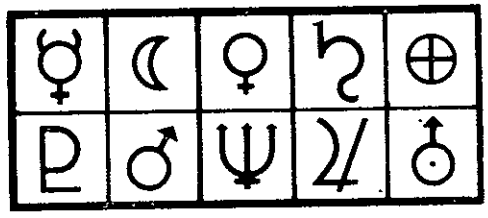

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INTERIM REPORT
DRY-HEAT STERILIZATION MODELING

J. P. Brannen
Planetary Quarantine Systems Studies
Division

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DRY-HEAT STERILIZATION MODELING

Section I

Introduction

A rational model for spacecraft sterilization requirements was proposed in [1] and introduced to the open literature in [2]. We shall understand a rational model to be one in which the parameters are physical in nature and which can be investigated independently of the phenomenon being modeled. This should not be confused with the rationale for a model. For example, the rationale for using, say, the log model for microbial sterilization may be "everyone else uses it," while the log model becomes a rational model provided the sterilization "mechanism" is a single "vital" molecule being destroyed by a first-order reaction. Here the physical parameters are T, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ related by the expression

$$k_r = \frac{kT}{h} \exp\left[(-\Delta H^{\ddagger} + T\Delta S^{\ddagger})/RT\right] ,$$

which gives the reaction-rate constant for the first-order reaction. The parameters T, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ can be investigated independently. (These terms are discussed and clarified in Section II.)

For spacecraft sterilization we consider a rational model to be desirable for the following reasons:

1. The 10-12 log drop in population is not practical in laboratory experimentation.

2. "Best predictability is obtained by introducing as much rationality as possible into all models used."[3].

The rational model of [1] and [2] was formulated with the above notions in mind under the four basic scientific assumptions:

1. Microorganisms are independently sterilized, i.e., the sterilization of a particular organism has no effect on the sterilization of another.
2. In a thermal environment, sterilization is the consequence of chemical reactions.
3. These reactions, which may be "in the small", have order.
4. There may be several competing "mechanisms" involved sterilization.

By "in the small" we mean that the reactant concentration may be as small as one molecule.

For sterilization modeling and applications we shall understand the "mechanism(s)" of sterilization to be known provided we know the physicochemical parameters of the reactions involved in sterilization. It is not necessary that we know the name of the "vital" structures being destroyed. It is sufficient that we know the orders of the reactions and the $\Delta H^{\ddagger}$'s and $\Delta S^{\ddagger}$'s mentioned above. This, of course, is under the assumption that sterilization is the consequence of chemical reactions.

Under the above assumptions, a model was developed which can faithfully represent the different types of survivor curves seen in dry-heat sterilization.

This general type of analysis was apparently first discussed extensively in 1943 by Otto Rahn [4]. Rahn's work was of a theoretical nature, and was not extended through the 1943-1968 era. There are two very basic differences between Rahn's work and that presented in [1] and [2]. First, Rahn allowed only first-order reactions in the inactivation of "vital" structures and second, he did not allow for competing sterilization mechanisms. Second-order reactions were allowed in [1] and [2], since some proteins are thermally destroyed by higher ordered reactions [5, p. 277] and microbes contain protein. Competing sterilization mechanisms are indicated as possible in the thermal sterilization of certain viruses [6, p. 197]. Thus, it seems reasonable to at least allow competitive mechanisms in the sterilization of bacteria. It should be emphasized that competitive systems and higher ordered reactions were allowed but not required in [1] and [2]. In particular, the sterilization mechanisms considered were a "vital" molecular structure A being degraded by the first-order reactions

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$

$$A \xrightarrow{k_2} C$$

or a "vital" structure D being degraded by the second-order reactions

$$2D \xrightarrow{k_3} C .$$

These were simply the sterilization mechanisms considered and were not intended as the outer bounds of those that could be utilized in the model.

Developments in modeling since the publication of [2] have followed five distinct lines; namely,

1. Generalization to include a menu of more reaction possibilities.
2. Conversion from Arrhenius formulation of the reaction-rate equation to the more general absolute reaction-rate theory.
3. Computerization of the model,
4. Model verification experiments (in the computer and in the laboratory), and
5. Theoretical developments related to the inclusion of other environmental parameters.

These developments are treated in this interim report.

It is clear that, under the four basic scientific assumptions, a great variety of specific models can be considered. Thus, for many purposes, the model is indeed excessively dynamic. For this reason, it is appropriate that the following notions concerning model utilization be stated.

1. For applications, it is not necessary to know the precise mechanism(s) of sterilization. It is sufficient to know the physicochemical parameters of the system.

   For example, suppose it is known that the precise mechanism of sterilization under certain circumstances is the denaturation of DNA by a first-order reaction with rate constant k. For application purposes it is sufficient to know that a "vital" molecular structure is being degraded by a first-order reaction with that rate constant.

2. With first-line modern computing equipment, it is feasible to couple the theoretical concepts of physical chemistry with biological observations to probe the probable ranges of physicochemical parameters which are of interest in sterilization.
3. The ranges of the physicochemical parameters can provide insight into the names of these heretofore nameless sterilization mechanisms.

The first and third of these statements are simply an assertion of agreement with the procedure followed by biologists for years. The second is of most interest, since it expresses an intent to utilize the capability of modern computing equipment to extend the range of reactions and systems to be considered. In fact, it includes the notion that, if one takes a sufficiently large collection of systems and reactions, the systems and reactions relevant to sterilization will be imbedded in this collection, and that, by peeling off the obscuring irrelevant reactions through computers, one may gain a clearer view of the relevant. (Those familiar with the recent development of the invariant imbedding technique in applied mathematics will recognize the analogy.)

Theoretical developments are treated in Section II. These include model generalization and analyses for both microbial water activity and pressure. Water activity is included because of the demonstrated importance of this parameter [7, 8, 9, 10, 11, 12] to the thermal sterilization of microorganisms. Pressure is included to aid in system studies related to the die-off of microorganisms in outer space. An additional interest in pressure comes from the importance of the $\Delta V^{\ddagger}$ term in the

analysis of sterilization mechanisms. (Here $\Delta V^{\ddagger}$ is the activation volume for a chemical reaction when absolute reaction-rate theory is used for obtaining the rate constant. It is discussed more thoroughly in Section II.)

In addition to these theoretical developments which are closely related to immediate planetary quarantine applications, a secondary study has been carried out concerning the cell constituents involved in sterilization. This study has had the objective of analysis technique development, as well as the actual identification of sterilization mechanisms. So far, this study has produced rather impressive evidence that DNA denaturation is responsible for wet-heat sterilization of Bacillus subtilis [43]. This same study also led to the formulation of the concept that genetic homogeneity of a population and chemical homogeneity of its DNA are not equivalent. This notion is used to explain some anomalous results concerning survival "tails." This is included in theoretical development.

Model verification experiments are discussed in Section III of the report. To test the validity of the model relative to a given environmental parameter, base-line data are obtained from certain laboratory experiments and the model is then "challenged" to predict survivors when that particular environmental parameter is allowed to vary. Model computerization is discussed in Section IV.

At this time, a number of conclusions are possible:

1. For obtaining spacecraft sterilization parameters, it is feasible to utilize a kinetic approach thereby including considerable rationality in the modeling.

2. The inclusion of water activity is accomplished in a kinetic sterilization model by means of absolute reaction-rate theory and the entropy of activation, $\Delta S^{\ddagger}$.

3. The inclusion of pressure in a kinetic sterilization model is accomplished by means of absolute reaction-rate theory and the volume of activation, $\Delta V^{\ddagger}$ [70].

4. From a kinetic viewpoint, there is little difference between experiments run at a pressure of 10^{-6} torr and 10^{-17} torr. Consequently, experiments run at 10^{-6} torr should be adequate for kinetic sterilization prediction related to an outer space environment [71].

5. The sterilization mechanism for Bacillus subtilis in wet heat is probably DNA denaturation. The sterilization mechanism for Bacillus subtilis in dry heat is probably DNA denaturation. However, the reactions on this substrate are not the same since the $\Delta V^{\ddagger}$'s observed for these distinct cases differ by several orders of magnitude. Other considerations lead to the conclusion that DNA is the likely substrate.

Section II

Theoretical Developments

A. a_w and Microbial Sterilization

It is well documented that water activity, a_w, has an important role in the thermal sterilization of microorganisms. (From a practical point of view, assuming equilibration of spore moisture with external environment, a_w = relative humidity/100.) The nature of this role is the subject of some debate and, at the present time, is unresolved. At one extreme is the assertion that the microbe is sterilized as a result of water loss and at the other extreme is the assertion that water serves as a vehicle for transporting "vital" substances away from "vital" sites during sterilization. We will take the intermediate viewpoint that either

(1) Water changes the rate at which sterilizing reactions occur, or

(2) The dominant "mechanisms" for sterilization change with changes in water activity.

If the second of these two viewpoints holds, then the overall modeling discussed in [2], in which competitive mechanisms for sterilization are allowed, should prove useful. If the first of these should hold, then a way must be found for treating this reaction-rate change. The word "or," used in the statements above, is not exclusive; thus, if both conditions hold, the inclusion of a_w as an environmental parameter will simply require a combination of competitive modeling and reaction-rate

effects. Incidentally, Rahn flatly states that there are separate mechanisms for wet-heat and dry-heat sterilization [13]. If he is correct, then one must allow competitive mechanisms in this modeling. The quantity of water inside the spore as a function of time is examined by Barrett in [14]. His modeling is in terms of the diffusion equation and essentially relates sterilization to water loss.

1. Effect of a_w on Reaction Rates. There is evidence that water molecules have an effect on the stability of various macromolecules [15]. If the stability of a macromolecule changes as a function of a_w, then one would expect the entropy of activation for destruction of that macromolecule to change as a function of relative humidity. It is known that the entropy of adsorption of water by macromolecules changes as a function of relative humidity [16] and, since entropies are additive, one would expect a correlation between reaction-rate change and entropy of adsorption, as functions of a_w. The relationship is poorly understood [17]. However, if one assumes entropy changes independent of temperature, the overall rate constant C' for a given surface reaction with adsorption may be written

$$C' = \frac{k'SL \exp\left[\left(\Delta S^* + \Delta S_A^o + \Delta S_B^o\right)/R\right]}{2h}$$

where k' is the reaction-rate constant, ΔS^* is the entropy of activation, and ΔS_X^o is the standard entropy change for component X. R and h are kinetic constants while S and L are constants related to adsorption sites' [18].

The important thing to observe about this formula is that the activation entropy and entropy of adsorption are additive relative to the rate constant. This, then, is the key to our incorporation of water activity as an environmental parameter in dry-heat sterilization.

This, of course, requires that we leave the Arrhenius formulation for the rate constant used in [1] and [2] and incorporate the absolute reaction-rate theory in our modeling.

The Arrhenius equation, which is basically empirical in nature, simply states that the rate constant, k_r, as a function of temperature is

$$k_r = A \exp[-E_a/T] ,$$

where A and E_a are constants and T is the temperature in degrees Kelvin. This formulation of the reaction-rate constant offers minimal nonempirical possibilities for extension to account for the changing stability of molecular structures as a function of a_w. On the other hand, the thermodynamic formulation of the rate constant derived from absolute reaction-rate theory offers some attractive possibilities, especially when one considers the stability implications of entropy changes. This formulation of the rate constant gives k_r as follows:

$$k_r = \frac{kT}{h} \exp[-\Delta F^{\ddagger}/RT] , \qquad (1)$$

where h is Planck's constant, k is Boltzmann's constant, R is the gas constant, and $\Delta F^{\ddagger}$ is the free energy of activation [5, p. 19]. Thermodynamics enters via the $\Delta F^{\ddagger}$ term, since

$$\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} ,$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are energy and entropy of activation, respectively. Entropies are additive; therefore, if one knew the "vital" structures being destroyed in the sterilization process and the integral entropy of adsorption of water, i.e., mean entropy per molecule [19], by these structures as a function of a_w, it would be a relatively simple matter to institute alterations to Equation (1) and the basic models of [2] to account for survivor changes as a function of both temperature and a_w.

2. Feasibility. An immediate question is: Are entropy changes observed in vitro sufficient to produce the rate changes as observed in, say, Figure 1? For the 120° curve in Figure 1, the D value ranges from approximately 0.01 min to 80 min, with the smaller value for wet-heat sterilization. Assuming that the organisms are being sterilized by the destruction of a single molecule being inactivated by first-order kinetics, the following relation exists between the D value and k_r: Let t be the time required to sterilize 90% of the population, i.e., t is the D value, then

$$t = \frac{-\log_e 0.1}{k_r} \qquad (2)$$

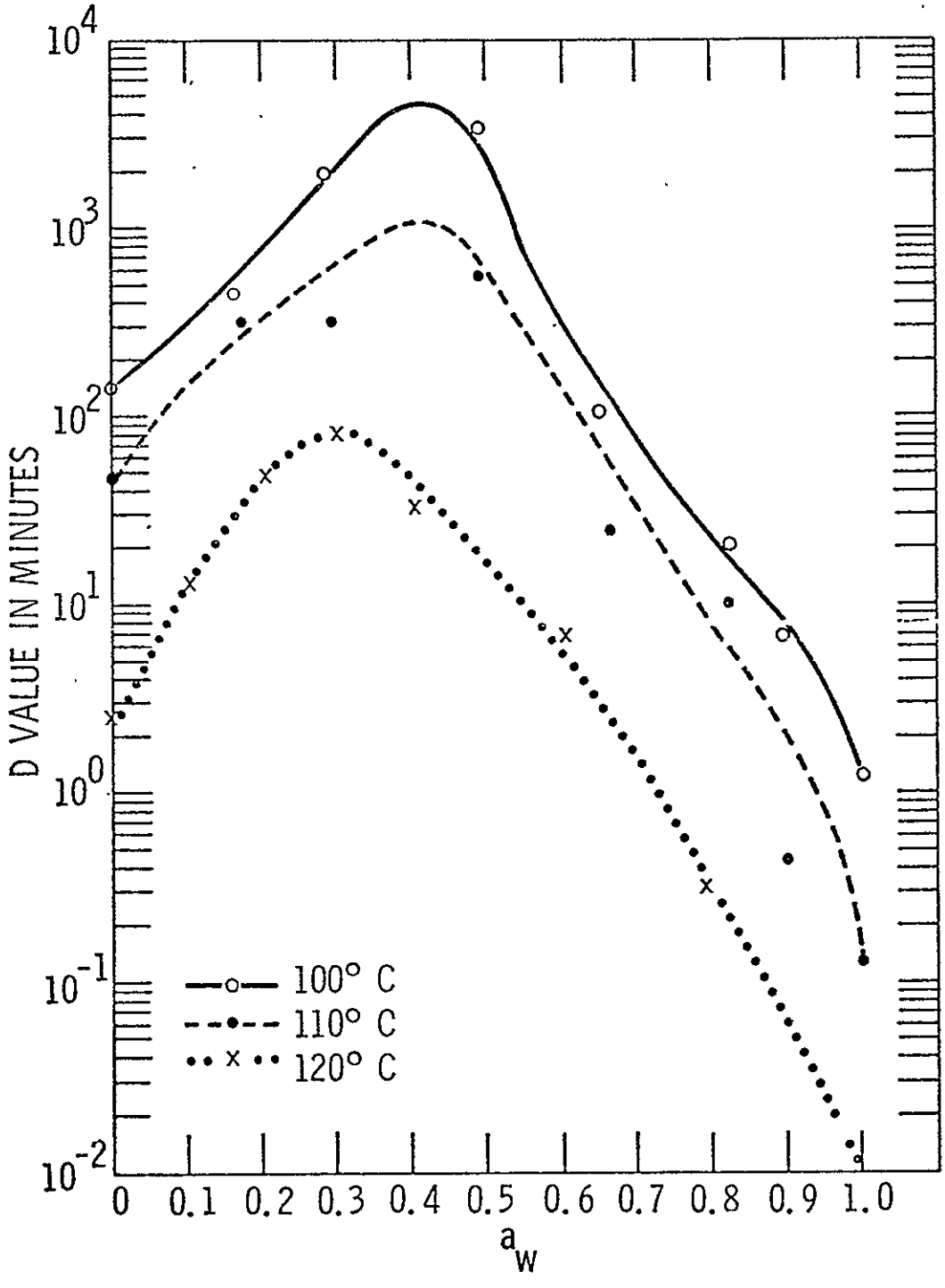

Figure 1. D values (time required to reduce the population by 90%) of Bacillus megaterium spores as a function of a_w. From Murrell and Scott [8].

From Equation (1), we can get the effect of changes in $\Delta S^{\ddagger}$ on k_r and, from Equation (2), we see the resulting alteration in D values. For a given temperature, D values are usually obtained from a linear regression analysis and, when an experimenter runs survivors at more than one temperature level, he does not commonly relate the D values by imposing the additional constraint of either the Arrhenius equation or the absolute reaction-rate theory. Consequently, published D values provide an extremely poor base for our analysis. D-value estimates from Figure 1 are entirely too rough for this analysis. Instead, data from a report, No. 14, of the Public Health Service Laboratories in Cincinnati [11], were used, since actual D values were reported. These data give D values at 125°C, 135°C, and 140°C for a_w's of 0, 0.03, 0.05, 0.07, 0.1, 0.2, 0.4, 0.6, 0.8, and 0.9. At 125°C, the D values range from a high of 364.8 min to a low of 250.2 min. In a short computer program, these values were converted to k_r's by Equation (2) and corresponding $\Delta F^{\ddagger}$'s computed by Equation (1). From the relationship $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, values were obtained for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ at $a_w = 0$. Since three values were available for both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (as $\Delta F^{\ddagger}$'s for only two temperatures are required to compute $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, the combination of three things, taken two at a time, gives us three values for $\Delta H^{\ddagger}$ and three for $\Delta S^{\ddagger}$), the averages were taken, which were $\Delta H^{\ddagger} = 26.897$ kcal/mole and $\Delta S^{\ddagger} = -8.85$ eu/mole. Using $\Delta H^{\ddagger} = 26.897$ kcal/mole and T = 398°K, nominal D values were then computed for $\Delta S^{\ddagger} = -8.85 \pm 2$ eu/mole in steps of 0.1. For $\Delta S^{\ddagger} = -8.65$ eu/mole, a D value of 225 min was obtained, and with $\Delta S^{\ddagger} = -9.65$ eu/mole, a D value of 372.5 min was obtained. Thus, over

the range of a_w's covered in the PHS report, a variation of 2 eu/mole is adequate to explain the D-value variation. The 120° data in Figure 1 show a variation of four orders of magnitude over the a_w range 0 to 1, and three orders of magnitude in the 0 to 0.9 range. (The 0 to 0.9 range is included, since many experimenters consider different sterilizing mechanisms for wet and dry heat [13].) By extending the range of $\Delta S^{\ddagger}$ in the above computations, it is found that a change of $\Delta S^{\ddagger}$ (dry) ±8 eu/mole is sufficient to change D by four orders of magnitude and that a change of $\Delta S^{\ddagger}$ (dry) ±4 eu/mole is sufficient to change D by three orders of magnitude. Since different organisms were used in the PHS report and by Murrell and Scott [8], a correlation was not attempted. It is clear, however, that entropy changes of only a few eu/mole are sufficient to explain the observed variations.

The next question is: Are observed <u>in vitro</u> entropy changes comparable with the <u>in vivo</u> changes implied by the above analysis? (Here, <u>in vitro</u> data are physicochemical data on cell constituents and <u>in vivo</u> data are those obtained from the organisms.) To this end, the PHS data were examined in the following manner: Using the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ computed for an a_w of 0 as a base, the entropies required to produce the observed D values for T = 398°K = 125°C and the various values of a_w were computed. A graph showing the entropy as a function of a_w required to give the observed D values, using a_w = 0 as a base, curve C, is given in Figure 2. In the same figure, <u>in vitro</u> integral entropy data for freeze-dried haemoglobin [16], over a somewhat reduced range of a_w's, curve B, and entropy of sorption as a function

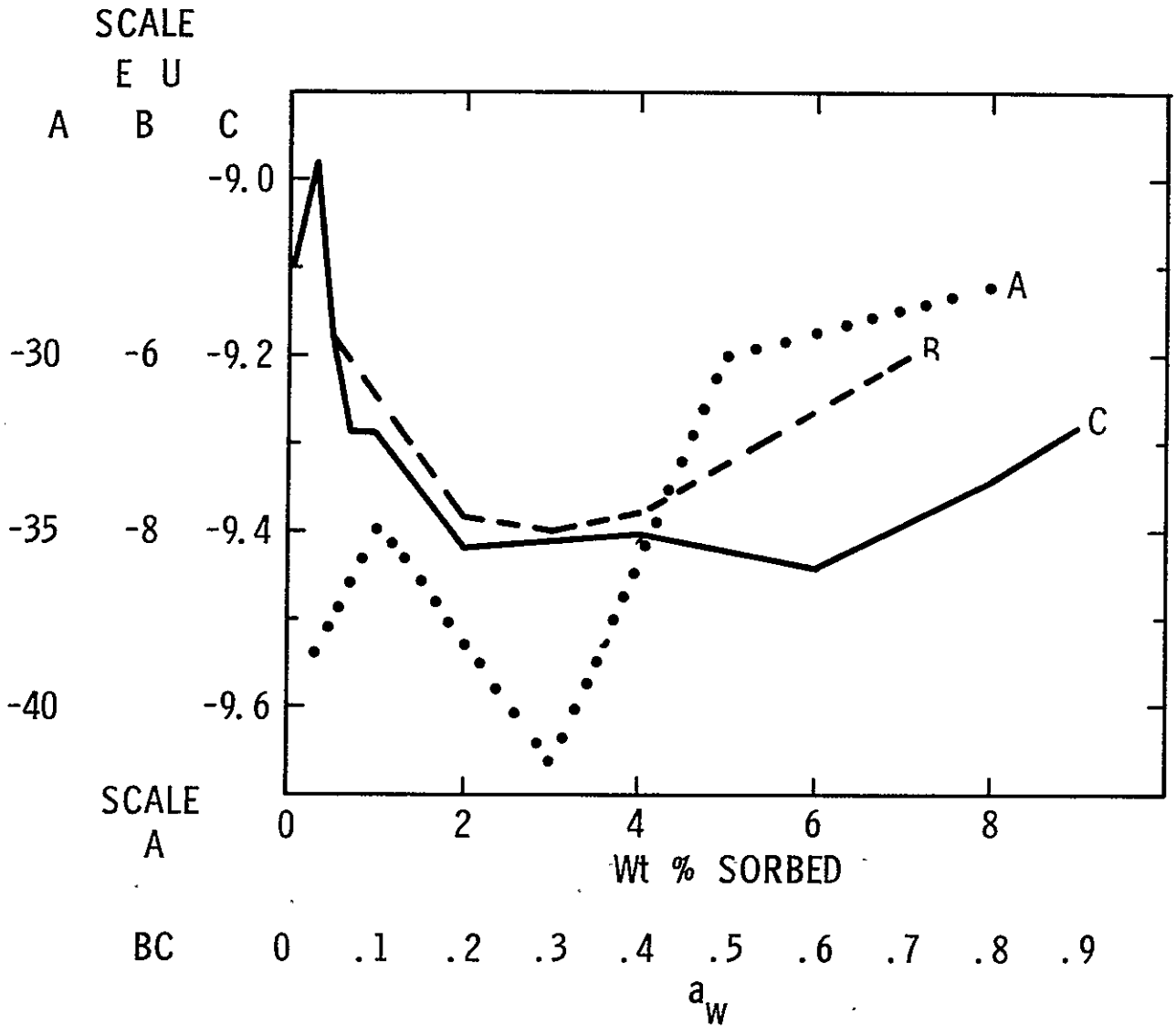

Figure 2. Relationship between entropy and observed D value for PHS data [11] curve B. Integral entropy as a function of a_w for haemoglobin [16] curve C. Entropy of sorption as a function of percent wt sorbed [20] curve A.

of percent wt sorbed for ribonuclease [20], curve A, are given. As can be seen in Figure 2, the general shapes of the entropy curves are quite comparable. Also, observe that for ribonuclease, the entropy of sorption varied 10 eu, while to cover the change observed from the data only 1 eu was required.

3. Conclusions. It is clear from the discussion that the suggested modifications of the models of [2] are sufficient to incorporate water activity as an environmental parameter. For applications, reporting of D values by experimenters is inadequate for efficacious utilization of these modifications. Instead, the raw data should be fit to a kinetic model as proposed in [2], subject to the constraints implied by the thermodynamic parameters of Equation (1). For example, if data are available at more than one temperature level for a fixed a_w, the best fit should be obtained by varying $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. In this way, the modifications suggested here can be easily employed.*

Since k_r is extremely sensitive to small changes in $\Delta S^{\ddagger}$, an analysis which computes the "theoretical" entropy as shown in curve B, Figure 1, could be useful in determining the structures being inactivated in sterilization. One might be able to distinguish the most likely mechanism from a number of candidates by comparing in vitro entropy of adsorption data to the theoretical entropy. A positive result of this form would go far in inducing biologists to recognize nonstatistical mathematical analysis as a research tool. An immediate application of these techniques lies in setting parameters for spacecraft sterilization.

*This work is in progress. See Section V.

B. Pressure and Microbial Sterilization*

The effects of pressure on the sterilization of microorganisms by heat is of interest for planetary quarantine applications. It is not reasonable to assume that the 10^{-17} torr pressure of outer space can be attained in a terrestrial laboratory for the purpose of observing the effect on microorganisms. However, it is possible to carry out an analysis, based on observations at greater pressures, which provides the parameters necessary for gaining some insight into the effect of heat on microorganisms in outer space. In particular, modeling of pressure effects on sterilization is necessary for the analysis of microbial die-off on orbiting hardware.

Some work has been performed on the consequences of pressure on microbial sterilization. Davis et al. [21] and Portner et al. [22] have investigated the effects of pressures in the 10^{-5} to 10^{-8} torr range on microorganisms. Some relevant experimentation was done by Hotchin et al. [23] via high-altitude balloon and rockets. These papers provide clues as to vacuum effects in sterilization, in that the organisms seem to die a little faster at a fixed temperature in vacuum than at ambient pressure. These experiments may all be considered as in the dry-heat mode. For wet-heat sterilization, experimentation under hydrostatic pressure has been carried out by Johnson and Zobell [24], which indicates that, as pressure increases up to 600 atm, survival of certain microorganisms is enhanced. Although the sterilization means may be different under wet- and dry-heat conditions, the pressure effects seem to be consistent, i.e., as pressure decreases, in both cases, death rate increases.

*Most of Section B will appear in [70].

In addition to experiments on microorganisms, there have been investigations into the pressure effects on reactions involving various proteins [25, 26, 27, 28]. A correlation between these observations and those mentioned above is of considerable interest, especially in view of the fact that activation volumes are considered more reliable than other thermodynamic parameters in studies of reaction mechanisms [29]. (The relationship of activation volume to the other kinetic parameters is discussed in the following subsection.)

A study has been made of the relationship between hydrostatic pressure and thermal denaturation of tobacco mosaic virus by Johnson et al. in 1948 [30], which included a kinetic analysis of the results. They found that under the assumption of a first-order sterilization mechanism, the sterilization rate decreased with increasing pressure up to a pressure of around 1000 atm. In 1949, Johnson and Zobell [24] found similar results for spores of Bacillus subtilis. In these two studies the sterilization was by wet heat and the pressure was elevated rather than depressed. Kinetic analyses for dry-heat sterilization under vacuum have apparently not been done.

1. Pressure, Reaction Rates, and Sterilization. The basis for our pressure analysis is the 1941 paper of Stearn and Eyring [31], in which the more complete formulation for the $\Delta F^{\ddagger}$ term in the transition state or absolute reaction-rate theory is given as

$$\Delta F^{\ddagger} = \Delta H_0^{\ddagger} - T\Delta S_0^{\ddagger} + p\Delta V^{\ddagger} ,$$

where $\Delta H_0^{\ddagger}$ is the activation enthalpy and $\Delta S_0^{\ddagger}$ is the activation entropy, both at 0 pressure, where p is the pressure and $\Delta V^{\ddagger}$ is the volume change as the reactant goes into the activated state.

Unless $\Delta V^{\ddagger}$ is quite large, it is sufficient to set $\Delta V^{\ddagger} = 0$, which effectively includes the pressure effect on the reaction-rate constant in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. However, data from Davis, Silverman, and Keller [21] suggest that, in some cases at least, the $p\Delta V^{\ddagger}$ term should not be neglected; for example, they report that, under a pressure of 10^{-8} torr and 60°C, only 40% of a population of B. subtilis survive after 5 days. This translates to 90% destruction in 12.5 days. On the other hand, Silverman [32] reported a $\Delta H^{\ddagger}$ of 34.6 kcal/mole and a $\Delta S^{\ddagger}$ of 14.65 eu for the same organism, under what one can presume to be a pressure of 1 atmosphere. Assuming, as did Silverman, one molecule's being inactivated by a first-order reaction as the sterilization mechanism, and, using his values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, it follows that 123 days should be required for the 90% reduction at 60°C. In the same report, Silverman gives $\Delta H^{\ddagger} = 25{,}000$ cal/mole and $\Delta S^{\ddagger} = -11.1$ eu for data generated by Koesterer [33]. Using these values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ leads to a value of 24.8 days for 90% destruction at 60°C and 1 atmosphere. From a 60°C, 1-atmosphere, 90% reduction value and a 60°C 10^{-8} torr D value, reaction-rate constants can be calculated by Equation (2),

$$k_r = -\log_e (0.1)/D \ .$$

An estimate for $\Delta V^{\ddagger}$ can now be obtained by means of the formula

$$\Delta V^{\ddagger} = \frac{RT}{(p_2 - p_1)} \log_e \left(k_{p_1} / k_{p_2} \right) , \qquad (3)$$

where p_1 and p_2 are pressures in atm, R is the gas constant in cc-atm/deg-mole, and k_{p_1} and k_{p_2} are the reaction-rate constants at pressures p_1 and p_2 [34].

In this manner, it was found that, with $\Delta H^{\ddagger} = 34.6$ kcal/mole and $\Delta S^{\ddagger} = 14.65$ eu,

$$\Delta V^{\ddagger} = 62{,}480.7 \text{ cc/mole} ,$$

while, if $\Delta H^{\ddagger} = 25$ kcal/mole and $\Delta S^{\ddagger} = -11.1$ eu,

$$\Delta V^{\ddagger} = 18{,}770.1 \text{ cc/mole} .$$

Additional analysis sans $\Delta H^{\ddagger}$'s and $\Delta S^{\ddagger}$'s can be carried out via Equation (3) and data presented by Silverman, Davis, and Keller in [35]. In this paper they report that, after 5 days at 60°C, there was 72% survival for B. subtilis at 1 atm and 40% survival at 10^{-6} torr. This results in a $\Delta V^{\ddagger}$ of 28.1 liters/mole. They also report that, after 5 days at 88°C, there was 0.01% survival for B. subtilis at 10^{-6} torr; and, at 90°C, there was 0.55% survival for one experiment and 0.09% survival for another. Assuming no great difference between 88°C and 90°C regarding survival, $\Delta V^{\ddagger} = 62$ liters/mole for the first experiment and 19.4 liters/mole for the second.

The positive value for $\Delta V^{\ddagger}$ indicates that the activated state of the "vital molecule" is less ionized than the inactive state. Since $\Delta V^{\ddagger}$ is generally positive for unimolecular reactions [34], the positivity of the $\Delta V^{\ddagger}$ computed from the Silverman-Koesterer data is consistent with the assumption of a 1-molecule sterilization mechanism.

Assuming a unimolecular sterilization reaction, it is possible to approximate $\Delta V^{\ddagger}$ for a very wide range of pressures by the formula

$$\Delta V^{\ddagger} = \frac{0.1 b^{\ddagger} V}{\sum b + r_1 + r_2 + 1},$$

where $b^{\ddagger}$ is the length of the bond that is broken, $\sum b$ is the sum of the bond lengths in the chain, V is molar volume, and r_1 and r_2 are the covalent radii of the terminal atoms [36]. This formula is based on the assumption that the volume change from reactant to activated state is all due to length changes and that the molecular cross section remains constant.

Suppose the "vital" molecule responsible for sterilization is DNA. To compare a $\Delta V^{\ddagger}$ estimate for bond breakage in DNA with the $\Delta V^{\ddagger}$ computed from the Silverman-Koesterer data, we will assume DNA has 20,000 nucleotide pairs [37], a molecular weight of 2.5×10^9 [38], bond lengths of 3 Å [39], and a specific volume of 0.55 ml/g [40].

Under these assumptions, the value of $\Delta V^{\ddagger}$ for DNA is approximately 7 liters/mole. This is in fair agreement with the $\Delta V^{\ddagger}$ computed from Koesterer's data, especially in view of the assumption that $\Delta V^{\ddagger}$ results solely from length changes. Since the volume of a mole of DNA is about 10^6 liters, these $\Delta V^{\ddagger}$'s represent a volume change of approximately 0.003%. On a percentage volume change, this is somewhat less than is observed for smaller molecules [5, p. 318].

There is some in vitro data available on pressure and DNA. Weida and Gill [28] found a $\Delta V^{\ddagger}$ of 4.5 cc/mole base pairs for DNA transition. This would require, for sterilization, the breakage of approximately 13,000 base pairs to be consistent with our analysis. If the unwinding of a large portion of the DNA is required for dry-heat sterilization, then the magnitude of the $\Delta V^{\ddagger}$ terms for the Silverman-Koesterer data is reasonable.

From data given by Bücker, Horneck, and Wollenhaupt [41], it was possible to compute a $\Delta V^{\ddagger}$ for Escherichia coli being sterilized by X-rays at 1 atm and at 10^{-6} torr, under the assumption that the X-ray damage was analogous to a first-order reaction. Interestingly enough, the $\Delta V^{\ddagger}$ obtained was 31 liters/mole, which lies in the range indicated above. We consider this to be interesting, but very weak, circumstantial evidence.

2. Kinetics of Sterilization at 10^{-6} Torr Relative to 10^{-17} Torr.* A question of immediate interest concerns the change in D value for a given $\Delta V^{\ddagger}$ as pressure goes from 10^{-6} torr (within the experimental range) to 10^{-17} torr (the pressure of outer space). To examine this, we rewrite Equation (3) as

$$\log_e \left(k_{p_1} / k_{p_2} \right) = \Delta V^{\ddagger} (p_2 - p_1) / RT$$

*This Subsection, 2, will appear in [71].

and let $\Delta V^{\ddagger}$ be as large as 10^5 cc/mole (a larger value than reason or any evidence dictates, so that we may examine a worst case). Converting p_1 and p_2 from 10^{-6} and 10^{-17} torr, respectively, to atm, we get

$$p_2 - p_1 = (1/7.6)(10^{-8} - 10^{-19}) \text{ atm}$$

$$= -10^{-8}(1 - 10^{-11})/7.6 \text{ atm} .$$

Examining $\log_e \left(k_{p_1}/k_{p_2}\right)$ in orders of magnitude, we get

$$\log_e \left(k_{p_1}/k_{p_2}\right) \approx -10^5(10^{-9}) = -10^{-8}$$

from which we get

$$e^{-0.00000001} \approx k_{p_1}/k_{p_2}$$

so that the ratio of k_{p_1}/k_{p_2} is very close to 1. Thus, we conclude that, although there is a great variation between 10^{-6} and 10^{-17} torr, from a kinetic viewpoint, dry-heat survivor data obtained at 10^{-6} torr should not vary greatly from those taken at 10^{-17} torr.

3. Applications. Having an approximation for $\Delta V^{\ddagger}$, pressure is easily incorporated as an environmental parameter in a kinetic sterilization model. One must be careful, however, in using the formula $\Delta F^{\ddagger}/RT = (\Delta H^{\ddagger} - T\Delta S^{\ddagger} + p\Delta V^{\ddagger})/RT$. Since $\Delta V^{\ddagger}$ is in cc/deg-mole, $\Delta S^{\ddagger}$ in cal/deg-mole, and $\Delta H^{\ddagger}$ in cal/mole, the gas constant R used with $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ should be in cal/deg-mole; while, with $\Delta V^{\ddagger}$, it should be in cc-atm/deg-mole.

It follows that the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values computed by Silverman, from the point of view expressed here, actually include $p\Delta V^{\ddagger}$. After $\Delta V^{\ddagger}$ has been determined, it is possible to compute corrected values for $\Delta H^{\ddagger}$, assuming $\Delta S^{\ddagger} = \Delta S_0^{\ddagger}$. For the Silverman data,

$$\Delta H_0^{\ddagger} = 33.087 \text{ kcal/mole}$$

and, for the Koesterer data,

$$\Delta H_0^{\ddagger} = 24.545 \text{ kcal/mole} .$$

Using these corrected values for $\Delta H^{\ddagger}$ and the previously determined $\Delta V^{\ddagger}$'s, one is in a position to examine D values as a function of pressure. Table 1 shows comparison of D values computed for Silverman's and Koesterer's data at a temperature of 125°C and for pressures from 1.3×10^{-11} to 4 atm. (It was found in the computation that, in the pressure range below 10^{-8} torr computer roundoff becomes dominant. However, in view of the preceding kinetic analysis on 10^{-6} versus 10^{-17} torr, this is not considered important.)

It is also of interest to examine D values at a low pressure as a function of temperature. Table 2 shows a comparison of calculated D values for Silverman's and Koesterer's data as a function of temperature at 1.3×10^{-11} atm.

The relationship between D value and temperature for fixed $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are shown for the various $\Delta V^{\ddagger}$'s obtained both theoretically and from observations as shown in Figure 3.

TABLE 1

Calculated 125°C D-Values as a Function of Pressure*

Pressure (atm)	D-Values in Minutes, Based on Silverman's Data	Koesterer's data
1.3×10^{-11}	4.3	35.5
0.2	6.3	40.2
0.4	9.2	45.5
0.6	13.5	51.4
0.8	19.8	58.2
1.0	29.0	65.8
1.4	62.3	84.3
2.0	196.4	122.0
2.4	422.3	156.1
3.0	1330.7	226.1
3.4	2860.3	289.3
4.0	9014.1	418.9

*All other environmental parameters are assumed constant.

TABLE 2

D-Values Calculated for a Pressure of 1.3×10^{-11} atm = 10^{-8} Torr as a Function of Temperature*

Temperature (°C)	D-Values in Days, Based on Silverman's Data	D-Values in Days, Based on Koesterer's Data
0	902,643.0	52,327.0
10	100,920.0	10,227.0
20	13,088.0	2,232.0
30	1,940.0	538.0
40	324.6	142.0
50	60.6	40.6
60	12.5	12.5
70	2.8	4.1
80	0.69	1.45
90	0.18	0.537
100	0.052	0.21

*All other environmental parameters are assumed constant.

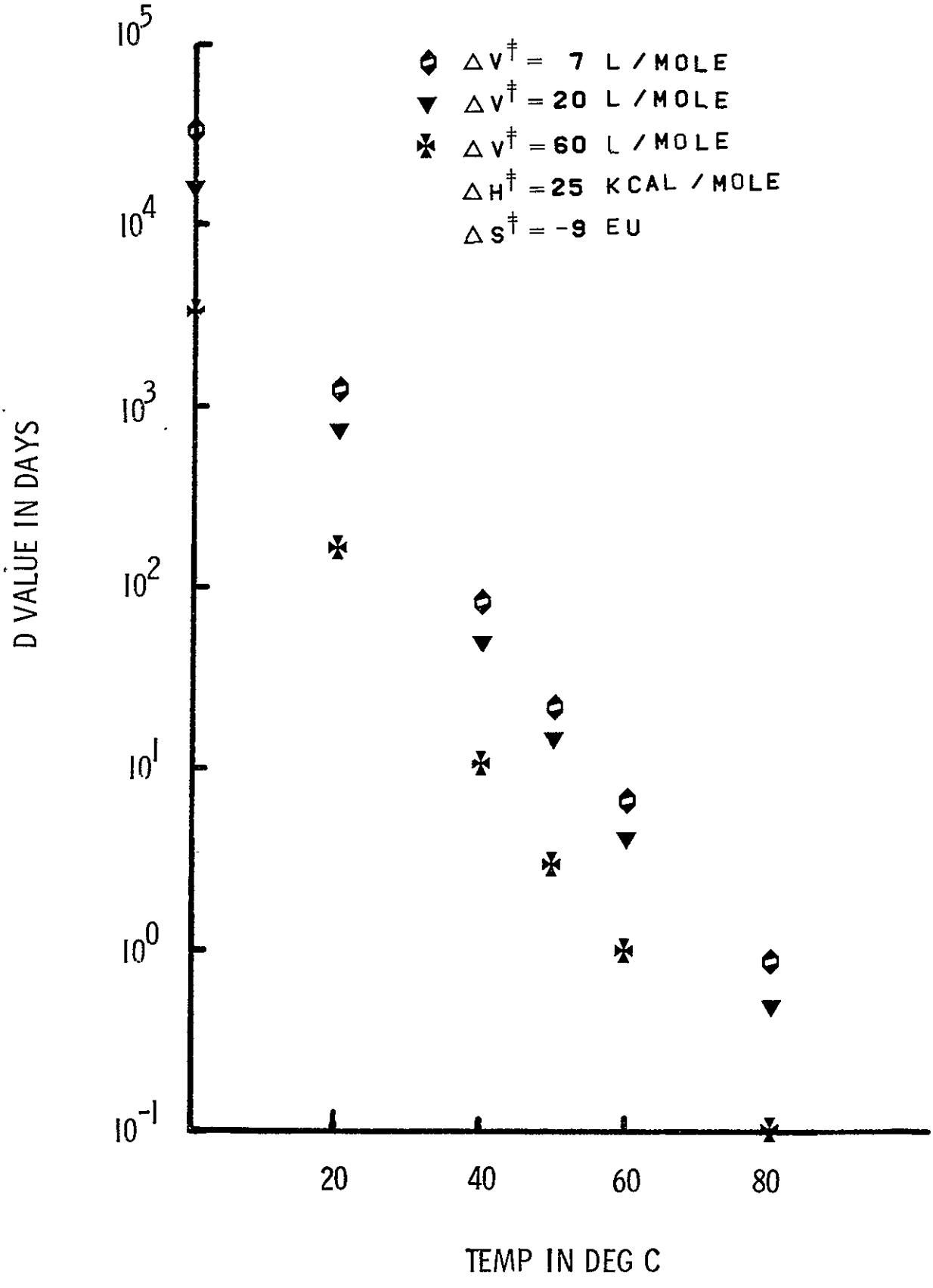

Figure 3. D value as a function of temperature for fixed $\Delta H^{\ddagger}$ = 25 kcal/mole and $\Delta S^{\ddagger}$ = -9 eu for various $\Delta V^{\ddagger}$'s.

4. Conclusions. The primary purpose of this analysis is the presentation of a model and a technique for considering pressure as an environmental parameter in dry-heat sterilization. The outcome of such calculations is entirely dependent on the input data, and the data used for this analysis were not generated with this analysis in mind. Consequently, one should hesitate to draw firm conclusions from this study. However, the $\Delta V^{\ddagger}$ value of 7 liters/mole derived theoretically for DNA is within an order of magnitude of the 15- to 65-liters/mole values computed for Silverman's and Koesterer's observations with Bacillus subtilis. Under these conditions, the agreement is good. The fact that the lower $\Delta V^{\ddagger}$ value was obtained for DNA raises the question of what else in the microbe could lead to a greater $\Delta V^{\ddagger}$ value. We then are led to agree with Wax [42] that, in the sterilization of Bacillus subtilis by heat, we are likely observing effects on DNA in vivo.

The input data for this analysis were dry-heat sterilization data. From these, it followed that D values increased with pressure. Within limits, it has been found that this is true in solutions under hydrostatic pressure [24].

An examination of Table 2 suggests that a significant decrease in a spacecraft's external bioburden can occur in outer space, provided the temperature can be maintained at above 50°C.

It is our belief that this analysis of pressure effects is far from complete, but we do think it can be useful in pointing out several areas for profitable investigation.

C. DNA, "Tails," and Wet-Heat Sterilization*

For years, there has been a controversy among microbiologists concerning the shape of survivor curves in various sterilizing environments [44, 45, 46]. Roughly, the question is: If the logarithm of survivors is plotted as a function of time of exposure to a sterilizing environment, then is the curve which is obtained a straight line? Although most experiments do result in a straight line, especially if one plays the confidence-limit game, there are enough results constantly appearing in the literature [4, 46, 47, 48, 49, 58] to keep the question open. These exceptional curves generally fall into two classes. In the first class are those curves which exhibit a lag phase, followed by a linear phase; in the second class are those which are concave in appearance. Simple rational explanations have long been available for curves in the first class; namely, multihit theories and redundancies in vital components [4, 5]. Those in the second class are actually the most controversial. The most common explanation is that the population being sterilized is a mixture of two or more populations having different levels of resistance to the sterilizing environment [46, 49, 50]. If this is the case, one should be able to cultivate, from a resistant strain, a population more resistant than the least resistant strain in the parent stock. It is the case, however, that the results from such experiments have been inconclusive [45, 51]. In some cases, it has been found that the population had indeed been nonhomogeneous, while in others, the stock cultivated from a resistant parent did not inherit a high degree of resistance.

*Most of the material in this section appeared in [43].

In this section, we show that it is quite likely that both factions of the dispute are in large measure correct. In so doing, we also present supportive evidence for the previously mentioned thesis by Wax [42] that, in the sterilization of Bacillus subtilis by heat, we are observing the action of heat on DNA in vivo.

1. Assumptions. The key to our analysis is the observation that, in a genetically homogeneous population, it does not follw that the DNA is chemically homogeneous. For genetic homogeneity, all that is required is that any two distinct DNA molecules replicate the same. Chemical homogeneity, on the other hand, requires that the structure, bonding, etc., of all molecules be the same.

   In 1968, Alberts and Doty [52] discovered that approximately 6% of a certain sample of B. subtilis DNA contains "naturally occurring" cross-linkages between complementary strands. (The authors used the term "naturally occurring" to denote linkages which appear to be intrinsic to DNA preparations from normal sources, and implied no commitment as to the origin of the cross-linkages.) Let us assume that some fraction of these cross-linkages are natural to a given genetically homogeneous B. subtilis population, and let us further assume that B. subtilis spores are sterilized in wet heat by the denaturation of DNA. This is in agreement with the Wax conclusion that the sterilization mechanism for a certain thymine-requiring strain of B. subtilis is probably genetic damage [42]. These rather strong assumptions are utilized for simplicity of analysis, and should not be taken as a belief that, in a sterilizing environment,

a single mechanism sterilizes all organisms. In fact, we will be very much surprised if it is ever shown that this is a fundamental principle of microbial sterilization. Also, we will assume that each organism in our population has two DNA molecules. The work of Yoshikawa [53] indicates that this is not too far out of line. At any rate, if we are off here by a unit either way, the net result in the analysis will be evidenced in the presence or absence of an initial lag phase. Finally, to complete the assumptions necessary for modeling, the sterilization mechanism, denaturation of DNA, will be by first-order kinetics.

2. Model. Under the above assumptions, using the kinetic models of [2], the probability that a given spore is viable at time t, p(t), is given by

$$p(t) = 1 - [1 - q(t)]^2 , \tag{4}$$

where q(t) is the probability that a given DNA molecule is "active" at time t.

Taking rough measurements from a graph of Alberts and Doty [52], we find that the fraction of B. subtilis DNA transforming activity after 1 minute in 0.1μ-potassium phosphate, 0.015μ-NaCl (pH 7.0) at 100°C is approximately 0.15. Under first-order kinetics, we approximate the denaturation rate constant k_1 by the formula

$$dC/dt = -k_1 C(t)$$

so that

$$C(t)/C(0) = e^{-k_1 t}$$

Since we are dealing in single molecules, we will interpret C (1 min) to be 0.15, with C(0) = 1; therefore,

$$0.15 = e^{-k_1}$$

so that $-k_1 = \frac{\ln 0.15}{\text{min}} \doteq -3.16 \times 10^{-2}/\text{sec}$, which is the denaturation rate constant for the less resistant fraction of the sample used by Alberts and Doty.

From the same graph we find that, after 3 minutes, the surviving fraction is approximately 2×10^{-2}. Assuming that a negligible portion of the less resistant fraction is still active and that the minor resistant fraction was initially 6% of the total, we find, in the same manner as above, that this fraction has a denaturation rate constant

$$k_2 = 6.1 \times 10^{-3}/\text{sec} .$$

Now, we construct a genetically homogeneous but chemically heterogeneous, relative to DNA, population and examine the expected survivor curve. We shall assume that 6% of the DNA in our population has denaturation rate constant at 100°C of 6.1×10^{-3}/sec, and that 94% has a denaturation constant at 100°C of 3.16×10^{-2}/sec. Since we have, in our model, two molecules of DNA, we will for simplicity assume that 88% of our population has two of the less resistant molecules and that the remaining 12% has one resistant molecule and one from the major fraction. Under these conditions, the expected fraction of survivors from the initial population N(0), after exposure to 100°C wet heat for time t sec, is given by N(t)/N(0), where

$$N(t) = N_1(t) + N_2(t) ,$$

with $N_1(t)$, the "normal" fraction, given by

$$N_1(t) = \left[1 - \left(1 - e^{-k_1 t}\right)^2\right] N_1(0) \qquad (5)$$

and $N_2(t)$, the resistant fraction, given by

$$N_2(t) = \left[1 - \left(1 - e^{-k_1 t}\right)\left(1 - e^{-k_2 t}\right)\right] N_2(0) . \qquad (6)$$

In accordance with our proportioning of the fractions, we set

$$N_1(0) = 0.88\, N(0) \text{ and } N_2(0) = 0.12\, N(0) .$$

Note that Equation (5) is an application of Equation (4), with q(t), the probability that a given molecule is active at time t, taken to be the ratio of concentration at time t to the initial concentration, given that the substance is degraded by a first-order reaction with reaction constant k_1. Equation (6) is an obvious generalization of these comments to the heterogeneous case.

Figure 4 shows the ratio N(t)/N(0) from our fabricated population. It has been recommended by Pflug and Schmidt [49] that, for experimental data of this type, a linear regression analysis should be given for the second linear portion of the curve, and the "apparent initial number" of organisms be recorded. Therefore, to observe our fabricated population's response to this analysis, we have shown a dashed-line "fit" to the second linear portion of the curve.

To note the effect of different ratios of $N_1(0)$ to $N_2(0)$, we have provided, in Figure 5, the survivor curves which result if $N_1(0) = 0.999\ N(0)$ and $N_2(0) = 0.001\ N(0)$, and $N_1(0) = 0.99\ N(0)$ and $N_2(0) = 0.01\ N(0)$.

3. DNA in Wet-Heat Sterilization. The DNA extraction of Alberts and Doty was from logarithmically growing cells. Thus, it is safe to assume that the DNA obtained was from vegetative cells. It is also the case that differences are to be found in Bacillus subtilis DNA, depending on whether it is extracted from spore or vegetative cells [54, 55, 56]. However, there are enough similarities to

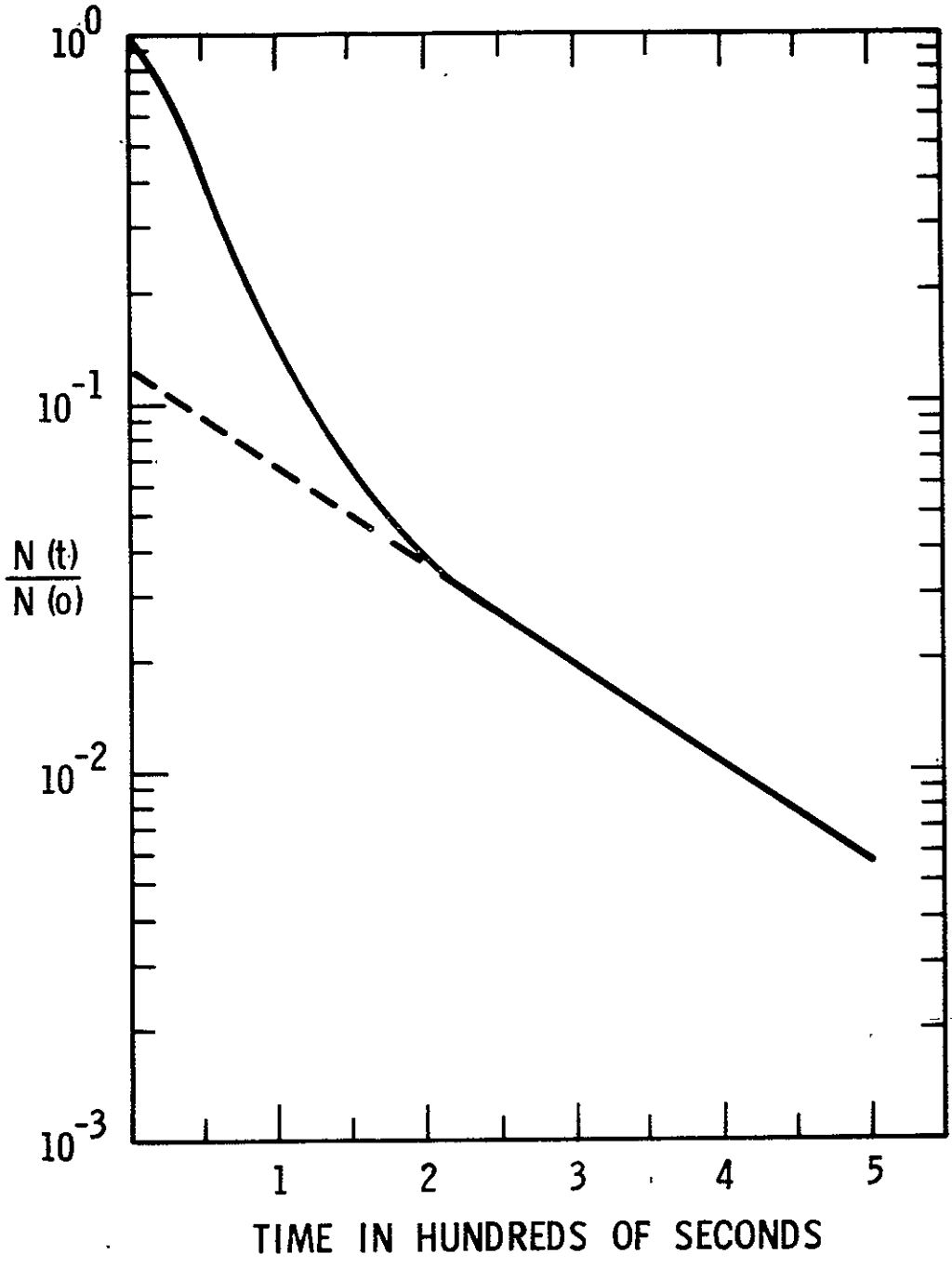

Figure 4. Hypothetical survivor curve. 88% of the population is inactivated as per Equation (2) and 12% per Equation (3). The dashed line gives the "apparent initial number."

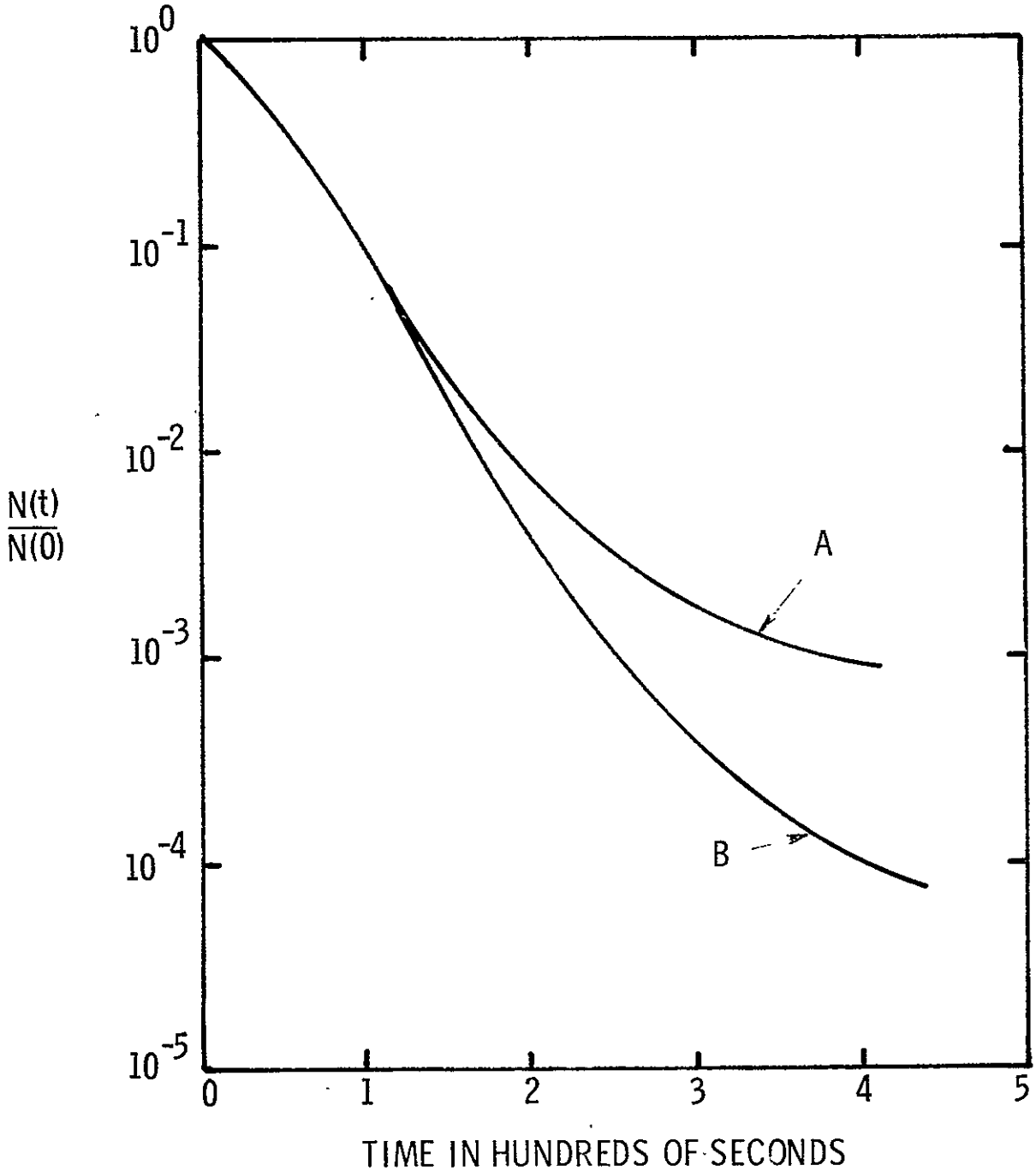

Figure 5. Hypothetical survivor curves. Curve A 99% of the population is normal and 1% resistant. Curve B 99.9% of the population is normal and 0.1% is resistant.

prompt an attempt at correlating expected survivors from a population being sterilized by DNA denaturation with observations.

Licciardello and Nickerson [57] gave survivor curves for B. subtilis var. niger in phosphate buffer (pH 7.0) at temperatures of 105°C and 95°C. Since the in vitro DNA denaturation was at 100°C, the in vivo data given at 95°C and 105°C must be converted to 100°C. Therefore, an Arrhenius plot was made to determine the approximate time for the linear portion of the 100°C survivor plot to diminish by 90%. It is extremely difficult to achieve any precision using published curves for such purposes; therefore, we simply state that the time required for the 90% decrease is between 60 and 90 seconds. In addition, Wax [42] provided an Arrhenius plot for the sterilization of the thymine-requiring B. subtilis strain in wet heat. From this plot we get a reaction-rate constant of approximately 2.5×10^{-2}/sec.

Neither the curves of Licciardello and Nickerson nor the curves of Wax exhibited a sigmoid pattern when plotted in the usual form, log [N(t)/N(0)] versus time, so we will not mix our population. Instead, we will use only Equation (5). However, we will compute two curves using the values of 3.16×10^{-2}/sec and 6.1×10^{-3}/sec for k_1. Thus, we compare the observed data with predictions based on the assumption that denaturation of DNA is the principle mechanism of wet-heat sterilization with rates of denaturation as determined from the data of Alberts and Doty. This comparison is shown in Figure 6. The solid curves are from the in vitro DNA data. Curve A was generated from

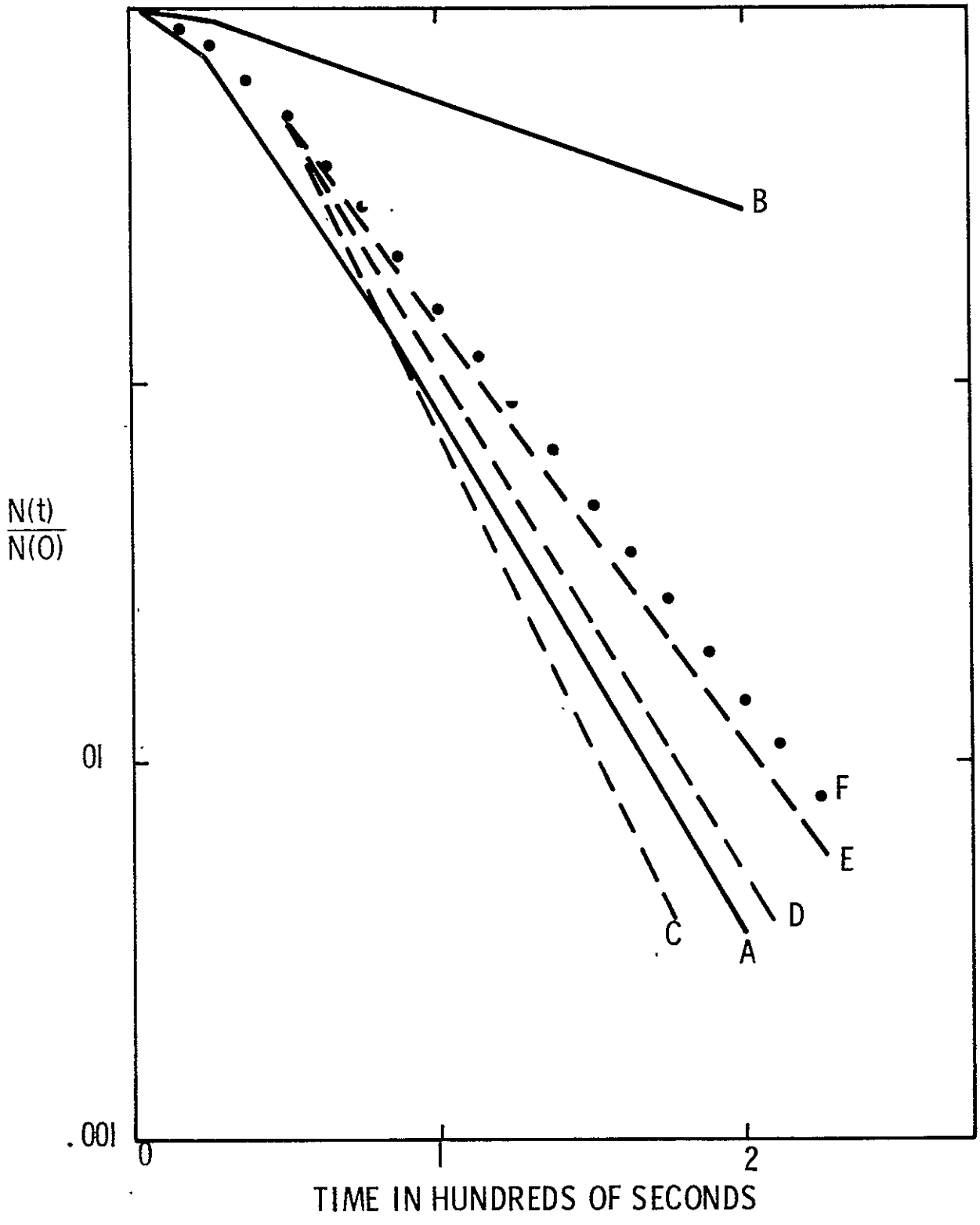

Figure 6. Comparison of survivors (Licciardello and Nickerson--dashed lines; Wax--dotted curve) to hypothetical populations inactivated according to the denaturation of DNA. Curve A is from the normal DNA fraction and curve B is from the resistant fraction.

Equation (2) with reaction-rate constant 3.16×10^{-2}/sec, and curve B was generated from Equation (6) with one "normal" molecule and one "resistant" molecule. Although the in vivo data of Licciaradello and Nickerson showed a pronounced lag phase, for simplicity we have given only the projected linear portions in Figure 6. The dashed lines represent these predictions. Time for 90% sterilization for curves C, D, and E are 60, 75, and 90 seconds, respectively. The dotted curve, F, is from Wax [42], using the 100°C reaction-rate constant.

4. Discussion and Conclusions. If it will be allowed that genetic homogeneity and DNA chemical homogeneity of a B. subtilis population are not necessarily equivalent, then the anomaly of nonlogarithmic death of seemingly homogeneous populations can be explained by variations in energy requirements for DNA denaturation. (The nonlinearity of the curves in Figures 4 and 5 are obvious.) This is established by relating the probability of single B. subtilis spore survival at a given time t to the probability that a given DNA molecule is "active" at time t. The relationship utilizes in vitro DNA denaturation data to determine the reaction-rate constants, and a mathematical model to couple the survival of B. subtilis to the "activity" of DNA. This is, of course, based on the assumption that the principal mechanism of wet-heat sterilization of B. subtilis is via DNA denaturation.

The analysis is meaningless unless a close correlation can be established between wet-heat sterilization of B. subtilis and DNA denaturation. This point was investigated by comparing in vivo data

for wet-heat sterilization to expected survivor curves obtained by using *in vitro* DNA denaturation data. It is interesting to observe the similarities in Figure 6 of the slopes of curves A, the *in vitro* data based curve, and curve D, one of the *in vivo* data based curves. This provides support for the conclusion of Wax that, in the sterilization of *B. subtilis*, we are likely observing the effect of heat of DNA *in vivo*.

Section III

Model Verification Experiments

In the absence of accepted standards for sterilization modeling, we have challenged the kinetic model of [2] on the basis of agreement with "acceptable" data. Since there is no criterion for ascertaining which data are "acceptable," it has been necessary that we make subjective judgments regarding data acceptability. The primary question here concerns the acceptability of nonlogarmithmic data. Our concern with nonlogarithmic data follows from the fact that the kinetic model and logarithmic data are trivially compatible. However, it was required that a number of microbiological tests be carried out to establish that the nonlogarithmic data were truly nonlogarithmic. Thus, our experiments in model verification cover a broad area of the intersection of the mathematical, chemical, and biological sciences.*

Through fortuitous circumstances, Mr. Statt was able to cultivate a spore stock of B. subtilis var. niger which exhibited both a "shoulder" and a pronounced "tail" in dry-heat sterilization (Figure 7). This spore stock enabled us to conduct a variety of model verification experiments. In addition to the stock cultivated by Statt, hereafter called UNM (University of New Mexico) stock, it was observed that a slight tail resulted from a B. subtilis stock obtained from the U. S. Biological Center at Fort Detrick, Maryland, hereafter called Fort Detrick stock. Most of the nonlogarithmic validation tests were run on this stock

*The microbial work pertaining to these model verification experiments was carried out by Mr. R. H. Statt of the University of New Mexico.

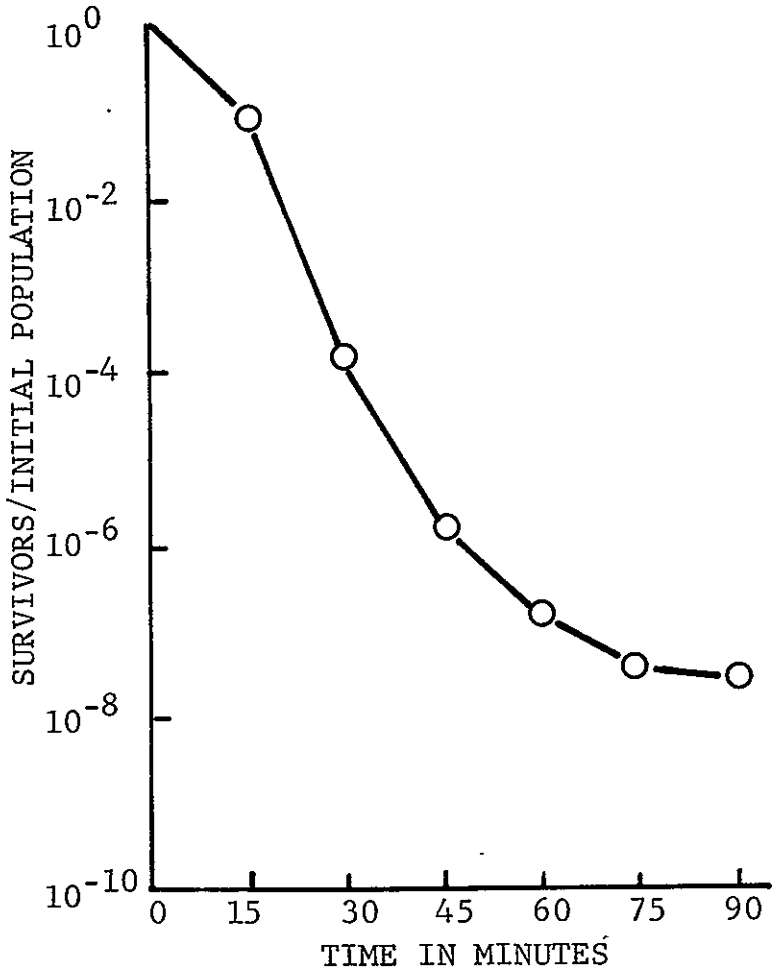

Figure 7. Tailing stock with shoulder.

as well, and the same conclusions were reached as with the UNM stock. (These experiments were carried out during the years 1968-1969. Concurrently, similar experiments were being carried out by the Cincinnati Research Laboratories of the Food and Drug Administration. They have recently found [58] similar nonlogarithmic survival for B. subtilis.) However, as indicated above, it was first necessary to verify that this nonlogarithmic survival was indeed nonlogarithmic.

A. Verification of Nonlogarithmic Stock

The first potential causative factor that was investigated concerned the rate at which the samples were cooled on removal from the oven. Since the rate of cooling has an effect on renaturation of DNA [59, 60], and DNA denaturation is a prime candidate for being one of the principal sterilization mechanisms [42, 43], we reasoned that rapid cooling of the samples would simplify the analysis by minimizing the effect of renaturation, as is done with in vitro DNA studies [60]. The survival curve shown in Figure 6 was obtained in this manner. To settle the question of whether the cooling rate was responsible for the "tail," the test was repeated; the only change being the rate at which the samples were cooled. The results are shown in Figure 8. Although the rapid cooling tended to depress the "tail," the "tail" was still present when the samples were allowed to cool slowly. We then concluded that the "tail" did not result from the rapid cooling procedure. These experiments were carried out with the UNM stock.

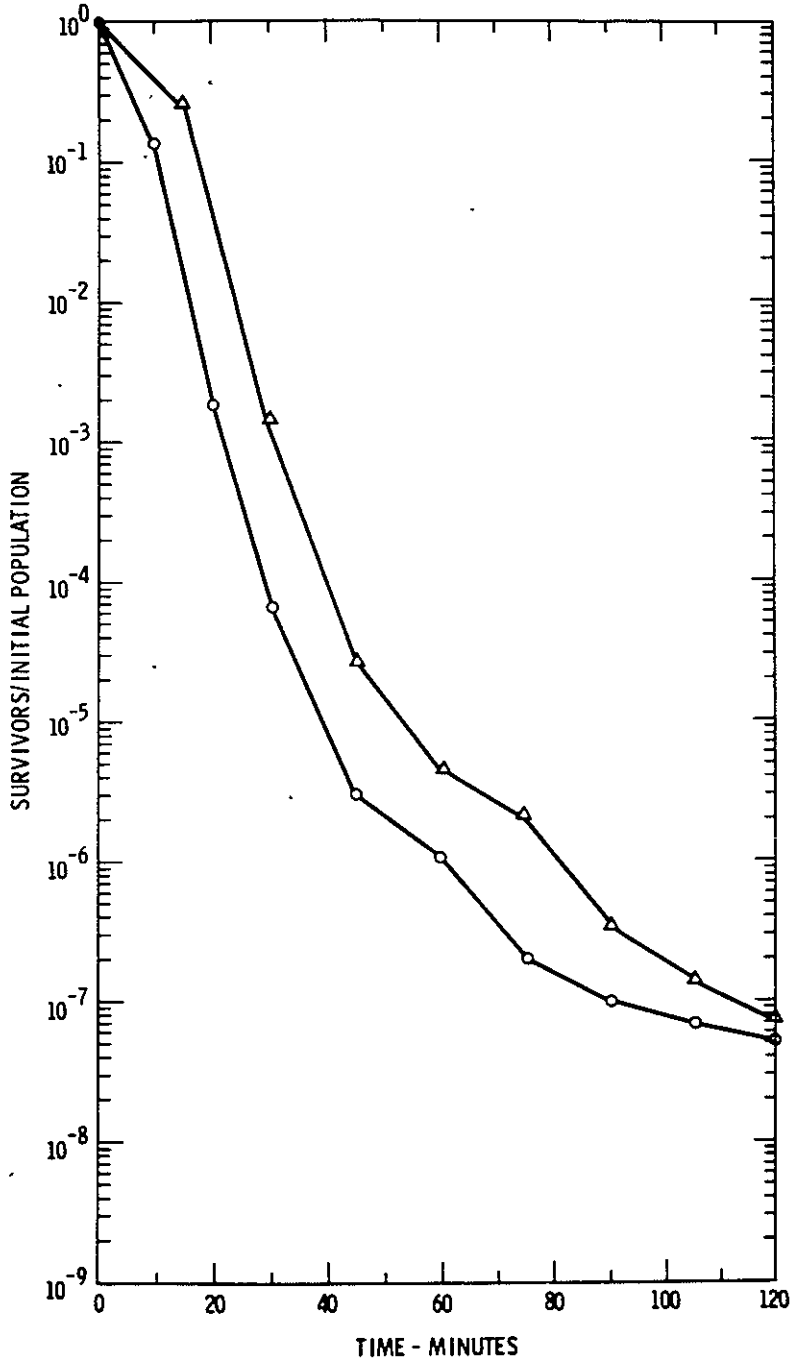

Figure 8. Comparison of rapid sample cooling, Δ, to slow sample cooling, O, for UNM B. subtilis stock. Temperature = 135°C.

The rapid versus slow cooling experiments raised the question of what happens if the samples are cooled slowly after an extensive exposure to the sterilizing environment. This was investigated in an experiment in which two sets of samples were exposed to 135°C. At the end of 1 hour, one set was cooled to ambient in 15 sec and the other cooled to ambient in 4 to 5 min. The samples were then reinserted in the oven for continued exposure. The results are shown in Figure 9. The time of exposure is the time that samples were in the 135°C environment. Our first observation is that the set of rapidly cooled samples continues to follow the previously observed curve, while the slowly cooled samples tend to reproduce the nontailing portion of the survivor curve. This indicated that the tailing portion of the survival curve was a function of the treatment history from the time of introduction to the oven to the time at which the cooling differences occurred. This, in turn, suggested that a genetic inhomogeneity was not a causative factor in the tailing.

To further investigate the genetic aspect of the tailing stock, innoculums were taken from the hardiest colonies, i.e., from colonies which were grown from survivors of the most extreme exposure. It was expected that, if a genetic inhomogeneity is involved in the tailing survivor curve, then stock cultivated from this "different" organism would produce a survivor curve roughly parallel to the tailing portion of the curve. Survivor curves were then obtained from this stock and the original simultaneously. From this it was found (Figure 10) that the new stock had a logarithmic survival-curve, while the survivor curve for the original stock was unchanged. From this, we draw three conclusions:

1. The original stock was genetically homogeneous.

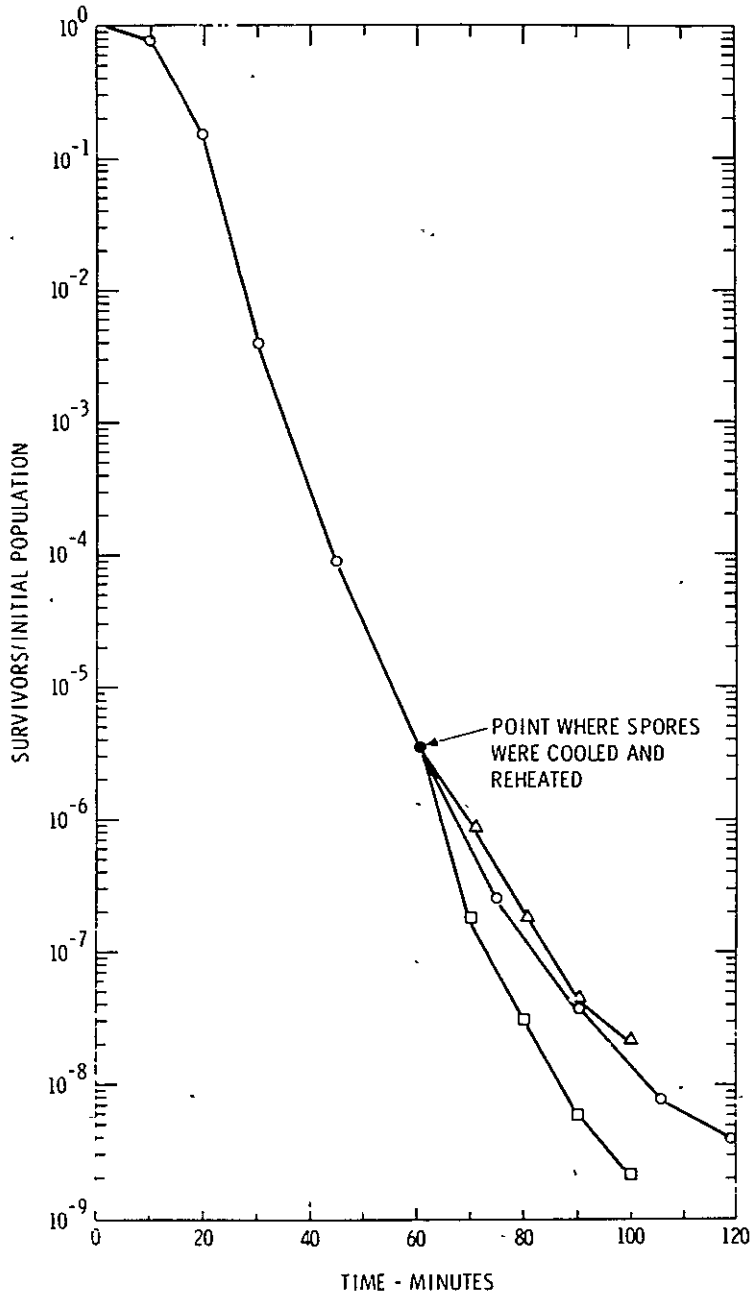

Figure 9. Removal of "tail" from Fort Detrick B. subtilis stock by slow cooling and reheating.
T = 135°C. □ = slow cool reheat.
Δ = rapid cool reheat. O = normal.

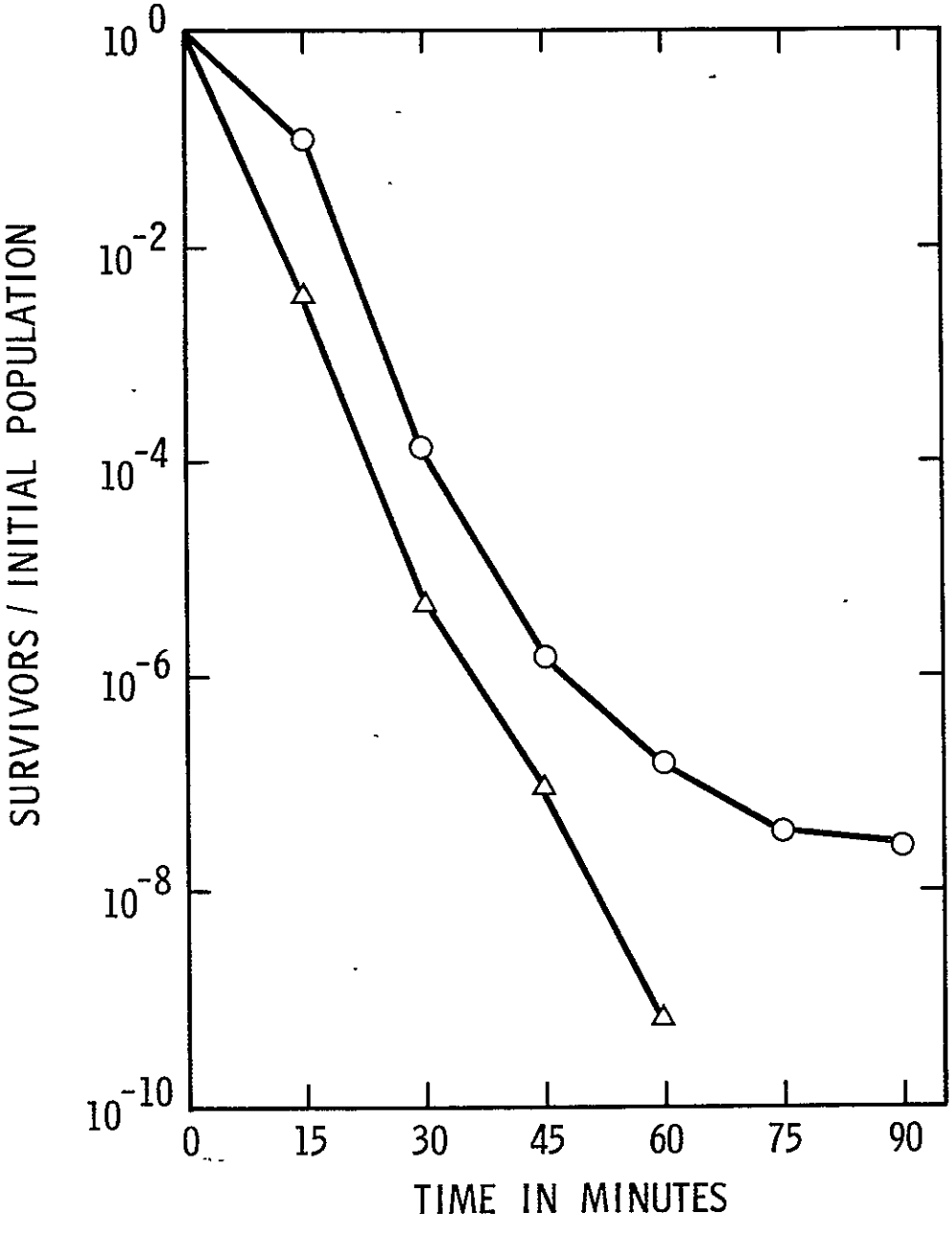

Figure 10. Population homogeneity tests.
○ = UNM B. subtilis stock.
△ = daughter stock cultivated from 90 min survivors.
T = 135°C.

2. Water activity is not a primary factor in the shape of the survivor curve of the original stock, since preconditioning of both stocks was the same.
3. The tailing effect was not the result of faulty laboratory technique.

There are intriguing subtleties pertaining to our first conclusion. In particular, as discussed in Section IIC, the genetic homogeneity of the original stock does not imply that it was chemically homogeneous.

It has long been known [61] that "clumping" of spores theoretically has an effect on the survivor curve, in that, if a clump is indistinguishable from a single organism, a clump is permanent, and deaths of individuals in a clump are independent; the survivor curve will then have a shoulder. Since our original nonlogarithmic stock had a shoulder, laboratory experiments were undertaken which were designed to test whether clumping had a significant effect on our survivor curves. This experiment involved the construction of an electrostatic deposition device which deposited spores on aluminum strips in a roughly uniform manner, so that spore-to-spore contact was minimized [62]. As can be seen in Figure 11, the effect of clumping on the survivor-curve shoulder was minimal, while the tail of the monolayered, nonclumped spores was depressed 1-1/2 to 2 log cycles over 9 logs, indicating some protection from close packing of spores. However, in the monolayered case, a tailing effect in the survivor curve is observed.

It was of interest in these experiments to use populations as large as feasible. This was based primarily on a concern with unnecessary extrapolation for planetary quarantine requirements and, in part, with our

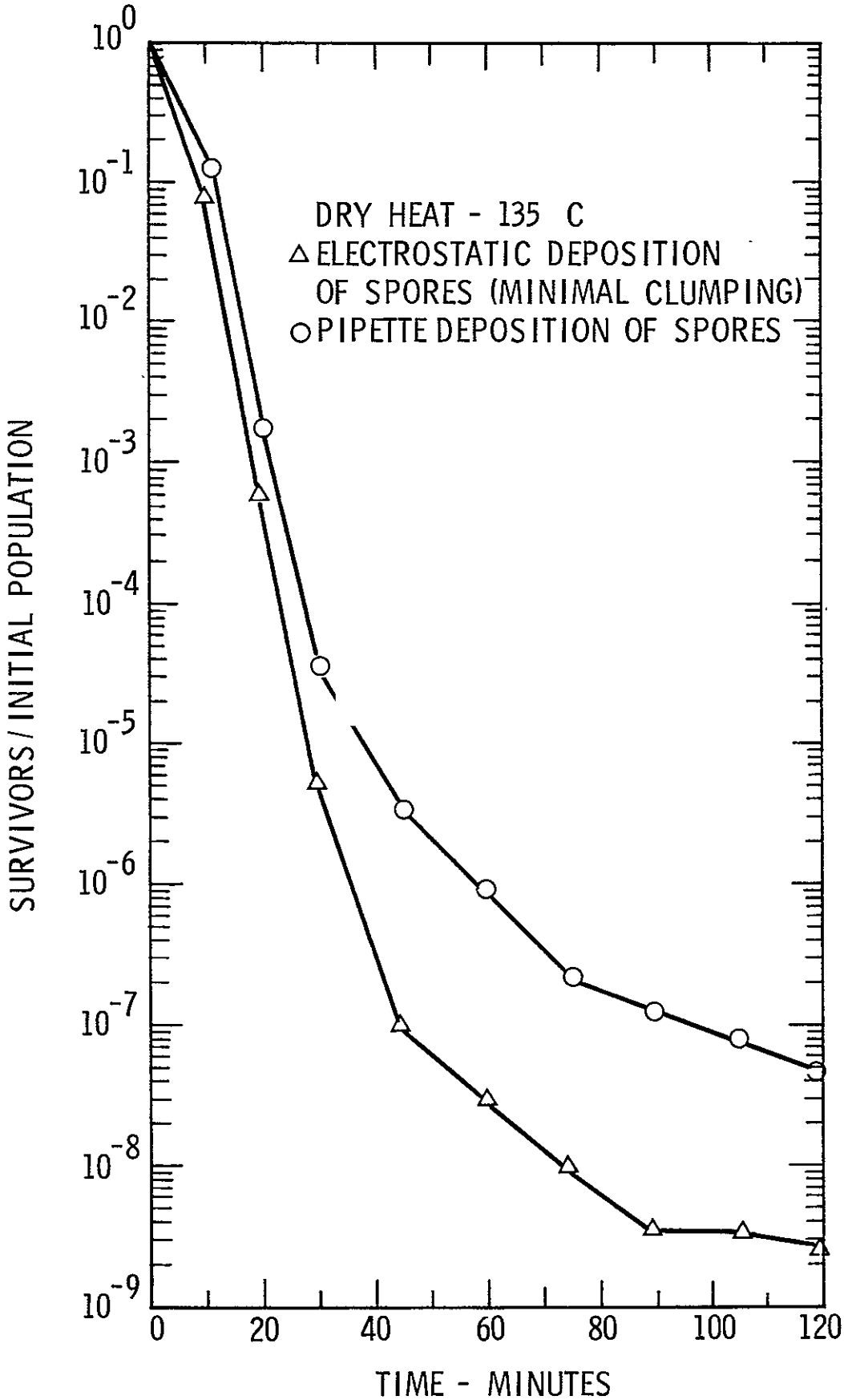

Figure 11. Effect of spore clumping on dry-heat inactivation, UNM stock.

desire to extract maximum information from each experiment. The monolayer-clump experiments did show that close packing of spores on sample strips did impart a degree of protection to the population. This effect was investigated in a series of experiments in which sample strips were loaded in the clumped mode at levels of 1×10^9, 1×10^{10}, and 8×10^{10}.

Survivor curves for these loadings are shown in Figure 12. It was decided, on the basis of these curves and those of Figure 11, that a sample loading at 1×10^9 would provide conservative survivor curves suitable for model verification purposes.

B. Model Base-Line Parameters

To establish base-line parameters utilizing either the Arrhenius equation or absolute reaction-rate theory for relating rate constants to temperature, it is theoretically sufficient to have survivor curves obtained at only two temperatures. Since our concern is with model validation, survivor curves for three temperatures were used as follows: Base-line parameters were obtained from two of the temperatures, and these parameters were used in the model to predict the third survivor curve. In obtaining a "best" fit, the parameters were subject to constraints of physical

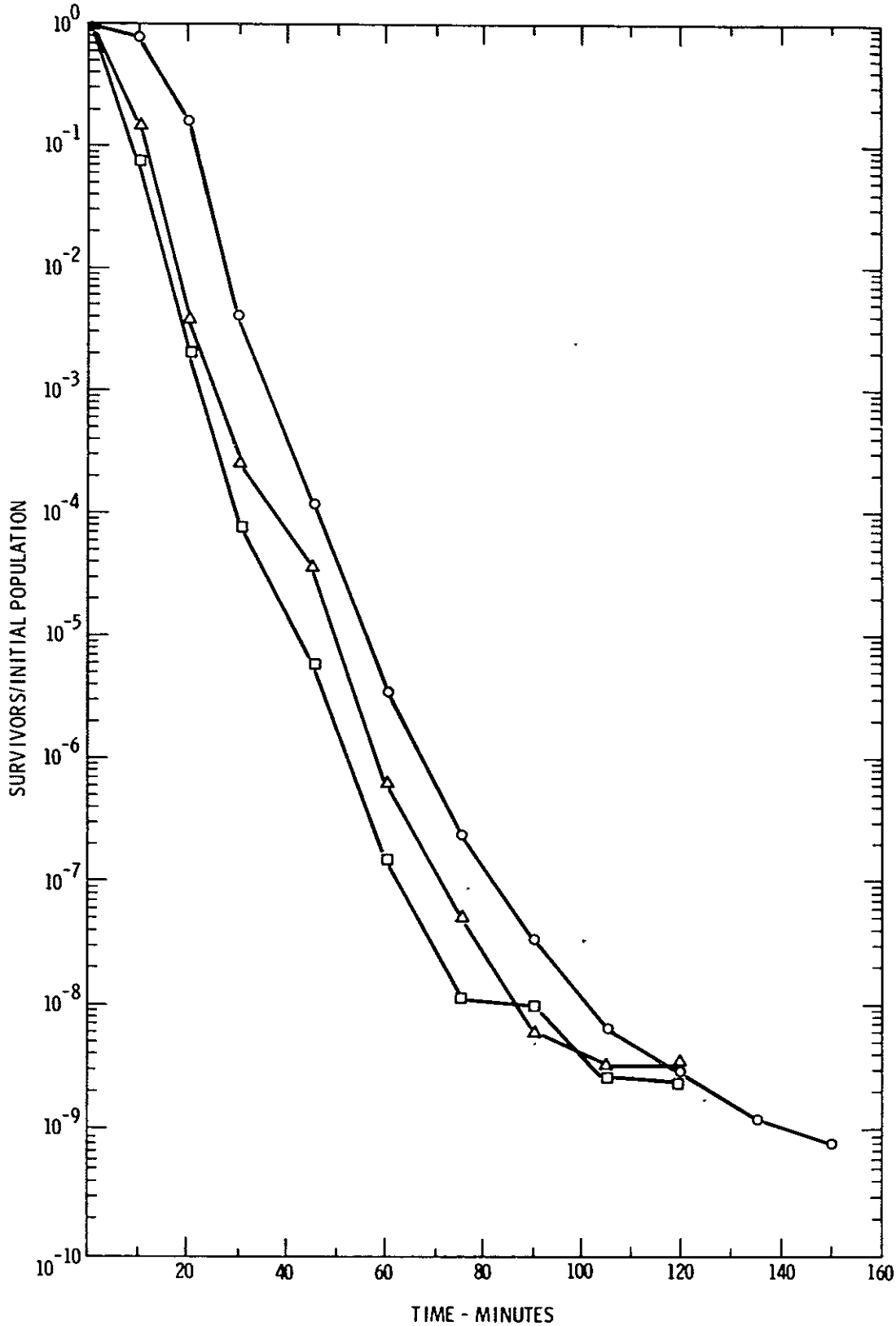

Figure 12. Effect of initial loading on Fort Detrick *B. subtilis* stock. Initial loads:

○ = 8×10^{10}, △ = 1×10^{10}, and

□ = 1×10^{9}. T = 135°C.

chemistry as well as "unit rule" constraints. Unit-rule constraints follow from the fact that the numbers of systems and subsystems must be integers. Thus, the procedure used is distinct from the simpler task of obtaining a "best" fit for a given model by varying the parameters.

1. University of New Mexico Stock. The ratio of observed survivors to initial population for 125°C, 135°C, and 145°C are shown in Figure 13 for the UNM stock by squares, X's, or circles. The solid curves in Figure 13 for 125°C and 135°C represent the fit obtained from the equation

$$p(t) = \left\{1 - \left[1 - \left(k_1 e^{-(k_1+k_{-1})t} + k_{-1}\right)^2\right]\right\} \times \frac{1}{1 + 50k_2 t} \sum_{j=6}^{50} \binom{50}{j} \left(50k_2\right)^{50-j} \tag{7}$$

by varying the thermodynamic parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the rate constants k_{-1}, k_1, k_2. The integers 2, 50, and 6 appearing in the equation were obtained by varying the number of first-order systems, second-order systems, and the number of second-order systems necessary for viability for the reactions

$$\begin{aligned} A &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \\ 2C &\xrightarrow[k_2]{} D \ , \end{aligned} \tag{8}$$

consistent with the earlier version [2] of the kinetic model.

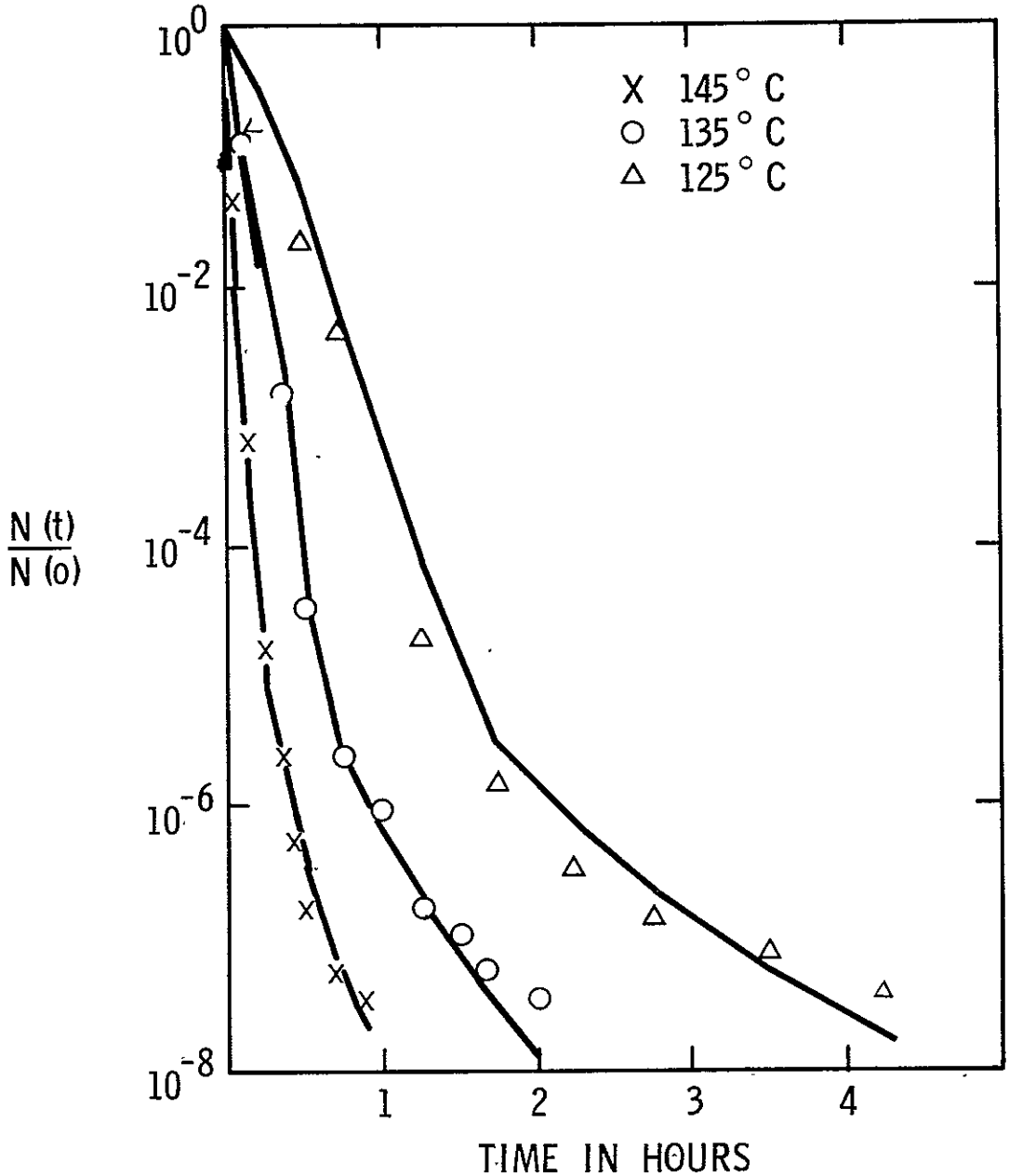

Figure 13. Base-line parameter fits for UNM B. subtilis stock. Fits were made to 125°C and 135°C data to predict 145°C curve. The X's, O's, and Δ's represent observations. The solid curves represent fits and prediction.

The parameter constants obtained for the 125-135°C fits were then used with the absolute reaction-rate equation, Equation (1), obtain the prediction for 145°C.

It is of interest to compare the values obtained for enthalpies and entropies of activation for the hypothetical reactions, Equations (8), to in vitro observations for known cell constituents. Comparisons of the values obtained for the rate constants k_{-1}, k_1, and k_2 with laboratory data for RNA, DNA, and various enzymes from Ginoza et al. [63], Bacher and Kauzmann [64], Rice and Doty [65], Guild and van Tubergen [66], and Johnson et al. [5] are given in Table 3. Any conclusions drawn from such comparisons should be tempered by the recognition that the assumption has been made that the reactions of Equations (8) accurately describe the sterilizing event. It would be surprising indeed if these reactions accurately describe sterilization, especially in view of the fact that a path has not been provided for exit from B except to A. This simplification was incorporated for our own computer experiments, since it was thought that the rapid cooling procedure would effectively allow us to monitor A for spore viability.

With the proper disclaimers in mind, we will offer the following observations about Table 3:

(1) Independent of all models, including ours, it appears that the $\Delta F^{\ddagger}$ terms for most of the denaturation reactions tend to group around the same value, while $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ vary over a wide range. Thus, recording of $\Delta F^{\ddagger}$ for sterilization experiments offers little toward solving the mechanism(s) riddle. Conversely,

TABLE 3

Free Energies of Denaturation of RNA,
DNA, and Various Enzymes

	Condition	$\Delta H^{\ddagger}$ kcal/mole	$\Delta S^{\ddagger}$ eu	$\Delta F^{\ddagger}$ kcal/mole T = 398°K
Bacher & Kauzmann (RNA)	0.5 N HCL	16	-26	26
	0.01 N HCL	22	-15	28
Ginoza et al.	pH			
(ϕx 174 DNA) single strand	5.4	29	17	22
	6.7	35	22	26
	7.5	35	21	27
(R17 RNA)	5.4	29	10	25
	6.7	25	-7	28
	7.5	29	5	27
Rice & Doty				
(calf thymus DNA) double strand	--	35	63	10
		40	75	10
		93	220	16
Guild & van Tubergen (catalase)	pH			
	7 in H_2O	87	191	12
	6.85 in D_2O	145	360	2.5
Johnson et al.	pH			
(entero kinase)	--	42.2	52.8	21
(trypsin kinase)	--	44.2	57.6	21
(proteinase)	--	37.8	40.6	22
(invertase-yeast)	5.7	52.3	27.3	41
(lipase)	--	24.2	-13.0	29

Theoretical Energies from Model Fit
Illustrated in Figure 13

Rate Constant	Associated Energies $\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
k_{-1}	0.18	-63	2.5
k_1	34.4	29.1	23
k_2	21.3	-5.8	24

utilization of absolute reaction-rate theory and kinetic modeling enables a determination of values for $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and, where appropriate, $\Delta V^{\ddagger}$, which, with their wider ranges of values, offer increased opportunities for focusing on mechanism(s).

(2) The values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, associated with the various model reaction-rate constants, are well within the ranges observed by most of the DNA-RNA preparations, and for some of the enzymes.

2. Fort Detrick Stock. Base-line parameters for the Fort Detrick stock were established for the reactions

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_2} C$$

$$A \xrightarrow{k_3} D$$

$$2E \xrightarrow{k_4} F$$

by altering the computer program used for the UNM stock to accommodate these reactions. [By the time we were prepared to investigate this stock, model computerization had progressed to the point that Equations (9) could be used in lieu of Equations (8). Note that Equations (8) are a very special case of Equations (9).]

For the computer experiments on the Fort Detrick data, the reactions of Equations (9) were used. To account for repair during incubation, the reverse reaction $A \xleftarrow[k_{-1}]{} B$ was allowed to continue for 12 hours at 37°C.

Survivor curves for this stock are shown in Figures 14a, 14b, and 14c for 105°C, 125°C, and 135°C, respectively. The isolated points represent the observations, while the curves represent a computer fit which minimizes the sum of the variation from the data at all three temperature levels. The thermodynamic parameters for the reactions are given in Table 4.

The fits for these data and Equations (9) are not as good as were obtained for the UNM stock and Equations (8). Observe the excessive tailing for the model at both 105°C and 125°C. This results from the extreme tailing of the 135°C data which could be caused by experimental error. In addition, these experiments were performed at widely separated times and a_w was not controlled.

It is of interest to observe the great flexibility of the reactions of Equations (9) for analysis of microbial sterilization. As k_{-1}, k_2, k_3, and k_4 go to zero, the model goes logarithmic; while, for the parameters of Table 4, a diphasic curve results.

Our next step is the consideration of reactions involving free radicals. This is indicated from a perusal of the literature. The methods for computerizing these reactions have been worked out and are discussed in the model computerization section. After programs have been modified, the UNM and Fort Detrick data will be analyzed, using these reactions.

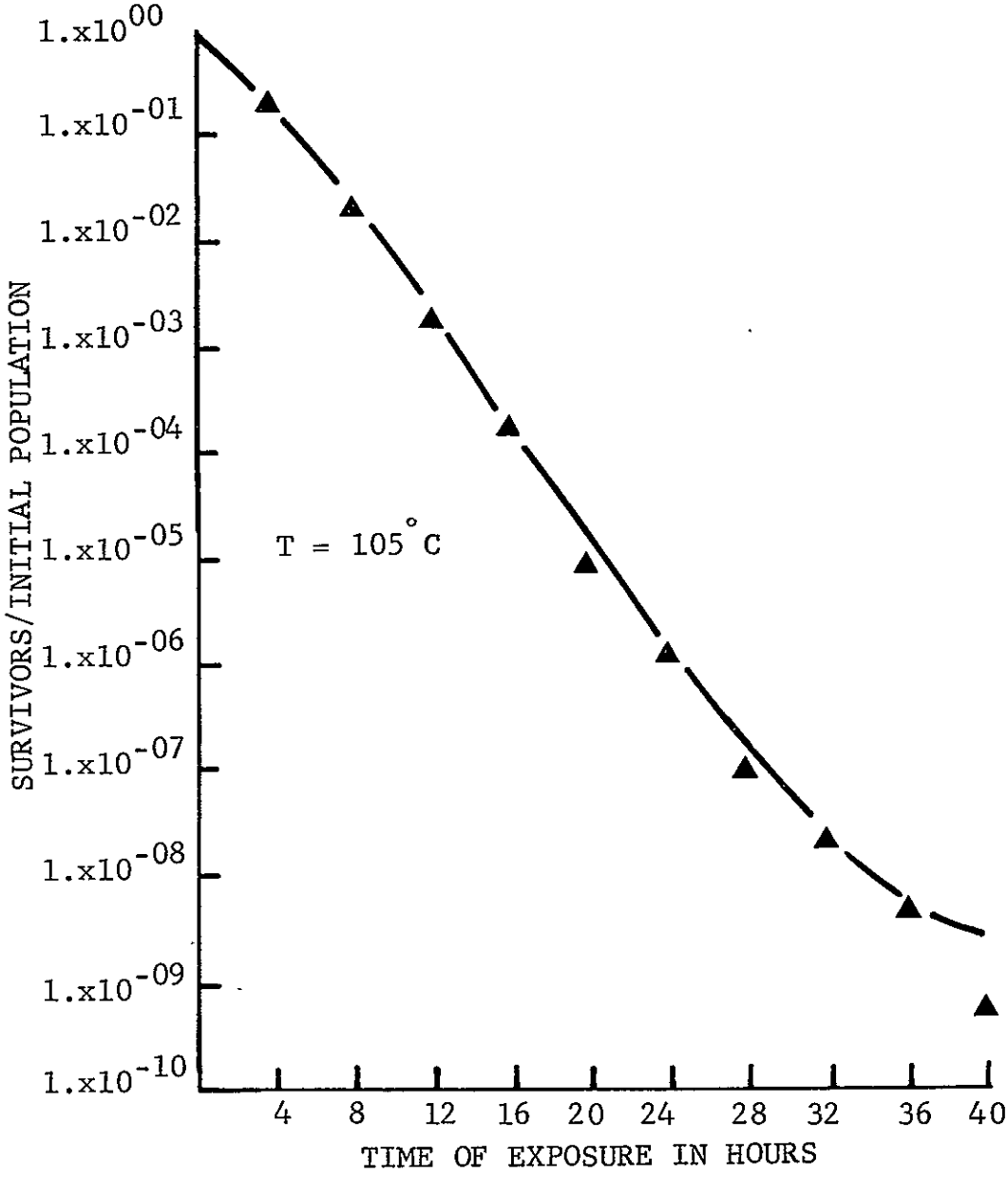

Figure 14a. Computer fit, solid curve to 105°C observations of Fort Detrick B. subtilis stock ▲.

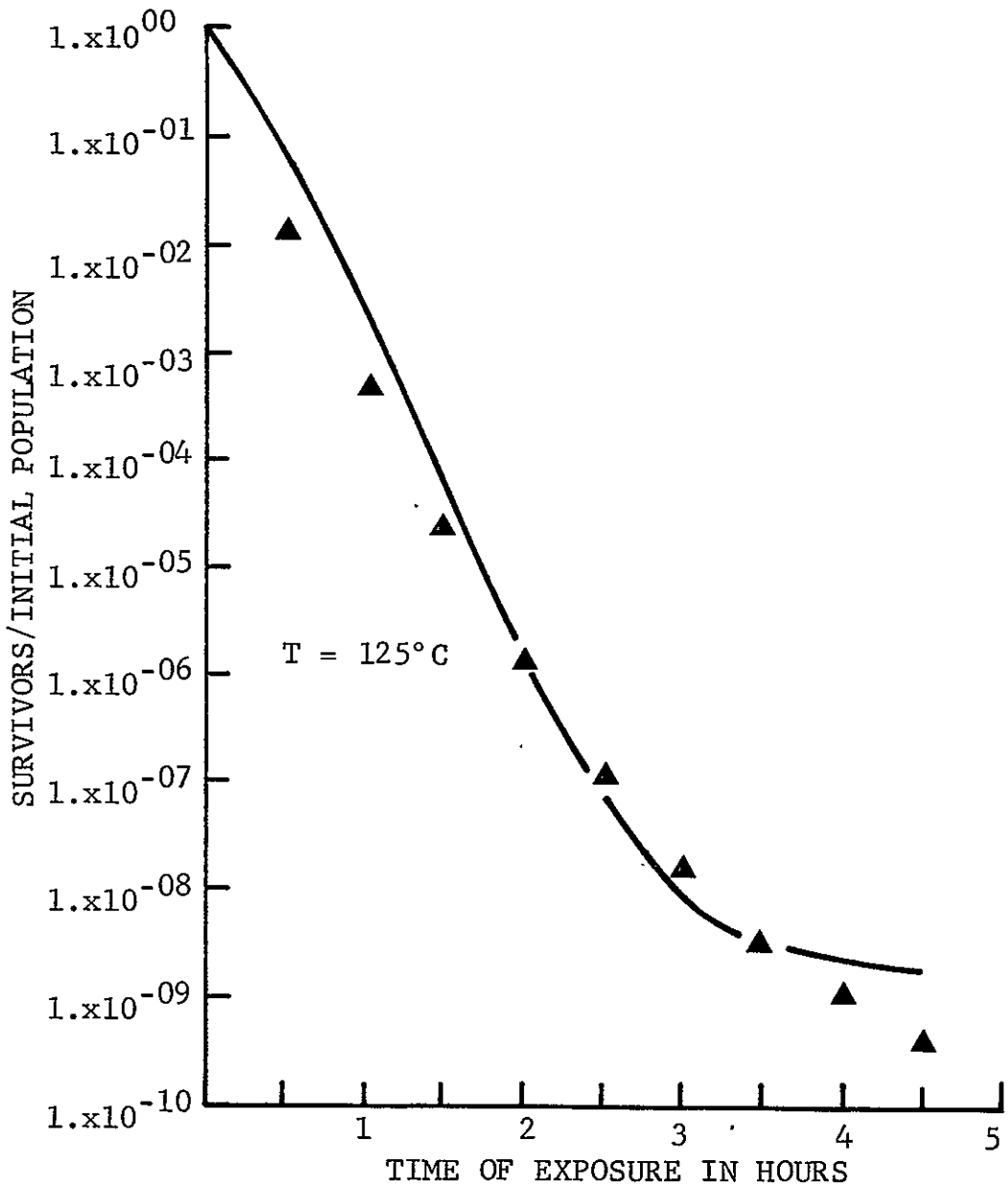

Figure 14b. Computer fit, solid curve to 125°C observations of Fort Detrick stock ▲.

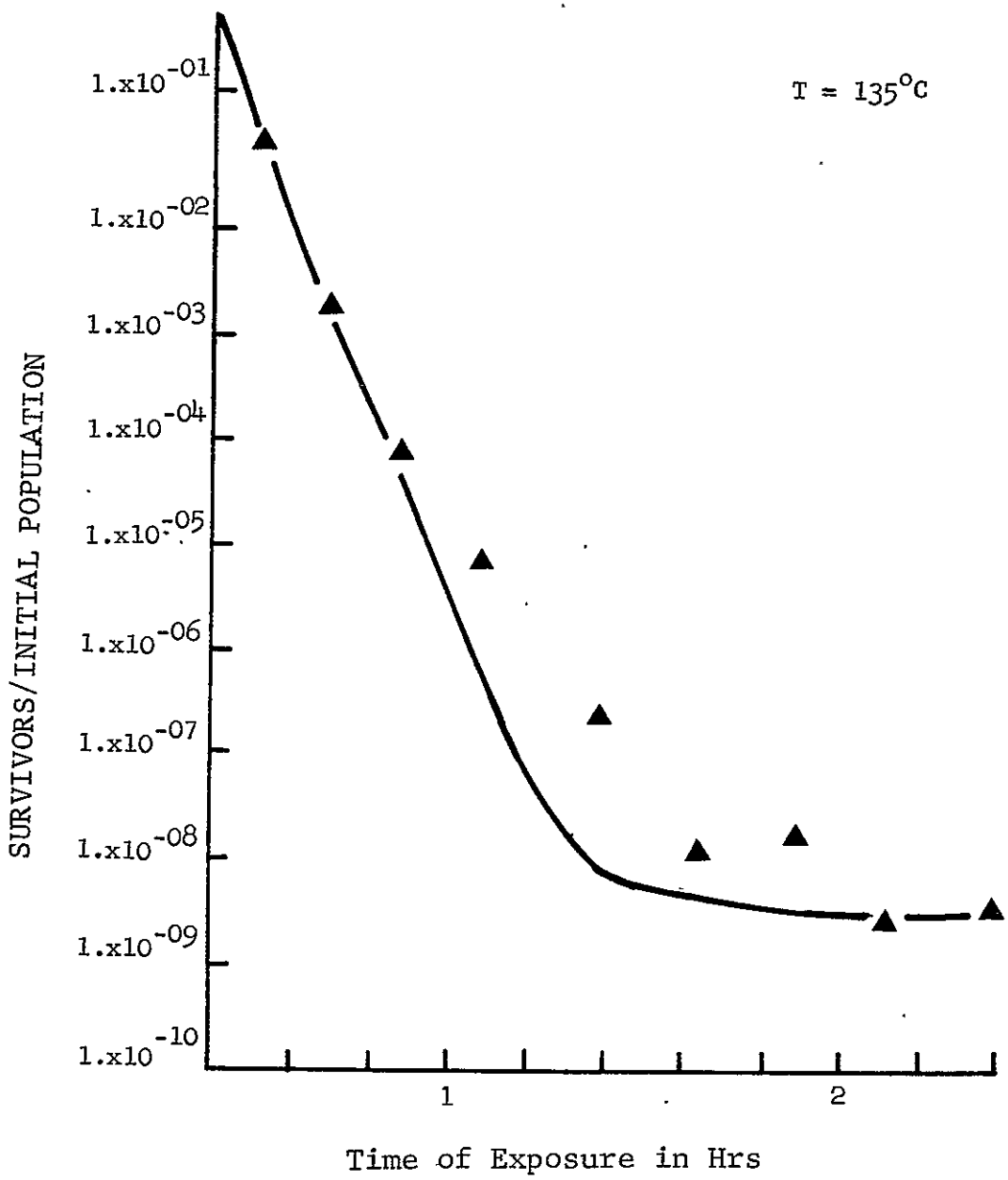

Figure 14c. Prediction, solid curve, for 135°C based on computer fits at 105°C and 125°C for Fort Detrick stock. Observations at 135°C are indicated by ▲'s.

TABLE 4

Thermodynamic Parameters
Associated with the Rate Constants of Equations (9)
which Gave the Fits and Prediction of Figure 14

Number of first-order systems = 2

Number of second-order systems = 2

Number of second-order units/system = 13

Necessary number of second-order units = 9

Rate Constant	$\Delta H^{\ddagger}$ kcal/mole	$\Delta S^{\ddagger}$ eu
k_{-1}	39.3	-12.8
k_1	35.9	19.0
k_2	44.0	-40.0
k_3	50.0	-10.0
k_4	40.2	14.4

C. Variable Temperature Experiments

Once base-line parameters are established for a given stock, a simple and obvious model verification experiment can be carried out by exposing organisms from that stock to a variable temperature profile. Then, model predictions can be compared to these observations.

In addition to model verification, this experiment provides a test of computer techniques for actual spacecraft sterilization since a nonconstant temperature profile is expected for this application. These techniques were discussed in [2], but at that time, the actual experiments had not taken place.

In the laboratory, organisms from the UNM stock were exposed to the temperature profile shown in Figure 15. The ratio of survivors to initial population is shown by the circles in Figure 16. Then, using the base-line parameters, $\Delta H^{\ddagger}$'s and $\Delta S^{\ddagger}$'s previously obtained for this UNM stock, expected survivors were computed for the temperature profile of Figure 15. That is, the base-line thermodynamic parameters were used to generate the rate constants for Equations (8) and the rate constants were changed with time as the temperature changed with time. The ratio of expected survivors to initial population is given by the solid curve of Figure 16.

For applications, it was of interest to know if the simpler Arrhenius equation would give comparable results. The computer run was repeated after making this replacement for the absolute reaction-rate equation. The results were essentially the same. Thus, for applications, we conclude that it is sufficient to use the Arrhenius equation.

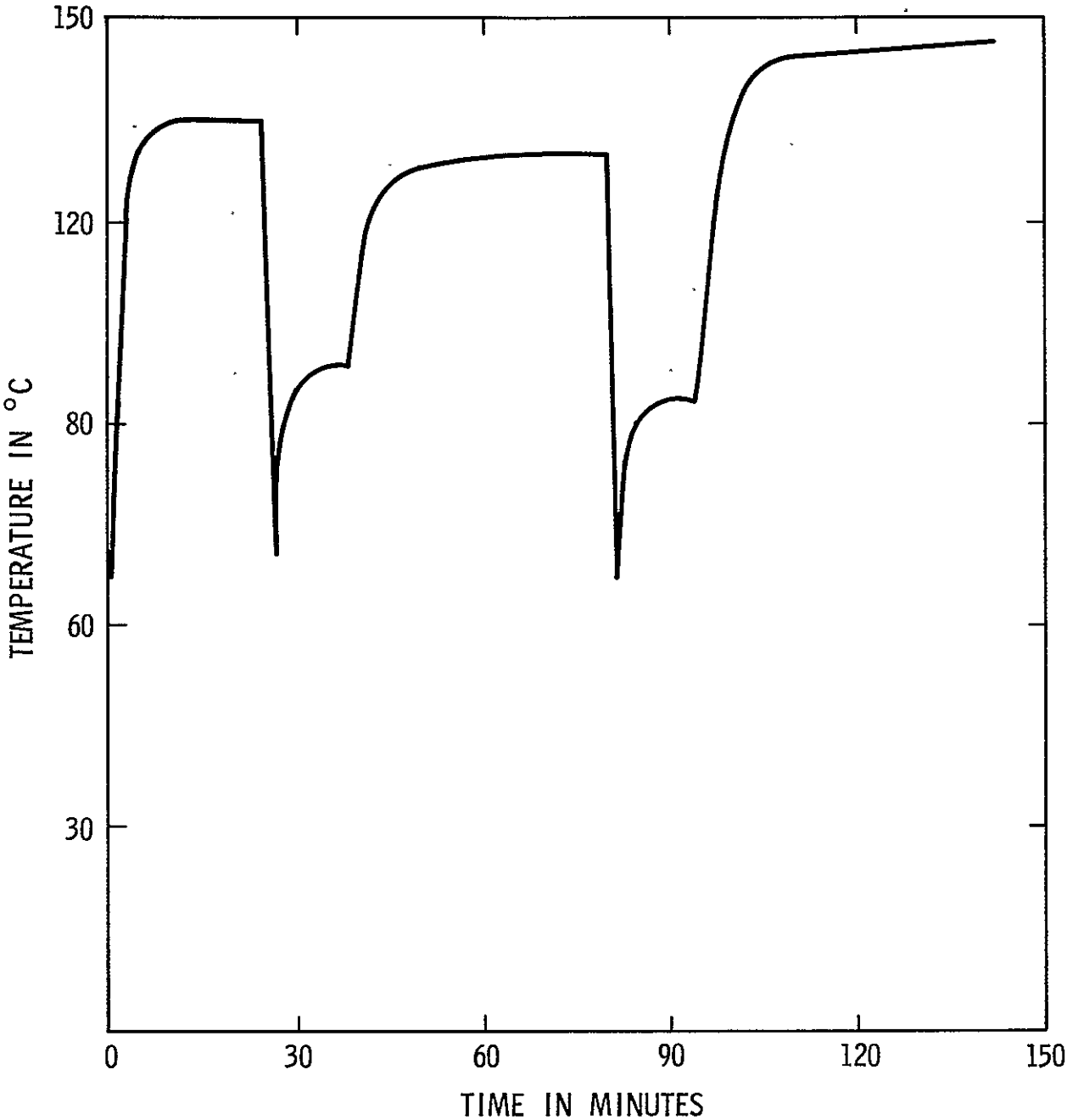

Figure 15. Temperature profile for variable temperature experiments.

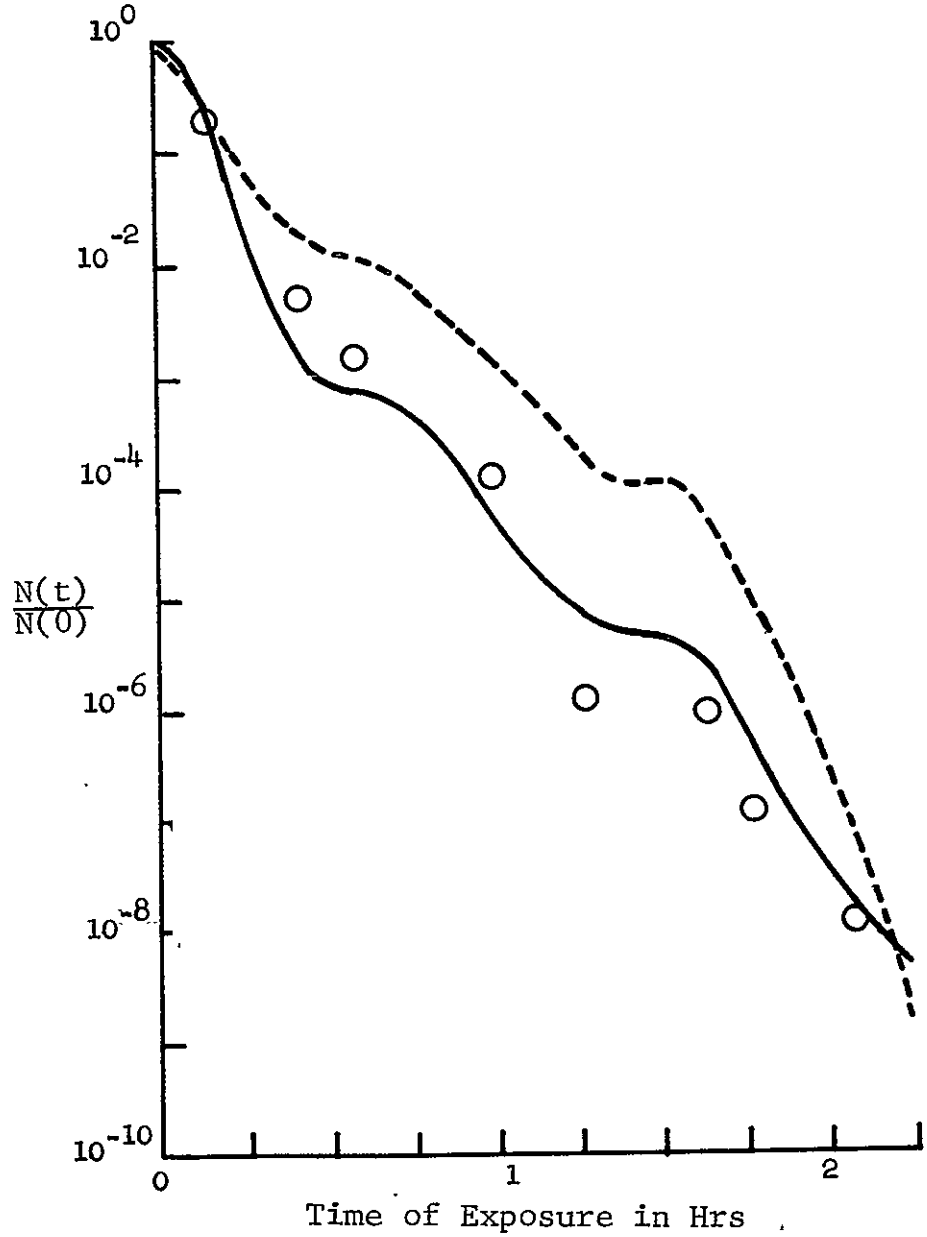

Figure 16. Predictions and ratio of survivors to initial population for variable temperature experiments.

—— = prediction via Equations (8)
--- = prediction via log model
C = observations

It was also of interest to compare these results with those obtained from the log model. To this end, a D value of 0.25 hr was assumed for the 135°C data and a value of 0.5 hr for the 125°C data. The above procedure was repeated with this model. The results are shown by the dashed curve in Figure 16.

Our conclusions from these experiments are:

1. The kinetic model of Equations (8) is consistent with observations.
2. The techniques we have developed are suitable for spacecraft sterilization applications.
3. For spacecraft applications the Arrhenius equation is suitable for variable temperature inputs.

D. a_w Experiments

It was pointed out in Section IIA that although a rough cut at the a_w-$\Delta S^{\ddagger}$ relationship could be made from D values alone, a more satisfactory analysis could be made from raw data, since with raw data it was possible to fit a model subject to reaction-rate theory constraints. Dr. J. E. Campbell of PHS, now FDA, Cincinnati, very kindly made available to us the raw data on which their report [12] was based. The data provided are indicated by X's in Table 5.

Note that data were not available for all three temperature levels at an a_w of 0. Therefore, we used the a_w = 0.03 data at the three temperature levels to fix $\Delta H^{\ddagger}$. To do this, we first found that the survivor curves

TABLE 5

Raw Data Available for

a_w Model Verification Experiments

a_w	Temperature		
	125	135	140
0	X		
0.03	X	X	X
0.05	X	X	X
0.07	X	X	X
0.1	X		
0.2	X		X
0.3		X	
0.4	X		X
0.6	X		X
0.8	X	X	X
0.9	X	X	X
1.0		X	X

closely approximated curves resulting when two molecules are being sterilized by a first-order reaction. It was determined that when $\Delta H^{\ddagger}$ = 26.975 kcal/mole and $\Delta S^{\ddagger}$ = -8.84 eu, the curves for 125°C, 135°C, and 140°C were closely approximated for a_w = 0.03. Using this $\Delta H^{\ddagger}$, the indicated model and the 125°C data, a theoretical entropy of activation curve was calculated, Figure 17. Figure 18 shows the fits for the 125°C, 135°C, and 140°C at 0.03 a_w data subject to kinetic constraints. The Cincinnati data were smoothed out for these calculations.

The first thing to note about Figure 18 is that the a_w = 0.03 data are not consistent with kinetic theory. Usually, microbiological data exhibit this consistency. An examination of the report by Angelotti et al. [67], which describes the experiments which provided the data, is necessary for an appreciation of the difficulty of the experiment. Perhaps it would have been wiser for us to have selected a different a_w for establishing base-line parameters. At around a_w = 0.4 the entropy change with a_w is not so great as at 0.03. However, data were not available for all three temperatures at this a_w. Therefore, we attempted our predictions with the a_w = 0.03 base-line parameters.

Typical results are shown in Figures 19 and 20. In Figure 19 we see the 125°C fit at a_w = 0.4. (Observations are dots and + signs.) The prediction for 140°C at that a_w is also shown. In Figure 20 we see the predictions and observations for a_w = 1 for 140°C and 135°C. Note in Table 5 that 125°C data were not available so that the $\Delta S^{\ddagger}$ = -8.7 eu for the a_w = 0.9 data were used for this prediction.

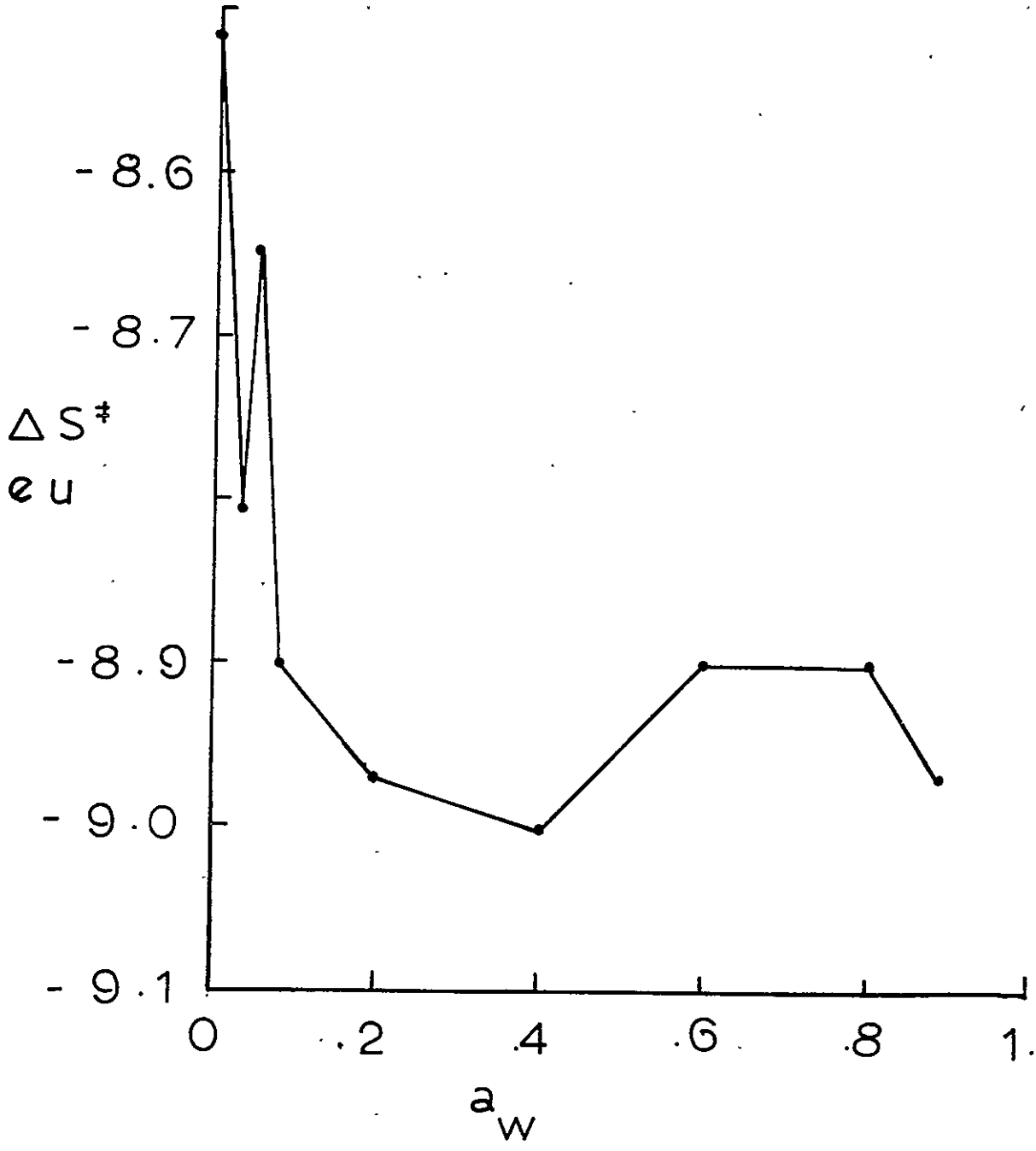

Figure 17. Theoretical entropy of activation as a function of a_w calculated from PHS data.

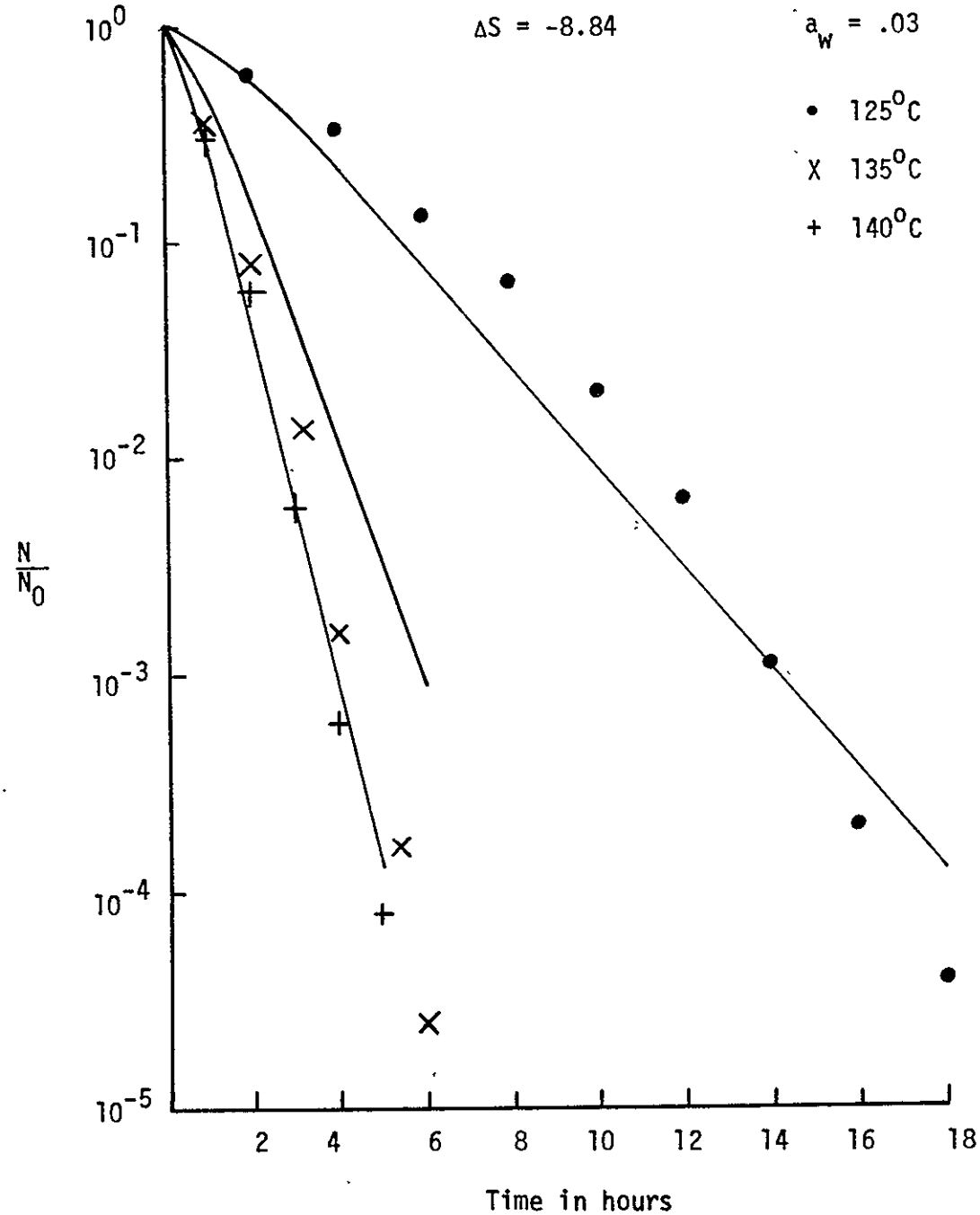

Figure 18. Computer fit to a_w = 0.03 data for base-line parameters.

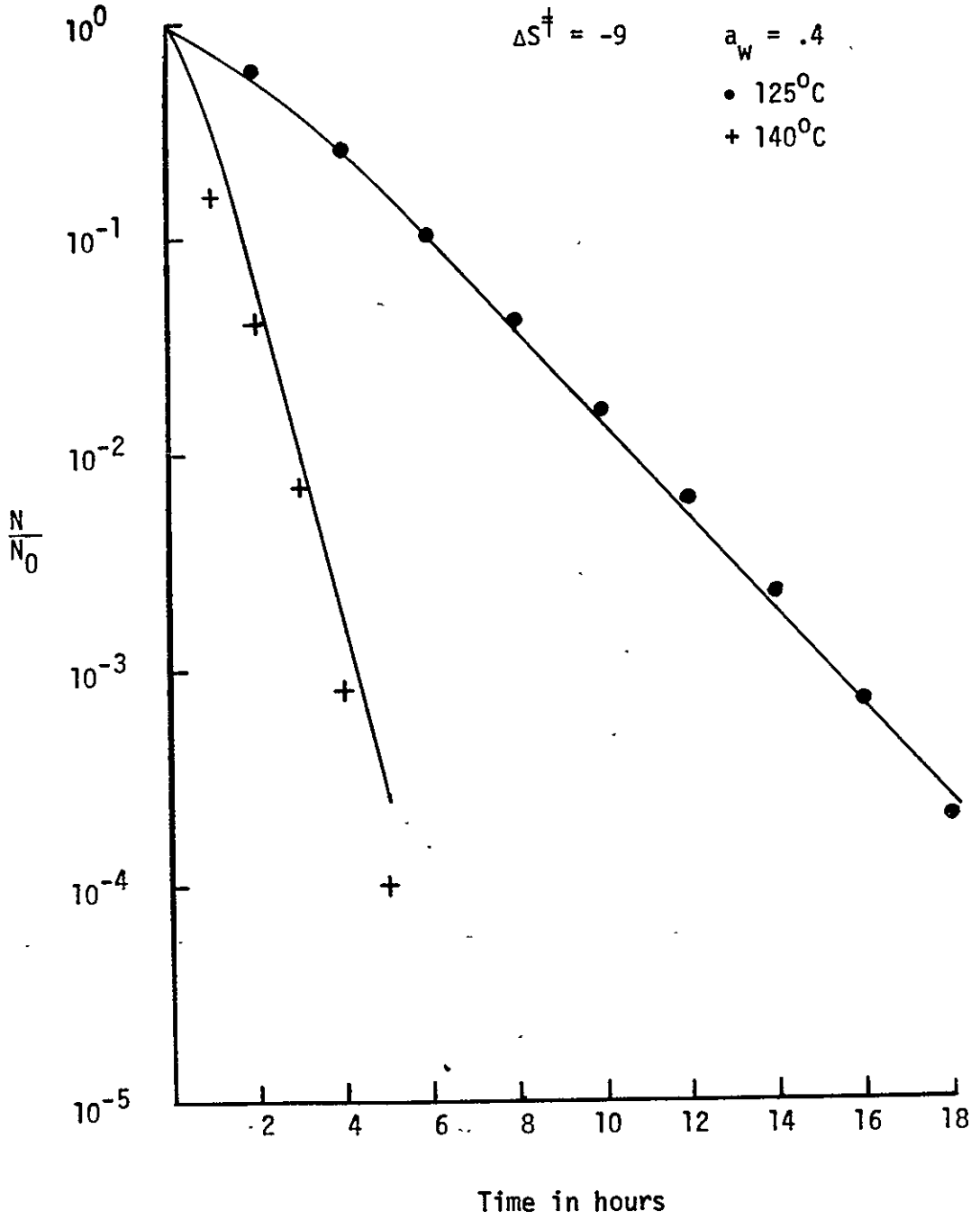

Figure 19. Comparison of predictions to observations for $a_w = 0.4$.

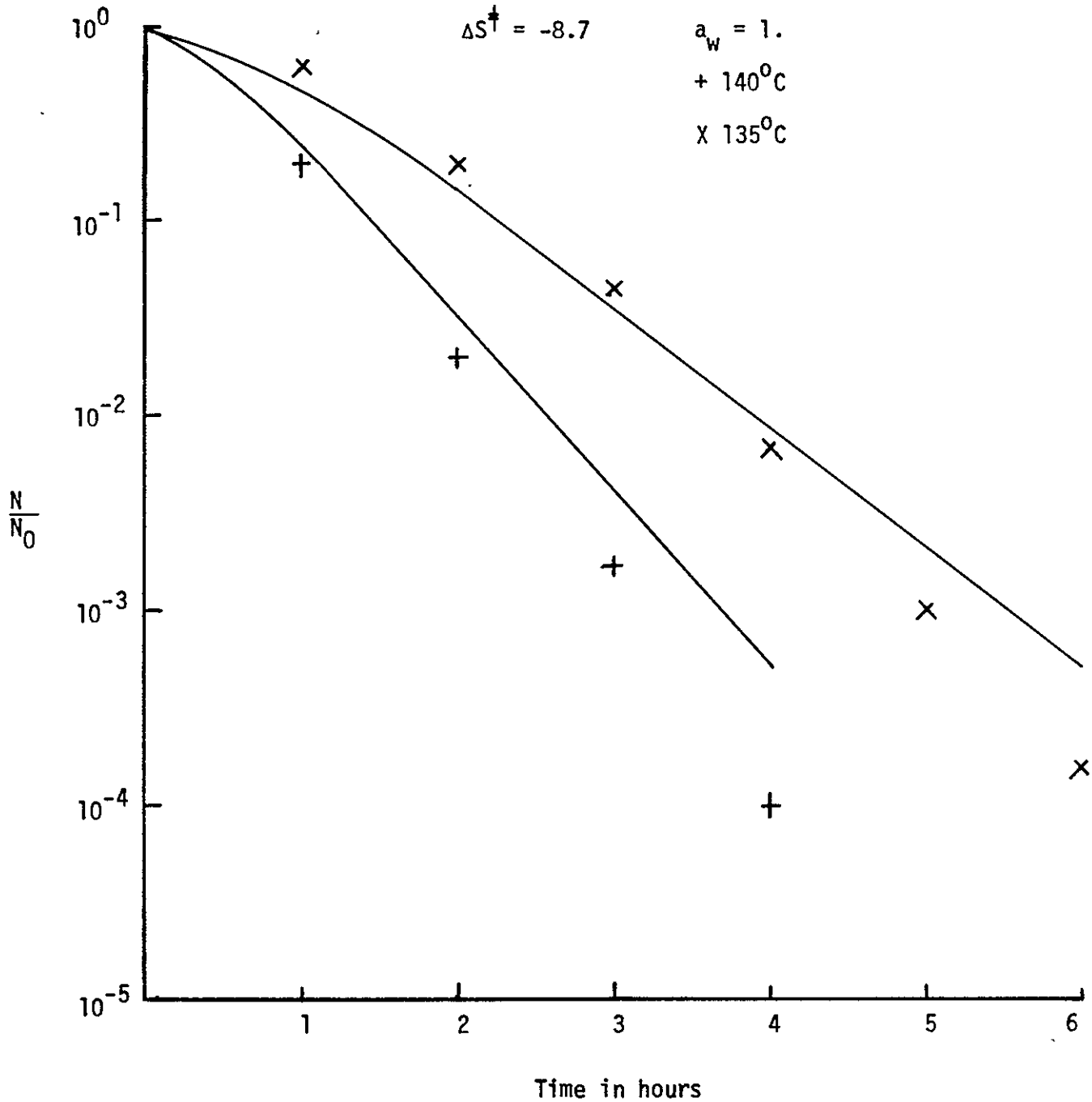

Figure 20. Comparison of observations to predictions for $a_w = 1.0$.

Our conclusions from these experiments are:

1. Our developments in modeling a_w are consistent with the available microbiological observations.
2. Our computer techniques for handling the model are acceptable for spacecraft sterilization applications.

Section IV

Computerized Modeling

The core of model computerization is the collection of reactions that will be allowed and the concentrations of the reactants. If one allowed only the simplest case,

$$A \xrightarrow{k} B \ ,$$

by a first-order reaction with N molecules of A present and microbial viability assured if any one of these N molecules is active, probability of single spore survival, p(t), is given by

$$p(t) = 1 - \left(1 - q(t)\right)^N$$

where q(t) is the probability that any given molecule of A is active at time t. As was discussed in [1] and [2], we allow q(t) to be given by

$$q(t) = A(t)/A_0$$

the ratio of the concentration of A at time t to the initial concentration of A.

Suppose, then, that this is the only reaction allowed and that we have survival data at two temperature levels, T_1 and T_2. These data are normalized or placed in the form N(t)/N(0) where N(t) is the number of survivors at time t and N(0) is the initial population. These data now are in the following form.

Temperature = ___°C

Time of Exposure	N(t)/N(0)
t_1	$N(t_1)/N(0)$
t_2	$N(t_2)/N(0)$
.	.
.	.
.	.
t_n	$N(t_n)/N(0)$

Again, as discussed in [1] and [2], we consider $p(t_i) = N(t_i)/N(0)$; i.e., the probability of single-spore survival at time t is the ratio of survivors at time t to the initial population. Call the reaction-rate constants for

$$A \xrightarrow{k} B$$

at temperature T_1 and T_2, k_1 and k_2, respectively, and consider the N molecules of A as N independently reacting systems, so that $q(t) = A(t)/1$.

The next step is to find values for k_1, k_2, and N which most nearly "fit" the data. Here, we relate k_1, k_2, and N to the reaction allowed, and to the observations, by finding the values of k_1, k_2, and N which minimize the sum

$$\sum_{j=1}^{2} \sum_{i=1}^{n} \left[\log p(t_{ij}) - \log N(t_{ij})/N_j(0)\right]^2 \qquad (10)$$

under the restriction that k_1 and k_2 are related by, say, the Arrhenius equation. Suppose we examine the $p(t_{ij})$ term more closely. For temperature T_1 and the observation taken at time t_i,

$$p(t_{i1}) = 1 - \left(1 - q(t_i)\right)^N$$

where

$$q(t_i) = e^{-k_1 t_i}$$

It is the job of the computer to find the Arrhenius-equation-related k_1 and k_2, and the integer N which minimizes the sum of Equation (10). Note that the fit is that which minimizes the square of the differences between log p and log $\left(N(t)/N(0)\right)$. If N is restricted to be 1 and the sums are done independently, with no requirement that k_1 and k_2 be related by the Arrhenius equation, then the familiar linear regression analysis has been performed on the separate data sets.

Suppose additional reactions are allowed. For example, suppose we allow competition between the simple first-order system given above and a second-order system,

$$2C \xrightarrow{v} D \ .$$

Our microorganism will be sterilized either by losing its A or some portion of its C. Let the second-order system, consisting of N_2 molecules of C, be functional if M of them are active.

Then

$$p(t_{il}) = \left\{1 - \left(1 - q(t_i)\right)^N\right\}$$

$$\times \left(\frac{1}{1 + N_2 v_1 t_i}\right)^{N_2} \sum_{j=M}^{N_2} \binom{N_2}{j} \left(N_2 v_1 t_i\right)^{N_2 - j} \qquad (11)$$

Again, the reaction rates v_1 and v_2 for temperatures T_1 and T_2 are related by the Arrhenius equation. Now the job of the computer is to minimize the sums of Equation (10) by finding the best k_1, k_2 and v_1, v_2 combinations, and the best nonnegative integer values for N, N_2, and M, with $N_2 \geq M$. Note that N can be 0, in which case, the first-order system is rejected; or N_2 can be 0, implying that the second-order system has been rejected.

Hopefully, the idea of this approach to kinetic modeling is made clear by the above simple examples. Data fitting is involved, but in a very special way, since the parameters are either reactant concentrations or reaction-rate constants, and all must obey certain assumptions from either physical chemistry, the Arrhenius equation, or simple logic, without negative or fractional molecules.

The procedure we use is somewhat more complex than that indicated by the simple preceding example. For example, the semiempirical Arrhenius equation is replaced by the more general absolute reaction-rate equation for relating rate constants to temperature, and reactions and systems leading to considerably

more complexity replace the relatively simple expressions for p(t). However, a general flow chart for the simple case will also include the more complex case. The important point to observe is that a computer is used to couple biological observations with physical chemistry and that, although an extensive class of reactions and systems is considered, no one reaction or system is required. The outcome of any computer run is a specific model complete in detail in regard to physicochemical parameters and concentrations.

Figure 21 is a general flow chart covering the procedure discussed above. The in vivo effects of a given environment on a given organism are uncertain; see block 2. (Here, in vivo is taken to mean the reactions occurring within the organism.) Survival data (block 3) are available for this organism and environment. In vitro data, data on reactions of typical cell constituents, and data on chemical kinetics (blocks 4 and 5) are utilized in formulating a general model (block 6). By means of high-speed computation, model parameters and the in vivo data are coupled (blocks 3, 6, and 7).

A. Kinetic Equations

It was pointed out in the preceding section that the reactions of Equations (8) are included in Equations (9). Thus, we will only derive the solutions required for the computer utilization of Equations (9).

Let A be a "vital" substance that is being destroyed by the following reactions:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_2} C$$

$$A \xrightarrow{k_3} D \ .$$

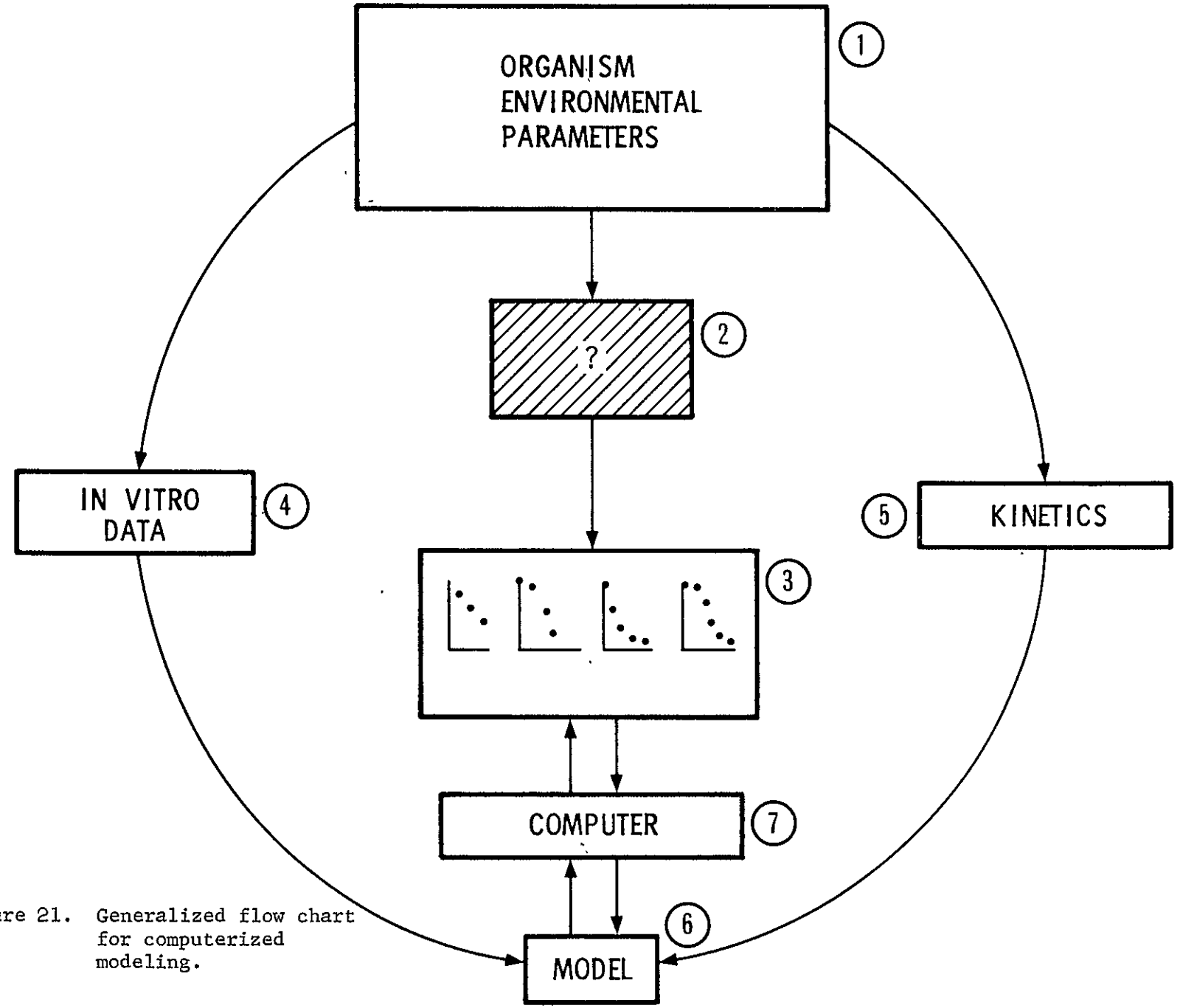

Figure 21. Generalized flow chart for computerized modeling.

Here, B represents an intermediate state from which the flow is either back to A or to a state C, from which repair (reversibility) cannot occur. The A to D reaction has no reversibility. All the reactions are first order and are based on the notion that the vital molecule may be destroyed either by a process like helical unwinding, which is reversible unless completely unwound, or an irreversible process like complete strand breakage.

The differential equations which describe this system are:

$$A'(t) = -(k_1 + k_3)A(t) + k_{-1}B(t)$$

$$B'(t) = -(k_{-1} + k_2)B(t) + k_1A(t) \ .$$

By differentiating the first of these two equations and making the proper substitutions, this system can be reduced to the second-order equation

$$A''(t) + \left[k_1 + k_3 + k_{-1} + k_2\right]A'(t)$$

$$+ \left[(k_1 + k_3)(k_{-1} + k_2) - k_1k_{-1}\right]A = 0 \ .$$

Fortunately, this is both homogeneous and linear, so that

$$A(t) = c_1 \exp\left[\left(-c + \sqrt{c^2 - 4d}\right)0.5t\right] \qquad (12)$$

$$+ c_2 \exp\left[\left(-c - \sqrt{c^2 - 4d}\right)0.5t\right] \ ,$$

where

$$c = k_{-1} + k_1 + k_2 + k_3$$

and (13)

$$d = (k_1 + k_3)(k_{-1} + k_2) - k_1 k_{-1}$$

For initial conditions, we will take A(0) = 1 (the molecules are degraded independently) and $A'(0) = -(k_1 + k_3)$ (the initial concentration of B is 0).

$$A(0) = 1 \Rightarrow c_1 + c_2 = 1 \ .$$

$$A'(0) = -(k_1 + k_3) \Rightarrow c_1\left(-c + \sqrt{c^2 - 4d}\right)0.5$$

$$+ c_2\left(-c - \sqrt{c^2 - 4d}\right)0.5 = -(k_1 + k_3) \ .$$

From these two relationships, we get

$$c_2 = (k_1 + k_3)\Big/\sqrt{c^2 - 4d} - 0.5c\Big/\sqrt{c^2 - 4d} + 0.5 \qquad (14)$$

and

$$c_1 = 1 - c_2 \ . \qquad (15)$$

In the manner described in the previous section, Equations (12) through (15) can be combined with the second term of Equation (11) to provide a mechanism menu of

$$\begin{array}{l} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \quad \overset{k_2}{\rightarrow} \\ A \qquad B \rightarrow C \\ \\ \overset{k_3}{\rightarrow} \\ A \rightarrow D \end{array} \tag{16}$$

i.e., Equations (9).

If k_{-1} is 0, there is no reversibility, so that the first two equations effectively reduce to

$$A \xrightarrow{k_1 + k_3} B \ .$$

Similar deductions can be made if other rate constants become 0. In addition, biological observations are made by heating samples for some specified time and recording survivors by counting colonies after some 48 hours of incubation. Thus, it seems reasonable to allow for repair during some portion of that 48 hours. When using the system of Equations (16), we are able to test the effect of the reverse reaction by allowing it to continue for various portions of the 48 hours.

Another system which is of interest is

$$A + B \xrightarrow{k_1} X$$

$$C \xrightarrow{k_2} B$$

$$A \xrightarrow{k_3} D \ .$$

Here, we examine the possibility that A be degraded by a combination with some substance B which is liberated by the breakup of the substance C, i.e., we examine the sterilization mechanism of destruction of "vital" molecules by free radicals.

The differential equations which describe the system are:

$$X'(t) = k_1 A(t) B(t) \tag{17}$$

$$C'(t) = -k_2 C(t) \tag{18}$$

$$D'(t) = -k_3 A(t) \ . \tag{19}$$

From material balance, we have

$$B(t) = C(0) - C(t) - X(t) \tag{20}$$

$$A(t) = A(0) - D(t) - X(t) \ . \tag{21}$$

Also, from Equation (18),

$$C(t) = C(0)\, e^{-k_2 t} \tag{22}$$

From Equations (17), (19), and (21),

$$A'(t) = -k_3 A(t) - k_1 A(t) B(t) \tag{23}$$

From Equations (20) and (22),

$$B(t) = C(0)\left(1 - e^{-k_2 t}\right) - X(t) \tag{24}$$

so that, from Equations (17), (23), and (24), the following system is obtained:

$$\begin{aligned} X'(t) &= -k_1 A(t)\left[C(0)\left(1 - e^{-k_2 t}\right) - X(t)\right] \\ A'(t) &= -k_3 A(t) - k_1 A(t)\left[C(0)\left(1 - e^{-k_2 t}\right) - X(t)\right]. \end{aligned} \tag{25}$$

Initial conditions are taken as $A(0) = 1$, $C(0) = 100$, and $X(0) = 0$.

The system of Equations (25) does not readily reduce to an expression for which closed-form solutions exist; however, numerical solutions are easily obtained from the form of Equations (25) by several methods. For example, the second-order Runge-Kutta method, where

$$X'(t) = f(t, X, A)$$

and

$$A'(t) = g(t, X, A)$$

for step-size h is given by

$$X_{n+1} = X_n + 0.5(u_1 + u_2)$$

$$A_{n+1} = A_n + 0.5(\ell_1 + \ell_2)$$

where

$$u_1 = hf(t_n, X_n, A_n)$$

$$\ell_1 = hg(t_n, X_n, A_n)$$

$$u_2 = hf(t_n + h, X_n + u_1, A_n + \ell_1) \ ;$$

$$\ell_2 = hg(t_n + h, X_n + u_1, A_n + \ell_1)$$

[68, p. 897].

It takes more machine time to manage systems which are not in closed form, but it can be done. The methods we have used here for systems like Equations (16) are experimental and have not been published; however, in the preparation of this report, we checked out the Runge-Kutta scheme above, and it works.

B. Rate Equations

The rate equation currently being used is from the absolute reaction rate or transition-state theory. The rate constant k is given by

$$k = \frac{\kappa T}{h} \exp(-\Delta F^{\ddagger}/RT) \qquad (26)$$

κ is Boltzmann's constant,

$$\kappa = 1.38045 \times 10^{-16} \text{ erg/deg} ,$$

h is Planck's constant,

$$h = 6.6252 \times 10^{-27} \text{ erg-sec} ,$$

R is the gas constant,

$$R = 1.98726 \text{ cal/deg mole} ,$$

and T is the temperature in degrees Kelvin. $\Delta F^{\ddagger}$ is the energy of activation, and is given by

$$\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

where $\Delta H^{\ddagger}$ is the enthalpy of activation and $\Delta S^{\ddagger}$ is the entropy of activation [36, p. 195]. Thus, if temperature is the only environmental parameter under examination, we vary $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ to vary k, so that, for fixed T_1 and T_2, the k's are related by Equation (26).

C. Minimization Subroutine

The minimization subroutine is similar to a procedure suggested by Hooke and Jeeves in [69]. This is a search routine which sequentially tests the various coordinate directions for minima from a base-line "guess." The routine is a modification of a Sandia Laboratories minimization package called CDC CO-OPID: H2 SAND MIN. The modifications include the option for

varying step size with coordinate direction and the option for considering only integer values for certain coordinate directions. The original H2 SAND MIN routine was programmed by Z. E. Beisinger of Sandia Laboratories, Division 5162, and was adapted for our needs by Mrs. Diane B. Holdridge of Sandia Laboratories, Division 1711.

Other methods for minimizing the function, Equation (10), were examined, but none matched the above routine for consistency and machine speed.

To use the minimization routine, initial guesses for the variables, upper and lower coordinate bounds, variable step sizes, and the number of times the step sizes will be reduced by a factor of 10 are required as inputs.

Section V

Current Efforts

At the time of this writing, the primary efforts are in the direction of model verification experiments for water activity. The PHS data used for model verification in this report was generated for an entirely different purpose and is marginal for our application. For example, in view of the pressure analysis of Subsection B Section II, there are reservations regarding the appropriateness of data obtained from closed systems for this verification. This and the paucity of a_w data required that we attempt to carry out these experiments ourselves, preferably in an open system.

Potential payoffs from such experiments include, in addition to model verification, an ideal a_w for dry heat sterilization. Notice that there is an initial ΔS rise followed by a decrease, then a general increase for the absorption of water by ribonuclease, Figure 2 Section II. This "peak" is associated with the monolayer state. According to the theory developed here, one should find an enhanced sterilization rate at this peak.

By means of a collection of ingenuous devices conceived and fabricated by Whitfield, Garst, and Lindell of Sandia's Division 1742, we have been able to attain "vernier" control of a_w in an open dry heat sterilization system.* The study is now in progress and will be the subject of a later report. We can say now that as a_w moves from .002 to .006, the sterilization rate at 105°C is approximately doubled.

*A preliminary report related to this system [72] is available. A more complete report will be published later.

We expect to use the equipment developed for this investigation in future studies for dry heat sterilization at lower temperatures and in open system studies of pressure effects.

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