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Dry Heat or Gaseous Chemical Resistance of *Bacillus subtilis* var. *niger* Spores Included Within Water-soluble Crystals

CHARLES L. MULLICAN AND ROBERT K. HOFFMAN
Department of the Army, Fort Detrick, Frederick, Maryland 21701

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Inclusion of spores of *Bacillus subtilis* var. *niger* in water-soluble crystals increased the resistance of the spores to dry heat and to a gaseous mixture of methyl bromide and ethylene oxide. Resistance of spores in glycine crystals to dry heat at 125 C was increased 5 to 24 times compared to unprotected spores. There appeared to be a positive correlation between the size of the crystal and the degree of resistance. The resistance to dry heat of spores included in sodium chloride crystals was about six times greater than unprotected spores. A gaseous mixture of methyl bromide (964 mg/liter) and ethylene oxide (642 mg/liter) at 37% relative humidity was ineffective in sterilizing spores enclosed within these water-soluble crystals, as was ethylene oxide alone. However, if the relative humidity was sufficiently high to dissolve the crystals during exposure to the vapor, viable-spore counts were drastically reduced or were negative. The surfaces of crystals grossly contaminated with dry spores were sterilized by exposure to gaseous ethylene oxide. Sterilization of heat-labile or moisture-labile materials with a critical requirement for sterility, as in planetary probes or drugs, may be complicated by the presence of spores in naturally occurring water-soluble crystals. This phenomenon is similar to the protection afforded spores entrapped in solid plastics.

Widespread use of ethylene oxide (ETO) has filled an urgent need for a means of sterilizing heat-labile and moisture-labile materials. However, ETO and other sterilizing gases are effective only if they can contact the microorganisms. Numerous substances can provide an impermeable barrier to these gases. Several investigators have observed the resistance of spores trapped in crystals to sterilization by gaseous chemicals. Kaye and Phillips (5) observed that spores suspended in NaCl solutions and dried under vacuum on metal or glass for 5 hr were more resistant to sterilization with gaseous ETO than spores dried from distilled water and subjected to the same treatment. Abbott, Cockton, and Jones (1) reported that spores included in Rochelle salt crystals resisted sterilization with formaldehyde and ETO. They also found that spores in glycine crystals were not sterilized when exposed to formaldehyde. Royce and Bowler (9) noted that bacteria in crystals of glucose, NaCl, and other pharmaceutical products were protected against sterilization by ETO. Phillips and Hoffman (7) reported that viable microorganisms could be recovered from the interior of some electronic components. Angelotti (*unpublished data*) found that microorganisms enclosed in plastic were 5 to 30 times more resistant to dry heat than unprotected microorganisms. Other investigators (2, 6) also demonstrated increased resistance to dry heat sterilization of spores in various types of solid materials. In a recent paper, Doyle and Ernst (3) reported that spores occluded in water-insoluble crystals of calcium carbonate were not sterilized by exposure to ETO and were 900 times more resistant to moist heat and 9 times more resistant to dry heat than unoccluded spores. They concluded that poor heat transfer within the crystal was the most likely explanation for the increased resistance to dry heat.

The primary purpose of this study was to determine the resistance of spores included within water-soluble crystals to dry heat at 125 C and to a gaseous mixture of methyl bromide (MeBr) plus ETO and ETO alone at 25 C.

Glycine and NaCl were selected as organic and inorganic crystals that met the following criteria: (i) melting point above 200 C, (ii) no water of crystallization, and (iii) moderate-sized crystals (1 mm or larger) readily formed.

MATERIALS AND METHODS

Preparation of crystals containing spores. Dry spores of *Bacillus subtilis* var. *niger* were suspended in sterile distilled water to give a concentration of 8×10^8 viable spores per ml. A saturated solution of glycine or NaCl was prepared with sterile distilled water at 56 C and held in a water bath at the same temperature. The spore suspension (1 ml) and 9 ml of the saturated solution were pipetted into a sterile 50-mm petri dish and were mixed by swirling the dish. The covered dish was placed in a 37 C incubator, and crystals were harvested when they had reached a suitable size (generally after 1 to 3 days of incubation). During the holding period, the dish was swirled at least once daily to resuspend settled spores. At harvest, crystals were blotted on tissue paper and held in closed petri dishes at room temperature until used.

Glycine crystals used in these tests ranged in size from $2 \times 1 \times 1$ mm (0.003 g) to $12 \times 9 \times 3$ mm (0.32 g). Viable spores recovered from these crystals, after washing to remove surface contaminants, ranged from approximately 1,000 to 900,000 with a mean of about 100,000. NaCl crystals ranged in size from $1 \times 1 \times 1$ mm (<0.005 g) to $7 \times 4 \times 3$ mm (0.175 g). Viable spores recovered, after washing, ranged from 300 to 9,000 with a mean of about 3,000. In each test, an exposed cytstal was matched as closely as possible by weight and size with an unexposed control crystal. The wide range in crystal sizes and numbers of included spores complicates direct comparisons of test results.

Clean glass microscope slides were inoculated with 0.01 ml of the spore suspension (approximately 8×10^6 spores per slide) and dried at 25 C. Recovery of viable spores from these slides was compared with recovery from the crystals.

Exposure of crystals with entrapped spores. Crystals and glass slides in an open petri dish were placed in a forced-draft electric oven which had been preheated at about 127 C. During the insertion of the crystals, the oven temperature usually dropped to about 121 C and then required 2 to 4 min to reach 125 C, at which time the exposure period was started. The exposure time ranged from 2 to 24 hr at 125 C.

Crystals containing spores to be exposed to gaseous sterilants were placed on a galvanized wire mesh screen in a vacuum-type desiccator jar similar to that described and illustrated by Gilbert et al. (4). With this system, relative humidity (RH) within the jar is adjusted, a partial vacuum is drawn in the jar, and the sterilizing gas is introduced, bringing the pressure within the jar almost up to atmospheric pressure.

Crystals of glycine or NaCl containing entrapped spores were exposed to a gaseous mixture of 40% MeBr and 60% ETO, by volume (964 mg of MeBr per liter and 642 mg of ETO per liter), at relative humidities above 75% at 25 C for 48 hr. In these tests, the high RH was obtained by placing a filter paper patch wet with distilled water into the sealed jar.

Glycine and NaCl crystals were also exposed to gaseous ETO at low (35 to 38%) and high relative humidities for 24 to 48 hr at 25 C. Concentrations of ETO ranged from 140 to 642 mg/liter.

Filter paper or cotton twill patches, each containing a dried inoculum of approximately 1 million spores, were compared with the crystals in these tests.

Exposure of spores on surface of crystals to gaseous ETO. Crystals of NaCl were either held at ambient (room) RH or dried at < 1% RH; then the surfaces of the crystals were grossly contaminated by rolling the crystals in a powder of dry spores (5×10^{11} viable spores per g) of *B. subtilis* var. *niger*. These crystals were exposed to ETO (319 to 362 mg/liter) at either 34 or 42% RH at 25 C for 24 hr.

Filter paper or cotton twill patches containing dried spores were exposed with the crystals in some tests.

Assay of viable spores. After exposure of the single crystals to either dry heat or gaseous sterilants, each crystal was aseptically transferred to a rubber-stoppered test tube containing sterile distilled water. Glass slides were aseptically transferred to bottles containing a sterile aqueous 0.01% solution of Tween 20 and were shaken for 5 min on a mechanical shaker. Unexposed (control) crystals were washed by swirling for about 1 min in sterile distilled water and rinsed in 100% ethyl alcohol before dissolving and sampling. Washing the unexposed crystals was designed to eliminate or drastically reduce the number of viable spores on the crystal surface so that essentially only spores within the crystal would remain. The washing procedure removed some of the surface layer of the crystals including some of the spores entrapped there.

After washing, each single control crystal was dissolved in a test tube containing sterile distilled water. In tests where filter paper or cotton twill patches containing spores were used for comparison, each patch was placed into a test tube containing 9.0 ml of sterile distilled water. Immediately prior to plating, each tube was vigorously shaken by hand.

Plate Count Agar (Difco) was used as the assay medium for viable spores in all tests. The pour plate method of assaying for viable spores was employed. All plates were incubated at 36 C for 48 hr before counting.

RESULTS

Recovery of viable spores from the interior of crystals exposed to dry heat at 125 C. Viable spores were consistently recovered from large glycine crystals heated at 125 C for periods up to and including 20 hr, but no viable spores were recovered after exposure for 24 hr. Recoveries from glycine crystals of various sizes after exposures of 2 to 20 hr at 125 C gave *D* values ranging from more than 1 to 6 hr, when compared with recoveries from unheated washed crystals.

Crystals of NaCl yielded viable spores after heating at 125 C for 4 hr or less, but no viable spores were recovered from NaCl crystals after 16 hr or longer. Calculated *D* values for spores heated in NaCl crystals at 125 C are generally in the range of 1 to 2 hr.

Table 1 presents the mean percentage of survival of spores in glycine and NaCl crystals exposed to dry heat at 125 C. Recovery of viable

TABLE 1. *Mean percentage of survival of viable spores of B. subtilis var. niger from crystals heated at 125 C*

Time of heating at 125 C	Crystal wt	Glycine crystals		NaCl crystals	
		Survival	*D* values	Survival	*D* values
hr	*g*	%	*hr*	%	*hr*
2	<0.01	4	1.4	5	1.5
	0.01–0.09	10	2.0	—	
	0.10–0.32	19	2.8	—	
4	<0.01	—	—	0	
	0.01–0.09	0.1	1.3	0.3	1.6
16	<0.01	0.2	5.8	—	
	0.01–0.09	—	—	0	
18	0.01–0.09	0.001	3.6	—	
	0.10–0.32	—	—	0	
20	0.10–0.32	0.05	6.0	—	
24	0.01–0.09	0		—	
	0.10–0.32	0		0	

TABLE 2. *Recovery of viable spores from glycine crystals of various sizes*

Crystal wt	Mean viable spores recovered from glycine crystals		Percentage survival[a]	*D* values
	Heated at 125 C for 2 hr	Unheated (washed) crystals		
g				*hr*
<0.01	270	7,400	4	1.4
0.01–0.09	2,300	22,000	10	2.0
0.10–0.32	28,000	147,000	19	2.8

[a] Percentage survival = 100 × heated/unheated.

spores from a glass slide heated at 125 C for 30 min was about 0.8%, which gives a *D* value of about 15 min. With one exception, no viable spores were recovered from glass slides exposed to 125 C for 1 hr or longer in repeated tests.

Crystal size is apparently a factor in recovery of viable spores from heated crystals, with the greater number of spores generally recovered from the larger crystals and the percentage of recovery also generally higher from larger crystals. These relationships are given in Table 2 for glycine crystals heated at 125 C for 2 hr.

Recovery of viable spores from the interior of crystals after exposure to sterilizing gases. Crystals of glycine or NaCl containing enclosed spores showed no reduction in viable spore counts after exposure to a gaseous MeBr-ETO mixture or gaseous ETO at about 37% RH at 25 C for up to 48 hr as compared with unexposed washed crystals. However, NaCl crystals exposed at high RH (>75%) to MeBr plus ETO or ETO alone for 48 hr either partially or completely dissolved and were either sterile or yielded only low numbers of viable spores upon assay.

Recovery of viable spores from the surfaces of crystals exposed to ETO. Surfaces of NaCl crystals, grossly contaminated with more than 10^7 viable spores by tumbling in a powder of dry spores, were generally sterilized when exposed to gaseous ETO at 34 or 42% RH for 24 hr at 25 C.

DISCUSSION

Spores included in glycine crystals were 5 to 24 times more resistant to dry heat than unprotected spores, and spores included in NaCl crystals were about 6 times more resistant. The apparent greater resistance of spores in glycine crystals over spores in NaCl crystals was probably due to the larger average size of the glycine crystals and not the chemical. The ninefold increase in dry-heat resistance of spores in water-insoluble crystals over nonprotected spores reported by Doyle and Ernst (3) is within the range of the increase in resistance of 5 to 24 times reported here for spores protected by water-soluble crystals of various sizes. This similarity is rather interesting considering the numerous differences between the test procedures, such as (i) use of water-soluble crystals versus water-insoluble crystals, (ii) heated in oven at 125 C versus heated in special aluminum block at 121 C, (iii) crystals 1 to 12 mm versus crystals considerably smaller than 1 mm, (iv) crystals formed rather slowly versus crystals formed rapidly, and (v) crystals dried at room temperature versus crystals dried at 90 C.

Interest in the MeBr-ETO mixture was enhanced by reports of a synergistic effect with these two gases by Richardson and Monro (8) and more recently by Russian representatives at the meetings of the Committee on Space Research in Vienna in May 1966 (10). However, exposure of water-soluble crystals to a gaseous mixture of MeBr and ETO (40% MeBr + 60% ETO by volume) did not kill spores within the crystals any more successfully than did ETO alone. Because ETO gas can penetrate some organic materials, such as various plastics and rubber, the glycine was used here to test the ability of the MeBr-ETO mixture and ETO alone to penetrate and kill spores within an organic crystal.

The inability to sterilize spores in crystals of glycine or NaCl by gaseous ETO agrees with similar observations in previous reports: Doyle and Ernst (3) with water-insoluble crystals, and Abbott, Cockton, and Jones (1) with crystals of Rochelle salt (and glycine crystals exposed to formaldehyde).

The protective effect provided against gaseous sterilization of spores entrapped in crystals is

probably caused by the inability of the gas molecule to penetrate the crystal lattice rather than competition for the gas between the crystalline material and the embedded spores.

The surfaces of NaCl crystals grossly contaminated with more than 10^7 viable spores were sterilized by exposure to gaseous ETO when the gas concentration, RH, and exposure time were adequate.

Doyle and Ernst (3) discussed several possible explanations for the increased resistance to dry heat of spores in crystals and concluded that poor heat transfer within the crystal was the most probable reason. Angelotti (*unpublished data*) thought that the protective effect against dry heat provided by embedding spores in solids was most plausibly explained by the prevention of spore desiccation. We believe that moisture is a factor in the increased resistance of entrapped spores, but investigations to date have not shown the precise nature of this role.

Because natural crystals in soils and elsewhere probably contain viable trapped spores, additional information on the resistance of these spores to sterilization should be of interest, especially in areas where the labile nature of the materials limits the choice of sterilization methods and there is a critical sterility requirement such as with planetary probes or drugs.

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