PSEUDORECOMBINANTS OF
CHERRY LEAF ROLL VIRUS

by

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ABSTRACT

Cherry leaf roll virus, as a nepovirus with a bipartite genome, can be genetically analysed by comparing the properties of distinct 'parental' strains and the pseudorecombinant isolates generated from them. In the present work, the elderberry (E) and rhubarb (R) strains were each purified and separated into their middle (M) and bottom (B) components by sucrose gradient centrifugation followed by near-equilibrium banding in cesium chloride. RNA was extracted from the separated components by treatment with a dissociation buffer followed by sucrose gradient centrifugation. Extracted M-RNA of E-strain and B-RNA of R-strain were mixed and inoculated to a series of test plants as were M-RNA of R-strain and B-RNA of E-strain. New local lesion types in *Nicotiana clevelandii* induced by these heterologous RNA combinations were passaged three times through local lesions on *N. clevelandii* in order to generate pure pseudorecombinant stocks. M-RNA determined serological specificity, the distribution of virus particle components, systemic symptoms in *N. clevelandii* and *N. tabacum* cvs. Samsun and Xanthi as well as the local and systemic symptoms in *Chenopodium amaranticolor* and *C. quinoa*. B-RNA determined the ability to induce systemic symptoms in *Gomphrena globosa* and the type of local lesion in *N. clevelandii*. When the procedure used for originally generating the pseudorecombinants from the parental strains was applied to the pseudorecombinant isolates themselves, isolates were obtained in the predicted manner that were identical to the original parental E- and R-strains.
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INTRODUCTION

In terms of genome organization, viruses may be assigned to one of three broad categories: a) single, unsegmented genome, b) segmented genome with all segments contained in one particle type, and c) segmented genome with segments distributed among different particle types. A majority of both DNA and RNA viruses of animals, plants and bacteria belong to the first category. Examples are adeno- and paramyxoviruses of animals (Fenner et al., 1974), caulimo-, tobamo- and luteoviruses of plants (Tremaine, 1977) and phages lambda, T4 and phi-6 of bacteria (Casjens and King, 1975). Viruses belonging to the second category include the double-stranded RNA animal and plant reoviruses (Fenner et al., 1974; Casjens and King, 1975) and the single-stranded RNA orthomyxoviruses of animals (Casjens and King, 1975). In the case of the orthomyxovirus, influenza A, the analysis of the genetic recombination 'rescues' of mutants defective in different capacities and genome segments has succeeded in establishing a complete genetic map (Ritchey et al., 1976). Almost all viruses of the third category are plant viruses (Casjens and King, 1975).

Among viruses of this third category, new isolates can be generated by artificially combining complementary genome segments of related isolates. If the separate components are by themselves uninfectious, but yield a new productive infection upon heterologous combination, the resultant new isolates are termed 'pseudorecombinant' (Gibbs and Harrison, 1974). 'Pseudorecombination' with viral genome segments is distinguished from 'recombination' (as occurs with influenza virus) by the fact that the different genome segments do not
come together in one particle but are encapsidated in different component particles. Deliberate pseudorecombination, like recombination with influenza virus, can be employed as a useful tool for genetic analysis if the following criteria are met: a) the viral genomic components can be separated to the extent that each component on its own is not, or only slightly, infectious but combined with the complementary components is highly infectious; the quantitative measure of success is termed the 'enhancement-of-infectivity' ratio, usually defined as the number of local lesions induced by a deliberate mixture of the separated components divided by the average number of local lesions (at equal particle concentration on equal leaf area) induced by each of the separated components alone (Bruening, 1977). Higher ratios indicate superior separation of components. b) there are related viruses or strains that are distinct from one another in a number of observable properties, and c) the new infection resulting from pseudorecombination is sufficiently productive that properties of the pseudorecombinants can be observed and compared with those of the contributing 'parental' isolates.

Several virus groups (and as yet ungrouped or monotypic viruses) appear on the basis of their divided genomes to be candidates for pseudorecombination. All plant viruses in this category are single-stranded RNA viruses of helical, isometric or bacilliform geometry (Tremaine, 1977). In what follows, the essential findings of pseudorecombination studies with plant viruses that possess two separately encapsidated RNA species whose combined activity is required for full infectivity ('functionally bipartite'), will be briefly reviewed.
Tobraviruses are helical plant viruses with bipartite genomes and are transmitted by trichodorid nematodes. They can be seen as counterparts of the isometric nepoviruses, which are transmitted by longidorid nematodes (Harrison et al., 1971; Shepherd et al., 1976). Tobraviruses were among the first viruses to be studied by pseudorecombination analysis. All studies to date have been performed with the type number, tobacco rattle virus (TRV), and strongly indicate that the coat protein is determined by the smaller of the two RNA species (Ghabrial and Lister, 1973). Symptomatology appears to be determined by either or both RNAs, depending on the particular host species (Saenger, 1968; 1969; Ghabrial and Lister, 1973).

All other plant viruses with divided genomes consisting of two separately encapsidated RNA species are of isometric geometry. The only members in this category are the monotypic carnation ringspot virus (CRSV) and the como- and nepovirus groups (Tremaine, 1977).

The genome of CRSV consists of two RNA species of 0.5 and 1.5 million daltons molecular weight. The bipartite nature of the genome is only apparent upon analysis of the extracted RNA since the genome is packaged in particles which sediment as a single nucleoprotein component (Dodds et al., 1977). From pseudorecombinant genetic analysis it appears that the larger RNA determines the coat protein (Dodds et al., 1977). Besides pea enation mosaic virus (PEMV), whose genome is functionally bipartite but contains a third RNA species which appears not to influence infectivity and is smaller than either of the two functional RNA species (Hull and Lane, 1973), CRSV is the only other divided-genome plant virus in which the coat protein is known to be determined by the largest functional RNA species.
As a group, the comoviruses have been favored objects of study by plant virologists (Bruening, 1977) and the type member, the yellowing strain of cowpea mosaic virus (CPMV-Sb), was one of the first subjects of pseudorecombinant genetic analysis (Bruening, 1969). Since each comovirus capsid contains 60 subunits of each of two polypeptide species (Wu and Bruening, 1971), the distribution of the genetic determinants between the two (middle and bottom) nucleoprotein component RNAs is of interest. All pseudorecombinant analyses performed to date indicate antigenic determination of the capsid protein by the smaller, middle component RNA (Kassanis et al., 1973; Moore and Scott, 1971; Thongmeearkom and Goodman, 1978). Recently, Gopo and Frist (1977) have shown the smaller polypeptide itself to be determined by the smaller RNA. The genetics of symptom determination among comoviruses appear to vary with the host species and the origin of the variant strain (de Jager and van Kammen, 1970; Wood, 1972; de Jager, 1976; Thongmeearkom and Goodman, 1978; Oxelfelt and Abdelmoeti, 1978).

Nepoviruses, like comoviruses, have a bipartite genome distributed between two differentially sedimenting classes of constant-diameter particles, but they differ from comoviruses in certain physical and biological properties. Nepoviruses possess only one coat-protein polypeptide species (with the possible exception of a few members whose status as nepoviruses is still a matter of debate). They are transmitted by the nematode genera Longidorus and Xiphinema and tend to have wide host ranges in which they cause ringspot and mottle symptoms, often followed by symptomless infection (Harrison and Murant, 1977a).

All nepoviruses (Shepherd et al., 1976; Harrison and Murant, 1977a) appear to have two main RNA species, the heavier one (RNA-1) close to
2.4 million daltons in all members and making up about 42% by weight of the bottom component nucleoprotein, and the lighter one (RNA-2) varying with the particular member of the group from 1.3 to 2.3 million daltons and thus ranging from about 27-40% by weight of the middle component nucleoprotein. Much smaller satellite RNAs (RNA-3) in addition to the two larger species have been reported in three nepoviruses: tobacco ringspot virus (TRSV) (Schneider et al., 1972), tomato black ring virus (TBRV) (Murant and Mayo, 1976) and myrobalan latent ringspot virus (MLRV) (Dunez et al., 1976). Recently, it has been shown that in at least two nepoviruses, TRSV and TomRSV, a small protein covalently linked (possibly at the 5'-end) to both main RNA species is required for infectivity (Harrison and Barker, 1978).

Until recently, the nepovirus capsid was described as being a T=1 icosochedral structure with 60 polypeptide subunits, the polypeptide subunit having a molecular weight of about 55,000 daltons (Harrison and Murant, 1977a). Some viruses, such as strawberry latent ringspot virus (SLRV), have, in most respects, properties similar to nepoviruses, but their capsids consist, like those of comoviruses, of two polypeptides with apparent molecular weights of 29,000 and 44,000 daltons (Mayo et al., 1974). Very recent analyses of the coat protein of TRSV by Chu and Francki (1979), using SDS-polyacrylamide gel electrophoresis preceded by dissociation in the presence of SDS, urea and 2-mercaptoethanol, and tryptic peptide mapping, have provided strong evidence that the molecular weight of the polypeptide subunit of nepovirus capsid proteins is about 13,000 daltons, only one-fourth the size previously described. In the light of this information, a reassessment of seemingly two-polypeptide viruses, tentatively assigned to the nepovirus group,
such as SLRV and cherry rasp leaf virus (CRLV), may reveal that they share with the definitive nepoviruses a basic 13,000 dalton polypeptide subunit.

Within the nepovirus group, subgroups based on serological relationships and the apparent molecular weight of the lighter RNA-2, have been defined (Quacquarelli et al., 1976). The type and English strains of raspberry ringspot virus (RRV) form a cluster of related serotypes as do potato black ring virus (PBRV) and the type and eucharis mottle strains of TRSV; the arabis mosaic virus (AraMV) cluster comprises grapevine fanleaf virus (GFV) and AraMV. The RNA-2 of the viruses of these three serotype clusters ranges in molecular weight from 1.4-1.5 million daltons. In addition, a portion of the nucleoproteins sedimenting in the bottom component consists of particles which possess two molecules of RNA-2 while the rest contain one molecule each of RNA-1.

Among nepoviruses that have an RNA-2 of 1.5 - 1.6 million daltons with only one RNA molecule per particle [possible exception, MLRV (Debos et al., 1976)], there are two serotype clusters. The TBRV subgroup comprises cocoa necrosis virus (CNV), grapevine chrome mosaic virus (GCMV), MLRV, and the potato bouquet and beet ringspot strains of TBRV, while artichoke Italian latent virus (AILV) appears so far to have no serologically close relatives.

Finally, among the definitive nepoviruses, tomato ringspot virus (TomRSV), peach rosette mosaic virus (PRMV) and cherry leaf roll virus (CLRV) have an RNA-2 only slightly smaller than RNA-1, allowing only one molecule of RNA per particle. The three viruses are not close serological relatives but a number of serologically closely related strains have been described for CLRV (Walkey et al., 1973).
The first evidence for a functionally bipartite genome in a nepovirus was obtained by Bancroft (1968) who demonstrated a 10-fold enhancement-of-infectivity when middle and bottom nucleoprotein components of tomato top necrosis virus (TomTNV), previously separated by sucrose gradient centrifugation, were combined in an inoculum. Jones and Mayo (1972), using similar methods with repeated cycles, obtained an enhancement-of-infectivity ratio of about 7 with CLRV. Schneider et al. (1974), working with TomRSV, also employed sucrose density-gradient separation of middle and bottom component nucleoproteins and obtained an enhancement-of-infectivity ratio of about 4. The relatively poor ratios obtained were attributed to poor separations of the two component nucleoproteins. The middle component nucleoproteins had sedimentation coefficients only slightly lower than those of the bottom component [102S, 115S and 119S respectively for TomTNV, CLRv and TomRSV compared to about 127S for each bottom component (Harrison and Murant, 1977a)] and sucrose density-gradient centrifugation does not readily resolve such closely sedimenting components.

With nepoviruses such as TRSV and RRV, the problem in obtaining high enhancement-of-infectivity ratios is not mainly due to poor resolution of middle and bottom nucleoprotein components but rather due to the presence of a full genome complement in bottom component particles; some bottom-component particles, contain one RNA-1 and others two RNA-2 molecules (Diener and Schneider, 1966; Murant et al., 1972). Murant et al. (1972), solved this problem by using RNA components as inoculum. The RNA preparations were heated in 8M urea to promote denaturation and were well resolved by subsequent polyacrylamide gel electrophoresis. Enhancement-of-infectivity ratios of about 80
were obtained with RRV RNA components separated in this way.

The first successful pseudorecombinations with nepoviruses were achieved by Harrison et al. (1972) using the serologically related but distinct English and Scottish strains of RRV. The two strains also differed in the systemic symptoms they induced in Petunia hybrida Vilm. Pseudorecombinants were made from gel electrophoresis-separated RNA-1 and -2 using the methods of Murant et al. (1972). Both serological relationships and systemic symptom expression were inherited through RNA-2. In a later study (Harrison et al., 1974a) the genetics of transmission of the Scottish strain by the nematode, Longidorus elongatus deMan were examined. RNA-2 was also found to determine this property. Taken together with the evidence for determination of serological properties by RNA-2 (Harrison et al., 1972), it clearly appeared that the smaller RNA contains (as with tobraviruses, the other specifically nematode-transmitted plant virus group) the genetic information for the coat protein.

This conclusion was confirmed by a more extensive study (Harrison et al., 1974a) where pseudorecombinants were generated among four serologically related strains: the English and Scottish strains of earlier work and the Lloyd George strain (capable of infecting the Lloyd George variety of raspberry) and the Dutch strain, serologically identical with the Scottish strain but inducing, as the English strain does, systemic yellowing in P. hybrida. Assignments of properties to pseudorecombinant RNA species were confirmed by backcrossing to generate the predicted parental phenotypes. In addition, RNA-1 was found to determine the ability to infect systemic leaves of Phaseolus vulgaris L. The determination of systemic yellowing symptoms in P. hybrida,
indicated in earlier work (Harrison et al., 1972) to be controlled by RNA-2, appeared more complex and was shown to be associated with ultrastructural changes in the chloroplasts. The RNA-2 of the English strain, shown previously to determine these symptoms, appeared to be suppressed in its expression of this property when it was associated with the RNA-1 of the Lloyd George strain.

Recently, Hanada and Harrison (1977), using similar materials and methods as before (Harrison et al., 1972; 1974b), have shown that seed transmissibility in chickweed (Stellaria media L.) of RRV and TBRV depends greatly on the transmissibility of the strain contributing the RNA-1. RNA-2, however, also exerts a measurable but smaller influence, the extent of which depends on the RNA-1 with which it is combined. This has been interpreted as suggesting that, in naturally-occurring strains, a selection for compatibility of RNA-1 and -2 with respect to seed transmission frequency has taken place.

A similar linking of expression of RNA-2 to the type of RNA-1 with which it associates has been observed in a study of the competitiveness in a mixed infection of different RRV genotypes (Harrison and Hanada, 1976). Different pseudorecombinants of RRV with one RNA in common were compared in mixed infections as to the rapidity with which they systemically infected Chenopodium quinoa Willd. plants. This property, the ability to 'dominate', was related to the rapidity with which systemic symptoms were induced in plants singly infected by each of the pseudorecombinant types. RNA-1 was found to be the major determining factor, though to the extent that RNA-1 'allowed' the expression of differences in competitiveness between RNA-2 from different strains, RNA-2 exerted a
minor influence on the competitiveness of different pseudorecombinant types. Though indirect, this evidence supports the notion gained from studies by Hanada and Harrison (1977), that RNA-1 principally determines the rate of seed transmission, since a more rapidly systemically invading virus would seem to have a better chance of infecting the seed forming tissues at an earlier stage.

The only other nepovirus, for which pseudorecombination genetic analyses have been reported to any extent, is TBRV. Its RNA-2, at 1.5 million daltons, like that of RRV, is considerably smaller than its 2.5 million dalton RNA-1. In contrast to RRV, however, TBRV bottom component particles contain only one molecule each of RNA-1 (Murant et al., 1973; Randles et al., 1977). TBRV was first shown to have a functionally bipartite genome by Murant et al. (1973), who found three RNA species of which only the larger two contributed to infectivity. Separated RNA-2 was only slightly infectious, RNA-1 moderately infectious. Combinations of dilute component inocula gave enhancement-of-infectivity ratios of over 30. The smallest RNA component, (RNA-3), of 0.5 million daltons apparent molecular weight, was neither intrinsically infective nor able to contribute to the infectivity of any combination of component RNAs. Instead, it appeared to inhibit lesion formation when added to fully infectious mixtures of RNA-1 and -2. This lesion-inhibiting ability disappeared when the RNA-3 was irradiated with ultraviolet light. If not included in the inoculum, it failed to be subsequently detected in the progeny virus, indicating its 'satellite' nature.

Randles et al. (1977) used both nucleoproteins separated by sucrose
density-gradient centrifugation and RNAs separated by polyacrylamide gel electrophoresis to generate a pseudorecombinant from serologically distinct, satellite-free isolates derived from the Scottish beet ringspot strain (A) and the German potato bouquet strain (G-12) of TBRV. As a preliminary step, they demonstrated that in both isolates middle component (M) nucleoproteins contained only RNA-2, while bottom component (B) nucleoproteins carried only RNA-1.

The combination of M nucleoprotein or RNA-2 of TBRV-G12 with respectively B nucleoprotein or RNA-1 of TBRV-A failed to enhance infectivity over the additive infectivity of the separated components, indicating poor compatibility. The reverse combination, however, of M nucleoprotein or RNA-2 of TBRV-A with respectively B nucleoprotein or RNA-1 of TBRV-G12 showed a level of infectivity enhancement about 12-25% of that observed in homologous component combinations. The pseudorecombinant, which was much less infectious than either parent and which induced smaller local lesions on C. quinoa, was serologically identical with the TBRV-A isolate that had contributed its RNA-2. This result, indicating coat protein determination by the smaller RNA, is in agreement with that obtained in pseudorecombination genetic analyses of RRV (Harrison et al., 1972; 1974b).

Harrison and Murant (1977b) used the same isolates to investigate the genetic determination of nematode transmissibility of TBRV. TBRV-A is specifically transmitted, as is the Scottish beet ringspot strain from which it is derived by L. elongatus, while TBRV-G12, like its original German potato bouquet source, is transmitted by L. attenuatus Hooper. As in the other study (Randles et al., 1977), only one of the two pseudorecombinations between these two parent isolates was achieved.
Specific nematode-transmissability was determined by RNA-2, although the pseudorecombinant was transmitted less frequently by L. elongatus than the TBRV-A parent which had contributed the RNA-2. Lesion type in C. quinoa was also determined by RNA-2, though the pseudorecombinant-induced lesions took longer to appear than those induced by either parent. This was interpreted as indicating that the property of speed of lesion appearance requires determinants in both RNAs.

Hanada and Harrison (1977) used the same parent and pseudorecombinant isolates to study seed transmission of TBRV in chickweed (Stellaria media L.). As with RRV (Hanada and Harrison, 1977), seed transmission appeared to be mainly determined by RNA-1. The possible secondary role of RNA-2 (as with RRV) in seed transmission could not be determined, given the inability to generate the reciprocal pseudorecombinant. In another series of experiments in the same study, RNA-2 rather than RNA-1 appeared to influence seed transmission in Xanthi-nc. tobacco but not in Samsun NN tobacco. In the absence of the reciprocal pseudorecombinant, however, these data are only suggestive.

Using the original Scottish beet ringspot (S) and German potato bouquet (G) strains which harbor a satellite RNA-3 for comparison, it was found (Hanada and Harrison, 1977) that RNA-3 was recovered upon seed transmission but did not appear in any way to affect the frequency of seed transmission in S. media. This is in contrast to RNA-3's effect on local-lesion formation; Murant et al. (1973) had previously observed that addition of RNA-3 to an infectious combination of the two larger RNAs inhibited local-lesion formation.

Of the nepoviruses that fall into the category of having an RNA-2 only slightly smaller than the RNA-1, such as peach rosette mosaic
virus (PRMV) and tomato ringspot virus (TomRSV), the most promising candidate for pseudorecombinant genetic analysis appears to be cherry leaf roll virus (CLRV). A number of distinct, well-characterized, serologically closely-related strains are known (Walkey et al., 1973). In addition, a number of successful purification protocols have been worked out. While the small difference in sedimentation coefficients between the bottom component (127S) and middle component (115S) nucleoprotein does not allow efficient separation of the two components by sucrose density-gradient centrifugation, there is at least no concern that the bottom component nucleoprotein might contain two molecules of RNA-2. Moreover, even with this difficulty, enhancement of infectivity of combined compared to separated nucleoproteins has been demonstrated (Jones and Mayo, 1972). The application of techniques for better separation of the nucleoprotein or RNA could be expected to open the way to successful pseudorecombination genetic analyses since the closely-related, naturally-occurring strains with reproducible, distinct genetic markers are at hand.

Cherry leaf roll virus was first described by Cropley (1961), though it had been reported earlier (Posnette and Cropley, 1955; Posnette, 1956) as a graft-transmissible agent causing severe leaf rolling in cherry trees. Cropley (1960), upon transferring it to Nicotiana and Chenopodium species, found symptom types similar to those observed with the soil-borne viruses, arabis mosaic, raspberry ringspot and tomato black ring. The reactions in some hosts, however, did distinguish cherry leaf roll from these three viruses (Cadman et al., 1960).

The localized distribution of the disease in orchards suggested
soil-borne transmission. Attempts to transmit the disease to herbaceous hosts by growing them in soil taken in soil taken from root zones of infected trees, however, were not successful (Cropley, 1960).

Virus purified from tobacco sap appeared, when fixed in formalin, as isometric particles with an average diameter of 32 nm, casting shadows similar to those of arabis mosaic, raspberry ringspot and tobacco blackring viruses (Harrison and Nixon, 1960).

The physical properties of the virus and its symptoms in infected plants suggested a similarity between cherry leaf roll virus and these three viruses, so serological and cross-protection relationships were examined but none were detected (Cropley, 1960). Jones and Murant (1971), using gel-diffusion serological tests showed that the 'type' cherry leaf roll virus isolated and described by Cropley (1960) was serologically related to 'elm mosaic virus' (Varney and Moore, 1952), 'golden elderberry virus' (Hansen, 1967) as well as a rhubarb isolate of cherry leaf roll virus (Tomlinson and Walkey, 1967). Since the evidence indicated close relations among the four isolates and the Cropley isolate (1960) had been the most studied, it was suggested that the elm, golden elderberry and rhubarb isolates should be considered strains of cherry leaf roll virus (CLRV) (Jones and Murant, 1971). Waterworth and Lawson (1973) isolated a virus from dogwood (Cornus florida L.) which, although it appeared consistently smaller than the Cropley type strain of CLRV (25 nm diameter compared to 28 nm), was found to be serologically related to CLRV type and elderberry strains, and distantly related to the elm strain.

Walkey et al. (1973) examined the comparative physical and biological properties of these five strains of CLRV. All strains had
coat protein with molecular weights of about 55,000 daltons, similarly sedimenting components of 52S, 114S and 132S and middle and bottom component nucleoprotein RNAs of 2.1 and 2.4 million daltons respectively, but serological data revealed different degrees of relatedness among the five strains. The dogwood and elm strains showed a close serological relationship, the cherry strain was more closely related to the dogwood than the elm strain while the rhubarb and elderberry strains were more closely related to each other than to the other three strains.

Since pseudorecombinants are generally easier to prepare from closely rather than distantly related strains (Bruening, 1977), the dogwood-elm or elderberry-rhubarb strain pairs would seem to be the best starting materials for pseudorecombination studies. The elm strain, however, has been reported as being more difficult to purify than the other strains (Fulton and Fulton, 1970).

The properties most accessible to examination in any such pseudorecombination genetic analysis would be serological relationships and symptomatology in a range of differential hosts. The results would be examined to see how they conform to the pattern established in previous genetic analyses with RRV (Harrison et al., 1972; 1974a; Harrison and Hanada, 1976; Hanada and Harrison, 1977) and TBRV (Randles et al., 1977; Hanada and Harrison, 1977).

In a brief report of preliminary work, Jones (1977) stated that pseudorecombinants had been made from the elderberry and rhubarb strains of CLRV. Serological properties were inherited through the middle component, while the ability to induce systemic symptoms in *Gomphrena globosa* L. appeared to be conferred by the bottom component of the rhubarb strain.
MATERIALS AND METHODS

Viruses

The dogwood (D), cherry (C), elderberry (E) and rhubarb (R) strains of CLRV, identical to those described by Walkey et al. (1973), were obtained in the form of sealed freeze-dried leaf samples from Dr. R. Stace-Smith. Apart from initial comparisons of symptoms done with all four strains, most experiments employed the elderberry (E) and rhubarb (R) strains, which were maintained by serial transfers in *Nicotiana clevelandii* Gray. host plants.

Host plants

Inoculations were carried out by grinding leaves from a source plant (usually *N. clevelandii*) infected with a given strain of CLRV in 0.01M potassium phosphate, pH 7.1 buffer and rubbing the buffered sap onto host plants that had been dusted with carborundum. The following host species and cultivars were examined for their ability to serve as differential hosts of the four strains: *Nicotiana clevelandii* Gray, *N. sylvestris* L., *N. glutinosa* L., *N. tabacum* L. cvs. Samsun and Xanthi, *Chenopodium amaranticolor* Coste & Reyn, *C. quinoa* Willd., *Capsicum annuum* L., *Phaseolus vulgaris* L. cvs. Bountiful and Pinto, *Vigna unguiculata* Erdl., *Datura stramonium* L. and *Zinnia elegans* L. Most later experiments, involving only the E- and R-strains and the pseudorecombinant isolates generated from them, employed the following range of differential hosts: *N. clevelandii*, *N. tabacum* cvs. Samsun and Xanthi, *C. amaranticolor*, *C. quinoa* and *Gomphrena globosa* L.
Purification methods

The methods of Mayo and Jones (1972) and Walkey et al. (1973)
were compared. In addition, the following method was developed and
was the procedure used in most experiments:

All procedures were carried out at about 4 C. Fresh systemically-
infected leaf tissue of N. clevelandii (harvested 10-14 days after
inoculation) or leaf and stem tissue of C. quinoa (harvested 6-10 days
after inoculation) was homogenized in two volumes per weight of tissue
of cold (4C) 0.25M (0.5M for C. quinoa) potassium phosphate pH 6.8
containing 0.2% mercaptoethanol and 0.1% sodium diethyldithiocarbamate.
The slurry was strained through cheese-cloth, and chloroform and
butanol (1:1 v/v) was added to the sap to a concentration of 6% (v/v).
The mixture was stirred for about one hour and the emulsified green
sap then centrifuged at 8,000 rpm for 5 min. (Sorvall GSA rotor). An
amber to yellow supernatant was collected to which 8% w/v polyethylene
glycol (PEG) 6000 (Fisher Carbowax) was added and allowed to fully
dissolve by stirring for about two hours. After centrifuging at 8,000
rpm for 20 min. at 4 C (Sorvall GSA rotor) the whitish pellet was
resuspended in 0.02M sodium phosphate pH 7.0 and allowed to stand for
at least one hour, before centrifuging at 10,000 rpm for 10 min (Sorvall
SS-34 rotor) to remove remaining crude flocculent host material. This
clarified concentrated sap was then centrifuged at high speed, depending
on volume, in either a Beckman No. 30 rotor at 27,000 rpm or a Beckman
No. 65 rotor at 35,000 rpm for two to three hours. After discarding
the supernatant, each pellet was overlaid with 1.0 mL of 0.02M sodium
phosphate pH 7.0 in No. 30 tubes or 0.5 mL in No. 65 tubes and allowed
to stand for one hour before being triturated in a ground-glass homogenizer. The triturate was then centrifuged at 8,000 rpm (Sorvall SS-34 rotor) and the supernatant, containing the partially purified virus preparation, collected.

To check the yield and biological activity of this preparation, the optical density of a 100-fold dilution in 0.02M sodium phosphate pH 7.0 buffer was measured against buffer alone at 260 and 280 nm in a Gilford Model 260 spectrophotometer and subsequently inoculated to a series of test plants. This series consisted of N. clevelandii, N. tabacum cvs. Samsun and Xanthi and C. amaranticolor. In some later experiments, C. quinoa and G. globosa were included in this series. In determining the virus yield, an extinction coefficient at 260 nm of 10.0 cm$^2$/mg was assumed on the basis of the similar extinction coefficient reported for TomRSV and the bottom component nucleoprotein of TRSV (Stace-Smith, 1970). Like CLRV, these are nepovirus particles containing about 40% RNA.

The partially purified preparation was stored at 4 C with a trace of crystalline chloro-butanol added as a preservative, until needed for other experiments.

**Sucrose density gradients**

Linear-log sucrose gradients were prepared manually for Beckman SW 41 centrifugation tubes using the formulae of Brakke and van Pelt (1970) and Jackson et al. (1973) and allowed to form a smooth gradient overnight at 4 C. Sucrose gradients for the purification of virus preparations were made with Fisher 'laboratory-grade' sucrose and 0.02M sodium phosphate pH 7.0 buffer. Sucrose gradients for the isolation
of infectious RNA were made with ribonuclease-free sucrose (Schwarz-Mann) and 0.02M sodium phosphate pH 7.0/0.15M NaCl (PBS) buffer containing 5 mg/mL of bentonite, a ribonuclease inhibitor (Fraenkel-Conrat et al., 1961).

Immediately before centrifugation, the top 0.5-1.0 mL of the gradient was removed and a corresponding volume of preparation carefully layered on. All rate-zonal sucrose gradient centrifugations of partially purified virus preparations were done at 5°C and 38,000 rpm with the Beckman SW 41 rotor. Most runs were of 105 min duration; runs to achieve better component resolution were of 135 min duration. The centrifuged gradients were then scanned at 254 nm with an ISCO UA-4 density-gradient monitor, and the components corresponding to the absorbance peaks were manually collected. In some experiments, samples from each component fraction were examined in the electron microscope to monitor the integrity of the purified virus.

**Preparative near-equilibrium CsCl density-gradient centrifugation**

Early experiments employed BDH 'Analar' grade but later experiments made use of BDH 'Technical' grade cesium chloride purified by a method adapted from Birnie (1978). A CsCl solution in distilled water (100 g/100 mL) was first filtered through Whatman No. 1 filter paper. A slurry of 10 mL of Dowex 50-mesh 'Chelating Resin' (Sigma) in distilled water was poured into a long narrow column (about 50 mL capacity) that had been plugged with glass wool and fitted with a Teflon stopcock. The filtered solution of technical CsCl was passed through the resin column at the rate of about 1 drop per second. The density of the eluted CsCl solution was determined by precisely weighing 5 mL in a tared
Teflon cup and was then made up to the desired stock density of 1.45 g·cm⁻³ and buffer strength of 0.02M sodium phosphate pH 7.0.

Preparative near-equilibrium CsCl density-gradient centrifugation was performed in SW 41 (Beckman) centrifugation tubes with self-forming gradients consisting of 9 mL of the CsCl stock solution on which 3 mL of sample were layered. The samples were usually 'separated' sucrose density-gradient fractions of either the middle or bottom nucleoprotein components. Centrifugation was usually for 18 h at 30,000 rpm (SW 41 rotor) and 5°C. No electromagnetic braking was applied at the end of this time, in order to limit the disruption of the density-gradient formed during the run.

As with sucrose density-gradients, the centrifuged CsCl gradients were scanned at 254 nm in an ISCO absorbance monitor with a 100% (w/v) solution of unpurified 'Technical' CsCl serving as pump fluid. Fractions of 0.25 mL each were collected (ISCO Model 270) from the leading edge to the apex of the middle component absorbance peak and from the apex to the trailing edge of the bottom component peak. The respective middle (M) and bottom (B) component fractions were pooled and in some cases observed under the electron microscope to determine particle integrity. In some runs, five or six fractions from different depths of the gradient were collected and the density of each of these fractions was determined with an Abbé refractometer.

In early experiments of this type, the resolved M and B nucleoprotein component fractions were diluted 50-200 fold with 0.02M sodium phosphate pH 7.0 buffer. These less plasmolytic, dilute preparations of separated nucleoprotein components were inoculated directly to plants, separately, and in near-equimolar combination, to determine the
enhancement-of-infectivity ratio and thus the efficiency of component resolution.

Isolation and analysis of nucleoprotein components

Middle and bottom component nucleoproteins were removed from CsCl by precipitation with PEG (10% w/v) and centrifuging the suspension at 10,000 rpm for 20 min (Sorvall SS-34 rotor). For later use, the blueish-white pellets were resuspended in a minimal volume (0.1 or 0.2 mL) of 0.02M sodium phosphate pH 7.0.

Absorbance profiles of diluted samples of such nucleoprotein preparations, without correction for light scattering, were obtained from optical density measurements at 5 nm intervals from 295-225 nm (Gilford Model 250 spectrophotometer).

Infectivity of the separated and combined nucleoprotein components was determined at $A_{260} = 0.1, 0.03,$ and 0.01. Both opposite half-leaves and individual plants of *C. amaranticolor*, *C. quinoa*, and Samsun and Xanthi tobacco were inoculated in order to measure the enhancement ratios of combined nucleoprotein components.

Isopycnic banding in the Model-E analytical ultracentrifuge

Purified preparations of the E- and R-strains were made to $A_{260} = 0.75$ in 1mL of 'Analar' CsCl solution with a density of 1.481 g·cm$^{-3}$ in 0.02M sodium phosphate pH 7.0. Determinations of isopycnic buoyant densities of the nucleoprotein components were made from the Schlieren optical diffraction patterns after centrifugation for at least 18 h at 44,770 rpm.
Preparation of RNA for polyacrylamide gel electrophoresis

The dissociation buffer employed for the preparation of RNA for electrophoresis adapted from that of Dodds et al. (1977), consisted of 0.08 M Tris-HCl pH 9.0, 0.004 M Na$_2$EDTA, 4.0 mg/mL bentonite, 4 M urea, 4% SDS, 16% sucrose and 0.2% mercaptoethanol. It was added to the nucleoprotein component suspensions described above, to achieve a final RNA concentration of about 1 mg/mL, assuming, consistent with other nepoviruses, an RNA content of about 40%. The nucleoproteins in dissociation buffer were heated in a water bath at 50°C for thirty minutes. Immediately before application of the samples to the gels, one drop of 1% bromo-phenol blue tracker dye was added. Samples of 40 and 80 μL were applied to 2.5% polyacrylamide gels using a Gilson 'Pipetman'. The gels were prepared in a procedure adapted from that of Dodds et al. (1977). A 'plug' gel of 0.5 mL of 10% polyacrylamide was first poured into Perspex tubes whose bottom ends had been sealed with Parafilm. The 10% polyacrylamide, sufficient for a run of 12 tubes, consisted of 2.07 mL 3E electrophoresis buffer (Dodds et al., 1977), 0.65 mL of 1% (v/v) N,N,N',N'-tetramethylethylene diamine (TEMED), 4.0 mL of 15% acrylamide/0.75% bis-acrylamide and 0.1 mL of 10% ammonium persulfate solution. The 3E buffer had been prepared by a 3:7 dilution with distilled water from a 10E stock containing 0.36 M Tris, 0.18 M sodium acetate trihydrate, 0.01 M Na$_2$EDTA and adjusted to pH 7.2 with glacial acetic acid. When the plug gel had set, 3 mL of 2.5% polyacrylamide solution consisting of 12.14 mL distilled H$_2$O, 9.95 mL of 3E buffer, 2.4 mL of 1% (v/v) TEMED, 5.01 mL of 15% acrylamide/0.75% bisacrylamide and 0.2 mL of 10% ammonium persulfate was poured into each tube. Before the solution had gelled, the top was carefully layered with 1E buffer.
to form a smooth gel top surface. Before use, the gels were pre-electrophoresed at 75V and about 8mA per tube (ISCO Model 490 power supply) for 30 minutes. Electrophoresis, using 1E tank buffer, was carried out for 150 min at 75V and about 8 mA per tube. At the end of the electrophoresis, gels were removed from the tubes, stained overnight in 0.01% toluidine blue and destained with several changes of distilled water.

**Preparation of RNA for inoculum**

The dissociation buffer employed for the preparation of RNA for inoculum, a modification of the buffer used by Dodds et al. (1977), consisted of 0.1M Tris-HCl pH 9.0, 1% SDS, 0.025M Na₂EDTA and 0.15M NaCl. It was added directly to the bluish-white nucleoprotein PEG pellets to give a resuspension volume of 0.5 mL. The resuspensions were heated at 50 C for thirty minutes in a water bath before being layered onto sucrose gradients prepared as previously described. The RNA preparations were centrifuged at 38,000 rpm and 5 C for 240 min in the SW41 rotor and scanned at 254 nm with an ISCO UA-4 density-gradient monitor in order to collect the RNA peak fractions. The density-gradient absorbance profiles of RNA prepared from separated nucleoprotein components, the remixed components and the originally partially-purified virus preparation were compared in this way.

In order to determine that the moiety corresponding to the absorbance peak was RNA rather than nucleoprotein, the $A_{260}/A_{280}$ ratios were measured. In one experiment, the absorbance profile from 320-220 nm of RNA prepared as just described was determined in a Cary Model 15 scanning spectrophotometer and compared with published profiles of RNA. The RNA
in sucrose from the density-gradient centrifugation was usually stored at -20°C until needed.

For inoculation, the frozen RNA component preparations were thawed and the necessary amounts of each sample diluted to $A_{260} = 0.1$ with PBS containing 5 mg/mL of bentonite. An extinction coefficient of 25 cm$^2$/mg at 260 nm was used to determine RNA concentration (Dodds et al., 1977).

To test the efficiency of RNA component separations and the specific infectivity of the RNA preparations, _C. amaranticolor_, _C. quinoa_, _N. clevelandii_, and Samsun and Xanthi tobacco plants were inoculated (using sterilized cotton swabs to limit ribonuclease activity) with either cold M-RNA, B-RNA, an equimolar combination of M-RNA and B-RNA, or RNA prepared directly from partially-purified virus preparations.

In some experiments, the infectivity of combined M- and B-RNA was compared to that of each separate component RNA using opposite half-leaves of _C. amaranticolor_, _C. quinoa_, and Xanthi and Samsun tobacco.

**Generation of pseudorecombinants**

Pseudorecombinants were generated from M- and B-RNA components of the E- and R-strains. M-RNA of the E-strain and B-RNA of the R-strain, both at $A_{260} = 0.1$, were mixed $\left(\frac{E}{R}\right)$ as were M-RNA of the R-strain and B-RNA of the E-strain $\left(\frac{R}{E}\right)$ and inoculated to _C. amaranticolor_, _N. clevelandii_, and Samsun and Xanthi tobacco. Inoculations of the individual component RNAs and homologous RNA combinations to individual plants and opposite half-leaves served as controls. New pseudorecombinant local lesion types appearing on _N. clevelandii_ were passaged three times through local lesions to generate pure pseudorecombinant lines with uniform symptomatologies in the plants of the above host.
range. The pseudorecombinant isolates were then maintained, like the parental isolates, in *N. clevelandii* by serially transferring young systemically infected leaves to young plants.

**Comparison of parental- and pseudorecombinant isolates**

**Component distribution**

The E-, R-, \( \frac{E}{R} \), and \( \frac{R}{E} \)-isolates were each propagated in *C. quinoa*, partially purified by the methods described earlier and subjected to linear-log sucrose density-gradient centrifugation to obtain particle component ratios. This process also served to produce purified virus preparations for subsequent comparisons of symptomatology and serological relationships.

**Symptomatology**

Purified virus of the four isolates was diluted to \( A_{260} = 0.1 \) and inoculated to *N. clevelandii*, Samsun and Xanthi tobacco, *G. globosa*, *C. quinoa* and *C. amaranticolor* to compare local and systemic symptom types. Opposite half-leaf inoculations of *C. amaranticolor*, *C. quinoa*, Samsun and Xanthi tobacco were done to confirm relations of local-lesion symptom types.

**Serology**

Antisera were prepared against the parent E- and R-strains from two young, white New Zealand rabbits each immunized with three intramuscular injections of either purified E- or R-strain (1mg/mL) emulsified 1:1 with Freund's complete adjuvant. The first and second injections were 2 weeks apart, with booster injections given at 10 weeks. After
the second injection, bleedings were done at weekly intervals for the next 11 weeks and tested by Ouchterlony double-diffusion serology to determine the titre of the antiserum against its homologous antigen at a concentration of $A_{260} = 1.0$.

Serological comparisons of the four isolates were done with Ouchterlony double-diffusion in agar gel on glass slides (Mansi, 1958). Each parent-strain antiserum, diluted to a concentration 25 times that of its titre dilution, was placed in a central well surrounded by eight peripheral wells which were filled in pairs with purified virus of the four isolates each at $A_{260} = 1.0$. The slides were then incubated at room temperature for 48 h in a moist chamber to obtain precipitin reactions.

Regeneration of parents from pseudorecombinants

The $R_E$ and $E_R$ pseudorecombinant isolates were propagated in $N. clevelandii$, purified, and the component RNAs of the two isolates prepared as described for the two parent strains. The M-RNA of the $R_E$ and the B-RNA of the $E_R$ pseudorecombinant isolates were mixed to generate a predicted E-strain parental combination, as were the reciprocal RNAs to generate a predicted R-strain combination. These combinations and separate component RNAs were inoculated to opposite half-leaves of $C. quinoa$, $C. amaranticolor$ and Samsun and Xanthi tobacco; $N. clevelandii$ was also inoculated with these combinations as well as the pseudorecombinants, to give direct symptom evidence of regeneration of parental from pseudorecombinant isolates.

The apical tips of $C. quinoa$ plants systemically infected with 'predicted-parent' RNA combinations were used as inoculum for propagation
in C. quinoa. The purified viruses obtained from this propagation were examined for component distribution, symptomatology and serology. These observations were compared with those made previously of known parental isolates to confirm that parents had been generated from pseudorecombinants and that the pseudorecombinants had the genetic make-up ascribed to them.
RESULTS

Comparative symptomatologies of CLRV strains

No obvious local or systemic symptoms were induced by the D-, C-, E- and R-strains of CLRV in *N. glutinosa*, *N. glauca*, *Zinnia elegans* and *Datura stramonium*. *N. sylvestris*, *N. rustica*, *Capsicum annum*, *Vigna unguiculata* cv. Blackeye, and *Phaseolus vulgaris* cvs. Bountiful and Pinto showed local and systemic symptoms when inoculated with the four strains but differences in symptom types among the four strains, if any, were neither clear nor easily reproducible. Local and systemic symptoms of the four strains on *N. clevelandii*, *N. tabacum* cvs. Samsun and Xanthi, *C. quinoa* and *C. amaranticolor* (Table 1) were sufficiently distinct and reproducible to indicate their usefulness as genetic markers. The systemic symptoms in Samsun and Xanthi tobacco appeared as various forms of radiating concentric curves of chlorosis and were described as 'map-contour' symptoms (Table 1). The long term (beyond 60 days after inoculation) systemic symptom types of the E- and R-strains in *C. amaranticolor* were so markedly different (Fig. 1) that, partly because of this valuable marker, they were the strains selected for pseudorecombination genetic analysis.

Purification methods

Significant losses of virus material are to be expected in the various procedures required for nucleoprotein-resolution and RNA-extraction. Purification procedures should therefore be chosen on the basis of their amenability to rapid (1 day or less) large scale purification of the different-CLRV strains.
Table 1: Comparative symptomatologies of the strains of CLRV in local and systemic infections

<table>
<thead>
<tr>
<th>Strain</th>
<th>Infection</th>
<th>Reactions on differential hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N. clevelandii</td>
<td>N. tabacum cv. Samsun</td>
</tr>
<tr>
<td>Dogwood</td>
<td>local</td>
<td>large necrotic disc lesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>necrotic rings</td>
</tr>
<tr>
<td></td>
<td>systemic</td>
<td>extensive necrosis, death</td>
</tr>
<tr>
<td></td>
<td></td>
<td>small, tight map-contour</td>
</tr>
<tr>
<td>Cherry</td>
<td>local</td>
<td>small necrotic ring lesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>'blurry' map-contour</td>
</tr>
<tr>
<td></td>
<td>systemic</td>
<td>fleck necrosis followed by recovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>'rippling' mottled chlorosis</td>
</tr>
<tr>
<td>Elderberry</td>
<td>local</td>
<td>small necrotic/chlorotic rings</td>
</tr>
<tr>
<td></td>
<td></td>
<td>necrotic/chlorotic rings</td>
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<tr>
<td></td>
<td>systemic</td>
<td>point necrosis followed by recovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fine-line map-contour</td>
</tr>
<tr>
<td>Rhubarb</td>
<td>local</td>
<td>large necrotic disc lesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>large necrotic lesions</td>
</tr>
<tr>
<td></td>
<td>systemic</td>
<td>severe fleck necrosis followed by recovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coarse-line map-contour</td>
</tr>
</tbody>
</table>

N. tabacum cv. Xanthi
C. amaranticolor
C. quinoa

necrotic point lesions
mottle, straight spindly growth
apical wilt, slow death
necrotic/chlorotic point lesions
mottle, fasciculate growth
severe wilt, slow death
chlorotic, pin-point lesions
apical tufting, dwarfing
chlorosis, survives and bears seed
necrotic disc lesions
mottle, fasciculate growth
severe wilt, rapid death
Fig. 1. Systemic symptoms of CLRV infection in C. amaranticolor.
A. inoculated with elderberry (E) strain: dwarfing and apical tufting after 60 days;
B. inoculated with rhubarb (R) strain: systemic mottle and partly fasciculate growth after 60 days
The purification procedure described as 'Method 2' by Jones and Mayo (1972) was the first attempted. Purified, infectious preparations of the C- and R-strains were obtained from systemically infected C. quinoa leaf and stem tissue. The need for overnight freezing of tissue sap (and for subsequent thawing) and the use of large quantities of fairly expensive ammonium sulfate, however, did not make it seem a particularly convenient method for the large-scale routine purification that would be required later in the work.

Walkey et al. (1973) had also described an effective purification method suitable for a number of CLRV strains, but the requirement for large volumes of chloroform (1 volume per weight of tissue) made it inconvenient for large-scale purifications. More seriously, the middle component nucleoprotein of the cherry strain appeared (as reflected in sucrose-gradient scanning profiles) to be particularly adversely affected; its concentration, nearly equimolar to the bottom component in other methods, was reduced to a mere shoulder at the base of the bottom component peak.

The method selected for partial purification involved homogenization with high-molarity phosphate buffer, clarification aided by small proportions by volume of chloroform/butanol, and concentration by PEG precipitation and differential centrifugation. It was rapid (less than one day), easily applied to small and large amounts of tissue and gave reasonable yields (20-40 mg/kg) of infectious particles. Virus purified by this method also stood up to the rigors of subsequent CsCl near-equilibrium centrifugations.

The $A_{260}/A_{280}$ ratios and infectivity assays on 100-fold dilutions of partially purified preparations were useful indicators of purity and
quality. The $A_{260}/A_{280}$ ratios usually ranged from 1.7 - 1.9; preparations with lower ratios still contained large proportions of host protein while higher ratios indicated host ribosome contamination as confirmed by subsequent sucrose gradient scanning profiles and electron microscopy. Preparations made from systemically infected tissues of *C. quinoa* rather than *N. clevelandii* tissue tended to have higher $A_{260}/A_{280}$ ratios (1.9 - 2.1) and higher levels of host ribosome contamination.

**Sucrose gradient centrifugation and scanning profiles of particles**

Sucrose density-gradient scanning profiles of partially purified preparations of the E- and R-strains propagated from *N. clevelandii* (Fig. 2) and *C. quinoa* (Fig. 3) revealed that while relative sedimentation values of the particle components of the two strains in the two hosts were constant, relative component distribution was a property of the virus strain as well as a function of the propagation host. The E-strain consistently showed a lower proportion of middle component than the R-strain but the difference was far more striking in preparations from *C. quinoa* than from *N. clevelandii*. In addition, the R-strain consistently produced detectable amounts of RNA-free top component particles (confirmed by electron microscopy) while such a top component was never detected in E-strain preparations (Figs. 2 and 3). The sucrose density-gradients were also simultaneously employed to preparatively purify the nucleoprotein and also achieve a first-order
Preparative near-equilibrium CsCl density-gradient centrifugation

Centrifugation of purified nucleoprotein components through CsCl ($\rho = 1.45 \text{ g cm}^{-3}$) for about 18 h to near-equilibrium gave much better resolution than rate-zonal sucrose-gradient centrifugation. (Fig. 4). In both the E- and R-strains, the near-equilibrium buoyant densities corresponding to the component absorbance peaks, calculated by interpolation of densities obtained for five or six 0.25 mL fractions from the lower (CsCl-containing) 9 mL of the gradient, were found to be $\rho = 1.44$ and $1.48 \text{ g cm}^{-3}$ for the middle and bottom components, respectively.

'Pure' middle and bottom component preparations obtained, respectively, by pooling leading leading edge to apex, and apex to trailing edge fractions of the two components were assessed for functional nucleoprotein-component resolution by infectivity measurements. The components in CsCl, diluted 50- to 200-fold with 0.02M sodium phosphate to render them non-plasmolytic, were inoculated separately and in one-to-one combination to host plants. In one experiment, preparations of 'pure' (i.e. fully resolved and separated) nucleoprotein components of the R-strain were diluted 50-fold with buffer and
Fig. 2. Absorbance scan patterns of sucrose density- 
gradients showing differences in particle 
component distribution of the elderberry 
and rhubarb strains of CLRV purified from 
N. clevelandii. 
A. middle (M) and bottom (B) component 
nucleoproteins of the elderberry strain; 
B. top (T) component RNA-free empty particles 
and middle (M) and bottom (B) component 
nucleoproteins of the rhubarb strain
Fig. 3. Absorbance scan patterns of sucrose density-gradients showing particle component distributions of the elderberry and rhubarb strains of CLRV purified from C. quinoa.
A. middle (M) and bottom (B) component nucleoproteins of the elderberry strain
B. top (T) component RNA-free particles and middle (M) and bottom (B) component nucleoproteins of the rhubarb (R) strain
Fig. 4. Absorbance scan patterns showing extent of separation of middle and bottom nucleoprotein components (rhubarb strain). In each scan, the peak at the greatest depth corresponds to the bottom component.

A. Linear-log sucrose gradient centrifugation;
B. 'Middle' component of 'A' after near-equilibrium CsCl density-gradient centrifugation;
C. 'Bottom' component of 'A' after near-equilibrium CsCl density gradient centrifugation
inoculated separately and in combination to *C. amaranticolor* plants at the four-leaf stage; near-perfect functional resolution was observed (Fig. 5). In all experiments of this type, the separated components appeared, on the basis of induced symptoms, much less infectious than their artificial one-to-one combination. In addition, local and systemic tissue of hosts inoculated earlier with pure middle component was almost never infectious, while pure bottom component was often only infectious at very low levels. Plants inoculated with one-to-one combinations, however, yielded highly infectious tissue, which, when used as inoculum induced 10-50 times higher numbers of local lesions on *C. amaranticolor* compared to similar tissue from hosts which had been inoculated with pure bottom component.

**Analysis of nucleoprotein components by analytical ultracentrifugation**

The determination of equilibrium buoyant densities of the two nucleoprotein components by analytical ultracentrifugation at 25°C was only possible with the R-strain; the E-strain appeared to form a precipitate-like aggregate within a short time of beginning the run. The buoyant densities of the middle and bottom components, calculated from the Schlieren diffraction pattern (Fig. 6) were respectively 1.471 and 1.508 g·cm⁻³. As the run progressed, the middle component appeared to decrease and its Schlieren diffraction pattern became wider than that of the bottom component.

**Isolation and analysis of nucleoprotein components**

For the further analysis of the nucleoprotein components, the extraction and purification of RNA and for medium-term storage of the
Fig. 5. *C. amaranticolor* plants 45 days after inoculation (at the four-leaf stage) with middle (M), bottom (B) and combined (M+B) nucleoprotein components of the rhubarb strain of CLRV. The components had been separated by sucrose gradient centrifugation followed by near-equilibrium banding in CsCl ($\rho=1.45 \text{ g cm}^{-3}$).
Fig. 6. Schlieren pattern from analytical ultracentrifugation of CLRV-rhubarb strain in CsCl density-gradient at equilibrium (44,770 rpm; 24h). Density increases from left to right. Middle component nucleoprotein appears as smaller density-band near the centre of the gradient; bottom component nucleoprotein appears as the larger density band to the right of the middle component.
components, it was desirable to have more concentrated preparations of separated nucleoprotein components that were also CsCl-free. The addition of PEG at 10% (w/v) to CsCl component preparations, followed by low-speed centrifugation achieved both concentration of the particles and removal of CsCl in a single step with minimal loss.

Comparative spectrophotometric analysis

The middle and bottom component nucleoproteins, respectively, of both the E- and R-strains showed $A_{260}/A_{280}$ ratios of 1.64-1.66 and 1.81-1.83. In contrast, the $A_{260}/A_{280}$ ratios of purified 'whole-virus' preparations (from N. clevelandii) of the two strains differed markedly: 1.76-1.77 for the E-strain and 1.71-1.72 for the R-strain. The $A_{260}/A_{280}$ ratios of the separated nucleoprotein components were consistent with their differences in absorbance profile, uncorrected for light scattering, between 225 and 295 nm (Fig. 7A). For both middle and bottom components the absorbance maximum was at 259 nm, the minimum at 239 nm. The $A_{\text{max}}/A_{\text{min}}$ ratios were 1.28 and 1.41 for the middle and bottom components, respectively.

Infectivity of nucleoprotein components

The relative infectivities, at defined concentrations, of the middle and bottom component nucleoproteins of the R-strain and their equi-absorbant combination were measured mainly in terms of number of local lesions induced and secondarily by their ability to induce systemic infections. Opposite half-leaves of C. amaranticolor and C. quinoa at the four-leaf stage and Xanthi tobacco at the two-leaf stage inoculated on one half with one component and on the other half with the equi-
Absorbance scan patterns of CLRV nucleoproteins extracted RNA, uncorrected for light scattering, from 220-295 nm.

A. separated middle component (---) and bottom component (—) nucleoproteins;
B. extracted RNA

Fig. 7. Absorbance scan patterns of CLRV nucleoproteins and extracted RNA, uncorrected for light scattering, from 220-295 nm.

A. separated middle component (---) and bottom component (—) nucleoproteins;
B. extracted RNA
absorbant component combination clearly showed enhancement of infectivity with the presence of both components. In Xanthi tobacco, at $A_{260} = 0.10$ and 0.03, the equiabsorbant combinations induced respectively, an average of 6.0 and 3.5 lesions per half-leaf while the pure components failed to induce any lesions on their inoculated half-leaves. C. amaranticolor and C. quinoa, more sensitive hosts, were each tested in duplicate plants by the opposite half-leaf method (Tables 2 and 3).

In sensitive hosts, such as C. amaranticolor, a low-level CLRV infection may fail to produce local lesions, but it may eventually produce systemic symptoms. To determine if component resolution had been obtained to the extent that neither component on its own could induce even such a low-level infection, separate C. amaranticolor plants at the four-leaf stage were inoculated on four half-leaves with middle, bottom and equiabsorbant combinations at three different component concentrations. At $A_{260} = 0.10$, plants inoculated with middle component, bottom component and their combination showed, respectively, totals of 16, 67 and 316 local lesions, indicating a moderate enhancement-of-infectivity ratio (7.6) similar to that obtained by opposite half-leaf measurement (11.0 from Table 2). At $A_{260} = 0.03$, the plant inoculated with middle component showed no local lesions and failed to later show any systemic symptoms; the bottom component induced 2 and the component combination 63 lesions, indicating, as with opposite half-leaves, that much higher ratios are obtained at lower component concentrations. Finally, at $A_{260} = 0.01$, neither plant inoculated with separated components showed any local lesions or later systemic infection, while the component combination induced 11 local lesions, suggesting functionally 'perfect' separation had been achieved.
Table 2: Enhancement of infectivity by complementation of separated middle (M) and bottom (B) nucleoprotein components, as measured by induction of local lesions (ls.), on opposite half-leaves (v) of *C. amaranticolor*.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Number of lesions on inoculated half-leaves</th>
<th>(A_{260} = 0.10)</th>
<th>(A_{260} = 0.03)</th>
<th>(A_{260} = 0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant 1</td>
<td>5 97 26 142 0 17 1 13 0 4 0 2</td>
<td>5 146 15 216 0 12 0 8 0 3 0 4</td>
<td>5 146 15 216 0 12 0 8 0 3 0 4</td>
<td>5 146 15 216 0 12 0 8 0 3 0 4</td>
</tr>
<tr>
<td>Plant 2</td>
<td>19 191 28 207 0 19 1 15 0 2 0 5</td>
<td>10 103 3 115 0 11 0 12 0 3 1 6</td>
<td>10 103 3 115 0 11 0 12 0 3 1 6</td>
<td>10 103 3 115 0 11 0 12 0 3 1 6</td>
</tr>
<tr>
<td>Totals</td>
<td>39 537 72 680 0 59 2 48 0 12 1 17</td>
<td>39 537 72 680 0 59 2 48 0 12 1 17</td>
<td>39 537 72 680 0 59 2 48 0 12 1 17</td>
<td>39 537 72 680 0 59 2 48 0 12 1 17</td>
</tr>
<tr>
<td>M+B ls.</td>
<td>537 + 680 (= 11.0)</td>
<td>59 + 48 (= 63.5)</td>
<td>12 + 17 (= 29)</td>
<td></td>
</tr>
<tr>
<td>M ls.+B ls.</td>
<td>39 + 72</td>
<td>0 + 2</td>
<td>0 + 1</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Enhancement of infectivity by complementation of separated middle (M) and bottom (B) nucleoprotein components, as measured by induction of local lesions (ls.), on opposite half-leaves (v) of *C. quinoa*.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Number of lesions on inoculated half-leaves</th>
<th>(A_{260} = 0.03)</th>
<th>(A_{260} = 0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant 1</td>
<td>0 7 1 10 0 3 0 6</td>
<td>0 17 0 17 0 2 0 3</td>
<td>0 17 0 17 0 2 0 3</td>
</tr>
<tr>
<td>Plant 2</td>
<td>0 2 0 3</td>
<td>0 4 1 3</td>
<td>0 4 1 3</td>
</tr>
<tr>
<td>Totals</td>
<td>0 24 1 27</td>
<td>0 11 1 15</td>
<td>0 11 1 15</td>
</tr>
<tr>
<td>M+B ls.</td>
<td>24 + 27 (= 51)</td>
<td>11 + 15 (= 26)</td>
<td></td>
</tr>
<tr>
<td>M ls.+B ls.</td>
<td>0 + 1</td>
<td>0 + 1</td>
<td>0 + 1</td>
</tr>
</tbody>
</table>
RNA of separated components in polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of RNAs extracted from concentrated (A_{260} = 10-15) preparations of separated nucleoprotein components of the R-strain provided physical evidence confirming that virtually complete separation of the nucleoproteins had been achieved (Fig. 8). The RNA isolated from middle component co-migrated with the smaller, faster migrating viral RNA of a partially purified preparation (Fig. 8A, B), while RNA from bottom component co-migrated with the larger, slower migrating RNA of a purified 'whole-virus' R-strain preparation (Fig. 8C, D).

RNA of separated components as inoculum

The moiety obtained by sucrose gradient centrifugation for use as inoculum was confirmed as being RNA rather than nucleoprotein by its A_{260}/A_{280} ratios of 2.05-2.25 and its absorbance profile, uncorrected for light scattering, from 220-295 nm (Fig. 7B). The absorbance maximum was at 259 nm and the minimum at 230 nm with an A_{max}/A_{min} = 2.20.

As an RNA control for analysis and inoculation, RNA was extracted from purified 'whole-virus' preparations of the E- and R-strains (propagated in N. clevelandii) by the same methods used to extract RNA from concentrated preparations of separated nucleoproteins (Fig. 9). Although resolution of CLRV RNA components in sucrose density-gradients is poor compared to the resolution of particles, it is possible to see that the RNA component distribution reflects the nucleoprotein component distribution: the R-strain nucleoprotein (Fig. 3B) and RNA (Fig. 9B) components are about equimolar while for the E-strain both nucleoprotein
Fig. 8. Polyacrylamide gel electrophoresis of RNA extracted from separated nucleoprotein components and 'whole-virus' preparations of CLRV.

A. RNA extracted from 'pure' middle component (M) nucleoprotein;
B. RNA extracted from a partially purified preparation of CLRV;
C. RNA extracted from fully purified CLRV;
D. RNA extracted from 'pure' bottom component (B) nucleoprotein
Fig. 9. Absorbance scan patterns of RNA obtained by sucrose density-gradient centrifugation. The RNA absorbance peaks are the doublets about one-third of the way down the gradient.

A. RNA extracted from the elderberry (E) strain of CLRV;
B. RNA extracted from the rhubarb (R) strain of CLRV
(Fig. 3A) and RNA (Fig. 9A) middle component are present in one third to one-half the amount of respective bottom component.

RNA made from concentrated (PEG-pelleted) separated middle (M) and bottom (B) component nucleoproteins gave mono-disperse $A_{254}$ peaks, their apices differing by 1.8-2.0 mm (SW 41 centrifugation tubes) in sedimentation depth (Fig. 10). When diluted to $A_{260} = 0.10$ with PBS-bentonite buffer, such RNA component preparations were readily used as inoculum. M-BNA or B-RNA prepared from their separated nucleoprotein components had very low levels of infectivity, but had high levels in equiabsorbant combination. When measured by local-lesion counts on inoculated half-leaves of C. amaranticolor at the four-leaf stage, enhancement-of-infectivity ratios ranged from 30-60. In the less sensitive Xanthi and Samsun tobacco hosts, each RNA component, on its own, usually failed to produce any symptoms, while equiabsorbant mixtures produced local lesions and systemic symptoms.

**Generation of pseudorecombinants**

The pseudorecombinant inocula, made by mixing the equiabsorbant ($A_{260} = 0.10$) RNA component preparations of one strain with complementary RNA component preparations of the other strain, might induce residual low-level infections of one of the component strains rather than true pseudorecombinant infections. To distinguish between these outcomes, C. amaranticolor at the four-leaf stage and Xanthi tobacco plants were inoculated with single RNA components, homologous RNA component combinations as well as the heterologous, pseudorecombinant RNA combinations (Table 4).
Fig. 10. Absorbance scan patterns of RNA (of the rhubarb strain of CLRV) obtained by sucrose density-gradient centrifugation, showing the monodisperse nature and slight differences in sedimentation velocity of RNA extracted from separated middle (M-RNA) and bottom (B-RNA) component nucleoprotein. The RNA absorbance peaks are about one-third of the way down the gradient.

A. RNA extracted from separated middle component nucleoprotein (M-RNA);
B. RNA extracted from a re-mixture of separated middle and bottom component nucleoproteins;
C. RNA extracted from separated bottom component nucleoprotein (B-RNA);
D. RNA extracted from a 'whole-virus' preparation
Table 4: Relative infectivities at $A_{260} = 0.10$ of middle (M) and bottom (B) RNA components of CLRV elderberry (E) and rhubarb (R) strains inoculated as separate components and in homologous and heterologous equiabsorbant combinations

<table>
<thead>
<tr>
<th>RNA inoculum</th>
<th>Sum of lesions on inoculated half-leaves</th>
<th>C. amaranticolor (4 half-leaves)</th>
<th>N. tabacum cv. Xanthi (2 half-leaves)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-RNA</td>
<td>B-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>E</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>E</td>
<td>69</td>
<td>4</td>
</tr>
<tr>
<td>R</td>
<td>-</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>R</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>R</td>
<td>69</td>
<td>17</td>
</tr>
<tr>
<td>R</td>
<td>E</td>
<td>51</td>
<td>4</td>
</tr>
</tbody>
</table>

The data indicating enhancement of infectivity by complementation with heterologous RNA components (Table 4) corresponded to observations of unique types of local-lesion induced by heterologous RNA combinations in some of the differential hosts (cf. Table 1). These new types were especially distinct in *N. clevelandii* and predominated over parental lesion types on leaves inoculated with heterologous RNA combinations. Pure stock infections of the two pseudorecombinant isolates were obtained by three local lesion passages in *N. clevelandii*; the infections were deemed to be pure after the third passage by virtue of their monotypic local-lesion symptoms after the second passage. The pseudorecombinant that had obtained its M-RNA from the E-strain and B-RNA from the R-strain was designated $'E' \ R$, the isolate from the reciprocal combination, $'R' \ E$. 
Comparison of the parental and pseudorecombinant isolates

Symptomatology

The local lesions induced on N. clevelandii 4-6 days after inoculation were the clearest, most reliable single markers to distinguish the E-, R-, $\frac{E}{R}$ - and $\frac{R}{E}$ - isolates. It is clear from the comparison of the local-lesion types induced in this host by each of the four isolates (Fig. 11), that although $\frac{E}{R}$ and $\frac{R}{E}$ are distinct from their parent E- and R-isolates, basic similarities in lesion morphology exist between parents and pseudorecombinants; the lesions induced by $\frac{E}{R}$ are similar to those induced by R, as those induced by $\frac{R}{E}$ are to E. With this indication that local lesion morphology, at least in N. clevelandii is determined by the larger RNA, which is derived from the bottom component nucleoprotein (B-RNA), other differential hosts (cf. Table 1) were examined in this regard. To ensure that results would be comparable, purified preparations (from N. clevelandii propagations) at a concentration of $A_{260} = 0.10$ of each of these four isolates were used. Opposite half-leaves of Xanthi tobacco and C. amaranticolor (Figs. 12 and 13, respectively), were inoculated with the two parental isolates, and parental versus pseudorecombinant isolates in order to examine the inheritance of local lesion determination in these hosts.

From Fig. 12C it appears $\frac{R}{E}$ -induced lesions have the morphology of those induced by the R-isolate, but $\frac{E}{R}$ -induced lesions (Fig. 12B) are so distinct from those induced by either parent (Figs. 12A,B), that no clear assignment of inheritance can be made. Good comparisons of local-lesion types on opposite half-leaves of Samsun are more difficult to
Fig. 11. Comparison of local-lesion symptom types induced by parental and pseudorecombinant isolates 4 days after inoculation on N. clevelandii.

A. E-strain (parent)  
B. R-strain (parent)  
C. E pseudorecombinant  
D. R pseudorecombinant
Fig. 12. Comparisons on opposite half-leaves of local-lesion symptom types induced by parental and pseudorecombinant isolates in Xanthi tobacco.

A. left half: R-strain (parent); right half: E-strain (parent);

B. left half: E \text{ pseudorecombinant}; right half: E-strain (parent);

C. left half: R \text{ pseudorecombinant}; right half: R-strain (parent)
Fig. 13. Comparisons on opposite half-leaves of local-lesion symptom types induced by parental and pseudorecombinant isolates in C. amaranticolor.
A. left half: R-strain (parent); right half: E-strain parent;
B. left half: E\_R-pseudorecombinant; right half: E\_strain parent;
C. left half: R\_E-pseudorecombinant; right half: R\_strain parent
obtain, but a very similar pattern of local lesion symptomatology to that in Xanthi tobacco is observed.

The pseudorecombinant isolates appear to inherit their local-lesion symptomatology in *C. amaranticolor* from the parent contributing the smaller RNA, which is derived from the middle component nucleoprotein (M-RNA) (Fig. 13); a similar pattern was also observed in *C. quinoa*.

The genetics of systemic-symptom determination in the differential hosts were also examined by comparing symptoms induced (at equal concentrations: $A_{260} = 0.10$) by purified parent and pseudorecombinant isolates.

Among the differential hosts of the genus *Nicotiana*, Samsun tobacco gave the clearest, readily distinguishable systemic symptoms. The pseudorecombinants appear to inherit their type of systemic 'map-contour' symptoms from the parent contributing the M-RNA (Fig. 14). The $E/R$ isolate, while a potent local-lesion inducer on *Nicotiana* spp. (cf. Table 4), is a relatively poor inducer of systemic symptoms and the inheritance does not appear as clear as in the $R/E$ pair (Figs. 14B,D). A similar pattern of inheritance is observed in *N. clevelandii* and Xanthi tobacco.

In *Chenopodium amaranticolor*, where particularly distinct systemic symptoms are induced by the parental isolates, a clear-cut determination of symptom inheritance is evident (Fig. 15). The $E/R$-isolate inherits the characteristic apical tufting and dwarfing from the parent E-isolate (Figs. 15A,C), though it can be distinguished from the parent by its consistent retention of dead apical leaves (Fig. 15C); the $R/E$-isolate inherits the paling leaf-mottle and spindly, partly fasciculate growth from the parent R-isolate.

This pattern of systemic-symptom determination by M-RNA is also
Fig. 14. Comparison of systemic symptoms induced in Samsun tobacco by the parental and pseudorecombinant isolates.

A. 'Fine-line' map-contour symptom induced by E-strain (parent);
B. 'Coarse-line' map-contour symptom induced by R-strain (parent);
C. 'Fine-line' map-contour symptom induced by $^{E}_R$-pseudorecombinant;
D. 'Coarse-line' map-contour symptom induced by $^{R}_E$-pseudorecombinant
Fig. 15. Comparison of systemic symptoms induced in *C. amaranticolor* 60 days after inoculation with:

A. buffered sap from healthy *C. amaranticolor*;
B. E-strain (parent);
C. R-strain (parent);
D. \( \frac{E}{R} \)-pseudorecombinant;
E. \( \frac{R}{E} \)-pseudorecombinant
observed in *C. quinoa*. Plants inoculated with the $\frac{E}{R}$ pseudorecombinant or the $E$ parent survive a systemic mottling infection to bear seed. In contrast, plants inoculated with either the $\frac{R}{E}$—or $R$—isolate rapidly succumb to an apical wilt.

Since Jones (1977) has indicated systemic symptoms in *C. globosa* are inherited through the B-RNA of the R-strain, the genetics of symptom determination in this host were also examined, although rather high ($A_{260} = 0.50$) doses of purified virus are usually needed to reliably induce levels of symptoms suitable for comparison. At $A_{260} = 0.50$, E-strain showed neither local nor systemic symptoms, while R-strain showed local 'fine-line' chlorotic rings and systemic flecking, vein necrosis and wilt. Of the pseudorecombinants, only the $\frac{E}{R}$—isolate induced systemic symptoms and they appeared similar to those induced by the parent R-strain. This pseudorecombinant also produced local lesions but, consisting of coarse anthocyanescent rings, were clearly different from the R-strain lesions. The $\frac{R}{E}$—isolate occasionally induced small numbers of faint fine-line local lesions that bore resemblance to parent R-strain local lesions, but never induced systemic infections. These observations tend to confirm those of Jones (1977) that B-RNA determines the ability to induce systemic symptoms in *C. globosa*.

**Particle component distribution**

Absorbance ($A_{254}$) scans of partially-purified (from *C. quinoa*) parental and pseudorecombinant isolates centrifuged through sucrose gradients show that particle component distribution is inherited through M-RNA (Fig. 16). The $\frac{E}{R}$—isolate has the profile of its $E$ parent, the $\frac{R}{E}$—isolate that of its $R$ parent.
Fig. 16. Absorbance scan patterns of sucrose density gradients showing relationships of particle component distributions among parental and pseudorecombinant isolates partially purified from *C. quinoa*.

A. E-strain (parent)  
B. R-strain (parent)  
C. \( E/R \)-pseudorecombinant  
D. \( R/E \)-pseudorecombinant
Serology

The agar-gel Ouchterlony double-diffusion serology of the parent and pseudorecombinant isolates is shown in Fig. 17. The precipitin-line patterns against each of the parent E- and R-strain antisera indicate, by their confluence, serological identity of the E- and $E_R^E$-isolates and of the R and $R_E^R$-isolates. Thus serology, like particle component distribution, appears determined by the smaller M-RNA.

Regeneration of parents from pseudorecombinants

Mixing of 'pure' M-RNA of the $E_R^E$-isolate with 'pure' B-RNA of the $R_E^E$-isolate was predicted to produce an isolate identical to the parent E-strain, as the reciprocal combination was to produce one identical to the parent R-strain. These predictions appeared to be fulfilled based on the available biological and physical evidence.

Symptom evidence

The differential hosts (cf. Table 1) inoculated with heterologous combinations of pseudorecombinant-isolate RNAs were predicted to induce symptoms characteristic of the parental strains. The local-lesion symptoms induced on C. quinoa and C. amaranticolor were indeed those of the predicted parents but since the local symptoms induced by E- and $E_R^E$-isolates are identical, as those induced by the R- and $R_E^R$-isolates are, additional confirming symptom evidence is required. Since the local-lesion symptoms on N. clevelandii are characteristic for each isolate, 'regenerated' parental RNA combinations were examined for the induction of such symptoms of the predicted parent-type infection (Fig.
Fig. 17. Double-diffusion agar gel serology showing genetic determination of coat protein antigenicity.

A. Central well contains a 1:100 dilution of antiserum (titre = 2560) made against purified E-strain. Peripheral wells contain preparations of purified E-strain\((\frac{E}{E})\) and R-strain \((\frac{R}{R})\) parents, and \(\frac{E}{R}\) - and \(\frac{R}{E}\) - pseudorecombinants at \(A_{260} = 1.0\);

B. Central well contains a 1:50 dilution of antiserum (titre = 1280) made against purified R-strain. Peripheral wells are as in Fig. 17A.
18). The local-lesion symptoms induced by the regenerated parental RNA combinations are indeed identical with those induced by inocula of the original parent strains (cf. Fig. 11). The local-lesion symptoms induced in Samsun and Xanthi tobacco, although less clear-cut and characteristic than those of *N. clevelandii*, conformed to this pattern.

Systemic symptoms induced on the differential hosts were also used to test for the identity of 'regenerated' parental RNA combinations. On all the differential hosts examined, the types of induced systemic symptoms conformed to the predicted pattern. The systemic symptomatology on *C. amaranticolor* was the most decisive evidence since the, otherwise similar, systemic pathology induced by the parent E- and pseudorecombinant E R-isolates can be distinguished on the basis of retention of dead apical leaves by the pseudorecombinant (cf. Fig. 15). The regenerated 'parental' RNA combination in consistently failing to retain dead apical leaves shows the systemic symptoms characteristic of the parent E-isolate.

Purified preparations of virus at A<sub>260</sub> = 0.50, propagated in *C. quinoa* from the regenerated parental RNA combinations, and inoculated to *G. globosa* at the four-leaf stage also produced the predicted parental-isolate local and systemic symptoms; the 'regenerated' E-isolate induced no symptoms while the symptoms of the 'regenerated' R-isolate were identical with those of the original parent R-strain inoculated under the same conditions.

**Particle component distribution evidence**

Absorbance scans at 254 nm after sucrose gradient centrifugation of purified preparations from *C. quinoa* propagations of the pseudorecombinants and the products of the regenerated parental RNA combinations
Fig. 18. Comparison of local-lesion symptoms on *N. clevelandii* demonstrating regeneration of parental from pseudorecombinant isolates.

A. Enlarged leaf shows symptoms induced by 'regenerated' E-strain RNA combination. Inset shows symptoms induced by the 'original' parent E-strain (from Fig. 11);

B. Enlarged leaf shows symptoms induced by 'regenerated' R-strain RNA combination. Inset shows symptoms induced by the 'original' parent R-strain (from Fig. 11).
are shown in Fig. 19. The product of the regenerated-parental RNA combinations showed the presence or absence of top component, and component distribution patterns that would be the properties predicted on the basis of earlier comparisons of parent strains (Fig. 2) and parent and pseudorecombinant isolates (Fig. 16). Thus, the regenerated E-isolate, like the natural E-strain, had no detectable top component and much more B- than M-nucleoprotein; the regenerated R-isolate, like the natural R-strain, has a small top component and about equal amounts of the two nucleoprotein components. The 'regenerated' parental isolates also show the same relationships in particle component distributions (inheritance through M-RNA) with pseudorecombinant isolates as do natural parental isolates (Figs. 16 and 19).

Serological evidence

Ouchterlony double-diffusion serology of purified virus at $A_{260} = 1.0$ of regenerated parental isolates and the pseudorecombinant isolates they were generated from, against antisera to the purified virus of the original parent strains, produced a pattern of precipitin lines identical to that obtained when comparing purified virus of the original parent strains and the pseudorecombinants that had been made from them (cf. Fig. 17). The precipitin line of the regenerated E-isolate was confluent only with that of the $E_R$ pseudorecombinant and spurred with that of the $R_E$ pseudorecombinant; correspondingly, the regenerated R-isolate was serologically identical to the $R_E$ pseudorecombinant it was generated from, but spurred with that of the $E_R$ pseudorecombinant.
Fig. 19. Absorbance scan patterns of sucrose density gradients showing predicted relationships of particle component distributions among pseudorecombinant and 'regenerated' parental isolates purified from \textit{C. quinoa}.

A. $\frac{E}{R}$ - pseudorecombinant  
B. 'regenerated' E-isolate  
C. $\frac{R}{E}$ - pseudorecombinant  
D. 'regenerated' R-isolate
DISCUSSION

The observations made in this work confirm and extend earlier work on the strains and functionally-bipartite genome of CLRV by Jones and Mayo (1972), Walkey et al. (1973) and Jones (1977).

The existence of related virus isolates with sets of properties that differ consistently is an essential requirement for any genetic analysis by pseudorecombination. In reporting consistent and significant differences among the elm, dogwood, cherry, elderberry and rhubarb strains of CLRV with respect to serology and particle immunoelectrophoresis, the work of Walkey et al. (1973) indicated some possible genetic markers. While the cherry and rhubarb strains also differed from the other three by their higher proportion of coat protein lysine, all five strains appeared virtually indistinguishable by particle sedimentation velocities or protein subunit and nucleic acid component molecular weights. Thus, these latter properties cannot serve as genetic markers of CLRV strains.

Of the possible markers of differences between related viral isolates, symptomatology in differential hosts is probably the most accessible to examination. It was a fortunate circumstance that some markedly different symptoms on the differential hosts were observed (cf. Table 1) when comparing the serologically-distinct but closely-related (Walkey et al., 1973) elderberry and rhubarb strains of CLRV; success in achieving pseudorecombination appears, generally, to increase with increasing serological relatedness of the isolates concerned (Jaspars, 1974; Bruening, 1977). Thus the strains of CLRV predicted to be most likely to form productive pseudorecombinants also had the
best differential symptom markers.

Differences between isolates in particle component distribution have been used as markers in pseudorecombination genetic analyses. For example, Hartmann et al. (1976), in analysing the genetics of alfalfa mosaic virus (AMV), which has four distinct RNA species and a functionally tripartite genome, found the particle component ratio to be inherited with the RNA that determines tryptic peptide fingerprints and thus the coat protein. Jones and Mayo (1972) have shown that with the elderberry strain of CLRV relative proportions of the particle components do not change with increasing time of infection. This has been confirmed for the rhubarb strain as well, in the course of the work reported here. This stability of particle component distribution with time allows the use of this property as a genetic marker with these two strains of CLRV, but may not be taken for granted in similar nepovirus systems; Schneider and Diener (1966) have shown that the duration of infection before harvesting of infected Phaseolus vulgaris cv. Black Valentine affects the relative proportions of purified particle components of two strains of TRSV.

The other essential requirement for successful pseudorecombination genetic analysis, the separation of components, nucleoprotein or RNA, that are complementary in infection, is relatively difficult to fulfill in CLRV because of the proportionally small differences in molecular weight and sedimentation velocity of the nucleoprotein and RNA components. Walkey et al. (1973), using single-cycle separation by sucrose density-gradient centrifugation, did not obtain separation of the two nucleoprotein components and found each 'component' to be highly infectious.
on its own. Jones and Mayo (1972) employed multiple-cycle sucrose
gradient centrifugation and obtained enhancement-of-infectivity ratios
from 4 to 8. Moreover, in some experiments, they succeeded in obtaining
middle component nucleoprotein free of infectivity. Based on the
persistent residual infectivity of bottom component nucleoprotein, however,
it appeared the technique was unable to produce bottom component
preparations entirely free of middle component.

Nucleoprotein components can often be more highly resolved by
separation techniques based on differences in particle density rather
than sedimentation velocity. Thus, bromoviruses, which sediment as
single components in rate-zonal sucrose gradients, can be resolved into
three closely-spaced equilibrium-density bands in CsCl (Lane and
Kaesberg, 1971). In CsCl equilibrium analytical ultracentrifugation,
the nucleoprotein components of the rhubarb strain of CLRV were clearly
resolved as bands at 1.471 (middle) and 1.508 (bottom) g·cm\(^{-3}\). The RNA
proportions by weight of the middle and bottom component nucleoproteins,
obtained by applying these equilibrium density values to the empirical
formula of Sehgal et al. (1970) are 38.5 and 42.3%, respectively.

Using values for nucleoprotein particle and RNA molecular weights of the
elderberry and rhubarb strains of CLRV, obtained by calculation from the
results of Walkey et al. (1973), and confirmed for the preparations of
these two strains actually used in this work (Ramsdell; unpublished
results), the middle component nucleoprotein consisted of 38.8%, the bottom
component 42.0% RNA by weight. This is in good agreement with the
results calculated from the buoyant densities.

The buoyant density values obtained in the preparative CsCl centri-
fugations are lower than those observed in analytical ultracentrifugation
because the nucleoprotein preparations were centrifuged through CsCl for about 18 h, sufficiently long to achieve good resolution. Longer centrifugation times were not used, so as to avoid particle disintegration, especially of the middle component, and the loss of particle infectivity. A true equilibrium density under the preparative conditions described would take over 400 h to achieve (Birnie, 1978). Thus the preparative separation in CsCl brings the components only to a near-equilibrium density.

In both strains examined in this work, the $A_{260}/A_{280}$ ratios of the purified middle and bottom component nucleoproteins were 1.64-1.66 and 1.81-1.83, respectively. These ratios and the nucleoprotein component ratios account together for the $A_{260}/A_{280}$ ratios of the purified 'whole-virus' preparations (from *N. clevelandii*) of the two strains. The rhubarb strain with its about equimolar proportions of nucleoprotein components has an $A_{260}/A_{280}$ ratio of 1.71-1.72 (in agreement with Walkey *et al.*, 1973), about half-way between the values for the separated components, while the elderberry strain with an approximately two-to-one preponderance of the bottom component (cf. Fig. 3) has a proportionally higher ratio of 1.76-1.77.

All the resolution of components was achieved by CsCl near-equilibrium centrifugation at the nucleoprotein level. The technique used for extracting the RNA ordinarily used for analytical purposes gave, in the $A_{254}$ scans of the sucrose gradients, information that could confirm that the extracted RNAs were monodisperse products of separate components, and indicate by the sharpness of the absorbance peak on the leading edge, the quality or lack of degradation in the RNA preparation
(cf Fig. 9). No additional resolution per se, however, was accomplished at this step.

The RNA preparations obtained by this technique are easy to measure for concentration by absorbance at 260 nm, immediately available in a form suitable for inoculation and are readily stored by freezing at -20 C without apparent loss of infectivity. In addition, this RNA-component preparation protocol, unlike the separation of RNA components by polyacrylamide gel electrophoresis (Murant et al., 1972), gives separated nucleoproteins as well as separated RNA-components in the same run. A major objection to the use of CsCl for preparative purposes, the high cost of suitable purified grades, is partially met by the use of a cheaper, technical grade sufficiently purified by filtration and chelation of contaminating divalent cations (Birnie, 1978).

The pseudorecombination genetics of CLRV described in this study are of a similar pattern to those observed in earlier work on other nepoviruses. As with RRV (Harrison et al., 1972; Harrison et al., 1974b) and TBRV (Randles et al., 1977; Harrison and Murant, 1977b) and preliminary work on CLRV (Jones, 1977), the smaller M-RNA determines serology and thus the antigenicity of the coat-protein polypeptide. Indeed, the only functionally multipartite-genome plant viruses known not to have properties of the coat protein determined by the smallest functional RNA species are CRSV (Dodds et al., 1977) and PEMV (Hull and Lane, 1973). The presence or absence of detectable amounts of RNA-free top-component particles was also found in this study to be inherited through M-RNA. Although the genetics of this property have not been examined with other nepoviruses, a similar pattern of inheritance through the smaller RNA was described in CPMV-yellow strain by Bruening
(1969), and interpreted as a function of capsid protein production and not as an allele of another gene on the smaller RNA.

The genetic determination of particle component distribution, found in this work to be associated with M-RNA, has not been examined in other nepoviruses. Such examinations have been carried out with PEMV (Hull and Lane, 1973), AMV (Hartmann et al., 1976), CPMV-yellow strain (Wood, 1972) and CPMV-severe strain (Thongneearkom and Goodman, 1978). In the first three cases, particle component distribution appears determined by the same RNA that determines coat protein, suggesting, that like the formation of top component, this property may be a function of the capsid protein. In the case of CPMV-severe strain, one of the pseudorecombinants had a particle component distribution unlike that of either parent (Thongmeearkom and Goodman, 1978). Since the two parent strains did not differ in particle component distribution, it was suggested that each RNA component might in some manner be determining the rate of synthesis or encapsidation of the other RNA.

The determination of symptom types by different RNAs, depending on host, and in the Nicotiana spp. on type of infection, local or systemic, lacks direct counterparts in earlier genetic analyses of other nepoviruses. It does correspond in pattern to the observation in RRV (Harrison et al., 1974b), where the larger RNA determines the ability to infect non-inoculated leaves of Phaseolus vulgaris L., while the smaller RNA (except when in association with the larger RNA of the Lloyd George strain) determines systemic yellowing symptoms in Petunia hybrida Vilm. The observation in the present work, that the larger RNA (B-RNA) of the rhubarb strain confers the ability to systemically infect G. globosa agrees with the preliminary report of Jones (1977).
Pseudorecombination genetic analysis can only be valid if the isolates prepared by mixing heterologous, complementing nucleoproteins or RNAs of two different viral isolates are truly pseudorecombinant and not merely fortuitously modified by the treatment undergone to exhibit altered physical and biological properties. Regenerating isolates, identical in all observable properties to the parental isolate that is the predicted outcome of the 'pseudorecombination applied to the pseudorecombinants', provides strong confirming evidence of the validity of the genetic analysis. Besides the present work, such a confirmation of nepovirus pseudorecombinant genetic analysis by backcrossing to regenerate the predicted parental types has been reported for RRV (Harrison et al., 1974b) but not for TBRV, the only other nepovirus hitherto reported in the literature to have undergone pseudorecombination genetic analysis (Randles et al., 1977). This is because only one of the two parental pseudorecombinants could be generated in the first place.

There are still many aspects of nepovirus genetics that await investigation by pseudorecombinant genetic analysis. For CLRV in particular, the genetics of nematode- and seed-transmissibility remain to be determined. Determination of the former is unlikely to be a particularly profitable line of inquiry. In the first place, the specific transmission of CLRV by nematodes has yet to be unequivocally demonstrated. Also, the evidence that specific nematode transmission is a function of the capsid protein (Harrison et al., 1974a) and the observed determination of this property in RRV (Harrison et al., 1974a,b) and TBRV (Harrison, 1977b) by the coat-protein determining smaller RNA, suggests that no new insight into nepovirus genetics would be gained. The genetic analysis of seed transmission is another matter, however,
since the analyses of this property in RRV and TBRV (Hanada and Harrison, 1977), while suggesting a predominant influence of the larger RNA, are not conclusive. Moreover, unlike TBRV (Hanada and Harrison, 1977), the possibility exists with CLRV to confirm results with at least two pseudorecombinant isolates and corresponding backcrosses that regenerate predicted parental isolates.

Recent discoveries about two definitive nepoviruses, TRSV and TomRSV, suggest exciting areas of future inquiry. Harrison and Barker (1978) have shown that a small protease-sensitive structure, covalently attached (probably at the 5'-end) to both RNA components, is required for infectivity. If, by oligopeptide analysis, or some other form of analysis capable of distinguishing small similar peptides, this RNA-peptide can be shown to be strain-specific, pseudorecombination analysis might reveal which RNA codes for it. Of course, enormous technical difficulties would exist, mainly in obtaining enough of this RNA-peptide to work with in the first place.

Probably beyond the reach of pseudorecombination analysis alone is the question: What are the immediate gene products coded for by the plant viral genome? It now appears likely, from the recent work of Chu and Francki (1979) that only one-quarter of the previously estimated coding capacity in the smaller RNA is required in nepoviruses for the coat protein polypeptide subunit. There is thus, as with all plant viruses, a great deal of genetic information to which we as yet can ascribe no specific product or function.
SUMMARY

The elderberry (E) and rhubarb (R) strains of cherry leaf roll virus, a nepovirus, were found to differ consistently in particle component distribution, serology and symptomatology in a number of indicator host species. The separation of the two genetic components, middle- (M) and bottom- (B) component RNA, of both E- and R-strains were confirmed by evidence from sucrose density-gradient centrifugation and polyacrylamide gel electrophoresis of RNA as well as by the much greater infectivity of combined-component compared to separated-component inocula. Stable pseudorecombinant isolates generated by heterologous combination of complementing RNA components, when compared with the original parental strains, indicated the genetics of the marker properties that were used to distinguish between the two strains. M-RNA determined serological specificity, particle component distribution, systemic symptoms in Nicotiana clevelandii and N. tabacum cvs. Samsun and Xanthi, and local and systemic symptoms in Chenopodium amaranticolor and C. quinoa. B-RNA determined the ability to induce systemic symptoms in Gomphrena globosa and the type of local lesion in N. clevelandii. This pattern of genetic determination is similar to that described for raspberry ringspot- and tomato black ring virus, other nepoviruses for which pseudorecombination genetic analyses have been reported. The genetic assignments determined in the present work were confirmed by regenerating isolates identical to the original parental strains, in a predicted manner, from the pseudorecombinant isolates.


