Effect of Preservatives on the Heat Sterilization of Sesame Oil

By ROBERT L. ROBISON* and MELVIN H. WEINSWIG†

There is little information available concerning heat sterilization of oils containing antibacterial agents. Two species of thermoresistant bacterial spores, B. subtilis and B. stearothermophilus, were used as positive controls to determine requirements for the dry heat sterilization of sesame oil containing 0.5 per cent phenol and 2 per cent benzyl alcohol. Sesame oil, polyethylene glycol (PEG), and air controls were also studied. Spores were impregnated on filter disks, dried, and sterilized in oil. Data were collected in the range of 100–160° at 0.5-hr. intervals to 2 hr. Phenol significantly reduced the sterilization requirements for B. subtilis; benzyl alcohol gave variable results. Controls survived higher temperatures in air than in oils. The sterilization requirements for B. stearothermophilus were not altered by the presence of phenol or benzyl alcohol. Sterilization in oils required higher temperatures than in air. Spores of both organisms were killed at lower temperatures in PEG than in the oils.

THE VIEW is widely held that most antibacterial agents have limited activity in anhydrous systems. Under normal conditions and within the context of the usage of preservative agents in oil vehicle injectables, there is ample evidence to support the premise that preservative agents are, indeed, ineffective if water is absent (1, 2).

Injectable grade fixed vegetable oils are normally anhydrous or contain only traces of moisture and it is unlikely that any currently acceptable parenteral preservative is of value in the usual sense unless microbial contamination is introduced into an ampul in a moist condition. The effectiveness of the preservative agent would be dependent upon its transfer from the oil to the aqueous phase where it would exert an effect if time and concentration values were adequate (3).

In an aqueous medium, the activity of antibacterial agents generally increases with rises in temperature. This increased activity is utilized in the sterilization process known as "heating with a bactericide" (4).

There is little information relating to the activity of antibacterial agents in oils at higher temperatures. This is probably due to the reported ineffectiveness of preservatives in oils which have been tested at ambient temperatures.

In reference to the sterilization of nonaqueous injections, the U.S.P. (5) states "non-aqueous injections in sealed containers may fail to be sterilized if the customary temperature and time are used, since in the absence of water vapor within the container the processing is only the equivalent of dry heat sterilization. Processing of such solutions must be accomplished by heating at higher temperatures in a dry heat sterilizer, the cycle being dependent upon the stability of the particular solution. The process should be selected only on the basis of previously demonstrated ability to sterilize representative samples of such products which have been inoculated with thermoresistant spores." The U.S.P. indicates that 2 hr. at 160° is a suitable dry heat sterilization cycle.

The requirements for the dry heat sterilization of injectable oils have not been studied extensively. Pasquale et al. (6) determined the time and temperature requirements for the sterilization of unpreserved vegetable oils inoculated with the spores of 3 species of aerobic bacilli and found that the requirements varied both with the species and the oils used. The maximum survival time observed was for B. subtilis in sesame oil and in corn oil, being 2 hr. at 150°. Perkins (7) recommends that oils be sterilized by heating for 2 hr. at 160°, the timing to start after the oil has reached full sterilization temperature. The B. P. recommends oils be sterilized by holding for 1 hr. at 150° (4).

It was the purpose of this investigation to determine if two commonly used preservatives would lower the time and temperature requirements for the dry heat sterilization of a commonly used injectable oil containing thermoresistant spores.

EXPERIMENTAL

Equipment—A circulating hot air oven[1] was used for all dry heat sterilization tests. The temperature of the oil was monitored with a battery operated potentiometer[2] with copper-constantan thermocouples.

Materials—Sesame oil U.S.P.,[3] polyethylene glycol 300,[4] polyethylene glycol 400,[5] phenol reagent, and benzyl alcohol reagent.

Organisms—A preliminary series of experiments was conducted to obtain data on the dry heat resistance characteristics of five strains of nonpathogenic aerobic bacilli. Four strains of mesophilic spore formers and a thermophilic strain were evaluated.

Mesophiles—Three strains of *Bacillus subtilis* and one strain of *Bacillus cereus* were obtained from the following sources: a spore suspension of *B. subtilis* from Dr. H. Allisbaugh, Eli Lilly and Co. (HA), a lyophilized spore culture of *B. subtilis* from Mr. R. Russomanno, Hoffmann-LaRoche, Inc. (HL), a lyophilized spore culture of *B. subtilis* (ATCC 9372), and a spore suspension of *B. cereus* (ATCC 13061) from Mr. R. P. Roman, Eli Lilly and Co. The spore suspensions were used as received. The lyophilized cultures were inoculated into media to grow a suitable number of spores for the study.

Thermophile—*Bacillus stearothermophilus* was obtained by culturing a commercial autoclave control[6] which contained spores of this organism. The strain has been identified in the literature as NIH No. 7953 (8).

Growth and Harvest of Spores—*Mesophiles*—The *B. subtilis* (HL) lyophilized culture was inoculated into fluid thioglycollate medium U.S.P. (BBL) from which a 24-hr. culture was transferred to nutrient agar and held 7 days at 30–32°. The agar culture was then held at room temperature for 1 week during which sporulation became nearly complete. A spore suspension was prepared by washing off the growth with normal saline. The suspension was heated in a water bath at 80° for 15 min. to kill any vegetative cells remaining. A count by the most probable number (MPN) method (9) in fluid thioglycollate indicated 240 million viable spores per ml.

A 20-day-old fluid thioglycollate culture of *B. subtilis* (ATCC 9372) was harvested by centrifuging and resuspending three times in sterile distilled water. The suspension which contained approximately 60% spores was heated for 15 min. at 80° to kill the remaining vegetative cells. The MPN count in fluid thioglycollate was approximately 70 million viable spores per ml.

Thermophile—*B. stearothermophilus* spore strips were transferred into fluid thioglycollate and incubated for 48 hr. at 60°. The growth was harvested by centrifuging and washing three times with distilled water. The cell suspension which was

comprised mostly of vegetative cells with a low percentage of spores was boiled 30 min. in a water bath. An inoculum from the heated suspension was then transferred two times in fluid thioglycollate followed each time by centrifuging, washing, and heating the cell suspension as before. The percentage of sporulation increased with each subculture and was approximately 50% on the third transfer. This procedure seemed to produce more synchronous sporulation and uniform morphology than other methods investigated and was perhaps a variant selective mechanism. Water replacement techniques (10) followed by agitation on a rotary shaker were unsuccessful as there was a marked tendency for this procedure to produce variable spore forms with low viable counts.

Preparation of Spore Disks—One-half inch filter paper disks[7] were sterilized by heating overnight at 105°. The sterile disks were then impregnated with appropriate dilutions of the spore suspensions using a 0.1-ml. capillary pipet. The disks were impregnated in a sterile enclosed stainless steel hood to prevent incidental air-borne contamination of the disks. They were then stored over silica gel in a desiccator for a minimum of 48 hr. prior to initial heat resistance studies.

Initial Resistance Studies—*Mesophiles*—Dried spore disks were placed in screw-cap bottles and heated in the preheated oven at 130° in 0.5-hr. increments to 2 hr. Disks were removed at the designated intervals and tested for growth in fluid thioglycollate medium U.S.P.

The results as shown in Table I indicated disks prepared from the *B. subtilis* (HL) and ATCC 9372 strains were the most resistant to dry heat.

Further evaluation of *B. subtilis* (HL) and *B. subtilis* ATCC 9372 indicated that the ATCC 9372 strain was somewhat more resistant. When drying was extended to 6 days the spores of ATCC 9372 survived 2 hr. at 130° while the spores of *B. subtilis* (HL) became less heat resistant. Results in tests described later show even greater resistance for the ATCC 9372 strain.

Initial Resistance Studies—*Thermophile*—The freshly harvested spore suspension of *B. stearothermophilus* was heated in a boiling water bath for 1 hr. to kill vegetative cells. The viable count by the MPN method was approximately 3,500,000 spores per ml. Dilutions of this stock suspension were made for the preparation of the disks.

A series of experiments were conducted with the spore disks to determine the proper load, the effect of drying on the resistance, and a suitable recovery medium for the spores of *B. stearothermophilus*. Since the spores of this organism are primarily used as an autoclave control, it was thought that it would also be of interest to compare its relative resistance to moist and dry heat.

Preliminary studies were conducted with disks containing approximately 50,000 spores each. In the initial experiment, the disks were dried for 2 days before testing for heat resistance and were cultured in fluid thioglycollate only. A second experiment was conducted with disks that had been dried for 6 days. Fluid thioglycollate with and without the addition of 0.1% soluble starch were used as recovery media.

TABLE I—DRY HEAT RESISTANCE OF MESOPHILIC SPORES[a]

Organism	Spores/Disk	Control, No Heat	0.5	1.0	1.5	2.0
				——130°C., hr.——		
B. subtilis (HA)	22,000	5/5[b]	5/5	0/5	0/5	0/5
B. subtilis (HL)	70,000	5/5	5/5	4/5	0/5	0/5
B. subtilis (ATCC 9372)	110,000	5/5	5/5	5/5	3/5	0/5
B. cereus (ATCC 13061)	100,000	5/5	0/5	0/5	0/5	0/5
	200,000	5/5	0/5	0/5	0/5	0/5
	400,000	5/5	0/5	0/5	0/5	0/5

[a] Disks were dried a minimum of 2 days over silica gel before use. [b] 5/5 = number of tubes showing growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 7 days at 30–32°.

TABLE II—EFFECT OF DRYING ON RESISTANCE IN MOIST HEAT
(B. stearothermophilus, 50,000 SPORES/DISK)

Days[a] Dried	Culture Medium	0	1	2	3	4	5	6	7	8	9	10
						—Time, min. at 121°C. Steam—						
2	FT[c]	2/2[b]	2/2	2/2	2/2	2/2	2/2	0/2	...	...	...	...
6	FT	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2
	FTS[d]	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2

[a] Dried over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. Incubated 4 days at 60°C.

TABLE III—EFFECT OF DRYING ON RESISTANCE IN DRY HEAT
(B. stearothermophilus, 50,000 SPORES/DISK)

Days[a] Dried	Culture Medium	0	15	30	45	60	75	90
				—Time, min. at 130°C. Dry Heat—				
2	FT[c]	2/2[b]	1/2	0/2	0/2	0/2	0/2	0/2
6	FT	2/2	2/2	1/2	0/2	0/2	0/2	0/2
	FTS[d]	2/2	2/2	2/2	0/2	0/2	0/2	0/2

[a] Dried over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. Incubated 4 days at 60°C.

The results as shown in Tables II and III indicated that additional drying had caused a significant increase in resistance, especially to dry heat. The slight increase in recovery of dry heated spores in fluid thioglycollate containing soluble starch was not considered significant because of the very limited number of disks tested at each temperature. The initial results demonstrated that the spore was inadequate and that a heavier load should be used in subsequent studies.

The composition of the recovery medium has an important influence on the germination and outgrowth of bacterial spores and thus will affect resistance values. This is particularly so if the spores have been damaged in some way such as by heat or chemicals since damaged cells may become fastidious and require specialized media (11). It was believed the reliability of the results would be improved if additional media were evaluated for the recovery of B. stearothermophilus. The U.S.P calls for the use of fluid thioglycollate medium in the official sterility test; however, the literature suggests that the inclusion of soluble starch or phytone in the medium might improve the recovery of heat damaged spores of B. stearothermophilus (8, 12–14). The three media evaluated to determine a suitable recovery medium were: fluid thioglycollate medium, fluid thioglycollate plus 0.1% soluble starch, formulated phytone–starch medium containing phytone peptone 1.5%, trypticase peptone 1.0%, dextrose 0.5%, soluble starch 0.1%, sodium chloride 0.15%, pH adjusted to 7.2.

Disks containing approximately 100,000 spores each were used to determine the effect of the increased spore load and to compare the three media. The results, as shown in Tables IV and V, indicated greater resistance from the heavier spore load and improved recovery with the fluid thioglycollate containing soluble starch. Growth by visual inspection was essentially the same with fluid thioglycollate medium with or without starch and was somewhat greater than with the phytone–starch medium. Visible growth occurred sooner, however, with the phytone–starch medium than with either of the thioglycollate media. The recovery after dry heat was slightly greater in fluid thioglycollate with starch than in the phytone–starch medium. Both media gave appreciably better recovery than the fluid thioglycollate without starch.

The fluid thioglycollate with soluble starch also gave the best recovery after steam heat. The phytone–starch medium showed a definite tendency toward incomplete recovery after sublethal time intervals in steam heat. These results tend to support the theory that moist and dry heat kill or damage bacteria by different mechanisms (15). It has been suggested that starch has an adsorption effect on substances in the medium that repress spore germination (16).

Oil Sterilization—Dried spore disks were inserted into 10 ml. size ampuls, filled with 5 ml. oil, and flame sealed. Oil sections included 10 ampuls each of sesame oil, sesame oil containing 0.5% phenol, and sesame oil containing 2% benzyl alcohol. Controls consisted of spore disks in PEG 300 and in air. The sealed ampuls were placed into the pre-

TABLE IV—EFFECT OF RECOVERY MEDIUM ON RESISTANCE IN MOIST HEAT
(*B. stearothermophilus*, 100,000 SPORES/DISK[a])

Culture Medium				Time, min. at 121°C. Steam					
	0	5	6	7	8	9	10	11	12
FT[c]	2/2[b]	5/5	5/5	5/5	4/5	0/5	0/5	0/5	0/5
FTS[d]	2/2	5/5	5/5	5/5	5/5	1/5	0/5	0/5	0/5
PS[e]	2/2	5/5	2/5	4/5	3/5	0/5	0/5	0/5	0/5

[a] Dried 2 days over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. [e] Phytone–starch medium. Incubated 4 days at 60°C.

TABLE V—EFFECT OF RECOVERY MEDIUM ON RESISTANCE IN DRY HEAT (*B. stearothermophilus*, 100,000 SPORES/DISK[a])

Culture Medium			Time, min. at 130°C. Dry Heat			
	0	15	30	45	60	75	90
FT[c]	2/2[b]	5/5	5/5	2/5	0/5	0/5	0/5
FTS[d]	2/2	5/5	5/5	4/5	2/5	0/5	0/5
PS[e]	2/2	5/5	4/5	4/5	1/5	0/5	0/5

[a] Dried 2 days over silica gel. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. [c] Fluid thioglycollate medium. [d] Fluid thioglycollate medium plus 0.1% soluble starch. [e] Phytone–starch medium. Incubated 4 days at 60°C.

heated oven at the designated temperature plus a 2° override to reduce the decrease in the rate of heat uptake that occurs as the temperature approaches final temperature. The controller was reset after the oil reached the designated temperature. The oil temperature was determined with a potentiometer which was connected to a thermocouple placed in an identical ampul containing 5 ml. sesame oil. The time required to reach the designated temperature (lag time) as well as the temperature during the run was monitored each time. The temperature range was from 100 to 160° in 10° increments. Ampuls were removed at 0.5, 1, 1.5, and 2 hr. after the oil reached temperature. The lag time of the oil varied only slightly from run to run, regardless of the sterilization temperature, ranging from a low of 45 min. to a high of 55 min.

Sterility Testing—Sterility tests were performed within 12 hr. of the time of sterilization. The ampuls were wiped with 5% phenol, flamed and broken open, and the oil allowed to drain. The disks were then aseptically removed with sterile-flamed tweezers, tapped lightly to remove excess oil, and transferred into fluid culture medium in 30 × 165 mm. tubes. The *B. subtilis* disks were tested in fluid thioglycollate medium U.S.P. Incubation was for 14 days at 30–32°. The *B. stearothermophilus* disks were tested in fluid thioglycollate medium containing 0.1% soluble starch. Incubation was for 4 days at 60–62°.

The tubes, which contained approximately 40 ml. of medium, were shaken vigorously by hand at the time the disks were placed on test and at periodic intervals throughout the incubation periods to disperse oil remaining on the disks and to effect transfer of the spores to the aqueous media.

RESULTS AND DISCUSSION

B. subtilis ATCC 9372—Interpretation of the results obtained with *B. subtilis* was complicated initially by a change in resistance that occurred during the approximately 5-week period in which the first series of oil tests was conducted. In the preliminary heat resistance studies, disks containing approximately 110,000 spores, which were dried for 2 days over silica gel prior to use, had a maximum resistance of 1.5 hr. at 130° in air. Disks that had been dried for 6 days were used for the initial oil sterilization at 130°. Ten disks per each time increment were included as air controls. The survival in air at this sterilization temperature was 2 hr., with nine of ten disks producing growth when cultured. Although this represented a change in resistance, the magnitude of the change was not recognized until later tests were conducted at 140°. With the first tests at 130° completed, it appeared that the preservatives had caused a decrease in the sterilization time and that the spores would survive less time in the oils or PEG 300 than in air.

As tests were conducted at the other temperatures designated for the study a rather irregular pattern of sterilization results emerged. Spores that initially survived only 1 hr. at 130° in sesame oil later survived 1 hr. at 140°. Spores that had been sterilized in 1.5 hr. at 120° in sesame oil with 2% benzyl alcohol later were not sterilized after 2 hr. at 130°. The 4 days additional drying perhaps accounted for the increased resistance first observed in air at 130°. The spore disks, however, continued to show changes in resistance which could not be related to drying of the disks as they had been stored in sealed jars after 6 days drying over silica gel.

It was noted that while sterilization in unpreserved sesame oil required 1.5 hr. at 130° and later 1.5 hr. at 140°, sterilization in sesame oil with benzyl alcohol had shown "skips" at 130° with minimal but definite survival at 140°. Repeat tests were then conducted in the range of the critical sterilization and sublethal temperatures and a quite different pattern of resistance was seen, especially as regarded the sections with benzyl alcohol in sesame oil.

The results obtained on spore disks less than 5 weeks of age are shown in Table VI. On the basis of these results alone, and relating the one survivor out of ten after 0.5 hr. at 140° to an extremely resistant spore, it would have been enticing to conclude that both the preservatives tested had a definite lowering effect on the sterilization requirements for sesame oil. Data acquired using spore disks which were aged more than 5 weeks showed that this was not the case with benzyl alcohol. The repeat tests indicated that the spores had an increased resistance to the action of benzyl alcohol but not to phenol.

The data in Table VII were extracted from all the *B. subtilis* test results without regard to the age of the spores, and represent the maximum resistance observed with each of the sterilization vehicles.

The results, although they do not help explain

TABLE VI—DRY HEAT STERILIZATION TESTS OF *B. subtilis* ATCC 9372 (110,000 SPORES/DISK[a])

Temp., °C.	Control, No Heat	0.5	1.0	1.5	2.0
			Air		
100	2/2[c]	5/5	5/5	5/5	5/5
110	2/2	5/5	5/5	5/5	5/5
120	2/2	5/5	5/5	5/5	5/5
130	2/2	10/10	10/10	10/10	9/10
140	2/2	5/5	4/5	2/5	3/5
150	2/2	0/5	0/5	0/5	0/5
160	2/2	0/5	0/5	0/5	0/5
			PEG 300		
100	5/5	0/5	0/5	0/5	0/5
110	5/5	0/5	0/5	0/5	0/5
120	5/5	0/5	0/5	0/5	0/5
130	5/5	0/5	0/5	0/5	0/5
			Sesame Oil		
100	5/5	8/10	8/10	10/10	10/10
110	5/5	10/10	10/10	10/10	10/10
120	5/5	10/10	8/10	9/10	6/10
130	5/5	7/10	2/10	0/10	0/10
140	5/5	4/10	1/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
			Sesame Oil + 0.5% Phenol		
100	5/5	8/10	6/10	4/10	5/10
110	5/5	0/10	1/10	3/10	2/10
120	5/5	0/10	0/10	0/10	0/10
130	5/5	0/10	0/10	0/10	0/10
140	5/5	0/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
			Sesame Oil + 2% Benzyl Alcohol		
100	5/5	9/10	10/10	7/10	7/10
110	5/5	9/10	8/10	6/10	7/10
120	5/5	3/10	1/10	0/10	0/10
130	5/5	0/10	0/10	0/10	0/10
140	5/5	1/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10

[a] Aged less than 5 weeks. [b] Time after sesame oil reached designated temperature. Air controls not corrected for difference in lag time between oil and air. [c] 2/2, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 14 days at 30–32°C.

why, point to a possible difference in mechanism of phenol and benzyl alcohol or to different mechanisms of spore resistance to the activity of the preservatives. It would also suggest that the resistance mechanism may be age dependent. Although there was no additional drying of the spore disks beyond 6 days, it is possible that a further loss in spore moisture content took place by equilibration with the disk material. If this hypothesis were accepted, then it could be suggested that the benzyl alcohol activity was dependent upon the moisture content of the spores whereas phenol activity was not dependent on moisture.

Additional tests were conducted at irregular intervals over a 8-month period with essentially the same findings, *i.e.*, aged spores continued to be resistant to benzyl alcohol but not to phenol.

Relation of Phenol Activity to Vehicle—Additional tests were run with another lot of sesame oil

and mineral oil since earlier results indicated that phenol markedly reduced the sterilization requirements for sesame oil containing spores of *B. subtilis*. Although these tests were conducted at only limited time and temperature ranges, the results point out the significant effect of the vehicle on the resistance of the spores and the effect of the vehicle on the preservative action. The results as shown in Table VIII indicate that the resistance of the spores was about 10° greater in light mineral oil than in sesame oil and that phenol exerts less effect in mineral oil than in sesame oil. The results obtained with the second lot of sesame oil did not vary appreciably from those of the first lot.

Effect of Drying on Resistance of B. subtilis to Benzyl Alcohol—A second batch of spore disks was prepared from the original *B. subtilis* spore suspension. The MPN count on the suspension, which was

TABLE VII—DRY HEAT STERILIZATION TESTS OF *B. subtilis* ATCC 9372 AT MAXIMUM RESISTANCE LEVEL (110,000 SPORES/DISK)

Temp., °C.	Control, No Heat	0.5	1.0	1.5	2.0
			Air		
100	2/2[b]	5/5	5/5	5/5	5/5
110	2/2	5/5	5/5	5/5	5/5
120	2/2	5/5	5/5	5/5	5/5
130	2/2	10/10	10/10	10/10	9/10
140	2/2	5/5	4/5	2/5	3/5
150	2/2	0/5	0/5	0/5	0/5
160	2/2	0/5	0/5	0/5	0/5
			PEG 300		
100	5/5	0/5	0/5	0/5	0/5
110	5/5	1/5	0/5	0/5	0/5
120	5/5	0/5	0/5	0/5	0/5
130	5/5	0/5	0/5	0/5	0/5
			Sesame Oil		
100	5/5	8/10	8/10	10/10	10/10
110	5/5	0/10	10/10	10/10	10/10
120	5/5	10/10	10/10	10/10	10/10
130	5/5	10/10	8/10	8/10	7/10
140	5/5	4/10	1/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
			Sesame Oil + 0.5% Phenol		
100	5/5	8/10	6/10	4/10	5/10
110	5/5	0/10	1/10	3/10	2/10
120	5/5	0/10	0/10	0/10	0/10
130	5/5	0/10	0/10	0/10	0/10
140	5/5	0/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10
			Sesame Oil + 2% Benzyl Alcohol		
100	5/5	9/10	10/10	10/10	10/10
110	5/5	10/10	10/10	10/10	10/10
120	5/5	10/10	9/10	8/10	8/10
130	5/5	10/10	10/10	7/10	6/10
140	5/5	1/10	0/10	0/10	0/10
150	5/5	0/10	0/10	0/10	0/10
160	5/5	0/10	0/10	0/10	0/10

[a] Time after sesame oil reached designated temperature. Air controls not corrected for difference in lag time between oil and air. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 14 days at 30–32°C.

TABLE VIII—EFFECT OF STERILIZATION VEHICLE ON PHENOL ACTIVITY (*B. subtilis*, 110,000 SPORES/DISK)

Temp., °C.	Time, hr.[a]			
	0.5	1.0	1.5	2.0
		Sesame Oil[c]		
130	10/10[b]	8/10	8/10	7/10
140	4/10	1/10	0/10	0/10
150	0/10	0/10	0/10	0/10
		Sesame Oil[d]		
130	...	7/10	...	5/10
	Sesame Oil[d] + 0.5% Phenol			
110	1/5	1/5	0/5	0/5
120	0/5	0/5	0/5	0/5
		Mineral Oil		
140	3/3	...	...	...
150	4/5	0/5	0/5	0/5
	Mineral Oil + 0.5% Phenol			
130	5/5	5/5	...	...
140	1/5	0/5	...	...
150	0/5	0/5	...	...

[a] Time after reaching designated temperature, dry heat. [b] 10/10, number of tubes with growth/number of tubes inoculated with disks. [c] Original lot of sesame oil. [d] A second lot of sesame oil. Fluid thioglycollate medium incubated 14 days at 30–32°C.

3 months old, was approximately 80 million per ml. and was considered to have shown no significant change. The disks were prepared to contain 200,000 spores each. They were then stored in a tightly sealed can over silica gel for 10 days, after which disks were removed and heated in sesame oil with 2% benzyl alcohol at 130° for periods of 0.5 and 1 hr. This procedure was repeated again after 20, 30, and 90 days of drying. The results in Table IX showed a similar trend to those obtained with the earlier batch (Tables VI and VII). However, the degree of maximum resistance during the 3-month drying interval was not so great as when the disks were dried only 6 days. These results did not clarify the relationship of age and degree of desiccation to resistance but they did confirm the same general pattern of increased resistance developing with time.

TABLE IX—EFFECT OF DRYING ON RESISTANCE OF *B. subtilis* SPORES IN SESAME OIL WITH 2% BENZYL ALCOHOL (200,000 SPORES/DISK)

Days Dried[a]	Time, hr. at 130°C. Dry Heat	
	0.5	1.0
10	2/10[b]	1/10
20	5/10	1/10
30	3/10	3/10
90	6/10	5/10

[a] Dried over silica gel. [b] 2/10, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium incubated 14 days at 30–32°C.

B. stearothermophilus—Disks containing approximately 100,000 spores each were held over silica gel for 6 to 8 days and then used for the sterilization tests. As shown in Table X the maximum survival of *B. stearothermophilus* spores in air was 1.5 hr. at 130°. The maximum survival time in oil occurred

in unpreserved sesame oil, being 1 hr. at 150° or 2 hr. at 140°. This increased resistance in sesame oil indicated the oil protected slightly against the effects of heat. Based on the maximum survival temperature alone, it would appear that benzyl alcohol and phenol caused a slight reduction in the sterilization requirements. Comparison of the results obtained with sublethal time intervals in the 130° and 140° temperature range, however, indicated neither 0.5% phenol nor 2% benzyl alcohol had any effect on the survival or death of the spores. In a repeat of the sterilization at 150°, there were no survivors in any of the oil sections and repeat tests at 140° did not give evidence of the preservatives having any effect. It would be tenuous, indeed, to relate any activity to the preservatives even though there was a slight increase in survival observed in the initial sterilization test of unpreserved oil at 150°. Sterilization of spores of *B. stearothermophilus* in PEG 300 occurred at significantly lower temperatures than in oil or air.

TABLE X—DRY HEAT STERILIZATION TESTS OF *B. stearothermophilus* (100,000 SPORES/DISK)

Temp., °C.	Control, No Heat	Time, hr.[a]			
		0.5	1.0	1.5	2.0
			Air		
100	2/2[b]	5/5	5/5	5/5	5/5
110	2/2	5/5	5/5	5/5	5/5
120	2/2	9/10	10/10	10/10	10/10
130	2/2	8/10	4/10	1/10	0/10
140	2/2	0/10	0/10	0/10	0/10
150	2/2	0/5	0/5	0/5	0/5
160	2/2	0/5	0/5	0/5	0/5
			PEG 300		
100	2/2	3/5	2/5	1/5	1/5
110	2/2	0/5	1/5	0/5	1/5
120	2/2	0/5	0/5	0/5	0/5
130	2/2	0/5	0/5	0/5	0/5
140	2/2	0/5	0/5	0/5	0/5
150	2/2	0/5	0/5	0/5	0/5
			Sesame Oil		
100	2/2	10/10	10/10	10/10	10/10
110	2/2	10/10	10/10	10/10	10/10
120	2/2	10/10	10/10	10/10	10/10
130	2/2	10/10	10/10	8/10	2/10
140	2/2	6/10	1/10	0/10	1/10
150	2/2	2/10	1/10	0/10	0/10
160	2/2	0/10	0/10	0/10	0/10
		Sesame Oil + 0.5% Phenol			
100	2/2	10/10	10/10	10/10	10/10
110	2/2	10/10	10/10	10/10	10/10
120	2/2	10/10	10/10	10/10	10/10
130	2/2	10/10	10/10	7/10	8/10
140	2/2	10/10	0/10	0/10	0/10
150	2/2	0/10	0/10	0/10	0/10
160	2/2	0/10	0/10	0/10	0/10
		Sesame Oil + 2% Benzyl Alcohol			
100	2/2	10/10	10/10	10/10	10/10
110	2/2	10/10	10/10	10/10	10/10
120	2/2	10/10	10/10	10/10	10/10
130	2/2	10/10	9/10	10/10	8/10
140	2/2	9/10	1/10	0/10	0/10
150	2/2	0/10	0/10	0/10	0/10
160	2/2	0/10	0/10	0/10	0/10

[a] Time after sesame oil reached designated temperature. Air controls not corrected for difference in lag time between oil and air. [b] 2/2, number of tubes with growth/number of tubes inoculated with disks. Fluid thioglycollate medium with 0.1% soluble starch incubated 4 days at 60°C.

Minimum Inhibitory Level of Phenol in Aqueous Media—Phenol was added to fluid thioglycollate in concentrations ranging from 1:1200 to 1:600. The tubes were then inoculated with dried spore disks of *B. subtilis* and *B. stearothermophilus* containing 110,000 and 100,000 spores, respectively. Growth of *B. subtilis* occurred with 1:1000 but not with 1:900 phenol. Growth of *B. stearothermophilus* occurred with 1:800 but not with 1:700 phenol.

The *B. subtilis* spores used in the oil sterilization study were killed at lower temperatures in oil containing phenol than in oil alone while the *B. stearothermophilus* spores were not significantly affected by phenol under the conditions of the test. The *B. stearothermophilus* was more resistant to phenol than the *B. subtilis* in aqueous media. This suggests a possible reason why phenol in oil did not reduce the sterilization requirements for *B. stearothermophilus*.

Polyethylene Glycol for Oil Sterilization Control—It has been reported that oils containing only a few spores will frequently pass the sterility test by the U.S.P. method of direct inoculation because the spores may not transfer from the oil to the aqueous medium. It has been suggested that spores be placed in a water miscible nonaqueous liquid such as polyethylene glycol (PEG) and that containers of spores in this vehicle be placed in the oven when sterilizing oils (17). After sterilization, the spore controls in the PEG are tested for sterility by the regular U.S.P. method. Since the vehicle is miscible with water, it will mix readily with the culture medium and any spores not killed will be detected. The results in this series of experiments did not substantiate that this is a satisfactory means of gauging the adequacy of an oil sterilization cycle.

One lot of PEG 300 was used throughout the initial series of oil sterilizations. Two additional lots of PEG 300 and 2 lots of PEG 400 were subsequently used in sterilization tests at 110° and 120°. PEG 300 gave results similar to those obtained in the earlier tests. PEG 400 caused nearly as great a reduction in the sterilization requirements as the PEG 300. The results in these tests confirmed that the combined effect of heat and polyethylene glycol is a marked reduction in sterilization time when compared to that in either oil or air.

SUMMARY

The spores of two thermoresistant species of nonpathogenic aerobic bacilli were heated in preserved sesame oil to determine the effect of the preservatives on the time and temperature requirements for dry heat sterilization.

B. subtilis ATCC 9372 and *B. stearothermophilus*, mesophilic and thermophilic species, respectively, were selected as test organisms. Spores of these organisms were impregnated on filter disks and heated in (*a*) sesame oil, (*b*) sesame oil with 0.5% phenol, (*c*) sesame oil with 2% benzyl alcohol, (*d*) PEG 300, and (*e*) air. Additional comparisons were made with mineral oil and PEG 400. Data were collected in the range of 100–160° in 10° increments at 0.5 hr. intervals to 2 hr.

The *B. subtilis* spores survived 2 hr. at 140° in air. The requirements for sterilization in the sesame oil and PEG 300 were lower than in air. Maximum survival time in unpreserved sesame oil was 1 hr. at 140°. The maximum survival time in sesame oil with 0.5% phenol was 2 hr. at 110°. Benzyl alcohol gave variable results which were related to an increase in resistance of the spores. It had only minimal effect against aged spores. Mineral oil required higher temperatures for sterilization than sesame oil. Phenol had less effect in mineral oil than in sesame oil.

The *B. stearothermophilus* spores survived 1.5 hr. at 130° in air. Higher temperatures were required for sterilization of the oils. There was limited survival after 1 hr. at 150° in unpreserved sesame oil. Sesame oil containing phenol and benzyl alcohol showed no survivors at 150°. Review of the survival data at sublethal temperatures as well as repeat tests indicated no significant effect from phenol or benzyl alcohol.

The spores of both organisms were killed at lower temperatures in PEG 300 than in oil. PEG 400 gave similar results.

The results of this study do not lead to any overall generalizations regarding the effect of preservatives on the heat sterilization of oils. It would appear, however, that phenol would have a wider application and would be more effective than benzyl alcohol as an injectable oil preservative.

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