Microbiological Aspects of Ethylene Oxide Sterilization

II. Microbial Resistance to Ethylene Oxide

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The death rate kinetics of several sporeforming and nonsporeforming microorganisms, including radiation-resistant cocci, were determined by exposing them to a mixture of ethylene oxide and dichlorodifluoromethane (500 mg of ethylene oxide per liter, 30 to 50% relative humidity, and 54.4°C). Spore survivor curves obtained from tests of inoculated and exposed hygroscopic and nonhygroscopic carriers showed that the spores of Bacillus subtilis var. niger are more resistant to ethylene oxide than are spores of Clostridium sporogenes, B. stearothermophilus, and B. pumilus. The decimal reduction times (expressed as D values at 54.4°C-500 mg of ethylene oxide per liter) obtained under the test conditions for B. subtilis var. niger spores on hygroscopic and nonhygroscopic carriers exceeded the values obtained for the other organisms considered, both sporeformers and nonsporeformers. The decimal reduction times for the vegetative cells of the radiation-resistant organisms (Micrococcus radiodurans and two strains of Streptococcus faecalis) and the ATCC strain of S. faecalis demonstrated comparable resistance to ethylene oxide with the spores of C. sporogenes, B. stearothermophilus, and B. pumilus, but not those of B. subtilis var. niger.

The rapidly growing interest in ethylene oxide as a sterilant, especially for spacecraft and disposable medical supplies, demands more information on the resistance of microorganisms to that agent. Investigations by R. Vondell (Ph.D. Dissertation, Univ. of Mass., 1962), H. El-Bisi, R. Vondell, and W. Esslen (Bacteriol. Proc., p. 13. 1963), Liu, Howard, and Stumbo (4), and R. Gammon, K. Kereluk, and R. Lloyd (Bacteriol. Proc., p. 16, 1968) demonstrated the resistance of several nonsporeforming and sporeforming bacterial species to ethylene oxide.

It has been found that bacterial spores can be up to 100,000 times more resistant to chemical agents than vegetative cells (5). However, Phillips (5) has also shown a comparatively narrow range of resistance to ethylene oxide between spores and vegetative cells.

This study developed quantitative comparative data on the death kinetics of several sporeforming and nonsporeforming bacteria after exposure to a mixture (w/w) of 12% ethylene oxide and 88% dichlorodifluoromethane.

MATERIALS AND METHODS

Test organisms. The sporeforming organisms selected for this study included Bacillus subtilis var. niger (Fr. Detrick strain), Clostridium sporogenes (ATCC 7955 and 3584), B. stearothermophilus (ATCC 7953), and B. pumilus (ATCC 7061).

Five nonsporeforming species were used, namely, Mycobacterium phlei (ATCC 11728), four strains of Streptococcus faecalis [ATCC 349 and strains no. A21, F6, and D1 (Ethicon Co., Somerville, N.J.)], and Micrococcus radiodurans. M. radiodurans and the Ethicon strains of S. faecalis were radiation resistant as described by Anderson et al. (1) and P. Borick (Ethicon Co., unpublished data), respectively.

Preparation of organisms. B. subtilis var. niger spores were grown on nutrient agar with 0.01% MnSO4. A medium developed by Wang et al. (7) 0.1% vitamin-free Casamino Acids, 0.25% glucose, 0.5% yeast extract, 0.01% MnSO4, 0.0001% FeSO4, 2% agar, and 100 ml of water; pH 6.8, was used to obtain B. stearothermophilus spores.

The two C. sporogenes strains were grown on TSP (8) agar (1.5% Trypticase, 0.5% sodium chloride, 0.25% KH2PO4, 0.2% glucose, 1.5% agar, and 100 ml of water; pH 7.2). Soybean agar (2% soytone, 0.05% KH2PO4, 0.01% MnSO4, 2.5% agar, and 100 ml of water; pH 6.8) was used to produce B. pumilus spores. In each case, the sporulation medium was placed in Roux flasks. The sporeformers were incubated at 37°C, except for B. stearothermophilus which was incubated at 55°C. The flasks of TSP agar, inoculated with C. sporogenes, were incubated...
in an anaerobic incubator (National Appliance Co., Portland, Ore.) charged with illuminating gas. After inoculation, the cultures were incubated until microscopic examination revealed 90% (or better) sporulation. The spores were then harvested, washed five times, and stored in sterile distilled water under refrigeration.

All of the nonsporeforming bacteria were grown on tryptone, yeast extract, glucose (1) agar (0.5% tryptone, 0.1% glucose, 0.3% yeast extract, 1.5% agar, and 100 ml of water; pH 7.0) in Roux flasks.

The M. phlei and S. faecalis cultures were incubated for 24 hr at 37°C; M. radiodurans was incubated at 30°C for 48 hr. After incubation, the cells were harvested with sterile water, washed twice, resuspended in sterile water, and then used immediately.

Preparation of organisms for testing. The spore and vegetative cell suspensions were prepared for the tests as previously described (2).

The strains of S. faecalis presented a problem because the cells died rapidly while being dried on the carriers. To avoid such population losses due to desiccation, plastic microcups (Bacti-Capalls, Clay-Adams, Inc., New York, N.Y.) were used as non-hygroscopic carriers. The cups were inoculated with an aqueous suspension (0.25 ml) containing 10⁶ cells which were then transferred (without drying) to individual envelopes and immediately exposed to ethylene oxide.

Exposure apparatus and procedures. The thermo-chemical death rate apparatus and exposure procedures previously described (2) were used in this study. The exposure conditions selected were 500 mg of ethylene oxide per liter, 54.4°C ± 3°C, and approximately 40% relative humidity. The exposure time of the

Fig. 1. Resistance of B. subtilis var. niger to ethylene oxide (500 mg/liter, 54.4 ± 3°C, 30 to 50% relative humidity). Carrier symbols: X, nonhygroscopic; O, hygroscopic.

Fig. 2. Comparative resistance of two strains of C. sporogenes and B. subtilis var. niger to ethylene oxide (500 mg/liter, 54.4 ± 3°C, 30 to 50% relative humidity). Symbols: □, C. sporogenes (ATCC 3584); ○, C. sporogenes (ATCC 7955); X, B. subtilis var. niger. Fig. 2a, nonhygroscopic carrier. Fig. 2b, hygroscopic carrier.

Fig. 3. Comparative resistance of B. stearothermophilus (ATCC 7953) and B. subtilis var. niger to ethylene oxide (500 mg/liter, 54.4 ± 3°C, 30 to 50% relative humidity). Symbols: □, B. stearothermophilus (ATCC 7953); X, B. subtilis var. niger. Fig. 3a, nonhygroscopic carrier. Fig. 3b, hygroscopic carrier.
inoculated carriers was the primary variable concerned.

Recovery and enumeration of survivors. The recovery procedures described in reference 2 were applied to the exposed spore carriers.

Exposed nonhygroscopic carriers, inoculated with cells of one of the nonsporeforming species, were transferred to 99 ml of sterile distilled water and shaken for approximately 20 min. After this, appropriate dilutions were prepared for survivor counts.

The recovery procedures used with exposed hygroscopic carriers inoculated with vegetative cells were similar to those used for the hygroscopic spore carriers.

Plate count agar (Difco) was used as the recovery medium for the aerobic sporeformers and nonsporeformers; TSP agar was used for C. sporogenes strains.

The dilution plates of the test organisms were incubated at their optimal growth temperatures for 48 hr, and survivor counts were prepared. The dilution plates of C. sporogenes, each overlaid with TSP agar, were incubated in an anaerobic incubator charged with illuminating gas.

RESULTS AND DISCUSSION

The results of this study are shown in Fig. 1–7 and in Table 1. The thermochemical survivor curves and decimal reduction values were prepared as described in the preceding paper (2).

Resistance of the sporeformers to the exposure conditions varied. The B. subtilis var. niger spores (Fig. 1) were the most resistant, particularly when dried on nonhygroscopic surfaces. Spores of the two C. sporogenes strains demonstrated similar resistance patterns (Fig. 2), as did the spores of B. stearothermophilus and B. pumilus (Fig. 4 and 5).

Among the nonsporeformers, three of the four S. faecalis strains (F6, A11, and the ATCC strain) were the most resistant to the ethylene oxide conditions (Fig. 8). The cells of S. faecalis (strain 31) and of M. phlei (Fig. 7) were the least resistant.

Table 1 shows the decimal reduction times derived from the curves. Decimal reduction values, obtained by destruction methods such as steam under pressure and dry heat, have been reported by other investigators (6) as the D value at temperature of exposure (e.g., D4.4 c). Since there is only one variable in these processes, that is sufficient for D-value reporting. However, for the D value to be meaningful to ethylene oxide sterilization, temperature and concentration of gas must be considered. Both of these factors can effect different D values independently. Therefore, ethylene oxide D values must include exposure
temperature and gas concentration (e.g., $D_{54.4}$ 0-500 mg/liter). The $D$ values of 6.0 and 4.55 min for the B. subtilis var. niger spores (dried on hygroscopic and nonhygroscopic surfaces, respectively) indicated greater resistance when compared to $D$ values of the other test organism.

Values obtained for M. radiodurans and the S. faecalis strains ATCC 349, F$_6$, and A$_2$I suggest that, except for B. subtilis var. niger, the vegetative cells of these nonsporeformers are almost as resistant to ethylene oxide as the spores of C. sporogenes, B. steaerotherophilus, and B. pumilus. These results simulate those of J. B. Opfell, J. L. Shannon, and H. Chan (Bacteriol. Proc., p. 13–14, 1967). They reported that dry Staphylococcus epidermidis cells were more resistant to ethylene oxide vapor than were B. subtilis var. niger spores and were capable of surviving in liquid ethylene oxide as well as ethylene oxide mixed with a solid propellant. (We were unable to obtain a culture of Dr. Opfell’s strain of S. epidermidis for testing; therefore, a comparative $D$ value with the other microorganisms tested is not available.) Though in agreement with the work of others, our results illustrate the relative variation in resistance among microorganisms when exposed to ethylene oxide and disclose differences when organisms are dried on nonhygroscopic and hygroscopic surfaces. Such data are of value when developing specific sterilization cycles for materials of known contamination levels and when extrapolating conditions for particular sterilization applications.

**LITERATURE CITED**

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**Table 1. Decimal reduction values of various sporeforming and nonsporeforming organisms exposed to ethylene oxide**

| Organism                        | NHS | HS  |
|--------------------------------|-----|-----|
| Bacillus subtilis var. niger    | 6.66| 4.30|
| Clostridium sporogenes( ATCC 3584) | 3.67| 3.30|
| C. sporogenes ( ATCC 7955)     | 3.25| 2.80|
| B. steaerotherophilus ( ATCC 7953) | 2.63| 2.63|
| B. pumilus ( ATCC 7061)        | 2.81| 2.21|
| Micrococcus radiodurans        | 3.00| 2.25|
| M. phlei ( ATCC 11728)         | 2.40| 1.40|
| Streplococcus faecalis (ATCC 349) | 3.04|     |
| S. faecalis (Ethicon F$_6$)    | 3.75|     |
| S. faecalis (Ethicon A$_2$I)   | 3.13|     |
| S. faecalis (Ethicon $\theta_{12}$) | 2.00|     |

*a Values are expressed as $D$ values at 54.4°C and a concentration of ethylene oxide of 500 mg/liter.  
*b Nonhygroscopic surface.  
*c Hygroscopic surface.
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