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Kinetics of Heat Activation of Spores of *Clostridium perfringens*

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Spores of *Clostridium perfringens* NCTC 8238 and NCTC 8798, free from cell debris, vegetative cells, and sporangia, but with ca. 3% phase-dark spores, were produced on a modification of Duncan-Strong medium in large quantities. Aqueous suspensions of spores were heated at temperatures from 55 to 75°C for various times. Activation was estimated as the percent increase in (i) colony-forming units or (ii) germination rate in 5 mM KNO₃. Activation rate, activation lag, and time (t_{max}) of heating required to achieve maximum activation were computed. With both strains, whether based on colony-forming units or germination rate, activation rate increased, while lag and t_{max} decreased, with increasing temperature. The energy of activation, μ , was ca. 3×10^5 J, consistent with involvement of protein denaturation as a prime event in activation. After exposure at 62°C or higher for 5 h, i.e., several hours beyond t_{max} , colony-forming units, but not germination rate, decreased from the maximum, suggesting inactivation of postgerminative development rather than of germination. Spores of another strain, *C. perfringens* ATCC 14810, were not heat activated at either 60 or 75°C.

Since the publication of Hobbs' (14) classical paper, *Clostridium perfringens* type A has been increasingly recognized as an important food-poisoning organism. During 1975, *C. perfringens* was exceeded only by *Staphylococcus* and by *Salmonella* as the bacterial agent confirmed as causing outbreaks of foodborne disease in the United States (6).

The increasing realization of the significance of *C. perfringens* in food-poisoning outbreaks coupled with the demonstration of the close relation between enterotoxin synthesis and sporulation (9) have stimulated research on the physiology of spores of this organism. *Bergey's Manual* (29) calls special attention to rare or sparse spore formation by this species in the usual bacteriological media. The difficulty in obtaining large quantities of clean spores has been a major deterrent to extensive and reliable studies on *C. perfringens* spore germination and other properties. Much effort has been expended in resolving this difficulty, but, of the host of media designed for *C. perfringens* sporulation (3, 8, 10, 18, 24, 25, 27), that described by Duncan and Strong (8) has probably been the most widely accepted. We have now modified this medium to give improved yield.

Some other investigators (2, 19) of germination of *C. perfringens* spores have cleaned spores by ultrasonic treatment. In separating spores from

other cell forms, we have used only methods which could confidently be assumed to have little or no effect on heat resistance or on germinability, avoiding the possibility of alteration of spore properties by ultrasonic treatment (4).

In this paper, we discuss, in some detail, the first step in the transition of spores to vegetative cells, i.e., activation. We have used a somewhat broader range of temperatures and times of heat activation than was reported by Ando (2), and we have used increases in colony formation and in germination rate as indexes of activation. Our eventual aim is to apply to cleaned *C. perfringens* spores the same sort of intensive study that has been accorded to sporeformers of the genus *Bacillus*.

MATERIALS AND METHODS

Organisms and preparation of spores. Spores of *C. perfringens* type A, NCTC 8238 and NCTC 8798, were prepared. These strains were isolated by Hobbs et al. (14) from salt beef and beef rissole involved in food-poisoning incidents and are classified (15) as serotypes 2 and 9, respectively.

Frozen stock cultures were thawed, heated at 75°C for 20 min, rapidly chilled, and inoculated (1%) into 120 ml of Baltimore Biological Laboratory (BBL) fluid thioglycolate medium (FTM). The FTM culture, after 12 h (NCTC 8238) or 17 h (NCTC 8798) at 37°C, was inoculated into 12 liters (in a 20-liter carboy) of

Duncan-Strong medium (8), modified to contain (grams per liter): phytone (BBL), 15.0 (NCTC 8238) or 10.0 (NCTC 8798); yeast extract (Difco), 4.0; soluble starch (Difco), 4.0; sodium thioglycolate (Difco), 1.0; and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 10.0. Of the four brands of soluble starch which were tested, the Difco brand gave the clearest solution and the best yield of spores.

The inoculated modified Duncan-Strong medium was flushed with N_2 and incubated for 22 h at 40°C (NCTC 8238) or at 37°C (NCTC 8798). The cultures, containing a mixture of cell forms (Table 1), were harvested by continuous-flow centrifugation and were cleaned by repeated (at least six times) manual scraping of the lower-density vegetative cells (Table 1) and debris from the surface of the pellets, rinsing of the surface with chilled water, and washing by suspension in chilled water and centrifuging at 4°C. The cleaned preparation was lyophilized (Table 1) and stored over silica gel under vacuum at 4°C. Lyophilization did not affect heat resistance or germinability of the spores. The batches of cleaned, lyophilized spores were pooled

and characterized (Table 1) as to spore density, heat resistance, and total P, Ca, N, and dipicolinic acid.

The frozen stock cultures (above), used for inoculating FTM, were prepared by inoculating 10 ml of modified Duncan-Strong medium in screw-cap tubes (20 by 125 mm) with 0.1 ml of a 15-h (37°C) FTM culture and incubating at 37°C for 22 h. Stock cultures were frozen and maintained at -20°C.

Glassware and chemicals. All chemicals were of chemically pure quality. Distilled water was deionized by sequential passage through columns of activated carbon and mixed bed resin (Continental Water Conditioning Corp.) and had a resistivity of $>3 \times 10^6$ ohms. All glassware was "leached" (1) to reduce the possibility of ionic contamination from glassware.

Heat activation. Heat activation of the two strains of *C. perfringens* was quantitated by two methods.

(i) CFU-based activation = percent increase in colony-forming units (CFU), or $[(\text{CFU}_t - \text{CFU}_0)/$

TABLE 1. *C. perfringens* NCTC 8238 and NCTC 8798 grown on modified Duncan-Strong medium.

Characteristics	NCTC 8238	NCTC 8798	Method (reference)
Culture			
Phytone, optimum, %	1.5	1.0	
Incubation temperature, °C	40	37	
Incubation time, h	22	22	
Bacterial form			
Free spores, %	64	43	
Germinated spores, %	5	6	
Spores in sporangia, %	3	7	
Vegetative cells, %	28	44	
Yield, plate count, spores/ml	10^8	6×10^7	
Biochemical			
Lactose fermentation	+	+	30
Nitrate reduction	+	+	30
Motility	-	-	30
Gelatin liquefaction	+	+	30
Lecithinase	+	+	30
Sulfite reduction	+	+	28
Washed, lyophilized spores			
Yield/12-liter carboy, g	1.8	0.98	
Yield, total, g	32.0	25.5	
Hemocytometer count, spores/mg	3.4×10^8	3.9×10^8	
Bacterial form			
Free spores, %	98	95	
Germinated (phase-dark) spores, %	2	5	
Sporangia and vegetative cells, %	0	0	
P, µg/mg	15.9	17.8	11, 21
Ca, µg/mg	46.4	44.4	21, 26
Mn, µg/mg	<1	<1	7, 21
N, µg/mg	85.6	88.3	23
Dipicolinic acid, µg/mg	136	143	16
$D_{90^\circ\text{C}}$, min ^a	130	80	
Density			
Spores, g/cm ³	1.347	1.343	31
Vegetative cells, g/cm ³	1.24	1.24	31

^a $D_{90^\circ\text{C}}$ = time at 90°C required for 90% reduction of viable count on thioglycolate medium 135C (BBL) + 1.5% agar, 24-h anaerobic incubation at 37°C.

$CFU_t / CFU_0 \times 100$, where CFU_0 and CFU_t were, respectively, colony counts from unheated spores and from spores heated for t minutes.

(ii) GR-based activation = percent increase in germination rate (GR), or $[(GR_t - GR_0) / GR_0] \times 100$, where GR_0 and GR_t were, respectively, the germination rates of unheated spores and of spores heated for t minutes.

CFU-based activation. Aqueous suspensions (5 mg/ml) of lyophilized spores were thoroughly mixed with a Vortex mixer and were filtered through a sintered-glass funnel (Pyrex, coarse) to remove large clumps. Generally, ca. 10% of the dry weight was removed by filtration, somewhat more being removed from NCTC 8798 than from NCTC 8238 suspensions. Filtered spores were diluted 1:10 into water at the appropriate heating temperature (55 to $75^\circ\text{C} \pm 0.05^\circ\text{C}$), and the resulting spore suspension (0.5 mg/ml) was heated with continuous magnetic stirring. Samples (1.0 ml) were withdrawn, immediately added to ice-chilled water (9.0 ml), and, after appropriate dilution in chilled water, plated (pour plates) in reinforced clostridial agar (13), which had the following composition (grams per liter): yeast extract (Difco), 3.0; Lab-Lemco-beef extract (Oxoid), 10.0; Bacto-peptone (Difco), 10.0; soluble starch (Difco), 1.0; D-glucose, 5.0; NaCl, 5.0; sodium acetate, 3.0; cysteine-HCl, 0.5; and agar, 15.0; the pH was adjusted to 6.8 with NaOH, and the medium was autoclaved for 20 min at 115°C . The medium was made fresh each day. CFU were counted after incubation at 37°C for 22 to 24 h under anaerobic conditions (BBL GasPak). It was important to put reinforced clostridial agar plates under anaerobic conditions immediately after inoculation to avoid marked reductions in count attributable, apparently, to sensitivity of germinated spores to aerobic conditions.

The plate counts from unheated spores of NCTC 8238 and of NCTC 8798 averaged ca. 4.6×10^7 and ca. 1.1×10^7 CFU/mg, respectively. Maximum colony counts of heat-activated spores of NCTC 8238 on reinforced clostridial agar indicated that there were ca. 1.5×10^8 spores/mg, <50% of the hemacytometer count (Table 1); the viable count of maximally heat-activated *C. perfringens* NCTC 8798 spores was only ca. 25% of the hemacytometer count. We attribute the discrepancy between plate and hemacytometer counts to the presence of many aggregates of two or three spores in suspensions of NCTC 8238 spores and somewhat larger spore clumps in suspensions of NCTC 8798. The counts from unheated spores and from heated spores were compared to calculate CFU-based activation (see above) for each temperature and time of heating.

GR-based activation. The definitive parameter of activation, however, is not increased colony formation, but rather an increased rate or extent of germination (12). As an alternative (and preferable) index of activation, we used germination rate at 30°C in 5 mM KNO_3 , a system which avoids the outgrowth necessary for colony formation. Spores of *C. perfringens* (2) and of *B. megaterium* QM B1551 (20) are capable of germination in KNO_3 or in other ionic solutions, without organic adjuvants. Thoroughly mixed aqueous suspensions of lyophilized spores of *C. perfringens* NCTC 8238 (6.5 mg/ml) or NCTC 8798 (7.8 mg/ml) were filtered through sintered glass, 1.0 ml of the filtered

suspension was added to 9.0 ml of water at the desired temperature, and the resulting spore suspensions were heated in the bath with continuous magnetic stirring. At appropriate time intervals, individual tubes were removed from the bath, and, after immediate chilling in an ice-water bath, were equilibrated at 30°C . Experiments were initiated by adding 2.5 ml of heated spore suspension to standard "leached" Klett tubes, containing 2.5 ml of 10 mM KNO_3 at 30°C . The initial Klett-Summerson colorimeter reading (no. 56 filter) was 250 to 270. Rubber stoppers, rinsed in four changes of boiling deionized water and covered with Parafilm, were used to plug the Klett tubes, which were read at 5-min intervals. No attempt was made to maintain anaerobiosis. The percent decrease in Klett reading was the index of germination. Germination rate was estimated from the slopes of plots of percent loss in optical density (OD) as a function of time of germination (22). GR-based activation was calculated (see above) for all temperatures and times of heating.

In one experiment, spores heated at 70°C were germinated in half-strength reinforced clostridial broth, a broth prepared like reinforced clostridial agar, but with the agar omitted. In this experiment, 2.5 ml of spore suspension was added at 30°C to an equal volume of full-strength reinforced clostridial broth in Klett tubes.

Kinetic constants. Plots of activation versus time of heating, one of which is represented diagrammatically in Fig. 1, permitted graphical and mathematical determination of a number of kinetic constants. After visually determining the range of points (x_1y_1 to x_2y_2) defining the rectilinear portion of the curve for each temperature, we computed (method of the sum of least squares) and constructed a regression line, $y = mx + b$ (Fig. 1). Activation rate (activation/minute of heating), activation lag, and t_{max} , the time required to achieve maximum activation (A_{max}), were computed by the indicated extrapolations and equations (Fig. 1).

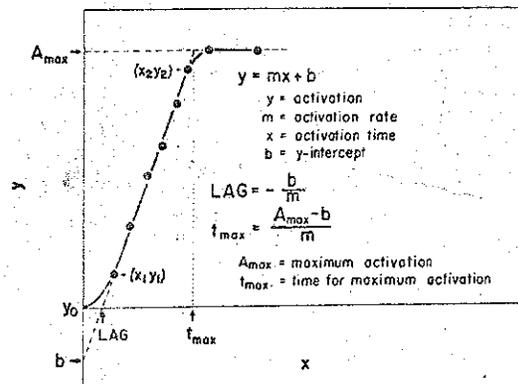


FIG. 1. Diagrammatic representation of heat activation of spores of *C. perfringens* as a function of time of heating. Similar plots and the indicated equations were used to determine activation rate, activation lag, and time (t_{max}) to achieve maximum activation.

RESULTS AND DISCUSSION

Kinetics of KNO_3 -supported germination. The rate and extent of germination of both strains of *C. perfringens* increased with increasing time of heat activation (Fig. 2). In the example shown, spores were heated at two of the activation temperatures: 65°C and 70°C. The slopes of the rectilinear portions of these curves represent the germination rate from which GR-based activation was calculated. Spores of NCTC 8238 germinated more rapidly and to a greater extent than did those of NCTC 8798. GR_u (dotted lines; Fig. 2) for unheated spores of the two strains were 0.19 and 0.13% OD loss/min, respectively; GR_u (65°C, 10 min) was 0.63% OD loss/min for NCTC 8238 and 0.32% OD loss/min for NCTC 8798, with calculated activations of ca. 230 and 150% increase in germination rate, respectively. After 5 min at 70°C, the germination rate of NCTC 8238 was 0.95% and that of NCTC 8798 was 0.49% OD loss/min, for respective activations of ca. 400 and 280% increase in germination rate.

Activation as a function of time of heating. CFU- and GR-based heat activation of both strains were similarly calculated for all temperatures. The results for 62, 65, and 70°C are repre-

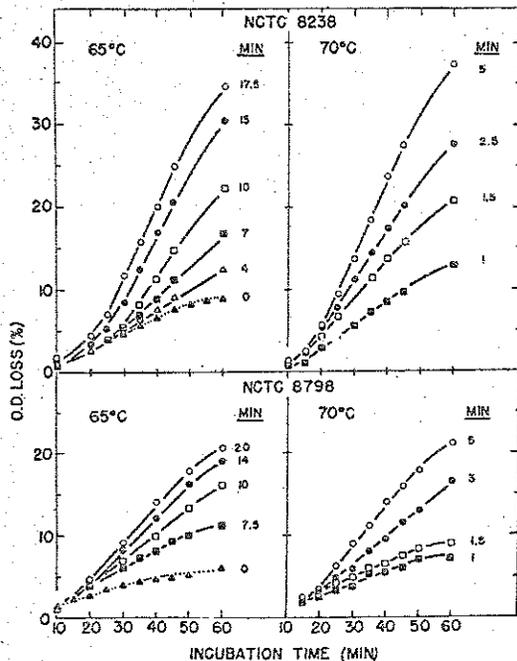


FIG. 2. Kinetics of germination in 5 mM KNO_3 at 30°C of spores of *C. perfringens* heat activated for various times at 65 and 70°C. Germination rate (as % OD loss/min) was estimated from the slopes of the rectilinear portions of the plots.

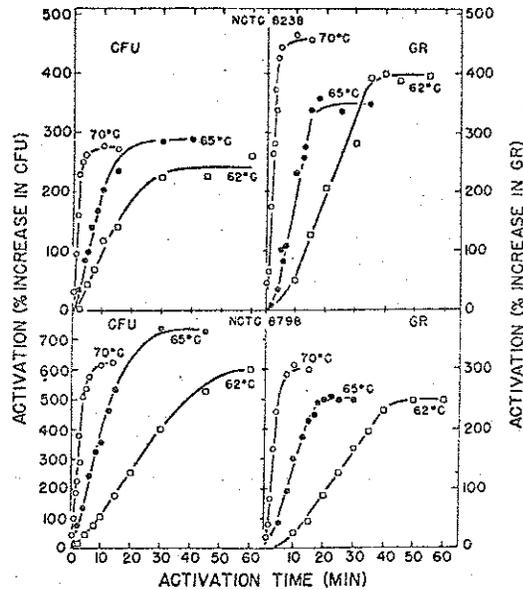


FIG. 3. Heat activation, at three representative temperatures, of spores of *C. perfringens* NCTC 8238 and NCTC 8798. Activation was based on increase in colony-forming units (CFU) or in germination rate (GR).

sented in Fig. 3. With NCTC 8238, GR-based activation was generally higher than that based on CFU; on the other hand, with NCTC 8798, CFU-based activation (note ordinate, Fig. 3) was generally higher. We attribute the significantly higher level of CFU-based activation of *C. perfringens* NCTC 8798 to a low CFU_u, a value which was used as a basis for calculation of activation. Nevertheless, similarities in the activation patterns of the two strains appear to us to be more impressive than the differences.

Similar curves were constructed for all experimental conditions, and, from such curves, the kinetic constants were computed (Fig. 1). It was noted, although not shown on the curves, that CFU-based activation declined when the time of heating was extended substantially beyond t_{max} .

Activation rate. The higher the temperature of activation, the greater was the activation rate (Fig. 4), regardless of activation criterion or *C. perfringens* strain. At 55 and 70°C, the NCTC 8238 CFU-based activation rates were ca. 1 and 130% increase in CFU/min of heating, respectively; GR-based activation rates at 58 and at 70°C were ca. 2 and 145% increase in germination rate/min of heating, respectively. The temperature characteristics or energies of activation, μ , approximate those for the activation of spores of *B. megaterium* for glucose-supported germination (22) and are of a magnitude

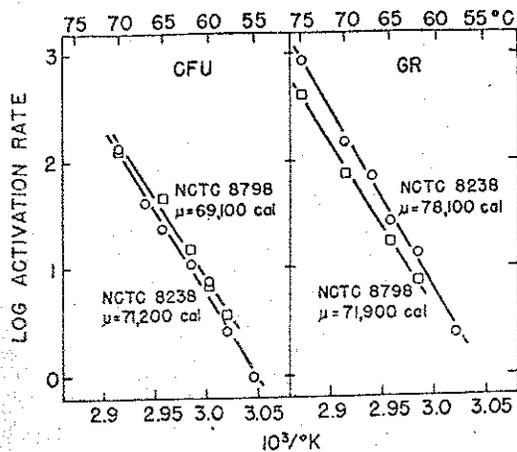


FIG. 4. Arrhenius plots of heat activation rates of spores of *C. perfringens* NCTC 8238 and NCTC 8798. The lower abscissa scale shows reciprocal ($\times 10^3$) of temperature ($^{\circ}\text{K}$) of heat activation; the upper scale shows the activation temperature as $^{\circ}\text{C}$. Activation was based on colony-forming units (CFU) or on germination rate (GR). The average energy of activation, μ , calculated from the slopes of these plots was ca. 72.5 kcal (3×10^5 J).

(average ca. 72.5 kcal, ca. 3×10^5 J) consistent with hypotheses suggesting involvement of macromolecular denaturation—breaking of bonds—in the activation process (5, 17, 22).

Activation lag. Regardless of the basic parameter, the activation lag of both strains decreased with increasing temperature of activation (Fig. 5). Heat activation appears to represent a continuous response through the range of test temperatures, agreeing with the conclusion of Busta and Ordal (5). These authors further concluded, and we agree (22), that "aging" (activation by storage of aqueous suspensions of spores for extended periods at low temperature) is a special case of heat activation. If the linearity of the log lag versus activation temperature curve (Fig. 5) is extended, we estimate that the GR-based activation lag of *C. perfringens* NCTC 8238 at 25 $^{\circ}\text{C}$ would be 6.3×10^4 min or ca. 1.5 months. Arrhenius plots were constructed with the reciprocal of the lag used as the rate function. Energies of activation, μ , calculated from these plots, were of sufficiently high magnitude (average 59.4 kcal, 2.5×10^5 J), regardless of strain or basic parameter of activation, to be consistent with an involvement of macromolecular denaturation in activation.

Time for maximum activation. The lower the temperature of activation, the greater was t_{\max} of both strains, whether CFU- or GR-based (Fig. 6). By assuming extended linearity of the plot of log t_{\max} versus activation temperature, we estimate

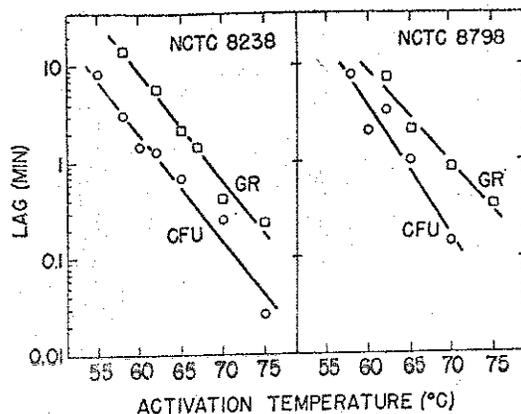


FIG. 5. Effect of heat activation temperature on activation lag of spores of *C. perfringens* NCTC 8238 and NCTC 8798. Lag = $-blm$ (Fig. 1), using curves such as those shown in Fig. 3.

GR-based t_{\max} of NCTC 8238 as ca. 0.15 s for spores heated at 95 $^{\circ}\text{C}$, ca. 3.2 years at room temperature (25 $^{\circ}\text{C}$), and ca. 1,000 years at refrigerator temperature (5 $^{\circ}\text{C}$). Recalling the activation lag at 25 $^{\circ}\text{C}$ of ca. 1.5 months, one would expect (if "aging" is a specialized form of heat activation) spores kept in aqueous suspension at room temperature for periods between 1.5 months and 3 years to be activated to varying degrees. This would imply the necessity for caution in interpretation of results obtained with spores stored as aqueous suspensions.

Maximum activation required about 15 min at 65 $^{\circ}\text{C}$ and about 1.0 min at 75 $^{\circ}\text{C}$ (Fig. 6). The 20-min activation at 75 $^{\circ}\text{C}$, recommended by other workers (8), probably exceeds that neces-

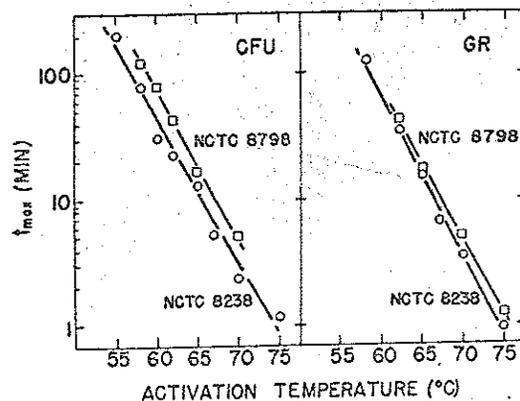


FIG. 6. Effect of heat activation temperature on the time (t_{\max}) required to attain maximum activation of spores of *C. perfringens* NCTC 8238 and NCTC 8798. $t_{\max} = (A_{\max} - b)/m$ (Fig. 1), using curves such as those shown in Fig. 3.

sary to assure maximum activation of spores of these strains of *C. perfringens*.

Germination in reinforced clostridial broth.

Heat activation, then, increases the rate and extent of germination of spores of *C. perfringens* NCTC 8238 and NCTC 8798. That increased colony counts on reinforced clostridial agar do indeed reflect enhanced germination of heated spores is evidenced by the data obtained with spores of NCTC 8238 heated at 70°C for various periods of time and germinated at 30°C in reinforced clostridial broth. There was substantial activation (1,028% increase in germination rate for spores heated for 2.5 min), with an activation rate of 629% increase in germination rate/min of heating, an activation lag of 0.77 min, and a t_{max} of 4.1 min.

Decline in CFU-based activation. All of the data to this point emphasize the similarity in GR- and CFU-based activation of both strains. When spores of NCTC 8238 were heated for a period of time substantially beyond that required to attain maximum CFU-based activation (Fig. 6), there was often a decline from the maximum. The demonstration of this decline in CFU-based activation depended on the temperature of activation. For example (Fig. 7), after heating for 5 h at 55 to 60°C, there was little decline from maximum activation. With temperatures from 62 to 75°C, however, the decline in CFU was significant. Spores heated at 70°C for 5 h lost ca. 80% of the increase in colony count representing maximum CFU-based activation, but even after 5 h at 75°C the colony count was not less than that of unheated spores. It is possible that only activated spores were susceptible to this type of inactivation, those capable of colony formation without prior heat activation being largely unaffected. However, maximum GR-based activation was fully retained during 5 h of heating at 70°C (Fig. 7). Thus, in contrast to the germination-related activation of spores resulting from limited exposure of aqueous suspensions to elevated temperatures, extended heating exerts its effect, not on germination, but rather on the ability of spores to undergo a postgerminative developmental stage or stages.

Temperatures recommended for pasteurization (62.8°C) and for institutional or military food serving lines are effective activation temperatures as well. If *C. perfringens* spores are present in these situations, they are likely to be activated and, if subsequently exposed to suitable growth conditions, to multiply and reach a population density constituting a hazard to the consumer. Perhaps this partly accounts for the prevalence of food service establishments as the place of origin of *C. perfringens* food-poisoning outbreaks (6).

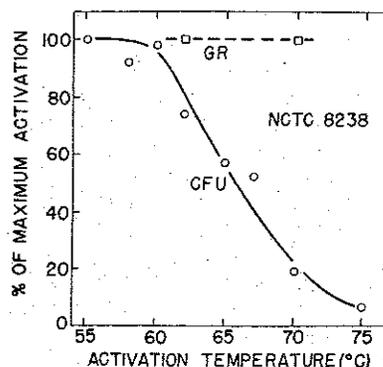


FIG. 7. Relative retention, with extended heating, of the maximum level of activation of spores of *C. perfringens* NCTC 8238. Activation was based on colony-forming units (CFU) or on germination rate (GR). Spores were heated for 5 h at various temperatures.

In continuing our efforts to describe the properties of *C. perfringens* spores, we are planning more extended studies on heat and radiation effects (including inactivation) and on germination and outgrowth. Where feasible, we intend to compare properties of the two heat-resistant food-poisoning strains described here with other heat-resistant and heat-sensitive strains, both enterotoxigenic and nonenterotoxigenic.

A very few experiments were done with *C. perfringens* ATCC 14810 (NCTC 10240, Hobbs' serotype 13), a strain which is more heat sensitive ($D_{90°C} = \text{ca. } 10 \text{ min}$) than either NCTC 8238 (8) or NCTC 8798. Spores of ATCC 14810 showed no evidence of heat activation either at 60°C or at 75°C. Undoubtedly, some problems remain unsolved.

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