Storage of Purified Plant Viruses in the Unfrozen State Free of Microbial Contamination

This communication describes a method of storage of purified plant viruses which precludes microbial growth and which preserves the virus in an unaltered state when judged by the criteria of electron microscopy, density gradient centrifugation and infectivity. Although similar to the filtration method recently described by PERRY and VINCENT[1] for animal viruses, the method described herein differs in that an evacuated tube is utilized in the filtration process and for storage of the virus.

Materials and methods. The viruses included in this study were alfalfa mosaic virus (AMV) and tobacco mosaic virus (TMV). The AMV was purified, subjected to density gradient centrifugation and assayed for infectivity as described previously[2]. The TMV was purified by a modification[3] of the celite-charcoal method of STEERE[4] and subjected to density gradient centrifugation and assayed for infectivity as previously described[3].

The AMV was suspended in 0.01 *M* phosphate buffer, pH 8.0, and stored for 5 weeks at 4 °C before testing. The TMV was suspended in double distilled water and in 0.02 *M* phosphate buffer, pH 7.5, and stored for 28 weeks at 4 °C before testing. Both viruses were also stored in the frozen state for comparative studies with unfrozen and freshly purified virus.

For electron microscopy the virus preparations were either sprayed onto grids followed by shadow casting with palladium metal or mixed with neutral phosphotungstic acid (PTA) and pipetted onto microscope grids. All specimens were examined in the RCA EMU-3B electron microscope.

The quality of the stored virus preparations was considered to be satisfactory, (a) if they were free of microbial growth, (b) if there was no increase in virus particle aggregation, (c) if there was no visible degradation of virus particles and (d) if the infectivity was maintained at a high level when compared with freshly purified virus.

Filtration and storage procedure. The suspensions of purified virus were placed in a steam sterilized glass syringe attached to a Millipore micro-syringe filter holder containing either a 0.22 μ or a 0.30 μ filter[5]. In our studies we used a 0.22 μ filter for AMV and a 0.30 μ filter for TMV. A sterile 18 guage needle was aseptically attached to the syringe with filter holder. The needle was then forced through the rubber stopper on a B–D Vacutainer blood collecting tube[6]. The surface of the rubber stopper was sterilized with 95% ethanol prior to insertion of the needle. The vacuum in the Vacutainer tube caused the virus suspensions to be drawn through the filter into the tube after which the needle was withdrawn from the rubber stopper. Use of the evacuated tube provided a sealed container for storage after filtration. The assembled apparatus is shown in Figure 1.

Precautions. To prevent filter membrane breakage with resulting contamination, the syringe apparatus should be autoclaved at a pressure of 15 p.s.i. for 20 min with slow exhaust of the autoclave. The plunger should be sterilized separate from the syringe and filter holder. After the virus suspension has been injected, and before the needle is removed from the Vacutainer tube, the plunger should be withdrawn very slowly from the syringe. Rapid removal will usually result in rupture of the filter membrane. The needle can then be removed from the rubber stopper. When working with large lots of Vacutainer tubes frequent change of the complete filter apparatus is recommended.

Results. Filtered lots of both viruses stored at 4 °C were found to be free of microbial growth when viewed macroscopically with the naked eye, when extensively examined in the electron microscope and when streaked on nutrient agar plates.

The density gradient centrifugation (DGC) studies of freshly purified preparations of both viruses when compared to stored preparations of the viruses indicated that

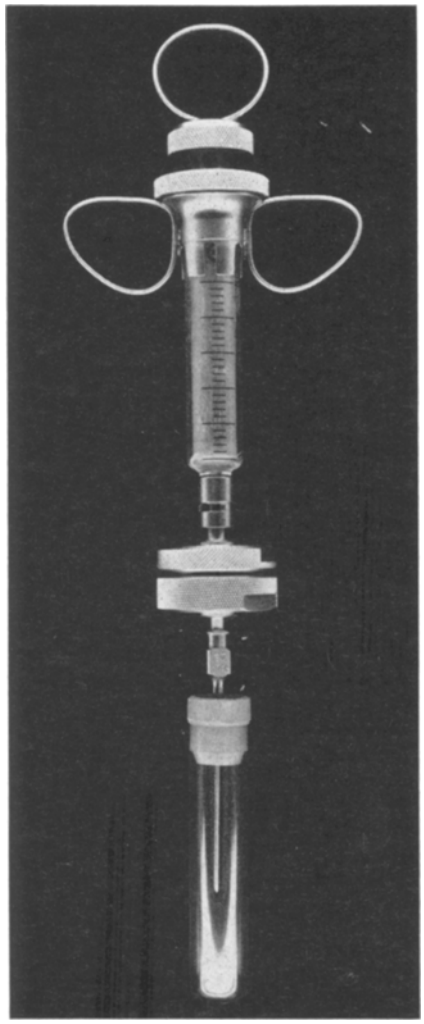

Fig. 1. Syringe with micro-syringe filter holder and needle attached and with the needle inserted into a sterile Vacutainer tube.

[1] V. P. PERRY and M. M. VINCENT, in *Methods in Virology* (Ed. K. MARAMOROSCH and H. KOPROWSKI; Academic Press, Inc., New York 1967), vol. 2, chapter 13, p. 367.

[2] P. R. DESJARDINS and R. L. STEERE, Arch. Virusforsch., in press.

[3] T. O. DIENER and P. R. DESJARDINS, Virology *29*, 15 (1966).

[4] R. L. STEERE, Science *140*, 1089 (1963).

[5] Millipore micro-syringe filter holder (Cat. No. XX30-025-000), with 0.22 or 0.30 μ pore size filter (MF type HA). Millipore Corporation, Bedford, Mass., 01730, USA (Similar syringe filters are available from the Gelman Instrument Company, Ann Arbor, Mich. USA and from the Scientific Systems Division, Amicon Corp., Lexington, Mass. USA).

[6] B-D Vacutainer blood collecting tube, 7 ml capacity (75 × 16 mm), with red stopper, Cat. No. VBD3202, Aloe Scientific, 1831 Olive St., St. Louis, Missouri USA.

no additional particle aggregation had occurred during storage. The DGC-UV scanning profiles of AMV are shown in Figure 2 and those for TMV in Figure 3. These findings were verified by the electron microscope studies.

The DGC-UV scanning profiles (Figures 2 and 3) and the electron microscope studies also indicated that there was no visible degradation of particles of both viruses when filtered and stored in the unfrozen state. The UV profile of frozen TMV in phosphate buffer (Figure 3) suggests a very slight decrease in the relative concentration of standard rod (300 nm) particles and the accumulation of a very small amount of material at the meniscus.

Both frozen and unfrozen lots of stored AMV (Table I) had a greater infectivity than freshly purified virus. It has been established[7] that the specific infectivity of this virus varies with the time of systemic infection. The specific infectivity of the virus preparation utilized for the storage studies was apparently initially higher than the particular lot of freshly purified virus used in the studies. Although the unfrozen and frozen lots of stored AMV came from the same original purified preparation, the stored, unfrozen virus was more infectious than stored, frozen virus when compared on opposite half leaves. The infectivity studies of TMV at two different concentrations (Table II) indicate that the infectivity of the stored, unfrozen virus remained at a level equal to that of freshly purified virus, which satisfies the infectivity criterion for satisfactory storage.

Discussion. Although the method described above is not necessarily recommended for prolonged periods of storage, for storage periods of intermediate length it is quite satis-

[7] C. W. KUHN and J. B. BANCROFT, Virology *15*, 281 (1961).

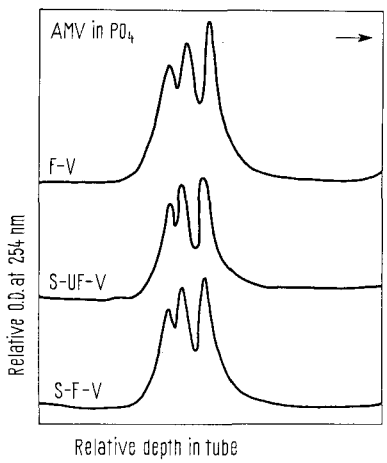

Fig. 2. Density gradient centrifugation profiles showing the 3 major components of 3 preparations of AMV. F-V denotes freshly purified virus; S-UF-V denotes stored, unfrozen virus; S-F-V denotes stored, frozen virus. The arrow indicates the direction of sedimentation. Gradient zones were scanned at 254 nm.

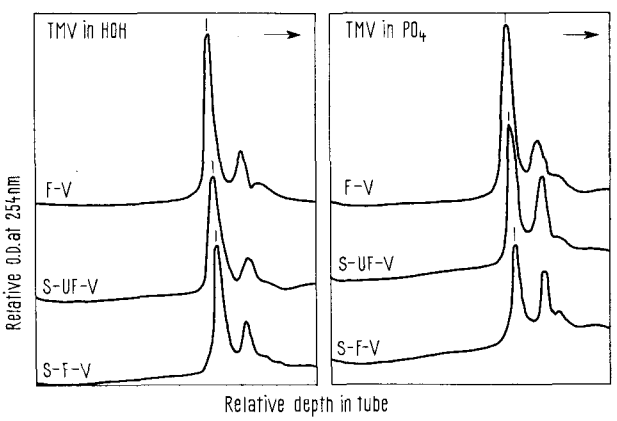

Fig. 3. Density gradient centrifugation profiles of 3 TMV preparations in water and 3 TMV preparations in PO_4 buffer. The letter designations for the individual curves are the same as in Figure 2. The vertical lines above the main peaks mark the position of 300 nm virus particles.

Table I. Infectivity of freshly purified and stored preparations of AMV on *Vigna sinensis* Endl. variety Ramshorn

Virus preparations[a]	Average number lesions per half leaf[b]	
	40 µg/ml[c]	20 µg/ml[c]
Fresh vs Stored unfrozen	21 / 85	11 / 52
Fresh vs Stored frozen	8 / 56	7 / 41
Stored unfrozen vs Stored frozen	73 / 35	43 / 18

[a] Virus preparations compared on opposite half leaves. [b] Average number of lesions on 8 half leaves of the assay plant. [c] 2 different virus concentrations used in the infectivity tests.

Table II. Infectivity of freshly purified and stored preparations of TMV on *Nicotiana tabacum* cv Xanthi-nc

Medium	Virus preparations[a]	Average number lesions per half leaf[b]	
		1 µg/ml[c]	0.2 µg/ml[c]
HOH	Fresh vs Stored unfrozen	42 / 44	45 / 55
	Fresh vs Stored frozen	77 / 72	41 / 32
PO_4 buffer	Fresh vs Stored unfrozen	71 / 95	107 / 99
	Fresh vs Stored frozen	151 / 168	66 / 68

[a] Virus preparations compared on opposite half leaves. [b] Average number of lesions on 10–14 half leaves of the assay plant. [c] 2 different virus concentrations used in the infectivity tests.

factory for the two viruses studied. The method should be useful when it is desirable to use a single preparation of purified virus for a series of experimental studies and especially useful when freezing is undesirable[8]. For any particular virus one would probably have to determine the optimum pH and ionic strength of the storage medium before the method could be successfully used.

Résumé. On décrit une méthode d'accumulation des virus végétaux. Elle ne comprend pas la congélation ou la conservation chimique. La contamination microbienne est évitée par filtration à travers un microfiltre aseptique placé dans un réservoir également aseptique.

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Department of Plant Pathology, University of California, Riverside (California 92502, USA),
5 November 1968.

[8] P. R. Desjardins and T. O. Diener, Phytopath. *57*, 809 (1967).

Methylated Cellophan Membranes for Possible Use in Multi-Membrane Electrodecantation

In multi-membrane electrodecantation[1], cellophan membranes spaced 1 mm apart are inserted in the separation chambers of the apparatus. This arrangement reduces the horizontal distance of migration of a protein before it meets a barrier. On this obstruction the protein concentrates and slides to the lower region of the vessel. The protein compound which is isoelectric at the pH of operation of the electrodecantator remains stationary and may be separated from the migrating fractions after completion of the experiment. If electrodecantation is conducted for long periods, concentration of the isoelectric component may occur in the lower regions of the separation cells. This may be due to one of two reasons. The pH of the buffer in which the protein mixture is contained may not be exactly isoelectric for the component to be purified, or, the buffer in the separation sections of the electrodecantator moves on account of electroendosmosis of the buffer through the cellophan membranes. At very low ionic strength this movement of the fluid is very noticeable. The electroendosmosis is due to the Helmholtz double layer effect in the pores of the cellophan. This migration of the electrolyte may be reduced by the addition of small amounts of Mg ions to the buffer. The divalent ions have a suppressing effect on the zeta potential in the double layer. Unfortunately divalent ions are insoluble on the alkaline side of neutrality and consequently will have no effect on the zeta potential.

As the major zeta potential in cellophan is due to dissociation of carboxyl groups which formed by oxidation during the manufacture of the membrane it follows that if these groups could be eliminated the zeta potential would be greatly diminished and the electroendosmosis in the membranes would be reduced to a minimum. An effective means of elimination of the carboxyl groups is to methylate the membranes prior to trimming them to the correct size for the electrodecantator.

The methylation is done by washing traces of glycerine from the surface of the cellophan followed by thorough dehydration in absolute alcohol. The remaining ethanol is removed with dry methanol. The membrane is then submerged in a mixture of dry methanol containing 4% by weight of dry HCl vapour. They are left in this mixture for 4 days at room temperature after which they are washed free of the methanol-HCl mixture. The methylation of the carboxyl groups seems to be complete after this treatment as additional methylation with diazomethane does not appear to have any further effect.

The effectiveness of removal of the charges is semi-quantitatively assessed by the electroendosmosis which the membranes show under standardized conditions.

The membranes are clamped between the flanges attached to 2 glass tubes and a potential gradient of 15 V/cm is applied across the membrane. The electrolyte solution was 0.014 *M* NaCl and the current density was 6.6 mA/cm². Reversible Ag/AgCl electrodes are used. The migration of the salt solution through the membrane was measured in capillaries attached to the electrode vessels. After passing the current for 1 h to allow for initial temperature equilibration of the apparatus readings were taken. The results are shown in the Table.

It is evident from the Table that the electroendosmosis shown by the treated membranes is much less than that shown by the untreated cellophan and that the removal of the carboxyl groups is complete after the first methylation. The residual electroendosmosis is probably due to normal Helmholtz double layer effect shown by any surface due to adsorption of ions. It would not be possible to eliminate this effect.

Electroendosmosis of cellophan medium 0.014 *M* NaCl potential gradient 15 V/CM, current density (6.6 mA/cm²)

Material	Rate of migration of 0.014 *M* NaCl in ml/min
Untreated cellophan	0.0125
Cellophan methylated with methanol HCl	0.00166
Cellophan methylated with methanol HCl followed by diagomethane treatment	0.0018

[1] A. Polson, Biochim. biophys. Acta *11*, 315 (1953).