Biophysical Properties of *Penicillium stoloniferum* Virus S.

By K. W. BUCK and GILLIAN F. KEMPSON-JONES

Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AY, England

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**SUMMARY**

*Penicillium stoloniferum* virus S has been fractionated into four particle classes, E, M, L and H, with sedimentation coefficients of 66, 87, 101 and 113 S, respectively. E particles were shown to be empty capsids, while M particles contained single-stranded RNA, L particles contained double-stranded RNA, and H particles contained both single-stranded RNA and double-stranded RNA. The mol. wt. of L particles was found from sedimentation and diffusion coefficients to be $6.0 \times 10^6$.

Evidence is presented that the M, L, and H particle classes each contain two components, M₁, M₂, L₁, L₂, H₁ and H₂, respectively. It has been shown that the M₁, M₂, L₁ and L₂ particles each contain only one molecule of RNA with mol. wts. of $0.47 \times 10^6$, $0.56 \times 10^6$, $0.94 \times 10^6$ and $1.11 \times 10^6$, respectively, while H₁ and H₂ particles each contain one molecule of double-stranded RNA of mol. wts. $0.94 \times 10^6$ and $1.11 \times 10^6$, respectively, together with single-stranded RNA.

**INTRODUCTION**

Polyhedral virus particles were first detected in *Penicillium stoloniferum* (strain ATCC 14586) by Ellis & Kleinschmidt (1967), and were isolated and purified by Banks et al. (1968), who showed that the particles contained double-stranded RNA. Later, Buck & Kempson-Jones (1970) showed that the isolates contained two serologically distinct viruses (PsV-S and PsV-F). Subsequently, Bozarth, Wood & Mandelbrot (1971) showed that both viruses were composed of several types of particle with different sedimentation rates and buoyant densities. It was reported that PsV-S contained only double-stranded RNA, while PsV-F contained double-stranded RNA as well as single-stranded RNA. Lhoas (1971) has shown that both PsV-S and PsV-F may be transferred to virus-free strains of *P. stoloniferum* by heterokaryosis, but after infecting protoplasts of the same strains with extracellular virus only PsV-S could be detected, indicating that this virus can replicate independently (Lhoas, 1971).

PsV-S has now been fractionated by equilibrium sedimentation in caesium chloride gradients and the biophysical properties of the virus components have been studied. Evidence is presented that PsV-S contains, in addition to empty capsids, particles containing only single-stranded RNA, particles containing only double-stranded RNA and particles containing both double-stranded RNA and single-stranded RNA.
METHODS

Abbreviations for buffers. P buffer: 0·03 m-sodium phosphate, pH 7·6; TAE buffer: 0·05 M-tris + 0·04 M-acetate + 0·0016 M-EDTA, pH 8·0; SSC: 0·15 M-NaCl + 0·015 M-Na citrate, pH 7·0.

Spectrophotometry. U.v. spectra were measured with a Cary 15 spectrophotometer in 1 cm silica cells. An extinction unit ($E_{260}$ U) is defined as the extinction at 260 nm in a 1 cm cell, multiplied by the sample volume.

Preparation and purification of virus. Penicillium stoloniferum was grown for 2 days in 60 l fermenters as described previously (Banks et al. 1968). Virus was obtained from the homogenized mycelium by co-precipitation with yeast RNA at pH 4·0 using the procedure described for Penicillium cyaneo-fulvum virus (Banks et al. 1969), except that the final virus pellet was taken up in P buffer (1 l), debris was removed by centrifuging at 20000 g for 30 min and the virus was again sedimented by centrifuging at 35000 g for 18 h.

Separation of PsV-S and PsV-F. Virus preparation (up to 125 $E_{260}$ U) in P buffer was adsorbed on to a column of DEAE-cellulose (Whatman DE 52, 1·5 × 10 cm) previously equilibrated with P buffer. The column was eluted with a linear gradient of 0 to 0·3 M-NaCl in P buffer (640 ml total volume) at a flow rate of 100 ml/h. The eluant was monitored at 254 nm with an LKB Uvicord I and 3·5 ml fractions were collected. A typical separation is shown in Fig. 1. Peak 1 (fractions 15 to 25) contained PsV-S and peak 2 (fractions 48 to 70) contained PsV-F.

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Fig. 1. Separation of PsV-S and PsV-F on a DEAE-cellulose column. $E_{260}$; $\text{molarity of NaCl}$ (calculated).
Gel electrophoresis. Polyacrylamide gels (4 %), containing 0.04 % methylene-bis-acryla-
mide in TAE or 2 x TAE buffer, were prepared in glass tubes (7.0 x 0.5 cm), essentially as
described by Loening (1967). Electrophoresis was carried out at 6 mA/tube for 1.5 to 4 h
using TAE or 2 x TAE as running buffer. RNA gels were stained with acridine orange
(Richards, Coll & Gratzer, 1965) and scanned at 490 nm using a Unicam SP Spectrophoto-
meter with a Gilford gel-scanning attachment. Virus gels were fixed and stained in 0.1 %
Coomassie Brilliant Blue, containing 10 % sulphosalicylic acid and 10 % TCA, for 20 min
and then transferred to a 5 % acetic acid solution, when the stained bands intensified.

Sedimentation coefficients. Determinations were made in a Beckman Model E analytical
ultracentrifuge equipped with a u.v. scanner. Virus suspensions in P buffer + 0.1 M-NaCl
and RNA solutions in SSC were centrifuged at 20000 rev/min and 40000 rev/min, respec-
tively, using cells with filled Epon double sector centrepieces in the AN-H rotor. Detection
of the moving boundary was made with the u.v. scanner at 265 nm and at the low concentra-
tions used (E260 0.5 to 0.9) the sedimentation coefficients determined were effectively s0
(Schumaker & Schachman, 1957). Values were corrected to standard conditions (s020,w)
using equation 63 of Svedberg & Pedersen (1940).

Diffusion coefficient. This was determined with the same ultracentrifuge, rotor and cells
as for sedimentation coefficient determinations, essentially as described by Möller (1964).
Virus preparations (about 100 µg/ml in P buffer + 0.1 M-NaCl) were centrifuged at 20000
rev/min for 15 to 20 min to establish a boundary before deaccelerating to 4400 rev/min at
which speed diffusion occurred. A constant temperature close to 20 °C was maintained and
u.v. scans were taken at 32 min intervals over a period of 384 min. Values of µ at 20 (80) %
were obtained from the boundary patterns at different times as described by Burness & Clothier (1970). Plots of µ2(1 - sw2t) against time were linear and from the slope of which
the diffusion coefficient was calculated from the relationship

\[ D = \frac{\bar{\mu}^2(1 - sw^{2t})}{4y^2t} \]

where \( y = 0.5951 \). Values of \( D \) were corrected to standard conditions using equation 176 of
Svedberg & Pedersen (1940).

Molecular weight. The mol. wt. of virus particles was calculated using equation 3(a) of
Svedberg & Pedersen (1940):

\[ M = \frac{RT \cdot s_{20,w}^0}{D_{20,\omega}(1 - V_p)} \]

Determination of virus buoyant density. Virus preparations were centrifuged to equilibrium
(18 to 24 h) in gradients of caesium chloride in P buffer at 36000 rev/min at 22 to 24 °C
using cells with double sector filled Epon centrepieces in the AN-F rotor in the Beckman
Model E analytical ultracentrifuge. Profiles were obtained with the u.v. scanner at 265 nm.
Average densities of caesium chloride solutions were calculated from refractive indices,
measured with a Bellingham and Stanley Abbe High Accuracy '60' refractometer (Ifft,
Voet & Vinograd, 1961). Virus buoyant densities were calculated by the method of Erikson
& Szybalski (1964).

Fractionation of PsV-S. Virus preparations (10 to 15 E260 U) were layered on pre-formed
gradients of caesium chloride (50 ml, 10 to 45 % (w/w) in P buffer) and centrifuged for 18 h
at 22000 rev/min at 4 °C in a Beckman SW 25 rotor. Fractions (0.5 ml) were collected using
an ISCO Model D gradient fractionator and u.v. analyser. Densities of caesium chloride
were calculated from refractive indices (Ifft et al. 1961). Further fractionations were
carried out by adjusting samples (1 to 1.5 E260 U) with CsCl to the required density and
centrifuging at 40000 rev/min for 18 h in a Beckman SW 50 rotor at 4 °C. Fractions (0·05 ml) were collected from the bottom of the gradient using a Büchler density-gradient fractionator.

Determination of RNA and protein contents of virus samples. RNA content was estimated from phosphorus analyses, carried out by the method of Bartlett (1959). An RNA phosphorus content of 9·6%, the value obtained here for PsV-S L RNA, was used in all calculations. Protein contents were estimated by amino acid analysis (Thomas, 1970) following acid hydrolysis of whole virus preparations. The amino acid composition of whole virus hydrolysates was very similar to that of the pure virus protein, except that the glycine value was raised in the former case, because of acid decomposition of the RNA. Hence the values for glycine (and also cystine, proline and tryptophan, which require separate determinations) were interpolated from the values obtained for pure PsV-S protein (K. W. Buck, G. F. Kempson-Jones & G. Ratti, unpublished results).

Preparation of virus RNA. Virus suspension in SSC, containing bentonite (4 mg/ml, prepared by the method of Fraenkel-Conrat, Singer & Tsugita, 1961), and SDS (1 %), was incubated at 40 °C for 15 min and then extracted with phenol, once at 40 °C and twice at 0 °C. The aqueous solution was then dialysed against SSC, in the presence of bentonite (4 mg/ml) to remove phenol. The bentonite was removed by sedimentation at 20000 g for 1 h and the RNA solution was stored at −20 °C.

Thermal denaturation of virus RNA. Melting profiles of RNA preparations were determined at 260 nm using a Beckman DK-2A spectrophotometer, equipped with a heated cell compartment. Thermal transition mid-points (Tm) were calculated as described by Marmur & Doty (1962). For thermal denaturation of RNA prior to gel electrophoresis, samples in 1 mM-EDTA, pH 7·0, were heated at 100 °C for 5 min and then rapidly chilled in a bath at −100 °C.

Action of ribonuclease on virus RNA. Virus RNA (40 μg/ml) was incubated with Boehringer pancreatic ribonuclease (0·2 μg/ml) in SSC at 25 °C for 2 h. The solutions were then examined by analytical sedimentation, or by polyacrylamide gel electrophoresis using 2 × TAE buffer in the gels and as running buffer.

Molecular weights of RNA. Mol. wts. of virus RNA components were determined by using polyacrylamide gel electrophoresis using the ten components of reovirus RNA as standards for double-stranded RNA (Shatkin, Sipe & Loh, 1968) and Escherichia coli 23, 16 and 5 S ribosomal RNAs (Bishop, Claybrook & Spiegelman, 1967) and denatured PsV-S L RNA as standards for single-stranded RNA.

Electron microscopy. Samples were negatively stained with 0·2 % potassium phosphotungstate, pH 7·0, and examined with a J.E.M. Model 7 electron microscope.

RESULTS

Properties of PsV-S E, M, L and H particle classes

Preparations of PsV-S, purified by DEAE-cellulose chromatography, gave a single precipitin line in gel diffusion tests with the antiserum prepared previously (Banks et al. 1968), which showed a reaction of identity with the precipitin line obtained with PsV-S preparations purified by gel electrophoresis. Moreover, preparations of PsV-S were electrophoretically homogeneous with a mobility identical with that of PsV-S purified by gel electrophoresis. The previously described tendency of PsV-S to separate into two bands in gel electrophoresis (Buck & Kempson-Jones, 1970) was shown only by ageing virus preparations; all the results in this paper refer to freshly prepared PsV-S, which was
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Fig. 2. Sucrose density gradient centrifugation of PsV-S. Virus sample (0.1 ml, $E_{260}$ 1 to 1.5) was layered on a preformed 10 to 45% (w/w) sucrose gradient in P buffer (5 ml) and centrifuged in a Beckman SW 50 rotor for 3 h at 4°C. After sedimentation the gradient was analysed with the ISCO Model D fractionator and u.v. analyser.

Fig. 3. Fractionation of PsV-S by centrifuging in 10 to 45% CsCl gradients. ISCO u.v. profile.
Fig. 4. Analytical equilibrium sedimentation of PsV-S fractions in CsCl density gradients: u.v. scanner tracings. (i) Unfractionated PsV-S; (ii) M particles; (iii) L particles; (iv) LI particles; (v) L2 particles; (vi) H particles; (vii) HI particles; (viii) H2 particles.

Electrophoretically homogeneous. Electron microscopy of negatively stained PsV-S preparations revealed isometric particles of uniform size (30 to 33 nm diam.).

When centrifuged in sucrose density gradients, PsV-S gave four bands, designated E, M, L and H, respectively (Fig. 2). In preparative isopycnic caesium chloride gradients PsV-S also gave four bands (Fig. 3). Similar caesium chloride gradient profiles were obtained with preparations of PsV-S isolated from filtrates of 5-day-old cultures of *P. stoloniferum* at pH 8·5 as described by Banks *et al.* (1968) and purified either by electrophoresis or by DEAE-cellulose chromatography. Examination of fractions from the sucrose gradients by sedimentation in caesium chloride gradients showed that E, M, L and H particles in Fig. 2 gave rise to the corresponding bands, respectively in Fig. 3. When E, M, L and H particles were separated by repeated centrifuging of appropriate fractions in caesium chloride
gradients, each class of particles sedimented in the analytical ultracentrifuge as a single boundary. Each particle class was completely stable to repeated sedimentation in caesium chloride gradients. Electron microscopy of negatively stained preparations showed that E particles were empty capsids, while the M, L and H classes all contained unpenetrated particles of approximately the same size (30 to 33 nm).

The physical properties of E, M, L and H particle classes are given in Table I. Although each particle class sedimented as a single boundary, examination by caesium chloride sedimentation in the analytical ultracentrifuge (Fig. 4ii–vi) showed that M particles were heterogeneous, and L and H particles each contained two components of closely similar densities designated L 1 and L 2, H 1 and H 2 particles, respectively. The proportions of the individual components depended on the degree of fractionation; in the preparations studied here the proportion of L 1 to L 2 particles was about 2 to 1 and H 1 to H 2 particles was about 3 to 2.

Properties of the RNAs from PsV-S M, L and H particle classes

L particle RNA

RNA from L particles sedimented as a single boundary (s_{20\text{w}} = 11.7 S). The thermal denaturation profile (Fig. 5ii) was typical of double-stranded RNA (Bellamy et al. 1967) with a T_m of 102.4 °C (in SSC), corresponding to a C+G content of 53 % (calculated by using the equation of Kallenbach, 1968), and a phosphorus content of 9.6 %. In polyacrylamide gel electrophoresis (Fig. 6ii), two clear components were separated with mol. wts. of 0.94 × 10^6 and 1.11 × 10^6. After treatment of L particle RNA with ribonuclease in SSC, the sedimentation coefficient and electrophoretic mobilities of the RNA were unchanged, confirming its double-stranded nature.

By repeated fractionation of L particles in caesium chloride gradients, small quantities of L 1 and L 2 particles were obtained which gave single peaks in caesium chloride analytical gradients (Fig. 4iv, v). RNA was prepared from the separated L 1 and L 2 particles and examined by gel electrophoresis. The results are given in Fig. 6 (iii, iv).

M particle RNA

RNA from M particles sedimented as a single boundary (s_{20\text{w}} = 16.4 S). After treatment with ribonuclease in SSC, sedimentation analysis showed that the RNA was completely degraded to small fragments, indicating its single-stranded nature. The thermal denaturation profile in SSC (Fig. 5i) showed a gradual increase in E_{260} with increasing temperature, also typical of single-stranded RNA, with a T_m of 63 °C. In polyacrylamide gel electrophoresis two discrete components were observed (Fig. 6i) which had the same mobilities as the two bands obtained from heat denatured L particle RNA, indicating that the mol. wts. were half those of double-stranded L particle RNA, namely 0.47 × 10^6 and 0.56 × 10^6. Values obtained, namely 0.50 × 10^6 and 0.60 × 10^6, using E. coli 23, 16 and 5 S ribosomal RNA’s as standards were in good agreement.

H particle RNA

RNA from H particles sedimented as two discrete boundaries (s_{20\text{w}} 11.7 and 15.3 S, respectively). The ratio of double-stranded RNA to single-stranded RNA was estimated to be approximately 2:1, calculated by assuming that the extinction coefficient of the single-stranded RNA was 10 % higher than that of the double-stranded RNA. After treatment of H particle RNA with ribonuclease in SSC the 11.7 S component was unchanged, but the 15.3 S component had been completely degraded to small fragments. This indicated that
the 11.7 S component is double-stranded RNA and the 15.3 S component is single-stranded RNA. The thermal denaturation profile of PsV-S H particle RNA (Fig. 5iii) showed the gradual increase in $E_{260}$ with increasing temperature typical of single-stranded RNA, followed by the steep rise in $E_{260}$ characteristic of double-stranded RNA. In gel electrophoresis (Fig. 6v), PsV-S H particle RNA gave (a) two discrete components with identical mobilities to those of PsV-S L particle RNA, not susceptible to ribonuclease in SSC, and (b) a diffuse band of low mobility, which could not be detected after treatment of the RNA with ribonuclease.

By repeated fractionation of PsV-S H particles in caesium chloride gradients, small amounts of H 1 and H 2 particles were obtained which gave single bands in analytical caesium chloride gradients (Fig. 4vii, viii). RNA was prepared from the separated H 1 and H 2 particles and examined by gel electrophoresis. The results are given in Fig. 6 (vi, vii).
Fig. 6. Polyacrylamide gel electrophoresis profiles of RNA from PsV-S fractions. RNA from:
(i) M particles; (ii) L particles; (iii) L1 particles; (iv) L2 particles; (v) H particles; (vi) H1 particles;
(vii) H2 particles. Electrophoresis was carried out for 2 h in TAE buffer.

**RNA from particles with densities intermediate between those of L and H particles**

Examination of the total PsV-S particles in analytical caesium chloride gradients (Fig. 4i) showed that there was a range of particles with densities intermediate between those of L2 and H1 particles. In order to examine the RNA from particles in this region of the gradient, PsV-S was sedimented in preparative caesium chloride gradients and fractions
A to F were collected as shown in Fig. 3. RNA, prepared from each fraction, was examined by analytical sedimentation and the derivative curves of the boundaries are given in Fig. 7.

DISCUSSION

The properties of PsV-S particles and their constituent RNA's are summarized in Table 1. The results show clearly that the heterogeneity of PsV-S is a result of the differing proportions of RNA within the particles. Electron microscopy showed E particles to be empty capsids and the low buoyant density and $E_{260}/E_{280}$ ratio are consistent with this. The properties of the RNA derived from the other three particle classes show that M particles contain single-stranded RNA, L particles contain double-stranded RNA and H particles contain both double-stranded RNA and single-stranded RNA.

The mol. wt. of L particles ($5.97 \times 10^6$), determined from the sedimentation and diffusion
<table>
<thead>
<tr>
<th>Particle class</th>
<th>E</th>
<th>M</th>
<th>L</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{20,W}^0$ (Svedberg units)</td>
<td>66.1</td>
<td>87.1</td>
<td>101.2*</td>
<td>112.6</td>
</tr>
<tr>
<td>$D_{20,W}$ (cm$^2$/sec $\times 10^7$)</td>
<td>ND</td>
<td>ND</td>
<td>1.39†</td>
<td>ND</td>
</tr>
<tr>
<td>Buoyant density (g/ml)‡</td>
<td>1.297 M 1 1.332 L 1 1.358 H 1 1.384</td>
<td>M 2 ND L 2 1.362 H 2 1.390</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{260}/E_{380}$</td>
<td>0.75</td>
<td>1.36</td>
<td>1.51</td>
<td>1.67</td>
</tr>
<tr>
<td>RNA (%)</td>
<td>8.7</td>
<td>15.5</td>
<td>24.4</td>
<td></td>
</tr>
<tr>
<td>$\upsilon$ (ml/g)§</td>
<td>0.732</td>
<td>0.716</td>
<td>0.704</td>
<td>0.688</td>
</tr>
<tr>
<td>Mol. wt. $\times 10^{-6}$</td>
<td>5.04</td>
<td>5.52</td>
<td>5.97</td>
<td>6.67</td>
</tr>
<tr>
<td>Total weight of RNA/particle $\times 10^{-6}$</td>
<td>ND</td>
<td>0.48</td>
<td>0.93</td>
<td>1.63</td>
</tr>
<tr>
<td>$S_{20,W}^0$ of RNA (Svedberg units)</td>
<td>ND</td>
<td>16.4 (ss)</td>
<td>11.7 (ds)</td>
<td>11.7 (ds) + 15.3 (ss)</td>
</tr>
<tr>
<td>Mol. wt. of RNA components $(\times 10^{-6})$</td>
<td>M 1 0.47 (ss) L 1 0.94 (ds) H 1 (a) 0.94 (ds) + ND (ss)</td>
<td>M 2 0.56 (ss) L 2 1.11 (ds) H 1 (b) 1.11 (ds) + ND (ss)</td>
<td>H 2 1.11 (ds) + ND (ss)</td>
<td></td>
</tr>
</tbody>
</table>

ND = Not determined.
ds = double-stranded.
ss = single-stranded.
* Standard deviation in 4 determinations was ±0.8.
† Standard deviation in 5 determinations was ±0.04.
‡ Standard deviation in 4 determinations was ±0.003.
§ Calculated from the RNA and protein composition, assuming $\upsilon$ (RNA) = 0.55 ml/g and $\upsilon$ (PsV-S protein) = 0.732, calculated from the amino acid composition of pure PsV-S protein (K. W. Buck, G. F. Kempson-Jones & G. Ratti, unpublished results).

coefficients, is an average value for L 1 and L 2 particles. The average weight of RNA/particle was calculated from the particle composition to be 0.93 $\times 10^6$. Since the mol. wt. of L particle RNA components were found from gel electrophoresis to be 0.94 $\times 10^6$ and 1.11 $\times 10^6$, it is clear that L particles contain only one molecule of RNA/particle. This was confirmed by gel electrophoresis of RNA from the separated L 1 and L 2 particles (Fig. 6iii, iv), from which it can be seen that L 1 particles contain RNA of mol. wt. 0.94 $\times 10^6$ and the more dense L 2 particles contain the RNA of mol. wt. 1.11 $\times 10^6$.

The mol. wt. of M and H particles were calculated from their compositions assuming that the weight of their capsid protein is the same as that of L particles (5.97 $\times 10^6$ – 0.93 $\times 10^6$ = 5.04 $\times 10^6$). This assumption seems reasonable because PsV-S was electrophoretically homogeneous, electron microscopy showed that M, L and H particles were all approximately the same size and no difference was detected in the amino acid compositions of M, L and H particles. From the mol. wt. of M particles (5.52 $\times 10^5$) and their composition, the average weight of RNA/particle was calculated to be 0.48 $\times 10^6$. The mol. wt. of M particle RNA components found from gel electrophoresis were 0.47 $\times 10^6$ and 0.56 $\times 10^6$. Hence it may be deduced that M particles contain only one molecule of RNA/particle. The caesium chloride density gradient profile of M particles (Fig. 4iii) and the gel electrophoresis scans of M particle RNA (Fig. 6i) are consistent with the presence of two particles, M 1, the major component containing the RNA of mol. wt. 0.47 $\times 10^6$, and M 2, the minor and more dense component, containing the RNA of mol. wt. 0.56 $\times 10^6$. The mol. wt. of M particle RNA components were half those of L particle RNA components and it is possible
that they consisted of one strand of the double-stranded RNA. Proof of this will require infectivity studies and hybridization studies with labelled RNA.

H particles contained both double-stranded and single-stranded RNA. The single-stranded RNA moved as a slow diffuse band in gel electrophoresis (Fig. 6v) and was probably aggregated; hence its mol. wt. could not be determined by this method. The two double-stranded RNA components had mol. wt. identical with those of L particle components. Comparison of the calculated amount of RNA/particle \((1.67 \times 10^6)\) with the mol. wt. of the two double-stranded RNA components \((0.94 \times 10^6)\) and \((1.11 \times 10^6)\) indicated that H 1 and H 2 particles each contained one molecule of double-stranded RNA together with sufficient single-stranded RNA to make up the difference. The ratio of double-stranded to single-stranded RNA in H particle RNA \((2:1)\) found from sedimentation analysis is consistent with this interpretation. Gel electrophoresis of H 2 particle RNA (Fig. 6vii) showed it to contain the RNA of mol. wt. \((1.11 \times 10^6)\) together with single-stranded RNA, while H 1 particle RNA (Fig. 6vi) contained both double-stranded RNA components in the ratio of 3:1 in favour of the lower mol. wt. component, together with single-stranded RNA. Further fractionation of H 1 particles did not increase this ratio. Hence it may be deduced that H 1 particles are not homogeneous and contain two types of particles (H 1a, H 1b), each having one of the double-stranded RNA components, but differing in the quantity of single-stranded RNA, so as to make the total quantity of RNA per particle, and hence the particle density the same. The results of sedimentation analysis of the RNA components with densities intermediate between those of L 2 and H 1 particles (Fig. 7) indicate that these particles contain the same amount of double-stranded RNA, but with the amount and sedimentation coefficient of single-stranded RNA increasing with the density of the particles.

Whether the single-stranded RNA in H particles is encapsidated virus or host RNA cannot be determined from the present data, but the density stability to repeated centrifuging in caesium chloride gradients makes it unlikely that the single-stranded RNA was carried on the exterior of the particles. It has been reported recently that PsV, presumably a mixture of PsV-S and PsV-F since no separation was carried out, contains an RNA polymerase as an integral part of the virus particles (Lapierre, Astier-Manifacier & Cornuet, 1970). Part of the RNA synthesized \textit{in vitro} by this polymerase was insensitive to ribonuclease, and it was suggested that the newly synthesized RNA may be in the interior of the capsid. This suggested that PsV-S H particles may be particles which are in the process of replicating their RNA.

PsV-S appears to be the first virus reported to contain particles with single-stranded RNA, particles with double-stranded RNA and particles with both double-stranded and single-stranded RNA. Bozarth \textit{et al.} (1971) reported that PsV-F contained both double-stranded and single-stranded RNA, but no fractionation studies were carried out. It has been suggested (Ratti & Buck, 1972) that multicomponent virus systems may be favoured in fungi, in which virus replication occurs in parallel with the growth of the organism and cell lysis and re-infection from without are not required. However, the biological functions and interrelations of the components of fungal viruses remain a challenge for the future.

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