Inactivation of Several Viruses by Liquid Ethylene Oxide. (17762)

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Sterilization of bacteriological media, as well as materials such as serum and milk, by liquid ethylene oxide has been recently demonstrated by Wilson and Bruno(1) to be a simple and efficient laboratory procedure. Not only is ethylene oxide able to kill a number of aerobic gram positive and gram negative organisms as well as aerobic and anaerobic spore-forming bacilli, but also the media can be readily rid of the sterilizing agent, and then once more be capable of supporting bacterial multiplication(1). Phillips and Kaye recently reviewed the literature on the sterilizing action of gaseous ethylene oxide and pointed out the wide spectrum of microorganisms for which it is lethal(2).

Following the demonstration(1) that 1% liquid ethylene oxide can inactivate vaccinia virus, it was of interest to determine whether this viral sterilizing property could be extended to a variety of other viruses. It is the purpose of this paper to report that viruses of the influenza-Newcastle disease group as well as neurotropic viruses of the mouse encephalomyelitis type are readily inactivated by this liquid chemical sterilizing agent.

Materials and methods. Viruses. The following viruses were employed: influenza A, PR8 strain; influenza B, Lee strain; Newcastle disease virus, Beaudette strain; Columbia MM mouse encephalomyelitis virus; Theiler's mouse encephalomyelitis virus, FA strain. These viruses shall be referred to as PR8, Lee, NDV, Col. MM, and FA, respectively. PR8, Lee and NDV were cultivated in the allantoic sacs of 9-11-day-old chick embryos. The PR8 and Lee viruses were stored between passages as infected allantoic fluid, and NDV was stored as a 10^{-1} dilution of infected allantoic fluid in heated normal horse serum. The Col. MM and FA viruses were cultivated by intracerebral inoculation of The Rockefeller Institute strain of albino Swiss mice, and stored as 10% mouse brain suspensions in 0.85% NaCl buffered at pH 7.2 (0.01 M phosphate). All virus suspensions were stored in a solid carbon dioxide cabinet at —70°C.

Virus Infectivity and Hemagglutination Titrations. The methods employed with Lee, PR8 and NDV viruses were carried out as previously described(1). Serial 10-fold dilutions of infected allantoic fluid were made in broth, and appropriate dilutions were inoculated into the allantoic sacs of chick embryos 9-11 days of age. Four chick embryos per dilution were employed. Following incubation for 2 days at 35°C and subsequent chilling, the allantoic fluid of each embryo was removed. Hemagglutination titrations with 1% chicken erythrocytes were carried out through a dilution of 1.128 to determine the presence of viral infection. The 50% infectivity titer was calculated by the method of Reed and Muench(3). Infectivity titrations with Col. MM and Theiler's FA viruses were carried out by intracerebral inoculation of 10-fold dilutions of infected mouse brain; 0.03 cc per mouse. Groups of 6 mice per dilution were employed. The 50% mortality titer(3) was determined at the end of 14 days in each instance.

Experimental. Inactivation of Viruses with Ethylene Oxide. The ethylene oxide employed and the technics of handling it were identical to those previously described in detail(1). The respective viruses were diluted 10-fold in sterile, heat-inactivated normal horse serum, and the mixtures placed at 4°C for 60 minutes. Then, to a 9.9 cc aliquot of each virus-serum suspension was added 0.1 cc of liquid ethylene oxide with a chilled pipette, and the mixture agitated to insure thorough mixing. Follow-

1. Wilson, A. T., and Bruno, P., *J. Exp. Med.*, 1950, v91, 449.

2. Phillips, C. R., and Kaye, S., *Am. J. Hyg.*, 1949, v50, 270.

3. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

TABLE I.
Inactivation of Newcastle Disease Virus by Ethylene Oxide.

Virus*	Ethylene oxide, vol. %	Mixture held at 4°C, hr	Mixture held at 35°C, hr	Virus dilution inoculated intra-allantoic .1 cc/embryo	Hemagglutination titers† of allantoic fluids‡ 1	2	3	4	Score +/t§	Infectivity titer E.I. 50‖
NDV 10^{-2}	0	1	24	10^{-6}	>128	>128	>128	>128	4/4	
				10^{-7}	>128	>128	>128	>128	4/4	
				10^{-8}	>128	0	>128	0	2/4	10^{-8} or >
"	1	1	24	10^{-2}	0	0	0	0	0/4	$<10^{-2}$
				10^{3}	0	0	0	0	0/4	

* Suspended in 99% normal horse serum.
† Expressed as the reciprocal.
‡ Harvested from embryos after incubation at 35°C for 48 hr.
§ Numerator represents the number of chick embryos infected; denominator represents the number of chick embryos inoculated.
‖ E.I. 50 = 50% embryo infectivity titer.

ing a further interval of 60 minutes at 4°C, the virus-ethylene oxide mixtures were transferred to an incubator regulated at 35°C where they remained for 24 hours. During this period of incubation the virus was acted upon by the sterilizing agent, and the agent itself disappeared by volatilization and combination. A second aliquot of each virus suspension was employed as a control, and carried through identical steps excluding the addition of ethylene oxide. Following the 24 hours incubation period at 35°C, infectivity titrations were carried out in appropriate hosts.

Inactivation of the Viruses of Influenza A and B and Newcastle Disease. In each instance liquid ethylene oxide in a concentration of 1 volume % was capable of causing complete inactivation of the virus as determined by the ability of the virus to infect the chick embryo. The results of a typical experiment employing NDV are shown in detail in Table I. It will be noted that even when more than 10^6 embryo infectious doses of virus was present initially, not a single embryo was infected when inoculated with the virus-ethylene oxide mixture. The results of experiments with NDV, PR8 and Lee are summarized in Table II, indicating the effectiveness of ethylene oxide in rendering these three distinctly different viruses non-infectious. It is of considerable interest and importance that dilution of the virus suspensions in 90-99% normal horse serum did not diminish the effectiveness of ethylene oxide. That various animal sera have a protective effect upon viruses in the presence of many inactivating substances as well as pH alterations is well known. Inactivation of even larger quantities of viruses by ethylene oxide could not be tested readily because of the necessity of incubating the mixtures for 24 hours at 35°C as part of the inactivation procedure. Incubation alone, in the absence of ethylene oxide, resulted in some viral inactivation in each instance.

Inactivation of Neurotropic Viruses. The infectivity of mouse encephalomyelitis viruses, Columbia MM and Theiler's FA, when suspended in normal horse serum was likewise completely inactivated by 1% liquid ethylene

TABLE II.
Inactivation of Various Viruses by Ethylene Oxide.

Virus	Ethylene oxide, %	Infectivity titer, E.I. 50*	Quantity virus inactivated, E.I. 50
Influenza B	0	$10^{-5.7}$	
	1	$<10^{-1}$	$10^{4.7}$ or $>$
Influenza A	0	10^{-4}	
	1	$<10^{-1}$	10^{3} or $>$
Newcastle disease	0	10^{-8}	
	1	$<10^{-2}$	10^{6} or $>$
		M.L. 50†	M.L. 50
Theiler's Fa	0	$10^{-4.6}$	
	1	$<10^{-2}$	$10^{2.6}$ or $>$
Col. MM	0	$>10^{-6.4}$	
	1	$<10^{-2}$	$10^{4.4}$ or $>$

* E.I. 50 = 50% embryo infectivity.
† M.L. 50 = 50% mouse mortality.

oxide. The results of these experiments are summarized also in Table II. It should be emphasized that neither with Col. MM nor FA did a single mouse inoculated intracerebrally with the ethylene oxide-treated virus show any evidence of infection by the neurotropic virus. Eighteen days following inoculation with the inactivated virus, the mice were challenged with 10^2 M.L.50 doses of the homologous active virus, and no evidence of immunity could be demonstrated in either instance.

Discussion. That liquid ethylene oxide can inactivate a variety of unrelated viruses is clearly indicated by the experimental data presented. Similar inactivation of the vaccinia virus also has been reported(1). That such viral inactivation can be accomplished with considerable reliability, ease, economy and safety is apparent. Viral inactivation was so complete, even in the case of NDV where more than 10^6 E.I.D. of virus was present, that not a single animal, chick embryo or mouse was infected. This is of particular significance with viruses tested in the chick embryo in which, in the case of the influenza viruses, infection is produced by about 10 viral particles or less(4). It is of considerable interest and importance that the ethylene oxide is active in the presence of 90-99% serum but causes no visible change in the serum. That some chemical alterations of the proteins do take place seems probable, but whether these changes affect the physiological activities of the serum proteins is not known. It has been clearly demonstrated(1), however, that the growth factors of bacteriological media are not sufficiently modified to prevent the subsequent growth and multiplication of fastidious bacteria in these media. The possibility is raised that the virus of homologous serum hepatitis in plasma, serum or other blood products might be inactivated by this procedure. Were this the case, it would indeed afford a simple and practical method of ridding these important therapeutic substances of a stable, troublesome, and dangerous virus.

Summary. Liquid ethylene oxide in a concentration of 1 volume % inactivates the viruses of influenza A, influenza B, and Newcastle disease as well as Columbia MM and Theiler's FA mouse encephalomyelitis viruses. These viruses were inactivated in the presence of 90-99% normal horse serum without any gross alterations in the serum.

4. Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, v79, 301.

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