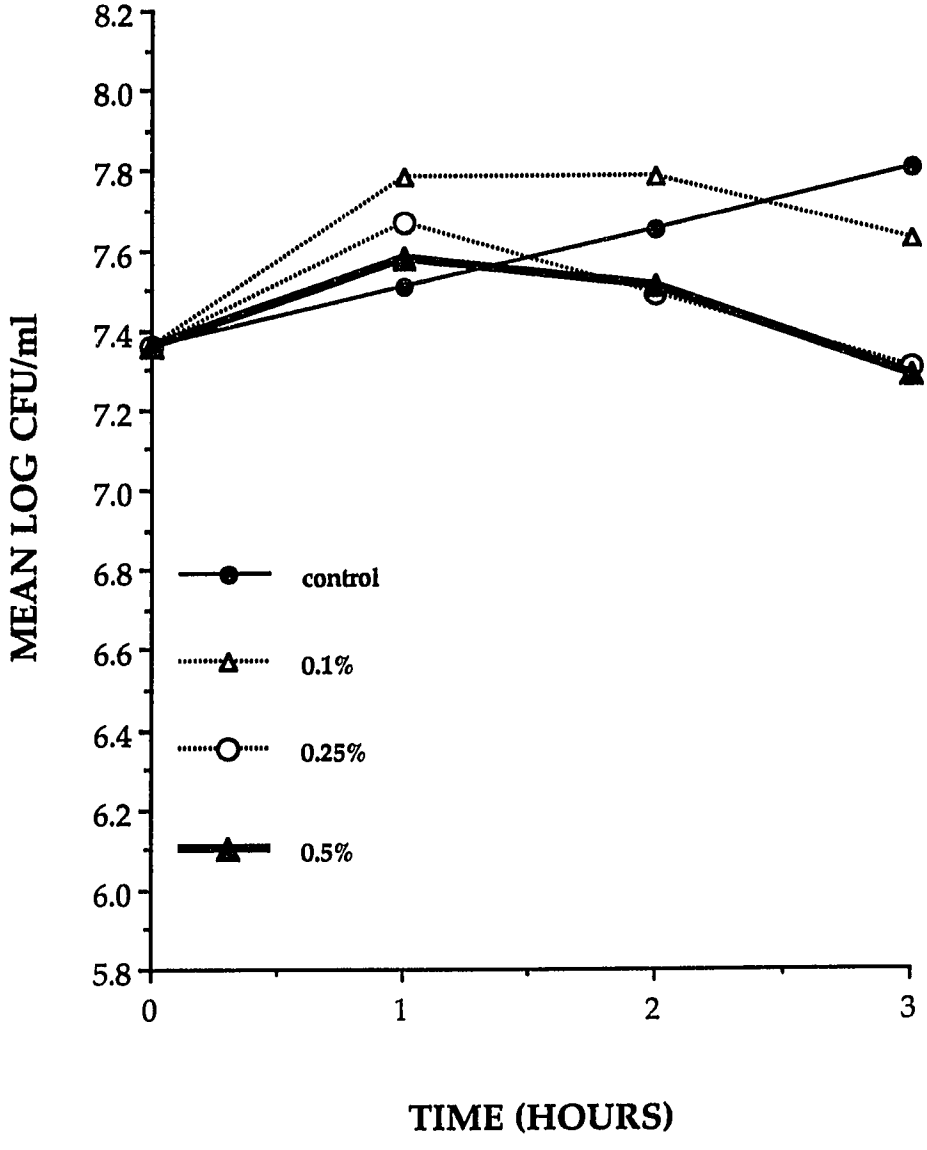


Figure 7. Effect of STPP on growth (mean log CFU/ml) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.05.

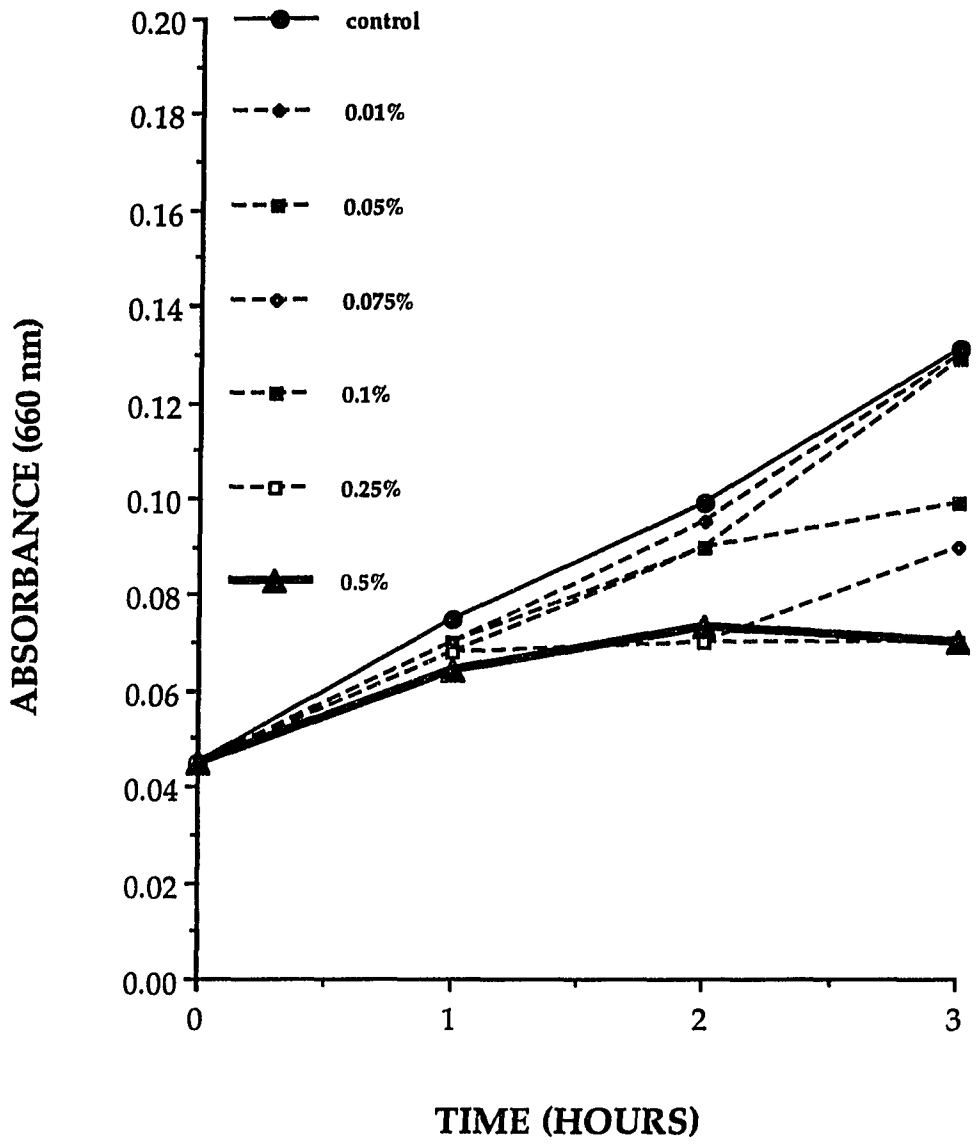


Figure 8. Effect of SAPP on growth (absorbance 660 nm) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.003.

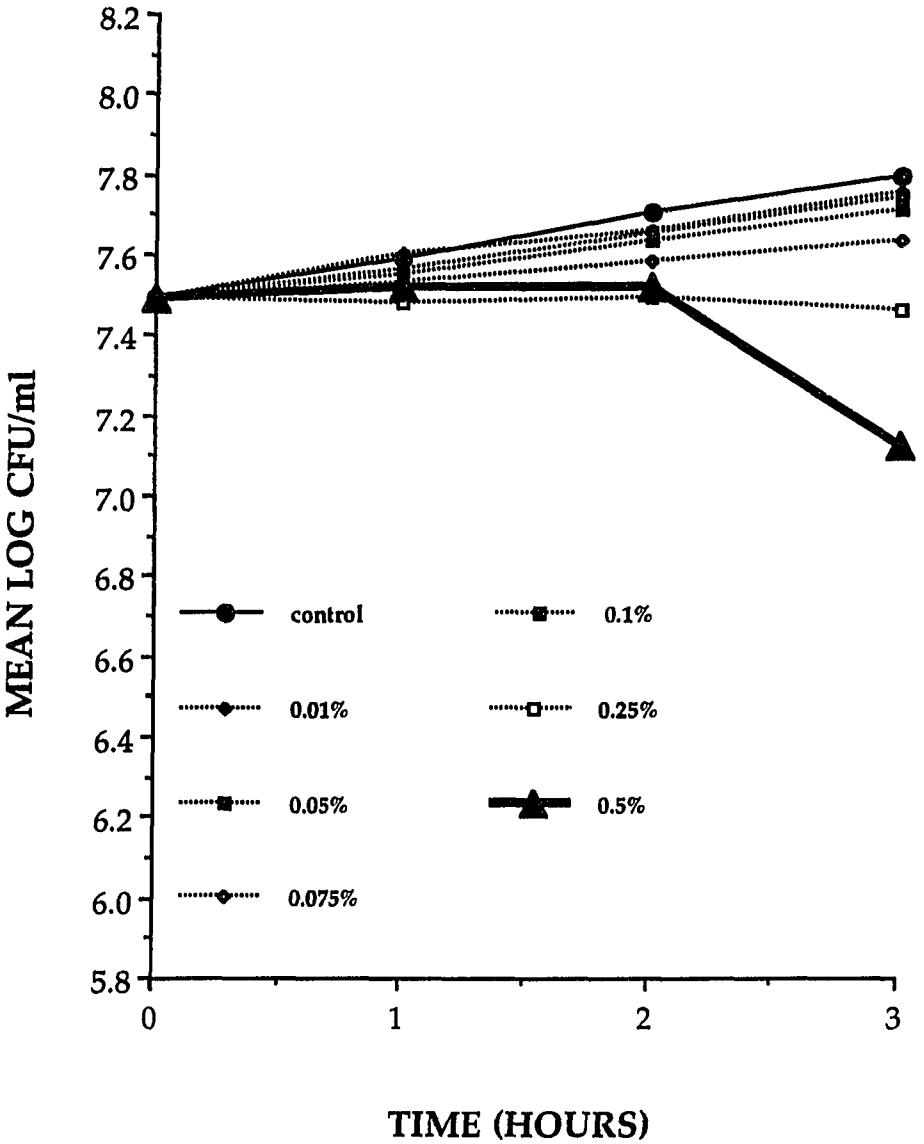


Figure 9. Effect of SAPP on growth (mean log CFU/ml) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.05.

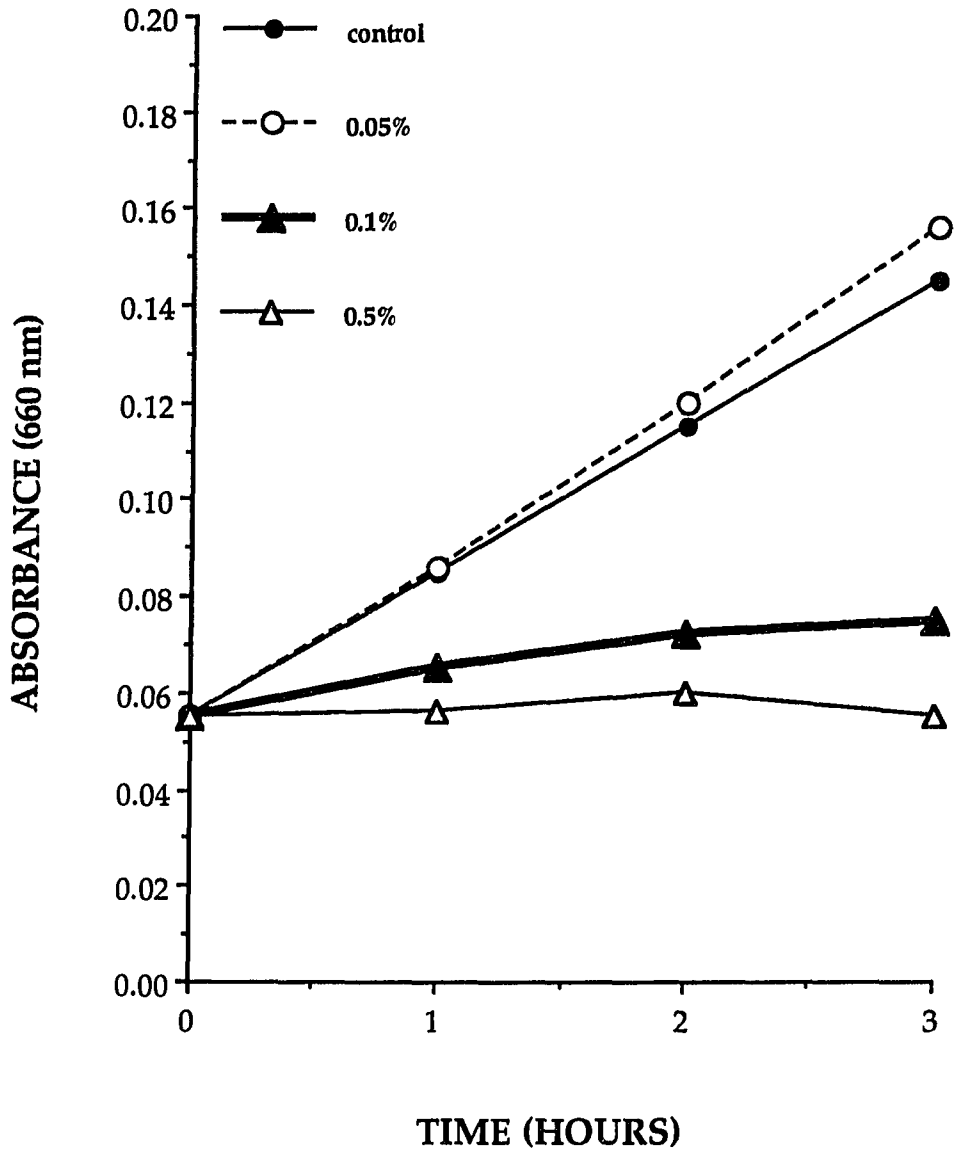


Figure 10. Effect of SPG on growth (absorbance 660 nm) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.01.

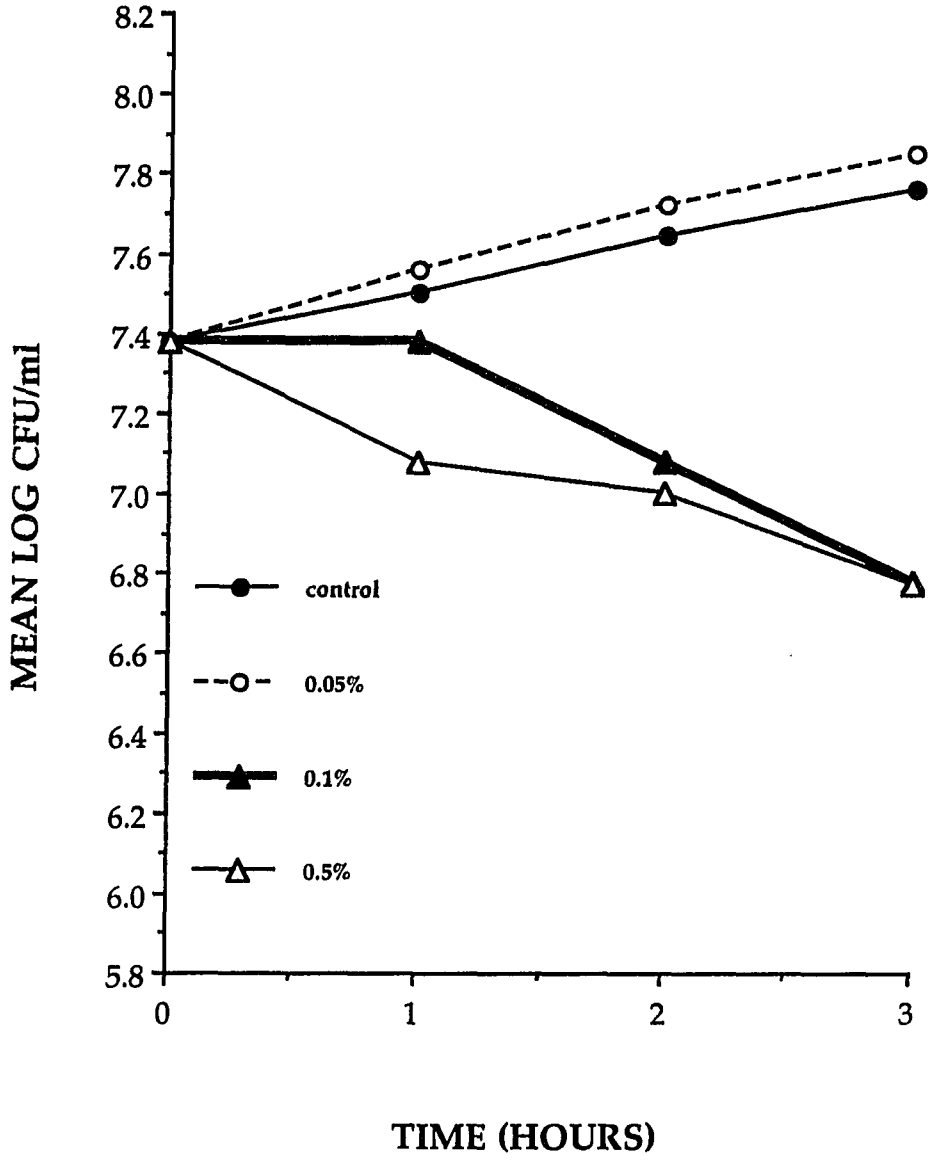


Figure 11. Effect of SPG on growth (mean log CFU/ml) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.07

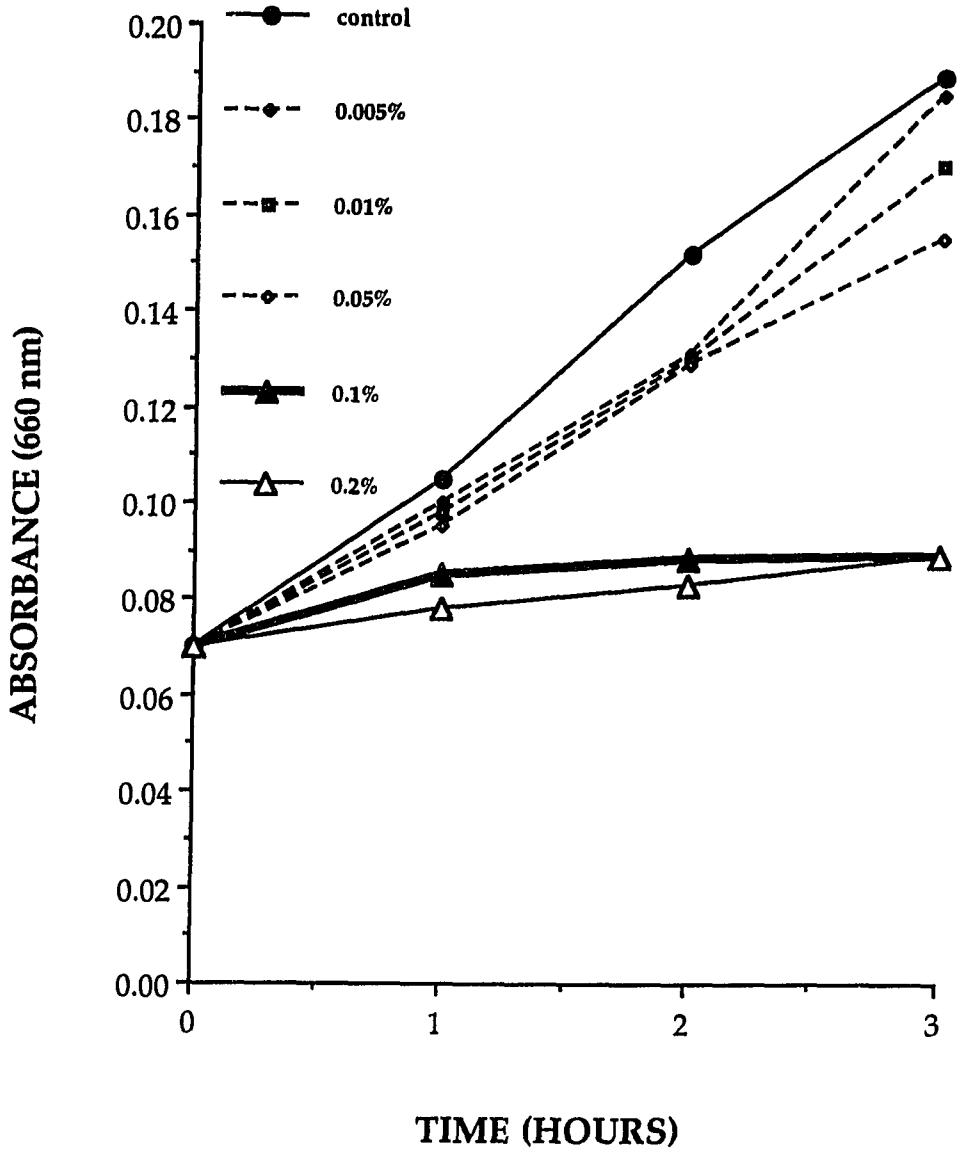


Figure 12. Effect of UP on growth (absorbance 660 nm) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.009.

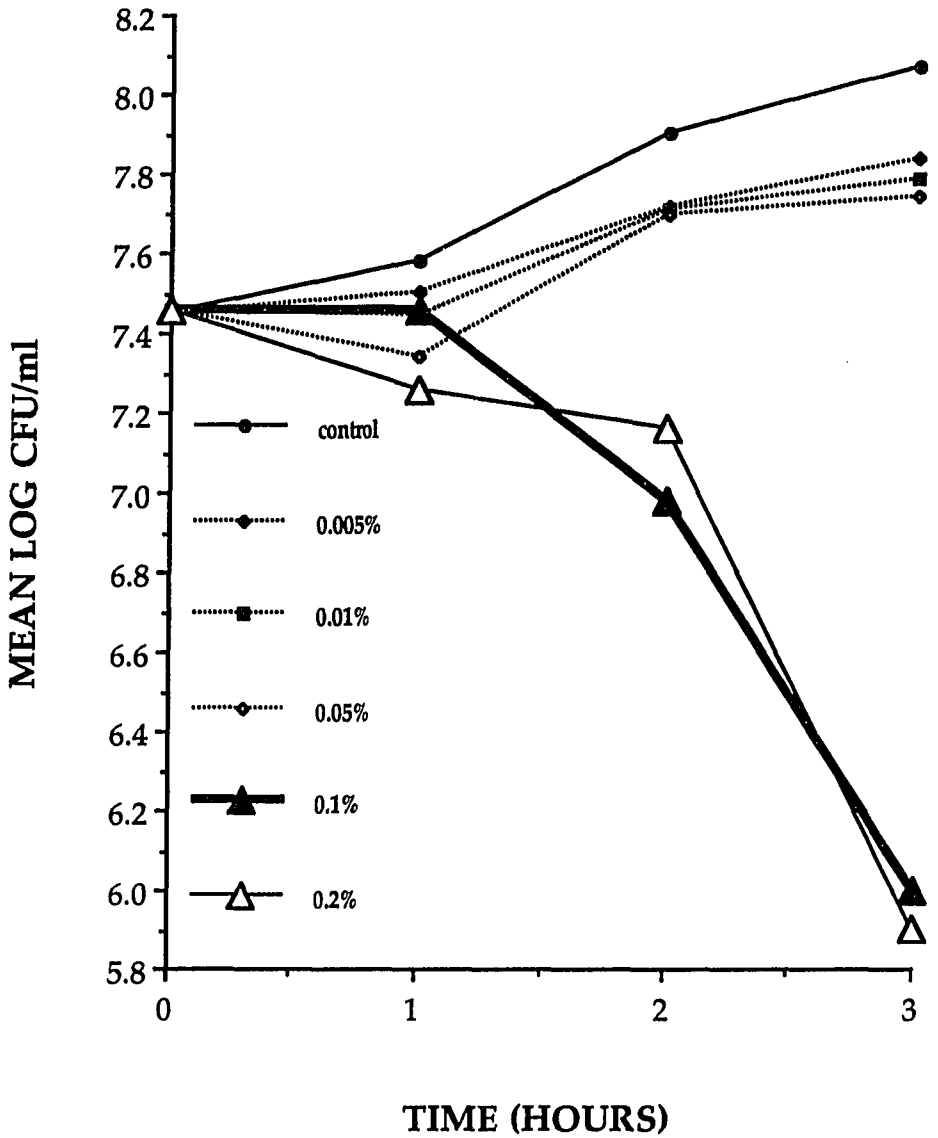


Figure 13. Effect of UP on growth (mean log CFU/ml) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.04.

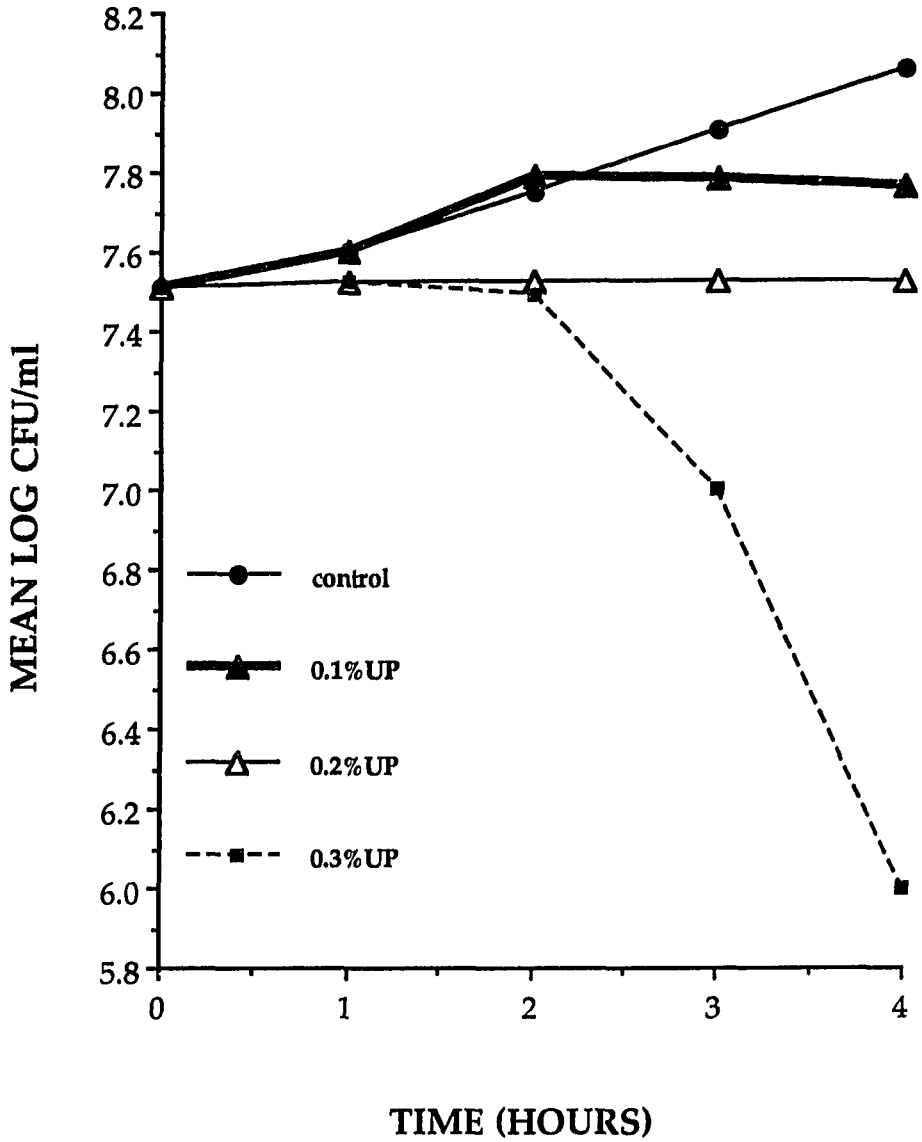


Figure 14. Effect of UP (overnight preparation) on growth (mean log CFU/ml) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.04.

were observed (Figure 7). Bacterial counts of 0.25% and 0.5% STPP treatments were significantly ($P<0.05$) lower than that of the control by 3 hours of incubation.

Concentration effects of SAPP are shown in Figures 8 and 9. When SAPP concentrations were increased (0.01%, 0.05%, 0.075%, 0.1%, 0.25%, and 0.5%), bacterial growth rates decreased. A bacteriostatic effect was observed at 0.25% SAPP. Bactericidal effects of SAPP were observed at 0.5% after 2 hours of incubation (Figure 9). Bacterial counts of 0.25% and 0.5% SAPP treatments were significantly ($P<0.05$) lower than that of the control by 3 hours of incubation.

Bacterial growth was slightly stimulated by 0.05% SPG (Figures 10, 11). *S. aureus* ISP40 was inhibited by higher SPG concentrations (0.1% and 0.5%). Bactericidal effects (Figure 11) were observed at 0.1% by 2 hours of incubation and at 0.5% by 1 hour of incubation. Bacterial counts of 0.1% and 0.5% SPG treatments were significantly ($P<0.05$) lower than that of the control by 2 hours of incubation.

The concentration effects of UP are shown in Figures 12 and 13. With increases of UP concentrations (0.005%, 0.01%, 0.05%, 0.1%, and 0.2%), bacterial growth decreased. Bactericidal effects (Figure 13) were observed at 0.1% after 1 hour of incubation and at 0.2% after 1 and 2, and especially 3 hours of incubation. Bacterial counts of 0.1% and 0.2% UP treatments were significantly ($P<0.05$) lower than that of the control by 1 hour of incubation.

UP stock solution (5%) was moderately soluble in water at room temperature. It was completely soluble in water after overnight (>16 hours) incubation at room temperature. However, hydrolysis of UP begins when water is added. Moreover, tridimensional ultraphosphates are rapidly degraded in

solution at 25°C (van Wazer and Holst, 1950b). In addition, pH shifts away from neutral values (pH 6) accelerate the hydrolysis of linear polyphosphates (Greenfield and Clift, 1975). Therefore, the antibacterial effect of the overnight UP preparation (Figure 14) was less than that of UP used within 3 hours (Figure 13). Bacteriostatic effects were observed at 0.2% of the overnight UP preparation (Figure 14). Bactericidal effects of the overnight UP preparation were observed at 0.3% after 2 hours of incubation. Bacterial counts of 0.2% and 0.3% of the overnight UP treatments were significantly ($P<0.05$) lower than that of the control by 2 hours of incubation.

The MIC value of freshly prepared UP was 0.1% (Figure 13) compared to 0.3% (Figure 14) when a UP preparation aged overnight was used.

The MIC levels of phosphates (Figures 5-14) on *S. aureus* ISP40 are compared in Figure 15. Bacterial counts of all phosphate treatments were significantly ($P<0.05$) lower than that of the control by 2 hours of incubation. Bacterial counts of TSPP and STPP treatments were not significantly ($P>0.05$) different from each other. Bacterial count of SAPP treatment was significantly ($P<0.05$) lower than those of TSPP and STPP treatments by 3 hours of incubation. Bacterial count of SPG treatment was significantly ($P<0.05$) lower than that of SAPP treatment by 2 hours of incubation. Bacterial count of UP treatment was significantly ($P<0.05$) lower than that of SPG treatment by 3 hours of incubation. In general, the longer the chain-length of phosphates, the greater the inhibition. Under the experimental conditions of the present studies, phosphates were bactericidal within the 0.5% legal use limit.

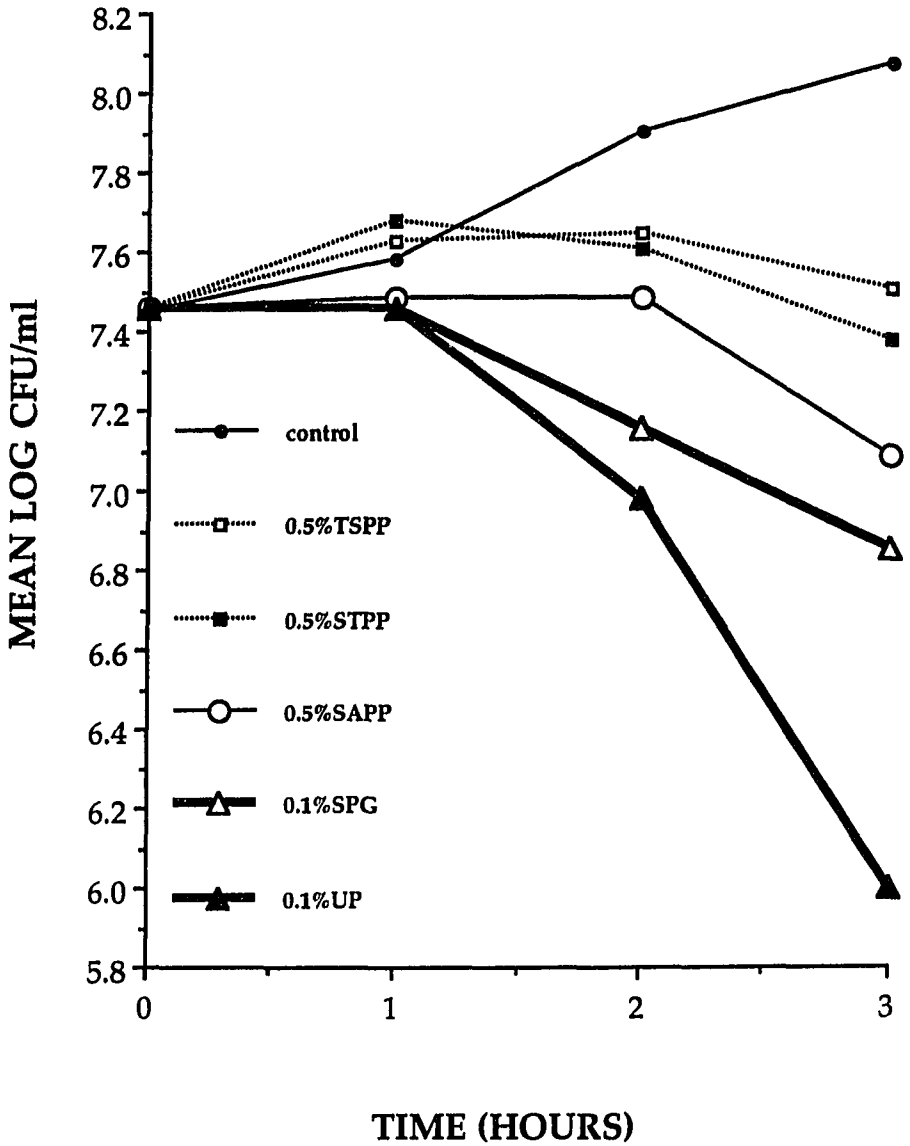


Figure 15. Effect of phosphates at MIC on growth (mean log CFU/ml) of early-exponential phase cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.07.

Leakage Studies

Selection of a suspending medium

Deionized water (Gray and Wilkinson, 1965), distilled water (Salton, 1951), and various buffers (Gray and Wilkinson, 1965; Greenway and Dyke, 1979) have been used as suspending media for leakage studies. In this study, a simple suspending medium was required for pH 6 adjustment and metal chelation studies. Because NaCl and sucrose are commonly used as osmotic stabilizers, several concentrations of NaCl (pH6) and sucrose (pH6) were tested. Figure 16 shows the absorbance 260 nm measurements when cells of *S. aureus* were suspended in several concentrations of NaCl (pH6) and sucrose (pH6) at 25°C. The leakage of cellular constituents in hypotonic suspending media is not uncommon (Salton, 1951). Moreover, *S. aureus* is known to release nucleic acids and proteins to the cell surface (Personal communication: Dr. Peter A. Pattee, Department of Microbiology, Immunology and Preventive Medicine, ISU). Therefore, it is expected that there would be a detectable absorbance reading at 260 nm for the control. When NaCl or sucrose concentrations were increased, absorbance 260 nm readings decreased. Because significantly ($P<0.05$) less leakage occurred in NaCl than in sucrose at the same percentage concentration, NaCl was deemed a better candidate for a suspending medium. In addition, 0.85% NaCl is commonly used as physiological saline for *S. aureus* (Personal communication: Dr. Peter A. Pattee, Department of Microbiology, Immunology and Preventive Medicine, ISU). Although higher concentrations of NaCl (2.34%/0.4 M and 3.5%/0.6 M) caused less bacterial leakage than 0.85% NaCl, these higher concentrations of NaCl were used in later studies to differentiate the target site of phosphate action.

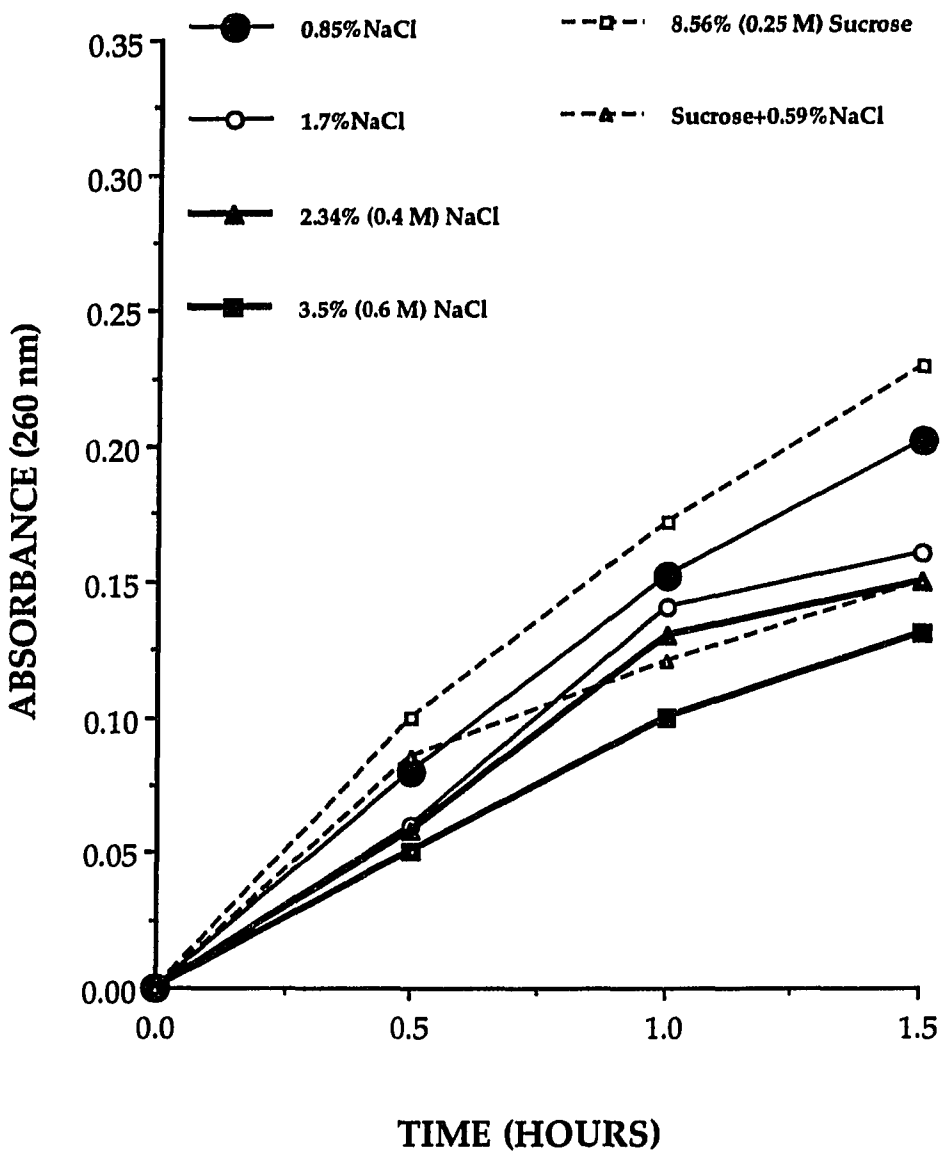


Figure 16. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 suspended in pH 6 NaCl and/or sucrose at 25°C. The mean standard deviation was 0.004.

Therefore, 0.85% NaCl (saline) was used as the suspending medium in most of the subsequent studies.

Bacteriolytic effect of phosphates

Because phosphates are bactericidal as well as bacteriostatic, a mechanism other than the inhibition of macromolecular synthesis must be involved. Bacterial leakage was tested. Bacterial leakage is defined as the leakage of cellular constituents, particularly intracellular nucleotides measured spectrophotometrically by absorbance at 260 nm.

As described above, there would be a detectable absorbance reading at 260 nm for the control. Leakage of 260-nm absorbing material was compared to the control when cells of *S. aureus* were treated with phosphates and disodium ethylenediamine tetraacetate (EDTA) at 25°C (Figure 17). All phosphates induced significantly ($P<0.05$) more bacterial leakage than the control by 1 hour of incubation. In general, the longer the chain-length , the greater the extent of bacterial leakage. EDTA was used as a positive control of bacterial leakage (Gray and Wilkinson, 1965). EDTA (0.0034 M) induced the greatest amount of bacterial leakage at 0.5 hour of incubation. Bacterial leakage from EDTA (0.0034 M), SPG (0.1%), and UP (0.1%) were comparable ($P>0.05$) at 1 hour of incubation. UP (0.1%) induced significantly ($P<0.05$) greater leakage than EDTA at 1.5 hours of incubation. As a control, the amount of 260-nm absorbing material present in a cell-free supernantant of a boiled (100°C, 15 minutes) and cooled cell suspension was measured (Greenway and Dyke, 1979). The total amount of 260-nm absorbing material released from the boiled control was 0.61 ± 0.04. Absorbance values from SPG, UP, and EDTA treatments were about half of those of the

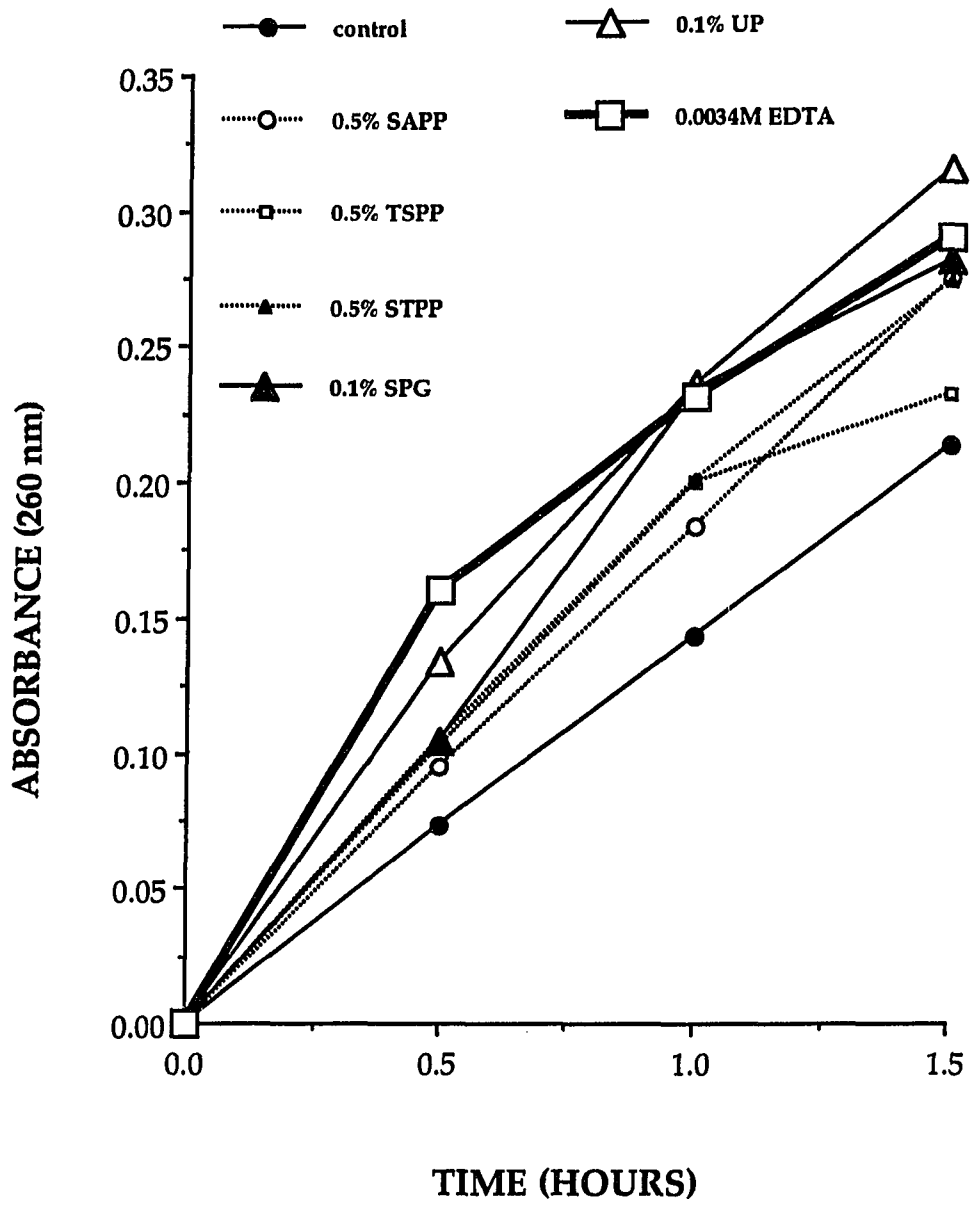


Figure 17. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 phosphates and EDTA (0.0034 M) at 25°C. The mean standard deviation was 0.0118

boiled control at 1.5 hours of incubation. These results were consistent with other researcher's findings (Gray and Wilkinson, 1965)

Microscopy

Nucleotides absorb strongly at absorbance 260 nm and proteins absorb strongly at 280 nm. Since absorbance 260 nm (Figure 16) and 280 nm (data not shown) readings were detected spectrophotometrically when cells were treated with phosphates, nucleotides and proteins were suspected to be released during cell lysis. To confirm the bacteriolytic property of phosphates, leakage study was conducted microscopically.

Massive gelatinous aggregates were observed microscopically when cells were treated with SPG (Figure 19) and UP (Figure 20), compared to the control (Figure 18). To further identify these massive gelatinous aggregates, enzymes (DNase, RNase and Proteinase K) were used to hydrolyze what was suspected to be nucleotides and proteins that had been released during cell lysis. There were less gelatinous aggregates from enzyme(s)-SPG-treated cells (Figures 21-24) than that of the SPG-treated cells (Figure 19). The presence of gelatinous aggregates was dependent on the type of enzyme(s) used. The effectiveness of enzyme(s) in dissolving the aggregates was as follows: DNase+RNase+Proteinase K (Figure 24) > DNase (Figure 21) > Proteinase K (Figure 23) > RNase (Figure 22). These results indicated that the massive gelatinous aggregates were nucleotides and proteins that had been released during cell lysis. The relative amounts were in the following descending order: DNA > Proteins > RNA. The study with microscopy confirmed that phosphates were bacteriolytic.

Figure 18. Microscopic view (1000X) of crystal violet stained early-exponential phase cells of *S. aureus* ISP40 suspended in pH 6 saline for 0.5 hour at 25°C.

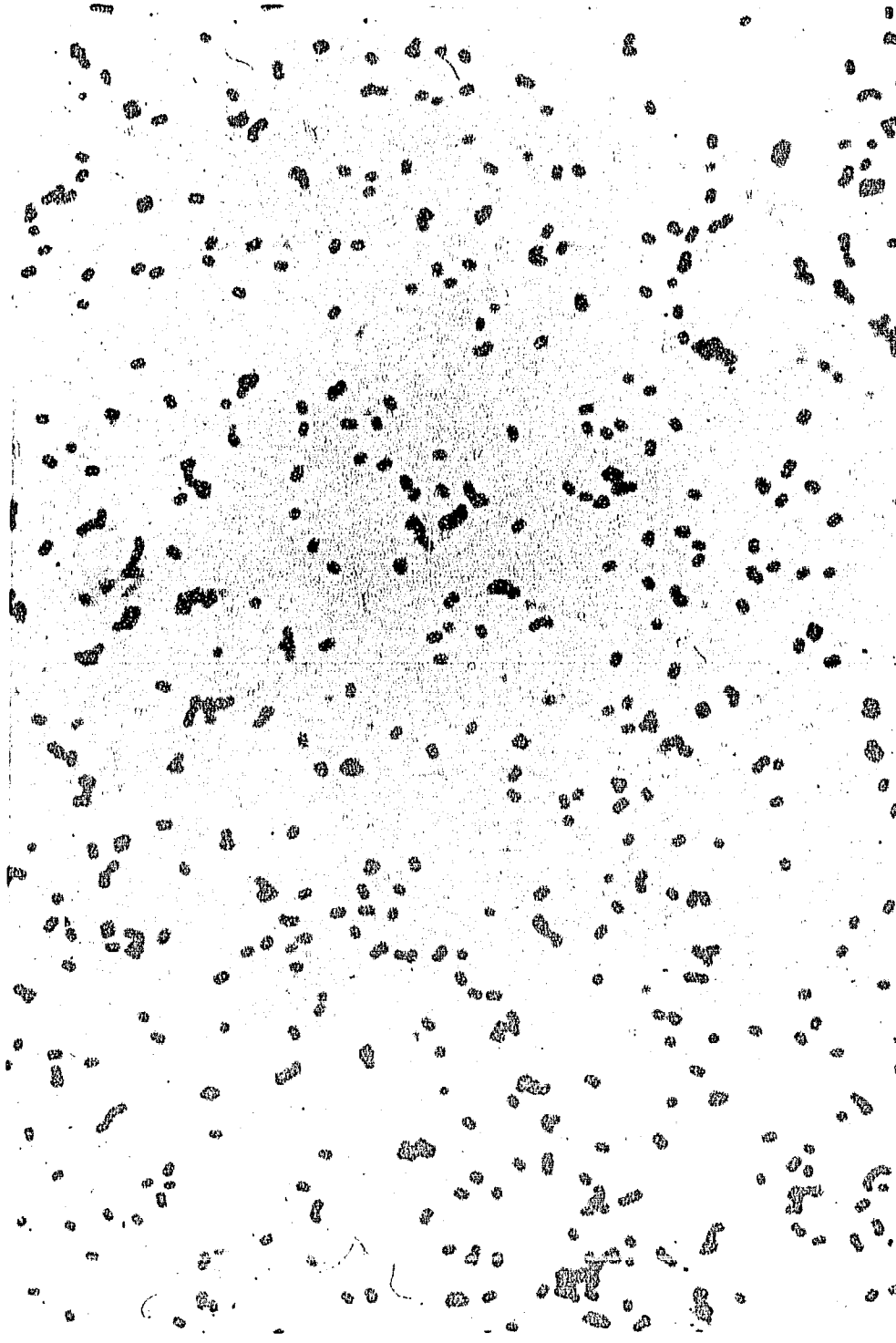


Figure 19. Microscopic view (1000X) of massive gelatinous aggregates in crystal violet stained early-exponential phase cells of *S. aureus* ISP40 treated with pH 6 SPG (0.1%) for 0.5 hour at 25°C.

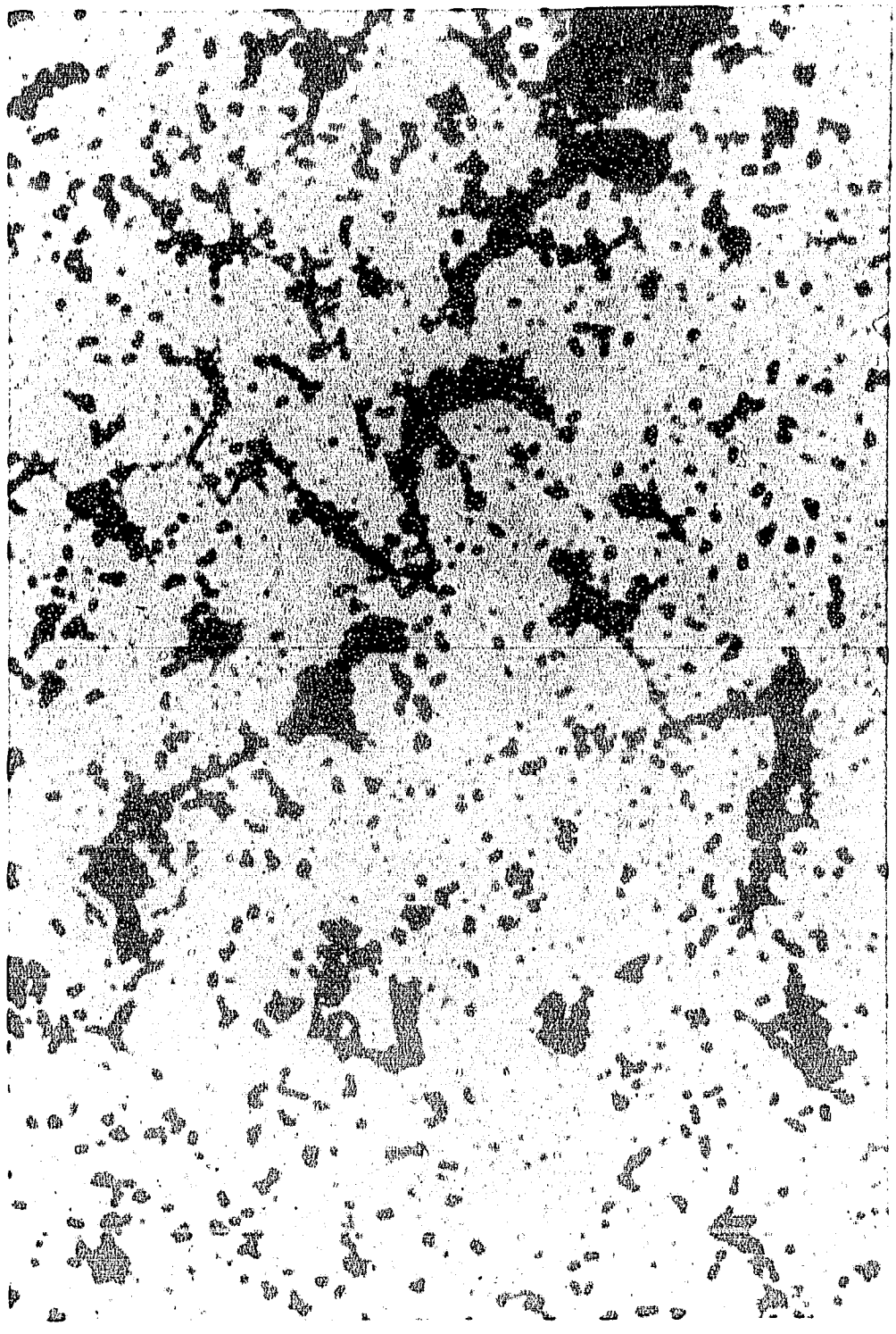


Figure 20. Microscopic view (1000X) of massive gelatinous aggregates incrystal violet stained early-exponential phase cells of *S. aureus* ISP40 treated with pH 6 UP (0.1%) for 0.5 hour at 25°C.

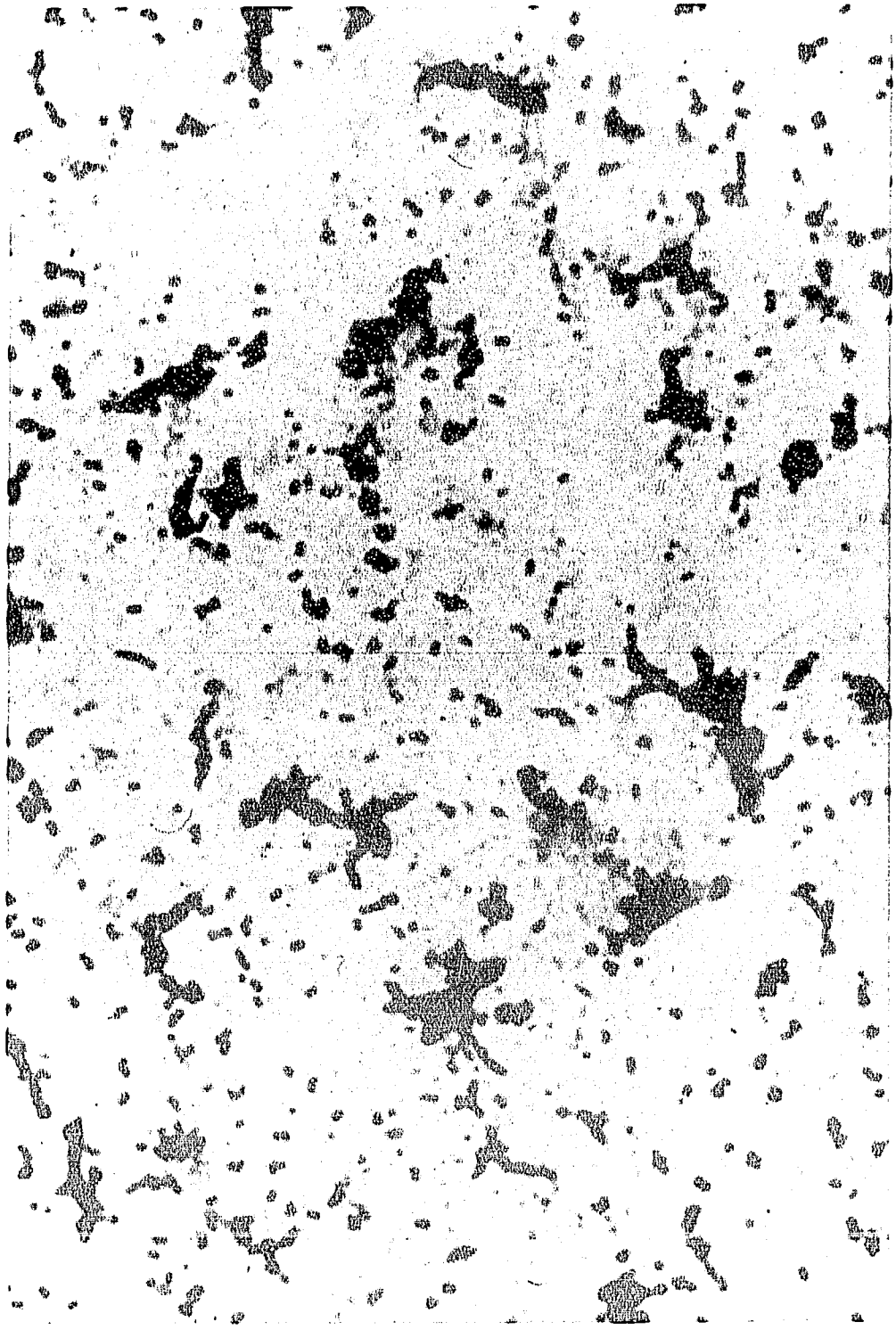


Figure 21. Microscopic view (1000X) of crystal violet stained early-exponential phase cells of *S. aureus* ISP 40 treated with pH 6 SPG (0.1%) and DNase for 1 hour at 25°C.

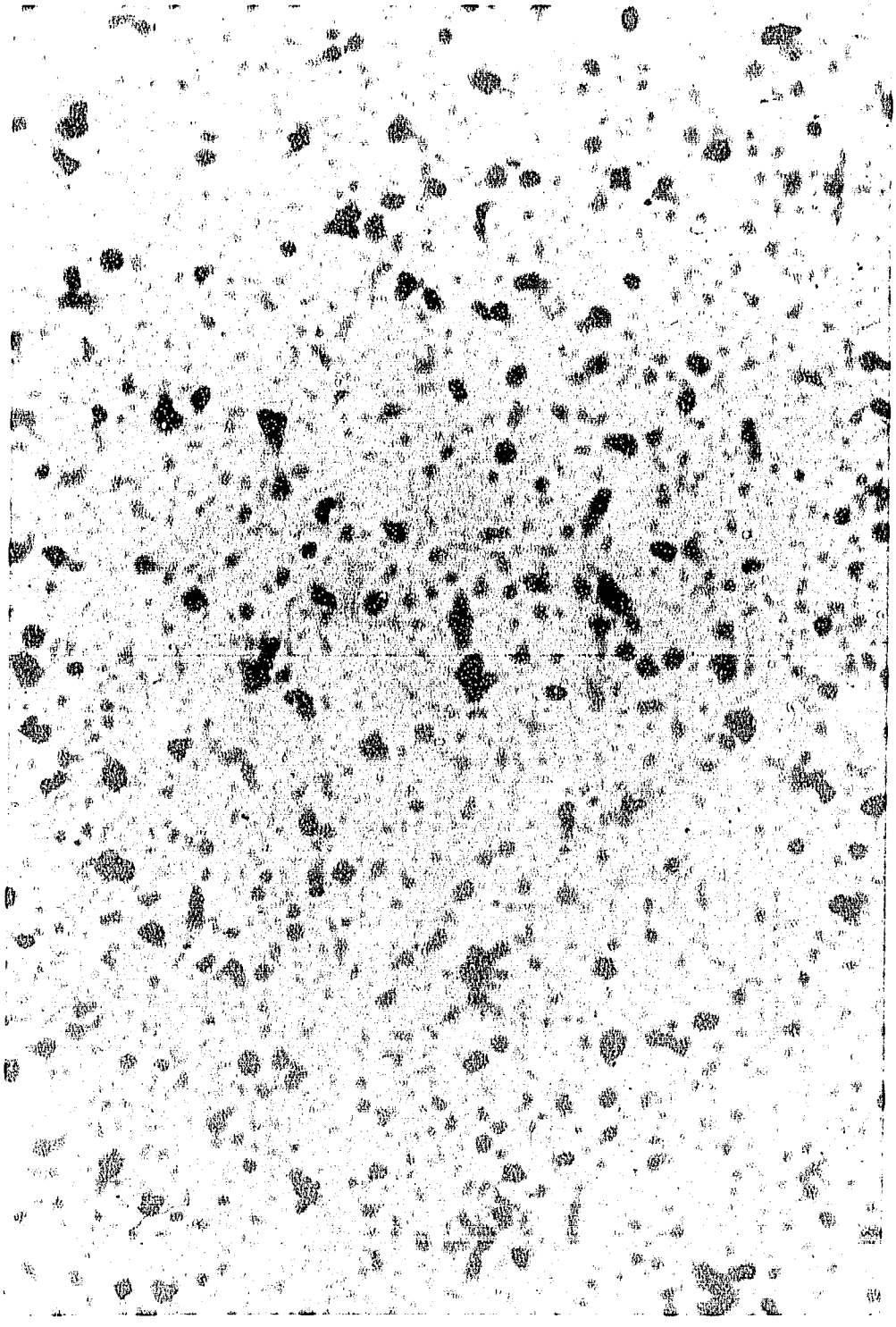


Figure 22. Microscopic view (1000X) of crystal violet stained early-exponential phase cells of *S. aureus* ISP 40 treated with pH 6 SPG (0.1%) and RNase for 1 hour at 25°C.

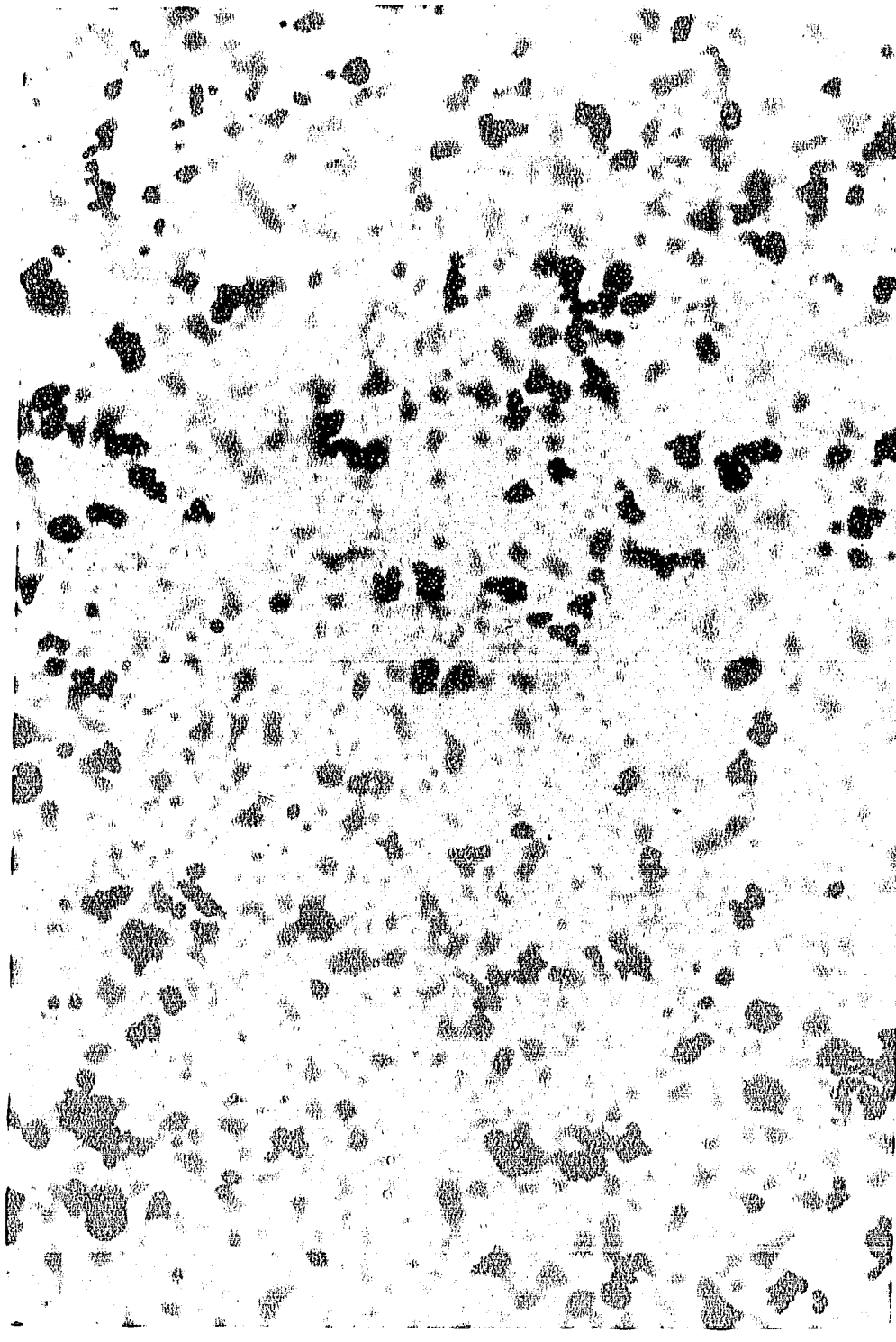


Figure 23. Microscopic view (1000X) of crystal violet stained early-exponential phase cells of *S. aureus* ISP 40 treated with pH 6 SPG (0.1%) and proteinase K for 1 hour at 25°C.

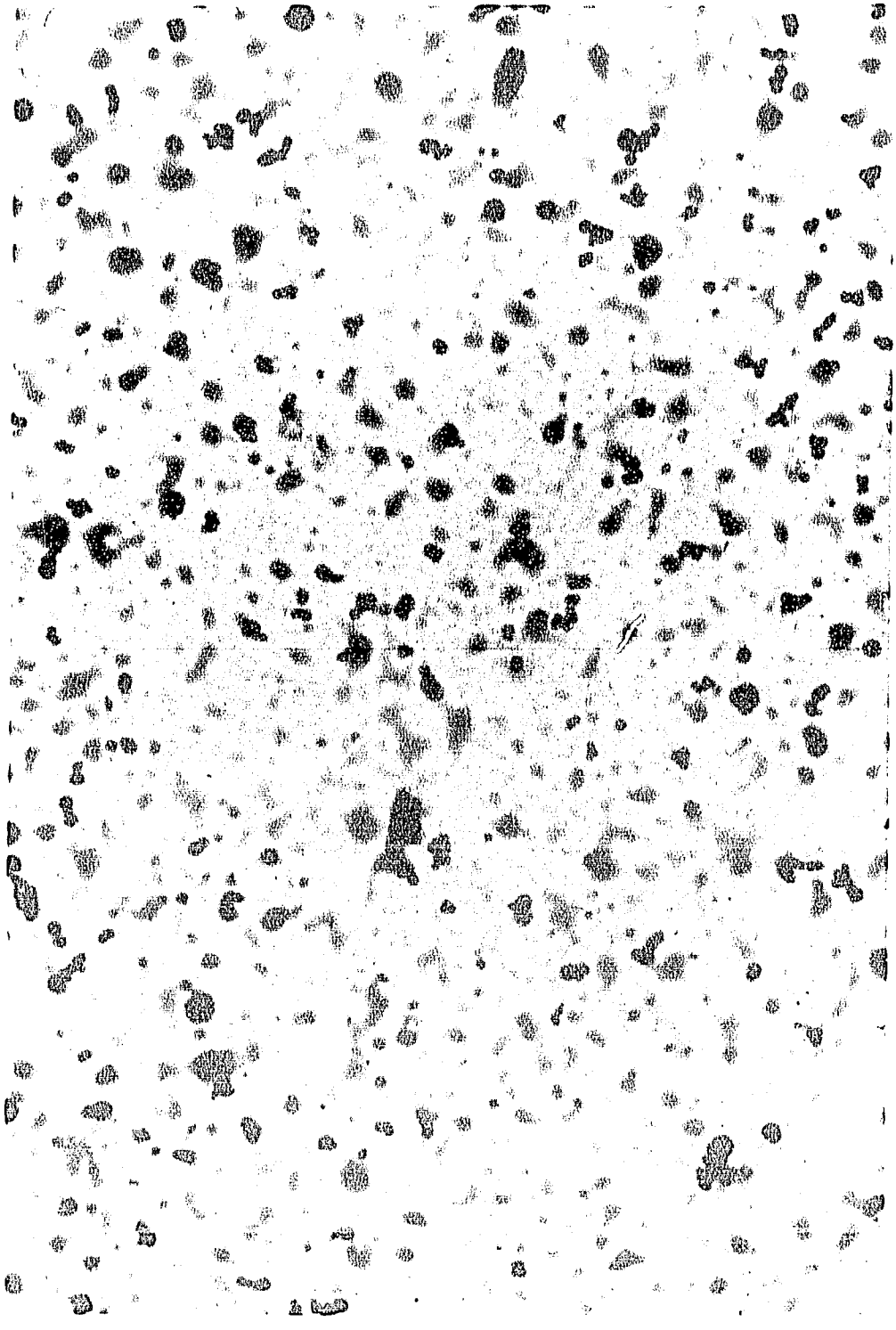
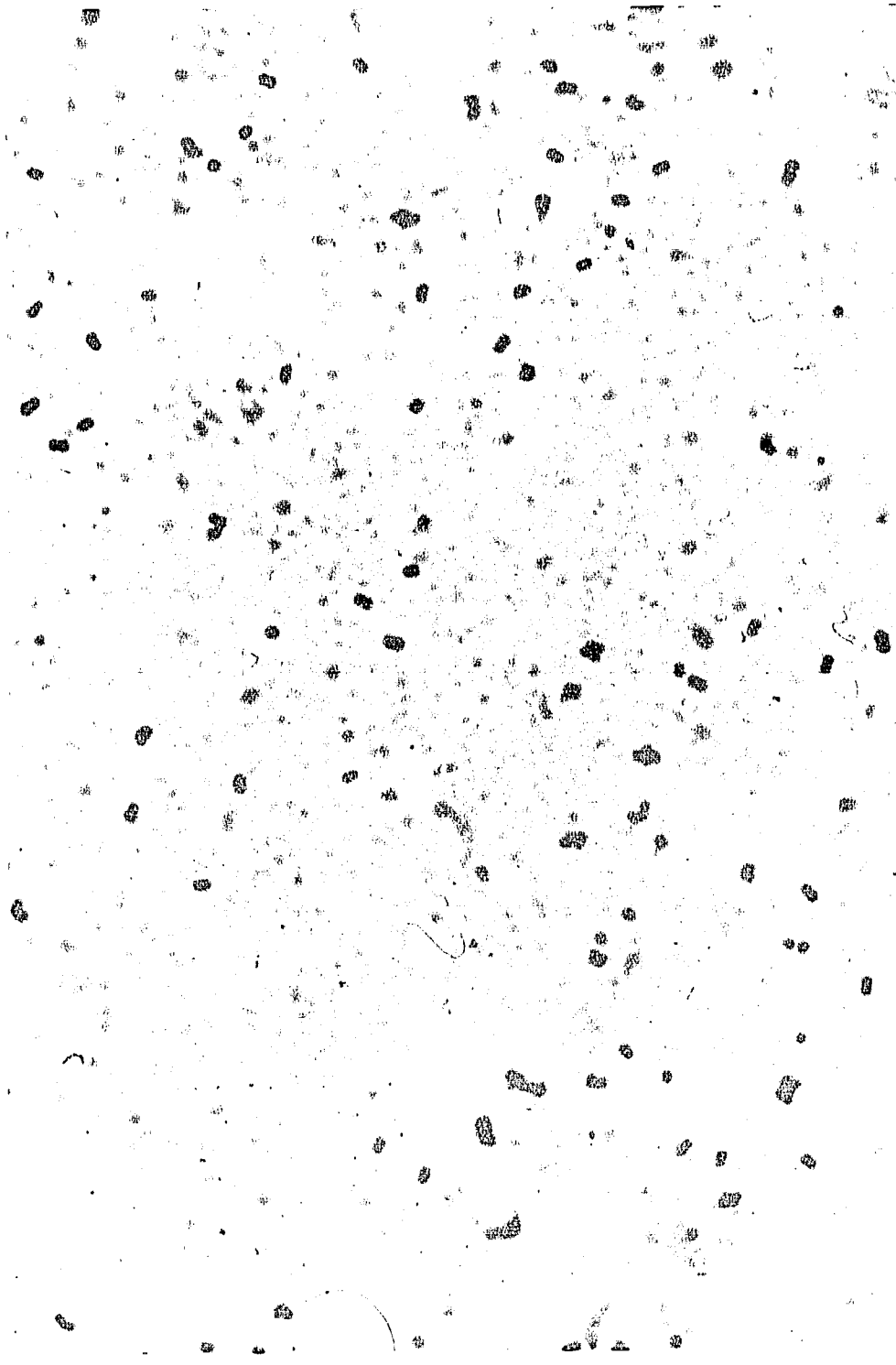


Figure 24. Microscopic view (1000X) of crystal violet stained early-exponential phase cells of *S. aureus* ISP 40 treated with pH 6 SPG (0.1%) and DNase, RNase, and proteinase K for 1 hour at 25°C.



Absorbance 260 nm readings were detected for the control which suggested the release of nucleotides from the bacteria (Figure 17). However, no massive gelatinous aggregates were observed at 0.5 hour or 1 hour of incubation (Figure 18). *S. aureus* is a known producer of protease(s) (Zayaitz and Ledford, 1982) and nucleases (Jay, 1986; Tatani et al., 1975; Zayaitz and Ledford, 1982). These enzymes hydrolyze the nucleotides and proteins being released from the cells, thereby no massive gelatinous aggregates were observed. On the other hand, phosphates are known to inhibit nucleases (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD). Therefore, it is suspected that the gelatinous aggregates accumulated because cellular constituents released during cell lysis were not hydrolyzed by nucleases in the presence of phosphates.

Target site of phosphates

The bactericidal effects shown in the growth studies and the bacteriolytic effects shown in the leakage studies indicated that the cell envelope (cell wall or cell membrane) was damaged in the presence of phosphates. To differentiate action between the cell wall and the cell membrane, phosphates were tested against *S. aureus* in the presence of high concentrations (0.4 M and 0.6 M) of NaCl (Gray and Wilkinson, 1965).

Polymyxin, a peptide antibiotic that causes cell membrane damage, was used as a positive control (Newton, 1954; 1956). Polymyxin (200 μg/ml) induced bacterial leakage (Figure 25). In the presence of high concentrations (0.6 M) of NaCl, polymyxin still induced significant ($P<0.05$) amounts of bacterial leakage.

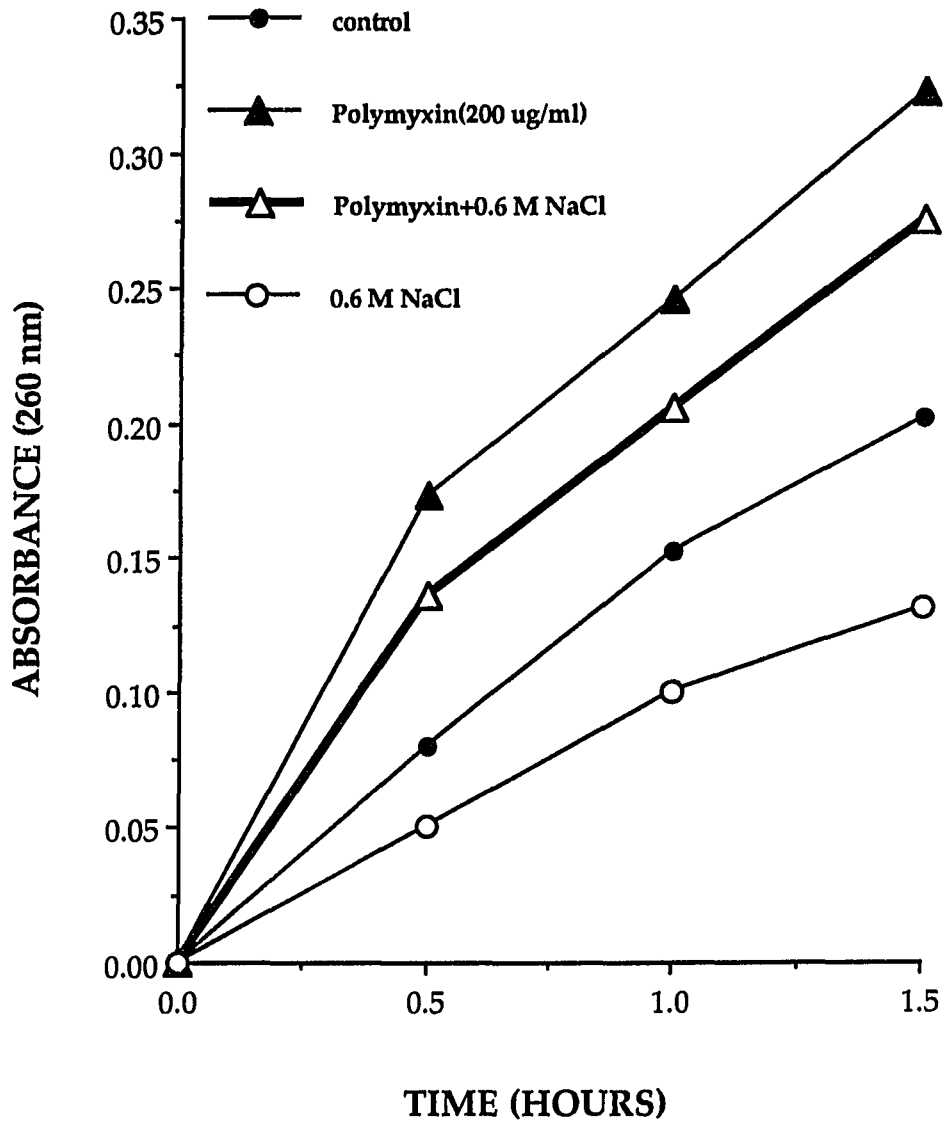


Figure 25. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 polymyxin B sulfate (200 ug/ml) and/or NaCl (0.6 M) at 25°C. The mean standard deviation was 0.01.

Because functions of the cell membrane (the osmotic barrier) were impaired, even high concentrations of NaCl could not completely prevent cell leakage.

EDTA, a well-known metal chelator that causes cell wall damage, was used as a positive control (Gray and Wilkinson, 1965). EDTA (0.0034 M) induced bacterial leakage (Figure 26). In the presence of high concentration (0.4 M) of NaCl, bacterial leakage by EDTA was significantly reduced and leakage was comparable ($P>0.05$) to that of the control. Since the cell membrane was not damaged by EDTA, high concentration of NaCl provided an isotonic environment that retarded bacterial leakage.

TSPP (0.5%) induced bacterial leakage (Figure 27). In the presence of 0.4 M NaCl, bacterial leakage by TSPP significantly ($P<0.05$) reduced to one third at 1 hour of incubation when compared to that of TSPP without NaCl protection. In the presence of 0.6 M NaCl, bacterial leakage by TSPP was prevented. Since high concentration of NaCl (0.6 M) prevented bacterial leakage by TSPP, the cell membrane was not damaged. These results suggested that the target site of TSPP was at the cell wall.

SPG (0.1%) induced bacterial leakage (Figure 28). In the presence of 0.4 M NaCl, bacterial leakage by SPG was prevented and comparable ($P>0.05$) to that of the control. These results suggested that the target site of SPG was at the cell wall.

UP (0.1%) induced bacterial leakage (Figure 29). In the presence of 0.4 M NaCl, bacterial leakage by UP was significantly ($P<0.05$) reduced. In the presence of 0.6 M NaCl, bacterial leakage by UP was prevented. These results suggested that the target site of UP was at the cell wall.

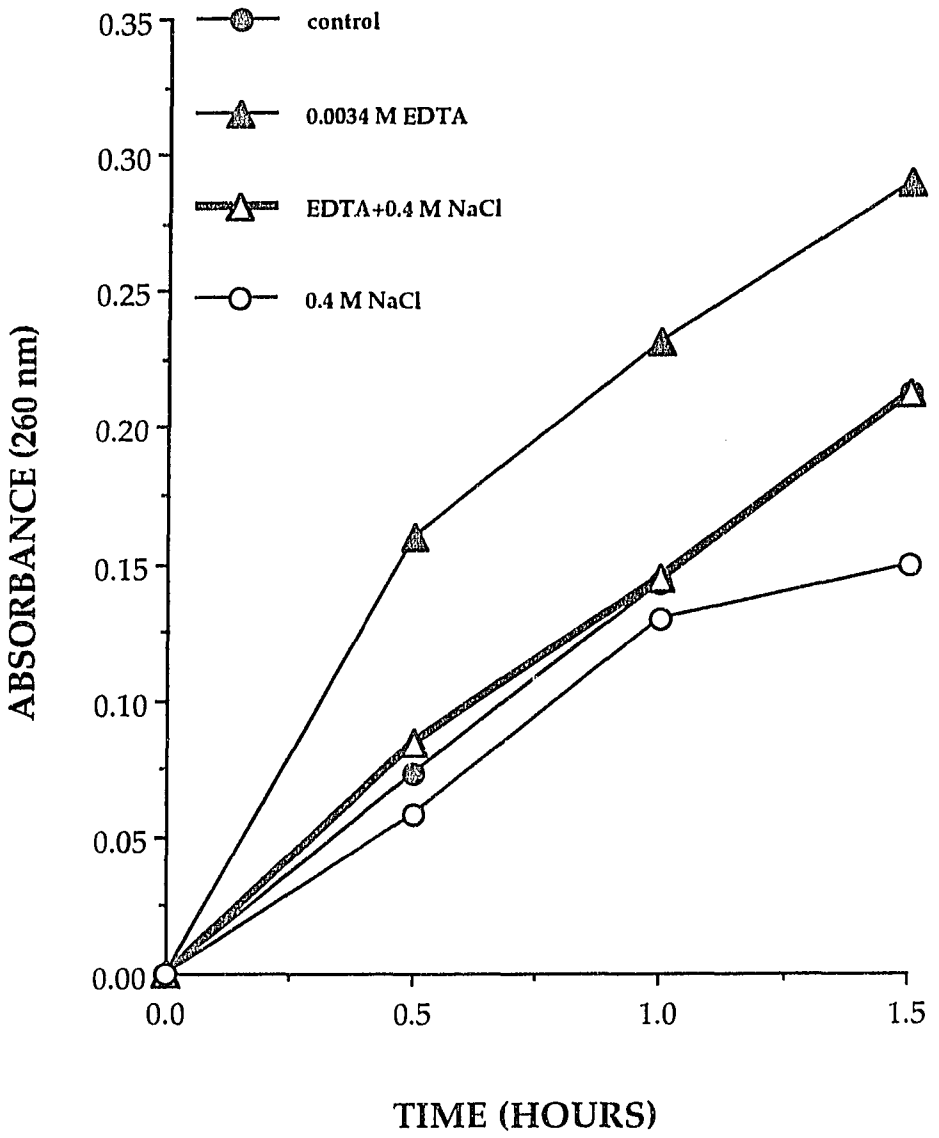


Figure 26. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 EDTA (0.0034 M) and/or NaCl (0.4 M) at 25°C. The mean standard deviation was 0.01.

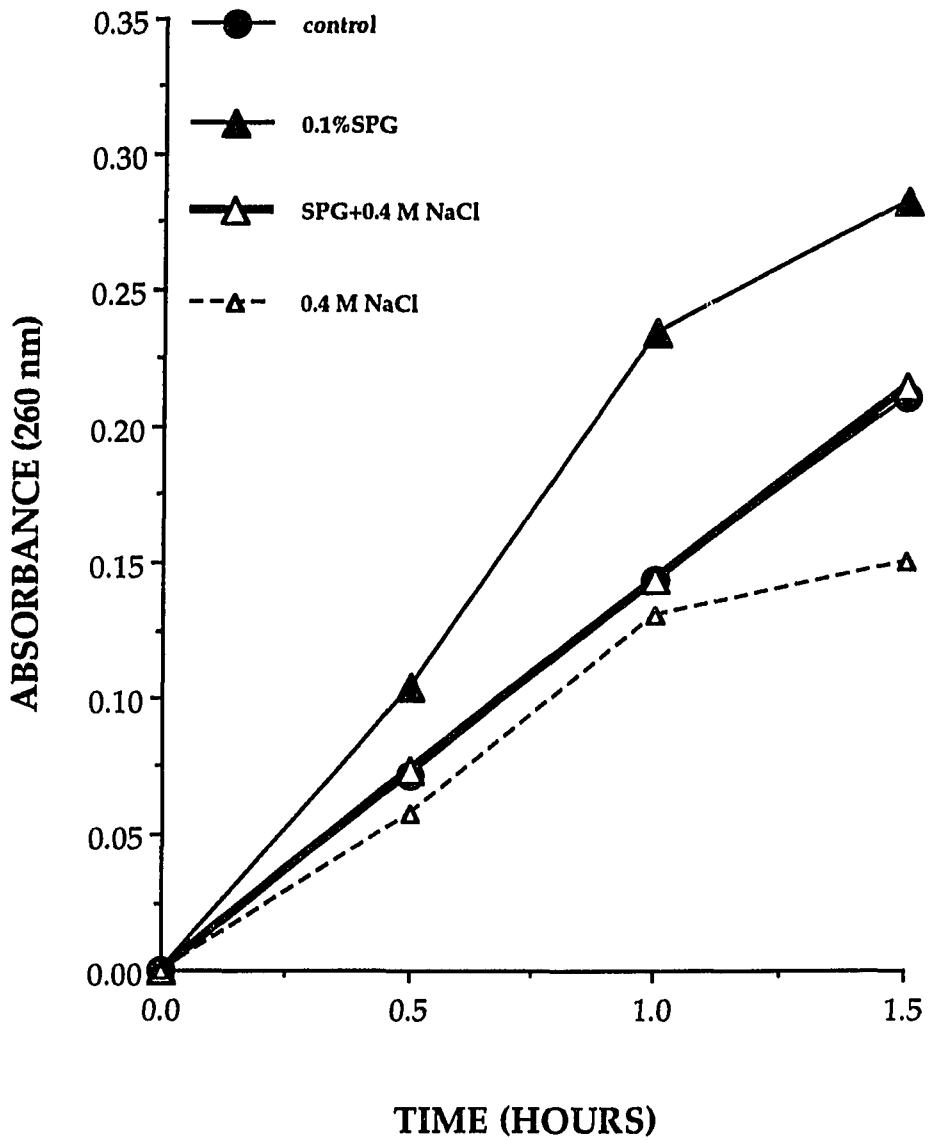


Figure 28. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 SPG (0.1%) and/or NaCl (0.4 M) at 25°C. The mean standard deviation was 0.002.

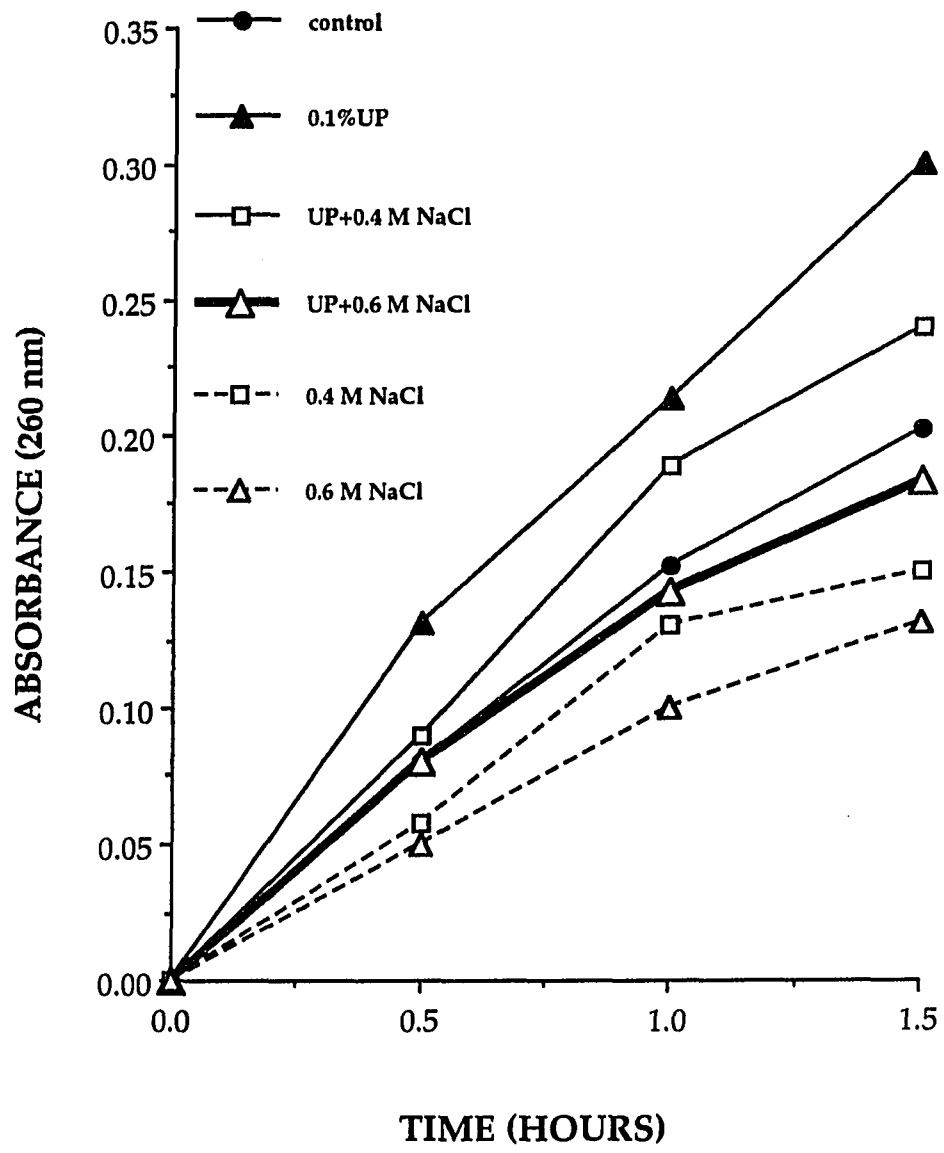


Figure 29. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 UP (0.1%) and/or NaCl (0.4 M and 0.6 M) at 25°C. The mean standard deviation was 0.01.

pH study for metal chelation mechanism

Results from previous studies have suggested that phosphates interact with the cell wall of *S. aureus* which results in the bactericidal and bacteriolytic effects. In addition, results of the leakage studies from EDTA-treated cells (Figures 17, 26) were very similar to those of phosphate-treated cells, particularly the SPG-treated cells (Figures 17, 28) and UP-treated cells (Figure 17, 29). Moreover, EDTA and phosphates are metal chelators. Therefore, phosphates are suspected to interact with the cell wall of *S. sureus* by a metal chelation mechanism. Calcium and magnesium are the major endogeneous metals found in the cell wall of Gram-positive bacteria (Beveridge et al., 1982). Since chelation of calcium and magnesium by long-chain polyphosphates should increase as pH increases (Steinhauer, 1983), pH study was pursued to confirm if a metal chelation mechanism might involve in the antibacterial mechanism by determining if higher pH would increase the bacteriolytic properties of phosphates.

Absorbance 260 nm measurements of controls at pH 6, 7 and 8 are shown in Figure 30. The controls at pH 6 and 7 had similar absorbance 260 nm readings. The control at pH 8 induced a greater leakage than the controls at pH 6 and 7 at 0.5 hour of incubation. Bacterial leakage from control at pH 8 was lower than that of the controls pH 6 and 7 at 1.5 hours of incubation. Figures 31-33 show that bacterial leakage in the presence of 0.5% TSPP (Figure 31), 0.1% SPG (Figure 32), and 0.1% UP (Figure 33) was significantly ($P<0.05$) greater at pH 8 than pH 6 by 1 hour of incubation. The results suggested that metal chelation was involved in the antibacterial mechanism.

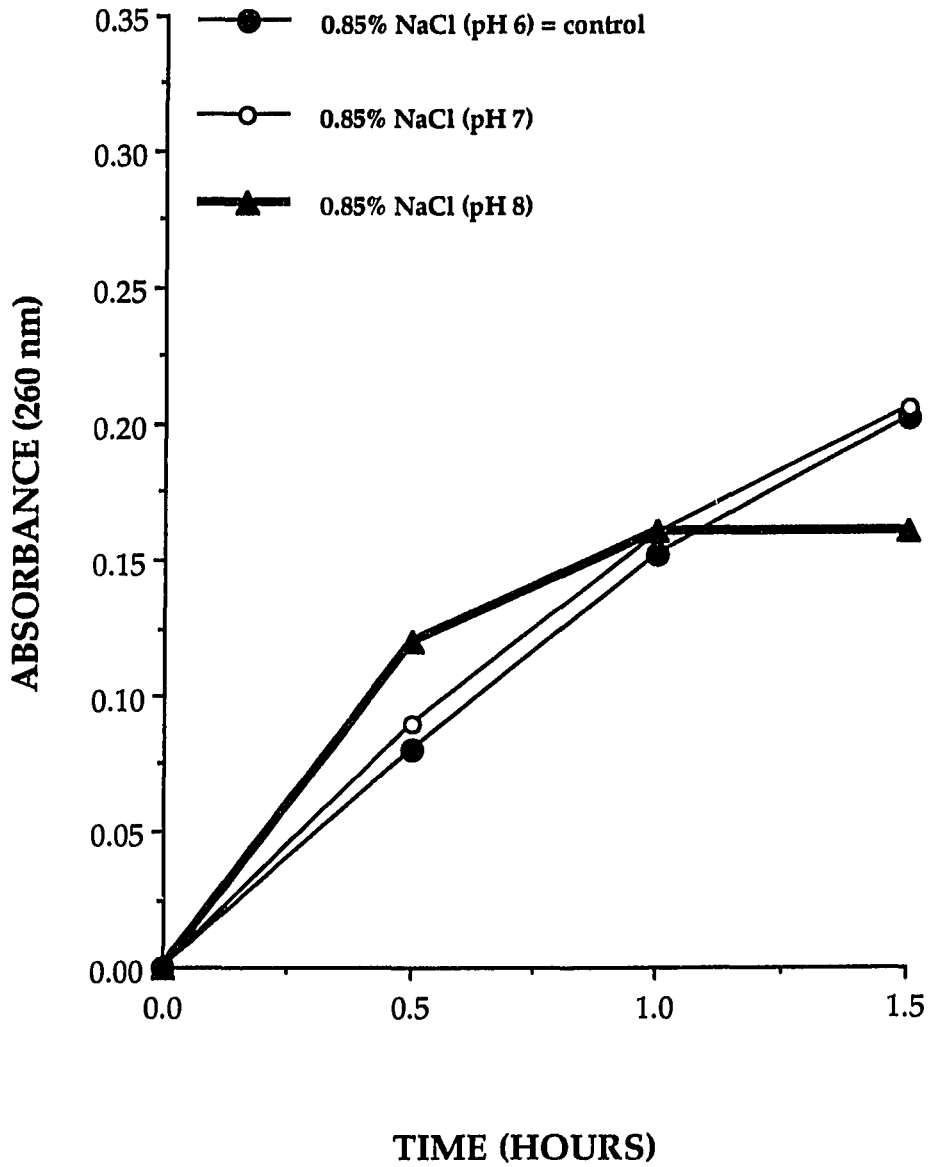


Figure 30. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 suspended in 0.85% NaCl (pH 6, pH 7, pH 8) at 25°C. The mean standard deviation was 0.005.

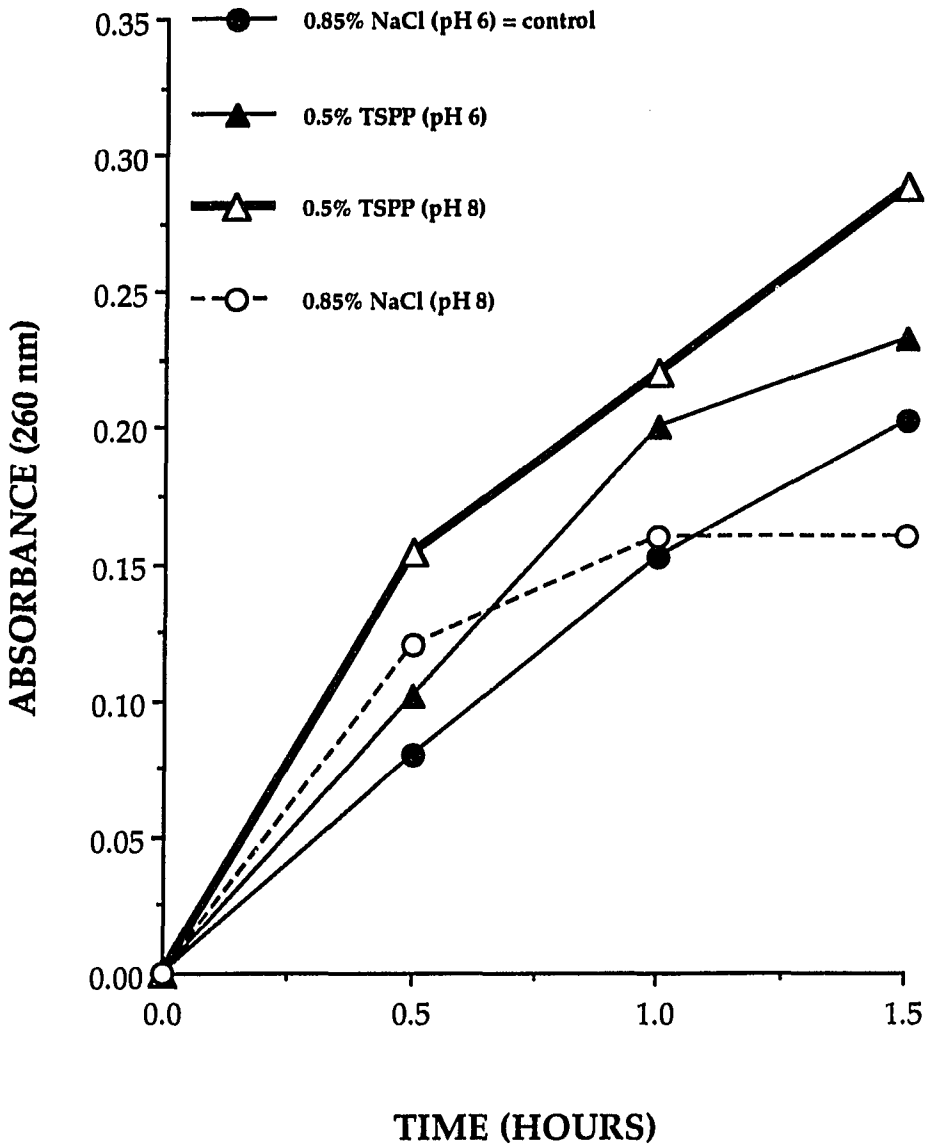


Figure 31. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with 0.5% TSPP (pH 6, pH 8) at 25°C. The mean standard deviation was 0.006.

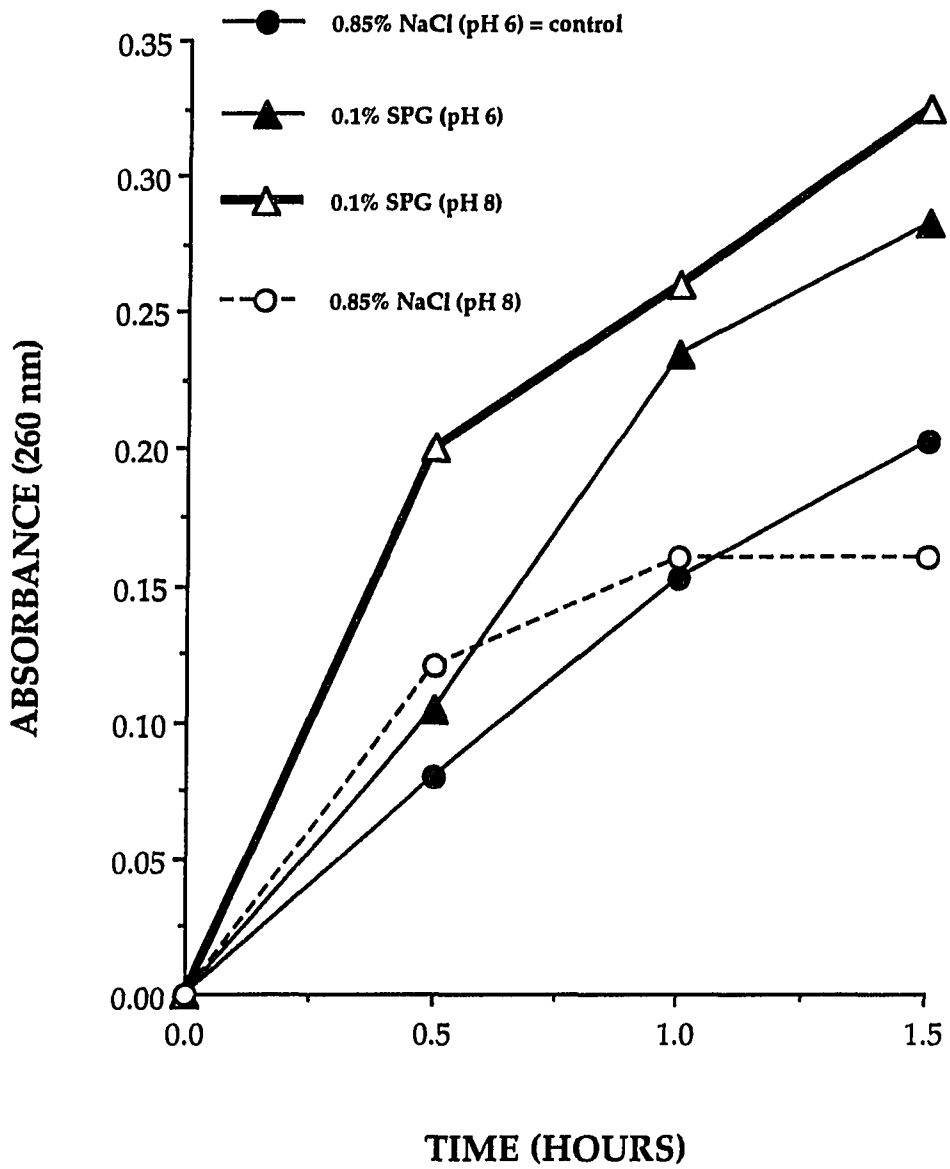


Figure 32. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with 0.1% SPG (pH 6, pH 8) at 25°C. The mean standard deviation was 0.004.

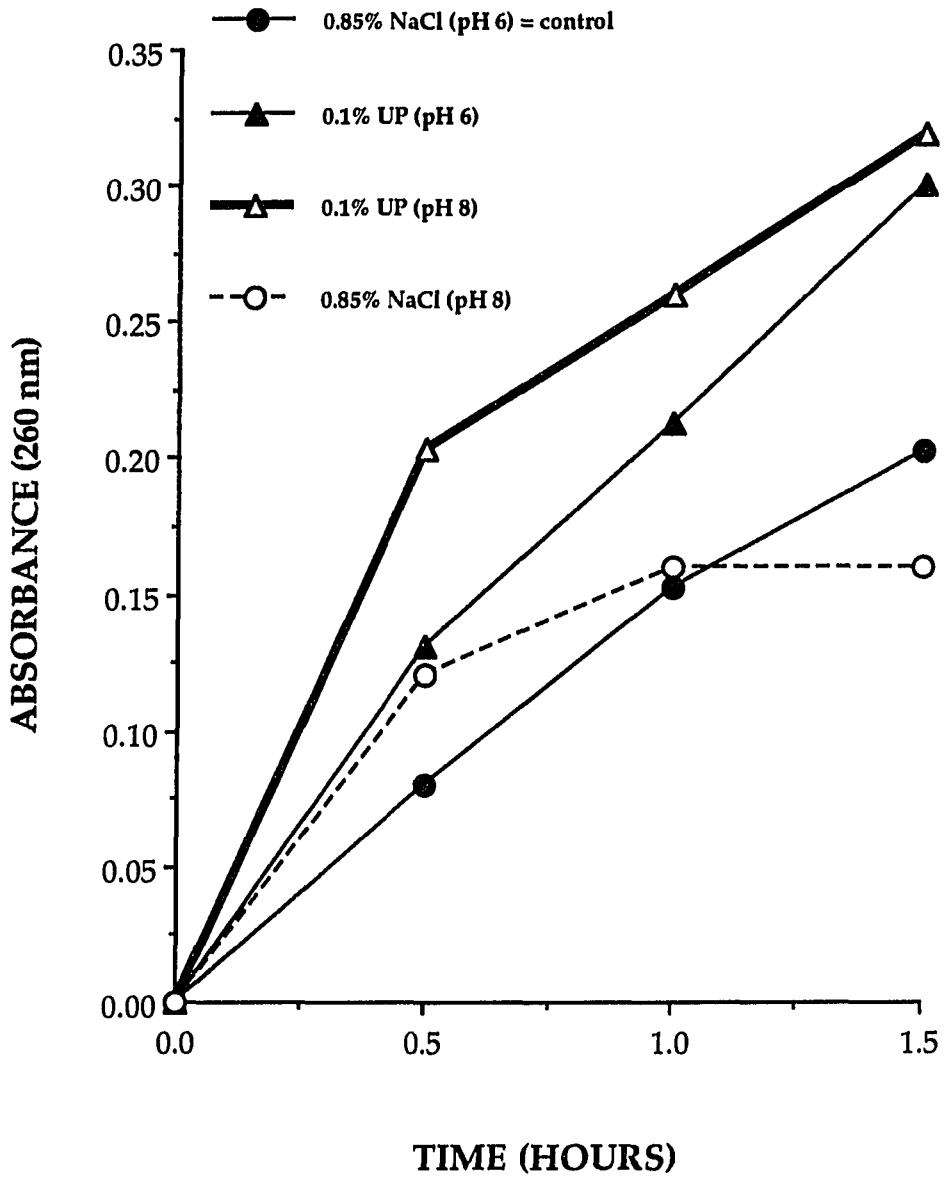


Figure 33. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with 0.1% UP (pH 6, pH 8) at 25°C. The mean standard deviation was 0.012.

Metal-Ion Studies

Reversal of growth inhibition by metal ions

Results of previous studies suggested that phosphates interacted with the cell wall of *S. aureus* by a metal chelation mechanism. Because Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{3+} are the major essential metals in bacteria (Beveridge and Murray, 1976b; Knabel et al., 1991), several concentrations (0.001, 0.01 and 0.1 M) of these metals were tested for their abilities to reverse growth inhibition in the presence of phosphates. The results indicated that 0.01 M Ca^{2+} or Mg^{2+} reversed the growth inhibition of 0.1% UP or SPG. The growth inhibition of 0.5% TSPP was reversed by 0.01 M Fe^{3+}.

Reversal of UP inhibition by metal ions

Reversal of bacteriolytic effect of UP by metal ions

Since 0.01 M Ca^{2+} or Mg^{2+} reversed growth inhibition of 0.1% UP, these metals were tested for their ability to reverse the bacteriolytic effect of UP. Absorbance 260 nm readings for the metal controls (0.01 M Ca^{2+} and Mg^{2+}) are shown in Figure 34. Both metal controls induced greater leakage than the 0.85% NaCl control at 0.5 hour of incubation and then decreased after 1 hour of incubation. Since both metal controls caused more leakage than the saline control at 0.5 hour of incubation, the reversal of bacteriolytic effect of UP by these metals would be confirmed if these metals reduced the leakage caused by UP. Figure 35 showed that both Ca^{2+} and Mg^{2+} significantly ($P<0.05$) decreased the leakage of intracellular nucleotides caused by UP in which Ca^{2+} had a significantly ($P<0.05$) greater reversal of leakage than Mg^{2+} at 0.5 hour of incubation. Mg^{2+} retarded leakage only at 0.5 and 1 hour of incubation.

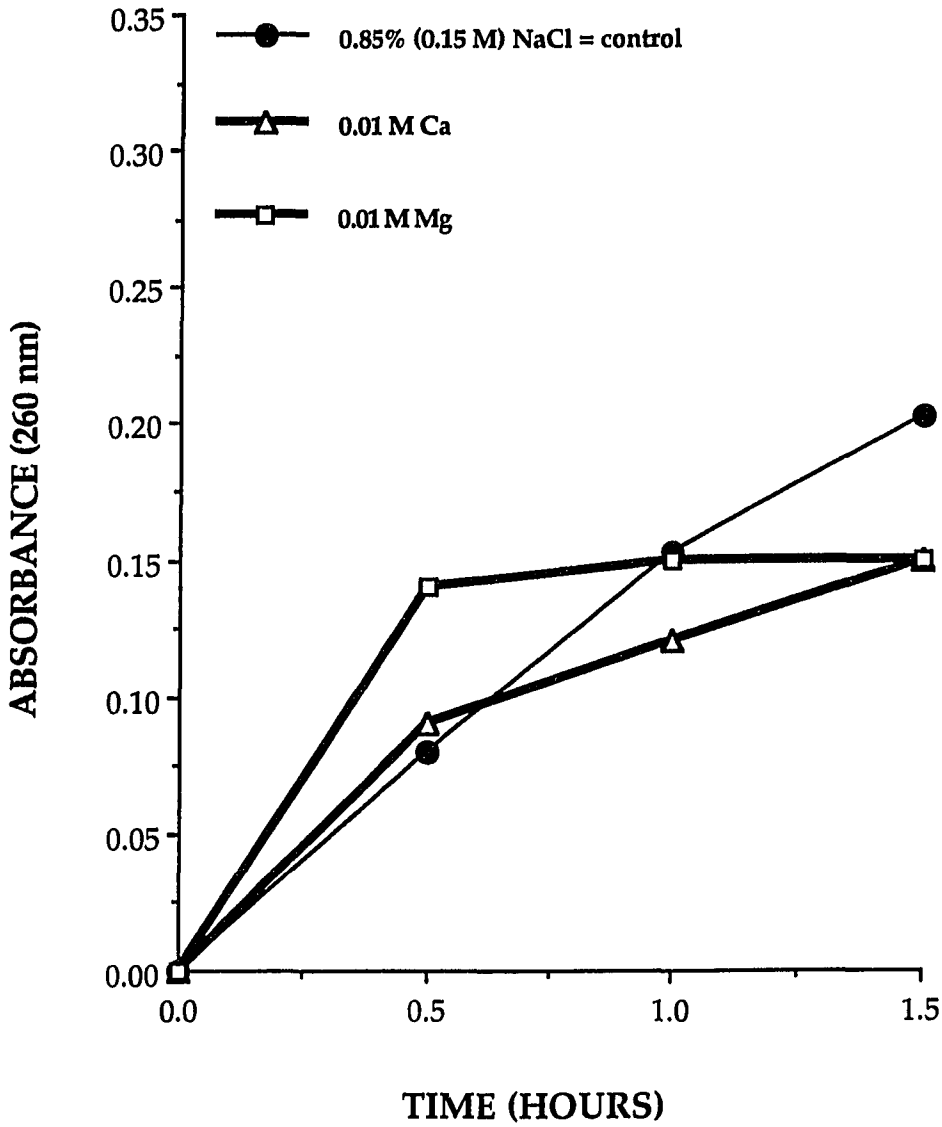


Figure 34. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 suspended in Mg^{2+} (0.01 M) and Ca^{2+} (0.01 M) solution at 25°C. The mean standard deviation was 0.006.

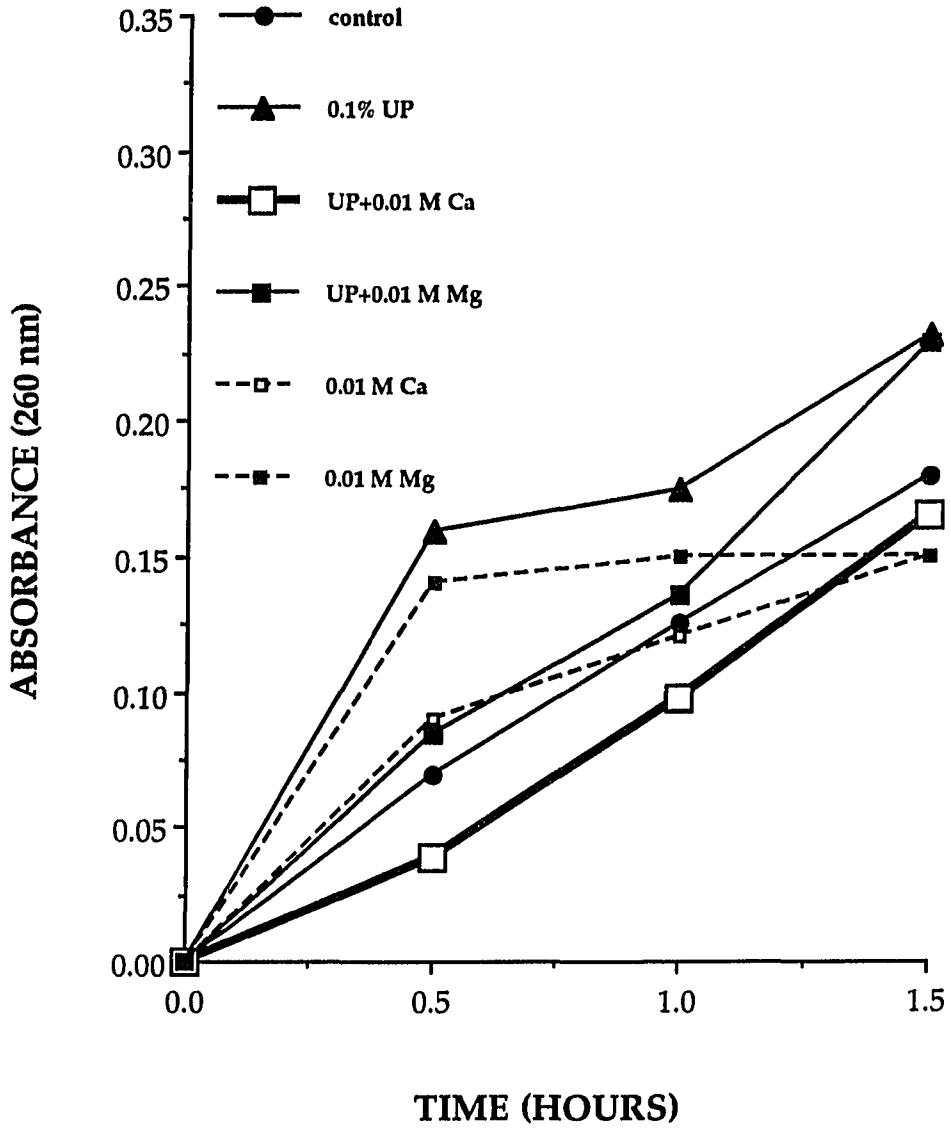


Figure 35. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 UP (0.1%), UP plus Ca^{2+} (0.01M), and UP plus Mg^{2+} (0.01M) at 25°C. The mean standard deviation was 0.017.

These results suggested that Ca^{2+} and Mg^{2+} reversed the bacteriolytic effects of UP.

Reversal of bactericidal effects of UP by metal ions

The reversal of the bactericidal effects of UP by metal ions was conducted to differentiate between metal-protection and metal-rescue studies, and to clarify each study. In the metal-protection study, cells were protected by metals one hour before exposure to UP. The bactericidal effects of UP (0.1%) on *S. aureus* cells was reversed when the cells were protected by the addition of Ca^{2+} (0.01M) or Mg^{2+} (0.01 M) one hour before the addition of UP (Figure 36). The rate of reversal was a little faster for Ca^{2+} than Mg^{2+}. However, the rates of reversal were not significantly ($P>0.05$) different from each other. Reversal of the bactericidal effect of UP (0.1%) on *S. aureus* cells also was shown by rescuing cells by Ca^{2+} (0.01 M) or Mg^{2+} (0.01 M) one hour after UP was added (Figure 37). Again, the rate of reversal was a little faster for Ca^{2+} than Mg^{2+}. However, the rates of reversal were not significantly ($P>0.05$) different from each other. The reversal of growth inhibition was complete by 4 hours of incubation. Metal-rescue (Figure 37) was slightly more effective than metal-protection (Figure 36). Furthermore, as shown in Figure 36, the bacteria grew more rapidly in the metal controls (0.01 M Mg^{2+} and Ca^{2+}) than in the control to which Mg^{2+} and Ca^{2+} had not been added. Apparently, the mCDS (pH6) medium was marginal in Mg^{2+} and Ca^{2+} content.

The addition of either Ca^{2+} (0.01 M) or Mg^{2+} (0.01 M) reversed growth inhibition. However, whether Ca^{2+} and Mg^{2+} were additive or synergistic in their effects was not known. Additive and synergistic efffects of these metals

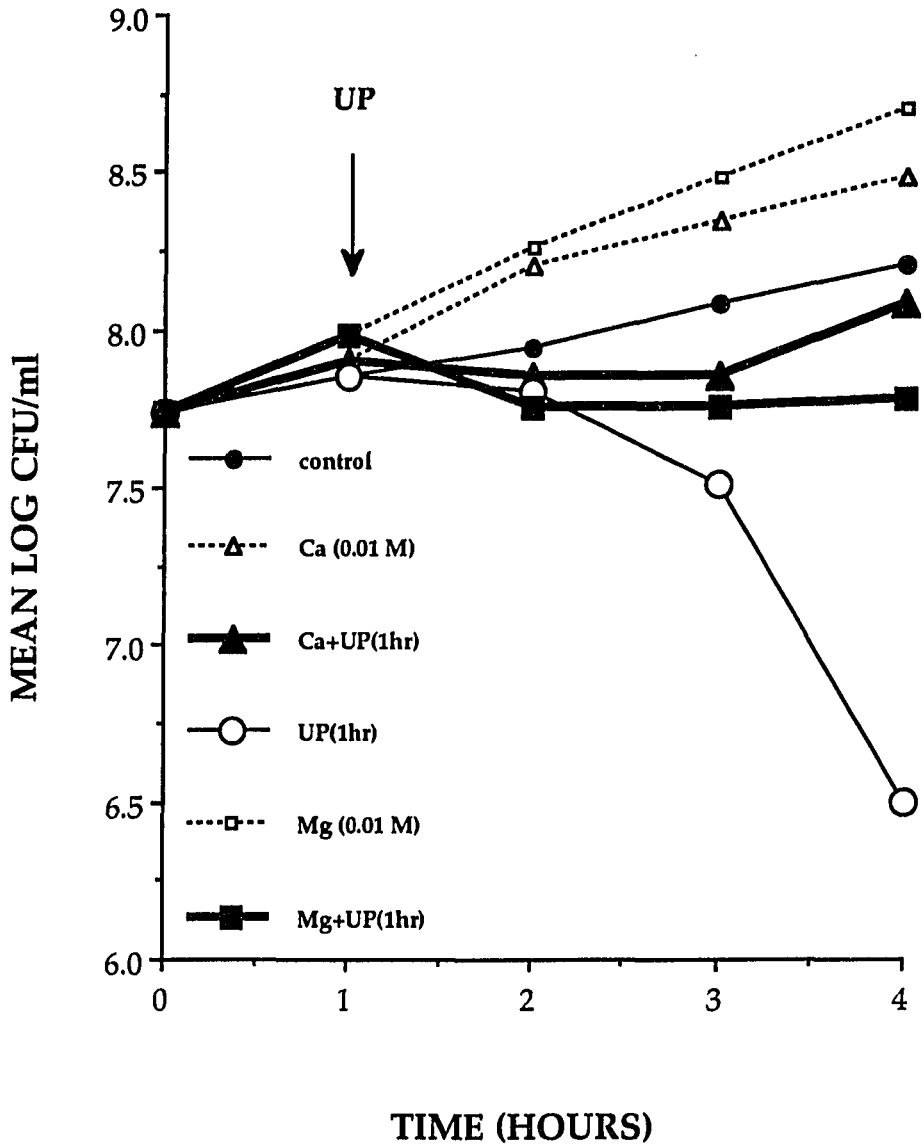


Figure 36. Reversal of bactericidal effect of UP (0.1%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by protecting cells with pH 6 Ca^{2+} (0.01M) or Mg^{2+} (0.01M) one hour before UP addition. The mean standard deviation was 0.19.

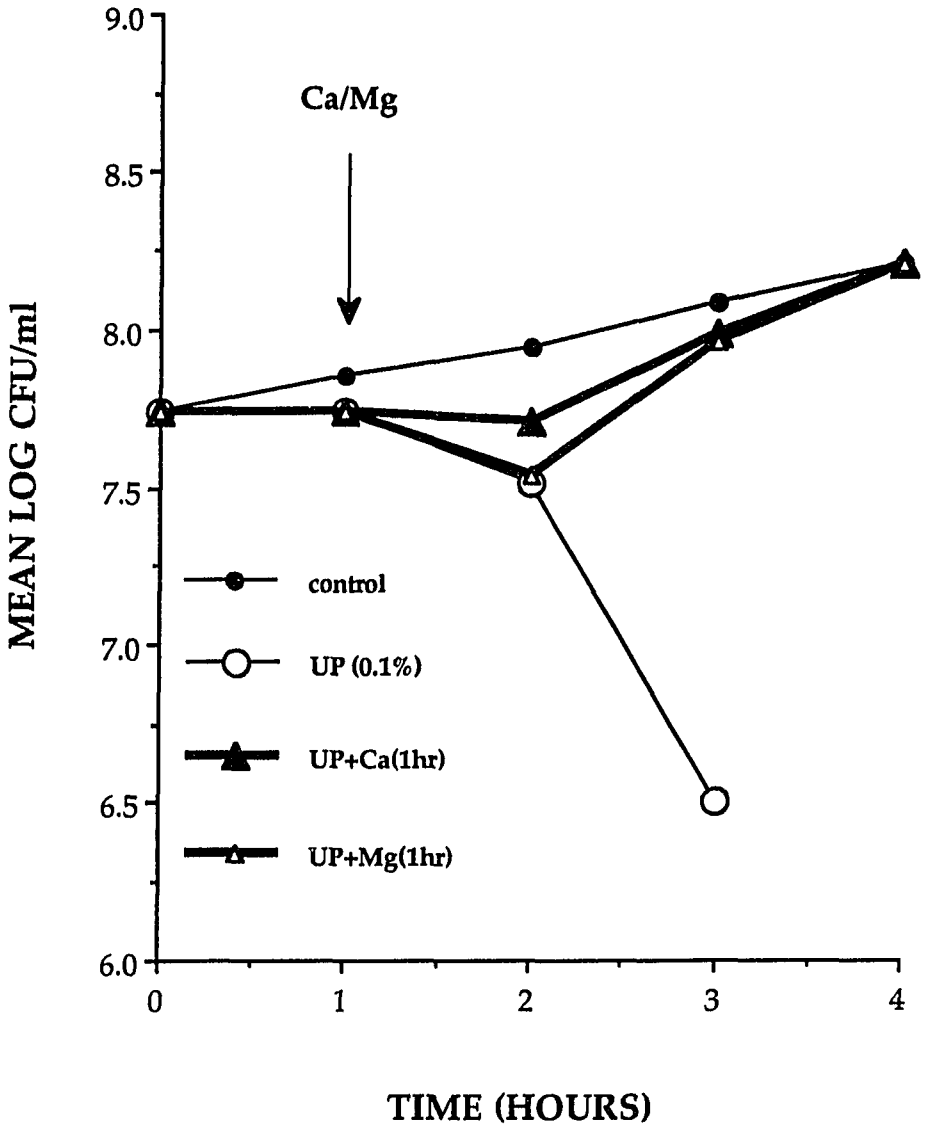


Figure 37. Reversal of bactericidal effect of UP (0.1%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by Ca^{2+} (0.01M) or Mg^{2+} (0.01M) rescue one hour after UP addition. The mean standard deviation was 0.15.

were tested to reverse the growth inhibition by the overnight UP preparations. The antibacterial effects of overnight UP preparations were less than that of the freshly prepared UP (Figures 13, 14) because of the hydrolysis of UP with time. Bacterial cells were not inhibited in the presence of 0.1% overnight UP preparation in the first 2 hours (Figure 38). Bacteriostatic effect was observed after 2 hours of incubation. Ca^{2+} (0.01 M) and Mg^{2+} (0.01 M) were added to rescue *S. aureus* 1 hour after the addition of UP (0.1%, overnight preparation). No reversal of growth inhibition by Ca^{2+} was observed. Also, Ca^{2+} and Mg^{2+} did not have any additive effects. Bacteriostatic effect of 0.2% overnight UP treatment was observed in Figure 39. Ca^{2+} reversed the bacteriostatic effect at 2 hours of incubation. However, the reversal of growth inhibition was stopped after 2 hours of incubation. Again, no additive effects of Ca^{2+} and Mg^{2+} were observed. Bactericidal effect of a 0.3% overnight UP preparation was observed in Figure 40. Ca^{2+} reversed the bactericidal effect by rescuing cells one hour after overnight UP addition.

Reversal of SPG inhibition by metal ions

Reversal of bacteriolytic effect of SPG by metal ions

Because 0.01 M Ca^{2+} or Mg^{2+} reversed growth inhibition caused by 0.1% SPG treatment, these metals were tested for their ability to reverse the bacteriolytic effect of SPG. Absorbance 260 nm readings for the metal controls (0.01 M Ca^{2+} and Mg^{2+}) are shown in Figure 34. Both metal controls induced greater leakage than the 0.85% NaCl control at 0.5 hour of incubation and then decreased after 1 hour of incubation. Since both metal controls caused more leakage than the saline control at 0.5 hour of incubation, the reversal of

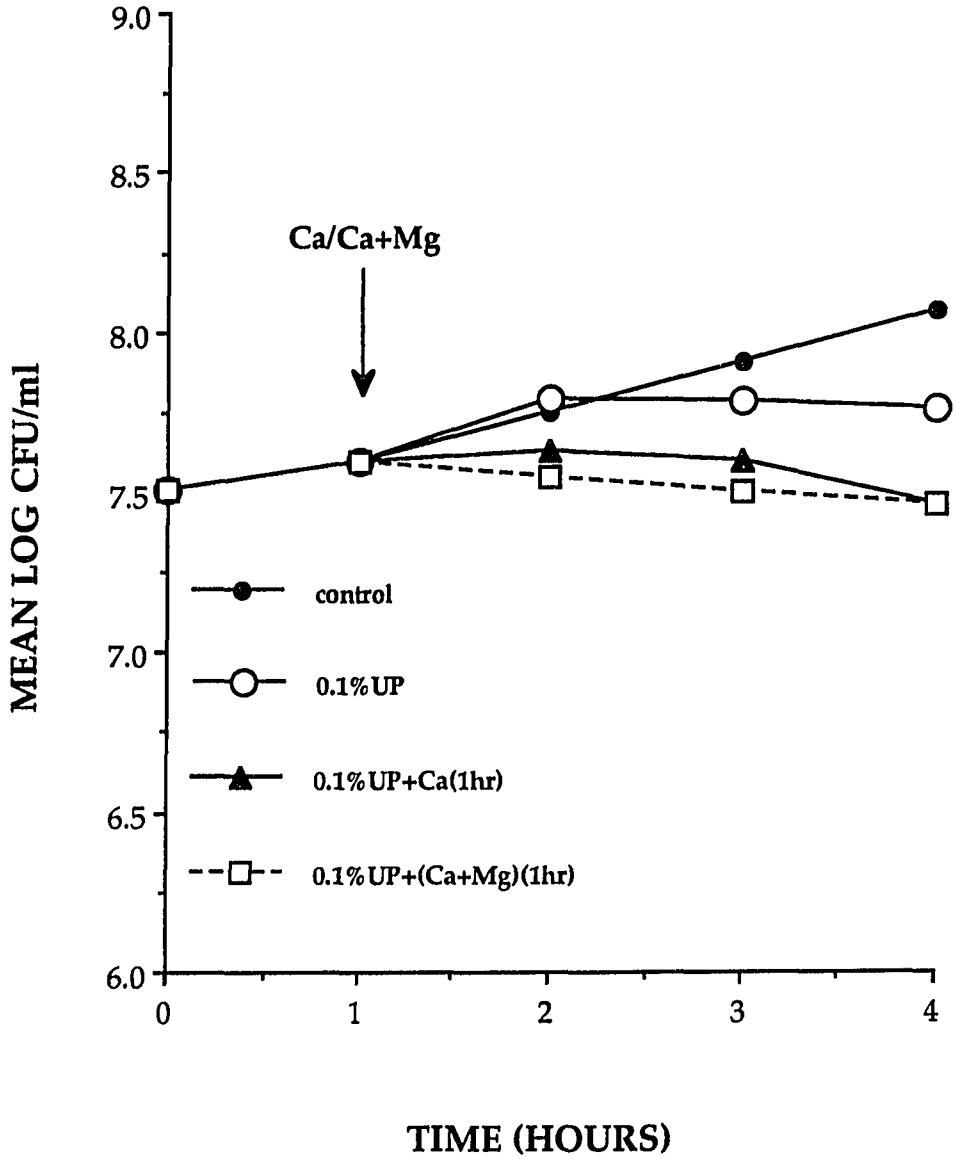


Figure 38. Effect of adding pH 6 Ca^{2+} (0.01M), and Ca^{2+} (0.01M) plus Mg^{2+} (0.01M) to UP (overnight preparation, 0.1%) treated early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.16.

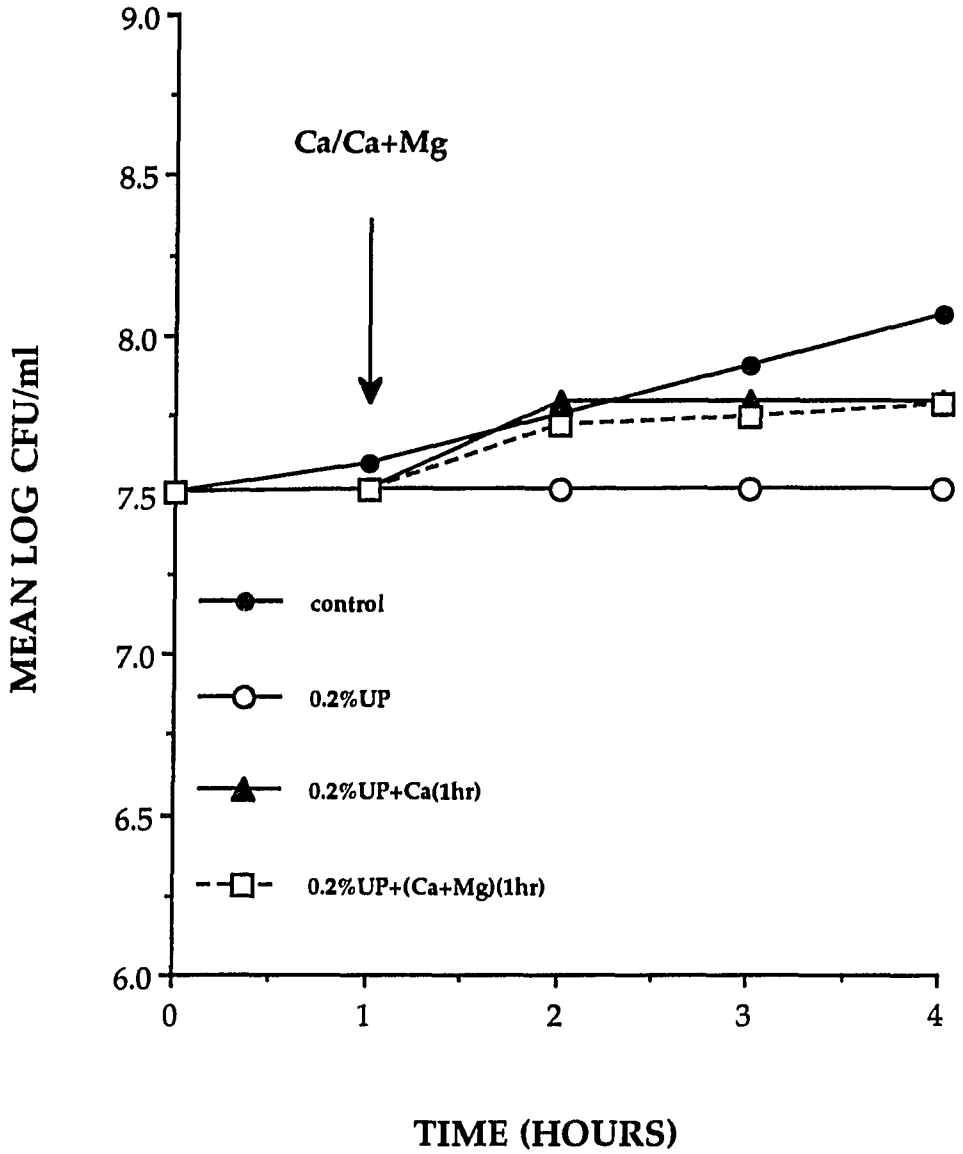


Figure 39. Reversal of bacteriostatic effect of UP (overnight preparation, 0.2%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by Ca^{2+} (0.01M) and Ca^{2+} (0.01M) plus Mg^{2+} (0.01M) rescue one hour after UP addition. The mean standard deviation was 0.04.

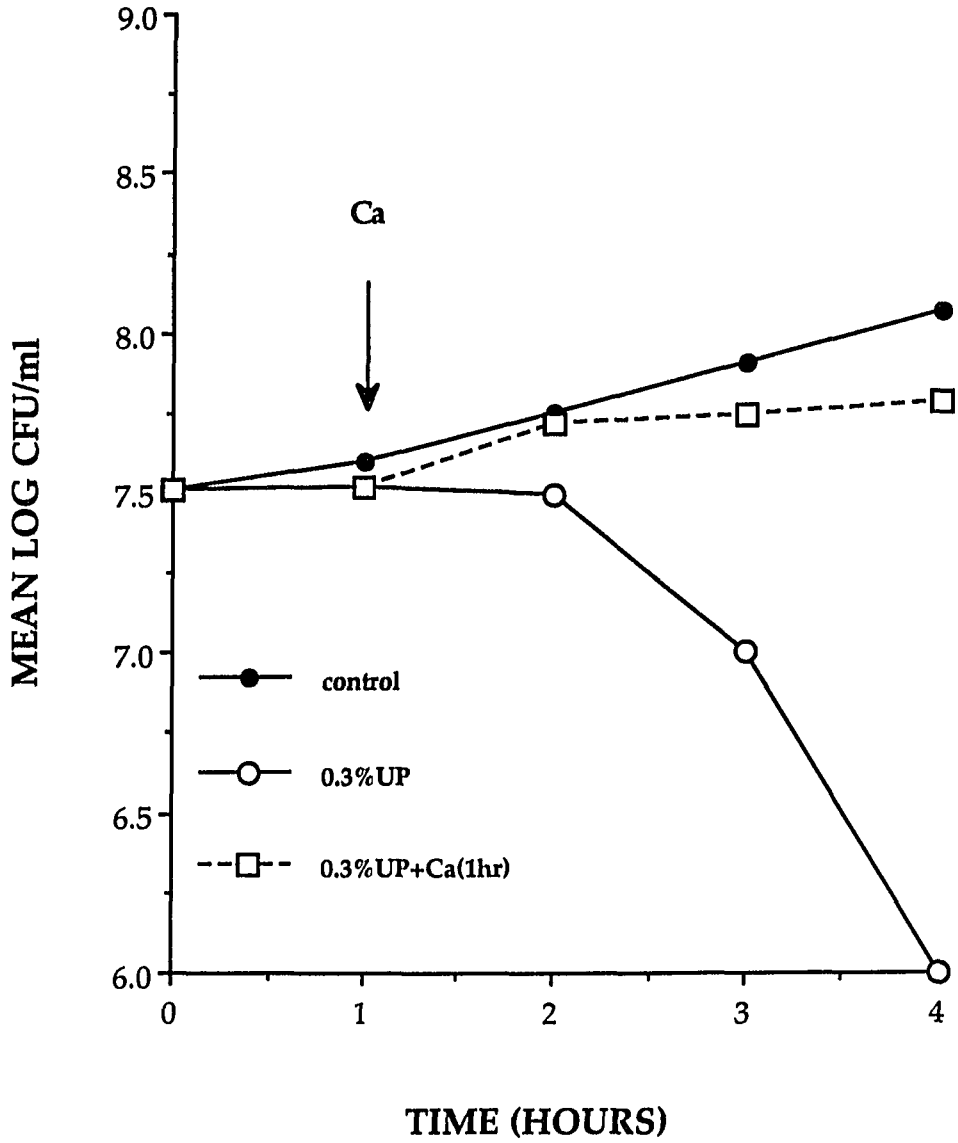


Figure 40. Reversal of bactericidal effect of UP (overnight preparation, 0.3%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by Ca^{2+} (0.01M) rescue one hour after UP addition. The mean standard deviation was 0.12.

bacteriolytic effect of SPG by these metals would be confirmed if these metals reduced the leakage caused by SPG. Figure 41 showed that both Ca^{2+} and Mg^{2+} decreased the leakage of intracellular nucleotides caused by SPG in which Mg^{2+} had a significantly ($P<0.05$) greater reversal of leakage than Ca^{2+}. Ca^{2+} retarded leakage only at 0.5 and 1 hour of incubation. These results suggested that Mg^{2+} and Ca^{2+} reversed the bacteriolytic effects of SPG.

Reversal of bactericidal effects of SPG by metal ions

The reversal of the bactericidal effects of SPG by metal ions was conducted to differentiate between metal-protection and metal-rescue studies, and to clarify each study. In the metal-protection study, cells were protected by metals one hour before exposure to SPG. The bactericidal effects of SPG (0.1%) on *S. aureus* cells was reversed when the cells were protected by the addition of Ca^{2+} (0.01M) or Mg^{2+} (0.01 M) one hour before the addition of SPG (Figure 42). The rate of reversal was significantly ($P<0.05$) faster for Ca^{2+} than Mg^{2+}. The significantly ($P<0.05$) faster growth rate of the Mg^{2+} control than the Ca^{2+} control suggested that more Mg^{2+} might be used by the cells than Ca^{2+} for metabolism. Therefore, more Ca^{2+} were suspected to be available than Mg^{2+} to protect the cells when SPG was added. Reversal of the bactericidal effect of SPG (0.1%) on *S. aureus* cells also was shown by rescuing cells by Ca^{2+} (0.01 M) or Mg^{2+} (0.01 M) one hour after SPG was added (Figure 43). In contrast, the rate of reversal was a little faster for Mg^{2+} than Ca^{2+}. Metal-rescue (Figure 43) was slightly more effective than metal-protection (Figure 42). Furthermore, as shown in Figure 42, the bacteria grew more rapidly in the metal controls (0.01 M Mg^{2+} and Ca^{2+}) than in the control to which Mg^{2+} and Ca^{2+} had not been added. Apparently, the

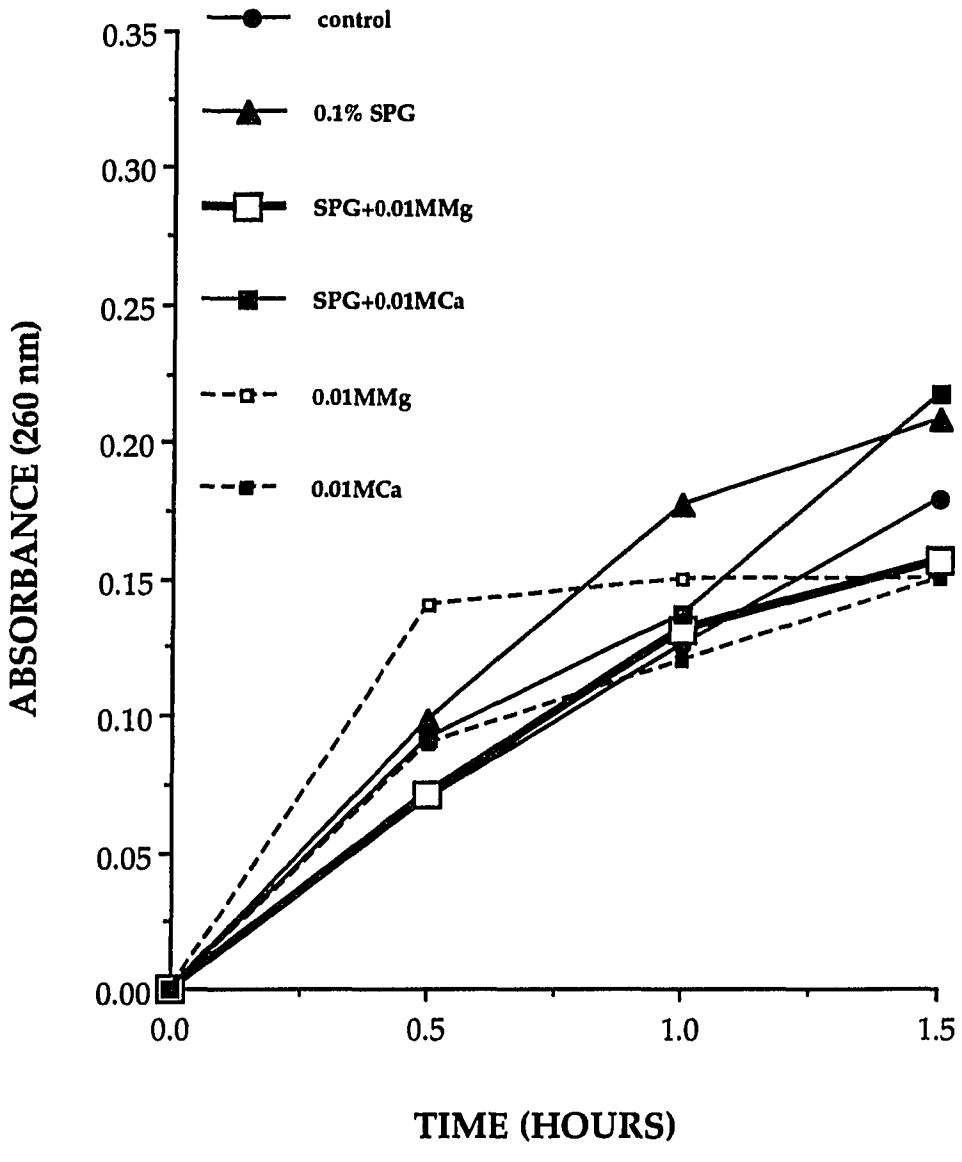


Figure 41. Leakage of intracellular nucleotides as determined at 260 nm from early-exponential cells of *S. aureus* ISP40 treated with pH 6 SPG (0.1%), SPG plus Mg^{2+} (0.01M), and SPG plus Ca^{2+} (0.01M) at 25°C. The mean standard deviation was 0.004.

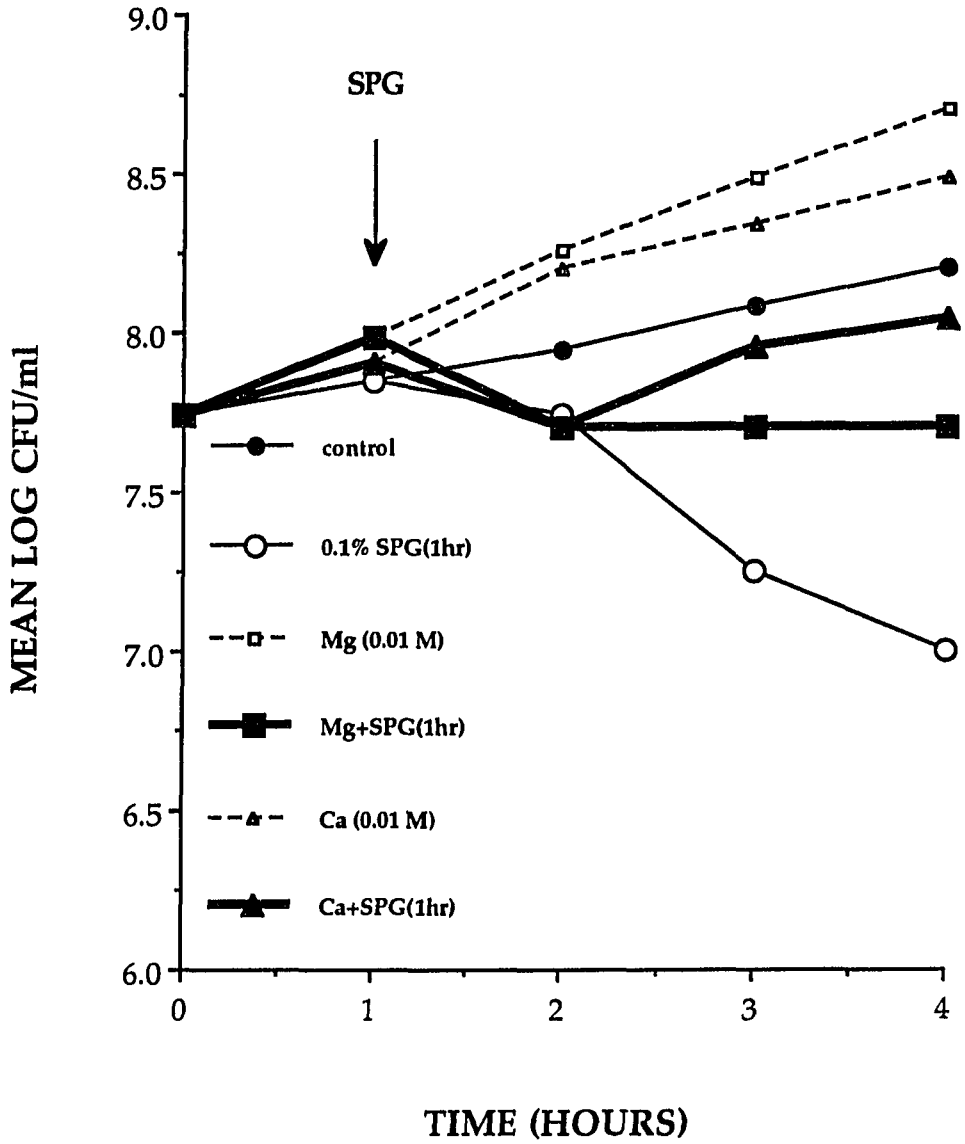


Figure 42. Reversal of bactericidal effect of SPG (0.1%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by protecting cells with pH 6 Mg^{2+} (0.01M) or Ca^{2+} (0.01M) one hour before SPG addition. The mean standard deviation was 0.06.

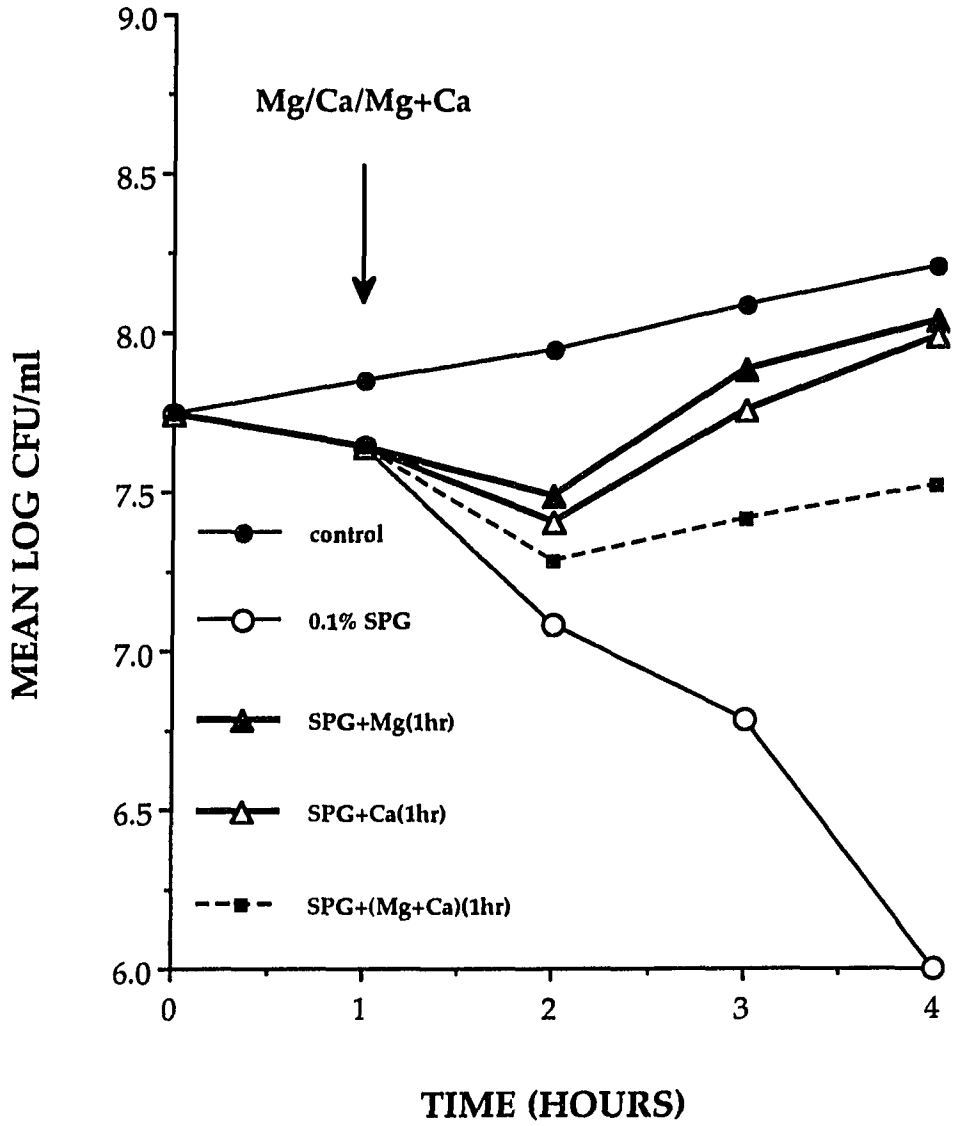


Figure 43. Reversal of bactericidal effect of SPG (0.1%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by Mg^{2+} (0.01M), Ca^{2+} (0.01M), and Mg^{2+} (0.01M) plus Ca^{2+} (0.01M) rescue one hour after SPG addition. The mean standard deviation was 0.07.

mCDS (pH6) medium was marginal in Mg^{2+} and Ca^{2+} content.

The addition of either Ca^{2+} (0.01 M) or Mg^{2+} (0.01 M) reversed growth inhibition. However, whether Ca^{2+} and Mg^{2+} were additive or synergistic in their effects was not known. Therefore, Ca^{2+} (0.01 M) and Mg^{2+} (0.01 M) were added to rescue *S. aureus* one hour after the addition of SPG (0.1%). Again, no additive of Ca^{2+} and Mg^{2+} effects were observed in the reversal of SPG inhibition (Figure 43) as well as UP inhibition (Figures 38, 39).

Reversal of TSPP inhibition by metal ions

The reversal of TSPP inhibition by metal ions was conducted to differentiate between metal-rescue and metal-protection studies, and to clarify each study. In the metal-rescue study, cells were rescued by adding Fe^{3+} one hour after exposure to TSPP. The effect of Fe^{3+} (0.01 M) on TSPP (0.5%) treated cells is shown in Figure 44. Ferric ions did not rescue cells from TSPP inhibition within 3 hours of incubation. In the metal-protection study, Fe^{3+} was added one hour before TSPP was added. The bactericidal effect of TSPP (0.5%) on *S. aureus* cells was significantly ($P<0.05$) reversed by 4 hours of incubation by protecting the cells with Fe^{3+} (0.01 M) one hour before TSPP (0.5%) was added (Figure 45). Therefore, it was concluded that TSPP inhibition of *S. aureus* was reversed by metal-protection with Fe^{3+}. As shown in Figures 44 and 45, growth rates in the metal controls (0.01 M Fe^{3+}) and the control without metal were not significantly different ($P>0.05$). Ferric ions added to protect cells might not be used by the cells as much as that of Mg^{2+} and Ca^{2+} (Figures 36 and 42). Therefore, most of the ferric ions (0.01 M) was available to protect cells against TSPP inhibition.

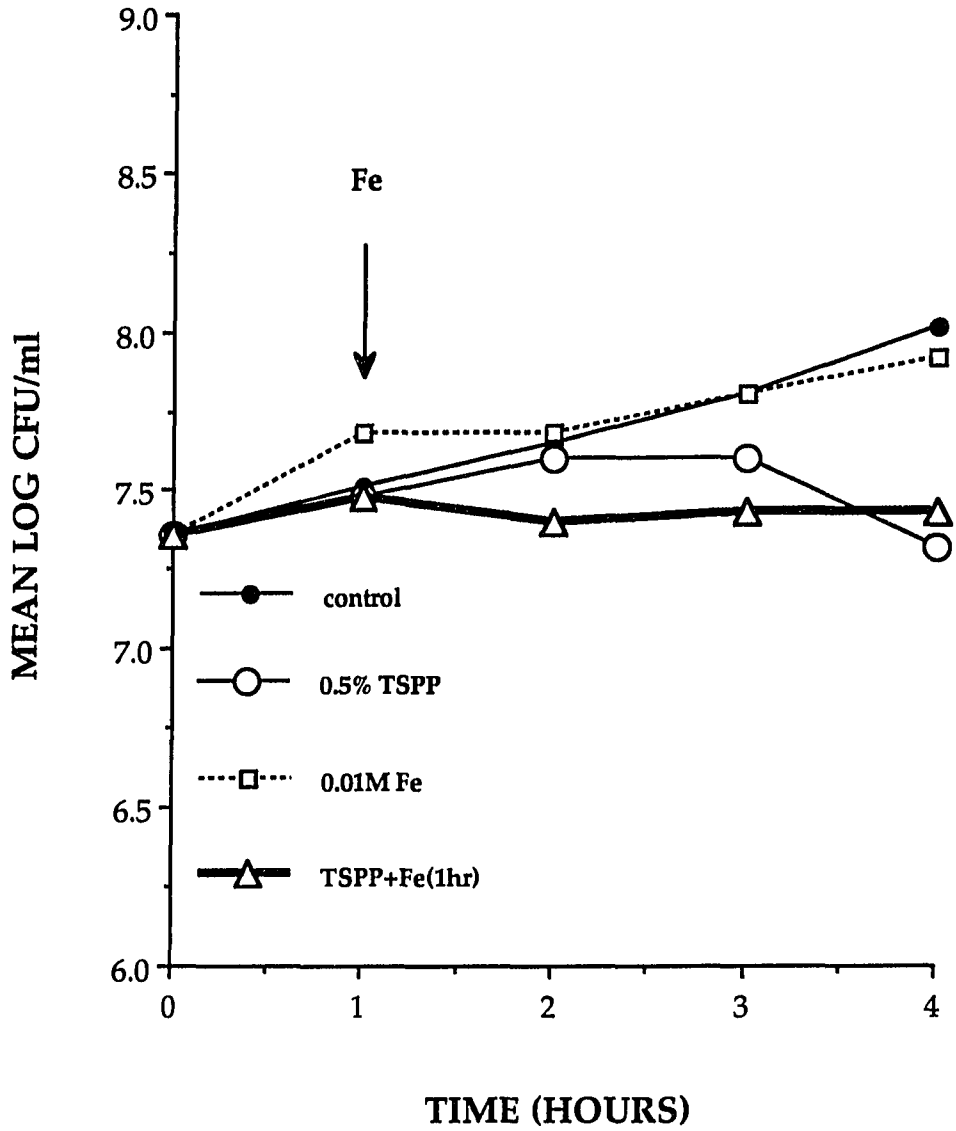


Figure 44. Effect of pH 6 Fe^{3+} (0.01M) to TSPP (0.5%) treated early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C. The mean standard deviation was 0.07.

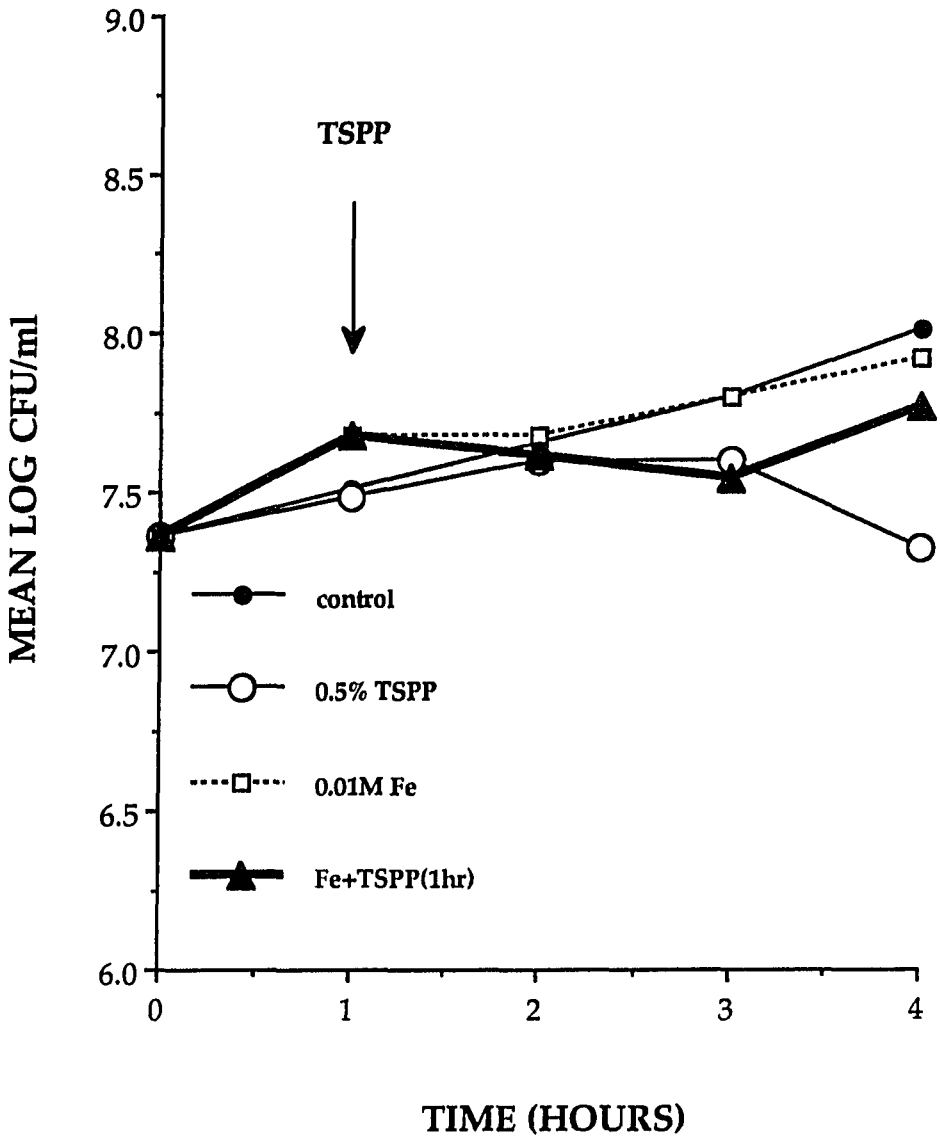


Figure 45. Reversal of growth inhibition of TSPP (0.5%) on early-exponential cells of *S. aureus* ISP40 in mCDS(pH6) medium at 37°C by protecting cells with pH 6 Fe^{3+} (0.01M) one hour before TSPP addition. The mean standard deviation was 0.07.

Free-Metal Dialysis Study

Results from previous studies provided microbiological evidence that showed phosphates interacted with *S. aureus* cell walls by a metal chelation mechanism. The addition of Ca^{2+} (0.01 M) or Mg^{2+} (0.01 M) one hour after treatment with UP (0.1%) or SPG (0.1%) rescued cells from the bactericidal action of the phosphates. The addition of Fe^{3+} (0.01 M) one hour before TSPP (0.5%) was added protected the cells from the bactericidal action of TSPP. A free-metal dialysis study was conducted to obtain chemical evidence to show that phosphates really chelated metals in the cell envelope. As free metals but not metal-phosphate chelates could pass through the dialysis membrane (MWCO 100), only free metals would be detected in samples taken from the dialysis beakers. If phosphates really interacted with *S. aureus* cell walls by metal chelation, metals would be bound by phosphates and levels of free metal in phosphate-treated samples would be lower than those of the controls.

When compared to the control, samples from UP (0.1%) and SPG (0.1%) treated cells were significantly ($P<0.05$) lower in free Ca^{2+} (Figure 46) and free Mg^{2+} (Figure 47). Apart from the microbiological evidence, these results provided chemical evidence to support the hypothesis that phosphates interacted with *S. aureus* cell walls by metal chelation. On the other hand, UP-treated cells had significantly ($P<0.05$) less free Ca^{2+} than that of the SPG-treated cells (Figure 46). UP had greater affinity than SPG for Ca^{2+}. These results correlated with the faster reversal of UP inhibition by Ca^{2+} than by Mg^{2+} (Figures 36, 37).

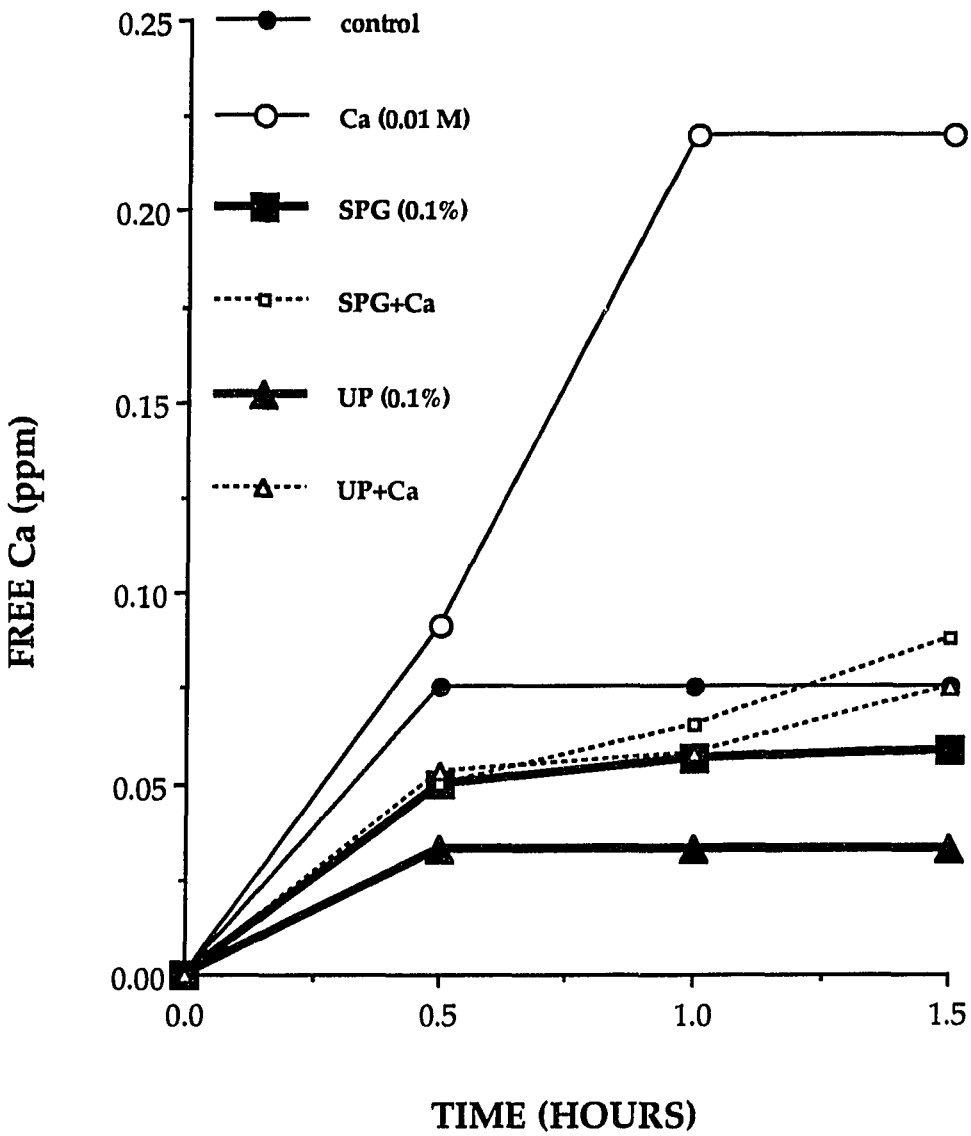


Figure 46. Free Ca^{2+} levels in dialysates of pH 6 UP (0.1%) and SPG (0.1%) treated early-exponential cells of *S. aureus* ISP40 at 25°C in the presence and absence of added Ca^{2+}. The mean standard deviation was 0.002.

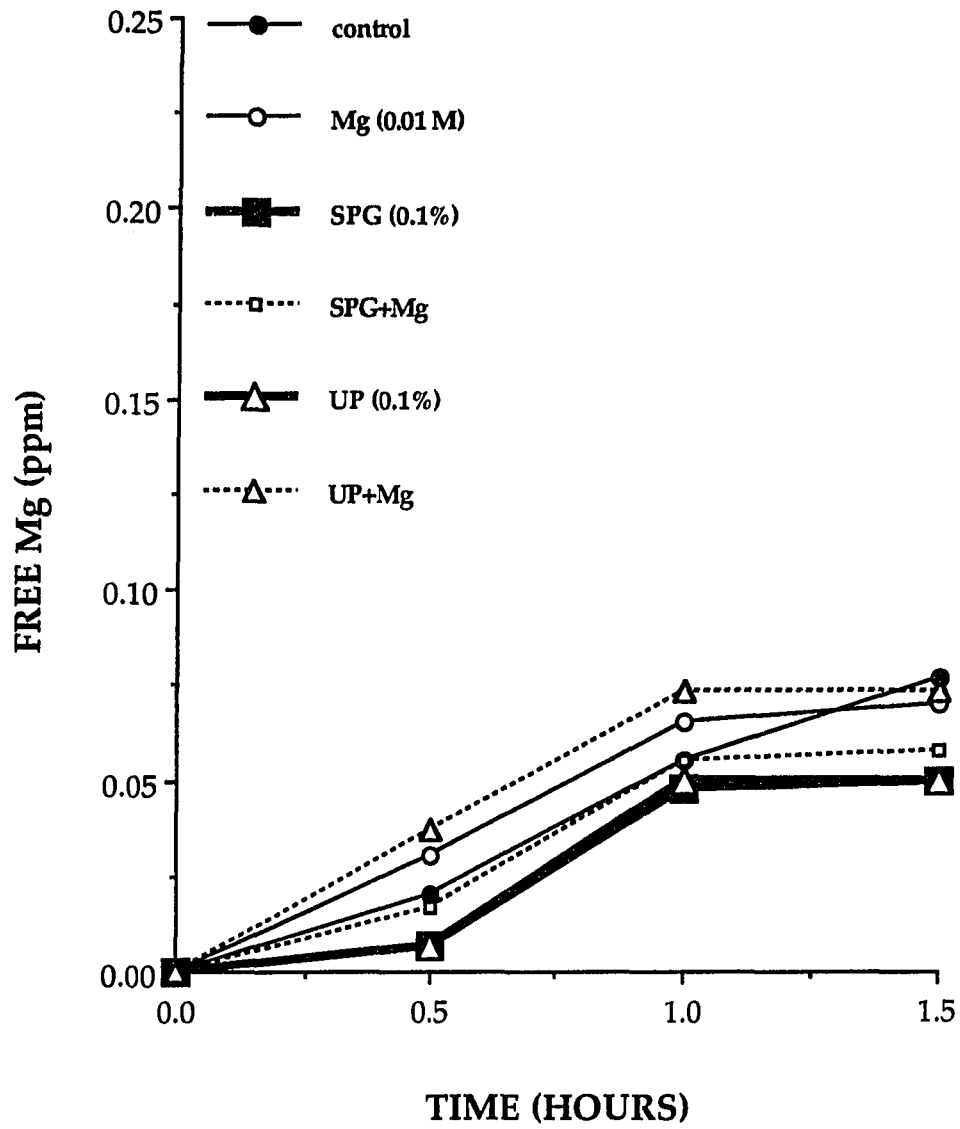


Figure 47. Free Mg^{2+} levels in dialysates of pH 6 UP (0.1%) and SPG (0.1%) treated early-exponential cells of *S. aureus* ISP40 at 25°C in the presence and absence of added Mg^{2+}. The mean standard deviation was 0.002.

Metal Leakage Study

A metal leakage study was conducted to answer the question of whether phosphates bound to the cell walls, chelated metals in the cell walls, and remained bound or phosphates released metals from the cell walls into the medium. After the cells were sedimented by centrifugation, the presence of excessive quantities of metals in the cell-free supernatant would suggest that phosphates chelated and released metals from the cell walls into the medium. In contrast, the presence of minimal quantities or absence of metals in the cell-free supernatant would indicate that phosphates chelated metals in the cell walls and remained bound to the cell walls.

Because bacterial leakage in the presence of polymyxin (Figure 25) was comparable to bacterial leakage in the presence of SPG and UP (Figure 17), polymyxin was used as a control. Figure 48 shows that almost no Ca^{2+} was detected in the cell-free supernatants of UP (0.1%), SPG (0.1%) or polymyxin-treated cells. Also, no Mg^{2+} was detected in cell-free supernatants of UP (0.1%) treated cells (Figure 49). A minimal quantity of Mg^{2+} was detected in cell-free supernatants of SPG (0.1%) treated cells. However, an excessive quantity of Mg^{2+} was detected in cell-free supernatants of polymyxin-treated cells. All metal measurements from phosphate-treated cells were significantly ($P<0.05$) lower than those of the control and polymyxin-treated cells. These results indicated that phosphates did not release metals from the cell walls into the medium. Therefore, it was concluded that phosphates bound on the cell walls of *S. aureus*, chelated metals in the cell walls, and remained bound.

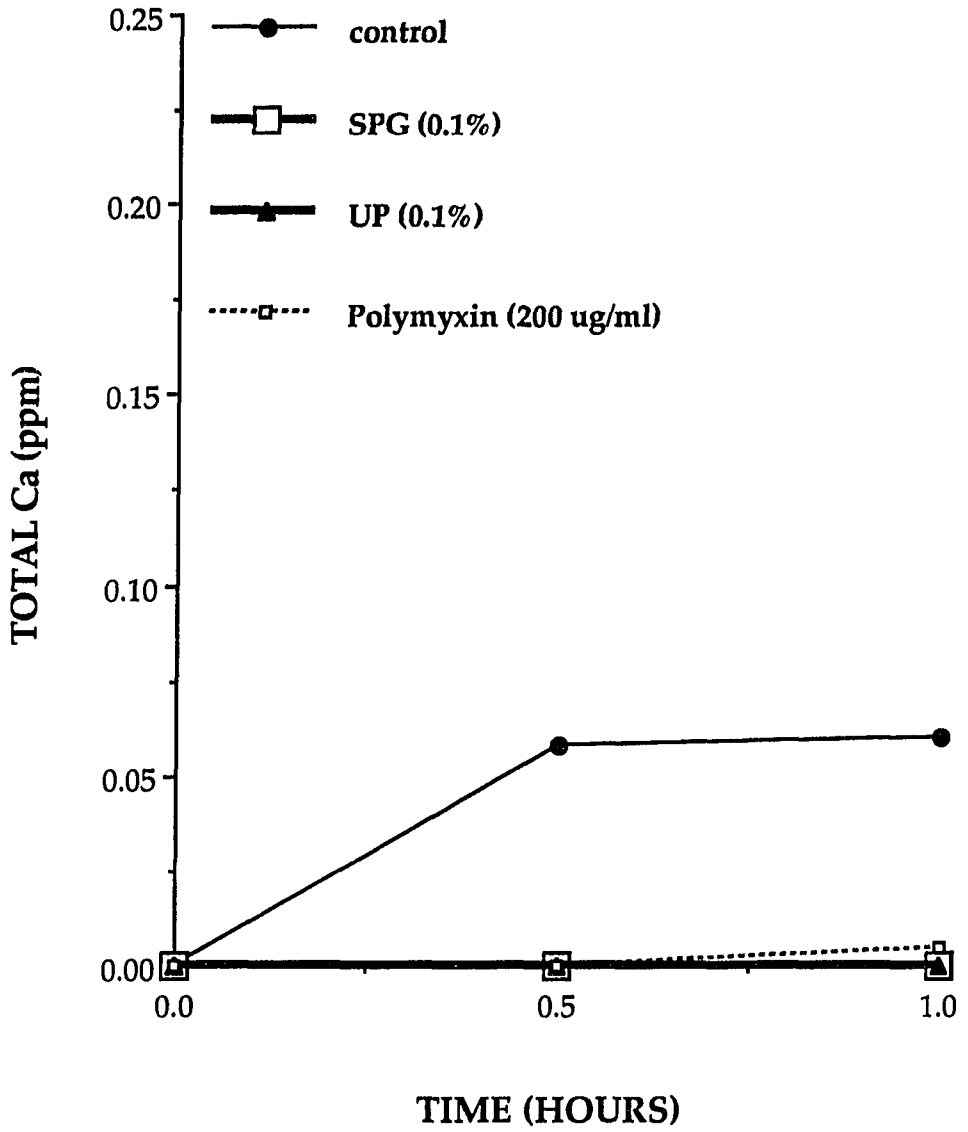


Figure 48. Total Ca^{2+} in cell-free supernantants of pH 6 UP (0.1%), SPG (0.1%) and polymyxin-treated early-exponential cells of*S. aureus* ISP40 at 25°C. The mean standard deviation was 0.003.

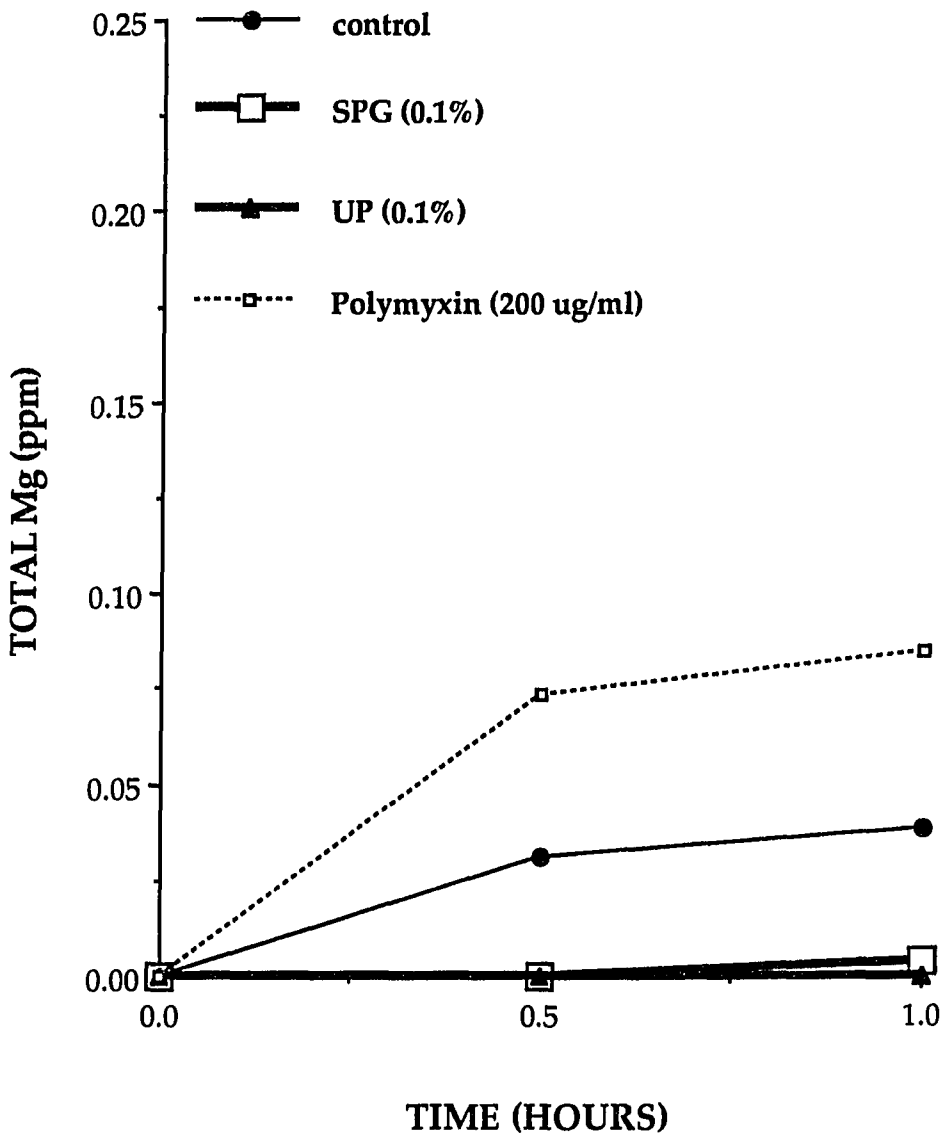


Figure 49. Total Mg^{2+} in cell-free supernantants of pH 6 UP (0.1%), SPG (0.1%) and polymyxin-treated early-exponential cells of*S. aureus* ISP40 at 25°C. The mean standard deviation was 0.004.

GENERAL DISCUSSION AND SUMMARY

The results of the present studies showed that the minimum inhibitory concentrations (MIC) of phosphates (0.1% sodium ultraphosphate, UP; 0.1% sodium polyphosphate glassy, SPG; 0.5% sodium acid pyrophosphate, SAPP; 0.5% sodium tripolyphosphate, STPP; and 0.5% tetrasodium pyrophosphate, TSPP) were bactericidal to early-exponential phase cells of *Staphylococcus aureus* ISP40 8325 in a pH 6 modified complete defined synthetic medium (mCDS). Concentration effects were observed for each phosphate in which the higher the concentration, the greater the inhibition. Chain-length effects also were observed for these phosphates in which the longer the chain-length, the greater the inhibition.

The bactericidal effect of phosphates indicated that a more lethal antibacterial mechanism than merely the inhibition of macromolecular synthesis was involved. Leakage of intracellular nucleotides was confirmed spectrophotometrically and microscopically; these could account for the bactericidal effect of phosphates.

The bactericidal and bacteriolytic effects of phosphates indicated that the cell envelope (cell wall or cell membrane) was damaged. Because high concentrations (0.4 M and 0.6 M) of NaCl protected the cells from leakage, the most likely target site of phosphates was the cell wall.

Because of the properties shared between phosphates and EDTA as metal chelators that cause bacterial leakage, phosphates were suspected to interact with the cell wall of *S. aureus* by a metal chelation mechanism. The results of the pH study also showed that metal chelation was involved as part of the antibacterial mechanism. Further, Ca^{2+} (0.01 M) or Mg^{2+} (0.01 M) reversed the growth

inhibition of UP (0.1%) or SPG (0.1%). Fe^{3+} (0.01 M) reversed the growth inhibition of TSPP (0.5%). Moreover, the bactericidal effects of UP or SPG were reversed by Ca^{2+} or Mg^{2+} in metal-rescue experiments. In these experiments, Ca^{2+} or Mg^{2+} was added to rescue cells one hour after UP or SPG addition. No additive effect existed between Mg^{2+} and Ca^{2+}. In contrast, growth inhibition of TSPP was reversed by Fe^{3+} when Fe^{3+} was added to protect cells one hour before the addition of TSPP. Apart from the microbiological evidence, results from the free-metal dialysis study provided chemical evidence to support the concept that long-chain polyphosphates (UP and SPG) interacted with *S. aureus* cell walls by a metal chelation mechanism. In addition, long-chain polyphosphates were shown to bind on the cell wall, chelate metals, and remain bound without releasing metals from the cell wall into the suspending medium.

A hypothesis is proposed in which the antibacterial mechanism of phosphates is caused by binding of long-chain polyphosphates on the cell wall of early-exponential phase cells of *S. aureus* ISP40 8325. The polyphosphates chelate structurally essential metals (Ca and Mg) of the cell wall, resulting in bactericidal and bacteriolytic effects. Metal ions are required for the integrity of bacterial cell walls and for the attachment of their superficial layers (Beveridge and Murray, 1976a). In Gram-positive bacteria, the phosphodiester groups of teichoic acids and the carboxyl groups of peptidoglycan are potent metal coordinators (Beveridge and Murray, 1976; Lambert et al., 1975ab). In *Staphylococcus aureus,* ribitol teichoic acid is the major component of the walls responsible for binding magnesium (Heptinstall et al., 1970) because the peptidoglycan is highly cross-linked (Archibald, 1972). Magnesium forms bridges across phosphate groups in adjacent ribitol teichoic acid chains in

Staphylococcus aureus cell walls (Lambert et al., 1975a). Therefore, the structurally essential metals probably form cross bridges between the teichoic acid chains in the cell walls of Gram-positive bacteria.

As calcium and magnesium are the major metals found in the cell wall (Beveridge et al., 1982) and these metals are best sequested by long-chain polyphosphates (Steinhauer, 1983), the antibacterial mechanism of long-chain polyphosphates is defined in the present study. Although short-chain phosphates (TSPP, STPP, and SAPP) also caused bacterial leakage, their antibacterial mechanism(s) are more difficult to define than the long-chain polyphosphates because short-chain phosphates chelate calcium and magnesium weakly. In addition, tripolyphosphate is the form of polyphosphate appropiate for transport through membranes of fungal cells and possibly cells of other organisms (Kulaev, 1979). Therefore, the target site(s) of these short-chain phosphates may be multiple. The target sites may be extracellular, intracellular, or both.

Short-chain phosphates are better iron chelators than long-chain polyphosphates (Irani and Morgenthaler, 1963). The chelation of iron in the cytochrome system (Molins, 1991) may be involved in the antibacterial mechanism of the short-chain phosphates. Short-chain phosphates may repress enzyme synthesis and inhibit enzyme activity inside the cells by chelating specific metal cofactors, Mg and Mn.

Another possible antibacterial mechanism of short-chain phosphates on enzyme inhibition is reflected from the works of Kornberg (1957) and Harold and Harold (1965). Short-chain phosphates degraded to orthophosphates with time. Harold and Harold (1965) found that external orthophosphates repressed the

synthesis of intracellular polyphosphate kinase in *Aerobacter aerogenes*, thereby inhibiting internal polyphosphate formation. Kornberg (1957) reported that external orthophosphates repressed an intracellular polyphosphate kinase in *E. coli* that catalyzed ATP formation from intracellular polyphosphate and ADP. These results suggested that external orthophosphates are involved in the inhibition of enzyme synthesis and activity essential for nucleic acid synthesis (Molins, 1991). Also, McKellar and Cholette (1984, 1985) reported that excess orthophosphates inhibited proteinase synthesis in *Pseudomonas fluorescens*. The partial reversal of inhibition of enzyme synthesis by calcium and manganese suggests that metal-ion chelation may be involved in the the antibacterial mechanism.

Apart from serving as an energy source in several reactions in pro- and eucaryotes (Baltscheffsky, 1967), inorganic pyrophosphates regulate enzyme synthesis and inhibit enzyme activities (Kleppe, 1966; Pina et al., 1975; Sawhney and Nicholas, 1978). For example, Tarr et al. (1969) demonstrated that SAPP inhibited a 5'-nucleotidase in Pacific cod fish muscle which catalyzes the hydrolysis of adenosinemonophosphate to adenosine and orthophosphate. Wagner and Busta (1985) reported that SAPP inhibited the toxin activating protease of *Clostridium botulinum* when SAPP was added to culture media. Later, the same authors attempted to define the antibotulinal mechanism of SAPP on protease activity and found that [^{32}P]-SAPP was associated with the RNA fraction (Wagner and Busta, 1986). They proposed that the synthesis of proteases mediated by RNA may be affected by the association of SAPP with RNA. That is, SAPP or other short-chain phosphates may inhibit macromolecular synthesis. Because short-chain phosphates are the major phosphates used in meat

processing, more study is needed to further clarify their antibacterial mechanism(s).

The knowledge of the antibacterial mechanism by which UP acts is important. UP is classified as hexametaphosphate (< 72% P_2O_5) and is therefore approved by the USDA for use in meat products. UP has excellent antimicrobial properties, particularly against Gram-positive bacteria, as described in a previous (Kung, 1991) and the present study. Because UP is a long-chain polyphosphate which is degraded by heat and with time, surface application of freshly prepared UP is suggested on cooked meat products. The literature on UP application is extremely limited and more study on the antibacterial effects of surface application of UP on cooked meat products is needed.

From the microbiological point of view, polyphosphates are generally more effective in cooked meats and fresh poultry than in fresh meats and seafoods. The presence of meat phosphatases in fresh meats and high sodium chloride content in seafoods may reduce the antibacterial effect of phosphates. To achieve maximum antibacterial effects of phosphates, hard water, metal containers, chemical additives containing divalent cations, and ingredients with high iron contents, such as blood and heart tissue, should be minimized.

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