ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

Nineteenth Quarterly Report of Progress

Research Project R-36-015-001

October 1, 1969 - December 31, 1969

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Introduction

In our last report we described a modified spore preparation procedure and gave data for thermal inactivation of Bacillus subtilis var. niger spores at 125°C under dry heat conditions with an initial spore concentration of 10^6 per test unit and final concentration after heating that approximated 10^2 per test unit. In this quarter we (a) performed preliminary studies on the inactivation of B. subtilis var. niger in the range of mean concentrations of 10^6 to 10^{-2} test organisms per sample using this modified system, (b) determined the amount of water in the system when spores were exposed to heat, and (c) characterized 1247 isolates from Apollo 12.

I. THERMAL INACTIVATION STUDIES

A series of exploratory experiments were done to determine spore survivors by dry heat inactivation in the range of 10^6 to 10^{-2} survivors per test unit with emphasis on the < 10 to 10^{-2} range of survivors. Bacillus subtilis var. niger spores suspended in 95% ETOH were diluted in sterile phosphate buffered water and were dispensed with a microburette in 0.01 ml amounts into sterile stainless steel cups so that there were approximately 10^6 spores per cup. The sample cups were fitted onto circular shelves which were placed in 206 x 300 tin cans. The cans, lids, and contents were dried in a vacuum oven for 75 min at 46-50°C at 1.5 in Hg pressure (absolute). After the initial drying, the spores were treated by purging the system five

times with dry nitrogen with a vacuum cycle between each purge. After this the spores were placed in a hood at < 3% relative humidity at 90°F and allowed to equilibrate overnight. Following this, the cans were sealed, brought out of the equilibration chamber, heated at 125°C for varying times, and cooled in an ice bath. Spore assays were made on survivor counts of > 10 per cup by sonication of the cup containing the spores in peptone water followed by conventional plating. When the survivor count was < 10 per cup, the sample cups were assayed by the most probable number method. This was done by adding 0.5 ml of tryptone glycose beef extract broth to each of the cups, scoring for growth after 7 days' incubation at 35°C, and calculating the most probable number of survivors.

The results of determinations done with survivors ranging between 10^6 to 10 per test unit approximated a straight line as did the results of assays with survivors ranging from < 10 to 10^{-3} per ml; however, the slopes of the lines were different. Since the number of survivors during a heating cycle represents a continuum rather than two distinct events with two rates, the points on the destruction rate curve were fitted with a second degree polynomial. Some other standard emperical model might have been as useful. This polynomial will fit the data with a R^2 near 1. This reduces the error in choice of model to the order of magnitude of the experimental error (replicate error) and allows one to detect small differences among groups of data. First a Bartletts test was performed to determine if gross differences among replicate variances existed. Next a

goodness of fit test was performed to see if the portion of the curve due to lack of fit in the residual error was significant compared with the experimental error. A test of homogeneity of regression lines was then performed. Two determinations of the inactivation of B. subtilis var. niger in the range of 10^{6} to 10^{-3} were done and examined to determine whether data from these determinations could be pooled. No significant differences between determinations could be detected between the sets of data at the $\alpha = 0.01$ level. The model used to determine poolability of data was $\log_{10} Y = b_o + b_1 X + b_2 X^2$; wherein Y is the number of survivors, b_o, b_1, and b_2 are regression coefficients and X is time. Values obtained from the two determinations are shown in Table 1. A graphical presentation of the data from one of these determinations is shown in Figure 1.

Since the determinations discussed above were done with approximately 0.25 μ of water per ml of head space air, we attempted to obtain data in a drier system. To do this, spores were prepared and dried using the procedure described above and the cans were exposed to heat with dry nitrogen flowing through the cans at a rate of 150-200 ml per min during the entire heating cycle. This increased the rate of spore inactivation in the initial phase of the heating cycle but resulted in greater stability of the spores to heat with survivors of < 10 per test unit (Figure 2).

II. STUDIES ON MOISTURE

After measuring the moisture content of dry sealed cans from several experiments, it was observed that the cans heated 300 min at 125°C had a slightly higher moisture content than the cans which had not been heated. Since the D value in each of the sealed can experiments "tailed" in the lower portion of the curve and knowing moisture affects the D value, it appeared desirable to eliminate this source of additional moisture. Heating pieces of mastic which were used to seal the lids of the cans indicated that some water was present but could probably be eliminated by heating the mastic overnight at 150°C. This heating temperature was incorporated into the procedure for drying the cans. Measurement of the cans for moisture after the procedure indicated that the additional water was still present. Table 2 shows this comparison. The average variation for each of these series of measurements was 20%. The stepwise procedure for preparing the cans and measuring their moisture content is given as follows:

PREPARATION PROCEDURE FOR TIN CANS PRIOR TO MEASURING MOISTURE CONTENT

1. Top and bottom of can are wire brushed for solder application.

2. Liquid flux is put on surface and a pool of solder applied with a soldering iron, in order to maintain a low temperature inside the can.

3. One-half of a 1/8" tube union is heated separately and placed on the solder.

4. The union on each end of the can is washed with warm water to remove any residual flux. The excess water is blown out of the unions by forced air and the unions rinsed with acetone to remove any residual water. The can is warmed in an oven to remove acetone and prevent moisture condensation on the cooled surface.

5. The can is cooled to ambient temperature and the excess solder is drilled from the inside of the union before puncturing.

6. The drilled union is tightly capped immediately after puncturing, then the union on the other end of the can is drilled and capped.

7. One end of the can is connected to the Moisture Analyzer via a teflon tube, then the dry nitrogen source is attached to the other end of the can.

8.. After the "free" water has been measured by the moisture analyzer, the can is heated between two heating mantles to a temperature of 150°C (thermistor sensor taped to the outside of the can) to determine if any bound water is present.

As the high temperature heating procedure did not eliminate the question about the influence of an additional amount of water on the "tailing," a flow-through system using the cans was designed. This consisted of attaching 1/8-inch copper tubing to each end of the can. This connection was made in the same manner as preparing the can for measuring moisture content. Dry nitrogen was passed through a manifold filled with magnesium perchlorate, then through each of the cans in the silicon oil bath. The flow of N_2 through each can was 100 ml/min.

As expected the initial D value was less than that obtained with the sealed cans containing water (0.3 μg/ml). However, the tailing portion appeared to have a flatter slope than that obtained in the sealed cans.

III. EXAMINATION OF CULTURES FROM APOLLO 12

Cultures were received in two lots. The first lot was accidentally shipped by Railway Express and required 9 days to arrive. The second lot came by air express. Because of the delay all cultures were subcultured to fresh Eugon agar slants. This appears to be a worthwhile step because the number of strains that failed to grow on subculture was markedly reduced. Gram stains were made from the original slants and the cultures divided into groups for further study. The results obtained are shown in Tables 3 and 4.

Table 1

Regression coefficients for two inactivation determinations of B. subtilis var. niger at 125°C with 0.25 μg of water per ml of head space air

Coefficients	Run Number			
	97	SE*	98	SE*
b_o	5.89165		5.76908	
b_1	-0.07179	0.00475	-0.07191	0.00501
b_2	0.00017	0.00002	0.00017	0.00002
R	0.984		0.984	

*Standard error of regression coefficient.

Table 2

Comparison of moisture content in tin cans after spore preparation by standard and high temperature heating procedures

Procedure	Date 1969	Average µg water per can: 0 min in oil bath — Before heating*	0 min in oil bath — After heating*	0 min in oil bath — Difference	300 min in oil bath — Before heating*	300 min in oil bath — After heating*	300 min in oil bath — Difference
Standard	11/17	15	42	27	43	56	13
	11/18	16	45	29	43	59	16
Mastic dried at 150°C	11/24	21	45	24	47	61	13
	12/12	24	44	20	44	55	10

* In moisture analyzer to 150°C.

Table 3

Results of Studies on Cultures from Apollo 10, (A series), 11 (B-series) and 12 (C-series)

	Apollo 10	Apollo 11	Apollo 12
Cultures that could not be completely identified because of failure to grow at some step in the identification procedure	386	174	120
Catalase positive, gram positive cocci	586	874	1034
Gram positive, non-sporeforming bacilli	103	80	72
Sporeforming bacilli	49	10	12
Gram negative bacilli	6	12	5
Catalase negative, gram positive cocci	4	0	0
Yeasts	5	0	4
Fungi	2	0	0
Total number of cultures	1141	1150	1247

Table 4

Types of Catalase Positive Cocci Identified

	Apollo 10 Number	10 %[a]	Apollo 11 Number	11 %	Apollo 12 Number	12 %
Staphylococcus						
Subgroup I[b]	48	8	22	3	1	≤ 1
Subgroup II[c]	121	21	221	25	217	21
Subgroup III	41	7	6	< 1	16	2
Subgroup IV	97	17	194	22	166	16
Subgroup V	103	18	192	22	123	12
Subgroup VI	93	16	142	16	197	19
Micrococcus						
Subgroup 1	19	3	31	4	33	3
Subgroup 2	1	< 1	10	1	15	1
Subgroup 3	2	< 1	6	< 1	52	5
Subgroup 4	0	0	0	0	1	< 1
Subgroup 5	0	0	0	0	8	< 1
Subgroup 6	0	0	0	0	1	< 1
Subgroup 7	51	9	34	4	204	20
Subgroup 8	0	0	1	< 1	0	0

[a] Approximate percentage

[b] Staphylococcus aureus

[c] Staphylococcus epidermidis

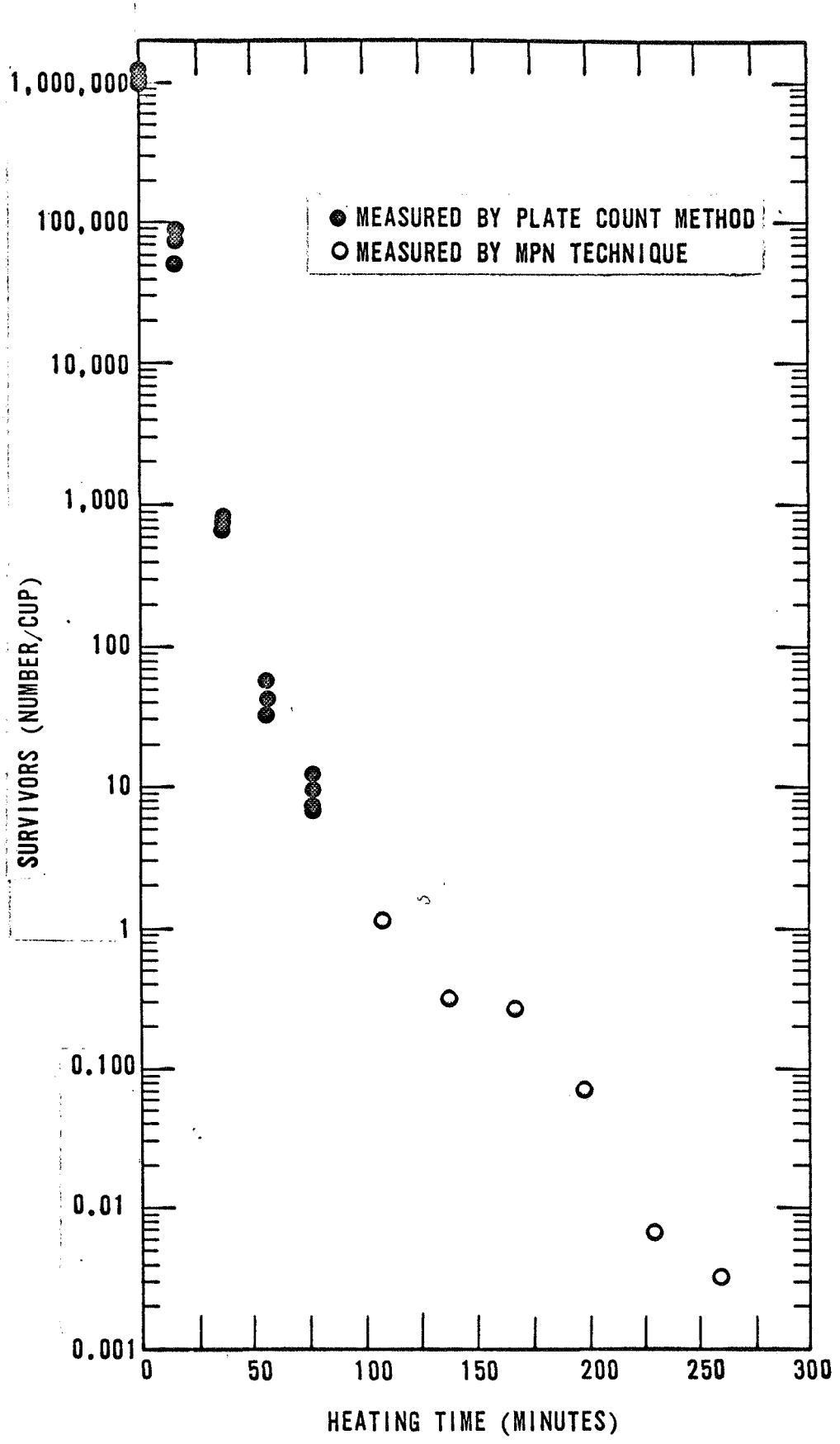

Fig. 1. THERMAL INACTIVATION OF B. Subtilis var. Niger AT 125°C. HEADSPACE WATER CONCENTRATION ~0.25 µg PER ml.

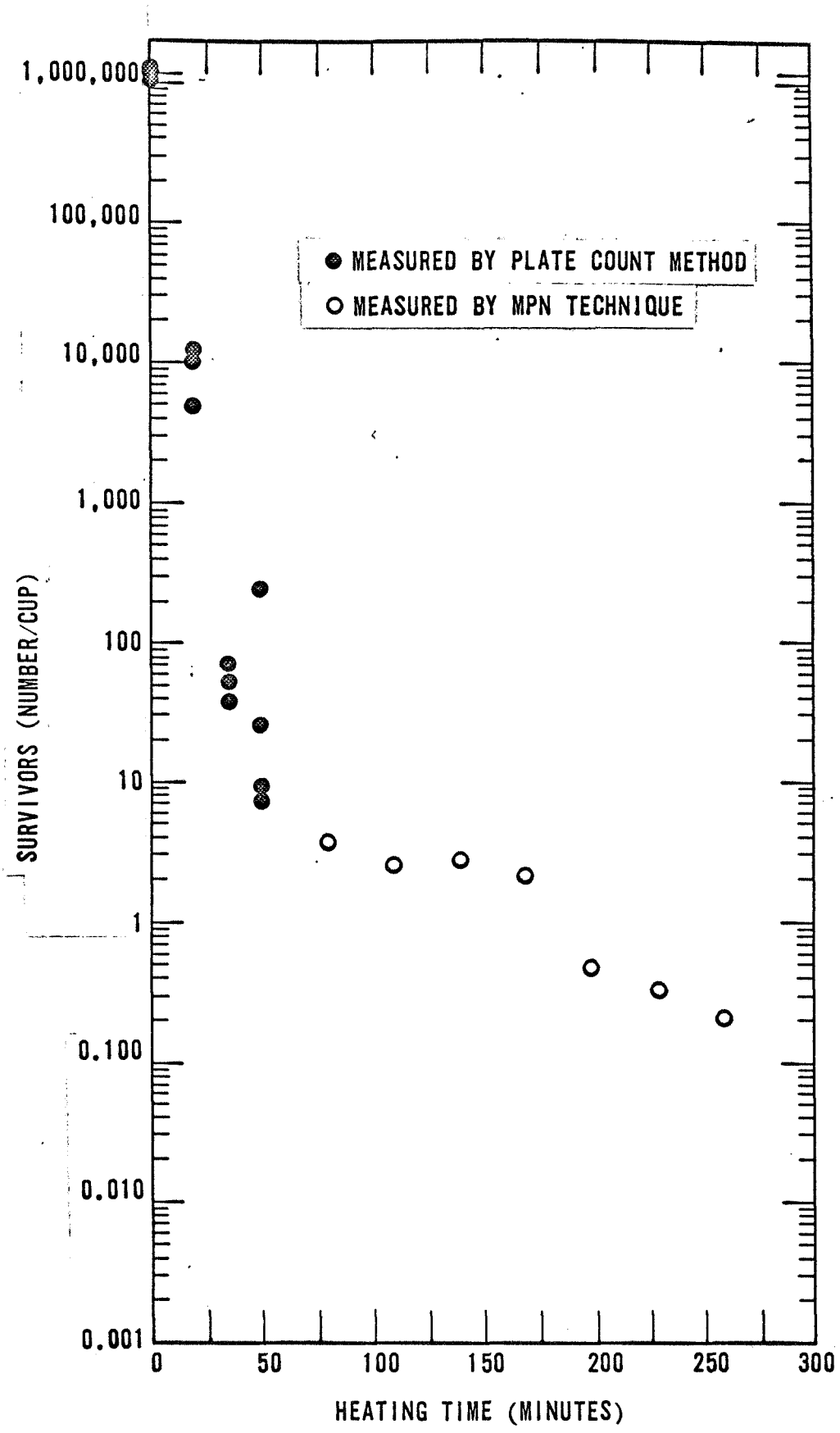

Fig. 2. THERMAL INACTIVATION OF B. Subtilis var. Niger AT 125°C IN FLOWING NITROGEN.