Effect of Delayed Germination by Heat-Damaged Spores on Estimates of Heat Resistance of *Clostridium botulinum* Types E and F

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ABSTRACT

The effect of delayed germination by heat-damaged spores on estimates of heat resistance of *Clostridium botulinum* types E and F was studied by analysis of data from thermal death time tube experiments. Type E strains were studied in crabmeat and nonproteolytic and proteolytic type F strains in phosphate buffer and crabmeat. The date each culture became positive was recorded and tabulated by week. The decimal reduction times (D) and z values for each strain were calculated at several different time intervals and compared. Although both D and z values increased during the incubation period, D values as much as fourfold, only proteolytic strains showed substantial increases in z values.

INTRODUCTION

DELAYED GERMINATION of heat-damaged spores was recognized many years ago as a problem in heat-resistance studies (Bigelow and Esty, 1920; Dickson et al., 1925). The heat resistance of PA3679 and *Clostridium botulinum* was compared in cultures for surviving spores which were incubated for at least 3 months before they were considered sterile (Townsend et al., 1938). A study of the effect of incubation temperature on the apparent heat resistance of spores of *C. botulinum* types A and B showed that differences caused by media were often eliminated by prolonged incubation (Williams and Reed, 1942).

In studies of the heat resistance of type E and both proteolytic and nonproteolytic type F, the nonproteolytic strains were found to have low heat resistance, whereas the proteolytic strains, like those of types A and B, were found to have high heat resistance (Lynt et al., 1977, 1979, 1981). Delayed germination of heat-damage spores required an incubation time of 10 wk in cultures for surviving spores in type E experiments and 6 months in all type F experiments. Obviously, data on the heat resistance of food spoilage bacteria, including *C. botulinum*, would be more readily obtainable if incubation times could be shortened. When solid media have been used for recovery, incubation times generally were much shorter (Odlaug and Pflug, 1977; Odlaug et al., 1978; Roberts et al., 1965; Stumbo et al., 1950). After exposure of *C. perfringens* spores to heat, recovery of the stress-damaged spores peaked in a few days. Although recovery declined when the spores were incubated in an agar medium, some spores were recovered throughout the 90-day incubation period in a broth medium (Futter and Richardson, 1972).

This study evaluated the effect of delayed germination of estimates of heat resistance by re-examining the data from previous experiments and using survivor data at various times during incubation of broth cultures.

MATERIALS & METHODS

Strains and spore stocks

The Alaksa, Beluga and G21-5 strains of type E *C. botulinum* and the nonproteolytic 190, 202 and 610 as well as the proteolytic 4YRC, PC and Langeland strains of type F were studied. Spore stocks were grown in 500-ml Wheaton bottles containing 350 ml of typticase-peptone-glucose-yeast extract (TPGY) broth. When the cultures were fully sporulated, the spores were harvested, washed three times in distilled water and concentrated by centrifugation. The spores were finally resuspended in a small amount of distilled water for seeding 150 ml of buffer or 454g of crabmeat to give a final count of 10^4–10^6 spores per ml or g. Each experiment was planned to cover a range of time intervals so that a gradation occurred between the last time surviving spores were observed in all tubes to the time beyond which no spores survived in any tube.

Preparation of buffer

Buffer was prepared from 0.067M solutions of Na_2HPO_4 and KH_2PO_4, which were combined with constant stirring and monitoring until a pH of 7.0 was reached. The buffer was dispensed in 150-ml quantities and sterilized by autoclaving at 121°C for 15 min. Buffer was refrigerated until used, at which time 1.0 ml of the spore stock was added to each 150 ml needed for the experiment. After being sealed and stored in an ice bath (or under refrigeration for proteolytic strains) for the duration of each experiment, at least three representative tubes of the seeded buffer were used to determine the actual spore count in that experiment. Spores were counted by the 3-tube most probable number (MPN) method, with TPGY as the culture medium. Vegetative cells were not considered to be present since the method of preparation of the spore stock makes this highly unlikely.

Preparation of crabmeat

Crabmeat was purchased in 1-lb (454g) cartons as needed from a local dealer as "fresh-picked crabmeat" and sterilized in 454-g portions by autoclaving 40 min at 121°C in flat, shallow pans. After being autoclaved, the crabmeat was refrigerated overnight; the following morning it was aseptically transferred to a sterile Waring Blendor in which the spore stock was uniformly blended into the meat to form a smooth paste. After being tubed and stored in an ice bath for the duration of the experiment, at least three representative tubes, each containing 1g of crabmeat, were used to determine the spore count by the 3-tube MPN method.

Experimental procedure

The thermal death time (TDT) experiments were conducted by the TDT tube technique at 160, 165, 170, 175, 180 and 185°F for the type E and the nonproteolytic type F strains and at 210, 215, 220, 225 and 230°F for the proteolytic type F strains. Four replicate experiments were performed for each set of conditions. The results were cumulated to give approximately 40 tubes per time interval for each temperature with each strain for calculations. For each experiment, 100 replicate 10 x 75 mm heat-resistant glass TDT tubes were filled with either 1.1 ml of seeded buffer from a Cornwall pipet, or 1 g of the seeded crabmeat from a specially adapted grease gun (Stumbo et al., 1945). The tubes were immediately flame-sealed and stored in an ice bath or refrigerated until needed. They were bundled into groups of 3 or 4, attached to a sinker to ensure complete immersion in the bath, and labeled with an identifying tag. The 100 tubes were dropped, in groups of 10, at each time interval into a 50-gallon (378.6-liter) water or silicone bath controlled at the desired temperature. Precision of the bath temperature was indicated by a straight line recording produced by the monitoring copper-constantan thermocouples on a potentiometer-recorder chart. Thermocouples used for this purpose had been previously checked against a calibrated thermometer. Accuracy of the thermocouples was checked periodically. All tubes were removed simultaneously

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at the end of the experiment and returned immediately to the ice bath or chilled in running tap water.

Tubes of the nonproteolytic strains were held in the ice bath until they were opened for culturing. Proteolytic type F strains were held at room temperature. Either 1 ml of seeded buffer or the entire contents of seeded crabmeat from each tube was cultured in a separate tube of TPGY broth, which had been boiled 10–15 min to exhaust dissolved oxygen just before inoculation. Each culture contained a Durham tube. All type E and nonproteolytic type F cultures were incubated at 26°C for 2 wk (or less if they showed growth). Proteolytic type F cultures were incubated at room temperature only. All tubes without growth in 2 wk were sealed with a layer of vaspar to maintain anaerobiosis and further incubated at room temperature for at least 6 months. TPGY broth is an anaerobic medium with enough thioglycolate to maintain anaerobiosis for at least 2 wk but not for extended incubation. If no growth was observed in 6 months, the tubes were considered to be sterile. Cultures from tubes having the longest heat exposure with growth in all 10 and those with growth at longer exposure times were tested for botulinum toxin by intraperitoneal injection of 0.5 ml of undiluted culture fluid into mice. Only toxic cultures were counted. The possibility of contamination from the handling involved in filling, sealing, opening and culturing the TDT tubes required proof that the culture was from spores surviving the heat treatment. Nontoxic cultures were rarely found.

Calculations

Correction of the time of exposure for thermal lag and lethality during lag was made by the graphic method (Anellis et al., 1954). The heat penetration curve for each temperature of the bath was plotted on inverted 3-cycle semilogarithmic paper as the average number of °F below bath temperature for at least five determinations. Temperatures were charted at 11-sec intervals by a potentiometer-recorder from a copper-constantan thermocouple located as close as possible to the geometric center of 1.1 ml of buffer or 1g of crabmeat in a TDT tube. The time to reach bath temperature, thus determined, and corresponding temperatures from the penetration curve and the uncorrected TDT curve were the basis for the corrections. Decimal reduction times (D) were calculated from the corrected data by Schmidt's probability method (1957). This method uses a modification of the general formula, D = U/log A-log B, in which D = LD_{50} (in min)/log A + 0.16, where log A is the log of the initial count per tube and log B, the log of the number of survivors per tube at LD_{50} (0.69), is −0.16. The Karber method of calculating the LD_{50} from the number of positive and negative cultures at each time interval was used. Because of skips and tailing in the data, use of the LD_{50} seemed more appropriate than methods using absolute end points. D values were calculated for each temperature for each strain.

The data obtained from these experiments were subjected to statistical analysis. The standard error of each 10-wk LD_{50} for type E and each 26-wk LD_{50} for type F was determined and the upper and lower 95% confidence limits were calculated. The mean logarithm of all initial spore counts of each strain was used to calculate D values at all temperatures for that strain. The D values thus calculated from the cumulated data of replicate experiments at each temperature were plotted and the heat-resistance curve was drawn as a straight line by visual best fit on semilogarithmic paper, with time on the logarithmic (vertical) axis and temperature along the arithmetic (horizontal) axis on the assumption that the destruction of spores by heat is logarithmic. For each of these curves the temperature increment in F° for one log cycle (z) was determined.

Delayed germination

The date when growth first appeared in each positive culture was recorded. Positive cultures were tabulated by week. Any culture requiring more than 1 wk to show evidence of growth was counted as delayed. D values were calculated at 1 wk, 2 wk and 4 wk in addition to the final incubation time for all strains and at 13 and 20 wk for the type F strains.

RESULTS & DISCUSSION

NORMAL SPORES of these 9 strains of *C. botulinum* types E and F grow out very quickly in TPGY broth. Spore counts of the stock suspensions made by the MPN method in this medium did not change after prolonged incubation. Therefore, for purposes of this study, any culture requiring more than 1 wk to grow out was considered delayed.

Tables 1, 2 and 3 show the percent of positive cultures exhibiting delayed germination in heat-resistance experiments with these organisms. Type E strains Alaska, Beluga and G21-5 showed the lowest percentages of delayed germination. The nonproteolytic strains of type F, 190, 202 and 610 showed much higher rates of delayed germination, but with neither the type E strains nor these did the extent of delay correlate with the temperature of exposure. Among the nonproteolytic type F strains delayed germination was high with strains 610 and 190, which were also the most and least heat-resistant strains, respectively, at these temperatures. Delayed germination was most extensive in experiments with the 3 proteolytic strains of type F, PC, Langeland and 4YRC, and among these strains it correlated with their heat resistance. But, like the nonproteolytic strains, they showed less delayed germination in crabmeat than in buffer.

Regardless of the percentage of cultures showing delayed germination, these cultures cannot be ignored in measuring

Table 1 — Percent of positive cultures for surviving spores showing delayed germination in heat-resistance experiments on 3 strains of Clostridum botulinum type E in crabmeat

Temp (°F)	Strain		
	Alaska (%)	Beluga (%)	G21-5 (%)
165	11	10	0
170	6	5	7
175	14	5	10
180	16	25	8
185		18	
Overall percentage at all temps	12	12	5

Table 2 — Percent of positive cultures showing delayed germination in heat-resistance experiments on 3 nonproteolytic strains of Clostridium botulinum type F

Temp (°F)	Strain			
	190	610	202	
	in buffer (%)	in buffer (%)	in buffer (%)	in crabmeat (%)
160	34		29	
165	77		25	
170	49	50	51	23
175	53	45	37	23
180	60	96	31	39
185		37		25
Overal percentage at all temps	55	58	33	26

Table 3 — Percent of positive cultures for surviving spores showing delayed germination in heat-resistance experiments on 3 proteolytic strains of Clostridium botulinum type F

Temp (°F)	Strain			
	PC	Langeland	4YRC	
	in buffer (%)	in buffer (%)	in buffer (%)	in crabmeat (%)
210	94	38	46	
215	97	94	71	72
220	95	66	62	74
225	73	50	47	55
230	80	68	70	53
Overal percentage at all temps	88	56	62	53

Table 4 — Effect of delayed germination on D and z values of Clostridium botulinum type E in crabmeat, calculated from survival data at intervals during incubation

Temp (°F)	D value (min) Alaska strain 1[a]	2	4	10
165	8.10	8.69	9.96	10.05 (± 2.15)[b]
170	2.66	2.66	2.82	2.82 (± 0.70)
175	1.33	1.47	1.47	1.49 (± 0.44)
180	0.55	0.65	0.71	0.71 (± 0.19)
185				
z value (F°)	10.5		11.0	11.0
	Beluga strain 1	2	4	10
165	10.77	11.22	11.22	11.22 (± 3.46)
170	2.80	2.88	2.91	2.94 (± 2.14)
175	1.18	1.19	1.19	1.19 (± 0.75)
180	0.37	0.53	0.84	0.84 (± 0.29)
185	0.15	0.15	0.33	0.33 (± 0.13)
z value (F°)	10.3		11.7	11.7
	G21-5 strain 1	2	4	10
165	6.15	6.15	6.15	6.15 (± 1.06)
170	1.53	1.62	1.70	1.70 (± 0.72)
175	0.66	0.70	0.71	1.09 (± 0.52)
180	0.45	0.49	0.49	0.51 (± 0.30)
z value (F°)	11.0		11.3	12.8

[a] Weeks of incubation.
[b] 95% confidence limits in parentheses.

Table 5 — Effect of delayed germination on D and z values of nonproteolytic strains of Clostridium botulinum type F in phosphate buffer, calculated from survival data at intervals during incubation

Temp (°F)	D value (min) Strain 190 1[a]	2	4	13	20	26
160	26.13	29.87	29.93	30.43	30.88	31.88 (± 16.60)[b]
165	6.66	7.29	7.51	8.49	8.80	9.07 (± 3.50)
170	0.59	1.00	1.17	1.53	1.60	1.66 (± 1.22)
175	0.27	0.43	0.44	0.83	0.91	1.03 (± 0.55)
180	0.13	0.14	0.16	0.22	0.24	0.25 (± 0.20)
185						
z value (F°)	7.5		8.0	9.3	9.5	9.7
Temp (°F)	Strain 202 1	2	4	13	20	26
160	36.87	39.60	39.74	41.84	42.41	42.41 (± 17.40)
165	9.72	11.49	11.78	12.21	12.68	12.68 (± 6.87)
170	2.63	2.90	3.12	3.94	4.29	4.29 (± 2.32)
175	0.60	0.70	0.77	0.89	0.91	0.93 (± 0.44)
180	0.20	0.23	0.23	0.31	0.33	0.33 (± 0.25)
185						
z value (F°)	8.7		8.8	9.4	9.5	9.5
Temp (°F)	Strain 610 1	2	4	13	20	26
160						
165						
170	3.62	4.75	5.03	6.06	6.47	6.64 (± 2.68)
175	1.28	1.53	1.63	2.05	2.10	2.12 (± 1.89)
180	0.33	0.46	0.57	0.78	0.78	0.84 (± 0.68)
185	0.17	0.25	0.26	0.32	0.36	0.37 (± 0.38)
z value (F°)	10.3		11.1	11.2	11.3	11.3

[a] Weeks of incubation.
[b] 95% confidence limits in parentheses.

heat resistance. The influence of delayed germination on the calculation of D values is shown in the remainder of the tables. Type E was studied in crabmeat only (Table 4). With the lowest percentages of delayed germination, type E nevertheless showed a general rise in D values during the incubation period until all surviving spores were accounted for. In some instances there was no increase, but in others, D values almost doubled during the incubation period because of delayed germination. The z values for type E remained virtually unchanged throughout the incubation period.

Among the nonproteolytic type F strains, D values reflected the greater percentage of cultures showing delayed germination. In the nonproteolytic type F strains in 0.067M phosphate buffer (Table 5), delayed germination accounted for up to 2-, 3- and 4-fold increases in D values over the 6-month incubation. Crabmeat seemed to offer protection to the spores of strain 202, giving higher D values than in phosphate buffer (Table 6), but the overall effect of delayed germination appeared to be the same. The greatest effect, however, at 180°F in crabmeat and 170°F in phosphate buffer, corresponded to the temperatures with the highest rate of delayed germination in each substrate. The z values for type F showed a slight increase.

Proteolytic strains of type F (Tables 7 and 8), showing the highest percentages of delayed germination, also showed 2-, 3- and 4-fold increases in D values over the 6-month incubation, but crabmeat afforded them no protection. The D values were approximately the same as in phosphate buffer. However, the z values of the corresponding heat-resistance curves increased over the 6-month incubation period in both phosphate buffer and crabmeat. The z value of strain 62A in phosphate buffer, based on a 3- to 6-wk incubation period in an agar recovery medium, was reported as 16.3 (Stumbo et al., 1950), which, although somewhat lower, is still very close to our results for the PC and Langeland strains at 4 wk in a broth recovery medium.

A least squares plot of the heat-resistance curves, which sometimes gives a better logarithmic fit to the D values, was tried for comparison of z values with those determined by visual best fit. The differences were small and not statistically significant, but for nonproteolytic stains were affected in the same manner as those determined by visual best fit. For the proteolytic strains, z values calculated by least squares reached the peak earlier, but extrapolated D_{250} values were approximately the same as those determined by visual best fit.

Occasionally, germination of a culture of either proteolytic or nonproteolytic type F was delayed until the last week of the 6-month incubation period; thus, further incubation might have yielded additional positive cultures. At the same time, however, with little or no difference between 20- and 26-wk D values, longer incubation for greater precision in estimates of D values seemed unwarranted. On the other hand, D values calculated at the end of a few days or

Table 6 — Effect of delayed germination on D and z values of Clostridium botulinum nonproteolytic type F strain 202 in crabmeat, calculated from survival data at intervals during incubation

Temp (°F)	D value (min) 1[a]	2	4	13	20	26
170	7.59	8.52	8.77	9.28	9.32	9.50 (± 4.64)[b]
175	2.85	3.14	3.33	3.61	3.63	3.64 (± 1.85)
180	0.68	1.03	1.17	1.18	1.18	1.20 (± 0.73)
185	0.41	0.47	0.49	0.53	0.53	0.53 (± 0.25)
z value (F°)	10.6		11.0	11.0	11.5	11.5

[a] Weeks of incubation.
[b] 95% confidence limits in parentheses.

1 wk may be underestimated by ½ to ¾ of that based on a 6-month incubation period. Translated into processing time, such an underestimate could have the effect of reducing a calculated 12D process to an actual 3D process, eroding the margin of safety and giving a false sense of security.

There is no indication that the usual heat processes for low-acid canned foods are not adequate tc destroy proteolytic type F strains; on the contrary, if the curves based on 6 months of incubation are extrapolated, the D_{250} would be from 0.14–0.23, or about the same as the proteolytic types A and B, which is the basis for safe processes. However, similar extrapolation at 1 wk gives an entirely different picture. With no delayed germination the D_{250} would be only 0.02–0.05 min, very low values in terms of heat processing. One might therefore conclude that type F is no problem, when actually it poses the same problem to heat-processing safety as types A and B (proteolytic). Elsewhere, heat-resistance studies with types A and B showed z values in food substrates to be <18, the z value by which F_0 is defined, and that such deviations may result in an underesimate of F_0 (Perkins et al., 1975). This also appears to be true if delayed germination is not accounted for by adequate incubation times.

The nonproteolytic strains would be readily destroyed by safe heat processes for low-acid canned foods, whether delayed germination is taken into account or not; nevertheless, where a low heat process or "pasteurization" is used, it is just as important that it be adequate to destroy spores of the nonproteolytic strains as for canning processes to destroy spores of the proteolytic strains. An underestimate of heat resistance could be equally disastrous. Unless adequate consideration is given to delayed germination of heat-damaged spores, the heat resistance of *C. botulinum* may be underestimated by both the D and z values.

Table 7 — Effect of delayed germination on D and z values of proteolytic strains of Clostridium botulinum type F in phosphate buffer, calculated from survival data at intervals during incubation

Temp	D value (min)					
	PC strain					
(°F)	1[a]	2	4	13	20	26
210	13.15	16.40	18.05	19.76	22.68	23.22 (± 5.23)[b]
215	5.20	8.45	9.70	11.78	11.96	12.02 (± 5.74)
220	2.72	3.45	4.11	5.44	6.24	6.33 (± 2.86)
225	0.76	1.69	2.59	3.24	3.33	3.33 (± 2.27)
230	0.93	1.10	1.30	1.76	1.82	1.82 (± 1.28)
z value (F°)	14.6		17.2	18.0	18.1	18.1
Temp	Langeland strain					
(°F)	1	2	4	13	20	26
210	12.46	16.12	17.50	19.18	19.28	19.38 (± 5.23)
215	5.18	5.95	7.48	8.73	8.76	8.76 (± 2.39)
220	2.37	3.04	3.80	4.64	4.72	4.74 (± 2.12)
225	1.33	1.66	2.17	2.60	2.60	2.60 (± 0.90)
230	0.72	1.35	1.59	1.78	1.79	1.79 (± 1.16)
z value (F°)	14.2		17.0	19.0	19.1	19.1
Temp	4 YRC strain					
(°F)	1	2	4	13	20	26
210	6.89	8.25	9.70	11.84	12.07	12.19 (± 4.16)
215	3.35	4.06	4.79	5.30	5.35	5.35 (± 2.42)
220	1.16	1.66	2.37	3.35	3.46	3.55 (± 2.04)
225	0.93	1.48	1.73	2.07	2.07	2.09 (± 1.00)
230	0.54	0.88	1.19	1.45	1.45	1.45 (± 0.70)
z value (F°)	17.5		21.8	23.2	23.7	25.3

[a] Weeks of incubation.
[b] 95% confidence limits in parentheses.

Table 8 — Effect of delayed germination on D and z values of proteolytic Clostridium botulinum type F strain 4YRC in crabmeat, calculated from survival data at intervals during incubation

Temp	D value (min)					
(°F)	1[a]	2	4	13	20	26
215	3.30	4.50	4.93	5.05	5.07	5.07 (± 2.80)[b]
220	2.19	3.36	3.65	4.02	4.02	4.02 (± 2.41)
225	1.32	1.71	2.06	2.14	2.14	2.14 (± 1.08)
230	0.42	0.99	1.21	1.35	1.35	1.35 (± 1.06)
z value (F°)	14.5		20.5	23.0	23.0	23.0

[a] Weeks of incubation.
[b] 95% confidence limits in parentheses.

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