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Conditions Affecting Germination of *Clostridium botulinum* 62A Spores in a Chemically Defined Medium[1]

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Spores of *Clostridium botulinum* type 62A were germinated in a chemically defined medium (8 mM L-cysteine, 11.9 mM sodium bicarbonate, 4.4 mM sodium thioglycolate; buffered with 100 mM TES, *p*H 7.0). The rate and extent of germination were increased when an aqueous spore suspension was heated sublethally (80 C, 60 min) before addition to the germination medium. Neither sublethal nor lethal doses of gamma radiation had any marked effect on subsequent germination. Maximum germination (>90% in 2 hr) in the defined medium occurred in the *p*H range of 6.5 to 7.5, at 30 to 37 C, with an L-cysteine level of 8 mM. Increasing L-cysteine to 32 mM increased the rate (over that with 8 mM L-cysteine) but not the extent of germination. The rate and extent of germination increased with $NaHCO_3$ addition to 8.3 mM, but increasing levels to 11.9 mM had no further effect. For maximum germination, 2.2 mM sodium thioglycolate was required and higher levels (to 8.8 mM) had no further enhancing or inhibitory effect. Under optimal conditions for germination, 97% of the spores had become heat sensitive; 98% had become sensitive to radiation; 88 and 91% had become phase dark and stainable, respectively, and the spore suspension had lost 46% of its initial optical density by 2 hr. Loss of heat resistance preceded loss of radiation resistance, acquisition of stainability, and phase darkening by about 12 min.

Of 16 individual amino acids screened, in preliminary experiments, for their effect on the germination of *Clostridium botulinum* 62A spores, only L-cysteine and L-alanine initiated significant germination (90%) of spores when incubated (37 C, 24 hr) in a medium containing the amino acid plus sodium bicarbonate and sodium thioglycolate buffered at *p*H 7.0. Germination in L-alanine alone or as a component of a mixture was previously described for *C. roseum* (12), *C. bifermentans* (7), and *C. sporogenes* 3679h (20). Although spores of *Bacillus* species germinate in L-cysteine (15), we are not aware of any published reports of the germination of *Clostridium* spores in this amino acid. In the present report we describe the influence of heat activation, gamma radiation, initial *p*H of the medium, concentration of constituents, and temperature of incubation on the extent and rate of germination of *C. botulinum* 62A spores in L-cysteine, sodium bicarbonate, and sodium thioglycolate.

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The sequence of various germination events (loss of heat and radiation resistance, loss of optical density, phase darkening, and acquisition of stainability) under the established optimal conditions is also reported.

MATERIALS AND METHODS

Spore preparation. Spores of *C. botulinum* type 62A were produced in a biphasic culture system similar to that of Bruch et al. (3). Actively growing vegetative cells were used as the inoculum for the liquid phase of the biphasic system.

All liquid media were steamed for 20 min to remove oxygen, and were rapidly cooled to 37 C just before inoculation. When filter-sterilized sodium bicarbonate ($NaHCO_3$) was used to enhance germination, it was added after cooling of the medium. The inoculation sequence was initiated by incubating 2 ml of a heated (80 C, 10 min) spore stock culture (Difco brain liver heart) in 18 ml of a germination-growth (G-G) medium (Rowley, Feeherry, and El-Bisi, Bacteriol. Proc., p. 35, 1968) at 30 C for 16 hr, during which time spores germinated and vegetative cells grew, but no spores were formed.

Subsequent build-up of the vegetative inoculum

was by short-term (3 hr) incubation in the sporulation (S) medium (5% BBL Trypticase, 0.5% Difco Peptone, 0.1% BBL Yeast Extract, and 0.125% K_2HPO_4, *p*H 7.5). This S medium (45 ml) was incubated at 37 C for 3 hr with 5 ml of the 16-hr vegetative culture (G-G medium), and 14 ml of the resulting S medium culture were incubated with 126 ml of fresh S medium at 37 C for an additional 3 hr to give the final inoculum. Fernbach-type culture flasks (2,500 ml) contained 600 ml of the S medium with 3.0% agar. The S agar medium was overlaid with 135 ml of 2.0% $(NH_4)_2SO_4$. The latter liquid phase of the biphasic system was inoculated with 15 ml of the final 3-hr vigorously growing vegetative culture; the atmosphere of the flask was flushed with pure nitrogen for 5 min to remove oxygen; the cultures were incubated at 37 C for 6 days and stored at 5 C for 17 hr before harvest.

Spores in the liquid phase were harvested by centrifugation and were washed 8 to 10 times with glass-distilled water by repeated suspension and 5-min centrifugation at 4,080 × *g* (5 C). The remaining vegetative cells and debris were eliminated by a modification of the lysozyme-trypsin enzyme treatment described by Grecz et al. (10). The washed pellet from 150 ml of the original culture was suspended in 50 ml of an enzyme solution (50 μg of trypsin and 100 μg of lysozyme per ml, in 0.05 M K_2HPO_4, *p*H 8.1) and incubated at 45 C in a water-bath shaker for 2 hr. The enzyme-treated spores were washed with glass-distilled water until free of debris.

Clean spores were suspended in glass-distilled water to a concentration of 2×10^9/ml, dispensed in screw-cap tubes (5 ml/tube), frozen in acetone-dry ice, and stored at −20 C. Microscopic examination of smears stained with 0.5% methylene blue showed that the final spore suspension was free of vegetative cells and contained 1 to 2% germinated spores.

Conditions for germination. Unless otherwise specified, spores were germinated in a chemically defined medium (CTB) containing 8 mM L-cysteine, 4.4 mM sodium thioglycolate, 11.9 mM $NaHCO_3$, buffered at *p*H 7.0 with 100 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES). To remove dissolved oxygen, all medium constituents except $NaHCO_3$ were steamed in standardized Klett colorimeter tubes for 20 min. The medium was cooled to 37 C; $NaHCO_3$ and then aqueous spore suspension were added. The tubes, containing a final volume of 10 ml (10^8 spores/ml), were plugged with serum stoppers, and the head space of ca. 4.5 ml was flushed with purified nitrogen (99.997%) for 2 min. All tubes were incubated in a 37 C water bath unless otherwise specified.

Estimation of germination. The extent of germination was routinely estimated as the percentage of spores (200 cells were counted) staining with 0.5% aqueous methylene blue, and the rate of germination was estimated by following the loss in optical density (Klett-Summerson colorimeter, no. 42 filter) of spore suspensions. When used as a measure of germination, the loss of refractility (phase dark spores) was examined under Zeiss dark-contrast phase optics. Loss of resistance to heat (80 C, 10 min) and gamma rays (0.05 Mrad) during germination was followed by conventional colony counts of survivors in tubes (12 by 200 mm). Membrane filter-sterilized 5% $NaHCO_3$ (0.3 ml) was added to each culture tube before the appropriate dilution of the spore suspension. Approximately 13 ml of Thiotone Agar (3.0% BBL Thiotone, 0.05% BBL Sodium Thioglycollate, 0.85% Oxoid Ionagar no. 2, adjusted to *p*H 7.0 with 5.5 N KOH) was added, allowed to solidify, and capped with 2 cm of the same medium; tubes were incubated at 37 C for 24 hr.

RESULTS

In preliminary experiments, we observed that approximately 90% of a heat-activated (80 C, 10 min) *C. botulinum* 62A spore population germinated in 24 hr at 37 C in a medium (*p*H 7.0) consisting of 11.9 mM sodium bicarbonate, 4.4 mM sodium thioglycolate, 10 mM sodium phosphate, and 7.4 mM L-cysteine. Under optimal conditions, maximum germination took place within 2 hr.

Activation. Exposure to sublethal heat (5), reducing agents (14), water vapor (13), *p*H values below 3.0 or 10.0 and above (8), or gamma radiation (9) have been reported to enhance the extent and rate of germination. Heating (80 C) an aqueous spore suspension before addition to the CTB medium increased the rate and extent of germination of *C. botulinum* spores (Fig. 1) from the 51% germination of unheated spores in a 2-hr period. Heat activation at 80 C for 60 min resulted in 93% germination in 2 hr. Spores heated at 80 C for periods up to 100 min remained viable and germinated at the same rate and to the same extent as those heated for 60 min. Heat treatment at 90 C for 30 min rendered 95% of the spores incapable of colony formation on a suitable medium and reduced the extent of germination to 16%. In all subsequent experiments, heat activation, when used, was done at 80 C for 60 min.

Exposure at 5 C ± 2 C of unheated aqueous spore suspensions to either sublethal or lethal doses of gamma radiation up to 1.2 Mrad did not appreciably alter either the extent (Fig. 2) or rate (*data not shown*) of germination. Neither the radiation nor the heat treatments tested caused any increase in the number of stainable spores before incubation in CTB.

pH of germination medium. Heat-activated spores were incubated at 37 C in the defined medium with various buffers at *p*H levels of 4.1 to 9.2. No germination occurred below *p*H 4.8 in the 2-hr test period (Fig. 3). The optimum *p*H range for germination (90 to 94%) was 6.5 to 7.5. Above *p*H 7.5, there was a decrease in germination with only about 12% germination at *p*H 8.9 to 9.2.

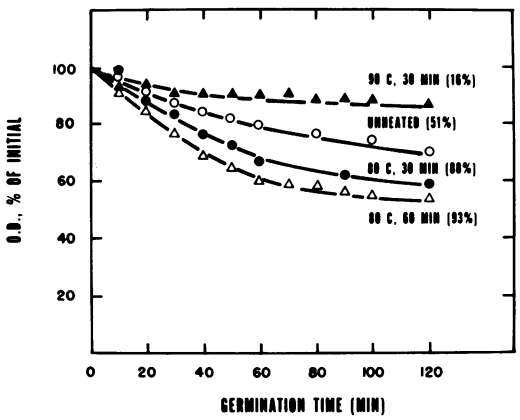

FIG. 1. *Effect of different heat treatments on subsequent germination of Clostridium botulinum spores. Distilled water suspensions of spores were either unheated (○), heated at 90 C for 30 min (▲), or at 80 C for 30 min (●) or 60 min (△). Untreated or treated spores (10^8/ml) were incubated at 37 C in the germination medium (8.0 mM L-cysteine, 4.4 mM sodium thioglycolate, and 11.9 mM $NaHCO_3$; buffered at pH 7.0 with 100 mM TES). Numbers in parentheses represent per cent germination (spores stainable with 0.5% methylene blue) after 120 min.*

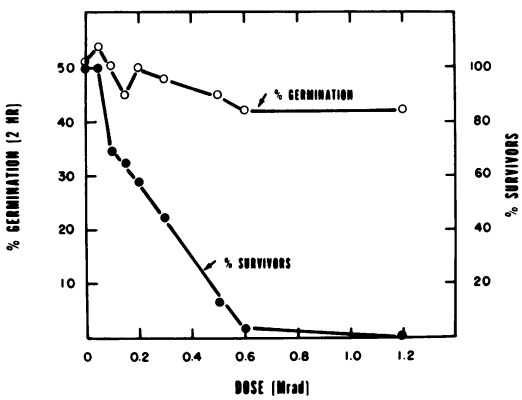

FIG. 2. *Effect of gamma radiation on the extent of germination of unheated spores of Clostridium botulinum 62A. Distilled water suspensions of spores were radiated at 5 C ± 2 C in a ^{60}Co source. Per cent germination (spores stainable with 0.5% methylene blue) was determined after 2 hr of incubation (37 C) in 8 mM L-cysteine, 4.4 mM sodium thioglycolate, 11.9 mM sodium bicarbonate; buffered at pH 7.0 with 100 mM TES. Per cent survivors were determined by conventional colony counts.*

Incubation temperature. Activated spores of *C. botulinum* were suspended in the CTB medium buffered at pH 7.0 and incubated for periods from 2 to 96 hr at different temperatures (Table 1). The optimal temperature for germination was 37 C. Although the extent of germination after 2 hr of incubation at 37 or 30 C was essentially the same, the rate of germination (optical density loss) was slower at 30 C (*data not shown*). If the incubation period were extended up to 4 days, more than 80% of the spores germinated at all temperatures tested. Thus, the rate but not the extent of germination depended on temperature.

Optimal concentration of L-cysteine, sodium bicarbonate, and sodium thioglycolate. Only 6% of the spores germinated over a 2-hr period in $NaHCO_3$ and sodium thioglycolate (Fig. 4). The extent of germination was not increased by the addition of 2 mM L-cysteine. The maximum extent of germination occurred with 8 mM L-cysteine, and this was reached in approximately 80 min of incubation at 37 C. With further increase in L-cysteine concentration to 16 and to 32 mM, there was a progressive increase in the rate of germination, so that 92% germination, the maximum attained, was reached in 70 and 50 min, respectively. The rate and extent of germination with 64 mM L-cysteine (*data not shown*) was the same as with 32 mM. The D-isomer was inactive as a germinant but inhibited the rate and extent of L-cysteine-initiated germination over a 2-hr test period (Fig. 5).

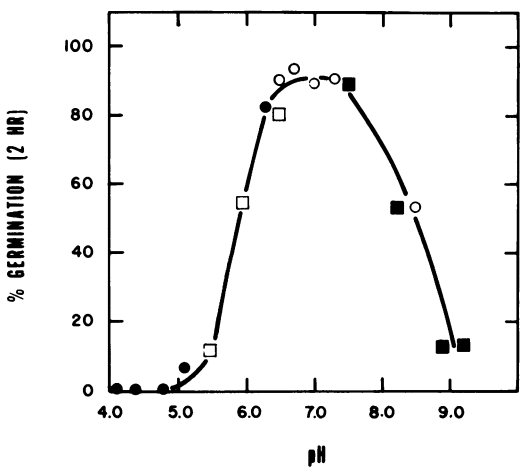

FIG. 3. *Effect of initial pH on extent of germination of Clostridium botulinum 62A spores. Aqueous spore suspensions were heated at 80 C for 60 min, cooled to 37 C, and suspended (10^8/ml) in the germination medium adjusted to indicated pH with 100 mM buffer. Sodium acetate buffer was used for pH 4.1 to 6.3 (●); 2-(N-morpholino)ethanesulfonic acid, for pH 5.6 to 6.5 (□); N-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid for pH 6.5 to 8.4 (○); and N,N-bis(2-hydroxyethyl)glycine for pH 7.5 to 9.2 (■). Per cent germination (spores stainable with 0.5% methylene blue) was determined after 2 hr of incubation.*

TABLE 1. *Effect of incubation temperature on the germination of Clostridium botulinum 62A spores*[a]

Incubation temp (C)	Percentage of germination[b]			
	2 hr	24 hr	48 hr	96 hr
4	3	24		81
10	9	74	91	
12	6	88		
16	13	92	96	
20	54	95		
23	79	94		
30	89	96		
37	93	—		
45	43	96		
70	9	53		84

[a] Aqueous spore suspensions were heated at 80 C for 60 min, cooled, suspended at a final concentration of 10^8/ml in a germination medium (8.0 mM L-cysteine, 11.9 mM sodium bicarbonate, 4.4 mM sodium thioglycolate; buffered at *p*H 7.0 with 100 mM TES), and incubated at various temperatures for the indicated times.

[b] Percentage of spores stainable with 0.5% methylene blue.

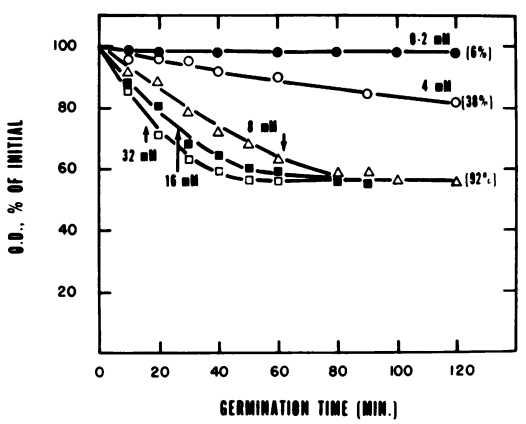

FIG. 4. *Effect of* L-*cysteine concentration on germination of Clostridium botulinum spores. Heat-activated spores, cooled to 37 C, were suspended (10⁸/ml) in a medium (4.4 mM sodium thioglycolate, 11.9 mM sodium bicarbonate; buffered at pH 7.0 with 100 mM TES) with* L-*cysteine in final concentrations of 0 to 2 mM (●), 4 mM (○), 8 mM (△), 16 mM (■), or 32 mM (□). Numbers in parentheses as indicated in Fig. 1.*

In the defined medium containing 8 mM L-cysteine and 4.4 mM sodium thioglycolate, without $NaHCO_3$, only 1% of the spores germinated in 2 hr (Fig. 6), and there was no further increase in germination over a 24-hr incubation period. As the concentration of $NaHCO_3$ was increased to 8.3 mM, there was a progressive increase in the rate and extent of germination, and these did not increase further with 11.9 mM $NaHCO_3$, the concentration in the CTB medium. Concentrations of $NaHCO_3$ lower than 8.3 mM supported he maximum extent of germination but at a

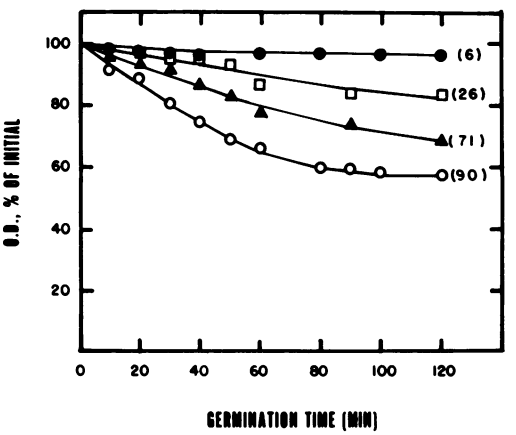

FIG. 5. *Inhibition of* L-*cysteine-initiated germination of Clostridium botulinum spores by* D-*cysteine. Spores were heated, cooled, and suspended in a medium (as in Fig. 4) with 8 mM* D-*cysteine (●); 8 mM* L-*cysteine alone (○); 8 mM* L-*cysteine plus 8 mM* D-*cysteine (▲); or 8 mM* L-*cysteine plus 16 mM* D-*cysteine (□). Numbers in parentheses as indicated in Fig. 1.*

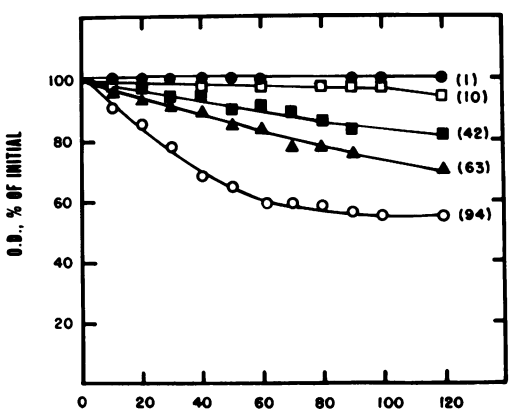

FIG. 6. *Effect of sodium bicarbonate concentration on germination of Clostridium botulinum 62A spores. Heat-activated spores, cooled to 37 C, were suspended (10⁸/ml) in a medium (8 mM* L-*cysteine, 4.4 mM sodium thioglycolate; buffered at pH 7.0 with 100 mM TES) with and without sodium bicarbonate. The final concentration of $NaHCO_3$ in the medium was none (●), 1.19 mM (□), 2.38 mM (■), 3.57 mM (▲), or 8.3 to 11.9 mM (○). Numbers in parentheses as in Fig. 1.*

slower rate (*data not shown*). Although only 42% of the spores germinated over a 2-hr period when the germination medium contained 2.38 mM $NaHCO_3$, maximum germination (92%) was attained by 24 hr.

With deletion of sodium thioglycolate, the defined medium supported germination of only 6% of the spores in 2 hr (Fig. 7). The maximum rate and extent of germination was obtained in the presence of 2.2 mM sodium thioglycolate. Further addition (up to 8.8 mM) of sodium thioglycolate neither enhanced nor inhibited the rate or extent of germination.

Sequence of germination events. When heat-activated spores of *C. botulinum* 62A were incubated in the CTB medium at 37 C for 2 hr, there was more than 90% germination. Although this medium did not support postgerminative development, the germinated spores were viable, as evidenced by growth and colony formation on G-G medium plus 0.85% Ionagar no. 2. This retention of viability permitted estimation of the sequence of such germination events as loss of heat and radiation resistance (Fig. 8). By 2 hr, 97% of the spores had become heat sensitive (80 C, 10 min); 98% had become sensitive to radiation (0.05 Mrad at 5 C ± 2 C); 88 and 91% had become phase dark and stainable, respectively. However, when the per cent completion of germination was normalized, related to germination at 2 hr, it was evident that loss of heat resistance preceded the other events. The 50% completion times for loss of radiation resistance, acquisition of stainability, and loss of phase brightness coincided and trailed loss of heat resistance by approximately 12 min.

DISCUSSION

The germination of *Clostridium* spores in chemically defined media is well documented (2, 7, 12, 20). However, little comparative information is available on the germination of spores of *C. botulium*. Spores of *C. roseum* germinated in L-alanine, L-phenylalanine, L-arginine, and sodium thioglycolate at *p*H 7 to 8, and it was suggested that spores of *C. botulinum* type A and B would germinate under the same conditions (12). In the same concentration of amino acids plus sodium bicarbonate, but no sodium thioglycolate, spores of *C. botulinum* 62A exhibited only 12% germination over a 4-hr incubation period (19). Our preliminary studies showed that 90% of a heat-activated (80 C, 10 min) *C. botulinum* 62A spore population germinated in 24 hr at 37 C in L-alanine or L-cysteine plus sodium bicarbonate and sodium

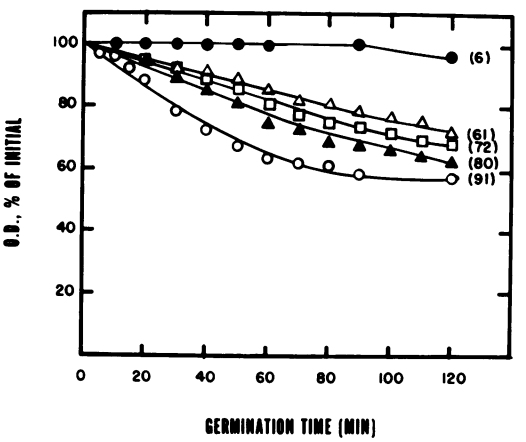

FIG. 7. *Effect of sodium thioglycolate concentration on germination of Clostridium botulinum 62A spores. Heat-activated spores, cooled to 37 C, were incubated (10^8/ml) in a medium (8 mM L-cysteine, 11.9 mM sodium bicarbonate; buffered at pH 7.0 with 100 mM TES) with and without sodium thioglycolate. The final concentration of sodium thioglycolate in the medium was none (●); 0.44 mM (△); 0.66 mM (□); 0.88 mM (▲); or 2.2, 3.5, 4.4, or 8.8 mM (○). Numbers in parentheses as in Fig. 1.*

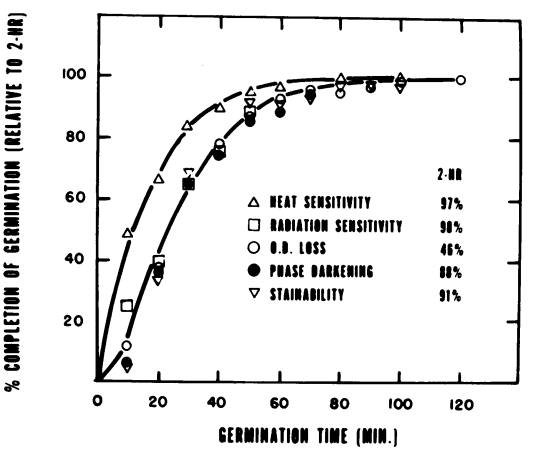

FIG. 8. *Sequence of optical density loss, complete phase darkening, acquisition of stainability, and loss of heat and gamma radiation resistance during germination of Clostridium botulinum 62A spores. Heat-activated spores, cooled to 37 C, were suspended in the germination medium (8 mM L-cysteine, 11.9 mM sodium bicarbonate, and 4.4 mM sodium thioglycolate; buffered to pH 7.0 with 100 mM TES) and incubated at 37 C for 120 min. Each germination event, examined at 10-min intervals, was considered to be essentially complete in 120 min. The maximum change (100% completion value) in 2 hr for each germination event was as indicated.*

thioglycolate (*p*H 7.0). Various conditions affect the germination of *C. botulinum* spores in such a chemically defined medium, and by optimizing these conditions one could induce type 62A spores to undergo maximal germination (>90%) within 50 min.

Other investigators have reported on the effects of prior heating or radiation on spore germination. The germination rate, but not the extent of germination, of *C. botulinum* 62A spores in 5% yeast extract plus 0.1% $NaHCO_3$ was enhanced by heating at 75 C for 20 min (19). The increase in plate counts of *C. botulinum* 33A spores, heated at 80 C for periods up to 240 min in phosphate buffer, was attributed to heat activation (11); heating in excess of 4 hr at this temperature markedly decreased the number of viable spores. Spore activation by ionizing radiation as the basis for accelerated spoilage of cured ham by *C. botulinum* type A and B (1) has not been conclusively demonstrated. Indeed, spore activation was ruled out as an explanation for the stimulatory effect of radiation on growth and toxin production by *C. botulinum* type F (22), and, in the present experiments, we found no evidence for radiation activation of spore germination. However, with *B. cereus* PX, inosine-induced germination was maximally activated both by heat and by ionizing radiation (9), maximal heat activation being attained without loss in viability and maximal radiation activation being observed only after 97% of the spores had been rendered nonviable. We find that maximal heat activation was attained after heating *C. botulinum* 62A spores at 80 C for 60 min, and there was no loss of viability even after extension of the heating time to 100 min. Heating at 90 C for 30 min resulted in 95% inactivation and markedly decreased germination in the defined medium. Radiation of spores, on the other hand, had no significant inhibitory effect on germination, even when radiation doses sufficient for 99.998% inactivation were used. Thus, it appeared that *C. botulinum* 62A spores were inactivated for germination by a lethal heat treatment, but not by a lethal radiation dose. Since viability (ability to form macrocolonies) is not a prerequisite for germination (4, 16, 18, 21), it is possible that a less severe lethal heat treatment would not decrease the extent of germination. The germination ability of *B. megaterium* spores was more resistant to both heat and radiation than was the ability to undergo postgerminative development (16).

Our results confirm a previous observation (19) that $NaHCO_3$ enhances the germination of spores of *Clostridium*. Reports on the effect of thioglycolate, which may function as a reducing agent or as a chelating agent, are conflicting. Although we find, in confirmation of the work of other investigators (12, 20), that sodium thioglycolate stimulates germination of *Clostridium* spores, others have indicated that sodium thioglycolate inhibited *C. bifermentans* (7) and *C. botulinum* 62A (19) spore germination in hydrolysates of casein.

Uehara and Frank (21) reported that the germination of PA 3679 spores occurred in two stages; changes in the spore cortex were followed by changes in the spore core. They suggested that the initial stage could be measured by absorbency decrease and loss of heat resistance, whereas stainability and complete phase darkening were measures of the final stage. During the germination of *C. botulinum* 62A spores in the CTB medium, the loss of heat resistance preceded the loss of radiation resistance which coincided with stainability, complete phase darkening, and decrease in optical density. It would thus appear that loss of heat resistance is a better indicator than optical density loss of an early stage in the germination process of *C. botulinum* 62A spores. Loss of heat resistance has also been reported as an early step in the germination of spores of *B. megaterium* (17) and *C. roseum* (23). Conceivably, the loss of radiation resistance which occurs concurrently with acquisition of stainability and complete phase darkening represents a terminal stage of germination. Recently Durban, Goodnow, and Grecz (6) concluded that loss of resistance to heat, to ultraviolet radiation, and to gamma radiation occurred simultaneously during the germination of *C. botulinum* 33A spores. However, their data do not support such a conclusion. Actually a loss in resistance to heat and radiation coincided with the onset of logarithmic growth and therefore should not be confused with events during germination.

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LITERATURE CITED

1. Anellis, A., D. Berkowitz, C. Jarboe, and H. M. El-Bisi. 1967. Radiation sterilization of prototype military foods. II. Cured ham. Appl. Microbiol. 15:166–177.
2. Bergère, J. L. 1969. Spore germination in *Clostridium tyrobutyricum*. I. Action of various compounds on the initial stage. Ann. Inst. Pasteur 117:179–195.
3. Bruch, M. K., C. W. Bohrer, and C. B. Denny. 1967. Adaptation of biphasic culture technique to the sporulation of *Clostridium botulinum* type E. J. Food Sci. 33:108–109.
4. Costilow, R. N. 1962. Fermentative activities of control and radiation-"killed" spores of *Clostridium botulinum*. J. Bacteriol. 84:1268–1273.
5. Curran, H. R., and F. R. Evans. 1945. Heat activation in-

ducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bacteriol. 49:335–346.
6. Durban, E., R. Goodnow, and N. Grecz. 1970. Changes in resistance to radiation and heat during sporulation and germination of *Clostridium botulinum* 33A. J. Bacteriol. 102:590–592.
7. Gibbs, P. A. 1964. Factors affecting the germination of spores of *Clostridium bifermentans*. J. Gen. Microbiol. 37:41–48.
8. Gibbs, P. A. 1967. The activation of spores of *Clostridium bifermentans*. J. Gen. Microbiol. 46:285–291.
9. Gould, G. W., and Z. J. Ordal. 1968. Activation of spores of *Bacillus cereus* by γ-radiation. J. Gen. Microbiol. 50:77–84.
10. Grecz, N., A. Anellis, and M. D. Schneider. 1962. Procedure for cleaning of *Clostridium botulinum* spores. J. Bacteriol. 84:552–558.
11. Grecz, N., C. A. Lin, T. Tang, W. L. So, and L. R. Sehgal. 1967. The nature of heat resistant toxin in spores of *Clostridium botulinum*. Jap. J. Microbiol. 11:384–394.
12. Hitzman, D. O., H. O. Halvorson, and T. Ukita. 1957. Requirements for production and germination of spores of anaerobic bacteria. J. Bacteriol. 74:1–7.
13. Hyatt, M. T., P. K. Holmes, and H. S. Levinson. 1966. Activation of *Bacillus megaterium* spore germination by water in the vapor phase. Biochem. Biophys. Res. Commun. 24:701–704.
14. Keynan, A., Z. Evenchik, H. O. Halvorson, and J. W. Hastings. 1964. Activation of bacterial endospores. J. Bacteriol. 88:313–318.
15. Krask, B. J. 1961. Discussion of the role of alanine dehydrogenase in the control of germination of aerobic spores initiated by L-alanine, p. 89–100. *In* H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis, Minn.
16. Levinson, H. S., and M. T. Hyatt. 1960. Some effects of heat and ionizing radiation on spores of *Bacillus megaterium*. J. Bacteriol. 80:441–451.
17. Levinson, H. S., and M. T. Hyatt. 1966. Sequence of events during *Bacillus megaterium* spore germination. J. Bacteriol. 91:1811–1818.
18. Powell, E. O. 1957. The appearance of bacterial spores under phase-contrast illumination. J. Appl. Bacteriol. 20:342–348.
19. Treadwell, P. E., G. J. Jann, and A. J. Salle. 1958. Studies on factors affecting the rapid germination of spores of *Clostridium botulinum*. J. Bacteriol. 76:549–556.
20. Uehara, M., and H. A. Frank. 1965. Factors affecting alanine-induced germination of clostridial spores, p. 38–46. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
21. Uehara, M., and H. A. Frank. 1967. Sequence of events during germination of putrefactive anaerobe 3679 spores. J. Bacteriol. 94:506–511.
22. Williams-Walls, N. J. 1969. Effects on growth and toxin production of exposure of spores of *Clostridium botulinum* type F to sublethal doses of gamma irradiation. Appl. Microbiol. 17:128–134.
23. Wooley, B. C., and R. E. Collier. 1965. Changes in thermoresistance of *Clostridium roseum* as related to the intracellular content of calcium and dipicolinic acid. Can. J. Microbiol. 11:279–285.