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Effects of phosphates on *Pseudomonas fragi* growth, protease production and activity

by

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ABBREVIATIONS

BHA	Butylated hydroxyanisole
BHI	Brain heart infusion
CFU	Colony forming units
DSP	Disodium phosphate
EDTA	Ethylendiamine tetracetate
EGTA	Ethyleneglycol-bis N, tetraacetic acid
MHA	Mueller hinton agar
MIC	Minimum inhibitory concentration
MSP	Monosodium phosphate
NA	Nutrient agar
SAPP	Sodium acid pyrophosphate
SEM	Scanning electron microscopy
SPG	Sodium hexametaphosphate, glassy
STPP	Sodium tripolyphosphate
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TSP	Trisodium phosphate
TSPP	Tetrasodium pyrophosphate
UP	Ultraphosphate
WHC	Water holding capacity

INTRODUCTION

Phosphates are added to meats primarily to increase water holding abilities, although their importance and functional capabilities in meats exceeds this single function. Currently, phosphates are not approved for use as food preservatives (Food and Nutrition Board, 1981; FAO, 1962), although they have been known to have antimicrobial effects as early as 1864 (Leuke, 1980). Several studies have shown that phosphates are inhibitory to the growth of pathogenic and spoilage organisms in a variety of meats.

Pseudomonads are the most dominant and most metabolically significant spoilage organisms at refrigerated temperatures. This is attributed to their ubiquitous nature and ability to tolerate low temperatures (Suhren, 1989; Dainty et al., 1983; Hurst and Collins-Thompson, 1979). *Pseudomonas fragi* is the most prevalent pseudomonad found in beef, pork, lamb and poultry (Shaw and Latty, 1984, 1982, 1981: Molin and Ternstrom, 1986, 1982). In red meat, eighty-five percent of the bacterial flora was characterized as *P. fragi* and *Pseudomonas lundensis*. In addition to their ease of colonization of and growth in meats, *P. fragi* produces an extracellular protease which can hydrolyze myofibrillar, sarcoplasmic and stroma meat proteins (Yada and Skura, 1981; Bala et al., 1979; Porzio and Pearson, 1980; Tarrant et al., 1973, 1971). These properties aid *P. fragi* in the spoilage of meats.

In our laboratory, phosphates have been analyzed for their antimicrobial ability in a variety of meat systems (Molins et al., 1987a,b, 1985, 1984; Marcy et al., 1988a,b,c). However a comprehensive study has never been done concerning the antimicrobial effects of ortho-, pyro-, and polyphosphates on a common meat spoilage organism and its extracellular protease. An effective inhibitor of the growth of *P. fragi*, the synthesis of the protease, or reduction of the proteolytic activity would contribute to the improved quality and shelf-life of the meat product.

The purpose of this study was to determine the antimicrobial effects of a variety of phosphates on: 1) *P. fragi* growth in laboratory media and in a meat system, 2) synthesis of *P. fragi* extracellular protease, and 3) the activity of the purified *P. fragi* extracellular protease.

LITERATURE REVIEW

Phosphates

History of phosphorus

In an attempt to make gold from urine, Hennig Brandt, in 1669, inadvertently discovered a white, waxy, transparent substance which glowed in the dark and spontaneously ignited at 30° C (Toy and Walsh, 1987). It was the first element isolated other than the metals and nonmetals. He had discovered phosphorus.

Brandt would never realize how extensively this element would be used in modern society. From its first commercial use as sodium pyrophosphate as a substitute for tartaric acid in baking powder during World War I (Greenfield and Clift, 1975), phosphorous-containing compounds have gone beyond functioning in a single capacity. Instead, they have been used in many diverse areas. Today, fertilizers account for about three-fourths of phosphate manufacture followed by detergents, animal feed, metal surface treatments, deflocculants, insecticides and oil additives to list a few examples (Emsley and Hall, 1976).

Phosphorus is unique in that it can function dichotomously being used in matches and in flame-retardant materials as well as in foods and in nerve gases.

Phosphorus and phosphate distribution

Phosphorus is found in igneous rock and meteorites. Igneous rock contains 0.1% phosphorus by weight and supplies the majority of phosphorus as apatites, $CaX_2 \cdot 3Ca_3(PO_4)_2$ where X=F, Cl or OH anions (Halmann, 1972). Meteorites contain large amounts of phosphorus although the overall phosphorous content, compared to igneous rock, is quite small (Emsley and Hall, 1976).

There are two cycles whereby phosphate is distributed in nature. The primary cycle involves the liberation of phosphorus from igneous rocks due to the weathering process which produce soluble phosphorus compounds. These compounds are carried by rivers and streams to the sea where they are precipitated as insoluble apatites and are deposited on continental shelves. The geological uplifting of the marine deposits complete the cycle and expose the phosphates to weathering (Emsley and Hall, 1976). This process is extremely slow and requires a faster, secondary cycle for the availability of phosphorus to, primarily, the marine environment. This cycle encompasses the terrestrial and aquatic ecosystems where phosphate is dissolved in soil, water, or in the sea. Plants take up the phosphates, the animals eat the plants and then excrete the phosphates. The plants and animals die and decay, releasing the phosphates into the soil. With the help of bacteria, the phosphates are regenerated into forms plants and animals can use. Ultimately, the rivers replenish the sea which has lost phosphate through the insolubilities of the apatites.

Phosphorus and human health

In humans, eighty percent of phosphorus is in the form of calcium fluorophosphate apatite found in teeth and bones (Tietz, 1982). The calcium fluorophosphate provides bone structure, and a large reserve for phosphorus and calcium. The remaining phosphorus is involved in both metabolic and anabolic functions in the body which include energy metabolism of ATP, GTP, FAD, NAD, nucleic acid synthesis, intracellular buffers and membrane phospholipids (Linder, 1985). Our body cannot synthesize phosphorus and must derive the mineral from food in the form of the phosphate ion (Krigsman, 1985). In the American diet, phosphorus can be obtained from processed food, soft drinks, and high protein food including meat, whole grains and cheese. The U.S. Recommended Daily Allowance (RDA) for phosphorus and calcium are both 800 mg/day. However, the amount of phosphorus consumed by Americans far exceeds this level. There is evidence that links high phosphorus diets to decreased bone mineralization (Draper and Bell, 1980). These researchers showed that ratios greater than 2:1 of phosphorous to calcium may result in an increased secretion of parathyroid hormone (PTH). PTH increases the activation of vitamin D which, if there is insufficient levels of calcium relative to phosphorous for absorption, will induce bone demineralization. Usually, the mineral content of bone begins to decline in early adulthood with the greatest rate of decline occurring in women who have gone through menopause. This decline can lead to

osteoporosis, a decrease in mineral content without a decrease in organic bone elements, or osteomalacia, a decrease in mineral content with an increase in organic bone elements. Osteoporosis is a common problem among elderly women in the United States. There may be some similarities between the problems the Canadian Eskimo experienced by changing to a high phosphorus diet and the current high phosphorous diet of Americans (Linder, 1985). The Eskimos have abandoned their traditional calcium sources such as bones from fish and mammal in their diets. As a result, the phosphorus to calcium ratios rose to the same levels as those of Canadian non-Eskimos. The unusually high incidence of osteoporosis that has been observed in both Eskimo men and women is speculated to be due to the higher phosphorus to calcium levels in their diet.

Structure and properties of inorganic phosphates

Phosphates encompass a large group of compounds with the orthophosphate anion, PO_4^{-3}, as the basic unit. The orthophosphate anion is made up of a phosphorous atom bonded to four oxygen atoms which form a tetrahedron structure. The orthophosphate anion may be bonded to hydrogen, a metal ion or another orthophosphate anion.

The classification of inorganic phosphates is based on the number of phosphorous atoms in the molecule. Orthophosphates are salts of orthophosphoric acid, H_3PO_4, and contain one phosphorous atom per molecule. Pyrophosphates contain two phosphorous atoms, tripolyphosphates have three and tetrapolyphosphates have four atoms

of phosphorous per molecule. The shorter chain phosphates are known as individual compounds while those that have longer chains exist as mixtures of phosphates made up of different percentages of phosphate species (Harold, 1966). The nomenclature often can be confusing as different classification systems have resulted in many different names for the same phosphate compound. Some common phosphates and their structures can be found in Figure 1.

Polyphosphates exist as highly crystalline salts or as amorphous glasses. Generally, polyphosphates are in the form of crystalline salts. They are amorphous when there is between 5-15 phosphorous atoms per molecule (Shimp, 1983a; Steinhauer, 1983).

Polyphosphates can exist as straight-chain, cyclic and branched forms and can have as few as three phosphorous atoms per molecule or as many as 10^4 phosphorous atoms per molecule such as in the case of Kuroll's and Madrell's salts (Harold, 1966).

Straight-chain polyphosphates, $H_{(n+2)}P_nO_{(3n+1)}$ where n = the number of phosphorus atoms in the chain, are produced from the elimination of one molecule of water per two molecules of orthophosphate units. Cyclic phosphates (metaphosphates) have the general formula $(HPO_3)_n$ where n = the number of phosphorus atoms in the ring. The ringed-compound, dimetaphosphate, with two orthophosphoric anions is unknown. Sodium hexametaphosphate is a misnomer since it is a long, straight-chained phosphate. The United States Department of Agriculture (USDA) has officially replaced the

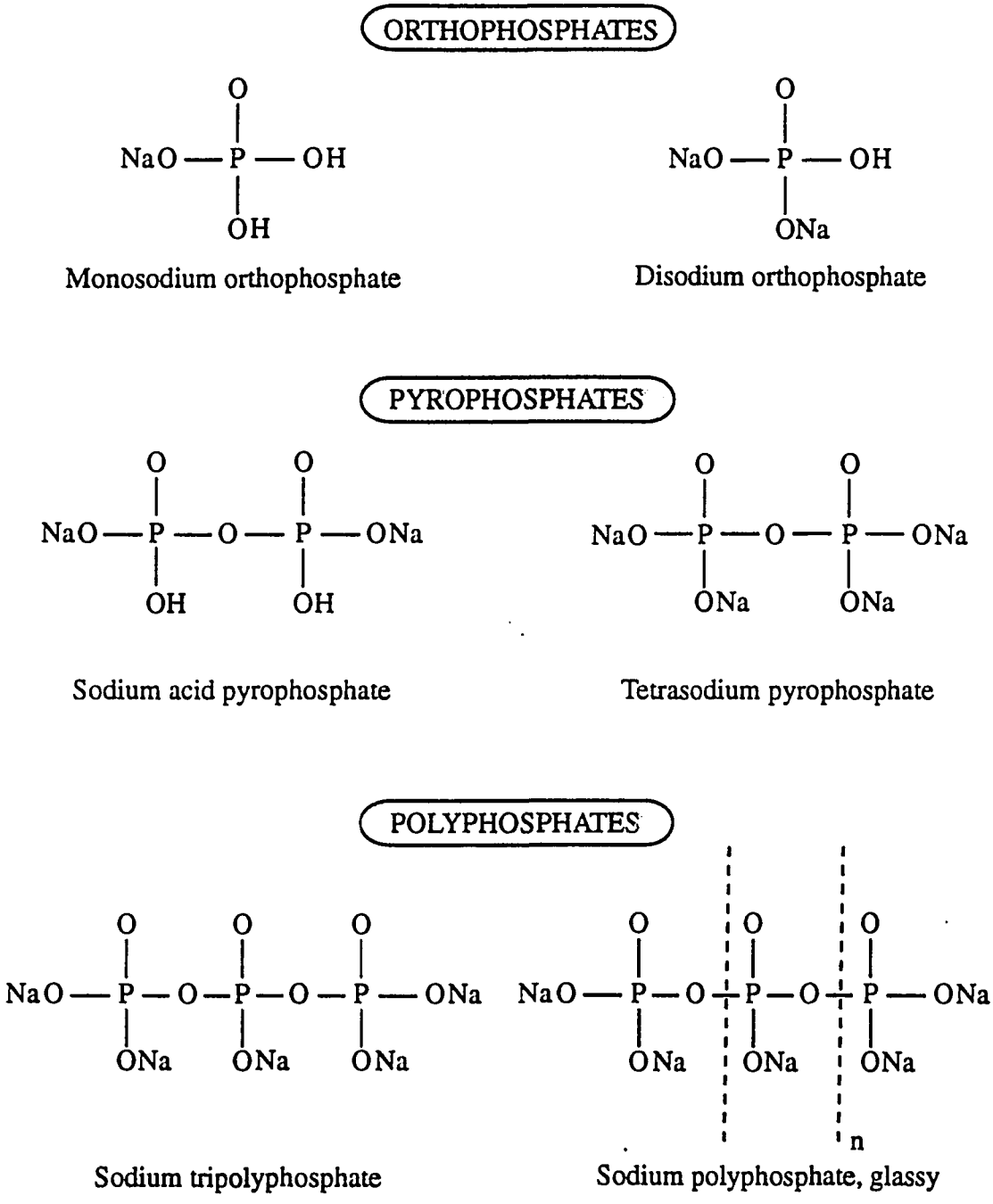


Figure 1. Structure and classification of some common sodium phosphates

sodium hexametaphosphate name with sodium polyphosphate, glassy (SPG). Branched phosphates, including both the linear and metaphosphates, are formed by the sharing of the three oxygen atoms in the phosphate molecule with neighboring phosphate groups. Ultraphosphates are highly branched phosphates and includes those phosphates with a tridimensional structure. Branched structures are highly unstable due to the nature of their bonding (Strauss et al., 1953) and are readily hydrolyzed in water.

Food-grade phosphates are made by a two-step thermal process (Toy and Walsh, 1987). Elemental phosphorous is heated until phosphoric anhydride, P_2O_5, is produced at which time water is added to the reaction to produce phosphoric acid. The phosphoric acid that is produced is 80%-90% pure and is further purified by the removal of arsenic impurities. Commercial polyphosphates contain variable numbers of orthophosphate units due to the condensation process during the manufacturing of phosphates. Therefore, the average chain lengths of commercial polyphosphates are usually given in place of an exact chain length (Ellinger, 1972). For example, commercial sodium polyphosphate, glassy (SPG) has an average chain length of 10-15 orthophosphate units (Davidson et al., 1983).

Properties of phosphates

The properties of phosphates determine their functionality and thus, their applications in the food industry. These properties include hygroscopicity, hydrolysis, pH, and sequestering abilities. The

amorphous polyphosphates, potassium tripolyphosphates and tetrapyrophosphates are highly hygroscopic (Krigsman, 1985). Water loss and subsequent caking and/or crystallization can occur upon storage.

Hydrolysis is the reaction between phosphate anions and water (Green, 1950). The hydrolysis of phosphates ultimately results in orthophosphate production (Toy and Walsh, 1987; Greenfield and Clift, 1975), although the rate and the route of hydrolysis can differ between phosphates (Corbridge, 1974). Bell (1947) determined that pyrophosphate hydrolysis resulted in two moles of orthophosphate and tripolyphosphate hydrolyzed to one mole of orthophosphate and one mole of pyrophosphate which in turn hydrolyzed to two moles of orthophosphate. Sodium hexametaphosphate, glassy (SPG) hydrolyzed by two simultaneous reactions. One reaction results in orthophosphate production and the other forms cyclic trimetaphosphate. Upon additional hydrolysis, trimetaphosphate is hydrolyzed to one mole of tripolyphosphate and ultimately to orthophosphate formation. The common hydrolytic end product contributes to some phosphates having similar functionalities. Several phosphates can be used interchangeably to give the same results in a food product (Toy and Walsh, 1987). This allows for a greater degree of flexibility in a particular product formulation.

Phosphates are generally stable at ambient temperatures but hydrolytic susceptibility of the phosphate ester bond can be accelerated

by increased temperatures (Bell, 1947; Corbridge, 1974; Molins et al., 1984), decreased pH (Thilo, 1962a,b), and presence of divalent cations (Greenfield and Clift, 1975), and enzymes or colloidal gels (Karl-Kroupa et al., 1957). In addition, increasing the chain length increases the rate of hydrolysis of phosphates (Bell, 1947).

Phosphates can undergo enzyme-catalyzed hydrolysis (Awad, 1968) by alkaline, acid and neutral phosphatases. The hydrolytic process of phosphatases in meat is very similar to that which occurs in water (Sutton, 1973). Phosphatase hydrolysis yields orthophosphate from the breakdown of pyro- and polyphosphates in fresh beef, pork and fish (Molins et al., 1985; Sutton, 1973; Awad, 1968). To determine whether hydrolysis of phosphates added to fresh meat or cooked meat were hydrolyzed enzymatically, Awad (1968) tested TSPP, STPP and SPG in fresh beef. He measured the accumulation of orthophosphate over time and observed a continuous increase in orthophosphate concentration with the fresh beef versus the cooked beef which did not increase. Molins and coworkers (1985) supported these findings while using SAPP and STPP in fresh pork. Phosphatases are found naturally in animal muscle and have been shown to significantly increase the rate of phosphate hydrolysis (Karl-Kroupa et al., 1957). Studies indicate that tripolyphosphatase activity is greater in meat than pyrophosphatase activity (Neraal and Hamm, 1977a,b; Awad 1968; Sutton, 1973). Phosphatases located in animal muscle were found to be completely inactivated at 60°C although increasing the temperature to 40°C (Awad,

1968) increased the tripoly- and pyro-phosphatase activities (Neraal and Hamm, 1977c).

Phosphates have been used in food applications to control pH by acting as buffers. The best buffering agents are the orthophosphates followed by the pyrophosphates with the buffer range occurring between pH 5.5-7.5. Buffering abilities decrease with an increase in chain length (Van Wazer and Holst, 1950; Steinhauer, 1983). Early studies by Van Wazer and Holst (1950) demonstrated that for every phosphorous atom in the orthophosphate unit, there was one strongly ionizable hydrogen. In addition, polyphosphates contained one weakly dissociated hydrogen at each end of the polymer. The polyphosphate charge was attributed to the negative charge on each phosphorous atom. It has been shown, through pH titration curves (Van Wazer, 1958), that orthophosphoric acid and short chain polyphosphoric acids such as pyro- and tripolyphosphoric acids are stronger acids than the longer chained phosphoric acids. After the titration of the first hydrogen ion, the long chain phosphoric acids exhibit weaker acid characteristics. Titration points confirm this where ortho-, pyro- and tripolyphosphoric acids have three titratable groups at pH 4.5, 7 and 10 whereas long chain polyphosphoric acids have titration points at 4.5 and 10. The ionization ability of a variety of phosphates was studied by Batra (1965) who demonstrated that an inverse relationship existed between chain length and the degree of ionization in aqueous solutions. He reported that the degree of ionization increased as follows: polyphosphate <

tetraphosphate < tripolyphosphate < pyrophosphate < orthophosphate. Furthermore, the same studies showed that calcium enhanced phosphate ionization and that the ionization was concentration dependent.

Phosphates have the unique ability to form soluble complexes or chelates with monovalent or polyvalent metal cations. The ability of phosphates to chelate metal ions has been utilized to a great extent in the treatment of water, although in the food industry it plays a significant role in food stabilization.

Chelation is a term used when a soluble complex is formed between competing anions, one precipitating and the other sequestering, for the same metal ion (Ellinger, 1972). Generally, chelation can be thought of as the temporary or permanent removal of cations (Molins, 1991). Chelates can be formed with any metal having an unshared electron pair, although formation of a five or six membered ring is necessary for a stable chelation complex.

The chelating abilities of phosphates depend upon pH, temperature, di- or trivalent metal ions and organic matter in the system (Krigsman, 1985). Chelating ability increases with an increase in pH due to the decreased competition between the metal ion and the hydrogen ion for the phosphate anion. In addition, elevated temperatures increase the chelating efficiency with certain metals (Irani and Callis, 1962). The chain length of the phosphate influences the ability of the phosphate to chelate the metal. Van Wazer and Campanella (1950) proposed that the ability of polyphosphates to

complex metal ions was proportional to the total number of phosphorus atoms in the phosphate molecule. Generally, the best sequestering agents for light metal cations (calcium and magnesium) are the long-chain polyphosphates while shorter-chain phosphates are better for heavy metal cations like copper and iron (Irani and Callis, 1962).

Regulation of phosphates

The addition of phosphates to meat products began in 1952 and were used in the curing of hams, bacon, picnics and loins primarily to increase moisture retention (Schmidt, 1983) but also for improvement of color and texture (Swift and Ellis, 1956). In 1982, the USDA expanded the addition of phosphates in cured and processed meat and poultry products (USDA, 1982) to help reduce the cooked out juices normally lost through processing in these products.

The United States Food and Drug Administration (FDA) classifies phosphates and their calcium, potassium, sodium and ammonium salts as GRAS (generally recognized as safe) ingredients. In meats, phosphate addition is regulated by the Meat Inspection Division (MID) Consumer and Marketing Service, U. S. Department of Agriculture (USDA). Sodium and potassium phosphates are allowed individually or in combination to a maximum concentration of 0.5 percent of the total meat product and are permitted in some of the following meat products: corned beef, pastrami, cooked and precooked sausages, pork, veal, mutton and poultry products. Phosphates are not permitted in fresh pork sausage, chopped beef, ground beef or pork, and hamburger

although they are allowed in fresh meats that will be frozen, cooked or further processed. Some phosphates allowed in meat products include monosodium phosphate (MSP), disodium (DSP), trisodium (TSP), sodium tripolyphosphate (STPP), tetrasodium phosphate (TSPP), sodium acid pyrophosphate (SAPP), sodium hexametaphosphate, glassy (SPG) and their blends. In the processing of red meats, STP is the most widely used phosphate (Mahon et al., 1970) and SPG, STPP, and SAPP are the most commonly used phosphates in phosphate blends.

Functions of phosphates in the meat industry

Phosphates are added to meat products for one or more of the following reasons: to increase water holding capacity, decrease cooking losses, enhance emulsification and binding, aid in color stabilization, improve texture, and to inhibit growth of microorganisms (Ellinger, 1972).

Water holding capacity Water retention in meat is desire able because it increases the quality of the meat product. To the consumer, water contributes to the juiciness and tenderness of the meat product. In addition, it is important economically to the meat industry since meat is sold by weight and the loss of water in a meat product results in a decreased amount of marketable product. The ability of meat to retain its natural or added water is defined as water holding capacity (WHC).

Water makes up 75% of the total weight of lean meat immediately after slaughter. There are three forms of water that is present in the

muscle: bound, immobilized, and free. Early studies by Hamm (1960) indicated that there was a limit to how much water was tightly bound to the muscle proteins and that this amount was too small to account for the increase in WHC. Therefore, it is believed that free water is responsible for the increased WHC of meats. This is because the majority of the water is in the free form and because it occupies the spaces between the thick and thin filaments of the myofibril, which is where the WHC of meat has been shown to take place (Offer and Trinick, 1983).

Consumer groups have placed some pressure on meat producers because they want lower salt levels in meat products as higher salt concentrations may contribute to hypertension (Pearson and Wolzak, 1982). Phosphates, used as a partial replacement for sodium chloride, may provide a means to reduce sodium chloride levels in meat products. The addition of NaCl with phosphates has been shown to have a synergistic effect on WHC of meats (Vollmar and Melton, 1981; Trout, 1982; Jones et al., 1980). WHC is increased at lower NaCl/phosphate concentrations than if the salt and phosphate were added singly (Lewis, 1981). Offer and Trinick (1983) found that 0.8 M NaCl resulted in maximum myofibril swelling whereas 0.4 M NaCl in the presence of tetrasodium pyrophosphate achieved the same effect. Thus, phosphate addition reduces the concentration of NaCl required for water retention in meat products.

The WHC of meat has been shown to increase with the addition of phosphates. Although there has been extensive research done in this area, the role phosphates play in increasing the WHC of meats remains unknown. It has been reported that phosphates act by a variety of mechanisms which include increasing the pH of the meat system, increasing the ionic strength, chelating divalent metal cations, binding phosphate anions to proteins, and the dissociation of the actin-myosin complex (Trout and Schmidt, 1983; Hamm 1960, 1970). These mechanisms can be placed into two general groups. One group encompasses a charge effect and the other deals with the removal of structural constraints. Both groups agree that an increase in WHC results in the swelling of the myofibril (Offer and Trinick, 1983).

The charge effect theory deals with the electrostatic repulsion between myofibrillar proteins with the same charge. This causes the space between the proteins to increase and allows for greater water penetration. Anything which reduces these repulsive forces between the proteins will reduce the WHC of the meat.

Hamm (1960, 1970) and Lewis et al. (1986) attributed the charge effect to pH. After normal rigor mortis, the pH of meat is near pH 5.4 which is the area where the WHC of meat is the lowest. It is also the area where the isoelectric point (pI) of actomyosin is located. The movement away from the pI, by the addition of phosphates, will cause a charge imbalance. At pH values greater than 5.0, the thick and thin filaments are negatively charged. When the pH is raised, the charges on

the proteins are increased which results in the repulsion of similarly charged protein groups. This charge repulsion is responsible for the increased WHC of meats (Hamm, 1960, 1970).

The pH range between 5.4 and 6.1 was found to be where the greatest WHC changes occurred (Puolanne and Matikkala, 1980). Therefore, phosphates that can raise the pH of the meat to the higher pH range may be the most effective in increasing the WHC of the meat. One percent solutions of phosphates approved for the use in meat products have the following pH values (Ellinger, 1972):

Phosphates	pH
Monosodium phosphate	4.4
Disodium phosphate	8.8
Tetrasodium pyrophosphate	10.2
Sodium acid pyrophosphate	4.2
Sodium tripolyphosphate	9.8
Sodium polyphosphate (glassy)	7.0

It has been demonstrated that when phosphates (0.2 to 0.5% sodium tripolyphosphate) are added to meat products the change in pH is very small (Mahon, 1961). This may be due to the buffering capacity of the meat (Knipe et al., 1985). Although the pH changes can be small, they can produce large effects on the WHC of the meat product (Puolanne and Matikkala, 1980).

In addition to the pH mechanism, the basis for the ionic strength mechanism is the charge repulsion between the myofibrillar proteins. The ionic strength between 0.4 and 0.6 appears to be the range where the greatest increases of WHC have occurred (Trout and Schmidt, 1983; Hellendoorn, 1962; Mahon, 1961). Phosphates alone do not have the ability to reach these high ionic strengths because they do not fully dissociate in solution (Van Wazer, 1958), however, they can be reached in combination with salt concentrations of 1-2% (Trout and Schmidt, 1983). The effectiveness of phosphate types may be explained by the degree of dissociation and the final ionic strength of the phosphate. Trout and Schmidt (1983) determined that longer chain phosphates did not dissociate as readily as the shorter chain phosphates. Taking this into consideration, the ionic strength decreases with an increase in chain length.

It was thought that the chelation of divalent cations by polyphosphates was a factor in increasing the WHC of meats. Hamm (1960) proposed that magnesium and calcium ions could bind to proteins which would reduce or shield the electrostatic repulsive charges between the meat proteins. The WHC of the meat could be increased if the divalent cations were removed by a chelating agent. This would allow the structure of the myofibril to expand and accommodate more water molecules. However, researchers have provided evidence which contradicts this hypothesis (Lewis et al., 1986; Hellendoorn, 1962). Hellendoorn (1962) did not observe an increase in

WHC with ethylene diamine tetraacetic acid (EDTA), a stronger chelating agent than phosphates, and Inklaar (1967) showed that calcium and magnesium were firmly bound to the meat and that polyphosphates did not remove these ions.

There is evidence that phosphate anions may bind specifically or nonspecifically to meat proteins leading to an increase in the net negative charge on the protein (Hamm, 1970). This then results in protein-protein repulsions and an increase in WHC. Specific binding sites such as myosin and actomyosin can bind two moles of pyrophosphate and one mole of pyrophosphate, respectively (Nauss et al., 1969). Nonspecific phosphate binding sites include positively charged protein side groups. It has been demonstrated that all phosphates are able to bind to proteins although the degree of binding depends upon the type of phosphate used and the protein present (Vandegrift and Evans, 1981).

The second group includes the removal of structural constraints of transverse structures within the myofibril like the M-line, Z-line, actin and myosin crossbridges, and titin (Offer and Trinick, 1983; Patterson et al., 1988). The removal of these constraints will allow the addition of water molecules to occupy the newly formed areas.

The major constituent of meat is actomyosin. In the presence of some phosphates, primarily pyrophosphate, actomyosin is able to dissociate into its components of actin and myosin (Grabowski and Hamm, 1979; Hamm, 1970; Fukazawa et al., 1961). Bendall (1954)

hypothesized that the increase in WHC by pyrophosphate was due to the dissociation of actomyosin in the presence of low concentrations of magnesium. It was thought that magnesium ions activates the dissociation of actomyosin. Although Bendall (1954) did not believe that pyrophosphate could cause complete dissociation of the actomyosin complex, studies have shown that pyrophosphate can dissociate actomyosin at low protein concentrations (Granicher and Portzehl, 1964).

Offer and Trinick (1983) hypothesized that salt and pyrophosphate increased the swelling of the myofibril through a cooperative effect which included electrostatic repulsive forces and the removal of structural constraints. As the salt concentration increased from 0.6 M to 1.0 M, the electrostatic repulsive forces increased causing dissociation of actomyosin to its components of actin and myosin. Pyrophosphate reduced the concentration of salt necessary for maximum swelling effects. In addition, there was greater extraction of the A-band as indicated by phase-contrast microscopy. Although the actomyosin cross bridges were the major mechanical constraint to swelling, Offer and Trinick (1983) believed the M-line and Z-line were important structures as well.

Recently Offer and coworkers (1989) revised their earlier hypothesis and proposed that salt-induced swelling was entropically rather than electrostatically driven, although the removal of structural constraints remained unchanged. Electrostatic repulsive forces between

the filaments, in the presence of high salt concentrations, were thought to be screened by the excess of sodium chloride ions located near the surface of the filaments. Therefore, swelling was reduced and the WHC decreased. The authors stated that salt weakens the interactions between proteins by increasing the solubility of the protein and/or by disrupting the protein structure. They suggested that salt depolymerizes the thick filaments thereby liberating the myosin tails. The myosin head is still attached to actin, although in the H-zone, myosin would diffuse away because crossbridges were never made with actin. Water molecules would expand the lattice and after swelling, the myosin tails would move to a higher entropic state. Pyrophosphate aids salt in the depolymerization of the thick filament (Harrington and Himmelfarb, 1972) and it can extract myosin at lower concentrations with salt compared to if salt were added singly. Higher levels of pyrophosphate are presumed detrimental to the swelling of the myofibril because it promotes the dissociation of actomyosin into myosin and actin. Dissociation of myosin from actin would reduce the swelling of the myofibril more than if it were still bound to actin. It is unknown how this theory affects the removal of other structural constraints, such as the M-line and Z-line, since it was not addressed in their paper.

Experimentation by Paterson et al. (1988) involving myofibril/cytoskeletal proteins, led these authors to conclude that titin was a significant structural constraint partially responsible for the regulation of increased WHC. Using SDS-PAGE gels and phase-

contrast microscopy, they showed that titin was extracted by pyrophosphate and salt and that it was associated with increased myofibrillar swelling and increased WHC.

The multiple conclusions derived by scientists in their search for the effects of phosphates on the WHC of meat are perhaps a direct result of the variety of levels, types of phosphates and the many different ways in which phosphates have been added to the meat system.

Improve texture Evidence indicates that in some meat systems, texture is improved upon the addition of phosphates. Ground chicken was smoother and less crumbly upon cooking when phosphate blends were added (Froning, 1966) although a rubbery texture resulted at levels of 2 % phosphate. Tenderness of lean beef increased upon the addition of DSP, STPP and SPG although Baldwin and DeMan (1979) did not observe an effect with MSP or sodium pyrophosphate. Frankfurter texture was improved slightly with SAPP. Hargett et al. (1980) observed that springiness and hardness were increased although this may have been due to better skin formation. Other researchers have found that meat product texture was improved by phosphate addition (Trout, 1984; Madril and Sofos, 1985; Keeton et al., 1984) In contrast, Knipe et al., (1985) found that firmness decreased with added phosphate in sausages. Ranken (1976) believes that the texture of meat changes from a more fibrous nature to a more gelatinous one when phosphates are added and when there is significant mechanical action. The actin-myosin complex becomes solubilized and forms an exudate

which moves from within the meat to the outside meat surface. This action, although important in cooked meat texture, is at the basis of restructured meat product binding.

Binding and emulsification Increased binding strength and cooking yield has been shown to correlate with myosin extraction (Turner et al., 1979). This was supported by Offer and coworkers (1989) who reported that polyphosphates solubilized myosin. The solubilization of myosin helped to form an exudate on the surface of the meat pieces so that upon heating, gelation of the exudate occurred. This aided in the binding of the meat pieces. The combined effectiveness of phosphates and sodium chloride in the binding of restructured beef rolls were found to be in the order of most to least effective: pyro- > tripoly- > tetra- > hexameta-phosphate (Trout and Schmidt, 1984). This indicated that as the phosphate chain length increased with salt present the binding effectiveness decreased. In addition, increasing the phosphate or sodium chloride concentrations increased the binding effectiveness only up to a maximum level.

Emulsions or batters are the result of finely comminuted meat systems that include an oil in water emulsion and a protein gel (Barbut, 1988). The fat particles are trapped in a protein matrix which prevents the particles from forming larger fat pockets.

Emulsion stability is increased upon the addition of phosphates in a variety of meat systems including high collagen emulsions with STPP (Ladwig et al., 1989) and lean meat mixtures (Knipe et al., 1990) using

combinations of phosphates (STPP and TSPP) with salt.. Additionally, Barbut (1988) reported that phosphates when added to reduced salt poultry batters restored emulsion stability. The degree of restoration was dependent upon the type of phosphate added to the meat mixture. He found that SPG was the most effective, followed by SAPP and STPP, for improving emulsion stability. The authors concluded that the phosphates increased protein extraction which caused increased and better coating of the fat particles.

In addition to emulsion stability, pyrophosphate (SAPP) lowered emulsion viscosity and held emulsion temperatures down thereby controlling the temperature increase during processing. This prevented frictional or viscous heat build up in the frankfurter emulsion (Hargett et al., 1980).

Decreased cooking losses Increased yields were observed by Vollmar and Melton (1981) when they tested hams treated with STPP, SPG and TSPP. They concluded that nonphosphate treated hams had greater cooking losses and the lowest sensory attributes as compared to phosphate-treated hams. Studies by other researchers (Wierbicki et al., 1976; Shults et al., 1972) indicate that alkaline phosphates, specifically pyro-, tripoly-, and sodium polyphosphate, glassy, increase yields or decrease shrink of pork or beef products.

Color stabilization Myoglobin is the principal protein responsible for the color in all red meats. In cured meats, nitric oxide combines with myoglobin to form nitrosylhemichrome, prior to

cooking, and nitrosylmetmyoglobin in thermally processed meats. This reaction occurs rapidly but may not go to completion during processing. The rate of color development in cured meats is reduced with alkaline phosphates and increased with acid phosphates (Schmidt, 1983). Increasing the pH of the meat using an alkaline phosphate will adversely affect the development of nitrosylmyoglobin to nitrosylmetmyoglobin (Fox and Thomson, 1963) whereas decreasing the pH will shift the reaction of nitric oxide and myoglobin to nitrosylmyoglobin. SAPP lowers the meat pH and therefore is used as a color accelerator in frankfurters, bologna, and similar processed meat products (Steinhauer, 1983; Hargett et al., 1980).

Inhibit microorganisms Currently, phosphates are not approved for use as food preservatives (Food and Nutrition Board, 1981; FAO, 1962), although they have been known to have antimicrobial effects as early as 1864 (Leuke, 1980). The food industry recognizes, to some extent, the antimicrobial properties of phosphates. But these properties are viewed as a secondary function, more as a benefit rather than a consideration as a primary function. Several studies have shown that phosphates have an inhibitory effect on the growth of spoilage and pathogenic organisms in both laboratory media and in various food systems.

In laboratory media, the effects one observes may not necessarily parallel those that are observed in a food system due to the simplicity of the laboratory media and/or the complexity of the food system.

However, laboratory media provides a more controlled system with less variables than a food system when gathering information. Early studies using laboratory media indicate that the antimicrobial properties of phosphates are more effective against Gram positive bacteria than Gram negative bacteria (Elliot et al., 1964; Post et al., 1963; Kelch and Buhlmann, 1958). Recent studies have supported this finding (Zessin and Shelef, 1988; Jen and Shelef, 1986; Wagner and Busta, 1985, 1986; Molins et al., 1984).

Molins and coworkers (1984) investigated the effects of heated and unheated food grade phosphates (TSPP, SAPP, STPP, SPG) in Trypticase Soy Agar (TSA) on *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and two lactic starter cultures. TSPP, in the unheated form, was found to be inhibitory to all the organisms tested, although the Gram negative organisms were less inhibited than the Gram positive organisms. Unheated STPP and SPG were also inhibitory to the bacteria analyzed. Upon heating, TSPP retained its antimicrobial abilities whereas STPP and SPG lost these abilities. This was attributed to the knowledge that longer chain phophates undergo more rapid hydrolysis than shorter chained phosphates (Bell, 1947). Interestingly, SAPP was only slightly inhibitory in the unheated form while it actually enhanced bacterial growth when it was heated.

Experimentation was undertaken to understand the sensitivities of Gram positive and Gram negative bacteria to polyphosphates, the

resistance of *Pseudomonas* strains in media with increasing polyvalent cations and protein contents in the presence of polyphosphates, and the effect of heat and filter sterilization of polyphosphates (Zessin and Shelef, 1988). It was found that polyphosphates (STPP, TSPP and SPG), at 0.5%, were inhibitory to seven of 13 Gram positive bacteria whereas the Gram negative bacteria were more resistant. Only three of 12 Gram negative organisms were inhibited by the phosphates. The antimicrobial abilities of the phosphates tested were found to be strain dependent with STPP and SPG being the most inhibitory. Protein content affected the growth of the *Pseudomonas* strain such that the antimicrobial activities were greater in low protein content media. The authors concluded that the higher protein content provides more protection to the bacterium. Liquid media having the most protein was Tryticase Soy Broth (TSB) followed by Brain Heart Infusion (BHI) and Nutrient Broth (NB) which contained the least amount of protein. The mineral content of each of these media influenced the antimicrobial activities of the polyphosphates. *Pseudomonas* strains were more sensitive to polyphosphates when in media having low mineral concentrations. It is thought that the low levels of cations essential for cell growth and maintenance were chelated by the polyphosphates which made them unavailable to the organism. The antimicrobial activities of heat sterilized polyphosphates were reduced when compared to filter sterilized polyphosphate treatments. Hydrolysis of the polyphosphate was implicated in the reduced effectiveness of the heated phosphates,

giving support to the findings of Molins et al. (1984). It was also suggested that heat-induced binding of phosphates to proteins may have contributed to the reduced antimicrobial activities.

Staphylococcus aureus 196E sensitivity to polyphosphates was studied by Jen and Shelef (1986) and Shelef and Wang (1989). Growth of *S. aureus* was inhibited with 0.5% phosphate (SPG and STPP) in BHI. Inhibition was not observed with SAPP or TSPP. Initial population size affected the number of survivors with 10^4 CFU/ml being the pivotal concentrations for growth of the organism. Supplementation of magnesium caused *S. aureus* to overcome its inhibition by 0.5% STPP while calcium and iron only partly eliminated the inhibition. Chelation of magnesium, calcium and iron is believed to be responsible for the inhibition of *S. aureus*. Magnesium is known to form a stable chelation complex with phosphate (Irani and Callis, 1962). Jen and Shelef (1986) believe that the addition of a cation which has a high stability constant, like magnesium, may aid in releasing essential chelated cations that possess lower stability constants. This would make these cations available to the organism. It was found that magnesium formed the most stable complex with STPP and SPG followed by calcium, iron, zinc and manganese. Therefore, if magnesium were in excess in the medium, it would cause the release of other cations with less stable complex constants. Inhibitory effects by STPP and SPG were found to be pH dependent. Raising the pH resulted in a reduction of antimicrobial activity which is speculated to be due to the chelation

complex becoming increasingly dissociated at elevated pH values. Enterotoxin A and extracellular protein production of *S. aureus* 196E was found to be inhibited by SPG, STPP and SAPP. The effectiveness of SAPP was increased at lower pH values (pH 5.5).

Knabel et al. (1991) determined that *Bacillus cereus*, *S. aureus*, *Listeria monocytogenes* and *Pseudomonas fluorescens* were inhibited by media containing 1% phosphate (TSPP, STPP, SPG or SAPP). The inhibition that resulted was attributed to the chelation, by phosphates, of essential metal ions originating from specific cation-binding sites within the Gram positive bacterial cell wall. Well-plate assays were performed whereby metal ions could be used to differentiate the metal growth requirements of a particular organism as well as the ability of phosphates to chelate the metal ion. Teichoic acids and/or teichuronic acids that are associated with the Gram positive cell wall possess cation-binding sites. The function of these cation-binding sites appear to be in maintaining the ionic charge of the cell wall so that the cation-dependent membrane systems continue to function efficiently. More teichoic acids have been shown to be synthesized in low magnesium ion concentrated environments, which indicates a high requirement for this metal. Knabel and coworkers (1991) believe that phosphates compete with the teichoic acids for magnesium ions and that the phosphates successfully bind magnesium due to their ability to form a more stable chelation complex (Irani and Callis, 1962). This results in the removal of magnesium, which is essential for Gram positive cell growth and

maintenance. Gram negative bacteria do not have teichoic acids and utilize different metal binding systems. Therefore Gram negative organisms are not as affected by phosphates. As one might expect, the degree of inhibition by polyphosphate chelation depends upon the unique metal requirements of each individual organism.

The effect of phosphate on the growth and toxin production of *Clostridium botulinum* was investigated to determine if phosphate could act as a replacement for nitrite. Nitrite is used in cured meats to prevent toxin formation of C. botulinum. However, nitrite can form a carcinogenic precursor, nitrosamine, at high temperatures which makes an alternative antimicrobial agent desirable (Wagner and Busta, 1984). SAPP, in a peptone-yeast extract broth, was found to delay the growth of most of the ten strains of *C. botulinum*. In addition, SAPP may be responsible for the inhibition of the protease involved in toxin activation (Wagner and Busta, 1985). Dual-substrate protease assay plates showed a reduction in zone sizes with 0.4% and 1% SAPP. It was speculated that SAPP binds to the proteinaceous toxin molecule itself or it inactivates the proteases responsible for the activation of the precursor toxin molecule.

In meat products, it is not surprising that phosphates have varied degrees of antimicrobial effects. The results are often confusing because a clear cut generalization cannot be made that one phosphate will work better than another phosphate in a particular meat system. In fact, between meat systems one phosphate may have an entirely different

antimicrobial effect when compared to another phosphate. Adding to the confusion is the variety of phosphate levels, phosphate type, conditions, (pH, temperature, atmosphere), meat system (beef, pork, poultry), and cut of meat (ground, processed, whole) used by each experimentor in addition to the complexity of the meat system itself.

Early studies on the use of phosphates as antimicrobial agents had been done primarily in poultry (Elliot et a.l, 1964; Steinhauer and Banwart, 1963; Spencer and Smith, 1962). Recently, a large amount of research has been done in fresh and processed pork and beef.

Aerobic mesophilic and psychrotrophic bacterial and Staphylococcus aureus Z 88 growth in uncooked bratwurst with SAPP, STPP, SPG and TSPP (0.5%) was unaffected (Molins et al., 1985) after seven days at 5° C. Although it did not significantly inhibit growth, SAPP had lower total aerobic counts as compared to the other phosphate treatments. The loss of inhibitory properties in a meat system, as compared to that in laboratory media (Molins et al., 1984), may have been due to natural meat phosphatases causing the hydrolysis of the phosphates to microbiologically inactive species. Therefore, to retain antimicrobial activities when using phosphates, it is advisable to cook the meat product immediately after phosphate addition to inactivate the phosphatases

Molins et al. (1985) investigated the effects of SAPP and STPP individually or in combination with low and high levels of sodium nitrite on the survival and growth of Clostridium sporogenes PA 3679

and in normal spoilage bacteria. Cooked, vacuum-packaged bratwurst were held under refrigeration (5° C) for seven days and at temperature-abuse conditions (24° C) for up to 48 hours. SAPP and STPP, alone or in combination with nitrite were able to reduce *Clostridial* and anaerobic counts after seven days at 5 C, but aerobic bacterial counts were unaffected. At the higher temperatures, SAPP alone and in combination with high levels of nitrite (100 ppm) effectively inhibited aerobic and anaerobic bacterial growth and *C. sporogenes* although, STPP appeared to lose its antimicrobial abilities after 24 hours, possibly due to its hydrolysis to orthophosphate. SAPP has demonstrated some inhibitory properties against *C. sporogenes*. With this in mind, Marcy et al. (1988a) compared the antimicrobial properties of an acid pyrophosphate (SAPP) to an alkaline pyrophosphate (TSPP). Phosphate-treated vacuum-packaged pork sausage was stored at 5° C for 21 days and at temperature-abuse conditions (7° C, 48 hours). After storage, it was microbiologically analyzed. No inhibitory effects were observed with either SAPP (0.4%), TSPP (0.4%), or a combination of the two at refrigeration temperatures. At temperature-abuse conditions, SAPP was able to lower mesophilic and facultative anaerobic organism growth after 48 hours. In addition, 0.2% SAPP/0.2% TSPP had no synergistic effects. Therefore, the antimicrobial abilities of SAPP at elevated temperatures were found to be different from those of TSPP. The reason for the lack of antimicrobial action is unclear but the authors do not believe that pH is the primary cause of the differences

between the alkaline and acid pyrophosphate antimicrobial abilities. A neutral pyrophosphate (Pyro-3) was compared to SAPP's antimicrobial action (Marcy et al., 1988b) in vacuum-packaged cooked pork sausage. Both phosphates inhibited natural mesophilic flora and facultative anaerobes at refrigeration (7° C) and room temperature (20 C). The main organism inhibited at the elevated temperature was *Streptococci.* SAPP was only slightly more effective than Pyro-3 in its antimicrobial abilities. Lastly, these authors evaluated four commercial phosphate blends (Brifisol 414, 414-B, 414-K, 515) and Pyro-3 in its antimicrobial abilities in cooked vacuum-packaged pork sausage (Marcy et al., 1988c). Increasing the phosphate levels up to 0.6%, resulted in lower mesophilic, psychrotrophic and facultative anaerobic bacterial counts at both refrigeration (7° C) and room temperature (20° C). However, the type of phosphate blend was not as important as the level used for bacterial inhibition.

Frozen beef patties with 0.4% STPP, TSPP or three phosphate blends were examined for their antimicrobial abilities (Molins et al., 1987a). The mesophilic, psychrotrophic, presumptive *S. aureus* and lactic acid bacterial counts were not reduced by any of the treatments after 90 days at -20° C whereas after 90 days, at elevated temperatures (24° C), TSPP and Brifisol 414-B inhibited bacterial growth.

In addition to meat systems, the antimicrobial abilities of phosphates are observed in fruits and vegetables, dairy products, breads

and egg products. For additional information, detailed reviews are given by Molins (1991), Sofos (1986), and Tompkin (1983).

Possible antimicrobial mechanisms of phosphates

There are a variety of proposed mechanisms which explain the antimicrobial abilities of phosphates (Knabel et al., 1991; Zessin and Shelef, 1988; Elliot et al., 1964; Post et al., 1963). These include the chelation of essential cations, pH effects, ionic strength increases, interactions with cell walls, interactions with cell membranes, and inhibition of various transport functions. Much of the antimicrobial action of phosphates in meat is proposed to occur by metal chelation. It may also be the mechanism responsible for the inhibition of microbial enzymes in foods containing phosphates.

Microbial proteases

Microbial enzymes are essential for the metabolic processes of the microorganism. Enzymes produced by the microorganism and secreted outside the cell membrane are called exoenzymes. They are used in catabolic reactions to degrade polymers that are unable to penetrate cell membranes. The polymers are degraded into smaller, transportable molecules (Frazier and Westohoff, 1978) by the exoenzymes. Exoenzymes include proteinases which catalyze the hydrolysis of proteins into long-chain polypeptides and peptidases which breakdown these polypeptides into amino acids. The amino acids and low molecular weight peptides can then be utilized for growth by many microorganisms. Proteases are found within the cells of all bacteria,

however, only a limited number of bacteria can synthesize them extracellularly. The bacteria that can produce extracellular proteases can be divided into aerobic or facultatively anaerobic bacteria that may or may not be spore-forming and anaerobic spore formers. Examples of bacteria that fall into these catagories are *Bacillus*, *Micrococcus*, *Clostridium*, *Aeromonas*, *Proteus* and *Pseudomonas*.

The enzymatic breakdown of proteins by proteases is both beneficial and undesirable. Some of the benefits that proteases provide the food industry are meat tenderization, solubilization of proteins present in beer, product modification of gelatin to prevent subsequent gelation in foods, coagulation of casein in cheese production, breakdown of gluten in bread dough to control viscosity during handling and improvement of the final texture and appearance of some food products (Gacesa and Hubble, 1987). On the other hand, proteases contribute to the spoilage of food products by the degradation of the proteins which result in poor texture, off-odors and off-flavors.

Pseudomonas fragi protease

Pseudomonads are psychrotrophic organisms which grow at 7° C or less and have optimal growth temperatures between 25° C and 35° C (Suhren, 1989). At refrigeration temperatures, Pseudomonads are the most dominant and most metabolically significant spoilage organisms. They are Gram negative, motile organisms and they are oxidase positive. They have extensive oxidative capabilities, utilize a wide variety of substrates as energy sources, grow rapidly at low

temperatures and in the pH range of meat (Dainty et al., 1983; Hurst and Collins-Thompson, 1979). Studies determined that *Pseudomonas fragi* was the most prevalent Pseudomonad in beef, pork, lamb and poultry (Shaw and Latty, 1984, 1982, 1981; Molin and Ternstrom, 1986; 1982) Together with *Pseudomonas lundensis*, *P. fragi* made up 85% of the bacterial flora in red meat (Molin and Ternstrom, 1986).

P. fragi has been found to produce an extracellular neutral protease (E.C. 3.4.4.-) with a molecular weight range of 40,000-50,000 Kd (Porzio and Pearson, 1975; Tarrant et al., 1973, 1971). Its enzyme production rate was found to vary inversely with the cell growth rate (Myhara and Skura, 1989). Less enzyme is produced when *P. fragi* is actively growing but when cell growth is slowed, as during stationary phase, the enzyme production is greatly increased. These authors believe that growth rate reduction precedes enzyme rate increases.

Tarrent et al. (1973) first partially purified the *P. fragi* protease and determined some of its properties. Its pH range was between 6.0-8.0, with optimal proteolytic activity between 6.5 and 7.5. The enzyme was also found to be sensitive to temperatures approaching 35°C. Porzio and Pearson (1975) further purified the *P. fragi* protease and determined that the protease was zinc activated and calcium stabilized. The authors concluded that the proteolytic activity of the extracellular enzyme was due entirely to it being a metalloenzyme because a loss of proteolytic activity was observed when the enzyme was in the presence of chelating agents (EDTA, ethyleneglycol-bis N,N'-tetraacetic acid

(EGTA), dithizone, 8-hydroxyquinoline and cyanide). Additionally, for maximal enzyme activity a minimum concentration of 2-5mM $CaCl_2$, and an ionic strength between 0-0.5M KCL was needed. Later studies defined the optimal culture conditions for the production of *P. fragi* protease to be 12.5° C, 38 hours incubation time, initial pH of 6.8, organic nitrogen concentration of 314 micromoles nitrogen/liter (glutamine), gas mixture of 16.4% oxygen and 7.42 ppm dissolved oxygen (Myhara and Skura, 1990). These authors found oxygen to be the major factor involved in the production of protease.

When *P. fragi* colonized muscle tissue, protrusions of the outer cell wall (blebs), were observed (Thompson et al., 1985; Tarrant et al., 1973; Dutson et al., 1971). Bleb formation was hypothesized to correlate with proteolytic activity. Using polyclonal rabbit antiserum against the purified protease and peroxidase reactions, Thompson et al. (1985) determined that the blebs originated near the cell wall and they contained the protease. The authors concluded that the extracellular protease was most likely synthesized at the cell membrane, secreted directly into a bleb, and then released into the surrounding environment. It is thought that the bleb is responsible for the controlled release of the protease for the hydrolysis of macromolecular proteins, resulting in a source of amino acids for cellular metabolism. The conditions at which blebs are produced were further characterized using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Myhara et al., 1990; Myhara and Skura, 1989).

Myhara and Skura (1989) observed that growth of *P. fragi* in liquid or on solid media gave startlingly different times for proteolytic enzyme production. On solid medium (TSA), *P. fragi* began producing extracellular protease at 4 hours while in liquid medium (TSB) protease production started at 24 hours. Electron micrographs showed bleb formation and bleb shedding (blebbing) were associated with protease activity in solid medium. However, a lack of bleb formation, but not protease activity, was observed with *P. fragi* growth in liquid medium. The authors attributed this result to the washing action of the liquid medium. Further experimentation (Myhara et al., 1990) demonstrated this to be true. Blebs were found in the culture supernatant and were shown to have proteolytic activity. Most of the proteinase activity was associated with the liquid culture medium (free proteinase) and a small amount with the blebs. There was no protease activity associated with the whole intact cells.

Pseudomonas fragi protease activity in meat

Lean muscle is composed of 15%-20% protein (Dainty, 1982). These proteins can be fractionated, based on solubility, into myofibrillar, sarcoplasmic and stromal components (Greer, 1989). Within each of these categories, numerous proteins exist. The proteolytic effects of *P. fragi* inoculated into whole or ground meat were determined in early studies. Later, they involved the purification of the enzyme and its effects on the various meat proteins. *P. fragi* has been shown to hydrolyze sarcoplasmic (Yada and Skura, 1981; Bala et

al., 1979; Hasegawa et al., 1970), myofibrillar proteins (Porzio and Pearson, 1980; Tarrant et al., 1973b, 1971; Dutson et al., 1971) and stroma proteins (Yada and Skura, 1981).

In sterile beef and beef extract, the color stability was shown to be reduced by *P. fragi* (Bala et al., 1979). SDS-PAGE gels and nonprotein nitrogen analysis indicated proteolysis of the sarcoplasmic proteins.

Yada and Skura (1981) found that urea-soluble proteins, urea-insoluble (stroma) proteins and salt-soluble proteins were degraded in beef muscle inoculated with *P. fragi* and refrigerated at 4° C for 12 days. When the sarcoplasmic fluids were removed, no proteolytic changes were observed in the beef samples. These authors suggested that the lack of extensive changes in the washed beef muscle were because the population of *P. fragi* was not high enough to produce proteolytic changes when the sarcoplasm was removed. Some researchers believe that for proteolysis of meat proteins to occur, bacterial populations must exceed 10^8 CFU/cm^2 (Gill and Newton, 1977; Dainty et al., 1975; Ingram and Dainty, 1971). Other factors may also contribute to the lack of observed proteolysis in the washed beef samples. These include the physical state of the muscle (intact or minced), myosystem specificity of bacterial proteases, or the type of electrophoretic method (SDS-PAGE or starch gel electrophoresis).

Pork muscle was inoculated with *P. fragi* and stored at 2° C and 10° C for 20 days (Borton et al., 1970a). In that time, the sarcoplasmic

and salt-soluble proteins were degraded, while the stroma proteins were unaffected by *P. fragi*. The salt-soluble proteins were analyzed further (Borton et al., 1970b) using starch-urea gel electrophoresis. The results supported earlier findings (Borton et al., 1970a) that the myofibrillar proteins were hydrolyzed.

In experiments done by Hasegawa and collaborators (1970), *P. fragi* had the highest proteolytic activity, in pork and rabbit, out of all the organisms tested. This experiment found the sarcoplasmic proteins to be significantly degraded. Some of these proteins, from pork, included aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, creatine kinase and hemoglobin.

Degradation of the H-zone, disruption of the A-band, and loss of Z-line proteins were observed by Dutson et al. (1971) using electron micrographs. Maximum muscle disruption occurred at the end of 8 days.

Tarrant et al. (1971) looked at the effects of *P. fragi* growth on ground pork using SDS-PAGE and electron micrographs. They observed considerable degradation of the salt-soluble proteins after 20 days at 10° C. Although this research did not differentiate between the myofibril proteins, myosin was believed to be the major protein degraded. The sarcoplasmic proteins were not degraded. Most of the proteolytic activity was observed to occur directly after, and not prior, to meat spoilage. In later studies, Tarrant et al. (1973) partially purified the protease produced by *P. fragi* and examined its proteolytic

activity on sarcoplasmic and myofibrillar proteins, purified myosin and crude preparations of G-actin. Electron micrographs indicated that after 72 hours (10° C), the Z-line was almost totally removed. This was not caused by autolysis because the uninoculated control did not show any degradation. The least susceptible of the muscle proteins to proteolysis by *P. fragi* were the sarcoplasmic proteins.

Porzio and Pearson (1980) used purified *P. fragi* protease and determined its proteolytic effect on rabbit myofibrils. These workers found myosin to be greatly hydrolyzed. In fact, heavy meromyosin, light meromyosin and premeromyosin were all found to be degraded.

Microbial proteases and chelating agents

Metalloenzymes can generally be inactivated by chelating agents (Beynon and Bond, 1989). Most of the synthetic inhibitors contain a negatively charged moiety designed to chelate or bind the catalytic metal atom. Some examples of synthetic chelating agents with their corresponding apparent inhibition constants (Auld, 1988) are listed in Table 1.

Few studies have been done examining the effects of chelating agents on *P. fragi* growth and fewer still on the effects of its protease. In fact, only one study (Porzio and Pearson, 1975) looked at some effects of chelating agents on *P. fragi* protease and this was done to elucidate the classification of the protease. Therefore, comparisons with

Table 1. Chelating agents and their inhibition constants

Compound	log K_1	log K_2	log K_3
a,a'-bipyridyl	5.3	4.5	3.8
1,10-phenanthroline	6.6	5.8	5.2
8-hydroxyquinoline	8.5	7.3	15.8
EGTA	12.9		
EDTA	16.4		

the effects of chelating agents on other extracellular proteases will be made. However, this does not necessarily preclude that *P. fragi* will give a similar response to the chelating agents.

The inactivation of *P. fragi* protease by chelating agents is similar to that reported by Sekine (1972). He found that two Zn^{++} activated-Ca^{++} stabilized neutral proteases from *Aspergillus sojae* were inactivated by EDTA, o-phenanthroline, 8-hydroxyquinoline and a,a'-dipyridyl. One of the proteases was found to be pH dependent in the presence of EDTA. It was suggested that the decreased metal-chelating ability of EDTA, as hydrogen ion concentration increased, was due to either steric hindrances or ionizable groups of EDTA.

Several studies were done to determine the effects of antimicrobial and chelating agents on bacterial growth and their ability to produce proteases. Venugopal et a. (1984) determined that butylated hydroxyanisole (BHA), propylhydroxy parabenzoate (paraben), and

sodium tripolyphosphate (STPP) were inhibitory for the growth and protease secretion of *Aeromonas hydrophila*. Furthermore, they found that protease secretion was inhibited at lower concentrations than that required for the inhibition of growth. Another study examined the extracellular protease production by *Pseudomonas fluorescens* in mineral salts medium (McKellar and Cholette, 1984). Maximum growth was obtained at 1.0 and 2.5 mM orthophosphate (K_2HPO_4) at 20° C and 5°C, respectively. For maximum protease production, 5 mM orthophosphate was required at both temperatures. Increasing the orthophosphate concentration was needed for protease production rather than for growth.

Explanation of dissertation format

The dissertation is divided into seven experiments, each with separate objectives. Experiment I examines the effects of phosphates (0 to 1%) on the growth of *Pseudomonas fragi* and the production of protease. Experiments II and III characterize the antimicrobial effects of phosphates on the growth of *P. fragi* in liquid and solid media. Experiments IV and V examine the antimicrobial abilities of phosphates and the effect of phosphates on protease production in media and in ground pork. Experiment VI studies the ability of *P. fragi* to synthesize protease in the presence of phosphates. Experiment VII attempted to examine the effects of phosphates on the purified protease.

EXPERIMENT I

Materials and Methods

This experiment was designed to determine the effects of seven different phosphates at five concentrations (0-1.0%) on *Pseudomonas fragi* growth and protease activity in a solid growth medium containing casein. Growth was monitored by measuring the diameter of the bacterial colony over an eight-day period of time. Proteolysis was monitored by the hydrolysis of the casein proteins whereby a zone of clearing around the bacterial colony was measured. The proteolysis was confirmed by flooding the nutrient/skim milk agar plates with a 1% solution of HCL at the end of the growth period. This was done at the end of the growth for two reasons. First, the HCL would interfere with the growth of the organism if it were added earlier and secondly, the cost and time needed to do the experiment with individual plates for each growth measurement period was not feasible. This experiment was done in duplicate and replicated two times.

Culture

Freeze-dried cells of *P. fragi* (ATCC 4973) were suspended in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) and incubated at 37° C for 48 hours. The culture was transferred to BHI broth and incubated at 25° C for 48 hours. The organism was subsequently maintained on nutrient agar (NA, Difco Laboratories).

P. fragi was incubated at 30° C and stock cultures were stored at 4° C with bimonthly transfers.

Test chemicals

Phosphates tested included monosodium (MSP), disodium (DSP), and trisodium phosphate (TSP) (Fisher Scientific Co., Fair Lawn, N.J.), sodium acid pyrophosphate (SAPP) (Stauffer Chemical Co., Westport, CT), tetrasodium pyrophosphate (TSPP) and sodium tripolyphosphate (STPP) (Monsanto, St. Louis, MO), and sodium hexametaphosphate, glassy (SPG) (BK-Ladenburg Corp., Cresskill, N.J.). Four percent (w/v) phosphate stock solutions were prepared using sterile, distilled water. The solutions were filter sterilized through a 0.22 μ Cameo IV filter unit (Micron Separations Inc., Westboro, MA).

Media

Nutrient/skim milk agar plates with phosphates were prepared as described in Table 2. Appropriate portions of the stock phosphate filtrates were aseptically added to bottles of sterile, specifically measured amounts of melted NA (Difco Laboratories), previously tempered to 45° C. The appropriate volumes from a 10% (w/v) stock of sterilized (121° C, 10 minutes) skim milk (BBL, Cockeysville, MD) were added to obtain the desired final concentration of 1% skim milk, 2.3% NA, and phosphate concentrations of 0, 0.2%, 0.4%, 0.6%, 0.8% and 1.0%. The ingredients were mixed into the melted agar by gently inverting the bottle several times. The contents were then poured into sterile plastic petri plates (Fisher Scientific, ca 20 ml per plate) and

allowed to dry overnight, in an inverted position, at room temperature (24° C).

Table 2. Nutrient agar/skim milk plate formulations with added phosphates

	Final phosphate percentage					
	0	0.2%	0.4%	0.6%	0.8%	1%
2.3% NA	180[a]	170	160	150	140	130
10% skim milk	20	20	20	20	20	20
4% phosphate	0	10	20	30	40	50

[a] Volumes are in milliliters (ml)

Inoculum

Plate inoculation was performed by using a sterile needle inoculated with *P. fragi* by piercing the agar at the center of the plate with the needle tip. The inoculated plates were incubated at 30° C for a maximum of eight days. Bacterial growth and protease production was monitored every two days, by measuring the colony diameter and the zone of clearing originating from the bacterial colony. Control plates were prepared using the appropriate amount of sterile water, in place of the added phosphate, NA and skim milk. On the eighth day of incubation, the zone of clearing was measured before and after a one

percent solution of HCL (Fisher Scientific) was added to flood the plates (American Public Health Association, 1972). This was done to confirm that the zones of clearing were due to proteolysis and not caused by acid produced by bacteria from the fermentation of sugars (Martley et al., 1970).

Results and Discussion

Growth

All phosphates were inhibitory to the growth of *Pseudomonas fragi* (Figures 2-4). The concentrations at which growth was inhibited differed between the phosphates. In general, all phosphate treatments, at 0.8% and 1%, showed an inhibitory effect on the growth of *P. fragi.*

Growth of *P. fragi* was dependent on the concentration of TSP, SAPP, TSPP and STPP (Figures 2-3). The other phosphates (MSP, DSP and SPG) show an inhibitory effect on *P. fragi* growth at certain levels, however, there is no apparent concentration effect. It is possible that MSP and DSP (Figure 2) may show an effect at higher concentrations than those tested in this experiment.

TSP was the only orthophosphate that showed an inhibitory effect on the growth of *P. fragi.* This may be due to the highly basic nature of TSP (pH 11, 1.0% aqueous solution) when compared to MSP (pH 4.4) or DSP (pH 8.8). Although TSP (Figure 2) was the most effective orthophosphate at reducing *P. fragi* growth, the the short-chain polyphosphates (Figure 3) at 1% and after eight days of growth,

were the most effective inhibitors of *P. fragi* overall. TSPP (Figure 3) was the most inhibitory phosphate because it reduced the apparent growth rate at 0.6%, 0.8% and 1.0%. A factor in TSPP's ability to inhibit the growth of *P. fragi* may be due to its high pH. TSPP, STPP and SAPP all have similar chain-lengths however the pH for each phosphate is different and may contribute to the different inhibitory abilities. The pH values of a 1.0% solution of TSPP, STPP and SAPP are 10.2, 9.8 and 4.2, respectively (Ellinger, 1972).

Chain length may be involved in the antimicrobial abilities of the phosphate with 2-3 phosphate units being the most effective inhibitors of *P. fragi* growth. The long-chain polyphosphate, SPG (Figure 4), was slightly inhibitory to *P. fragi* growth. It is possible that SPG partially hydrolyzed to orthophosphate and this may have contributed to the reduced inhibitory abilities. It is known that SPG hydrolyzes by two simultaneous reactions which ultimately results in orthophosphate formation (Bell, 1947). Accumulation of orthophosphate by SPG hydrolysis would presumably show an inhibitory effect similar to that of MSP, DSP or TSP, however, this was not observed. This may indicate that SPG was not completely hydrolyzed to orthophosphates or even short-chained polyphosphates, such as STPP, which did exhibit a reduction in growth of *P. fragi*.

Protease production

The zone of clearing surrounding the *P. fragi* colony was measured and used as an indication of protease production. At the

higher phosphate concentrations (0.8%-1.0%), production of the protease is reduced or completely inhibited (Figure 5), although in most cases this may have been a result of reduced *P. fragi* growth (Figures 6-9).

Of the orthophosphates, MSP (Figure 6) was the most effective inhibitor of protease production. Media treated with MSP did not show protease hydrolysis of casein at 0.8% and 1.0%. Growth, corresponding to 0.8% and 1.0%, had been reduced at these levels of MSP (Figure 6) which may have contributed to the inhibition of protease production. Protease production was reduced by DSP (Figure 7) and TSP (Figure 8) at 1.0% (8 days). At 1.0% TSP, protease production was delayed, with the hydrolysis of casein first observed 4 days after inoculation.

SAPP (Figure 9) reduced the production of protease at all concentrations and completely inhibited its production at 0.8% and 1.0% after 8 days of incubation. No casein hydrolysis was observed with TSPP, STPP, and SPG until after 1% HCL was added (day 8) to the plates (Table 3). After HCL addition, no cleared zones were observed at 0.8% and 1% in TSPP, STPP and SPG. This indicated that protease production was absent. The inhibition of protease activity with STPP at 0.8% and 1% corresponds with the reduced growth of *P. fragi* at the same concentrations of STPP (Figure 3). However, the reduction of protease production by SPG can not be explained by a decline of *P. fragi* growth (Figure 4) at 0.8% and 1%.

Table 3. Hydrolysis zones after the addition of HCL

% Phosphate	TSPP	STPP	SPG
0	0.92[a]±0.10	0.94±0.08	0.78±0.20
0.2	0.90±0.28	1.20±0	0.30±0.28
0.4	0.50±0	1.20±0.14	0.30±0.28
0.6	0.15±0.21	0.60±0.14	0.10±0.14
0.8	NP	NP	NP
1.0	NP	NP	NP

[a]Hydrolysis zones measured in millimeters.
NP=no observed proteolysis.

P. fragi protease production showed complete inhibition with TSPP, STPP, SPG and SAPP at 0.8% and 1%. Except for SPG, each of these phosphates reduced *P. fragi* growth at 0.8% and 1.0%. A reduction in growth may have led to the decline in protease production. This contradicts the observation of Myhara and Skura (1989) who determined that enzyme production rate was inversely related with cell growth rate. In the current experiments, enzyme production generally paralleled growth. Therefore, a decrease in growth resulted in a decrease in enzyme production. Interestingly, SPG had a high amount of growth but enzyme production was completely inhibited.

The most effective inhibitor of protease production for the orthophosphates was MSP and for the short-chain polyphosphates it

was SAPP. It is possible that the acidic nature of these phosphates caused the denaturation of the protease. Additional experimentation needs to be done to determine the exact effects of phosphates on protease production.

The nutrient/skim milk agar plates were adequate in the gathering of preliminary data. However, there were limitations with this experimental technique in terms of determining protease production. To confirm protease production, the plates had to be flooded with a protein precipitating reagent, HCL (1%, v/v). For studying growth and proteolysis over time this method was not feasible for the large amount of treatments studied in this experiment. Therefore, protease production was confirmed at the end of the growth period (day 8). This presented some problems with several phosphates (TSPP, STPP, and SPG) because hydrolysis of casein was not observed until after HCL addition. Martley et al. (1970) explains that casein hydrolysis actually occurs but the first stage of casein hydrolysis, the formation of a white precipitous haze, can not be adequately distinguished from the rest of the medium.

Conclusions

Preliminary results indicate that the growth of *Pseudomonas fragi* can be reduced by all the phosphates tested, although the extent of inhibition will depend upon the concentration of the phosphate and the chain-length of the phosphate.

Protease production was completely inhibited by MSP, TSPP, STPP, SPG and SAPP at 0.8% and 1.0%. Protease production may be influenced by the pH or the chain-length of the phosphate..

Figure 2. Effects of orthophosphates on the growth of *Pseudomonas fragi*

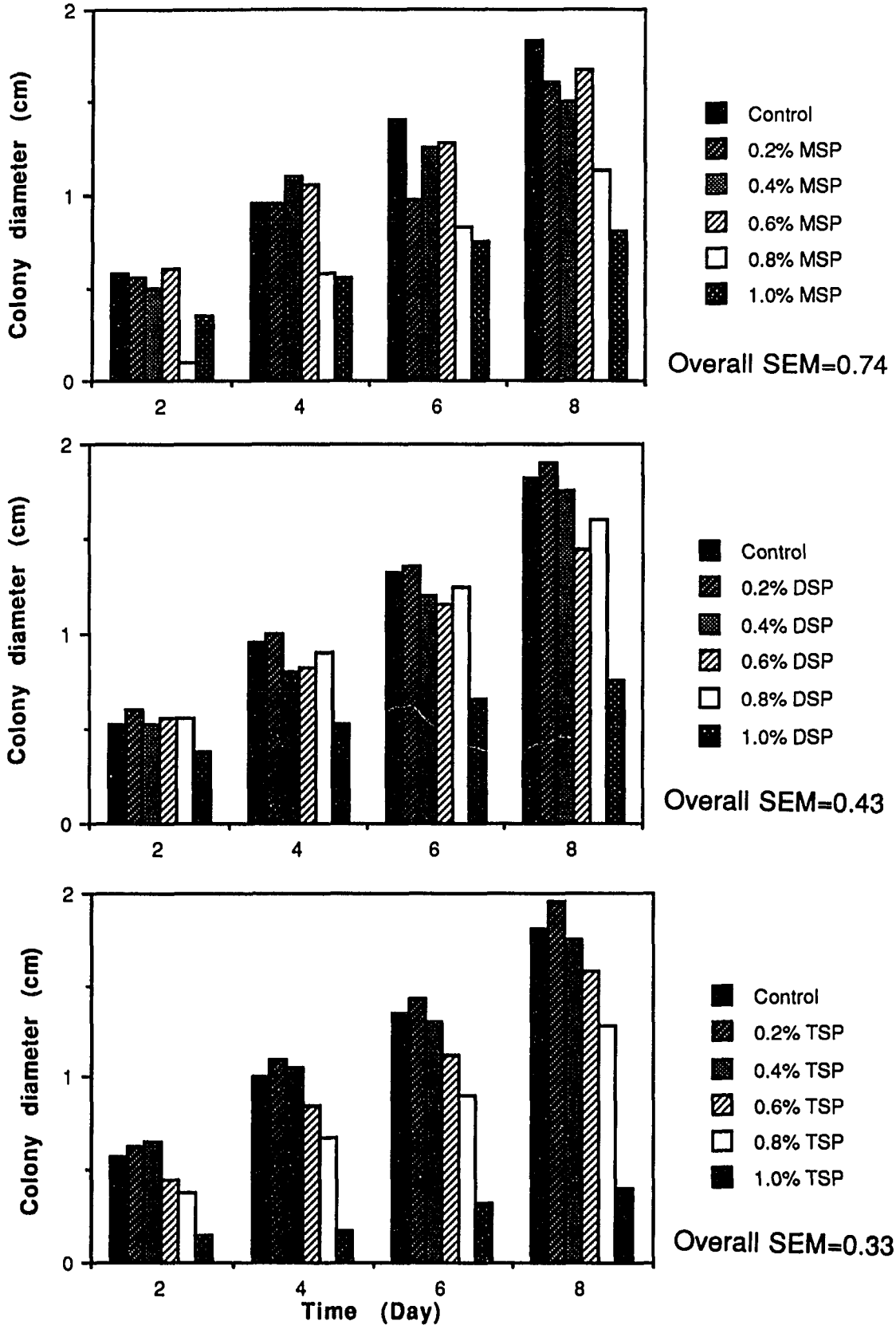

Colony diameter (cm)
2
1
0
2
4
6
8
Control
0.2% MSP
0.4% MSP
0.6% MSP
0.8% MSP
1.0% MSP
Overall SEM=0.74
Colony diameter (cm)
Control
0.2% DSP
0.4% DSP
0.6% DSP
0.8% DSP
1.0% DSP
Overall SEM=0.43
Colony diameter (cm)
Control
0.2% TSP
0.4% TSP
0.6% TSP
0.8% TSP
1.0% TSP
Overall SEM=0.33
Time (Day)

Figure 3. Effects of pyro- and polyphosphates on the growth of *Pseudomonas fragi*

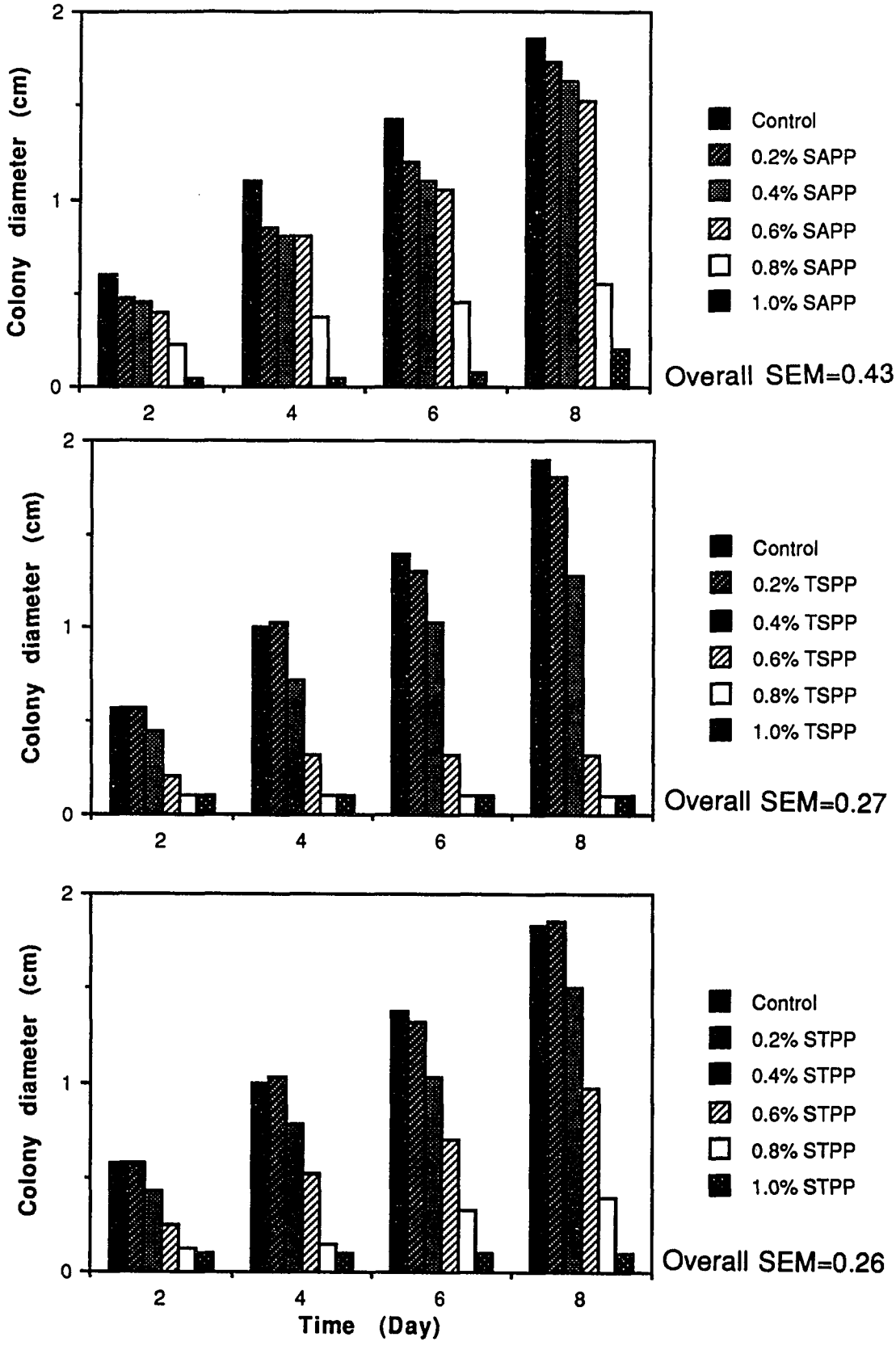
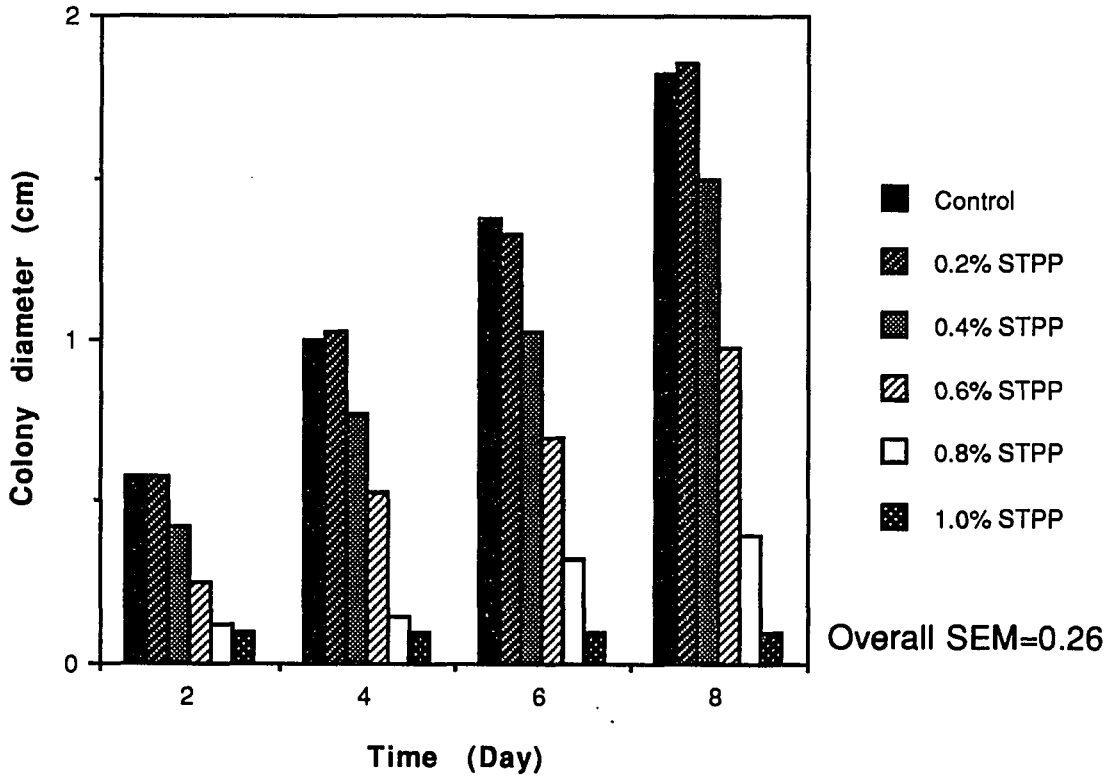

Colony diameter (cm)
2
1
0
2
4
6
8
Control
0.2% SAPP
0.4% SAPP
0.6% SAPP
0.8% SAPP
1.0% SAPP
Overall SEM=0.43
Colony diameter (cm)
Control
0.2% TSPP
0.4% TSPP
0.6% TSPP
0.8% TSPP
1.0% TSPP
Overall SEM=0.27
Colony diameter (cm)
Control
0.2% STPP
0.4% STPP
0.6% STPP
0.8% STPP
1.0% STPP
Overall SEM=0.26
Time (Day)

Figure 4. Effects of polyphosphates on the growth of *Pseudomonas fragi*


2
1
0
Colony diameter (cm)
2
4
6
8
Time (Day)
Control
0.2% STPP
0.4% STPP
0.6% STPP
0.8% STPP
1.0% STPP
Overall SEM=0.26

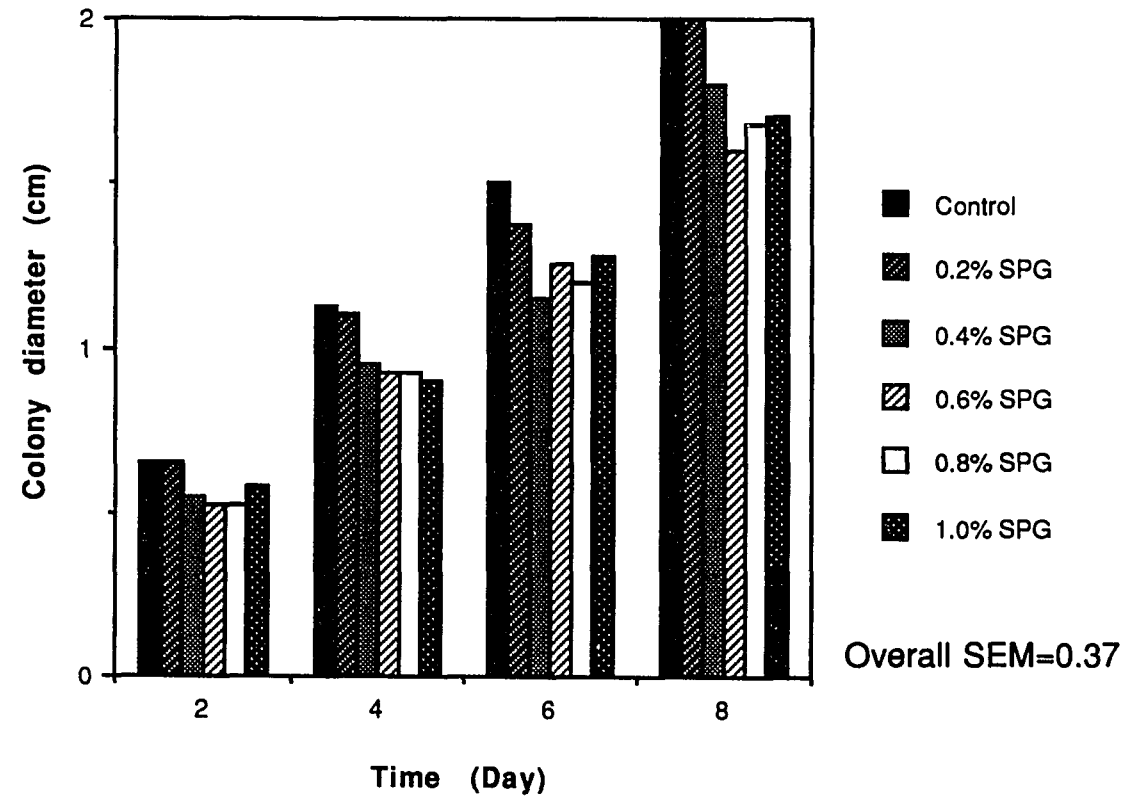

2
1
0
Colony diameter (cm)
2
4
6
8
Time (Day)
Control
0.2% SPG
0.4% SPG
0.6% SPG
0.8% SPG
1.0% SPG
Overall SEM=0.37

Figure 5. Effects of 1% phosphates on the growth of *Pseudomonas fragi*

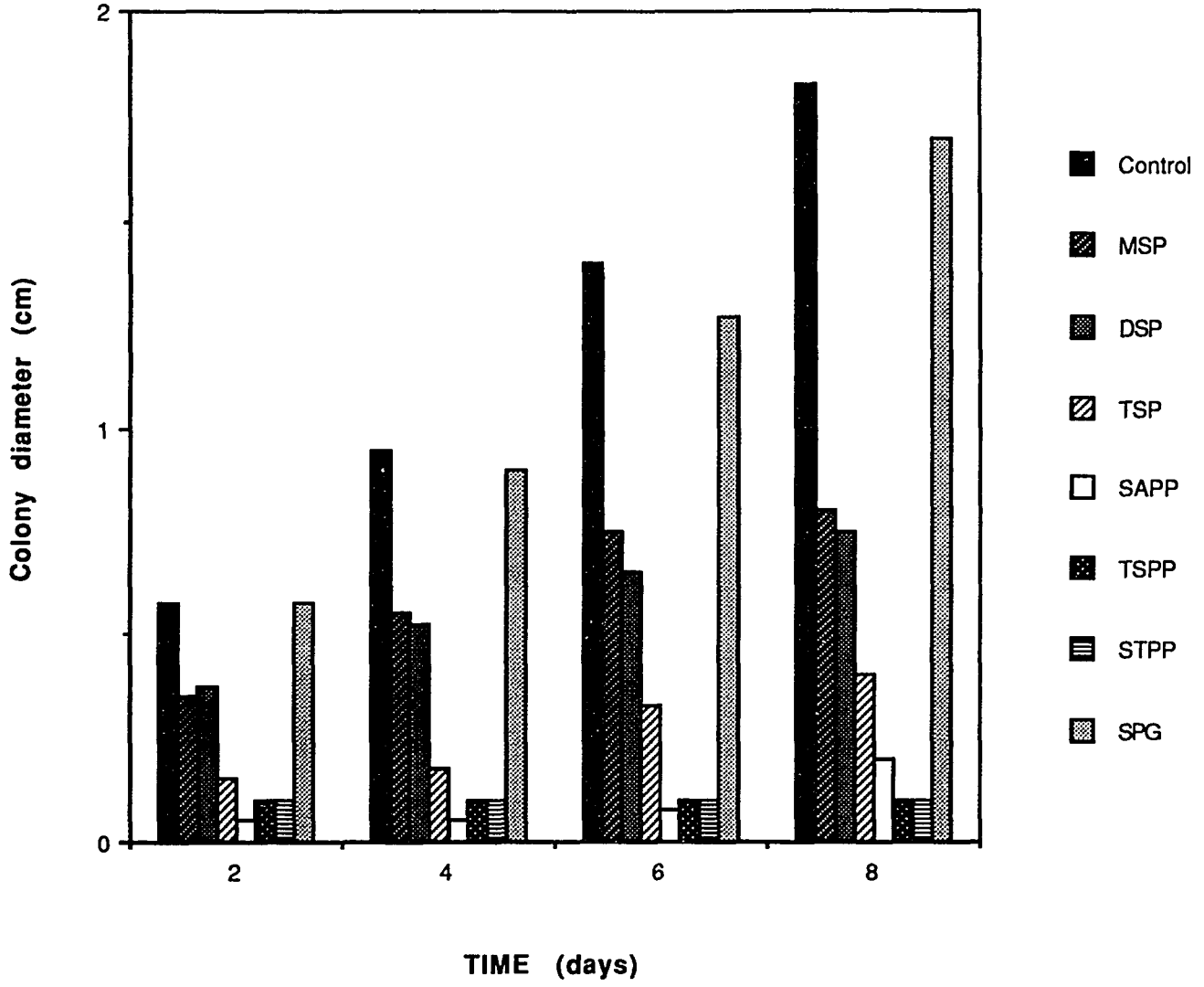
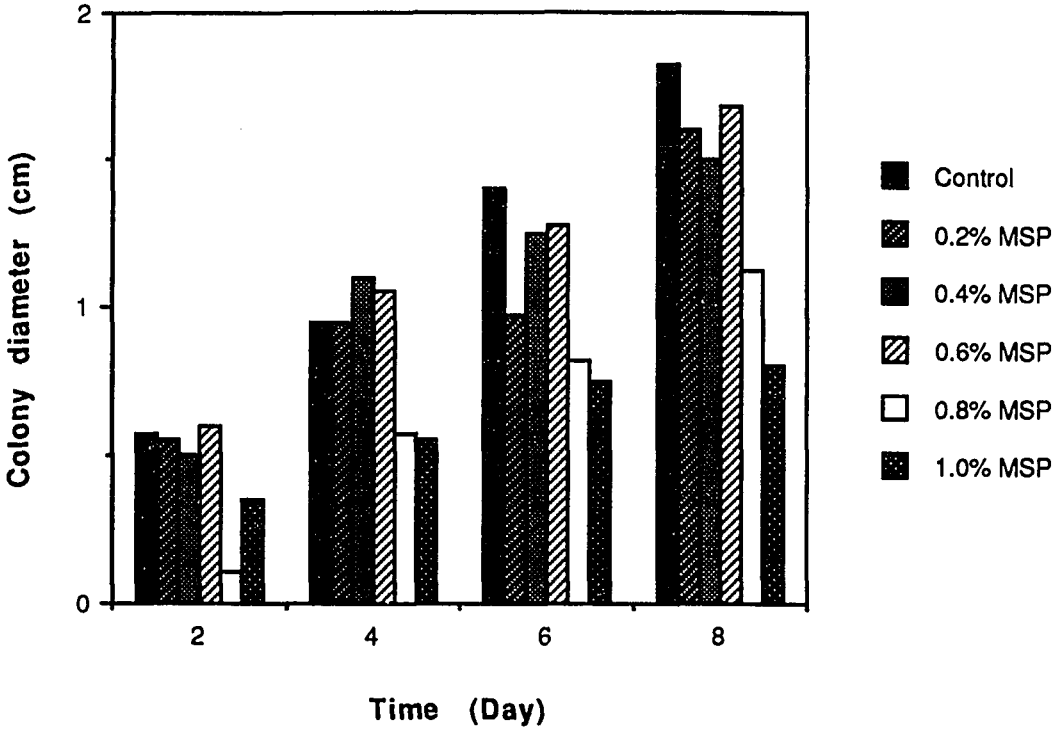

Colony diameter (cm)
2
1
0
2
4
6
8
TIME (days)
Control
MSP
DSP
TSP
SAPP
TSPP
STPP
SPG

Figure 6. Effects of MSP on the growth of *Pseudomonas fragi* (top) and the hydrolysis of casein (bottom)


Colony diameter (cm)
2
1
0
2
4
6
8
Time (Day)
Control
0.2% MSP
0.4% MSP
0.6% MSP
0.8% MSP
1.0% MSP

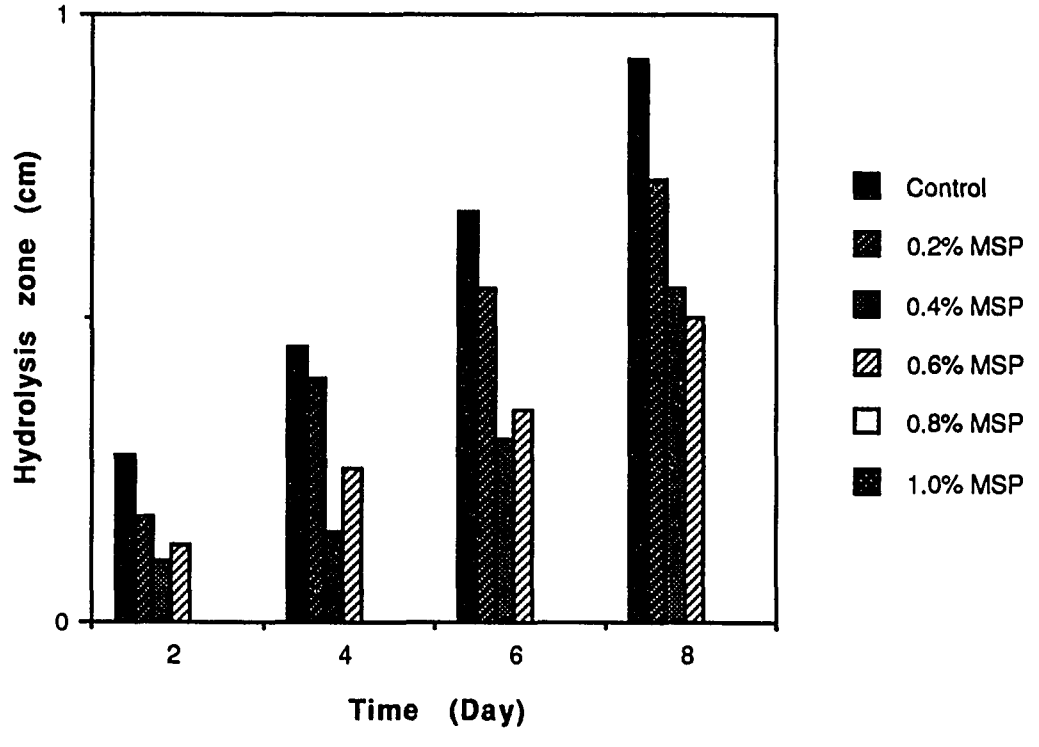
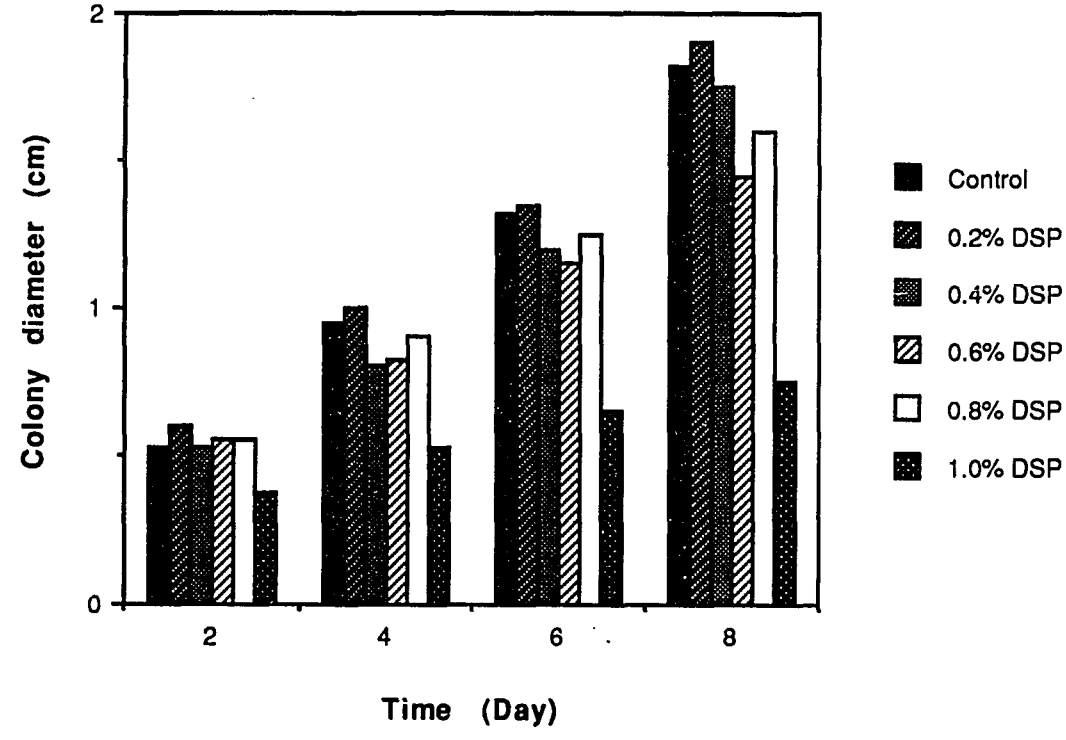

Hydrolysis zone (cm)
1
0
2
4
6
8
Time (Day)
Control
0.2% MSP
0.4% MSP
0.6% MSP
0.8% MSP
1.0% MSP

Figure 7. Effects of DSP on the growth of *Pseudomonas fragi* (top) and the hydrolysis of casein (bottom)


Colony diameter (cm)
2
1
0
2
4
6
8
Time (Day)
Control
0.2% DSP
0.4% DSP
0.6% DSP
0.8% DSP
1.0% DSP

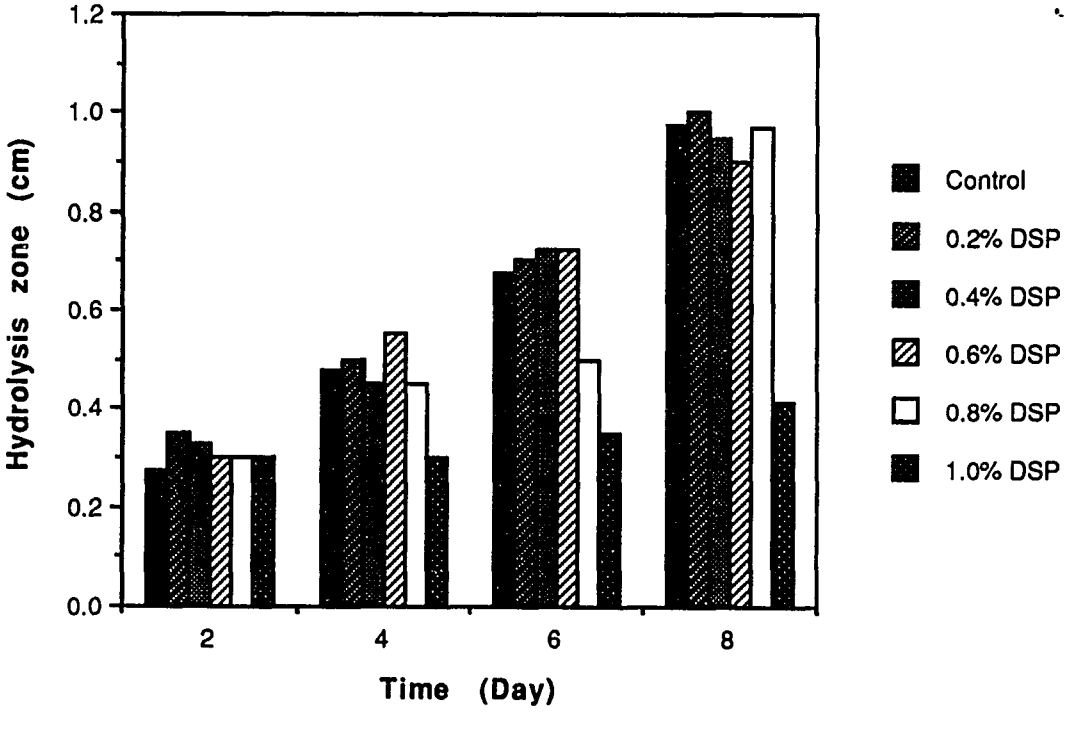
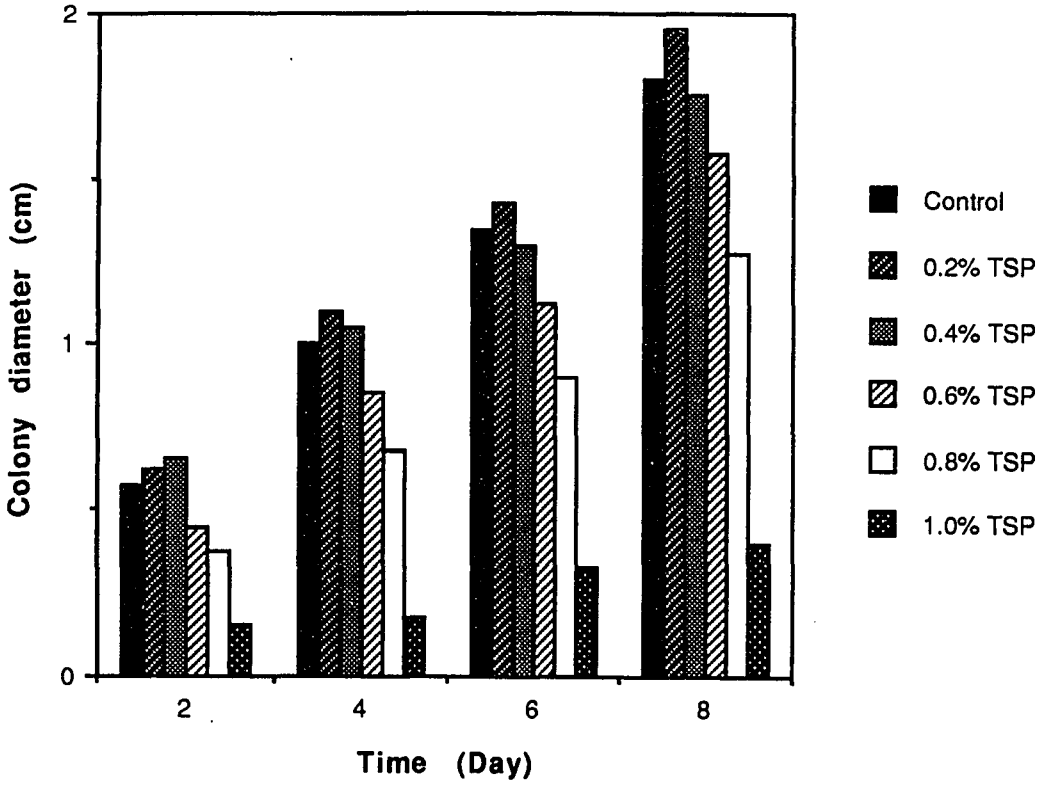

Hydrolysis zone (cm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
Time (Day)
Control
0.2% DSP
0.4% DSP
0.6% DSP
0.8% DSP
1.0% DSP

Figure 8. Effects of TSP on the growth of *Pseudomonas fragi* (top) and the hydrolysis of casein (bottom)


Colony diameter (cm)
2
1
0
2
4
6
8
Time (Day)
Control
0.2% TSP
0.4% TSP
0.6% TSP
0.8% TSP
1.0% TSP

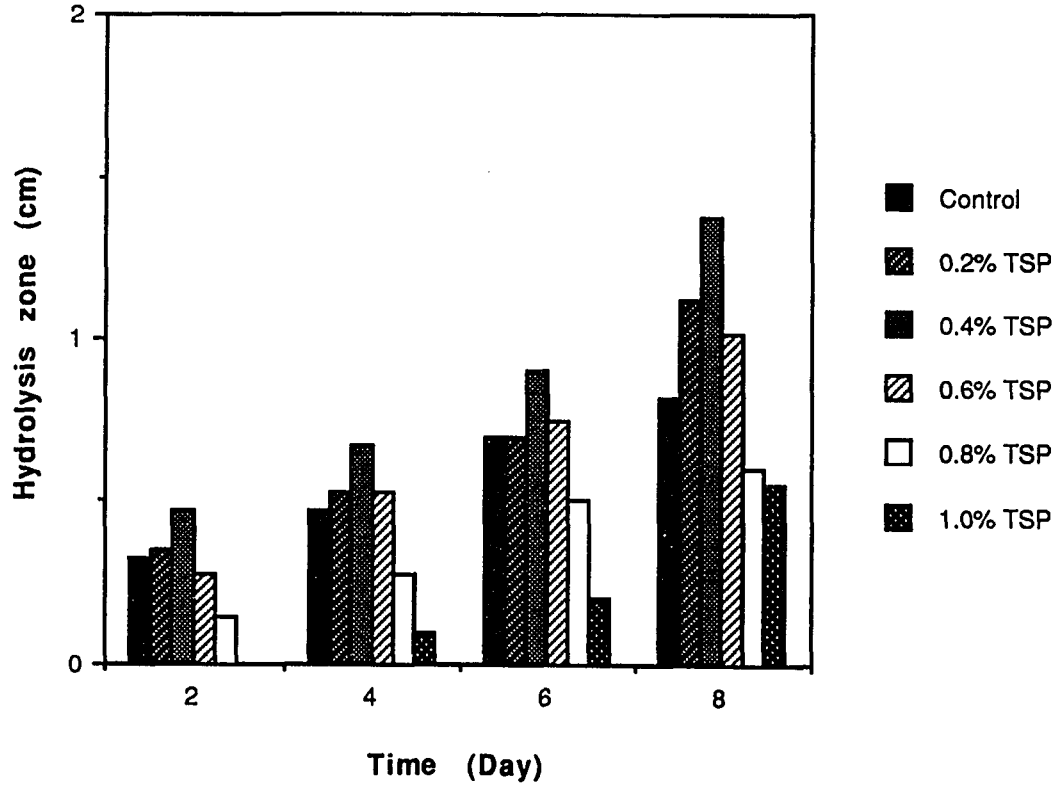
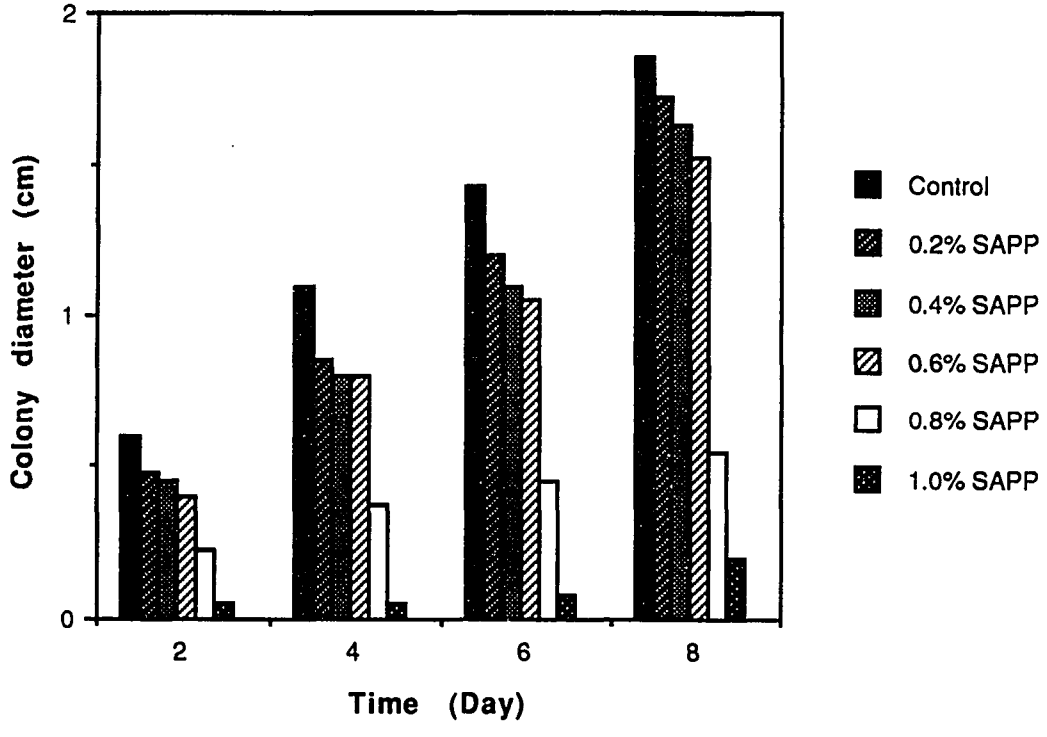
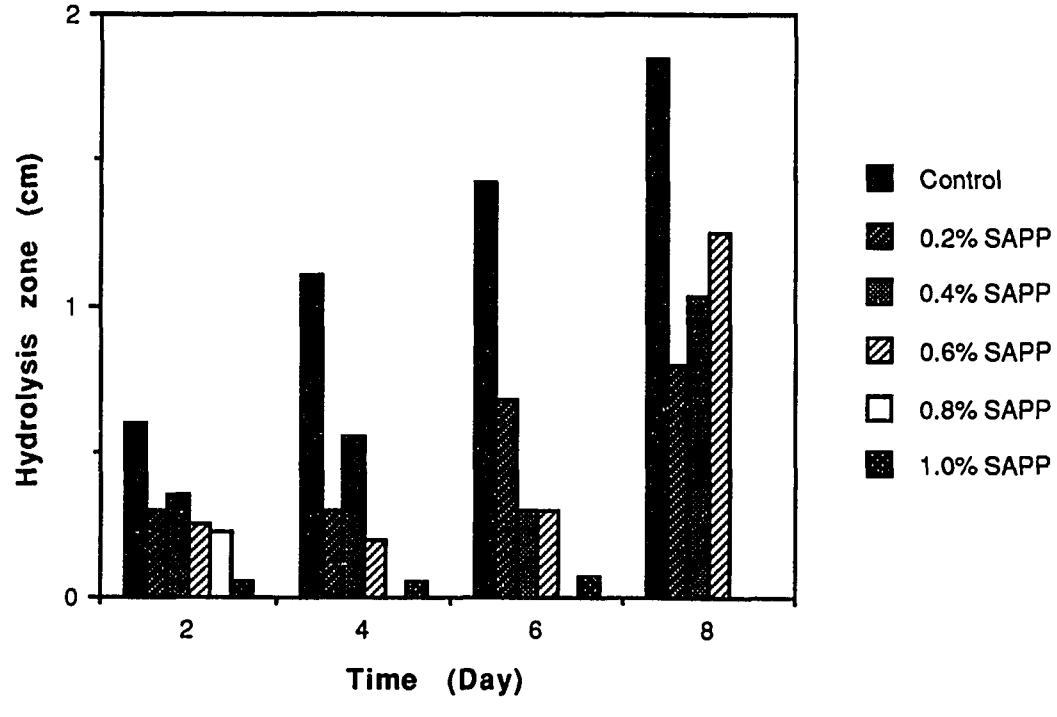

Hydrolysis zone (cm)
2
1
0
2
4
6
8
Time (Day)
Control
0.2% TSP
0.4% TSP
0.6% TSP
0.8% TSP
1.0% TSP

Figure 9. Effects of SAPP on the growth of *Pseudomonas fragi* (top) and the hydrolysis of casein (bottom)


Colony diameter (cm)
2
1
0
2
4
6
8
Time (Day)
Control
0.2% SAPP
0.4% SAPP
0.6% SAPP
0.8% SAPP
1.0% SAPP


Hydrolysis zone (cm)
2
1
0
2
4
6
8
Time (Day)
Control
0.2% SAPP
0.4% SAPP
0.6% SAPP
0.8% SAPP
1.0% SAPP

EXPERIMENT II

Materials and Methods

The purpose of this experiment was to characterize the antimicrobial effects of phosphates in liquid media. The minimal inhibitory concentration (MIC) was determined in synthetic broth medium using the kill curve method of Schoenknecht et al. (1985). The MIC is the lowest concentration without growth using serial dilutions. Seven different phosphates were used at two different pH values (pH 5.5 and 7.0). This experiment looked at the inhibitory effects of phosphates as influenced by pH in a liquid medium. Trypticase soy broth (TSB) was used to simulate a meat system because of its high protein content.

Inoculum

A subculture of *Pseudomonas fragi* ATCC 4973 was streaked onto nutrient agar (Difco Laboratories) and incubated overnight at 25° C. A tube containing 3 ml of TSB (BBL) was inoculated with the above mentioned culture and allowed to incubate overnight at 25° C. An aliquot of 0.1 ml was transferred to 10 ml of TSB (BBL) and incubated for 5-6 hours at 25° C on a rotary shaker (Laboratory Rotator-Model G-2, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) until turbid. The inoculum was standardized to equal a 0.5 McFarland turbidity standard (ca 5 x 10^8 organism per ml).

Test chemicals

The following phosphates were used: MSP, DSP, and TSP (Fisher Scientific), SAPP (Stauffer Chemical Co.), TSPP and STPP (Monsanto), and SPG (BK-Ladenburg). Phosphate stock solutions of 8% (TSPP, SAPP, STPP), 16% (DSP, TSP) or 32% (MSP, SPG) (w/v) were prepared by adding the appropriate amounts of selected phosphate to distilled water. The solutions were filter sterilized through a 0.22μ Cameo IV filter unit (Micron Separations, Inc.).

Media

TSB (BBL) was adjusted to pH 5.5 or 7.0 (4 N NaOH or 1 N HCL) using a Zeromatic SS-R pH meter (Beckman, Inc., Fullerton, CA). The solutions were sterilized and dispensed in 2 ml aliquots. Two-fold dilutions of the phosphate solutions were made by adding 2 ml phosphate stock solution to 2 ml of the pH adjusted TSB (pH 5.5 or 7.0). The pH of the resulting phosphate solutions can be found in Table 4. The *P. fragi* inoculum (0.1 ml) was added to the medium, tubes were inverted for adequate mixing and samples were taken at 0, 4, 8, 24, and 48 hours for bacterial count enumeration. Appropriate serial dilutions, followed by pour-plating with TSA (BBL), were made by following standard methods. The plates were incubated for 24 hours at 25° C.

Table 4. The final pH values of trypticase soy broth after phosphate addition

Phosphate	Final Percentage	TSB adjusted to pH 5.5	TSB adjusted to pH 7.0
MSP	8	5.3	5.2
	16	4.9	5.0
DSP	4	8.4	8.3
	8	8.6	8.7
TSP	1	11.4	11.2
	2	11.5	11.4
SAPP	2	5.6	5.6
	4	5.1	5.3
TSPP	2	9.0	8.8
	4	9.1	9.2
STPP	2	8.7	8.6
	4	8.7	8.7
SPG	8	6.5	6.5
	16	6.3	6.4
UP	0.25	3.7	4.1
	0.5	3.6	4.0
EDTA	0.5	5.2	5.7
	1	5.3	5.9

Results and Discussion

The kill curve method assessed the killing effect of phosphates on the growth of *Pseudomonas fragi*.

Two additional treatments were added in this experiment. Ultraphosphate (UP) was used because its antimicrobial properties had never been tested. It is a highly branched experimental polyphosphate which is not available as a food ingredient. EDTA was used for the comparison of phosphates to a known chelating compound. This was important to include because metal chelation is the favored mechanism for inhibition of microorganisms by polyphosphates (Knabel et al., 1990; Zessin and Shelef, 1988; Elliot et al., 1964).

Initially, the objectives of this experiment were 1) to determine the MIC's of phosphate in a liquid medium, 2) to find the stage of growth where *P. fragi* was most affected by phosphates, and 3) to determine the effect of pH on the antimicrobial properties of phosphates. The pH of the media was adjusted to pH 5.5 and 7.0 prior to phosphate addition. This was done to simulate the addition of phosphates to a meat system.

The initial starting inoculum concentration was 10^8 organisms per ml. The bacterium counts at time zero exhibited some variability immediately after the phosphate-treated medium was inoculated. This is attributed to environmental shock.

Minimum inhibitory concentration

The MIC's could not be determined since growth occurred at the highest phosphate concentrations tested, after 24 hours. Twenty-four hours is the time normally used to calculate the MIC's.

All phosphates (Figures 10-16, 19, 20) were bacteriostatic to *P. fragi*. In addition, at the concentrations tested, most of the phosphates were bactericidal. The phosphates that showed only bacteriostatic and not bactericidal effects included: 8% and 16% MSP (Figure 10), 8% DSP (pH 8.7) (Figure 11), 1% and 2% TSP, except 2% TSP (pH 11.4) (Figure 14), 2% TSPP (pH 9.0) (Figure 14), 0.25% UP (pH 3.7, 4.1) and 0.5% UP (pH 4.0) (Figure 19).

Of the phosphates tested at 2% (Figures 17), no one phosphate consistently reduced *P. fragi* growth. Two percent TSP (pH 11.4) killed *P. fragi*, however, 2% TSP (pH 11.5) did not remain bactericidal after 24 hours. This may have been due to the selection of resistant *P. fragi*. Comparing MSP, DSP and SPG at 8% (Figures 18), MSP was consistently bacteriostatic. However, 8% DSP (pH 8.6) and 8% and 16% SPG were bactericidal after 24 hours.

At 0.25% and 0.5%, except for 0.5% (pH 3.7), UP (Figure 19) was initially better at reducing the growth of *P. fragi* up to 24 hours after inoculation when compared to EDTA (Figure 20) at the same concentrations.

Effects of phosphate concentration were observed with MSP (Figure 10), TSP (Figure 12), SAPP (Figure 13) and TSPP (Figure 14) after 48 hours of growth. SPG (Figure 16) showed early concentration effects, up to 24 hours, however they did not last to 48 hours.

Growth

All treatments were inoculated with a culture of *P. fragi* in mid-logarithmic growth phase. This can be seen in the inoculated controls (pH 5.5 and 7.0) of TSB in the absence of phosphates. Late logarithmic-early stationary phase was shown to occur between 8-16 hours after which time full stationary phase began.

The growth of *P. fragi* was affected by all the phosphate treatments at the logarithmic phase, which was either shortened or absent. Stationary phase was observed in SAPP (Figure 13) after initial inoculation of *P. fragi*. However, after 24 hours, death phase commenced. The onset of death phase occurred within 4 hours in the presence of MSP, TSPP, and STPP, after 8 hours with SPG, and at 24 hours with DSP (pH 5.5) and SAPP. Recovery of *P. fragi* growth occurred with UP and TSP.

pH

Although the pH was initially adjusted to 5.5 or 7.0 to simulate a model meat system, the addition of the phosphates altered the pH of the medium (Table 3). The final pH of the solution was directly related to the individual phosphate added to the medium. No one pH was consistently more inhibitory than another pH. This can be seen in Figures 19 and 20 where, at a single percentage, an acidic to basic pH range was achieved by a variety of phosphates yet no pH value was consistently detrimental to the growth of *P. fragi*. Additionally, although MSP (Figure 10) and SAPP (Figure 13) exhibit a pH effect,

other treatments (UP and EDTA) under acidic conditions do not show the same effect . Therefore, pH alone can not explain the differences in the antimicrobial abilities of the various phosphates.

It is possible that after a period of time a pH effect may not be observed. This is because over time the pH can change from one value which shows a pH effect to another pH value that does not show an effect. A change in pH away from the initial pH may be due to bacterial metabolic products secreted into the environment. This possibility was not tested as the pH of the medium was not followed throughout the time course of the experiment. However, early time points, such as after 10 hours, where the accumulation of metabolic products may not be a factor showed pH effects with MSP, SAPP and UP. Thus, UP may show a pH effect that was not observed.

Conclusions

Growth of *Pseudomonas fragi* was bacteriostatic in the presence of all phosphates tested and in most instances, the phosphates were bactericidal. Phosphates affected the logarithmic phase of *P. fragi* which was either shortened or absent. Some phosphates (MSP, TSP, SAPP, TSPP, and SPG) exhibited concentration effects on the growth of *P. fragi.* Additionally, MSP and SAPP showed a pH effect although it was concluded that pH alone could not explain the differences in the antimicrobial abilities of the various phosphates. Overall, no one pH was consistently more inhibitory than another pH.

Figure 10. Effects of MSP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.69)

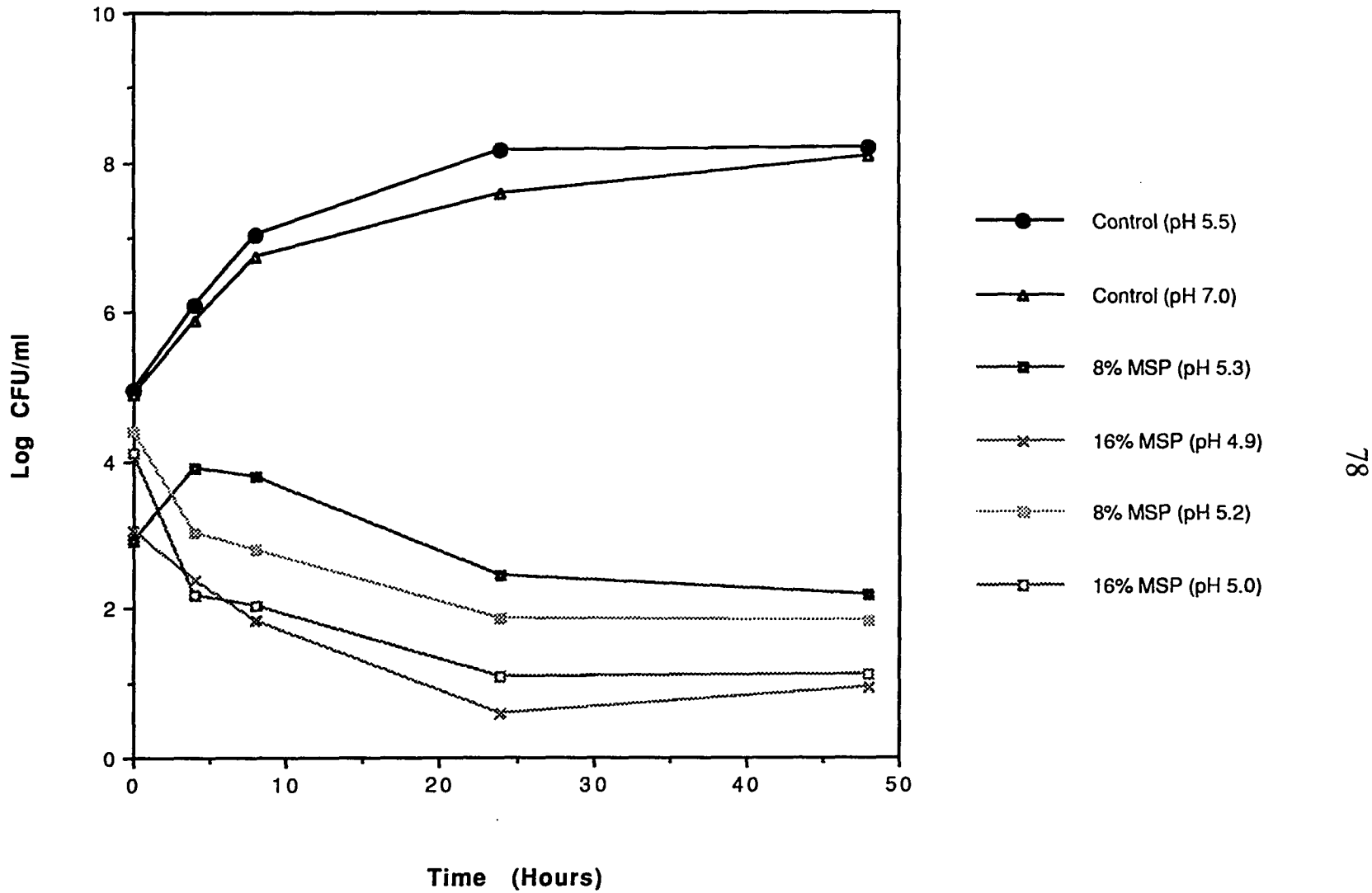

Log CFU/ml
10
8
6
4
2
0
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
8% MSP (pH 5.3)
16% MSP (pH 4.9)
8% MSP (pH 5.2)
16% MSP (pH 5.0)

Figure 11. Effects of DSP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.89)

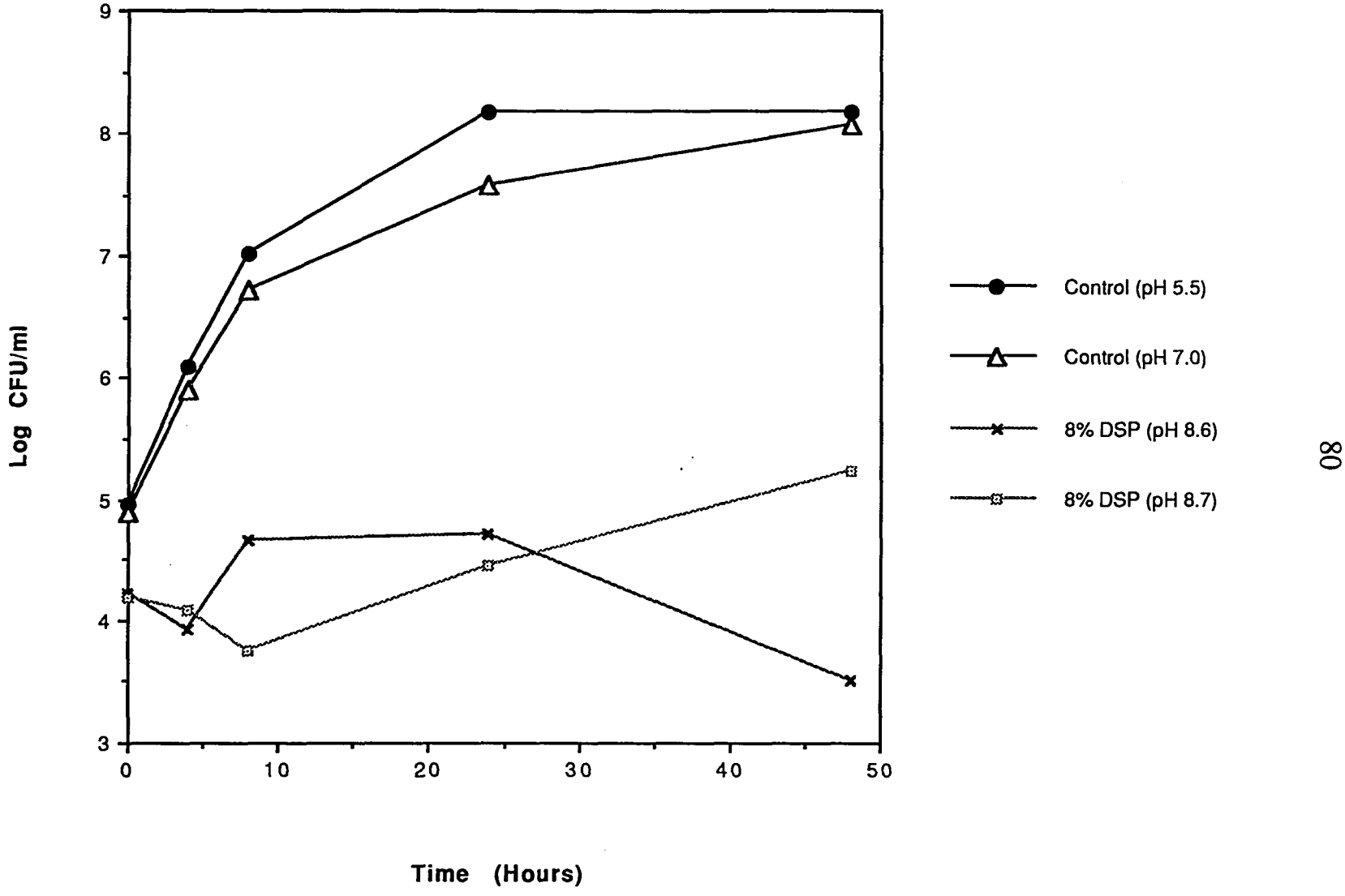

Log CFU/ml
9
8
7
6
5
4
3
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
8% DSP (pH 8.6)
8% DSP (pH 8.7)

Figure 12. Effects of TSP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.50)

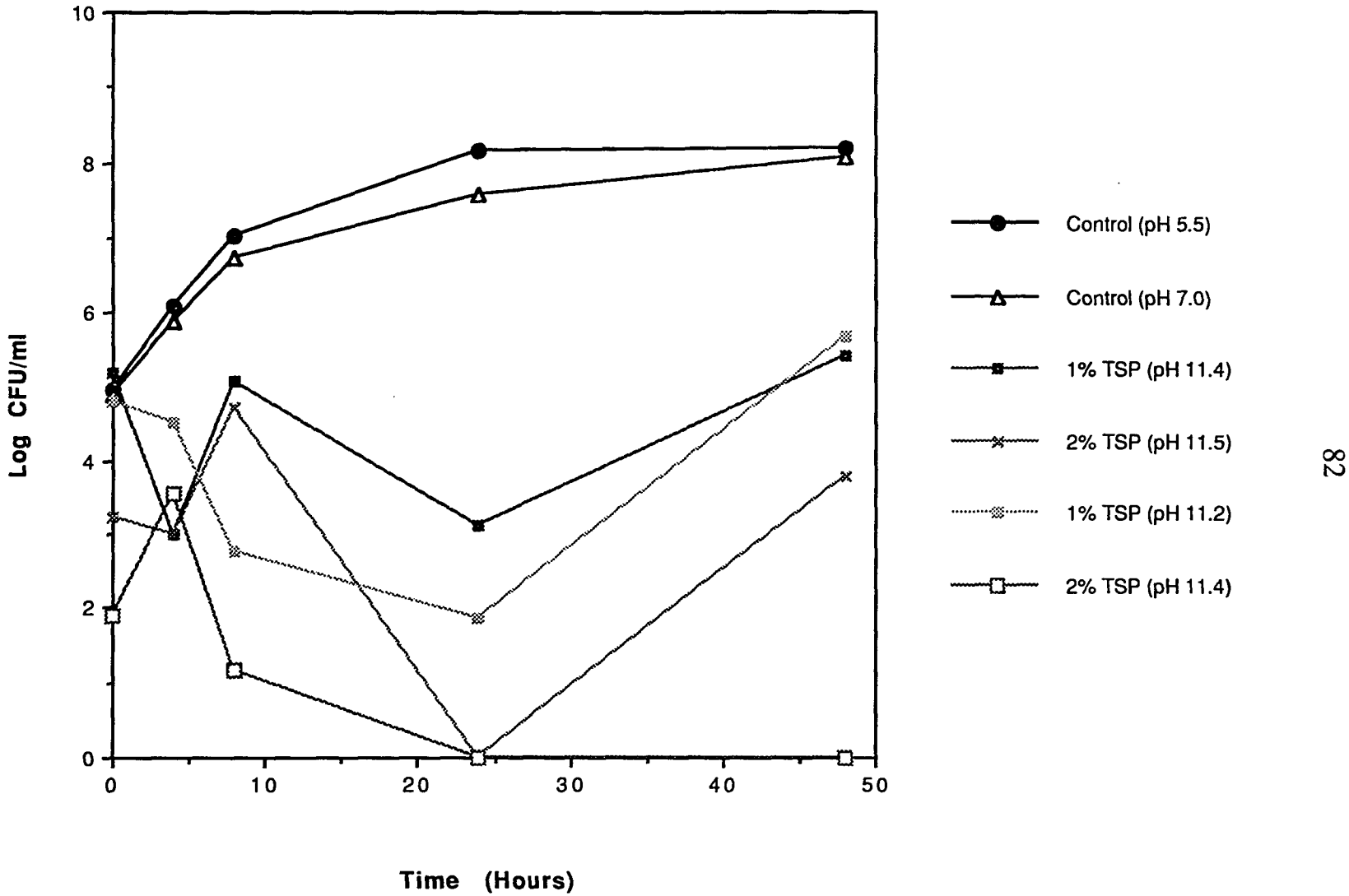

Log CFU/ml
10
8
6
4
2
0
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
1% TSP (pH 11.4)
2% TSP (pH 11.5)
1% TSP (pH 11.2)
2% TSP (pH 11.4)

Figure 13. Effects of SAPP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.53)

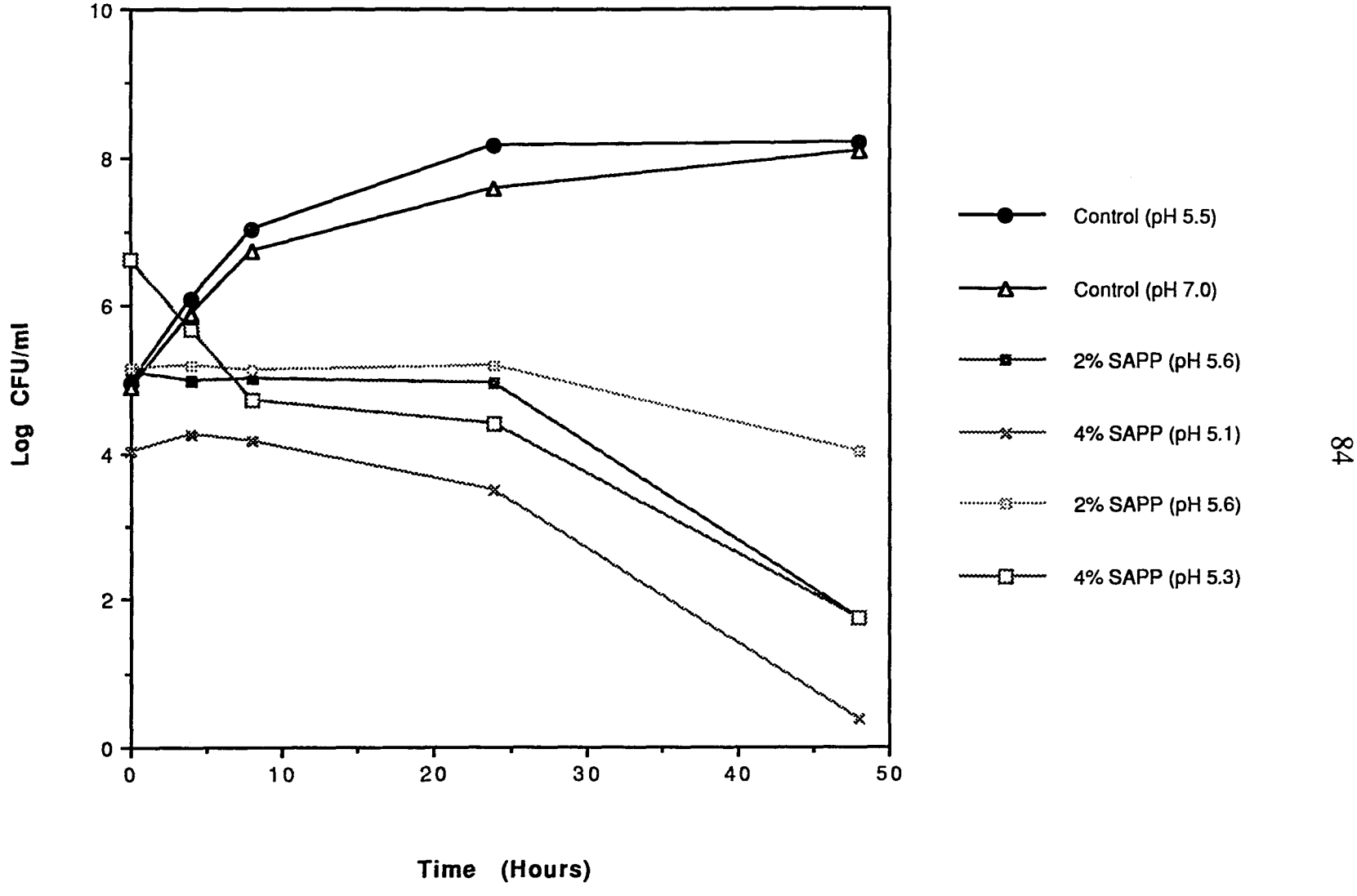

Log CFU/ml
10
8
6
4
2
0
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
2% SAPP (pH 5.6)
4% SAPP (pH 5.1)
2% SAPP (pH 5.6)
4% SAPP (pH 5.3)

Figure 14. Effects of TSPP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.74)

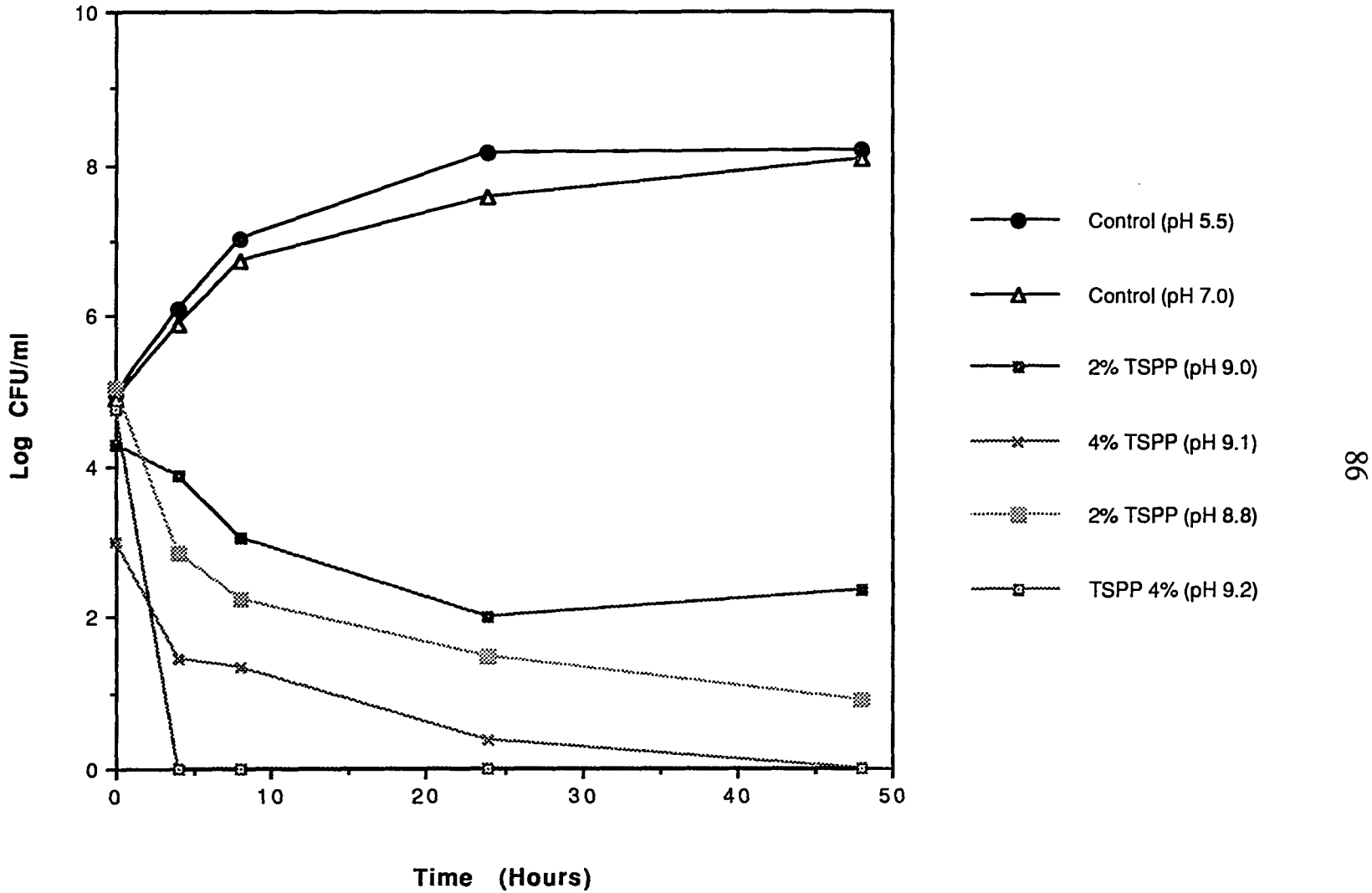

10
8
6
4
2
0
Log CFU/ml
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
2% TSPP (pH 9.0)
4% TSPP (pH 9.1)
2% TSPP (pH 8.8)
TSPP 4% (pH 9.2)

Figure 15. Effects of STPP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.48)

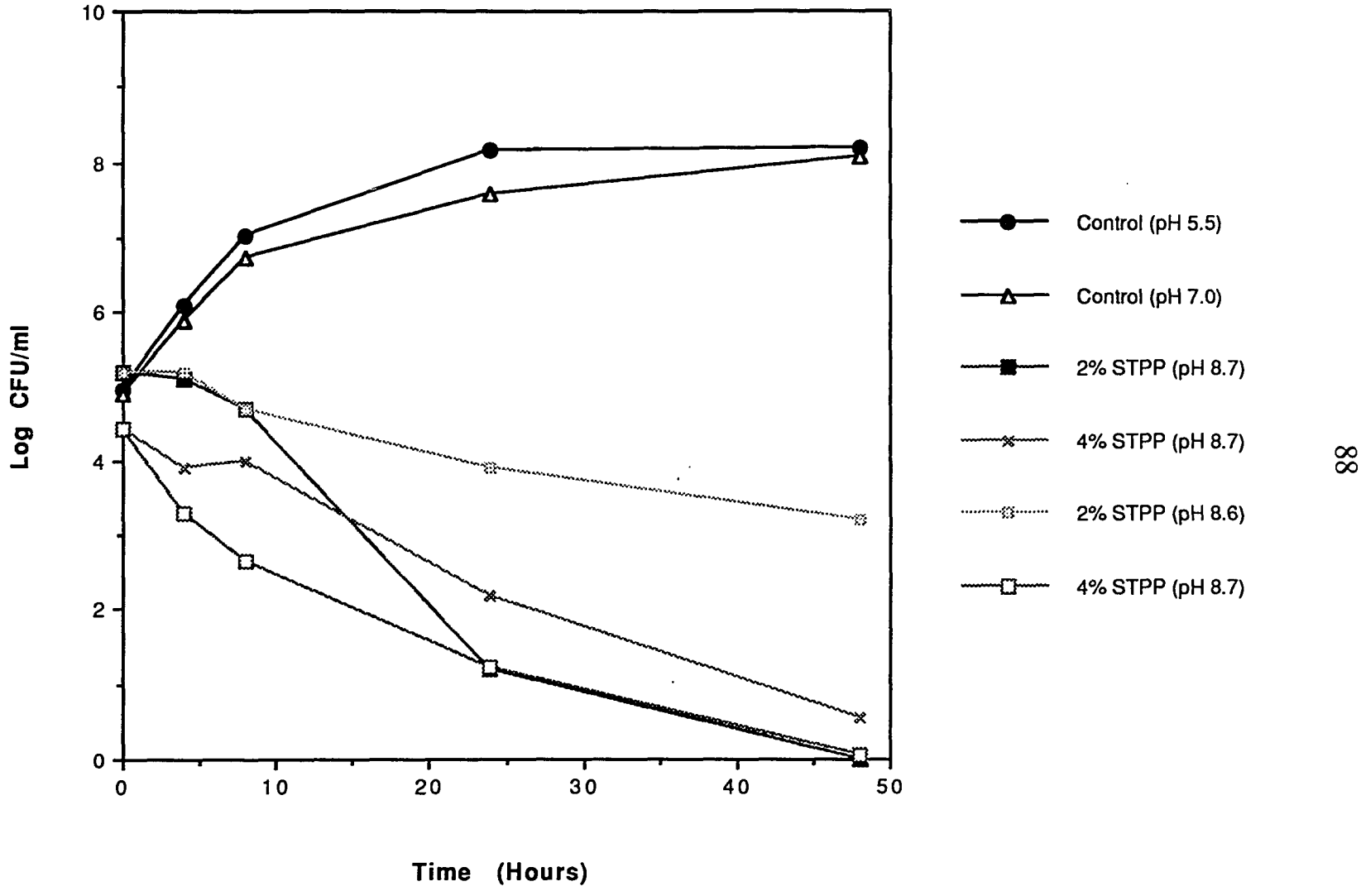

Log CFU/ml
10
8
6
4
2
0
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
2% STPP (pH 8.7)
4% STPP (pH 8.7)
2% STPP (pH 8.6)
4% STPP (pH 8.7)

Figure 16. Effects of SPG on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.54)

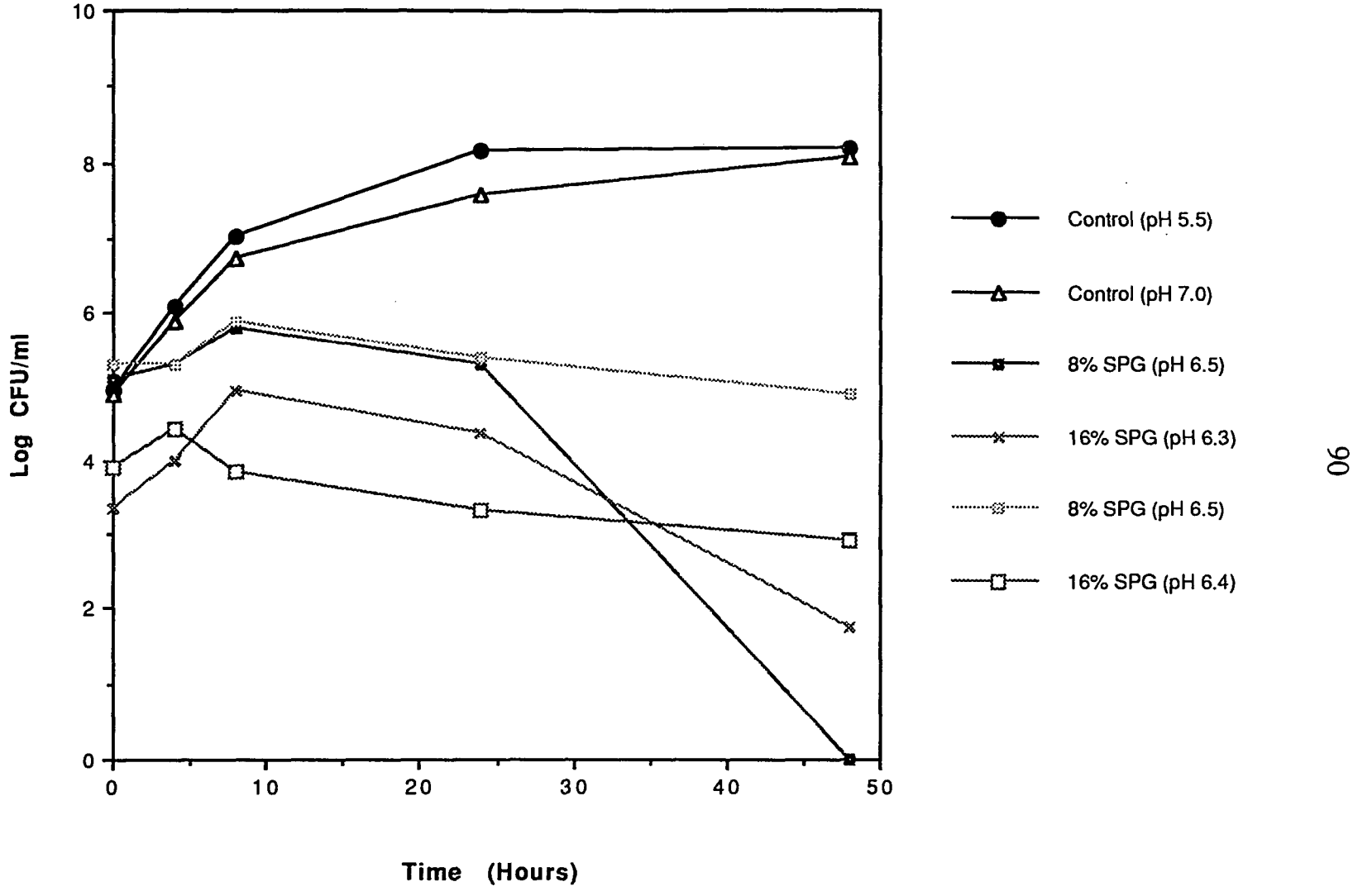

10
8
6
4
2
0
Log CFU/ml
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
8% SPG (pH 6.5)
16% SPG (pH 6.3)
8% SPG (pH 6.5)
16% SPG (pH 6.4)

Figure 17. Effects of 2% SAPP, TSPP and STPP on the growth of *Pseudomonas fragi* using the kill curve method

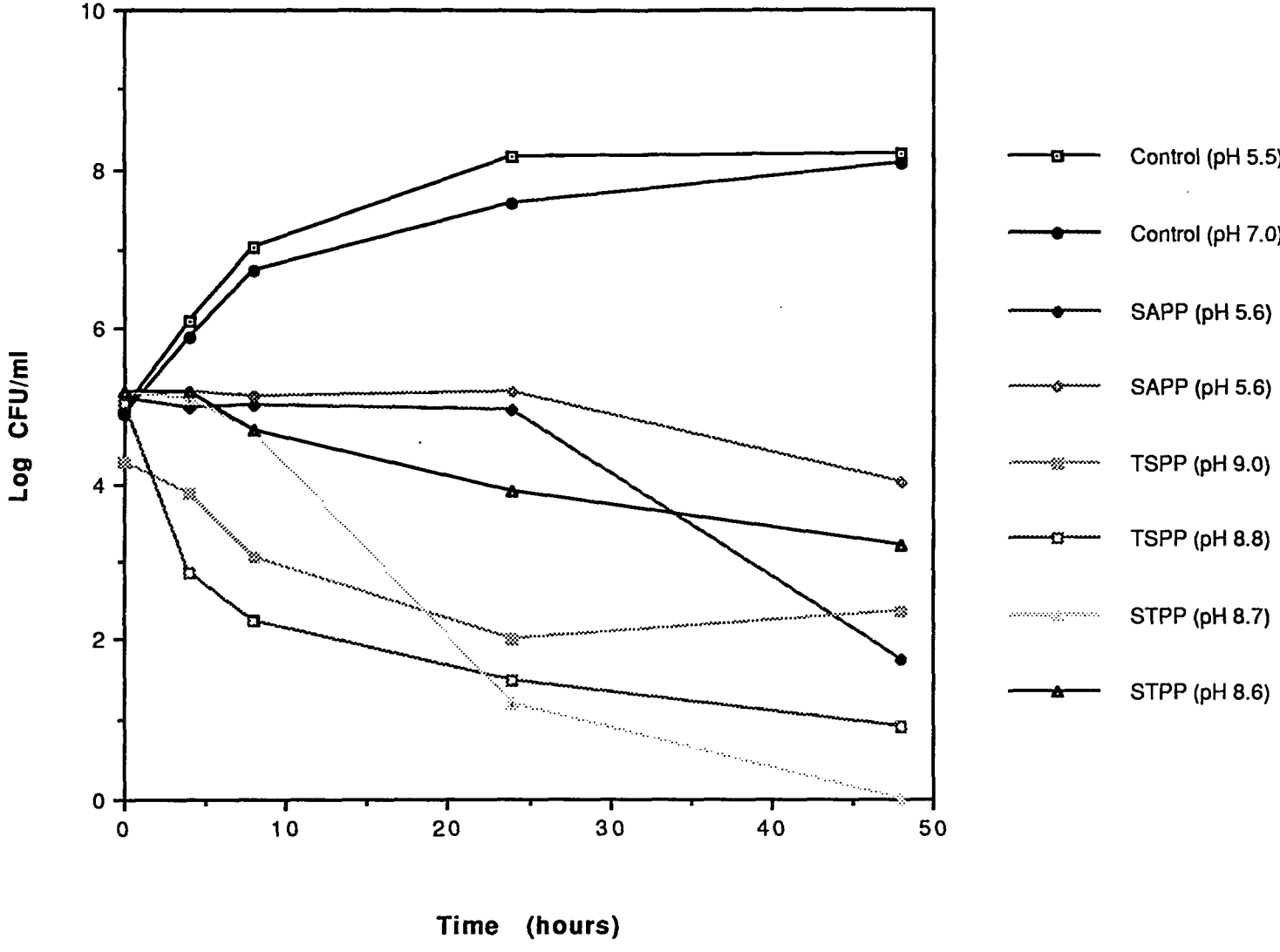

Log CFU/ml
10
8
6
4
2
0
0
10
20
30
40
50
Time (hours)
Control (pH 5.5)
Control (pH 7.0)
SAPP (pH 5.6)
SAPP (pH 5.6)
TSPP (pH 9.0)
TSPP (pH 8.8)
STPP (pH 8.7)
STPP (pH 8.6)

Figure 18 Effects of 8% MSP, DSP and SPG on the growth of *Pseudomonas fragi* using the kill curve method

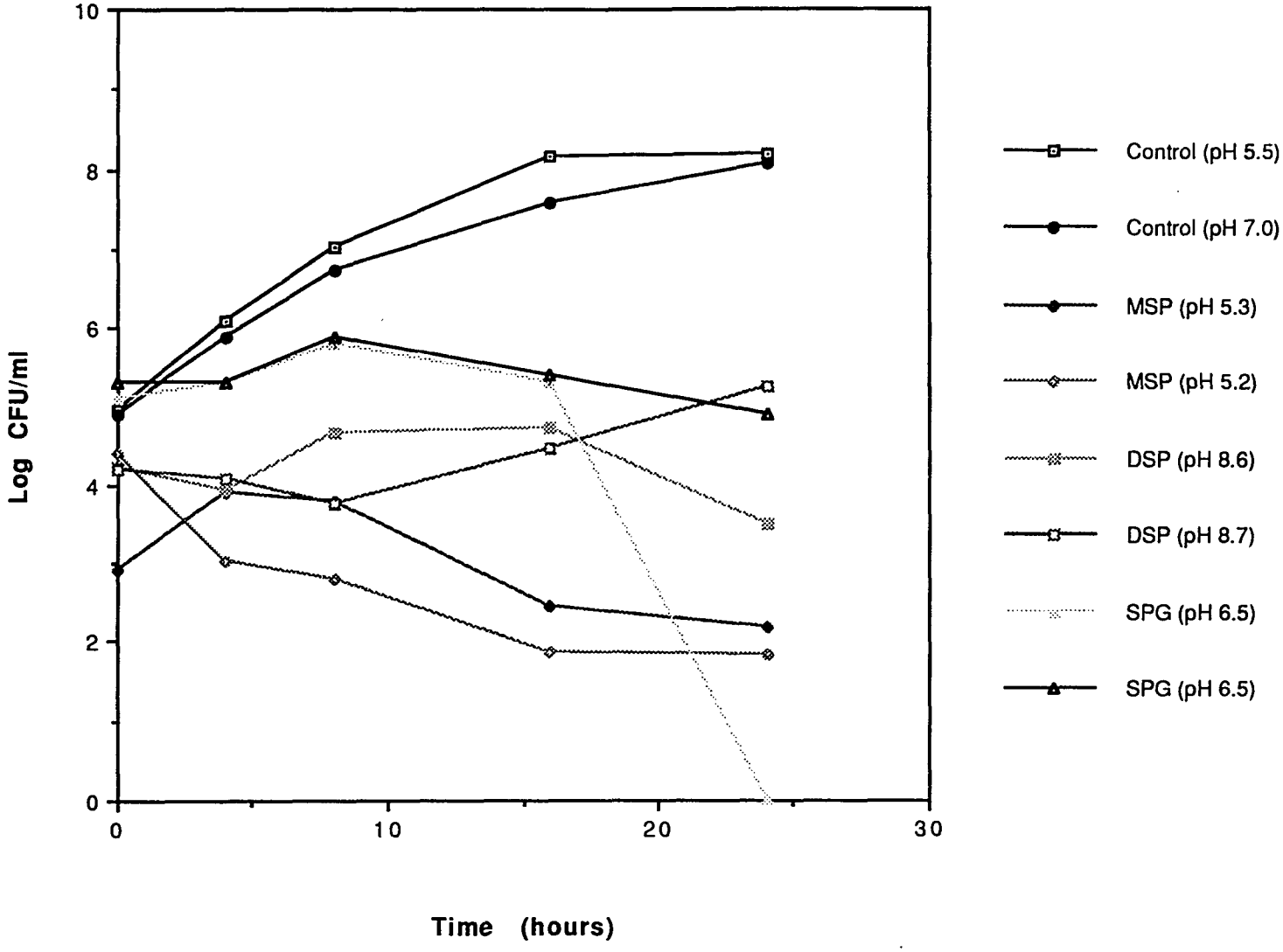

Log CFU/ml
10
8
6
4
2
0
0
10
20
30
Time (hours)
Control (pH 5.5)
Control (pH 7.0)
MSP (pH 5.3)
MSP (pH 5.2)
DSP (pH 8.6)
DSP (pH 8.7)
SPG (pH 6.5)
SPG (pH 6.5)

Figure 19. Effects of UP on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.19)

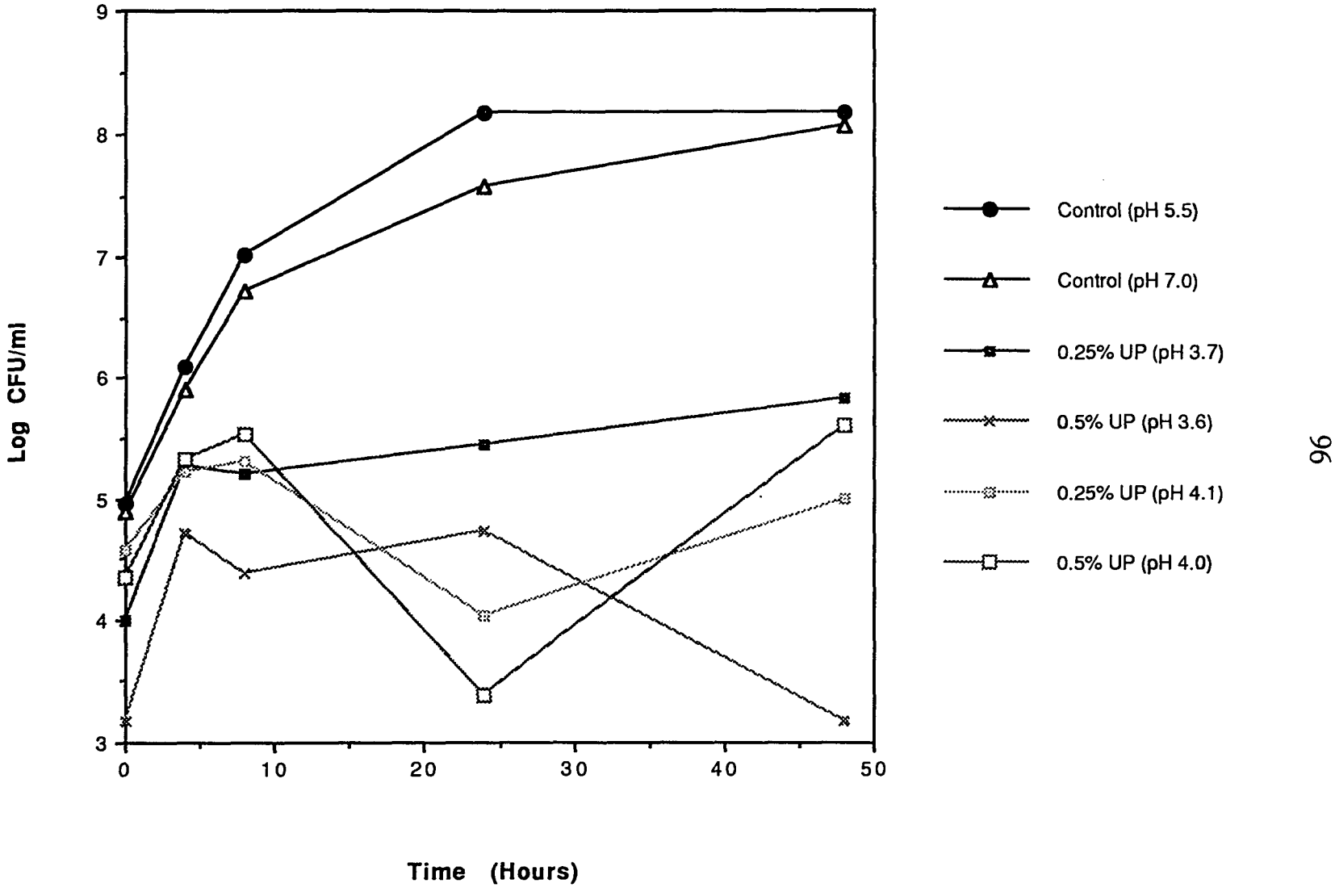
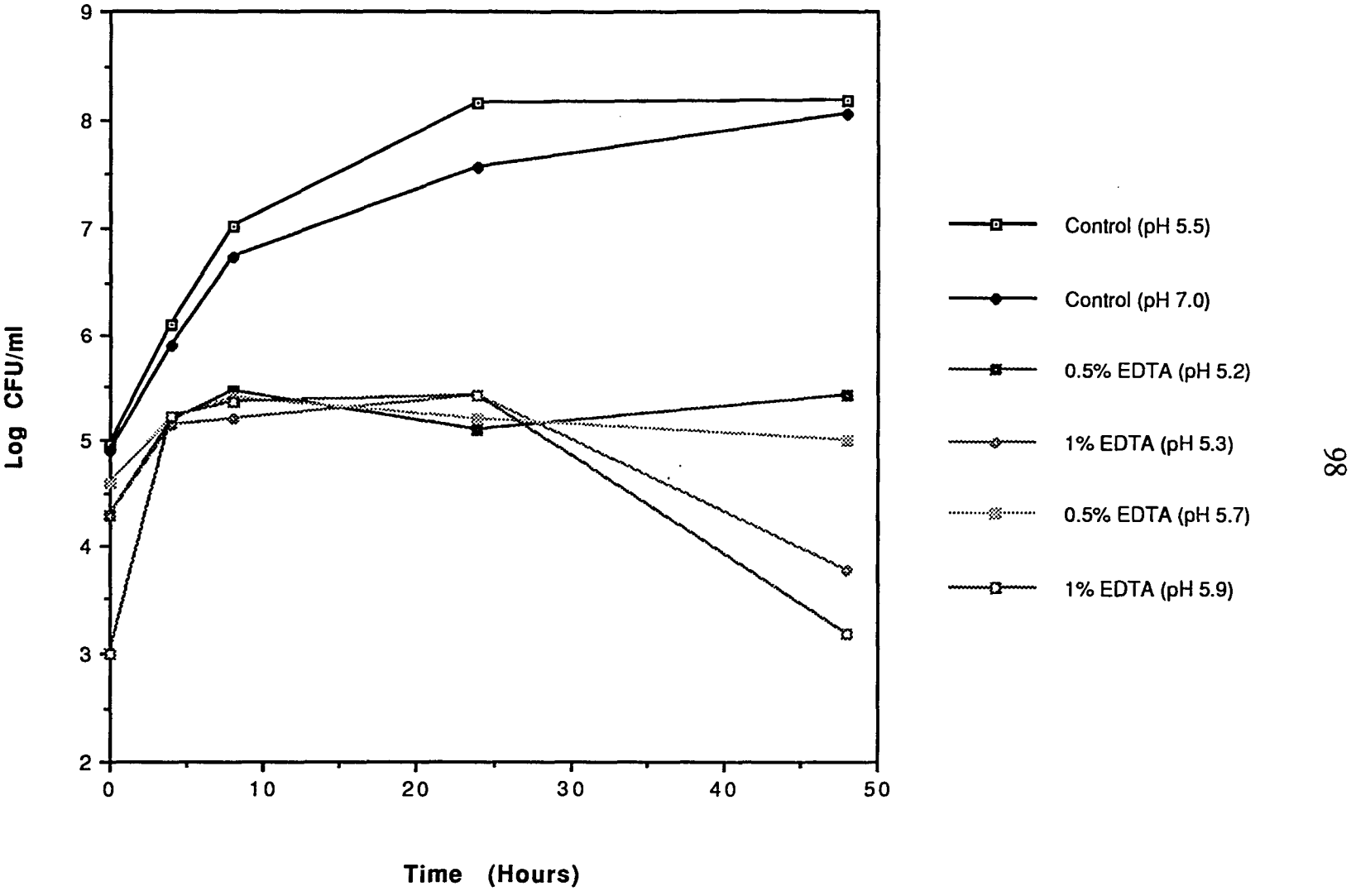

Log CFU/ml
9
8
7
6
5
4
3
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
0.25% UP (pH 3.7)
0.5% UP (pH 3.6)
0.25% UP (pH 4.1)
0.5% UP (pH 4.0)

Figure 20. Effects of EDTA on the growth of *Pseudomonas fragi* using the kill curve method (Overall SEM=0.19)


Log CFU/ml
9
8
7
6
5
4
3
2
0
10
20
30
40
50
Time (Hours)
Control (pH 5.5)
Control (pH 7.0)
0.5% EDTA (pH 5.2)
1% EDTA (pH 5.3)
0.5% EDTA (pH 5.7)
1% EDTA (pH 5.9)

EXPERIMENT III

Materials and Methods

The susceptibility of *Pseudomonas fragi* growth to phosphates was examined using the agar dilution method (Washington, 1985). Dilution tests are commonly used to determine the minimal concentration of an antimicrobial agent required to inhibit or kill a microorganism. The minimal inhibitory concentration (MIC) is the lowest concentration without visible growth. The MIC was determined using seven different phosphates at eight levels (0-1.6%). The pH of the medium was pH 7.3 prior to phosphate addition. This experiment was done in duplicate and replicated three times.

Additional MIC's were determined using eight phosphates and EDTA at eight levels (0-3.2%). In the second part of this experiment, the pH of the medium was adjusted to pH 5.5 and 7.0 prior to sterilization and addition of phosphates. This part of the experiment was done in duplicate and replicated five times.

Inoculum

Stock cultures of *P. fragi* ATCC 4973 growing on nutrient agar (Difco Laboratories) at 4° C were used in this experiment. Four or five discrete *P. fragi* colonies, representative of the organism, were aseptically inoculated into 5 ml of trypticase soy broth (TSB, BBL) and adjusted with TSB to the turbidity of a McFarland 0.5 standard (ca

5 x 10^8 organisms per ml). A 1:20 dilution was made, from which the agar plates were inoculated.

Test chemicals

The phosphates tested included MSP, DSP, and TSP (Fisher Scientific), SAPP (Stauffer Chemical Co.), TSPP and STPP (Monsanto), and SPG (BK-Ladenburg). Two additional test chemicals were used in the pH adjusted experiments. These were ultraphosphate (UP) (BK-Ladenburg) and EDTA (Fisher Scientific). Stock solutions of phosphates were as follows: 32% (MSP, SPG), 16% (DSP, TSP, STPP), 8% (SAPP, TSPP), and 2% (UP, EDTA) (w/v). The highest concentration of phosphate used was dependent upon the phosphate's solubility in distilled water. The stock solutions were prepared by adding the appropriate amounts of selected phosphate to distilled water. The solutions were filter-sterilized through a 0.22μ Cameo IV filter unit (Micron Separations, Inc.) and two-fold serial dilutions were made according to the procedure by Washington (1985).

Media (no pH adjustment)

Aliquots from the serial dilutions were taken and added to tubes containing 45 ml Mueller Hinton Agar (MHA, Difco Laboratories) Final phosphate/agar plate concentrations were 0.025-0.8%, 1.6% or 3.2%, depending upon the initial stock phosphate concentrations used. The phosphate solution was mixed into the agar by gently inverting the tube several times. The contents were then poured into sterile petri plates (ca 20 ml per plate). Control plates contained sterile water, in

place of the phosphate aliquot, and MHA. Petri plates containing the agar-phosphate mixture were allowed to solidify and then dried in an inverted position at 25° C for 24 hours. The surface of the plates were spot inoculated (2 µl), without spreading, using short-length microcaps (Drummond Scientific Co.). The inoculated plates were allowed to sit for one half hour and then incubated in an inverted position at 25° C for 24 hours at which time the MIC was determined. The MIC represents the range of phosphate concentrations, in a series of consecutive dilutions, where growth is last observed and where complete inhibition first occurs. A negative result includes a single colony or barely visible, very fine haze.

Media (pH adjusted)

Aliquots (5 ml) from the serial dilutions were added to tubes containing TSA (ca 42 ml). Duplicate tubes were made for each serial dilution. Adjustment of the phosphate/agar to pH 5.5 or 7.0 was made at this time. One of the duplicated dilution series was used to determine the amount of acid (5 N HCL) or base (4 N NaOH) required for the adjustment to pH 5.5 or 7.0. This known volume of acid or base was then added aseptically to the tubes from the other duplicated dilution series. Sterile TSB was added (not more that 1 ml) to bring the total volume to 50 ml. The tubes were gently inverted for even mixing. The contents were then poured into sterile petri plates (ca 20 ml per plate). Control plates contained sterile water, in place of the phosphate aliquot, and were adjusted to pH 5.5 or 7.0 as mentioned

previously. The remaining part of the procedure was the same as that for the agar dilution method for the unadjusted pH.

Results and Discussion

The phosphate concentrations used in liquid media, as in Experiment II, were higher than the phosphate concentrations found to inhibit *P. fragi* growth (Table 5) in the present experiment. This is because liquid medium was found to have a buffering and protective effect on the microorganism when compared to solid medium (Schoenknecht et al., 1985). Additionally, when compared to the results from Experiment I, done in solid medium, the phosphate concentration necessary to inhibit growth was lower. This may be due

Table 5. Minimum inhibitory concentration (MIC) results of *P. fragi* with selected phosphates using the agar dilution method

Phosphate	MIC (%, w/v)[a]
MSP	0.8 - 1.6
DSP	0.8 - 1.6
TSP	0.2 - 0.4
SAPP	0.2 - 0.4
TSPP	0.1 - 0.2
STPP	0.1 - 0.2
SPG	0.8 - 1.6

[a]Mueller Hinton agar was used as the test medium.

to the the size of the inoculum which was not standardized in the first experiment. The inoculum was not standardized since the first experiment was used primarily for a preliminary indication of whether phosphates had any effects on the growth of P. fragi and/or the production of its protease.
previously. In the current experiment, the inoculum was standardized to a final concentration of 5 x 10^8 organisms/ml.

Media (no pH adjustment)

The most inhibitory phosphates were TSPP and STPP, with MIC's between 0.1%-0.2%. These were followed by TSP and SAPP with MIC's between 0.2% and 0.4%. SPG, MSP and DSP all had similar MIC's which were between 0.8% and 1.6%. The short-chain polyphosphates were more inhibitory than the longer-chain phosphate (SPG) tested and two (MSP, DSP) of three orthophosphates. As in the previous experiment, TSP is more inhibitory to *P. fragi* compared to the other orthophosphates.

Media (pH adjusted)

The following experiment considered pH as an inhibitory factor. The pH was adjusted to pH 5.5 or pH 7.0 before the addition of the phosphates to the medium (TSA). Although Mueller Hinton agar was one of the recommended media used for the agar dilution method (Washington, 1985), this medium is used primarily for the recovery of human pathogenic bacteria (*Neisseria gonorrhoeae* and *Neisseria meningitis*). MHA was subsequently replaced with trypticase soy agar

which is commonly used in the recovery of food spoilage organisms. Additionally, Zessin and Shelef (1988) found that trypticase soy broth (TSB) provided the lowest inhibitory effect when compared with nutrient broth (NB) and brain heart infusion broth (BHI). They found that TSB had an increased protein content which may provide bacteria with additional protection from phosphate treatments. This was beneficial because TSB could provide a high protein environment for the simulation of a model meat system.

The following MIC results (Table 6) were obtained after the pH of the medium, containing the selected treatment, had been adjusted to pH 5.5 or 7.0. The lowest MIC's were with STPP, TSPP, SAPP, and SPG. The most inhibitory phosphate was STPP with MIC's of 0.2%-0.4% (pH 5.5) and 0.4%-0.8% (pH 7.0). The other phosphate MIC's, MSP, DSP, TSP, UP (both pH's) and SAPP and TSPP (pH 7.0), could not be determined due to the limitations of the solubilities of the phosphate and EDTA stock solutions. MSP, DSP and TSP, however, were not able to inhibit *P. fragi* growth up to 3.2%, indicating a high MIC. In previous experiments, SPG was weakly inhibitory to *P. fragi* growth whereas the MIC determined in this experiment was lower than expected.

P. fragi was able to grow at both pH's in the absence of phosphate. However, with phosphates present, the MIC's at pH 5.5 were lower compared to pH 7.0. Thus, there was some pH effects

Table 6. Minimum inhibitory concentration (MIC) results of *P. fragi* with selected phosphates at pH 5.5 and pH 7.0 using the agar dilution method

	MIC (%, w/v)[a]	
Phosphate	pH 5.5	pH 7.0
MSP	>3.2[b]	>3.2
DSP	>3.2	>3.2
TSP	>1.6	>1.6
SAPP	0.4-0.8	>0.8
TSPP	0.4-0.8	>0.8
STPP	0.2-0.4	0.4-0.8
SPG	0.4-0.8	1.6-3.2
UP	>0.2	>0.2
EDTA	0.0125-0.025	0.025-0.05

[a]Trypticase soy agar was used as the test medium.
[b]The '>' is used to denote that growth occurred at the highest concentration tested, therefore the MIC may be higher.

between phosphates at pH 5.5 and pH 7.0. Possibilities for the decreased MIC values at pH 5.5 may include an impaired permeability system or mineral transport system as a result of the phosphates or phosphatases may be active at pH 7.0. The phosphatases could hydrolyze the phosphates to orthophosphates which appear not to be as inhibitory as the other phosphates.

The MIC of EDTA was very low, which was expected with such a powerful chelator. Interestingly, the MIC at pH 7.0 was higher than that at pH 5.5. The change in pH from pH 5.5 to pH 7.0 would not affect the ionization of EDTA since EDTA is fully ionized near pH 4.0. Therefore the change in pH would not have an effect on the metal chelation abilities of EDTA. The metal chelation abilities of EDTA would remain the same at both pH 5.5 and pH 7.0. The loss of inhibitory abilities of EDTA at pH 7.0 may be explained by the formation of insoluble metal-EDTA complexes at pH 5.5, which would make metal cations less available for the growth of *P. fragi.*

Increasing the phosphate chain length from one (orthophosphate) to two or more phosphate groups has a greater inhibitory effect on the growth of *P. fragi.* The MIC's of the orhthophosphates were quite high, therefore less inhibitory, when compared to the other phosphates. The results obtained with the orthophosphates support the metal chelation theory. Orthophosphates do not have chelating abilities, therefore, more metals are available for the bacterium to grow. The MIC's at pH 7.0 could not be conclusively determined although STPP was more inhibitory compared to all phosphate treatments, except UP, which has the possibility of having an MIC lower than STPP (0.4%-0.8%).

Conclusions

In media without the pH adjusted, the most inhibitory phosphates were TSPP and STPP; with MIC's between 0.1% and 0.2%. As in experiment I, the short-chain polyphosphates were more inhibitory than the longer chain phosphate (SPG) or the two orthophosphates (MSP, DSP).

In media with the pH adjusted to 5.5 or 7.0, the lowest MIC's were with STPP, TSPP, SAPP and SPG. STPP was the most inhibitory. Additionally, there were decreased MIC values at pH 5.5 which indicate possible pH effects.

The chain-length of the phosphate contributes to its antimicrobial ability. Increasing the phosphate chain length to two or three phosphate groups increases the inhibitory effect on *P. fragi* growth.

EXPERIMENT IV

Materials and Methods

The effects of phosphates and EDTA, at four different levels, on the growth and protease production of *P. fragi* in a synthetic medium were examined. After the addition of the phosphates to the growth medium, the pH was adjusted to pH 5.5.

Inoculum

TSB (BBL) was inoculated with *P. fragi* ATCC 4973, from a stock culture grown at 4° C on nutrient agar (Difco Laboratories) and incubated overnight at 30° C. An aliquot (0.3 ml) was transferred to sterile TSB (30 ml, BBL) and incubated for 16 hours at 30° C. The inoculum contained approximately 10^8 organisms per ml.

Test chemicals

The following test chemicals were used: MSP, DSP, and TSP (Fisher Scientific), SAPP (Stauffer Chemical Co.), TSPP and STPP (Monsanto), and SPG and UP (BK-Ladenburg), and EDTA (Fisher Scientific). Phosphates were added, in solid form, to TSB (BBL) to achieve final concentrations of 2%, 4% and 6% (w/v), with the exception of UP and EDTA which were used at 1%, 2% and 4% (w/v).

Media

The solutions were prepared by adding the appropriate amount of test chemical to sterile TSB (BBL). The pH was adjusted to pH 5.5

(5 N HCL or 4 N NaOH), brought up to volume (50 ml) with TSB (BBL) and then filter sterilized using a 0.22μ Cameo IV filter unit (Micron Separations Inc.) into sterile screw cap tubes (20 ml). A control consisted of TSB (BBL) adjusted to pH 5.5. Tubes were inoculated (0.1 ml) with the *P fragi* inoculum) and incubated for 48 hours at 30° C. Samples were taken at 0, 4, 8, 16, 24 and 48 hours for microbiological examination, protease activity (Porzio and Pearson, 1975), and protein determination. The experiment was replicated three times.

Microbiological analysis

Aerobic mesophilic bacterial populations were determined by using standard procedures. Serial dilutions in 0.1% peptone (BBL) were made and pour plates (TSA, BBL) were performed in duplicate at each dilution.

Protease activity assay

Proteolytic activity was determined according to the procedure of Porzio and Pearson (1975) using a 2% (w/v) Hammersten casein (U.S. Biochemical Co., Cleveland, OH) made with 0.1 M Tris•HCL (pH 7.2). The reaction tube included prewarmed (35° C) 2% casein (5 ml) and 1 ml enzyme sample. The reaction was allowed to incubate for 10 minutes at 35° C after which time 5 ml of a precipitating reagent was added to the tube. The precipitating reagent was made up of 0.11 M Trichloroacetic acid (TCA) (Fisher Scientific), 0.22 M sodium acetate (Fisher Scientific) and 0.33 M acetic acid (Fisher

Scientific). The tubes were vortexed and allowed to stand at room temperature (24° C) for 30 minutes. The supernatant was collected after centrifugation (8,000 x g) for 10 minutes at 4° C. Absorbance was measured at 275 nm and was compared to a tyrosine standard curve (five-fold serial dilutions of 1 μmol tyrosine per ml 0.2 N HCL). One protease activity unit was expressed as 1μg of tyrosine equivalent liberated per ml enzyme solution per minute at 35° C

Protein determination

Protein concentration was measured using the BCA protein asssay method (Pierce, Rockford, IL) and expressed in mg of protein. The experiment was replicated three times.

Analysis of data

Statistical analysis was performed using the statistical analysis system's (SAS Institute, 1979) analysis of variance (ANOVA) and general linear models (GLM) procedures. When more than two treatments were being compared, Duncan's multiple range test (Steel and Torrie, 1980) was used.

Results and Discussion

This experiment was done to compare the antimicrobial abilities of phosphates in liquid medium at concentrations slightly above those allowed by law in meat products. The pH was adjusted to 5.5 after the addition of phosphates. The pH of 5.5 was chosen over pH 7.0 because pH 5.5 was closer to the pH of post-rigor meat. In addition, the pH

was adjusted after phosphates had been added to the medium since it is believed that the low concentrations of phosphates, when added to meat, would not cause a change in the overall pH of the meat due to its natural buffering capacity.

In trypticase soy broth, the growth of *P. fragi* was significantly reduced by all phosphates tested at all levels: MSP, DSP, TSP, SAPP, TSPP, STPP, SPG and UP (Figures 21-24), with the exception of 2% MSP (Figure 22).

At 1%, UP lost some of its inhibitory abilities after 24 hours. When compared to EDTA, UP was not significantly different from EDTA until after 24 hours. Of the orthophosphates at 2% (Figure 22), MSP did not show any inhibitory effects of *P. fragi* growth. Two percent DSP and TSP showed similar inhibitory patterns. SAPP (2%) (Figure 22) was less inhibitory than TSPP and STPP, at the same concentration. After 24 hours, STPP was observed to increase its inhibitory abilities by becoming bactericidal. There was no significant difference between STPP, UP and SPG. Two percent UP and SPG (Figure 22) were bacteriostatic and were not significantly different from each other. At 4 %, all phosphates were bacteriostatic with no one phosphate being more inhibitory than the other (Figure 23). Orthophosphates were not as inhibitory to *P. fragi* growth after 8 hours of incubation (Figure 23). Six percent phosphates (Figure 24) exhibited similar inhibitory action as that at 4% (Figure 23). At

higher concentrations (6%), the phosphates were more inhibitory than at the lower concentrations.

EDTA was bactericidal at all concentrations tested after 24 hours incubation time (Figures 21-23). When the phosphates were compared with EDTA, the pyrophosphates and polyphosphates were found to not be significantly different from EDTA. This suggest that these phosphates may have the same inhibitory mechanism, this is metal chelation, on the growth of *P. fragi* as EDTA.

The polyphosphates (STPP, SPG, and UP) were more inhibitory than the pyrophosphates which were more inhibitory than the orthophosphates.

Protease activity, reported as activity/ mg protein (Figures 25-28), was reduced by all phosphates, except MSP (4%), TSP (6%), and SPG (4%, 6%) after 48 hours. These exceptions actually increased the protease activity. This may indicate an inductive response of the protease to these phosphates. Reporting protease activity per mg protein may not have been as sensitive due to the large contribution of protein found in the medium. The high protein content would make it harder to detect small changes in the production of enzyme. For this reason, protease activity was also reported as activity/ organism (Figures 29-32). However, protease activity was not affected by any phosphate at each of the levels tested based on statistical analysis.

Conclusions

All phosphates significantly reduced the growth of *P. fragi* in trypticase soy broth at all levels tested (up to 6%), with the exception of 2% MSP. Chain-length was a factor in the antimicrobial effects of the phosphates. The longer the phosphate, the more inhibitory the effect.

Protease activity (activity/mg protein) was reduced by most phosphates however, MSP (4%), TSP (6%), and SPG (4%, 6%) increased the activity which may indicate an inductive response.

Figure 21. Effects of 1% Up and EDTA on the growth of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.47)

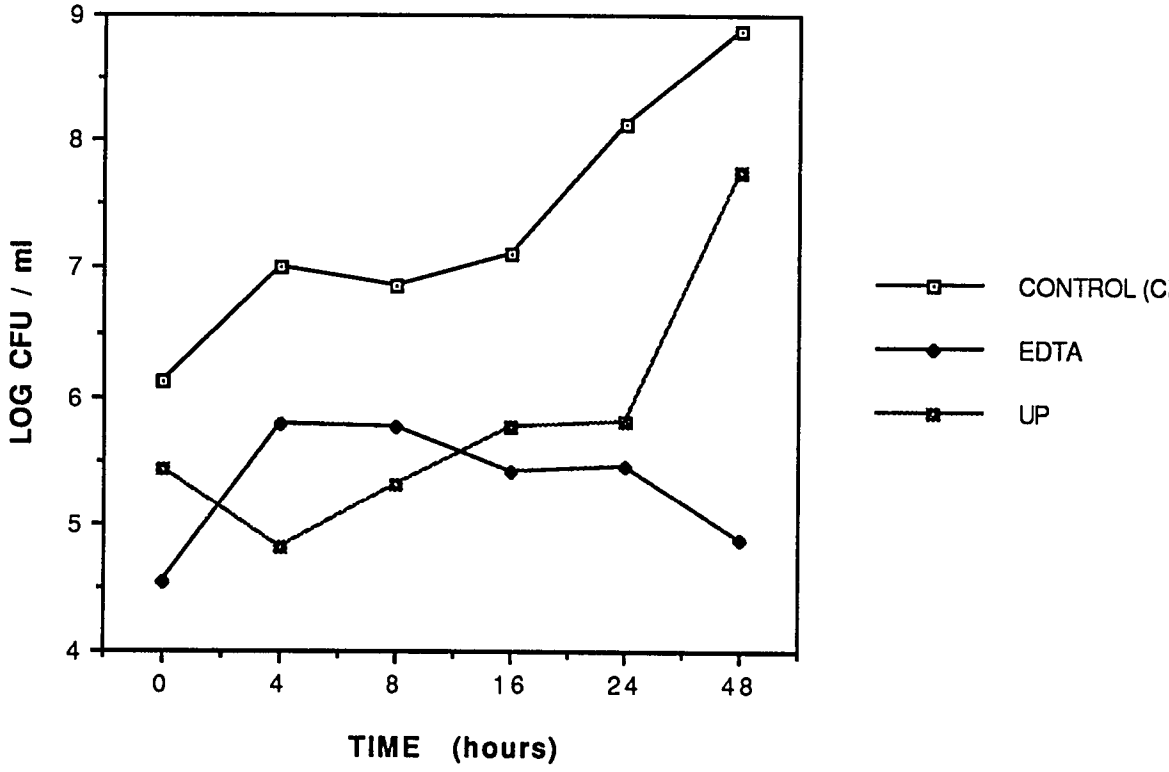

9
8
7
6
5
4
LOG CFU / ml
0
4
8
16
24
48
TIME (hours)
CONTROL (CI)
EDTA
UP

Figure 22. Effects of 2% phosphates on the growth of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.34)

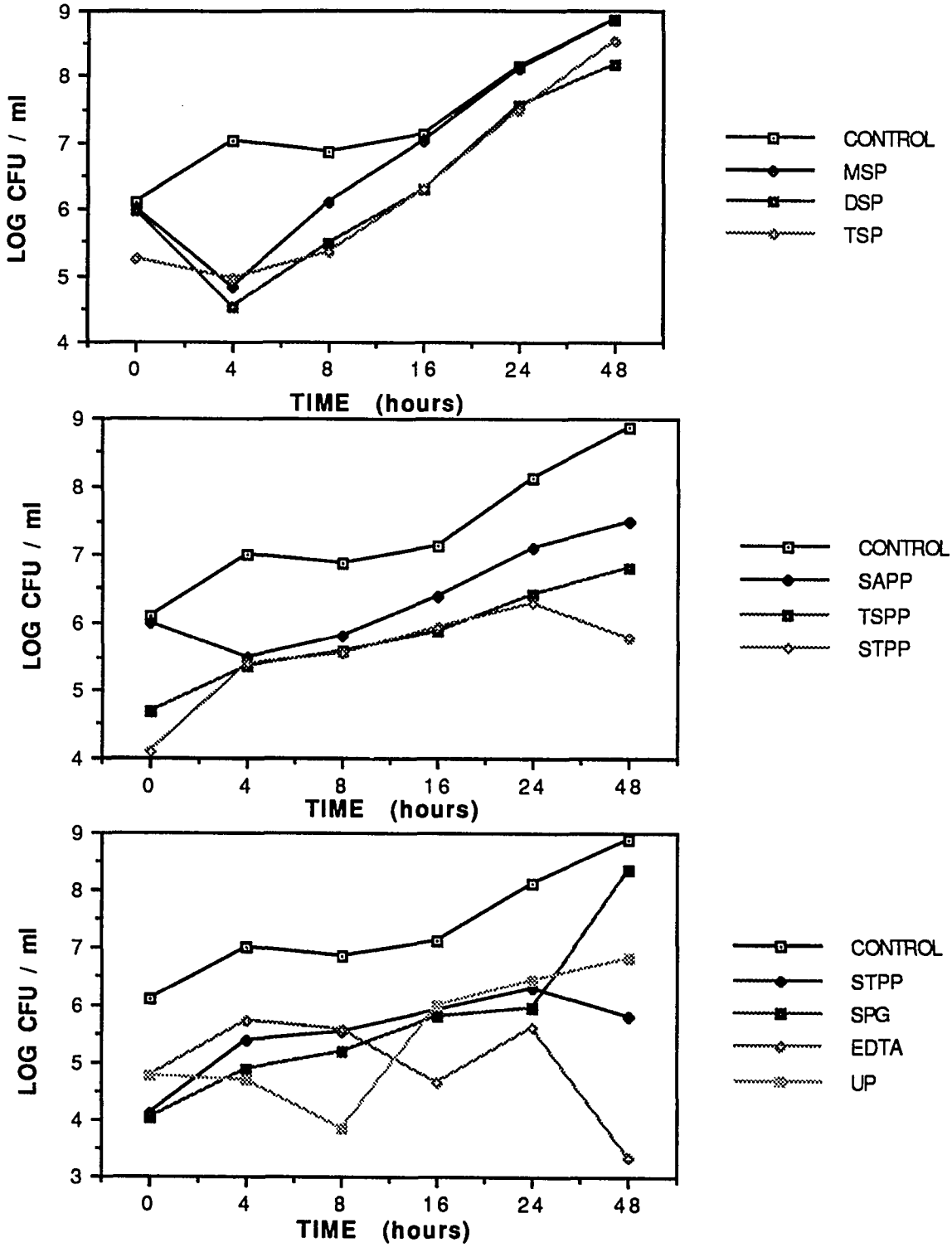

LOG CFU / ml
TIME (hours)
CONTROL
MSP
DSP
TSP
LOG CFU / ml
TIME (hours)
CONTROL
SAPP
TSPP
STPP
LOG CFU / ml
TIME (hours)
CONTROL
STPP
SPG
EDTA
UP

Figure 23. Effects of 4% phosphates on the growth of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.31)

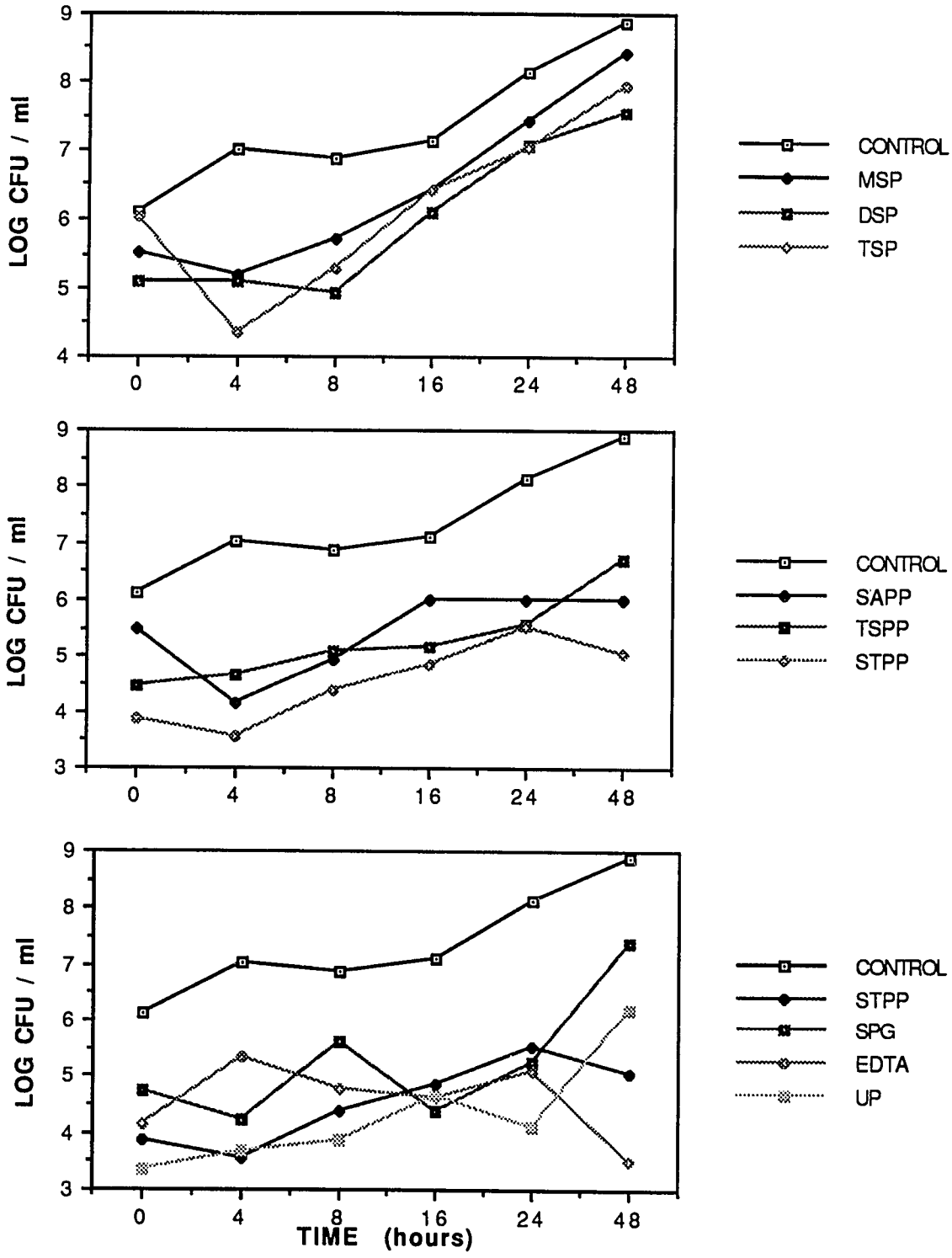
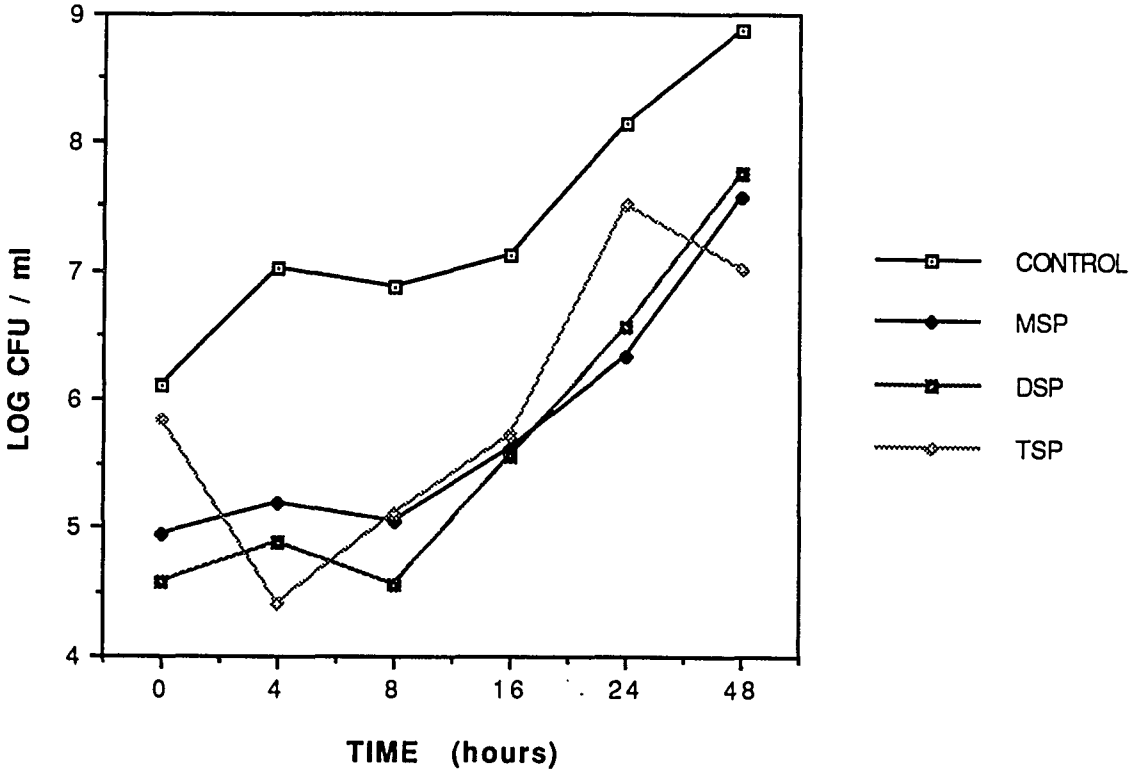

LOG CFU / ml
9
8
7
6
5
4
0
4
8
16
24
48
CONTROL
MSP
DSP
TSP
LOG CFU / ml
9
8
7
6
5
4
3
0
4
8
16
24
48
CONTROL
SAPP
TSPP
STPP
LOG CFU / ml
9
8
7
6
5
4
3
0
4
8
16
24
48
TIME (hours)
CONTROL
STPP
SPG
EDTA
UP

Figure 24. Effects of 6% phosphates on the growth of *Pseudomonas fragi* in a synthetic medium (Overall SEM=.50)


LOG CFU / ml
9
8
7
6
5
4
0
4
8
16
24
48
TIME (hours)
CONTROL
MSP
DSP
TSP

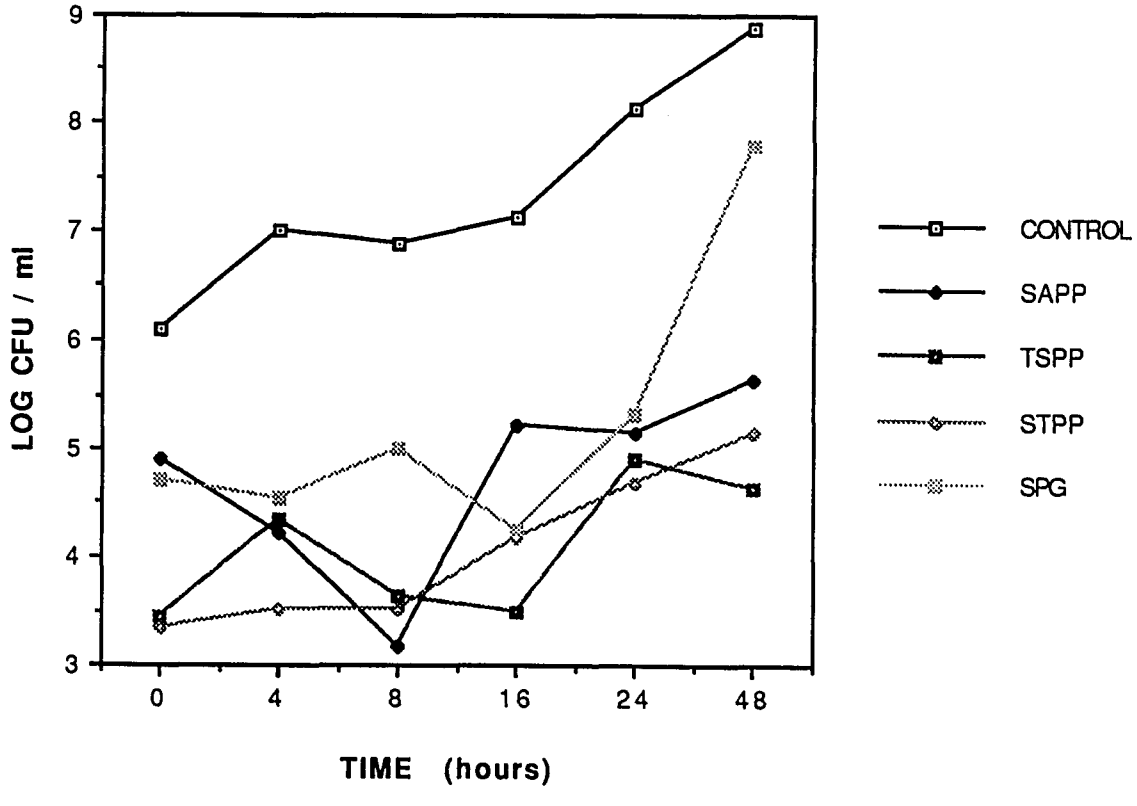

LOG CFU / ml
9
8
7
6
5
4
3
0
4
8
16
24
48
TIME (hours)
CONTROL
SAPP
TSPP
STPP
SPG

Figure 25. Effects of 1% Up and EDTA on the activity (units/protein) of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.07)

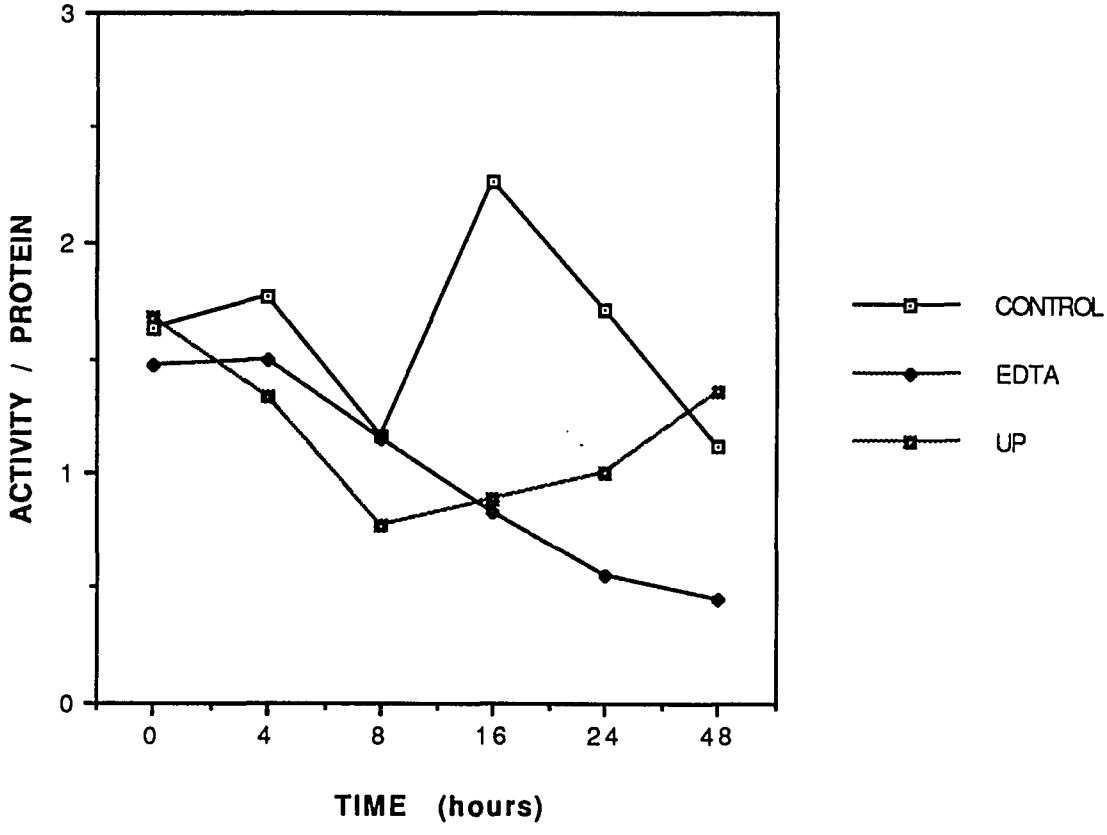

3
2
1
0
ACTIVITY / PROTEIN
0
4
8
16
24
48
TIME (hours)
CONTROL
EDTA
UP

Figure 26. Effects of 2% phosphates on the activity(units/protein) of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.06)

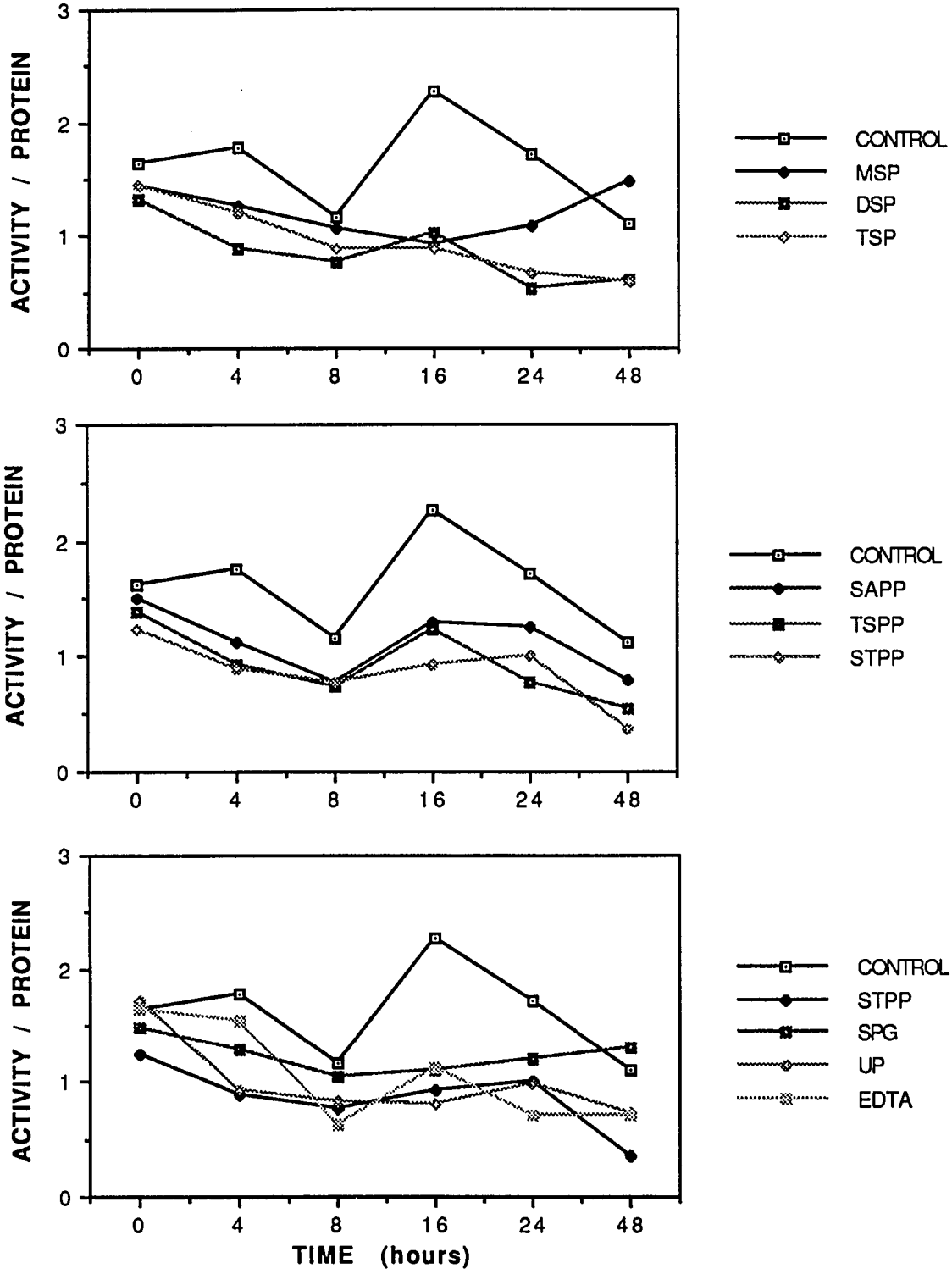

ACTIVITY / PROTEIN
3
2
1
0
0
4
8
16
24
48
CONTROL
MSP
DSP
TSP
ACTIVITY / PROTEIN
CONTROL
SAPP
TSPP
STPP
ACTIVITY / PROTEIN
CONTROL
STPP
SPG
UP
EDTA
TIME (hours)

Figure 27. Effects of 4% phosphates on the activity (units/protein) of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.07)

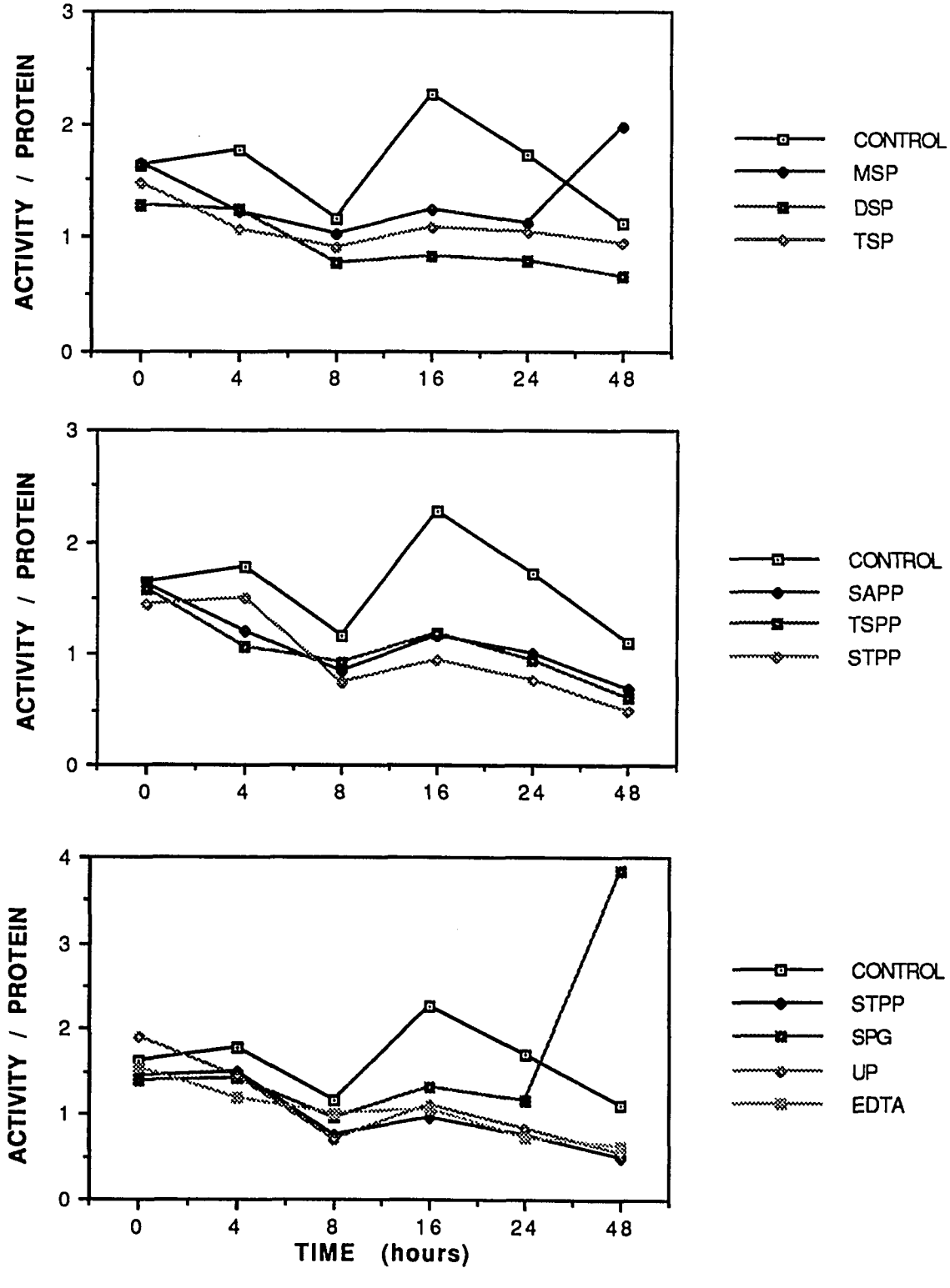
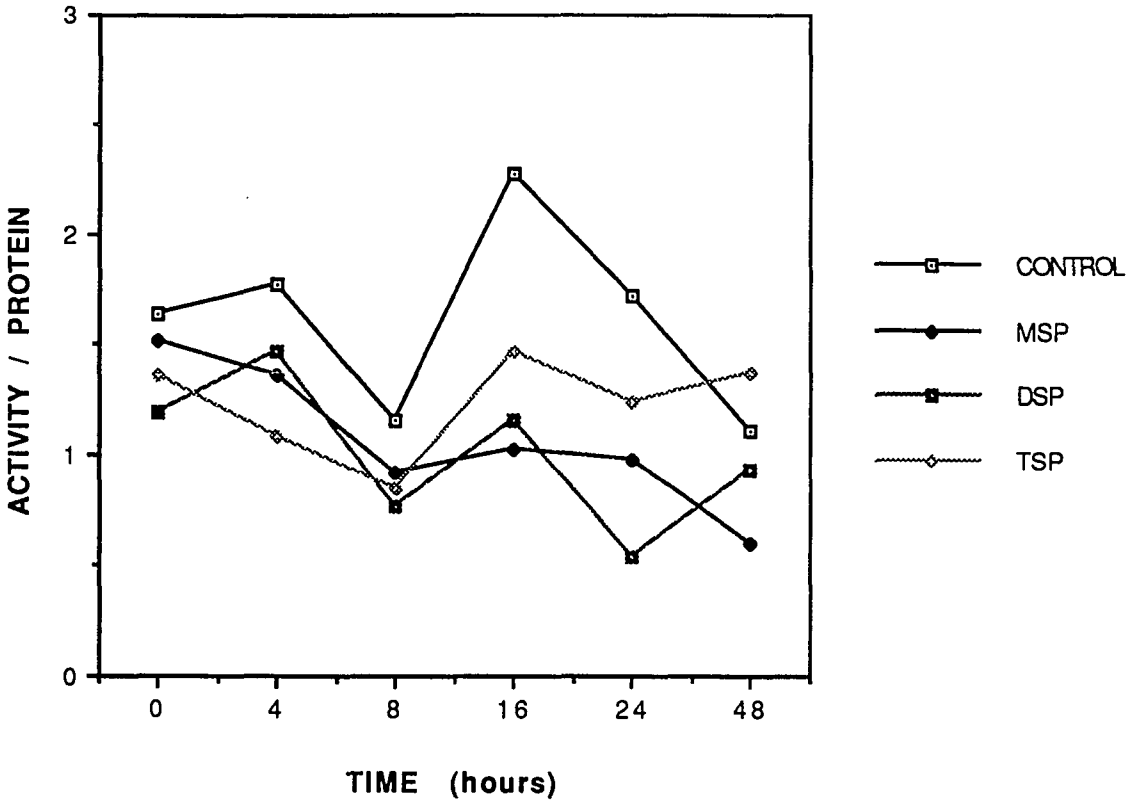

ACTIVITY / PROTEIN
3
2
1
0
0
4
8
16
24
48
CONTROL
MSP
DSP
TSP
ACTIVITY / PROTEIN
CONTROL
SAPP
TSPP
STPP
ACTIVITY / PROTEIN
4
3
2
1
0
CONTROL
STPP
SPG
UP
EDTA
TIME (hours)

Figure 28. Effects of 6% phosphates on the activity (units/protein) of *Pseudomonas fragi* in a synthetic medium (Overall SEM=0.06)


ACTIVITY / PROTEIN
3
2
1
0
0
4
8
16
24
48
TIME (hours)
CONTROL
MSP
DSP
TSP

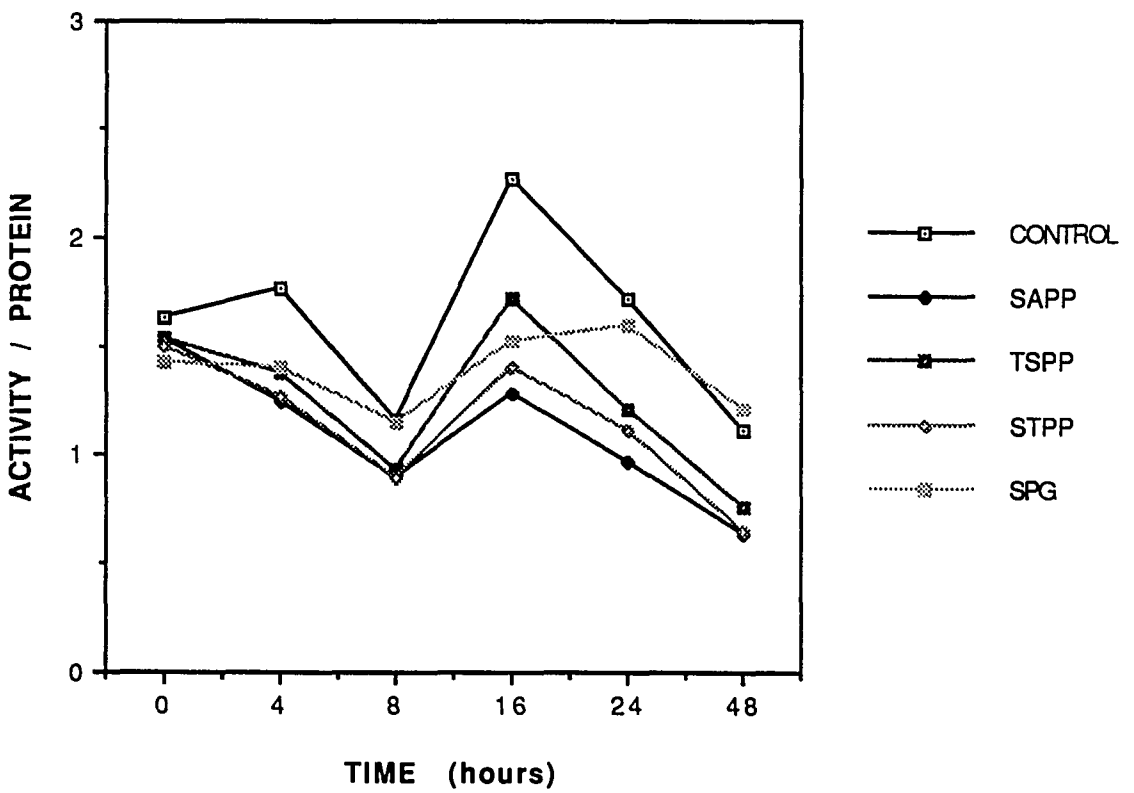

ACTIVITY / PROTEIN
3
2
1
0
0
4
8
16
24
48
TIME (hours)
CONTROL
SAPP
TSPP
STPP
SPG

Figure 29. Effects of 1% Up and EDTA on the activity (units/organism) of *Pseudomonas fragi* in a synthetic medium

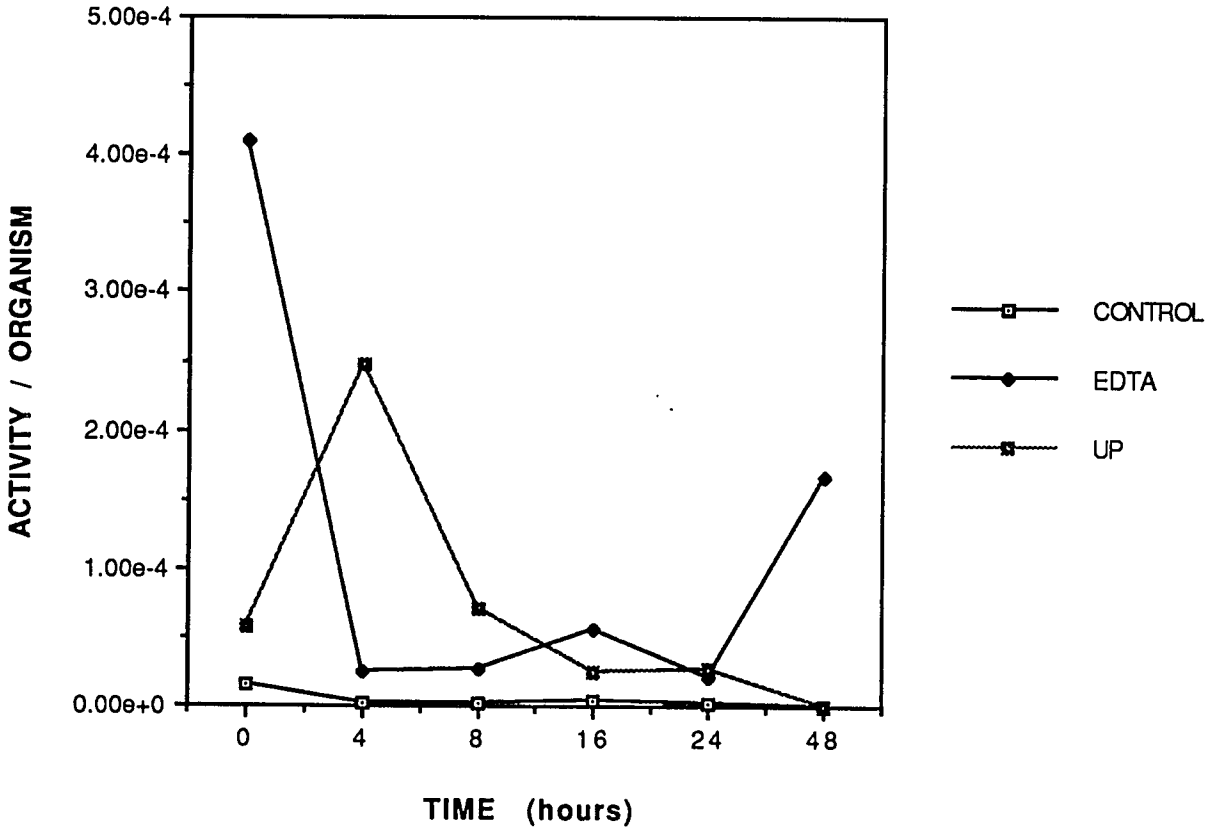

5.00e-4
4.00e-4
3.00e-4
2.00e-4
1.00e-4
0.00e+0
ACTIVITY / ORGANISM
0
4
8
16
24
48
TIME (hours)
CONTROL
EDTA
UP

Figure 30. Effects of 2% phosphates on the activity (units/organism) of *Pseudomonas frag* in a synthetic medium

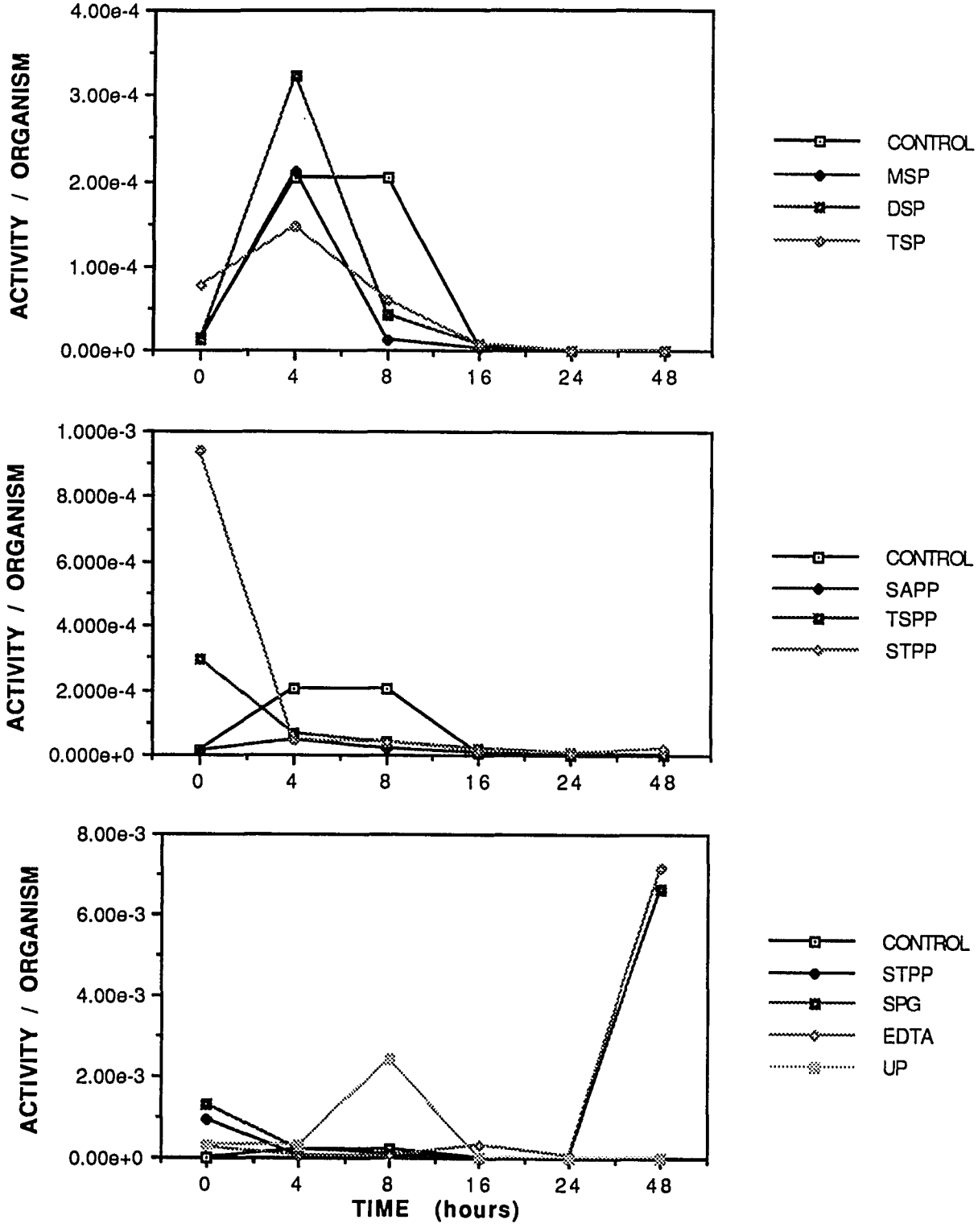

4.00e-4
3.00e-4
2.00e-4
1.00e-4
0.00e+0
ACTIVITY / ORGANISM
0
4
8
16
24
48
CONTROL
MSP
DSP
TSP
1.000e-3
8.000e-4
6.000e-4
4.000e-4
2.000e-4
0.000e+0
ACTIVITY / ORGANISM
CONTROL
SAPP
TSPP
STPP
8.00e-3
6.00e-3
4.00e-3
2.00e-3
0.00e+0
ACTIVITY / ORGANISM
CONTROL
STPP
SPG
EDTA
UP
TIME (hours)

Figure 31. Effects of 4% phosphates on the (units/organism) of *Pseudomonas fragi* in a synthetic medium

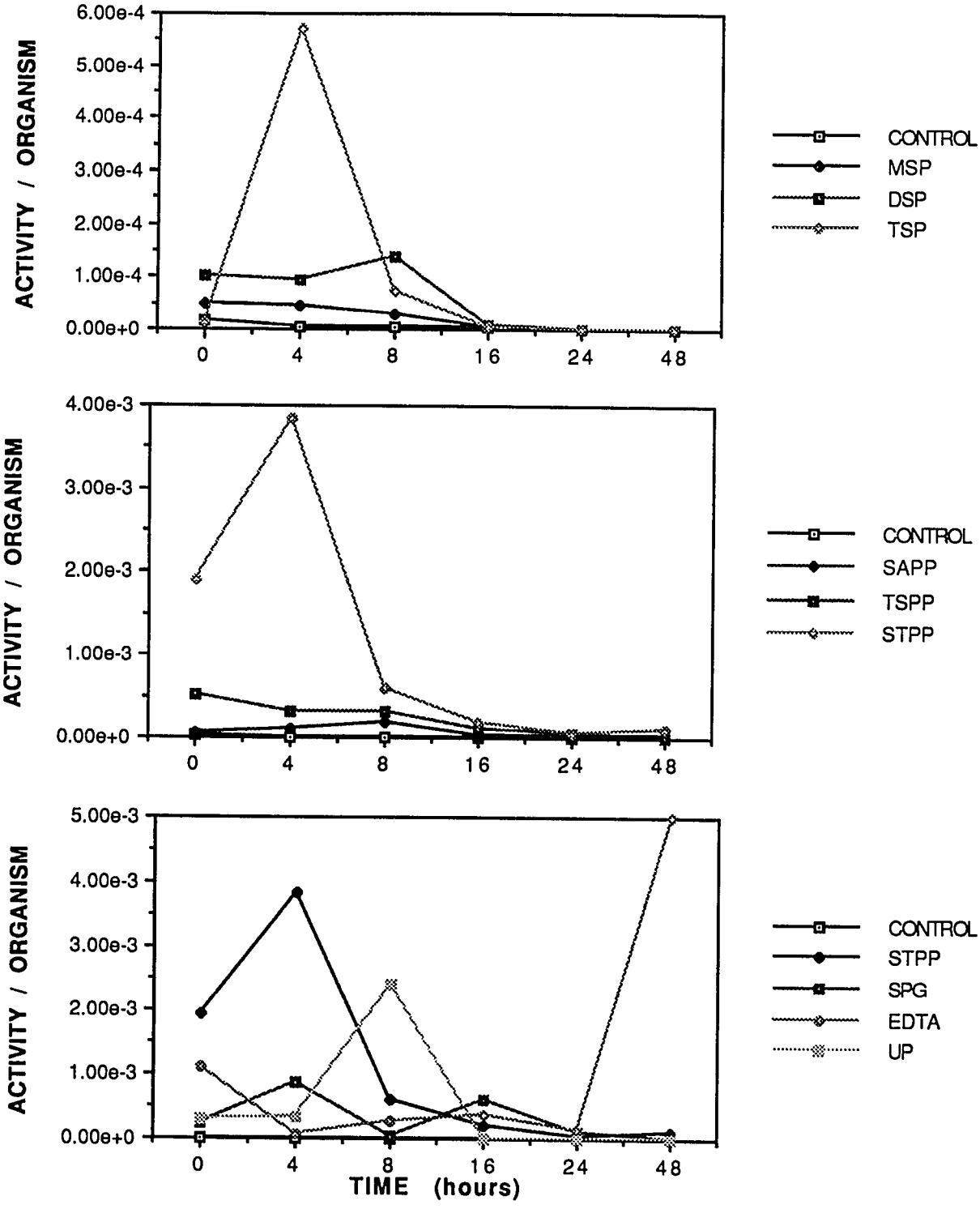
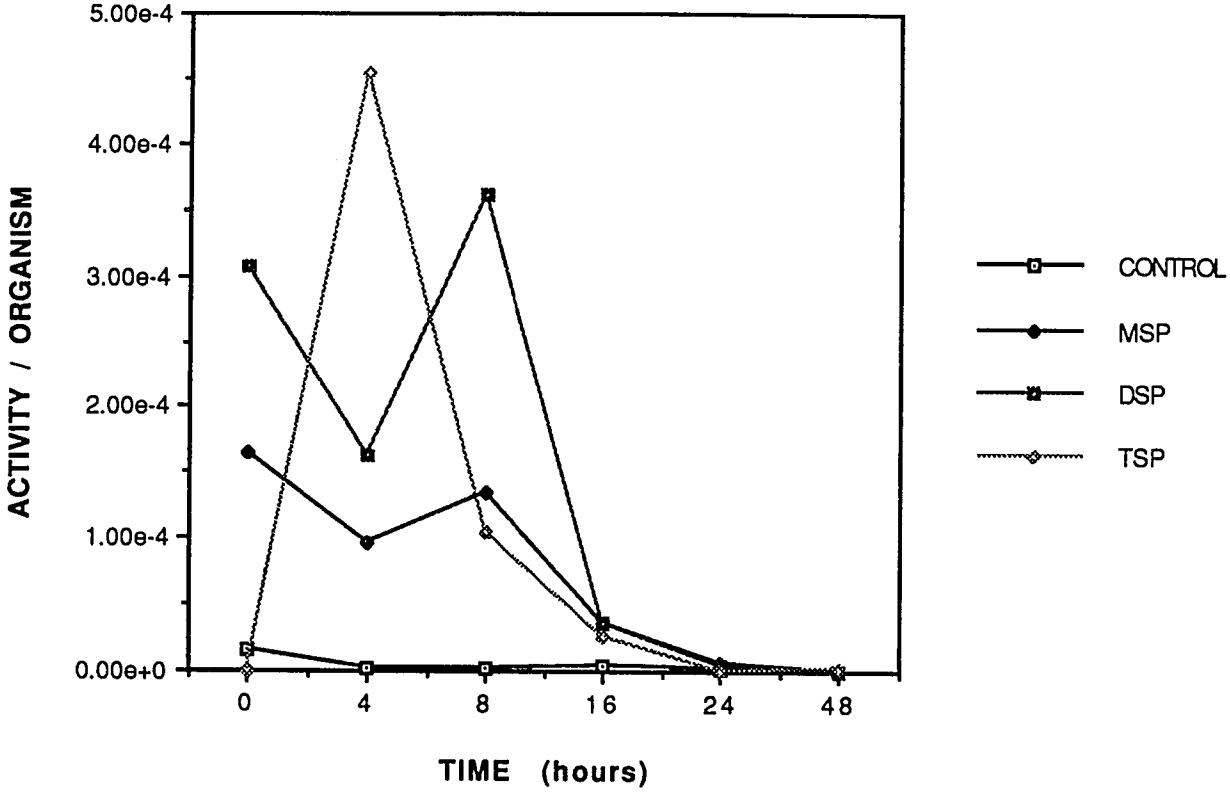
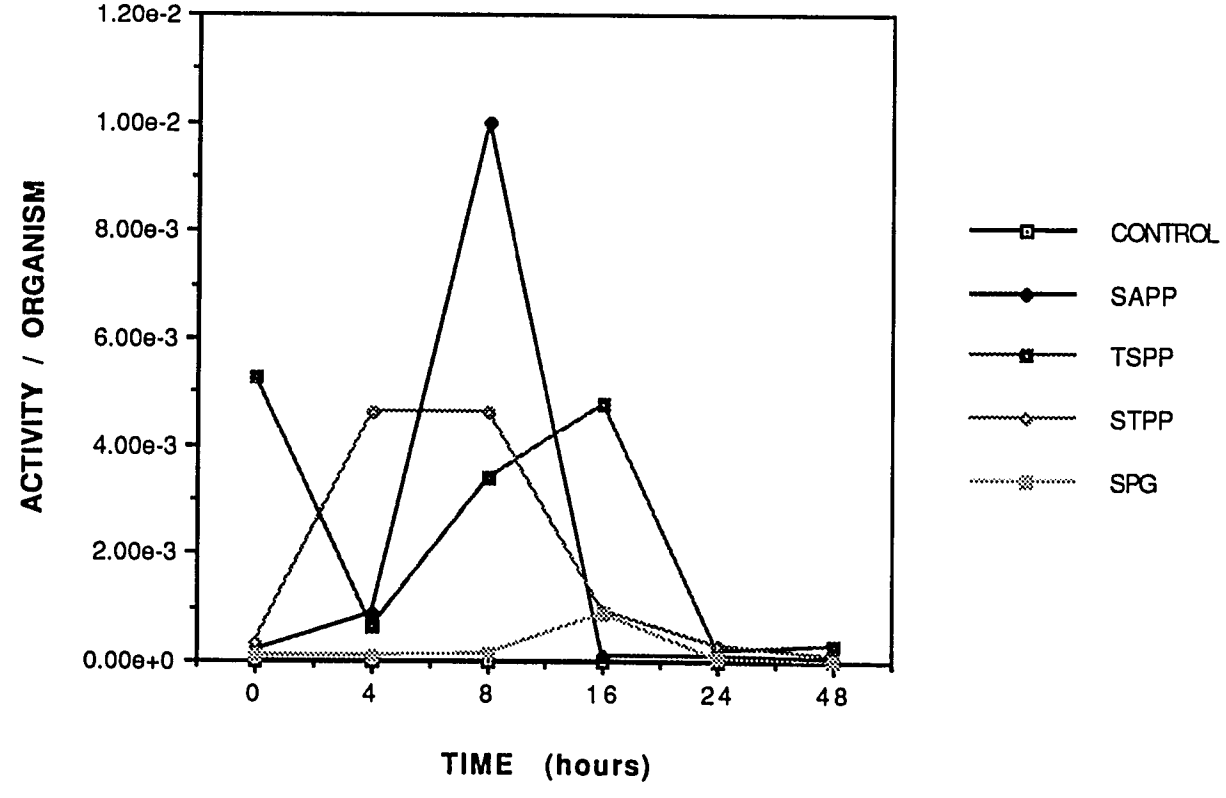

ACTIVITY / ORGANISM
6.00e-4
5.00e-4
4.00e-4
3.00e-4
2.00e-4
1.00e-4
0.00e+0
0
4
8
16
24
48
CONTROL
MSP
DSP
TSP
ACTIVITY / ORGANISM
4.00e-3
3.00e-3
2.00e-3
1.00e-3
0.00e+0
0
4
8
16
24
48
CONTROL
SAPP
TSPP
STPP
ACTIVITY / ORGANISM
5.00e-3
4.00e-3
3.00e-3
2.00e-3
1.00e-3
0.00e+0
0
4
8
16
24
48
TIME (hours)
CONTROL
STPP
SPG
EDTA
UP

Figure 32. Effects of 6% phosphates on the (units/organism) of *Pseudomonas fragi* in a synthetic medium


5.00e-4
4.00e-4
3.00e-4
2.00e-4
1.00e-4
0.00e+0
ACTIVITY / ORGANISM
0
4
8
16
24
48
TIME (hours)
CONTROL
MSP
DSP
TSP


1.20e-2
1.00e-2
8.00e-3
6.00e-3
4.00e-3
2.00e-3
0.00e+0
ACTIVITY / ORGANISM
0
4
8
16
24
48
TIME (hours)
CONTROL
SAPP
TSPP
STPP
SPG

EXPERIMENT V

Materials and Methods

The effects of phosphates (MSP, SAPP, TSPP, STPP and SPG) at two levels (0.5% and 2%) were examined on the growth and proteolytic activity of *Pseudomonas fragi* in ground pork.

Inoculum

A *P. fragi* (ATCC 4973) inoculum was prepared as described in Experiment IV. A 100-fold serial dilution was prepared from the initial inoculum. The 100-fold dilution contained approximately 10^6 organisms per ml and was used as the meat inoculum.

Test chemicals

Stock phosphate solutions (2% and 8%, w/v) of MSP (Fisher Scientific), SAPP (Stauffer Chemical Co.), TSPP and STPP (Monsanto) and SPG (BK-Ladenburg) were filter-sterilized through a 0.22 Acrodisc syringe filter (Gelman Scientific, Ann Arbor, MI).

Meat

A 25 pound batch of picnic shoulders were obtained from the Iowa State University Meat Laboratory and ground (1/8th plate) and vacuum-packaged (150 g) to 1 kPa using a Multivac MG-2 (Sepp Haggenmueller KG, West Germany) in Curlon 892 (Curwood Inc., New London, WI) vacuum packages. The ground pork was stored at -28° C until needed. Meat was allowed to thaw prior to experimentation (15° C) for 48 hours. Phosphate stock solutions were

added to the meat to obtain a total phosphate concentration of 0.5% and 2% of the meat weight. Sterile distilled water was used in place of the phosphate solutions for the control. The added aqueous phase amounted to 25% of the ground pork. The meat was inoculated (0.01 ml/g) and the bags were massaged to mix the contents evenly. Meat samples were dispensed (50 g) in sterile mason jars and stored at 10° C. Samples were taken on days 0, 3 and 6 for microbiological examination, protease activity (Porzio and Pearson, 1975) and protein determination (BCA, Pierce). The experiment was replicated four times.

Microbiolgical examination

Meat samples were weighed (30 g) and blended in a sterile stomacher bag (Teckmar, Cincinnati, OH) and blended with sterile 0.1% peptone (BBL) water (270 ml) for two minutes using a Stomacher Lab Blender 400 (Tekmar Co., OH). Serial dilutions were made according to standard procedures. Plate count agar (Difco Laboratories) was used to pour plate aerobic mesophilic and psychrotrophic (30° C, 48 hours and 5 ° C, 10 days, respectively) bacterial populations.

Protease activity assay

The same procedure was used as that in Experiment IV. One protease activity unit was expressed as 1μg of tyrosine equivalent liberated per ml enzyme solution per minute at 35° C.

Protein determination

Protein concentration was measured using the BCA protein asssay method (Pierce) and expressed in mg of protein.

Analysis of data

After logarithmic transformation, statistical analysis was performed using the statistical Analysis System's (SAS Institute, 1979) analysis of variance (ANOVA) and general linear models (GLM) procedures. When more than two treatments were being compared, Duncan's multiple range test (Steel and Torrie, 1980) were used.

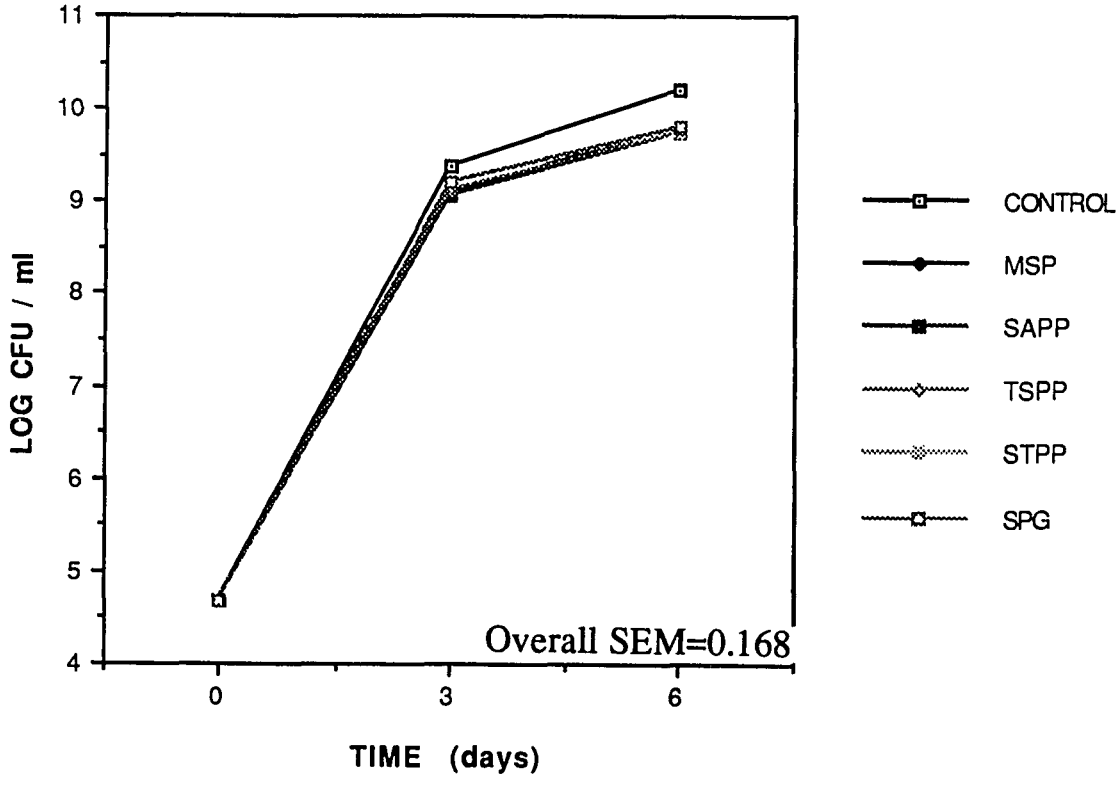
Results and Discussion

Phosphates, when blended with ground pork at 0.5% and 2%, did not have an inhibitory effect on mesophilic and psychrotrophic bacterial populations (Figures 33-34). In addition, protease activity was not affected at either phosphate concentration (Figure 35). The lack of inhibitory abilities of the phosphates may be attributed to the hydrolysis of phosphates by naturally occurring phosphatases in the meat or the buffering capacities of the meat did not allow the full efffect of the antimicrobial abilities of the phosphate.

Conclusions

The results of this study indicate that phosphate concentrations allowed by law, 0.5% of the final meat product, do not inhibit the psychrotrophic or mesophilic growth of ground pork inoculated with *P. fragi*.

Figure 33. Effects of 0.5% (top) and 2% (bottom) phosphates on the mesophilic growth of *Pseudomonas fragi* in ground pork


LOG CFU / ml
11
10
9
8
7
6
5
4
0
3
6
TIME (days)
Overall SEM=0.168
CONTROL
MSP
SAPP
TSPP
STPP
SPG

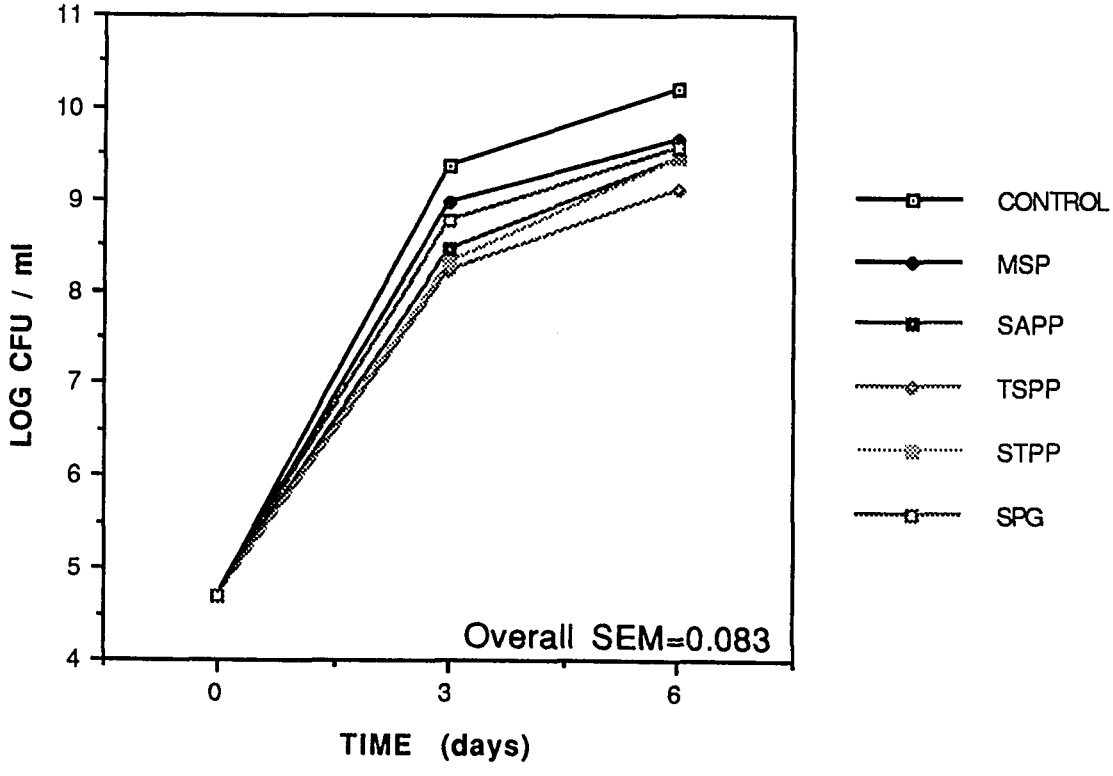
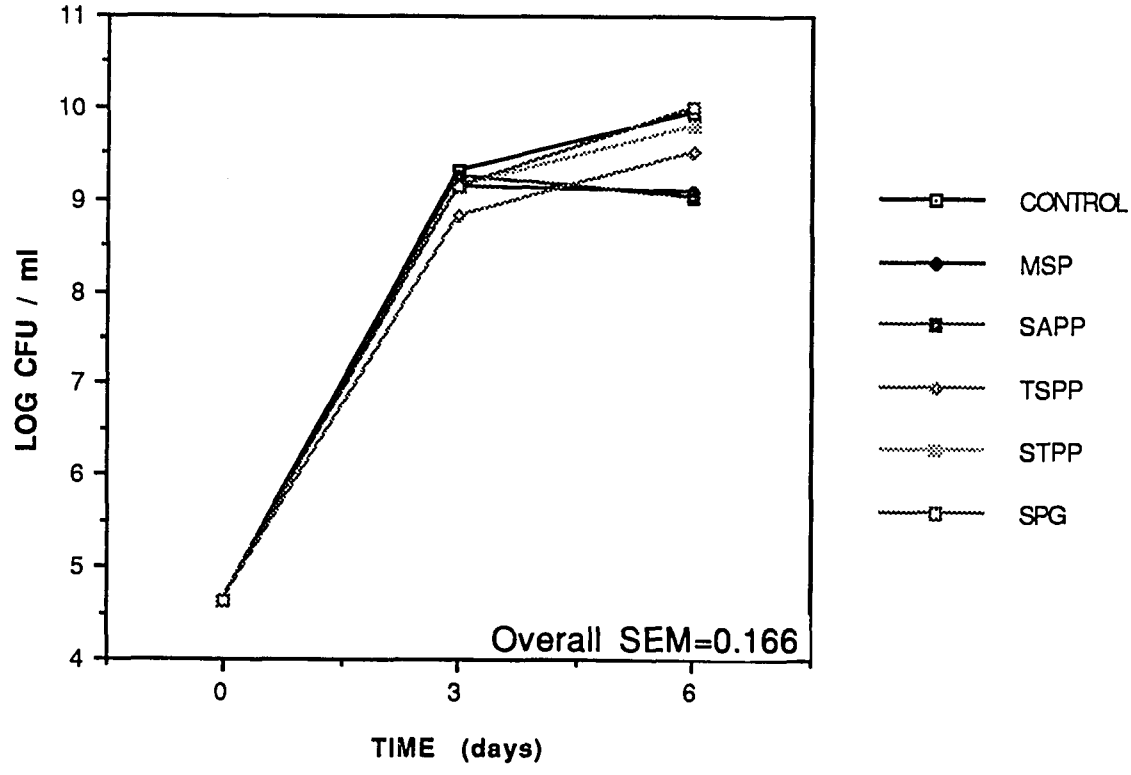

LOG CFU / ml
11
10
9
8
7
6
5
4
0
3
6
TIME (days)
Overall SEM=0.083
CONTROL
MSP
SAPP
TSPP
STPP
SPG

Figure 34. Effects of 0.5% (top) and 2% (bottom) phosphates on the psychrotrophic growth of *Pseudomonas fragi* in ground pork


LOG CFU / ml
TIME (days)
Overall SEM=0.166
CONTROL
MSP
SAPP
TSPP
STPP
SPG

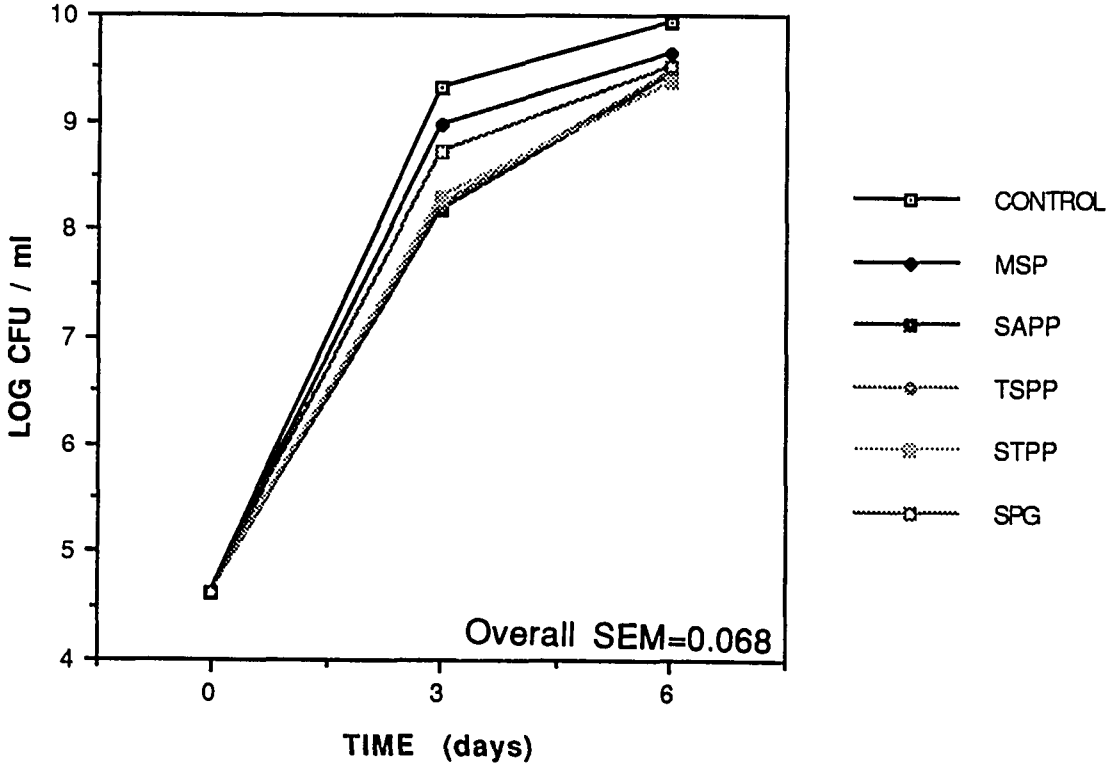
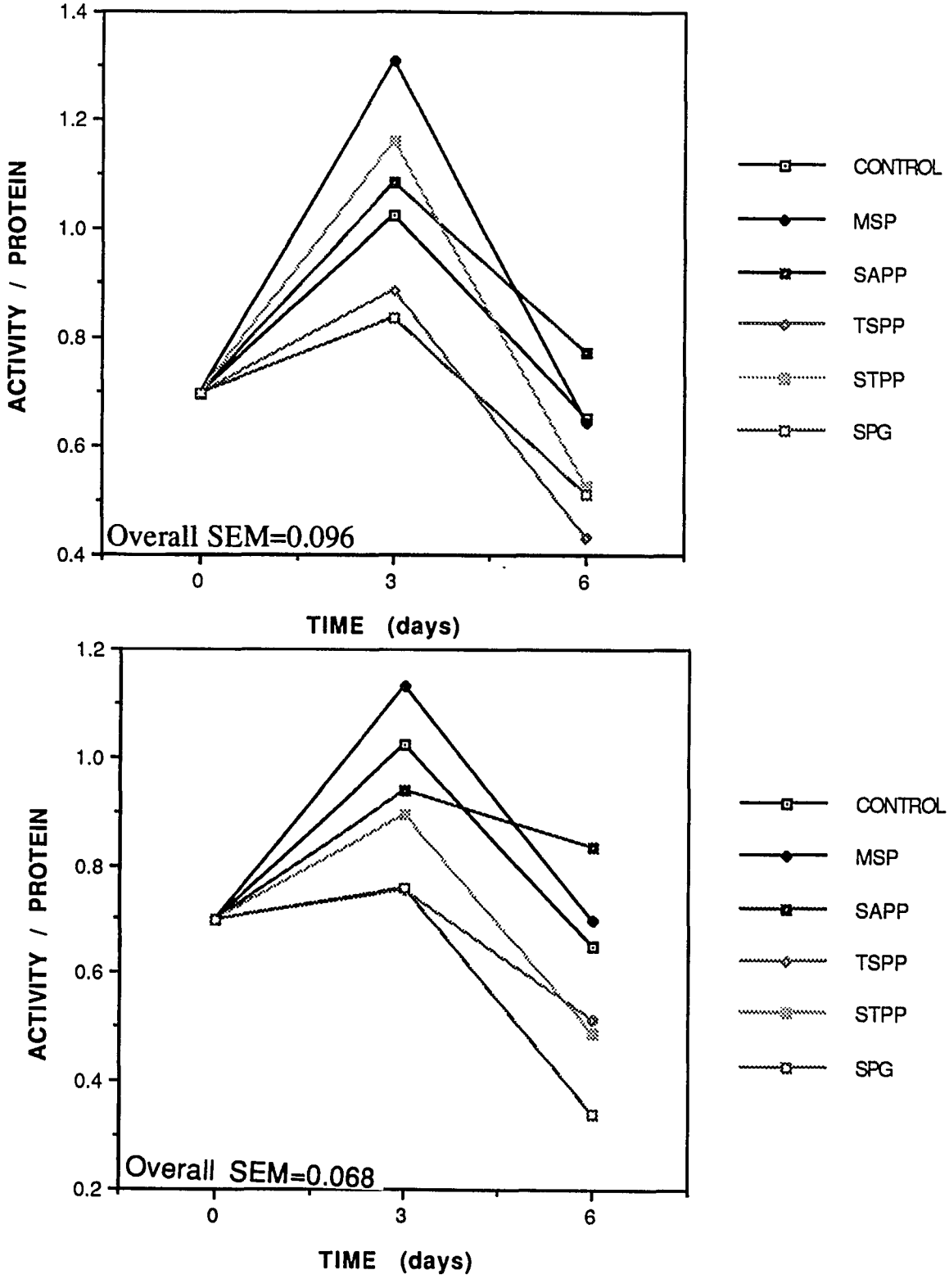

LOG CFU / ml
TIME (days)
Overall SEM=0.068
CONTROL
MSP
SAPP
TSPP
STPP
SPG

Figure 35. Effects of 0.5% (top) and 2% (bottom) phosphates on the activity (units/protein) of *Pseudomonas fragi* in ground pork


1.4
1.2
1.0
0.8
0.6
0.4
ACTIVITY / PROTEIN
Overall SEM=0.096
0
3
6
TIME (days)
CONTROL
MSP
SAPP
TSPP
STPP
SPG
1.2
1.0
0.8
0.6
0.4
0.2
ACTIVITY / PROTEIN
Overall SEM=0.068
0
3
6
TIME (days)
CONTROL
MSP
SAPP
TSPP
STPP
SPG

EXPERIMENT VI

Materials and Methods

The ability of Pseudomonas fragi to synthesize protease in the presence of eight phosphates and EDTA at three levels was examined. Growth, protease activity and protein determinations were made.

Inoculum

Pseudomonas fragi (ATCC 4379), from a stock culture grown on nutrient agar (Difco Laboratories) at 4° C, was inoculated into TSB (10 ml, BBL,) and incubated overnight at 30° C. An aliquot (0.3 ml) was transferred to sterile TSB (30 ml) and incubated for 16 hours at 30° C. The following procedure was done twice: the culture was pelleted (5,000 x g) for 30 minutes and the precipitate was washed with 50 mM Tris•HCL/5mM $CaCl_2$ buffer (pH 7.5).Cells were suspended with 50 mM Tris•HCL/5 mM $CaCl_2$ buffer (pH 7.5) to an absorbance at 600 nm of 0.67 (ca 10^8 organisms/ml).

Test chemicals

The phosphates tested included MSP, DSP, and TSP (Fisher Scientific), SAPP (Stauffer Chemical Co.), TSPP and STPP (Monsanto), and SPG and UP (BK-Ladenburg), and EDTA (Fisher Scientific).

Media

Phosphates were added in solid form to TSB (BBL) at concentrations of 0.5%, 2% and 4% (w/v). The pH was adjusted to pH

pH 5.5 (5 N HCL or 4 N NaOH), brought up to volume (50 ml) with TSB and the final pH measured. The solutions were filter sterilized using a 0.22μ Cameo IV filter unit (Micron Separations, Inc.) and dispensed (20 ml) into sterile screw cap tubes. An uninoculated and inoculated *P. fragi* control consisted of distilled water, used in place of the test chemical, and TSB adjusted to pH 5.5. Tubes were inoculated (0.1 ml *P. fragi* inoculum) and incubated for 48 hours at 30° C. Samples were taken and examined at 0, 4, 8, 16 and 24 hours for microbiological examination, protease activity and protein determination. The experiment was replicated three times.

Microbiological examination

Aerobic mesophilic bacterial populations were determined by using standard procedures. Serial dilutions in 0.1% peptone (BBL) were made and pour plates (TSA, BBL) were performed in duplicate at each dilution.

Protease activity assay

The procedure was described in Experiment IV. One protease activity unit was expressed as 1μg of tyrosine equivalent liberated per ml enzyme solution per minute at 35° C.

Protein determination

Protein concentration was measured using the BCA protein asssay method (Pierce) and expressed in mg of protein.

Analysis of data

After logarithmic transformation, statistical analysis was performed using the statistical analysis system's (SAS Institute, 1979) analysis of variance (ANOVA) and general linear models (GLM) procedures. When more than two treatments were being compared, Duncan's multiple range test (Steel and Torrie, 1980) was used.

Results and Discussion

The preformed protease was removed and the cells were exposed to new media with or without phosphates. The phosphate concentrations used in this experiment were 0.5%, 2% and 4%. The concentration of 0.5% was used since it is the maximum level allowed by regulation in meat products.

Growth was significantly affected by selected phosphates over time and by concentration. As time increased, more phosphates became inhibitory. This is shown in Figures 36-38. At 0.5% and at time 24 hours, EDTA, SPG and UP significantly reduced *P. fragi* growth, whereas, at time 4 hours, only SPG significantly reduced growth. At a concentration of 2% and at time 4 hours, only SPG, UP and STPP inhibited growth while at 24 hours all phosphates, except MSP and TSP, reduced growth. At 4% and at 4 hour, all phosphates except MSP significantly reduced growth whereas at 24 hour all phosphates reduced *P. fragi* growth. In addition, as the concentration of the phosphate increased, more phosphates are able to decrease *P.*

fragi growth. At 0.5%, after 24 hours, EDTA, SPG and UP significantly reduced *P. fragi* growth (Figure 36). At 2% (Figure 37), more phosphates had an inhibitory effect after 24 hours. These treatments included SPG, UP, STPP, EDTA, SAPP, DSP and TSP (Figures 37-39). All phosphate treatments were able to reduce growth at 4% (Figure 38).

P. fragi protease production was monitored by using activity/ mg protein (Figures 39-41). The results indicated that after 24 hours, protease activity was not affected, although it was reduced by all phosphates at time 8 hours. This time period corresponded to the logarithmic growth phase (Figure 37) of *P. fragi.* At late logarithmic growth phase (hour 16), several phosphates were able to reduce the activity of the protease. These included TSPP and STPP (0.5%), TSPP, STPP and EDTA (2%), and only EDTA at 4%. These results indicated that early logarithmic growth phase was most affected by the phosphates when compared to the control. The organism became more resistant to the phosphates late in the growth cycle which may indicate that the phosphates are affecting something necessary for the growth of the organism. This may include the chelation of essential metal cations or the phosphates may be affecting mineral transport systems.

The activity/ organism (Figures 42-44) was not significantly affected by phosphate at any concentration which is in agreement with the results found in experiment IV.

Conclusions

Pseudomonas fragi growth was affected by both the concentration of phosphates and the length of time the organism was in contact with the phosphate. All phosphate treatments were able to reduce growth at 4%. The longer *P. fragi* was in the presence of the phosphates, the more the phosphates became inhibitory.

Protease production is affected by phosphates at the early logarithmic growth phase. However, at later stages of growth *P. fragi* is resistant to the phosphates.

Figure 36. Effects of 0.5% phosphates on the growth of *Pseudomonas fragi* in a synthetic medium

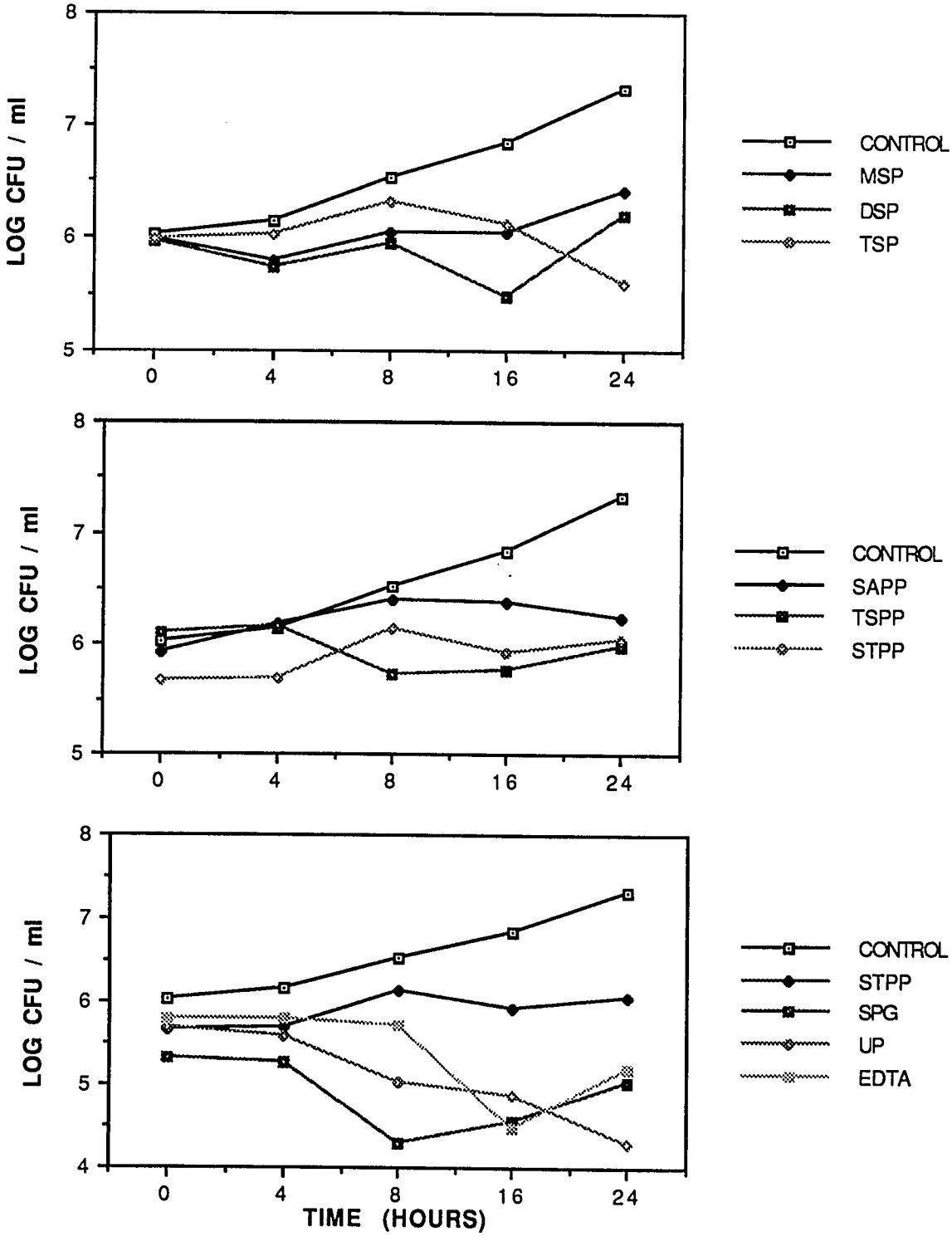

LOG CFU / ml
8
7
6
5
0
4
8
16
24
CONTROL
MSP
DSP
TSP
LOG CFU / ml
CONTROL
SAPP
TSPP
STPP
LOG CFU / ml
4
CONTROL
STPP
SPG
UP
EDTA
TIME (HOURS)

Figure 37. Effects of 2% phosphates on the growth of *Pseudomonas fragi* in a synthetic medium

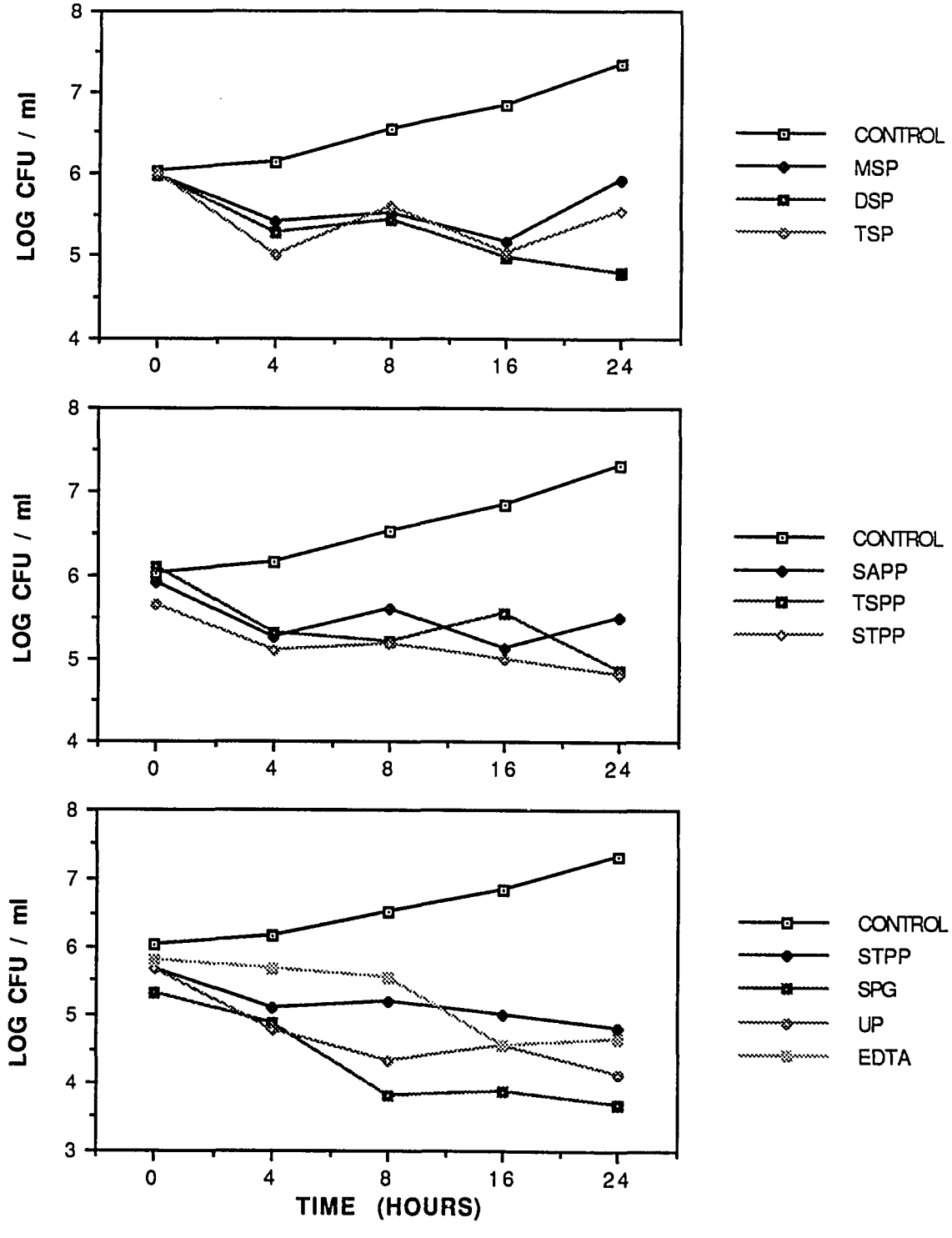

LOG CFU / ml
8
7
6
5
4
0
4
8
16
24
CONTROL
MSP
DSP
TSP
LOG CFU / ml
CONTROL
SAPP
TSPP
STPP
LOG CFU / ml
3
CONTROL
STPP
SPG
UP
EDTA
TIME (HOURS)

Figure 38. Effects of 4% phosphates on the growth of *Pseudomonas fragi* in a synthetic medium

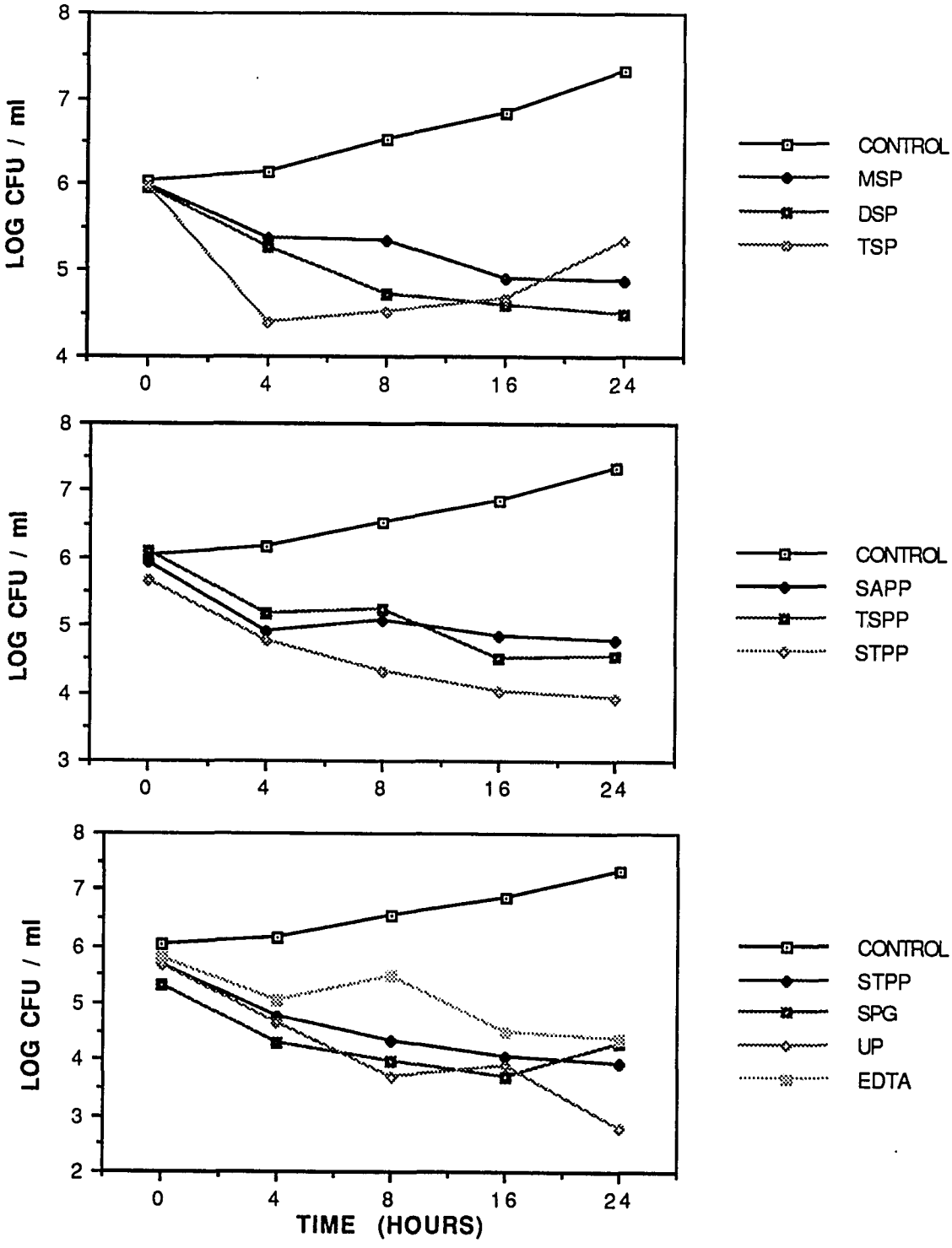

LOG CFU / ml
8
7
6
5
4
0
4
8
16
24
CONTROL
MSP
DSP
TSP
LOG CFU / ml
8
7
6
5
4
3
0
4
8
16
24
CONTROL
SAPP
TSPP
STPP
LOG CFU / ml
8
7
6
5
4
3
2
0
4
8
16
24
TIME (HOURS)
CONTROL
STPP
SPG
UP
EDTA

Figure 39. Effects of 0.5% polyphosphates on the*Pseudomonas fragi* protease (activity/protein) in a synthetic medium

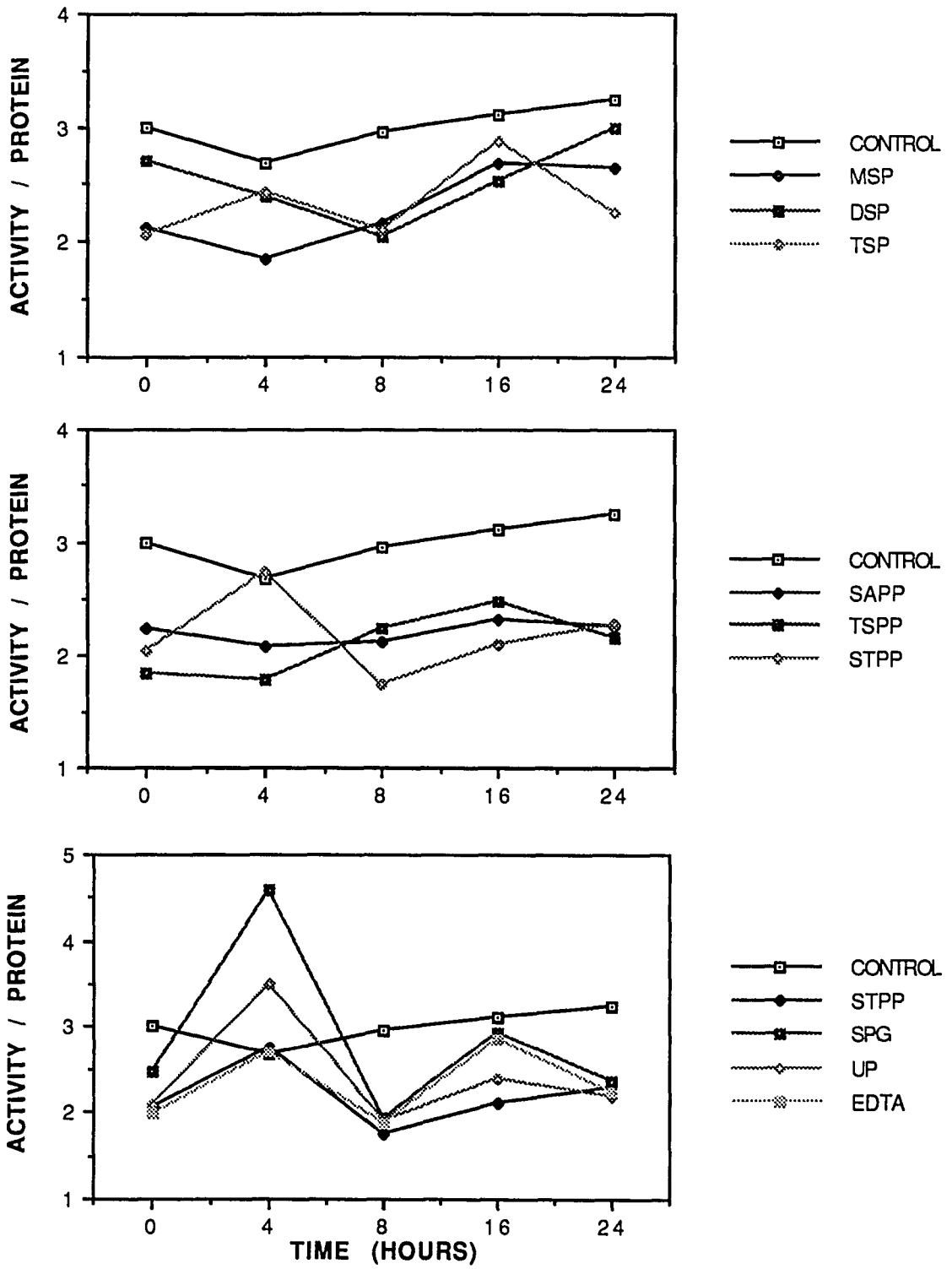

ACTIVITY / PROTEIN
4
3
2
1
0
4
8
16
24
CONTROL
MSP
DSP
TSP
ACTIVITY / PROTEIN
CONTROL
SAPP
TSPP
STPP
ACTIVITY / PROTEIN
5
CONTROL
STPP
SPG
UP
EDTA
TIME (HOURS)

Figure 40. Effects of 2% phosphates on the *Pseudomonas fragi* protease (activity/protein) in a synthetic medium

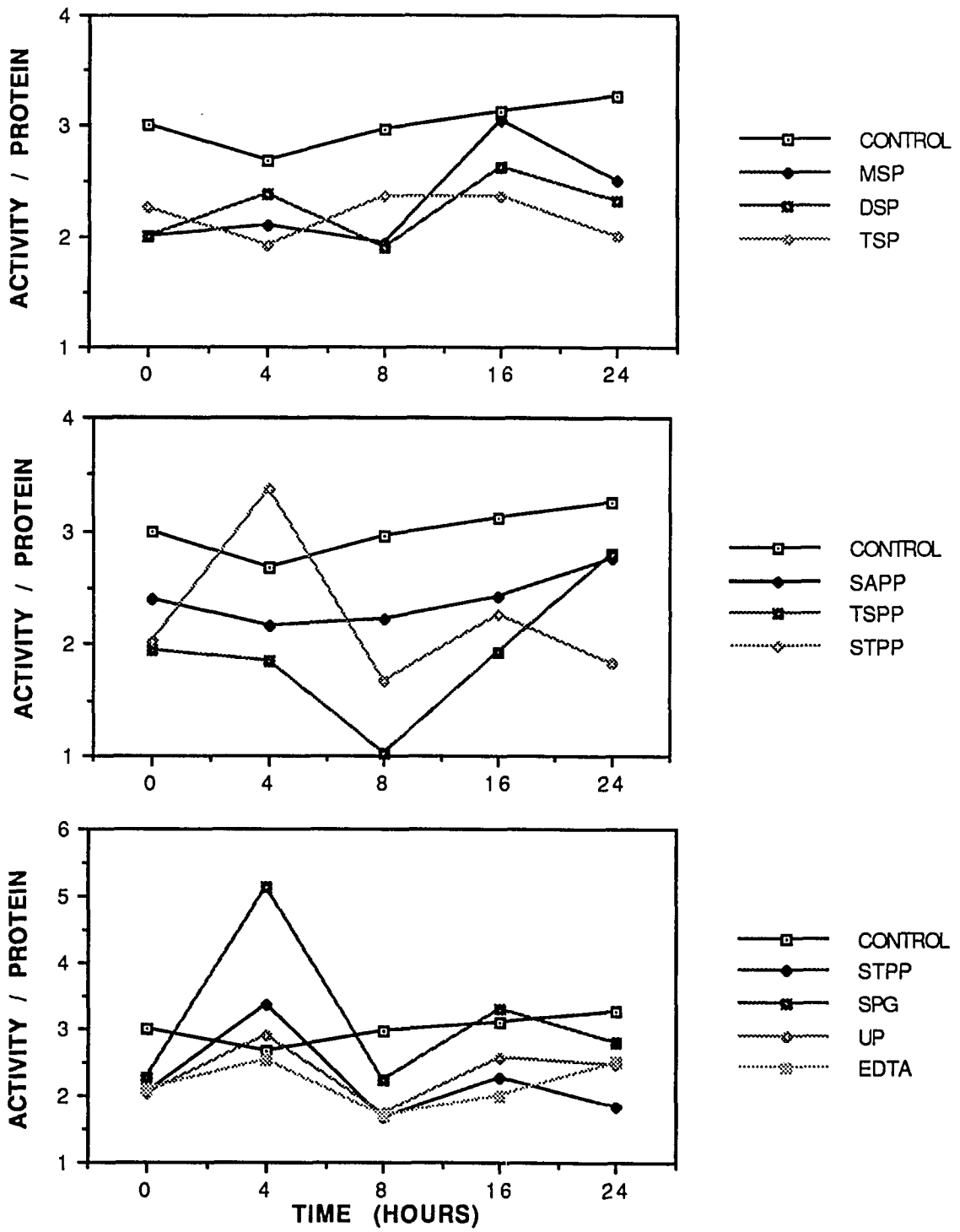

ACTIVITY / PROTEIN
4
3
2
1
0
4
8
16
24
CONTROL
MSP
DSP
TSP
ACTIVITY / PROTEIN
CONTROL
SAPP
TSPP
STPP
ACTIVITY / PROTEIN
6
5
4
3
2
1
CONTROL
STPP
SPG
UP
EDTA
TIME (HOURS)

Figure 41. Effects of 4% phosphates on the *Pseudomonas fragi* protease (activity/protein) in a synthetic medium

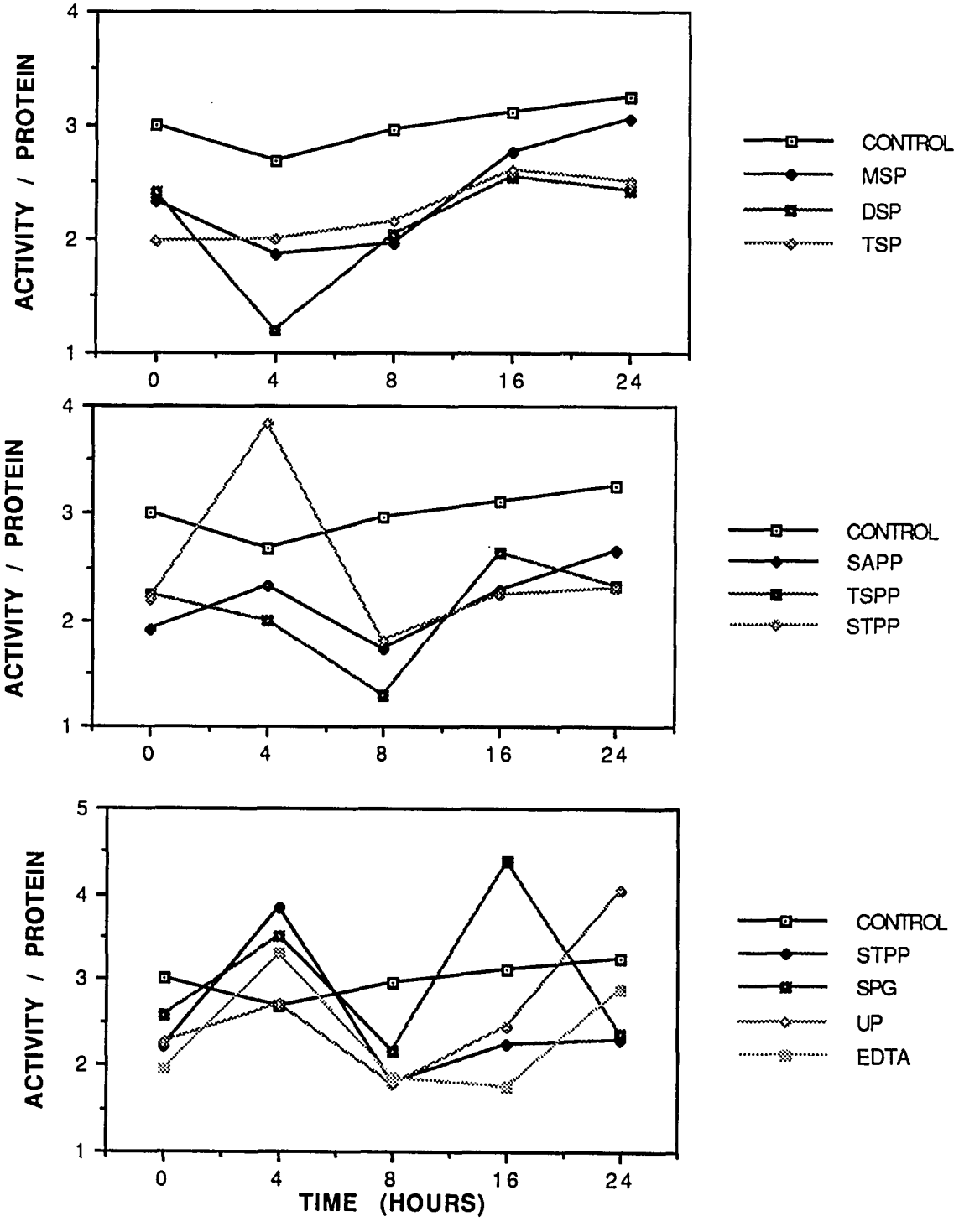

ACTIVITY / PROTEIN
4
3
2
1
0
4
8
16
24
CONTROL
MSP
DSP
TSP
ACTIVITY / PROTEIN
CONTROL
SAPP
TSPP
STPP
ACTIVITY / PROTEIN
5
CONTROL
STPP
SPG
UP
EDTA
TIME (HOURS)

Figure 42. Effects of 0.5% phosphates on the*Pseudomonas fragi* protease (activity/organism) in a synthetic medium

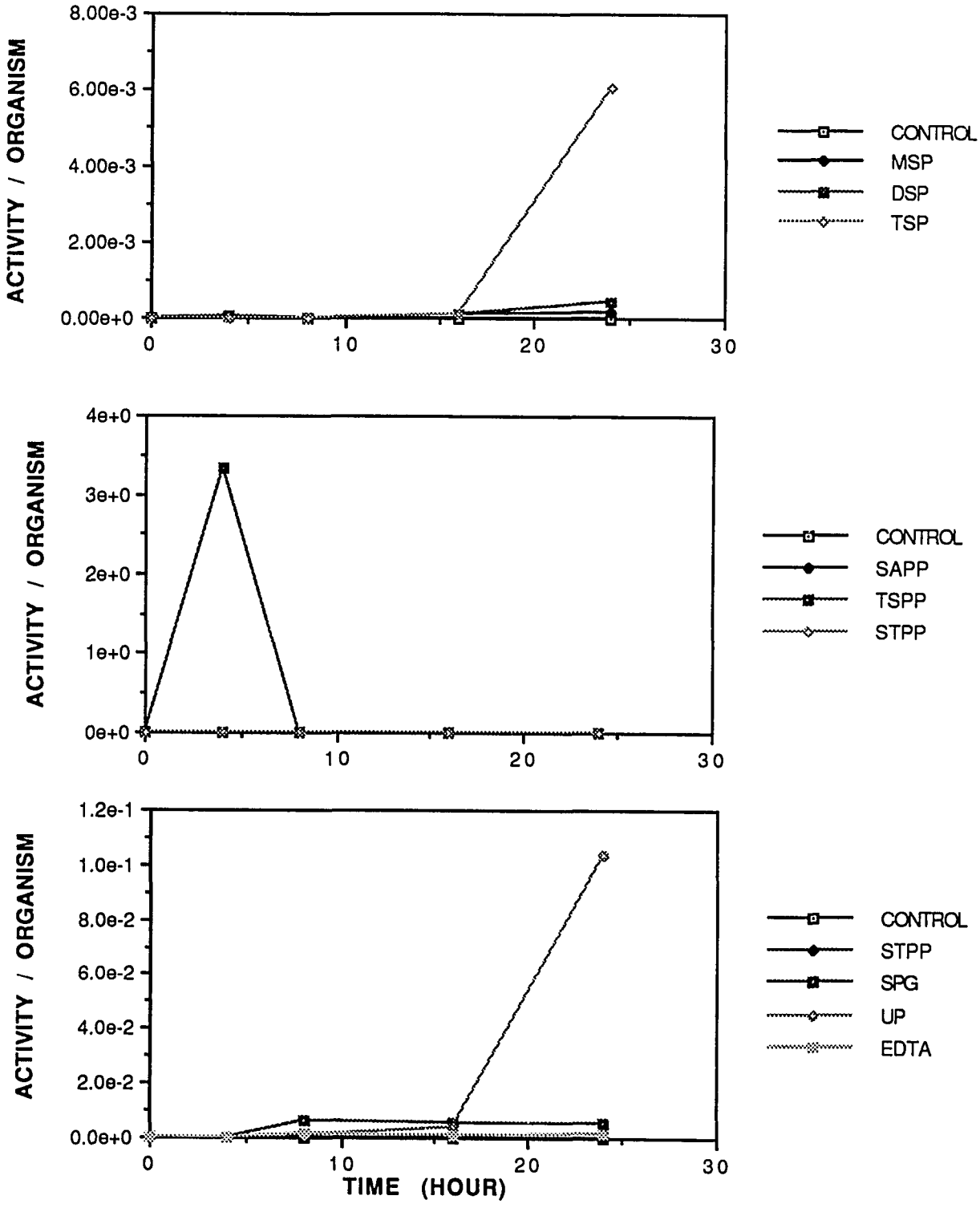

ACTIVITY / ORGANISM
8.00e-3
6.00e-3
4.00e-3
2.00e-3
0.00e+0
0
10
20
30
CONTROL
MSP
DSP
TSP
ACTIVITY / ORGANISM
4e+0
3e+0
2e+0
1e+0
0e+0
0
10
20
30
CONTROL
SAPP
TSPP
STPP
ACTIVITY / ORGANISM
1.2e-1
1.0e-1
8.0e-2
6.0e-2
4.0e-2
2.0e-2
0.0e+0
0
10
20
30
TIME (HOUR)
CONTROL
STPP
SPG
UP
EDTA

Figure 43. Effects of 2% phosphates on the *Pseudomonas fragi* protease (activity/organism) in a synthetic medium

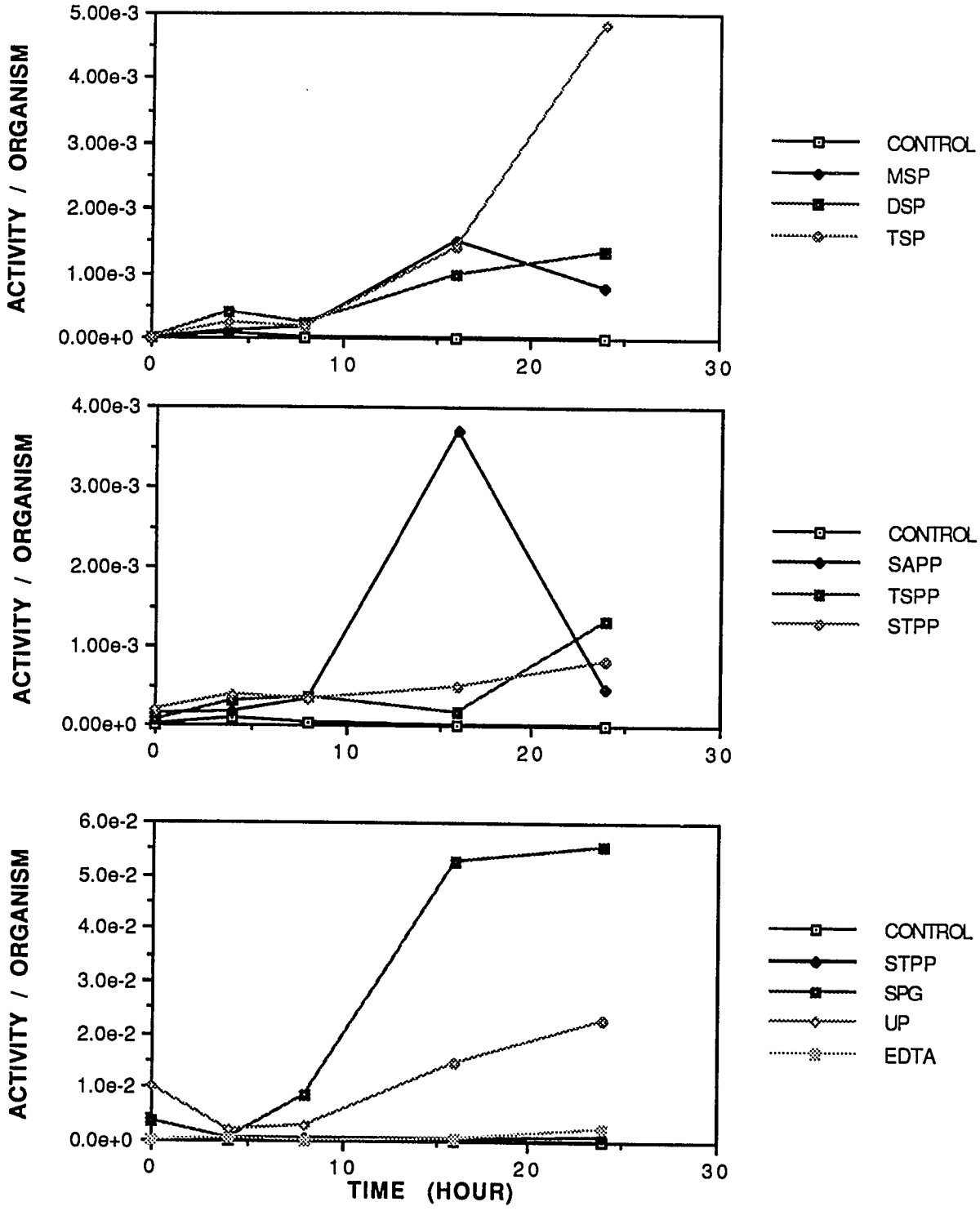
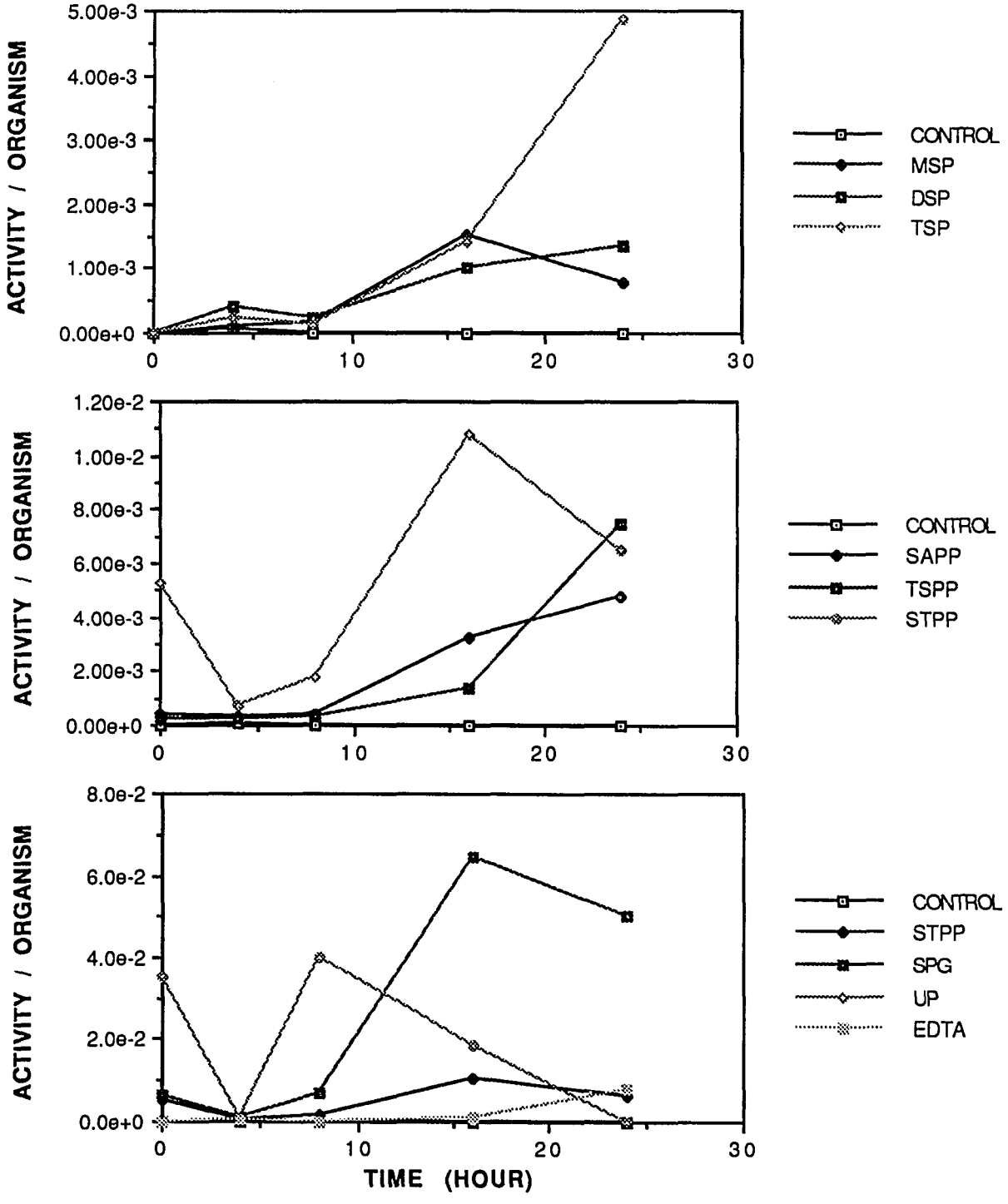

ACTIVITY / ORGANISM
5.00e-3
4.00e-3
3.00e-3
2.00e-3
1.00e-3
0.00e+0
0
10
20
30
CONTROL
MSP
DSP
TSP
ACTIVITY / ORGANISM
4.00e-3
3.00e-3
2.00e-3
1.00e-3
0.00e+0
0
10
20
30
CONTROL
SAPP
TSPP
STPP
ACTIVITY / ORGANISM
6.0e-2
5.0e-2
4.0e-2
3.0e-2
2.0e-2
1.0e-2
0.0e+0
0
10
20
30
TIME (HOUR)
CONTROL
STPP
SPG
UP
EDTA

Figure 44. Effects of 4% phosphates on the *Pseudomonas fragi* protease (activity/organism) in a synthetic medium


ACTIVITY / ORGANISM
5.00e-3
4.00e-3
3.00e-3
2.00e-3
1.00e-3
0.00e+0
0
10
20
30
CONTROL
MSP
DSP
TSP
ACTIVITY / ORGANISM
1.20e-2
1.00e-2
8.00e-3
6.00e-3
4.00e-3
2.00e-3
0.00e+0
0
10
20
30
CONTROL
SAPP
TSPP
STPP
ACTIVITY / ORGANISM
8.0e-2
6.0e-2
4.0e-2
2.0e-2
0.0e+0
0
10
20
30
TIME (HOUR)
CONTROL
STPP
SPG
UP
EDTA

EXPERIMENT VII

Materials and Methods

An attempt was made to purify the *P. fragi* protease and use it for inhibition studies with selected phosphates.

Inoculum

TSB (100 ml) was inoculated with *P. fragi* ATCC 4973 and grown at 30° C. TSB (1.5 L) was inoculated to 4% with the overnight culuture. The inoculated medium was aseptically, and equally dispensed between three 2-L flasks. The culture was incubated for 72 hours at 15° C using an orbit rotary shaker (200-230 rpm) (Lab-Line, Melrose, IL). After 72 hours, growth was about $1x10^9$ CFU/ml.

Protease purification

Procedures of Porzio and Pearson (1975) and Thompson et al. (1983) (Figures 45 and 46, respectively) were followed with the exception that the DEAE-Sephadex step was left out.

Protease activity assay

The procedure was described in Experiment IV. One protease activity unit was expressed as 1µg of tyrosine equivalent liberated per ml enzyme solution per minute at 35° C.

Protein determination

Protein concentration was measured using the BCA protein asssay method (Pierce) and expressed in mg of protein.

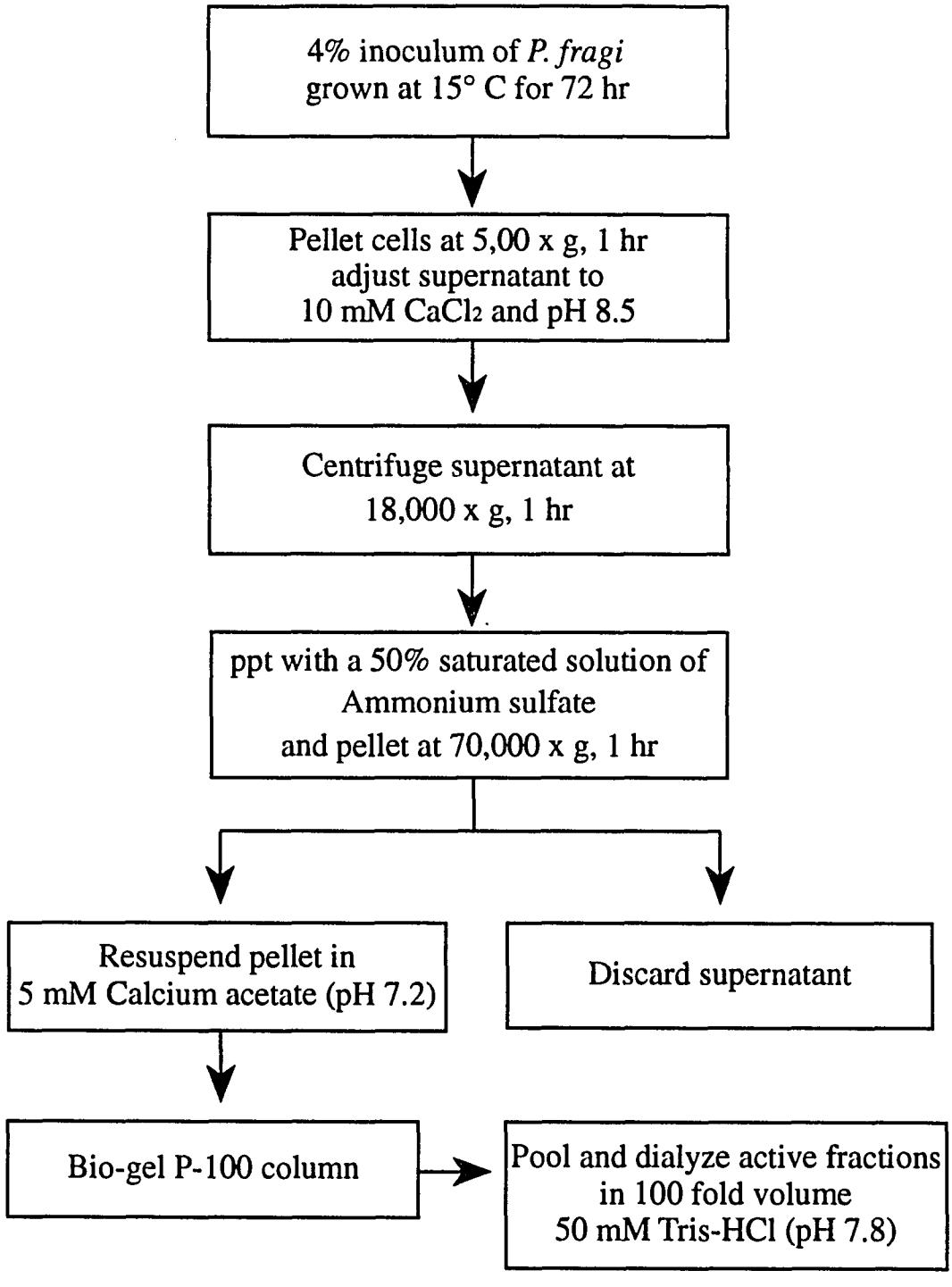


Figure 45. Porzio and Pearson (1975) P. fragi protease purification scheme.

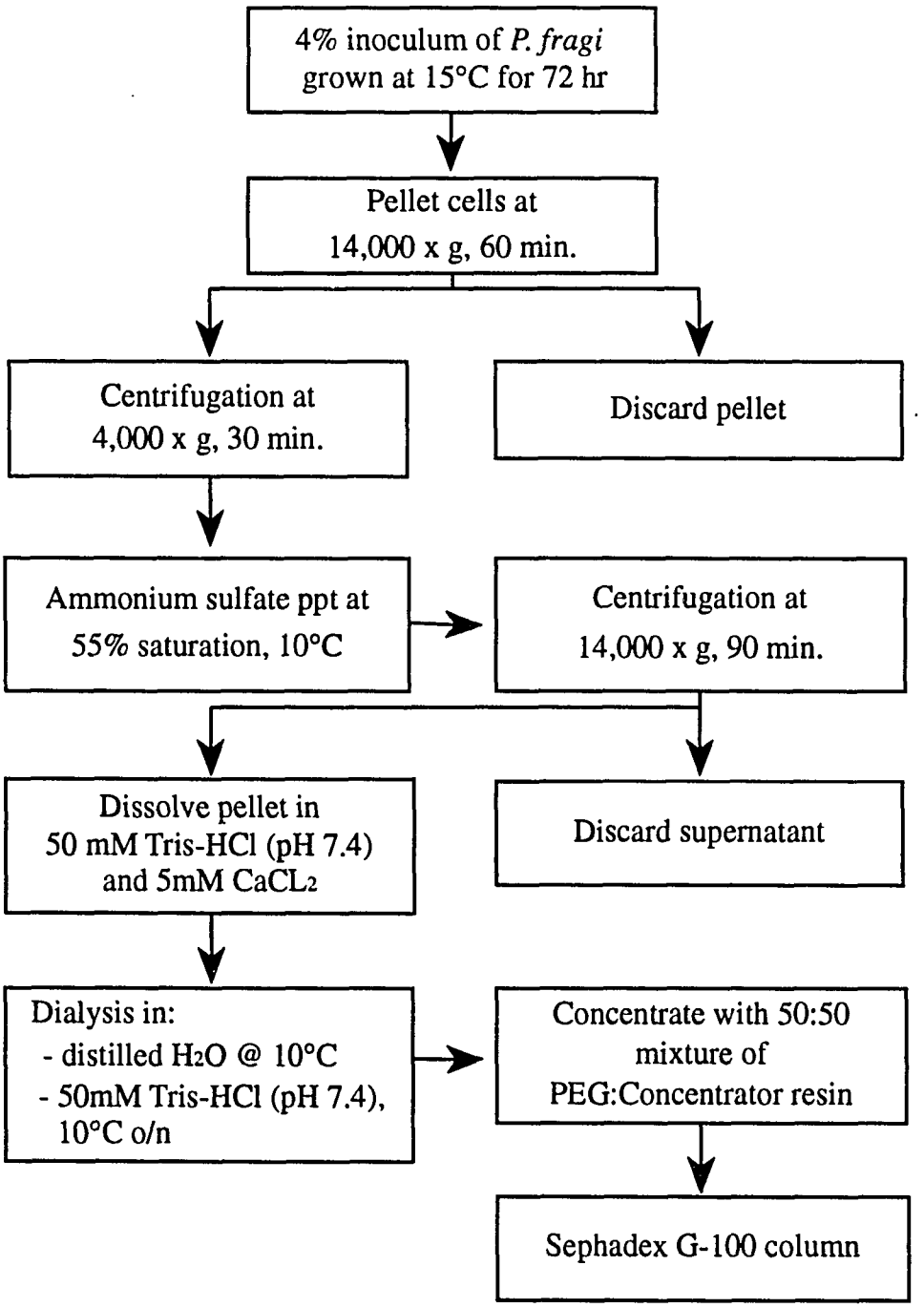


Figure 46. Thompson et al. (1983) *P. fragi* protein purification scheme.

Electrophoresis

SDS-PAGE gels were performed using 7.5% acrylamide/0.18% bis-acrylamide at pH 8.9 according to the procedure of Davis (1964). Protein was stained with Coomassie brilliant blue reagent (0.25%, Sigma) and destained in 10% (v/v) acetic acid/40% (v/v) methanol.

Enzyme reactivation with zinc

Two concentrations of zinc chloride (0.5 mM and 0.05 mM) (Fisher Scientific) were used in this experiment. Aliquots were added (0.5 ml) to the resuspended ammonium sulfate precipitate enzyme preparation (0.5 ml). The zinc chloride was allowed to react with the enzyme for 0, 1, 2, 5, 10, 15, 20 and 30 minutes. At each time, 1 ml was removed and assayed for protease activity, as described in Experiment IV. A control consisted of 0.5 ml enzyme preparation and 0.5 ml deionized water.

Results and Discussion

The enzyme was not able to be purified to 38-fold or 60-fold according to the methods of Thompson et al. (1985) or Porzio and Pearson (1975). The enzyme lost activity after precitation with ammonium sulfate in both procedures (Figures 45 and 46). SDS-PAGE gels suggested purification of the enzyme since the appearance of a single band on the gel (bands 1 and 2, Figure 47). However, there was no corresponding increase in activity. In fact there was no activity. It was thought that the zinc component of the metallo-enzyme

was becoming dissociated possibly at the desalting step of the purification procedure. Zinc chloride was added to the enzyme preparations that had no activity to see if reactavation would occur. After zinc was added, activity was restored (Figure 48) but only briefly. Possibilities to the loss of activity include an inappropriate enzyme assay or that the enzyme was autoproteolytic. Inhibition studies using the purified enzyme in the presence of phosphate could not be done.

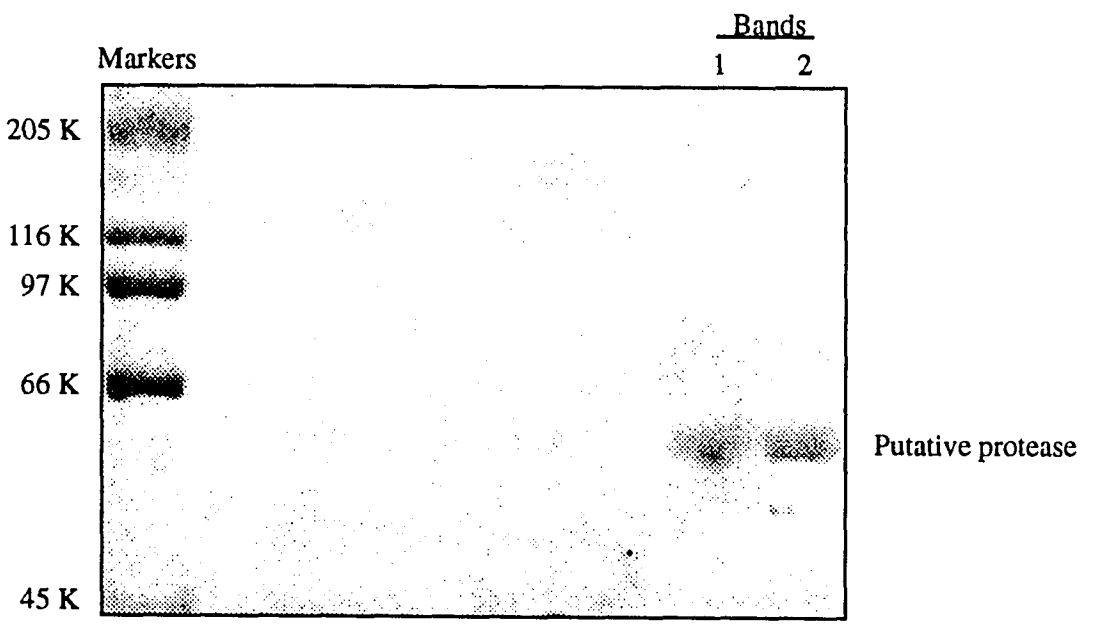


Figure 47. Sodium dodecyl sulfate-polyacrylamide gel of *P. fragi* protease

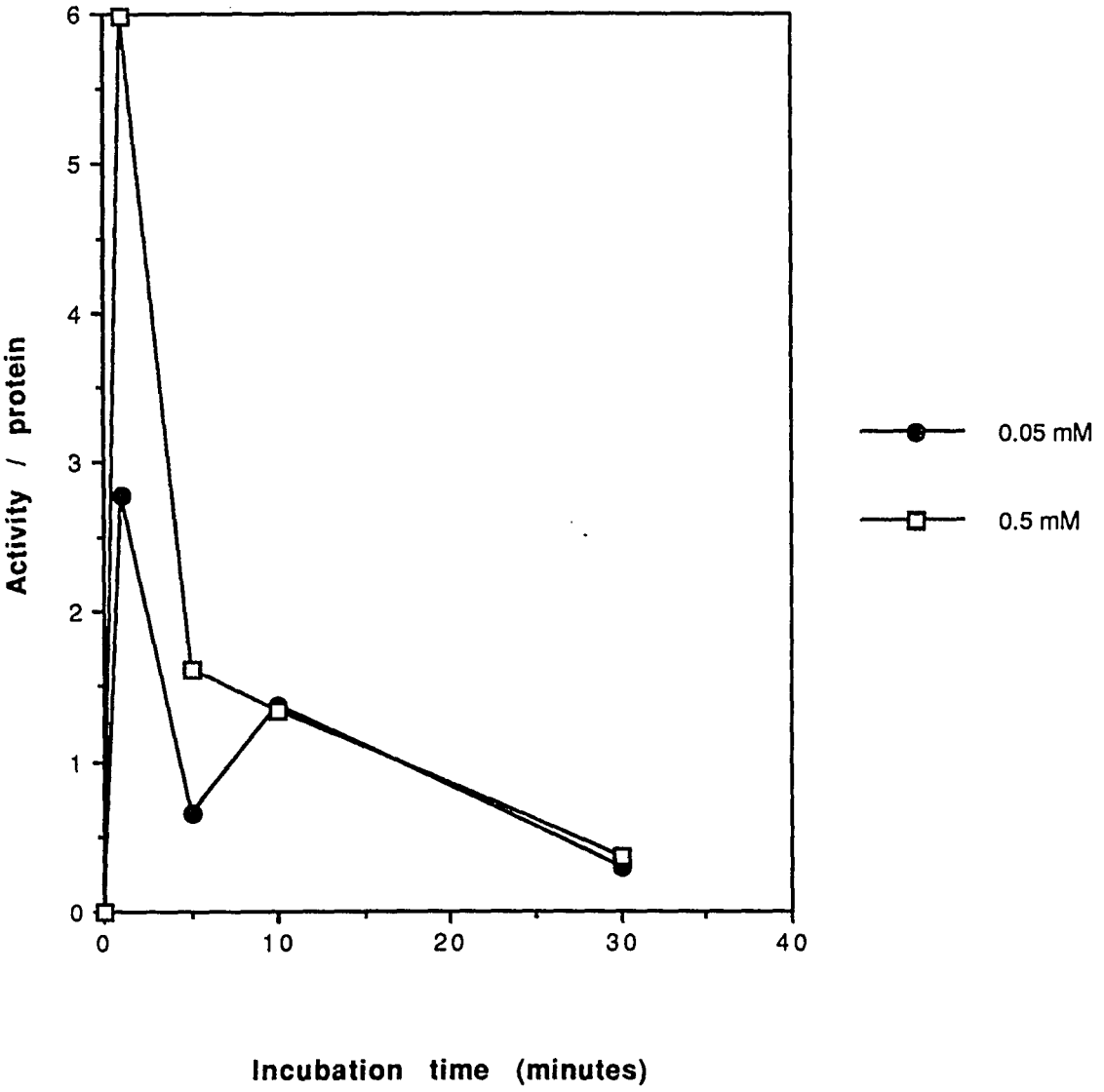


Figure 48. Addition of zinc chloride to *P. fragi* purification preparations

Conclusions

Enzyme activity was lost.during the purification tests.and could not be restored. SDS-PAGE results suggest increased purification however no corresponding enzyme activity supported this data.

Recommendations

The intent of this research project was to determine the antimicrobial activities of phosphates on the growth and protease production and activity of *Pseudomonas fragi.* It was not to determine the mechanism of action of the phosphates. However, future research may include the elucidation of the phosphate's mechanism on the growth of this organism, especially since it is a Gram-negative organism. Very little research has dealt with the mechanism of phosphates on the growth of Gram-negative bacteria. Future research may initially include looking at the types of phosphatases present in *P. fragi.* This would help to determine the phosphate hydrolysis capabilities of this organism. Additionally, experiments designed to determine the effects of phosphates on the cell wall and cell membrane, including transport systems, may be done.

Completing the experiments dealing with the effects of phosphates on the purified enzyme would be beneficial. It is recommended that the buffers contain zinc chloride to reactivate the enzyme during the purification procedures.

The effects of phosphates on meat proteins, as determined by SDS-PAGE, would be helpful in understanding the proteolytic abilities of the protease. Although research has been done on the effects of the protease on meat proteins, in a variety of meat systems, the activity of the protease in meat, in the presence of phosphates, has not been studied.

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