PURIFICATION OF POTATO VIRUS X AND PREPARATION OF INFECTIOUS RIBONUCLEIC ACID BY DEGRADATION WITH LITHIUM CHLORIDE*

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Potato virus X (PVX) was one of the first plant viruses to be partially purified (Bawden and Pirie 1938). Purification by precipitation with salt solutions, however, resulted in aggregation of the particles which became entwined to form extensive rope and netlike structures (Kleczkowski and Nixon 1950). Markham (1959) suggested that the problem of aggregation and the formation of gels when the virus is treated with salt solutions has prevented PVX from being extensively studied.

Reichmann (1959) described a method for the purification of non-aggregated PVX by dialysis of infected plant sap against sodium citrate, followed by differential ultracentrifugation of the dialysate. Corbett (1961) found that virus prepared by Reichmann's method was poorly infective and thus he developed a method which involved clarification with activated charcoal and rate zonal density-gradient centrifugation. However, this method has the limitation that only relatively small amounts of virus can be handled. Difficulties have also been reported in preparing PVX-RNA by the standard phenol extraction procedure although Reichmann and Stace-Smith (1959) isolated infectious PVX-RNA by denaturation with guanidine.

This paper describes a rapid method of preparing large quantities of relatively unaggregated PVX and a convenient method of isolating infectious viral RNA by degradation with lithium chloride.

Experimental

The virus used throughout this study was identified as PVX by its host range, thermal inactivation point, and the presence in leaf extracts of flexuous rods about 500 mμ in length. Virus was propagated in *Nicotiana glutinosa* L. and infectivity assays were carried out on leaves of *Gomphrena globosa* L.

Systemically infected leaves of *N. glutinosa* were homogenized in 1·5 volumes of 0·2M Na_2HPO_4 and the extract was clarified by adsorption with charcoal and DEAE-cellulose and filtration through celite, as used for the purification of lettuce necrotic yellows virus (McLean and Francki 1967). The virus was concentrated by centrifugation in a Spinco model L centrifuge at 44,000 *g* for 90 min. The pellets were resuspended in distilled water and emulsified with an equal volume of chloroform for 5 min. The emulsion was centrifuged at 12,000 *g* for 10 min and the aqueous layer containing the virus was removed with a hypodermic syringe. The virus was sedimented at 160,000 *g* for 30 min, resuspended in distilled water, and the chloroform extraction and sedimentation steps were repeated. The final virus preparation in distilled water was stored at 4°C until required.

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Homogenates of PVX-infected leaves lost less than 50% of the virus when clarified, whereas virtually all chloroplast material was removed. After centrifugation of the filtrate the small, pale yellow pellets, when resuspended in distilled water and examined in the electron microscope, were observed to contain numerous characteristic virus particles with very little contaminating material, presumably fraction I protein (Fig. 1).

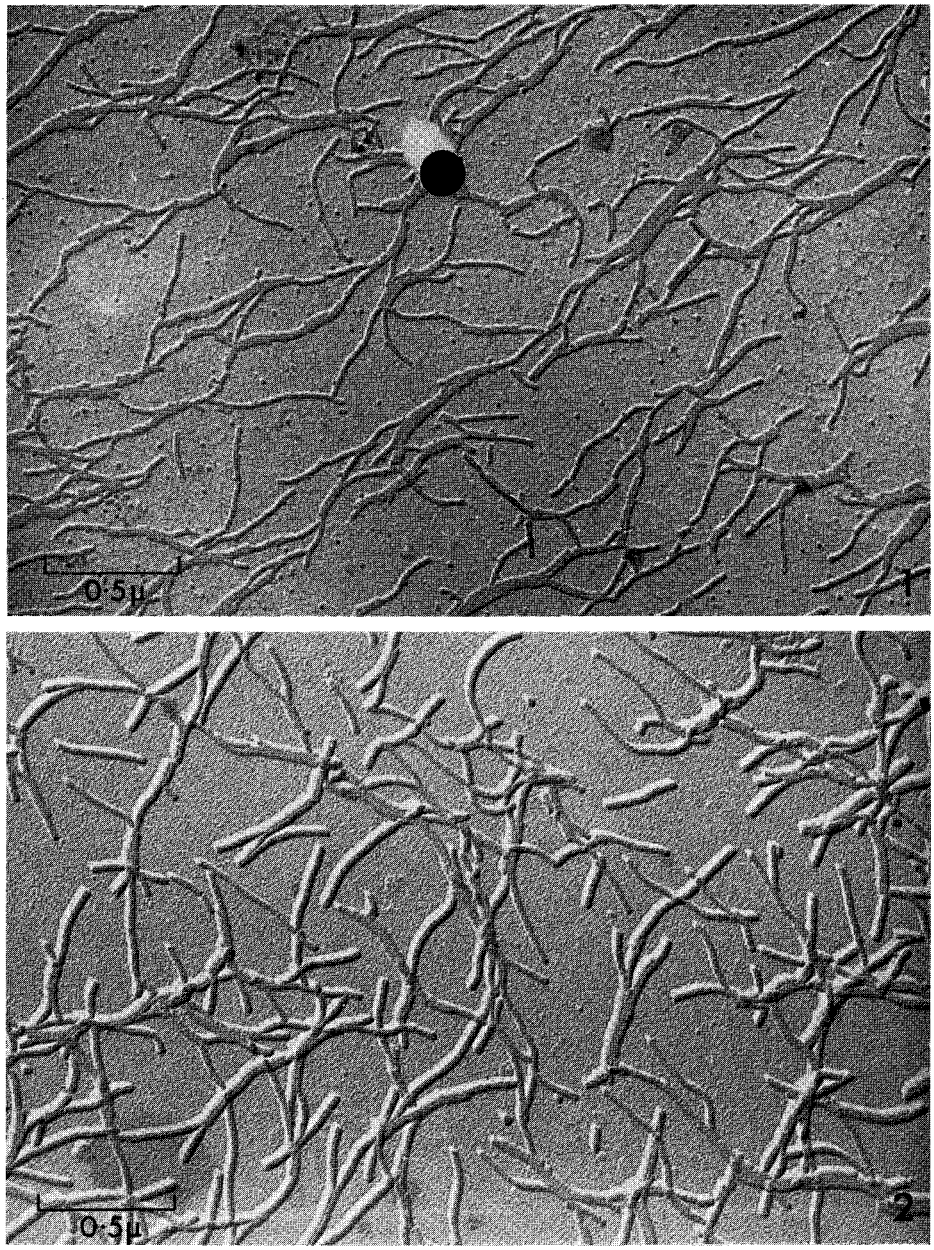
Virus preparations purified by the entire procedure outlined above were highly infectious, producing 50–100 lesions on leaves of *G. globosa* when diluted to a concentration of 10 μg/ml. No significant amounts of materials contaminating the virus were detected by either electron microscopy (Fig. 2) or by serological gel-diffusion (Figs. 3 and 4). In the analytical ultracentrifuge only a single peak was observed which was homogeneous when virus was suspended in water but showed a small shoulder when suspended in 0·1M phosphate buffer, pH 7·2 (Fig. 5). Absorption spectra of virus preparations between 230 and 300 mμ were characteristic of a nucleoprotein. Size distribution of virus particles measured from electron micrographs varied slightly from preparation to preparation, and 60–70% of the virus material consisted of particles between 450 and 550 mμ long. Concentrated virus preparations, when stored in distilled water at 4°C for several weeks, did not show any tendency to form gelatinous suspensions and, even at concentrations of 25 mg/ml, were not unduly viscous. Yield of virus varied widely in experiments and appeared to depend on the conditions under which the plants were grown. However, usually at least 150 mg of virus was obtained from 1 kg of *N. glutinosa* leaves.

PVX-RNA was isolated from the virus by the degradation procedure using lithium chloride (Francki *et al.* 1966) but it was found that freezing for at least 3 hr at -10 to -15°C was necessary for the complete separation of virus protein and RNA (Fig. 6). For the routine preparation of PVX-RNA we have found it convenient to freeze PVX in water or in 0·02M phosphate buffer, pH 7·2, with an equal volume of 4M LiCl overnight. On thawing, the mixture was centrifuged for 10 min at 3000 *g* and the RNA was recovered in the pellet, whereas the protein remained in the supernatant. The RNA was resuspended in 0·02M phosphate buffer, pH 7·2, and was then precipitated with 2 volumes of ethanol to which a few drops of 0·1M $MgCl_2$ were added. The precipitate was centrifuged for 10 min at 3000 *g* and the pellet again resuspended in 0·02M phosphate buffer, pH 7·2. The resulting preparation showed specific absorption typical of RNA between 220 and 300 mμ.

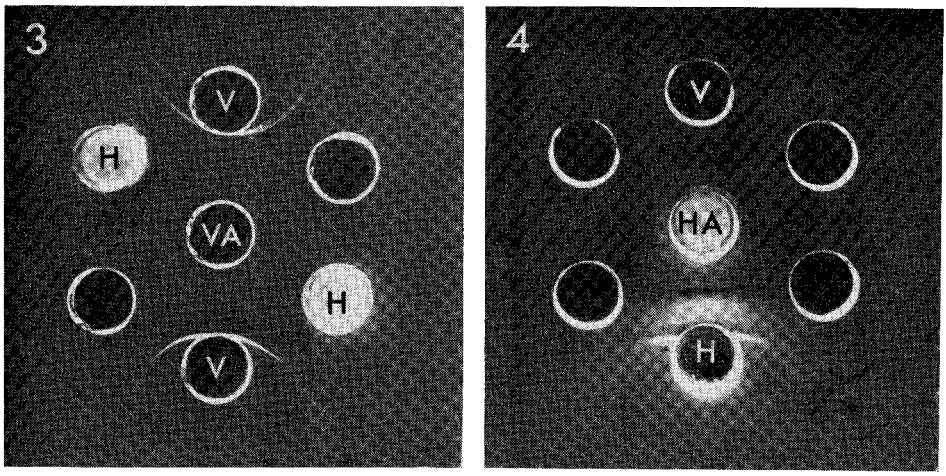
Results

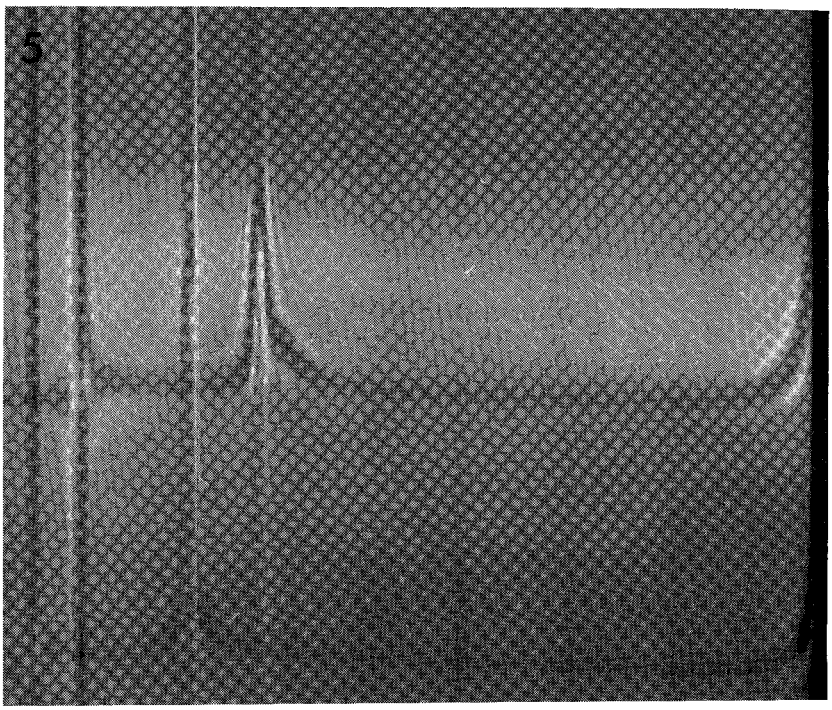
Results of experiments in which the infectivity of undegraded virus and PVX-RNA prepared by degradation with lithium chloride were compared are summarized in Table 1. The results of one experiment in which PVX-RNA was prepared by the phenol extraction procedure (Gierer and Schramm 1956) are also included. Thus PVX-RNA prepared by the lithium chloride method was less than 1% as infectious as undegraded virus containing an equal amount of RNA. In subsequent experiments phenol-prepared RNA was inoculated to *G. globosa* leaves at concentrations ranging from 35 to 185 μg/ml but no local lesions were produced. Table 2 summarizes the results of a series of experiments in which the properties of RNA preparations made by degradation with lithium chloride and by extraction with phenol were compared.

PVX-RNA preparations isolated by these two methods were analysed by sucrose density-gradient centrifugation and found to behave similarly [Fig. 7(*a*)]. Lithium chloride-prepared PVX-RNA was compared with TMV-RNA prepared by extraction with phenol and the peaks were found to reach the same position in the gradients [Fig. 7(*b*)] although PVX-RNA contained considerably more material sedimenting ahead of the main peak. This behaviour of PVX-RNA has been consistent, although the proportion of material sedimenting ahead of the main peak has



Figs. 1 and 2.—Electronmicrographs of PVX preparations examined in a Philips 100B electron microscope after shadowing with platinum–carbon. *1*, Virus preparation after initial sedimentation of clarified leaf extracts. In addition to elongate virus particles numerous small spherical particles of fraction I protein are seen. Shadowing at 30°. ×36,000. *2*, Virus preparation after purification by the complete purification procedure as described. Shadowing at 20°. ×36,000.





Figs. 3 and 4.—Serological reactions by gel-diffusion tests. *3*, Antiserum to purified preparation of PVX (*VA*) tested against the homologous antigen (*V*) and *N. glutinosa* leaf antigen (*H*). *4*, Antiserum to *N. glutinosa* leaf antigen (*HA*) tested against purified PVX (*V*) and homologous antigen (*H*).

Fig. 5.—Schlieren diagram of a PVX preparation containing 2 mg/ml of virus in 0·1M phosphate buffer, pH 7·2, (top pattern) and in distilled water (bottom pattern). Photograph taken at bar angle of 65°, 20 min after reaching a speed of 19,160 r.p.m. in the AnD rotor of a Spinco model E ultracentrifuge. Sedimentation from left to right.

varied from preparation to preparation. It has also been observed that the proportion of fast-sedimenting material increases with the amount of RNA that is applied to the density gradient. Infectious material was recovered from gradients in positions of the main peak and ahead of it, but not behind it.

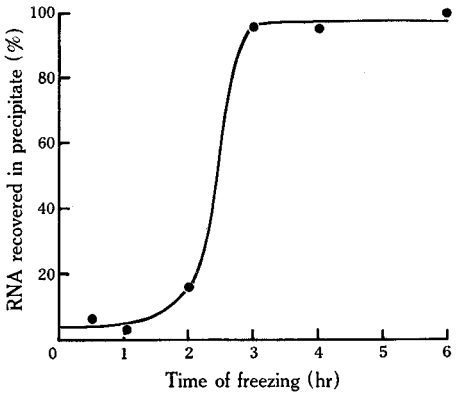


Fig. 6.—Recovery of PVX-RNA in the precipitate after freezing for various times in 2M lithium chloride. Each sample was frozen for the specified time and then thawed at 4°C for 30 min.

PVX-RNA prepared by degradation with lithium chloride is sensitive to pancreatic ribonuclease. RNA at a concentration of 360 μg/ml incubated at room temperature for 90 min with 0·01 μg/ml of the enzyme produced only one lesion on 14 leaves of *G. globosa*, whereas RNA alone produced 287 lesions.

TABLE 1

INFECTIVITY OF INTACT PVX COMPARED WITH ISOLATED VIRAL RNA

Experiment	Viral Nucleoprotein Inoculum		Viral RNA Inoculum		
	RNA Concn. (μg/ml)*	Lesions per Leaf†	Method of Preparation	RNA Concn. (μg/ml)‡	Lesions per Leaf†
1	0·5	7	Phenol	100	0
2	0·5	59	LiCl	50	4
3	0·4	65	LiCl	89	4
4	0·5	30	LiCl	55	19
5	0·7	37	LiCl	720	35

* Concentration of RNA inside intact PVX protein determined by assuming PVX to contain 5% RNA and having an absorbancy index of 2·7 at 260 mμ in a cell of path length 1 cm (Reichmann and Stace-Smith 1959).

† In each experiment virus and RNA inocula were compared on 8–16 opposite leaves of *G. globosa* plants.

‡ Determined by assuming the absorbancy index of PVX-RNA to be 25·6 at 260 mμ in a cell of path length 1 cm (Reichmann and Stace-Smith 1959).

Viral protein was recovered from the supernatant after freezing PVX with lithium chloride and removing the precipitated RNA by centrifugation. The solution was dialysed and concentrated with ammonium sulphate and found to be serologically active when tested against a PVX antiserum by gel-diffusion. PVX protein prepared

in this way must have been of relatively low molecular weight as it failed to sediment into sucrose density gradients when centrifuged under conditions used for the analysis of RNA preparations.

TABLE 2

YIELDS AND SPECTRAL PROPERTIES OF RNA PREPARATIONS ISOLATED FROM PVX BY THE LITHIUM CHLORIDE AND PHENOL METHODS

Results were obtained from six experiments, in each of which half of the virus preparation was used for preparing RNA by the lithium chloride method and half by the phenol method

Property	LiCl-prepared RNA		Phenol-prepared RNA	
	Range	Mean	Range	Mean
Yield (%)	30–71	42	8–35	20
Absorbance ratio*	1·74–2·22	2·08	1·79–2·21	1·97
Absorbance ratio†	1·59–1·88	1·77	1·36–2·21	1·84

* Ratio of absorbance at 260 mμ to that at 280 mμ.

† Ratio of absorbance at 260 mμ to that at 230 mμ.

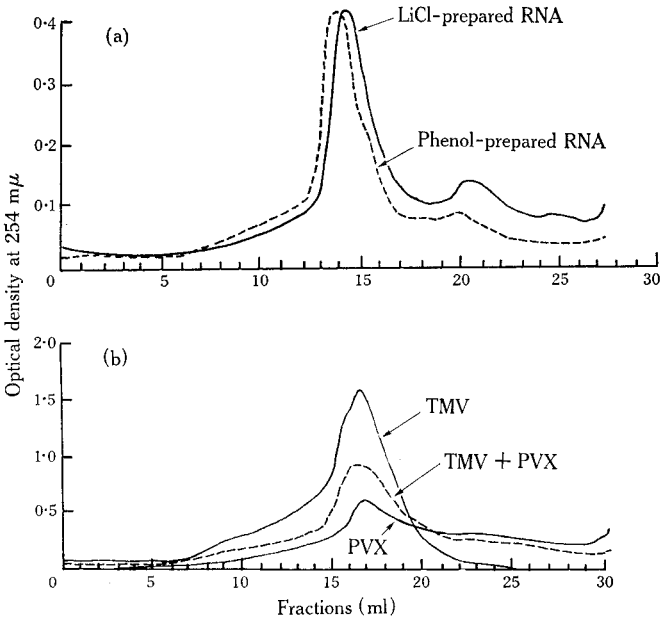


Fig. 7.—Sedimentation in sucrose density gradients of (*a*) 112 μg of lithium chloride- and phenol-prepared PVX-RNA and (*b*) lithium chloride-prepared PVX-RNA (400 μg), phenol-prepared TMV-RNA (400 μg), and a mixture of the two preparations (230 μg PVX-RNA and 165 μg TMV-RNA). Linear density gradients were prepared in 30-ml Spinco SW25 tubes using 5 and 25% sucrose in 0·02M phosphate buffer, pH 7·2. RNA preparations, layered over the gradients, were centrifuged at 24,000 r.p.m. for 16 hr and analysed in an ISCO model D fractionator and densitometer (Brakke 1963).

Discussion

The procedure described above for the purification of PVX is suitable for large-scale production of virus. The method is not time-consuming and the virus is highly infective and free of host materials. The procedure appears to avoid the problem of excessive particle aggregation encountered by some earlier workers (Markham 1959). Initial clarification of plant extracts by adsorption on charcoal and DEAE-cellulose and filtration through celite appears to be better than procedures involving heat coagulation or the use of organic solvents. It is probably relevant that chloroplast materials are removed without being solubilized to release materials which may be potentially capable of adsorption to virus (Ginoza, Atkinson, and Wildman 1954). The method used for purifying PVX may find a far wider application. So far it has been successfully used for routine preparation of TMV and *Cymbidium* mosaic virus in this laboratory; we have obtained as much as 1 g of highly purified TMV from 1 kg of infected tobacco leaves.

The method of preparing PVX-RNA by degradation with lithium chloride is simple to perform, and the virus so obtained is infectious. It has an additional advantage in that the virus protein can be recovered retaining some of its immunological properties. The method may have wider application since we have used it for the preparation of cucumber mosaic virus RNA (Francki *et al.* 1966) and also *Cymbidium* mosaic virus RNA (Francki, unpublished results).

Although it is not known why a fraction of the PVX-RNA sediments ahead of the main peak in sucrose density gradients, we consider that it may be due to aggregation of the RNA. This view is supported by the observation that the proportion of this fast-sedimenting material increases with the amount of RNA which is layered on the gradient. It seems unlikely that this behaviour of PVX-RNA is produced by lithium chloride as (1) very little fast-sedimenting material was observed when lithium chloride-prepared cucumber mosaic virus was subjected to similar analysis, and (2) PVX-RNA prepared by phenol extraction behaved similarly to that isolated by degradation with lithium chloride [Fig. 7(*a*)]. It seems likely that the presence of the fast-sedimenting material is characteristic of PVX-RNA because very little material sedimented ahead of the main peak when phenol-prepared TMV-RNA was analysed by density-gradient centrifugation [Fig. 7(*b*)].

The sedimentation rate of PVX-RNA appears to be very similar to that of TMV-RNA [Fig. 7(*b*)] and hence their molecular weights must be similar. The molecular weight of infectious TMV-RNA has been reported to be 2×10^6 (Friesen and Sinsheimer 1959).

Acknowledgments

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