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Bacteriostatic Action of Sorbic Acid: A Hypothesis

Induction of a Stringent-type Response in Putrefactive Anaerobe 3679 by the Effects of the Protonophoric Activity of Sorbic Acid

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

AUGUST 1985

By

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I want to thank my husband Ronald J. Ricupero for his love, patience, encouragement, and continued support, and to thank my daughter Gina for joy and inspiration.

I want to thank Dr. Hilmer Frank for his guidance, lively and stimulating discussions, patience, encouragement, and friendship.

My gratitude also goes to Dr. David Karl, Dr. Christopher Winn, and Ms. Ursula Magaard for their advice with the nucleotide experiments, with a special thanks to Dr. Karl for the use of his laboratory and equipment.

I want to thank Dr. Arlan Hinchee for instructing me on the use of the electron microscope and the Department of Botany, University of Hawaii, for the use of its electron microscope.

My gratitude also goes to Drs. Kim Bridges and Tom Pitcher for discussions on the statistical analysis of data.

Special thanks goes to Ms. Thelma Narita for her invaluable assistance in helping type and retype preliminary versions of this document.

However, for life itself, I thank God who has also given me strength, wisdom, faith, and hope to continue.
ABSTRACT

The inhibitory effect of potassium sorbate on bioenergetics, amino acid uptake, protein synthesis, cell regulation, and morphology was examined in Putrefactive Anaerobe (PA) 3679. Undissociated sorbic acid appeared to act as a protonophore, lowering the intracellular pH and dissipating the protonmotive force of the membrane. Sorbate and benzoate inhibited uptake of the amino acid, phenylalanine. Further studies on the effects of sorbate on PA 3679 revealed altered patterns of phosphorylated nucleotide accumulation with increased rates of formation of GTP, ppGpp, and an unidentified compound, possibly pppGpp, and a decreased rate of protein synthesis. The addition of a noninhibitory amount of tetracycline released the sorbate-induced inhibition of growth in PA 3679. Based on these results, it is concluded that inhibition of PA 3679 by sorbate resulted from a stringent-type response to the protonophoric activity of sorbic acid.

Similar morphological changes were observed when PA 3679 was treated with sorbate or inorganic acid, HCl. In both cases, the cells became filamentous with bends and bulges and had thickened cell walls. Sorbate-induced filaments had fewer septa than did the acid-treated cells. In contrast nitrite-treated cells also became filamentous
with bends, but the cells had many septa, did not have thickened cell walls, and did not bulge.

Low concentrations of sucrose and sodium chloride antagonized the inhibitory effects of sorbate on the growth of PA 3679; however, a low concentration of nitrite acted synergistically with sorbate. Nitrite alone increased the intracellular pH and protonmotive force in untreated as well as sorbate-treated cells.
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LIST OF ABBREVIATIONS AND SYMBOLS

PA Putrefactive Anaerobe
DMO dimethyloxazolidinedione
pmf protonmotive force
pH$_i$ intracellular pH
pH$_o$ extracellular pH
pH proton gradient
membrane potential
TPPBr$^+$ triphenylmethylphosphonium bromide
TCA trichloroacetic acid
ATP adenosine triphosphate
GTP guanosine triphosphate
ppGpp guanosine tetraphosphate
pppGpp guanosine pentaphosphate
dpm disintegrations per minute
CFU colony forming units
DAP diaminopimelic acid
I. INTRODUCTION

Sorbic Acid as a Preservative: Literature Review

Sorbic Acid and/or its potassium salt are widely used in the preservation of various food products, e.g. dairy and bakery products, fruit and vegetable products, beverages, fish and meat products (Sofos and Busta, 1981). In 1945, C.M. Gooding of The Best Foods, Inc. was awarded a U.S. patent for sorbic acid as a fungistat, currently its primary use. In the 1940's the antibacterial properties of sorbic acid were discovered but further investigation indicated that its activity against bacteria is not as comprehensive as it is against yeasts and molds. However, the recent desire to decrease the nitrite added to meat products due to the possible formation of carcinogenic or mutagenic compounds has resulted in increased interest in sorbic acid/sorbate as a complete or partial replacement for nitrite in processed meat products (Robach and Sofos, 1980, 1981; Marriott et al., 1981; Huhtanen, 1984). Of further interest are the effects of sorbic acid in combination with other compounds that are added to cured meat products, e.g. sodium chloride, sucrose, and reduced amounts of sodium nitrite. Prior studies have indicated that the antibacterial effect of sorbic acid is synergistic with these compounds (Busta, 1982).

Surprisingly, despite extensive research (over 3,000 papers by 1976), the mechanism of action has remained unknown although its elucidation would be very useful in
the application of sorbic acid as a food preservative. Until recently, research with sorbic acid/sorbate has been primarily of an applied nature and literature on the mechanism of action has consisted of speculation based on generalizations extracted from limited research (Sofos and Busta, 1981, 1982, 1983).

Early studies on the possible mechanism of action of sorbic acid suggested direct inhibition of various enzymes or enzyme systems, e.g. dehydrogenases involved in beta oxidation of fatty acids in molds (Melnick et al., 1954), fumarase (York and Vaughn, 1955), aspartase, succinic dehydrogenase, yeast alcohol dehydrogenase (Martodiprawito and Whitaker, 1963; Whitaker, 1959; York and Vaughn, 1964), enolase (Azukas et al., 1969), proteinase (Dahle and Mordal, 1972), enzyme reactions involving coenzyme A (Harada et al., 1968), catalase or synthesis of catalase (Troller, 1963), and others. There is little or no agreement among the hypotheses derived from these studies and the enzymes inactivated by sorbic acid are not present in all the organisms whose growth is inhibited (e.g. the growth of PA 3679 is inhibited but the organism is catalase negative and does not have a tricarboxylic acid cycle).

In an attempt to explain the activity of sorbic acid, general mechanisms of action for fatty acids have been proposed but no hypothesis, supported by tangible evidence, has been presented for sorbic acid. In their work with lipophilic acid preservatives, Freese et al. (1973) and
Sheu et al. (1975) stated that sorbic acid uncouples both oxidative phosphorylation and substrate transport from the electron transport system but did not explain how this occurred or what effects it would have on growth. In a study on the resistance mechanism of *Saccharomyces bailii* to weak acid preservatives, Warth (1977) suggested that sorbic acid may uncouple active transport from an energy supply but noted that work on the mechanism of resistance to these preservatives was hampered by the lack of understanding of their mechanism of action. Based on amino acid uptake experiments with *Pencillium chrysogenum*, Hunter and Segel (1973) postulated that sorbic acid, by diffusing through the cell membrane in the undissociated state and ionizing in the more alkaline interior, can discharge the proton gradient involved in energizing membrane transport. In a study using bacterial vesicles, Eklund (1980), reported that sorbic acid inhibited the uptake of various amino acids in whole cells and vesicles of *Escherichia coli*, *Bacillus subtilis*, and *Psudomonas aeruginosa* and suggested that inhibition was probably due to the protonophoric activity of sorbic acid. This explanation was based on speculation because the possible effects of sorbic acid on the intracellular pH had not as yet been tested. Eklund could not explain how the amount of inhibition of amino acid uptake could result in the degree of growth inhibition seen in living cells.
Recently, Salmond et al. (1981) using $^{14}\text{C}$-dimethylloxazolidinedione (DMO) (also used in this study, page 38), measured the internal pH of *E. coli* after treating the cells with various food preservatives, including sorbic acid. Their data suggest that the potency of weak acid food preservatives is related to their capacity to reduce the intracellular pH. The authors did not explain how the decreased intracellular pH could lead to the observed degree of growth inhibition. Eklund (1985) studied the effects of sorbic acid and other preservatives on the protonmotive force (pmf) in *E. coli* vesicles by measuring the magnitude of the pH gradient and the membrane potential across vesicular membranes. In order to determine the proton gradient, $^{14}\text{C}$-benzoic acid was used to measure the intravesicular pH (a variation of the DMO method), and methyl-$^3\text{H}$-triphenylmethylphosphonium bromide, a membrane permeable ion, was used to measure the membrane potential. Although the protonmotive force was a function of the concentration of the preservatives, Eklund stated that he was unable to correlate the complete dissipation of the pmf of the vesicles with the limited growth inhibition seen in whole cells. The author apparently did not consider the buffering capacity of the cytoplasm of the whole cells or the ability of living cells to maintain their pmf by regulating both their proton gradient and their membrane potential (Skulachev, 1981).
Tuncan and Martin (1985) studied the effects of pH, temperature, and potassium sorbate concentration on amino acid uptake in *Salmonella typhimurium* by measuring the amount of radiolabelled amino acids in treated and untreated cells after incubation for one minute without the addition of a protein synthesis inhibitor. Unfortunately, since protein synthesis was not taken into consideration and radiolabelled amino acids are incorporated into protein only a few seconds after addition to the medium (Schlief, 1967), the results they obtained reflect both uptake and protein synthesis. Since an incubation period of one minute is too long to study uptake yet too short for protein synthesis, their results are difficult to interpret. However, the authors obtained fewer counts per minute with sorbate-treated cells and suggested that sorbic acid inhibits amino acid uptake by competitively binding to the amino acid permeases. However, sorbic acid has been shown to affect the uptake of many different amino acids (Eklund, 1980) which utilize different, specific permease systems. It seems unlikely that these permeases, which have the capacity to differentiate among different amino acids, cannot detect the difference between a specific amino acid and sorbic acid. Sorbic acid was not mentioned as a possible protonophore, and any possible connection between the observed effects and cellular bioenergetics was not discussed.
Objective

The primary objective of this dissertation was to study the antimicrobial activity of sorbic acid/sorbate particularly with regard to its possible use as a nitrite substitute for the preservation of processed meat products. The effects of sorbic acid on aspects of bacterial growth, bioenergetics, amino acid uptake, protein synthesis, and cell regulation were examined in order to explain its mechanism of action. A better understanding of how sorbic acid inhibits bacterial growth would improve its application as a food preservative. The effects of sorbic acid on bacterial growth in combination with nitrite, sodium chloride, and sucrose also were examined since these compounds are often used in processed meat products and have been reported to be synergistic with sorbic acid (Busta, 1982).

Sorbic Acid, the Preservative

Sorbic acid is a weak lipophilic, 2,4-hexadienoic monocarboxylic aliphatic acid (CH₃-CH=CH-CH=CH-COOH) whose carboxyl group reacts readily to form salts and esters. The potassium salt, which was used in this study, is especially important in applications because of its high solubility (58.2%) in water as compared with that of sorbic acid (0.25%). The relationship between the pH and the amount of undissociated sorbic acid (pKₐ' of sorbic acid = 4.76, dissociation constant = 1.73 x 10⁻⁵) is particularly important because the antimicrobial activity of sorbic acid
has been shown to be pH dependent, inhibition increasing with decreasing pH values (Robach and Sofos, 1980, 1981; Marriott et al., 1981; Huhtanen, 1984).

Organism Used to Study, Putrefactive Anaerobe 3679

Prior studies have demonstrated that sorbate inhibits the growth of *Clostridium botulinum*, a primary concern in the preservation of meat products (Sofos et al., 1979; Robach, 1980; Sofos and Busta, 1980, 19891; Marriott et al., 1981, Robach and Sofos, 1982). However, because of laboratory risks involved in working with *C. botulinum*, which produces an extremely potent neurotoxin, a non-toxic substitute was used. The organism used in this study was originally isolated by the National Canners Association Research Laboratories and given the name Putrefactive Anaerobe (PA) 3679. PA 3679, a Gram positive, sporeforming rod, belongs to the genus *Clostridium* and is considered by some to be *C. sporogenes* (Williams, 1940; Sognefest and Benjamin, 1944). PA 3679 is non-pathogenic to human beings but has sufficient cultural similarities to proteolytic *C. botulinum* to be an accepted experimental substitute.

Since botulinal toxin production is associated with vegetative growth, vegetative cultures of PA 3679 were used in this study. In growth inhibition experiments using colony formation as an indication of viability, it was possible to determine the levels of sorbate that exhibited bactericidal or bacteriostatic effects on PA 3679. Because such information could not have been obtained with commonly
used methods of turbidity measurement for dilute inocula (10^3 cells ml^{-1}), colony counts were used for all vegetative cell number determinations often in conjunction with direct microscopic counts with a Petroff-Hausser counting chamber. Small inocula of about 3 \times 10^3 cells ml^{-1} were used in growth inhibition experiments because they more accurately simulated the likely number of clostridia surviving in a processed meat product.

Although PA 3679 is a strict anaerobe, it is quite aerotolerant, and if a medium is sufficiently reduced, it will grow in an aerobic environment although at a reduced rate. This characteristic allowed all manipulations and certain short experiments to be performed without a constant, totally anaerobic atmosphere.

**Protonophores and Intracellular pH (pH_i)**

By definition, a protonophore increases the concentration of protons within a cell. Such activity and its significance to cellular energetics were first postulated by Mitchell (1961a,b). A weak lipophilic acid can act as a protonophore by passing through a cell membrane in its undissociated form and dissociating after entering the more alkaline interior. Because the membrane is highly impermeable to protons, the continued influx of an undissociated acid will increase the concentration of protons within the cell. Therefore, a sufficiently high external concentration of undissociated acid should decrease the internal pH (pH_i) of a cell.
Because sorbic acid has been shown to act as a protonophore in E. coli cells (Salmond et al., 1984) and vesicles (Eklund, 1985), and nitrous acid formed from nitrite has been suggested to act similarly (Meijer et al., 1979), the internal pH (pH\textsubscript{i}) of sorbate- or nitrite-treated and untreated PA 3679 cells was determined in this study. The pH\textsubscript{i} in PA 3679 was measured by the distribution of radiolabelled 5,5-dimethyl-2,4-oxazolidinedione (DMO), a procedure developed by Waddell and Butler (1959) for studies on skeletal muscle; currently, it is widely used for measuring pH\textsubscript{i} of both procaryotic and eucaryotic cells (Rottenberg, 1975; Maloney et al., 1975; Roos and Keifer, 1982). DMO is a weak acid whose undissociated form equilibrates readily across the cell membrane. Since the degree of ionization depends on the pH, the concentration of DMO will increase inside the cell as the pH\textsubscript{i} decreases. Although other methods have been developed for determining pH, measuring the distribution of weak acids such as DMO or benzoic acid, or weak bases such as methylamine, remains the most sensitive method available for determining the pH\textsubscript{i} of bacteria (Ross and Keifer, 1982).

**Protonmotive Force**

The importance to cellular energetics of maintaining a proton gradient across a membrane became apparent when Mitchell published his chemiosmotic theory (Mitchell, 1961a). In this classic paper, Mitchell explained how electron transport is coupled to oxidative phosphorylation.
According to his theory, electrons transported down the electron transport chain lead to a concomittant efflux of protons creating a proton gradient across the mitochondrial membrane such that the concentration of protons is greater on one side. This disequilibrium of proton concentration causes protons to flow back through a membrane bound ATPase system resulting in the phosphorylation of ADP to ATP. The protonic potential across the membrane consists of two major components, a chemical one, $\Delta \text{pH}$ (proton gradient), and an electrical one, $\Psi$ (membrane potential). The magnitude of the protonic potential, or protonmotive force (pmf), is given by the sum of its components according to the equation,

$$\text{pmf} = \Delta \Psi - 2.303 \frac{RT}{F} \Delta \text{pH},$$

where pmf and $\Delta \Psi$ are in mV, pH is in pH units, R is the gas constant, T is absolute temperature, and F is the Faraday constant. Later research by others has supported Mitchell's hypothesis and also extended it to include bacterial cell membranes. The pmf also was shown to have multiple functions in addition to coupling electron transport to membrane-linked ATP synthesis (Skulachev, 1975a, 1981; Kell, 1979).

To accurately determine the pmf, it is necessary to measure the pH$_i$ and the membrane potential simultaneously in the same cells. This is impossible to do with current methodology. However, an alternative method would be to
measure the pHᵢ and membrane potential separately by determining the distribution of radiolabelled compounds in cells from the same culture at the same time under the same conditions. This can be accomplished by using a weak acid such as DMO to measure the pHᵢ and triphenylmethylphosphonium bromide (TPPBr⁺), a membrane permeable ion (Schuldiner and Kaback, 1975), to measure the membrane potential. In a preliminary experiment, TPPBr⁺ was used to determine the membrane potential of PA 3679 cells in order to calculate the pmf or sorbate-treated cells. However, TPPBr⁺ was too reactive for use under the conditions employed in this study. Fortunately, bacterial flagella are energized by the pmf and therefore the rate of motility can be used to monitor the pmf in flagellated bacteria.

Investigations have shown that either a positive or negative pmf and one or both of its components, ΔpH and Ψ, can drive the flagellum (Manson et al., 1980; Khan and McNab, 1980). Studies with a motile strain of Streptococcus, whose flagellar motor turns in response to ΔpH or Ψ, showed that the rotation rate is linearly proportional to the pmf (Manson et al., 1980; Berg et al., 1982). Many studies on the energetics of flagellar rotation in other bacteria, e.g., Rhodospirillum rubrum (Skulachev, 1975b; Belyakova et al., 1976; Glagolev and Skulavhev, 1978) and Bacillus subtilis (Matsuura et al., 1977, 1979), have supported this hypothesis. According to Berg et al. (1982), "It is now clearly established that the
immediate source of energy for motility is a protonmotive force (Δp)." Therefore, in this study, the relative rate of motility in PA 3679, a highly motile organism, was used as an indicator of changes in the magnitude of the pmf of bacteria treated with sorbate and/or nitrite.

**Amino Acid Uptake**

Bacteria are capable of taking up amino acids from a medium against a large concentration gradient. The molecular organization that facilitates such a translocation across the cytoplasmic membrane is called an amino acid transport system. This system consists of a carrier, an intrinsic membrane protein, that binds the amino acid specifically, and anisotropically translocates and releases it across the membrane while coupled mainly to chemiosmotic energy or ATP in a few systems (Anraku, 1982). In a study on maintenance and exchange of the aromatic amino acid pool in *E. coli*, Brown (1971) demonstrated that energy was required for pool maintenance as well as formation. She reported that the aromatic amino acids circulate rapidly between the inside and the outside of the cells. In order for a sufficient amount of amino acids to be available within the cells for protein synthesis, the rate of energy-requiring influx has to be greater than the efflux. Without a constant, adequate energy source, the amino acids rapidly efflux. Therefore, anything that can dissipate the pmf, such as protonophore, will inhibit the transport of most amino acids and results in a reduction in
the amino acid pool. Since sorbic acid acts as a
protonophore (Salmond et al., 1984; Eklund, 1985) and can
inhibit amino acid uptake in E. coli (Eklund, 1980), the
effect of sorbic acid on amino acid uptake was studied in
PA 3679 cells. Because the PA 3679L strain used in this
study (Campbell and Frank 1956), has an absolute
requirement for seven amino acids (other strains require
still more), amino acid uptake is important for continued
growth.

Although radiolabelled amino acids are used routinely
to determine their concentrations within bacterial cells,
the metabolism of the bacteria must be considered before
these tracers can be used in studies of amino acid uptake
and protein synthesis. Research on amino acid uptake and
protein synthesis in E. coli using radiolabelled amino
acids has been complicated by the ability of E. coli cells
to synthesize amino acids from other nitrogen sources and
carbohydrates, necessitating the use of amino acid
auxotrophs or membrane vesicles for uptake studies. In PA
3679, such studies are complicated because the organism is
proteolytic and catabolizes amino acids for energy and as
precursors for the biosynthesis of other compounds as well
as for protein. Campbell and Frank (1956) reported that,
although PA 3679L required a medium containing many amino
acids for cell division, there was an absolute requirement
for seven (phenylalanine, isoleucine, tyrosine, valine,
serine, arginine, and cysteine). Because of this, it
seemed possible that at least one of the seven amino acids would go directly into protein synthesis, if present in low concentration in an otherwise amino acid rich medium. Phenylalanine and isoleucine were chosen for this study of amino acid uptake and protein synthesis because it has been reported for E. coli that these amino acids do not share a common transport system as do phenylalanine and tyrosine (Piperno and Oxender, 1968), and that their uptake is coupled directly to the energy of the protonomotive force (Anraku, 1982).

In order to study amino acid uptake in cells, it is necessary to distinguish between the radiolabelled amino acid present as free amino acid in the cell cytoplasm and that which is incorporated into protein and transfer RNA. One procedure that has been employed frequently in studies of amino acid uptake by whole bacterial cells involves the addition of chloramphenicol to inhibit protein synthesis. However, in this study, chloramphenicol was not added because PA 3679 exhibited residual protein synthesis in high concentrations of chloramphenicol. This antibiotic also has been shown to have other effects such as antagonism to the stringent response (see page 25).

The size of the amino acid pool in the bacterial cytoplasm during any phase of growth can readily be determined from the difference between the total amount of radiolabelled amino acid within the cells and that which is incorporated into the trichloroacetic acid (TCA) insoluble
cell fraction (Britten and McClure, 1962). However, Brown (1970) reported that the amino acid pools in fast growing *E. coli* were smaller than in slower growing cultures, possibly because of rapid removal of the amino acids by high rates of protein synthesis. Therefore, the effect of sorbic acid on amino acid uptake in PA 3679 was studied by a method suggested by Ingraham et al., (1983, p. 368) based on a study of protein synthesis by Schlief (1967). The relative amounts of radiolabelled, free amino acid (i.e. the amounts transported into the cells), were determined by sampling during the several seconds of lag time following the addition of labelled amino acid into the medium but preceding its incorporation into protein and transfer RNA.

**Nutrient Deprivation and the "Stringent Response"**

It has long been believed that the relationship between bacterial growth and nutritional circumstances in the medium could be expressed by the following equation,

\[ \mu = \mu_{\text{max}} \cdot \frac{K}{K + C} \]

where \( \mu \) is the specific growth rate constant in a medium with a given concentration of a growth limiting nutrient, \( \mu_{\text{max}} \) is the maximum value of \( \mu \) at saturating concentrations of the nutrient, \( C \) is the concentration of the limiting nutrient, and \( K \) a saturation constant for solute transport (Herbert et al., 1956). This equation assumes that the bacterial growth rate will steadily decrease with a
steadily decreasing concentration of growth limiting nutrient; but this equation is now believed to be not entirely correct. According to Verseveld et al. (1984), accepted methods of calculating molar growth yields and maintenance energy demands also are no longer valid due to specific intracellular mechanisms which can sense low nutrient concentrations and change the patterns of bacterial metabolism and growth.

When the environment becomes too stressful, e.g. when nutrients become limited or certain antimicrobial substances are present, bacteria stop replicating but may not die; death being defined as a loss of their basic integrity as cells and their metabolic capabilities. As Ingraham et al. (1983, p. 1) have stated, "Bacteria... as a group they emerge from over two billion years of evolution as masters of rapid growth in many environments. Their existence today is a function largely of their ability to grow rapidly when conditions permit and to survive when conditions prevent growth." The ability to survive without replication, an ability which is not limited to sporeformers, is particularly important in food technology since one of the basic principles of food preservation is preventing or retarding the growth of organisms but not necessarily destroying them. One way that this may be accomplished is by the addition of substances called food preservatives such as sorbic acid. For purposes of this document, bacteria which are alive but
do not reproduce will be said to be in a "conserved" state, conservation being defined as a programmed management of resources to prevent destruction. This term accurately describes the activities of such cells since it is now known that this biostasis is not simply the cessation of metabolic activities but rather the result of major intracellular readjustments that allow the bacteria to utilize more efficiently the resources which are still available for survival.

Although it has long been known that bacteria can regulate their internal activities, most intracellular regulation was thought to be due mainly to feedback-type mechanism. However, it is now known that cell regulation is extremely complex, involving low molecular weight compounds whose functions so resemble those of hormones in higher animals that Ames and his associates named them "alarmones" (Stephens et al., 1975). These compounds appear to play crucial roles in a myriad of cell regulation activities, including intracellular readjustments needed for bacteria to enter and remain in the conserved state.

Bacteria can be induced to enter a conserved state by a variety of environmental stresses, including amino acid deprivation (Gallant and Lazzarini, 1976; Gallant, 1979). The effects of amino acid deprivation are important to a study involving sorbic acid which has been reported to inhibit amino acid uptake (Eklund, 1980; Tuncan and Martin, 1985). The major intracellular readjustments seen in
bacteria during amino acid starvation have been well studied and are referred to as the "stringent response." These studies demonstrated the existence of alarmones and their possible functions.

Research on stringent regulation began with a study by Sands and Roberts (1952) with a tryptophan-histidine auxotroph of E. coli. In the absence of its required amino acids, this mutant decreased its rate of protein synthesis by 90% in the absence of required amino acids while its other metabolic activities and respiration continued but at less reduced rates. Borek et al. (1955a, b), also working with amino acid auxotrophs of E. coli, observed mutants with two different responses to deprivation of their required amino acid. One mutant accumulated RNA but the other did not, while the latter organism remained viable longer under amino acid starvation conditions than the former. In 1961 Stent and Brenner suggested that this accumulation of RNA (now termed "stringent control") in E. coli auxotrophs was a function of a specific gene. It is now known that more than one gene is involved in the stringent response with rel A, B, C, and SpoT being implicated. Mutations in the rel genes relax the stringency of the amino acid control on RNA synthesis whereas mutations in SpoT inhibits rapid catabolism of ppGpp needed for quick release of the response. The rel mutants, called relaxed strains, continue to accumulate RNA in the absence of a required amino acid (Engel et al.,
In studies of possible affectors of the stringent response, Cashel and Gallant (1969) implicated two phosphorylated compounds called Magic Spots I and II, which appeared on thin layer chromatograms from extracts of cells of a stringent strain of *E. coli*. These Magic Spots, so named because of their rapid appearance in cells undergoing stringent response, were later identified as guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) respectively (Cashel and Kalbacher, 1970; Sy and Lipmann, 1973; Que et al., 1973). In 1971, Lazzarini and Cashel studied the intracellular levels of ppGpp in an isogenic pair of relaxed stringent strains of *E. coli* during balanced growth, carbon depletion, and amino acid deprivation. Basal levels of ppGpp were found in both strains during carbon depletion or step-down transitions while only the stringent strain accumulated ppGpp during amino acid deprivation. The authors concluded that ppGpp participates in a mechanism controlling ribosomal RNA synthesis in both the stringent and relaxed cells. In a study on the relationship between RNA synthesis and ppGpp levels within the cells of a strain of *E. coli* with a temperature sensitive valyl-tRNA synthetase to regulate degrees of amino acid starvation, Fiil et al. (1972) stated that the variations in ppGpp during balanced growth are too small to be the only element controlling the wide range of stable RNA synthesis. The authors concluded that ppGpp could not be the main effector but acts rather as a
modulator of a yet unknown factor and that a second control mechanism is probably superimposed on ppGpp control of RNA synthesis.

Although it is now known that ppGpp participates in decreasing the rate of protein synthesis and improving its accuracy during amino acid starvation, the mechanism is still unknown. Pingoud et al. (1983) showed that ppGpp binds to the elongation factor EF-Tu, thereby depleting the pool of EF-Tu GTP. Since the ternary complex EF-Tu ppGpp aa-tRNA does not bind significantly to the ribosome due to the higher affinity of EF-Tu GTP aa-tRNA, it is not clear how this activity can produce the observed effects on protein synthesis. Studying the effects of ppGpp on specific promoters, Kajitani and Ishihara (1984) observed that while ppGpp represses certain promoters it also activates others. Apparently ppGpp exerts multiple control on transcription. This is further complicated by the fact that ppGpp has been reported to directly affect enzymes of catabolism by increasing or decreasing the activity of various glycolytic enzymes in an allosteric manner (Taguchi et al., 1978). The regulator mechanisms of ppGpp are not as yet known nor is the extent of its participation in the myriad of cellular readjustments of the stringent response.

Although originally the term "stringent response" was used in reference to the lack of RNA accumulation in response to amino acid deprivation in E. coli, it now refers to a large number of intracellular adjustments made
in response to an imbalance of tRNA charging in any bacterial species (Gallant, 1979), e.g. *Salmonella* (Stephens *et al.*, 1975), *Bacillus* (Swanton and Edlin, 1972), *Klebsiella* (Riesenber and Kari, 1981; Riesenbert *et al.*, 1982), *Streptomyces* (Riesenbert *et al.*, 1984), and *Paracoccus* (Verseveld *et al.*, 1984). Therefore, what has been learned about stringent response from studies with *E. coli* is probably applicable to a broad range of bacteria (Gallant, 1979). *E. coli*, the most widely studied organism, exhibits the stringent response when any species of aminoacyl-tRNA falls below the minimum level required for protein synthesis due to amino acid deprivation or inactivation of an aminoacyl-tRNA-synthetase. Among the ensuing major intracellular readjustments is a reduced rate of RNA accumulation, due in part to a 10-20 fold decrease in the net rate of synthesis of stable ribosomal and transfer RNA. Since the decrease in instantaneous global rate of RNA synthesis is greater than can be accounted for by the reduction in the rate of stable RNA must also be effected (Gallant and Lazzarini, 19765; Gallant, 1979). O'Farrell (1978) studied the effect of the stringent response on the rates of synthesis of over 300 different proteins and found that it stimulated 25% and inhibited 25%. Although the exact origin of these effects was not determined, it is apparent that stringent control effects the whole pattern of transcription and, not surprisingly, a large variety of adjustments are seen in cellular
metabolism. The stringent response inhibits the transport of various precursors and effects the synthesis of nucleotides, glycolytic intermediates, carbohydrates, lipids such as the membrane phospholipids, fatty acids, polyamines, and peptidoglycan (Gallant, 1979). The total effect of the stringent response is growth inhibition without cell death, i.e. a state of cellular conservation, provided there is a minimal amount of nutrients available for cell maintenance and to maintain the stringent response since it also requires energy. Addition of the limiting nutrient will release the stringent control, and the cells quickly adjust to the pre-stringent response rate of growth (Verseveld et al., 1984).

The stringent response also is characterized by the appearance of various unusual phosphorylated nucleotides (alarmones), such as ppGpp discussed previously, as well as pppGpp and ppGp which have not been well studied but which also appear to play a role in cell regulation. Further studies with E. coli and other bacteria have revealed a variety of phosphorylated nucleotides, some or all of which may have roles in cell regulation (Gallant, 1979). In Bacillus, ppGpp and pppGpp are synthesized when the cells are deprived of a limiting nutrient or are at the end of exponential growth in a semi-synthetic medium (considered to be condition of ill-defined nutrient depletion) but prior to sporulation. Although ppGpp and pppGpp do not appear to play a role in sporulation, other unusual
phosphorylated nucleotides such as pppApppp, pppAppp, ppApp and ppZpUp (Z = undetermined sugar) also accumulate and have been implicated as regulators of sporulation (Rhaese et al., 1972, 1975a, b, 1976, 1977; Nishino et al., 1979). To complicate matters further, it is known that in Bacillus, ppGpp can be synthesized by at least two distinct enzymes (Sy, 1979), and its degradation proceeds by at least two different pathways (Richter, 1979) suggesting the possibility of multiple control mechanisms on the regulatory compound itself. Only within the last 10-15 years has the true complexity of a bacterium's response to its environment become appreciated fully. A growth downshift due to carbon or amino acid deprivation is the sum total of a very large number of complex intracellular readjustments which involve the accumulation of ppGpp in most bacteria studied.

The production of alarmones (rel A independent) has been reported with energy source shiftdowns (Cashel, 1975) with increased production of ppGpp observed in bacterial cells which are shifted down from growth on rich carbon and energy sources to poorer ones (Lazzarini et al., 1971; Winslow, 1971; Hansen et al., 1975). Intracellular levels of ppGpp also increased after E. coli was treated with cyanide which interferes with energy production (Edlin and Donini, 1971). Gallant et al. (1976) reported that, while the level of ppGpp increased, another nucleotide termed the "phantom spot" decreased when E. coli was treated with the
uncoupler carbonyl cyanide metachlorophenylhydrazone or shifted from aerobic to anaerobic growth. Since a decrease in the pmf is a form of energy depletion, these studies suggest that anything which decreases the pmf could cause the cells to respond by cell regulation mechanisms which involve alarmones. Since protonophores cause energy depletion and amino acid deprivation in amino acid requiring bacteria, the addition of a protonophore such as sorbic acid would provide two forms of stress to PA 3679, either one of which might be expected to trigger a stringent-type response. Therefore, the relative rates of ATP, GTP, and the alarmone ppGpp accumulation in PA 3679 were examined and compared in sorbate-treated and untreated cells. Since large decreases in the rates protein synthesis are also a characteristic of stringent-type responses (Gallant, 1979), relative rates of protein synthesis in untreated and sorbate-treated PA 3679 cells were compared.

Studies have shown that release of the stringent response is readily accomplished by returning cells to more favorable growth conditions or by the addition of certain inhibitors of protein synthesis such as chloramphenicol, fusidic acid, rifampicin (Cashel, 1975; Ikehara et al., 1984), lincomycin (Ochi and Freese, 1983), and sparsomycin (de Boer et al., 1971). According to a hypothesis by Kurland and Maaloe (1969) and later supported by Kaplan et al. (1973), when bacterial cells are starved for amino
acids, a small amount of amino acids produced from autoproteolysis is used to maintain a low level of aminoacylated tRNA resulting in an increased production of ppGpp. If the minimal consumption of amino acids by residual protein synthesis is inhibited by certain protein synthesis inhibitors, the concentration of charged tRNA increases and causes a decrease in the intracellular level of ppGpp, releasing the stringent response. However, not all bacteria respond uniformly to all protein inhibitors. In a study with *B. subtilis*, Rhaese et al. (1975) observed that when chloramphenicol was added, ppGpp and pppGpp accumulated within the cells. Chloramphenicol was not used as an inhibitor of protein synthesis in the amino acid uptake studies because chloramphenicol has been reported to have varied effects, either triggering the production of ppGpp which inhibits glycolysis, transport of various compounds, etc., or releasing the stringent response (Rhaese et al., 1975; Gallant, 1979).

Tetracycline also is an inhibitor of protein synthesis that releases the stringent response, but its action does not depend on levels of tRNA charging. Silverman and Atherly (1978) have shown that tetracycline inhibits the stringent factor in the absence of ribosomes, thereby appearing to interfere directly with ppGpp metabolism and function. The authors suggested that tetracycline and its derivatives are in some way analogous to ppGpp, since high concentrations mimic ppGpp by specifically inhibiting rRNA
synthesis but low concentrations prevent the synthesis of ppGpp. Hence tetracycline appears to bind to the same component as does ppGpp within the cell. Since tetracycline affects the level of ppGpp more directly than other protein synthesis inhibitors and appears to act uniformly on all bacteria tested, tetracycline was used in this study to examine the effects of sorbate on PA 3679. A release of sorbate-induced growth inhibition by tetracycline would show that the inhibition by sorbate in PA 3679 is due to a stringent-type response, possibly involving increased levels of ppGpp.
II. MATERIALS AND METHODS

Organism

The organism used was Putrefactive Anaerobe 3679, originally obtained from Dr. E.S. Wynne and designated PA 3679L (Campbell and Frank 1956).

Preservatives

Potassium sorbate, potassium nitrite, sodium nitrite, and sodium benzoate were purchased from Calbiochem (San Diego, California).

Media

a. Broth

The culture medium used for vegetative cell inocula was a slight modification of that described by Uehara et al. (1965). Six percent Trypticase (BBL) and 0.1% glucose were dissolved in distilled water, sterilized (121°C, 20 min), and preincubated anaerobically for 1-2 days. Immediately prior to use, 0.05% L-cysteine·HCl (U,S, Biochemical Corp., Cleveland, Ohio) was added, the pH adjusted to the desired value with either NaOH or HCl, and the broth sterilized by filtration through a 0.45μ membrane filter (Nalgene). All pH value determinations were made with a Microprocessor Ionalyzer 901 (Orion Research, Cambridge, Massachusetts).

b. Agar

A solid medium was developed to support PA 3679 colony formation when inoculated by the pour plate method. A mixture of 6% Trypticase, 0.1% glucose, and 1.5% Bacto-
Agar (Difco) in distilled water was allowed to boil until the agar was completely dissolved. L-cysteine·HCl was then added to a final concentration of 0.15%. The pH was adjusted to 6.5 with NaOH, but it increased to 7 with autoclaving. Approximately 350 ml of the hot agar medium was dispensed into 500 ml media storage bottles (Bellco, Inc., New Jersey) and sterilized (121°C, 25 min). After autoclaving, the caps of the media bottles were tightened immediately to prevent oxidation of the medium and stored at room temperature. The medium was used within 1-2 weeks of initial preparation; during this time the medium did not darken due to oxidation and retained its ability to support colony formation by PA 3679. Just prior to use, the medium was melted either by placing bottles in boiling water or in a microwave oven and placed in a 50°C water bath to cool without solidifying. To prevent oxidation of the medium, the bottle caps were not loosened until immediately prior to use. Approximately 35 ml of medium was poured into 100 x 15 mm Petri dishes containing inocula and incubated anaerobically. After 2 days of incubation, 2-3 mm diameter PA 3679 colonies could be counted easily throughout the medium.

c. Defined Medium

Unless indicated otherwise, the medium used was a slight modification of that described by Campbell and Frank (1956) (Table 1). Stock solutions and the complete medium were filter sterilized through 0.22 µm membrane filters.
All amino acids and vitamins were purchased from U.S. Biochemical Corp. (Cleveland, Ohio) except L-alanine and L-histidine which were purchased from Calbiochem.
Table 1: Defined Medium for PA 3679*

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (mg)</th>
<th>Constituent</th>
<th>Amount (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tryptophan</td>
<td>10</td>
<td>Glucose, 25%</td>
<td>1.00</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>600</td>
<td>Salts A</td>
<td>1.00</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>200</td>
<td>Salts B</td>
<td>0.10</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>25</td>
<td>Salts C</td>
<td>0.25</td>
</tr>
<tr>
<td>L-Valine</td>
<td>100</td>
<td>Salts D</td>
<td>0.25</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>150</td>
<td>Vitamin solutions</td>
<td></td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>90</td>
<td>1, 2, 3, and 4</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Lysine</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>84</td>
<td>Distilled water</td>
<td>to 100</td>
</tr>
<tr>
<td>L-Cysteine·HCl</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Salts A: $K_2HPO_4$, 25 gm; $KH_2PO_4$, 25 gm; distilled water, 250 ml.

Salts B: $MgSO_4·7H_2O$, 10 g; NaCl, 0.5 g; $FeSO_4·7H_2O$, 0.5 g; $MnSO_4·4H_2O$, 0.5 g; distilled water, 250 ml.

Salts C: $ZnSO_4·7H_2O$, 800 mg; $CuSO_4·5H_2O$, 450 mg; $CoCl_2·6H_2O$, 1.65 g; $CaCl_2$, 600 mg; $(NH_4)_{6}Mo_7O_{24}·4H_2O$, 225 mg; distilled water, 250 ml.

Salts D: $Na_2B_4O_7·10H_2O$, 1.4 g; distilled water, 250 ml.

Vitamins 1: Thiamine, 4 mg ml$^{-1}$; Pyridoxal, 5 mg ml$^{-1}$.

Vitamins 2: Biotin, 5 μl ml$^{-1}$; p-Aminobenzoic acid, 100 μl ml$^{-1}$.

Vitamin 3: Folic acid, 100 μl ml$^{-1}$.

Vitamin 4: Pantothenic acid, 10 mg ml$^{-1}$.

*Modification of defined medium as described by Campbell and Frank (1956).
Anaerobic Incubation

Cultures of PA 3679 were incubated at 34°C in a commercial anaerobic incubator (National Appliance Co., Oregon). Anaerobiosis was achieved by evacuating the incubation chamber with a vacuum pump and subsequently filling with commercial 99.9% nitrogen gas. This procedure was done three times to insure a final atmosphere of nearly pure nitrogen gas. Prior to gassing, a beaker containing 6 gm pyrogallol and 2 gm sodium carbonate in 100 ml water was placed in the chamber (Carlquist, 1959) to absorb residual oxygen while also serving as a visual indicator of anaerobiosis. The sodium carbonate provides carbon dioxide to stimulate the growth of the organism.

Spore Inocula

A fresh spore culture was prepared according to a modified technique of Rowley and Feeherry (1970) from a PA 3679L spore culture provided by Dr. H.A. Frank (Dept. of Food Science and Human Nutrition, University of Hawaii, Honolulu, Hawaii). A drop (0.25 ml) of the spore culture was added to 10 ml of broth medium, heated at 80°C for 20 min to activate germination and incubated anaerobically until the vegetative cells had sporulated (about 2 days). One-tenth ml of the fresh spore culture was then added to 100 ml broth medium, heated for 20 min at 80°C, and incubated anaerobically overnight. The 100 ml vegetative culture was then added to 1.5 l of broth medium and incubated until cells sporulated (about 2-3 days). The
spore culture was centrifuged (15,000 x g, 30 min, 5°C) and washed twice with cold (5°C), sterile, distilled water. The spores were resuspended in 150 ml cold water, divided into 3 aliquots of 50 ml, and stored at 5°C for 2 days. Each aliquot was then centrifuged, washed four times with cold water, resuspended in 50 ml cold water and stored overnight at 5°C. Each aliquot was then centrifuged 4,000 x g for 10 min at 5°C to remove debris from the preparation, washed once with cold water and centrifuged (15,000 x g, 30 min, 5°C). Each aliquot was resuspended in 50 ml fresh, cold, filter-sterilized enzyme solution containing 100 μg ml⁻¹ lysozyme (Calbiochem) and 50 μg ml⁻¹ trypsin (Calbiochem) in 0.05M K₂ HPO₄ at (pH 8.1) and left overnight at 5°C to digest remaining traces of sporangia. The spores were then centrifuged (15,000 x g, 30 min, 5°C), washed twice in cold water, resuspended at a concentration of about 2 x 10¹⁰ cells ml⁻¹, dispensed into sterile, screw-cap culture tubes, and stored at 5°C until needed. The spore culture was examined microscopically and appeared to consist entirely of highly refractile, i.e. dormant, mature spores.

Viability Tests

Nine and nine-tenths ml of broth medium with and without test substances were added to sterile, 150 x 20 mm screw-cap tubes and inoculated with 0.1 ml containing 3 x 10⁴ cells (direct microscopic counts) for a final concentration of 3 x 10³ cell ml⁻¹. The inoculated medium
was incubated anaerobically and, at designated times, duplicate 0.1 samples were taken and diluted when necessary for colony counts in the agar medium.

**Microscopy**

a. **Light Microscopy**

Direct microscopic examinations were done with a standard Zeiss WL microscope using phase contrast optics. Phase contrast photomicrographs were taken with a Zeiss camera plus attachments on Kodak FX-135 film.

b. **Electron Microscopy**

Vegetative cells were grown under test conditions as described above and prepared for electron microscopy by a slight modification of methods described by Hinchee and Haskins (1980). To fix the cells, 25% glutaraldehyde was added to 2.5 ml of PA 3679 culture for a final concentration of 3% and left at room temperature for 3 hrs. Fixed cells were centrifuged (15,000 x g, 4°C) and washed three times with ice cold 0.02 M Sorensen's buffer at pH 6.8. A small drop (about 0.03 ml) of warm (45°C), melted 2% agar in medium was added to the pellet and transferred to a clean glass slide and allowed to harden. Once hard, 1 mm blocks were cut with a scalpel blade and washed overnight in buffer at 4°C. The blocks were removed and the cells post-fixed with 1% OsO₄ in buffer at 4°C for 1 hr and then washed three times for 15 min in cold buffer. After washing, the material was dehydrated through an ice cold ethanol series (30%, 50%, 70%, 95%, 100%, 100%), 5 min
each, transferred into propylene oxide and embedded according to the method of Luft (1961). The blocks were treated twice with propylene oxide for 15 min each and then a mixture of propylene oxide and Epon 812 (1:1) for 2 hrs followed by a 1:3 mixture of propylene oxide and plastic overnight. The blocks were subsequently removed, put into fresh, pure plastic and allowed to polymerize in a vacuum oven (60°C). The embedded material was cut with glass knives on a Porter-Blum II microtome with serial sections placed on 200 mesh copper grids. The bacteria were stained with 3% uranyl acetate (Kellenberger et al., 1958) for 10 min and washed with double distilled water. The grids were examined with an Hitachi HS-8-1 electron microscope at 50kv and photographed on Kodak EM 4480 Estar thick base film.

Internal pH (pHᵢ) Measurement

a. Internal water volume

Internal water volumes needed to calculate pHᵢ were determined from the difference between the wet and dry weights of cells, as first mentioned by Winkler and Wilson (1966); extracellular water was determined with ³H-sorbitol, to which PA 3679 is impermeable.

A 30 ml vegetative cell culture was centrifuged (15,000 x g, 15 min) in a dry, sterile centrifuge tube preweighed on a Mettler H20T balance. Cells were resuspended in 10 ml fresh medium (pH 7) containing 10 mM unlabelled sorbitol to prevent non-specific binding of labelled sorbitol, and incubated for 10 min at 27°C in a
shaking water bath. $^3$H-sorbitol (0.05 Ci ml$^{-1}$, New England Nuclear) was added to the culture which was vortexed and incubated for 2 min in a shaking water bath to insure uniform distribution of the labelled compound. Duplicate 0.1 ml samples were taken and put into scintillation vials for radioassay. The remaining culture was centrifuged (15,000 x g, 15 min), and the supernatant was carefully removed by pipette and discarded. The inside of the centrifuge tube was wiped thoroughly with sterile cotton swabs to remove excess moisture. More then half the pellet was then removed with a clean, flame-sterilized spatula and placed in a clean, dry, preweighed scintillation vial. Both the centrifuge tube and the vial containing portions of the pellet were weighed to obtain wet weight of cells. The bacteria in the centrifuge tube were resuspended in 10 ml fresh medium and duplicate 0.1 ml samples were taken for colony counts. The pelleted cells in the scintillation vial were dried to constant weight at 104°C in a drying oven and dissolved in 0.1 ml 8 M urea. Ten ml scintillation cocktail (Aquasol-2, New England Nuclear) were added to all vials and the contents were radioassayed for tritium in a Packard Tri-Carb 4640 liquid scintillation system. Calculations used for determining cell water volume of $10^7$ PA 3679 cells are described below. (See Figure 1).
1. Extracellular water volume of pelleted cells (a) =
   \[
   \frac{\text{DPM}^* \text{ of pellet}}{\text{DPM of 0.1 ml whole cell suspension} \times 0.1 \text{ ml}}
   \]
2. Wet weight of pellet in tube (b) [or in vial (c)] =
   \[
   \text{[weight of tube (or vial) + pellet] - weight of tube (or vial)}
   \]
3. Dry weight of pellet (d) =
   \[
   c - [(\text{weight of vial} + \text{dry pellet}) - \text{weight of vial}]
   \]
4. Total water weight of pellet (e) = b - d
5. Assuming 1 g water = 1 ml:
   \[
   \text{Total water volume of cells pellet of vial (f) = e - a}
   \]
6. Total number of cells in pellet of vial (g) =
   \[
   \frac{\text{number of cells in pellet of tube}}{c \times b}
   \]
   *disintegrations per minute
Figure 1. Flow diagram for estimating cell water volume
7. Cell water volume of $10^7$ cells = $\frac{f \times 107}{g}$

b. Internal pH ($pHi$)

Thirty ml of vegetative cell culture were centrifuged (15,000 x g, 15 min) and resuspended to a final concentration of $2 \times 10^{10}$ cells ml$^{-1}$ in fresh medium containing 10 mM unlabelled sorbitol (pH 7.0 or 6.5). Two-tenths ml of the concentrated cell suspension was added to 1.8 ml of fresh medium containing 10 mM unlabelled sorbitol, preservatives at final concentrations and pH values as indicated, and incubated for 10 min in a shaking water bath. After $^3$H-sorbitol and $^{14}$C-DMO (New England Nuclear) were added each to a final concentration of about 0.05 Ci ml$^{-1}$, the suspensions were incubated for 1 min. Incubations and all manipulations were done at a constant 27°C because the final concentration of DMO within the cells is dependent on the $pK_a$ of the DMO which decreases with increasing temperature (Addanki et al., 1968). Duplicate 0.1 ml samples were taken with an automatic pipettor and put into scintillation vials for radioassay. Duplicate 0.2 ml samples were taken and filtered through 13 mm diameter, 0.45 μm, dry membrane filters (Millipore) using negative pressure. Clean, dry filter holders (Gelman) were fitted on a manifold and negative pressure was applied by a vacuum pump. Dry filters and holders were necessary to prevent efflux of DMO from the cells. Filters
were removed immediately after filtration, the undersides blotted to remove excess medium, placed in scintillation vials and allowed to dry. Ten ml of scintillation cocktail (Aquasol-2) were added to all vials and the samples were radioassayed for $^3$H and $^{14}$C in a Packard Tri-Carb-4640 liquid scintillation system. Duplicate 0.1 ml samples were taken and diluted for colony counts. Intracellular pH values were calculated according to the method described by Maloney et al. (1975, pg 29) from the equation of Waddell and Butler (1959):

$$pH_i = pK_a' + \log \left[ \frac{C_t}{C_e} \left( 1 + \frac{V_e}{V_i} - \frac{V_a}{V_i} \right) \times \left[ 10^{(pH_o-pK_a')} + 1 \right] - 1 \right]$$

Where: $pK_a'$ of DMO at 27°C = 6.3 (Addanki et al., 1968)

- $V_i$ = intracellular water volume
- $V_e$ = extracellular water volume
- $C_t$ = concentration of DMO in cells
- $C_e$ = concentration of DMO in extracellular water
- $pH_i$ = intracellular pH
- $pH_o$ = pH of external medium

Cell Motility as an Indicator of Protonmotive Force (pmf)

Cell suspensions were prepared as described above in $pH_i$ determinations except that incubation was extended to 20 min and no radiolabelled compounds were added. At the end of incubation the cells were wet mounted and examined microscopically using phase contrast optics. The rates of
motility were observed and evaluated on a scale ranging from 0 (non-motile) to 4 (highly motile). The motility rate of cells treated with preservatives and/or at pH values below 7 were compared with that of cells without preservatives at pH 7 which had been arbitrarily given a motility score of 3. Duplicate 0.1 ml samples of the cell suspensions were taken for colony counts to show that a lack of motility did not necessarily reflect cell death.

**Amino Acid Uptake and Protein Synthesis**

The methods used to examine amino acid uptake and patterns of protein synthesis were modifications of Brown (1970). A 100 ml overnight, vegetative culture of PA 3679 was centrifuged (15,000 x g, 15 min), washed three times in defined medium (pH 7) lacking the amino acid monitored, and starved for that amino acid for 30 min. The cells were then centrifuged and resuspended in specific amino acid-deficient medium to a final concentration of 5 x 10⁹ cells ml⁻¹ as determined by direct cell counts. Bacterial suspensions prepared in this manner were used as inocula for amino acid uptake and protein synthesis studies.

In both series of experiments, tritiated amino acids were used because their specific activity is over 200 times greater than that of commercially available ¹⁴C-labelled amino acids, permitting detection of much smaller quantities of radiolabelled compounds.
a. Amino Acid Uptake

Two and one half ml each of the prepared bacterial inocula were added to 50 ml Erlenmeyer flasks containing 22.5 ml phenylalanine-deficient medium (pH 7) with preservatives at concentrations indicated, for a final cell concentration of $5 \times 10^8$ ml$^{-1}$. After incubating for 10 min in a shaking water bath (34°C), magnetic bars were added and the flasks placed on magnetic stirrer. After placing the filling tube of an automatic Cornwall syringe in the swirling cell suspension, $^3$H-phenylalanine (New England Nuclear) was added to a final concentration of 0.17 µCi ml$^{-1}$. One ml samples were taken with the automatic syringe at 6 and 12 seconds and filtered through 0.45µm membrane filters (Millipore). To insure that the labelled amino acid within the cells was not incorporated into protein or transfer RNA, a 1 ml sample was taken at 13-14 seconds, added to 1 ml cold, 10% trichloroacetic acid (TCA), held at 4°C for 30 min, and filtered. Immediately after filtration, the whole and TCA-treated cells were washed with 10 ml phenylalanine-deficient defined medium or 5% cold TCA, respectively. The filters were placed in scintillation vials and allowed to dry. Ten ml scintillation cocktail (Aquasol-2) was then added to each vial and the samples radioassayed for tritium in a Packard Tri-Carb 4640 liquid scintillation system.
b. Protein Synthesis

Two ml of bacterial inoculum added to 18 ml of specific amino acid-deficient medium with and without preservatives for a final cell concentration of $5 \times 10^8$ ml$^{-1}$ and a final concentration of preservatives as indicated (pH 7). After incubating for 10 min in a shaking water bath (34°C), $^3$H-phenylalanine or $^3$H-isoleucine ($3.3 \times 10^{-2}$ μCi ml$^{-1}$, New England Nuclear) were added. At 0, 5, 10, 15, 20, and 25 min, 1 ml samples were taken and filtered through 0.45μm membrane filters (Millipore) and washed with 10 ml amino acid-deficient medium. The filters were prepared and samples radioassayed as described above.

To determine the percentage of intracellular $^3$H-amino acid incorporated into protein and transfer RNA, duplicate 1.5 ml samples were taken after the 25 min interval, and each added to 1.5 ml cold, 10% TCA, and left for 30 min at 4°C. Two milliliter TCA treated samples were then filtered and washed with 10 ml cold, 5% TCA. The filters were placed in scintillation vials, dried, and the samples radioassayed as described above.

Possible catabolism of the radiolabelled amino acids was checked by the method of Karl et al. (1981). At the 25 min. interval, 0.1 ml samples were pipetted into each of four scintillation vials. Ten ml of Aquasol-2 were added immediately to each of 2 vials while 2 were placed in 60°C drying oven. After being dried the samples were resuspended in 0.1 ml distilled water, 10 ml Aquasol-2 was added,
and radioassayed as described above. Since catabolism of tritiated amino acids by PA 3679 could be expected to yield $^{3}\text{H}_2\text{O}$, $^{3}\text{H}_2$, and $^{3}\text{H}_3$ which would go off into the atmosphere during drying, a lower count in the dried samples than in the hydrated one would be indicative of such breakdown processes.

**Nucleotide Determinations**

**a. Radiolabelling and Extraction**

A 100 ml overnight PA 3679 vegetative culture was centrifuged (15,000 x g, 15 min), washed three times, and resuspended at a concentration of 5 x $10^9$ cells ml$^{-1}$ in defined low-phosphate (1mM) medium (pH 6.85). Three ml of the cell suspension were added to each of two 50 ml Erlenmeyer flasks containing 27 ml of defined low-phosphate medium and $^{32}\text{PO}_4$ (carrier free, New England Nuclear) for a final concentration of 7.5 or 15 $\mu$Ci ml$^{-1}$. After the bacteria had been incubated for 10 min in a shaking water bath (34°C), a 5 ml sample was pipetted from each flask and added to tubes containing 0.5 ml cold, 10 M $\text{H}_3\text{PO}_4$ for extraction of nucleotides and dilution of the excess $^{32}\text{PO}_4$ remaining in the medium (Karl and Bossard, 1985).

Immediately after this initial sampling, potassium sorbate was added to one flask for a final concentration of 200 mM which increased the pH to 7. A drop of diluted NaOH was added to the second flask, increasing the pH of that cell suspension to 7. The bacteria were then reincubated and sampled at 5, 10, 15, and 20 min as described. Immediately
after the 20 min sampling, a small aliquot from each flask was removed for microscopic examination and a direct cell count. Acid treated samples were stored overnight at 4°C.

b. Purification and Concentration

Sorbic acid precipitates at very low pHs. Since it was possible that the differences seen in this study between untreated and sorbate treated cells could be due to interference by the precipitate in the purification procedure, sorbate was added to a final concentration of 200 mM to all tubes of cell extract previously not containing sorbate. After 1 hr at 4°C all cell extracts were filtered through glass fiber filters (GF/F, WhatmanR) to remove the precipitated sorbic acid, washing the filters once with 5 ml 1 M H3PO4. The extracts were then purified and concentrated according to the method of Cashel et al. (1969). Fifty mg charcoal were added to each tube of extract and mixed vigorously for 5 min at room temperature to allow adsorption of the nucleotides to the charcoal particles. The suspension was centrifuged and the charcoal washed twice with 0.01 M HCl. The nucleotides were eluted from the charcoal by resuspending the particles in 10 ml of a mixture of ethanol-H2 0-NH4 OH (150:80:1) and mixing vigorously for 30 min at room temperature. The mixture was filtered through glass fiber filters to remove the charcoal, washing the filters once with 2 ml of the ethanol mixture. The purified extracts were then dried in a vacuum
evaporator, redissolved in 100 μl of distilled water, and stored at -20°C.

c. Nucleotide Separation and Radioassay

The nucleotides were separated by a two step, one dimensional separation on plastic backed (20 x 20 cm) sheets coated with polyethyleneimine (PEI) impregnated cellulose (MN 300 PEI; Brinkman Instrument Co., Westbury, N.Y.) (Randerath and Randerath, 1966). Ten or 20 μl of the extracts were applied 2 cm from the bottom edge of the plate along with 8 μl each of 2.5 mg ml⁻¹ unlabelled adenosine triphosphate (ATP), guanosine triphosphate (GTP), and guanosine tetraphosphate (ppGpp) (Sigma) as uv markers. After applied samples were dried with cool air, the plates were washed in two consecutive distilled water baths (5 min each) to remove salts and residual ³²P₀₄, and washed once in methanol (2 min) to dehydrate the cellulose layer (Bochner and Ames, 1982). The plates were air dried at room temperature and developed in 0.2 M formic acid to the top of the plate further removing residual ³²P₀₄ while the tri- and tetraphosphates remain at the origin. The plates were washed twice in distilled water and once in methanol, and air dried before final development in the same direction with 1.5 M potassium phosphate, pH 3.4 (Cashel et al., 1969). Both ascending TLC developments were done in closed glass tanks at room temperature, each development taking about 2 to 2-1/2 hrs to complete. The plates were then washed twice in distilled water and once in methanol for
rapid air drying. The ATP, GTP, and ppGpp spots were located by using short wavelength uv light and circled with a soft lead pencil. The spots and the areas between them were cut out and placed in scintillation vials. The nucleotides were eluted from the PEI cellulose by adding 1 ml 0.7 M MgCl₂ (Karl and Bossard, 1985) and shaking vigorously for 1 hr at room temperature. Ten milliliters of Aquasol-2 were added to each vial and the samples radioassayed for ³²P⁰⁴ with a Packard Tri-Carb 4640 liquid scintillation system.
III. RESULTS

Viable Counts

The amount of cysteine was crucial in obtaining colony counts that corresponded with cell number of the inoculum determined by direct microscopic counts with a Petroff-Hauser counting chamber (Figure 2). The Trypticase at the 6% concentration used in the medium does not contain sufficient cysteine for optimal growth of PA 3679. Cysteine is a required nutrient for the organism and probably also helps to keep the medium reduced. Agar medium containing 0.15% cysteine gave the highest counts, therefore, this concentration was used.

Protonophoric Activity of Sorbic Acid

Activity of the undissociated acid

Viability studies were conducted on the effects of potassium sorbate on the growth of PA 3679 at three concentrations of undissociated acid at pH 6 and 5.5. Figure 3 shows typical dose response curves where the antibacterial activity increased with increasing concentrations of undissociated sorbic acid.

Internal pH ($pH_i$)

Riebling et al. (1975) found that DMO penetrated Clostridium pasteurianum within 45 seconds and the internal concentration of DMO remained stable for at least 6 minutes. In this study, DMO penetrated PA 3679 cells within one
Figure 2. Effect of L-cysteine·HCL on estimation of PA 3679 by colony counts in an agar medium. Column at left represents a direct cell count using a Petroff-Hausser counting chamber.
Figure 3. Inhibitory effect of undissociated sorbic acid on the growth of PA 3679. Three concentrations of undissociated sorbic acid (undissoc. acid) were obtained from 6 concentrations of potassium sorbate (KS) at pH 6.0 and 5.5.

* Colony forming units
minute and remained stable for 10 minutes. Although DMO is a protonophore, the concentrations used in this procedure are too low to have any appreciable effect on the pH\textsubscript{i} measurements in PA 3679 cells.

The intracellular water volume of PA 3679 was 0.026 ± 0.014 per 10\textsuperscript{7} cells and was identical to that observed by Clarke \textit{et al.} (1982) with \textit{Clostridium pasteurianum}. Using this value and the results from the DMO uptake experiments (Table 2), the effect of adding sorbate on the pH\textsubscript{i} of PA 3679 was determined at pH 7 and 6.5 (Tables 3, 7). Decreasing the external pH (pH\textsubscript{o}) to 6.5 reduced the pH\textsubscript{i} of the cells. However, the addition of sorbate lowered the pH\textsubscript{i} of PA 3679 cells when the pH\textsubscript{o} was 7 and 6.5.

**Protonmotive Force (pmf)**

The effect of sorbate on the pmf of PA 3679 was examined by using the rate of motility (rate of motion when swimming in a straight line) as an indicator of the relative magnitude of the pmf. Table 4 shows that a high concentration (11 mM) of undissociated sorbic acid resulted in a complete loss of motility, an effect which was easily reversed by diluting the sorbate with added medium or by decreasing the concentration of undissociated acid through increasing the pH.
Table 2. Effect of potassium sorbate (KS, 200 mM) at pH 7 and 6.5 on DMO uptake in PA 3679 cells. These results were used to calculate $pH_i$ in Table 3.

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>Average dpm*</th>
<th>Intracellular water (μl) of filtered cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>$^{3}$H</td>
</tr>
<tr>
<td>Untreated pH 7</td>
<td>14,996</td>
<td>10,718</td>
</tr>
<tr>
<td>KS, pH 7</td>
<td>15,788</td>
<td>9,457</td>
</tr>
<tr>
<td>Untreated pH 6.5</td>
<td>15,302</td>
<td>11,943</td>
</tr>
<tr>
<td>KS, pH 6.5</td>
<td>16,983</td>
<td>10,680</td>
</tr>
</tbody>
</table>

| Trial 2                  |              |                |               |
|-------------------------|--------------|----------------|
| Untreated pH 7           | 17,369       | 11,497         | 1,706        | 816              | 0.6838        |
| KS, pH 7                | 15,516       | 9,878          | 1,657        | 864              | 0.5928        |
| Untreated pH 6.5         | 16,548       | 10,755         | 1,729        | 738              | 0.5122        |
| KS, pH 6.5              | 16,676       | 9,983          | 1,686        | 783              | 0.4628        |

| Trial 3                  |              |                |               |
|-------------------------|--------------|----------------|
| Untreated pH 7           | 14,431       | 17,668         | 1,491        | 1,355            | 0.7462        |
| KS, pH 7                | 12,064       | 12,362         | 1,372        | 1,206            | 0.7514        |
| Untreated pH 6.5         | 13,426       | 17,346         | 1,594        | 1,229            | 0.7566        |
| KS, pH 6.5              | 13,390       | 14,986         | 1,449        | 1,218            | 0.6162        |

| Trial 4                  |              |                |               |
|-------------------------|--------------|----------------|
| Untreated pH 7           | 15,207       | 16,317         | 1,708        | 1,469            | 0.6006        |
| KS, pH 7                | 14,215       | 12,592         | 1,732        | 1,367            | 0.5304        |
| Untreated pH 6.5         | 15,871       | 16,668         | 1,724        | 1,172            | 0.5226        |
| KS, pH 6.5              | 15,793       | 14,179         | 1,940        | 1,277            | 0.5096        |

* disintegrations per minute
Table 3. The internal pH (pHᵢ) of untreated and potassium sorbate (KS, 200mM)-treated PA 3679 cells as measured by the distribution of DMO with extracellular pH (pHₒ) values of 7.0 and 6.5. The results presented here and in Table 7 on the effects of sorbate on the pHᵢ when the pHₒ was 7 (n = 8) were analyzed statistically by a two-tailed t test and found to be significant (p = 0.00732). Sample size for the effects of pHₒ 6.5 in combination with KS on pHᵢ proved to be too small for meaningful statistical analyses. However, since these effects were equal to or greater in magnitude than that of KS at pHₒ 7, they are probably also significant.

<table>
<thead>
<tr>
<th>pHₒ</th>
<th>Trial no.*</th>
<th>pHᵢ untreated</th>
<th>KS</th>
<th>Concn. undissociated sorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>1</td>
<td>7.66</td>
<td>7.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.66</td>
<td>7.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.61</td>
<td>7.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.63</td>
<td>7.45</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>1</td>
<td>7.70**</td>
<td>7.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.53</td>
<td>7.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.48</td>
<td>7.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.55</td>
<td>7.49</td>
<td></td>
</tr>
</tbody>
</table>

* Identical trial numbers represent PA 3679 cells taken from the same culture.

** This value is probably higher than it should be due to a slight filtration problem.
Table 4. Effect of potassium sorbate (200mM) on the motility of PA 3679 with external pH (pH₀) values of 7, 6.5, and 6.

<table>
<thead>
<tr>
<th>Relative motility rate</th>
<th>potassium sorbate</th>
<th>concn. undissociated sorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH₀</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>3* 2</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>6.5</td>
<td>3 1</td>
<td>3.6 mM</td>
</tr>
<tr>
<td>6.0</td>
<td>2 0</td>
<td>11.0 mM</td>
</tr>
</tbody>
</table>

*arbitrarily designated motility rate to which all other rates were compared.

Amino Acid Uptake

Table 5 shows that the amount of radiolabelled phenylalanine taken up by sorbate- or benzoate-treated PA 3679 cells was always less than in the untreated cells; benzoate is more inhibitory than sorbate.

Stringent-type Response

a. Phosphorylated nucleotides

Figure 4 illustrates the degree of separation among ATP, GTP, ppGpp, and the original extract spot on the chromatograms after final development with phosphate (1.5 M, pH 3.4).

The effect of sorbate on the intracellular accumulation rates of ATP, GTP, and ppGpp in PA 3679 cells were examined. Figure 5 shows that treating PA 3679 cells with sorbate resulted in an increased intracellular
Table 5. Effect of potassium sorbate and sodium benzoate on the uptake of phenylalanine. Amounts of $^3$H-phenylalanine* in untreated, potassium sorbate (KS, 200 mM), and sodium benzoate (NaB, 200 mM)-treated PA 3679 cells after 12 seconds incubation at pH 7. The results were analyzed statistically by two-tailed t tests and found to be significant ($P<0.001$).

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>untreated</th>
<th>KS</th>
<th>NaB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>572</td>
<td>162</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>582</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>309</td>
<td>114</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>519</td>
<td>399</td>
<td>163</td>
</tr>
<tr>
<td>5</td>
<td>340</td>
<td>208</td>
<td>122</td>
</tr>
</tbody>
</table>

* Amounts of $^3$H-phenylalanine are presented as the number of disintegrations per minute. Counts obtained at 6 seconds were too low. No counts were obtained from TCA insoluble cell material at 13-14 seconds incubation, indicating that all counts obtained represent uptake only.
Figure 4. Locations of guanosine tetraphosphate (ppGpp), guanosine triphosphate (GTP), and adenosine triphosphate (ATP) on PEI cellulose chromatography sheets after final development with 1.5 M phosphate (pH 3.4).
Figure 5. Effect of potassium sorbate on the accumulation rates of $^{32}\text{P}$-labelled ATP, GTP, ppGpp, and an unidentified compound in PA 3679 cells. Rates are given as dpm* from nucleotide spots on PEI cellulose chromatograms developed with 1.5 M phosphate, pH 3.4, from 5, 10, 15, and 20 min sampling times relative to time "0" (immediately prior to the addition of 200 mM potassium sorbate, KS, at pH 7). Dpm were $10^4$-$10^5$ for ATP, $10^3$-$10^4$ for GTP, $10^2$-$10^3$ for ppGpp and the unidentified compound. Untreated and sorbate-treated cells in both experiments (1a,b and 2a,b) had been preincubated (10 min) with $^{32}\text{P}$-orthophosphate prior to time "0". Rates of ATP and GTP accumulation are given in 1a and 2a and of the accumulation of ppGpp and the unidentified compound (unknown) in 1b and 2b.

* disintegrations per minute
1a
- ATP-Control
- ATP-KS
- GTP-Control
- GTP-KS

1b
- ppGpp-Control
- ppGpp-KS
- Unknown-Control
- Unknown-KS

2a
- ATP-Control
- ATP-KS
- GTP-Control
- GTP-KS

2b
- ppGpp-Control
- ppGpp-KS
- Unknown-Control
- Unknown-KS

ACCUmULATION RATES

MINUTES
accumulation of GTP, ppGpp, and an unidentified compound (possibly pppGpp) which appeared between the original extract spot and the ppGpp spot on the developed chromatogram. The amount of ppGpp and the unidentified compound increased also in the untreated cells but at a more reduced rate. The amount of ATP did not decrease, indicating that changes in the formation rates were not due to cell death.

Figure 6 shows that at time "0" (just prior to the addition of sorbate), the ratios of ATP to GTP and GTP to ppGpp are each about ten to one. Although GTP and ppGpp accumulated within sorbate-treated cells, the amount of GTP never exceeded that of ATP and although the initial rate of ppGpp formation was more rapid than that of GTP, the total amount of ppGpp did not exceed that of GTP during the entire incubation period.

b. Protein Synthesis

The patterns of protein synthesis in untreated sorbate- or benzoate-treated PA 3679 cells were examined. Tritiated isoleucine and phenylalanine were chosen for this study, but since isoleucine was readily catabolized, especially by the preservative treated cells, 

3H-phenylalanine was used in the amino acid uptake experiments and in a study of the relative rates of protein synthesis. The results show that both sorbate and benzoate inhibit protein synthesis in PA 3679 cells, with benzoate being more inhibitory than sorbate (Figure 7).
Figure 6. Effect of potassium sorbate on the rate of accumulation of $^{32}$P-labelled ATP relative to ppGpp, ATP to GTP, and ppGpp to GTP in PA 3679 cells. In the two experiments presented (1 and 2), the ratios ATP/ppGpp (1a, 2a), ATP/GTP (1b, 2b), and ppGpp/GTP (1c, 2c) were obtained from dpm* (adjusted for the number of phosphates in each compound) from nucleotide spots developed on PEI cellulose chromatograms with 1.5 M phosphate at pH 3.4. The dpm obtained from each spot were $10^4$-$10^5$ for ATP, $10^3$-$10^4$ for GTP, and $10^2$-$10^3$ for ppGpp. The cells had been preincubated (10 min) with $^{32}$P-orthophosphate prior to the time "0" sampling. Potassium sorbate (200 mM, pH 7) was added immediately after the initial time "0" sampling.

* disintegrations per minute
Figure 7. Effect of potassium sorbate and sodium benzoate on the rate of protein synthesis. Relative rates of protein synthesis in untreated (O), potassium sorbate (KS, 200 mM) and sodium benzoate (NaB, 200 mM) treated PA 3679 cells (pH 7) are presented as dpm obtained from the incorporation of $^{3}$H-phenylalanine. Since a low concentration of $^{3}$H-phenylalanine was used, dpm represents the amount of amino acid incorporated into protein and transfer RNA.
c. Effects of Tetracycline on Sorbate-Induced Growth Inhibition

Figure 8 shows the effect of a small amount of tetracycline on sorbate-induced growth inhibition of PA 3679. PA 3679 cells treated with sorbate alone were inhibited but those treated with sorbate and tetracycline continued to grow slowly.

Morphology

Normally growing PA 3679 cells in late exponential phase, are about 0.3-0.7 x 2.8-7.1 μ and appear as shown in Figures 9 and 10. However, earlier in exponential phase, a rapidly growing PA 3679 culture consists of very long filaments (Figures 11, 12) that can be 30 μ or more in length. These filamentous cells are quite straight yet flexible and undulate as they move through the liquid medium.

When PA 3679 was grown overnight in medium containing inhibitory concentrations of sorbate, the cells were elongated, bulged in some areas, and bent (Figures 13, 14, 15). When these cells were subcultured in medium without sorbate, the morphology of the cells in the growing culture was that of normal clostridial rods. With electron microscopy, the filamentous PA 3679 cells formed due to sorbate treatment either showed no evidence of septation or had occasional abnormal septation with the formation of a minicell (Figure 14). The inner layer of the cell wall appeared thickened in some areas while the outer layer
Figure 8. The effect of tetracycline (Tetra) on potassium sorbate (KS) induced growth inhibition of PA 3679 at pH 6.

* Colony forming units
Figure 9. Untreated PA 3679 cells late in exponential phase, as seen with phase contrast optics.
Figure 10. Untreated PA 3679 cells in late exponential phase; a, cell membrane; b, inner cell wall; c, outer cell wall. X 42,000
Figure 11. Untreated filamentous PA 3679 cells in exponential phase, as seen with phase contrast optics.
Figure 12. Untreated filamentous PA 3679 cells in exponential phase. X 19,600
Figure 13. Filament formation in PA 3679 treated with potassium sorbate (50 mM, pH 6.5), as seen with phase contrast optics. Cell is bent and bulging in several areas.
Figure 14. Filament formation in PA 3679 cells treated with potassium sorbate (50 mM, pH 6.5). There are no septa except for the formation of an abnormal minicell. Arrows indicate areas of thickened inner cell wall. Outer wall appears to be lacking in many areas. X 37,500
Figure 15. Filament formation in PA 3679 cell treated with potassium sorbate (50 mM, pH 6.5). Cell is bent and bulging. X 37,500
appeared to have pulled away from the inner layer and in other areas appeared to be lacking entirely. (Figure 14, 15). Some of the cells appeared to have fallen apart, and in others the cell interior appeared to consist mainly of dark clumped and fibrillar material (Figure 16).

Filaments formed in HCl-treated cultures of PA 3679 (Figures 17, 18, 19) showed evidence of septation at irregular intervals, with distances longer between septa than those in untreated short cells. The appearance of the HCl-treated cell wall was similar to that in the sorbate-treated cells. The inner wall appeared thickened in some places while the outer wall appeared to be absent in some areas. Many of the cells were seen to be falling apart with clumped, dark material and some fibrillar material remaining in the cell interior.

When PA 3679 was grown overnight in 1 mM sodium nitrite (pH 7), the cells became filamentous (Figures 20, 21, 22). Some of the filaments had septa (Figure 21) while others did not (Figure 22). Unlike sorbate-or acid-treated cells, nitrite-treated cells did not appear to have thickened inner cell walls and although many were bent, they did not appear to bulge. Many of the nitrite-treated cells appeared to have come apart and the interiors of some appeared to consist mainly of clumped and fibrillar material (Figure 23).
Figure 16. Filament formation in PA 3679 cells treated with potassium sorbate (50 mM, pH 6.5). The interior consists of dark clumped and fibrillar material and the inner cell wall (a) has pulled away from the cell membrane (b). X 32,500
Figure 17. Filament formation in PA 3679 cells treated with acid (HCl, pH 5), as seen with phase contrast optics. Cells are bent and thickened in some areas.
Figure 18. Filament formation in PA 3679 cells treated with acid (HCl, pH 5). Cells are bent and have septations. The inner cell wall is thickened (arrows) and outer cell wall is absent in some areas. X 23,250
Figure 19. Filament formation in PA 3679 cells treated with acid (HCl, pH 5). Septa formation and mesosomes (arrows). Many cells have fallen apart. X 24,500
Figure 20. Filament formation in PA 3679 cells treated with sodium nitrite (1 mM, pH 7), as seen with phase contrast optics. Cells are bent in some places.
Figure 21. Filament formation in PA 3679 cells treated with sodium nitrite (1 mM, pH 7). Elongated cell with irregularly spaced septa. X 14,000
Figure 22. Filament formation in PA 3679 cells treated with sodium nitrite (1 mM, pH 7). Cells lack septa. Many cells have fallen apart. X 19,600
Figure 23. Filament formation in PA 3679 cells treated with sodium nitrite (1 mM, pH 7). Interior of elongated, septated cell consists of clumped and fibrillar material. X 19,600
Application to Food Preservation

a. Effect of 1% sucrose on sorbate induced growth inhibition

Figure 24 shows that while 1% sucrose alone appeared to have no effect on the growth of untreated PA 3679 in combination with an inhibitory concentration of sorbate (30 mM, pH 6), it appeared to have a very slight antagonistic effect to the preservative.

b. Effect of 3% and 1% sodium chloride on sorbate-induced growth inhibition

At a concentration of 3%, sodium chloride alone had a slightly inhibitory effect on the growth of PA 3679 (Figure 25) but in combination with sorbate the inhibition of growth was greater than with either compound alone. At a 1% concentration, sodium chloride alone had no apparent effect on the growth of PA 3679 (Figure 26); in combination with sorbate, 1% sodium chloride antagonized the inhibitory action of the preservative.

c. Effect of a low concentration of sodium nitrite on sorbate-induced growth inhibition

A concentration of 100 M sodium nitrite alone appeared to have no appreciable effect on the growth of PA 3679 (Figure 27). However, in combination with sorbate, sodium nitrite was synergistic to the antibacterial action of the sorbate. This combination not only prevented the growth of PA 3679 but killed the cells within a few days.
Figure 24. Effect of 1% sucrose on potassium sorbate (KS) induced growth inhibition of PA 3679 at pH 6.

* Colony forming units
Figure 25: The effect of 3% NaCl on potassium sorbate (KS) induced growth inhibition of PA 3679 at pH 6.

* Colony forming units
Figure 26. Effect of 1% NaCl on potassium sorbate (KS) induced growth inhibition of PA 3679 at pH 6.

* Colony forming units
Figure 27. Effect of sodium nitrite (NaNO₂) on potassium sorbate (KS) induced growth inhibition of PA 3679 at pH 6.

* Colony forming units
d. Effect of nitrite on $pH_i$

Table 7 shows the results of measuring the $pH_i$ (calculated from using the results in Table 6) of PA 3679 cells treated with nitrite alone and in combination with sorbate. Nitrite increased the $pH_i$ of the cells.
Table 6. Effect of potassium sorbate (KS, 200 mM) and nitrite (sodium nitrite, NaNO₂, 40 mM, or potassium nitrite, KNO₂, 40 mM) alone and in combination on the uptake of DMO in PA 3679 cells (pH 7). These results were used to calculate pHᵢ in Table 7.

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<td>¹⁴C</td>
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* disintegrations per minute
Table 7. Effect of sodium nitrite (NaNO₂, 40 mM) or potassium nitrite (KNO₂, 40 mM), alone or in combination with potassium sorbate (KS, 200 mM) at pH₀ 7, on the intracellular pH (pHᵢ) of PA 3679 as measured by the distribution of DMO. The results presented here and in Table 3 on the effects of KS on the pHᵢ when the pH₀ was 7 (n = 8), were analyzed statistically by a two-tailed t test and found to be significant (p = 0.00732). Sample sizes for the effects of NaNO₂ and KNO₂ and their combination with KS on pHᵢ proved to be too small for meaningful analyses. However, since these effects were equal to or greater in magnitude than that of KS at pH₀ 7, they are probably also significant.

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IV. DISCUSSION

Protonophoric Activity of Sorbic Acid

Activity of the undissociated acid

Prior research has shown that the antimicrobial activity of sorbic acid/sorbate is pH dependent, its effectiveness as a preservative increasing with decreasing pH (Robach and Sofos, 1980, 1981; Marriott et al., 1981; Huhtanen, 1984). It has been suggested that the active component might be the undissociated acid since its concentration increases under acid conditions and being an uncharged, lipophilic molecule it could more readily transverse the lipid of the cell membrane. Although this hypothesis has been discussed, it has never been clearly demonstrated experimentally. Figure 3 shows that sorbate-induced growth inhibition of PA 3679 appears to be due to the activity of the undissociated acid.

Internal pH (pHi)

The proton gradient (pH) across the bacterial membrane is given as the difference between the pH of the cell interior (pHi) and that of the external medium (pH0). The range of the proton gradients for untreated PA 3679 cells at pH 7 was 0.54-0.70 pH unit (inside alkaline) (Tables 3, 7) and is very similar to the 0.4-0.8 range reported for C. pasteurianum (Riebling et al., 1975) and the 0.63-0.75 range observed with E. coli (Padan et al., 1975). The internal pH of a cell varies with its metabolic
activities, e.g. the pH$_i$ of *E. coli* is lower when the organism is growing anaerobically (Padan *et al.*, 1975), and *Streptococcus faecalis* maintains a larger proton gradient when fermenting glucose than it does with arginine (Harold, 1970). Therefore, differences in the pH$_i$ can be expected between cultures and at different times during the growth cycle of a given organism.

The pH$_i$ of PA 3679 cells treated with sorbate was always lower than that of untreated, control cells (Table 3). Similar observations were reported by Salmond *et al.* (1984) and Eklund (1985) who tested the effects of sorbate on the pH$_i$ of *E. coli* cells and vesicles respectively. Although the addition of sorbate always reduced the proton gradient of PA 3679, it never totally collapsed it (Tables 3, 7). This observation was not unexpected because 200 mM potassium sorbate at pH 7 represents only 1.2 mM undissociated sorbic acid, a concentration that is only slightly inhibitory to PA 3679 (Figure 3) and represents the amounts often added to food. Most of the experiments were conducted at pH 7 to minimize the effect of external acidity. Nevertheless, even at this low concentration of undissociated acid at pH 7, sorbate had protonophoric capabilities (Tables 3, 7).

Since a high pH$_i$ in neutrophilic organisms such as *E. coli* reflects a more efficient proton extrusion system, i.e. in aerobic respiration (Padan *et al.*, 1975), cells with higher pH$_i$ values should be more efficient in
maintaining their proton gradient in the presence of a low concentration of a protonophore. This hypothesis was tested in PA 3679 by plotting (Figure 28) the pHi of untreated cells versus the magnitude of decrease in the pHi of sorbate-treated cells from the same culture. The decreasing pHi values of untreated cells between 7.70-7.61 and 7.54 reflect a loss in efficacy of their proton extrusion system to maintain the proton gradient. The small decrease in pHi of the sorbate-treated cells with initial pHi values of 7.57 and 7.59 may be the result of the small sampling size (n = 8; point at pHi(control) 7.66 represents identical results from 2 trials). One possibility for the dichotomy seen at pH 7.57 and 7.59 may be due to changes in respiratory systems regulated by growth conditions (Ingledew and Poole, 1984). Another possibility is that there is a change in the energy patterns of PA 3679 as the pHi values decrease. As first proposed by Mitchell in 1961, ATP is generated by a reversible proton pump in H+-ATPase systems such that the proton gradient can be used to drive the phosphorylation of ADP for the production of ATP. When the proton gradient is too low, the reaction runs in reverse, using energy from the hydrolysis of ATP to generate a proton gradient i.e. by effluxing protons (Skulachev, 1981). Possibly, untreated cells with a pHi of 7.57 and 7.59 are utilizing ATP to create a proton gradient that increases the efflux of protons brought in by sorbic acid. Also, perhaps this
Figure 28. The magnitude of the decrease in the intracellular pH ($pH_i$) of PA 3679 cells with the addition of potassium sorbate [$pH_i$(control) - $pH_i$(KS), in pH units] as a function of the initial $pH_i$, i.e. the $pH_i$ of untreated cells from the same culture [$pH_i$(control)]. The $pH_i$ values were taken from tables 3 and 7.
method of maintaining a $\Delta \text{pH}$ in PA 3679 cells is not efficient at pH 7.54 so that the addition of sorbate collapsed the $\Delta \text{pH}$ to 0.1 pH unit.

Recent interest in $\text{pH}_i$ and the effects that small changes (0.2 pH unit or less) may either cause or reflect intracellular activities has been confined primarily to eucaryotic cells (Nuccitelli and Deamer, 1981). One reason for this may be that the $\text{pH}_i$ of the larger cells is easier to measure. However, the results from the present study indicate that such research with clostridial cells might be helpful in elucidating some of their intracellular activities.

Since many foods are acidic, the effects of lowering the external pH ($\text{pH}_o$) to 6.5 on the $\text{pH}_i$ were examined in untreated and sorbate-treated cells of PA 3679. The observed decrease in the $\text{pH}_i$ of PA 3679 cells grown at $\text{pH}_o$ 6.5 (Table 3) produced no discernable effect on the growth of the organisms, probably because the proton gradient, pH, was actually increased by lowering the external pH. Although lowering the external pH increases the $\Delta \text{pH}$, there is a minimum internal pH below which bacteria will not grow. In studies on the effects of various preservatives, Salmond et al. (1984) reported that $\text{E. coli}$ failed to grow when the $\text{pH}_i$ fell below 7.1, regardless of the $\text{pH}_o$.

Protonmotive Force (pmf)

The proton gradient, $\Delta \text{pH}$, is important to the bioenergetics of cells but is only one component of the pmf
which powers cellular activities such as the active transport of various compounds necessary for growth and motility. A cell can maintain a high pmf by increasing its membrane potential even when its proton gradient decreases (Skulachev, 1975a, 1980, 1981). A lack of motility in sorbate-treated PA 3679 cells (Table 4) suggests that the pmf has been dissipated.

**Amino Acid Uptake**

Eklund (1980) studied the effect of various preservatives on the uptake of several amino acids in vesicles from *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* and reported that sorbate and benzoate inhibited uptake of all the amino acids tested in vesicles from all three organisms, but the amount which gave 50% inhibition varied. Benzoate was more effective than sorbate in inhibiting phenylalanine uptake in *E. coli* vesicles while the reverse was true for *P. aeruginosa* vesicles (*B. subtilis* vesicles were not tested for phenylalanine uptake). Although Eklund believed that amino acid uptake was due to the protonophoric effects of sorbate and benzoate, he could not explain how this affected the growth inhibition observed with whole cells. In the present study, sorbate-induced inhibition of phenylalanine uptake (Table 5) varied among different cultures of PA 3679, with the percentage inhibition at 12 seconds ranging from 13% to 84%. This variability in the inhibitory
concentration of sorbate may be due to differences in the ability of sorbate to reduce the pH (Table 3, 7).

**Stringent-type Response**

**Phosphorylated Nucleotides**

When faced with a limited intracellular concentration of amino acids for protein synthesis, many types of bacteria undergo a series of major intracellular readjustments that have been called the "stringent response" (Gallant, 1979). This response occurs when amino acids are not available in the ratios required, or if only one amino acid becomes limiting. In these organisms, the stringent response is characterized by many alterations within the cell, e.g. changes in the triphosphate pool, increased levels of guanosine tetraphosphate (ppGpp) and other alarmones such as guanosine pentaphosphate (pppGpp) in some organisms (Cashel, 1975; Gallant, 1979). Because of the possibility that sorbate-induced growth inhibition in PA 3579 involves a stringent-type response, the relative rates of $^{32}$P-labelled ATP, GTP, and ppGpp formation were determined.

Figure 5 shows that the addition of sorbate results in a dramatic change in the pattern of nucleotide formation. In sorbate-treated cells, the rates of formation of GTP, ppGpp, and an unidentified compound increased rapidly, resulting in the accumulation of larger amounts of these compounds than in the untreated cells. The amounts of GTP,
ppGpp, and an unidentified phosphorylated compound in sorbate-treated cells were greater in experiment 2 (Figure 5). Although GTP and pppGpp are believed to be involved in the metabolism of ppGpp, ATP is considered to be the initial source of the phosphate groups (Sy et al., 1979). Sorbate dissipates the pmf which energizes ATP production via the H^+-ATPase system. Perhaps a lower rate of ATP production in the sorbate-treated cells in experiment 1 was responsible for the smaller amount of ppGpp produced than in sorbate-treated cells of experiment 2. However, since the turnover rate was not determined for ATP during the sampling times, and the metabolic pathways of ppGpp in PA 3679 are unknown, further research would be required to determine the relationship between the formation of ATP and the other phosphorylated compounds in this organism.

Guanosine tetraphosphate is quite labile, decomposing at a rate of 1% per day at room temperature, and is sensitive to acid resulting in losses of perhaps as much as 50-80% during the extraction procedure (Lagosky and Chang, 1978). Therefore, the levels of ppGpp in living cells are probably higher and the difference between untreated and sorbate-treated cells would be greater than the results of this study indicate.

The amount of ppGpp in the untreated cells increased during the sampling period. This was probably due to the fact that the growth conditions employed for the experiment were not ideal for PA 3679; the defined medium used in this
study does not support growth quite as well at Trypticase broth, the cell suspensions were very concentrated \((5 \times 10^8\text{ cells ml}^{-1})\), and aerobic incubation was used to permit sampling at 5 minute intervals. Under such conditions, division in the untreated cells stopped shortly after 35-45 minutes. Nevertheless the differences in the pattern of nucleotide formation between untreated and sorbate-treated cells were readily discernable.

The levels of radioactivity on the chromatography sheets were checked for the presence of unidentified compounds in the areas between the origin, ppGpp, GTP, and ATP spots. An increased level of radioactivity was observed in the area between the origin and the ppGpp spot developed from extracts made from sorbate-treated cells (Figure 5). The separation methods used in this study would result in pppGpp remaining in this area of the chromatograph as well as other unusual nucleotides reported to be involved in the regulation of sporulation in *Bacillus subtilis* (Rhaese et al., 1972, 1975). Since sorbate-treated cells do not sporulate, it would seem more likely that this unidentified compound was pppGpp rather than other unusual nucleotides. Although the identity of this compound is not known, its appearance in sorbate-treated cells, as well as the observed nucleotide changes, typify the responses seen in cells adjusting to environmental stresses such as nutrient and energy deprivation.
Using an *E. coli* strain with a temperature labile valyl-tRNA synthetase, Fiil *et al.* (1972) reported that during valine starvation, the level of ATP rose within the cells and then fell below prestarvation levels. The level of GTP fell, and the ppGpp level rose sharply in 3-5 minutes and subsequently fell but remained at a higher level than was seen initially. Accumulation of ppGpp has also been reported in cells with energy depletion (Edlin and Donini, 1971; Cashel, 1975; Gallant *et al.* 1976; Molin *et al.*, 1977). Edlin and Donini (1971) treated *E. coli* cells with cyanide to block respiration and deplete their energy. They observed that the intracellular level of ppGpp rose sharply, and in contrast to amino acid deprivation, the level remained high. In *E. coli* cells treated with the uncoupling agent carbonyl metachlorophenylhydrazone, Gallant *et al.* (1976) observed only a small decrease in the ATP level, a small but definite increase in ppGpp level, a sharp reduction in protein synthesis, and the disappearance of an unusual and unidentified nucleotide called the "phantom spot". The authors speculated that this phantom spot was a form of GTP with a modified imidazole portion of the purine ring and that it plays a role in the regulation of protein synthesis.

In *B. subtilis* partially deprived of amino acids, increased levels of ppGpp and pppGpp induced by the stringent response occurred immediately prior to the
initiation of sporulation (Ochi et al., 1981). A later study showed that sporulation induced by the stringent response in *B. subtilis* is caused by a decrease in the GTP level but not by an increase in the level of ppGpp (Ochi et al., 1982). In sorbate-treated PA 3679 cells the rates of GTP and ppGpp formation increased; although the sorbate-treated cells stopped dividing as expected in the stringent response, they did not sporulate. However, since sorbate has been reported to reduce energy levels within cells as well as limit the uptake of available amino acids, sorbate-treated PA 3679 cells were probably responding to energy and amino acid deprivation simultaneously. Therefore, the response to sorbate observed in PA 3679 will be referred to as "stringent-type" because although it is the result of stringently controlled regulation mechanisms, it is not identical to the classical stringent response which refers exclusively to the effects of amino acid deprivation.

Protein Synthesis

Another feature of stringently controlled cell regulation is a sharp reduction in the accumulation of RNA, and this is reflected in a corresponding reduction in the rate of protein synthesis which is determined directly by the number of ribosomes present in the cell (Cashel, 1975; Gallant, 1979). Both sorbate and benzoate inhibited protein synthesis in PA 3679 cells, with benzoate being
more effective than sorbate, as would be anticipated from the results obtained in the amino acid uptake experiments (Table 5). In all trials with sorbate-treated cells, there was a period of about 5 to 10 minutes when protein synthesis essentially stopped, while in untreated cells a similar period occurred at about the 15 to 20 minute interval (Figure 7). In the phosphorylated nucleotide study (Figures 5, 6), the amount of ppGpp and the ratio of ppGpp to GTP in sorbate-treated cells increased sharply within the first 5 to 10 minutes of incubation while the amount of ppGpp and the ratio of ppGpp to GTP in untreated cells increased gradually and in 15 to 20 minutes reached the same level as in the cells treated with sorbate for 5 to 10 minutes. Although the cells in the protein synthesis study had been preincubated with sorbate for 10 minutes prior to time "0", the cells had been starved for phenylalanine, a required amino acid, and had ceased replicating before addition of the sorbate. These cells did not become fully active metabolically in the presence of sorbate until the radiolabelled phenylalanine was added at time "0". Therefore, since the periods of little or no protein synthesis appear to occur simultaneously with the periods of increased levels ppGpp and a high ppGpp to GTP ratio, it seems plausible that ppGpp is involved in regulation of protein synthesis in PA 3679 cells.
Release of Sorbate-Induced Stringent-type Control

When protein synthesis and cell replication in bacteria have ceased due to stringent control, the cells do not necessarily die but instead remain in a conserved state (page 17) for extended periods and can resume normal growth when environmental conditions are favorable and stringent control has been released. A low concentration of tetracycline has been shown to release the stringent control by effecting perhaps directly, the metabolism of ppGpp (Cashel, 1975; Silverman and Atherly, 1978). The continued slow growth of PA 3679 in a medium containing an inhibitory concentration of sorbate and a small amount of tetracycline (Figure 8), is similar to that seen with relaxed mutants of E. coli amino acid auxotrophs lacking the stringent response. When the amino acid concentration is reduced, these mutants continue growing at a slow rate and utilize the low concentration of nutrients available. The results of this experiment with PA 3679 indicated that sorbate-induced growth inhibition is a result of stringently controlled cell regulation in which ppGpp is probably involved. Continued growth of PA 3679 in the presence of tetracycline and an otherwise inhibiting concentration of sorbate also shows that energy and amino acids need not be totally depleted within a bacterium before cell division ceases.
Morphology

Stringently controlled cell regulation responses involve major intracellular readjustments. The alarmones produced as a result of such regulation have a broad range of effects throughout the cells, directly or indirectly controlling many enzymatic reactions. It is not surprising that the early literature suggested that the mechanism of action of sorbic acid was based on inhibition of so many different enzymes. One important system among the myriad of cellular activities under stringent control is the biosynthesis of peptidoglycan (Ishiguro and Ramey, 1976). Bacteria subjected to nutrient limiting conditions have been reported to change their size and shape (Ishiguro and Ramey, 1976; Ron et al., 1977; Chesbro et al., 1979; Reichhardt and Morits, 1982; Grossman et al., 1982; Verseveld et al., 1984).

In a study of cell length, growth, and division in E. coli, Donachie et al. (1976), reported that the bacteria divided upon reaching a maximum length of twice their minimum size, irrespective of their rate of growth. Although Sargent (1975), also reported similar findings with B. subtilis, it should not be assumed that this generalization is true for all bacteria. Henrici (1928) studied the length of B. megaterium during growth on agar slants and reported that cell length increased with the rate of growth and that during the period of most rapid growth, the cells were six times as long as cells in the
original inoculum. Filament formation during rapid growth of *E. coli* and *Proteus vulgaris* also was reported by Kleneberger-Nobel (1947) who wrote, "The differences in the sizes of the cells are so great that the unbiased onlooker may doubt the purity of the culture from which the preparation was made." In a study on bacterial swarming, Turner and Eales (1941) reported filament formation in *Clostridium botulinum, Clostridium novyi, Clostridium tetani,* and *Clostridium septicum,* while Henrichsen (1972) reported similar findings in *Proteus mirabilis, Clostridium tetani,* and *Bacillus alvei.* These latter three authors considered the filamentous cell to be a normal form of the organism which could be observed during periods of rapid colony swarming. Henrichsen (1972) reported that the filamentous cells had unusually large numbers of flagella and were present on the leading edge of swarming colonies. Filament formation in PA 3679 was observed previously by Frank and Lum (1969) but considered to be a normal phase of growth for these organisms in batch culture and resembled those in Figures 11 and 12.

The peptidoglycan of *C. sporogenes* was reported to be identical to that of *C. botulinum* (Takumi and Kawata, 1976) and similar to that of *E. coli* and *B. subtilis* (Schleifer and Kandler, 1972). The peptidoglycans of these organisms consist of repeating units of N-acetylglucosamine and N-acetylmuramic acid in which the D-lactate moiety of each muramic acid residue is substituted by a short peptide
chain. Although some variations exist in the peptide chains of the peptidolycans of *C. sporogenes*, *E. coli*, and *B. subtilis*, all have direct crosslinkages between the meso-diaminopimelic acid (DAP) residue of one peptide chain and the terminal D-alanine of another. It is believed that these peptidoglycans are synthesized in the same way, with synthesis occurring in three stages; in the cytoplasm, membrane bound, and wall bound (Mirelman, 1979).

Ishiguro and Ramey (1976) reported that peptidoglycan synthesis in *E. coli* is under stringent control. Using $^3$H-DAP with a stringently controlled, lysine and DAP auxotroph of *E. coli*, Ramey and Ishiguro (1978) showed that amino acid deprivation did not inhibit the synthesis of monosaccharide-pentapeptide or disaccharide-pentapeptide derivatives of the lipid intermediate but did prevent the incorporation of the disaccharide-pentapeptide into the existing cell wall. The amount of labelled peptidoglycan in the amino acid deprived cells was only 20-44% of that synthesized in the presence of amino acids. Ishiguro and Ramey (1976) reported that, although the stringently controlled *E. coli* cells had been deprived of amino acid, cell division continued for 30-40 minutes without detectable increase in mass, resulting in smaller than normal cells, indicating that septation is under stringent control. They reported that walls of these smaller cells were thickened but unfortunately did not publish electron photomicrographs. Treatment of PA 3679 cells with sorbate
resulted in a thickening of the cell wall (Figures 14, 15) as was described in amino acid-deprived stringently controlled E. coli (Ishiguro and Ramey, 1976). However, unlike E. coli cells which became smaller during the stringent response, sorbate-treated PA 3679 cells were elongated and, except for a few unusual forms, had no septa.

In a study on the role of the protonmotive force in the synthesis of peptidoglycan and teichoic acid in B. subtilis, Harrington and Baddiley (1984) reported that the pmf maintained the activity of the cell wall synthetic enzymes. Since sorbate reduces the pmf as well as inducing a stringent-type response in PA 3679 cells, sorbate would have multiple effects on cell wall biosynthesis. However, from the effects of sorbate observed with PA 3679, it appears that a reduction in the pmf and/or a stringent-type response had a greater effect on the rate of septation than on the rate of elongation.

Although the cell walls of sorbate-treated PA 3679 cells appear thickened, the filaments which formed were bent and had areas that appeared to bulge (Figure 13, 14, 15). This could be due to a decrease in the number of crosslinkages in the peptidoglycan, resulting in a general loss of cell rigidity. When E. coli DAP auxotrophs were deprived of the DAP needed for peptidoglycan cross-linking, the cells elongated and bulged (Bayer, 1967). Warth and Strominger (1972) reported that peptidoglycan produced
during sporulation of *B. subtilis* was different than that of vegetative cells, one difference being a lower degree of cross-linking. Since sporulation occurs after the vegetative cell has undergone a stringent response (Ochi et al., 1981), it is tempting to think that the stringent response can trigger production of a different kind of peptidoglycan with fewer crosslinkages. Another possibility is that the thickened areas of the cell wall are excessively crosslinked, causing constricted patches which distort the shape of the filaments. Harkness and Ishiguro (1980) reported that peptidoglycan of amino acid-deprived *E. coli* exhibited 95% crosslinking as compared with only 40-50% crosslinking in the peptidoglycan of cells grown in the presence of amino acids.

Morphological changes observed in PA 3679 treated with sorbate and acid are very similar (Figures 13, 14, 15, 17, 18, 19), but since both are known to lower the pH, this is not surprising. Since the addition of acid lowers both intracellular and extracellular pH, whereas sorbate reduces only the pH, some differences could also be expected. Such differences are apparent when the degree of septation is compared. Filaments formed in acid-treated PA 3679 cells appear to have septa, whereas the only evidence of septation seen in filaments of sorbate-treated cells are those involved in the formation of the abnormal minicells. Nitrite-treated cells are different from either sorbate- or acid-treated cells. The addition of nitrite leads to the
formation of filaments which may (Figure 21) or may not (Figure 22) have septa and, unlike sorbate- or acid-treated cells, do not have thickened cell walls nor do they appear to bulge. Further research would be needed to explain the morphological changes observed in sorbate-, acid-, and nitrite-treated PA 3679 cells.

Applications to Food Preservation

When sorbic acid/potassium sorbate is used as a preservative, it is generally employed in combination with other compounds that may or may not have added effects. Three ingredients commonly used in processed meat products, i.e. sucrose, sodium chloride, and low levels of sodium nitrite, were tested for possible effects on sorbate-induced growth inhibition of PA 3679.

It has been reported that high concentrations of sucrose usually inhibit the growth of microorganisms while low levels appear to have no adverse effects (Busta, 1982). However, in this study, 1% sucrose in combination with sorbate, appeared to antagonize slightly the effect of the preservative (Figure 24). The sugar probably served as an additional nutrient for the organisms.

While 3% sodium chloride enhanced the inhibitory effect of sorbate on the growth of PA 3679 (Figure 25), 1% appeared to be antagonistic to the inhibitory effects of the preservative (Figure 26). This antagonism could have been due to the role that Na⁺ might have in the
bioenergetics of the organism. According to Skulachev (1980), Na\(^+\) helps stabilize the proton gradient, while K\(^+\) helps stabilize the membrane potential; together the gradients established by both ions serve as a buffer for the protonmotive force. Thus, in PA 3679, the added Na\(^+\) may help stabilize the pmf which had been reduced by the protonophoric activity of potassium sorbate. This view is supported by the observation that sodium chloride increased the relative rate of motility from a 3 to a 4 untreated PA 3679 cells and also increased the rate of motility in sorbate-treated cells. Another explanation is that Na\(^+\) may have aided directly in the transport of various amino acids. The sodium gradient energized the transport of glutamate in E. coli (Anraku, 1982). Other investigators observed that Na\(^+\) stimulated the anaerobic transport of glutamate, aspartate, serine, cysteine, and lactate in a non-NaCl-requiring Alteromonas putrefaciens (Stenberg et al., 1984) and that of L-leucine in Pseudomonas aeruginosa (Hoshino and Kageyama, 1979). Perhaps the added Na\(^+\) stimulated directly the transport of amino acids in PA 3679, thereby antagonizing sorbate-induced inhibition of amino acid uptake.

Although high concentrations of either sucrose or sodium chloride may act synergistically with sorbate to inhibit microbial growth, the results obtained in this study show that low concentrations may antagonize inhibition by sorbate. Therefore, the concentrations of
both sucrose and sodium chloride used in foods must be considered carefully whenever sorbate is intended for use as a preservative.

The synergistic action of sorbate and nitrite has been studied widely (Marriott et al., 1981; Busta, 1982). Low concentrations of sodium nitrite, in combination with an inhibitory concentration of potassium sorbate, were very effective against PA 3679 (Figure 27). To date, the synergistic action of sorbate and nitrite has been unclear because the mechanisms of action of either preservative were not known. Nitrite is known to bind to many iron containing compounds such as ferredoxin, which serves as the active site for electron transport in aerobic and anaerobic respiration, and to rubredoxin which is involved in anaerobic respiration in clostridia (Benedict, 1980). Nitrite has been reported to inhibit respiratory-energy coupling in Pseudomonas putida, Paracoccus denitrificans, and Pseudomonas aeruginosa (Rake and Eagon, 1980). Nitrite has also been reported to inhibit the phosphoroclastic system in C. sporogenes (Woods et al., 1981) and C. botulinum (Woods and Wood, 1982) with a concomitant increase in the intracellular concentration of pyruvic acid. In the phosphoroclastic system, coenzyme A reacts with pyruvic acid, with the aid of an oxidoreductase, to form acetyl CoA and CO₂. Electrons from pyruvic acid are passed through an electron transport chain that contains ferredoxin to a hydrogenase where they combine with two
protons to form $H_2$. Therefore, blocking this reaction would inhibit respiration in organisms having this system as well as inhibiting substrate level phosphorylation when acetyl CoA reacts with phosphate to form CoA and acetyl phosphate and then reacts with ADP to form ATP and acetate. If nitrite inhibits respiration i.e. proton efflux, and sorbate increases proton influx, then their synergistic effects would be understandable. If the proton flow into the cell could not be pumped out fast enough while the intracellular concentration of pyruvic acid was increasing, the intracellular pH could be expected to drop dramatically. However, as Table 7 indicates, this was not the case. Surprisingly, nitrite increased the $pH_i$ of PA 3679 cells as well as increasing their rate of motility. Evidently, nitrite disturbs the normal flow of protons across the cell membrane but the mechanism of action is still unknown.

**Conclusions**

Sorbic acid, as do other lipophilic weak acids tested (Chipley, 1983; Doores, 1983; Kabara, 1983), is inhibitory to a wide range of eucaryotic and procaryotic micro-organisms. Their undissociated forms have been implicated as the active agent, and they appear to interfere with cellular energetics and transport. Thus, it appears that the primary mechanism of inhibitory action is due to a common element that affects a common cell system in most if not all microorganisms. This appears to be the
protonophoric ability that dissipates the protonmotive force of the cell and results in a loss of the energy necessary for growth. Based on the results obtained in this study, I propose that undissociated sorbic acid enters PA 3679 cells and reduces the proton gradient and, in turn, the protonmotive force as well. This loss in energy reduces the uptake of amino acids required for energy and for biosynthesis. Bacteria possessing the proper regulatory mechanisms can detect the reduction in energy and/or the intracellular levels of amino acids available for protein synthesis and can respond by major intracellular readjustments, i.e. stringent-type response, that result in a cessation of cell division.

Although lipophilic weak acids such as sorbic and benzoic appear to act as protonophores, they differ in effectiveness as antimicrobial agents. Also, variations exist among different organisms in response to the growth inhibition by each acid. These variations are due to a number of factors that modulate the primary mechanism of action, including:
1. The hydrophobicity of the acid.
2. The permeability of the cell membranes to each acid.
3. The dissociation constant of the acid.
4. The presence of ions or other substances which may act synergistically or antagonistically with the acid.
5. The efficiency of the cell's proton pumps, i.e. its ability to respond to increased proton influx.
6. Possible effects of the anionic portion of the dissociated acid. This portion may be easily metabolized by the cells or may itself be toxic.
7. The regulatory mechanisms of the organisms i.e. how the cells respond to the direct effects of the acid.

Since so many factors can determine the effectiveness of a lipophilic weak acid, it is desirable to investigate the antimicrobial activity of each preservative under the conditions prevailing during its use, i.e. the specific food, temperature, packaging, added ingredients, etc., and the likely organisms for which it is intended.
SUMMARY

The inhibitory effects of potassium sorbate on bioenergetics, amino acid uptake, protein synthesis, cell regulation, and morphology were examined in PA 3679 in order to elucidate its mechanism of action.

Viability studies employing different concentrations of sorbic acid suggest that the undissociated form is the active antimicrobial agent. Measurements of the intracellular pH (pHᵢ) in sorbate-treated PA 3679 cells revealed that sorbate reduced the pHᵢ by acting as a protonophore. Using the rate of motility as an indicator, sorbate dissipated the pmf, thereby depleting the energy required for active transport of nutrients such as amino acids. The uptake of radiolabelled phenylalanine was inhibited by the protonophoric activity of sorbate under conditions where the amino acid was not catabolized by PA 3679.

The relative rates of accumulation of phosphorylated nucleotides were studied in order to determine whether a stringent-type response to amino acid and energy depletion was responsible for biostasis during inhibition by sorbate. Altered patterns of phosphorylated nucleotide formation were observed similar to those seen in stringently controlled bacterial cells. In sorbate-treated cells, the intracellular concentrations of GTP, ppGpp, and an unidentified phosphorylated compound possibly pppGpp,
increased. Using radiolabelled phenylalanine in a study of the relative rates of protein synthesis in untreated and sorbate- or benzoate-treated PA 3679 cells, sorbate like benzoate, inhibited protein synthesis. The periods when no apparent protein synthesis occurred corresponded to those periods with increased levels of ppGpp in untreated and sorbate-treated cells, suggesting that ppGpp may be involved in the regulation of protein synthesis. In a viability study, the addition of a small amount of tetracycline to an inhibitory concentration of sorbate released the growth inhibition in PA 3679, and reproduction resumed slowly. Since tetracycline is known to release the stringent response by acting on ppGpp metabolism, these results suggest that sorbate-induced inhibition of growth is due to a stringent-type response that probably involves ppGpp. Continued growth with sorbate in the presence of tetracycline also indicates that the cells need not be totally depleted of energy or amino acids before cell division is inhibited.

Under examination by light and electron microscopy, sorbate-treated cells appeared filamentous, had bends and bulges and usually had no septation, except where occasional minicells were formed. Some areas of the inner cell wall were thickened, and the outer cell wall appeared to be absent in some places. The morphology of acid-treated PA 3679 cells was similar to that seen in sorbate-treated cells, except that septation occurred.
Since thickening of the cell wall and changes in the rates of elongation and septation have been reported previously in *E. coli* cells undergoing stringent response, the changes in morphology with sorbate-treated PA 3679 cells are consistent with the effects expected from a cell undergoing stringent response.

Based on the results of this study, it is proposed that undissociated sorbic acid acts as a protonophore that reduces the pH$_i$ of PA 3679 and dissipates the pmf, an important form of energy for the cells. A reduction in the pmf restricts amino acid uptake which, coupled with a depletion in energy, can trigger a stringent-type response with changes in the patterns of phosphorylated nucleotide formation, the production of alarmones, reduction in protein synthesis, and cessation of cell replication.

Viability studies with low concentrations of sucrose and sodium chloride in combination with sorbate were antagonistic to the inhibitory effects of sorbate on PA 3679. Therefore, knowledge of the amounts of sugar and salt added to food is important when considering the use of sorbate as a preservative. On the other hand, a low concentration of sodium nitrite in combination with sorbate acted synergistically in their antimicrobial effect on PA 3679. Since nitrite alone, or in combination with sorbate, increased the pH$_i$ of the cells while simultaneously increasing the motility rate of the cells, the mechanism of growth inhibition by nitrite probably affected the
bioenergetics of the cells. Morphological changes in nitrite treated PA 3679 cells were also different than those treated with sorbate. Nitrite-treated cells were elongated and bent but had septa and did not have thickened cell walls. Further studies are needed to determine the mechanism of action of nitrite.
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