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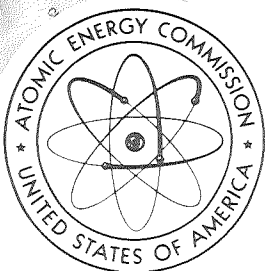
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Sandia Laboratories Quarterly Report - Planetary Quarantine Program

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Planetary Quarantine Department
Sandia Laboratories, Albuquerque, New Mexico

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Summary of Activities

Bacterial Spore Inactivation Modeling. The effect of pressure upon dry heat sterilization has been incorporated into the kinetic inactivation model. The inclusion of this parameter offers potential for estimating bacterial inactivation rates in deep space, where pressures are many orders of magnitude lower than current experimental capabilities, and explaining anomalies of and estimating inactivation rates for embedded and mated organisms. The volume of activation, of absolute reaction rate theory, becomes a critical parameter when attempting to address problems such as these, and it must be more fully understood before confident answers may be obtained.

The inclusion of pressure as a parameter also suggests that, should current values for the volume of activation be approximately correct, the molecules involved in inactivation must be quite large.

Thermoradiation Sterilization Experimentation and Modeling. Experimental efforts this quarter have been concentrated primarily on assessing the effect of radiation dose rate upon organism inactivation rate at 105°C. Inactivation at this temperature is demonstrated to be sensitive, not only to total dose, but also to dose rate.

A model, based upon the kinetic model for thermal inactivation and the thermoradiation experimental data, has been developed which faithfully represents the experimental data taken between 23°C and 135°C and 0 Krad/hr. to 64 Krad/hr. This model is interpretable in terms of free radical reactions and the previous kinetic model for thermal inactivation.

Inactivation Mechanism Studies. Inactivation agents can function either by interfering with the spore's ability to transform into the germinated state or by interfering with the germinated spore's capacity for macromolecular synthesis and

and cell division. Since these stages are biochemically and physiologically distinct, information about the mechanism of spore inactivation using various agents must begin by identifying the spore state at which the agents exert their influence.

Results this quarter suggest that dry heat, γ -radiation and ultraviolet radiation all interfere primarily with the spore's capacity for macromolecular synthesis and cell division rather than with its ability to transform into a germinating spore.

Bioburden Experimentation and Modeling. Models for the estimation and prediction of spacecraft bioburdens in assembly environments yielding predictions and estimates about which statistical confidence statements may be made are needed.

The notion of "clumping" of organisms permits one to include both contact and fallout in a model by treating them as both different cases of depositing clumps of organisms. Under these circumstances, a general Markov process governs the distribution of the bioburden on a surface as a function of time. Knowing only three parameters allows one to predict future bioburdens and to compare these predictions with estimations derived using a steady state version of the same general process. A general description of the prediction model and its application is presented here.

Computerized Identification Scheme. The PHS bacterial identification scheme was simulated on a computer in an attempt to see if computerized identification compared at all favorably with current practices. Obviously, whenever identification criteria can be specified precisely, a computer simulation of these criteria is possible. Thus, a comparison between a computer simulation and current practices is a comparison between a defined set of identification criteria and whatever additional judgement may be used in current identification practices. Using a specified set of rules, agreement with current practices was obtained for more than 85% of the colonies identified from Apollo 11.

BACTERIAL SPORE INACTIVATION MODELING

- A. Description. The objective of this activity is to develop a physically based model of bacterial spore inactivation capable of exhibiting behavior paralleling all known forms of bacterial spore survival data and consistent with known facts about spores. A subsidiary objective has been to rationally relate environmental parameters to spore inactivation through the physical parameters occurring in the model.
- B. Progress. During this past quarter, progress has been made toward including pressure as an environmental parameter in sterilization modeling. Prospects are good for this inclusion. Theoretically this can be achieved by the more complete formulation,

$$\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger + p\Delta V^\ddagger,$$

for the free energy term in Eyring's absolute reaction rate equation, where p is pressure and $\Delta V^\ddagger$ is the volume change as the reactant goes into the activated complex.

Usually 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 & Keller (1962) suggests 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 five days. This translates to 90% destruction in 12.5 days. On the other hand, Silverman (1966) report a $\Delta H^\ddagger$ of 34.6 kcal/mole and a $\Delta S^\ddagger$ of 14.65 e.u. for the same organism under what one can presume to be a pressure of 1 atmosphere. Assuming as did Silverman, 1 molecule 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 $\Delta S^\ddagger = -11.1$ e.u. for data generated by Koesterer (1964).

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 6°C , 1-atmosphere D value, and a 60°C , 10^{-8} torr D value, reaction-rate constants can be calculated by the formula

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

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

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

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

(Johnson, Eyring & Polissar, 1954, p 305).

In this manner it was found that, with $\Delta H^\ddagger = 34.6$ kcal/mole and $\Delta S^\ddagger = 14.65$ e.u.,

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

while, if $\Delta H^\ddagger = 25$ kcal/mole and $\Delta S^\ddagger = -11.1$ e.u.,

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

The $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values computed by Silverman for his and Koesterer's data roughly represent the extremes which one sees for B. subtilis. Thus, the value of $\Delta V^\ddagger$ probably lies between 15 and 65 liters/mole.

The positive value for $\Delta V^\ddagger$ indicates that the activated state of the "vital molecule" is less ionized than the unactive state. Since $\Delta V^\ddagger$ is generally positive for unimolecular reactions (Johnson, Eyring & Polissar, 1954, p.306), the positivity of the $\Delta V^\ddagger$ computed from the Silverman-Koesterer data is consistent with the assumption of a 1-molecule sterilization mechanism.

For unimolecular reactions involving relatively small molecules, molecular weight in the hundreds, excellent correlations with experimental data have been obtained over a pressure range of up to 3000 atm by the formula

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

where $b^\ddagger$ is the length of the bond that is broken, Σb is the sum of the bond lengths in the chain, V is the molar volume, and r_1 and r_2 are the covalent radii of the terminal atoms (Glasstone, Laidler & Eyring, 1941, p. 473). 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.

The magnitude of the estimated $\Delta V^\ddagger$ for the Silverman data suggests a molecule much larger than those used in Glasstone et al. However, it is of interest to use the above formula for larger molecules for an order of magnitude $\Delta V^\ddagger$ estimate. 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 (White, Haudler & Smith, 1964, p. 173), a molecular weight of 2.5×10^9 (Watson, 1955, p. 85), bond lengths of 3 Å (Pauling, 1960, p. 503), and a specific volume of 0.55 ml/g (Katz & Schachman, 1955). Under these assumptions, the $\Delta V^\ddagger$ calculated by the above formula is 7 liters/mole which is within an order of magnitude of the estimates from Silverman's data. Incidentally, this 62 liters/mole value for $\Delta V^\ddagger$ represents a volume change of approximately .003% of DNA molar volume, which is consistent with percentage changes known to take place for smaller molecules.

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 $\Delta F/RT = (\Delta H^\ddagger - T\Delta S^\ddagger + p\Delta V^\ddagger)/RT$. Since $\Delta V^\ddagger$ is in cc/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.

The sign and magnitude of $\Delta V^\ddagger$ can be of considerable importance in planetary quarantine applications. For example, if $\Delta V^\ddagger$ is positive, a monomolecular reaction is indicated and a decrease in pressure would cause the reaction rate to rise so that for a fixed temperature D values would decrease with pressure. The amount of the decrease would depend on the magnitude of $\Delta V^\ddagger$. Conversely a negative $\Delta V^\ddagger$ would indicate a multimolecular sterilization mechanism and pressure decreases would result in a D value increase. The importance of the $\Delta V^\ddagger$ magnitude is indicated in Table I. There D values are given as a function of temperature and $\Delta V^\ddagger$ with $\Delta H^\ddagger = 25$ kcal/mole and $\Delta S^\ddagger = -9$ e.u.

TABLE I
 $\Delta H^\ddagger = 25$ kcal/mole, $\Delta S^\ddagger = 9$ e.u.

Temp. °C	$\Delta V^\ddagger = 7$ l/mole	$\Delta V^\ddagger = 20$ l/mole	$\Delta V^\ddagger = 60$ l/mole
0	32,706.	18,306.	3,070.
20	1,340.	780.	148.
40	82.	50.	10.
50	23.	14.	3.1
60	7.	4.	1.
80	.8	.5	.1

The $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values selected are roughly those calculated for some data from the University of Minnesota. The important thing to note in Table I is that at the temperatures one expects to see on a space vehicle in transit, there is an order of magnitude difference between the D value associated with $\Delta V^\ddagger = 7$ l/mole and that associated with $\Delta V^\ddagger = 60$ l/mole. Also observe that if $\Delta V^\ddagger$ is around 60 l/mole there should be a population reduction of 36 logs per year at only 40°C. This suggests that some careful experimentation to determine $\Delta V^\ddagger$ more accurately could pay excellent dividends to planetary quarantine.

In addition to its potential usefulness in a deep space environment, the inclusion of pressure as a parameter affecting the inactivation of bacterial spores may, in part, offer an explanation for the slower rate of inactivation of embedded and mated spores.

Finally, values of $\Delta V^\dagger$ on the order of 10 to 60 liters/mole if correct, suggest that, should only a single molecule be involved in spore inactivation, that molecule must be quite large in order that $\Delta V^\dagger$ represent a percentage volume change consistent with the fractions of percentages experimentally known for smaller molecules.

THERMORADIATION STERILIZATION

A. Description. The thermal degradation of spacecraft as a result of present dry heat sterilization is a problem still not completely resolved. There is some concern that silver-zinc batteries, high value mylar capacitors, vidicon tubes, tantalum capacitors (10 to 15 μf), solid propellant (especially PBAA), culture media, photometers, and other scientific instruments will not withstand present dry heat sterilization cycles without excessive degradation. The purpose of the thermoradiation study is to explore possible techniques that will reduce the dry heat time and temperature requirements for sterilization of spacecraft hardware. More specifically, this study will explore the feasibility of using combinations of heat and radiation where synergistic effects may be available to achieve effective sterilization of spacecraft.

B. Progress. The work this quarter was directed toward the determination of the sensitivity of thermoradiation lethality to inoculation technique of the test organism, and secondly, work was directed at determination of gamma radiation dose rate effects.

Inoculation technique sensitivity. Because of the differing wetting characteristics of metal surfaces used for samples in thermoradiation experiments, it was necessary to assess the effects of small changes in sample area for fixed test organism populations. This information was necessary to supplement the data reported in QR 14 where sample loading, for a fixed sample area, was varied from 10^6 to 10^9 B. subtilis var. niger spores per sample. Experiments this quarter considered a fixed population of

10^6 *B. subtilis* spores per sample with the sample areas of 0.2, 0.7 and 1.2 square inches. Experimental samples were prepared by pipeting 0.1 ml. of *B. subtilis* spores (suspended in ethanol, 10^7 spores per ml) onto 0.0015 inch thick X 1.2 inch diameter disks cut from biological grade aluminum foil. The inoculum was confined to a desired area by the appropriate sized depression stamped into the foil. Samples were desiccated over Drierite in vacuo for 1 hr. and then assembled with a cover foil between two .025 inch aluminum strips with four samples per strip. This assembly operation was completed in air at a relative humidity of less than 5%. Samples were again placed in a desiccator in vacuo for 15 hrs. before exposure to the sterilization environment. One strip containing four samples represents a single data point. Sample strips were exposed to the thermoradiation environment of 105°C and a gamma dose rate of 36 Krads per hr. for periods up to 4 hrs. Ambient humidity was controlled at $30 \pm 2\%$ RH at 75°F .

Results are shown in Figure 1 where little difference is indicated between the three sample configurations. The somewhat higher survivors at the three hour period for the .7 and .2 square inch samples must be rechecked to determine if an area effect is beginning to emerge at this point.

Effects of variable gamma dose. The purpose of this series of experiments was to begin to evaluate the thermoradiation lethality of *B. subtilis* under variable gamma dose rates. This data is needed to begin optimization of heat and radiation levels to accomplish effective sterilization with minimum environmental stress to spacecraft componentry. These

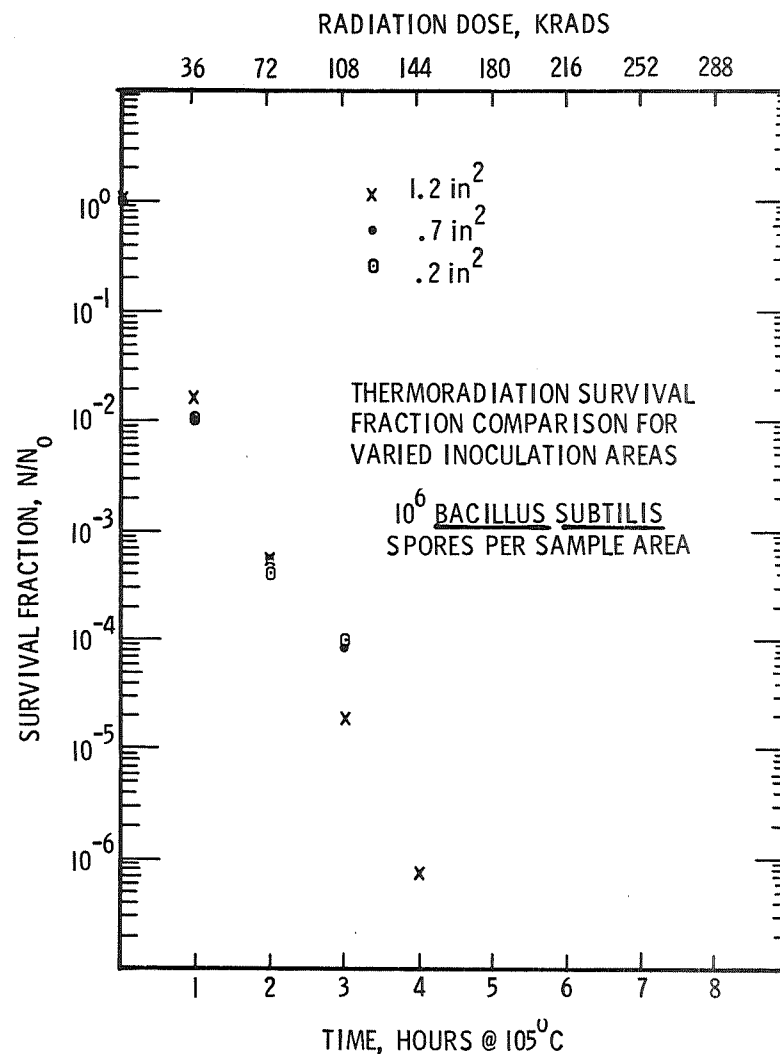


FIGURE 1

first experiments were performed at a single temperature of 105°C and at four rates of radiation exposure. Dose rates were 5, 13, 22 and 36 Krads per hour. Relative humidity was controlled at an ambient of $30 \pm 2\%$ RH at 75°F . Experimental samples were prepared in the same manner as previously described with the exception of sample area which was maintained at 1.2 square inches.

Single experiments were run at the 13 and 22 Krad per hour exposures but multiple experiments were run at the extremes of 5 and 36 Krad per hour dose rates. Results are shown in Figures 2, 3 and 4.

Several interesting observations can be made from these data. First of all, at the lowest level of 5 Krad per hour, the lethality curves have a tendency to tail. Tailing occurs up to what appears to be a critical dose of around 25 Krads, then the curves suddenly changes to become coincident with the original slope. This behavior at low radiation doses will be further explored to find if there is, in fact, a lower threshold of thermoradiation effectiveness at this temperature. A plot of D values, Fig. 5, taken from these four exposure rates tend to further indicate that the lethality is more sensitive to dose rates below 5 Krad per hour at 105°C . In addition, Figure 5 indicates marginal gains are available at the higher dose rates above 20 Krads per hour. Thus, one can select a more or less optimum dose rate of about 12 Krads per hour at 105°C . This results in a D value of 1.1 hrs. or based on the 12 log reduction, a sterilization cycle of 13 hrs. at 105°C with a nominal gamma dose of 156 Krads. This 13 hr. cycle is a substantial reduction from the more common¹ 55 to 60 hrs. for dry heat at 105°C .

¹Spacecraft Sterilization Technology, NASA SP-108, p. 217.

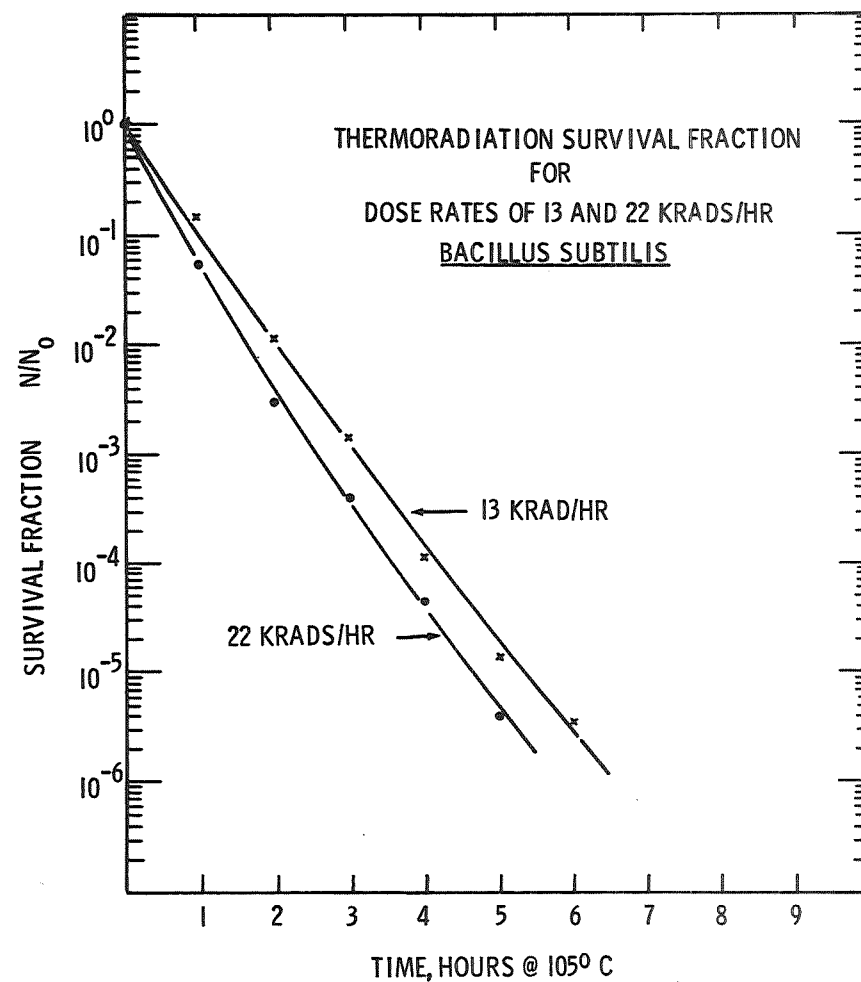


FIGURE 2

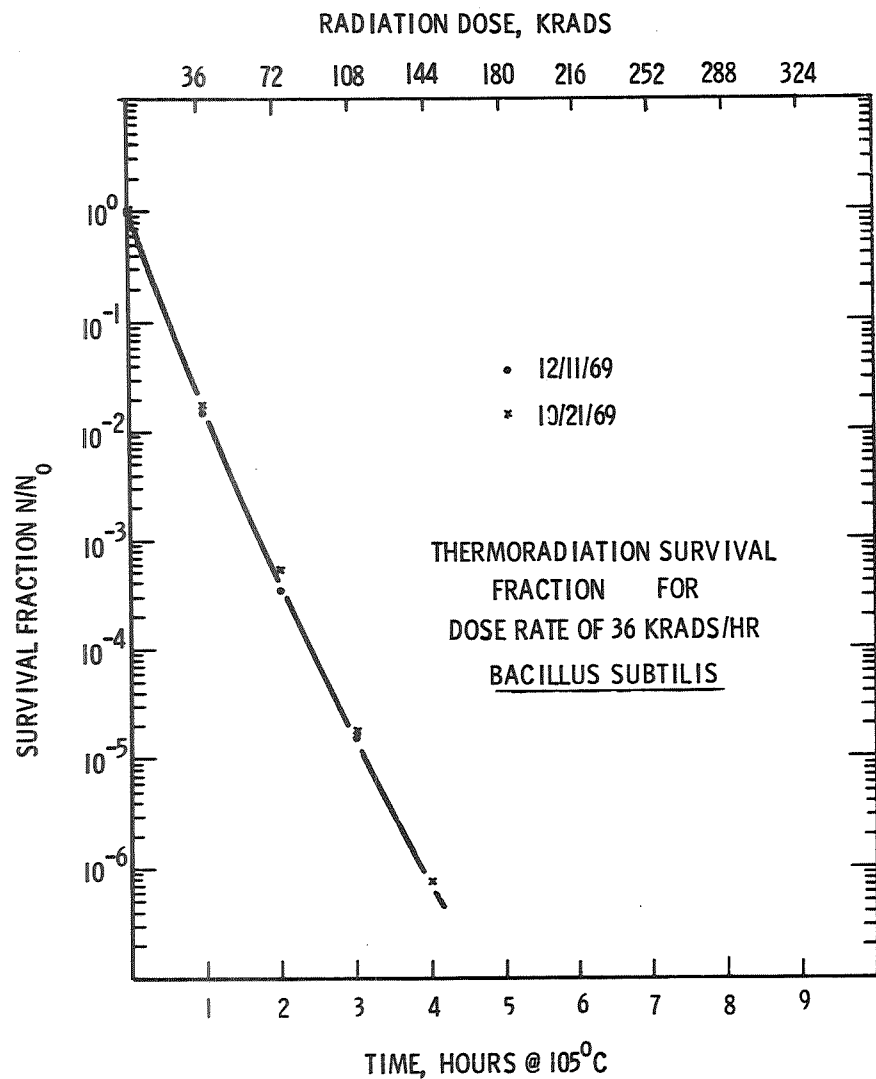


FIGURE 3

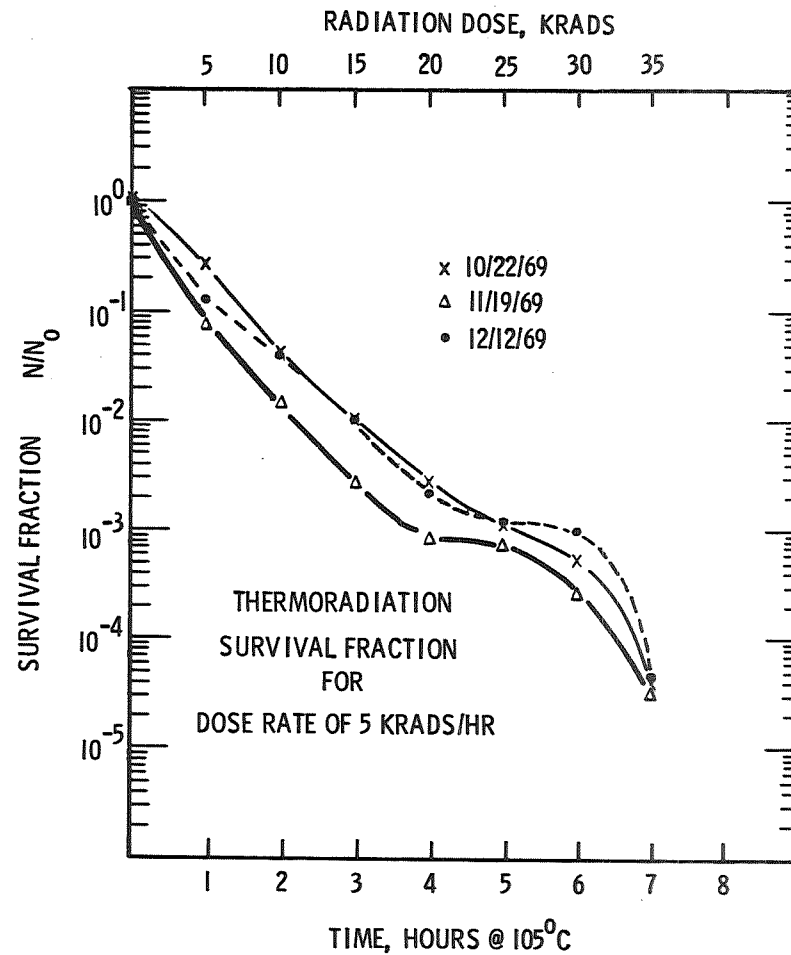


FIGURE 4

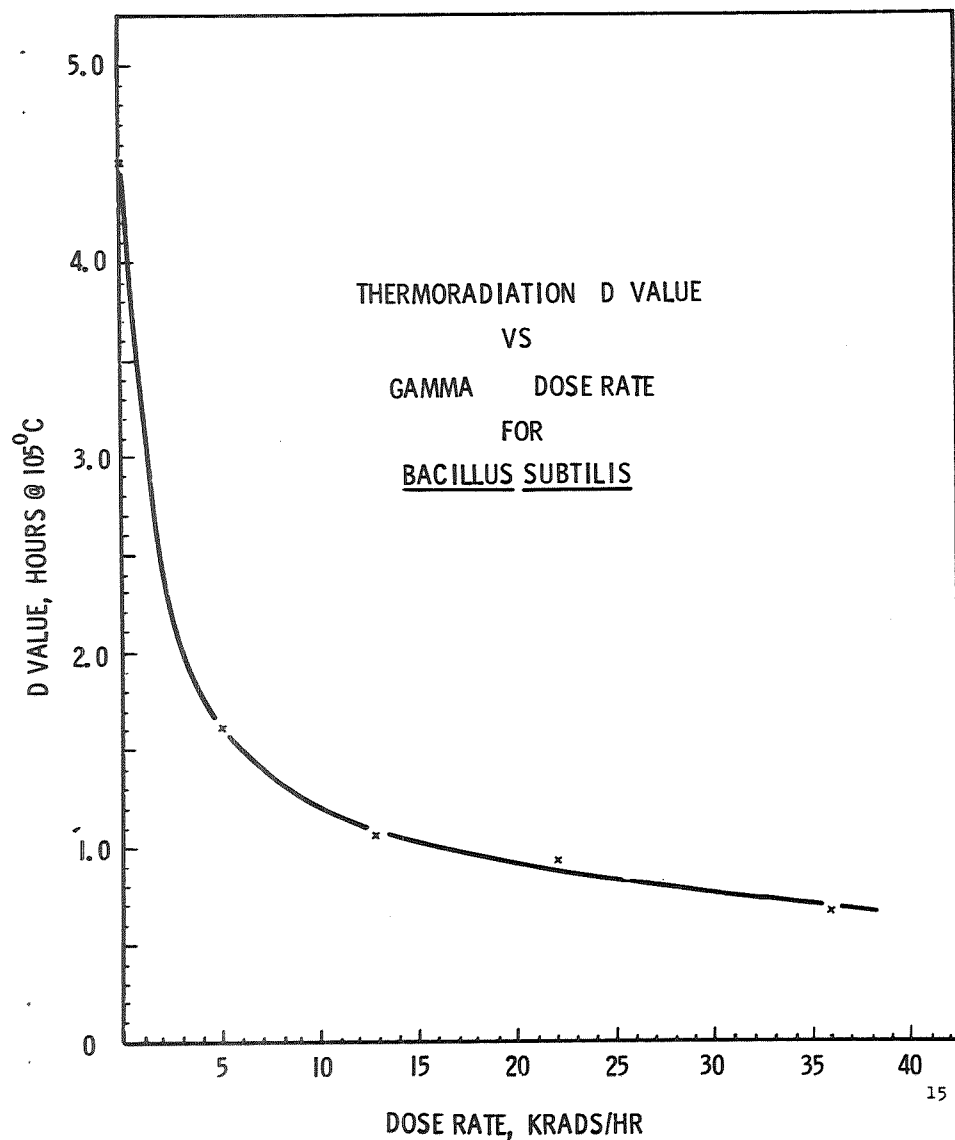
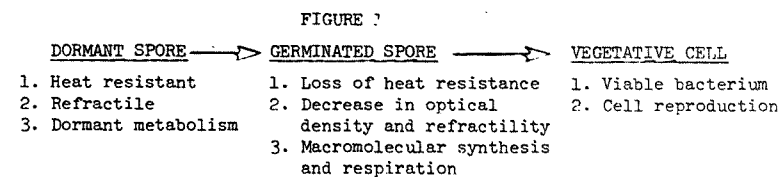


FIGURE 5

STUDIES ON SPORE INACTIVATION

A. Description: The requirement for spacecraft sterilization using techniques not deleterious to spacecraft components has lead to studies on the effect of heat and radiation on spore viability. This work has established that heat and ionizing radiation inactivate spores in a synergistic manner. The basis for such a synergism may lie in the different mechanisms by which these sterilization treatments effect microbial death. It is the mechanistic basis of how various sterilization treatments cause spore inactivation which is the subject of current study. The working hypothesis is that ionizing radiation effects inactivation by both breaking covalent bonds in critical spore molecules and by promoting free radical reactions in the spore, while heat exerts its sterilizing effect by a thermal denaturation of macromolecules. In the course of this study, it became apparent that use of ultraviolet radiation (U.V. radiation) offered advantages in studies on spore response to sterilization treatments. The advantages are related to the use of a similar sterilization technique and experimental accessibility.

Insight into the mechanism of spore inactivation by various treatments may be obtained by studying the effect of such treatments on the stages involved in the transformation of a spore into a vegetative cell. These stages together with some of their properties are as follows:



Inactivation treatments can function either by interfering with the spore's ability to transform into the germinated state or by interfering with the germinated spore's capacity for macromolecular synthesis and cell division. Since these stages are biochemically and physiologically distinct, information about the mechanism of inactivation by various techniques must begin by identifying the spore state at which the treatments exert their influence. Such studies have been initiated.

B. Progress.

1. Irradiation of spores, deposited as a uniform layer using an electrostatic deposition device, was accomplished with a Jarrell-Ash ultra-violet lamp. The spore survivor curve as a function of U.V. dose (Fig. 2) shows that "tailing" had occurred to a significant degree. "Tailing" in survivor curves may be attributed to at least two causes-- a resistant spore sub-population, or the presence of a functional repair mechanism in the microbial system. Light-dependent and light-independent (dark repair) repair systems are known to function in spores damaged by exposure to U.V. irradiation. That the light-dependent repair system was not a factor was shown by repeating the work in the absence of light and again observing "tailing" in the survivor curve.

A model relating the probability of spore survival to the competing processes of spore damage and damage repair has been proposed. This model predicted a dependence of spore survivor level ("tailing") on radiation dose rate. Figure 2 shows that such a dependence was experimentally observed. Figure 2 also bears on the question of

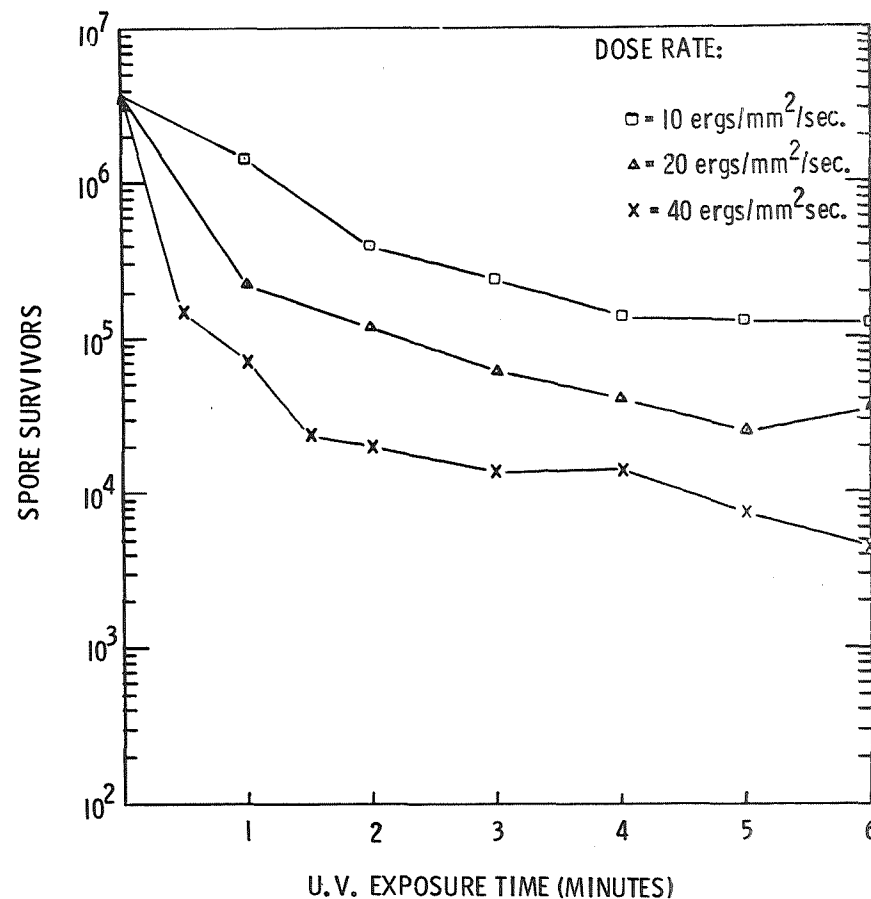


FIGURE 2. U.V. SURVIVAL CURVES AT VARYING DOSE RATES

whether "tailing" may be due to a resistant spore sub-population. If such a sub-population were present, then extrapolation of the "tailing" portion of the survivor curve to the ordinate should yield a common intercept point for all dose rates. Such a common intercept at the ordinate, which would indicate a fixed percentage of resistant spores in the population, is not observed. These results suggest that spores of *B. subtilis* var. *niger* have a functional repair system that can be of practical concern in efforts to achieve low levels of contamination.

"Tailing" of survivor curves has been observed in dry heat and thermo-radiation sterilization of spores but at much lower survival levels than observed with U.V. irradiation. This suggests the possibility of studying the "tailing" phenomena using the U.V. system as an experimentally approachable model.

"Tailing" was still observed when spores were treated with heat or ionizing radiation and then subjected to U.V. irradiation. Heating of spores before or after U.V. irradiation did not change the characteristic "tailing" of the U.V. survivor curve. Identification of a possible synergism between the sterilization treatments is difficult due to this observed tailing phenomena.

2. As noted in Figure 1, the conversion from dormant spore to germinated spore can be followed by observing the change in optical density of a spore suspension. This technique was used to ascertain which spore state was being affected by the various inactivation treatments.

No change in the germinating capacity of the spores was observed after treatment of the spores with amounts of ionizing or U.V. radiation

sufficient to prevent at least 90% of the spores from becoming vegetative cells (forming colonies). Inactivation of 90% of a spore population by heat resulted in a slowing of the germination rate to about one-half the unheated control rate, but germination was still observed. These studies indicate that the sterilizing techniques of gamma radiation, U.V. radiation, and heat do not prevent dormant spores from becoming germinated spores. These sterilizing treatments must function by preventing the germinated spores from becoming vegetative cells.

During the course of this study it was observed that ethanol at very low levels (0.7%) caused inhibition of spore germination. The spores did not germinate in a media containing low levels (0.7%) of ethanol. If, however, the spores were removed from the ethanol containing germinating medium by centrifugation and then resuspended in germinating media free of ethanol, then germination proceeded normally. The low levels of ethanol required for inhibition and the reversal of this inhibition by ethanol removal suggests that perhaps a spore enzyme, responsible for the initiation of the events associated with spore germination, had been reversibly inhibited by the ethanol. The observation that spore inactivation by heating caused a reduced germination rate is also consistent with the presence of such a spore enzyme, since thermal denaturation may have reduced the effective enzyme concentration and, therefore, lowered the germination rate. An alternate possibility is that the reduced rate of spore germination after heating may have resulted from thermal decomposition of spore

structure which induced germination by a mechanical process. This possibility was studied by adding ethanol (0.7%) to the heat inactivated spores and noting the effect on germination. Ethanol inhibited spore germination in the heat-inactivated spores as it had in the viable spore population. This finding is consistent with the premise that the slower germination rate of heat-inactivated spores is due to a lower spore enzyme concentration rather than to a heat induced rupture of the spore.

These germination studies have shown that the effect of ionizing radiation, heat, and U.V. irradiation in spore inactivation is at the level of macromolecular synthesis and microbial replication and not at the level of conversion of the spore from dormant to germinated state. The presence of an enzyme system required to effect the transformation from dormant spore to germinated spore is also indicated.

MODELING OF THERMO-RADIATION SYNERGISM

- A. Description. The objective of this activity is to develop a physically based model representing bacterial spore inactivation in lethal environments combining both heat and ionizing radiation. The modeling effort is based upon experimental activity of the same nature described elsewhere in this report. The first phase of this activity has been an attempt to combine the rational model of spore inactivation in thermal environments previously developed, with an empirical model representing the effects of radiation.
- B. Progress. Significant progress toward the phase one objective has been made this quarter. The modeling has been facilitated by the fact that all of our survivor data in combined thermo-radiation environments has been logarithmic in character. Thus, the expected number of survivors $E(n(t))$ has been given by an expression of the form

$$E(n(t)) = n(0)10^{-t/D} = n(0)e^{-kt}$$

where $n(0)$ is the initial population, D is the D value, and k is the reaction rate of an assumed first order inactivating reaction.

The modeling effort to date has consisted of determining k as a function of temperature T and radiation dose rate r_d . The rationale used to develop such an expression for k was, in part, the following. First, it was assumed that the activation enthalpy of the combined heat-radiation rate process must be the same as that associated with heat alone. Thus, the enthalpy term in k is of the form

$$e^{-\Delta H^\ddagger/RT}$$

where the activation enthalpy $\Delta H^\ddagger$ is that associated with heat alone (i.e., $r_d=0$). Secondly, it was assumed that this enthalpy term must be multiplied by some expression containing

$$\frac{KT}{h} e^{\Delta S^\ddagger/R}$$

where $S^\ddagger$ is the activation entropy for heat only. Such a multiplicative expression must reduce to this entropy term when $r_d=0$, so that k in hr^{-1} must take the form

$$k = \left[3600 \frac{KT}{h} e^{\Delta S^\ddagger/R} f(r_d) \right] e^{-\Delta H^\ddagger/RT}$$

where 3600 appears to compensate for the appearance of sec^{-1} in Boltzman's constant K , and where $f(r_d)$ is some function of radiation dose rate that becomes zero when r_d is zero. Additionally, one expects $f(r_d)$ to be dependent upon temperature because of the observed synergism in inactivation in a combined heat and radiation environment.

The function $f(r_d)$ was determined empirically using thermo-radiation data for *Bacillus subtilis* var. *niger* presented elsewhere in this report. For this data it was found to be of the form

$$f(r_d) = e^{6.15 e^{14115/T} r_d^{218/T}}$$

These same data indicate that for the spore stock being used, $\frac{\Delta H^\ddagger}{R} = 16890$ and $\frac{\Delta S^\ddagger}{R} = 6.36$ when only heat is used as the inactivating agent.

Letting $3600 = e^{8.19}$, k then takes the form

$$k = \left(\frac{KT}{h} e^{14.55} e^{6.15 e^{14115/T} r_d^{218/T}} \right) e^{-16890/T}$$

where

k is in hr^{-1}

K is Boltzman's constant

h is Planck's constant

T is in degrees Kelvin, and

r_d is the radiation dose in kilorads/hr.

This model provides a good fit of all experimental data obtained to date. This data has been taken in the ranges where T was between 23°C and 125°C and r_d varied between 0 and 62.5 kilorads per hour. Figure 1 shows the agreement to date along with some other predictions of k as a function of temperature and dose rate. The solid lines represent model predictions, and triangles the data obtained so far. Further experimentation will be carried out to assess the correctness of other predictions.

Reaction Rate of *Bacillus subtilis* var. *niger* Spore
Inactivation as a Function of Temperature and
Radiation Rate.

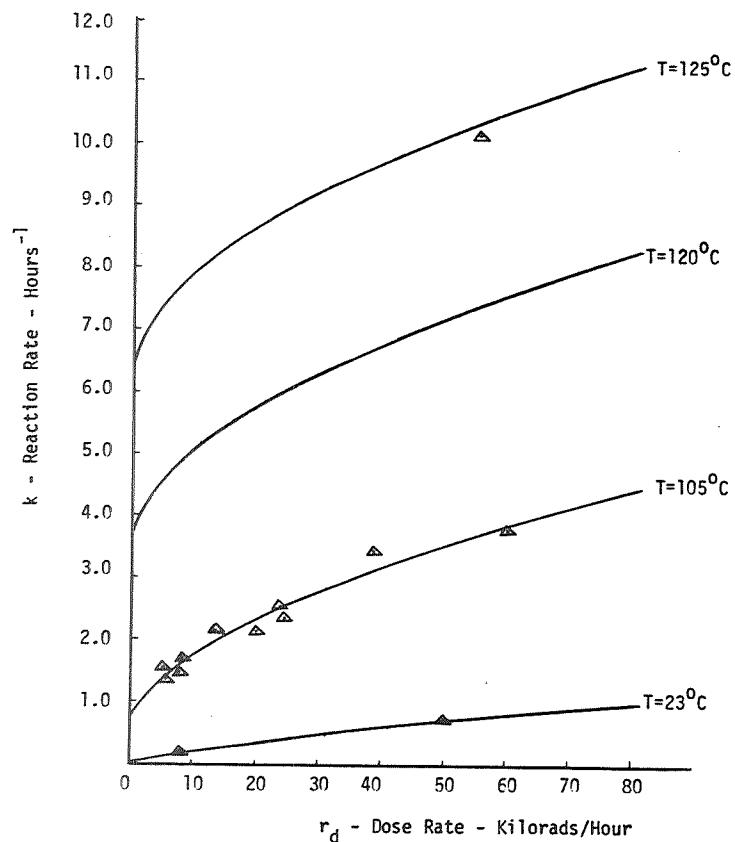


Figure 1

It is interesting to note that there is a definite correlation between this model and data describing the processing of commercial polymers using heat and/or radiation. Polymeric materials are formed chiefly by a free-radical type reaction, and the reaction rate is characterized by activation energies much smaller than that of ordinary chemical reactions. For temperatures below about 50°C , the thermo-radiation model presented here has a dominant term

$$e^{6.15} e^{-2775/T_d^{218/T}}$$

the first part of which may be thought of as

$$\frac{KT}{h} e^{-\Delta F^{\ddagger}/RT}$$

where $\Delta F^{\ddagger}$, the activation energy, is quite small compared to the activation energy of heat inactivation alone. Also, the dependence of the reaction rate upon a fractional power of the radiation dose rate parallels the behavior of polymerization by a free-radical mechanism. These observations suggest that spore inactivation in thermo-radiation environments may be due to free-radical reactions when temperature is not the dominant inactivation agent. There have been other recent reports in the literature which suggest that degradation of macromolecules in a radiation environment may result primarily from free-radical reactions, lending credence to the model, and suggesting possible approaches for physically interpreting $f(r_d)$.

Bioburden Experimentation and Modeling

- A. Description. Models for the estimation and prediction of spacecraft bioburdens are needed for use in spacecraft assembly environments. Ideally, these models should have bioburden outputs about which some statistical confidence statement can be made, and, of course, they should be capable of rigorous verification.
- B. Progress. This quarter a prediction model to complement the estimation model already developed and used in the Apollo program has been developed. An experimental program to investigate a parameter common to both of these models is underway. In general, the bioburden of a spacecraft may be divided into two categories: that which is deposited by the contact of humans and tools, and that which is not. The latter portion is said to be "fallout" from the environment. On the other hand, humans may, and undoubtedly are, the major source of environmental organisms in controlled assembly environments. In order to incorporate both types of bioburden into estimation and prediction models some assumptions must be made.
1. When rigorous clothing requirements are set on and work procedures followed by assembly personnel, most normal human flora, and particularly organisms not indigenous to human beings that are deposited by contact are organisms that came from the environment, first to a glove or tool, and, thence, to a surface. This should be true of spores, for example.
 2. Organisms in an environment tend to occur in clumps. It is assumed that the distribution of organisms (of whatever class one is considering) on these environmental clumps is the same whether the clumps be in the environment, on a surface, glove or tool.

With these assumptions, let the random variable $X(t)$ represent the number of microorganisms on a given surface at time t . Assume that the surface which we are discussing is chosen such that it sees a spatially uniform environment that may vary with time and has uniform removal characteristics (i.e. same material, same temperature, same humidity, etc.). Let $N_{\max}$ be some maximum number of microorganisms we could possibly find on a surface. We know that $X(t)$ takes on values with the range $0, 1, 2, \dots, N_{\max}$. Define $P_{ij}(\tau, t) = \text{Prob} \{X(t) = j \mid X(\tau) = i\}$. Assumptions 1) and 2) imply that microorganisms either fall or are placed on the surface in clumps and that they are for all practical purposes removed from the surface by either death, decontamination, or other physical means in clumps. Let us further assume that we are considering particles of like "deposition" and "removal" characteristics. Define Y to be the random variable representing the number of microorganisms per clump and let

$$q_j = \text{Prob.}\{Y = j\}$$

for

$$j = 0, 1, 2, \dots, N_{\max}.$$

Assumption 2) allows us to conclude that the stochastic process governed by $P_{ij}(\tau, t)$ is a Markov process.

Let $\lambda_m(t)h+O(h)$ be the probability that a deposition of a particle occurs between t and $t+h$ when $X(t) = m$ and $\mu_m(t)h+O(h)$ be the probability that a particle is removed from the surface between t and $t+h$ when $X(t) = m$.

To be physically reasonable, we see that $\lambda_m(t)$ should not depend upon the number of organisms, m , present on the surface. Thus, we denote it by $\lambda(t)$. If we let the mean of the random variable Y be γ , then we shall assume

$$\mu_m(t) = \mu(t) \frac{m}{\gamma},$$

that is, the removal shall depend on the expected number of clumps, not the expected number of microorganisms, and this dependence takes the form that might reasonably be encountered in the "death" of these particles.

We now observe that

$$\lim_{h \rightarrow 0} \frac{1 - P_{jj}(t, t+h)}{h} = (\lambda(t) + (t) \frac{1}{\gamma})(1 - q_0)$$

$$\lim_{h \rightarrow 0} \frac{P_{jk}(t, t+h)}{h} = \begin{cases} \lambda(t) q_{k-j} & \text{if } k \geq j \\ \mu(t) \frac{1}{\gamma} q_{j-k} & \text{if } k < j. \end{cases}$$

Using the usual derivation of the Chapman-Kolmogorov equation, we obtain the Kolmogorov Forward Equations, namely,

$$\frac{\partial P_{ik}(\tau, t)}{\partial t} = -(\lambda(t) + \mu(t) \frac{k}{\gamma})(1 - q_0) P_{ik}(\tau, t) + \sum_{j=k+1}^{N_{\max}} \mu(t) \frac{1}{\gamma} P_{ij}(\tau, t) q_{j-k} + \sum_{j=0}^{k-1} \lambda(t) P_{ij}(\tau, t) q_{k-j} \quad (1)$$

where

$$P_{ik}(\tau, \tau) = \begin{cases} 1 & \text{if } k=i \\ 0 & \text{otherwise} \end{cases}$$

Several observations are in order:

1. If $\lambda(t) = \lambda$ and $\mu(t) = \mu$, then the Markov process is homogeneous.
2. There is a nonzero probability of $X(t)$ going between any two states.
3. If 1 is true, then, 2. implies that

$$\lim_{t \rightarrow \infty} P_{ik}(\tau, t) = \pi_k.$$

independent of the initial conditions and these can be obtained by solving the equations obtained from (1) by setting $\frac{\partial P_{ik}(\tau, t)}{\partial t} = 0$.

This is true under more general conditions.

4. If $q_i = \begin{cases} 1 & j=1 \\ 0 & \text{otherwise} \end{cases}$, then (1) reduces to the standard birth and death equations.
5. If $\lambda(t)$ and $\mu(t)$ are bounded and continuous then the solutions of (1) are also bounded.

A solution $P_{ij}(\tau, t)$ of the system of equations (1) yields a statistical prediction of the bioburden at time t when some initial conditions (bioburden at time τ) are prescribed. Thus, one might hope to use these solutions as a prediction model for bioburdens.

With this observation, the implementation of this approach may be accomplished as follows. Suppose that the entire assembly procedure takes place between t_0 and t_f and that samples will be taken at the times $t_0, t_1, \dots, t_f$. Assume that after t_f the surface cannot be further sampled prior to sterilization. Let us assume that on each subinterval $[t_k, t_{k+1}]$ $\lambda(t)$ and $\mu(t)$ have some prescribed form (possibly at first a constant). If q_j is a known distribution which has been measured independently in a lab-

oratory, then, since we can assume that the number of clumps residing on a surface follows a birth and death process, we can obtain the distribution on a surface at a fixed time using a mixing procedure. For example, if Y is Poisson distributed and $N_{\max}$ is large, the steady state solution to the system (1) takes a form like the estimation model already developed and reported on in Sandia Laboratories Research Report, SC-RR-69-23, January, 1969. Sampling at the times t_k allows us to obtain the parameters of this distribution. Using this, we would then have $S_j(t_0)$ and $S_j(t_1)$ for $j=0,1,\dots,N_{\max}$, where $S_j(t) = \text{Prob}\{X(t) = j\}$.

Using optimization procedures we could then obtain $\lambda^{(1)}(t)$ and $\mu^{(1)}(t)$ which cause the solution of (1) to assume these boundary conditions or would approximate them in some norm. At time t_2 , we would then have $S_j(t_2)$ for $j = 0,1,\dots,N_{\max}$.

We could then try $\lambda^{(1)}(t)$ and $\lambda^{(2)}(t)$ to see if the solution of (1) on the interval $[t_1, t_2]$ assumed the appropriate boundary conditions. If it does, let $\lambda^{(2)}(t) = \lambda^{(1)}(t)$ and $\mu^{(2)}(t) = \mu^{(1)}(t)$. If it does not, we obtain $\lambda^{(2)}(t)$ and $\mu^{(2)}(t)$ so that the solution of (1) assumes or approximates the boundary values $S_j(t_1)$ and $S_j(t_2)$.

We also may wish to obtain $\hat{\lambda}^{(2)}(t)$ and $\hat{\mu}^{(2)}(t)$ on $[t_0, t_2]$ in the same family so that we minimize

$$\sup_j \sum_{i=1}^2 |P_{ej}(\tau, t_i) - S_j(t_i)|^2$$

This would take into account our sampling errors.

We would continue in this fashion through $t=t_f$.

We would then have two sets of functions. The first are functions which are in a prescribed class on each subinterval (such as a constant on each subinterval). Thus we would have

$$\lambda(t) = \lambda^{(k)}(t), \quad t_{k-1} \leq t \leq t_k, \quad k = 1, \dots, f$$

(2)

and

$$\mu(t) = \mu^{(k)}(t), \quad t_{k-1} \leq t \leq t_k, \quad k = 1, \dots, f$$

which have been determined such that

$$P_{ij}(\tau, t_k) = S_j(t_k), \quad k = 0, 1, 2, \dots, f; \quad j = 0, 1, \dots, N_{\max},$$

or such that $P_{ij}(\tau, t_k)$ approximates $S_j(t_k)$.

The second set of functions are functions from this class (e.g., a constant). They are chosen to be

$$\lambda(t) = \hat{\lambda}^{(f)}(t)$$

(3)

and

$$\mu(t) = \hat{\mu}^{(f)}(t)$$

which have been determined to minimize

$$\sup_j \int_{e=0}^f |P_{ij}(\tau, t_e) - S_j(t_e)|^2$$

over all functions of this class.

Then, depending on what the spacecraft experiences after t_f , we can choose $\lambda(t)$ and $\mu(t)$ to be those given in (3) for use in our predictions down stream from t_f or, we could choose a "deposition" to be an upper bound on the $\lambda(t)$ in (2) and the "removal" to be a lower bound on the

$\mu(t)$ in (2). The choice of $\lambda(t)$ and $\mu(t)$ could be based on a comparison of the results of environmental sampling and the amount of activity after t_f and that on each subinterval before t_f .

Some other observations about the model may be made.

1. If we could find the forms of $\lambda(t)$ and $\mu(t)$ in the laboratory, then we could use equation (1) to determine the t_k 's $k = 1, 2, \dots, f-1$.

To do this we would want to limit the amount of change we would permit in $P_{ij}(t, t)$ on each interval $[t_k, t_{k+1}]$.

2. Knowing $\lambda(t)$ and $\mu(t)$ we can then get confidence intervals on our parameters which would allow us to work at the 95%, 99%, etc. confidence limits.
3. Knowing the forms of $S_j(t_k)$ we can also obtain information regarding the number of samples required at each sampling period. This is illustrated in SC-RR-69-23 referenced above.

In both this prediction model, and the previous estimation model, it is necessary to know Y and its mean. This is necessary for making any a priori predictions for planning, using the prediction model as outlined above, and calculating required numbers of samples from the estimation model. In order to begin to investigate the random variable Y , instrumentation has been developed and is being tested to measure the number of microorganisms per fixed number of particles for given size ranges. The hypothesis that the samples taken are from a Poisson distribution can also be tested.

The instrument that has been developed is a Royco particle counter placed in parallel with a modified Andersen sampler. The Royco counts

and sizes particles and the Andersen sampler sizes and enumerates organism-bearing particles (clumps).

A sequence of tests was conducted using the apparatus (QR 14) designed to measure the mean number of microorganisms per particle. The device consisted of a Royco particle counter in series with an Andersen sampler. The tests showed that it was impossible to collect a large enough sample of organism-bearing particles to be meaningful before the plates of agar on the Andersen sampler dried.

Two modifications of the tests were considered. Either experiments could be performed in which the air to be sampled was heavily loaded with organism-bearing particles by dusting with an air jet, or a sampler could be devised which sampled considerably larger volumes of air. Since the particle size distribution and the distribution of microbes per particle might be changed by dusting with an air jet, it was decided to attempt to modify the Andersen sampler to make it sample considerably larger volumes of air.

The Andersen sampler was modified in that absorbent pads replaced the nutrient agar normally used as a collection surface. An electronically-controlled water level system keeps the pad wet at all times during the sampling period. This modification enables the sampler to be used for an extended period of time.

An evaluation of this instrument is underway.

Computerized Identification Scheme

A. Description. Sandia was asked to investigate the feasibility of computerizing the identification of colonies sampled during the outbound contamination inventory of Apollo spacecraft with a view to possible applications to future Apollo missions. This identification was to be performed by the use of the colony test data available in QUALSUM reported on in the previous Quarterly Report in conjunction with the identification procedures described in "Identification Schemes for Microorganisms Isolated from Apollo Spacecraft", USPHS, NCDC, Phoenix.

B. Progress. This activity was completed during the past quarter. The overall activity will be discussed in the following three categories:

1. Problem analysis,
2. Programming, and
3. Results.

In analyzing the problem, present practices were considered. For the outbound contamination inventory program, microorganisms were isolated from Apollo spacecraft as colonies to be identified. These colonies were to be classified as belonging to one of 95 possible categories. To perform this classification the results of (possibly up to 64) tests performed by the Bioassay Unit of the Public Health Service are recorded on a form. At present the Bioassay Unit also determines the category to which the colony belongs and records it on the form. As part of the Lunar Information System the data from these forms is processed and is available in the computer subroutine QUALSUM. Thus, the problem was: could a program be written which

could take the data, say from QUALSUM, and determine the appropriate category for the sample? This essentially amounts to simulation of the PHS identification system in a computer program. The PHS identification system consists of two essentially different parts. The first part is a flow diagram which uses the results of five tests to enter the second portion of the system. This second part consists of thirteen schemes (schemes A through M) which permit an identification to be made on the basis of certain tests selected by the outcomes of the five tests used in the first part. The schemes used in the second part vary from complicated keys relating a number of tests and organisms, to simple keys which admit an identification based only upon the five tests used in the first part of the scheme.

When the first portion of the identification system is analyzed in terms of the data reported by the PHS, it seems to have the form of a decision tree in which each decision is essentially of a yes-no character. Because of this, the simulation of this portion was quite straightforward. Thus, simulation of the identification system reduced to reading the five results necessary to determine which of the thirteen schemes of the second part should be entered and a simulation of each of these thirteen schemes. In general, the schemes of the second part of the system consisted of a matrix relating (through test results) tests and the identify of the organisms associated with the scheme. Hence, given a colony known to belong to some scheme, the outcomes of the required tests could be read from QUALSUM as a Test Vector and compared with the scheme matrix until a match was obtained which would provide the desired identification.

In exercising the program with data, it developed that frequently an exact match did not occur between the unknown Test Vector and results associated with any organism category in the scheme to which it presumably belonged. As a result the "best possible" match (or identification) was considered. This could occur in a number of ways. An identification could occur with an exact match except for a single test result which disagreed with that anticipated for the organism category. It might happen that there was a single disagreement with the results to be anticipated for two distinct organism categories or three or more organism categories. Similarly, there may be disagreement on two tests for one or more organism categories. As a result of these considerations, the computerized system indicates the level at which an identification is made in accordance with the following table.

No. of Test Disagreements	No. of Categories	Computer Statement of RESULT
0		EXACT IDENT
1	1	ONE DISAGREEMENT
1	2	2 SINGLE DISAGREEMENTS
1	3	3 SINGLE DISAGREEMENTS
2	1	TWO DISAGREEMENTS
Other		NO ID POSSIBLE

As indicated above these options imply that multiple identifications will occur e.g. 3 SINGLE DISAGREEMENTS with three organism categories being equally possible using these selection rules. The level at which an identification is made could be varied at will. The above was selected as representing current PHS practice with the Apollo data.

In accordance with the above considerations a program was written which simulated the PHS identification system. To determine the feasibility of this approach the program was exercised with the Apollo 11 data as obtained from QUALSUM. Figure 1 is a sample page of the computer output. The first group of columns under the heading SCHEME A TESTS in Figure 1 are (in order) the test results as reported by the PHS. The group of columns headed TEST VECTOR are the results of interest in Scheme A after conversion for computer use. They are obtained from the particular column of the SCHEME A TESTS headed by the digit indicating their order of occurrence. This TEST VECTOR is compared with the columns of the matrix representing the Scheme A key. The results of this comparison are contained in the MATCH VECTOR columns. These columns contain the number of tests which disagreed with the TEST VECTOR for each of the fourteen organism categories of Scheme A. Finally, the column headed TEST ID lists the category number of the PHS identification while that headed SCHEME ID lists those category numbers assigned by the program. When the SCHEME ID column contains, among its choices, the TEST ID assigned by the PHS, the scheme was considered to agree with the PHS identification even though the SCHEME ID may not have been unique. This is illustrated below.

Most of the Apollo 11 data was identified as Staphylococcus or Micrococcus which used Scheme A of the Identification System. A summary of the computer identification for Scheme A data is as follows:

Figure 1. Sample Output of Computerized

No. of Test		Organism Category Number														Total
		Disagree	Categ.	1	2	3	4	5	6	7	8	9	11	13	14	
0			12	261	4	59	78	77	41	11	14	13	11	0	581	
1	1		7	0	0	157	128	40	16	2	1	1	59	1	412	
1	2		3	82	0	18	2	30	13	67	18	9	5	0	247	
1	3		5	1	2	3	0	1	108	0	0	0	0	0	120	
2	1		0	0	0	2	0	0	2	2	1	2	2	0	11	
No ID			0	3	0	2	1	5	26	19	2	0	27	0	85	
Not A			1	8	0	1	6	1	1	2	0	1	0	0	21	
Total			28	355	6	242	215	154	207	103	36	26	104	1	1477	
Disagree			4	1	1	23	5	3	20	1	10	2	11	0	81	5.5%
No ID			0	3	-	2	1	5	26	19	2	0	27	0	85	5.7%
Not Scheme A			1	8	0	1	6	1	2	0	1	0	0	0	21	1.4%
Total			5	12	1	26	12	9	47	22	12	3	38	0	187	12.7%

Summary of Computer Identification for Scheme A with Apollo 11 Data

The above table gives the frequency with which multiple identifications occurred as well as summarizing the agreement with the PHS identification. There was disagreement with PHS for 12.7% of the data given, 83.7% agreement including, in about one third of these cases, a multiple identification from the computerized system which contained the PHS identification.

A more complete summary of the computerized system status is as

follows:

		Apollo 11						
Scheme	Scheme Outcomes	Samples Identified	Program Status	Disagree	No ID	Not Scheme	Total	% Ident
A	14	1,477	Done	81	85	21	187	87.3
B	4	5	Not Done#					
C	18	66	Done	22	0	0	22	66.7
D	1*	183	Done	0	0	0	0	100.0
E	1	0	Immediate	-	-	-		
F	4	12	No Scheme	-	-	-		
G.	1	0	Immediate	-	-	-		
H	1	0	Immediate	-	-	-		
I	40	0	Not Done#	-	-	-		
Subtotal		1,743		103	85	21	209	87.9

* D has four outcomes solely dependent upon the General Scheme.

Only 5 samples of Viridans

‡ No data

(Con't)	Apollo 11	
	Samples	
Scheme	Identified	
J	7	} Insufficient Data or No Scheme
K	0	
L	8	
M	34	
Added	248	
Subtotal	297	
TOTAL	2,040	

Agreement with PHS Identification was obtained for 87.9% of the Apollo 11 data. Again, it is emphasized that for agreement, it is only necessary that the PHS identification be among those listed by the scheme when multiple identification was given (which occurred about one third of the time). As can be seen, the schemes of primary interest for the Apollo data were programmed and run. In some cases either no identification scheme was available or insufficient data was recorded in QUALSUM to enable an identification to be made. Discussions with PHS indicate that some of these difficulties could be removed.

The program also provides a tool which can be used to obtain insight into the identification processes. It can be used to obtain a measure of the effectiveness with which various tests group microorganisms into categories. Thus, the relative effectiveness of various schemes and tests can be estimated.

Scheme C is currently being changed by the PHS. The changes will be incorporated into the computer program and the revised program will then be exercised with Apollo 12 data.

PUBLICATIONS

1. M. C. Reynolds, "The Feasibility of Thermoradiation Sterilization of Spacecraft - A Preliminary Report", SC-RR-69-857, December 1969.
2. J. P. Brannen, "On the Role of DNA in Wet Heat Sterilization of Microorganisms", accepted for publication by the JOURNAL OF THEORETICAL BIOLOGY.

PRESENTATIONS

1. H. D. Sivinski prepared talk presented by W. J. Whitfield entitled, "Present Day Usage of Clean Rooms in Medical and Life Science Research", at the Instrument Society of America Annual Conference, Houston, Texas, Oct. 1969.
2. J. P. Brannen presented a colloquium talk entitled, "Some Applications of Biophysics to Spacecraft Sterilization", University of New Mexico, Department of Physics and Astronomy, October 31, 1969.
3. M. C. Reynolds presented a paper entitled "The Clean Room - A Vital Element in Contamination Control", at the Instrument Society of America, Annual Conference, Houston, Texas, October 27-30, 1969.
4. W. J. Whitfield, "Instrumentation and Methodology in Measurement of Viable and Non-Viable Contamination", International Meeting of the Instrument Society of America, Houston, Texas, October 28, 1969.
5. W. J. Whitfield, "Principles of Contamination Control", Rochester Institute of Technology, Rochester, New York, October 14, 1969.

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