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

Fourth Quarterly Report of Progress

on

Research Project R-36-015-001

January 1 - March 31, 1966

Conducted by

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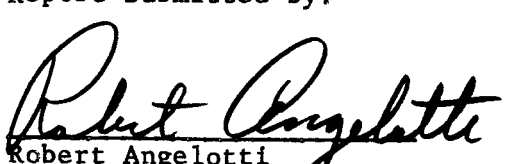

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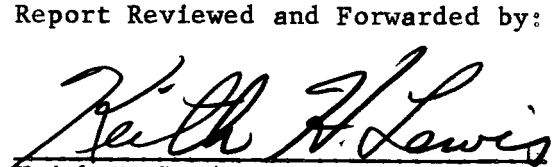

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SUMMARY

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The thermal resistance of Bacillus globigii spores dried on paper strips and encapsulated in Lucite rods has been determined at a dry heat exposure temperature of 125°C. The $D_{125°C}$ value for these spores dried on paper strips was found to be 1.72 hours with a 95% confidence interval of 1.61 to 1.83 hours. In duplicate experiments performed with these spores encapsulated in Lucite, the $D_{125°C}$ values were: experiment 1, 3.08 hours, 95% confidence interval of 2.55 to 3.61 hours; experiment 2, 3.44 hours, 95% confidence interval of 2.98 to 3.89 hours.

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INTRODUCTION

The general goals of this project and the related need for a system to quantitatively enumerate the bacterial spores encapsulated in plastics or other insoluble materials have been discussed in the First Quarterly Report of Progress. In order to pursue these goals, a decision was made to carry out the initial work in a model system using Bacillus globigii and Lucite. In the Third Quarterly Report of Progress methods were described by which acetone suspensions of spores could be added to Lucite rods and recovered quantitatively by a combination of acetone dissolution and Seitz filtration. Statistical analysis of data relating to the reproducibility of the rods revealed that: (a) no difference in count, on the average, occurred in rods prepared from separate batches of plastic on different days; (b) approximately the same number of spores, on the average, was present in every part of any rod when an inoculum level of at least 7×10^6 spores per gram was used and (c) that the number of viable organisms encapsulated in the polymerized rod does not change with respect to time over a period of several days storage at room temperature.

The research planned for the Fourth Quarter in connection with the evaluation of grinding systems for recovering spores from non-soluble plastics was abandoned at the request of the Contract Officer in favor of the development of data on the dry-heat inactivation of Bacillus globigii spores encapsulated in Lucite and exposed to 125°C. Accordingly, all work presented in this report relates specifically to the generation of these data.

EXPERIMENTAL

Effect of acetone on the dry-heat resistance of B. globigii spores.

Initial experiments in which acetone suspensions of B. globigii spores were encapsulated in Lucite rods revealed that a 5-log cycle reduction in numbers of viable spores occurred after 3-hours of exposure to a dry heat temperature of 125°C. This was followed by an additional log cycle reduction after 6-hours of exposure. This extremely rapid die-off was contrary to the resistance of B. globigii spores encapsulated in plastics that Koesterer (1) reported. These observations prompted a series of experiments to determine the normal heat resistance of the B. globigii spore crop produced in this laboratory (First Quarterly Report of Progress) and the effects of the plastic fabrication and recovery methods on the heat resistance of these spores.

To evaluate these factors, a portion of the stock aqueous spore crop was divided into two fractions. One fraction was used to inoculate paper strips (1" x 3/8") by applying 0.03 ml amounts to each strip by means of a RGI Inc. Micrometer Buret. The second fraction was diluted in acetone to obtain the desired concentration of spores and 0.1 ml portions of the acetone suspension were inoculated onto similar filter paper strips. The inoculated paper strips were placed in sterile cotton stoppered borosilicate TDT tubes (10 mm x 100 mm) and dried in a dessicator overnight at room temperature. The following morning the tubes were sealed in an oxy-gas flame and placed in an oil bath operating at 125°C. At various intervals tubes were removed, cooled in an ice bath and opened aseptically. The paper strips were examined bacteriologically by grinding each strip in a Waring blendor with 100 ml of phosphate buffer dilution water and plating the survivors in TGE agar. The plates were

incubated for 48 hours at 35°C. These data are presented in Table 1 and indicate that pre-treatment of spores with acetone renders them more sensitive to dry heat inactivation. In another experiment, plastic rods were heated after inoculation with spores separately prepared in water and acetone. The data were comparable to the paper experiment as fewer acetone prepared spores survived the heating in plastic.

Because acetone affected the heat resistance of spores, it was decided to determine whether acetone also affected the outgrowth of spores that survived a heat treatment. Experiments were performed with paired paper strips that were inoculated and dried as previously described with either acetone or water prepared spores. The strips were subjected to 125°C (dry heat) for 1 hour in sealed TDT tubes and then examined for survivors. One pair of paper strips was ground and diluted in sterile acetone and the second pair was ground and diluted in sterile water. No difference in the numbers of spores recovered was noted from the papers ground and diluted in either diluent indicating acetone did not affect outgrowth of heat-sensitized spores. In each instance, however, fewer of the acetone prepared spores survived the heat treatment than water prepared spores, confirming the observation that acetone sensitizes spores to heat (see Table 2).

These data indicated that a new method of inoculating and fabricating Lucite rods was necessary in order to prevent sensitization of spores with acetone. Accordingly, the following procedure was developed: 1-ml of the aqueous stock spore suspension is distributed over 50 grams of Lucite powder contained in a sterile drying pan. The pan is placed in a forced-air drying oven for 30 minutes at 50°C. The pan is removed from

the oven and the powder is placed in a sterile motar and ground by hand until the dried spore inoculum appears to be evenly distributed throughout the powder. The powder is returned to the drying pan and heated for an additional 30 minutes in the oven followed by a second grinding in the motar. To each 50 grams of inoculated and dried powder, 50 ml of methymethacrylate is added. The inoculated, unpolymerized plastic is fabricated into rods, from this point on, identically to the manner described in the Third Quarterly Report of Progress.

The analysis for the distribution of spores in rods that was described in the Third Quarterly Report of Progress was applied to the rods fabricated by the new method. This analysis revealed that no significant differences ($\alpha = 0.05$) in spore concentrations occurred between rods or at different locations within a rod.

Dry-heat resistance of B. globigii spores encapsulated in Lucite rods or dried on paper strips.

Dry-heat inactivation experiments at 125°C were performed to determine the resistance of Bacillus globigii spores encapsulated in Lucite rods and dried on filter paper strips. Lucite rods were inoculated and fabricated as described in the preceding section of this report. Sterile paper strips (1" x 3/8") were inoculated by means of the micrometer buret with 0.01 ml amounts of the same aqueous stock spore suspension used to inoculate the plastic. Following inoculation, the paper strips were placed in sterile cotton stoppered TDT tubes. Because the fabrication of inoculated plastic rods requires a one-hour exposure to 50°C in a forced air oven to dry the inoculum on the granulated plastic and is followed by a second heat-treatment in a water bath (2 hours at 50°C) to polymerize the plastic, the TDT tubes containing the inoculated paper

strips were subjected to these same conditions simultaneously to the plastic powder and polymerizing rods. After completion of the polymerization, all tubes containing paper or plastic were removed from the water bath. The cotton stoppers were removed and the tubes sealed in an oxy-gas flame. After sealing, all the tubes (paper and plastic) remained at room temperature overnight (approximately 16 hours).

At various intervals throughout 24 hours, two TDT tubes containing plastic and two containing paper were dropped simultaneously into a circulating oil bath operating at 125°C (± 0.1°C). All the experimental tubes were removed simultaneously from the oil bath and dropped into a circulating cold water bath (4°C, ± 0.5°C). The tubes were allowed to cool for 15 minutes after which they were removed from the cold bath, wiped dry and placed in a refrigerator until examined (see below).

The heat-treated TDT tubes containing the Lucite were examined bacteriologically as described in the Third Quarterly Report of Progress with the following exceptions: (a) two 1/2" wide cuts instead of four were made through each rod; and (b) the 200 ml of acetone-plastic solution was poured directly into a one-liter Erlenmyer flask containing 800 ml of sterile phosphate buffered dilution water while the flask contents were being vigorously agitated by means of a magnetic stirrer. Additional serial dilutions were prepared of the flask contents and plated out in duplicate in tryptone, glucose, beef extract agar. This latter modification eliminated the need for the Seitz filtration step described formerly.

The heat-treated TDT tubes containing the paper strips were scored and opened aseptically. The paper strip was dropped into a micro Waring

blendor cup containing 100 ml of sterile phosphate buffered dilution water and blended for 2 minutes at slow speed. Further serial ten-fold dilutions of the blend were prepared in the same buffer and plated in duplicate in tryptone, glucose, beef extract agar.

Because the heat exposure experiment was conducted in an unconventional manner (tubes placed in the bath at separate intervals but all removed simultaneously; tubes refrigerated for several hours before sampling), it was necessary to make the following determinations as controls, (a) the persistance of spores encapsulated in plastic and dried on paper when sealed in TDT tubes and stored at room temperature for various periods; and (b) whether a change occurred with time of the number of detectable spores in plastic or on papers that had been heated and then stored in the refrigerator before sampling. Accordingly, a number of extra TDT tubes containing inoculated plastic rods or papers were similarly prepared at the same time as those employed in the heating experiment. These extra tubes were held at room temperature and duplicate tubes of each type were examined bacteriologically by the above method at the various time intervals corresponding to the heat exposure intervals of the experimental tubes. Another set of extra TDT tubes of each type was subjected to exposure in the 125°C oil bath for 1 hour after which the tubes were removed, dried and cooled as above, and stored in the refrigerator at 4°C. At various intervals throughout 72 hours these tubes were removed from the refrigerator and examined bacteriologically according to the methods described above.

The initial count of the number of spores per gram of plastic or per paper strip was obtained as the "zero time" counts of the duplicate plastic and paper TDT tubes in the non-heat-treated control series. All

plates derived from either the contents of experimental or control TDT tubes were incubated for 48 hours at 35°C and then counted.

The experiments described above were performed on two separate occasions using separate batches of plastic and paper strips. The spore inoculum used in each experiment was obtained from the same aqueous stock spore suspension that was maintained continually at 5°C.

No significant ($\alpha = 0.05$) change in spore counts per gram of plastic or per paper strip was noted when non-heat-treated control samples, stored at room temperature, were examined at various time intervals. This experiment demonstrates that a predictably constant number of spores was heat treated in the plastic and on the paper at each time interval.

Additionally, no differences in spore counts were observed between the heat-treated control rods and papers examined immediately after heating, compared with similarly heated but subsequently refrigerated rods and papers sampled after varing storage times up to 72 hours. These data indicated that refrigerated storage after heating did not cause any change in numbers of heat sensitized spores during refrigerated storage.

The plots of the survival points for B. globigii spores encapsulated in Lucite are shown in Figures 1 and 2, and present the data from the first and second experiments, respectively. Each point in these plots is a mean value obtained by averaging the duplicate plate count values for each rod. In observing these data, a non-logarithmic order of death is apparent. If these points were connected, a tri-phasic curve would result that would be characterized by an approximately 99% reduction in numbers

within the first 30 minutes of exposure, followed by a flattening of the curve between 30 minutes and 4 hours and finally a logarithmic die-off from 4 hours onward. This type of curve could result from a population of spores with non-homogeneous or mixed heat resistance. That the heat resistance of the spores in the original aqueous stock spore suspension was homogeneous is established by observing the thermal survival curve in Figure 3 obtained from spores dried on paper strips. This figure presents all the points obtained from the duplicate plate count values for each of the paired paper strips examined at each time interval in both experiments. A weighted linear regression was calculated from these data such that 93% of the sums of squares of deviations could be explained by this linear regression. This regression is presented as the survival curve in Figure 3 and reveals that a straight line could be fitted from the survivor counts obtained on paper from 0 hours through 14 hours exposure. Surviving spores were not detected for all samples collected at 16, 20 and 24 hours of exposure. The calculated $D_{125°C}$ value for this curve is 1.72 hours with 95% confidence intervals of 1.61 hours to 1.83 hours. The true D value may be expected, 95% of the time, to lie between the above range. Since spores placed on the paper originated from the same spore suspension encapsulated in plastic, the two distinct heat-resistant spore populations in the plastic most probably arise as the effects of encapsulation. The majority of the spores (99%) encapsulated in the plastic are heat labile in comparison to the heat resistance of the same spores heated on paper. The remainder of the spores in the plastic are exceptionally heat resistant in comparison to those heated on paper. These two populations are easily discernable in Figures 1 and 2 as those dying rapidly in the first 30 minutes of heating and those

dying at a significantly slower rate from 4 hours through 24 hours of heating. The flattened portion or mid-section of the curve is explainable as the combined die-off rate of heat-sensitive and heat-resistant spores.

Figures 4 and 5 present the thermal resistance data from the first and second experiments of the most heat-resistant spore populations encapsulated in plastic. The number of survivors (4 values - duplicate plate count values for each of two rods) are plotted for each exposure interval from 4 hours through 14 hours. Because the limit of reproducible recovery for this procedure is between 10^2 and 10^3 spores per gram, survival points of fewer numbers than these have not been plotted. A separate weighted linear regression was calculated for all the points obtained between 4 and 14 hours in each experiment and they are separately presented in Figures 4 and 5. These regressions explain 86% and 89%, respectively, of the sums of squares of deviations of the first and second experiments. Because of the differences in numbers of surviving spores present after 4 hours exposure in the two experiments, it was not possible to combine the data from the two experiments, as was done with the paper data, and present a single line based on a linear regression of all the data. Though the two lines have different intercepts the slopes of both are comparable. The $D_{125°C}$ values and corresponding 95% confidence intervals for the two experiments are: experiment 1, $D_{125°C}$ = 3.08 hours, 95% confidence interval of 2.55 to 3.61 hours; experiment 2, $D_{125°C}$ = 3.44 hours, 95% confidence interval of 2.98 to 3.89 hours. The 95% confidence interval for the linear regression line are represented as the dashed lines in Figures 4 and 5.

Though it may be a bit premature, at this point, to speculate on the causal mechanism responsible for the unusual shape of the heat inactivation curves for spores in plastic, certain explanations appear plausible

and warrant further investigation. One explanation, though probably the least likely, is that during the polymerization process a certain chemical entity or entities in the casting syrup react with the spores and predispose the majority to heat inactivation, whereas these same entities react differently with a minor portion of the population rendering it heat resistant. A second explanation may relate to the distribution of spores in the plastic during polymerization. Data collected in this laboratory and reported upon previously indicate that an approximate one-log cycle reduction in numbers of spores occurs during the polymerization step. Upon completion of polymerization, no further reduction in numbers of spores occurs in rods stored for several days at room temperature. From this it may be assumed that the active toxic moiety present in plastic is only free to react with the spore when the plastic is in the unpolymerized state. Consequently, it may also be true that the agent responsible for predisposing spores to heat inactivation is active only in the liquid plastic and that the longer the contact time between the spore and this active agent, the less heat resistant the spore becomes. Therefore, spores located in the outer areas of the plastic rod would be more heat resistant than those centrally located because polymerization occurs first at the tube wall-plastic interface and progresses inward requiring approximately 2 hours for completion. Thus spores peripherally located have experienced only a few moments contact time with the active agent, whereas centrally located spores may be in contact with the active agent for as much as 2 hours. A third explanation may be that of the existance of heat resistant mutants present in small numbers in the original population. To achieve a final inoculum of $1x10^{8}$ spores per gram in a polymerized rod requires that a total of approximately $1x10^{11}$ spores be added to each 50

grams of granulated powder that is dried in the oven yielding an initial inoculum of $1x10^9$ spores per gram. During the drying step and the following polymerization step, the number of detectable spores per gram is reduced to approximately $1x10^8$. During the first 4 hours of heating at 125°C this number is further reduced to approximately $1x10^5$ spores per gram. A naturally occurring mutation frequency rate of one mutant in $1x10^4$ to $1x10^6$ cells is not uncommon among bacterial species and such a genetic basis may be responsible for the results observed in plastic. Because only 50 to 70 x 10^6 spores are inoculated onto each paper strip, one would not expect to encounter similar heat resistant mutants on the paper. It is anticipated that as time permits, these hypotheses will be subjected to experimentation in an effort to explain the nature of the thermal inactivation curves obtained in plastic.

Projected Research for Fifth Quarter

Activities during the fifth quarter will be largely devoted to obtaining thermal resistance data on B. globigii spores encapsulated in Lucite and exposed to dry heat temperatures of 115°C and 135°C.

References

1. Koesterer, M. G., NASA Contractor Report CR-191, March 1965.

Table 1

Effect of pre-treatment with acetone on the dry heat inactivation of Bacillus globigii spores at 125°C

Exposure Time in Hours	Acetone Prepared Spores Dried on Paper	Aqueous Prepared Spores Dried on Paper
	No. of Survivors Recovered per Strip	No. of Survivors Recovered per Strip
0	295×10^6	290×10^6
3	4.25×10^6	5.05×10^6
6	750	5.7×10^4

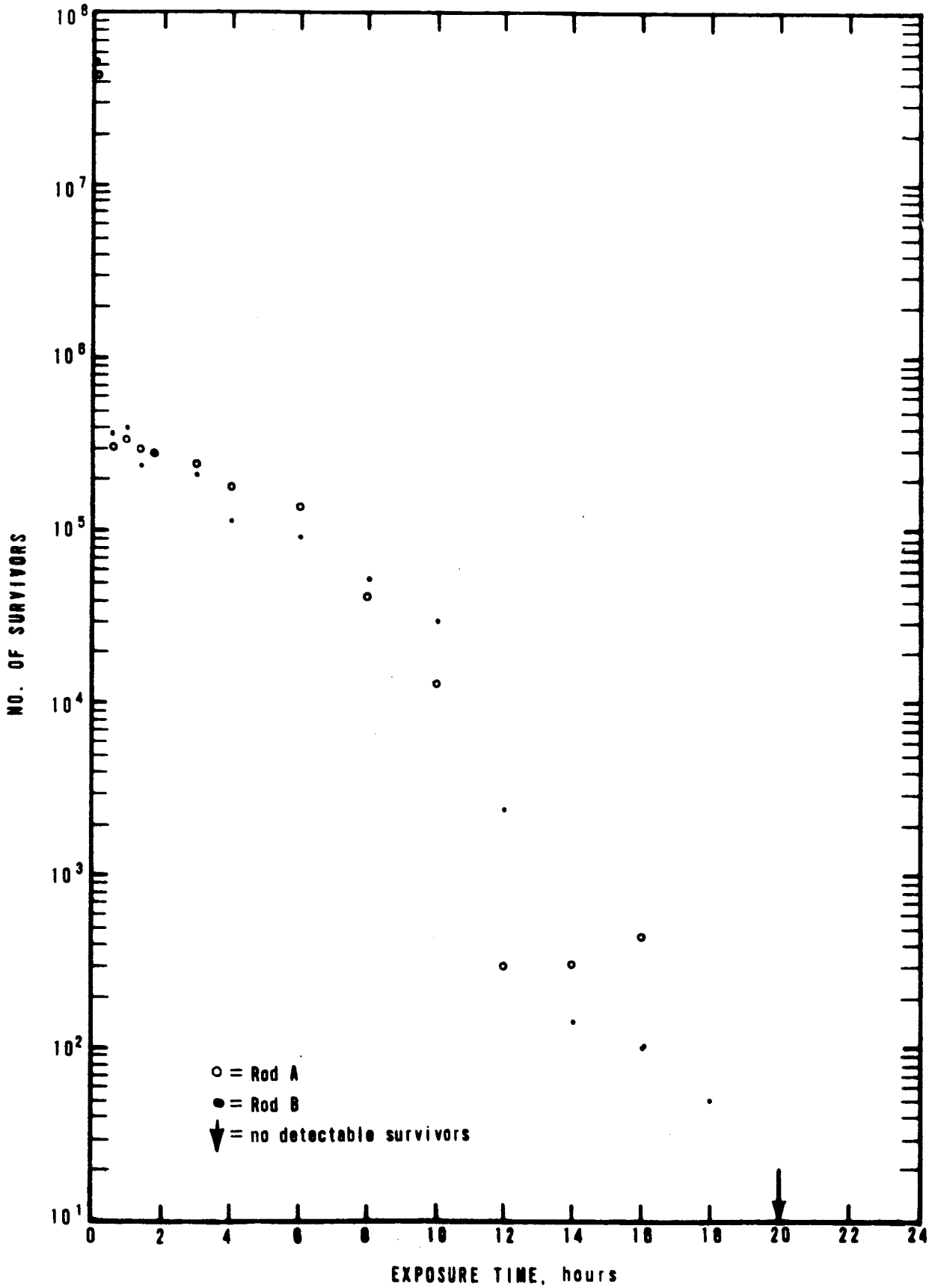
Table 2

Number of B. globigii spores on paper strips surviving one hour exposure to 125°C dry heat in sealed TDT tubes

Spores prepared in water and dried on paper*		Spores prepared in acetone and dried on paper*	
Blended and diluted in acetone	Blended and diluted in water	Blended and diluted in acetone	Blended and diluted in water
13.5×10^7	17×10^7	8×10^7	9×10^7

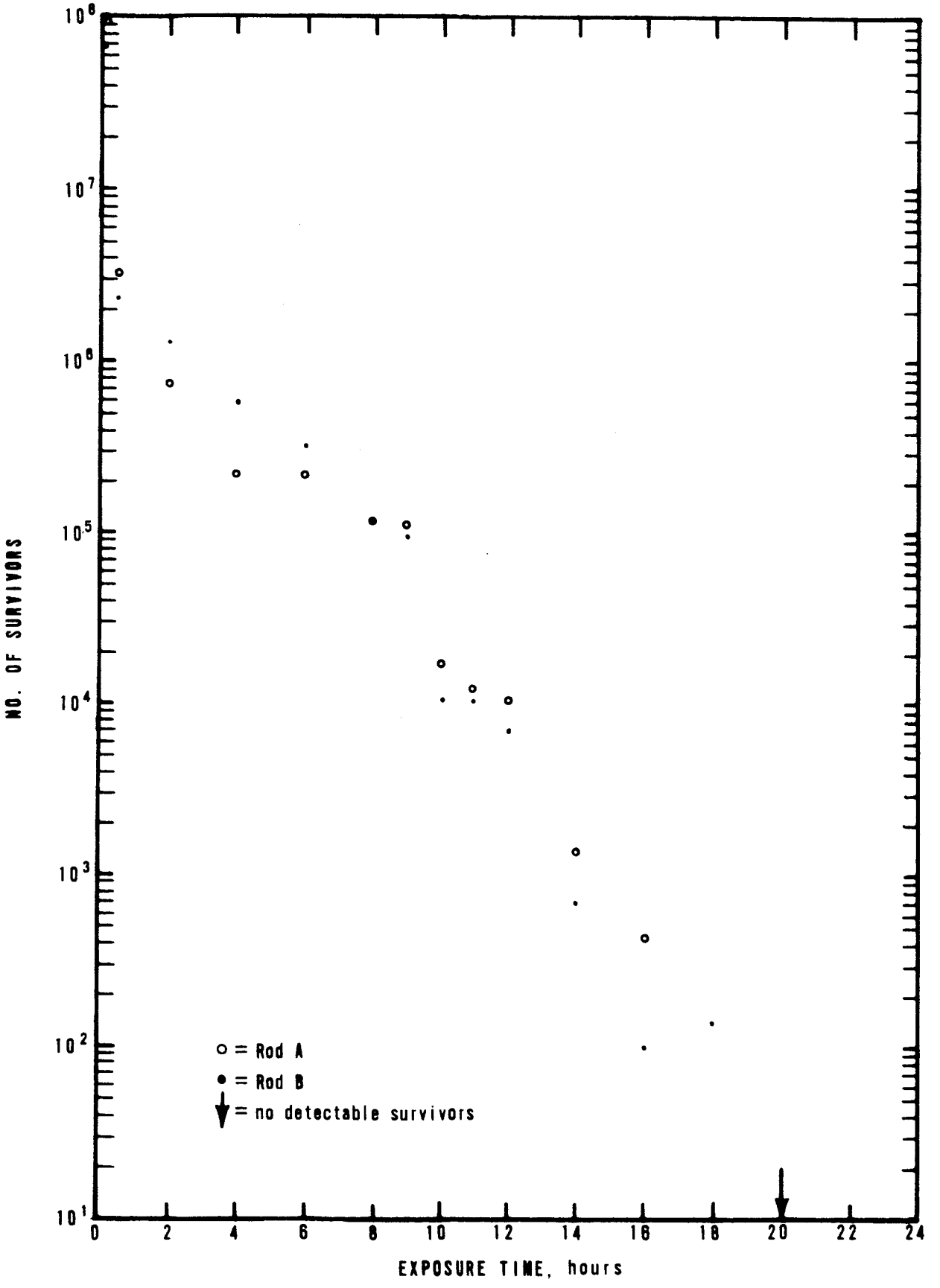
*Number of spores per strip of paper:
paper inoculated from water suspension = 20×10^7 per strip
paper inoculated from acetone suspension = 15×10^7 per strip

Fig. 1



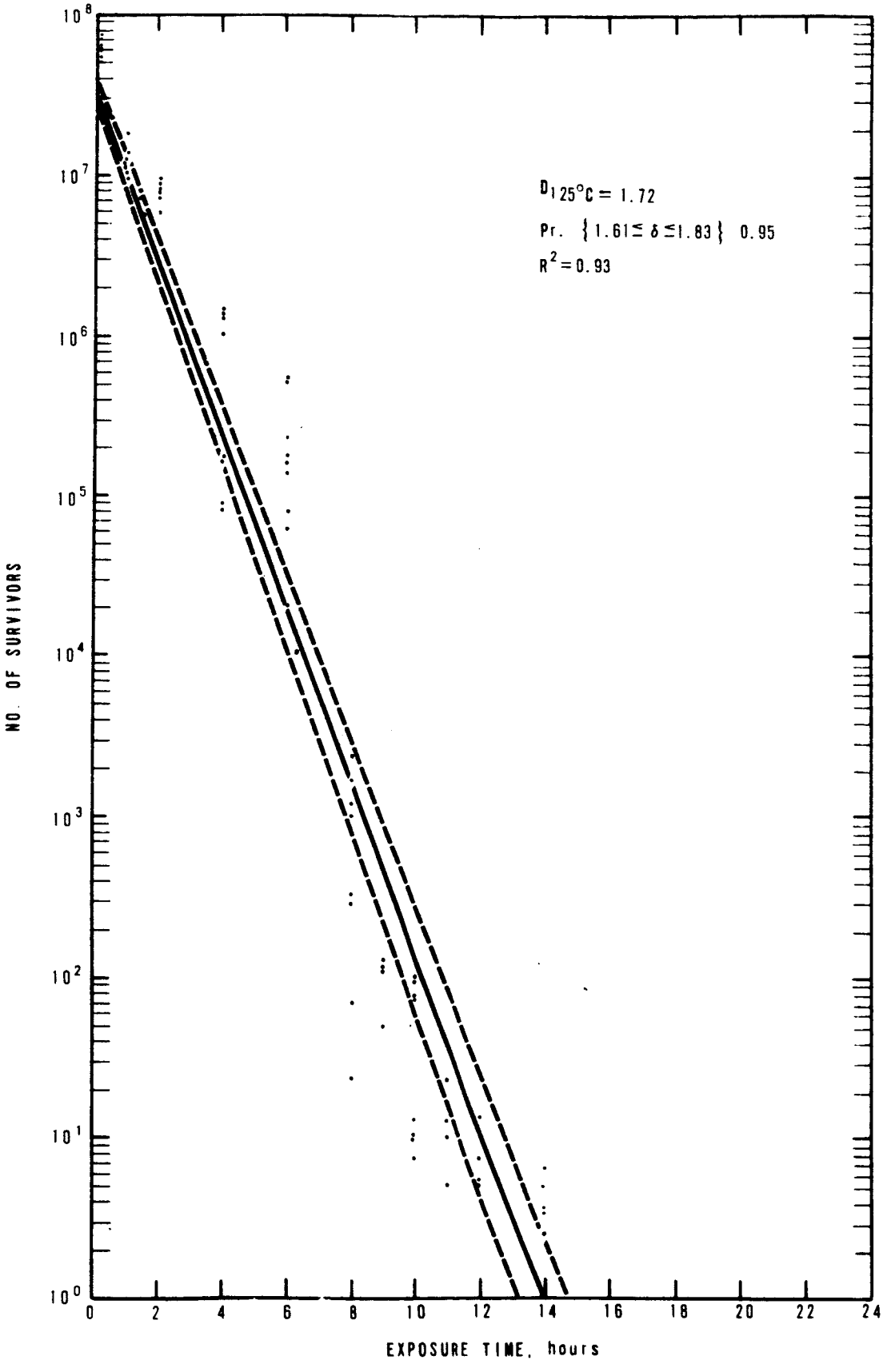
Dry Heat Inactivation at 125°C of Bacillus globigii Spores Encapsulated in Lucite Rods
(Experiment I)

Fig. 2



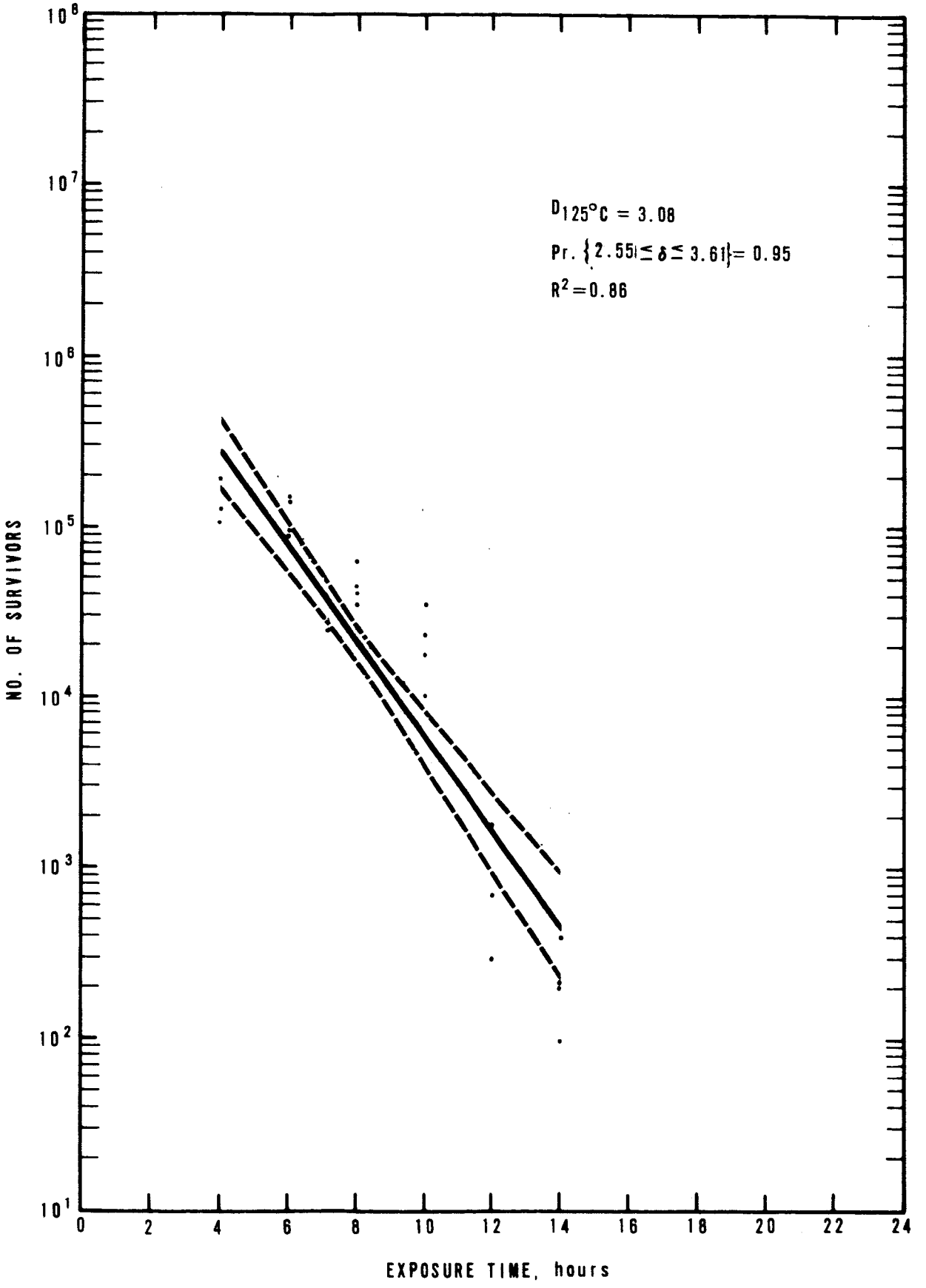
Dry Heat Inactivation at 125°C of Bacillus globigii Spores Encapsulated in Lucite Rods
(Experiment II)

Fig. 3



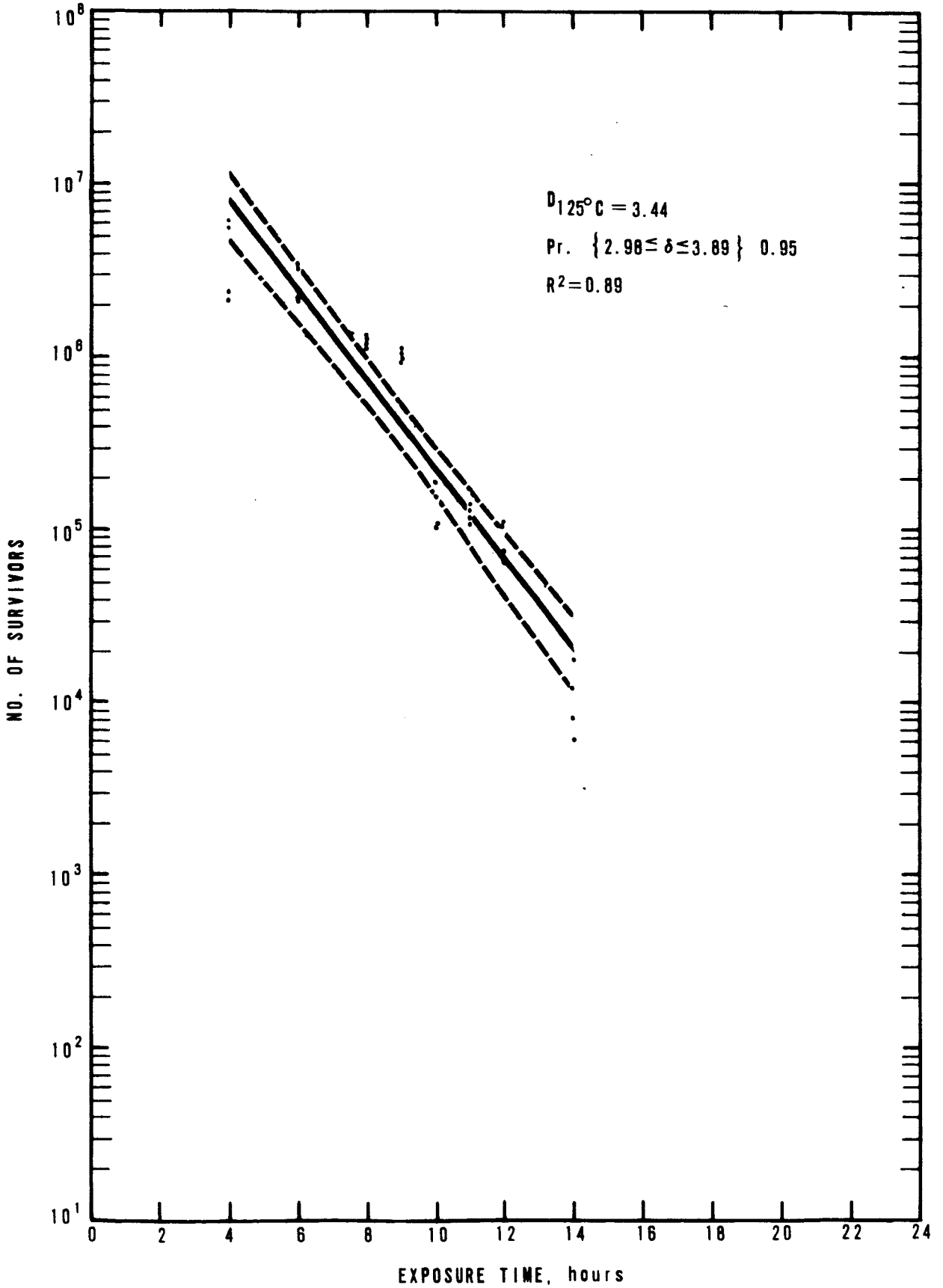
Dry Heat Inactivation at 125°C of Bacillus globigii Spores Dried on Paper Strips

Fig. 4



Dry Heat Inactivation at 125°C of the Most Resistant Population of Bacillus globigii Spores Encapsulated in Lucite (Experiment I)

Fig. 5



Dry Heat Inactivation at 125°C of the Most Resistant Population of Bacillus globigii Spores Encapsulated in Lucite (Experiment II)