Comparative Properties of Necrotic Ringspot Virus from Peach and Cherry

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ABSTRACT

Peach and cherry isolates of the necrotic ringspot virus have similar herbaceous host ranges, component types, sedimentation coefficients, buoyant densities in CsCl, and particle diameters. The peach isolate reacted with antisera of the cherry isolate. The data support the conclusion that the ringspot diseases of peach and cherry are caused by closely related strains. Phytopathology 61:532-537.

Additional key words: biophysical properties, component virus, serology.

The ringspot virus of peach occurs widely throughout peach plantings of the United States, and is seed transmitted in a number of peach cultivars. Cochran et al. (5) considered it to be identical to or at least in the same group as necrotic ringspot virus of cherry.

In this paper, infectivity on a number of herbaceous test plants and some biophysical properties of isolates from peach (NRSV-P) and cherry (NRSV-C) are compared.

MATERIALS AND METHODS.—The peach isolate of necrotic ringspot virus (NRSV-P) was originally isolated from a seedling of *Prunus persica* (L.) Batsch 'Kingston Indian Red Leaf' by Flemion et al. (7). The cherry isolate of necrotic ringspot virus (NRSV-C) and antisera were obtained from W. R. Allen, Canada Department of Agriculture, Vineland Station, Ontario.

Virus transmissions were made by rubbing tissue homogenates or purified extracts over the leaf surface of test plants which had been dusted previously with Carborundum (600 mesh). The viruses were maintained in cucumber (*Cucumis sativa* [L.] 'Marketer'). Bioassays were made on *Momordica balsamia* (L.) which developed necrotic local lesions 4-6 days following inoculation.

Virus was purified by the hydrated calcium phosphate gel (HCP) method of Fulton (9), the ethylene glycol method of Venekamp & Mosch (16), or the method of Van Regenmortel & Engelbrecht (15). The HCP method most consistently yielded infective preparations which were relatively free of host proteins.

Cotyledons of cucumber were harvested 5 days after inoculation and blended in 0.02 M phosphate buffer pH 8.0 containing 0.01 M sodium dithiocarbamate and 0.02 M sodium thioglycolate in the ratio of 2 g tissue to 3 ml buffer. Following low-speed centrifugation at 6,000 *g*, the supernatant solution was decanted and mixed with HCP prepared previously according to Fulton (9) in the ratio of 1 ml of solution to 0.6 ml HCP. The mixture was separated by centrifugation at 6,000 *g* and the supernatant solution treated a second time with one-half the original quantity of HCP and again separated by centrifugation at 6,000 *g*. Two cycles of differential centrifugation, in which the virus was pelleted at 70,000 *g* for 1.5 hr and 144,000 *g* for 1.0 hr, resulted in a glassy-clear pellet which was resuspended in a small quantity of 0.02 M potassium phosphate buffer pH 8.0.

Sucrose density-gradients were performed in a Model L Spinco ultracentrifuge at 25,000 rpm for 4 hr using 10-30% linear sucrose density gradients prepared with 0.02 M phosphate buffer pH 8.0. Density gradients were scanned by piercing the bottom of the gradient tubes and pumping the contents of the gradient column through a broad spectrum ultraviolet monitor.

Ultraviolet absorption spectra were determined in a Beckman DB spectrophotometer. Analytical ultracentrifugation was performed in a Beckman Model E ultracentrifuge using schlieren or ultraviolet optics as appropriate.

Electron micrographs were made in a Zeiss EM 9A electron microscope at a working magnification of ×18,000 calibrated with a carbon grating replica.

For negative staining, carbon-coated Formvar grids were floated on drops containing virus particles. After draining, they were floated on a drop of formaldehyde solution, drained, dipped quickly in a solution of 2% sodium phosphotungstate (Na PTA) pH 7.0, and drained immediately. Preparations to be shadowed were sprayed onto Formvar-coated grids with a Nebulizer (Vaponefrin Co., New York, N.Y.) and subsequently shadowed with platinum (80%) palladium (20%) (Pt-Pd).

Centrifugations in CsCl density gradients were made according to the procedures of Vinograd & Hearst (17). Virus samples were layered on CsCl solutions at 1.352 g/cm^3 and centrifuged 16 to 24 hr at 36,000 rpm (125,000 *g*) in an SW 39 rotor. Columns were fractionated by piercing the bottom of the tubes and collecting drops. Densities of every fourth tube were determined from the refractive index of a 10-μliter sample as described previously (4). Optical density at 260 mμ was determined by adding 1 ml buffer to each sample and reading absorbancy in a spectrophotometer.

Serology was performed using the Ouchterlony double-diffusion technique on Hyland Immuno-Plates (Hyland, Los Angeles, Calif.) using the procedures described by Ball (2).

RESULTS.—*Host range.*—NRSV-P and NRSV-C produced similar symptoms on cucumber (*Cucumis sativa* L. 'National Pickling'), buttercup squash (*Cucurbita pepo* L. 'Buttercup'), butternut squash (*Cucurbita pepo* L. 'Butternut'), pumpkin (*Cucurbita pepo* L. 'Big Tom'), and *Momordica balsamia* L.

Chlorotic spots were produced on inoculated cotyledons of cucumber in 3-5 days, followed by severe

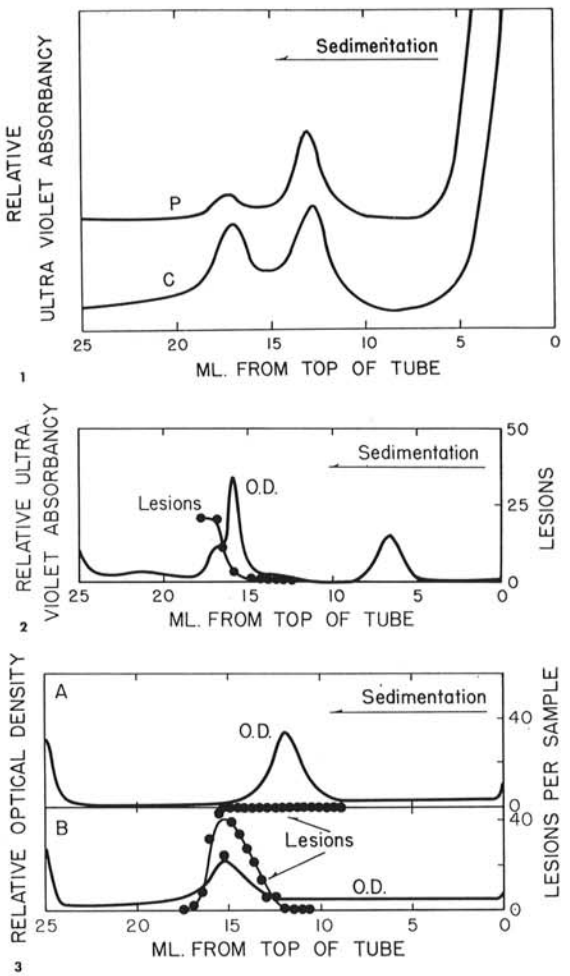


Fig. 1-3. **1)** Typical absorbancy profile of the peach isolate (NRSV-P) and the cherry isolate (NRSV-C) of necrotic ringspot virus following rate-zonal sucrose density-gradient centrifugation. **2)** Ultraviolet absorbancy profile and infectivity of fractions of partially purified peach virus isolate (NRSV-P) following 16 hr centrifugation at 25,000 rpm in a 10-60% sucrose density gradient. **3)** Ultraviolet absorbancy and infectivity (lesions of *Momordica balsamia*) of the slow (A) and fast (B) components of the cherry isolate of necrotic ringspot virus following sucrose density-gradient centrifugation of the components which had been previously purified by sucrose density-gradient centrifugation.

mosaic and necrosis in the first true leaves. If the inoculated plants did not die they remained severely distorted and grew little.

Necrotic rings and spots appeared on inoculated cotyledons of squash in 3-6 days, but no symptoms appeared on true leaves. When true leaves were inoculated, angular necrotic lesions appeared in 3-6 days. The lesions expanded, became necrotic, and virus was recovered from them. Inoculated cotyledons of butternut squash failed to show symptoms, but true leaves produced a severe mosaic 6-8 days after inoculation of the cotyledons. Neither buttercup nor butternut squash was consistently infected.

Pumpkin developed a severe chlorosis on inoculated leaves 5-6 days after inoculation.

Momordica balsamia L. produced necrotic local lesions when inoculated with NRSV-C, and chlorotic and necrotic local lesions when inoculated with NRSV-P.

Cowpea (*Vigna sinensis* L. 'Blackeye'), pea (*Pisum sativum* L. 'Perfected Wales'), tobacco (*Nicotiana tabacum* L. 'Samsun'), tomato (*Lycopersicon esculentum* Mill.), petunia (*Petunia hybrida* Vilm.), and *Gomphrena globosa* L. showed no symptoms when inoculated, and recovery tests to cucumber were negative.

Component analysis by sucrose density-gradient centrifugation.—Rate zonal sucrose density-gradient cen-

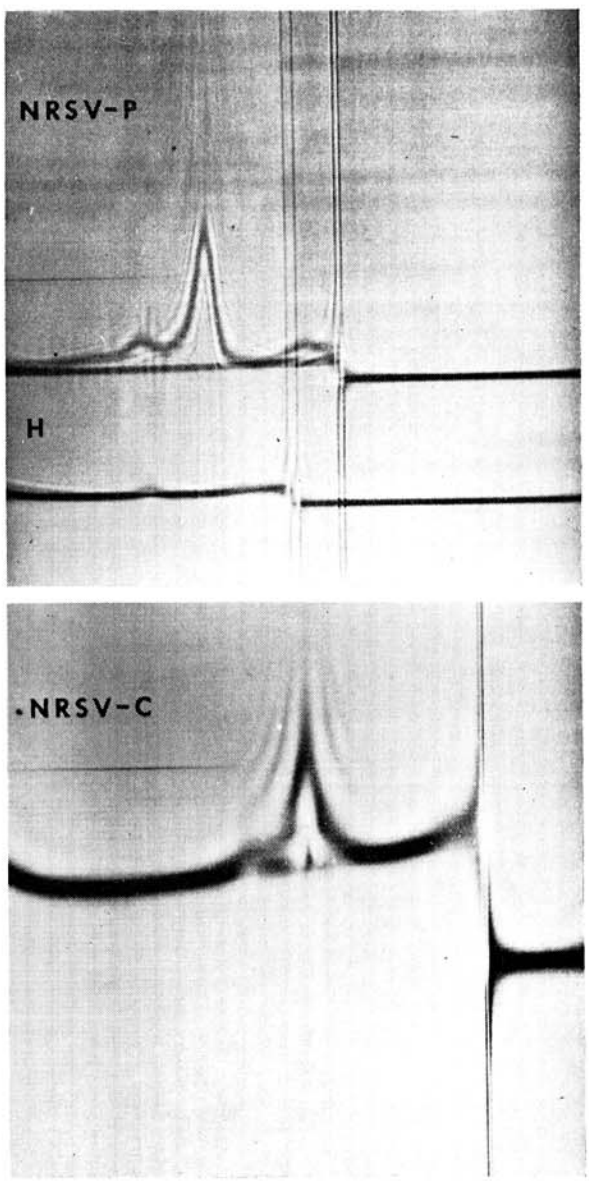


Fig. 4. Schlieren pattern during analytical ultracentrifugation of the peach virus isolate (NRSV-P) and healthy control (H) and the cherry isolate (NRSV-C) of necrotic ringspot virus.

trifugation followed by scanning of the column effluent shows that both NRSV-P and NRSV-C have two similarly sedimenting components (Fig. 1). In addition, both preparations contain a significant amount of slow-sedimenting material which bands near the top of the centrifuge tube and which is also found in preparations from healthy cucumber tissue. This latter contaminating material could be eliminated from the preparations by acidification to pH 5.0 with no apparent physical damage to the virus particles. However, acidification usually resulted in loss of infectivity and was, therefore, discontinued.

When NRSV-P was subjected to equilibrium density-gradient centrifugation in a 10-60% sucrose density gradient for 16 hr at 25,000 rpm, the components were closer together than following rate-zonal centrifugation. Bioassay of the fractionated sample showed that only the faster sedimenting component was infective (Fig. 2).

When each of the two peaks of NRSV-C obtained from density-gradient centrifugation was concentrated and subjected to a second rate-zonal centrifugation, the slow component produced a symmetrical peak (Fig. 3-A), indicating little, if any, contamination with the fast component. In assays on *M. balsamia*, there was no infectivity associated with the peak. The fast com-

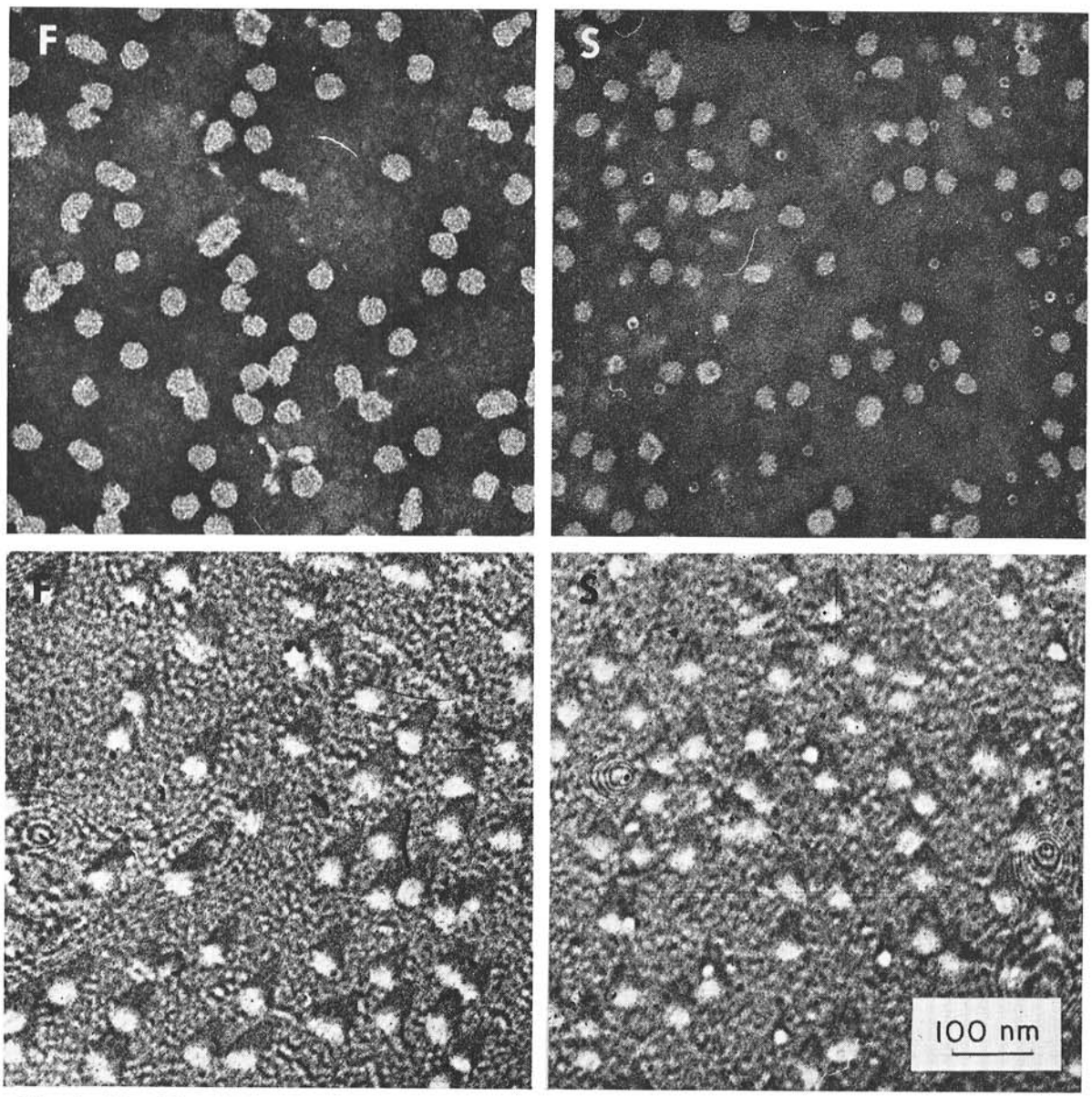


Fig. 5. Fast (F) and slow (S) component of the peach virus isolate of necrotic ringspot fixed with 1% glutaraldehyde and negatively stained with sodium phosphotungstate (above) and shadowed with platinum (80%) palladium (20%) (below). Scale is 100 mμ.

ponent showed some heterogeneity, as indicated by a slight shoulder on the right side of the peak (Fig. 3-B). Infectivity tests of fractions showed that infectivity was associated only with the fast peak.

Sedimentation velocity.—Schlieren patterns obtained during analytical ultracentrifugation are shown in Fig. 4. Both NRSV-P and NRSV-C had two components, followed by a peak in the region of 20 S that was considered to be host protein. Sedimentation measurements ($S_{20,w}$) with ultraviolet optics were: NRSV-P = 69.8 (slow); 101.7 (fast); NRSV-C = 69.5 (slow); 102.3 (fast).

Particle diameter.—Both NRSV-P and NRSV-C collapsed badly when negatively stained for electron microscopy. Pretreatment with 1% formaldehyde or 1% glutaraldehyde had little if any beneficial effect. Figure 5 shows the fast (F) and slow (S) components of NRSV-P after they were pretreated with 1% formaldehyde and negatively stained with 2% Na PTA. It was difficult to select particles for measurement, but particles of the fast and slow components which appeared least-collapsed had diam of 35 and 28 nm, respectively. Particles from the fast and slow peaks of NRSV-P both have diam of 28 nm if shadowed with Pt-Pd. Particles from the fast peak of NRSV-C, when negatively stained and shadowed with Pt-Pd, appear similar and are of the same size as those of NRSV-P (Fig. 6).

Buoyant density in CsCl.—Optical density profiles of NRSV-P and NRSV-C preparations centrifuged to equilibrium in CsCl are shown in Fig. 7. Both preparations had symmetrical peaks at a density of 1.352 g/cm^3. Nucleoprotein with RNA content of 20% would be expected to band at about this density. Both preparations had large peaks at 1.25 g/cm^{-3}, where protein is expected to band. There was no suggestion that the two virus components had different densities in CsCl.

Ultraviolet absorption spectra.—Virus obtained from the peak of the CsCl gradient was diluted with 0.02 M phosphate buffer pH 8.0 and used for ultraviolet spectra. Both NRSV-P and NRSV-C had typical nucleoprotein spectra, with max at 260 mμ and min at 242 mμ (Fig. 8). The 280:260 ratios, following light scattering correction, of about 0.59 for both viruses are compatible with a nucleic acid content of about 20%, according to the method of Paul (13).

Serology.—The results of Ouchterlony double-diffusion tests are shown in Fig. 9. Both NRSV-P and NRSV-C antigen reacted in a single line with the NRSV-C antisera. There was no reaction with a similar extract of healthy cucumber tissue.

DISCUSSION.—Early works relating peach ringspot virus to the necrotic ringspot virus of stone fruit were based on host range and symptomatology. Helton (11) considered that the graft transmission experiments of Parker & Cochran (12) established that ringspot of peach and necrotic ringspot of cherry were the same. Cochran et al. (5) also considered them to be the same. Heinis & Milbrath (10) transmitted 20 of 22 isolates of necrotic ringspot virus and peach ringspot virus to cucumber. They suggested that the viruses transmitted to cucumber were strains of stone fruit ringspot virus. The herbaceous host ranges of NRSV-P and NRSV-C were similar; both were similar to Fulton's (8) cherry necrotic ringspot virus (isolate G). Later authors have described properties of various isolates of necrotic ringspot virus (3, 6, 14, 15, 18), but none has made a comparison of the properties of isolates from peach and cherry.

NRSV-P is similar to NRSV-C in that both have two sedimentation components, and both band in CsCl at the same density and have identical ultraviolet absorbancy spectra. Furthermore, negatively stained electron micrographs of both viruses show severely collapsed particles, with the fast component apparently

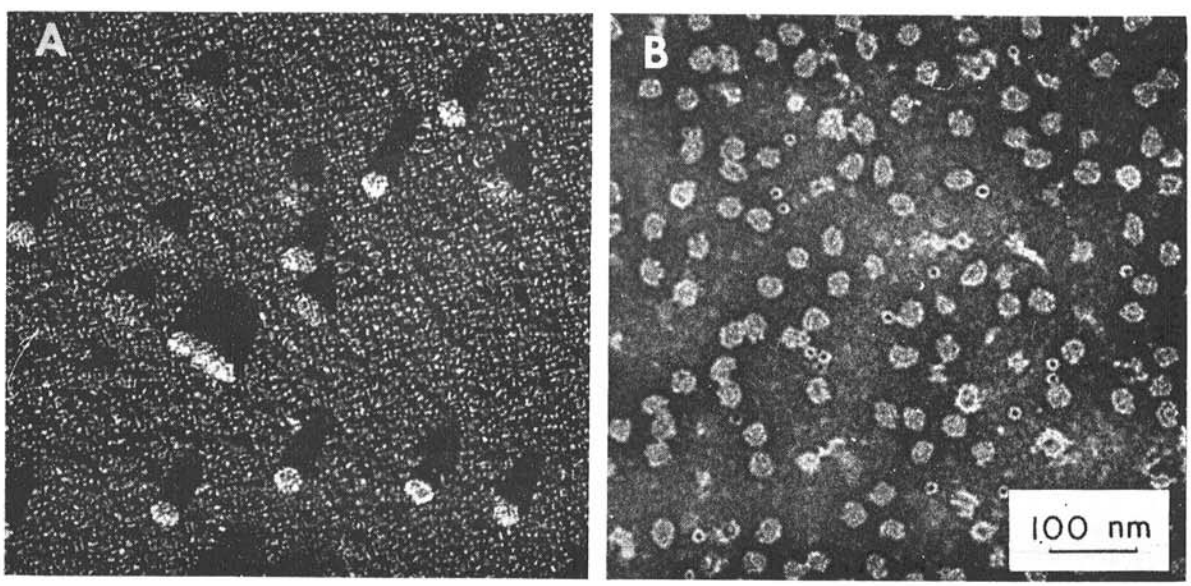
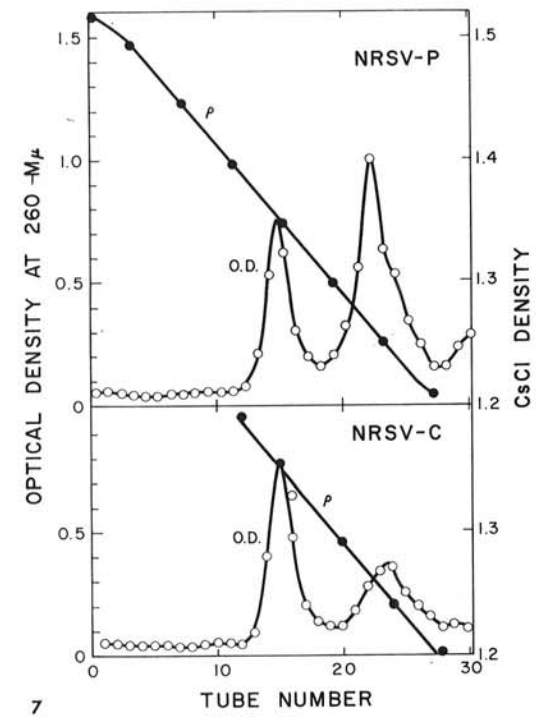


Fig. 6. The cherry isolate (fast component) of necrotic ringspot virus (NRSV-C) shadowed with platinum (80%) palladium (20%) (A), and negatively stained with sodium phosphotungstate (B).

larger than the slow component. Shadowed preparations of both viruses show spherical particles of the same size which are not collapsed. The NRSV-P and NRSV-C react strongly with antisera prepared against NRSV-C. Therefore, on the basis of those properties studied here, it is concluded that these peach and cherry isolates are closely related strains. These results confirm those of Parker & Cochran (12) and Helton (11), who based their conclusions on host range and symptomatology.

The emergence of necrotic ringspot virus as a component virus is a matter of some interest. Tolin (14) reported two zones associated with a recurrent form of necrotic ringspot virus with sedimentation velocities of approx 95 and 117 S. Both were infective. De Sequeira (6) found two zones associated with an apple isolate as well as a cherry isolate of necrotic ringspot virus. He reported the sedimentation velocity to be 88 and 117 S for the two components of the apple isolate and only the fast sedimenting species was infective. Furthermore, Bock (3) found two sedimenting species (79 and 107 S) associated with a strain of necrotic ringspot virus isolated from hop. He also reported that only the fast component was infective. These reports, together with the finding that NRSV-P and NRSV-C each has two components, would seem to establish necrotic ringspot as a two-component virus. Earlier reports of Willison et al. (18) and Van Regenmortel & Engelbrecht (15) on NRSV failed to mention the fast component. It is possible that the component ratio was such that a small quantity of the fast-sedimenting component was not observed due to the relative insensitivity of schlieren optics. The schlieren plates published by the latter authors support this suggestion. De Sequeira (6) and Bock (3) both worked with isolates which appeared to have a relatively high proportion of the fast component (about 50% judging from their schlieren plates). In this laboratory as well as that of Tolin (14), a very sensitive technique was used to scan sucrose density-gradient columns. Hence, a comparatively small quan-



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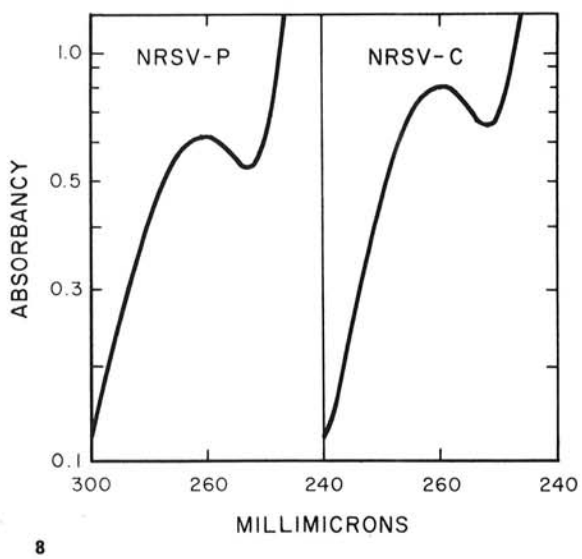

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Fig. 7-8. **7)** Optical density profile and density of the peach isolate (NRSV-P) and the cherry isolate (NRSV-C) of necrotic ringspot virus following equilibrium density-gradient centrifugation in CsCl. **8)** Ultraviolet spectrum of the peach isolate (NRSV-P) and the cherry isolate (NRSV-C) of necrotic ringspot virus.

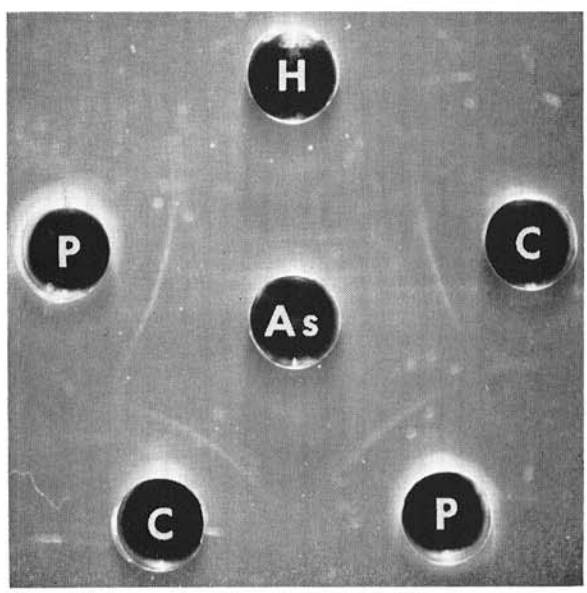

Fig. 9. Ouchterlony double-diffusion test. Center well contains antiserum (As) to the cherry isolate of necrotic ringspot virus. The outer wells, starting at the top and reading clockwise, contain extract of healthy cucumbers (H), a purified preparation of NRSV-C (C), a purified preparation of NRSV-P (P), NRSV-C (C), and NRSV-P (P).

tity of fast sedimenting component could be easily detected.

Considering the susceptibility of necrotic ringspot viruses to collapse (3, 6, 15) when prepared for electron microscopy, the various particle diam reported are in agreement. A diam of 28 nm for a Pt-Pd-shadowed preparation is reasonably close to the 23 nm reported by Fulton (9) and by Van Regenmortel & Engelbrecht (15). It also agrees with the hydrated particle diam of 30 nm ± 10 calculated by Allen & Tremaine (1) and the 29 nm hydrated diam of Van Regenmortel & Engelbrecht (15). The reports of Bock (3) and de Sequeira (6), that the fast component particles seem to have greater diam than the slow component particles, agree with results reported here for NRSV-P.

Because only one density species was found in CsCl density-gradient centrifugation, it is possible that the two components represent only differences in structure resulting from the breakage of certain bonds within the particle. This may result in susceptibility of the nucleic acid of the slower sedimenting species to nucleases; hence, they were found to be noninfective in this work as well as by Bock (3) and de Sequeira (6). In this respect, these isolates differ from that reported by Tolin (14).

LITERATURE CITED

1. Allen, W. R., & J. H. Tremaine. 1965. Characteristics of antigens and antibodies associated with the Prunus necrotic ringspot virus immune system. Virology 25: 122-132.
2. Ball, E. M. 1961. Serological tests for the identification of plant viruses. Amer. Phytopathol. Soc., St. Paul, Minn. 16 p.
3. Bock, K. R. 1967. Strains of Prunus necrotic ringspot virus in hop (Humulus lupulus L.). Ann. Appl. Biol. 59:437-446.
4. Bozarth, R. F., & C. C. Chow. 1966. Pea enation mosaic virus: purification and properties. Contrib. Boyce Thompson Inst. 23:301-309.
5. Cochran, L. C., L. M. Hutchins, J. A. Milbrath, G. L. Stout, & S. M. Zeller. 1951. Ring spot. USDA Agr. Handbook 10:71-80.
6. De Sequeira, O. A. 1966. Purification and serology of an apple mosaic virus. Virology 31:314-322.
7. Flemion, F., J. Beardow, & H. Mee. 1965. A virus in physiologically dwarfed peach seedlings. A preliminary report. Contrib. Boyce Thompson Inst. 23:135-138.
8. Fulton, R. W. 1957. Properties of certain mechanically transmitted viruses of Prunus. Phytopathology 47: 683-687.
9. Fulton, R. W. 1959. Purification of sour cherry necrotic ringspot and prune dwarf viruses. Virology 9: 522-535.
10. Heinis, J. L., & J. A. Milbrath. 1954. Strain relationships of ring spot virus on cherry, peach, and cucumber. Phytopathology 44:492 (Abstr.).
11. Helton, A. W. 1962. Incidence of ringspot and prune dwarf and the relationship of these conditions to prune decline in Idaho. Phytopathology 52:159-162.
12. Parker, K. G., & L. C. Cochran. 1951. Similarities of symptoms produced by the viruses causing ring spot of peach and necrotic ring spot of sour and sweet cherry. Phytopathology 41:942 (Abstr.).
13. Paul, H. L. 1959. Die Bestimmung des Nucleinsäuregehaltes pflanzlicher Viren mit Hilfe einer spektrophotometrischen Methode. Z. Naturforsch. 14b:427-432.
14. Tolin, S. A. 1963. Sedimentation properties of two stone fruit viruses. Phytopathology 53:891 (Abstr.).
15. Van Regenmortel, M. H. V., & D. J. Engelbrecht. 1963. Purification and biophysical properties of necrotic ringspot virus of stone fruits. South Africa J. Agr. Sci. 6:505-515.
16. Venekamp, J. H., & W. H. M. Mosch. 1963. Chromatographic studies on plant viruses. I. The isolation of potato virus X by means of various systems of adsorption chromatography. Virology 19:316-321.
17. Vinograd, J., & J. E. Hearst. 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. Fortschr. Chem. Org. Naturstoffe 20:372-422.
18. Willison, R. S., J. H. Tremaine, & M. Weintraub. 1961. Serological and physical properties of some stone-fruit viruses: non-virus particles associated with infection. Can. J. Bot. 39:1447-1452.