J. gen. Virol. (1976), **31**, 93–100
Printed in Great Britain

Cauliflower Mosaic Virus: an Improved Purification Procedure and Some Properties of the Virus Particles

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(*Accepted* 28 *November* 1975)

SUMMARY

A new procedure for the purification of cauliflower mosaic virus (CaMV) which involves the use of Triton X-100 and urea results in higher and more reliable yields. CaMV particles have a $s_{20,w}$ of 208S, a $D_{20,w}$ of $0{\cdot}75 \times 10^{-7}$ cm²/s, contain 17 % nucleic acid and have a mol. wt. of $22{\cdot}8 \times 10^6$. Virus precipitated by Mg^{2+}, low pH or ethanol at moderate salt concentrations, or by polyethylene glycol, is not easily resuspended.

INTRODUCTION

Cauliflower mosaic virus (CaMV) was the first plant virus shown to contain DNA. The most popular purification technique and the current estimates of many of the parameters of the virus were summarized by Shepherd (1970). Not published are the difficulties often encountered in purification of this virus, with variable and often extremely low yields from large quantities of leaf tissue. In this paper the development is described of a new purification technique which gives reliable yields of CaMV. Using material purified by this technique some of the physical properties of the virus have been determined or reassessed.

METHODS

Cabbage B, CM4-184, Invergowrie, Wellesbourne and John Innes (JI) isolates of cauliflower mosaic virus were used. The following method of virus purification, similar to that used by Gömec (1973) for dahlia mosaic virus, was developed. Infected leaves of turnip (*Brassica rapa* L. cv. Just Right) 2½ to 3½ weeks after inoculation were chilled and then blended at 4 °C in 0·5 M-potassium phosphate buffer, pH 7·2, containing 0·75 % sodium sulphite (1 ml/g leaf tissue). After filtration through cheese cloth, Triton X-100 and urea were added to the sap to give 2·5 % and 1 M respectively; the sap was then stirred overnight. The supernatant fluid from low speed centrifugation (10 min, 5000 rev/min in a Sorval GSA rotor) was subjected to high speed centrifugation (1·5 h at 27000 rev/min in a Spinco R30 rotor or 2·5 h at 20000 rev/min in a Spinco R21 rotor) and the pellets dispersed in water overnight. After low-speed centrifugation (10 min at 7000 rev/min in a Sorval SS34 rotor) the virus suspension was passed down 10 to 40 % sucrose gradients (in 0·01 M-potas-

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sium phosphate buffer, pH 7·2; 2 h at 23000 rev/min in Spinco SW 25·1 rotor) and the virus-containing band collected using an ISCO ultraviolet scanner and fractionator. The virus in sucrose was diluted 1:1 with water and pelleted by high speed centrifugation (1 h at 45000 rev/min in Spinco R50 rotor), the final pellets being resuspended in water. The yield of Cabbage B and Invergowrie isolates were 6 to 10 mg/kg leaf tissue and that of other strains somewhat less.

A less laborious alternative procedure was developed for recovering virus from large quantities of leaf material. The extracted sap, prepared as above, was subjected to low speed centrifugation (10000 rev/min for 15 min in a Sorval GSA rotor) and the pellets resuspended in one-tenth the original volume of 0·1 M-phosphate buffer containing 2·5 % Triton X-100 and 1 M-urea. This was then stirred at 4 °C overnight and the virus purified as described above. The yield was about 70 % of that obtained by the original technique and some preparations were contaminated by green material; however the modification overcame the necessity of high-speed centrifuging of large volumes.

The effects of various agents on the virus were examined by treating the virus, usually for 10 min at room temperature, subjecting the samples to low speed centrifugation (7000 rev/min for 10 min in a Sorval SS34 rotor) and either reading the E_{260} of the supernatant fluids in a spectrophotometer or loading them on to 10 to 40 % sucrose gradients (in 0·01 M-potassium or sodium phosphate buffer unless otherwise specified). The gradients were then centrifuged for 2 h at 23000 rev/min in Spinco SW 25·1 rotor and the distribution of the virus in each gradient examined using an ISCO scanner.

Analytical sedimentation was performed in a Spinco model E centrifuge as described by Hull, Hills & Markham (1969). For dry weight determinations, samples of purified virus which had previously been dried at 70 °C *in vacuo* were weighed using a Kahn Vacuum electro-balance at 25 °C until constant weight was achieved.

Diffusion coefficients were measured by laser light scattering using a Malvern system 4300 Photon Correlation Spectrometer (Precision Devices & Systems Ltd., Malvern) and an Argon laser interfaced to a CAI minicomputer. Some experiments were also performed using a Hewlett Packard auto-correlator and an experimental arrangement described previously (Harvey, 1973; Farrell, Harvey & Bellamy, 1974).

Partial specific volumes were measured on a DMA 02C digital precision density meter (Anton Paar K.G., Graz, Austria) with temperature controlled by a PTC 40 temperature controller (Tronac Inc., Orem, Utah) equipped with a quartz thermometer 2801A (Hewlett Packard).

Virus preparations were negatively stained in 2 % ammonium molybdate or 2 % methylamine tungstate (Fabergé & Oliver, 1974) and were examined in a Siemens Elmiskop 1A electron microscope. Crystalline catalase was used as a marker for measurements of particle diameter.

Nucleic acid content of the virus was estimated from the phosphorus content (Nakamura, 1952) and by the diphenylamine reaction (Burton, 1956).

For lipid estimation 2 mg virus were extracted with chloroform which was then evaporated. After methylation with boron trifluoride + methanol and extraction with hexane, the samples were analysed for triglycerides and phospholipids on a HI-EFF-2BP gas liquid chromatography column.

Table 1. *Effect of various treatments on yield of cauliflower mosaic virus*

Treatment	Yield as % control*
Clarification	
Butanol (8 % v/v)	17
Chloroform (equal vol.)	34
Butanol/chloroform (equal vol.)	25
1 % Triton for 30 min	—†
19 h	101
41 h	88
2·5 % Triton for 30 min	52
41 h	74
Concentration	
PEG precipitation (10 % w/v)	38
2nd high speed centrifugation	72

* Control extraction was in 2·5 % Triton for 19 h, followed by one cycle of centrifugation. Yield was assessed from the area under the peak of virus in sucrose gradient obtained using an ISCO u.v. monitor.
† Green material obscured virus band.

RESULTS

Factors affecting yield of virus

Because previous methods of purifying cauliflower mosaic virus (Pirone, Pound & Shepherd, 1960; Shepherd, Bruening & Wakeman, 1970) gave poor yields, a new purification technique was developed. In a study of how yield was related to time of infection (plants grown in glasshouse at 25° ± 5 °C and artificial lighting to give a daylength of 16 h) it was found that hardly any virus could be extracted 11 days after infection and that the maximum virus yield was reached at about 24 days and maintained until at least 35 days after infection. The yield from turnip (cv. Just Right) was greater than that from mustard (*Brassica perviridis* cv. Tendergreen) or from Chinese cabbage (*B. pekinensis* cv. Wong Bok) at 24 and 35 days after infection. Some of the other factors which affected virus yield are listed in Table 1 from which it can be seen that the use of butanol was probably one of the main factors involved in the relatively low and variable yields using previous techniques. No virus was obtained after clarification using 25 % ethanol (sap in 0·1 M-phosphate buffer) or bentonite (sodium or magnesium, using the technique of Dunn & Hitchborn, 1965). The virus yield was decreased by the extraction of sap in water; in 0·1 M- or 1·0 M-phosphate, pH 7·2; in 0·2 M-borate, pH 8·2; or by extraction and treatment with Triton X-100/urea at room temperature.

Physical properties of the virus

CaMV purified by the new technique sedimented in 0·1 M-NaCl, 0·01 M-phosphate buffer, pH 7·2, as a single major component with an $s_{20,w}$, at infinite dilution, of 208·2 ± 1·1 S (10 samples measured); there was sometimes a small amount of material sedimenting at about 300 S, probably polymers of the virus. The $s_{20,w}$ of the major peak had a concentration dependence of 8 S/mg/ml up to at least 3 mg/ml (Fig. 1).

The measured diffusion coefficient also showed a marked concentration dependence in low ionic strength solvents, an effect which has been observed by laser light scattering from suspensions of particles of other viruses (Pusey *et al.* 1972; Harvey, 1973). The apparent diffusion coefficient (calculated from the slope of a semi-logarithmic plot of the autocorrelation function of the scattered light intensity at 90° angle) is shown as a function of concentra-

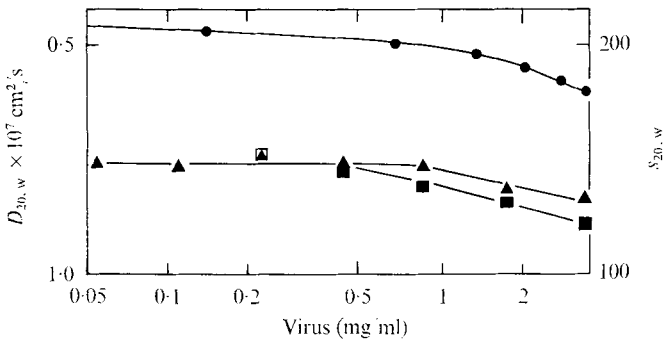

Fig. 1. Concentration dependence of $s_{20,w}$ and $D_{20,w}$ of cauliflower mosaic virus. The $s_{20,w}$ values (●—●) were measured in 0·01 M-phosphate, pH 7·2, 0·1 M-NaCl and the $D_{20,w}$ values were measured in H_2O (▲—▲) and in 0·002 M-phosphate buffer (■—■).

tion in Fig. 1. It is apparent from Fig. 1 that the interparticle interactions significantly affect both sedimentation and diffusion coefficients down to approx. 0·1 mg/ml in low ionic strength solvents. The measured diffusion coefficient ($D_{20,w}$) was $0{\cdot}75 \pm 0{\cdot}04 \times 10^{-7}$ cm²/s, its reproducibility probably being limited by the presence of differing quantities of dimers in the preparations. These contaminants could just be detected by the curve fitting procedures used to extract the decay constant of the autocorrelation function, and the corresponding Q parameter defined by Pusey *et al.* (1974) varied from 1 % to 7 % for the preparations on which these measurements were performed. The apparent partial specific volume did not vary over the concentration range of 1·2 to 0·12 mg/ml and a value of $0{\cdot}704 \pm 0{\cdot}007$ g/ml was determined.

Purified preparations of CaMV examined in the electron microscope contained spherical particles with little or no contaminating plant material. There was some variation in the shape of particles especially those not surrounded by other particles. As this was thought to be due to flattening of the particles when drying down on the grid, only those particles surrounded by others were measured. The mean diam. of particles negatively stained in either ammonium molybdate or methylamine tungstate was $50{\cdot}3 \pm 1{\cdot}4$ nm (mean of 50 particles for each stain).

When CaMV preparations were examined in the ultraviolet spectrophotometer, light scattering was not eliminated even after heating with 1 % SDS in boiling water for 5 min. Therefore allowance for light scattering was made using the procedure described by Englander & Epstein (1957). From the E_{260} and the average dry weight of 4 samples from a preparation, an extinction coefficient ($E_{260}^{0{\cdot}1\%}$) of 4·36 extinction units/mg/ml was determined. If allowance was not made for light scattering the $E_{260}^{0{\cdot}1\%}$ was close to the uncorrected value of 7·0 given by Shepherd (1970).

Phosphorus determinations of dry samples of purified virus showed that CaMV contained 1·63 % phosphorus which indicated a nucleic acid content of 17·1 % (using the base ratio analysis published by Shepherd *et al.* 1970). The diphenylamine reaction indicated a DNA content of 15·9 %.

The lipid assay suggested that the virus contained less than 0·1 % fatty acids (triglycerides or phospholipids).

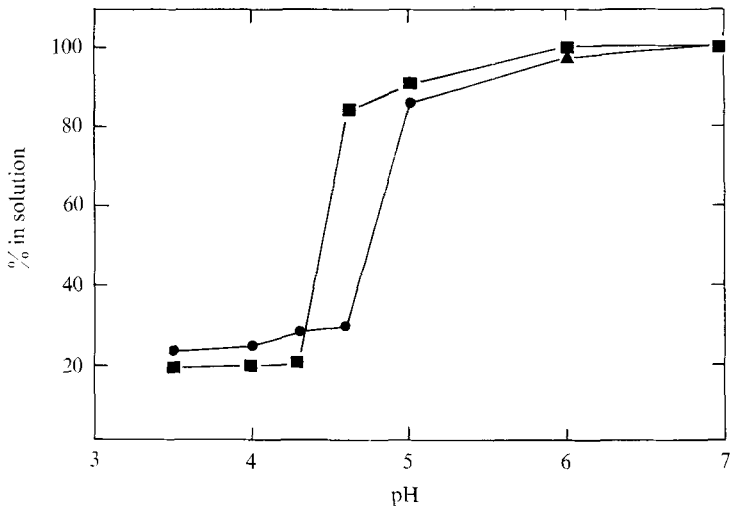

Fig. 2. Precipitation of Cabbage B (■—■) and JI (●—●) isolates of cauliflower mosaic virus at low pHs. Samples of virus (0·15 mg/ml) were adjusted to various pHs using 0·05 M-phosphate buffer for pHs 7 and 6 and 0·1 M-acetate buffer for pH 6 and below. After standing at room temperature for 10 min the samples were centrifuged at low speed and the E_{260} of the supernatant fluids were measured.

Effects of various agents

During experiments to develop a purification technique, information was obtained on the effects of various agents on CaMV. CaMV isolates Cabbage B and JI precipitated between pHs 4 and 5, the 50 % precipitation point of JI isolate being at a slightly higher pH than that of Cabbage B isolate (Fig. 2); the virus particles of both isolates also precipitated at pH 3·5. Only 25 to 30 % of the precipitated virus redissolved in buffer at pH 7·0. Electron microscopy of the precipitated virus indicated that it was not degraded.

CaMV (Cabbage B isolate) was also precipitated by Mg^{2+} (Fig. 3). It can be seen from Fig. 3 that in low ionic strength buffer (0·01 M-sodium phosphate, pH 7·1) the greatest proportion of virus was precipitated at 50 mM-Mg^{2+} and that a greater proportion was precipitated from the more concentrated virus suspension. When the ionic strength was raised (0·01 M-phosphate buffer, 0·1 M-NaCl) relatively little virus was precipitated over the range of Mg^{2+} concentrations examined. This ionic strength effect would explain why less virus was precipitated from solutions in low ionic strength buffer at Mg^{2+} concentrations of 100 mM and above.

The effects of ethanol on CaMV (Cabbage B isolate) were the opposite to those of Mg^{2+} (Table 2). In low ionic strength buffer at 4 °C the virus remained in solution at the highest ethanol concentration tested whereas in the presence of 0·1 M-NaCl it was precipitated by 50 % ethanol. Virus which had been treated with 90 % ethanol in low ionic strength buffer reached a similar depth after density gradient centrifugation as untreated virus and had a similar specific infectivity when tested at various dilutions on turnip plants.

CaMV (Cabbage B isolate) was precipitated in 5 % polyethylene glycol 6000 (PEG) in 0·2 M-NaCl but not in 4 % PEG. When the salt concentration was 0·05 M the virus was precipitated at 10 % but not at 8 % PEG. Not more than 25 % of the precipitated virus resuspended in water or 0·01 M-phosphate buffer, pH 7·1, with or without 0·1 M-NaCl. However when such precipitates were left in water or phosphate buffer at 4 °C for periods

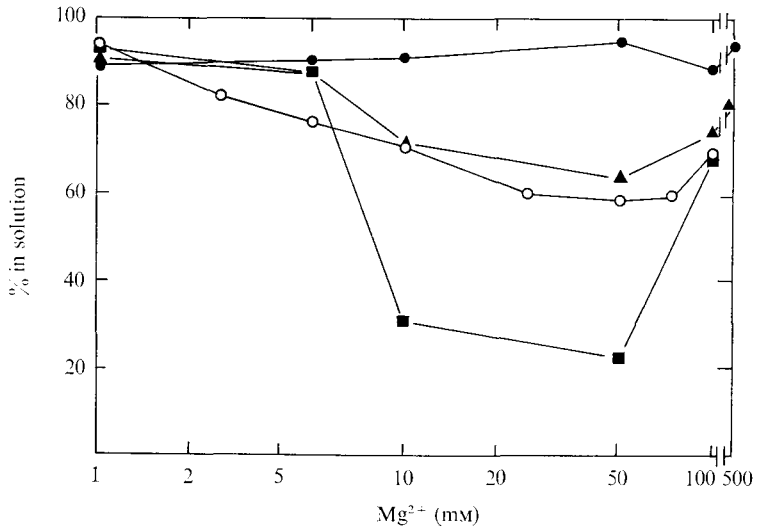

Fig. 3. Precipitation of Cabbage B isolate of cauliflower mosaic virus by Mg^{2+}. Expt. 1 (○—○), virus concentration 0·2 mg/ml, 0·01 M-phosphate buffer; Expt. 2 (▲—▲), virus concentration 0·2 mg/ml, 0·01 M-phosphate buffer; (●—●), virus concentration 0·2 mg/ml, 0·01 M-phosphate buffer + 0·1 M-NaCl; Expt. 3 (■—■), virus concentration 1·0 mg/ml, 0·01 M-phosphate buffer.

Table 2. *Effect of ethanol on cauliflower mosaic virus**

Ethanol (%)	Virus remaining in suspension (% control)		
	Expt. 1†	Expt. 2	Expt. 3
10	98	—	98
25	—	—	97
33	104	—	—
50	97	92	2
66	85	70	—
90	—	69	—

* Virus was mixed with ethanol and centrifuged at low speed; the supernatant fluid was then layered on a 10 to 40 % sucrose gradient which was scanned after centrifugation using an ISCO gradient u.v. monitor.
† Salt concentrations: Expt. 1 and 2 0·01 M-NaCl; Expt. 3 0·1 M-NaCl.

of 7 days or longer 75 to 80 % of the virus resuspended. This probably explains the necessity for a long period for resuspension (1 to 2 weeks) of PEG precipitates recommended by Shepherd *et al.* (1970). CaMV precipitated using $(NH_4)_2 SO_4$, (40 % saturation) also did not resuspend easily. The difficulty of resuspending CaMV after precipitation precluded the use of these treatments during purification.

DISCUSSION

It seems likely that, as pointed out by Fujisawa *et al.* (1967), one of the main reasons for the difficulty in purification of CaMV is the localization of the virus particles within inclusion bodies. In the modification of the purification technique described in this paper, it was assumed that the inclusion bodies were pelleted by the initial low speed centrifugation. The

partially purified inclusion bodies of cabbage B isolate seem to be degraded by Triton X-100 and urea; overnight incubation of sap with Triton/urea seems about the optimum time for the release of virus from the inclusions (Table 1).

The variability in yield between different isolates cannot be fully explained by differences in abundance of inclusion bodies or of virus contained within the inclusions. Although Cabbage B isolate, which gives a relatively high yield, has larger inclusions than other isolates examined, similar yields are obtained from the Invergowrie isolate which has relatively small inclusions (R. Hull & A. Plaskitt, unpublished observation). The yields from JI, Wellesbourne and CM4-184 isolates, the inclusions of which have the same size, frequency of occurrence and virus content as those of the Invergowrie isolate, are consistently lower than those from the Invergowrie isolate. The inclusions of the CM4-184 isolate are known to be considerably more stable than those of other isolates. Hence it seems probable that stability of the inclusions is partially responsible for the differences in yield between isolates.

The $s_{20,w}$ value of CaMV particles reported in this paper is close to that of 206S reported by Itoh, Matsui & Hirai (1969) and is somewhat lower than the 220S given by Pirone, Pound & Shepherd (1961); it is considerably lower than the value determined for dahlia mosaic virus (Brunt, 1971) which is serologically closely related to CaMV.

The diffusion coefficient of CaMV particles indicates an effective hydrated average diam. of 57 nm (calculated from the Stokes–Einstein relationship). The diam. which we have determined by electron microscopy for the dried virus is similar to the value reported by Pirone *et al.* (1961) and is somewhat larger than the 45 nm reported by Hills & Campbell (1968) and by Kelly, Cooper & Walkey (1974). From the hydrated diam. and the anhydrous ('hard sphere') diam. (determined from the particle mol. wt. and partial specific volume to be 37 nm) the water content of CaMV particles can be calculated to be 1·9 ml/g anhydrous material (for example of calculation see Harvey, Farrell & Bellamy, 1974) which is a relatively high value for a plant virus.

The value for partial specific volume obtained by direct measurement is close to the value estimated using the proportions of nucleic acid and protein and values of $\bar{v}$ for DNA of 0·50 (measured in very low ionic strength medium, D. W. Grundwedel, personal communication; Cohen & Eisenberg, 1968) and for protein of 0·736 (calculated from the gross amino acid analysis given by Brunt *et al.* 1975).

Using the Svedberg equation the mol. wt. of CaMV particles can be calculated to be $22{\cdot}8 \pm 1{\cdot}4 \times 10^6$ comprising $3{\cdot}9 \pm 0{\cdot}2 \times 10^6$ nucleic acid and $18{\cdot}9 \pm 1{\cdot}2 \times 10^6$ protein. This estimate of the nucleic acid mol. wt. compares with that of $4{\cdot}4 \times 10^6$ made using electron microscopy (Shepherd & Wakeman, 1971) and of $4{\cdot}7 \times 10^6$ based on electron microscopy and ultracentrifugation (Russell *et al.* 1971).

The effects of pH and Mg^{2+} suggest that CaMV might have an isoionic point between pH 4 and 5; this would agree with the direction of migration of the virus in gels at about neutral pH (Lung & Pirone, 1973). These authors also reported differences between isolates in their mobility in gels and this would be compatible with slight difference in pH precipitation behaviour of the two strains referred to in Fig. 2. As CaMV appears to have a net negative charge at neutral pH, many of the lysine residues, of which the virus has a high content (Brunt *et al.* 1975), are probably in internal parts of its capsid and associated with the nucleic acid.

The assistance of Dr D. W. Gruenwedel in determining the partial specific volume, that of Dr N. Packter in the lipid estimation and the technical assistance of Mr R. J. Wakeman is gratefully acknowledged. This work was supported by National Science Foundation Grant GB 39959.

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(*Received* 5 *September* 1975)