Preservation of Enteroviruses by Freeze-Drying

TRYGVE O. BERGE, RONALD L. JEWETT, AND WILLIAM O. BLAIR
American Type Culture Collection, Rockville, Maryland 20852

Received for publication 16 August 1971

A method was developed for freeze-dry stabilization of poliovirus type 3. An ultrafiltration procedure was used to remove salts from infected tissue culture fluid, and the virus was freeze-dried after suspension in an alkaline organic buffer. This method was further tested with other picornaviruses including poliovirus types 1 and 2, coxsackieviruses A9, A20, B2, and B5, echovirus 11, and the encephalomyocarditis virus. Freeze-dried preparations of the poliovirus could be shipped to distant laboratories at ambient temperature with excellent retention of infectivity. Data are presented showing effects of freeze-drying as well as results of exposure to temperatures to 37 C.

The preservation of enteroviruses by freeze-drying has had little application because of generally unsuccessful attempts using empirical methods. Picornaviruses, with the exception of the rhinoviruses, have been found not to be easily preserved by lyophilization, with most of the original infectivity lost through this procedure (1). George and Gharpure (3) reported that lyophilization of coxsackievirus B4-infected muscle suspension and echovirus 7-infected tissue culture fluid resulted in 98.4 to 99% reduction in infectivity. Hammon (4) stated that laboratory evidence indicated inactivation of poliovirus after drying. Kraft and Pollard (5) found that poliovirus type 2-infected mouse brain tissue could be dried in the cold in various media with survival of up to one-third of the virus activity. However, their data indicated that residual infectivity of the dried virus preparation decreased sharply when the materials were held for as little as 20 min at temperatures of 30 C or higher.

At the American Type Culture Collection (ATCC), preservation of viruses and other biologic agents by freeze-drying, where it can be done successfully, is preferred to maintenance in the frozen state because of the greater convenience of handling and storage of dried cultures and the lower shipping costs involved in their distribution. This is an important consideration where enteroviruses comprise approximately 20% of more than 500 animal viruses maintained by ATCC, with distribution made to all parts of the world.

In a search for an effective stabilizer before the presently reported method was developed, many additives were tested for their effect on retention of infectivity of the tissue culture virus in the freeze-drying process. Bovine serum albumin (BSA), which has been used regularly in concentrations of 4.0 to 7.5% as a protective compound in the lyophilization of arbovirus preparations, was detrimental to poliovirus at a concentration of 1.0%. Poliovirus freeze-dried in the presence of 4.0% BSA was completely inactivated. Addition of cryoprotective agents including high- and low-molecular-weight dextrins (2 or 5%), polyvinylpyrrolidone (2, 5, or 10%), polyethylene glycol (2%), or dimethyl sulfoxide (2 or 5%) resulted in complete loss of poliovirus infectivity during the freeze-drying process.

The incorporation of 10% skim milk in the medium before drying permitted some survival of virus, although more than 99.9% of the infectivity was lost. Greater retention of infectivity was found when any of a series of sugars or organic salts was added to the preparation. These included glucose, sucrose, lactose, the sucrose PG additive of Bovarnick et al. (2), mannitol, inositol, sorbitol, sodium glycerophosphate, sodium glutamate, and calcium lactobionate. Sucrose PG or 10% lactose protected best against the adverse effects of drying, but even these permitted a 90% or greater loss in infectivity (unpublished data).

An additional problem, apparently not directly related to the freeze-drying process, was lack of stability of residual virus infectivity when the dried product was stored at temperatures higher than 4 C, regardless of additive employed. Pretreatment of the poliovirus-infected tissue culture fluid by dialysis against distilled water or 1 M tris(hydroxymethyl)aminomethane (Tris) buffer, or by removal of electrolytes and other low-molecular-weight substances by ultrafiltration, resulted in marked increase in virus stability after drying. The method reported here permits the
freeze-drying of some enteroviruses with minimal loss of infectivity and good stabilization of the dried virus at ordinary storage temperatures.

MATERIALS AND METHODS

Preparation of virus pools. Poliovirus type 3 (Saukett) was obtained from the Center for Disease Control and propagated in KB cells (ATCC CCL 17) at 37°C by using Eagle's minimal essential medium (MEM) with 2% fetal bovine serum (FBS). Identity of the virus was confirmed in tissue culture neutralization tests with specific antiserum received from the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases. Other viruses were from ATCC stocks. Poliovirus type 1 (VR 192, Chat) and type 2 (VR 61, MEF 1); coxsackievirus A9 (VR 186, P.B.), B2 (VR 29, Ohio 1), and B5 (VR 185, Faulkner); and echovirus 11 (VR 41, Gregory) were grown in LLC-MK₂ cells (CCL 7) with Melnick's B medium. Coxsackievirus A20 (VR 180, I.H.) was grown in a cloned derivative of Chang conjunctiva cells (CCL 20.2) by using MEM with 10% FBS. Encephalomyocarditis virus (VR 129, EMC) was grown in BHK-21 cells (CCL 10) by using Leibovitz-15 medium with 2% FBS. All infected tissue culture pools were frozen and thawed twice, centrifuged at 3,000 rev/min for 15 min at 5°C, dispensed in small portions, and stored frozen at -60°C until tested.

Organic buffer. Tris buffer (1 M) was made up to 80% of total volume in triple-distilled water. The pH was adjusted to 8.5 by carefully adding concentrated, reagent grade HCl. Triple-distilled water was added to bring the solution to final volume, and the solution was sterilized by filtration through a membrane filter (Millipore Corp.).

Ultrafiltration. Removal of salts from the virus pool was accomplished by ultrafiltration through a PM-30 membrane in a Diaflo model 52 cell (Amicon Corp.) under 50 psi positive pressure. The membrane and ultrafiltration cell were rinsed by forcing triple-distilled water through the membrane. Virus was resuspended to original volume in Tris (pH 8.5), dispensed in 0.5-ml amounts in ampoules or serum bottles, and shell- or plug-frozen in an alcohol-dry ice bath.

Drying cycle. Two different freeze-drying machines were used; both gave comparable results. One was an American Sterilizer freeze-dryer model L-3 on which the shelf temperature was lowered to -40 to -55°C before serum bottles containing frozen virus material were loaded into the chamber. After a high vacuum was established, refrigeration was turned off and the samples were allowed to come slowly to ambient temperature (20 to 22°C) during the drying cycle. The bottles were sealed with nontoxic butyl rubber stoppers under high vacuum. The second machine was a Virtis modified horizontal manifold type with an alcohol-dry ice moisture trap and refrigerated alcohol circulated through troughs for control of product temperature. The initial temperature of the alcohol circulating in troughs bathing the ampoules was -30 to -40°C. The system was evacuated with a Precision 300 double stage vacuum pump. After a high vacuum was obtained, the refrigerating unit was turned off, allowing the temperature to rise slowly to 20 to 22°C. The ampoules were flame-sealed at atmospheric pressure after filling with dry nitrogen gas. Total drying time with both machines was 24 hr.

Conditions of test and assays. With few exceptions, at least two separate lots of each virus preparation were dried and tested with results reported as geometric mean titers. In cases where duplicate tests did not show agreement, additional lots were tested.

Dried samples were rehydrated using 0.5 ml of chilled, triple-distilled water. The rehydrated material was further diluted in serial 10-fold dilutions in the appropriate medium for the cell system being used in assays. Titers of samples were determined before and after freeze-drying and routinely after 5 days at 37°C. Other storage times and temperatures were occasionally tested.

All titrations were made in the tissue culture cell systems and media used for viral propagation except for poliovirus 3, which was tested in LLC-MK₂ cells maintained with Melnick's B medium. Four tubes were used per dilution, and an inoculum of 0.2 ml per tube containing 0.8 ml of maintenance medium was used. Final readings for complete cytopathic effect were made 12 days after inoculation. The median tissue culture infective dose (TCID₅₀) was calculated according to the method of Reed and Muench (6).

Shipping trials. Samples of a freeze-dried preparation of poliovirus 3 were shipped by airmail without refrigeration to a series of cooperating laboratories for testing. Upon arrival in the laboratories, specimens were tested either on the same day or after storage in an ordinary refrigerator for periods ranging from 1 to 390 days. Titrations were made in primary or secondary monkey kidney cell cultures from rhesus or cynomolgus macaques or African green monkeys, as routinely employed in the individual participating laboratories.

RESULTS AND DISCUSSION

Repeated tests showed that poliovirus type 3 preparations could be lyophilized by the method outlined with relatively little loss of infectivity in contrast to results obtained with techniques previously employed. Geometric mean titer of frozen and thawed tissue culture seed virus preparations was 10⁷.⁷ TCID₅₀ per 0.2 ml; that of the freeze-dried materials was 10⁷.⁴ TCID₅₀. A specimen stored at 4°C for 24 weeks showed no decrease in titer.

A test lot of the type 3 poliovirus, prepared as described above in October 1968, was tested in shipping trials to determine stability of the dried virus under actual conditions without refrigeration. Results are summarized in Table 1. Titer of the original virus pool was 10⁷.⁵ TCID₅₀ per 0.2 ml and 10⁷.⁴ after freeze-drying. Titters of samples tested by the receiving laboratories within 1 month after arrival were reported to range from 10⁶.₃ to 10⁷.⁵. A single specimen stored in a refrigerator for 390 days after receipt was found
TABLE 1. Tissue culture infectivity of freeze-dried preparations of poliovirus type 3 after shipment at ambient temperature to designated laboratories

| Testing laboratory | No. of days en route | No. of days stored at 4 to 10 C | Test system<sup>a</sup> | TCD<sub>50</sub> (log 10) |
|--------------------|----------------------|--------------------------------|------------------------|--------------------------|
| Tokyo, Japan; M. Kitaoka | 5                   | 26                             | Pr. cyno. MkK          | 7.0/0.2 ml               |
|                      |                     | 26                             | Pr. A.G. MkK           | 7.3/0.2 ml               |
| Vancouver, B.C.; D. M. McLean | 3               | 1                              | Pr. cyno. MkK          | 7.3                      |
|                      |                     | 390                            | Pr. Rh MkK             | 6.5/0.1 ml               |
| Berkeley, Calif.; N. J. Schmidt | 1            |                                | Pr. Rh MkK             | 6.5/0.1 ml               |
| Houston, Tex.; J. L. Melnick | 2               | 0                              | Pr. A.G. MkK           | 7.2/0.1 ml               |
| Kansas City, Kan.; P. S. Kamitsuka | 2              | 16                             | Pr. MkK                | 7.5/0.1 ml               |

<sup>a</sup> Abbreviations: pr., primary; sec., secondary; cyno., cynomolgus; MkK, macaques monkey kidney; Rh, rhesus; A.G., African green.

<sup>b</sup> Median tissue culture dose (TCD<sub>50</sub>). Prelyophilization titer, 10<sup>7.5</sup> TCD<sub>50</sub>/0.2 ml; titer after drying, 10<sup>5.9</sup> (test system LLC-Mk<sub>2</sub> cells).

TABLE 2. Infectivity titer of enteroviruses before and after freeze-drying

| ATCC VR no. | Virus     | TCD<sub>50</sub> log 10/0.2 ml | Dried virus held 5 days at 22°C | Dried virus held 5 days at 37°C |
|-------------|-----------|--------------------------------|---------------------------------|---------------------------------|
| 192         | Poliovirus 1 | 8.0                            | 7.3                              | 5.2                             |
| 61          | Poliovirus 2 | 8.5                            | 7.3                              | 5.0                             |
| 186         | Poliovirus 3 | 7.7                            | 7.4                              | 5.2                             |
| 180         | Coxsackie A9 | 6.5                            | 3.8                              | 2.1                             |
| 180A        | Coxsackie A20 | 8.3                          | 7.1                              | 5.6                             |
| 29          | Coxsackie B2 | 5.5                            | 5.0                              | 4.0                             |
| 185         | Coxsackie B5 | 8.0                            | 7.0                              | 4.3                             |
| 41          | Echo 11     | 7.0                            | 5.7                              | 3.3                             |
| 129         | Encephalomyocarditis | 6.8                       | 5.3                              | 3.7                             |

<sup>a</sup> Median tissue culture dose.

to titer 10<sup>6.5</sup> at one of the laboratories. These results indicate that such dried virus preparations can be shipped successfully at ordinary ambient temperatures to any geographic area with sufficient retention of infectivity to ensure recovery of virus.

When the above method was applied to the freeze-dry preservation of eight additional enteroviruses, results were considered to be generally satisfactory except for coxsackievirus A9, which lost 2.7 log<sub>10</sub> TCD<sub>50</sub> upon drying (Table 2). In all instances, virus could be recovered from specimens held at 37°C for 5 days in accelerated deterioration tests. This temperature was chosen in part to simulate adverse conditions which might be encountered in tropical areas during shipment, but chiefly because residual infectivity of enteroviruses freeze-dried by other techniques was rapidly destroyed at this temperature and period of time.

Loss of infectivity at 37°C of the virus preparations dried by usual techniques was attributed to the presence of inorganic salts in the original tissue culture medium. When the infected tissue culture fluid was diluted 1,000-fold with distilled water to reduce concentration of compounds contained in the medium, that virus infectivity which remained after freeze-drying showed greater stability at 37°C than did the undiluted product. The same effect was found without concomitant reduction in virus titer imposed by dilution when the virus suspension was dialyzed against either cold Tris or distilled water prior to drying. Removal of salts was simplified by Diaflo filtration as described.

Tris buffer (1 M) provided better protection against loss of virus activity during the drying cycle than did any other material tested. Lower concentrations of Tris were less effective. The pH of Tris-virus mixture appeared to have little influence on virus infectivity retained during freeze-drying over the range of pH 7.5 through 10.0. Residual infectivity, however, showed greatest stability at 37°C in the products dried at pH 8.5 to 9.0, with 8.5 giving the most consistent protective effect.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI 07318 from the National Institute of Allergy and Infectious Diseases and contract no. PH 43-68-675.
LITERATURE CITED

1. Andrewes, C., and H. G. Pereira. 1967. Viruses of vertebrates, 2nd ed. The Williams & Wilkins Co., Baltimore, Md.

2. Bovarnick, M. R., J. C. Miller, and J. C. Snyder. 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. 59:509-522.

3. George, S., and P. V. Gharpure. 1964. Lyophilization of enteroviruses. Indian J. Pathol. Bacteriol. 1:46-53.

4. Hammon, W. M. 1954. In F. W. Hartman, F. L. Horsfall, and J. G. Kidd (ed.), The dynamics of virus and rickettsial infections, p. 188. Blakiston, New York.

5. Kraft, L. M., and E. C. Pollard. 1954. Lyophilization of poliomyelitis virus. Heat inactivation of dry MEF 1 virus. Proc. Soc. Exp. Biol. Med. 86:306-309.

6. Reed, L. J., and H. Muench. 1938. Simplified method of estimating fifty per cent endpoints. Amer. J. Hyg. 27:493-497.