APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1983, p. 1360–1368
0099-2240/83/041360-09$02.00/0

Vol. 45, No. 4

Hypochlorite Injury of *Clostridium botulinum* Spores Alters Germination Responses†

PEGGY M. FOEGEDING‡ AND F. F. BUSTA*

Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108

Received 23 August 1982/Accepted 19 January 1983

Clostridium botulinum spores were sublethally damaged by exposure to 12 or 28 μg of available chlorine per ml for 2 min at 25°C and pH 7.0. The damaging dose was 2.7×10^{-6} to 3.1×10^{-6} μg of available chlorine per spore. Damage was manifested by a consistent 1.6 to 2.4 log difference between the most probable number enumeration of spores (modified peptone colloid medium) and the colony count (modified peptone yeast extract glucose agar); this did not occur with control spores. Damaged spores could be enumerated by the colony count procedure. Germination responses were measured in several defined and nondefined media. Hypochlorite treatment altered the rate and extent of germination in some of the media. Calcium lactate (9 mM) permitted L-alanine (4.5 mM) germination of hypochlorite-treated spores in a medium containing 12 or 55 mM sodium bicarbonate, 0.8 mM sodium thiosulfate, and 100 mM Tris-hydrochloride (pH 7.0) buffer. Tryptose inhibited L-alanine germination of the spores. Treatments with hypochlorite and with hydrogen peroxide (7%, 25°C, 2 min) caused similar enumeration and germination responses, indicating that the effect was due to a general oxidation phenomenon.

Vegetative cells or spores may be damaged after exposure to environmental stresses (6, 18). Recognition of sublethally damaged organisms is imperative for the accurate interpretation of microbiological results. Understanding the mechanisms of bacterial spore injury would heighten understanding of spore physiology and resistance and provide information on the mechanisms of inactivation.

The use of chlorine for the destruction of microorganisms is well recognized; however, most studies are conducted with vegetative cells or aerobic sporeformers. Sanitizers, such as hypochlorite, may sublethally injure vegetative cells (27). Information on the effect of chlorine on anaerobic spores is limited because of the inherent difficulty of working with anaerobes compounded by the complexity of bacterial spores. The toxic nature of *Clostridium botulinum* is another factor which limits work with this organism.

Chlorine treatment slows or prevents the germination of *Clostridium bifermentans, Bacillus cereus*, and *Bacillus subtilis* (32). Yet treatment of slowly germinating mutants of *C. bifermentans* spores with hypochlorite increases the germination rate of these spores (31). Others have indicated that treatment with hypochlorite, sodium hydroxide, and other substances may alter the permeability barrier of *Bacillus* and *Clostridium* spores (13, 22, 30). The altered germination responses and spore permeability may be related. Other halogens and oxidizing agents increase the germination rate of spores (8) and the number of colonies formed from them (9). No reports of which we are aware indicate that hypochlorite treatment may sublethally injure bacterial spores.

The objectives of this report are to demonstrate hypochlorite injury of *C. botulinum* spores, to describe altered germination responses and requirements of hypochlorite-treated *C. botulinum* spores, and to provide information on the chemical nature of the injury.

(This information was presented in part at the 42nd Annual Meeting of the Institute of Food Technologists, Las Vegas, Nev., 22 to 25 June 1982.)

MATERIALS AND METHODS

Spore inoculum. *C. botulinum* 62A and 12885A were used in these experiments. The original cultures of each *C. botulinum* strain were provided as frozen spore suspensions by the Swift and Co. Research Center (Oak Brook, Ill.). Spore crops of the cultures were prepared by the method of Christiansen et al. (7) and were held at 4°C in distilled water.

† Paper no. 13,044, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minn.

‡ Present address: Department of Food Science, North Carolina State University, Raleigh, NC 27650.

Enzymatic cleaning of spore crops. Spores were enzymatically cleaned by a modification of the method of Rowley and Feeherry (26). The washed pellet from approximately 200 ml of the original spore suspension was aseptically mixed with 75 ml of lysozyme solution and incubated for 30 min in a 45°C water bath with stirring. After 30 min, 75 ml of trypsin solution was added, and the incubation was continued with stirring for 2 h. The solutions of lysozyme (U.S. Biochemical Corp., Cleveland, Ohio) and trypsin (U.S. Biochemical) (200 and 100 mg/ml, respectively) were made with 0.05 M potassium phosphate buffer (KH_2PO_4 and K_2HPO_4), pH 8.1, and were filter sterilized (pore size, 0.45 μm). Enzymatically treated spores were washed 10 times with distilled water by centrifugation at 4,100 × *g*, 4°C.

Cleaned washed spores were suspended in distilled water at a concentration of about 10^7 to 2×10^7 spores per ml and were held at 4°C. Microscopic observation confirmed that the final spore suspension was essentially free of vegetative cells and contained about 7 to 10% phase-dark spores.

Compared with repeated washing (20 times), the enzymatic treatment had no measurable influence on the germination of spores of strains 62A and 12885A in modified peptone yeast extract glucose broth (PYEG) or modified peptone colloid medium (MPCM) (P. M. Foegeding, Ph.D. thesis, University of Minnesota, St. Paul, 1982). Enzymatic cleaning could not be replaced by extensive washing to remove microscopically observed debris on spores.

Heat activation. Before all experiments, spores (10^7 to 2×10^7 per ml) were heat shocked in distilled water for 15 min at 80°C (±2°C). Heat shocking immediately preceded hypochlorite or H_2O_2 treatment. Heat activation was within 3 h of enumeration or germination.

Hypochlorite treatment. Hypochlorite solutions were made within 1 h of use by diluting 5% sodium hypochlorite (University of Minnesota Chemical Storehouse) with 100 mM potassium phosphate buffer, pH 7.0, to the desired concentrations. The pH of the hypochlorite solutions was checked and adjusted with 1 to 2 drops of ca. 3 M HCl per 50 to 100 ml of hypochlorite solution to assure a pH of 7.0. Concentrations of available chlorine (AC) were 12 and 28 μg/ml. Filter-sterilized potassium phosphate buffer (100 mM, pH 7.0) was used as a control. Hypochlorite concentrations reported are those actually in contact with the spores. Treatments are reported as micrograms of AC per milliliter, as determined by iodometric titration (1).

Reagents (hypochlorite solutions, buffer, sodium thiosulfate neutralizer) and spore suspensions were tempered to 25 ± 0.2°C. Spores were treated with the selected concentration of hypochlorite for 2 min at 25°C. The treatments were done by rapidly adding the hypochlorite to a test tube or a 12-ml conical centrifuge tube containing the spores and mixing rapidly. Timing was begun at the beginning of the mixing. After 2 min, excess hypochlorite was neutralized with filter-sterilized 0.4% sodium thiosulfate in either 100 mM Tris-hydrochloride buffer, pH 7.0, or 100 mM potassium phosphate buffer, pH 7.0. The buffer in the sodium thiosulfate neutralizer system was the same as that used for the test germination media. If the spores were not used for germination studies, either buffer was used. After the treatment and neutralization, the spore suspensions were held in an ice water bath until needed.

The volume ratio of spore suspension (10^7 to 2×10^7 spores per ml) to the selected hypochlorite solution to neutralizer was 6:1:5. The sodium thiosulfate added was in excess of that needed to neutralize the full amount of hypochlorite added.

Hydrogen peroxide treatment. Hydrogen peroxide solutions were made within 1 h of use by diluting 30% H_2O_2 (Fisher Scientific Co., Pittsburgh, Pa.) with 100 mM potassium phosphate buffer, pH 7.0, to the desired concentrations. Concentrations reported are those actually in contact with the spores. Concentrations from 0 to 10% H_2O_2 were used and were verified by pH measurement (28).

Reagents (H_2O_2 solutions, buffer, catalase neutralizer) and spore suspensions were tempered to 25 ± 0.2°C. Spores were treated with the selected H_2O_2 concentration for 2 min at 25°C. The treatments were done by rapidly adding the H_2O_2 to a 12-ml conical centrifuge tube containing the spores and mixing rapidly. Timing was begun at the beginning of the mixing. After 2 min, excess H_2O_2 was neutralized with a sterile catalase solution (46,000 U/ml; Worthington Diagnostics, Freehold, N.J.), and the tubes were placed in an ice water bath. The volume ratio of spore suspension (10^7 to 2×10^7 spores per ml) to the selected H_2O_2 solution to neutralizer was 6:2:1.5.

Washing spores. The spores were washed to remove the added chemicals to prevent interference with subsequent experimentation. Spore suspensions were washed three times with 2 to 5 ml of cold sterile distilled water. Washing did not alter the responses of the spores (data not shown).

Enumeration procedures. All dilutions were made in 0.1% peptone (Difco Laboratories, Detroit, Mich.) water. Direct microscopic counts of the spore suspensions made in Petroff-Hausser counting chambers were used as population estimates (25).

Colony counts were determined with Lee tubes (24) containing PYEG (peptone yeast extract agar without vitamin K_1 and with 0.1% glucose [16] and with $NaHCO_3$ added after autoclaving to obtain 14 mM $NaHCO_3$ in the medium). Preparation and use of PYEG Lee tubes was as follows. Five g of peptone (Difco), 5 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 10 g of yeast extract (BBL), 1 g of dextrose, 15 g of agar (Difco), 40 ml of salts solution (16), 4 ml of 0.025% (wt/vol) resazurin in distilled water (Eastman Kodak Co., Rochester, N.Y.), and 1 liter of distilled water were mixed, adjusted to pH 7.0 with ca. 1 N HCl or 1 N NaOH, and steamed 20 min at 100°C. After steaming and cooling to ca. 50°C, 10 ml of 0.05% (wt/vol) hemin in 0.01 N NaOH solution and 0.5 g of cysteine hydrochloride-water (Sigma Chemical Co., St. Louis, Mo.) per liter of distilled water were added. The medium was next mixed, and 14.5 ml was dispensed into each Lee tube. The medium was prepared 1 day before use and was held at 4°C overnight. Before inoculation, the PYEG Lee tubes were steamed 15 min and tempered to 46 to 48°C, and 0.2 ml of freshly prepared, filter-sterilized $NaHCO_3$ (86 mg/ml; Matheson, Coleman and Bell, Norwood, Ohio) per tube in distilled water was aseptically added. Duplicate or triplicate PYEG Lee tubes were inoculated with 0.1 ml of the appropriate culture dilution while the tubes were sitting on a warm hot

TABLE 1. Germination media

Code	Component	Concn[a]
A	L-Alanine (Sigma)	4.5 mM
B	Sodium bicarbonate (Matheson, Coleman and Bell)	12 mM
B^+	Sodium bicarbonate (Matheson, Coleman and Bell)	55 mM
C	L-Cysteine hydrochloride-water (Sigma)	9 mM
G	Sodium thioglycolate (Difco)	4 mM
L	DL-Calcium lactate (Fisher)	9 mM
T	Tryptose (Difco)	1.8%
X	Tris-hydrochloride buffer (THAM, Fisher), pH 7.0 at 35°C	100 mM
Z	Potassium phosphate buffer (KH_2PO_4, K_2HPO_4; Mallinkrodt, Inc., St. Louis, Mo.), pH 7.0	100 mM
	Sodium thiosulfate (Fisher)[b]	0.8 mM

[a] Concentrations listed have taken into account dilution owing to inoculation.

[b] Sodium thiosulfate was present in each medium and is not designated by a code.

plate (low setting; temperature, ca. 45°C). The tubes were mixed and incubated at 35°C for 20 to 26 h. Colonies were counted with the aid of a Quebec colony counter. Salt solution components were 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 119 mM $NaHCO_3$, 5.7 mM K_2HPO_4, 7.4 mM KH_2PO_4, and 34 mM NaCl.

Enumeration was also carried out by a most probable number (MPN) procedure in MPCM as described by Sofos et al. (29). Serial dilutions of heat-shocked spores were inoculated into five MPCM tubes, and the tubes were capped with Vaspar and incubated at 35°C for 7 days. Positive MPCM tubes, indicated by blackening and gas production, were used to calculate the MPN and the 95% confidence interval.

Germination procedures. Components of the germination media, their concentrations, and the codification system are detailed in Table 1. The germination media were prepared from the dry components immediately before use, filter sterilized (pore size, 0.45 μm), and held in an ice water bath until the incubation was begun. Buffers were prepared up to 2 months ahead of use and were stored at 4°C. The pH values of all the media were between 6.97 and 7.36.

Germination experiments were performed by aseptically adding 0.5 ml of each germination medium to separate sterile glass test tubes (10 by 75 mm). Each tube of germination medium was inoculated with 2 drops of the desired spore suspension (ca. 10^7 to 5×10^7 spores per ml of suspension) from a Pasteur pipette. Tubes of germination media were placed in an ice water bath during inoculation and until incubation was begun. Racks containing ≤ 35 tubes were incubated at 35°C in a water bath. The germination media reached 35°C in approximately 30 s. Sampling was done at selected times by aseptically removing 1 drop of each inoculated germination medium and placing it onto a clean flamed cover slip. Cover slips were held on a hot plate (ca. 50°C) before and during sampling. Samples dried in ca. 15 s. The total time to sample tubes from one rack was ≤ 5 min, so the actual sampling time may have ranged from the indicated time to the indicated time plus 5 min.

Cover slips were placed onto microscope slides coated with ca. 0.5 ml of 0.2% purified agar or onto 1 drop of distilled water on microscope slides. The smears were examined by phase-contrast microscopy at magnifications of ×1,000 or ×1,250 (Nikon Labophot, Nippon Kogaku K.K., Tokyo, Japan, with blue filter or Zeiss Universal, Carl Zeiss, Oberkochen, West Germany, with green filter). A total of 100 to 200 spores were counted. During counting, spores were recorded as phase bright or phase dark. Only fully bright spores were counted as phase bright. Spores appearing either fully or partially dark were recorded as phase-dark spores. Of the spores recorded as phase dark, generally 10% were of the partially dark variety.

The negative controls for the germination experiments were the spore treatments placed in the appropriate buffer bases (100 mM potassium phosphate, pH 7.0, or 100 mM Tris-hydrochloride, pH 7.0) corresponding to the buffer of the test germination medium and were incubated at 35°C with the germination media. Results are reported as percentages of germination, i.e., the percentage of phase-dark spores in the test germination medium less the percentage of phase-dark spores in the negative control. Generally, the controls contained about 7 to 10% phase-dark spores, regardless of sampling time.

Amino acid analysis. Total amino acids in tryptose were determined with a Dionex D300 microbore column kit (Dionex Corp., Sunnyvale, Calif.). Free (soluble) amino acids in tryptose were determined as follows. To 1 ml of 27 mg of tryptose per 100 ml of water was added 0.8 ml of 5 M norleucine (internal standard) in water and 2.7 ml of 13% sulfosalicylic acid in water. The mixture was centrifuged, and the supernatant fluid (0.676 ml) was removed and titrated to pH 2.2 with 0.5 N NaOH. The volume was brought to 1 ml with water. Free amino acids were determined in the titrated diluted supernatant fluid.

RESULTS

Enumeration. Enumeration data for *C. botulinum* 12885A and 62A spores treated with 0, 12, and 28 μg of AC per ml are presented in Table 2. Colony counts from the PYEG Lee tubes consistently were about one-half log cycle higher than the MPN data in MPCM for buffer-treated spores of strains 12885A and 62A (0 μg of AC per ml) and for strain 12885A spores treated with 12 μg of AC per ml. The average population of buffer-treated *C. botulinum* 12885A spores enumerated with PYEG Lee tubes was 9.4×10^6 CFU/ml, which agreed well with the calculated number of spores added based upon direct microscopic count of the phase-bright spores in the stock suspension. The calculated number of *C. botulinum* 12885A spores added was 10^7 per ml. Agreement of the average population enumerated by PYEG Lee tubes and the calculated population from the direct microscopic count of the stock suspension for buffer-treated strain 62A

TABLE 2. Enumeration of buffer-treated and hypochlorite-treated *C. botulinum* spores

C. botulinum strain	AC concn (μg/ml)	Log population	
		Colony counts (CFU/ml)[a]	MPN procedure (MPN/ml)[b]
12885A[c]	0	6.97	6.28 (5.94–6.52)
	12	6.94	6.19 (5.74–>6.45)
	28	6.37	3.97 (3.64–4.28)
62A[d]	0	6.70	6.14 (5.60–6.48)
	12	5.73	4.13 (3.85–4.67)
	28	3.72	2.12 (1.81–2.52)

[a] PYEG Lee tube procedure.
[b] MPCM five-tube MPN procedure; numbers in parentheses show 95% confidence interval.
[c] Data represent three replicate trials.
[d] Data represent three or four replicate trials.

spores was excellent. The population was 5.0×10^6 per ml by each of the methods.

Populations of *C. botulinum* 12885A spores treated with 28 μg of AC per ml and of strain 62A spores treated with 12 and 28 μg of AC per ml were 2.4, 1.6, and 1.6 logs, respectively, lower by the MPN procedure than by the PYEG Lee tube procedure (Table 2). *C. botulinum* 62A spores were apparently inactivated by treatment with 28 μg of AC per ml since the populations recovered by both enumeration procedures were about 3 to 4 log cycles less than buffer-treated spores. By both enumeration procedures, the population of *C. botulinum* 12885A spores remained essentially constant for treatment with 0 to 12 μg of AC per ml. For spores treated with 28 μg of AC per ml, some inactivation was indicated by the small decrease of the population (colony count enumeration) as compared with buffer-treated controls. MPCM with added agar placed in Lee tubes was no more productive than the medium used in MPN tubes overlaid with Vaspar for the recovery of buffer-treated or hypochlorite-treated spores (data not shown).

Germination. Germination of hypochlorite-treated and buffer-treated *C. botulinum* 12885A spores was evaluated in several media. Figures 1 and 2 show representative germination responses of the spores in media designated ABLX and TB^+Z, respectively (see Table 1). The data presented in Fig. 1 and 2 are each examples of one representative trial in two of the four primary media studied. Data from replicate trials in each of the media studied frequently differed in the exact percentage of germination observed, but the relative amounts of germination and the trends observed were consistent within replicates. Consequently, relative responses were compared. This avoided overemphasizing the actual numerical extents of germination.

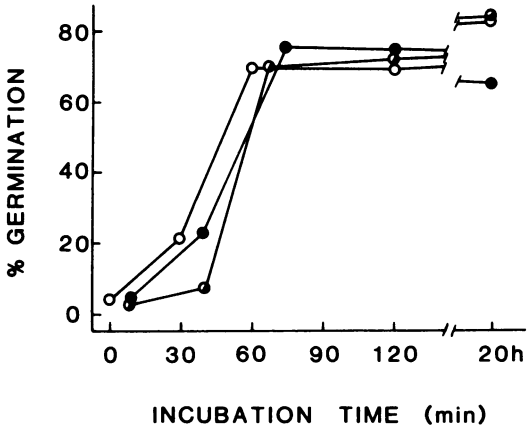

FIG. 1. Germination of buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores in medium ABLX (see Table 1). Data represent one trial. AC concentrations, 0 (○), 12 (◑), and 28 (●) μg/ml.

In medium ABLX (Fig. 1), the rates and extents of germination were similar for spores treated with 0, 12, or 28 μg of AC per ml. However, in medium TB^+Z (Fig. 2), increased hypochlorite treatment decreased both the rate and extent of spore germination. Data in Fig. 1 and 2 show that the effect of the hypochlorite treatment on the spore germination response varied in different media and was evident at 120 min of incubation. Consequently, subsequent comparisons were based upon the extent of germination at 2 h.

Germination responses of buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores in the four primary germination media studied are presented in Fig. 3. Treatment of the spores with hypochlorite reduced the extent of

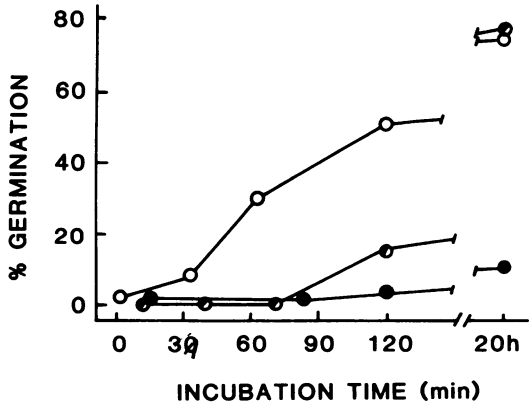

FIG. 2. Germination of buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores in medium TB^+Z (see Table 1). Data represent one trial. Symbols as in legend to Fig. 1.

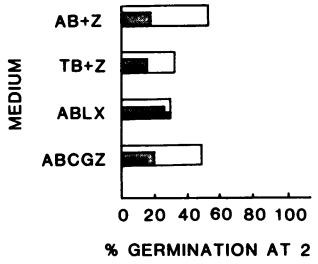

FIG. 3. Extent of germination after 2 h for buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores in AB^+Z, TB^+Z, ABLX, and ABCGZ germination media (see Table 1). Data represent averages of three to seven trials. AC concentrations, 0 (□), 12 (▦), and 28 (■) μg/ml.

germination at 2 h compared with that of buffer-treated spores in the media designated AB^+Z, TB^+Z, and ABCGZ (see Table 1). However, hypochlorite treatment did not reduce the extent of germination after 2 h in medium ABLX compared with that of buffer-treated spores. To determine which medium components supported or interfered with the germination of hypochlorite-treated spores, components were added, deleted, or exchanged, and germination responses were evaluated in these reformulated media. Because essentially equivalent extents of germination of hypochlorite-treated and buffer-treated spores occurred only in medium ABLX, components which were present only in or absent only from medium ABLX were targeted for further study. These components were Tris-hydrochloride buffer and calcium lactate.

The influences of the buffer system, sodium bicarbonate concentration, and the presence or absence of sodium thioglycolate, cysteine hydrochloride, and sodium thiosulfate were examined in several medium formulae (data not shown). The influences of each of these variables on the germination of buffer-treated and hypochlorite-treated spores were minimal, except that 55 mM sodium bicarbonate generally increased the extent of germination (ca. 30%) in 2 h compared with 12 mM sodium bicarbonate. In no case did the presence, absence, or change in concentration of these components result in equal extents of germination of spores treated with 0 or 28 μg of AC per ml.

The influence of DL-calcium lactate on the germination of buffer-treated and hypochlorite-treated spores of *C. botulinum* 12885A in four media was studied (Fig. 4). The presence of calcium lactate generally increased the germination of buffer-treated spores by 20 to 40% after 2 h. Only in medium AB^+X, when compared with medium AB^+LX (see Table 1), did the presence of calcium lactate have little effect (7% difference) on the germination of buffer-treated spores.

The presence of calcium lactate also increased the extent of germination of hypochlorite-treated spores in each of the four basic media presented in Fig. 4. In some media the increase was negligible (3 to 12% for TB^+X versus TB^+LX and ABCGX versus ABCGLX). The beneficial effect of calcium lactate on the germination of hypochlorite-treated spores in medium AB^+LX was extensive. This was readily apparent when the extents of germination of buffer-treated and hypochlorite-treated spores were compared in the media without (AB^+X) and with (AB^+LX) calcium lactate. In medium AB^+X, a stepwise decrease in germination at 2 h resulted from the treatment of spores with increasing amounts of hypochlorite. However, in medium AB^+LX, with calcium lactate present, the extent of germination was essentially equivalent regardless of hypochlorite treatment. Merely adding calcium lactate to the germination media ABCGX and TB^+X, forming ABCGLX and TB^+LX, did not result in equivalent germination of hypochlorite-treated and buffer-treated spores. Rather, for media ABCGX and TB^+X as well as for their calcium lactate-containing counterparts, ABCGLX and TB^+LX, there was a stepwise decrease in the extent of germination at 2 h with increasing hypochlorite treatment. The data presented in Fig. 4 indicate that the influence of calcium lactate on germination of hypochlorite-treated spores is small in media ABCGLX and TB^+LX compared with media ABCGX and TB^+X, intermediate in medium TAB^+LX compared with TAB^+X, and large in medium AB^+LX compared with AB^+X.

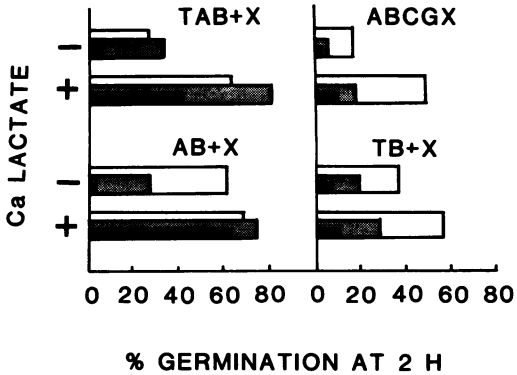

FIG. 4. Influence of the presence (+) or absence (−) of DL-calcium lactate on the germination of buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores. Data represent averages of three, one, four, and four trials in TAB^+X, ABCGX, AB^+X, and TB^+X medium systems (see Table 1), respectively. Symbols as in legend to Fig. 3.

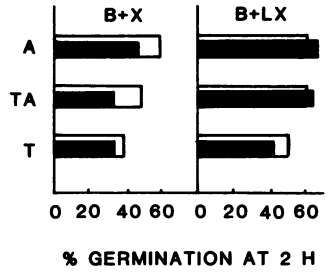

FIG. 5. Influence of L-alanine on the germination of buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores in media without and with tryptose and without and with calcium lactate. Data represent averages of three trials. Symbols as in legend to Fig. 3.

The only difference in three of the basic media shown in Fig. 4 was the amino acid or protein source present. The media TAB^+X (TAB^+LX), AB^+X (AB^+LX), and TB^+X (TB^+LX) (see Table 1) differed by the presence of tryptose plus L-alanine, L-alanine alone, or tryptose alone. A comparison of media formulated with these three components without and with added calcium lactate is presented in Fig. 5. In the media without added calcium lactate, L-alanine supported the highest and tryptose supported the lowest germination for buffer-treated spores of strain 12885A. This trend was also generally true for hypochlorite-treated spores of strain 12885A in the media without calcium lactate. The data on extent of germination, $T \leq TA < A$, indicate that (i) the presence of tryptose or a component of tryptose interfered with L-alanine germination; and (ii) if the L-alanine content of tryptose were larger, tryptose would have supported more germination of hypochlorite-treated and non-hypochlorite-treated spores. As shown in Fig. 4, when calcium lactate was present along with L-alanine, buffer-treated and hypochlorite-treated spores showed essentially equal extents of germination in 2 h. These data illustrate the importance of a combination of L-alanine plus calcium lactate in permitting the germination of hypochlorite-treated spores. They further demonstrate the interference by tryptose or a tryptose component(s) in the L-alanine germination of the spores.

The concentrations of free and total amino acids contributed by tryptose to the germination media are presented in Table 3. The free alanine concentration owing to tryptose in the media was 1.85 mM, whereas 4.5 mM L-alanine was added to the media. The total alanine concentration due to tryptose in the media was 6.28 mM.

Hydrogen peroxide. The effect of hydrogen peroxide on the enumeration and germination of *C. botulinum* 12885A spores was investigated for comparison with the effect of hypochlorite. Enumeration data for hydrogen peroxide-treated spores are presented in Table 4. By both enumeration methods, the spore population remained essentially constant, about 6×10^6 CFU/ml or 2×10^6 MPN/ml for H_2O_2 treatments up to 5%, indicating that no inactivation resulted from these levels of H_2O_2. Spores treated with 7% H_2O_2 were differentially recovered by the two enumeration procedures. There was a difference of approximately 2.5 log cycles in the results by the two procedures, and the PYEG Lee tube results were higher than the MPN data. Treatment with 10% H_2O_2 resulted in apparent inactivation since the population recovered by either procedure was much less (>3 log cycles) than for spores treated with 0% H_2O_2.

Germination data for spores treated with H_2O_2 or hypochlorite are presented for comparison in Fig. 6. Treatment of the spores with 7% H_2O_2 resulted in 32 to 37% less germination in 2 h than treatment with 0 or 5% H_2O_2 in medium AB^+Z. In medium ABLX, the extent of germination was essentially equal despite H_2O_2 treat-

TABLE 3. Free and total amino acid concentrations in germination media owing to tryptose[a]

Amino acid	Concn (mM)	
	Free[b]	Total[c]
Alanine	1.85	6.28
Arginine	1.30	3.35
Aspartic acid	1.30	5.98
Carboxymethyl cysteine	—[d]	—
Cysteic acid	—	—
Cysteine (half cystine)	—	—
Glutamic acid	1.48	11.90
Glycine	1.17	8.89
Histidine	4.39[e]	1.44
Isoleucine	1.13	3.47
Leucine	4.86	7.15
Lysine	—	4.84
Methionine[f]	1.49	0.36
Phenylalanine	2.52	3.02
Proline	—	7.79
Serine	2.07	4.64
Threonine	1.30	3.94
Tryptophan	5.74	≥5.74[g]
Tyrosine	2.30	3.28
Valine	2.09	5.56

[a] Based on amino acid analysis of tryptose and presence of 1.8% tryptose in media.

[b] Based on amino acid analysis of tryptose components not precipitated by sulfosalicylic acid.

[c] Based on amino acid analysis of tryptose hydrolysate.

[d] —, None detected.

[e] Peak shifted, so positive identification as histidine was not possible.

[f] Small peptides may have eluted with the free methionine or some methionine may not have been stable to hydrolysis.

[g] Tryptophan was not stable to hydrolysis.

TABLE 4. Enumeration of buffer-treated and hydrogen peroxide-treated *C. botulinum* 12885A spores

H_2O_2 concn (%)	Log population	
	Colony counts (CFU/ml)[a]	MPN procedure (MPN/ml)[b]
0	6.71	6.29 (5.93–6.57)
2	6.68	6.38 (5.97–6.62)
5	6.87	6.20 (5.73–6.54)
7	6.74	4.35 (3.83–4.59)
10	3.37	1.80 (1.38–2.23)

[a] PYEG Lee tube procedure; data represent duplicate trials.

[b] MPCM five-tube MPN procedure; numbers in parentheses show 95% confidence interval; data represent duplicate trials.

ment. Similar results have been described for hypochlorite-treated spores and are presented in Fig. 6 for comparison.

DISCUSSION

C. botulinum 12885A and 62A spores were injured under these experimental conditions by limited exposure to sodium hypochlorite. Exposure of enzymatically cleaned spores to 12 or 28 μg of AC per ml for 2 min at pH 7.0 and 25°C resulted in sublethal damage or injury of spores of strains 62A and 12885A. The injury was manifested by a large consistent difference in colony count (PYEG Lee tubes) versus MPN enumeration of spores in MPCM; this difference did not occur with non-hypochlorite-treated spores. To the best of our knowledge, this is the first report identifying sublethal hypochlorite injury of bacterial spores. Scheusner et al. (27) indicated that hypochlorite treatment causes sublethal injury of vegetative cells; some 29 to 60% of treated *Escherichia coli* cells (10^9 cells per ml) were injured by 1 μg of active hypochlorite per ml (0°C, 60 s, pH 7.2). Several reports have documented that spores of *Clostridium* species are generally less resistant to chlorine than are those of *Bacillus* species (19, 23).

Under our experimental conditions, *C. botulinum* 62A and 12885A spores were injured by 12 and 28 μg of AC per ml, respectively. The damaging hypochlorite doses were 2.9×10^{-6} to 3.1×10^{-6} μg of available chlorine per spore for strain 12885A and 2.7×10^{-6} μg of available chlorine per spore of strain 62A. These doses were lower than those used by Ito et al. (20) to inactivate *C. botulinum* spores. Ito and coworkers (20) reported that 4.5 μg of AC per ml (pH 6.5, 25°C) reduced 99.99% of types A and B *C. botulinum* spore populations (10^4 spores per ml) in 3 to 8 min. Their dose which inactivated 99.99% of the spores was 4.5×10^{-4} μg per spore, with a longer exposure time and lower pH. The AC per spore must be considered since hypochlorite activity is reduced by high organic loads.

Germination responses of hypochlorite-treated *C. botulinum* 12885A spores were similar to or different from those of buffer-treated spores depending on the formulations of the media. The germination response was a sensitive measure of injury. *C. botulinum* 12885A spores treated with 12 μg of AC per ml germinated less than controls in some media (TB^+Z, ABCGZ, etc.) but not all (ABLX); nevertheless, the spores were essentially equivalently enumerated.

The rates and extents of germination in some media were altered by hypochlorite treatment. This has been observed with *C. bifermentans*, *B. cereus*, and *B. subtilis* spores treated with chlorine (32). Similar extents of germination at 2 h for hypochlorite-treated and buffer-treated spores occurred only in the medium containing 4.5 mM L-alanine, 12 mM sodium bicarbonate, 9 mM DL-calcium lactate, 100 mM Tris-hydrochloride buffer (pH 7.0), and 0.8 mM sodium thiosulfate. An evaluation of the influences of medium components on the germination of hypochlorite-treated and buffer-treated spores indicated that increasing the sodium bicarbonate concentration or adding calcium lactate to the medium resulted in a general increase in germination of the spores, whereas adding sodium thioglycolate or cysteine hydrochloride, substituting Tris-hydrochloride for potassium phosphate buffer, or eliminating sodium thiosulfate from the medium had a minimal effect on spore germination. These findings are in general agreement with other reports on germination requirements of *Clostridium* spores (2, 14, 17, 21, 33). However, researchers have indicated that ions, particularly potassium and phosphate ions, may cause or enhance the germination of *Clostridium*

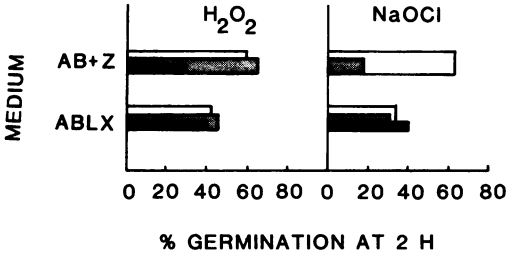

FIG. 6. Extent of germination after 2 h of buffer-treated, hydrogen peroxide-treated, and hypochlorite-treated *C. botulinum* 12885A spores in AB^+Z and ABLX media (see Table 1). Data represent averages of duplicate trials. Hydrogen peroxide concentrations for hydrogen peroxide-treated spores, 0% (□), 5% (▦), and 7% (■); AC concentrations for hypochlorite-treated spores as in legend to Fig. 3.

spores (F. E. Feeherry and H. S. Levinson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, I10, p. 88; 3); nevertheless, our data did not indicate enhanced germination owing to potassium phosphate buffer.

The full possible extent of germination of the hypochlorite-treated spores (an extent of germination at 2 h equivalent to that of spores treated with 0 μg of AC per ml) required both DL-calcium lactate and L-alanine. Tryptose inhibited L-alanine germination of both buffer-treated and hypochlorite-treated spores.

The free alanine concentration in tryptose-containing media was 1.85 mM, less than one-half the L-alanine concentration (4.5 mM) added to the germination media. Other free amino acids were present in similar concentrations, and free amino acids such as glycine and L-arginine can inhibit germination (12, 15). D-Amino acids also are known to inhibit L-amino acid-initiated germination (15). Rowley and Feeherry (26) screened 16 amino acids for their effect on *C. botulinum* 62A germination. They reported that only L-cysteine and L-alanine significantly initiated germination in a medium containing sodium bicarbonate, sodium thioglycolate, and the amino acid at pH 7.0. Inhibition of germination owing to some of the free amino acids in tryptose (35 mM total) was likely. Increasing the free alanine concentration partially overcame the inhibitory effect of tryptose (medium T versus TA; see Fig. 5), suggesting that the inhibition may be competitive in nature.

Several explanations exist for the observed germination requirement of hypochlorite-treated spores for calcium lactate plus L-alanine. Calcium lactate may be a germinant, a precursor to a germinant, or an enhancer of the ability of L-alanine to germinate the spores by activator-type activity. These questions are addressed elsewhere in this journal (10, 11).

Because hypochlorite is a strong oxidizing agent, the observed injury of the spores may have been the result of indiscriminant oxidation. Hydrogen peroxide, another oxidizing agent, was selected for comparison. *C. botulinum* 12885A spores were sublethally damaged by treatment with 7% H_2O_2, resulting in enumeration and germination responses similar to those of the hypochlorite-damaged spores. The similar results obtained by treating spores with either sodium hypochlorite or H_2O_2 suggest that these observed effects were the result of a general oxidation mechanism rather than being specific to the chemical treatment. Others have reported similar effects from the treatment of spores with H_2O_2 and sodium hypochlorite (4, 31, 32), although *B. subtilis* spores produced by different procedures did not show the same relative resistance to these two oxidizing agents (5).

ACKNOWLEDGMENTS

This research was supported in part by University of Minnesota Agricultural Experiment Station project 18-59. P.M.F. was supported by General Foods-Institute of Food Technologists and Ralston Purina Food Science Fellowships.

We thank Cindy L. Nelson and Lorraine B. Smith for invaluable assistance.

LITERATURE CITED

1. **American Public Health Association, American Water Works Association, and Water Pollution Control Federation.** 1975. Chlorine (residual). B. Iodometric method I, p. 316–318. *In* M. C. Rand, A. E. Greenberg, and M. J. Taras (ed.), Standard methods for the examination of water and wastewater, 14th ed., part 409. American Public Health Association, Washington, D.C.
2. **Ando, Y.** 1971. The germination requirements of spores of *Clostridium botulinum* type E. Jpn. J. Microbiol. **15**:515–525.
3. **Ando, Y.** 1974. Ionic germination of spores of *Clostridium perfringens* type A. Jpn. J. Microbiol. **18**:433–439.
4. **Bayliss, C. E., and W. M. Waites.** 1976. The effect of hydrogen peroxide on spores of *Clostridium bifermentans*. J. Gen. Microbiol. **96**:401–407.
5. **Bayliss, C. E., W. M. Waites, and N. R. King.** 1981. Resistance and structure of spores of *Bacillus subtilis*. J. Appl. Bacteriol. **50**:379–390.
6. **Busta, F. F., P. M. Foegeding, and D. M. Adams.** 1981. Injury and resuscitation of germination and outgrowth of bacterial spores, p. 261–265. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
7. **Christiansen, L. N., R. W. Johnston, D. A. Kautter, J. W. Howard, and W. J. Aunan.** 1973. Effect of nitrite and nitrate on toxin production by *Clostridium botulinum* and on nitrosamine formation in perishable canned comminuted cured meat. Appl. Microbiol. **25**:357–362. (Erratum **26**:653).
8. **Cochran, S. A., and Z. J. Ordal.** 1973. Oxidative activation of *Bacillus cereus* spores. Appl. Microbiol. **25**:173–179.
9. **Cousins, C. M., and C. D. Allan.** 1967. Sporicidal properties of some halogens. J. Appl. Bacteriol. **30**:168–174.
10. **Foegeding, P. M., and F. F. Busta.** 1983. Proposed role of lactate in germination of hypochlorite-treated *Clostridium botulinum* spores. Appl. Environ. Microbiol. **45**:1369–1373.
11. **Foegeding, P. M., and F. F. Busta.** 1983. Proposed mechanism for sensitization by hypochlorite treatment of *Clostridium botulinum* spores. Appl. Environ. Microbiol. **45**:1374–1379.
12. **Foerster, H. F.** 1981. L-Arginine inhibition of the initiation of germination in spores of *Thermoactinomyces thalpophilus*, p. 289–293. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Association for Microbiology. Washington, D.C.
13. **Galanina, L. A., L. L. Mityushina, V. I. Duda, and M. N. Bekhtereva.** 1979. Electron microscopic study of *Bacillus anthracoides* spores under normal conditions and upon exposure to chloroactive disinfectant. Microbiology (Engl. Transl. Mikrobiologiya) **48**:368–372.
14. **Heiligman, F., N. W. Derosier, and H. Broumand.** 1956. Spore germination. I. Activators. Food Res. **21**:63–69.
15. **Hills, G. M.** 1950. Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain and conditions of growth. J. Gen. Microbiol. **4**:38–47.
16. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
17. **Holland, D., A. N. Barker, and J. Wolf.** 1969. Factors affecting germination of clostridia, p. 317–323. *In* L. L.

Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
18. **Hurst, A.** 1977. Bacterial injury: a review. Can. J. Microbiol. **23**:935–944.
19. **Ito, K. A., and M. L. Seeger.** 1980. Effects of germicides on microorganisms in can cooling waters. J. Food Prot. **43**:484–487.
20. **Ito, K. A., D. J. Seslar, W. A. Mercer, and K. F. Meyer.** 1967. The thermal and chlorine resistance of *Cl. botulinum* types A, B, and E spores, p. 108–122. *In* M. Ingram and T. A. Roberts (ed.), Botulism 1966. Chapman & Hall, Ltd., London.
21. **King, W. L., and G. W. Gould.** 1971. Mechanism of stimulation of germination of *Clostridium sporogenes* spores by bicarbonate, p. 71–83. *In* A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spore research 1971. Academic Press, Inc. (London) Ltd., London.
22. **Kulikovski, A. V.** 1976. Structural and biochemical changes in spores of *Bacillus cereus* produced by caustic soda and hypochlorite. Microbiology (Engl. Transl. Mikrobiologiya). **45**:113–118.
23. **Odlaug, T. E., and I. J. Pflug.** 1976. Sporicidal properties of chlorine compounds: applicability to cooling water for canned foods. J. Milk Food Technol. **39**:493–498.
24. **Ogg, J. E., S. Y. Lee, and B. J. Ogg.** 1979. A modified method for the cultivation and enumeration of anaerobic bacteria. Can. J. Microbiol. **25**:987–990.
25. **Pusch, D. J., F. F. Busta, W. A. Moats, and A. E. Schulze.** 1976. Direct microscopic count, p. 132–151. *In* M. L. Speck (ed.), Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, D.C.
26. **Rowley, D. B., and F. Feeherry.** 1970. Conditions affecting germination of *Clostridium botulinum* 62A spores in a chemically defined medium. J. Bacteriol. **104**:1151–1157.
27. **Scheusner, D. L., F. F. Busta, and M. L. Speck.** 1971. Injury of bacteria by sanitizers. Appl. Microbiol. **21**:41–45.
28. **Smith, Q. J., and K. L. Brown.** 1980. The resistance of dry spores of *Bacillus subtilis* var. *globigii* (NCIB 8058) to solutions of hydrogen peroxide in relation to aseptic packaging. J. Food Technol. **15**:169–179.
29. **Sofos, J. N., F. F. Busta, and C. E. Allen.** 1979. Sodium nitrite and sorbic acid effects on *Clostridium botulinum* spore germination and total microbial growth in chicken frankfurter emulsions during temperature abuse. Appl. Environ. Microbiol. 37:1103–1109.
30. **Waites, W. M., L. R. Wyatt, and B. Arthur.** 1972. Effect of alkali treatment on the germination and morphology of *Clostridium bifermentans*, p. 430–436. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
31. **Wyatt, L. R., and W. M. Waites.** 1973. The effect of hypochlorite on the germination of spores of *Clostridium bifermentans*. J. Gen. Microbiol. **78**:383–385.
32. **Wyatt, L. R., and W. M. Waites.** 1975. The effect of chlorine on spores of *Clostridium bifermentans, Bacillus subtilis* and *Bacillus cereus*. J. Gen. Microbiol. **89**:337–344.
33. **Wynne, E. S., and J. W. Foster.** 1948. Physiological studies on spore germination, with special reference to *Clostridium botulinum*. III. Carbon dioxide and germination, with a note on carbon dioxide and aerobic spores. J. Bacteriol. **55**:331–339.