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Temperature dependence of thermal inactivation rate constants of bacterial spores in a glassy state

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SUMMARY

Differential scanning calorimetry data obtained from corn embryos is consistent with the hypothesis of their glassy state. This work extends that hypothesis to explain the speculation about the high heat resistance of bacterial spores. By considering the protoplast to be in a glassy solid-state, it can be assumed that the configurational rearrangements of the key life dependent polymer chain backbones (DNA, etc.) are extremely slow, thereby ceasing thermal motions. It is assumed that at the glass transition temperature, the spore protoplast undergoes a discontinuity in the thermal expansion coefficient, and above this critical temperature, the rate of thermal inactivation of spores is free volume dependent and can be described adequately by the William, Landel and Ferry (WLF) equation. Glass transition temperatures for *Bacillus stearothermophilus* and *Clostridium botulinum* spores, obtained by fitting the inactivation rate data to the WLF equation, indicate a decrease in the inactivation rates with increasing glass-transition temperatures.

INTRODUCTION

Resistance of bacterial spores to heat inactivation has been studied by microbiologists for more than a century because of its importance to the field of sterilization. Gombas [10], Lindsay et al. [14], Murrell [18] and Gerhardt and Marquis [9] presented comprehensive papers examining various theories on development and maintenance of the heat-resistant state of bacterial spores. Common consensus is that spore survival at high temperature depends upon stabilization of all vital, potentially labile, components. Traditionally the heat resistance of a spore is attributed to partial dehydration of the protoplast and molecular stabilization caused by the presence of spore-specific compounds such as dipicolinic acid (DPA) and its calcium chelate (CaDPA) [17]. Although individual components may have specific adaptations, a general mechanism for stability is required. The stability of a spore protoplast in a glassy state would offer such a mechanism and would not be mutually exclusive from the traditional heat stability theories. Reduction in water content could increase interactions between protoplast components, resulting in specific stabilization in addition to increasing the glass transition temperature. DPA and CaDPA are believed to play a critical role in the stabilization of the spore [11,14,17].

There are several factors found related to the high heat resistance of spores that are consistent with an amorphous glassy state. A glassy solid-state results from an increase in the viscosity of an aqueous, amorphous, rubbery system such that there is no flow during a reasonable time scale. The temperature of transition from a rubbery liquid to a glassy solid-state is often labelled T_g and is moisture-dependent, decreasing with increasing moisture [13]. Formation of a glassy solid-state results in significant arrest of translational molecular motion, and chemical reaction becomes very slow.

Murrell [18] concluded that there was little evidence of order within the spore protoplast from the X-ray diffraction pattern of spores of *Bacillus subtilis* [12] and those generated from coat material of *B. stearothermophilus* [10]. The crystalline structure observed by X-ray diffraction by Kadota and Iijima [12] has been attributed to the spore coats [10].

Studies on the dielectric conductivity of spores, have indicated that ions and small solutes within the spore are immobilized prior to germination [4]. Extremely limited mobility of solutes is consistent with a glassy state [13].

^{31}P-NMR studies by Cornell (cited in Murrell [17]) indicated that the presence of DPA or CaDPA reduced the normal torsional motion of the DNA. At higher concentrations of DPA or CaDPA, no motion of DNA was observed, even at an a_w of 0.98, indicating a stiff polynucleotide backbone, again consistent with the glassy state theory of a polymer [13].

Studies by Lindsay and Murrell (cited in Murrell [17]), on the melting profile behavior of salmon sperm DNA (strand separation as observed by spectrophotometry), indicated an increase of 20 °C, when 20 μg ml^{-1} DPA/CaDPA was added. At very high concentrations of DPA, the strands could not be separated. This suggests that these components interact

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with the protoplast nucleic acids forming a condensed very restricted glassy solid-state, thereby raising the temperature required for strand separation.

Heat activation of a spore probably occurs at the glass transition temperature of the core, making it accessible to germinants, which if present, initiate the events leading to its normal vegetative cycle, because just above the glass transition temperature, there is a dramatic increase in the free volume, with a large drop in viscosity [8]. However, in the absence of these germinants the spore reverts back to its dormant state. Thus it seems activation would increase the permeability and molecular diffusion processes and explain the increase in the possibility of germination of these spores as observed by Beaman et al. [3]. Heating of spores in a dry environment causes removal of moisture from within the spore, thereby preventing rapid plasticization of the matrix and subsequent inactivation. It is well known that T_g increases as moisture decreases [22]. This behavior would also explain the increased resistance of spores heated in a dry medium such as oil [1].

These cited studies are indicative of a glassy state of the spore protoplast, with DPA and CaDPA playing a key role in its maintenance. Analysis of the spore protoplast alone, for its glass transition temperature by the conventional methods would involve its isolation. However, in spores, this disruption is often characterized with the release of spore-specific components and destruction of the native structure, which would thereby prevent a successful analysis. Studies in our lab using a Dupont DSC 1100 and by Gerhardt (personal communication, 1991) fail to reveal any T_g when whole spores were examined. This could be because events in the outer cortex and coats mask the changes in specific heat changes taking place within the protoplast. Alternatively the hypothesis of the glassy solid-state of bacterial spores could be supported if the thermal inactivation rates of bacterial spores followed the behavior of common polymers above their glass transition temperature. Thus the objective of this work was to utilize kinetics describing reaction rates above the glass transition temperature to determine if rates of spore death by heat at different temperatures followed this pattern.

MATERIALS AND METHODS

Arrhenius kinetics used to describe the temperature dependence of chemical reactions including the thermal inactivation rate constant k (1/time) of bacterial spores is given by:

$$k = Ae^{-\frac{Ea}{RT}} \tag{1}$$

where A is the Arrhenius or frequency constant (1/time), E_a is the activation energy (J mol^{-1}), R the gas constant (J mol^{-1} K) and T the absolute temperature (K). However, above the glass transition temperature, the free volume, interpreted as the presence of a critical void volume required for the movement of a polymeric segment, is considered to be the limiting factor for any reactions to take place [5]. It is therefore appropriate to describe the dependence of the reaction rate constant on the free volume in an Arrhenius form such as:

$$k = Ae^{-\frac{B}{f}} \tag{2}$$

where A and B are constants and f is the fractional free volume. Since the magnitude of α, the coefficient of expansion of free volume is small, it may be assumed that f increases linearly with temperature [1] according to:

$$f = f_g + \alpha(T - T_g) \tag{3}$$

where subscript g refers to the parameter values at the glass transition temperature. Taking the differential of Eqn 2 at T_g, substituting Eqn 3 for α and rearranging, we get:

$$\ln\left(\frac{k_g}{k}\right) = -\frac{C_1(T - T_g)}{C_2 + (T - T_g)} \tag{4}$$

where $C_1 = B/f_g$ and $C_2 = f_g/\alpha$. This has the same form as the WLF equation. For many linear amorphous polymers, independent of chemical structure, constants $C_1 = 40.2$ and $C_2 = 51.6$ [25].

Thermal inactivation rate constants for *B. stearothermophilus* and *C. botulinum* were compiled from published data and were fitted to Eqn 4 with the above constants by nonlinear regression using the Marquardt–Levenberg algorithm [19], generating the optimum values for the glass transition temperature along with the inactivation rates at that temperature.

RESULTS AND DISCUSSION

Figs 1 and 2 show the fit of the WLF equation to the experimental data for heat inactivation of *B. stearothermophilus* [6,7,16,23,24] and *C. botulinum* type A and type E spores [1,15,20,21]. In all the cases it can be shown that the

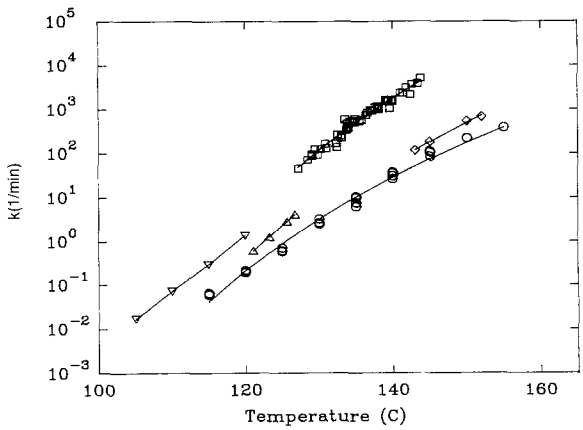

Fig. 1. Experimental data and predicted curve using the WLF equation, for thermal inactivation rate constant of *B. stearothermophilus* spores. Glass transition temperatures obtained by nonlinear regression of different data sets are: T_g = 80 °C (▽) [23]; 90 °C (○) [6]; 98 °C (◊) [7]; 98 °C (△) [16]; 99 °C (□) [24].

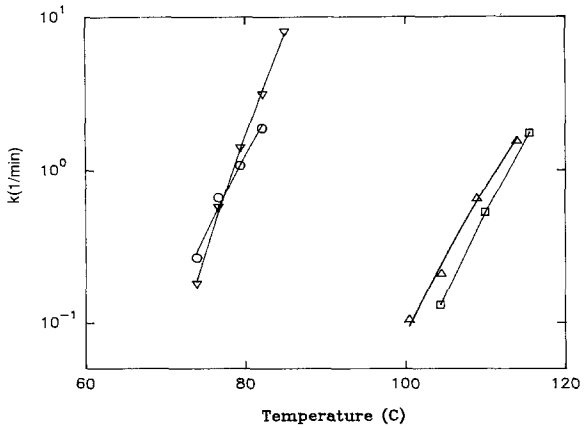

Fig. 2. Experimental data and predicted curve using the WLF equation, for thermal inactivation rate constant of *C. botulinum* spores. Glass transition temperatures obtained by nonlinear regression of different data sets are: T_g = 35 °C (○) [21]; 52 °C (▽) [15]; 60 °C (△) [1]; 67 °C (□) [20].

WLF equation describes the behavior of thermal inactivation rates as a function of temperature very well. The thermophilic spores of *B. stearothermophilus* display a higher glass transition temperature than the mesophilic spores of *C. botulinum*, and the more resistant Type A spores of *C. botulinum* indicate a higher glass-transition temperature than the less-resistant type E spores. Where data was available over a broad range, the WLF equation provided a better fit than did the Arrhenius equation as is shown in Fig. 3 [6]. It can also be observed that the rate constants, predicted by the extrapolation of the Arrhenius equation to higher temperatures, will give high values.

CONCLUSION

The WLF equation derived from the Arrhenius type relation between the specific rate constant and free volume adequately describes the temperature dependence of the rate constant and shows a higher T_g with higher heat resistance. It is clear that thermophilic spores of *B. stearothermophilus* display a higher glass transition temperature than mesophilic spores of *C. botulinum* and the more resistant Type A spores of *C. botulinum* indicate a higher glass transition temperature than the less resistant Type E spores. More importantly it may reveal the physical state of the bacterial spores.

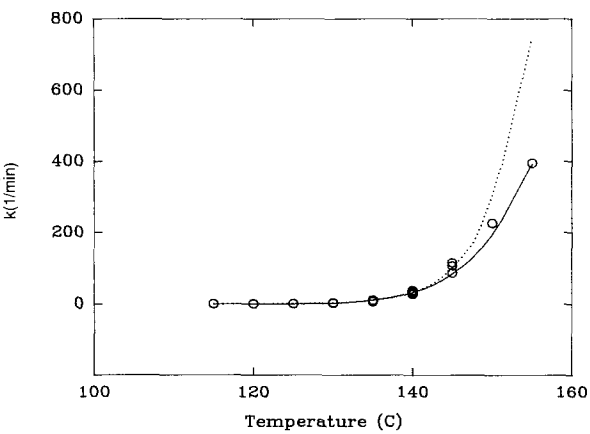

Fig. 3. Experimental data and predicted curve using the WLF (—) and the Arrhenius (...) equation, for thermal inactivation rate constant of *B. stearothermophilus* spores (○) [6].

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