A Comparative Procedure for Evaluating Antimicrobial Activity of Gaseous Agents

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The increasing use of gaseous sterilization (Nordgren, 1939; Salle and Korzenovsky, 1942; Ingram and Heinse, 1949; Phillips, 1949; Kolb and Schneiter, 1950; Logrippo et al., 1955; Newman, Colwell, and Jameson, 1955; Hoffman and Warshowsky, 1958) has called the attention to the need for a uniform, reproducible procedure to investigate the antimicrobial activity of gaseous agents. The classical methods for testing disinfectant activity are designed for aqueous agents and thus do not give adequate consideration to some of the factors involved in gas exposure techniques. The criterion used for measuring the effect of a gas or gases on organisms should be a reproducible function of the concentration and duration of exposure.

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Apparatus to study this has been designed and tested. The technique requires exposing a thin layer of organisms collected on Millipore filter paper to the gaseous agent, transferring the bacteria to the growth medium, and determining the growth lag by change in optical density.

MATERIALS AND METHODS

Apparatus for exposure of organisms to gases. Figure 1 is a diagrammatic drawing of an all glass chamber for single filter exposures. It is made from a Pyrex Petri dish (100 by 15 mm) with a 7 mm outside diameter tubing fused into the base. A 12/5 socket connects to the gas line and an equal orifice quadrapole outlet, blown inside the base, provides uniform dispersion of the gases. Imperfections in fit of the Petri dishes suffice to provide an exit for the gases.

Figure 2 is a diagram showing a cross section of the multiple (4 filters) exposure chamber. It is con-
structed of stainless steel (diameter, $6\frac{3}{4}$ by $1\frac{7}{8}$ in.) with a gas inlet tube (diameter, $5\frac{1}{4}$ in.) at the top of which the exposure plugs are seated. The plugs are made from 1-in. thick Bakelite plastic (outside diameter $2\frac{3}{4}$ in.). The first offset is $3\frac{3}{8}$ in. from the bottom and the diameter is $2\frac{1}{4}$ in. This serves as a seat or rest on top of the chamber. The second offset is $3\frac{1}{2}$ in. from the bottom and the diameter is $1\frac{3}{8}$ in. Sixteen small holes ($\frac{1}{6}$ in.) are drilled entirely through the plugs along this edge and serve as an outlet for the gases.

The inlet tube is $3\frac{3}{4}$-in. diameter Bakelite plastic about $3\frac{3}{4}$ in. in length, with a $3\frac{1}{4}$-in. hole drilled up to the four $\frac{1}{4}$-in. orifices through which the gas enters the chamber. The upper end is threaded about $\frac{1}{2}$ in. to fit into the plastic baffle and $1\frac{1}{2}$ in. at the lower end for screwing into the chamber and to permit sealing a glass tube with a $12/5$ socket joint into it for connecting to the gas train. A rubber ring insures a tight seal. The entire chamber can be disassembled for cleaning and can be sterilized.

**Apparatus for growth and absorbance measurement of organisms on Millipore filters (figure 3).** This is a two piece Pyrex glass vessel with a 45/50 standard taper joint. The lower half has both a diameter and a height of 2 in., and is designed to hold a Millipore filter, up to 10 ml of culture medium, and be shaken in a Dubnoff shaker without wetting the vent plug or splashing on the upper half. A vent and a matched cuvette (13 by 100 mm) are sealed into the cover or upper half. Optical density readings are made by inverting the entire vessel in the plane and side away from the vent.

**Apparatus for metering and mixing gases.** The gas train used for metering and mixing of the gases is shown in figure 4, using ethylene oxide and carbon dioxide as an example. The flow rates were 10 and 190 ml per min, respectively, through separate flow meters no. o8F-1/16-16- and no. o1N-150-A.\(^2\) Methyl bromide

\(^2\) Fischer and Porter Company, Hatboro, Pennsylvania.
air, and nitrogen dioxide-air mixtures were similarly prepared. The gases were led through two concentric inlet tubes (3 mm and 8 mm diameters) into a 500-ml mixing flask. Back pressure was avoided by making the inner tube 23 mm shorter. The gas mixture then entered the 5-L equilibration flask and from thence into the exposure chamber.

The gaseous formaldehyde was prepared by bubbling commercial compressed air, at a flow rate of 100 ml per min, through 35 ml of formalin (30.3 per cent) contained in a 150-ml round bottomed flask. The air-formaldehyde mixture was passed through a trap to prevent entrainment of liquid aerosols and finally equilibrated as usual.

The formaldehyde-gas mixture was analyzed by the method of Tanenbaum and Bricker (1951), whereas the other gas mixtures were analyzed by gas chromatography. All work on the gases was done at room temperature (25 C).

Cultures and media. Escherichia coli strain K-12 (ATCC 10789) was used primarily as the test organism. The composition for the mineral glucose medium is as follows: NH₄Cl, 2 g; Na₂HPO₄, 6 g; KH₂PO₄, 3 g; NaCl, 3 g; Mg (as MgCl₂), 0.010 g; S (as Na₂SO₄), 0.026 g; glucose, 5 g; and distilled water, 1 L. For slants and plates, agar is added.

Other testing organisms, Bacillus cereus, Bacillus megaterium, Bacillus licheniformis, and Staphylococcus aureus were grown, harvested, and resuspended in the same way except Trypticase soy medium (BBL)³ was used.

The 18-hr culture was removed from a mineral glucose agar slant with saline. The organisms were washed once with saline, centrifuged, and resuspended in saline to 0.06 optical density with a 660 mμ filter (Klett and Summerson photoelectric colorimeter).⁴ Seven milliliters of the suspension were delivered to a sterilized Millipore filter paper (HA 047 mm).⁵ The suspension was added slowly to avoid any loss of organism due to spattering or upward fluid surges. A Pyrex glass filter funnel and fritted glass base were used. Water suction with a negative pressure of 30 mm mercury, as measured by an attached manometer, was applied. The uniformity of this slight suction is essential to hold an even amount of moisture in the pad as well as to avoid excessive adhesion of the bacteria to the filter. The suction was stopped immediately after the filtration is completed so that the organisms could subsequently be quantitatively removed.

The organisms, after spreading out on the Millipore filter paper, were transferred together with the filter pad to the Petri dish top (single exposure) or the Bakelite plugs (multiple exposure). Two-tenths milliliter of saline suffices to hold the filter and pad on the plug of Petri dish top for about 1 hr. More saline may be used if longer exposures are desired.

The gas train, including the exposure chamber with four blank plugs, but without Millipore filters, was equilibrated for approximately 2 hr prior to starting an exposure. This permits more precise regulation of flow and gas analyses. At the starting time, organism laden filters on separate Petri dish tops or Bakelite plugs were interchanged with the blanks.

Following exposure, the Millipore filter without the pad was washed. Because of the varying water solubility of different gases as well as the different exposure times, it was felt that more uniform results would be obtained by introducing two 10-ml aliquots of saline washing following exposure. Such large volumes may not always be required. The washing was done in the funnel in the usual manner except for a rubber ring inserted between the filter paper and the filter funnel to prevent it from touching the organism. The inner

![Figure 6. Inhibition of growth of Escherichia coli after exposure to ethylene oxide-carbon dioxide mixture for varying time intervals. Aliquots from the same batch of cells are used for each curve. Viable count before exposure = 2.5 × 10⁶ per ml. Concentration (c) = 2 mM per L; time (t) of exposure is in minutes.](http://aem.asm.org/)

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² Baltimore Biological Laboratory, Inc., Baltimore, Maryland.
⁴ Millipore Filter Corporation, Watertown, Massachusetts.

<table>
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<th>Time (min)</th>
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* Optical density.
diameter of the rubber ring is slightly greater than the diameter of the layer of bacteria. Care must be exercised in the placement of the filter and the ring to avoid touching the organism with a consequent loss. Since the objective has been to place and retain the organisms on the filter, the flow of wash solutions has been maintained as nearly as possible equal to the flow of filtrate by suction.

The filter then was transferred to the lower half of the growth-absorbance vessel, and 7 ml of glucose-mineral medium were added. The vessel was closed and shaken for 1/2 min to wash the bacteria from the filter, inverted, and the initial optical density was read. The filter remained inside the vessel throughout the experiment. Preliminary experiments showed that this initial optical density was the same as before filtering, i.e., 0.06 at 660 μ. The vessel was incubated and shaken at 37 C in a Dubnoff® metabolic shaking incubator. Optical densities were read every 30 min at the same wave length.


**Figure 6.** Inhibition of growth of *Escherichia coli* after exposure to formaldehyde-air mixture for varying time intervals. Viable count before exposure $6 \times 10^6$ per ml. Concentration (c) of formaldehyde = 0.137 mM per L; time (t) of exposure is in minutes.

**Figure 7.** Inhibition of growth of *Escherichia coli* after exposure to methyl bromide. Viable count before exposure = $6 \times 10^6$ per ml. Concentration (c) of methyl bromide = 2 mM per L; time (t) of exposure is in minutes.

**Figure 8.** Comparison of inhibition of growth of several organisms after 1 hr. exposure to ethylene oxide-carbon dioxide mixture (c \times t = 120). Viable counts on aliquots of unexposed starting suspensions of the same optical density: *Escherichia coli* $212 \times 10^8$ per ml; *Bacillus licheniformis* $212 \times 10^8$ per ml; *Bacillus megaterium* 1.0 $\times 10^8$ per ml; *Bacillus cereus* 7.0 $\times 10^8$ per ml.

**Figure 9.** Comparison of inhibition of growth of several organisms after exposure to formaldehyde-air mixture (c \times t, 2.74). Viable counts on aliquots of unexposed starting suspensions of the same optical density: *Escherichia coli* = $2.2 \times 10^8$ per ml; *Bacillus megaterium* = $5 \times 10^8$ per ml; *Bacillus licheniformis* = $6.7 \times 10^7$ per ml; *Staphylococcus aureus* = $1.2 \times 10^8$ per ml.
RESULTS
Prior to using this technique for evaluating the antimicrobial activity of gaseous agents it was necessary to know the variations to be expected from handling the organisms and their exposure to air or CO₂. The consistency observed with any one batch of organisms is demonstrated by a typical set of observations shown in table 1. Similar results have been obtained after

![Figure 10. Comparison of the effect of ethylene oxide to Escherichia coli using (A) the percentage survival as the criterion by plate count method and (B) the length of growth lag as the criterion by Millipore filter technique.](image1)

![Figure 11. Comparison of inhibition of growth of Escherichia coli by different gases. Viable counts on aliquots of unexposed starting suspension 1.3 × 10⁸ per ml. Concentration of ethylene oxide = 2 mM per L; methyl bromide = 2 mM per L; formaldehyde = 0.157 mM per L; exposure time in minutes.](image2)

![Figure 12. Comparison of inhibition of growth of Escherichia coli using two different media after exposure to ethylene oxide-carbon dioxide mixture. S = synthetic (glucose-mineral) medium, -----; O = organic (tryptocase-soy) medium, ---.](image3)

![Figure 13. Effect of sodium thiosulfate on growth of Escherichia coli after exposure to ethylene oxide-carbon dioxide mixture. Na₂S₂O₃ is added to give a concentration of 0.05 per cent. ○——○ Unexposed (control), without addition of Na₂S₂O₃; Δ——Δ exposed (c × t = 60), without addition of Na₂S₂O₃; X——X exposed (c × t = 60), with addition of Na₂S₂O₃; •——• exposed (c × t = 120), with addition of Na₂S₂O₃.](image4)
exhibited no change in lag time or starting optical density as long as the organisms were not touched in the placement or removal of the filter and rubber ring.

Figure 14. Effect of growth of *Escherichia coli* exposed to an oxidant type pollutant mixture (Gast, Schwartz, and Estes, 1957). Exposure time 15 min, oxidant concentration equivalent to 3.7 μg O₃ per L; rate of air flow 200 ml per min. Synthetic medium used for growth measurement.

Figure 15. Effect of additives on growth of *Escherichia coli* exposed to an oxidant type pollutant mixture. Exposure time 30 min, oxidant concentration 5.6 μg O₃ per L. Curve A exposed to pollutant mixture, Casamino acid (Difco) added to 0.1 per cent concentration. Curve B exposed to pollutant mixture, yeast extract (Difco) added to 0.1 per cent concentration. Curve C exposed to air, without additive (control). Curve D exposed to pollutant mixture, without additive.

Figure 5 is an example of the results obtained by varying the concentration-time product (c × t) of ethylene oxide toward *E. coli*. Similar results are produced with different c × t for formaldehyde (figure 6) and methyl bromide (figure 7).

Typical responses of different bacteria to a single concentration-time product of gaseous agents are demonstrated in figures 8 and 9. Figure 8 shows the effects of ethylene oxide on four different species of organisms and figure 9 shows the effects of formaldehyde. The variation in lag time of the different organisms depends on the species of organisms present, the concentration, duration of exposure, and the nature of the gas.

The correlation between the percentage of surviving organisms and growth lag to the log c × t of the gaseous agent is shown in figure 10. Both of them are linear functions of log c × t. Rough comparison of the antimicrobial activities of methyl bromide, formaldehyde, and ethylene oxide are illustrated in figure 11. The growth lag resulting from each gas was found by projecting the intersection of tangents drawn from the two sectors of each growth curve to the x-axis. By comparison of the growth lags of ethylene oxide (9.3 hr, c × t = 60), methyl bromide (4.2 hr, c × t = 60), and formaldehyde (21.5 hr, c × t = 2.7) and calculation to the corresponding value of c × t = 60, the antimicrobial activities of the three gases were found to be 100, 2:1:106, respectively, toward *E. coli* under our experimental conditions.

Figure 12 illustrates the growth lags obtained from the same batch of exposed organisms subsequently grown in synthetic and in organic media. It is interesting to note that when sodium thiosulfate was added to the synthetic medium the growth lag was greatly reduced (figure 13).

Application of this technique to the evaluation of an unknown mixture is illustrated in figure 14 with the exposure of the organisms to a synthetic oxidant type air pollution mixture. The addition of either amino acids or yeast extract hastens the growth rate tremendously. Figure 15 shows a comparison of the effect of these additives on organisms exposed to the pollutant mixture.

**DISCUSSION**

The primary purpose of this study is to develop a procedure for comparison of the antimicrobial activities of the gaseous agents toward a particular species of bacteria or of the susceptibilities of different species of bacteria to a specific gas. Effects of humidity and temperature effects have not been investigated.

The method is to expose, as directly as possible, the bacteria to the gas. To achieve this objective, the amount of fluid required to maintain the bacteria in the surviving state throughout the experiment is reduced to the minimum. Uniformity of gas mixture in
their compositions, flows, and distributions are deliberately considered in designing the apparatus.

Results from figure 5 to 10 lead to the conclusion that the growth lag is a function of the product of concentration and exposure time and that the susceptibilities of each bacteria varies with different gases. Likewise, the effect of each gas varies with different bacteria. Thus it is difficult to set up an antimicrobial index of gases in a general manner.

The growth lag of this dynamic Millipore filter method is correlated with per cent of the bacterial population killed, a common criterion employed in gas sterilization. A 90 per cent kill corresponds to about a 12-hr lag under our conditions.

In the study of antimicrobial activity where plate count is used, it is a common practice to choose the most favorable medium, usually an organic complete medium, for plating. Data in figure 12 show that under such conditions, the impaired cells which subsequently recovered have been ignored and counted as living cells. In other words, the part played by the gas on the impaired cells is neglected. In the method presented, the cells suffering from a chemical block are also evaluated which gives a relatively complete measurement of the total toxicity of the gaseous agents.

Mechanism of the action of ethylene oxide toward biological products has drawn the attention of numerous investigators (Hawk and Mickelsen, 1955; Windmuller, Ackerman, and Engel, 1956, 1959; Windmuller, Ackerman, and Bakerman, 1959). The reverse action of amino acids and yeast extract (figure 15) against the effect of the unknown pollutant mixture furnishes a possible access to the investigation of the mode of action of gas sterilizations. Different precursors of proteins, vitamins, nucleic acids, or energy sources can be added to the exposed organisms. Any reverse action demonstrated by the additives as evidenced by a marked decrease in lag time, suggests the possibility of the metabolic block or other impairment along the line of the precursor or precursors added.

Acknowledgments
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Summary
A dynamic method for comparison of the antimicrobial activities of gaseous agents is proposed. Specially designed apparatuses for multiple exposure and growth of organisms have been described and the validity of the method has been discussed.

Three gases, ethylene oxide, methyl bromide, and formaldehyde, and five bacteria, Escherichia coli, Bacillus cereus, Bacillus megaterium, Bacilluslicheniformis, and Staphylococcus aureus, have been tested and compared; typical data were presented to demonstrate the results. Application of this dynamic method for the investigation of the mechanism of action of gas sterilization has been suggested.

REFERENCES
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