Comparative Properties of Type, Nasturtium Ringspot and Petunia Ringspot Strains of Broad Bean Wilt Virus

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SUMMARY

The purification and characterization of the type, nasturtium ringspot and petunia ringspot strains of broad bean wilt virus are described. The use of sucrose throughout purification reduced losses due to aggregation of the virus particles. Electron microscopy of virus preparations revealed particles which were apparently hexagonal in outline and approx. 27 nm in diam. Each virus had three sedimenting components, top (T), middle (M) and bottom (B), indistinguishable from those of cowpea mosaic virus (CPMV) in co-sedimentation experiments. The sedimentation coefficients, $s_{20,W}$ of PRSV were 56S (T), 95S(M) and 116S(B). The buoyant densities of M and B components of BBWV, NRSV and PRSV in CsCl+2·34% (w/v) Igepon T73 were approx. 1·40 and 1·44 g/ml respectively. B components of the viruses contained a single RNA species (RNA-1) of mol. wt. 2·0 ± 0·1 x 10^6 and approximate molar percentage base ratio of 26% guanine, 30% adenine, 17·5% cytosine and 26·5% uracil. M components of the viruses contained a single RNA species (RNA-2), mol. wt. 1·5 to 1·6 x 10^6. The base ratios of RNA-1 and RNA-2 of BBWV were very similar. Mixtures of RNA-1 and RNA-2 (or M and B components) were more infective than the separate RNA species (or virus components). Mol. wt. of the formaldehyde-denatured RNA species of BBWV, NRSV and PRSV were very similar to those of CPMV. BBWV, NRSV and PRSV contained two species of polypeptide, mol. wt. 42000 and 26000.

These properties in conjunction with serological evidence indicate that the viruses are closely related strains. The similarity between some of the properties of BBWV, NRSV and PRSV and those of CPMV is discussed.

INTRODUCTION

Broad bean wilt virus (BBWV) and nasturtium ringspot virus (NRSV) are multicomponent icosahedral plant viruses (Taylor et al. 1968; Sahambi, Milne & Phillips, 1970) and are transmitted by aphids in the non-persistent manner (Smith, 1950; Stubbs, 1960). Petunia ringspot virus is transmitted by aphids and is thought to be spherical (Rubio-Huertos, 1968). The viruses have extensive and similar host ranges (Schmelzer, 1960; Doel, 1972; Taylor & Stubbs, 1972; Doel, 1973) and appear to be serologically identical (Cook & Gibbs, 1971; Doel, 1972; Frowd & Tomlinson, 1972; Murant & Goold, 1972; Doel, 1973; Sahambi et al. 1973). BBWV and NRSV do not appear to be serologically related to numerous isometric plant viruses (Taylor et al. 1968; Verhoyen & Autrique, 1968). Cells infected with BBWV, NRSV or PRSV contain similar types of inclusion bodies (Rubio-Huertos, 1968; * Present address: Animal Virus Research Institute, Pirbright, Surrey, England.

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This paper reports further properties of BBWV, NRSV and PRSV, shows that they cannot be readily distinguished and indicates that they have some similarities to the comoviruses.

METHODS

**Virus propagation and infectivity assay.** The following virus isolates were used: (1) BBWV, the isolate described by Stubbs (1947); provided by Mr P. R. Smith, Victorian Plant Research Institute, Burnley, Australia. (2) NRSV, the isolate described by Smith (1949); provided by Dr R. Hull, John Innes Institute, Norwich. (3) PRSV, the isolate described by Rubio-Huertos (1959); provided by Dr R. Hull. (4) CPMV, yellow strain; provided by Professor A. Van Kammen, Wageningen, The Netherlands.

The viruses were propagated by grinding infected leaves in 0.05 M-potassium phosphate buffer, pH 7.0, and rubbing the extracts onto 'Celite'-dusted leaves of 2- to 4-week-old *Chenopodium quinoa* Willd. plants, grown in a greenhouse at approx. 21 °C, under 360 W high pressure sodium lamps.

Virus preparations were assayed for infectivity on 'Celite'-dusted whole leaves of 6-week-old *Chenopodium quinoa* plants. RNA preparations were inoculated on to half-leaves of similar plants, using 'cotton buds'.

**Virus purification.** Both inoculated and systemically infected leaves of *Chenopodium quinoa* were harvested 6 to 8 days after inoculation and homogenized with an equal quantity (v/w) of 0.5 M-potassium phosphate buffer containing 25 % (w/v) sucrose and 0.01 M-cysteine-HCl, pH 7.0. The extracts were clarified by the dropwise addition of 8.5 % (v/v) n-butanol, and subsequent centrifuging at 5000 rev/min for 10 min. The supernatant fluids were centrifuged at 50000 rev/min for 4 h in a Beckman 60Ti rotor at 4 °C and the virus pellets further purified by one of the following methods: (1) The pellets were resuspended in 0.5 M-potassium phosphate buffer, pH 7.0, and samples (0.5 to 1.0 ml) containing 1 to 3 mg virus were floated on CsCl density gradients made by layering 3 ml samples of CsCl solutions of densities 1.31, 1.35, 1.39 and 1.43 g/ml in 0.05 M-potassium phosphate buffer, pH 7.0. Gradients were centrifuged at 32000 rev/min for 24 h in a Beckman SW40 rotor at 4 °C. The separated virus components were removed through the side of the centrifuge tube with a syringe and dialysed against distilled water. Virus prepared in this way was insoluble and was used for polyacrylamide gel electrophoresis of the proteins and base ratio analysis of the nucleic acids. (2) The pellets were resuspended in either 0.004 M (BBWV), 0.12 M (NRSV)- or 0.02 M (PRSV)-potassium phosphate buffer, pH 7.0, and 0.5 to 1.0 ml samples (1 to 3 mg virus) floated on 10 to 50 % (w/v) linear sucrose density gradients in 0.004, 0.12 or 0.02 M-potassium phosphate buffer, pH 7.0, respectively. The gradients were centrifuged in a Beckman SW41 rotor at 36000 rev/min for 90 min at 20 °C and the components removed either through the side of the tube or by piercing the base and collecting drops. Virus prepared in this way was soluble and was used for spectrophotometry, polyacrylamide gel electrophoresis of the nucleic acids of separated virus components and infectivity experiments.

**Serology.** The following antisera were used: (1) BBWV antiserum; provided by Mr P. R. Smith, Victorian Plant Research Institute, Burnley, Australia. (2) Parsley virus 3 (PV3) antiserum; provided by Dr J. A. Frowd, Canada Department of Agriculture, Research Station, Vancouver, Canada. (3) PRSV antiserum; prepared by injecting a rabbit intramuscularly with 0.5 mg of PRSV in Freund's incomplete adjuvant (Difco) and intravenously with 1 mg of PRSV in 0.14 M-NaCl. Five more intravenous injections were given over a period of 30 days. The rabbit was bled 7 days after the final injection.
Gel diffusion experiments were done in 0.7% (w/v) Ionagar (Oxoid) gels containing 0.85% (w/v) NaCl, 0.01% (w/v) sodium azide and 0.1 M-potassium phosphate buffer, pH 7.0. Undiluted BBWV, PRSV or PV3 antiserum was added to the central well and extracts of healthy and virus-infected plants in 0.5 M-potassium phosphate buffer, pH 7.0, were added to the peripheral wells. Intragel cross-absorption experiments were done, as described by Van Regenmortel (1967), using partially purified samples of each virus. BBWV, NRSV or PRSV were initially used in excess to absorb homologous antibodies. Subsequent gel diffusion was conducted with optimal proportions of the viruses and antisera.

Electron microscopy. Samples of the partially purified viruses were negatively stained with 2% (w/v) potassium phosphotungstate, pH 6.4, and photographed at 30,000 and 100,000 times magnification in a Jeol 100B electron microscope. The magnification of the instrument was calibrated with platinum phthalocyanine crystals which have a lattice spacing of 1.194 nm (Markham et al. 1964). The maximum diam. of regular particles were measured from electron micrographs and the means and standard errors calculated.

Analytical ultracentrifugation. Virus preparations were examined in a Beckman Model E ultracentrifuge, using Schlieren optics. The sedimentation coefficients of PRSV components were determined by the method of Markham (1960). Also, BBWV, NRSV and PRSV were each co-centrifuged with CPMV. The buoyant densities of M and B components of the viruses were determined in CsCl + 2.34% (w/v) Igepon T73 (Brakke, 1959). Approx. 0.2 to 0.5 mg virus was mixed with the CsCl/detergent solution and centrifuged at 44,000 rev/min for 22 h at 20°C in the Beckman An-D rotor. Buoyant densities were derived from subsequent measurements as described by Dirks (Beckman Instruments Inc. publication).

Spectrophotometry. Samples of the virus components were removed from sucrose density gradients and scanned in a Unicam SP800A ultraviolet spectrophotometer.

RNA base ratio analysis. RNA was obtained from B components of BBWV, NRSV and PRSV and M component of BBWV by two overnight extractions with n-HCl at 20°C. The RNA was hydrolysed at 100°C for 1 h and the products separated by paper chromatography as described by Smith & Markham (1950). The solvent contained 700 ml t-butanol, 132 ml 11.7 N-HCl and 168 ml water. Molar percentage base ratios were calculated using the molar extinction coefficients given by Markham & Smith (1951).

SDS – polyacrylamide gel electrophoresis of the virus proteins. The stock electrophoresis buffer referred to in this section comprised 108 g tris, 55 g boric acid and 9 g Na2-EDTA in 1 l of distilled water, pH 8.3 (Peacock & Dingman, 1967).

Proteins were prepared by dissolving approx. 1 mg virus (M and/or B component) or 1 mg virus protein (the residue from n-HCl extraction of the RNA) or 5 mg of each of the marker proteins in 10 ml of a solution consisting of 1 g sucrose, 4 ml 10% (w/v) SDS, 5 ml water and 2 ml of the stock electrophoresis buffer. The following were used as mol. wt. markers: ovalbumin (chicken egg white), gamma globulin (bovine), carbonic anhydrase (bovine erythrocytes) and myoglobin (sperm whale) (Weber & Osborne, 1969). Prior to electrophoresis, 10 μl samples of the virus protein solution were mixed with 10 μl 2-mercaptoethanol, 5 μl 10% (w/v) SDS and 2.5 μl carbonic anhydrase solution and heated at 90°C for 10 min. Mixtures of the marker proteins were similarly treated. Polyacrylamide gels (10% w/v) containing 8 M-urea, 0.1% (w/v) SDS in a fivefold dilution of stock electrophoresis buffer were prepared in glass tubes (7 cm long × 0.5 cm internal diam.). Proteins were electrophoresed for 13 h at 20 V in a buffer which contained 400 ml stock electrophoresis buffer, 600 ml water, 10 g SDS and 1 ml mercaptoethanol. The protein bands were stained with 0.25% (w/v) Coomassie brilliant blue R250 in 227 ml methanol/
46 ml acetic acid/227 ml water, and mol. wt. estimated by comparing the mobilities of the virus proteins with those of the proteins of known mol. wt.

**SDS--polyacrylamide gel electrophoresis of virus RNA.** Two buffer systems were used in the following work. Buffer A is described in the previous section (Peacock & Dingman, 1967). Buffer B comprised 43.4 g tris, 47.6 g NaH₂PO₄·2H₂O and 3.7 g Na₂-EDTA·2H₂O in 1 l water, pH 7.6 to 7.8 (Loening, 1969). RNA was prepared by two methods: (1) RNA was extracted by emulsifying 2 to 5 mg virus in 2 ml water with 5 ml water-saturated phenol (containing 10 % (v/v) m-cresol and 0.1 % 8-hydroxyquinoline), 0.25 ml 10 % (w/v) SDS and 0.5 ml bentonite suspension (40 mg/ml, Fraenkel-Conrat, Singer & Tsugita, 1961). The mixture was briefly heated to 50 °C before thorough agitation for 5 min. The emulsion was broken by centrifuging and the supernatant fluid thoroughly washed with ether. RNA was precipitated with 2.5 volumes of absolute ethanol + 4 drops of 3 M-acetate buffer, pH 4.7, and the sedimented pellets dissolved in a fivefold dilution of absolute ethanol + 4 drops of 3 M-acetate buffer, pH 4.7, and the sedimented pellets dissolved in a fivefold dilution of stock electrophoresis buffer B containing 0.1 % (w/v) SDS and 7.5 % (w/v) succrose. RNA was frozen until required. (2) Separated M and B components were heated at 60 °C for 5 min in 50 to 100 μl of a buffer which comprised 1 g SDS, 0.5 ml 2-mercaptoethanol, 0.04 g Na₂-EDTA, 0.58 g NaH₂PO₄ and 0.16 g Na₂HPO₄, 2H₂O in 100 ml water, pH 7.5. Formaldehyde solution was added to a final concentration of 4 % and the samples heated at 80 °C for 10 min.

Polyacrylamide gels (2.6 % w/v) were made in either a fivefold dilution of buffer B or a tenfold dilution of buffer A and cast in glass tubes (7 cm long × 0.5 cm internal diam.). The formaldehyde-treated RNA species (0.1 to 1.0 μg samples) were electrophoresed at 100 V for 2.5 h at room temperature in a tenfold dilution of buffer A + 0.05 % (w/v) SDS. Gels were stained in 0.1 % (w/v) toluidine blue in methanol/acet acid and, after de-staining, were scanned on the Joyce–Loebl 'Polyfrac' scanner at 265 nm. Mol. wt. of BBWV, NRSV and PRSV RNA species were determined by comparing their mobilities with those of the formaldehyde-treated RNA species of *Escherichia coli* ribosomes and tobacco mosaic virus. Mol. wt. of the formaldehyde-treated marker RNA species were taken as 0.56 × 10⁶, 1.1 × 10⁶ and 2.0 × 10⁶ (*E. coli* 16S and 23S RNA and TMV RNA, respectively, Boedtker, 1971).

Samples (20 μg) of native RNA of BBWV, NRSV and PRSV required for infectivity experiments, were heated at 50 °C for 5 min and electrophoresed in a fivefold dilution of buffer B + 0.05 % (w/v) SDS at 60 V for 2.5 h at room temperature. Gels were briefly stained in 0.1 % (w/v) toluidine blue in 40 % (v/v) methanol to locate the bands. Bands were cut from the gels and homogenized in 2 ml tris-phosphate buffer (0.98 g o-phosphoric acid—6.07 g tris in 100 ml distilled water, pH 8.7) and 0.1 ml bentonite suspension. Homogenates were immediately inoculated onto *Chenopodium quinoa*.

**RESULTS**

*Virus purification*

When CsCl density gradient sedimentation was used as the final step in virus purification, the virus particles aggregated. A need for samples of soluble BBWV, NRSV and PRSV prompted experiments with sucrose density gradients.

Preliminary investigations showed that yields of BBWV, NRSV and PRSV increased when 25 % (w/v) sucrose was included in the extraction buffer. An indication of these increases is given by the relative sizes of the Schlieren peaks produced by partially purified samples of NRSV in the analytical ultracentrifuge (Fig. 1). It is also apparent that the yield of NRSV T component increased disproportionately to those of M and B components.

Turbidimetric experiments indicated that the solubility of pure BBWV, NRSV and PRSV
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Fig. 1. Analytical ultracentrifugation of partially purified NRSV. Virus was extracted from tissue either in the presence (upper Schlieren image) or absence (lower Schlieren image) of 25% (w/v) sucrose. T, top component; M, middle component; B, bottom component. Sedimentation is from left to right. Rotor speed 35,000 rev/min. Schlieren bar angle 60°.

Table I. Infectivity of M and B components of NRSV and PRSV, separated by sucrose density gradient sedimentation

<table>
<thead>
<tr>
<th>Component</th>
<th>Optical density of inoculum/ml</th>
<th>Lesion number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRSV M</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>NRSV B</td>
<td>0.001</td>
<td>1.9</td>
</tr>
<tr>
<td>NRSV M+B</td>
<td>0.01</td>
<td>12</td>
</tr>
<tr>
<td>NRSV M</td>
<td>0.01</td>
<td>7</td>
</tr>
<tr>
<td>NRSV B</td>
<td>0.01</td>
<td>77</td>
</tr>
<tr>
<td>NRSV M+B</td>
<td>0.01</td>
<td>162</td>
</tr>
<tr>
<td>PRSV M</td>
<td>0.001</td>
<td>0.16</td>
</tr>
<tr>
<td>PRSV B</td>
<td>0.001</td>
<td>0.06</td>
</tr>
<tr>
<td>PRSV M+B</td>
<td>0.01</td>
<td>1.5</td>
</tr>
<tr>
<td>PRSV M</td>
<td>0.01</td>
<td>10</td>
</tr>
<tr>
<td>PRSV B</td>
<td>0.01</td>
<td>46</td>
</tr>
<tr>
<td>PRSV M+B</td>
<td>0.01</td>
<td>94</td>
</tr>
</tbody>
</table>

* Mean number of lesions per leaf. 10 whole leaves per sample.

in sucrose density gradients was critically affected by slight changes in sucrose concentration, ionic strength of the buffer and, to a lesser extent, temperature (Doel, 1973). Ethylene glycol, glucose or glycerol were as effective as sucrose in preventing aggregation of the purified viruses.

M and B components of each of the viruses were removed from sucrose density gradients and assayed for infectivity. Table I shows that mixtures of M and B components of NRSV and mixtures of M and B components of PRSV were approx. 4 to 12 times more infective than the separate components. Similar results were obtained with components purified by CsCl density gradient sedimentation. Although infectivity enhancement was not obtained with M and B components of BBWV, Taylor et al. (1968) reported enhancement in two out of five experiments with this virus.
Serology

BBWV, NRSV and PRSV formed a single continuous precipitation line in gel diffusion experiments, regardless of which of the three antisera was placed in the central well. Fig. 2 shows a typical gel. Intragar cross-absorption experiments, which have been used to detect highly specific antigenic determinants (Van Regenmortel, 1967), did not show differences between the viruses. Therefore, the viruses appear to be antigenically identical.

Electron microscopy

Samples of the partially purified viruses contained particles which were roughly hexagonal in outline. PRSV was morphologically indistinguishable from BBWV and NRSV, although the absence of fine structure in our micrographs made comparisons difficult. All of the virus preparations contained 'empty' particles, i.e. particles penetrated by the stain (potassium phosphotungstate). Similar observations have been made by Taylor et al. (1968) with BBWV and Sahambi et al. (1973) with NRSV. The maximum diameters, i.e. the distance between the opposite vertices of the hexagonal particles, were measured for 20 to 50 particles of each virus and were 27.4 ± 0.1 nm (BBWV), 26.8 ± 0.1 nm (NRSV) and 26.5 ± 0.2 nm (PRSV). These values do not differ greatly from those given by Taylor et al. (1968, BBWV, 25 nm) and Sahambi et al. (1973, NRSV, 26 nm) whereas Frowd & Tomlinson (1972) reported a value of 28 to 32 nm for the parsley virus 3 (PV3) isolate of NRSV.

Analytical ultracentrifugation

Each of the viruses sedimented as three components, top (T), middle (M) and bottom (B). The sedimentation coefficients, $s_{20,w}$ of the components of PRSV were 56S, 95S and 116S respectively. Although the sedimentation coefficients of BBWV and NRSV were not determined, the components of BBWV, NRSV and PRSV were indistinguishable from those of CPMV in co-sedimentation experiments (Fig. 3). The reported values for the latter are 58S (T), 95S (M) and 115S (B) (Van Kamen, 1967). My results differ somewhat from the values reported by Taylor et al. (1968) for BBWV (63S, 100S and 126S) and Sahambi et al.
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Fig. 3. Co-sedimentation of BBWV, NRSV or PRSV with CPMV in the analytical ultracentrifuge. (a) Upper Schlieren image, BBWV + CPMV; lower Schlieren image NRSV + CPMV. (b) PRSV + CPMV. In each case equal amounts of the two viruses were used. T, top component; M, middle component; B, bottom component. Sedimentation is from left to right. Rotor speed 35 600 rev/min. Schlieren bar angle 60°.

Table 2. Molar percentage base ratios (± standard error) of RNA species extracted from B components of BBWV, NRSV and PRSV and M component of BBWV

<table>
<thead>
<tr>
<th>No. of experiments</th>
<th>Guanine</th>
<th>Adenine</th>
<th>Cytosine</th>
<th>Uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBWV M</td>
<td>9</td>
<td>25·1 ± 0·12</td>
<td>30·1</td>
<td>17·5 ± 0·16</td>
</tr>
<tr>
<td>BBWV B</td>
<td>14</td>
<td>25·3 ± 0·19</td>
<td>30·1</td>
<td>17·8 ± 0·15</td>
</tr>
<tr>
<td>NRSV B</td>
<td>15</td>
<td>26·1 ± 0·12</td>
<td>29·7 ± 0·13</td>
<td>17·9</td>
</tr>
<tr>
<td>PRSV B</td>
<td>9</td>
<td>25·6 ± 0·06</td>
<td>31·1 ± 0·08</td>
<td>16·8</td>
</tr>
</tbody>
</table>

(1973) for NRSV (58 S, 99 S and 124 S) but are in good agreement with those of Sahambi et al. (1970) for a strain of NRSV (58 S, 98 S and 113 S).

The sedimentation coefficients of the components of PRSV were used to estimate the RNA contents of M and B components by the method of Reichmann (1965), assuming that the T, M and B components of PRSV contain the same amount of protein and differ only in their proportions of RNA. Values of 26 % (M) and 35 % RNA (B) were calculated which compare with 24 % (M) and 33 % RNA (B) reported by Van Kammen (1970) for CPMV. The buoyant densities and $E_{260/280}$ ratios of M and B components of BBWV, NRSV and PRSV (given below) are consistent with the estimated RNA contents of M and B components of PRSV.

CsCl density gradient sedimentation of partially purified samples of the viruses revealed extensive aggregation of the separated components. A detergent, Igepon T73 (Brakke, 1959), was partially successful in dispersing the aggregates and consequently was included in the CsCl solution used for buoyant density determinations. The buoyant densities of M components were 1·39 g/ml (BBWV), 1·40 g/ml (NRSV and PRSV) and those of B component were 1·44 g/ml (BBWV, NRSV and PRSV).
Fig. 4. Electrophoretic separation of the large (L) and small (S) polypeptides of BBWV in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS. Approx. 10 µg protein was electrophoresed at 13 V for 20 h in each gel. The arrow indicates the direction of migration of the polypeptides.

**Spectrophotometry**

The u.v.-absorption spectra of the separated components of each of the viruses suggested that M and B components were nucleoproteins and the T component a protein containing little or no nucleic acid. $E_{260/280}$ of M components were 1.69 (BBWV), 1.73 (NRSV) and 1.70 (PRSV) whereas those of B components were 1.79 (BBWV and PRSV) and 1.78 (NRSV). The approximate $E_{260/280}$ of T component of NRSV was 1.0. However, the spectrum from which this value was derived showed considerable light-scattering.

**RNA base ratios**

The base ratios of the nucleic acids extracted from B components of BBWV, NRSV and PRSV were very similar (Table 2). Base ratios of the nucleic acids of M components of NRSV and PRSV were not determined but would not be expected to differ greatly from the values obtained with their corresponding B components in view of the close relationship of the three viruses and the fact that the base ratio of BBWV M component RNA was essentially identical to that of BBWV B component RNA (Table 2).

These results conflict with those of Taylor et al. (1968) who, using a similar experimental approach, reported values of 21.8% guanine, 30% adenine, 17.6% cytosine and 30.6% uracil for RNA extracted from a mixture of M and B components of BBWV.

**Polyacrylamide gel electrophoresis of the virus proteins**

When electrophoresed in SDS-polyacrylamide gels, protein preparations from M or B components of BBWV, NRSV and PRSV each contained two polypeptides. On the basis of 6 to 9 determinations for each polypeptide, the mol. wt. were calculated to be 42,300 and...
Fig. 5. Electrophoresis of formaldehyde-treated RNA of PRSV, TMV and Escherichia coli ribosomes in 2.6% (w/v) polyacrylamide gels at 100 V for 2.5 h. a, E. coli 16 S RNA; b, E. coli 23 S RNA; c, PRSV RNA-2; d, PRSV RNA-1 and TMV RNA. The arrow indicates the direction of migration of the nucleic acids.

26400 (BBWV, Fig. 4); 41700 and 25500 (NRSV) and 41700 and 26000 (PRSV). From these mol. wt. figures it is unlikely that the large (L) polypeptides are dimers of the small (S) polypeptides, although the amino acid compositions of the S and L polypeptides of NRSV are similar (Doel, 1973). Moreover, tryptic digestion of the unfractionated proteins of BBWV, NRSV or PRSV yielded approx. 70 peptides (Doel, 1973), a figure which is consistent with that predicted from the amino acid compositions of the S and L polypeptides of NRSV, assuming that the amino acid sequences in the S and L polypeptides are different.

In co-electrophoresis experiments, the S and L polypeptides of CPMV, which have mol. wt. similar to those given above (Wu & Bruening, 1971), were indistinguishable from the S and L polypeptides of BBWV.
Fig. 6. Distribution of RNA species of PRSV in selected fractions from a sucrose density gradient. The middle (M) and bottom (B) components of PRSV were separated by sedimentation through a 10 to 50% (w/v) linear sucrose density gradient for 1.5 h at 36000 rev/min in the Beckman SW41 rotor. The gradient was fractionated and fractions monitored for $E_{260}$. RNA was extracted from particular groups of fractions (a, b, c, d, e), treated with formaldehyde and electrophoresed in 2.6% (w/v) polyacrylamide gels. The gels were stained with toluidine blue O to detect the RNA species and scanned on the Joyce–Loebl ‘Polyfrac’ at 265 nm. Selected portions of the five scans are shown on the right of the figure. The types of RNA species (RNA-1 or RNA-2) in each group of fractions were identified by reference to TMV and *Escherichia coli* ribosomal RNA species. Sedimentation of the virus components and migration of the RNA species are from right to left.

**Polyacrylamide gel electrophoresis of virus RNA**

Electrophoresis of native RNA extracted from mixtures of M and B components of BBWV, NRSV and PRSV showed that each virus contained two RNA species, mol. wt. about $2.1 \times 10^6$ (RNA-1) and $1.5 \times 10^6$ (RNA-2). However, the RNA-2 bands in polyacrylamide gels were diffuse. This was attributed to the presence of a range of configurations of RNA-2 molecules with varying degrees of secondary structure. Attempts to sharpen the bands included electrophoresis at 4, 20 and 40°C, in 2 to 8 M-urea, in different ionic strengths of electrophoresis buffers A and B, and heating of the RNA samples, prior to electrophoresis, in the presence of 8 M-urea (5 to 10 min at 50°C) or formaldehyde (10 min at 80°C). Only formaldehyde significantly sharpened the RNA-2 (and RNA-1) bands (Fig. 5). Consequently, mol. wt. of the RNA species were estimated with formaldehyde-
Table 3. Infectivity of RNA species of BBWV, NRSV and PRSV separated by polyacrylamide gel electrophoresis

<table>
<thead>
<tr>
<th>RNA species</th>
<th>BBWV</th>
<th>NRSV</th>
<th>PRSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>6.0</td>
<td>3.8</td>
</tr>
<tr>
<td>1 + 2†</td>
<td>5.0</td>
<td>19.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Average of 12 half-leaves per sample.
† The proportion of RNA-1 and RNA-2 in mixtures for infectivity assays was the same as that in polyacrylamide gels.

denatured material (5 determinations/RNA species). The calculated mol. wt. of RNA-1 and RNA-2 were $2.0 \times 10^6$ and $1.53 \times 10^6$ (BBWV), $2.0 \times 10^6$ and $1.55 \times 10^6$ (NRSV) and $2.0 \times 10^6$ and $1.55 \times 10^6$ (PRSV). The position of each RNA-1 band was indistinguishable from that of TMV RNA (Fig. 5).

When CPMV RNA was treated with formaldehyde and electrophoresed with TMV RNA and *Escherichia coli* RNA, the larger of the two RNA species of CPMV ran identically with TMV RNA. The mol. wt. of the formaldehyde-treated RNA species of CPMV (estimated to be $2.0 \times 10^6$ and $1.53 \times 10^6$) conflict with the reported mol. wt. which are $2.58 \times 10^6$ and $1.45 \times 10^6$ (Van Kammen & Van Griensven, 1970). However, the latter values were derived from the sedimentation coefficients of native RNA species. It may be concluded that the apparent mol. wt. of formaldehyde-denatured RNA species of CPMV, BBWV, NRSV and PRSV are identical.

Samples of BBWV, NRSV and PRSV were separated into their respective components by sucrose density gradient sedimentation, and polyacrylamide gel electrophoresis used to identify the RNA species in selected fractions. B components contained predominantly RNA-1 whereas M components contained RNA-2 (e.g. PRSV, Fig. 6). However, significant quantities of RNA-2 were detected in those fractions corresponding to the leading edge of the band of B component and, to a lesser extent, the centre of the band of B component. It is possible that the RNA-2 in B component fractions was a contaminant and originated either from dimers of M component particles or virus components which contained two RNA-2 molecules.

Infectivity experiments with the native RNA species removed from polyacrylamide gels were hindered because of the diffuse nature of the bands. Nevertheless, mixtures of the RNA-1 and RNA-2 species of each virus were four to eight times more infective than the separate RNA species (Table 3).

These results are consistent with those of previous experiments which showed that M and B components contained predominantly RNA-2 and RNA-1, respectively, and that mixtures of M and B components were more infective than the separate components. Two results in Table 3 suggest that pure samples of RNA-1 and RNA-2 may be non-infective. This is not unexpected in view of the results of Van Kammen & Van Griensven (1970) with CPMV, which is structurally similar to BBWV, NRSV and PRSV; they reported that highly purified samples of B or M components of CPMV were virtually non-infective, whereas mixtures were highly infective.
DISCUSSION

Although minor differences among BBWV, NRSV and PRSV might be expected in view of their widely differing origins (Australia, England and Spain respectively), the results presented here suggest that the viruses are serologically indistinguishable from each other. Other workers have been unable to distinguish between BBMV and NRSV on the basis of serology, and limited physical properties. For example, Frowd & Tomlinson (1972) and Sahambi et al. (1973) were unable to detect spurs in gel diffusion experiments. The present work confirms the findings of these authors. In addition, PRSV is seen to be serologically indistinguishable from BBWV and NRSV and the three viruses are also indistinguishable in a number of their physico-chemical properties. However, the following evidence indicates that the viruses may differ from each other in certain minor respects. J. A. Frowd (cited by Taylor & Stubbs, 1972) reported that broad beans infected with NRSV or PV3 developed milder symptoms than those infected with BBWV. Electron microscopy also reveals differences among BBWV, NRSV and PRSV (R. Hull & A. Plaskitt, unpublished data) and between BBWV and NRSV (Sahambi et al. 1973) with regard to the morphology of inclusion bodies in cells infected with each of the viruses. The latter results and those described in this paper support the classification scheme of Taylor & Stubbs (1972) in which NRSV and PRSV are regarded as strains of BBWV (the first of the viruses to be isolated).

There is little doubt that BBWV and its strains NRSV and PRSV constitute a new virus group. They do not appear to be serologically related to numerous spherical viruses, including representatives of the structurally similar comoviruses and nepoviruses (Taylor et al. 1968, Verhoyen & Autrique, 1968). They are transmitted by aphids in the non-persistent manner which also serves to distinguish them from the beetle-borne comoviruses and nematode-borne nepoviruses, although there is an isolated report of the transmission of tobacco ringspot virus by aphids (Stace-Smith, 1970). In direct contrast to the differences between the biological properties of the BBWV-type viruses and the comoviruses, there are distinct similarities between the physical properties of the two groups. The nepoviruses will not be considered further for the following reasons. With the exception of strawberry latent ringspot virus, members of this group differ from BBWV, NRSV and PRSV in having a single species of coat protein, mol. wt. 50000 to 60000, and a bottom component which contains significantly more RNA than the bottom components of the BBW viruses. Hence the high buoyant densities and sedimentation coefficients of nepovirus bottom components (1.52 g/ml and 130S for raspberry ringspot virus, Mayo, Murant & Harrison, 1971; Murant et al. 1972). Although classified as a nepovirus, strawberry latent ringspot has two species of coat protein, mol. wt. 44000 and 29000 (Mayo et al. 1974) and in this respect resembles BBWV. However, it does not react with antisera to the PV3 isolate of BBWV and various members of the comovirus and nepovirus groups (Lister, 1964; Mayo et al. 1974).

Prior to the work described in this paper, the comoviruses were regarded as being distinct from other groups of plant viruses in possessing two coat polypeptides instead of one (e.g. CPMV, Wu & Bruening, 1971). This paper shows that the BBWV-type viruses also possess two coat proteins, with mol. wt. very similar to those of CPMV (Wu & Bruening, 1971; Geelen, Van Kammen & Verduin, 1972). Both the BBWV-type viruses and CPMV contain two RNA species which have essentially identical mol. wt. when treated with formaldehyde and electrophoresed in polyacrylamide gels. Although the base compositions of the RNA species of the two groups are dissimilar (e.g. CPMV, B + M components, 21.8% guanine, 28.5% adenine, 18.3% cytosine, 31.5% uracil; Van Kammen & Van Griensven, 1970), it is interesting that the cytosine to adenine ratios of both groups are similar, i.e. approx. 0.5.
Broad bean wilt virus strains

It is also interesting to note that base ratios vary significantly within the comovirus group. For example, squash mosaic virus (Campbell, 1971) contains 23% guanine, 32% adenine, 16% cytosine and 30% uracil (B+M components). Finally, the BBWV-type viruses and CPMV have essentially identical sedimentation coefficients and buoyant densities in CsCl; Wood (1971) gives values of 1.39 and 1.44 g/ml for the M and B components of CPMV.

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