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Technology Branch  
Communicable Disease Center  
Public Health Service  
U.S. Department of Health, Education, and Welfare

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1. Studies were continued to develop a reliable standard model system for evaluating the recovery of viable microorganisms from the interior of plastics. Emphasis was placed on the methyl methacrylate system. In order to determine the effect of polymerization and subsequent storage on survival, spores of Bacillus subtilis var. niger were incorporated into unpolymerized methyl methacrylate and assays were performed before and after polymerization. The results of 5 experiments demonstrated a considerable die-away during the process of polymerization. This reduction in the viable spore population slightly exceeded one log in all cases. After the completion of polymerization the rate of die-away was less dramatic and appeared to decrease with time. Spores also were inoculated into liquid (unpolymerized) methyl methacrylate whose preservative had been removed previously by a 2 percent sodium hydroxide wash. At room temperature no significant decrease in the number of spores was noted (Figure 1).

The value of this model system is that the seeded plastic can be dissolved in acetone and assayed by means of a solvent-resistant membrane filter (Gelman alpha 6). Consequently in determining percent recovery, compensation can be made for any die-away that occurred during polymerization and storage. Several experiments were performed to determine the number of spores destroyed by the grinding technique (Blender-Mill) and the number still embedded in the plastic after grinding. The number of viable spores per gram of plastic was determined by dissolving the sample in acetone and assaying the suspension by means of a solvent-resistant membrane filter. Other samples were ground for 1 minute in the Blender-Mill and the resultant suspension was assayed by both pour plates and standard membrane filters. Aliquots of the same suspensions were passed through standard membrane filters and each filter was dissolved in acetone. This suspension, which included those spores freed from the plastic particles by dissolution, was assayed with a solvent-resistant membrane filter. Preliminary results showed that about 85 percent of the spores were destroyed by the grinding procedure. Of the spores that survived approximately half could not form colonies because they were still embedded in plastic (Table 1). Efforts will be made to increase the efficiency of recovery by suitable sample manipulations.

2. Studies were continued on using ultrasonic energy for recovering microbial contaminants from surfaces. Emphasis was placed on testing surfaces contaminated with natural contamination resulting from human handling or aerial fallout and also aerosolized spores of B. subtilis var. niger. Comparisons were made between ultrasonication and mechanical agitation. In all cases more microorganisms were recovered from surfaces of stainless steel and glass (Tables 2 and 3) by ultrasonication than by mechanical agitation and in most the difference was significant statistically. When percent recovery, rather than total organisms recovered, was used as the criterion for comparison, ultrasonication effected significantly higher percent recoveries than mechanical agitation. However, when natural contamination systems were employed evaluations based solely on percent recovery were of limited value because of unknown variation in clumping encountered with natural microbial contamination.

It was noted again that in some instances recovery was significantly higher when the contaminated surface faced the ultrasonic energy source than when the contaminated surface faced away from the source (Table 4).

To date 3 ultrasonic baths having the same frequency and made by the same manufacturer have been compared with each other and with mechanical agitation. Although slight differences were noted among the 3 baths all were consistently more effective in recovering microorganisms from surfaces than mechanical agitation.

3. Studies were continued on the die-away of bacterial spores on surfaces of methyl methacrylate (lucite) and polystyrene. Earlier tests showed that die-away rates for spores of B. subtilis var. niger were significantly greater at 0 percent R.H. (no detectable moisture using an electric hygrometer) than at 46 percent R.H. when the organisms were seeded onto polystyrene granules and lucite powder. Similar results were obtained in a second series of experiments (Figure 2). In addition discs of lucite and polystyrene were inoculated with spores and stored at 0 and 46 percent R.H. These studies, in contrast to those using powdered or granulated plastic, showed that significant die-away occurred only on lucite discs at 0 percent R.H. (Figure 3). In neither case was the die-away on the discs as rapid as on the granulated or powdered plastic at 0 percent R.H. Since more surface area was exposed to the spores when the powder was used than smooth discs the inoculum per se may have protected individual spores. In any case it appears that high death rates observed on powdered or granulated plastic was caused by the combination of low moisture content and the chemical properties of the surface.

In order to determine whether out-gassing of bactericidal substances from the plastics was responsible for the rapid die-away of 0 percent R.H. one set of seeded polystyrene granules and lucite powder was stored at 0 percent R.H. under vacuum. No significant die-away was observed in the evacuated system. However, die-away did occur in the non-evacuated environment (Figure 4).

4. Studies of the comparative levels and types of microorganisms among hospital operating rooms and industrial clean rooms were continued. Air samples were taken in two operating rooms with slit samplers (Reyniers) using 30-minute clock mechanisms. The highest levels of airborne viable particles that have been measured by this laboratory in any environment were noted during this period (171 per cubic foot). Colonies were selected randomly from the

air sampling plates after incubation and pertinent morphological and biochemical tests were performed for identification. Results showed that most of the microorganisms detected in both operating rooms were sporeforming bacteria, yeasts, molds and actinomycetes which are associated with soil. Very few microorganisms indigenous to humans were found. As reported earlier the vast majority of microorganisms detected by air samplers in industrial clean rooms were those associated with humans. Very low numbers of microorganisms associated with soil were detected in clean rooms (Table 5).

5. Efforts were continued to develop a technique for injuring bacterial spores with dry heat so that various culture media can be evaluated for maximum recovery of sublethally damaged spores. The first phase was to obtain typical destruction curves for spores at various temperatures so that subsequent tests could be performed on spore populations that have a high ratio of dead spores to viable spores. In the course of these experiments extremely long "tails" were noted in the destruction curves.

The following experimental procedure was used. Each of a series of tubes was inoculated with 0.05 ml of an ethanol suspension of B. subtilis var. niger so that the final concentration per tube was about  $1 \times 10^7$  viable spores. The tubes were placed in a desiccator containing silica gel and evacuated over night (at least 16 hours). The tubes were then capped and all except the control tubes were placed in an oil bath at 120 C. Assays were performed at 0, (controls), 2, 4, 8, 12, 24, 48, and 72 hours and after 7, 14, and in some cases 21 days. At each interval the appropriate number of tubes (3-5) was removed and placed in an ice bath. Five milliliters of sterile buffered distilled water were added to each tube along with three sterile glass beads. The tubes were ultrasonicated for 8 minutes. Appropriate dilutions of the suspension were made and 1 ml portions were plated with trypticase soy agar. Incubation was for 48 hours at 32 C. The results showed that the spore populations decreased to  $10^4$  to  $10^3$  per tube after 12 hours and remained at this level for periods up to 21 days (Figure 5). It was determined that this "tail" was not an artifact due to background contamination because sterile tubes processed in the same manner were consistently negative. In addition assays were performed in a laminar flow clean bench. Organisms which had survived 14 days at 120 C were isolated and a new spore crop was made. When this population was tested against the parent strain no differences were noted in the rate of destruction or the level of the "tail". In another series of tests one set of tubes was inoculated with the same number of spores but the inoculum was dispersed about the sides of the tube by using a tissue culture roller drum. No differences were noted in the

destruction curves or the formation of "tails" between spores in these tubes and those which were inoculated in the normal manner (Figure 6). Consequently physical protection of the spore population did not seem to be an important factor. However, when the same number of spores were inoculated onto paper discs which were placed in tubes and subjected to 120 C no "tails" were observed. Studies will be continued to determine what factors are responsible for these phenomena.

Other tests showed that heat activation of spores of B. subtilis strain 5230 could be eliminated when the plating medium was supplemented with calcium dipicolinate (Table 6). Studies will be made to determine if the recovery of heat injured spores can be enhanced using such a system.

6. Studies were continued to assess the levels of microbial contamination on lunar spacecraft and the environments in which they are tested and assembled. Continuous 8-hour air samples were taken from Building AE, A-IMP solar ray test facility, A-IMP spin test facility, Building AO, Surveyor fuel loading room, Surveyor sterilization and assembly laboratory, Hangar S, Lunar Orbiter fuel loading room, Lunar Orbiter camera room, and operating suites 1 and 2 of the Bioastronautics Hospital. Lowest levels of airborne and fallout microbial contamination were found in those areas employing laminar air flow. In the conventional clean rooms and non-controlled areas the levels of airborne contamination were proportional to the density and activity of personnel (Tables 7, 8, and 9, Figures 7, 8, 9, 10, 11, and 12). Surface samples of Lunar Orbiters C, 4, and 5 and Surveyor 2 were performed using cotton swabs. The results showed that the Lunar Orbiters had 258, 125, and 125 aerobic mesophilic microorganisms per square foot respectively (Table 10). Surveyor 2, which was sampled on 6 occasions during 41 days, had 117, 3156, 3311, 820, and 45,297 aerobic mesophilic microorganisms per square foot. It appeared that a buildup of microorganisms on Surveyor occurred. After the spacecraft was cleaned prior to launch a marked reduction in the levels of surface contaminants (252 per square foot) was observed (Table 11).

To determine the source of variance in the stainless steel strip sampling technique, 42 strips were exposed to the intramural environment of the operating suite of the Bioastronautics Hospital. After 4 weeks the strips were retrieved and assayed in the usual manner. The data indicated that the variation between the mean levels among individual strips was greater than the variation between samples from the same strip.

The effect of hydrogen ion concentration of trypticase soy agar on the recovery of bacteria and molds by slit samplers was determined. Two sets of media, one at pH 5.6 and the other at 7.0, were employed. The results showed that there was no significant difference between the two media in recovering molds. Although higher number of bacteria were recovered at pH 7 than at pH 5.6, the difference was not statistically significant.

7. The bioclean room support facility was largely completed and invitations to bid on the bioclean room package were issued.

TABLE 1. RECOVERY OF SPORES OF BACILLUS SUBTILIS VAR. NIGER FROM POLYMERIZED METHYL METHACRYLATE.

Experimental factors	Viable spores per gram of methyl methacrylate	
	Test 1	Test 2
Initial number of spores per gram of methyl methacrylate (prior to polymerization)	$5.0 \times 10^5$	$5.0 \times 10^5$
Sample dissolved in acetone and suspension assayed with a solvent resistant membrane filter <sup>1</sup> (after polymerization)	$3.7 \times 10^4$	$3.7 \times 10^4$
Sample ground for 1 minute in a Blender-Mill and the resultant suspension:		
A. Assayed with a standard membrane filter	$2.4 \times 10^3$	$4.4 \times 10^3$
B. Collected on a standard membrane filter <sup>2</sup> which was dissolved subsequently in acetone. Assays were then performed with solvent resistant filters	$5.4 \times 10^3$	$5.7 \times 10^3$
C. Assayed by standard pour plate technique	$2.4 \times 10^3$	$2.2 \times 10^3$

1. Gelman alpha 6 or 8, 0.45 and 0.2 micron pore size, respectively.

2. Millipore, HA, 0.45 micron pore size.

TABLE 2. COMPARISON OF MECHANICAL AGITATION AND ULTRASONICATION IN RECOVERING MICROBIAL CONTAMINANTS FROM STAINLESS STEEL STRIPS.

Test	Criterion	Ultrasonication	Mechanical agitation	Probability factor
A <sup>1</sup>	Avg. No. microorganisms recovered	392	441	< 0.02
	Percent recovery	98.9	99.3	> 0.2
B <sup>1</sup>	Avg. No. microorganisms recovered	382	197	< 0.001
	Percent recovery	99.4	98.5	< 0.05
C <sup>2</sup>	Avg. No. microorganisms recovered	826	441	< 0.01
	Percent recovery	99.1	99.3	> 0.3
D <sup>2</sup>	Avg. No. microorganisms recovered	591	197	< 0.001
	Percent recovery	99.4	98.5	< 0.001
E <sup>3</sup>	Avg. No. microorganisms recovered	170	112	< 0.001
	Percent recovery	99.4	85.0	< 0.001
F <sup>4</sup>	Avg. No. microorganisms recovered	136	99	< 0.001
	Percent recovery	99.9	93.7	< 0.001

1. Natural aerial fallout on strips, contaminated surface facing away from ultrasonic source.
2. Natural aerial fallout on strips, contaminated surface facing ultrasonic source.
3. Aerosolized spores of Bacillus subtilis var. niger air-dried on strips, contaminated surface facing ultrasonic source.
4. Aerosolized spores of B. subtilis var. niger heat-fixed on strips, contaminated surface facing ultrasonic source.



TABLE 3. COMPARISON OF MECHANICAL AGITATION AND ULTRASONICATION IN RECOVERING NATURAL MICROBIAL CONTAMINANTS, RESULTING FROM HANDLING, FROM GLASS SURFACES.<sup>1</sup>

Subject	Criterion used for comparison	Ultrasonication	Mechanical agitation	Probability factor
A.	Avg. No. microorganisms recovered	24	26	> 0.8
	Percent recovery	99.5	99.0	> 0.5
B.	Avg. No. microorganisms recovered	194	178	> 0.7
	Percent recovery	96.0	98.3	< 0.01
C.	Avg. No. microorganisms recovered	1,887	1,678	> 0.7
	Percent recovery	98.1	99.4	< 0.01

1. 1" x 2" glass strips.

TABLE 4. COMPARISON OF THE RECOVERY OF MICROBIAL CONTAMINANTS BY  
ULTRASONICATION FROM STAINLESS STEEL STRIPS IN TWO POSITIONS.<sup>1</sup>

Test	Criterion	Position		Probability factor
		Up <sup>2</sup>	Down <sup>3</sup>	
A	Avg. No. microorganisms recovered	892	826	> 0.7
	Percent recovery	98.9	99.1	> 0.7
B	Avg. No. microorganisms recovered	382	591	< 0.05
	Percent recovery	99.4	99.9	< 0.01

1. Natural aerial fallout on 1" x 2" stainless steel strips.
2. Contaminated surface facing away from ultrasonic source.
3. Contaminated surface facing ultrasonic source.

TABLE 5. TYPES OF AEROBIC MESOPHILIC MICROORGANISMS IN THE INTRAMURAL AIR OF TWO OPERATING ROOMS AND A CLEAN ROOM.

Types of microorganisms	Hospital A - O.R. 2		Hospital B - O.R. 2		Clean Room B	
	No.	%	No.	%	No.	%
<u>Staphylococcus epidermidis</u>	8	5.4	5	4.5	59	70.2
<u>Staphylococcus aureus</u>	1	0.7	4	3.6	5	5.9
<u>Micrococcus</u> spp.	23	15.4	23	20.7	3	3.6
<u>Sarcina</u> spp.	0	0.0	1	0.9	0	0.0
<u>Gaffkya</u> spp.	0	0.0	0	0.0	1	1.2
<u>Bacillus</u> spp. (sporeformers)	13	8.7	14	12.6	0	0.0
Misc gram-positive bacilli*	7	4.7	7	6.3	8	9.5
<u>Streptococcus</u> spp.	0	0.0	0	0.0	1	1.2
Actinomycetes	10	6.7	26	23.4	1	1.2
Molds	51	40.9	19	17.2	1	1.2
Yeasts	22	14.8	5	4.5	0	0.0
<u>Flavobacterium</u> spp.	1	0.7	0	0.0	0	0.0
<u>Pseudomonas-Achromobacter</u> spp.	3	2.0	7	6.3	3	3.6
Unidentified	0	0.0	0	0.0	2	2.4
Total	149	100	111	100	84	100

\* Representative of genera: Brevibacterium, Corynebacterium, Lactobacillus and Arthrobacter.

TABLE 6. EFFECT OF SUPPLEMENTING TRYPTICASE SOY AGAR WITH CALCIUM  
 DIPICOLINATE ON THE ENUMERATION OF SPORES OF BACILLUS SUBTILIS  
 VAR. NIGER AND B. SUBTILIS STRAIN 5230, WITHOUT HEAT ACTIVATION.

Plating medium	<u>Bacillus subtilis</u> var. <u>niger</u> Avg. No. of colonies	<u>Bacillus subtilis</u> strain 5230 Avg. No. of colonies
Trypticase soy agar	127	88
Trypticase soy agar plus calcium dipicolinate <sup>1</sup>	154	306

1. Obtained by adding appropriate amounts of sterile calcium chloride and sodium dipicolinate to give a final concentration in the medium of 50 and 40 mM, respectively.

TABLE 7. SUMMARY OF AIR SAMPLING DATA IN FACILITIES USED FOR TEST AND ASSEMBLY  
OF THE A-IMP.

Area	Date	Volume of Air Sampled (cubic feet)	Number of Microorganisms Detected	Viable Particles/ft <sup>3</sup>
Hangar AE clean room	5-19-66	330	7	0.0210
	5-26-66	420	17	0.0400
	5-2-66	420	7	0.0167
	5-3-66	240	20	0.0833
	6-6-66	240	5	0.0208
	6-7-66	240	12	0.0500
	6-8-66	240	4	0.0167
	6-9-66	240	3	0.0125
	6-10-66	240	9	0.0375
	6-13-66	240	1	0.0042
	6-14-66	240	5	0.0208
	6-15-66	240	0	0.0000
	6-16-66	240	0	0.0000

TABLE 7, continued

SUMMARY OF AIR SAMPLING DATA IN FACILITIES USED FOR TEST AND ASSEMBLY  
OF THE A-IMP.

Area	Date	Volume of Air Sampled (cubic feet)	Number of Microorganisms Detected	Viable Particles/ft <sup>3</sup>
Portable downflow room	5-19-66	180	4	0.0220
	5-26-66	420	2	0.0047
	6-2-66	240	1	0.0042
	6-3-66	120	1	0.0083
	6-6-66	120	0	0.0000
	6-7-66	120	0	0.0000
	6-8-66	120	0	0.0000
	6-9-66	120	0	0.0000
	6-10-66	120	0	0.0000
	6-13-66	120	0	0.0000
	6-14-66	120	0	0.0000
	6-15-66	120	1	0.0083
	6-16-66	120	0	0.0000
Solar ray test facility	6-7-66	360	944	2.6220
	6-14-66	1,290	2,743	2.1260
Spin test facility	8-18-66	900	3,510	3.9000

TABLE 8. SUMMARY OF AIR SAMPLING DATA IN FACILITIES USED FOR TEST AND ASSEMBLY OF SURVEYOR.

Area	Date	Volume of Air Sampled (cubic feet)	Number of Microorganisms Detected	Viable Particles/ft <sup>3</sup>
Sterilization and assembly lab, North bay	6-8-66	180	25	0.1388
	7-20-66	960	1,385	1.4437
Sterilization and assembly lab, South bay	6-8-66	180	20	0.1111
	7-12-66	960	268	0.2791
Sterilization and assembly lab, Air lock	6-8-66	120	5	0.0417
Fuel loading room	6-8-66	120	82	0.6833
	6-29-66	780	455	0.5833
Hangar A0 clean room	6-10-66	960	70	0.0729

TABLE 9. SUMMARY OF AIR SAMPLING DATA IN FACILITIES USED FOR TEST AND ASSEMBLY OF LUNAR ORBITER.

Area	Date	Volume of Air Sampled (cubic feet)	Number of Microorganisms Detected	Viable Particles/ft <sup>3</sup>
Hangar S clean room	5-6-66	240	32	0.1333
	6-23-66	900	61	0.0677
Explosive safe area	7-5/6-66	1380	61	0.0442
	7-16-66	960	4	0.0042
Hangar S camera room	8-12-66	900	1,129	1.2500



TABLE 10. LEVELS OF MICROBIAL CONTAMINATION ON THE SURFACE OF LUNAR ORBITER:

Spacecraft	Date	Area sampled (in <sup>2</sup> )	No. of microorganisms per ft. <sup>2</sup>			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Lunar Orbiter C (mock-up)	7-14-66	47.4	258	76	137	0
Lunar Orbiter 4	7-28-66	57.4	125	38	25	25
Lunar Orbiter 5	7-29-66	57.4	125	38	0	0

TABLE 11. LEVELS OF MICROBIAL CONTAMINATION ON THE SURFACE OF SURVEYOR.

Spacecraft	Date	Area sampled (in <sup>2</sup> )	No. of microorganisms per ft. <sup>2</sup>			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Surveyor 2	8-2-66	80	117	63	9	0
Surveyor 2	8-17-66	68	3,156	583	21	0
Surveyor 2	8-23-66	80	3,311	2,456	27	0
Surveyor 2	9-6-66	72	820	100	50	0
Surveyor 2	9-12-66	80	45,297	15,784	100	0
Surveyor 2	9-15-66 <sup>1</sup>	80	252	45	9	0

1. Samples were taken within 1 hour after the spacecraft was cleaned.

- Figure 1. Average slopes of die-away curves of spores of B. subtilis var. niger in unpolymerized (liquid; preservative removed) and polymerized methyl methacrylate.
- Figure 2. Survival of spores of B. subtilis var. niger on polystyrene granules and lucite powder at 0 and 46 percent R.H. and 50° C.
- Figure 3. Survival of spores of B. subtilis var. niger on discs of lucite and polystyrene at 0 and 46 percent R.H. and 50° C.
- Figure 4. Survival of spores of B. subtilis var. niger on lucite powder and polystyrene granules at 0 percent R.H. and 50° C with and without vacuum.
- Figure 5. Destruction curve of spores of B. subtilis var. niger at 120° C (dry heat). The regression line of data points after 24 hours is shown.
- Figure 6. Effect of dispersing the inoculum of spores of B. subtilis var. niger on death rates and "tailing" at 120° C.
- Figure 7. Effect of personnel density and activity on the levels of airborne microbial contamination in the Surveyor Sterilization and Assembly Laboratory.
- Figure 8. Effect of personnel density and activity on the levels of airborne microbial contamination in Hangar S (Lunar Orbiter).
- Figure 9. Levels of airborne microbial contamination which accumulated on stainless steel surfaces exposed within Building A0.
- Figure 10. Levels of airborne microbial contamination which accumulated on stainless steel surfaces exposed within the Surveyor Fuel Loading Room.

Figure 11. Levels of airborne microbial contamination which accumulated on stainless steel surfaces exposed within the Surveyor Sterilization and Assembly Laboratory.

Figure 12. Levels of airborne microbial contamination which accumulated on stainless steel surfaces exposed within Hangar S.

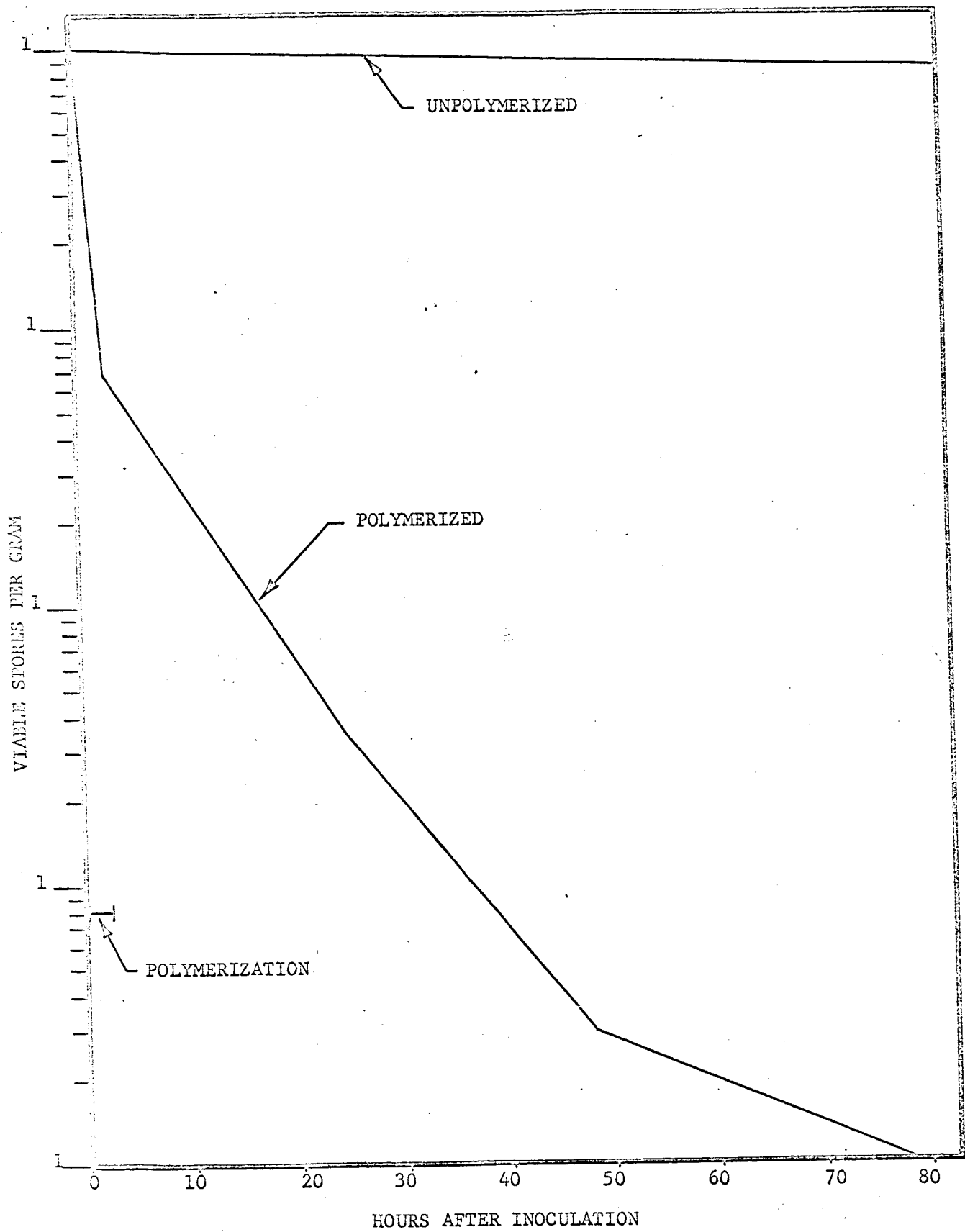


Figure 1.

Figure 2.

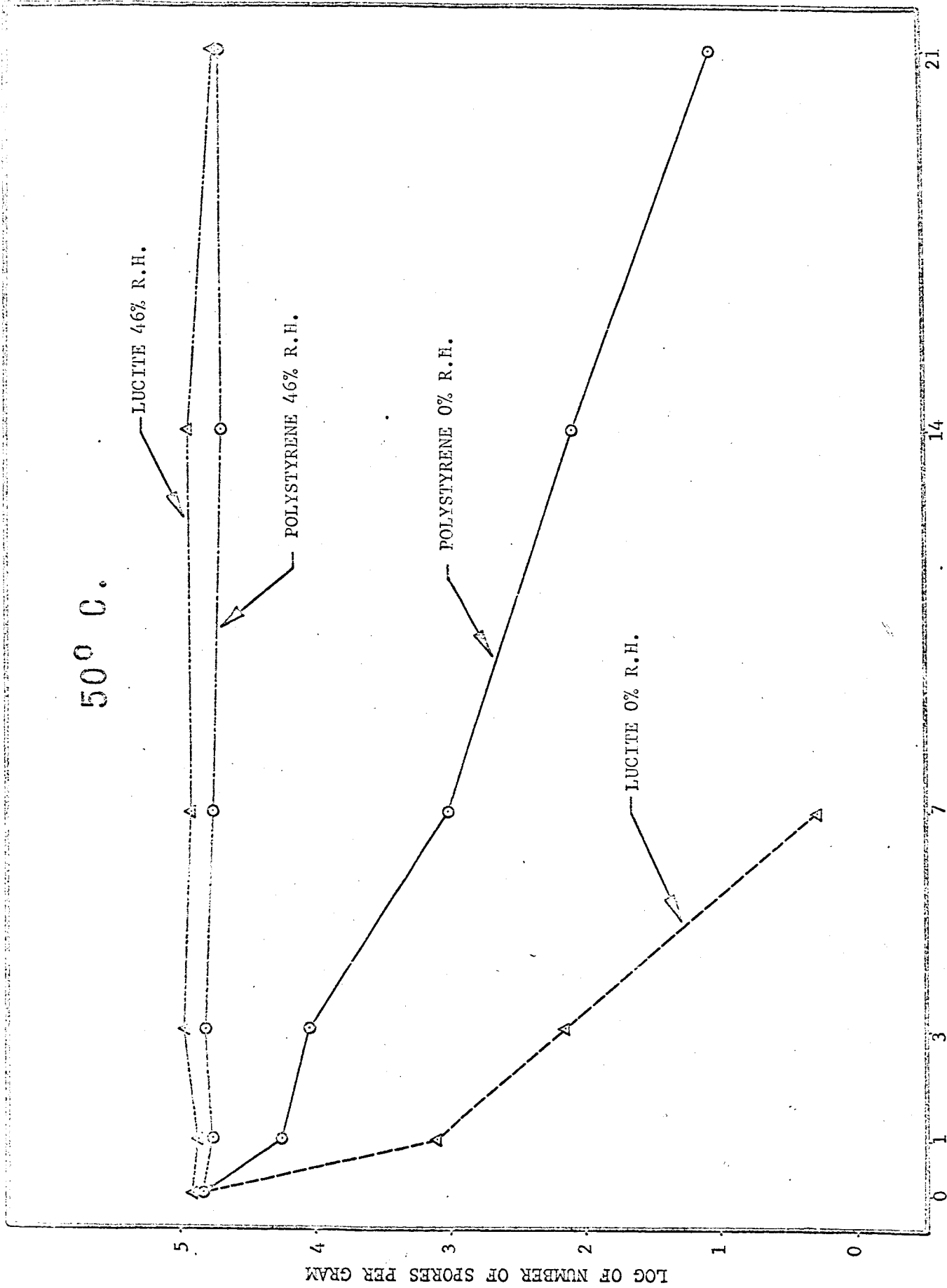


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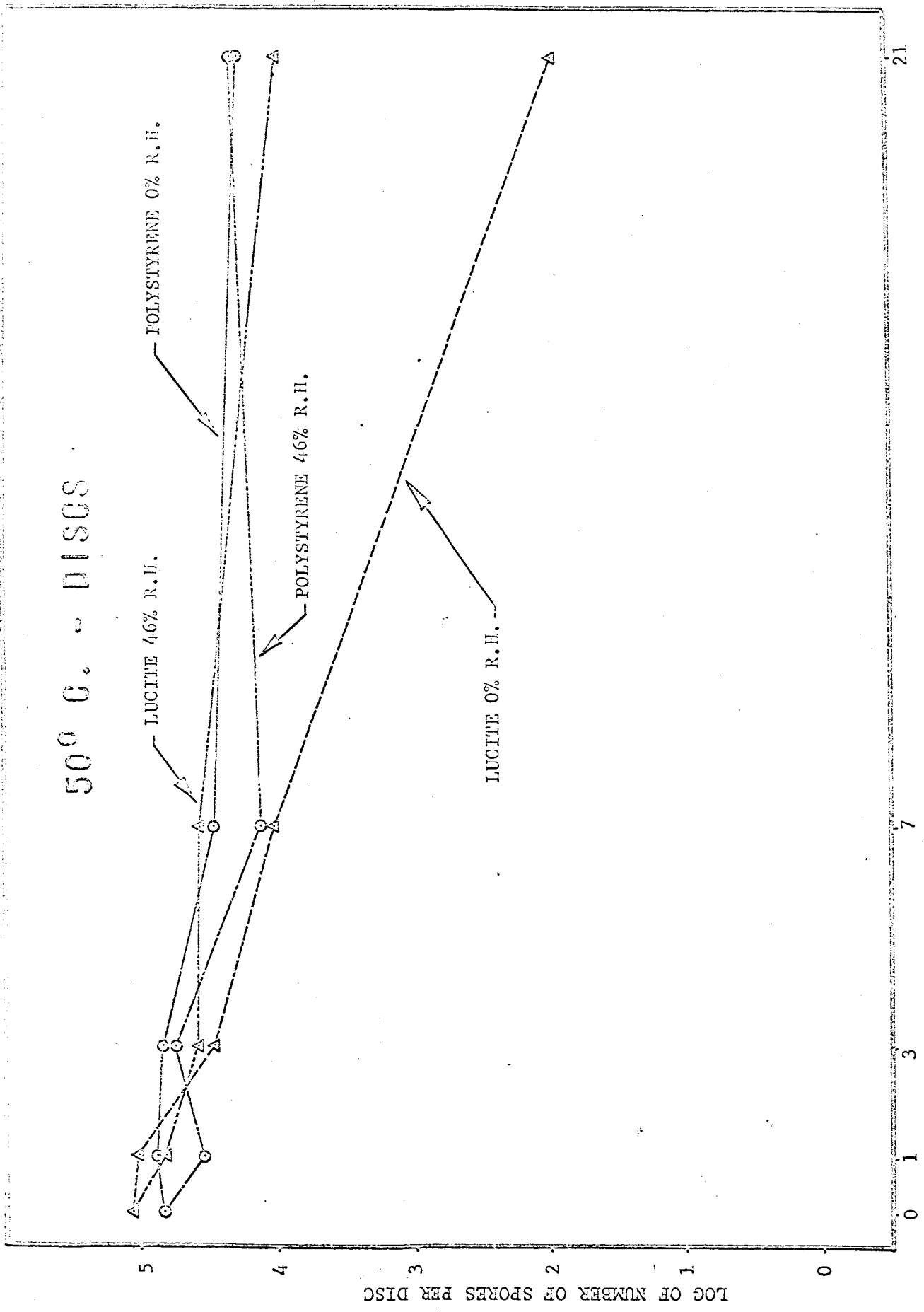
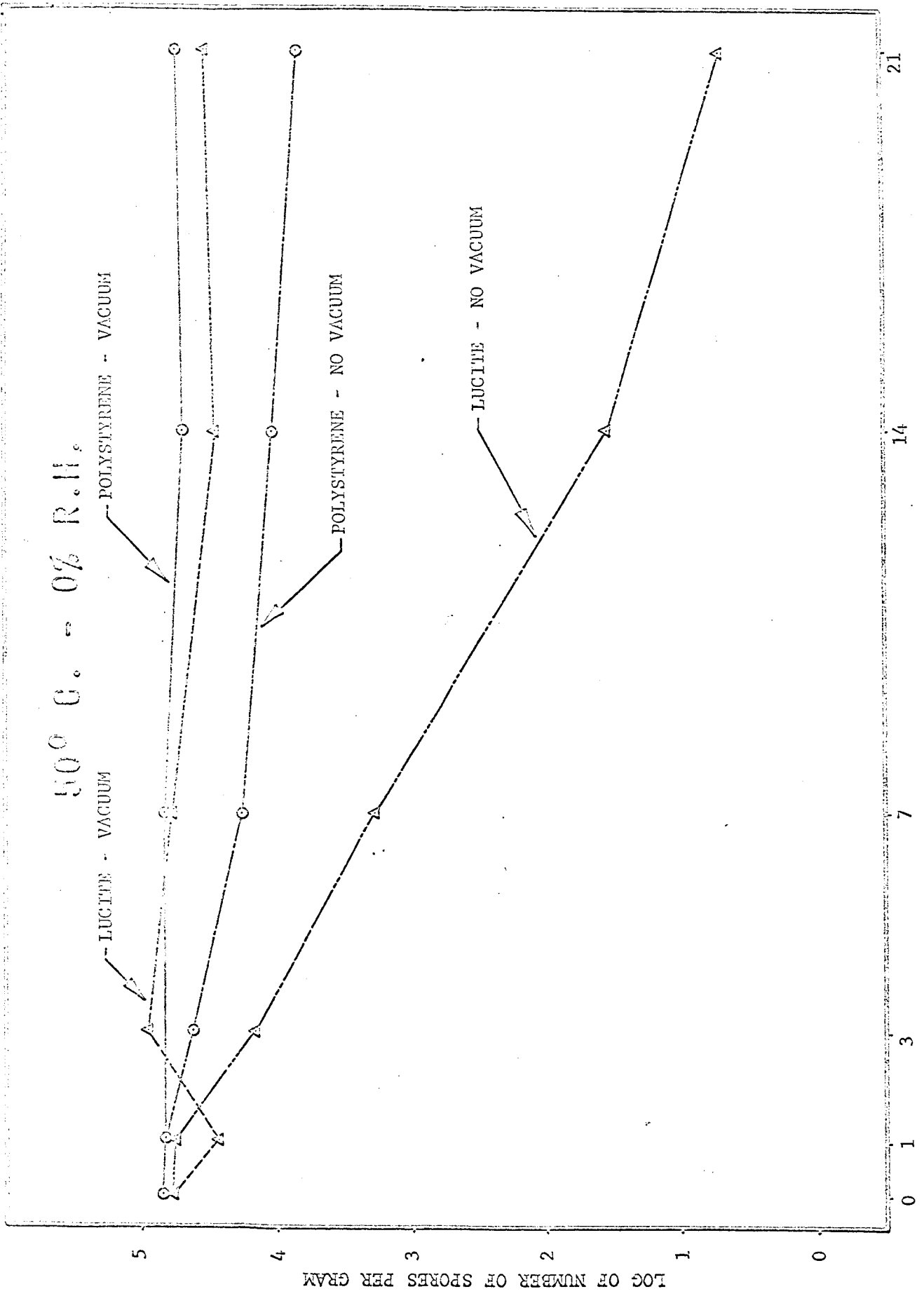


Figure 4.





120 C. - B. SUBTILIS VAR. NIGER

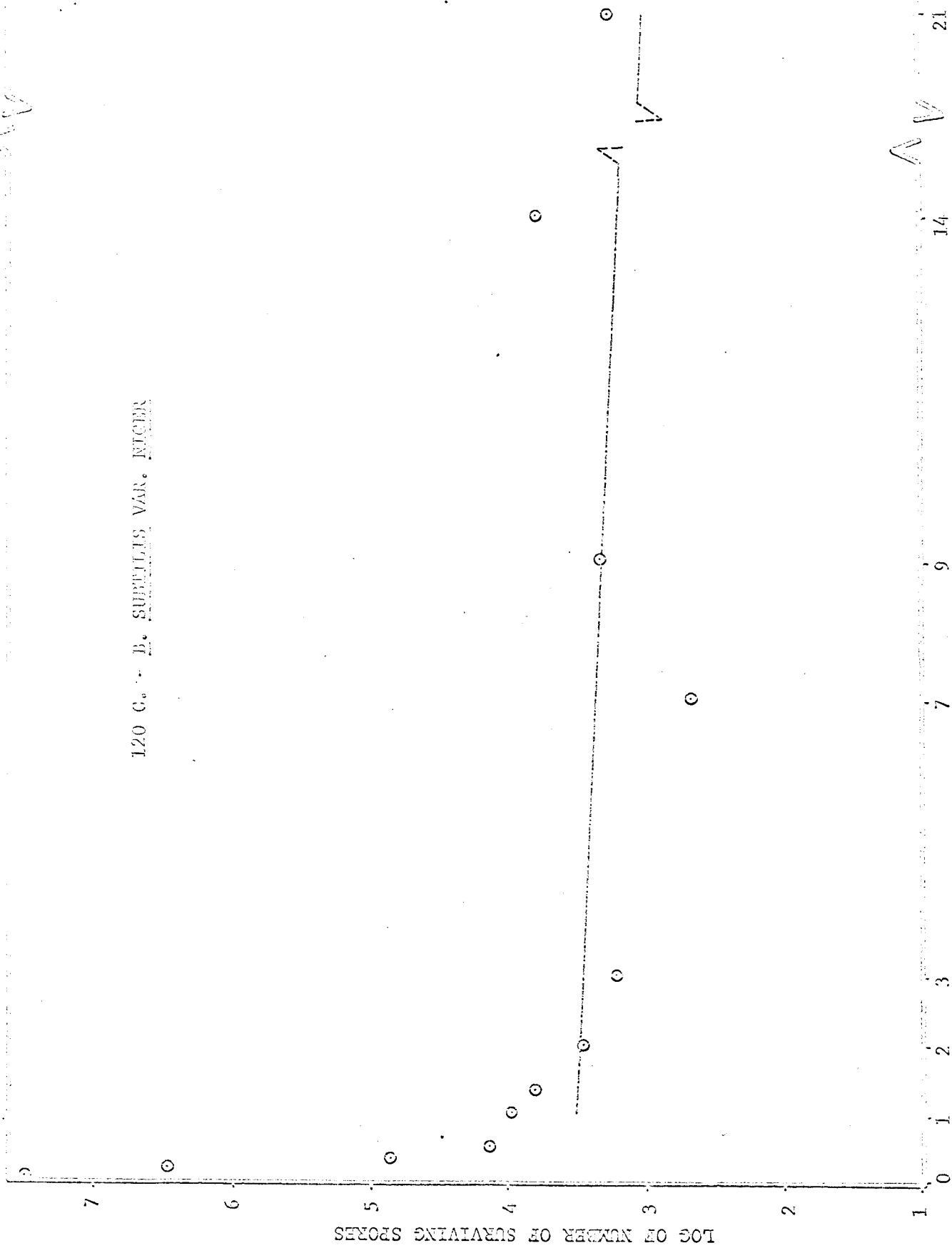


Figure 5.

120 C. · B. SUBTILIS VAR. NIGER

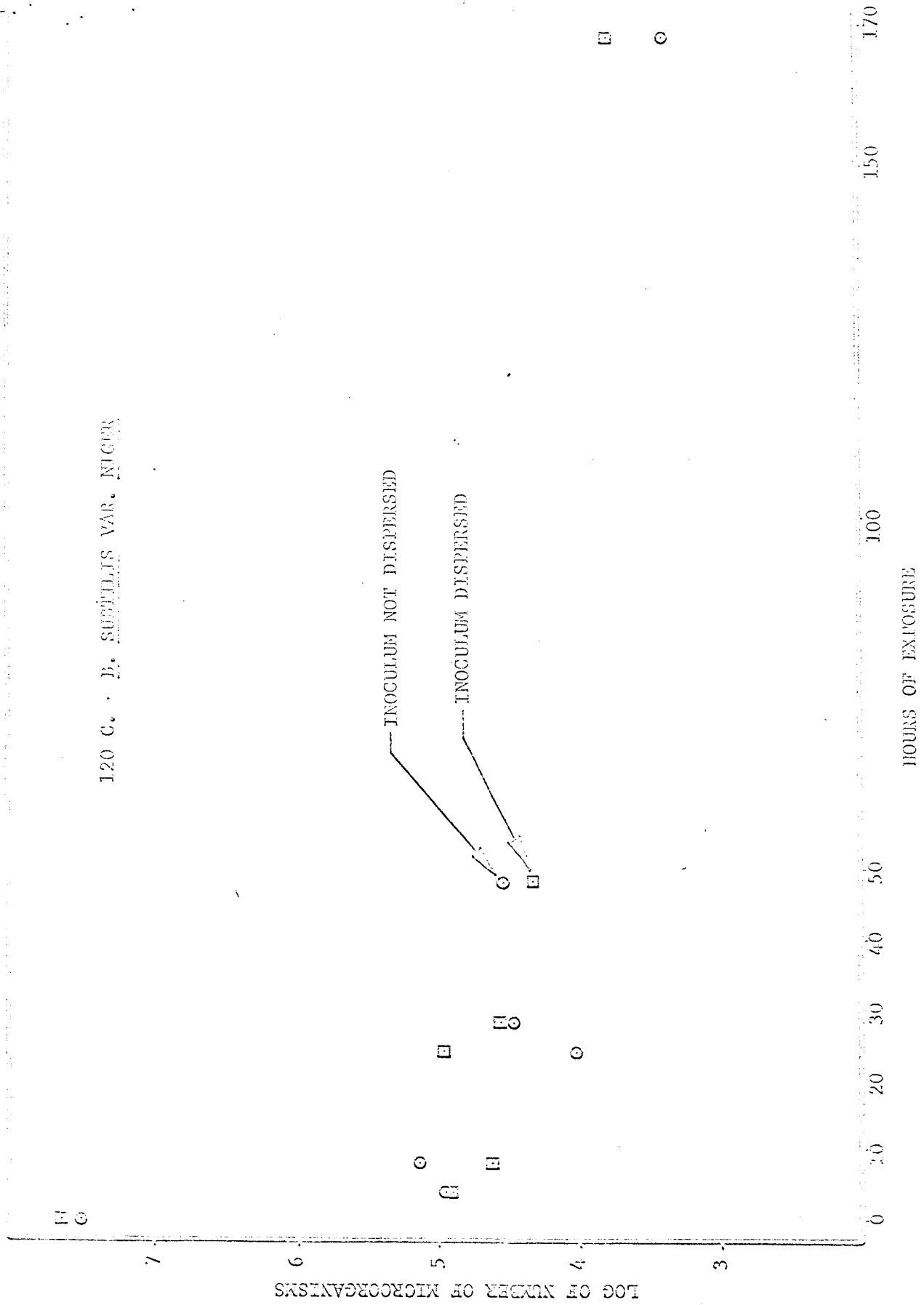


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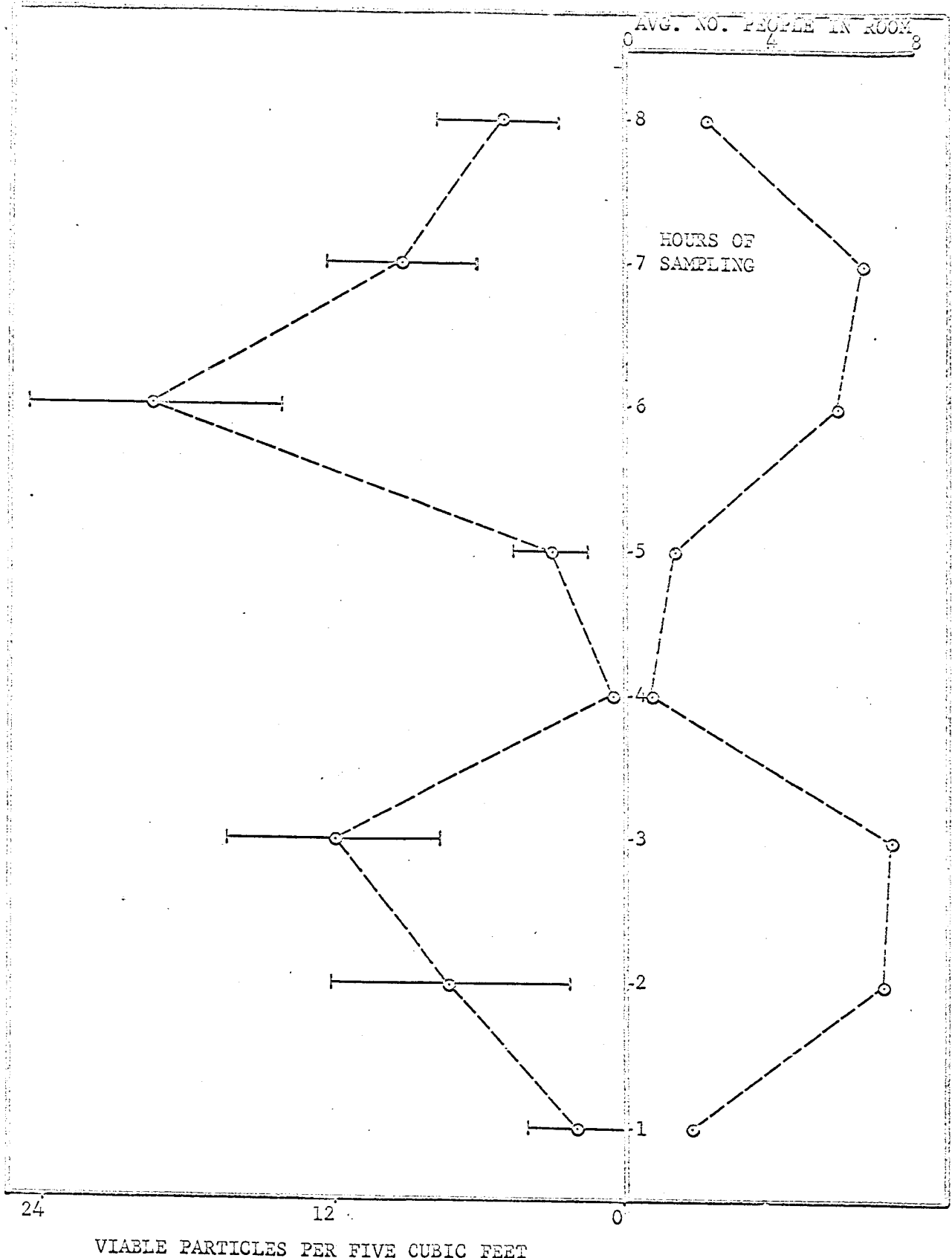


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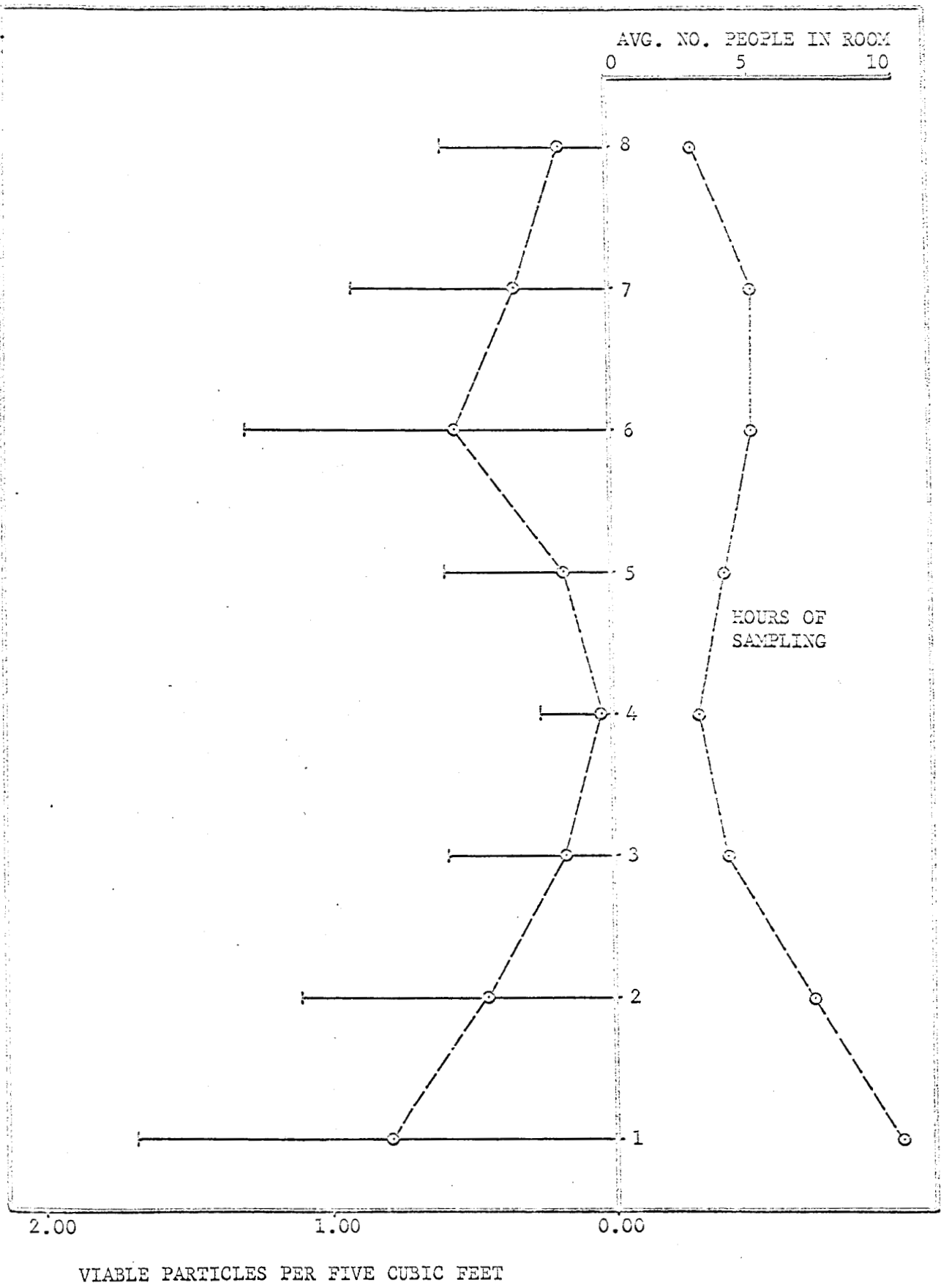


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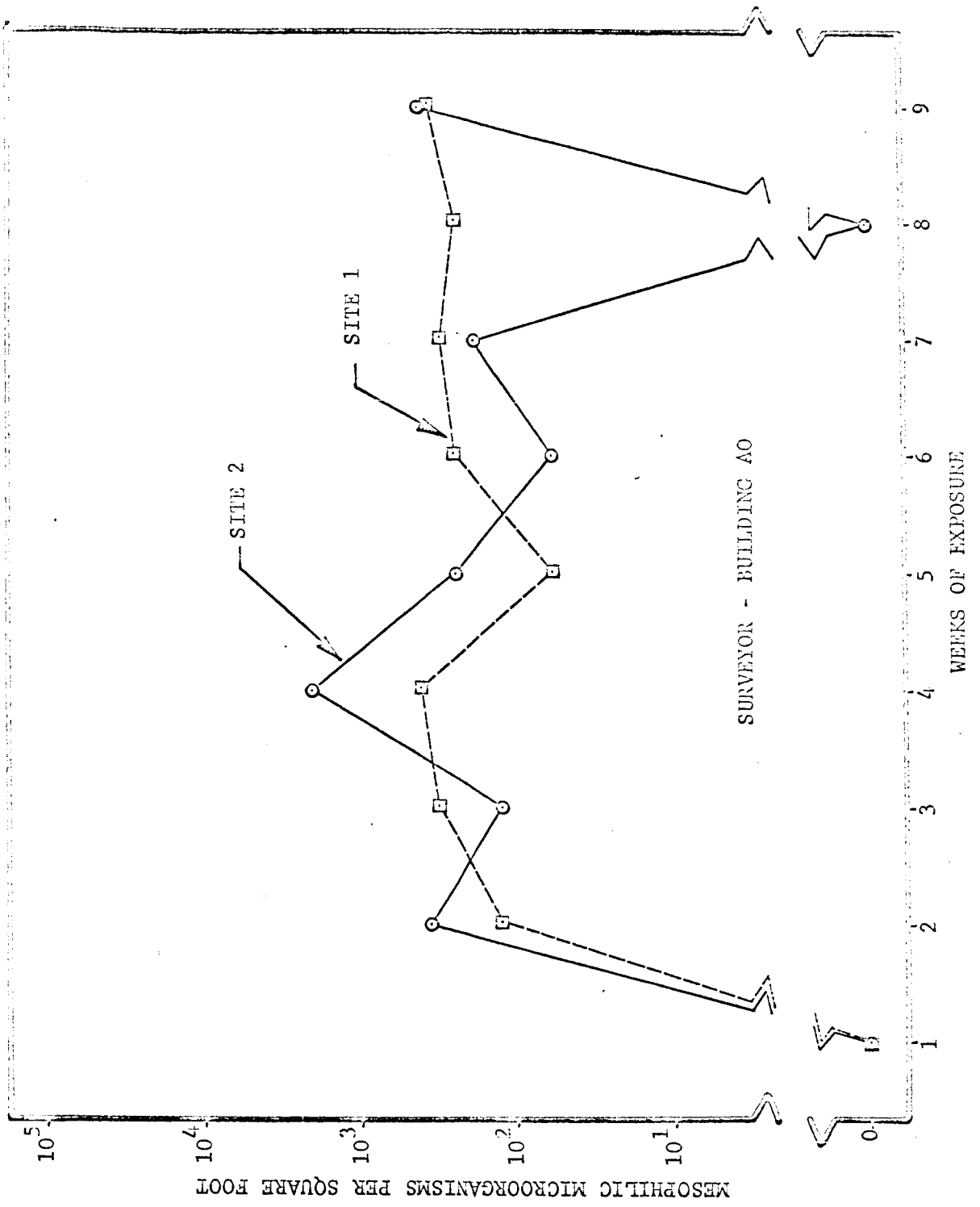


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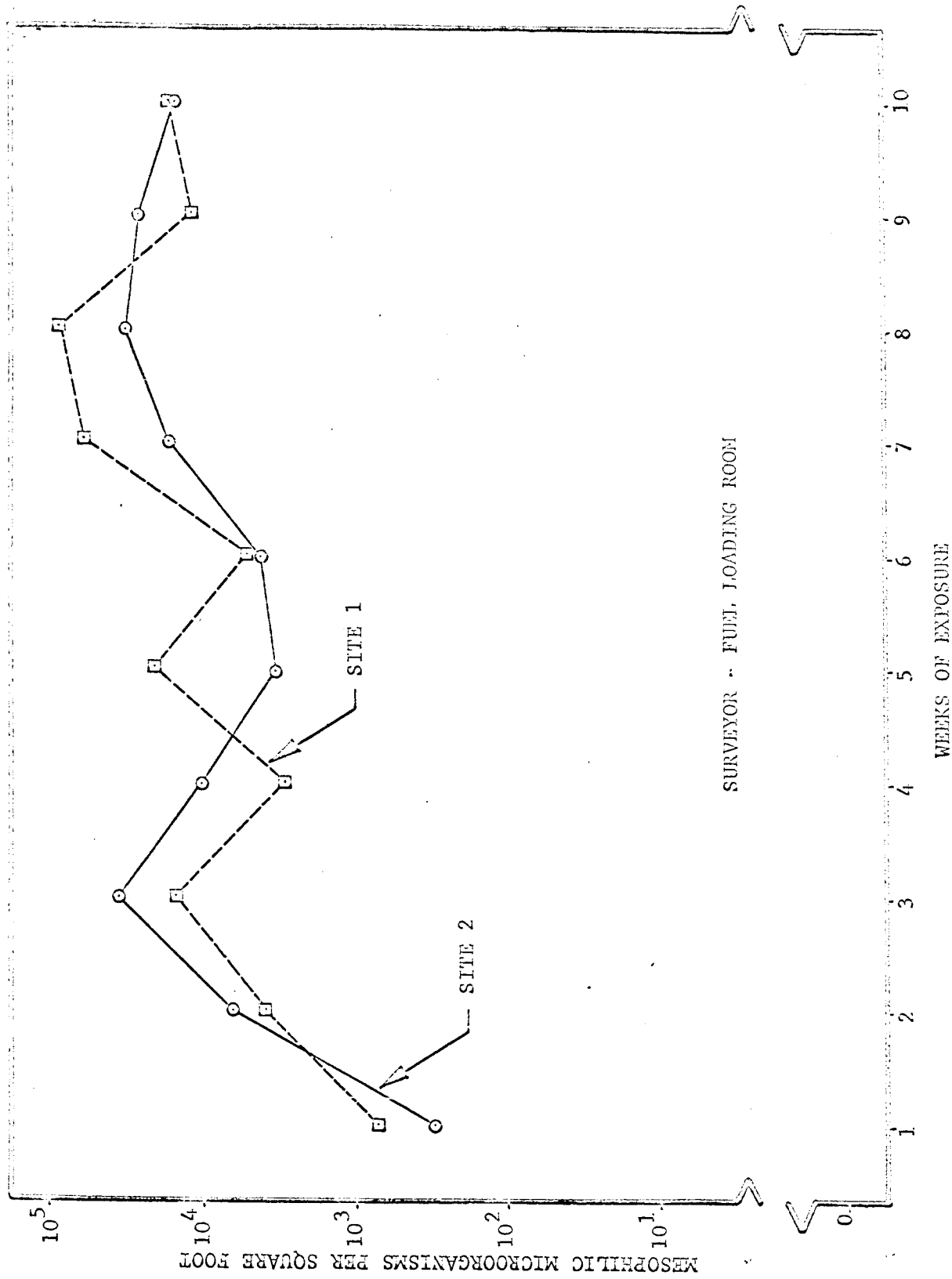


Figure 10.

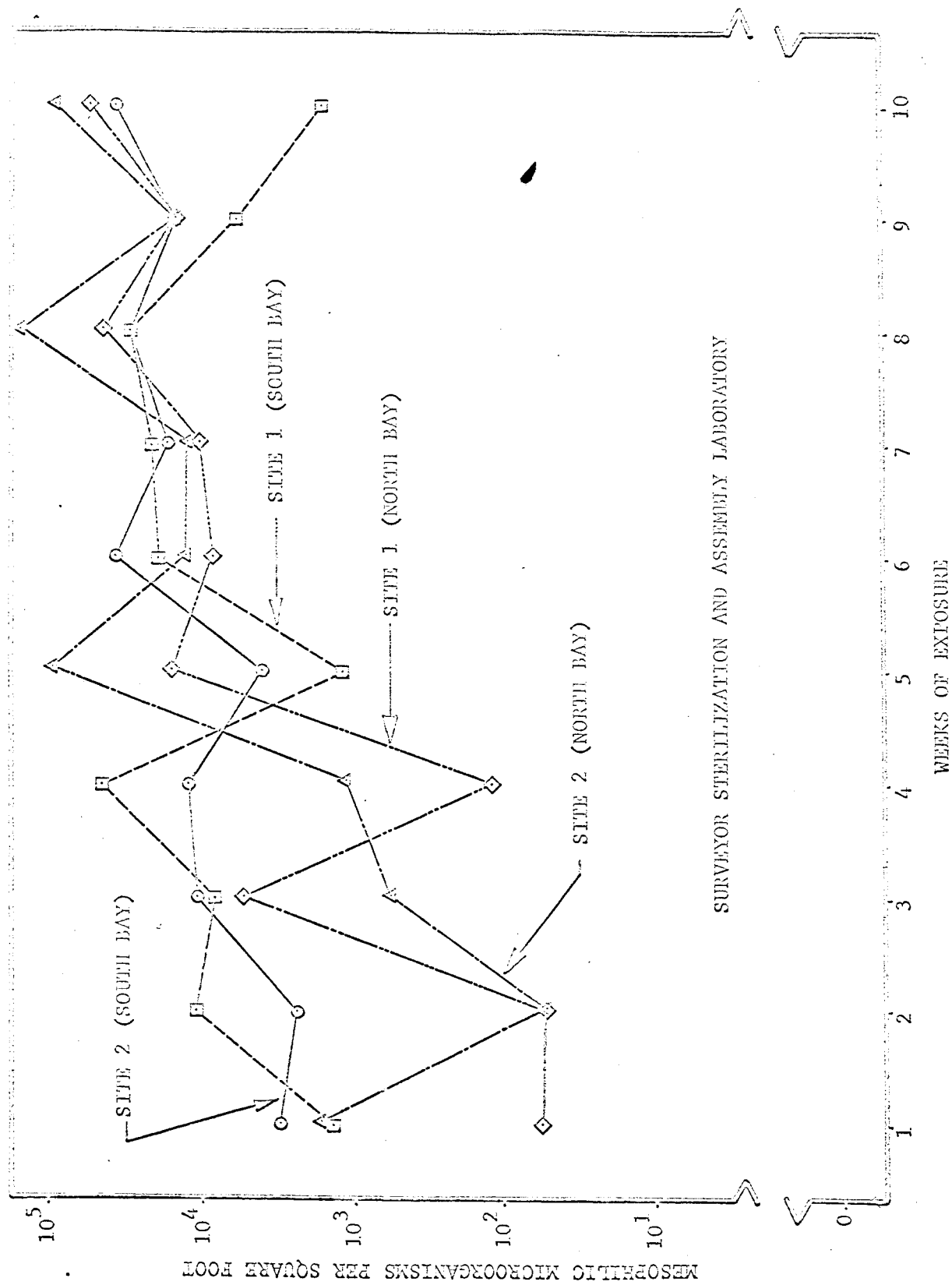


FIGURE 11.

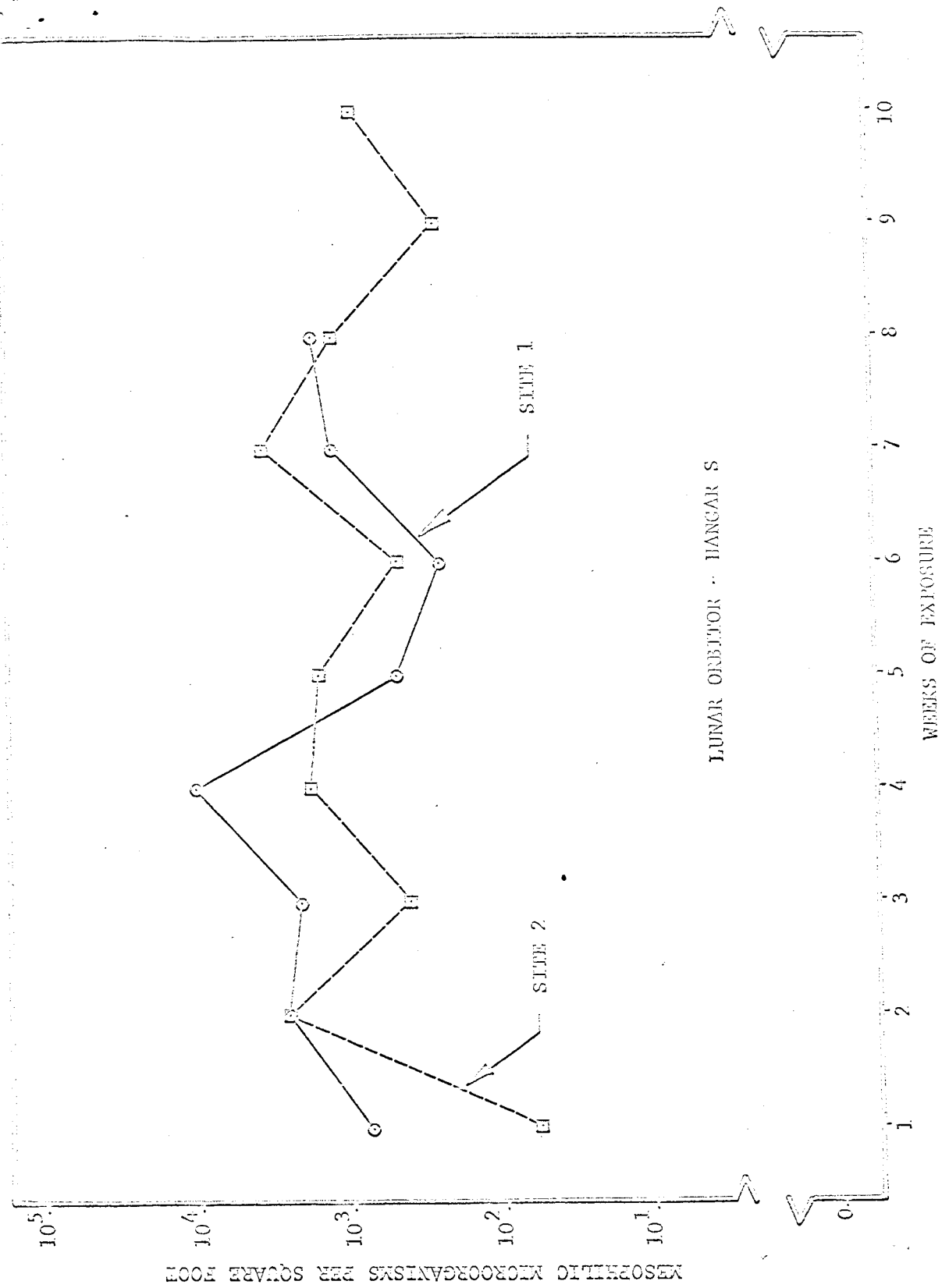


Figure 12