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VIRUS INACTIVATION BY ETHYLENE OXIDE CONTAINING GASES

By

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JORDY, ANGELIKA, RIKKE HOFF-JØRGENSEN, ANNETTE FLAGSTAD and EBBA LUND: *Virus inactivation by ethylene oxide containing gases.* Acta vet. scand. 1975, *16*, 379—387. — Gases containing ethylene oxide mixed with carbon dioxide alone (Etox®) or together with methyl formate (Etoxiat®) were employed for virus treatment in a way that has been shown efficient for the killing of bacteria. A number of viruses selected for their capacity to withstand chemical or physical treatments were tested under varying conditions, including in a dried state in the presence of high amounts of organic matter (animal spillings). The viruses tested were enteroviruses, paramyxovirus (NDV), poxvirus and parvovirus, and they were all inactivated to a high degree.

ethylene oxide; virus inactivation.

In a previous study (*Hoff-Jørgensen & Lund* 1972) it was found that enterovirus, vaccinia virus and Newcastle Disease virus may be inactivated employing a treatment, which was found satisfactory from a bacteriological point of view. As the resistance towards spontaneous inactivation for some of the viruses differed to some degree from what was expected, these were reinvestigated. In addition the very stable parvovirus, feline panleucopaenia virus, was included. This study had as the previous one the purpose of evaluating the virus inactivation under difficult, but still realistic conditions, but not of looking into the nature of the reaction.

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MATERIAL AND METHODS

Virus

Coxsackie virus. A strain of Coxsackie virus B3, which has been employed previously (*Hoff-Jørgensen & Lund* 1972), and which has been isolated from Copenhagen sewage, was employed. The titer in HeLa cell cultures was around 10^7 ID50/ml.

ECHO virus. The same strain of ECHO virus type 11, which had been employed previously and was isolated from sewage, was employed. The titer was around 10^7 ID50/ml in HeLa cell cultures.

Newcastle Disease virus. The same F strain (Weybridge) adapted to growth in HeLa cells, which was employed previously, was employed in the present experiment. The titer was around 10^6 ID50/ml.

Vaccinia virus (strain A 583) was kindly supplied by dr. J. Leerhøy, The State Serum Institute. Also this virus was grown in HeLa cells, where the titer was around 10^5 ID50/ml.

The feline panleucopaenia virus was isolated from a cat with clinically diagnosed panleucopaenia. The virus was propagated in feline kidney cell cultures, where the titer was around 10^3 ID50/ml.

Cell cultures

The HeLa cells employed were grown in Hanks' salt solution to which was added 20 % calf serum, 0.5 % lactalbumin hydrolysate and antibiotics (100 i.u. of penicillin and 100 µg of streptomycin per ml). The maintenance medium contained 3 % calf serum and antibiotics on the base of Eagle's Minimal Essential Medium.

The feline cells employed for panleucopaenia virus were secondary cultures of feline kidney cells grown in Hanks' solution containing 0.5 % lactalbumin hydrolysate, 0.01 % yeast extract, the antibiotics as for the HeLa cells and 10 % foetal calf serum. The cells were seeded in Leighton tubes.

Virus titrations

For the viruses which were titrated using HeLa cells the titrations were carried out employing serial ten-fold dilutions and inoculation of 0.1 ml in each of three tubes containing 2 ml of fluid. Final reading of CPE was usually done on the 10th day after the inoculation, and the titers were expressed in ID50/ml.

The panleucopaenia virus was titrated using Leighton tubes, and the inoculations were made about 4 hrs. after the seeding with cells. Ten-fold dilutions of virus were each inoculated using 0.1 ml inocula in four cultures from which the fluid was removed prior to inoculation. After allowing virus to adsorb to the cells during 1 hr. at room temperature 2 ml of medium was added. The tubes were inocubated at 37°C, and the medium was renewed 18 hrs. after inoculation, as the virus suspensions seemed to be toxic to the cells.

The virus titer was found in the following way: The cultures were examined after fixing and staining on the third day after inoculation. Some cultures were fixed in methanol and stained with haematoxylin eosin, and some cultures were fixed in acetone and treated with FITC (fluorescein isothiocyanate) conjugated immunoglobin (*Flagstad* 1975). The haematoxylin eosin stained cultures were examined for intranuclear inclusion bodies and the other for immunofluorescence.

The titer was roughly estimated in the haematoxylin eosin stained preparations, and the final end-point was estimated using the fluorescein stained preparations. At the titration end-point only few infected cells could be demonstrated, and it was found faster and more reliable to find these cells employing the immunofluorescence rather than the haematoxylin eosin stained preparations.

Experimental procedure

As previously described (*Hoff-Jørgensen & Lund*) six types of samples were prepared for each virus under investigation:

1. One ml of virus suspension was left for 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 for 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 for 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 was prepared in four parts. Three parts were sent by special delivery from Copenhagen to Frankfurt, where the two samples, unstoppered, were submitted to treatment, and the third sample served as untreated transport control. The fourth sample was kept in Copenhagen at —20°C and was considered representing the original virus suspension. Upon arrival back in the laboratory in Copenhagen 9 ml of Hanks' solution was added to each sample. The Hanks' solution was supplemented with 1000 i.u. of penicillin, 1000 µg 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. Occasionally the samples were stored at —20°C before titration.

Ethylene oxide treatment

The gas treatment was carried out at Degesch, Frankfurt. Two different gas mixtures were employed:

Etox®, the registered trade name of Degesch (Deutsche Gesellschaft für Schädlings-Bekämpfung mbH, 6 Frankfurt/Main) for a 90 % ethylene oxide, 10 % carbon dioxide gas mixture.

Etoxiat®, the registered trade name of Degesch for a 45 % ethylene oxide, 45 % methyl formate, 10 % carbon dioxide gas mixture.

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 mixtures was 1.25 g/l. The fumigation vessel and the whole equipment were evacuated down to 20 mm of mercury. The gas 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 hrs. period at 25°C the gas mixtures were removed and sterile, fresh air was let in.

RESULTS

The results obtained are collected in Tables 1—5. The virus titres are expressed in approximate numbers indicating that no high titration accuracy has been attempted or desired.

Table 1. Titre in ID50/ml of Coxsackie virus B_3 after treatment.

Sample no.	Nature of sample*	Titre in sample			
		A	B	C	D
1	virus suspension	neg.	10^1	10^5	10^6
2	virus suspension dried on plastic	neg.	neg.	neg.	10^2
3	virus suspension mixed with spillings etc.	neg.	neg.	10^4	10^6
4	as 3, but left to dry in a tube	neg.	neg.	10^2	10^6
5	as 3, but left to dry on a metal surface	neg.	10^1	10^2	10^3
6	as 3, but left to dry on a plastic surface	neg.	10^1	10^2	10^3

* See under experimental procedure.

A Etox® treated sample.
B Etoxiat® treated sample.
C Transported, but otherwise untreated sample.
D Untreated sample, which was kept at —20°C.

The results indicate that the Coxsackie virus does not withstand drying very well. Some virus activity is lost during transportation even in the ordinary virus suspension. Etox treatment inactivated the virus completely, but apparently the Etoxiat treatment was slightly inferior in efficiency. Dried virus seemed more resistant than virus in suspension.

The ECHO virus was, as may be seen from Table 2, inactivated to a high degree by drying, except in the case where virus

Table 2. Titre* of ECHO virus in ID50/ml after treatment.

Sample no.	A	B	C	D
1	neg.	neg.	10^6	10^6
2	neg.	neg.	10^2	10^3
3	neg.	neg.	10^6	10^6
4	neg.	neg.	10^6	10^6
5	neg.	neg.	10^2	10^5
6	neg.	neg.	10^2	10^4

* For explanation of sample nos. and symbols see Table 1.

was mixed with dirt and left to dry in a tube. The efficient inactivation by Etox was confirmed in all samples. Also the Etoxiat effectively inactivated the virus of all sample types.

The Newcastle Disease virus was inactivated by drying. From Table 3 may be seen that the Etox and Etoxiat treatments could inactivate the virus remaining infectious after transportation as suspensions.

Table 3. Titre* of Newcastle Disease virus in ID50/ml after treatment.

Sample no.	A	B	C	D
1	neg.	neg.	10^2	10^4
2	neg.	neg.	neg.	neg.
3	neg.	neg.	10^2	10^2
4	neg.	neg.	neg.	neg.
5	neg.	neg.	neg.	neg.
6	neg.	neg.	neg.	neg.

* For explanation of sample nos. and symbols see Table 1.

From Table 4 may be seen, that vaccinia virus does not withstand drying and transportation very well, except when much organic matter is present. However, the inactivation of virus was completed for all samples using Etox and Etoxiat irrespective of the sample type.

Table 4. Titre* of vaccinia virus after treatment.

Sample no.	A	B	C	D
1	neg.	neg.	10^4	10^4
2	neg.	neg.	neg.	neg.
3	neg.	neg.		
4	neg.	neg.		
5	neg.	neg.	10^2	10^3
6	neg.	neg.	10^2	10^3

* For explanation of sample nos. and symbols see Table 1.

The results obtained using panleucopaenia virus are seen in Table 5. The virus withstood drying quite well. In fact it was the only virus tested that withstood drying without added organic matter without a reduction in titre. The virus was somewhat

Table 5. Titre* of panleucopaenia virus after treatment.

Sample no.	A	B	C	D
1	neg.	neg.	10^3	10^4
2	neg. or 10^1	neg.	10^3	10^4
3	neg.	neg.	neg.	10^3
4	neg.	neg.	10^2	10^3
5	neg.	neg.	10^1	10^3
6	neg.	neg.	10^2	10^3

* For explanation of sample nos. and symbols see Table 1.

reduced in titre during transportation and was inactivated completely by the Etox or the Etoxiat treatment, except in one case: In one experiment some active virus was left in the dried out virus suspension without any addition of dirt.

DISCUSSION AND CONCLUSIONS

The viruses of the present and previous report (*Hoff-Jørgensen & Lund* 1972) were chosen among such types, which are considered resistant to physical and chemical inactivation.

Enteroviruses are rather resistant to a number of chemical disinfectants, but are usually reported (*Andrewes & Pereira* 1972) to lose infectivity on drying. Therefore the results of the previous report especially as regards ECHO virus were found so remarkable that it was decided to repeat these experiments. The results of the present report indicate that the examined enteroviruses, especially where high concentrations of organic matter are present, withstand drying quite well. The residual humidity may probably vary in the different experiments, so the spread in titres obtained does not seem surprising.

Contrary to other paramyxoviruses the Newcastle Disease virus is usually from epidemiological evidence considered quite resistant to drying, and this is why the virus was included in the study. However, neither in the previous nor the present report any signs of resistance to inactivation through drying were found. The experiments with NDV were repeated several times, because the activity was lost completely during transportation, and the results of Table 3 are in fact the ones with the highest titer obtained. There might thus be reason to reevaluate the epidemiological evidence.

Poxvirus including vaccinia virus is reported to withstand drying and may be lyophilized to a very stable vaccine. However, as may be confirmed by the present report, the stability is very much dependent on the presence of organic matter, and drying of an ordinary suspension of virus over 24 hrs. at room temperature completely inactivates the virus. The organic matter of spillings etc. protects the virus well.

Panleucopaenia virus was included in the present report because of its pronounced resistance to a number of treatments. *Andrewes & Pereira* reported that "the results on the resistance to desiccation are conflicting". It is said to withstand lyophilization, but to be readily inactivated when dried at room temperature. On the other hand, formates seem to retain infectivity for months under natural conditions". The results of the present work indicate a high resistance to desiccation.

In general the results of the present report confirm the previous ones: Ethylene oxide gases are very efficient in inactivating even chemically resistant viruses like enterovirus, poxvirus and even parvovirus, when a treatment satisfactory from a bacteriological point of view is employed. This seems to be true, even when the virus is dried in the presence of high concentrations of organic matter.

Using Etoxiat treatment on some of the Coxsackievirus samples and the Etox treatment on dried panleucopaenia virus a small remaining titre of active virus was still demonstrable after treatment. This serves as a warning to indicate that the treatment employed is on the borderline under certain circumstances and should not be reduced neither in the time nor in the concentration employed.

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SAMMENDRAG

Virus inaktivering ved hjælp af etylenoxydholdige luftblandinger.

Luftblandinger indeholdende etylenoxyd med kuldioxyd alene (Etox®) eller sammen med methylformiat (Etoxiat®) blev anvendt til at behandle virus på en måde, som har vist sig effektiv over for bakterier. Et antal virustyper, som blev udvalgt på grund af deres accepterede evne til at modstå fysisk eller kemisk behandling, blev undersøgt under forskellige betingelser indbefattet behandling af indtørrede præparater med højt indhold af organisk materiale (hentet ved rengøring af kaninbure). De undersøgte virustyper: Enterovirus, paramyxovirus (NDV), koppevirus og parvovirus blev alle inaktiveret i høj grad.

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