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Activation and Injury of *Clostridium perfringens* Spores by Alcohols

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The activation properties of *Clostridium perfringens* NCTC 8679 spores were demonstrated by increases in CFU after heating in water or aqueous alcohols. The temperature range for maximum activation, which was 70 to 80°C in water, was lowered by the addition of alcohols. The response at a given temperature was dependent on the time of exposure and the alcohol concentration. The monohydric alcohols and some, but not all, of the polyhydric alcohols could activate spores at 37°C. The concentration of a monohydric alcohol that produced optimal spore activation was inversely related to its lipophilic character. Spore injury, which was manifested as a dependence on lysozyme for germination and colony formation, occurred under some conditions of alcohol treatment that exceeded those for optimal spore activation. Treatment with aqueous solutions of monohydric alcohols effectively activated *C. perfringens* spores and suggests a hydrophobic site for spore activation.

Clostridium perfringens is one of the most common bacterial agents in foodborne-disease outbreaks. The vehicle of infection is frequently a cooked, high-protein food in which spores of this organism will survive cooking temperatures and germinate so that multiplication occurs during post-cook handling (36). Sublethal heat will activate spores of many, but not all, strains of *C. perfringens* (28) and promote germination under conditions not permissive for the germination of dormant spores. Barnes et al. (8) reported that heat treatment activated spores of *C. perfringens* in meat so that colony formation increased 25 times on a plating medium. Roberts (33) reported that only 0.13 to 3.6% of spores of "food-poisoning" strains grew without heat. Heat treatment of an inoculum containing *C. perfringens* spores resulted in a culture with an enhanced ability to produce enterotoxin during a subsequent sporulation event (39).

Heat activation is routinely used as a prerequisite for studies of bacterial spore germination and, by use of plating procedures, for the enumeration of spores from food products and other environmental sources. Time-temperature conditions for maximum activation may vary with strains of *C. perfringens* (17).

Treatment with ethanol (ET) has been reported to increase the germination rate of *Bacillus megaterium* spores (21, 22) and to be an effective technique for the selective isolation of sporeforming bacteria from mixed cultures (24). In this study, we compared the efficiency and kinetics of heat activation with those of alcohol activation of *C. perfringens* spores and report on the conditions of alcohol exposure that injure spores.

MATERIALS AND METHODS

Organisms. The *C. perfringens* type A strain used in this study, NCTC 8679, was obtained from O. Giger. Stock strains were maintained in Cooked Meat Medium (Difco Laboratories, Detroit, Mich.) at room temperature.

Medium preparation. The media for growth and sporulation of *C. perfringens* strains were prepared as previously described (14). Spores were prepared in Duncan-Strong (17) sporulation medium plus 1.0 mM caffeine (DSC). The observation that many spores of strain NCTC 8679 were formed in this medium with but not without caffeine is similar to results reported for this strain in a defined sporulation medium (26).

Spore preparation. From stock cultures, 0.1 ml was transferred into 10 ml of thioglycolate medium. The inoculated medium was heated at 75°C for 20 min and incubated for 16 h at 37°C. A 0.2-ml portion from this culture was transferred into 10 ml of thioglycolate medium and incubated for 16 h. A 1.0% inoculum from this last 16-h culture was delivered into 3.0 liters of DSC. Incubation was carried out at 37°C for 18 h. All cultures were incubated without provisions for anaerobiosis. Spores were harvested by centrifugation at 4°C and cleaned by repeated washing with cold deionized water. The cleaned preparation containing 95% refractile spores, 4% phase-dark spores, and <1.0% vegetative cells was lyophilized and stored desiccated at −18°C.

Spore activation treatments. Lyophilized spores of strain NCTC 8679 were rehydrated with cold deionized water, mixed with a vortex mixer (Scientific Industries, Inc., Springfield, Mass.), and sonicated briefly to disrupt spore clumps. The spore suspensions were adjusted with deionized water to a given spore count as determined by measuring the A_{600} on a Beckman model 25 spectrophotometer. They were diluted 10-fold in water or aqueous alcohol solutions at a temperature in the range of 10 to 90°C to give the desired alcohol concentration. Incubation was carried out for up to 120 min. In another experiment, DSC cultures were incubated at 37°C for up to 24 h, diluted, and treated in water at 75°C for 20 min (heat shock) or in alcohol at 37°C for 60 min. Controls demonstrated that vegetative cells in these cultures were killed by the treatments.

Recovery and enumeration of spores. Treated spore suspensions were cooled in ice water and diluted serially in 0.1% peptone water. Dilutions were plated on tryptose-sulfite-cycloserine agar (TSC) without egg yolk (19) and with or without 1.0 μg of lysozyme per ml. TSC compares favorably to a nonselective agar base in recovering heat-activated and heat-injured *C. perfringens* spores (7, 27). In our experiments, this medium gave highly reproducible results. Plates were incubated anaerobically with the GasPak system (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 24 h. For spores heated at 75°C for 20 min, CFU were not increased by incubating plates for 48 or 72 h. As determined experimentally, the dilution of samples to give

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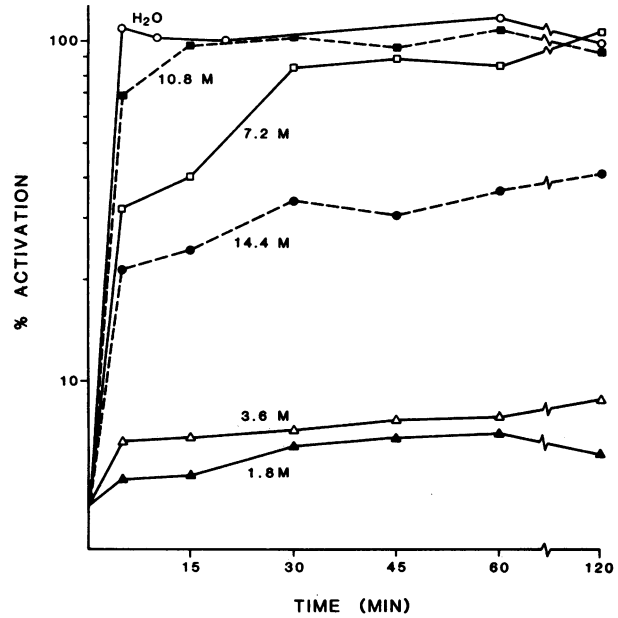

FIG. 1. Percent activation of *C. perfringens* NCTC 8679 spores after heat treatment in water at 75°C or in 1.8, 3.6, 7.2, 10.8, or 14.4 M ET at 37°C. The number of CFU on TSC after heating the spores at 75°C for 20 min was considered to represent 100% activation.

colony counts of 20 to 200 CFU per plate eliminated the effects of alcohols on colony formation. The percent activation was determined with the formula $(N/N_0) \times 100$, where N is the number of CFU on TSC or TSC plus lysozyme after a given treatment, and N_0 is the number of CFU on TSC after heating of the spores in water at 75° for 20 min. The mean N_0 for all experiments was $(1.4 \pm 0.3) \times 10^8$ CFU/ml. The mean number of refractile spores as determined with phase microscopy and a Petroff-Hausser counting chamber was $(2.3 \pm 0.5) \times 10^8$/ml. The values reported in the tables and figures are the means of duplicate samples. Data were analyzed by the General Linear Models Procedure (SAS Institute, Cary, N.C.).

Chemicals. The following chemicals were purchased from the indicated companies: ET, U.S. Industrial, Cincinnati, Ohio; methanol, Burdick and Jackson Laboratories, Inc., Muskegon, Michigan; 1,2-ethanediol (ethylene glycol), Matheson, Coleman and Bell, Norwood, Ohio; isopropanol, Mallinckrodt, Inc., St. Louis, Mo.; *tert*-butanol and 1,2-propanediol (propylene glycol), J. T. Baker Chemical Co., Phillipsburg, N.J.; 2-propyn-1-ol (propargyl alcohol), 1-propanol, *sec*-butanol, allyl alcohol (AL), tetrahydrofurfuryl alcohol, and benzyl alcohol, Aldrich Chemical Co., Inc., Milwaukee, Wis.; 2-ethyl-2-propanol (*tert*-amyl alcohol), 1,4-butanediol, 1,5-pentanediol, 1,2,6-hexanetriol, and phenyl-1,2-ethanediol, Chem Service, Inc., West Chester, Pa.

RESULTS

Activation of *C. perfringens* spores by alcohol treatment. Spores of *C. perfringens* NCTC 8679 required activation for maximum germination and colony formation on TSC. Colonies formed by control spores not exposed to heat or alcohols represented only 4 to 6% of those formed by spores subjected to heat shock (75°C for 20 min) in water. At 75°C, maximum activation occurred within 5 min, with little change after exposure for 120 min. Spores were also activated by treatment with ET at 37°C (Fig. 1). The extent and rate of activation increased with increasing concentrations of ET up to 10.8 M. At this concentration, complete activation occurred after 15 min of exposure. Exposure of spores to a higher concentration (14.4 M) resulted in suboptimal activation.

The activation of *C. perfringens* NCTC 8679 spores by ET was temperature as well as concentration dependent (Fig. 2). The activation of spores was not apparent in water at temperatures of 10 to 40°C, but the CFU increased after exposure to 50°C, with maximum numbers occurring at 70 to 80°C. The portion of the curve (Fig. 2) exhibiting a rapid increase in activation was shifted to lower temperatures with increasing concentrations of ET up to 10.8 M. The optimal activation temperature was lowered as much as 30°C, from 70 to 80°C in water to 40 to 50°C in 10.8 M ET, whereas the activation temperature was lowered to a lesser extent with an ET concentration of 14.4 M.

The efficiency of other monohydric alcohols in activating NCTC 8679 spores was also determined (Table 1). A treatment consisting of exposure of the spores to each alcohol for 1 h at 37°C was selected because, after this exposure, spores were activated by ET (Fig. 1) and bacterial vegetative cells were killed (data not shown). At optimal concentrations, all monohydric alcohols activated spores, ranging from 47% of heat shock activation for 2-propyn-1-ol to 107% for ET.

The concentration of monohydric alcohols required to produce maximum activation at 37°C decreased with their increasing lipophilicity (apolar character) (Fig. 3). A linear relationship was observed when the log of the alcohol concentration giving maximum activation from Table 1 was plotted against its *n*-octanol–water partition coefficient.

The polyhydric alcohols examined, with the exception of phenyl-1,2-ethanediol, were more polar in character and more variable in their ability to activate NCTC 8679 spores than the monohydric alcohols were (Table 2). Only three of the six polyhydric alcohols were effective in activating spores to levels 25% or greater than those after heat shock

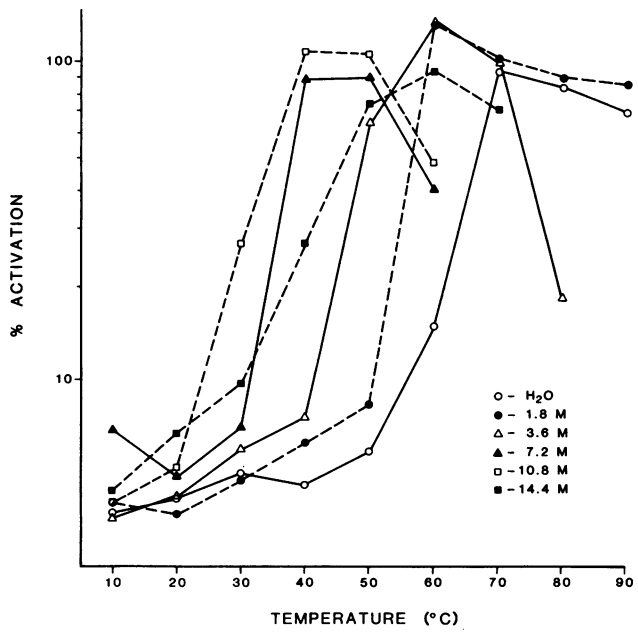

FIG. 2. Effect of temperature on the percent activation of *C. perfringens* NCTC 8679 spores in water or 1.8, 3.6, 7.2, 10.8, or 14.4 M ET. The heating time was 15 min.

TABLE 1. Percent activation of *C. perfringens* NCTC 8679 spores after 60 min at 37°C in aqueous solutions of monohydric alcohols

Alcohol	Lipophilicity[a]	Concn (M)[b]	% Activation[c]
None (control)[d]			6
Methanol	−0.66	4 (16)	8
		8 (32)	8
		12 (49)	20
		16 (65)	63
		18 (73)	96
		20 (81)	93
		22 (89)	49
ET	−0.32	1.8 (11)	7
		3.6 (22)	8
		7.2 (44)	86
		10.8 (67)	107
		14.4 (89)	36
Isopropanol	−0.04	2 (15)	8
		4 (31)	79
		8 (61)	104
		10 (77)	68
AL	0.17	1 (7)	6
		2 (14)	15
		4 (27)	72
		8 (54)	0.008
1-Propanol	0.37	1 (7)	9
		2 (15)	54
		4 (30)	65
		6 (45)	20
tert-Butanol	0.37	2 (11)	6
		4 (21)	21
		8 (43)	98
		12 (64)	51
sec-Butanol	0.61	0.6 (5)	26
		0.8 (7)	45
		1.0 (8)	62
		1.2 (10)	72
2-Ethyl-2-propanol	0.89	0.6 (7)	8
		0.8 (9)	8
		1.0 (11)	48
		1.2 (13)	51
Benzyl alcohol	1.10	0.1 (1)	8
		0.2 (2)	10
		0.3 (3)	40
		0.4 (4)	89
Tetrahydrofurfuryl alcohol		1 (10)	14
		2 (20)	27
		4 (39)	103
		8 (78)	94
2-Propyn-1-ol		1 (6)	17
		2 (12)	41
		4 (24)	47
		8 (47)	<0.006

[a] Reported as the Log_{10} *n*-octanol–water partition coefficient.
[b] Numbers in parentheses represent the percent volume.
[c] Calculated by the formula given in the text.
[d] Spores were held in water at 4°C for 60 min.

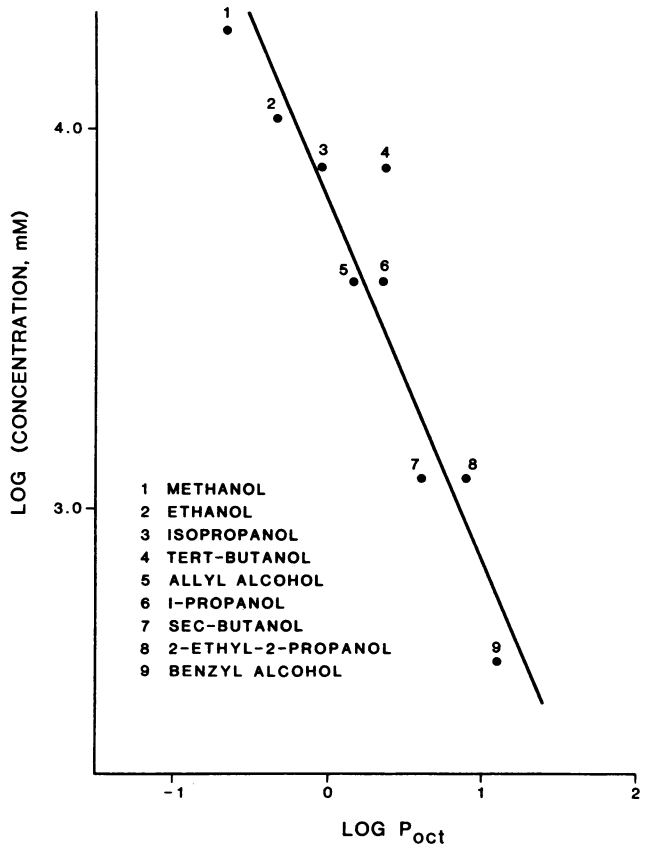

FIG. 3. Plot of the log of monohydric alcohol concentrations producing optimum spore activation at 37°C for 60 min versus the log *n*-octanol–water partition coefficients of the alcohols (LOG P_{oct}).

activation. When the log of the optimum concentration of each of these three alcohols was plotted against its *n*-octanol–water partition coefficient, a linear relationship was observed, but with a slope different from that of the line in Fig. 3 (data not shown).

Appearance of resistance to alcohols in a sporulating culture. Figure 4 shows data for the development in a DSC culture of *C. perfringens* NCTC 8679 of resistance to various activation treatments. Heating the culture at 75°C or treatment with 8 M *tert*-butanol or 10.8 M ET at 37°C killed vegetative cells. Spores resistant to the two alcohols appeared after 4.5 h, but no resistance to heat shock in water was detected at this time. The presence of spore forms was confirmed by phase-contrast microscopy. Spores resistant to alcohol treatment were more numerous by more than 1 log factor than spores resistant to heat treatment in the 5-h culture. Differences decreased with the age of the culture until, by 24 h, the numbers of spores resistant to alcohol and to heat were essentially equal.

Injury by alcohol treatment. The recovery of spores after treatment with higher concentrations of AL, 2-propyn-1-ol, and phenyl-1,2-ethanediol at levels below the 4 to 6% activation level of untreated spores indicated death or injury or both (Tables 1 and 2). Table 3 shows the recovery of spores treated with these alcohol concentrations and plated on TSC with or without lysozyme, which has been shown to permit the germination and recovery of *C. perfringens* spores in which the germination lytic enzyme is apparently missing (12) or is inactivated (1, 16). As in Tables 1 and 2, the

TABLE 2. Percent activation of *C. perfringens* NCTC 8679 spores after 60 min at 37°C in aqueous solutions of polyhydric alcohols

Alcohol	Lipophilicity[a]	Concn (M)[b]	% Activation[c]
1,2,6-Hexanetriol	−2.09	1 (12)	8
		2 (24)	7
		4 (49)	8
		6 (73)	5
1,2-Ethanediol	−1.93	8 (45)	11
		10 (56)	17
		12 (67)	19
		14 (78)	76
		16 (90)	4
1,2-Propanediol	−1.41	4 (29)	6
		6 (44)	7
		8 (59)	10
		10 (73)	32
		12 (88)	18
1,4-Butanediol	−1.38	2 (18)	12
		4 (35)	17
		6 (53)	11
		8 (71)	20
		10 (89)	2
1,5-Pentanediol	−0.99	2 (21)	10
		4 (42)	11
		6 (63)	9
		8 (84)	15
Phenyl-1,2-ethanediol	1.16	0.125 (2)	9
		0.25 (3)	12
		0.5 (7)	57
		1 (14)	32
		2 (28)	0.06
		4 (55)	<0.006

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.

recovery of heat-shocked spores on TSC was considered to represent 100% activation. The addition of lysozyme to TSC permitted the apparent increased recovery of an additional 5% of heat-shocked spores and an additional 5% of untreated spores. An analysis of variance of 20 spore samples indicated that this small increase in CFU with lysozyme is significant ($P < 0.01$) for untreated and heat-shocked spores. Spore counts on TSC after treatment with the alcohols shown in Table 3 were <0.006 to 0.01% of heat-shocked spore counts. With lysozyme, the recovery of spores treated with AL and phenyl-1,2-ethanediol approached that comparable to the 5% level of activation. The recovery of spores treated with 2-propyn-1-ol was improved on TSC with lysozyme, but to a lesser extent than with the other alcohol treatments, indicating more extensive damage to the spores by this alcohol. The data in Table 3 and in Fig. 5 (to be discussed later) indicate that ca. 5% of NCTC 8679 spores are sensitive to germination by lysozyme.

Additional studies on the effects of AL, which caused injury to spores at the 8 M concentration (Tables 1 and 3), were undertaken. As observed before, 5 to 6% of spores formed colonies on TSC without heat or alcohol treatment (Fig. 5). The rate of activation increased with increasing concentrations of AL from 2 to 8 M, an effect similar to that observed for ET in Fig. 1. Activation at 37°C to near optimal levels was observed in 2 M AL after 120 min, 4 M AL after 5 min, and 8 M AL after 1 min. Little or no injury occurred after treatment with 2 and 4 M AL under the experimental conditions (i.e., counts were similar with or without lysozyme). With 8 M AL, spore survival was linear, with counts on TSC dropping to less than 1% after 7.5 min, to 0.06% after 30 min (data not shown), and to 0.006% after 60 min (data not shown). With lysozyme, the survival curve for spores exposed to 8 M AL was biphasic; the counts were similar to the counts on TSC without lysozyme after 5 min of exposure. From 7.5 to 120 min, counts declined on TSC with lysozyme at a much slower rate than on TSC without lysozyme, with greater than 1% survival. When a straight line was plotted by the least-squares method through the portion of the biphasic curve showing recovery after the exposure of spores to 8 M AL for 15 to 120 min (Fig. 5), the intercept with the ordinate was 4.8%, which is similar to the 5% of spores that would be naturally sensitive to lysozyme, as indicated by the data in Table 3.

As observed for ET, AL has a sparing effect on the temperature for activation of *C. perfringens* NCTC 8679 spores, with the optimal temperature being lowered with increasing concentrations of AL (Fig. 6). The 2 M concentration lowered the optimal activation temperature by 20°C, as compared with 10°C for 1.8 M ET (Fig. 2). The 8 M concentration lowered it by 50°C, as compared with 30°C for 7.2 to 10.8 M ET. The greater sparing effect of AL as compared with ET on the activation temperature and the injury of spores at 37°C by AL but not by ET (Table 1 and Fig. 5) indicate a greater reactivity of AL with the spore components responsible for activation or injury or both. This effect is consistent with the greater lipophilicity of AL (Fig. 3). Even though ET did not injure spores at 37°C, it did produce injury (as indicated by a dependency on lysozyme for colony formation) when the optimal activation temperature at a particular concentration (Fig. 2) was greatly exceeded (data not shown). A similar effect was noticed for AL (but not shown in Fig. 6). For instance, injury resulted when the optimal activation temperatures for 7.2 to 10.8 M ET and 8 M AL were exceeded by 30 and 20°C, respectively.

DISCUSSION

Curran and Evans (H. R. Curran and F. R. Evans, J. Bacteriol. 47:437, 1944) demonstrated that sublethal heat could induce dormant bacterial spores to germinate. Subsequent research has concentrated on the activation of spore germination by heat. Activation is known to change the qualitative and quantitative requirements for the induction of germination, to decrease germination lag (20, 40), to increase the overall rate of germination (23), to increase the rate of commitment to germinate (37), and to activate certain enzymes in resting spores (10, 13). Duncan and Strong (17) reported that heating at 75°C for 20 min, which is recommended to kill vegetative cells, is in the range of the optimal heat shock requirement for the germination of *C. perfringens* spores. Our results for spores of strain NCTC 8679 indicate that 75°C is optimal for activation in water (Fig. 2).

A variety of chemicals that induce structural changes in macromolecules cause activation of bacterial spores, but many are less efficient than heat (9). The activation of *B. megaterium* spores by aqueous alcohol or by heat suggested a common mechanism (21). Our results indicate that, like heat, alcohols activate or injure *C. perfringens* spores. Alcohols have a sparing, and not additive, effect on the temperature requirement for activation by lowering the

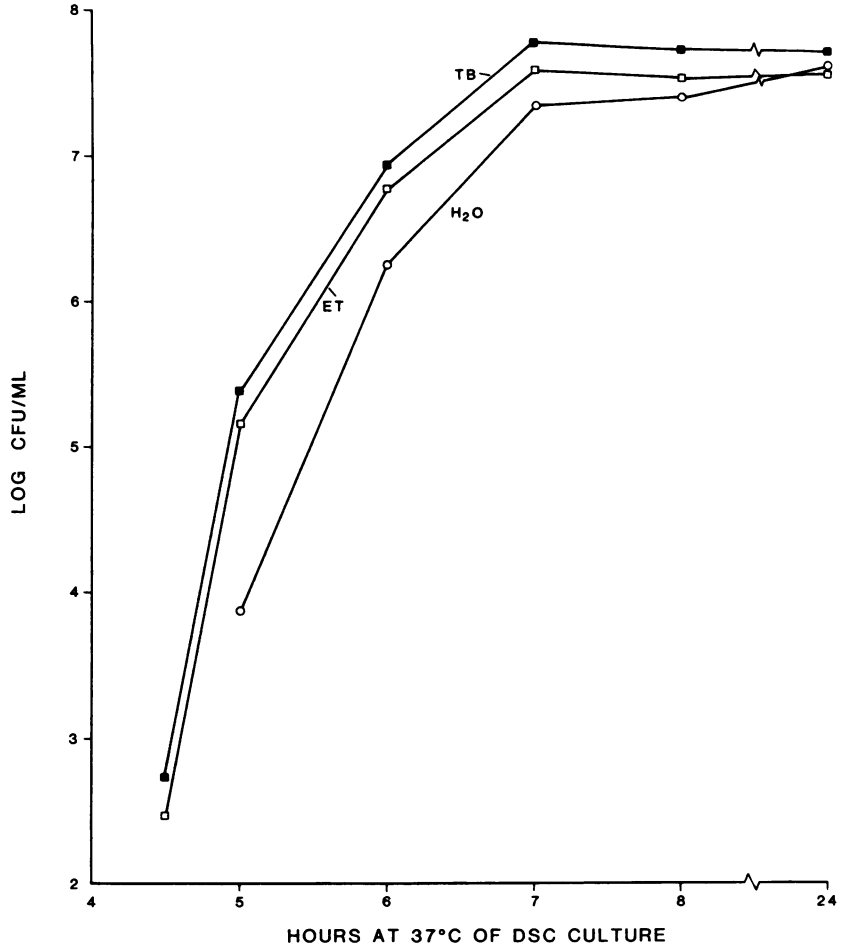

FIG. 4. Appearance of spore forms resistant to heating at 75°C for 20 min in water or to treatment at 37°C for 60 min in 10.8 M ET or 8 M *tert*-butanol (TB) in a *C. perfringens* NCTC 8679 culture in DSC.

optimal activation temperature. Increasing the alcohol concentration lowers the activation temperature until, beyond a certain concentration (e.g., 10.8 M ET at 37°C), the effectiveness of alcohols as sparing agents decreases. Levinson and Hyatt (29) reported that *B. megaterium* spores exposed at 30°C to 8 to 15 M ET, but not concentrations below or above this range, germinated completely in a glucose solution, as determined by a decrease in the optical density of spore suspensions. The disinfectant action of alcohols as well as their denaturing effect on proteins has a similar alcohol-water concentration range for optimum effectiveness; ET is most effective in a 50 to 70% (8 to 11 M) aqueous solution and ineffective below 10 to 20% and near 100% (15). The kinetics of alcohol activation as observed in this study are similar to those described for heat activation of *C. perfringens* spores (28) in that the rate and extent of CFU-based activation increased with either increasing temperature or increasing alcohol concentration at a given temperature. Also, when the length of exposure to heat or alcohol substantially exceeded the optimum, CFU-based activation declined. The failure to observe spore counts near 100% of the heat-shocked spore counts of NCTC 8679 spores exposed to some of the monohydric alcohols may have been because the competitive nature of the activation and inactivation of *C. perfringens* spores as described by Roberts (33) may have favored more inactivation. Alternatively, the optimum concentrations of the alcohols may not have been tested.

TABLE 3. Effect of lysozyme on the recovery of *C. perfringens* NCTC 8679 spores after treatment in water or aqueous alcohol

Treatment	% Activation[a]	
	Without lysozyme	With lysozyme (1.0 μg/ml)
Water (75°C for 20 min)	100	105
Water (4°C for 60 min)	5	10
8 M AL (37°C for 60 min)	<0.006	7
8 M 2-Propyn-1-ol (37°C for 60 min)	<0.006	0.004
2 M Phenyl-1,2-ethanediol (37°C for 60 min)	0.01	8
4 M Phenyl-1,2-ethanediol (37°C for 60 min)	<0.006	8

[a] See Table 1, footnote *c*.

The energy of activation calculated from heat activation data for *C. perfringens* spores is consistent with protein denaturation being a prime event in activation (28). In developing their model for the denaturation of proteins by organic cosolvents, Brandts and Hunt (11) proposed two effects: melting of ordered water structures and preferential solvation of the hydrophobic group by the organic cosolvent (ET) in aqueous mixtures. They reported that ET acts to

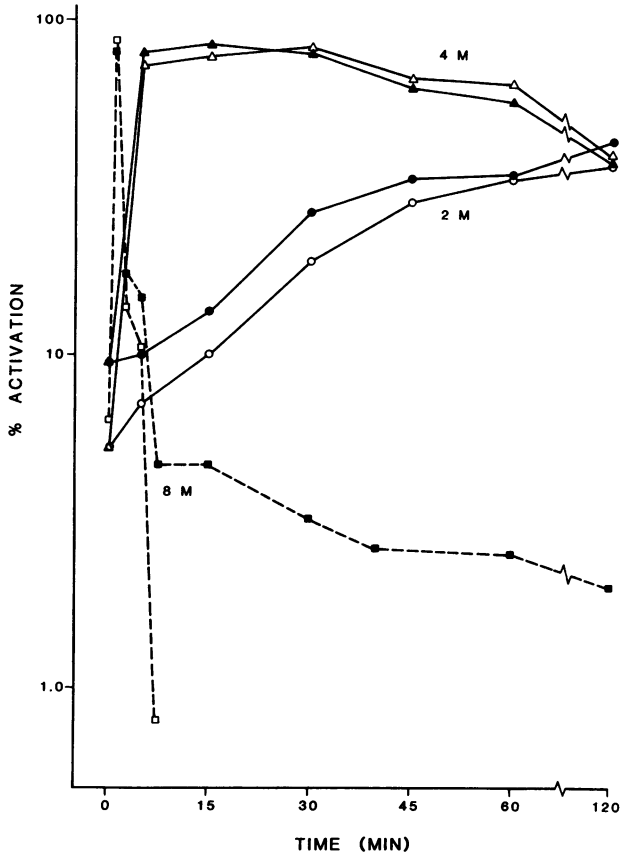

FIG. 5. Percent activation of *C. perfringens* NCTC 8679 spores in 2, 4, or 8 M AL at 37°C. Treated spores were enumerated on TSC with (closed symbols) or without (open symbols) 1.0 μg of lysozyme per ml.

decrease the free energy of denaturation and that the more prominent effects of ET on the thermodynamics of denaturation may arise from changes in the solvation of exposed hydrophobic side chains in the denatured protein. Our results showed that a linear relationship exists between the ability of the monohydric alcohols to lower the activation temperature and their lipophilicity. This relationship is generally thought to indicate a hydrophobic action site and has been reported for the effect of alcohols on fungal spores (38) and on other biological systems (18, 30, 32, 35). In explaining the inhibition of the germination of *Bacillus subtilis* spores by low concentrations of alcohols, Yasuda-Yasaki et al. (42) proposed a hydrophobic interaction between alcohols and a hydrophobic region on the spore near the receptor site for the germinant. The effectiveness of alcohols (15) and aliphatic diols (5) as disinfecting agents increases with increasing chain length (i.e., lipophilicity). The similar effective alcohol concentrations for spore activation and protein denaturation, the kinetics of spore activation by alcohols, and the relationship of spore activation to alcohol lipophilicity are consistent with protein denaturation being the activation event. Whether the *C. perfringens* spore sites for activation by heat or aqueous alcohol are indeed the same or whether they reside in the spore coat protein, as suggested by Duncan et al. (16), in membrane-associated protein, or at some other macromolecular site awaits additional evidence.

The evidence reported here and elsewhere does not indicate that the mechanism of activation differs for alcohols and heat. However, evidence has been presented that in developing *Bacillus* spores, the acquisition of resistance to heat and to alcohol may differ. Aronson and Fitz-James (6) reported that *B. megaterium* spores stripped of their spore coats retained dipicolinic acid, heat resistance, and refractility but became sensitive to solvents such as octanol. Octanol resistance develops at stage V of sporulation (34), can be attributed to newly synthesized spore coats (31), and develops before heat resistance, which is an indicator of complete spore maturation (41). Our results indicated that the resistance of *C. perfringens* spores to ET and *tert*-butanol develops before the resistance to heat. Consequently, alcohol activation may permit greater detection of *C. perfringens* spores that are immature as a result of mutation or interrupted sporulation. For example, Labbe (25) reported that *C. perfringens* NCTC 8235 appeared to be blocked at a late sporulation stage, formed a high percentage of nonrefractile spores and large quantities of enterotoxin, but produced few spores resistant to heating at 75°C.

Our results demonstrated that alcohols could injure spores at temperatures exceeding those for optimum activation but not previously reported to cause injury. Injury was manifested as a dependence on lysozyme in the plating medium for the germination of spores and colony formation. The addition of lysozyme to the plating medium was previously reported to increase the recovery of *C. perfringens* spores exposed to NaOH or temperatures above 100°C (1, 3, 16). Alcohols not only lowered the optimum activation temperature for NCTC 8679 spores but also had a sparing effect on the temperatures that injured the spores. Spores were not injured by being heated in water at temperatures as high as 90°C but were injured at 70°C in 10.8 M ET and at 40°C in 8 M AL. Spores were also injured at 37°C in 8 M 2-propyn-1-ol and 2 to 4 M phenyl-1,2-ethanediol. Because survival in our experiments was based on the ability to form colonies, we

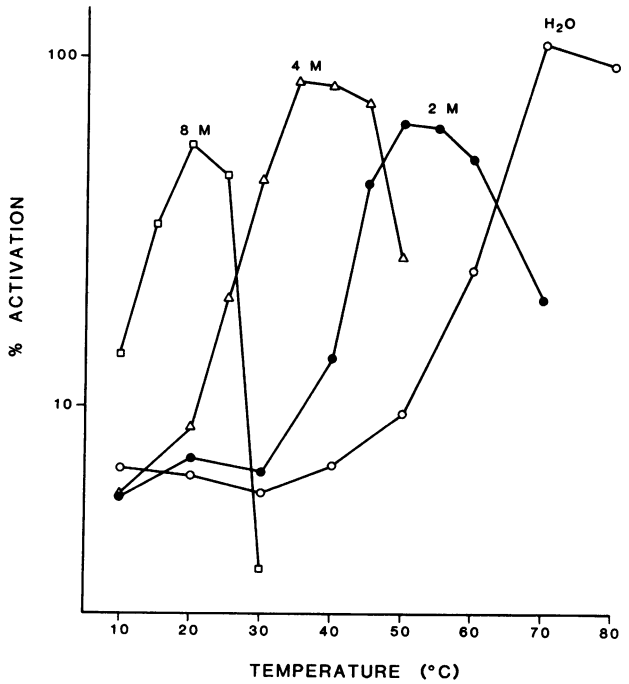

FIG. 6. Effect of temperature on the percent activation of *C. perfringens* NCTC 8679 spores in water or 2, 4, or 8 M AL. The heating time was 15 min.

could not determine with certainty whether injury involved some component of the germination or postgermination system. We did observe that, with spores treated with alcohols under conditions that produced lysozyme dependency, germination as determined spectrophotometrically was retarded, as compared with the germination of heat-activated spores (results not shown). The ability of these spores in the presence of lysozyme to regain the ability to form CFU at levels approximating those of the untreated spore population and the biphasic concave nature of the survival curve on TSC with lysozyme (Fig. 5) for spores treated with 8 M AL were similar to the results reported by Adams (3) for ultrahigh temperature injury of *C. perfringens* spores. His results indicated that the steeper portion of the survival curve represented inactivation of the normal germination system, whereas the shallow latter portion of the survival curve represented damage to the outgrowth system of a subpopulation of spores sensitive to germination by lysozyme. This sensitive spore subpopulation represented 1 to 2% of *C. perfringens* NCTC 8798 spores (3). Our results for the recovery of alcohol-injured spores suggested that a subpopulation normally sensitive to lysozyme represents about 5% of heat-activated NCTC 8679 spores.

Duncan et al. (16) proposed that injury to *C. perfringens* spores was caused by the inactivation of a normal germination lytic enzyme. Lysozyme apparently functions in a manner similar to the missing enzyme through degradation of the spore cortex. They reported that, because a normal lytic enzyme is inactivated or removed by alkali, a close association or location of a critical component of the germination system (e.g., the cortex-lytic enzyme release mechanism) and the alkali-soluble coat protein may exist.

The use of lysozyme for the recovery of spores with damaged germination systems implies that the injury is bypassed rather than repaired (4). As nongerminated spores are not metabolically active, repair based on the resynthesis of damaged molecules or structures would not be expected. Because the germination mechanism of *C. perfringens* spores may be injured by chemicals or heat, the inclusion of lysozyme in plating media, the sensitization of injured spores to lysozyme (2), or other methods for the recovery of injured spores should be considered for the detection and recovery of *C. perfringens* from foods or other environmental sources likely to contain injured spores.

Our preliminary analyses with a limited number of food samples shows that an activation treatment with *tert*-butyl alcohol may yield *C. perfringens* spore counts up to three times higher than heat activation does. Alcohol treatment may be a useful procedure for detecting *C. perfringens* spores in foods and for studying the mechanisms of spore activation and injury.

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