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Limitations of Thioglycolate Broth as a Sterility Test Medium for Materials Exposed to Gaseous Ethylene Oxide[1]

JOHN E. DOYLE, WILLIAM H. MEHRHOF, AND ROBERT R. ERNST
Research Laboratory, Castle Company, Rochester, New York 14623

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Although ethylene oxide is a reliable sterilizer, the process may be limited by diffusion. Thus, situations may exist where microorganisms are protected from the sterilizing gas. It is possible that the exterior of a substance may be sterilized, whereas the interior is not. We investigated three general types of materials in which this limitation of diffusion could occur: the bore of glass and plastic tubing, the center of cotton balls, and plastic adhesive film/paper backing interface. These materials were contaminated as close to their geometric center as possible with *Bacillus subtilis* var. *niger* spores occluded in crystals of sodium chloride. After exposure of the contaminated materials (except aluminum foil) to ethylene oxide, thioglycolate broth (a standard sterility-test medium) indicated sterility, whereas Trypticase Soy Broth indicated nonsterility. It is likewise possible that aerobic microorganisms, surviving in or on material after exposure to dry heat or steam sterilization processes, would not be recovered by thioglycollate broth. Entrapped aerobic organisms will probably not grow out in the low oxygen tension zone of an anaerobic medium such as thioglycollate broth. It is recommended than an aerobic medium such as Trypticase Soy Broth be used concurrently with thioglycolate broth for sterility testing.

A significant contribution to microbiological control was made by Brewer (3) when he developed a liquid medium capable of supporting both aerobic and anaerobic growth. Many modifications have been made since then (10, 11). The final result was the thioglycolate broth now recommended as a standard sterility test medium by the United States Pharmacopeia, the National Formulary, the National Institutes of Health, and the Food and Drug Administration.

Ethylene oxide is a reliable sterilizing agent, but its action is extremely complex (6). The principal concomitant factors are moisture availability and diffusion, that is, the diffusion of ethylene oxide, moisture, and heat into the contaminated sites.

In addition, in situations where microorganisms are protected from the sterilizing process, such as occlusion in crystals (1, 4) or contact with protective agents (7), either may react with the moisture or the ethylene oxide, thus preventing inactivation.

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This study was performed to determine whether viable aerobic microorganisms present in materials after exposure to ethylene oxide will grow out in thioglycolate broth.

MATERIALS AND METHODS

Organism. Bacillus subtilis var. *niger* 356 S.C. no. 4 N.R. Smith strain was used (12).

Culture methods. The culturing methods have been described previously (4).

Preparation of test materials. The following materials were investigated: aluminum foil (3.81 by 0.63 cm), aluminum foil (3.81 by 0.63 cm) rolled up in the center of cotton balls (Johnson & Johnson, New Brunswick, N.J.), Teflon tubing (0.083-cm outside diameter by 11.4 cm; Cadillac Plastic & Chemical Co., Detroit, Mich.), glass melting-point capillary tubing (0.8 to 1.2 mm, inside diameter, by 100 mm) Kimble no. 34502 (Will Scientific Inc., Rochester, N.Y.), and plastic surgical adhesive film (1.27 by 1.27 cm, Vi-Drape; Parke, Davis & Co., Detroit, Mich.).

Initially, the test materials were inoculated with *B. subtilis* var. *niger* spores in isotonic saline. Later, a similar suspension containing 1% hydroxyethylcellulose to bind the inoculum and salt crystals to the test materials was used.

The test materials were inoculated with 10^5 spores

per test material with one drop from a syringe and a 25-gauge needle (a reproducible 0.01-ml inoculum).

Glass and Teflon tubing were inoculated in the following manner. The droplet inoculum was placed on polyethylene film; then the tubing was placed over the drop so that the inoculum was drawn into the tubing by capillary action and into the center of the tubing by suction.

The plastic surgical adhesive film was inoculated as follows. The paper backing was removed, and the inoculum was placed on the adhesive side of the film. The paper backing was replaced after the inoculum dried. This provided protected organisms at the plastic film/paper backing interface.

All materials were dried at 55 C until visibly dry. Salt crystals always formed upon drying.

Ethylene oxide procedures. Procedures similar to those described by Ernst and Shull (8) were used. Ethylene oxide conditions were determined that would sterilize all portions of the materials except the protected area in the geometrical center. The spore count was repeatedly reduced from 10^5 to 10^3 per test piece, as ascertained by standard plate count procedures after 1 hr of exposure to 1,200 mg of ethylene oxide per liter at 40% relative humidity and 54 C. High resistance of spores inoculated from isotonic saline on aluminum foil was also obtained by Beeby and Whitehouse (2). Similar material contaminated with 10^5 *B. subtilis* var. *niger* spores in distilled water was inactivated under these conditions in 10 min; therefore, 1-hr exposure times were used throughout this study. In this way, nonsterile materials (10^3 spores per test piece) were obtained in approximately their geometric center.

Recovery methods. After exposure to the ethylene oxide process, half of the exposed samples were transferred to Thioglycollate Medium (BBL) and half to Trypticase Soy Broth (BBL) and incubated at 32 C for 14 days. At least 200 pieces of each test material were treated in this manner.

RESULTS

The initial test pieces were inoculated with *B. subtilis* var. *niger* spores dried from isotonic saline. Although there was a distinct difference in recovery from Thioglycollate Medium and Trypticase Soy Broth, there was a relatively high percentage of recovery in Thioglycollate Medium with the aluminum foil in the center of the cotton balls and with the glass capillary tubing (Table 1). The data are reported as percentage of recovery, derived from the number of pieces producing growth divided by the number of pieces tested.

Salt crystals containing occluded spores may have been dislodged from the inoculated surfaces during manipulation. Thus, protection from ethylene oxide sterilization could have been occasionally produced near or close to the exterior of the material, and internal contamination was not truly represented. Therefore, a suspension containing hydroxyethylcellulose was used to bind the inoculum to the test pieces.

TABLE 1. *Comparison of recovery of viable organisms after exposure to ethylene oxide*[a]

Test material[b]	Percentage of recovery	
	Trypticase soy broth	Thioglycolate broth
Aluminum foil	100	100
Aluminum foil in center of cotton balls	100	27
Teflon tubing	100	0
Glass capillary tubing	100	50
Plastic surgical adhesive film/paper backing	100	10

[a] After 1 hr of exposure to 1,200 mg of ethylene oxide per liter at 40% relative humidity and 54 C.

[b] Each test piece was contaminated with 10^5 *B. subtilis* var. *niger* spores in isotonic saline (reduced to 10^3 spores per test piece after ethylene oxide exposure).

TABLE 2. *Comparison of recovery of viable organisms after exposure to ethylene oxide*[a]

Test material[b]	Percentage of recovery	
	Trypticase soy broth	Thioglycolate broth
Aluminum foil only	100	100
Aluminum foil in center of cotton balls	100	0
Glass capillary tubing	100	0
Plastic surgical adhesive film/paper backing	100	10

[a] After 1 hr of exposure to 1,200 mg of ethylene oxide per liter at 40% relative humidity and 54 C.

[b] Each test piece was contaminated with 10^5 *B. subtilis* var. *niger* spores in isotonic saline and 1% hydroxyethylcellulose (reduced to 10^3 spores per test piece after ethylene oxide exposure).

Growth from these materials developed in the Trypticase Soy Broth but not in the Thioglycollate Medium (Table 2).

DISCUSSION

In these experiments, thioglycolate broth did not support growth of *B. subtilis* var. *niger* spores when entrapped or held, so that the organisms could not be released into an environment in which they will grow, i.e., containing high oxygen tension.

One of the principal functions of thioglycolate broth is to produce a low oxygen tension zone in which anaerobes will grow. However, strict aerobic organisms held in this zone probably will not grow. It is possible that aerobic organ-

isms that are least accessible to an ethylene oxide process will not grow when cultured in thioglycolate broth. Many disposable materials processed with ethylene oxide are composed of absorbent materials, such as cotton, plastic tubing materials, and plastic film/paper interface materials.

It is possible that materials exposed to dry heat or steam-sterilizing conditions may fail to show growth when cultured into thioglycolate broth. For example, there are limitations of time, heat transfer, and in the case of steam, air pockets, superheat, etc., that can exist in material resulting in nonsterility in the center (5).

Koesterer (9) reported that Trypticase glucose yeast extract broth (an aerobic medium) gave better recovery than thioglycolate broth from soil exposed to dry heat. Perhaps the surviving organisms were heat-fixed to the particles of soil and unable to grow in the low oxygen tension zone of thioglycolate broth.

It is recommended that an aerobic medium, such as Trypticase Soy Broth, be used concurrently with thioglycolate broth in sterility testing.

LITERATURE CITED

1. Abbott, C. R., J. Cockton, and W. Jones. 1956. Resistance of crystalline substances to gas sterilization. J. Pharm. Pharmacol. **6**:709–721.
2. Beeby, M. M., and C. E. Whitehouse. 1965. A bacterial spore test piece for the control of ethylene oxide sterilization. J. Appl. Bacteriol. **28**:349–360.
3. Brewer, J. H. 1940. Clear liquid mediums for the "aerobic" cultivation for anaerobes. J. Am. Med. Assoc. **115**:598–600.
4. Doyle, J. E., and R. E. Ernst. 1967. Resistance of *Bacillus subtilis* var. *niger* spores occluded in water-insoluble crystals to three sterilization agents. Appl. Microbiol. **15**:726–730.
5. Ernst, R. R. 1968. Sterilization by heat, p. 703–740. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization, and preservation, 1st ed. Lea & Febiger, Philadelphia.
6. Ernst, R. R., and J. E. Doyle. 1968. Sterilization with ethylene oxide: a review of chemical and physical factors. Biotech. Bioeng. **10**:1–31.
7. Ernst, R. R., and J. E. Doyle. 1968. Limiting factors in ethylene oxide gaseous sterilization. Develop. Ind. Microbiol. **9**:293–296.
8. Ernst, R. R., and J. J. Shull. 1962. Ethylene oxide gaseous sterilization. I. Concentration and temperature effects. Appl. Microbiol. **10**:337–341.
9. Koesterer, M. G. 1964. Thermal death studies on microbial spores and some considerations for the sterilization of spacecraft components. Dev. Ind. Microbiol. **6**:268–276.
10. Linden, B. A. 1941. Fluid thioglycollate medium for the sterility test. Natl. Inst. Health Bull. Bethesda, Md.
11. Pittman, M. 1946. A study of fluid thioglycollate medium for the sterility test. J. Bacteriol. **51**: 19–32.
12. Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. Aerobic spore forming bacteria. U.S. Dept. Agr. Monograph No. 16.