Growth Inhibition of Putrefactive Anaerobe 3679 Caused by Stringent-Type Response Induced by Protonophoric Activity of Sorbic Acid†

IRENE E. RONNING AND HILMER A. FRANK*

Department of Food Science and Human Nutrition, University of Hawaii, Honolulu, Hawaii 96822

Received 2 September 1986/Accepted 12 February 1987

The inhibitory effects of potassium sorbate on the bioenergetics, phenylalanine uptake, protein synthesis, and certain aspects of cell regulation were examined in putrefactive anaerobe 3679. Undissociated sorbic acid appeared to act as a protonophore by lowering the intracellular pH and dissipating the proton motive force of the membrane. Sorbate inhibited the uptake of phenylalanine, decreased the rate of protein synthesis, and altered patterns of phosphorylated nucleotide accumulation, resulting in increased intracellular concentrations of GTP, ppGpp, and an unidentified compound (possibly pppGpp). The addition of a noninhibitory amount of tetracycline released the inhibition of growth by sorbate. Based on these results, we concluded that the inhibition of putrefactive anaerobe 3679 by sorbate resulted from a stringent-type regulatory response induced by the protonophoric activity of sorbic acid.

Sorbic acid, a weak lipophilic acid, is widely used as a food preservative. Although several studies have shown that potassium sorbate inhibits the growth of Clostridium botulinum (41, 42, 50–53), the mechanism of action has remained uncertain.

Based on the ability of sorbate to inhibit amino acid uptake in Penicillium chrysogenum (20) and in whole cells and vesicles of Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa (11), the authors of these studies postulated that sorbic acid could act as a protonophore. Being a weak lipophilic acid, undissociated sorbic acid could diffuse through the cell membrane, ionize in the more alkaline cell interior, and increase the intracellular proton concentration. This process could dissipate the transmembrane proton gradient, one component of the proton motive force (PMF) that energizes transport of compounds such as amino acids. In fact, studies have shown that sorbic acid decreased the internal pH in cells (45) and vesicles (12) of E. coli and that the PMF across the vesicular membrane decreased when the concentration of this food preservative was increased. To date, the protonophoric activity of sorbic acid has not been demonstrated in the clostridia. Furthermore, a relationship between the protonophoric activity of sorbic acid and growth inhibition has not been established for any bacterial species.

Using a strain of putrefactive anaerobe 3679 (PA 3679), a proteolytic species of Clostridium (probably C. sporogenes), we studied the protonophoric activity of sorbic acid and its effect on growth. However, to measure accurately the PMF in PA 3679, it is necessary to calculate the proton gradient (ΔpH) and the membrane potential (V) simultaneously in the same cells; but this is impossible with available methods. Also, current methods used to measure membrane potential are questionable because they require alteration of the test conditions that also can affect the PMF itself (9, 13, 28, 43). Since the rate of bacterial motility is directly correlated with the PMF (2, 3, 18, 25, 27, 48), we elected to use relative rates of motility to measure the magnitude of the PMF in sorbate-treated PA 3679 cells.

Prior studies with E. coli amino acid auxotrophs have led to the discovery of an intracellular mechanism which can help certain bacteria survive conditions of limited amino acid availability (6, 17). This mechanism, known as the stringent response, involves a series of major intracellular adjustments which can result in the cessation of replication without causing cell death. Because sorbate has a bacteriostatic effect in foods, we thought that this regulatory mechanism also might be involved in the inhibition of PA 3679 cells by potassium sorbate.

MATERIALS AND METHODS

Preservatives. Potassium sorbate and sodium benzoate were purchased from Calbiochem (San Diego, Calif.). Organism, media, and culture conditions. The bacterium used was PA 3679 strain L, obtained originally from E. S. Wynne and studied by Campbell and Frank (5). Vegetative cells were grown in a modification of the medium of Uchara et al. (57). A solution containing 6% Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and 0.1% glucose was autoclaved (121°C, 20 min) and preincubated anaerobically at ambient temperature for 1 or 2 days in an anaerobic incubator (National Appliance Co., Portland, Oreg.) with a 99.9% nitrogen atmosphere and a beaker containing a pyrogallol-sodium carbonate solution to ensure the reduced state of the medium. Immediately before inoculation, 0.05% L-cysteine hydrochloride (U.S. Biochemical Corp., Cleveland, Ohio) was added, the pH was adjusted to 7.0 (unless indicated otherwise), and the medium was sterilized through a 0.45-μm membrane filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.).

 Colonies counts were obtained by the pour plate method with a solid medium consisting of 6% Trypticase, 0.1% glucose, 1.5% agar (Difco Laboratories, Detroit, Mich.), and 0.15% L-cysteine hydrochloride. The pH of the medium was adjusted to 6.5 but increased to 7.0 during autoclaving. The medium was stored at room temperature in 350-ml samples.
in 500-ml screw-cap bottles kept tightly sealed until the medium was used.

Unless indicated otherwise, the chemically defined medium used in this study (Table 1) was a slight modification of that described by Campbell and Frank (5). Stock solutions and the complete medium were sterilized with 0.22-μm membrane filters (Nalgene). Amino acids and vitamins were purchased from U.S. Biochemical Corp., except l-alanine and l-histidine which were purchased from Calbiochem.

All cultures were incubated at 34°C under anaerobic conditions as described above.

Active vegetative cell inocula were prepared by adding 1 drop of a fresh spore culture to 10 ml of liquid medium, heat shocking the suspension for 20 min at 80°C, and incubating the tube anaerobically for 18 h at 34°C.

Fresh spore cultures were prepared by a modification of the technique of Rowley and Feeherry (44). One drop of a stock spore suspension was added to 10 ml of liquid medium, heated at 80°C for 20 min, and incubated anaerobically about 2 days at 34°C to allow sporulation. A 0.1-ml sample of this suspension was added to 100 ml of liquid medium, heated for 20 min at 80°C, and incubated anaerobically overnight. The resulting active vegetative culture was added entirely to 1.5 liter of liquid medium and incubated for 2 or 3 days until all cells had sporulated, as determined by microscopic observation.

The spore suspension was centrifuged (15,000 × g, 30 min, 5°C) and washed twice with cold (5°C), sterile, distilled water, suspended again in cold water, and stored at 5°C for 2 days. The spores were centrifuged, washed four times with cold water, washed, stored, and incubated at 5°C, and centrifuged (4,000 × g, 10 min, 5°C) to remove sporangial debris. The preparation was washed once, centrifuged (15,000 × g, 30 min, 5°C), and suspended in 150 ml of a fresh, cold, filter-sterilized enzyme solution (100 μg of lysozyme [Calbiochem] per ml and 50 μg of trypsin [Calbiochem] per ml in 0.05 M K₂HPO₄, pH 8.1), and kept overnight at 5°C to digest remaining tracts of sporangia. The spores were centrifuged (15,000 × g, 30 min, 5°C), washed twice, and suspended in cold water at a concentration of about 2 × 10⁸ cells per ml (direct microscopic count), in sterile, screw-cap culture tubes and stored at 5°C until used. The spore suspensions consisted entirely of highly refractile, dormant spores.

**Viability tests.** Sterile liquid medium (9.9 ml) was inoculated with 0.1 ml of vegetative cell suspension at a final concentration of 3 × 10⁵ cells per ml (microscopic count). The inoculated medium, with or without test substances, was incubated anaerobically, and at designated times duplicate samples were removed for colony counts in the agar medium.

**pH.** The intracellular pH (pHi) was determined by the dimethyloxazolidinedione uptake method (58). A 30-ml sample of vegetative cell culture was centrifuged (15,000 × g, 15 min) and suspended to a final concentration of 2 × 10¹⁰ cells per ml in fresh medium containing 10 mM unlabeled sorbitol (pH 7) to prevent non-specific binding of labeled sorbitol. A 0.2-ml sample of the concentrated cell suspension was added to 1.8 ml of fresh medium containing 10 mM unlabeled sorbitol, with and without potassium sorbate (final concentration of 200 mM, pH 7), and incubated for 10 min in a water bath shaker. Incubations and manipulations were done at 27°C. [³H]Sorbitol and [¹⁴C]dimethyloxazolidinedione (0.05 μCi/ml; New England Nuclear Corp., Boston, Mass.) were added to the suspensions and incubated for 1 min. Duplicate 0.1-ml samples were taken and put into scintillation vials for radioassay. Duplicate 0.2-ml samples also were removed and filtered through 3-mm 0.45-μm, dry membrane filters (Millipore Corp., Bedford, Mass.). The filter membranes were placed in scintillation vials and allowed to dry. Aquasol-2 (10 ml; New England Nuclear) was added to each vial, and the samples were assayed for ³H and ¹⁴C in a Packard Tri-Carb 4640 liquid scintillation system (Packard Instrument Co., Inc., Rockville, Md.). Intracellular pH values were calculated according to the method described by Maloney et al. (24).

**Motility as an indicator of PMF.** Cell suspensions were prepared as for pH determinations, except that incubation was extended to 20 min and no radiolabeled compounds were added. A small sample of cells was placed on a slide, covered with a glass slip, and examined microscopically with...
phase contrast optics. Motility rates observed for bacteria moving in a straight line were graded on a scale of 0 (nonmotile), 1 (slow moving), 2 (moderately motile), and 3 (highly motile). Untreated cells at pH 7 were arbitrarily assigned a motility rate of 3. Nonmotile cell suspensions also were tested for viability (plate counts) to determine whether a lack of motility indicated that the cells were dead.

**Amino acid uptake and protein synthesis.** A 100-ml sample of an overnight vegetative cell culture was centrifuged (15,000 × g, 15 min), washed three times, and suspended for 30 min in defined medium without phenylalanine to starve the cells for that amino acid. The cells were centrifuged again and suspended (5 × 10⁸ cells per ml) in phenylalanine-deficient medium; 2.5 ml of this inoculum was added to flasks containing 22.5 ml of phenylalanine-deficient medium (pH 7), with and without preservatives, and incubated for 10 min at 34°C. [³H]phenylalanine (0.17 μCi/ml; New England Nucleon) was added to the suspensions which were swirled to ensure thorough mixing. After 12 s, 1-ml samples were removed and filtered through 0.45-μm membranes (Milli-pore). To determine whether labeled phenylalanine within the cells was incorporated into protein or transfer RNA, another 1-ml sample was taken at 13 to 14 s, added to 1 ml of cold 10% trichloroacetic acid, homogenized at 4°C for 30 min, and filtered. Immediately after filtration, the whole or trichloroacetic acid-treated cells were washed with 10 ml of phenylalanine-deficient medium or 5% trichloroacetic acid, respectively. After the filters were dried in scintillation vials, 10 ml of Aquasol-2 was added, and the samples were radioassayed for [³H] in a Packard Tri-Carb 4640 liquid scintillation system.

To determine the relative rates of protein synthesis in treated and untreated PA 3679, the cells were prepared as described above, except that the final concentration of [³H]phenylalanine was decreased to 3.3 × 10⁻³ μCi/ml and samples were taken at 0, 5, 10, 15, 20, and 25 min.

Possible catabolism of the [³H]-amino acid was checked by the method of Karl et al. (22). Samples of the test suspensions containing labeled phenylalanine were dried and assayed for [³H], and the counts were compared with those of hydrated samples. Volatile, tritiated products of catabolism are lost to the atmosphere during drying and result in lower radioactive counts.

**Nucleotide determinations.** A 100-ml suspension of vegetative cells was centrifuged (15,000 × g, 15 min), washed three times, and suspended at a concentration of 5 × 10⁸ cells per ml in low-phosphate (1 mM), chemically defined medium (pH 6.85). Three milliliters of the suspension was added to each of two 50-ml Erlenmeyer flasks containing 27 ml of low-phosphate defined medium and [³²P]carrier free; New England Nucleon) for a final concentration of 7.5 and 15 μCl/ml. After incubation for 10 min in a water bath shaker (34°C), a 5-ml sample was removed from each flask and added to tubes containing 0.5 ml of cold 10 M H₃PO₄ to extract nucleotides and dilute the remaining excess [³²P] (21). Immediately after sampling, potassium sorbate was added to one flask (final concentration, 200 mM), thereby increasing the pH to 7, and a drop of dilute NaOH was added to the second flask to raise its pH to 7. The bacteria were then reincubated in a water bath at 34°C and sampled as described above at 5, 10, 15, and 20 min. Immediately after the sample was taken at 20 min, a small sample from each flask was examined microscopically for direct cell counts. The acid-treated samples were stored overnight at 4°C.

Sorbic acid precipitates at very low pH. Since the differences between untreated and sorbate-treated PA 3679 cells could have been due to interference by the precipitate in the purification procedure, sorbate was added (200 mM) to all tubes of cell extract without sorbate. After 1 h at 34°C all cell extracts were filtered through glass fiber filters (GF/F; Whatman Inc., Clifton, N.J.) to remove precipitated sorbic acid, and the filters were washed once with 5 ml of 1 M HP₃PO₄. The extracts were purified and concentrated by the method of Cashel et al. (7). Fifty milligrams of charcoal was added to each tube of extract and mixed vigorously for 5 min at room temperature to allow adsorption of the nucleotides, centrifuged, and washed twice with 0.01 M HCl. The nucleotides were eluted from the charcoal by suspending the particles in 10 ml of ethanol-H₂O-NH₄OH (150:80:1) and mixing vigorously for 30 min at room temperature. The charcoal was removed by filtering the mixture through glass fiber filters and washing the filters once with 2 ml of the ethanol-H₂O-NH₄OH mixture. The purified extracts were dried in a vacuum evaporator, dissolved in 100 μl of distilled water, and stored at −20°C.

The nucleotides were separated by a two-step, one-dimensional separation method (34) on 20- by 20-cm plastic-backed sheets coated with polyethyleneimine-impregnated cellulose (MN 300 PEI; Brinkmann Instrument Co., Westbury, N.Y.). Samples (10 or 20 μl each) of the extracts were applied 2 cm from the bottom edge of the plate along with 8 μl each of 2.5-mg/ml unlabeled ATP, GTP, and ppGpp (Sigma Chemical Co., St. Louis, Mo.) as UV markers. After the applied samples were dried with cool air, the plates were washed in two consecutive distilled water baths (5 min each) to remove salts and residual [³²P], and once in methanol (2 min) to dehydrate the cellulose layer. The plates were air dried at room temperature and developed in 0.2 M formic acid to the top of the plate to remove residual [³²P], while leaving the triphosphates and tetraphosphates at the origin. The plates were washed twice in distilled water, once in methanol, and air dried before final development in the same direction with 1.5 M potassium phosphate, pH 3.4 (7). Both ascending thin-layer chromatography developments were done at room temperature in closed glass tanks. Each development was completed in about 2 to 2.5 h. The developed plates were washed twice in distilled water and once in methanol for rapid air drying. The ATP, GTP, and ppGpp spots were located by using short-wavelength UV light and circled with a soft lead pencil. The spots and the areas between them were cut out separately and placed in scintillation vials. The nucleotides were eluted from the polyethyleneimine-cellulose with 1 ml of 0.7 M MgCl₂ (21) and shaken vigorously for 1 h at room temperature. A 10-ml sample of Aquasol-2 was added to each vial, and the samples were radioassayed for [³²P] with a Packard Tri-Carb 4640 liquid scintillation system.

**RESULTS AND DISCUSSION**

**Protonophoric activity of sorbic acid.** (i) Effects of undissociated sorbic acid on growth. Prior studies have shown that the antimicrobial activity of sorbic acid increases with decreasing pH, suggesting that the active component is the undissociated acid (19, 23, 26, 41, 42, 51, 52). In the present investigation, viability studies were used to measure the inhibitory effects of potassium sorbate on growth of PA 3679 at three concentrations of undissociated acid. Inhibition of PA 3679 by sorbate corresponded to the concentration of undissociated acid (Fig. 1).

(ii) pH₇. Previous studies have shown that sorbate can reduce the pH₇ of E. coli cells (45) and vesicles (12). The effect of potassium sorbate on the pH₇ of PA 3679 was
studied at an extracellular pH (pHₐ) of 7.0 by using the intracellular concentration of dimethyloxazolidinedione to determine pHᵢ. In each of eight trials, PA 3679 cells treated with potassium sorbate had lower pHᵢ than untreated cells (Table 2).

(iii) PMF. The ΔpH and the membrane potential (Ψ) are both components of the PMF, which provides energy for cellular activities such as bacterial motility (2, 3, 18, 25, 27, 48) and active transport of various essential compounds such as amino acids (1). However, if the proton gradient decreases, a cell can maintain its PMF by increasing the Ψ (43, 49). Therefore, the effect of sorbate on the PMF of PA 3679 was examined by measuring changes in motility rate. The rate of motility decreased as the concentration of undissociated acid was increased, and a complete loss of motility occurred at 11 mM undissociated acid (Table 3), indicating that sorbic acid had dissipated the PMF. Acidity per se exerted only a slight inhibitory effect on motility. Sorbate-induced decreases in motility were reversed easily by diluting the suspension with fresh medium or by raising the pH to reduce the concentration of undissociated acid.

(iv) Amino acid uptake. By employing vesicles from E. coli, P. aeruginosa, and B. subtilis, Eklund (11) showed that

<table>
<thead>
<tr>
<th>Cell culture no.</th>
<th>pHᵦ</th>
<th>pHᵢ (untreated) − pHᵢ (KS treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.70</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>7.67</td>
<td>0.09</td>
</tr>
<tr>
<td>3</td>
<td>7.66</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>7.63</td>
<td>0.18</td>
</tr>
<tr>
<td>5</td>
<td>7.61</td>
<td>0.23</td>
</tr>
<tr>
<td>6</td>
<td>7.59</td>
<td>0.20</td>
</tr>
<tr>
<td>7</td>
<td>7.57</td>
<td>0.10</td>
</tr>
<tr>
<td>8</td>
<td>7.54</td>
<td>0.44</td>
</tr>
</tbody>
</table>

* The concentration of undissociated sorbic acid was 1.2 mM.
* Dimethyloxazolidinedione used for pH determinations penetrated the cells within 1 min and remained at a constant level for at least 10 min. For pH calculations, the internal water volume determined from the weight difference between wet and dry cells (59) was 0.026 μl per 10⁷ cells, being identical with that reported for C. pasteurianum (8).

**TABLE 2.** The pH of PA 3679 cells treated with potassium sorbate (KS, 200 mM) at pHₐ, 7.0

**TABLE 3.** Effect of potassium sorbate (200 mM) on the motility rate of PA 3679 at three pHₐs

<table>
<thead>
<tr>
<th>pHₐ</th>
<th>Relative motility rate</th>
<th>Undissociated sorbic acid (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>3*</td>
<td>2</td>
</tr>
<tr>
<td>6.5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6.0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Arbitrarily designated motility rate used as a basis for comparison.

sorbate and benzoate (a recognized protonophore) inhibited uptake of all three amino acids tested. Tuncan and Martin (56) reported that sorbate inhibited the uptake of two amino acids in *Salmonella typhimurium*. Energy provided by the PMF and its components (ΔpH and Ψ) would be needed for the uptake of several amino acids required for growth in PA 3679 strain L (5). Sorbate and benzoate inhibited the uptake of [³H]phenylalanine (Table 4), an amino acid that is specifically required by PA 3679 strain L (5). Inhibition by sorbate ranged from 12 to 84% among the five cultures tested. [³H]isoleucine, another specifically required amino acid, was unsuitable for amino acid uptake studies because it was readily catabolized by PA 3679.

Based on these results, we believe that undissociated sorbic acid enters the cell and reduces the proton gradient and, in turn, the PMF. The resulting loss in energy inhibits the uptake of amino acids required for biosynthesis and energy in PA 3679.

**Growth inhibition.** When amino acids are not available in specific ratios or when one amino acid becomes limiting, many bacteria undergo a series of major readjustments known as the stringent response (6, 17). The overall effect of these alterations is that the cells stop replicating but do not die. Because they have instituted a system of programmed management of available resources to prevent destruction, we consider such cells to be in a "conserved" state. Bacteria in this conserved state remain viable and can resume growth at a normal rate whenever the limiting amino acids are restored above the intracellular levels required for protein synthesis.

Neither and Brown (46) reported that growth inhibition of E. coli by parquat, a herbicide, and nitrofurantoin, an antibiotic, was induced by the stringent response. Both compounds are believed to block biosynthetic pathways of specific amino acids and cause amino acid starvation in the cells. Adding the necessary amino acids to the medium released the growth inhibition. Because sorbate inhibited the uptake of required amino acids in PA 3679 (Table 4), we
considered it likely that sorbate-induced growth inhibition also was due to the stringent response.

(i) *Protein synthesis*. The stringent response is characterized by a sharp reduction in RNA accumulation and a corresponding reduction in the rate of protein synthesis (6, 15, 17). Sorbate and benzoate inhibited protein synthesis in PA 3679 cells, with benzoate being more effective (Fig. 2).

(ii) *Phosphorylated nucleotides*. The stringent response is also characterized by changes in the phosphorylated nucleotide pool, with increased levels of ppGpp and other unusual compounds such as pppGpp (6, 17). The addition of sorbate to PA 3679 resulted in a drastic change in the accumulation pattern of several nucleotides (Fig. 3 and 4). Since the amount of ATP did not decrease, changes observed in the rates of nucleotide accumulation were not considered to be caused by cell death. The amounts of GTP, ppGpp, and another unidentified phosphorylated compound increased rapidly in sorbate-treated cells. In the study by Seither and Brown (46), where the stringent response in E. coli was induced by paraquat and nitrofurantoin, the level of ppGpp also increased. Although in Fig. 3 sorbate-treated cells accumulated GTP, ppGpp, and the unidentified compound more rapidly in experiment 2, the similarity in the patterns of the nucleotide accumulation ratios of both experiments (Fig. 4) indicates that the responses were similar.

The increased amount of ppGpp in the untreated cells during the sampling period probably was due to the growth conditions employed for the experiment. Possibly, the combined effects of a defined medium, a heavy cell suspension (5 \( \times \) 10^8 cells/ml), and the aerobic incubation (to permit sampling every 5 min) all contributed to reduced growth for PA 3679 and eventual cessation of cell division in the untreated cells after about 35 to 45 min. Because the stringent response also is believed to be involved in the onset of the stationary phase in batch cultures of certain bacteria (30, 34-37), the increased amounts of ppGpp could be expected in the untreated cells before division ceased.

Unidentified compounds were sought by measuring radioactivity in the areas of the chromatographs with the origin, ppGpp, GTP, and ATP spots. An increased level of radioactivity was found in the area between the origin and the ppGpp spot of extracts obtained from sorbate-treated cells. With the separation methods used in this study, ppGpp and unusual nucleotides involved in regulating sporulation in *B. subtilis* (35, 38, 39) would be expected in this area of the chromatograph. Since sorbate-inhibited PA

---

**FIG. 3.** Effect of potassium sorbate (KS) on the accumulation rates of 32P-labeled ATP, GTP, ppGpp, and an unidentified compound (possibly ppGpp) in PA 3679 cells. Rates are given as disintegrations per minute in nucleotide spots on polyethyleneimine-cellulose chromatograms developed with 1.5 M phosphate (pH 3.4) from 5-, 10-, 15-, and 20-min sampling times relative to time 0 (immediately before the addition of 200 mM potassium sorbate, pH 7). Counts were 10^2 to 10^3 dpm for ATP, 10^3 to 10^4 dpm for GTP, and 10^4 to 10^5 dpm for ppGpp, and an unidentified compound located where ppGpp is reported to develop with this chromatography method. Untreated and potassium sorbate-treated cells in both experiments (1 and 2) were preincubated (10 min) with 32P before time 0. Rates for ATP and GTP accumulation are given in 1a and 2a, and the accumulation of ppGpp and the unidentified compound (possibly ppGpp) is given in 1b and 2b.

**FIG. 2.** Inhibition of protein synthesis in PA 3679 by potassium sorbate (KS) and sodium benzoate (NaB). Relative rates of protein synthesis in untreated, potassium sorbate (200 mM)-treated, and sodium benzoate (200 mM)-treated cells (pH 7) are shown as disintegrations per minute obtained from incorporation of [3H]phenylalanine. Since PA 3679 did not catabolize phenylalanine under the test conditions, the disintegrations per minute represent the amount of label incorporated directly into protein.
3679 cells did not sporulate, it is likely that the unidentified compound was ppGpp.

The pattern of phosphorylated nucleotide accumulation in sorbate-treated PA 3679 cells was not identical to that seen in valine-starved *E. coli* cells during the stringent response (14). In *E. coli*, ATP rose and then fell below prestarvation levels, whereas the GTP level fell. Also the level of ppGpp rose sharply and then fell but remained at a higher level than was seen initially.

Accumulation of ppGpp also was reported in energy-depleted cells (6, 10, 16, 29). In *E. coli* cells treated with cyanide to block respiration and deplete energy (10) the intracellular level of ppGpp rose sharply and remained high, in contrast to amino acid deprivation where ppGpp fell after the initial increase. *E. coli* cells treated with the uncoupling agent carbonyl metacholorphenylhydrazone (16) had only a small decrease in the ATP level and a small increase in ppGpp. However, there was a sharp reduction in protein synthesis, and an unusual and unidentified nucleotide called the "phantom spot," believed to play a role in regulation of protein synthesis, disappeared from the chromatographs.

When *B. subtilis* was partially deprived of amino acids, increased levels of ppGpp and ppGpp were induced by the stringent response that occurred immediately before the initiation of sporulation (32). In a later study, Ochi et al. (31) showed that sporulation in *B. subtilis* induced by the stringent response was attributed to a decrease in GTP but not to the increased level of ppGpp. In sorbate-treated PA 3679 cells, the rates of GTP and ppGpp accumulation increased (Fig. 4 and 5), and the cells stopped dividing but did not sporulate. Since sorbate can reduce intracellular energy levels in cells as well as limit amino acid intake, sorbate-treated PA 3679 cells probably were responding simultaneously to energy and amino acid deprivation. Because the stringent response is restricted to the effects of amino acid deprivation, we consider this set of very similar intracellular readjustments in sorbate-inhibited PA 3679 cells as being a stringent-type response.

(iii) **Effects of tetracycline on sorbate-induced growth inhibition.** A low concentration of tetracycline has been shown to cause release of the stringent response, probably by affecting directly the metabolism of ppGpp (6, 47). Growth of PA 3679 cells was inhibited by potassium sorbate, but cells treated with sorbate and tetracycline grew slowly (Fig. 5). The continued slow growth of PA 3679 in a medium containing an inhibitory concentration of sorbate and a small amount of tetracycline resembles that observed with relaxed mutants of *E. coli* amino acid auxotrophs lacking the stringent response (6, 15, 54, 55). When the amino acid concentration was reduced, these mutants continued to grow slowly and utilized the low concentration of nutrients present. Continued growth of PA 3679 in the presence of tetracycline and an otherwise inhibitory concentration of sorbate also shows that energy and amino acids need not be totally depleted within a bacterium before cell division ceases.

The finding that sorbate-induced inhibition is due to intra-
cellular regulatory mechanisms is important when sorbate is being considered as a food preservative. Because the stringent response in bacteria can be released easily, it may be possible for small changes in the ingredients or storage conditions (or both) to permit the resumption of bacterial growth in sorbate-treated foods.

Based on the results of this study, we propose that the loss of energy and the resulting inhibition of amino acid uptake due to the protonophoric activity of undissociated sorbic acid induced a stringent-type response in PA 3679 cells. Since such a regulatory mechanism can affect numerous systems (17), it is not unexpected that previous studies have reported that sorbate inhibits many different microbial enzymes (51).

Factors affecting antibacterial activity. The antibacterial effectiveness of lipophilic weak acids such as sorbic and benzoic acting as protonophores can be influenced by a number of factors. These factors include the properties of the acids and other substances in the environment as well as differences among the organisms in question.

In addition, subtle differences may exist among apparently similar cultures of the same organism. For example, the $\Delta pH$ ($pH_i - pH_j$) values in untreated PA 3679 cells of pH 7 covered the fairly narrow range of 0.54 to 0.70 pH units (Table 2), being similar to those found in other neutrophils such as E. coli (33) and C. parvum (40). Nevertheless, the effect of sorbate on the $pH_j$ and $\Delta pH$ varied considerably for these supposedly similar cells. Sorbate reduced the $pH_i$ of untreated cells having a $pH_i$ of 7.70 by only 0.03 pH units, while reducing the $pH_j$ of cells with a lower $pH_i$ of 7.54 by 0.44 pH units, resulting in almost total collapse of the $\Delta pH$. Because the $pH_j$ of normal bacterial cells is affected by metabolic, respiratory, or ion transport activities that help maintain $pH$ homeostasis (4), there may be a relationship between the $pH_i$s of untreated PA 3679 cells and the ability of sorbate to reduce the $pH_i$. A high $pH_i$ may indicate a more effective proton efflux system or a buffering system that can maintain an adequate $\Delta pH$ when challenged with the sudden influx of protons provided by a protonophore. Because of possible homeostatic effects, the large variation in the effect of sorbate on $pH_i$ among similar cell cultures is important when considering sorbate as an antimicrobial agent in foods. Slight changes in growth conditions within a food could alter the intracellular activities of the contaminating organisms and have large effects on the efficacy of sorbate as a food preservative.

Since many factors can determine the effectiveness of lipophilic weak acids, the antimicrobial activity of each preservative should be tested under the conditions prevailing during its use, i.e., the specific food, storage temperatures, packaging, etc.

ACKNOWLEDGMENTS

We thank David M. Karl, Department of Oceanography, University of Hawaii, for helpful advice and use of his laboratory for the nucleotide studies. We also thank Thelma Naria for secretarial assistance.

LITERATURE CITED


