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Effect of Combined Heat and Radiation on Microbial Destruction[1]

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A series of experiments at several levels of relative humidity and radiation dose rates was carried out using spores of *Bacillus subtilis* var. *niger* to evaluate the effect of heat alone, radiation alone, and a combination of heat and radiation. Combined heat and radiation treatment of microorganisms yields a destruction rate greater than the additive rates of the independent agents. The synergistic mechanism shows a proportional dependency on radiation dose rate, an Arrhenius dependency on temperature, and a dependency on relative humidity. Maximum synergism occurs under conditions where heat and radiation individually destroy microorganisms at approximately equal rates. Larger synergistic advantage is possible at low relative humidities rather than at high relative humidities.

One of the more intriguing subjects of the Planetary Quarantine research has been the discovery of the synergistic effect that results from the combination of heat and radiation for bacterial spore destruction. Koesterer (2) observed this effect while carrying out exploratory sterilization studies for the National Aeuronautics and Space Administration (NASA). Scientists at the Sandia Laboratories carried out extensive laboratory and feasibility studies on the use of combined heat and radiation for spacecraft sterilization (5).

This report describes studies carried out at the University of Minnesota to investigate the sterilization attributes of the thermoradiation process. Destruction rate tests were carried out at a number of radiation levels, temperatures, and relative humidities. Both wet- and dry-heat conditions were used. We have attempted not only to develop destruction rate data but also to determine the mechanistic basis for the synergism displayed by these seemingly independent lethal agents.

MATERIALS AND METHODS

Biological procedures. *Bacillus subtilis* var. *niger* spores grown at 32°C in synthetic sporulation medium-10 (3) were used in this study. The spores were cleaned by exposure to ultrasonic energy and by repeated washing with deionized distilled water and centrifugation.

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For the wet-heat studies, the spores were suspended in 5 ml of Sorensen 0.067 M phosphate buffer (pH 7.0) in screw-capped glass test tubes. The population density of the suspension was approximately 10^6 spores per ml. After inoculation the tubes were refrigerated at 4°C until treated. After treatment the tubes were placed in an ice bath until assayed. In the assay procedure, the sample was mixed and a 1-ml portion was diluted in buffered distilled water (described in references 4), and duplicate portions were plated using Trypticase soy agar (BBL).

For the dry-heat studies, a 0.01-ml portion of an ethanol suspension of the spores was deposited on stainless steel planchets (12.7 by 12.7 mm, 10^6 spores per planchet). The planchets were then equilibrated at 22°C, 50% relative humidity, for at least 24 h before treatment. Samples were moved as needed to the University of Minnesota Gamma Irradiation Facility for testing. After treatment, the planchets were placed in ice-cooled flasks until assayed using NASA Standard Procedures (4). In the assay procedure, buffered distilled water was added to each flask, the flask was suspended in an ultrasonic (25 kHz, 0.35 W/cm^2) tank filled with an aqueous solution containing 0.3% Tween 80 (described in reference 4) for 2 min, and duplicate portions of the eluate were plated using Trypticase soy agar.

All inoculation and recovery procedures were carried out in a class 100 clean room. Colony-forming units were counted after 48 h of incubation at 32°C.

Radiation system. The University of Minnesota Gamma Irradiation Facility uses a cylindrical array of cesium-137 sources of approximately 10,000 Ci. The radiation field was mapped using Fricke Dosimetry as a primary reference and a calibrated Victoreen rate meter as a secondary reference.

Before biological testing, areas within the radia-

tion field with the desired intensities were identified. When all of the environmental support equipment was in place in the radiation facility, selected locations were retested using Fricke dosimetry samples in the environmental chambers. The results of these tests indicated that radiation intensities were not significantly affected either by absorption from environmental chamber walls or back scattering from support equipment. Exposure dose rates of 20, 10, and 5 krd/h were used.

Heating systems. For wet-heat experiments, samples sealed in test tubes were placed in an exposure chamber of the type shown in Fig. 1. This chamber was a tubular thin-walled aluminum vessel through which water was circulated from a constant temperature bath. The system heated the sample within 1°C of bath temperature within 50 s after start-up. This time lag was short relative to treatment times used in the experiment.

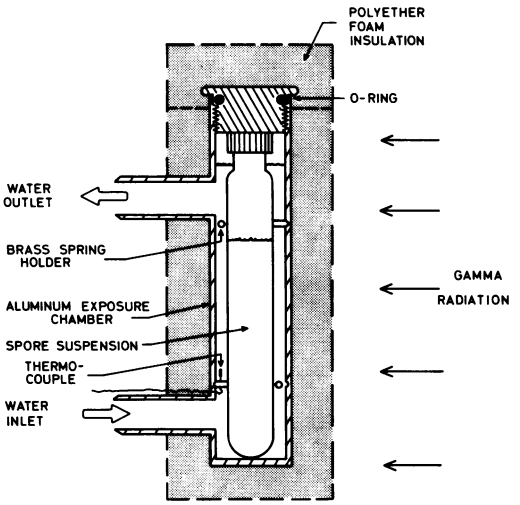

FIG. 1. *Sectional view of exposure chamber used in wet-heat thermoradiation study.*

For dry-heat experiments, the inoculated planchets were clamped in an environmental chamber at positions of known radiation intensities. Temperature control was provided through heaters located along the base of the chamber. The humidity of the air was controlled by controlling the dew point of the air circulating through the chamber; a diagram of the environmental control system is shown in Fig. 2.

The start-up and end procedures of each test were selected to apply or remove both biological stress factors simultaneously. The start or end of the radiation treatment was determined by the position of the radiation source elevator. The end point of the thermal treatments was accomplished by making step changes in the thermal stress environment. For wet-heat experimentation, start was taken as the time when circulation of the heated water was started. The samples were quenched in cool water at the end of the test. For dry-heat experiments, start was the time when the humidity conditions were changed rapidly producing a step change from nonlethal to lethal conditions. At the end of the test a reverse change in humidity was made.

Treatment temperatures were monitored throughout the tests using thermocouples located at each sample site; they were recorded using a temperature-recording potentiometer. Psychrometric conditions were monitored using wet- and dry-bulb thermocouples located in a chamber at the rear of the environmental chamber.

Several spore samples were treated simultaneously. Treatment periods were successive to one an-

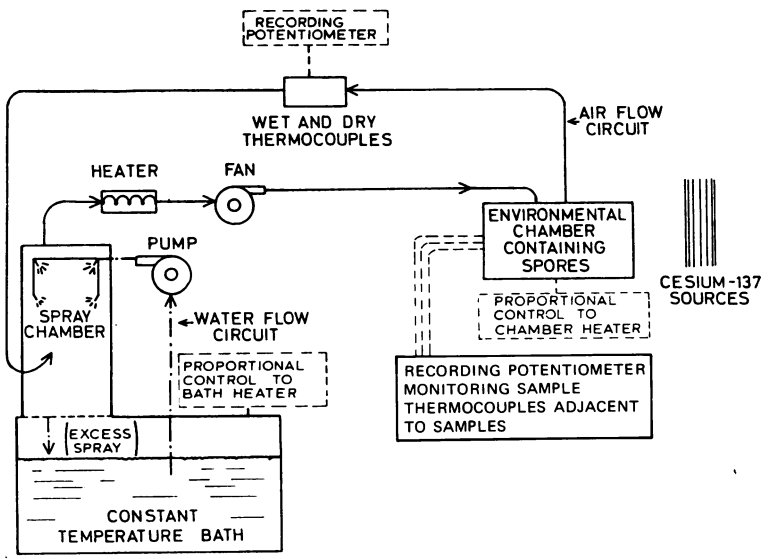

FIG. 2. *Schematic flow chart for the environmental system in thermoradiation experiments.*

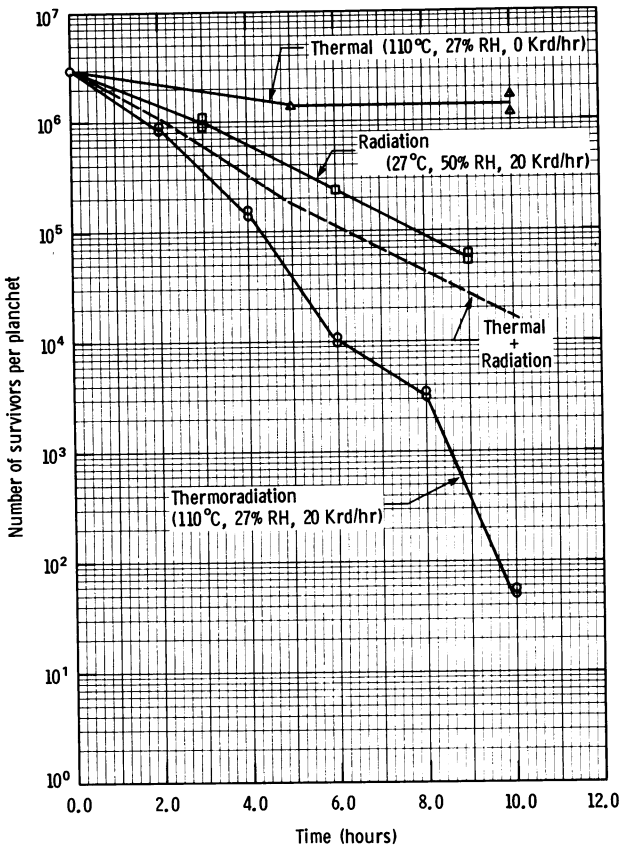

FIG. 3. *Survivor curves for dry radiation, dry-heat thermal, and thermoradiation of B. subtilis var. niger (AAHK) at 110°C, 27% relative humidity, and 20 krd/h.*

TABLE 1. *D-values (hours) at various temperatures and exposure rates for wet-heat thermoradiation of Bacillus subtillis var. niger (AAHF)*

Temp (°C)	D-values (h)			
	20[a]	10	5	0
22	7.0	13.9	28.3	1,141[b]
45	7.6	17.0	37.8	−851[b]
60	11.6	15.7	29.5	2,564[b]
75	6.1	8.8	16.0	67[c]
80	2.3	2.8	3.7	6.1
85	1.0	1.3	1.6	2.1
90	0.35	0.41	0.44	0.50

[a] Dose rates (krd/h).
[b] Essentially infinite for time span of experiments.
[c] Estimate (insufficient data for accurate analysis).

TABLE 2. *D-values at various temperatures and exposure rates for dry-heat thermoradiation of Bacillus subtilis var. niger (AAHK)*

Temp (°C)	Relative humidity (%)	D-values[a] (h)			
		20[b]	10	5	0
27	50	4.4	8.0	17.4	
75	52	3.2	6.8	13.9	1,089
90	28	3.0	6.2	13.0	207
90	55	2.9	6.2	12.2	103
90	82	2.5	3.3	4.3	6.5
105	17	1.9	3.4	4.2	225
105	32	2.4	4.7	11.4	42.1
105	58	1.8	3.4	4.1	4.9
105	70	0.77	0.99	0.89	0.98
110	27	2.1	3.8	8.1	49.5
110	49	1.4	2.6	3.5	11.7
110	59	0.81	1.4	1.8	3.3
125	25	0.78	1.2	1.9	2.6

[a] Geometric mean of two to six *D*-value determinations from separate experiments.
[b] Dose rates (krd/h).

other, and a randomized treatment sequence was followed.

RESULTS

Semilogarithmic survivor curves were constructed from the data gathered at each test condition. The survivor curves for one set of thermoradiation conditions are shown in Fig. 3; the synergistic effect is illustrated by this graph. The destruction rate obtained using the

simultaneous application of heat and radiation is greater than the additive microbial destruction rate of the individual agents.

The D-values obtained from wet- and dry-heat thermoradiation experiments are shown in Tables 1 and 2. The D-value is the reciprocal of the slope of the straight-line semilogarithmic survivor curve; it is measured as the treatment time necessary to reduce the number of spores by 90% and is calculated from the results of the linear regression analysis of spore survivor data. D_T, D_R, and D_{TR} are specific D-values for temperature, radiation, and combined temperature and radiation.

It is convenient to quantify the synergism in terms of a synergism index (SI):

SI = thermoradiation destruction rate/
thermal destruction rate
+ radiation destruction rate

The SI as a function of the relative rates for the thermal and radiation destruction tests for

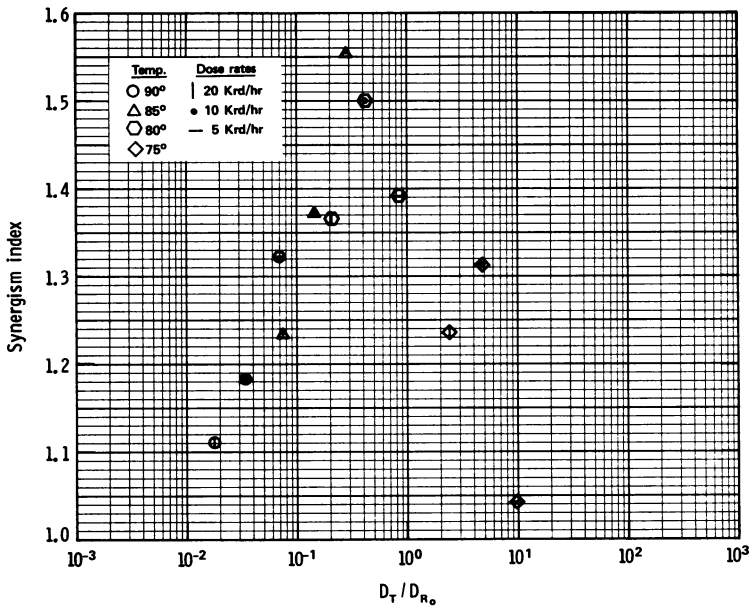

FIG. 4. *Values of SI calculated from experimental wet-heat thermoradiation study.*

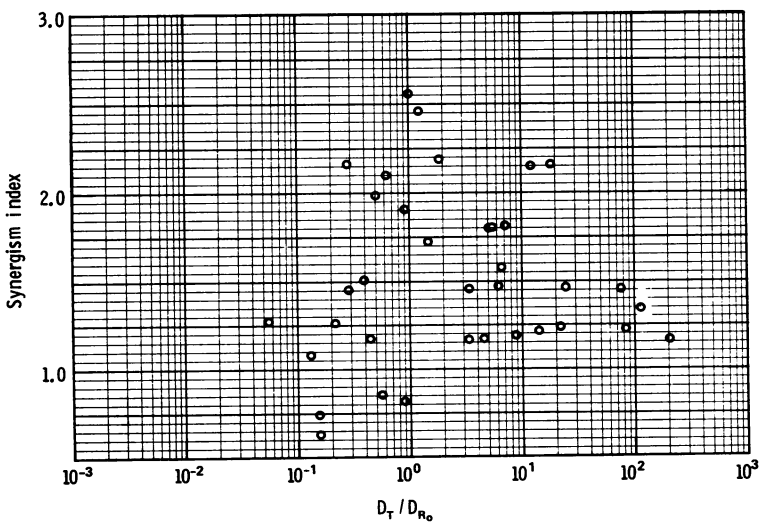

FIG. 5. *Values of SI calculated from experimental dry-heat thermoradiation study.*

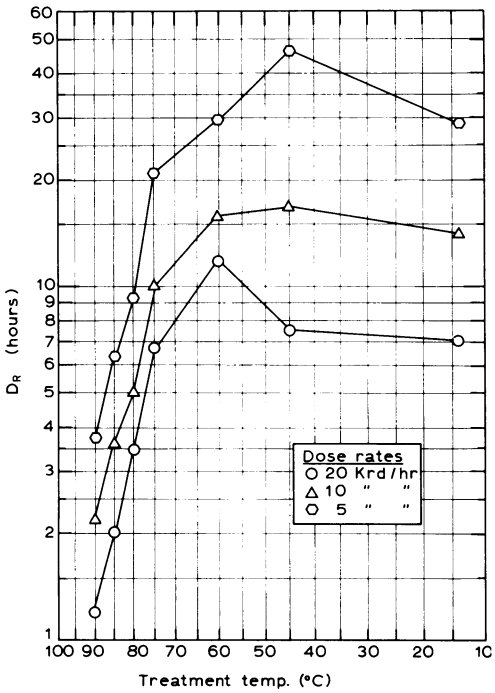

FIG. 6. *Arrhenius plot of the D-value (hours) for radiation-induced death for wet-heat thermoradiation.*

wet heat are shown in Fig. 4 and for dry heat in Fig. 5. (D_{R_0} is the D-value obtained from radiation tests carried out at ambient temperatures.)

DISCUSSION

Insights into the thermoradiation mechanism can be gained by examining the relative experimental response as temperature, radiation dose rate, and psychrometric conditions are varied.

Radiation-induced microbial destruction kinetics are expected to have a temperature dependency of the Arrhenius form: $D_R = ke^{(E_A/RT)}$; where k = preexponential coefficient, e = Naperian base (2.71828 . . .), E_A = activation energy, R = gas constant, and T = absolute temperature. The measured energy of activation was 110 cal/mol over the range −143 to 36°C in *Bacillus megaterium* (6). An Arrhenius plot of D-values for radiation-induced destruction (D_R) from wet-heat thermoradiation experiments is shown in Fig. 6. The wet-heat contribution to microbial destruction has been subtracted using the following equation: $1/D_R = 1/D_{TR} - 1/D_T$; where D_R = D-value for radiation-induced mechanisms, D_{TR} = D-value from thermoradiation experiments, and D_T = D-value from thermal experiment performed at same temperature as thermoradiation experiment. Plotted in this form, the graph would be a straight line if only one mechanism were present with an Arrhenius temperature dependency. In reality, the curves remain approximately horizontal over the lower range of temperatures but break sharply downward over the higher temperature range. This behavior suggests that different mechanisms are dominant over different temperature ranges. At high temperatures, the synergistic mechanism becomes effective.

Wet-heat starts to be an effective sterilization tool at about 75°C. The D-value at that temperature is approximately 67 h. This is the temperature where the synergistic effect also becomes noticeable. When we examined all of our data where synergism was apparent, heat by itself was a lethal agent.

Curves in Fig. 6 corresponding to different radiation levels are similar in shape. Over the range of temperatures studied, the vertical distance between any two adjacent curves remains approximately equal to log 2 (0.301). Thus the preexponential coefficient of the kinetic "constant" is proportional to radiation dose rate. The energy of activation calculated over the temperature range of 75 to 90°C for radiation-induced spore destruction in 27.3 kcal/mol (ca. 114.3 KJ/mol) compared with 65.3 kcal/mol (ca. 273.4 KJ/mol) for thermal destruction over the same temperature range.

In dry-heat thermoradiation experiments, the amount of synergism depends on the relative humidity. A graph of D-values for radiation-induced destruction from dry-heat experiments as a function of relative humidity is shown in Fig. 7. Once again, the thermal contribution has been uncoupled from the total destruction rate. The resulting D-value for D_R shows a dependency on relative humidity. This dependency is of the same form as isothermal D-value data for thermally induced mechanisms (Fig. 8), although it is somewhat weaker. Vertical distance between curves for a 2× dose rate difference is approximately equal to log 2 (0.301).

Comparison of D_R with D_{R_0} shows that D_R is consistently less than D_{R_0}. The differences between D_R and D_{R_0} represent the destruction caused by the synergism mechanism.

The location of the maximum point for synergism is vital in order to take the maximum advantage of thermoradiation as a sterilization method. Optimum advantage is gained when SI is maximized; i.e., thermoradiation death rate is maximized relative to the additive rates of heat and radiation. As seen in Fig. 4 and 5,

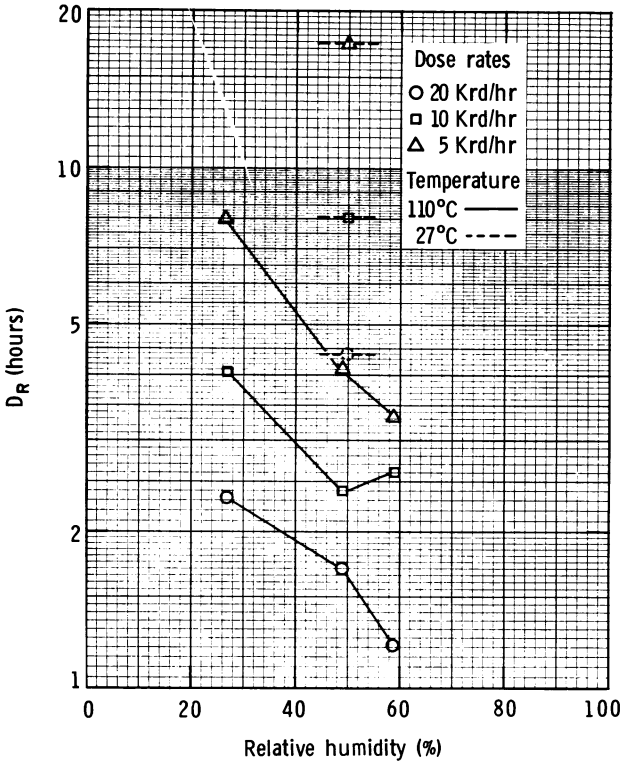

FIG. 7. *D_R-values calculated from thermoradiation treatment of B. subtilis var. niger (AAHK) at various relative humidities and 110°C. (Dashed lines are results from radiation treatment at 27°C and 50% relative humidity.)*

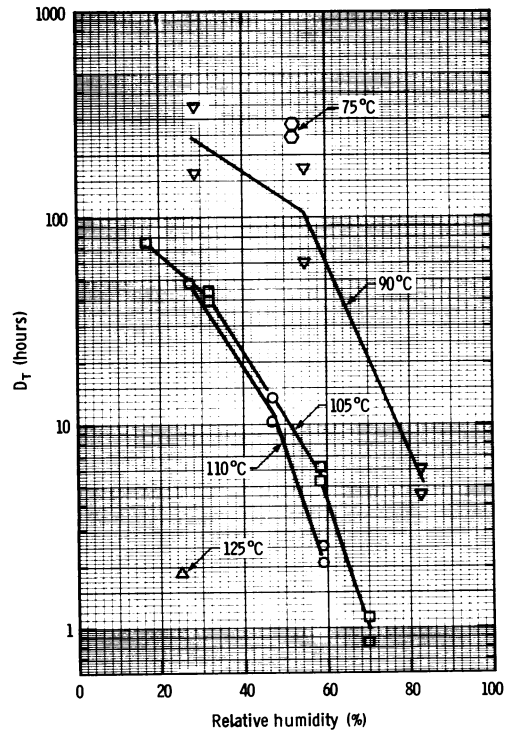

FIG. 8. *D_T-values for thermal treatment of B. subtilis var. niger (AAHK) at various treatment temperatures over a range of relative humidities.*

maximum synergism occurs when heat and radiation are approximately equally effective sterilization agents.

The maximum synergistic advantage depends on the spore water level. Dry-heat thermoradiation gains more synergistic advantage than does wet heat. SIs of 2.5 are possible with dry heat, whereas with wet heat, the maximum SI is limited to about 1.5. An implicit result is that a larger synergistic advantage is possible at low relative humidities rather than at high relative humidities.

We believe that the synergistic effect is a consequence of the need to degrade a single or pair of vital macromolecules at a multiple of locations in order to "kill" the microbial spore. A mathematical model describing such a process has been developed which a priori predicts the experimental phenomena (1).

Conclusions. From this study we conclude the following. (i) Radiation and heat display a synergistic effect in the destruction of microbial spores. (ii) No synergism is possible unless each physiological stress is great enough to effectively destroy spores by itself. (iii) The synergistic mechanism has characteristics resembling each of the constituent agents; a proportional dependency on radiation dose rate, an Arrhenius dependency on temperature, and it is affected by relative humidity. (iv) Maximum synergism occurs at those conditions where heat and radiation are equally effective as sterilization agents.

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LITERATURE CITED

1. **Fisher, D. A., and I. J. Pflug.** 1975. Semiannual Progress report no. 14, NASA grant NGL 24-005-160. Environmental microbiology as related to planetary quarantine. University of Minnesota, Minneapolis.
2. **Koesterer, M. G.** 1965. Thermal death studies on microbial spores and some considerations for the sterilization of spacecraft components. Dev. Ind. Microbiol. 6:268–276.
3. **Lazzarini, R. A., and E. Santangelo.** 1967. Medium-dependent alteration of lysine transfer ribonucleic acid in sporulating *Bacillus subtilis* cells. J. Bacteriol. 94:125–130.
4. **National Aeronautics and Space Administration.** 1968. NASA standard procedures for the microbiological examination of space hardware, NHB 5340.1A. National Aeronautics and Space Administration, Washington, D. C.
5. **Sivinski, H. D.** 1972. Final report on planetary quarantine activities, N72-30064#; NASA-CR-127835. SC-RR-72 0516. Sandia Laboratories, Albuquerque, New Mex.
6. **Webb, R. B., C. F. Ehret, and E. L. Powers.** 1958. A study of the temperature dependence of radiation sensitivity of dry spores of *Bacillus megaterium* between 5°K and 309°K. Experimentia 14:324–326.