Microbiological Aspects of Ethylene Oxide Sterilization

I. Experimental Apparatus and Methods

K. KERELUK, R. A. GAMMON, AND R. S. LLOYD

Research Department, American Sterilizer Company, Erie, Pennsylvania 16512

Received for publication 16 September 1969

A specially built thermochemical death-rate apparatus is described which can be used to determine the resistance of microorganisms to ethylene oxide under controlled conditions. The apparatus was designed to provide instantaneous exposure of microorganisms to ethylene oxide and to eliminate variables that could result in errors when death kinetic reaction rates are calculated. The apparatus is used to obtain ethylene oxide resistance data which are useful in evaluating and developing sterilizing cycles for materials with known bacterial concentrations, as well as for calculating probability factors on which a given test condition can be expected to provide sterilization.

Since 1949, when Phillips and Kaye (8) first published their work on the sterilizing capabilities of ethylene oxide, there have been numerous other reports on various aspects of this subject (1, 2, 6, 7; H. El-Bisi, E. Thompson, and J. J. Perkins, Bacteriol. Proc., p. 34, 1962). Various techniques and apparatuses have been employed in investigating the conditions required for ethylene oxide sterilization. Ernst and Shull (1) used a cylindrical pressure vessel in their studies of the effects of temperature and concentration on ethylene oxide sterilization. Other investigators have used anaerobic jars (5, 6), as well as vapor-phase resistometers (H. El-Bisi, E. Thompson, and J. J. Perkins, Bacteriol. Proc., p. 34, 1962; T. Liu, C. R. Stumbo, and G. L. Howard, Ph.D. dissertation, Univ. of Mass., 1966) to study bacterial death with ethylene oxide.

This paper presents details of the methods and apparatus used in our laboratories to study the resistance of sporeforming and nonsporeforming bacteria to ethylene oxide. Preliminary data are presented on the use of this apparatus in studying the thermochemical death rate of Bacillus subtilis var. niger spores. Subsequent papers will describe studies concerning the resistance of other microorganisms to ethylene oxide and the effects of certain environmental factors (sterilant concentration, relative humidity, spore moisture content, temperature, and packaging materials) on ethylene oxide sterilization.

MATERIAL AND METHODS

Organisms. Both sporeforming and nonsporeforming microorganisms were used in these studies. The sporeformers were grown on selected sporulation media (9), and the nonsporeformers on appropriate media. In both cases, once a suitable population was obtained, the organisms were suspended in sterile distilled water, washed by centrifugation, resuspended in sterile distilled water, and stored at 4°C prior to exposure. The details of growth, conditions of incubation, media used, and procedures of harvest and storage for specific microorganisms will be described as pertinent in subsequent articles.

Selection of carrier. Studies have suggested that spores on a hygroscopic carrier are less resistant to ethylene oxide than are spores on a nonhygroscopic carrier (4, 6). To establish comparative data, we chose to work with both surface types.

Two types of nonhygroscopic carriers were considered. These were glass beads, 4 mm in diameter, and 0.635-cm square, glazed, ceramic tiles. Each carrier was prepared as follows.

Glass beads. B. subtilis var. niger spores (in a 2.5-ml distilled-water suspension) were placed in a 250-ml polyethylene bottle containing 1,000 sterile glass beads. The bottle was connected to a drive shaft which rotated the unit (on its side) at 35 rev/min. During rotation, dry air at 45°C was circulated through the bottle to facilitate drying of the spores on the beads.

Ceramic tiles. Each tile in three groups of 50 tiles each was inoculated with 0.1 ml of a B. subtilis var. niger spore suspension. The inoculated tiles were dried in a hot-air oven at 55°C for 1.25 hr. Viable spore counts were then prepared.

The results of this study (Table 1) led to the rejection of glass beads as a suitable nonhygroscopic carrier, in favor of the ceramic tiles.

The procedures developed and used to recover inocula from the glass beads and ceramic tiles will be described in detail.

Strips 0.635 by 0.375 cm of filter paper (no. 40E,
Table 1. Comparison of spore counts of B. subtilis var. niger recovered from ceramic tiles and glass beads

| Carrier type          | Group 1 Population\(^a\) | Log value | Group 2 Population | Log value | Group 3 Population | Log value | F ratio\(^b\) |
|-----------------------|--------------------------|-----------|--------------------|-----------|--------------------|-----------|--------------|
| Ceramic tile          | 268 × 10\(^3\)          | 5.42      | 252 × 10\(^3\)     | 5.40      | 258 × 10\(^3\)     | 5.41      | 0.65         |
| Glass beads           | 16.7 × 10\(^3\)         | 4.22      | 8.40 × 10\(^3\)    | 3.92      | 3.20 × 10\(^3\)    | 3.51      | 7.50         |

\(^a\) Values represent the average spore counts obtained from 50 carriers per group.

\(^b\) Ratio of two estimates of the sample variance with the second estimate in the numerator.

![Diagramatic representation of the thermochemical death apparatus and a reaction container, with auxillary and recording equipment.](image)

Schleicher & Schull Co., Keene, N.H.) were used as the hygroscopic carriers. They were placed in glass petri dishes and sterilized in a hot-air oven for 2 hr at 170°C. Subsequently, each sterilized carrier was inoculated with 0.01 ml of the spore- or vegetative-cell suspension which contained approximately 10\(^9\) organisms per ml. The spore-inoculated carriers were dried at 55°C for 1.25 hr; those inoculated with nonsporeforming organisms were air dried at ambient temperature.

Test apparatus. Figure 1 shows the main components of the test apparatus used to determine the resistance of the microorganisms on the carriers to ethylene oxide.

Sterilizer. The sterilizer was a Cryotherm (American Sterilizer Company, Erie, Pa., model 1016). The chamber (25.4 cm in diameter by 40.64 cm in length), provided and maintained the required levels of temperature, humidity, and ethylene oxide concentration for the tests. Thermostatically controlled strip heaters
affixed to the exterior chamber walls were the heat source. The chamber also included a pressure relief valve (Fig. 1A), and a hand valve for admitting the sterilant (Fig. 1B).

Instrumentation for the chamber included a pressure gauge, graduated in increments of 0.2 psi; a vacuum gauge, graduated in increments of 0.25 cm of mercury; and a humidity indicator, with a sensor inside the chamber.

An adaptor (Conax Co., Buffalo, N.Y.) was installed in the chamber door to facilitate removal of gas samples while the door was closed.

Manifold. A metal manifold tube equipped with a shutoff valve (Fig. 1, C) and covered with double-element heating tape (Glas-Col Apparatus Co., Terre Haute, Ind.) was connected to the sterilizer exhaust line and extended over the water bath. This tube contained six quick-disconnect couplers (Fig. 1, E) for the reaction containers. Each coupler (Snap-Tite Co., Oil City, Pa.) was fitted with a hand-valve shutoff (Fig. 1, D).

Heated water bath. The 25-gal water bath was augmented by a constant temperature heater and circulator (Precision Scientific Co., Chicago, Ill., no. 66567 and 66540, respectively).

Reaction containers. The containers shown in Fig. 2, hold the inoculated carriers for exposure to the gaseous atmosphere contained in the sterilizer chamber. The containers are brass cylinders 125 mm long and 4 mm thick with an inside diameter of 44 mm. One end of each container is closed with the opposite end threaded to receive a standard pipe cap. The pipe cap, with four 0.375-cm openings on the top, was equipped with two 0.375-cm adaptors (Conax Co.), a 0.375-cm shutoff valve, and a compound pressure-vacuum gauge. A copper–constantan thermocouple for sensing the temperature within the container was inserted through one of the Conax adaptors. The other Conax adaptor was used for the removal of gas samples from the container for chromatographic analysis. In operation, the equipped pipe cap was screwed onto the reaction container (with inoculated carriers inside) until a seal was formed against a rubber O-ring located on the container. For connection to and release from the gas manifold, the shutoff valve on the reaction container pipe cap was equipped with a quick-disconnect coupling. This arrangement provided for two shutoff valves between the gas manifold and each reaction container.

**Monitoring equipment.** The temperature inside the gas chamber was determined by thermocouples attached to the inside of the chamber. These thermocouples and those in the reaction containers were connected by wire leads to a multipoint recorder (Minneapolis-Honeywell Co., Philadelphia, Pa.) for direct temperature monitoring throughout the tests.

Chamber humidity was determined by a humidity sensor connected to a humidity indicator (El-Tronics, Inc., Mayfield, Pa., model 1106) on top of the chamber. The desired relative humidity within the chamber was attained by injecting a predetermined amount of distilled water into the chamber prior to each test run.

A Lira infrared gas analyzer (Mine Safety Appliance Co., Pittsburgh, Pa., model 300), connected to the gas chamber, was used to analyze the ethylene oxide concentration during the tests.

Sterilant supply. The sterilant used for the tests was a gaseous mixture of 12% ethylene oxide and 88% dichlorodifluoromethane by weight, contained in a 145-lb. cylinder (Pennsylvania Engineering Company, Philadelphia, Pa.).

**Exposure procedure.** The test procedures used with the experimental exposure apparatus were as follows, in the order stated. (i) The sterilizer chamber, manifold, and water bath were brought to 34.4 ± 3 C. (ii) The gas analyzer was calibrated according to operating instructions. (iii) Distilled water (Table 2) was added to the chamber to provide the desired relative humidity. (iv) Five inoculated hygroscopic carriers and five inoculated nonhygroscopic carriers in individual, sterile, glassine envelopes, were placed in each of six reaction containers. The containers were sealed, connected to a vacuum pump, and
TABLE 3. Comparison of recovery from nonhygroscopic and hygroscopic carriers subjected to various recovery procedures

| Carrier | Recovery procedures | Time shaken | No. of glass beads in dilution blanks§ | Time in sonicator | Recovery |
|---------|---------------------|-------------|----------------------------------------|-------------------|----------|
| Nonhygroscopic | 1 | 1 | 6 | 0 | 63.0 |
| | 2 | 2 | 6 | 0 | 66.0 |
| | 3 | 4 | 6 | 0 | 68.0 |
| | 4 | 15+ | 6 | 0 | 66.0 |
| | 5 | 1 | 6 | 5 | 88.0 |
| | 6 | 2 | 6 | 5 | 72.0 |
| | 7 | 4 | 0 | 5 | 90.0 |
| | 8 | 15+ | 6 | 5 | 93.0 |
| | 9 | 15+ | 12 | 10 | 99.0+ |
| Hygroscopic | 1 | 0 | 0 | Water§ | 100 |
| | 2 | 1 | 0 | Water§ | 100 |
| | 3 | 1 | 0 | Darvan§ | 100 |

§ Minimum of five carriers was used for each set of conditions.

§ Dilution blanks contained 1% Darvan in 99 ml of distilled water.

Substrate.

evacuated to 67.58 cm of mercury. They were then connected to the manifold via the quick-disconnect couplers and suspended in the water bath. The shutoff valves on the containers and their respective connections were closed. Each container with inoculated carriers was suspended in the water bath and heated to 54.4°C (approximately 15 min) before the contents were exposed to ethylene oxide. (v) The shutoff valve between the chamber and the manifold was opened. The chamber and the manifold were then evacuated to 67.58 cm of mercury and charged to a preselected pressure with the sterilant. (vi) After a 6-min stabilization period, the sterilant concentration, chamber temperature, and humidity were measured by the instrumentation noted previously. Deviations of these factors from the preselected test conditions were adjusted at this time. (vii) Once the preselected test conditions were established, the sterilant was transferred to each pretempered reaction container by opening the shutoff valve leading from the manifold to the reaction container and the shutoff valve on the reaction container. This initiated the exposure period for each container.

At predetermined intervals during the exposure period, the two shutoff valves between the manifold and one of the reaction containers were closed. This reaction container was then disconnected from the manifold and chilled in ice water (5 to 10 min) while being evacuated, returned to atmospheric pressure, and opened. The inoculated carriers were then removed for survivor counts. Initial total viable spore counts were prepared from inoculated carriers placed in one of the reaction containers and removed from the manifold and water bath prior to introducing the sterilant into the remaining containers.

Recovery procedures. Comparative studies were performed to develop procedures which would yield maximal recovery of viable organisms from the inoculated carriers.

Inoculated nonhygroscopic carriers were transferred to dilution blanks containing 99 ml of 1% aqueous Darvan (R. T. Vanderbilt Co., New York, N.Y.) and a variable number of 4-mm diameter glass beads. Darvan, a polymerized sulfonic acid salt, was used to enhance the dispersion of the inoculum. The dilution blanks were placed in a reciprocating shaker and then in a sonic disintegrator for various time intervals. Sonic treatment was employed for further dispersion of the inoculum from the carrier surface.

To obtain a 99.9% recovery of viable inoculum from the inoculated hygroscopic carriers (filter paper strips), a 2-min mixing in a Waring blendor micropip was required.

As a result of these studies (Table 3), the following recovery procedures were adopted for treating inoculated carriers after exposure to the test conditions.

Immediately after exposure, each treated nonhygroscopic carrier was transferred to a dilution blank containing 99 ml of aqueous 1% Darvan solution and 12 (4 mm in diameter) glass beads. The dilution blanks were placed in a reciprocating shaker, in water at 4°C, and shaken overnight. The dilution blanks were then placed in a sonic disintegrator and exposed to 20 kc/sec for 10 min.

The treated hygroscopic carriers were transferred to 99 ml of sterile, distilled water in a Waring blendor micropip and were blended for 2 min. After these recovery procedures, serial dilutions were then prepared and viable cell counts were determined in various plating media.

RESULTS AND DISCUSSION

An analysis of the variance (3) among the viable spore count values obtained from three different groups of the glass beads demonstrated
statistically the inconsistency of using the beads as inoculum carriers.

Gross viable spore count irregularities were found among the bead groups. This did not occur among the ceramic tiles, as is indicated by viable spore counts and the derived \( F \) ratio. When the same statistical test was applied to the viable counts from three groups of tiles, the advantage of this type of carrier was clearly demonstrated as the variance among the three groups was negligible.

The \( F \) ratio is the ratio of two estimates of the sample variance with the second estimate in the numerator (3). This type of analysis is one in which observations are classified into groups on the basis of a single property, for example, carrier populations. There were three population groups for each carrier. The sample variance for each group was obtained and the three values were averaged to give the first estimate of the variance; the sample variance for the three mean values of the three groups was also calculated to give the second estimate of the variance. The variance values were used to calculate the \( F \) ratio. Large \( F \)-value ratios would indicate significant differences among the three populations.

The tiles had an \( F \) ratio of 0.65 as compared to 7.50 for the glass beads as noted in Table 1.

Ernst and Shull (1) noted the variation in the count of individual beads and tried to minimize this variation by using three beads per tube since they did not utilize the bead carriers quantitatively.

The use of the specially designed thermo-chemical death rate apparatus aided in reducing some of the possible errors in death kinetic studies, once the conditions of exposure were established in the main chamber of the apparatus. The transfer of these conditions to the reaction container was instantaneous. Similarly, once the exposure period for a group of inoculated carriers was completed, the design of the apparatus allowed the immediate removal of the carriers from the test environment.

Chromatographic analyses of samples of the chamber atmosphere demonstrated that once the main chamber and reaction containers had been charged with a specific concentration of ethylene oxide, it did not vary greatly from one reaction container to another. No difficulty was encountered in maintaining constant humidity and temperature within the sterilizer chamber and reaction containers.

From these investigations, a recovery procedure was developed which yielded 99% (or better) viable inocula from the inoculated and exposed carriers (Table 3).

Survivor curves were prepared for spores and cells of microorganisms dried on both hygroscopic and nonhygroscopic surfaces and exposed to ethylene oxide in the test apparatus. Each point used in preparing the curves represents an average number of survivors. The straight-line portion of all curves was located by linear regression. Analysis of death kinetic reaction rates, when plotted on semilogarithmic paper, is usually expressed in terms of the decimal reduction, or \( D \) value. The \( D \) value is the time required to destroy 90% of the bacterial cell or spore population under a given set of conditions (9). This value can be useful in determining the theoretical probability of bacterial cells or spores surviving a given sterilizing agent or process. From the curves, the decimal reduction times (\( D \) values at 54.4 C-concentration of ethylene oxide in milligrams per liter) were taken as the time (in minutes) to kill 90% of the spores. Survivor curves of \( B. \ subtilis \ var. \ niger \) spores, deposited on both hygroscopic and nonhygroscopic surfaces and exposed to ethylene oxide, are presented in Fig. 3, which represents typical data obtained.

LITERATURE CITED

1. Ernst, R. R., and J. J. Shull. 1962. Ethylene oxide gaseous sterilization I. Concentration and temperature effects. Appl. Microbiol. 18:337–341.

2. Gilbert, G. L., V. M. Gambil, O. R. Spinner, R. K. Hoffman,
and C. R. Phillips. 1964. Effects of moisture on ethylene oxide sterilization. Appl. Microbiol. 12:496-503.
3. Hoel, P. G. 1960. Elementary statistics. John Wiley & Sons, Inc., New York.
4. Kay, S., and C. R. Phillips. 1949. The sterilizing action of gaseous ethylene oxide. IV. The effect of moisture. Amer. J. Hyg. 50:296-306.
5. Liu, T., G. L. Howard, and C. R. Stumbo. 1968. Dichlorodifluoromethane-ethylene oxide mixture as a sterilant at elevated temperatures. Food Technol. 22:86-89.
6. Opfell, J. B., J. P. Honmann, and A. B. Latham. 1959. Ethylene oxide sterilization of spores in hygroscopic environments. J. Amer. Pharm. Ass. 48:280-282.
7. Phillips, C. R. 1952. Relative resistance of bacterial spores and vegetative bacteria to disinfectants. Bacteriol. Rev. 16:135-138.
8. Phillips, C. R., and S. Kaye. 1949. The sterilizing action of gaseous ethylene oxide. I. Review. Amer. J. Hyg. 50:270-279.
9. Stumbo, C. R. 1965. Thermobacteriology in food processing. Academic Press Inc., New York.