The Two Nucleoprotein Particles of Cherry Leaf Roll Virus

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SUMMARY
Purified preparations of the golden elderberry strain of cherry leaf roll virus contain two spherical nucleoprotein components with sedimentation coefficients of 115s and 128s. Purification of the components by centrifuging several times through sucrose density gradients decreased their specific infectivity. The original specific infectivity of unfractionated virus was restored by mixing the separated components, indicating that both components are necessary for maximum infectivity. The components were indistinguishable serologically, in their extinction spectra and in their appearance in the electron microscope. Polyacrylamide gel electrophoresis of preparations from each component indicated that both contained a single protein species of 54,000 mol. wt, but that whereas the 115s component contained a RNA molecule of $2.1 \times 10^6$ mol. wt the 128s component contained one of $2.4 \times 10^6$. These and other properties suggest that cherry leaf roll virus can be considered a member of the nepovirus group. The present cryptogram of the golden elderberry strain is R/1 : 2.1/40 + 2.4/43 : S/S : S/*.

INTRODUCTION
Cherry leaf roll virus (CLRV, */* : */* : S/S : S/Ne) described by Cropley (1961), occurs naturally in several plant species in Europe and North America (Cropley & Tomlinson, 1971). In its herbaceous host range, symptomatology and physical properties (Cropley, 1961) it closely resembles members of the nepovirus group (Cadman, 1963) and it has been reported to be transmitted by nematodes (Xiphinema spp.) (Fritzche & Kegler, 1964; Flegg, 1969). However, difficulties in purification of the virus have hindered its detailed characterization and therefore its membership in the nepovirus group has been tentative (Harrison et al. 1971). Recently, Hansen (1967) and Hansen & Stace-Smith (1971a) described a virus from American elder which they called golden elderberry virus and which was later shown to be a strain of cherry leaf roll virus (Jones & Murant, 1971). Unlike other reported strains of CLRV that have been studied, the golden elderberry strain is relatively stable. Some of its properties have already been described (Hansen & Stace-Smith, 1971b). In this paper, we describe some further properties and indicate that it is an acceptable member of the nepovirus group.

METHODS
Virus isolate. The $S_3$ isolate of the golden elderberry strain of cherry leaf roll virus was used. This isolate is serologically indistinguishable from the isolate of golden elderberry virus studied by Hansen & Stace-Smith (1971b) (Jones & Murant, 1971) and is similar in physical properties and host range. The virus was cultured in Chenopodium quinoa Willd. in glasshouses kept between 15 and 25°C.
Infectivity assays. Local lesion assays were made in *Nicotiana tabacum* L. cv. Xanthi-nc by applying inoculum on a muslin pad to half-leaves previously dusted with carborundum (600 mesh/inch).

Separation of components. Following purification, virus was separated into two components by centrifuging several times through sucrose density gradients. Sucrose density gradients were prepared in Spinco SW 27 tubes by allowing layers of 5 ml. of 40%, and 10 ml. of 30, 20 and 10% (w/v) sucrose (in 0.006 M-neutral phosphate buffer unless otherwise stated) to diffuse overnight at 4°. Partially purified virus (0.6 to 0.8 ml.) was then layered on each, centrifuged for 90 min. at 25,000 rev./min., the gradients displaced upwards with 50% sucrose and E254 recorded. Fractions were collected manually and, for further sucrose density gradient runs, were diluted three to fivefold in either 0.006 M-phosphate buffer (pH 7.0) or 0.01 M-tris-HCl buffer (pH 7.0) and centrifuged for 150 min. at 180,000 g. Pellets were resuspended in 0.006 M-phosphate buffer and re-run through sucrose density gradients.

Equilibrium sedimentation in caesium chloride. Partially purified virus was centrifuged in caesium chloride solution (initial density 1.5 gm./cm.³) for 24 hr at 35,000 rev./min. in a Spinco SW 39 rotor at 5°. The gradient was fractionated through an ISCO recording absorptiometer and the refractive indices of the fractions determined.

Polyacrylamide gel electrophoresis. Virus protein was prepared by boiling approximately 0.1% virus for 1 min. in 1% (w/v) sodium dodecyl sulphate (SDS) + 1% (v/v) 2-mercaptoethanol and examined by electrophoresis for 5 hr at 4 v/cm. in 7.5% acrylamide gels, using 0.1 M-sodium phosphate buffer pH 7.0. The mol. wt of the proteins were estimated by comparing their rates of migration with those of proteins of known mol. wt. These were bovine serum albumin (Calbiochem), ovalbumin, carbonic anhydrase and chymotrypsinogen (Sigma Chemical Co.) and tobacco mosaic virus protein. Protein bands were stained with Coomassie brilliant blue.

Virus nucleic acid was prepared by resuspending purified virus at about 0.1% in pronase (1.5 mg./ml., Calbiochem) and SDS (5 mg./ml.) in 0.15 M-sodium chloride + 0.015 M-sodium citrate which had been pre-incubated at 37° for 30 min. Virus was incubated at 37° for 16 hr and the nucleic acid liberated was either layered directly onto the surface of gels or recovered by precipitation with 2.5 vol. of ethanol. Gels (2×2 cm) were prepared from purified monomers as described by Loening (1967). Bands of RNA in gels were detected using a u.v. densitometer (Joyce–Loebl Ltd.) and mol. wt were estimated by comparing mobilities with those of ribosomal RNA of known mol. wts (Loening, 1968).

Electron microscopy. Purified virus preparations were stained in uranyl formate/NaOH (Barnett & Murant, 1970) and examined in a Siemens Elmiskop IA. Photographs were taken at a fixed magnification of ×40,000 (±5%).

RESULTS

Purification

Several purification methods were assessed for yield and purity of virus. Initial purification experiments used virus extracted from systemically infected leaves of *Nicotiana clevelandii* Gray, but later it was found that *C. quinoa* Willd. was a better host. Three principal purification techniques were used, those for tomato ringspot (Gooding, 1963, method 1; and Stace-Smith, 1966, method 2) and that for CLRV, golden elderberry strain (Hansen & Stace-Smith, 1971b, method 3).

All except (1) gave acceptable yields of virus but also contained quantities of host plant
components. An experiment to compare the relative merits of methods 2 and 3 indicated that method 3 gave three times the virus yield of method 2, but contained more host plant material. When samples were centrifuged through sucrose density gradients in distilled water, preparations from method 3, but not those from method 2, were degraded. Although preparations from both purification methods were stable in buffered gradients, the disruption of virus from method 3 in unbuffered gradients suggested that this purification method damaged the virus. Method 2 was therefore adopted as the standard purification technique. Infective sap was extracted in 0.5 M-boric acid buffer (1 g. leaf: 2 ml. buffer), frozen overnight, thawed, and clarified by centrifugation for 15 min. at 10,000 g. Granulated ammonium sulphate was then added to the supernatant fluid (15 g./100 ml. sap) and allowed to stir overnight at 4°C. After centrifuging at 10,000 g for 15 min., the supernatant fluid was further clarified and concentrated by two cycles of differential sedimentation.

The interval between inoculation and virus purification affects the relative proportions of virus components obtained when tobacco ringspot virus is purified (Schneider & Diener, 1966). When C. quinoa leaves infected with CLRV were harvested 2, 5, 7 and 9 days after inoculation, the virus yield increased progressively, reaching a maximum at 9 days, but the relative proportions of the components, as assessed by extinction profiles from sucrose density gradients, did not alter appreciably.

**Analytical ultracentrifugation**

Preparations of CLRV in 0.006 M-phosphate buffer (pH 7.0) contained two major components, having sedimentation coefficients (S20w) at infinite dilution of 115s and 128s. This compares with values of 114s and 128s obtained by Hansen & Stace-Smith (1971b). Although CLRV does not seem to form stable RNA-free protein shells (top), the 115s and 128s components are here called middle (M) and bottom (B) component respectively.

**Equilibrium sedimentation**

Two light scattering zones were formed when CLRV was centrifuged to equilibrium in caesium chloride solution. The densities, 1.47 and 1.50 g/cm³ presumably refer to the M and B components respectively.
Table 1. Infectivity of mixtures of purified middle and bottom components of CLRV

<table>
<thead>
<tr>
<th>Expt</th>
<th>E260</th>
<th>Component</th>
<th>Infectivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01</td>
<td>M</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M + B</td>
<td>214</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLRV</td>
<td>158</td>
</tr>
<tr>
<td>2</td>
<td>0.001</td>
<td>M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M + B</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLRV</td>
<td>172</td>
</tr>
<tr>
<td>3</td>
<td>0.001</td>
<td>M</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M + B</td>
<td>351</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLRV</td>
<td>362</td>
</tr>
</tbody>
</table>

* Total number of lesions in four half leaves of *Nicotiana tabacum* cv. Xanthi-nc.

Infectivity of components

When the two components of CLRV were separated by centrifuging in sucrose density gradients, the infectivity in the gradient was largely associated with component B (Fig. 1), suggesting that M is either not infective or much less infective than B. To confirm this, the components were separated by centrifuging two further times through sucrose density gradients and assayed for infectivity. After the third separation, the total number of lesions in four half-leaves of *N. tabacum* cv. Xanthi-nc produced by preparations having *E*260 values of 0.06 was M, O and B, 86. This indicates that as the purity of each component increases (Fig. 2), the infectivity decreases, the greater purity of M being reflected in a lower infectivity.

Further results showed that the decrease in the infectivity of the purified components was not caused by damage produced by repeated sedimentation, because the original high specific
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Table 2. Effect of ribonuclease on infectivity of CLRV and its nucleic acid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infectivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus-containing extracts in 0·006 m-phosphate buffer</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>328</td>
</tr>
<tr>
<td>+ RNase, 0·25 mg./l.</td>
<td>386</td>
</tr>
<tr>
<td>+ RNase, 0·25 μg./l.</td>
<td>290</td>
</tr>
<tr>
<td>Nucleic acid extracts in 0·006 m-phosphate buffer</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>56</td>
</tr>
<tr>
<td>+ RNase, 0·25 mg./l.</td>
<td>0</td>
</tr>
<tr>
<td>+ RNase, 0·25 μg./l.</td>
<td>43</td>
</tr>
<tr>
<td>Nucleic acid extracts in 0·006 M-phosphate buffer + NaCl, 0·2 M</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>31</td>
</tr>
<tr>
<td>+ RNase, 0·25 mg./l.</td>
<td>0</td>
</tr>
<tr>
<td>+ RNase, 0·25 μg./l.</td>
<td>30</td>
</tr>
</tbody>
</table>

* Total number of lesions in two half-leaves of Nicotiana tabacum cv. Xanthi-nc.

Infectivity of unfractionated virus was restored by mixing equal amounts (as judged by \( E_{260} \) measurements) of purified M and B components (Table 1). Thus, the infectivity of the components was four to eight times greater when mixed than when separate. These results indicate that both M and B are necessary for maximum infectivity.

When virus from highly purified preparations of B component was passed through two cycles of single lesion isolations in N. tabacum cv. Xanthi-nc and the final isolate purified, preparations contained two sedimenting components corresponding to M and B. One explanation of this is that the low infectivity of purified preparations of B is due to the inability of our technique of separation to produce preparations of B entirely free of M.

Effect of ribonuclease on nucleic acid extracts

Nucleic acid preparations were made from CLRV-infected C. quinoa leaves using method B of Loening & Ingle (1967) (Murant et al. 1972), and were divided into two equal samples. One was resuspended in 1 ml. 0·006 m-phosphate buffer at pH 7 and the other in 1 ml. 0·2 M-NaCl in 0·006 m-phosphate buffer at pH 7. Samples (0·25 ml.) of each were stored for 90 min. at 2° with either 0·25 ml. of 0·006 m-neutral phosphate buffer, or buffer containing pancreatic ribonuclease (5 × crystallized, salt and protease-free; Type 1-A Sigma Chemical Company) heated for 5 min. at 90° before use to inactivate any deoxyribonuclease present. CLRV-containing C. quinoa extracts were used as controls and all samples were assayed for infectivity.

RNase (0·25 mg./l.) did not affect the infectivity of intact virus but abolished that of nucleic acid extracts even in a medium of high ionic strength (Table 2), suggesting that CLRV contains single-stranded RNA.

U.v. extinction spectrum

Purified preparations of CLRV had spectra typical of nucleoprotein. From eight measurements the mean \( E_{260}/E_{280} \) ratio was 1·62 and the mean \( E_{320}/E_{260} \) ratio was 0·061. Spectra of separated M and B components were similar to those for unfractionated preparations.

Electron microscopy

The particles in purified preparations of M and B differed little in size or appearance. Both contained a proportion of particles penetrated by the stain and almost all such particles showed breakages at one or more points (Fig. 4). The mean diameter of particles from unfractionated CLRV preparations was 26 nm.
Fig. 3. Double-diffusion test in 1% agarose gel. Wells contain: A antiserum to CLRV, golden elderberry strain diluted 1/16; M purified middle component of CLRV; B purified bottom component of CLRV.

Fig. 4. Particles of CLRV in uranyl formate.

Serology

Purified preparations of CLRV and of M and B components, each with E260 values of 0.2 were titrated against antiserum to CLRV, golden elderberry strain in micro-precipitin tests. All reacted with the antiserum to a dilution of 1/512 (its precipitation end-point). In 1% agarose gel diffusion plates, M and B reacted with the antiserum to golden elderberry virus without the formation of spurs (Fig. 3) indicating that they are serologically indistinguishable.

Protein

Protein preparations from M component, B component, a mixture of both, and unfractionated virus, contained a single similar sized polypeptide with an estimated mol. wt of 54,000 (mean of 20 determinations). The protein could not be resolved from that prepared from raspberry ringspot virus, confirming that its estimated mol. wt is 54,000 (Mayo, Murant & Harrison, 1971). This estimate was not altered when CLRV was disrupted with 8 M-urea, 1% SDS and 1% 2-mercapto-ethanol in 0.07 M-tris-HCl (pH 8.3), or when such protein was subsequently alkylated by incubating with 0.3 M-iodoacetamide at 37° for 20 min.

RNA

Two bands of RNA were resolved in preparations from unfractionated virus in 2.2% polyacrylamide gels (Fig. 5a). Preparations from M contained predominantly the smaller RNA species with an estimated mol. wt of 2.1 x 10^6, whereas B contained predominantly...
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the larger RNA species with an estimated mol. wt of $2.4 \times 10^6$. The relative amounts of the RNA species reflected the sedimentation profile of the virus preparation (Fig. 5). RNA from B component could not be resolved from the larger RNA (RNA-1) of raspberry ringspot virus (Murant et al. 1972).

**Particle weight calculations**

The estimated % RNA determined from the buoyant density values in caesium chloride (Sehgal et al. 1970) is M 38 % and B 42 %. Another estimate was obtained from an expression derived from the Svedberg equation relating percent RNA, particle radius, RNA mol. wt and sedimentation coefficient (unpublished). Using our data, including the radius of the dehydrated particle, the estimates were 41.8 and 43.3. Allowance for hydration will slightly decrease this estimate, thus the true value is probably about 40 % and 43 %.

From these data, estimated particle weights were $5.3 \times 10^6$ and $5.6 \times 10^6$. Alternatively, assuming a partial specific volume of 0.55 for RNA and of 0.74 for protein and a hydrated radius of 14 nm., using the Svedberg equation (Markham, 1967a) and that for diffusion coefficient (Markham, 1967b) estimates of $5.4 \times 10^6$ and $5.9 \times 10^6$ were obtained. The weights of protein in the two components are therefore M, 3.2 to $3.3 \times 10^6$ and B, 3.2 to $3.5 \times 10^6$ and the virus particles probably contain 60 asymmetric units, each of a single poly-peptide of mol. wt 54,000.

**DISCUSSION**

These results show that highly purified preparations of each of the two components of cherry leaf roll virus have little or no infectivity individually but have a high specific infectivity, similar to that of unseparated virus, when mixed. This contrasts with the findings of Hansen & Stace-Smith (1971) who concluded that each component was highly infective alone; tests to detect enhanced infectivity from mixing the separated components were not reported. This discrepancy probably arises from differences in the techniques of separating the components. Absorptiometer traces support this conclusion (Fig. 2), indicating that at least three separations through sucrose density gradients are necessary to achieve reasonable purity of the components. Although our results do not entirely exclude the possibility that one or other component is infective alone, we interpret the low specific infectivity observed in some highly purified preparations of the components as an indication of the inability of our technique to produce entirely homogenous preparations, particularly with B. We suggest that neither component is infective alone. This situation seems the more likely in view of recent findings which indicate that in several other multicomponent virus systems the virus genome is contained within two or more separate particles, all of which are necessary for normal virus synthesis (Matthews, 1970).

Hansen & Stace-Smith (1971) concluded that because the two components had similar spectra and were very infective, each contained the same amount of RNA, and they suggested that the slower sedimenting component was deficient in protein. However, the results of polyacrylamide gel electrophoresis of the nucleic acid of each component indicate that the difference in their particle weights, estimated from sedimentation coefficients, can be accounted for by the difference between the estimated mol. wt of their RNA molecules. This difference in RNA content (2 to 3 %), is not sufficient to produce an appreciable change in the spectrum. Moreover, the proteins of the two components were indistinguishable serologically and in size suggesting that the protein shells of the two components are identical, and that the difference between them in sedimentation coefficient reflects the difference in their RNA content.

The existence of at least two RNA species has recently been established for some nepo-
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viruses (Diener & Schneider, 1966; Schneider & Diener, 1968; Harrison, Murant & Mayo, 1972; Murant et al. 1972) and the protein shell of at least three nepoviruses is probably composed of 60 units, each of a single polypeptide of mol. wt 5 to 6 x 10^4 (Mayo et al. 1971). Our data on the size of CLRV protein and the size of and need for more than one RNA species to initiate normal virus synthesis, indicates that CLRV conforms to the general pattern of the nepovirus group and thus extends previous observations of this affinity (Cropley, 1961; Cadman, 1963).

However CLRV differs from most other nepoviruses in that its slower sedimenting nucleoprotein component sediments more rapidly than the corresponding component of the others (Murant et al. 1972). This is reflected in the size of its RNA molecule which is considerably larger than that reported for tobacco ringspot (Diener & Schneider, 1966; Schneider & Diener, 1968), arabis mosaic and raspberry ringspot (Mayo et al. 1971; Murant et al. 1972) viruses. Nevertheless, this difference in the size of the small RNA need not preclude CLRV from membership of the group. There are precedents for variation in the size of the smaller RNA of multicomponent viruses, for example among strains of tobacco rattle virus (Harrison & Woods, 1966).

The present cryptogram for the golden elderberry strain of CLRV is therefore,

\[ R/1 : 2:1/40 + 2:4/43 : S/S : S/(Ne). \]

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REFERENCES


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