Concentration of Enteroviruses on Membrane Filters

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Enteroviruses can be made to adsorb or to pass through membrane filters by manipulation of the suspending medium. Salts facilitate virus adsorption, but membrane-coating components (MCC) interfere. Because cells release MCC into the culture medium during viral growth, MCC must be removed before virus can be adsorbed to membranes. Adsorbed virus can be eluted with diluents containing MCC (cell extracts or serum) or agents that reduce surface tension (sodium lauryl sulfate). By membrane adsorption and elution, enteroviruses can be readily concentrated and quantitatively recovered from crude virus harvests.

To avoid loss of virus by adsorption during filtration studies, Elford (1) recommended that viruses should be suspended in proteinaceous diluents. However, even when crude virus harvests containing proteins and other organic materials are filtered through membranes with pores much greater in diameter than the size of the virus under test, only a small fraction of the original virus may be found in the filtrate (2), sometimes as little as 10^{-4} . Viruses can actually be quantitatively adsorbed to membranes if the viruses are suspended in salt solution free from organic compounds (6). The present study is concerned with the dynamics of enterovirus adsorption and elution from membranes, and the application of these new findings to virus concentration.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys from young rhesus monkeys were trypsinized and grown in Melnick medium A containing 0.2 mM AlCl_3 to suppress adventitious agents (3).

Viruses and their assays. A plaque-purified line of type 1 virulent poliovirus (Mahoney) was used as a model. The virus stock used in these studies was grown in a single cycle in MK cells maintained with Melnick medium B without serum and had a titer of $10^{8.0}$ plaque-forming units (PFU) per ml. The virus was assayed by the PFU method. Overlay medium consisted of Earle's salt solution, 0.4% NaHCO_3 , 1:60,000 neutral red, 0.1% skim milk, 1.5% agar (Difco), and 25 mM MgCl_2 to enhance enterovirus growth (4). Other viruses used in this study were type 1 attenuated poliovirus (LSc), echovirus type 7 (Wallace), and coxsackie virus B3 (Nancy). These viruses were also grown and assayed in MK cells.

Filtration procedures. All experiments were carried out with membrane filters from Millipore Corp., Bedford, Mass. The filters were either in 25-mm microsyringe holders (no. XX 3002500) or in 142-mm

laboratory sterilizing filter holders (no. YY 3014200). The clarifying pads and membranes, which had pore diameters at least 10 to 20 times larger than the diameters of the enterovirus particles, included the AP 20 clarifying pad, the HA membrane with 0.45 μ porosity, and the GS membrane with 0.22 μ porosity. Filtration experiments were carried out with nitrogen pressure at 2 psi unless otherwise indicated.

Control harvests. To simulate the physical and chemical environment of a harvest fluid, poliovirus was diluted to contain 100 PFU/0.1 ml in control tissue culture fluids. Such control fluids were prepared from normal virus-free cultures maintained for 48 hr with medium B. The cultures were frozen, thawed, and centrifuged at 2,000 rev/min for 10 min to yield the control harvest fluid.

Anion resin columns. Dowex 1-X8 (Cl^-) 100-200 mesh was placed in a column, and the resin was washed with distilled water until the filtrate obtained was at pH 4 to 5. The resin was then washed with 0.15 M NaCl until the filtrate yielded fluids having the same pH as the saline used for washing. The column was sterilized by autoclaving and, when cool, was again washed with sterile saline until the filtrate had the pH of the saline used for washing. After being autoclaved and rewashed with saline, the column was treated with nitrogen at 10 psi to force all free fluid from the column. The virus suspension was then passed through the resin column at 2 to 5 psi until the input volume was recovered as filtrate.

RESULTS

Filtration of poliovirus. In preliminary experiments, purified virus free from extraneous organic material and suspended in saline was found to be adsorbed to membranes. If the membranes were pretreated with control harvest fluids, the membranes became coated and virus passed into the filtrate. These findings were in essential agreement with Elford's original studies (1). The membrane-coating components will be referred to as MCC.

When poliovirus was used as present in crude virus harvests, it did not adsorb to the membrane. However, when the virus was diluted in saline and then filtered, the major portion of the virus population was adsorbed to the membrane.

The following experiments were performed to determine (i) the relative concentration of MCC in virus harvest fluids and (ii) the effects of Earle's salt solution and distilled water on the adsorption of virus to membranes in the presence of decreasing amounts of MCC. Virus harvest fluid was diluted in 10-fold steps in distilled water; a second series was diluted in Earle's salt solution. Samples (5 ml) of undiluted virus and virus at each dilution were filtered through 25-mm membranes with a pore size of 0.45 μ and were assayed immediately. The results are shown in Fig. 1, in which all values of virus infectivity are calculated back to the original undiluted fluid. Filtration of the undiluted sample decreased its titer fourfold. Poliovirus at a 10^{-1} dilution in distilled water yielded a filtrate that decreased 100-fold in titer, but a 10^{-2} dilution in water yielded a filtrate with only a 10-fold loss in titer. As the virus was further diluted to 10^{-3} through 10^{-5} , no loss of infectivity was detectable in the filtrates. In contrast, poliovirus diluted in Earle's salt solution yielded filtrates that always decreased markedly in titer regardless of the dilution of the harvest that was filtered. At 10^{-3} to 10^{-5} dilutions, no detectable infectivity was evident

TABLE 1. *Effect of salt concentration on poliovirus adsorption to membranes*

Salt concn	Avg no. of PFU/0.1 ml			
	NaCl		MgCl ₂	
	Control	Filtrate	Control	Filtrate
<i>M</i>				
0.12	84	0	92	0
0.03	89	2	90	0
0.0075	96	54	90	11
0.002	78	83	85	46
0.0005	87	90	79	80

in the filtrates. The experiments showed that harvest fluids had to be diluted 1,000-fold in salt solution to escape the influence of the MCC contained therein.

From the results above, it was evident that salt solutions enhance the adsorption of virus to membranes. The influence of a monovalent and that of a divalent salt were studied. Virus was diluted 10,000 times in distilled water, well beyond the effect of the MCC in the harvest. This suspension was then further diluted 10 times in different molarities of NaCl and MgCl₂ to contain plating stocks of about 100 PFU/0.1 ml. Samples of 5 ml were filtered as described above, and assayed. As shown in Table 1, poliovirus was effectively adsorbed in the presence of a concentration as low as 0.03 M for NaCl and of 0.0075 M for MgCl₂.

Surface tension plays a role in the adsorption of poliovirus. In the experiments described below, tension reducers were found to be active in eluting adsorbed viruses from membranes. Poliovirus was diluted to contain 100 PFU/0.1 ml in Earle's salt solution, and 5-ml volumes were filtered through 0.45- μ membranes. Under these conditions, the virus is completely adsorbed (see Fig. 1), so that each membrane contained about 5,000 PFU. The membranes were then treated with a variety of solutions, and the eluates were tested for virus. Sodium lauryl sulfate (1%), 0.1% saponin, and 10% bovine serum in medium B were the most effective eluents, yielding 100% recovery of virus. Distilled water yielded only 50% recovery, and Earle's salt solution and 0.1 M ethylenediamine-tetraacetic acid failed to elute any virus. (Lauryl sulfate and saponin are toxic to MK cells. To remove these agents prior to plating, the eluates were passed through a 5-g anion resin column, and the resin filtrates were assayed.)

Effect of various components of harvest fluids on the virus adsorption to membranes. A poliovirus harvest was diluted 100,000 times in salt solution

FIG. 1. *Salt solution (ESS) facilitates adsorption of poliovirus to 0.45- μ membrane. Virus titers calculated on the basis of the original undiluted fluid.*

to contain about 100 PFU/0.1 ml. Samples of 5 ml each were brought to pH 7.5 and filtered through 0.45- μ membranes pretreated with control harvest fluids or the different materials present in medium B. Virtually all of the virus passed into the filtrate if the membranes had been pretreated with extracts of cells that had been frozen in medium B. Membranes pretreated with diluted solutions of amino acids, vitamins, or salt adsorbed the virus completely, as did membranes pretreated with extracts of cells that had been frozen in water. It is interesting to note that not only viruses but also MCC adsorb to membranes in the presence of salts.

The following experiment was performed to determine whether MCC are released spontaneously from living cells or are released only by rupturing the cells. Normal cell cultures were maintained in Earle's salt solution at 37 C, and extracellular fluids were obtained over a 3-day period. Fluids were removed and centrifuged at 2,000 rev/min for 30 min to remove cells, and the clarified supernatant fluid was collected and frozen until used. Similarly, whole cultures (cells and nutrient fluid) maintained for the same periods were frozen until used. Membranes were treated with 5-ml volumes of the whole tissue culture harvests and of the extracellular fluids. The treated membranes were then used to filter poliovirus diluted in salt solution, and filtrates were assayed. The results of such tests (Table 2) indicate that MCC are released into the maintenance fluid after 16 hr of incubation.

Removal of MCC. In view of the results shown above, it is apparent that organic components in virus suspensions will have to be removed or altered before poliovirus can be concentrated on membranes. The following experiment was performed to remove the proteins present in virus harvests that adsorb to membranes and prevent virus adsorption. Undiluted poliovirus was treated with a final concentration of 0.01% protamine sulfate (salmine; 7) for 30 min at 25 C with gentle agitation on a rotary shaker. The voluminous precipitate was then removed by sedimentation at 2,000 rev/min for 15 min. A portion of supernatant fluid was assayed, and a 5-ml sample was filtered through a 0.45- μ membrane and assayed. The protamine treatment did not decrease the virus titer. The sample filtered through a membrane yielded a filtrate which was decreased in titer by 1.5 log units. A control sample of untreated virus passed through a membrane was only decreased in titer by 0.4 log. Higher concentrations of protamine (0.1%) decreased the virus titer prior to filtration, and lower concentrations (0.001%) failed to remove MCC, so that filtrates were not significantly decreased in titer.

TABLE 2. Release of membrane coating components into extracellular fluid^a

Time of harvest <i>hr</i>	Avg no. of PFU/0.1 ml in filtrates of poliovirus passed through membranes treated	
	With extracellular fluid	With whole culture harvest
0	0	84
4	0	
8	0	
16	32	87
24	89	
48	94	
72	90	80

^a Unfiltered control stock yielded a titer of 95 PFU/0.1 ml.

To examine the effect of lipids, virus harvests were treated with chloroform. Undiluted poliovirus was treated with 25% chloroform at 25 C for 30 min on a rotary shaker. Supernatant fluids were not decreased in titer, but a 5-ml sample of the treated virus passed through a membrane yielded a filtrate now decreased in titer by 2.1 log units.

In a previous study, we showed that enteroviruses could not be photosensitized with heterotricyclic dyes in the presence of organic medium (5). However, virus suspensions passed through anion resins yielded filtrates that were readily photosensitized with dyes. These resins were tested to see whether they could remove MCC from virus harvests. Samples (6 ml) of undiluted poliovirus were filtered through 2.5-, 5.0-, 7.5-, and 10-g anion resin columns (contained in 10 × 500 mm tubes); the resin filtrates were assayed. No virus was lost by this treatment. Samples (5 ml) of each resin filtrate were then filtered through 0.45- μ membranes and were assayed again. A ratio of 1 g of resin to 1 ml of undiluted virus removed a large fraction of MCC from the virus stock.

The effect of pH on adsorption of poliovirus to membranes was also examined. A 40-ml amount of undiluted poliovirus was placed in a beaker, and the pH of the virus suspension was brought to 8.0 with NaOH. A sample was obtained for assay, 5 ml was filtered through an 0.45- μ membrane, and the filtrate was assayed. The remainder of the virus suspension in the beaker was adjusted to pH 7 with HCl; a sample was assayed, and 5 ml was filtered as above. Samples were tested as above through pH 3. At pH 5, maximal retention of virus occurred, and only 0.05% appeared in the filtrate.

It is evident from the above experiments that no

single treatment was adequate for complete removal of MCC. In fact, when increased volumes of virus were used, the individual effects of protamine, chloroform, anion resins, or pH were diminished, owing to the cumulative effects of the residual MCC not affected by the individual treatment. However, when poliovirus was treated in series with protamine, chloroform, and anion resins, and then the pH was lowered to 5, efficient adsorption of virus from undiluted virus harvests was achieved.

Recovery and concentration of virus from tissue culture harvests. To concentrate large volumes of poliovirus on a 25-mm membrane, prefiltration of the virus stock was necessary, since only 20 ml of clarified, undiluted virus could be passed through a 0.45-μ membrane without clogging the membrane pores. Preliminary experiments showed that, because of the presence of MCC, 10-ml volumes of undiluted poliovirus could be serially filtered through three freshly prepared 25-mm, 0.45-μ membranes without detectable decrease in titer of the filtrate. Thus, preliminary filtration of virus through 0.45-μ membranes of larger surface could be performed by using 12 cm² of surface for each 10 ml of virus. This procedure yielded a filtrate that could be subsequently passed through 25-mm membranes in larger volumes. The following formula was used: volume of virus to be filtered/10 × 12 cm² = maximal surface of 0.45-μ membrane needed for clarification. In a number of experiments with different virus harvests, this procedure proved to be useful for achieving clarification without detectable loss of virus on the membrane.

After clarification and serial treatment with protamine sulfate, chloroform, resin, and pH adjustment, final concentration of the virus on membranes was performed by use of 25-mm, 0.22-μ membranes, which provided additional surface area for virus adsorption from the clarified suspension. In this test, 130 ml of undiluted poliovirus was clarified through a 142-mm, 0.45-μ membrane (100 cm²). As shown in Table 3, the

virus titer was not decreased by this procedure. A 15-ml sample of this filtrate was then filtered through a 25-mm, 0.22-μ membrane at 5 psi with no loss in titer, indicating that there was still sufficient MCC in the virus suspension. All 110 ml of the remaining virus suspension was treated with 1.1 ml of 1% protamine sulfate (in distilled water) to give a final concentration of 0.01%. The virus-protamine suspension was placed in a rotary shaker for 30 min at 25 C, and a sample was reserved for assay. A 30-ml amount of chloroform was added to the virus-protamine mixture, and the mixture was shaken for 30 min on a rotary shaker. The chloroform was removed in a separatory funnel, and then the protamine-chloroform treated virus was filtered through 100 g of anion resin (contained in a 25 × 600 mm tube); the resin filtrate was adjusted to pH 5. The 100 ml of treated virus was filtered through a 25-mm, 0.22-μ membrane at 5 psi, and the filtrate was assayed, along with the samples of virus that had been obtained after each treatment described above. The 25-mm, 0.22-μ membrane was treated with 1 ml of whole fetal serum to elute the virus from the membrane (this treatment consisted of addition of 1 ml of serum to a 2-ml syringe attached to the filter holder). The serum was allowed to remain in contact with the membrane for 5 min. The serum was forced through and then the serum filtrate was twice recirculated through the membrane before it was assayed. The results of this test are shown in Fig. 2. The treatment of poliovirus by clarification through a membrane in the presence of MCC and then by addition of protamine sulfate, chloroform, anion resins, and HCl (pH 5) did not reduce the virus titer. When the serially treated 100 ml of virus was passed through a 25-mm, 0.22-μ membrane, no detectable infectivity was in the filtrate. The membrane was treated with 1 ml of serum to obtain a volume concentration of 100 times, and a parallel virus concentration of 63 times was achieved.

Concentration of other enteroviruses. The fol-

TABLE 3. Concentration of enteroviruses

Viruses	Log ₁₀ virus titer (PFU/ml)					
	Untreated, unfiltered control	142-mm membrane filtrate, 0.45 μ porosity	After protamine, chloroform, resin, and pH treatments	25-mm membrane filtrate, 0.22 μ porosity	Eluate concentrated 100 times into 1 ml	Virus concn achieved ^a
Polio						
Mahoney.....	8.1	8.0	8.0	0.0	9.8	63×
LSc.....	7.5	7.5	7.5	2.2	9.5	100×
Echovirus type 7.....	7.7	7.7	7.6	0.0	9.6	80×
Coxsackievirus B3.....	7.2	7.0	7.0	0.0	8.9	80×

^a Based on difference between columns 2 and 5.

lowing viruses were concentrated on and recovered from membranes by the methods described above: attenuated type 1 poliovirus (LSc), echovirus type 7, and coxsackievirus B3.

Each virus was processed as described above for virulent type 1 poliovirus, with the use of 130 ml of undiluted virus initially and the concentration of 100 ml of the treated virus on a 25-mm, 0.22- μ

FIG. 2. Flow chart of poliovirus concentration and recovery.

membrane. The results of these tests are shown in Table 3.

DISCUSSION

Membranes pretreated with cell extracts or serum do not significantly adsorb enteroviruses, for MCC in virus fluids or serum adsorb to membranes and prevent virus adsorption. However, when viruses are diluted in salt solution and passed through membranes, they are virtually all adsorbed. If diluted in distilled water, the virus is found in the filtrate. Not only are salts required for adsorption of viruses to membranes, but the MCC contained in cell extracts and serum also require an isotonic saline environment for their adsorption.

There seem to be a number of different types of organic components in a virus harvest that occupy membrane sites and preclude virus adsorption. One type could be removed by adsorption to anion resins; the effect of a second type could be minimized by adjusting the suspension to pH 5. Interfering proteins were removed with protamine sulfate and lipids were removed with chloroform. Large volumes of undiluted enterovirus harvests could be serially treated with these reagents without a significant decrease in titer of the virus sample. The entire procedure requires only 90 min. After passage of the treated sample through membranes having a porosity nine times greater in diameter than the virus under test, the filtrates contained no infectivity or only an insignificant level of detectable virus. Virus could be recovered quantitatively from the membranes and, in virtually all cases, 80- to 100-fold concentrations were achieved.

This method of selectively adsorbing enteroviruses on membranes should be useful for producing virus concentrates for a variety of

purposes. As an example, viruses which are usually present in sewage in amounts too small to be detected could be readily concentrated on membranes by the method described (6). In this study, covering a period of 7 months, only four virus isolates were detected in raw sewage by direct inoculation of sewage in cultures, as compared with 2,795 isolates obtained by adsorption and elution from membranes.

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