FINAL REPORT

STERILIZATION OF SPACE PROBE COMPONENTS

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I ABSTRACT

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The research presented herein constitutes a study of methods of sterilization for space probe components which can be utilized in an effort to avoid the inadvertent contamination of extraterrestrial bodies. The methods investigated were dry heat, irradiation, and other techniques including the use of chemical sterilants (liquid and gaseous) and aseptic assembly. Emphasis has been placed on the use of dry heat sterilization processes. Since some electronic components are damaged by temperatures higher than 120°C and since little data exists on the effectiveness of dry heat for sterilization below 150°C, most of the investigation has been centered on temperatures in this lower range.

The studies reported here have attempted to define the various biological, chemical, and physical factors that could influence the effectiveness of dry-heat as a sterilizing process. A list of the areas of investigation follows: (1) the screening, isolation and growth of organisms resistant to dry heat, (2) the effects of time, temperature, strain of microorganism, and concentration on the effectiveness of dry heat sterilization; (3) the effect of the physical carrier (paper strip, glass tube, sand, and vermiculite) on the effectiveness of dry heat sterilization; (4) the effects of air, vacuum, inert gases, entrapment of organisms in non-aqueous liquid and on solids; (5) methods for

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sterility testing of components after inoculation with spores of known resistance to dry heat and the subsequent application of adequate sterilization cycles.

A summary of the findings to date is as follows:

1 - Mesophilic aerobic spore-formers are, in general, more resistant to dry heat than are the anaerobic and thermophilic spore-forming bacteria.

2 - The type of carrier markedly affects the dose requirements for dry heat sterilization. Soil samples are the most resistant, with sand, vermiculite, glass and paper following in that order.

3 - The gaseous environment also markedly influences the time required for sterilization. Samples in air are the most resistant with samples under helium and under low vacuum ($10^{-1}$$10^{-2}$ mm Hg) being less resistant respectively.

4 - Entrapment of dry bacterial spores in solids definitely increases the dose of dry heat required for sterilization.

5 - These data raise a question concerning the adequacy of the proposed 24 hour dry heat cycle at a temperature of 125°C.

II INTRODUCTION

Since the first space satellite was launched four years ago, the question of the origin of life and the possibility of extraterrestrial life have been revived. Bejuki (3) listed the prevention of biological contamination between planets as one of the challenges of astrobiological or exobiological exploration. As Lederberg (15) has pointed out, the best approach to the study of extraterrestrial life involves microorganisms. Cameron (6) further states the most logical environment in which microorganisms might be found would be soil, whether they be indigenous or merely temporary contaminants. Moreover, as Davies and Communtzis (11) report, biological contamination might distort the findings of samplings of prebiotic organic substances on a celestial body even if no living forms are found. It is obvious then that such studies would be seriously jeopardized by contamination of celestial bodies with terrestrial microorganisms deposited as a result of hard landings of space probes.

The hazards of such biological contamination have been assessed by CETEX (Committee on Contamination for Extraterrestrial Exploration of the International Council of Scientific Unions), and certain covenants regarding space exploration have been formulated by this group. Thus the sterilization of space probes is required by international agreements designed to prevent contamination of extraterrestrial sites by terrestrial microbes.

Investigations by Phillips and Hoffman (21) and Cordaro and Wynne (9) have shown that some of the electronic components presently employed in the construction of space probes are contaminated with microorganisms. Studies by Davis et al (12) further show that the simulated environmental conditions of outer space will not necessarily sterilize probes constructed with such components.

Several methods for obtaining sterile space probes have been suggested by Davies and Communtzis (11), Phillips and Hoffman (21), Wynne (31), Nowitzski (17), Opfell (18) and Jaffe (14) including sterile assembly, built in or self-sterilization, and terminal sterilization or any combination of such processes. The use of heat, irradiation, and chemicals have been suggested for terminal sterilization. Our primary research task has been the investigation of the use of dry heat for the terminal sterilization of electronic components prior to assembly of the space probe. At the time of launch, the entire space probe is presently surface sterilized with gaseous ethylene oxide.

Jaffe (14) has recently recommended that the preferred technique should be, if at all possible, dry heat in the final sealed container with no access permitted or mechanically possible thereafter, except with complete resterilization by heat.

Our research has followed the lines of accepted thermal death studies so as to define a dry heat process in terms of achieving the sterilization requirement. The report by Jaffe (14) examines

the sterilization requirements for unmanned lunar and planetary missions and evaluates the required degree of assurance necessary so as not to contaminate extraterrestrial environments.

III-A REVIEW OF LITERATURE ON DRY HEAT STERILIZATION

A brief review of the literature on dry heat sterilization has revealed that there is a dearth of information on this subject, especially at temperatures below 150°C. While dry heat is used for the sterilization of hospital supplies, the sterilizing cycles thus employed have been based on the destruction of spores from pathogenic microorganisms. Perkins (19), in an analysis of dry heat sterilization for hospital supplies, has shown the serious inconsistencies in results obtained by earlier investigators of the dry heat resistance of spores of several bacterial species.

Recently, Darmady, Hughes, and Jones (10) recommended 45 min. at 160°C and 18 min at 170°C for the dry heat sterilization of small hospital instruments. These values were based on the destruction of tetanus spores, which were more resistant to dry heat than spores of Bacillus stearothermophilus. Small samples of soil were more resistant to sterilization than the bacterial spore preparations, but they concluded that the instruments would have been damaged by sterilizing cycles based on the sterilization time for soil.

Nearly all of the fundamental work on mechanisms and kinetics of thermal death of microorganisms has been provided or supported by the food industry. Until 15 years ago moist heat was the chief

sterilizing agent of that industry; but the advent of aseptic canning procedures in which cans and covers are sterilized by superheated steam or other hot gases have renewed interest in the study of microbial resistance to dry heat.

Collier and Townsend (8) reported a comparison of bacterial resistance to superheated steam and gases of low water content. They found that, although dry heat is usually considered as one entity, microorganisms are more resistant to destruction by hot gases of low water content than to superheated steam.

Pflug also (20) has determined the resistance of spores of Bacillus subtilis strain 5230 to superheated steam.

Vera (30) has reported on methods and techniques regarding sterility testing and the control of efficiency of sterilization techniques, including dry heat at several temperatures below 150°C.

Extrapolations of graphical plots from the data of these workers with specific microorganisms indicated that 10-25 hour cycles at 120°C were necessary to kill populations of 1 million to 1 billion spores. Until more data becomes available hot air, hot inert gases, dry heat in a vacuum, and superheated steam should not be accepted as equivalent sources of dry heat sterilization. In addition, the need for more basic thermal resistance studies at temperatures around 120-125°C was quite obviously in order to confirm the above extrapolation. The

reason for choosing temperatures in this range, or below, is that temperatures above this level have deleterious effects on some of the electronic components presently used in the assemblies of space probes.

III-B REVIEW OF DOSE CALCULATIONS FOR DRY HEAT STERILIZATION

The data obtained from any research on sterilizing processes must be expressed in meaningful terms. The terminology and concepts that have been developed by the food industry for moist heat by Ball and Olsen (2) and for moist heat and radiation sterilization by Schmidt (25,26) are applicable to data obtained from studies on dry heat sterilization. Any calculation of dose requirements for dry heat sterilization should be based, until proven otherwise, on concepts similar to those. A thorough review of mechanism of death, order of death, and mathematical considerations for dose calculations for microbial death cannot be given in this report. More contact with these concepts can be obtained from the chapter on thermal resistance of microorganisms by C. F. Schmidt in Reddish (25).

In Appendix A of this report a brief discussion is presented on dose calculations and a comparison of the experimental and actual dose levels for some of the bacterial spores investigated in this study.

In this report only D values (time to reduce a given microbial population 90% or one log in count), F values (time to sterilize a given microbial population at a given temperature), and z values (slope of the thermal death time curve expressed in degrees F to traverse one log cycle) will be used to report our results and the following is presented by way of introduction.

Dry heat D values may be calculated from partial survival data by the equation that Stumbo (27) has applied to such data from moist heat studies:

$$D = \frac{t}{\log A - \log B}$$

where A is the total number of samples heated multiplied by the number of spores per sample,

B is calculated by assuming one surviving spore per sample when less than the total number of samples show survival,

and t is the exposure time at a given temperature that gives sterilization of some but not of all of the samples.

It is also possible to calculate D values from survivor curves that show a logarithmic order of death. The D value from a logarithmic survivor plot is the reciprocal of the "k" value obtained from the slope of the curve.

F values can be determined experimentally or can be derived from D values by the following equations of Schmidt (26):

(1) $F = D (\log A+2)$

where A is the number of spores per replicate, or

(2) $F = D (\log M+1)$

where M is equal to the number of spores per replicate times the number of replicates.

The latter formula has been employed in our calculations.

IV PREPARATION OF BIOLOGICAL INDICATORS ON VARIOUS CARRIERS

A. Pure Culture Spore Preparations: Organisms which have been reported as having some degree of resistance to various sterilization agents as well as some of the organisms commonly employed as biological indicators were selected to be included in this study. The variety of organisms (see Appendix B) covers many of the types of microorganisms reported to be resistant to heat and includes: aerobes, anaerobes, mesophiles, thermophiles, molds and resistant isolates, most of which happen to fall into the gram positive sporeforming bacillus group. After the initial screening only those organisms which proved to be the most resistant were used in the extended studies. It is an accepted fact that the bacterial spore is more heat resistant than the vegetative cell form. Thus in the use of the more resistant bacterial spores as biological indicators for sterilization there is an inherent safety factor regarding non-spore producing microorganisms.

Washed stock spore suspensions of all aerobic sporeformers except B. subtilis var. niger, B. stearothermophilus strain 1518, and B. coagulans were obtained from growth on nutrient agar with one ppm Mn. B. subtilis var. niger was sporulated in the casein acid digest broth of Roth, Lively, and Hodge (25), B. stearothermophilus strain 1518 on nutrient agar with 10 ppm Mn, and B. coagulans on the agar medium of Rice and Pederson (24).

Spores of the clostridial species were obtained from 10% trypticase broth according to Brown, Ordal, and Halvorson (4) or modifications of that medium. Aspergillus niger and Streptomyces griseus were sporulated on Cook's No. 2 Agar and glucose yeast extract agar, respectively. All spore suspensions were harvested by washing them off or centrifuging them from the sporulating medium, washing at least three times with sterile distilled water, heat shocking respectively as noted in Appendix B and then held refrigerated in distilled water. Working stock suspensions, diluted to produce the desired concentrations of spores, were also held refrigerated in distilled water. Several preparations of each organism were made to back up the program in case of decrease or heterogenicity in resistance of any one preparation.

In our studies with various bacterial sporeformers, we attempted to simulate various environmental conditions and achieve the maximum resistance possible for any one specific organism. Since there was no data on the effect of physical carriers on microbial resistance to dry heat sterilization, several carriers were included in the studies. The types and preparation of each are given below:

1 - Spore preparations dried on paper strips: Small samples (0.01 ml) of washed spore suspensions of known count were in-

oculated on to sterile bibulous filter paper strips (0.25 x 2 in) and air dried. The air-drying procedure was standardized for all strips and the home-made dryer sterilized between preps so as to eliminate cross contamination. Standard conditions for drying were 45°C dry bulb and 26°C wet bulb for 5 min. These strips were then bottled, coded, assayed by blending several strips with 99 ml of sterile water each in a Waring blender cup for 3 min, heat shocking a 5 ml aliquot, and plating on the appropriate medium. The lot was then held for thermal resistance studies.

2 - Spore preparations dried in glass tubes: Equivalent amounts of the same spore suspensions were pipetted onto the bottom of 150 x 16 mm screw cap sterile pyrex tubes and, with caps removed, the tubes were placed in a desiccator at room temperature for 2 days. These preps were then capped with sterile dry screw caps and several were assayed by washing off the glass by placing 10 ml of sterile water into each of the tubes, after which the tubes were mechanically agitated for 10 min., heat shocking a 5 ml aliquot, and then plating on the appropriate medium.

3 - Spore preparations dried in sand: Small samples (0.10 ml) of the same washed spore suspensions used for paper strips and tubes (but diluted 10 fold) were inoculated as evenly as possible into 2 g quantities of sterile clean play sand in 150 x 16 mm pyrex screw cap tubes. The inoculum was added as slowly as possible and

the sand continuously mixed to assure some degree of uniform dispersion. These tubes were then placed, with caps removed, in a desiccator at 50°C for 2 days. These preps after being dried were then capped with sterile screw caps. Several samples were assayed by mechanically shaking for 10 min. with 9.9 ml sterile water, heat shocking a 5 ml aliquot, and then plating on the appropriate medium. The rest of the lot was held for thermal resistance studies.

4 - Spore preparations dried in vermiculite: Small aliquote (0.10 ml) of the same diluted washed spore suspensions used for preparations dried in sand were inoculated as evenly as possible onto 0.1 g quantities of sterile particulate granules of vermiculite in 150 x 16 mm screw cap tubes. This type of preparation was then dried, etc. in the same manner as the preps dried in sand.

Thus biological indicators were prepared in the above manner so that each had equivalent numbers of spores per carrier from each stock spore suspension. Assays were performed to establish the levels of spores in the washed, heat shocked, stock suspensions as well as on the final preparations at various intervals throughout the course of this work. The dry spore preparations did not fall off significantly.

B - Soil Samples: Samples of six soils were obtained from various

sources. An evaluation of their natural microbial flora which provided a spectrum of organisms with respect to variety and heat resistance as biological indicators was undertaken (1) since spil may protect organisms against agents which might kill unshielded cells and (2) spores in soil were shown by Darmady et al (10) to require longer times to sterilize than other types of biological indicators. Samples included a variety of soils chosen from desert and dry areas; farm yards where horses, cows, and chickens were kept; and soil taken from an active garden.

Each of the six soils was passed through a screen (about 40 mesh) after being dried at 50°C for one week and the large pieces of debris had been removed. Each sample was coded as a separate lot and stored in a dry screw-capped bottle at room temperature during the course of this study. One-tenth gram quantities of these soils were weighed out into sterile 150 x 16 mm screw cap test tubes and capped tightly until used.

In addition to using the soil with its natural flora, we isolated the predominantly more resistant organisms from several soils requiring the longest times to sterilize. Such isolates were handled then the same as the other pure cultures and similar preparations dried on each of the carriers. The relative dry heat resistance of these organisms was approximately the same as that of the more resistant known organisms.

V STUDIES ON THE RESISTANCE OF DRY MICROBIAL SPORES TO MOIST AND DRY HEAT

In earlier studies we had investigated the relative resistance of dry spores of B. subtilis var. niger and B. stearothermophilus strain 1518 to moist and dry heat sterilization. Spore strips were placed in 150 x 16 mm screw-cap tubes, with the latter loosened, were then heated in test tube ports in the cylindrical aluminum block with a covering lid. (Appendix C. figure 2.) After the heating period, the spore strips were aseptically removed from the tubes and placed in trypticase soy broth (BBL)[1] and incubated at 32°C for B. subtilis var. niger and at 55°C for B. stearothermophilus strain 1518. Thermal death times (F values) were determined at intervals of 18°F in the temperature range of 248-356°F. The thermal death time curves that were plotted from these data are shown in Figure 1.

A direct comparison of the F values from these curves is not possible because the concentration of spores for B. subtilis var. niger was 1×10^6 per strip, while the spore concentration for B. stearothermophilus strain 1518 was 5×10^5 per strip. D values, which are independent of initial spore concentration, were calculated and showed that dry spores of B. subtilis var. niger was 3.5 to 5 times more resistant to dry heat than dry spores of B. stearothermophilus strain 1518.

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1. Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

In a correlated study with moist heat, where the spore strips were placed in glassine envelopes and positioned centrally in a thermocouple-monitored autoclave, the spore strips for B. stearothermophilus strain 1518 required 25 min. at 250°F for sterilization, whereas the spore strips of B. subtilis var. niger were sterilized in less than 5 min. at 230°F. The dry spores of B. stearothermophilus strain 1518 were far more resistant than the dry spores of B. subtilis var. niger to moist heat sterilization. In contrast, however, the dry spores of B. subtilis var. niger were 3 to 5 times more resistant than the dry spores of B. stearothermophilus strain 1518 to dry heat sterilization in the temperature range studied.

VI STUDIES ON RESISTANCE OF MICROBIAL SPORES TO DRY HEAT

A. DRY HEAT STUDIES AT 120°C:

The studies with dry heat were concerned in ascertaining whether or not a dry heat cycle of 125°C for 24 hours would be adequate to sterilize electronic components. This was the cycle which NASA recommended at that time based on an extrapolation of previously mentioned published data at higher temperatures. The temperature of 125°C had been selected because of the known sensitivity of certain electronic components to higher temperatures.

Our first efforts were designed to confirm the sterilization of small soil samples, and spores of known bacterial species held on several carriers at temperatures of 120°C and 125°C in the cylindrical aluminum blocks.

Six soils that had been prepared as noted in Section IV-B were also screened for their resistance to dry heat sterilization at 120°C. Sterilization times of from 35 to 60 hours were obtained. The two most resistant soils, FG and CO, were selected for sterilization studies in the temperature range of 120-160°C. These results are given in Section VI-F.

In attempting to confirm the reliability of the extrapolated sterilization cycle, our research required the performance of partial survival tests employing no less than 5, and usually 6, samples in any one run for each contaminated carrier and for most

of the organisms listed in Appendix B at various temperatures. At least three separate runs were made for each organism on any one carrier. The recovery medium employed in each case is listed in Appendix B.

The data for the dry heat resistance of several mesophilic sporeformers on the various carriers at 120°C are given in Table 1. An analysis of these D values by organisms showed that B. subtilis var. niger, B. coagulans, and soil isolate-69C were the most resistant members in this test series. It should be noted that the spore harvest of B. subtilis 5230 shows high heat resistance was obtained from growth on nutrient agar with 10 ppm Mn and 0.05% glucose as compared with the less resistant harvest from nutrient agar with one ppm Mn. The unidentified sporeformers isolated from heat treated samples of soil that had high resistance to dry heat sterilization possessed heat resistance comparable to that observed for known sporeformers.

An analysis of Table 1 by carriers shows that the spore samples on sand were always more heat resistant than the samples on paper or glass. Although the vermiculite samples are listed in Table 1, it should be noted that they frequently tended to be more resistant to sterilization than the sand samples, but the variation in sterilization time for the vermiculite samples made the calculated D values difficult to interpret. The inclusion

of thermocouples in the samples showed an increased lag of only a few minutes for the sand and vermiculite samples. Some unknown physical factor or factors are responsible for the difference in heat resistance of the spores on the various carriers.

The dry heat resistance of several clostridia and B. stearothermophilus strain 1518 on the various carriers at 120°C is given in Table 2. What is immediately striking is that this group of sporeformers has 1/3 to 1/4 of the dry heat resistance of the mesophilic aerobic sporeformers described in Table 1. The increased resistance of the spore samples on sand as compared to the samples on paper and glass is similar to the results noted in Table 1. Spores of Aspergillus niger and Streptomyces griseus have also been assayed for their dry heat resistance, and the data indicates that their resistance is less than that of the other organisms in Tables 1 and 2. Specific values for A. niger are not listed, but those which were obtained were similar to those for S. griseus.

VI-B DRY HEAT STUDIES AT 125°C:

In order to confirm and back-up the data at 120°C, as noted in the previous section, a series of studies was undertaken to determine the heat resistance of the same organisms on the same carriers at 125°C.

The results of these studies are given in Tables 3 and 4. The same trends and conclusions were reaffirmed, i.e., the mesophilic aerobic sporeformers were more resistant to dry heat sterilization than were the spores from thermophilic or anaerobic sporeformers, and spores carried on samd were more resistant to heat than those carried on paper strips or glass.

VI-C DRY HEAT STUDIES AT TEMPERATURES OTHER THAN 120°C:

Studies have recently been initiated to determine the resistance of several of the more resistant sporeforming organisms at temperatures above and below 120°C, particularly at 80°C and 100°C and at 130-160°C range. These studies however are conducted only with a single carrier. It is hoped the resulting data will yield curves which will define the sterilization time required at any given temperature in the range noted.

Preliminary results of such studies are given in Tables 5 and 6. These values when sketched on a curve with those already established at 120°C and 125°C do show a linear relationship at temperatures above 120°C, however, the curve is slightly askew below 120°C. Work is in progress which should further illuminate this problem.

VI-D DRY HEAT STUDIES EMPLOYING VARIOUS ENVIRONMENTS:

An examination of the chemical and physical conditions that may pertain, because of materiel characteristics and manufacturing conditions, during the dry heat sterilization of electronic components indicated the need for investigating the effect of vacuum, inert gases, or entrapment of spores within solids (Section VI-E) on sterilization by dry heat.

In preliminary studies, spores of the various organisms on the four carriers and soil were subjected to dry heat sterilization in a vacuum oven[1], preheated to 120°C. Vacuum oven data were uncorrected for heat and vacuum lags. Heat lags, times necessary to reach the desired temperature (in this case 120 C) were in the range of 1.5 hrs. Vacuum lags (to get to 10^{-1}-10^{-2} mm Hg) were found to be approximately 20 min. The preliminary data obtained from this system is given in Table 7. The results indicate, when compared with the sterilization times in air, a definite trend of decreased sterilization times for all samples, except the soils, when heated in a vacuum.

The vacuum that has been employed in our work is low (10^{-1}-10^{-2} mm Hg). Even with this limitation the increased resistance of the spore samples on sand noted in Tables 1 and 2 is decreased to

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1 Freas Vacuum Oven, Precision Scientific Co., Chicago, Illinois.

levels of resistance found for spores on paper strips sterilized in air. Although a shorter time for sterilization in the presence of a low vacuum than in air at 120°C has been found for all of the species of bacterial spores tested on paper strips, this effect is not exhibited by all spore crops for a given organism.

Davis, Silverman, Goldblith, and Keller (12) have studied the viability of microbial spores at ultrahigh vacuums (10^{-8} to 10^{-9} mm Hg) at temperatures from -110C to +88C. At the higher temperatures they found a definite decrease in the thermal resistance of microbial spores in the presence of a high vacuum as compared to their thermal resistance at atmospheric pressure.

Portner et al. (22) reported that the viability of B. subtilis and some other microbial species was not affected by a 5 day exposure to pressures as low as 2×10^{-10} mm Hg at room temperature. Bakanauskas (1) reported that exposing organisms, including B. subtilis to prolonged treatment at 1×10^{-5} to 5×10^{-7} mm Hg for periods up to 32 days, with the exception of B. cereus, did not kill them. Brueschke et al (5) however, reported that exposure to pressures of 8×10^{-8} to 6×10^{-9} mm Hg for 30 days would render his test organisms, including B. subtilis nonviable. Conversely, Morelli (16) et al has reported that B. subtilis would survive pressures of 10^{-8} mm Hg for a period of 35 days. Hence it was deemed necessary to investigate the combined effect

of an elevated temperature and vacuum on microbial spores and to elucidate these quantitatively.

To eliminate the rather large lags in the vacuum oven system another system (Fig. 1 of Appendix C) was designed in which these lags were greatly decreased, if not eliminated, and allowed periodic removal of samples without altering the conditions in the rest of the system and samples. This system was designed to accommodate dry inert gases as well.

The only biological indicators employed in the extended work were spore strips of the following organisms: B. subtilis var. niger, B. coagulans, Clostridium sporogenes and Clostridium perfringens.

Due to the relatively long periods of time required to sterilize spores of B. subtilis var. niger (5 hrs.) and B. coagulans (19 hrs.) at 120 C, it was possible to employ the survivor curve method of investigation.

Quantitative recovery of organisms from spore strips was accomplished by blending the strips in 250 ml Waring semimicro metal blender cups for 4 min at low speed, diluting in sterile distilled water and plating aliquots in triplicate. B. subtilis var. niger was assayed in tryptose agar[1] and B. coagulans in Rice-Pederson agar (23) using the triple layer plating technique.

The clostridial species, due to their lesser heat resistance, lent themselves more readily to a partial survival method of analysis, and were cultured directly in thioglycollate broth.[2]

1,2 Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland.

A manifold, such as is used in freeze-drying, containing ports for 24 rubber hoses was used in conjunction with a 120 C constant circulating magnetically stirred oil bath[1]. The test tubes containing the test spore strips were connected to the manifold by vacuum tubing adapters. The test tubes were then completely immersed in the oil bath. Spore strips were placed aseptically in individual 100 x 13 mm sterile cotton-plugged bacteriological test tubes and at least 6 tubes were included in any one run. The cotton plug acted as an individual air filter for each test sample and prevented contamination from entering with changes in atmosphere.

This system could be evacuated, filled with a gaseous atmosphere and moreover allowed the periodic removal of the tubes containing treated samples without altering the conditions in the remaining tubes. The temperature of the system was monitored at several points potentiometrically by a Honeywell Brown Electronic Recorder[2] and iron-constantan thermocouples. The temperature was maintained at 120 C± 0.5. Vacuum measurements were made by two McLeod gauges attached to the manifold. Vacuum in the system varied between 50 and 200 u of mercury. Only very small decreases in vacuum were noticed when a sample tube was removed.

Heat lags in this system after immersion in the 120°C oil

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1 Blue M Electric Co., Blue Island, Ill.

2 Minneapolis-Honeywell Co., Philadelphia, Penna.

bath were approximately 4 min for both gas filled and evacuated tubes thus eliminating, for all practical purposes, this lag factor. Vacuum lag was completely eliminated by evacuation of the system before attachment of the sample tubes and their immersion in the oil bath.

Inert gas experiments necessitated the removal of air by drawing a vacuum, and replacement with dried helium. Vacuum-helium displacement-replacement flushings were repeated three times before the final admission of helium. During an exposure a small amount of heated gas was allowed to flow continuously through the system to insure against air leaks that would cause helium displacement in the tubes containing the samples. A water trap acted as a safety valve, allowing the excess inert gas to bubble out and prevented contaminated air from inadvertently entering the system. When required the system could be easily sterilized by using hot gases.

Exposures under atmospheric conditions were conducted by attaching the cotton plugged test tubes containing the test spore strips to the manifold and opening the system to the atmosphere. Upon removal of the tubes from the system the exposed spore strips were aseptically withdrawn from the tubes, and appropriately cultured as soon as possible.

Survivor curves comparing the effects of vacuum, helium and

air at 120 C on B. subtilis var. niger and B. coagulans are given in Figs. 2 and 3. All points in Figs. 2 and 3 represent a mean value of at least four separate exposures. The effect of vacuum produced the greatest reduction in time required to attain sterility. A time reduction of 80% was noted for B. subtilis var. niger and as much as 40% of B. coagulans. The effect of helium in decreasing resistance of both organisms was about half that of vacuum as compared to such times in an atmosphere of air. The combined effects of the different atmospheres with dry heat followed a logarithmic pattern of viable spore destruction, after an initial lag phase in each case. The lag phase was greatest for air, less for helium, and negligible, if any, for vacuum.

Similar effects on decrease in resistance of C. sporogenes and C. perfringens spores were observed, and are presented in terms of D values in Table 8. Because of the relatively low resistance of the spores of these two organisms and hence the relatively short thermal death time, and since this made it difficult to follow viable spore destruction by plate counts, partial survival data was obtained. The level of spores per strip was 6.8×10^6 for C. perfringens and 4.0×10^6 for C. sporogenes.

The trends of decreasing heat resistance for the anaerobic spores in atmospheres of air, helium, and vacuum in that order were quite similar to those found for the mesophilic aerobic

sporeformers. The quantitation of the effects on resistance appears to approximate that for the latter also.

It should be noted that other studies in which air was displaced, such as spores under mineral oil and glycerol (5% water present), indicated no significant increase in dry heat resistance of B. subtilis var. niger and B. coagulans. No data is presented here because problems in the recovery of spores from the mineral oil were not encountered. Further work on resistance in mineral oil is in progress.

Some studies on desiccated spore strips have shown no increase in dry heat resistance at 120 C. Small scale studies have been started to determine whether spore strips exposed to various relative humidities before dry heat sterilization are more susceptible to destruction.

Studies employing freeze-dried spores of several of the test organisms employed in this report indicated that such preparations were very much less resistant to dry heat treatment.

VI-E DRY HEAT RESISTANCE OF BACTERIAL SPORES ENTRAPPED IN SOLID COMPOUNDS OR ON COMPONENTS

The entrapemnt of spores within solids provides an analogous situation to that existing in actual components, i.e., the spores are in a constant limited atmosphere and a heat penetration factor is introduced. Spores of B. subtilis var. niger have been entrapped in plaster of Paris, asbestos patching cement, solid rocket propellant, and various dental materials. The times for sterilization and the approximate D values are listed in Table 9 for cylindrical pieces of the solids that were exposed in test tubes in the heated aluminum blocks. The samples were of a constant weight for each material, usually about 1 gram. After the heat treatment the samples were aseptically ground as finely as possible using a stainless steel mortar and pestle, and added to tubes of sterile trypticase soy broth. This work was done in sterile hoods. Untreated inoculated control samples were also assayed to assure the level of contamination. Adequate control tests were made to insure the absence of extraneous contamination during the assay procedures and lack of toxicity from the solids added to the sterility test medium.

Some of the D values in Table 9 are the highest yet found for any known organism in or on any known carrier. The variation in D values indicates that the ingredients of solids giving low D

values probably react with the spores during the heat cycle, increasing the rate of destruction. Since the lag in heat penetration was approximately the same for all of these materials and negligible in view of the times required for sterilization (typical times are listed in Table 10), the increased D values found for the spores in most of the solids could only result from the presence of a protecting stable physical-chemical environment around the spore. This situation would be in marked contrast to the condition of a spore in a vacuum and may explain in part why a heat treatment in vacuum is more deleterious than a heat-treatment in air.

Limited initial studies have been made on the dry heat sterilization of actual electronic components inoculated with spores of B. subtilis var. niger. The effects of heat treatments of various times on the survival of these spores entrapped in selected electronic components is given in Table 11. The spore concentration was greater than a million per component. These components were spot-contaminated in the laboratory, i.e., taken apart, contaminated, allowed to dry, then sealed over, or sealed together again and were not contaminated during actual manufacture. The level of contamination was high as compared to the level of contamination expected for such components. Even so, it took no longer than 12 hours at 120°C to sterilize them; with the single

exception of one resistor.

A very brief survey of two general types of components on which sterility checks were made showed that three out of eight resistors were contaminated and that both of the two capacitators checked were contaminated but not to a very high degree. These results are in agreement with the results of the screening programs on the sterility (or contamination) of actual components by the Fort Detrick group (21) and the Air Force group (9).

VI-F STUDIES ON THE RESISTANCE OF MICROORGANISMS IN SOILS TO DRY HEAT

Comparative observations were made of cultures of treated soils in thioglycollate broth with analogous control cultures based on the types of organism predominating in the soil. It was readily determined whether these were predominantly aerobic or anaerobic and since duplicate samples were incubated at 32°C and at 55°C it was easy enough to further note whether such organisms were mesophilic or thermophilic. No specific selection for fungi was made in the sterility tests.

The results of screening various soils with their different natural microbial populations with respect to the resistance of such materials to dry heat as a sterilizing agent are given in Tables 12 and 13. These results indicated the following: (a) soil was the most difficult material to sterilize; (b) the mesophilic aerobic sporeformers were the most resistant group of organisms in soil (as well as on all the other carriers investigated); and (c) the variation of microorganisms and their resistance in various soils to dry heat does not appear to be due to the selective nature or climatic environment of the soil but more likely to the protective effect of the organic matter, especially since garden soil as contrasted to dry desert soil, which is exposed to a severe environment, appears to have the

more resistant microflora. The commonly referred to "resistant fungi" were not observed in the soils screened.

As mentioned previously, the two most resistant soils FG and CO were selected as a result of the initial screening for further resistance studies through the temperature range of 120 to 160°C.

The times for sterilization of 0.1 g samples of soils FG and CO at several temperatures are given in Table 12. If one plots the D values on the log scale against temperature on the linear scale for both of these soils on the same sheet of semi-log paper, a fairly linear plot is obtained with a z value of approximately 46. Only the results at 120 C are askew. These data show that a cycle of 24 hrs. at 125 C may sterilize some soils, but would be borderline for other more resistant soils or soils containing a higher level of resistant organisms.

VII DISCUSSION

It has been the major objective in this study to investigate dry heat at relatively low temperatures as a sterilization process. Bacterial spores are recognized as the most resistant of all living organisms in their capacity to withstand destructive agents. The order of death by heat in a bacterial population is supposedly logarithmic and is a function of the time-temperature relationship employed when a population of organisms is exposed to a lethal degree of heat; the population decreases in an orderly and predictable manner until nearly all of the population is extinct. Exceptions are noted in that a very few organisms of the population may survive longer than the predicted or calculated time which would indicate that thermal death time-temperature values are dependent upon a variety of factors and as a consequence it is difficult to obtain consistent data.

Not the least important of these factors is the thermal resistance of the microbial population involved. Resistance varies widely from species to species and even within a species and also within a given spore population. The unusual resistance of bacteria, and in particular bacterial spores, to dry heat sterilization has long been known. A review of the literature has shown that not only is there a lack of systematic study on dry heat death time-temperature relationships, but that this is especially true

at temperatures below 150°C.

Since the principles and applications of dry heat for effective sterilization should be based on the resistance of microorganisms the expansion of this area of knowledge is of foremost importance.

The only single practical criterion of the resistance of microorganisms is the death of the organisms or their failure to reproduce when, to the best of existing knowledge, conditions suitable for reproduction are provided. Thus, when an organism fails to grow (or show evidence of growth) it is considered dead. This is the primary assumption involved in such studies. In the case at hand we are not so much concerned with elaborating what led to the death of the organism as we are with establishing the reliability of its kill with dry heat.

Employing the time-elaborated concepts of thermal death time, we attempted to obtain data in the temperature range mentioned which could then be used to postulate reliable sterilization cycles.

We have employed the conventional terms and values initiated by the food industry to describe the resistance of the organisms studied.

Specifically, this study elaborates the resistance of several bacterial spores in air at temperatures from 80° to 160°C on various carriers, under various environments. The D and F values

do support each other and lend credence to the logarithmic death rate concepts. Comparisons of the calculated F values with the experimentally found sterilization endpoints show good agreement for spore samples on paper strips, but the soil samples and spore samples on glass or sand occasionally had sterilization endpoints smaller than the calculated F values.

Clearly, the presence or lack of a gaseous environment surrounding microbial spores during dry heat treatment influences the efficiency of dry heat as a sterilizing agent. The composition and stability of the environment of the spores is importand and may be the factor responsible for the high dry heat resistance of spores on sand, in soil samples, and imbedded in solids.

The adequacy of the proposed 24 hr dry heat cycle at 125°C is to be questioned, in the light of the times it takes to sterilize the more resistant soil samples and the degree of assurity desired so as not to contaminate extraterrestrial bodies. The other primary factor to be concerned with the adequacy of such a cycle, as with any cycle, is that of heat penetration. Any significant barrier to heat penetration would prove the proposed cycle inadequate. It must also be mentioned that although the spore samples on sand and vermiculite and spores imbedded in solid materials tend to approach the values of resistance of soil samples there is still quite a gap which could be construed perhaps as a

safety factor. These factors can be quantitated and taken into account when formulating a desired cycle. The data for the range of temperatures investigated will allow some latitude in evaluating such time-temperature regimens which can be relied upon to accomplish the degree of sterility desired.

Another concept concerning the death of bacteria which should be kept in mind is the concept of partial sterility based on the fact that sterilities are additive. If the proposed cycle is to be applied to components of space craft prior to assembly of the space craft and if the entire space craft is to be terminally treated with such a cycle then the adequacy of any combination of cycles can be interpolated mathematically.

It is readily apparent from all of the studies which have been made, including this one, that there are a great number of factors which play a part in the thermal resistance of micro-organisms to dry heat and that these are not easy to elucidate or definitively evaluate. Studies in such areas would appear to be insurmountable but are nonetheless vital to an understanding of the phenomena and the reliable application of thermal cycles to achieve sterility under varied conditions for a variety of materials.

VIII FUTURE WORK

Since the completion of this contract, a new contract (NASw-550) extending the investigations reported here has been entered into and is currently underway.

The areas of investigation under this new contract are mentioned in the Statement of Work of that contract but are briefly outlined below:

1 - Conduct thermal-death-time, including survivor curve studies with

(a) pure cultures at various temperatures within the range of interest and primarily at 125, 135, and 145°C.

(b) soils contaminated with the more resistant organisms, both pure and mixed cultures at various levels of contamination (including some that are normally contaminated).

(c) the above samples under various environmental conditions, in order to better evaluate the reliability of applying any heat cycle for the purpose of sterilization.

2 - Conduct sterilization studies on contaminated materials of construction or actual electronic components to verify any considered cycles, only as needed to back up the above.

3 - Conduct study on materials of construction and actual components so as to group or classify them as to applicable method of sterilization.

4 - Another area of investigation which was initiated near the end of the previous contract and extended into the new one was to determine how much, if any, better than additive effect might be achieved by combining a dry heat and irradiation treatment. Further work on this might prove fruitful in the development of an alternate sterilization procedure for certain components.

5 - To continue investigation of the biological factors that influence the dry heat resistance of spores such as the growth and sporulation media, recovery media, etc.

IX FIGURES AND TABLES

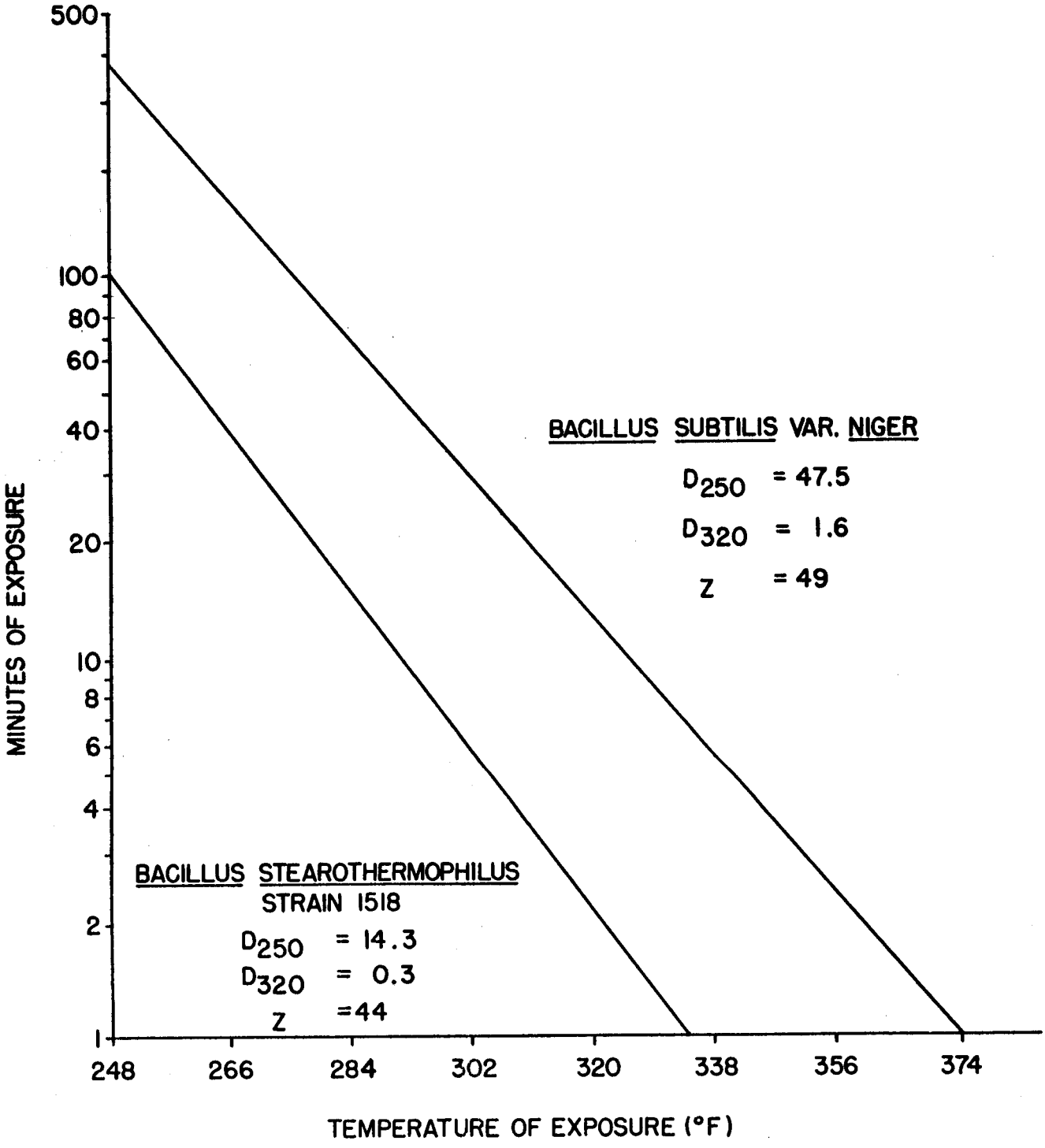

FIGURE 1. RESISTANCE OF DRY SPORES FROM TWO BACTERIAL SPECIES TO DESTRUCTION BY DRY HEAT.

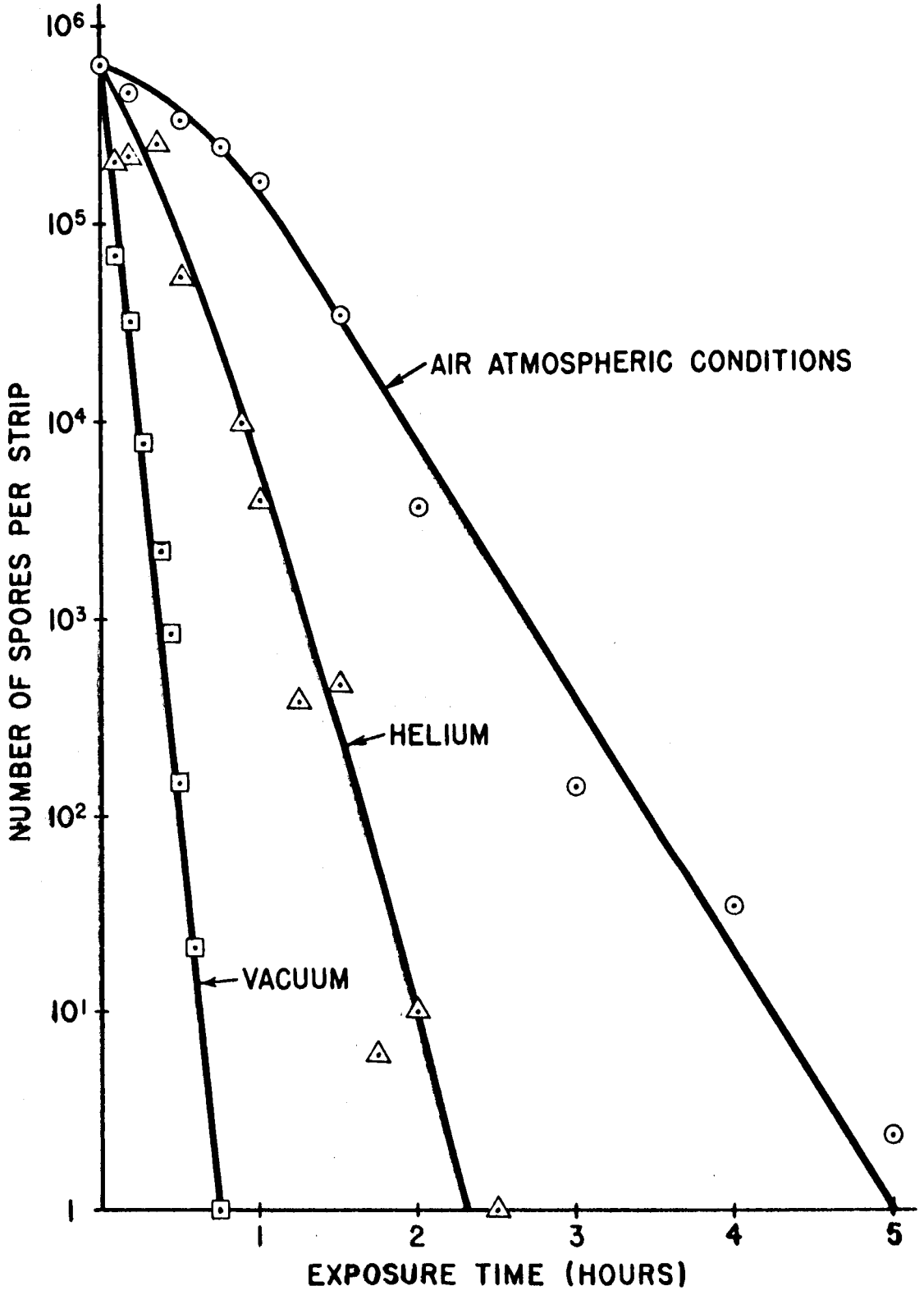

FIG. 2. SURVIVOR CURVES AT 120°C FOR BACILLUS SUBTILIS VAR. NIGER SHOWING EFFECTS OF HELIUM AND AIR AT ATMOSPHERIC PRESSURE AND VACUUM

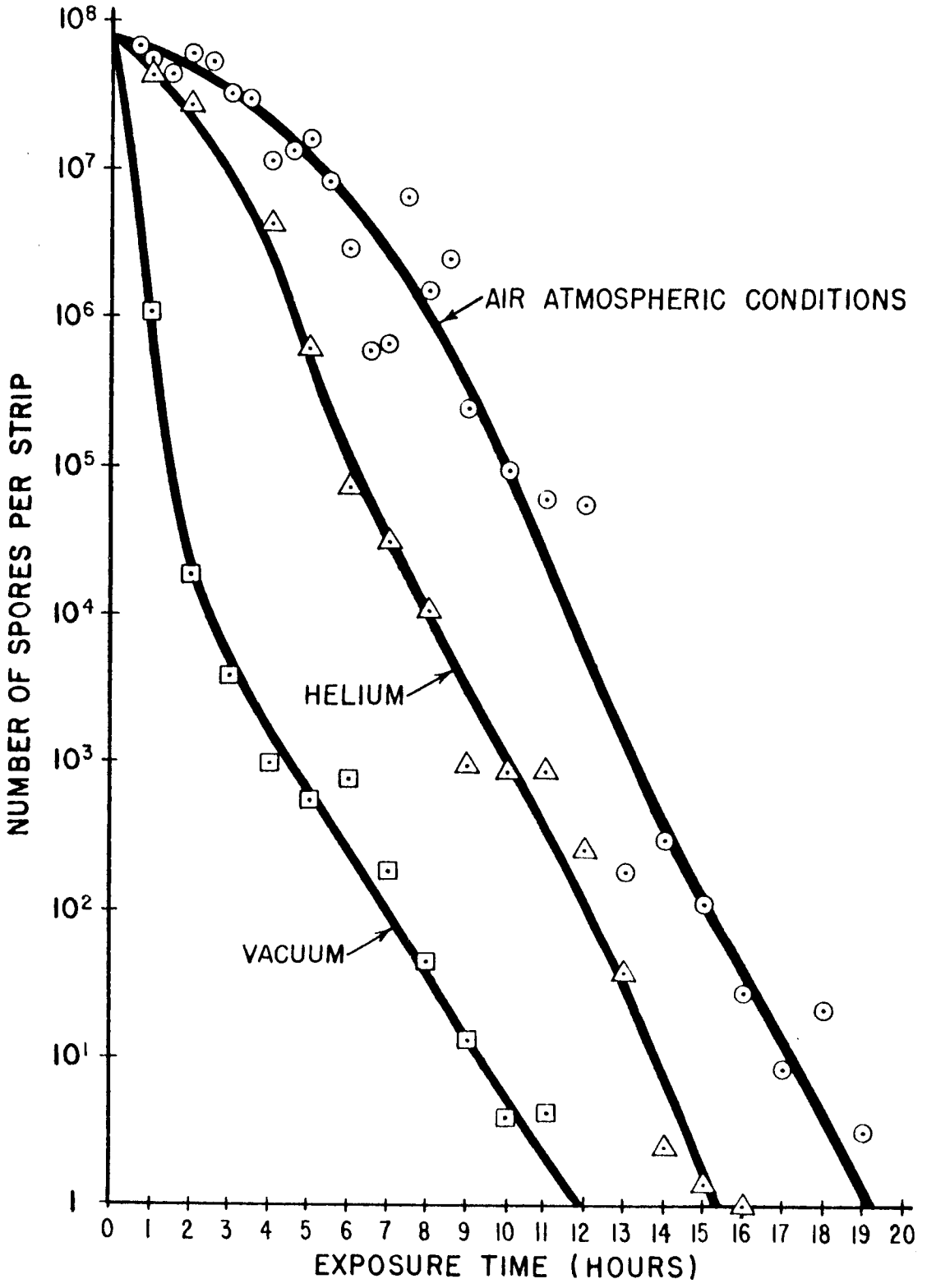

FIG. 3. SURVIVOR CURVES AT 120°C FOR BACILLUS COAGULANS SHOWING THE EFFECTS OF HELIUM AND AIR AT ATMOSPHERIC PRESSURE AND VACUUM.

TABLE 1

Thermal death times (F values) and D values (in hours) for dry heat sterilization at 248F (120C) of spores of mesophilic aerobic bacteria dried on four carriers.

Organisms	Carriers							
	Paper strip		Glass tube		Sand		Vermiculite	
	D value[1]	F value[2]	D value	F value	D value	F value	D value	F value
B. subtilis var. *niger*[3]								
Spore harvest A	1.03	7.2	0.95	6.6	1.5	10.5	1.5	10.5
Spore harvest B	0.91	6.4	0.94	6.6	1.8	13.0	1.9	13.6
Spore harvest C	0.63	4.4	0.72	5.0	1.3	9.1	1.5	10.5
B. subtilis 5230								
Spore harvest A	0.51	3.6						
Spore harvest B	0.74	5.2	0.38	2.6	1.8	13	1.6	11
Spore harvest C	0.48	3.4	0.50	3.5	1.1	7.7	1.5	10.5
B. subtilis 120	0.39	2.7	0.29	2.0	1.0	7.0	1.3	9.0
B. coagulans WH-9[3]	2.2	15.6	2.1	14.7	2.9	20.5	3.6	25
Soil isolate-44X								
Spore harvest A	0.75	5.3	1.04	7.3	1.3	9.0		
Spore harvest B	0.43	3.0	0.84	5.9	1.15	8.1	1.0	7.0
Soil isolate-69C	1.00	7.0	1.4	10.1	2.4	16.8	2.3	16.0
ETO isolate-TG[4]	0.74	5.2	0.89	6.2	1.73	12.1	1.70	11.9

1 D values were calculated from the equation of Stumbo (27)

2 F values were calculated from the equation of Schmidt (25). These F values are for the destruction of one million (10^6) spores on the various carriers.

3 The recovery medium for these organisms was trypticase soy broth. Thioglycollate broth was used for the sterility assays of the other mesophilic sporeformers.

4 Organism with high resistance to gaseous ethylene oxide sterilization.

TABLE 2.

Thermal death times (F values) and D values (in hours) for dry heat sterilization at 248F (120C) of spores of several fungi and anaerobic and thermophilic bacteria dried on four carriers.

Organisms	Carriers							
	Paper strip		Glass tube		Sand		Vermiculite	
	D value[1]	F value[2]	D value	F value	D value	F value	D value	F value
B. stearothermophilus strain 15183								
Spore harvest A	0.31	2.2	0.28	1.9	0.47	3.2	0.57	3.6
Spore harvest B	0.32	2.2	0.21	1.4	0.31	2.2	0.32	2.2
Spore harvest C	0.27	1.9	0.18	1.2	0.28	1.9	0.41	2.9
C. sporogenes (PA 3679)								
Spore harvest A	0.27	1.9	0.26	1.8	1.8	12.5	1.8	12.5
Spore harvest B	0.08	0.52	0.04	0.3			0.07	0.5
C. sporogenes (Vera strain)								
Spore harvest A	0.10	0.72	0.27	1.9	>1.5	>11	>1.5	11
Spore harvest B	0.12	0.85	0.08	0.52	0.12	0.82	0.12	0.82
C. tetani[4]	0.34	2.4	0.15	1.05	0.15	1.05	0.15	1.03
C. perfringens (type A)	0.18	1.3	0.03	0.21	0.06	0.43	0.06	0.44
S. griseus[3]	0.26	1.8	0.12	0.82	0.20	1.4	0.20	1.4

1 D values were calculated from the equation of Stumbo (27).

2 F values were calculated from the equation of Schmidt (25). These F values are for the destruction of one million (10^6) spores on the various carriers.

3 The recovery medium for these organisms was trypticase soy broth. Thioglycollate broth was used for the sterility assays of the anaerobic sporeformers.

4 Non-toxigenic strain.

TABLE 3

Thermal death times (F values) and D values (in hours) for dry heat sterilization at 257F (125C) of spores of mesophilic aerobic bacteria dried on four carriers.

Organisms	Carriers							
	Paper strip		Glass tube		Sand		Vermiculite	
	D value[1]	F value[2]	D value	F value	D value	F value	D value	F value
B. subtilis var. *niger*[3]								
Spore harvest A	no runs made at 125°C.							
Spore harvest B	0.60	4.2	0.63	4.4	1.4	9.6	1.5	10.6
Spore harvest C	0.48	3.3	0.49	3.4	1.06	7.4	1.2	8.5
B. subtilis 5230								
Spore harvest A	no runs made at 125°C.							
Spore harvest B	0.50	3.5	0.12	0.9	0.51	3.6	0.51	3.6
Spore harvest C	0.24	.17	0.12	1.0	0.49	3.4	0.74	5.2
B. subtilis 120	0.26	1.8	0.25	1.8	0.38	2.7	0.52	3.6
B. coagulans WH-9[3]	1.2	8.4	1.1	7.6	1.6	11.3	2.2	15.2
Soil isolate-44X								
Spore harvest A	0.53	3.7						
Spore harvest B[3]	0.44	3.1	0.37	2.6	0.57	4.0	0.73	5.1
Soil isolate-69C	0.70	4.9	0.95	6.7	1.4	9.8	1.5	10.8
ETO isolate-TG[4]	0.36	2.5	0.71	4.9	1.0	7.0	1.3	9.0

1 D values were calculated from the equation of Stumbo (27)

2 F values were calculated from the equation of Schmidt (25). These F values are for the destruction of one million (10^6) spores on the various carriers.

3 The recovery medium for these organisms was trypticase soy broth. Thioglycollate broth was used for the sterility assays of the other mesophilic sporeformers.

4 Organism with high resistance to gaseous ethylene oxide sterilization.

TABLE 4

Thermal death times (F values) and D values (in hours) for dry heat sterilization at 257F (125C) of spores of several fungi and anaerobic and thermophilic bacteria dried on four carriers.

Organisms	Carriers							
	Paper strip		Glass tube		Sand		Vermiculite	
	D value[1]	F value[2]	D value	F value	D value	F value	D value	F value
B. stearothermophilus strain 1518[3]								
Spore harvest A	0.10	0.67						
Spore harvest B	0.29	2.0						
Spore harvest C	0.14	0.97	0.04	0.3	0.13	0.92	0.13	0.92
C. sporogenes (PA 3679)								
Spore harvest B	0.04	0.26	0.05	0.3	0.07	0.49	0.28	2.0
C. sporogenes (Vera strain)								
Spore harvest B	0.04	0.3	0.03	0.2	0.03	0.2	>0.13	>0.94
C. tetani[4]	0.18	1.2	0.07	0.48	0.14	0.97	0.15	1.05
C. perfringens	0.15	1.03	0.12	0.85	0.03	0.21	0.12	0.83
S. griseus[3]	0.15	1.05	0.08	0.54	0.29	2.0		

1 D values were calculated from the equation of Stumbo(27).
2 F values were calculated from the equation of Schmidt (25). These F values are for the destruction of one million (10^6) spores on the various carriers.
3 The recovery medium for these organisms was trypticase soy broth. Thioglycollate broth was used for the sterility assays of the anaerobic sporeformers.
4 Non-toxigenic strain.

TABLE 5

D values (in hours) for dry heat sterilization at temperatures below 120°C (248°F) of spores of several bacterial species dried on paper strips.

Organisms	Temperature 80°C (176°F) D value*	F value**	100°C (212°F) D value	F value
B. subtilis var.niger Spore harvest A	70	480	9.5	66
B. stearothermophilus 1518 Spore harvest A	29	210	3.3	23.3
B. coagulans WH-9			11	77
C. sporogenes-(PA 3679) Spore harvest B	13	112	1.6	14.5

* D values were calcualted from the equation of Stumbo (27).

** F values were calculated from the equation of Schmidt (25)

TABLE 6

D values (in hours) for dry heat sterilization at temperatures above 120°C (248°F) of spores of several bacterial species dried on paper strips.

Organisms	Temperature 140°C (284°F) D value*	F value**	160°C (320°F) D value	F value
B. subtilis var.niger				
Spore harvest D	0.11	0.77	0.02	0.13
B. stearothermophilus 1518				
Spore harvest C	0.04	0.3	0.009	0.06
B. coagulans WH-9	0.11	0.76	0.03	0.20

* D values were calculated from the equation of Stumbo (27).

** F values were calculated from the equation of Schmidt (25)

TABLE 7

Effect of vacuum during dry heat sterilization on the destruction of bacterial spores at 120°C (248°F).

Organisms	D values (in hours)	
	Air and Atmospheric pressure	Vacuum* (10^{-1} to 10^{-2}mm H_9)
Paper strips		
B. subtilis var. *niger*		
Spore harvest B	0.91	0.30
Spore harvest C	0.63	0.61
B. subtilis 5230		
Spore harvest C	0.48	0.24
B. coagulans WH-9	0.96	0.60
Soil isolate 44-X		
Spore harvest B	0.43	0.26
ETO isolate-TG	0.74	0.47
Sand		
B. subtilis var *niger*		
Spore harvest C	1.3	0.60
B. coagulans WH-9	1.2	0.56
Soils		
FG	8.9	9.4
CO	8.7	9.3

* The samples were exposed in a pre-heated vacuum oven and the times of exposure were based on total time in oven. No correction has been made for lag time to reach exact temperature and vacuum.

TABLE 8.
D values (in minutes) for dry heat sterilization at 248F (120C) of Clostridial spores dried on paper strips under various environments

Organism	D value (min.) in Environment*		
	Air Atmospheric pressure	Dry Helium Atmospheric pressure	Vacuum 100-200μ Hg
C. sporogenes (PA 3679)	16.9	9.1	5.7
C. perfringens	8.3	4.0	2.0

* D Values were calculated from the equation of Stumbo (27)

TABLE 9

Thermal death times and D values (in hours) at 248F(120C) for spores of B. subtilis var. niger entrapped in several solids.

Compound	Time to sterilize	D value*
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	36	3.6
Bridge model material	30	1.6

* The D values were calculated from levels of spore contamination found by assay of the solid materials. The weight of samples for a given solid was held constant and was in the range of 0.5 to 1.5 g for all materials. The spore counts in these materials ranged from 2×10^6 to 5×10^7 per gram of dry material. Samples solidified around thermocouples showed that all solids reached temperature in 10 min.

TABLE 10

Heat penetration studies for the dry heat sterilization at 248F(120C) of the various carriers and samples employed in this study.

Type of Sample.	Average time (in hours) for sample to reach 120C from ambient in tested aluminum blocks.
Spore strip in tube c cap	0.03-0.07
Glass tube c cap	0.03-0.07
0.1 g sand in tube c cap	0.07-0.09
2.0 g vermiculite in tube c cap	0.08-0.10
1.0 g cylinders of solids in tube c cap	0.165
3.0 g cylinders of solid propellant in tube c cap	0.34
6 inch cubes of solid propellant	6.0 (calculated)

* Tests were made employing a Minneapolis-Honeywell Brown Universal Electronic potentiometer and recorder (type 153) with the use of iron-constantan thermocouples embedded in the sample and under actual experimental conditions.

TABLE 11

Effect of dry heat treatments at 248F(120C) on the survival of spores of B. subtilis var. niger in selected electronic components.*

Component		Time of heat treatment		
Type	Code	12 hours	18 hours	24 hours
Resistor	1 meg ½ (1W5%)	0/2**	0/2	0/2
"	47 ½ (1W5%)	0/1	0/2	0/2
"	CDIR (715 ½)		0/2	0/2
"	CDM1 (1%-50K)		0/1	0/2
"	1K (1W5%)	1/2	0/2	0/3
Rectifier	1N 538		0/2	0/2
Capacitor	118P2559652			0/2
"	118P2059252			0/1
"	150D156X0035R2			
"	CM20C-510J	0/2	0/2	0/2
	Gas Connector			0/1
	Switch			0/1
	Starter			0/1

* From the quantities of spore preparation added, it is estimated that each component was contaminated with more than 1 million spores.

** Results are given as number of positive samples per total number of samples treated.

TABLE 12

Thermal death time values (in hours) for mesophilic microbial populations of six soils in the temperature range of 120-160 C (248-320F) in air.

Soils	Level of spores per g	Observation	120 (248)	125 (257)	130 (266)	140 (284)	150 (302)	160 °C. (320) °F
FG	3.1×10^6	Survivors	48	24	18	6	2	1
		Sterile	61	24-30	24	8	4	4
IN-1	3.1×10^5	Survivors	24	20				
		Sterile	30	24				
IN-2	2.2×10^5	Survivors	48	18	6	4	1	0.5
		Sterile	55	24	12	8	4	2
CH	4.5×10^5	Survivors	48	12	10	4	0.5	0.5
		Sterile	55	18	18	8	2	2
CO	1.4×10^6	Survivors	48	24	12	6	2	1
		Sterile	61	30	18	8	6	4
HO	3.6×10^7	Survivors	48	12	8	4	2	0.5
		Sterile	55	24	18	8	4	2

TABLE 13

Thermal death time values (in hours) for thermophilic microbial populations of six soils in the temperature range of 120-160°C (248-320°F) in air.

Soils	Level of spores per g	Observation	120 (248)	125 (257)	130 (266)	140 (284)	150 (302)	160°C (320)°F
FG	2.5×10^4	Survivors	48				1	0.5
		Sterile	61	18	15	6	3	2
IN-1	7.5×10^3	Survivors	44					
		Sterile	61	24				
IN-2	3×10^4	Survivors	48				1	
		Sterile	55	18	15	8	4	1
CH	1.5×10^4	Survivors	44			1	0.5	0.25
		Sterile	55	24	15	6	2	1
CO	3×10^5	Survivors	48					
		Sterile	61	24	15	4	2	1
HO	3.5×10^5	Survivors	48					
		Sterile	55	24	15	8	3	1

TABLE 14

Summary of thermal resistance for the mesophilic spore populations of six soils and a fine dust in the temperature range 120-160°C (248-320°F) in air.

Soil	Thermal resistance values (in hours)	Temperature 120 (248)	125 (257)	130 (266)	140 (284)	150 (302)	160°C (320)°F
FG	D value[1]	8.9	4.0	2.6	1.1	0.41	0.13
	F value[2]	62.5	28	21	7.6	3.3	0.91
IN-1	D value	5.0					
	F value	35					
IN-2	D value	9.7	3.8	1.2	0.88	0.21	0.11
	F value	68	27	8.7	6.2	1.5	0.77
CH	D value	8.4	3.2	1.9	0.69	0.11	0.05
	F value	59	22.4	13	4.8	0.74	0.34
CO	D value	8.7	4.1	2.4	1.1	0.39	0.19
	F value	60	29	17	7.7	2.8	1.4
HO	D value	3.4	2.5	1.1	0.60	0.27	0.07
	F value	24	17.5	7.9	4.2	1.9	0.52
Vacuum Cleaner Dust	D value	0.84					
	F value	5.8					

1. The D values for each soil and fine dust were calculated from a mesophilic spore count obtained after a heat shock of 10 minutes at 80C. The counts for each soil are given in Table 12. The count for the fine dust was 3×10^5 spores per g. Assays for sterility were made in Thioglycollate broth. D values were calculated from the equation of Stumbo (27).
2. F values were calculated from the equation of Schmidt (25). These F values are for the destruction of one million (10^6) spores.

X ACKNOWLEDGMENT

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APPENDIX A

DOSE CALCULATIONS FOR DRY HEAT STERILIZATION

Any calculation of dose requirements for dry heat sterilization should be based, until proven otherwise, on concepts similar to those applied to the thermal sterilization of foods. A thorough review of mechanism of death, order of death, and mathematical considerations for dose calculations for microbial death cannot be given in this brief discussion. More contact with these concepts can be obtained from the chapter on thermal resistance of microorganisms by C. F. Schmidt in Reddish (25).

Esty and Meyer (13) reported the data upon which is based the "classical" thermal death time curve for the maximum resistance of spores of C. botulinum in phosphate buffer. The maximum resistance of spores of C. botulinum from the classical thermal death time curve was equal to 2.78 minutes at 250°F. Later Townsend, Esty and Baselt (28) corrected this figure to 2.45 minutes by applying a correction factor to account for come-up time in the original determinations. Based upon the 2.78 value, it was then, and since has been accepted in the canning industry that the minimum process lethality for nonacid canned foods (above pH 4.5) shall be equivalent to 3.0 minutes at 250°F as calculated from heat penetration data.

In more recent years much of the work in thermal resistance

has been expressed in terms of D values or the time required to reduce a population 90%, assuming approximately logarithmic rates of destruction. We may now estimate the D_{250} value for the classical curve since the determinations of Esty and Meyer (13) were made at a spore concentration of 60 billion per tube. While the number of tubes used for each time-temperature was not specified, if only one tube were used the spore load would be $10^{10.7}$; if several tubes were used it would be 10^{11} or slightly greater. Since the F value of a thermal death time curve represents a time at which less than one spore would survive, we may say that the F value represents the destruction of approximately 10^{12} spores. In other words, the F value, as an expression of the resistance of the suspension, in the classical curve represents the heat treatment sufficient to reduce the inoculum through 12 log cycles. As stated, perhaps more briefly by Townsend et al. (29) in A Laboratory Manual for the Canning Industry, while the number of spores used by Esty and Meyer (13) in their TDT runs was 6×10^{10}, their thermal death times were based on complete destruction. It is now customary to assume their classical TDT curve resulted in reducing 1×10^{12} spores to one spore or that $F = 12D$. From $F = 12D = 2.45$ minutes we may calculate that the classical $D_{250} = 0.204$ minutes.

If it was desired that this one survivor from the inoculum of 10^{12} spore should have only one chance in 10,000 of surviving,

a total inactivation dose of 16 D (16 x 0.204 minutes) or 3.26 minutes at 250 F would have to be given. Actually, the load of spores of C. botulinum in a can of food before thermal processing rarely, if ever, exceeds a 1000 to 10,000 spores. Thus, if the can receives a dose of 12 D, the probability of a survivor is 10^{-8} to 10^{-9}. This interpretation will explain why there has not been any deaths from C. botulinum in foods canned in the U. S. in the last thirty-five years.

Dry heat D values may be calculated from partial survivor data using the equation that Stumbo (27) has applied to data from moist heat studies:

$$D = \frac{t}{\text{Log A - log B}}$$

where A is the total number of samples heated multiplied by the number of spores per sample, B was calculated by assuming one surviving spore per container when less than the total containers showed survival, and t was exposure time at a given temperature.

This equation has been used quite extensively for the calculation of moist heat and radiation D values by current investigators and has been applied to data in the literature which are amenable to calculation. The application of this formula to partial survivor data assumes an approximately logarithmic rate of destruction. It is acknowledged that survivor curves from moist heat or radiation studies have been obtained which do not show a logarithmic order of death, but it is known that many survivor curves show this

order or a very close approximation of it. The determination by survivor curves of the order of death for dry heat has been investigated in our dry heat studies but only for a limited number of organisms and only on one carrier (paper strips). These curves after a short lag phase do show logarithmic death rates. The determination of a survivor curve introduces many additional factors which may determine survival or destruction of the spores in addition to the exposure to the lethal agent. Furthermore, it is not certain and probably indeterminable whether the survivor curve obtained by subculture corresponds to the survivor curve of the organism in the presence of the surrounding product. In general, those moist heat and radiation survivor curves which do not show a logarithmic order of death have the major distortion from linearity in the first log cycle of reduction. If this is the case, then D values determined from partial survival after 8-9 log cycles of reduction may provide a reasonably good estimate of resistance and have valid predictive value. Schmidt and Nank (26) also suggested that the validity of radiation D value calculations from end point data (the use of the Stumbo equation) was supported by showing that the same radiation D values could be calculated from initial inocula at least 100 fold (2 logarithms) different. With one suspension in whole kernel corn and brine they found the same calculated D values for inocula of 100 million spores per can and 1 million spores per can. It is

this later criterion which has been employed in our studies to validate the D value concept for prediction of the sterilizing dose for dry heat sterilization. Further support for this dose prediction will be made by doing survivor curves in the near future for other dry-heat resistant microorganisms, possibly on various carriers or under various environments.

The data in Table 1 of this appendix show a comparison of D values for 3 levels of spore inocula for B. subtilis var. niger. There is good agreement among D values for each level of temperature. These results support the argument that the survival curve for this organism is approximately logarithmic. In addition, F values (time for sterilization) were calculated from these data using the equation that Schmidt has described in his chapter in Reddish (25)

$$F = D\ (\text{Log } A+2)$$

where A equals the number of spores per replicate.

Schmidt (personal conversation) has expressed this equation in a more general form:

$$F = D\ (\text{Log } M+1)$$

where M is equal to the number of spores per replicate times the number of replicates.

Our data show fairly good agreement between the calculated F values and those found experimentally.

The data in Table 2 of this appendix show a comparison of D values for different levels of spore inocula for B. coagulans WH-9 found at various temperatures. This supplements the agreement obtained among D values found in Table 1 for B. subtilis var. niger and supports the argument that the survival curve for these organisms is approximately logairthmic and the validity of applying such calculations for predicting sterilization values. It should be noted that although D values are independent of spore concentration, they are dependent on the type of spore carrier.

In Table 3 the estimated time for sterilizing with dry heat (F values) in air are given for various contamination levels, for several microorganisms and soils. These are the predicted times required at 125°C (257°F) to sterilize the preparation noted. The estimated F values are dependent both on the spore carrier and the level of spore contamination. All of the more resistant bacterial spore preparations at the higher level of spore contamination investigated can be sterilized in less than 24 hrs. However, the two soils noted cannot be sterilized at the lower level of spore contamination let alone the higher level.

The estimated times (F values) required to sterilize the two more resistant soils with dry heat in air are given in Table 4. They are given for two levels of spore contamination and for various temperatures in the range 125-160°C (257-320°F). These values should hold reasonably well for the destruction of any spore forming organisms at these levels of contamination and temperature.

Another very important aspect in evaluating the thermal resistance of microorganisms are the recovery conditions and the recovery media employed for the treated cells. In this study we utilized the standard sterility test medium -- thioglycollate broth, except in cases where it was shown not to be the optimum recovery medium. We have made a comparison study of two media employing several of the more resistant organisms. The data is presented in Table 5 of this section and indicates that trypticase soy broth gives better recoveries (i.e. larger D values) than does thioglycollate broth for the mesophilic aerobic organisms. Investigation of these factors is being continued.

TABLE 1

Comparison of dry heat D values and experimental and estimated dose levels for spores of B. subtilis var.niger determined from different spore inoculum levels on paper strips.

Level of Spore Inoculum	Sterilizing dose level Temperature °C	°F	Time (in hours)	D value* (hours)	F value** (hours)
1×10^7	120	248	8-9	0.98	8.6
1×10^6	120	248	6.5-7.5	0.92	7.1
3.5×10^4	120	248	4.5-5.5	0.85	5.3
1×10^7	125	257	5.5-6.5	0.67	5.9
1×10^6	125	257	5-6	0.64	5.0

* D values were calculated from partial survival data using the equation of Stumbo (27).

* F values were calculated using the equation of Schmidt (25) and are for the specific level of inoculum noted.

TABLE 2

Comparison of dry heat D values and experimental and estimated dose levels for spores of B. coagulans WH-9 determined from different spore inoculum levels on paper strips.

Level of spore inoculum	Sterilizing dose level Temperature °C	°F	Time (hours)	D value (hours)	F value (hours)
1×10^3	100	212	42-44	12.0	44
1×10^8	100	212	96-120	11.0	107
1×10^3	120	248	6.5-7.5	1.7	7.3
1×10^8	120	248	17-18	1.9	18.5

* D values were calculated from partial survival data using the equation of Stumbo (27).

* F values were calculated using the equation of Schmidt (25) and are for the specific level of inoculum noted.

TABLE 3

Estimated dry heat sterilizing times in air for various contamination levels and carriers for several microorganisms and soils at 125°C (257°F)

Preparation	Level of spore contamination	Approximate D values* (hours)	Estimated time for sterilization (F value)** (hours)
Paper strips			
B. subtilis var. *niger* Spore harvest B	1×10^6	0.62	4.3-5.0
	1×10^9		6.2-6.8
B. coagulans WH-9	1×10^6	1.2	8.4-9.6
	1×10^9		12-13.2
Soil isolate 44-X Spore harvest B	1×10^6	0.44	3.1-3.5
	1×10^9		4.4-4.8
Sand			
B. subtilis var. *niger* Spore harvest B	1×10^6	1.2	8.4-9.6
	1×10^9		12-13.2
B. coagulans WH-9	1×10^6	1.6	11.2-12.8
	1×10^9		16-17.6
Soil isolate 44-X Spore harvest B	1×10^6	0.57	4.0-4.6
	1×10^9		5.7-6.3
Soils (mesophilic population)			
FG	1×10^6	4.0	28-32
	1×10^9		40-44
CO	1×10^6	4.1	29-33
	1×10^9		41-44

* D values calculated using the equation of Stumbo (27).

** F values calculated using the equation of Schmidt (25).

TABLE 4

Estimated dry heat sterilizing times in air for two soils at various temperatures.

Soil	Level of mesophilic spore contamination	Temperature °C	Temperature °F	Approximate D value* (hours)	Estimated time for sterilization (F value)** (hours)
FG	1×10^6	125	257	4.0	28-31.5
		130	266	2.6	18.3-21
		140	284	1.1	7.7-9.0
		150	302	0.41	2.9-3.3
		160	320	0.13	0.9-1.1
	1×10^9	125	257	4.0	40-44
		130	266	2.6	26-28.6
		140	284	1.1	11-12.1
		150	302	0.41	4.1-4.5
		160	320	0.13	1.3-1.5
CO	1×10^6	125	257	4.1	28.6-32.7
		130	266	2.4	16.8-19.2
		140	284	1.1	7.7-8.8
		150	302	0.39	2.7-3.1
		160	320	0.19	1.3-1.5
	1×10^9	125	257	4.1	41-45
		130	266	2.4	24-26.4
		140	284	1.1	11-12
		150	302	0.39	3.9-4.3
		160	320	0.19	1.9-2.1

* D values calculated using equation of Stumbo (27).
* F values calculated using equation of Schmidt (25).

TABLE 5

Comparison of D values (in hours) obtained for several bacterial spores employing different recovery media.

Temperature	Organisms	Recovery medium	Carriers			
			Paper strip	Glass tube	Sand	Vermiculite
120°C(248F)	B. coagulans WH-9	Thioglycollate broth[1]	1.2	1.1	1.8	2.9
		Trypticase soy broth[2]	2.2	2.1	2.9	3.6
	Soil isolate-44X Spore harvest B	Thioglycollate broth	0.43	0.04	1.15	1.0
		Trypticase soy broth	0.82	0.82	0.86	1.13
125°C(257F)	B. coagulans WH-9	Thioglycollate broth	0.8	0.73	0.94	
		Trypticase soy broth	1.2	1.1	1.6	2.2
	Soil isolate-44X Spore harvest B	Thioglycollate broth	0.22	0.30	0.40	
		Trypticase soy broth	0.44	0.37	0.57	0.73

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Standard operating procedures for organisms employed in this study.

Organism	Origin or source	Sporulation medium	Incubation temperature	Sterility test medium	Assay medium	Heat shock treatment for spore assays	
						Temp.	Time
B. subtilis var. niger	Fort Detrick 356 O.V. strain	Casein acid digest glucose broth	32°C	Trypticase soy broth (BBL)[1]	Plate count or tryptose agar	65°C	30 min.
B. subtilis 5230	C.F. Schmidt, Continental Can Co. Chicago, Ill.	nutrient agar with 10 ppm. mn.	37°C	Thioglycollate broth (BBL)[2]	Dextrose tryptone agar	100°C	15 min.
B. subtilis 120	University of Wisconsin Culture collection	nutrient agar	37°C	Thioglycollate broth	Plate count agar	65°C	30 min.
B. subtilis-TG	ethylene oxide resistant isolate	nutrient agar with 1 ppm mn	37°C	Thioglycollate broth	Plate count agar	65°C	30 min.
B. coagulans WH-9	ATCC #8038	Rue and Pederson agar (23)	37°C	Thioglycollate and trypticase soy broth	Pederson growth agar	95°C	5 min.
Soil isolate-44X	aerobic mesophilic heat resistant organism isolated from FG soil	Plate count agar	32°C	Thioglycollate and trypticase soy broth	Plate count agar	65°C	30 min.
Soil isolate-69C	aerobic mesophilic heat resistant organism isolated from CO soil	Plate count agar	32°C	Thioglycollate and trypticase soy broth	Plate count agar	65°C	30 min.
B. stearothermophilus-strain-1518	NCA 1518 strain received from Dr. Z.J. Ordal U. of Ill., Urbana, Ill.	nutrient agar with 30 ppm mn.	55°C	Trypticase soy broth	Dextrose tryptone agar	100°C	5 min.

Soils-mesophiles	soils from several sources		32°C	Thioglycollate broth	Plate count agar	80°C	10 min.
Soils-thermophiles	soils from several sources		55°C	Thioglycollate broth	Plate count agar	100°C	5 min.
C. sporogenes-Vera	#11437 ATCC	10% trypticase broth	37°C	Thioglycollate broth	Yeast extract starch agar in Prickett tubes	80°C	10 min.
C. sporogenes-PA 3679	ATCC #7955	10% trypticase broth	37°C	Thioglycollate broth	Yeast extract starch agar in Prickett tubes	80°C	10 min.
C. tetani	received from Dr.L.S. McClung Univ. of Indiana strain 505	10% trypticase broth	37°C	Thioglycollate broth			
C. perfringens	received from Dr.R.Angelotti Tapt Sanitary Eng. Center, Cincinnati, Ohio strain 580	10% trypticase broth	37°C	Thioglycollate broth	Brain-heart infusion or Eugon agar	80°C	10 min.
Aspergillus niger	ATCC #6275	Cook's No.2 agar	room temp.	trypticase soy broth (acidified to pH 4)	Cook's No.2 agar	no heat shock	
Streptomyces griseus	Waksman strain obtained from U. of Wisconsin culture collection	glucose yeast extract agar	room temp.	glucose yeast extract broth	glucose yeast extract agar	no heat shock	

NOTE: The formulations for all media are available upon request.

1,2 Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland.

DESIGN OF THERMAL TEST UNIT

A cylindrical aluminum block equipped as shown in Figure 2 of Appendix C, with a covering lid, was the major test unit employed in this work. It was equipped with a central heating element and a temperature regulator. It can hold six 150 x 16 mm screw-cap test tubes. The temperature variation in the unit when set at any temperature from 100°C to 160°C was in the range of ± 0.5°C. The temperature was uniform throughout the entire tube and in all six replicate tubes and was monitored routinely with thermocouples and/or thermometers which were compared against a certified thermometer.

Other equipment and test units are described in this report in the section in which they were specifically employed.

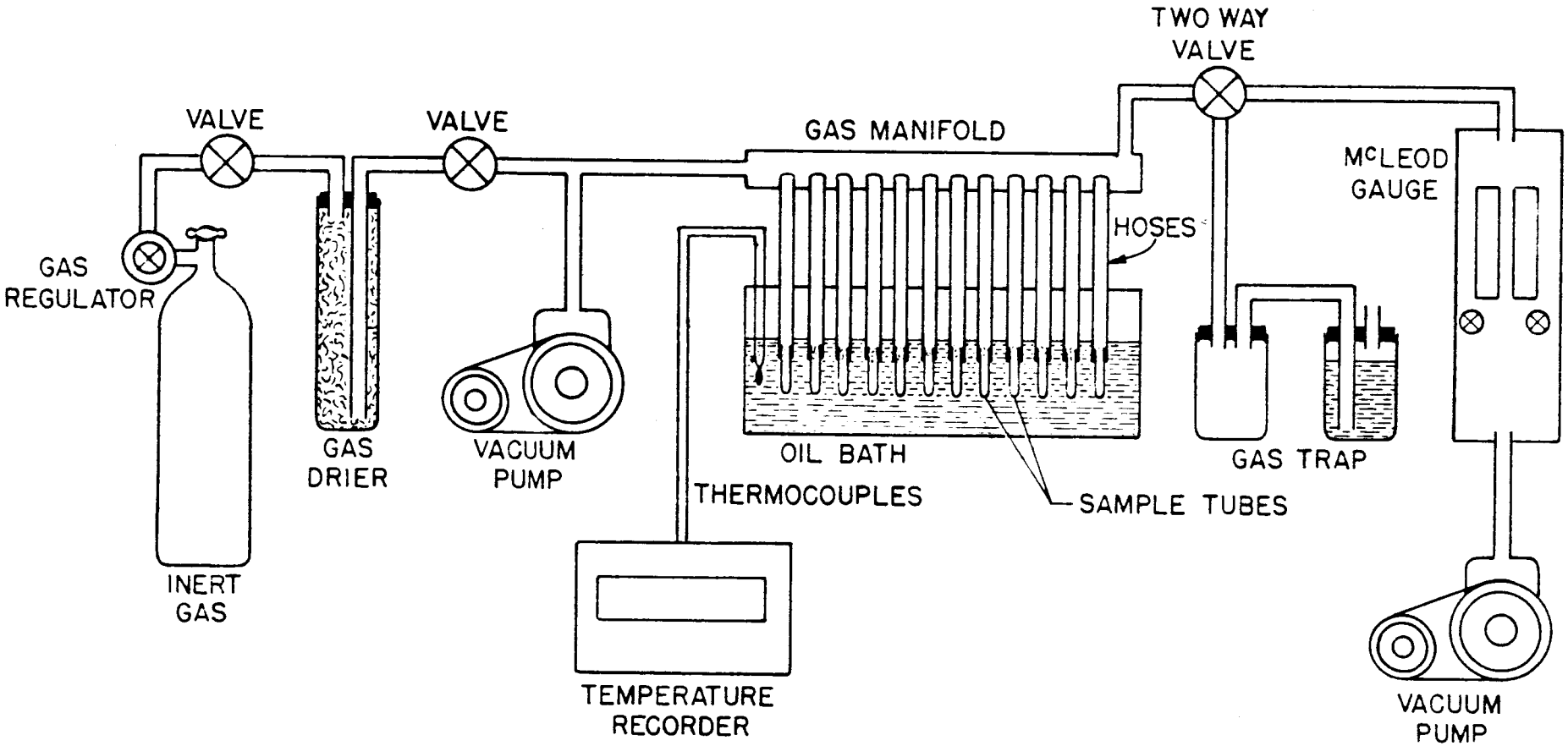

FIG. 1. SCHEMATIC DRAWING OF VACUUM – INERT GAS APPARATUS.

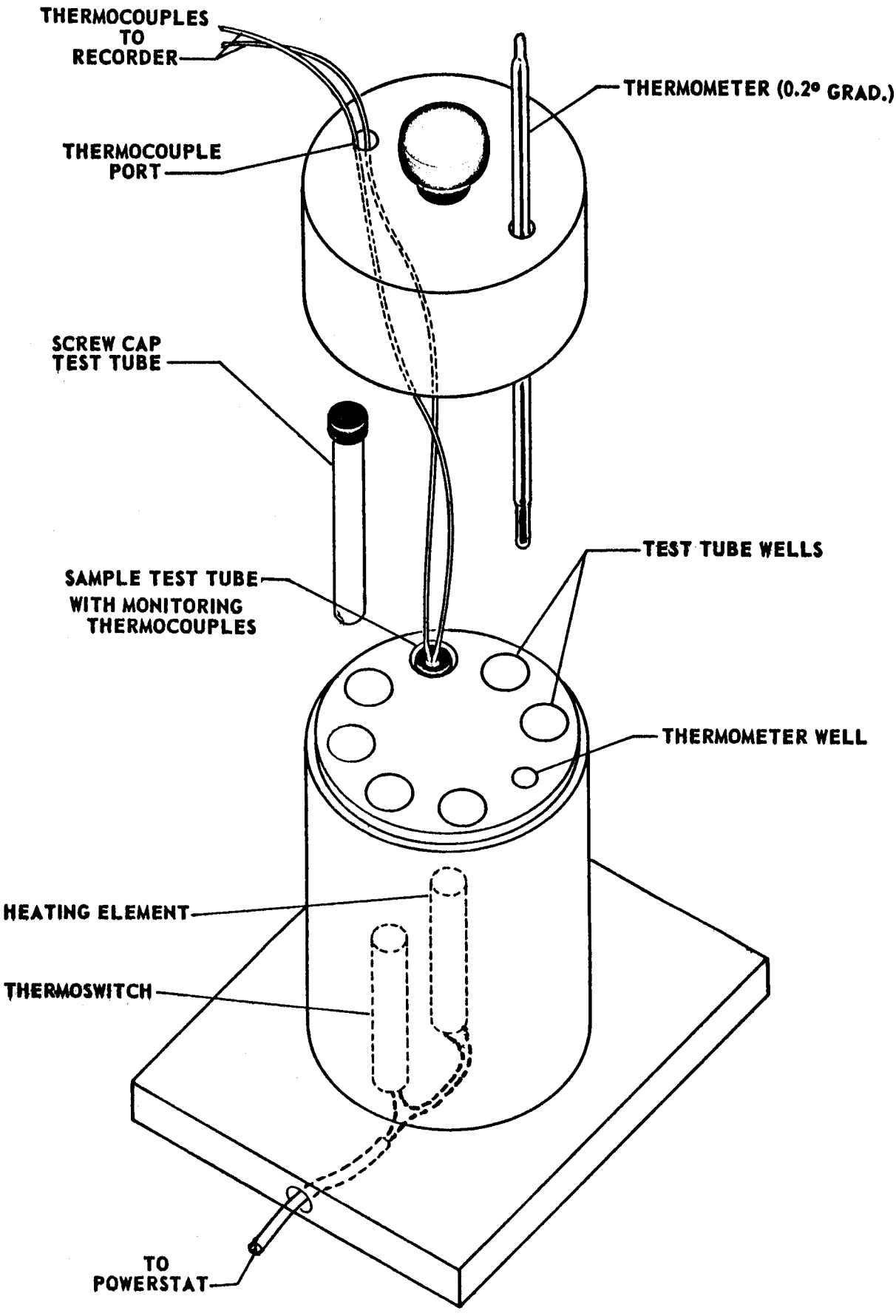

FIG. 2. CYLINDRICAL ALUMINUM BLOCK–DRY HEAT TEST UNIT

Several papers, oral and written, which are based on the work included in this report have been presented during 1961 and 1962. These are listed below:

Bruch, C. W., M. G. Koesterer, and M. K. Bruch. 1961. Studies on dry heat for the sterilization of components of astrobiological space probes. Paper given before the Central New York Branch, American Society for Microbiology, at Geneva, New York, November 18, 1961.

Koesterer, M. G., C. W. Bruch, and S. Feasy. 1961. Resistance of bacterial spores to sterilization by moist and dry heat. Paper given before the Central New York Branch, American Society for Microbiology, at Geneva, New York, November 18, 1961.

Bruch, C. W., and M. G. Koesterer. 1962. Studies on dry heat for the sterilization of electronic components of astrobiological space probes. (Abstr.) Bacteriol. Proc. A 47. Paper read at the 62nd Annual Meeting of the American Society for Microbiology at Kansas City, Missouri, May 9, 1962.

Koesterer, M. G., and C. W. Bruch. 1962. Resistance of dry bacterial spores to sterilization by moist and dry heat. (Abstr.) Bacteriol. Proc. A 44. Paper read at the 62nd Annual Meeting of the American Society for Microbiology at Kansas City, Missouri, May 9, 1962.

Bruch, C. W., M. G. Koesterer, and M. K. Bruch. 1962. Dry heat sterilization: Its development and application to components of exobiological space probes. Developments in Ind. Microbiol. 14 (In press).

Smith, F. and M. G. Koesterer. 1962. The effect of various environments on the resistance of dry bacterial spores to dry heat. (In press).