Purification and Concentration of Influenza Types A and B by Chromatography on Calcium Phosphate

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Received for publication 4 February 1969

A rapid chromatographic method for the isolation of types A and B influenza virus from allantoic fluid was described. The adsorbent was prepared from calcium dihydrogen orthophosphate monohydrate \([\text{Ca}(\text{H}_2\text{PO}_4)_2\cdot\text{H}_2\text{O}]\) by alkali treatment. The addition of sodium trimetaphosphate to the influenza-infected allantoic fluid afforded a 67 to 100\% viral recovery and a 26 to 43-fold increase in purity.

A number of methods have been developed which free influenza virus from much of the extraneous protein found in the allantoic fluid harvested from the influenza-infected chick embryo (11). Currently, adsorption and elution from chick red cells and high speed centrifugation (Sharples) are used to isolate virus for vaccine production. Both of these methods have been criticized (10, 2), and the need still exists for a rapid method to isolate all of the influenza strains.

The high resolution made possible by chromatography has been used by Tiselius and co-workers to purify proteins on calcium phosphate columns (7, 8). This method was adapted to the isolation and purification of virus from influenza-infected allantoic fluid. Taverne et al. (6) reported a 30- to 100-fold purification of PR-8 influenza A with a recovery of 50 to 80\% from columns of brushite \([\text{CaHPO}_4\cdot\text{H}_2\text{O}]\). Kibardin and Boldasov (3) recovered 60\% of PR-8 influenza A and 62\% of influenza A\(_2\) with a 16-fold purification from columns of hydroxylapatite \([\text{Ca}_8(\text{PO}_4)_6\text{OH}]\). Neither of these investigators reported any experience with the other strains of influenza or attempted to purify large quantities of influenza-infected allantoic fluid.

Our lack of success in obtaining purified influenza type B in good yield from brushite or hydroxylapatite columns turned our attention to exploring the usefulness of other calcium phosphates (9). The objective of the present investigation was to develop a suitable adsorbent from calcium dihydrogen orthophosphate monohydrate, \([\text{Ca}(\text{H}_2\text{PO}_4)_2\cdot\text{H}_2\text{O}]\).

MATERIALS AND METHODS

Viruses. The following A and B strains of influenza virus, grown in chick embryos, were selected for study: PR-8/A, Ann Arbor/A\(_1\), Asian 170/A\(_2\), Asian 305/A\(_2\), Great Lakes/B, and Maryland/B.

Infected allantoic fluid. Ten-day chick embryos were inoculated in the allantoic sac with diluted seed virus in 0.1 ml of Brain Heart Infusion (Difco). After reinoculation for 48 hr at 33\(^\circ\)C, the eggs were cooled to 4\(^\circ\)C and the infected allantoic fluids were collected aseptically. The pooled fluid from each viral strain was adjusted with merthiolate to a concentration of 1:10,000 and refrigerated at 4\(^\circ\)C until used.

Viral assay. The chicken red cell agglutination value (CCA) for influenza was determined by the method of Miller and Stanley (5). The reference influenza virus used as the standard for the CCA values was obtained from the Division of Biological Standards, National Institutes of Health, Bethesda, Md.

Protein determination. Protein was determined colorimetrically (4). The purity of the virus was calculated from the specific activity (CCA per milligram of protein).

Reagents. All chemicals used were analytical reagent grade. Sodium trimetaphosphate (TMP) was obtained from Monsanto Co., St. Louis, Mo.

Preparation of adsorbent. Calcium dihydrogen orthophosphate monohydrate was prepared by the method of Jensen and Rathlev (1) and stored wet with acetone. The adsorbent was prepared in a sterile tank fitted with stirrer, thermometer, pH electrode, inlet ports for the addition of sterile solutions and water, and an outlet port for removing supernatant fluids and transferring adsorbent by positive air pressure to a sterile glass chromatography column. Calcium dihydrogen orthophosphate monohydrate (9 kg) was added to 36 liters of distilled water and the mixture was stirred vigorously for 15 min at
room temperature. The pH was increased from 2.1 to 6.4 by adding, with continuous stirring over a period of 30 minutes, 18.8 liters of a cold (4 C) solution of 3 n sodium hydroxide. The absorbent was stirred for 1 hr at room temperature and allowed to settle; the supernatant fluid was decanted. The absorbent was first washed with 30-liter portions of distilled water until the pH was 7.1, then with 0.03 M TMP (pH 9) until it reached 7.9. The absorbent was then transferred to a 9-inch diameter glass column (Table 1, experiment 4). The experimental variations in the adsorbent are recorded in Table 1.

**Purification of virus.** Maryland/B influenza-infected allantoic fluid (102 liters) was diluted with water 1:5, adjusted to pH 8, made 0.03 M in sodium trimetaphosphate, and allowed to flow through the adsorbent bed at the rate of 75.9 liters per hour. The column was washed with 30 liters of 0.03 M TMP (pH 9), and the virus was eluted with 0.5 M phosphate buffer (pH 6.8). The opalescent fraction eluted from the adsorbent (17 liters) contained the purified virus in suspension (Table 2, experiment 4). A similar sequence of steps, with some experimental variations, was incorporated in the purification experiments of the other strains.

**RESULTS AND DISCUSSION**

The investigation started with the evaluation of known calcium phosphate adsorbents for their usefulness in purifying types A and B influenza. Little difficulty was experienced in repeating and scaling-up the brushite columns of Taverne (6) to purify 16 to 18 liters of influenza PR-8/A-infected allantoic fluid. The experience with the purification of type B influenza was disappointing; only 10% of the virus (Great Lakes) was recovered. The difficulty was traced to the poor adsorption of the virus on the brushite. Hydroxylapatite proved equally disappointing as an adsorbent for Great Lakes influenza.

Since brushite and hydroxylapatite had only limited applicability, the investigation turned to other calcium phosphates. When calcium dihydrogen orthophosphate monohydrate was treated according to the method outlined above, a new adsorbent was obtained that would effectively isolate the A and B strains of influenza from influenza-infected allantoic fluid.

The addition of TMP to the allantoic fluid substantially improved the adsorption and subsequent recovery. There are a number of possible

| Expt | C{|(H_2PO_4)|_2}+H_2O | Initial pH | NaOH treatment pH | Water pH | TMP wash mol-arity | Final pH | Size of column |
|------|----------------|------------|------------------|----------|-------------------|----------|----------------|
| 1    | 3.1            | 2.3        | 6.6              | 7.1      | 0.005             | 8.0      | 6 by 14.5      |
| 2    | 5.7            | 2.2        | 6.5              | 7.0      | 0.01              | 9.0      | 6 by 15        |
| 3    | 7.3            | 2.2        | 6.9              | 7.3      | 0.01              | 7.9      | 9 by 12        |
| 4    | 9.0            | 2.1        | 6.4              | 7.1      | 0.03              | 7.9      | 9 by 12        |
| 5    | 7.3            | 2.1        | 6.7              | 7.2      | 0.01              | 7.9      | 9 by 8.2       |
| 6    | 8.5            | 2.1        | 6.4              | 7.1      | 0.01              | 8.2      | 9 by 9.7       |

* Refers to pH of C{(H_2PO_4)|_2}+H_2O and water mixture, 1:4 (w:v).

b Amount of TMP solution (pH 9), at molarity indicated, was used to adjust the adsorbent water mixture to the pH indicated in the next column.

c Adsorbent bed volume, diameter by height (inches).

**Table 2. Chromatographic purification of influenza**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Virus-infected allantoic fluid put through column</th>
<th>Virus recovered in eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain</td>
<td>Volume</td>
</tr>
<tr>
<td>1</td>
<td>PR-8/A</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>Asian 305/A_{2}</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>Great Lakes/B</td>
<td>332.5</td>
</tr>
<tr>
<td>4</td>
<td>Maryland/B</td>
<td>510</td>
</tr>
<tr>
<td>5</td>
<td>Asian 170/A</td>
<td>310</td>
</tr>
<tr>
<td>6</td>
<td>Ann Arbor/A_{1}</td>
<td>385</td>
</tr>
</tbody>
</table>

* Influenza-infected allantoic fluid diluted with water (1:5).

a Expressed in liters per hour.

b Purification factor is based on increase in specific activity of the purified virus.

c Specific activity is expressed in CCA per milligram of Lowry protein.
explanations to account for the effect of this cyclic polyphosphate. It may act by reversing the egg protein-influenza virus complex, thus releasing the viral particle, or it may complex with the egg protein, viral particle, and adsorbent in a manner favoring the adsorption of the viral particle.

The studies have indicated that considerable latitude in the chromatographic conditions is possible; the preparation of the adsorbent (Table 1) and the treatment of the influenza-infected allantoic fluid (Table 2) were varied widely without jeopardizing the recovery and purification of the influenza virus. The versatility of this method is shown by the 67 to 100% recovery of influenza A and B from the columns and by a 26- to 43-fold increase in purity (Table 2). The flow rates shown in the table represent the processing of 7 to 15 liters per hour of undiluted infected allantoic fluid. This is a considerable increase in the efficiency of isolating influenza over that of the Sharples method.

ACKNOWLEDGMENT

I appreciate the skillful technical execution of this study by Dennis Austin, James Sillaman, and supporting staff.

LITERATURE CITED