THE STERILIZATION OF BACTERIOLOGICAL MEDIA AND OTHER FLUIDS WITH ETHYLENE OXIDE

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Sterilization by heat cannot be used when it is necessary to preserve heat-labile substances. Sterilization by bacterial filtration fails when materials are used which plug the filter, when critical constituents are adsorbed onto the filter, or when elements which should be removed pass through the filter (filterable viruses). Most chemical sterilizing agents, such as the phenols, the heavy metal salts, and the detergents, are persistent in their effect and materials treated with them are permanently rendered incapable of supporting bacterial growth.

The purpose of this paper is to describe the use of ethylene oxide as an alternative to sterilization by heat and filtration. Media to which the proper proportions of ethylene oxide have been added are temporarily bactericidal, but the action of the chemical is of short duration, the media regaining in a few hours their ability to grow bacteria. Ethylene oxide gas has been widely used in the food industry and as an insecticide and fumigant. Schrader and Bossert (1) were the first to use ethylene oxide to destroy microorganisms. Roberts, Allison, Prickett, and Riddle (2) demonstrated its ability to sterilize soil. Hansen and Snyder (3) used the gas to sterilize straw before incorporation into media. Phillips and Kaye (4–7) have recently reviewed the journal and patent literature on ethylene oxide and have discussed its effectiveness in gaseous form as a sterilizing agent. So far as we have been able to discover, no previous description of the use of liquid ethylene oxide in the manner and for the purpose advocated here has been published.

Methods and Materials

Sterilization with Ethylene Oxide.—The ethylene oxide used in these experiments was obtained from the Eastman Kodak Co., Rochester, New York. It was furnished in sealed ampoules containing 100 ml. of the liquid substance. The ampoule was chilled before opening. A stock supply was kept in the cold in a glass-stoppered bottle, the stopper being lubricated with vaseline. Ethylene oxide was always measured and dispensed in the liquid form and the quantities mentioned in this paper refer to the amount of liquid ethylene oxide in milliliters added to 100 ml. of medium.

A measured quantity of the fluid to be sterilized was placed in an Erlenmeyer flask of suit-

able size, which was plugged with cotton and placed in the refrigerator or cold room at approximately 0°C. for 1 hour. One part of liquid ethylene oxide was added by means of a chilled pipette or chilled tuberculin syringe and needle to 100 parts of the fluid to be sterilized and the flask was agitated briskly to assure thorough admixture of the two components. After standing in the cold room for an additional hour, the flask was transferred to a 37°C. air incubator and allowed to stand for 24 hours. At the end of this time the fluid was sterile and was ready for use as a culture medium.

Ethylene oxide is a liquid which vaporizes at 10.7°C. It is explosive and care must be taken in its management to prevent accumulation of the vapor in the cold room, refrigerator, or laboratory air. It is toxic to many forms of life including man, and care must be taken to prevent inhalation of the vapor or aspiration of the liquid. For this reason, the use of syringe and needle is preferable to the pipette for measurement whenever possible.

The amount of ethylene oxide added to various fluids has been varied from time to time. For routine purposes 0.5 per cent and 1.0 per cent by volume have most often been used. No fluids have been encountered which have not been sterilized by 1 per cent ethylene oxide. As little as 5 ml. of serum has been treated by adding 0.05 ml. of ethylene oxide by means of a 0.1 ml. pipette graduated in hundredths.

Strains.—In many of the experiments, a strain (327W) of hemolytic streptococcus, group A, type 1, was used. It was originally isolated from the throat of a patient during a food-borne epidemic. Strains of beta hemolytic streptococcus, group B (090R), group C (K106), and group D (D76) and a green streptococcus (C699) were obtained from Dr. R. C. Lancefield.

The following strains were obtained from the American Type Culture Collections: *Eberthella typhosa* (167), *Bacillus subtilis* (9789), *Clostridium tetani* (9155), *Salmonella typhimurium* (9148), *Sarcina lutea* (272), *Serratia marcescens* (60), and *Lactobacillus casei* (7469).

Other organisms were a strain of *Escherichia coli*, recovered from the urinary tract of a patient with pyelonephritis, a coagulase-positive strain of *Staphylococcus aureus* isolated from a patient's throat, a commercial compressed cake preparation (Fleischmann) of bakers' yeast, and commercial calf lymph vaccinia virus (Parke-Davis).

RESULTS

Effectiveness of Sterilization.—50 ml. portions of heat-sterilized tryptose phosphate broth, milk, or other suitable media were inoculated with the following organisms: *Staphylococcus aureus*, beta hemolytic streptococci of groups A, B, C, and D, green streptococcus, *Sarcina lutea*, *Bacillus subtilis* spores, *Serratia marcescens*, *Lactobacillus casei*, *Escherichia coli*, *Salmonella typhimurium*, *Eberthella typhosa*, *Shigella paradysenteriae*, bakers' yeast, and two unidentified fungi which were isolated as contaminants of laboratory media. The flasks were incubated at 37°C. for 24 hours or more to achieve heavy growth. They were then treated with 0.5 per cent ethylene oxide by the standard technique described above, after which material from the flasks was inoculated into tryptose phosphate broth and onto sheep blood agar plates. No bacterial growth occurred in either medium during a 7 day period of observation. The cultures had been sterilized.

To give the method a more severe trial, flasks of tryptose phosphate broth were heavily contaminated with the following materials: throat washings, feces, laboratory floor sweepings, and soil. 1 per cent ethylene oxide produced sterili-

zation of the heavily contaminated media, whereas 0.5 per cent ethylene oxide was in some cases inadequate.

Three flasks containing 50 ml. of Brewer's fluid thioglycollate medium (thioglycollate content: 0.03 per cent) were inoculated with 0.1 ml. of an old culture of *Clostridium tetani* containing many spores. One of the flasks was treated with 0.5 volume per cent ethylene oxide, another was treated with 1.0 volume per cent ethylene oxide, and the third flask was not treated. All 3 flasks were incubated for 1 week at 37°C.

Excellent growth of *Clostridium tetani* occurred in the untreated flask, whereas no growth occurred in the ethylene oxide–treated flasks. It is apparent that ethylene oxide was able to destroy the anaerobic spores.

The ability of ethylene oxide to destroy vaccinia virus was tested in the following way:—

Calf lymph vaccinia virus was inoculated onto the dropped chorioallantoic membrane of a 10 day old chick embryo. After 48 hours' incubation, a section of the chorioallantoic membrane was removed and ground in 10 ml. of 10 per cent inactivated sterile rabbit serum–water in a sterile mortar with sand. The virus suspension was divided into 2 equal parts, one of which was then treated with 1 volume per cent ethylene oxide in the usual way. The untreated suspension was placed in the cold and in the incubator overnight along with the treated suspension, so that the two were exposed to the same conditions except for the treatment of one of them with ethylene oxide. The destruction of virus was tested by titration of the treated and untreated suspensions on chorioallantoic membranes and by dermal inoculation in a rabbit.

No visible lesions were produced on the membranes of four 12 day old chick embryos which received 0.1 ml. of the ethylene oxide-treated virus suspension. The titre of the untreated suspension was approximately 32,000 particles per ml. 10^{-5} ml. or more of the untreated suspension produced a lesion on injection into the skin of a rabbit. No lesions were produced by the injection of 0.1 ml. of undiluted ethylene oxide–treated suspension, which was the largest quantity injected. It is concluded that ethylene oxide was able to sterilize a fresh suspension of vaccinia virus in 10 per cent rabbit serum.

Time Factors.—Experiments were performed to discover the time required after the addition of ethylene oxide for sterilization of contaminated materials to be achieved and the time required for the medium to recover its ability to promote growth.

Four flasks each of boiled milk and tryptose phosphate broth were inoculated with a culture of group A streptococcus, so that each milliliter of the milk or medium received approximately 75 organisms. To the flasks in each set was added 0.5 per cent, 0.75 per cent, 1.0 per cent, and 2.0 per cent of liquid ethylene oxide by volume. The flasks stood at 0°C. for 1 hour and were then incubated at 37°C. The bacterial content of each flask was estimated at hourly intervals during incubation by pouring sheep blood agar plates inoculated with 1.0 ml. of the treated fluids.

Table I shows that the streptococci were destroyed by 2 per cent ethylene oxide within 2 hours and by 0.5 per cent ethylene oxide within 6 hours.

A similar experiment was performed in which unsterilized fresh market milk was treated with the same amounts of ethylene oxide. It was shown that within 4 hours the natural bacterial flora of the milk was destroyed by as little as 0.5 per cent ethylene oxide (Table I).

The period required for ethylene oxide–treated broth to recover its ability

TABLE I

Time Required for Ethylene Oxide to Sterilize

	Time of incubation of ethylene oxide-treated media	Concentration of ethylene oxide			
		0.5 per cent	0.75 per cent	1.0 per cent	2.0 per cent
		Colonies per ml.			
	hrs.				
Sterile boiled milk inoculated with group A streptococcus 327W. 75 organisms per ml.	1	62	70	82	74
	2	95	66	48	0
	3	29	5	1	0
	4	3	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	7	0	0	0	0
Tryptose phosphate broth inoculated with group A streptococcus 327W. 75 organisms per ml.	1	73	68	82	59
	2	66	77	56	0
	3	60	15	1	0
	4	5	0	0	0
	5	2	0	0	0
	6	0	0	0	0
	7	0	0	0	0
Fresh pasteurized market milk. Not sterilized	4	0	0	0	0
	7	0	0	0	0
	16	0	0	0	0
	24	0	0	0	0

to support growth was determined by sterilizing several flasks of neopeptone-meat infusion broth with 1 per cent ethylene oxide, and inoculating them with strain 327W at various time intervals after addition of the sterilizing agent. No growth occurred in the flasks which were inoculated after incubation for 12 hours or less. When inoculated after 16 hours, growth occurred but was delayed. After 24 hours' incubation, the broth had recovered its maximum ability to support growth.

Comparison of Growth Yields of Media Sterilized by Various Methods.—A comparison was made of the final bacterial yield from broth sterilized by ethylene oxide, by heat, and by bacterial filtration.

A meat infusion–neopeptone broth was divided into 3 parts. One was sterilized by 0.5 per cent ethylene oxide, another was sterilized by passage through Selas 02 filters, and the third was sterilized by autoclaving at 121°C. for 20 minutes. Each was adjusted to pH 7.4 after sterilization and was given an inoculum of 0.1 ml. of a blood broth culture of strain 327W. Growth was allowed to proceed for 96 hours, at which time measured portions were centrifuged in Bauer-Schenck sediment tubes to determine bacterial yield.

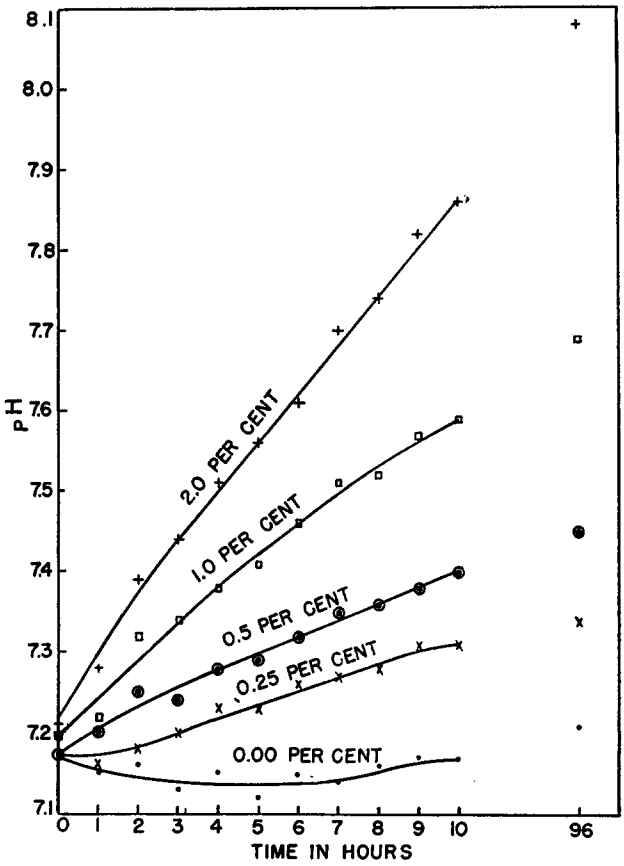

FIG. 1. Effect of various concentrations of ethylene oxide on pH of tryptose phosphate broth during incubation at 37°C.

The ethylene oxide–treated broth yielded 2.1 ml. of packed cells per liter of broth; the filtered broth yielded 2.3 ml. per liter, and the heat-sterilized broth yielded 2.4 ml. per liter. The differences in yield are probably not significant, since the packed-cell method is subject to large error. In another experiment, the bacterial yield of heat-sterilized broth was 1.7 ml. per liter whereas the yield from broth sterilized with 0.5 per cent ethylene oxide was 2.1 ml. per liter at 91 hours.

Effect on pH.—When ethylene oxide was added to chilled milk, broth, or serum, a slow rise in pH occurred during subsequent incubation. The rise depended on the nature of the fluid treated and on the amount of ethylene oxide

TABLE II

Effect of Ethylene Oxide on pH of Various Fluids

Fluid	Concentration of ethylene oxide				
	0.00 per cent	0.25 per cent	0.50 per cent	1.0 per cent	2.0 per cent
M/15 phosphate buffer	7.5	7.5	7.5	7.6	7.6
Tryptose phosphate broth	7.2	7.4	7.5	7.8	8.2
Milk	6.6	6.9	7.0	7.4	8.3
Normal rabbit serum	7.8	8.2	8.6	8.8	9.1

50 ml. quantities of the fluids were treated with the indicated quantities of ethylene oxide. Incubated at 37°C. for 24 hours before making pH determinations. pH readings were made with the Cambridge pH meter.

TABLE III

Toxicity of Ethylene Oxide–Treated Broth for Mice

Duration of incubation of mixture	Fate of mice				
	1	2	3	4	5
hrs.					
0	D2	D2	D2	D2	D3
1	D2	D2	D2	D2	D24
2	D2	D2	D2	D3	D3
3	D2	D2	D3	D5	D6
4	D3	D3	D3	D3	S
5	D5	D24	S	S	S
6	D24	S	S	S	S
7	S	S	S	S	S
8	S	S	S	S	S
9	S	S	S	S	S
10	S	S	S	S	S

1.0 ml. ethylene oxide was added in the cold to 100 ml. of chilled tryptose phosphate broth and allowed to stand in the cold 1 hour. Flask then removed to 37°C. incubator and 0.5 ml. inoculated intraperitoneally into 5 mice as indicated. D = died; figures = No. hours after injection until death; S = surviving 1 week after injection.

added. Fig. 1 shows the pH rise during a 10 hour period after adding varying proportions of ethylene oxide to tryptose phosphate broth. Maximum rise occurred by 24 hours, and no significant changes in pH took place thereafter. The effect of similar quantities of ethylene oxide on the pH of various fluids is shown in Table II. All pH measurements were made with the Cambridge pH meter.

Since the pH rise is of appreciable magnitude, it must be taken into account when fluids intended for bacterial growth are sterilized with ethylene oxide. The initial pH of milk is about 6.7, and after sterilization with 0.5 per cent ethylene oxide it rises to 7.0–7.4, which is satisfactory for a bacteriological medium. Fluids which have a high initial pH may exceed the optimal value considerably and it may be necessary to readjust them with sterile acid to the proper pH.

Toxicity of Ethylene Oxide–Sterilized Medium.—Preliminary experiments indicated that the ethylene oxide–media mixtures were highly toxic for mice immediately after admixture, but on the passage of several hours toxicity waned.

100 ml. of tryptose phosphate broth was treated with 1.0 ml. of liquid ethylene oxide by the technique described. At hourly intervals, 5 mice were injected with 0.5 ml. of the treated medium intraperitoneally. Mice were inoculated every hour for 10 hours in this manner.

The results are shown in Table III. It is seen that early in the course of contact with ethylene oxide the mixture was highly toxic for mice, usually causing death in a few hours, that after the ethylene oxide and medium had stood in the incubator for more than 6 hours the toxicity for mice had disappeared.

It has been suggested that ethylene oxide may be cumulative in its effect.

A group of 5 mice, therefore, was treated repeatedly by intraperitoneal inoculation of 1.0 ml. of ethylene oxide–sterilized broth which was stored in the refrigerator. These mice were inoculated at 3 day intervals for a total of 3 inoculations. A control group of 5 mice received the same medium sterilized by heat instead of ethylene oxide.

All the mice in both groups survived, suggesting that under these circumstances the medium sterilized by ethylene oxide did not possess cumulative toxic activity.

DISCUSSION

Our interest in ethylene oxide arose from the necessity of obtaining sterile milk which had not been subjected to heat, since boiling destroys the heat-labile antistreptococcal substance in milk. Filtration through Berkefeld, Coors, or Seitz filters was unsatisfactory, because very little material passed through the filters before they became plugged. Ethylene oxide, however, worked very well for the purpose. Streptococci of all serological groups grew well in milk which had been sterilized by that method, and we have been using the procedure for approximately a year. The antistreptococcal property of the milk was not destroyed.

In previous work on sterilization with ethylene oxide, the agent has been used in gaseous form and has been applied chiefly to the sterilization of solid materials. That bacteriological media might be sterilized with ethylene oxide appears not to have been appreciated. Although Hutner and Bjerknes (8) listed

it as an agent to be considered for the purpose, they do not appear to have investigated its possibilities further. It is highly likely that ethylene oxide introduced as a gas into a pressure chamber containing bacteriological media, or bubbled through media, would accomplish the same purpose as the liquid oxide. We have not explored the possibility. The manipulation of ethylene oxide in liquid form has certain mechanical advantages, and is not too cumbersome for small scale operations. Workers who wish to use the process on a large scale would probably find it economical to use tanks of the compressed gas. Practical methods of sterilizing in such a manner would have to be worked out.

Because of the possible broad applications of this sterilization procedure, we investigated its effectiveness in treating several types of fluid contaminated with a variety of microorganisms. It was shown that liquid ethylene oxide, added in adequate quantity to fluid media, destroyed a number of diverse microorganisms including Gram-positive and Gram-negative bacteria, aerobic and anaerobic spores, fungi, and at least one virus. It sterilized media heavily contaminated with throat washings, dust, soil, and feces. No organism was encountered which resisted its action.[1]

Since serum and plasma were readily sterilized with ethylene oxide, and since vaccinia virus was destroyed, the possibility of sterilizing human plasma containing hepatitis virus should be investigated. It is possible that the side effects of ethylene oxide on the constituents of plasma would limit its usefulness for that purpose. Preliminary experiments have indicated that fibrinogen is not affected by the procedure, but that prothrombin and complement are destroyed. It cannot be used to sterilize and preserve whole blood, since red blood cells are hemolyzed.

The mode of action of ethylene oxide is unknown. Considering its wide range of action, which includes all microscopic forms of life studied as well as laboratory animals and insects, it is apparent that it attacks some very fundamental life process. The suggestion has been made that ethylene oxide is converted to ethylene glycol on contact with water, and that it is the ethylene glycol which is bactericidal. That this is not the case is indicated by the effective sterilizing action of 0.5 per cent ethylene oxide on hemolytic streptococci in broth, whereas Robertson, Appel, Puck, Lemon, and Ritter (9) have shown that 15 per cent ethylene glycol is required to kill streptococci under similar circumstances.

Ethylene oxide is highly reactive chemically. It combines slowly with water to form ethylene glycol. This reaction is accelerated in the presence of acid. Ethylene oxide combines directly with many acids in aqueous solution. Fur-

[1] Since this paper was submitted for publication, we have demonstrated that liquid ethylene oxide also kills *Mycobacterium tuberculosis*, when added to a culture of that organism in Dubos' medium.

thermore, it has the remarkable property of combining simultaneously with H and Cl ions in neutral solution (10). The direct combination with acids in neutral and acid solution accounts for part of the pH rise observed when ethylene oxide is used to sterilize biological fluids. Fraenkel-Conrat (11) has studied its reaction with proteins and amino acids. Ethylene oxide combines with carboxyl, amino, sulfhydryl, and phenolic groups. When it combines with dissociated carboxyl groups the acidity of the latter is suppressed, whereas the basic character of the amino group is not suppressed by combination.

Considering its extensive reactivity, it is rather surprising that ethylene oxide does not irreversibly destroy the activity of some of the essential growth factors in bacteriological media, but we encountered no evidence suggesting that such occurred in the strains we studied.

CONCLUSIONS

Ethylene oxide is an effective sterilizing agent for bacteriological broths, milk, and serum. A short time after it has exerted its sterilizing effect, the treated fluids again become capable of supporting bacterial growth.

Ethylene oxide has been shown to destroy a number of aerobic Gram-positive and Gram-negative organisms, aerobic and anaerobic spore-forming bacilli, fungi, and vaccinia virus. No organisms have been encountered which withstand its action.

The quantity of ethylene oxide necessary for sterilization depends to some degree on the character of the medium sterilized. None of the materials tested required more than 1 volume per cent of liquid ethylene oxide.

Ethylene oxide is highly reactive chemically, and its reactions with many of the components of complex biological fluids cannot be fully anticipated. Regardless of the chemical changes produced, ethylene oxide did not permanently alter the essential qualities of the growth factors in broth and milk which are required by fastidious organisms such as group A streptococci and others. However, its effect on the biological activity of any particular organic compound should be assayed before it can be assumed not to have a deleterious effect.

The sterilization of media by ethylene oxide is accompanied by a rise in pH. Fluids which have a relatively high initial pH may require adjustment with sterile acid to return them to the optimal pH for bacterial growth.

A mixture of ethylene oxide and broth was toxic to mice for 6 hours after admixture. Toxicity disappeared on incubating the mixture longer, and no residual toxicity could be demonstrated after 6 hours, even on repeated intraperitoneal inoculations.

As a means of sterilizing bacteriological media and other biological fluids, ethylene oxide deserves consideration when heat and filtration cannot be used.

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