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Chromatographic purification of plant viruses depending on the properties of the virus particles

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In the investigations on purification of viruses the ultracentrifuges played a particular important role. Since the work of Svedberg a number of plant viruses have been purified with the aid of the ultracentrifuge. In this procedure many substances sedimented very easily along with the virus. Therefore the pellet should be resuspended. A low speed centrifugation removed the undissolved denatured proteins. A second ultracentrifugation of the supernatant yielded a sediment containing more or less pure virus. If necessary this last treatment could be repeated. Notwithstanding the loss of a relatively large amount of virus a pure virus preparation could be obtained in many cases. For example tobacco mosaic virus and potato virus X has been purified according to this procedure. The loss of some virus was not very serious when large amounts of the virus were present in the homogenate. Enough had been left to collect. However, a further purification may be necessary in cases when the virus was not separated from some of the normal cell constituents. In other cases the plant material contained a very small amount of virus particles and then a loss of virus during the purification procedure might be too serious.

Brakke [9] developed the well-known density-gradient centrifugation method. During the high speed centrifugation the virus sedimented against a gradient of increasing sugar concentrations until the density of the solvent was too high for the virus particles to settle down further, on. The result was a well-defined layer more or less remote from the surface.

Besides these methods also other techniques have been applied in the virus purification. On the Virus Conference at Wageningen, The Netherlands, a review of the chromatographic techniques was given [55]. Some remarks should be added. Adsorption chromatography on calcium phosphate gel was applied by Fulton [21] to purify sour cherry necrotic ringspot and prune dwarf viruses, and by McLean and Francki [32] to isolate lettuce necrotic yellows virus. Kubo *et al.*, [27] used methylated albumin kieselguhr columns to study the constituents of tobacco mosaic virus. Ralph *et al.*, [37] isolated the double-stranded replicative form of viral RNA from tobacco leaves infected with tobacco mosaic virus and from Chinese cabbage leaves infected with turnip yellow mosaic virus by use of methylated bovine serum albumin on kieselguhr.

Levin [29] fractionated tobacco mosaic virus and potato virus X by stepwise elution from the anion-exchanger DEAE-cellulose. Tyoda *et al.*, [49] used this anion-exchanger in their experiments on isolating rice dwarf virus and Salama and Lyman [41] to purify internal cork virus of sweet potato. Purified tobacco mosaic virus was separated into several components by stepwise elution of a cation--exchange resin (Amberlite IR-120) and Ecteola-cellulose columns by Taniguchi [47, 48]. The adsorption chromatography on calcium phosphate gel as well as that on DEAE-cellulose were studied by Venekamp and Mosch [50]. From their experiments on purification of potato virus X they concluded that both techniques did not lead to a complete purification of the virus, because the same chromatograms were found with healthy plant material. Probably the success of the application of these techniques depended on the previous treatment of the crude sap before a chromatographic column was used.

Steere and Ackers [42, 43], Steere [44] and Ackers and Steere [1] applied agar-gel columns for purification of plant viruses. These columns contained agar gel grains of uniform size. Because these grains were porous, the lower molecular weight substances penetrated into the gel. The ability of the substances to penetrate depended on the concentration of the agar solution used for the preparation of the grains. The virus particles were too big and percolated trough the column along with the eluting solvent. This resulted in a separation of the virus from many other substances.

Another means in virus purification is electrophoresis. This is an important technique to isolate viral nucleic acids [4, 15, 38]. Van Regenmortel [38] suggested that zone electrophoresis is a very efficient method to obtain pure virus preparations suitable for immunological studies. Van Regenmortel *et al.*, [39] separated potato virus X, alfalfa mosaic virus, and tobacco mosaic virus from a mixture of these viruses by density gradient zone electrophoresis. Also Brakke [8] used a similar technique in his studies on potato yellow-dwarf virus. The technique of continous free-flow electrophoresis was successfully applied by Streeter and Gordon [45] for separation of two strains of tobacco mosaic virus and by Zaitlin [57] for a partical purification of a stone fruit virus isolated from cucumber. Weintraub *et al.*, [56] described a method of electrophoresis in agar which allowed ready sampling of fractions from crude sap for electron microscope and infectivity tests.

Precipitation was always an important aid in the separation of certain protein fractions. Mostly ammonium sulphate was used. During the last few decennia many new substances were synthesized. Especially polymers were prepared in relation to plastics and nylons. The development of these modern artificial products influenced also to a certain extent the virus purification technique.

Independently of our work with polyethylene glycol Hebert [23] used this polymer to precipitate some viruses. Different amounts of polyethylene glycol and sodium chloride were necessary in the purification of soil-borne wheat mosaic, tobacco-mosaic, tobacco ringspot, and bean pod mottle viruses. Leberman [28] also applied this procedure to four different viruses and found that turnip crinkle virus precipitated at pH 6 with 7% polyethylene glycol and 0.3 M sodium chloride.

Turnip yellow mosaic virus precipitated with 6.8% polyethylene glycol and 0.48 M sodium chloride, tobacco mosaic virus with 0.53% polyethylene glycol and 0.56 M sodium chloride, and bacteriophage T-4 with 6.4% polyethylene glycol and 0.37 M sodium chloride. The plant homogenate should be clarified before the application of polyethylene glycol and sodium chloride. Kanarek and Tribe [25] precipitated certain myxoviruses from suspensions in balanced 0.9 molar salt solutions with 7.5% polyethylene glycol.

Albertsson [2] studied aqueous high polymer two-phase systems. Mixtures of polyethylene glycol and dextran or dextran sulphate solutions in certain ratios are immiscible. High molecular weight substances, such as nucleoproteins, have reproducible partition coefficients in these systems. In many cases virus particles collected at the interface between the upper and bottom phase. This was especially true when sodium chloride was present in the system. Venekamp and Mosch [51] used these two-phase systems to clarify the crude plant extract. The chloroplasts collected in the dextran-rich bottom phase. In the presence of 4.5% glucose tobacco mosaic virus concentrated in the upper phase. Caudwell [11] purified the virus of the "Courtnoué" in the vine. For this purpose he homogenized the leaves in a mixture of 2.5% polyethylene glycol and 1% dextran. Centrifugation at 10,000 rpm during ten minutes resulted in a virus-containing supernatant. Addition of polyethylene glycol to a final concentration of 8% precipitated the virus. A second centrifugation collected the virus in a pellet that could be resuspended in a buffer solution.

In the chromatographic virus purification technique of Venekamp and Mosch [52] the homogenate was applied on top of a column of cellulose between two sand layers. Percolation of the first solvent containing 5% polyethylene glycol, 2% sodium chloride, 4.5% glucose, 0.004 M magnesium chloride and 0.01 M buffer solution, pH 7, liberated low molecular weight compounds including proteins. The chloroplasts, chloroplast fragments and the virus remained in the column. Percolation of another solvent containing less polyethylene glycol and no sodium chloride, but similar percentages of glucose, magnesium chloride, and buffer solutions, resulted in the elution of the virus. Automatic recording at 254 mn of the effluents with a LKB-Uvicord absorption metter, detected the presence of ultraviolet-absorbing substances.

The occurrence of virus in the effluents was confirmed by ultraviolet absorption and by biological assay on plants. Typical virus peaks failed when the same technique was applied to healthy plant material. In this way a specific difference between healthy and virus-diseased plants was demonstrated.

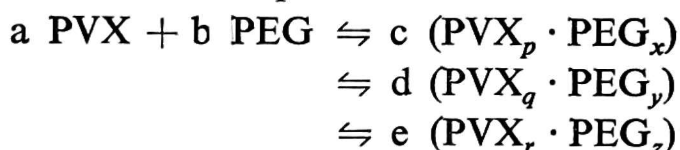
Because of theoretical considerations potato virus X was used in these first experiments. This virus could be propagated very easily in tobacco plants. One or two weeks after inoculation considerable amounts of the virus occurred in the plants. These amounts were comparable with those of tobacco mosaic virus in tobacco. However, the virus X particles have a stronger tendency to aggregate than tobacco mosaic virus. This made purification of potato virus X according to the current techniques more difficult. Aggregation is a frequently occuring phenomenon of macromolecules that interferes every purification procedure.

As the experiments with potato virus X were successful it was obvious to apply the same procedure [52] for purification of other plant viruses. The result was that viruses of a quite different type could be purified in a similar way. A list of these viruses was published [53]. The shape of the virus particles whether these were spherical or rod shaped, was not essential. Banttari [3] purified oat blue dwarf virus according to the same procedure.

Liberation of a virus, for example potato virus X or tobacco mosaic virus, could be clearly observed. This visibility depended on the amount of virus originally present in the homogenate. When the virus-carrying effluent was observed more precisely, then very small particles could be distinguished. These gave the liquid a "cloudy" appearance and were very big in size with respect to a single virus particle. In the case of potato virus X an addition of sodium chloride to a final concentration of 10 or 15% reduced the cloudiness of the solution completely and the liquid became opalescent. Ultracentrifugation of the preparation precipitated the virus. Dissolving of the precipitate in a diluted buffer solution resulted in a typical opaque virus solution. Analysis with the analytical ultracentrifuge yielded a very sharp peak. This pointed to pure and unaggregated virus. Moreover the sedimentation constant S_{20} appeared to agree with that of 117.7 given by Reichmann [40]. De Bokx [6] used this virus solution to prepare antiserum and this had a titer of 1,024 after one injection of the rabbit. Moreover this antiserum failed to react with healthy plant material.

Besides the liberation from the column of potato virus X in very big particles, the virus eluted in a few different fractions when the column was subjected to a serial passage of solvent mixtures in which the polyethylene glycol concentration was reduced stepwise from 2 to 1.5, 1.0, 0.5 and 0%. The solvents with 2, 1.5 and 1% polyethylene glycol yielded the virus-carying effluents.

The explanation of the partition of the virus in these fractions may be found in the formation of complexes of potato virus X with polyethylene glycol. It is believed that the virus particles adsorb the polyethylene glycol molecules. This is a slow reaction represented as follows:



$$\begin{aligned} \text{a PVX} + \text{b PEG} &\leftrightharpoons \text{c } (\text{PVX}_p \cdot \text{PEG}_x) \\ &\leftrightharpoons \text{d } (\text{PVX}_q \cdot \text{PEG}_y) \\ &\leftrightharpoons \text{e } (\text{PVX}_r \cdot \text{PEG}_z) \end{aligned}$$

It is assumed that $r > q > p > 1$ and $z > y > x > 1$. This equation represents the formation of gradually bigger complexes. Probably the complex $\text{PVX}_r \cdot \text{PEG}_z$ eluted with the solvent containing a lower polyethylene glycol content than the complexes $\text{PVX}_q \cdot \text{PEG}_y$ and $\text{PVX}_p \cdot \text{PEG}_x$. To calculate the coefficient p, q and r, and x, y and z it is necessary to estimate the amounts of potato virus X and of polyethylene glycol in the complexes of the different fractions. Till now a technique to determine small amounts of polyethylene glycol has not been realized. The reactions mentioned in the equation were indicated as equilibrium processes because the ratios of the virus concentrations in the corresponding fractions in a number of experiments were almost the same. Moreover, the complex formation was easily to abolish by addition of sodium chloride to a final concentration of 10 or 15%.

However an addition of sodium chloride to an effluent containing complexes of tobacco mosaic virus and polyethylene glycol did not induce dissolving of the virus. In this case breaking of the complexes was possible according to the following two procedures.

Ultracentrifugation sedimented the complexes. These gradually dissolved after suspending of the pellet in a diluted buffer solution, e. g. 0.01 M phosphate buffer, pH 7. A low speed centrifugation at 10,000 rpm during 15 minutes resulted again in a pellet still containing most of the virus. Resuspending of this pellet in diluted buffer solution yielded a preparation of almost all the virus particles in solution. Another low speed centrifugation removed the last contaminations.

A second method was the addition to the complex-containing effluent of polyethylene glycol and sodium chloride to final concentrations of 5 and 2%, respectively. This precipitated the complexes completely and a low speed centrifugation of 10,000 rpm during 15 minutes was enough to sediment the virus. The sediment was resuspended in diluted buffer solution, but low speed centrifugation still sedimented most of the virus. Resuspending of this pellet permitted to disslove the virus particles.

In both cases the first suspending of the pellet in diluted buffer solution removed the polyethylene glycol. The second suspension did not contain polyethylene glycol and therefore allowed the virus to dissolve.

The final colour of the virus preparation is the result of the amount of the first solvent containing 5% polyethylene glycol and 2% sodium chloride that percolated through the column before the elution of the virus by another solvent. After passage of an insufficient amount of the first solvent the final preparations of tobacco mosaic virus had a brown-yellow colour. Coloured compounds from the original homogenate were apparently adsorbed by the virus particles. Besides polyethylene glycol also substances originally present in the homogenate, such as lipoids, carotenoids, xanthopylls and polyphenols, were able to form complexes with the virus. Because of the apolar character of these compounds an addition of sodium chloride to the virus-containing effluent had no effect.

The adsorption of normal substances on the virus particles could be demonstrated by the use of radioactive isotopes. Potato virus X was isolated from a mixture of radioactive (P^{32} or C^{14}) healthy plants and non-radioactive diseased plants. Though a synthesis of a new virus in the mixed homogenate from radioactive substances of the healthy plant material was not possible, the isolated virus, however, appeared to contain a very small amount of radioactivity [55]. It was concluded that besides polyethylene glycol the normal substances from the homogenate were able to form complexes with the virus particles. The properties of these substances determined in which way such complexes could be abolished. Hydrophobic substances, such as fats, could not be displaced from the virus particles by ions. In this respect polyethylene glycol is a better tool to achieve this displacement. It was therefore recommended to elute all the low molecular weight substances completely from the column with an excessive amount of the first solvent before liberation of the virus.

The size of the complex particles is determined by the concentration of the virus particles, the concentration of other substances in the homogenate and the concentration of polyethylene glycol. The more molecules enter the attraction sphere of each other the bigger complexes will be formed. In this reaction the character of the particles is not determinant but the mass is of importance. All the macromolecules are concerned in the complex formation, and therefore also the high molecular weight carbohydrates. Consequently the cellulose used as column material in this procedure, participates in the compex formation. This explains the combination of the virus and other high molecular weight compounds with the column material when the solvent contains polyethylene glycol.

In all the experiments described in this paper the polyethylene glycol was of the quality 6,000. Batches of this quality contain polyethylene glycol molecules with molecular weights ranging from 5,500 to 6,500 but the average molecular weight is 6,000.

In crude extracts potato virus S formed complexes with poleythylene glycol 6,000 which were too heavy to elute the virus from the column with a next solvent containing no polyethylene glycol. In this case the use of polyethylene glycol 4,000 or polyethylene glycol 3,000 was more successfull and some virus could be collected in the effluent obtained with the next solvent mentioned above (Table 1).

Table 1

The retention of some viruses on a cellulose column by solvents containing 5% polyethylene glycol (PEG) of different quality, 1% NaCl, 4.5% glucose, 0.01 M Tris buffer, pH 7, and 0.004 $MgCL_2$

Type PEG	Retention by solvent containing 5% polyethylene glycol				Liberation by solvent without polyethylene glycol			
	PVA	PVS	PVY	AMV	PVA	PVS	PVY	AMV
400	—	—	—					
1,540	—	—	—					
3,000	—	±	±			+	+	
4,000	±	+	+		+	+	+	
6,000	+	+	+	—	+	—	+	
20,000				+[1]				+

[1] Data of Clark [13].

Presence of virus indicated by +. Ability of a second solvent containing 4.5% glucose, 0.01 M Tris buffer, pH 7 and 0.004 M $MgCL_2$, to liberate the virus from the column indicated by +.

PVA — potato virus A; PVS — potato virus S; PVY — potato virus Y; AMV — alfa mosaic virus.

Polyethylene glycol 6,000 was useful in the purification of potato viruses A and Y. However, polyethylene glycol 4,000 produced smaller complexes with these viruses and therefore the liberation from the column was easier.

Moreno [34] studied the chromatographic purification of alfalfa mosaic virus. Here the virus particles were too small to be retained in the column by a solvent with polyethylene glycol. Even solvents with higher concentrations of polyethylene glycol 6,000 were not able to establish the adsorption of this virus on the cellulose. Clark [13] precipitated the virus from clarified sap with 8% polyethylene glycol

20,000 in 0.2 M sodium chloride to remove contaminating substances before the chromatographic procedure. The mass of polyethylene glycol 20,000 was big enough to form sedimentable complexes with the very small virus particles.

The combination of virus and other high molecular weight compounds with cellulose was amplified by the ions in the solvent. The presence of ions stimulated the complex formation. The explanation of this effect might be found in the attraction of water by the ions and less free water was left. The high molecular weight particles were able to approach each other more easily.

Reducing of the content of ions and polyethylene glycol might break the complexes. However, this breaking was more difficult when the complexes were very big. This occured in a mixture containing besides the virus large amounts of chloroplasts, ribosomes, nucleic acid and proteins. The size and the composition of the complexes was therefore determinant for the liberation of the virus particles. The liberation of virus particles was easier from smaller complexes. It was of importance to keep the high molecular weight particles in the original suspension as far from each other as possible. A dilution of the suspension favoured the limitation of the complex formation under the influence of polyethylene glycol and salt. However, dilution was not always practical becuase percolation of the liquid through the column took a longer time. It was considered to mix the original suspension with cellulose or sand to achieve a spatial separation of the particles. After the suspension was mixed with the added cellulose or sand polyethylene glycol and salt could be added. The system now consisted of very small compartments and each compartment contained a small number of high molecular weight particles concerned in the formation of complexes. The size of the complex particle would be limited by the number of particles in each compartment. In the most ideal case the compartments should by extremely small that each contained only one macromolecular particle forming a complex with polyethylene glycol. This could not be realized in practice but it appeared that addition of cellulose or sand to the homogenate resulted in an important increase of the virus yield.

In the above the effect of the partial separation has been suggested too simply. Addition of too much cellulose or sand to the homogenate induced a decrease in the amount of virus that could be isolated. This was the case when 50 g of fresh plant material were mixed with more than 800 g of sand or 50 g of cellulose. The effect of the partial separation was more favourable when the original extract had been clarified before an extra amount of cellulose or sand was added. The combination of chloroplast-cellulose (or sand) — polyethylene glycol had a too strong tendency to adsorb the virus. Then it might be almost impossible to liberate the virus.

This could be demonstrated with apple chlorotic leaf spot virus. *Chenopodium amaranticolor* was used as a host plant [16, 17]. Five days after inoculation chlorotic spots on the inoculated leaves were visible and later-on the upper leaves showed the systemic chlorotic spots. Inoculation of the virus on *Chenopodium quinoa* resulted in very small necrotic lesions of the inoculated leaves and therefore this plant was used as a test plant. In attempts of Maat [31] to purify this virus, he clari-

fied the homogenate by use of ether and carbontetrachloride. Fifty grams of leaves were homogenized with 100 ml of 0.02 M *Tris* buffer, pH 9, containing 0.1% thioglycolic acid, and 50 ml of ether and 50 ml of carbontetrachloride. A centrifugation at 6,000 rpm during 20 min separated the mixture in three layers. The upper layer was collected and the pH was adjusted to 9. Inoculation of this solution on *Chenopodium quinoa* yielded a large number of local lesions.

For the chromatographic purification of this virus polyethylene glycol ammonium acetate, glucose and $MgCl_2$ to final concentrations of 5%, 0.5%, 4.5% and 0.004 M, respectively, were dissolved in the clarified solution mentioned above. This mixture was percolated through the column. After washing the column with a solvent containing 5% polyethylene glycol, 0.5% ammonium acetate, 4.5% glucose, 0.01 M *Tris* buffer, pH 9 and 0.004 M $MgCl_2$ the virus eluted with the solvents in which the ammonium acetate, glucose $MgCl_2$ and pH 9 *Tris* buffer concentrations were held at a constant level while the polyethylene glycol concentration was reduced stepwise from 2 to 1 and 0%. The virus-carrying effluents were ultracentrifuged at 50,000 rpm during 1.5hr. Inoculation of the suspended pellet in 1 ml of 0.01 M *Tris* buffer, pH 9, resulted in a large number of local lesions.

When infected leaves were homogenized in the first solvent and this crude homogenate was placed in the column directly, then the virus-containing effluents were not very infectious (Table 2).

Table 2

Relative infectivities of apple chlorotic leaf spot virus in fractions obtained from *Chenopodium amaranticolor* by chromatographic purification on cellulose columns

Percentage of polyethylene glycol in effluent	Number of local lesions per leaf of *Chenopodium quinoa*	
	homogenate directly on column	homogenate clarified by treatment with ether-CCl_4 before application on the column
2	0	54
1	11	67
0	17	32

Solvents contained 0.5% ammonium acetate, 4.5% glucose, 0.01 M Tris buffer, pH 9, 0.004 M $MgCl_2$, and different amounts of polyethylene glycol. Virus-carrying effluents were ultracentrifuged (50,000 rpm, 1.5 hr); pellets were dissolved in 1 ml of 0.01 M Tris buffer, pH 9, and inoculated.

It was concluded that this striking difference in numbers of local lesions was a consequence of the combination of the virus with chloroplast and polyethylene glycol. This could also be demonstrated by another experiment. Leaves of *Chenopodium amaranticolor* infected with apple chlorostic leaf spot virus were homogenized with a solvent containing 0.5% ammonium acetate, 4.5% glucose, 0.01 M *Tris* buffer, pH 9, and 0.004 M $MgCl_2$. Centrifugation at 10,000 rpm during 25 min resulted in a clear solution with a brown-greenish colour. This supernatant was divided in four equal portions. To the first portion 1% polyethylene glycol was

added, to the second portion 3%, and to the third portion 5% polyethylene glycol. The fourth portion was a reference. Ultracentrifugation of equal amounts of each portion at 50,000 rpm during 1.5 hr yielded supernatants and pellets. The supernatants as well as the pellets dissolved in 0.5 ml of Tris buffer, pH 9, were inoculated on *Chenopodium quinoa*. The reference and the pellet of the first portion gave rise to considerable numbers of local lesions (Table 3).

Table 3

The influence of polyethylene glycol on the infectivity of apple chlorotic leaf spot virus

Final concentration of polyethylene glycol (%)	Number of local lesions per leaf of *Chenopodium quinoa*	
	supernatant	pellet
0	0	64
1	0	45
3	0	25
5	0	2

Homogenate was prepared from 15 g of *Chenopodium amaranticolor* in 100 ml of a solution cont·ining: 0.5% ammonium acetate, 4.5% glucose, 0.01 M Tris buffer, pH 9 and 0.004 M $MgCl_2$. The mixture was centrifuged (10,000 rpm, 25 min). Different, amounts of polyethylene glycol were added to equal portion of the supernatant. Samples of these were ultracentrifuged (50,000 rpm, 1.5 hr). Supernatants and pellets dissolved in 0.5 ml of Tris buffer, pH 9, were inoculated on *Chenopodium quinoa* (12 leaves per sample).

These results gave a direct indication of the inhibiting effect of polyethylene glycol. In a similar experiment with an extract clarified by ether and carbontetrachloride no effect of polyethylene glycol could be found. Therefore even the presence of small amounts of chloroplasts was responsible for this inhibiting effect with polyethylene glycol. Moreover the chloroplasts were able to adsorb the virus irreversibly when polyethylene glycol was not present because the infectivity decreased after standing for one day or more. In this experiment a homogenate of 10 g of leaves of *Chenopodium amaranticolor* in 80 ml of a solvent containing 0.5% ammonium acetate, 4,5% glucose, 0.01 M *Tris* buffer, pH 9, and 0.004 magnesium chloride was centrifuged at 10,000 rpm during 25 min and yielded the first supernatant. Centrifugation at 10,000 rpm during 25 min of the resuspended pellet in 50 ml of the above mentioned solvent resulted in a second supernatant. This washing was repeated once more. Inoculation of the three supernatants on the same day gave rise to large numbers of local lesions on *Chenopodium quinoa*. When the same preparations were inoculated on the next day, the first two supernatants were completely inactive and the third yielded a few lesions. A treatment with ether and carbontetrachloride stabilized the supernatants (Table 4).

These data demonstrated the almost complete inactivation of the virus after one day. It was possible that the chloroplasts adsorbed the virus particles without the presence of polyethylene glycol. In this case reaction took a longer time. The cause of this adsorption was removed by the treatment with ether and carbon tetrachloride.

Dunez [19] discussed the clarification of plant sap by an emulsion of *n*-butanol--chloroform and ether-carbon tetrachloride. He used three viruses of fruit trees:

Table 4

The infectivity of apple chlorotic leaf spot virus in crude extracts

Preparation	Number of local lesions per leaf of *Chenopodium quinoa*	
	inoculation on the first day	inoculation on the second day
Original homogenate	69	0
First supernatant	43	1
Second supernatant	64	0
Third supernatant	71	12
Supernatant treated with ether and CCl_4		85

Homogenate of 10 g of leaves of *Chenopodium amaranticolor* in 80 m of a solvent containing 0.5% ammonium acetate, 4.5% glucose, 0.01 M Tris buffer, pH 9 and 0.004 M $MgCl_2$ was centrifuged (10,000 rpm, 25 min); pellet was washed twice. Supernatants were inoculated on *Chenopodium quinoa* on the same and on the next day (12 leaves per sample). On the first day 70 ml of the combined supernatants were clarified with ether and carbontetrachloride and inoculated on the next day.

necrotic ringspot virus, prune dwarf virus and tobacco mosaic virus. It was concluded from these experiments that only the procedure of clarification by ether-carbon tetrachloride did not result in a loss of infectivity.

In the investigations of macromolecules polyethylene glycol is a noteworthy and very important product. It has already been mentioned that polyethylene glycol was able to displace hydrophobic compounds from virus particles. The attraction of macromolecules to polyethylene glycol gave rise to complex formation which seemed an impeding co-phenomenon but appeared to be the cause of the protecting action of polyethylene glycol (see also [2]). Numerous unstable macromolecules were stable in a polyethylene glycol solution and consequently easier to approach in further investigations.

This question yielded another important aspect concerning tobacco rattle virus. Harrison and Klug [22] distinguished the particles of this virus in two main groups, the long and the short particles. Frost *et al.*, [20] suggested that both types of particles had a special function in the infection process. However, only one type of particles was found after the chromatographic purification on cellulose columns. The length of these particles equalled about the sum of the length of a long and a short particle. The electron micrographs gave almost the same impression as those of tobacco mosaic virus and consequently Bos [7] tested this preparation on a number of different host plants to compare the symptoms with those caused by tobacco mosaic virus. However, the typical host plants showed the symptoms of tobacco rattle virus. This question was not investigated further on but it was possible that the tobacco rattle virus occurred in the plant as one type of particles. In the usual conditions of analysis the virus particles might break into pieces and then it seemed as if the two types of particles were present in the plant.

The behaviour of the new strain of potato virus Y [5] in the chromatographic procedure was different. Sometimes a small amount of this virus could be succesfully isolated but on the next day the isolation of this virus from the same plant material failed completely. Electron micrographs of the most infectious prepara-

tions showed different types of virus particles. A few of these were complete and their lenght was in agreement with that given in literature. However, many other particles occurred in the micrograph. All of these were much smaller than the complete virus particles. Moreover these small particles were localized in groups and some of them even in a line. It is possible that each group of small particles represented a complete virus particle. It was concluded that during the procedure of the preparation the virus particles were unstable and desintegrated. It seemed that many virus particles "exploded".

In the usual chromatographic purification procedure of apple chlorotic leaf spot virus a lesser amount of sodium chloride (not more than 0.5%) in the first solvent was very favourable to collect infectious fractions. During the percolation of the virus-eluting solvents the absorptio-meter did not indicate the liberation of virus particles. In spite of this the presumable virus-carrying fractions were ultra-centrifuged at 50,000 rpm during 1.5 hr. These yielded no visible pellets. Solutions of these pellets were highly infectious on *Chenopodium quinoa.*

Different techniques to demonstrate the presence of virus particles were attempted. Concentrated hydrochloric acid hydrolyzed the protein of the virus and yielded amino acids. Usually the ninhydrin reaction with amino acids resulted in purple coloured products. Although this reaction is one of the most sensitive reactions this procedure did not yield any results [35].

Further it was possible to label the virus by presenting P^{32} to the host plant. The virus containing fractions prepared from these plants by column chromatography gave rise to a large number of local lesions on *Chenopodium qiunoa* but Noordink [36] could not determine the radioactivity of the virus. Apparently the concentration of apple chlorotic leaf spot virus in the preparations was still too small to obtain positive results by the use of the two techniques mentioned above.

In studies of Cropley [17] apple chlorotic leaf spot virus was partially purified by differential centrifugation of sap from inoculated *Chenopodium quinoa* leaves. Electron micrographs of the final preparation showed flexuous rodshaped particles. The lengths of these particles were very variable and it seemed that many had broken during the preparation process. By using of the "dip-method" Kegler and Schmidt [26] obtained electron micrographs of a latent virus from apple. In these micrographs a few flexible rods of about 750 mμ occurred. The "dip-method" was probably more suitable to obtain complete particles in the electron micrographs because purification of the virus was omitted and thus the riks of damaging the virus particles. It was also possible that other strains of apple viruses were more stable. Lister *et al.*, [30] studied some apple viruses and classified these into two groups. Partially purified preparations contained flexuous filamentous particles. The lengths of these particles were close to 620 mμ for type 2 viruses and in the range of 500-700mμ for type 1 viruses. Probably the particles of type 1 viruses underwent some degradation during the isolation procedure. Those of type 2 viruses did not. Our electron micrographs of apple chlorotic leaf spot virus revealed some very small particles in groups resembling the particles in the micrographs of potato virus Y. Before the application of the virus on the grids for electron microscopy the polyethylene

glycol was removed by ultracentrifugation. During drying the preparation on the grids the virus particles missed the protecting action of polyethylene glycol and could be desintegrated.

In the above the protecting or stabilizing action of polyethylene glycol was discussed. However, many other macromolecules have similar effects on viruses. Diener and Desjardins [18] found that an addition of pancreatic ribonuclease, β-lactoglobuline, trypsin, or pronase, but not of ovalbumin, lysozyme, or bovine albumin, protected purified tobacco mosaic virus from degradation when the virus-protein mixtures were exposed to 0.005 M borate buffer, pH 9.8, for one hour at 25°C. Brunt and Kenten [10] used protein to protect cocoa-swollen-shoot virus during the purification of this virus from cocoa leaves.

In the beginning of the column chromatographic purification procedure the low molecular weight products could be thoroughly removed by percolation of an excess of the first solvent. In this solvent the chloroplast and perhaps some other types of cell constituents had the tendency to coagulate. These coagulated particles remained in the upper part of the column during the whole purification procedure. However, it should be mentioned that elution of the low molecular weight substances was rather difficult. In the beginning large amounts of these substances were liberated from the column immediately but the column was not clean soon after this. It took a long time and the line indicated by the absorption meter, declined very slowly to the beginning value. One reason for this was the tendency of the chloroplasts to adsorb many different substances. Desorption of the low molecular weight substances from the chloroplasts by the first solvent took more time than a simple displacement of dissolved substances from the homogenate. Moreover it was very likely that the chloroplasts underwent a slow change during the procedure. Built-in compounds of low molecular weight released and left the column. This was easy to understand when the chloroplasts were considered more precisely. Besides green pigments these cell constituents contain lipoids, proteins, ribonucleic acids, deoxyribonucleic acids and even more compounds. Therefore it would not be surprising if certain viruses might be found also in the chloroplasts.

According to Cech [12] the tobacco mosaic virus particles were passively entrapped into the chloroplasts vacuoles or vesicles *via* invagination. In electton microscope studies Cocking [14] found that isolated tomato fruit protoplasts took up both tobacco mosaic virus and ferritin by the process of pinocytosis. Very close association existed between the nucleus and chloroplasts and channels extending from the plasmolemma into the cytoplasm were present. Sometimes Milne [33] saw tobacco mosaic virus particles in invaginations or vacuoles in chloroplasts. Sun [46] found evidence that virus particles could penetrate into the chloroplasts of *Abutilon striatum*.

Mostly Whatman standard grade cellulose powder was used as column material. In some experiments potato virus Y was purified on white sand (approximately 100 mesh). A difference in results could not be found when this sand was used instead of cellulose. The same held for the purification of chlorotic leaf spot virus on a column of glass beads (approximately 80 mesh, The British Drug Houses, Ltd). Clark [13]

preferred kieselguhr as column material to cellulose to purify alfalfa mosaic virus because of a non-specific adsorption of the virus by cellulose. Moreover he found a better retention and separation of virus components with kieselguhr than with cellulose.

Different aspects of the virus purification according to our chromatographic system have been reviewed. These are the formation of complexes with polyethylene glycol or with compounds in the homogenate, the liberation of the virus in several fractions, the protecting action of polyethylene glycol, the decomposition of virus particles and finally the problem of the occurrence of a virus in chloroplasts.

It may be concluded that complex formation is necessary to protect the virus and to allow chromatographic purification. The size of these complexes should be within certain limits. The removal of chloroplasts is recommended especially when the plant material contains a small amount of the virus to be purified.

Finally the automatic performance of our chromatographic virus purification procedure may be mentioned. The effluent coming from the column through a continuous flow cuvette of the absorption meter, is fractionated in a fraction collector. A number of glass vessels contain various solvents. These vessels are connected with the top of the column by a silicon tubing closed by an electromagnetic clamp. At the start of the procedure an electric clock induces electric power on the magnetic soil of the first clamp to open the first vessel during a chosen length of time. Now the first solvent is allowed to percolate trough the column. After the chosen time has expired the mechanism of the clock induces the impuls to close the first clamp and to open the clamp of the second vessel simultaneously. After expiring of this period the clamp of the second vessel is closed and that of the third vessel is opened. In this way an almost unlimited number of vessels may be connected to the clock. Independently of the apparatus mentioned above, Hicks and Nalevac [24] developed a similar instrument. Succesive solvents of various compositions may pass the column automatically when the vessels of the apparatus have been filled with liquids in the right sequence. The absorption meter records on a chart the ultra-violet light absorbance at 254 mμ of the effluent passing the column through the continous flow cuvette. On the same chart the fractions are recorded so that the virus-containing fractions can easily be collected at the end of the procedure.

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