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Iowa State University, 1992

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Mechanism of inhibitory action of potassium sorbate in *Escherichia coli*

by

Aubrey Francis Mendonca

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of

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In Charge of Major Work

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For the Graduate College

Iowa State University
Ames, Iowa

1992

DEDICATION

To my dear wife Ingrid, for her love and incredible patience throughout the past ten years. To my children Trevor, Michelle and Michael who kindly accepted my absence from a few of our important holiday activities.

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LITERATURE REVIEW

Introduction

Sorbic acid (2,4-hexadienoic acid; CH_3-CH=CH-CH=CH-COOH) and its salts are collectively known as sorbates. Over the last forty years sorbates have been tested and extensively used for preserving a variety of food products throughout the world. During that time their antimicrobial effects and safety of concentrations used in foods have been well established (Leuck, 1980; Sofos and Busta, 1981; Sofos and Busta, 1983). Sorbic acid and potassium sorbate are the forms most widely used in food preservation. They are also commercially important in the preservation of animal feeds, cosmetics, pharmaceuticals and in other industrial applications (Sofos and Busta, 1981). In the United States sorbate is considered a Generally Regarded as Safe (GRAS) food additive and its use in any food product in which preservatives are allowed may be requested. The amount of sorbate added to food products varies from 0.01 to 0.3% (Sofos, 1989). Those concentrations of sorbate commonly used in food preservation (0.1%-0.3%) usually cause growth stasis in microorganisms but higher concentrations can be lethal.

The precise mechanism by which sorbate inhibits microbial growth is not well understood (Sofos, 1981). An understanding of the antimicrobial mechanism of action of sorbate is important for optimizing its use and for development of new, safe and effective food preservative systems. The need for such systems in present and future food processing and the recent developments in new types of food

products, stimulated great interest in the use of antimicrobials such as sorbate (Sofos, 1981).

Properties of Sorbate

Reactivity

Sorbic acid is a trans-trans unsaturated, straight-chain, monocarboxylic C_6 fatty acid with the following structural formula: CH_3-CH=CH-CH=CH-COOH. It is volatile in steam but does not decompose; therefore, this property is exploited to isolate it from foods during analysis (Lueck, 1976). The chemical behavior of sorbic acid is determined primarily by its chain length, carboxyl group and conjugated double bonds. The reactions of sorbic acid via its carboxyl group are similar to those of other carboxylic acids and thus salts and esters are readily formed (Lueck, 1980). The reactions of sorbic acid via its conjugated double bonds are not uniform and often yield mixed reaction products from different modes of addition at those points of unsaturation. For example, hydrogenation can occur at different positions in the molecule, depending on the presence of catalysts and other conditions.

Both double bonds of sorbic acid can be attacked by molecular oxygen; however, in practice sorbic acid and its derivatives in a dry form are highly resistant to oxidation (Lueck, 1976). Reactions of sorbic acid involving the conjugated double bonds are not uniform and can result in mixtures of various products. Sorbic acid can react with dienophiles, such as maleic anhydride and styrene, via the Diels-Alder

reaction to form complex end products. Different modes of addition for example, the shifting of double bonds, isomerization or other rearrangements contribute to the formation of complex end products.

Solubility

Sorbic acid is relatively insoluble (0.15% w/v) in water at 20°C. It dissolves readily in hot water, in alcohols (especially ethanol), and in anhydrous acetic acid. Its solubility increases with increasing pH because of the partial change of the acid to its more soluble salts (Sofos, 1989). Apart from alcohols and acids, the presence of certain compounds such as alginate, methylcellulose, pectin, propylene glycol and polysorbate increases the solubility of sorbic acid (Ionescu-Stoian et al., 1967). Three times as much sorbic acid is soluble in edible lipids than in water (Chichester and Tanner, 1972). Knowledge of the solubility of sorbic acid in different solvents is necessary to determine how to add it to foods and how to optimize its retention in solution (Sofos, 1989).

Potassium sorbate is very useful in food preservation because of its high water solubility which is 58.2% (w/v) at 20°C. It hydrolyzes in water to produce sorbic acid which is the more active antimicrobial form. Concentrated water solutions (about 50% w/v of potassium sorbate) can be added to liquid food products or diluted and used in immersion or spray treatments in food preservation processes. When potassium sorbate is used, its activity on an equal weight basis is 74% that of sorbic acid. Therefore, about four parts of potassium sorbate are

approximately equal to three parts of sorbic acid for equivalent preservation. The solubility of sodium sorbate in water is 28 to 32% (w/v) at 20°C whereas calcium sorbate is sparingly soluble in water (1.2% w/v) and insoluble in lipids (Sofos, 1989).

Oxidation and decomposition

Oxidation and decomposition of sorbate are caused by a variety of conditions and can affect its antimicrobial properties. Increased moisture accelerates oxidation (Bolin et al., 1980). Aqueous solutions of sorbic acid are relatively sensitive to oxidation and degrade by first-order reaction kinetics (Melnick et al., 1954). Generally, the double bonds of sorbic acid are more prone to attack by molecular oxygen when the compound is in an aqueous state than in a dry form (Smith and Rollin, 1954). Oxidation of the olefinic bonds of sorbate results in the formation of peroxides followed by their breakdown and polymerization. Sorbate salts in solution polymerize upon prolonged storage and develop a yellow color (Chichester and Tanner, 1972).

Acidic conditions can also increase the oxidation rate of sorbate in solution. Acids, such as hydrochloric, sulfuric, acetic, trichloroacetic and trifluoroacetic, accelerate oxidation of sorbate solutions. However, glucono-delta lactone, citric acid and malic acid in appropriate amounts can stabilize sorbate solutions or may have no significant effect on sorbate degradation (Collins and Moustapha, 1969; Perry and Lawrence, 1960). Differences in the oxidation of sorbate by various acids are affected by temperature and pH; generally, the type of acid is more

important than the effect of pH on the oxidation of sorbate (Sofos, 1989).

Proper storage conditions for sorbate solutions are crucial to their stability. The rate of oxidation of sorbate solutions is faster in the presence of light than in the dark (Arya, 1980; Saxby et al., 1982; Vidyasagar and Arya, 1984). Sorbates decompose faster at high storage temperatures or with prolonged heating (Petropavlovski and Ustinova, 1967). Autoxidation of sorbate can be restricted by short-term storage of solutions in cool, dark conditions and by the use of antioxidants such as propyl gallate (Marx and Sabalitschka, 1965). Direct sunlight and high storage temperatures should always be avoided to retard oxidation and decomposition of sorbate solutions.

Antimicrobial Spectrum of Sorbate

Sorbate inhibits the growth of microorganisms from many genera. Studies of the various microbial species and strains affected by sorbate are too numerous to be discussed completely in this dissertation. Only brief reviews of some important food spoilage and pathogenic microorganisms controlled by sorbate and instances of microbial resistance to sorbate are presented here.

Bacteria

When present at appropiate levels in culture media or in food products, sorbate inhibits the growth of spoilage bacteria, such as *Pseudomonas fluorescens* (Robach, 1978), *Pseudomonas fragii*

(Moustapha and Collins, 1969), *Alteromonas putrefaciens* (Robach, 1979), *Arthrobacter* spp., *Moraxella* spp. (Chung and Lee, 1982), *Aerobacter aerogenes* (Wallhaeusser and Lueck, 1972), *Proteus morganii* and *Klebsiella* that produce histamine (Taylor and Speckhard, 1984), and *Clostridium sporogenes* (Vareltzis et al., 1984).

Many pathogenic bacteria, including *Clostridium botulinum* (Sofos et al., 1986), *Salmonella* spp. (Larocco and Martin, 1981; Park and Marth, 1972), *Staphylococcus* spp. (Lahellec et al., 1981), *Escherichia coli* (Wallhaeusser and Lueck, 1972), *Vibrio parahaemolyticus* (Robach and Hickey, 1978; Beuchat, 1980), *Yersinia enterocolitica* (Tsay, 1989) and *Listeria monocytogenes* (El-Shenawy and Marth, 1988) are also inhibited by sorbate concentrations of 0.05% to 0.3%. Sorbate seems to inhibit bacterial growth by delaying the onset of the logarithmic growth phase (Chung and Lee, 1981; Chung and Lee, 1982; Greer, 1982; Zamora and Zaritzky, 1987a; Zamora and Zaritzky, 1987b).

Sporeforming bacteria Sorbate is an inhibitor of bacterial spore germination. Spore germination in *B. cereus* T and *C. botulinum* 62A was inhibited by 0.39% and 0.52% potassium sorbate, respectively (Smooth and Pierson, 1981). Sorbate inhibits the germination of *C. botulinum* spores in the presence or absence of sodium nitrite (Sofos et al.,1979a; Sofos et al., 1979b; Sofos et al., 1980). Higher sorbate concentrations are needed to inhibit spore germination than to inhibit vegetative cell growth (Lund et al., 1987). Factors such as pH, sorbate concentration and species and strain of bacteria are variables that must

be considered when determining the inhibition of spore germination by sorbate.

Sorbate (0.26%) inhibits cell division of *C. botulinium* types A and B in culture media (Blocher and Busta, 1983). Higher levels of sorbate (1.0 to 2.0%) inhibit emergence, elongation and cell division of vegetative cells from germinating *C. botulinium* type E spores in agar microslide cultures at pH values of 5.7 to 5.8 (Seward et al., 1982). Using phase contrast microscopy, Blocher,1984 observed that potassium sorbate inhibited the outgrowth of cells from *C. botulinium* spores. Abnormally long cells, defective in cell division and which lyse easier than normal cells, have been observed at pH 7.0-7.2 in the presence of 1.0 to 2.0% sorbate (Seward et al., 1982). Sodium sorbate (0.015 to 0.05%) prevents cell division in vegetative cells that emerge from spores of *Bacillus* spp. Higher sorbate concentrations restrict shedding of the spore wall (Gould, 1964).

Resistance to sorbate Some bacteria are resistant to commercially used levels of sorbate (0.01% - 0.3%) and some may even metabolize that compound depending on growth conditions (Edinger, 1985; Liewen and Marth, 1985). *Staphylococcus* spp., *Streptococcus* spp. and certain *Pseudomonas* spp. are more resistant to sorbate than other bacteria such as *C. perfringens* (Kodicek, 1958), *E. coli* and *E. aerogenes* (Wallhaeusser and Lueck, 1972).

Generally, *Staphylococcus aureus* is less sensitive than other bacteria to sorbate inhibition, but such resistance depends on pH and sorbate concentration. *S. aureus* is inactivated by 0.1% sorbate at pH 5.0

but at pH values of 7.0 to 8.0 concentrations of sorbate up to 10% seem to have little inhibitory effect (Doell, 1962). At pH 6.0, 0.25% sorbate does not significantly inhibit *S. aureus*, but that same concentration of sorbate is inhibitory at pH 5.5 (Parada et al., 1982).

Certain lactic acid bacteria, for example species of *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, can metabolize relatively low sorbate concentrations (0.02% or less) under conditions of high contamination in spoiled wines (Crowell and Guymon, 1975; Horwood et al., 1981; Edinger and Splittstoesser, 1986a). *Lactobacillus plantarum* and *Streptococcus lactis* can utilize sorbate as a carbon source (Raduchev and Rizanov, 1963). Some lactic acid bacteria, particularly *Leuconostoc* spp., can reduce sorbate to hexadienol (sorbic alcohol) which chemically reacts with ethanol to produce compounds such as 1-ethoxy-2,4-hexa-diene and 2-ethoxy-3,5-hexa-diene. Those substances seem to contribute to a "geranium-like" odor in spoiled wines (Crowell and Guymon, 1975).

The tolerance of lactic acid bacteria to sorbate varies widely. At sorbate concentrations of 0.03 to 0.1% there is variable inhibition of strains of *Leuconostoc oenos* but *Lactobacillus* strains are inhibited. Strains of both *Leuconostoc oenos* and *Lactobacillus* spp. can grow in culture media with conditions (pH, alcohol) similar to those of wine and with sorbate levels of 0.02 to 0.03% (Edinger, 1985; Edinger and Splittstoesser, 1986a; Edinger and Splittstoesser, 1986b). It has been proposed that those strains of lactic acid bacteria that metabolize sorbate have an aldehyde dehydrogenase which is absent from other strains (Edinger and Splittsoesser, 1986a; 1986b). However, tests

utilizing cell-free extracts from sorbate-metabolizing strains indicated that the activities of two separate enzymes may be necessary for sorbate metabolism. (Edinger, 1985).

In general, certain lactic acid bacteria can metabolize sorbate but other bacterial species that can metabolize sublethal concentrations of sorbate include *Clostridium* spp. (York and Vaughn, 1954), and *Acetobacter* spp. (Raduchev and Rizanov, 1963).

Yeasts

The growth of most yeasts can be inhibited by 0.01 to 0.2% sorbate. Many factors, (for example, pH; water activity; the presence of sodium chloride, sucrose, and/or organic acids; temperature; species and strain of yeast; and the amount of oxygen present) can affect the inhibitory concentration of sorbate (Sofos, 1989). In cucumber pickle fermentations where the pH is low, the growth of undesirable yeasts is effectively controlled by sorbate without inhibiting the desirable lactic acid fermentation (Jones and Harper, 1952; Costilow et al., 1957). With low pH and increased sodium chloride concentration, less sorbate is required to inhibit yeasts common in cucumber fermentations (Costilow et al., 1955). Sorbic acid (0.01 to 0.1%), in combination with acetic acid (0.5 to 0.1%) and sucrose (2 to 40%), is strongly inhibitory to yeasts isolated from spoiled sweet cucumber pickles (Sheneman and Costilow, 1955). Strains of several yeasts in wines are inhibited by 0.01 to 0.03% sorbate (Watanabe and Iino, 1984), but higher levels of sorbate (0.1% or

greater) are required to inhibit *Saccharomyces* spp. and other useful yeasts.

The D values of six genera of yeasts (*Saccharomyces, Debaromyces, Candida, Pichia, Rhodotorula* and *Kloeckera*) were reduced by the use of potassium sorbate and sodium benzoate at 0.05 to 1.0% plus heating and reduced water activity at pH 4.5 (Beuchat, 1981b). The nature of solute used to lower the water activity of the heating medium determines the extent of synergism of those preservatives with heat. In contrast to potassium sorbate and sodium benzoate, sucrose and sodium chloride increase the heat resistance of those yeasts (Sofos, 1989). Sorbate also acts synergistically with heat to inactivate yeast and mold conidia (Shibasaki and Tsuchido, 1973), thus the shelf life of products such as fruit salads, apple cider and fruit slices are increased when combinations of sorbate and mild heat are used (Robinson and Hills, 1959). Potassium sorbate has a stronger inhibitory effect on heat treated-yeasts than on non heat-treated yeasts. Heat-treated yeasts failed to form colonies in recovery medium in the presence of 0.1% potassium sorbate (Beuchat, 1981a).

Sorbate's inhibitory action against yeasts is affected by the presence of oxygen. *Candida albicans* is more sensitive to sorbate in anaerobic than in aerobic conditions. Detoxification of sorbate by that yeast under aerobic conditions seems to reduce inhibition which varies with medium composition. The addition of cysteine or glutathione to the medium increased the duration and extent of inhibition by retarding the detoxification of sorbate (Deak and Novak, 1968).

Resistance to Sorbate Certain yeasts, particularly osmotolerant types, are resistant to or develop resistance to inhibition by sorbate. Most osmotolerant yeasts belong to the genus *Saccharomyces* and include species such as *S. rouxii, S. bailii, S. acidifaciens* and *S. bisporus*. Even though high sucrose concentrations (20 to 70%), low pH, and increasing sorbate concentrations potentiate the inhibition of many yeasts, osmotolerant yeasts can develop resistance to sorbate (Bills et al., 1982; Pitt, 1974; Pitt and Richardson, 1973; Restaino et al., 1983; Restaino et al., 1982; Warth, 1977).

The inhibitory effects of sorbate concentrations less than 0.12%, against osmotolerant yeasts such as *S. rouxii* are determined by the level of water activity and substrate pH. When the water activity is decreased, *S. rouxii* becomes resistant to increasing sorbate concentrations (Restaino et al., 1983) even though lowered water activity and low pH potentiate the inhibitory effects of sorbate on most yeasts (Baird-Parker and Kooiman, 1980) and other microorganisms.

Resistance of osmotolerant yeasts to inhibition by sorbate may be an acquired property when such yeasts are pre-exposed to sorbate. Previous exposure of *S. rouxii* to 0.1% sorbate enhances that yeast's resistance to inhibition by sorbate in culture media and in food. However, preconditioning of the *S. rouxii* cells in 60% sucrose with no sorbate gives no advantage to growth of those cells when inoculated into growth media containing 60% sucrose and 0% sorbate (Bills et al., 1982).

A mechanism of resistance of *S. rouxii* to inhibition by sorbate has been proposed (Bills et al., 1982; Restaino et al., 1983). At low water activity, the shrinkage of yeasts cells and reduction of membrane pore size may restrict the entry of sorbate into the cell. The uptake of sorbate is greater at higher water activities and the increased intracellular accumulation of sorbate inhibits the yeast.

S. bailii becomes resistant to high concentrations of sorbate or benzoate after being preconditioned to growth in moderate amounts of those preservatives (Warth, 1977). Starved cells concentrate the preservatives intracellularly but upon the addition of glucose to the growth medium, the intracellular concentrations of the preservatives are reduced. The resistance of *S. bailii* may be linked to an inducible, energy-requiring system which transports sorbate out of the cell. That property is induced when the yeast has been previously grown in subinhibitory amounts of sorbate (Warth, 1977).

Molds

Sorbate effectively inhibits the growth of molds, including mycotoxin-producing types, in culture media and in food products (Liewen and Marth, 1984; Sofos and Busta, 1983). The minimum inhibitory concentration of sorbate for most molds ranges from 0.001 to 0.1% and is influenced by pH, species, strains and other factors (Bandelin, 1958; Przybylski anb Bullerman, 1980). The concentrations of sorbate (0.05 to 0.3%) permitted in cheeses inhibit the growth of most molds in those products (Liewen and Marth, 1985). Sorbate

inhibits molds at all stages of their life cycle, including spore germination, growth initiation, and mycelial growth (Bullerman, 1983; 1984; 1985). The viability of conidia of molds such as *Penicillium roqueforti* and *Aspergillus parasiticus*, is decreased by sorbate (Liewen and Marth, 1983; Przybylski and Bullerman, 1980). Heat-treated (55°C, 15 min.) mold spores are more susceptible than irradiation-treated (50 Krad) spores to inhibition by sorbate (Tsai et al., 1984).

Sorbate inhibits the production of mycotoxins, including aflatoxins, patulin, citrinin, sterigmatocystin and ochratoxin (Bhattachrya and Majumdar, 1984; Bullerman, 1983; 1984; 1985; Lennox and McElroy, 1984; Reiss, 1976; Tong and Draughon, 1985; Tsai et al., 1984). The degree of inhibition of mycotoxin production by sorbate can vary with the type of mycotoxin; for example, sorbate inhibits aflatoxin B_1 production to a greater extent than that of aflatoxin G_1 (Yousef and Marth, 1983). The inhibition of mold growth and mycotoxin production is generally greater with increasing sorbate concentrations (Bullerman, 1983; 1985; Liewen and Marth, 1984). At higher pH values, increased sorbate levels are necessary to inhibit mold growth and mycotoxin production (Marshall and Bullerman, 1986; Tsai et al., 1984). In some instances, sorbate may inhibit mold growth but fail to prevent the production of mycotoxins, including patulin and aflatoxin (Ankerstrand and Anderson, 1979; Yousef and Marth, 1981).

Mycotoxin production may be stimulated by subinhibitory concentrations of sorbate under certain conditions. The production of aflatoxin B_1 and T-2 toxin, by *Aspergillus flavus* and *Fusarium*

acuminatum, respectively, is stimulated by sorbate concentratious of less than 0.05% (Bullerman and Olvigni, 1974). Such stimulation of mycotoxin production depends on species and other factors, such as injury and storage temperature. More aflatoxin is produced in the presence of 0.05% sorbate by *Penicillium patulin* from uninjured spores than from injured spores (Tsai et al., 1984). Ochratoxin production by *Aspergillus ochraceus* is stimulated by sorbate (0.05%) at 25°C but inhibited by that same concentration of sorbate at 12°C (Bullerman, 1985).

The inhibition of mycotoxin production by sorbate may be associated with inhibition of nutrient transport into the cell (Yousef and Marth, 1983). Stimulation of mycotoxin production is possibly due to reduction in activity of the tricarboxylic acid cycle by levels of sorbate near the minimum inhibitory concentration. This may result in the intracellular accumulation of acetyl coenzyme A, an important intermediate in biosynthesis of certain mycotoxins (Gareis et al., 1984).

Resistance to sorbate Some strains of molds are resistant to sorbate, and variation in sensitivity to sorbate among molds has been associated with the ability of some molds to metabolize sorbate (Sofos, 1989). The normal degradation of fatty acids by molds yields unsaturated fatty acids as intermediates. Sorbate can be viewed as an intermediate of caproic (saturated) acid in supplying carbon for mold growth, just as crotonic acid is related to butyric acid (Melnick et al., 1954).

Certain stains of *Aspergillus niger* and other mold species degrade sorbate (Lukas, 1964a; 1964b). Some *Penicillium* strains isolated from natural and processed cheddar cheese treated with sorbate, grow in the presence of 0.71% potassium sorbate (Marth et al., 1966). Potassium sorbate (0.3%) did not inhibit the growth of 70% of the molds isolated from moldy cheese (Bullerman, 1977). This may indicate that sorbate-resistant mold strains may be selected for at cheese factories where sorbate is used as a food additive (Liewen and Marth, 1964).

Mechanisms of Antimicrobial Activity of Sorbate

Spore germination

Sorbate is a potent inhibitor of bacterial spore germination. The actual mechanism of spore germination and associated reactions are not well defined; thus, it is difficult to determine the precise mechanism by which sorbate inhibits spore germination (Sofos, 1989).

The trigger or initiation reaction in spores seems to be the first event in the germination process and during that reaction, spores become committed to germinate. The initiation reaction is followed by a lag period in which the "connecting reactions" occur. Those reactions result in visible signs of germination such as loss of spore refractility and resistance (Blocher and Busta, 1985; Sofos et al., 1986).

Germination can be initiated by exposing activiated spores to L-alanine, but exposure of spores to D-alanine competitively inhibits initiation. D-alanine, however, does not affect spores that are already committed to germinate. The use of the L-alanine germination system

facilitated studies on germination inhibition of *Clostridium* and *Bacillus* spores in pure culture (Blocher and Busta, 1985; Smooth and Pierson, 1981).

Site and stage of inhibition Potassium sorbate inhibits *Bacillus cereus* T and *Clostridium botulinum* 62A spore germination at some point during the initiation process of germination. The inhibition is reversible. Potassium sorbate seems to be a competitive inhibitor of bacterial spore germination induced by certain amino acids (eg. L-alanine) and affects the trigger or initiation reaction (Smoot and Pierson, 1981).

The commitment of *C. botuliium* 62A spores to germinate in L-alanine with or without potassium sorbate (0.39 to 0.52%) and with D-alanine as a competitive inhibitor of L-alanine binding, has been investigated (Blocher, 1984). The data from that study indicate that potassium sorbate inhibits initiation of germination and that the inhibition depends on both the concentration of sorbate and pH.

It was postulated that direct competition exists between sorbate and the L-amino acid germinant for a binding site on the germination-initiation receptor site or for an active site on some important enzyme associated with the germination process (Smoot and Pierson, 1981). The data for germination were expressed as change in absorbance at different amino acid germinant concentrations with or without sorbate. Lineweaver-Burke reciprocal plots of the data resulted in two straight lines of different slopes but with the same Y intercept. Such plots indicate competitive inhibition (Segel, 1976), and it was concluded that

sorbate is a competitive inhibitor of amino acid-induced spore germination (Smooth and Pierson, 1981).

In both of those studies (Blocher, 1984; Smoot and Pierson, 1981), absorbance changes or reduction in spore refractility were followed to examine germination. However, those changes indicate relatively late events in the process of germination (Gould and Dring, 1972). The use of such late events as a basis for kinetic analysis of spore germination may not reflect the kinetics of the initiation reaction but may represent the kinetics of the connecting reactions (Stewart et al., 1981). Therefore, the inhibition of spore germination by sorbate, reported by Smoot and Pierson (1981), occurred after initiation or commitment to germination, and during the connecting reactions (Sofos, 1989).

Blocher and Busta, (1985) exposed *C. botulinum* and *B. cereus* spores to 100 mM L-alanine or L-cysteine at pH 7.0 for specified lengths of time, after which samples of spores were transferred to 1.8 M D-alanine at pH 7.0 with or without sorbate. The spores were then viewed under phase contrast microscopy. The following important observations were made: (i) The commitment of spores to germinate in L-alanine occurred quickly (0.5 minutes) and, after 15 to 25 minutes, the first completely germinated spores, were detected; (ii) D-alanine treatment inhibited germination of untriggered spores but spores already committed to germination continued that process; (iii) sorbate plus D-alanine caused a greater inhibition of spore germination than D-alanine alone.

If sorbate prevented L-alanine from binding to the trigger site of the spores, then the combined effects of D-alanine and sorbate should have been the same as the effect of D-alanine by itself. Since that effect did not occur, it is possible that sorbate inhibited germination of spores that were uninhibited by D-alanine (Sofos, 1989) which only interferes with germination of untriggered spores (Blocher and Busta, 1985; Smoot and Pierson, 1981). Therefore, sorbate does not seem to affect the triggering reaction or complete with the amino acid germinant L-alanine for a specific binding site on the spore. However, sorbate does seem to interfere with the connecting reactions that follow initiation of germination (Blocher and Busta, 1985; Sofos et al., 1986).

Results of studies on the inhibition of spore germination by acetate, lactate or hexanoate indicate that interference with the connecting reactions in spore germination may be a common mechanism of action of various fatty acids (Blocher and Busta, 1985).

Possible mechanisms of inhibition The connecting or post-trigger reactions of spore germination are not precisely understood (Keynan, 1978; Setlow, 1981; Smoot and Pierson, 1981). However, possible mechanisms of how sorbate may interfere with connecting reactions and cause inhibition of spore germination have been presented by Blocher and Busta (1985).

Metabolic reactions, including electron and cation transport or lytic processes, may be associated with the early stages of germination (Blocher and Busta, 1985; Setlow, 1981). Another viewpoint is that electron and proton transport in spores may be functional only in the

late events of germination (Setlow, 1981). Since sorbate seems to inhibit the initial stages of germination, interference with transport functions seems an unlikely primary lesion (Blocher and Busta, 1985). Although the early stages of spore germination may involve metabolic reactions, some data support the occurrence of a structural or allosteric change on the spore during the trigger reaction of germination (Blocher and Busta, 1985; Smoot and Pierson, 1981). Sorbate may, however, alter spore membrane permeability and cause inhibition of cation transport functions in the later stages of germination (Blocher and Busta, 1985).

Cell membrane permeability may be altered during the initiation reaction of spore germination, thus making the membrane a site that is susceptible to the action of sorbate (Vary, 1978; Smoot and Pierson, 1981). Since decreased fluidity of the inner spore membrane is important in germination (Racine et al., 1981), sorbic acid may inhibit spore germination by interaction with spore membranes and interfering with their fluidity (Sheu and Freese, 1972; Sheu et al., 1975).

Cortex hydrolysis and loss of refractility in spores probably result from the action of lytic enzymes in the initial stages of germination (Blocher and Busta, 1985; Boschwitz et al., 1983; Johnstone and Ellar, 1982; Labbe et al., 1981; Setlow, 1981). Some of the lytic enzymes found in spores are serine or sulfhydryl proteases. Sorbate inhibits spore germination and the activity of hexosaminidase, an enzyme isolated from the spores of *B. cereus* (Brown and Sanford, 1984). Since spore germination involves the activity of proteases, and sorbate can

inhibit sulfhydryl enzymes (Sofos and Busta, 1985; Whitaker, 1959), sorbate may interfere with the connecting reactions of spore germination by inhibiting the activity of proteases (Blocher and Busta, 1985; Sofos et al., 1986).

To date, the available data on the inhibition of spore germination by sorbate indicate that sorbate interferes with the post-trigger connecting reactions in the spore and exerts its inhibitory action on proteases and/or spore membranes.

Cell morphology

Exposure of microbial cells to sorbate results in changes in their morphology and appearance under certain conditions. Abnormally-long outgrowths of vegetative cells are observed when *C. botulinum* type A spores are treated with 0.26% potassium sorbate at pH 5.85 (Wagner and Busta, 1985). *C. botulinum* type E cells become distorted (for example, elongated with bulbous formations) and do not divide when 1 to 2% sorbate is used in spore microcultures at pH values of 7.0 to 7.2. At lower pH values (5.7 to 5.8), the same concentrations of sorbate (1 to 2%) inhibit the outgrowth of vegetative cells from *C. botulinum* spores or elongation and division of emerged cells. Those defective cells lyse readily and are 3 to 5 times longer than normal cells (Seward et al., 1982).

When yeast cells are exposed to 0.025 to 0.05% sorbic acid, irregular nuclei, many mitochondria of different sizes, dense phospholipoprotein granules and vacuoles are observed

(Alimukhamedova, 1977). The mechanism by which sorbate causes changes in cell morphology and appearance is not known. Sorbate may be interfering with reactions associated with the formation of the cell wall and cell division. Also, sorbate may incorporate into certain cell structures and stimulate or inhibit specific biosynthetic events in the cell (Sofos et al., 1986).

Membrane integrity

Changes in the integrity and function of cell membranes have been suggested as the ways by which sorbate inhibits microbial growth. At pH 7.0, 5% potassium sorbate caused outer membrane damage in *Alteromonas putrefaciens* (Statham and McMeekin, 1988). The death of microbial cells in the presence of high concentrations of sorbate and other preservatives was attributed to increased porosity of the cell membrane (Freese and Levin, 1978; Reinhard and Radler, 1981).

Anderson and Costilow (1963) discredited the hypothesis that sorbate affects yeast cells by inhibiting membrane permeation via disruption because sorbic acid is not surface active and the anaerobic evolution of carbon dioxide remains uninhibited in yeast cultures exposed to sorbate. Also, sorbic acid did not cause any significant efflux of intracellular substrates in *Candida utilis* (Tsuchido et al., 1972). Apart from sorbate's potential influence on cell walls and membranes, which may alter their integrity and permeability, sorbate may affect other processes. Examples of these processes are electron transport,

substrate uptake, and the proton-motive force, all of which are associated with cell membranes (Sofos, 1989).

Genetic material

There is a scarity of information on the effects of sorbate on microbial DNA, RNA and ribosomes. In addition, evidence for the effect of sorbate on genetic mechanisms is almost non-existent (Eklund, 1980). Sorbate (0.4%) inhibited the synthesis of DNA and RNA in *P. fluorescens* (Nose, 1982); however, sorbate did not inhibit nucleic acid synthesis in cells of *C. utilis* (Tsuchido et al., 1972). The effect of sorbate on DNA-dependent RNA polymerase is discussed in the section on enzyme inhibition by sorbate. No mutagenic activity has been attributed to sorbate itself but it may produce mutagenic products on reaction with sodium nitrite or sulfur dioxide under certain conditions (Sofos, 1989).

Cell metabolism

Sorbate has inhibited cell metabolism by reducing microbial assimilation of carbon from many substrates. The decrease in oxidative assimilation of many substrates, such as fumarate, glucose, pyruvate, lactate, -ketoglutarate, ethanol, acetate, succinate and acetaldehyde, has been correlated to growth inhibition by sorbate in bacteria, yeasts and molds (Sofos, 1989). Sorbate (0.01% - 0.1%) inhibited glucose assimilation in *Pseudomonas aeruginosa* (York and Vaughn, 1964) but failed to inhibit oxygen uptake by that same organism with glucose and malate as substrates (Nose et al., 1982). Differences in experimental

conditions, such as pH and type of culture media used, may have contributed to lack of inhibition of sorbate. Sorbate's inhibition of cell metabolism in those studies may be related to inhibition of enzymes, nutrient uptake and/or various transport systems (Sofos, 1989).

Inhibition of enzymes The inhibition of certain enzyme systems as the mechanism by which sorbate inhibits microbial growth has been postulated; however, there is little or no consensus opinion among investigators on the enzyme system which represents the actual site of growth inhibition. It was proposed that certain dehydrogenases associated with oxidation of fatty acids are inhibited by sorbic acid (Sheu et al., 1975). The oxidation of fatty acids by molds yields unsaturated fatty acids as intermediate products. The accumulation of such intermediates, particularly from the addition of sorbic acid, would inhibit the function of dehydrogenases. Subsequent metabolism and growth of molds would be inhibited. That hypothesis was derived from studies on sorbic acid degradation by molds (Sheu et al., 1975).

Most enzymes that have sulfhydryl groups at their active sites are inhibited by sorbate (York and Vaughn, 1954). Sorbate's inhibition of sulfhydryl enzymes has been linked to decreases in the amount of active sulfhydryl groups on the enzymes by the binding of sorbic acid to those groups. The basis for that conclusion is that the addition of cysteine to the reaction mixture prevented the inhibitory action of sorbate. Cysteine may have inactivated sorbate by reacting with it (York and Vaughn, 1964; Rehm, 1967). In other instances, the addition

of cysteine enhances (Rehm, 1963) or fails to reverse enzyme inhibition by sorbate (Whitaker, 1959).

The inhibition of yeast alcohol dehydrogenase involves either covalent bond formation between the sulfur of important sulfhydryl groups or the $ZnOH^-$ of the enzyme and the and/or carbon of the sorbate ion (Martoadiprawito and Whitaker, 1963). The inhibition of sulfhydryl enzymes by sorbate may be attributed to the formation of a thiohexenoic acid derivative: CH_3-CH=CH-RSCH-CH_2-COOH (Whitaker, 1959). The reaction of sorbic acid with cysteine via an addition reaction with the sulfhydryl group of that same amino acid was presented as the mechanism by which sorbate inhibits sulfhydryl enzymes (York and Vaughn, 1964).

The inhibition of synthesis or activity of catalase has been proposed as a mechanism of growth inhibition in *A. niger* by sorbate (Taylor and Speckhard, 1984). Sorbate's inhibitory effect on catalase may result from the formation of sorbyl peroxides from the autoxidation of sorbic acid. Sorbyl peroxide either inhibits catalase or the activity of other enzymes essential to cell development (Troller, 1965). Since sorbate is inhibitory to certain catalase-negative bacteria (for example clostridia), but has little inhibitory effect on others (for example lactic acid bacteria), different mechanisms of inhibition may be active in various microbial systems.

It was proposed that sorbate (0.05%-0.1%) competes with acetate at the site of acetyl coenzyme A formation and inhibits respiration in

yeast (Troller, 1965). It may competitively bind with coenzyme A and consequently inhibit oxygen uptake and microbial growth.

Other suggested mechanisms of inhibitory action by sorbate include inhibition of protein synthesis in *P. fluorescens* (Nose, 1982) and *C. utilis* (Tsuchido et al., 1972), or RNA synthesis via inhibition of DNA-dependent RNA polymerase from *E. coli*, but sorbate only mildly inhibited the in vitro activity of that enzyme (Yamada, 1977).

Inhibition of transport functions Sorbate interferes with substrate and electron transport functions in microbial cells. Sorbate and other lipophilic acid food preservatives inhibit the entrance of substrate molecules by uncoupling their transport from the electron transport system (Deak et al., 1970a; Deak et al., 1970b; Sheu and Freese, 1972; Sheu and Freese, 1973; Sheu et al., 1972; Sheu et al., 1975). Growth inhibition is caused by cell starvation due to uptake inhibition of compounds such as amino acids, organic acids and phosphates (Freese and Levin, 1978).

Concentrations of sorbate ranging from 0.1 to 1.0% inhibited amino acid uptake in *Bacillus subtilis*, *E. coli*, *P. aeruginosa* (Eklund, 1980), *Salmonella typhimurium* (Tuncan and Martin, 1985) and *Penicillium chrysogenum* (Hunter and Segel, 1973). Inhibition of amino acid uptake may be attributed to sorbate's effect on amino acid permeases that transport amino acids into the cell (Tuncan and Martin, 1985).

Kodicek (1958) viewed interference of cellular uptake of substrate by unsaturated acids as a physiochemical mechanism. Unsaturated

fatty acid molecules are absorbed into transport-active areas in the lipoprotein layer of the cytoplasmic membrane. The double bonds of those fatty acids create pressures that result in steric disorganization of active membrane transport proteins, resulting in defective transport functions that cause inhibition of nutrient uptake. Various mechanisms for nutrient uptake inhibition by sorbate have been presented and several hypotheses have been proposed to explain those mechanisms. Those mechanisms include neutralization of the proton-motive force (Freese and Levin, 1978); inhibition of the electron transport system, inhibition of ATP synthesis or depletion of ATP, inhibition of transport enzymes, and inhibition of metabolic energy utilization by amino acid transport systems (Sofos, 1989).

Effect on ATP Reduction in cellular ATP levels has resulted in the inhibition of microbial growth and respiration (Anderson and Costilow, 1963; Deak, 1969; Harada et al., 1968; Hunter and Segel, 1973). In the presence of sorbate (0.22% or 0.34%), ATP concentrations in conidia of *Aspergillus parasiticus* are rapidly lowered (Przybylski and Bullerman, 1980). They proposed proposed mechanisms for ATP depletion by sorbate. When sorbic acid dissociates in the cell cytoplasm, the intracellular cation concentration increases. In an attempt to maintain ion balance, the cell utilizes ATP because the primary sodium/hydrogen pump is directly associated with ATP hydrolysis. If the influx of hydrogen ions exceeds the pumped efflux, a charge shift occurs, resulting in a decrease in the net negative intracellular charge. This change discharges the pH gradient required for regenerating ATP

according to the chemiosmotic mechanism of oxidative phosphorylation. It is thus possible that sorbate-induced ATP depletion may be one mechanism responsible for the inhibition and death of molds.

The view that depletion of the ATP content of cells is responsible for growth inhibition by chemical preservatives is contradicted by the studies of Sheu et al. (1975). Those investigators demonstrated that most lipophilic acids including sorbate (0.1 to 1.0%) and related compounds, inhibitory to the facultative anaerobe *E. coli*, caused little or no decrease in the quantity of ATP per cell.

The protonmotive force A popular explanation of the mechanism of microbial growth inhibition by sorbate is that lipophilic weak acid preservatives starve microbial cells by inhibiting entry into the cell of compounds that are actively transported by the protonmotive force (Eklund, 1980; Eklund, 1983; Eklund, 1985; Freese and Levin, 1978; Freese et al., 1973; Hunter and Segel, 1973; Sheu and Freese, 1972; Sheu and Freese, 1973; Sheu et al., 1973; Sheu et al., 1975). The ability of weak acid preservatives, like sorbic acid, to lower the cytoplasmic pH and diffuse the transport-driving protonmotive force forms the basis for that explanation.

The mechanism by which bacteria maintain pH homeostasis is not clearly understood, but it is generally accepted that bacteria try to maintain relatively constant cytoplasmic pH although the extracellular pH of their environment may vary (Salmond et al., 1984). As the extracellular pH of the cell decreases, the antimicrobial activity of sorbate increases because of the formation of undissociated sorbic acid.

The acid form of sorbate is the more inhibitory component. The dissociated molecule is about 10 to 600 times less active than the undissociated species (Eklund, 1983).

The pKa of sorbic acid is 4.76. A larger amount of sorbic acid enters the cell and dissociates in the cytoplasm when the extracellular pH is much lower than the intracellular pH (Oka, 1960; Oka, 1964; York and Vaughn, 1964). That action increases the cytoplasmic hydrogen ion concentration, which acidifies the cytoplasm, inhibits metabolic processes, and diffuses the proton gradient across the cytoplasmic membrane. The proton gradient is a component of the protonmotive force, which energizes the cytoplasmic membrane to transport compounds such as amino acids and keto acids (Ronning and Frank, 1987; Salmond et al., 1984). The loss of the proton gradient across the cytoplasmic membrane is believed by some investigators to be the main mechanism by which sorbate and other weak acid preservatives inhibit microbial growth (Bell et al., 1959; Eklund, 1980; 1983; 1985; Lueck, 1980; Nomoto et al., 1955; Ronning and Frank, 1987). However, this loss of the proton gradient may not be a result of a reduction of cellular ATP, according to the findings of Sheu et al., (1975) who reported that there was no significant decrease in ATP levels when *E. coli* was treated with sorbate.

Based on the results of studies with whole cells and membrane vesicles of bacteria, the antimicrobial activity of sorbate is possibly associated with inhibition of nutrient transport into the cell (Eklund, 1980; Freese and Levin, 1978; Freese et al., 1973; Ronning and Frank,

1987). Studies by Eklund (1985) confirmed that food preservatives, such as sorbate, benzoate, propionate at concentrations of 0.1 to 1.1% and esters of p-hydroxybenzoic acid at concentrations of 0.01 to 0.1%, act as transport inhibitors. However, that same author questioned whether transport inhibition adequately explained microbial growth-inhibition in all instances. The transport of weak acids into the cell to cause acidification of the cytoplasm and consequent growth inhibition (Baird-Parker, 1980) cannot explain the growth inhibitory action of sorbate on bacteria and yeasts at a neutral pH value (Eklund, 1983).

Both the proton gradient and the electrical potential across the cytoplasmic membrane make up the protonmotive force (Mitchell, 1972). The protonmotive force provides energy for uptake of certain nutrients by the cell. An accumulation of sorbic acid in the cytoplasm of the cell reduces the intracellular pH and, consequently, destroys the proton gradient. Elimination of the proton gradient disrupts many of the chemiosmotic related processes of the cell (Garland, 1977; Mitchell, 1972), particularly amino acid transport (Eklund, 1980; Freese et al., 1973). According to Ramos and Kaback (1977), the electrical potential difference by itself is adequate to drive the cellular uptake of several substances. Eklund (1980) used that finding as a basis for suggesting that the elimination of the proton gradient is insufficient to totally inhibit nutrient uptake in microbial cells.

Weak acids, including sorbic and propionic which are potent inhibitors of microbial growth, lower the intracellular pH to a greater extent than other weak acids of similar dissociation constant (Salmond

et al., 1984). Thus, microbial growth inhibition by weak acid preservatives may consist of two components - a generalized inhibition that results from intracellular acidification, and a specific inhibition of a particular metabolic function by the undissociated acid. Although cytoplasmic acidification is a significant cause of growth inhibition, the latter effect may be of greater importance (Salmond et al., 1984).

Disruption of the protonmotive force as the main mechanism of microbial growth inhibition by sorbate can be contradicted when one considers that many sugars and sugar alcohols, including glucose, frutose, mannose, N-acetyl glucosamine, mannitol, sorbitol and lactose, enter the bacterial cell by group translocation (Stryer, 1988) and do not depend on the protonmotive force for their transport into the cell.

Disruption of the protonmotive force by sorbate inhibits amino acid uptake which causes a stringent response in bacteria. Sorbate induces a stringent type regulatory response which causes growth inhibition in *Clostridium sporogenes* P.A. 3679 (Ronning and Frank, 1987). A stringent response refers to the metabolic readjustments that occur in bacterial cells, when one or more amino acids is unavailable. This phenomenon was recognized when a mutant strain of *E. coli*, in which the stringent response is absent, was discovered. In that mutant *E. coli*, the phenotypic characteristic with regard to the stringent response was described as relaxed (Rel) and its mutant gene was called relA. Unlike the wild-type *E. coli*, the relA mutant does not readily accumulate specific nucleotide derivatives under nutrient-limiting conditions. It is known that bacterial cells increase intracellular levels

of certain compounds in response to nutritional adversity (Cashel, 1975). Those compounds are derivatives of guanosine diphosphate and guanosine triphosphate, containing pyrophosphate residues at the three prime position, and are abbreviated as ppGpp and pppGpp, respectively. The intracellular accumulation of ppGpp and pppGpp seems to inhibit RNA synthesis by interfering with the functioning of RNA polymerase (Cashel and Rudd, 1987). The stringent response inhibits cell replication but the cells retain their viability (Cashel, 1975; Gallant, 1979; Ronning and Frank, 1987). When the limiting amino acids are supplied, the cells are able to resume protein synthesis and replication. Since various degrees of amino acid uptake inhibition by sorbate may be attributed to differences between amino acid transport systems (Tuncan and Martin, 1985), more than one mechanism of action is probably operating under different or similar conditions (Sofos, 1989).

Summary

Many hypotheses on sorbate's antimicrobial mechanism of action have been presented in this literature review. It seems that the antimicrobial activity of sorbate, especially the site of the initial lesion, cannot be fully attributed to any single mechanism known to date. The literature reviewed indicates that precise mechanisms by which sorbate inhibits bacterial growth are not clearly understood. Generally, the possible targets for the antimicrobial action of food preservatives such as sorbate can be categorized as: (a) the cytoplasmic membrane, (b) genetic material, and (c) cellular enzymes. Limited data are available

on the effect of sorbate on genetic material, and evidence for the interaction between sorbate and microbial genetic mechanisms is almost nonexistent (Eklund, 1980). A summary of proposed mechanisms of antimicrobial action of sorbate is presented in Table 1.

Research Objective

The objective of this research was to investigate the antimicrobial mode of action of potassium sorbate by determining whether growth inhibition by sorbate in bacteria is closely associated with inhibition of a particular metabolic function. The specific objectives were to determine the relative sensitivities of selected metabolic functions of *E. coli* to inhibition by subbacteriostatic, growth-inhibitory concentrations of potassium sorbate.

Table 1. Proposed mechanisms of the antimicrobial action of sorbate

Mechanism	Organism; pH; sorbate (%)	Reference
Inhibition of dehydrogenases	*P. roqueforti*; pH 5.5; 0.01-0.03%	Melnick et al. (1954)
Inhibition of sulfhydryl-containing enzymes	*E. coli;* pH 6.2 0.01-0.07%	York and Vaughn (1964)
Inhibition of catalase	*A. niger*; pH 5.0; 0.05-0.10%	Troller (1965)
Inhibition of respiration at the site of acetyl CoA formation	*S. cerevisiae*; pH 4.0; 0.21%	Harada et al. (1968)
Inhibition of carbohydrate uptake	*Candida spp.*; *Saccharomyces spp.*; pH 5.5; 0.03-0.07%	Deak and Novak (1972a; 1972b)
Inhibition of amino acid uptake	*B. subttilis*; *E. coli*; pH 6.5; 0.56%	Freese et al. (1973)
Elimination of the proton gradient that energizes membrane transport	*P. chrysogenum*; pH 4.5; 0.01%	Hunter and Segel (1973)
Uncoupling of active transport from cellular energy production	*S. bailii* pH 3.67; 0.02%	Warth (1977)

Table 1. (continued)

Mechanism	Organism; pH; Sorbate (%)	Reference
Depletion of ATP	*A. parasiticus*; pH 4.75; 0.22%	Przybylski and Bullerman (1980)
Induction of a stringent-type response	*C. sporogenes* PA 3679; pH 7.0; 2.24%	Ronning and Frank (1987)

MATERIALS AND METHODS

Bacterial Cultures and Media

The organisms used in this study were *Escherichia coli* 15TAU (CGSC #4907) and *Escherichia coli* K12. *E. coli* 15TAU, a derivative of *E. coli* K12, was obtained from Dr. Barbara Bachman at the Department of Biology, Yale University. *E. coli* 15TAU is a triple auxotroph which must be supplied with thymine, arginine and uracil for growth. *E. coli* K12 was obtained from the culture collection of the Department of Microbiology, Immunology and Preventive Medicine, Iowa State University. Both organisms were maintained by monthly transfer on Brain Heart Infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) and stored at 4°C. *E.coli* 15TAU was grown in a complete defined synthetic (CDS) medium. *E.coli* K12 was grown in CDS medium, glucose-salts (GS) medium or glycerol-salts (GLS) medium.

The composition of CDS medium is shown in Table 2. GS medium consisted of glucose (1.0 g/l), KH_2PO_4 (9.0 g/l), K_2HPO_4 (1.4 g/l), $(NH_4)_2SO_4$ (1.0 g/l), and $MgSO_4$ (0.05 g/l). GLS medium consisted of glycerol (1.0 g/l) and the same concentration of each salt as stated for GS medium. CDS, GS and GLS media were adjusted to pH 5.70 with 5N HCL, filter sterilized, and used on the same day of preparation. Both growth media were filter sterilized through Gelman Supor 200 membrane filters (0.2um pore size, 47 mm diameter). Media (CDS or GS) with potassium sorbate were prepared by making stock solutions of potassium sorbate in CDS or GS medium. The pH of those stock

Table 2. Composition of defined synthetic medium[a]

Ingredient	Amount[b]	Ingredient	Amount[b]
Glucose (g) (100x)[c]	5.0	Amino acids (mg) (100x)	
		L-glutamic acid	100
Salts (g) (5x)		L-serine	30
K_2HPO_4	7.0	L-methionine	3
KH_2PO_4	2.0	L-tyrosine	50
Na_3 citrate.H_2O	0.4	L-alanine	60
$MgSO_4$	0.05	L-lysine	50
$(NH_4)_2SO_4$	1.0	L-threonine	30
		L-phenylalanine	40
Vitamins (mg) (1000x)		L-histidine	20
Thiamine	1.0	glycine	50
Niacin	1.2	L-tryptophan	10
Biotin	0.005	L-isoleucine	30
Ca pantothenate	0.25	L-valine	80
		L-leucine	90
Purines & pyrimidines (mg) (200x)		L-aspartic acid	90
Adenine	10	L-arginine	10
Guanine	10	L-proline	80
Cytosine	10	L-cystine	20
Uracil	10		
		N-acetylglucosamine (mg) (100x)	20
Thymine (mg) (50x)	10		
Deionized water (ml)	1000		

[a] Synthetic media were made by mixing appropriate volumes of each stock. The final mixture was sterilized by filtration through Gelman Supor 200 membrane filter (0.2 μm pore size).

[b] Final concentration of nutrient per liter.

[c] Numbers in parentheses represent stock concentrations from which media were made; all stocks were autoclaved (except for vitamins) at 121°C for 15 minutes and stored at 4°C.

solutions was adjusted to 5.70 with 5N HCL. Two-fold dilutions of potassium sorbate stocks were made to give CDS or GS media with the following sorbate concentrations (ug/ml): 125, 250, 500 and 1000. CDS or GS medium (pH 5.70) was used to dilute the stock solutions. All media containing sorbate were filter sterilized before use.

Chemicals

Potassium sorbate, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and polymyxin B sulfate were obtained from Sigma Chemical Company (St. Louis, Missouri). The following radiochemicals were also obtained from Sigma Chemical Co.: thymine-2-^{14}C (57 mCi/mmole) and N-acetyl-D-^{14}C-glucosamine (57 mCi/mmole). The following radiochemicals were obtained from ICN Biomedicals, Inc., (Costa Mesa, California): L-(U-^{14}C) arginine (146 mCi/mmole), D-(U-^{14}C) glucose (332mCi/mmole), L(U-^{14}C) glutamic acid (250 mCi/mmole), ^{35}S-sodium sulfate (43 mCi/mg), and ^{32}P-dipotassium phosphate (1000 mCi/mg). All radiochemicals were delivered as aqueous solutions and stored at 2 to 4°C prior to use.

Growth Studies

To determine the sensitivities of *E. coli* 15TAU and *E. coli* K12 to potassium sorbate in synthetic media (pH 5.70) at 37°C, a two-fold tube dilution test was used. Stock solutions of potassium sorbate (3 mg/ml and 4 mg/ml) in CDS were prepared and the pH was adjusted to 5.70 with 5N HCL. Two-fold dilutions of both stocks were made by serial dilution of 20

ml of stock with 20 ml of CDS (pH 5.70) to give the following potassium sorbate concentrations (ug/ml): 3000, 2000, 1500, 1000, 750, 500 and 250. Each sorbate soultion was filter sterilized through a membrane 0.2 μm pore filter (Gelman Supor 200) (Gelman Sciences Inc., Ann Arbor, MI). Ten milliliters of each solution were aseptically transferred to sterile, screw-capped test tubes. Each tube was inoculated with 0.1 ml of a diluted overnight culture of *E. coli* 15TAU or *E. coli* K12. The concentration of cells in each tube was approximately 5.5×10^7 viable cells per ml. All tubes were incubated for 18 hours in a water bath at 37°C. After incubation, the lowest potassium sorbate concentration that prevented visible tubidity was noted as the minimum inhibitory concentration (MIC). A separate test tube containing 10 ml of sterile CDS (pH 5.70) was also inoculated and placed at 2°C for 18 hours. This tube was used to compare its turbidity with those of other tubes. Concentrations of potassium sorbate that were less that the MIC were used in subsequent growth studies.

A 250-ml Erlenmeyer flask containing 60 ml of sterile CDS medium (pH 5.70, 37°C) was inoculated with 2.0 ml of an overnight (18-hour) culture of *E. coli* 15TAU or *E. coli* K12. The cell suspension was incubated at 37°C in a Fisher Model 129 shaker water bath reciprocating at 120 strokes per minute. Changes in the optical density (OD) of the culture were followed by periodically transferring 4.0 ml of culture into a standard culture tube (13 X 10 mm) and reading the percent transmittance (%T) at 660 nm in a Bausch and Lomb Spectronic 20

spectrophotometer. The equation, Absorbance = 2 - $\log_{10}$ %T was used to convert %T values to Absorbance.

Viable counts were determined by making serial dilutions of 1.0-ml samples of culture in sterile peptone (0.1%) water and plating appropriate dilutions in BHI agar via the pour plate method. The BHI agar plates were incubated at 37°C and colonies were counted after 48 hours of incubation. The counts obtained were expressed as $\log_{10}$ colony-forming-units (CFU) per ml. The data from four replications of the above procedure were used to construct a standard curve of Absorbance versus $\log_{10}$ CFU/ml. The standard curve was used to estimate the viable count of cell suspensions used as inocula in growth inhibition experiments.

Experiments on growth inhibition of *E. coli* 15TAU or *E. coli* K12 by potassium sorbate in CDS or GS medium were conducted as follows. Two milliliters of an overnight culture of *E. coli* were transferred into 60ml of sterile medium (pH.5.70) at 37°C. The culture was grown under identical conditions to those as described previously. When the culture was in the exponential phase and attained an Absorbance (660 nm) of 0.19, which represented about 1.7 X 10^8 viable cells per ml, a 10-ml sample was inoculated into each of five 250-ml Erlenmeyer flasks. Each flask had 20 ml of sterile medium (pH 5.70) at 37°C with or without potassium sorbate. The desired concentration of potassium sorbate (0, 125, 250, 500 or 1000 ug/ml) in each flask was attained upon addition of 10 ml of culture to each flask. The growth media were well buffered so that the addition of 10 ml of cell suspension (pH 5.68) to 20 ml of fresh medium did not change the pH of the medium from 5.70. All flasks were immediately

incubated at 37°C in a shaker water bath reciprocating at 120 strokes per minute.

At 0, 10, 20, 40, 60 and 80 minutes of incubation, Absorbance (660 nm) measurements were made on 3.0-ml samples of cell suspension taken from the flasks. Also, 1.0-ml samples were used for determining bacterial viability via serial dilution in sterile peptone (0.1%) water and plating of appropriate dilutions in BHI agar. Data from three replications of growth inhibition tests were used to construct separate standard curves (Absorbance versus $\log_{10}$CFU/ml) for each treatment and the control. The standard curves were used to estimate the viable counts of bacteria in samples of culture during investigations involving the use of radiolabeled components in the growth medium.

Measurements of Macromolecular Synthesis

Cells of *E. coli* 15TAU or *E. coli* K12 were grown in CDS media and transferred to fresh media with or without sorbate, exactly as described in the section on growth studies and under identical conditions. However, the control and the treatment flasks contained 4.0 µCi/flask of a particular radiolabeled precursor, depending on the type of macromolecule (DNA, RNA, protein, cell wall) that was being investigated. For example, 4.0 µCi of radiolabeled thymine were used in 30 ml of cell suspension to follow the synthesis of DNA in the presence or absence of potassium sorbate.

To determine the effect of potassium sorbate of the rate of macromolecular synthesis in *E. coli*, 4.0-ml samples from the control and

each treatment flask were removed at 0, 10, 20, 40 and 60 minutes of incubation. Each 4.0-ml sample of cell suspension was dispensed into a separate, coded culture tube (13 x 10 mm). The Absorbance (660 nm) was quickly determined for each sample and 4.0 ml of ice cold 10% trichloroacetic acid (TCA) were immediately added to each sample to precipitate the cellular macromolecules and stop further incorporation of precursor molecules. All samples were placed on ice for one hour. Each sample was filtered through a separate presoaked Whatman Glass Microfiber filter (GF/F, 0.7 um particle retention size, 25 mm diameter) under mild suction. Each filter was presoaked with cold TCA (10%) which contained 0.1% of the nonradioactive of the appropriate radiolabeled precursor that was used in the experiment. The trapped, TCA-insoluble precipitate on each filter was washed three times with 3.0 ml of ice cold 10% TCA. Each filter was carefully removed from its filter holder by means of filter forceps and dried under an infrared heat lamp for 45 minutes. Each filter was placed into a coded 22-ml scintillation vial which contained 10 ml of a liquid scintillation counting cocktail (Scintiverse BD). The vials were tightly capped and placed in a LKB Wallac (Model 1217) RackBeta liquid scintillation counter for measuring the level of radioactivity in each sample.

The amount of radioactivity in each sample represented the extent of incorporation of the labeled precursor into its respective macromolecule. The time course of incorporation of a labeled precursor into its respective macromolecule is an index of the rate of synthesis of that macromolecule. A separate flask containing 30 ml of CDS medium

(pH 5.70, 37°C) without bacterial cells and spiked with 4.0 µCi of a radiolabeled precursor was used for samples that were tested for background radioactivity. Those samples were processed in the same manner as described for samples that contained precipitated bacterial macromolecules.

Scintillation counts derived from cold TCA-insoluble precipitates were considered as gross counts per minute (cpm). The value for background cpm was subtracted from each value for gross cpm to give the net cpm. The net cpm for each sample was divided by the number of viable cells present at each sampling time to obtain the cpm per cell at 0, 10, 20, 40 and 60 minutes.

The molar amount of each labeled precursor incorporated into its respective macromolecule was calculated by dividing the net cpm per cell by the known specific activity (cpm per picomole) of the labeled precursor. The data on rate of macromolecular synthesis were expressed as picomoles of labeled precursor per 10^8 cells as a function of time in minutes.

The percent inhibition of the rate of macromolecular synthesis in the presence and absence of potassium sorbate was calculated by using the following equation: Inhibition (%) = [(incorporation without sorbate-incorporation with sorbate) ÷ (incorporation without sorbate)] X 100, where incorporation is expressed as picomoles of labeled precursor per 10^8 cells.

Nutrient Uptake Studies

Cells of *E. coli* 15TAU or *E. coli* K12 were grown in CDS or GS medium and transferred to the same medium with or without potassium sorbate. The cultures were grown under identical conditions as described in the preceeding sections on growth and macromolecular synthesis. Four 250-ml Erlenmeyer flasks, each containing 60 ml of sterile medium (pH 5.70), were used for growing exponential phase cells. Each of the four flasks was inoculated with 2.0 ml of an overnight (18-hr.) culture of *E. coli* 15TAU or *E. coli* K12. The inoculations were timed to be 20 minutes between each of the flasks. The flasks were coded with the time of inoculation and incubated at 37°C. When the culture attained an Absorbance (660 nm) of 0.19, 10 ml were transferred to 20 ml of CDS or GS (pH 5.70), with or without sorbate and prewarmed at 37°C. At specific time intervals (0, 30, 60, 90, 120, and 150 seconds), 4.0 ml of cell suspension were removed and filtered through Whatman Glass microfiber filters (0.7 um, 25mm) under mild suction. The filters were presoaked with phosphate-buffered saline (PBS) that contained 0.1% of the unlabeled form of the radioactive nutrient being investigated. The trapped cells on the filters were washed three times with 3.0 ml of PBS (37°C). The filters were removed and dried under an infrared heat lamp for 45 minutes. Each filter was placed in a coded scintillation vial that contained 10 ml of Scintiverse BD liquid scintillation counting cocktail. The radioactivity of samples was then measured by liquid scintillation counting.

The samples on the filters consisted of washed whole cells; thus, the scintillation counts obtained represented radioactivity of labeled

substrates present in both the macromolecules and the metabolic pool of the cells.

The data were handled in the same manner as described in the procedure for measurement of macromolecular synthesis. The data were expressed in picomoles of nutrient per 10^8 cells as a function of time in seconds. The percent inhibition of nutrient uptake by potassium sorbate was calculated by use of the following equation: Inhibition (%) = [(uptake without sorbate - uptake with sorbate) ÷ (uptake without sorbate)] X 100, where uptake is expressed as picomoles of labeled nutrient per 10^8 cells.

Oxygen Consumption

Cell preparation

A 1000-ml Erlenmeyer flask containing 500 ml of sterile CDS medium (pH 7.0) was inoculated with 5.0 ml of an overnight culture of *E. coli* K12. The cell suspension was incubated for 18 hours at 37°C on a rotary shaker at 200 revolutions per minute. After incubation, the culture was transferred into two (250-ml) centrifuge bottles and the cells were sedimented in a Sorval RC-2 refrigerated centrifuge (25°C) at 7000 x g for 10 minutes. The supernatant was discarded and each cell pellet was washed twice with citrate-phosphate buffer (pH 5.70). The cells were washed by resuspending each pellet in 200 of ml citrate-phosphate buffer. The cells were sedimented (7000 X g, 10 minutes) and the supernatant was discarded. Each pellet was resuspended in 20 ml of citrate-phosphate buffer (pH 5.70). The cell suspensions were pooled and adjusted to a final volume of 50 ml that contained 3.0 X 10^{10} viable cells

per ml as determined by serial dilution and plating in BHI agar. A 1/10 dilution of that cell suspension was made in citrate-phosphate buffer (pH 5.70) to give a cell concentration of 3.0 X 10^9 cells per ml.

Measurement of oxygen consumption

The oxygen consumption of *E. coli* K12 cells was measured polarographically at 37°C by means of Clark-type oxygen electrode fitted to a YSI Model 5300 Biological Oxygen Monitor (YSI Co. Inc. Yellow Springs Ohio). The instrument was attached to a Kipp and Zonen flatbed recorder set at a chart speed of 1.0 mm per second. The two-channel feature of the instrument allowed simultaneous measurement of a control and a treated sample (with sorbate) in separate chambers. The oxygen electrode was calibrated with air saturated buffer at 37°C and the initial oxygen concentration was 250 µM.

Citrate-phosphate buffer (2.8 ml) at pH 5.70 was added to the electrode chamber and allowed to equilibrate to 37°C. An *E. coli* K12 cell suspension (220 ul) was added to the buffer and was vigorously mixed by means of a small magnetic stirrer in the oxygen chamber. The oxygen electrode was carefully inserted into the chamber to completely cover the surface of the stirred cell suspension, thus displacing any air in the headspace. The background oxygen consumption rate was recorded and then 50 ul of a 10% glucose solution were added to the cell suspension through a small hole at the top of the electrode by means of a Hamilton spring-loaded syringe. The addition of glucose was done through a small hole at the top of the electrode. The oxygen consumption rate in the

presence of glucose was recorded. When a constant oxygen consumption rate was established, 200 ul of potassium sorbate were injected into the cell suspension. The new oxygen consumption rate was then recorded. The above procedure was done simultaneously with *E. coli* K12 cell suspensions in two chambers except that one chamber was injected with 200 ml of citrate phosphate buffer (pH 5.70) and the other with the same volume of potassium sorbate at the same pH. Data on oxygen consumption rates were expressed as μmoles of oxygen consumed by 2.0 X 10^8 cells as a function of time in seconds.

Leakage of Radioactive Cell Materials

An aliquot (0.6-ml) of an overnight culture of *E. coli* K12 in GS medium was transferred to 60 ml of fresh GS medium (pH 7.0) containing 10 μCi of uniformly labeled ^{14}C glucose. The culture was incubated at 37°C for 20 to 22 hours in a shaker water bath at 120 reciprocal strokes per minute. After incubation, the culture, which contained approximately 1.9 X 10^9 viable cells per ml, was transferred to two sterile centrifuge tubes and sedimented by centrifugation (7,000 X g, 10 minutes) in a Sorval RC-2 refrigerated centrifuge at 25°C. The supernatant was discarded and the pelleted cells were washed twice with citrate-phosphate buffer (pH 5.70). The cells were washed by resuspending them in buffer, centrifugation (7,000 X g, 10 minutes) and decanting the supernatant. The cells were resuspended in a small amount of citrate-phosphate buffer (pH 5.70) which contained 1.0% NaCl. The cell suspensions were pooled and adjusted to a final volume of 20 ml by

adding appropriate amounts of that same buffer. One milliliter of the washed cell suspension was added to each of six 250-ml Erlenmeyer flasks; each flask contained 54 ml of citrate-phosphate 1% NaCl buffer (pH 5.70, 37°C) with or without potassium sorbate. The cell concentration in each flask was about 2.0 X 10^8 cells/ml. The control flask contained no potassium sorbate; one flask, which contained polymyxin B (15 ug/ml), served as a positive control. Each of the remaining four flasks contained potassium sorbate at a concentration of 10, 125, 250 or 500 ug/ml.

All flasks were incubated in a shaker water bath at 37°C and 120 reciprocal strokes per minute. At specific intervals thereafter (10, 30, 60, 90 and 120 minutes), 10 ml of cell suspension were removed from each flask and placed into separate coded disposable centrifuge tubes. The cells were removed by centrifugation (10,000 X g, 10 minutes), and 5.0 ml of supernatant from each tube were carefully transferred to coded liquid scintillation vials. Each vail contained 10 ml of Scintiverse BD liquid scintillation counting cocktail. The vials were tightly capped and the radioactivity of the samples was measured by liquid scintillation counting. The radioactivity from each sample was expressed as counts per minute (cpm) per ml of supernatant as a function of time in minutes.

Permeability of Cells to H^+ ions

Cells from an overnight culture (500 ml) of *E. coli* K12 in CDS (pH 7.0) were harvested by centrifugation (7,000 X g, 10 minutes). The cells were washed twice in a glycylglycine-salts medium (pH 6.5) consisting of 50 mM KCl, 2 mM $MgCl_2$ and 1mM glycylglycine (Harold and Baarda,

1968) and resuspended in 50 ml of that same medium to give a viable cell count of 3.0 X 10^{10} cells per ml. Twenty milliliters of a 1/10 dilution of the cell suspension were prepared in the same medium (pH 6.5) and a 1.5-ml portion of the diluted culture was used in each experiment. Stock solutions of potassium sorbate (460, 920, and 1840 µg/ml), carbonyl cyanide m-chlorophenylhydrazone (CCCP, 55 µg/ml), and hydrochloric acid (HCl, 0.1N) were maintained at 37°C in a water bath set at that temperature. The method of Mitchell and Moyle (1967) was used to determine the conduction of protons.

To an oxygen electrode chamber with a magnetic stir bar, 2.5 ml of glycylglycine-salts medium (pH 6.5) were added, followed by 1.5 ml of *E. coli* K12 cell suspension. A pH electrode was immersed in the mixture. The temperature in the chamber attained maintained at 37°C by a Cole Parmer Model 1261-05 polystate circulator. When the cell suspension in the chamber was 37°C, 20 ul of 0.1N HCl were added. The pH of the cell suspension was quickly lowered from pH 6.50 to pH 5.68. When the pH reached 5.70, 1.5 ml of glycylglycine-salts medium (pH 5.70) without sorbate were added. The slow rise in pH, which represented the entry of protons in untreated cells, was recorded over a time period of 10 minutes. The chamber was emptied and rinsed three times with deionized water. The same amount of medium (pH 6.5) and *E. coli* K12 cell suspension were added to the chamber and allowed to attain a temperature of 37°C. After the addition of 20 µl of HCl (0.1N), the pH of the cell suspension was lowered to pH 5.68 and it subsequently began to rise slowly. When the pH reached 5.70, 1.5 ml of potassium sorbate stock

solution (460, 920, or 1840 µg/ml; pH 5.70) or CCCP (55 µg/ml; pH 5.70) was added to the cell suspension. Changes in pH of the cell suspension were recorded for 10 minutes. Using a Radiometer Model PHM84 Research pH meter attached to a Kipp and Zonen Flatbed recorder with a chart speed of 0.2 mm per second. CCCP, a known protonophore, was used as a positive control. Both sorbate and CCCP stock solutions were prepared to yield final concentrations of sorbate (125, 250, or 500 ug/ml) and CCCP (15 ug/ml) respectively on addition of 1.5 ml of appropriate stock solution to 4.0 ml of cell suspension. The data were expressed as changes in pH as a function of time in minutes.

Each of the afore mentioned experiments was repeated three times. Each set of data shown in the results and discussion section represents the means of three replicate experiments.

RESULTS AND DISCUSSION

Growth Inhibition of *Escherichia coli* by Potassium Sorbate The minimum inhibitory concentration (MIC) of sorbate for *E. coli* 15TAU or *E. coli* K12 was 1500 ug/ml by means of the tube dilution test. That MIC represented the lowest concentration of sorbate that prevented growth of *E. coli* (5.0 x 10^7 CFU/ml) for 24 hours in CDS (pH 5.70) at 37°C. Concentrations of sorbate commonly used to inhibit bacteria in foods are in the range of 0.05% (500 μg/ml) to 0.3% (3000 μg/ml). Those concentrations are usually bacteriostatic whereas higher concentrations may be lethal (Sofos et al., 1986; Sofos, 1989). The effect of potassium sorbate on the growth of *E. coli* was studied in more detail by the use of shake-flask cultures. Five concentrations of sorbate lower than the MIC were tested for their effect on the growth of exponential cells of *E. coli* in CDS. The two highest concentrations of sorbate (1000 μg/ml and 1200 μg/ml) were bacteriostatic and were not used in subsequent experiments. Preliminary growth studies revealed that, in the presence of those two sorbate concentrations, the viable count of *E. coli* (5.0 x 10^7CFU/ml) remained essentially constant for several hours. When growth is inhibited in that manner, the biosynthesis of cellular macromolecules quickly ceases and it is difficult to determine the primary site of inhibition. The remaining sorbate concentrations (125, 250, and 500 μg/ml) effectively decreased the growth rate of exponential cells of *E. coli*, and growth inhibition increased with higher concentrations of sorbate (Figures 1 and 2).

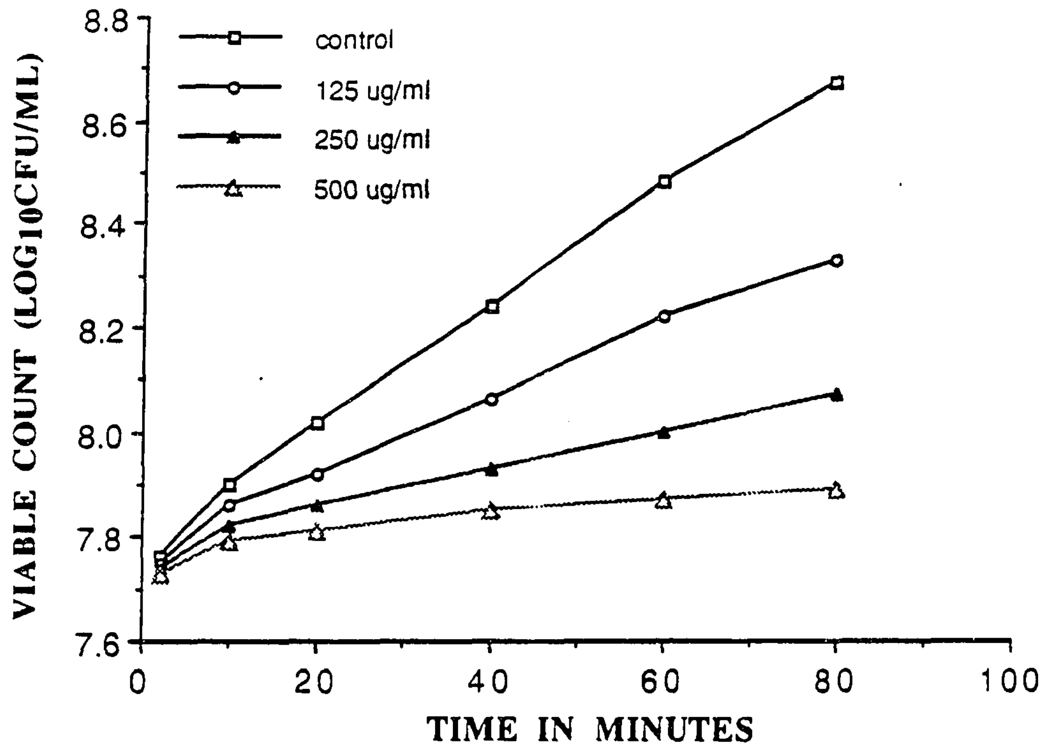

Figure 1. Effect of potassium sorbate on growth of exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C (Standard error: 0.08)

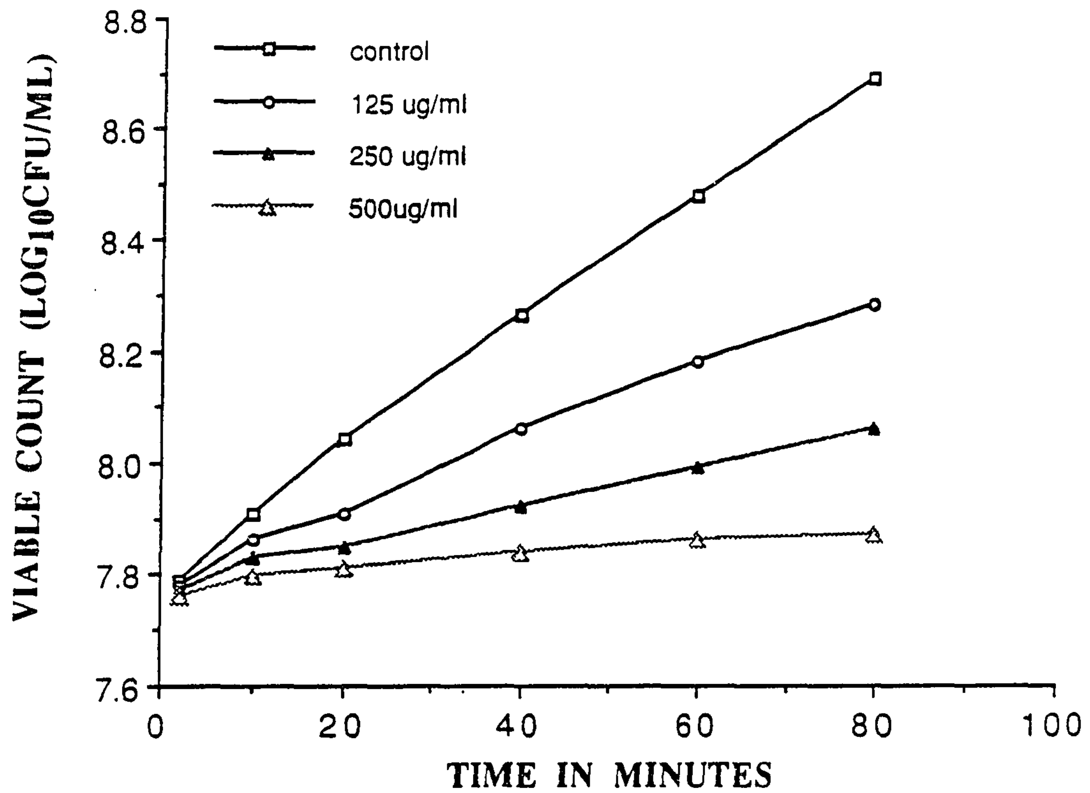

Figure 2. Effect of potassium sorbate on growth of exponential-phase cells of *E. coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C (Standard error: 0.10)

The generation times of *E. coli* 15TAU and *E. coli* K12 in CDS media under the experimental conditions used were estimated to be 28 minutes and 25 minutes, respectively. When those cultures were exposed to subbacteriostatic concentrations of potassium sorbate (125, 250, and 500 μg/ml), their growth rates were effectively reduced, and decreased systematically with increasing concentrations of sorbate (Figures 1 and 2). Potassium sorbate seemed to have an almost immediate effect on the growth rate of exponential cells of *E. coli*. The results of growth inhibition studies indicate that the primary cellular site inhibited by sorbate was affected rapidly and the extent of inhibition increased with higher sorbate concentrations.

Effect of Potassium Sorbate on the Synthesis of DNA, RNA, Protein and Cell wall in *Escherichia coli*

It is a well known fact that when the synthesis of cellular macromolecules is inhibited, microbial growth slows down or soon ceases. The primary cellular site of inhibition by an antimicrobial agent can be determined by kinetic studies on the incorporation of specific radiolabeled precursors into their appropriate macromolecules (Davis, 1980). That approach was used in this study to find out whether the synthesis of DNA, RNA, protein or cell wall was inhibited first and to a relatively large extent by sorbate. The effect of sorbate on macromolecular synthesis in *E. coli* was determined at the earliest possible time after the cells were in contact with sorbate. It was necessary to do this because growth inhibition occurred rapidly after

exponential cells of *E. coli* were treated with sorbate. The synthesis of DNA, RNA, and protein in the triple auxotroph *E. coli* 15TAU was studied without complications from endogenous synthesis of thymine, uracil and arginine.

Figures 3 to 6 show the effects of sorbate on the synthesis of DNA, RNA, protein, and cell wall in *E. coli* 15TAU. The synthesis of nucleic acids, protein, and cell wall in control cultures of *E. coli* 15TAU proceeded rapidly. The amount of radioactivity incorporated into the TCA-insoluble cell fraction of untreated cultures increased 8 to 9 fold for each precursor at 40 minutes of incubation. In sorbate-treated cultures, the inhibition of DNA, RNA, or protein synthesis was evident as early as 2.0 minutes of incubation. At that same time, the lowest growth-inhibitory concentration of sorbate (125 µg/ml) inhibited the synthesis of DNA, RNA, protein and cell wall by 22%, 30%, 23% and 6%, respectively. At 2.0 minutes of incubation, there was only a slight difference (1%) in the inhibition of synthesis of DNA and protein by sorbate (125 µg/ml). However, at that same time, higher concentrations of sorbate (250 µg/ml or 500 µg/ml) inhibited protein synthesis more than DNA synthesis (Table 3). Compared to the synthesis of other macromolecules, cell wall synthesis was inhibited the least (6, 9 and 24%) by sorbate concentrations of 125, 250, and 500 µg/ml, respectively, at 2.0 minutes. RNA synthesis was most sensitive to sorbate. At 2.0 minutes, RNA synthesis was inhibited 30%, 51% and 63% by sorbate at 125, 250, and 500 µg/ml, respectively (Table 3.).

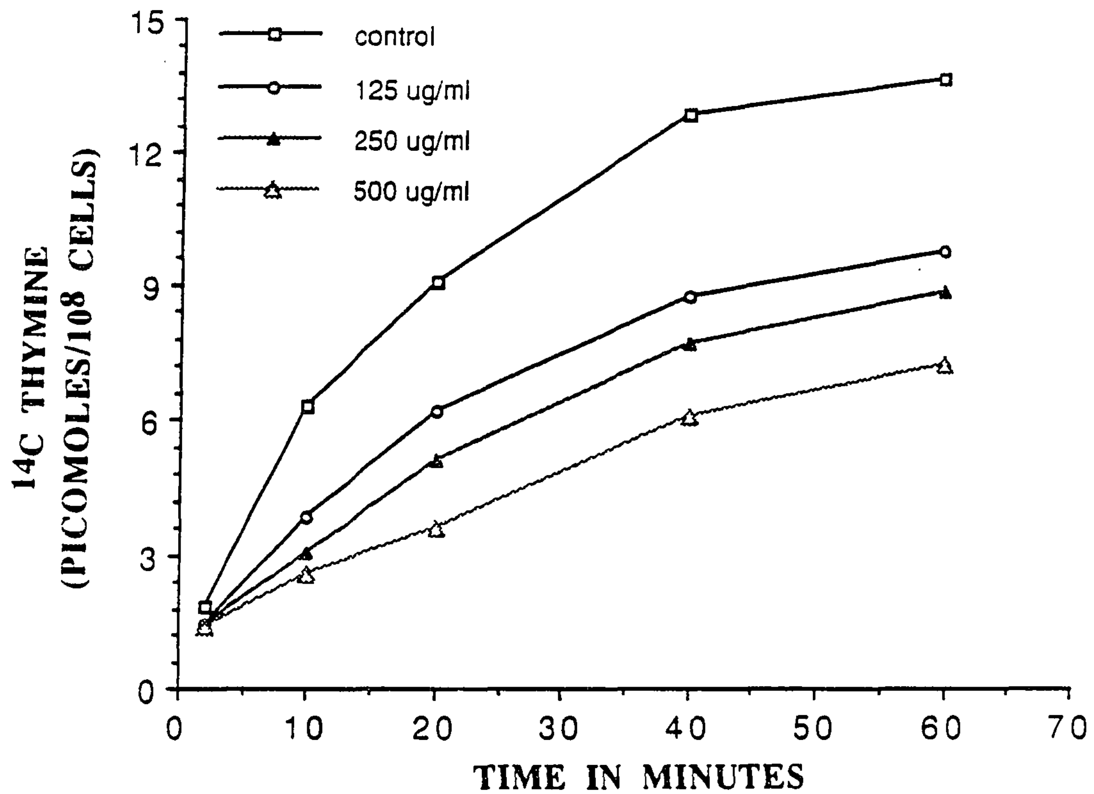

Figure 3. Effect of potassium sorbate on ^{14}C thymine incorporation into the DNA of exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.28)

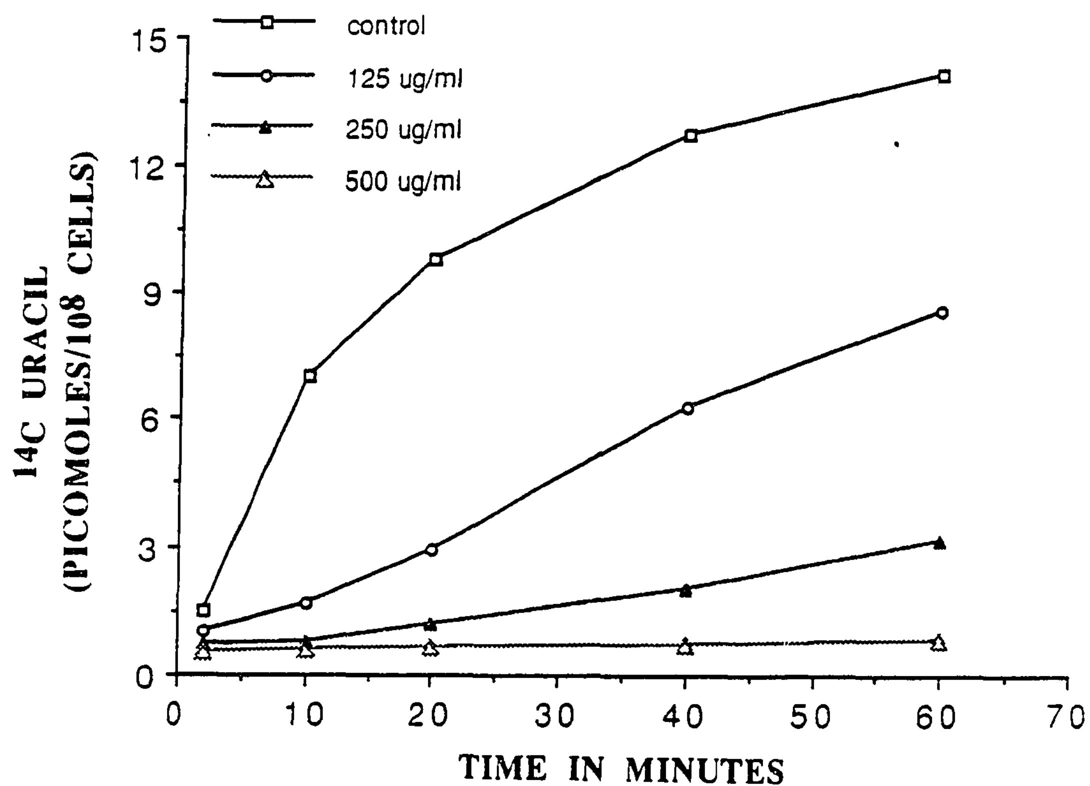

Figure 4. Effect of potassium sorbate on ^{14}C uracil incorporation into the RNA of exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.23)

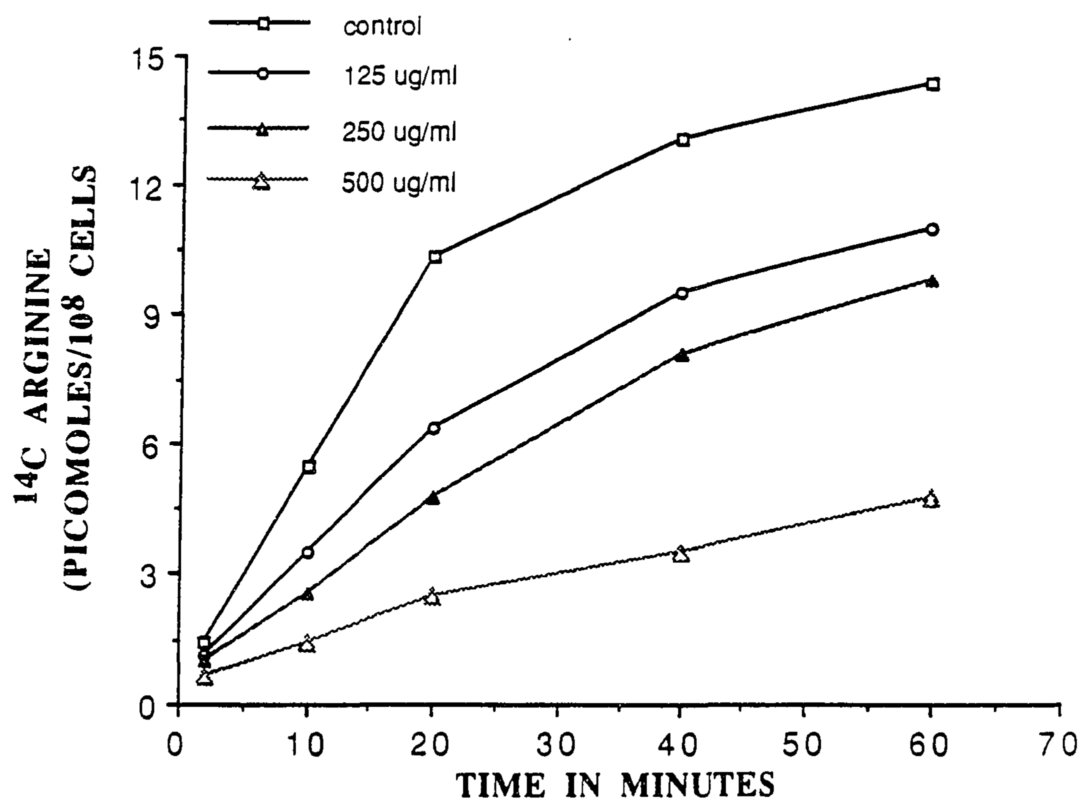

Figure 5. Effect of potassium sorbate on ^{14}C arginine incorporation into the protein of exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.26)

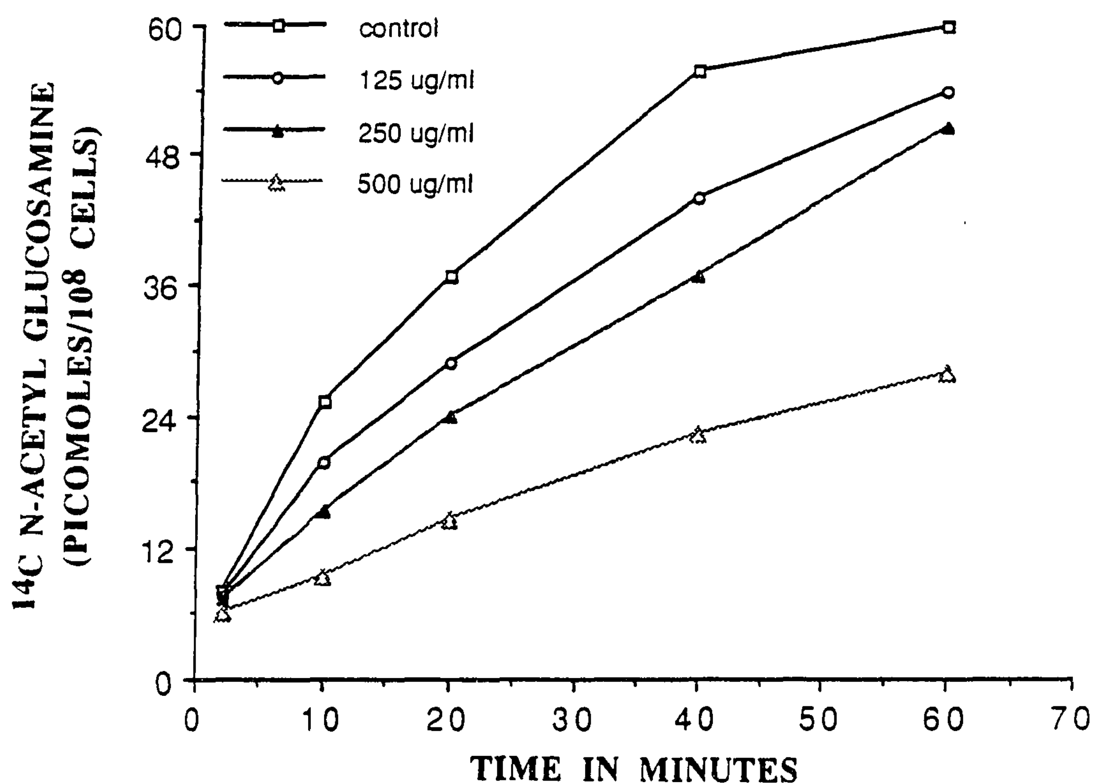

Figure 6. Effect of potassium sorbate on ^{14}C N-acetyl-D-glucosamine in corporation into the cell wall of exponential-phase cells of *E coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.30)

Table 3. Percent inhibition of growth and macromolecular synthesis in exponential-phase cells of *E. coli* 15 TAU after 2.0 minutes of exposure to potassium sorbate in complete defined synthetic medium (pH 5.70) at 37°C [a]

	Potassium sorbate (μg/ml)			
	125	250	500	S.E.M.[b]
Growth (CFU/ml)[c]	2.3	4.3	6.6	0.10
Macromolecular synthesis				
DNA	22.0	23.0	26.0	0.26
RNA	30.0	51.0	63.0	0.20
Protein	23.0	30.0	55.0	0.28
Cell wall	6.0	9.0	20.0	0.18

[a] Means of three replicate experiments.
[b] Overall standard error.
[c] Colony forminfg units per ml.

To ascertain that the observed inhibitions of DNA, RNA, and protein synthesis by sorbate were not unique to the triple auxotroph *E. coli* 15 TAU, the effect of potassium sorbate on the synthesis of those macromolecules was studied in *E. coli* K12, a wild type strain. The experiments utilizing E. *coli* K12 were done under conditions identical to those used for *E. coli* 15TAU.

The effect of sorbate on the synthesis of DNA, RNA, and protein in *E. coli* K12 is shown in Figures 7 to 9. Rapid synthesis of DNA, RNA, and protein occurred in control cultures of *E. coli* K12. There was approximately an 8-fold increase in the incorporation of each labeled precursor into the TCA-insoluble cell fraction at 40 minutes of incubation. The inhibition of DNA, RNA, and protein synthesis by all three concentrations of sorbate in *E. coli* K12 was similar to inhibition of synthesis of those same macromolecules in *E. coli* 15TAU. Sorbate (125 µg/ml) inhibited DNA, RNA, and protein synthesis in *E. coli* K12 by 20%, 31%, and 21% respectively at 2.0 minutes of incubation. Sorbate (250 µg/ml) inhibited RNA synthesis more than DNA or protein synthesis (Table 4). At a sorbate concentration of 500 µg/ml, both RNA and protein synthesis were inhibited more than DNA synthesis. However, the difference in inhibition between RNA and protein synthesis was smaller (Table 4). That difference was evident in both *E. coli* 15TAU and *E. coli* K12.

By direct chemical analysis of sorbate-treated cultures, Nose et al., (1982) demonstrated that potassium sorbate (4000 µg/ml)

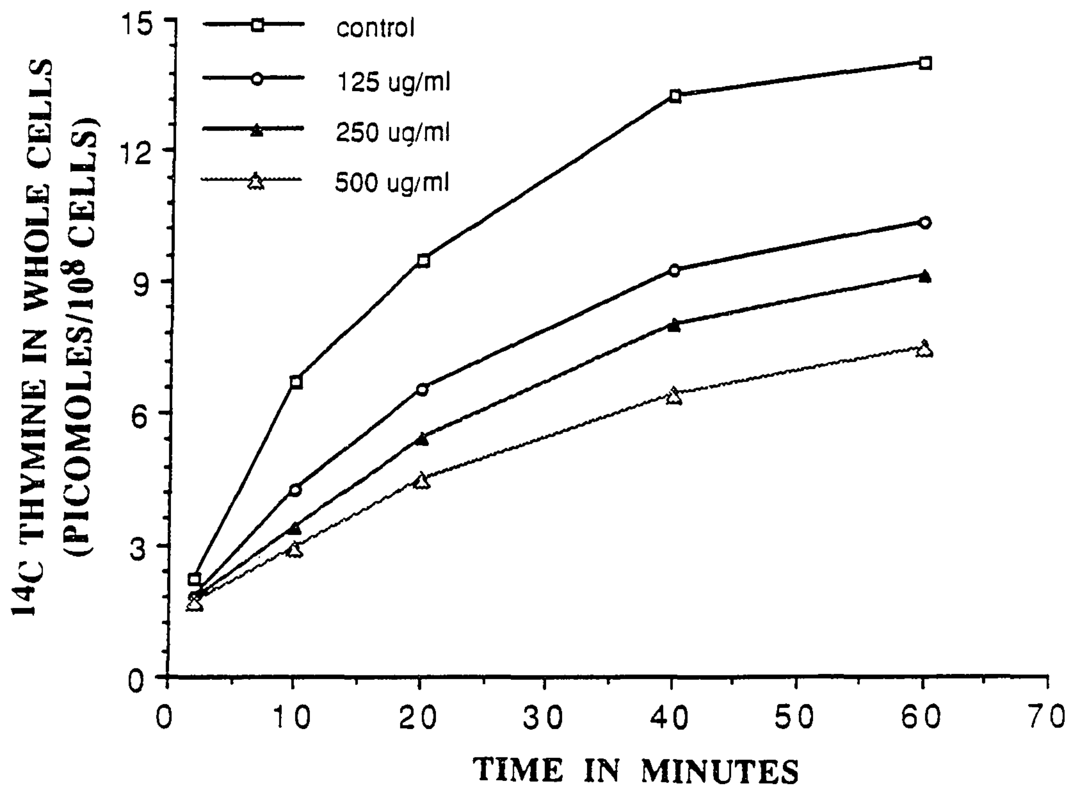

Figure 7. Effect of potassium sorbate on ^{14}C thymine incorporation into the DNA of exponential-phase cells of *E. coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.16)

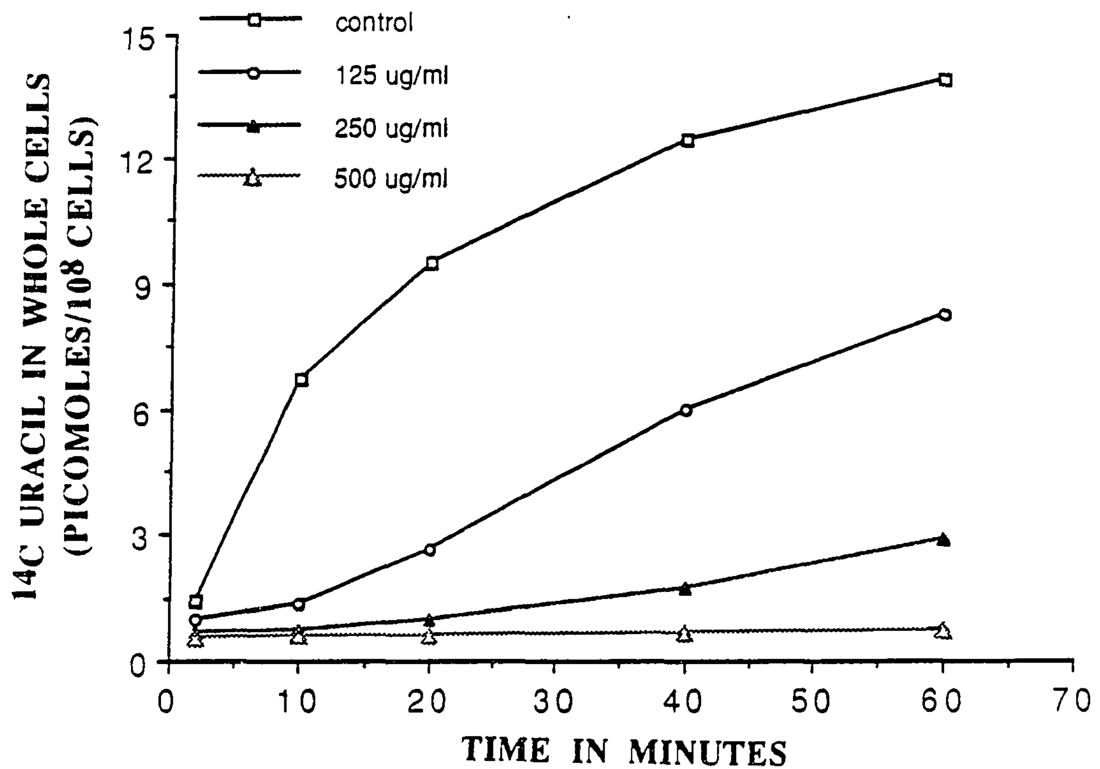

Figure 8. Effect of potassium sorbate on ^{14}C uracil incorporation into the RNA of exponential-phase cells of *E. coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.26)

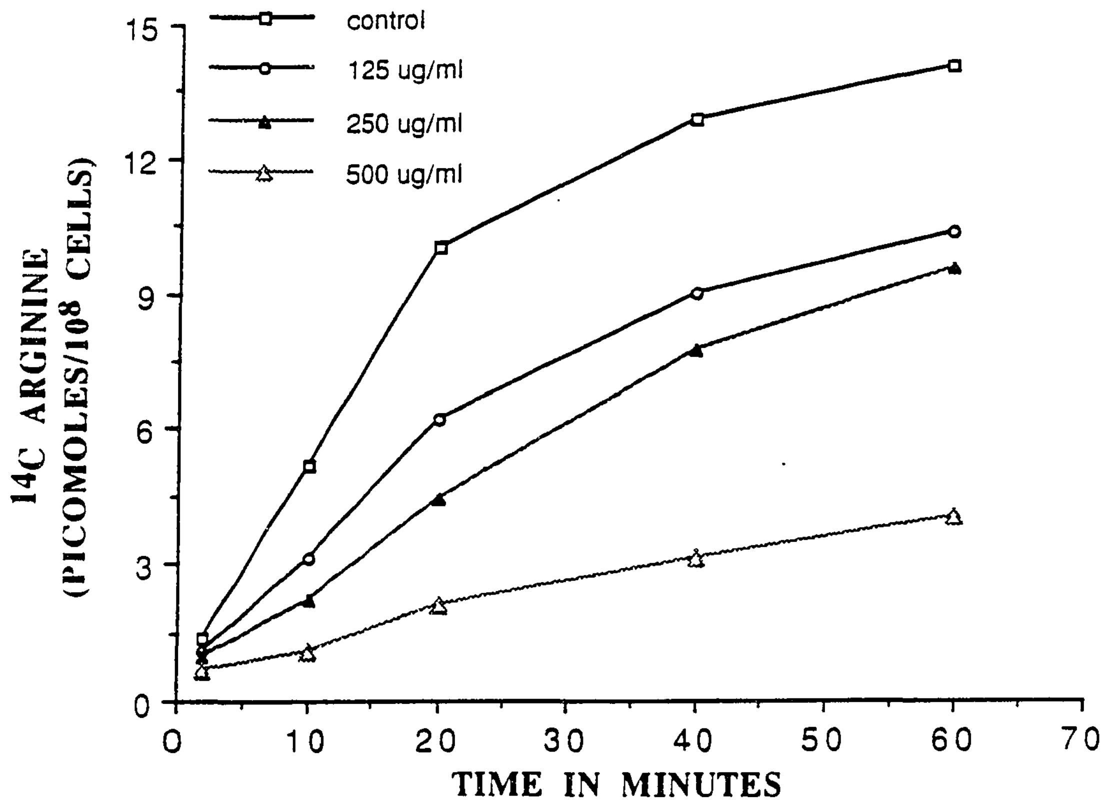

Figure 9. Effect of potassium sorbate on ^{14}C arginine incorporation into the protein of exponential-phase cells of *E. coli* K12. in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity in the cold trichloroacetic acid insoluble cell fraction was measured (Standard error: 0.22)

Table 4. Percent inhibition of growth and macromolecular synthesis in exponential-phase cells of *E. coli* K 12 after 2.0 minutes of exposure to potassium sorbate in complete defined synthetic medium (pH 5.70) at 37°C [a]

	Potassium sorbate (μg/ml)			
	125	250	500	S.E.M.[b]
Growth (CFU/ml)[c]	2.5	4.6	7.0	0.11
Macromolecular synthesis				
DNA	20.0	22.0	25.0	0.22
RNA	31.0	53.0	60.0	0.27
Protein	21.0	28.0	51.0	0.25

[a] Means of three replications.
[b] Overall standard error.
[c] Colony forming units per ml.

inhibited the synthesis of DNA, RNA and protein in *Pseudomonas fluorescens*. In that study, there was little difference in the extent of inhibition of those macromolecules by sorbate. In my studies, there were negligible differences in the extent to which the synthesis of DNA, RNA, or protein in *E. coli* was inhibited by sorbate at concentrations (1000 μg/ml and 1200 μg/ml) near the MIC (1500 μg/ml). By using radioisotopic tracer techniques, it was possible to determine the relative sensitivities of DNA, RNA, protein and cell wall syntheses to subbacteriostatic, growth-inhibitory levels (125, 250, and 500 μg/ml) of sorbate. In the present investigation, it was impractical to determine the inhibition of macromolecular synthesis by sorbate precisely at time zero because the time lapse between removal of four culture samples and precipitation by the addition of TCA to stop further biosynthetic incorporation was approximately 1.5 to 2.0 minutes in all instances of sampling. The results of the present investigation indicate that sorbate may be non-specific in its inhibitory action on macromolecular synthesis in *E. coli* because the syntheses of all four macromolecules tested were inhibited. However, the synthesis of each macromolecule was inhibited to a different extent within 2.0 minutes of exposure of *E. coli* to sorbate. Those differences in amount of inhibition may reflect unique sensitivities of certain macromolecular synthetic systems to sorbate, or secondary effects of some primary lesion induced by sorbate. The synthesis of RNA was of particular interest because sorbate exerted the greatest inhibitory effect on the synthesis of RNA in both *E. coli* 15TAU and *E. coli* K12 (Figures 4 and 8). It was anticipated

that the inhihition of RNA synthesis in *E. coli* resulted from an interference by sorbate with one or more processes by which RNA is synthesized. The cellular production of RNA is catalyzed by the enzyme DNA-dependent RNA polymerase (RNA polymerase). To make RNA, the enzyme uses one strand of DNA as template and any ribonucleoside triphosphate (ATP, UTP, GTP, or CTP) as a substrate. Inhibition of RNA polymerase may not be how sorbate inhibits RNA synthesis because sorbate failed to inhibit the activity of RNA polymerase isolated from *E. coli* (Yamada, 1977).

During balanced growth in bacteria, the rate of RNA synthesis is strongly coupled to the rate of protein synthesis (Neidhardt and Magasanik, 1960). The results of the present study indicate that when sorbate is added to exponentially growing bacterial cells, the closely regulated synthesis of RNA and protein is disrupted. Figures 4 and 5 show that the rates of RNA and protein synthesis in control cultures of *E. coli* 15TAU were very similar during 20 minutes of incubation; however, in sorbate-treated cultures, the rates of RNA and protein synthesis were inhibited disproportionately in that same time frame with RNA synthesis being inhibited to a greater extent. Similar results were obtained for *E. coli* K12 (Figs. 8 and 9). There are a number of ways by which the rate of RNA synthesis can be uncoupled from that of protein synthesis in bacteria. Those ways include the addition of antibiotic inhibitors of protein synthesis (Takeda et al., 1960), starvation of an amino acid auxotroph of *E. coli* (Borek et al., 1955), and depletion of intracellular potassium ion (K^+) levels in certain *E. coli*

mutants (Ennis and Lubin, 1961). Since sorbate does not inhibit *E. coli* RNA polymerase activity (Yamada, 1977), it is possible that sorbate inhibited RNA synthesis by starving *E. coli* 15 TAU of uracil and/or arginine for which that same organism is auxotrophic. *E. coli* 15TAU lacks the capacity to synthesize thymine, arginine, or uracil. If limited quantities of these nutrients are available in the cell, the synthesis of DNA, RNA, or protein would be inhibited. To test this hypothesis, the effect of sorbate on the uptake of selected nutrients by *E. coli* 15TAU was investigated.

Effect of Potassium Sorbate on Nutrient Uptake by Whole Cells of *Escherichia coli*

E. coli 15TAU does not grow in the absence of exogenous supplies of thymine, arginine, and uracil. The effects of potassium sorbate on the uptake of those nutrients by *E. coli* 15TAU were investigated to determine whether the inhibition of growth and macromolecular synthesis in that organism was due to interference of nutrient uptake in the presence of sorbate. Since inhibition of growth and macromolecular synthesis in exponential cells of *E. coli* was detected within 2.0 minutes after treatment with sorbate, the effects of sorbate on nutrient uptake were measured aafter 0, 0.5, 1.0, 1.5, 2.0, and 2.5 minutes of exposure of the cells to sorbate.

The uptake of nutrients by control cultures of *E. coli* 15TAU was rapid. In treated cultures of the organism, sorbate (125 µg/ml) failed to alter the rate of uptake of thymine or uracil for up to 1.5 minutes of

incubation and only slightly inhibited (8.4 and 8.6%) the uptake of those nutrients at 2.0 minutes (Figures 10 and 11). Uptake inhibition of arginine or glutamic acid occurred as early as 0.5 minutes and increased dramatically throughout the incubation period (Figures 12 and 13). At 2.0 minutes, sorbate (125 µg/ml) inhibited the cellular uptake of arginine or glutamic acid approximately four times as much as the uptake of thymine or uracil. The use of higher concentrations of sorbate (250 and 500 µg/ml) resulted in greater uptake inhibition for all nutrients tested. The extent of nutrient uptake inhibition in *E. coli* 15TAU by each of the three sorbate concentrations is shown in Table 5.

The effects of sorbate on the uptake of thymine, uracil, arginine or glutamic acid in the wild-type organism *E. coli* K12 (Figures 14 to 17), was similar to the uptake inhibition of those same nutrients observed in *E. coli* 15TAU. Sorbate (125 µg/ml) did not affect the rate of thymine or uracil uptake during 1.5 minutes of incubation (Figures 14 and 15), whereas inhibition of arginine or glutamic acid uptake occurred at 0.5 minutes (Figures 16 and 17). In *E. coli* K12, the percent uptake inhibition of all nutrients tested also increased with increasing sorbate concentration (Table 6). With both *E. coli* 15TAU and *E. coli* K12, sorbate (125, 250 or 500 µg/ml) in CDS medium (pH 5.70) effectively inhibited amino acid uptake as compared with the uptake inhibition of the nucleic acid precursors, thymine and uracil. The results of the present investigation indicate that the lowest growth inhibitory concentration of sorbate (125 µg/ml) strongly inhibited the uptake of arginine and glutamic acid in whole cells of *E. coli*. Uptake

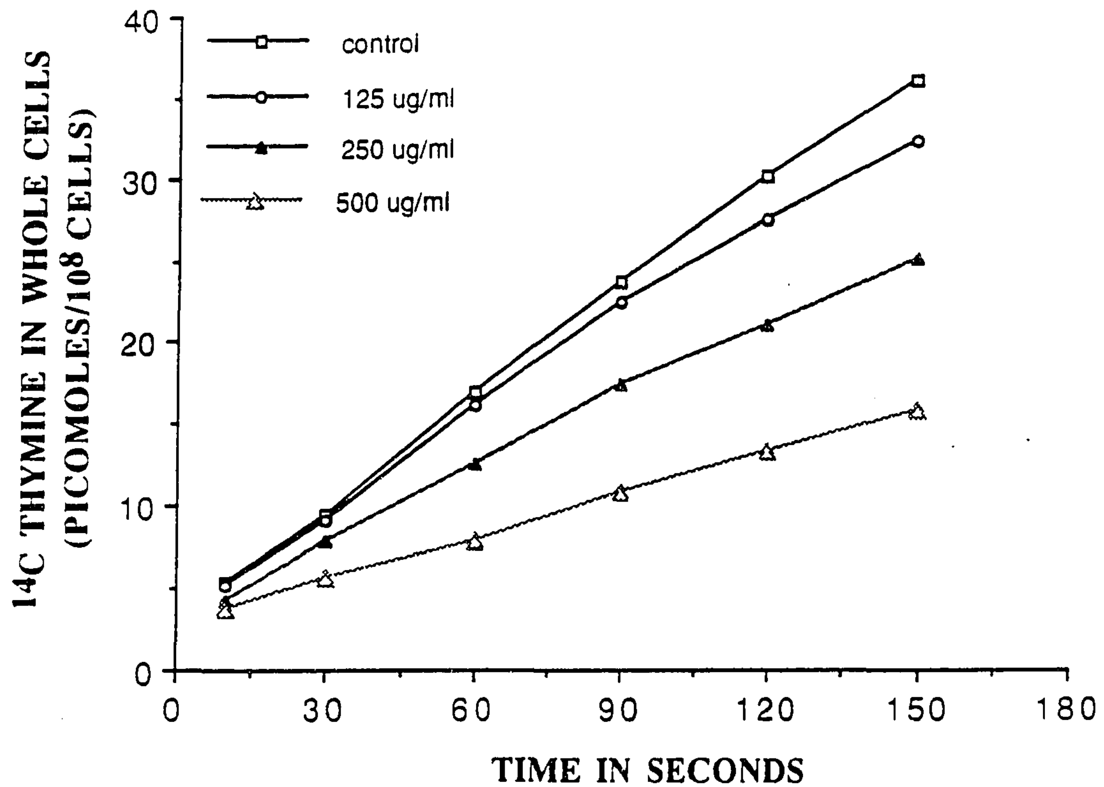

Figure 10. Effect of potassium sorbate on uptake of ^{14}C thymine by exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.20)

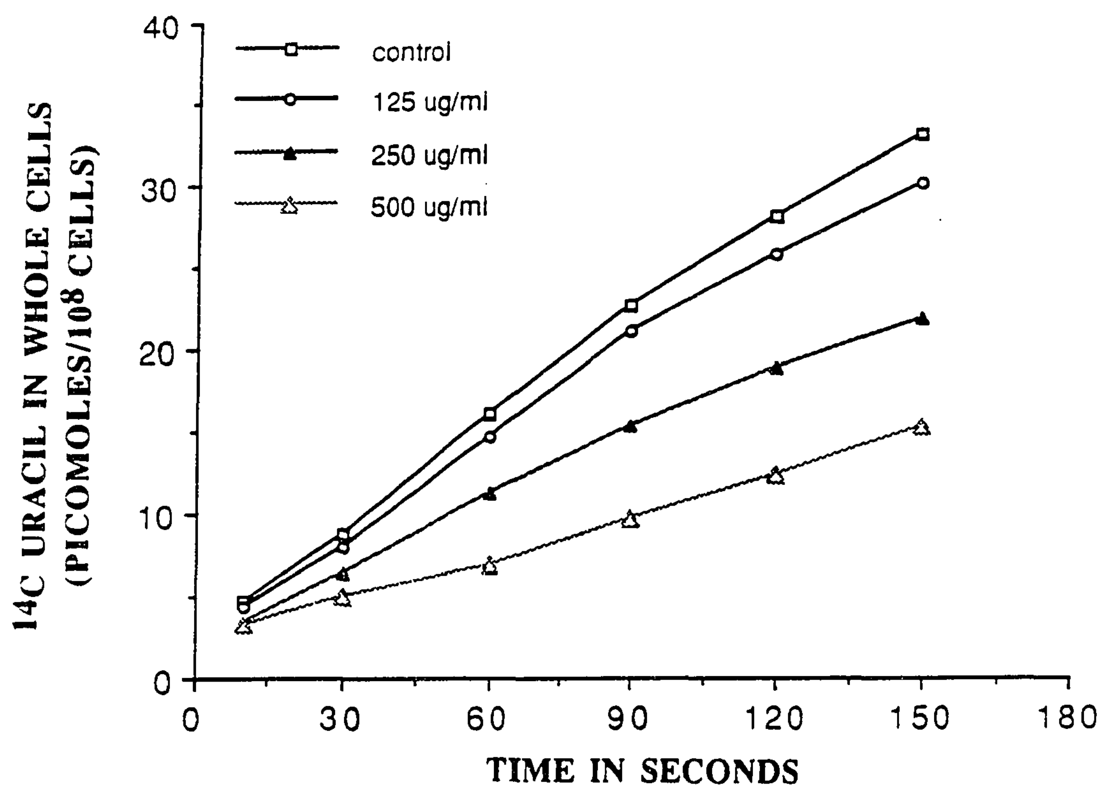

Figure 11. Effect of potassium sorbate on uptake of ^{14}C uracil by exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.18)

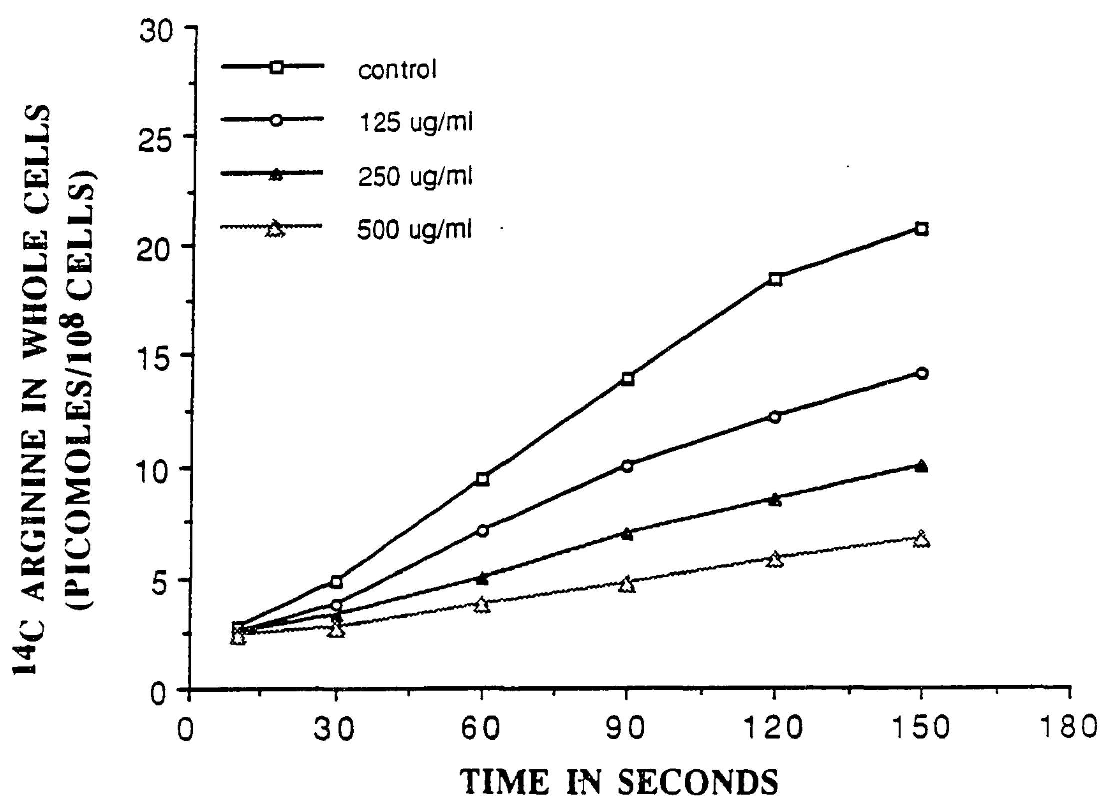

Figure 12. Effect of potassium sorbate on uptake of ^{14}C arginine by exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.22)

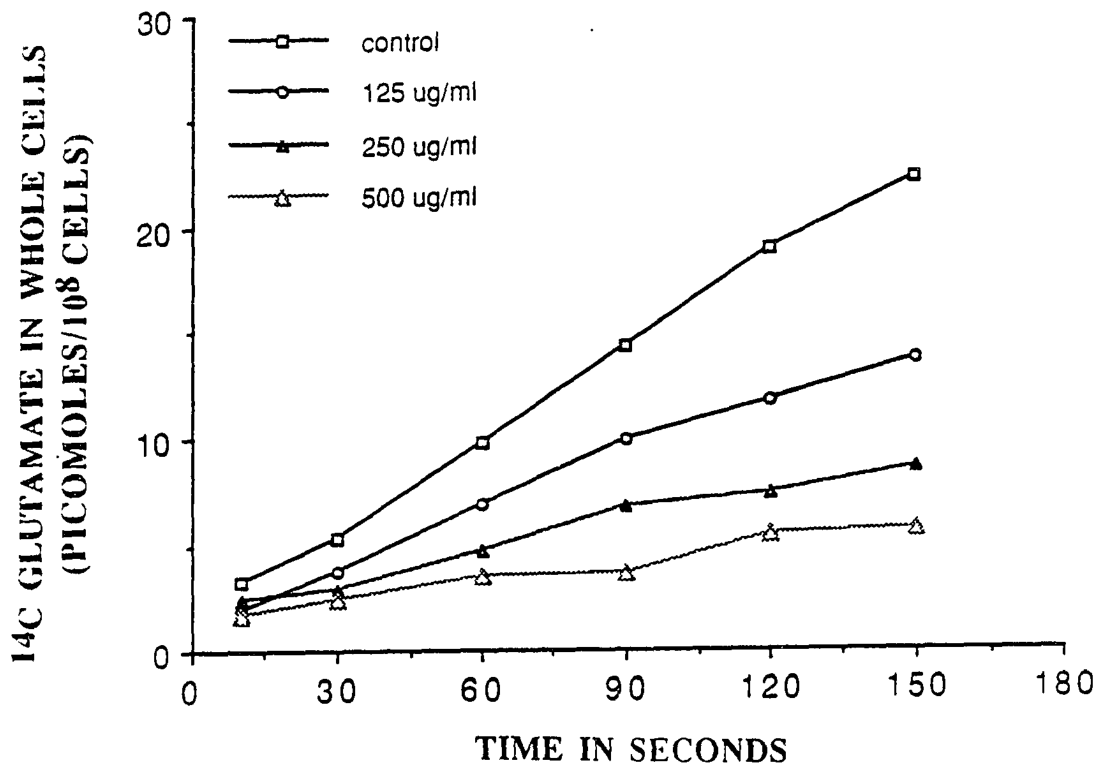

Figure 13. Effect of potassium sorbate on uptake of ^{14}C glutamic acid by exponential-phase cells of *E. coli* 15TAU in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.26)

Table 5. Percent uptake inhibition of selected nutrients in exponential-phase cells of *E. coli* 15 TAU after 2.0 minutes of exposure to potassium sorbate in complete defined synthetic medium (pH 5.70) at 37°C [a]

	Potassium sorbate (µg/ml)			
	125	250	500	S.E.M.[b]
Nutrient uptake				
Thymine	8.4	30.1	55.5	0.18
Uracil	8.6	33.1	56.4	0.15
Arginine	33.0	53.0	65.4	0.17
Glutamic acid	38.5	61.2	71.0	0.22

[a] Means of three replications.
[b] Overall standard error.

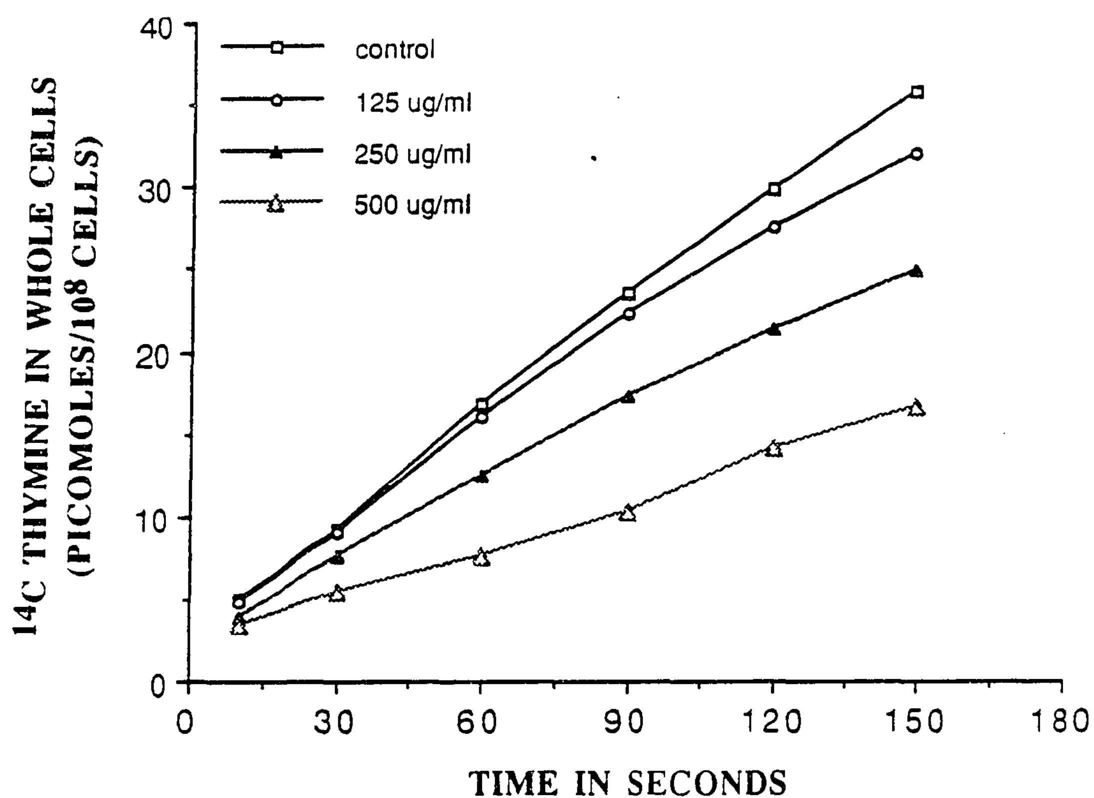

Figure 14. Effect of potassium sorbate on uptake of ^{14}C thymine by exponential-phase cells of *E. coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.16)

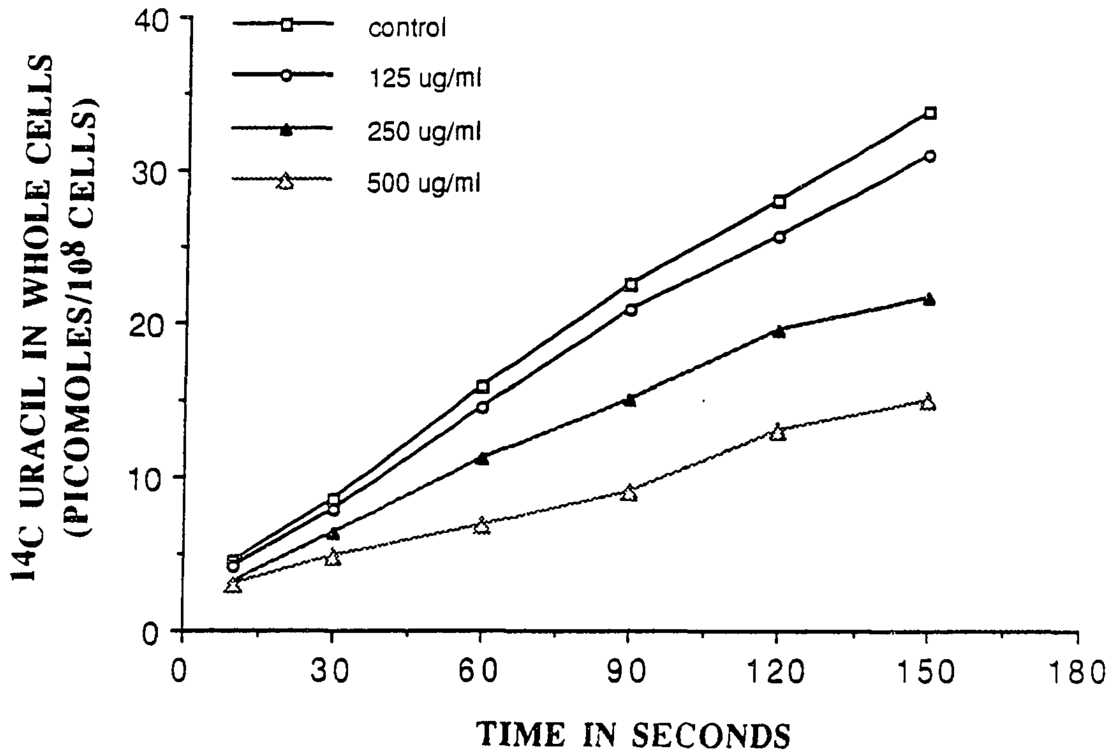

Figure 15. Effect of potassium sorbate on uptake of ^{14}C uracil by exponential-phase cells of *E. coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.10)

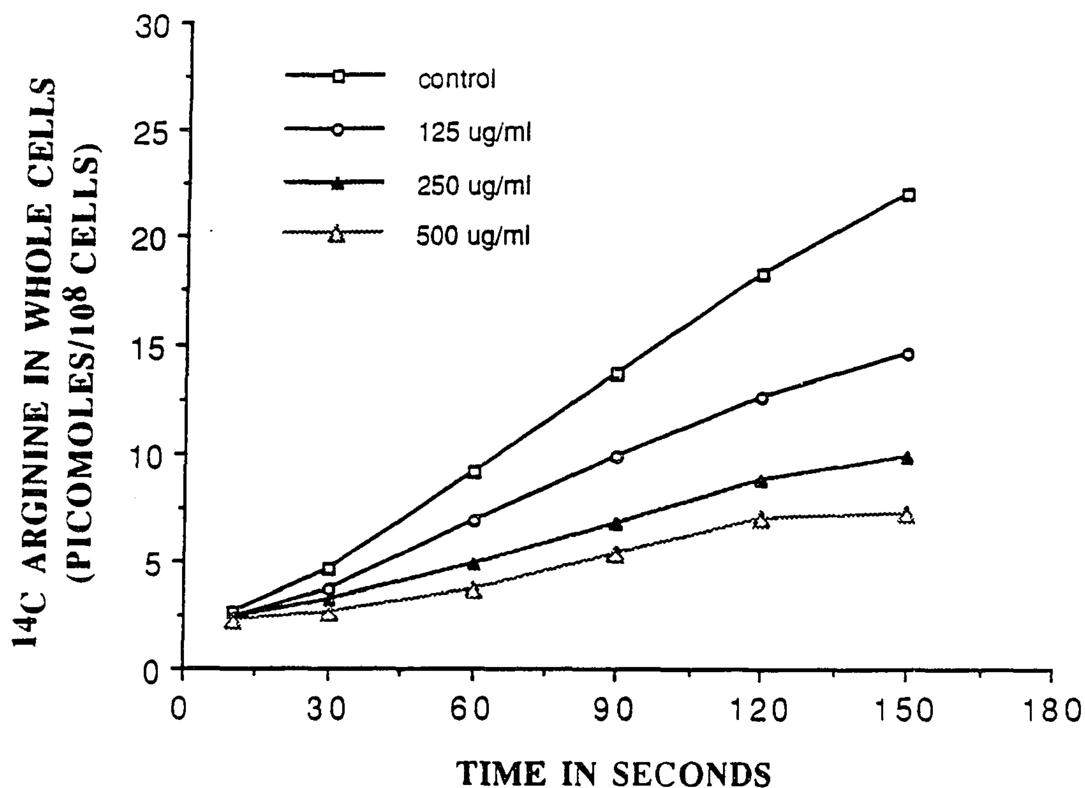

Figure 16. Effect of potassium sorbate on uptake of ^{14}C arginine by exponential-phase cells of *E coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.15)

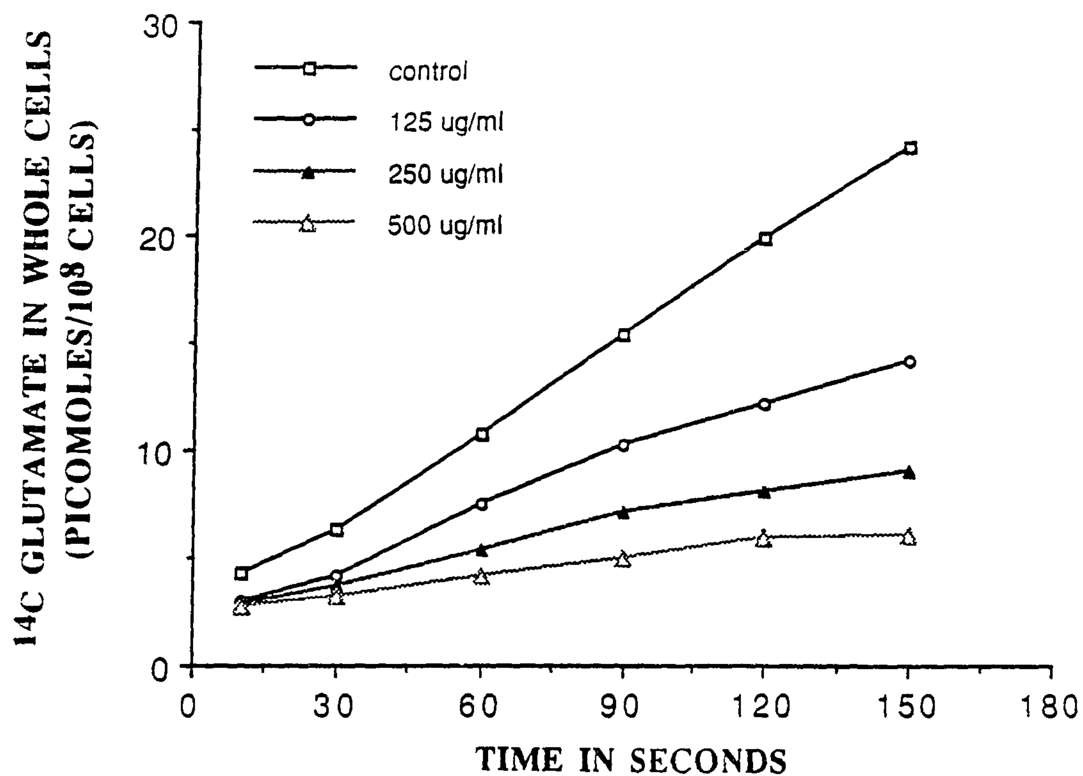

Figure 17. Effect of potassium sorbate on uptake of ^{14}C glutamic acid by exponential-phase cells of *E. coli* K12 in complete defined synthetic medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate-buffered saline (Standard error: 0.18)

Table 6. Percent inhibition of growth and uptake of selected nutrients in exponential-phase cells of *E. coli* K 12 in glucose-salts medium (pH 5.70) or complete defined synthetic medium (pH 5.70) at 37°C [a]

	Potassium sorbate (ug/ml)			
	125	250	500	S.E.M.[b]
Growth (CFU/ml)[c]				
GSM[d]	3.2	5.1	7.9	0.12
Nutrient uptake				
Thymine[e]	7.8	28.6	52.5	0.17
Uracil[e]	8.2	30.4	53.3	0.20
Glucose[f]	7.0	16.8	28.3	0.22
Arginine[e]	31.5	52.2	61.4	0.18
Glutamic acid[e]	39.8	59.0	70.1	0.20
Sulfate[f]	37.8	50.2	57.6	0.15
Phosphate[f]	41.7	62.4	71.9	0.23

[a] Means of three replications; [b] overall standard error.
[c] Colony forming units per ml; [d] glucose-salts medium.
[e] In complete defined synthetic medium.
[f] In glucose-salts medium.

inhibition of those amino acids occurred rapidly (0.5 minutes), whereas inhibition of uracil or thymine uptake was noted only after 1.5 minutes. Cellular uptake of uracil was generally uninhibited by sorbate (125 µg/ml); therefore, it was doubtful whether the strong inhibition of RNA synthesis in sorbate-treated cells could be mainly attributed to a lack of intracellular uracil. The fact that sorbate (125 µg/ml) also inhibited RNA synthesis in *E. coli* K12, an organism that can endogenously synthesize its own uracil, supports the notion that sorbate-inhibition of RNA synthesis was not a consequence of the reduction of intracellular uracil levels via inhibition of uracil uptake. In the present study, the physiological response of *E. coli* cells treated with sorbate seemed similar to the response of cells which had been transferred from a nutrient rich medium to a minimal medium. Maaløe and Kjeldgaard (1966) carried out shift-down experiments in which cells of *Salmonella typhimurium* growing in nutrient broth at 37°C were transferred to glucose-minimal medium. In those studies, the rate of RNA synthesis abruptly decreased immediately after transfer of the cells to glucose minimal media. Conversely, in shift-up studies done by those same researchers, RNA synthesis sharply increased when *S. typhimurium* cells growing in glucose-minimal medium at 37°C were transferred to nutrient broth. RNA synthesis, compared to DNA synthesis and cell growth, was highly responsive to nutritional changes in the growth medium. It is likely that the presence of sorbate in growth media quickly creates some sort of nutritional restriction in *E. coli*. The results of the present study show that sorbate strongly inhibited the uptake of

arginine and glutamic acid in *E. coli* 15TAU and *E. coli* K12. These results are consistent with those of previous studies in which sorbate inhibited amino acid uptake in bacterial cells. Sorbate (0.1 to 1.0%) effectively inhibited the uptake of alanine, serine and phenylalanine in whole cells and vesicles of *B. subtilis, E. coli, and P. aeruginosa* (Eklund, 1980). Sorbate (0.1%) also inhibited the uptake of serine and histidine in whole cells of *S. typhimurium* and seemed to act as a noncompetitive inhibitor of amino acid uptake (Tuncan and Martin, 1985). One of the hypotheses that has been proposed to explain the mechanism of microbial growth inhibition by sorbate is that sorbate inhibits growth by inhibiting nutrient uptake (Freese et al., 1973). To discover if inhibition of amino acid uptake by sorbate was mainly responsible for growth inhibition in *E. coli,* the effects of sorbate on growth of *E. coli* K12 in a medium devoid of amino acids were investigated. *E. coli* K12 can endogenously synthesize its required amino acids from the simplest of precursors. *E. coli* K12 was grown at 37°C in GS medium (pH 5.70) or glycerol-salts medium (pH 5.70) with or without potassium sorbate. The generation times for that organism in GS medium and glycerol-salts medium were approximately 75 and 102 minutes, respectively. Sorbate (125, 250 or 500 µg/ml) inhibited the growth of *E. coli* K12 in GS medium (Figure 18) and in glycerol-salts medium (Figure 19). If the inhibition of amino acid uptake by sorbate was the primary mode of growth inhibition in bacteria, then the growth of *E. coli* K12 in GS or glycerol-salts medium should not have been inhibited by sorbate

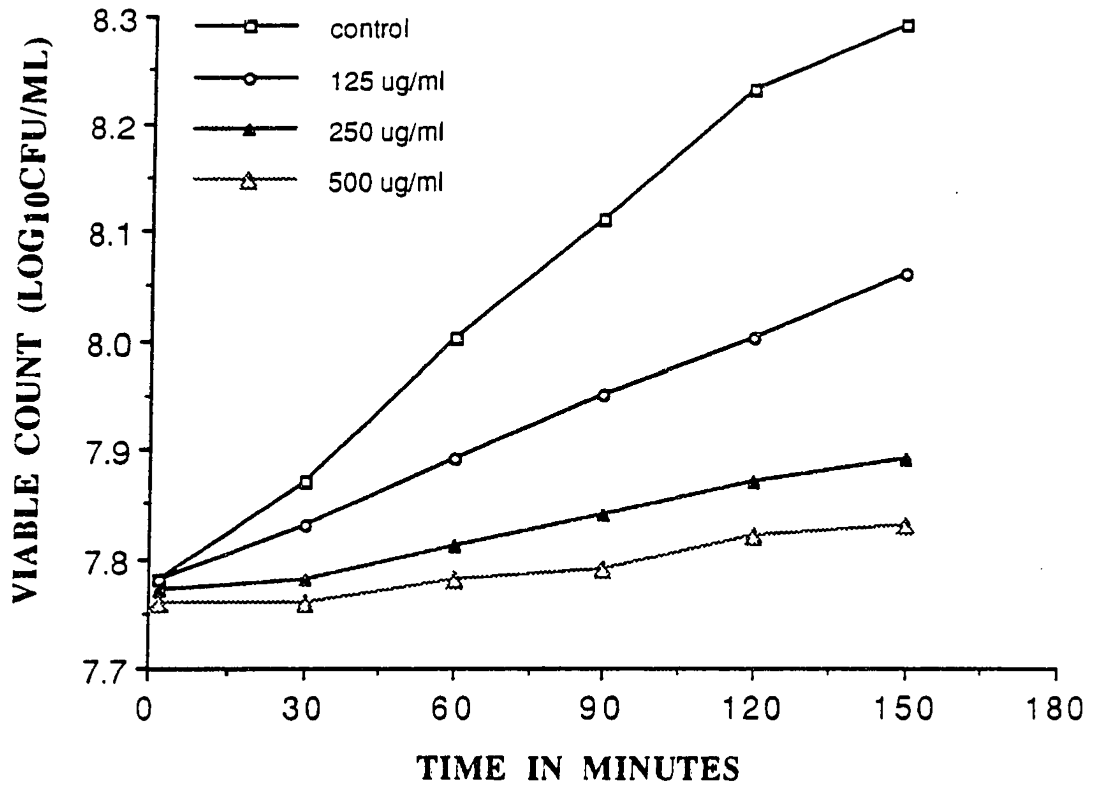

Figure 18. Effect of potassium sorbate on growth of exponential-phase cells of *E. coli* K12 in glucose-salts medium (pH 5.70) at 37°C (Standard error: 0.06)

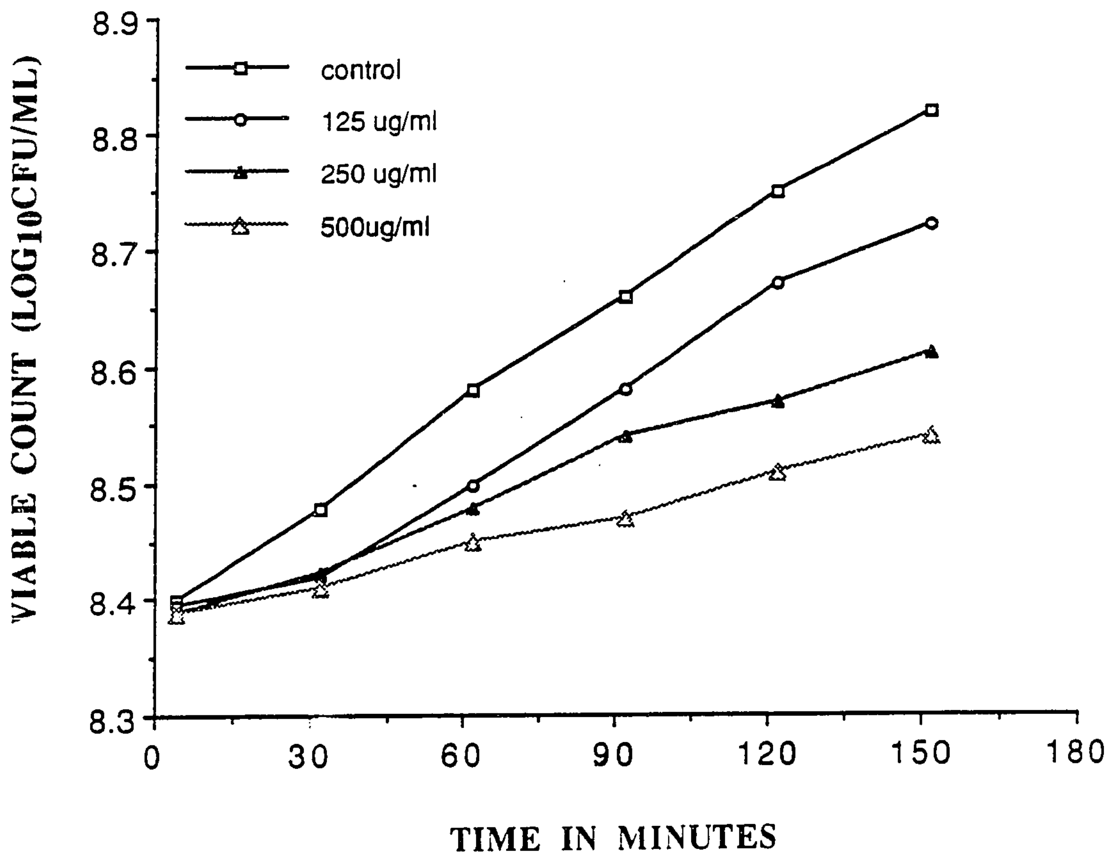

Figure 19. Effect of potassium sorbate on growth of exponential-phase cells of *E. coli* K12 in glycerol-salts medium (pH5.70) at 37°C (Standard error: 0.18)

because no amino acids were present in those media and *E. coli* K12 has the capacity to synthesize its required amino acids. In glycerol-salts medium, the growth of *E. coli* K12 was inhibited by all three concentrations of sorbate used. However, after 30 minutes of incubation, cells treated with the lowest concentration of sorbate (125 µg/ml) seemed to overcome growth inhibition and started to grow at a rate similar to that of the untreated cells (Figure 19). At present no precise explanation can be offered for this observation. Results of earlier studies have indicated that growth inhibition of *B. subtilis* or *E. coli* correlated with the inhibition of membrane transport of amino and keto acids by short-chain lipophilic acids (Sheu et al., 1972; Freese et al., 1973). In those studies, *E. coli* NF161, which was auxotrophic for methionine and arginine, or *B. subtilis* 60015, auxotrophic for methionine and tryptophan, was used as the test organism. The growth medium was either nutrient medium or minimal medium supplemented with the amino acids which the organisms were unable to synthesize. The uptake inhibition of amino acids for which those organisms were auxotrophic may have largely contributed to the growth inhibition of *B. subtilis* and *E. coli* reported in those studies. However, in the present study, the growth inhibition of the prototroph *E. coli* K12 by sorbate in minimal media cannot be explained in terms of amino acid uptake inhibition.

Generally, lipophilic acid food preservatives, such as sorbate, seem to inhibit microbial growth by starving the cells of nutrients, such as amino acids, organic acids and phosphates. Cell starvation occurs via

uptake inhibition of nutrients (Freese and Levin, 1978). High concentrations of acetate or other short-chain fatty acids (C_6 or less) inhibited the uptake of sugars by *Aspergillus nidulans* (Romano and Kornberg, 1969) as well as phosphate uptake by yeast (Samson et al., 1955). In the present investigation, it was assumed that sorbate might have inhibited the growth of *E. coli* K12 in GS medium by interfering with the uptake of glucose and/or inorganic nutrients. Therefore, the effects of sorbate on the uptake of glucose, sulfate and phosphate by *E. coli* K12 in GS medium (pH 5.70) was investigated. This was done by following the uptake of radioisotopic forms of those nutrients by whole cells of *E. coli* K12 over a 2.5-minute period.

The effects of sorbate on the uptake of glucose, by *E. coli* K12 in GS medium (pH 5.70) are presented in Figure 20. The uptake of glucose in *E. coli* K12 remained uninhibited by sorbate (125 μg/ml) for 1.0 minute after exposure of the culture to that concentration of sorbate. Uptake inhibition increased slightly after 1.0 minute. Higher sorbate concentrations (250 or 500 μg/ml) inhibited glucose uptake earlier than 1.0 minute and to a greater extent throughout incubation (Figure 20). Many sugars, including glucose, fructose, mannose, N-acetyl glucosamine and lactose, are transported into the bacterial cell by group translocation (Stryer, 1988). Group translocation is a mechanism that chemically modifies a substrate as that substrate crosses the cytoplasmic membrane. For example, in *E. coli* glucose is modified to glucose-6-phosphate and mannitol to mannitol-1-phosphate by specific

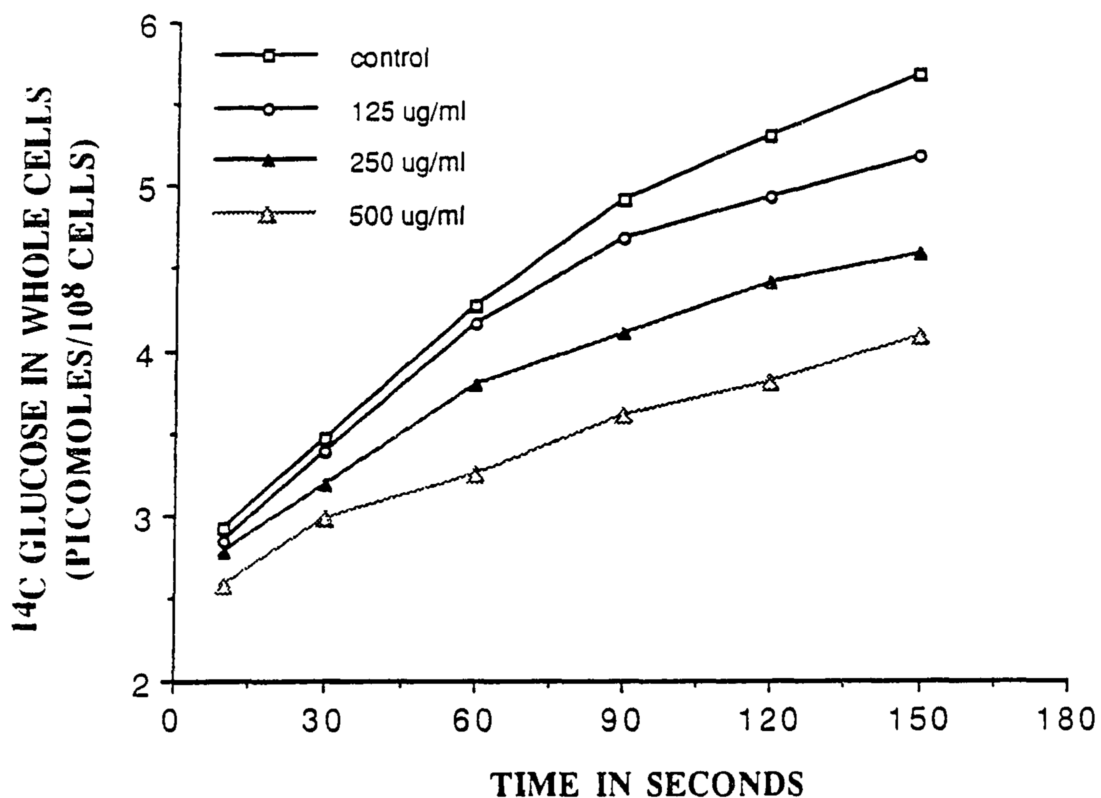

Figure 20. Effect of potassium sorbate on uptake of ^{14}C glucose by exponential-phase cells of *E. coli* K12 in glucose-salts medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate buffered saline (Standard error: 0.20)

phosphotransferase systems (Postma and Lengeler, 1985). Similarly, phosphoribosytransferases, which phosphorylate purine or pyrimidine bases as those nutrients enter the bacterial cell, are considered as group translocation systems (Neidhardt et al., 1990). Generally, glucose enters *E. coli* by the phosphotransferase system. That type of group translocation was first described by Kundig and Roseman (1971), and is present in both Gram-positive and Gram-negative bacteria. In addition high concentrations of glucose can be transported into cells via facilitated diffusion, and glucose is phosphorylated by the enzyme glucoskinase when glucose enters the cell (Freese et at., 1970). Both phosphotransferase system and facilitated diffusion are not active transport systems and do not depend on the protonmotive force as an energy source.

In the present study, the relative lack of inhibition of glucose uptake in *E. coli* K12 by sorbate (125 µg/ml) suggests that the translocation system responsible for the cellular uptake of glucose was only moderately inhibited by low, growth-inhibitory concentrations of sorbate. The same argument may apply to the very weak inhibitory effect of sorbate (125 ug/ml) on the uptake of thymine and uracil in *E. coli* 15TAU and *E. coli* K12 because like glucose, pyrimidine bases enter the bacterial cell by group translocation (Neidhardt et al., 1990). The data presented here confirm those of Eklund (1980), who reported that the inhibitory effect of sorbate (0.1%) on glucose uptake in membrane vesicles of *E. coli* was relatively weak (~20%) compared to the uptake

inhibition (50, 85, and 60%) of the amino acids serine, alanine and phenylalanine, respectively.

The effects of sorbate on the uptake of sulfate and phosphate by *E. coli* K12 are shown in Figures 21 and 22, respectively. All three concentrations of sorbate almost immediately inhihited the uptake of those inorganic nutrients. The percentages of uptake inhibition of glucose, sulfate, and phosphate by sorbate (125 µg/ml) at 2.0 minutes of incubation were 7%, 38%, and 42% respectively. The effects of sorbate on the transport of inorganic nutrients such as sulfate, nitrate and phosphate in bacteria have received only limited attention, unlike the transport of other nutrients such as sugars and amino acids. The electrical potential across the cytoplasmic membrane in bacteria is generally inside-negative; thus, the cellular uptake of anionic nutrients can only be achieved by active transport systems (Lanyi, 1987). Active transport systems for sulfate have been identified in *E. coli* (Ellis, 1964) and *Salmonella* (Pardee et al., 1966). Medveczky and Rosenberg (1970, 1971) described in detail the phosphate transport system in *E. coli* and showed that it was an active process.

Active transport is an energy-driven process by which a membrane-bound carrier (permease) combines with a specific nutrient and transports it inside the bacterial cell. The nutrient, chemically unchanged, is released into the cytoplasm. Some nutrients which are taken up by the bacterial cell via active transport are lactose, amino acids, organic acids and a number of inorganic ions such as sulfate, phosphate, nitrate, and potassium (Maloney, 1987; Lanyi, 1987).

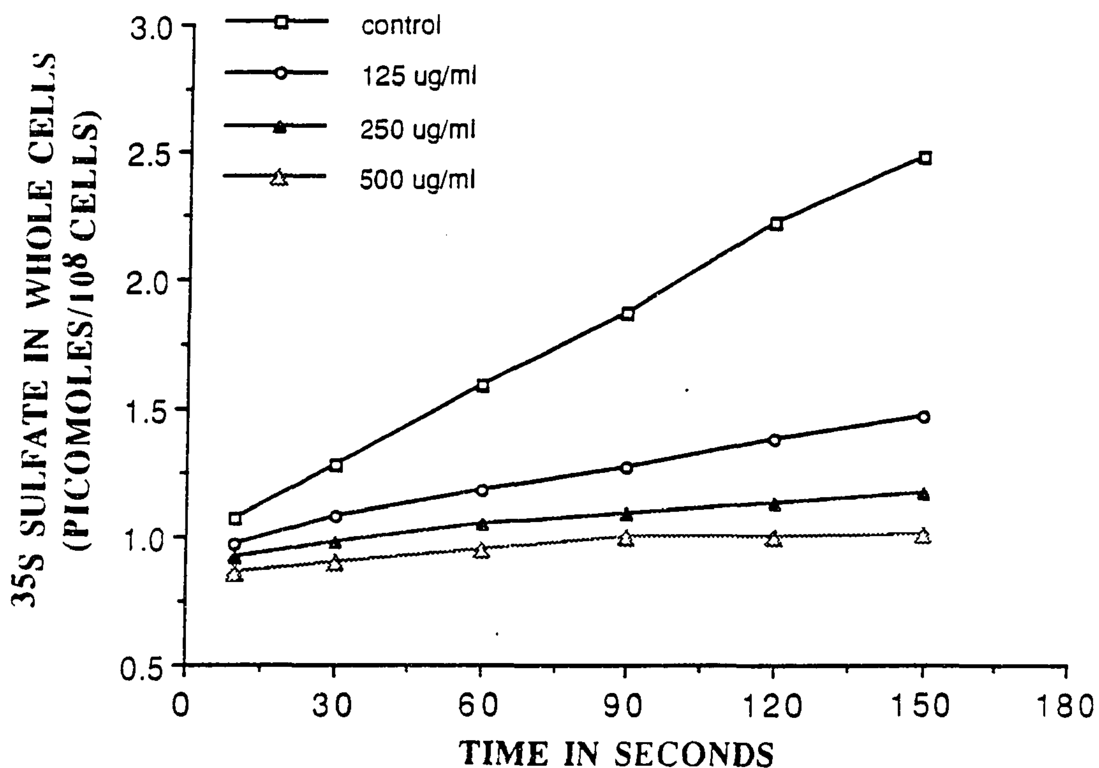

Figure 21. Effect of potassium sorbate on uptake of ^{35}S sulfate by exponential-phase cells of *E. coli* K12 in glucose-salts medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate buffered saline (Standard error: 0.24)

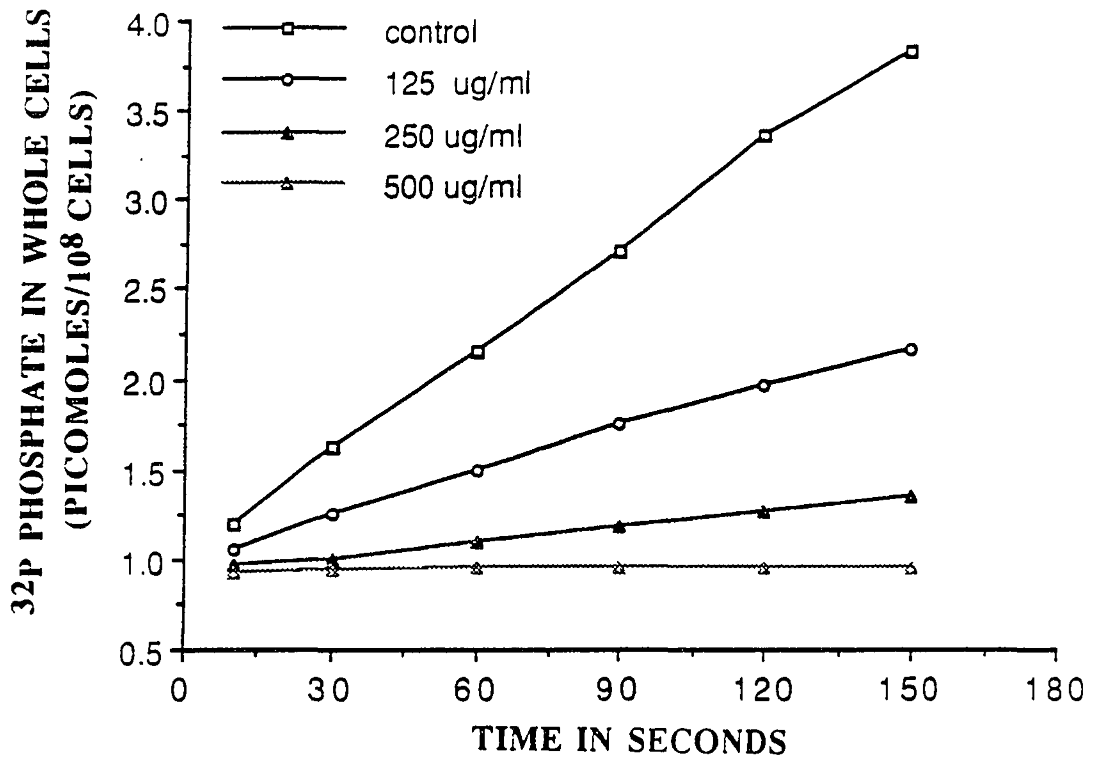

Figure 22. Effect of potassium sorbate on uptake of ^{32}P phosphate by exponential-phase cells of *E. coli* K12 in glucose-salts medium (pH 5.70) at 37°C. Radioactivity was measured in cells washed with phosphate buffered-saline (Standard error: 0.16)

In the present investigation, the uptake of amino acids (arginine and glutamic acid), sulfate, and phosphate by *E. coli* K12 was inhibited earlier and to a larger extent than uptake of glucose or the pyrimidine bases (thymine and uracil). This suggests that sorbate is more inhibitory to the uptake of nutrients which enter the cell via active transport than on the uptake of nutients which enter the cell via group translocation. In a previous study by Sheu et al., (1972), acetate inhibited the uptake of methylglucoside or fructose in whole cells or membrane vesicles (with phosphoenolpyruvate as an energy source for transport) of *B. subtilis* to a lesser extent than the uptake of amino acids. Those same authors suggested that the PEP-phosphotransferase system of sugar transport was less sensitive to acetate than the transport system coupled to the electron transport chain.

Oxygen Consumption

Sorbate was tested for its ability to inhibit oxygen consumption in *E. coli* K12. All three concentrations of sorbate (125, 250, 500 μg/ml) inhibited oxygen consumption by that organism in 0.1M citrate-phosphate buffer (pH 5.70) with glucose as substrate (Figure 23). The percentages of inhibition of oxygen consumption by sorbate concentrations of 125, 250, and 500 μg/ml at 2.0 minutes were 24%, 32% and 38%, respectively.

According to Freese et al. (1973), many weak lipophilic acids, including sorbate (5600 μg/ml), inhibited oxygen consumption in whole cells of *B. subtilis* but did not inhibit NADH oxidation by isolated

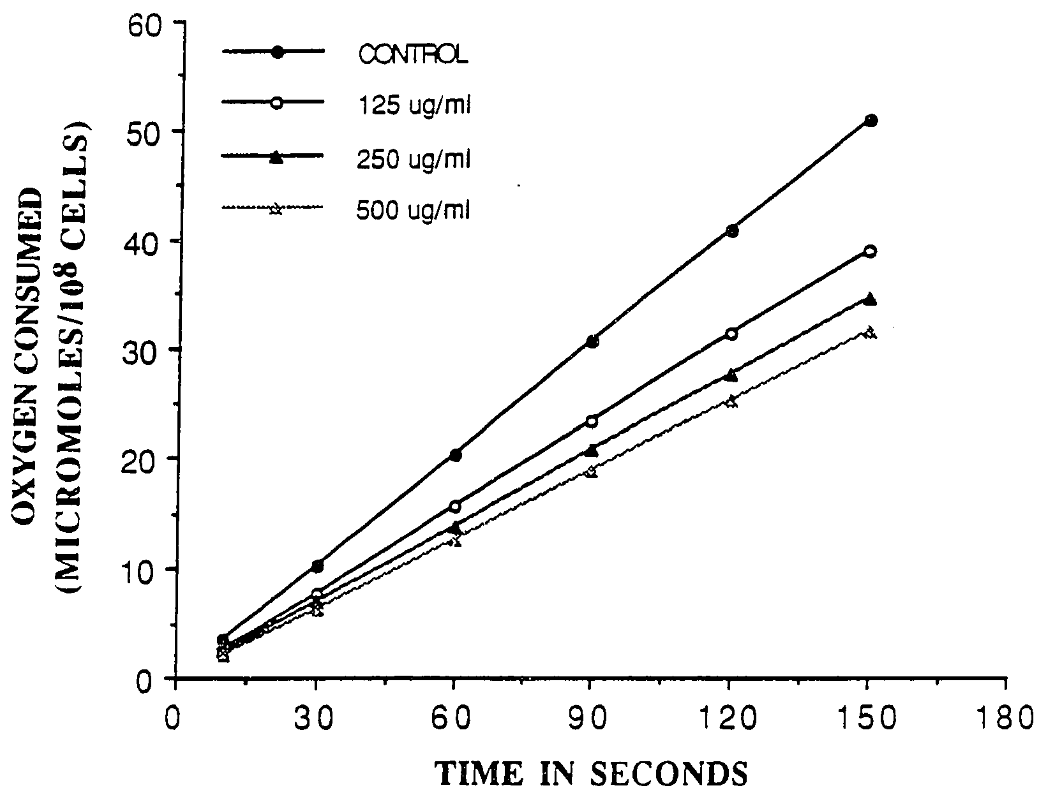

Figure 23. Effect of potassium sorbate on respiration (oxygen consumption) in *E. coli* K12 in citrate-phosphate buffer (pH 5.70) with glucose as substrate (Standard error: 0.02)

membrane vesicles of that same organism. Their interpretation of those findings was that weak lipophilic acids inhibit transport functions that supply reducing compounds to the electron transport chain. In membrane preparations of *B. subtilis*, neither NADH oxidation nor the reduction of cytochromes by NADH were inhibited by short chain (C_1 - C_6) fatty acids that inhibited the growth of *B. subtilis* (Sheu et al., 1972). In the presence of oxidizable energy sources, for example, glycerol phosphate, sorbate (1.0%), inhibited oxygen consumption in isolated membrane vesicles of *E. coli* and in whole cells of the same organism by 58% and 85%, respectively (Sheu et al., 1975). The inhibition of bacterial growth in nutrient media by sorbate and other fatty acids, possibly results from uptake inhibition of amino acids and other compounds whose uptake is energized by electron transport. The low intracellular concentrations of those nutrients can reduce the amount of electrons that pass through the electron transport chain and, consequently, inhibit oxygen consumption (Sheu et al., 1972). In the present study, the glucose added to *E. coli* K12 cells in citrate-phosphate buffer represented the sole carbon and energy source that could be metabolized by the cells. Since sorbate (125 μg/ml) did not initially inhibit glucose uptake in GS medium where glucose was also the only source of carbon and energy for *E. coli* K12, the observed inhibition of oxygen consumption in citrate-phosphate buffer with glucose may have resulted from uptake inhibition of some other nutrient (for example, phosphate) needed for cellular metabolism. Therefore, decreased oxygen consumption may have been a direct consequence of lowered

metabolic rate in the cell. Nose et al. (1982) reported that oxygen consumption in *P. aeruginosa* (with glucose or malate and substrates) was not inhibited by sorbate (0.4%). In an earlier investigation by York and Vaughn (1964), glucose assimilation in *P. aeruginosa* was inhibited by sorbate (0.1%). It is difficult to explain those conflicting results, but differences in experimental conditions such as type of growth medium, pH, and concentration of sorbate used may have contributed to lack of inhibition of oxygen consumption.

Leakage of ^{14}C labeled Cell Material

Because sorbate is lipophilic and inhibited nutrient uptake in *E. coli* more than it inhibited certain other cellular processes, it was assumed that it interfered with the structural integrity of the bilipid layer of the bacterial cytoplasmic membrane. One consequence of disruption of membrane integrity is altered membrane permeability. Figure 24 shows the effect of sorbate on leakage of ^{14}C-labeled intracellular material from radiolabeled cells of *E. coli* K12. Concentrations ranging from 10 µg/ml to 500 µg/ml did not result in increased leakage of ^{14}C-labeled material from labeled cells of *E. coli* K12 in citrate-phosphate buffer (pH 5.70). Throughout the 2.0 hours of incubation the level of radioactivity in the supernatant from each sorbate-treated cell suspension was approximately the same as that from untreated cells. Cells treated with polymyxin B sulfate (15 µg/ml) increasingly released ^{14}C-labeled material during the 2.0 hours

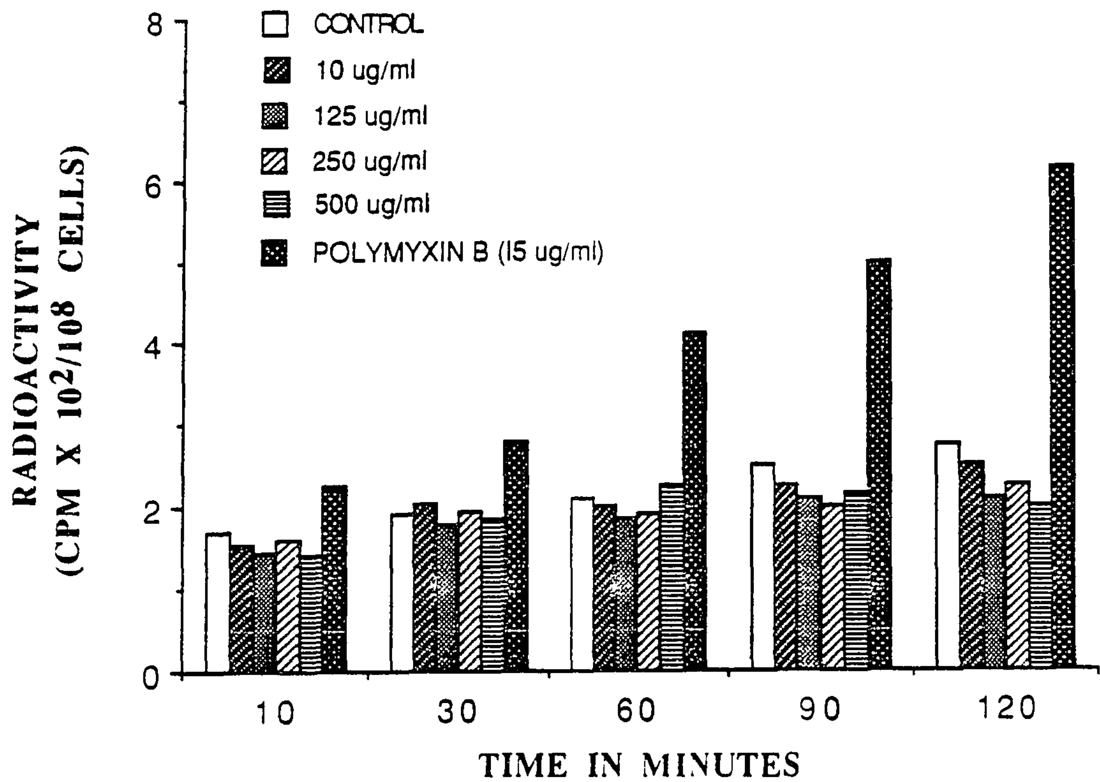

Figure 24. Effect of potassium sorbate on leakage of ^{14}C intracellular material from *E. coli* K12 in citrate-phosphate buffer (pH 5.70) at 37°C (Standard error: 0.35)

of incubation (Figure 24). Polymyxin B (produced by *Bacillus polymyxa*) is a cylic polypeptide which has basic groups and a fatty acid side chain. It causes direct membrane damage by a detergent-like action which results in leakage of intracellular constituents such as nucleotides and inorganic ions.

In previous studies, high concentrations of lipophilic acid food preservatives, including sorbate, increased the porosity of the cytoplasmic membrane and caused death of bacteria and yeasts (Freese and Levin, 1978; Reinhard and Radler, 1981). At pH 7.0, the outer membrane of *Alteromonas putrefaciens* was damaged by 5% sorbate (Statham and McMeekin, 1988). In those studies, the sorbate concentrations that caused membrane damage far exceeded the concentrations of sorbate used in the present investigation. It seems that microbial membrane damage (detected by the efflux of intracellular constituents) occurs primarily in the presence of high sorbate concentrations but not at low sorbate concentrations. In a recent study by Buazzi and Marth (1991), no leakage of 260-280 nm-absorbing intracellular materials occurred when cells of *Listeria monocytogenes* were treated with 0.1, 0.5, or 1.0% sorbate for 30 minutes at 35°C.

Permeability of *Escherichia coli* Cells to Protons

The intrinsic low permeability of the bacterial cytoplasmic membrane to protons averts the rapid pH equilibrium that can occur between the outside and inside of the cell. As a consequence, a pH

gradient is established across that membrane. Mitchell and Moyle (1967) reported that 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) enhanced the passage of protons across mitochondrial and other cell membranes. By employing their procedure in the present study, it was demonstrated that sorbate (125, 250 or 500 µg/ml) facilitated proton translocation into cells of *E. coli* K12. Those cells were suspended in a medium (pH 6.50) which consisted of 50mM KCL, 2mM $MgCl_2$, and 1mM glycylglycine. The same medium devoid of bacterial cells was used as an additional control. When 0.1N HCl was added to that suspension of *E. coli* K12 cells, the pH decreased sharply to about 5.67 and then slowly increased as protons entered the cells. When the pH of the cell suspension reached 5.70, an appropriate amount of sorbate stock solution (pH 5.70) was added to give a final sorbate concentration equivalent to 125, 250, or 500 µg/ml. CCCP (pH 5.70), a known protonophore, was used as a positive control. The addition of sorbate (125 µg/ml) resulted in an immediate rise in pH. The rise in pH of the cell suspension was more pronounced when higher concentrations of sorbate (250 µg/ml, 500 µg/ml) or CCCP (15 µg/ml) were used (Figure 25). However, the addition of sorbate to the no-cell control (pH 5.70) to give sorbate concentrations of 125, 250, or 500 µg/ml, did not change the pH from 5.70. At approximately 1.0 minute after the addition of sorbate (125 µg/ml) to the cell suspension, the pH increased from 5.70 to 5.76. The pH values at that same time for higher concentrations of sorbate (250, 500 µg/ml) and CCCP (15 µg/ml) were 5.80, 5.85 and 6.05, respectively. The increases in pH of *E. coli* cell

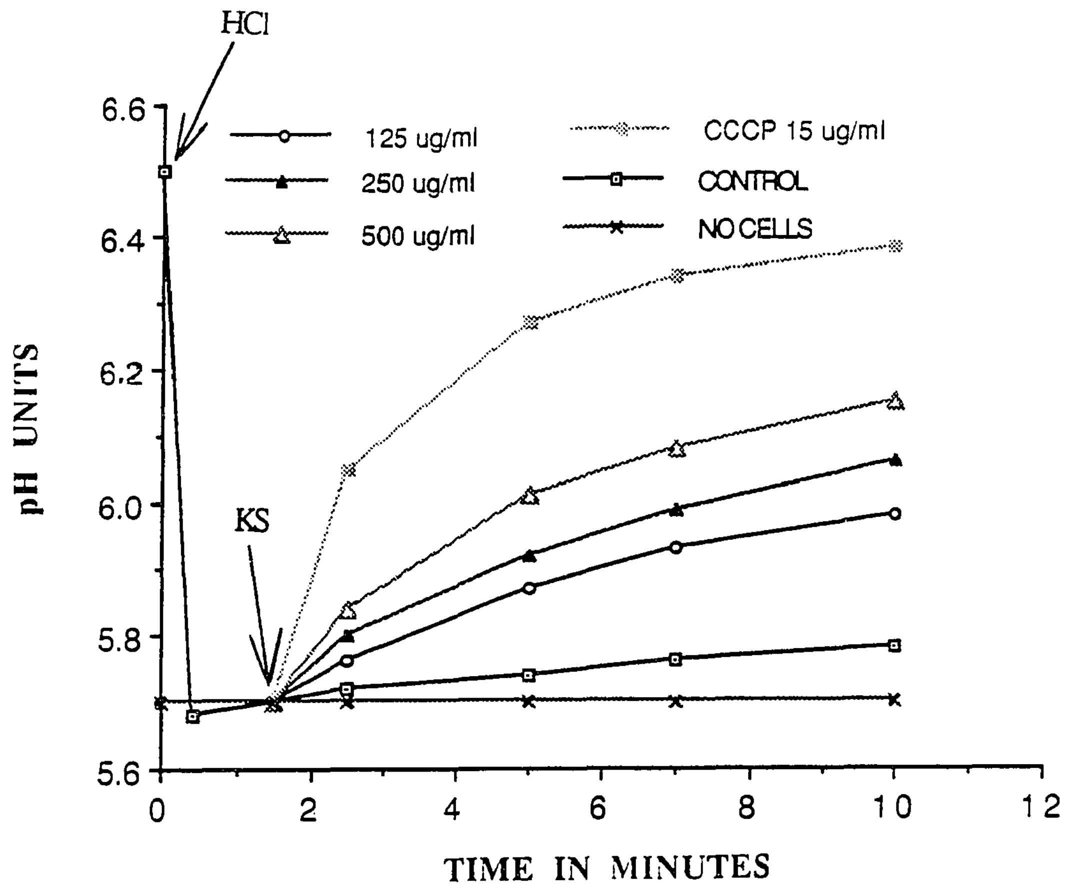

Figure 25. Effect of potassium sorbate on uptake of protons by *E. coli* K12 cells suspended in KCl (50mM), $MgCl_2$ (2mM) glycylglycine (1mM) at 37°C. Each arrow indicates the addition of hydrochloric acid (HCl) or potassium sorbate (KS) (Standard error: 0.02)

suspensions after the addition of sorbate might have resulted from either a rapid influx of protons into the cells or pH equilibrium between the cytoplasm of the cells and the suspending menstrum via increased permeability of the cytoplasmic membrane. *E.coli* is known to maintain its intracellular pH (pH_i) at 7.4 to 7.6 over an extracellular pH (pH_o) range of 5.0 to 9.0 (Padan et al., 1981). Therefore, if sorbate interfered with the cytoplasmic membrane to make it permeable to small ions, it is likely that apart from an influx of protons into the cells, intracellular ions may have freely leaked from the cells to alter the pH of the cell suspension. The results of the present investigation indicate that sorbate facilitates proton translocation in whole cells of *E. coli*; however, no evidence is provided to precisely explain the mechanism by which sorbate causes this action. Mitchell (1966) proposed that a principal feature of oxidative phosphorylation and membrane transport is the separation of hydrogen ions and hydroxide ions on opposite sides of an impermeable membrane. The resulting protonmotive force (proton gradient plus membrane potential) drives the synthesis of ATP during oxidative phosphorylation and also the transport of nutrients by associated exchange diffusion systems. In addition, uncouplers (for example CCCP) facilitate the movement of protons across the membrane, resulting in the destruction of the proton gradient. Based on the results of the present investigation, it is clear that sorbate somehow increases the permeability of bacterial cells to protons. When this happens, the proton gradient, which couples metabolic energy to transport of nutrients across the cytoplasmic membrane, is destroyed. This may

explain why sorbate inhibited the uptake of amino acids, sulfate and phosphate by *E. coli* in the present study. Those nutrients enter the cell via active transport (Anraku, 1980; Pardee et al., 1966; Rosenberg et al., 1979) which is highly dependent on the capacity of the intact cytoplasmic membrane to maintain a proton gradient (Mitchell, 1972). Sorbate (125 µg/ml) did not inhibit the uptake of nutrients (glucose, uracil or thymine) whose transport into the cell is achieved by group translocation which is not directly linked to the protonmotive force.

Salmond et al. (1984) have shown that sorbate (0.01%) lowered the intracellular pH (pH_i) of *E coli* in minimal medium at pH 5.5 by 0.84 pH units. Sorbate-treated (2.0%) cells of *Clostridium sporogenes* PA 3679 had lower pH_is than untreated cells in a defined synthetic medium at pH 7.0 (Ronning and Frank, 1987). The lowering of the cytoplasmic pH of bacterial cells in the presence of sorbate may be the result of an increased concentration of sorbic acid in the cytoplasm or interference by sorbate with the structural integrity of the membrane, thus, allowing extracellular protons to accumulate in the cytoplasm. Sorbate is a lipophilic compound and its undissociated form (sorbic acid) is about three times more soluble in lipids than in water (Gooding, 1955). At pH 5.70, which was used in the present investigation, about ten percent of potassium sorbate is present as sorbic acid (Banwart, 1981). It is likely that sorbate interacted with the bilipid layer of the cytoplasmic membrane to increase its permeability to protons. If the unhindered passage of protons across the membrane of *E. coli* cells actually occurred in the presence of sorbate, one can speculate that

sorbate may have allowed uncontrolled movement of other small ions, such as sodium and potassium, across the cytoplasmic membrane. This action can result in the elimination of ionic gradients other than the proton gradient. Other ion gradients in bacteria can serve as energizers of nutrient transport; for example, the sodium ion (Na^+) gradient has been shown to drive the uptake of melibiose (Stock and Roseman, 1971; Tsuchiya et al., 1982), glutamate (Tsuchiya et al., 1977) or proline (Stewart and Booth, 1983) in *E. coli*. Generally, the elimination of ion gradients, including the proton gradient, could result in destruction of the proton gradient as well as the membrane potential, both of which are components of the protonmotive force.

Eklund (1980) suggested that the destruction of the pH gradient alone might be inadequate to totally inhibit nutrient uptake by bacteria. The basis for the suggestion was that the membrane potential can energize the uptake of several nutrients (Ramos and Kaback, 1977). Eklund (1985) investigated the effects of sorbate and esters of p-hydroxybenzoic acid on the protonmotive force in membrane vesicles of *E. coli* in potassium phosphate buffer (pH 6.6). Sorbate (0.1% and 1.0%) completely neutralized the pH gradient; the membrane potential was inhibited to a lesser extent. About two-thirds and one-third of the initial membrane potential remained in vesicles treated with 0.1% and 1.0% sorbate, respectively. Eklund (1985) also concluded that inhibition of nutrient uptake by sorbate, although important, cannot be the only mechanism of action of sorbate because the membrane potential which

remained after sorbate treatment of membrane vesicles could still energize the uptake of nutrients.

When the transmembrane proton gradient is lowered, the bacterial cell strives to maintain its protonmotive force by increasing the membrane potential (Rottenberg, 1975; Salmond et al., 1984) and the membrane potential alone can drive the uptake of some nutrients, including certain amino acids (Ramos and Kaback, 1977). Niven and Hamilton (1974) concluded that basic, neutral and acidic amino acids behaved as cationic, uncharged and anionic substrates, respectively, with respect to their uptake by bacterial cells. Those same groups of amino acids accumulated in *S. aureus* in response to: (a) the membrane potential, inside negative (b) the total protonmotive force; and (c) the transmembrane pH gradient, inside alkaline.

The effects of potassium sorbate on the uptake of glutamic acid and arginine by *E. coli* were determined under identical conditions in the present study. The lowest growth-inhibitory concentration of sorbate used (125 ug/ml) inhibited the uptake of glutamic acid and arginine by *E. coli* K12 by 40% and 32%, respectively (Table 6). In *E. coli* 15TAU that same concentration of sorbate inhibited the uptake of glutamic acid and arginine 39% and 33%, respectively (Table 5). If sorbate inhibited the transmembrane pH gradient alone, the uptake of glutamic acid should have been inhibited to a larger extent than that of arginine because glutamic acid is an acidic amino acid and depends on the pH gradient for entry into the cell. The membrane potential energizes the cellular uptake of basic amino acids such as arginine and

lysine (Niven and Hamilton, 1974); therefore, one would expect the uptake of arginine to be stimulated as a result of a cellular increase in membrane potential to compensate for the reduction of the pH gradient.

In the present study, the difference in the uptake inhibition of glutamate and arginine by sorbate (125 µg/ml) was 8% in *E. coli* K12; in *E. coli* 15TAU, that difference was 6%. Since those differences are relatively small, the following assumption seems appropriate. In the present study, the transmembrane pH difference was probably inhibited slightly more than the membrane potential. However, the remaining membrane potential might have been insufficient to energize the uptake of arginine in sorbate-treated whole cells. The relatively small difference in uptake inhibition of glutamate and arginine in *E. coli* by sorbate would suggest that sorbate lowered both the proton gradient and the membrane potential to extents that diminished their capacity to energize the transport of nutrients that enter the cell via active transport.

GENERAL SUMMARY AND DISCUSSION

Concentrations of sorbate used commercially (0.05%-0.3%) are normally bacteriostatic. During bacteriostasis, cells stop multiplying but remain viable. Therefore, it is logical to expect that sorbate primarily inhibits some metabolic function vital to bacterial growth. If bacterial cells are prevented from synthesizing cellular macromolecules, growth would be inhibited. This concept was used as the basis for first investigating the effects of sorbate on growth and macromolecular synthesis in *E coli*. Sorbate is a lipophilic compound and may permeate the bilipid layer of the bacterial cytoplasmic membrane. Interaction of sorbate with that membrane may result in the interference of membrane-associated cellular functions that are crucial to bacterial growth. This concept formed the basis for investigating the effects of sorbate on nutrient transport, respiration (oxygen consumption), cell leakage, and proton conduction in *E. coli*.

The growth-inhibitory concentrations (125, 250, and 500 ug/ml) of sorbate used in the present study were subbacteriostatic concentrations. The reason for using those concentrations was that metabolic effects which occur at growth-inhibitory sorbate concentrations much lower than concentrations which result in bacteriostasis normally represent cellular sites that are very sensitive to sorbate. Those metabolic effects might give a better indication of the nature of the primary lesion. The use of high concentrations of almost any antimicrobial agent can result in an array of metabolic effects,

many of which are secondary lesions that tend to obscure the primary growth-inhibitory action of the test agent.

In the present study, sorbate (125, 250 or 500 ug/ml) rapidly decreased the growth rate of exponential-phase cells of *E. coli.* Those same sorbate concentrations strongly inhibited RNA synthesis in *E. coli.* It is likely that sorbate-treated bacteria experience a "shift-down" type of change in their metabolism. RNA synthesis is known to be very sensitive to that kind of change. In a "shift-down" metabolic state, bacterial cells decrease their rate of macromolecular synthesis (particularly RNA synthesis). This condition can be achieved by transferring cells from a nutrient-rich medium to a poorer medium (Maaloe and Kjeldgaard, 1966). It seems that the presence of sorbate caused bacterial cells to behave as if they were shifted to a nutrient-poor medium. In the present study, sorbate inhibited the growth of *E. coli* 15TAU and *E. coli* K12 in a defined synthetic medium. This suggests that sorbate restricted the uptake or assimilation of nutrients from that medium. Restricted cellular uptake of nutrients by sorbate may explain the decrease in respiration (oxygen consumption) reported in the present investigation because Freese et al. (1973) discovered that sorbate (50mM) inhibited oxygen consumption in whole cells of *B. subtilis* but did not inhibit NADH oxidation by isolated membrane vesicles of that same organism. Therefore, the inhibition of oxygen consumption by sorbate in *E. coli* in the present study may be attributed to a decrease in the cellular metabolic rate via inhibition of nutrient uptake from the growth medium.

The inhibition of uptake of amino acids (glutamic acid and arginine) and inorganic nutrients (sulfate and phosphate) occurred earlier and to a greater extent than inhibition of macromolecular synthesis in sorbate-treated cells of *E coli*. It is a well known fact that restricted uptake of essential nutrients in bacteria secondarily inhibits synthesis of cellular macromolecules. As a consequence, growth and multiplication decrease. Therefore, inhibition of RNA synthesis in *P. fluorescens* (Nose, 1982) and in *E. coli* in the present study may not be the primary lesion that resulted in growth inhibition by sorbate. Inhibition of RNA synthesis in sorbate-treated bacteria may be explained by the stringent response phenomenon.

The stringent response in bacteria is triggered whenever their growth rate is inhibited by almost any sort of nutritional restriction, for example: a change from a rich to a minimal medium, starvation of an amino acid auxotroph for its needed amino acid; a change to a poor carbon and energy source; or a shift from a rich to a poorer nitrogen source (Neidhardt, 1990).

Ronning and Frank (1987) concluded that a stringent-type regulatory response in *C. sporogenes* PA 3679 treated with 200mM sorbate was responsible for growth inhibition of that organism. Those same authors stated that the stringent response in *C. sporogenes* PA 3679 was induced by the protonophoric activity of sorbic acid.

In the present study, relatively low growth inhibitory concentrations of sorbate (125, 250, or 500 μg/ml) at pH 5.70 increased the permeability of *E. coli* K12 cells to protons and strongly inhibited

the cellular uptake of glutamic acid, arginine, phosphate and sulfate. In contrast, the uptake of glucose, thymine or uracil, not directly associated with a transmembrane ion gradient, was only slightly inhibited. These results suggest that the growth-inhibitory action of sorbate in *E. coli* is linked mainly to inhibition of active uptake of nutrients. The growth of both aerobic and anaerobic bacteria is inhibited by sorbate (Sofos, 1989). Both groups of bacteria depend on the energy provided by the protonmotive force and its components (the pH difference and membrane potential) for uptake of several nutrients, including amino acids.

The membrane potential is created by the separation of ions by the cytoplasmic membrane to form a charge difference across the membrane, inside negative. The cellular uptake of basic amino acids, such as arginine and lysine, is driven by that charge difference. Amino acids, such as glutamic acid and aspartic acid, depend only on the transmembrane pH difference for entry into the cell. In the present study, sorbate inhibited the uptake of glutamic acid and arginine; thus, sorbate probably interfered with both the pH difference and the charge difference across the cytoplasmic membrane of *E. coli*. Generally, sorbate seems to interfere with the transport functions of microorganisms, particularly active transport (Sheu and Freese, 1972; Freese and Levin, 1978; Eklund, 1980). This action by sorbate may be attributed to an increase in the transmembrane movement of protons and other small ions. The selective separation of ions on opposite sides of the cytoplasmic membrane contribute to the pH gradient and the

charge difference both of which energize the active uptake of metabolites by the cell. The results of the present investigation indicate that sorbate increased the permeability of *E. coli* cells to protons. It is suggested, therefore, that the destruction of the proton gradient (and possibly other ion gradients) represent the dominant antimicrobial effect of sorbate in *E. coli*, resulting in severely reduced capacity of the cytoplasmic membrane to function in the active transport of nutrients crucial to growth of the microbial cell.

POTENTIAL AREAS FOR FUTURE RESEARCH

Based on the results of the present investigation, the following seem to be potential areas for future research to elucidate the mechanism by which sorbate inhibits microbial growth.

Cellular Localization of Sorbate

It is widely assumed that sorbate enters the bacterial cell in its undissociated form (sorbic acid). Sorbic acid probably accumulates in the cell to cause growth inhibition via acidification of the cytoplasm. Results of the present investigation indicate that sorbate increases the permeability of *E. coli* cells to protons. It is not clear whether sorbate incorporates into the cell membrane and increases its porosity to protons or serves as a source of protons itself by entering the cytoplasm as sorbic acid. Therefore, it is necessary to determine where sorbate is predominantly localized in sorbate-treated cells. This can be done by treating bacterial cells with radiolabeled sorbate and measuring the radioactivity in the cell fractions (membrane, cell wall, and cytoplasm).

Effect of Sorbate on Ion Gradients

In this study it is suggested that sorbate inhibits bacterial growth by destroying the proton gradient and possibly other ion gradients. No evidence is presented to directly show that ion gradients (other than the proton gradient) across the cell membrane are diffused in sorbate-treated cells.

Bacterial cells can be preloaded with a radiolabeled potassium, washed, and then treated with growth-inhibitory concentrations of sorbate. The measured radioactivity of the supernatants from suspensions of treated and untreated cells could give useful information with regard to the effect of sorbate on the ability of the bacterial cell to effectively maintain ion gradients across the cytoplasmic membrane.

Effect of Glycerol on Sorbate Action

The antimicrobial effectiveness of sorbate in the presence of glycerol warrants further investigation. The results of this study indicate that *E. coli* K12 cells treated with sorbate (125 μg/ml) seem to eventually overcome growth inhibition when grown in a glycerol-salts medium. No explanation is offered for this observation. However, it was reported that the presence of glycerol in sorbic acid solutions accelerates the degradation of sorbic acid (Arya, 1980). Sorbate might be interacting with glycerol to lower the concentration of free sorbate necessary to exert a marked growth-inhibitory effect on cells growing in the presence of glycerol.

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