Use of Ethylene Oxide for Preparation of Stable Non-Infective Influenza and Mumps Soluble Antigens. (19858)

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Ethylene oxide assumed importance as a biological agent in 1928 when it was described as a powerful insecticide against a large number of insect pests(1). Its use for this purpose spread rapidly. Later its activity against molds was reported(2). It soon became widely used for the sterilization of spices and other materials in a vacuum chamber process(3,4). Schrader and Bossert(5) first described the use of ethylene oxide as an antibacterial agent. This work was extended with the reports of its use to sterilize soil(6) and various culture media(7). In all the above reports, however, ethylene oxide was employed in the gaseous form. The first use of liquid ethylene oxide for the sterilization of media and other fluids was described by Wilson and Bruno(8). Later it was reported that 1% by volume of liquid ethylene oxide inactivated numerous viruses(9).

A method for the preparation of stable non-infective soluble influenza antigen involving the use of formaldehyde followed by its neutralization prior to lyophilization has been described previously(10). If other agents such as liquid ethylene oxide could be used for this purpose the procedure would be simplified. Consequently, an investigation was made to seek conditions of treatment with ethylene oxide by which stable dry non-infective soluble antigens could be prepared.

Materials and methods. The soluble antigen was prepared from the chorioallantoic membranes of 13-day-old chick embryos which had been infected with the PR8 strain of influenza A virus by the allantoic route 2 days previously. To the pooled membranes was added 1.5 ml of saline per membrane and the mixture was ground for 2 minutes in a Waring blendor. After a preliminary centrifugation for 10 minutes at 2000 r.p.m., the supernatant suspension was stored in the cold room at 4°C for 4 days. Any precipitate formed was then removed by another centrifugation at low speed, after which the supernatant suspension was centrifuged for one hour at 15,000 r.p.m. The supernatant liquid was removed and used as the soluble antigen. Infectivity tests on untreated controls and samples treated with ethylene oxide were conducted by direct inoculation of undiluted and 10^{-2} diluted suspensions into the allantoic cavity of 10-11-day-old chick embryos, with a subsequent incubation for 72 hours. Samples which after 2 successive passages yielded allantoic fluids free of specific hemagglutinins were considered to be non-infective. The following experiment was conducted to determine the optimal experimental conditions of ethylene oxide concentration, temperature, and time of treatment required to yield a soluble antigen which could be lyophilized and stored, and which when redissolved would be non-infective and would have undergone a minimal loss of antigenicity. A 50% solution of ethylene oxide (Eastman Kodak) was freshly prepared in distilled water at 4°C. This solution was added to 3 samples of a common pool of PR8 soluble antigen, precooled to 4°C, to give final concentrations of 1%, 3%, and 5% by volume of ethylene oxide. The ethylene oxide was added in the form of a 50% aqueous solution to facilitate accurate transfer with precooled glassware. Aliquots of each antigen sample were then placed in cotton-stoppered test tubes in water baths at 37°C, 25°C, and in the cold room at 4°C. Samples were removed after various periods of time and were lyophilized immediately in an Edwards centrifugal freeze-dryer, thereby removing all ethylene oxide. The dried antigens were reconstituted to their original volume with distilled water and tested for specific antigenicity in the complement-fixation test.

Results. It was found from these equipments that: 1) the samples of antigen stored at 4°C up to 24 hours showed no decrease in titer, 2) with the samples stored at 25°C, the

TABLE I. Effect of Various Conditions of Ethylene Oxide Treatment on PR8 Soluble Antigen.

Ethylene oxide vol, %	Temp., °C	Time of treatment (hr)	No. antigens tested	No decrease in titer	50% decrease in titer	>50% decrease in titer	No. of antigens containing live virus after treatment
5	4	16	9	7	1	1	1
5	4	24	22	21	1	0	0
5	4	48	13	12	1	0	0
5	25	2	5	3	1	1	1
3	25	2	17	13	4	0	3
3	25	24	14	8	5	1	0
3	37	2	20	10	10	0	1
10	4	24	8	8	0	0	0

antigen containing 5% ethylene oxide showed a progressive decrease in titer with time but the antigens containing 3% and 1% ethylene oxide underwent no significant decrease, 3) with the samples at 37°C, a decrease in titer was observed after 2 hours with the 3 concentrations of ethylene oxide used and the antigenicity was almost completely destroyed after 24 hours.

This type of experiment was then repeated using several different lots of PR8 soluble antigen to which there was deliberately added viable PR8 virus which resulted in suspensions with hemagglutinin titers of 1280 units per ml or greater. After lyophilization, the ID_{50} of 4 different untreated antigens were found to be approximately $10^{-8.6}$ to $10^{-9.8}$ per ml by allantoic titration. Only ethylene oxide concentrations and temperatures which had been found previously not to cause a significant loss of antigenicity were used in this experiment. The samples stored at 4°C were loaded into ampoules at room temperature, the total time at room temperature being 30 minutes. This step was introduced for the convenience of handling large lots. In an additional experiment, a concentration of 10% by volume of liquid ethylene oxide was employed, obtained by direct addition of the oxide in the undiluted form to the antigen. After lyophilization, these antigens were reconstituted and tested for antigenicity and infectivity as described above. The results are summarized in Table I.

From Table I it can be seen that several combinations of experimental conditions come close to fulfilling the desired criteria for a method of rendering these antigens non-infective with little or no loss of antigenicity. Treatment with 3% ethylene oxide at 37°C for 2 hours destroyed the infectivity in all cases except one, but half of the treated antigens showed a 50% decrease in complement-fixation titer. A concentration of 3% ethylene oxide at 25°C for 24 hours was found to be effective for destroying the infectivity but there was a decrease of antigenicity in some antigens. However, it is evident that the best results in preparing non-infective antigens can be obtained by the addition of 5% by volume of liquid ethylene oxide and treatment at 4°C for 24 or 48 hours, followed by a 30-minute period at room temperature to allow for ampoule filling and then immediate lyophilization. Since one sample was found to be still infective after 16 hours, it is considered that the 48-hour period of treatment represents the method of choice as it provides a wider margin of safety than the 24-hour treatment.

These experiments were repeated on 10 different lots of mumps soluble antigen to which were added viable mumps virus to give hemagglutinin titers of 640 to 1280 units per ml. Here it was found that only the treatment with 5% ethylene oxide for 24 hours at 4°C was successful in destroying the infectivity in all cases while the antigenicity was not decreased. The period of treatment could also be extended to 48 hours with no loss of titer except in one case where the antigen had been stored for 11 months in a deep-freeze at —20°C prior to its present use.

Discussion. In the biological field in the past, ethylene oxide has been employed primarily as a fungicide, insecticide, and germicide, that is, as a disinfectant. In the present investigation conditions of ethylene oxide treatment have been sought which would

permit the destruction of viral infectivity while at the same time preserving the antigenicity. No reaction conditions below a 24-48-hour period of treatment have been found which would achieve this double objective. However, treatment of the soluble antigens of influenza and mumps with 5% by volume of liquid ethylene oxide at 4°C for 48 hours followed by a 30-minute period at room temperature to allow for ampoule filling and then immediate lyophilization has been found to yield antigens which when reconstituted to their original volume with distilled water are non-infective and have undergone no significant decrease in complement-fixation titer in the process.

At the time of writing, antigen samples treated as described above have been stored in the cold room at 4°C for 3 months without significant loss of titer.

Summary. An investigation has been conducted of the use of liquid ethylene oxide for the preparation of stable non-infective soluble antigens of influenza and mumps. It has been found that treatment of these antigens with 5% by volume of liquid ethylene oxide at 4°C for 48 hours, then for 30 minutes at room temperature, followed by immediate lyophilization, is a satisfactory method to accomplish this purpose.

1. Cotton, R. T., and Roark, R. C., *Ind. Eng. Chem.*, 1928, v20, 805.

2. Kirby, G. W., Atkin, L., and Frey, C. N., *Food Industries*, 1936, v8, 450, 470, 488.

3. Griffith, C. L., and Hall, L. A., *Sterilization Process U. S. Patent*, 1940, 2,189,947.

4. Baer, J. M., *Method of fumigation of organic products U. S. Patent*, 1941, 2,229,360.

5. Schrader, H., and Bossert, E., *U. S. Patent* 1936, 2,037,439.

6. Roberts, J. L., Allison, L. E., Prickett, P. S., and Riddle, K. B., *J. Bact.*, 1943, v45, 40.

7. Hansen, H. N., and Snyder, W. C., *Phytopathology*, 1949, v37, 369.

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

9. Ginsberg, H. S., and Wilson, A. T., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 614.

10. Polley, J. R., Burr, M., and Gillen, A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 330.

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Influence of Nitrogen Mustard upon Reactions to Bacterial Endotoxins: Shwartzman Phenomenon and Fever.* (19859)

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Becker(1) found that the Shwartzman phenomenon could not be elicited in rabbits a few days after injection of nitrogen mustard (hereafter referred to as HN_2) or benzol or after total body x-ray irradiation. This effect has been confirmed by Schlang(2) and by Stetson and Good(3). Although Becker postulated that the effect of HN_2, benzol and x-ray was due to the production of an "anergic" state in cells of the vascular endothelium. Stetson pointed out that all 3 of these forms of treatment are followed in a few days by a profound neutropenia. His experiments showed that the period of Shwartzman non-reactivity after HN_2 or benzol coincides with the time of maximum depression of circulating granulocytes. Furthermore, prevention of leukopenia by aortic compression in HN_2-treated animals or by administration of sulfapyridine in benzol-treated rabbits eliminated the period of Shwartzman non-reactivity. Stetson concluded that the presence of an adequate number of circulating granulocytes is necessary for the elicitation of the Shwartzman reaction and that the inhibitory effect of HN_2 and benzol is due to suppression of these cells. Weis-

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