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STUDIES ON THE INACTIVATION OF VIRUSES BY ETHYLENE OXIDE

By

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HOFF-JØRGENSEN, RIKKE and EBBA LUND: *Studies on the inactivation of viruses by ethylene oxide.* Acta vet. scand. 1972, *13*, 520—527. — Under conditions designed to imitate heavy natural contamination of equipment, animal cages, etc. virus was exposed to ethylene oxide treatment.
The effect on strains of the enterovirus types Coxsackie B3 and ECHO 11, of vaccinia virus and of Newcastle disease virus was studied. The viruses were all inactivated by a treatment which was considered sufficient from a bacteriological point of view.

ethylene oxide; virus inactivation.

Due to the low boiling point of ethylene oxide gas it is possible to use this compound for sterilizing purposes in connection with heat sensitive material e.g. medical equipments and special foodstuffs by treatment at low temperatures. There are thus definite advantages connected with the use of this compound. The practical difficulties that may be encountered due to the low boiling point and inflammable nature of the compound may be overcome by using a gas tight vacuum chamber.

The literature concerning the virus inactivating capacity of ethylene oxide is rather limited (e.g. *Mathews & Hofstad* 1953), but the reports published mention the poor effect on dried preparations of e.g. FMV (foot and mouth disease virus). If dried preparations of FMV were rehumidified before ethylene oxide treatment the virus was, however, inactivated (*Tessler & Fellows* 1961).

The handling of equipment, e.g. in connection with the breeding of SPF animals, requires sterilizing methods. It was with such application in mind that the work to be presented was

carried out. The purpose was to imitate conditions that may be corresponding to natural contamination of equipment and to test the virus inactivating effect of an ethylene oxide treatment which seems satisfactory from a bacteriological point of view.

Each time an attempt is made to determine rates of inactivation of viruses in connection with disinfection procedures, the number of parameters involved at the same time cause difficulties. In this work no attempt was made to determine the rate of inactivation as a function of ethylene oxide concentration, humidity and temperature, however important such determination may be, but only to examine if an empirically set treatment might cause apparent loss of active virus under various conditions. Four different virus strains were selected to cover a wide range of stabilities towards chemical and physical treatment.

MATERIAL AND METHODS

Virus types

A human enterovirus, a strain of Coxsackie virus type B 3 was isolated in HeLa cell cultures from human sewage in this laboratory. This strain of virus was selected, because it had withstood sewage treatment and consequently must be a relatively resistant strain under "natural" conditions. The titer was around 10^6 TCID/ml. For the same reason another enterovirus isolated from sewage in HeLa cell cultures, a strain of ECHO virus type 11 was selected. The titer in HeLa cell cultures was around 10^7 TCID50/ml.

A strain of vaccinia virus had kindly been supplied from the State Serum Institute, Copenhagen, by dr. J. Leerhøy. The strain (WRCE, L III) was adapted to a human amniotic cell line, where the titer was around 10^4 TCID50/ml.

The Newcastle disease virus (NDV) used in the experiment was an F-strain (Weybridge) kindly supplied by dr. Grete Velling of the Poultry Disease Laboratory, Copenhagen, and adapted in the laboratory to growth in human amniotic cells giving a titer of around 10^6 TCID50/ml.

Cell cultures

The HeLa cells used were originally obtained from the State Serum Institute and have been grown for several years in this laboratory in a 20 % calf serum (Flow laboratories) medium

containing 1.4 % NaHCO$_3$, 0.5 % lactalbumin hydrolysate (Sigma) with 200 i.u. of penicillin and 200 γ of streptomycin per ml in Hanks' basic salt solution.

The cultures of human amniotic cells were originally purchased from Flow laboratories. They were grown in Eagle's Min. Essential Medium (MEM) with 4.4 % NaHCO$_3$, 20 % calf serum and the same antibiotic as the medium for the HeLa cell cultures.

The maintenance medium was for both cell lines MEM containing 3 % calf serum, 4.4 % NaHCO$_3$ and antibiotics.

Virus titrations

The virus titrations were carried out in tube cell cultures. Each of serial ten-fold dilutions of the virus samples to be titrated were inoculated in three cultures using 0.1 ml per tube containing 2 ml of medium. Final reading of CPE was done on the 10th day of incubation at 37°C and the titer expressed in TCID50/ml.

Experimental procedure

All virus suspensions used were the crude harvests from cell cultures. For each virus type investigated six types of samples were prepared:

1. One ml of virus suspension was left 24 hrs. at room temperature in a rubber stoppered glass tube.

2. One ml of virus suspension was placed on a piece of polyethylene and left to dry by air 24 hrs. protected against contamination. The sample was then placed in a polycarbonate tube which was closed.

3. One ml of virus suspension was thoroughly mixed with about the double volume of a disintegrated mixture of rabbit spillings, straw and sawdust in a tube, which was closed and kept 24 hrs. at room temperature.

4. This sample was prepared as 3, but left open to dry at room temperature for 24 hrs.

5. This sample was prepared as 3, but was placed on a piece of metal from an animal cage and left to dry for 24 hrs. at room temperature.

6. This sample was corresponding to 5, except that the cage material was of the same plastic material as for sample 2.

Each sample except no. 1 was prepared in three parts. The parts A and B of the samples were sent by special delivery to Dr. A. Jordy, Degesch, Frankfurt, Germany, who exposed the part A to ethylene oxide treatment during which the tubes were unstoppered. The parts A and B were then returned to Copenhagen by special delivery. The total transportation was completed within three to six days under uncontrolled conditions. For this reason the sample B was included in the experiment serving as a control for spontaneous inactivation of virus. The third sample (C) was kept in the laboratory at —20°C and served as a control which was examined in parallel with the two other samples.

Upon arrival in the laboratory 9 ml of Hanks' solution was added to all three parts (A, B and C). The Hanks' solution was supplemented with 1000 i.u. of penicillin, 1000 γ of streptomycin and 1000 i.u. of mycostatin per ml. The samples were centrifuged at 4000 r.p.m. (2200 × g) for 10 min. and the supernatants tested for infectious virus as described. In some cases the supernatants were stored at —20°C before titration.

Ethylene oxide treatment

The ETO (ethylene oxide) treatment was performed at Degesch, Frankfurt, using a procedure that has proved efficient for the killing of spores of Bac. subtiles (*Jordy* 1970, *Kristensen* 1970). The treatment was carried out using a vacuum chamber with a capacity of 35 l. The temperature was kept at 25°C for 6 hrs. The concentration of gas was 1.5 g ETO/l. The fumigation vessel and the whole equipment were evacuated down to 20 mm of mercury. The ETO vapour was produced by means of a specially designed evaporizer and allowed to stay in the fumigation chamber for the time desired. At the end of the 6 hr. period at 25°C the ETO was removed and sterile, fresh air was let in.

RESULTS

The results of the experiments are compiled in Table 1. From the table may be seen that drying reduced the infectivity of Coxsackie virus, so that no virus was demonstrable. The ETO treatment inactivated the virus. This effect could not be due to spontaneous inactivation during transportation, where little apparently was lost. Drying hardly affected the ECHO virus, nor did the transportation. The ETO treatment, however, inactivated the ECHO virus of all samples.

Table 1. ETO-treatment of various virus types (Virus titer expressed in TCID50/ml).

No. of sample	Nature of sample*	Coxsackie B$_3$ A**	B†	C††	ECHO 11 A	B	C	vaccinia A	B	C	NDV A	B	C
1	virus suspension		10^5	10^6		lost	10^7		10^3	10^4		10^2	10^6
2	virus suspension dried on plastic	nd†††	nd	nd	nd	nd	nd	neg.	neg.	neg.	neg.	neg.	neg.
3	virus suspension mixed with spillings etc.	neg.	10^6	10^6	neg.	10^6	10^7	neg.	10^2	10^2	neg.	neg.	neg.
4	as 3, but left to dry in a tube	neg.	10^5	10^6	neg.	10^7	10^7	neg.	neg.	10^3	neg.	neg.	neg.
5	as 3, but left to dry on a metal surface	neg.	neg.	neg.	neg.	10^6	10^6	neg.	neg.	neg.	neg.	neg.	neg.
6	as 5, but left to dry on a metal surface	neg.	neg.	neg.	neg.	10^5	10^6	neg.	neg.	10^3	neg.	neg.	neg.

* See experimental procedure.

** Ethylene oxide treated. See Material and Methods for further details.

† Transportation control. „ „ „ „

†† Untreated control. „ „ „ „

††† Not done.

In the experiment using vaccinia virus it was found that mere drying of a virus suspension may inactivate the material unless there is a high concentration of organic matter present (3 and 4). Somehow the metal surface contributed to the virus inactivation (5). The titer of the control suspension (1 C) was, however, quite low (10^4 TCID50/ml).

In no ETO treated vaccinia virus samples could virus be demonstrated.

The experiments using NDV were characterized by few virus containing samples. All dried samples, also of the laboratory control material (part C), were without demonstrable amounts of virus. The otherwise untreated sample (1) lost three log units of infectivity just by the transportation procedure. The ETO treatment also in this case inactivated the virus.

From Table 1 may be seen that the viruses selected were stable (even NDV to some degree) as suspensions during transportation and that ETO treatment in all these cases inactivated virus.

Suspensions of NDV and vaccinia virus dried at room temperature as suspensions on a plastic surface lost their infectivity. If mixed with the dirt from animal cages the virus suspensions retained their infectivity except for NDV. By exposure of this material to drying vaccinia virus lost its infectivity. If the drying was carried out in a tube rather than on a surface the Coxsackie virus could retain its infectivity, while the ECHO strain tested withstood all types of drying tried. No dried samples could resist ETO treatment.

DISCUSSION AND CONCLUSIONS

In selecting viruses for the present work it was attempted to find virus types which should be among the ones considered resistant in general to physical and chemical inactivation and also cover a wide range of the virus classes.

Enteroviruses are relatively resistant to chemical treatment and the two virus strains selected were wild strains isolated from urban sewage after biological treatment. Thus they must be considered relatively resistant to spontaneous inactivation, an impression which was supported by their resistance to drying, in the experiments reported here, especially found for the ECHO 11 strain.

It is regrettable that the sample containing only the dried

virus suspensions was not included in the test using ECHO virus, because this virus might have proved more sensitive to drying under conditions, where less organic matter was present than in the samples containing faecal matter etc. The high resistance towards drying found in the present experiment must be considered unexpectedly high (*Andrewes & Pereira* 1967), but little work of this nature has been reported.

Vaccinia virus and NDV are, especially from epidemiological evidence, accepted as being resistant to drying. Therefore the results of the present report are quite surprising, but are to some degree supported by the findings of *Sidwell et al.* (1969). More experiments using other strains of the viruses are indicated to elucidate this point.

Because of the reported resistance of FMV towards inactivation by physical means (*Burbury* 1927) and the reports on resistance towards ETO treatment, it would have been desirable to include this virus in the test. The laboratory is not equipped in such a way that this would be permissible, so in this connection the work of *Tessler & Fellows* (1961) must be referred to, even if the experimental conditions in their report are somewhat different.

As also pointed out by *Mathews & Hofstad* (1953) "lyophylized samples desiccated in vacuum are not comparable to the type of virus infection dried under atmospheric conditions encountered on instruments, feed sacks, clothing, notebooks and laboratory equipment." For these reasons lyophilisation was not employed in the present work. The experiments were designed to get results that would correspond to what one might expect under "real life" situations as e.g. in an animal room. The samples rather correspond to such conditions as one may expect if extremely soiled articles were to be treated. The transportation controls were included so that the effect of spontaneous inactivation could not be misinterpreted as being due to ETO treatment. The ETO used was one that has been considered satisfactory from a bacteriological point of view (*Jordy* 1970, *Kristensen* 1970). Thus the whole experiment had a purely practical, empirical purpose, and no information regarding the nature of ETO inactivation or actual inactivation rates was sought.

From the results of the present experiment it is indicated that even under extremely difficult conditions, i.e. in presence of high concentrations of organic matter from animal cages,

where the samples were left to dry in the air before treatment, ETO inactivated Coxsackie virus B_3, ECHO virus Type 11, vaccinia virus and Newcastle disease virus, so that no active virus was demonstrable after treatment. These results do not seem to be in conflict with reports on the treatment of artificially dried virus suspensions. Such dried preparations may contain virus resistant to the treatment, but the results of the present report seem more relevant for "real life" situations.

Only four virus strains were tested, and especially the poor stability of the selected NDV and vaccinia virus strain towards drying makes further work with such viruses warranted.

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SAMMENDRAG

Undersøgelser af virusinaktivering med ætylenoksyd.

Under forsøgsbetingelser, som søgte at efterligne betingelser ved naturlig stærk forurening af instrumentarium, dyrebure etc., blev forskellige virus udsat for ætylenoksyd behandling.

Effekten på enterovirustyperne Coxsackie B_3 og ECHO 11, på vaccinia virus og Newcastle disease virus blev undersøgt.

Gennem behandlingen blev alle fire virustyper inaktiveret.