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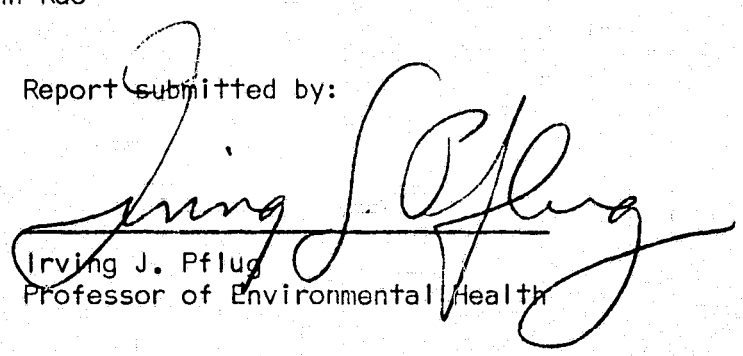

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SUMMARY

During the period covered by the current report, the Environmental Sterilization Laboratory, Space Science Center, University of Minnesota has continued experimental work related to dry heat resistance of microorganisms. One phase of this research has been concerned with the viability and dry heat resistance of indigenous microflora associated with small soil particles. A report on the progress we have made in this area is included in this report.

The second part of this report is an analysis of the present status of dry heat sterilization. In this analysis we have attempted to integrate results for both laboratory grown spores and spores in soil.

DRY HEAT EFFECTS ON SURVIVAL OF INDIGENOUS SOIL PARTICLE MICROFLORA AND PARTICLE VIABILITY STUDIES OF KENNEDY SPACE CENTER SOIL

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INTRODUCTION

The NASA Planetary Quarantine Program has been concerned with various facets of microbial contamination and sterilization problems relevant to space exploration research. One area of interest is in the small soil particles and their associated natural microorganisms as a source of contamination for space probes. In earlier reports Koesterer (1963) and Bond et al. (1970) have discussed the heat resistance of soil microflora and suggested that this phenomenon should be recognized in the design of sterilization cycles. Bond and associates (1973) in studies of Kennedy Space Center soil, reported the isolation of a Bacillus sp (ATCC 27380) which was extremely resistant to dry heat sterilization. In a recent publication, Reynolds et al. (1974) have also reported that a certain fraction of the soil microbial spore population may be quite resistant to heat treatment. Thus, additional information about dry heat effects on soil particulate fractions and the viability of their natural organisms appears desirable.

During the period covered by this report, we have been investigating the effects of dry heat on the natural in situ microbial population of soil, especially the effect of heat treatment on soil particle viability profiles. This work has involved studies of both single soil particles as well as heat treatment of multiple particle samples. It is anticipated that data from these experiments will be helpful in establishing effective heat treatment cycles required to inactivate in situ soil particle microflora and to better understand the soil contamination problem.

Experimental data included in the present report were obtained from our most recent studies of microscopic sized particles of Kennedy Space Center soil fractions. This research is one phase of our continuing laboratory investigations concerned with the dry heat effects on micro-

bial spores and related phenomena relevant to the spacecraft sterilization programs. The project is part of a more extensive program on heat resistance of microorganisms conducted by the Environmental Sterilization Laboratory, Space Science Center, University of Minnesota.

OBJECTIVES

In this investigation, research efforts were concentrated on attempts to obtain data concerning the dry heat resistance of particle microflora in Kennedy Space Center soil samples. Specific objectives for the project were: (1) to determine the *in situ* dry heat resistance profiles at selected temperatures for the aggregate microflora on soil particles of certain size ranges. (2) to compare viability profiles of older with more recently stored soil samples and (3) to investigate the effect of increased particle numbers on viability profiles after dry heat treatment. These soil particle viability data for various temperatures and times provide information on the soil microflora response to heat treatment and may be useful in making selections for spacecraft sterilization cycles.

MATERIALS AND METHODS

All aspects of this investigation have been concerned with the natural, mixed microbial populations of soil particles. The experimental work was done directly with soil particles and their naturally associated microflora. This approach seemed to be desirable because it provided a system which appeared to be reasonably representative of natural soil particle contamination.

Soil Particles

In our studies of heat effects on survival of soil particle microflora, two samples of Kennedy Space Center soil were used. One of these was a 1970 soil sample (Identification Code WAJJ Series) that had been stored in our laboratory following completion of earlier work. This sample had been collected in June 1970 by personnel of the NASA Spacecraft Bioassay Laboratory, Kennedy Space Center. The dried soil was

received by our laboratories in September 1971 and initially stored at 4°C. In December 1971, this sample was separated into particle size ranges and stored at room temperature since that time. In 1973, a second soil sample was collected at the NASA Kennedy Space Center; this sample was delivered to our laboratory in June 1973. This sample was designated by code as WAKM series and was also stored at room temperature in the dried state. This "new" soil has been used extensively in some of the recent particle viability profile analyses.

When the soil samples were received by our laboratory, they were processed in a Ro-Tap soil separator to fractionate each sample into a series of particle size ranges using ASMT standard sieves (See University of Minnesota, School of Public Health NASA Report for December 1972 - May 1973). Each soil fraction collected from the sieving process was stored at ambient laboratory conditions in a clean, covered glass jar until analyzed. Among the soil fractions stored, the smallest particle range separated was 44-53 μm and the largest fraction size range was 105-125 μm. Most of the viability profile studies were done with the intermediate sized 74-88 μm soil particles.

The soil particle viability analyses were run with either a series of randomly selected particles or a series of dark particles. In the random series, particles were transferred to test cups without any conscious selection for structure, color, texture, etc. However, for the dark particle series, a special effort was made to select the more dark, organic or clay type soil particles while quartz-like particles were specifically rejected.

Microbiological Media

In the experiments to determine the survival of microflora on soil particles following heat treatment, Trypticase Soy Agar (TSA) modified by the addition of triphenyltetrazolium chloride (TTC) was used. The TSA-TTC modified medium had been found to aid in the detection of microbial growth and determination of particle viability (University of Minnesota, School of Public Health, NASA Progress Report June-November 1973). Use of the redox indicator dye was particularly helpful in facilitating recognition of microbial colony development emanating from the

microscopic sized soil particles. The red precipitate formed by microbial growth in the medium provided a sharp contrast to the particles and normal background.

Soil Particle Viability Profiles

The soil particle viability experiments were done using dry heat treatment at 110°C or 125°C for selected times. Individual soil particles were placed in separate, sterile, stainless steel TDT cups and heated on temperature controlled hot plates in our clean room facility. The TDT Cup-Aluminum Boat-TTC procedure was used in all experiments. For all these studies ranging from analyses of one particle to 25 particles per cup, the soil particles were selected and transferred to TDT cups by micromanipulation with the aid of a stereoscopic microscope. A detailed description of the methodology has been reported previously (University of Minnesota, School of Public Health, NASA Progress Report June - November 1973).

After appropriate heating periods and cooling of test units, TTC media was added directly to the particles in the TDT cups. All cups were then incubated at 32°C for two weeks. Following incubation, the cups were carefully checked under a stereoscopic microscope for any evidence of growth and the number of particles or cups demonstrating growth was recorded. From these data the proportion of cups with viable microorganisms after each heating time was calculated for all test units. The resulting values were used to plot the viability profiles for each series of soil fractions investigated.

Because of the meticulous and tedious nature of the microscopic work as well as personnel limitations only certain particle size fractions of soil have been analyzed. In most experiments, only randomly selected particles were studied. However, for certain particle investigations, series of random or series of dark soil particles were also selected for analysis and comparison of data.

Additional information concerning the procedures have been outlined in the University of Minnesota, School of Public Health, NASA Progress Report June - November 1973.

RESULTS AND DISCUSSION

Results obtained from the investigations of dry heat effects on survival of microflora associated with soil particles are reported in the following sections. Included are studies of various soil fractions, single and multiple particle samples and analyses of soils stored for different times. All the experimental work was concentrated on the response of the *in situ* heterogeneous, aerobic, mesophilic microorganisms associated with the particles.

Particle Size Effect on Viability Profile

The earlier studies dealing with particle viability analyses using TSA-TTC media were confined to preliminary experiments with the 74-88 μm fraction of WAKM series soil. More recently, we have completed a viability analysis for the WAKMG (88-105 μm) particles as well. Random single soil particles were used in this experiment and the particles were heated at 125°C for time intervals from 15 to 120 minutes. Experimental data obtained in this study are listed in Table I and the particle viability profile is shown in Figure 1.

Table I

Soil Particle** Viability After Dry Heat Treatment

On Clean Room Hotplate at 125°C

WAKMG Soil (88-105 μm) Proportion Positive Data*

Experiment Number	Heating Time (Minutes)	Proportion Positive	
		Fraction	Decimal
OR4003A	0	67/74	0.905
OR4003B	15	24/74	0.324
OR3354A	30	17/74	0.230
OR4004A	45	8/74	0.108
OR3354B	60	3/74	0.040
OR4004B	80	4/74	0.054
OR4023A	100	1/74	0.014
OR4023B	120	0/74	0.000

* Refers to fraction of particles with viable microorganisms

** Particles selected randomly

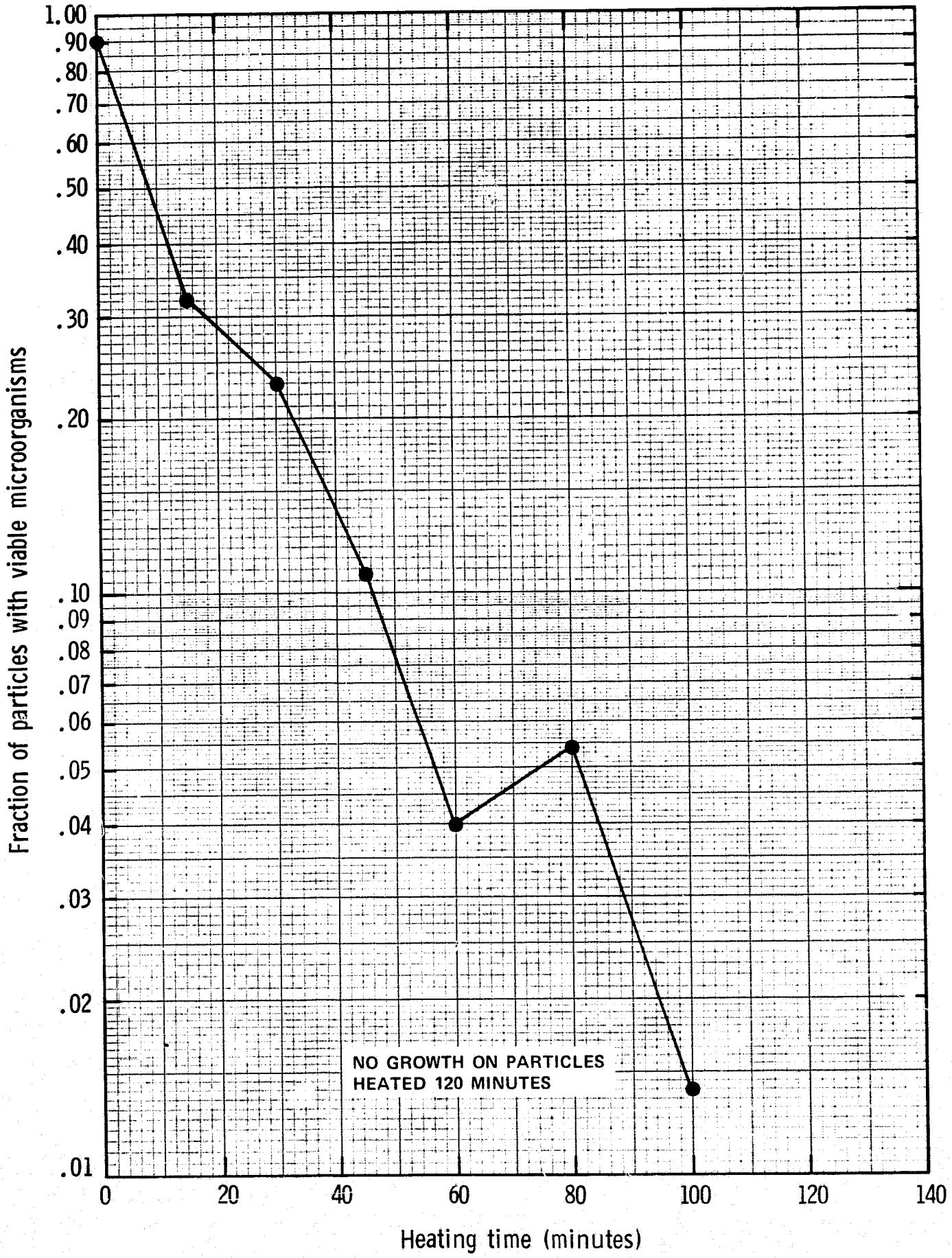

FIG. 1 VIABILITY OF WAKMG (88–105 μm) SOIL PARTICLES, 125°C DRY HEAT — CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC

Among the unheated WAKMG particles tested, 67 of 74 demonstrated microbial growth. Apparently, some particles did not retain microorganisms which were capable of growth under the test conditions or these particles did not have viable forms associated with them. The viability response of the unheated particles was slightly greater than 90 per cent. After dry heat treatment for 100 minutes at 125°C, only one particle out of 74 tested showed evidence of viability. No growth was observed on particles tested after 120 minutes heating time.

For purposes of comparison, the viability profile for random particles of the WAKMG (88-105 μm) soil fraction has been graphed together with similar data for WAKMF (74-88 μm) soil particles (Figure 2). These data suggest that the larger sized particles retain a viable microflora for a somewhat longer period of time when heated at the same temperature. This effect of particle size is similar to that observed earlier for WAJJ series soil studied by Moore et al. (University of Minnesota, School of Public Health NASA Progress Report, December 1972 - May 1973).

Random vs. Dark Particle Profiles

As part of the experimental soil work, it was also of interest to run a comparative viability profile series to note any differences in the profiles when only single dark particles were used as opposed to single randomly selected ones. For this experiment a series of 74-88 μm particles of both WAKMF and WAJJF soil samples were tested. Particles were treated with dry heat at 110°C for various time intervals up to 24 hours for some cups. The proportion of particles retaining viable microorganisms after each heating interval was determined and recorded for viability profile plots.

Experimental data from the dark particle study with WAKMF particles are listed in Table II. The corresponding graph illustrating the fraction of particles with surviving microflora at the various heating time intervals has been plotted in Figure 3. These data show that of the 74 unheated particles tested, 67 particles had viable, detectable microorganisms associated with them. This proportion constitutes about 90 per cent of the tested dark particles. After eight hours of heat treatment at 110°C, only one particle of the 74 tested retained viable microflora. No growth was observed on WAKMF dark particles heated for time intervals longer than eight hours.

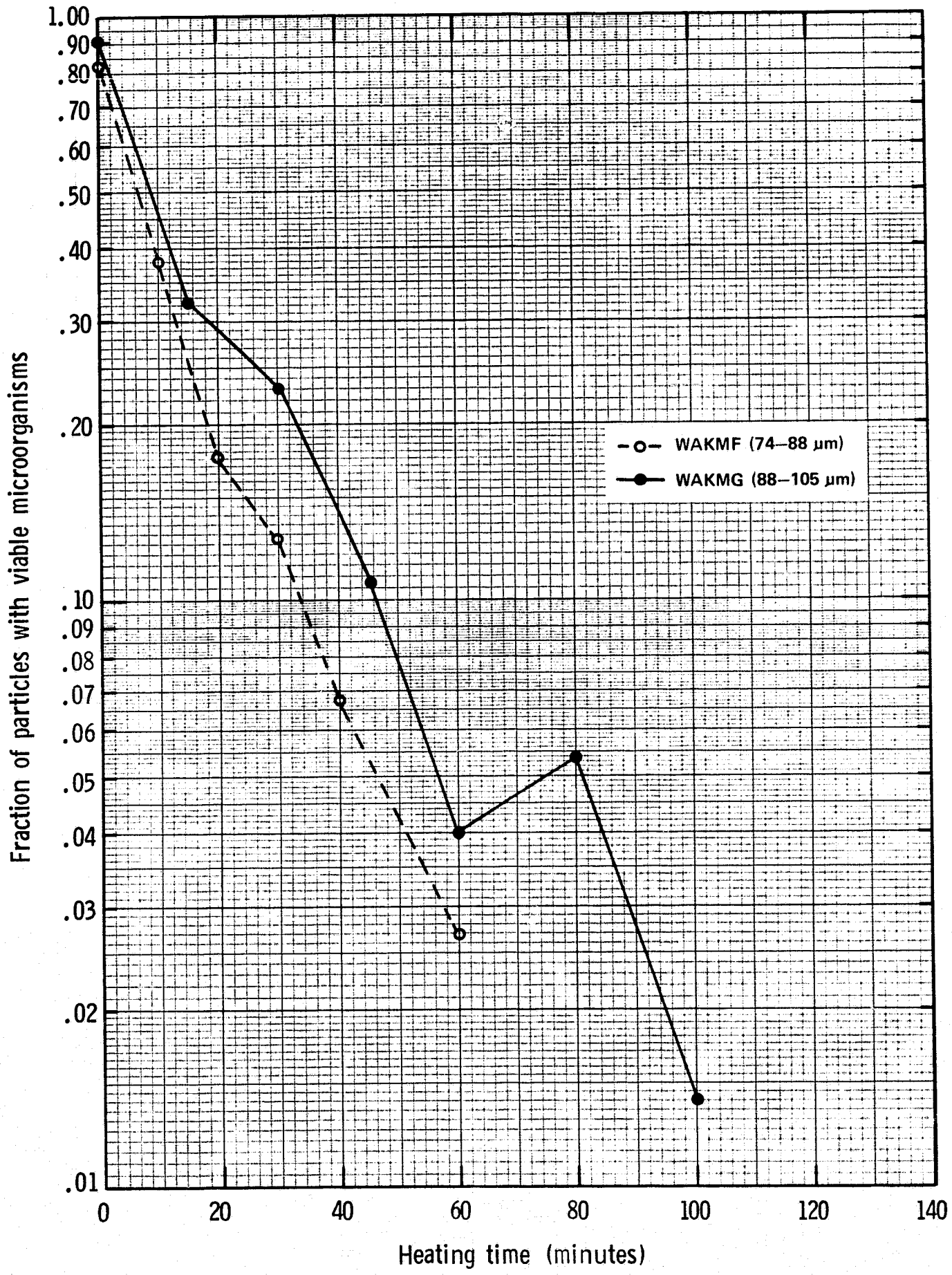

FIG. 2 COMPARATIVE VIABILITY DATA FOR RANDOM CAPE KENNEDY SOIL PARTICLES, 125°C DRY HEAT –– CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC

Table II

Soil Particle Viability After Dry Heat Treatment on Clean Room Hotplate at 110°C. WAKMF (74-88 μm) Dark Particles. Proportion Positive Data*

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4039A	0	67/74	0.905
OR4039B	1	37/74	0.500
OR4039C	2	17/74	0.230
OR4045A	4	7/74	0.095
OR4045B	6	5/74	0.068
OR4045C	8	1/74	0.014
OR4052A	10	0/74	0.000
OR4052B	12	0/74	0.000
OR4050A	16	0/74	0.000
OR4050B	24	0/74	0.000

* Refers to fraction of particles with viable microorganisms

The graph in Figure 4 shows the viability profile of the WAKMF dark particles compared with a profile for WAKMF randomly selected particles determined in an earlier experiment. In this case the two profiles are quite similar in slope and no growth was detected upon the analysis of either set of particles after 10 hours heating time. Although no differences in viability profiles are evident in the results of this experiment, it is recognized that these data are limited in that they represent only one experiment for each series of WAKMF particles. Additional work may reveal differences not detected in this experiment.

On the basis of the limited data available for WAKMF particles it appears that dark particles and randomly selected ones yield a similar inactivation response to dry heat effects. Because light colored particles, with generally fewer organisms per particle, constitute part of

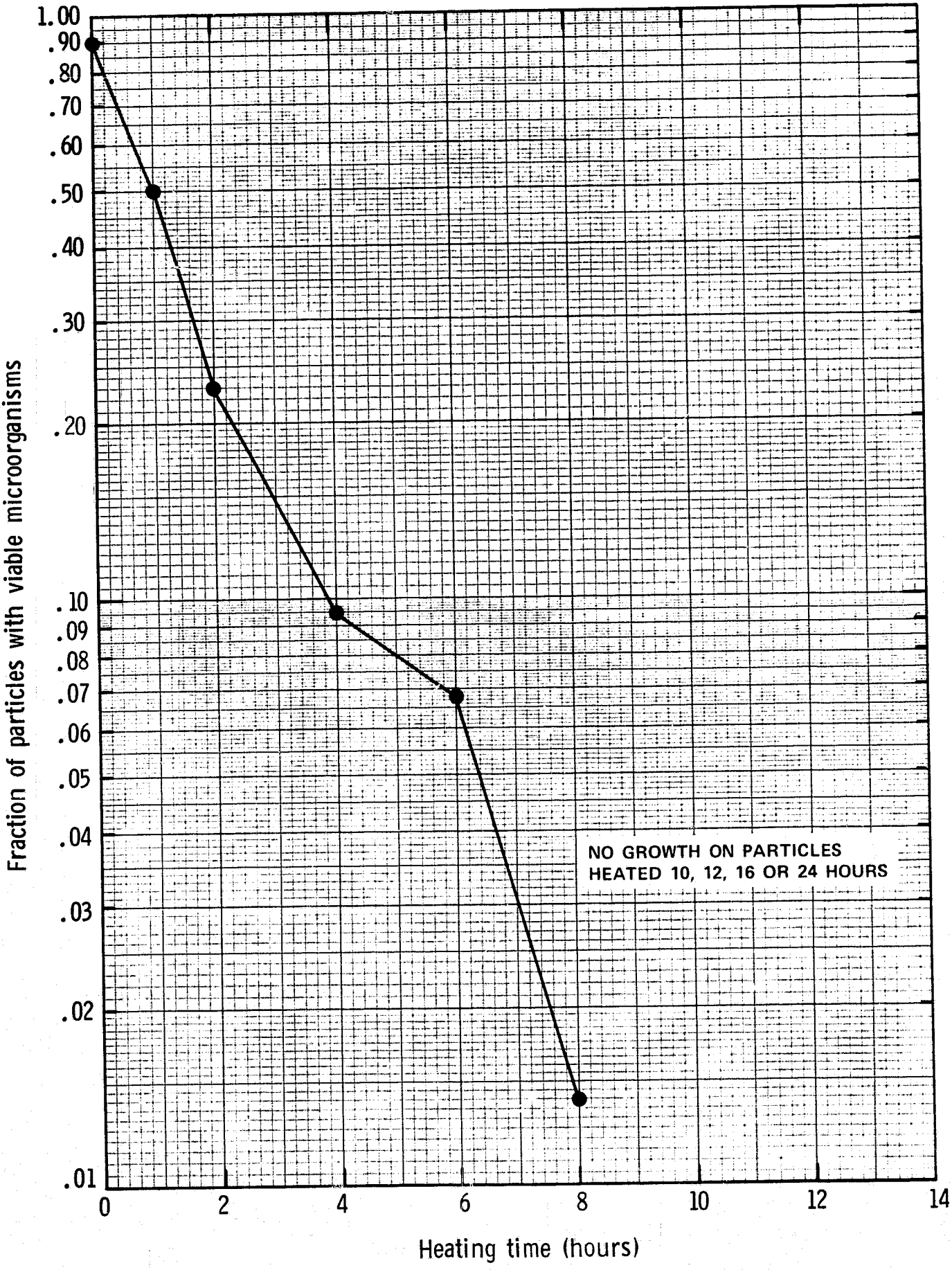

FIG. 3 VIABILITY OF WAKMF (74–88 μm) DARK SOIL PARTICLES, 110°C DRY HEAT — CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–ALUM BOAT–TTC (Expts. OR4039–4052)

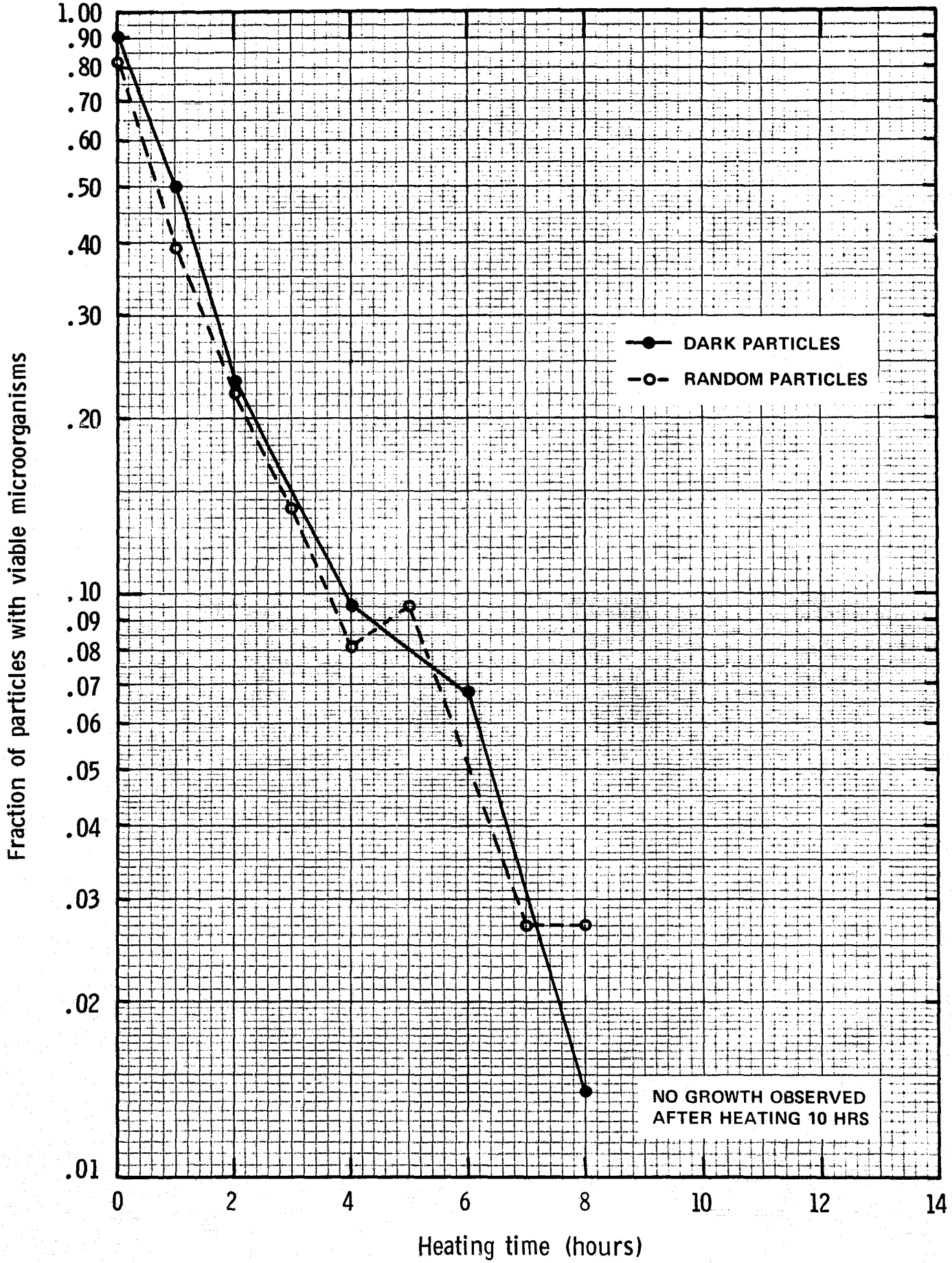

FIG. 4 VIABILITY PROFILES OF WAKMF (74–88 μm) RANDOM AND DARK SOIL PARTICLES, 110°C DRY HEAT –– CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC (Expts. OR3296–3311; OR4039–4052)

the random particle series, one might have expected some effect of selection on the viability profile. It is also conceivable that, due to special coincidence, the random particle set for the current experiment may have contained a higher proportion of darker particles than generally occur in such selections.

A second experiment with single random versus dark particles was carried out with the 74-88 μm WAJJF "old" soil fractions. These particles were also heated at 110°C for various time intervals. The proportion of random particles with associated microflora capable of surviving heat treatment at each time interval are listed in Table III. A viability profile plot for these random WAJJF particles is shown in Figure 5. For this particular experiment, approximately 73 per cent of the unheated random particles showed evidence of growth. It was also observed that about four per cent of the particles heated for four hours still retained viable organisms. However, after six hours or more of heat treatment, none of the random particles from this series demonstrated viability in the test medium.

Table III

Soil Particle Viability After Dry Heat Treatment At 110°C On Clean Room Hotplate WAJJF (74-88 μm)

Single Random Particles Proportion Positive Data*

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4247A	0	54/74	0.730
OR4247B	1	14/74	0.189
OR4248A	2	7/74	0.095
OR4248B	4	3/74	0.040
OR4249A	6	0/74	0.000
OR4249B	8	0/74	0.000
OR4249C	10	0/74	0.000

* Refers to fraction of particles with viable microorganisms

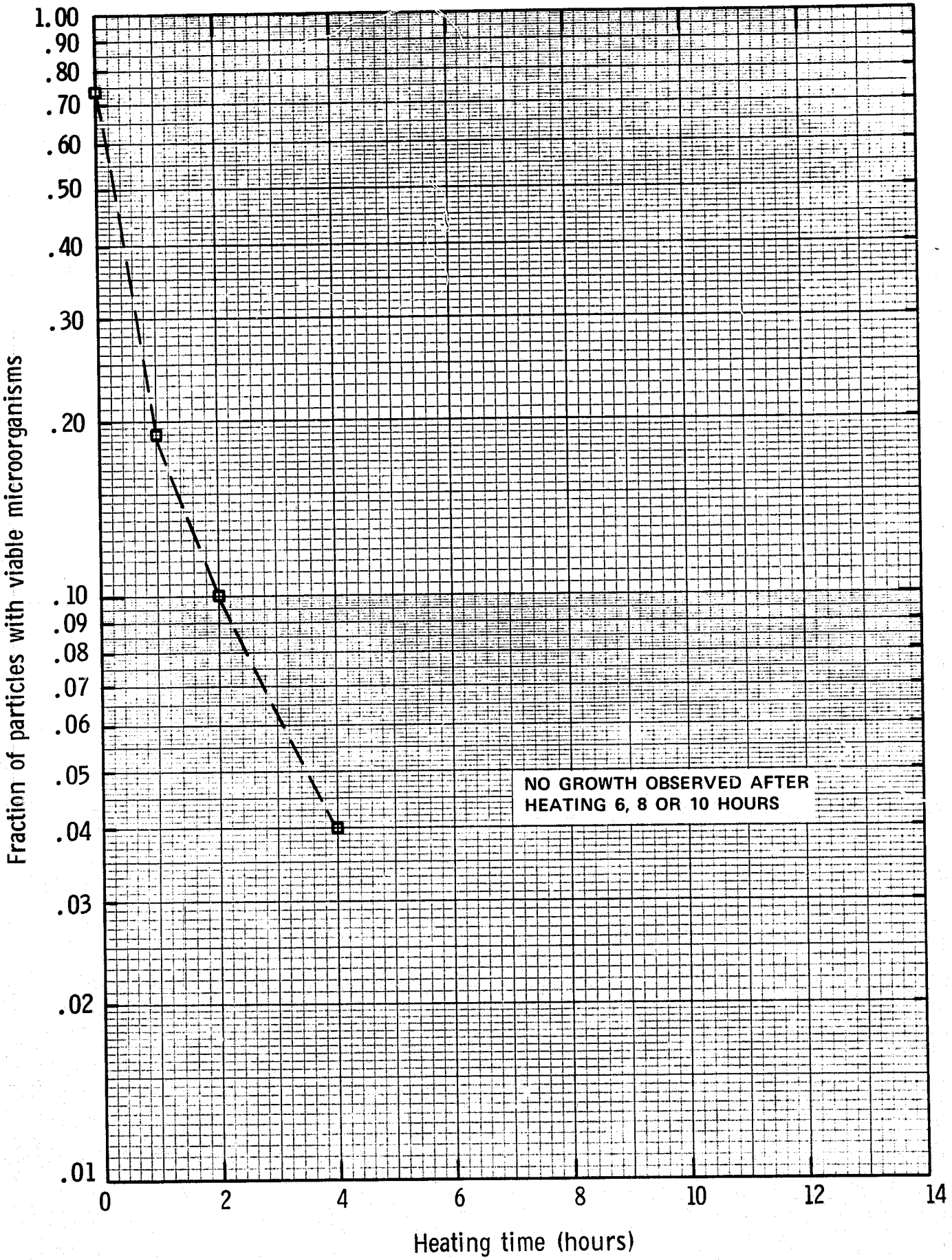

FIG. 5 VIABILITY OF WAJJF (74–88 μm) SINGLE RANDOM SOIL PARTICLES, 110°C DRY HEAT — CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC (Expts. OR4247A–49C)

A viability profile for WAJJF dark particles, together with the random particle profile is shown in the graph of Figure 6. The experimental data from which the profile of the WAJJF dark particle response was drawn are listed in Table VII, page 22. In this experiment, comparison of data from dark and random particle analyses indicates that for all heating time intervals the dark series had a higher proportion of particles that retained viable microflora through eight hours of heating time.

The results from studies of random versus dark particles are inconclusive. Although the data obtained from the WAJJF series suggest that a difference in viability profiles can be expected, this phenomenon was not demonstrated with the WAKMF soil fraction. Whether or not this is due to the inherent variability in particle selection is unknown at this present time.

Viability Profile Reproducibility

During the initial investigations of WAKMF soil particles and the response of associated microorganisms to dry heat treatment, the question of viability profile reproducibility occurred. Therefore, plans were made to run a replicate series of three experiments to provide information on how well the viability profiles for a soil fraction would be reproduced from one experimental series to another. Throughout these experiments, Kennedy Space Center WAKMF single dark particles were used in three similar dry heat treatment series at 110°C. In total this study alone involved the selection, heat treatment and analysis of 1,628 individual soil particles for the combined replicate series experiments.

Results obtained from the first replicate experiment analyses were presented earlier in Table II, p. 9. Data which were obtained from the two additional replicate series determinations with individual WAKMF particles are listed in Table IV and Table V. Figure 7 shows the viability profiles that have been plotted for the initial series of soil particles (Series I) and the replicate experiments (Series II and Series III).

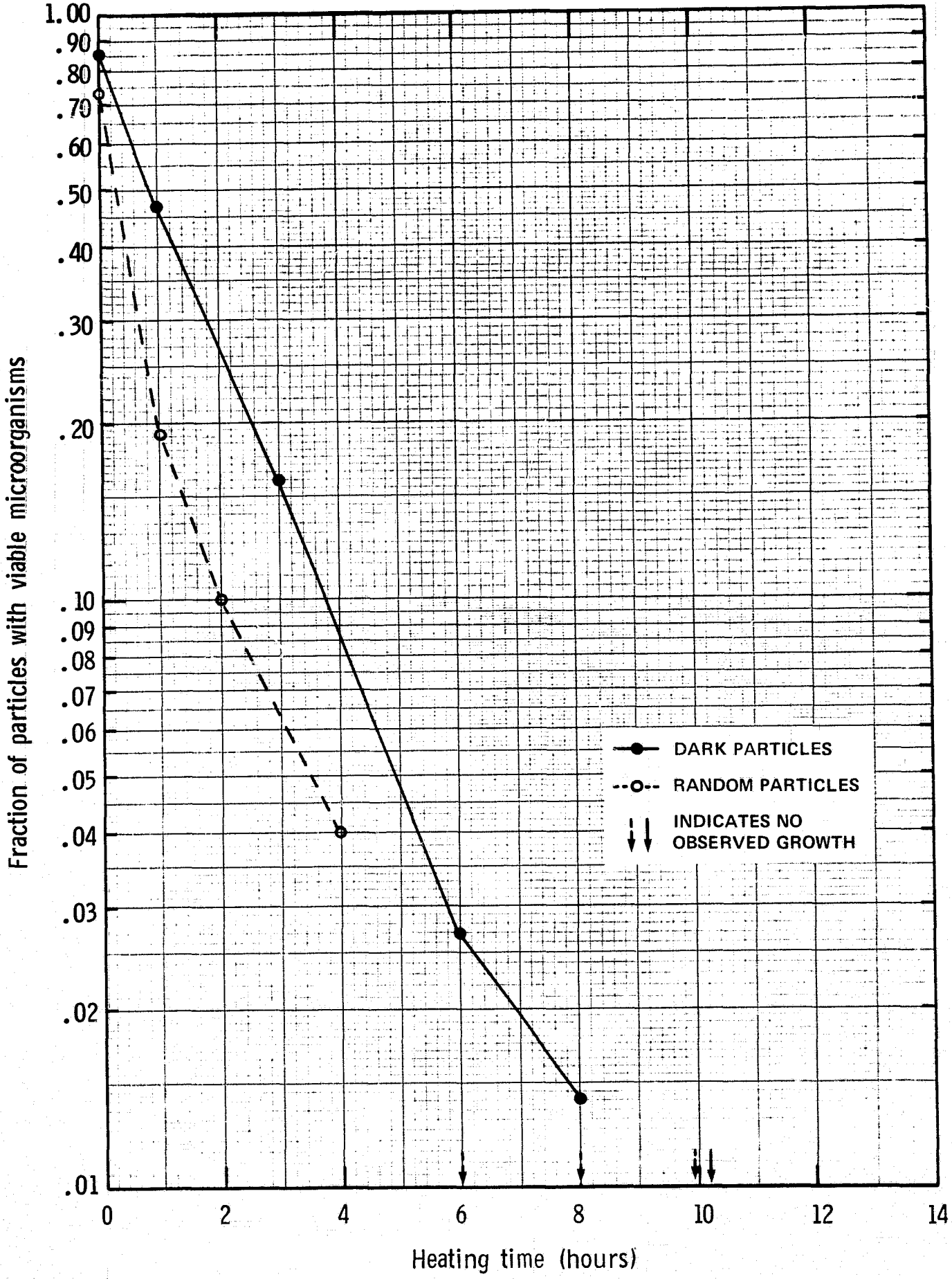

FIG. 6 VIABILITY PROFILES OF WAJJF (74–88 μm) SINGLE RANDOM AND DARK SOIL PARTICLES, 110°C DRY HEAT — CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC (Expts. OR4085–4113; OR4247–4249)

Table IV

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C WAKMF (74-88 μm) Dark Particles. Replicate II Proportion Positive Data

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4059A	0	66/74	0.892
OR4067A	2	21/74	0.284
OR4059B	4	8/74	0.108
OR4067B	6	3/74	0.041
OR4059C, 4071A	8	1/148	0.007

Table V

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C. WAKMF (74-88 μm) Dark Particles. Replicate III Proportion Positive Data

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4255A	0	72/74	0.973
OR4255B	1	33/74	0.446
OR4255C	2	19/74	0.257
OR4256A	4	8/74	0.108
OR4256B	6	3/74	0.040
OR4262A	8	4/74	0.054
OR4262B	10	1/74	0.014

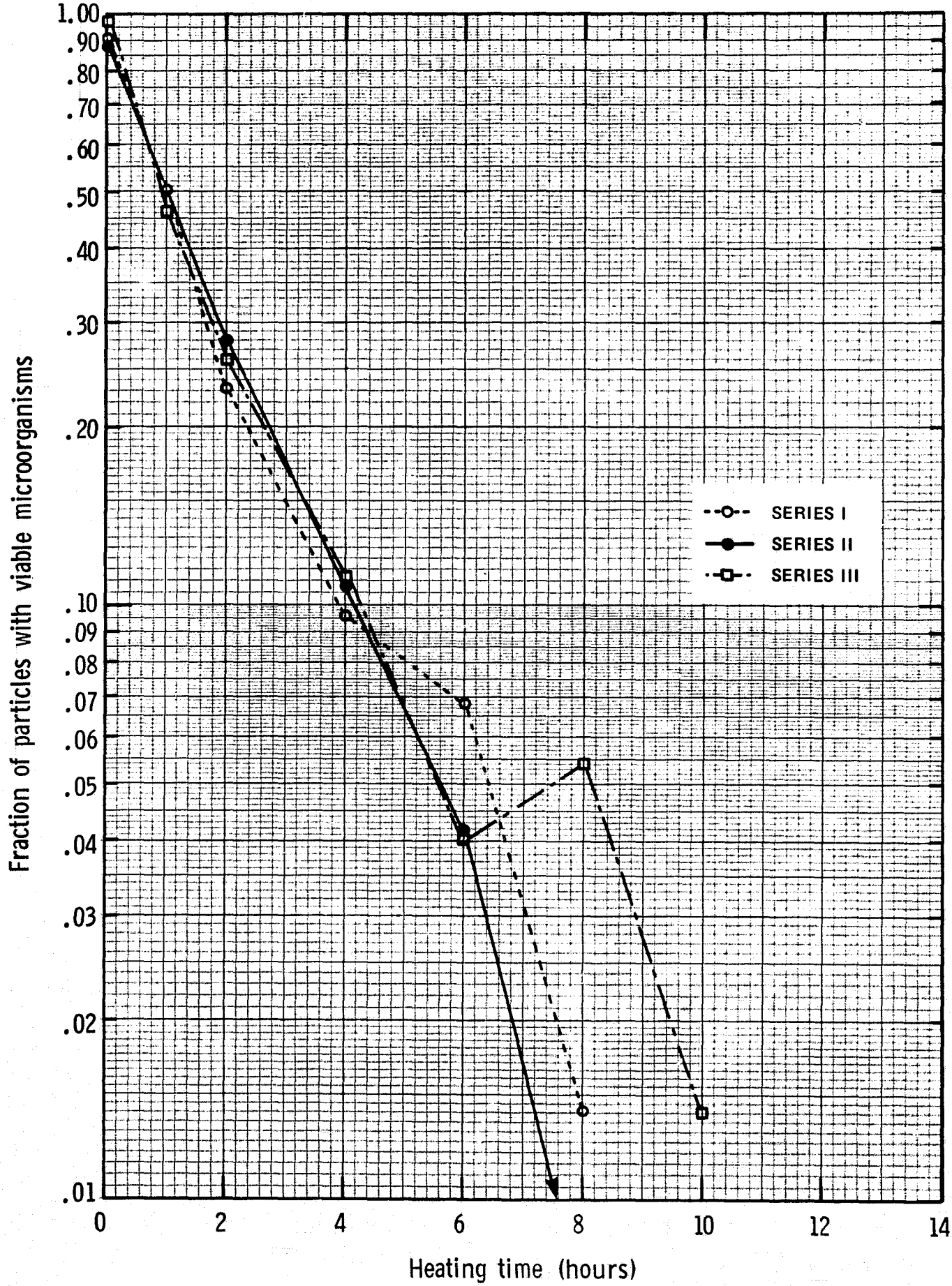

FIG. 7 COMPARISON OF SOIL PARTICLE VIABILITY DATA. REPLICATE EXPERIMENTAL SERIES. DRY HEAT TREATMENT ON CLEAN ROOM HOTPLATE AT 110°C WAKMF (74–88 μm) DARK PARTICLES. PROPORTION POSITIVE. SS CUP–BOAT–TTC

It is especially noteworthy to point out that the data for these replicate studies are in remarkably good agreement. This is particularly interesting because we are dealing with an initial heterogeneous population of soil microflora plus the fact that these experiments include the attendant problems of selection, manipulation and treatment of microscopic size particles. Despite these problems, the replicate experimental series yielded results that are quite similar. These data suggest that with random sampling and meticulous attention to technique, reasonable reproducibility of viability profiles for soil particle microflora from a sample can be obtained. It should be recognized that this experiment was limited to only one soil fraction because laboratory time requirements precluded additional work with other soil particle sizes. This is generally the situation whenever time consuming micromanipulation is required for the particle studies.

Viability Profiles of Stored Soil Particles

Another aspect of the soil particle microbiology problem has been the effect of long term storage on particle viability. For this reason some experimental studies were done to obtain data relevant to the viability of microorganisms associated with soil particles which had been stored in our laboratory for several years. In these experiments, individual WAJJF (74-88 μm) dark soil particles were subjected to heat treatment. These particles had been stored in covered glass jars at ambient laboratory conditions for approximately 2.5 years. Viability profiles at 125°C and 110°C have been determined for these soil particles.

For comparative purposes, a similar profile at 110°C for WAKMF (74-88 μm) dark soil was also determined during the same period. The WAKMF particles had been stored in our laboratories for approximately one year at the time of heat treatment and analysis.

Results from the analyses of WAJJF "old" particles heated at 125°C are listed in Table VI and plotted on the graph of Figure 8. These data show that, despite the 2.5 year storage period, more than 75 per cent of the soil particles still retained viable microorganisms as demonstrated by the response of the unheated series. After 60 minutes of heat treatment at 125°C, most of the microbial population on the particles was rendered

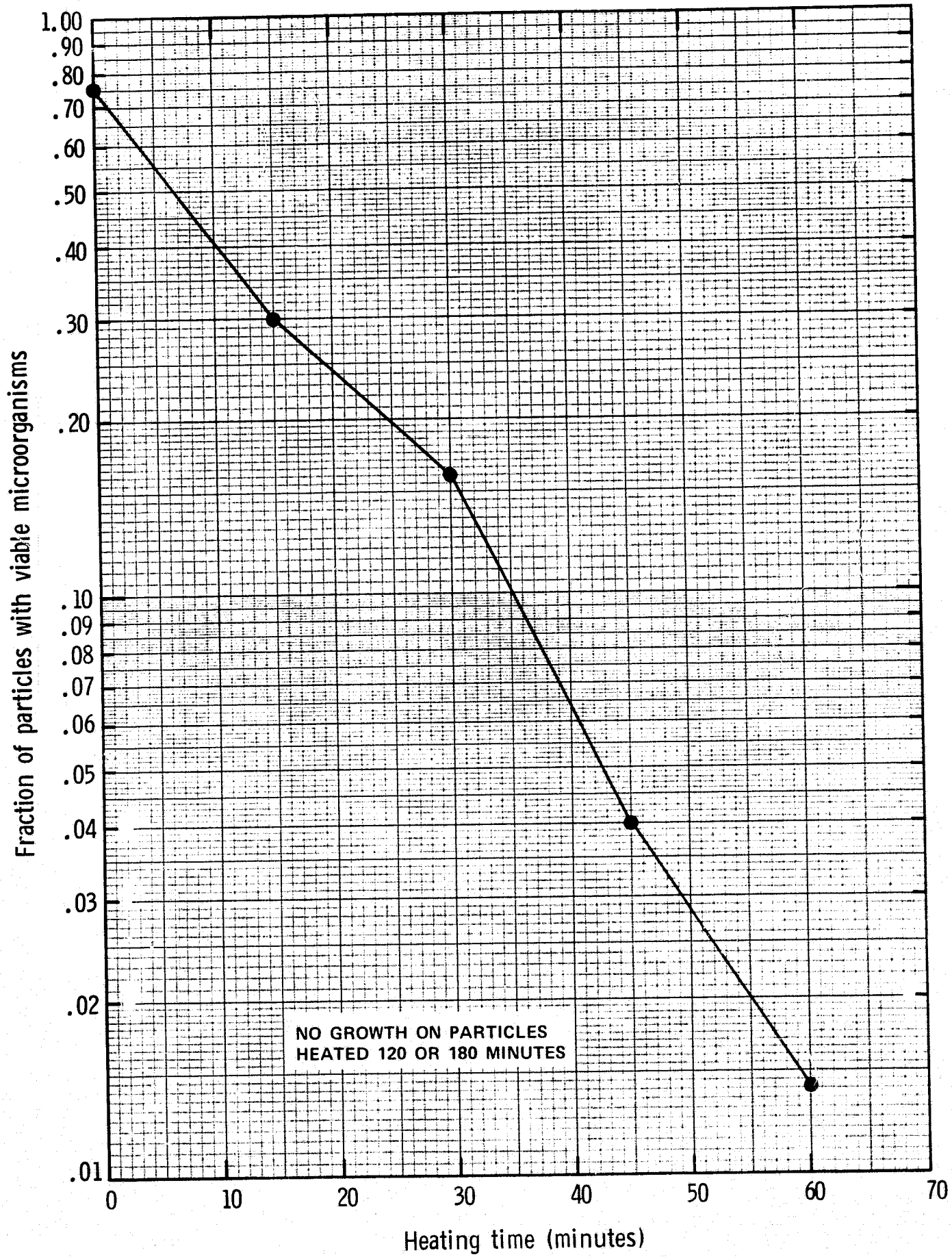

FIG. 8 VIABILITY OF WAJJF (74–88 μm) DARK SOIL PARTICLES, 125°C DRY HEAT — CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC (Expts. OR4177–79)

non-viable. Only 1.4 per cent of the particles heated for one hour showed growth under test culture conditions. None of the particles tested demonstrated viability after 120 minutes heating at 125°C. The viability plot in Figure 8 shows the trend of the particle inactivation curve with time.

Table VI

Soil Particle Viability After Dry Heat Treatment at 125°C on Clean Room Hotplate. Single WAJJF (74-88 μm) Dark Particles Proportion Positive Data*

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4177A	0	56/74	0.757
OR4177B	15	22/74	0.297
OR4177C	30	12/74	0.162
OR4179A	45	3/74	0.040
OR4178B	60	1/74	0.014
OR4178B	120	0/74	0.000
OR4178C	180	0/74	0.000

* Refers to fraction of particles with viable microorganisms

Particle viability data from the 110°C dry heat treatment of stored WAJJF dark particles are presented in Table VII. Analyses of the unheated particles indicated that approximately 85 per cent of the individually tested particles still retained viable microorganisms. Figure 9 shows the particle inactivation profile which was obtained from this experiment with the "older" soil. The time required to inactivate the particles at 110°C was at least eight times longer than the time observed for 125°C. After eight hours of heat treatment at 110°C, slightly over one per cent

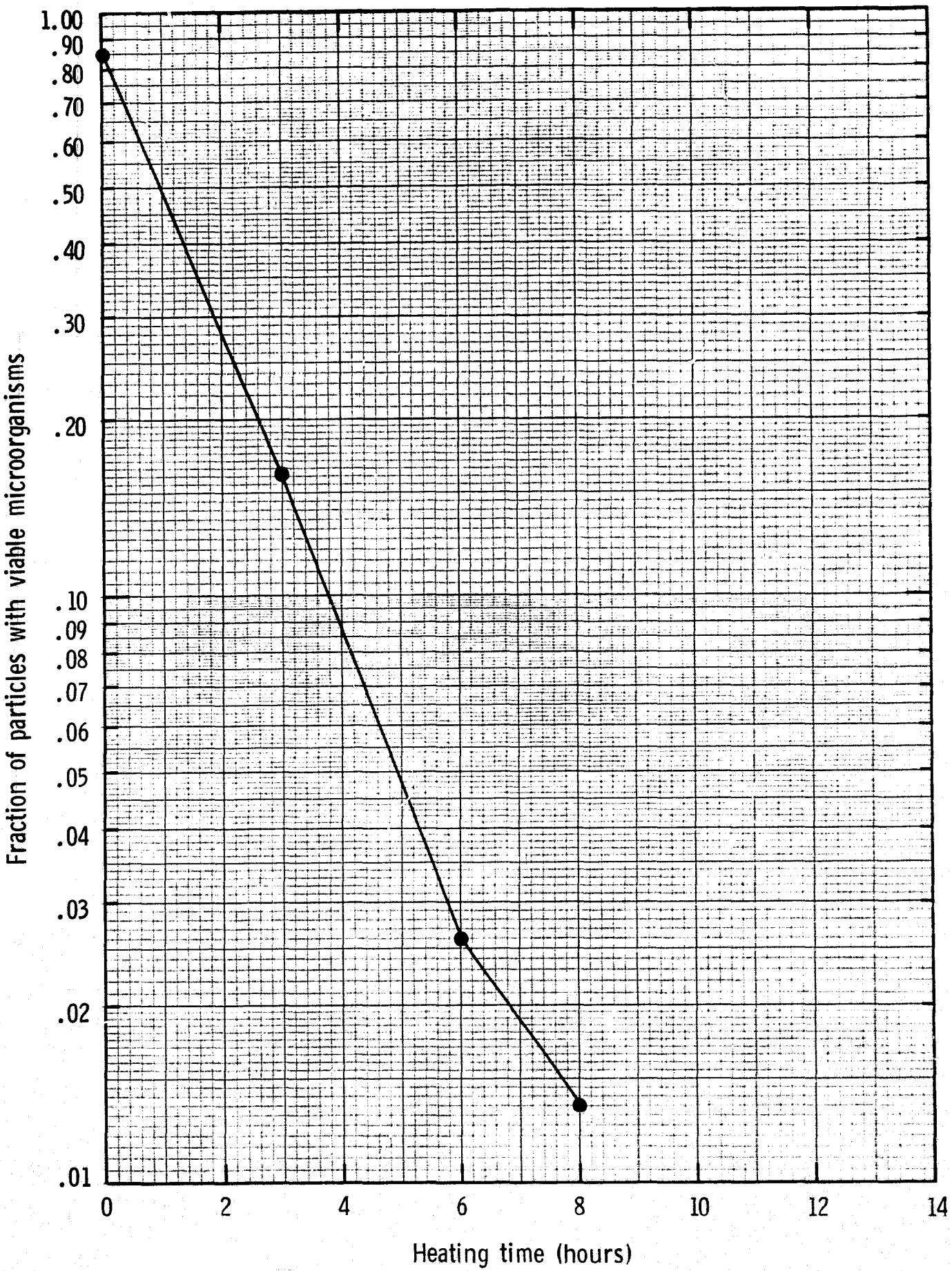

FIG. 9 VIABILITY OF WAJJF (74–88 μm) DARK SOIL PARTICLES, 110°C DRY HEAT — CLEAN ROOM HOTPLATE PROPORTION POSITIVE. SS CUP–BOAT–TTC (Expts. OR4085, 86, 113)

of the treated particles still demonstrated the presence of viable microorganisms.

Table VII
Soil Particle Viability After Dry Heat Treatment at 110°C on Clean Room Hotplate. WAJJF (74-88 μm)
Dark Particles Proportion Positive Data*

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4113A	0	63/74	0.851
OR4086A	1	35/74	0.473
OR4085	3	12/74	0.162
OR4086B	6	2/74	0.027
OR4113B	8	1/74	0.014

* Refers to fraction of particles with viable microorganisms

Figure 10 provides a comparison of the particle inactivation profiles obtained when dark particles of WAJJF (old) and WAKMF (new) soil fractions were treated at 110°C. It is of interest to note that, despite the longer storage time of approximately 2.5 years for WAJJF soil particles, both particle viability profiles appear to be quite comparable. Thus, the results suggest that the dry heat survival response of these soil sample microflora are apparently similar regardless of the longer storage time for WAJJF soil. Furthermore, these data also suggest that there was no marked loss of heat resistant forms from the particles during the storage period. These experiments indicate that aging of the soil, under dry conditions in the laboratory, has not caused any great difference in the viability profile for resistant organisms of Cape soil heated at 110°C. Apparently, under these conditions, the spores may be very old and still retain their viability and heat resistance. This is not an unusual phenomenon with spore forming bacteria;

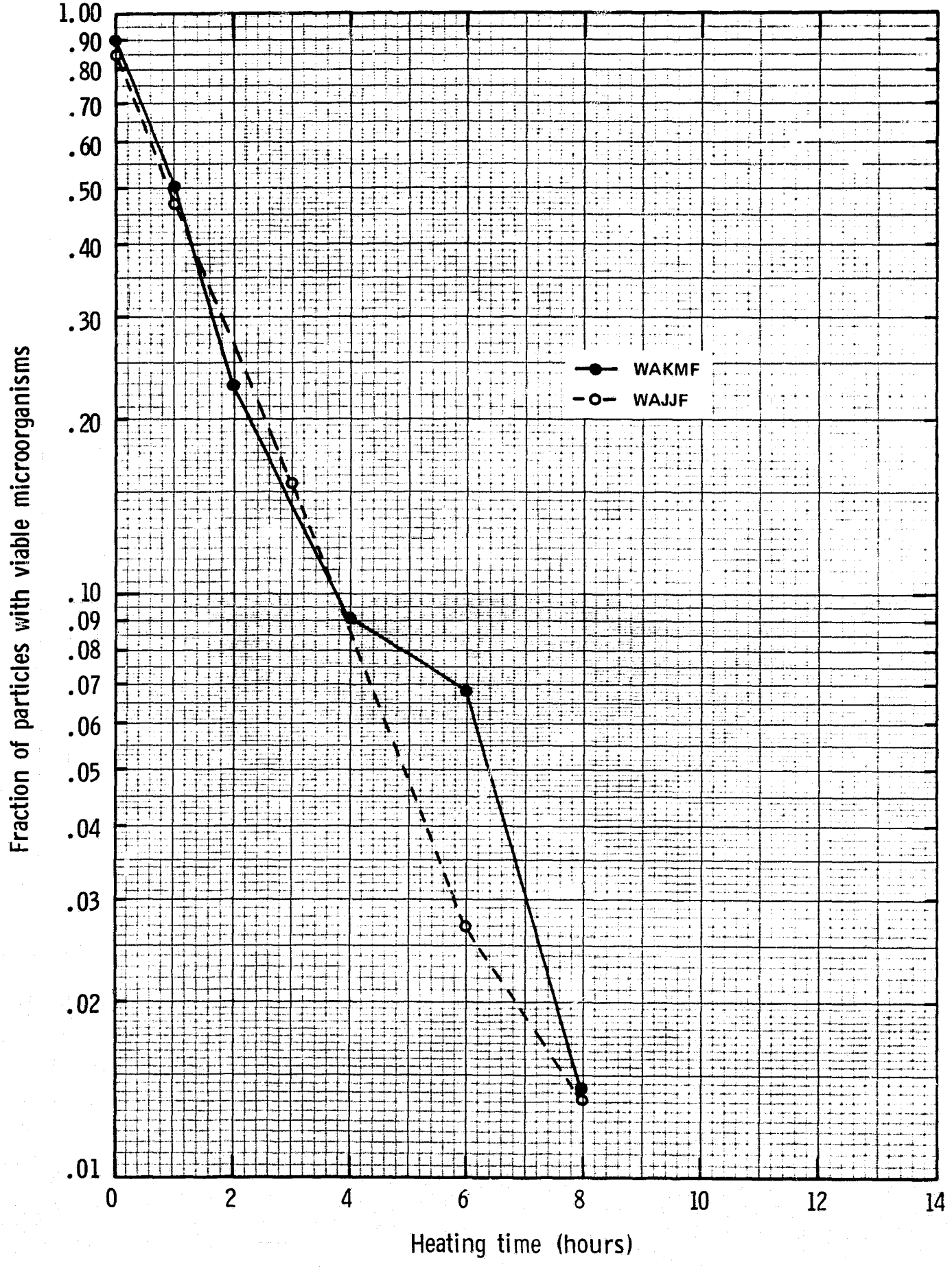

FIG. 10 COMPARISON OF PARTICLE VIABILITY FOR WAKMF (NEW) VS. WAJJF (OLD) SAMPLES OF CAPE KENNEDY SOIL. (74–88 μm) DARK PARTICLES. DRY HEAT 110°C CLEAN ROOM HOTPLATE. SS CUP–BOAT–TTC

since some forms have been found to remain viable for many decades in dried soils. Anthrax spores are a classic example and these forms have been reported to survive for many years in certain soils of Europe and the United States.

Effect of Particle Number on Viability Profile

As the various investigations of soil particle microflora were carried on, an interest was also developed with regard to any effects on particle viability that might result from an increased particle density per unit area. Of particular concern was the question of how an increase in the number of soil particles per TDT cup would influence the configuration of the viability profile graph. An extensive series of experiments was completed in order to obtain the data relevant to the effect of particle load on the soil particle viability profiles.

In these experiments, numerous series of 74-88 μm sized random particles, drawn separately from the "new" WAKMF and "old" WAJJF soil samples, were subjected to dry heat treatment. For each of the soil samples studied, analyses were done to obtain viability profiles for concentrations of one, ten and 25 soil particles per TDT cup.

The randomly selected soil particles were placed in each TDT cup and series of 74 cups were heated at 110°C for each selected time interval. In aggregate, the test groups for both soils were comprised of a total of more than 22,000 individual particles. The particles were generally separated into sequences of six to seven experimental heating time series. This number of heating times furnished a sufficient number of points to allow graphing of the viability response curves and note trends in time required for inactivation.

Results obtained in the series of experiments with WAKMF soil particles are presented in Tables VIII, IX and X. The tabulated data refer to the proportion of cups which retained viable soil particles after each interval of heating time. These data have also been plotted as viability profiles for one, ten and 25 particles per cup. Graphs of these data are shown in Figure II and provide an interesting comparison of viability profiles for the increasing loads of WAKMF soil particles per cup.

Table VIII

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C. WAKMF (74-88 µm) Random Particles--1 Particle/Cup Proportion Positive Data*

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR3296A	0	60/74	0.811
OR3296B	1	29/74	0.392
OR3298A	2	16/74	0.216
OR3311A	3	10/74	0.134
OR3298B	4	6/74	0.081
OR3311B	5	7/74	0.095
OR3311C	7	2/74	0.027
OR3303A	8	2/74	0.027
OR3303B	16	0/74	0.000
OR3305A	24	0/74	0.000

* Refers to fraction of particles with viable microorganisms

Table IX

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C. WAKMF (74-88 µm) Random Particles--10 particles/Cup Proportion Positive Data

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4081A	1	72/74	0.973
OR4066A, 91A	2	91/111	0.820
OR4066B	4	28/74	0.378
OR4081B	6	8/74	0.108
OR4074A	8	2/74	0.027
OR4074B	12	0/74	0.000

Table X

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C. WAKMF (74-88 μm) Random Particles--25 Particles/Cup Proportion Positive Data

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4212A	1	74/74	1.000
OR4212B	2	73/74	0.986
OR4213A	4	64/74	0.865
OR4213B	6	34/74	0.459
OR4218A	8	18/74	0.243
OR4218B	10	2/74	0.027
OR4218C	12	0/74	0.000

Inspection of the graphs in Figure 11 suggests that there was an influence on the particle viability curve as the number of particles per cup was increased. In the series using ten particles per cup, the inactivation of the associated microorganisms showed an initial lag up to about two hours of heating time. Following this period, the rate of particle inactivation apparently increased and, after ten hours of heat treatment, no growth was detected in any of the cups tested.

Results from using single particles of WAKMF soil per cup did not demonstrate any evidence of delayed inactivation of particles. The viability profile for the single particles was of approximately uniform slope and no growth was found in these cups at heating time intervals that were longer than eight hours. Although these single particles generally appeared to undergo more rapid inactivation, a few retained viable organisms for the same length of heating as was observed with the ten particle per cup series.

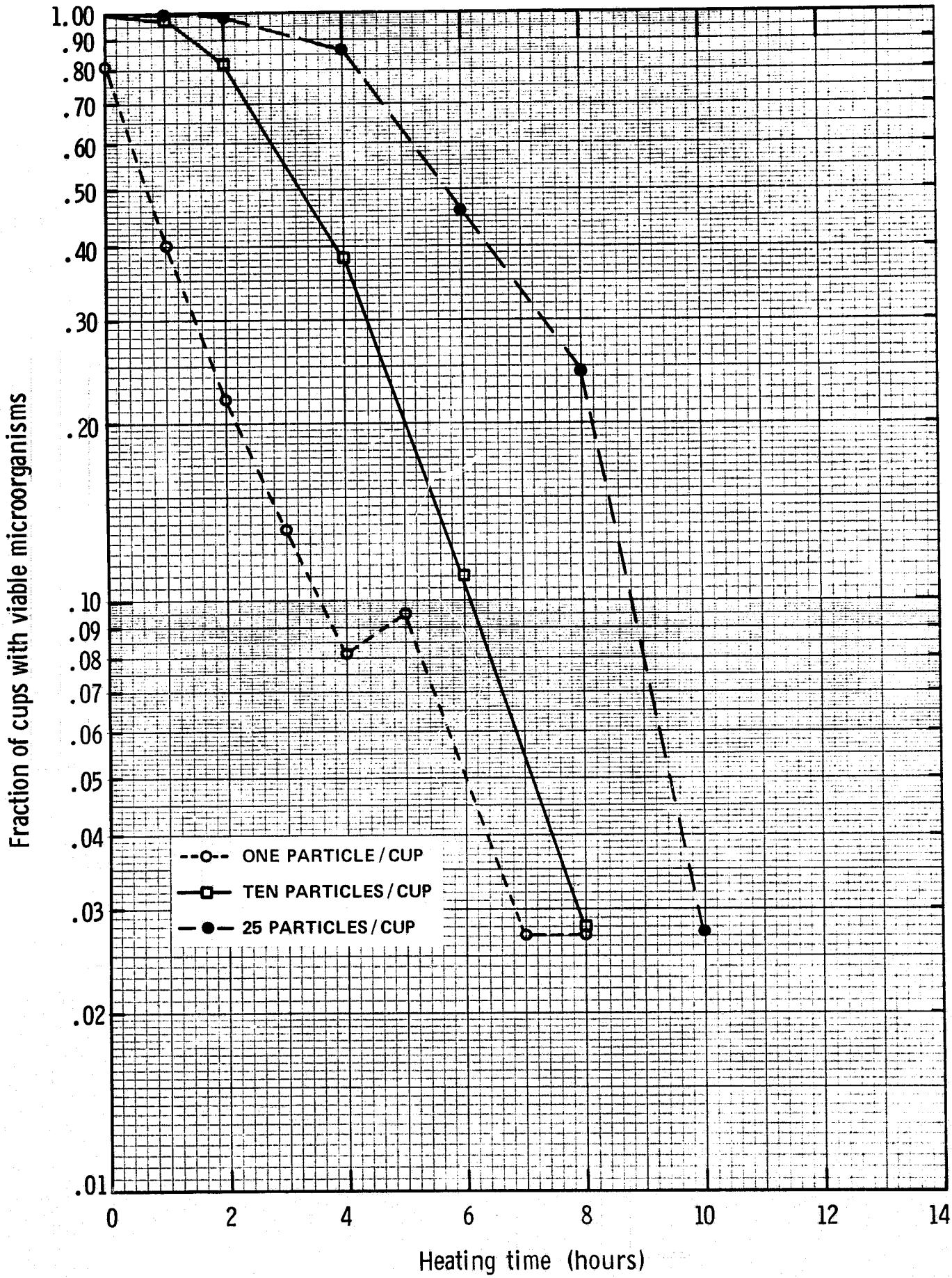

FIG. 11 VIABILITY PROFILES — WAKMF (74–88 μm) RANDOM SOIL PARTICLES. 110°C DRY HEAT CLEAN ROOM HOTPLATES. SS CUP–BOAT–TTC (Expts. OR3296–3311; 4066–91; 4212–18) PROPORTION POSITIVE

Of the three groups of experiments for WAKMF soil particles, the most noticeable effect was shown by the studies with 25 particles per cup. In this case no marked increase in the slope of the viability profile was observed until after four hours heating time. The lag in the inactivation rate was clearly more pronounced than that observed for ten particles per cup. Some particles of this group remained viable through ten hours of heat treatment. However, after 12 hours heating time none of the cups from this series showed any growth response on TSA-TTC media under test conditions.

Similar experimental work with the "old" WAJJF soil particle fraction revealed a somewhat similar trend to that found for the WAKMF soil sample. However, in the case with the WAJJF soil particles, the differences were not as marked and the graphed data was not as clear cut.

The data from the experimental series using ten and 25 random particles per cup of WAJJF soil are shown in Tables XI and XII. Results from the work with one random particle per cup have been listed in Table III, page 12 of this report. The three viability profiles obtained for the different particle loads of WAJJF soil per cup are drawn in the graphs of Figure 12.

Table XI

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C. WAJJF (74-88 µm) Random Particles--10 Particles/Cup Proportion Positive Data

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4191A	1	67/74	0.905
OR4191B	2	55/74	0.743
OR4192A	3	53/74	0.716
OR4192B	4	25/74	0.338
OR4197A	6	3/74	0.040
OR4190A	8	2/74	0.027
OR4180B	10	2/74	0.027

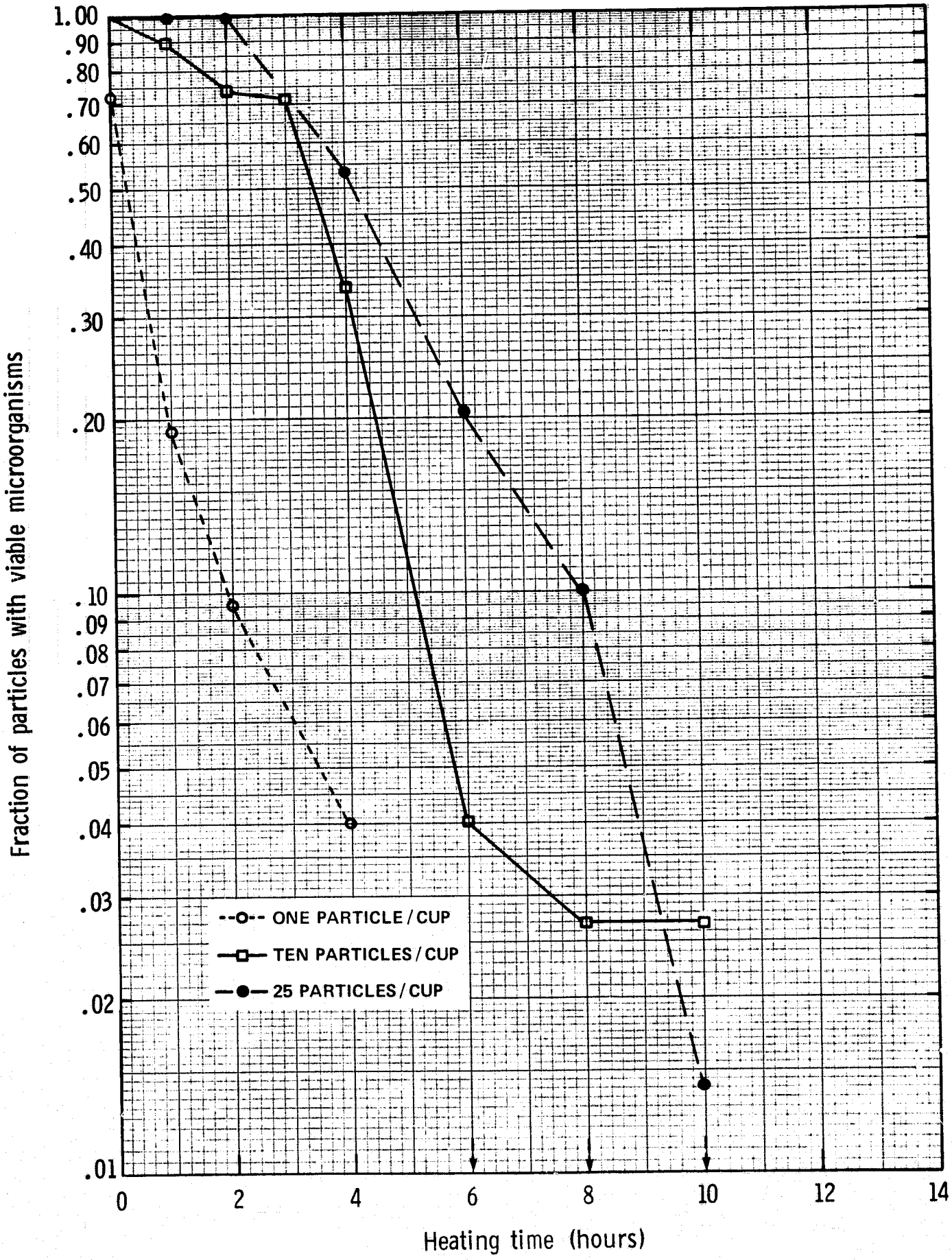

FIG. 12 VIABILITY PROFILES — WAJJF (74–88 μm) RANDOM SOIL PARTICLES. 110°C DRY HEAT CLEAN ROOM HOTPLATE. SS CUP–BOAT–TTC (Expts. OR4190–97; 4234–4241; 4247–49)

Table XII

Soil Particle Viability After Dry Heat Treatment On Clean Room Hotplate at 110°C. WAJJF (74-88 μm) Random Particles--25 Particles/Cup Proportion Positive Data

Experiment Number	Heating Time (Hours)	Proportion Positive	
		Fraction	Decimal
OR4235A	1	74/74	1.000
OR4235B	2	73/74	0.986
OR4234A	4	39/74	0.527
OR4234B	6	15/74	0.203
OR4241A	8	7/74	0.095
OR4241B	10	1/74	0.014
OR4241C	12	0/74	0.000

These data reveal a response which is analogous to that found with the WAKMF particles. The viability profile for single, random particles of WAJJF soil per cup indicated that particle inactivation occurred without any noticable lag period. Data in Table III (page 12) shows that none of the particles produced microbial growth after four hours heating time. Tests run after heating times of six, eight and ten hours, and representing a total of 222 individual particles, showed no evidence of viability.

In contrast to the single particle data, the results with 25 particles of WAJJF soil per cup showed a readily detectable lag in the viability profile plot. Furthermore, some of the particles in these cups retained viable microorganisms through ten hours of heat treatment. Both of these results are similar to those found with WAKMF soil studies.

The ten particle per cup profile for WAJJF soil showed a somewhat intermediate response but was not as clearly defined as those for one particle or 25 particles. Some of the microflora associated with particles in these cups were also found to survive ten hours heating time.

Data from the two experimental series with WAKMF and WAJJF soil fractions indicate that as the particle load per cup is increased, a lag in the inactivation rate occurs in the early heating periods. Furthermore, as the particle load per cup was increased from one to 25 particles, the time required to achieve inactivation of all cups was increased. These data suggest that increasing the particle load per cup beyond the number currently tested may extend the lag effect and prolong the time for inactivation.

Results from the experiments with WAKMF soil particles were also utilized to develop a composite microbial destruction curve from the data shown in Tables VII, IX and X. It was necessary to make several assumptions in order to carry out this additional analysis step. For purposes of this calculation, it was assumed that there were initially five viable spores per particle of unheated soil and that, following heat treatment, each positive cup represented one surviving spore. It was now possible to calculate an initial number and a surviving number of spores, and, therefore, the survival ratio, N/N_o, based on these assumptions. The results in terms of N/N_o as a function of heating time at 110°C are shown in Figure 13.

The determinations of viability profiles for soil particles subjected to dry heat has depended extensively on microscopy for particle selection, transfer and microbial growth detection. Microscopy was an essential part of these studies because of the small particle sizes involved (44 to 125 μm). The fact that micromanipulation techniques are required has to some extent limited the number of experiments that could be completed in this reporting period. However, it is noteworthy that viability data on microbial survival presented in this report has required manipulation of large numbers of particles. The data presented here are based on results obtained from the processing and analysis of more than 40,000 individual Kennedy Space Center soil particles of microscopic size. These analyses have provided an extensive series of viability profiles for particle types at selected temperatures, including comparison of stored soil samples, effect of the size and number of particles on the time required for heat inactivation of the microflora, reproducibility of viability profiles, etc.

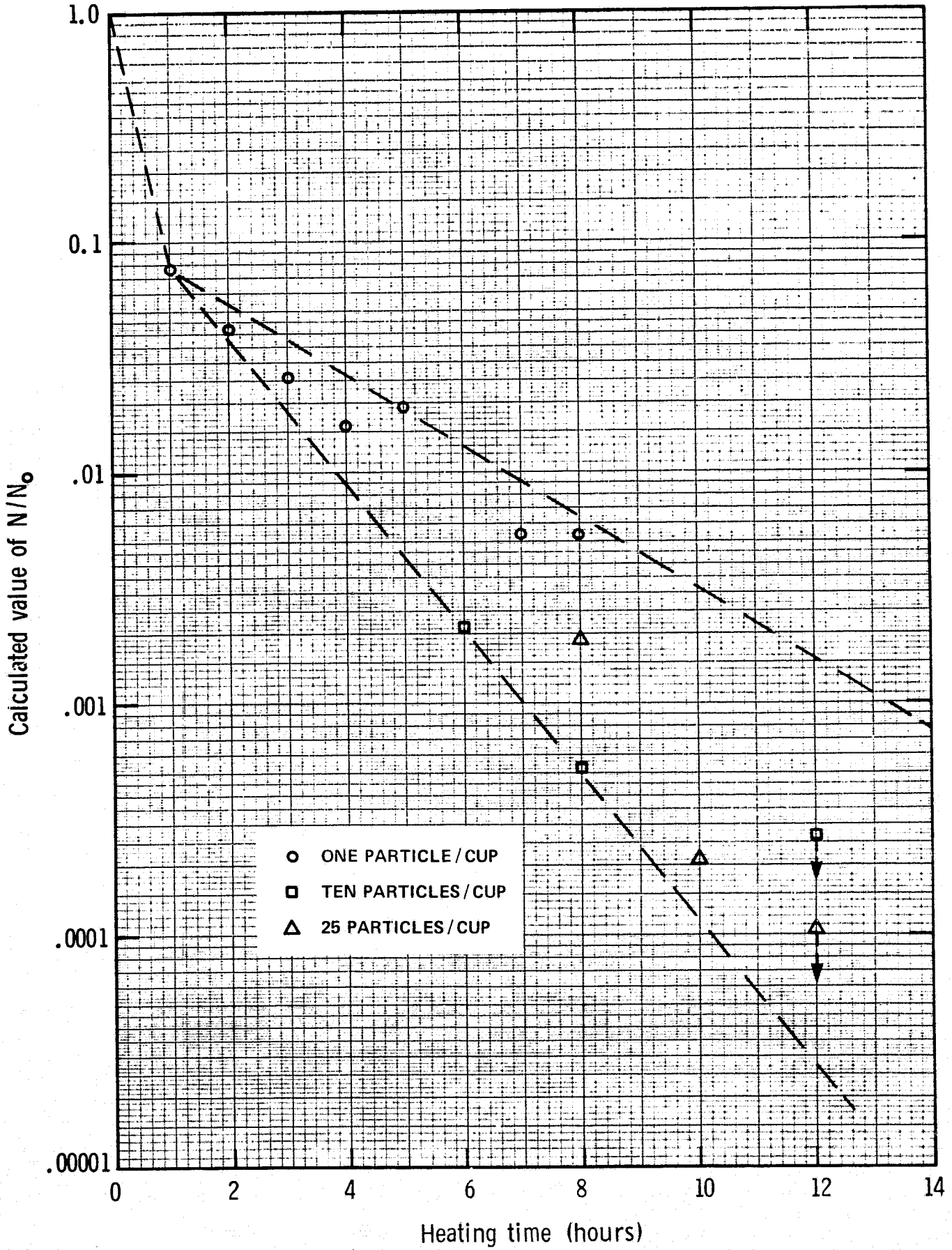

FIG. 13 CALCULATED RATIOS FOR N/N_0 ASSUMING 5 SPORES PER PARTICLE AND THAT A POSITIVE CUP MEANS ONE SPORE SURVIVED. KENNEDY SPACE CENTER SOIL PARTICLES WAKMF (74–88 μm) — DATA FROM ANALYSES OF 1, 10 OR 25 PARTICLES/CUP

CONCLUSIONS

The laboratory studies completed recently with Kennedy Space Center soil particles have provided additional data regarding the response of *in situ* particle microflora to heat treatment. Results from these microbiological analyses of soil particles support the following conclusions:

1. Data from soil particle viability analyses suggest that as the particle size increases, a viable microflora is retained for a longer time under dry heat treatment conditions.

2. Viability profiles for random *versus* dark WAKMF (74-88 μm) soil particles were similar. No microbial growth was observed from these particles (74 particles per test group) after dry heat treatment at 110°C for ten hours. Analyses of the WAJJF series of random *versus* dark particles yielded viability profiles indicating that the dark particles manifested a more prolonged particle inactivation time than the random particles. Whether or not the observed difference in response is due to variability in particle selection, soil type or some other factor is not known.

3. Replicate experiments with three separate series of WAKMF (74-88 μm) dark particles treated at 110°C showed good reproducibility of the viability profile for these soil particles.

4. Analyses of unheated WAJJF soil fractions showed that at least 75 per cent of the 128 individual dark particles tested still retained viable microorganisms after several years storage in the laboratory. After 120 minutes of heat treatment at 125°C none of the 128 particles tested showed evidence of microbial growth. At 110°C approximately one per cent of particles tested still retained viable microorganisms after eight hours heat treatment.

5. Soil particles which had been stored in the laboratory for 2.5 years and tested at 110°C were found to produce a viability profile similar to the profile obtained with more recently acquired soil particles in the 74-88 μm size fraction.

6. Experimental data from studies using one, ten and 25 particles per cup suggest that the particle load may influence the configuration of the particle viability profile for dry heat treatments. With an increase in particle load per unit area, a lag in the particle inactivation was observed and the time required for inactivation was extended.

FUTURE WORK

1. Further investigation concerned with the effect of particle load on the viability profiles for soil particles.

2. Analyses of particles to obtain viability profiles for anaerobic, mesophilic microflora.

3. Experiments to obtain viability profiles for soil particles from the 44-53 μm and 105-125 μm size fractions treated at 110°C

4. Exploration of statistical procedures for treatment of the viability profile data.

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FACTORS AFFECTING THE DRY HEAT DESTRUCTION OF MICROORGANISMS

I. J. Pflug

Dry heat has been used throughout the world for sterilizing glassware and hospital supplies that could not be sterilized in saturated steam. It is amazing that with this wide usage of dry heat so little is known about its overall effectiveness. Bruch (1966) notes that the United States Pharmacopeia specifies that containers for pharmaceutical products be held at 170°C for two hours for dry heat sterilization; in contrast, the British Pharmacopeia states that 150°C for one hour is satisfactory for the vessels and containers to be used in parenteral injections. Lastly, the State Pharmacopeia of the Union of Soviet Socialist Republics indicates that heating in a drying cabinet for one hour at 160 to 170°C is adequate for containers to hold various pharmaceutical preparations. These variations in dry heat recommendations undoubtedly reflect the fact that dry heat is not so easily defined as wet heat. Wet heat sterilization is a relatively easily defined condition in that the relative humidity (RH) is 1.00, or 100% depending on whether we are expressing RH as a ratio or percentage.

If the relative humidity is 100%, then some water is present in the liquid state. Dry heat is the sterilization condition where water is not present in the liquid state. Therefore, in dry heat sterilization the relative humidity of the system will be less than 100%; it can be any value between 0 and 100%. Since the destruction rate of dry microbial cells is a function of their water content, which is in turn determined by the RH of the atmosphere surrounding the cells, the destruction rate will vary with the relative humidity of the system. Therefore, in dry heat sterilization the relative humidity conditions must be specified in addition to temperature to establish the relative sterilization effect.

Relative humidity is used because the response of biological materials more nearly parallels vapor pressure than water content, and vapor pressure of the gas atmosphere surrounding dry microbial cells is more easily measured and controlled than the water content inside the microbial cell.

The term relative humidity in gaseous systems corresponds to water activity in liquid systems. The water vapor pressure in the gas surrounding the spore can be measured by psychrometric methods. It can be reported in several ways, but relative humidity is the most widely used format.

At equilibrium, the relative humidity of the atmosphere surrounding the microbial cell is theoretically equal to the water availability inside the cell, which is called water activity (A_w).

The concept of water activity of solutions adopted by Scott (1957) has been extended in some cases to microbial cells and spores. Since it is possible to confuse terms, the following are suggested to clarify the description of water in dry microbial systems: Relative humidity is a real, physical unit that is the ratio of two measured quantities; the actual water vapor pressure in a system and the saturated water vapor pressure at the same temperature. It is used with gaseous systems, for example, to describe the water condition in the atmosphere surrounding bacterial cells or spores. Water activity, A_w, is a term used to describe the relative water availability inside a microbial cell or spore. It is a theoretical term that cannot be measured directly. If the cell or spore is in equilibrium with the surrounding atmosphere, theoretically the water activity of the spore is equal to the external relative humidity.

In discussing and reporting on dry heat test data, it is suggested that: if in a microbial destruction rate test the relative humidity is measured and controlled, then the results should be reported as a function of relative humidity, not water activity. Whenever a relative humidity value is reported, the temperature at which the relative humidity was measured should be included; for example, 0.2% RH(110°C).

Dry Heat Destruction of the Natural Microorganisms Associated With Soil or Soil Particles

When an object is in contract with people and ambient air, it will accumulate a microflora that can, in general, be divided into two groups: 1) naked or unprotected microbial cells from human or other animal origin; individual or clumps of naked cells that are a residue from food or other organic media that remain on the objects to be sterilized, and 2) spores associated with particles of dirt or dust that fall upon the object to be

sterilized either from the air or from the activity of persons or apparatus working near the objects to be sterilized. These spores will have originated in the soil and will have been transported to the objects by air or human carrier. In general, the naked microbial cells are easily killed by a dry heat sterilization treatment. However, some of the microflora associated with the soil particles are very difficult to kill.

It often seems that we know the least about those things close by. It has just been in recent years that we have been learning about the microflora associated with soil particles. The decision by the National Aeronautics and Space Administration (NASA) to use dry heat for the terminal sterilization cycle of the Viking Lander stimulated a large research effort regarding the dry heat resistance of microbial spores. The results of the findings to date regarding the dry heat destruction characteristics of the microflora associated with soil particles suggest that: 1) The normal microflora of soil is widely variable; the semi-logarithmic survivor curve for microorganisms in soil is non-linear. A typical survivor curve for microorganisms in soil is shown in Figure 14.

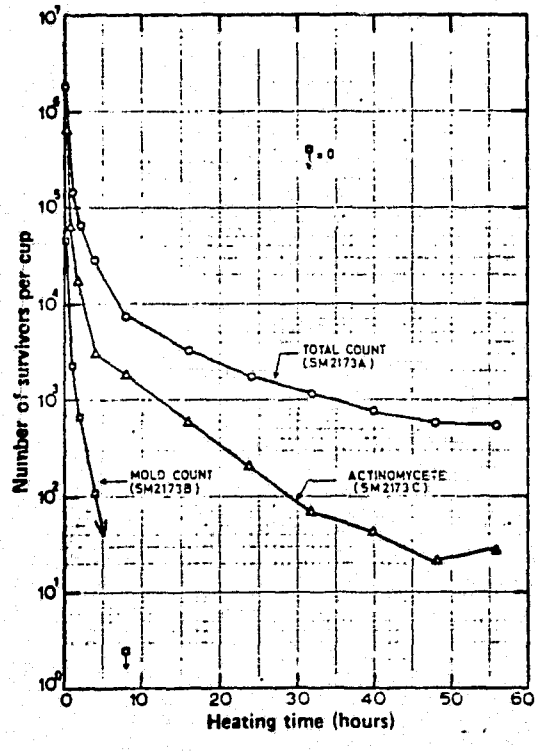

Fig. 14: Survival characteristics of bacterial, mold and actinomycete spores associated with dry Minnesota soil, heated at 125°C, 0.1 g soil per TDT cup.

2) There are some very dry heat resistant soil organisms. One such organism, Bacillus xerothermo durans was isolated by Bond et al. (1973) and found to have a $D_{125°C}$ value of 139 hours. 3) That moderately resistant organisms such as Bacillus subtilis can be very dry heat resistant when encapsulated in particles. 4) The best estimate today is that in soil, one spore in between 10^3 and 10^5 is very dry heat resistant. 5) That the fallout dirt or dust in a facility are part of the area dirt that has become airborne and is either carried into the plant by air or on clothing or equipment that moves into the facility. 6) The role of the ambient relative humidity on the rate of destruction of spores in soil heated in open systems has been studied but the results are not conclusive. It is possible that the bound water in the soil particles over-shadows the effect of the ambient humidity level on the spore destruction rate.

The normal microflora of soil is widely variable and includes both vegetative cells and spores. The survivor curve in Figure 14 at 125°C is for 0.1 gram samples of Minnesota soil. As is evident in Figure 14, this Minnesota soil contains a large fraction of very dry heat resistant spores. Not only are there large numbers of dry heat resistant bacterial spores in this soil but there are also large numbers of relatively dry heat resistant actinomycetes.

Koesterer (1965a, b), reports on studies of the survival of organisms in 0.1 gram of a sample of dry soil. Survivor curves for temperatures in the range of 120 to 160°C are shown in Figure 15.

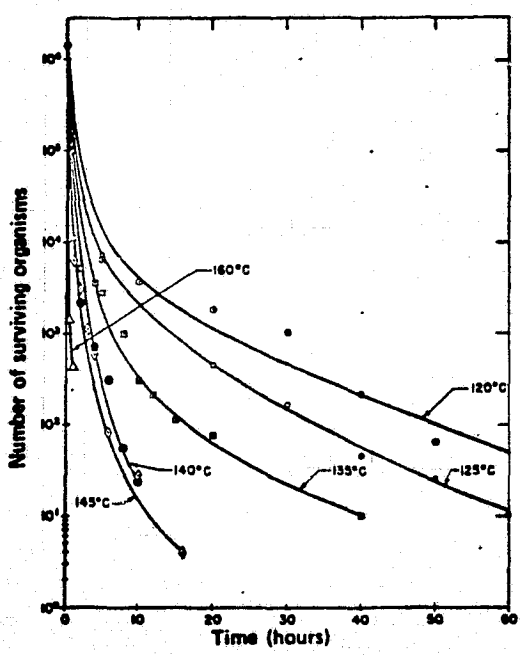

Fig. 15: Survivor curves for the indigenous mesophilic population in 0.1 g samples of dry FG soil to dry heat in the temperature range 120°C to 160°C (Koesterer, 1965a)

There is surprising similarity between the 125°C dry heat survivor curves for Koesterer FG soil from upstate New York and the Minnesota soil, even though the Minnesota soil has a larger resistant population.

At the present time there is no survivor curve model for microorganisms in soil. Therefore the D-value cannot be used because it is a parameter of the straight line semi-logarithmic survivor curve. The semi-logarithmic survivor curve for microorganisms in soil has been described as being biphasic; in general, soil contains a large low resistant population and a small high resistant population of microorganisms. However, neither the low nor high resistance population is homogeneous.

Soil contains some very dry heat resistant organisms. If the surviving organisms of dry heat tests are cultured in the laboratory, spore crops with high dry heat resistance can be produced. Koesterer (1965), Bond et al. (1970), and Campbell (1974) have all cultured resistant organisms from soil. The resistance of these "hardy organisms" is variable; D(125°C) values range from 5 to 139 hours.

When some of the surviving organisms of dry heat tests are cultured in the laboratory, the resulting spore crops have resistance levels of the order of Bacillus subtilis var. niger. Both Doyle and Ernst (1967) and Mullican and Hoffman (1968), have shown that when spores are encapsulated in crystals the dry heat resistance can be increased more than ten-fold. In many soil particles there are crystals with large waters of hydration that may cause organisms that are normally of low dry heat resistance to become super-resistant spores.

In soil the possible variations in numbers and species of microflora and the physical conditions of their location are almost unlimited; the spores can be of a range of species all produced under widely varying unknown conditions and the soil particles can vary in size and composition. The spores can be located at any point on or within the soil particles. It seems probable that due to soil wetting and drying cycles spores can be completely encapsulated in soil particles which in itself could increase dry heat resistance by a factor of ten.

Studies carried out by Puleo and co-workers (1974) suggest that the relative number of resistant spores is small. We estimate that one spore in between 10^3 and 10^5 will survive a dry heat treatment of about 30 hours at 100°C.

When objects are manufactured under ambient air conditions, with adequate opportunity for soil particles to be deposited on the units to be sterilized, then the sterilization cycle will have to be based on the resistance of the spores in or on the soil particles. If manufacturing is carried out under closely controlled conditions such as in a Class-100 clean room, then it is possible that the number of soil particles per object will be reduced to the point where they are not the critical design criteria. The naked microorganisms from the people working with the objects or grown in the product will become the critical microbial load and the sterilization cycle will be based on the resistance of these naked organisms rather than organisms associated with soil particles. Weather can have a major effect on dry-heat sterilization requirements. Humid conditions reduce and dry conditions increase soil particle movement. High wind velocities and dry soil and air conditions will increase the number and size of soil particles that move into production areas. This will increase the number of soil particles and accompanying microbial load on objects to be sterilized.

Dry Heat Destruction of Laboratory Grown Spores

During the last decade, because of the potential use of dry heat for sterlizing space hardware, NASA has supported a great deal of research in the dry heat area. A large part of this work involved model systems. Bacillus subtilis var. niger spores were used extensively. The results of these studies led to certain general conclusions regarding the dry heat destruction of homogeneous cultures of spores: 1) the semi-logarithmic survivor curves are, in general, straight lines if we exclude the N_o point from the analysis. 2) The destruction rate of the spores starting at time zero is greater than the steady state destruction rate and increases with a decrease in spore water content.

In discussing the mode of action of dry heat there are three primary variables and three secondary variables. The primary variables are: temperature, water content and time; the secondary variables are open and closed systems, physical and chemical properties of the microorganism and adjacent support, and the gas atmosphere. The secondary variables all play an important role in determining the microbial water content during heating and will be discussed as part of that section.

Effect of Temperature

Temperature, which is the measure of the heat energy level is the most important variable in the dry heat destruction of microorganisms; its action is a function of time. Thermal destruction curves for Bacillus subtilis var. niger spores in five different systems (Data of Angelotti et al., 1968a) are shown in Figure 16.

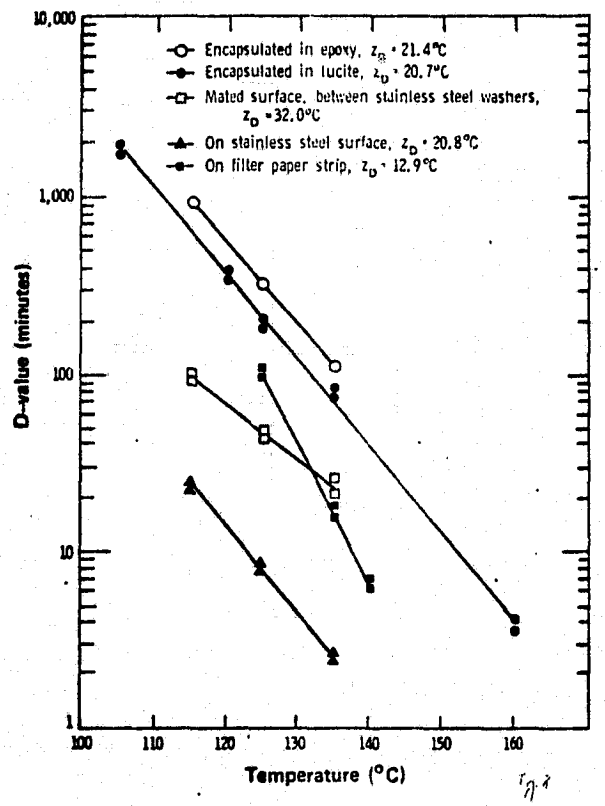

Fig. 16: Thermal destruction curves for Bacillus subtilis var. niger spores (Angelotti et al., 1968a)

Dry heat resistance values for several species of microorganisms are shown in Table XIII.

Table XIII

Dry Heat Resistance Values

Organism	D_{300} min.	z°F	Testing conditions	Source
B. subtilis (5230 or 15U)	5.2 - 7.3	33	hermetic cup-TDT can system five gasses, He, N_2, O_2, CO_2 and air	Pheil et al. (1963)
Cl. sporogenes (PA 3679)	6.0 - 8.8	39-41	hermetic cup-TDT can system three gasses, He, CO_2 and air	
B. subtilis (niger)	4.8	49	filter paper strips in 150 x 16 mm screw cap tubes in aluminum block	Bruch et al. (1963)
B. stearothermophilus (FS 1518)	1.2	44		
B. subtilis (niger)	8.7 - 21	42	added to or trapped in solids	Bruch et al. (1963)
B. subtilis (5230 or 15U)	7.0	42	superheated steam in thermo-resistometer (300 to 350°F)	Pflug (1960)

The z-value of the Bigelow (1921) model is one measure of the change in destruction rate with temperature. Reported z-values for dry heat destruction of microbial spores range from about 15 to 30°C (27 to 54°F) with Q_{10} values of 4.6 to 2.2. In the temperature range of 105 to 135°C, the average z-value is about 21°C, Q_{10} is 3.0. A z-value of 21°C has been adopted by NASA (1969). The z-values for microbial spores subjected to wet heat is in the range of 8 to 10°C with Q_{10} values of 17.8 to 10. It is this major difference in z or Q_{10} value that is the basis for the assumption that the mechanism of dry heat destruction is different from the mechanism of wet heat destruction.

Effect of Microbial Water Content

The role of water in the heat inactivation of intracellular molecules has long been a subject for speculation among microbiologists. Recently, evidence has been provided that water has a direct influence on microbial resistance to destruction by dry heat.

The effect of water on the dry heat destruction rate of spores was first reported by Murrell and Scott (1957). This initial report has been supported by additional studies by Murrell and Scott (1966) and Angelotti et al. (1968a). In addition to these studies dealing directly with water and dry heat destruction, there have been a large number of studies in recent years reporting dry heat destruction rates for specific conditions.

Pflug (1960); Jacobs et al. (1965); Pheil et al. (1967); Paik et al. (1967); Green et al. (1967); Fox and Pflug (1968); Silverman (1968); Hoffman et al. (1968); Bruch and Smith (1968); and Bond et al. (1970).

At this time it appears that the dry heat destruction rate of microbial spores is a function of the quantity of water in the cell at the time of heating. The quantity of water in the cell during heating will be constant only under certain conditions. In the great majority of conditions, the moisture content of the cell can change so that initial cell water content, the physical and chemical properties of the cell and adjacent support, and the water vapor pressure of the surrounding gas atmosphere may act as variables and cause confusion in the analysis of reseach results.

The movement of water to or from microorganisms on surfaces will be determined by the water vapor pressure in the atmosphere surrounding the cell (Pflug, 1960). By increasing the humidity in air passing over microbial spores from near zero to 0.20, Silverman (1968) was able to consistently increase the D-value by a factor of a hundred and his data suggest that the maximum was not reached.

In the temperature range of 100 to 135°C, spores of an intermediate moisture content (equilibrated at relative humidities between 0.1 and 0.6) are more resistant to the effects of heat (larger D-values) than spores of either greater or lesser moisture content. According to Marshall et al.

(1963) who measured the moisture content of spores of six bacterial species equilibrated to various water levels at 25°C, the critical moisture content is between 5.5 and 12.4 percent of the dry weight of the spores depending on the species. (See Table XIV.)

Table XIV

Moisture Content of Six Bacterial Species of Spores Equilibrated to Various Relative Humidities (Data from Marshall, et. al., 1963)

Relative Humidity	Range of Water Content (Per cent dry weight)
0.1	4.8 - 8.2
0.2	5.5 - 10.2
0.4	7.3 - 12.4
0.6	9.5 - 16.0
0.8	12.1 - 25.5
0.9	38.5 - 57.0

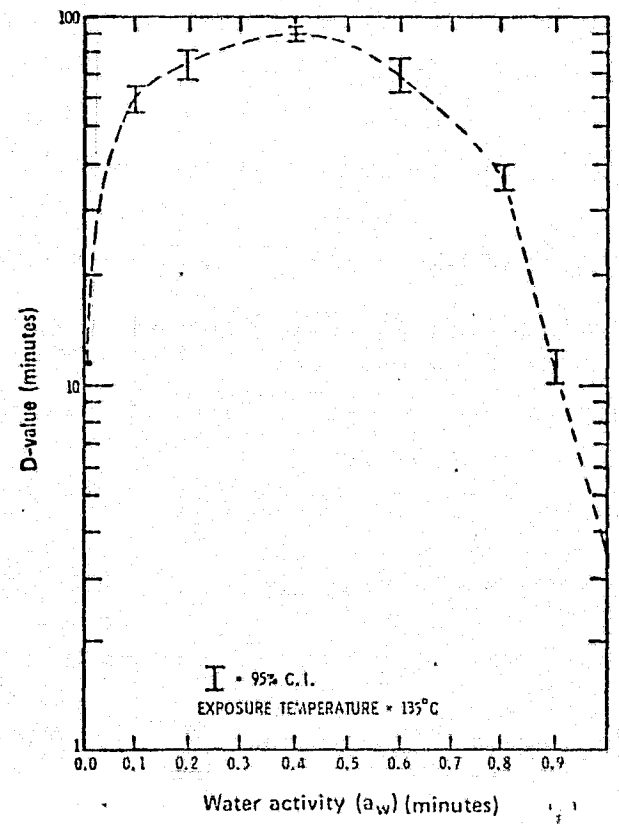

Fig. 17: Influence of water activity on dry-heat resistance of Bacillus subtilis var. niger spores encapsulated in Lucite (Angelotti et al., 1968a).

In Figure 17 is shown a curve of D-value versus spore water content; the details from Angelotti et al. (1968a). The zero value is for an open system; it is added here along with the dotted line to indicate the probable D-value when spore water content approaches zero.

Brannen and Garst (1972) determined D-values at 105°C for Relative Humidities from 3×10^{-4} to 2×10^{-3}. They found that when log D was plotted vs log RH between an RH of 1×10^{-3} ans 1×10^{-2}, the data points formed a straight line. Jacobson and Pflug (1972) studied the dry heat destruction rates of Bacillus subtilis var niger spores at 90, 110 and 125°C at atmospheric water contents from about 5 to 13,000 ppm, volume per volume (at 100°C this is a RH range from 7×10^{-6} to 7×10^{-3}). They found that in the range studied, the D-values decreased continuously as the RH calculated at test temperature decreased. When log D was plotted as a function of log RH at each test temperature, the data points formed straight lines. The lines for data at 90, 110 and 125°C were parallel. A decrease in the RH from 1×10^{-2} to 10^{-6} resulted in a 90% reduction in the D-value. The z-value was about 20°C and appeared to be constant over the temperature and RH ranges studied.

It is generally agreed that at heating temperatures from 90°C to 125°C the maximum D-value occurs at an RH between 0.2 and 0.5 and the z-value is about 21°C in this area and is in the range of 8°C to 10°C when RH is 1.00 (wet heat). On the basis of the data reported, it appears that in the temperature range from 90°C to 125°C the z-value does not change as the RH decreases below about 0.35.

Control of Spore Water Content During Heating

The terms "open system" and "closed system" were suggested by Pflug (1968) to indicate the relative control of the heating environment on spore water loss or gain. In general, in an open system, the spore water content will be determined by the environmental atmosphere surrounding the spore while in a closed system, the spore water content is a function of conditions inside an enclosure and are not influenced by the heating environment.

Closed system. In the closed system (shown diagrammatically in Figure 10), water movement and water availability to the cells are restricted. The quantity of water that is available, or that can be transferred to or from the cell is limited by the quantity of water initially present in the enclosure.

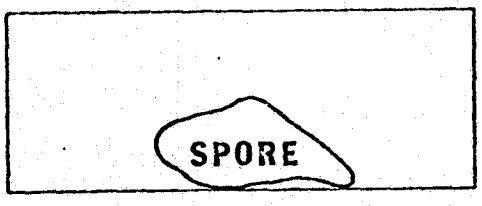

Fig. 18: Diagrammatic concept of a microorganism in a closed system (Pflug, 1968).

There are two important parameters regarding cells in a closed system: These are initial water content and enclosure volume. The water concentration in the cell during heating will be determined by the relative humidity

of the atmosphere in the enclosure at the time of sealing the enclosure, and the total volume of the enclosure. Changing either the quantity of water initially in the enclosed volume or the enclosed volume itself will alter the relative humidity, and in turn, the water content in the spore during the heating cycle.

Open system. In the open system (shown diagrammatically in Figure 19), microbial cells are heated during which time water can be lost or gained by the cells almost without limit.

Fig. 19: Diagrammatic concept of a microorganism in an open system (Pflug, 1968).

In an infinite time the cells will be in equilibrium with the water condition of the environment. This definition places no restrictions on the rate of water transfer; the cells may lose or gain water either rapidly or slowly which will in turn be determined by the nature of the spore and the physical system.

Encapsulation of Spores in Crystals and Solids

Doyle and Ernst (1967) and Mullican and Hoffman (1968) both produced crystals in which Bacillus subtilis var. niger spores were encapsulated. Doyle and Ernst reported a nine-fold increase in the dry heat resistance of spores encapsulated in calcium carbonate crystals compared to the non-encapsulated controls. Mullican and Hoffman found that spores in glycine crystals were 5 to 24 times as resistant and in sodium chloride crystals six times more resistant than the non-encapsulated spore controls. Encapsulating the spores increased the wet heat resistance by 900 times. It was not possible to sterilize the crystals with ethylene oxide.

The spores inside crystals were in a "closed system" compared to the spore controls which were in an "open system." The vapor tightness of the crystals is verified by the fact that ethylene oxide did not sterilize even after 48 hours.

The "wet heat" results suggest that wet heat conditions existed on the outside of the crystal but that inside the crystal (closed system) dry heat conditions existed. The results of the encapsulation-in-crystal studies are similar to the results of Angelotti et al. (1968a) where the survival times were longer when spores were encapsulated in Lucite. The very high dry heat resistance of encapsulated spores is probably due to the presence of the optimum amount of water in the spores to give near-maximum dry heat resistance.

Bruch et al. (1963) reported D-values for spores of Bacillus subtilis deposited on paper strips, sand and glass tubes. The D-values were lowest when spores were deposited on the paper strips and highest when deposited on sand. They also studied the effect of the material in which the spores were entrapped.

Table XV

Thermal Death Times and D Values (In Hours) at 248°F (120°C) For Spores of B. subtilis var. niger Entrapped In Several Solids

(From Bruch, Koesterer and Bruch, 1963)

Compounds	Time to Sterilize	D values (a
Solid rocket propellant	24	2.5
Asbestos patching cement	20	2.1
Plaster of Paris	12	1.7
Glue-base marble patching plaster	30	4.0
Dental materials:		
Inlay investment A	4.5	0.6
Inlay investment B	30	3.2
Inlay die material	30	3.6
Bridge model material	15	1.6

(a The D values were calculated from levels of spore contamination found by assay of the solid materials. The wt of samples for a given solid was held constant and was in the range of 0.5-1.5 g for all materials. Samples solidified around thermocouples showed that all solids reached temperature in 10 min.

Table XV includes data from Bruch et al. (1963) for Bacillus subtilis var. niger entrapped in several solids. (For reference, the resistance of the suspension of Bacillus subtilis var. niger spores on a paper strip was 0.91 hours.) It would be of considerable value to know if these heat resistance values correlate with water activity of the particular material or the water activity of the micro-area where the spores may be contacting the carrier. Are these differences a reflection of the hygroscopic characteristics of these carriers and as such do they act as protectors of spore death in the manner that Greaves (1960) has described as a protective colloid and buffer system to maintain a minimum moisture level in freeze-dried spores?

Gas Atmosphere

Pheil et al. (1967) evaluated the effect of the gas atmosphere surrounding dry spores of Bacillus subtilis strain 5230 and Clostridum sporogenes strain PA 3679 over the temperature range, 121.1 to 160°C (250 to 320°F). In all cases they found that the effect of the different dry gases was small; Bacillus subtilis showed slightly more resistance in the inert gases nitrogen and helium than in oxygen, air and carbon dioxide. The

PA 3679 exhibited highest resistance in helium, least resistance in air, with carbon dioxide generally between the two but nearer the resistance in helium rather than in air. The z-value was 18.3°C (33°F) for Bacillus subtilis in the five gases tested, 21.7°C (39°F) for PA 3679 in helium and carbon dioxide, and 22.8°C (41°F) in air. Bruch (1966) included data from Koesterer (1962) where microorganisms showed the greatest heat resistance in an air atmosphere with decreasing resistance in a helium atmosphere and in a vacuum. The reported relative D-value effect of air and helium is approximately 2 to 1, whereas Pheil et al. (1967) found a relative air to helium D-value effect of 5 to 6. These differences may be due to the type of experimental system: Pheil et al. (1967) used a closed system while Koesterer (1962) used an open system.

Simko et al. (1971) determined the dry heat resistance of Bacillus Subtilis var. niger spores on mated surfaces in an air, nitrogen and helium atmosphere. They found no difference in the resistance of the spores in the two dry gases, nitrogen and helium. The spores had a signficantly greater resistance in air. Since ambient air was used, its higher water content was probably responsible for the higher spore resistance in air.

Davis et al. (1963) found that the survival of dry spores at 60°C and high vacuum was consistently lower than for dry spores held at atmospheric pressure for the same length of time, whereas at 25°C there was little difference between atmospheric pressure and a high vacuum; at a temperature of 88°C growth (upon subculturing) was considerably less than at 60°C and 100°C; in general there was no growth (upon subculturing) after four to five days at high vacuum. The relative vapor pressure of water at these temperatures are: 25°C, 23.756 mm of mercury; 60°C, 149.38 mm; 88°C, 487.1 mm; and at 100°C, 760 mm. Comparison of the vapor pressure values, a measure of the rate of dehydration when measured against a hard vacuum, points out that the drying rate is more than 6 times faster at 60°C than at 25°C; more than 3 times faster at 88°C than at 60°C; and 50% faster at 100°C than at 88°C. Destruction rates seem to approximately parallel drying rates. These data appear to parallel data of Marshall et al. (1963) and Murrell and Scott (1957) who have reported that spore viability decrease when spores are exposed to a near zero water activity or in other words subjected to a severe drying stress.

Bruch et al. (1963) observed when discussing their data and the data of Davis et al. (1963) that "Clearly, the presence or lack of gaseous environment surrounding microbial spores during dry heat sterilization influences the rate of spore destruction. The composition and stability of the gaseous atmosphere also may be important and may be the factor responsible for the high dry heat resistance of spore samples on sand and soil samples."

When microbial spores are heated in a superheated steam atmosphere they are subject to dry heat conditions, at equilibrium there will be no liquid water present and the relative humidity will be less than 1.00. It is perhaps confusing, but true, that we can have a 100% water vapor atmosphere and still have a relative humidity below 100% and dry heat conditions. In a 100% superheated steam atmosphere the relative humidity is determined by the pressure and temperature of the system. The saturated vapor pressure is determined by the temperature of the system. The vapor pressure will be the actual pressure of the system. Reducing the pressure while holding temperature constant will reduce the relative humidity.

Discussion of Dry Heat Destruction of Microorganisms

Lea et al. (1950) demonstrated the critical nature of relative humidity or water activity on certain chemical reactions associated with dry living cells, mainly those involving proteins in work directed toward preservation of cells by freeze-drying. These authors found reaction rates expressed as Q_{10} (in the range 20-30°C) to range from 4.8 to 8.5. They found that the low reaction rates are associated with low water contents, increasing reaction rates with increasing water contents. The temperature coefficients that these authors found for the rate of loss of amino groups in dry human blood plasma containing added glucose and citrate are not materially different from those that are being found for dry heat destruction of microorganisms. This work suggests that the z-value of the thermal destruction curve will decrease with increased moisture.

Greaves (1960) suggested that the beneficial effect of glucose in the drying medium for the preservation of freeze-dried cultures was due to its buffering effect on the residual moisture content. If the organisms were dried too far, they were killed during the drying operation; on the other hand, if they were not dried far enough, they survived poorly on storage. Greaves (1962) concluded that the most important factor in determining the percentage survival both immediately and after drying and for long-term storage was the medium in which the organisms are suspended for drying. In reviewing this area, he noted that Fry and Greaves (1951) suggested the use of a serum which they felt acted as a protective colloid and as a support medium to give a final dried cake and that the glucose in this serum exerted its effect by acting as a buffer on the residual moisture content preventing the organisms from becoming too dry.

Greaves (1960) concluded that a drying media for bacteria should contain: 1) a protective colloid, for example, 5% dextran containing no glucose; 2) a buffer to control the residual moisture content to around 1%, for example, a buffer could be 5 to 10% sucrose or 5 to 10% sodium glutamate; 3) a neutralizer of carbonyl groups, for example, broth or 1% sodium glutamate.

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