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Freeze-Drying of Foot-and-Mouth Disease Virus and Storage Stability of the Infectivity of Dried Virus at 4 C

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ABSTRACT

FELLOWES, O. N. (Plum Island Animal Disease Laboratory, U.S. Department of Agriculture, Greenport, N.Y.) Freeze-drying of foot-and-mouth disease virus and storage stability of the infectivity of dried virus at 4 C. Appl. Microbiol. **13**:496–499. 1965.—Foot-and-mouth disease virus, type A, strain 119, propagated in cultures of calf kidney cells and in the tongue epithelium of cattle was used. The process of freeze-drying was conducted in two cycles on unit volumes of 4 ml in Pyrex ampoules, averaging 150 ampoules per run, and was studied separately from the problems of storage. Ampoules containing freeze-dried virus were flame-sealed for either immediate study or storage at 4 C for later reference. Tissue-culture virus dried with various additives had a mean processing loss of 0.8 log LD_{50} per ml for six different preparations. Virus freeze-dried in tissue suspension had a mean loss of 0.8 log LD_{50} per ml for three different preparations. A second set of preparations was processed and specifically studied for storage quality at 4 C. The virus in 14 freeze-dried tissue-culture preparations had a mean loss of 0.75 log LD_{50} per ml while stored at 4 C for 1 year. Virus in four freeze-dried tissue suspensions had a mean loss of 0.05 log LD_{50} per ml held at 4 C for 1 year. None of the specific additives used for conservation of the virus during the freeze-drying process or during storage at 4 C contributed significantly to the stability of the virus preparations over and above that observed with the normal growth medium of the tissue culture or the ordinary diluents used in making suspensions of tissue virus.

It is sometimes desirable to freeze-dry viruses, virus vaccines, or antiviral serums to reduce the volume of the biological product for storage or shipment, to raise the usual low temperature requirements for preservation of such products, and to better the keeping qualities of the biological as compared with the same material in the liquid state. Greaves (1960) has indicated certain general principles which serve as guidance in the preservation of bacteria and viruses in the frozen and dried state. The addition of dextran or albumin to the preparation will cushion the shock of freeze-drying. The addition of sucrose will hold water content of the product at the 1% level. The inclusion of sodium glutamate to the mixture will neutralize the carbonyl groups of the proteins during storage.

This study is directed toward the preservation of foot-and-mouth disease virus (FMDV) by freeze-drying and subsequent storage for 1 year at 4 C with the least overall loss of infectivity.

MATERIALS AND METHODS

Virus. FMDV, type A, strain 119, was used. The tissue-culture virus in the 87th, 88th, and 89th passages in calf kidney cell cultures and tissue-suspension virus in the 8th, 9th, and 10th bovine passages were prepared as test viruses to be dried. The 10% suspensions of bovine epithelial tissue and the tissue-culture fluid were centrifuged at 900 × *g* so that gross particles were removed from suspension.

Supporting substances. The following materials were added singly to, or in combination with, the virus in tissue culture or in tissue suspension in the indicated concentrations: for tissue-culture virus, 5% bovine serum, 5 and 2% sucrose, 2% dextrin, 2% gelatin, 5% nonfat dried skim milk, 5 and 1% sodium glutamate; for tissue-suspension virus, Tryptose Phosphate Broth, 5% bovine serum, 5% nonfat dried skim milk, 5% sodium glutamate, LC fluid (1% lactalbumin hydrolysate, 2% bovine serum, 0.1% phenol red, and Hanks' salt solution).

Procedure. The virus was grown in calf kidney cell cultures in Povitsky bottles and harvested as whole culture; after centrifugation at 900 × *g* for 20 min, 250 units of penicillin G and 250 μg of dihydrostreptomycin per ml were added to the cultures. Infected bovine tongue epithelium was ground in a mortar, and diluents, as described above, were added. The supernatant fluid of this

suspension, after centrifugation at 900 × *g* for 20 min, became the source of tissue-suspension virus and was considered a 10% suspension. Antibiotics were added, as above, to the tissue-culture virus. Additives or diluents were prepared for addition to the basic virus preparation by filtration or steam sterilization, where appropriate. Mixtures were made with regard for volume so that percentage relationships of mixtures were as described above. Individual preparations of virus and additive(s) were made, adjusted to *p*H 7.5, and distributed in 4-ml volumes into 10-ml Pyrex ampoules. The ampoules were plugged with loose cotton pledgets. These containers were placed in the centrifuge compartment of an Edward's freeze-dry unit (model 3 P.S./A.; W. Edward and Co., Ltd., London, England). With the condenser temperature at −50 C, the centrifuge was started, and the compartment was evacuated to a partial air pressure not exceeding 120 μ in 20 min. This treatment enabled the virus preparation in the ampoules to freeze in a slanted position, presenting a large surface for drying. The primary cycle of desiccation was continued for 24 hr at a partial air pressure not exceeding 80 μ.

After 24 hr, the ampoules were removed from the drying chamber and attached to the secondary drying manifolds on the back of the machine. Connections between ampoules and manifolds were accomplished by means of rubber nipples. Drying was continued under a partial air pressure of not more than 40 to 50 μ at room temperature (23 C) over a large tray of P_2O_5 in the main manifold. The secondary cycle of the drying lasted 24 hr. All ampoules were checked on the nipples after flame-sealing with a high frequency induction coil to detect leaks. Satisfactory ampoules were removed for immediate testing of contents or for storage at 4 C. No additional heat input was employed to hasten the drying. The residual moisture content of the final product was determined by loss of weight of a 1-g sample in a weighing bottle in a vacuum desiccator over fresh P_2O_5 at a partial pressure of not greater than 50 μ of mercury at room temperature and to a constant weight. Three samples were used for moisture determination.

Virus assay. Virus assay was primarily concerned with the virus concentration in a preparation before and after treatment. However, other assays were made during the period of processing the virus to a dry state and during the year of storage at 4 C. It is known that all such type assays do not follow a straight line whether virus or vaccine is being tested, as most recently shown by Randall, Binn, and Harrison (1964). Some occasional values out of line were found in this study, and were considered examples of uneven drying if the virus assay at the time interval before and after was in line. The process of drying virus was repeated three times with different preparations of the same materials described above. Materials for storage at 4 C were prepared four times and observed for 1 year in each instance. The contents of an ampoule were restored to the liquid state by the addition of 4 ml of distilled water. Appropriate 10-fold dilutions were made of each sample in LC fluid medium. Mice, 7 to 9 days old, were each inoculated intraperitoneally with 0.05 ml; 10 mice were used per dilution. The LD_{50} values were calculated by the method of Reed and Muench (1938).

TABLE 1. *Survival of virus during freeze-drying process*

Form of virus* and additive	Mouse LD_{50} per ml		
	Original mixture	After primary drying	After secondary drying
TC + no additive	8.0†	7.3	7.1
TC + 5% sucrose	7.8	6.3	7.3
TC + 2% gelatin	7.8	6.8	6.5
TC + 2% dextrin	7.9	6.1	6.6
TC + 2% sucrose	7.5	6.9	7.2
TC + 5% dry skim milk	7.7	7.5	7.1
TS + Tryptose Phosphate Broth	5.6	5.4	5.4
TS + LC fluid	6.2	6.0	5.5
TS + 5% dry skim milk	6.0	5.0	4.5

* TC = tissue culture; TS = tissue suspension.
† Logarithm to the base 10.

RESULTS

Twenty different preparations of tissue-culture virus, with and without additives, and seven preparations of tissue-suspension virus, with additives or diluents, were studied in either the freeze-drying process or in storage at 4 C for 1 year. The mean loss in virus concentration in six tissue-culture preparations, while being frozen and dried, was 0.8 log mouse LD_{50} per ml; under the same conditions, three tissue-suspension preparations of virus had a mean loss of 0.8 log mouse LD_{50} per ml (Table 1).

During storage of the virus for 1 year at 4 C, 14 freeze-dried tissue-culture virus preparations had a mean virus loss of 0.75 log mouse LD_{50} per ml (Table 2). The four freeze-dried preparations of virus in tissue suspension had a storage loss of 0.05 log mouse LD_{50} per ml. The reference point for virus stored at 4 C was the titer "before storage" and "after freeze-drying."

In summary, it is apparent that no special additive contributed more than any other to the preservation of the virus during the freeze-drying process and subsequent storage at 4 C for 1 year. In fact, the culture medium in which the infected calf kidney cells were grown or the ordinary diluents used in the preparation of tissue-virus suspensions were as effective as the special additives. Residual moisture in the product at the end of the freeze-drying process was found to be 1% in most instances.

TABLE 2. *Survival of FMDV after freeze-drying and storage at 4 C*

Form of virus* and additive	Mouse LD_{50} per ml†								
	Before storage	Days of storage							
		30	60	90	120	180	240	300	360
TC + no additive	7.2	6.7	6.9	6.6	5.7	6.2	6.5	6.7	6.7
TC + 5% sucrose	7.1	6.7	5.6	6.4	6.6	5.8	5.7	5.8	6.3
TC + 2% gelatin	6.6	7.0	6.4	6.1	6.5	—	5.6	—	6.2
TC + 5% dry skim milk	7.3	5.6	5.4	7.0	7.5	7.6	7.7	—	7.2
TC + 2% dextrin	6.9	5.9	5.4	5.6	5.7	5.3	4.7	—	5.0
TC + 5% sucrose + 1% glutamate	7.2	7.1	6.7	6.8	6.7	—	—	6.2	6.0
TC + 2% gelatin + 5% glutamate	6.7	7.3	6.7	6.3	6.5	—	—	5.0	5.8
TC + 5% dry skim milk + 5% glutamate	7.3	7.3	6.5	6.2	6.3	—	—	6.5	6.5
TC + 2% dextrin + 5% glutamate	6.8	7.2	7.0	7.1	6.9	—	—	6.5	6.1
TC + 5% bovine serum + 5% sucrose + 1% glutamate	7.3	6.8	7.1	6.8	7.0	5.9	—	5.8	6.0
TC + 5% bovine serum + 5% sucrose	6.9	7.0	6.8	6.8	6.1	5.8	—	5.9	6.2
TC + 5% bovine serum + 1% glutamate	6.9	7.5	7.1	6.9	7.1	5.9	—	6.9	6.3
TC + 5% bovine serum	6.9	6.8	6.7	6.5	5.8	6.4	—	6.3	6.5
TC + 1% glutamate	7.0	7.0	6.6	6.8	6.6	6.8	—	6.8	6.8
TS + Tryptose Phosphate Broth	4.7	4.5	4.0	3.8	2.9	—	3.5	4.0	4.6
TS + LC fluid	4.9	3.0	3.8	4.5	5.0	4.5	4.8	—	5.0
TS + LC fluid	3.6	3.8	—	3.5	—	—	—	3.5	3.5
TS + 50% bovine serum + 5% glutamate	3.6	5.3	3.9	4.5	4.8	—	—	4.4	4.1

* TC = tissue culture; TS = tissue suspension.
† Logarithm to the base 10; — = not done.

DISCUSSION

From the data presented, it can be seen that FMDV in both tissue culture or in tissue suspension was resistant to loss of activity by freezing and drying by storage for 1 year. The fact that the infectivity of the preparations was retained as well as it was indicated that the conditions of drying for the volume of virus used, such as vacuum, temperature of condenser and drying chamber, and length of drying period, were about optimal.

It may be noted that tissue-culture virus in its own growth medium, without additional support, appeared to freeze-dry and store well at 4 C. Therefore, proteins or carbohydrates are not needed in the various viral preparations being dried and stored. The possibility exists that the conditions of preservation described in this study were most conducive to a small loss of infectivity of the virus involved. A change in any of the processing or storage conditions might introduce an increased loss of infectivity in the protected preparations and a great loss of infectivity in the unprotected biological material. It is, therefore, better to protect the biological's activity where unwanted or unanticipated changes in the process of freeze-drying or in storage may harm the product.

At this time, there is no indication of what special additive would be the best for the stated purpose of preservation, since none of those tested contributed more than the simple medium and diluents described. By the methods of assessment of titer or concentration of virus in the samples employed in this study, two end points would have to differ by more than 1 log of viral activity to be significant. This difference is not apparent between any of the preparations with or without specific additives.

Relatively few investigators have recorded their experiences with freeze-drying of FMDV. Sergeev (1958) attempted to freeze-dry FMDV in a tissue suspension of infected mouse muscle. He felt that egg yolk, defatted cow's milk, or

gelatin plus sucrose aided the survival of the virus in processing and helped in subsequent long-term storage. However, the virus in storage lost 2 to 3 logs of infectivity during observation for 1 year, and the unsupported virus control was not recoverable at the 6-month level. It appeared that virus in mouse muscle was not very stable when freeze-dried and stored under the described conditions. There were no details of the freeze-drying process given. In other instances, Ludwig (1961, 1964) reported studies on freeze-drying and storage of FMDV. She used natural virus (infected tongue epithelium) suspended in phosphate buffer, albumin solution, skim milk, horse serum, and horse meat bouillon. After processing and storage, no differences in preserving or stabilizing ability were noted among the various supporting additives to the virus. Under proper conditions of storage, which included no loss of vacuum, the virus was 100% stable at 4 and 23 C for 4.5 years. No changes in characteristics of virus were observed due to lyophilization. Ceccarelli (1960), in freeze-drying FMDV cultivated in bovine kidney cells, found that storage of the dried product was accompanied by survival of the virus at 5 C for 4 months and at −79 C for 8 months. No other information was given.

It is highly desirable that the details of the freeze-drying process be given when reporting its use in the preservation of various microorganisms.

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