STUDIES ON ROSE MOSAIC VIRUS AND P. SYRINGAE

FROM SOUTH AUSTRALIAN ROSES

by

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STATEMENT

This thesis has not previously been submitted for an academic award to this or any other University, and is the original work of the author, except where due reference is made in the text.

A.A. Basit
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SUMMARY

Viruses from rose plants in South Australia showing mosaic symptoms have been transmitted to peach seedlings [Prunus persica L. Batsch cv. Elberta] by grafting and then by mechanical inoculation to herbaceous indicator plants. In some cases, a latent bacterial pathogen was isolated together with virus. The two pathogens were separated by passage through differential host plants. The virus isolates recovered after passage through Chenopodium quinoa were identified as Rose Mosaic Virus (RMV) and the bacterial pathogen obtained after passage through Mormordica balsamina was identified as a strain of Pseudomonas syringae. It was observed that South Australian isolates of RMV have a very narrow host range whereas other properties of the virus are very similar to RMV described from North America.

A plant pathogenic P. syringae was also isolated from cherry leaves and flower petals infected with Prunus necrotic ringspot virus. Comparative studies with three isolates of P. syringae from rose, cherry and lemon and one isolate of P. pisi from pea showed that the symptoms produced by these isolates were similar on M. balsamina, C. tetragonoloba and cowpea. Serological investigations revealed that isolates of P. syringae from rose and cherry have some antigens in common with six
bacteria belonging to several different serotypes of *P. syringae* from California.

RMV was consistently isolated from pollen of infected *C. quinoa* and cucumber but not from rose pollen, although the virus was shown to be transmitted through rose seed.

Isolates of RMV were purified from cucumber plant extracts by emulsification with anhydrous ether followed by differential centrifugation and by sucrose density gradient centrifugation. It was observed that RMV purified from infected *C. quinoa* leaves contained less virus and more host material than that obtained from cucumber. Partially purified RMV preparations both from cucumber and *C. quinoa* had ultra-violet absorption spectra characteristic of nucleoprotein. Purified preparations of RMV were found to be degraded upon prolonged storage or by incubation with ribonuclease, and could not be subjected to CsCl2 density gradient centrifugation without extensive damage to the virus particles. Infectious RNA was prepared by degradation of virus with LiCl but not with phenol.

It has been confirmed that RMV is serologically related to apple mosaic virus, *Prunus* necrotic ringspot virus and Danish plum line pattern virus and a serological relationship between RMV and cherry rugose mosaic virus has been established.
During sucrose density gradient centrifugation RMV preparation sedimented as three components with sedimentation coefficients of approximately 86, 98 and 119S; only the 119S component was infectious. The three components of RMV could be separated from one another by centrifugation through sucrose density gradients and T and B components were obtained with very little contamination after two successive cycles of density gradient centrifugation. Difficulties were experienced with purification of M component as its sedimentation is not very different from the other two components. Purified T and B components have ultraviolet absorption spectra characteristic of nucleoproteins and it appears that they contain similar proportions of nucleic acid. The sizes of T and B components are different; T component is 22.5 nm and the B component 28.5 nm in diameter. In preparation of purified B component, bacilliform particles, similar to those of alfalfa mosaic virus, were also observed. No antigenic differences were observed between T and B components when tested against RMV antiserum.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to Dr. R.I.B. Francki for suggesting this problem, his guidance throughout the course of the investigation, and a critical reading of this manuscript. My thanks are also due to Dr. J.W. Randles for his advice during the experimental work. I wish to thank Dr. A. Kerr and Dr. A.C. Hayward for carrying out biochemical tests on P. syringae reported in this thesis and Dr. Kerr for his advice on bacteriological matters. I would like to thank Drs. R.W. Fulton, R. Cropy, R.G. Grogan and H. English for generous gifts of antisera; Dr. A. Kerr for isolates of Pseudomonas, Dr. J. Fisher for collecting some rose material, and Mr. P.R. Smith for isolates of Prunus necrotic ringspot virus. I also wish to thank Mrs. L. Wichman for the preparation of illustrations, Mr. B. Palk for the photography, Dr. E.M. Davison and Mr. A. Soeffky for the electron micrographs, Mr. C. Grivell and Mr. D. Coleman for some technical assistance, Mr. K. Jones for the supply and maintenance of plants in the glasshouse and Miss T. Siekmann for typing the text. Finally I wish to thank the staff of the Plant Pathology Department and all postgraduate students for their friendly cooperation.

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INTRODUCTION

Under the name of "mosaic", a virus disease of Rosa setigera Michx. was first described from the United States by McWhorter (1931), who recognised the disease by a wide variety of symptoms, including mosaic, yellowing, chlorosis, blanching of the veins and leaf crenations. Since then an even greater variety of disease symptoms have been reported by many workers (Thomas and Massey, 1939; Brierly and Smith, 1940; Meyer, 1960; Fry and Hunter, 1956; Hunter, 1965) but difficulties were experienced in the transmission of the viruses from rose to herbaceous hosts by mechanical inoculation. More recently, however, several viruses isolated from roses showing "mosaic" symptoms have been transmitted to herbaceous hosts and characterised, including rose mosaic virus (Fulton, 1952), Prunus necrotic ringspot virus (Cochran, 1950; Kirkpatrick et al., 1962), tomato ringspot virus (Halliwell and Milbrath, 1962), strawberry latent ringspot virus (Cammack, 1966), arabis mosaic virus (Harrison, 1967) tobacco ringspot virus (McDaniel et al., 1971) and tobacco streak virus (Fulton, 1970), which shows the complexity of the term "mosaic" when referred to disease symptoms on rose.

The only references to virus-like symptoms on roses in South Australia are those by Crowley (1963, 1965). Virus was
detected in many varieties of rose (Crowley, 1963) and tentatively identified as tobacco ringspot virus (TRSV). However, these reports do not give any experimental details.

Earlier attempts at identifying virus isolates from rose have been based on biological properties, such as host range, symptomatology and to some extent on transmission studies. More recently, Fulton (1967b, 1968) has purified rose mosaic virus (RMV) and determined some of its physical, chemical and serological properties. The work on the isolation and characterisation of virus from roses in South Australia was initiated in 1967, before the data published by Fulton (1967b, 1968) was available. RMV was isolated from several varieties of rose and from some varieties it was isolated together with a bacterial pathogen which has been shown to be a strain of Pseudomonas syringae (Van) Hall. RMV was separated from P. syringae (Basit, Francki and Kerr, 1970) and a method of purifying RMV was developed independently of the one reported by Fulton (1967b) and has already been published (Basit and Francki, 1970). This thesis is a detailed report on the studies carried out on RMV isolated in South Australia and on the strain of P. syringae which was found to be present in some isolates of RMV.
SECTION I

MATERIALS AND METHODS

I. Isolation of viruses and bacteria from rose plants

a. Mechanical. Young terminal leaves, flower petals and roots from several rose varieties showing disease symptoms were used as a source of inoculum in attempts to transmit viruses to some herbaceous plants. Inoculum was prepared by grinding the leaves, petals and roots separately in 0.01M phosphate buffer, pH 7.5, with a pestle and mortar. Sodium diethyl-dithiocarbamate (DIECA), thioglycollic acid (Tremaine et al., 1964), nicotine base (Caíman, 1959), ascorbic acid, cysteine hydrochloride and sodium sulphite (Fulton, 1967a) were used in different combinations to aid virus transmission. The extracts were inoculated to carborundum-dusted leaves of Vigna sinensis (Torn.) Savi, Phaseolus vulgaris L., Chenopodium quinoa Willd, and cucumber seedlings.

b. Grafting. Small pieces of bark from young rose shoots were grafted near the stem base of healthy peach seedlings (Prunus persica (L) Batsch cv. Elberta). Three grafts were made to each seedling and two or three seedlings were used for testing each rose variety. Presence of virus in the peach
seedlings was tested by mechanical inoculation to cucumber 30-40 days after grafting. Young leaves from peach seedlings were ground in water with a pestle and mortar and the extract was mechanically inoculated to carborundum-dusted cotyledons of cucumber. The inoculum was washed with tap water soon after inoculation.

c. **Maintaining stock cultures of viral and bacterial pathogens isolated from rose.** Stock cultures were maintained throughout this study in peach seedlings which were kept in pots for up to three years and later transferred into the field. Peach seedlings were tested for the presence of the pathogens after every 2-3 months and they could be recovered throughout the study. It was found that in spring when new leaves developed, pathogens were very easy to recover but transmission became more difficult as the season progressed. Isolates were maintained in glasshouse in cucumber seedlings which were reinoculated at weekly or fortnightly intervals.

II. **Host range studies**

Sap from infected cucumber plants was mechanically inoculated to a range of test plants grown in an insect-proof glasshouse. The plants were observed for symptoms and after 15-25 days were indexed by mechanical inoculation to cucumber...
III. Bacteriological studies

a. Preparation of bacterial suspensions for infectivity studies. Bacterial suspensions for routine mechanical inoculation to plants were prepared by suspending bacteria grown for 24-48 hours on nutrient agar plates in 10 ml of 0.01M phosphate buffer, pH 7.5. The suspension was centrifuged at 3,000 rpm for 10 minutes, the pellet was re suspended in 5-7 ml of sterile distilled water (SDW) and the bacterial cells were separated by vigorous shaking. The O.D. at 306 nm was adjusted to between 50-60 to produce a bacterial concentration of approximately $10^8$ cells/ml (New, 1972).

b. Preparation of bacterial antigens for serology. Bacteria cultured for 48 hours on nutrient agar were suspended in SDW and centrifuged at 3,000 rpm for 10 minutes. The pellet was washed three times with 0.1M phosphate buffer in 0.85% NaCl, pH 7.2. The final bacterial concentration was adjusted to approximately $10^8$ cells/ml.

c. Preparation of antigens for gel diffusion tests. Eight isolates of Pseudomonas were each grown in 250 ml conical flasks containing 100 ml of a solid medium made up as follows:
Casamino Acid (Difco) 2 gm
Glycerol 15 ml
K₂HPO₄ 1.5 gm
MgSO₄ 0.4 gm
Bacto-agar (Difco) 20 gm
Distilled water 1 litre

Bacteria cultured for 48 hours on this medium were harvested by adding to each flask 10 ml of 0.05M phosphate buffer, pH 7.2, in 0.85% NaCl. The resulting suspension was centrifuged at 10,000 g for 10 minutes and the pellet was resuspended in 0.05M phosphate buffer, pH 7.2, in 0.85% NaCl. Centrifugation was repeated and the final pellet was resuspended in 0.05M phosphate buffer, pH 7.5 (approximately 1 gm of bacteria/10 ml buffer). The samples were then sonicated at maximum intensity for 15 minutes in a MSE ultrasonic unit. Each suspension thus obtained was divided into three equal parts. One was autoclaved for 15 minutes, the second was heated for 1 hour at 100°C, while the third portion received no further treatment. The resulting suspensions were combined for each bacterial isolate to form composite antigens. This method of preparing composite antisera is similar to that used by Otta and English (1971).
d. **Sero logical techniques.** Antisera prepared against 8 serotypes of *P. syringae* (Otta and English, 1971) were kindly supplied by Dr. H. English of the University of California, Davis. Antiserum to the bacterial pathogen of rose isolated in this study was prepared in rabbits by two intramuscular injections followed by one intravenous injection at weekly intervals. One ml of the bacterial suspension cultured for 24-48 hours emulsified with 1 ml of Freund’s complete adjuvant was administered intramuscularly and the intravenous injection was given without adjuvant. Serum was collected by bleeding the rabbit 14 days after the last injection and was stored at -20°C. The titre was 1/1024 in precipitin tube test with homologous antigen.

i. **The tube precipitin test**

To titrate antisera in tubes two-fold dilutions of serum were prepared in 0.14M NaCl and an equal volume of diluted bacterial suspension was pipetted into each tube and incubated at 37°C. Observations of precipitation were recorded after 2 hours.

ii. **Slide agglutination test**

Slide agglutination tests (Perlasca, 1960) were carried out under a light microscope. A single drop of freshly prepared
bacterial suspension was placed on a clean microscope slide and examined. When a drop of antiserum was added clumping and aggregation of cells was observed. No clumping or aggregation occurred when normal serum was tested in the same way. All bacterial cultures isolated in this work were tested by this method.

iii. Gel diffusion test

Sero logical test was carried out by the gel diffusion technique using 0.75% agar in 0.05M phosphate buffer, pH 9.0, containing 0.14M NaCl and 0.02% sodium azide.

e. Electron microscopy. Bacteria cultured for 24 hours on nutrient agar were suspended in 0.05% bovine serum albumin, mounted on Formvar-coated electron microscope grids and stained with 2% PTA, pH 6.7. The grids were examined immediately in a Philips 100B electron microscope.

f. Preparations of media. Following two media were used for studying various aspects of P. syringae:
i. **Fluorescenic production medium** *(King et al., 1954)*

- Proteose peptone NO₃ (Difco) 20 gm
- Glycerol 10 ml
- K₂HPO₄ (anhydrous) 1.5 gm
- MgSO₄ 1.5 gm
- Bacto-agar (Difco) 15 gm
- Distilled water 1 litre

ii. **Nutrient agar** *(New, 1972)*

- Nutrient agar (Difco) 23 gm
- Distilled water 1 litre

IV. **Virus studies**

a. **Infectivity assay.** As no suitable local lesion host could be found for RMV, infectivity was assayed by determining the proportion of inoculated cucumber seedlings which developed symptoms *(Diener and Weaver, 1959)*. Although this method is not as accurate as a local lesion assay, satisfactory dilution curves were obtained (see, for example, Figure 20), and the assay was considered adequate for the work described in this thesis.

b. **UV absorption studies.** The relative concentrations of virus preparations were measured in a Schimadzu QR.50 spectrophotometer at 260 nm.
c. **Sucrose density gradient centrifugation.** Diluted samples of virus (0.2 - 0.3 ml) were layered over linear sucrose density gradients prepared in 5 ml Spinco 39SW tubes, using 10-40% sucrose dissolved in 0.01M phosphate buffer, pH 7.5. The tubes were centrifuged at 37,000 rpm for 120 minutes and the contents scanned and fractionated with an ISCO model D gradient fractionator and flow densitometer assembly (Brakke, 1963). When large amounts of virus were handled, 5-25% linear sucrose density gradients were prepared in 25SW tubes. The tubes were centrifuged at 24,000 rpm for 4 hours.

d. **Serological techniques.** Serological studies were carried out by gel diffusion tests using 0.75% agar in 0.05M phosphate buffer, pH 9.0, containing 0.14M NaCl and 0.02% sodium azide.

Antisera prepared against rose mosaic virus (RMV), cherry necrotic ringspot virus (NRSV), Danish plum line pattern virus (DLPV) and apple mosaic virus (PAMV), were kindly supplied by Dr. R.W. Fulton of the University of Wisconsin; against NRSV, DLPV and PAMV by Dr. R. Cropley of East Malling Experimental Station, Kent; and against cherry rugose mosaic virus (CRMV), arabis mosaic virus (AMV), tomato ringspot virus (TomRSV), tobacco ringspot virus (TRSV) and strawberry latent ringspot virus (SLRV) by Dr. R.G. Grogan of the University of California, Davis.
Antisera to TRSV and the Q strain of cucumber mosaic virus (CMV) were prepared in this laboratory (Randles and Francki, 1965; Francki et al., 1966).

Antisera to the three isolates of RMV used in this work were prepared by injecting rabbits intravenously at weekly intervals with 2 ml samples of purified virus recovered from sucrose density gradients without prior dialysis or concentration. After 3 injections, the antiserum had a titre of 1/4 - 1/8 in gel diffusion tests and further booster injections failed to increase the titre above 1/8. The antisera were stored at -20°C.

To test for the presence of antibodies to host antigens, healthy cucumber antigens were prepared by grinding 50 g of cucumber seedlings with 50 ml of 0.01M phosphate buffer, pH 7.5, and 10 ml of 0.01% ascorbic acid. The pulp was strained through a double layer of muslin and the extract was centrifuged at 15,000 g for 20 minutes. The supernatant was emulsified with ether, and the phases were separated by centrifugation at 15,000 g for 10 minutes. The buffer phase was sedimented by centrifugation at 105,000 g for 90 minutes and the pellet suspended in one-tenth of the original volume in 0.01M phosphate buffer, pH 7.5. This procedure is similar to that finally adopted for the initial steps of purifying RMV (see Figure 21).
e. **Electron microscopy.** Purified samples of virus preparations were examined in a Siemens Elmiskop I electron microscope. Carbon-coated electron microscope grids were floated for 30 seconds on a drop of virus suspension and then grids were transferred to a drop of 2% uranyl acetate (UAc), pH 4.8, or 2% phosphotungstic acid (PTA) adjusted with KOH to pH 6.8. The excess stain was removed with filter paper and the grids were air dried.
SECTION II

TRANSMISSION OF VIRUS-LIKE PATHOGENS FROM ROSE TO HERBACEOUS PLANTS AND THEIR IDENTIFICATION

Mechanical transmission of RMV from rose to herbaceous plant is known to be difficult (Halliwell and Milbrath, 1962). Fulton (1952) managed to transmit RMV by mechanical inoculation from *Rosa setigera* Michx. to cucumber and other plant species; this allowed him to study its host range and later to achieve its purification (Fulton, 1967b). In this section are described the results of experiments in which two virus-like pathogens were transmitted from rose plants showing virus-like symptoms.

I. Virus-like symptoms observed on roses in South Australia

a. Field observations. Until the present, virus diseases of rose occurring in South Australia have been identified on the basis of their producing symptoms similar to those described abroad. Fry and Hunter (1956) and Hunter (1965) described three types of symptoms on roses from New Zealand, which they called rose mosaic virus (RMV). These symptoms are line pattern (Figure 1a), vein banding (Figure 1d) and chlorotic mottle (Figure 1c). All three types of symptoms have been observed on roses in South Australia and often two or three types of
**Figure 1.** Disease symptoms on rose.

a. Line pattern on cv. Peace.

b. Chlorotic ringspot on cv. Peace.

c. Chlorotic mottle on cv. Cuba.

d. Vein banding on cv. Peace.
symptoms occur on different leaves of the same plant. Chlorotic rings (Figure 1b) observed on some plants are considered to be a variant of line pattern symptoms. The symptoms observed on roses in South Australia usually appear in early or late spring and then disappear in late summer and autumn. However, the symptoms may sometimes remain on some of the older leaves throughout the year. Although no comparisons have been made, growth of the rose plants infected with virus is not noticeably affected and reasonable crops of flowers and seeds are produced.

b. Symptom expression

i. Line pattern

The symptoms appear on leaves as white, creamy or yellow coloured lines scattered irregularly on the leaf surface (Figure 1a). These lines may be narrow or broad and may extend over the entire surface of the leaf, often assuming an irregular oak leaf pattern. Chlorotic concentric rings are also observed which could be a variant of line pattern symptoms (Figure 1b).

ii. Vein banding

In leaves with vein banding symptoms, white creamy lines are formed along the leaf veins and often cover the entire vein system of the leaf (Figure 1d). Vein banding and line pattern symptoms were sometimes observed on the same plant. Usually these types of symptoms appear on leaves in late summer.
iii. Chlorotic mottle

Great variation was observed in the development of chlorotic mottle on rose leaves. Initially, the disease manifests itself as white or creamy coloured mottle. This may lead to the formation of large blotchy areas resulting in mosaic. Puckering and twisting of leaves was commonly associated with the mottle. In addition to the above symptoms, rasp leaf, leaf deformation, leaf tattering, yellowing, chlorosis and colour break in flowers have been observed in South Australian roses.

II. Transmission of virus-like pathogens from rose to herbaceous plants

a. Mechanical transmission from leaves, petals and roots of rose. Several attempts were made to transmit virus directly from rose leaves, flower petals and roots by mechanical inoculation. Sodium diethylthiocarbamate (DIECA), thioglycollic acid (Tremaine et al., 1964), nicotine base (Cadman, 1959), ascorbic acid, cysteine hydrochloride and sodium sulphite (Fulton, 1967a) were used to aid virus transmission. However, all attempts to transmit virus-like pathogens directly from rose plants to herbaceous plants were unsuccessful.

b. Graft transmission to peach seedlings. Virus-like pathogens were readily transmitted mechanically from the young
Figure 2. Leaves of grafted peach seedlings showing systemic mosaic symptoms due to a virus-like pathogen transmitted from diseased rose.
Figure 3. Symptoms produced by various isolates transmitted from rose to peach seedlings, then to cucumber.

A. Isolate A causing chlorotic and necrotic lesions on inoculated cucumber cotyledons; isolates B and C also cause similar symptoms (Table 1).

D, E and F. Isolates causing chlorotic lesions and systemic mosaic symptoms on cucumber cotyledons.
leaves of peach seedlings which were patch bark-grafted with rose material showing virus-like symptoms. The infectivity of virus-like pathogens in peach seedlings was tested at 7-day intervals, and they could be isolated 30-40 days after grafting. The grafted peach seedlings did not show any symptoms in the first year but during the second and third years, mosaic symptoms developed on some of the older leaves (Figure 2), and pathogens were recovered from such leaves. No symptoms were observed on healthy peach seedlings of the same age kept as controls. Not all grafts from rose plants yielded pathogens. Only six virus-like pathogens were transmitted from ten rose plants which represented eight different varieties. Virus-like pathogens were transmitted from rose plants which showed line pattern and chlorotic ringspot symptoms, but none were isolated from those which showed chlorotic mottle and vein banding symptoms (Table 1).

On mechanical inoculation to cucumber seedlings with extracts of young peach leaves, the cucumber cotyledons developed chlorotic lesions within 3 days and systemic mosaic symptoms developed on young leaves a few days later (Figure 3).

c. Separation of two distinct pathogens from rose. Isolate A (Table 1) consistently induced two types of lesions on inoculated cucumber cotyledons (Figure 3A). On mechanical inoculation of
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<td>-</td>
</tr>
<tr>
<td>6 Queen Elizabeth</td>
<td>LP, CM</td>
<td>&quot;</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7 Ophelia</td>
<td>LP, CM</td>
<td>&quot;</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8 Doctor</td>
<td>LP</td>
<td>&quot;</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 Crimson Glory</td>
<td>LP, CM</td>
<td>&quot;</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 Rosa multiflora</td>
<td>LP</td>
<td>&quot;</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Total number of plants infected after graft/total number of plants grafted.

LP: Line pattern symptoms
CR: Chlorotic rings symptoms
VB: Vein banding symptoms
CM: Chlorotic mottle symptoms

+ Positive reaction on cucumber
- Negative reaction on cucumber
**Mormordica balsamina** with an extract of cucumber cotyledons infected with isolate A, small brown necrotic lesions developed on the inoculated leaves (Figure 4A). The same inocula induced mottling, chlorotic rings and line pattern symptoms on young leaves of *Chenopodium quinoa* (Figure 4B). When the pathogens were transferred back to cucumber seedlings, the symptoms produced by the inocula from the two plant species were quite different (Figure 5A, B). These experiments indicated that the peach seedlings had been infected with two pathogens, both of which had been transferred from rose material to peach seedlings by grafting, and thence to cucumber by mechanical inoculation.

The pathogen isolated by passage through *M. balsamina* will be referred to as pathogen M, whereas the pathogen recovered by passage through *C. quinoa* will be referred to as pathogen Q (Figure 5C). The separated pathogens were maintained in an insect-proof glasshouse on cucumber seedlings by weekly to fortnightly transfers.

Ungrafted healthy peaches of the same age as those which were used for grafting with infected rose material were kept as controls and tested several times by mechanical inoculation to cucumber seedlings, but neither pathogen M nor pathogen Q was ever detected.
Figure 4. Reaction on *Mormordica balsamina* and *Chenopodium quinoa* after mechanical inoculation with sap from cucumber seedlings infected with isolate A.

A. Local necrotic lesions on inoculated leaves of *M. balsamina*.

B. Systemically infected leaves with rings, line pattern and mosaic symptoms on leaves of *C. quinoa*.
Figure 5. Symptoms produced by isolate A on cucumber after passage through differential hosts.

A. Necrotic local lesions with yellow margins on inoculated cotyledons after inoculation from infected M. balsamina.

B. Chlorotic lesions on inoculated cotyledons and systemic mosaic symptoms after inoculation from C. quinoa.
Figure 5. C. Schematic presentation of isolation and separation of two different pathogens from rose (isolate A).
DIAGRAMMATIC REPRESENTATION OF EXPERIMENT TO DEMONSTRATE THAT ISOLATE OF RMV WAS INFECTED WITH TWO DIFFERENT PATHOGENS

Large chlorotic and small necrotic lesions and systemic mosaic

Cucumber

Necrotic local lesions

Mormordica balsamina

Rings and mosaic on new leaves

Chenopodium quinoa

Necrotic local lesions

Cucumber

C. tetragonoloba

Local necrotic lesions

Cowpea

C. quinoa

No infection

No infection

Rings and mosaic

Pathogen M

C. tetragonoloba

Local necrotic lesions

Cowpea

C. quinoa

No infection

No infection

Pathogen Q
d. **Demonstration of pathogen M in roses.** When isolate A was shown to consist of two pathogens, as described above, it was considered necessary to check the other virus-like pathogen isolates transmitted from rose (isolates B-F, Table 1) for the presence of pathogen M. Inocula of isolates B-F were screened by mechanical inoculation to *M. balsamina* which was the test plant found to be most susceptible to pathogen M. Pathogen M was detected in isolates B and C but not in isolates D, E and F.

III. **Host range and symptomatology**

a. **Host range of pathogen M and Q.** Twenty-eight plant species belonging to nine families were inoculated mechanically with extracts from cucumber infected with pathogens M and Q. Plant species previously reported to be hosts of RMV (Fulton, 1952; Halliwell and Milbrath, 1962) were included in the tests. Results of these experiments (Table 2) showed that only two species of herbaceous plants, viz. *Cucumis sativus* and *Chenopodium quinoa* (Figure 4B, 5B) became infected with pathogen Q. However, pathogen Q also induced line pattern symptoms on *C. amaranticolor* when inoculated with sap from *C. quinoa*, but not with sap from infected cucumber (Figure 6). The reason for this surprising result remains obscure. Repeated attempts to infect
Figure 6. Symptoms produced by pathogen Q on Chenopodium amaranticolor.

Systemically infected leaves showing mottling, line pattern and rings.
<table>
<thead>
<tr>
<th>Plant species</th>
<th>No. of trials</th>
<th>Symptoms produced by two pathogens</th>
<th>Pathogen N</th>
<th>Pathogen Q</th>
<th>Pathogen N</th>
<th>Pathogen Q</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amaranthaceae</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Gomphrena globosa</em> L.</td>
<td>6</td>
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<tr>
<td><strong>Apocynaceae</strong></td>
<td></td>
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<tr>
<td><em>Vinca rosea</em> L.</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Chenopodiaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chenopodium quinoa</em> Willd.</td>
<td>6</td>
<td>-</td>
<td></td>
<td>LP,SM,SR</td>
<td></td>
<td>SM,CL</td>
</tr>
<tr>
<td><em>Chenopodium arenanthicolor</em> (Coste &amp; Reyn.)</td>
<td>4</td>
<td>-</td>
<td></td>
<td>LP,SM</td>
<td></td>
<td>SM,CL</td>
</tr>
<tr>
<td><em>Beta vulgaris</em> L.</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Spinacia oleracea</em> L.</td>
<td>3</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Cucurbitaceae</strong></td>
<td></td>
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<tr>
<td><em>Cucurbita pepo</em> L.</td>
<td>3</td>
<td></td>
<td></td>
<td>SM</td>
<td></td>
<td>CL,SM</td>
</tr>
<tr>
<td><em>Mormordica balsamina</em> L.</td>
<td>6</td>
<td>NL</td>
<td></td>
<td>NL+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mormordica charantia</em> L.</td>
<td>6</td>
<td>NL</td>
<td></td>
<td>NL+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Compositae</strong></td>
<td></td>
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<td><em>Helianthus annuus</em> L.</td>
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<tr>
<td><em>Zinnia elegans</em> Jacq.</td>
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<tr>
<td><strong>Malvaceae</strong></td>
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<tr>
<td><em>Gossypium hirsutum</em> L.</td>
<td>2</td>
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<tr>
<td><strong>Cruciferae</strong></td>
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<tr>
<td><em>Brassica oleracea</em> L.</td>
<td>3</td>
<td></td>
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<tr>
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<td></td>
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<tr>
<td><em>Cyanopsis tetragonoloba</em> (L) Taub.</td>
<td>6</td>
<td>NL</td>
<td></td>
<td>NL+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fissum sativum</em> L.</td>
<td></td>
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<tr>
<td><em>Glycine max</em> (L.) Merr.</td>
<td>4</td>
<td>-</td>
<td></td>
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<tr>
<td><em>Phaseolus vulgaris</em> L.</td>
<td>6</td>
<td>NL</td>
<td></td>
<td>NL+</td>
<td></td>
<td></td>
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<tr>
<td><em>Phaseolus mungo</em> L.</td>
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<td></td>
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</tr>
<tr>
<td><em>Vicia sinesis</em> (Torn.) Savi</td>
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<td>NL</td>
<td></td>
<td>NL+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Solanaceae</strong></td>
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<tr>
<td><em>Datura stramonium</em> L.</td>
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<tr>
<td><em>Petunia hybrida</em> Vilm.</td>
<td>4</td>
<td>-</td>
<td></td>
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</tr>
<tr>
<td><em>Nicotiana tabacum</em> L.</td>
<td>6</td>
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<tr>
<td><em>Nicotiana glutinosa</em> L.</td>
<td>6</td>
<td>-</td>
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<tr>
<td><em>Physalis floridana</em> Rydb.</td>
<td>4</td>
<td>-</td>
<td></td>
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<tr>
<td><em>Capsicum frutescens</em> L.</td>
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<td>-</td>
<td></td>
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</tr>
<tr>
<td><em>Lycopersicon esculentum</em> Mill</td>
<td>4</td>
<td>-</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CL</td>
<td>Chlorotic lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>Line pattern symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>Systemic mosaic symptoms</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SR</td>
<td>Systemic rings</td>
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<tr>
<td>NL</td>
<td>Necrotic lesions</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>+</td>
<td>Positive reaction on cucumber after return transmission</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Negative reaction on cucumber after return transmission</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Cynoglossus tetragonoloba, Vigna sinesis, Petunia hybrida, Phaseolus vulgaris, Nicotiana glutinosa and Mormordica balsamina with pathogen Q failed. All these plants have been reported to be hosts of RMV (Fulton, 1952, 1967b). Isolates D, E and F transmitted from rose plants (Table 1) were found to have host ranges similar to that described for pathogen Q. However, during this study some minor differences were observed in symptom expression by these isolates on the susceptible plant species. These are summarised in Table 3.

Pathogen M was readily transmitted to C. tetragonoloba, V. sinesis, P. vulgaris and M. balsamina. The symptoms induced by pathogen M on C. tetragonoloba (Figure 7A) were very similar to those reported for RMV (Fulton, 1952) and on M. balsamina (Figure 4A) to those reported for Prunus necrotic ringspot virus (NRSV) (Fulton, 1957).

b. Symptomatology of pathogen M.
i. Cucumis sativus L. (var. Polaris)

Pathogen M induced local necrotic lesions with yellow margins on the inoculated cotyledons after 5-7 days (Figure 5A). First true leaves which developed after a further 3-5 days were symptomless but the pathogen could be detected in these leaves by mechanical inoculation to cucumber cotyledons.
<table>
<thead>
<tr>
<th></th>
<th>Isolates A, B and C</th>
<th>Isolate D</th>
<th>Isolates E and F</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cucumis sativus</em>  (var. Polaris)</td>
<td>Large irregular blotchy lesions produced 48-72 hours after inoculation. First true leaves developed characteristic symptoms with curling and distortion on one side. Infected plant stunted, growing point became necrotic (occasionally) and plant died within 20-25 days after inoculation</td>
<td>Small pale chlorotic spots developed on inoculated cotyledons but plant showed severe systemic mosaic; stunting in plant rarely occurred and growing point never became necrotic.</td>
<td>Small pale green chlorotic spots developed on inoculated cotyledons, later plant showed systemic mosaic.</td>
</tr>
<tr>
<td><em>Chenopodium quinoa</em></td>
<td>Few chlorotic spots developed on inoculated leaves 7-9 days after inoculation, chlorotic rings and line pattern symptoms developed in new leaves up to flowering stage.</td>
<td>Small chlorotic lesions developed (or may not develop) on inoculated leaves, but small rings mottling and mosaic symptoms developed on new leaves up to flowering time.</td>
<td>No chlorotic lesions developed on inoculated leaves. First noticeable sign of symptoms appeared as mottling on leaves and later small chlorotic spots developed on new leaves up to flowering time.</td>
</tr>
<tr>
<td><em>Chenopodium amaranticolor</em></td>
<td>Few chlorotic spots appeared on inoculated leaves; later line pattern symptoms developed on young leaves.</td>
<td>Very mild mottling symptoms developed on new leaves.</td>
<td>No symptoms.</td>
</tr>
</tbody>
</table>
Figure 7. Symptoms produced by pathogen M on *Cvamopsis tetragonoloba* and *Vigna sinensis*.

A. Small necrotic lesions on inoculated leaves of *C. tetragonoloba*.

B. Small sunken local lesions on inoculated leaves of *V. sinensis*. 
ii. Mormordica balsamina L. and M. charantia L.

Both species of Mormordica were susceptible to pathogen M. Numerous small brown pin-point lesions developed on the inoculated leaves within 3-5 days (Figure 4A). Sometimes these lesions were large with yellow margins but no systemic infection developed.

iii. Cyamopsis tetragonoloba L. (Taub.)

Small necrotic lesions with yellow margins developed on the inoculated leaves after 9-11 days (Figure 7A). No systemic infection developed.

iv. Vigna sinensis (Torn.) Savi

Pin-point sunken lesions developed after 7-9 days (Figure 7B), systemic infection developed rarely with yellowing and deformation of leaves.

v. Phaseolus vulgaris L.

No noticeable symptoms were observed but the pathogen was consistently recovered from inoculated plants by transmission to cucumber.

c. Symptomatology of pathogen Q.

i. Cucumis sativus L. (var. Polaris)

Pale green chlorotic spots which sometimes joined together to form big blotchy patches developed on inoculated cotyledons. These were visible 48-72 hours after inoculation. Systemic mosaic
symptoms developed later with puckering and distortion on one side of the leaf (Figure 5B). Infected plants died generally within 20-45 days after inoculation.

ii. *Chenopodium quinoa* Willd.

Chlorotic lesions developed on the inoculated leaves 7-9 days after inoculation. Yellow coloured rings, chlorotic lines, streaks, bands and systemic mosaic symptoms developed on young systemically infected leaves (Figure 4B). These symptoms remained on the leaves for 5-9 days and became less pronounced as the leaves expanded. Young leaves developing in leaf axils showed symptoms up to flowering time.

iii. *Chenopodium amaranticolor* (Coste & Reyn.)

Pathogen Q induced a few necrotic lesions on inoculated leaves after 7-9 days. Line pattern symptoms later developed on new systemically infected leaves (Figure 6). Line pattern symptoms remained on leaves for another 9-11 days and became less pronounced as the leaves expanded.

IV. Physical properties of sap extracts of pathogens M and Q

Physical properties of the two pathogens such as dilution end point, thermal inactivation point, longevity *in vitro*, the effect of pH and stability in organic solvents were determined
in cucumber leaf extracts prepared in 0.01M phosphate buffer, pH 7.5.

Results of these experiments are summarised in Table 4. Pathogen M was inactivated after heating at 54°C but not at 52°C. It was infectious at a dilution of 1:10,000. It retained its infectivity after being kept at 25°C for at least 48 hours. Pathogen Q was inactivated after being heated at 56°C but not at 54°C, it was found to be infectious at a dilution of 1:100 but not at 1:1,000 and it was completely inactivated between 4-6 hours at 25°C. The infectivity of pathogen M was not greatly affected by acidification to pH 4.5-5.5 as nearly all the infectivity was recovered from the pellet after resuspension in 0.01M phosphate buffer, pH 7.5, whereas the infectivity losses of pathogen Q were higher at pH 4.5-5.5 but were only slightly affected between pH 6.0-7.5 (Figure 8). Pathogen M was inactivated within 50 minutes in organic solvents such as chloroform, carbon tetrachloride and ether, while pathogen Q was relatively stable in such organic solvents.

V. Identification of pathogen M

a. Attempts to purify the pathogen. It was at first thought that pathogen M was a virus. Attempts were therefore
Figure 3. Effect of pH on the infectivity of pathogen Q in cucumber leaf extracts.
Table 4
In vitro properties of the two pathogens isolated from rose cv. Peace

<table>
<thead>
<tr>
<th>Properties</th>
<th>Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pathogen M</td>
</tr>
<tr>
<td>Dilution end point</td>
<td></td>
</tr>
<tr>
<td>1 : 1</td>
<td>Not counted</td>
</tr>
<tr>
<td>1 : 10</td>
<td>198&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 : 100</td>
<td>68</td>
</tr>
<tr>
<td>1 : 1,000</td>
<td>46</td>
</tr>
<tr>
<td>1 : 10,000</td>
<td>8</td>
</tr>
<tr>
<td>Thermal inactivation point (°C)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>98</td>
</tr>
<tr>
<td>52</td>
<td>68</td>
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<tr>
<td>54</td>
<td>12</td>
</tr>
<tr>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>58</td>
<td>0</td>
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<tr>
<td>Longevity in vitro at 25°C</td>
<td></td>
</tr>
<tr>
<td>0 hrs</td>
<td>124</td>
</tr>
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<td>2 hrs</td>
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<td>4 hrs</td>
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<td>6 hrs</td>
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</tr>
<tr>
<td>8 hrs</td>
<td>76</td>
</tr>
<tr>
<td>pH</td>
<td>Stable at pH 4.5-5.5</td>
</tr>
<tr>
<td>Organic solvents</td>
<td>Unstable</td>
</tr>
</tbody>
</table>

a. Numbers represent the percentage of infected plants. In each experiment 40-50 cucumber seedlings were used for each dilution.

b. Numbers represent the average number of lesions per pair of cucumber cotyledons. In each experiment 10 cucumber seedlings were used.
made to purify the pathogen from cucumber extract by standard virological techniques. It was found that commonly used clarification procedures either greatly decreased the infectivity of the extracts or failed to clarify them satisfactorily. Several organic solvents known to be good for the initial clarification of extracts from virus infected tissues, completely inactivated the pathogen and the addition of chemicals used for the preservation of viruses (Fulton, 1967a) did not help to prevent this inactivation. Acidification of extracts to pH 4.8-5.0, followed by resuspending the pellet in 0.01M phosphate buffer, pH 7.5, removed most of the green material and often yielded preparations that were more infectious than the original extract. Precipitation of the infectious material with ammonium sulphate or ethanol was not successful and approximately 80% of infectious material was lost after the addition of salt. When all efforts to purify the pathogen failed, it was suspected that the pathogen was not a virus and, therefore, further experiments were carried out to identify it.

b. Filtration experiments. A single cucumber plant infected with pathogen M was ground with 5 ml sterile distilled water (SDW) and the sap was extracted through a double layer of muslin and passed through a 0.22 μ bacteriological filter. Samples of the
filtrate and the unfiltered material from the top of the filter were diluted with SDW and assayed on cucumber cotyledons. Sap from healthy cucumber plants was similarly extracted and passed through a bacterial filter as a control.

Table 5
Retention of pathogen M by a bacterial filter

<table>
<thead>
<tr>
<th>Extracts from</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/10</td>
</tr>
<tr>
<td>Infected plants</td>
<td>0/112²</td>
</tr>
<tr>
<td>Healthy plants</td>
<td>0/0</td>
</tr>
</tbody>
</table>

a. Numerator is the mean number of lesions per cotyledon induced by a filtered extract while the denominator is the number produced by an unfiltered extract. Each assay was carried out on four cucumber cotyledons.

Results of this experiment (Table 5) show that all the infectious material was retained by the bacterial filter.

c. Isolation of pathogenic agent on nutrient agar. Well separated single lesions on cucumber cotyledons were excised under a dissecting microscope. The tissue was suspended in a drop of SDW and streaked on King's B medium (King et al., 1954). Bacterial colonies appeared on the agar within 24 hours, six
colonies were selected and subcultured on nutrient agar. Each bacterial culture formed dirty white, flat, smooth surfaced colonies on agar.

d. Infectivity tests of agar cultured bacteria. The bacteria cultured for 24 hours on nutrient agar were suspended in SDW and mechanically inoculated to cucumber, C. tetragonoloba, V. sinesis and M. balsamina. Local lesions indistinguishable from those produced by sap from infected cucumber cotyledons were observed after 5-10 days, suggesting the isolated bacterium was identical to pathogen M.

Sap from infected cucumber and a bacterial suspension from nutrient agar cultures were inoculated to cucumber seedlings by several different techniques and control inoculations were carried out with both water and healthy cucumber sap.

Results presented in Table 6 indicate that when cultured bacteria or sap from infected cucumber cotyledons were inoculated to cucumber seedlings either by stem puncture or by dipping the roots in inocula, about 90% of the plants showed wilting symptoms and characteristic lesions on the cotyledons (Figure 9). When the two types of inocula were rubbed on carborundum-dusted cotyledons no wilting was observed but lesions appeared on the inoculated cotyledons.
Figure 9. Symptoms produced by pathogen M on cucumber seedlings inoculated by injecting a bacterial suspension into the stem. (Note wilting and drooping of cotyledon on one side.)
Table 6
Infectivity of pathogen M inoculated to cucumber seedlings by various techniques

<table>
<thead>
<tr>
<th>Methods of inoculation</th>
<th>Inocula</th>
<th>Experiment Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Stem puncture</td>
<td>i. Plant culture</td>
<td>5/8^a</td>
</tr>
<tr>
<td></td>
<td>ii. Bacterial culture</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>iii. Control</td>
<td>0/8</td>
</tr>
<tr>
<td>Roots dipped</td>
<td>i. Plant culture</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>ii. Bacterial culture</td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td>iii. Control</td>
<td>0/8</td>
</tr>
<tr>
<td>Half cotyledon dipped</td>
<td>i. Plant culture</td>
<td>3/8</td>
</tr>
<tr>
<td></td>
<td>ii. Bacterial culture</td>
<td>5/8</td>
</tr>
<tr>
<td></td>
<td>iii. Control</td>
<td>0/8</td>
</tr>
<tr>
<td>Cotyledons rubbed</td>
<td>i. Plant culture</td>
<td>32^b</td>
</tr>
<tr>
<td></td>
<td>ii. Bacterial culture</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>iii. Control</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Total number of plants wilted/total number of plants inoculated.
b. Average number of lesions per pair of cucumber cotyledons. Four plants were used for each culture.

e. Electron microscopy. Pathogen M cultured for 24 hours on nutrient agar was suspended in 0.05% bovine serum albumin, stained with PTA and examined immediately in a Philips 100B electron microscope. Bacilliform cells with bunches of bipolar flagella were observed (Figure 10).
Figure 10. Electron micrograph of pathogen M showing a bacilliform bacterial cell with bipolar flagellae.
f. **Biochemical tests on cultured pathogen.** Dr. A. Kerr performed the biochemical tests on the bacterial culture of pathogen M. It utilized glucose oxidatively (Hugh and Leifson, 1953), was oxidase negative (Kovacs, 1956), 3-ketolactose negative (Bernaerts and DeLey, 1963), did not hydrolyse starch and did not produce fluorescent pigment on King’s B medium. On the basis of these tests the bacterium was identified as a non-fluorescent species of *Pseudomonas*. The culture was sent to Dr. A.C. Hayward, at the Department of Microbiology, University of Queensland, who, after further experiments identified it as being closely related to *Pseudomonas syringae* (Van) Hall.

VI. **Identification of pathogen Q**

a. **Seroology.** Pathogen Q extracted from cucumber leaves and concentrated by centrifugation at 105,000 g for 90 minutes was tested by gel diffusion against an antiserum prepared against RMV from Wisconsin. Two precipitin lines were observed between the wells containing antigen and antiserum. By placing healthy concentrated cucumber sap in an adjacent well, only one of the precipitin lines was shown to be virus specific (Figure 11).
Figure 11. Serological test of pathogen Q in agar gel.
The symbols denote the preparations added to the wells in the agar gel:
A/S Antiserum to RMV.
D Healthy cucumber sap.
H Pathogen Q preparation.
The *in vitro* properties of pathogen Q in cucumber leaf extracts such as dilution end point, thermal inactivation point, the longevity in vitro at 25°C and its pH tolerance (Table 4) are very similar to those reported for RMV (Fulton, 1952, 1967b).

VII. Conclusions

In the experiments described above, the presence of a plant pathogenic bacterium together with a virus was demonstrated in rose. One of the pathogens was shown to be a virus very similar to the RMV described in North America and the other one was shown to be a bacterium belonging to the genus *Pseudomonas*. These experiments illustrate the dangers of identifying a pathogen as a virus on the basis of virus-like symptoms produced on test plants inoculated by standard virological techniques. The *P. syringae* isolate from rose (pathogen M) appears to be similar to that isolated from pear petals and cherry buds by Ramaswamy and Garrett (1970). A similar misidentification of a bacterium for a plant virus has previously been reported by Yarwood *et al.* (1961). They demonstrated that the disease initially described as beet latent virus (Smith, 1951) was actually caused by *Pseudomonas aptata*. A similar confusion has been reported by animal virologists where *P. aeruginosa* was found to form virus-like plaques in a human tissue culture system (Ludovici and
Symptom expression on rose and some properties of the virus isolated from rose in South Australia are very similar to those reported for RMV from North America (Fulton, 1952). However, the South Australian isolates differ in their reactions on the herbaceous hosts studied. It appears that South Australian isolates of RMV have a very narrow host range and their failure to infect some plant species (*C. tetragonoloba*, *M. balsamina*, *V. sinesie*), susceptible to RMV in North America, is puzzling. The *P. syringae* isolate from rose produced symptoms on *C. tetragonoloba* which were very similar to those reported for RMV (Fulton, 1952), and the symptoms on *M. balsamina* were similar to those reported for NRSV (Fulton, 1957). One possible explanation of these apparent differences in the host range of RMV isolates from South Australia and North America may be that the North American isolates were contaminated by a *Pseudomonas* sp. which produces symptoms similar to the South Australian *M* isolates.
SECTION III

TRANSMISSION OF ROSE MOSAIC VIRUS

Several viruses are known to infect pollen as well as being carried in the seed. Bennett (1969) recorded 53 seed transmitted viruses in about 132 plant species, and includes NRSV amongst the viruses which are both pollen and seed transmitted. It has already been shown by Fulton (1967b, 1968) and in this thesis (Table 26) that RMV is a member of the Prunus necrotic ringspot virus group and yet no information is available about the seed transmission properties of RMV.

The present study was undertaken to investigate the association of RMV with pollen and seeds of rose, cucumber and C. quinoa.

a. Tests for infection of pollen by RMV. Samples of pollen from cucumber, C. quinoa and rose flowers were each ground with 0.3 ml of 0.01M phosphate buffer, pH 7.5, and the extracts were assayed for infectivity. Results of these tests (Table 7) show that virus was readily recovered from pollen collected from infected cucumber and C. quinoa but not from infected roses.
Table 7
Tests for the presence of RMV in pollen

<table>
<thead>
<tr>
<th>Pollen collected from infected plants of:</th>
<th>Infectivity of buffer extract on</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cucumber</td>
<td>C. quinoa</td>
<td></td>
</tr>
<tr>
<td>Rose</td>
<td>0/20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/20</td>
<td></td>
</tr>
<tr>
<td>Cucumber</td>
<td>17/20</td>
<td>13/20</td>
<td></td>
</tr>
<tr>
<td>C. quinoa</td>
<td>11/20</td>
<td>4/20</td>
<td></td>
</tr>
</tbody>
</table>

<br>

<sup>a</sup>. Numerator represents total number of plants infected while the denominator represents the total number of plants inoculated.

Several attempts were made to transmit virus from rose pollen. Ascorbic acid (Fulton, 1967a), nicotine base (Cadman, 1959), thioglycollic acid and diethylidithiocarbamate (DIECA) (Tremaine <i>et al.</i>, 1969) were used in an endeavour to aid virus transmission. However, all attempts to transmit virus from rose pollen to herbaceous hosts were unsuccessful.

An experiment was carried out to test if pollen can transmit RMV to cucumber plants. Twelve unopened female flowers were selected and covered with paper bags. Control flowers were labelled and left open for natural pollination.
The pollination with pollen from infected flowers was carried out by placing pollen on the stigma using a fine camel-hair brush. Flowers were covered again after pollination and the covers removed when fruits were set and had grown to a length of 1½ - 2 inches. Fruits were collected when fully mature.

Three out of the 12 hand-pollinated flowers developed normal fruits and the remaining 9 did not develop and died within 15 days of pollination. Results of this experiment are presented in Table 8. There was marked effect on the quality and quantity of seeds produced by each fruit. Control fruits produced 10 - 12 times more seed.

Table 8
Test for RMV transmission through pollen to cucumber seeds

<table>
<thead>
<tr>
<th>No. of fruit</th>
<th>Seeds harvested from each fruit</th>
<th>Total No. of seeds</th>
<th>Percentage of seeds germinated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seed sown</td>
<td>Seed germinated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>44 a</td>
<td>42</td>
<td>134</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>16</td>
<td>68</td>
</tr>
</tbody>
</table>

a. Defective seeds did not germinate.
b. Total numbers of seeds obtained from each fruit.
Seeds obtained were sown and observed for virus symptoms. No virus symptoms were detected in any of the seedlings raised from these seeds.

b. Tests for transmission of RMV through seeds from infected plants. Seed transmission tests could not be carried out on cucumber seeds from RMV infected plants because the majority of infected seedlings died within 30 days of inoculation, and those that survived set fruit without seeds (Figure 12). With rose and C. quinoa two experiments were carried out to test for the presence of virus in seed. In the first, seeds were ground in 0.01M phosphate buffer, pH 7.5, and the extracts assayed for infectivity on cucumber. No virus was detected by this method in any of the tests carried out.

In the second experiment, rose seedlings raised from seeds collected from infected plants were observed at weekly intervals for the development of symptoms. In one of two such experiments, 3 rose seedlings out of a total of 46 which survived, showed disease symptoms (Table 9). These seedlings showed line pattern and chlorotic ring symptoms 15 days after germination (Figure 13) which were similar to those observed on the parent plant (Figure 1a). The presence of RMV in these
Figure 12.  

A. Fruit harvested from RMV infected cucumber seedling showing mosaic symptoms.  

B. Two halves of the fruit showing that there was no seed formation.
Figure 13. Virus symptoms on rose seedling 15 days after germination.
Table 9
Tests of RMV transmission through seeds

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Seeds obtained from infected plants</th>
<th>No. of seeds sown</th>
<th>No. of seeds germinated</th>
<th>No. of plants survived</th>
<th>No. of plants developed virus symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rose</td>
<td>112</td>
<td>34</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>C. quinoa</em></td>
<td>300</td>
<td>240</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Rose</td>
<td>138</td>
<td>52</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>C. quinoa</em></td>
<td>400</td>
<td>328</td>
<td>300</td>
<td>0</td>
</tr>
</tbody>
</table>

Plants was confirmed by graft transmission to peach seedlings, followed by mechanical transmission to cucumber seedlings. In a parallel experiment no virus symptoms were observed on *C. quinoa* seedlings.

It has been shown (Table 7) that RMV is present in the pollen of infected *C. quinoa* plants, but virus transmission through seeds of infected *C. quinoa* could not be demonstrated. This is surprising because most viruses which are carried by pollen are also seed transmitted (Matthews, 1970). In order to obtain some relevant information about this phenomenon, samples of flowers (sepals, petals, anthers and stigma), immature seeds and dry seeds were collected from infected *C. quinoa* plants.
Each individual sample which consisted of 20 flowers and seeds was ground with 0.3 ml of 0.01M phosphate buffer, pH 7.5, and the extract was assayed for infectivity on 20 cucumber seedlings. Results of the experiments (Table 10) show that virus was readily recovered from flowers and immature seeds, but no virus was detected in dry seeds.

**Table 10**

Detection of RMV in tissue of *C. quinoa* reproductive organs

<table>
<thead>
<tr>
<th>Tissue extract from</th>
<th>Infectivity assay on cucumber seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flower</td>
<td>a17/20</td>
</tr>
<tr>
<td>Immature seed</td>
<td>6/20</td>
</tr>
<tr>
<td>Dry seed</td>
<td>0/20</td>
</tr>
</tbody>
</table>

a. Numerator is the total number of plants infected while the denominator is the total number of plants inoculated.

c. Return transmission of virus from herbaceous plants to woody hosts. There are remarkably few reports on the successful return transmission of viruses from herbaceous plants to their original woody hosts and yet this is necessary to fulfil Koch's postulate in showing the pathological nature of
a virus isolate. Therefore, it was considered necessary to attempt to return RMV to rose and peach seedlings. Various methods were employed (Table 11), and rose seedlings at the 6-8 leaf stage were selected for the experiment.

Table 11
Attempts to transmit RMV to rose and peach seedlings

<table>
<thead>
<tr>
<th>Technique employed</th>
<th>Infectious material recovered from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rose</td>
</tr>
<tr>
<td>Mechanical inoculation with concentrated virus</td>
<td>8/4</td>
</tr>
<tr>
<td>Mechanical inoculation with partially purified virus</td>
<td>0/4</td>
</tr>
<tr>
<td>Grafted with infected cucumber tissue</td>
<td>0/4</td>
</tr>
<tr>
<td>Approach grafted with infected C. quinoa*</td>
<td>0/4</td>
</tr>
</tbody>
</table>

a. Numerator represents total number of seedlings that became infected while denominator represents total number of seedlings inoculated.

* C. quinoa seedlings were inoculated with concentrated virus.

All attempts to return RMV to rose seedlings were unsuccessful. However, virus was returned to peach seedlings by approach grafts between RMV infected C. quinoa and healthy peach seedlings (Figure 14).
Figure 14. Approach graft between RMV-infected *C. quinoa*
and healthy peach seedling demonstrating virus return.
d. Tests for stylet-borne transmission of RMV. In this study a single insect species, *M. persicae*, was selected as a vector to test if RMV could be transmitted by aphids. QCMV was also used in an experiment as it is known to be readily transmitted by the aphid. *M. persicae* fasted for 2–3 hours were fed on the infected cucumber plants for 20, 40 and 60 minutes. Four aphids were transferred to each healthy cucumber seedling and allowed to feed 2–4 hours after which the aphids were killed with a systemic insecticide and the plants were kept for symptom development.

Results of the experiment (Table 12) show that QCMV was readily transmitted by *M. persicae*, whereas RMV was not under the same experimental conditions.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Feeding time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 min.</td>
</tr>
<tr>
<td>Rose mosaic virus (RMV)</td>
<td>50/32</td>
</tr>
<tr>
<td>Cucumber mosaic virus (QCMV)</td>
<td>8/24</td>
</tr>
</tbody>
</table>

a. Numerator is the total number of seedlings that became infected while the denominator is the total number of seedlings on which aphids were fed.
e.  **Attempts to transfer RMV through soil.** To check the possibility that RMV is transmitted through soil, two groups of soil were selected and used without sterilization.

A. Soil which contained RMV infected cucumber plants grown and maintained for at least 60 days.

B. Soil samples collected within a radius of 6"-12" from infected roses.

Cucumber seeds were sown as "bait plants" in pots containing the soils. Twelve to 16 seeds were sown in each pot and 24 pots were used for each soil type. The experiment was repeated twice but no virus symptoms developed on any of the cucumber seedlings.

f.  **Conclusions.** The preliminary experiments described above present evidence that RMV can be detected by infectivity assay in pollen of two infected plant species, but not in that from roses. It seems likely that the ability of RMV to invade pollen resembles that reported previously for other viruses belonging to the *Prunus* necrotic ringspot virus group. For example, Das *et al.* (1961) reported up to 0.5% virus transmission through the seeds from plants of *Cucurbita maxima* L. pollinated by NRSV infected pollen, and Way and Gilmer (1958) obtained up to 27.8% virus transmission through the seed of flowers pollinated
by infected pollen of *Prunus cornus*. Das and Milbrath (1961) reported the transmission of NRSV in squash fruit through pollen, and Gilmer (1965) reported that the mother plant itself became infected through pollen. The failure of RMV to infect cucumber seeds, but to cause sterility appears to be similar to that reported by Crowley (1957) for tomato aspermy virus, and as suggested by Allard (1915) for tobacco mosaic virus.

Limited information is available regarding the inability of viruses to invade seeds (Bennett, 1969). The present results show that RMV is apparently capable of infecting *C. quinoa* seeds, and its behaviour appears to be somewhat similar to the case described previously by Cadman (1965), with apple chlorotic leaf spot virus. He reported that virus can be detected in pollen of infected *C. quinoa* and *C. amaranthicolor*, but found no seed transmission in either of the species of *Chenopodium*. The fact that RMV can be detected in immature seeds of *C. quinoa* seems to be similar to the case reported with southern bean mosaic virus (Cheo, 1955); this virus can be isolated from the embryo of immature bean seeds but by the time the seed dries, virus can no longer be detected. A similar phenomenon was observed by Ford (1966) with pea streak virus in peas.
SECTION IV

ISOLATION, TRANSMISSION AND IDENTIFICATION OF

P. SYRINGAE (VAN) HALL.

It has been described in the first section of this thesis that RMV is often isolated from roses together with a strain of P. syringae which produces virus-like symptoms on certain host plants (Figures 4A, 5A, 7A, 7B). The reported host ranges of RMV (Fulton, 1952) and NRSV (Fulton, 1957) are very similar. Some symptoms on several plants reported as hosts of both RMV and NRSV coincide with the symptoms shown in this thesis to be caused by P. syringae isolated from rose (Figures 4A, 5A, 7A, 7B). It was therefore decided to test if P. syringae could be isolated from cherry trees suspected of being infected with NRSV. In this section of the thesis are described results of experiments on the isolation and some properties of P. syringae from rose plants and cherry trees.

a. Isolation of bacteria pathogenic to cucumber from rose and cherry wood. Young wood from eight rose and five cherry was tested for the presence of plant pathogenic bacteria in stem tissue. Small pieces of the material, swabbed in 95% alcohol, were surface sterilized by flaming, bark was removed and pieces of the wood tissue transferred to plates of King's B medium.
Bacterial colonies were observed within 24 hours and selected smooth surfaced colonies were subcultured on nutrient agar.

The pathogenicity of individual sub-cultures from nutrient agar was tested by mechanical inoculation to cucumber. Bacteria, both pathogenic and non-pathogenic to cucumber, were isolated from rose and cherry wood. Non-pathogenic bacteria generally produced more than one type of colony on nutrient agar. Pathogenic bacterial isolates from both rose and cherry wood had very similar properties to those obtained from rose by grafting rose bark to peach seedlings followed by mechanical inoculation to cucumber seedlings.

b. **Simultaneous isolation of Prunus necrotic ringspot virus and Pseudomonas from cherry trees.** Samples of petals and young leaves were collected from 19 trees of 5 cherry varieties showing ringspot symptoms. Extracts of the material were mechanically inoculated to leaves of cucumber, *M. balsamina* and *C. amaranticolor*. Results of these tests are summarized in Table 13.

From the symptoms produced on cucumber and *M. balsamina* inoculated with extracts from *S. s* and *WF* trees (Table 13), it was suspected that *P. syringae* was present as similar symptoms were observed previously when *P. syringae* was shown to contaminate
Table 13
Isolation of *Prunus* necrotic ringspot virus and *Pseudomonas syringae*
from cherry trees to three herbaceous plant species

<table>
<thead>
<tr>
<th>Cherry variety</th>
<th>Code</th>
<th>Reactions on test plants</th>
<th>Presence of <em>P. syringae</em></th>
<th>Presence of NRSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>William's favorite</td>
<td>WF</td>
<td>Chlorotic and necrotic lesions, mottling</td>
<td>Few necrotic lesions on inoculated leaves</td>
<td>+</td>
</tr>
<tr>
<td>Delta</td>
<td>DT</td>
<td>Chlorotic mottling systemic mosaic</td>
<td>No infection</td>
<td>-</td>
</tr>
<tr>
<td>Morello</td>
<td>MO</td>
<td>Systemic mosaic</td>
<td>No infection</td>
<td>-</td>
</tr>
<tr>
<td>Eagle</td>
<td>BG</td>
<td>Systemic mosaic</td>
<td>No infection</td>
<td>-</td>
</tr>
<tr>
<td>Seedling 6</td>
<td>S6</td>
<td>Necrotic lesions and systemic mosaic</td>
<td>Few necrotic lesions on inoculated leaves</td>
<td>+ +</td>
</tr>
</tbody>
</table>

a. Shown by isolation on nutrient agar and re-inoculation to cucumber and *M. balsamina*.
b. Shown by serological test.
c. + denotes presence of pathogen.
- denotes absence of pathogen.
isolates of RMV (see Figures 3A, 4A). Difficulties were encountered in the direct isolation of the pathogen on nutrient agar from the tissue under the lesions on M. balsamina. Isolation was much easier when the culture from M. balsamina was transmitted to cucumber before isolation on nutrient agar.

The identification of the bacterial pathogen isolated from the $S_6$ extract was confirmed as P. syringae by Dr. A.C. Hayward of the Department of Microbiology, University of Queensland. Both the $S_6$ and WF bacterial isolates were also identified as P. syringae by their symptoms on indicator hosts, their reaction with antiserum to the P. syringae isolated from rose, and their growth characteristics on nutrient agar.

All six virus isolates obtained from cherry (Table 13) were identified as NRSV by concentrating leaf extracts (see Materials and Methods) from infected cucumber plants and testing against NRSV antiserum from Wisconsin in gel diffusion plates.

A procedure developed for the purification of RMV (Figure 21) was successfully employed for the purification of the cherry isolates of NRSV from infected cucumber, and partially purified virus preparations had ultraviolet spectra characteristic of nucleoprotein. Samples of the virus preparation analysed by sucrose density gradient centrifugation separated into three
Figure 15. Symptoms produced by 3 isolates of *Pseudomonas*
on cucumber seedlings.

A. *Pseudomonas pisi* (PP).

B. *Pseudomonas syringae* (IS).

C. *Pseudomonas syringae* (CS).

D. Not inoculated (control).
ultraviolet absorbing zones similar to those obtained when RMV was subjected to the same procedure (Figures 23, 24A, 24D and 24F). Material collected under the ultraviolet absorbing zones was highly infectious.

c. The host range of the *Pseudomonas syringae* isolates from rose, cherry and six other *Pseudomonas* cultures.

The *P. syringae* isolate from rose induced local lesions on several plant species (Figures 4A, 5A, 7A, 7B). This isolate designated RS, was compared to the isolate from cherry (CS), two other isolates of *P. syringae* and 4 other species of *Pseudomonas* on four herbaceous plants on which RS produced local lesions.

The results summarized in Table 14 show that the RS, CS and LS isolates of *P. syringae* produced similar lesions on susceptible test plants whereas the VS isolate did not produce symptoms on any of the four plant's tested. VS had been kept in culture for some 15 years at the Victorian Plant Research Institute and hence it may have lost its pathogenicity. Although RS, CS and LS all produced local lesions on the four indicator plants used (Table 14), there were some significant differences in the lesion type and the time of their appearance (Figure 15). From the other species of *Pseudomonas* tested, only *P. pisi* (PP) was able to produce local lesions on the four hosts tested.
### Table 14
Pathogenicity tests of different *Pseudomonas* isolates by mechanical inoculation to selected hosts

<table>
<thead>
<tr>
<th>Pseudomonas isolate*</th>
<th>Original source of isolation</th>
<th>Abbreviation</th>
<th>Experimental test plants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. syringae</strong></td>
<td>Rose</td>
<td>RS</td>
<td>Small necrotic lesions within 5-7 days; limited systemic symptomless infection (Figure 5A)</td>
</tr>
<tr>
<td><strong>P. syringae</strong></td>
<td>Cherry</td>
<td>CS</td>
<td>Similar to RS (Figure 15C)</td>
</tr>
<tr>
<td><strong>P. syringae</strong></td>
<td>Lemon</td>
<td>LS</td>
<td>Necrotic flecking within 3 days became more pronounced later (Figure 15B)</td>
</tr>
<tr>
<td><strong>P. syringae</strong></td>
<td>?</td>
<td>VS</td>
<td>No infection</td>
</tr>
<tr>
<td><strong>P. vitis</strong></td>
<td>Pea</td>
<td>PP</td>
<td>Necrotic flecking and lesions within 3 days (Figure 15A)</td>
</tr>
<tr>
<td><strong>P. phaselicola</strong></td>
<td>Bean</td>
<td>SB</td>
<td>No infection</td>
</tr>
<tr>
<td><strong>P. fluorescens</strong></td>
<td>Soil</td>
<td>SS</td>
<td>No infection</td>
</tr>
<tr>
<td><strong>P. coronafacalis</strong></td>
<td>?</td>
<td>SC</td>
<td>No infection</td>
</tr>
</tbody>
</table>

* Isolates of *Pseudomonas*, vis. LS, PP, SB, SS and SC were obtained from Dr. A. Kerr of this Department and isolate VS was obtained from the Victorian Plant Research Institute, Burnley, Victoria.
Figure 16. Symptoms produced by two isolates of

*P. syringae* on cowpea.

A. Small dark necrotic lesions produced by

*P. syringae* (LS) 3 days after inoculation.

B. Small dark necrotic lesions produced by

*P. pisi* (PP) 3 days after inoculation.
d. **Serology of P. syringae and comparison with other isolates of Pseudomonas.** A composite antigen preparation from RS prepared as described in "Materials and Methods" was tested against homologous antiserum in a gel diffusion test. Three precipitin lines were observed between the wells containing antigen and antiserum. There were no major differences observed between RS and CS antigens when tested against RS antiserum but RS antiserum failed to react with isolates VS, SB, SS and SC (Figure 17a). However, the number of precipitin lines varied when antigens from RS and CS and 6 other Pseudomonas isolates were tested against antisera to six *P. syringae* serotypes occurring in California. No serological reaction was observed between antigens from the 8 isolates of Pseudomonas and antiserum to *P. syringae* serotype II (Figures 17h, 18).

Three isolates of *P. syringae*, RS, CS and LS, which appeared to be similar in their ability to produce local lesions on 4 test plants were tested simultaneously against 8 antisera of *P. syringae* in a gel diffusion test. It appears from the results (Figure 16) that isolates RS and CS reacted serologically with serotypes IIIa and IVb showing confluent immunodiffusion bands, and there was no obvious crossing of bands. However, small differences were observed between RS and CS when antigens from
Figure 17. Serological reactions of 8 isolates of *Pseudomonas* with antisera of 8 *P. syringae* serotypes. Central wells contain antisera to *P. syringae* serotypes (a) RS, (b) IIIa, (c) IIIb, (d) IIIc, (e) IV, (f) IVa, (g) IVb, and (h) II. Outer wells in each pattern contain composite antigens of 8 isolates of *Pseudomonas* RS (1), LS (2), VS (3), PP (4), CS (5), SB (6), SC (7) and SS (8).
Figure 13. Serological reactions of 3 isolates of *P. syringae* with antisera to 8 *P. syringae* serotypes. Central wells contain antisera to *P. syringae* serotype IVb, IIIa, II, RS, IVa, IV, IIIb and IIIc. Outer wells contain composite antigens of 3 isolates of *P. syringae* RS (A), LS (B) and CS (C).
these two bacteria were tested in adjacent wells against serotype IV (Figure 18). Other antigenic differences may have gone undetected as RS and CS were not tested in adjacent wells against serotypes IIIc, IVa and IIIb.

e. Return transmission of *P. syringae* to woody hosts. It was necessary to return the *P. syringae* isolated from rose (RS) back to rose in order to fulfil Koch's postulate. RS was also inoculated to other woody plants.

Small seedlings of rose, pear, peach and cherry were used for the experiment. Freshly cultured bacterial suspensions in SDW were mechanically inoculated on carborundum-dusted leaves, followed by two stem puncture inoculations near the base of the seedling stems. This procedure was repeated after 2 days. A total of 2 cherry, 4 pear, 4 rose and 12 peach seedlings were inoculated by this technique. One plant of each type kept as control with the experiments was inoculated by the same procedure with SDW.

After 3–4 weeks, young leaves from the inoculated seedlings were macerated with small amounts of SDW and mechanically inoculated to carborundum-dusted leaves of cucumber and *M. balsamina*.Extracts prepared from pear and cherry leaves were non-infectious. *P. syringae* was recovered successfully from 3
Figure 12. Leaves of rose and peach seedlings showing bacterial lesions.

A. Necrotic lesions with yellow margins similar to those observed on cucumber, developed on rose 65 days after inoculation with RS cultured on nutrient agar.

B. Necrotic lesions on peach leaves, developed 45 days after inoculation with RS cultured on nutrient agar.
rose and 10 peach seedlings. It was also observed that a few necrotic lesions with yellow margins developed 45-65 days after inoculation on some of the peach and rose seedlings (Figure 19A, 19B). Bacteria were recovered from these lesions by inoculation to cucumber seedlings and the identity of the pathogen was confirmed by transfer of the bacteria to nutrient agar and then by inoculation of bacterial suspensions to selected test plants.

f. **Conclusions.** The experiments described above show that several isolates of *Pseudomonas* produce virus-like symptoms on selected experimental test plants which are similar in many respects to those produced by *P. syringae* isolated from rose. It appears that *M. balsamina* is the most suitable test plant for detecting the pathogenic *Pseudomonas* regardless of their host of origin.

Evidence has been presented to show that *P. syringae* and NRSV can be isolated simultaneously from cherry trees in the same way as that described for rose (Figures 4A, 5A, 7A and 7B). An isolate of *P. syringae* from cherry leaves and buds which induced virus-like symptoms on cucumber and cowpea has been reported by Ramaswamy and Garrett (1970). The presence and systemic distribution of *P. syringae* in cherry and in other deciduous stone fruit trees has been reported by Wilson (1935) and Cameron
(1962, 1970). It was shown that \textit{P. syringae} can be eliminated from cultures of RMV by passage through \textit{C. quinoa} (Figure 4B); similarly it appears that the bacterium can be eliminated from NRSV cultures by passage through \textit{C. amaranthicolor}.

Identification of bacteria by serology and their separation into serological sub-groups is difficult as has previously been shown using agglutination tests (Mushin and Lahovary, 1958; Perlasca, 1960) and gel diffusion tests (Loverkovich and Klement, 1961; Loverkovich \textit{et al.}, 1963; Lucas and Grogan, 1969; Otto and English, 1971). In the present study gel diffusion test with RS and CS has shown that the two isolates have very similar antigenic properties when tested against RS antiserum. Serological relationships were also observed when the two isolates were tested against antisera to six Californian serotypes of \textit{P. syringae} (IIIa, IIIb, IIIc, IV, IVa and IVb). However, RS and CS appear to be unrelated to \textit{P. syringae} of serotype II. Isolate LS of \textit{P. syringae} and an isolate of \textit{P. pisi} (PP) were shown to have some antigens in common with six bacteria belonging to different serotypes of \textit{P. syringae} but could be differentiated serologically from both RS and \textit{P. syringae} serotype II. These results suggest that serology cannot be used as a test for the identification of \textit{P. syringae} to species level because other species of \textit{Pseudomonas}
were also shown to cross react with antisera to \textit{P. syringae}.
A similar conclusion has been reached by Otta and English (1971),
who observed that \textit{P. syringae} serotype IIIb was serologically
related to 4 different species of \textit{Pseudomonas}: \textit{P. antirrhini},
\textit{P. savastanoi}, \textit{P. tomato} and \textit{P. mori}.
SECTION V
PURIFICATION AND SOME PROPERTIES OF RMV

The viruses isolated from rose showing line pattern symptoms and described in the first section of this thesis have been identified as rose mosaic virus (RMV) by serology using an antiserum prepared against RMV from Wisconsin. In most of the previously reported investigations, no attempts appear to have been made to characterise the virus in detail. However, Fulton (1967b, 1968) reported that purified preparations of RMV were heterogeneous when subjected to sucrose density gradient centrifugation, that they contained polyhedral particles 25 nm in diameter, and that they shared some antigens with NRSV.

In this section are reported the results of experiments carried out in developing a method of purifying RMV different to that used by Fulton (1967b). Most of this work was carried out before Fulton's paper was available. However, some of the steps of his method were tested later but, in my hands, they were found to be less satisfactory than the methods described here. Some properties of purified RMV were investigated and are also reported in this section.

I. Purification of the virus

a. Rate of increase of virus in infected cucumber tissue and a suitable time of harvest for purification. To determine the rate of increase of virus in infected tissue and the best time
to harvest the infected material for purification, two batches each of 28 plants were inoculated. Each individual seedling was ground with 0.5 ml of 0.01M phosphate buffer, pH 7.5. The extract was assayed on 5 cucumber seedlings without dilution. This sampling and assay procedure was commenced two days after inoculation and was repeated at daily intervals until the tenth day. Later, samples were taken at two-day intervals.

**Table 15**

Rate of increase of RMV in systemically infected cucumber plants

<table>
<thead>
<tr>
<th>Days of inoculation</th>
<th>Infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td>2 days</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 days</td>
<td>24</td>
</tr>
<tr>
<td>4 days</td>
<td>38</td>
</tr>
<tr>
<td>5 days</td>
<td>68</td>
</tr>
<tr>
<td>6 days</td>
<td>82</td>
</tr>
<tr>
<td>7 days</td>
<td>98</td>
</tr>
<tr>
<td>8 days</td>
<td>98</td>
</tr>
<tr>
<td>9 days</td>
<td>99</td>
</tr>
<tr>
<td>10 days</td>
<td>92</td>
</tr>
<tr>
<td>12 days</td>
<td>90</td>
</tr>
<tr>
<td>14 days</td>
<td>82</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of seedling developing symptoms. Each assay was carried out on 50-40 plants.

Results of two such experiments (Table 15) show that virus was detected on the second day after inoculation and maximum infectivity of extract was reached about 7 days after infection.
In all subsequent experiments infected material was harvested between 7 and 12 days after inoculation.

b. **Longevity of virus in cucumber tissue stored at 4°C.**

To investigate the longevity of virus in cucumber tissue upon storage at 4°C, two groups each of 28 infected plants with similar appearance, were harvested 7 days after inoculation. The whole aerial portion of each plant was ground with 0.5 ml of 0.01M phosphate buffer, pH 7.5, and assayed on 5 cucumber plants without dilution. Two plants were assayed each day for 7 days.

<table>
<thead>
<tr>
<th>Days of storage at 4°C</th>
<th>Infectivity²</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment I</td>
<td>Experiment II</td>
<td></td>
</tr>
<tr>
<td>same day</td>
<td>98²</td>
<td>100²</td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>98</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>96</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>4 days</td>
<td>97</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td>86</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>6 days</td>
<td>68</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>62</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>

a. Percentage of seedlings developing symptoms. Each assay was carried out on 30-40 plants.
Results of two such experiments (Table 16) show that the amount of virus extracted from stored cucumber did not decrease significantly over a period of at least 4 days.

c. Stabilization of virus in plant extracts. Usually 0.01M - 0.05M phosphate buffers were used for extracting the virus from cucumber tissue. Like some other unstable polyhedral

<table>
<thead>
<tr>
<th>Chemical supplement</th>
<th>Concentration of chemicals</th>
<th>Infectivitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.5%</td>
<td>98</td>
</tr>
<tr>
<td>Cysteine-HCl</td>
<td>0.5%</td>
<td>98</td>
</tr>
<tr>
<td>2 Mercaptoethanol</td>
<td>0.2%</td>
<td>90</td>
</tr>
<tr>
<td>Sodium sulphite</td>
<td>0.5%</td>
<td>92</td>
</tr>
<tr>
<td>Sodium diethyl-</td>
<td>0.1%</td>
<td>83</td>
</tr>
<tr>
<td>dithiocarbamate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylene diamine-</td>
<td>0.1%</td>
<td>76</td>
</tr>
<tr>
<td>tetracetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioglycollic acid</td>
<td>0.1%</td>
<td>92</td>
</tr>
<tr>
<td>Ascorbic acid +</td>
<td>0.2%</td>
<td>98</td>
</tr>
<tr>
<td>Cysteine-HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid +</td>
<td>0.2%</td>
<td>90</td>
</tr>
<tr>
<td>Sodium sulphite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>96</td>
</tr>
</tbody>
</table>

a. Percentage of seedlings developing symptoms. Each assay was carried out on 40-50 plants.
### Table 18
Effect of chemical additives on the infectivity of RMV in cucumber extract

<table>
<thead>
<tr>
<th>Chemical supplements</th>
<th>Infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration of chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>Cysteine-hydrochloride</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>Thioglycolic acid</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>Ethylene diaminetetra-acetic acid (EDTA)</td>
<td>98</td>
<td>90</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of seedlings developing symptoms. Each assay was carried out on 40–50 plants. Extracts were kept in an ice bucket for 30 minutes before inoculation.

Viruses (Fulton, 1967a), RMV loses its infectivity in cucumber sap within 4 hours at 25°C (Table 17). To achieve its purification it was considered essential to maintain the infectivity of the virus for longer periods, and hence various chemicals reported as virus stabilizing agents were tested. The initial extract was treated with a range of concentrations and combinations of such chemicals, and infectivity assays were carried out on cucumber seedlings at intervals of 2 hours.

Results of two such experiments (Tables 17 and 18) show
that infectivity was best stabilized by the addition of 2 ml of 0.01% ascorbic acid per 10 ml of cucumber extract. In all future experiments this concentration of ascorbic acid was used in extraction buffers.

d. **Routine procedure for extraction of RMV from cucumber plants.** In further experiments aerial parts of cucumber seedlings were harvested 7-12 days after inoculation and stored at 4°C until required (up to 4 days). One hundred grams of tissue was ground with 100 ml of 0.01M phosphate buffer, pH 7.5, and 20 ml of 0.01% ascorbic acid in a Waring blender for 3-5 minutes. The Waring blender, tissue, buffer and ascorbic acid were pre-cooled to 4°C. The pulp was strained through a double layer of muslin and centrifuged at 15,000 g for 20 minutes. The sediment was discarded.

e. **Clarification of extract.** Attempts were made to clarify the virus extract with calcium phosphate gel (HCP) as used by Fulton (1959), but variable and unpredictable losses were encountered (Table 19), hence several organic solvents were tested as clarifying agents (Figure 20). Butanol, Freon 113 and chloroform all caused serious virus losses and, therefore, were considered unsatisfactory. Emulsification of the extract with either anhydrous ether or carbon tetrachloride caused only slight virus losses (Figure 20) and
Figure 20. Effect of various clarifying agents on the infectivity of RMV in cucumber extract.

- control
- ether
- carbon tetrachloride
- calcium phosphate gel
- chloroform
Table 19
Effect of HCP on RMV in cucumber extracts

<table>
<thead>
<tr>
<th>Infectivity comparison</th>
<th>Experiment number</th>
<th>Dilution 1/1</th>
<th>1/10</th>
<th>1/100</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCP treated virus</td>
<td>1</td>
<td>68a</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>61</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>100</td>
<td>76</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96</td>
<td>68</td>
<td>18</td>
</tr>
</tbody>
</table>

a. Percentage of seedlings developing symptoms. Each assay was carried out on 50-60 plants.

Ether was selected as the most satisfactory clarifying agent as it was also efficient at removing host plant materials. Therefore, as a routine procedure the extract was shaken for one minute with an equal volume of ether, the emulsion was centrifuged at 15,000 g for 10 minutes and the lower buffer phase was collected either by using a hypodermic syringe or by carefully discarding the ether phase.

f. Concentration by differential centrifugation. The buffer phase after ether clarification was centrifuged at 105,000 g in a Spinco 30 rotor for 60, 90 and 120 minutes. The pellets were resuspended in one-tenth of the original volume in 0.01 M phosphate...
buffer, pH 7.5, and assayed at several dilutions on 20 cucumber seedlings.

<table>
<thead>
<tr>
<th>Centrifugation for:</th>
<th>Infectivity at dilution:</th>
<th>1/1</th>
<th>1/4</th>
<th>1/16</th>
<th>1/64</th>
<th>1/256</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 minutes</td>
<td></td>
<td>18/20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13/20</td>
<td>12/20</td>
<td>5/20</td>
<td>0/20</td>
</tr>
<tr>
<td>90 minutes</td>
<td></td>
<td>20/20</td>
<td>19/20</td>
<td>19/20</td>
<td>18/20</td>
<td>4/20</td>
</tr>
<tr>
<td>120 minutes</td>
<td></td>
<td>20/20</td>
<td>20/20</td>
<td>20/20</td>
<td>18/20</td>
<td>6/20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numerator is the number of plants infected and the denominator the number of plants inoculated.

Results of these tests (Table 20) showed that centrifugation for 90 minutes was adequate to sediment virus in good yield, and this was adopted as a routine procedure. The virus was then resuspended in 0.01M phosphate buffer, pH 7.5, and clarified by centrifugation at 15,000 g for 20 minutes. The virus at this stage of purification will be referred to as "Pellet I virus".

The virus preparations at this stage of purification were usually light green or yellow in colour and contained appreciable amounts of host plant material. Differential centrifugation of
virus was carried out by sedimentation at 225,000 g in a Spinco 50Ti rotor for 30 minutes and clarification at 15,000 g for 10 minutes. Preparations of RMV purified by 3-4 cycles of differential centrifugation will be referred to as "partially purified virus preparations".

g. Routine preparation of partially purified RMV. The routine procedure for purification of virus is outlined in a flow diagram (Figure 21).

II. Properties of partially purified virus preparation

a. Absorption spectrum. Partially purified preparations of RMV had ultraviolet absorption spectra characteristic of a nucleoprotein (Figure 22). There was maximum absorption at 260 nm and a minimum at 240 nm. Preparations from equivalent amounts of healthy plant material processed by the same method showed relatively little absorption in ultraviolet light (Figure 22). Partially purified preparations of all the isolates of RMV (Table 1, isolate A-F) had indistinguishable absorption spectra between 230 and 300 nm.

b. Sucrose density gradient centrifugation. After density gradient centrifugation the contents of the tubes were analysed with an ISCO apparatus which showed that they contained three bands
Figure 21. Schedule for the purification of RMV.
Buffers: .01M phosphate buffer pH 7.5 containing .01% ascorbic acid at the rate of 2 ml per 10 ml of sap.

Procedure: cucumber tissue homogenized W/V in above buffer

Clarified at 15,000 g for 20 minutes

supernatant
mixed with equal volume of ether
clarified at 15,000 g for 10 minutes
buffer phase (clarified sap)
ether phase (discarded)
centrifuged at 105,000 g for 90 minutes
pellet
resuspended in .01M phosphate buffer
clarified at 15,000 g for 20 minutes
supernatant (discarded)
virus suspension (Pellet I)
centrifuged at 225,000 g for 30 minutes
pellet
resuspended in .01M phosphate buffer
clarified at 15,000 g for 10 minutes
supernatant (discarded)
virus suspension (Pellet II virus)
centrifuged at 225,000 g for 30 minutes
pellet
resuspended in .01M phosphate buffer
clarified at 15,000 g for 10 minutes
supernatant (discarded)
virus suspension (Pellet III virus)
clarified at 15,000 g for 20 minutes

partially purified virus
Figure 22. Absorption spectrum of partially purified RMV compared with that of material from healthy sap prepared by the same procedure.
of ultraviolet absorbing material: top (T), middle (M) and bottom (B) component (Figure 23). No significant variation was observed in the proportion of the virus components from different batches of plants grown during the period of the investigation. The middle component always showed as a very small peak, the bottom component varied between 1/3 - 1/4 of the top and was about four times bigger than the middle component. When material from healthy cucumber plants was prepared by the same procedure no corresponding bands were visible, and the analysis of the gradient showed that they contained relatively little absorbing material (Figure 23). When 0.25 ml fractions of the gradients were collected from the ISCO apparatus and each assayed on 10 cucumber seedlings, infectivity was detected in the gradient in a position corresponding to that of the ultraviolet absorbing bands, but not above or below the bands. All isolates (Table 1) of RMV sedimented in sucrose density gradients in the same relative positions and their distances from the meniscus were similar. The experiment was repeated at several different times of the year and approximately the same absorption patterns were observed (Figure 24A, 24D and 24F).

c. Variation in yield of RMV from cucumber seedlings. It was observed during the study that all isolates of RMV induced only
Figure 23. Density gradient centrifugation profiles of partially purified preparation of RMV (isolate A) with a similar preparation from healthy cucumber plants.
Figure 24. Sucrose density gradient centrifugation profile of three isolates of RMV.

A. RMV isolate A (Peace).
B. RMV isolate D (Chrysler Imperial).
C. RMV isolate F (First Love).
relatively mild systemic symptoms in winter and yielded very low amounts of virus by the purification method described above. No significant difference in yield of virus was obtained from plants infected between 10-15 days. To obtain improved yields of virus, various techniques were tested to increase the amount of virus in the cucumber tissue. The efficiency of infection was assayed by counting the chlorotic lesions and later estimating the amount of virus in purified preparations spectrophotometrically at 260 nm.

The results (Table 21) indicate that double inoculation of plants and selection of leaf tissue for the preparation of the inoculum, by excising lesions, were the most suitable methods for inoculation and, therefore, similar techniques were used in preparing the material for purification in most of the subsequent work.

d. Attempts to purify RMV from infected *C. quinoa* leaves.

RMV purified from infected cucumber yielded low amounts of virus which were considered to be inadequate for further studies. Therefore, an attempt was made to purify RMV from infected *C. quinoa* leaves, the only alternate herbaceous plant species susceptible to RMV. In this experiment virus was purified as described in Figure 21. To compare virus yield from the two hosts equal weights of leaf tissue from infected cucumber and
### Table 21
Effect of inoculation methods on virus infectivity and yield

<table>
<thead>
<tr>
<th>Method of inoculation</th>
<th>Lesions induced on cucumber</th>
<th>Relative yield of virus as estimated O.D/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. I</td>
<td>Exp. II</td>
</tr>
<tr>
<td>Control (glasshouse temperature)</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control at 25&lt;sup&gt;o&lt;/sup&gt;C</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Double inoculation</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Inoculation on both sides of leaf</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Tissue under lesion</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Pellet I virus</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Celite in inoculum</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Bentonite in inoculum</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mg trisilicate in inoculum</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a. Average number of chlorotic lesions per inoculated cucumber cotyledons. In each experiment 20 plants were used.

b. Plants kept in temperature controlled room under constant illumination.

c. Plants were inoculated as usual and were again inoculated with fresh inoculum 2 days later.

d. Each cucumber cotyledon was inoculated on both the upper and lower surfaces.

e. Inoculum was prepared from selected tissue showing severe symptoms.
C. quinoa were processed at the same time. The UV spectra of the two virus preparations were compared (Figure 25) and then diluted with 0.01M phosphate buffer, pH 7.5, to give suspensions of equal optical densities at 260 nm. These suspensions were then compared for infectivity on cucumber plants and two-fold dilutions were tested against RMV antisera by gel diffusion. The two preparations were subjected to sucrose density gradient centrifugation and an additional gradient was run in which the two preparations were mixed in a ratio of 1:1.

Table 22a

Effect of dilution on infectivity of RMV purified from infected cucumber and Chenopodium quinoa leaves

<table>
<thead>
<tr>
<th>RMV purified from</th>
<th>Dilutiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/10 1/20 1/40 1/80 1/160 1/320 1/640 1/1280</td>
</tr>
<tr>
<td>Cucumber</td>
<td>20/20b 20/20 16/19 18/20 19/19 16/20 7/19 3/20</td>
</tr>
<tr>
<td>C. quinoa</td>
<td>18/20 12/19 11/19 4/20 0/19 0/20 0/20 0/20</td>
</tr>
</tbody>
</table>

a. Preparations from both hosts were equalized with respect to O.D. at 260 nm before dilution.

b. Numerator is the total number of plants infected and the denominator is the total number of plants inoculated.
Figure 25. Absorption spectra of partially purified RMV preparations from two infected plant species.

A. RMV purified from infected C. quinoa.
B. RMV purified from infected cucumber.
Table 22b
The serological titration of RMV purified from infected cucumber and C. quinoa leaves as determined by agar gel diffusion test

<table>
<thead>
<tr>
<th>Antigens purified from</th>
<th>Antiserum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/1</td>
</tr>
<tr>
<td>Cucumber</td>
<td>+</td>
</tr>
<tr>
<td>C. quinoa</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Plus signs indicate the positive reaction.
- Minus signs indicate negative reaction.

Virus purified from cucumber was infective at a dilution of 1 : 1280 and reacted with homologous antiserum at an antigen dilution of 1 : 8 whereas virus purified from C. quinoa was infectious at a dilution of 1 : 80 and reacted with RMV antiserum at an antigen dilution of 1 : 1 (Table 22a, 22b).

Sucrose density gradient sedimentation profiles of the two virus preparations and a mixture of the two preparations are shown in Figure 26. Virus prepared from cucumber showed three bands of ultraviolet absorbing material and infectivity was detected in the gradient in a position corresponding to those bands. Virus
Figure 26. Sucrose density gradient centrifugation profiles of RMV purified from infected cucumber and infected *C. quinoa* leaves by a similar procedure.

A. Virus purified from cucumber.

B. Virus purified from *C. quinoa*.

C. A mixture of two virus preparations.
prepared from *C. quinoa* showed two bands of ultraviolet absorbing material and infectivity was associated where there was very little ultraviolet material seen, but in the position where bottom component was usually observed in virus preparations from cucumber. When material from healthy *C. quinoa* was prepared by the same procedure a similar profile was observed, but no infectivity was detected anywhere in the tube. These experiments indicate that RMV preparations from *C. quinoa* contain much host contaminating material which was not removed during purification; whereas from cucumber, much purer virus was obtained. Therefore, in subsequent studies purified RMV was always prepared from cucumber seedlings.

e. **Degradation of virus in CsCl₂.** To investigate the possibility of separating RMV components and determining their buoyant densities, partially purified virus preparations were subjected to isopycnic density gradient centrifugation in CsCl₂ at 49,000 rpm for 16 hours in a Spinco 50SW rotor. After centrifugation, three distinct light scattering zones were observed and the material from each zone was recovered with a hypodermic syringe by puncturing the side of the tube. The fractions were dialysed against 0.01M phosphate buffer, pH 7.5, for 4 hours, changing the buffer several times. The material in
each of the three fractions was subjected to sucrose density gradient centrifugation. There were no detectable peaks of optical density when the material from the two fractions of lower densities (zones T and M) were centrifuged and analysed in the ISCO apparatus. However, a diffuse double peak was detected when the densest of the three fractions (zone B) was analysed (Figure 27). The ultraviolet absorption spectrum of zone T was not characteristic of nucleoprotein suggesting that it was a virus degradation product; however, the other two fractions had ultraviolet absorption spectra characteristics of nucleoproteins (Figure 28).

Table 23
Infectivity of the fractions containing ultraviolet absorbing material after RMV was centrifuged in a CsCl₂ density gradient

<table>
<thead>
<tr>
<th>Fractions</th>
<th>O.D. at 260 nm</th>
<th>Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Zone</td>
<td>0.34</td>
<td>0</td>
</tr>
<tr>
<td>M Zone</td>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>B Zone</td>
<td>0.34</td>
<td>19</td>
</tr>
</tbody>
</table>

Figures are total numbers of infected plants. Each fraction was assayed on 20 plants.
Figure 27. Sucrose density gradient analysis of the fraction of highest density recovered after RMV was subjected to isopycnic density gradient centrifugation in CaCl₂. The material was dialysed prior to sucrose density gradient centrifugation.
Figure 28. Ultraviolet absorption spectra of fractions recovered from a density gradient after isopycnic centrifugation in CsCl₂.

- - - - M zone of highest density.

- - - - B zone of intermediate density.

- - - - T zone of least density.
The infectivity of each fraction was determined after equalizing them with respect to optical density at 260 nm. The data summarized in Table 23 show that only the densest fraction (zone B) was infectious. From the experiments described it appears that RMV could not be subjected to centrifugation in CsCl₂ without extensive virus degradation.

f. Degradation of RMV upon storage at 4°C. It was observed that the sucrose density gradient profiles of RMV (Figure 23) did not change noticeably after storage at 4°C during the first 10 days. However, precipitates were usually observed in stored virus preparation after this period and there was a noticeable loss of infectivity. The sedimentation properties of such preparations were also affected (Figure 29) suggesting that the virus was being degraded.

g. Separation of RMV protein and RNA with LiCl. A virus preparation in 0.01M phosphate buffer, pH 7.5, was mixed with an equal volume of 4M LiCl at 0°C and stored for 16 hours at -20°C. The frozen mixture was thawed and the precipitate was collected by centrifugation at 6,000 g for 30 minutes. The precipitate was resuspended in a volume of 0.01M phosphate buffer, pH 7.5, equal to that of the original virus preparation and centrifuged again at 6,000 g for 20 minutes. The supernatant was recovered
Figure 29. Degradation of RMV on storage. Purified virus kept at 4°C for 10 days was centrifuged on sucrose density gradient.
and 2 volumes of cold ethanol were added to precipitate the RNA at 0°C for 1 hour. The precipitate was recovered by centrifugation at 6,000 g for 20 minutes and the pellet resuspended in 0.01M phosphate buffer, pH 7.5. The ethanol precipitation procedure was repeated once more and the final precipitate thus obtained was suspended in 0.5 - 1 ml of 0.01M phosphate buffer, pH 7.5.

Samples from both the original supernatant containing most of the viral protein and the washed precipitate containing RNA were diluted and their ultraviolet spectra were determined. The supernatant fraction had an ultraviolet absorption spectrum with a maximum at 280 and a minimum at 250 nm. The precipitate fraction had an ultraviolet absorption spectrum with a maximum at 260 nm and a minimum at 230 nm (Figure 30). This shows that LiCl has a similar effect on RMV in separating the RNA and protein as that described for QCMV by Francki et al. (1966).

The infectivity of both the RNA and protein fractions was assayed by inoculation to cucumber seedlings, after equalizing the optical density of both at 260 nm. The results (Table 24) show that most of the infectious material in the virus preparation treated with LiCl was precipitated.

RNA prepared by LiCl preparation from RMV was layered on a 5-25% linear sucrose density gradient and centrifuged at
Figure 30. Absorption spectra of RMV RNA and protein separated by LiCl.
A VIRAL PROTEIN  
(LiCl supernatant)

B RNA  
(LiCl resuspended pellet)

OPTICAL DENSITY

WAVE LENGTH IN nm
Table 24
Infectivity of RMV RNA prepared by LiCl precipitation

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Preparation</th>
<th>O.D. at 260</th>
<th>Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supernatant (Protein)</td>
<td>0.32</td>
<td>1/20 a</td>
</tr>
<tr>
<td></td>
<td>Precipitate (RNA)</td>
<td>0.31</td>
<td>18/20</td>
</tr>
<tr>
<td>2</td>
<td>Protein</td>
<td>0.24</td>
<td>Not determined</td>
</tr>
<tr>
<td></td>
<td>RNA</td>
<td>0.21</td>
<td>14/20</td>
</tr>
</tbody>
</table>

a. Numerator is the total number of plants infected and the denominator is the total number of plants inoculated.

105,000 g for 4 hours in a Spinco 50SW rotor. A sucrose density gradient profile of the RMV-RNA showed three ultraviolet absorbing peaks (Figure 31).

h. Isolation of RNA from RMV. RNA from purified virus was isolated using the phenol extraction method of Gierer and Schram (1956), the single phenol phase extraction method of Diener and Schneider (1968) and a modification of the LiCl salt method used by Francki et al. (1966) to compare the product obtained by 3 methods.
Figure 31. UV absorption profile (254 nm) of sucrose density gradient of RMV RNA prepared by LiCl precipitation.
Preparation of RNA by the modified LiCl method was carried out as follows: To 1 ml of a virus suspension in 0.01M phosphate buffer, pH 7.5, was added 1 ml of 4M LiCl at 4°C. The mixture was frozen for 16 hours, thawed and the precipitate was collected by centrifugation at 6,000 g for 30 minutes. The sediment was taken up in 1 ml of 0.01M phosphate buffer, pH 7.5, and centrifuged again for 10 minutes at 6,000 g. The pellet was discarded and 2 ml phenol, 1 ml of 4% SDS and 1 ml of 0.005% EDTA were added and the mixture was agitated for 30 minutes. The resulting emulsion was centrifuged at 6,000 g for 10 minutes and the upper buffer layer was collected and the RNA was precipitated by adding two volumes of cold ethanol. The precipitated RNA was collected by centrifugation at 6,000 g for 10 minutes and taken up in 0.5 ml of 0.01M phosphate buffer, pH 7.5. The phenol extraction procedure was repeated once more and finally the precipitate was washed with acetone and dried by passing a jet of air over it. The RNA isolated by this procedure was either kept dry in powder form at room temperature under low pressure in a vacuum desiccator or suspended in 0.1 - 0.2 ml of 0.01M tris HCl buffer, pH 7.6, containing 1-2 sterilized sucrose crystals at -20°C.

The RNA extracted by the three different procedures had
**Figure 32.** Absorption spectra of isolated RMV RNA.

A. Prepared by a modification of the LiCl as described in the text.

B. Prepared by the phenol procedure described by Gierer and Schram (1956).

C. Prepared by the single phase phenol procedure described by Diener and Schneider (1968).
Figure 32. Sucrose density gradient centrifugation of RMV RNA isolated with different procedure.

A. Prepared by a modification of the LiCl as described in the text.

B. Prepared by the phenol procedure described by Gierer and Schram (1956).

C. Prepared by the single phase phenol procedure described by Diener and Schneider (1968).
absorption spectra as shown in Figure 32. The ratio of absorption at 260 nm to 250 nm varied from preparation to preparation but was always between 1.7 and 1.9. All RNA preparations were non-infectious when assayed on C. quinoa and cucumber.

Sucrose density gradient analysis of RMV RNA prepared by the three different methods (Figure 33) showed considerable heterogeneity suggesting that in all the preparations the RNA was degraded and also perhaps aggregated to some degree.

i. **Effect of RNase on the infectivity of RMV**

The effect of RNase on the infectivity of RMV nucleoprotein was tested by the addition of the enzyme to give a final concentration of 0.01 µg/ml. Both enzyme treated and untreated virus samples were kept at 25°C, two-fold dilution series were prepared at intervals of time up to 2 hours in 0.01M phosphate buffer, pH 7.5, and infectivity was assayed by inoculation to cucumber plants. Results of the experiment are summarized in Table 25 and show that RMV nucleoproteins are sensitive to the enzyme.

j. **Approximate estimation of the sedimentation coefficients of the RMV components.** Tobacco ringspot virus (TRSV) is a small icosahedral three component virus (Randles and
Table 25
The effect of RNase on infectivity of RMV

<table>
<thead>
<tr>
<th>Dilution Time</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
<th>1/32</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNase Control</td>
<td>RNase Control</td>
<td>RNase Control</td>
<td>RNase Control</td>
<td>RNase Control</td>
</tr>
<tr>
<td>0 minutes</td>
<td>9 10</td>
<td>9 10</td>
<td>8 8</td>
<td>8 9</td>
<td>8 9</td>
</tr>
<tr>
<td>20 minutes</td>
<td>10 10</td>
<td>7 9</td>
<td>6 9</td>
<td>6 8</td>
<td>7 8</td>
</tr>
<tr>
<td>40 minutes</td>
<td>6 10</td>
<td>7 9</td>
<td>6 9</td>
<td>6 8</td>
<td>4 8</td>
</tr>
<tr>
<td>60 minutes</td>
<td>7 10</td>
<td>5 9</td>
<td>6 9</td>
<td>4 9</td>
<td>2 9</td>
</tr>
<tr>
<td>120 minutes</td>
<td>4 10</td>
<td>4 8</td>
<td>3 8</td>
<td>2 8</td>
<td>1 8</td>
</tr>
</tbody>
</table>

Figures are total numbers of infected plants out of 10 inoculated.

Francki, 1965). the components having approximate sedimentation coefficients ($S_{20,W}$) of 57, 99 and 136S. This virus was used as a marker to determine the approximate $S$ values of the RMV components. Aliquots of partially purified preparations of TBSV, RMV and a mixture of the two viruses were layered on a 10-30% sucrose density gradient and centrifuged at 37,000 rpm for 90 minutes in a Spinco 39SW rotor. The contents of each tube was analysed with an ISCO apparatus. The tube containing TBSV showed three ultraviolet absorbing peaks (Figure 34) and the distance of each zone moved from the meniscus was plotted against their known $S$ value (Figure 35). RMV preparations also showed three
Figure 34. Optical density traces of RMV and TRSV and a mixture of two in sucrose density gradients. RMV purified as described in Figure 21. TRSV purified by procedure of Randles and Francki (1965).

A mixture of purified RMV and TRSV.
Figure 35. Calculation of S values for components of RMV using TRSV as a standard. Approximate S values of T, M and B of RMV were calculated to be 86, 98 and 103.
ultraviolet absorbing peaks and their approximate S values were determined by plotting their position on the standard curve derived from the TRSV component markers. With this technique the three components of RMV were calculated to have approximate S values of 86, 98 and 109S (Figure 35). This was further confirmed when the tube containing a mixture of the two viruses showed that the H component of TRSV sedimented between the T and B components of RMV (Figure 34).

k. **Serological investigations.** Concentrated virus (Pellet I preparations) of all isolates of RMV were tested against antisera to RMV, NRSV, DLPV and PAMV from North America and antisera to NRSV, DLPV and CRMV from Great Britain in gel diffusion tests. Results of these tests are summarised in Table 26. Reactions with some of the antisera produced two bands such as those with Wisconsin RMV, DPLV and NRSV. One of the bands was virus specific and the other was shown to be due to the presence of antibodies to host plant material (Figure 11). Virus specific precipitin lines were curved and close to the antigen well (Figures 11 and 36), whereas host specific lines had little curvature and were midway between the wells (Figure 11). Alfalfa mosaic virus did not react when tested against antiserum to RMV, and RMV did not produce any precipitin
Figure 36. Serological test of isolate A in agar gel.
The symbols denote the preparation added to the wells of the agar gel.
lines when tested against antisera prepared to arabis mosaic, tomato ringspot, tobacco ringspot, strawberry latent or cucumber mosaic viruses (Table 26).

Table 26
SEROLOGICAL REACTIONS IN GEL DIFFUSION TESTS OF RMV TESTED AGAINST ANTISERA TO VARIOUS VIRUSES

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Source of antiserum</th>
<th>Reaction to healthy plant antigen</th>
<th>Reaction to RMV antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rose mosaic virus (RMV)</td>
<td>Prepared in this laboratory</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rose mosaic virus (RMV)</td>
<td>Dr. R.W. Fulton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Danish plum line pattern virus (DPLV)</td>
<td>Dr. R.W. Fulton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Necrotic ringspot virus (NRSV)</td>
<td>Dr. R.W. Fulton</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Apple mosaic virus (PAMV)</td>
<td>Dr. R.W. Fulton</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cherry rugose mosaic virus (CRMV)</td>
<td>Dr. R. Grogan</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tomato ringspot virus (TomRSV)</td>
<td>Dr. R. Grogan</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Strawberry latent virus (SLRV)</td>
<td>Dr. R. Grogan</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabis mosaic virus (AMV)</td>
<td>Dr. R. Grogan</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tobacco ringspot virus (TRSV)</td>
<td>Dr. R. Grogan</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cucumber mosaic virus (CMV)</td>
<td>Dr. R. Francki</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Positive reaction in agar gel plates.
- Negative reaction in agar gel plates.
1. Electron microscopy. Purified preparations of isolates A, D and F of RMV recovered from sucrose density gradient columns were examined. Preparations stained with 2% UAc showed roughly spherical particles ranging from 23 nm - 30 nm and some bacilliform shaped particles were also present (Figure 38a-d). In preparations stained with 2% PTA similar particles were observed but the majority showed signs of disintegration. The electron micrograph from three isolates of RMV stained with UAc are shown in Figure 37 (A, D and F). No significant difference in size distribution of the particles was found between the three isolates of RMV. The mean diameter of the particles was 25 nm which is very similar to that reported for RMV from North America (Fulton, 1967b).

III. Conclusions

Fulton (1967b) reported the stabilization of RMV by the addition of both 0.02M 2-mercaptoethanol and 0.02M sodium diethyldithiocarbamate (DIECA) to extracts from infected cucumber leaves, and clarified the extracts with hydrated calcium phosphate. He also mentioned that Gold's (1961) suggestion for using the gamma globulin fraction of an antiserum to normal host proteins was useful in precipitating contaminating host material from RMV preparations. In this work the addition of ascorbic
Figure 37. Electron micrographs of RMV preparations stained with 2% uranyl acetate.

A. RMV isolate A (Peace).
B. RMV isolate D (Chrysler Imperial).
C. RMV isolate F (First Love).
Figure 38. Electron micrographs of selected RMV particles from a purified virus preparation stained with uranyl acetate.
acid to the extracting buffer effectively protected the virus from inactivation during extraction and clarification. Other chelating and reducing agents were not as effective (see Tables 17 and 18). The use of hydrated calcium phosphate gels to clarify leaf extracts containing RMV was not very satisfactory as virus losses were usually very considerable (see Figure 20). Bock (1966) and De Sequeira (1966) have used ether–carbon tetrachloride emulsification to remove contaminating material during the purification of NRSV isolates. In this study plant extracts containing RMV were clarified by emulsification with anhydrous ether which was found to be an efficient method of removing some host material without an excessive loss of virus. Further purification of RMV was achieved by differential and sucrose density gradient centrifugation which yielded relatively pure preparations of virus as assessed by density gradient centrifugation (Figure 23), electron microscopy (Figure 37) and serology as no antibodies to host plant materials were detected in antisera prepared against purified RMV (Figure 36).

Infected C. quinoa leaves have been used as source material for the purification of NRSV (Casper and Schulze, 1971) and several other plant viruses (Saksena and Mink, 1969;
Converse and Lister, 1969). It was found that RMV purified from this plant was contaminated with much host material and only very poor yields of virus were obtained.

When RMV preparations were subjected to analysis in an ISCO apparatus after centrifugation in sucrose density gradients, three ultraviolet absorbing peaks (T, M and B) were observed. Similar T, M and B peaks were observed in preparations from the other three isolates of RMV. The proportion of material in each peak varied slightly from experiment to experiment but there appeared to be consistent differences in their proportion between different isolates of the virus; a similar observation has been reported by Fulton (1967b). T, M and B have sedimentation coefficients of 86, 98 and 109S respectively (Figure 35). These figures were obtained by comparing the sedimentation velocities of the RMV components to those of TRSV in linear sucrose density gradients and are therefore only approximate.

Infectious RMV RNA was isolated by degradation of nucleoprotein by LiCl as described for QCMV (Francki et al., 1966), but such preparations were not suitable for analysis by polyacrylamide gel electrophoresis. Phenol-extracted RMV RNA prepared as described by Gierer and Schram (1956), and Diener
and Schneider (1968) had characteristic absorption spectra, but the preparations were not infectious. The behaviour of these preparations, when centrifuged in sucrose density gradients, suggest that the RNA was degraded. Clark and Lister (1971) have recently reported difficulties in the preparation of undegraded RNA from tobacco streak virus and it seems probable that a similar problem may be involved in the preparation of RMV RNA.

Preparations of all isolates of the RMV studied, reacted in gel diffusion tests against antisera to apple mosaic virus (PAMV), Prunus necrotic ringspot virus (NRSV), cherry rugose mosaic virus (CRMV) and Danish plum line pattern virus (DLPV). RMV has an average particle diameter of 25 nm which is similar to that reported for RMV by Fulton (1967b). Similar scanning patterns after sucrose density gradient centrifugation, and the serological cross reactions suggest a close relationship between the various isolates of RMV from South Australia and RMV from North America.
SECTION VI

STUDIES ON THE SEPARATED COMPONENTS OF RMV

Purified preparations of RMV are heterogeneous and separate into three zones on sucrose density gradient centrifugation. Such centrifugal heterogeneity has been reported in the literature for some other small isometric plant viruses as well as for some rod-shaped viruses (Sanger, 1968; Bancroft, 1968). The significance of these components is not understood in many cases (Van Kammen, 1968).

This section describes the experiments concerned with the infectivity and properties of T and B components of RMV which were separated from one another by sucrose density gradient centrifugation.

I. Separation of components and their infectivity

Purified preparations of all three isolates of RMV are heterogeneous (Figure 24A, B and C) and were separated into three components by sucrose density gradient centrifugation. When material from each zone was collected, concentrated and refractionated on a sucrose density gradient, each component was obtained with some contamination by material from the adjoining component (Figure 39). The T and B components
**Figure 39.** Optical density profiles and infectivity distribution in sucrose density gradient columns after centrifugation of separated components of RMV. Each fraction of 0.25 ml was assayed on 10 cucumber seedlings.

B. T component of RMV.

C. M component of RMV.

D. B component of RMV.
could be obtained with very little contamination when subjected to two cycles of sucrose density gradient centrifugation (Figure 40A and B). Difficulties arose with M component material because its rate of sedimentation is not greatly different from that of the other two components and it usually occurs in relatively small amounts; hence pure material was not obtained.

Infectivity of RMV components which had been separated after one and two successive fractionations by density gradient centrifugation was determined. Each 0.25 ml fraction collected from a gradient was assayed for infectivity on cucumber seedlings. Figure 39B shows that infectivity was not detected under the main peak of optical density when T component was analysed, but some infectivity was detected on the gradient in the expected position of B component. Similarly, Figure 40A shows that no infectivity was detected from any part of the gradient when a preparation of highly purified T component was assayed. On analysis of relatively pure B component, a good correlation between optical density and infectivity was observed (Figure 40B). Partially purified M component (one cycle of density gradient centrifugation) sedimented as a relatively inhomogeneous peak and the distribution of
Figure 40. Optical density profiles and infectivity distribution in columns after centrifugation of purified T and B components of RMV, and a mixture of the two. Each fraction of 0.25 ml was assayed on 10 cucumber seedlings.

A. T component of RMV.
B. B component of RMV.
C. A mixture of T + B components of RMV.
infectious material could not be correlated specifically
with either M or B component (Figure 39C).

In an experiment in which T and B components were
mixed in a ratio of 1:1, the mixture sedimented as two
well separated zones in positions characteristic of T and B
components (Figure 40C). Infectivity was correlated with the
B component only (Figure 40B) and the addition of T component
in various proportions failed to enhance significantly the
infectivity of B component (Table 27).

Table 27
The effect of T component on the infectivity
of B component

<table>
<thead>
<tr>
<th>Components of RMV and their combination</th>
<th>O.D. at 260 nm of undiluted preparations</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/4 1/8 1/16 1/32 1/64 1/128 1/256 1/512</td>
</tr>
<tr>
<td>T only</td>
<td>0.12</td>
<td>0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>B only</td>
<td>0.11</td>
<td>23 21 16 9 6 5 2 2</td>
</tr>
<tr>
<td>T+B (1:1)</td>
<td>0.23</td>
<td>25 24 19 11 7 5 3 2</td>
</tr>
<tr>
<td>T+B (2:1)</td>
<td>0.35</td>
<td>25 24 21 13 9 7 3 3</td>
</tr>
</tbody>
</table>

Figures are total number infected plants, each
dilution assayed on 25 plants in 2 separate
experiments.
II. **Absorption spectra of T and B components**

The ultraviolet absorption spectra of T and B components are shown in Figure 41. The spectrum of T component has minimum absorption at 245 nm and maximum at 260 nm with an $E_{max}/E_{min}$ of 1.24. The B component has a similar spectrum with an $E_{max}/E_{min}$ of 1.21. These spectra indicate that both T and B components contain nucleic acid and it appears that the proportions of nucleic acid to protein are similar in both components.

III. **Reaction of T and B components with antiserum prepared against unfractonated virus**

Concentrated preparations of T and B components were tested against antiserum prepared against unfractonated RMV in gel diffusion tests. The tests revealed no difference in the reactions produced by two components (Figure 42). This suggests that possibly there are no differences in the components as far as antigenic properties are concerned. It seems likely that they have a similar surface structure.

IV. **Electron microscopy of T and B components**

Preparations of T component stained with 2% UAe contained spherical particles ranging from 21.5 - 23.8 nm in diameter with an average of 22.5 nm. These measurements were
Figure 41. Absorption spectra of purified T and B components of RMV.
Figure 42. Serological test on isolated T and B components of RMV in agar gel. The symbols denote the preparations added to the wells in the agar gel.

A/S  Antiserum to RMV.
T    Purified T component.
B    Purified B component.
based on 150 apparently intact particles (Figure 44). B component contained both spherical and bacilliform shaped particles. Particles which were roughly spherical were slightly larger in diameter than those of the T component, ranging from 23.8 to 29.8 nm with an average of 26.5 nm. These measurements are based also on 150 particles (Figure 45). Photographs of B component were taken at the same time as that of the T component preparation. The distribution of particle size in T and B components are shown in Figure 43. The differences observed in the size of the spherical particles of T and B components of RMV are remarkably similar to those observed by Seneviratne and Posnette (1970) with three virus isolates from plum causing decline, line pattern and ringspot symptoms.

Bacilliform shaped particles varied in length, some being as long as 82 nm and resembled those observed in preparations of alfalfa mosaic virus (Hull et al., 1969). The ratio of bacilliform shaped to spherical particles in B component was roughly 1 : 20 and bacilliform shaped particles were observed in the B component of all three isolates of RMV.

V. Virus prepared from plants infected with purified B component only

Symptoms induced on cucumber by inoculation with
Figure 43. Particle diameter distribution of RMV.
Figure 44. Electron micrograph of T component of RMV.

A. Preparation stained with 2% PTA.

B. Preparation stained with 2% UAc.
Figure 45. Electron micrograph of B component of RMV.
A. Preparation stained with 2% PTA.
B. Preparation stained with 2% UAe.
homogeneous B component, isolated by two cycles of density
gradient centrifugation, were compared with those produced by
an unfractionated virus preparation, but no differences were
observed. Virus purified from plants inoculated with a
preparation of pure B component, when subjected to sucrose density
gradient centrifugation, contained T, M and B components
sedimenting in exactly the same positions as in preparations
from plants inoculated with unfractionated virus.

VI. Inoculations from single lesions to detect stable and
unstable virus

Results of the studies on T and B components so far
reported indicate that the particles were different in size,
each containing similar proportions of RNA to protein, and that
T component alone is not infectious and apparently does not
enhance the infectivity of B component. It seems worth
considering the possibility that the T component of RMV may have
a similar function to that of the short particles of tobacco
rattle virus (Sanger, 1968). In the case of tobacco rattle virus,
it has been demonstrated that the short particles have genetic
information required for viral protein synthesis. An experiment
was therefore carried out to test if T component in the inoculum is
necessary for the synthesis of complete virus particles.
Twenty widely separated chlorotic lesions produced by purified B component on cucumber cotyledons inoculated with dilute inocula were selected. Two cucumber plants were inoculated with an extract from each lesion prepared by grinding the tissue in 2–3 drops of 0.01M phosphate buffer, pH 7.5; a similar experiment was performed at the same time using the same technique with lesions induced by an unfractionated virus preparation.

Table 28
Infectivity of extracts from local lesions induced by inocula consisting of unfractionated RMV and isolated B component

<table>
<thead>
<tr>
<th>Transmission of virus from tissue containing a lesion from</th>
<th>Infectivity assayed on cucumber</th>
<th>Calculated percentage(^a) of stable and unstable virus infections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stable infection</td>
</tr>
<tr>
<td>B component</td>
<td>18/40</td>
<td>46%</td>
</tr>
<tr>
<td>Unfractionated virus</td>
<td>36/40</td>
<td>90%</td>
</tr>
</tbody>
</table>

\(^a\) Lesions from which infectious virus was transmitted by mechanical inoculation are defined as "stable infections" whereas those from which no virus could be transmitted are defined as "unstable infections".

\(b\) Numerator represents total number of seedlings that assayed positively for virus while denominator represents total number of seedlings assayed.
Results of the experiment (Table 28) show that less than 50% of the lesions induced by inoculum of separated B component produced stable infections, whereas preparations of unfractionated virus resulted in a greater frequency of stable infections.

VII. Conclusions

The infectivity of RMV appears to be dependent upon the presence of the B component. When T and B components were separated after two successive fractionations by sucrose density gradient centrifugation, the infectivity was associated only with the B component. T component alone was not infectious and the results in Table 27 and Figure 40 indicate that it does not enhance the infectivity of B component.

For other plant viruses which have more than one particle type, several mechanisms have been proposed to explain the interaction of the components in producing infection (Bancroft, 1968; Sanger, 1968). With tobacco rattle virus evidence has been obtained (Lister, 1966; Frost et al., 1967) that the two nucleoprotein components are needed for the synthesis of complete virus particles. Results of the experiments summarized in Table 28 indicate that stable and unstable forms of infection can be detected by transfer of single lesions from cucumber.
cotyledons produced by inoculum of either the B component or the unfractionated virus. The percentage of lesions with the unstable form of infectious material produced by B component was much greater than that produced by the unfractionated virus preparation. An interpretation of these data could be that lesions produced by B component alone contain viral RNA with no coat protein and hence extracts lose infectivity rapidly. However, virus purified from plants systemically infected by inocula containing only B component, contained T, M and B components sedimenting in exactly the same position as in preparation from plants inoculated with unfractionated virus (Figure 23). This could perhaps result from small amounts of T and M components contaminating the preparations of B component and this could be expected to build up in concentration in systemically infected plants.

The preliminary data presented in this thesis on the infectivity associated with RMV components suggest that T component, although not infectious alone, may help inocula containing B component in inducing infection by complete virus particles. It seems that future work should test the hypothesis that RMV-T component has a similar function in infection to that of the short particles of tobacco rattle virus (Lister, 1966),
namely that of containing the cistron coding for viral protein synthesis (Sanger, 1968).

Judging from their ultraviolet absorption spectra, the T and B component particles appear to contain similar proportions of nucleic acid. The gel diffusion test between T and B components and antiserum to RNV failed to detect any antigenic differences between the two components. This suggests that they have coat proteins constructed from similar protein.

Electron microscopic examination of preparation of T and B components show that these particles are similar to those of other small multi-component plant viruses and that their differences in particle diameter are also approximately similar to those found in apple mosaic virus (De Sequeira, 1966), NRSV (Bock, 1967; Bozarth, 1971), and the viruses isolated from plum line pattern, decline and ringspot (Seneviratne and Posnette, 1970). In each case only the larger particles were shown to be infectious.
SECTION VII

GENERAL DISCUSSION

RMV was isolated only from roses showing line pattern symptoms on leaves. However, it seems very likely that other viruses of rose are also present in South Australia. RMV was isolated from several varieties of rose and in some cases a bacterial pathogen was isolated together with the virus (Basit et al., 1970) which has been identified as a strain of P. syringae. A similar bacterial pathogen was also isolated from cherry infected with NRSV. The virus and bacterial pathogen from both rose and cherry were separated by passage through differential hosts.

M. balsamina has been used as a standard indicator plant for both RMV and NRSV (Fulton, 1952, 1957; K. Paulechova, 1967; Bozarth, 1971). In the present work I have found M. balsamina to be immune to the South Australian isolates of both RMV from rose and NRSV from cherry. However, the symptoms produced by P. syringae on M. balsamina have been shown to be very similar, if not identical, to those produced by RMV and NRSV on this host in other laboratories (Fulton, 1952, 1957; K. Paulechova, 1967; Bozarth, 1971). Similarly, P. syringae from both rose and cherry as well as some other isolates of Pseudomonas, have been shown to produce local lesions on cowpea and C. tetragonoloba leaves not unlike those reported to be produced by RMV and NRSV (Fulton,
1952, 1957). Again, the RMV and NRSV isolated in South Australia did not infect either cowpea or G. tetragonoloba. At present it is difficult to explain the apparent differences in the susceptibility of M. balsemina, G. tetragonoloba and cowpea to the local and North American isolates of RMV and NRSV. One possibility that immediately comes to mind is that the North American isolates may have been contaminated by P. syringae. Nevertheless, whatever the explanation, it seems advisable to avoid the use of these plants as indicators for RMV and NRSV.

Yarwood (1957), reported the return transmission of NRSV from infected bean to peach seedlings. This was confirmed by Seneviratne and Poanette (1970) where decline and ringspot viruses of plum were returned to Prunus seedlings by approach grafts between infected G. quinoa and peach seedlings. However, although RMV was transmitted to peach, attempts to return it to rose were unsuccessful and hence Koch's postulate has not been completely satisfied. It seems that the most likely method of achieving the transmission of RMV to rose would be to graft scions of infected peach seedlings to rose plants.

With the exception of apple chlorotic leaf spot virus (Cadman, 1965) pollen transmitted viruses have also been shown to be seed transmitted. In the present studies RMV could not be
detected in rose pollen but its seed transmission has been demonstrated (Figure 13). It is difficult to explain this unexpected observation, especially as the virus could readily be detected in pollen in both *C. quinoa* and cucumber. It seems most unlikely that RMV is transmitted by rose pollen but could not be detected either because it was present in very low concentration or because rose pollen also contains potent virus inhibitors.

The method of RMV purification described in this thesis (Figure 21) is relatively simple and reproducible; it has been used successfully here for the purification of NRSV and may be applicable to other viruses. The present investigations on the physical properties of RMV isolates from South Australia show that it is similar to RMV from North America described by Fulton (1967b). Data included in this thesis and also that reported elsewhere (Basit and Francki, 1970), leave little doubt that RMV is related to NRSV and several similar viruses (Gibbs, 1969). Harrison *et al.* (1971) have recently defined sixteen groups of plant viruses but none appear to be suitable for the inclusion of either RMV or NRSV. It seems that there is sufficient data available now to define a distinct virus group to include RMV, NRSV and several other reasonably well characterized viruses;
it should perhaps be called the *Prunus* necrotic ringspot virus group. On the basis of various degrees of relationship among RMV related viruses, Fulton (1968) suggested two serotypes using the term as proposed by Kassanis (1961). In serological cross absorption tests RMV and apple mosaic virus (PAMV) were shown to be indistinguishable, whereas NRSV and DLPV were shown to be distinct (Fulton, 1968). Nyland and Lowe (1964) showed that the viruses causing cherry rugose mosaic virus (CRMV) and almond calico mosaic virus (ACMV) are serologically related to NRSV. In this study the serological relationships between RMV, PAMV, NRSV, DLPV and CRMV have been confirmed. DLPV has been shown to be serologically unrelated to plum line pattern virus isolates from some other localities (Paulsen and Fulton, 1969; Seneviratne and Posnette, 1970) but these viruses can probably still be included in the *Prunus* necrotic ringspot virus group by virtue of their similar physical properties. Prune dwarf virus, tobacco streak virus and tulare apple mosaic virus (Fulton, 1967a; Gibbs, 1969) may perhaps also be included in the group. The suggestion made by Halliwell and Milbrath (1962), that TomRSV is related to RMV, however, seems unjustified as its properties indicate that it belongs to the tobacco ringspot virus group (Stace and Smith, 1966).
The structure of RMV particles and those of the other members of the *Prunus* necrotic ringspot virus group is interesting because of the striking similarity on the one hand to cowpea chlorotic mottle virus, brome mosaic virus (Bancroft, Hills and Markham, 1967), broad bean mottle virus (Finch and Klug, 1967), and cucumber mosaic virus (Francki *et al.*, 1966; Finch, Klug and Van Regenmortel, 1967) and, on the other hand, to alfalfa mosaic virus (Hull, Hills and Markham, 1969). The similarity of RMV to alfalfa mosaic virus (Figure 38a–d) may be due to artefacts, as it seems possible that RMV particles may degrade and reassemble during preparations for electron microscopy. RMV, although differing in the number of the components, has many characteristics in common with cucumber mosaic virus. Like QCMV, infectious RNA could be prepared by degradation of the nucleoprotein with LiCl (Francki *et al.*, 1966) showing that the capsomeres are held together mainly by electrovalent bonds. It was also found that RMV degraded during centrifugation in CsCl density gradients similarly to that of QCMV (Randles, 1965). Infectivity of RMV nucleoprotein has also been shown to be reduced by the addition of pancreatic RNase; this behaviour is similar to that reported for QCMV (Francki, 1968), NRSV (Diener and Weaver, 1959) and cowpea
chlorotic mottle virus (Bancroft et al., 1967).

The preliminary results reported in this thesis on the infectivity of RMV components and the suggestion that the RNA of B component contains the genetic information for viral RNA synthesis while that of the T component contains information for viral protein synthesis require further study. Further work is required to characterise the M component before one can establish any mechanism of interaction between the three RMV components in the infection process. The main problem in this type of work is the preparation of pure components; both the relatively low yields of virus and the small differences in sedimentation coefficients (68, 98 and 1098) are serious technical problems.

Preparations of RNA by phenol extraction have so far yielded preparations which lack infectivity. This is interesting as Clark and Lister (1971) also experienced difficulties in the preparation of RNA from tobacco streak virus which has some properties in common with RMV. Although infectious RNA has been prepared from RMV by degradation the virus with LiCl, such preparations contain some protein and hence are not altogether satisfactory for further work. It will be necessary in the future to devise methods of preparing infectious RNA from RMV free of protein.
ADDENDUM I

**NOTE:**
This publication is included in the print copy of the thesis held in the University of Adelaide Library.

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ADDENDUM II

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