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INVESTIGATIONS INTO A DIFFUSION MODEL OF DRY HEAT STERILIZATION

Interim Report

Contract NASw-1734
for
National Aeronautics and Space Administration
Office of Biosciences

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May 5, 1969

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TRSR-041

N70-31176
(ACCESSION NUMBER)
(PAGES)
CR-110611
(NASA CR OR TMX OR AD NUMBER)
(THRU)
1
(CODE)
24
(CATEGORY)
FACILITY FORM 602

ABSTRACT

The analytical model described in this study formalizes the hypothesis that dry heat inactivation of microorganisms is closely related to the water content of the spore and its micro-environment. Experimental data are examined relative to this model and it appears to be valid. This model is aimed at overcoming the well known deficiencies of the logarithm model.

INTRODUCTION

Several recent investigations have related environmental conditions other than temperature to microbial spore survival under heat sterilization. Murrell and Scott, Angelotti, and Reid have shown strong dependency of dry heat death rates on ambient relative humidity and on water content of the spore*. Hunnell and Ordall have found that heat causes spores to exude calcium and dipicolinic acid (DPA) prior to death, while Alderton, Thompson, and Snell show that spores in aqueous suspension have improved heat resistance when calcium ions are present.

In addition to these correlations with environmental conditions during sterilization, spore survival shows correlations with environmental conditions that prevailed when the spore formed. Vinter shows heat resistance is affected by calcium and cystine availability during sporulation; Murrell and Warth show correlations of heat resistance with five different substances found in the spore.

These investigations show the importance of environment on heat sterilization characteristics. A simple chemical reaction does not appear to be the complete mechanism for spore destruction. Rather, a sequence of transport of chemicals, notably water, occurs and modulates the rate at which chemical reactions destroy the viability of the spore.

In this report we present a diffusion-denaturation model of spore heat resistance that attempts to correlate with the water effects observed, and to provide a basis for determining the efficiency of proposed dry-heat sterilization plans. Such a model is immediately useful in a dry-heat sterilization program, and also offers promise of eventual understanding of the remarkable resistance of spores to heat.

*References are listed at the end of this report.

DIFFUSION MODEL

A model to predict the water content of a spore as a function of time, temperature and initial water concentration within the spore has been developed. The assumptions made are that heat deactivation in spores is due to protein denaturation and that the rate of this reaction is controlled by the water content of the spore core.

A spore is composed of an outer coat (cortex plus spore coats) and a central core (cytoplasm) which is the dormant micro-organism. The cortex protects the core from physical damage, chemical contamination and rapid wetting by the environment. The cortex is mainly composed of mucopeptide polymers (Warth et al, 1963) with the chains twisted and coiled or interwoven (Mayall and Robinow 1957). The coat is mainly protein with a high cystine disulphide bond content (Vinter 1961).

The heat resistance of spores is assumed to rest in the ability to control the amount of water internal to the spore. A contractile cortex system (Lewis, Snell and Burr 1960) would provide a mechanism to dehydrate the cytoplasm and maintain it in this state. Chemical variation in the mucopeptide of the cortex and in the amount of Cu ++ or Ca-DPA (Young 1959) binding to the mucopeptide which may cause the contraction, could result in differences in the degree of contraction and therefore in the final water content of the spore, resulting in marked difference in heat resistance.

In the development of this diffusion model we have assumed a simplified spore structure composed of an outer coat of negligible thickness at a radius surrounding a spherical, homogeneous one.

Water transport thru the spore can be described by the well known diffusion equation

$$D\nabla^2 C = \frac{dC}{dt} \qquad (1)$$

where C is the concentration of water/cm^3, as a function of position and time. D is a diffusion coefficient depending on the medium.

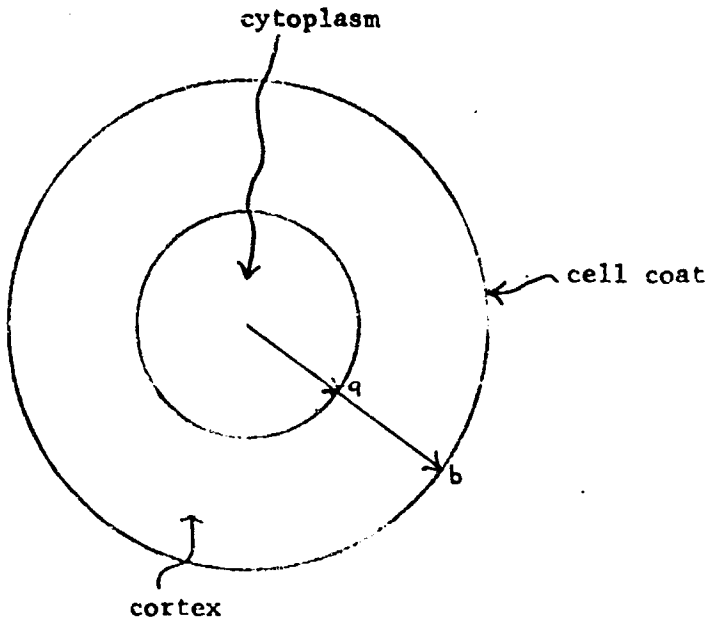

Figure 1. Model of Spore

For a simple example, assume the initial water content in a spherical spore is distributed radially according to

$$rC(r) = \frac{b}{\pi}\left[\sin\frac{\pi r}{b} - \tfrac{1}{2}\sin\frac{2\pi r}{b}\right] \qquad (2)$$

where C(r) is the water concentration at a distance r from the center of the spore, and b is the radius of the spore (see Fig 1). This expression, as seen in Figure 2, corresponds to a distribution that is peaked in the outer portion of the spore, and can be considered as approximating the case where a spore contains more water than an optimum amount. This excess water is stored in the cortex.

Applying the diffusion equation to this initial distribution results in the time variation shown in Figure 3. Water diffuses inward to the cytoplasm, and the spore gradually loses water to the medium. As a result, the initially-dry central region reaches a peak concentration of water that occurs around a time t given by

$$\frac{D\pi^2 t}{b^2} = 1.5 \qquad (3)$$

where D is the diffusion coefficient of the system. This simple model assumes D to be independent of position. As a result, it approximates the situation when the spore is buried in an appropriate medium, since normally one would expect the diffusion coefficient of water outside a spore to differ from D inside the spore.

Data have been reported by Angelotti (1966) for the thermal sterilization of spores in lucite. These data (Fig 4) indicate the death rate to be greatest at about 1.5 hours after commencing to heat the spores at 125°C. Using this value for t, in the above equation, we arrive at an estimate for the diffusion coefficient at 125°C:

$$D = 1.2.10^{-6} \ (cm^2/sec) \qquad (4)$$

This estimate is in reasonable agreement with values for diffusion of protein molecules in water (Clark, p. 138). Our example requires the opposite: water molecule diffusion in protein, which has an unknown diffusion coefficient,

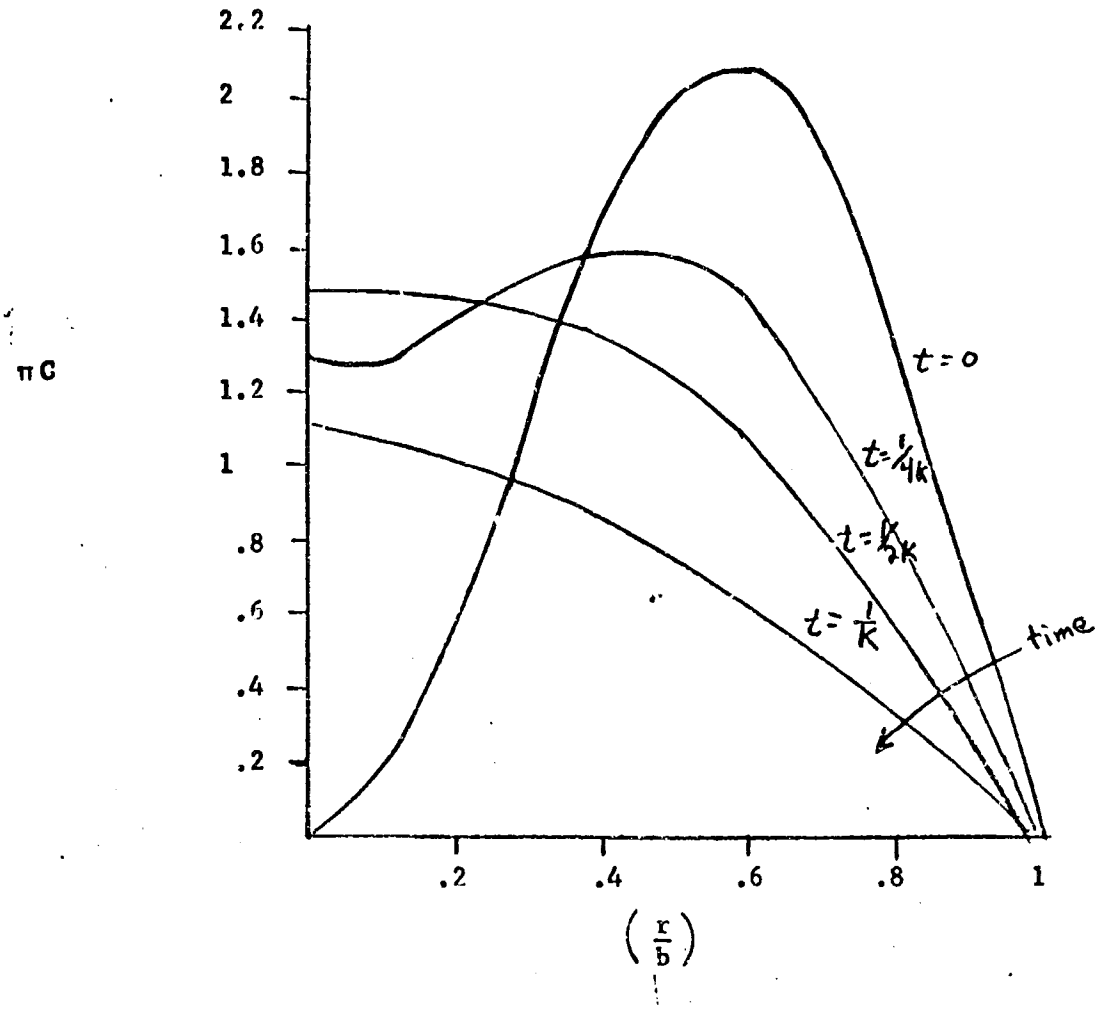

Figure 2. Water Distribution in the Spore

For $t = o$, $\quad rC = \frac{b}{\pi}\left[\sin\frac{\pi r}{b} - \frac{1}{2}\sin\frac{2\pi r}{b}\right]$

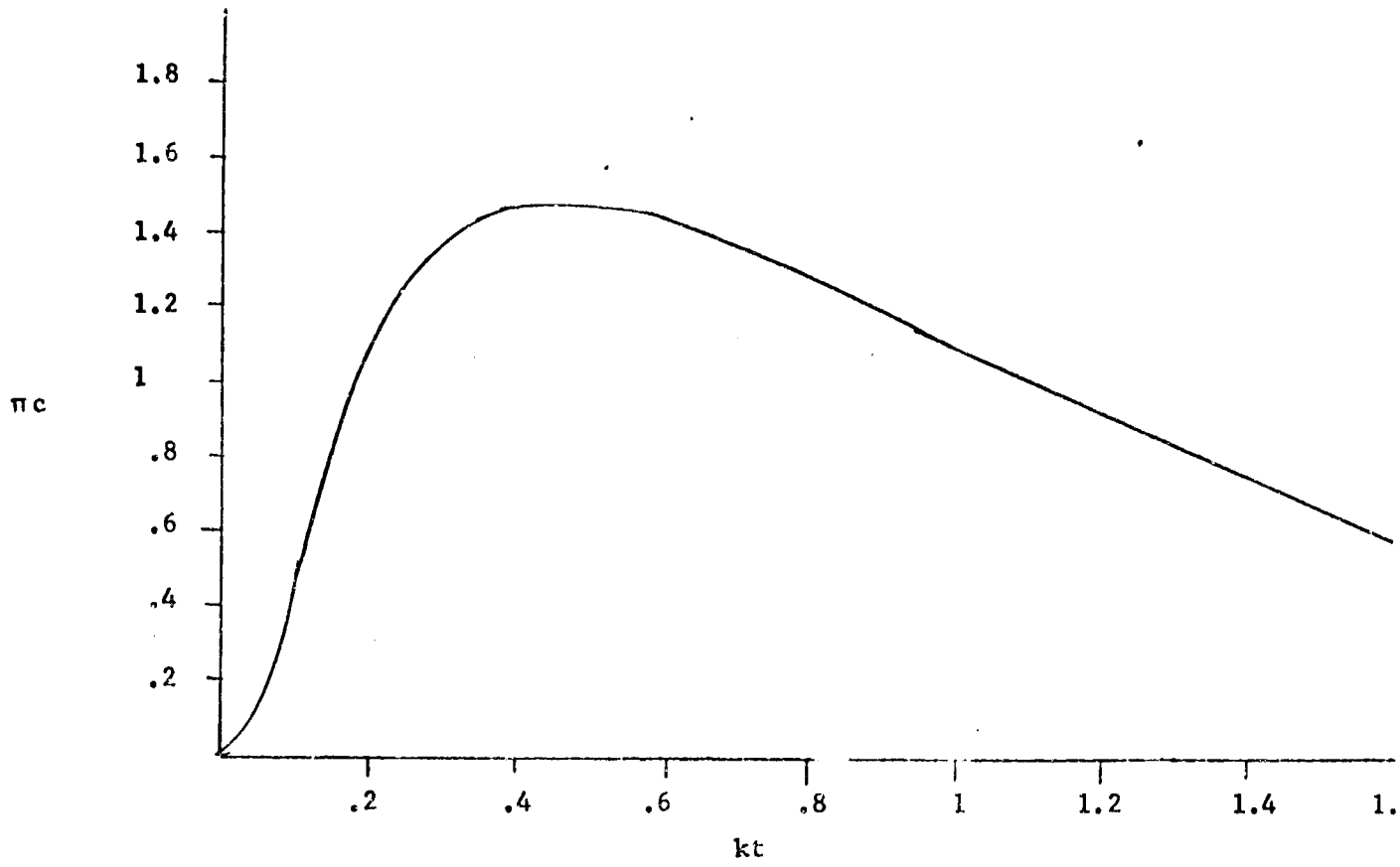

Figure 3. Concentration of Water at center of spore, as a function of heating time.

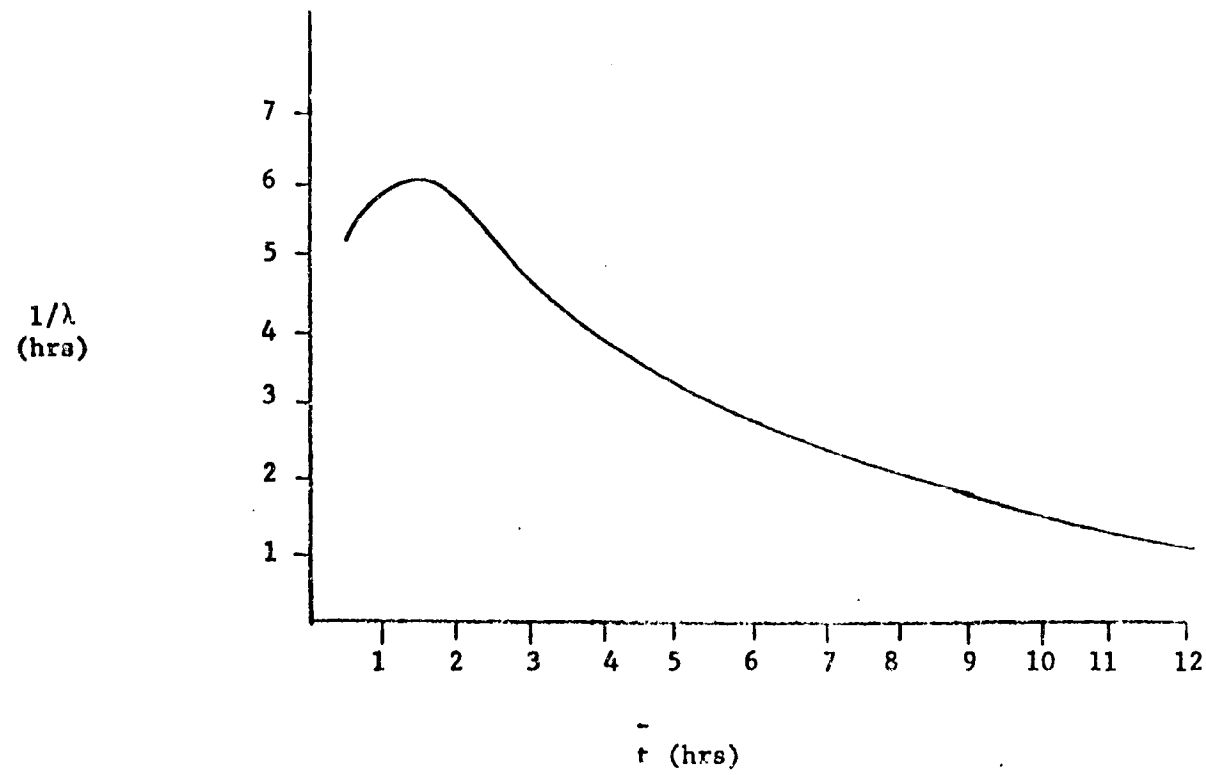

Figure 4. Apparent change in resistance of B. globigii in lucite after times $\bar{t}$ in hours at 125° C

and further assumes this coefficient does not differ greatly from water diffusion in lucite.

One method of evaluating the diffusion coefficient, which is necessary if a diffusion model is to be applied to dry sterilization, is to look at wet sterilization curves. Such curves are shown in Figure 5 for four temperatures. In wet heat, the spores should absorb water to some maximum. Then, the denaturation of proteins in the spore, with this water present, results in a straight logarithmic curve for survivors, as a function of time of heating.

Such a description fits the curves shown. The knee of each curve represents the time at which the cytoplasms of the spores are in equilibrium with the external water. For a purely exponential buildup of water, this occurs about when three relaxation times have passed, according to diffusion kinetics. The diffusion coefficient calculated by this method can be fitted to a formula

$$D = D_o e^{H/RT}, \text{ where } \begin{cases} H = 60 \text{ K cal} \\ R = 1.98 \\ T = \text{temperature } (^oK) \\ D_o = \text{Constant} \end{cases} \qquad (5)$$

Results of the calculations are shown in Figure 6, together with the diffusion coefficient calculated as Eqn. 5. The two sets of experiments seem to be in fair agreement, and indicate initial success in applying a diffusion model to the data.

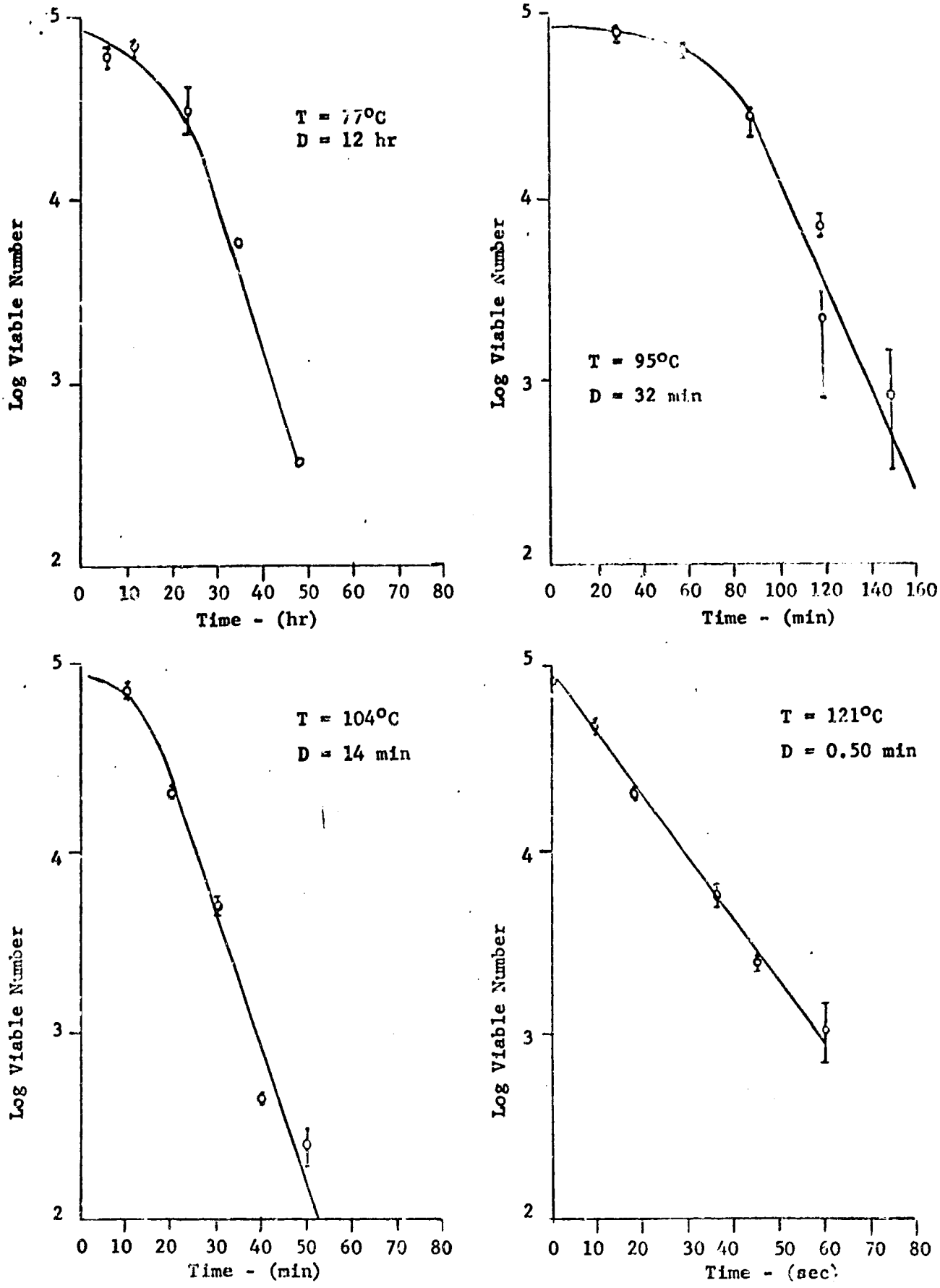

Figure 5. Wet Sterilization Curves (Data from Fox, Eder and Pflug 1967)

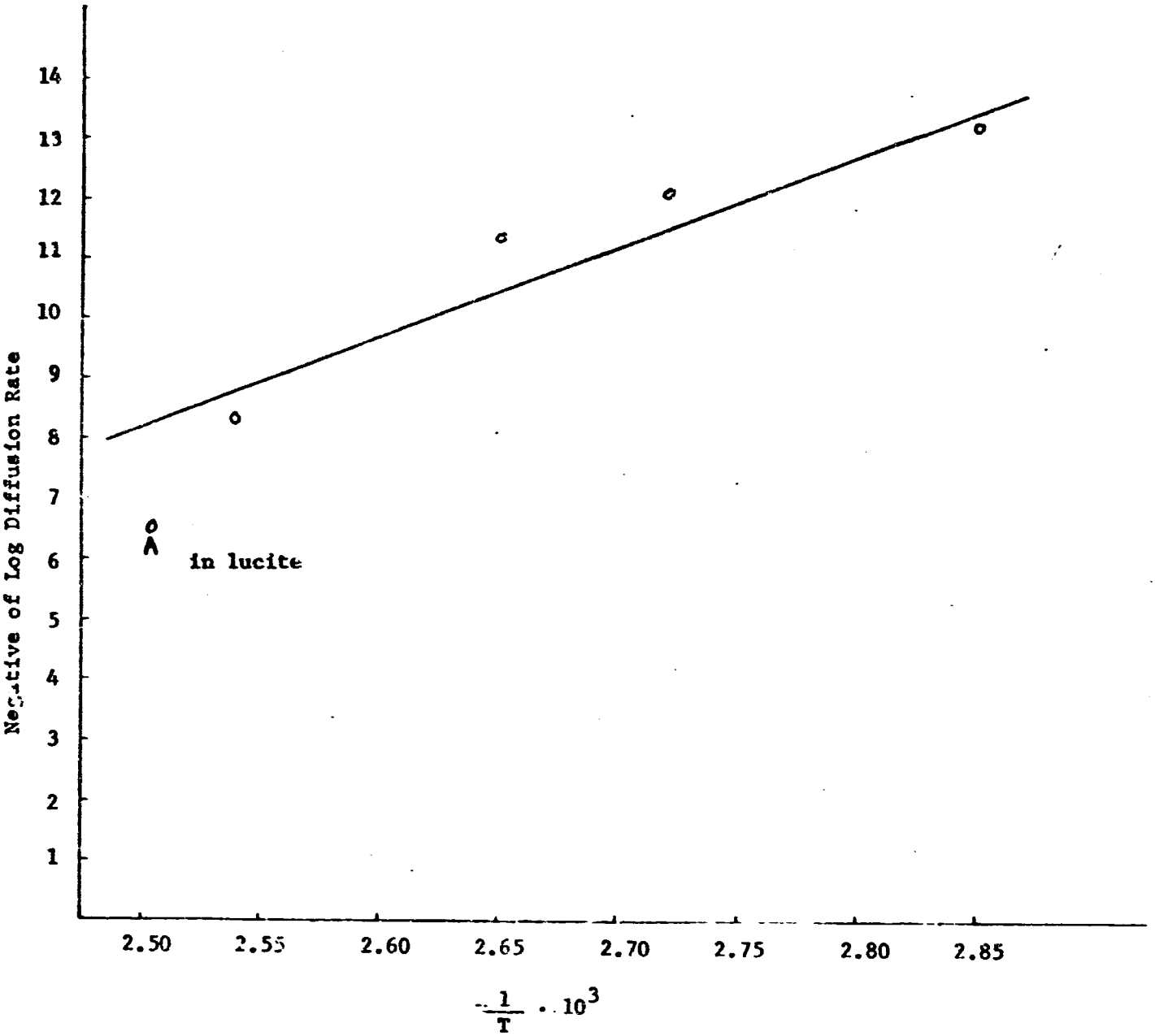

Figure 6. |log D| plotted versus 1/T
(Estimates based on wet heat data by Fox, Eder, Pflug)
(A: estimate from dry heat, by Angelotti)

IMPLICATIONS OF THE MODEL

The correlation of water activity with spore resistance indicates that the death mechanism is a denaturation of some vital protein. Much the same correlation has been observed between protein denaturation and water content. Furthermore, the reaction is generally of first-order kinetics, resulting in a logarithmic curve such as is frequently seen for thermal die-off of spore populations.

The vital protein is clearly in the cytoplasm, since the outer portions of the spore, shedded during germination, are not vital. This outer portion, the cortex, appears to squeeze water from the cytoplasm during the formation of the spore, and thereby provides that there will be a residual concentration that is near the optimum for heat resistance.

Part of the heat resistance of spores has been shown to be due to calcium dipicolinate, manufactured during the formation of the spore, and present in the cytoplasm. Ca-DPA is a chelating agent. Presumably, the vital protein has sensitive bonds that are protected by Ca-DPA and other sensitive bonds protected by water molecules.

Germination of the spore presumably requires that the protein be rid of these protections. Excess water can remove the Ca-DPA; the attached water molecules may separate thermally (heat shock). These separations, if reversible, would be of little help to germination unless the protecting molecules stripped from the protein were to leave the cytoplasm. This is the argument for diffusion: it allows proteins to react with enzymes, etc., in the germination process without hindrance from the former bond-protecting agents. At the same time, the proteins become more sensitive to heat.

Diffusion is the random movement of molecules. It is characterized by straight-line paths between molecule interactions, and arbitrary change of direction after the interaction. The net result of the random motion is a movement of molecules from regions of high density to regions of low density. Frequently, these interactions are mere collisions. In such a case, the diffusion constant D is proportional to

temperature. A less frequent situation is where the collisions involve chemical reactions. The molecule moves in a straight line, collides and "sticks" to a fixed obstacle, is freed by the action of heat, and moves off in an arbitrary direction. This kind of diffusion, characterized by a diffusion constant as given in Eq. 5, appears to fit the process of water diffusing through the spore cortex.

The environmental conditions prevailing during spore formation have been shown to affect the subsequent resistance of the spore to heat. This, too, is as the model would imply. There is no known mechanism by which the spore can control the water concentration in the cortex and spore coats. As a result, the moisture content of these regions will vary so that they are in equilibrium with external conditions. When the spore is heated, the contents of these regions can diffuse inward and outward to affect the heat sensitivity of the spore.

These two mechanisms - diffusion and chemical reactions of proteins - appear responsible for the survival probability of spores as a function of humidity, temperature and time. Much work remains in the analysis of their quantitative aspects. What are the equilibrium moisture contents of spores? What is the water distribution inside the spore? What are the surface transfer properties? Does the Ca-DPA diffuse, too? What is the protein denaturation reaction that occurs? How many protein molecules must denature before the spore becomes nonviable? The answers to these questions require further study. Many previous experiments, unfortunately, are of little help since not all the pertinent variables were measured.

RECOMMENDATIONS FOR FUTURE WORK

1. Moisture Content Analysis

Measurements of the moisture content of spores are needed. For ease in comparing results of different environments, only one species (B. subtilis var. niger is the accepted norm) should be used and standard harvesting, washing, and drying procedures employed. Selection of procedures is a subject for discussion, but standardization will permit studies of the results to be concentrated on the environmental effects.

The environments to which the spores are equilibrated can vary in temperature, in humidity, and in length of time of equilibration. Down-side and up-side equilibration need separate studies, in view of the "hysteresis" effect observed. The rate at which spores give off water during an analysis, together with the cumulative water emission, should be measured.

These measurements should be analyzed to see whether they conform to the hypotheses discussed in this report. Evaluations of diffusion coefficients and surface transfer effects should be possible.

2. Correlation of Moisture and Sterilizability

Dry heat sterilization, with moisture content as a parameter, has been measured with uncertain results. The understanding gained from the analysis above should provide benchmarks for future measurements of spore sterilization rates. The early work sometimes suffered from a lack of determination of the pressure or the relative humidity during sterilization. The substrate deserves attention: if it has good water transference properties it can affect the experiment significantly.

With knowledge of moisture transfer properties in the spore, on its surface, and through the environment, it should be possible to

design crucial experiments where the moisture content and temperature of the spores are known quantities. The die-off of spores under these conditions should be measured. Such experiments include:

(a) Spores in vacuum. The moisture transfer outside the spores is a relatively easy calculation.

(b) Spores in non-permeable materials (e.g. epoxy). With different initial moisture contents, spores hould show different die-offs, but each experiment should provide nearly logarithmic curves.

(c) Spores in air. Moisture transfer is affected by the relative humidity and this can be varied in a sequence of experiments.

(d) Spores of different initial A_w in air.

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