Lyophilizing Foot-and-Mouth Disease Virus at Low Drying Temperature

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SUMMARY

Drying tissue-culture virus at low temperatures (+4 to +6 C.) and adding 10% lactose before freezing were useful procedures in the lyophilization of foot-and-mouth disease virus (FMDV).

Freeze-dried virus kept reasonably well when stored at low temperatures (—25 or +2 C.) but lost most of its infectivity in relatively short periods at 37 C. and at ambient room temperature (23 to 26 C.). This loss of activity may be due to ribonuclease activity in the presence of residual moisture.

The lyophilization of picornaviruses, which include FMDV, often leads to considerable loss of viral activity.[1]

Before 1965, several investigators reported their experiences with freeze-drying FMDV: Michelsen and Mikkelsen[8] in 1945, Sergeev[13] in 1958, Ceccarelli[3] in 1960, and Ludwig[6,7] in 1961 and 1964. In 1965, while our work with Edwards* freeze-driers was in progress, a report by Fellowes[4] was published; he used equipment manufactured by the same firm and reported experimental results similar to our findings. The objective of the present report will therefore be limited to the description of matters not previously reported.

Materials and Methods

Virus. — Unmodified Iranian field strains of types A and O grown in monolayers of calf kidney or lamb testis cells (2nd or 3rd passages) were used. The cells were grown in Hanks' medium with 10% normal bovine serum, which was replaced by VM 3 as maintenance medium shortly before the cells were infected. This medium[11] was developed in an effort to eliminate the phosphate-buffer system which damaged FMDV on freezing and thawing.[5,14] This and similar mediums permit repeated freezing and thawing of FMDV without significant loss of activity.[12] Before drying, the frozen and thawed cultures were clarified by centrifugation.

Freeze-Drying and Virus Assay. — For lyophilization, an Edwards primary centrifugal drier* and an Edwards secondary drier,** in which drying was done with phosphorus pentoxide, were used separately or in combination.

The drying procedure was similar to that described by Fellowes[4] except that 3 experiments were carried out at ambient temperatures (+26.0 and 38.5 C.) and 10 at cold-room temperature (+4 to +6 C.). The drying time at ambient temperature was varied between 22 and 46 hours, and at cold-room temperature, between 22 and 68 hours. When only the

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*Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex, England.

*Model 30 P.I., Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex, England.

**Model 30 S.I., Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex, England.

secondary drier was used, the contents of the ampules were shell-frozen in a mixture of dry ice, acetone, and alcohol before they were attached to the drying manifolds. Four tests were made with the primary centrifugal freeze-drier alone (compressor temperature, —55 C.; vacuum, 70 to 75 μ; drying time, 22 to 41 hours), 4 tests with the secondary drier alone (vacuum, 75 μ; drying time, 24 to 68 hours), and 2 tests in which the primary and secondary driers were used in succession, each for 24 hours. In every instance, the driers were kept in cold rooms, and the samples contained 10% lactose.

The 5-ml. ampules containing 2 ml. of tissue culture (TC) fluid, with or without adjuvants (10% lactose, 10% sucrose, or NaCl in saturation), were sealed under vacuum after lyophilizing and were assayed for virus in calf-kidney cells with VM 3 as diluent and maintenance medium. In titrating the losses of activity due to freeze-drying, 7 or 8 test tube cultures were inoculated with each decimal dilution. For determining the losses during storage, the number of test tube cultures was reduced to 5 or 6 tubes per dilution.

Infectivity titers were calculated according to the method of Reed and Muench[9] and expressed in the text and table as negative $\log_{10}$ of the median tissue culture infective doses ($TCID_{50}$).

Results

Influence of Adjuvants. — Lactose (10%), sucrose (10%), and NaCl (saturated solution) were tested as stabilizers in comparison with untreated TC fluid; satisfactory results were obtained only with lactose. In 11 tests, including drying at ambient and cold-room temperatures, the mean titer decrease by freeze-drying was 0.98 log in samples containing 10% lactose and 1.15 log with untreated TC fluid. The addition of lactose prevented frothing, which was most marked in samples of TC fluid saturated with NaCl, but occasionally occurred in untreated TC fluid as well.

TABLE 1 — Loss of Activity by Storage at Different Temperatures

Test No.	Time of storage (days)	Loss by drying (log)	Loss (log) by storage at			
			—25 C.	+2 C.	+23 to 26 C.	+37 C.
1	11	0.3	0	0	...	>3.0
2	13	0.3	...	0.7	2.0	>4.0
3	13	0.2	...	0.8	2.1	~4.1
4	15	0.9	...	0.1	...	>2.8
5	15	0.9	...	0.4	...	>2.8
6	15	1.0	...	0	...	~2.5
7	23	0.3	...	0.5	...	>3.3

Influence of the Drying Temperature. — In the first 3 experiments with drying temperatures close to ambient temperature, viral samples containing 10% lactose had losses of infectivity (caused by drying) of 1.0 log at 26.0 C. and 1.9 log at 38.5 C. When the driers were placed in cold rooms at +4 to 6 C., the mean infectivity loss in samples with lactose was only 0.65 log.

Influence of Drier Equipment. — The mean titer decreases were 0.45 log in the primary drier, 0.87 log in the secondary drier, and 0.6 log when both were combined. It appears therefore that all 3 methods are suitable for lyophilizing FMDV.

Influence of Storage at Different Temperatures. — In the current experiment with samples containing 10% lactose, the storage periods were rather short (Table 1). The tests were discontinued when the work of Fellowes[4] became known. At that time, our results were in agreement with the reports by Ludwig[7] and Fellowes; *i.e.*, storage at low temperatures preserved the infectivity

of lyophilized virus reasonably well. However, storage at 37 C. soon led to a great loss of activity. Even storage at ambient room temperatures, between 23 and 26 C., for 13 days effected significant reduction of the infectivity titers.

Discussion

Although Ludwig[6] and Fellowes[4] did not see any advantage in the addition of stabilizing substances to the viral suspensions that were freeze-dried, Sergeev[13] reported on a certain stabilizing effect by egg yolk, defatted cow's milk, and gelatin plus sucrose, as cited by Fellowes. As far as we know, the first to use lactose as adjuvant in the lyophilization of FMDV was H. Miehler,* who observed a definite stabilizing effect by this sugar.

Results of the present experiment indicate that adding lactose and drying at low temperature are useful procedures in the lyophilization of FMDV. Since lactose prevents frothing during lyophilization, it can be recommended for this reason alone. However, our experiments did not solve a major problem that occurs in warm climates, namely, storage of the dried product at high ambient temperatures without significant loss of activity.

This low heat resistance may be correlated with the activity of ribonuclease and residual moisture, which we did not measure but was found by Fellowes to approximate 1% of the dried material even after 2 cycles of freeze-drying (primary plus secondary). At ambient temperature, this may permit sufficient ribonuclease activity to destroy most of the infectivity. According to Bachrach *et.al.*,[2] particles of FMDV are fragile and may readily lose their infectious nucleic acid, even under gentle chemico-physical treatment, in the laboratory. It is not surprising that the complement-fixing antigen, which consists essentially of virus-specific protein and is not affected by ribonuclease, resists freeze-drying and storage at room temperature for at least 40 months even without vacuum.[10]

Further work should be directed towards reduction of the residual moisture, which may be a difficult procedure, and chemical inhibition of ribonuclease activity in freeze-dried preparations.

It also remains to be determined whether or not freeze-drying of TC virus leads to changes in type-specificity as were observed by Michelsen and Mikkelsen[8] with lyophilized vesicular epithelium.

*H. Miehler, Munich, West Germany: Personal communication to E. Traub, 1944.

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