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PURIFICATION OF POTATO VIRUS X, WHITE CLOVER MOSAIC VIRUS, TOBACCO MOSAIC VIRUS AND RIBOSOMES BY COLUMN CHROMATOGRAPHY

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SUMMARY

Chromatographic procedures for the isolation on cellulose columns of potato virus X, tobacco mosaic virus and ribosomes from tobacco plants, and of white clover mosaic virus and ribosomes from bean plants, are described. The yields obtained by using procedures involving the use of ten different solvents were compared. The first step consisted of the extraction of homogenates, each prepared from 100 g of fresh plant material, by using columns packed with 20 g of ground chromatographic paper and elution with ten different solvent systems. The compositions of these solvents differed in their contents of dextran, sucrose, tris(hydroxymethyl)-aminomethane–acetic acid buffer (pH 7), magnesium chloride, magnesium acetate, sodium diethyldithiocarbamate, urea and polyethylene glycol.

The second step consisted of fractionation of viral and ribosomal nucleoproteins and other UV-absorbing substances in the effluents from the first columns. Elution was carried out on columns containing 10 g of Whatman cellulose powder in which a stepwise reduction in polyethylene glycol, sodium chloride, sodium acetate, ammonium acetate and magnesium acetate was applied. This procedure resulted in the complete separation of virus particles and ribosomes. Using the UV absorption of the effluents as a measure, the most effective procedure for the recovery of viruses and ribosomes was found. The extraction solvent in the first step contained 1% dextran, 0.4 *M* sucrose, 0.01 *M* tris(hydroxymethyl)aminomethane–acetic acid buffer of pH 7 and 0.1% sodium diethyldithiocarbamate. In the second step, the omission of magnesium acetate resulted in the liberation of ribosomes.

INTRODUCTION

In previous papers, VENEKAMP AND MOSCH[1–3] described the purification of

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some plant viruses by using chromatography on cellulose columns with solutions containing polyethylene glycol as solvents.

The presence of phenolic compounds, however, may interfere in the purification of viruses. LOOMIS AND BATTAILE[4] reported the reversible adsorption of phenolic compounds on proteins by hydrogen bonding. After oxidation of the phenols, this adsorption became irreversible by covalent condensation. Oxidation can nevertheless be avoided by the elimination of polyphenol oxidase. Several investigators purified plant viruses with more success by using sodium diethyldithiocarbamate to eliminate this enzyme; for example: prune dwarf and sour cherry necrotic ringspot viruses were purified by HAMPTON AND FULTON[5,6], Tulare apple mosaic virus by MINK AND BANCROFT[7], cucumber mosaic virus by HARRISON AND PIERPOINT[8,9], cocoa swollen-shoot virus by BRUNT AND KENTEN[10], infectious preparations from fruit tree petals by TREMAINE *et al.*[11], potato virus Y by DELGADO-SANCHEZ AND GROGAN[12] and Sharka virus by RANKOVIC AND JORDOVIC[13].

HIEBERT *et al.*[14] and DAMIRDAGH AND SHEPHERD[15] assumed that the aggregation of virus particles interferes in purification procedures. They found that low concentrations of urea had a favorable effect on the isolation of tobacco etch virus and potato virus Y. DOYLE *et al.*[16], however, reported that urea modified the conformation of proteins and abolished hydrogen bonds.

Following the findings of the above workers, sodium diethyldithiocarbamate and urea were included in the present studies.

VENEKAMP[17] successfully extracted infectious thread-like viruses, such as potato virus Y and apple chlorotic leaf-spot virus, by use of a dextran solution. In these experiments, the adsorption of the green material on the cellulose was intensified by the addition of 0.4 *M* sucrose to the extracting solvent. These results enabled the effects of extracting solvents containing dextran and sucrose on the final yields of the viruses and ribosomes purified by the use of the procedures described in this paper to be tested.

KLIFFEN[18] purified ribosomes from very young pea plants by means of a modification of the procedure of VENEKAMP AND MOSCH[2]. The ribosomes were eluted from the column with a solvent that contained polyethylene glycol but without magnesium ions. Magnesium ions were also omitted in some of the present procedures.

Because of the deleterious effect of sodium chloride on the stability of ribosomes[18], the influence of ammonium acetate on the adsorption of the viruses on cellulose was studied.

This paper describes modifications to chromatographic procedures for the purification of viruses. Solvents of different compositions were used in order to establish procedures that would give optimum yields of potato virus X, white clover mosaic virus, tobacco mosaic virus and ribosomes.

MATERIALS AND METHODS

Eight-weeks-old "White Burley" tobacco plants were mechanically inoculated with potato virus X and with a mixture of potato virus X and tobacco mosaic virus. Six-weeks-old bean plants ("Bataaf") were mechanically inoculated with white clover

mosaic virus. Ten days after inoculation, 100 g of fresh leaves were taken for each procedure described below.

In three experiments, nine different chromatographic procedures were compared with the original procedure of VENEKAMP AND MOSCH[2], which is referred to as procedure 0.

For each experiment, fresh plant material was harvested, cut into small pieces of about 1 cm², randomized and then divided into ten portions, each containing 100 g of plant material. One portion was used for procedure 0. Another portion was homogenized in a mixture of 100 ml of double-concentrated and 150 ml of single-concentrated solvent, the composition of which is indicated in Table I under procedure 1. Each of the other portions was homogenized with the solvent of the corresponding procedure as in Table I. Then each homogenate was filtered through nylon cloth (Monodur normal 500, Verseidag, Krefeld, G.F.R.) and the filtrate was used.

TABLE I

COMPOSITION OF SOLVENTS FOR THE FIRST SERIES OF COLUMNS FOR THE TWO-STEP CHROMATOGRAPHIC PROCEDURES

First columns: 20 g of ground Whatman No. 1 chromatographic paper, 5-cm diameter, 10-cm height. Dextran = dextran T500; Tris = tris(hydroxymethyl)aminomethane–acetic acid buffer; Na-DDC = sodium diethyldithiocarbamate; PEG = polyethylene glycol 6000.

Chemical		*Procedure*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Dextran	(%)	1	1	1	1	1	1	1	1	1
Sucrose	(*M*)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Tris, pH 7	(*M*)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
$MgCl_2$	(*M*)	0.004								
Mg acetate	(*M*)		0.004	0.004	0.004	0.004	0.004			
Na-DDC	(%)					0.1	0.1	0.1	0.1	0.1
Urea	(%)								1	1
PEG	(%)									0.5

One sheet of Whatman No. 1 chromatographic paper was cut into pieces of about 10 cm² and ground in 500 ml of water with an electric stirrer (Heidolph electro kg, Kelheim, laboratory stirrer Type RZR). The slurry was poured into tubes of 5-cm diameter containing a plug of cotton-wool at the bottom. The height of the settled columns was 10 cm. Each column was washed with 400 ml of 0.1% sodium ethylenediaminetetraacetate solution followed by 400 ml of water. Finally, the columns were equilibrated with 200 ml of the solvent described in Table I.

The filtrates from the homogenates were applied on to the corresponding columns. During the elutions, the first 150 ml of the effluent were discarded. The elution was continued with the corresponding solvent (Table I) until the volume of the effluent was 900 ml.

Polyethylene glycol was added to each effluent up to a final concentration of 5%. To the effluent obtained by using the first procedure, sodium chloride was added to give a final concentration of 2%. Sodium acetate was added to the effluent by applying the second procedure up to a final concentration of 2%. To the effluent obtained by using the third procedure, ammonium acetate was added up to a final

TABLE II

COMPOSITION OF SOLVENTS FOR THE SECOND SERIES OF COLUMNS FOR THE TWO-STEP CHROMATOGRAPHIC PROCEDURES

The first steps are extractions of homogenates, each of 100 g of fresh plant material, by solvents containing 1% dextran T500, 0.4 *M* sucrose and the substances given in Table I.

The second step is a fractionation of viral and ribosomal nucleoproteins and other UV-absorbing substances in the effluents from the first columns.

Second columns: 10 g of Whatman CF 11 cellulose powder between 1-cm layers of ground Whatman No. 1 chromatographic paper, 3-cm diameter, 10-cm height.

PEG = polyethylene glycol 6000; NaOAc = sodium acetate; NH_4OAc = ammonium acetate; $Mg(OAc)_2$ = magnesium acetate; Na-DDC = sodium diethyldithiocarbamate; Tris = tris(hydroxymethyl)aminomethane–acetic acid buffer, pH 7.

Procedure	*Solvent number*	*PEG (%)*	*NaCl (%)*	*NaOAc (%)*	*NH_4OAc (%)*	*Glucose (%)*	*Na-DDC (%)*	*$MgCl_2$ (M)*	*$Mg(OAc)_2$ (M)*	*Tris (M)*
1	1	5	2			4.5		0.004		0.01
	2	5	1			4.5		0.004		0.01
	3	5				4.5		0.004		0.01
	4	3				4.5		0.004		0.01
	5	2				4.5		0.004		0.01
	6	1				4.5		0.004		0.01
	7					4.5		0.004		0.01
	8									
2	1	5		2		4.5			0.004	0.01
	2	5		1		4.5			0.004	0.01
	3	5				4.5			0.004	0.01
	4	3				4.5			0.004	0.01
	5	2				4.5			0.004	0.01
	6	1				4.5			0.004	0.01
	7					4.5			0.004	0.01
	8									
3	1	5			2	4.5			0.004	0.01
	2	5			1	4.5			0.004	0.01
	3	5				4.5			0.004	0.01
	4	3				4.5			0.004	0.01
	5	2				4.5			0.004	0.01
	6	1				4.5			0.004	0.01
	7					4.5			0.004	0.01
	8									
4	1	5			1	4.5			0.004	0.01
	2	5				4.5			0.004	0.01
	3	3				4.5			0.004	0.01
	4	2				4.5			0.004	0.01
	5	1				4.5			0.004	0.01
	6					4.5			0.004	0.01
	7									
5	1	5			1	4.5	0.1		0.004	0.01
	2	5			1	4.5			0.004	0.01
	3	5				4.5			0.004	0.01
	4	3				4.5			0.004	0.01
	5	2				4.5			0.004	0.01
	6	1				4.5			0.004	0.01
	7					4.5			0.004	0.01
	8									
6, 7, 8, 9	1	5			1	4.5	0.1		0.004	0.01
	2	5			1	4.5			0.004	0.01
	3	5				4.5			0.004	0.01
	4	5				4.5				0.01
	5	3				4.5				0.01
	6	2				4.5				0.01
	7	1				4.5				0.01
	8					4.5				0.01
	9									

concentration of 2%. Ammonium acetate was added to each of the other effluents to a final concentration of 1%. Also, magnesium acetate was added to the effluents obtained by using procedures 7, 8 and 9 to a final concentration of 0.004 *M*.

A second set of columns was prepared by grinding up chromatographic paper as described earlier. Ten milliliters of this slurry were poured into a tube of 3 cm diameter with a plug of cotton-wool at the bottom. Excess water was removed and a suspension of 10 g of Whatman CF 11 cellulose powder in 100 ml of water was poured into the tube. After allowing the tubes to stand for 15 min, part of the excess water was percolated, allowing the cellulose to become compacted. A further 10 ml of ground chromatographic paper were added. After complete percolation of the excess water, the columns were washed with 100 ml of 0.1% sodium ethylenediaminetetraacetate solution and then with 100 ml of water. The columns were equilibrated with 50 ml of the corresponding first solvents (Table II).

The effluents from the first columns were then applied on to the second columns, as described above. After percolation of the effluents, the second columns were washed with 200 ml of corresponding solvents indicated by the number 1 in Table II. Finally, 200-ml portions of solvents listed in Table II were passed successively through the columns.

The UV absorbance of each effluent was measured in a Beckman DB-G spectrophotometer with a Sargent recorder. Each of the corresponding solvents was used as reference. Table III gives the following characteristics of the spectra of viruses and ribosomes: wavelengths of maximum and minimum absorbances, the ratios of maximum and minimum absorbances, and the ratios of the absorbances at 280 nm and the maximum absorbances.

TABLE III

CHARACTERISTICS OF THE UV ABSORPTION SPECTRA OF THE VIRUSES AND RIBOSOMES PURIFIED BY THE TWO-STEP CHROMATOGRAPHIC PROCEDURES ON CELLULOSE COLUMNS

Chromatographic procedures are given by Figs. 1–3. The results are averages from the measurements on the virus- and ribosome-carrying effluents.

Nucleoprotein	*Wavelength of maximum absorption (nm)*	*Wavelength of minimum absorption (nm)*	*Ratio of maximum and minimum absorbances*	*Ratio of absorbance at 280 nm and maximum absorbance*
Potato virus X	260	246	1.15	0.83
White clover mosaic virus	260	246	1.15	0.82
Tobacco mosaic virus	258	248	1.08	0.80
Tobacco ribosomes	255	236	1.30	0.60
Bean ribosomes	258	236	1.54	0.58

The criteria for the occurrence of viruses in the effluents from the second columns are based on electron microscopic studies, absorption spectra and infectivity for susceptible host plants. The criteria for the presence of ribosomes are limited to electron micrographs and absorption spectra.

In the fourth experiment, two identical filtrates were used, each prepared from 100 g of leaves from eight-weeks-old "White Burley" tobacco plants. The plants were inoculated with potato virus X 10 days before harvesting. The first filtrate was

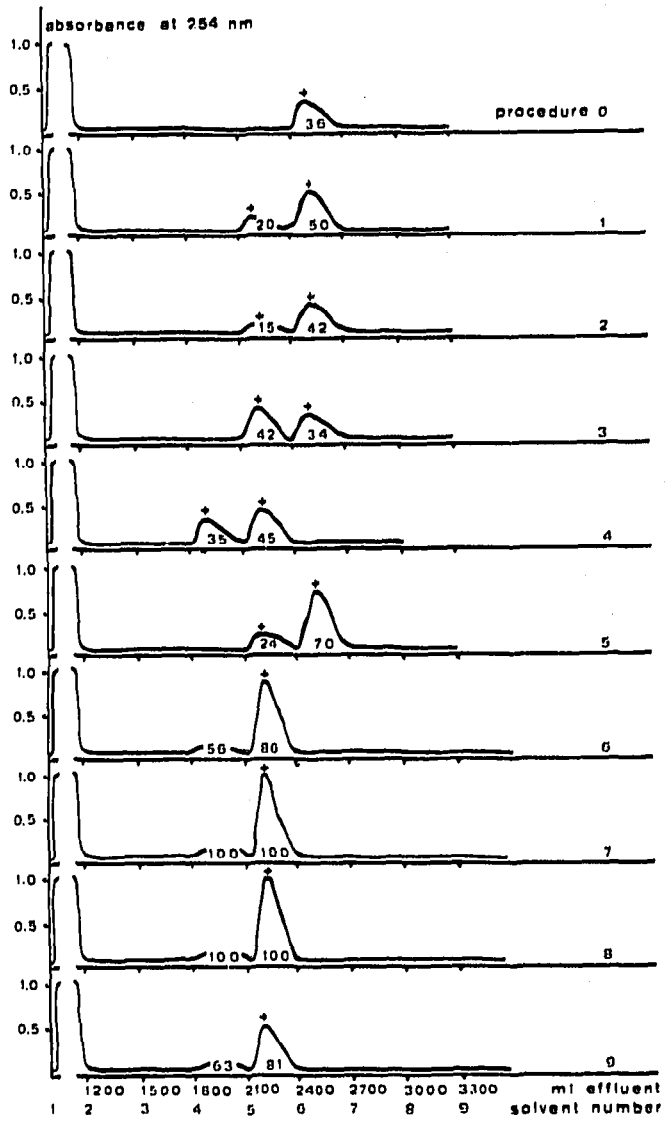


Fig. 1. Fractionation of potato virus X and ribosomes from "White Burley" tobacco plants by the second step of the two-step chromatographic procedures on cellulose columns. The first steps are extractions of homogenates, each of 100 g of fresh plant material, using columns of 20 g of ground Whatman No. 1 chromatographic paper and solvents containing 1% dextran T500, 0.4 *M* sucrose and the substances given in Table I. The second steps are fractionations of viral and ribosomal nucleoproteins and other UV-absorbing substances in the effluents from the first columns. The second columns contain 10 g of Whatman CF 11 cellulose powder between 1-cm layers of ground chromatographic paper (3-cm diameter, 10-cm height). Compositions of the solvents numbered along the abscissa are indicated in Table II. The yield of virus in the effluents is indicated as a percentage of the total amount of virus in procedure 7. The same holds for the yield of ribosomes in the effluents. A virus content of 100% is equivalent to an absorbance/ml/g fresh weight of 1.48. A ribosome content of 100% is equivalent to an absorbance/ml/g fresh weight of 2.32. Fractions with a plus sign contain highly infectious virus giving typical symptoms on "White Burley" tobacco plants. The absorbances of the effluents at 254 nm were recorded with an LKB Uvicord absorptionmeter.

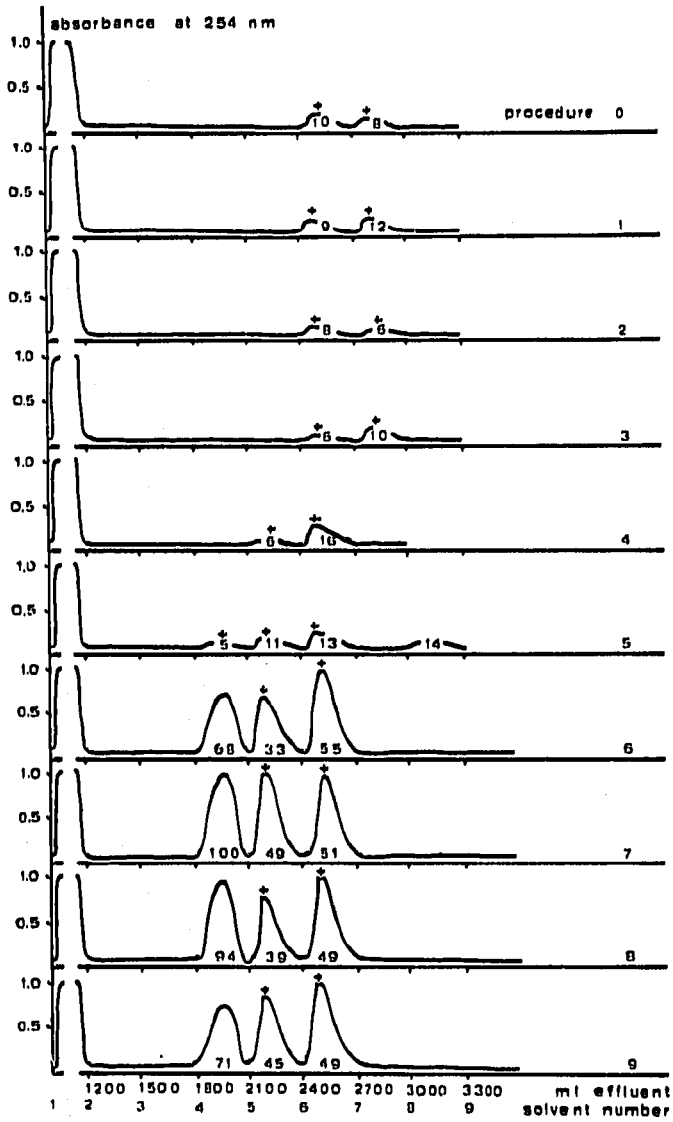


Fig. 2. Fractionations of white clover mosaic virus and ribosomes from bean plants ("Bataaf") by the second step of the two-step chromatographic procedures on cellulose columns. For explanations, see Fig. 1. Fractions with a plus sign contain highly infectious virus giving typical symptoms on "Bataaf" bean plants. A virus content of 100% is equivalent to an absorbance/ml/g fresh weight of 3.92. A ribosome content of 100% is equivalent to an absorbance/ml/g fresh weight of 12.48.

chromatographed according to the above-mentioned procedure 7, and the results are shown later in the upper part of Fig. 4. The second filtrate was chromatographed according to procedure 7 except that the 200-ml portions of the successive solvents with 3%, 2% and 1% of polyethylene glycol contained 0.004 *M* magnesium acetate. These percolations were continued by the serial passage of 200-ml volumes of solvents

containing 1% and 0% of polyethylene glycol without magnesium acetate and finally by the passage of 200 ml of water. The results from this procedure are given later in the lower part of Fig. 4. The UV absorbance of the effluents was measured spectrophotometrically, as described above.

RESULTS

The effluents from the first columns, 0, 1, 2, 3, and 4, had a brown colour. The presence of sodium diethyldithiocarbamate in the extraction solvent induced a yellow colour in the effluent.

The first effluents from all of the second columns contained low-molecular-weight substances, including phenolic compounds. These effluents had a very high absorption at 260 nm.

The following conclusions can be drawn from the results obtained from the experiments shown in Figs. 1–3.

Extraction with a solvent containing 1% of dextran yielded much more potato virus X and tobacco mosaic virus than was obtained in the original procedure (number 0). This solvent, however, was no more effective in extracting white clover mosaic virus.

The use of acetate ions, instead of chloride ions, in the first solvent in the second column did not improve the yield (compare procedure 2 with procedure 1).

When the first solvent of the second column contained ammonium ions instead of sodium ions, the final result was improved in the case of tobacco plants infected with potato virus X (compare procedure 3 with procedure 2).

The first solvent, containing 1% ammonium acetate, did not yield more virus (compare procedure 4 with procedure 3).

The presence of sodium diethyldithiocarbamate in the extraction solvent in the first column and in the first solvent in the second column resulted in a larger amount of virus (compare procedures 5, 6, 7, 8 and 9 with procedures 0, 1, 2, 3 and 4). Only tobacco mosaic virus was an exception. In procedure 5, white clover mosaic virus was successfully eluted with three successive solvents.

Omission of magnesium ions from the solvent in the second column resulted in the elution of ribosomes (compare procedures 6, 7, 8 and 9 with procedure 5). This yielded more white clover mosaic virus, but not more potato virus X or tobacco mosaic virus.

More important is the elution of potato virus X in the experiment shown in Fig. 1 in one fraction, and the elution of white clover mosaic virus in two fractions. When the tobacco plants were infected with tobacco mosaic virus and potato virus X, both viruses were eluted with two successive solvents (compare procedure 6 with procedure 5). In procedure 5 of the experiment shown in Fig. 2, a small amount of ribosomes was eluted with water.

Omission of magnesium ions from the extraction solvent in the first column yielded more ribosomes (compare procedure 7 with procedure 6) and an even larger amount of potato virus X and white clover mosaic virus. Bean plants contained 5–6 times as many ribosomes as tobacco plants.

Urea in the extraction solvent in the first column had almost no influence on the yields of ribosomes and viruses (compare procedure 8 with procedure 7).

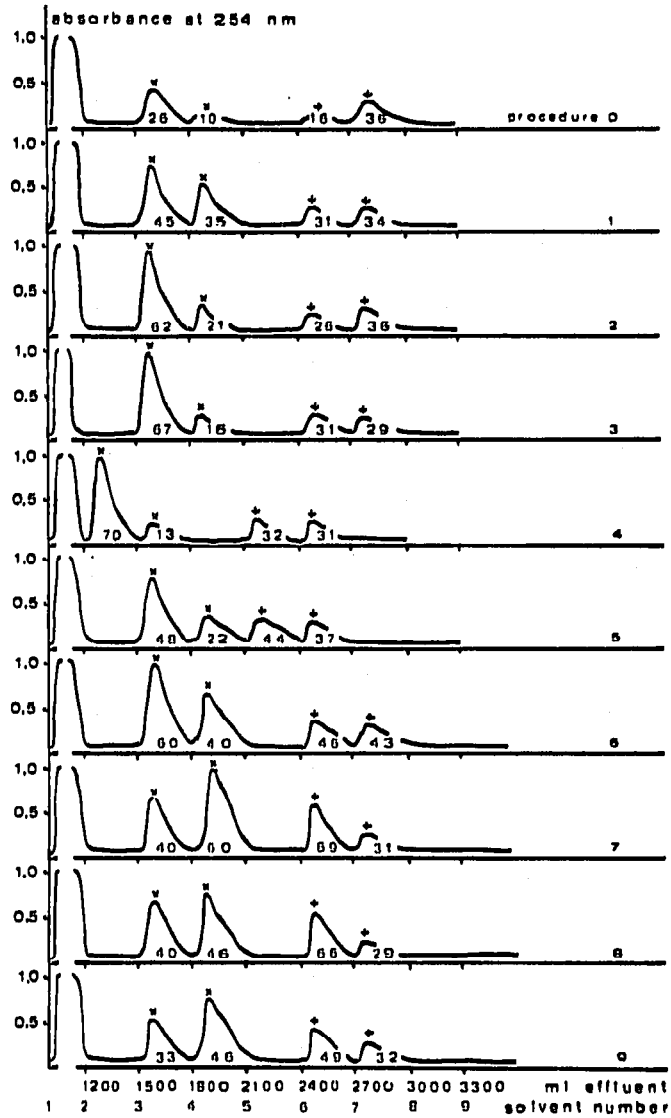
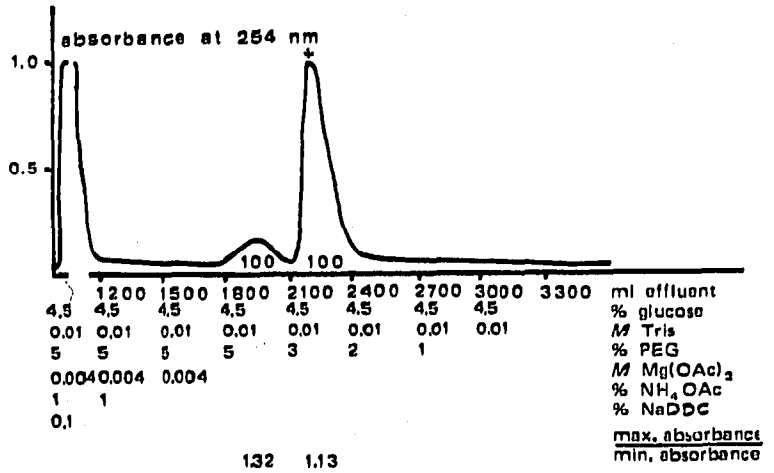


Fig. 3. Fractionations of tobacco mosaic virus and potato virus X from "White Burley" tobacco plants by the second step of the two-step chromatographic procedures on cellulose columns. For explanations, see Fig. 1. Fractions with a multiplication sign contain highly infectious tobacco mosaic virus giving typical symptoms on "White Burley" tobacco plants and fractions with a plus sign contain highly infectious potato virus X giving typical symptoms on "White Burley" tobacco plants. A tobacco mosaic virus content of 100% is equivalent to an absorbance/ml/g fresh weight of 11.38. A potato virus X content of 100% is equivalent to an absorbance/ml/g fresh weight of 1.94.



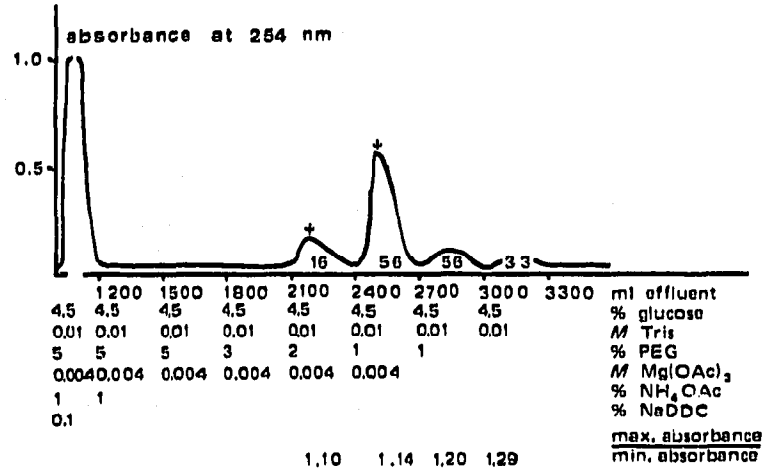


Fig. 4. Fractionations of potato virus X and ribosomes from "White Burley" tobacco plants by the second step of the two-step chromatographic procedures on cellulose columns. For explanations, see Fig. 1. A virus content of 100% is equivalent to an absorbance/ml/g fresh weight of 1.26. A ribosome content of 100% is equivalent to an absorbance/ml/g fresh weight of 2.07.

The presence of 0.5% polyethylene glycol in this solvent decreased the final yields of viruses and ribosomes (compare procedure 9 with procedure 8).

In the experiment shown in Fig. 1, procedures 7 and 8, and in the experiments shown in Figs. 2 and 3, procedure 7 resulted in the highest yields of viruses and ribosomes.

The measured absorbances of the effluents were averaged. Table III gives the characteristics of the absorption spectra of ribosomes and viruses.

Tobacco ribosomes had a smaller ratio of maximum and minimum absorbances than bean ribosomes.

In the experiment in Fig. 4, the elution of potato virus X prior to that of ribosomes was compared with procedure 7 in Fig. 1. In this case, potato virus X was eluted by two successive solvents. The total yield of this virus was lower than in procedure 7. When magnesium ions were omitted from the solvent, the ribosomes were eluted from the column. Moreover, the first ribosome fraction contained some virus particles according to the low ratio of maximum and minimum absorbances.

DISCUSSION

Precipitation of plant viruses by a mixture of polyethylene glycol and sodium chloride was reported by HEBERT[19]. VENEKAMP[17] used this technique in order to purify potato virus X. The UV absorption spectrum of the final preparation, however, was not typical of this virus in spite of the biological activity.

ZEPPEZAUER AND BRISHAMMAR[20] purified glycoproteins by using polyethylene glycol as a precipitant. POLSON *et al.*[21] sedimented proteins with polyethylene glycol. In both instances the concentrations of this polymer were similar to those required for the purification of viruses. Therefore, the impurity of precipitated potato virus X could be explained as the result of coprecipitation of the virus with other cell components. This was the reason why the present authors preferred cellulose columns to separate viruses and other nucleoproteins.

The techniques described in this paper are considered to be chromatographic procedures. For the second column, ground chromatographic paper could be used instead of cellulose powder. Large particles, such as chloroplasts or even whole cells suspended in sucrose solutions, passed through this column easily. Viruses in solvents that contained polyethylene glycol and ammonium acetate (or sodium chloride or sodium acetate), however, could not pass through this column because of adsorption by the cellulose. Cellulose powder was used in the second columns to obtain a regular pattern in the chromatograms.

The use of sodium diethyldithiocarbamate resulted in a higher yield of potato virus X and white clover mosaic virus because of the reversibility of the adsorption of reduced phenols on the virus particles. Irreversible adsorption of oxidized phenols hindered to some extent the liberation of virus particles from the column. Sodium diethyldithiocarbamate had, on the contrary, no effect on the yield of tobacco mosaic virus.

In all instances, omission of magnesium ions resulted in isolation of ribosomes, except when tobacco mosaic virus was present. Synthesis of this virus seemed to reduce appreciably the ribosome content in a plant.

The omission of magnesium ions also induced the elution of potato virus X and white clover mosaic virus with solvents containing a higher concentration of polyethylene glycol. Therefore these viruses were eluted earlier. The presence of microsomal material in the column slowed down the liberation of potato virus X and white clover mosaic virus. The experiment shown in Fig. 4 supports this observation.

In the procedures described in this paper, aggregation of the virus particles

did not interfere in the liberation from the columns, because urea had no effect on the elution.

The ribosomes from tobacco have the same ratio of maximum and minimum absorbances as those of fig fruits as found by MAREI AND ROMANI[22]. Bean ribosomes have a similar ratio to that of clover ribosomes[23]. The ratios of these absorbances of tobacco and bean ribosomes may indicate a difference in purity or in composition.

The similarity of the ratios of the absorbances at 280 and 258 nm of the ribosomes from tobacco and from bean plants indicates no difference in the adsorption of phenolic compounds.

All the procedures yielded fairly pure viruses. Corresponding fractions isolated from healthy plant homogenates were free from UV-absorbing materials. Electron microscopic studies of the preparations failed to show the presence of impurities. The absorption spectra of the preparations were typical of purified viruses.

All the purified viruses induced typical symptoms when inoculated into susceptible hosts. Ribosomes from healthy plants could also be isolated after the omission of magnesium ions. The electron micrographs and the absorption spectra of the ribosomes isolated in the experiments described were typical of purified nucleoproteins.

From the above considerations it is evident that in procedure number 7, the optimum conditions to give the highest yields of virus particles and ribosomes were realized.

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