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Inhibition of Germinant Binding by Bacterial Spores in Acidic Environments†

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Commitment to germinate occurred in both *Clostridium botulinum* and *Bacillus cereus* spores during 0.5 min of exposure to 100 mM L-alanine or L-cysteine, measured by the inability of germination inhibitors (D form of amino acid) to inhibit germination. Spore germination at pH 4.5 was inhibited because the germinant did not bind to the trigger sites. *C. botulinum* spores exposed to 100 mM L-alanine or L-cysteine at pH 4.5 remained sensitive to D-amino acid inhibition at pH 7, indicating that no germinants had bound to the trigger site at pH 4.5. Inhibition of germinant binding at pH 4.5 was reversible but lagged in commitment to germinate upon transfer to pH 7. Spores sequentially exposed to pH 4.5 buffer and pH 7 buffer with the germinant also demonstrated a lag in commitment to germinate. The pH at which binding was inhibited was not significantly affected by composition of the buffer or by reduced germinant concentrations (10 mM). Nonspecific uptake of L-[^{3}H]alanine by *C. botulinum* spores was not inhibited at pH 4.5. Inhibition of germinant binding in acidic environments appeared to be due to protonation of a functional group in or near the trigger site. This may represent a general mechanism for inhibition of spore germination in acidic environments.

Many food preservation processes use acidic conditions to inhibit bacterial spores. The pH limiting growth from spores varies with species and strain, incubation temperature, and concentration of specific germinant (3). Research has focused primarily on inhibitory conditions so that little is known about the mechanisms of inhibition. The most frequently studied species has been *Clostridium botulinum*. Knowledge of the mechanisms of inhibition of *C. botulinum* at low pH would assist in reconciling the different pH values reported to limit growth of this organism (3).

Spore germination is the breaking of dormancy by degradative biochemical reactions (15). It is divided into two phases: (i) the trigger reaction which initiates germination (12) and (ii) the connecting reactions which occur after triggering and lead to the first visible signs of germination (10). Low pH seems to have a critical effect on the trigger reaction (11, 22). In an L-alanine-induced germination system, D-alanine specifically inhibits the triggering reaction without affecting the connecting reactions (11, 13). Spores become insensitive to the inhibitory action of D-alanine after brief exposure to L-alanine. Loss of sensitivity to D-alanine defines commitment to germinate, the first irreversible reaction in the trigger mechanism (22).

The purpose of this study was to determine the effect of acid environments on the germination-triggering mechanism of *C. botulinum* and *Bacillus cereus* spores. The specific sensitivity of triggering to D-amino acids was used to study and separate triggering from connecting reactions.

MATERIALS AND METHODS

Spore inoculum. Spores of *C. botulinum* 62A and 12885A were obtained originally from Swift and Co. Research Center (Oak Brook, Ill.). Spore suspensions were prepared by the method of Christiansen et al. (5), enzymatically cleaned (8), and held at 4°C in distilled water. Two strains of *B. cereus*, F4810/72 (vomiting type) and F2769/77 (diarrheal type), were obtained from the Food Hygiene Laboratory, London, England. The reference strain, *B. cereus* T (also called F1248), was obtained from the culture collection of the Department of Food Science and Nutrition, University of Minnesota. Spores of the three strains of *B. cereus* were prepared, harvested, and cleaned as described by Johnson et al. (14). Spores were heat activated for 15 min in distilled water immediately before use: *C. botulinum* at 80°C and *B. cereus* at 70°C. Viable spores were counted as colonies formed in Lee tubes (18) containing peptone-yeast extract-glucose agar (8). Direct microscopic counts of the spore suspensions were made in a Petroff-Hauser counting chamber (19).

Medium preparation. L-Alanine (10 or 100 mM) or L-cysteine (100 mM) was used as germination trigger, and D-alanine (1.8 M) or D-cysteine (0.9 M) was used as the competitive inhibitor of triggering (concentrations are after inoculation). Germinants and inhibitors were solutions in 100 mM phosphate-citrate or 100 mM 2-(*N*-morpholino)ethanesulphonic acid (MES; United States Biochemical Corp., Cleveland, Ohio) buffers.

Stock solutions of 100 mM dibasic potassium phosphate, citric acid, or MES were held at 4°C for up to 1 month. Sodium bicarbonate (55 mM), used in all experiments with *C. botulinum*, was added to the stock solutions. When MES was used as the buffer solution, the pH was adjusted with 6 N NaOH or HCl. Phosphate-citrate buffers were made by combining the necessary proportions of 100 mM phosphate solution with the 100 mM citric acid solution to obtain solutions ranging from pH 4.5 to 7.1 as needed. The final buffer system varied from ca. 54 mM citrate and ca. 46 mM phosphate at pH 4.5 to ca. 13 mM citrate and ca. 87 mM phosphate at pH 7.0 (17). Triggering media were prepared by dissolving the appropriate germinant in the correct stock buffers followed by pH adjustment. Solutions of D-alanine or D-cysteine were prepared in phosphate-citrate buffer. Further pH adjustment after media stabilized for ca. 15 min was

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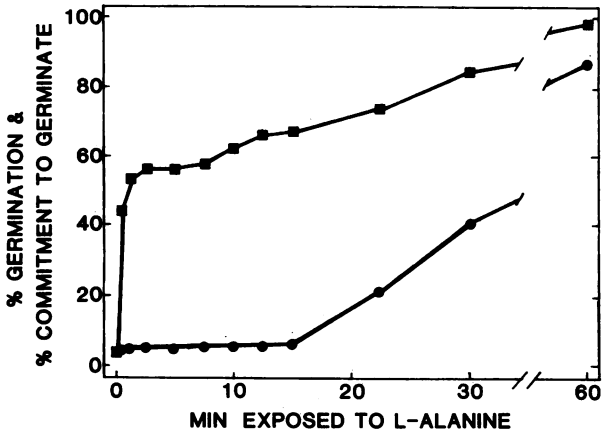

FIG. 1. Comparison of the time for commitment to germinate and germination for *C. botulinum* 62A spores in 100 mM L-alanine at pH 7. Data represent averages of three to five duplicate trials. Symbols: ■, commitment to germinate; ●, germination.

made when necessary (Orion 620 pH meter with a glass combination electrode).

The pH-adjusted media were filter sterilized (0.45-μm pores) and stored at 4°C. The pH values after filter sterilization were within ±0.1 unit of the values reported. Sterile media were aseptically dispensed into glass test tubes (10 by 75 mm) and capped with rubber stoppers. Media were tempered to 35°C for 15 min before inoculation.

Commitment and germination measurements. Heat-shocked spores were added to the treatment germination medium at a final concentration of ca. 10^8/ml, immediately mixed, and incubated at 35°C in a water bath. At designated intervals 50 μl was transferred to 450 μl of the appropriate D-amino acid solution at pH 7.1, mixed immediately, and incubated for 1 h at 35°C. The addition of 50 μl of buffer with germinant at pH 4.5 altered the pH of the D-amino acid solution by less than 0.1 pH unit. After the second incubation period, duplicate samples were removed from each tube and 1 drop of inoculated medium was placed on a clean, flamed cover slip at ca. 50°C. Samples dried in less than 1 min. Cover slips were later examined after rehydration with 1 drop of dilute methylene blue on a microscope slide at × 1,000 (Nikon Labophot with blue filter, Nippon Kogak K.K., Tokyo, Japan). A total of 100 spores were counted on each cover slip and classified as refractile or nonrefractile. Only fully refractile spores were classified as refractile; all others were classified as nonrefractile. The methylene blue solution was used to help differentiate residual amino acid crystals from spores. In experiments with cysteine, 0.1 N HCl was used to rehydrate samples to aid in dissolving cysteine crystals.

Percent commitment to germinate was defined as the percentage of spores which were nonrefractile after 1 h of incubation in the appropriate D-amino acid. The addition of excess D-amino acid to the spore-plus-germinant mixture competitively inhibited further triggering of germination (D/L ratio of 180:1 for alanine and 90:1 for cysteine) but did not inhibit completion of the connecting reactions in spores which had been triggered. Spores which had been triggered to germinate before transfer to the D-amino acid solution completed the connecting reactions during the 1-h incubation at 35°C. Exposure of spores to the germinant at pH 4.5 followed by transfer to a D-amino acid solution at pH 7.1 allowed the effects of low pH on triggering to be measured independently from the effect of low pH on the connecting reactions. Percent germination was defined as the percentage of nonrefractile spores in samples which had been dried immediately after a given exposure to germinant.

Spores were exposed to buffer alone or to D-amino acid solutions for 1 or 2 h in each experiment. The percent nonrefractile spores increased <3% over initial values in buffer-treated controls and <5% over initial values in spores exposed to the D-amino acids. The initial percentage of nonrefractile spores ranged from 3 to 8%.

Recovery of commitment to germinate procedures. Recovery of commitment was measured by inoculating spores held at pH 4.5 with or without germinant for 1 h into media with germinant at pH 7. Commitment to germinate or germination was then measured.

Measurement of L-[^{3}H]alanine uptake. Phosphate-citrate buffer and 1 mM unlabeled L-alanine were prepared as described above. After filter sterilization 2 μCi of L-[^{3}H]alanine (72.4 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added per 45 μl of uninoculated medium. Media were inoculated with ca. 2×10^9 spores per ml of medium and incubated for 15 min at 35°C. Retention of labeled alanine was measured before and after a 1-h exposure to 1.8 M D-alanine solution. Duplicate 50-μl portions were filtered through a 0.45-μm-pore-size filter (previously soaked in 100 mM L-alanine), washed with 20 ml of pH 7 phosphate-citrate buffer, and dried at ca. 50°C. Dried filters were placed in vials with 10 ml of Aquasol II liquid scintillation counting cocktail (New England Nuclear) and counted on a Beckman LS-250 liquid scintillation system with a tritium Iso-set. The counting efficiency for ^{3}H was determined to be 41.04%, and this factor was used to convert counts per minute data to disintegrations per minute. Data reported are counts retained after background counts were subtracted. Filters without spores retained ca. 146 dpm and were used to determine the background count. In all cases, the amount of label retained by the spores was significantly greater ($P < 0.05$) than the background count.

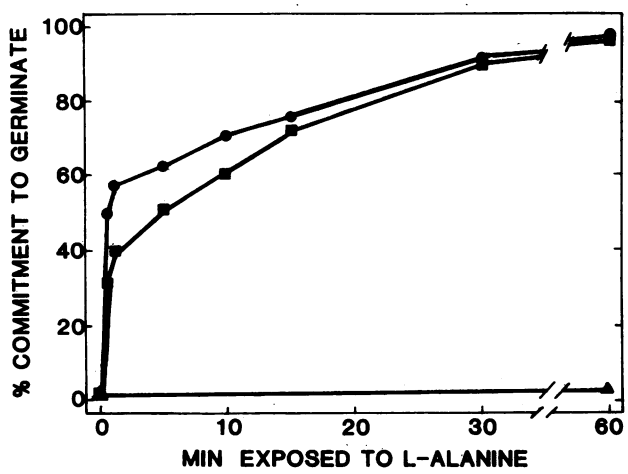

FIG. 2. Inhibition and recovery of commitment to germinate at pH 4.5 in 100 mM L-alanine for *C. botulinum* 62A spores. Data represent averages of four duplicate trials. Spore treatments were as follows: 100 mM L-alanine at pH 7 for exposure time indicated followed by 1 h in 1.8 M D-alanine (●); 100 mM L-alanine at pH 4.5 for 1 h followed by 100 mM L-alanine at pH 7 for exposure time indicated followed by 1 h in 1.8 M D-alanine (■); 100 mM L-alanine at pH 4.5 for exposure time indicated followed by 1 h in 1.8 M D-alanine (▲).

TABLE 1. Enumeration of *C. botulinum* 62A spores exposed to 100 mM L-alanine at pH 4.5 followed by 1.8 M D-alanine at pH 7 at 35°C

Treatment	Population[a]	
	Unheated	Heated[b]
None		8.52
100 mM L-alanine at pH 4.5 for 1 h	8.64	8.66
100 mM L-alanine at pH 4.5 for 1 h followed by 1.8 M D-alanine at pH 7 for 1 h	8.67	8.65

[a] Log CFU per milliliter from Lee tube procedure with peptone-yeast extract-glucose agar.
[b] Heated for 15 min at 80°C in final suspension.

RESULTS

Commitment to germinate in L-alanine. The time course of commitment to germinate was compared with that of germination for spores of *C. botulinum* 62A at pH 7 (Fig. 1). Commitment occurred rapidly (ca. 50% after 0.5 min). Nonrefractile spores were first detected after 22.5 min.

Commitment to germinate at pH 4.5 and 7 is compared in Fig. 2 for spores of *C. botulinum* 62A. Spores initially exposed to L-alanine at pH 7 demonstrated rapid commitment to germinate, whereas spores exposed to L-alanine at pH 4.5 for 1 h remained sensitive to D-alanine at pH 7. Enumeration data indicated no loss of viability or loss in heat resistance in spores exposed to pH 4.5 in the presence of 100 mM L-alanine for 1 h with or without a subsequent 1 h at pH 7 with 1.8 M D-alanine (Table 1). Spores exposed to pH 4.5 in the presence of 100 mM L-alanine for 1 h with subsequent transfer to pH 7 and 100 mM L-alanine became insensitive to D-alanine more slowly than spores exposed directly to L-alanine at pH 7 with no pretreatment (Fig. 2).

Similar curves for the three variables presented in Fig. 1 were obtained with spores of *C. botulinum* 12885A and *B. cereus* F4810/72, T, and F2769/77. These data are summarized in Tables 2 and 3. No commitment to germinate was observed in spores exposed to 100 mM L-alanine at pH 4.5 followed by transfer to pH 7. Inhibition of commitment to germinate was reversible in all strains examined. Recovery responses of spores of *C. botulinum* 12885A and the three *B. cereus* strains held at pH 4.5 followed by transfer to pH 7 and 100 mM L-alanine were similar to responses of spores of *C. botulinum* 62A, i.e., commitment occurred after a short lag period (data not shown).

Commitment to germinate in L-cysteine. Commitment in 100 mM L-cysteine occurred more slowly than in 100 mM L-alanine at pH 7 (Table 4). No commitment to germinate was observed at pH 4.5. Spores exposed to pH 4.5 with or without 100 mM L-cysteine and subsequently transferred to pH 7 and 100 mM L-cysteine demonstrated a lag in commitment to germinate.

Commitment to germinate at pH 7 after exposure to pH 4.5. Spores exposed to pH 4.5 buffer followed by recovery at pH 7 in buffer before L-alanine exposure and spores exposed to 100 mM L-alanine with no other treatment responded essentially identically (Fig. 3). Spores transferred from pH 4.5 buffer directly into 100 mM L-alanine at pH 7 exhibited a short lag in their commitment response.

Effect of buffer system and germinant concentration on commitment to germinate. Effects of different buffer systems and decreased germinant concentration on inhibition of commitment were investigated with spores of *C. botulinum* 12885A. Previous work had indicated that germination of strain 12885A had a sharper lower pH limit than that of strain 62A; consequently, strain 12885A was selected over strain 62A for these experiments (J. C. Blocher, Ph.D. dissertation, University of Minnesota, Minneapolis, 1985). In Fig. 4, the extent of commitment to germinate after 15 min of exposure to germinant is plotted versus pH. For commitment in 100 mM L-alanine, the buffer system did not significantly alter the effect of pH (MES versus phosphate-citrate). In both buffer systems, a reduction in pH from 6.3 to 5.9 reduced commitment from ca. 80 to 90% to ca. 30%. Inhibition of commitment occurred in the same pH range (6.3 to 5.9) at both L-alanine concentrations (10 and 100 mM).

Uptake and retention of labeled L-alanine. Nonspecific uptake of L-alanine by *C. botulinum* 62A spores was com-

TABLE 3. Inhibition and recovery of commitment to germinate in L-alanine for *B. cereus* spores at 35°C

Treatment pH			% Spores committed to germinate[a]		
Initial[b]	Recovery[b]	Final[c]	Strain F4810/72[d]	Strain T[e]	Strain F2769/77[f]
7.0 (2 h)		7.0 (1 h)	80	87	82
4.5 (1 h)		7.0 (1 h)	12	10	10
4.5 (1 h)	7.0 (2 h)	7.0 (1 h)	72	71	79

[a] Data represent average of two duplicate trials.
[b] Spores exposed to 100 mM L-alanine at pH indicated.
[c] Spores exposed to 1.8 M D-alanine at pH indicated.
[d] 8% nonrefractile spores present initially.
[e] 5% nonrefractile spores present initially.
[f] 6% nonrefractile spores present initially.

TABLE 2. Inhibition and recovery of commitment to germinate in L-alanine for *C. botulinum* spores at 35°C

Treatment pH			% Spores committed to germinate[a]	
Initial[b]	Recovery[b]	Final[c]	Strain 62A[d]	Strain 12885A[e]
7.0		7.0	96	98
4.5		7.0	7	6
4.5	7.1	7.0	95	95

[a] Data represent average of three duplicate trials.
[b] Spores exposed to 100 mM L-alanine for 1 h at pH indicated.
[c] Spores exposed to 1.8 M D-alanine for 1 h at pH indicated.
[d] 3% nonrefractile spores present initially.
[e] 6% nonrefractile spores present initially.

TABLE 4. Inhibition and recovery of commitment to germinate in L-cysteine for *C. botulinum* 62A spores at 35°C

Treatment pH			% Spores committed to germinate[a]
Initial[b]	Recovery[b]	Final[c]	
7.0		7.0	96
4.5		7.0	3
4.5	7.0	7.0	74
4.5[d]	7.0	7.0	75

[a] Data represent average of two duplicate trials, 3% nonrefractile spores present initially.
[b] Spores exposed to 100 mM L-cysteine for 1 h at pH indicated except as noted in footnote [d].
[c] Spores exposed to 0.9 M D-cysteine for 1 h at pH indicated.
[d] Spores exposed to buffer for 1 h at pH indicated.

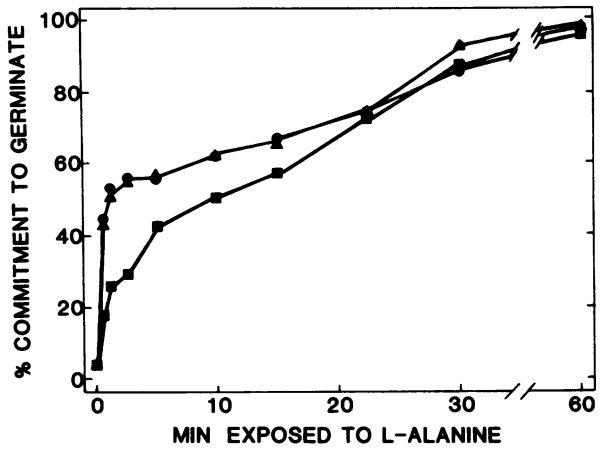

FIG. 3. Effect of recovery in pH 7 buffer on the lag in commitment to germinate induced in *C. botulinum* 62A spores by exposure to pH 4.5 buffer. Data represent averages of two to four duplicate trials. Spore treatments were as follows: 100 mM L-alanine at pH 7 for exposure time indicated followed by 1 h in 1.8 M D-alanine (●); 1 h in pH 4.5 buffer, 1 h in pH 7 buffer, and then 100 mM L-alanine at pH 7 for exposure time indicated followed by 1 h in 1.8 M D-alanine (▲); 1 h in pH 4.5 buffer and then 100 mM L-alanine at pH 7 for exposure time indicated followed by 1 h in 1.8 M D-alanine (■).

pared at pH 7, 5.5, and 4.5 by using L-[^{3}H]alanine (Fig. 5). Spores exposed to L-alanine for 15 min at pH 7 and 5.5 retained equally low levels of labeled L-alanine before and after 1 h of exposure to 1.8 M D-alanine. Spores exposed to 1.8 M D-alanine retained ca. 70% of the initial labeled L-alanine content. Spores exposed to L-alanine at pH 4.5 appeared to retain significantly more labeled L-alanine after 15 min of exposure ($P < 0.05$). After 1 h of exposure to 1.8 M D-alanine at pH 7, there were no significant differences in labeled L-alanine retention at the three pH levels.

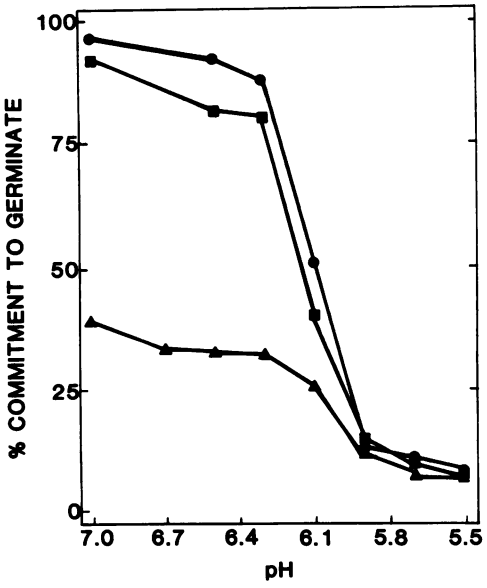

FIG. 4. Influence of pH on the extent of commitment to germinate after 15 min of exposure to 100 or 10 mM L-alanine for *C. botulinum* 12885A spores. Data represent averages of duplicate trials for 100 mM L-alanine and one trial for 10 mM L-alanine. Symbols: ■, 100 mM L-alanine in 100 mM phosphate-citrate buffer; ●, 100 mM L-alaine in 100 mM MES buffer; ▲, 10 mM L-alanine in 100 mM phosphate citrate buffer. All commitment data were collected after incubation for 1 h at pH 7 in 1.8 M D-alanine.

DISCUSSION

Commitment to germinate at pH 7 occurred during 0.5 min of exposure to germinants, but the first visible germination occurred 15 to 20 min after commitment (Fig. 1). This delay represents the duration necessary for the completion of the connecting reactions that lead to loss of refractility (10). Similar results have been reported for *Bacillus terminalis* (13), *Bacillus licheniformis* (11), and *Bacillus megaterium* (21, 22, 25). A biophysical model of triggering proposed by Stewart et al. (22) suggests that germination could be inhibited by acid conditions at three points: (i) inhibition of germinant binding at the trigger site, (ii) inhibition of the formation of an activated complex between the germinant and the trigger site, or (iii) inhibition of the connecting reactions leading to loss of refractility.

C. botulinum and *B. cereus* spores exposed to L-amino acids at pH 4.5 remained refractile and sensitive to D-amino acid inhibition at pH 7 (Tables 2 and 3), indicating that germinant binding had not been completed at pH 4.5. If binding had occurred at pH 4.5, formation of the activated germinant-trigger site complex would occur on transfer to pH 7, and commitment would have been observed despite the presence of the D-amino acid inhibitor. Similarly, if the critical concentration of the activated complex had been formed at pH 4.5, the connecting reactions could have been completed after transfer to pH 7, leading to loss of refractility.

Germinant binding could be inhibited by altering the germinant or the spore. Most of the alanine molecules exist as dipolar ions at pH 4.5 (99.3%) or at pH 7 (99.9%); thus, shifting the pH from 7 to 4.5 did not significantly alter the charge of most of the L-alanine molecules. Decreased L-alanine concentration (100 to 10 mM in Fig. 4) altered the

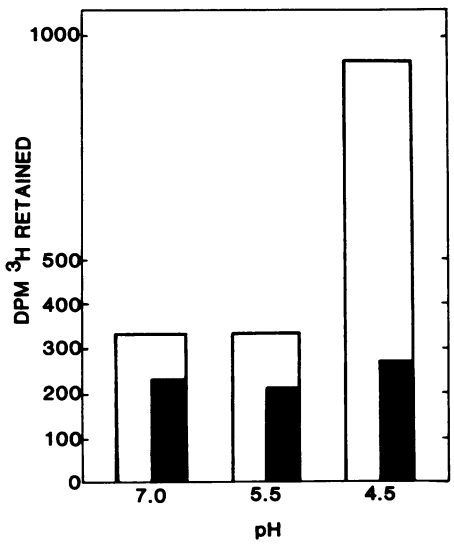

FIG. 5. Uptake and retention of L-[^{3}H]alanine by *C. botulinum* 62A spores in phosphate-citrate buffer at pH 7.0, 5.5, and 4.5. Data represent averages of four duplicate trials. Open bars represent L-alanine uptake after 15 min of exposure; solid bars represent L-alanine retained after 1 h at pH 7 in 1.8 M D-alanine.

rate of commitment but did not affect the pH threshold at which inhibition of commitment was observed. This indicated that inability to bind germinants at an inhibitory pH and not changes in germinant concentration or charge inhibited commitment.

Inhibition of commitment to germinate was similar in two different buffer systems. The lag associated with recovery of commitment was induced by treatment at pH 4.5 with or without germinants being present (Table 4; Fig. 3). Alteration of the germinant-binding capacity did not require spores to interact with a specific buffer system or with germinants. This suggested that titration of sites on the spores with H^+ altered the ability of the spores to bind germinants and inhibited commitment. The lag associated with recovery of commitment in spores transferred from pH 4.5 to pH 7.0 may represent a time delay in the return of these sites to the original charge. This mechanism is consistent with the previous reports on commitment to germinate in *B. megaterium* spores (22) and may represent a generalized mechanism by which bacterial spore germination is inhibited in acid environments.

Use of ^{3}H-labeled L-alanine suggested that L-alanine uptake with *C. botulinum* spores occurred at pH 4.5 (Fig. 5). L-Alanine uptake occurs by passive diffusion in *B. cereus* spores (1, 2). Factors which affect solute uptake include molecular weight, charge, and lipid solubility (9). None of these factors change significantly for L-alanine as the pH decreases from 7 to 4.5; thus, uptake should be similar (Fig. 5). Nonspecific uptake of L-[^{3}H]alanine has been reported for *Bacillus subtilis* spores (6, 23). The amount of nonspecific uptake was linearly related to the L-alanine concentration (6) and was not inhibited by D-alanine (6, 23). Downing and Dawes (6) concluded that L-alanine is trapped but not bound to the spore. Solute taken up by passive diffusion may be localized and associated with the peripheral membranes external to the core of the spore (1). Measurement of commitment as insensitivity to D-amino acid inhibition ensured that carry-over of the germinant from pH 4.5 to 7 did not influence the results.

Acidic conditions (pH 4.5) did not appear to inhibit nonspecific uptake of L-alanine; therefore, inhibition of commitment to germinate at pH 4.5 may involve a change in spore properties more specific than an alteration in the overall ion-exchange properties of the spore as previously suggested (22). The trigger site has been associated with the spore cytoplasmic membrane or the inner spore coat layer (4, 7, 16, 20, 24, 26, 27). Inhibition of germinant binding at pH 4.5 may involve protonation of ionizable groups associated with the trigger site. This could prevent binding by altering the configuration of the binding site or by blocking access of the germinant to the site. Changes in sites associated with the inner spore coat or cytoplasmic membranes should not alter nonspecific uptake of germinants in the outer regions of the spore (9).

Inhibition of germinant binding under acidic conditions was demonstrated for spores of *C. botulinum* and *B. cereus* and appeared to be similar in germination initiated by L-alanine and L-cysteine. This may represent a general mechanism for inhibition of bacterial spore germination at low pH. Uptake or retention of germinants did not appear to be altered.

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