Purification and serology of the W strain of cucumber mosaic virus

BY J. A. TOMLINSON, ANNE L. CARTER
ELIZABETH M. FAITHFULL AND M. J. W. WEBB

National Vegetable Research Station, Wellesbourne, Warwick

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SUMMARY

During studies on the purification of cucumber mosaic virus (strain W) it was found that preparations were most infective and stable when made from tobacco leaves (10–12 days after inoculation) homogenized in phosphate buffer containing EDTA and thioglycollic acid and clarified with diethyl ether.

The preparations were further purified by centrifugation in sucrose density gradients containing EDTA at pH 9·0 and were then stable at 2 °C for > 100 days. When mounted in neutralized ammonium molybdate they were shown to consist of predominantly intact particles.

In tube and ring precipitin tests and in agar gel-diffusion tests, specific precipitation with homologous antiserum occurred only in media containing alkaline adjusted solutions (ammonium molybdate and dipotassium hydrogen phosphate).

INTRODUCTION

A detailed study was made of an isolate of cucumber mosaic virus (CMV), designated strain W (Tomlinson, Carter, Dale & Simpson, 1970) following outbreaks of the virus in recent years in lettuce, spinach and cucurbits. The isolate was typical of many others obtained from lettuce and was shown subsequently to be seed transmitted in Stellaria media (Tomlinson & Carter, 1970).

The objects of the study were to find a method of preparing large amounts of purified virus, to develop an antiserum to the virus and to study its serological reactions, particularly in agar gel-diffusion tests.

MATERIALS AND METHODS

Virus. The isolate, obtained from infected lettuce (Lactuca sativa) plants in Worcestershire in 1968, was sap-transmitted to and maintained in tobacco (Nicotiana tabacum cv. White Burley) by inoculating three Celite-dusted leaves (c. 14 cm long) of young plants with an extract (infectivity end point $10^{-3}$ to $10^{-4}$) of infected tobacco leaf, ground (1 g/l ml) in 1 % $\text{K}_2\text{HPO}_4$. Inoculated leaves were immediately air-dried (Yarwood, 1963) and plants were kept humid overnight. The experiments were made between September and April, infected plants being grown in a glasshouse at 20 ± 3 °C with supplementary lighting from mercury vapour lamps (Philips, HLRG, 400 W) giving a 15 h photoperiod.
Virus assay and multiplication. The relative infectivities of preparations were determined by inoculating Celite-dusted leaves of Chenopodium quinoa or those of cowpea (Vigna sinensis cv. Blackeye obtained from E. J. Lyng Co., Inc., Modesto, California) using a muslin pad soaked in the extract. The resultant local lesions were counted respectively 4 and 6 days later. Serial tenfold dilutions were compared using eight to ten leaves (or half leaves) per dilution sample.

In tests of the infectivity of sap from N. tabacum cv. White Burley and N. rustica plants during 24 days after inoculation, a disk 1 cm diam. was taken from each of three leaves of ten inoculated plants on alternate days. Each sample of thirty disks was homogenized with a pestle and mortar in 1\% K$_2$HPO$_4$ (1 g/1 ml), filtered through muslin and the sap assayed on cowpea.

Serology. To prepare antiserum, 2.5 ml of a purified virus preparation (infectivity dilution end point $10^{-6}$) was emulsified in 2.5 ml Freund's incomplete adjuvant (Difco Laboratories, Detroit, Mich.) and one half was injected intramuscularly into each hind leg of a rabbit. After 7 days an intravenous injection was given with 1.5 ml of the same virus preparation. After 14 days the rabbit was bled and the serum stored in an equal volume of glycerol at 3 °C. For use in tests, antiserum and normal serum were dialysed at 3 °C for 3 days against 0.01 M phosphate buffer, pH 7.5, containing 0.02% sodium azide. Tube tests were made at 20 °C in 8 x 75 mm test-tubes containing 0.5 ml virus preparation and 0.5 ml antiserum diluted in a suitable diluent to prevent non-specific precipitation and examined every 12 h for 2 days. Virus in normal serum and in the diluent alone were used as controls.

Ring tests were made in 5 x 50 mm test-tubes by placing 0.2 ml of various dilutions of antiserum containing 10% (v/v) glycerol in each tube and floating on the surface a similar volume of virus preparation. Tubes were kept at 20 °C and after 4–5 h examined with a thin beam of light in a dark room to assess the amount of precipitate at the interface.

Agar gel diffusion tests were made in 90 mm plastic Petri dishes, containing 3 mm layers (17 ml) of 0.75% agar (Ionagar No. 2, Oxoid Ltd., London). Antiserum and antigen wells were 5 mm diam. and 6 mm apart. A central well containing antiserum was surrounded by eight peripheral wells containing the virus suspension. Agar plates were incubated at 20 °C in a moist chamber.

Purification studies

The methods of Tomlinson, Shepherd & Walker (1959), Scott (1963) and Murant (1965) for the purification of CMV (Y) differed from each other mainly in the type of extraction buffer and the type of organic solvent used to clarify the leaf extracts. Later the value of ethylene diamine tetraacetic acid (EDTA) was established for use in the purification of CMV (Tomlinson, 1963; Takanami & Tomaru, 1969). All methods, however, had given virus preparations that were not entirely satisfactory. Thus, the dilution end-point of those prepared by Tomlinson (1963) rarely exceeded $10^{-4}$; those made by Scott (1963, 1968) were apparently weakly immunogenic and precipitated in weak salt solutions. The preparations made by Murant (1965) were reported to be not homogenous and to disintegrate in negative stain.

Various aspects of these methods were compared to determine their effects on the
infectivity, stability and other attributes of preparations of CMV (W). As a result of these studies, the following method was selected for the purification of the virus.

Tobacco leaves (200–300 g), 10–12 days after inoculation, were homogenized (1 g/1 ml) in a blender in ice-cold 0.5 M potassium phosphate buffer pH 7.5 containing 0.001 M-EDTA and 0.1 % thioglycollic acid (TGA) and the homogenate filtered through muslin. Pulp was scraped off the muslin, re-extracted with 50 ml of the buffer and filtered. The combined filtrates were emulsified in an equal volume of diethyl ether for 20 min and centrifuged for 15 min at 2000 rev/min (MSE Super-Minor centrifuge). The aqueous (lower) phase was retained and aspirated for 20–30 min at 20 °C to remove excess ether. The suspension was centrifuged in a Beckman No. 30 rotor for 30 min at 8000 rev/min and the supernatant fluid centrifuged for 2 h at 30000 rev/min.

![Text-fig. 1. The relative infectivity of leaf homogenates of Nicotiana tabacum cv. White Burley (●—●) and N. rustica (○—○) at various intervals after inoculation with CMV (W).](image)

The pellets were each dispersed in 1 ml 0.005 M disodium tetraborate containing 0.005 M-EDTA adjusted with 0.1 N-NaOH to pH 9.0 (borate/EDTA buffer), pooled and stored at 4 °C for 20 h. The preparation was centrifuged for 10 min at 5000 rev/min and the supernatant fluid withdrawn with a fine Pasteur pipette (partially purified virus).

Further fractionation was done in density gradient columns (Brakke, 1960). Prepared gradients were stored for 24 h before centrifugation in an SW 27 rotor. Tubes, 3.5 x 1 in, contained 7 ml each of 10, 20 and 30 % and 15 ml of 40 % (w/v) sucrose dissolved in borate/EDTA buffer, pH 9.0. One ml of the virus preparation was floated on each gradient and centrifuged for 90 min at 24,000 rev/min. After centrifugation, most virus occurred in a zone 15–25 mm beneath the meniscus and was withdrawn from above, in a 5 ml sample per tube by a syringe with a 70 mm hypodermic needle. The pooled fractions (50–60 ml) from twelve tubes were then dialysed against 2 l
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borate/EDTA buffer, pH 9.0 for 40 h, the dialysing buffer being renewed after 16 h. After dialysis, the preparation was centrifuged for 2 h at 30000 rev/min and the pellets were each resuspended in 1 ml borate/EDTA buffer, pH 9.0 at 1 °C (purified virus).

In tests of the different stages of the purification method the following were found to be the most critical: (i) time of harvest of inoculated leaves of two tobacco species tested, (ii) type of extraction buffer and the presence or absence of EDTA and TGA, (iii) type of organic solvent used in clarification, and (iv) presence of EDTA during density gradient centrifugation.

Assessments of the infectivity of leaves of Nicotiana tabacum cv. White Burley and N. rustica made at intervals after inoculation showed that most virus per unit weight of leaf was obtained from N. tabacum leaves and that it increased to a maximum 10–12 days after infection (Text-fig. 1).

Table 1. Infectivity of CMV (W) purified from phosphate, citrate and borate buffered homogenates*

| Dilution of | Local lesions per leaf† |
| preparation‡ | Phosphate | Citrate | Borate |
| 10⁻³ | > 250 | > 250 | 160 |
| 10⁻² | > 250 | 153 | 0 |
| 10⁻¹ | 186 | 79 | 0 |
| 10⁻⁰ | 14 | 2 | 0 |
| 10⁰ | 2 | 0 | 0 |

* Infected tobacco leaves, divided into three equal lots were homogenized (1 g/1.5 ml) in 0.5 M potassium phosphate, 0.5 M sodium citrate and 0.5 M sodium borate (boric acid/NaOH) buffers at pH 7.5. Each buffer contained 0.001 M-EDTA and 0.1% TGA. Homogenates were emulsified in an equal volume of diethyl ether for 20 min. The aqueous phase was given one cycle of low- and high-speed centrifugation. Pellets were each resuspended in 1 ml borate/EDTA buffer, pH 9.0.
† Diluted in borate/EDTA buffer, pH 9.0.
‡ Mean of eight C. quinoa leaves.

Table 2. Infectivity of CMV (W) prepared with and without EDTA and TGA

<table>
<thead>
<tr>
<th>Solutions used for leaf homogenization*</th>
<th>Dilution of preparation‡</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>10⁻³</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10⁻²</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate with EDTA</td>
<td>10⁻³</td>
<td>67</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>10⁻²</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate with TGA</td>
<td>10⁻³</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10⁻²</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate with EDTA + TGA</td>
<td>10⁻³</td>
<td>145</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>10⁻²</td>
<td>56</td>
<td>25</td>
</tr>
</tbody>
</table>

* The homogenates were emulsified in an equal volume of diethyl ether for 20 min and centrifuged at 20000 rev/min. The aqueous phase was given one cycle of low- and high-speed centrifugation and the pellets each resuspended in 1 ml borate/EDTA buffer, pH 9.0. Solutions contained 0.001 M-EDTA and 0.1% TGA.
† Diluted in borate/EDTA buffer, pH 9.0.
‡ Mean of eight C. quinoa leaves.
Comparative results of the infectivity of partially purified preparations made from leaves extracted in a range of buffers, with and without EDTA and TGA and after clarification with several organic solvents are shown in Tables 1, 2 and 3.

As shown in Table 4, the presence of EDTA was necessary both during the initial stages of purification and in the density gradient solutions. It gave highly infective preparations containing numerous intact particles, whereas in its absence virus particles were few and mostly disrupted (Plate, fig. 2).

Table 3. Infectivity of CMV (W) in homogenates clarified with butanol, chloroform and diethyl-ether*

<table>
<thead>
<tr>
<th>Dilution of preparation†</th>
<th>Clarifying agent</th>
<th>Butanol</th>
<th>Chloroform</th>
<th>Diethyl-ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-2}$</td>
<td></td>
<td>154‡</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td></td>
<td>15</td>
<td>140</td>
<td>&gt; 250</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td></td>
<td>0</td>
<td>64</td>
<td>179</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td></td>
<td>0</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* The clarifying agents, n-butanol (8.5 %, v/v) chloroform (1:1, v/v) or diethyl-ether (1:1, v/v) were added to homogenates of infected leaves in 0.5 M-phosphate, pH 7.5, containing 0.001 M-EDTA and 0.1 % TGA. The sample containing butanol was stirred for 1 h at 3°C. The aqueous phase of the chloroform-treated sample was dialysed for 24 h at 3°C against borate/EDTA buffer, pH 9.0. The diethyl-ether treated sample was emulsified for 20 min at 3°C and the aqueous phase obtained after centrifugation at 2000 rev/min. Preparations were given one cycle of low- and high-speed centrifugation and the pellets each resuspended in 1 ml borate/EDTA buffer, pH 9.0.
† Diluted in borate/EDTA buffer, pH 9.0.
‡ Mean no. lesions per leaf in eight C. quinoa leaves.

Table 4. Infectivity of CMV (W) in preparations with and without EDTA

<table>
<thead>
<tr>
<th>Method*</th>
<th>Type of gradient</th>
<th>Treatment</th>
<th>Dilutions†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no.</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>Phosphate containing</td>
<td>+ EDTA</td>
<td>1</td>
<td>310§</td>
</tr>
<tr>
<td>0.001 M EDTA‡</td>
<td>−EDTA</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td>Phosphate only</td>
<td></td>
<td>+ EDTA</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>−EDTA</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* Leaf homogenates were made in 0.5 M phosphate with or without EDTA and the virus in each was partially purified by the phosphate/ether method. One ml samples of the two preparations were floated on 10-40 % (w/v) sucrose columns in borate buffer, pH 9.0, with or without 0.005 M-EDTA. After centrifugation for 90 min at 24000 rev/min, the virus zones were removed and dialysed for 48 h against borate buffer, pH 9.0, with or without 0.005 M-EDTA.
† Preparations were all diluted in borate buffer, pH 9.0.
‡ Pellets resuspended in borate/EDTA buffer, pH 9.0.
§ Mean no. lesions per leaf in eight C. quinoa leaves.
|| Pellets resuspended in borate buffer, pH 9.0.

PROPERTIES AND PURIFIED PREPARATIONS

The yield of virus was about 10 mg/kg infected leaf. Samples of purified virus formed a single prominent light-scattering zone when centrifuged in sucrose density gradients, contained a single component with a sedimentation coefficient of 96 S at infinite dilution (Plate, fig. 3) and their A 260/280 (uncorrected for light scattering) was about
Preparations containing about $2 \times 10^{-14}$ particles/ml had an infectivity end point of $10^{-6}$. The virus particles remained intact when negatively stained in $2\%$ ammonium molybdate, pH 7.0 (Plate, fig. 1), but not when $2\%$ potassium phosphotungstate, pH 7.2, was used (Plate, fig. 4).

**SEROLOGICAL REACTIONS**

*Tube and ring precipitin tests.* Salt solutions normally used for serological tests have been shown by previous workers to cause non-specific precipitation of certain preparations of CMV. Francki, Randles, Chambers & Wilson (1966) found that CMV (Q) precipitated above $0^\circ C$ in $0.85\%$ NaCl and in other salt solutions in which viruses are normally stable. Scott (1968) obtained similar results with CMV (Y) but partly avoided such precipitation by the use of $0.05\, M$ phosphate buffer, pH 8.0, at $4^\circ C$.

In the present work, CMV (W) was shown to precipitate non-specifically at $25^\circ C$ if virus or serum were diluted in solutions containing various concentrations of sodium chloride, sodium tetraborate or potassium phosphate, with or without EDTA. Precipitation was more intense at $30^\circ C$. It was found, however, that if the virus preparations, normal serum or antiserum were diluted with $0.01-M$ ammonium molybdate solution adjusted to pH 7.2 with NaOH and the tests were carried out at $20^\circ C$, non-specific precipitation was prevented for up to 65 h. In ring precipitation tests made in this way, specific precipitation occurred in 3 h and the antiserum titre was $1/2048$. Tube tests were less sensitive and gave a titre of $1/512$.

**Table 5. Effect of potassium phosphate concentration in gel-diffusion tests between CMV-infected tobacco leaf extracts and CMV antiserum**

<table>
<thead>
<tr>
<th>Concentration of K$_2$HPO$_4$ in agar†</th>
<th>0.1 M (pH 8.0)</th>
<th>0.05 M (pH 7.7)</th>
<th>0.01 M (pH 7.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of leaf extract*</td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
</tr>
<tr>
<td>0.1 M, undiluted (pH 6.9)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.05 M, undiluted (pH 6.5)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.05 M, diluted 1/4 (pH 7.3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.01 M, undiluted (pH 5.9)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.01 M, diluted 1/4 (pH 6.0)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Infected leaf was ground (1 g/ml) in $0.005\, M$-K$_2$HPO$_4$ solutions containing $0.005\, M$-EDTA and $0.02\%$ sodium azide, filtered and the filtrate tested undiluted or diluted 1/4 in the same extraction solution.

† Agar (0.75\%) contained $0.01-0.1\, M$-K$_2$HPO$_4$, $0.005\, M$-EDTA and $0.02\%$ sodium azide. Agar pH determined on molten samples after 1/10 dilution in distilled water.

‡ Precipitation line intensity is indicated by the number of + signs; - indicates no precipitation line.

**Agar gel diffusion tests.** Reactions in these tests were affected by the salts dissolved in the agar and by pH, and a series of experiments was made to determine the best method. The pH was adjusted by adding predetermined volumes of saturated tris solution to the various salt solutions in which the agar was dissolved. Increasing pH up to pH 8.0 increased the intensity and sharpness of the precipitation lines. Plate,
Cucumber mosaic virus

The study of Cucumber mosaic virus (CMV) revealed that figs. 5-7 show this effect in agar containing 0.01 M ammonium molybdate, 0.001 M EDTA and 0.02% sodium azide. Further tests showed that the use of 0.05 M-KH₂PO₄, pH 7.8, with 0.005 M-EDTA and 0.02% sodium azide gave the best precipitation lines. This was adopted as the standard method and gave an antiserum titre of 1/32. The use of 0.85% sodium chloride and 0.005 M-EDTA (pH 5.9) gave only faint precipitation lines and a titre of 1/4. Other salt solutions were similarly less satisfactory.

**Gel diffusion tests with infective sap.** It was found that the production of diagnostic precipitation lines between the CMV (W) antiserum prepared as described and sap from infected plants was affected by the concentration of phosphate in the solution in which the leaf tissue was extracted, by the dilution of the sap and by phosphate concentration in the agar (Table 5). The method selected for routine tests was to use the standard phosphate agar (above), containing 0.05 M-KH₂PO₄ and 0.005 M-EDTA, to extract infected leaves in the same phosphate/EDTA solution and to dilute the extract 1/4 with this solution. In such tests positive reactions were obtained with extracts of infected marrow (Cucurbita pepo) and cucumber (Cucumis sativus) but not with extracts of symptomless but infected leaves of Stellaria media plants which had been inoculated with CMV (W) 10, 20, or 30 days previously.

**Relationship between the W and Y strain of CMV.** CMV (W) antiserum reacted in agar gel-diffusion tests with homologous antigen and with a purified preparation of the Y strain of CMV (Sill, Burger, Stahmann & Walker, 1952). Precipitation spurs showed that the viruses were serologically related but not identical.

**DISCUSSION**

As the isolate of CMV (W) used in these studies was pathologically typical of many found in Britain it was a matter of some importance to obtain stable and infective preparations of the virus and to develop satisfactory methods of working with them. It became clear, however, during experiments on this subject, that the yield and infectivity of the virus during purification, the stability of its particles in preparations made for electron microscope examination and the effectiveness of serological tests were greatly affected by the composition of the media in which the various processes were carried out.

These studies enabled suitable methods to be developed, and although very-high-titred antisera were not obtained and the yield of virus per g infected leaf was low it is considered that these may be intrinsic properties either of the W strain or possibly of CMV strains in general. Little information is available on the effects of the various factors studied on other strains of CMV but it was found that infective stable preparations of the CMV (Y) strain could be obtained by the methods described. As concluded by Van Regenmortel (1967), however, different strains of the virus may require further modifications of the purification method.

The results obtained may explain some of the difficulties encountered by previous workers. Francki et al. (1966) obtained antisera of only very low titre (1/2 in agar gel diffusion tests) but this may have been attributable, not to the antiserum, but to the effect on the virus of the medium in which the tests were made. Kaper, Diener & Scott (1965) found that CMV (Y) preparations showed more than one sedimentation
peak in sucrose density gradients but their solutions did not contain EDTA. The work of Takanami & Tomaru (1969), who obtained single sedimentation peaks with CMV (Y) when EDTA was added to the sucrose solutions, and the present study, emphasize the importance of EDTA at many stages in the purification of CMV and the investigation of its properties.

Francki & Habili (1972) have recently demonstrated enhanced immunogenicity of CMV (Q) injected into mice when the virus was treated with formaldehyde. This was considered to be due to increased stabilization of the protein capsid. If this method could be combined with the use of an alkaline medium and a suitable salt (e.g. ammonium molybdate or \( \text{K}_2\text{HPO}_4 \)) in the test solutions, as in the present studies, further improvements in antiserum test methods may be developed. Studies of these aspects are now in progress.

We wish to thank Mr R. D. Woods, Rothamsted Experimental Station, for examining preparations in the analytical ultracentrifuge.

REFERENCES


EXPLANATION OF PLATE

Fig. 1. Particles of CMV, mostly intact and of uniform size in 2% ammonium molybdate (pH 7·0) after preparation of the virus with EDTA. (Bar represents 100 nm).

Fig. 2. Disrupted particles of CMV in 2% ammonium molybdate (pH 7·0) after preparation of the virus without EDTA. (Bar represents 100 nm).

Fig. 3. Ultracentrifuge schlieren pattern of a preparation of CMV in borate/EDTA buffer (pH 9·0) after centrifugation for 12 min at 29500 rev/min. Sedimentation is from left to right.

Fig. 4. Particles, many of which are disrupted, of CMV in 2% potassium phosphotungstate (pH 7·2) after preparation of the virus with EDTA. (Bar represents 100 nm).

Figs. 5–7. Gel-diffusion tests with purified CMV (outer wells) and its antiserum (central wells). The medium contained 0·75% agar, 0·01 M ammonium molybdate, 0·001 M EDTA and 0·2% sodium azide. The pH of the agar was respectively 5·0 (Fig. 5), 6·5 (Fig. 6) and 8·0 (Fig. 7).