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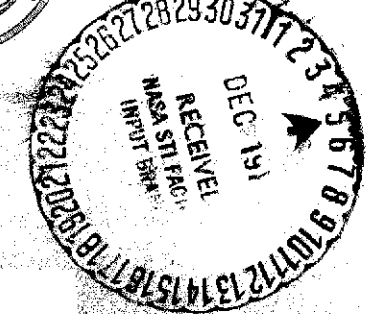

SUMMARY OF PROGRESS IN THE PROJECT ENVIRONMENTAL MICROBIOLOGY AS RELATED TO PLANETARY QUARANTINE

December 1, 1972 through May 31, 1973

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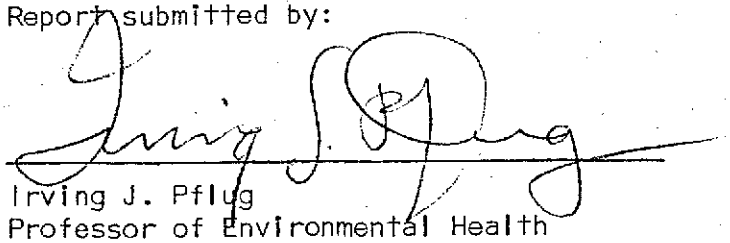

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SUMMARY

During the period December 1, 1972 through May 31, 1973, the period covered by the current report, the Environmental Sterilization Laboratory, Space Science Center, University of Minnesota has continued experimental work related to the dry heat resistance of microorganisms and to the combination of heat and radiation.

The work on the effect of combined heat and radiation on microbial destruction is covered by an abstract of the recent effort and also the conclusions of these experiments.

During this reporting period we have also evaluated the dry heat resistance of selected bacterial spore crops other than Bacillus subtilis var. niger. The results of these studies indicate that the different strains of Bacillus stearothermophilus may demonstrate marked differences in dry heat resistance. It was also found in these studies that conditioning and heating the spores in a very dry atmosphere significantly increased the dry heat destruction rate in some spores over the values observed in clean room tests. For the more resistant strains, differences between the dry box experiments and the clean room experiments were small.

A study has been initated to determine the effect of storage time, suspending medium, storage temperature and spore crop cleaning procedures on the dry-heat survival characteristics of Bacillus subtilis var. niger over a long period of time.

Some preliminary work dealing with the dry heat resistance of natural microflora on soil particles is included in this report. These studies have dealt with the effects of dry heat at 125 deg. C on viability of microorganisms associated with soil particles.

I. EFFECT OF COMBINED HEAT AND RADIATION ON MICROBIAL DESTRUCTION

Donald A. Fisher and Irving J. Pflug

This study on the effect of combined heat and radiation on microbial destruction was initiated in June 1971 and is now being terminated. The environmental system developed in our laboratory for use in the dry heat thermoradiation study has been described previously (see Progress Reports #7 and #8). In Progress Report #9 we described work on the effect of combined wet heat and radiation.

We have also attempted to determine the mechanistic basis of the synergism which results upon addition of these two seemingly independent lethal agents.

The objectives of this project were: (1) to investigate the synergistic effect which results when bacterial spores are subjected to simultaneous heat and gamma irradiation, thereby enabling us to specify thermoradiation cycles, and (2) to derive a clearer understanding of the underlying mechanism which leads to non-viability of bacterial spores.

The conclusions of these experiments are:

1. Dry-heat thermoradiation experiments show a synergistic effect, the degree of which depends on radiation intensity, temperature, and relative humidity.
2. A physical and mathematical model has been derived which, first of all, predicts a synergistic effect, and secondly, displays a priori many of the salient features experimentally observed. This model is useful for the execution of further thermoradiation experiments and relates to future testing for synergistic effects between other combinations of physical stresses.

2. DRY HEAT RESISTANCE STUDIES OF SELECTED BACTERIAL SPORE CROPS

O. R. Ruschmeyer, G. Smith, I. J. Pflug, R. Gove, and Y. Heisserer

INTRODUCTION

Spacecraft sterilization programs have been concerned primarily with the spore forming bacteria as indicators for determining the efficacy of sterilization procedures. Among the most heat resistant micro-organisms known are the aerobic spore formers of the genus Bacillus. Of particular interest to the NASA program is the response of various spore forming bacteria to dry heat sterilization processes and the thermal resistance patterns of their spores.

Variations in the heat resistance of spore forming strains cultured or stored in different laboratories may be a problem when selecting certain spores as biological indicators for monitoring sterilization cycles. This is true not only for spacecraft sterilization programs, but also in applications for hospital sterilization procedures and for quality control operations in the food industry as well.

The work reported here has been concerned with the dry heat resistance and response characteristics of selected strains of aerobic spore formers obtained from various sources. Survival patterns of the spore crops were determined at 110° and 125°C. The influence of conditioning and heating at two humidity levels and the effect on dry heat destruction rates of the spores was also investigated.

OBJECTIVES

The objectives of this investigation were as follows: (1) To obtain survivor curve data and calculated D-values to indicate the dry heat destruction rates for special spore crops from selected strains of thermophilic and mesophilic Bacillus cultures. (2) To determine the influence of conditioning and heating at low humidity (less than 1.0 per cent R.H.)

on the thermal resistance of the selected spore crops as contrasted with treatment at 50 per cent R.H.

MATERIALS AND METHODS

Spore Strains

Spore crops from four different strains of bacteria were investigated in an attempt to obtain additional information on dry heat destruction rates of aerobic spore formers. Three of the organisms studied were selected strains of Bacillus stearothermophilus, a thermophilic, aerobic, spore former. The fourth organism was a special strain of Bacillus subtilis isolated in food industry studies. A description of these spore crops, together with the assigned spore codes and the source of the isolates, is provided in Table 2.1.

Culture Methods and Spore Harvest

Isolates of each organism investigated were cultured in our laboratories for spore production. Bacillus stearothermophilus strains were grown on a sporulation medium of nutrient agar supplemented with $MnSO_4$ (5 mg/liter) and incubated at 55°C. Sporulation media for Bacillus subtilis consisted of nutrient agar supplemented with $MnSO_4$ (1 mg/liter). This organism was incubated at 32°C.

Spore crops were harvested from the culture media with distilled water washing and centrifugation. Additional washing with distilled water and centrifugation was used to clean the spores of vegetative cell debris. Following this treatment, spores were resuspended in deionized, double-distilled water. These suspensions were stored at 4°C until tested.

Heat Treatment Procedures

Dry heat destruction rates of the spores were determined with the Planchet-Boat-Hot Plate Method described in detail previously (see University of Minnesota, School of Public Health, NASA Report #3). For experiments reported here, two 1/2" x 1/2" sterile, stainless steel planchets were placed in each boat and three boats were treated for each of six heating time intervals. All preparatory work was done in our laminar

Table 2.1

Description and Identification of
Spore Crops Tested

Spore Identification	Description
ECFF	Bacillus subtilis strain. Schmidt, Continental Can Company, Chicago #5230. Cultured in laboratory, spore crop harvested and stored in deionized, distilled water at 4°C in refrigerator.
PBBF	Bacillus stearothermophilus. ATCC strain #7953. Cultured in laboratory, spore crop harvested and stored in deionized, distilled water at 4°C until tested.
QHAF	Bacillus stearothermophilus. Original source was a sterility test spore strip from a hospital. Cultured in laboratory, spores harvested and stored in deionized, distilled water at 4°C until tested.
PCFF	Bacillus stearothermophilus. Strain supplied by Dr. C. F. Schmidt of Continental Can Company. Cultured in laboratory, spores harvested and stored in deionized, distilled water at 4°C until tested.

downflow clean room facility.

Aliquots of the stored spore suspensions were diluted with distilled water so that the final test suspension spore concentration was approximately 10^6 spores per 0.02 ml volume. The diluted samples were mixed in a Vortex unit for about five to ten seconds and then 0.02 ml quantities of the test spore suspension were deposited on each planchet with an Eppendorf pipette. This procedure provided an initial test concentration of approximately 10^6 spores per planchet. Spore deposits were left to dry and then equilibrated overnight in the test environment before being subjected to heat treatment.

Hot plate temperatures used in these studies were 110°C and 125°C. Boat positions on the hot plates were randomly selected. Heating time intervals were chosen arbitrarily because no information on the response of these particular spore crops to dry heat treatment was available. Hot plate and boat temperatures were monitored during each experiment with thermocouple probes and a Hewlett-Packard multi-channel data acquisition system.

Plate Counts and Data Analysis

Following the heat treatment, planchets from each boat were dropped into previously numbered, sterile flasks. Suitable amounts of sterile, buffered dilution water were added and each flask was placed in a sonicator (25 Khz/sec.) for two minutes to elute spores from the planchets. If required, appropriate dilutions of the sonicated spore suspensions were made and then 10 ml, 1.0 ml, and 0.1 ml aliquots of each dilution were plated in duplicate using Trypticase Soy agar media. After 48 hours incubation at 55°C for B. stearothermophilus or 32°C for B. subtilis, colonies on the plates were enumerated with a Bactronic colony counter. Unheated spore deposits on the planchets of three boats served as zero time controls. These unheated planchets were processed and plated in the same manner as the heated spore series.

Plate counts provided information on the number of surviving spores at each heating time. For each spore test series data were obtained from three boats processed for each of six heating times plus the values from the zero time controls. Plate count data were fed into the university

computer for analysis. From the computer output, points representing the mean number of survivors for each heating time were plotted on semi-logarithmic paper to reflect the trend of the survivor curve. The computer program also provided a calculation of a least squares regression analysis (omitting N_o) to estimate the D-value and y-intercept for each spore crop test series.

Clean Room and Dry Box Environments

During the first series of experiments, all of the dry heat resistance determinations were done on hot plates in our laminar downflow clean room. The clean room air system was controlled at a temperature of 22.2°C with a relative humidity of 50 per cent. All spore suspensions tested on clean room hot plates were deposited on the planchets and allowed to dry. The spore deposits were then allowed to equilibrate in the clean room for 18 hours or more before heat treatment. During the conditioning period, the spore deposits were protected from direct air flow with a stainless steel tray elevated on supports. After the period of conditioning, spore deposits were heated on the 110°C or 125°C hot plates and the planchets were processed for plating.

Following the initial series of thermal resistance tests in the clean room atmosphere, a second series of experiments was carried out to test the response of the spore crops to heating in dry atmospheres as compared to the relatively more moist atmosphere of the clean room. The dry atmosphere tests were run in a converted glove box and antechamber which were modified to provide a dry gas environment. A constant, controlled flow of dry nitrogen gas was passed through the unit and a humidity control system was included in the gas train. The glove box humidity was recorded continuously by a moisture monitor. Hot plates in the glove box were operated at 110°C and 125°C with temperature monitor systems similar to those used in the clean room.

The dry glove box atmosphere was controlled at approximately 27°C with a 0.28% R.H. Additional data concerning the calculated humidities for the spores in the conditioning and heating atmospheres are shown in Table 2.2. Further details of the dry atmosphere system have been de-

Table 2.2

Relative Humidity in Conditioning Atmospheres and in Atmospheres Surrounding Spores Treated on Hot Plates

Conditioning Atmospheres

	Dry Box	Clean Room
Temp.	27°C	22.2°C
R.H. (%)	0.28	50
Moisture (PPM)	100	13,000

Calculated Relative Humidities of Atmospheres Surrounding Spores on Hot Plates

Temp.	Dry Box Hot Plate R. H.	Clean Room Hot Plate R. H.
110°C	0.007%	1.0%
125°C	0.004%	0.6%

scribed in a previous report (See University of Minnesota, School of Public Health, NASA Report #9, Appendix A).

With the use of the clean room and dry box systems, it was possible to subject the spore crops to conditioning and heating in both a dry atmosphere and in the relatively higher moisture conditions of the clean room. For comparative studies in the dry box and the clean room, spore suspensions were first deposited on two series of planchets in the clean room area and allowed to evaporate to dryness for a period of two hours. When the spore deposits were dry, those samples to be run in the dry atmosphere were transported to the glove box antechamber, held there for 30 minutes, and then transferred to the main chamber of the dry box. These samples were conditioned and equilibrated in the dry box overnight at 27°C and 0.28% R.H. for a period of 17-18 hours. The spores were then heated on the dry box hot plate at the test temperature.

A second set of equal numbers of boats and planchets were conditioned overnight (17-18 hours) in the clean room at 22°C and 50 per cent R.H. These spores were heated the next day on the clean room hot plate at the test temperature. Thus, we were able to prepare two sets of the same spore deposits that were conditioned and heated nearly simultaneously, but at two different moisture levels.

RESULTS AND DISCUSSION

Data from the tests of dry heat destruction rates of spores heated in the clean room series indicated that there were marked differences in the resistance of these spore crops to thermal treatment. This effect was readily noticeable when comparisons were made of D-values obtained for the three strains of Bacillus stearothermophilus spores studied. The dry heat D-values for these clean room experiments are listed in Table 2.3.

Among the thermophilic strains tested, the QHAF spore crop demonstrated the greatest resistance to dry heat destruction. For most of the test series carried out at 125°C, D-values for the QHAF strain spores were approximately four to five times greater than the corresponding

Table 2.3

Summary of Dry Heat D-values Obtained in Clean Room Hot Plate Studies with Selected *Bacillus sp* Spore Crops

Bacterial Species and Strain Spore Code	D_{110} (min.)	D_{125} (min.)
B. stearothermophilus		
QHAF	69.8	6.3
	63.0	13.0
	118.0	13.5
	--	12.7
	($\bar{x}$ = 83.3)	($\bar{x}$ = 11.4)
PCFF	27.6	3.4
	28.4	3.2
	37.6	2.2
	--	2.5
	($\bar{x}$ = 31.2)	($\bar{x}$ = 2.8)
PBBF	--	2.3
B. subtilis		
ECFF	--	13.6
	--	12.2
		($\bar{x}$ = 12.9)

D-values obtained for the other two B. stearothermophilus spore crops (PCFF and PBBF). The average D_{125} value for the QHAF spore crop was 11.4 minutes as compared to a D_{125} value for 2.3 minutes for PBBF spores and an average value of 2.8 minutes for spore crop PCFF.

Two clean room dry heat survivor experiments were also completed at 125°C with B. subtilis (ECFF) spores. The average D_{125} value for this spore crop was 12.9 minutes (see Table 2.3). It is of interest to note that the spore crop of this mesophilic species was approximately as resistant to dry heat destruction at 125°C as was that of the thermophile, B. stearothermophilus (QHAF). Furthermore, the B. subtilis spore crop was also much more resistant to heat effects at 125°C than the PCFF and PBBF strains of B. stearothermophilus spores.

At 110°C only the QHAF and PCFF spore strains were investigated. The data in Table 2.3 indicate that the QHAF spore crop had an average D_{110} of 83.3 minutes as compared to a value of 31.2 minutes for PCFF spores. On the basis of these D-value data, it is clear that the QHAF spore strain was also considerably more resistant to heat destruction at 110°C than was the PCFF spore crop.

It is noteworthy that the dry heat survival studies completed to date suggest that there are definite strain differences in the three spore crops of Bacillus stearothermophilus cultures we have studied. Despite the fact that all three cultures were grown on the same media, that spores were harvested by the same procedures, and that storage of spores was under identical conditions, differences in thermal resistance to dry heat were readily detected. Differences in the genetic composition of these spores is one factor that could account for these observations. It is important to recognize that these differences in strain resistance to dry heat sterilization occur and this phenomenon must be taken into account when selecting bacterial species for use as indicators in sterilization processes.

Survivor curves plotted from the data obtained in the initial clean room hot plate studies with spore crops PCFF and QHAF are reproduced in Figure 2.1 through 2.10. The points on the graphs represent the mean values of the results obtained from duplicate plate counts for each of

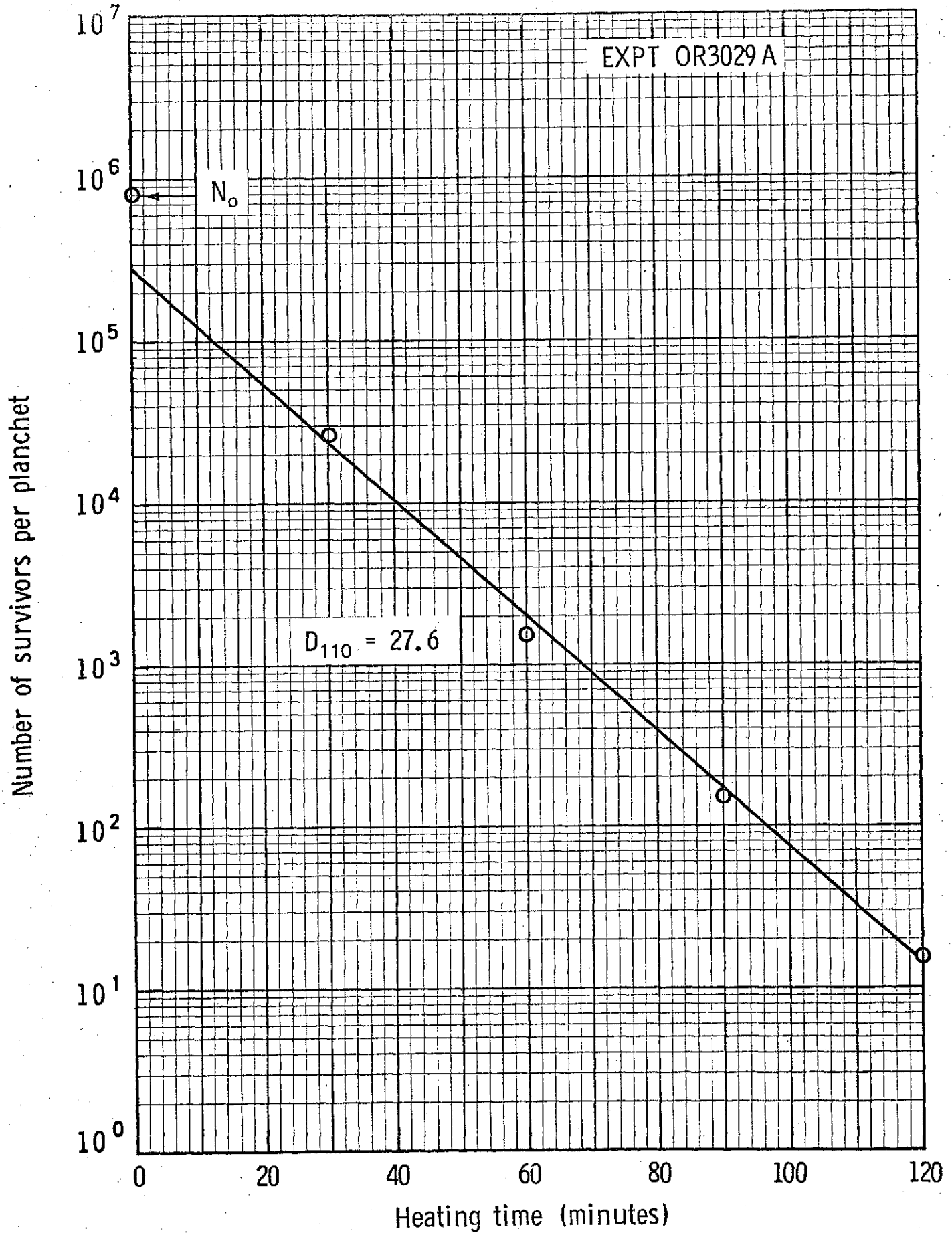

Fig. 2-1 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 110°C. (Clean Room Hotplate).

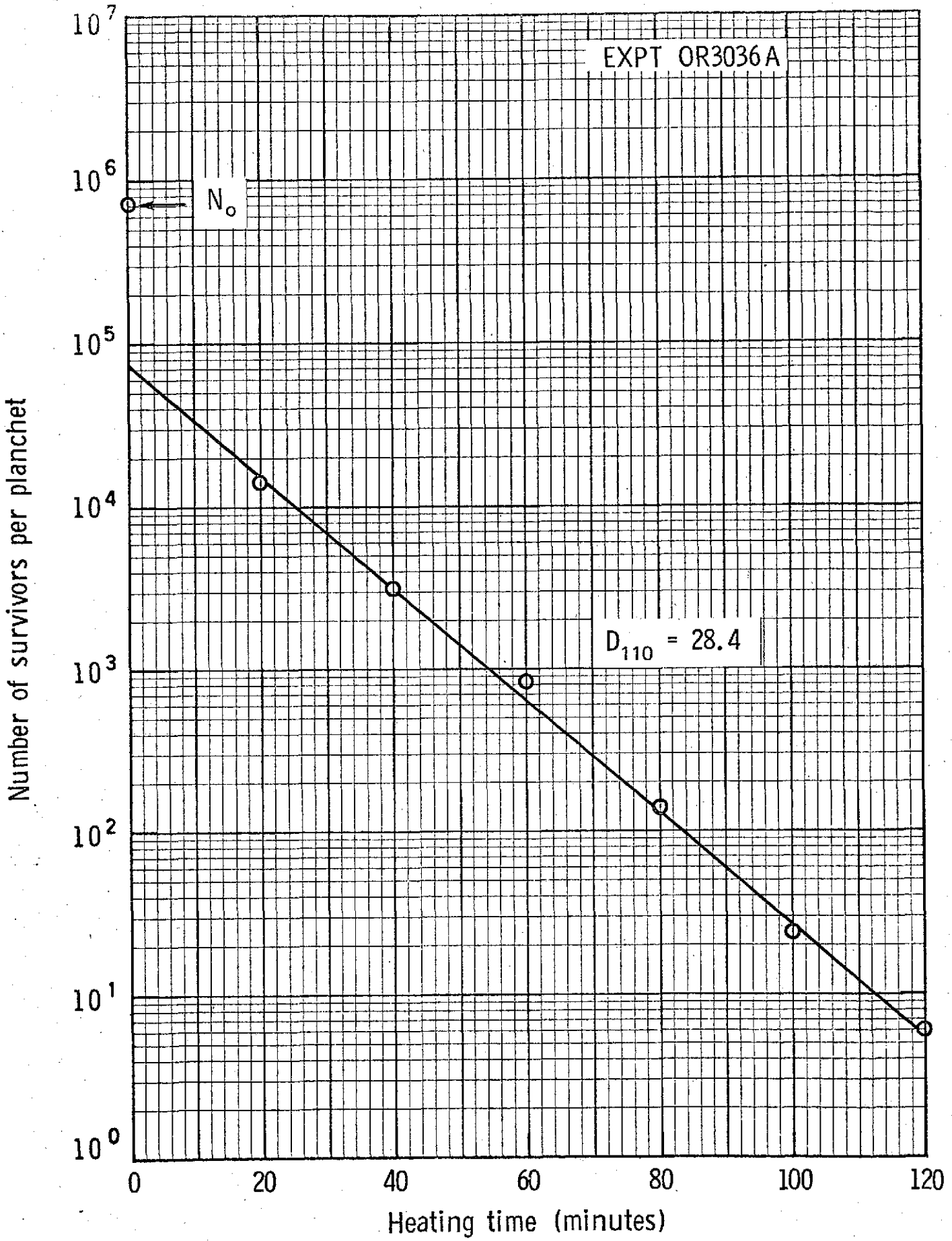

Fig. 2-2 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 110°C. (Clean Room Hotplate).

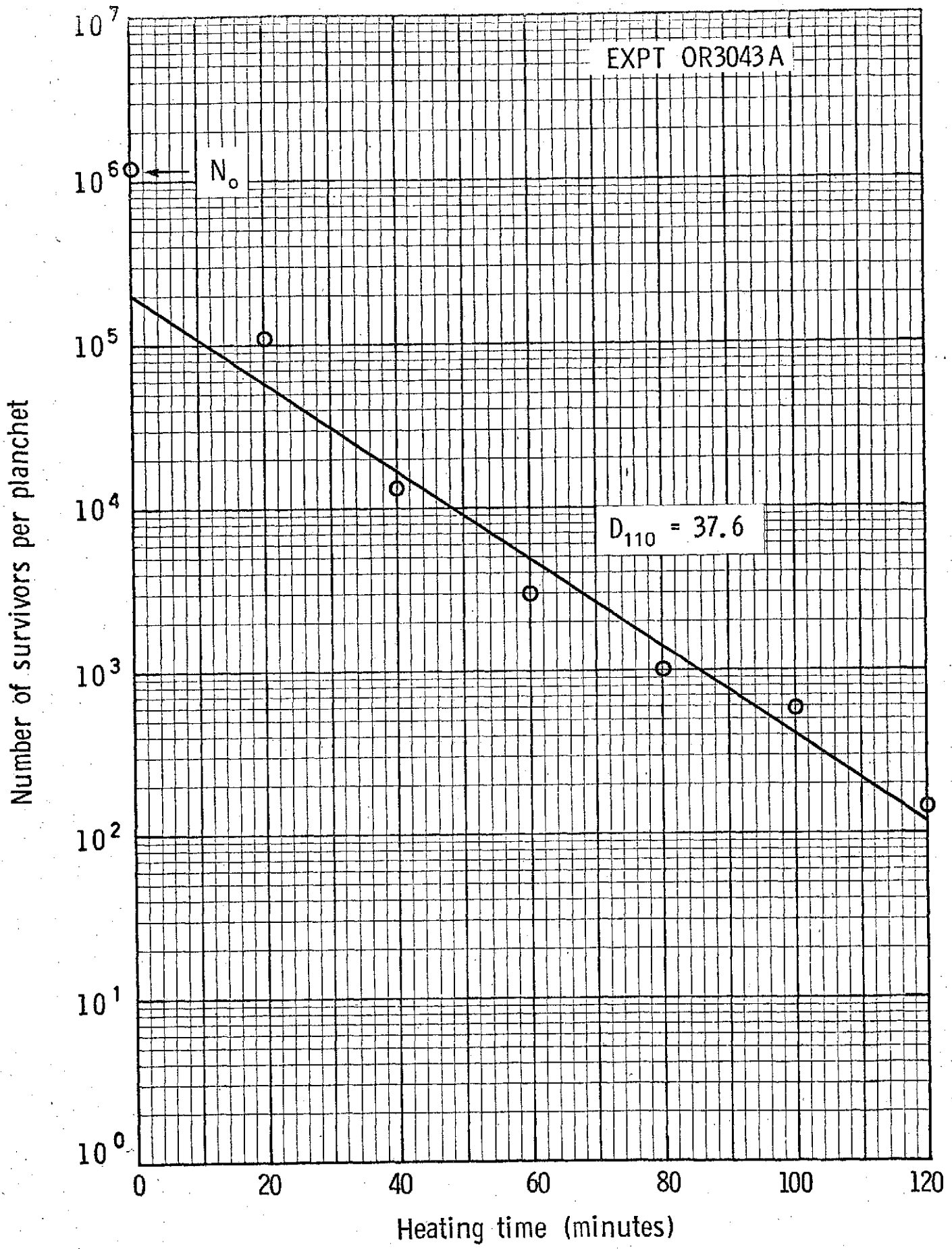

Fig. 2-3 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 110°C. (Clean Room Hotplate).

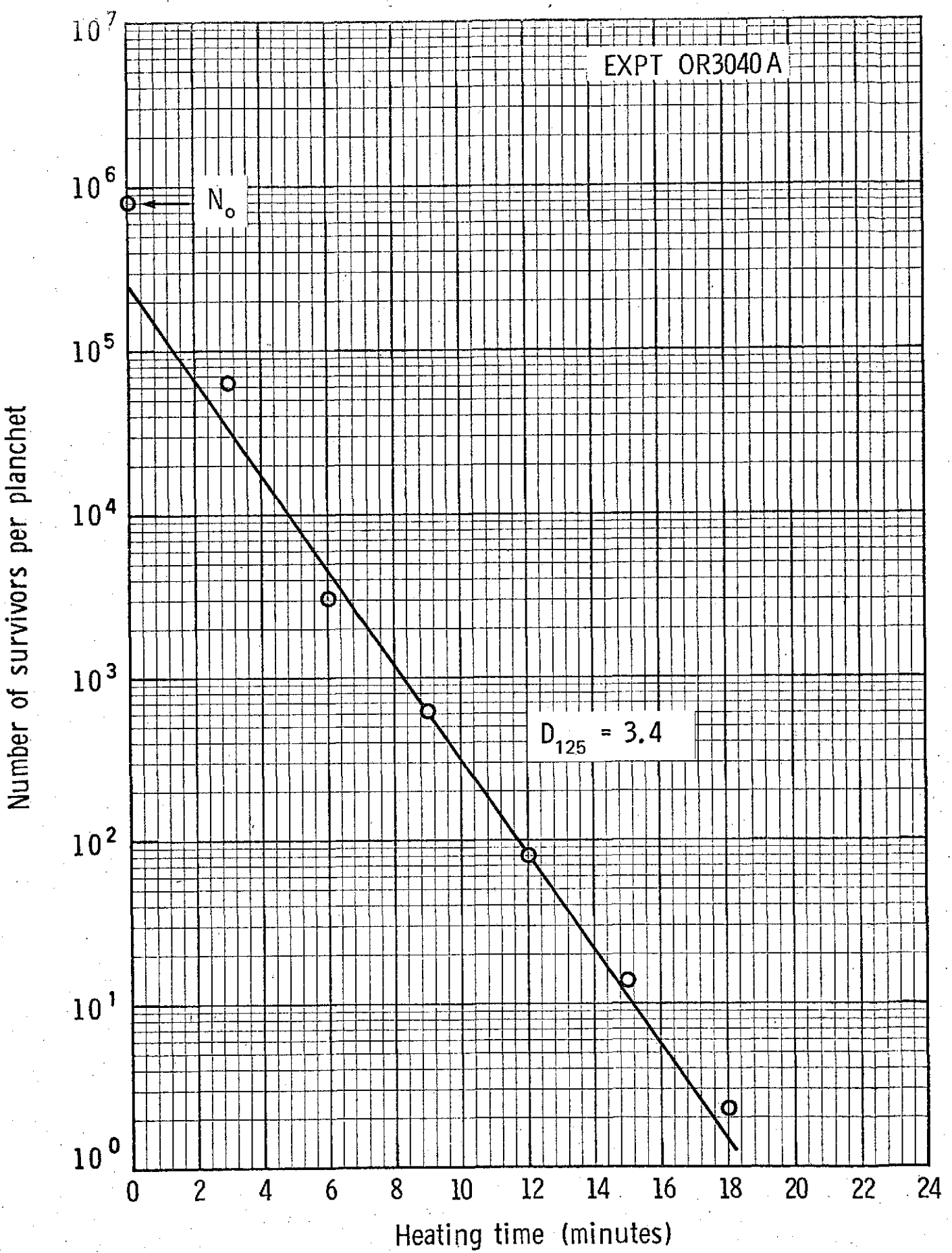

Fig. 2-4 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 125°C. (Clean Room Hotplate).

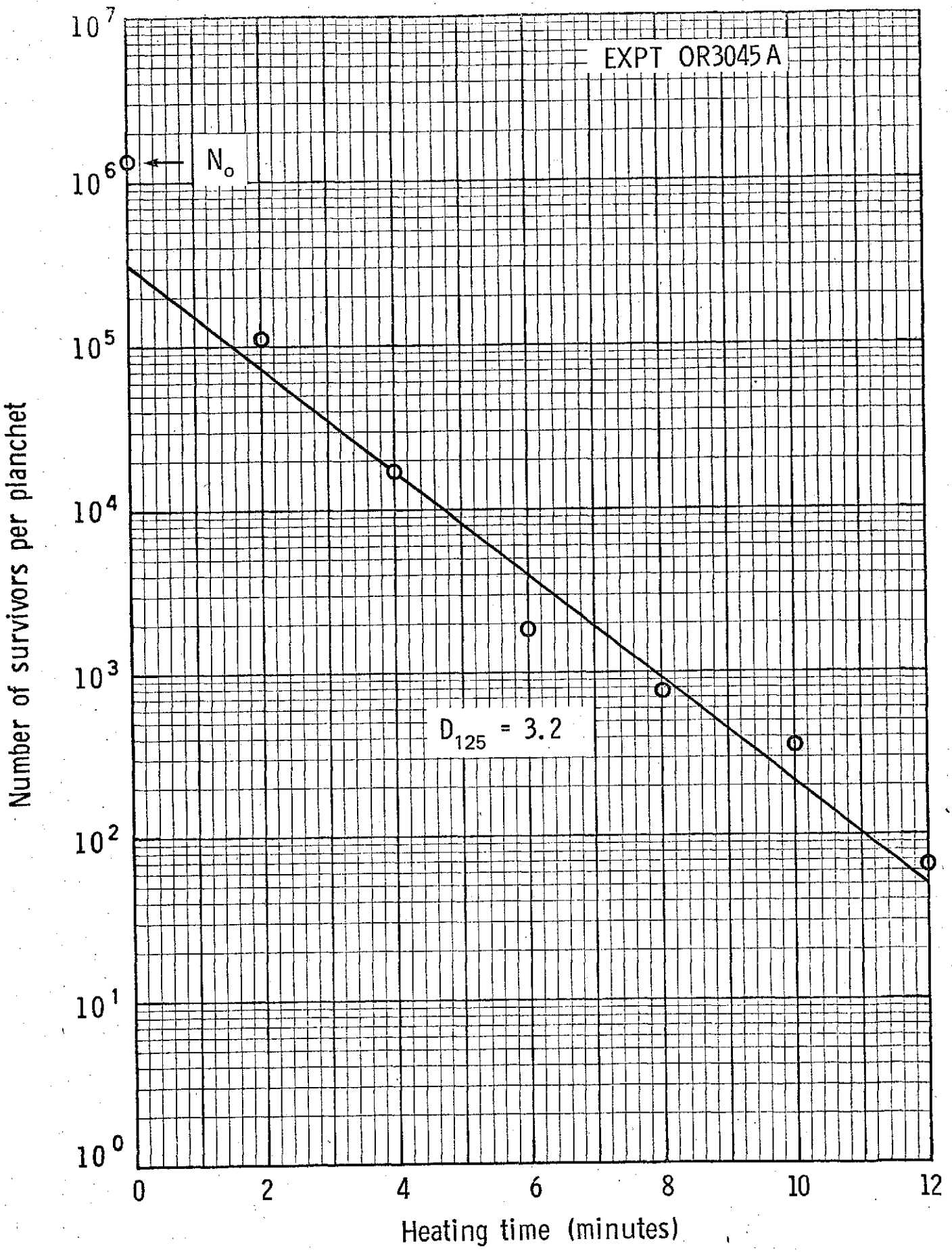

Fig. 2-5 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 125°C. (Clean Room Hotplate).

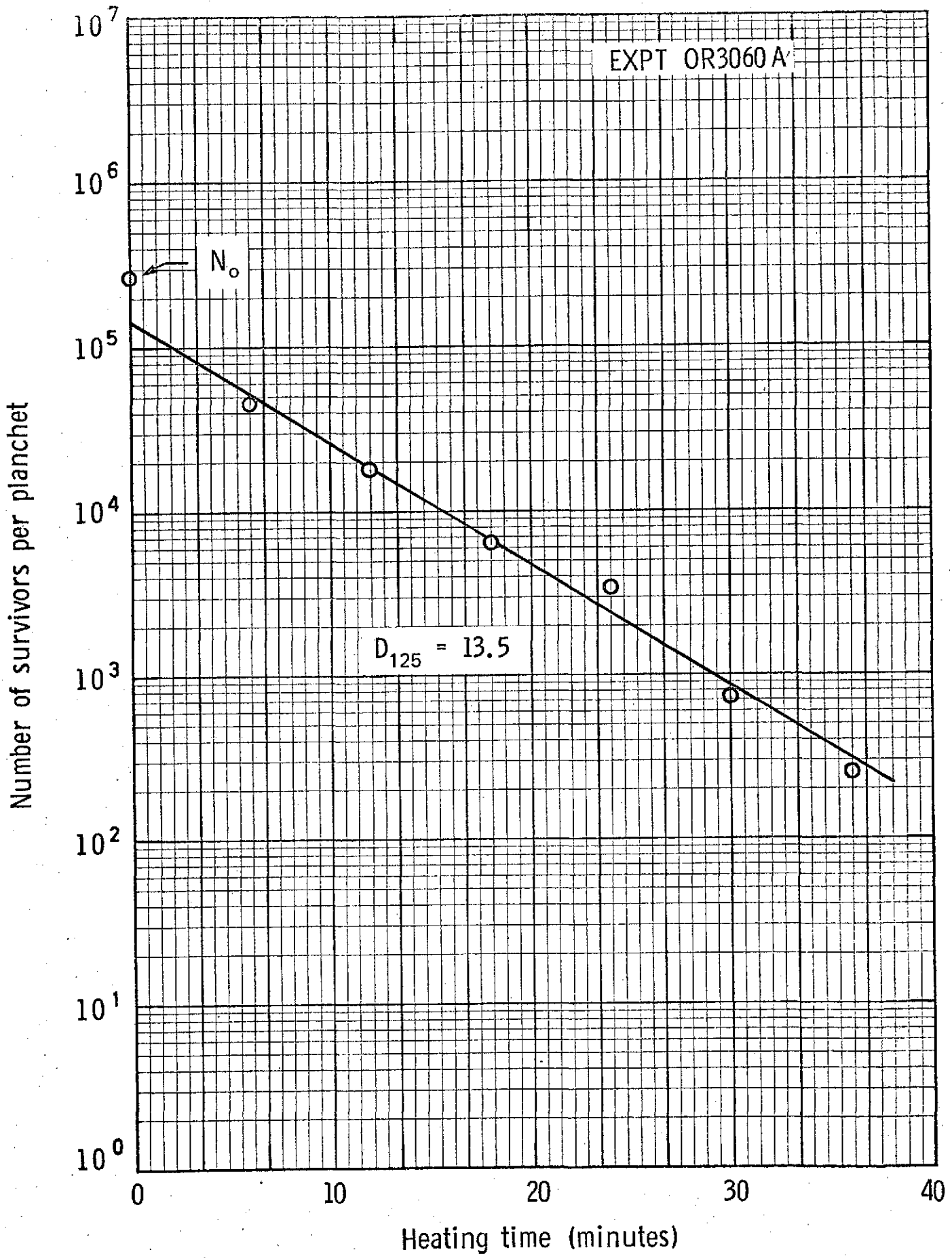

Fig. 2-6 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 125°C. (Clean Room Hotplate).

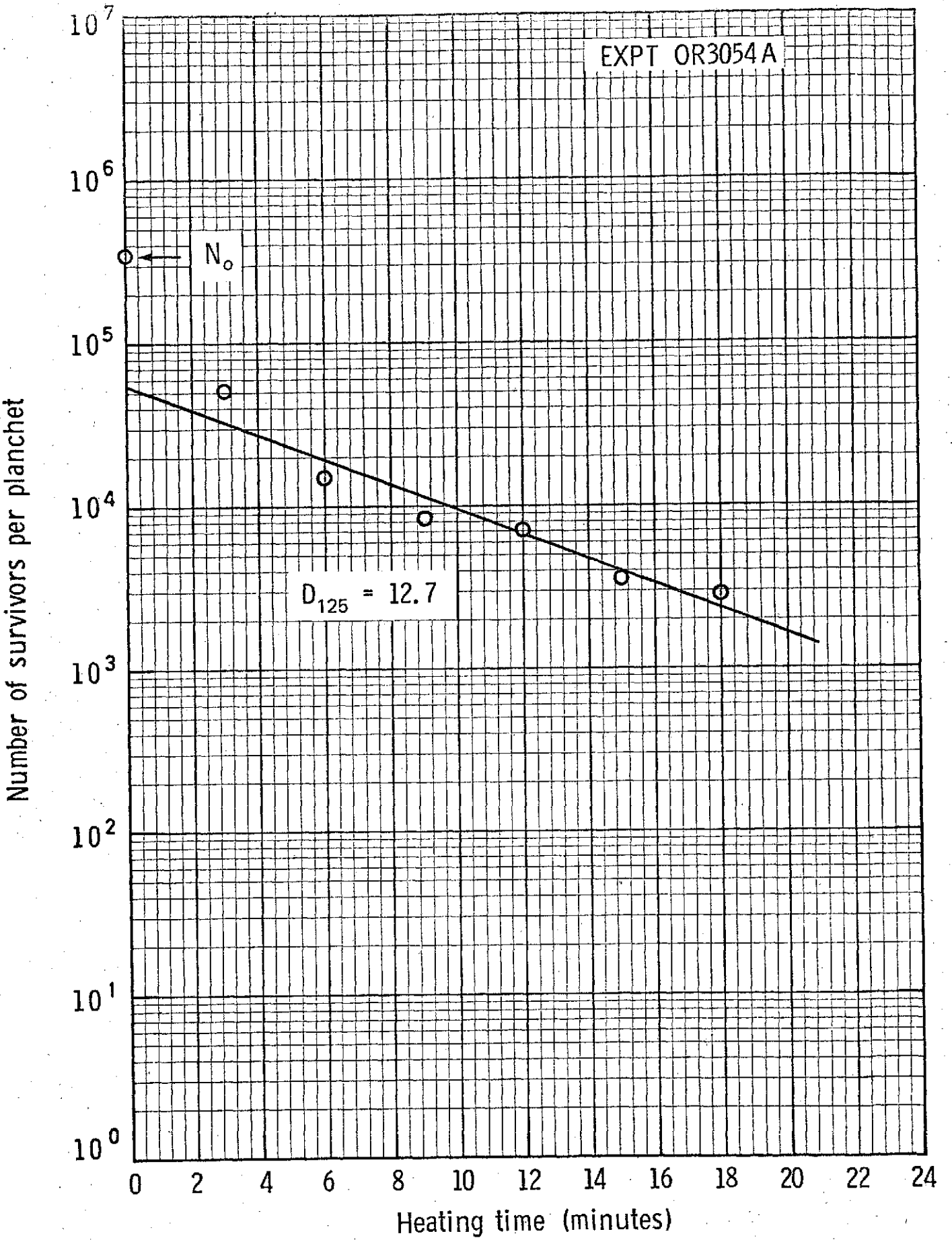

Fig. 2-7 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 125°C. (Clean Room Hotplate).

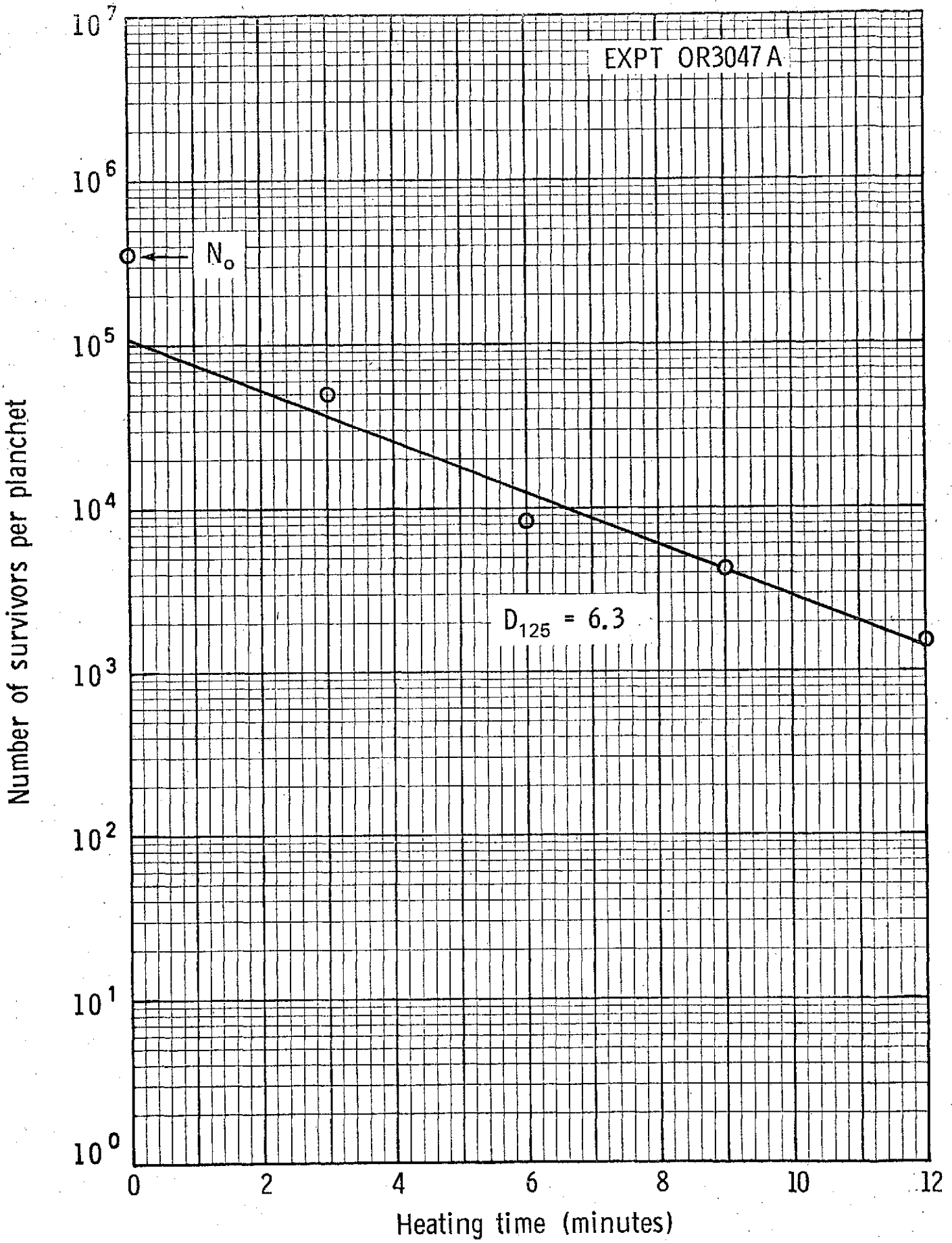

Fig. 2-8 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 125°C. (Clean Room Hotplate).

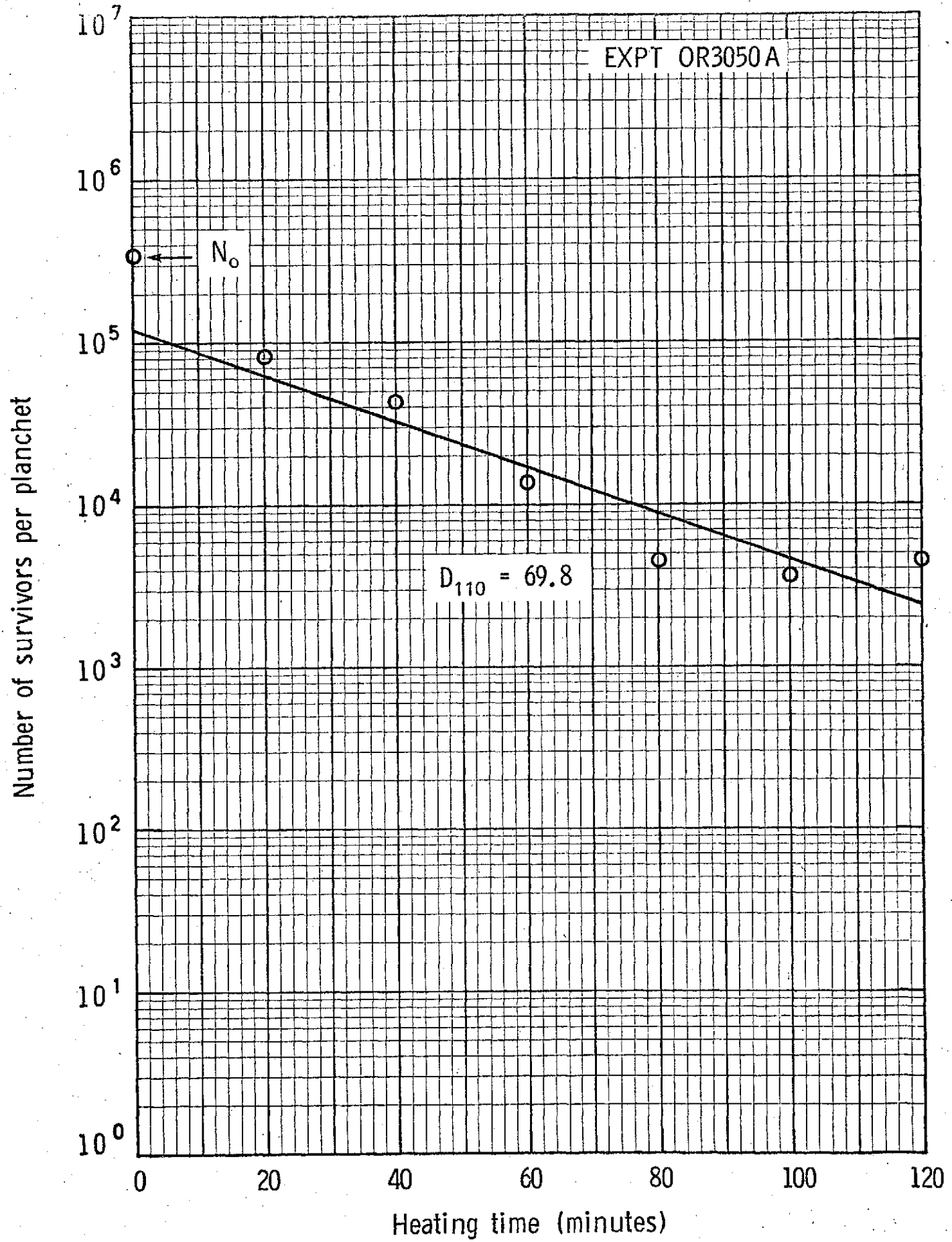

Fig. 2-9 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 110°C. (Clean Room Hotplate).

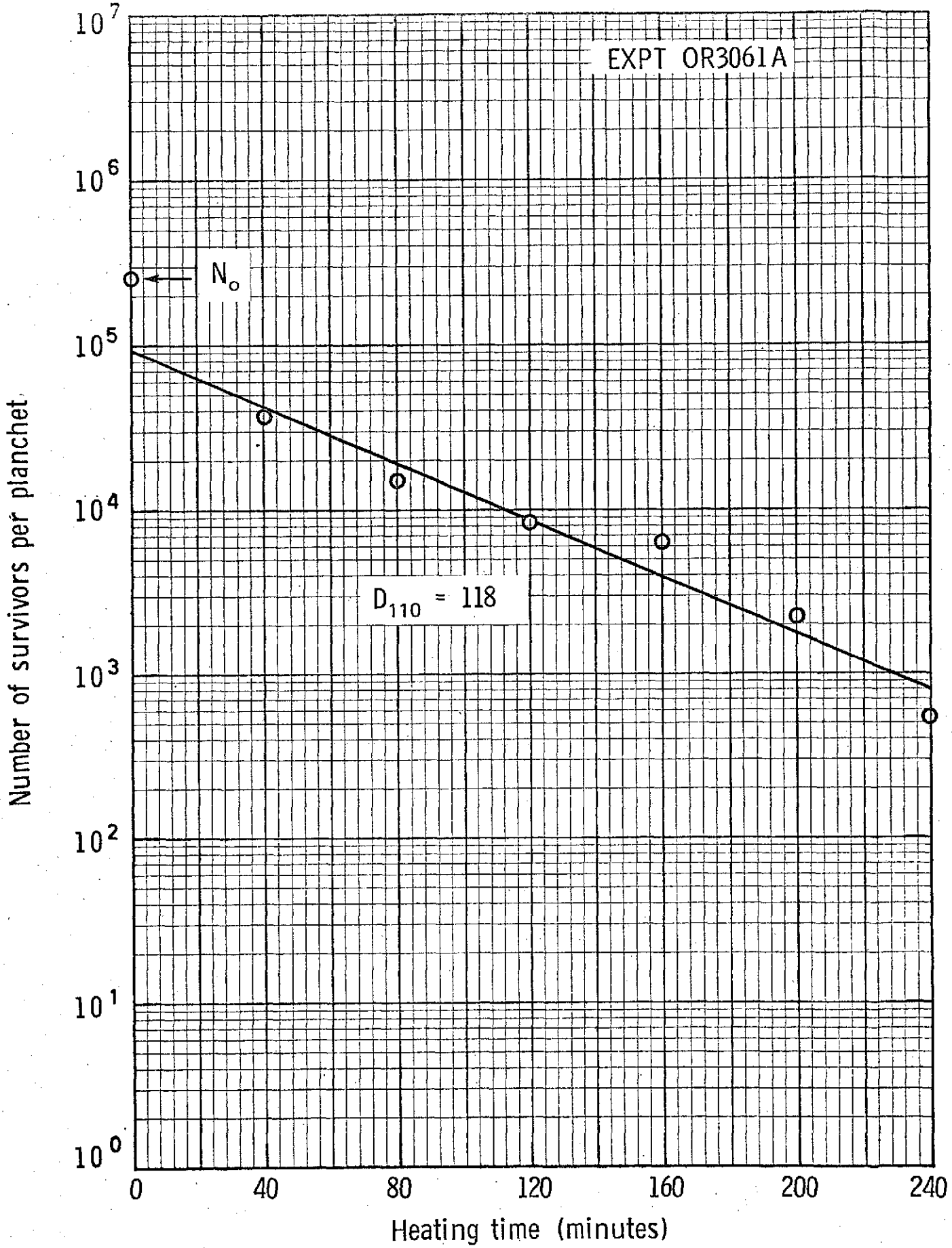

Fig. 2-10 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 110°C. (Clean Room Hotplate).

three boats run at each heating time. The regression line has been plotted on each graph and was calculated from the plate count data by means of our laboratory computer program, CTRJ. D-values determined for each set of experimental data are also included on the graphs.

A limited number of clean room versus low humidity dry box experiments were also done to compare the effects of these two environments on the heat resistance and survivor curves of the selected spore crops. The data accumulated from these experiments are summarized in the D-values listed in Table 2.4 and the survivor curves are shown in Figures 2.11 through 2.17.

For the QHAF spores, the clean room versus dry box hot plate series yielded results that appear to show similar trends at both 110°C and 125°C. At both temperatures, the data indicate that conditioning and heat treatment of QHAF spores in the glove box promoted a greater destruction rate of spores in only the very first phase of heating. Following this initial period, the destruction rates were nearly similar for spores conditioned and treated in either the clean room or dry box systems (see Figures 2.11 and 2.12). Table 2.4 shows that the D-values obtained at each particular temperature were nearly similar for the two environments. From the survivor curve data, it appears that conditioning in the dry box may render a proportion of these spores somewhat more susceptible to dry heat destruction in the first interval of heat treatment. Following this initial increased rate of destruction, the shapes of the survivor curves for the QHAF spores were nearly identical for both dry box and clean room environments.

The data for the two least resistant spore crops of B. stearothermophilus (PBBF and PCFF) indicate that, in dry box treatments, the dry heat destruction rates of these spores were increased to some extent. In each of the experiments with these spores, the D_{125} values were reduced when spores were conditioned and heated in the dry box as contrasted with clean room results. The data of Table 2.4 indicate that dry atmosphere conditioning and heat effected as much as a two to four fold reduction in D-values in some of the experiments at 125°C.

The two clean room-dry box experiments with Bacillus subtilis (ECFF) produced variable results. In both trials, the spores treated in the dry

Table 2.4

Comparison of Dry Heat Hot Plate D-value Obtained with Selected Bacillus sp Spore Crops Tested Simultaneously in Clean Room Versus Low Humidity Dry Box Conditions

Bacterial Species and Spore Code	D_{125}(min.)		D_{110}(min.)	
	Clean Room	Dry Box	Clean Room	Dry Box
B. stearothermophilus				
QHAF	13.0	14.4	63	65
PCFF	2.2	1.5	--	--
PCFF	2.5	1.0	--	--
PBBF	2.3	0.5	--	--
B. subtilis				
ECFF	12.2	10.2	--	--
ECFF	13.6	6.1	--	--

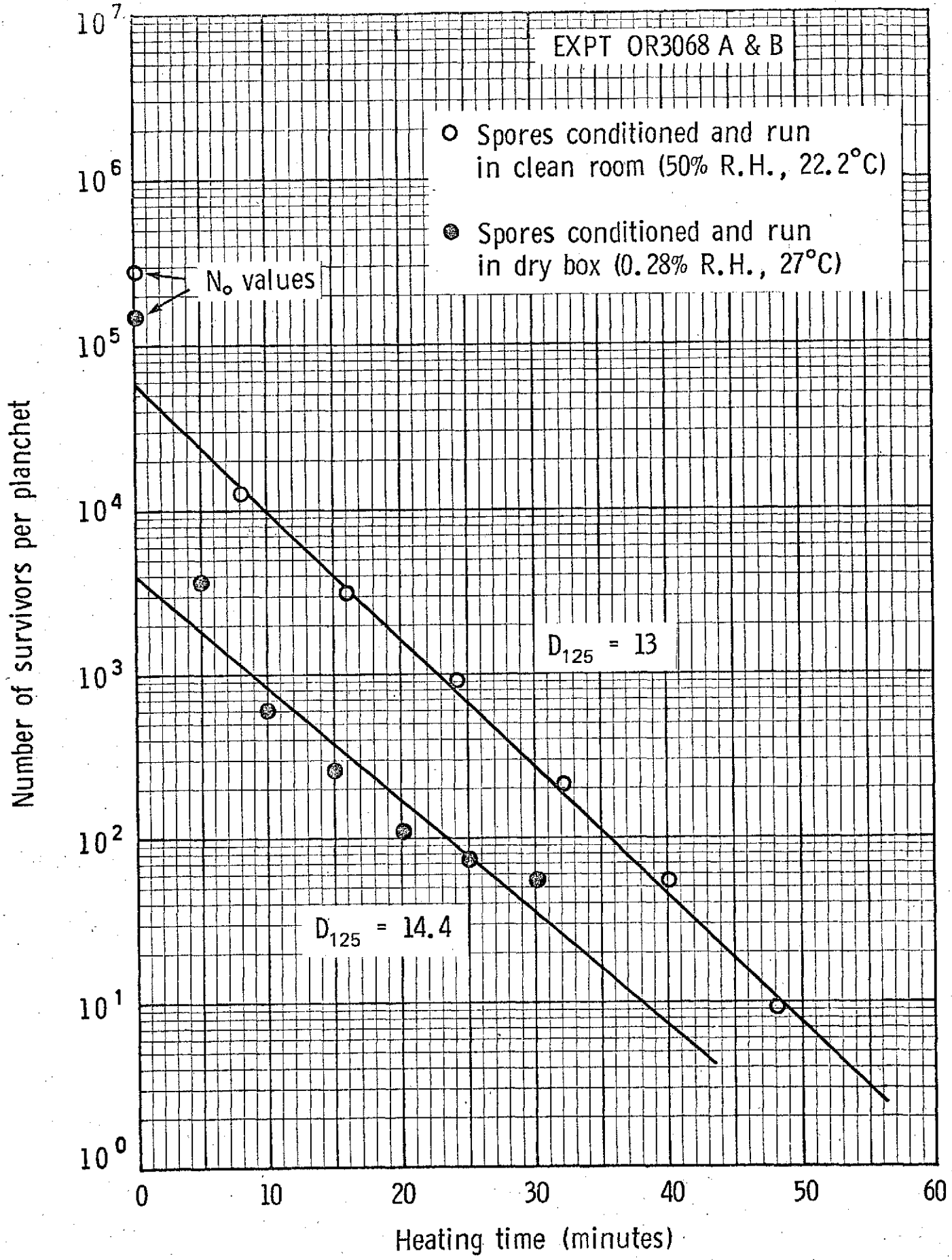

Fig. 2-11 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 125°C. (Dry Box & Clean Room Series).

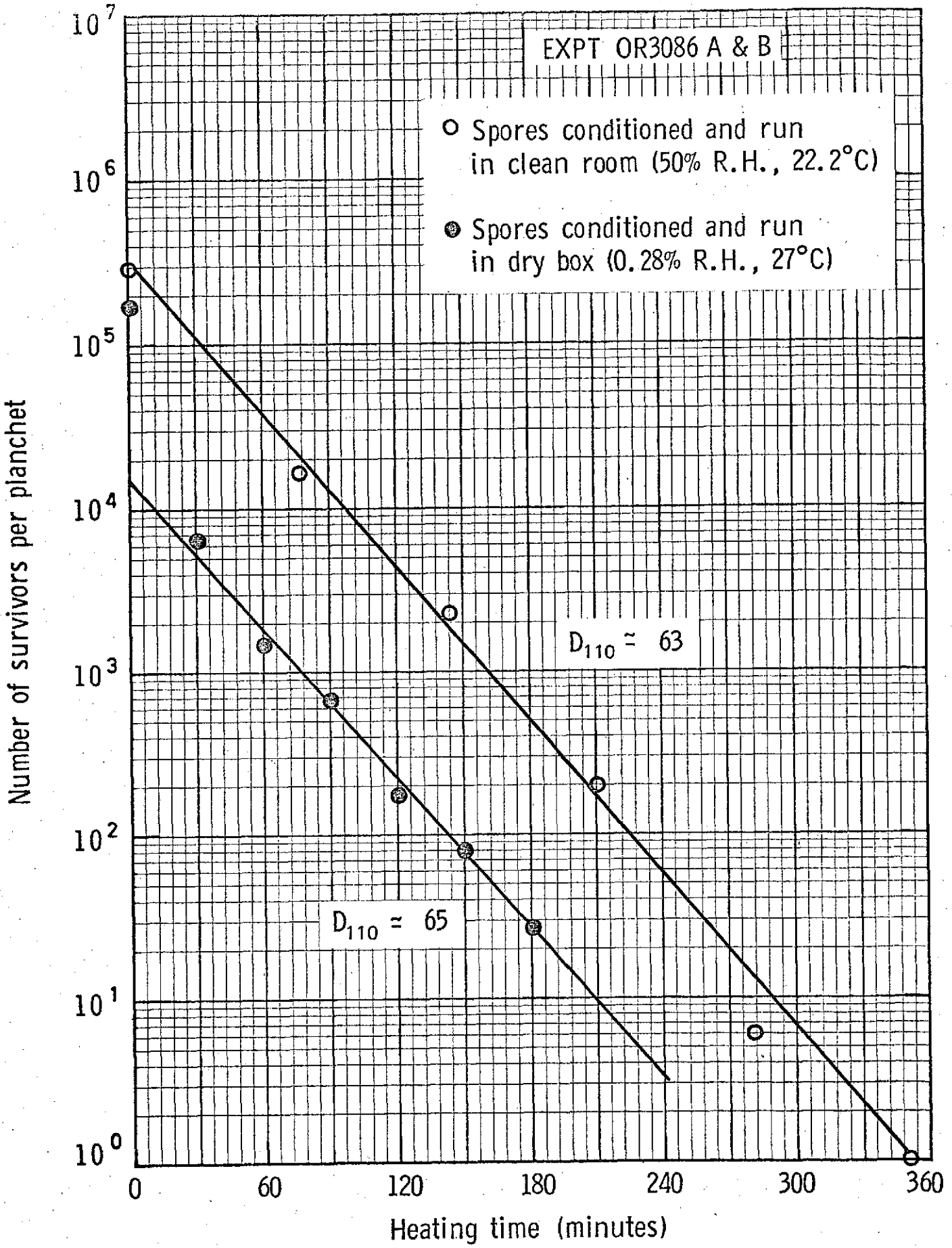

Fig. 2-12 Survivor Curve of Bacillus stearothermophilus Spores (QHAF) in Dry Heat at 110°C. (Dry Box & Clean Room Series).

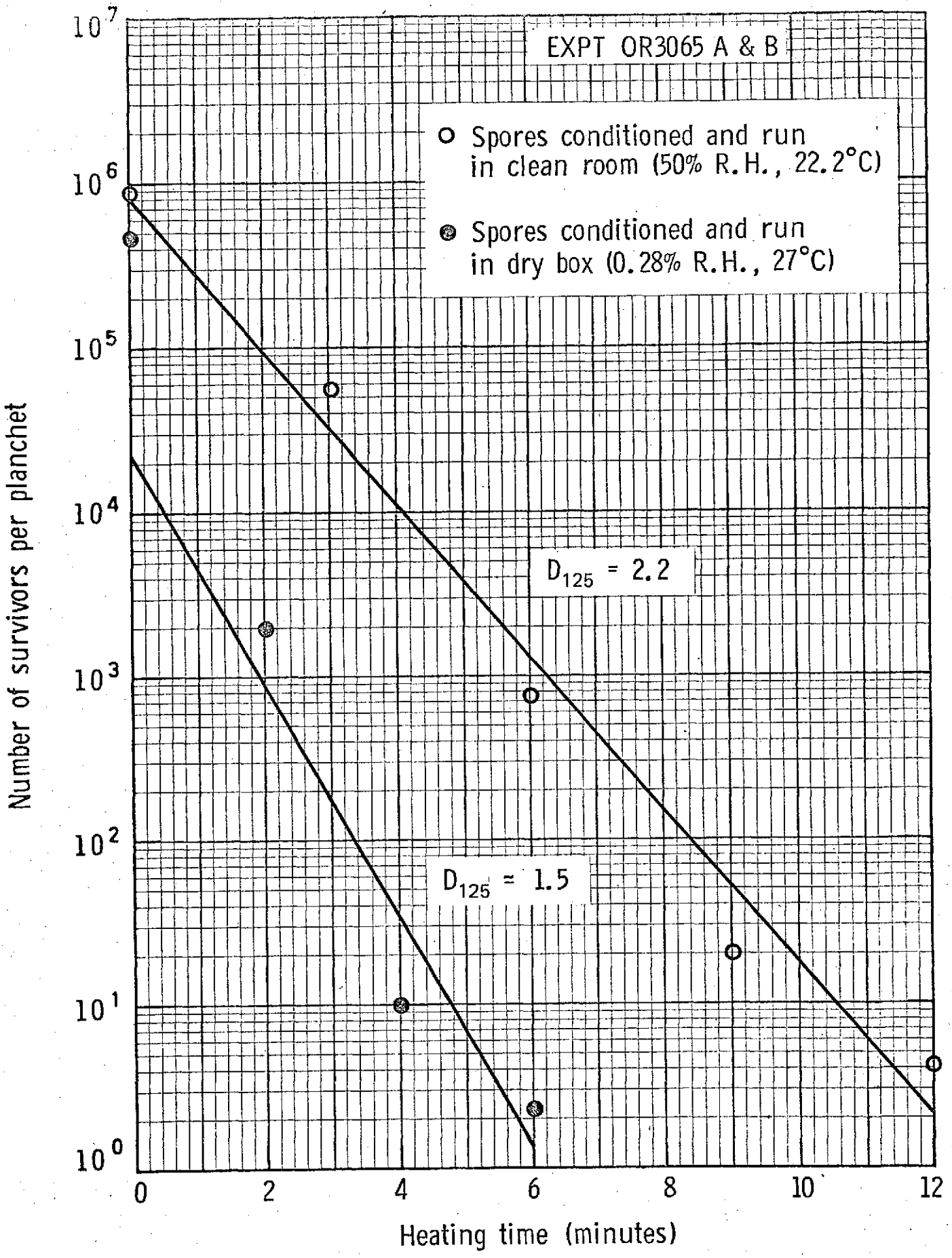

Fig. 2-13 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 125°C. (Dry Box & Clean Room Series).

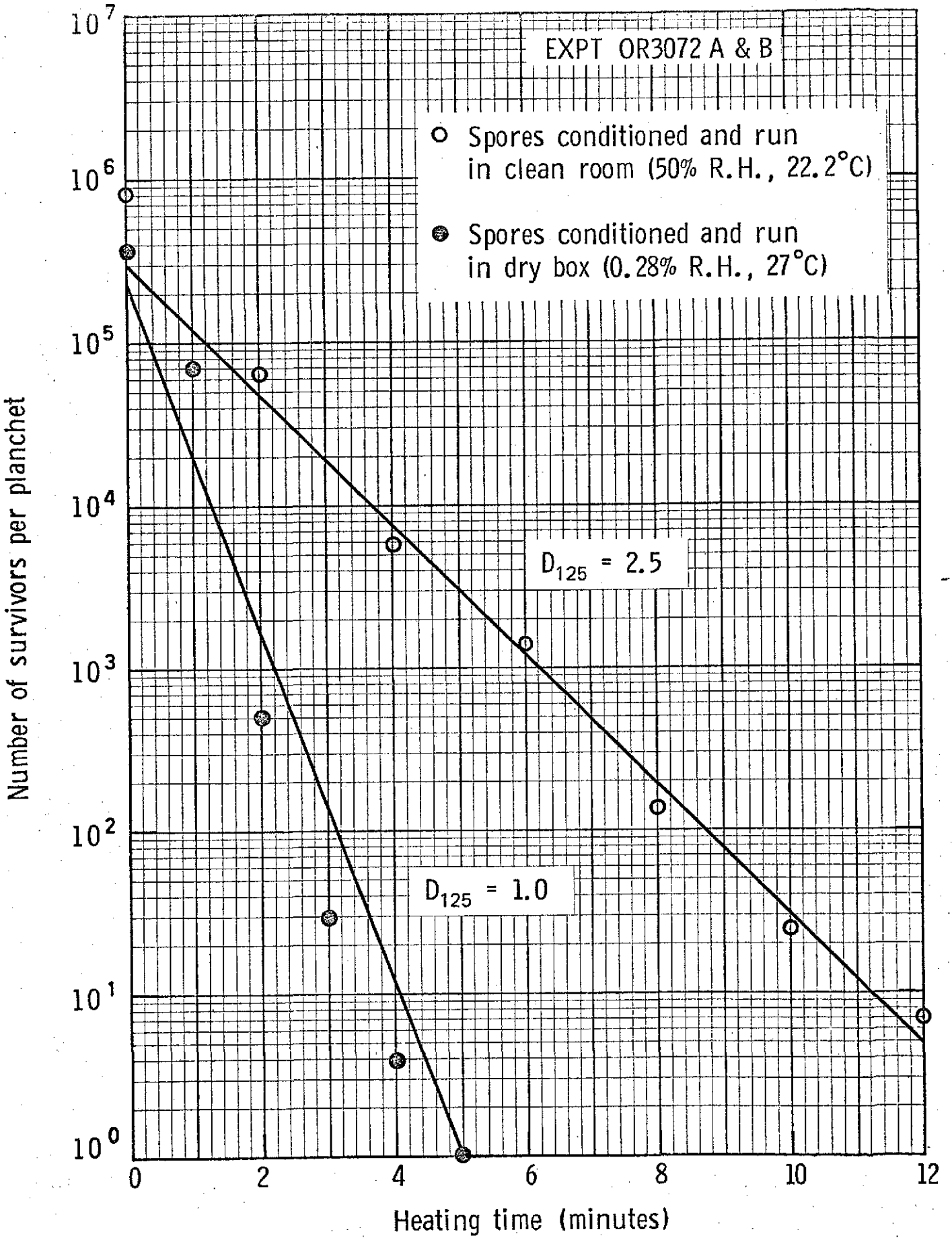

Fig. 2-14 Survivor Curve of Bacillus stearothermophilus Spores (PCFF) in Dry Heat at 125°C. (Dry Box & Clean Room Series).

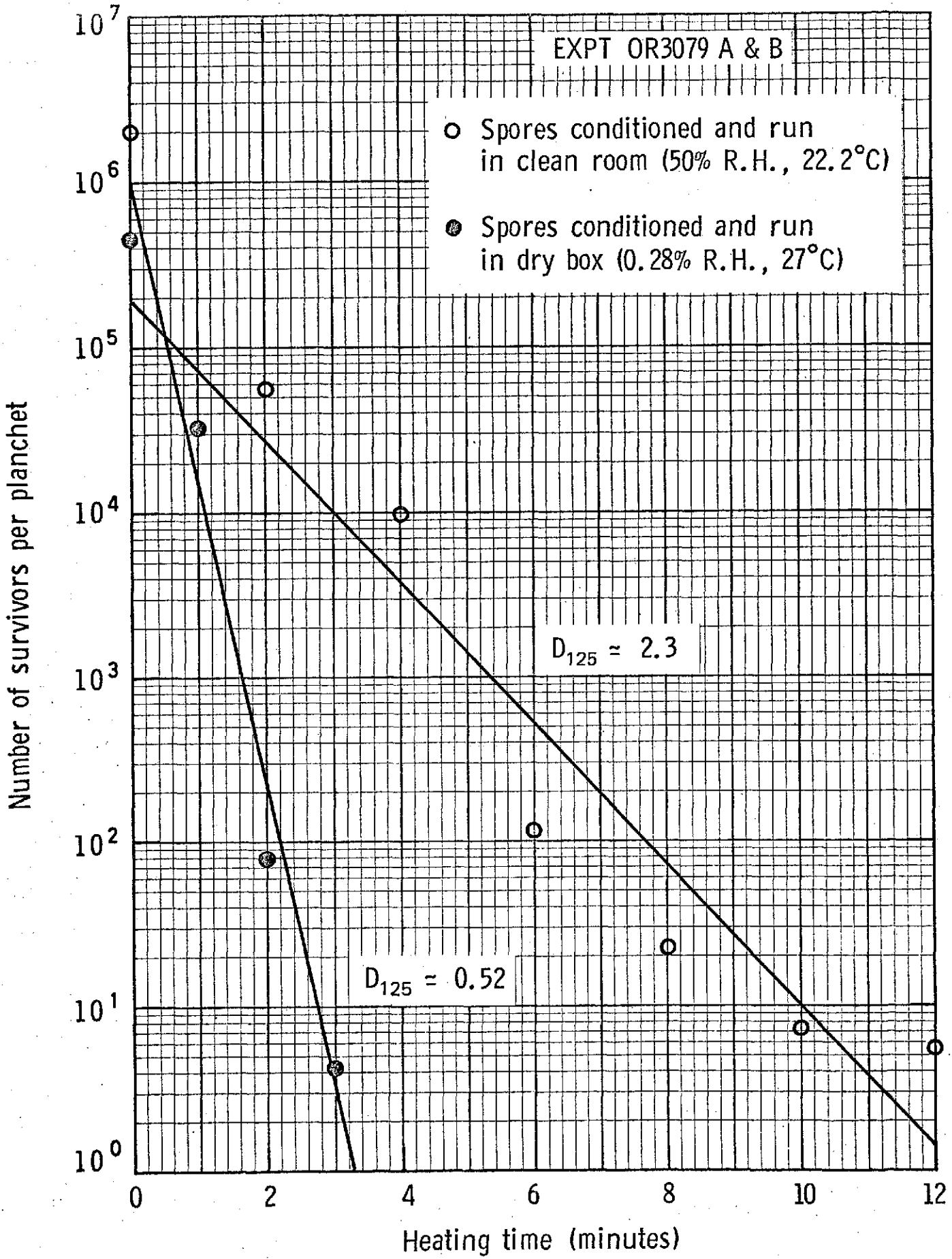

Fig. 2-15 Survivor Curve of Bacillus stearothermophilus Spores (PBBF) in Dry Heat at 125°C. (Dry Box & Clean Room Series).

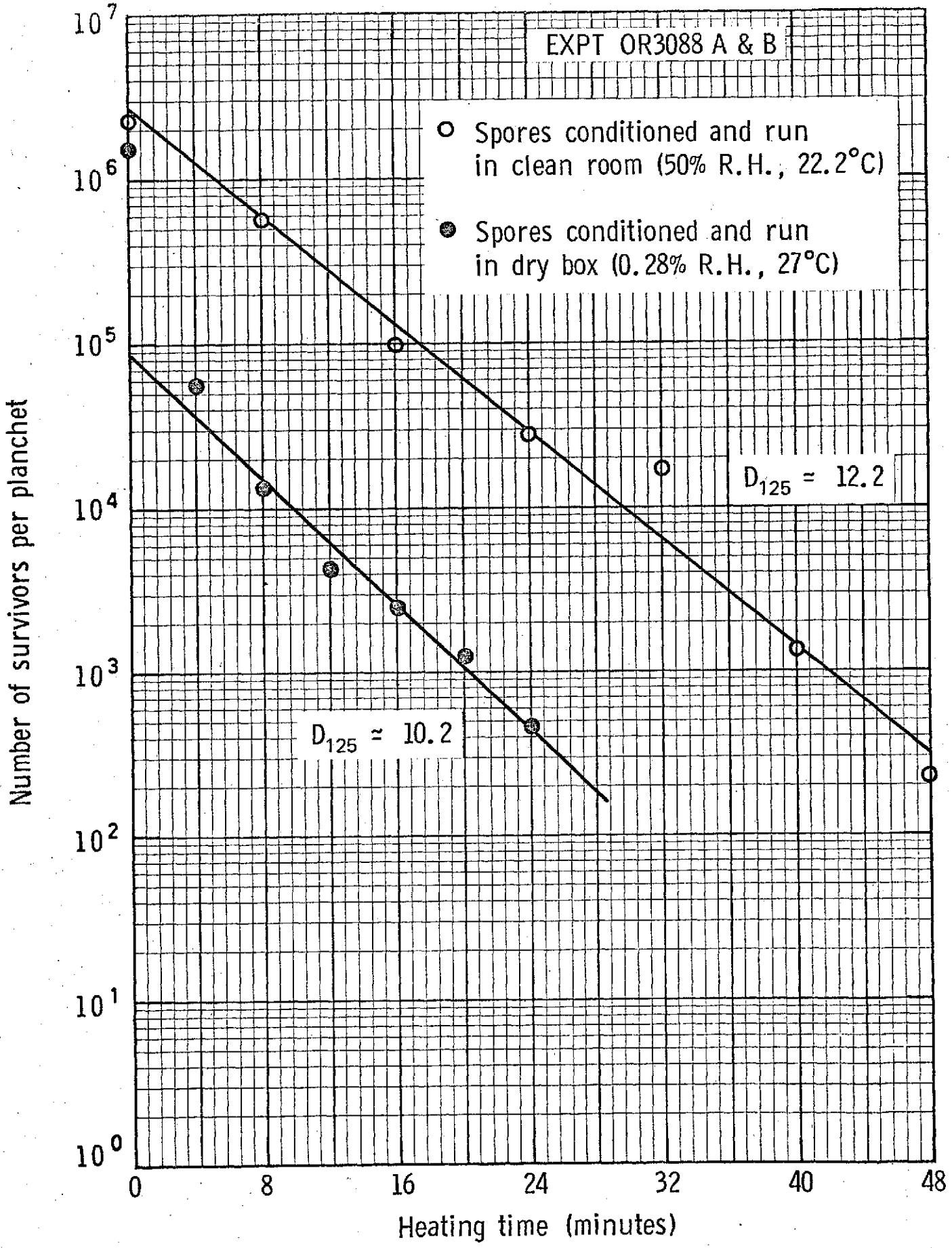

Fig. 2-16 Survivor Curve of Bacillus subtilis (ECFF) Spores in Dry Heat at 125°C. (Dry Box & Clean Room Series).

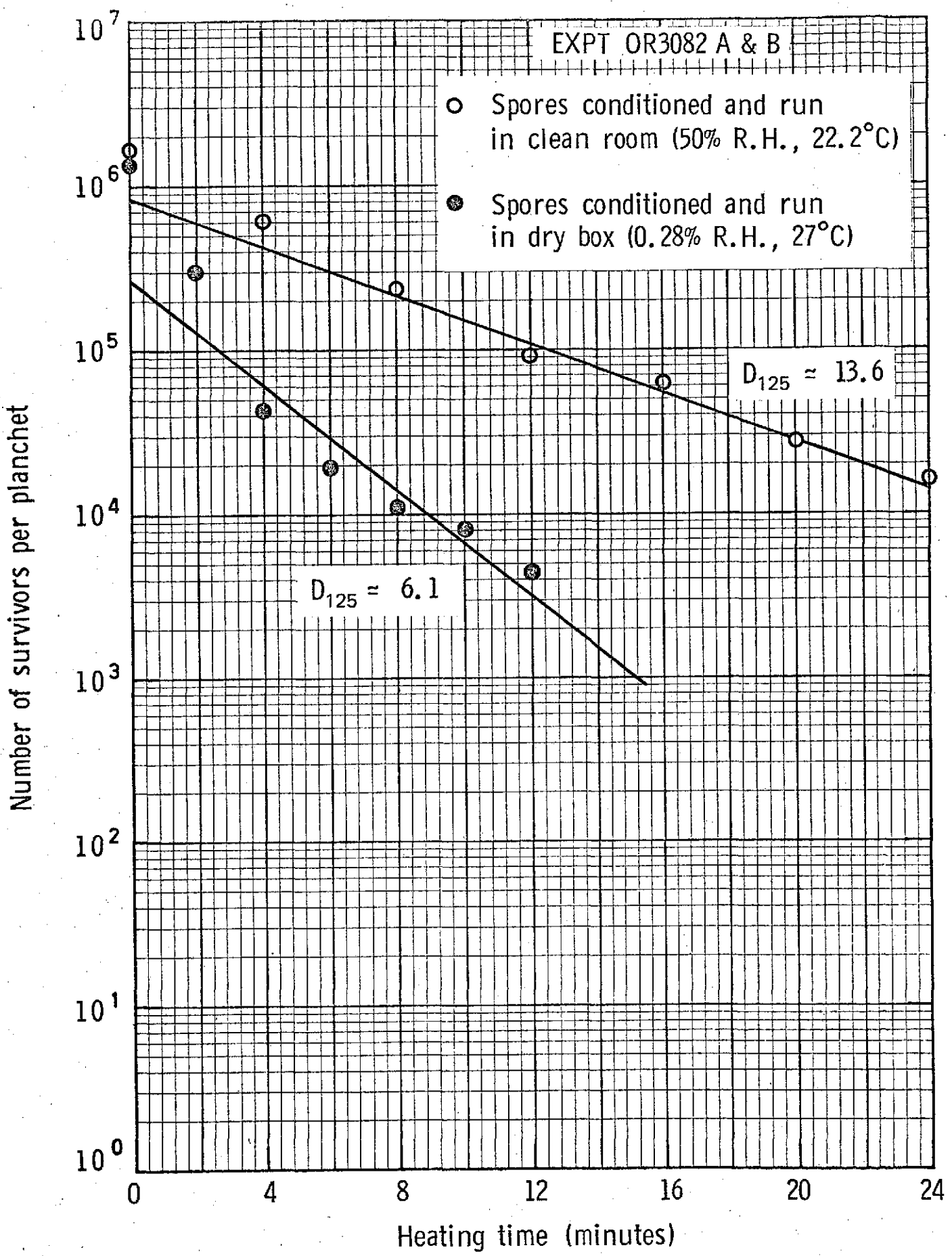

Fig. 2-17 Survivor Curve of Bacillus subtilis (ECFF) Spores in Dry Heat at 125°C. (Dry Box & Clean Room Series).

box demonstrated greater rates of heat destruction in the first interval of the heating time (see Figures 2.16 and 2.17). These graphs also show that the survivor curve data and D-values for the two clean room experiments were consistent. However, the two dry box results were not in agreement, yielding D-values that showed an approximately two fold difference between runs. At the present time, the reason for this difference in response is not known. Additional experiments will be required to determine the characteristics of the survivor curve for ECFF spores treated in dry box atmospheres.

SUMMARY

1. Spores of different Bacillus stearothermophilus strains, which were grown, harvested and stored, using identical procedures, demonstrated marked differences in dry heat resistance in tests at 110°C and 125°C.

2. B. stearothermophilus (QHAF) and B. subtilis (ECFF) were the most heat resistant of the spores tested. Spore crops PCFF and PBBF, also both strains of B. stearothermophilus, were much less resistant to dry heat destruction.

3. Conditioning and heating the two least resistant spore crops (PCFF and PBBF) in a dry box atmosphere increased the dry heat destruction rates over those observed in clean room tests.

4. For the more heat resistant strain of the thermophile, B. stearothermophilus (QHAF), D-values obtained from dry box experiments were approximately similar to those from clean room tests. However, conditioning in the dry atmosphere apparently rendered a proportion of these spores more susceptible to dry heat destruction during the first heating time interval. Similar conditioning effects were observed in dry box experiments with B. subtilis (ECFF) spores, but results and D-values were not consistent.

FUTURE WORK

In future investigative work, the following areas might be explored to provide additional information concerning the dry heat resistance of

aerobic spore forming species.

1. Additional studies on the dry heat resistance of other thermophilic spore crops would be helpful in determining the range of heat tolerance in this group of bacteria.

2. Further work remains to be done with spore crops currently in our collection. Among these studies are certain analyses of thermophilic spore crop responses to dry heat at 110°C.

3. Another area of interest would be the analysis of dry heat resistance characteristics of other selected mesophilic, Bacillus species isolated from Minnesota soils or contaminated food products.

4. Supplementary studies to determine the response of other spore crops to heat treatment in dry box atmospheres.

3. DRY HEAT DESTRUCTION RATE OF BACTERIAL SPORES

Bliss Moore, Ronald Jacobson, and Irving J. Pflug

INTRODUCTION

Most of our earlier work has been concerned with laboratory cultured cleaned spore suspensions, mainly because of simplicity of handling and precision of experimental control. However, we have also been interested for some time in the dry heat resistance of the indigenous soil microflora as they occur in their natural environment, i.e. associated with soil and dust particles. Some studies have been directed toward investigating the dry heat resistance of particles < 43 μm in ethanol and water suspensions (University of Minnesota, School of Public Health, NASA Progress Report #6, June 1971, pp. 26-31 and NASA Progress Report #7, December 1971, pp. 13-31). More recent work was concerned with a given mass of dry (ambient condition) soil particles (University of Minnesota, School of Public Health, NASA Progress Report #8, June 1972, pp. 25-38). However, we have done no work until now on individual dry soil particles > 43 μm. Therefore, it was decided that we should attempt to more nearly simulate actual spacecraft conditions by using the dry soil and individual particles. The particles were sized in an effort to associate a certain level of dry heat resistance with the different groups of soil particles.

OBJECTIVES

The objectives of these studies were: (1) to develop a workable, reliable test system for soil particles, and (2) to examine the dry heat resistance characteristics at 125°C of individual Cape Kennedy soil particles of four different homogeneous sizes.

MATERIALS AND METHODS

A sample of Cape Kennedy soil (WAJJA), received from the Spacecraft Bioassay Laboratory in December 1971, was separated into particle sizes

by shaking on a Ro-tap machine for 30 minutes. The Ro-tap unit included a series of seven clean sieves to separate the soil into seven fractions. The standard ASMT-NBS sieves used from top to bottom were NBS numbers 140, 170, 200, 230, 270, and 325. These sieves had openings of 105 μm, 88 μm, 74 μm, 63 μm, 53 μm, and 44 μm respectively (see Table 3.1). Subsequently seven more fractions were collected by sifting the 105 μm soil fraction through larger sieves: NBS numbers 60, 70, 80, 100, 120 and 140. The openings of these sieves were 250 μm, 210 μm, 177 μm, 149 μm, 125 μm, and 105 μm respectively (see Table 3.1). All these fractions were given code numbers and stored in clean, sealed jars at room temperature.

Initially four fractions were chosen for study. These were a 44-53 μm fraction, the smallest defined size group; a 105-125 μm fraction, the largest size that might still be considered dust; and two intermediate sizes, 74-88 μm and 88-105 μm, that could be conveniently handled. A small amount of soil of a given size was spread out in a petri dish from which individual particles were separated with a fine wire (.005" chromel) inoculating needle. By means of natural static charges on the needle and careful manipulation, these individual particles were transferred to sterile, stainless steel cups (O.D. = 5/16", h = 3/8") or to the wells (O.D. = 3/16", d = 3/32") of stainless steel boats (l = 2 1/2", w = 2 1/2", h = 1/4").

It was thought that organic conglomerate type of particles would be the most likely ones to have the most spores, so only the darker, non-crystalline, non-sand-like particles were used in these studies. All the experiments reported here were carried out in the clean room (22°C at 50% RH or about 13,000 ppm water).

Most of the experiments were carried out using the Cup-Boat-TSB (trypticase soy broth) system. The stainless steel cups were set in 5 place copper boats (l = 3", w = 3/4", h = 3/8") for heat treatment studies. Groups of four or more boats were heated on a 125°C hot plate for selected time periods. After heating, the boats were cooled on a cold plate (circulating cold tap water) for three minutes; the cups were removed aseptically from the boats with sterile forceps; each cup was placed in a TSB (.005%

alanine) tube and incubated 14 days at 32°C. Positive tubes were determined by the slightest presence of turbidity, then smeared, Gram stained, and subcultured for confirmation. Any typical Gram negative rods or Gram positive cocci were considered as contamination and designated negative.

A few experiments were done using the Well-Boat-TSA (trypticase soy agar) system. In this system, individual soil particles were placed in the small wells (3/16" dia x 3/32" deep) drilled in a 2 1/2" square stainless steel boat (100 wells per boat). The boat was placed on the hot plate for a given time interval, cooled in the same manner as the copper boats, and then TSA was added directly to the wells. The boat was then placed in a petri dish with water in the bottom to prevent the agar from drying out during the two-week incubation period at 32°C.

RESULTS

A number of the experiments carried out were concerned with comparing the dry heat resistance of four different sizes of particles, i.e., 44-53 μm, 74-88 μm, 88-105 μm, and 105-125 μm. The method initially used for these preliminary studies was the Cup-Boat-TSB system previously described under Materials and Methods. Data obtained from these initial experiments with individual soil particles tested by using the Cup-Boat-TSB system are presented in Tables 3.2 through 3.6. In these studies either 20, 40, or 80 particles were used per heating time.

Tentative estimates of D_{125}-values for each particle size category are listed in Table 3.2. These estimates were obtained in two different ways. The first method was to average the D-values taken from N_o to each data point. Although the N_o point was not always exactly 1.0 for each data point, N_o was assumed to be 1.0 for these calculations. The second way of estimating a D-value was simply by connecting the first and last data points and determining the slope.

Some work was also initiated to compare the two treatment procedures and assay systems previously described under Materials and Methods. One was the Cup-Boat-TSB system where particles were put into stainless steel cups, placed in a copper boat, and heated. After cooling, each cup was

Table 3.1

Particle Size Ranges Separated By Standard Sieve Series

Sieves(#)	Fractions (μm)	Sieves(#)	Fractions (μm)
140	> 105	60	> 250
170	88-105	70	210-250
200	74-88	80	177-210
230	63-74	100	149-177
270	53-63	120	125-149
325	44-53	140	105-125
Bottom	< 44	Bottom	< 105

Table 3.2

D-Value Data Obtained In Viability Studies of Various Particle Size Ranges. Cup-Boat-TSB Method

Code	Size (μm)	Ave. 2 pt. D-value (from N_o)	2 pt. D-value (1st pt. to last pt.)
WAJJC	44-53	75.6	148
WAJJF	74-88	121.9	122
WAJJG	88-105	140.6	345
WAJJH	105-125	284.8	284

Table 3.3

Viability of Cape Kennedy Soil Particles

After Dry Heat Treatment at 125°C WAJJC Soil (44-53 µm) Cup-Boat-TSB Method

Experiment Number	Heating Time (min)	Proportion Positive[a]	
		Fraction	Decimal
BM2364A	15	8/20	0.400
BM2364A	30	6/20	0.300
BM3026, 3075	60	9/120	0.075
BM3026	120	1/40	0.025
BM3080	180	4/80	0.050
BM3094	240	1/80	0.012

[a] Refers to fraction of particles with viable microorganisms.

Table 3.4

Viability of Cape Kennedy Soil Particles

After Dry Heat Treatment at 125°C WAJJF Soil (74-88 µm) Cup-Boat-TSB Method

Experiment Number	Heating Time (min)	Proportion Positive[a]	
		Fraction	Decimal
BM2364B	15	14/20	0.700
BM2364B	30	17/20	0.850
BM3005, 3068	60	17/80	0.212
BM3005	120	3/40	0.075
BM3033, 3040	180	1/160	0.006
BM3096	240	1/80	0.012

[a] Refers to fraction of particles with viable microorganisms.

Table 3.5

Viability of Cape Kennedy Soil Particles

After Dry Heat Treatment at 125°C WAJJG Soil (88-105 μm) Cup-Boat-TSB Method

Experiment Number	Heating Time (min)	Proportion Positive [a]	
		Fraction	Decimal
BM3012	60	5/40	0.125
BM3012	120	4/40	0.100
BM3047	180	11/80	0.138
BM3053	240	3/80	0.038

[a] Refers to fraction of particles with viable microorganisms.

Table 3.6

Viability of Cape Kennedy Soil Particles

After Dry Heat Treatment at 125°C WAJJH (105-125 μm) Cup-Boat-TSB Method

Experiment Number	Heating Time (min)	Proportion Positive [a]	
		Fraction	Decimal
BM3059	60	21/40	0.525
BM3059	240	2/40	0.050
BM3061	360	6/80	0.075
BM3089	480	1/80	0.012

[a] Refers to fraction of particles with viable microorganisms.

Table 3.7

Results from Trials with "Boat-TSA" and "Cup-Boat-TSB" Assay Systems. Cape Kennedy Soil Particles (105-125 μm). Dry Heat 125°C for One Hour--Clean Room Hot Plate

Experiment Number	TEST SYSTEM			
	Boat-TSA		Cup-Boat-TSB	
	Proportion Positive	D_{125} (min) from N_o	Proportion Positive	D_{125} (min) from N_o
BM3103A	38/100	142	11/40	105
BM3103B	48/100	187	11/40	105
BM3136A	52/100	214	11/40	105
Mean		181		105

aseptically transferred to a Trypticase Soy broth tube (.005% alanine) and incubated. The other system consisted of a stainless steel boat with 100 wells to which particles were added. After heating and cooling the boat, Trypticase Soy agar was added directly to each well before incubation. For a comparative study of these two systems, three experiments were done in the laminar downflow clean room using the largest particle size group, 105-125 μm (WAJJH). Results from these experiments are shown in Table 3.7.

DISCUSSION

The somewhat erratic results in the proportion-positive survivor data for the four particle sizes (Tables 3.3 through 3.6) are not surprising considering the extremely low initial number of particles used, i.e. in some cases only 20 particles per time period. However, there appears to be a trend suggesting that the larger particles may be a little more heat resistant than the smaller particles. This trend is reflected in the D-values of Table 3.2. There are three possible explanations. First, the larger particles may have a significantly higher population of organisms than the smaller ones, thereby increasing the survival time under treatment according to logarithmic death theory. Second, more dry heat resistant organisms may be associated with the larger particles than with the smaller ones. Third, the larger particles may afford some sort of protection to the organisms associated with them (e.g. water content). At this time we have no evidence which supports any one of these three possibilities. In fact, more work needs to be done to substantiate the apparent greater heat resistance of the larger particles.

The results from the particle viability detection system comparison study definitely show a difference between the two systems (see Table 3.7). In each of these experiments, the test system using TSA media yielded consistently higher numbers of particles with viable microorganisms (proportion positive values) than was found with the TSB system. A similar trend is shown by the D_{125}-values estimated from these results for 0-60 minutes. Since there is no known appreciable difference between the two heating systems, the difference in numbers of survivors was most

probably due to the media. That is, either better growth occurred with the TSA than with the TSB, or growth was more easily detected in the TSA than in the TSB, or both. However, other possibilities have not been ruled out and further investigations are planned.

CONCLUSIONS

1. The larger particles appear to be somewhat more dry heat resistant than the smaller ones. This same pattern was found in some of our previous soil investigations (see NASA Progress Report #7, December 1971, pp. 14-20).

2. In the comparison of two different test and sssay systems, the Stainless Steel Cup-Copper Boat-Trypticase Soy Broth system yielded consistently fewer positives than did the Well-Stainless Steel Boat-Trypticase Soy Agar system. The most probable reason for this difference was the media. Apparently, the TSA either promoted better growth than did the TSB, or better facilitated growth detection, or both.

FUTURE WORK

1. Further work is planned in developing a treatment and assay system that is relatively simple and reliable.

2. More complete characterizations of the survivor curves of the various sized particles is planned.

3. Comparison of the dry heat resistance between some newly acquired Cape Kennedy soil (WAKMA) and the Cape Kennedy soil received in 1971 (WAJJA) which were used in these studies will be made.

4. Efforts will be made to examine any effects a dry atmosphere, i.e., 110 ppm water, during heating might have on the dry heat resistance of these particles as opposed to the clean room ambient atmosphere, i.e., 13,000 ppm water.

4. LABORATORY CONTROL AND STATISTICAL ANALYSIS

Geraldine M. Smith and Irving J. Pflug

A study has been initiated to determine the effect of storage time, suspending medium, storage temperature and spore crop cleaning procedures on the dry heat survival characteristics of Bacillus subtilis var. niger spores. A spore crop was produced in SSM-10 broth in sufficient quantity to make it possible to assay samples at 110 and 125 deg. C at six-month intervals over a three year period. Two suspending media (distilled water and 95% ethanol), and two storage temperatures (-10 and 4 deg. C) are being evaluated. At each of these conditions, spores that were stored without performing any cleaning procedures are being compared with spores that were cleaned, using insonation to free spores and break up vegetative debris, followed by several washings with deionized distilled water. The planchet-boat hotplate system of heating is being used. To date tests have been carried out for storage times of approximately 90 days and 300 days.

The experiments to determine media variation, where samples from different batches and lots of the same type of media are used to assay replicates of a single dry heat test, have been continued. The results of these tests indicate no significant difference between batches of the same lot (mfg.'s lot #) of media. However, when two different lots of media from the same manufacturer were compared, a significant difference was detected.

The objectives of these experiments were: (1) to improve laboratory control through long range studies of the effect of storage conditions on the survival characteristics of Bacillus subtilis and (2) to evaluate the day-to-day variation of media prepared in the laboratory.