Purification and Fractionation of Alfalfa Mosaic Virus with Polyethylene Glycol

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SUMMARY

Preparations of alfalfa mosaic virus made by precipitation with polyethylene glycol were as infective and contained the same relative proportions of components as virus preparations made by ultracentrifugation. A column chromatographic procedure using a continuously decreasing concentration gradient of polyethylene glycol was employed to fractionate the nucleoprotein components of the virus. By this procedure a partial sorting of the virus components into three groups according to particle length was achieved. Particles in the three groups are thought to correspond to top component \(a\), top component \(b\), and a mixture of middle and bottom components.

INTRODUCTION

Alfalfa mosaic virus is unusual among plant viruses in that purified virus preparations contain several nucleoprotein components having different lengths. A model for the structure of the larger particles was proposed by Gibbs, Nixon & Woods (1963). Recent investigations (Kelley & Kaesberg, 1962; Gibbs et al. 1963) suggest that each type of particle sediments at a different rate. Attempts have been made to separate the various types of particle by means of sucrose density gradient centrifugation but with only partial success because of their relatively close rates of sedimentation. Recently, polyethylene glycol solutions have been employed at various strengths to precipitate virus particles (Hebert, 1963; van Kammen, 1967), although its mode of action in precipitating virus is not fully understood (Leberman, 1966). This paper reports on the use of polyethylene glycol both to prepare alfalfa mosaic virus and to fractionate the virus by a chromatographic procedure which has afforded some success in separating three of the four known components.

METHODS

**Virus.** The strain of alfalfa mosaic virus used was isolated from white clover in New Zealand, and has been propagated in *Nicotiana tabacum* L. var. Samsun N N and *N. glutinosa* L. Virus was extracted from systemically invaded leaves of *N. tabacum* or *N. glutinosa* 8 to 14 days after inoculation. A mortar and pestle were used to grind leaves in 0.2 M-phosphate buffer, pH 6.5 (1.5 ml./g. leaf), containing 0.5% mercaptoethanol. The slurry was squeezed through muslin and shaken with 20% (v/v) chloroform, followed immediately by centrifugation for 5 min. at 14,000 rev./min. in the SS 34 rotor of a Sorvall RC 2 B refrigerated centrifuge. Excess chloroform was removed by blowing air on to the surface of the continuously stirred liquid. The virus was then...
either sedimented at 39,000 rev./min. for 1 hr using a Spinco no. 40 rotor, or precipitated with 8% polyethylene glycol, mol. wt. = 20,000 (L. Light and Co. Ltd) in 0.2M-NaCl and centrifuged at 14,000 rev./min. for 10 min. Precipitated virus was resuspended in water, but virus prepared by ultracentrifugation was resuspended in 0.01 M-ammonium acetate, pH 6.5.

Local lesion assays were made on fully expanded primary leaves of *Phaseolus vulgaris* L. var. Topcrop, 4 to 8 half-leaves being inoculated per sample. Lesion numbers were recorded 4 to 6 days after inoculation. Serological tests were made by the precipitin method in tubes.

Chromatography. Virus to be chromatographed was reprecipitated with 8% polyethylene glycol, mol. wt. = 20,000, and 0.2M-NaCl, and mixed with about 2 g. fine-grain acid-washed kieselguhr (British Drug Houses). The slurry was introduced under pressure into a conical column (maximum diameter 2.5 cm.) over a 1 cm. bed of Celite 545. The packed column was washed with 10% polyethylene glycol, mol. wt. = 6000, 0.2M-NaCl and 0.05 M-phosphate buffer, pH 6.5, until no change in u.v. absorbence by the eluate was observed as determined with a continuous flow ISCO Model UA2 u.v. analyser. Virus was eluted from the column by means of a nonlinear decreasing gradient of polyethylene glycol, mol. wt. = 6000, in the presence of 0.2M-NaCl and 0.05 M-phosphate buffer, pH 6.5. Approximately 2.5 ml. fractions were collected. Buffers of pH values less than 7.0 gave better separation than those greater than pH 7.0. After suitable dilution, absorbence of the fractions was determined at 260 nm. with a Beckman DU spectrophotometer.

Electron microscopy. Virus suspensions to be examined by electron microscopy were treated with 1% formaldehyde for 10 min. (Gibbs et al. 1963) before mixing them with potassium phosphotungstate. The suspensions were sprayed on carbon-coated grids and examined and photographed at a magnification of ×9000 (Philips EM 200). Particle length measurements were made from electron micrographs with a calibrated light microscope. A leaf-dip preparation of tobacco mosaic virus, 2890 Å length (Markham et al. 1964), was used to calculate the actual size of the alfalfa mosaic virus particles.

RESULTS

Preparation of virus using polyethylene glycol

Comparisons were made by analytical ultracentrifugation, by electron microscopy and by infectivity tests between alfalfa mosaic virus prepared by ultracentrifugation and by polyethylene glycol precipitation. Clarified sap from 10 g. infected *Nicotiana glutinosa* leaves was divided into two equal portions. Alfalfa mosaic virus was extracted from one portion by sedimentation at 39,000 rev./min. for 1 hr, and from the other portion by treatment with polyethylene glycol, mol. wt. = 20,000, followed by sedimentation of the resuspended precipitate to remove residual polyethylene glycol. The pellets were resuspended in equal quantities of 0.01 M-ammonium acetate and examined in a Spinco Model E analytical ultracentrifuge. Infectivity measurements were made with two dilutions of each preparation as well as with equivalent dilutions of the original clarified sap (Table 1). Virus prepared by polyethylene glycol precipitation was at least as infectious as virus prepared by centrifugation alone. Only slight differences were observed between the two preparations by area measurement of the schlieren profiles of the viral components, although low-molecular-weight constituents present
in the centrifuged preparation were not apparent in the preparation made by precipitation. The two preparations were examined by electron microscopy and particle lengths measured (Fig. 1 D). Four distinct lengths of particle were observed in both preparations, the average lengths being 280, 360, 440 and 580 Å. These four types of particle presumably correspond to the top component a, top component b, middle component and bottom component, respectively, described by Kelley & Kaesberg (1962).

Table 1. Infectivity of alfalfa mosaic virus in clarified sap and in preparations made by ultracentrifugation and by precipitation

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Clarified sap</th>
<th>Method of virus preparation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Ultra-centrifugation</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>223*</td>
<td>192</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>26</td>
<td>8</td>
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* Total numbers of lesions produced. Each sample inoculated on four half-leaves.

Fig. 1. Particle length distribution of unfractionated AMV and of AMV fractionated by column chromatography. (A) fraction A from column, (B) fraction B from column, (C) fraction C from column, (D) unfractionated virus.
Separation of nucleoprotein components

In initial experiments using column chromatography to separate the nucleoprotein components, virus was precipitated from clarified sap by the addition of 8% polyethylene glycol, mol. wt = 20,000, in 0.2 M-NaCl. The precipitated virus was mixed with a small amount of Celite 545 (1 to 2 g.) and packed in a conical column. After washing, virus was eluted from the column as described in Methods.

The u.v. absorbence profile of the eluate varied from experiment to experiment. The virus was usually eluted in two major fractions. Electron microscopy showed that the first fraction to be released from the column contained mainly the smaller virus particles with lengths < 330 Å and in the range 330 to 390 Å. A small number of 400 to 500 Å particles were present and these also occurred in the second fraction from the column. However, the bulk of the particles present in the second fraction were 520 to 640 Å in length. Examination in the Spinco Model E confirmed these results, i.e. that the first fraction obtained consisted of mainly top a and top b component particles while the second fraction contained mainly bottom component with middle component particles occurring in both fractions.

Subsequent attempts to obtain better fractionation were made by using a finer grade of Celite, because experiments with other viruses had indicated that both with coarser grades of Celite and with cellulose, elution of virus from the column began before the virus was completely resuspended from the precipitated state. In the following experiment, typical of several, virus was precipitated from clarified sap and centrifuged. The sediment was resuspended in dilute buffer and clarified by centrifuging for 5 min. at 14,000 rev./min. After adjustment to 0.2 M-NaCl, virus was again precipitated by addition of solid polyethylene glycol, mol. wt = 20,000, to a concentration of 8% and

![Graph showing the separation of alfalfa mosaic virus components by column chromatography using a decreasing concentration gradient of polyethylene glycol.](image-url)
Fractionation of alfalfa mosaic virus

mixed with about 2 g. fine grade kieselguhr. The slurry was introduced into a conical column over a thin layer of kieselguhr and washed free of contaminating substances with 10% polyethylene glycol, mol. wt = 6000, 0.2 M-NaCl, and 0.05 M-phosphate buffer, pH 6.3. Virus was eluted from the column using a nonlinear 10% to 0% continuous gradient of polyethylene glycol, mol. wt = 6000 (Fig. 2). The high background of u.v. absorbance was due principally to the presence of polyethylene glycol itself. It was not possible to reduce the u.v. absorbance by the polyethylene glycol by dialysis, solvent precipitation, ether extraction or mixing with activated charcoal. Polyethylene glycol, mol. wt = 6000, was used for the gradient rather than polyethylene glycol, mol. wt = 20,000, as the u.v. absorbance of the latter was several-fold greater than that of polyethylene glycol, mol. wt = 6000.

The eluate contained three major groups of particles from which virus components were removed by sedimentation. The pellets were suspended in dilute ammonium acetate and examined in the electron microscope. For each group the lengths of all particles in each of several electron micrographs were measured (Fig. 1 A–C). The data confirmed the results of earlier experiments that the particles had been sorted by size. The smaller classes of particle were partially separated from each other and from the two larger-sized particles. All three fractions reacted with antiserum to unfractionated virus.

DISCUSSION

The unusual structure of alfalfa mosaic virus among plant viruses has recently generated considerable interest among workers in plant virology. The instability of this virus and the difficulty of adequately separating the various nucleoprotein components have resulted in only slow accumulation of information on their properties. In contrast to earlier reports (Bancroft & Kaesberg, 1960; Frisch-Niggemeyer & Steere, 1961) more recent investigations (Kelley & Kaesberg, 1962; van Domelen & Beeman, 1962; Gibbs et al. 1963) indicate that components with different sedimentation rates are in fact particles with different lengths. This conclusion is supported by the present results.

The use of polyethylene glycol to precipitate virus from clarified or partially clarified sap may result in a more rapid removal of virus from inhibitors and degradative enzymes than is possible by ultracentrifugation alone. No tests were made to ascertain the stability of the virus in the precipitated state, but there was no immediately obvious deleterious effect on virus infectivity. More efficient precipitation of virus occurred with polyethylene glycol, mol. wt = 20,000, than with polyethylene glycol, mol. wt = 6000, but high u.v. absorbance by solutions of the greater mol. wt polyethylene glycol precluded its use as an eluent during chromatography. In tests with polyethylene glycol, mol. wt = 20,000, from various sources, a considerable variation in u.v. absorbance by equivalent strength solutions was observed. Presumably the u.v. absorbance of these solutions was due to the presence of impurities. The impurities themselves may adversely affect virus stability. None of the purification steps used significantly altered the u.v. absorbance of the polyethylene glycol solution.

Several plant viruses have been purified by column-chromatographic procedures in which polyethylene glycol solutions were used as eluents (Venekamp & Mosch, 1963, 1964; Venekamp, Mosch & Noordink, 1966). Cellulose was used to retain the precipitated virus in the column. In the present experiments kieselguhr was used in
preference to cellulose because of the possibility of nonspecific adsorption of virus to cellulose. In addition better retention and separation of virus components was obtained with kieselguhr than with cellulose or the coarser Celite, both of which allowed elution of virus components from the column to begin before complete resuspension had occurred. The kieselguhr seemed to act as a filter for the finely dispersed precipitate, separation of the components depending on differential solubility in the polyethylene glycol-containing eluent.

Although complete separation of the nucleoprotein components of alfalfa mosaic virus was not obtained by column chromatography, the results were sufficiently good to encourage further investigations on possible modifications to the chromatographic system.

The three fractions obtained from the column consisted mainly of particles thought to correspond to top a component, top b component and a mixture of middle and bottom components. No tests were made to determine recoveries of the various components. In experiments in which all fractions were combined and rechromatographed there was a considerable reduction in the amounts of top a and top b components. Whether this loss was due to incomplete precipitation or to degradation of these components is not known. However, nearly quantitative recoveries of middle and bottom component were achieved, indicating that the chromatographic system could be of use in investigations in which it is necessary to separate the two smaller from the two larger components.

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REFERENCES


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