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Mechanisms of Sorbate Inhibition of *Bacillus cereus* T and *Clostridium botulinum* 62A Spore Germination

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The mechanism by which potassium sorbate inhibits *Bacillus cereus* T and *Clostridium botulinum* 62A spore germination was investigated. Spores of *B. cereus* T were germinated at 35°C in 0.08 M sodium-potassium phosphate buffers (pH 5.7 to 6.7) containing various germinants (L-alanine, L-α-NH_2-n-butyric acid, and inosine) and potassium sorbate. Spores of *C. botulinum* 62A were germinated in the same buffers but with 10 mM L-lactic acid, 20 mM sodium bicarbonate, L-alanine or L-cysteine, and potassium sorbate. Spore germination was monitored by optical density measurements at 600 nm and phase-contrast microscopy. Inhibition of *B. cereus* T spore germination was observed when 3,900 μg of potassium sorbate per ml was added at various time intervals during the first 2 min of spore exposure to the pH 5.7 germination medium. *C. botulinum* 62A spore germination was inhibited when 5,200 μg of potassium sorbate per ml was added during the first 30 min of spore exposure to the pH 5.7 medium. Potassium sorbate inhibition of germination was reversible for both *B. cereus* T and *C. botulinum* 62A spores. Potassium sorbate inhibition of *B. cereus* T spore germination induced by L-alanine and L-α-NH_2-n-butyric acid was shown to be competitive in nature. Potassium sorbate was also a competitive inhibitor of L-alanine- and L-cysteine-induced germination of *C. botulinum* 62A spores.

Sorbic acid and its potassium salt have been used as fungistatic agents in the food industry for many years (3, 14). Only recently have the sorbates been recognized as potential antibotulinal agents in foods, particularly the cured meats (20). The effectiveness of the sorbates against *Clostridium botulinum* is thought to be due to the inhibition of spore germination (19). However, earlier studies on the inhibitory effect of sorbate on bacterial spore germination have been inconclusive (1, 2, 8). We have found that potassium sorbate inhibits the germination of *Bacillus cereus* T and *C. botulinum* 62A spores in chemically defined media and prevents the loss of spore heat resistance (L. A. Smoot and M. D. Pierson, J. Food Sci., in press). The mechanism of potassium sorbate inhibition of spore germination, however, still remains unknown. The purpose of this investigation is to determine the nature of potassium sorbate inhibition of *B. cereus* T and *C. botulinum* 61A spore germination.

MATERIALS AND METHODS

Spore preparation. Clean spores of *B. cereus* T and *C. botulinum* type 62A were prepared, harvested, and stored as described previously (Smoot and Pierson, in press).

Conditions for germination. Unless specified otherwise, *B. cereus* T spores were germinated in sterile sodium-potassium phosphate buffers (0.08 M, pH 5.7 to 6.7) to which 0.1 mM L-alanine and 0.1 mM inosine had been added. Spores were heat activated at 70°C for 20 min and added to germination media to give final optical density readings at 600 nm (OD_{600}) of 0.26 to 0.36 (7×10^6 to 1×10^7 spores/ml).

Unless specified otherwise, *C. botulinum* 62A spores were germinated in sterile sodium-potassium phosphate buffers (0.08 M, pH 5.7 to 6.7) to which 40 mM L-alanine, 10 mM L-lactic acid, and 20 mM sodium bicarbonate had been added. To remove dissolved oxygen, the buffers in sterile screw-cap tubes were heated in boiling water for 20 min and cooled to room temperature before the remaining medium constituents were added. Spores as aqueous suspensions were added to germination media to give final OD_{600} readings of 0.30 to 0.35 (1.8×10^7 to 2.4×10^7 spores/ml). Spores were heat activated in buffer (pH 6.7) at 80°C for 60 min before each set of experiments.

All germination experiments were conducted in spectrophotometer cells with 10-mm light path. The final volume of the germination solution contained in each cell was 3 ml. Germination was monitored at 35°C, and all germination media were pretempered to 35°C before the spores were added.

Estimation of germination. Germination was estimated by monitoring the decrease in OD_{600} of the spore suspensions as measured by a Perkin-Elmer Coleman 124 double-beam spectrophotometer (Coleman Instruments, Maywood, Ill.) equipped with a temperature-controlled automatic cell changer and a model 56 strip chart recorder (Coleman Instruments). The cell changer was connected to a Lauda model WB-20/R constant-temperature water bath (Brinkmann Instruments Inc., Westbury, N.Y.) to ensure maintenance of the desired incubation temperature. Overall germination was calculated and plotted as percentage of the initial OD value. Rates of germination for *B. cereus* T spores were calculated as the percentage fall in OD per minute for the first 5 min of spore incubation in the germination medium. Rates of germination for *C. botulinum* 62A were calculated for the first 20 min of spore incubation.

The extent of *B. cereus* T spore germination was determined as the percentage of 150 to 200 spores that were not refractile in phase-contrast microscopy after 30 min of incubation. The extent of *C. botulinum* 62A spore germination was determined similarly, except that spores were considered to be germinated if they were stainable with 0.5% aqueous methylene blue after 120 min of incubation.

Potassium sorbate. Potassium sorbate (Monsanto Industrial Chemicals Co., St. Louis, Mo.) was prepared as a 10% (wt/vol) stock solution in buffer of the appropriate pH value (5.7, 6.2, or 6.7) and filter sterilized (0.22 μm; Millipore Corp., Bedford, Mass.). Fresh stock solutions of potassium sorbate were prepared on the day of use.

Inhibition of germination. The influence of potassium sorbate on the germination of *B. cereus* T and *C. botulinum* 62A spores was examined by adding various concentrations of the chemical to the germination medium. The fall in OD, the rate of germination, and the percentage of germination at pH 5.7, 6.2, and 6.7 (35°C) were the parameters used to measure the effect of potassium sorbate on spore germination.

Duration of sensitivity to potassium sorbate. Potassium sorbate was added to germination media (pH 5.7, 35°C) containing spores of either *B. cereus* T or *C. botulinum* 62A, and the fall in OD was monitored. In experiments with *B. cereus* T spores, additions of potassium sorbate to a final concentration of 3,900 μg/ml were made at various time intervals during the first 2 min of spore incubation. In experiments with spores of *C. botulinum* 62A, additions of potassium sorbate to a final concentration of 5,200 μg/ml were made at various time intervals during the first 30 min of spore incubation.

Reversibility of potassium sorbate inhibition. Spores were suspended in the appropriate germination medium (pH 5.7, 35°C) containing the appropriate inhibitory concentration (3,900 μg/ml for *B. cereus* T; 5,200 μg/ml for *C. botulinum* 62A) of potassium sorbate. After incubation for at least 1 h, the spores were removed from the potassium sorbate by membrane filtration (0.45 μm; Millipore Corp.). The spores were immediately suspended in the appropriate germination medium free from potassium sorbate. Changes in OD of the suspensions were monitored throughout the entire experiment.

Competitive inhibition of germination. Preliminary experiments using *B. cereus* T spores were performed with buffer (pH 5.7) containing 0.1 mM inosine to determine the effect of various concentrations (3 to 20 μM) of L-alanine on spore germination rates. Plots of one over the germination rate versus one over the L-alanine concentration gave straight lines similar to Lineweaver-Burk reciprocal plots. Similar experiments using *B. cereus* T spores were conducted with buffers (pH 5.7 and 6.7) containing various concentrations of L-alanine (0.1 to 1.0 mM) plus potassium sorbate (0, 50, or 100 μg/ml) or various concentrations of L-α-NH_2-*n*-butyric acid (2 to 10 mM) plus potassium sorbate (0, 100, or 1,000 μg/ml). The germination rates obtained were plotted as described above. The L-alanine and L-α-NH_2-*n*-butyric acid used in these experiments were prepared as filter-sterilized (0.22 μm; Millipore Corp.) 10% (wt/vol) stock solutions in buffer of the appropriate pH value. All experiments were performed at 35°C in duplicate.

Experiments with spores of *C. botulinum* 62A were conducted by using buffers (pH 5.7 and 6.7) containing 10 mM L-lactic acid, 20 mM sodium bicarbonate, and various concentrations of L-alanine (0.2 to 2.0 mM) plus potassium sorbate (0, 50, or 200 μg/ml) or various concentrations of L-cysteine (5 to 30 mM) plus potassium sorbate (0, 50, or 200 μg/ml). Reciprocals of the calculated germination rates were plotted versus the reciprocals of the germinant concentrations. The L-alanine and L-cysteine used in these experiments were prepared as described above. All experiments were conducted at 35°C in duplicate.

RESULTS AND DISCUSSION

Inhibition of germination. Table 1 shows the effect of potassium sorbate on the germination of *B. cereus* T spores incubated in various germination media (pH 5.7 to 6.7) at 35°C. The rate of germination, the fall in OD, and the percentage of germination decreased in the pres-

TABLE 1. *Effect of potassium sorbate on the germination of B. cereus T spores at 35°C*[a]

pH	Potassium sorbate (μg/ml)	Germination rate[b] (%/min)	% Fall in OD after 30 min	% Germination[c]
5.7	0	6.8	45.2	84
	3,900	0.0	0.0	3
6.2	0	8.1	50.0	90
	3,900	3.7	35.7	76
6.7	0	9.2	52.6	93
	3,900	7.1	48.7	86

[a] Heat-shocked spores were suspended in 0.08 M sodium-potassium phosphate buffer (pH 5.7 to 6.7) containing 0.1 mM L-alanine and 0.1 mM inosine with or without potassium sorbate.

[b] Percentage fall in OD per minute for the first 5 min of spore incubation in the germination medium.

[c] Percentage of phase-dark spores after 30 min of incubation.

ence of potassium sorbate. The effectiveness of potassium sorbate decreased as the pH of the medium was increased from 5.7 to 6.7. This is consistent with reports that sorbate is a more effective microbial inhibitor at acidic pH levels than at pH levels near neutrality (7, 8). Complete inhibition of *B. cereus* T spore germination by 3,900 μg of potassium sorbate per ml was observed only in the pH 5.7 medium. Table 2 shows the effects of potassium sorbate on *C. botulinum* 62A spore germination in various media (pH 5.7 to 6.7) at 35°C. These results are similar to those found for *B. cereus* T spores; however, a potassium sorbate concentration of 5,200 μg/ml was necessary to completely inhibit *C. botulinum* 62A spore germination at pH 5.7.

We have found that the concentrations of potassium sorbate necessary to inhibit germination also prevent the loss of *B. cereus* T and *C. botulinum* 62A spore heat resistance in the same media (Smoot and Pierson, in press). Loss of heat resistance is known to be a prime event in the germination process of *Bacillus* and *Clostridium* spores (4, 10, 13, 22). These data suggest that potassium sorbate may inhibit bacterial spore germination at some point during the process of initiation.

Time addition of potassium sorbate and germination inhibition. When potassium sorbate (3,900 μg/ml) was added after 0- to 2-min exposure of the *B. cereus* spores to the germination system, subsequent germination was inhibited (Fig. 1). In studies by Scott et al. (17), it was found that spores of *B. megaterium* KM became committed to germination after only 1.5 min of exposure to L-alanine. Based on this observation, they concluded that the initial binding of L-alanine to a site on the spore triggers an immediate response which results in the commencement of germination. This may explain the relationship between the time of addition of potassium sorbate and the inhibition of germination. We propose that as the time between the exposure of the spores to L-alanine and the addition of potassium sorbate is increased, the percentage of spores committed to germination is increased. This also suggests that potassium sorbate may either compete with L-alanine for a receptor site on the spore, combine with L-alanine making it unavailable to the spore, or inhibit an enzyme on the spore that initiates L-alanine metabolism. Similar results were obtained for *C. botulinum* 62A spores (Fig. 2). The exact mechanism by which L-alanine triggers spore germination, either allosterically or metabolically, has not been satisfactorily determined. The different approaches used to elucidate this mechanism have been reviewed (Smoot and Pierson, J. Food Protect., in press).

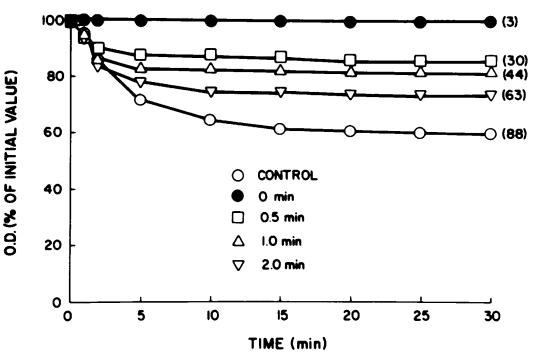

FIG. 1. *Influence of the time addition of potassium sorbate (3,900 μg/ml) on inhibition of germination of B. cereus T spores in 0.08 M phosphate buffer (pH 5.7) containing 0.1 mM L-alanine and 0.1 mM inosine at 35°C. The periods (minutes) on the figure represent the time interval between spore exposure to the medium and the addition of the potassium sorbate. Numbers in parentheses represent percentage of germination (spores phase dark) after 30 min.*

TABLE 2. *Effect of potassium sorbate on the germination of C. botulinum 62A spores at 35°C*[a]

pH	Potassium sorbate (μg/ml)	Germination rate[b] (%/min)	% Fall in OD after 120 min	% Germination[c]
5.7	0	0.50	32.5	74
	5,200	0.0	5.0	5
6.2	0	0.75	37.5	90
	5,200	0.15	18.0	8
6.7	0	0.98	41.3	94
	5,200	0.30	32.0	62

[a] Heat-shocked spores were suspended in 0.08 M sodium-potassium phosphate buffer (pH 5.7 to 6.7) containing 40 mM L-alanine, 10 mM L-lactic acid, and 20 mM sodium bicarbonate with or without potassium sorbate.

[b] Percentage fall in OD per minute for the first 20 min of spore incubation in the germination medium.

[c] Percentage of spores stainable with 0.5% methylene blue after 120 min.

Reversibility of sorbate inhibition. The inhibition of germination for both *B. cereus* T and *C. botulinum* 62A spores by potassium sorbate was reversed when the spores were removed from the sorbate-containing medium and suspended in germination media without sorbate (Fig. 3 and 4). This indicated that sorbate inhibition did not result in permanent alterations related to germination. Similar experiments demonstrating the reversible inhibition of *B. subtilis* spore germination by various alcohols have been reported by Trujillo and Laible (21). They concluded from their results that the al-

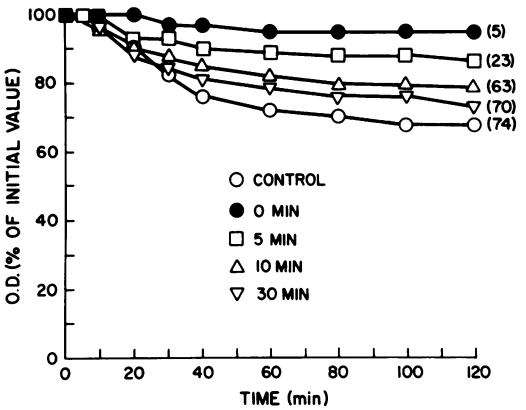

FIG. 2. *Influence of the time addition of potassium sorbate (5,200 μg/ml) on inhibition of germination of C. botulinum 62A spores suspended in 0.08 M phosphate buffer (pH 5.7) containing 40 mM L-alanine, 10 mM L-lactic acid, and 20 mM sodium bicarbonate at 35°C. The periods (minutes) on the figure are as indicated in Fig. 1. Numbers in parentheses represent percentage of germination (spores stainable with 0.5% methylene blue) after 120 min.*

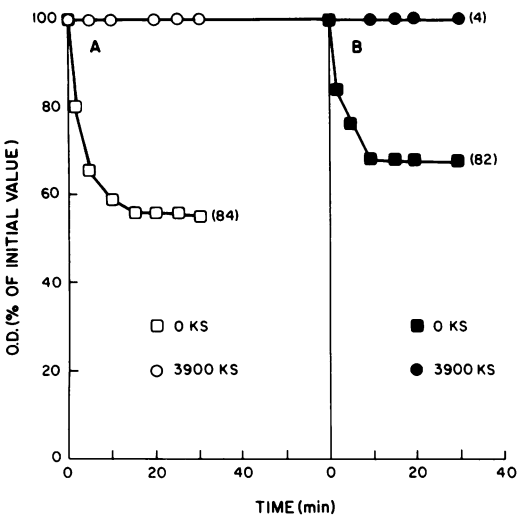

FIG. 3. *Reversibility of potassium sorbate-induced inhibition of B. cereus T spore germination. (A) Spores were suspended in 0.08 M phosphate buffer (pH 5.7) containing 0.1 mM L-alanine and 0.1 mM inosine at 35°C with or without potassium sorbate. (B) After 60 min, the spore suspension incubated with potassium sorbate was membrane filtered and suspended in the pH 5.7 germination medium at 35°C with or without potassium sorbate. KS, potassium sorbate (concentration in micrograms per milliliter). Numbers in parentheses represent percentage of germination (spores phase dark) after 30 min.*

cohols were functioning by inhibiting an enzyme(s) required for germination.

Competitive inhibition of germination.

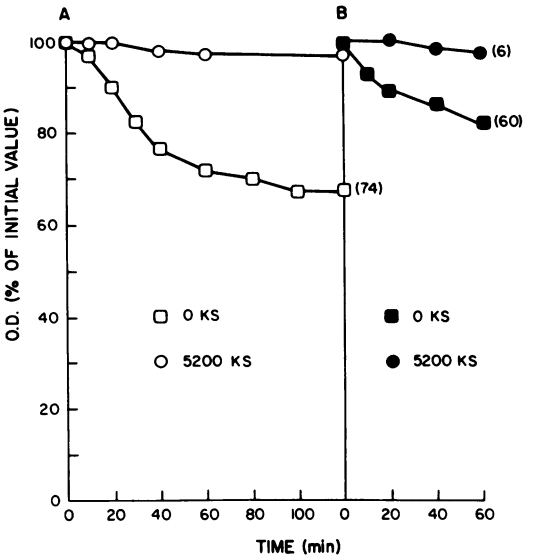

FIG. 4. *Reversibility of potassium sorbate-induced inhibition of C. botulinum 62A spore germination. (A) Spores were suspended in 0.08 M phosphate buffer (pH 5.7) containing 40 mM L-alanine, 10 mM L-lactic acid, and 20 mM sodium bicarbonate at 35°C with or without potassium sorbate. (B) After 120 min, the spore suspension incubated with potassium sorbate was membrane filtered and suspended in the pH 5.7 germination medium at 35°C with or without potassium sorbate. Numbers in parentheses represent percentage of germination (spores stainable with 0.5% methylene blue) after 120 min.*

Figure 5 shows the effect of potassium sorbate on germination rates of *B. cereus* T spores suspended in buffer (pH 5.7) containing various concentrations (0.1 to 1.0 mM) of L-alanine at 35°C. In the absence of potassium sorbate, the rate of germination for *B. cereus* T spores increased as the concentration of L-alanine increased. This is in agreement with the results of other workers (23). When potassium sorbate (50 μg/ml) was added to the germination medium, the germination rates induced by the lower concentrations of L-alanine were greatly decreased, but as the concentration of L-alanine was increased, the effect of potassium sorbate on the germination rate decreased. The reciprocal plot of the data for germination in the absence and presence of sorbate resulted in two straight lines with different slopes but the same *y* intercept (Fig. 5). Lineweaver-Burk reciprocal plots of this nature indicate competitive inhibition (18). Potassium sorbate appeared to function as a competitive inhibitor of L-alanine-induced germination. A similar competitive inhibition of L-alanine-induced spore germination of *B. cereus*, using D-alanine as the inhibitor, was reported by O'Connor and Halvorson (16). With the same

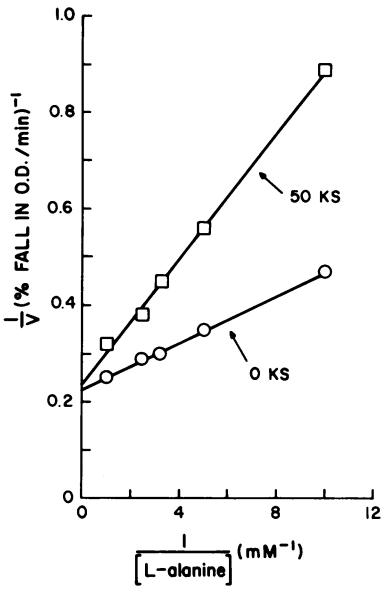

FIG. 5. *Competitive inhibition of* L-*alanine-induced germination of B. cereus T spores by potassium sorbate. Spores were suspended in 0.08 M phosphate buffer (pH 5.7) with various concentrations of* L-*alanine at 35°C with or without potassium sorbate. KS, potassium sorbate (concentration in micrograms per milliliter).*

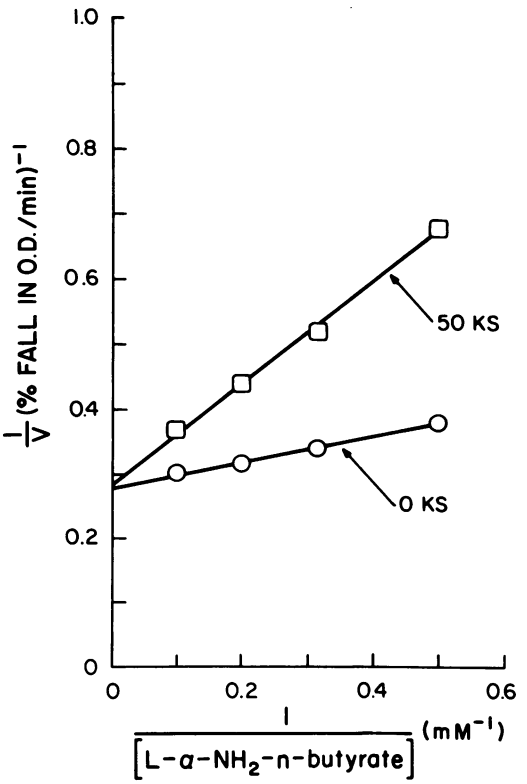

FIG. 6. *Competitive inhibition of* L-α-NH_2-*n-butyric acid-induced germination of B. cereus T spores by potassium sorbate. Spores were suspended in 0.08 M phosphate buffer (pH 5.7) containing various concentrations of* L-α-NH_2-*n-butyric acid at 35°C with or without potassium sorbate. KS, potassium sorbate (concentration in micrograms per milliliter).*

medium at pH 6.7 (data not shown), a higher concentration (100 μg/ml) of potassium sorbate was required to produce an inhibitory effect similar to that of 50 μg/ml at pH 5.7. Competitive inhibition of germination was also seen at pH 6.7. The effect of pH is consistent with the results discussed earlier in this study and those of Gould (8). He demonstrated that sorbate is a more effective inhibitor of *Bacillus* spore germination at pH 6.0 than pH 7.0.

To determine whether potassium sorbate is a competitive inhibitor specific for L-alanine-induced germination of *B. cereus* T spores, a similar experiment was conducted with buffer (pH 5.7) containing various concentrations of L-α-NH_2-*n*-butyric acid (Fig. 6). In the absence of sorbate, germination rates increased as the concentration of L-α-NH_2-*n*-butyric acid increased. In the presence of sorbate, germination rates were markedly decreased at the lower concentrations of the L-α-NH_2-*n*-butyric acid, but as the level of the germinant increased, the effect of potassium sorbate decreased. Potassium sorbate was a competitive inhibitor of L-α-NH_2-*n*-butyric acid-induced germination, indicating that sorbate was not a specific competitive inhibitor for L-alanine-induced germination. With the same germination system at pH 6.7 (data not shown), a higher concentration of potassium sorbate (1,000 μg/ml) was required to produce an inhibitory effect similar to that of a lower level of sorbate (50 μg/ml) at pH 5.7.

These results show that potassium sorbate is a competitive inhibitor of L-alanine- and L-α-NH_2-*n*-butyric acid-induced germination of *B. cereus* T spores. Exactly how L-alanine and other similar germinants induce spore germination is unknown (11). Some researchers feel that germinants trigger germination by causing a structural change at some unidentified locus of the spore, i.e., an allosteric change in a surface or membrane protein (9, 24). If this is true, potassium sorbate may be competing with L-alanine or L-α-NH_2-*n*-butyric acid for a specific receptor site. Other researchers contend that the trigger reaction is metabolic in nature (5). If this is the case, potassium sorbate may be inhibiting the activity of an enzyme that initiates the metabolism of L-alanine or L-α-NH_2-*n*-butyric acid. The enzyme L-alanine dehydrogenase was at one time thought to be the first binding site of L-

alanine (6, 15). L-Alanine dehydrogenase has been isolated from spores of *B. cereus* T, and L-alanine and L-α-NH_2-*n*-butyric acid have been shown to be substrates for this enzyme (16). Potassium sorbate may be competing with the germinants for the active site on the L-alanine dehydrogenase enzyme. Lehninger (12) states, "The hallmark of competitive inhibition is that the inhibitor can combine with the free enzyme in such a way that it competes with the normal substrate for binding at the active site." He also indicates that the inhibitor can react reversibly with the enzyme. Reversible inhibition by potassium sorbate has been demonstrated. Which of the above proposed scenarios is the true mechanism for potassium sorbate competitive inhibition of L-alanine or L-α-NH_2-*n*-butyric acid induced germination has yet to be determined.

Potassium sorbate was a competitive inhibitor of L-alanine-induced germination of *C. botulinum* 62A spores (Fig. 7). This competitive inhibition was pH dependent (data not shown) in that at pH 6.7 a higher concentration (200 μg/ml) of potassium sorbate was required to obtain an inhibitor effect comparable to that of 50 μg of sorbate per ml at pH 5.7. This is consistent with results of similar experiments discussed earlier in this report. In an additional experiment (Fig. 8), potassium sorbate competitively inhibited *C. botulinum* 62A spore germination induced by L-cysteine. This inhibition was also pH dependent (data not shown). The results suggest a similar mechanism of sorbate inhibition of germination of *B. cereus* T and *C. botulinum* 62A spores.

This investigation was conducted with a model system to demonstrate the inhibition of bacterial spore germination by potassium sorbate. Although potassium sorbate was shown to be a strong inhibitor of spore germination, the exact mechanism by which potassium sorbate inhibits spore germination is still unknown. Evidence presented here suggests that this inhibition is similar for the genera *Clostridium* and *Bacillus*, that it occurs at some point during the

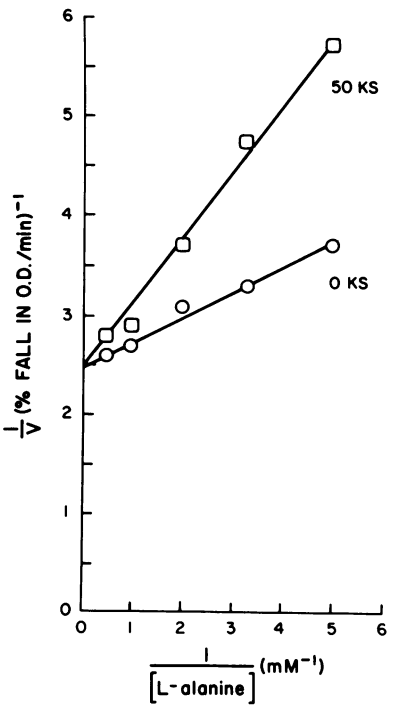

FIG. 7. *Competitive inhibition of L-alanine-induced germination of C. botulinum 62A spores by potassium sorbate. Spores were suspended in 0.08 M phosphate buffer (pH 5.7) containing 10 mM L-lactic acid, 20 mM sodium bicarbonate, and various concentrations of L-alanine at 35°C with or without potassium sorbate. KS, potassium sorbate (concentration in micrograms per milliliter).*

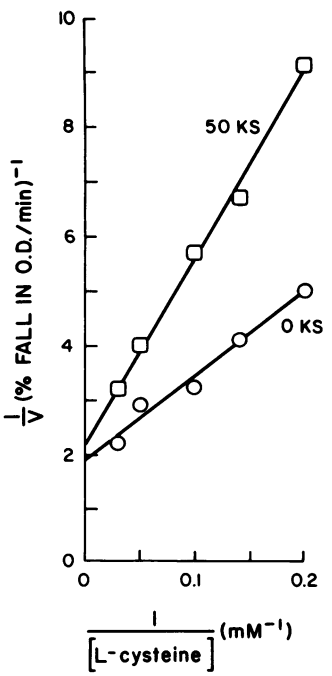

FIG. 8. *Competitive inhibition of L-cysteine-induced germination of C. botulinum 62A spores by potassium sorbate. Spores were suspended in 0.08 M phosphate buffer (pH 5.7) containing 10 mM L-lactic acid, 20 mM sodium bicarbonate, and various concentrations of L-cysteine at 35°C with or without potassium sorbate. KS, potassium sorbate (concentration in micrograms per milliliter).*

initial stages of the germination process, and that it is competitive in nature. The mechanism(s) by which potassium sorbate inhibits spore germination will become more evident once the exact nature of the trigger reaction for bacterial spore germination has been elucidated.

ACKNOWLEDGMENTS

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