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DEVELOPMENT OF A BIOLOGICAL INDICATOR FOR DRY HEAT STERILIZATION

JPL CONTRACT NO. 951001

FINAL SUMMARY REPORT

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ABSTRACT

A biological sterility indicator was designed for dry heat sterilization at 135 C. Tablets composed of cleaned, well-dried spores of Bacillus subtilis WC18, a notably heat resistant organism, were hermetically sealed under dry nitrogen in a Teflon container. The container was surrounded by various metallized thermostable films and sealed in Aclar film. Each tablet contained about 1×10^{10} spores.

Thermal studies were conducted for exposures of 2 - 24 hours at 135 C. Only one-third of the indicators survived the constraint of survival for 18 hours at 135 C. It was found that dispersion and agitation significantly increased the incidence of positive cultures in trypticase soy broth, but a 100 percent reliable indicator was not achieved. The environmental and nutritional requirements for recovering thermally injured spores of the test organism must be established.

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INTRODUCTION

The Castle Company conducted research and development on a biological sterility test system to indicate sterility after application of dry heat sterilization. The objectives are noted with the paragraph numbers used in Article 1, Statement of Work, JPL Contract 951001.

1.(a)(1)(i)	Isolate an organism resistant to dry heat.
1.(a)(1)(ii)	Determine a proper carrier.
1.(a)(1)(iii)	Determine the organism's specific resistance, both with and without the carrier.
1.(a)(1)(iv)	Formulate a system operating procedure and functional method for practical usage.
1.(a)(1)(v)	Conduct for JPL approval performance testing to demonstrate the adequacy of the test system and the basic sterilization cycle. Such testing was to include, but not necessarily be limited to, the accumulation and recording of survival-kill data to establish that the test system indicates sterilization efficiency of the specified dry heat sterilization cycle.

The objectives were to be accomplished in accordance with the following constraints and requirements:

1.(b)(1) The dry heat sterilization cycle shall be considered as one continuous period of twenty-four hours duration at a temperature of 135 C in an atmosphere of dry nitrogen. Cycle timing shall commence when all exposed materials have been equilibrated at the specified temperature.

1.(b)(2) The prepared system of organism and carrier shall:

1.(b)(2)(i) Based on the stated cycle, show organism survival for eighteen to twenty hours and no survival after approximately twenty-three hours.

1.(b)(2)(ii) Provide stability to the extent that there shall be no demonstrable decrease in heat resistance for a minimum shelf storage period of thirty days at a temperature of 70-80 F.

1.(b)(2)(iii) Provide simplicity of operation and function, convenience of usage and performance and be

sufficiently small in physical size to allow free and easy placement and retrieval from congested internal areas of assembled electronic and mechanical gear.

1.(b)(2)(iv)
By composition, be capable of variation to meet dry heat sterilization specifications of other time/temperature relationships. This constraining condition is very desirable, but is not a mandatory requirement and is secondary to the other stated constraints.

1.(b)(2)(v)
Be contained in a sealed system having an atmosphere of dry nitrogen at a pressure slightly greater than ambient.

1.(b)(3) The test organism shall be:

1.(b)(3)(i)
Non-pathogenic.

1.(b)(3)(ii)
Cultured in a simple manner from the carrier using readily available media.

1.(b)(3)(iii)
Easily discernable as to growth.

1.(b)(3)(iv) Of such form and type to allow summary identification, having some characteristic that can be easily recognized. This constraining condition is very desirable, however it is not a mandatory requirement and is secondary to the other stated constraints.

1.(b)(4) The carrier shall be non-toxic to both the user and the test organisms.

This report provides a record and summation of the accomplishments of the research and development program. All data necessary to support the contract requirements, the conclusions and recommendations are included.

All phases of the work were coordinated with Mr. Alexander S. Irons, JPL Cognizant Engineer. Mr. Victor J. Magistrale, formerly acting supervisor of the Sterilization Group, JPL, also participated in technical discussions at the Castle Company.

The principal individuals associated with this research at the

Castle Company were: Dr. Norman S. Davis, Project Supervisor, James A. Rowe and Larry E. Neal, Microbiologists, and Richard R. Landwehrle, Technician.

1.(a)(1)(i) ISOLATION OF AN ORGANISM RESISTANT TO DRY HEAT

Spores of several Bacillus species known to be resistant to dry heat were selected as potential sterility indicator organisms. The principal test organism, B. subtilis WC18, satisfied the following criteria:

1) Notable dry heat resistance reproducible with different spore crops.

2) Non-pathogenic.

3) Readily grown on available media.

4) Massive sporulation without concurrent spore germination.

5) Autolysis of sporangia and low incidence of residual vegetative cells so as to provide free spores.

6) Spores readily washed free of cell fragments, residual vegetative cells, culture medium nutrients and metabolic products.

7) Spores maintain the non-germinated state after washing and drying as indicated by refractility when examined by phase contrast microscopy and non-staining by crystal violet demonstrated with bright field microscopy.

8) Spore viability maintained in the dry

state as indicated by agreement with initial assay values after at least a thirty day storage period at 70-80 F.

9) Spore heat resistance maintained in the dry state as indicated by agreement with initial assay values after at least a thirty day storage period at 70-80 F.

10) Dried spores germinate promptly, preferably without heat activation, and vegetative cells proliferate on available culture media.

11) The organism produces a characteristic color, growth appearance, or significant change in the culture medium.

12) No toxicity or inhibition of outgrowth observed when about 10 unheated spores were inoculated into recovery culture medium containing 10^{9} - 10^{10} dry heat-killed spores.

The cultures selected for these studies were:

1) Bacillus coagulans ATCC 8038

2) Bacillus coagulans WH-9

3) Bacillus subtilis WC18

4) Bacillus subtilis var niger Fort Detrick strain

Spores of these organisms were obtained on a) yeast extract-V-8 juice agar, b) tomato juice broth with 2 percent agar and c) TAM sporulation agar. Media (b) and (c) are commercial items. The spores were washed with sterile distilled water, cleansed by separating spore and debris layers in centrifuge cups, and assayed from distilled water suspension on trypticase soy agar.

Spores were air dried on 0.25 x 1.0 inch Whatman No. 3 MM chromatography paper strips to provide 10^5 - 10^7 spores per strip in repetitive studies with several spore crops. Each lot of spore strips was assayed to establish the average number of spores per strip. The range of spore numbers on strips in each lot was within normal variation and did not significantly influence interpretation of the heat resistance tests. Spore strips were assayed by blending the strips in sterile distilled water and plating appropriate dilutions on tryptone glucose yeast extract agar.

For heat resistance studies, spore strips were placed in

loosely capped 16 x 150 mm glass tubes which were inserted in aluminum block heaters maintained at 135 C. Tubes were withdrawn at intervals and the strips placed in individual trypticase soy broth tubes. Cultures were incubated at 37 C for 2 days. The incidence of survival with respect to time at 135 C was obtained by comparing the number of cultures showing characteristic growth of the test strain with the total number of cultures prepared from strips exposed for the same time interval at 135 C. This so-called partial survival technique was a convenient way to compare the resistance of different strains, and was adopted for the evaluation of sterility indicator performance.

Some typical thermal resistance data is presented in Table 1. Similar survival patterns were observed in other trials with these organisms. Bacillus subtilis WC18 was selected as the primary heat resistant strain during the early months of the program and consistently proved to be of high resistance whenever high quality, ungerminated spores were tested.

TABLE 1

THERMAL RESISTANCE OF SPORES ON PAPER STRIPS EXPOSED TO 135 C IN AIR

Organism	Spores per Strip	Hours at 135 C							
		1	2	3	4	5	6	7	8
		Survival Fraction							
B. coagulans 8038	1.4×10^7	-	-	6/6	6/6	4/6	0/6	-	0/6
B. coagulans WH-9	8.8×10^5	6/6	0/6	0/6	0/6	0/6	-	-	-
B. subtilis WC18	4.9×10^6	6/6	6/6	6/6	6/6	6/6	5/6	-	0/6
B. subtilis var. niger Fort Detrick strain	1.5×10^6	-	6/6	-	24/24	6/12	0/6	0/6	-

This strain was isolated from soil at the Castle Research Laboratories. A culture has been sent to JPL. The morphological and biochemical characteristics of the organism described in Table 2 were determined by methods recommended in Aerobic Sporeforming Bacteria, Smith, Gordon and Clark, 1952, Agriculture Monograph No. 16, U.S. Department of Agriculture, and in Bergey's Manual of Determinative Bacteriology, Seventh Edition, 1957, The Williams and Wilkins Co., Baltimore.

Methods for sporulating B. subtilis WC18 and the procedures used to obtain clean ungerminated spore powder are described in the Appendix. The characteristic surface growth was a feature important in establishing that a culture was "positive" since large numbers of spores cause immediate turbidity in culture media and might mask proliferation of a less hydrophobic organism.

TABLE 2

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

OF *BACILLUS SUBTILIS* WC18

Vegetative rods-	0.8 μ by 2.0 to 3.0 μ; not in chains; ends rounded; uniformly stained; no capsules; motile; Gram-positive.
Sporangia-	Very little, if any, swelling by spores.
Spores-	0.8 μ by 1.2 μ; oval; central; thin-walled; many in 48 hours.
Colonies-	Rough; opaque; spreading; offwhite.
Nutrient agar slants-	Growth abundant; echinulate; opaque
Nutrient broth-	Clear with heavy, wrinkled, waxy, tough offwhite pellicle.
Sodium chloride broth-	Growth at 7 and 10 per cent NaCl in trypticase soy broth.
Utilization of citrate-	positive
Hydrolysis of starch-	positive
Production of acetylmethyl-carbinol-	positive
Fermentation tests-	Acid without gas from glucose

TABLE 2 (CONTINUED)

Hydrolysis of gelatin-	Positive; wide zone of hydrolysis
Hydrolysis of casein-	Positive; wide zone of hydrolysis
Reduction of nitrate to nitrate-	Positive
Anaerobic production of gas from nitrate-	Negative
Anaerobic growth in glucose broth-	Slight growth; pH at 5.4 at 14 days
Anaerobic growth on nutrient agar slants-	Negative
Temperature for growth-	Good growth at 23, 37, 55 C; no growth at 60 C.

1.(a)(1)(ii) DETERMINE A PROPER CARRIER

The term substrate represents that component of the sterility indicator upon which or within which the test organisms have been placed. The carrier is the container within which the substrate is sealed. The prepared system of organism(substrate) and carrier that comprises the sterility indicator includes any flexible film outerwrap, insulation, gaseous atmosphere, thermocouples, or other items that are part of the device.

The carrier design required:

1) Simplicity of operation.

2) Convenience of usage and performance.

3) Size sufficiently small to allow free and easy placement and retrieval from congested internal areas of assembled electronic and mechanical gear.

4) Stability to the dry heat time/temperature specification.

5) Retention of a dry nitrogen atmosphere.

6) Ready separation from the test organism.

7) Selection of materials that are non-toxic to the user and the test organism.

Materials. A survey of plastics, insulating materials, silicones and ceramics was completed. Those types having properties necessary for proper fabrication, handling, and stability of the indicator during storage and during thermal exposure were procured for laboratory evaluation. Several materials were determined to be useful for different concepts of the carrier. The materials which appeared to be most promising are described in Table 3. Other thermostable materials such as the ultrahigh temperature polyimides available as films, sheets, and molding materials and marketed as Nomex (sheet) or H-film by DuPont, and Kynar (polyvinylidene fluoride), a Pennsalt Chemicals Corporation product, were not evaluated because of sealing and flexing problems. Samples of Lexan polycarbonate resin films (General Electric) were received but were not evaluated because of the heat distortion point near 130 C and permeability to gases.

Mylar polyester film (DuPont) could not be heat sealed and was not evaluated in the clear film state. On the other hand, aluminized Mylar is an excellent thermal radiation barrier by

virtue of its high reflectivity. Layers of metallized plastic films are excellent insulators when spacers are provided between layers to prevent or limit conduction. A wrinkling technique yields an embossed film which can be used without spacers and provide a system having more suitable thermal properties and lower weight than probably any other insulation. Samples of this material were obtained from a converter (Hastings and Co.) and evaluated in combination with the Teflon carrier model.

Kodar polyester film (Eastman Chemical Products) possessed excellent thermal characteristics, but was considered too permeable to gases for this application.

Carrier development proceeded from rather simple systems to well-insulated devices intended to delay substrate arrival at 135 C. The latter approach was necessary because of difficulties encountered in demonstrating organism survival for eighteen to twenty hours and no survival after approximately twenty-three hours.

TABLE 3

SOME THERMOSTABLE MATERIALS USED IN STERILITY INDICATOR DEVELOPMENT

TRADE NAME	CHEMICAL NATURE	FORM TESTED	SOURCE	SPECIAL PROPERTIES
Teflon TFE	fluorocarbon	rod, tubing	DuPont	low gas permeability
Teflon FEP	fluorocarbon	film	DuPont	low gas permeability; heat sealable; cementable
Penntube II (Teflon FEP)	fluorocarbon	tubing	Pennsylvania Fluorocarbon Co.	low gas permeability; heat sealable; transparent; shrinkable
Penntube III	trifluoro-chloroethylene	tubing, rod	Pennsylvania Fluorocarbon Co.	ibid
Aclar 22 C	fluorohalocarbon	film, 2 mil.	Allied Chemical Corp.	low gas permeability; heat sealable; thermoformable
Aluminized Aclar	fluorohalocarbon	film	Allied Chemical Corp.	low gas permeability; heat sealable
Aluminized Mylar	polyethylene terephthalate	film, embossed 0.25 mil.	Hastings & Co., Inc.	low gas permeability; excellent insulation

TABLE 3 (CONTINUED)

TRADE NAME	CHEMICAL NATURE	FORM TESTED	SOURCE	SPECIAL PROPERTIES
Nycote 7-11	nylon	liquid coating	Nycote Laboratories	tough coating, provides hermetic seal; cures at room temperature
Torr Seal	epoxy resin	two paste components	Varian Associates	provides hermetic seal; cures at room temperature; nontoxic
Vultafoam	urethane	two liquid components	United States Plastic Corp.	foamed in place; readily shaped after expansion
COHRlastic	silicone	sponge sheet	Connecticut Hard Rubber Co.	cushion; insulation
Silastic 50	silicone	gasket	Dow Corning Corp.	low compression set

Unitized sterility indicator-survival test system. If the sterility indicator and the culture medium required to demonstrate viability were sealed in a single package, the opportunity for contamination from external sources would be at a minimum, and confidence in "positive" cultures would be high. This approach was discarded in favor of separate sterility indicator and recovery systems because of the prolonged research effort and least likelihood of success.

Flexible film packets. Strip, disc and tablet substrate configurations were sealed in various films to evaluate this type of system. Although 0.5-1.0 inch packets were readily made, they exhibited the following limitations, and were eventually excluded from consideration.

1) Provided no thermal protection for the substrate.

2) Provided poor mechanical protection for the substrate.

3) Gas expansion during thermal exposure tended to cause the packet to bulge, compromising the gas atmosphere.

4) Required considerable manual dexterity to cut open and transfer substrate to culture medium in a flexible film isolator fitted with rubber gloves.

5) Pinhole-free films could not be assured.

6) Film thickness in test packets depended on the properties of the material. The same film thickness could not be tested for all materials because of film sealing requirements and flexibility.

7) Potential toxicity of plasticizers and volatile decomposition products trapped within the packet during sealing could not be evaluated.

Sealed rigid tubing. Sections of plastic tubing sealed at the ends were an advance over the film packet concept. Mechanical protection was provided the substrate by the inflexible tube wall. The Penntube (Pennsylvania Fluorocarbon Company) series of thermoplastics was well-suited for this application. Dimensional control over the carrier was easily maintained. Miniaturization was limited only by substrate size. Flattened ends formed in bar sealing favored unsupported placement in the thermal test oven as well as in the proposed application for the device. Thermocouple wires

could be sealed through one end of the tube, maintaining the position of the thermocouple junction without compromising the hermetic seal. The principal limitation of this device was the tendency of the tubing to "pinch" when an end was cut off to remove the substrate. It was difficult to manipulate these small carriers in a flexible film isolator with rubber gloves. Sealed tubing carriers received only incidental attention when the silicone rubber gasketed Teflon carrier was adopted as the primary carrier model for thermal studies with the tablet substrate.

Rigid carrier models. A 10 x 40 mm glass vial sealed with Torr Seal (Varian Associates) served as the carrier in some early experiments. This low vapor pressure epoxy resin did not appear to kill spores contained in the vial during resin curing at room temperature and did not enhance lethality at 135 C. All silicone sealants tested, including a medical grade product, produced toxic volatiles during curing.

Electric units for sealing glass ampules do not appear to be

available. Gas flame sealers could not be used since water formed in gas combustion would prevent assembly of the indicator under dry conditions. The glass vial system was time-consuming to prepare. Vial indicators occasionally burst under test at 135 C. Thermal expansion of the cured resin probably induced fracture in the glass.

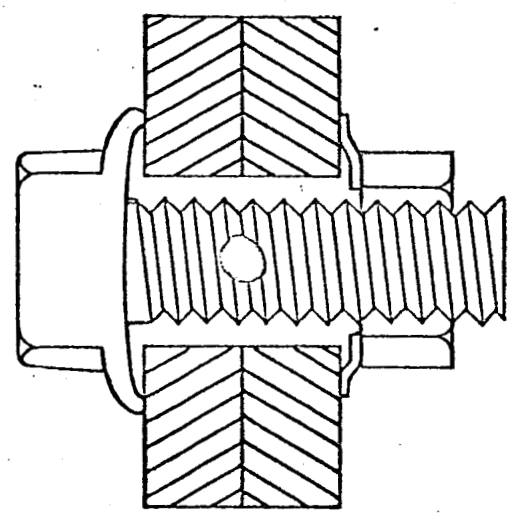
Teflon rod and tubing were used in several core-sleeve models. The basic designs consisted of a) a Teflon rod with a hole drilled transversely through the rod or b) a depression obtained by drilling partially through the rod. The substrate in disc or tablet form was contained in the space. The closely-fitting Teflon sleeve maintained the position of the substrate and provided protection against possible mechanical damage. This device provided simplicity of usage since it permitted rapid transfer of substrate to the microbial culture system. A simple ejecting tool would force the rod out of the sleeve the proper distance to permit the substrate to drop into the culture medium without intermediate handling. This sterility indicator system would be hermetically sealed by coating the

device with a liquid nylon preparation (Nycote 7-11) or by sealing it within a suitable flexible film.

The principal disadvantage of the injector-type indicator was the high potential reject rate for a device lacking a backup gas retention system in the event the hermetic seal failed. Undetected entry and absorption of moisture would reduce the reliability of the substrate to a rather low order. These sterility indicators were sealed under slight positive pressure and resisted finger pressure compression. Certain films, however, are known to absorb gases such as carbon dioxide from the atmosphere. Packets prepared from such films will build up an internal pressure sufficient to cause the initially flat packets to distend.

A modification of the injector type indicator provided a positive seal at the ends of the device. The rod component was made longer than the sleeve and the ends were threaded to fit Bartite Seal-Fast tension nuts (L.J.Barwood Manufacturing Co., Inc.). These tension nuts have a thermo-

stable polymeric sealant. The nuts were obtainable only in metal and required heavy-wall Teflon tubing in the sleeve component of the indicator. The manner in which these units were sealed is indicated schematically in Figure 1. The rod in this instance was shown threaded over its entire length and the sleeve wall thickness is exaggerated.

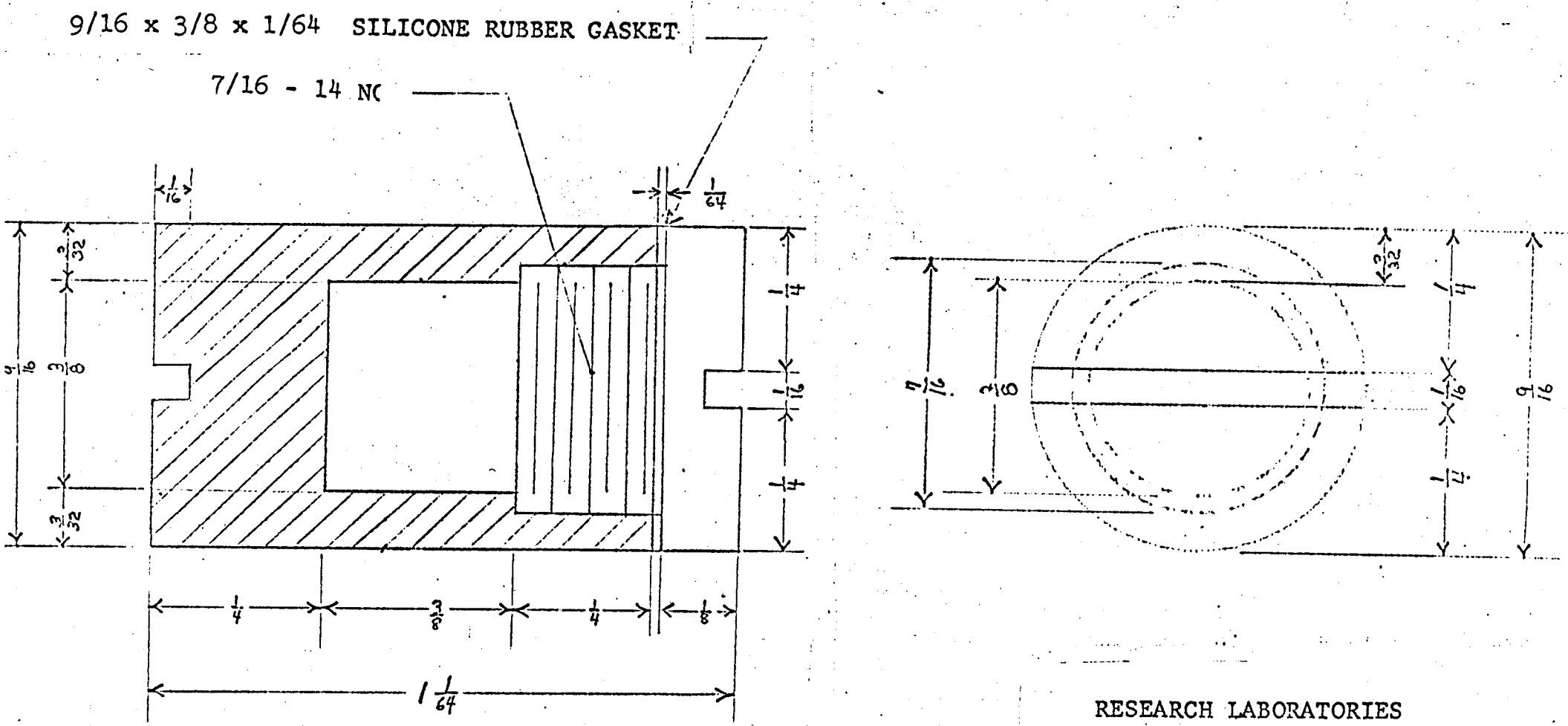


THE TENSION NUT-TEFLON CARRIER SYSTEM

FIGURE 1

The principal carrier model consisted of a Teflon hollow rod and threaded cap container (Figure 2). The central cavity held the tablet-type substrate sandwiched between silicone foam rubber pads. These pads immobilized the tablet and prevented tablet abrasion. A silicone rubber gasket under the cap head provided the primary hermetic seal. The overall dimensions of this cylindrical carrier (9/16 x 1 1/64 inch or 1.4 x 2.6 cm) were considered suitable for the intended application. The container is easily sealed or opened by hand. The container ends are slotted so that a thin metal bar may be used to aid manipulations with gloves.

The sterility indicator was easily assembled in a flexible film isolator maintained under slight positive pressure of nitrogen. A second hermetic seal was provided by a 2-mil Aclar film outerwrap. Most of the tests were run with the Teflon container-tablet-Aclar film model. This indicator required little manipulation to recover the spore tablet for assessment of viability. Films were usually sealed with a Weldotron model 8HT thermal impulse sealer.



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HERMETICALLY SEALABLE TEFLON CONTAINER

FIGURE 2

1.(a)(1)(iii) DETERMINE THE ORGANISM'S SPECIFIC RESISTANCE, BOTH WITH AND WITHOUT THE CARRIER

The number of spores necessary for an effective indicator could not be estimated from existing data. The thermal resistance of spores on surfaces and on paper strips could not be extrapolated to situations in which spores are not freely exposed to the heat transfer medium. Microorganisms appear to survive thermal exposures for longer periods when they are embedded in materials. One protective substrate system considered of a tablet of inert or nutrient material containing an inert disc holding dried spores.

Thermal death studies at 135 C were carried out with B. subtilis WC18 spores on paper strips and with cleaned spores compressed into tablets. Specific resistance data obtained in repetitive experiments with spores on paper strips were in agreement for strips prepared from the same and different spore lots. These spore suspensions consisted almost entirely of highly refractile spores when examined by phase contrast microscopy. The heat exposure and culture methods were the same as in section 1.(a)(1)(i) of this report. Spore strips holding 5×10^6 spores gave D and F values of 0.70 and 5.94 hr respectively.

The constraints on sterility indicator performance suggested that a thermal barrier system and an easily manipulated substrate were primary objectives in substrate design. Several types of tablets were considered to represent the most feasible form for the substrate. Tablets were prepared with:

1) Spores dried on inert discs which were sealed within tablets.

2) Spores mixed with various binders.

3) Spore powder as the only component.

Tablet substrate attributes. Tablet design and composition was studied from the standpoints of:

1) Selection of thermostable compressible binder materials.

2) Appearance, wettability, solubility and dispersibility of unheated and heated (105, 135, and 160 C) materials before and after compression.

3) Resistance of tablet to powdering and chipping.

4) Retention of structural integrity and appearance after heating the tablet for 24 hours at 135 C.

5) Absence of toxicity to spores in the dry state and in culture media when tested unheated and after heating at 135 C for 24 hours.

Tablet production. Dried materials were formed into tablets in a flexible film isolator having a dry nitrogen atmosphere. Tablets were fabricated with a Stokes Model E, 1/4 inch standard concavity tablet die, and with 3/16 and 1/4 inch dies which were designed to permit 1/8 inch discs to be placed in the geometric center of the tablet. Tablets prepared from both 1/4 inch dies were crowned for maximum strength. Dies holding suitable quantities of material were placed in an FC 1646 drill jig (N.A.Woodworth Co., Detroit). A Craftsman (Sears) model 4464 torque wrench was used to apply 10-50 tons per square inch final pressure for 5-10 seconds to form the tablets. This operation did not produce differences in assay data between weighed spore powder and spores in tablets. Satisfactory tablets composed entirely of spores were formed at 30 tons/in^2 pressure.

Material properties. Many thermostable materials do not have the proper powder structure to permit their use as tablet components. A variety of sugars, salts, amino acids and organic chemicals were evaluated (Table 4). Most materials were eliminated from further consideration if good tablets could not be formed at pressure up to 50 tons per square inch.

Although high purity materials were tested, some substances discolored severely at temperatures well below their melting or decomposition points.

Microcrystalline cellulose (Avicel) (FMC Corp., American Viscose Div.) was the best tablet-forming agent. Although Avicel is insoluble, this property was not significant when large numbers of spores were mixed with Avicel in tablets. It was noted previously in this report that the substantial number of spores in these experiments cause culture media to become turbid when the tablets are dispersed. Avicel was also useful in combination with materials such as sodium chloride which did not make good tablets without an auxiliary binder.

TABLE 4

NONTOXIC MATERIALS CONSIDERED FOR THE TABLET SUBSTRATE

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
Organics					
Saccharin (Blake)	229-30	+[3]	very slightly darkened	slightly darkened	good; 30 tpsi
Saccharin sodium (Key)	---	+	unchanged	---	good; 30 tpsi
Saccharin sodium-Avicel (75:25)	---	partly soluble	unchanged	---	good; 30 tpsi
Polymers					
Avicel (microcrystalline cellulose) (FMC)	---	-	unchanged	slightly darkened	excellent; 10-19 tpsi
Methocel (methylcellulose) (Dow)	---	+	unchanged	---	good; 11, 22, 33 tpsi
Polyvinyl-pyrrolidone 360 (Sigma)	---	+	slight yellowing	---	fair; 33 tpsi

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
PVP- NaCl (5:95)	---	+	slight yellowing	---	fair; 33 tpsi
PVP- Avicel (50:50)	---	partly soluble	slight yellowing	---	fair; 25 tpsi
Gelvatol 20-30 (polyvinyl alcohol) (Shawinigan Resins)	---	+	hardened	---	fair; 17 tpsi
Gelvatol 20-30 - Avicel (50:50)	---	partly soluble	hardened	---	fair; 17 tpsi
Gelvatol 1-30	---	+	hardened	---	fair; 17 tpsi
Gelvatol 1-30 - Avicel (50:50)	---	partly soluble	hardened	---	fair; 17 tpsi
Starch	---	+	unchanged	---	---
Gum arabic (Meer)	---	+	charred	---	---
Gum guar (Stein Hall)	---	partly soluble	slightly darkened	darkened	---

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
Gum acacia (Stein Hall)	---	+	darkened hardened	charred hardened	---
Gum acacia-Avicel (75:25)	---	partly soluble	charred	charred	---
Gum tragacanth (Stein Hall)	---	+	darkened hardened	charred hardened	---
Gum karaya (Meer)	---	+	charred	charred	---
Ezon (amylose, amylopectin, MgO) (Seamless Rubber)	---	---	unchanged	---	fair; 22 tpsi
Ezon-Avicel (90:10)	---	---	---	---	fair; 22 tpsi
Ezon-Avicel (50:50)	---	---	---	---	good; 22 tpsi
Amino Acids					
L-Glycine	233 d.	+	slight browning	---	good; 25 tpsi

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
L-Glycine - Avicel (85:15)	---	partly soluble	slight browning	---	good; 50 tpsi
D,L-Tryptophan (Nutritional Biochemicals)	289 d.	-	browned	---	---
L-Proline (Sigma)	220-2 d.	+	browned	---	---
L-Histidine (Calbiochem)	277 d.	+	unchanged	---	poor; 24 tpsi
L-Histidine - Avicel (50:50)	---	partly soluble	unchanged	---	fair; 24 tpsi
L-Threonine (Nutritional Biochemicals)	255-7 d.	+	unchanged	---	poor
L-Threonine - Avicel (90:10)	---	partly soluble	unchanged	---	fair; 22 tpsi
L-Threonine - Avicel (50:50)	---	partly soluble	unchanged	---	good; 22 tpsi
L-Glutamic acid (Sigma)	208 d.	+	melted	---	---

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
L-Alanine (Nutritional Biochemicals)	279 d.	+	unchanged	---	fair; 22 tpsi
L-Alanine - Avicel (90:10)	---	partly soluble	unchanged	---	fair; 22 tpsi
L-Alanine - Avicel (50:50)	---	partly soluble	unchanged	---	fair; 22 tpsi
L-Phenylalanine (Nutritional Biochemicals)	283-4 d.	-	unchanged	---	---
D,L-Phenylalanine (Eastman)	318-20 d.	-	---	---	---
D,L-Serine (Nutritional Biochemicals)	246 d.	+	slight browning	---	---
L-Valine (Nutritional Biochemicals)	315 d.	+	unchanged	---	---
D,L-Isoleucine (Nutritional Biochemicals)	292 d.	+	slight browning	---	---

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
Organic Acids					
Ascorbic acid (Merck)	191-2	+	charred	charred	---
Ascorbic acid - Avicel (75:25)	---	partly soluble	charred	charred	---
Dipicolinic acid (Aldrich)	226 d.	partly soluble	unchanged	---	good; 27 tpsi
Dipicolinic acid - Avicel (90:10)	---	partly soluble	unchanged	---	good; 27 tpsi
Carbohydrates					
Lactose (Baker)	201.6	+	unchanged	slight charring	fair; 33 tpsi
Arabinose (Calbiochem)	164.5	+	---	---	fair; 17 tpsi
Arabinose - Avicel (50:50)	---	partly soluble	---	---	fair; 17 tpsi

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
Cellobiose (Calbiochem)	225	+	---	---	poor; 17 tpsi
Cellobiose - Avicel (50:50)	---	partly soluble	---	---	fair; 17 tpsi
D-Mannitol (Calbiochem)	166.1	+	---	---	poor; 17 tpsi
Mannitol - Avicel (50:50)	---	partly soluble	---	---	fair; 17 tpsi
D-Xylose (Calbiochem)	153	+	---	---	poor; 17 tpsi
D-Xylose- Avicel (50:50)	---	partly soluble	---	---	fair; 17 tpsi
Sucrose (Baker)	186 d.	+	yellowed	---	---
Sodium Alginate (Kelco)	---	+	light brown	charred	---
Salicin (Difco)	198-202	+	unchanged	---	---

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
Inorganics					
Sand	---	---	unchanged	unchanged	---
Sand - Avicel (75:25)	---	---	unchanged	unchanged	---
Sand - Avicel (50:50)	---	---	unchanged	slightly darkened	---
Sand - Avicel (25:75)	---	---	unchanged	slightly darkened	---
Silica Aerogel (Cab-O-Sil M-5, Cabot)	---	---	unchanged	---	---
Glass beads, 297-177 μ (Micro-beads Div., Cataphote Corp.)	---	---	unchanged	unchanged	poor; 25 tpsi
Glass beads - Avicel (85:15)	---	---	unchanged	slightly darkened	poor; 81 tpsi

TABLE 4 (CONTINUED)

TYPE MATERIAL	M.P. C	SOLUBILITY[1]	APPEARANCE AFTER 24 HR 135 C	160 C	TABLET QUALITY[2]
Na_2CO_3	851	+	unchanged	unchanged	poor; 17 tpsi
Na_2CO_3 - Avicel (50:50)	---	partly soluble	unchanged	slightly darkened	poor; 17 tpsi
Na_2SO_4	884, tr.	+	unchanged	unchanged	poor; 17 tpsi
Na_2SO_4 - Avicel (50:50)	---	partly soluble	unchanged	slightly darkened	poor; 17 tpsi
NaCl	801	+	unchanged	---	poor; 25 tpsi
NaCl - Avicel (50:50)	---	+	---	---	fair; 25 tpsi

1 0.1 g in 10 ml trypticase soy broth.

2 Excellent: thermostable; compressible; soluble or dispersible; non-powdering; non-chipping with normal handling.
Good: thermostable but one or more of the "excellent" criteria is of slightly lower quality.
Fair: tablet does not retain structural integrity or abrades with normal handling.
Poor: non-compressible or adheres to the dies.

3 + : soluble; - : insoluble.

Tablets containing spores on discs. Teflon discs 1/8 inch in diameter were punched from 5- and 15 mil sheet (Cadillac Plastics), cleaned and sterilized at 160 C. Distilled water suspensions of cleaned B. subtilis WC18 spores were placed on the discs through a 25 ga hypodermic needle and air-dried. About 1-4 x 10^6 spores were deposited on each disc in various experiments. Similar discs were prepared from Reeve Angel 934-AH glass fiber paper. Discs were stored over silica gel until they were embedded in tablets.

The rate of temperature rise to 135 C and spore survival. Six-hour temperature rise. The rate of temperature rise to 135 C appears to influence the incidence of positive cultures. The phenomenon was demonstrated in an exploratory experiment with glycine-Avicel (85:15) tablets. This mixture produced satisfactory tablets which disintegrated promptly in the culture medium. Two Teflon discs each holding 1.78 x 10^6 B. subtilis WC18 spores were sealed with spore surfaces adjacent in 3/16 inch tablets of the glycine-Avicel mixture. An analogous experiment utilized single glass fiber discs sealed within Avicel tablets.

Tablet formation. A die that formed a shallow central depression in a half-tablet was used to partially compress one-half the required amount of powder. The discs were then placed in the depression and additional powder was added. The tablet was then completed with a normal tablet die. Teflon disc tablets were completed by applying a pressure of 50 tons/in^2 for 5-6 seconds while glass fiber discs were sealed in Avicel at 15 tons/in^2.

Experimental. Tablets were sealed individually in 10 x 40 mm glass vials with Torr Seal epoxy resin. The resin was cured for one day in the nitrogen atmosphere isolator used for all operations. The preparations were removed from the isolator and placed in an aluminum block heater at room temperature. A voltage controller enabled the temperature to rise linearly in 6 hours to 135 C. Two samples were removed from the heater when the temperature reached 135 C, and one or two samples were retrieved at two-hour intervals during the next 12 hours. Tablets were transferred immediately to preincubated (37 C) trypticase soy broth tubes and examined for growth after 24 and 48 hours incubation.

Results and discussion: Drops of spore suspension were easily placed on the small Teflon discs but most of the spores accumulated at the periphery of the evaporating drop. The spores dried from water tended to form a ring pattern. Drying spores from acetone suspension gave a more uniform distribution over the disc surface. The incidence of recovery after thermal exposure was not studied with respect to the distribution pattern of spores on Teflon discs. The principal disadvantage of Teflon for this application is its tendency to cold flow at room temperature after plastic deformation under load. Teflon disc tablets appeared normal at the time they were made but some stored tablets were found to have split at the disc plane.

All Teflon disc tablets removed from the heat unit within six hours after the block reached 135 C had viable spores. Cultures from broth tablets retrieved at the sixth hour had no evidence of growth at 24 hours but were positive after an additional day incubation. The culture from one of two tablets at 135 C for 8 hours was turbid and had the characteristic pellicle on the second day of incubation.

No additional cultures had growth in three weeks incubation.

The recovery pattern obtained at 6 and 8 hours in this experiment contrasted with the failure of Teflon disc tablets from the same lot to show viability after being placed directly at 135 C for 6 and 8 hours. This experiment did not include data for the 0-4 hour interval.

Data for the glass fiber disc tablets were similar to that obtained for Teflon disc tablets (Table 5). Only two of the four cultures from tablets at 135 C for 6 hours had growth in three weeks incubation. It must be recognized that these experiments served only to determine whether a disc-tablet substrate might be suitable, and there was no advantage gained by using more tablets. The D and F values are not well-defined.

The results indicate that a tablet containing approximately one million shielded spores is superior to an exposed spore strip but thermal barriers would be essential to upgrade survival time.

TABLE 5

Incidence of positive cultures at 24 and 48 hr incubation after 6 hr temperature rise to 135 C and after direct exposure to 135 C. Avicel tablets contained 1.2×10^6 *B. subtilis* WC18 spores on glass fiber discs.

Hours at 135 C	Positive cultures / Total incubated			
	6-hr to 135 C		Directly at 135 C	
	24 hr	48 hr	24 hr	48 hr
0	4/4	-	-	-
4	4/4	-	-	-
5	-	-	1/3	1/3
6	0/4	2/4	0/3	0/3
8	0/4	0/4	0/3	0/3
12	0/4	0/4	-	-
	F: 7.2 hr D: 0.9 hr		F: 5.8 hr D: 0.8 hr	

Tablets composed of spores mixed with a binder. A 1:10 mixture was prepared with dry B. subtilis var niger spores and oven-dried Avicel. Tablets (3/16 in) were formed under nitrogen at a pressure of 20 tons/in^2. The tablets were maintained for one week in a desiccator under continuous evacuation with a vacuum pump. The vacuum was broken with dry nitrogen and tablets were sealed in Aclar pouches under 5 mm nitrogen pressure. Tablets assayed about 6×10^9 spores, equivalent to 1.6×10^{11} spores per g of mixture. Pouches were placed at 135 C for 8 and 12 hours and the tablets transferred to trypticase soy broth for incubation at 32 C. None of these cultures had growth in 5 days incubation (Progress Report No.6).

Twenty-four hour temperature rise. One-fourth inch glycine-Avicel (85:15) tablets were made with two Teflon discs which each held 3.8×10^6 B. subtilis WC18 spores. Tablets were formed at 35 tons per in^2. Each tablet was sealed in a Teflon carrier coated with Nycote. Spore tablets were heated at a constant rate so as to reach 135 C in 24 hours. From 3-8 samples were removed from the oven at appropriate intervals after the oven temperature reached 135 C. Another

group of sealed tablets were placed directly at 135 C. Tablets were cultured as described for the six-hour experiment.

Results and discussion: Only one of eight samples processed at the time the oven reached 135 C showed growth. All other samples recovered after 2-12 hours at 135 C following the 24 hour warmup appeared to have been sterilized with the exception of a single anomalous positive culture from a group of four subjects at 135 C for 8 hours (Progress Report No.5). Spores were at 115 C and higher for 4 hours during the traverse from room temperature to 135 C.

All groups placed directly at 135 C for up to 4 hours had at least one positive culture. Two of the seven preparations which had been at 135 C for 4 hours, for example, had viable spores. None of the cultures from tablets at 135 C for 4.5 - 12 hours had growth.

Tablets composed entirely of spores.

Resistance of spore tablets and the need for a thermal barrier. Experiments with tablets partially constituted

with spores demonstrated that substantial numbers of spores and a thermal barrier were probably required for an indicator to resist 18-20 hours at 135 C. Pure spore tablets were sealed in several materials and exposed to 135 C for 10-24 hours in a challenge experiment aimed at determining whether this indicator conception warranted further development.

B. subtilis WC18 spore powder (see Appendix) was formed into tablets at a pressure of 30-tons/in^2. Pure spore tablets were of excellent quality. Spore Lot 1 was used to form 3/16 inch tablets while 1/4 inch tablets were made from Spore Lot 2. Lot 1 assayed 3.28×10^{11} spores per g while Lot 2 assayed 7.36×10^{11} spores per g. Tablet weights (Progress Report No.7) for 3/16 inch tablets ranged from 0.0162 - 0.0381 g, averaging 0.0238 g for 53 tablets. Tablet weights for twelve 1/4 inch tablets ranged from 0.0263 - 0.0539 g and averaged 0.0404 g.

Tablets were sealed in the following materials:

a) 2 ml Aclar 22 C.

b) Aclar overwrapped with 10 layers embossed aluminized one side 1/4 mil Mylar. The aluminized surface was outermost. The packet was then sealed in Aclar.

c) As in b) but with 30 layers of aluminized Mylar.

d) Teflon rod-tube carriers sealed in Aclar.

Tablets were incubated in trypticase soy broth at 37 C. Cultures were examined daily for growth during three weeks incubation.

Results and discussion: A summary of the data (Progress Report No.7) is given in Table 6. Aclar did not protect spores in tablets unless a thermal barrier was present. Five of the ten shielded tablets which had been in the 135 C oven for 18 hours gave positive cultures. The erratic recovery data was due in part to the small number of samples in each exposure interval.

Most of the positive cultures (11 of 16) required 6-8 days incubation. There did not appear to be any relationship between tablet weight and the incubation day at which growth was detected. Only one culture (3/16 tablet, Aclar only, 18 hr) was positive on the second day, while at the other extreme, one culture (30 layers aluminized Mylar, 18 hr)

TABLE 6

SURVIVAL RATIOS OF B. SUBTILIS WC18 SPORES IN TABLETS SEALED IN 2 MIL ACLAR 22 C AND VARIOUS THERMAL BARRIERS AFTER HEATING AT 135 C

Hours in 135 C Oven	1/4 inch Tablet	3/16 inch Tablet				Totals
		Thermal Barrier				
	None	None	10 layers Al Mylar Aclar	30 layers Al Mylar Aclar	Teflon Aclar	
10	1/3	-	-	-	-	1/3
12	-	-	2/6	1/3	-	3/9
14	0/3	-	2/3	1/3	-	3/9
16	-	0/3	2/3	-	0/2	2/8
18	0/3	1/3	1/3	2/4	2/3	6/16
20	-	0/3	-	-	1/2	1/5
22	0/3	0/3	-	-	1/3	1/9
24	-	0/3	-	-	0/3	0/6
Totals	1/12	1/15	7/15	4/10	4/13	7/65
Average tablet Weight	0.0404 g	0.0231 g	0.0268 g	0.0247 g	0.0205 g	

required 11 days to show growth. The prolonged incubation necessary for these cultures is consistent with the concept that thermally injured spores cannot proliferate until repair permits normal cell growth.

Ten - and thirty-layer aluminized Mylar appeared equivalent in maintaining spore viability. One Teflon carrier unit survived 22 hours at 135 C but required six days for outgrowth. Spores in negative cultures were almost all swollen and nonrefractile under phase contrast microscopy. Only one refractile spore might be found in ten fields. Vegetative cells were not seen.

Dispersion and oxygen requirement for growth. B.subtilis WC18 is a strict aerobe. Although broth tubes are satisfactory for routine cultivation of this organism, the available oxygen in cultures prepared with heated spore tablets may be depleted by the dead spores. Viable spores, likely in the minority, might not encounter sufficient oxygen. An experiment was devised to determine whether dispersion of the spores and increased oxygen tension favored an increase in the incidence of positive cultures from heated spore tablets (Progress Report No. 8).

Materials and methods: Freeze dried B. subtilis WC18 spore powder was formed into 3/16 inch tablets at 30 tons/in^2 pressure. Each tablet contained about 3×10^{10} spores. Twelve tablets were sealed in Aclar packets under dry nitrogen and exposed to 135 C. Four tablets were removed from the oven after 10, 14, and 16 hours. Two tablets from each exposure period were placed in tubes containing 10 ml sterile distilled water. The tubes were shaken mechanically for 10 min to disperse the spores. Portions of the suspensions were added to 250 ml screw cap flasks containing 50 ml trypticase soy broth and to tubes with 10 ml of the medium. Flask cultures were shaken during incubation at 32 C while tube cultures were undisturbed. Portions of two serial tenfold dilutions were also inoculated to trypticase soy agar slants and to broth tubes. The six remaining tablets were placed in diphasic culture tubes consisting of trypticase soy agar slants with 2 ml trypticase soy broth.

Results and discussion: Every culture prepared from dispersed spores had growth after 16 hour incubation. The diphasic cultures, on the other hand, had growth only in the two cultures containing tablets heated for 10 hr at 135 C.

Growth first appeared on the fourth day of incubation in both cultures. The diphasic cultures containing spore tablets heated for 14 and 16 hr. did not have growth in 27 days incubation. Heated B. subtilis WC18 spores appear to require dispersion and more oxygen than is available in unagitated culture medium.

Recovery of heated spores in shake flask cultures. A series of experiments supported the observation that the incidence of spore recovery was greater in shaken cultures than in static tube cultures. An experiment with B. subtilis WC18 Spore Lot 7 (Progress Report No. 8) demonstrated that spore recovery data is quite dependent on the dispersion-agitation requirement.

Tablets (3/16 in) were sealed in the Teflon carrier and over-wrapped with Aclar in an isolator containing a dry nitrogen atmosphere. Indicators were removed from the 135 C oven after 10, 16, and 18 hours. The tablets were transferred to 250 ml screw cap flasks containing 50 ml trypticase soy broth. These cultures were shaken at 37 C. The survival ratios were: 10 hr, 8/8; 16 hr, 2/4; 18 hr, 3/4. All positive cultures

were scored within 72 hours incubation. It is probable that the recovery patterns reported previously for spores recovered in broth tubes would have been improved had an agitated culture system been used.

Thermal barriers. Materials which might serve as thermal barriers were evaluated in a model heat transfer system placed in an oven at 135 C or in trials with sterility indicator units containing the spore substrate. The experimental details and the data have been discussed in various Progress Reports and will be described briefly.

Urethane foam - Aclar - aluminum foil models. A thermostable, open cell, soft urethane foam (Olympic Chemical Co.) was easily cut into 5/8 x 6 inch cylinders. Fine wire iron-constantan thermocouples were inserted longitudinally through the centers of the cylinders. The thermocouple junction was 5/8 inch from one end. The junction temperature was 101.4 C in 30 sec on a potentiometric recorder plot when the cylinders were placed in the 135 C oven. The temperature was 134.2 C in 90 sec for this poor insulating material. Cylinders were also sealed in a) 2-mil Aclar 22 C film (Allied Chemical),

b) Aclar surrounded by a bright household aluminum foil shield and c) Aclar covered with the dull surface of the aluminum foil outermost. None of these materials presented any significant barrier to heat penetration. All models were at 134 C within 90 sec.

Cooling rates were monitored when the models were removed from the oven. All barriers imposed some restriction on heat loss. The model with the outer dull aluminum foil surface required 4-5 minutes to reach room temperature (24.8 C). It is clear that an indicator system should be cooled rapidly if retrieved from a test fixture. A system which includes controlled cooling might cool the carrier and surrounding thermal barriers but heat could be retained in an insulated substrate. Lethal temperature could exist in the substrate for some time after the indicator was removed from the test chamber unless cooling time was integrated into the time-temperature program.

Rigid urethane foam. Closed cell urethane foam is a good thermal barrier. A foamed in place urethane (Vultafoam, United States Plastic Corp.) did not consistently form good rigid foams. The product did not contain a filler and would be best used in a thick cross-section surrounding the Teflon indicator

model. It would also be necessary to prepare cylinders under controlled conditions to obtain sections with appropriate cell dimensions.

Thermal properties of Teflon. The heat transfer properties of Teflon were used to calculate the rate of temperature rise in a phantom model simulating the Teflon carrier. The model system was a cylindrical Teflon rod with dimensions 1.4 x 2.4 cm, the size of the Teflon carrier (Progress Report No. 8). It was found that a cylinder placed directly at 135 C would be at 131.5 C in 5 min. and would be at 135 C within 10 min. The hollow Teflon carrier would not serve as a primary thermal barrier. Silicone rubber pads and the nitrogen gas within the carrier should not interfere significantly with heat transfer to the spore tablet. The spore mass must have a high order of inherent resistance in the absence of any insulation external to the Teflon carrier.

Sterility indicator performance upon direct exposure to 135 C - cumulative data. The incidence of positive cultures in various experiments with spore tablet - Teflon carrier indicators was totaled for each exposure period at 135 C. (Table 7). Most

TABLE 7

Cumulative incidence of positive cultures from experiments with *B. subtilis* WC18 tablet-Teflon carrier indicators placed directly at 135 C and recovered in trypticase soy broth; 10^9- 10^{10} spores per tablet.

HOURS AT 135 C	SURVIVAL RATIO POSITIVE CULTURES/TOTAL CULTURES	PERCENT RECOVERED
2	22/22	100
4	19/21	91
5	1/3	33
6	9/15	60
8	11/24	46
10	16/65	25
12	3/13	23
14	6/24	25
16	24/77	31
18	24/72	33
20	2/35	6
22	1/9	11
23	0/20	0
24	0/6	0

of this data was obtained with undisturbed broth cultures and a series of spore crops. About one-third of the units at 135 C for 18 hours had viable spores.

Although agitation during incubation provided a much higher order of recovery, a proper system must be 100 percent reliable. Statistical analysis may show that a finite number of sterility indicators of high reliability in a given thermal process could be considered equivalent to a lesser number of a hypothetical 100 percent reliable indicator. This compromise may be necessary since an extensive research effort may be required to establish the specific environmental and nutritional requirements for recovery of spores subjected to prolonged thermal stress. There is no assurance that a fully reliable system can be achieved without major alterations in the substrate and carrier.

Sterility indicator performance after controlled temperature rise to 135 C - cumulative data. Repetitive experiments in which the duration of temperature rise to 135 C was varied have been summarized in Table 8. It appears that the rate at which the temperature is raised will at least indirectly

affect the lethality attributed to heat. Surviving spores were detected through the tenth hour at 135 C in experiments involving a six-hour warmup to the test temperature. One positive culture was obtained after eight hours at 135 C when 24 hours were required to reach this temperature. The data for the 4 and 24 hour warmup suggests that more samples are required for proper evaluation of cycles likely to be unfavorable for spore viability. Furthermore, the occurrence of positive cultures in the 24 hour warmup experiment serves to illustrate that thermal death is not exclusively a thermal absorption effect.

Rapid vaporization of residual water in well-dried spores and retention of vapors may create a micro-autoclave in which spores are killed after brief exposure to an elevated temperature. Perhaps spores placed directly at 135 C and spores encountering critical temperature ranges during rapid temperature excursions are destroyed in this manner.

TABLE 8

Cumulative incidence of positive cultures from experiments with *B. subtilis* WC18 tablet-Teflon carrier indicators heated at 135 C after warmup from room temperature; 10^9- 10^{10} spores per tablet.

Hours at 135 C	Survival Ratio Positive Cultures/Total Cultures	Percent Recovered
	A. 4 hr to reach 135 C	
4	0/3	0
7	0/3	0
8	0/3	0
9	0/3	0
10	0/3	0
	B. 6 hr to reach 135 C	
0	13/15	87
2	1/1	100
3	2/2	100
4	6/6	100
5	1/7	14
6	5/13	38
7	1/8	13
8	2/8	25

TABLE 8 (CONTINUED)

Hours at 135 C	Survival Ratio Positive Cultures/Total Cultures	Percent Recovered
10	1/2	50
12	0/2	0
	C. 24 hr to reach 135 C	
0	1/4	25
2	0/4	0
6	0/4	0
8	1/4	25
12	0/4	0

Absence of toxicity in broth cultures containing heat-killed spores. Since a spore tablet might have few viable spores after being heated, it was necessary to determine whether heated tablets contained toxic materials which could interfere with growth of B. subtilis WC18 (Progress Report No. 7). The culture medium was trypticase soy broth with dispersed heated spore tablets that had not developed into positive cultures. Vegetative cells were not present in the turbid tubes which each had about 10^9 dead spores.

Twenty-four spore-broth tubes were inoculated with ca. 5 B. subtilis WC18 spores in 0.5 ml water to give a concentration of 0.5 spore per ml. After 20 hours incubation at 37 C, 19 tubes had a thick, white, opaque, wrinkled pellicle which extended as a ring about 1/4 inch above the surface of the medium. A clear zone extended about 1/2 inch below the luxuriant surface growth. The remainder of the culture was turbid with unsedimented spores.

The five tubes which did not have obvious growth were turbid throughout. Two of these cultures were found to have motile and nonmotile vegetative cells while the other 3 tubes had

no evidence of vegetative cells in 20 hours incubation. Control tubes of trypticase soy broth inoculated in the same manner all had thin white surface films and translucent ground-glass rings extending 1/4 inch above the medium surface.

The dead spores in the reinoculated tubes contributed nutrients or growth stimulants since growth was superior in these cultures than in the control cultures which did not have dead spores. The excellent growth responses from small inocula demonstrated that heated spores do not interfere with germination and development of unheated B. subtilis WC18 spores. It is probable that recovery of sublethally-heated spores might be favored in an environment rich in dead spores.

Some factors which influence spore death and the recovery of spores injured by heat. The total thermal exposure profile: warmup, duration at maximum test temperature, and cooldown interval will be the overriding factor in microbial lethality. It is evident that lethality probably occurs over a broad temperature range during the transition from

room temperature to 135 C. The rate at which organisms are heated to test temperature will influence the apparent thermal resistance because of intra- and inter-cellular phenomena which occur at various temperature ranges. Some of these phenomena are the rate of water loss, loss of volatiles, enzyme inactivation and structural changes at the cell or spore surface.

Volatiles released from the spore mass during heating are confined within the carrier and could exert some unknown action on the spores. The phenomenon might be expressed as a) lethality; b) pregermination and consequent heat death; c) inhibition interpreted as lethality when growth does not occur; or hopefully, d) germination triggered only when the spore encounters the culture medium.

It is probable that <u>any</u> test organism will have particular environmental or nutritional requirements for germination and outgrowth of unheated and heated spores. Adoption of a more heat resistant organism may not provide any advantage. The complexity of these interrelations may be illustrated

by the auto-inhibition observed in the germination of thick spore suspensions of B. globigii. The first spores to germinate release D-alanine which then inhibits germination of the remaining spores.

1.(a)(1)(iv) FORMULATE A SYSTEM OPERATING PROCEDURE AND FUNCTIONAL METHOD FOR PRACTICAL USAGE

These procedures are to be considered guidelines for a sterility indicator which has the desired performance characteristics in a given temperature program. It is assumed that the recovery method will provide the proper nutritional and physical environment for germination and outgrowth of all viable spores. It is beyond the scope of this effort to deine the specific laboratory operations for indicator code control and processing event sequence.

Quality Assurance. Twenty sterility indicators from each lot of indicators received from the vendor are to be placed in a dry heat oven set at 135 C. Ten units are to be removed after 18 hours and the remaining ten units are to be removed after approximately 23 hours. All twenty indicators are to be processed in the same manner as indicators being subjected to the specific sterilization requirement.

If sterility indicator specifications require a 100 percent reliable device, then all 10 units which had been at 135 C for 18 hours are to produce positive cultures characteristic

of the indicator organism. All 10 units which had been at 135 C for approximately 23 hours are to produce negative cultures in which there is no microbial growth detected by visual,microscopic and subculture techniques. Any deviation from these results shall be sufficient to classify the particular lot of sterility indicators not acceptable for use.

A general quality assurance provision requires that every indicator be subjected to an inspection regimen certified to have been performed by the vendor prior to being packaged in individual tamper-proof containers. The specific inspection and test procedures will be dependent on the structure of the sterility indicator and the quality control during indicator fabrication.

A general quality assurance provision not described herein will specify the manner in which sterility indicators are packaged for shipment by the vendor, the inclusion of temperature indicators which change color or structure should they encounter a given environmental temperature during package transit, the delivery means, and inspection and storage

upon receipt at the test laboratory.

Post-test Transport Container. This container will consist of a wide-mouth glass screw cap bottle. A plastic bottle may not be used because the hydrophobic nature of the material interferes with the germicidal treatment. The vertical dimension of the bottle will be at least 50 percent greater than the vertical space occupied by all indicators to be held in the container. The indicators to be contained will include all positive and negative control indicators and not more than an optimum number of indicators, possibly twenty-five, subjected to the thermal sterilization process.

Approximately 20 - 24 hours prior to use the washed bottle is rinsed with germicide on all surfaces. The bottle is then filled to two-thirds capacity with germicide solution, capped, and agitated to wet the entire interior surface.

Between six and ten hours prior to its use as a transport container the bottle is inverted, agitated briefly, and maintained on its side in a pan containing sufficient

germicide to cover the bottle and the cap. The bottle is turned at hourly intervals to wet all parts of the bottle wall.

The bottle is removed from the germicide pan, wiped with a sterile towel and placed in a sterile metal safety can for subsequent transfer to the site at which sterility indicators are being used.

Germicide. A sporicide such as a mixture of 5 parts 5 percent formalin and 95 parts 70 percent ethyl or isopropyl alcohol (v/v) is recommended.

Controls. A negative control on the recovery procedure shall consist of three coded sterility indicators which have been heated in a 160 C oven for three hours.

A positive control on the recovery procedure shall consist of three coded unheated sterility indicators.

The positive and negative controls are to be from the same

lot as the indicators subjected to the thermal sterilization process.

The positive and negative controls are placed in the Post-test Transport Container which will be used to return the indicators subjected to the thermal sterilization process to the laboratory.

Some Concepts for Biological Sterility Indicator Use.

1. Place sterility indicators and thermocouples in a spacecraft model undergoing heat transfer tests. Position each indicator near a thermocouple. Apply a proposed heat sterilization process. Culture the biological sterility indicator.

   If the indicators are sterile, adequate heat was applied to sterilize the indicator spore population. Thermal data with respect to time for each thermocouple to reach process sterilization temperature and duration at temperature can receive biological confirmation with respect to attainment of sterilizing conditions.

   If indicators show viability, insufficient heat was applied to that position of the spacecraft model, suggesting the need for redesign to promote heat transfer. The time-temperature relation is uncertain.

2. The same system as in 1 above is followed with respect to temperature control. In this case, sterility

indicators are in the same positions but thermocouples are not used.

If any indicators are now viable at sites which previously yielded sterile indicators, it is probable that heat transfer along the thermocouple wire was an important mechanism in introducing heat to the vicinity of the indicator. Furthermore, viability would indicate that heat conduction along thermocouple wire gave higher temperature readings than actually existed in the vicinity of the thermocouple junction.

3. Determine the degree of insulation that compromises indicator application in a given heat sterilization process.

4. Biological check on the application of a heat sterilization process to a spacecraft undergoing terminal sterilization. Place sterility indicators with several degrees of insulation in the same oven containing the flight capsule sealed in its

canister. Insulate sterility indicators so that one group has less insulation than the most difficult to heat area of the spacecraft, one group has insulation equivalent to that in the most difficult to heat area of the spacecraft, and one group has considerably more insulation than the worst area of the spacecraft. These units would be adjacent to a spacecraft undergoing terminal sterilization. The indicators would be retrieved after the sterilization process and cultured. Growth should occur if at all only in the indicators with more insulation than the spacecraft. Note that the flight capsule does not contain sterility indicators.

1.(a)(1)(iv) CONDUCT FOR JPL APPROVAL PERFORMANCE TESTING TO DEMONSTRATE THE ADEQUACY OF THE TEST SYSTEM AND THE BASIC STERILIZATION CYCLE.

The requirement that the test system show survival of the test organism for eighteen to twenty hours and no survival after approximately twenty-three hours at 135 C has been met by thirty-three percent of the 72 indicators exposed for eighteen hours (Table 7), six percent of the 35 indicators exposed for 20 hours and eleven percent of the 9 indicators exposed for 22 hours. Definition of the proper nutritional and physical environment for germination and proliferation of heated spores would increase the incidence of recovery, but there is no assurance that a 100 percent reliable indicator can be developed.

Data from experiments in which indicators were retrieved after 22-24 hours at 135 C tended to support the adequacy of the basic sterilization cycle. All but one of the 35 indicators in this series (Table 7) failed to grow. The mass of about 1×10^{10} spores is considerably greater than the population likely to be present in a spacecraft assembled under controlled conditions.

APPENDIX

SPORE PRODUCTION

1.0 Organism. Bacillus subtilis WC18

2.0 Primary Stock Culture. The parent stock culture of the organism is streaked on Plate Count Agar in three (3) Petri dishes. The cultures are incubated at 37 C for 20-24 hours and are examined visually for obvious or suspect contaminants and for the presence of morphologically typical colonies of the organism.

2.1 A single well-isolated colony is selected from the best acceptable Petri dish culture. Transfers are made to three (3) Plate Count Agar plates. These cultures are incubated for 20-24 hours at 37 C and examined for colony homogeneity. Morphological colony variants must not be present on the selected culture.

2.2 A single well-isolated colony is selected and inoculated to three (3) Plate Count Agar slants in screw cap culture tubes. The cultures are incubated at 37 C for 20-24 hours. One culture having typical growth is designated the Primary Stock Culture.

2.3 The Primary Stock Culture is checked for purity by preparing three (3) subcultures on Plate Count Agar.

2.4 The Primary Stock Culture is subjected to the determinative tests for B. subtilis WC18 (Table 2). All characteristics must conform to the description of the parent strain.

3.0 Reserve Stock Cultures. The two residual cultures from 2.2 are designated Reserve Stock Cultures and are stored at room temperature or refrigerated for possible replacement of the Primary Stock Culture.

4.0 Working Stock Culture. Two Plate Count Agar slant subcultures are made from the Primary Stock Culture. These cultures are incubated at 37 C for 20 hours. Should both cultures appear to have the characteristics of the organism, one culture is processed while the other is held in reserve and discarded if not required for another Spore Lot.

4.1 The Working Stock Culture is examined by phase contrast microscopy for spore uniformity, spore location in sporangia, and presence of vegetative cells which do not appear typical of B. subtilis WC18.

4.3 Subcultures are made to Plate Count Agar plates for purity check.

5.0 Sporulation Medium. Tomato Juice Broth (Difco Code 0517) with two (2) percent agar.

6.0 Roux Bottle Preparation. Three (3) liters of the sporulation medium are prepared for each Spore Lot. The medium is dispensed while hot in 150 ml portions to nineteen (19) Roux bottles.

6.1 The Roux bottles are plugged loosely with non-absorbent cotton covered with two layers of gauze. The plugged bottle mouths are covered with "dust caps" of heavy duty household aluminum foil.

6.2 The bottles are sterilized erect at 121 C and 15 lb steam pressure for 20 minutes. The medium is solidified at room temperature after the Roux bottles have been placed on their sides.

6.3 The bottles are incubated at 37 C for one day. Eighteen (18) bottles which do not show any evidence of contamination are reserved for inoculation.

7.0 Active Inoculum. The growth in the 20-hour Working Stock Culture (4.0) is washed off the slant with 10 ml sterile distilled water. The tube is agitated gently to break up any major clumps.

7.1 Each Roux bottle (6.3) is inoculated with 0.5 ml of this suspension taken directly from the Working Stock Culture tube. The Roux bottles are rocked gently to distribute the inoculum over the wet surface of the medium.

8.0 Sporulation and Autolysis. The Roux bottles are incubated flat at 45 C for seven days. The agar medium is on the lower side of the vessel during incubation.

8.1 Each seven-day Roux bottle culture is inspected for obvious contamination. Samples are taken from each bottle for Gram stains and examination by phase contrast microscopy. Only cultures which contain primarily free, refractile spores and empty sporangia are retained. In general, vegetative cells, if present, will not interfere with subsequent operations.

9.0 Harvesting Spores. The critical requirements for B. subtilis WC18 spores are: a) prompt removal of spores in water suspension from the culture medium to minimize leaching out germinating agents, b) cold

sterile distilled water, and c) gross dilution of the harvest to prevent incipient germination and consequent loss of heat resistance.

9.1 Two six-liter flasks containing two liters distilled water and a Teflon-covered magnetic stirring bar are sterilized, cooled, and stored in a refrigerator. Each flask will receive the spore harvest from nine Roux bottle cultures. When required for use, the flasks are placed on magnetic stirrers set at moderate speed.

9.2 Approximately 80 ml cold sterile distilled water is added to a Roux bottle culture. A sterile bent glass rod is moved gently across the surface of the culture medium to dislodge any adherent growth.

9.3 The spore suspension is passed through a sterile funnel holding two layers of gauze and a glass wool pad, and collected in one of the water flasks (9.1).

9.4 When the first flask has received its nine harvests, sufficient cold sterile distilled water is added to give a final volume of about five (5) liters of dilute spore suspension. The flask is wrapped with a towel soaked in ice water and the suspension stirred for one hour.

9.5 Procedure 9.4 is followed for the remaining nine Roux bottle cultures.

9.6 The suspensions (9.4 and 9.5) are passed through a continuous flow refrigerated centrifuge set to provide an effluent temperature of 4 C and 12,100 x G or 10,000 rpm for the Servall centrifuge. The flow rate is adjusted to maintain a clear effluent. The effluent is autoclaved and discarded.

9.7 The top layer of the spore pellets in the centrifuge cups separates readily from the packed spore mass and is discarded. This layer ordinarily consists of sporangia and vegetative cells (ca 1 percent) and about 50:50 refractile and nonrefractile spores.

9.8 The spore pellets are washed into a six liter flask containing four liters cold sterile distilled water and a stirring bar. The flask contents are stirred for five minutes and the flask is then refrigerated for 15-20 hours.

9.9 The centrifuge accessories are sterilized, washed, and sterilized for use.

9.10 The suspension (9.8) is removed from the refrigerator, wrapped in a wet towel and stirred for one hour.

9.11 The suspension is treated to procedures 9.6 and 9.7.

9.12 The spore pellets are suspended in about 100 ml cold sterile distilled water and subjected to procedures 9.6 and 9.7.

9.13 The cleaned spore pellets are suspended in about 100 ml cold sterile distilled water and reserved in the refrigerator for freeze-drying.

9.14 Crystal violet stained slides and unstained preparations from the clean spore suspension (9.13) are examined by bright light and phase contrast microscopy for an estimation of the proportion of ungerminated spores (unstained, bright refractile), germinated spores (stained, grey or dark refractile, swollen), and cell debris.

9.15 An acceptable spore crop should have at least 90 percent ungerminated spores and little evidence of cell debris and vegetative cells. These procedures have consistently given high quality spore crops.

10.0 Freeze-Dried Spore Powder. The general principles found to yield a quality spore powder are described. These techniques can be modified for any equipment that can perform the same functions.

10.1 Approximately 50 ml of the spore concentrate from 9.13 is placed in a sterile Roux bottle. The bottle is stoppered and placed on its side in a freezer operating at -20 C for about three hours.

10.2 The remainder of the spore concentrate is added to the Roux bottle in 10.1 and frozen onto the other side of the bottle.

10.3 An equipment train is set up to include in sequence: a) Roux bottle with frozen spores, b) cold trap charged with dry-ice-Freon 11, c) oil diffusion pump and d) rotary vacuum pump.

10.4 The Roux bottle with frozen spores is set in an insulated container in which the Roux bottle is surrounded by a 15 percent sodium chloride solution previously cooled to -10 C.

10.5 The rotary pump is turned on and the oil diffusion pump is operated when 500 μ pressure is reached. Pressures of 4×10^{-3} torr have been achieved in this vacuum system. The freeze-drying process requires 2-3 days.

10.6 The brine temperature is controlled with dry ice. When most of the water has been removed from the spores, the brine temperature is raised to -5 to -3 C. The Roux bottle containing essentially dry spores is maintained at room temperature during the final hours of drying.

10.7 The evacuated Roux bottle containing dry spores is isolated from the vacuum train by a clamp and the remainder of the system is brought to room temperature and pressure.

10.8 The sealed Roux bottle is transferred to a sterilized flexible film isolator. The vacuum is broken in a dry nitrogen atmosphere. The spore crust in the Roux bottle disintegrates into a fluffy spore powder which is readily formed into tablets.

10.9 The spore powder may be stored in a sealed container in a -20 C freezer until required.

10.10 The spore powder is placed under continual evacuation for one week.

10.11 Procedure 10.8 is followed, and the spores are formed into tablets without further delay.

11.0 Quality Assurance. One tablet is subjected to biological assay to determine the number of viable refractile spores, per gram of spore powder, and to assume that the Spore Lot is of suitable thermal resistance.

11.1 The tablet suspension from 11.0 is diluted to give a spore concentration suitable for applying approximately 1×10^6 spores to a group of 0.25 x 1.0 inch filter paper strips. The strips are air dried and subjected to routine thermal resistance tests at 135 C.