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CHROMATOGRAPHIC PURIFICATION OF PLANT VIRUSES ON CELLULOSE COLUMNS WITH POLYETHYLENE GLYCOL CONTAINING SOLUTIONS AS SOLVENTS[1]

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Rod-shaped and spherical viruses were purified from crude homogenates by chromatography on cellulose columns in two successive steps. The first step was a chromatographic clarification and the second step was a chromatographic purification. Passage through the cellulose column of a solvent containing 5% polyethylene glycol, 0.05% dextran, 4.5% glucose, 2% NaCl, 0.004 M $MgCl_2$ and 0.01 M phosphate buffer, pH 7, eluted proteins and low molecular-weight substances. Using a similar solution minus polyethylene glycol and NaCl resulted in the release of the viruses. Polyethylene glycol and NaCl were added to the virus-carrying effluents to give final concentrations of 5% and 2% respectively to precipitate the viruses on the cellulose for rechromatography. Subsequent percolations of solvents containing stepwise decreasing concentrations of polyethylene glycol and NaCl induced a separation of the viruses from host components. With tobacco mosaic virus the procedure could be simplified by applying only of the clarification chromatography.

INTRODUCTION

For the purification of potato virus X VENEKAMP & MOSCH (1963) described a chromatographic procedure using cellulose as column material and mixtures of polyethylene glycol and NaCl solutions as solvents. A prior removal of cellular constituents, such as nuclei, mitochondria and chloroplasts was necessary before the chromatographic purification. Clarification by shaking with chloroform using the technique of SCHNEIDER (1953) resulted in a considerable loss of active virus particles. VENEKAMP & MOSCH (1964) investigated the partition of chloroplasts and tobacco mosaic virus in high polymer two-phase systems described by ALBERTSSON (1960). As a consequence of the addition of glucose to the systems the chloroplasts collected in the dextran-rich bottom-phase and most of the virus moved into the polyethylene glycol-rich upperphase.

These studies were initiated in an attempt to develop a simple chromatographic separation of plant viruses from chloroplast materials using eluting solvents similar to the above upper phase.

MATERIALS AND METHODS

Six-week-old tomato plants were mechanically inoculated with tobacco mosaic virus, potato virus X, potato virus Y^N (DE BOKX, 1961) or tobacco rattle virus. Under the same conditions *Phaseolus vulgaris* var. 'Beka' was used for white clover mosaic virus inoculations. Controls were uninoculated. Seven days after inoculation 25 g of fresh leaves were taken for virus purification. Carnation ringspot virus and carnation mottle virus occurring in carnation plants (var. 'William Sim') from a nursery were also purified.

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The fresh leaves were homogenized at 0°C in 25 ml of a solution containing 10% polyethylene glycol 6000 (PEG) ("Carbowax", Carbide and Carbon Chemicals Company, U.S.A.), 4.5% glucose, 4% NaCl, 0.008 M $MgCl_2$ and 0.02 M phosphate buffer, pH 7.

Ten grams of dry cellulose (Whatman cellulose powder, standard grade) were mixed to form a slurry in 5% PEG, 0.05% dextran 500 (D) (viscosity number dl/g 0.48; Pharmacia, Uppsala, Sweden), 4.5% glucose, 2% NaCl, 0.004 M $MgCl_2$ and 0.01 M phosphate buffer, pH 7. This was poured into a 3 cm diameter chromatographic tube to form a 5 cm column between sand layers of 2 cm. The tube had a mantle for circulation of 2°C alcohol to keep the column cooled during the whole procedure.

The homogenate was applied on the column and incubated for 30 minutes before elution to allow the chloroplasts to precipitate. Clean sand was mixed with the sedimented chloroplasts to give a solvent flow rate of about 2 ml per minute. The initial effluent was brown colored. This cleared as 250 ml of the above solution passed through the column. Next this solvent was modified by eliminating the PEG and the NaCl and 150 ml of the modified solution eluted the viruses as they passed through the column.

PEG (5%) and NaCl (2%) were added to these virus-carrying eluates. The eluates were next passed through a second solumn (prepared in the same manner as the first). The viruses were again precipitated in the cellulose. The column was subjected to the serial passage of 100 ml amounts of solvent mixtures in which the PEG, D, glucose, $MgCl_2$ and pH 7 phosphate buffer concentrations were held at a constant level while the NaCl concentration was reduced stepwise from 2, to 1.5, 1.0, 0.5, 0.1 and 0.0 percent. These percolations were continued by passage of 100 ml amounts of solvent mixtures in which the D, glucose, $MgCl_2$ and pH 7 phosphate buffer concentrations were held at a constant level but the PEG concentration was reduced stepwise to 3 and 0 percent.

Addition of 0.0001 M mercaptoethanol and 0.01 M Na-diethyldithiocarbamate to the homogenate and to all the above solvents prevented serious aggregation of potato virus Y^N.

A solution containing 8% PEG and 3% NaCl was used instead of the solution containing 5% PEG and 2% NaCl to purify tobacco necrosis virus from 'White Burley' tobacco plants. These plants were inoculated with this virus under the same conditions as the plants mentioned above.

The presence of U.V.-absorbing substances including viruses in the effluents was recorded automatically at 254 mμ with a LKB-Uvicord absorption meter.

The occurrence of the viruses in the effluents was confirmed by biological assay on plants: tobacco mosaic virus on *Nicotiana glutinosa*, potato virus X on *Gomphrena globosa*, potato virus Y^N on *Solanum demissum* var. 'A6', tobacco rattle virus on 'White Burley' tobacco, white clover mosaic virus on *Phaseolus vulgaris* var. 'Beka', tobacco necrosis virus on 'White Burley' tobacco, carnation ringspot virus on *Chenopodium amaranticolor* and carnation mottle virus on *Gomphrena globosa*.

The results are summarized in Table 1. In these procedures the presence of NaCl in some of the above solutions precipitated the viruses on the cellulose and stimulated the initial coagulation of the chloroplasts.

The first chromatographic procedure resulted in retention of chloroplasts on the column. Moreover a large amount of low molecular-weight substances and proteins including brown colored materials eluted by the first solvent and were separated from the viruses occurring in the second effluent.

The second chromatographic procedure permitted a further purification of the viruses. Apparently substances adsorbed on the virus particles were released by passage of the first solvent through the second column.

The presence of $MgCl_2$ in the solvents reduced the amount of U.V.-absorbing substances from healthy plant homogenates to the small fraction eluted by the 3% PEG containing solvent. The total absorbancy of this fraction was not more than 1% of the total absorbancy of corresponding virus-carrying effluents.

TABLE 1. The occurrence of U.V.-absorbing substances with (+) or without (–) different virus activities in effluents obtained by the chromatographic purification procedure on cellulose columns with mixtures of 0.05% dextran, 4.5% glucose, 0.004 M $MgCl_2$, 0.01 M phosphate buffer (pH 7) and varying concentrations of polyethylene glycol (PEG) and NaCl as solvents.

	Column 1			Column 2								
Plant Material / % PEG / % NaCl	8[1] 3[1]	5 2	0 0	8[1] 3[1]	5 2	5 1.5	5 1.0	5 0.5	5 0.1	5 0	3 0	0 0
Tomato: healthy		–	–		–						.[2]	
Tomato infected with:												
tobacco mosaic virus		–	+		–				+	+	+	
potato virus X		–	+		–					+	+	+
potato virus Y^N		–	+		–						+	+
tobacco rattle virus		–	+		–					+	+	
Phaseolus vulgaris:												
healthy		–	–		–						.	
P. vulgaris infected with:												
white clover mosaic virus		–	+		–							+
Tobacco: healthy	–		–	–							.	
Tobacco infected with:												
tobacco necrosis virus	–		+	–		+						
Carnation: healthy		–	–		–						.	
Carnation infected with:												
ringspot virus		–	+		–					+	+	
mottle virus		–	+		–				+	+	+	

[1] Solution used for chromatography of tobacco homogenates.
[2] The absorbancy of effluents indicated by . is less than 1% of the absorbancy of corresponding virus-carrying effluents.

Two methods to remove PEG, D and glucose from the virus-carrying effluents permitted the study of the absorption spectra and the electron micrographs of the purified viruses.

The first method was applied to tobacco mosaic virus, potato virus X, potato virus Y^N and white clover mosaic virus. The viruses were precipitated from the effluents by the addition of PEG and NaCl to give final concentrations of 5% and 2% respectively. The pellets obtained by centrifugation at 10,000 rpm for 10 minutes were resuspended in 5 ml of pH 7 0.1 M NH_4-acetate solution. A final centrifugation at the same speed and time yielded a virus-carrying supernatant.

The second method was applied to tobacco rattle virus, tobacco necrosis virus, carnation ringspot virus and carnation mottle virus. Passage of the virus-carrying effluents through a DEAE-cellulose column (VENEKAMP & MOSCH, 1963) allowed to adsorb the viruses. Percolation of 100 ml of 0.02 M phosphate buffer, pH 7, removed PEG, D and glucose. Passage of 50 ml of 0.8 M NaCl solution in 0.02 M phosphate buffer, pH 7, eluted the viruses. Dialysis of the virus solution against water overnight removed excess ions.

The absorption spectra are typical for nucleo-proteins with absorption maxima between 260 and 262 mμ and minima between 245 and 248 mμ. The ratio E max/min was between 1.12 and 1.20.

Preliminary electron micrographs failed to demonstrate impurities. These micrographs will be published later.

CONCLUSION

Three facts suggest that these procedures are yielding rather pure virus. First, corresponding fractions isolated from healthy plant homogenates were mostly free of U.V.-absorbing materials. Second, the absorption spectra of the preparations were typical for purified viruses. Third, preliminary electron microscope studies of the preparations failed to show the presence of many impurities.

With tobacco mosaic virus the procedure could be simplified. After collecting the virus-carrying effluent from the first column the addition of 2% NaCl precipitated the virus. This was concentrated by centrifuging at 10,000 rpm for 10 minutes. The pellets from this step were resuspended in a small amount of pH 7 0.1 M ammonium acetate solution. A final centrifugation at the same speed and time yielded a blue opalescent supernatant with a slight greenish pellet consisting of unwanted impurities.

The application of the same techniques to comparable healthy plants yielded only small amounts of materials which had low absorbance at 254 mμ.

The faint green color accompanying the virus is probably caused by the adsorption of chloroplast material on the viral surfaces. This could occur because of possible close association of virus and chlorophyll within the chloroplasts. COCHRAN (1963) and COCHRAN *et al.* (in preparation) have shown that infective tobacco mosaic viral nucleic acid and whole tobacco mosaic viral particles can be synthesized in isolated chloroplasts and nuclei.

The adsorbed green material appeared to be completely removed by the final treatment of the virus-carrying effluent. These results suggest that the isolated virus is in a highly purified condition. An electron micrograph (shown in Fig. 1) of a 1:10 dilution appears to substantiate this conclusion.

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