RELEASE OF BACTERIAL SPORES FROM INNER WALLS OF A STAINLESS STEEL CUP SUBJECTED TO THERMAL STRESS

1st Quarterly Report, 1973-74

NASA Contract W13450

Task No. 193-58-62-13-10

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The work reported here is an extension of that presented by Chatigny, Wolochow and Hebert (1973). In the earlier report thermal stresses, simulating those expected on a Mars Lander, dislodged approximately 0.01% of an aerosol-deposited surface burden, as did a "landing" shock of 8-10 G deceleration. This work confirmed earlier results and demonstrated that release rate is not dependent on surface burden.

This work was to confirm earlier efforts and to evaluate the effects of reduced bioburden on the test cup surfaces. Early tests were done with unit bioburdens 10^7 ± c.f.u. whereas in these tests burden was reduced to 10^5 ± c.f.u. in a number of trials. In this report we present evidence which confirms the earlier work, (i.e., ca. .01% release) extending the number of temperature cycles to more than 80 in several trials and running tests with low bioburden per unit area. We have also determined that the viability of spores of Bacillus subtilus var. niger (BG spores), deposited on surfaces of stainless steel and held for extended times under very dry conditions, is not constant. In addition, resistance of spores to conventional heat shock (67 C for 15 min) declined slightly over a 60-day period under the conditions of these tests.

METHODS AND MATERIALS

1. Test cup procedures

Essentially the same procedures and materials for aerosol generation and loading of the test cups were used in this study as in the prior one (Chatigny, et al., 1973). Temperatures were cycled between about +60 C and -60 C. Cam switches, driven by synchronous motors, were used to provide power to a 3-way valve which changed fluid (methyl cellosolve) circulating through the test units from one temperature to the other (Fig. 1). Either a 1-hour or 2-hour total cycle (hot-cold) could be selected. A large dry ice-cellosolve bath with about 10 ft of 3/8" copper tubing was used for attaining the lower temperature; a smaller water bath with an immersion heater connected through a temperature controller (thermistor sensor) and its copper tube heat exchanger serves for the elevated temperature. About 6 min were required for temperature to come to within 90% of equilibrium (as measured at the test unit).

2. Heat stability procedures

Degreased, stainless steel squares (2.5 cm x 2.5 cm) in metal pans, were placed near the bottom of the 1500 1 settling chamber. An aerosol of BG spores in an alcohol suspension, (Chicago atomizer) was produced in the chamber. After about 20 minutes settling time, the pan covers were replaced, and the pans removed. Each square was then placed in a small sterile plastic petri dish, containing 4 holes in the lid. These dishes were held in metal pipette cylinders in the dry box (3-5 ppm H_2O). At intervals 4-8 petri dishes were removed and the metal squares assayed for spore count. Each square was placed in 50 ml sterile water and sonicated for 30 sec at 100 watts/min. The sonicate was plated on casitone agar and incubated for 24 hr at 37 C. In the last 2 time intervals the sonicate was heated to 67 C for 15 min and replated as above.

3. Particle evaluation

A number of trials was carried out to assess the number of spores per particle in those particles which reached, and were held, by the test unit. Millipore filters (47 mm-0.45μ pore size) were cut into quarters, sterilized and affixed (sterile lanolin on edges) to the inner surface of a test unit. A drop of sterile normal saline was placed on each section to dissipate static charges. A 1:30 dilution of stock alcoholic suspension of BG spores (1×10^{10}/ml was used as spray material. Alternatively, a 1:50 dilution of stock was made in a heat-killed stock suspension to provide fewer viable spores, without change of total solids. The test units were exposed to aerosol for 4-6 secs, and capped off. Two of the 4 sections of filter were placed on Casitone agar previously moistened with nutrient broth. Incubation at 37 C was continued until colonies were countable under 10x magnification. The other 2 sections were each suspended in 50 ml sterile water and sonicated for 1 min at 100 watt/min. Aliquots of the sonicate were filtered through membrane filters (0.45μ pore dia.) and treated as above. Estimates of particle size and size distribution were obtained by examining glass cover slips on which aerosol had settled. Both light microscope (transmission-oil immersion) and scanning electron microsocpy of gold shadowed specimens were carried out.

RESULTS

1. Handling artifacts

In the course of three trials (Tables 4, 6 and 10) assessments were made of the release of spores from the sampling disc by the act of changing it. In one trial (Table 4) 4 such changes were made prior to temperature cycling and 3 after 49 temperature cycles. With an initial burden of 2 to 3 x 10^5 spores, 0-6 spores were collected per

sample change. There were somewhat fewer spores collected after temperature cycling. The same results were obtained in a second trial (Table 6). In the third trial (Table 10, Unit III) from 0 to 13 spores were recovered in 7 samples, obtained over the period required for 89 temperature cycles.

In two trials (Table 9, Unit IV, Table 10, Unit IV) test vessels which had not received an aerosol burden were "sampled" along with three others, disc changes being made at the same time intervals. The number of spores recovered ranged from 0-10 per disc.

In one trial (Table 9) the number of airborne c.f.u. from within the test cup ranged from 44/1 of air as an initial burden, decreasing with time and temperature cycles to less than 0.1/1. In the succeeding trial the count of airborne c.f.u. ranged markedly lower. We suspect that the "airborne" spores recovered were MF disc contamination from handling other spore-burdened test vessels within the same test chamber. When the air mass inside the test chamber was sampled, it was found that the maximum count was about 0.015 c.f.u./1 (Table 11).

2. Spore release as a function of temperature cycling

The results from the series of trials differed but little from those previously reported. In all cases the tendency was for most of the releasable burden (caught on the settling disc) to be released in the first few cycles. However, inspection of the data in the accompying tables reveals that there were a number of exceptions. Similarly the numbers of airborne c.f.u. recovered on membrane filters tended to be higher at the beginning of a run. When recognition is given to contribution of the handling artifacts, the numbers of airborne c.f.u. is not impressive.

3. Number of spores per particle deposited on test vessel surfaces

In order to load the test units with 10^6 or less c.f.u., it was necessary to dilute our stock BG suspension of 10^{10} spores/ml. When this dilute suspension was made up in undiluted heat-killed spore suspension, the ratio of spores (viable and heat-killed)/particle ranged from 74 to 510. (Viable counts ranging from 1.5 to 10 spores/particle).

4. Number of spores per particle released from test vessel

Counts were determined by viable assay of membrane filters placed on top of settling disks. From Table 12, it can be seen that the spore/particle ratio varied from 2 to 200, with values averaging 12 neglecting a single very high reading.

5. Estimates of particle size

Light microscopy gave a number median diameter of about 6 µm for

particles produced from undiluted suspension (Fig. 2). Scanning electron microscopy analysis of particles produced from a 1:30 dilution of stock spores yielded a number median diameter of ca 1 μm (primarily single spores). Fig. 3 shows the spores and spore aggregates.

6. Survival of spores on metal surfaces under dry conditions

Over a 62-day period the number of spores/coupon fell from approximately 2×10^6 to 1×10^5 (Fig. 4).

7. Thermal stability of spores aged on metal

Shock heating (wet) tended to reduce viable recovery somewhat but this could not account for losses noted in (6) above.

DISCUSSION AND SUMMARY

The numbers of colony forming units (c.f.u.) per test cup in these trials are unrealistically high. These large burdens were required to obtain countable numbers of c.f.u. released during temperature cycling. Indeed, if lower burden had been used the contribution of handling artifacts ("noise") would have precluded meaningful results.

In general, results from these trials confirm those reported earlier. The number of spores (and c.f.u.) released was greatest at the beginning of a temperature cycling series.

The average number of spores per particle in the aerosol deposited on the test cup surfaces ranged from 74 to 510, whereas the same ratio for particles released during cycling averaged 12. This suggests that the smaller particles (lower spore/particle ratios) were preferentially released and sampled.

The median number diameter of particles deposited, as found by light microscopy was ca. 6μm, whereas with scanning electronmicroscopy this number was 1μm. This disparity in size may be an artifact stemming from "loss" in counting single spores by transmission light microscopy.

Over a 62 day period there was a reduction of about 20-fold in number of viable spores which had been deposited on metal coupons held under dry conditions. The loss in resistance of these spores to heat shock (wet, 67C/15 min) was insufficient to account for the reduction in numbers.

LITERATURE CITED

Wolochow, H., M. A. Chatigny and J. Hebert. 1973. Release of bacterial spores from inner walls of a stainless steel cup subjected to thermal stresses and mechanical shock. Naval Biomedical Research Laboratory, Univ. of Calif., Berkeley. 48th Tech. Prog. Rep. pp. 363-386.

KEY FOR TABULATED DATA-TABLES, 1 THRU 10

Table & Trial	Burden Hi Lo	Hot - Cold Cycles 30'	60'	Room temp. (Static temp) hrs.	Effluent test Yes - No	Techniques checks
1.	Hi		23	---	Yes - glass filter	
2.	Lo		16.5	---	No	---
3.	Hi	18	17	66	No	---
4.	Lo	40	9.	18	No	7
5.	Lo	10.5		---	Yes MF	- Hi humid.
6.	Lo	101	10	---	Yes MF	3
7.	Lo	1	89	---	Yes MF	---
8.	Hi		1	---	Yes MF	---
9.	Hi	83	2	---	Yes MF	1 blank unit
10.	Hi	87	2	---	Yes MF	1 non-cycled sample 1 blank unit

11. Organisms collected from dry box - vent air.
12. Spores/particle (settling on MF)
13. Spores/particle (settling on MF)
14. Spores/particle settling on vessel walls.

TYPICAL TEMPERATURE CYCLE & SAMPLING TIME

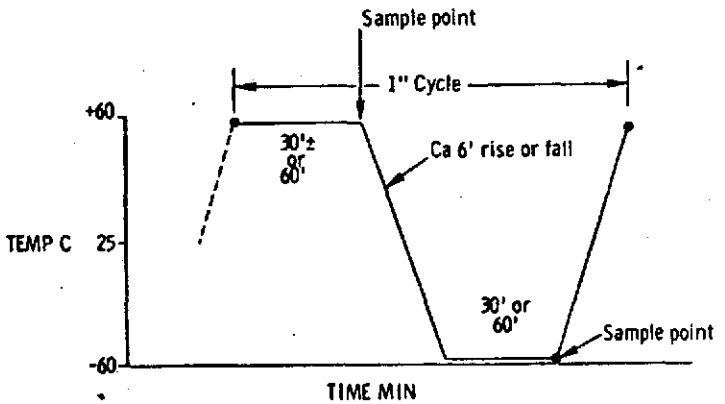

Table 1. Release of spores from test cups during temperature cycling (-60 to 60 C); 23.5 cycles

Sample	Spores per settling disc, from cup unit I	II	No. of time's at indicative temperature -60 C	+60 C
1	1,330	3,730		1
2	64	83	1	
3	27	82		2
4	1,036	41	2	
5	18	790		3
6	13	149	3	
7	2,130	4,600	12	11
8	10	460	24	22
Burden[1] Spores:	1.69×10^6	1.01×10^7		

[1] Burden = BG spores/unit at end run.

Table 2. Release of spores from test cups during temperature cycling (-60 to 60 C); 16.5 cycles

Sample	Spores per settling disc, from cup unit I	II	60 min/half cycle -60 C	+60C
1	100	0		1
2	32	58	1	
3	6	0		1
4	3	138	1	
5	47	7		1
6	12	12	9	9
7	0	0	5	5
Burden[1] Spores:	2.92×10^4	3.45×10^4		

[1] Burden = BG spores/unit at end run.

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Table 2. Release of spores from test cups during temperature cycling (-60 to 60 C); 16.5 cycles

Sample	Spores per settling disc, from cup unit I	II	60 min/half cycle -60 C	+60C
1	100	0		1
2	32	58	1	
3	6	0		1
4	3	138	1	
5	47	7		1
6	12	12	9	9
7	0	0	5	5
Burden[1] Spores:	2.92×10^4	3.45×10^4		

[1] Burden = BG spores/unit at end run.

Table 3. Release of spores from test cups during temperature cycling (-60 C to 60 C); 37 cycles

Sample	Spores per settling disc from cup unit I	II	Cycle Data *60 min **30 min -60 C	+60 C
1	90	45	*1	
2	360	122	*	1
3	62	140	*1	
4	78	203		1
5	3,120	768	*1	
6	52	204	*9	10
7	90	162	*5	6
			**8	8
8	444	40	**4	4
9	1	0	66 hr room temp.	
10	0	0	**6	6
11	3	4	*1	1
Burden Spores:	1.13×10^6		3.27×10^7	

Table 4. Release of spores from test cups during temperature cycling (-60 to 60 C); 49 cycles

Sample	Spores per settling disc, from cup unit I	II	Cycle Data *60 min **30 min -60 C	+60 C
1	2	4	"Handling" +	
2	4	6	"	
3	4	0	"	
4	1	6	"	
5	0	6	18 hr o/r/c at 22C	
6	90	5	*1	
7	1	9	*	1
8	0	5	*1	
9	2	6	*	1
10	0	0	*1	
11	4	4	**8	8
			*7	7
12	2	0	**8	9
13	2	2	**6	5
14	0	0	18	18
15	1	1	"Handling" +	
16	0	0	"	
17	0	5	"	
Burden Spores:	2.0×10^5		3.42×10^5	

+ "Handling" - sequential changing of sampling discs; no temperature cycling.

Table 5. Release of spores from test cups during temperature cycling (-60 to 60 C); 10.5 cycles

	Spores per settling disc and cfu*/MF from cup unit					
	I		II		Cycling Data, 30/30	
Sample	SD	MF	SD	MF	-60 C	+60 C
1	0	0	84	1	1	
2	6	6	4	9		1
3	7	34	5	23	1	
4	3	19	16	1	9	9
Burden Spores:	2.41×10^5		1.65×10^5			

Note: (1) Thin film of rime noted on outer walls of M.L. units during first -60 C hold periods

*cfu - Colony Forming Units

Table 6. Release of spores from test cups during temperature cycling (-60 to 60 C); 111 cycle

	Spores per settling disc and cfu*/MF from cup unit				Cycle Data (min/1/2 cycle) * = 60 min ** = 30 min	
	I		II			
Sample	SD	MF*	SD	MF*	-60C	+60C
1	8	14	2	2	*	1
2	2	14	0	0	* 1	
3	2	6	6	6	*	1
4	3	0	0	0	* 1	
5	8	4	2	2	**19	19
6	2	4	6	7	**23	23
7	6	6	2	3	**33	33
					* 8	8
8	0	2	0	1	**26	26
9	2	1	0	0	"Handling" ***	
10	1	1	2	0	"	
11	0	0	1	0	"	
Burden Spores:	1.70×10^5		2.35×10^5			

*Membrane filter used to assay number of airborne spores inside cup unit. Sampling rate 100 ml/min constant flow.

***"Handling" - sequential changing of sampling discs; no temperature cycling.

Table 7. Release of spores from test cups during temperature cycling (-60 to 60 C); 85 cycles

	Spores per settling disc and cfu*/MF from cup unit				Cycle Data (min/1/2 cycle) * = 60 min ** = 30 min	
	I		II			
Sample	SD	MF*	SD	MF*	-60C	+60C
1	12	9	5	4	*	1
2	2	9	6	3	* 1	
3	1	5	0	3	**	1
4	5	5	4	1	**1	
5	8	8	4	2	17	17
6	11	5	1	1	25	25
7	4	9	3	9	24	21
8	0	3	1	0	25	25
Burden Spores:	3.92×10^5		3.09×10^5			

*Colony Forming Units/Membrane Filter used to assay number of airborne spores inside cup unit. Sampling rate 100 ml/min.

Table 8. Release of spores from test cups during temperature cycling (-60 to 60 C); 1 cycle

	Spores per settling disc and cfu*/MF from cup unit								Cycle Data 60 min/ 1/2 cycle	
	I		II		III**		IV			
Sample	SD	MF*	SD	MF*	SD	MF*	SD	MF*	-60C	+60C
1	2.86×10^3	130	2.60×10^5	136	4.24×10^2	33	12	2	1	1
Burden Spores	2.96×10^6		1.89×10^7		1.09×10^7		0.0			

*Colony forming units/membrane filter used to assay number of airborne spores inside cup unit. Sampling rate 100 ml/min.

**Not cycled.

Table 9. Release of spores from test cups during temperature cycling (-60 C to 60 C); 85 cycles

	Spores per settling disc and cfu*/MF from cup unit								Cycle Data Units I-II * 1 hr **30 min	
	I		II		III		IV			
Sample	SD	MF*	SD	MF*	SD	MF*	SD	MF*	-60 C	+60 C
1	3.7×10^3	134	5.88×10^2	20	10	15	4	50	* 1	
2	1.96×10^3	0	2.52×10^5	5	4	50	6	40	* 1	1
3	1.06×10^2	16	16	11	16	40	10	43	*	1
4	5	3	16	3	14	30	6	15	**19	19
5	16	3	6	0	1	0	0	1	**20	20
6	14	1	2	0	1	7	0	0	**24	24
7	0	0	138	2	3	2	6	2	**20	20
Burden Spores	2.88×10^7		1.76×10^7		2.86×10^7		0.0			

*Colony forming units/Membrane Filter used to assay number of airborne spores inside cup unit. Sampling rate 100 m/min.

**Not cycled--Units III & IV.

Table 10. Release of spores from test cups during temperature cycling (-60 C to 60 C); 89 cycles

	Spores per settling disc and cfu*/MF from cup unit								Cycle Data Units[1] I-II *60[1] **30[1]	
	I		II		III[1]		IV[3]			
Sample	SD	MF[2]	SD	MF	SD	MF	SD	MF	-60 C	+60 C
1	1.06×10^2	3	2.12×10^2	-	13	2	5	1		* 1
2	9.50×10^2	66	8.9×10^4	-	3	2	1	1	* 1	
3	16	1	4	-	16	1	2	2	* 1	
4	1.080×10^3	2	9.6×10^2	-	4	1	3	2	**17	17
5	6	1	8	-	2	1	3	0	**24	24
6	1	0	1	-	0	0	1	1	**23	23
7	0	0	0	-	0	1	1	7	**22	23
Indicative Burden Spores:	5.1×10^6		5.55×10^6		1.11×10^7		5.0			

[1]Unit III - static; no temperature cycling

[2]Colony forming units/Membrane Filter used to assay number of airborne spores inside cup unit. Sampling rate 100 ml/min.

[3]Unit IV - Not cycled and no added spore burden.

Table 11. Number of colony forming units (cfu) per liter of air during trial summarized in Table 10

Sample #	Sample data	cfu/MF	liter air (x 10^{+3})	cfu/liter (x 10^{-3})
	Organisms collected from dry box effluent ventilating air			
1	15 hr unit. Drying period	28	12.8	2.19
2	1 hr Pre-removal S.D. #1	2	0.75	2.67
3	1 hr Pre-removal S.D. #2	11	0.75	14.7
4	2 hr Pre-removal S.D. #3	28	1.50	1.87
5	14 hr Pre-removal S.D. #4	8	12.80	0.625
6	24 hr Pre-removal S.D. #5	222	18.0	12.3
7	23 hr Pre-removal S.D. #6	122	17.3	7.05
8	22.5 hr Pre-removal S.D. #7	8	16.9	0.473

Table 12. Collection of spore-bearing particles released from surface of cup units. Settlement on membrane filters: Particles and spores/particle (One temperature cycle of 60 min per half-cycle)

Trial No.	Cup Unit#	cfu;* non-sonicated**	cfu; sonicated	Spores/ Particles	Unit Burden, spores
I	1	153	3.08×10^4	201	2.09×10^6
	2	3	95	32	2.97×10^6
	3	3	17	6	3.78×10^6
	4	1	7	7	1.70×10^6
II	1	1	10	10	8.14×10^6
	2	2	15	7.5	2.32×10^6
	3	3	68	23	1.59×10^6
	4	8	130	16	1.62×10^6
III	1	6	40	6.7	1.73×10^7
	2	Samp -	fast.	---	1.74×10^7
	3	11	88	8.0	1.94×10^7
	4	6	13	2.2	1.83×10^7

*Colony forming units; MF incubated on casitone agar

**cfu after sonication of filter in water; supernatant filtered through membrane filter and treated as above.

Table 13. Spores per particle deposited on walls of cup units. Stock suspension, diluted 1:50, used for spray suspension

	TRIAL 1					TRIAL 2					TRIAL 3				
	Settling MF* CFU**	Settling $\bar{X}$ cfu	Sonicated MF CFU	Sonicated $\bar{X}$ cfu	Org. per particle	Settling MF CFU	Settling $\bar{X}$ cfu	Sonicated MF CFU	Sonicated $\bar{X}$ cfu	Org. per particle	Settling MF CFU	Settling $\bar{X}$ cfu	Sonicated MF CFU	Sonicated $\bar{X}$ cfu	Org. per part.
A	891		3,475			855		1,463			264		650		
I		862		3,156	3.66		830		2,007	2.42		469		925	1.97
B	833		2,837			804		2,550			673		1,200		
A	1,306		3,088			546		2,075			1,477		3,400		
II		1,286		4,275	3.32		644		1,744	2.71		1,496		3,325	2.22
B	1,276		5,362			742		1,413			1,545		3,150		
A	1,397		2,988			866		2,775			1,232		4,700		
III		1,348		2,950	2.19		897		2,825	3.15		1,280		4,667	3.65
B	1,298		2,912			927		2,875			1,328		4,633		
A	3,338		6,313			346		1,200			532		2,050		
IV		3,193		6,663	2.09		393		1,075	2.74		650		2,109	3.24
B	3,048		7,012			429		950			768		2,167		

*Membrane filters (1/4 of 47 mm disc) affixed to inner surface of cup units

**See note to Table 12

Table 14. Spores per particle on walls of cup units. Stock suspension of spores diluted 1:50 in autoclaved stock suspension was used as spray material

	TRIAL 1					TRIAL 2					TRIAL 3				
	Settling MF* CFU**	cfu $\bar{X}$	Sonicated MF CFU	cfu $\bar{X}$	CFU per particle	Settling MF CFU	cfu $\bar{X}$	Sonicated MF CFU	cfu $\bar{X}$	CFU per particle	Settling MF CFU	cfu $\bar{X}$	Sonicated MF CFU	cfu $\bar{X}$	CFU per particle
A	1,606		3,831			1,302		3,350			234		250		
I		1,621		3,937	2.43 (124)**		1,512		3,850	2.55 (130)		254		369	1.45 (74)
B	1,635		4,042			1,722		4,350			274		488		
A	821		5,600			320		2,750			622		3,288		
II		839		4,308	5.13 (262)		222		2,225	10.1 (515)		719		3,473	4.83 (246)
B	857		3,016			123		1,700			815		3,663		
A	231		1,475			841		2,550			675		2,137		
III		326		1,271	3.90 (199)		927		2,500	2.70 (138)		660		2,656	4.02 (205)
B	421		1,067			1,012		2,450			645		3,175		
A	550		733			312		1,100			584		2,175		
IV		528		1,129	2.14 (109)		350		3,500	10.0 (510)		554		2,063	3.72 (190)
B	505		1,525			388		5,900			524		1,950		

*See notes to Table 13

**Numbers in parentheses: Total number of spores (viable and heat-killed) per particle.

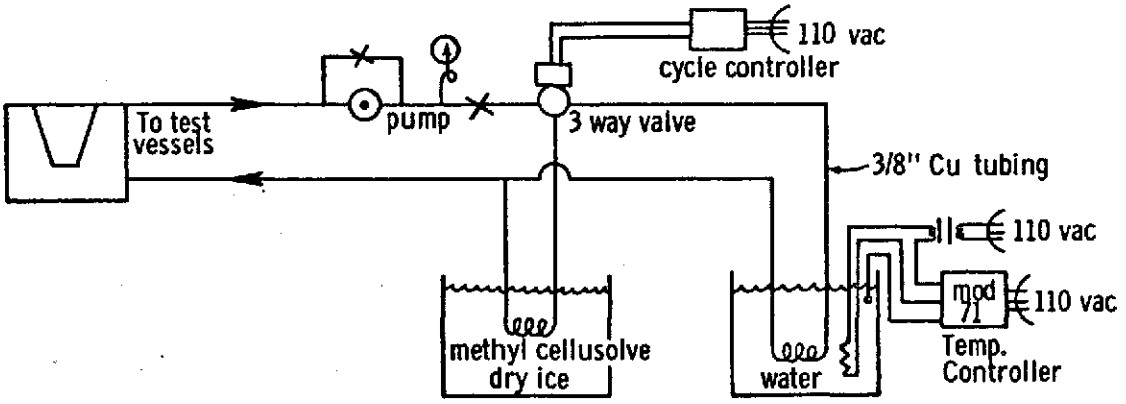

Fig. 1. Temperature cycling setup
Pump: Viking gear pump, Model FH32, with carbon bushings. 1/2 HP 1750 rpm motor. Coker Pump and Equipment Co. Oakland, California
3-way Valve: ASCO, Cat. No. 8300C72V, 3/8" Universal Config.
Cycle controller: Repeat cycle timers, Western Electrico-Mechanical Co., Inc., Oakland, CA. 1RPH and 1/2 RPH Motor. Cams set for 50% off/on.
High temperature controller: Yellow Springs Model 71, Thermistor sensor. Powerstat, 15 amp, in series with heater and normally open contacts. 600 watt immersion heater.

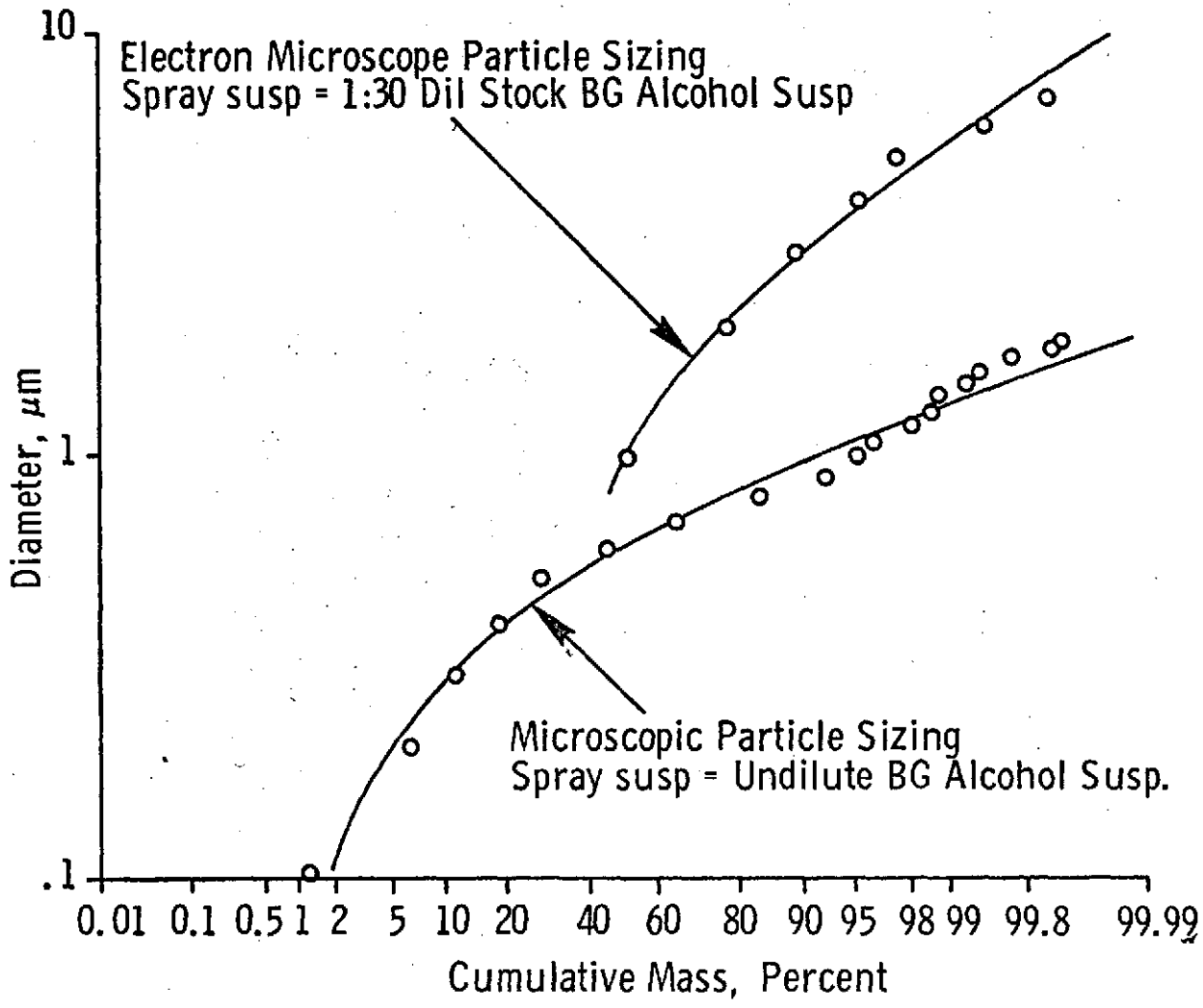

Fig. 2. Particle-sizing of aerosol depositing on glass cover slips on cup units.

Fig. 3A (x 840)

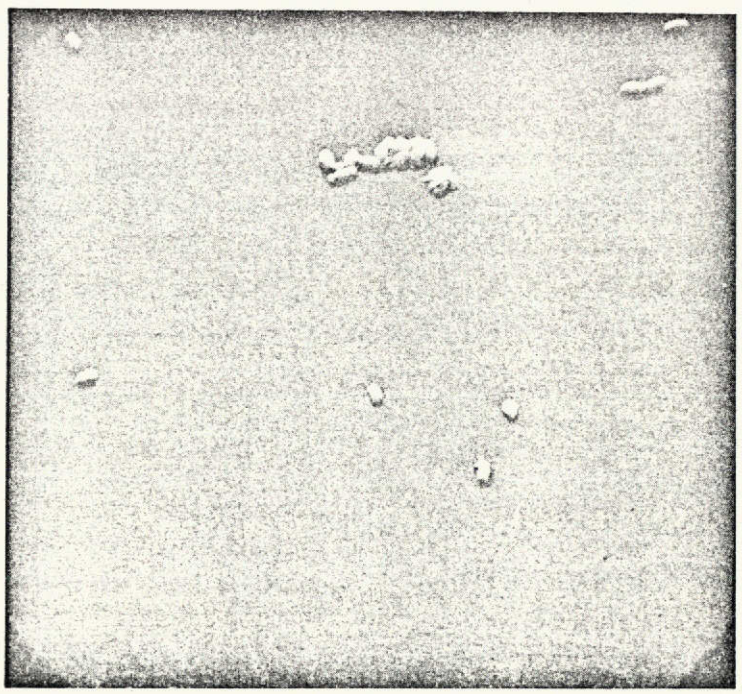

Fig. 3B (x 1350)

Scanning electron microscope photos of spores deposited from aerosol onto glass cover slips. 1:30 dilution of stock spores used for spray material.

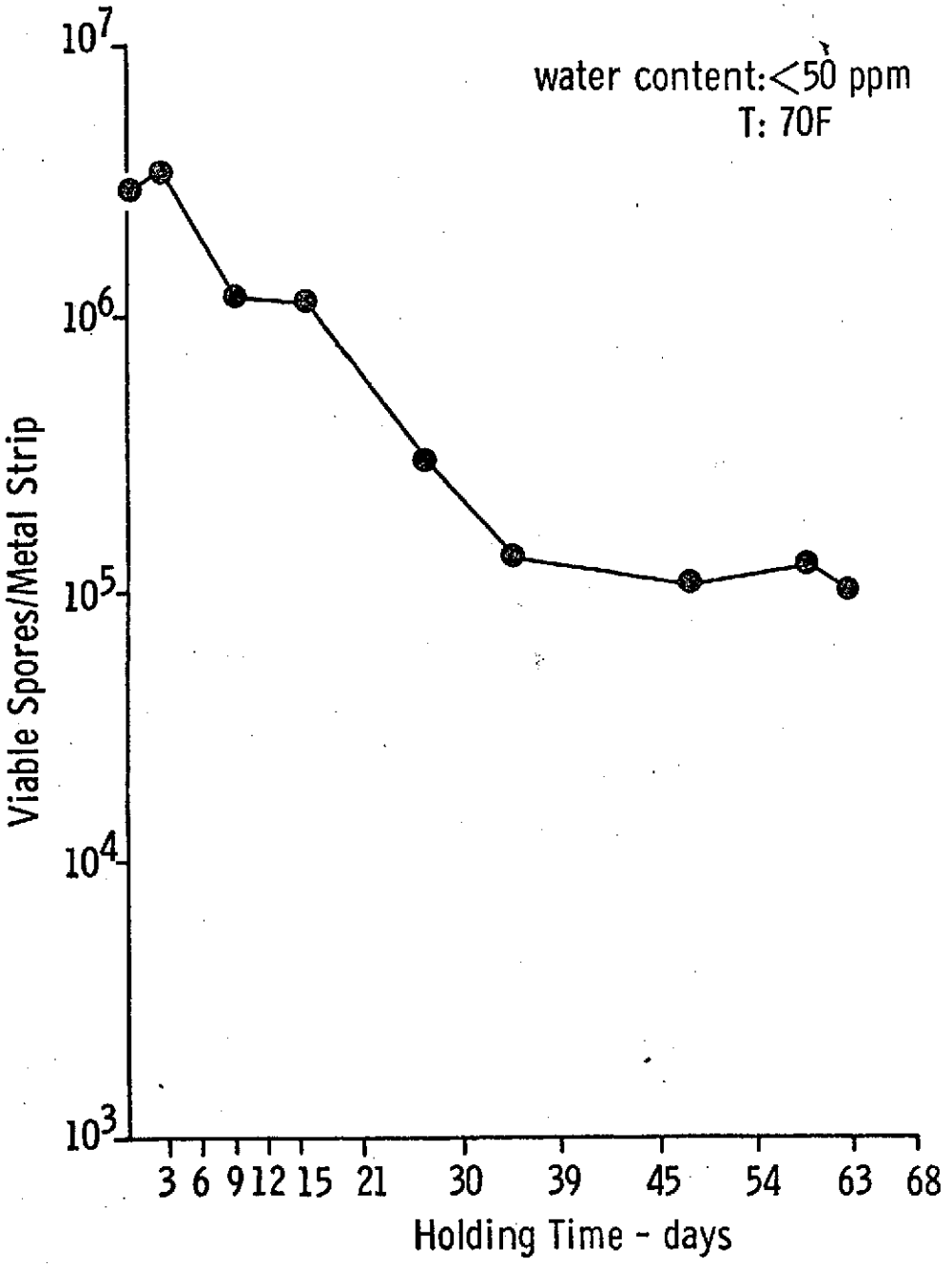

Fig. 4. Survival of B.G. spores on metal strips.