ETHYLENE OXIDE STERILISATION—SOME EXPERIENCES AND SOME PRACTICAL LIMITATIONS

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Causes of failure to sterilise using ethylene oxide have been investigated. Organisms can occur naturally in conditions in which they are "protected" from the action of the gas, and in consequence its action on organisms which have been artificially protected has been studied. The work is discussed with particular reference to moisture content and relative humidity, and means of overcoming the difficulties of sterilising "protected" organisms are suggested. Bacteriological controls have been found unreliable without safeguards.

SINCE the work of Phillips and Kaye (1949) many papers have been published on ethylene oxide sterilisation. Most have attested its effectiveness, but Walter and Kundsin (1959), Barwell and Freeman (1960) and Znarmirowski, McDonald and Roy (1960) reported that sterilisation of test objects was not always obtained. Similar cases of non-sterilisation of various materials and bacteriological controls occurring over the last 10 years are summarised in this paper and we have given some account of the laboratory investigations connected with them.

Many inter-related factors govern an ethylene oxide sterilisation. These include the time concentration product of the gas, the temperature and the relative humidity, the moisture content of the materials, the sorptive capacity of the materials, and the accessibility of the organisms to the gas.

Almost every worker has his own combination of the inter-related variables, time, gas concentration and temperature. Some treatments advocated in the literature are many times more stringent than others. Royce and Bowler (1959) suggested sorption of the gas can account for some of the variations in treatment practiced.

Kaye and Phillips (1949) noted that organisms dried from salt solutions were more difficult to sterilise than those dried from water, and that organisms dried on hard impervious surfaces were more difficult than those dried on absorbent surfaces. This they attributed to dry crust formation composed of protein or salts which prevented access of the gas to the organisms. Royce and Sykes (1955) mentioned that organisms in dried broth films were not always sterilised and again Royce (1959) drew attention to the fact that micro-organisms could be trapped inside crystals of various substances and were thus protected from ethylene oxide sterilisation. Abbott, Cockton and Jones (1956) working with bacterial spores noted that their inclusion in the crystals of many substances protected them from sterilisation by formaldehyde and ethylene oxide.

EXPERIMENTAL

Ethylene oxide at 10 per cent v/v gas concentration in air at ambient temperature (approximately 20°) and humidity (45–80 per cent) and

atmospheric pressure for an exposure period of about 20 hr. was employed for most of this work. This is referred to subsequently as the "standard treatment".

Naturally occurring "protected" organisms. Initial experiments using soil-dust-spore preparations, showed efficient sterilisation by the standard treatment. Further preliminary experiments with a range of pharmaceutical powders demonstrated sterilisation in most instances, but a few batches of glucose, lactose and sulphanilamide were notable exceptions. Later some batches of soil-dust-spores also gave occasional surviving organisms. Tests showed that this was not because of loss of ethylene oxide owing to reaction with or absorption by the materials. Progressive increments in the severity of the treatment, involving raising the gas

TABLE I

THE EFFECT OF ETHYLENE OXIDE ON MICRO-ORGANISMS IN THIN DRIED FILMS OF NUTRIENT BROTH

Organisms	Viable count (orgs/piece)	
	Before treatment	After treatment
Glass rods		
Staph. aureus	2×10^4	6×10^3
B. subtilis	10^4	Nil
Cl. sporogenes	5×10^2	Nil
Glass coverslips		
Staph. aureus	2×10^4	10^3
B. subtilis	10^4	2×10^2
Cl. sporogenes	5×10^2	Nil
Filter paper strips		
Staph. aureus	+(2)	−(4)
B. subtilis	+(2)	−(4)
Cl. sporogenes	+(2)	−(4)

+ = Growth − = No growth ( ) = Number of pieces tested

concentration (up to 100 per cent and even under increased pressure), extending the exposure period (up to 3 days) and raising the temperature (up to 80°) were ineffective, except once or twice with lactose and soil-dust-spores.

Many of the surviving organisms were tested to determine their inherent resistance to ethylene oxide by drying their cultures on filter paper strips and then subjecting them to the standard treatment. The initial viable counts were in the range 10^7–10^8 cells per strip, and in every instance they were sterilised.

Artificially protected organisms on different surfaces. Experiments using artificially protected organisms revealed that those in dried nutrient broth films on glass were rarely sterilised, but the same organisms dried from serum suspension were much more susceptible. In further studies nutrient broth cultures of *Staphylococcus aureus*, *Bacillus subtilis* and *Clostridium sporogenes* were distributed (*a*) in thin films, by dipping short lengths of glass rod and carefully draining them; (*b*) in thick films, by spreading large drops on glass coverslips, and (*c*) in absorbed films on filter paper strips. The preparations were dried over phosphorus pentoxide for 24 hr. and then given a standard treatment, after which the the glass test pieces were washed in tubes of broth and viable counts

made on the broth suspensions; the strips were cultured intact. The results of these tests are given in Table I.

In similar tests cultures of organisms which had survived ethylene oxide treatment were washed off agar slopes with saline. Part of each saline suspension was filtered through a Ford Sterimat (F.C. grade), which was then dried, and the remainder was absorbed in kieselguhr, which was also dried. Six of the 9 pad preparations, but none of the kieselguhr preparations, were sterilised by the standard treatment.

A final combined experiment was made to compare broth and normal saline suspensions of the same micro-organisms deposited or dried on different surfaces. The two vegetative organisms *Staph. aureus* and *Escherichia coli* were used, 18 to 24 hr. cultures from agar slopes being

TABLE II

THE EFFECT OF ETHYLENE OXIDE ON DIFFERENT BACTERIOLOGICAL CONTROLS PREPARED FROM THE SAME CULTURES

Surface used	Number sterile/Number tested			
	Staph. aureus in		*E. coli* in	
	Broth	Saline	Broth	Saline
Glass coverslip	0/5	0/5	2/5	0/5
Asbestos Sterimat	5/6	4/6	6/6	4/6
Kieselguhr powder	4/6	5/6	5/6	5/6
Filter paper	6/6	6/6	6/6	6/6

suspended in nutrient broth or in saline and 0·2 ml. amounts separately dried on: (*a*) $\frac{3}{4}$ in. diameter glass coverslips; (*b*) filter paper strips (Whatman No. 1); (*c*) $\frac{3}{4}$ in. squares of Sterimat pads (Ford F.C. grade), and (*d*) 2 g. quantities of kieselguhr. Six replicates of each preparation were given a standard ethylene oxide treatment and subsequently cultured individually, with the results shown in Table II.

Artificially protected organisms in powders. Glucose monohydrate was infected with an aqueous suspension of *B. subtilis* spores and air dried. It was further dried to remove most of the water of crystallisation and yield a preparation consisting mainly of anhydrous glucose. Tests showed this treatment had little effect on the viable count of contaminating spores. Some of this anhydrous material was then partly rehydrated with 5 per cent water, and some was more than fully rehydrated by adding 12 per cent water. Samples of the original contaminated monohydrate, the anhydrous, the partially and fully rehydrated materials were subjected to a standard ethylene oxide treatment. Results are presented in Table III. A similar experiment using sodium citrate, which is also a hydrated crystal, gave similar results.

Procaine penicillin also yields occasional contaminants. As before, cultures of contaminants dried on filter paper strips were sterilised by the standard ethylene oxide treatment. Portions of the cultures were added to separate aqueous solutions of sodium penicillin and procaine hydrochloride, after which the contaminated solutions were mixed and the precipitated procaine penicillin was collected, washed and dried. After

7 days storage at room temperature the preparation contained 4×10^7 organisms per gram, and after standard ethylene oxide treatment yielded an average survivor count of 3×10^5 organisms per gram.

In other work saline suspensions of contaminants were ground with sodium chloride, glucose, soil and sand and air dried. This procedure produced powders having counts of 10^6–10^7 organisms per gram. After ethylene oxide treatment the soil and sand preparations yielded only 10–10^2 organisms per gram, but the glucose and sodium chloride yielded 10^4–10^5 organisms per gram.

Effect of moisture and humidity. Using the same preparations and varying the moisture content from "high dried" up to 5 to 6 per cent moisture revealed no effect with the sodium chloride and glucose, but

TABLE III

THE EFFECT OF ETHYLENE OXIDE ON MICRO-ORGANISMS IN CRYSTALLINE AND ANHYDROUS GLUCOSE

Substances	Original count (orgs./g.)	Count after treatment (orgs./g.)
Glucose monohydrate	2×10^7	$1{\cdot}5 \times 10^4$
Glucose anhydrous	3×10^6	$1{\cdot}5 \times 10^2$
Glucose anhydrous (partially rehydrated)	3×10^6	$1{\cdot}2 \times 10^4$
Glucose anhydrous (fully rehydrated)	3×10^6	$5{\cdot}5 \times 10^4$

gave a higher probability of kill with the sand and soil preparations at the higher moisture levels. Again more severe ethylene oxide treatments produced no significant improvement.

Experiments were made with gross moisture additions of 25 and 33 per cent, which converted the preparations into sludges, and then adding liquid ethylene oxide (1 per cent) after the method of Wilson and Bruno (1950) for sterilising culture media. The sand and soil preparations, with their small amounts of easily soluble crystalloids, were sterilised, but the glucose and sodium chloride sludges, which consisted largely of undissolved crystals containing organisms were not sterilised and yielded high counts after treatment: a similar experiment with procaine penicillin also yielded a high count.

In confirmation of these observations, naturally infected samples of soil-dust-spore mixture, fuller's earth and bentonite, none of which had been sterilised initially by ethylene oxide, were readily sterilised after being well washed and dried. Tests showed that very few organisms were lost by washing and viable counts both before and after washing were 10^6–10^7 organisms per gram.

Further light was thrown on the problem when attempts were made to apply the washing technique to various sulphonamides and penicillin salts of differing solubilities in water. Spore infected preparations were made by washing bacterial spores from a slope with distilled water and incorporating in the powder by trituration in a mortar and subsequent drying. Samples (except sodium penicillin) were separately washed in water, recollected and dried. Washed and unwashed materials were given standard ethylene oxide treatments. Results are in Table IV.

Contaminations which may occur on surgical instruments, glassware and apparatus are likely to be present as dried films. Some of the crystalloids present in such films may be hygroscopic, and at high humidities may pick up enough moisture to solubilise the film partially or completely. Experiments were therefore devised using moist and dry films at various humidities.

TABLE IV

THE EFFECT OF ETHYLENE OXIDE ON UNWASHED AND WASHED CONTAMINATED SULPHONAMIDES AND PENICILLIN SALTS

Substance and treatment	Initial count (orgs./g.)	Count after treatment (orgs./g.)
Sodium Penicillin (very soluble)		
Unwashed	$2{\cdot}5 \times 10^8$	$1{\cdot}5 \times 10^4$
Sulphanilamide (soluble 1/170)		
Unwashed	6×10^7	5×10^3
Washed	1×10^7	$1{\cdot}3 \times 10^6$
Procaine penicillin (soluble 1/200)		
Unwashed	8×10^6	1×10^2
Washed	$1{\cdot}8 \times 10^6$	$1{\cdot}5 \times 10^3$
Sulphathiazole (soluble 1/2,500)		
Unwashed	$1{\cdot}4 \times 10^8$	6×10^4
Washed	6×10^7	4×10^5
Benzathine Penicillin (soluble 1/6,000)		
Unwashed	2×10^8	Nil
Washed	3×10^7	Nil

TABLE V

THE EFFECT OF ETHYLENE OXIDE ON WET AND DRIED BACTERIAL FILMS ON GLASS

Preparation	Viable count (orgs./coverslip)	
	Before treatment	After treatment
B. subtilis broth culture		
Wet	$5{\cdot}4 \times 10^8$	Nil
Dried	Not done	2×10^3
B. subtilis spores in saline		
Wet	$4{\cdot}5 \times 10^7$	Nil
Dried	Not done	5×10^5
Staph. aureus broth culture		
Wet	1×10^8	Nil
Dried	Not done	2×10^5

0·1 ml. quantities of 18–24 hr. nutrient broth cultures of *Staph. aureus* and *B. subtilis* and 0·1 ml. quantities of a suspension of *B. subtilis* spores in normal saline were spread on glass coverslips. Counts were made of the various preparations. Some were dried over phosphorus pentoxide, and then treated with ethylene oxide, others were transferred immediately to the ethylene oxide chamber whilst still wet and were given an immediate treatment. All treatments were at 50–65 per cent relative humidity. The wet preparations dried out in the ethylene oxide chamber within 1 hr. Results are given in Table V.

Dried film preparations of the broth cultures of *Staph. aureus* and *B. subtilis* were variously treated before standard ethylene oxide treatment. In each experiment four *Staph. aureus* and four *B. subtilis* coverslips were treated as follows.

(*a*) Dry prepared coverslips were placed in the gas chamber, sufficient moisture was added to give slightly super-saturated conditions at 20° and

the chamber and contents were heated to 45° to vaporise the added moisture. The coverslip preparations remained hard and dry. The chamber was then cooled to ambient temperature, "dew" was precipitated in the chamber and the preparations became moist and sticky.

(*b*) Sufficient moisture was added to the chamber to produce 100 per cent humidity at 20° and the chamber and contents were allowed to stand at room temperature for 3 days to equilibrate and were not heated.

(*c*) As (*b*) but ethylene oxide was added immediately with no equilibration period. Eight *B. subtilis* spore preparations in normal saline dried on coverslips were also included in this experiment.

All the test coverslips were sterilised.

A final experiment was made using process (*a*) again but this time after "dew" had moistened the preparations, the chamber and contents were raised to 55° and given an ethylene oxide treatment at 1,500 mg./l. (approximately 75 per cent ethylene oxide v/v) for 2 hr. at 55°. (This treatment is more stringent than the standard treatment.) Relative humidity during the sterilisation period was 60–70 per cent. None of the coverslips was sterilised.

Discussion

The differences shown by the results are clearly not due to differences between organisms in their resistance to ethylene oxide; they do show that organisms can be so prepared that they are protected from the action of the gas.

It is noteworthy that serum with its high protein content fails to protect, whereas nutrient broth, with its higher content of soluble amino-acids and other crystalloids and low protein content, is a good protecting material. A compact spherical organism, *Staph. aureus*, is more easily protected than the rod shaped *E. coli* and is generally more difficult to sterilise than the larger bacterial spores. The same cultures are protected when dried on glass but not when dried on filter paper (Tables I and II).

Organisms in saline solutions can be dried on filter paper and sterilised by ethylene oxide, when dried on asbestos filter pads or kieselguhr, they are sometimes sterilised, or when dried mixed with soluble powders such as glucose or sodium chloride, they are not sterilised. Thus, whilst organisms dried on hard surfaces are more difficult to sterilise than those on absorbent surfaces, not all organisms dried on absorbent surfaces can be sterilised (Table II).

The experiments with the hydrated glucose and sodium chloride crystals indicate that the organisms are included in the crystals, and, with hydrated crystals, can be released by drying off the water of the crystallisation under mild conditions. The inclusion of organisms in other materials has been demonstrated more elegantly by Abbott, Cockton and Jones (1956) using the electron microscope. The practical significance of crystal protection is well illustrated by the occurrence of such protected organisms in materials. We have shown that vegetative organisms as well as bacterial spores can be protected in crystal preparations and can survive for some weeks under such conditions.

Bacteriological controls do not provide satisfactory proof of effective sterilisation, since, as we have indicated (Table II), they can be prepared to show either sterilisation or non-sterilisation at will.

In pharmaceutical practice, for powder sterilisation the importance of ensuring that any crystallisation or precipitation stage is carried out aseptically is clearly demonstrated with procaine penicillin. Further, washing slightly soluble substances can convert a surface contamination, which could be eliminated by ethylene oxide, into an internal one that cannot.

Thus in routine operations with ethylene oxide it is possible to sterilise successively many batches of a given material and then to encounter a a batch which contains "protected" organisms which cannot be sterilised. Increasing the stringency of the ethylene oxide treatment is usually ineffective. This implies that constant surveillance of procedures with tests on the materials treated is an essential part of ethylene oxide sterilisation.

It is however, possible to devise a means of circumventing at least some of the difficulties. Thus for insoluble powders, merely washing with water can liberate the "protected" organisms and render the material, after redrying, sterilisable by ethylene oxide.

The experiments at 100 per cent relative humidity show that organisms in dried films can be effectively sterilised. Any substantial amounts of free water should be avoided because of the high sorptive capacity of water for ethylene oxide. More work is required on this aspect.

For absorbent materials such as paper and fabrics, it appears that where a fluid containing organisms is completely absorbed by the fibres the organisms which presumably remain on the fibre surface do not become protected. If more fluid is present than can be absorbed and this dries off in the free spaces between fibres, then "protected" organisms can occur and the material cannot be sterilised by ethylene oxide. Such organisms would probably be liberated at high humidities and there is an obvious application which might be further pursued, to dressing sterilisation.

Phillips (1961) in work on moisture in ethylene oxide sterilisations has shown that for a "naked" organism the moisture content of the organism corresponding to equilibration at 33 per cent R.H. approximately is optimum for sterilisation. High moisture impedes the sterilisation process, but lower moisture very markedly interferes and induces resistance to ethylene oxide sterilisation. He has also shown that high dried organisms require extended periods of equilibration at high humidities to make them susceptible again to ethylene oxide or, alternatively, require to be physically wetted, when they are speedily rendered sterilisable by ethylene oxide.

We agree with Phillips conclusions, but in practice protected organisms can occur. If organisms are enclosed inside crystals of insoluble materials, no equilibration at any relative humidity will affect them and they remain unsterilisable. With water soluble crystals, freeing of the organisms can take place if the partial pressure of water vapour exceeds the vapour pressure of a saturated solution of the substance enclosing the organism. In such cases, or if they are physically wetted as, for example, by "dew"

deposition, the crystal dissolves, the organism is liberated and although the small amounts of extra moisture slightly impede the action of the gas they are readily sterilised.

Mayr (1961) and Perkins and Lloyd (1961) in advocating equilibrations at 95 per cent relative humidity, and at 55–60 per cent relative humidity, with various holding periods seem to be concerned (as is Phillips, 1961) with the state of hydration of the organisms themselves, although Perkins and Lloyd also mention the adsorption of moisture by solid surfaces. Equally, however, their findings can be explained by the solution of protecting substances as we have shown. Sodium chloride will pick up moisture at relative humidities above 75 per cent and we have observed that broth films become moist and sticky at lower humidities, about 50–60 per cent. Perkins and Lloyd also report that at 50–60 per cent relative humidity they were unable to sterilise *Cl. sporogenes* on a porous medium at levels greater than 10^5 organisms per 0·15 ml. but could sterilise the same organism at 10^6–10^7 organisms per 0·15 ml. when grown in a synthetic medium and dried on a solid surface. We have shown that porous media can be made to function as hard surfaces (and yield protected organisms) by exceeding their absorptive capacity with culture fluids containing organisms. It is thus apparent that the varying amounts of crystalloids, derived from different culture media, enclosing the organisms could explain these findings.

In stressing certain difficulties (many of which can be overcome) in the use of ethylene oxide, we do not wish to imply that ethylene oxide is other than a most valuable sterilising agent. Frequently it may be the only practicable method available, and in most cases in our own experience satisfactory sterilisation has been the rule and the "protected" organism the exception.

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