CHARACTERISATION OF
THREE ISOMETRIC VIRUSES
INFECTING DAPHNE

A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Science in Microbiology at
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by

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ERRATA

p.16	Figure 7 : 'recrotic' should read necrotic
p. 23 & 26	'innoculation' mis-spelt should read inoculation
p. 27,62 & 78	The names of families should begin with capital letters e.g. Solanaceae
p. 29	mistake associated with the formula 'w = rev/min = 39,000' should read: $w = 2\pi \dfrac{rev/min}{60}$, rev/min = 39,000
p. 31	Table 2. 'HaOH' should read NaOH
p. 32	line 15 and 17 'distilled water' should read borate
p. 34	line 3 'Lot et al.,' should read Lot et al., 1972;
p. 44	Figure 13 in the heading the numbers '45,235,165' should read 4S, 23S, 16S
p. 53	Sentence begining 'nucleotide base ratios - - -' should not be a new paragraph.
p. 62	heading 2.6 'PHYSICAL PROTERTIES' should read PHYSICAL PROPERTIES - - -
p. 75	'additional tests' should read additional test

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PREFACE

The poor vigour of many cultivars belonging to the genus Daphne (Thymelaeaceae) has been attributed to viral infection (Chamberlain, 1954) and several viruses including alfalfa mosaic virus and cucumber mosaic virus have been isolated (Chamberlain, 1954; Milbrath & Young, 1956; and Schmelzer, 1968).

Concern about daphne 'virus' problems expressed by nurserymen, has led to several programmes aimed at improving stock plants. Recent survey work on daphne viruses (Forster & Milne, 1975; Sutton & Taylor, 1974; and Sweet & Campbell, 1973) has therefore been prompted by this requirement for high health and virus-free plants.

In a survey of viruses infecting Daphne species and cultivars in New Zealand, Forster and Milne, (1975) isolated four previously described viruses (alfalfa mosaic virus, arabis mosaic virus, cucumber mosaic virus and tobacco ringspot virus) plus seven partially characterised viruses (daphne isometric viruses 1,2 & 3, daphne-tobacco mosaic virus, daphne virus S, daphne virus X and daphne virus Y). The latter three anisometric viruses (DVS, DVX, DVY) have been further characterised (R.L. Forster & K.S. Milne, pers. comm., 1975), while the remaining isometric viruses (DIV-1, DIV-2,& DIV-3) are the subject of this study.

Several isolates of each of the three isometric viruses were obtained from their respective hosts and extensively characterised. DIV-1, DIV-2 and DIV-3 could be readily differentiated from each other by host range and symptomatology in differential hosts and more detailed study led to their separate identification.

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CHAPTER 1

CHARACTERISATION OF CUCUMBER MOSAIC VIRUS STRAIN D

An apparently distinctive isometric virus was isolated from *Daphne odora* Thunb. 'Leucanthe Variegata' and tentatively designated daphne isometric virus -1 (DIV-1) by Forster and Milne (1975). Several isolates with properties similar to DIV-1 were obtained in the present study from 'Leucanthe Variegata'. Detailed characterisation established that DIV-1 is a distinctive strain of cucumber mosaic virus (CMV) which can be distinguished on the basis of symptoms and serology from the 'typical' form of CMV. Characterisation of DIV-1 isolates hereafter designated cucumber mosaic virus strain D (CMV-D), and comparison with three 'typical' or common CMV isolates from daphne, designated CMV-C (isolates A,B and C) formed part of the present study.

CMV (R/1:1/18:S/S:S/Ap) is the type member of the cucumovirus group (Harrison et al., 1971) which includes peanut stunt virus (PSV), tomato aspermy virus (TAV) and chrysanthemum mild mottle strains (CMMV). Reports of serological relationships between members of the group have been conflicting (Grogan et al., 1963; Lawson, 1967) and have been attributed to the use of antisera prepared from antigen mixtures (Mink, 1975). CMV is an icosahedral virus ca. 30nm diameter which has a characteristic single sedimenting component (98S) containing 18% RNA with 4 major species/length (Kaper & West, 1972). Similar nucleotide base ratios are characteristic of the cucumoviruses viz; guanine 23: adenine 24: cytosine 23: uracil 29. The protein coat consists of 2 subunits each with a distinct molecular weight. Physical properties in crude sap include a dilution end point (DEP) of 10^{-3} to 10^{-5}, longevity in vitro (LIV) of a few days and a thermal inactivation point (TIP) of 70C (Gibbs & Harrison, 1970). CMV is transmitted by many aphid species (Kennedy et al., 1962) in a non persistent manner and low frequency 2.0-20.0% seed transmission has been reported for several hosts (Tomlinson & Carter, 1970). There are also reports of sap or mechanical transmission in the field (Brierley, 1962) and in the laboratory CMV is transmitted to a large number of herbaceous species.

CMV was first recognised by Dolittle (1916) and Jagger (1916) causing a mosaic disease of cucumber and is now reported as the incitant of disease in many horticultural crops including fruit, ornamental and vegetable species. Infection of these crops and numerous weed hosts, together with a facility for rapid transmission by over 60 aphid species, insures ubiquity for CMV.

Reports of CMV infecting woody plants are somewhat limited but do include *Berberis* (Wilkinson, 1953), *Buddleia* (Smith, 1952), *Nandina* (Barnett & Baxter, 1974), *Prunus* spp (Willison & Weintraub, 1957), *Ribes* (Schmelzer, 1962), and *Rubus* (Harrison, 1958).

Previous to the study Forster and Milne (1975) CMV was regarded as the most prevalent virus in *Daphne* spp. Reports of CMV in *Daphne* spp., include in *D. odora* (Chamberlain, 1954; Milbrath & Young, 1956; Schmelzer, 1968; Sutton, 1974); *Daphne mezereum*. L. (Smith, 1952) and (Forster, 1974) recorded CMV in *D.odora*, *Daphne cneorum* L. and *Daphne x burkwoodii* Turrill.

1.1 MATERIALS AND METHODS

Plant propagation

Plants were propagated in a sand-peat fertilizer medium in glasshouses maintained between 15-25C and shaded during the summer months. Disease and pest control were maintained using the insecticide spray methomyl (Lannate^R) and ethazol (Terrazole^R) were incorporated into the soil mix.

Sap inoculation and symptomatology

Virus inoculum for sap transmission was prepared by grinding plant tissue in 2-3mls of buffer plus a small quantity of the abrasive Celite, using a pestle and mortar. The buffer hereafter referred to as Yarwoods solution, consisted 0.5% each of K_2HPO_4 and bentonite (Yarwood, 1972). Inoculations were made with the pestle immediately after dipping into the macerate. Inoculated leaves were washed with water (ca. 3 seconds) to remove celite and sap residues.

Indicator plants were examined daily and symptom expression recorded. Virus infections resulting from sap, seed or vector transmission experiments were verified by examination of negatively stained squash homogenates in the electron microscope and by back inoculation to *Chenopodium quinoa* Willd.

Vector transmission

Studies on non persistent transmission by aphids were conducted using Myzus persicae Sulz. maintained on Brassica pekinensis (Lour.) Rupr. (chinese cabbage) and Macrosiphum euphorbiae Thom. maintained on Sonchus oleraceus L. (sowthistle). Non persistent aphid transmission experiments were conducted as follows: using a small brush aphids were placed on moist filter paper in a petri plate for preacquisition starvation (optimal starvation period determined in separate experiments). Aphids were then removed individually and transferred to a detached virus-infected leaf under a binocular microscope and allowed to probe for 20 seconds. Probing was stopped by disturbing the aphid, generally by touching their antennae. They were then placed on receiver hosts, caged for 24 hours and killed with an insecticide.

Physical properties in crude sap

The TIP was determined from centrifuged (10,000g/10min) macerated infected plant tissue in minimal volume of distilled water. One ml samples of this extract were pipetted into thin walled 8x1.5cm tubes previously equilibrated at appropriate temperatures. Tubes were incubated for 10min, at 5C intervals over the range 50-90C and assayed. For DEP determination the extracts were diluted with distilled water. Assays were conducted using half leaves of C.quinoa or Phaseolus Vulgaris L. 'Top Crop'.

Electron Microscopy

Negative staining: samples of plant tissue were prepared for electron microscopy using the squash homegenate technique of Walkey and Webb, (1968). A segment of tissue was macerated with a few drops of negative stain on a spotting tile. Then a 300 mesh formvar-carbon-coated copper grid was held face down onto the macerate for a few seconds, after which the excess solution on the grid was removed by touching the grid to filter paper. The grids were scanned within 24h of preparation using a Philips^R EM-200^R electron microscope at a magnification of ca.X20,000. Purified preparations of virus were negatively stained using a 1:1 ratio (virus:stain) and sprayed onto the grids.

Negative stains used were 2% solutions of ammonium molybdate (AM), phosphotungstic acid (PTA), uranyl acetate (UA) and uranyl formate (UF). The pH of the various stains were adjusted using either 1M KOH or NH_4OH.

Virus purification

Differential centrifugation was employed for virus purification using a Sorvall RC 5^R centrifuge and a MSE Superspeed 65^R ultracentrifuge. Detailed procedures and methods are described for each virus. Final purification procedures using zone electrophoresis (van Regenmortel, 1964) and density gradient centrifugation (Brakke, 1960) were tried.

Analytical ultracentrifugation and spectrophotometry

The number of sedimenting virus components and their sedimentation coefficients were determined from an appropriate (1-5mg/ml) concentration of virus preparation using a 12mm single sector cell in an AnD rotor on a Beckman Model E^R analytical ultracentrifuge with Schlieren optics. Sedimentation coefficients were calculated following the method of Markham, (1962).

UV absorption spectra were obtained for dilutions of virus preparations using a Pye Unicam SP 800^R UV spectrophotometer. Virus concentration, A max/min and A 260/280 were also determined (Noordam, 1973).

Isolation of viral RNA

RNA was extracted from purified virus, using the phenol-sodium dodecyl sulphate method of Peden and Symons, (1973). In the presence of 0.3% sodium dodecyl sulphate (SDS) and 0.3M sodium acetate, 10mg of virus was extracted with an equal volume of cold, water-saturated phenol (redistilled) as prepared by Ralph, (1967). The extraction mixture was shaken vigorously for 10min in a polypropylene centrifuge tube. The emulsion was broken by centrifugation at 12.000g for 5min, resulting in a clear upper phase containing the RNA and a cloudy lower phenolic phase containing protein. The upper phase was removed and 2 volumes of cold 95% ethanol was added together with a few drops of 1M sodium acetate/acetic acid buffer pH 5.0. On standing at 0C for 20min the RNA became flocculant and was then precipiated by centrifugation at 12,000g for 10min. The RNA pellet was dissolved in 1ml of distilled water and stored at -20C under 4ml of 95% ethanol plus a drop of the pH5 acetate buffer. Concentration determinations were made by measuring the absorbance at 260nm using an extinction coefficient

$$E_{0.1\%,\ 1cm,\ 260nm} = 30$$

Preparation of RNA standards

Escherichia coli (Migula) Castellani and Chalmers., ribosomal RNAs (23S and 16S) were prepared from isolated *E. coli* ribosomes using phenol-SDS RNA extraction. The ribosomes were purified (Kurkland, 1971), as follows: *E. coli* was obtained from a 2 liter log phase culture by centrifugation at 15,000*g* for 15min. The bacterial paste obtained was frozen and thawed in 10ml of tris buffer (10mM Tris, 3mM succinic acid, and 10mM $MgCl_2$). This was then expressed through a cold french press at 8.000-15,000 psi twice and the resultant crude extract clarified by centrifugation at 25,000*g* for 30min. The clarified extract was made up to 10ml with the tris buffer, at 4C, 2.1g NH_4SO_4 was added slowly with stirring for 3min. This was then centrifuged for 10min at 25,000*g* and the supernatant retained and a further 2.1g NH_4SO_4 added slowly and recentrifuged. The pellet was dissolved in the Tris buffer and dialyzed overnight against 2 liters of Tris buffer. Finally the ribosomes were concentrated by centrifugation for 2h at 150,000*g*, resuspended in 1ml of buffer and stored frozen until required for RNA extraction.

Two other extracted RNA standards, rat liver ribosomal RNA (28S and 18S) and rat liver transfer-RNA (4S) were kindly provided by Dr. J.W. Tweedie (Massey University).

SDS-polyacrylamide gel electrophoresis of extracted RNA

Gel preparation: agarose-acrylamide composite gels were prepared essentially according to Peacock and Dingman, (1968). The gel buffer consisted of 40mM Tris base, 20mM sodium acetate and 3mM disodium ethylenediamine tetra-acetate (EDTA) pH 7.6. The agarose was melted in a buffer-glycerol solution using a boiling water bath, then equilibrated to 40C along with a separate flask containing the acrylamide, Bis[1], TEMED, buffer solution. Both solutions were then mixed together with the catalyst ammonium persulphate and poured into quartz 0.8 x 8cm glass tubes.

The poured gels were allowed .to stand at 4C for 2min then polymerised at room temperature for 1h. Gels were then partially extruded from the tubes using a pipette bulb, cut with a scalpel to produce a flat surface and subsequently redrawn into the tubes.

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[1] abbreviations

Bis = N,N' - methylenebisacrylamide

TEMED = N,N,N, 'N' - tetramethylethylenediamine

Preparation of 8 gels (2.4% acrylamide 0.6% agarose)

agarose solution	agarose	120mg
	gel buffer	14ml
	glycerol	2ml
acrylamide solution	acrylamide	480mg
	Bis	480mg
	TEMED	20µl
	gel buffer	4ml
catalyst	ammonium-persulphate	12mg

Electrophoresis: the gel buffer plus 0.3% SDS was placed in an electrophoresis vessel (Adesnik, 1971) and preps run at room temperature as described by Loening, (1967). Gels were pre-electrophoresed, before adding the RNA samples, for 1.5h at 6ma per tube to give a flat baseline for UV scanning. RNA samples were diluted and layered onto the gels as follows: each RNA sample was diluted to a 1mg/ml concentration in distilled water, 50-100µl were mixed with a single drop of glycerol then a 50-75µl sample of this mixture (containing 20-50 µg of RNA) was layered onto geach gel. Within these RNA samples several RNA standards of known molecular weight were included. After layering all RNA samples, (one/gel) preps were electrophoresed for 1.5h at 4ma per tube. A voltage ca. 100_V was used to maintain this current as above this heating of the gels occurred and caused RNA degradation. The gels were scanned at 260nm, using either a Beckman^R Acta III spectrophotometer fitted with a Beckman^R 2 gel scanner or a Joyce-Loebl^R Chromoscan. From the trace obtained on scanning, the migration (cm) of each RNA peak was measured. A standard curve was constructed using the migration of RNA standards (Log molecular weight versus migration) and from this the molecular weight of each viral RNA species was determined.

Immunology and serology

Antigens: purified virus preparations (1-5mg/ml in a serological buffer, were used as antigens. Infected sap was also used from the following: sap from infected; *Chenopodium quinoa*, *Cucurbita pepo* 'Small Sugar', *Nicotiana clevelandii* L., *Nicotiana tabacum* L. 'White Burley'.

Appropriate controls, namely healthy sap, purified preparations of healthy tissue and sap from plants infected with other plant viruses unrelated to CMV were also used as controls in some tests.

Antisera: were prepared by immunization of rabbits with 2-3 intramuscular injections, each of ca. 5mg virus emulsified with Freunds Incomplete Adjuvant (Difco Bacto^R), at weekly intervals. These were followed by a 1ml intravenous injection containing ca. 5mg virus, 3-4 days before bleeding. Blood obtained by heart puncture was allowed to clot for 1h at room temperature and held overnight at 4C before centrifugation at 1,000g for 5min. The sera obtained were stored with either 0.02% sodium azide and frozen or mixed with 1:1 glycerol and stored at -20C. Other antisera used in this study are listed with sources in appendix 1.

Serological buffers: several buffers were used in the serological tests conducted.

Tomlinsons serology buffer/TSB (Tomlinson et al., 1973)
0.05M K_2HPO_4, pH 7.8 with 5mM EDTA,
Hollings serology buffer/HSB (Hollings & Stone, 1975)
0.03M K-K_2 phosphate buffer pH 7.6;
Phosphate buffered saline/PBS (Ball, 1974)
0.01M K-K_2 phosphate pH 7.0 with 0.15M NaCL.

Gel diffusion: Ouchterlony double diffusion tests (Ball, 1974) were conducted in either 0.75% or 0.9% Davis agar in one of the serological buffers with 0.02% sodium azide in 9cm plastic petri dishes. Several well patterns were tried, with a pattern of 6 or 8 peripheral wells around a central well, (all wells 4-5mm in diameter and 4-6mm apart) generally being preferred. Tests were conducted with several antisera dilutions and incubated in high humidity at 25C. The development of precipitin lines was observed with the aid of dark ground illumination and recorded over several days.

Microprecipitin: determination of prepared antisera titres by microprecipitin tests (Noordam, 1973) were carried out using Cooke Microtiter^R trays. One of the serological buffers listed was used as a diluent for two fold antigen and antisera dilutions. Volumes used were standardized as single drops from calibrated (tip diameter) pasteur pipettes. Appropriate controls, with and without antisera or antigen, normal serum, healthy antigen and buffer blanks were included in each test. The trays were incubated at 37C, covered with plastic film for 4h, and results recorded using a dissection microscope with dark ground illumination.

1.2 ISOLATION FROM DAPHNE

Cucumber mosaic virus isolate D (CMV-D) was obtained from *Daphne odora* Thunb. 'Leucanthe Variegata' flower or young leaf tissue

(Fulton, 1966) ground in Yarwoods solution plus celite by inoculation to *Chenopodium quinoa* Willd. A second virus tentatively named daphne isometric virus -2 (DIV-2/Forster & Milne, 1975) was frequently present and in *C. quinoa* masked CMV-D symptons. Separation of CMV-D from DIV-2 was carried out by several systemic passages through *Nicotiana glutinosa* L. and *N. Tabacum*.

1.3 HOST RANGE

Four isolates of CMV-D were inoculated to species from 15 families. Inoculum comprised CMV-D infected tobacco leaves ground in Yarwoods solution plus celite. The following host reactions were very similar for the four isolates of CMV-D tested.

AIZOACEAE

Tetragonia tetragonoides (Pall) A. Ktze (NZ. spinach): ca. 2mm chlorotic local lesions (5 days) become necrotic; faint systemic mottling.

AMARANTHACEAE

Amaranthus caudatus L. 'Love Lies Bleeding': Irregular local chlorotic blotches (7 days), darken to a red brown color; white systemic blotches and flecking develop (15 days) (Figure 1).

Celosia argentia L. 'Forest Fire': chlorotic local lesions 2mm diameter (3-5 days) rapidly coalesce until, the whole leaf becomes chlorotic; systemic chlorotic flecking follows (5-8 days) developing into a striking mosaic. Local and systemic red ring lesions also developed on occasions.

Gomphrena globosa L. 'Little Buddy' (globe amaranth): occasionally 2 mm local and systemic red lesions developed more frequently indistinct chlorosis and symptomless systemic infection. Virus readily recovered by back inoculation to *C. quinoa*.

CARYOPHYLLACEAE

Dianthus barbatus L. 'Indian Carpet' (sweet william): infrequent and symptomless infection; difficult to recover on back inoculation to *C. quinoa*.

Dianthus chinensis L. 'Bravo': local chlorosis and mild systemic veinal chlorosis; recoverable virus concentration low in *C. quinoa*.

Gypsophila elegans Bieb.: faint local and systemic veinal chlorosis, virus recoverable in low concentration.

Saponaria vaccaria L. 'Pink Beauty' (cowcockle): mild local and systemic veinal chlorosis, virus recoverable in low concentration.

CHENOPODIACEAE

Beta vulgaris L. 'Yates Early Wonder (red beet): red local lesions (6 days); systemic flecking.

Chenopodium amaranticolor Coste and Reyn.: 0.5-1mm chlorotic local lesions (4 days) develop with pin-point necrotic centres (6 days). No systemic infection observed or detected following numerous back inoculations to *C. quinoa*.

Chenopodium quinoa Willd.: 1 mm bright yellow local lesions (2-5 days) rapidly coalesce when inoculum concentration high; systemic light yellow chlorotic flecking (10-12 days) and leaf curling (Figure 2).

Spinacia oleracea L. 'Royal Denmark' (spinach): 1-2mm chlorotic local lesions (6 days) coalesce; systemic interveinal chlorotic rings and line patterns (infrequent).

COMPOSITAE

Calendula officinalis L. (calendula): chlorotic local lesions; symptomless systemic infection.

Lactuca sativa L. 'Calmar' (lettuce): local chlorosis; systemic mottling and veinal necrosis.

Senecio cruentus D.C. (cineraria): local and systemic mottle or symptomless infection.

Zinnia elegans Jacq. 'Cactus flowered': symptomless local infection; systemic mosaic and chlorotic flecking.

CONVOLVULACEAE

Ipomoea leptophylla Torr. (morning glory): local chlorotic blotches; systemic mosaic.

CRUCIFERAE

Arabis sp.: symptomless local and systemic infection, virus recoverable in low concentration.

Brassica oleracea L. var *capitata* 'Drumhead' (cabbage): local and systemic symptomless infection, low virus concentration recoverable in *C. quinoa*.

Brassica pekinensis (Lour) Rupr. 'Chi Hi Li' (chinese cabbage): local chlorosis; systemic infection not detected by back inoculation to *C. quinoa*.

Matthiola incana (L) R-Br. (stock): symptomless local and systemic infection virus recoverable in low concentration.

CUCURBITACEAE

Citrullus vulgaris Schrad, 'Golden Honey' (watermelon): chlorosis on inoculated cotyledons; no systemic infection.

Cucumis sativus L. 'Polaris' and 'Crystal Apple' (cucumber): 3-4mm local chlorotic blotches; systemic chlorotic flecking and veinal chlorosis (Figure 4).

Cucurbita maxima Duch. 'Buttercup' and 'Butternut' (pumpkin) faint chlorotic lesions on cotyledons; systemic chlorosis, on occasions chlorotic blotches and leaf curling.

Cucurbita pepo L. 'Small Sugar' (pumpkin): 1mm chlorotic local lesions on cotyledons (5 days); systemic chlorotic flecking and severe mosaic develops with necrotic flecks (Figure 5).

Momordica balsamina L. (balsam apple): chlorotic local lesions 2-3mm (4 days); systemic chloretic flecks, blotches and rings (5-7 days) followed by conspicuous systemic mosaic (Figure 3).

LABIATAE

Ocimum basilicum L. (basil): mild local chlorosis; symptomless systemic infection.

LEGUMINOSAE

Dolichos biflorus L.: symptomless local and systemic infection.

Phaseolus Vulgaris L. 'Top Crop', 'Prince' and 'Red Kidney' (french bean): pin point chocolate necrotic local lesions, most frequent autumn and spring months; symptomless systemic infection was detected occasionally on back inoculation to C. quinoa.

Pisum sativum L. 'Greenfeast' and 'Bonneville' (garden pea): 2-5mm irregular chlorotic and necrotic patches (5 days) on inoculated leaves, also local veinal necrosis. The top half of systemically infected leaves becomes chlorotic, front effect.

Vicia faba L. 'Coles Early Dwarf' (broad bean): 1mm chocolate local lesions or larger 3-4mm brown blotches and rings appear (4-5 days); symptomless systemic infection, virus recoverable in high concentration on back inoculation to C. quinoa.

Vigna unguiculata (L) Walp, subsp. cylindrica (L). van Eseltine ex Verdc. (catjang): 1mm brown local lesions on primary leaves (4 days); faint systemic mottling. Virus readily recoverable from systemically infected areas in high concentration.

Vigna unguiculata (L). Walp. subsp. unguiculata 'Blackeye' (cowpea): 1-2mm chocolate local lesions (5 days) on primary leaves; systemic mottling. High concentration of virus in systemic infection.

NYCTAGINACEAE

Mirabilis jalapa L. (four o'clock): local and systemic veinal chlorosis.

POLEMONIACEAE

Phlox drummondii Hock. (phlox): local and systemic veinal chlorosis.

SCROPHULARIACEAE

Antirrhinum majus L. (snapdragon): local chlorosis, virus recoverable from systemic leaves in high concentration.

SOLANACEAE

Capsicum frutescens L. 'Sweet Capsicum' (tobasco pepper): large ca. 5mm chlorotic blotches (7 days) inoculated leaves subsequently becoming necrotic along veins; systemic mosaic follows.

Datura stramonium L. (jimson weed): local chlorotic rings (7 days) rapidly become necrotic; systemic mottling and subsequent leaf strapping.

Lycopersicon esculentum Mill. 'Potentate' (tomato): mild chlorosis locally and systemically.

Nicotiana clevelandii Gray: diffuse 2mm local lesions (4 days) later encompassing whole leaf; systemic chlorosis of interveinal areas and stunting follows. Etching and oak-leaf patterns also occurred.

Nicotiana debneyi Domin: chlorotic local lesions (5 days); systemic bright yellow mosaic (9 days) with leaf strapping, curling; subsequently, chlorotic line patterns and pin point necrotic lesions form ring and line patterns along the leaf veins.

Nicotiana glutinosa L.: chlorotic local lesions (5 days) later enveloping whole leaf; systemic chlorotic rings, flecking and mosaic (10 days).

Nicotiana glutinosa x N. clevelandii (hybrid): local chlorotic blotches; systemic chlorotic flecking and blotches with veinal chlorosis following.

Nicotiana rustica L. 'Pavionii': faint chlorotic local lesions (5 days), whole leaf later chlorotic; systemic chlorotic flecking and mottle.

Nicotiana sylvestris Speg and Comes: faint chlorotic local ring lesions and systemic mosaic.

Nicotiana tabacum L. 'Burley 21', 'Havana 423', 'Samsun', 'White Burley': chlorotic local lesions 2-3mm (4 days) followed by a range of symptoms including necrosis; circular lesions, etch patterns, oak leaf patterns and target lesions; systemic symptoms were also diverse and variable including; striking mosaic, chlorotic line patterns, necrotic oak leaf patterns and raised green islands with chlorotic margins (Figure 6).

Petunia hybridia Vilm. 'Rose of Heaven ' and 'Rosy Morn': chlorotic local lesions (3 days); leaf becomes bright yellow as lesions coalesce; systemic mottling and chlorotic flecking, occasionally necrosis.

Physalis franchetii Mast.: local chlorotic lesions quickly forming 2-3mm necrotic ring lesions with white centres and dark irregular, somewhat star shaped margins (Figure 7): systemic mottling with virus recoverable from systemic tissue in low concentration.

Solanum melongena L. (Egg Plant): few 6-7mm chlorotic local blotches; systemic mottling with virus recoverable in low concentration.

TROPAEOLACEAE

Tropaeolum majus L. (nasturtium): faint chlorosis or symptomless infection virus recoverable from systemic tissue in low concentration.

UMBELLIFERAE

Apium graveolens L. 'Dulce DC' (celery): chlorotic mottling and veinal chlorosis, virus recoverable systemic tissue.

The following plants were not able to be infected with isolates of CMV-D: *Callistephus chinensis* (L) Nees. (Aster), *Helianthus annus* L. (sunflower), *Phacelia minor* (How.) Thell., *Salvia patens* Cov., *Tithonia speciosa* Hook.

Isolates of CMV-D could be distinguished from Daphne CMV-C (common type) isolates by the systemic infection of a number of hosts namely; *C. quinoa*, *Dolichos biflorus*, *Momordica balsamina*, *Vicia faba*, *Vigna unguiculata* subsp. *cylindrica*, *vigna unguiculata* subsp. *unguiculata* and *Zinnia elegans*. In *C. quinoa*, *Momordica balsamina* and *Zinnia elegans* CMV-D produced conspicuous systemic symptoms in contrast to CMV-C isolates which only produced localised infection. In the leguminous hosts *Dolichos biflorus*, *Vicia faba Vigna unguiculate* subsp., *cylindrica* and subsp., *unguiculata*, infections were either latent or produced only mild symptoms; however back inoculation to *C. quinoa* revealed high concentrations of CMV-D. In contrast CMV-C isolates did not cause any systemic infection in these legumes. The systemic infection of *C. quinoa* by CMV is not a common phenomena but has been previously recorded (Barnett & Baxter, 1974). Systemic infections of leguminous hosts are characteristic of legume isolates of CMV (Bird et al., 1974) but have also been recorded for an isolate of CMV from *Euonymus japonicus* 'Microphyllus' (Barnett & Baxter, 1974). Definitive hosts of CMV in the

FIGURE 1. CMV-D infection in *Amaranthus caudatus*

(a) local red-brown blotches (9 days)

(b) systemic chlorotic flecking and blotches (16 days)

FIGURE 2. Systemic chlorotic flecking and leaf curling (induced by CMV-D in Chenopodium quinoa (13 days).

FIGURE 3. Systemic chlorotic flecking in Momordica balsamina induced by CMV-D (7 days).

FIGURE 4. Systemic veinal chlorosis and flecking in *Cucumis sativus* 'Crystal Apple' induced by CMV-D (8 days).

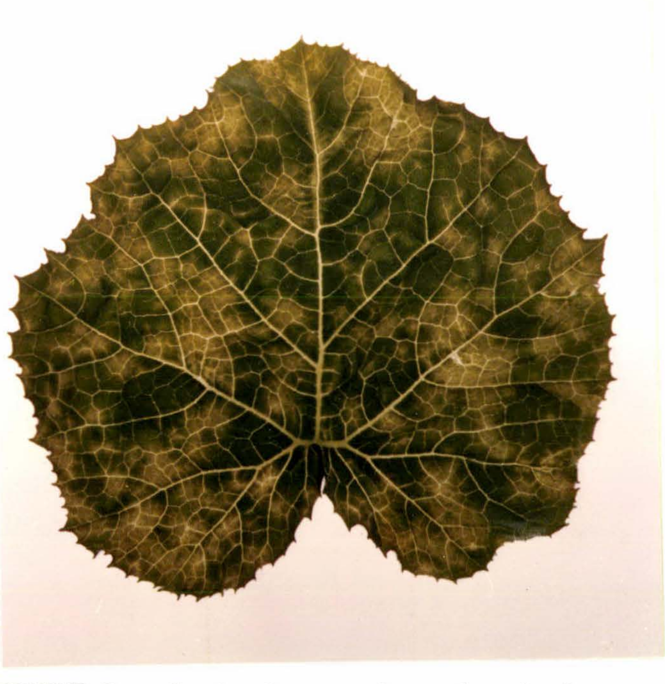


FIGURE 5. Systemic mosaic and veinal chlorosis in *Cucurbita pepo* 'Small Sugar' produced by CMV-D (10 days).

FIGURE 6. Local recrotic line patterns induced by CMV-D *Nicotiana tabacum* 'Burley 21'.

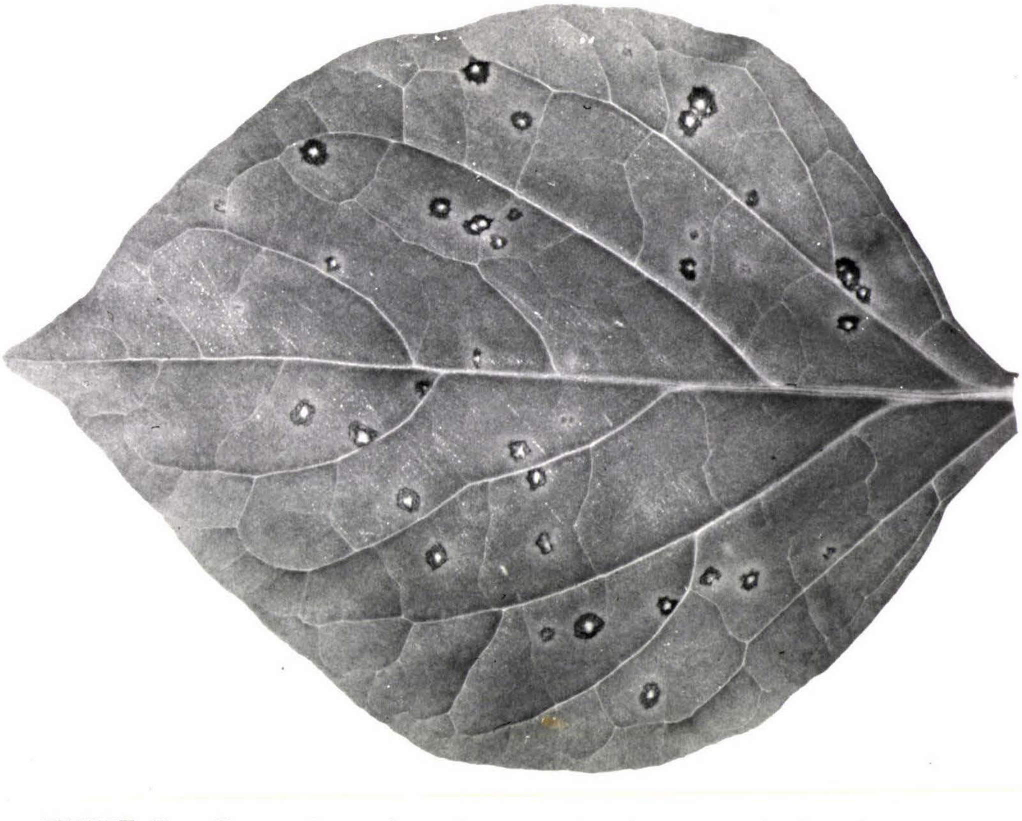


FIGURE 7. Irregular-shaped recrotic lesions induced on CMV-D inoculated *Physalis franchetii* leaf.

Cucurbitaceae and Solanaceae react similarly to all Daphne CMV isolates tested in this study and in accordance with reactions for other CMV isolates (Barnett & Baxter, 1974; Bhargava, 1951; Brierley & Travis, 1958; Price, 1940).

1.4 VECTOR TRANSMISSION

Aphid non persistent transmission

Determination of optimal starvation period: an experiment was conducted to determine the optimal preacquisition starvation period for the aphids *Myzus persicae* and *Macrosiphum euphorbiae*. Aphids were placed in petri dishes on moist filter paper and starved for 0.5, 1, 2h and overnight periods. After each period twenty aphids were placed on detached leaves and examined with a binocular dissection microscope; ten aphids were placed on a detached leaf of *S. oleracea*. Pre-probe times were recorded for each aphid and similar information was recorded for ten aphids on *N. tabacum* 'Havana'. The duration of the first probe was also timed. It was found both aphid species behaved similarly; after 0.5h starvation the first probe occurred within 5-20sec; after 1h the first probe was usually within 1min; after longer periods (2h and overnight) aphids were very restless and did not probe reliably within three minutes. *S. Oleracea* leaves were found preferrable to 'Havana' as the lack of leaf hairs enabled the aphids to settle more readily and find a site to probe. *M. euphorbiae* probed reliably for 20sec after 0.5h starvation, then withdrew and moved on, while M. persicae usually probed for a longer period. For transmission experiments it was decided to starve both aphid species for 0.5h; after 20sec the probes were interrupted by touching the antennae, although this was generally not required for *M. euphorbiae*.

Results of non persistent aphid transmission experiment: three experiments were conducted with CMV-D, two using *M. persicae* and one using *M. euphorbiae*. In all cases the identity of the transmitted virus was confirmed to be CMV-D by definitive host inoculation tests. Within 15 days local and systemic symptoms were observed in all the innoculated 'Havana' plants. In the second and third transmission experiments *M. persicae* and *M. euphorbiae* were used respectively. Ten aphids of the one species were transferred after 20sec probes on detached CMV-D infected spinach leaves, to each of two *S. oleracea* and two 'White Burley' tobacco plants. In the experiment with *M. persicae* all plants became infected, while in the experiment with *M. euphorbiae* both *S. oleracea* plants and only one 'White Burley' plant became detectably infected.

The transmission of CMV-D in a non persistent manner by *M. euphorbiae* and *M. persicae* is consistent with that reported for CMV (Kennedy et al., 1962).

1.5 PHYSICAL PROPERTIES IN CRUDE SAP

The thermal inactivation point (TIP) of CMV-D in crude sap from infected *N. rustica* leaves assayed on *C. quinoa* was 60-65C. A dilution end point (DEP) of 10^{-3} was obtained from systemically infected 'Havana' tobacco assayed on 'Top Crop' bean.

These results are consistent with those reported for CMV: TIP of 50-70C, DEP of 10^{-3} - 10^{-5} (Gibbs & Harrison, 1970).

1.6 ELECTRON MICROSCOPY

Negative staining of infected plant tissue

Using the squash homogenate negative staining technique (Walkey & Webb, 1968) small isometric particles ca. 30nm could be detected with either phosphotungstic acid (PTA) pH 4.0 or a 1:1 mixture of ammonium molybdate pH 5.3 and phosphotungstic acid pH 7.0 (pH of mixture 5.5) (Bennett, 1975). Ammonium molybdate pH 5.3 alone had good spreading but poor contrasting properties while PTA at lower pH's (pH 4-5) contrasted well but gave poor spreading. At higher pH's PTA is known for its disruption of CMV particles and this, together with its poor spreading properties, made it inappropriate to use alone. A 1:1 mixture of AM and PTA, proved a satisfactory compromise.

Due to the heterogenous nature of squash homogenates from daphne flower and leaf tissue, CMV-D was difficult to resolve in this host. However in several inoculated herbaceous hosts including *C. quinoa*, cucumber, momordica and several tobacco species CMV-D particles were more readily detected.

Negative staining of purified virus preparations

Negatively stained purified prepartions of CMV-D were examined in the electron microscope to observe contrast, spreading and disruptive properties of several stains. The effects produced were observed in relation to several factors: the negative stain used, its pH and the presence or absence of a fixative or chelating agent in the buffer used for resuspending the purified preparation. With this information a procedure was standardized to elucidate the quality of virus preparations with respect to the number of intact particles and the relative concentration of plant protein contaminants (F_1 protein) (van Regenmortel, 1966).

There are several earlier reports noting difficulties with negative staining of CMV. Murant (1965) found poor contrast and disruption of many CMV particles in PTA pH 6.0, while Francki et al., (1966) observed penetration and disruption of particles in PTA pH 7.0. In the analytical ultracentrifuge Francki et al., (1966) also observed the disruption evidenced by the broadening of the normal homogeneous single sedimention peak of CMV-Q strain preparations in PTA pH 7.0. The disruption of virus particles by neutral PTA has been observed with other icosahedral viruses including arabis mosaic (Murant, 1970) and carrot mottle (Murant et al., 1969). Difficulties with virus particle dispersion has also been reported for several negative stains with CMV and other viruses (Brenner & Horne, 1959).

Several suggestions have been proposed to overcome these difficulties. The use of fixatives to facilitate staining of CMV has been recommended. Francki et al., (1966) used the fixative osmium tetroxide with the negative stain uranyl acetate, and later formaldehyde was used as a fixative with PTA 7.0 (Francki & Habili, 1972). It was postulated that the disruptive qualities of negative stains on tobacco necrosis virus were simply a pH effect (Finlay & Teakle, 1969) and therefore low pH negative stains were recommended rather than neutral pH ones. However the stability of CMV in negative stains appears to involve more than just a simple pH effect. Forster (1974) and Tomlinson et al., (1973) demonstrated that the chelating agent disodium ethylene diamine tetra-acetate (EDTA) had a marked beneficial influence on particle stability in the presence of some neutral pH stains. Tomlinson et al., (1973) observed that EDTA prevented disruption of CMV particles in neutral AM but not in neutral PTA. However in the absence of EDTA broken CMV particles were also found in neutral AM.

To overcome dispersion problems with virus particles in negative stains on electron microscope grids, the use of wetting agents has been proposed. The most commonly used is bovine serum albumen (BSA) at a concentration of 0.5% mixed with the virus preparation before staining and grid spraying (Valentine, 1961). Other wetting agents include sucrose, glycerol, propylene glycol, Triton X-100 and sodium dodecyl sulphate. More recently bacitracin at a 50µg/ml concentration has been used, and good spreading of virus particles has been obtained with five common negative stains (Gregory & Pirie, 1973). Virus spreading difficulties may also be overcome by manipulation of the stain: virus ratio. Brenner & Horne (1959) noted that satisfactory spreading of virus particles on electron microscope grids was dependent on the ratio of specimen particle concentration to embedding material concentration.

Not unexpectedly, problems in maintaining the structural integrity of CMV-D preparations in negative stains were experienced. In the absence of EDTA, CMV-D particles were completely penetrated and disrupted by both PTA pH 7.0 and pH 4.0 in AM pH 5.3 broken particles also occurred (Figures 8 & 9). The fixative formaldehyde at 0.5% was tested, but although particles were stabilized in PTA pH 7.0 dispersion of particles on the grid was poor. These difficulties were not entirely prevented by the addition of BSA, SDS or bacitracin wetting agents. Following the experience of Forster (1974) and Tomlinson et al., (1973) EDTA was incorporated into the buffer used in the purification of CMV-D. Purified preparations of CMV-D were resuspended in 5mM borate buffer pH 9.0 with several levels of EDTA (0, 0.5, 1 and 5mM) and negatively stained using several negative stains including: AM pH 5.3, PTA pH 4.0 and pH 7.0, uranyl formate (UF) pH 4.0, uranyl acetate (UrAc) pH 4.0 and a 1:1 AM pH 5.3 : PTA pH 7.0 mixture. The results of this experiment are summarized in Table 1 and in Figures (8 & 9).

TABLE 1. The effects on CMV-D particle stability of several negative stains and different EDTA concentrations.

Negative stain	concentration of EDTA			
	0mM	0.5mM	1mM	5mM
AM pH 5.3	B	B	S	S
PTA pH 4.0	B	–	S	S
PTA pH 7.0	U	U	U	S
AM pH 5.3 & PTA pH 7.0 (1:1)	–	–	S	–
UAc natural pH	–	–	S	–
UF natural pH	–	–	S	–

S = stable (90% particles intact); B = broken (75% particles intact); U = unstable (50% particles intact).

Although the results are not quantitative the experiment was repeated on several occasions using purified preparations of CMV-D and similar results were obtained.

These results indicated that the more disruptive the negative stain the higher the concentration of EDTA required to stabilize CMV-D particles i.e. with PTA pH 7.0, 5mM EDTA is required to stabilize virus particles compared to AM pH 5.3 where only 1mM EDTA is required (Figures 8 & 9).

Further to these results it was found that AM gave excellent dispersion of CMV-D particles on electron microscope grids whereas PTA stains resulted in poorer spreading and UrF or UrAc resulted in extremely poor spreading. Where virus particles dispersion was poor a wetting agent (0.05% BSA or bacitracin) was added, or the 1:1 ratio of negative stain to virus preparation was diluted with distilled water. Some improvement in CMV-D dispersion was observed on dilution but neither of the wetting agents proved of value. Higher spraying pressures (>25psi) and spraying at closer ranges to the grids were tried, as well as post-staining of unstained, sprayed preparations. These methods did not improve results sufficiently to warrant their continuation.

From the results obtained it was decided to resuspend all purified preparations of CMV-D in EDTA and examine in the electron microscope using the negative stain ammonium molybdate pH 5.3 Excellent contrast and spreading was obtained reliably and repeatedly. Particles remained intact and the presence of F_1 protein could be determined where and when it contaminated the purified virus preparation. Unlike the results with squash homogenates, AM does appear to give sufficient contrast with purified virus preparations and consequently the presence of PTA is not required.

Because the cation environment around CMV-D particles differs markedly in purified preparations compared to squash homogenates it is not possible to critically compare the two in relation to negative staining. However by ignoring the cation environment some guarded comparison is possible. The negative stain mixture AM:PTA 1:1 which proved desirable for squash homogenates of CMV-D infected tissue, disrupts CMV-D particles in purified preparations. But the level of disruption observed with stained purified virus preparations was only small ca. 10-20% breakage of CMV-D particles. It is quite probable some CMV-D particles in squash homogenates are disrupted by the AM:PTA mixture but the number of particles would be of minor significance. The staining techniques were developed separately to suit the requirements of either squash homogenates of purified virus preparation. The mixture AM:PTA was not chosen for purified virus preparations in EDTA buffer because adequate contrast was obtained using AM alone.

A hypothesis for the observed requirement of EDTA in negative stained CMV-D purified preparations is outlined:

Proteins require divalent cations, in particular Mg^{2+} (Gergely, 1966) to maintain a tertiary and quaternary conformation. However the presence of excess divalent cations may result in binding between the separate protein entities themselves and thus precipitation will occur. Such a precipitate however is readily solubilized by the removal of the divalent cations and use has been made of this in purification of plant viruses (Dunn & Hitchborn, 1965) where chelating agents such as EDTA are used to remove the Mg^{2+} cations, thus facilitating the resuspension of the virus precipitate. The Mg^{2+} ions within proteins can be replaced by other divalent cations or even monovalent cations where the latter are in great excess. Replacement of divalent cations by monovalent cations does not however maintain protein conformation as monovalent cations cannot bind between two separate sites.

Degradation of CMV by monovalent ions has been reported frequently. Kaper et al., (1965) found disruption of CMV by KCl and Gibbs and Harrison (1970) emphasized the problem of non specific precipitation by NaCl in serological tests. Francki et al., (1966) likewise found CMV-Q destabilized by monovalent ions from LiCl.

The negative stain PTA is prepared from its potasium salt and its pH is usually adjusted by potassium or sodium hydroxide. It is therefore hypothesized that PTA as a negative stain causes an excess monovalent ion concentration around virus particles, replacing divalent cations and destabilizing the coat, resulting in disrupted particles. PTA pH 7.0 has a greater concentration of free K^+ ions than PTA pH 4.0 thus PTA pH 7.0 is more disruptive. The pH effect as proposed by Finlay and Teakle, (1969) therefore appears to be due to the availability of disruptive cations at different pHs. AM has a lesser ability to destabilize virus particles than PTA because ammonium ions do not compete as well as K^+ ions at divalent cation replacement. However if the virus preparation is resuspended in an adequate level of the chelating agent EDTA before negative staining, excessive monovalent ion concentrations are removed thus leaving the Mg^{2+} ions firmly bound within the tertiary or quaternary protein structure. The virus particles within the preparations therefore remain intact. The inefficiency of EDTA in chelating monovalent as compared to divalent cations would necessitate a reasonably high level of EDTA to stabilize the capsid of CMV, e.g. CMV-D preparations require 5mM EDTA to stabilize virus particles in the negative stain PTA pH 7.0.

Particle morphology and size

Isometric particles were observed in several innoculated hosts and purified CMV-D preparations, 50 particles stained in AM had an average diameter of 29nm.

These results on electron microscopy of CMV-D are not only in accordance with those reported for CMV but more fully illustrate a relationship between CMV particle disruption, negative stains and chelating agents.

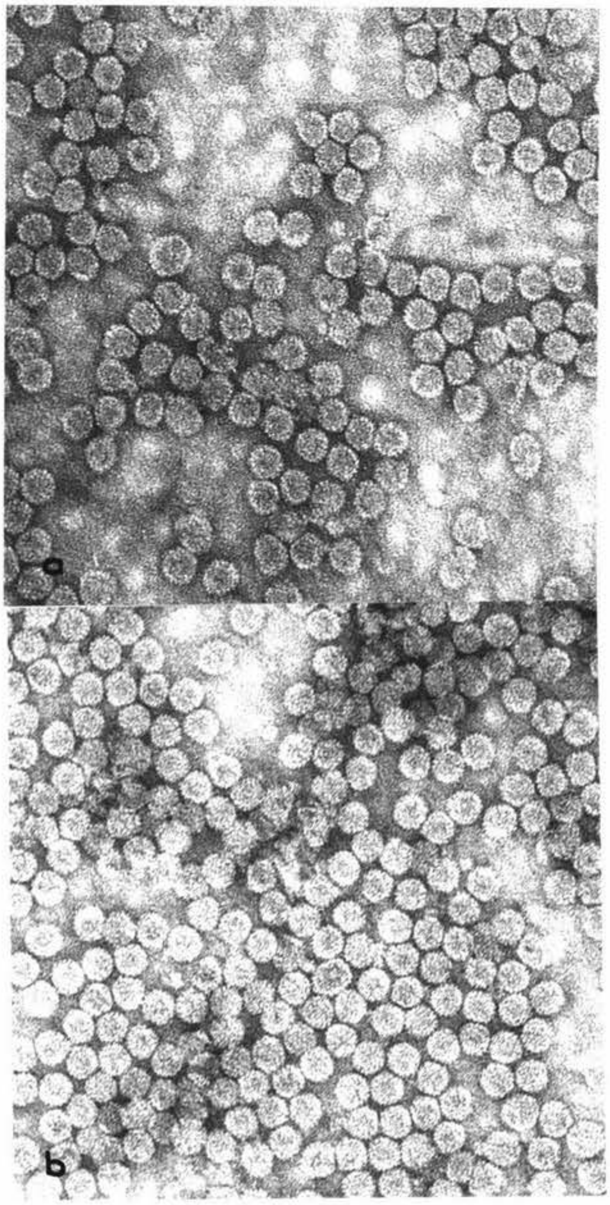


FIGURE 8. Purified preparations of CMV-D resuspended in (a) 0mM EDTA/5mM borate buffer pH9.0 (b) 1mM EDTA/5mM borate buffer pH9.0, negatively stained by ammonium molybdate pH5.3 (Mag. 166,500). Observe the greater level of disrupted virus particles in the suspension without EDTA.

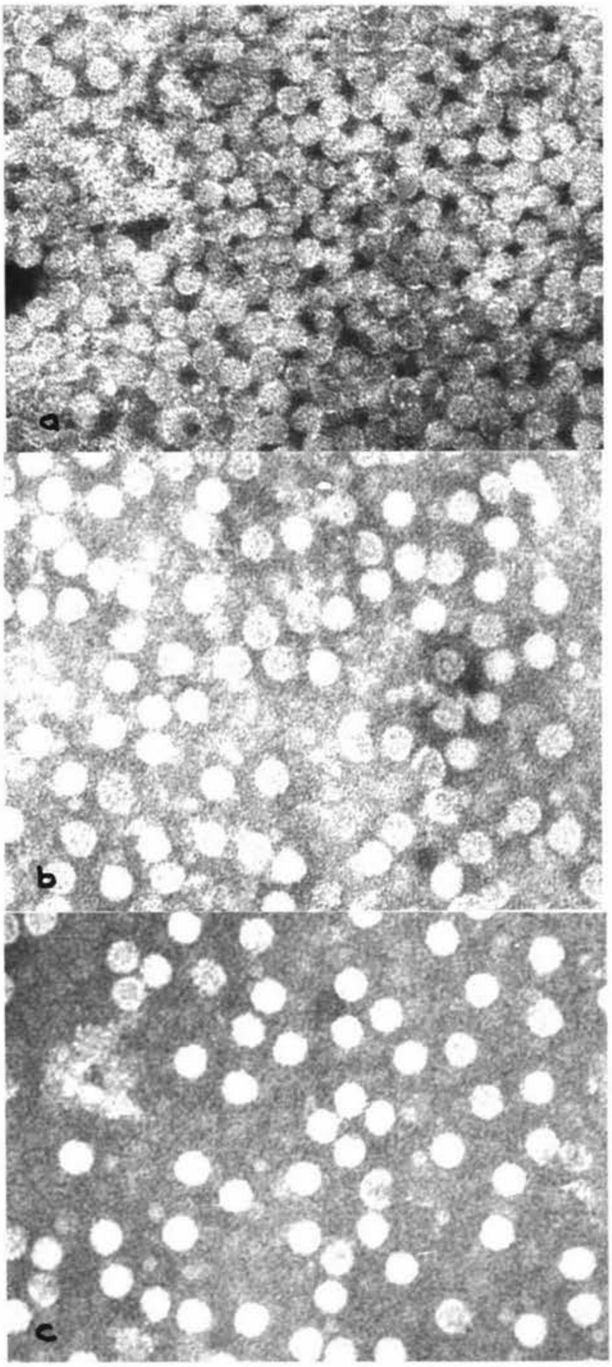


FIGURE 9. CMV-D purified preparations resuspended in 5mM borate buffer pH9.0 with (a) 0mM EDTA (b) 1mM EDTA (c) 5mM EDTA, negatively stained by phosphotungstic acid pH7.0 (Mag. 166,500. Observe the increased stability of CMV-D particles with increasing EDTA concentration.

1.7 PURIFICATION

A prerequisite to detailed virus characterisation is a reliable purification procedure producing homogeneous infectious preparations. Numerous methods have been reported for CMV purification including those by: Hollings (1968); Lot et al., (1972); van Regenmortel (1964); Scott (1963) and Tomlinson et al., (1973) and in part these different procedures have been developed because of problems with virus instability (Francki et al., 1966).

A purification procedure for CMV-D was developed enabling further characterisation and comparison with daphne common CMV (CMV-C). Several aspects of a purification procedure for CMV-D were examined, including: virus source; extraction medium; clarification; virus precipitation; resuspension medium and further purification. Analyses of homogeneity of final preparations were made with respect to (i) infectivity, (ii) serology, (iii)electron microscopy, (iv) analytical centrifugation, (v) UV spectrophotometry.

Virus source

The source of CMV has been demonstrated to be important in purification procedures and Hollings (1968) obtained highest and cleanest yields from *N. clevelandii* with poorer results from *N. glutinosa*. Varieties of *N. tabacum* (tobacco) are frequently used including 'Samsun', (Scott 1963) 'White Burley', (Tomlinson et al., 1973) 'Xanthi',(Lot et al., 1972) and good yeilds of CMV are obtained. van Regenmortel (1967) recommends *Cucurbita pepo L.* (squash) in preference to tobacco as with the use of electrophoresis for further purification better separation between virus and plant contaminants was obtained. Other workers have preferred *C. sativus* (cucumber) to tobacco (Peden & Symons, 1973; Habili & Francki, 1974).

The age of infection by CMV before harvest is also important. Tomlinson et al., (1973) demonstrated peak infectivity in tobacco occurred 10-12 days after innoculation while Lot et al., (1972) recommended harvesting 6 day old locally infected tobacco for CMV purification.

In this study with CMV-D, infected (local and systemic) plant tissue was harvested for purification ca. 12 days after innoculation. Several virus sources were tried: *N. clevelandii* gave highest virus yields (30-40mg/100g infected tissue) with little contamination by plant components (serologically undetectable). *N. tabacum* varieties

'Burley 21', 'Havana 423', 'Samsun' and 'White Burley' all gave clean preparations with good virus yields (ca. 20mg/100g infected tissue). Cucumber C. sativus 'Crystal Apple' and 'Polaris' also gave similar high yields and clean CMV preparations. Contrary to the results of Forster (1974) and Hollings (1968) N. glutinosa tissue gave reasonable virus yields (ca. 15mg/100g) which were not highly contaminated with F_1 protein. N. debneyi a fleshy species like N. clevelandii of the tobacco family solanaceae gave CMV yields of ca. 10mg/100g and preparations reasonably free of F_1 protein. Overall the purification procedure for CMV-D resulted in reasonable virus preparations from either tobacco or cucumber.

Extraction medium

The ionic environment in which CMV is extracted in from infected plant tissue undoubtedly influences the loss of virus by aggregation and attachment to cellular plant material (Hollings, 1968; Sill et al., 1952).

A range of extraction buffers have been used and recommended for CMV. Scott (1963) and Tomlinson et al., (1973) recorded greater infectivity using phosphate buffer for extraction compared to borate. Citrate buffer (van Regenmortel, 1964; Lot et al., 1972) has also been shown to give good results. Since Lot et al., (1972) showed greater infectivity with 0.5M citrate buffer compared to 0.05M citrate and Tomlinson et al., (1973) demonstrated 0.5M $K-K_2$ phosphate was better than 0.05M phosphate, high molarity extraction buffers have been commonly used. However van Regenmortel (1964) demonstrated with a high molarity extraction buffer (citrate 0.5M) that although the infectivity was higher than extraction in 0.05M citrate or distilled water, greater levels of F_1 plant protein contaminant were also present.

Several buffer additives have been tested. Reducing agents and chelating agents are frequently included in extraction systems with the aim of preventing virus degradation by plant phenolics. On extraction plant tissues release many substances including polyphenols and enzymes which catalyze oxidation. Problems with virus inactivation and precipitation during extraction have been attributed to these oxidized polyphenols, and the function of certain reducing and chelating agents which appear to inhibit the above have been hypothesized (Kosuge, 1965). Reducing agents such as thioglycollic acid (TGA) and mercaptoethanol (ME) apparently inhibit the oxidation of polyphenols by oxidase enzymes while certain chelating agents such as EDTA or sodium

Diethyldithiocabamate (DIECA) are purported to inactivate polyphenoloxidase enaymes by the removal of Cu^{++} cations from the prosthetic groups of these enzymes. Whereas EDTA has a marked chelating capacity for divalent and monovalent cations, it is most efficient at binding CA^{++} and Mg^{++}: DIECA chelates more specifically for larger cations such as Cu^{++}.

Relationships between polyphenoloxidases and virus instability have been demonstrated for CMV in tobacco leaf extracts (Harrison & Pierpont, 1963). By incorporation DIECA, infectivity of virus extracts was increased but the addition of Cu^{++} ions along with DIECA resulted in low infectivity.

TGA has frequently been included in extraction buffers for CMV purification (Hollings, 1968; Lot et al., 1972; Takanami & Tomaru, 1969), however Tomlinson et al., (1973) reported increased stability and infectivity during CMV purification by the incorporation of EDTA, whereas Hollings (1968) observed no marked benefit. Recently a CMV isolate from Nandina in this laboratory was demonstrated to give increased virus yields when extracted with EDTA, rather than without (pers, comm., Dr. K.S. Milne, 1974) supporting the work of both Takanami and Tomlinson.

In this study CMV-D was extracted in 0.5M K-K_2 phosphate buffer pH 7.0 with 0.1% TGA and increased virus yields were obtained when 1-5mM EDTA was present.

Clarification

Clarification of CMV buffer extracts is usually done by way of organic solvents including diethylether, chloroform, butanol, or a chloroform: butanol mixture. Using 8.5% n-butanol Hollings (1968) found increased infectivity with CMV on incubating the sap-butanol homogenate overnight at 2C or 20C. Longer standing, up to 14 days, further increased the virus yield, after which infectivity decreased. Clarification of sap from CMV-W infected tobacco with chloroform or diethyl ether (1:1 with expressed sap) gave a higher infectivity than clarification with 8.5% n-butanol (Tomlinson et al., 1973). Most CMV purification procedures involve clarification with chloroform (Lot et al., 1972; Peden & Symons, 1973; Takanami & Tomaru, 1969), although occasionally acidification has been used (Grogan et al., 1963). Bentonite (Dunn & Hitchborn, 1964) does not appear to have been tried.

In this laboratory chloroform has commonly been used for clarifying sap containing CMV, including isolates from daphne and nandina. Because of the satisfactory results obtained chloroform was also used in the purification of CMV-D. The incubation time of the chloroform homogenates, one hour with periodic shakings (Forster, 1974), was modified to vigorous shaking for 20min as Mink et al., (1969) found this resulted in less F_1 protein contaminating PSV purified preparations.

Virus precipitation

After breaking the solvent-extract emulsion by low speed centrifugation, the supernatant is usually subjected to ultracentrifugation to pellet the virus (Peden & Symons, 1973; Scott, 1963; Tomlinson et al., 1973). Precipipation of CMV by coacervation with polyethylene glycol (PEG) also resulted in good virus recovery (Lot et al., 1972). Lot et al., (1972) found ca. 6% PEG was a suitable level for precipitation of CMV. Coacervation is dependent on the ratio of the coacervant to monovalent cations (Herbert, 1963) and therefore ca. 0.15M NaCl is added to 6% PEG for CMV precipitation (Lot et al., 1972). To obtain purification CMV is usually subjected to several cycles of differential centrifugation or PEG precipitation.

Both procedures were tried with CMV-D, ultracentrifugation at 100,000g for 90min and coacervation by overnight stirring with 6% PEG (MW 20,000) and 0.15M NaCl, followed by pelleting with low speed centrifugation.

The period required to pellet CMV-D by ultracentrifugation in a 25ml tube, (MSE 8X25 titanium rotor), was calculated from:

$$T = \frac{1/S\ (\log_e \text{Rmax} - \log_e \text{Rmin})}{w^2}$$

where T = time required to pellet CMV

S = sedimentation coefficient CMV ($98 \times 10^{-13}\ \text{sec}^{-1}$)

Rmax = 9.415cm (for 8 X 25 rotor)

Rmin = 4.358cm

w = rev/min = 39,000

Calculated T = 1.309 hours

Therefore a period of 90min centrifugation was used to allow time for acceleration to 39,000rpm.

A level of 6% PEG MW 20,000 was used with CMV-D. It was found that 0.15M NaCl was not required in the first PEG precipitation cycle and this was probably attributable to the high levels of monovalent cations extracted from plant tissue and the high levels of Na^+ ions in the extraction buffer. Contrary to the experiences of (Forster (1974) all daphne CMV isolates tested could be concentrated with PEG to give satisfactory yields of purified virus. However in a comparison between ultracentrifugation and PEG precipitation of CMV-D approximately one third higher virus yield, with less F_1 protein contaminant, was obtained by ultracentrifugation.

Resuspension medium

Many different buffers have been used to resuspend pelleted CMV. Early workers (Murant (1965), (Tomlinson et al., (1959) used dilute phosphate disregarding virus stability and aggregation problems. Scott (1963) overcame these problems by dialysis and resuspension in dilute borate buffer, pH 9.0. Dilute citrate buffer also prevents CMV aggregation and has been used by van Regenmortel (1967). Lot et al., (1972) improved dilute citrate buffer by including Triton X-100, obtaining higher infectivity and preparations freer from plant contaminants. The prevention of aggregation of CMV with EDTA buffers was demonstrated by Takanami and Tomaru, (1969) who concluded its function was that of chelating free divalent cations which appear to aggregate CMV particles. Subsequently various EDTA levels (Peden & Symons, (1973) - 0.5mM EDTA; Tomlinson et al., (1973) - 5mM EDTA have been incorporated into resuspension buffers (frequently with 5mM borate pH 9.0; Tomlinson et al., 1973).

Several different buffers, at different pHs with and without EDTA, were compared in this study on CMV-D purification. Infected tobacco tissue was extracted and after ultracentrifugation the separate virus pellets were resuspended in different buffers. Following resuspension for an hour the solution was sampled before being subjected to a further cycle of differential centrifugation. The final virus pellet was resuspended in distilled water, again sampled and appropriate dilutions prepared and scanned in a UV spectrophotometer. The apparent virus concentration at each resuspension was calculated from the absorbance at 260nm (using E 0.1% = 5) and from this the percentage virus recovery was calculated. Absorbance maximum/minimum ratios were also calculated for the final virus resuspension. The results are summarized in (Table 2).

In Table 2 the buffers marked with asterisks appear to give lower % virus recovery. However use of these buffers results in A max/min values (ca. 1.40) more typical for CMV pure preparations (Lot et al., 1972), whereas the other buffer resuspensions have A max/min values (ca. 1.70) characteristic of preparations contaminated by plant material (Lot et al., 1972). It is probable that the apparent higher yields with these buffers is due to contaminating plant material. With the exception of citrate buffer, the buffers marked by asterisks contain EDTA and these virus resuspensions appear from A max/min values to be cleaner.

TABLE 2. Comparison of resuspension buffers used in CMV-D purification

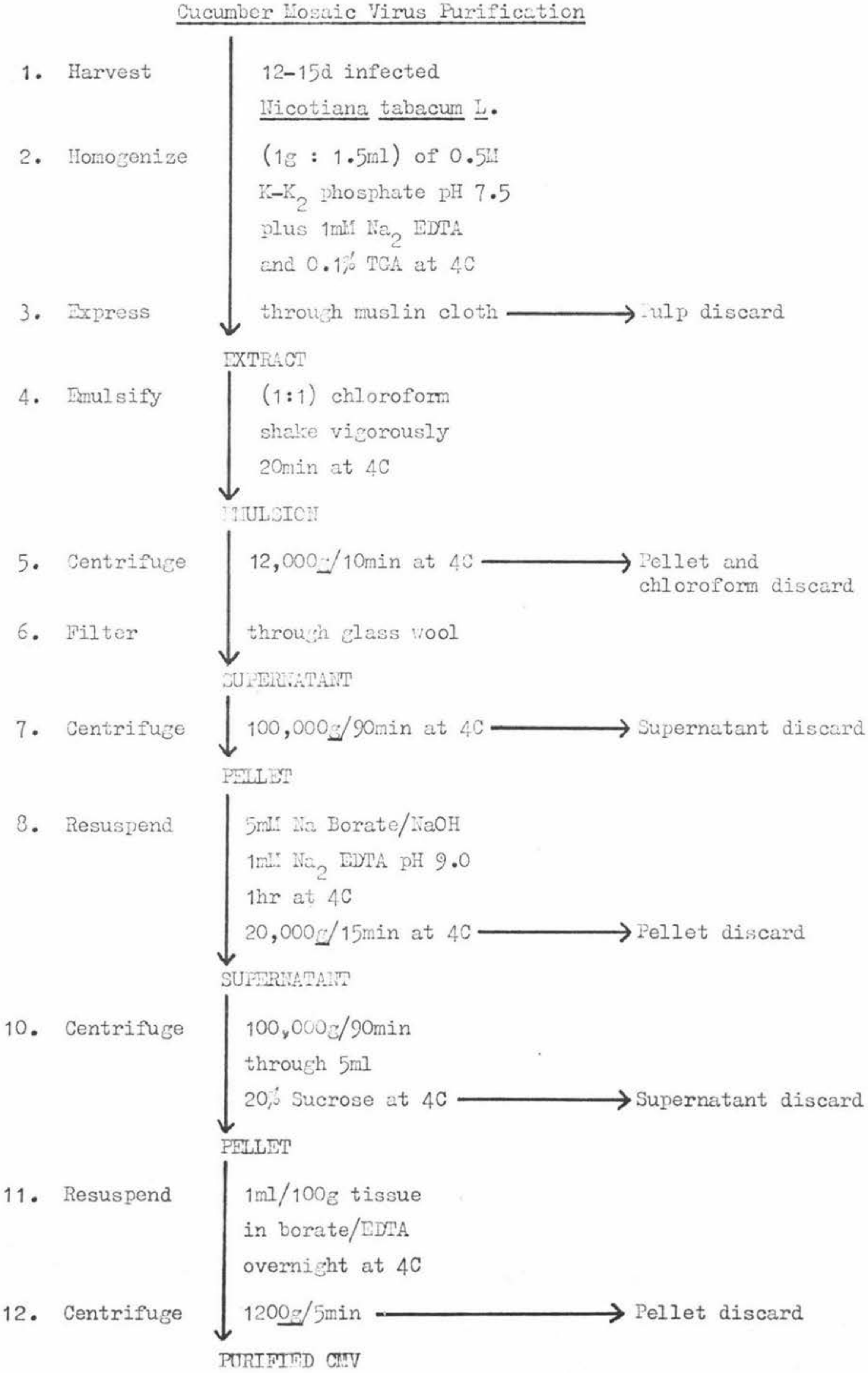
buffer		EDTA	pH	% virus recovery	absorbance max/min
distilled water		-	6.5	15.4	1.74
distilled water		+	5.0	12.2*	1.39
$K-K_2$ phosphate	10mM	-	7.0	14.8	1.71
$K-K_2$ phosphate	10mM	+	7.0	15.0*	1.37
Na citrate/citric acid	10mM	-	7.0	10.3*	1.39
Tris-HCl	20mM	-	8.0	13.0	1.64
Tris-HCl	20mM	+	8.0	6.2*	1.41
Na borate/NaOH	5mM	-	9.0	15.0	1.71
Na borate/NaOH	5mM	+	9.0	11.7*	1.43
Na bicarbonate/ carbonate	10mM	-	9.5	10.4	1.49

* apparent lower % virus recovery but 'cleaner' preparations (see text).

Experiments with purification of nandina CMV in this laboratory have shown that the apparent increased virus yields in resuspension buffers not containing EDTA is a result of greater contamination by plant components. This was demonstrated by the extraction of CMV infected and 'healthy' uninfected tobaccos using a buffer with and without EDTA (pers, comm., H, Neilson 1975). The ability of EDTA buffers to provide cleaner virus resuspensions is evident from these results. However, it does appear that dilute citrate (without EDTA) can adequately substitute for EDTA buffers.

On the basis of this work the buffer chosen for CMV-D resuspension was 5mM borate plus 1mM EDTA pH 9.0. Virus pellets were resuspended on an orbital shaker for one hour before clarifying by low speed centrifugation (15,000g/15min). The final virus pellet, after a second cycle of differential centrifugation, was also resuspended in the above borate/EDTA buffer (1ml/100g harvested tissue). The suitability of this borate/EDTA buffer for final CMV-D pellet resuspension was confirmed by work on negative staining for electron microscopic examination and analytical centrifugation of virus preparations. It was found that the level of EDTA was critical to ensure intact CMV particles within different negative stains. Two preparations of CMV-D were examined by analytical centrifugation, one suspended in distilled water and the other in borate/EDTA buffer. Aggregation of CMV-D occurred in the distilled water-virus suspension, two broad sedimenting peaks being observed (Figure 11), while the CMV-D suspension in borate/EDTA showed no aggregation, as evidenced by a single homogeneous sedimenting peak (Figure 12). This aggregation effect has been previously observed (Takanami & Tomaru 1969) with CMV in borate buffer without EDTA, during analytical centrifugation.

The action of EDTA in CMV purification appears to be four fold. Firstly during plant tissue extraction EDTA apparently chelates Cu^{++} ions necessary for activity of polyphenoloxidase enzymes, thus preventing oxidation of polyphenols and subsequent inactivation of CMV (Harrison & Pierpont, 1963). Secondly by chelating other free divalent cations EDTA removes the possibility of CMV aggregation (Takanami & Tomaru, 1969). Thirdly this same binding of divalent cations prevents virus particles aggregating with plant proteins (F_1). Consequently less F_1 protein is pelleted with the virus on centrifugation and the preparation is therefore 'cleaner'. The fourth function involves EDTA's chelation of monovalent ions which appear to be able to disrupt virus particles, removal of these monovalent ions by chelation maintains intact CMV particles (see discussion page 21).



Cucumber Mosaic Virus Purification

1. Harvest — 12-15d infected *Nicotiana tabacum* L.
2. Homogenize — (1g : 1.5ml) of 0.5M K-K_2 phosphate pH 7.5 plus 1mM Na_2 EDTA and 0.1% TCA at 4C
3. Express — through muslin cloth → Pulp discard

EXTRACT

4. Emulsify — (1:1) chloroform shake vigorously 20min at 4C

EMULSION

5. Centrifuge — 12,000g/10min at 4C → Pellet and chloroform discard
6. Filter — through glass wool

SUPERNATANT

7. Centrifuge — 100,000g/90min at 4C → Supernatant discard

PELLET

8. Resuspend — 5mM Na Borate/NaOH 1mM Na_2 EDTA pH 9.0 1hr at 4C 20,000g/15min at 4C → Pellet discard

SUPERNATANT

10. Centrifuge — 100,000g/90min through 5ml 20% Sucrose at 4C → Supernatant discard

PELLET

11. Resuspend — 1ml/100g tissue in borate/EDTA overnight at 4C
12. Centrifuge — 1200g/5min → Pellet discard

PURIFIED CMV

Figure 10. Procedure for the purification of cucumber mosaic virus

Further purification

A variety of techniques have been used to further purify CMV preparations: density gradient centrifugation (Tomlinson et al., 1973); further cycles of differential centrifugation (Lot et al., Peden & Symons, 1973); and zone electrophoresis (van Regenmortel, 1964).

Tomlinson et al., (1973) used 10-40% sucrose gradients to separate CMV from plant components, but low yields 10mg virus/kg of infected leaf were obtained with this procedure. Further differential centrifugation cycles were used by Lot et al., (1972) and Peden and Symons (1973) in CMV purification. On the basis of UV spectrophotometric data Lot et al., (1972) obtained very clean preparations with CMV yields of 380-475mg/kg leaf tissue. Zone electrophoresis has also been recommended for CMV final purification by van Regenmortel (1964) who, on the basis of serological data, reported that very clean virus preparations can be prepared using this method.

In the present study density gradient centrifugation of CMV-D on (5-30%) sucrose gradients was tried, but as with Tomlinson et al., (1973) very low (10%) virus recovery was obtained. It was therefore felt that manual fractionation of gradients is unsatisfactory with respect to the low recovery of virus. Some improvement of A max/min ratios occurred after subjecting CMV-D preparations to a third cycle of differential centrifugation, however ca. 50% loss of virus occurred, and therefore this technique was discontinued. Preparations of CMV-D were run in a zone electrophoresis apparatus (van Regenmortel 1964) as modified by Uyemoto (pers, comm., Dr K.S. Milne, 1975). However poor separation of CMV-D from plant components occurred as a result of difficulty in maintaining an adequate current. The CMV-D preparations electrophoresed were extracted from tobacco, a host which van Regenmortel (1964) does not recommend, as the virus and plant components have similar electrophoretic mobility. Squash, recommended by van Regenmortel (1964) as a source of CMV for zone electrophoresis was not tried.

The procedure adopted for further purification of CMV-D, involved the pelleting of the virus in the second cycle of differential centrifugation, through sucrose. Five ml of 20% sucrose was injected with a syringe below the virus suspension in the centrifuge tube, to form an interface. The sucrose impeded the sedimentation of F_1 plant protein and other plant components, hence avoiding contamination of the final CMV pellet. Such 'cushions' of sucrose have also been used along with polyethylene glycol in the purification of hop mosaic virus, (Probasco & Skotland, 1974) thereby effectively excluding F_1 protein contamination. This sucrose 'cushion' procedure was preferred to other techniques because of its simplicity, while still producing 'clean' CMV-D preparations without undue loss of virus.

The purification procedure for CMV-D developed in this study is outlined in Figure 10.

Analysis of homogeneity

(i) Infectivity

Infectivity tests on purified virus preparations are used extensively, however although these tests give an idea of concentration of intact infective units no information on the level purity of the virus preparation is gained. It is unfortunate that most comparative work on CMV purification involves the use of infectivity tests without regard to other techniques which reflect the purity of preparations.

A dilution end-point infectivity of ca. 10^{-6} is frequently reported for CMV purified preparation procedures (Scott, 1963; Tomlinson et al., 1973). In the current study a comparible dilution end-point of infectivity between 10^{-5} and 10^{-6} was obtained for CMV-D preparations assayed on <u>C. quinoa</u>.

(ii) Serology

CMV preparations were frequently examined with gel double diffusion tests using 'healthy' antisera to determine the level of F_1 protein contamination (Hollings, 1968; van Regenmortel, 1964). No 'healthy' plant components could be detected in CMV-D purified preparations diluted 1/2 and tested against 'healthy' antisera. However strong reactions with CMV-D preparations diluted as far as 1/1000 were observed against several CMV antisera.

(iii) Electron microscopy

Examination of CMV purified preparations negatively stained in the electron microscope enables determination of their quality, the presence of contaminating F_1 plant protein and the homogeneity of virus particles (Forster, 1974; Hollings, 1968). CMV-D purified preparations were examined using the procedure developed. Negatively stained preparations resuspended in EDTA revealed good contrast and spreading of intact virus particles with very little apparent contamination by F_1 protein.

(iv) Analytical ultracentrifugation

In the analytical ultracentrifuge purified CMV preparations reveal a characteristic single sedimenting peak, with a sedimentation coefficient ($s_{20,w}$) of ca. 98S (Gibbs & Harrison, 1970). The sedimentation coefficient of CMV has been found to be independent of the buffer the virus is suspended in, providing that the buffer does not cause aggregation of the virus. A marked concentration dependence has been found however (Francki et al., 1966), and is expressed by the equation.

$$s_{20,w} = 98.6-1.04C$$

where C is the virus concentration (mg/ml).

Several preparations of CMV-D, each at a concentration of about 4mg/ml, were examined by Schlieren optics in an analytical ultracentrifuge. It was found necessary to suspend CMV-D preparations in a buffer containing EDTA, otherwise aggregation of the virus resulted (Figure 11). (See discussion on CMV-D purification). From three analytical runs, at a speed of 23,150rpm using photographic plates exposed at 8min intervals, sedimentation coefficients for CMV-D were calculated using

$$S = dx/dt \times 1/w^2$$

where w = speed in radians per second ($2\pi/60$ rpm) and dx/dt is determined as the slope from a graph plotting the actual distance the virus peak migrates against time intervals the migration was measured over.

A single sedimenting virus peak was observed (Figure 12) during three analytical runs of CMV-D. A 'healthy' F_1 plant protein peak ($s_{20,w}$ ca. 24S) was present on one occasion, as the CMV-D preparation had not been purified through a sucrose 'cushion'. The sedimentation coefficients calculated for these three CMV-D runs were 96, 99 and 94S (uncorrected for concentration). Averaging these three values and upon correcting for concentration (Francki et al., 1966) the sedimentation coefficient for CMV-D was determined to be

$$s_{20,w} = 99 \times 10^{-13} \text{ sec}^{-1} \text{ or } 99S$$

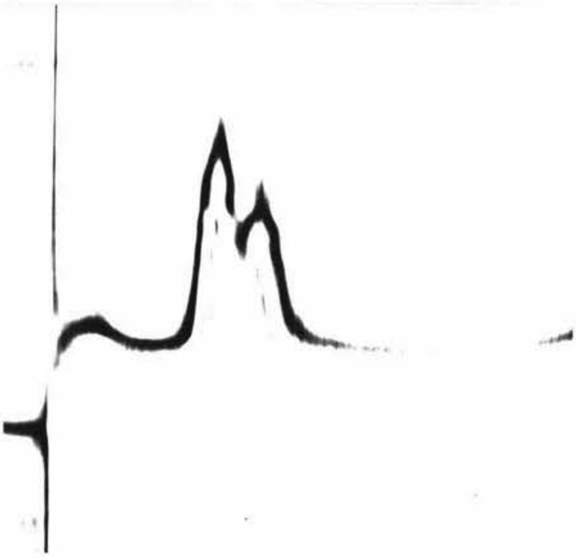


FIGURE 11. Analytical sedimentation pattern, for purified preparation of CMV-D resuspended in 5mM borate pH9.0 (without EDTA). Aggregation of the virus is evidenced by the two broad sedimenting peaks. Schlieren angle 45°, sedimentation is from left to right.

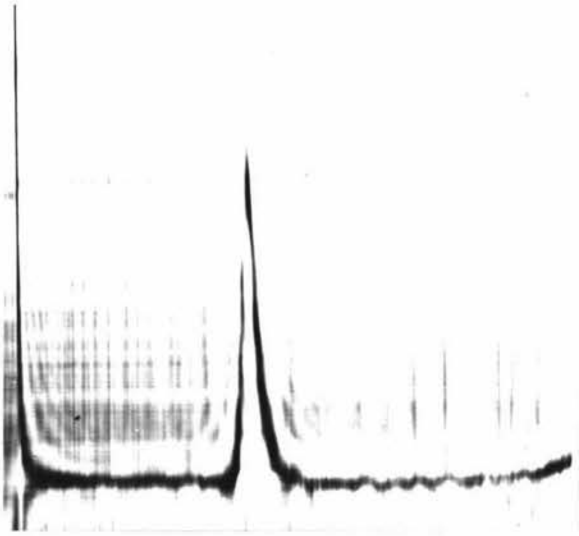


FIGURE 12. Analytical sedimentation pattern, for purified preparation of CMV-D resuspended in 5mM borate pH9.0 plus 1mM EDTA. A single sedimenting homogeneous, virus peak is evident. Schlieren angle 45°, photograph taken after 16 mins. at 23,150rpm.

(v) UV spectrophotometry

The concentration of CMV and the level of contamination by plant components can be determined by scanning virus preparations in a UV spectrophotometer (Noordam, 1973). CMV concentration is determined from the extinction coefficient E 0.1% = 5 from absorbance at 260nm for 1cm light path, while the level of contamination by plant components in purified CMV preparations can be determined from A max/min and A 260/280 ratios, (Noordam, 1973). Typical values for A max/min and A 260/280 ratios for pure CMV preparations are 1.40 and 1.65 respectively, while contaminated preparations have ratios above or below these, depending upon the type of contaminating material (Lot et al., 1972). The importance of including 'healthy' preparations in virus UV scans as controls is stressed by Demaire and Kummert (1969) as preparations from healthy tissue can give deceivingly virus-like UV absorption spectra.

Dilutions from CMV-D preparations were routinely scanned between 200-350nm and virus concentration, A max/min and A 260/280 values were calculated. All dilutions were made in distilled water to avoid the increased levels of light scattering resulting with many buffer solutions. Several pure preparations of CMV-C and 'healthy' (uninfected) tobacco, the source of most CMV purified preparations in this study, were also scanned. A summary of the UV spectrophotometric data obtained in this study along with reported CMV values is presented in Table 3.

The A max/min ratio range (1.34-1.44) obtained for purified preparations of CMV-D and CMV-C is in accordance with that reported by other workers (1.35-1.40), while the A 260/280 ratio range (1.75-2.00) is higher than that reported (1.60-1.70). This does not necessarily reflect more contamination by plant components, as the usual plant contaminating material in virus preparations is proteinaceous. Accordingly one would expect a lower A 260/280 ratio in 'dirty' preparations as the protein absorption maxima occurs at 280nm. Exactly this point was illustrated by healthy preparations scanned in this study where low A 260/280 ratios (1.38) were obtained. Whether nucleic acid could remain intact and significantly contaminate the virus preparation is questionable. Later in this study some contamination of virus preparations by plant nucleic acid was detected, but its significance in relation to the A 260/280 ratio is unknown.

TABLE 3. Summary of UV absorption spectrophotometric data for purified CMV.

Preparation	A max/min	A 260/280	Report
CMV-D^{+}	1.22 - 1.60	1.75 - 2.00	this study
CMV-D*	1.37 - 1.44	1.75 - 1.87	"
CMV-C*	1.34 - 1.43	1.75 - 1.90	"
'Healthy' tobacco*	1.17	1.38	"
CMV^{+}	1.30 - 1.40	1.60 - 1.70	Lot et al., 1972
CMV*	1.35 - 1.40	1.70	"
'Healthy' fraction $^{\neq}$	1.60 - 1.76	-	"
CMV-W*	-	1.65	Tomlinson et al., 1973
CMV-Q*	1.09 - 1.39	1.54	Francki et al., 1966
CMV-S*	1.38	1.67	van Regenmortel, 1967
CMV-Y*	1.43	1.69	Takanami & Tomaru, 1969

$^{+}$ partially pure preparation; * pure preparation

$\neq$ plant component contaminating fraction of CMV preparation.

Partially pure preparations of CMV-D revealed a wider range for both A max/min and A 260/280 ratios on UV absorption scanning, which is consistent with other reported results (Lot et al., 1972).

Scans of 'healthy' preparations showed virus-like absorption spectra, but A max/min and A 260/280 ratios were considerably lower than those of CMV preparations. It was observed that when 'healthy' preparations were diluted by the same amount as virus preparations, the spectra obtained were insignificant, at most an absorbance of 0.1 compared to 1.0 for the same dilution of a virus preparation.

1.8 PHYSICAL PROPERTIES OF PURIFIED VIRUS

Sedimentation coefficient

A sedimentation coefficient of $s_{20,w} = 99 \times 10^{-13}$ sec^{-1} or 99S[1] was determined for CMV-D from three separate analytical centrifuge runs. A photograph revealing a single homogeneous peak of one of these runs is shown (Figure 12). Purified preparations of two isolates of CMV-C were also examined in the analytical ultracentrifuge and sedimentation coefficients of 100 and 97S[1] were obtained respectively. These values are in accordance with those reported (98S[1]) for CMV (Francki et al., 1966).

UV absorption spectrum

Purified preparations of CMV-D gave the following UV absorption spectral characteristics; A max/min ca. 1.40 and A 260/280 ca. 1.80. Similar values were obtained with preparations of CMV-C. The A max/min ratios obtained are consistent with that reported for CMV (1.35-1.40 Lot et al., 1972) while the A 260/280 ratio for CMV-D and CMV-C are somewhat higher than that reported (1.70, Lot et al., 1972). The latter discrepancy in A 260/280 ratio possibly indicates nucleic acid contamination. An alternative explanation is that these values are simply a reflection of the system used in this study, therefore critical comparison with results obtained in different systems is not strictly valid. For this reason in comparisons of UV absorption spectrum data trends were looked for rather than absolute differences.

1.9 CHEMICAL COMPOSITION

SDS Polyacrylamide gel electrophoresis of isolated viral RNA

Extracted RNA from CMV-D purified preparations was electrophoresed in polyacrylamide-agarose gels to determine the number of RNA

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[1] corrected to infinite dilution or zero CMV concentration.

species and their respective molecular weights. At the same time comparison with CMV-C isolate A (representative of daphne common CMV) was conducted to see if differences in molecular weight between corresponding RNA species of CMV-D and CMV-C could be detected.

The RNA of CMV constitutes ca. 18% of the particle weight, giving it an average content per particle of about $1x10^6$ daltons. Poor separation of CMV RNA components has been acheived by sedimentation analysis (Diener et al., 1964; Kaper et al., 1965; May et al., 1969). However four major RNA species have been resolved by polyacrylamide gel electrophoresis of CMV RNA preparations (Nelson, 1970 and Kaper & West, 1972) with a pattern similar to that shown by RNA isolated from members of the bromovirus group (Bancroft, 1971; Hull, 1972; Lane & Kaesburg, 1971). Separation of the four major components of CMV RNA and subsequent recombination has demonstrated that only the three largest RNA's (1 +2+3) are required for infectivity (Lot et al., 1974). However, translation in a wheat embryo cell free system of fractionated CMV RNA resulted in numerous products from RNA's (1+2), the coat protein and two minor products from RNA 3 and the coat protein as a single product from RNA 4 (Schwinghamer & Symons, 1975). Several other viruses possess co-virus characteristics (multipartite genome) including alfalfa mosaic, cowpea mosaic, tobacco rattle, tobacco streak viruses (Jaspars, 1974) bromoviruses (Hull, 1972) and another cucumovirus, tomato aspermy virus (Habili & Francki, 1974).

The separation of RNA species in acrylamide gels is dependent upon the RNA species size (molecular weight), the degree of crosslinking within the acrylamide matrix (determined by the acrylamide: bis concentration) and the period electrophoresed at a standard current (Loening, 1967). Some dependence on the RNA secondary structure (conformation) is also predicted. However with CMV RNA it was demonstrated using the formamide system of Staynov (1972) that such dependence was insignificant (Peden & Symons, 1973).

The determination and comparison of molecular weights of RNA species from CMV-D and CMV-C was carried out using 2.0% acrylamide gels electrophoresed along with RNA standards for 90min. Gels of 2.0% acrylamide were chosen after poor separation of CMV RNA species was obtained in 2.4% gels. The period of 90min electrophoresis was chosen because the migration of the four major RNA components were maintained within the top half of gels in this period. Hence the 4S RNA standard would not be electrophoresed off the end of a gel.

Several RNA standards were included in each polyacrylamide gel experiment: rat liver ribosomal RNA's (28 & 18S); rat liver transfer RNA (t RNA), (4S); and E. coli ribosomal RNA's (23 & 16S). After electrophoresis the gels were scanned at 260nm, measurements of the migration of each RNA peak were taken from the scan trace, and a standard curve was plotted from the migration of the RNA standards (Log molecular weight RNA versus migration cm) (Figure 13). The molecular weight of each CMV RNA peak/species was determined from the standard curve (Figure 13).

Extracted RNA from CMV-D and CMV-C revealed the separation of four major RNA peaks (RNA species) within the acrylamide gels (Figure 14). The results of several experiments are summarized along with reported CMV RNA values in Table 4. The RNA peaks/species are numbered as in Figure 14 i.e. the largest molecular weight RNA or slowest migrating is labelled number 1.

Several smaller molecular weight species of RNA were also present (0.06 and 0.11 x 10^6 daltons). Peden and Symons (1973) attributed these small RNA's to breakdown products or pieces of the larger RNA species.

The results obtained show good correlation with those reported for CMV (Peden & Symons, 1973). No difference is detected between the RNA's of the two different daphne CMV's compared. These results indicate that the distinctive host range and serological differences between CMV-D and CMV-C are not significant enough to be reflected in easily detectable different genome sizes.

TABLE 4. Molecular weights of CMV RNA species determined by SDS polyacrylamide gel electrophoresis

		average mol. wt. ($\times 10^6$ daltons) RNA peak				number of determinations
experiment	preparation	1	2	3	4	
1	CMV-D RNA	1.45	1.05	0.88	0.46	2
2	"	1.35	1.20	0.78	0.30	1
3	"	1.38	1.21	0.76	0.33	2
4	"	1.30	1.10	0.79	0.42	3
1	CMV-C RNA	1.45	1.15	0.87	0.46	2
2	"	1.35	1.15	0.72	0.30	1
3	"	1.36	1.18	0.75	0.30	2
4	"	1.30	1.11	0.80	0.43	3
Report						
Habili & Francki (1974)	CMV-Q RNA	1.26	1.10	0.77	0.34	-
Kaper & West (1972)	CMV-S RNA	1.07	0.95	0.69	0.33	-
Peden & Symons (1973)	CMV-Q RNA	1.30	1.13	0.78	0.34	-

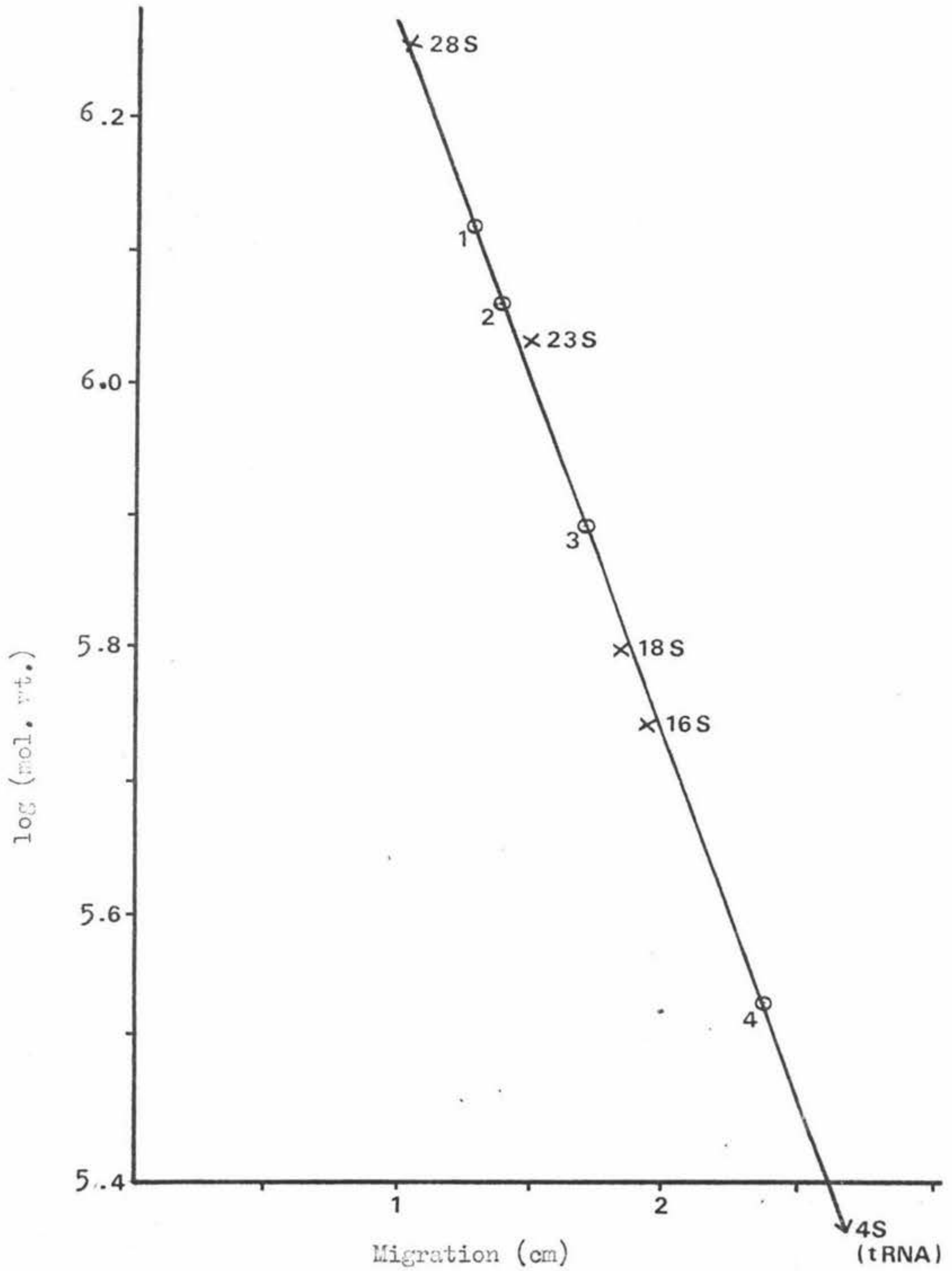

FIGURE 13. Standard curve for molecular weight determination of CMV RNA species by polyacrylamide gel electrophoresis. (28S, 18S = rat liver r RNAs; 4S = rat liver t RNA; 23S, 16S = E. coli r RNAs - used as standards; see text) (numbers 1-4 = CMV RNA species; see Figure 14).

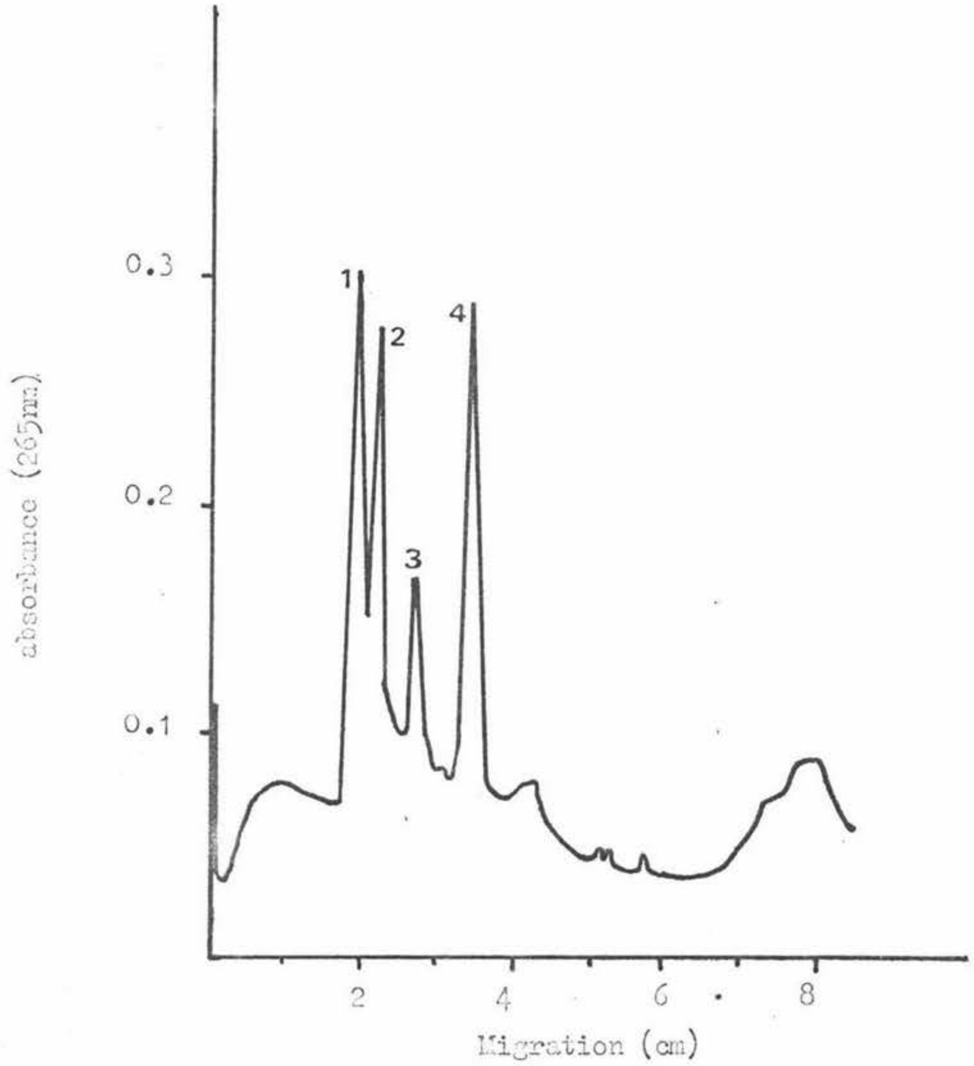

FIGURE 14. Densitogram showing four CMV-D RNA species, separated by polyacrylamide gel electrophoresis. RNA species (1-4) numbered in order of decreasing molecular weight. Gels scanned at 265nm in a Joyce-Loebl Chromoscan.

1.10 IMMUNOLOGY

The poor immunogenicity of CMV is well known (Francki et al., 1966; Scott, 1968) and has been attributed to the instability of the virus. CMV is readily degraded by salt solutions (Kaper et al., 1965; Francki et al., 1966) pancreatic ribonuclease (Francki, 1968; Kaper & Geelen, 1971), and neutral PTA (Francki et al., 1966). Enhanced immunogenicity of CMV has been observed with stabilization of the virus capsid with formaldehyde (Francki & Habili, 1972). Hollings and Stone (1962) likewise observed enhanced antibody response with formaldehyde-treated plant virus antigens although Scott (1968) failed to achieve such an effect with CMV-Y.

The effect of formaldehyde on proteins involves the formation of methylol groups on free amino groups, with probable further condensation of other functional groups (Kabat & Mayer, 1961). With CMV the overall effect is a probable cross-linking of reactive sites between adjacent subunits, making the virus capsid more stable.

Antisera to CMV-D and CMV-C (isolate A) were prepared using the procedure described earlier, including both fixed (0.5% formaldehyde) and unfixed virus preparations in the injection series. Formaldehyde fixed virus preparations were included with the aim of increasing the immunogenicity of CMV. However it is probable that the increased stability observed with resuspension of CMV in EDTA buffers (page 20) may result in increased immunogenicity of CMV without formaldehyde.

The titre of both CMV antisera (anti CMV-D, anti CMV-C) were determined as 1/512 using the microprecipitin procedure (Noordam 1973), while a suitable dilution of these antisera for gel diffusion was found to be 1/8.

1.11 SEROLOGY

Certain problems often become manifest with serology involving CMV: frequently only low titre antisera are available because of the poor immunogenicity of this virus (Francki et al., 1966); many of these antisera react with broad specificity, having been made to antigen mixtures (Mink et al., 1975); and in serological tests it is not uncommon to have problems with CMV non specific precipitation (Gibbs & Harrison, 1970). Tomlinson et al., (1973) avoided some of these problems using an EDTA buffer (50mM K_2HPO_4 + 5mM EDTA pH 7.8) for CMV serology, encountering no non specific precipitation problems.

The source of virus antigen can markedly affect serological systems, as agglutinating compounds call lectins occur in many plants (Lis & Sharon, 1973) and many antisera are contaminated with antibodies to 'healthy' plant components (van Regenmortel, 1966). The most frequent contaminant in antisera is claimed to be antibodies to fraction 1 (F_1) plant protein, this protein being ubiquitous in the plant kingdom (Dorner et al., 1958). This problem illustrates the requirement for proper controls in any serological system. Healthy plant sap is frequently included in serological tests as a control. However the concentration of plant antigens in sap from diseased and healthy plants may differ significantly, higher levels of F_1 protein have been found in plants infected with grapevine fanleaf virus (Martelli & Hewitt, 1963) than uninfected plants. Because of this Wetter, (1965) recommends the inclusion of sap from a plant infected with another unrelated virus, as a control. Van Regenmortel (1966) warned that this practise may not however be entirely adequate as metabolic variations with different virus-host combinations have been observed. The use of antihost or 'healthy' antisera is widely used to ascertain whether virus antigen preparations are contaminated by plant antigens (van Regenmortel, 1966), providing an excellent control in serological systems.

Serological relationships between members of the cucumovirus group have been recorded (Lawson, 1967; Mink, 1969) but Mink (1975) considers these relationships are a result of antisera containing antibody mixtures, resulting from the use of mixed virus isolates as antigens. Using 'narrow spectrum' ascites fluid, prepared against single virus isolates, Mink (1975) observed no serological reactions between cucumovirus group members.

In this study dilutions of CMV purified preparations were used as antigens, healthy antisera, healthy sap and sap from plants infected with an unrelated virus were routinely included in serological tests as controls. The identity of CMV-D was confirmed by serological reactions to several CMV antisera in gel diffusion tests.

Comparisons between CMV-D and CMV-C (daphne common CMV) isolates were conducted using the two homologous and several other CMV antisera in gel double diffusion tests. CMV-D antiserum reacted against all isolates of CMV-C (a, b, c) and the homologous CMV-D without spur formation (Figure 15.1). However reciprocal tests using CMV-C antiserum resulted in spur formation (Figure 15.2) and indicate CMV-C possess antigenic determinants lacking in CMV-D. Three further CMV antisera (common strain CMV, Holland; nandina CMV, this laboratory; CMV, University California Davis) also produced similar precipitin reactions to those obtained against CMV-C and CMV-D antigens in adjacent wells (Figure 16.1). Rearrangement of the antigen wells, revealed that the CMV-C isolates were serologically equivalent (Figure 16.2).

The most frequent reactions in gel diffusion tests were bands curved around the antigen wells, indicating the serological reaction was between whole virus and antibodies (Scott, 1968). The buffer used in gel diffusion serological tests contained 5mM EDTA, hence CMV was stabilized and reactions between virus coat subunits and antibodies did not usually occur.

No serological precipitin reactions were observed between CMV-D antigens and antisera to peanut stunt and tomato aspermy viruses. Also CMV-D and CMV-C antisera did not react against tomato aspermy virus (a chrysanthemum isolate). This indicates that CMV-D and CMV-C antisera were of 'narrow spectrum' type (Mink, 1975) i.e. were not produced by immunization with antigen mixtures.

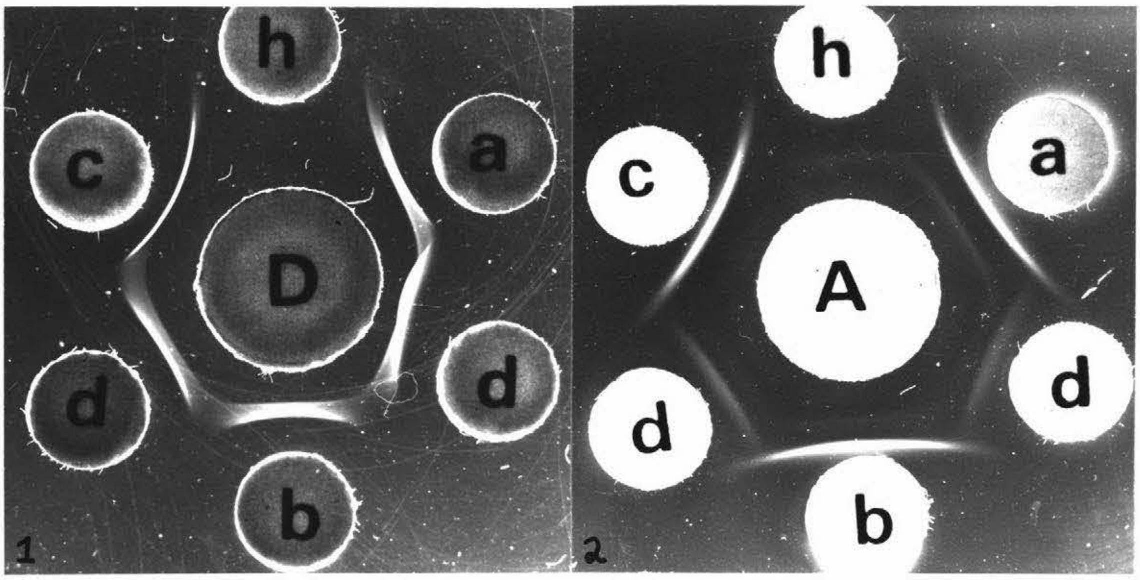

FIGURE 15. Serological comparison of CMV-C and CMV-D in gel double diffusion tests.

(1) CMV-D antiserum; fusion of precipitin lines formed by CMV-C and CMV-D isolates.

(2) CMV-C antiserum; precipitin spur formation (partial intersection) by CMV-C antigens over CMV-D antigens.

(antisera - centre well; D = CMV-D, A = CMV-C isolate a : antigens - outer wells; a, b and c = CMV-C isolates, d = CMV-D).

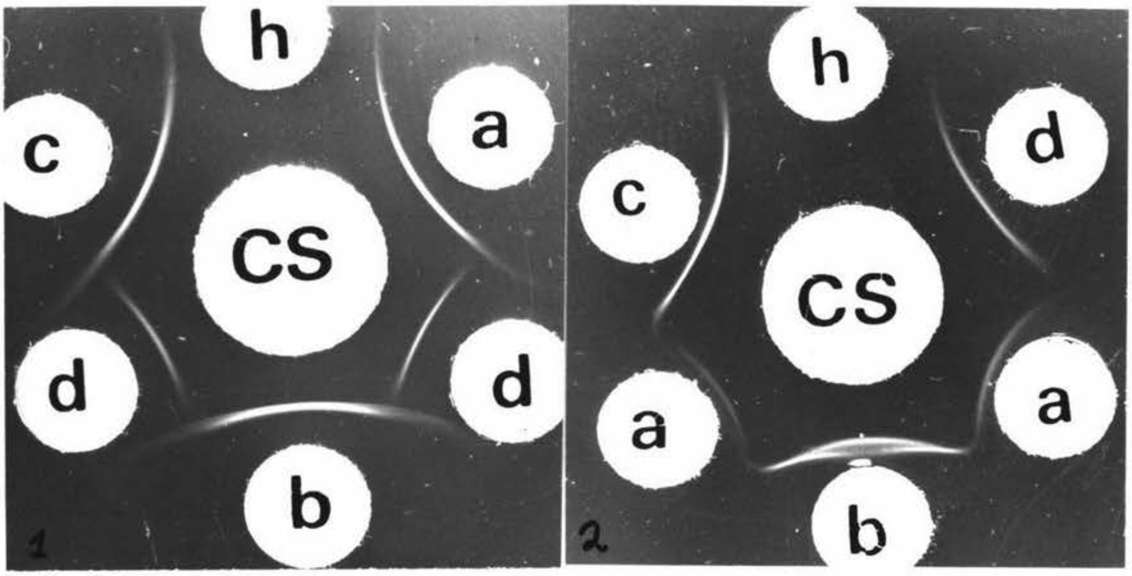

FIGURE 16. Serological comparison of CMV-C and CMV-D in gel double diffusion tests using CMV-CS (Holland) antiserum.

(1) and (2) different antigen arrangements.
(antisera - centre well; CS = (Holland) CMV-CS antiserum : antigens - outer wells; a, b and c = CMV-C isolates, d = CMV-D).

1.12 DISCUSSION

In all but two characteristics found in this study CMV-D exhibits properties conforming with CMV-C, which in turn possesses properties in general accord with other isolates of CMV. The two exceptional characteristics of CMV-D are its ability to systemically infect several hosts, which most CMV isolates do not and a serologically distinct capsid lacking some antigenic determinants.

Habili and Francki (1974b) used a virus 'hybrid' comprising CMV coat protein and TAV RNA to demonstrate that the ability of TAV to systemically infect several hosts (which CMV could not) is a genome function and is independent of the virus capsid. It would therefore be expected that the capability of CMV-D to infect several hosts systemically, is likewise a genome function i.e. CMV-D should have differences in its genome compared to CMV-C and other CMV isolates. In this study no such differences were detected in genome size of corresponding RNA species of CMV-D and CMV-C, reflecting that any differences, if they occur, are small or are sequential within the nucleotides. In contrast differences in molecular weight of corresponding RNA species have been detected between CMV and TAV (Habili & Francki, 1974 a). The simpler serological capsid or coat proteins of CMV-D are also likely to be reflected in minor genome differences. Only about one fifth of the virus genome in small RNA viruses is represented in the virus capsid and as only about one tenth of the virus capsid is exposed antigenically, it would appear serological tests involve expression of only ca. 2% of the virus genome (Fenner et al., 1974). Hence serological differences between viruses are likely to be reflected in even less of the virus genome.

As only two characteristic differences were detected with CMV-D after comprehensive characterisation and comparison with CMV-C, it is quite possible that these two characteristics may in some way be related. Such a relationship could be explained if the two characters occurred on the same RNA species. Schwinghamer and Symons (1975) demonstrated by translation of fractionated CMV RNA species that the coat protein and two other products (Mol. wt. ca. 27,000) were yielded from RNA 3, while RNA 4 only yielded the coat protein. CMV-D coat differences should therefore be coded on RNA 3 and perhaps the different systemic infection abilities are also coded on this RNA species.

Although this latter point is only speculative, a situation similar to this has been found with cowpea chlorotic mottle virus (CCMV) by Bancroft et al., (1972). With a temperature sensitive mutant and wild type isolates of CCMV functions have been assigned to different RNA species. CCMV is a member of the bromovirus group having 4 RNA species and therefore resembles cucumoviruses CMV and TAV. The coat proteins of CCMV and its ability to systemically infect several hosts were found coded for on RNA 3 (Bancroft et al., 1972).

CMV-D has been considered a specific virus strain of CMV because of characteristic differences observed when compared to common CMV isolates. It is unfortunate however that many isolates of CMV have been designated 'strain', without differentiation as such; these isolates should in fact be merely labelled as 'isolates'. The term 'strain' in virology is probably misleading in other respects because single infectious units are not used as in bacteriology but rather populations which must contain levels of heterogeneity.

CHAPTER 2

CHARACTERISATION OF DAPHNE CARNATION MOTTLE VIRUS

An isometric virus, tentatively designated daphne isometric virus-3 (DIV-3) by Forster and Milne, (1975), was isolated from Daphne x Burkwoodii Turrill 'Variegata'. Further isolates of this virus were obtained in this laboratory from Daphne odora Thunb. 'Rubra' by K.S. Milne. Several isolates from both Daphne species were selected in this study and following detailed characterisation DIV-3 was identified as carnation mottle virus. DIV-3 hereafter designated daphne-carnation mottle virus (D-CarMV)[1] could not be distinguished from carnation isolates of CarMV. The stability of CarMV and its ability to be transmitted mechanically on hands, required comprehensive proof of isolation from daphne and this is provided in the following studes.

CarMV (R/1 : 1.3/20 : S/S : S/*) has been tentatively included in the tombusvirus group (Harrison & Murant, 1970-74) which comprises turnip crinkle virus (TCV), tomato bushy stunt virus (TBSV, including; artichoke mottled crinkle, carnation Italian ringspot, pelargonium leaf curl and petunia asteroid mosaic strains) and tentatively CarMV, carnation ringspot virus (CarRV) and pelargonium flower-break virus (PFBV). Viruses in the tombusvirus group possess many common characteristics, but most notably have a single sedimenting virus component containing a single RNA species of about 1.5 million daltons; they are however not serologically related.

CarMV is an icosahedral virus ca. 28nm with a sedimentation coefficient ca. 122S (Hollings & Stone, 1970) containing 20% RNA of one molecular weight species (1.3 million daltons - Kaper & Waterworth, 1973).

Nucleotide base ratios of the CarMV genome are: guanine 27: adenine 30: cytosing 19: uracil 24 (Tremaine, 1970). The virion coat consists of ca. 240 subunits of molecular weight 26,200 each with

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[1] prefix D (daphne) is used because this virus is only an isolate of CarMV and not a distinctive strain.

ca. 243 amino acid residues (Tremaine, 1970). Physical properties of CarMV in crude sap include DEP of 10^{-5}, TIP of 90C and LIV of several months (Hollings & Stone, 1970). Transmission of CarMV by vectors, seed or dodder has not been found although the virus is readily mechanically transmitted by sap inoculation and on hands (Hollings & Stone, 1970).

CarMV was first recorded by Kassanis (1955) in carnation, and subsequently the virus has only been found naturally infecting this host. Infection of carnation by CarMV is very widespread, many commercial cultivars being wholly infected (Hollings & Stone, 1964).

2.1 MATERIALS AND METHODS

With the following additions the materials and methods used with D-CarMV are the same as outlined under section 1.1.

Electron microscopy of sectioned material

Ultrathin sections were prepared as follows: infected plant tissue was cut into 1x4mm strips and vacuum infiltrated in a cold aldehyde fixative (Karnovsky, 1965) (3% gluteraldehyde, 2% formaldehyde in 0.1M Na_2/KPO_4 buffer pH 7.2). These tissue pieces were washed in (0.1M Na_2/KPO_4, pH 7.2) buffer for 5-15min, and postfixed for 3-5h at 4C in 1% osmium tetroxide in buffer, washed and dehydrated in a graded ethanol series (25, 50, 75, 90 and 100%) and subsequently placed in two changes of propylene oxide. Tissue was then transferred through an ascending series of epoxy resin concentrations in propylene oxide (descending concentration) and finally embedded in 100% resin in gelatine capsules and cured for 2-3 days at 60C. Ultrathin sections were cut with an LKB ultratome and picked up on 200-mesh copper grids. They were then stained in saturated uranyl acetate in 50% ethanol for 3min, washed in 50% ethanol 30 seconds, then in distilled water and stained with lead citrate (Venable & Coggeshall, 1965) for 5-6min. Stained sections were thoroughly washed with distilled water and observed in the electron microscope.

Immunoelectrophoreses

Immunoelectrophoresis (Hollings & Stone, 1975) was conducted using microscope slides coated with a 1.5mm layer of 0.9% agar in one of the serological buffers HSB or TSB of, Hollings and Stone, (1975) Tomlinson et al., (1973). Two origin wells were punched on each slide with an LKB^R gel punch. Wells were filled with viral antigens and slides placed in an immunoelectrophoresis tank. The ends of each slide were connected by paper wicks to buffer channels through which platinum wire ran to opposing terminals. Buffer channels were filled with the same buffer as used in the medium coating the slides. Electrophoresis was carried out at 4C for periods of 1, 2, 3 and 4h using a constant voltage supply (Hewlett Packard^R) of 80V giving ca. 16V across each agar coated slide.

After electrophoresis a central strip of agar was cut and removed from between the two wells and the appropriate antiserum was placed in it. This was incubated for 24h at 25C in a humid chamber and subsequently examined for precipitin lines. The migration of antigens were recorded following Hollings and Stone, (1975).

2.2 PROOF OF ISOLATION FROM DAPHNE

The uniqueness and implications arising from finding CarMV in daphne are such that queries of doubts may arise. Consequently further experimentation with D-CarMV was required to prove its origin.

D-CarMV was consistently isolated by several different workers in this laboratory from different Daphne cultivars: P.R. Bennett, D. odora 'Leucanthe', R.L. Forster, D. x burkwoodii 'Variegata'; K.S. Milne, D. odora 'Rubra'; and the author D. x burkwoodii 'Variegata' and D. odora 'Rubra'.

Although initial inoculations of macerated daphne flower or leaf tissue to C. quinoa did not always produce symptoms, invariably back inoculation tests to further C. quinoa did reveal infection by D-CarMV. Direct inoculations to C. quinoa from daphne tissue produced either a few large 3-5mm local chlorotic spots or symptomless infection. Particular care was taken to ensure the detection of D-CarMV infection was not caused accidentally by contamination.

The repetitive isolation of D-CarMV from daphne after special precautions involving double autoclaving of all inoculation instruments and media, the use of isolated glasshouse space and extra care when inoculating, indicates that the virus came from daphne. Furthermore, no problems of CarMV contamination occurred while three workers in this laboratory experimented with at least 10 other viruses.

An experiment was conducted to demonstrate the presence of D-CarMV in daphne. Leaf and flower tissue was harvested separately from D. burkwoodii 'Variegata'. Flower tissue was largely obtained from a New Plymouth nursery[1] and leaf tissue from home gardens.. The material from the two harvests was used to purify any viruses present, following a procedure similar to that used for CMV-D.

The purified preparations were negatively stained with AM and examined in the electron microscope, and in preparations from both leaf and flower tissue small isometric and rod shaped particles were observed. The rod virus was apparently daphne virus-S (Forster & Milne, 1975), while the identity of the sphere could not be resolved by electron microscopy. Only 5-10 particles were observed in each droplet impacted on the electron microscope grid, there being probably 30 or 40 droplets on the grid. In gel diffusion tests specific serological reactions were observed against D-CarMV and CarMV (VPRI) antisera, while no reactions were observed against 'healthy' antiserum. Preparations were also inoculated to C. quinoa where they produced characteristic CarMV symptoms. Sap from these plants gave a positive reaction against CarMV antiserum.

The confirmed presence of CarMV in these preparations along with the consistency of isolation is presented as proof of isolation of CarMV from daphne.

Similar difficulties have been reported which revealed subliminal infections of tomato mosaic virus (TMV) in a number of hosts including: apple (Gilmer & Wilks, 1967; Kirkpatrick & Linder, 1964), cotton (Cheo, 1970), tulip (Mokra et al., 1973) and white ash (Nana & Agrios, 1974). Although a different virus TMV is similar to CarMV in a number of aspects, particularly stability, transmission by handling and lack of known arthropod, nematode or fungal vectors.

CarMV infected carnation usually contains a high concentration of virions, apparent on electron microscopic examination, and dilution end-point infectivity tests. However low levels of CarMV have been found in heat treated carnations (Hollings & Stone, 1964) and have been called 'attenuated' CarMV. Poupet et al., (1972) observed similar low levels of apparently 'attenuated' CarMV, but these carnations had not been subjected to heat treatment. The infections were not detectable serologically and isolation was only achieved after several serial back inoculations to C. quinoa from the primary host. Characteristics

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[1] Duncan and Davies Ltd.

similar to those of 'attenuated' CarMV were observed with D-CarMV in daphne.

2.3 TRANSMISSION FROM DAPHNE

Experiments were conducted to improve the transmission of D-CarMV from daphne. Several primary inoculation hosts were tried but C. quinoa, C. quinoa Variant[1] and S. vaccaria were found to be most reliable, with S. vaccaria being apparently the most sensitive to infection although displaying less distinctive symptoms. In one experiment a whole range of additives to the 0.01M K-K_2 phosphate, pH 7.0 buffer were tested. Several batches of daphne flower tissue were used as inoculum and assayed on C. quinoa. The following additives were tested separately and in several combinations: 0.5% bentonite, 1% caffeine, 0.01M Dieca, 1mM EDTA, 1% mercaptoethanol, 1% nicotine, 10% phenol, 1% PEG, 1% polyvinyl pyrollidone, 0.1% TGA. Only inoculum in the bentonite - phosphate buffer produced chlorotic local lesions directly on the primary inoculation host (C. quinoa). The other additives and combinations allowed transmission to the primary host but the symptomless infections had to be verified by transmission to a further C. quinoa.

Daphne flower tissue or young leaf tissue was preferred to older leaf tissue for isolating D-CarMV. Direct inoculation using cut daphne leaf surfaces and rubbing them on C. quinoa leaves did not result in any symptoms, although back inoculation tests again revealed the presence of D-CarMV. The transmission difficulties with D-CarMV from daphne further illustrate the low infection levels present and emphasises the care that must be taken when indexing plants for 'high-health' programmes.

2.4 HOST RANGE

Two isolates of D-CarMV, from D. odora 'Rubra' and D. x burkwoodii 'Variegata' respectively, were inoculated to species from 18 families. Inoculum comprised D-CarMV infected leaves of C. quinoa, N. clevelandii, or G. elegans macerated in Yarwood's solution plus celite. Both isolates produced very similar host reactions excepting that D. odora 'Rubra' isolate produced somewhat more severe symptoms compared to D. x burkwoodii

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[1] sensitive strain selected on basis of leaf shape.

'Variegata' isolate.

AIZOACEAE

Tetragonia tetragonoides: local chlorotic blotches occassionally necrotic local lesions; systemic mottling.

AMARANTHACEAE

Amaranthus caudatus: 'Love Lies Bleeding': symptomless local infection.

Gomphrena globosa 'Little Buddy': local chlorotic blotches; no systemic infection detected.

CAMPANULACEAE

Lobelia erinus L.: symptomless local and systemic infection, readily recovered on back inoculation to C. quinoa.

CARYOPHYLLACEAE

Dianthus barbatus. 'Indian Carpet': infrequent local symptomless infection.

Dianthus chinensis. 'Bravo': local veinal chlorosis; systemic infection symptomless. Virus readily recovered on back inoculation to C. quinoa.

Gypsophila elegans: local and systemic veinal chlorosis, high virus concentration recoverable.

Saponaria vaccaria. 'Pink Beauty': local and systemic veinal chlorosis and leaf curling, high virus concentration recoverable.

CHENOPODIACEAE

Chenopodium amaranticolor: 1mm local chlorotic lesions (5 days); no systemic infection.

Chenopodium quinoa: 1mm chlorotic local lesions (4 days); systemic chlorotic flecks and blotches (14 days) with stunting and leaf curling (Figure 17).

Spinacia oleracea 'Royal Denmark': local chlorotic mosaic; symptomless systemic infection.

COMPOSITAE

Calendula officinalis: symptomless local and systemic infection.

Callistephus chinensis (aster): local symptomless infection; systemic mottle.

Helianthus annus (Sunflower): symptomless local and systemic infection.

Lactuca sativa 'Calmar': symptomless local and systemic infection.

Zinnia elegans 'Cactus Flowered': local and systemic mottling.

CUCURBITACEAE

Cucumis sativus 'Marketer' and 'Polaris': local chlorosis; systemic blotches and veinal chlorosis.

Cucurbita pepo 'Small Sugar': local chlorotic blotches; systemic mottling.

Momordica balsamina: symptomless local and systemic infection.

LABIATAE

Ocimum basilicum: symptomless local and systemic infection.

LEGUMINOSAE

Cassia occidentalis L.: few local necrotic lesions or symptomless infection; systemic mottle, chlorotic fleck and occasionally necrotic line patterns.

Dolichos biflorus: few local 1mm chocolate colored lesions; symptomless systemic infection.

Phaseolus vulgaris 'Top Crop': local chlorotic mottle; systemic chlorotic and necrotic flecking or symptomless infection.

Pisum sativum 'Greenfeast': local and systemic mottling or symptomless infection.

Vicia faba 'Coles Early Dwarf': 1mm chocolate local lesions; systemic mottle and margin necrosis.

Vigna unguiculata subsp. cylindrica: 1mm chocolate colored local lesions (4 days) or symptomless local infection; systemic infection symptomless.

Vigna unguiculata subsp. unguiculata: 1-2mm chlorotic local lesions later necrotic; systemic veinal chlorosis or symptomless infection.

SCROPHURLARIACEAE

Digitalis purpurea L. (foxglove): symptomless local and systemic infection.

SOLANACEAE

Datura stramonium: local chlorosis; systemic mottle or symptomless infection.

Lycopersicon esculentum 'Money Maker' local chlorotic blotches; systemic chlorotic flecking.

Nicotiana clevelandii: irregular chlorotic local lesions, dark brown on aging; systemic veinal chlorosis and mottle.

Nicotiana debneyi: local and systemic symptomless infection.

Nicotiana glutinosa: local chlorosis; systemic infection detectable, symptomless.

Nicotiana glutinosa x *Nicotiana clevelandii*: faint chlorotic local lesions; systemic mottle.

Nicotiana rustica: local chlorosis; systemic chlorotic blotches or symptomless infection.

Nicotiana tabacum 'Havana', 'Samsun', 'White Burley': faint local chlorotic blotches or symptomless local infection; systemic infection symptomless, just detectable on back inoculation to *C. quinoa*.

Petunia hybrida 'Rose of Heaven': chlorotic local lesions; systemic mild mottle and chlorotic flecking or symptomless infection.

Physalis franchetii: local and systemic symptomless infection.

TROPAEOLACEAE

Tropaeolum majus L. (nasturtium): local veinal necrosis; systemic mottle and chlorotic fleck.

VERBENACEAE

Verbena hybrida Voss.: Local and systemic necrotic fleck and leaf curling.

Infection of the following hosts were not detected:

Antirrhinum majus, *Apium graveolens*, *Arabis* sp., *Brassica oleraceae* var *botrytis* or var *capitata*, *Brassica pekinensis*, *Primula malacoides*, *Tithonia speciosa*, *Torenia fournieri* and *Vinca rosea*.

FIGURE 17. Chenopodium quinoa infected with D-CarMV. Systemic chlorotic flecking, leaf curling and deformation (16 days).

These results reveal D-CarMV has the ability to infect a diverse range of plants, although many are only infected locally. Back inoculations to C. quinoa demonstrated very low levels of D-Car MV in many of the systemically infected hosts, but particularly those in the compositae, cucurbitaceae, leguminosae and solanaceae.

The host range of D-CarMV appears more extensive than that of CarMV but the diagnostic species for CarMV - Chenopodium amaranticolor, Chenopodium quinoa, Dianthus chinensis, Gomphrena globosa, Nicotiana clevelandii and Tetragonia tetragonoides - all reacted to D-CarMV in accordance with reports on CarMV (Hollings & Stone, 1964; Kowalska, 1972; Smith, 1972).

Because CarMV can be readily transmitted by handling infected plant material, particular care was taken with D-CarMV to ensure contamination did not occur with infection-verification tests i.e. back inoculations to C. quinoa.

The back inoculation tests to C. quinoa frequently resulted in a few (3-10) chlorotic local lesions hence further back inoculations were made from the primary C. quinoa to verify CarMV infection. Poupet et al., (1972) observed a similar situation with an apparent 'attenuated' strain of CarMV, but low levels of virus infection in primary inoculated C. quinoa, N. tabacum 'Samsun', 'Xanthi', N. glutinosa and Saponaria vaccaria were difficult to detect. Infections could only be verified by back inoculation to C. quinoa because of lack of symptoms and the unreliability of serological tests.

2.5 VECTOR TRANSMISSION

No aphid transmission of D-CarMV was achieved in three experiments, two using M. persicae, and one M. euphorbiae. Aphids were allowed to probe for 20 seconds on detached S. oleracea (spinach) and transferred to, two plants each of C. quinoa, Gypsophila elegans, Saponaria vaccaria and S. oleracea (10 aphids/plant). All plants were back inoculated to C. quinoa after 2 and 4 weeks. The back inoculation tests were all negative, indicating no aphid transmission.

These results are consistent with that reported for CarMV, no natural vector having been reported (Hollings & Stone, 1970).

2.6 PHYSICAL PROTERTIES IN CRUDE SAP

The TIP of D-CarMV in crude sap, determined from infected

C. quinoa leaves assayed on C. quinoa, was 90-95C. A DEP of ca. 10^{-6} was obtained from both locally infected C. quinoa and Saponaria vaccaria assayed on C. quinoa. In carnation, CarMV has a reported TIP of 90C and DEP of 10^{-5} (Hollings & Stone, 1970).

2.7 ELECTRON MICROSCOPY

Negative staining

In D-CarMV infected daphne leaf or flower tissue, isometric particles were extremely difficult to resolve with the electron microscope. Because of the extremely heterogeneous nature of such negatively stained squash homogenates. However D-CarMV infected C. quinoa, G. elegans, S. vaccaria and N. clevelandii tissue, when similarly prepared and viewed in the electron microscope, revealed numerous isometric particles frequently in closely associated arrays (Figures 18 & 19). The high concentrations of virus particles in negatively stained infected tissue is quite characteristic for CarMV (Robleda, 1973) and other Tombusvirus group members (Martelli et al., 1971).

Virus particles were stable in PTA stains with a pH range from 4 to 7 and in AM pH5.3, but the negative stain mixture AM-PTA (1:1) was preferred because of the combination of good virus contrast and particle dispersion provided. Purified preparations of CarMV revealed high concentrations of isometric particles, where AM alone was used as a negative stain because of the satisfactory results obtained.

D-CarMV isometric particles had an average diameter of ca. 30nm (50 particles). The reported particle size for CarMV is ca. 28nm (Hollings & Stone, 1970).

Ultrathin sections

Ultrathin sections of both locally and systemically D-CarMV infected C. quinoa tissue was examined with the electron microscope. The cytoplasm of infected cells contained a great many virus particles randomly distributed around cell organelles; chloroplasts and mitochondria. The tonoplast was frequently broken and numerous virus particles could be detected in the vacuole (Figure 20a). Vesicles containing virus particles bulging into the vacuole through the tonoplast were observed (Figure 21a) and detached vesicular sacs containing virions could be found free within the vacuole (Figure 21b). Larger protrusions containing arrays of virus particles were also observed (Figure 20b). Similar effects were seen in sectioned D-CarMV infected N. clevelandii and S. vaccaria.

Verification that the isometric particles observed in thin sections were virus particles was obtained by their size (ca. 29nm), and similarity to particles reported in material infected with other isometric viruses (De Zoeten & Gaard, 1969; Martelli & Russo, 1972; Rubio-Huertos & Garcia Hidalgo, 1971) and the absence of such particles in healthy C. quinoa tissue. Virions were also readily differentiated from cellular ribosomes by the size and uniformity of staining (Figure 20a).

Similar results for CarMV have been reported by Robleda, (1973) who found numerous particles in the cytoplasm, vacuole and within nuclei of vascular tissue in; Atriplex hortensi L., Chenopodium spp. and Dianthus spp. Bennett, (1956) and Schneider and Worley, (1959) suggest the presence of virus particles in the vascular tissue indicates long distance translation of small isometric viruses is achieved through vascular tissues.

The presence of vesicles containing virus particles at the tonoplast has been reported for tomato bushy stunt viruses (Russo et al., 1968; Russo & Martelli, 1972). The function of these vesicles has been proposed as mechanisms for virus disposal beyond the membrane system of the cell, (Russo et al., 1968). When advance stages of infection are reached the virus particles tend to occupy all the available space in the cell and this 'pressure' created possibly results in the cell disposing of virions by vesicular egression into the vacuole.

It is unlikely that the observed vacuolar localization of CarMV is an artifact resulting from tonoplast damage because of the discovery of whole vesicles in the vacuole containing virons. Furthermore, the virus particles were not just randomly scattered and the tonoplast was still intact in most cases.

With tomato bushy stunt virus, particles are frequently found accumulated in the cell nuclei (Russo & Martelli, 1972) an effect observed by Robleda (1973) for CarMV. Some particles of D-CarMV were observed associated with cell nuclei within the nuclear membrane. Members of the tombusvirus group in which CarMV is tentatively included, appear to display similar effects on plant ultrastructure and virus localization.

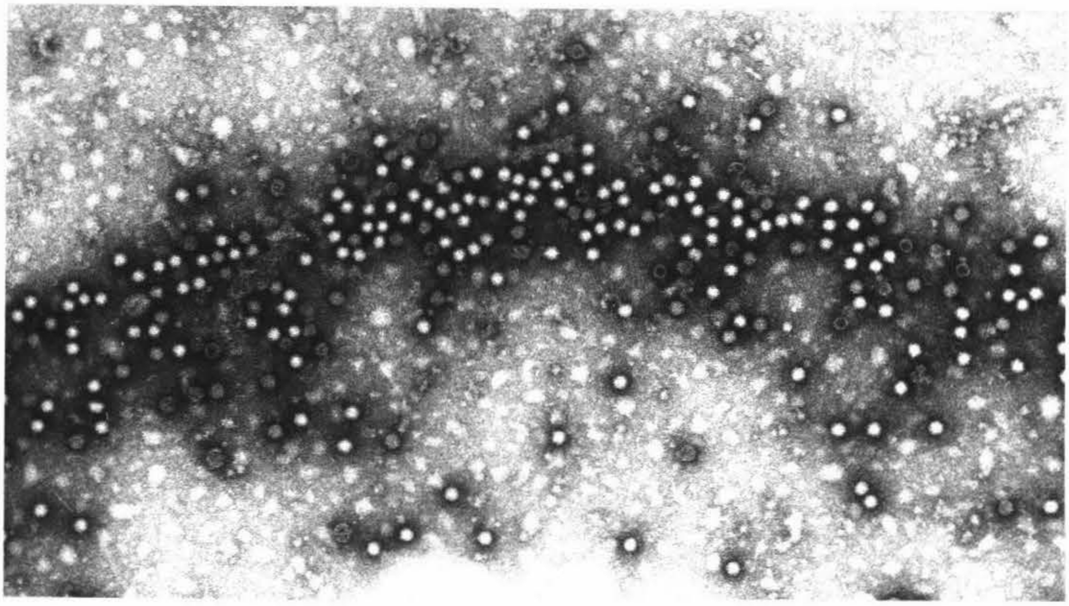


FIGURE 18. Isometric particles of D-CarMV in a squash homogenate from locally infected *Chenopodium quinoa*, negatively stained with AM-PTA. Mag. 55,000.

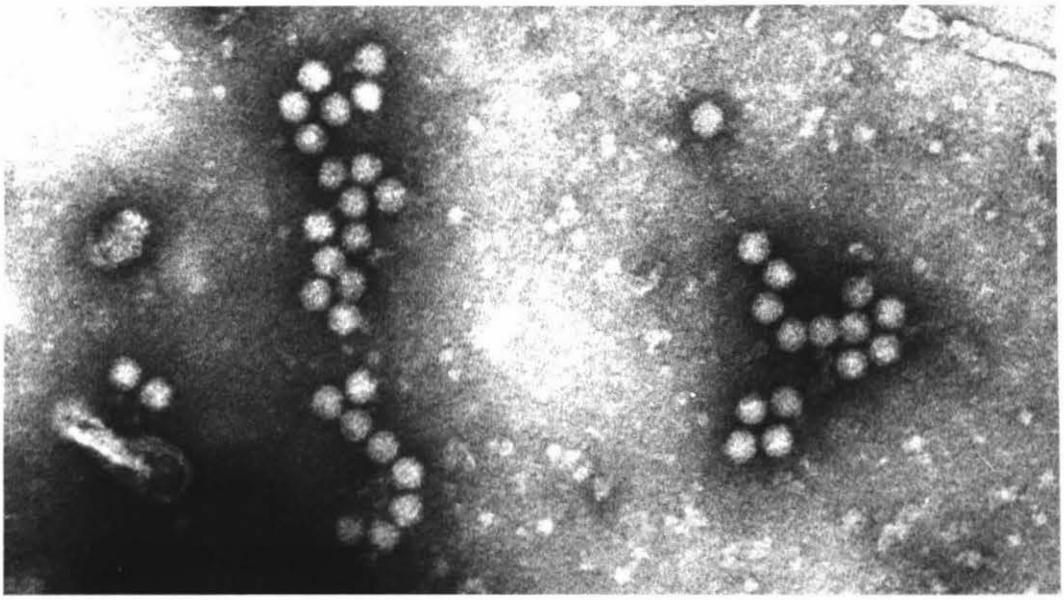
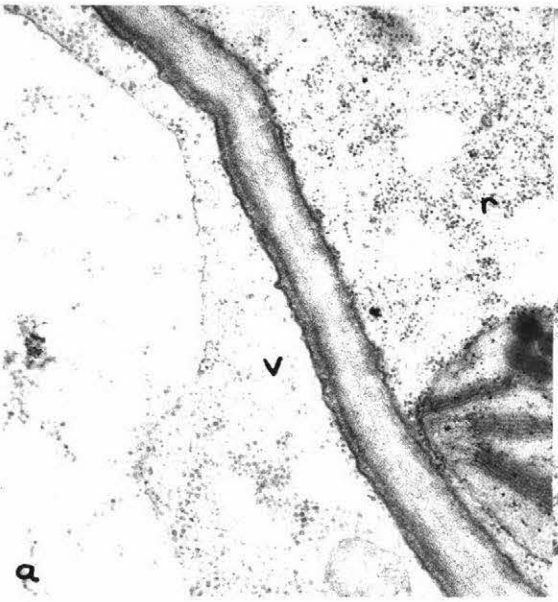


FIGURE 19. Aggregates of D-CarMV particles in a squash homogenate from locally infected *Nicotiana clevelandii*, negatively stained with AM-PTA. Mag. 166,500.



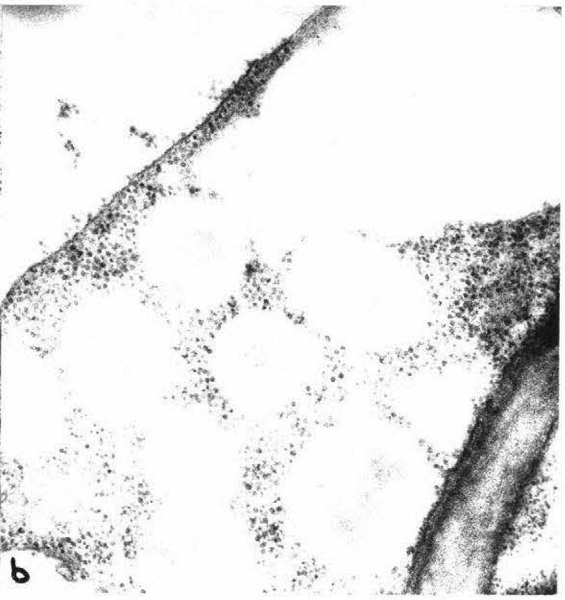
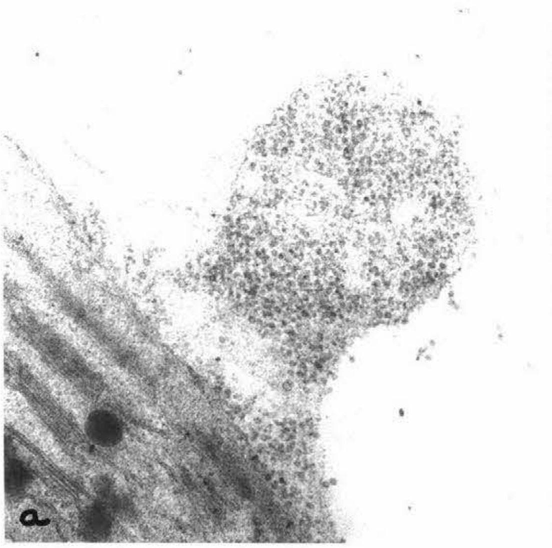


FIGURE 20a Differentiation of virus particles and ribosomes in thin sectioned D-CarMV infected *Chenopodium quinoa* leaf tissue. Note virus particles (v) near cell wall and irregular darkly stained ribosomes (r). Mag. 18,500.

FIGURE 20b An array of D-CarMV particles protruding into the cell vacuole of *Chenopodium quinoa* leaf mesophyll cell. Mag. 18,500.



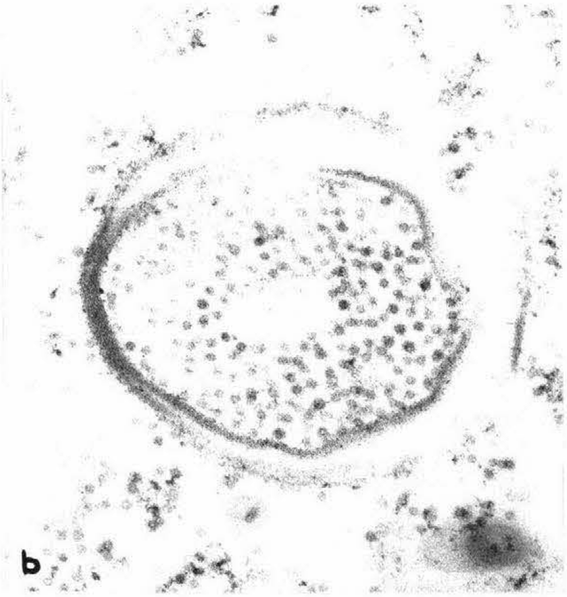


FIGURE 21a A vesicle containing D-CarMV particles, protruding into the vacuole of a *Chenopodium quinoa* mesophyll cell. Mag. 22,000.

FIGURE 21b A membrane-bound vesicle, containing virus particles of D-CarMV, within a *Chenopodium quinoa* leaf mesophyll cell vacuole. Mag. 23,000.

2.8 PURIFICATION

The high levels of CarMV infection observed in several hosts and the viruses relative stability (Hollings & Stone, 1970) enable the purification and concentration of large amounts of virus quite readily.

A procedure similar to the schedule of Hollings and Stone, (1964) was used to purify D-CarMV. Ten to 15 old CarMV infected (local & systemic) *C. quinoa* or *N. clevelandii* leaves were harvested and homogenized with 0.5M K-K$_2$ phosphate pH7.5 buffer (1.5ml : 1 gm tissue) including the additives 0.1% TGA and 1mM EDTA. After expression of the sap-buffer homogenate through cheesecloth emusification overnight with 8.5% n-butanol at 4C was carried out. The emulsion was then broken by low speed centrifugation (12,000g/10min) and the supernatant retained after filtering through glass wool. The supernatant was then subjected to ultracentrifügation (100,000g/70min) or PEG precipitation overnight (6% PEG + 0.15M NaCl) and low speed centrifugation to pellet the virus. The pellet was resuspended in 40ml 0.05M K-K$_2$ phosphate and 0.5mM EDTA buffer pH7.5 for 1h at 4C and then subjected to a further cycle of differential centrifugation. In the final centrifugation cycle D-CarMV was pelleted (100,000g/80min) through a 5ml 'cushion' of 20% sucrose to remove additional contaminating plant components. The final purified virus pellet was washed with distilled water and resuspended in 1mM EDTA pH5.0 (1ml/100g infected tissue) overnight at 4C. The period of ultracentrifugation (70-80min) was determined similarly to CMV-D (section 1-7) excepting that a sedimentation coefficient $122X10^{-13}$ sec^{-1} was used for CarMV-D.

As this isolate of CarMV reached a high concentration in *N. clevelandii* purified virus preparations were generally less contaminated by plant components this host was preferred to *C. quinoa* as a virus source for purification. *N. clevelandii* D-CarMV purified preparations contained fewer F$_1$ protein particles when viewed in the electron microscope and did not react against 'healthy' antisera when diluted 1/2. Hollings and Stone, (1975) also recommend *N. clevelandii* for the purification of another tombusvirus, tomato bushy stunt for the same reasons.

Butanol clarification was used in preference to chloroform on the recommendation of Demaire and Kummert, (1969) who observed, by spectrophotometry cleaner virus preparations. Preparations of D-CarMV obtained by differential centrifugation were generally superior to PEG preparations because of the lower levels of contamination.

2.9 PHYSICAL PROPERTIES OF PURIFIED VIRUS

Sedimentation coefficient

Three separate analytical centrifuge runs each revealed a single homogeneous sedimenting virus peak (Figure 22) for D-CarMV purified preparations with sedimentation coefficients (uncorrected for virus concentration) of 126, 124 and 130 S respectively. The sedimentation coefficient for D-CarMV was thus determined as $S = 120 \times 10^{-13}$ sec^{-1} or $120S^1$. This value is in general agreement with that reported for CarMV : 122S (Hollings & Stone, 1970).

UV Absorption spectrum

Preparations of purified D-CarMV had the following UV absorption spectral characteristics: A max and A min occurring at 260 and 240nm respectively, A max/min = 1.29, A260/280 = 1.59. These values are consistent with that reported for CarMV (Demaire & Kummert, 1969) and other tombusvirus group members e.g. tomato bushy stunt A260/280 = 1.62-1.66 (Martelli et al., 1971).

Summary of Physical properties

Purified preparations of D-CarMV yielded ca. 500mg virus/kg tissue harvested, had an infectivity dilution end-point of ca. 10^{-7} and maintained infectivity for several weeks. Preparations did not react against 'healthy' antisera but reacted strongly against CarMV antisera at dilutions down to 1/1000. Examination of negatively stained preparations in the electron microscope particles ca. 29nm with little contamination by F_1 plant protein particles. Preparations examined in the analytical centrifuge had a single homogeneous sedimenting virus peak with a sedimentation coefficient of ca. 120 S (Figure 22). Spectrophotometry revealed a characteristic absorption spectrum with values of A 260/280 = 1.59 and A max/min = 1.26.

2.10 CHEMICAL COMPOSITION

SDS-Polyacrylamide gel electrophoresis of isolated viral RNA

RNA extracted from D-CarMV purified preparation was electrophoresed in SDS polyacrylamide agarose gels to determine the number of

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[1] corrected for zero virus concentration

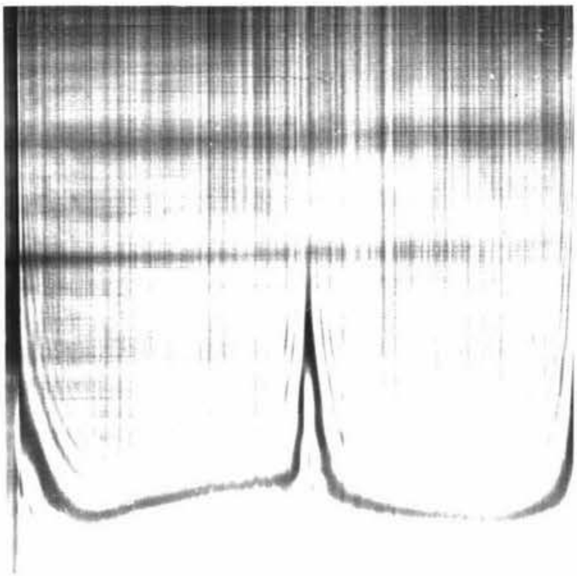

FIGURE 22. Analytical sedimentation pattern, for purified preparation of D-CarMV resuspended in 1mM EDTA pH5.0. The single virus peak is sedimenting from left to right. Schlieren angle 45°, photograph taken after 4 minutes at 35,600rpm.

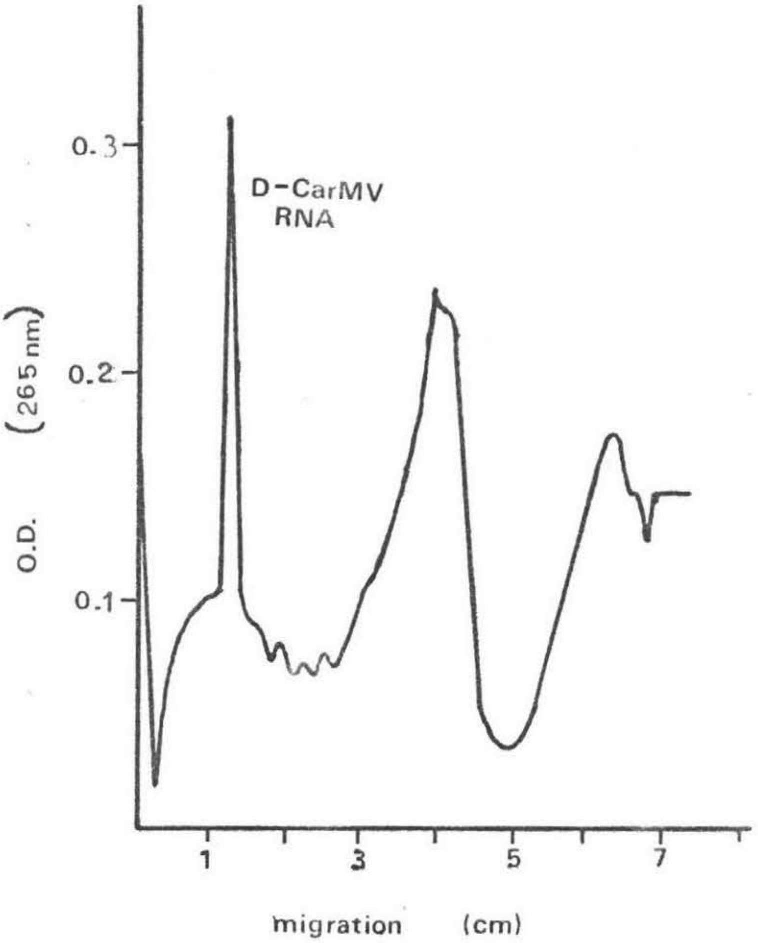

FIGURE 23. Densitogram showing a single D-CarMV RNA species, separated by polyacrylamide gel electrophoresis. Following the virus peak is a broad peak of heterogeneous RNA probably of plant origin. Gels scanned at 265nm in a Joyce-Loebl Chromoscan.

RNA species and their respective molecular weights, that this virus encapsulates. Polyacrylamide gels of 2.0 and 2.4% were used in the elucidation of D-CarMV RNA species along with a period of 90min electrophoresis. Several RNA standards were included and a standard curve similar to Figure 13 was constructed from which D-CarMV RNA molecular weights could be determined.

Tremaine (1970) determined the RNA or genome content of CarMV as 19.7%, thus a RNA molecular weight of ca. 1.31-1.52 million daltons can be calculated. A genome, comprising a single RNA species was determined for CarMV by Kaper and Waterworth (1973) it constituted c.a. 18% of the particle weight. Two empirical methods were used to determine the single RNA species molecular weight: sedimentation velocity ultracentrifugation; and polyacrylamide gel electrophoresis. Using these, values of 1.37 and 1.33 million daltons were obtained for the respective methods.

A single RNA species was revealed for D-CarMV extracted RNA on electrophoresis in SDS-polyacrylamide agarose gels (Figure 23). Values of 1.51 and 1.38 x 10^6 daltons were obtained in two separate experiments, for the single RNA species molecular weight. A large heterogeneous (broad) peak was also observed on the scanned gel traces (Figure 23), this was found to correspond with a similar broad peak obtained with RNA extracted from partially purified 'healthy' plant tissue. It probably comprised of a mixture of partially degraded or RNA fragments of plant origin.

The results obtained with D-CarMV reveal good correlation with that reported for CarMV (Kaper & Waterworth, 1973). The characteristic single RNA species (c.a. 1.5 million daltons) of CarMV is typical for other tombusviruses (Martelli et al., 1971) distinguishing this virus group from many others.

2.11 IMMUNOLOGY

Unlike CMV, CarMV is a good immunogen, (Hollings & Stone, 1970) a result largely attributable to the virus high stability (Hollings & Stone, 1964) and therefore specific and high titer antisera are easily prepared.

Antiserum to D-CarMV was prepared by a series of three intramuscular injections and two intravenous injections at weekly intervals before bleeding the rabbit. A dilution of 1/30-1/60 was determined as most suitable for gel diffusion tests. No reaction against concentrated 'healthy' preparation was observed with D-CarMV antiserum when diluted 1/2 or lower.

2.12 SEROLOGY

D-CarMV identity was confirmed by serological reactions against CarMV antisera in Ouchterlony gel diffusion tests (Crowle, 1973). Purified D-CarMV reacted producing a single curved precipitin line against the CarMV VPRI[1] antiserum. No reactions were observed between the antiserum and healthy plant sap, a concentrated 'healthy' preparation or plant sap infected separately by arabis mosaic virus, CMV, and tobacco ringspot virus. Serological reactions were also obtained using N. clevelandii plant sap infected respectively with D-CarMV and a carnation isolate of CarMV. No serological reactions were observed with other antisera to tombusviruses including: carnation ringspot virus, and three antisera to tomato bushy stunt virus (PLCV-232, TBSV-GCRI, TBSV-type).

A comparison between daphne and carnation isolates of CarMV was conducted by gel diffusion and immunoelectrophoresis tests using: two isolates of D-CarMV; two carnation CarMV isolates; antiserum prepared against D-CarMV and CarMV VPRI antisera. Appropriate controls, similar to those used in the carMV identification tests, were included in these serological comparison tests. The gel diffusion tests revealed that D-CarMV and CarMV-carnation isolates were serologically complementary, fusion of curved precipitatin lines resulting with both antisera. Similarly immunoelectrophoresis tests revealed the antigens were complementary, a single antigen component was found for D-CarMV and CarMV-carnation. However the D-CarMV isolates or antigens did migrate slightly further than the CarMV-carnation antigens

D-CarMV was serologically indistinguishable from carnation isolates of CarMV although strains of CarMV have been differentiated serologically (Kemp & Fazekas, 1966). The 'attenuated' form is however indistinguishable from the 'type' strain (Hollings & Stone, 1970). D-CarMV is similar to the 'attenuated' form of CarMV, with very low infection levels in its natural host, and as a consequence D-CarMV could perhaps be expected to be serologically similar to common carnation isolates of the virus.

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[1] VPRI abbreviation 'Victoria Plant Research Institute'

2.13 DISCUSSION

The virus D-CarMV isolated from daphne, was identified as a tombusvirus on several characteristics: limited host infection; high stability in crude sap; a single sedimenting virus component of ca. 120S; and a single RNA species of ca. 1.3 million daltons. The final identity of D-CarMV was confirmed by serological reactions with CarMV VPRI antiserum and the reaction of D-CarMV antiserum against carnation isolates of CarMV. Further interpretation of D-CarMV host reactions added to this identification.

The uniqueness of this report, the isolation of CarMV from a host other than carnation, required comprehensive proof of isolation from daphne. This proof was demonstrated by way of direct purification of D-CarMV from daphne leaf and flower tissue separately.

The infection of daphne by D-CarMV appears to involve low virus concentrations, because little D-CarMV was recovered by direct purification, and the virus was difficult to isolate to C. quinoa. Similar low infection levels have been observed by Hollings & Stone, (1964) with heat treated carnations, 'attenuated' CarMV being barely detectable. Likewise Poupet et al., (1972) observed low infection levels of apparent 'attenuated' CarMV in mediterranean carnations after extensive serial back inoculation programmes.

Thus low infection levels of CarMV and the difficulties involved in isolation as experienced by Poupet et al., (1972) suggest wider natural infection of carnations and possibly other hosts may be expected. This situation appears to occur in daphne, subsequent to the initial isolation of CarMV from D. x burkwoodii 'Variegata' this virus has been isolated from a further two cultivars and isolation from even more cultivars is likely.

The difficulties involved in isolation of D-CarMV relate to the requirement of back inoculation tests on primary inoculated hosts. This practise could easily be overlooked or treated lightly and thus the virus could remain undetected. Difficulties similar to those experienced here have occurred in proving TMV infection in several woody hosts.

Symptomless infection of CarMV in both hosts and the ability of the virus to be transmitted between carnations by handling, a feature which may occur in daphne, insures wide host infection. Clean stock programs would require heat treatment and/or tissue culture to remove

CarMV infection. Unfortunately problems with cleaning up carnations by these techniques have met with a number of difficulties (Hollings & Stone, 1964).

The problems involved in indexing daphne for viral infection become self evident after this study. Indexing for CarMV could not be done by way of direct electron microscope examination and mechanical transmission tests would require a comprehensive programme of serial back inoculations. Perhaps direct serological testing could be performed, but this seems unlikely after the experiences of Poupet et al., (1972) who found low infection levels of CarMV were not serologically detectable in carnation.

CHAPTER 3

CHARACTERISATION OF DAPHNE LATENT RINGSPOT VIRUS

A virus or 'virus-like' agent was isolated from Daphne odora 'Leucanthe Variegata' by Forster, (1974). After the determination of isometric virus particles earlier in the present study the virus was tentatively named daphne isometric virus-2 (DIV-2) by Forster and Milne, (1975). Three isolates of this virus were selected and subjected to characterisation. On the basis of several characteristics, including: host symptomatology; seed transmission; and multiple sedimenting components the virus was renamed daphne latent ringspot virus (DLRV) (*/* : */* : S/S : S/*) and is considered a probable member of the nepovirus group (Harrison & Murant, 1970-1974). Proof that DLRV is a new virus was not obtained in this study. However many viruses, which have some properties in common with DLRV, were eliminated because of the distinctive characteristics revealed by DLRV.

3.1 MATERIALS AND METHODS

The materials and methods used in the characterisation of DLRV are the same as outlined under section 1.1 with one exception: isolation and analysis of DLRV RNA was not carried out, but an additional tests, seed transmission, was included.

Seed transmission

Two DLRV-systemically infected C. quinoa plants were allowed to produce seed and this was collected after drying-off. The seed was planted out and 3 week old seedlings were macerated, either singly or in groups of 10, inoculated to C. quinoa and any infection noted.

3.2 TRANSMISSION FROM DAPHNE

DLRV was isolated from a single daphne cultivar D. odora 'Leucanthe Variegata'. No symptoms could be attributed to infection by this virus. The virus could be isolated from either flower or young leaf tissue macerated in Yarwood's buffer and celite and inoculated to C. quinoa. Separation of DLRV from CMV-D, which was also found infecting this daphne cultivar, was achieved by systemic passage of DLRV through C. amaranticolor. In this host CMV-D infection was localised.

DLRV was easily and consistently isolated using the above procedure. Three isolates were selected for characterisation two being obtained by the procedure outlined and a third was an original isolate from R.L. Forster.

3.3 HOST RANGE

Three isolates of DLRV were sap inoculated to species from 13 families. Inoculum comprised DLRV-infected C. quinoa or N. Clevelandii leaves macerated in Yarwood's solution plus celite. All isolates produced very similar host reactions.

AMARANTHACEAE

Amaranthus caudatus 'Love Lies Bleeding': local chlorosis followed by chlorosis and stunting of the whole plant with leaf epinasty; systemic white flecks and tip necrosis.

Celosia argentia 'Forest Fire': local chlorosis occasionally 2-3mm red ring spot local lesions; systemic mosaic with tip necrosis and epinasty of laterals.

Gomphrena globosa 'Little Buddy': symptomless local and systemic infection.

CARYOPHYLLACEAE

Gypsophila elegans: local and systemic veinal streaking and irregular shaped ringspot lesions (Figure 24).

Saponaria vaccaria 'Pink Beauty': local and systemic veinal chlorosis and necrosis (Figure 25).

CHENOPODIACEAE

Beta vulgaris 'Yates early Wonder': local 1-2mm red lesions; systemic mosaic.

Chenopodium amaranticolor: 1mm chlorotic local lesions (4-6 days); systemic chlorotic flecking and mosaic with leaf reflexing (Figure 27).

Chenopodium quinoa: 1-2mm chlorotic local lesions or 2-3mm chlorotic ring lesions (3-5 days); rapid systemic chlorotic front in top leaves (4-7 days) with necrosis of young lateral shoots and tip (Figure 26a). Regreening of leaf veins follows while interveinal areas remain chlorotic - chlorotic vein-net (Figure 26b).

Spinacia oleracea 'Royal Denmark': 2mm chlorotic local lesions (4 days) rapidly coalesce; systemic veinal chlorosis (6 days) with the whole of top leaves becoming chlorotic, followed by veinal regreening.

COMPOSITAE

Calendula officinalis: chlorotic local lesions; systemic tip necrosis.

Senecio cruentus (Cineraria): local and systemic chlorosis or symptomless infection.

Zinnia elegans 'Cactus Flowered': symptomless infection or systemic mottling.

CRUCIFERAE

Arabis sp: symptomless local and systemic infection.

CUCURBITACEAE

Citrullus vulgaris 'Golden Honey': local chlorosis on cotyledons; systemic chlorotic blotches and symptomless infection.

Cucumis sativus 'Marketer' and 'Polaris': chlorotic local blotches; systemic chlorotic flecking on lower true leaves, the rest symptomlessly infected.

Cucurbita pepo 'Small Sugar': local and systemic chlorosis.

LEGUMINOSAE

Cassia occidentalis: no local reaction; systemic chlorotic blotches and mosaic followed by necrotic flecking.

Dolichos biflorus: symptomless local and systemic infection.

Phaseolus vulgaris 'Top Crop': necrotic local lesions (1-2mm) and veinal necrosis (Figure 28); systemic infection symptomless or chlorotic flecking.

Pisum sativum 'Greenfeast': symptomless local infection; systemic chlorosis of leaf tips (12 days).

Vigna unguiculata subsp. cylindrica: faint 2-3mm chlorotic local lesions (6 days) become necrotic; systemic veinal chlorosis.

Vigna unguiculata subsp. unguiculata: 1mm chlorotic local lesions (6 days) become necrotic; systemic white chlorotic flecking or symptomless infection.

SCROPHULARIACEAE

Digitalis purpurea L: symptomless local infection.

SOLANACEAE

Lycopersicon esculentum 'San Marzano': local chlorosis; systemic infection.

Nicotiana clevelandii: faint chlorotic local lesions (4-5 days) irregular necrotic lesions and ringspots follow; systemic veinal chlorosis.

Nicotiana glutinosa: local chlorotic blotches; systemic chlorotic fleck or symptomless infection.

Nicotiana glutinosa x *N. clevelandii*: chlorotic local lesions; systemic veinal chlorosis, chlorotic and necrotic flecking.

Nicotiana rustica: local and systemic chlorosis.

Nicotiana tabacum 'Havana', 'Samsun', and 'White Burley': local chlorosis, occasionally necrotic etch ring lesions; symptomless systemic infection. DLRV recovered from systemically infected tissue in low concentration.

Petunia hybrida 'Firechief' and 'Rosy Morn': either chlorotic local lesions, systemic veinal chlorosis or symptomless infection; or grey brown necrotic local ring lesions, systemic chlorotic blotches and ring lesions with concentric ring lesions forming on flower petals. This second reaction occurred less frequently.

Physalis franchetii: symptomless local and systemic infection.

UMBELLIFERAE

Apium graveolens 'Dulce DC': local and systemic chlorosis.

No infection of the following hosts was detected: *Antirrhinum majus*, *Brassica oleraceae* var *capitata*, *Brassica pekinensis*, *Capsicum frutescens*, *Datura stramonium*, *Dianthus barbatus*, *Dianthus chinensis*, *Matthiola incana*, *Ocimum basilicum*, *Phlox drummondii*, *Salvia splendens*, *Torenia fournieri*, *Vicia faba* and *Vinca rosea*.

Several distinctive host reactions were observed with DLRV in hosts belonging to the amaranthaceae, caryophyllaceae, chenopodiaceae, cucurbitaceae, leguminosae and solanaceae. Ringspot symptoms were observed in several hosts, viz; *Celosia argentia*, *Chenopodium*

FIGURE 24. Local veinal streaking and irregular-shaped ringspot lesions induced by DLRV in *Gypsophila elegans*.

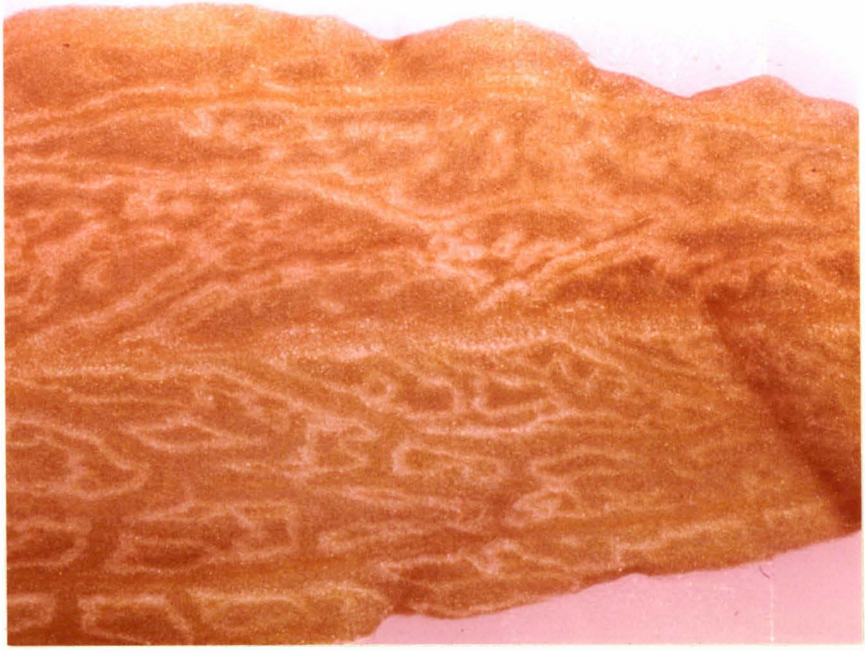

FIGURE 25. Local veinal chlorosis induced by DLRV in *Saponaria vaccaria*.

FIGURE 26a Systemic chlorotic front with tip necrosis induced by DLRV in *Chenopodium cuinoa* (7 days).

FIGURE 26b The same *Chenopodium cuinoa*, showing veinal regreening (11 days).

FIGURE 27. Systemic chlorotic flecking and mosaic induced by DLRV in *Chenopodium amaranticolor* (7 days).

FIGURE 28. Necrotic local lesions and veinal necrosis on DLRV infected *Phaseolus vulgaris* 'Top Crop' leaf (6 days).

quinoa and Petunia hybrida.

The characterstic host reactions observed were remarkably similar to those reported for nepoviruses and several other viruses: broad bean wilt, elderberry latent, elm mottle and robinia mosaic (Harrison & Murant, 1970-74). Host reactions of two nepoviruses (arabis mosaic, and tobacco ringspot), and broad bean wilt were observed in this laboratory. DLRV host reactions were distinct from isolates of these three viruses and generally milder. However, the identification of DLRV as a new virus on the basis of symptomatology is not possible.

3.4 VECTOR TRANSMISSION

Two attempts to transmit DLRV by aphids in a nonpersistent manner failed. In the first experiment aphids (M. persicae) were fed for 20 seconds on detached DLRV-infected Spinacia oleracea leaves and transferred to three Saponaria vaccaria and three S. oleracea plants (10 aphids/plant). In the second experiment aphids (M. euphorbiae) were similarly treated and transferred to two Gypsophila elegans, two S. oleracea plants respectively. Back inoculation tests to C. quinoa 2-3 weeks later failed to reveal any aphid transmission of DLRV.

There are several isometric viruses with similar host ranges to DLRV, including broad bean wilt (BBWV), robinia mosaic (RMV), some nepoviruses, elderberry latent virus (EldLV) and elm mottle virus (ElmMV).

Broad bean wilt virus (BBWV) can be eliminated because it is readily aphid transmitted in a nonpersistent manner by M. persicae (Taylor & Stubbs, 1972). Robinia mosaic virus (RMV) is also aphid transmitted (Schmelzer, 1971) so can possibly be excluded, although its transmission rate by M. persicae is rather low. With the exception of tobacco ringspot (Stace-Smith, 1971) no nepoviruses are reported to be transmitted by aphids and elderberry latent and elm mottle viruses are also in this category (Jones, 1974).

No attempts were made to transmit DLRV by nematode species, the natural vectors of nepoviruses (Harrison et al., 1971).

3.5 SEED TRANSMISSION

Infection of seedlings by DLRV, grown from seed collected from DLRV-infected C. quinoa plants was verified by characteristic host reactions. Seed transmission of DLRV was determined in the order of 95%. In two consecutive tests 10 seedlings from DLRV-infected C. quinoa's were separately back-inoculated to C. quinoa test plants, and in all but one case seed transmission of DLRV was verified. Seedlings infected by DLRV through seed transmission revealed veinal chlorosis and mottling.

With few exceptions seed transmission of plant viruses, reported in the order of 80-90% is restricted to members of the tobacco ringspot virus group or nepoviruses (Bennett, 1969).

3.6 PHYSICAL PROPERTIES IN CRUDE SAP

The TIP of DLRV, determined from infected C. quinoa sap assayed on C. quinoa, was 70-75C. Although this TIP value contributes to DLRV characterisation it is not particularly distinctive as it is only slightly higher than TIP's of the majority of isometric viruses.

3.7 ELECTRON MICROSCOPY

Negative staining

DLRV particles ca. 30nm in diameter first observed in negatively stained partially purified preparations but not in squash homogenates of daphne tissue. In other herbaceous hosts such as C. quinoa, N. clevelandii and S. vaccaria DLRV particles were extremely difficult to resolve despite the use of several different negative stains and staining procedures. Several PTA stains (pH 4, 6 and 7), AM (pH5.3) and AM: PTA mixture (previously described) were tried. Both squash homogenates (Walkey & Webb, 1968) and leaf dips (Brandes, 1957) were also tested. Isometric particles ca. 30nm were revealed after extensive scanning in the electron microscope, DLRV particles usually occurred in small clumps of about a dozen particles (Figure 31). The AM:PTA mixture (1:1) with the squash homogenate procedure was preferred to other techniques as this gave the best contrast.

Many squash homogenate preparations from seed, apical and lateral tips of DLRV-infected C. quinoa and N. clevelandii, failed to reveal virus tubules, characteristic of a number of nepoviruses (Roberts & Harrison, 1970; Walkey & Webb, 1970).

With the exception of the tombusviruses (Martelli et al., 1971) most isometric plant viruses are not found in squash homogenates in very high concentrations (Harrison & Murant, 1970-74). However among the viruses stable in most negative stains, few are as difficult to resolve as DLRV. During this study arabis mosaic, broad bean wilt, cucumber mosaic, tobacco ringspot and tomato aspermy viruses were all found more readily in negatively stained squash homogenates than DLRV.

Purified preparations of DLRV revealed many 30nm isometric virus particles, when negatively stained and viewed in the electron microscope. Good contrast and dispersion of DLRV particles was observed in AM pH5.3 (Figure 29). Similarly with PTA pH7.0 strong contrast occurred, although particles tended to aggregate more than in AM (Figure 30). Several positively stained DLRV particles were observed in both negative stains (Figures 29 & 30), indicating empty virions were present. Twenty DLRV particles measured had an average diameter of 30nm.

The presence of empty particles in negatively stained virus is a characteristic shared by a number of isometric viruses: comoviruses, nepoviruses, tymoviruses and several other viruses including; broad bean wilt and elderberry latent (Harrison & Murant, 1970-74). Verification that the positively stained DLRV particles were 'empty' was demonstrated after analytical ultracentrifugation analysis of DLRV preparations.

Ultrathin sections

Isometric particles distinguishable from ribosomes were observed within the cell cytoplasm of thin sectioned DLRV-infected tissue. No tubules or other distinctive features were observed in this sectioned material.

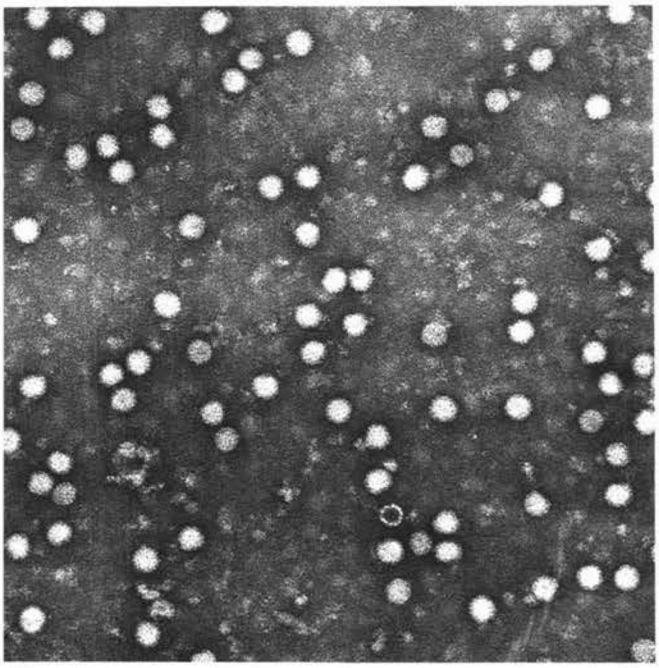

FIGURE 29. DLRV purified preparation negatively stained in ammonium molybdate pH5.3. Mag. 68,500.

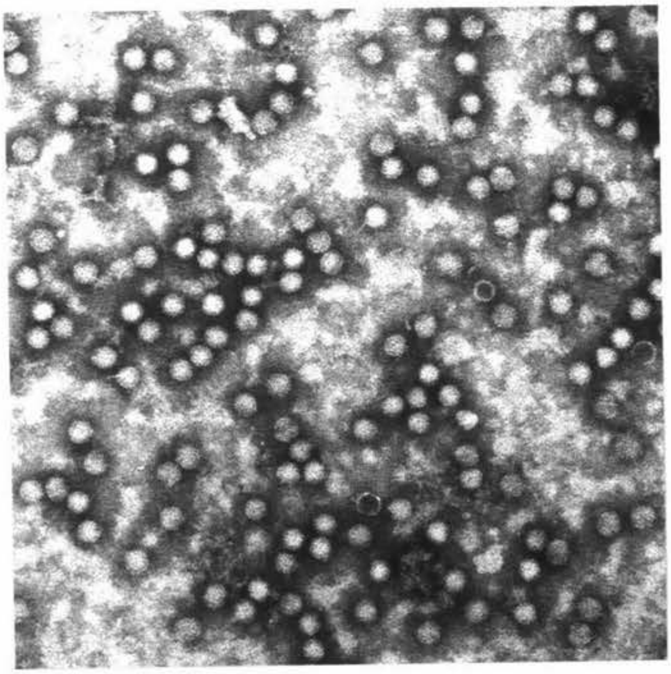

FIGURE 30. DLRV purified preparation negatively stained in phosphotungstic acid pH7.0. Mag. 68,500.

3.8 PURIFICATION

Difficulties were experienced in obtaining adequate yields of purified DLRV for further characterisation. Several different purification procedures similar to those of Harrison and Nixon, (1960), Hollings and Stone, (1964) and Peden and Symons, (1973) were tried, none of which proved outstanding.

The procedure finally adopted for DLRV purification was a modification of that used by Hollings and Stone, (1975) for purification of tomato bushy stunt virus. Two week old DLRV infected *C. quinoa*, *N. clevelandii* or *N. rustica* (local and systemically infected tissue) was harvested and homogenized with 0.5M K-K_2 phosphate, pH7.5 buffer (1.5ml : 1g tissue) including 0.1% TGA, 1mM EDTA and 10mM Dieca additives at 4C. This sap-buffer-homogenate was expressed through cheese-cloth and then emulsified with 8.5% n-butanol overnight at 4C. The emulsion was broken by low speed centrifugation (12,000*g*/10min) and the supernatant retained after filtering through glass wool. The virus was then pelleted by ultracentrifugation (100,000*g*/90min) and resuspended in 30ml of either 0.05M K-K_2 phosphate plus 0.5mM EDTA buffer pH7.5 or 20mM Tris-HCl buffer, pH8.5, for 1hr at 4C. DLRV was finally concentrated by ultracentrifugation (100,000*g*/80min) and resuspended in 1ml of 1mM EDTA pH5.0, or 10mM Tris-HCl pH8.5 buffers.

Based upon electron microscopy preparations of DLRV from *N. clevelandii* or *N. rustica* contained less F_1 plant protein than preparations from *C. quinoa*. Yields of 2-5mg of purified DLRV were obtained from 100g infected tissue, a result determined spectrophotometrically using $E_{0.1\%} = 12.0$ (Noordam, 1973). The DLRV purified preparations did not react against 'healthy' antisera and contained few F_1 protein particles when negatively stained preparations were observed in the electron microscope (Figure 29). The low DLRV yields obtained limited further characterisation studies of this virus.

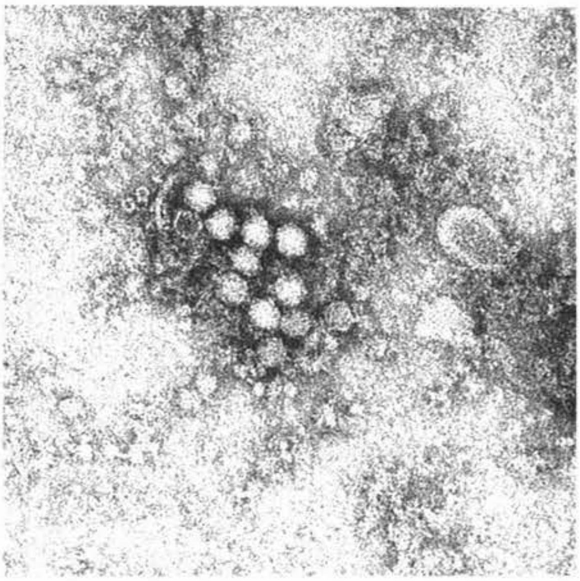

FIGURE 31. An aggregate of DLRV particles, in a squash homogenate from C. quinoa, negatively stained with [illegible]-PTA. Mag. 110,000.

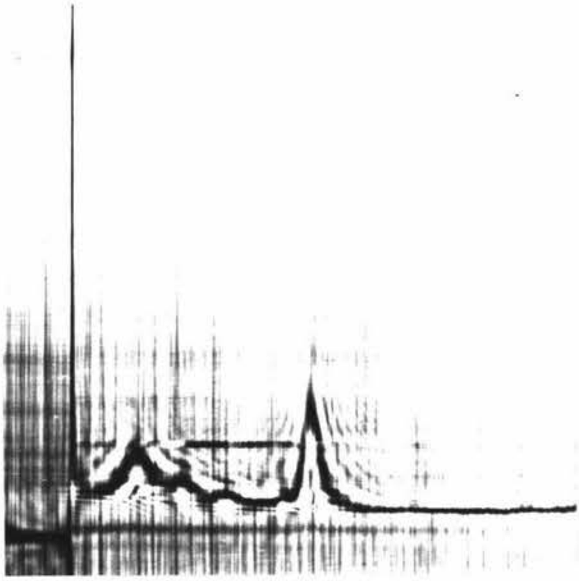

FIGURE 32. Analytical sedimentation pattern for a partially purified of DLRV. The four peaks from left to right are: F_1 plant protein; top (T); middle (M); and bottom (B) virus components. Schlieren angle 45°, photograph taken after 16 minutes at 23,150rpm. (sedimentation left to right).

3.9 PHYSICAL PROPERTIES OF PURIFIED VIRUS

Sedimentation coefficients

In two separate analytical ultracentrifugation runs with purified DLRV preparations, four sedimenting peaks were observed (Figure 32). The top or lightest component was found to be F_1 plant protein and had an sedimentation coefficient of 33S[1]. The three virus sedimenting components labelled top (T) middle (M) and bottom (B), in order of sedimentation, had sedimentation coefficients of 54, 76, and 118S[1] respectively. By correction for zero virus concentration these values would increase slightly, the B component increasing the most because it was observed in a higher concentration as indicated by the largest peak (Figure 32).

Three sedimenting virus components with sedimentation coefficients similar to those reported for DLRV have been recorded for several viruses: comoviruses; several nepoviruses; broad bean wilt; elm mottle; and tobacco streak viruses (Harrison & Murant, 1970-74).

UV absorption spectrum

Preparations of purified DLRV had the following UV absorption spectral characteristics: A max/min = 1.43-1.54 and A 260/280 = 1.69-1.90. Similar values to these are reported for both comoviruses and nepoviruses (Harrison & Murant, 1970-74).

3.10 IMMUNOLOGY AND SEROLOGY

In this study the antiserum prepared against DLRV was found to be unsatisfactory because of the accidental inclusion of D-CarMV antigens in the injection series. Not only did this antiserum contain antibodies to D-CarMV but high levels of antibodies against 'healthy' plant components were found. This poor antiserum arose partly because of early difficulties experienced in developing a purification procedure for DLRV.

Specific serological (gel diffusion) reactions could be obtained between the prepared antiserum and purified DLRV. The purified virus did not react against healthy antisera.

In gel diffusion tests (Crowle, 1973) no serological reactions were obtained with purified preparations of DLRV against several dilutions of antiserum to each of the following viruses: apple mosaic;

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[1] uncorrected for zero virus concentration

arabis mosaic*; broad bean wilt; carnation mottle; carnation ringspot; cherry leaf roll; cucumber mosaic; peanut stunt; prunus necrotic ringspot; raspberry ringspot*; strawberry latent ringspot; tobacco necrosis; tobacco ringspot*; tomato ringspot; and 'healthy' antisera.

The antisera marked with asterisks were included because the viruses had some properties resembling those determined for DLRV in this study.

3.11 DISCUSSION

Whether DLRV is a new virus or not remains unresolved at present. Comparison of DLRV characteristics with those reported for other 30nm isometric viruses does eliminate many possibilities. On the basis of DLRV host range, seed transmission and three characteristic sedimentation components all but some nepoviruses and several ungrouped isometric viruses can be eliminated. Gel diffusion serological tests with antisera to 14 viruses failed to reveal the identity of DLRV so far. Several other viruses including elm mottle, grapevine chrome mosaic, and grapevine fanleaf have some properties resembling DLRV but antisera to these viruses were not available for testing against DLRV. Further serological tests are therefore required to confirm the identify DLRV.

The nepoviruses are characterised by: distinctive and wide host ranges; nematode transmission; high rates of seed transmission; TIP's 55-70C, LIV few days or weeks, ca. 30nm isometric particles; 2 or 3 sedimenting virus components; single stranded RNA (G24:A23:C22:U31); 2RNA species of 2.5 and 1.5 million daltons; one protein subunit (excepting strawberry latent ringspot virus); all are good immunogens; and several produce virus tubules (Harrison & Murant, 1970-74).

On the characteristics revealed in this study with DLRV, it appears to belong to the nepovirus group (Harrison & Murant, 1970-74). Unfortunately no information on nematode transmission is known for DLRV, so its membership in this group cannot be verified.

APPENDIX 1. Antisera used in this study.

Antisera	Source	
Apple mosaic virus	R.W. Fulton	University of Wisconsin Madison, Wisconsin, U.S.A.
Apple mosaic virus (rose)	R. Casper	Biologische Bundenstalts Inst., fur Virusserologie Braunshweig W. Germany.
Arabis mosaic virus	R.A. Goold & B.D. Harrison	Scottish Horticultural Research Station, Invergowrie, Scotland.
Arabis mosaic virus (cucumber)	M. Hollings	Glasshouse Crops Research Inst., Littlehampton, England.
Arabis mosaic virus (carnation)	P.R. Bennett	Massey University, Palmerston North, New Zealand.
Arabis mosaic virus (tamarillo)	W. Thomas	Department of Scientific & Industrial Research. Plant Diseases Division, Auckland, New Zealand.
Broad bean wilt virus (type 1)	J.K. Uyemoto	Cornell University, Geneva, New York, U.S.A.
Broad bean wilt virus	J. Sutton	Victorian Plant Research Inst., Burnley, Melbourne, Australia.
Carnation mottle virus (carnation)	J. Sutton	
Carnation mottle virus	B.A.M. Morris-Krsinich	Massey University, Palmerston North, New Zealand.
Carnation ringspot virus	P.R. Fry	D.S.I.R. Plant Diseases Div., Auckland, New Zealand

APPENDIX 1. continued

Cherry leaf roll virus	J.K. Uyemoto	
Cherry leaf roll virus	R.A. Goold & B.D. Harrison	
Cucumber mosaic virus (nandina)	K.S. Milne	Massey University, Palmerston North, New Zealand
Cucumber mosaic virus	-	University of California Davis, U.S.A.
Cucumber mosaic virus (D strain daphne)	B.A.M. Morris-Krsinich	
Cucumber mosaic virus (C strain daphne)	"	
Cucumber mosaic virus (Q strain pepper)	R.I.B. Francki	Waite Agricultural Research Institute, University of Adelaide, Australia
Cucumber mosaic virus (Common strain - CS)	D.H.M. Van Stogteren	Laboratorium Voor Bloembollender zoek, Lisse, Netherlands.
Daphne latent ringspot virus	B.A.M. Morris-Krsinich	
Healthy 'White Burley'	K.S. Milne	
Peanut stunt virus	G.I. Mink	Washington State University, Washington, U.S.A.
Prunus necrotic ringspot	J. Sutton	
Prunus necrotic ringspot	R. Casper	
Raspberry ringspot virus	R.A. Goold & B.D. Harrison	
Strawberry latent ringspot virus	"	

APPENDIX 1. continued

Tobacco necrosis virus	J.K. Uyemoto	
Tobacco ringspot virus	"	
Tobacco ringspot virus (horse radish)	W. Thomas	
Tobacco streak virus	R.W. Fulton	
Tomato aspermy virus (chrysanthemum)		D.S.I.R. P.D.D. Auckland, New Zealand
Tomato aspermy virus (chrysanthemum)	D.H.M. Van Slogteren	
Tomato black ring virus	R.A. Goold & B.D. Harrison	
Tomato bushy stunt virus antisera to three separate isolates: type strain, GCRI 273, & PLCV 232.	M. Hollings	
Tomato ringspot virus	J.K. Uyemoto	

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