Concentration and Purification of Poliovirus by Ultrafiltration and Isopycnic Centrifugation

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A method is described by which poliovirus can be rapidly and simply concentrated by the use of a Diaflo XM-50 ultrafilter membrane. Freon-extracted ultrafilter concentrates banded in CsCl provided a 1,724-fold volumetric concentration of poliovirus. During concentration, trypsin-digested cellular material can pass through the ultrafilter membrane, thus providing a versatile means of degrading and eliminating extraneous contaminating proteins. The ultrafilter concentration system is compared with the CsCl cushion system of poliovirus concentration, and both systems are further compared by banding virus and virus capsid material in CsCl by use of isopycnic centrifugation.

The recent studies of viral structural components and of the development of vaccines have created a demand for large amounts of purified, concentrated virus. Many methods of achieving this end have been described (2, 4, 11, 12). We have developed a consistently reliable, efficient means of concentrating poliovirus by use of centrifugation and ultrafiltration. The virus concentrated by this means can be further purified by the use of isopycnic centrifugation. The advantages of this procedure include an economy of time and effort with the production of a concentrated, relatively pure virus preparation.

MATERIALS AND METHODS

Production of poliovirus. Stocks of poliovirus (type I, Mahoney) were prepared in serum and Methocel-free (Methocel HG, Dow Chemical Co., Midland, Mich.) suspension cultures of HEp-2 cells as previously described (6). The preliminary concentration of newly produced poliovirus was achieved by concentrating the infected cells during virus synthesis but before massive virus release. Approximately 10^8 HEp-2 cells from suspension cultures were infected at a multiplicity of 1 plaque-forming unit (PFU)/cell in 100 ml. After a 1.0-hr adsorption period at 36 C, infected cells were resuspended up to their original concentration of 10^8 cells/ml. After an additional 2.5-hr incubation (3.5 hr postinfection) on the gyratory shaker, cells were concentrated by centrifugation and finally were resuspended in calf serum-free minimal essential medium for suspension cultures (MEM-S, Grand Island Biological Co., Grand Island, N.Y.) at a 10:1 volume reduction. The infected cells were replaced on the gyratory shaker and were harvested at 14 hr postinfection. The cells were frozen and thawed three times, and the fluid was clarified by centrifugation at 16,300 x g for 10 min in a Sorvall GSA rotor at 4 C.

When tritium-labeled virus was needed, cells were infected as described above and concentrated at 2.5 hr postinfection by a 10:1 volume reduction in phenol red and serum-free MEM-S medium. At this time, the infected-cell suspension received 1.28 mCi of tritide-6-\(^{3}H\)/ml (10^7 cells; specific activity, 28 Ci/m mole; Schwarz BioResearch, Inc., Orangeburg, N.Y.). At 14 hr postinfection, the infected cells were harvested by centrifugation and treated as described above to obtain a clarified virus suspension. Unincorporated \(^{3}H\)-uridine was removed by dialyzing the virus preparation for 24 hr against two changes of 0.01 m tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.2.

Fluorocarbon extraction of poliovirus. After preliminary concentration by volume reduction, the poliovirus suspension was mixed with an equal volume of the fluorocarbon Freon 113 (duPont de Nemours & Co., Inc., Wilmington, Del.) to remove nonviral proteins and the remaining cellular debris (10). The mixture was homogenized for 3 min in a Waring Blendor and was centrifuged at 4,080 x g in a Sorvall GSA rotor for 10 min. The supernatant fraction was collected, and the pelleted material was re-extracted with approximately 10 ml of 0.01 m Tris buffer (pH 7.2). All supernatant fractions from the above extractions were pooled and used for further concentration and purification of the poliovirus preparation.

Ultrafiltration and ultracentrifugation concentration. Freon 113-extracted virus suspensions were further concentrated either by ultrafiltration or by ultracentrifugation onto a dense cushion of cesium
chloride. Ultrafiltration was accomplished by forcing the Freon 113-extracted virus suspension with 15 psi of nitrogen gas against an XM-50 Diaflo ultrafilter membrane (Amicon Corp., Lexington, Mass.) mounted in an Amicon 200-ml ultrafiltration cell equipped with a magnetic stirring bar and held at 4 C. By this procedure, 100 ml of Freon 113-extracted virus-containing material could be concentrated to a 10-ml volume within 4 hr.

Freon 113-prepared virus was also concentrated by centrifugation of 33-ml amounts at 27,000 rev/min (131,000 x g, maximum) in a Beckman SW 27 rotor (2.5 by 8.9 cm tubes) for 2 hr onto a 5-ml 44% (w/w) cesium chloride cushion (density, 1.486 g/cc).

**Isopycnic centrifugation.** Further concentration and purification of the poliovirus preparations were accomplished by isopycnic centrifugation (5). A 4.6-ml portion was removed from the ultrafiltrate concentrate or from the cesium chloride cushion and was mixed with cesium chloride to give a total concentration of 2.38 g per 5 ml of solution. The mixture was placed into a cellulose nitrate tube (5 by 1.3 cm), overlaid with mineral oil, and centrifuged in a Beckman SW 39 rotor at 35,000 rev/min (99,972 x g, average) for 22 hr.

**Fraction collection and gradient analysis.** Gradients were harvested by collecting fractions from a hole in the bottom of the tube, and the flow rate was controlled by pumping mineral oil onto the top of the gradient with a Buchler peristaltic pump. Twenty-five to thirty fractions were collected from each 5-ml gradient, and four criteria were used in the analysis of each fraction. For gravimetric density measurements, a 100-μl sample was removed from each fraction with a lambda pipette. This sample was then used to determine the number of poliovirus PFU in each fraction. Another 100-μl sample was removed from each fraction, diluted 1: 40 in 0.01 M Tris buffer (pH 7.2), and measured for ultraviolet absorption at 280 and 260 nm in a Gilford model 2400 spectrophotometer read against a distilled-water blank. A 10-μl sample was also removed from each fraction, added to 10 ml of Bray's solution, and analyzed for the incorporation of H-uridine by use of a Packard Tri-Carb model 3375 liquid scintillation spectrometer.

On some occasions, crude virus concentrates were treated with 0.25% trypsin for 1 hr at 37 C before Diaflo ultrafiltration. Degraded proteins not associated with the complete virion, trypsin, and trypsin cleavage products were removed from the concentrate by ultrafiltration. Lowry protein determinations (8) were made on samples from the various steps described.

**RESULTS**

**Ultrafiltration versus ultracentrifugation.** Figure 1 displays a flow diagram of the virus concentration and purification processes described above. Total recovered virus in the ultrafilter concentrate was somewhat greater than in the cushioned virus preparation (Table 1).

Some difficulty with precipitation was encountered when CsCl was added to the Diaflo ultrafilter concentrate preparatory to the isopycnic centrifugation. It was found that the Methocel and calf serum components of the suspension culture medium were unable to pass through the Diaflo XM-50 membrane and were concentrated along with the virus. Subsequently, calf serum and Methocel were not used during the production of virus for concentration.

Typical banding patterns of virus obtained from isopycnic centrifugation of an ultrafilter concentrate and of virus concentrated by sedimenting on the CsCl cushion are shown in Fig. 2. In addition to the virus band (V), a lighter band appeared in the material prepared by ultrafiltration. This lighter band (labeled C) had a buoyant density of 1.31 g/cc. This density falls within the range of values previously reported for the poliovirus procapsid (1, 7).

Analysis of the gradients is shown in Fig. 3. Each fraction harvested from the gradient was measured for density, ultraviolet absorption at 280 and 280 nm, number of PFU, and amount of tritium-labeled virus. Maximal activities were found in fractions 14 and 15, which corresponded with the position of the center band (V) in the tubes shown in Fig. 2. The average density of the fractions within this peak was 1.34 g/cc, which is the density of poliovirus previously reported by others (3, 7).

As an index of purification, the amount of protein at the various steps in the concentration procedure (Table 1) was measured by the Lowry method (8). The PFU relative to protein concentration after isopycnic centrifugation was twofold greater from the preparation concentrated by ultrafiltration than from the preparation concentrated by the cushion method. In addition, the efficiencies of concentration (the ratio of the total PFU recovered in the isopycnic band to the total PFU in the original cell culture) of the Diaflo ultrafiltration method as compared with the ultracentrifugation cushion method were 2.95 and 1.59, respectively.

**Trypsin treatment prior to ultrafiltration.** One advantage of the ultrafiltration method of virus concentration is that degradation and subsequent removal of the cellular products through the membrane can be accomplished. This was demonstrated through the use of trypsin (molecular weight, 24,000), which after use can freely pass through the Diaflo XM-50 ultrafilter membrane and thus facilitate the removal of protein contaminants. The band near the top of the gradient in Fig. 4 (K) was
Cells concentrated to 200 ml by centrifugation at 600 × g for 10 min at 3.5 hr postinfection; incubation continued at 36°C.

Freeze-thaw three times at 14 hr postinfection.

Centrifuge at 16,300 × g for 10 min.

Supernatant fluid (discard)

Supernatant, 200 ml

Pellet (discard)

Freeze-thaw three times at 14 hr 16,300 × g postinfection for 10 min.

Centrifuge at 4,080 × g for 10 min.

Supernatant, 200 ml

Freon extraction and centrifugation at 4,080 × g for 10 min.

Aqueous phase, 200 ml

Freon phase (discard)

Ultrafiltration (Diaflo XM-50 filter) of 100 ml

Ultrafiltrate (discard)

Concentrate (10 ml)

Cell cushion (combined), 16 ml

Supermatant (discard)

Ultracentrifugation of 100 ml onto three 5-ml CsCl cushions, 131,000 × g (maximum).

Cell cushion (combined), 16 ml

Supermatant (discard)

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TABLE 1. Purification and concentration of poliovirus

| Step                                | Diaflo prepn |              | Cushion prepn |              |
|-------------------------------------|--------------|--------------|---------------|--------------|
|                                     | Vol (ml)     | PFU/100 μl      | Counts per min per 100 μl | PFU/mg of protein | Purification factor* | Vol (ml) | PFU/100 μl      | Counts per min per 100 μl | PFU/mg of protein | Purification factor* |
| Cell infection...                   | 1,000        | 8.64 × 10^4    | 9,611          | —             | 1               | 1,000        | 8.64 × 10^4    | 9,611          | —             | 1               |
| Infected-cell concentration...      | 100          | 8.66 × 10^7    | 6,959          | 2.13 × 10^4   | 1               | 100          | 8.66 × 10^7    | 6,959          | 2.13 × 10^4   | 1               |
| Freon 113 extraction...            | 100          | 2.93 × 10^7    | 4,388          | 1.08 × 10^4   | 0.507           | 100          | 2.93 × 10^7    | 4,388          | 1.08 × 10^4   | 0.507           |
| Secondary concentration by ultrafiltration... | 16       | 5.19 × 10^6    | 15,205         | 1.85 × 10^14  | 86.8            | 32           | 1.62 × 10^6    | 7,990          | 1.4 × 10^14  | 65              |
| Equilibrium density gradient centrifugation... | 0.58       | 4.4 × 10^15    | 23,426         | 1.57 × 10^11  | 737             | 1.16         | 1.19 × 10^15   | 23,398         | 7.4 × 10^15  | 347             |

* The purification factor is based on increase in PFU per milligram protein over crude culture lysate.

The volume represents the pooled peak fraction (0.166 ml) from several gradient tubes (i.e., the number of gradients it would take to centrifuge the entire 16 or 32 ml when 4.6 ml is used for each gradient).

Another advantage of the Diaflo system was demonstrated by monitoring levels of 3H-uridine removed by the trypsin treatment (Fig. 4, tryp). This is indicative that the trypsin and its cleavage products were small enough to pass through the ultrafilter membrane. Although 0.25% trypsin did not alter the infectivity of poliovirus (unpublished data), free viral capsid proteins are susceptible to its action (9, 13). This explains the complete disappearance of the top band after trypsin treatment (Fig. 4).
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FIG. 2. Isopycnic banding patterns in CsCl of the products from ultrafiltration and cesium chloride cushioning of poliovirus preparations after Freon 113 extraction. UF, ultrafiltration; CsCl, cesium chloride cushion; V, virus; and C, poliovirus capsid material. Centrifugation at 99,972 x g (average) for 22 hr in a Beckman SW 39 rotor as described in the text.

dine before and after ultrafiltration and washing (dialysis) of the ultrafiltration concentrate. Through this means, it was possible to show that the efficiency of removal of low-molecular-weight material was greater (52% removal) by Diaflo ultrafiltration than by ordinary dialysis (49.2% removal). Removal of this excess label in the Diaflo preparation was accomplished by passing 1 liter of 0.01 M Tris buffer over the concentrate and through the ultrafilter membrane over a period of 12 hr.

DISCUSSION

Ultrafiltration provides an efficient and rapid method for the concentration of poliovirus. The procedure is easily adaptable to other viruses because retention depends only on the molecular weight and size of the virus. In addition, this method has the added advantage of retaining the larger capsid proteins that have not been incorporated into the virion when the proper size of ultrafilter membrane is used. For degradation and subsequent removal of capsid and nonviral proteins, the enzyme trypsin proved to be an ideal choice, because it is eliminated from the concentrate by passing through the XM-50 Diaflo membrane.

One further advantage of ultrafiltration is that simultaneous dialysis of the concentrate can be accomplished to the same extent as by

Fig. 3. Distribution of poliovirus in isopycnic cesium chloride gradients after concentration, as measured by PFU, ultraviolet absorbance, distribution of incorporated 3H-uridine, and density. Centrifugation as in Fig. 2. Left, Virus concentrated by ultracentrifugation onto a cesium cushion; right, virus concentrated by ultrafiltration.
ultrafiltration of filtration is useful when thelatter period comparable to that required for the latter procedure. This method is therefore useful when hypertonic states are detrimental to the virus.

A disadvantage encountered with this ultrafiltration system was the interference of certain substances such as calf serum and Methocel. These were retained by the Diaflo XM-50 ultrafiltration membrane and had to be removed by other means prior to concentration of the virus.

After comparing two techniques for the concentration of poliovirus, one involving the ultracentrifugation of a virus suspension onto a CsCl cushion and the other involving volume reduction by Diaflo ultrafiltration, we have concluded that the latter method offers more advantages and more versatility for subsequent purification and characterization procedures.

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