Ethylene Oxide Resistance of Nondesiccated and Desiccated Spores of *Bacillus subtilis var. niger* Hermetically Sealed in Various Polymeric Films

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The resistance to destruction of spores of *Bacillus subtilis var. niger* hermetically sealed in various polymeric films and exposed to ethylene oxide with and without relative humidity was determined. The effect of desiccation was also determined. The order of increased resistance to sterilization with regard to type of polymeric film was found to be: polyethylene equal to polyvinyl chloride, less than nylon, less than cellophane/polyethylene laminate, less than phenoxy, less than mylar/polyethylene laminate. Desiccated spores sealed in various polymeric films were much more resistant to ethylene oxide sterilization than nondesiccated spores. Relative humidity was an important factor in ethylene oxide sterilization with spores not sealed in polymeric films. However, with spores hermetically sealed in polyethylene, added relative humidity was an insignificant factor in the sterilization process.

Materials sterilized by ethylene oxide are usually wrapped in such a manner as to maintain sterility after sterilization. A variety of packaging materials are currently being used; these include paper, polyethylene, polyvinyl chloride, nylon, cellophane/polyethylene laminate, and mylar/polyethylene laminate. These packaging materials can be barriers to ethylene oxide, moisture, or both. The important criteria is that the packaging materials maintain sterility. Although there is considerable information published on the permeability of the commonly used polymeric films to ethylene oxide and moisture (1, 7, 10, 11, 13, 14), little of this data can be applied to determine sterilization parameters. Recently, (9), it was reported that polyethylene is an adequate packaging material for ethylene oxide sterilization. Hennessy et al. (8) indicated that care must be taken in interpreting results of permeability data which have been obtained under conditions not relevant to actual conditions of usage. Since the destruction of bacteria is the most important criteria for evaluating a sterilization process, this investigation centered on the determination of inactivation of bacterial spores in various thicknesses of polymeric films.

**Preparation of test samples.** Paper strips (1.5 by 0.25 inch, ca. 3.81 by 0.64 cm) were inoculated with 10⁶ spores of *B. subtilis var. niger* and allowed to dry. Pouches, approximately 2 by 0.75 inch, ca. 5.08 by 1.92 cm) were made of the various polymeric films with one end open. Five spore strips were placed in each pouch and, where indicated, were desiccated over calcium chloride for 48 hr. After desiccation, the pouches were hermetically sealed immediately after removal from the desiccator. The nondesiccated samples were simply prepared under ambient relative humidity and temperature.

**Determination of resistance to ethylene oxide.** Apparatus and procedures similar to those reported by Ernst and Shull (6) were used. Triplicate experiments were performed at 10-min intervals for each test condition, and test samples were described. Data from experimentation of this nature provided the following pattern of: (i) time intervals in which all test samples survived the test conditions (total survival), (ii) time intervals in which only part of the test samples survived the test conditions (partial survival or partial kill), and (iii) time intervals in which all of the test samples are killed (total kill). The degree of partial survivors decreases as one proceeds from the maximum total survivor time to the minimum total kill time. Thus, for each test condition and test samples, a pattern of resistance can be observed.

**RESULTS**

Figure 1 shows the comparative resistance of *B. subtilis var. niger* spores hermetically sealed in various polymeric films. In general, the order
of increasing resistance was polyethylene = polyvinyl chloride < nylon < cellophane/polyethylene laminate < phenoxy < mylar/polyethylene laminate.

Occasionally, large partial survival zones were observed. The phenomenon of large survival zones has been observed previously (3) due to relatively unclean spores. The partial survival zones observed in this study are not of this nature, but they could explain why "skips" occur occasionally in sterility testing.

Figure 2 demonstrates the difference in resistance of *B. subtilis* var. niger spores on paper to ethylene oxide with and without added relative humidity. This figure demonstrates the necessity of having moisture in close proximity to the spores. In the situation where relative humidity was not added, the spores (and the paper) would tend to lose moisture during the process. In the case of the situation where humidity was added, the spores would tend to gain moisture during the process. As was reported by Ernst and Doyle (5), it is important that moisture diffuses toward the spores to achieve optimal sterilization conditions. Figure 3 compares the resistance of desiccated and nondesiccated *B. subtilis* var. niger spores in paper hermetically sealed in polyvinyl chloride film. As the thickness of the film was increased above three thicknesses, an increase in resistance to sterilization was observed. The primary significance of this figure is that a high degree of resistance was observed where desiccated conditions were produced; approximately a five-fold increase in resistance was observed between nondesiccated and desiccated spores hermetically sealed in polyvinyl chloride.

Figure 4 demonstrates the comparative resistance of nondesiccated and desiccated *B. subtilis* var. niger spores on paper hermetically sealed in nylon film. Once again, as the barrier to the steri-
larization process was increased above three thicknesses, resistance to the agent was also increased. Desiccation, however, was the major factor in increased resistance. A very broad partial survival time was also observed.

Figure 5 shows the comparative resistance of desiccated and nondesiccated *B. subtilis* var. *niger* spores on paper hermetically sealed in polyethylene film with and without added relative humidity. Once again, as the film thickness was increased above four thicknesses, the resistance to sterilization was also increased. Desiccation as compared to nondesiccation also shows that it was a significant factor in increased resistance. Greater partial survival times were also observed when the spore strips were desiccated. What is very significant in Fig. 5 is the comparison of the processes with and without added relative humidity. It appears that with a polyethylene barrier, added relative humidity was not a factor in reducing ethylene oxide resistance. Although it has been reported (4, 5) that relative humidity appears to increase in hermetically sealed polyethylene after the introduction of ethylene oxide, it does not appear to affect the resistance of microorganisms to this agent.

**FIG. 3.** Comparative resistance of desiccated and nondesiccated *B. subtilis* var. *niger* spores on paper hermetically sealed in polyvinyl chloride film. Ethylene oxide concentration, 900 mg/liter; relative humidity, 40%; temperature, 130 F. (A) Nondesiccated strips, 1-mil polyvinyl chloride, one to three thicknesses. (B) Nondesiccated strips, 1-mil polyvinyl chloride, four thicknesses, or one thickness of 8-mil polyvinyl chloride. (C) Desiccated strips, 1-mil polyvinyl chloride, one to two thicknesses. (D) Desiccated strips, 1-mil polyvinyl chloride, three to four thicknesses.

**FIG. 4.** Comparative resistance of nondesiccated and desiccated *Bacillus subtilis* var. *niger* spores on paper hermetically sealed in nylon film. Ethylene oxide concentration, 900 mg/liter; relative humidity, 40%; temperature, 130 F. (A) Nondesiccated strips, 1-mil nylon, one to three thicknesses. (B) Nondesiccated strips, 1-mil nylon, four thicknesses. (C) Desiccated strips, 1-mil nylon, one thickness. (D) Desiccated strips, 1-mil nylon, two to four thicknesses.

**DISCUSSION**

In these experiments, spore strips sealed in various polymeric films were compared as to their resistance to ethylene oxide sterilization. In order of ethylene oxide resistance, we found the following: polyethylene equal to polyvinyl chloride and, in order of increasing resistance, nylon, cellophane/polyethylene laminate, phenoxy, and mylar/polyethylene laminate. As the thickness of the polymeric film was increased beyond a certain level, the resistance to ethylene oxide also increased. Furthermore, as the thickness increased, the partial survival zone also increased. The phenomenon of increased partial survival zone could account for "skips" which are occasionally observed in sterility testing in which a portion of the spore strips tested survive.
FIG. 5. Comparative resistance of desiccated and nondesiccated *B. subtilis* var. *niger* spores on paper hermetically sealed in polyethylene film, with and without added relative humidity. Ethylene oxide concentration, 900 mg/liter; temperature, 130 F. (A) Nondesiccated strips, 1-mil polyethylene, one to four thicknesses, or 4-mil polyethylene, one to two thicknesses, and 40% relative humidity. (B) Nondesiccated strips, 1-mil polyethylene, one to four thicknesses, no relative humidity added (ca. 0%). (C) Nondesiccated strips, 4-mil polyethylene, three to four thicknesses, 40% relative humidity. (D) Nondesiccated strips, 4-mil polyethylene, three to four thicknesses, no relative humidity added (ca. 0%). (E) Desiccated strips, 1-mil polyethylene, one to two thicknesses, 40% relative humidity. (F) Desiccated strips, 1-mil polyethylene, three to four thicknesses, 40% relative humidity. (G) Desiccated strips, 1-mil polyethylene, one to four thicknesses, no relative humidity added (ca. 0%).

If the spore strips sealed in polymeric film were desiccated prior to ethylene oxide exposure, a high increase in resistance to ethylene oxide was observed. This phenomenon demonstrates once again the importance of moisture in close proxim-

ity to the bacterial spores in ethylene oxide processes.

When desiccated and nondesiccated spore strips sealed in polyethylene film were compared in regard to resistance to ethylene oxide with and without relative humidity, a significant result was obtained. Once again the resistance increased as the film thickness increased, and desiccated spores were much more resistant than nondesiccated spores. What was of primary significance, however, was that added relative humidity to the process was not a significant factor in the sterilization. Previously (4, 5), moisture permeation was observed in polymeric films after ethylene oxide introduction by using various humidity recording devices hermetically sealed in the film. The moisture that permeates polymeric films apparently does not contribute to the sterilization process per se.

However, the introduction of moisture has another effect in ethylene oxide processes; it also helps heat the materials that are being processed. As steam is introduced into the chamber under vacuum, the moisture introduced tends to lose its heat to the materials being processed, hence assisting the load in gaining heat. Since the load reaches higher temperatures more quickly, ethylene oxide sterilizes at a faster rate. Furthermore, many packaging materials such as paper or cloth are permeable to moisture, and prehumidification would then be effective in the ethylene oxide process.

Consequently, it is recommended that prehumidification remain a necessary part of ethylene oxide sterilization processes. With material hermetically sealed in polyethylene, the packaging environment should be humidified.

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