AD NUMBER

AD837909

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Operational and administrative use; Jul 1968. Other requests shall be referred to Department of the Army, Fort Detrick, Attn: Technical Release Branch/TID, Frederick, MD, 21701.

AUTHORITY

BDRL, D/A ltr, 29 Sep 1971

AD

TECHNICAL MANUSCRIPT 471

RESISTANCE OF BACILLUS SUBTILIS VAR. NIGER SPORES IN WATER-SOLUBLE CRYSTALS TO DRY HEAT OR GASEOUS CHEMICALS

Charles L. Mullican
Robert K. Hoffman

JULY 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

14

DDC AVAILABILITY NOTICES

Qualified requesters may obtain copies of this publication from DDC.

Foreign announcement and dissemination of this publication by DDC is not authorized.

Release or announcement to the public is not authorized.

DISPOSITION INSTRUCTIONS

Destroy this publication when it is no longer needed. Do not return it to the originator.

The findings in this publication are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 471

RESISTANCE OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES IN WATER-SOLUBLE CRYSTALS TO DRY HEAT OR GASEOUS CHEMICALS

Charles L. Mullican

Robert K. Hoffman

Physical Defense Division
COMMODITY DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B622401A072

July 1968

ACKNOWLEDGMENT

Crystals used during the early phases of this investigation were grown by Charles Borland, and his method of growing the crystals was used throughout this study.

ABSTRACT

Inclusion of spores of Bacillus subtilis var. niger in water-soluble crystals increased their resistance to sterilization with dry heat or with a gaseous mixture of methyl bromide and ethylene oxide.

Resistance of spores in glycine crystals to dry heat at 125 C was increased five to 24 times compared with unprotected spores. Size of the crystal and degree of resistance appear to be positively correlated. The dry-heat resistance of spores included in sodium chloride crystals was about six times greater than that of unprotected spores.

A gaseous mixture of methyl bromide (964 mg/liter) and ethylene oxide (642 mg/liter) 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.

Crystal surfaces 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 drugs or planetary probes, 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.

I. INTRODUCTION

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[1] observed that spores suspended in sodium chloride (NaCl) solutions and dried under vacuum on metal or glass for 5 hours were more resistant to sterilization with gaseous ETO than spores dried from distilled water and subjected to the same treatment. Abbott, Cockton, and Jones[2] 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[3] noted that bacteria in crystals of glucose, NaCl, and other pharmaceutical products were protected against sterilization by ETO. Phillips and Hoffman[4] reported that viable microorganisms could be recovered from the interior of some electronic components. Angelotti[5] found that microorganisms enclosed in plastic were five to 30 times more resistant to dry heat than unprotected microorganisms. Other investigators[6,7] have also demonstrated increased resistance to dry-heat sterilization of spores in various types of solid materials. In a recent paper, Doyle and Ernst[8] 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 nine 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, to a gaseous mixture of methyl bromide (MeBr) and ETO at 25 C, and to 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.

II. MATERIALS AND METHODS

A. 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 using sterile

distilled water at 56 C and held in a water bath at the same temperature. One ml of the spore suspension and 9 ml of the saturated solution were pipetted into a sterile 50-mm petri dish and 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' 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 x 1 x 1 mm (0.003 g) to 12 x 9 x 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 x 1 x 1 mm (<0.005 g) to 7 x 4 x 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 crystal 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.

B. EXPOSURE OF CRYSTALS WITH ENTRAPPED SPORES

Crystals and glass slides in an open petri dish were placed in a forced-draft electric oven that had been preheated to about 127 C. During the insertion of the crystals, the oven temperature usually dropped to about 121 C; 2 to 4 minutes were required to reach 125 C, at which time the exposure period was started. The exposure time ranged from 2 to 24 hours 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.[9] 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 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 MeBr/liter + 642 mg ETO/liter) at RH above 75% at 25 C for 48 hours. 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 RH for 24 to 48 hours 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 one million spores, were compared with the crystals in these tests.

C. 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 hours.

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

D. ASSAY OF VIABLE SPORES

After exposure of the 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 minutes on a mechanical shaker. Unexposed (control) crystals were washed by swirling for about 1 minute in sterile distilled water and rinsed in 100% ethanol 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 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 used. All plates were incubated at 36 C for 48 hours before counting.

III. RESULTS

A. RECOVERY OF VIABLE SPORES FROM INTERIOR OF CRYSTALS EXPOSED TO DRY HEAT

Viable spores were consistently recovered from large glycine crystals heated at 125 C for periods to and including 20 hours, but no viable spores were recovered after exposure for 24 hours. Recoveries from glycine crystals of various sizes after exposures of 2 to 20 hours at 125 C gave D values (decimal reduction time or time to kill 90% of the spores) ranging from more than 1 hour to 6 hours, when compared with recoveries from unheated washed crystals.

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

Table 1 presents the mean per cent survival of spores in glycine and NaCl crystals exposed to dry heat at 125 C. Recovery of viable spores from a glass slide heated at 125 C for 30 minutes was about 0.8%, which gives a D value of about 15 minutes. With one exception, no viable spores were recovered from glass slides exposed to 125 C for 1 hour 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 per cent recovery also generally higher from larger crystals. These relationships are shown in Table 2 for glycine crystals heated at 125 C for 2 hours.

B. RECOVERY OF VIABLE SPORES FROM INTERIOR OF CRYSTALS EXPOSED TO 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 hours as compared with unexposed washed crystals. However, NaCl crystals exposed at high RH to MeBr + ETO or ETO alone for 48 hours either partially or completely dissolved and were either sterile or yielded only low numbers of viable spores upon assay.

TABLE 1. MEAN PER CENT SURVIVAL OF VIABLE SPORES OF B. SUBTILIS VAR. NIGER FROM CRYSTALS HEATED AT 125 C

Hours Heated at 125 C	Crystal Weight, g	Survival, %		D Value, hours	
		Glycine	NaCl	Glycine	NaCl
2	<0.01	4	5	1.4	1.5
	0.01 to 0.09	10	-[a]	2.0	-
	0.10 to 0.32	19	-	2.8	-
4	<0.01	-	0	-	-
	0.01 to 0.09	0.1	0.3	1.3	1.6
16	<0.01	0.2	-	5.8	-
	0.01 to 0.09	-	0	-	-
18	0.01 to 0.09	0.001	-	3.6	-
	0.10 to 0.32	-	0	-	-
20	0.10 to 0.32	0.05	-	6.0	-
24	0.01 to 0.09	0	-	-	-
	0.10 to 0.32	0	0	-	-

a. - = no data available.

TABLE 2. RECOVERY OF VIABLE SPORES FROM GLYCINE CRYSTALS OF VARIOUS SIZES

Crystal Weight, g	Mean Viable Spores Recovered from Crystals		Survival, % $(100 \times \frac{\text{Heated}}{\text{Unheated}})$	D Value, hours
	Heated at 125 C for 2 hours	Unheated (Washed)		
<0.01	270	7,400	4	1.4
0.01 to 0.09	2,300	22,000	10	2.0
0.10 to 0.32	28,000	147,000	19	2.8

C. RECOVERY OF VIABLE SPORES FROM SURFACES OF CRYSTALS EXPOSED TO ETO

Surfaces of NaCl crystals grossly contaminated with more than 1×10^7 viable spores by tumbling in a powder of dry spores were generally sterilized when exposed to gaseous ETO for 24 hours.

IV. DISCUSSION

Spores included in glycine crystals were five to 24 times more resistant to dry heat than unprotected spores, and spores included in NaCl crystals were about six times more resistant. The apparent greater resistance of spores in glycine crystals over spores in NaCl crystals was probably because of 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[8] is within the range of the increase in resistance of five 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:

1) Use of water-soluble crystals vs. water-insoluble crystals,

2) Heating in oven at 125 C vs. heating in special aluminum block at 121 C,

3) Crystals 1 to 12 mm vs. crystals considerably smaller than 1 mm,

4) Crystals formed rather slowly vs. crystals formed rapidly,

5) Crystals dried at room temperature vs. 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[10] and more recently by Russian representatives at the meetings of the Committee on Space Research in Vienna in May 1966. 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 crystal of glycine or NaCl by gaseous ETO agrees with similar observations in previous reports: Doyle and Ernst[8] with water-insoluble crystals and Abbott, Cockton, and Jones[2] 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 due to inability of the gas molecule to penetrate the crystal lattice, rather than to competition for the gas between the crystalline material and the embedded spores.

The surfaces of NaCl crystals grossly contaminated with more than 1×10^7 viable spores were sterilized by exposure to gaseous ETO when the gas concentration, RH, and exposure time were adequate.

Doyle and Ernst[8] 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[5] felt that the protective effect against dry heat provided by embedding spores in solids was most plausibly explained by the prevention of spore desiccation. The present authors feel 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 as with drugs or planetary probes.

V. CONCLUSIONS

Viable spores of Bacillus subtilis var. niger enclosed in water-soluble crystals were five to 24 times more resistant to dry heat than unprotected spores.

Crystal size and degree of resistance to dry heat for spores in crystals appear to be correlated.

A gaseous mixture of MeBr and ETO (964 mg MeBr/liter + 642 mg ETO/liter) was ineffective in sterilizing spores enclosed in water-soluble crystals, as was ETO alone.

The surfaces of NaCl crystals heavily contaminated with spores were sterilized with gaseous ETO.

LITERATURE CITED

1. Kaye, S.; Phillips, C.R. 1949. The sterilizing action of gaseous ethylene oxide: IV. The effect of moisture. Amer. J. Hyg. 50:296-306.

2. Abbott, C.F.; Cockton, J.; Jones, W. 1956. Resistance of crystalline substances to gas sterilization. J. Pharm. Pharmacol. 8:709-720.

3. Royce, A.; Bowler, C. 1961. Ethylene oxide sterilization: Some experiences and some practical limitations. J. Pharm. Pharmacol. 13:87T - 94T.

4. Phillips, C.R.; Hoffman, R.K. 1960. Sterilization of interplanetary vehicles. Science 132:991-995.

5. Angelotti, R. 1967. Protective mechanisms affecting dry-heat sterilization. Paper presented at the IQSY/COSPAR symposium on sterilization techniques for instruments and materials as applied to space research. London, England, July 1967.

6. Bruch, C.W.; Koesterer, M.G.; Bruch, M.K. 1963. Dry-heat sterilization: Its development and application to components of exobiological space probes. Develop. Ind. Microbiol. 4:334-342.

7. Koesterer, M.G. 1964. Thermal death studies on microbial spores and some considerations for the sterilization of space craft components. Develop. Ind. Microbiol. 6:268-276.

8. Doyle, J.E.; Ernst, R.R. 1967. Resistance of Bacillus subtilis var. niger spores occluded in water-insoluble crystals to three sterilization agents. Appl. Microbiol. 15:726-730.

9. Gilbert, G.L.; Gambill, V.M.; Spiner, D.R.; Hoffman, R.K.; Phillips, C.R. 1964. Effect of moisture on ethylene oxide sterilization. Appl. Microbiol. 12:496-503.

10. Richardson, L.T.; Monro, H.A.U. 1962. Fumigation of jute bags with ethylene oxide and methyl bromide to eradicate potato ring rot bacteria. Appl. Microbiol. 10:448-451.

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)
Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
RESISTANCE OF BACILLUS SUBTILIS VAR. NIGER SPORES IN WATER-SOLUBLE CRYSTALS TO DRY HEAT OR GASEOUS CHEMICALS

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)
Charles L. Mullican
Robert K. Hoffman

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
July 1968	14	10

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1B622401A072	Technical Manuscript 471
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT
Qualified requesters may obtain copies of this publication from DDC.
Foreign announcement and dissemination of this publication by DDC is not authorized.
Release or announcement to the public is not authorized.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Department of the Army Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

Inclusion of spores of Bacillus subtilis var. niger in water-soluble crystals increased their resistance to sterilization with dry heat or with a gaseous mixture of methyl bromide and ethylene oxide.

Resistance of spores in glycine crystals to dry heat at 125 C was increased five to 24 times compared with unprotected spores. Size of the crystal and degree of resistance appear to be positively correlated. The dry-heat resistance of spores included in sodium chloride crystals was about six times greater than that of unprotected spores.

A gaseous mixture of methyl bromide (964 mg/liter) and ethylene oxide (642 mg/liter) 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.

Crystal surfaces 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 drugs or planetary probes, 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.

14. Key Words

*Bacillus subtilis var. niger
Spores
*Resistance
*Sterilization
*Crystals (water-soluble)
Glycine
Sodium chloride
Heat
Ethylene oxide
Methyl bromide
Gases