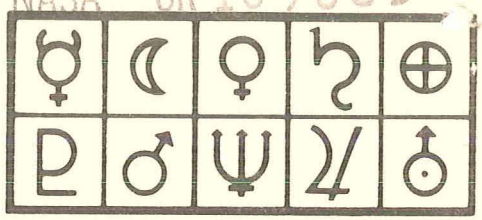

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A MATHEMATICAL MODEL FOR THE THERMORADIATION INACTIVATION OF DRY BACILLUS SUBTILIS VAR. NIGER SPORES

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ABSTRACT

This paper first presents the development of an empirically based kinetic model which describes the synergistic inactivation of dry Bacillus subtilis var. niger spores by a combined heat and gamma radiation environment. The mechanism of this inactivation is investigated by comparing the resulting empirical model parameters with analogous parameters of a free-radical mediated polymerization reaction. A theoretical chemical kinetic model of bacterial inactivation is then derived assuming a free-radical reaction. This theoretical model demonstrates the same form as the empirically based model and is capable of predicting a method for obtaining additional synergistic gain. This predicated method was subsequently tried and the prediction was experimentally verified, lending additional credence to the theoretical kinetic model.

April 1970

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A MATHEMATICAL MODEL FOR THE THERMORADIATION INACTIVATION OF DRY BACILLUS SUBTILIS VAR. NIGER SPORES

Introduction

The observation that prolonged dry-heat sterilization cycles compromise certain heat-sensitive elements of spacecraft hardware has prompted the search for sterilization mechanisms which rely on lower temperatures and shorter sterilization periods. One very promising discovery is that of a combined environment of dry heat and ionizing gamma radiation from a Co^{60} source. The basis for this optimism is related to the fact that the sterilizing action of radiation within the elevated temperature environment is greater than the sums of the sterilizing action of radiation in a nonlethal heat environment and of the action of a lethal temperature environment without the presence of radiation.[1] The observed phenomenon may be classed as a synergism, where one agency is defined as gamma radiation married with a temperature dependency and the other agency is defined as lethal temperature.

The existence of this synergistic effect when sterilizing dry populations of Bacillus subtilis var. niger spores in the above-mentioned composite environment has been definitely demonstrated.[1,2] The current bulk of experimental evidence has shown that the inactivation of the spores in the composite environment follows a straight-line log plot with a slope which increases in absolute value for increases in both temperature and/or radiation dose rate. This inactivation also appears to be rather insensitive to changes in the relative humidity in the range from 20 to 60 percent when measured in the ambient condition exterior to the heating chamber at a temperature of 105°C.

Since the composite gamma radiation/dry-heat environment does exhibit the synergistic effect on the inactivation of the dry spores, the possibility of accomplishing some required sterilization program within an acceptable time period with an acceptable upper temperature limit and radiation dose is entirely feasible. This feasibility has initiated further studies to elucidate the observed phenomenon with the intent of discovering the range of application of the sterilizing environment.

In an effort to augment the experimental effort, a modeling program has also been initiated. This program has as its goal the development of a rational mathematical model which will predict the inactivation properties of a combined heat and gamma radiation environment and will reflect an image of the physical properties of the inactivating mechanism within the microenvironment of the spore. The modeling effort is also intended to provide a base for sequential experimental design, for storage of understood and/or postulated physical inactivation properties, and for a projected design of a final sterilization program for some set of spacecraft hardware. Hopefully, a basic understanding of the complete model and its constituents will provide an insight into and a confidence in the existence of this synergistic death phenomenon for many and varied classes of biological systems.

The Development of an Empirical Model

The first postulate on which this initial phase of the modeling effort was based is that the inactivation of the spore proceeds by some event or chain of events which have the property(ies) of a chemical reaction and, thereby, will obey the theories of reaction rate kinetics. This general philosophy has been shown to be sound and to provide an excellent model base when the inactivating environment consists only of dry heat.[3, 4] In line with this first postulate, the modeling has been facilitated by the fact that all survivor data for dry B. subtilis var. niger spores in combined heat-radiation environments have been logarithmic in nature,[1, 2] as shown in Figure 1.

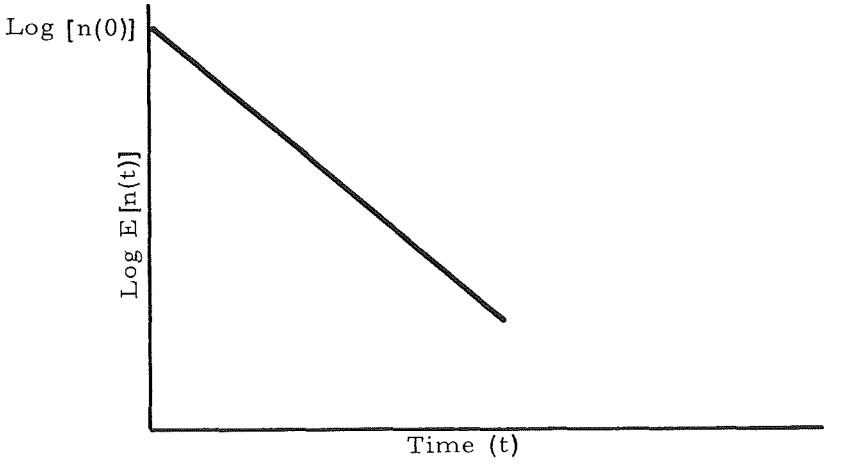

Figure 1. General form of an inactivation curve for a composite heat and radiation environment

Therefore, the expected number of survivors $E[n(t)]$ basically follows a first-order reaction which is described by

$$E[n(t)] = n(0)10^{-t/D} = n(0)\, e^{-kt} \tag{1}$$

where n(t) is the population at time t hours, D is the "D-value" in hours, and k is the reaction rate parameter in $hour^{-1}$.

The approach to the problem is, then, to describe k in terms of physical and environmental parameters. Proceeding in this way, two assumptions can be made concerning the nature of k. First, based on the experimental evidence,[2] k is a function of radiation dose rate r_d. This is to say that not only does the reaction rate increase or the "D-value" decrease if the dose rate is increased, but moreover, if two identical spore populations are both irradiated at the same temperature but at different radiation dose rates, the two surviving fractions will be different even though both populations are given the same dose. Secondly, k must equal k_T when no radiation is present ($r_d = 0$). Here k_T is the absolute reaction rate parameter for dry-heat inactivation given by[5]

$$k_T = \frac{KT}{h} e^{\Delta S^{\ddagger}/R} \cdot e^{-\Delta H^{\ddagger}/RT} \tag{2}$$

where

K = Boltzmann's constant: 1.38045×10^{-16} ergs/degree

h = Planck's constant: 6.6252×10^{-27} erg·seconds

T = temperature in degrees Kelvin

$\Delta S^{\ddagger}$ = activation entropy for dry-heat inactivation

$\Delta H^{\ddagger}$ = activation enthalpy for dry-heat inactivation

R = the gas constant: 1.98726 calories/degree·mole

A generic form of k which satisfies the preceding two assumptions is

$$k = k_T + F(r_d, T) \tag{3}$$

where

$$F(0, T) = 0 \, . \tag{4}$$

The function $F(r_d, T)$ depends on temperature, since its magnitude varies with temperature and since the dose-rate dependence is observed to vary with changes in temperature.[1,2]

A form of $F(r_d, T)$ ultimately found to meet these stipulations is

$$F\left(r_d, T\right) = k_s = r_d^{\alpha/T} \cdot e^{\beta} \cdot e^{-\gamma/(RT)} \tag{5}$$

where r_d is radiation dose rate in kilorads/second, T is temperature in degrees Kelvin, α, β, and γ are constants to be determined, and the unit of k_s is second^{-1}. It may be seen that Eq. (4) is satisfied by this formulation and that k_s roughly retains the structure of a reaction rate equation with γ analogous to energy of activation. The form of $F(r_d, T)$ given in Eq. (5) was chosen over several other generic forms after an extensive numerical investigation into its capabilities for fitting the established data. It was the only generic form found which would fit the restriction of Eq. (4) and the general form of a reaction rate equation, and which would provide a consistent fit over the range of available data. Hence, the final form for the reaction rate parameter k is

$$k = k_T + k_s = \frac{KT}{h} e^{\Delta S^{\ddagger}/R} \cdot e^{-\Delta H^{\ddagger}/(RT)} + r_d^{\alpha/T} \cdot e^{\beta} \cdot e^{-\gamma/(RT)} . \tag{6}$$

The determination of parameter values for Eq. (6) proceeded in the following manner using data obtained in the previously mentioned experimental work.[1] First, values for $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ were obtained from the reaction rate parameters, k_T, produced when the inactivating mechanism was dry heat alone. These values are

$$\Delta S^{\ddagger} = 12.63 \text{ eu} \tag{7}$$

and

$$\Delta H^{\ddagger} = 33.59 \text{ kilocalories/mole} . \tag{8}$$

Next, values for α, β, and γ were determined by a fitting technique using two experimentally determined values of k at 23°C and different dose rates, and one experimentally determined value of k at 105°C with a defined dose rate. The values obtained

for these constants are $\alpha = 218°K$, $\beta = 6.15$, and $\gamma = 5.46$ kilocalories/mole. The derived form of the reaction rate equation is then

$$k\left(sec^{-1}\right) = k_T + k_s = \frac{KT}{h} e^{12.63/R} \cdot e^{-33590/(RT)} + r_d^{218/T} \cdot e^{6.15} \cdot e^{-5460/(RT)}. \quad (9)$$

If r_d is expressed in kilorads/hour rather than in kilorads/second and if the other two terms of the equation are multiplied by 3600 to convert from sec^{-1} to $hour^{-1}$, then the equation may be more conveniently written as

$$k\left(hour^{-1}\right) = k_T + k_s = \frac{KT}{h} e^{14.55} \cdot e^{-16890/T} + r_d^{218/T} \cdot e^{6.15} \cdot e^{-2775/T}. \quad (10)$$

This model provides a good fit of all experimental data obtained to date. These data have been taken in the ranges where T was between 23° and 125°C and r_d varied between 0 and 62.5 kilorads/hour. Figure 2 shows the agreement along with some

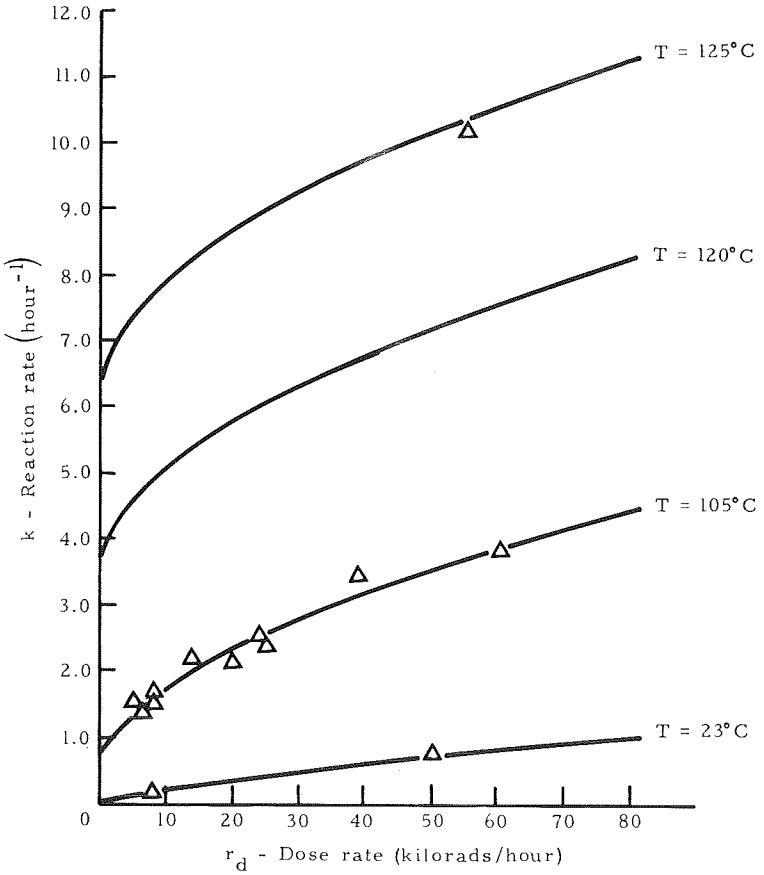

Figure 2. Reaction rate of Bacillus subtilis var. niger spore inactivation as a function of temperature and radiation dose rate

other predictions of k as a function of temperature and dose rate. The solid lines of Figure 2 represent model predictions, and triangles represent reaction rate values for the experimental data reported so far.[1,2] As Figure 2 demonstrates, the correspondence between model predictions and experimental data is very good for a number of radiation dose rates other than the one used for obtaining parameters at 105°C and the one reported dose rate at 125°C.

Several properties may be observed by an investigation of Eqs. (9) and (10). First, k is a monotonically increasing function of both T and r_d. Second, the constant γ in k_s which is analogous to energy of activation is very low. Values of this parameter for ordinary chemical reactions are typically at least an order of magnitude larger than the 5.46-kilocalories/mole value which was obtained. Finally, the dependence on dose rate is that of a fractional power for temperatures above 218°K or -55°C. At 218°K the power of the dose-rate factor of k_s is 1; therefore, the process is no longer dose-rate dependent in the sense that different dose rates but equal doses at -55°C provide equal inactivated fractions. In this connection, an ambient temperature of 23°C or 296°K provides

$$k_s = r_d^{0.737} \cdot e^{6.15} \cdot e^{-2775/T} \text{ hour}^{-1} . \qquad (11)$$

Here the power of r_d is close enough to 1.0 that variations in dose rate may not provide easily recognizable differences in the inactivated fractions when the total dose is held constant by corresponding variations in the time of irradiation. This may account for some previous failures to recognize a dose-rate dependence in bacterial inactivation.

The properties evidenced by the empirical model of Eqs. (1) and (10) have not only the intrinsic interest associated with their very nature, but they also suggest a convenient analogy between spore inactivation and polymerization. This analogy is presented in the following section.

Spore Inactivation Compared with Synthetic Polymers Under Gamma Radiation

In the previous section, the examination of the empirical rate function, k_s, brought out two points which form some very interesting and informative analogies when compared with the properties of synthetic polymers synthesized by exposure to gamma radiation. First of all, the k_s term of Eq. (10), which is the dominant term for temperatures below 100°C, is dependent upon a fractional power of radiation dose rate or radiation intensity. This power is approximately 0.737 at 23°C

and 0.548 at 125°C. The second point drawn from the k_s term is that the apparent activation energy (γ = 5.46 kilocalories/mole) is much smaller than that of ordinary chemical reactions. A conclusion which may be drawn from the dose-rate dependence of the reaction rate of spore inactivation is that the dominant inactivating mechanism does not consist totally of a breakage phenomenon. This is true since breakage is usually associated with a single hit of a photon of ionizing radiation; therefore, the total accumulated damage is only dependent upon the absorbed dose. Since a sole dose dependence has not been observed, then some other type of inactivating mechanism must be present.

In looking for different mechanisms, it has been found that the rate of formation of polymers at ambient temperature conditions is approximately proportional to the square root of radiation dose rate.[6, 7] More exactly, the dose-rate dependence has been found to vary between the 0.25 power and the 0.95 power of dose rate depending upon the type of polymer being formed. Also, activation energies of approximately 4.25 kilocalories/mole are noticed for methyl methacrylate and 6.7 kilocalories/mole are noticed for polystyrene when exposed to gamma irradiation.[6]

Several other prominent similarities have been observed between radiation initiated polymerization and radiation inactivated biological systems. Many biological systems show an increased sensitivity to radiation when the concentration of oxygen present is increased.[8] Polymer formation also exhibits this increased sensitivity.[8] Similarly, if a dry population of B. subtilis var. niger spores is irradiated with gamma radiation at room temperature (23°C) and is subsequently heated at an elevated temperature (105° to 125°C), the initial rate of inactivation is much greater than it would be without the preirradiation. However, the rate tends to slow down and approach the inactivation rate of the elevated temperature alone after the process has operated for some time.[1] Polymers also exhibit this feature in that the rate of polymerization of a preirradiated monomer is faster at the beginning of the polymerization process than it is sometime later.[9]

Since these very compatible similarities do exist between synthetic polymerization and dry spore inactivation under an environment of gamma radiation, it is not unreasonable to think of these as being analogous processes mediated by the same type of reaction mechanism. It has been well documented that the formation of many synthetic polymers[6] (polystyrene, methyl methacrylate, etc.) proceeds basically by a free-radical mechanism at temperatures above 20°C. Herein lies the strong suggestion that a large portion of the bacterial spore inactivation due to gamma radiation may be attributed to either a free-radical mechanism or at least something behaving very similar to a free-radical mechanism.

There have recently been other strong indications that radiation damage in biological macromolecules might also proceed by a free-radical mechanism. Zimmer[10] has found that the breakage of DNA molecules in phage when exposed to ionizing radiation accounts for only 20 to 40 percent of the apparent inactivation, and that the energies of activation at temperatures above 0°C are only several kilocalories per mole. Also, electron spin resonance studies demonstrated the presence of free radicals in all constituents of dry phage when exposed to ionizing radiation. Kurzinger[11] has explained how diffusible hydrogen radicals can damage intact molecules within the biological system by hydrogen abstraction. He has found also that the inactivation rate of T1 phage is proportional to the 0.82 power of dose rate at ambient temperature.

On the basis of the preceding evidence, the assumption is made that free-radical mechanisms have an important part in the process of inactivation of dry bacterial spores. Since the rate of inactivation of spores and the rate of formation of polymers is proportional to a fractional power of radiation dose rate, the implication that the concentration of free radicals present is proportional to the same fractional power of dose rate is established. Therefore, in view of the results of the modeling effort just presented and the experimental evidence which has been cited, it seems reasonable to investigate a free-radical inactivation mechanism from "first principles." An expansion of this complete rationale is provided by a simple chemical kinetic model in the next section.

Chemical Kinetic Modeling of Free-Radical Inactivation

Assume that a population of dry bacterial spores is exposed to a composite environment of lethal temperature, T, and gamma radiation at a dose rate, r_d. Also, assume that each bacterium contains a critical substrate A which is inactivated at a rate k_T due to the elevated temperature and at a rate k_1 due to free-radical interference. The descriptive reaction equations are

$$A \xrightarrow{k_T} D \tag{12}$$

and

$$A + R \xrightarrow{k_1} X \tag{13}$$

where R is the normalized free-radical concentration as a function of time. D and X represent inactivated states of the critical substrate A and infer death of the bacterium. Let R(t) be represented by the equation

$$R(t) = C_R\left(r_d, T\right) \cdot \left(1 - e^{-k_3 t}\right) + C_2\left(r_d, T\right) \cdot e^{-k_2 t} \tag{14}$$

where $C_R(r_d, T)$ represents an equilibrium concentration of free radicals for a constant r_d and T and where $C_2(r_d, T)$ represents a "pool" or bulk of free radicals formed by a preirradiation treatment; that is, they are formed prior to the introduction of the spores to the elevated temperature. Each of the k_i ($i = 1, \ldots, 3$) is assumed to be of the form of an absolute reaction rate equation which is a function of absolute temperature as in Eq. (2). The description of R(t) in Eq. (14) has the general form of a "birth and death" process with the exception of k_2 and k_3 being different from one another. To simplify the formulation here k_3 will be assumed to be very large; that is to say that the introduction of the preirradiated samples into the composite environment very quickly establishes the equilibrium concentration of free radicals. This allows R(t) to be approximated by

$$R(t) = C_R\left(r_d, T\right) + C_2\left(r_d, T\right) \cdot e^{-k_2 t} . \tag{15}$$

Here k_2 is the rate at which the free-radical concentration due to preirradiation is utilized.

The differential equation which describes the schematics of Eqs. (12) and (13) is

$$\frac{dA}{dt} = -k_T A - k_1 AR . \tag{16}$$

Substituting R(t) from Eq. (15) gives

$$\frac{dA}{dt} = -\left\{k_T + k_1 \cdot \left[C_R\left(r_d, T\right) + C_2\left(r_d, T\right) \cdot e^{-k_2 t}\right]\right\} A . \tag{17}$$

The solution to this first-order equation is:

$$A(t) = A(0)\, e^{-K(t)} \tag{18}$$

where

$$K(t) = k_T t + C_R\left(r_d, T\right) \cdot k_1 t + \frac{k_1}{k_2}\left[C_2\left(r_d, T\right)\right] \cdot \left(1 - e^{-k_2 t}\right) . \tag{19}$$

Recalling the assumed one-to-one correspondence between substrate A and bacterial cell, Eq. (18) may be written

$$E\left[n(t)\right] = n(0)\ e^{-K(t)} \tag{20}$$

where $E\left[n(t)\right]$ is the expected population as a function of time, and $n(0)$ is the initial population at time $t = 0$. The reference starting point $t = 0$ is located just after the preirradiation period and just before the beginning of the composite lethal environment.

Now consider the case for which no preirradiation is used. This situation requires that $C_2(r_d, T) = 0$ and is the case for which all of the data of Figure 2 applies. With $C_2(r_d, T) = 0$

$$K(t) = \left[k_T + C_R\left(r_d, T\right) \cdot k_1\right]t = kt . \tag{21}$$

Therefore, comparison with Eq. (9) or (10) provides

$$k_s = C_R\left(r_d, T\right) \cdot k_1 . \tag{22}$$

Drawing from the fact that free-radical concentration in polymers is a function of a fractional power of radiation dose rate and once again comparing k_s of Eq. (22) with k_s of Eq. (10) provides

$$C_R\left(r_d, T\right) = \xi r_d^{(218/T)} \tag{23}$$

where ξ is an undetermined proportionality constant. Continuing the comparison gives

$$k_1 = \xi' e^{-5460/(RT)} \tag{24}$$

which is intuitively pleasing since k_1 has the general form of an Arrhenius equation and is only a function of temperature. The resulting requirement on the constants which were used is

$$\xi\xi' = e^{6.15} . \tag{25}$$

If the sample is preirradiated and then heated without any simultaneous radiation, then $C_R(r_d, T)$ of Eq. (19) is zero,

$$K(t) = k_T t + \frac{k_1}{k_2}\left[C_2\left(r_d, T\right)\right] \cdot \left(1 - e^{-k_2 t}\right), \tag{26}$$

and the rate of the process is

$$\frac{dE[n(t)]}{dt} = -\left[k_T + k_1 \cdot C_2\left(r_d, T\right) \cdot e^{-k_2 t}\right] n(t) . \tag{27}$$

Inspection of Eq. (27) shows that the inactivation proceeds faster at the initiation of the heating cycle than would the inactivation without preirradiation. Also the rate of the preirradiated sample inactivation approaches that of the unirradiated sample after a sufficient period of time. Investigation of Eqs. (20) and (26) for $t \gg 1/k_2$ provides

$$E[n(t)] = n(0) \exp\left[-k_T t\right] \cdot \exp\left[-\frac{k_1}{k_2} C_2\left(r_d, T\right)\right] \tag{28}$$

and shows that the additional inactivated fraction when preirradiation is used is

$$\exp\left[-\frac{k_1}{k_2} C_2\left(r_d, T\right)\right] . \tag{29}$$

This is consistent with the experimental data for bacterial spore death and with radiation-initiated polymerization.

If the spore sample is preirradiated and then exposed to a composite environment, then both C_R and C_2 are nonzero. For this case, the increased rate of the

reaction is given by the quantity in braces in Eq. (17). Also, the inactivated fraction is less than that provided solely by lethal temperature by the factor

$$\exp\left\{-\left[C_R(r_d, T) \cdot k_1 t + \frac{k_1}{k_2} C_2 \cdot \left(1 - e^{-k_2 t}\right)\right]\right\} \tag{30}$$

and is less than that provided by the composite environment by the factor

$$\exp\left\{-\left[\frac{k_1}{k_2} C_2 \cdot \left(1 - e^{-k_2 t}\right)\right]\right\} \tag{31}$$

for any time t.

To verify the preceding kinetic model prediction, a population of dry B. subtilis var. niger spores was preirradiated at a dose rate of approximately 12 kilorads/minute for 3 minutes. Immediately following the preirradiation, the spores were subjected to a composite environment of 19 kilorads/hour and 105°C. The results[12] are shown in Figure 3. Time, t = 0, again refers to the beginning of the composite cycle. Notice that the preirradiation did provide an increased initial rate of death and a larger inactivated fraction at all times. Therefore, the generalized assumptions upon which the model was based do seem to be substantiated. The single set of data does not provide enough information to calculate values for k_1, k_2, C_R, and C_2 at 105°C, but good initial approximations for k_1 and k_2 at 105°C on this basis are $k_1 = 2.05$ hour^{-1} and $k_2 = 2.40$ hour^{-1}.

The rational kinetic model derived in this section must be regarded as being at least as valid as the earlier empirical model, since the two have precisely the same form when $C_2(r_d, T) = 0$. In other words, when there is no preirradiation, both models provide the same prediction. On the other hand, the rational kinetic model has a capability for predicting the results when preirradiation is used in addition to the combination environment, and the experimental verification of this prediction in a situation where data had not previously been taken lends additional support for the model's validity.

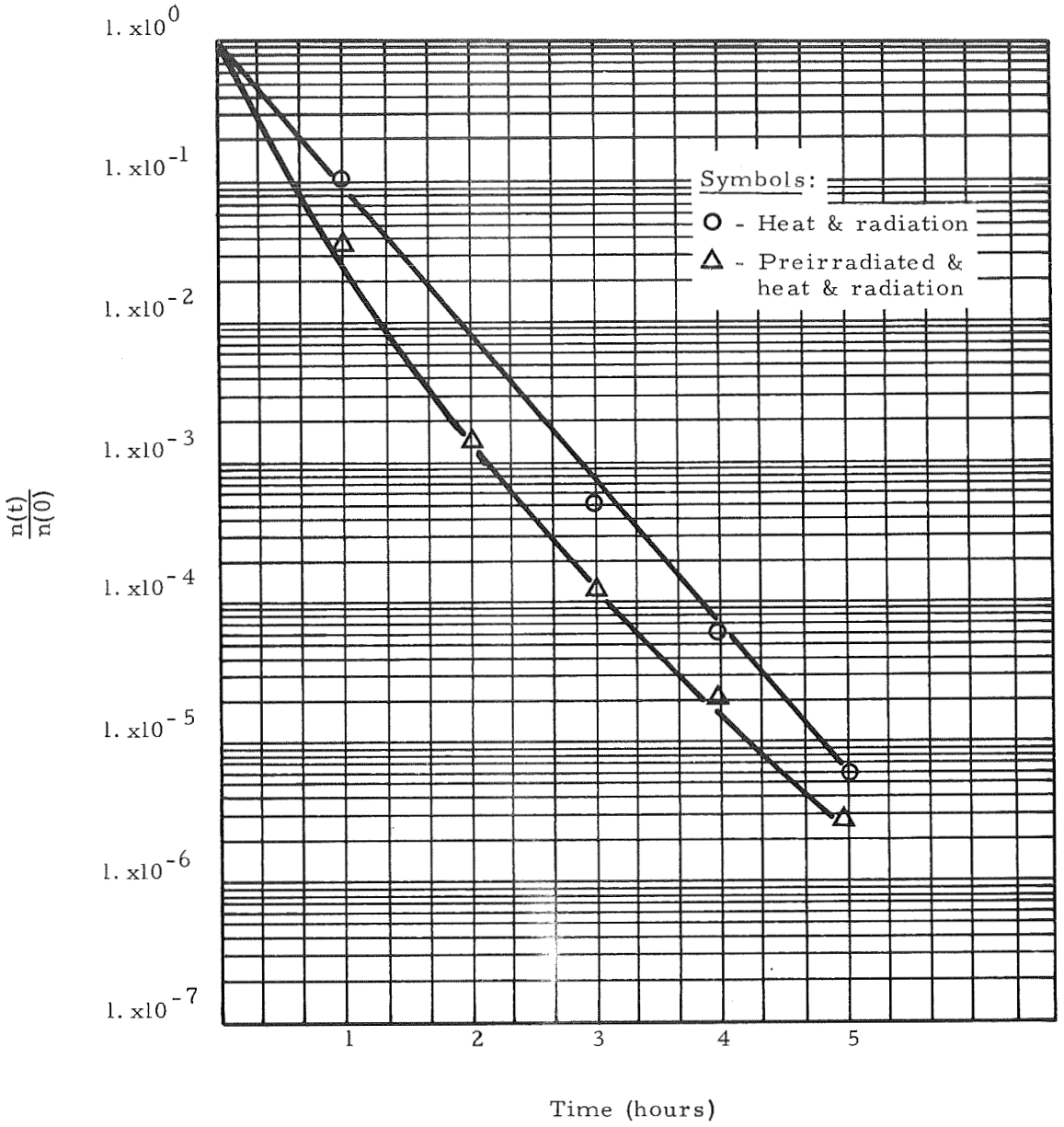

Figure 3. Effect of preirradiation on inactivation kinetics[12]

Desiderata

Additional experimental work is now being performed which will be used to either verify the empirical reaction rate model at other temperatures and radiation dose rates or to suggest changes which will improve its reflection of the true physical process. Also, additional effort will be directed toward the improvement of the chemical kinetic model and the understanding of its parameters. A more thorough experimental investigation will accurately project the life time of the free-radical population and will provide information for a descriptive rate equation for k_2 and k_1.

The kinetic model also suggests that a pulsed radiation source may be more efficient than a constant source in the sense of more death per kilorad. This possibility should also be investigated.

In the formulation of the kinetic model, it was assumed that the same critical substrate was acted upon by both the lethal temperature and by the free-radical intervention. This was done only to simplify the presentation, since two independent critical substrates would give the same forms for Eqs. (19) and (20) based on a probability argument. Further experimentation may point out which of these is truly correct.

Continued efforts in these directions will hopefully lead to a better understanding of inactivation mechanisms and further gains in sterilization efficiency.

Summary and Conclusions

This paper has presented the development of an empirical model which describes the inactivation of dry spores by a combined heat and gamma radiation environment. The resulting parameter values and properties of this empirical model are compared with analogous values and properties of the polymerization of nonbiological organic materials under the influence of gamma radiation. The values of these parameters are found to be in excellent agreement and thereby lead one to speculate that the forms of the chemical reactions in the two situations are very similar. Since polymerization proceeds by a free-radical mediated chemical reaction and since the literature has cited free-radical effects in the inactivation of biological systems when exposed to gamma radiation, the logical step of investigating free-radical inactivation of dry spores when exposed to the radiation environment is taken.

With this supporting rationale, a theoretical chemical kinetic model based on the free-radical inactivation of a critical substrate is developed. Although independently derived from first principles, the form of the theoretical kinetic model is identical to that of the semiempirical model for the data situations upon which the latter was founded. Also, an extension in the rationale of the theoretical model does suggest a greater synergistic effect by preirradiation of samples to then be heated and irradiated simultaneously--a situation which had not been investigated experimentally. This additional synergism was subsequently demonstrated experimentally, lending credence to the validity of the rational kinetic model.

The rational kinetic model which has evolved from this effort has proved to be very accurate in predictions of exposed B. subtilis var. niger spore inactivation rates for temperatures between 23° and 125°C and radiation rates between 0 and 62.5 kilorads/hour.

It is hoped that further improvements can be made upon the work presented here, that the combination experimental research/model effort will continue to augment one another, and that a clearer physical picture of the death of biological systems when exposed to lethal heat and radiation environments will emerge.

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