Effect of Moisture on Ethylene Oxide Sterilization

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Abstract

Gilbert, George L. (Fort Detrick, Frederick, Md.), Vernon M. Gambill, David R. Spinner, Robert K. Hoffman, and Charles R. Phillips. Effect of moisture on ethylene oxide sterilization. Appl. Microbiol. 12:496–503. 1964.—Bacterial cells dehydrated beyond a critical point no longer react uniformly to ethylene oxide sterilization. The percentage of cells resistant to the lethal effect of ethylene oxide after desiccation is often as small as 0.1 to 0.001%. However, 5% resistant cells were observed with one type of microorganism dried in broth. The presence of organic matter increases the percentage of cells that become resistant to ethylene oxide after dehydration. The phenomenon is produced by exposing cells to a vacuum or a chemically desiccated atmosphere. It is not a permanent change, because the resistant cells rapidly become susceptible if wetted with water. On the other hand, mere exposure to a high relative humidity (RH), i.e., 75 to 98%, after desiccation requires 6 and 4 days, respectively, to overcome this resistance. Moisture studies showed that there is less water in bacterial cells that have been desiccated and then equilibrated to successively high RH values up to 100% RH, than in cells that have not been desiccated, but allowed to dry naturally until equilibrated to the same RH values.

Dry sterilization with ethylene oxide gas has achieved importance within the past decade. Phillips and Kaye (1949) published a review on this subject, but at the time the technique had few applications. That ethylene oxide is bactericidal in the vapor state was then relatively unknown, even though several patents and a number of papers had been published on the subject (Gross and Dixon, 1937; Griffith and Hall, 1940; Yesair and Williams, 1942; Velu, Lepigne, and Belloq, 1942; Salle and Korzenovsky, 1942). Almost all applications had been in treating dry foodstuffs, particularly spices. The review of Phillips and Kaye (1949) was followed by a summary of 6 years of research on ethylene oxide (Phillips, 1949; Kaye, 1949), indicating that the effects of time, temperature, and concentration upon the sterilization process were uncomplicated; but this was not true of residual moisture effects (Kaye and Phillips, 1949). These data have been substantiated by the considerable practical experience accumulated in the last decade. Phillips (1949, 1952) indicated that the effect of extraneous organic material and variation between bacterial species was of less importance with ethylene oxide than with other chemical disinfectants. We confirmed this.

The “dry” sterilization process is dry only in the sense that liquid water is not involved. The ethylene oxide sterilization process is not carried out under completely anhydrous conditions, particularly in nonevacuated systems. In practice, some water vapor is present in the air surrounding the material being sterilized. Thus, the microorganisms have a moisture content that varies with the relative humidity (RH) of the atmosphere and the conditions prevailing at the time of sterilization.

Early investigators had divergent views on the effect of moisture in ethylene oxide sterilization (Phillips and Kaye, 1949). Experience over the past decade has confirmed this finding (Mathews and Hofstad, 1953; Newman, Colwell, and Jameson, 1955; Friedl, Ortenzio, and Stuart, 1956; Sampson and Ludwig, 1956; Opfell, Hoffmann, and Latham, 1959; Znamirowski, McDonald, and Roy, 1960; Tessler and Fellows, 1961). Our study was undertaken because most of the reported difficulties with ethylene oxide sterilization could be traced to a lack of understanding of the role of moisture, particularly when highly desiccated or lyophilized material, such as blood plasma, was involved. Not only the RH of the atmosphere during the ethylene oxide treatment was found important, but also the moisture to which the microorganisms had been subjected prior to exposure was even more important.

The following factors affecting the germicidal action of ethylene oxide were investigated: (i) effect of varying RH values during sterilization of microorganisms preconditioned to the same or different RH values; (ii) effect of dehydration of bacterial spores by chemical desiccation or vacuum on the rate at which they are killed at various RH values; (iii) time and RH of exposure required to overcome the resistance developed in desiccated microorganisms; (iv) effect of the nature of the supporting surface on the rate at which microorganisms are killed at various RH values; (v) effect of desiccation of various microbial species on the rate at which they are inactivated; (vi) effect of organic matter surrounding the microorganisms during desiccation on the rate at which they are killed at various RH values; (vi) effect of concentration on the rate at which
desiccated microorganisms are killed; (viii) means of breaking
the resistance to ethylene oxide in desiccated cells.

Materials and Methods

Preparation of test microorganisms and samples. The
activity of ethylene oxide was demonstrated by the rate at
which microorganisms were killed on surfaces when ex-
posed at various RH values.

The major portion of this investigation was performed
with Bacillus subtilis var. niger (B. globigii) spores; how-
ever, a few supporting experiments were performed with
Staphylococcus aureus, Mycobacterium smegmatis, Asper-
gillus fumigatus, and T-1 bacteriophage. The B. subtilis
spores were washed, resuspended in water, and heat-
shocked at 60 C for 30 min to kill vegetative cells. A 0.05-ml
portion of spore suspension was placed on each of a number
of clean sterile cotton cloth patches [diameter, ½ in.
(1.58 cm)] or Whatman no. 42 filter paper, and was
stored at a predetermined RH at 21 to 27 C. The concen-
tration of viable spores in the suspension was regulated so
that 500,000 to 5,000,000 organisms could be recovered
from a patch. Aside from the cloth and Whatman ashless
filter paper, glass and a filter paper containing some as-
bestos fibers (Chemical Corps Type 5 paper) were also in-
cluded to hold the microorganisms during treatment.

The S. aureus and M. smegmatis cultures were grown in
Tryptose Broth for 24 hr; 0.05 ml of this broth suspension
was placed on each of a number of patches that were in
turn treated as described above. The A. fumigatus cultures
were grown on Tryptone Agar; the spores were suspended
in water containing Tween 20 (a nonionic wetting agent),
and were treated in the same manner as were the other
microorganisms. The T-1 bacteriophage was obtained by
growing Escherichia coli in nutrient broth, inoculating
with phage, and shaking for 4 hr. The phage suspension
was purified by differential centrifugation, and patches
were inoculated.

Constant-humidity jars. Before exposure to ethylene ox-
ide, the contaminated patches were conditioned in stan-
dard desiccators to constant humidities maintained by the
following saturated salt solutions at 25 C: potassium di-
chromate, 98; potassium chloride, 85; sodium chloride, 75;
nickel chloride, 53; magnesium chloride, 33; potassium
acetate, 22; and lithium chloride, 11 % RH.

Anhydrous calcium sulfate was used in another desic-
cator to achieve a RH of less than 1 %.

Ethylene oxide exposure chamber. All exposures to ethy-
lene oxide were carried out in desiccators at 25 C. These
vacuum-type desiccators were modified at the top (Fig. 1)
so that air could be flushed through the jar. Contaminated
patches to be exposed to ethylene oxide were quickly tran-
ferred from the preconditioning constant-humidity jars to
these chambers, and air at the desired RH was flushed
through the jar. The RH of this air was controlled by mix-
ing two air streams in the proper proportions. One air
stream had less than 1 % RH after passing through calcium

FIG. 1. Ethylene oxide exposure chamber.
coli, mixing, and overlaying on agar in a petri dish. The plates were incubated for 5 hr at 37 C before counting.

With the technique of plating 5 ml of the total 10-ml sample or a one-half dilution, one or two residual microorganisms may be missed; but, if as many as three or more organisms remain viable, not all are likely to be missed. This was particularly so in our assays, because three patches were used in each exposure, and all the runs were repeated two to four times. Each value on the graphs is an arithmetic average of 6 to 12 individual observations. For the longer exposures to ethylene oxide, additional patches were used. These patches were placed directly into broth blanks and were incubated to check for sterility. This all-or-nothing technique is used only as supporting evidence, because it does not yield quantitative results.

RESULTS

Because 33 % RH approaches the most effective humidity level to kill microorganisms on cloth patches with ethylene oxide (Phillips and Kaye, 1949), this same RH was used in our studies for reference. The low ethylene oxide concentration (120 mg per liter at 25 C) was deliberately selected so that about 8 hr would be required for sterilization and valid intermediate points could be obtained.

Figures 2 and 3 show the effect of RH on the rate B. sub-

![Graph 1](image1)

**Fig. 2. Bacillus subtilis spores on cotton patches exposed to ethy-
lene oxide (180 mg per liter) at 85 C.**

![Graph 2](image2)

**Fig. 3. Bacillus subtilis spores on cotton patches exposed to ethy-
lene oxide (180 mg per liter) at 85 C.**

These spores are killed by ethylene oxide when the contaminated patches were dried, preconditioned, and exposed to ethylene oxide at the same RH. The effect of RH is very evident from these results. At RH values below 33 %, sterilization was not attained, even with prolonged exposure times. A check on the retention of the ethylene oxide in the chamber for the 24- to 72-hr exposure revealed no significant loss due to leakage. Therefore, nonsterility at these low RH values was not due to a lowered ethylene oxide concentration.

The sterilization rates are not just a simple function of the RH (Fig. 4). Preliminary drying of organisms by exposure to a low RH before subjecting to ethylene oxide produced a small percentage of resistant cells, even though the actual ethylene oxide exposure was carried out at the optimal 33 % RH. Even drying over a desiccant for only 1 hr produced highly resistant microorganisms, although the number was less than one in a million. The same effect was produced by exposure to vacuum (Fig. 5). The greater the desiccation effect, the greater was the percentage of organisms resistant to ethylene oxide. However, it should be noted that even after extreme conditioning for 24 hr in a vacuum at 54 C the number of resistant cells was less than 1 %.

To overcome the resistance to ethylene oxide sterilization produced by dehydration, samples were allowed to rehydrate at various RH values. The cells must be actually
wetted if the effect is to be overcome immediately (Fig. 6). Merely equilibrating to a high RH (75 to 98%) required 4 to 6 days to break the effect; conditioning at 33% RH produced little effect. Even after 12 months at this RH, resistant organisms were found.

In addition to the effect of desiccation, a study was made to determine whether the type of surface upon which the microorganisms were dried had an effect on the rate of cell inactivation by ethylene oxide. It is more difficult to kill on impervious surfaces (Fig. 7). Whatman filter paper was also included in these studies, but is not plotted because the results coincided with cotton cloth. The type-5 filter paper, developed for use in gas masks, contains impervious asbestos fibers. The deleterious effect of impermeable surfaces can be partially overcome by increasing the RH at which the organisms are dried and exposed to the oxide.

The development of an increase in resistance to ethylene oxide sterilization on extreme drying is not peculiar to B. subtilis spores. The death rates of A. fumigatus spores and cells of S. aureus, M. smegmatis, and T-1 bacteriophage upon exposure to ethylene oxide at 33% RH after equilibration to 1% RH are shown in Fig. 8.

The longer time required to kill vegetative cells dried from broth, as compared with B. subtilis spores dried from a distilled water suspension, prompted an investigation of the effect of organic matter on the death rate due to ethylene oxide under optimal conditions (drying and exposing at 33% RH on an adsorbent surface). The vegetative cell is not more resistant than spores if it is free from organic contamination when treated with ethylene oxide (Fig. 9). As expected, the higher the concentration of organic

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**Figures:**

- FIG. 4: Bacillus subtilis spores on cotton patches exposed to ethylene oxide (180 mg per liter) at 25°C.
- FIG. 5: Bacillus subtilis spores on cotton patches exposed to ethylene oxide (180 mg per liter) at 25°C.
- FIG. 6: Rehydration effect on Bacillus subtilis spores on cotton patches, exposed to ethylene oxide at 180 mg per liter at 25°C for 8 hr. All patches were first dried at less than 1% relative humidity (RH).
matter, the slower is the death rate produced by ethylene oxide. The differences in resistance due to increasing amounts of organic matter are not great. It was noted by Schley, Hoffman, and Phillips (1960) that ethylene oxide is less likely to be inactivated by a large excess of organic material than are the other decontaminants. Furthermore, Phillips (1952) showed that, although there are differences in the sensitivity of spores and vegetative cells to ethylene oxide, these differences are not as marked as with many other disinfectants.

The above data were obtained in experiments with 120 mg of ethylene oxide per liter of air. The effect of higher concentrations of the oxide on the death rate of previously dried B. subtilis spores is shown in Fig. 10. Increasing the concentration from 120 to 950 mg per liter increases the death rate slightly, but it is insufficient to overcome completely the resistance to ethylene oxide built up in a population previously subjected to extreme desiccation.

The differences in resistance to ethylene oxide of non-desiccated and once highly desiccated spores, even when exposed to the oxide at the same RH, led to the determination of the moisture content in the cells under the two types of conditions. Figure 11 shows the water content of B. subtilis spores as a function of the RH at 25°C. Two curves are shown; the top one is the desorption or dehydration curve, and the bottom is the adsorption or hydration curve. It is evident that the equilibrium moisture content of the cells at any particular RH is different, depending on whether the equilibrium was reached by hydrating thoroughly dry organisms or by dehydrating wet organisms. The same difference in moisture content between hydrated and dehydrated cells at the same RH was noted earlier for Serratia marcescens (Bateman et al., 1962).

It is only when dealing with cells that have been exposed to a desiccating action and then treated with ethylene oxide that the resistance is noted. Once the cells have been dried below some critical point, they do not regain all the moisture they once possessed upon rehydration until they are actually wetted or placed in essentially 100% RH. The average difference is only about 4% over the RH range of 20 to 80%. The finding that desiccated cells do not entirely regain their moisture seems critical for ethylene oxide sterilization. Certain sites in the cells susceptible to ethylene oxide may still not be rehydrated, even though the cells gained an average of 5 to 15% of their gross weight in moisture during rehydration.

**DISCUSSION**

The bactericidal effect of ethylene oxide was attributed to its chemical activity as an alkylating agent (Phillips,
1949, 1952). A large body of literature concerning the biological activity of alkylation agents has appeared in recent years (Wheeler, 1962). One particular interest has been the mutagenic activity of these compounds and their effect on the deoxyribonucleic acid (DNA) molecule. These investigations are furnishing a new insight into genetic mechanisms on a molecular scale, but the information is not applicable to the present problem for two reasons. First, these studies have dealt primarily with DNA and other materials in an aqueous system, whereas our investigation was concerned with a gaseous system: the effect of residual water in the vapor of adsorbed phase. Second, although mutagenic effects can only arise through action on the DNA molecule, it is not certain that the bactericidal action is due to alkylation on this site within the cell. It was proposed by Phillips (1949, 1952) that bactericidal action of ethylene oxide was by the alkylation of various essential proteins, such as enzymes, within the cell. Protein alkylation can as easily cause death of the cell as the induction of a lethal mutation. Indeed, the repeated failure to observe mutations among surviving cells after ethylene oxide treatment indicates that damage to the cell is direct. If death were the result of a lethal mutation, many non-lethal mutations should have been observed. In contrast, when bacterial spores were suspended in liquid sulfur mustard, an alkylationing agent whose mutagenic effect has been frequently noted, some survived for as long as 3 weeks, but all colonies growing from treated cells toward the end of the exposure period were abnormal (Phillips, unpublished data). We believe that we are dealing with an alkylation phenomenon, probably occurring at various rates on all positions within protein and other molecules capable of being alkylated, and occurring in the absence

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**FIG. 9.** Staphylococcus aureus in concentrations of Tryptose Broth indicated, on Whatman no. 42 filter paper, dried and exposed at 33% relative humidity (RH) to ethylene oxide (180 mg per liter) at 35°C.

**FIG. 10.** Bacillus subtilis on Whatman no. 42 filter paper exposed to ethylene oxide at 33% relative humidity (RH) and 35°C. Spores were previously dried at <1% RH.

**FIG. 11.** Moisture content of Bacillus subtilis spores as a function of relative humidity at 35°C.
of any liquid, although all traces of water have not necessarily been removed from the system. When a sufficient number of sites within the microorganism have been alkylated, death ensues. It is impossible at this time to say exactly which sites are involved.

The resistance to sterilization under investigation, which we interpret as a resistance to alkylation, occurs only when the moisture level has at some time been brought below a certain critical level—around 30% RH in most of these tests. There is some indication that this level may vary somewhat as test conditions vary. This phenomenon of resistance induced by desiccation was observed in all organisms investigated, but was most closely studied with *B. subtilis* spores.

Although the presence of extraneous organic matter, and drying upon an impervious surface (which may be related, in that soluble organic matter coats the cell rather than being taken up by adsorbent cellulose fibers), also produce some resistance to ethylene oxide sterilization, these effects are not as marked as is the resistance induced by desiccation, and do not appear to be related to it.

The resistance induced by desiccation is not permanent. The resistant spores react normally once fully rehydrated. This recovery is almost instantaneous if the spores are directly wetted with water, but is quite slow if the spores are exposed to a moist or even essentially saturated atmosphere. Because the same cells can be changed from resistant to normal states by manipulating their internal moisture, evidently mutation is not involved; and resistance does not appear in the progeny of cells that have resisted sterilization.

The most interesting observation is that all cells are not equally affected. Although a population of nondesiccated cells exhibits a uniform normal exponential death rate when exposed to ethylene oxide, the death-rate curves for desiccated cells exposed to the oxide are no longer linear when plotted on a semilog scale.

One of the most obvious explanations for these abnormal death rates is that a nonhomogeneous population is involved. This is strikingly suggested in these examples. All cells are certainly not resistant, because as many as 90% or more appeared to be killed at the normal rate. A small percentage of cells, however, exhibited a marked resistance. Increasing the time of exposure or the concentration of ethylene oxide did not appear to affect them materially.

It should be noted that each of the points in all the figures represented is an average of 6 to 12 observations. Past analysis of plate counting techniques used in our laboratory showed that the experimental error is constant within bounds of random sampling variation. The fact that the points, although each the result of separate experiments, fall into relatively smooth curves would not normally require statistical analysis. However, one such analysis was performed in an attempt to obtain information on the type of nonhomogeneity involved with the desiccated populations.

Two types of heterogeneity could be involved. Desiccation could produce a dichotomous population in which most of the cells are normal after partial rehydration; but a small percentage could be so affected that their death-rate constants are markedly lower or even approximately zero. In other words, this could be a hit-or-miss phenomenon in which the misses were considerably greater than the hits.

A second effect could also be conceived in which desiccation followed by partial rehydration produced a truly mixed population, with death-rate constants ranging from normal (the greatest number) through all stages of increased resistance until some cells were almost totally resistant.

The first hypothesis would indicate a crucial site which, if protected by direct cross-bonding in the absence of loose-bonding through adsorbed water molecules, is not available to alkylation. The second would indicate multiple alkylation sites, which, when some are protected, result in increasingly lower death-rate constants until enough are protected so that the death-rate constant approaches zero.

To settle the conflict between these two views, a statistical analysis of data similar to that in Fig. 4 was made by Theodore W. Horner (associated with Booz-Allen Applied Research, Inc., Bethesda, Md.).

Curves A and D in Fig. 4 were repeated, with 12 determinations made for each point and a greater number of intermediate time values included, so that smoother typical resistant and nonresistant curves were obtained. Several mathematical models were then set up to see whether these data would support the dichotomous population theory of two types only, i.e., resistant and nonresistant. This theory only was tested, because a good fit to the resistant curve could always be obtained if a large series of varying reaction rate constants was assumed.

With the resistant curve following very closely upon a straight line, a good fit was obtained with the normal death-rate equation:

\[
\frac{N}{N_0} = e^{-kt}
\]

Then, assuming that the resistant curve represented a dichotomous population, we tested only it to see whether it fit the model.

\[
Y = (1 - p)X + pe^{-k't}
\]

where \(X\) represents \(e^{-kt}\) in the first equation or the fraction \(N/N_0\) which would be expected to survive among the normal organisms having the normal death rate constant \(k\), and \(Y\) representing the fraction which did survive in the mixed population where a fraction \(p\) had a lower death rate constant \(k'\).

The normal death rate constant, \(k\), for the nonresistant 33% RH curve was 3.43121. A good fit was obtained, with the resistant curve giving values for \(p = 0.14245\) and \(k' = 0.16451\). Thus, the data would not contradict the
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The hypothesis that only two distinct populations were present rather than a continuously varying population.

Exactly what is happening is thus impossible to say. Not enough is known about the position of water molecules in dry but not completely dehydrated protein or other complex molecules, nor how the location is affected by dehydration-rehydration cycles. One is tempted to speculate that certain cross-linkages are formed through water molecule bridges either in these complex molecules or between adjacent molecules. When all or almost all of the water molecules are removed, direct cross-linkages could be established in their place, blocking what might otherwise be alkylation sites. These direct cross-linkages, once established, are then broken only upon complete dehydration. But the data do not establish how many sites are involved nor where they might be located.

The phenomenon is real enough so that one should avoid desiccating conditions in routine ethylene oxide sterilization. It does not arise too often in practice, probably because the total fraction of resistant organisms is low; but most of the reports on ethylene oxide sterilization skips can, in retrospect, be attributed to this cause. There is a parallel and also unexplained phenomenon with heat sterilization where the presence of moisture is also highly critical. Moist heat sterilization can be accomplished in an autoclave within 15 min at a temperature of about 124 °C, but sterilization with dry heat requires about 4 hr and approximately 160 °C.

Even though we do not understand why moisture plays such a role in heat sterilization, the phenomenon exists, and air-pockets in autoclaves where the steam would not reach are avoided. Similarly, knowing that a desiccation problem exists with ethylene oxide sterilization enables one to design equipment and establish procedures to avoid the phenomenon, even though it is not fully understood.

LITERATURE CITED


