Enterovirus Concentration on Cellulose Membranes

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Cellulose nitrate membranes were used as one of the adsorbents in concentrating viruses from water. For adsorption to occur, salts were required. With increase in valency of salt, less salt was necessary for enhanced virus adsorption to membranes. Trivalent salts were more effective because they could be used at only 1% the concentration required for divalent salts. Thus, 0.5 mM AlCl₃ was as effective as 50 mM MgCl₂. For testing 500 gal of water, only 0.24 kg of AlCl₃ was required in contrast to 20 kg of MgCl₂. Virus could then be eluted from such membranes, having an area of 486 cm², with 250 ml of pH 11.5 buffer. Lowering the pH of the eluate and adding AlCl₃ permitted the virus to be quickly readsorbed on a smaller cellulose membrane, i.e., 4 cm². Virus for assay was eluted from the small membrane in 1 ml. This procedure has provided the basis for concentrating minute amounts of virus from large volumes of water.

Cliver (2, 3) and Wallis and Melnick (9) reported on the use of cellulose membranes for the concentration of viruses. The parameters for virus adsorption to and elution from cellulose membranes were delineated (10), and such membranes were employed for detection of viruses in natural waters (11). Other investigators have adopted the method for concentrating viruses from water (4-7).

An apparatus was recently described which can concentrate virus from 400 gal of water onto cellulose membranes in 1 hr (8). The procedure required a 5-gal vessel containing 4 M MgCl₂. The salt was injected into the flowing water to make a final concentration of 0.05 M, which is necessary for virus adsorption to the membrane. Thus, 20 kg of MgCl₂ had to be used for each test. This salt had been selected because of its high solubility. The current investigation was undertaken to determine the effects of other highly soluble salts on virus adsorption to cellulose membranes in order to make the concentration of virus from large volumes of water more practical.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys obtained from immature vervet monkeys were trypsinized and grown as described (4).

Virus and virus assays. A plaque-purified line of poliovirus type 1 (Mahoney strain) was used in all experiments, unless otherwise indicated. Other viruses used were attenuated poliovirus type 1 (LSc strain), echoviruses type 1 (Farouk) and type 7 (Wallace), and coxsackieviruses A9 (Grigg) and B3 (Nancy). Stock viruses were grown in MK cells using an input of 1 to 10 plaque-forming units (PFU)/cell and stored at -70 C. Virus assays were made by the PFU method as used in this laboratory (4).

Viruses adsorbents. Cellulose membranes (Millipore Corp.) with a porosity of 0.45 μm were used throughout this study. The diameter of membranes is 25 mm, having an available surface area of 4.0 cm², unless otherwise indicated. The method used for concentrating viruses on cellulose membranes has been described (10, 11).

Viruses eluents. Proteins, as in serum (10) and beef extract (1), that adsorb to cellulose nitrate membranes, and wetting agents (10, 11) exchanged for virus and elute it. The membrane-coating components which are used in the eluent are then found in the eluate, where they interfere with recombination of virus on smaller surface membranes. Thus, another eluate had to be found. Protein-free salt solutions at pH levels from 2.0 to 8.5 are required for adsorption of viruses to membranes; above pH 8.5 the efficiency of adsorption is decreased. Enteroviruses and myxoviruses can also be eluted with a nonprotein solution at pH 11.5 without detectable loss of infectivity (8, 12). The suspension containing eluted virus can then be adjusted to acidic pH levels (4.0 to 4.5), AlCl₃ can be added (0.0005 M), and the virus can then be readsorbed to cellulose membranes. This cycle can be performed repeatedly with 100% recovery of virus. The recommended eluent is 0.05 M glycine buffer which can be adjusted to pH 11.5 with NaOH or pH 2 with HCl, without inacti-
vating enteroviruses in short-term exposures (5 to 10 min). Buffers that have Mg or other cations present which react with NaOH to form a gel should not be used, nor should buffers be used which contain anions which complex with Al ions.

**Purified water.** Water containing no more than 0.01 μg of dissolved solids per ml and no detectable organics was obtained by passing tap water (see below) through a water purification system (Carborundum Co., Niagara Falls, N.Y.), and was used in place of distilled water.

**Tap water.** Tap water contained 457 ppm dissolved solids and 350 ppm suspended solids. When used, it was dechlorinated with 1–3 ppm sodium thiosulfate.

**Clarifying filters.** To prevent clogging of cellulose membranes, AP20 fiberglass pads (Millipore Corp.) were sometimes used as clarifiers to remove suspended solids and ferric complexes from tap water. To prevent virus adsorption, the pads were first treated with 1% Tween 80 (100 ml for each 100 cm² surface); residual Tween was removed by thorough washing of the pad.

**RESULTS**

**Effects of different pH levels on elution of virus from membranes with glycine buffer.**

Table 1 shows the results of eluting poliovirus (Mahoney) from cellulose membranes at different pH levels. At pH 11 to 12, virtually 100% of the virus was recovered from the membrane. In these tests, 5 ml of glycine-NaOH buffer was used to elute virus from 25-mm cellulose membranes; the membrane was then washed with 5 ml of glycine-HCl buffer at pH 2.0 to 4.4, thus yielding a total volume of 10 ml of neutralized eluate.

The minimum volume required for elution of virus was found to be 0.5 ml for each 4-cm² surface. Thus, virus adsorbed to a 25-mm membrane (4 cm²) could be eluted with 0.5 ml of buffer (pH 11.5), immediately followed by 0.5 ml of pH 2 neutralizing wash, to make a final recovery volume of 1.0 ml.

**Effects of salts on virus adsorption to cellulose membranes.** Several monovalent, divalent, and trivalent salts dissolved in purified water were tested to determine the lowest concentration required for virus adsorption. The experimental procedures and results are shown in Table 2. Trivalent aluminum salts proved best, and AlCl₃ and Al₂(SO₄)₃ were just as effective at about 1% the concentration of MgCl₂. Thus, the amount of salt which would have to be transported to the field is significantly lower for AlCl₃ than for MgCl₂. Only 0.24 kg of aluminum salts would be required for adsorption of poliovirus to membranes possessing 500 gal of water, whereas 20 kg of MgCl₂ would be necessary (Table 3).

**Effects of MgCl₂ and AlCl₃ on virus suspended in tap water.** Since our goal is to concentrate virus in the field from natural water, Houston tap water was used to determine the effects of Mg and Al ions on virus adsorption. Figure 1 shows the experimental procedures and results. Again, AlCl₃ was at least 200 times more efficient than MgCl₂ in facilitating poliovirus adsorption.

**Effects of AlCl₃ on large volumes of tap water.** The experiments described above required that only 5-ml samples be passed through the cellulose membrane. When large volumes of tap water were treated with AlCl₃, the aluminum hydroxide gel which formed clogged the filter. However, enteroviruses can

### Table 1. Effects of pH on elution of poliovirus from cellulose membranes

| Samples tested | pH of original eluent (glycine-NaOH) | pH of neutralizing fluid (glycine-HCl) | Final pH | Avg no. of PFU/0.1 ml |
|----------------|-------------------------------------|---------------------------------------|----------|------------------------|
| Control virus  |                                     |                                       |          |                        |
| Membrane wash  |                                     |                                       |          |                        |
| 1              | 12.0                                | 2.0                                   | 7.1      | 76                     |
| 2              | 11.5                                | 2.0                                   | 6.5      | 71                     |
| 3              | 11.0                                | 2.5                                   | 7.0      | 78                     |
| 4              | 10.5                                | 3.0                                   | 7.1      | 66                     |
| 5              | 10.0                                | 3.8                                   | 7.3      | 39                     |
| 6              | 9.5                                 | 4.4                                   | 7.1      | 12                     |

* Stock poliovirus (Mahoney) was diluted 100,000-fold in tris(hydroxymethyl)aminomethane-buffered saline, and 10-ml samples were filtered through each of six 25-mm cellulose membranes (0.45 μm pore size). The membrane filtrates were collected, pooled, and assayed (control virus, membrane filtrate). Each membrane was then washed with 10 ml of saline at pH 6.9, and the membrane washes were pooled and assayed. Each membrane was then treated with 5 ml of the eluent at the indicated pH, and the membrane was washed with 5 ml of buffer made acidic with HCl so that the total 10 ml recovered would be neutral.
was aminomethane-buffered assayed for unadsorbed virus. Control indicated. Representativesamples (5 ml) were filtered through HAMembranes (0.45 µm), and filtrates were able adding AlCl₃ then the natural pH range of 4.0, 3.5, 3.0, and 2.5. Water was from clogging of membranes. Required pressure. AlCl₃ was used in the experiment to determine flow rates of tapwater containing 0.0005 M AlCl₃ through membranes at different pH levels.

Tap water was filtered through 90-mm membranes (0.45 µm pore size) at constant pressure. Tap water at 5 psi, free from Al ions, required 24.4 min for filtration of 10 liters. Tap water containing 0.0005 M AlCl₃ at pH 7 clogged the filter before 1 liter passed the membrane. Similarly, at pH 6 and 5, immediate clogging occurred. However, at pH 4.5, 4.0, 3.5, and 3.0, the flow rate was faster (13.6 min/10 liters) than the control sample free from AlCl₃. The increased flow rate is attributable to the fact that the acid dissolved some of the natural gels present in tap water, especially ferric hydroxide. All subsequent experiments were carried out by adjusting virus-tap water mixtures to pH 3.0 with HCl and then adding AlCl₃ to give a final concentration of 0.0005 M AlCl₃. The water must be made acid before addition of the Al ions.

**Effect of AlCl₃ on adsorption of enteroviruses to cellulose membranes.** A number of enteroviruses were studied (Table 4). All viruses tested (polioviruses, echoviruses, and coxsackieviruses) were adsorbed to the membranes with 0.0005 M AlCl₃ and were quantitatively eluted with pH 11.5 buffer.
Reconcentration of poliovirus adsorbed to cellulose membranes. Virus recovered from membranes as outlined above can be readily readsorbed to new membranes, since the eluate does not contain any membrane-coating components (10). This is done merely by adjusting the pH levels and adding AlCl₃. Under conditions which require the processing of large volumes of water at high flow rates, 293-mm membranes (0.45 μm pore size) require 200 to 300 ml for virus elution. The virus can then be readsorbed on a 25-mm membrane (0.45 μm pore size) at acidic pH levels and eluted from this membrane with as little as 0.5 ml of pH 11.5 buffer.

An experiment was conducted to show that by pH control and salt addition poliovirus can be recycled on and off cellulose membranes. A total of 1,300 PFU of poliovirus was adsorbed to a 293-mm membrane in the presence of 0.0005 M AlCl₃ at pH 4.0. The membrane was washed with saline to remove residual Al ions to avoid subsequent gel formation with the basic eluent and thus to prevent clogging of membranes. The 293-mm membrane was then treated with 300 ml of pH 11.5 eluent. The eluate was collected in 300 ml of pH 2 buffer to yield 600 ml of virus suspension at pH 6.7. [If the suspensions are not acidic (pH 4.0 to 4.5) at this point, they should be made acidic with HCl before addition of AlCl₃,] AlCl₃ was added to yield a final concentration of 0.0005 M to enhance virus readsorption to a freshly prepared 25-mm membrane (0.45 μm pore size). Virus was then eluted from the membrane with 0.5 ml of pH 11.5 buffer, and the membrane was washed with 0.5-ml volume of pH 2 buffer. The 1 ml of suspension now contained 1,250 PFU of poliovirus, virtually all of the virus initially contained in the 5-gal test volume. This type of concentration and elution of poliovirus was carried out many times with 90 to 100% recovery of the virus in the concentrate.

Processing of large volumes of tap water. These new findings were then applied to recovery of virus from large volumes of tap water. Five gallons of 0.05 M AlCl₃ in 0.5 M HCl acid was placed in a 5-gal pressure vessel. The salt solution was mixed into running tap water (20 psi) by nitrogen pressure (50 psi) and a metering valve so that the mixture was diluted 1:100 in the water to give a final concentration of 0.0005 M AlCl₃ and a pH of 3.0. A pH meter was used to monitor the effluent, and the metering device was adjusted to produce a flow that gave the desired pH of 3.0. Upstream a second vessel with 5 gal of purified water containing poliovirus, sodium thiosulfate, and 0.04% phenol red was connected to the nitrogen pressure. These fluids were also diluted 100-fold in the running tap water to give a final concentration of virus as indicated in Table 5 (i.e., 0.5 PFU/gal when 250 PFU input was used for 500 gal), 2 μg of thiosulfate per ml, and a faint pink color. Immediately before the downstream pressure vessel, a sight glass was inserted in the tubing to monitor the color of the fluids, and in this way the quantity of virus and thiosulfate being added was controlled. The results of three consecutive tests in which 500-gal batches of water were processed by adsorption on a 293-mm cellulose membrane (0.45 μm pore size) and then reconcentrated on a 25-mm membrane are shown in Table 5.

Additional experiments have shown that methyl orange can serve as an indicator to determine pH, since the dye changes from light yellow to bright red at pH 3.0. A solution of 0.5 M HCl, 0.05 M AlCl₃, and 0.1% methyl orange was prepared in a 5-gal pressure vessel so that 1:100 dilution would yield a pH of 3.0.

| TABLE 4. Effects of AlCl₃ on the adsorption of entroviruses to membranes |
|-----------------------------|-----------------|-----------------|-----------|
| Enteroviruses*              |                |
|                             | Unfiltered      | Membrane        |
|                             | control         |                 |
|                             | Filtrate        | Elute           |
| Poliovirus 1 (Mahoney)      | 142             | 0               | 131       |
| Poliovirus 1 (LSC)          | 71              | 0               | 65        |
| Echovirus 1                 | 96              | 0               | 90        |
| Echovirus 7                 | 80              | 0               | 81        |
| Coxsackievirus A9           | 101             | 0               | 92        |
| Coxsackievirus B3           | 85              | 0               | 82        |

* Viruses were diluted in pH 3.5 tap water containing 0.0005 M AlCl₃, and 10-ml samples were filtered through 25-mm cellulose membranes (0.45 μm pore size). The membranes were washed with 10 ml of saline and then treated with 5 ml of pH 11.5 eluent followed by 5 ml of pH 2 neutralizing buffer.

| TABLE 5. Processing of large volumes of tap water |
|----------------------------------|-----------|-----------|--------|
| Expt | Total virus (PFU) with various procedures | Per cent virus recovered |
|      | Total input virus/500 gal | Eluate obtained* |        |
| 1    | 250                    | 235         | 94     |
| 2    | 2,500                  | 2,200       | 88     |
| 3    | 25,000                 | 23,500      | 94     |

* A 500-ml amount of eluate was obtained, reconcentrated on a 25-mm membrane, and eluted into a final volume of 5 ml.
480 WALLIS, HENDERSON, AND MELNICK APPL. MICROBIOL.

0.0005 M AlCl₃ and 0.001% methyl orange. The concentrate was forced into running tap water under nitrogen pressure until the color changed from yellow to red. Testing the effluent by a pH meter confirmed that pH 3.0 had been obtained and also assured that the concentrate was being delivered into the running water at a dilution of 1:100. At this dilution, methyl orange was not toxic to the tissue culture cells (thus the effluent containing dye could be assayed without dilution), nor was the dye virucidal to poliovirus.

With Houston tap water (pH 8.2), the concentration of HCl indicated above was required. However, for other areas, the water under test must be titrated to determine the amount of HCl required to bring the water to pH 3.0. The concentration needed must then be determined so that a 100-fold dilution of the salt-dye-acid stock can be injected into the water to yield the optimal salt-pH concentrations.

DISCUSSION

The concentration of viruses from large volumes of water in the field is now feasible with the use of aluminum salts for adsorption of viruses to cellulose membranes. Aluminum salts can be used in 1% the concentration of magnesium salts. For virus to adsorb from a 400-gal sample, 45 lb of MgCl₂ is needed. On the other hand, with AlCl₃, 1,000 gal of water can be processed with only 1 lb of this trivalent salt.

In this report, we have also described a method for concentrating enteroviruses by serial adsorption to and elution from cellulose membranes. By manipulating hydrogen ion levels, viruses in 400- to 500-gal samples can be adsorbed to 293-mm membrane surfaces at pH 3 to 4 at high flow rates, eluted at pH 11.5 with 250 to 300 ml of protein-free buffer, and readSORBED to a small surface membrane (25 to 47 mm). Viruses can then be removed into a final eluate of 1 to 5 ml, which can be conveniently assayed.

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