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Microbiological Aspects of Ethylene Oxide Sterilization

I. Experimental Apparatus and Methods

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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		Group 2		Group 3		
	Population[a]	Log value	Population	Log value	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.

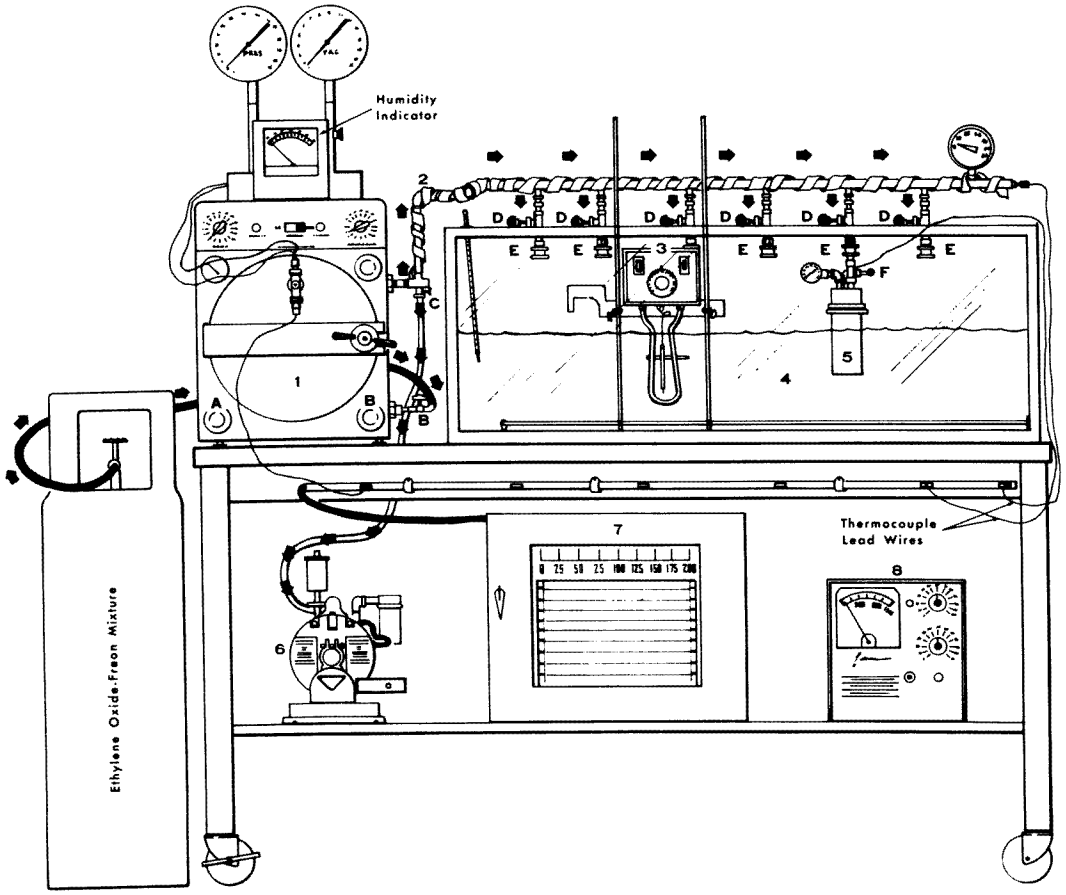

FIG. 1. *Diagramatic representation of the thermochemical death apparatus and a reaction container, with auxillary and recording equipment. Key: 1, conditioning chamber; 2, manifold and heating tape; 3, water heater; 4, water bath; 5, reaction container; 6, vacuum pump; 7, potentiomenter recorder; 8, gas analyzer.*

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 10^8 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

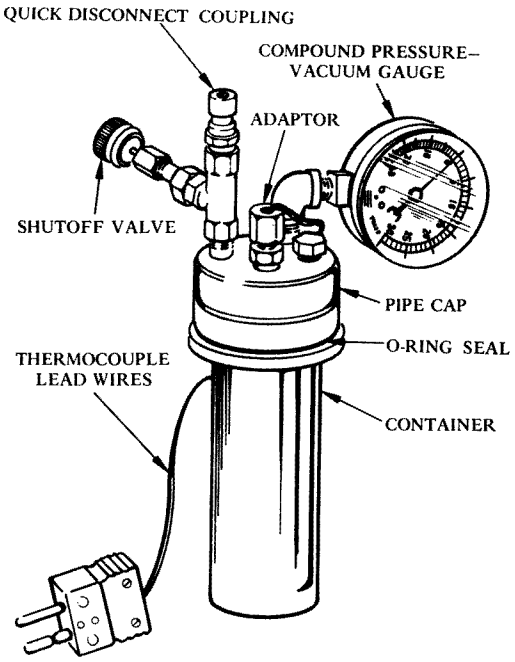

FIG. 2. *Diagramatic representation of a reaction container used with thermochemical death apparatus.*

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

TABLE 2. *Amount of water required in chamber to obtain a specific relative humidity in exposure system*

Humidity	Distilled water
%	*ml*
15	1.0
30	2.0–3.0
50	3.0–5.0
60	6.0
90	15.0

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 54.4 ± 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[a]	Recovery procedures	Time shaken	No. of glass beads in dilution blanks[b]	Time in sonicator	Recovery
		hr		*min*	%
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[c]	100
	2	1	0	Water[c]	100
	3	1	0	Darvan[c]	100

[a] Minimum of five carriers was used for each set of conditions.
[b] Dilution blanks contained 1% Darvan in 99 ml of distilled water.
[c] 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 dispersement 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 microcup 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 nonhydroscopic 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 microcup 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

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