Microbiological Aspects of Ethylene Oxide Sterilization

III. Effects of Humidity and Water Activity on the Sporicidal Activity of Ethylene Oxide

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An investigation determined the effects of environmental moisture content or water activity (Aw), exposure humidity, and sterilant concentration on the resistance of microbial spores. Decimal reduction values [expressed as D values at 54.4°C-specified concentration (milligrams per liter) of ethylene oxide] were determined from spore destruction curves of Bacillus subtilis var. niger dried on hygroscopic and nonhygroscopic surfaces. Four groups of spore preparations were preconditioned in one of four Aw environments (<0.1, 0.1, 0.5, 0.95) for 2 weeks or longer and were exposed to 500 mg of ethylene oxide per liter at 54.4 ± 3°C and 10, 50, and 95% relative humidity in a specially designed thermochemical death rate apparatus. A fifth group did not receive any preconditioning treatment and was exposed immediately after preparation, in the same apparatus at the same temperature, to ethylene oxide concentrations of 200, 400, 600, 800, and 1,200 mg/liter and relative humidities of 15, 30, 50, 60, and 90%. The resistance of the spores on both types of surfaces to ethylene oxide increased proportionately with the Aw of the conditioning environment. The study also showed that moisture in the exposure system was not as critical a variable as the ethylene oxide concentration. The spore destruction rates, irrespective of the carrier types at all concentrations and at different humidities, varied little from one another. The decimal reduction values were reduced as the ethylene oxide concentration increased, and no optimal exposure humidity concentration was observed.

It has generally been accepted that moisture is essential to sterilization with ethylene oxide vapors. In fact, Kaye (3) recently reported that water must be present, not simply as an adjuvant but as a necessity, and that ethylene oxide with water is a nonspecific toxic agent for all microorganisms. The influence of moisture on gas sterilization has been the subject of numerous studies (1, 2, 4, 5, 12). These studies have considered the effect of moisture on microbial cells when exposed to ethylene oxide as well as on the sterilization of products and materials by this agent.

Lea et al. (8) demonstrated the critical relation of cell moisture content to certain chemical restrictions associated with living cells. Their work suggested that the Z value (reciprocal of the decimal reduction value) will decrease as the moisture content of the cell increases. Murrell and Scott (9, 10) investigated the effect of cell moisture content on the rate of microbial death at dry-heat temperatures from 70 to 120°C. They demonstrated that Bacillus spores were most resistant to dry heat when preconditioned and exposed at 20 to 40% relative humidity. Gilbert et al. (2), in studying the effects of moisture on ethylene oxide sterilization, speculated that certain cross-linkages are formed through water molecule bridges within cell proteins or between adjacent proteins which make alkylating sites more accessible to ethylene oxide.

Kaye and Phillips (4) reported 20 to 40% relative humidity as optimal for the destruction of microorganisms by ethylene oxide. Kelsey (5) noted that insufficient moisture (very low humidities) prevented the bactericidal action of ethylene oxide, whereas an excess of moisture caused ethylene oxide to hydrolyze to the more ineffective compound ethylene glycol. Ernst and Shull (1) noted how barriers to diffusion of moisture could limit ethylene oxide effectiveness. They also reported that prehumidification of materials prior to exposure enhanced the penetration of water vapor through absorbent materials and thus decreased the time for sterilization.

In this study, relative humidity is expressed as the ratio between the density of the actual water vapor existing and the density of saturated vapor.
at the existing temperature (11). Since both densities are proportional to pressure, it is more often referred to as the ratio of actual vapor pressure to the saturated pressure at the existing temperature. This definition describes relative humidity as being independent of the total pressure in the area and independent of the characteristics of the other gases in the area. Water activity (Aw) is a fundamental property of an aqueous solution and by definition is equal to the ratio of the vapor pressure of a solution to that of a solvent (13). Aw is numerically equal to the corresponding relative humidity expressed as a fraction of 100. The term "water activity" describes the status of water in a substrate in preference to relative humidity which applies more strictly to the surrounding atmosphere.

The primary aim of this work was to consider how Aw and varied relative humidities affected the sporicidal nature of ethylene oxide. A second aim was to consider the relationship between ethylene oxide concentration and relative humidity.

**MATERIALS AND METHODS**

**Test organism preparation.** *Bacillus subtilis* var. *niger* (Pt. Detrick strain) was used for all tests. The groups of carriers inoculated with spore suspensions of this organism were prepared as previously described (7). Five groups of inoculated carriers (both types) were prepared. Four of the groups were placed in preconditioning environments before testing. The fifth group received no special preconditioning. Immediately after inoculation, the carriers of this group were transferred to sterile glassine envelopes and were exposed to the test conditions.

**Preconditioning.** Four environments, each with a different Aw value were used to precondition the
inoculated carriers. The values were <0.1, 0.1, 0.5, and 0.95 Aw, respectively. To obtain an Aw of <0.1, a desiccator containing CaCl₂ was used. An atmosphere with an Aw of 0.1 was prepared by placing a saturated aqueous solution of LiCl·H₂O in a desiccator. An Aw of 0.5 was prepared with a saturated aqueous solution of KNO₂ in a desiccator. A saturated aqueous solution of KNO₂ in a desiccator was used to obtain an Aw of 0.95. Each desiccator was equipped with a relative humidity sensor (El-Tronics, Inc., Mayfield, Pa.) attached to a portable hygrometer (El-Tronics, Inc.). The desiccators were maintained at 21 ± 3 °C and conditioned for a minimum of 1 week before use. At the end of this period, the inoculated carriers (in sterile petri dishes) were placed in the preconditioning desiccators and conditioned for at least 2 weeks before use. Prior to testing, the carriers were aseptically transferred from the desiccators to sterile glassine envelopes.

Exposure apparatus and procedure. The thermochemical death rate apparatus and exposure procedures previously described were used (6). Death kinetics were studied with the gas sterilant at 54.4 ± 3 C.

Humidity. Relative humidities of 10, 15, 30, 50, 60, 90, and 95% were obtained in the exposure system (6).

Ethylene oxide concentrations. Ethylene oxide concentrations were 200, 400, 500, 600, 800, 1,000, and 1,200 mg/liter. These concentrations were obtained by adjusting the pressure of the gaseous mixture within the test chamber.

Exposure time. The inoculated carriers were exposed at intervals of 0 to 35 min.

Tests performed. (i) The four groups of prehumidified inoculated carriers were exposed to an ethylene oxide concentration of 500 mg/liter at 54.4 ± 3 C. Various exposure times, and relative humidities of 10, 50, or 95% were used. (ii) The fifth group of inoculated carriers (not prehumidified) was exposed to various ethylene oxide concentrations (200, 400, 500, 600, 1,000, and 1,200 mg/liter), various exposure humidities (15, 30, 50, 60, 90% relative humidity), and various exposure periods at 54.4 ± 3 C.

Recovery and enumeration of survivors. The re-

Fig. 2. Spore-survivor curves of B. subtilis var. niger exposed to various ethylene oxide concentrations at relative humidities of 15, 30 and 50%. Symbols: ⊗, 200 mg/liter; ○, 400 mg/liter; □, 600 mg/liter; △, 800 mg/liter; ●, 1,000 mg/liter; ▲, 1,200 mg/liter.
covery and plating procedures previously described (6) were applied to the exposed spore carriers.

RESULTS AND DISCUSSION

Survivor curves for B. subtilis var. niger spores inoculated on both hygroscopic and nonhygroscopic surfaces and exposed to the previously described conditions are presented in Fig. 1, 2, and 3. The thermochemical survivor curves and the decimal reduction values (Tables 1 and 2) were prepared as previously described (6).

These results demonstrated the effect of three factors on the sporicidal activity of ethylene oxide during exposure of the spores in the test apparatus. These factors were preconditioned spores, various relative humidities, and various ethylene oxide concentrations.

The resistance of B. subtilis var. niger spores to ethylene oxide can be influenced by the water activity of the microenvironment surrounding the spores prior to exposure. Generally, as the Aw of the preconditioning environment increased the resistance of spores on both types of carriers increased. This was demonstrated by the higher decimal reduction values (expressed as D values at 54.4 C-500 mg of ethylene oxide per liter). However, the survivor patterns (Fig. 2 and 3) as well as the decimal reduction values in Table 2, signify that moisture in the exposure system is not as critical a variable as ethylene oxide concentration. It will be noted that at all concentrations, irrespective of the carrier types, spore destruction rates varied little at the different exposure humidities. An increase in the sporicidal effect was observed as the ethylene oxide concentration increased. The most marked effect was noted when the concentration of ethylene oxide was increased from 200 to 400 mg/liter. Considering all data at these two concentrations, irrespective of carrier materials and relative

**Table 1. Decimal reduction values of B. subtilis var. niger spores preconditioned at various water activities (Aw) and exposed to ethylene oxide**

| Water activity | Decimal reduction values, relative humidities of exposure* |
|---------------|----------------------------------------------------------|
|               | 10%           | 50%           | 95%           |
|               | NHSb         | HS*           | NHS           | HS            | NHS           | HS            |
| <0.1          | 3.3          | 2.3           | 2.3           | 3.0           | 3.7           | 2.6           |
| 0.1           | 3.8          | 3.3           | 3.9           | 3.3           | 4.0           | 2.8           |
| 0.5           | 3.8          | 3.6           | 3.8           | 3.8           | 4.5           | 3.6           |
| 0.95          | 5.5          | 3.6           | 4.2           | 3.6           | 3.7           | 3.7           |

* Expressed in minutes as D at 54.4 C-500 mg of ethylene oxide per liter.

b Nonhygroscopic surface.

c Hygroscopic surface.

**FIG. 3. Spore-survivor curves of B. subtilis var. niger exposed to various ethylene oxide concentrations at relative humidities of 60 and 90%. Symbols: ◆, 200 mg/liter; ○, 400 mg/liter; □, 600 mg/liter; △, 800 mg/liter; ●, 1,000 mg/liter; ▲, 1,200 mg/liter.**
humidity, there was an average decrease in the
decimal reduction values of 2.57 min. None of the
other gas concentration increments was as dra-
matic in its effect on the decimal reduction values.

Our results also provided some insight into
the effect of moisture on the resistance of *B. subtilis* var. *niger* spores to ethylene oxide from
two aspects—the moisture content Aw of the
microenvironment of the spores and the moisture
content (relative humidity) during ethylene oxide
exposure.

The effect of moisture on the sterilizing action
of ethylene oxide seems to lie either in the mois-
ture content of the bacterial cell at the time it
contacts the sterilant or in the moisture condi-
tions of the atmosphere (or microenvironment)
surrounding the individual cells. Kelsey (5) sug-
gested that the moisture content of the atmos-
phere surrounding the organism and the water
content of the organism itself are more important
than the overall humidity of the atmosphere sur-
rounding the material being sterilized. Gilbert et
al. (2) demonstrated that desiccated or highly
dried bacterial spores which were difficult to
sterilize with ethylene oxide lost their resistance
when prehumidified in an atmosphere of 75 to
98% relative humidity. Kaye and Phillips (4),
however, suggested that a zone of high moisture
about a spore will have a diluting effect (dilu-
tion theory) on ethylene oxide and reduce its
availability to the spore. This theory, as well as
others, would give credence to our findings that
the moisture content (Aw) of the atmosphere
had more effect on the resistance of *B. subtilis*
var. *niger* spores than did the overall relative
humidity in the exposure chamber.

It is concluded from these studies that the
water content or Aw of the microenvironment
of the spores influenced the ethylene oxide re-
sistance more than the overall relative humidity
in the sterilizing atmosphere. On the other hand,
the remarkable decrease in the spore resistance
noted in the presence of increased ethylene oxide
concentration was strong evidence that gas con-
centration is an essential variable in ethylene
oxide sterilization. Our study also disclosed no
optimal humidity for the destruction of spores of
*B. subtilis* var. *niger* when exposed under the
aforementioned test conditions. The small varia-
tions observed in the decimal reduction values
at various humidities were considered of little
consequence in commercial gas sterilization;
however, moisture can become a critical factor
when packaging materials interfere with moisture
and ethylene oxide diffusion or transmission.

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| Amt of ethylene oxide (mg/liter) | Decimal reduction values at relative humidities of exposure* |
|----------------------------------|----------------------------------------------------------|
|                                  | 15% | 30% | 50% | 60% | 90% |
| 200                              | NHS | HS  | NHS | HS  | NHS |
| 400                              | 5.75 | 6.75 | 7.50 | 7.50 | 6.25 |
| 600                              | 4.00 | 3.50 | 3.75 | 3.75 | 3.75 |
| 800                              | 4.25 | 2.75 | 3.75 | 3.75 | 3.75 |
| 1,000                            | 2.75 | 2.50 | 3.25 | 2.25 | 3.25 |
| 1,200                            | 2.50 | 1.75 | 2.25 | 2.00 | 2.00 |

* Expressed in minutes as D values at 54.4 C-specified concentration of ethylene oxide.

* Nonhygroscopic surface.

* Hygroscopic surface.
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