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Mathematical Model of Thermal Destruction of *Bacillus stearothermophilus* Spores

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The experimental survival curves of *Bacillus stearothermophilus* spores in aqueous suspension, for six constant temperatures ranging from 105 to 130°C, displayed an initial shoulder before a linear decline. To interpret these observations, we supposed that, before the heat treatment, the designated spore suspension contained a countable and mortal N_0 population of activated spores and an M_0 population of dormant spores which remained masked during spore counting and had to be activated before being destroyed by heat. We also hypothesized that the mechanisms of both activation and destruction are, at constant temperature, ruled by first-order kinetics, with velocity constants k_A and k_D, respectively. Mathematical analysis showed that this model could represent not only our experimental survival curves, but also all other shapes (linear and biphasic) of survival curves found in the literature; also, there is an inherent symmetry in the model formulation between the activation and destruction reactions, and we showed that the dormancy rate ($\tau = M_0/N_0$) is the only parameter which permits a distinction between the two reactions. By applying the model to our experimental data and considering that the dormancy rate is not dependent on the treatment temperature, we showed that, for the studied suspension, the limiting reaction was the activation reaction.

Heat destruction of microbial spores is generally considered to be a unimolecular reaction, ruled by first-order kinetics. Mathematically, the reaction is expressed by an equation of the type:

$$N = N_0\, e^{-kt} \text{ or } N = N_0\, 10^{\frac{-t}{D}} \tag{1a}$$

where N is the number of surviving spores after an isothermal treatment of t (minutes) in a suspension which initially contained N_0 spores, and k is the velocity constant of the reaction from which Katzin et al. (15) extracted the decimal reduction time which Ball and Olson (2) dubbed symbol D. The D value is obtained from the following equation:

$$D = \frac{\ln 10}{k} = \frac{2.3}{k} \tag{1b}$$

The plot of $\ln(N/N_0)$ versus time is a straight line, referred to as a survival curve (Fig. 1, curve A). Nevertheless, there are numerous documented instances of thermal destruction curves deviating from linearity such as those illustrated in Fig. 1 (curves B, C, and D) (20, 21, 23).

Biphasic curves (Fig. 1, curve B) express an advance in the effective reduction of spores when compared with the reduction expected from a first-order model. These curves are generally held to represent a mix of two species or strains of different heat resistance. The first straight portion of the curve mainly describes death of the organism of lower resistance and the second portion describes death of the more resistant one. Cerf (6) discussed a number of publications which report this phenomenon, which can be described as two parallel reactions of a logarithmic order, characterized by different slopes (k_1 and k_2):

$$N_1 = N_{01}\, e^{-k_1 t} \tag{2a}$$

$$N_2 = N_{02}\, e^{-k_2 t} \tag{2b}$$

$$N_0 = N_{01} + N_{02} \tag{2c}$$

$$N = N_1 + N_2 = N_{01}\, e^{-k_1 t} + N_{02}\, e^{-k_2 t} \tag{2d}$$

In curves C and D (Fig. 1), the shoulder or lag expresses a lag time in the destruction of the microorganisms when compared with first-order kinetics.

By analogy with a lethal effect of ionization on single cell populations, ranging from bacteria to mammalian cells, a multihit model, mathematically expressed by an equation of the following form:

$$N = N_0 [1 - (1 - e^{-kt})^n] \tag{3}$$

has sometimes been associated with type C curves, characterized by a zero initial destruction rate (1, 3, 19, 24). This model would be adequate to describe the behavior of a nonhomogeneous suspension in which aggregates of two or more spores would coexist with isolated spores. In equation 3, n may express the mean number of spores in the aggregates and is measured at the intersection of the extrapolation of the logarithmic portion of the curve with the ordinate. Nevertheless, the model remains inadequate to explain type D curves, which disclose an increase in the number of countable spores at the beginning of treatment. The high temperature level and the short duration of the heat treatment prevent any interpretation of an increase in population as growth. Type D curves have been associated with the presence of uncountable dormant spores which could, during the heat treatment, be activated and then destroyed successively. Reactions of both activation and destruction would obey first-order kinetics, characterized by velocity constants k_A and k_D, respectively. Mathematically, Shull et al. (22) expressed this model with the following equation:

$$N = N_0 [\alpha\, e^{-k_D t} + (1 - \alpha)\, e^{-k_A t}] \tag{4}$$

where α is a constant dependent on the two velocity constants k_A and k_D and on the ratio of the dormant spores in the initial suspension (22). Scharer (J. M. Scharer, Ph.D. thesis,

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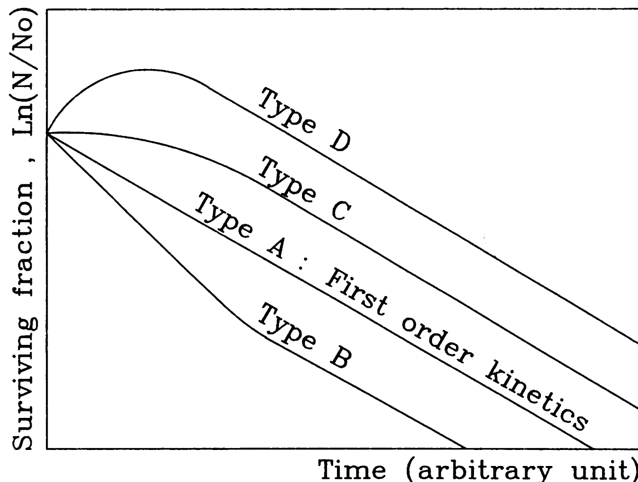


FIG. 1. Different types of survival curves. Type A, First-order kinetics; type B, biphasic curve; types C and D, shoulder.

University of Pennsylvania, Philadelphia, 1965) and Feeherry et al. (12) believe that the curvilinear part of survival curves comes from the existence of an intermediate state of thermal susceptibility between the normal viable state and death, but do not propound any mathematical model.

If experimental data show a curvilinear portion in the survival curves, the choice of an adequate mathematical treatment for data exploitation raises questions. Workers commonly ignore the curvilinear part of the curve and only take the linear part associated with the destruction into account, but this method is unsatisfactory. The model described by Shull et al. (22) permits us to consider the existence of the curvilinear portion and hence define the very constants of the microorganisms studied.

The aim of this paper was to deal with a mathematical analysis of the model proposed by Shull et al. and to validate it by applying it to experiments of inactivation of *Bacillus stearothermophilus* spores at several constant temperatures.

MATERIALS AND METHODS

Spore preparation. Spores of *B. stearothermophilus* ATCC 7553 were produced in Roux bottles, using medium of the following composition (in grams per liter): meat extract (Institut Pasteur Production [IPP]), 10.0; yeast extract (IPP), 2.0; agar (IPP), 23.0, to which 40 μg of $MnSO_4 \cdot H_2O$ per liter was added. Incubation was at 61°C for 4 days and then 1 week at room temperature (4).

Cultures from five Roux bottles were harvested by scraping the agar surface with glass balls and rinsing off the growth with sterile distilled water. The suspension was centrifuged at 1,000 × *g* for 15 min. The supernatant was discarded, and the pellet was washed three times by suspension in sterile distilled water followed by recentrifugation. The pellets were subsequently suspended in water (approximately 30 ml) and heated at 93°C for 1 h. Spores were enumerated by plating 1 ml of decimal dilutions in Muller-Hinton medium, incubating at 61°C for 20 h, and then diluting into sterile water to obtain a spore concentration of 3 to 4 10^6 per ml. Storage was at 4°C until spores were put in capillary tubes.

Capillary tube preparation. Microhematocrit tubes of 75-mm length, 1.15-mm inside diameter, and 0.2-mm glass thickness were used. The capillary tubes were sterilized (2 h; 180°C, dry heat), filled with 30 μl of the spore suspension, constantly stirred, and sealed (17). The spore population of one capillary tube was around 10^5 spores. Many capillary tubes (200 to 300) were prepared, and they were stored at 4°C until used.

Heat treatment. Capillary tubes were submitted to six temperatures (105, 110, 115, 121, 126, and 130°C) for various times. Three capillary tubes were plunged together into an oil bath (Huber T301) set up at the desired temperature. Thermal transfer modeling led us to assess the capillary thermal time at 2.8 s. Come-up time can therefore be deemed negligible with respect to heating times applied. The longest treatment times were chosen to ensure a spore destruction at least equivalent to two decimal reductions; a greater number of short treatment times were used to give a more precise quantification of the shoulder. After an adequate time, capillary tubes were brought out and chilled by immersion in cold water (0°C). Adequate external cleaning with soap solution and rinsing with distilled sterile water were done. Heated capillary tubes were stored at 4°C and used for enumeration of surviving spores within a maximum of 2 weeks after treatment.

Enumeration of surviving spores. Capillary tubes, without or after treatment, were crushed in recovery medium of the following composition (in grams per liter): Bio-trypticase (Bio-Mérieux), 3.70; Bacto-soytone (Difco Laboratories), 5; dextrose, 1.25; NaCl, 1.25; bromcresol purple, 0.020. The pH was adjusted to 7.5 by adding 1 N NaOH (around 3 ml/liter). The suspension was stirred for 10 min, and 200 μl/well was distributed into a microdilution plate with an automatic pipette. If necessary, i.e., for nonheated or short-term heated capillary tubes, dilutions with the same medium were made. After incubation, the total viable population of spores (N) was obtained through the equation of Halvorson and Ziegler (13) modified by Michiels et al. (17):

$$\mathrm{N} = n\ 2.303\ D \log \left(\frac{n}{q} \right)$$

where n is 96 (number of wells in the plate); D is dilution factor, if any; and q is number of wells showing no culture, i.e., purple color of the medium (when culture was positive, medium became yellow because of acidification due to fermentation of dextrose). Some 24 ± 1 h of incubation at 61°C was necessary to get a constant number of yellow cultured wells.

RESULTS

Thermal inactivation curves. The mean initial countable population (N_0) in the capillary tubes was $16 \times 10^4 \pm 3 \times 10^4$ (14 replicates). Surviving population after heat treatment was expressed as $\ln(N/N_0)$ and was plotted against time.

The kinetics of thermal death of *B. stearothermophilus* spores in aqueous suspension at 115°C is shown in Fig. 2. All time-temperature plots represented the average of two to three replicates. Excluding experimental plots corresponding to zero or positive values and taking measurement inaccuracies into account, other plots could be placed on a straight line and presented a positive value when extrapolation of the line cross the y axis. Yet numerous experimental plots showed that the countable spore population increased at the beginning of treatment. Similar observations could be made for other treatment temperatures (data not shown).

At a given temperature, the totality of the experimental

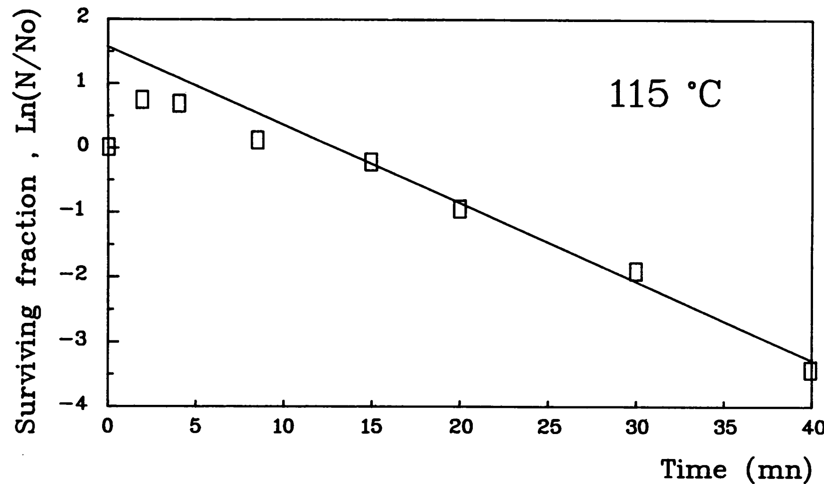


FIG. 2. Thermal destruction curve of *B. stearothermophilus* spores at constant temperature (115°C). Straight line was obtained by linear regression on experimental plots of negative values. The equation allowed obtaining values of the slope and the ordinate at the origin which were associated with k_∞ and $\ln(y_0)$, respectively.

plots could be mathematically approximated by a formula similar to that proposed by Shull et al (22):

$$\frac{N}{N_0} = y_0\, e^{-k_\infty t} + (1 - y_0)\, e^{-k_0 t}$$

with

$$k_\infty < k_0$$

where N/N_0 is the proportion of surviving spores and t is the holding time. Linearization by the least-squares method of the experimental plots of the final part of thermal death curves allowed us to obtain k_∞ and $\ln(y_0)$ values which are the slope and the ordinate at the origin of the straight line, respectively. Correlation coefficients were significant at a 95% probability level at least (Table 1 and Fig. 2 illustrate this for treatment at 115°C).

Owing to measurement inaccuracies, particularly at high temperatures, it was more difficult to evaluate a k_0 value which determined the shape of the curvilinear part of the curve, accurately. We determined two limit values which, with previous experimental k_∞ and y_0 values, allowed us to draw two curves encircling the totality of the experimental plots (Table 1 and Fig. 3 illustrate this for treatment at 115°C).

TABLE 1. Values for model constants obtained from experiments at different temperatures

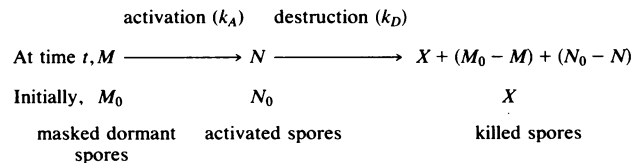
Temp (°C)	k_∞ (min^{-1})[a]	y_0[a]	r test (P)	k_0 (min^{-1})[b]
130	11	6.3	<0.05	20–40
126	3.5	5.5	<0.05	6–12
121	0.8	4.2	<0.001	1.5–3
115	0.12	4.7	<0.01	0.25–0.5
110	0.022	2.8	<0.05	0.06–0.12
105	0.008	2.0	<0.05	0.025–0.05

[a] k_∞ and y_0 were obtained from the slope and the ordinate at the origin of the straight line plotted with the experimental plots of negative values. (See also Fig. 2.).

[b] The k_0 value is between the two limit values, which allowed us to draw two curves, encircling the totality of the experimental plots. (See also Fig. 3.)

Mathematical aspect of the model of Shull. (i) Model formulation. We suppose that, before the heat treatment, the involved spore suspension contained a countable and mortal N_0 population of activated spores and an M_0 population of dormant spores which remained masked during enumeration, i.e., not capable of colony formation on a suitable medium and having to be activated before being destroyed by heat. We also make the hypothesis that mechanisms of both activation and destruction are, at constant temperature, ruled by first-order kinetics, with velocity constants k_A and k_D, respectively.

The whole scheme can be represented in the following form:



The evolution of the populations is given by the following differential equations:

$$dM = -M\, k_A\, dt \tag{5}$$

$$dN = dN_D + dN_A = -N\, k_D\, dt + M\, k_A\, dt \tag{6}$$

N being the only parameter available to measurement, it will be more convenient to have it under an independent integrated form, taking initial conditions into account.

From equation 5, we get:

$$M = M_0\, e^{-k_A t} \tag{7}$$

which, when transferred into equation 6, give the following equation:

$$\frac{dN}{dt} + N k_D = k_A\, M_0\, e^{-k_A t} \tag{8}$$

Integrating equation 8 leads to:

$$N = \left(\frac{k_A}{k_D - k_A}\right) M_0\, e^{-k_A t} + \left(N_0 - \frac{k_A}{k_D - k_A} M_0\right) e^{-k_D t} \tag{9}$$

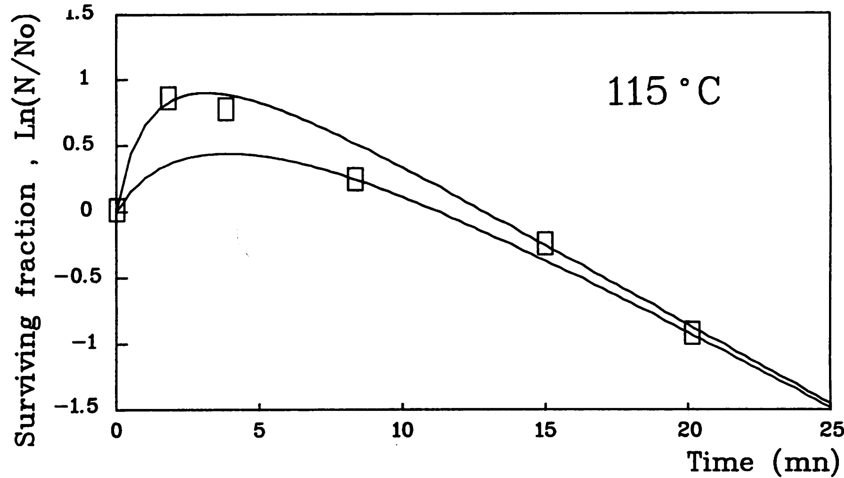


FIG. 3. Encircling survival curves obtained for treatment at 115°C with the two limits of k_0 (0.25 and 0.5 min^{-1}) and previously determined k_∞ and y_0 values (0.12 min^{-1} and 4.7 respectively).

Let us make the following definitions:

$$\tau = \frac{M_0}{N_0} \text{ (dormancy rate)} \quad (10)$$

$$\text{and } \alpha = 1 - \frac{k_A}{k_D - k_A}\tau \quad (11)$$

Equation 9 may be written in the following form:

$$\frac{N}{N_0} = (1 - \alpha)\, e^{-k_A t} + \alpha\, e^{-k_D t} \quad (12)$$

(ii) Initial velocity. Derivation of equation 12 leads to the expression of velocity:

$$v = \frac{d\frac{N}{N_0}}{dt} = -k_A(1 - \alpha)\, e^{-k_A t} - k_D\, \alpha\, e^{-k_D t} \quad (13)$$

which, at zero time, amounts to:

$$v_0 = -k_A(1 - \alpha) - k_D\,\alpha \quad (14)$$

Replacing α by expression 11, we get:

$$v_0 = k_A\tau - k_D \quad (15)$$

Thus, the evolution of the countable population at the beginning of treatment depends on the relative values of the three following characteristics: k_A and k_D velocity constants characterizing spore heat resistance and τ (dormancy rate) characterizing the considered suspension.

If $\tau > k_D/k_A$, velocity v_0 is positive: the initial countable spore population increases; if $\tau = k_D/k_A$, velocity v_0 equals zero: during the beginning of the treatment, heat is without any effect on the countable population; and if $\tau < k_D/k_A$, velocity v_0 is negative: the countable spore population decreases from the beginning of heating onwards.

(iii) Limiting phenomena. As duration of treatment increases, the reduction in the number of countable spores is limited by the slower of the two reactions activation and destruction.

Mathematically, we can write that, if the limiting phenomenon is destruction:

$$k_A > k_D \text{ and } \frac{N}{N_0} \xrightarrow[t \to \infty]{} \alpha\, e^{-k_D t}$$

The linear part of the thermal death curve follows the equation:

$$\ln\left(\frac{N}{N_0}\right) = \ln \alpha - k_D t$$

The slope gives the velocity constant of the reaction of destruction (k_D), and the ordinate at the origin is $\ln \alpha = \ln\left(1 - \frac{k_A}{k_D - k_A}\tau\right)$; it is always positive or zero. Thus, the destruction curve is always above the straight line representing the currently assumed first-order kinetics (Fig. 4, curves b and c) or superimposes it (Fig. 4, curve a) when τ is zero; i.e., the dormant spores do not exist.

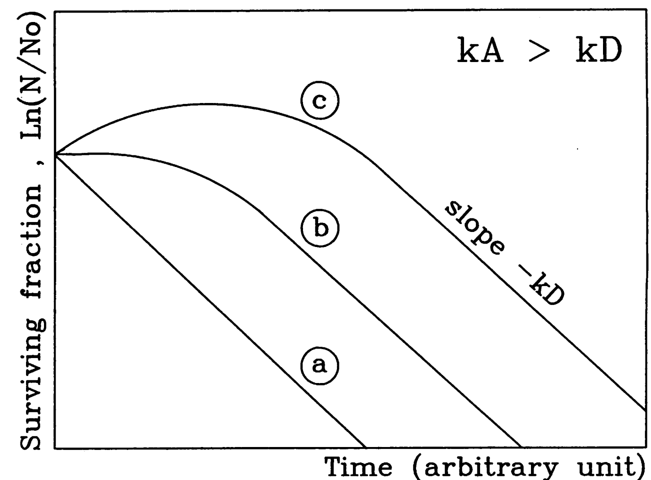


FIG. 4. Different shapes of the theoretical survival curves obtained when the limiting factor is destruction. Slope of the straight portion of the curves is $-k_D$. a, Classical first-order kinetics obtained when $\tau = 0$; b and c, shoulder: b is curve obtained when $\tau = k_D/k_A$ and initial velocity is zero, and c is curve obtained when $\tau > k_D/k_A$ and initial velocity is positive.

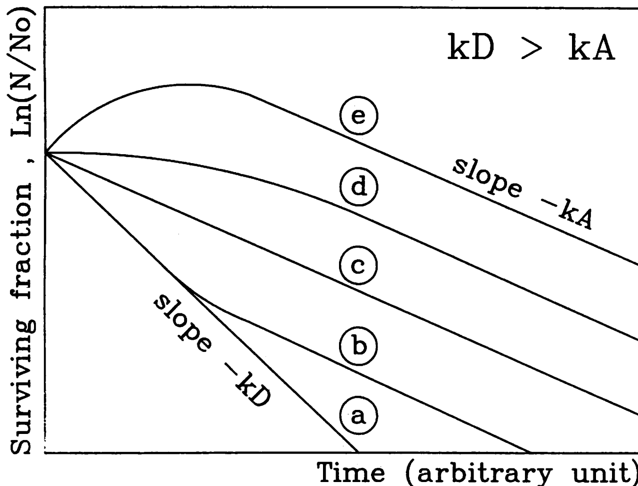


FIG. 5. Different shapes of the theoretical survival curves obtained when the limiting factor is activation. a, Classical first-order kinetics obtained when $\tau = 0$; b, biphasic curve obtained when $0 < \tau < (k_D/k_A) - 1$; c, straight line of slope $-k_A$ obtained when $\tau = (k_D/k_A) - 1$; d and e, shoulder: d is curve obtained when $\tau = k_D/k_A$ and initial velocity is zero, and e is curve obtained when $\tau > k_D/k_A$ and initial velocity is positive.

If the limiting phenomenon is activation:

$$k_D > k_A \text{ and } \frac{N}{N_0} \xrightarrow[t \to \infty]{} (1 - \alpha)\, e^{-k_A t}$$

Then, the linear part of the thermal death curve can be equated as follows:

$$\ln\left(\frac{N}{N_0}\right) = \ln(1 - \alpha) - k_A t$$

The slope no longer allows us to get the velocity constant of the reaction of destruction (k_D), but rather that of the reaction of the dormant spore activation. The ordinate at the origin is $\ln(1 - \alpha) = ln \left(\frac{k_A \tau}{k_D - k_A}\right)$; it may be positive, negative, or zero. It becomes equal to zero when $\tau = (k_D/k_A) - 1$. In these conditions, the thermal death curve is perfectly linear, with slope $-k_A$ (Fig. 5, curve c). For smaller τ values, the ordinate at the origin is negative and the destruction curve is below the previous straight line. When $\tau << (k_D/k_A) - 1$, the initial velocity tends to k_D and we can observe the succession of two linear portions: the first one with slope $-k_D$ and the second with slope $-k_A$ (Fig. 5, curves a and b). In contrast, when $\tau > (k_D/k_A) - 1$, the ordinate at the origin is positive and the destruction curve is above the straight line with slope $-k_A$ (Fig. 5, curves d and e).

TABLE 2. Dormancy rate calculated for $k_\infty = k_D$ (spore destruction is the limiting phenomenon) and for $k_\infty = k_A$ (spore activation is the limiting phenomenon) for lower and upper values of the second constant

Temp (°C)	$\frac{(1 - y_0)(k_\infty - k_0)}{k_0}$ ($k_\infty = k_D$)	$\frac{y_0(k_0 - k_\infty)}{k_\infty}$ ($k_\infty = k_A$)
130	2.4–4.0	5–17
126	1.9–3.2	4–13
121	1.5–2.3	4–12
115	1.9–2.8	5–15
110	1.2–1.5	6–14
105	0.68–0.84	4–10.5

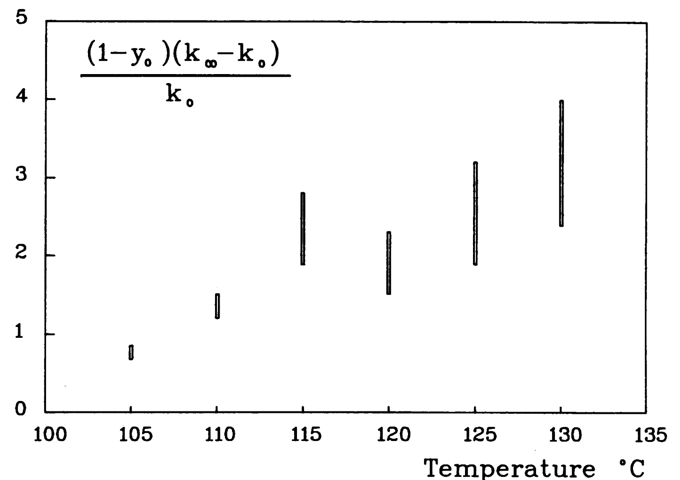


FIG. 6. Calculated τ-value limits from the two limits of k_0 values, when k_∞ is associated with destruction, at different temperature treatments. This hypothesis is incompatible with a τ-value constant and independent of the temperature.

(iv) Conclusion. The analysis of initial velocity and limiting phenomena showed that the developed model accounts for all destruction curve shapes shown in Fig. 1. This analysis also showed that the mathematical model presents a symmetry between destruction and activation reactions. Then, consideration of a curve is not enough to associate the determined velocity constants with destruction or activation. To make correct associations, we have to take the dormancy rate into consideration. The dormancy rate characteristic of the suspension before treatment can be calculated from the velocity constants and the ordinate at the origin (equation 11) and should exhibit a constant value, not dependent on the temperature, for the different curves obtained from one suspension.

Constant identification. We applied the theoretical model

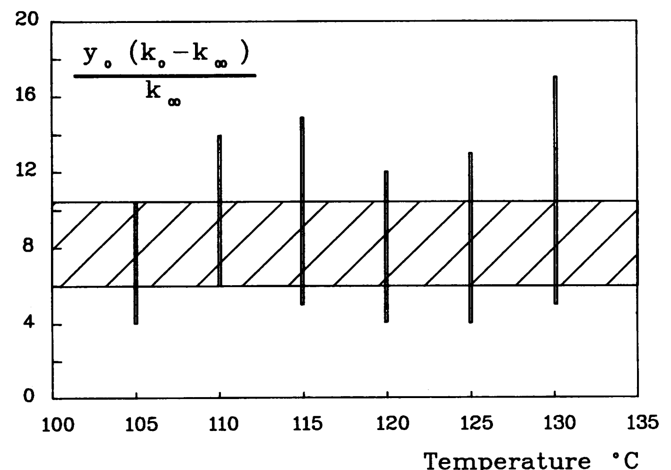


FIG. 7. Calculated τ-value limits from the two limits of k_0 values, when k_∞ is associated with activation, at different temperature treatments. This hypothesis is compatible with a τ-value constant and independent of the temperature.

TABLE 3. Calculated k_D (k_0) values from experimental k_A (k_∞) and (1 − α) (y_0) values and a mean constant τ = 8

Temp (°C)	k_D (min^{-1})
130	25
126	8.6
121	2.3
115	0.32
110	0.085
105	0.040

to our measurements. For different temperatures, we tried to associate k_∞ with the activation or destruction constants. When k_∞ was associated with destruction, then k_0 and y_0 corresponded to k_A and α of equation 12, respectively, and τ values were calculated from $[(1 - y_0)(k_\infty - _{k_0})]/k_0$. When k_∞ was identified to activation, k_0 and y_0 were associated with k_D and (1 − α) of equation 12, respectively, and the τ values were obtained through the formula $[y_0 (k_0 - k_\infty)]/k_\infty$. For the different treatment temperatures, Table 2, illustrated by Fig. 6 and 7, gives the calculated τ values for both assumptions and from the two k_0 limit values. Results showed that it was only when k_∞ was assimilated to the activation constant (k_A)

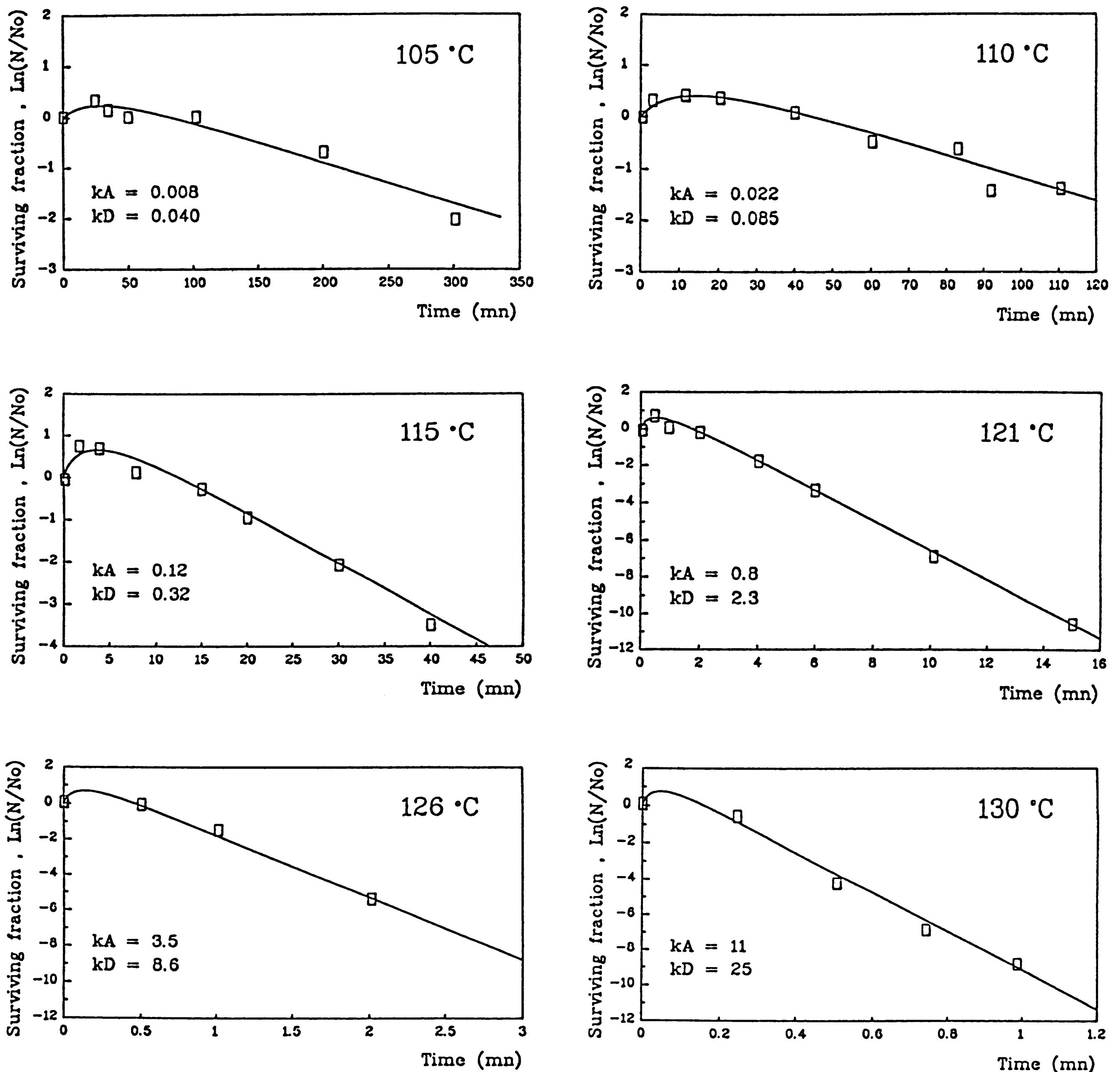


FIG. 8. Experimental plots and reconstituted survival curves at constant-temperature treatments from the developed model with calculated k_D and k_A values and a constant τ value of 8.

that the mathematical theory of the system was corroborated and that a constant dormancy temperature-independent rate between 6 and 10.5 could be obtained.

Confirmation of the model. To reconstitute destruction curves from the model, a coherent range of k_D values (k_0) was calculated from experimental values of k_A (k_∞) and (1 − α) (y_0) and a mean τ value of 8 (Table 3). The reconstituted survival curves for the different temperature treatments and the experimental plots are shown in Fig. 8.

DISCUSSION

According to Cook and Gilbert (9), heat treatment of spores of *B. stearothermophilus* suspended in MacIlvaine citric acid-phosphate buffer at pH 7 or Sörensen phosphate buffer (0.067 M) at pH 7 showed an exponential death rate corresponding to a first-order kinetics. It is a general finding, though, that, when spores of *B. stearothermophilus* are suspended in water, survival curves present an initial curvilinear portion preceding a logarithmic decline (5, 7–12), independent of the composition of the recovery medium (8) or of the water activity of the suspension medium (14). Nevertheless, Perkin et al. (18) suggested that heating time might be responsible for the delay in the destruction when the treatment was short and treatment temperature was high, and they proposed a method of calculation of the real treatment times which resulted in a survival straight line. However, he stated that, when immersion is 5 s or above and temperature is below 135°C, the heating period in capillaries has a negligible effect on the nonlinearization of the thermal death curve. In our experimental conditions, the heating time calculated was negligible with respect to duration of treatment. Our results were in accordance with the curvilinear form of the curves, indicating that the first-order law was not always valid.

Different hypotheses to explain deviations from the first-order law were expressed. Thus, the existence of spore aggregates which retard destruction might be described by the multihit model, developed to describe the destruction of spores subjected to radiation (1, 19). Nevertheless, this model would be unable to describe the totality of the observed survival curve shapes. In addition, Feeherry et al. (12) showed that, after filtration of spore suspension to remove spore aggregates, the survival curves always presented a curvilinear form. These authors believed that spores were injured by the heat treatment and that an injured spore population capable of repairs accounted for the curvilinear portion of survival curves. This mechanism seems to be consistent only with a type C curve and not with a type D curve (Fig. 1), which shows an increase in spore population at the beginning of treatment; a typical survival curve of *B. stearothermophilus* cannot always be described.

The curvilinear portion of the survival curves was also attributed to an activation process counteracting the destruction process. This phenomenon could be considered valid for thermophilic bacteria, such as *B. stearothermophilus*, for which activation and destruction began approximately at the same temperature, 100 to 105 and 110 to 115°C, respectively (16). A 1-h treatment at 93°C, performed during spore preparation to destroy the remaining vegetative forms, would not be sufficient to activate spores, so two populations of spores would be obtained: one of dormant spores and another of activated spores. Shull et al. (22) gave a mathematical model consistent with this phenomenon that could yield such curves.

Our mathematical analysis of the model showed that, first, all of the survival curves found in Fig. 1 (curves A, B, C, and D) could be described and, second, it was possible to know the nature of the limiting phenomenon by considering that the dormancy rate is not dependent on the treatment temperature.

Experimentally, application of the model showed that, for a spore suspension of *B. stearothermophilus*, the limiting phenomenon would be activation, as opposed to what is classically thought. Consequently, the spore heat resistance through determination of the *D* value would be overestimated.

Knowledge of the law of spore thermal destruction is necessary to calculate or predict the lethal effect of industrial sterilization treatments. When the hypothesis of destruction ruled by a first-order kinetics is verified, the lethal effect of the treatments is calculated by the numerical integration method developed by Bigelow or by an analytical method proposed by Ball and Olson (2). Nevertheless, when the kinetics of destruction does not obey first-order kinetics, the methods of Bigelow and Ball become inapplicable and the determination of spore heat resistance through calculation of the *D* value becomes meaningless. We are studying how to take a more complex kinetics into account to calculate the F_0 value of nonisothermal treatments.

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