A Chromatographic Procedure for the Purification of Influenza Virus

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SUMMARY
Owing to the difficulties of achieving consistently good recoveries of virus from allantoic fluid containing influenza virus by chromatography on calcium phosphate columns the factors determining optimum yield were systematically investigated. It was found that calcium phosphate prepared by precipitation at a controlled pH of 7.5 gave best results provided that eluting buffers were adjusted to the same pH. Although calcium phosphate fractions were still evidently contaminated with non-viral material, further purification could be obtained by chromatography in agarose-gel columns. It appeared that separation of the virus on these columns was probably achieved as a result of the binding of virus surface groups on to the gel. A minimum overall purification factor of about 800-fold was achieved.

INTRODUCTION
The purification of influenza virus has classically been approached in two ways: (i) non-specific concentration from allantoic fluid by preparative ultracentrifugation, (ii) specific adsorption to and elution from red blood cells or inhibitors coupled to cellulose (Curtain, 1954). These methods are frequently combined with conventional chromatographic methods such as CaHPO₄ (Taverne, Marshall & Fulton, 1958) or DEAE, to provide a final product of maximum purity. However, various strains of virus, notably the inhibitor-sensitive A2 strain, give poor yields in this type of procedure because (a) the elution rate from red blood cells is very low (Choppin & Tamm, 1960) and (b) the amount of irreversible adsorption on calcium phosphate columns is high. The present work was undertaken in order to improve the yield and purity of the product and to reduce the processing time.

METHODS
Preparation of calcium phosphate. Calcium phosphate was prepared (Tiselius, Hjerten & Levin, 1956) and standardized (Main, Wilkins & Cole, 1959) as follows: 100 ml. of 0.5M-CaCl₂ was added to 120 ml. of 0.5M-phosphate buffer at the rate of 1 drop per sec. with constant stirring at room temperature, and the calcium phosphate recovered by decantation after standing.

Preparation and calibration of agarose columns. A column of agarose beads (Hjerten, 1962) [4% (w/v) gel, 120 mesh] was prepared and calibrated with Escherichia coli.
(strain CL260), bacteriophage φX174 and horse serum α-2 macroglobulin (γ-inhibitor). Assuming the *E. coli* to be completely excluded from the gel beads and the absence of non-specific adsorption effects, the column dimensions were determined as indicated in Table 1.

*Propagation of influenza virus strains.* Allantoic fluids in capillaries thawed from −60° were diluted in nutrient broth containing crystalline penicillin (2500 u./ml.) and streptomycin hydrochloride (3000 μg./ml.) and inoculated into 11-day-old chick embryos. The embryos were incubated for 49 hr at 36°, and then chilled overnight at 4° before the allantoic fluids were harvested and pooled.

<table>
<thead>
<tr>
<th>Table 1. Dimensions of agarose column used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of beads and solvent (V₀ + V₁ + V₂) = 47·0 ml.</td>
</tr>
<tr>
<td>Internal volume of solvent (V₁)          = 30·0 ml.</td>
</tr>
<tr>
<td>Void volume of solvent (V₂)             = 16·0 ml.</td>
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</table>

*Virus strains.* Two stock strains of influenza virus were used: (a) A2 Singapore/57 'sensitive'—a γ-inhibitor-sensitive substrain derived in this laboratory from the A2/Singapore/57 variant originally obtained from Dr J. C. N. Westwood at M.R.E. Porton, in 1958; (b) PR 8—a subline of the classical type A virus strain, maintained in this laboratory since 1948. Both these strains were maintained as stock seeds with infrequent allantoic passage.

*Haemagglutination titration.* Virus haemagglutination (HA) was assayed using the standard ‘pattern’ method. Twofold dilutions of virus material were made in 0·5 ml. volumes of borate-buffered physiological saline (pH 7·6) in the cups of a WHO plastic tray. One half ml. of 0·5 % (v/v) fowl red cell suspension standardized densitometrically was added to each dilution with an automatic syringe and the test was then allowed to stand for 1 hr on the bench at room temperature. The end-point was defined in terms of an arbitrary partial agglutination pattern. For the screening of samples derived from a column, 0·5 ml. of each sample was pipetted undiluted into the cup of a plastic tray, and 0·5 ml. of 0·5 % (v/v) fowl cell suspension added to each sample, as before. Positive samples were then titrated for haemagglutinating potency.

*Haemagglutination inhibition assay.* The ‘pattern’ test was also used for assaying haemagglutination inhibition. Twofold dilutions of material for assay were made in 0·25 ml. volumes of borate-buffered physiological saline in plastic trays. To each dilution, 0·25 ml. of challenge virus diluted to contain 8 agglutinating doses was added, followed by 0·5 ml. of 0·5 % (v/v) fowl red-cell suspension. The test was read after 1 hr using the same pattern end-point as in the haemagglutination assay. The challenge dose used was checked by including a titration of the challenge material with each set of inhibition assays.

*Preparation of bacteriophage φX 174.* Sensitive *Escherichia coli* strain CL260 was inoculated into nutrient broth, and after 4 to 5 hr incubation 5 ml. of culture was inoculated with 0·5 ml. of undiluted stock preparation of bacteriophage φX 174, kindly made available by Professor G. G. Meynell of the Lister Institute of Preventive Medicine. After further incubation for 18 hr the host culture was centrifuged at c. 3000 rev./min. for 10 min. and then filtered through a collodion membrane (Oxoid).
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of sterilizing grade. The filtrate was assayed by a plaque count adaptation of the Miles & Misra (1938) method for surface viable counts.

Sample assay for φX174. Column samples were spotted with a bacteriological loop on to nutrient agar plates, seeded with young broth culture of Escherichia coli cl.260, and the plates then incubated overnight at 37°.

Sample assay for Escherichia coli. Column samples were streaked with a bacteriological loop on MacConkey plates, followed by overnight incubation. The test was used merely to detect presence or absence of the organism, and no attempt at quantitation was made.

Horse serum α₂-macroglobulin (γ-inhibitor). This material was prepared by the method previously described (Biddle, Pepper & Belyavin, 1965) and was used as a solution containing 20 mg./ml. in physiological saline. The preparation used was known from previous analytical ultracentrifugation experiments to contain both monomer and dimer.

Preparation of samples for electron microscopy. Fractions were centrifuged at 35,000 rev./min. in an SW39 rotor for 30 min., dialysed against 0.15M-ammonium acetate overnight at 4° and negatively stained on carbon-coated grids with 2% sodium phosphotungstate previously adjusted to pH 7.0 with KOH. Preparations were examined at a magnification of 40,000.

RESULTS

Determination of optimal conditions for preparation of calcium phosphate

Using the standard method of mixing the basic reagents, batches of calcium phosphate were precipitated in a series of buffers in the range pH 6.7 to 8.8; the resulting precipitate was washed several times by decantation in 0.005M-buffer and then packed into columns of 2.5 cm. diameter with glass wool bottom plugs, giving column lengths of 6 to 12 cm., depending on the pH of precipitation. Calcium phosphate

Table 2. Calcium phosphate chromatography of influenza virus: the variation of overall yield of virus HA with pH of precipitation of calcium phosphate

<table>
<thead>
<tr>
<th>pH of precipitation (CP no.)</th>
<th>6.7</th>
<th>7.0</th>
<th>7.3</th>
<th>7.6</th>
<th>7.9</th>
<th>8.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield of HA in model exp.</td>
<td>13%</td>
<td>40%</td>
<td>47%</td>
<td>52%</td>
<td>5%</td>
<td>12%</td>
</tr>
</tbody>
</table>

batches prepared at different pH values were designated CP followed by the appropriate pH value. Ten ml. volumes from the same batch of infected allantoic fluid were layered on to each column, washed in with 1.25 void volumes of 0.2M-buffer and finally eluted with 1.25 void volumes of 0.9M-buffer. All buffers used for washing and elution were of the same pH as that used for precipitating the calcium phosphate sample. All fractions were titrated for haemagglutinin (Table 2). On the basis of these results, all subsequent work was done at pH 7.5. The skewness of the pH virus recovery curve represents, in part at least, the error of using elution buffers of constant molarity instead of constant ionic strength. Thus the ionic strength of 0.9M, pH 6.7 buffer is 1.66 compared to that of 0.9M, pH 7.6 buffer which is 2.48. More virus can be removed from a CP 6.7 column by washing with pH 7.6 buffer, but this procedure should not be used because the structure of the calcium phosphate probably changes
under these conditions (manifest by a change in volume). The phosphate precipitates were observed to change continuously in packed volume from 30 ml. at pH 6.7 to 60 ml. at pH 8.8 with a uniform decrease in the flow rate. Main et al. (1959) showed the denser form (CP 6.7) to be secondary calcium orthophosphate (CaHPO₄) and the lighter form (CP 8.8) to be brushite (CaHPO₄·2H₂O), the latter form being metastable with respect to the anhydrous form above pH 7.0; for this reason the CP 7.5 preparation should always be freshly prepared.

![Fig. 1. The separation of allantoic cell fragments from influenza virus on agarose beads.](image)

**Adsorption to and elution from calcium phosphate of influenza virus**

Fifty ml. of packed CaHPO₄ prepared as described was suspended in 1 l. of allantoic fluid adjusted to 0.15M-PO₄, pH 7.5, by the addition of a calculated amount of solid NaH₂PO₄ + Na₂HPO₄. The CP 7.5 was then packed into a small column and washed with one void volume only of the same buffer (0.2M-PO₄). The virus was then eluted in a narrow yellow band with 0.9M-PO₄, buffer pH 7.5 and collected by the formation of a density gradient of 0.9M-buffer underneath 0.2M-buffer. The fraction containing virus (about 15 ml.) was centrifuged for 30 min. at 39,000 rev./min. in a Spinco SW39 rotor (at 20°) when the egg-yolk lipoprotein floated to the top of the buffer and the virus, together with some allantoic cell fragments, was sedimented. When resuspended in 0.5 ml. of borate saline, virus thus concentrated consistently had an HA titre of >0.5 x 10⁶ and was stable for 24 hr if freshly prepared.
Fractionation of virus concentrate on agarose

The crude virus concentrate prepared by calcium phosphate adsorption and elution as described in the previous section was layered on top of a column of 4 % (w/v) agarose beads in a column 1.6 x 23 cm. and eluted at 25 ml./hr with 0.15M-NaCl containing 0.02 % (w/v) NaN₃. Two peaks of protein were observed (Fig. 1). The first peak, which appeared at the void volume (Kᵥ = 0.00) and which was devoid of haemagglutinating activity, was found on electron microscopy to contain only amorphous particles, presumably cell fragments; the second peak (Kᵥ = 0.19) contained active haemagglutinin and under the electron microscope only typical virus particles were seen. This process was repeated using PR8 and another preparation of A2 (inhibitor-sensitive) virus prepared in the conventional way from red-cell eluates and calcium phosphate. The haemagglutinin activity eluted in exactly the same volume (Kᵥ = 0.19) and the contaminating low molecular weight protein at Kᵥ = 0.88. Owing to the low virus content of these samples it was not possible to determine the optical density accurately for PR8; however, the proportion of viral to contaminating protein in the original preparation was estimated as being approximately 1:3.

DISCUSSION

There is evidence that calcium phosphate prepared under different conditions may give products of varying Ca/P ratio but with similar lattice structures. These non-stoichiometric precipitates (with varying amounts of phosphate deficiency in the lattice) also have a varying particle size and therefore surface area. The affinity for any phosphate containing molecules in solution (Kibardin, 1965; Bernardi, 1965) will therefore vary with the condition of preparation. The optimum for chromatography of the influenza virus must represent a balance between two opposing factors such as very strong binding and irreversible adsorption on the one hand and very weak binding and competition by other phospholipids in the allantoic fluid on the other.

Several workers have used agar molecular sieves to purify viruses (Steere & Ackers, 1962a, b; Steere, 1963; Bengtsson & Philipson, 1964), but the larger tobacco mosaic virus and influenza viruses have probably never been resolved by this method as they usually appear at the void volume (Cech, 1962; Bengtsson & Philipson, 1964). The pore radius of agarose gels may be roughly estimated from the data for agar (Polson, 1961; Hjerten, 1962, 1964; Ackers & Steere, 1962) to be 200 Å in a 4 % (w/v) gel ranging up to around 1200 Å in a 1 % (w/v) gel. Thus influenza virus (500 Å radius) behaves anomalously on 4 % (w/v) gel in not eluting at the void volume (determined by Escherichia coli to be 34 % of total column volume, compared with a theoretical value of 27 % for tightly packed spheres). This behaviour may be confirmed by estimating the apparent Stokes radius of the virus by comparison with well-characterized molecules which have been run immediately before and after the virus on the same column. This was done by the method of Siegel & Monty (1966), giving an apparent value of 242 Å (Table 3). This is so far removed from the known value of 500 Å radius that, even allowing for the errors of the extrapolation, the virus is clearly behaving anomalously.

Agar normally contains about 3 % (w/v) of sulphonate groups present either as
the half or full ester; \( R_1-\text{SO}_3-\text{H} \) and \( R_2-\text{SO}_3-\text{R}_2 \) of amylopectin, of which only the former gives rise to ionic adsorption phenomena. Therefore, in the sample of agarose used with total sulphonate content < 0.2 \( \% \) (w/w), the amount of adsorbing sulphate was negligible and the retardation of influenza virus was unlikely to be due to such an ion-exchange mechanism.

Table 3. **Estimation of apparent Stokes radius of influenza virus from agarose gel filtration data based on column elution using 4% (w/v) gel**

<table>
<thead>
<tr>
<th>Substance</th>
<th>A 2 virus</th>
<th>( a_2-\text{MG} ) dimer</th>
<th>( \phi X174 ) monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_s^* )</td>
<td>0.190</td>
<td>0.226</td>
<td>0.464</td>
</tr>
<tr>
<td>Approx. Stokes radius†</td>
<td>242 Å</td>
<td>227 Å</td>
<td>150 Å</td>
</tr>
</tbody>
</table>

* Using the relation \( K_s = (V_E - V_0)/V_t \), where \( V_E \) is the eluted volume of any given component.
† Determined by the method of Siegel & Monty (1966).

Table 4. **Specific activities observed in the successive purification steps of influenza virus expressed as haemagglutination units per µg. protein**

### Calcium phosphate step: 1000 ml. of allantoic fluid (320 HA/ml.) adsorbed in 0.2M- and eluted in 0.9M-PO₄ buffer, centrifuged and resuspended in 0.5 ml.

<table>
<thead>
<tr>
<th>Sample on</th>
<th>Protein (mg.)</th>
<th>Total activity (HA units)</th>
<th>Specific activity (HA/µg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,720</td>
<td>320,000</td>
<td>0.118</td>
<td></td>
</tr>
<tr>
<td>Recovered total</td>
<td>6.5</td>
<td>330,000</td>
<td>50.8</td>
</tr>
</tbody>
</table>

Purification factor: 430

### Agarose step: 0.025 ml. of concentrate (40,000 HA/ml.) eluted in 2 hr and 9 active fractions pooled, total volume 7.8 ml.

<table>
<thead>
<tr>
<th>Sample on</th>
<th>Protein (mg.)</th>
<th>Total activity (HA units)</th>
<th>Specific activity (HA/µg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.325</td>
<td>1000</td>
<td>3.08</td>
<td></td>
</tr>
<tr>
<td>Recovered total</td>
<td>0.071</td>
<td>430</td>
<td>6.06</td>
</tr>
</tbody>
</table>

Purification factor: 1.97

The separation of the (chemically) similar virus and cell fragment impurities points to a more specific mechanism, of which the most likely would involve neuraminidase and/or haemagglutinin on the virus surface interacting with galactose residues in the gel matrix (galactose or its acetylated hexosamine are almost invariably the sugars to which sialic acid, the substrate of neuraminidase, is linked in glycoproteins). A similar retardation has been noted (Squire, 1964) for other carbohydrates on dextran columns and experiments in this laboratory with free neuraminidase from *Vibrio cholerae* indicate that it elutes with a \( K_s \) of 1.50, clearly indicating a specific adsorption to the agarose column.

The determination of overall purification factors was hampered by the extreme lability of highly concentrated influenza virus preparations; these factors were therefore calculated on HA titres observed immediately before and after each experiment (Table 4). It is interesting to note that an overall purification factor of 860-fold (assuming no lability of HA activity) would give a material of specific activity
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100 HA/μg. protein compared with the theoretical limit of 200 HA/μg., assuming a particle/HA ratio of 10⁷ and a protein content of 75%.

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REFERENCES


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