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# Chromatographic Separations of Alphavirus Strains by Hydroxylapatite

P. B. JAHRLING* AND J. L. BEALL

*U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701*

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Hydroxylapatite column chromatography methods were developed to characterize selected alphavirus populations. Different conditions of pH and phosphate molarity were required to obtain satisfactory elution profiles and separations for Western equine encephalomyelitis virus strains, compared with Eastern equine encephalomyelitis virus and Semliki Forest virus strains. Raising the pH of the buffers effected earlier elutions of all viruses. Selection of phosphate gradients with more gentle slopes and adjustment to the proper pH effected better separations of virus subpopulations. Elution profiles were not affected by 0.85% NaCl, 10% fetal calf serum, or 1% bovine serum albumin, which are common constituents of virus-stabilizing diluents. Passage of Western equine encephalomyelitis and Semliki Forest viruses in BHK-21, Vero, or duck embryo cell cultures or in suckling mouse brains did not usually affect elution profiles, unless passage also resulted in a shift in the plaque size marker. Essentially all infectious virus applied to the column was recoverable in appropriate fractions. This permitted accurate determinations of heterogeneity within alphavirus populations. For Western equine encephalomyelitis large-plaque (LP) and small-plaque (SP) virus populations, it was possible to detect ratios of 1 LP in a population of $10^6$ SP, and 1 SP in $10^3$ LP by using linear phosphate gradients. When stepwise elution procedures were used, it was possible to detect ratios of 1 SP in a population of $10^5$ LP. Hydroxylapatite column chromatography therefore appears to be a useful tool for characterizing alphaviruses and for isolating minority subpopulations of viruses of biological or epidemiological importance from apparently homogeneous virus stocks.

The applications of column chromatography methods, using calcium phosphate or its derivatives, to the purification and characterization of numerous viruses have been reported (4, 5, 7, 11-13). This paper describes procedures using the hydroxylapatite form of calcium phosphate to effect satisfactory separations of selected alphavirus strains including Western equine encephalomyelitis virus (WEE), Eastern equine encephalomyelitis virus (EEE), and Semliki Forest virus (SF) strains. Different conditions of pH and phosphate molarity were required for different alphaviruses. Previously published methods (12) for chromatographic separations of Venezuelan equine encephalomyelitis virus (VEE) on the brushite form of calcium phosphate were not useful for separating EEE or SF strains. In the present study hydroxylapatite was substituted for brushite since it was reported to afford better recoveries of some viruses, especially under alkaline conditions (5). This report establishes the utility of hydroxylapatite column chromatography for differentiating among selected alphaviruses and for detecting low levels of heterogeneity within apparently homogeneous virus stocks. Potentially practical applications of these procedures are discussed.

## MATERIALS AND METHODS

**Virus preparations and assays.** Large-plaque (LP) and small-plaque (SP) clones of WEE, derived from strain 72V1880, were described previously (8). EEE strains Panama and NJ 1945 were selected, as representative of North American and South American serotypes, and were obtained from Robert Shope, Yale Arbovirus Research Unit, New Haven, Conn., in first and eighth mouse brain passage, respectively. SF strains A774.C1.L1 and L10.C1 were selected because of their reported differences in virulence for laboratory rodents (3) and were provided by Claude Bradish, Microbiological Research Institute, Porton Down, England. Unless otherwise stated, viruses were passaged once in BHK-21 cells grown in 800-$cm^2$ roller bottles. Infected cultures were incubated with $^{32}P$ as monopotassium phosphate to label the viruses; cell culture supernatants were harvested, concentrated, and purified as described previously (10). Essentially all of the radioactive label in these preparations was associated with the virus particles. For one experiment, primary duck embryo cells (DEC) and Vero (African

green monkey kidney) cells were substituted for BHK-21 cells to prepare labeled virus. In that experiment, suckling mice were also inoculated intracerebrally with the WEE or SF viruses to prepare unlabeled virus suspensions. Infectious virus was assayed by counting plaque-forming units (PFU) in DEC monolayers maintained in 10-cm² wells of plastic plates under medium containing 1% agarose as described previously (9). Virus samples containing $^{32}P$ activity were also assayed by counting 50- or 100-$\mu$l samples, dissolved in 6 ml of Scintolute and Scintosol (4:1), in a scintillation counter. Quenching of $^{32}P$ activity was not a problem.

**Column chromatography technique.** Commercially prepared hydroxylapatite (Bio-Rad, HTP, lot 14607) was suspended in 6 volumes of starting buffer. After removal of the fine particles, the slurry was diluted to 2 volumes of starting buffer per g of hydroxylapatite, poured into columns (Pharmacia K-9, 0.9 cm in diameter), and allowed to settle. The packed bed was adjusted to 10 cm in length, and 45 ml of starting buffer was washed through the column. In preliminary experiments, columns were checked for channeling or skewing with bromocresol purple. The void volumes of these columns ranged from 5.5 to 5.8 ml. It was not necessary to use the indicator in routine experiments. Virus samples, usually suspended in 0.5 ml of NTE buffer (0.1 M NaCl-0.02 M tris(hydroxymethyl)aminomethane-hydrochloride-0.001 M ethylenediaminetetraacetic acid), were mixed with an equal volume of starting buffer and allowed to enter the column bed; 1.0 ml of starting buffer was then applied, and the gradient maker (Pharmacia, GM-1) was attached, producing a linear phosphate gradient. A total of 100 ml of buffer was used for each run. Columns were run under gravity (85-cm head pressure), and flow rates ranged from 1.2 to 1.5 ml/min. Elution buffers were prepared with $K_2HPO_4 \cdot 3H_2O$ and $KH_2PO_4$, dissolved in water at the proper concentrations and proportions to produce buffers of the desired pH's and molarities. Occasionally, for special purposes, 0.85% NaCl, 10% fetal calf serum, or 1% bovine serum albumin was added to the phosphate buffer solutions. Twenty 5.0-ml fractions were collected from each column with a photoelectric drop counter. Phosphate concentrations were determined colorimetrically by an adaptation of the method of Fiske and Subbarow (6) for determination of inorganic phosphorus (Sigma kit no. 670). Samples of each fraction were counted in the scintillation counter. If infectious virus assays were to be performed, 0.5 ml of fetal calf serum was added to each fraction to stabilize infectivity, and each sample was passed through a sterile 0.45-$\mu$m membrane filter (Millipore) to remove bacteria before titration on DEC monolayers. Columns were used only once before repacking and for only one virus at a time (except for the artificial mixtures described in the text). The figures are composites, with the elution profiles for two runs with different viruses plotted on the same axes.

## RESULTS

The adsorption and elution of the alphaviruses selected for this study from hydroxylapatite columns depended on two principal factors, the pH and the ionic strength of the phosphate buffers. Fig. 1 illustrates the effect of pH on the elution profiles of the LP and SP clones of WEE virus. The dimensions of the phosphate gradients were the same for all four pH values tested (0.05 to 0.40 M phosphate). At pH 6.0, neither virus clone was completely eluted from the column. At pH 6.5, the LP clone was eluted in a broad peak with a maximum at fraction 6 (0.13 M), whereas the SP clone was incompletely eluted. At pH 7.0, the LP clone was eluted in a sharp peak concentrated in fraction 2 (0.05 M), whereas the SP clone was eluted in a broad peak (maximum, fraction 10, 0.20 M). At the highest pH tested, 7.5, both viruses were eluted as sharp peaks, LP in fraction 2 (0.05 M) and SP in fraction 6 (0.13 M). Thus, when these viruses were chromatographed under high-pH conditions they were eluted earlier and in sharper peaks than they were under lower pH conditions.

A similar relationship between pH and elution profiles was observed for the SF and EEE viruses studied. However, the conditions for satisfactorily eluting these viruses were somewhat different from the conditions defined for the WEE viruses above. At pH 7, using a 0.05 to 0.40 M phosphate gradient, neither of the two SF virus strains was completely eluted from the column (Fig. 2A). To elute and satisfactorily separate SV-A774 from SF-L10, it was necessary to raise either the pH (Fig. 2B) or the phosphate molarity (Fig. 2C) of the gradient. It was not necessary to raise both pH and ionic strength simultaneously. A better separation of the elution peaks for the two SF virus strains was obtained by using a more shallow gradient (0.1 to 0.4 M), as opposed to the steeper gradient (0.1 to 0.8 M), at pH 8.0 (cf. Fig. 2B and D). Adjustment of the pH did not affect the positions of the two SF virus peaks relative to each other when gradients of similar phosphate dimensions were used (cf. Fig. 2C and D). Thus, better separations in the elution peaks of two

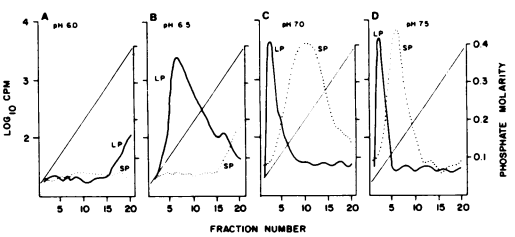


FIG. 1. *Elution profiles for $^{32}P$-labeled WEE LP and SP viruses on hydroxylapatite columns run at pH 6.0, 6.5, 7.0, and 7.5. Linear phosphate gradients began at 0.05 M and ended at 0.40 M.*

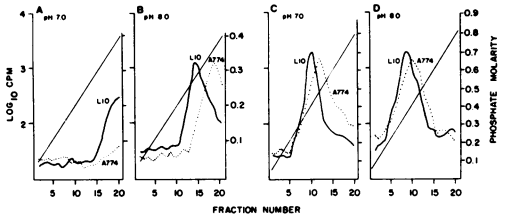


FIG. 2. *Elution profiles for $^{32}$P-labeled SF virus strains A774 and L10 on hydroxylapatite columns run at pH 7.0 and 8.0, with linear phosphate gradients ranging from 0.05 to 0.40 M and from 0.05 to 0.80 M.*

similar viruses were obtained by using less steep gradients, but not by altering the pH.

The following empirically developed procedure was found to be satisfactory for defining conditions to separate strains of virus whose chromatographic behavior was unknown. The approach is illustrated with two strains of EEE virus (Fig. 3). First, a shallow gradient (0.1 to 0.4 M, pH 7.0) was run. These conditions are often satisfactory for eluting many alphavirus strains (e.g., 42 strains of VEE [P. B. Jahrling, submitted for publication] and 2 WEE strains). However, in the case of EEE, one of the two strains was not completely eluted (Fig. 3A). The second step was to ensure elution of both viruses by raising both the pH and the ionic strength (Fig. 3B). At pH 8.0, using a steep gradient, both viruses were eluted, although the peaks were not well separated. Therefore, the third step was to use a less steep gradient at pH 8.0, whose extremes were defined by phosphate molarities slightly lower than the first peak and slightly higher than the second peak to be separated. On this basis, from Fig. 3B, a gradient whose dimensions were 0.3 to 0.6 M phosphate was selected. Use of this less steep gradient resulted in better separation of the elution peaks for the two EEE viruses (Fig. 3C).

The general applicability of hydroxylapatite chromatography to practical separations and characterizations of alphavirus populations in different laboratories depends on the extent to which certain variables influence the elution and recovery of infectious virus from the column. Anticipated variables would include the host cell origin and passage history of the virus and the presence of salts or serum in the virus suspension medium. To test the influence of these variables, elution profiles were determined for four viruses under a variety of conditions. The viruses tested were the LP and SP clones of WEE and the two strains of SF virus. All elution profiles were determined with 0.05 to 0.40 M phosphate gradients, adjusted to pH 7.0 for the WEE viruses and to pH 8.0 for the SF virus strains.

The addition of NaCl (final concentration, 0.85%), fetal calf serum (10%), or bovine serum albumin (1%) to the phosphate elution buffers did not significantly alter the elution profiles. Likewise, one passage of each of these viruses in Vero cells, DEC, or suckling mouse brain did not affect the elution profiles. In one experiment the LP clone of WEE was serially passed five times in DEC. The elution profile of this passaged virus was indistinguishable from that of the SP clone. However, this shift in elution profile was also accompanied by a shift in plaque morphology from LP to SP.

Figure 4 compares the elution profiles for the LP and SP clones of WEE virus, as determined by two methods, infectious virus (PFU) and $^{32}$P activity. The total activity of the label was much lower than that for infectious virus. However, the rapidly obtainable $^{32}$P profiles accurately reflected the infectious virus activities and could be reliably used to predict the fractions containing maximum infectious virus.

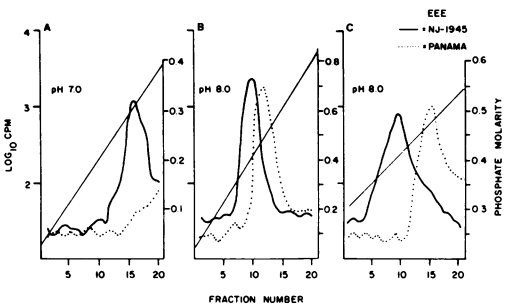


FIG. 3. *Elution profiles for $^{32}$P-labeled EEE virus strains NJ-1945 and Panama on hydroxylapatite columns run at pH 7.0 and 8.0, with linear phosphate gradients ranging from 0.05 to 0.40 M and from 0.30 to 0.55 M.*

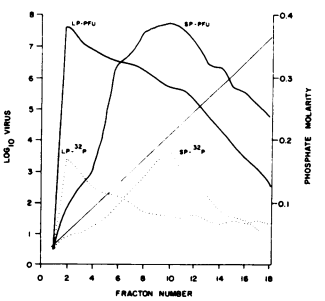


FIG. 4. *Comparison of infectious virus assays versus $^{32}$P label assays for determining elution profiles of WEE LP and SP viruses on hydroxylapatite columns, run at pH 7.0 with 0.05 to 0.40 M linear phosphate gradients.*

Greater than 90% of the total virus activity applied to the column, as measured by both PFU and $^{32}P$, was recovered in fractions 2 to 4 for the LP virus and 7 to 12 for the SP virus.

The recovery of essentially all input virus from predictable column fractions permitted an accurate determination of plaque size heterogeneity among SP and LP WEE virus populations. As seen in Fig. 4, the level of SP virus in fraction 2 was less than $10^{-5}$ the level in fraction 10, whereas more than half of the total LP virus applied to the column appeared in fraction 2. Since 1 LP is easily detectable in a population of 10 SP by standard plaquing procedures, it should be possible to detect 1 LP in a population of $10^6$ SP simply by plaquing fraction 2. To test the validity of these assumptions, an artificial mixture of LP and SP WEE virus clones was prepared, containing 4.4 $\log_{10}$ PFU of LP:9.9 $\log_{10}$ PFU of SP, and applied to the column. Fraction 2 was titrated on DEC and found to contain 4.3 $\log_{10}$ PFU of LP and 4.2 $\log_{10}$ PFU of SP. Thus, 4.3 $\log_{10}$ LP were detected in a virus population originally containing 9.9 $\log_{10}$ SP. The chromatography method was also useful but much less sensitive for detecting SP virions in a predominantly LP WEE virus population. Referring again to Fig. 4, in fractions 7 to 12 the level of LP virus was reduced to between 1 and 10% of the original proportion, and, thus, these fractions were proportionately enriched between 10- and 100-fold for SP virus. Since it is reasonable to detect 1 SP in a population of 10 LP, the 100-fold enrichment of the original LP virus population for SP virus in fractions 7 to 12 permitted detection of 1 SP in a maximum population of approximately 1,000 LP. The validity of these calculations was confirmed with artificial mixtures of LP and SP WEE viruses.

The sensitivity of the chromatographic technique to detect WEE SP in a predominantly LP population was enhanced still further by using a stepwise series of elution buffers. The elution behavior of WEE LP and the separation of a mixture of the two viruses by a stepwise procedure is presented in Table 1. The column was first eluted with 100 ml of 0.15 M buffer, pH 7.0, collected in 20 5-ml fractions. When 9.0 $\log_{10}$ PFU of WEE LP was applied, 8.9 $\log_{10}$ PFU was recovered in fraction 2. In the last fraction of the 0.15 M eluate, the LP virus titer was reduced by more than 4 $\log_{10}$ PFU. In contrast, when 9.0 $\log_{10}$ PFU of WEE SP was applied, no virus was recovered in any of the 0.15 M eluate fractions. However, when the buffer was shifted to 0.30 M, greater than 95% of the SP virus was recovered in fractions 2 and 3. When LP and SP viruses were mixed in a proportion of $10^7$:$10^2$ and subjected to stepwise elution, almost all of the LP virus was eluted early, so that fractions 2 and 3 of the 0.30 M eluate contained only 2.3 and 2.0 $\log_{10}$ PFU of LP, respectively. This permitted detection of the SP population in these fractions. Thus, a population of WEE SP virus was detected in the presence of WEE LP virus when the original proportion of SP to LP was 1:100,000. Stepwise elution is therefore the method of choice for detecting subpopulations of virus that elute later than the predominant populations. However, it is essential to first determine the elution peaks of the two viruses to be separated with a continuous gradient, to indicate the appropriate phosphate concentrations to use in this elution procedure.

## DISCUSSION

The data reported suggest that the technique of hydroxylapatite chromatography is a useful tool for detecting heterogeneity among alphavirus populations and for isolating virus subpopulations with biological properties different from the predominant population. The technique may also be useful for differentiating among different alphavirus strains. With appropriate minor modifications, the techniques should be broadly applicable to a wide range of studies of alphaviruses.

The methods used were developed empirically, adapted from the procedures initially used to separate encephalomyocarditis viruses

TABLE 1. *Recovery of infectious WEE LP and SP virus from hydroxylapatite columns by stepwise elution procedures*

| Phosphate buffer[a] | Fraction no. | Total PFU recovered ($\log_{10}$) | | | |
|---|---|---|---|---|---|
| | | LP[b] | SP[b] | Mixture (LP + SP)[c] | |
| | | | | LP | SP |
| 0.15 M | 1 | 3.2 | <0.7 | 3.0 | <0.7 |
| | 2 | 8.9 | <0.7 | 6.7 | <0.7 |
| | 3 | 7.4 | <0.7 | 6.4 | <0.7 |
| | 20 | 4.6 | <0.7 | 2.5 | <0.7 |
| 0.30 M | 1 | 4.5 | 2.2 | 2.2 | <0.7 |
| | 2 | 5.1 | 8.7 | 2.3 | 1.6 |
| | 3 | 4.9 | 8.4 | 2.0 | 1.7 |
| | 20 | 3.1 | 5.5 | 1.7 | <0.7 |

[a] Phosphate buffer (100 ml), pH 7.0, collected in 20 5-ml fractions.

[b] 9.0 $\log_{10}$ PFU of either WEE LP or WEE SP applied to the column.

[c] Mixture containing 7.0 $\log_{10}$ PFU of WEE LP plus 2.0 $\log_{10}$ PFU of WEE SP.

(4, 5) and subsequently adapted to VEE viruses (12). To obtain satisfactory elution profiles and separations for different alphaviruses, it was necessary to use different phosphate gradients at different pH values. Steep gradients ranging to high phosphate molarities usually effected complete elution of alphaviruses but poor separations between peaks. Raising the pH effected earlier elution of viruses at lower phosphate molarities; selection of phosphate gradients at the proper pH with more gentle slopes effected better separations of virus peaks. Alphavirus infectivities were stable under the conditions used, and it was possible to recover essentially all infectious virus applied to the column in appropriate fractions.

All studies were performed with a single lot of hydroxylapatite. Our experience has been that different lots of hydroxylapatite, even from the same manufacturer, have varying flow rates. If a simple phosphate gradient maker is used, variations in flow rate will influence the shapes of the phosphate gradients generated, and, thus, the elution profiles will also change. This difficulty can be circumvented by using a more elaborate apparatus to ensure that identical phosphate gradients are generated regardless of the flow rate or, more simply, by using a single lot of hydroxylapatite.

Since elution profiles are strongly influenced by changes in pH or gradient dimensions, it is difficult to compare our results with those reported by other investigators who have used similar, but not identical, techniques for alphaviruses. Pedersen et al. (12) reported that the elution peaks for VEE strains eluted from calcium phosphate columns at pH 7.4 ranged from 0.25 to 0.32 M phosphate. Fleming (7) reported that SF virus strains were eluted from hydroxylapatite columns by 0.6 to 0.7 M phosphate at pH 8.0 to 8.2, similar to our results (Fig. 2). He further described the influence of temperature on the elution profile of SF virus, a variable that we did not investigate. Elution profiles have not previously been reported for EEE strains. By carefully controlling the experimental conditions, it is possible to obtain very reproducible elution profiles, and, in fact, we have used these profiles to classify VEE viruses into groups which correlate closely with classification schemes using antigenic criteria (14; Jahrling, submitted for publication).

Further investigations of fresh fluid isolates and laboratory prototype alphavirus strains should be facilitated by the observation that passage in vertebrate cells did not influence elution profiles, unless, as in the case described for WEE LP, passage also resulted in a shift in the plaque size marker. However, whereas the elution characteristics of viruses on hydroxylapatite undoubtedly depend on surface charge, these determinants are not well defined (2). It is possible that passage of alphaviruses through cells other than those tested, especially mosquito cells, might impart different charge properties to the viruses, which would be reflected by altered elution profiles.

A further logical adaptation and extension of the method includes scaling down the volumes of the column and buffers. We have successfully duplicated the elution profiles for WEE LP and SP (Fig. 1C) with 2-ml columns and 25 ml of each buffer. Use of a scaled-down procedure might be necessary to conserve virus or to detect very low levels of virus that might go undetected if larger volumes of elution buffers were used.

Hydroxylapatite chromatography thus appears to be a useful tool that should provide information on the heterogeneity of alphavirus populations and may, perhaps, be adapted to the characterization of other viruses as well. It is anticipated that examination of fresh alphavirus isolates will reveal interrelationships among strains and heterogeneity within strains and may contribute to our understanding of the natural history of alphaviruses. Currently in this laboratory we are using the technique with the following objectives: (i) differentiation of antigenically distinct strains of VEE virus and examination of selected strains for detection of subpopulations with different antigenic properties; (ii) characterization of viruses isolated from individuals after immunization with live attenuated VEE vaccine, strain TC-83 (1), and comparison of these isolates with the virulent parent VEE strain and with the TC-83 inoculum; (iii) examination of TC-83 vaccine virus for heterogeneity; (iv) examination of low-passaged WEE virus strains with low virulence but high "reversion" frequency for the presence of virulent subpopulations of virus; and (v) isolation of candidate vaccine virus strains from virulent alphavirus stock suspensions.

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