Concentration and Purification of Viruses by Adsorption to and Elution from Insoluble Polyelectrolytes

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Received for publication 31 December 1970

Acid-resistant, nonenveloped viruses belonging to the enterovirus, reovirus, and adenovirus groups were readily concentrated on PE60, an insoluble cross-linked polyelectrolyte based on isobutylene maleic anhydride. Hydrolysis of PE60 by NaOH increased its capacity to adsorb viruses. Hydrogen ion levels played an important role in virus concentration; optimal pH levels for maximal virus adsorption were between pH 3.0 and 4.5. Undiluted virus was easily concentrated from large volumes on PE60, and the adsorbed virus was readily eluted at pH 8 to 9.

Divinylbenzene cross-linked styrene/maleic anhydride copolymers were found to adsorb highly purified tobacco mosaic virus (TMV) and poliovirus (3). Purified TMV was adsorbed to polyelectrolytes in salt solution, whereas purified poliovirus was adsorbed to these polymers in distilled water. The effect of organic material on the adsorption of viruses to the polymers was not investigated in the preliminary study by Johnson, Fields, and Darlington (3). Viruses as found in nature or as grown in the laboratory are present in fluids along with salts and organic material. Therefore, conditions for the concentration of viruses from natural or laboratory-grown fluids require more definitive data as to the role of organic and inorganic factors in the adsorption of viruses to polyelectrolytes.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys obtained from immature green (vervet) monkeys were trypsinized and grown in Melnick's medium A containing 2% calf serum.

Viruses. A plaque-purified line of virulent type 1 poliovirus (Mahoney) with a titer of 10^8 plaque-forming units (PFU)/ml was used for most of these studies. Other agents used were representative viruses from a number of different taxonomic groups. These consisted of plaque-purified lines of type 1 attenuated poliovirus (LSc vaccine strain); echovirus types 1 (Farouk strain), 7 (Wallace strain), and 12 (Travis strain); coxsackievirus types A9 (Grigg strain) and B3 (Nancy strain); adenovirus (SV13 strain); and reovirus type 1 (716 strain). Virus stocks were prepared by a single-cycle passage in MK cultures maintained with Melnick's medium B (0.5% lactalbumin hydrolysate, Earle's salt solution, 0.22% NaHCO_3, and penicillin-streptomycin mixture, 100 units or 100 μg per ml). Viruses were harvested from serum-free cultures and from cultures maintained on media which do not contain detergent. Serum or detergent (such as Tween 80 used in medium 199) elute virus adsorbed to PE60.

Virus assays. Quantification of virus was performed by the PFU method. Overlay medium consisted of Earle's salt solution, 0.4% NaHCO_3, 1:60,000 neutral red, amino acids as used in Eagle's minimal essential medium, and 1.5% agar (Difco). To enhance plaque formation, 25 mM MgCl_2 was included in overlays for enteroviruses (9), 400 μg of proline sulfate per ml was used in overlays for adenovirus (9), and 1:60 pancreatin (Oxoid) in overlays for reovirus (11).

Insoluble polyelectrolyte 60 (PE60). PE60, available from the Monsanto Co., St. Louis, Mo., was provided in the form of a 100-mesh powder. It is an insoluble cross-linked copolymer of isobutylene maleic anhydride.

Preparation of PE60. The polyelectrolyte (PE) was prepared for use by making a 1% suspension in distilled water. The suspension in a centrifuge bottle was shaken on a rotary shaker for 0.5 hr; the vessel was then centrifuged at 2,000 rev/min for 5 min, and the supernatant fluid was discarded along with a small amount of nonsedimentable, or nonwettable, product. The packed PE was then washed twice with distilled water. After the last washing, the pellet of PE was resuspended in 0.1 volume of distilled water to make a 10% suspension. The suspension in portions of 1 ml was then distributed into tubes; the tubes were centrifuged as described, and the supernatant fluids were discarded. The packed PE, 100 mg per tube, was treated by addition of a given volume of virus to the tube, or the packed PE was transferred to virus fluids by interchange of virus fluid into the packed PE tube.
and back to the vessel used for stirring or shaking the virus-PE mixture.

Tubes or beakers containing packed PE were treated with virus fluids on a rotary shaker or on magnetic stirrers to afford a good collision efficiency between the virus and the PE. After 1 hr of rotation or stirring at 25 C, the virus-PE mixtures were centrifuged at 2,000 rev/min for 5 min, and the supernatants fluids were obtained and assayed for unadsorbed virus.

**Borrate buffer.** A 0.05 m solution of sodium borate was made in distilled water and adjusted to pH 9.0.

**RESULTS**

**Adsorption of poliovirus to hydrolyzed PE60.** Poliovirus harvested diluted 100,000-fold in distilled water, saline, or phosphate buffer (pH 6 or pH 7) avidly adsorbed to PE60 (which had been treated by washing with water as described in Materials and Methods). On the other hand, poliovirus did not adsorb to PE60 in buffers above pH 8 or in any diluent containing serum or tissue culture extracts, regardless of pH. Thus, poliovirus will adsorb to PE in diluents free from proteins at neutral or acid pH levels.

Experiments with PE60 and undiluted poliovirus at different pH levels (2.5 to 8) failed to give any significant adsorption of this virus to the PE, apparently because of the nonviral proteins present in the harvest fluids.

During the course of these experiments, it was noted that PE60 suspended in water, stored at 25 C, and used from day to day began to improve in its ability to adsorb undiluted virus at low pH ranges. Such an increase in the number of reactive sites for virus adsorption could result from slow hydrolysis of the PE during storage. Therefore, freshly prepared PE60 was treated with HCl or NaOH to expedite hydrolysis. The adsorption of poliovirus to hydrolyzed PE60 is shown in Fig. 1, which is typical of several experiments that were carried out. Poliovirus did not effectively adsorb to water-washed PE60 at pH 2.5 to 8.0. PE60 treated with HCl gave results almost identical to those obtained with water-washed PE, and these data were omitted from the figure for the sake of clarity. However, NaOH-washed PE adsorbed virus from suspension with increased avidity. Repeated experiments indicated that the optimal pH range for virus adsorption was pH 2.5 to 4.5 for NaOH-washed PE60; consequently, pH 3.5 was used for subsequent experiments.

A number of parameters were examined to determine the optimal degree of PE60 hydrolysis for virus adsorption. As shown in Fig. 2, treatment of PE60 with NaOH was most effective between 0.03 and 0.3 N. When the NaOH concentration was increased to 3 N, virus was no longer adsorbed to the hydrolyzed PE.

The 0.5-hr treatment time used in the above ex-

![Fig. 1. Adsorption of poliovirus to hydrolyzed PE60. One gram of PE60 was washed with 50 ml of 0.1 N NaOH for 0.5 hr on a rotary shaker. The PE was then packed at 2,000 rev/min for 5 min and repeatedly washed (five times) with 200 ml of distilled water to remove residual NaOH. The suspended PE in distilled water was then distributed into tubes giving a concentration of 100 mg of PE per tube. The tubes were centrifuged, the water was discarded, and the packed PE was treated with 5 ml of undiluted virus for 1 hr on a shaking machine at the pH indicated (HCl or NaOH was used to adjust pH levels). After 1 hr of mixing, the tubes were centrifuged, and the supernatants were obtained and assayed for unadsorbed virus. Control virus (PE-free) at the pH levels indicated showed no decrease in infectivity. A duplicate test as described above was performed with PE washed only with distilled water (water-washed PE).](attachment:fig1.png)
poliovirus was added to each 100 mg of packed PE, and the pH was adjusted to 3.5 with HCl. The samples were rotated mechanically for 1 hr. The tubes were centrifuged at 2,000 rev/min for 5 min, and the supernatant fluids were assayed for infectivity. As shown in Fig. 4, treatment of the PE at temperatures over 60 C caused excess hydrolysis, and less virus was removed. Low temperatures (4 and 25 C) did not produce sufficient hydrolysis; the optimal temperature for NaOH treatment of the polymer was 37 to 55 C.

**Elution of poliovirus from PE60.** Since poliovirus adsorption to PE60 was negligible in isotonic salt solutions at high pH levels, or in the presence of serum, such diluents were used to elute adsorbed virus. The test was performed by adsorbing poliovirus to NaOH-washed PE60, and then the PE-virus complex was distributed into tubes so that each tube contained 100 mg of PE and 5 × 10^6 PFU of poliovirus. The packed PE-virus complex was treated with different solvents, and the eluents were assayed. The experimental procedures and results of these tests are described in Table 1. Eluents used at low pH levels (7.5 and below) failed to elute the virus. However, virus could be efficiently recovered from the PE with phosphate buffer at pH 8 or with borate buffer at pH 9. Thus, virus can be efficiently recovered with eluents that by themselves are nonantigenic.

The elution efficiency depended on the pH achieved after the eluent was added to the packed PE. Borate at pH 9 added to a small amount of packed PE was affected by the acidic polymer, and pH levels of the PE-eluent mixture averaged 7.5 to 8.5. When 3 ml of borate buffer was added to 1 g of packed acidic PE which had

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**Fig. 2.** Effects of NaOH concentration on hydrolysis of PE60. Samples (100 mg) of PE60 were treated with 5 ml of NaOH at the concentration indicated for 0.5 hr at 25 C. The NaOH-washed PE preparations were then repeatedly washed with distilled water to remove residual base, and the packed PE preparations (100 mg per tube) were then treated with 5 ml of undiluted poliovirus at pH 3.5 for 1 hr on a rotary shaker. The tubes were then centrifuged, and the supernatant fluids were assayed for unadsorbed virus. Control virus held at pH 3.5 for 1 hr at 25 C on the rotary shaker did not lose titer.

Experiments had been arbitrarily chosen. To determine the effects on PE60 of different times of treatment with NaOH, 100-mg samples of PE60 were treated with 5 ml of 0.1 M NaOH at 25 C for the times indicated in Fig. 3. The results of this experiment indicate that treatment with NaOH for more than 0.5 hr did not greatly increase virus attachment to the PE. However, prolonged treatment (5 hr or longer) produced excess hydrolysis, and less poliovirus was adsorbed to the hydrolyzed products. Repeated experiments confirmed the above results and indicated that 0.5- to 3.5-hr NaOH treatment of PE60 at 25 C gave essentially the same results.

Since hydrolysis proceeds more rapidly at elevated temperatures, the effects of increased temperatures were determined. PE60 (100-mg samples) was treated with 0.1 M NaOH for 0.5 hr at 4, 25, 37, 50, 60, 70, and 80 C. The NaOH-treated PE preparations were then washed five times with large volumes of distilled water to remove residual NaOH. A 5-ml amount of undiluted

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**Fig. 3.** Effect of time of NaOH treatment on PE60. Same procedure as described in Figure 2, except that 0.1 M NaOH was used to treat PE60 for different periods of time at 25 C.
been used to adsorb virus at pH 4, the eluate was brought to acidic ranges, and elution was poor. Therefore, after addition of eluents to the PE, the mixture was monitored and readjusted to pH 9 to obtain efficient elution.

Effect of time and PE concentration on poliovirus adsorption. Under the optimal conditions for hydrolysis of the PE as described above, an experiment was conducted to determine the capacity of 100 mg of PE to adsorb excess virus. In duplicate experiments, the reaction of 400 mg of PE60 with 20 ml of undiluted virus was compared with the reaction of the virus with 25% of the amount of PE previously used (100 mg/20 ml of virus). The time was varied, as shown in Fig. 5. Although the 400 mg of PE60 used to adsorb the virus contained in the 20 ml of undiluted harvest removed virus from the supernatant more effectively, the 100 mg concentration adsorbed 99% of the virus within the first hour. When 100 ml of undiluted virus was treated with 100 mg of PE60 for 2 to 4 hr, again 99% of the virus was adsorbed.

This avoided the use of excess PE which would require undesirably large volumes of eluate.

Concentration of large volumes of poliovirus on PE60. Under the optimal conditions for adsorbing poliovirus to PE60 as described above, 1 liter of poliovirus (undiluted harvest containing 10⁸ PFU per ml) was concentrated, as shown in Fig. 6. PE60 (1 g) was added to 1 liter of virus at 25°C, the pH was adjusted to 3.5, and the mixture was stirred gently for 4 hr. The titer of unadsorbed virus recovered was 5.7 log₁₀, indicating adsorption of greater than 99% of the total virus population. Virus eluted from the PE reflected a

| Poliovirus treatment | Log₁₀ titer (PFU/ml) | Per cent adsorbed or eluted |
|----------------------|----------------------|-----------------------------|
| Control, untreated virus | 8.0 | >99.99% adsorbed |
| Virus-PE supernatant | 3.9 |  |
| Eluents | Eluate titer |
| Distilled water, pH 6.8 | <2.0 | 0% eluted |
| Saline, pH 6.8 | <2.0 | 0% eluted |
| Phosphate buffer, pH 8.0 | 7.8 | 63% eluted |
| Borate buffer (0.05 M), pH 9 | 7.9 | 81% eluted |
| Fetal serum (10%) in saline, pH 7.5 | 6.2 | 16% eluted |
| Fetal serum (10%) in pH 8 phosphate | 7.9 | 81% eluted |

* Undiluted poliovirus (30 ml) was added to 600 mg of PE60 which had been washed with NaOH as described above. The suspension was adjusted to pH 3.5 with HCl, and then was placed on a magnetic stirrer for 1 hr at 25°C. The suspension was centrifuged at 2,000 rev/min for 5 min, and the supernatant fluid was collected for assay. The packed PE-virus complex was resuspended in 30 ml of distilled water, and 5 ml of the well-mixed suspension was distributed into each of six tubes, giving a final concentration per tube of 100 mg of PE and 5 x 10⁶ PFU of poliovirus bound to PE. The tubes were centrifuged, and the supernatant fluids were discarded. The packed PE-virus complex was treated with 5 ml of the eluents indicated by vigorously shaking the PE-eluent mixture for a few seconds. The pH of the PE eluent was checked on a pH meter, and adjustments were made with NaOH to attain the original pH of eluent. The mixture was then centrifuged as described above, and the supernatant fluids were assayed for eluted virus.

b Sorensen's phosphate, NaH₂PO₄, 0.05 M.
Concentration of other acid-resistant viruses on PE60. A number of other acid-resistant viruses were treated with PE60 as described above. The experimental procedures and results are shown in Table 2. All viruses tested (coxsackievirus, echovirus, reovirus, and adenovirus) were adsorbed to the PE, and efficient elution of the viruses was accomplished in 0.1 volume. Thus, all viruses examined could be concentrated on and eluted from PE60 by use of a simple procedure.

Since acid conditions are required for maximal virus adsorption, conditions must be worked out

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\text{ASSAYS}
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\log _{10} \text{PFU/ml}
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+1 \text{ g of NaOH-hydrolyzed PE60, stirred 4 hr at pH 3.5, 25 C}
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\text{Filter through coarse pad to trap the PE}
\]

\[
\text{Filtrate}
\]

\[
\text{Trapped PE recovered and suspended in 3 ml of eluent}
\]

\[
\text{Eluate}
\]

\[
10.3
\]

\[
8.0
\]

\[
5.7
\]

60% recovery and a 200-fold concentration per ml.

Counterimmunoelectrophoresis gave precipitin lines at a dilution of 1:4 with the original virus harvest against antiserum made in rabbits against normal tissue culture extracts (MK). The 200× concentrate (eluate) failed to manifest a precipitin line even when tested without dilution against the same antiserum. Thus, although the virus was concentrated 200 times, the nonviral proteins were not concentrated, as they were not detectable in the eluate under the conditions of the test.

The adsorption of virus to the PE was enhanced by the filtration procedure used (see Fig. 6). Virus that had not collided with PE and become adsorbed during stirring was passed through a PE pad, thus increasing the efficiency of virus adsorption.
TABLE 2. Concentration of other acid-resistant viruses on PE60

| Viruses          | Log10 virus titer (PFU/ml) of | Virus-polycel- | No. of |     |     |
|------------------|------------------------------|----------------|--------|-----|-----|
|                  |                              | trolate mixture | Supernatant | Eluate |     |
| Type 1 poliovirus, LSc | 7.7                          | 2.5            | 8.6     | 8   |     |
| Echoviruses      |                              |                |         |     |     |
| Type 1           | 7.4                          | <2.0           | 8.3     | 8   |     |
| Type 7           | 8.2                          | 4.1            | 9.0     | 6   |     |
| Type 12          | 6.9                          | <2.0           | 7.8     | 8   |     |
| Coxsackieviruses |                              |                |         |     |     |
| A9               | 7.7                          | <2.0           | 8.7     | 10  |     |
| B3               | 7.6                          | <2.0           | 8.6     | 10  |     |
| Reovirus, type 1 | 7.5                          | 2.9            | 8.3     | 6   |     |
| Adenovirus, SV15 | 8.1                          | 3.9            | 9.0     | 8   |     |

* Undiluted virus (5 ml) was treated with 100 mg of PE60 (preshewed with NaOH as described) for 1 hr at 25 C at pH 3.5 on a magnetic stirring apparatus. The suspensions were centrifuged at 2,000 rev/min for 5 min, and supernatant fluids were assayed for unadsorbed virus. The packed PE was treated with 0.5 ml of borate buffer (pH 9.0) by vigorously mixing the PE-eluent mixture for a few minutes, and the supernatant fluids were assayed for eluted virus.

for acid-sensitive viruses (e.g., herpesvirus, myxovirus, vaccinia virus, and rhinovirus).

DISCUSSION

The concentration of acid-resistant viruses (enteroviruses, reoviruses, and adenoviruses) on exchange resins or adsorbents has not been practical as a routine laboratory procedure. In many cases, the methods employed are laborious and complicated. For example, the concentration of poliovirus on exchange resins (Dowex-1, anion) required that the virus harvest be exhaustively dialyzed (for 3 days) to remove interfering components that would exchange on the resin and prevent virus exchange (5), and then only 2.25 ml of virus could be passed through the resin per hour to obtain efficient concentration. The adsorption and elution of 10 enteroviruses to bentonite was dependent on a number of requirements (4): three strains were adsorbed at acid pH levels, but the degree of adsorption varied. Elution of the viruses required different solutions, and thus no single procedure was established for concentration and elution of the 10 viruses tested. Similarly, adenovirus concentration on diethylaminoethyl-cellulose columns required dialysis of the virus stocks, and then elution of exchanged virus at specific molarities of salts (2). Different serotypes required different molarities for elution off the column.

In the current study, we have shown that acid-resistant viruses preferentially adsorb to an insoluble PE in the presence of tissue culture proteins, salts, amino acids, and vitamins, ultimately yielding a virus concentrate in the form of an eluate low in nonviral protein. The method is simple; the requirements for adsorption to and elution from the PE of all viruses tested are essentially identical. Although each of the tables and figures describes a single experiment, the data are representative of many experiments carried out with each virus, which yielded essentially the same results. NaOH-treatment of PE enhanced adsorption of certain viruses, but such hydrolysis did not deleteriously affect the PE and did not prevent adsorption of those viruses which do not require NaOH treatment of the PE. Thus, hydrolysis of PE60 could be routinely performed so that all acid-resistant viruses could be concentrated by a single procedure. These methods have been adapted to routine use, and Table 3 indicates the parameters for concentration on PE60 of acid-resistant viruses present in different fluids. Table 3 also indicates the conditions for concentration of influenza viruses.

TABLE 3. Optimal conditions for adsorption of acid-resistant viruses and influenza viruses to PE60

| Virus fluids          | PE treatment | Optimal pH of PE-virus mixture | Reference    |
|-----------------------|--------------|-------------------------------|--------------|
| Acid-resistant viruses |              |                               |              |
| In tissue culture     | NaOH         | 2.5-4.5                        | Current study|
| harvests.....         |              |                               | 6            |
| In sewage.....        | Water only   | 4.0-6.0                        | 10           |
| In tap waters...      | Water only   | 4.0-8.5                        | 10           |
| In feces and urine...| Water only   | 4.0-5.0                        | 6            |
| Influenza             |              |                               |              |
| PR 8 strain in allantoic fluid | Water only | 5.5-6.0                      | —e           |
| Hong Kong strain in allantoic fluid | NaOH      | 5.0-5.5                      | —e           |

* In the absence of excess organics and in hypotonic fluids, PE60 adsorbs viruses at a wide pH range (10).

b Viruses stabilized against deleterious effects of excess hydrogen ions with ammonium sulfate (A. Homma et al., in preparation).

e A. Homma et al, in preparation.
The concentration of viruses on collodion membranes (8) is more laborious and requires more equipment than the procedures described in the present paper. Adsorption of viruses to aluminum salts (7), also used for virus concentration, presents a problem in that only limited quantities of fluids can be used before the alumina gel clogs the filtration system; further, the tissue culture harvests must be diluted 10-fold before use to reduce the protein concentration of the virus suspension because Al(OH)₃ binds proteins and depletes the available sites for virus adsorption.

One application of this study is the purification of viruses by adsorption to and elution from polyelectrolytes in the preparation of vaccines of good antigenicity with low reactivity as the non-viral proteins are removed. Another application is in the detection of viruses contained in natural fluids where they are often present in subinfectious quantities. Concentration of virus on PE60 from sewage, urine, feces, and tap waters has already been reported (1, 6, 10).

**ACKNOWLEDGMENT**

This investigation was supported by a grant from the Monsanto Co., St. Louis, Mo.

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