Carnation Yellow Fleck Virus

M. Smookler and G. Loebenstein

Virus Laboratory, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

The authors thank U. Karo and B. Bar Tov, Department of Biochemistry, Bar Ilan University, for the analytical ultracentrifugation; Y. Cohen for help with the electron microscopy; and M. Bar-Joseph for valuable comments.

Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. 1973 Series, No. 312-E.

Accepted for publication 8 February 1974.

ABSTRACT

Carnation yellow fleck virus (CYFV), which causes yellow mottling, streaking, flecking, necrosis, and purple discoloration of carnation leaves, was isolated from *Dianthus caryophyllus* and transferred to *D. barbatus* and *D. chinensis*. *Myzus persicae* transmitted CYFV semipersistently after an acquisition feeding of 30 min, but not after 10 min. CYFV was transferred mechanically, but with extreme difficulty. Virus was purified using polyethylene glycol precipitation, sucrose density-gradient- and differential centrifugation. Purified preparations, examined in the analytical ultracentrifuge, were heterodisperse. The sedimentation coefficient ($S_{20,w}$) of the virus calculated from different preparations was estimated as 122-128 S at infinite dilution. Electron microscopy of CYFV revealed filamentous flexuous particles measuring 1,250×13 nm. Although CYFV morphologically resembled beet yellows virus (BYV) and beet yellow stunt virus, it did not react with BYV antiserum, nor did it infect some of the major indicator host plants of those viruses.

Phytopathology 64:979-984.

Commercial stocks of carnation in Israel are wholly infected with carnation mottle virus and partially infected with carnation etched ring virus (12). Recently, we isolated an elongated virus from carnation that differs from carnation latent and carnation vein mottle viruses, the two most widespread elongated viruses in carnations. This paper describes some of the properties of this virus, which we have tentatively named carnation yellow fleck virus (CYFV). A preliminary report of the occurrence of this virus in Israel has been published (12). While this work was in progress, Inouye and Mitsuhata (10) described an elongated carnation virus from Japan that apparently has very similar host range and aphid transmission characteristics to the one described here, but different with respect to particle length.

MATERIALS AND METHODS.—The virus was originally isolated from a spray carnation (*Dianthus caryophyllus* L. 'Cerise Royallet') and all the subsequent work used this isolate.

Preparations for electron microscopy were examined in a Jem 7A electron microscope. Particle-size determinations were made from preparations obtained by the dip method, whereby freshly cut infected leaf edges were drawn through a drop of 1% uranyl acetate placed on a carbon-coated formvar grid. Purified virus preparations were allowed to sediment on the carbon-coated grid for 2-60 min and were stained with 1% uranyl acetate. Where necessary, the glycerol float method of Brakke and van Pelt (3) was used to remove either sucrose or polyethylene glycol 6,000 (BDH Chemicals Ltd., Poole, England) from virus preparations. These preparations were later stained with 1% uranyl acetate before they were viewed in the electron microscope.

Determination of particle length was done from prints at a magnification of 66,200 using a grating replicate, 1,134 line/mm (28,800 lines/inch) (Ernest F. Fullam, Inc., N.Y.), as a standard. Particle width measurements were made directly from the plates using a binocular microscope.

Sucrose density gradients were prepared in Spinco SW25 tubes by layering 4 ml of 10%, and 7 ml of 20%, 30%, and 40% (w/v) sucrose in 0.02 M sodium phosphate buffer (pH 8.0) and allowing diffusion to occur overnight at 4 C. Sucrose gradients were fractionated after

centrifugation by upward displacement with 60% sucrose and analyzed using an ISCO density-gradient fractionator equipped with a 254-nm recording ultraviolet absorptiometer. Analytical ultracentrifugation was done in a Spinco Model E equipped with Schlieren optics.

All test plants, except *D. caryophyllus*, which was vegetatively propagated, were grown from seed in a glasshouse. Later, when required for testing, they were transferred to a 21-22 C air-conditioned plastic chamber.

RESULTS.—*Symptoms in carnation.*—In the carnation cultivars Cerise Royallet, 'Gus Royallet', and 'Joker', which also contained carnation mottle virus, symptoms appeared about 3-5 wk after aphid transmission. Generally, the distal halves of infected leaves showed yellow mottling, streaking, and flecking (Fig. 1). Later, these symptoms spread to the rest of the infected leaf, which ultimately turned yellow, became necrotic, and died. In some infected spray carnations, the basal leaves showed purplish-red discolorations (Fig. 2), while in others, where the infection had apparently been established for about a year, the older leaves of infected plants displayed whitish-grey necrotic spots (Fig. 3). In some plants after the initially infected leaves had died, no additional symptoms appeared, but virus could occasionally be recovered. Obvious flower damage was not seen in infected plants.

Host range.—At the present time the virus has been found to infect three *Dianthus* spp. only. In Chinese carnation (*Dianthus chinensis* L. 'Imperialis plenus') and sweet william (*Dianthus barbatus* L. 'Red Empress'), symptoms of CYFV were characterized, 3-5 wk after infection, by vein-yellowing and flecking in the infected leaves (Fig. 4, 5). Eventually the infected leaves turned yellow, became necrotic, and died, after which no other symptoms of virus infection were visible in the plants.

Numerous attempts to infect the following species by means of viruliferous aphids were unsuccessful: *Beta vulgaris* L. (beet, sugar beet, cow beet), *Chenopodium capitatum* (L.) Asch., *C. amaranticolor* Coste & Reyn., *C. hybridum* L., *Gomphrena globosa* L., *Nicotiana clevelandii* Gray, and *Tetragonia expansa* Murr.

Mechanical transmission.—The virus was transmitted mechanically with extreme difficulty. Mechanical cross-inoculations from carnation, Chinese carnation, and Sweet William to carnation, Chinese carnation, and Sweet William were carried out by grinding infected leaves in cold 0.05 M sodium phosphate buffer (pH 7.3) and wiping a muslin pad soaked with inoculum over leaves previously dusted with Carborundum. A total of 45 plants were inoculated and only one plant, Chinese carnation from carnation, became infected.

Attempts to transmit CYFV by rubbing the freshly cut edges of infected leaves onto test plants previously dusted with Carborundum were not successful. Carnation mottle virus (when present) was transmitted, but CYFV was not transmitted in 20 attempts. Similarly, all attempts to obtain infection using partially purified and purified virus preparations were unsuccessful.

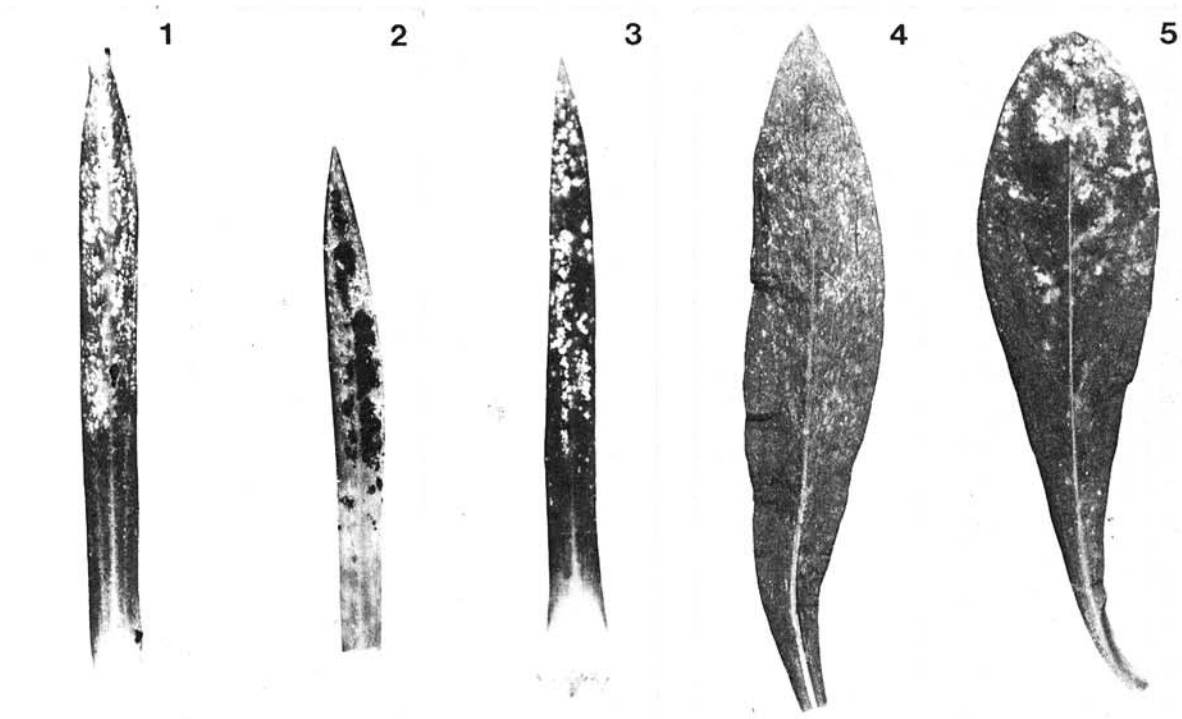


Fig. 1-5. 1) Leaf symptoms on carnation (*Dianthus caryophyllus*) caused by carnation yellow fleck virus (CYFV) and carnation mottle virus showing yellow mottling, streaking, and flecking. 2) Doubly-infected carnation leaf showing purple discolorations. 3) Doubly infected carnation leaf showing whitish-grey necrotic spots. 4) CYFV-infected leaf of *D. chinensis* showing vein-yellowing and flecking. 5) CYFV-infected leaf of *D. barbatus* showing vein-yellowing, flecking, and mottling.

Aphid transmission.—Preliminary studies indicated that CYFV was transmitted by the green peach aphid (*Myzus persicae* Sulz.). Nonviruliferous aphids were reared on Chinese cabbage (*Brassica chinensis* L.). Aphids were either starved for 2 h and given acquisition access periods of 2, 10, and 30 min on infected detached *D. chinensis* leaves kept moist in petri dishes, or they were placed directly, without starving, on detached leaves for 4 and 24 h. Ten aphids were subsequently transferred to each healthy *D. chinensis* test plant and 24 h later the aphids were killed. Virus was detected 3-5 wk later by both symptom observation and electron microscopy.

In a number of different trials, acquisition access feedings of 2, 10, and 30 min, and 4 and 24 h, led to infection in 0/10, 0/14, 2/14, 5/10, and 29/56 plants, respectively. Thus, in the present trials, the minimum acquisition feeding period necessary to transmit the virus was 30 min and groups of ten aphids were capable of transmitting the virus to 52% of the test plants when given 24-h acquisition access feedings.

Trials on the retention of CYFV by *M. persicae* were carried out using groups of ten aphids. Aphids were given a 24-h acquisition feeding and then transferred in daily serial transfers to healthy Chinese carnation plants. In a number of trails, the virus was transmitted after the first, second, third, and fourth daily serial transfer to 5/11, 2/11, 0/11, and 0/11 test plants, respectively. These results indicated that *M. persicae* retained CYFV for 2 days after the acquisition feeding, and that the virus apparently was semipersistent in its aphid vector.

In a separate experiment, single aphids were given a 24-h acquisition feeding on detached infected leaves of *D. chinensis* and then transferred to healthy leaves for 24 h. Single aphids transmitted the virus in two out of ten attempts.

Purification.—Infected leaves of *D. chinensis*, *D. barbatus*, or *D. caryophyllus* were ground in a mortar with 0.05 M sodium phosphate buffer, pH 7.3 (1.0 g/10 ml), and then squeezed through muslin. The extract was centrifuged, first for 10 min at 4,000 *g* and, after removing the pellet, again for 10 min at 8,000 *g*. Four g of polyethylene glycol 6,000 (PEG) and 1.17 g NaCl were added, with stirring, to every 100 ml of the supernatant. The suspension was stirred for 30 min and then centrifuged for 10 min at 16,000 *g*. The supernatant was discarded and the pellet resuspended in 20 ml 0.05 M sodium phosphate buffer (pH 7.3) per 100 ml of original supernatant. Resuspension was carried out, with gentle stirring, for 1 h. The suspension was clarified by centrifugation for 10 min at 4,000 *g* followed by 10 min at 8,000 *g*, and then the supernatant was spun at 29,000 rpm for 2 h in a No. 30 Spinco rotor. The pellet was resuspended in 0.02 M sodium phosphate buffer (pH 8.0) in 1/25th of the original volume, by gentle stirring overnight. After centrifugation, 10 min at 4,000 *g* followed by 8,000 *g* for 10 min, 1-4 ml of the supernatant was layered onto sucrose gradients and centrifuged for 2.5 h at 24,000 rpm in a Spinco SW25 rotor.

Most preparations formed a single light-scattering zone about 24 mm below the meniscus, but a few

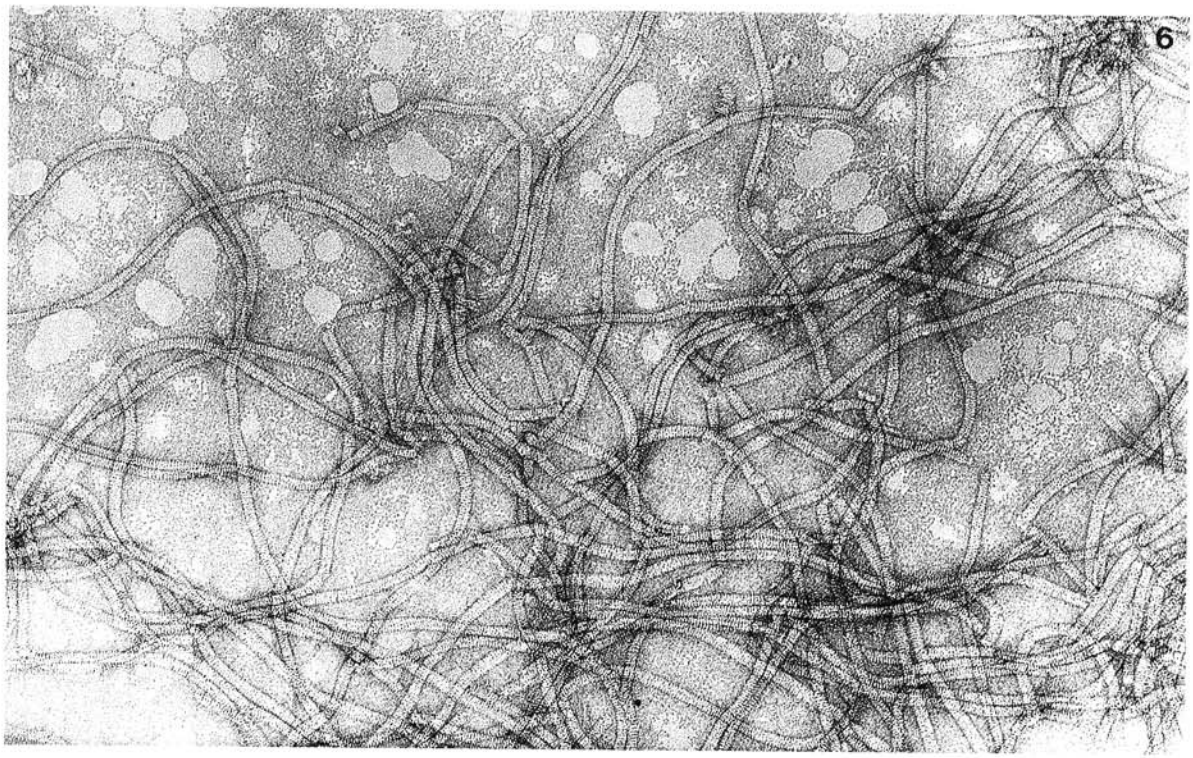


Fig. 6. Electronmicrograph of purified particles of carnation yellow fleck virus (× 96,000).

(presumably due to aggregation) produced broad zones extending almost to the bottom of the tube. Ultraviolet absorptiometer traces at 254 nm and electron microscopy of the fractions showed a correlation between maximum absorbance, relative concentration of particles, and the light-scattering zone.

Peak virus fractions were collected, pooled, and diluted 4- to 5-fold with 0.02 M sodium phosphate buffer (pH 8.0) and the virus was reconcentrated by centrifugation at 108,000 *g* for 90 min. The pellet was then resuspended, with gentle stirring for 30 min, in 1/50th of the original volume and the virus was clarified by centrifugation at 5,000 *g* for 10 min.

In some cases, after sucrose density-gradient centrifugation, where the pellet from the reconcentration step was green, the virus was further purified using PEG. After the pellet had been dispersed and clarified, 0.4 g PEG and 0.117 g NaCl were added, with stirring, to every 10 ml. The suspension was stirred for a further 30 min, and then centrifuged for 15 min at 16,000 *g*. The supernatant was discarded and the pellet resuspended in 1.0 ml 0.02 M sodium phosphate buffer, pH 8.0, per 100 ml original solution, stirred for 30 min, and then centrifuged at 5,000 *g* for 10 min. A green precipitate settled out, leaving the virus behind in suspension.

The preparation from either the reconcentration step or PEG purification was further subjected to another two cycles of high- and low-speed centrifugation. Care was taken to resuspend the high-speed precipitates adequately, and the final pellet was suspended in a sufficient volume of either 0.02 M sodium phosphate buffer (pH 8.0) or double-distilled water. All purification stages were done at 4 C. Preparations obtained in this way were colorless, unaggregated, and contained many intact and fragmented particles (Fig. 6). However, the numbers of broken particles in electronmicrographs from purified preparations did not appear to be much greater than in electronmicrographs from leaf-dip preparations.

Analytical ultracentrifugation of purified preparations in 0.1 M KCl revealed that the preparations were uncontaminated but heterodisperse. Such preparations gave one main Schlieren peak immediately after reaching 26,000 rpm in the analytical ultracentrifuge (Fig. 7). Dissociation of the peak occurred during the run and a diffuse Schlieren pattern with a much reduced major peak (Fig. 8) was observed. The same general pattern was seen for different preparations. An average sedimentation coefficient ($S_{20,w}$) of 122-128 S at infinite dilution was calculated. It seems likely that the heterodispersity of the preparations, and the long flexuous nature of the virus, contributed to this diffuse Schlieren pattern.

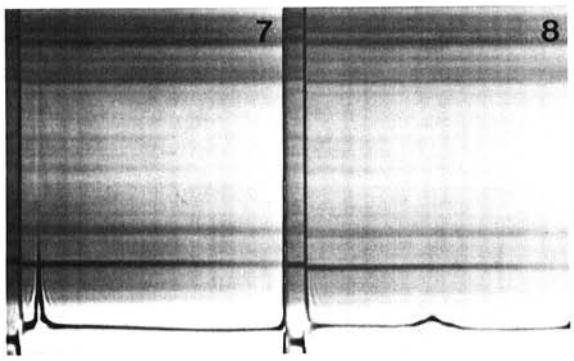


Fig. 7-8. Sedimentation pattern of carnation yellow fleck virus in 0.1 M KCl. **7)** Photograph taken soon after a speed of 26,000 rpm was reached (Schlieren angle 55°). **8)** Photograph taken 16 min after reaching 26,000 rpm (Schlieren angle 50°). Sedimentation was from left to right.

Virus yields ranged from 1.5 to 3.0 OD_{260} units/ml for 25 g of leaf material, depending on the source. *D. chinensis* and *D. barbatus* both gave higher yields than *D. caryophyllus*.

Electron microscopy.—In dip preparations from the leaves of infected *Dianthus* spp., elongated filamentous particles were readily seen. Such preparations characteristically showed bundles of aggregated particles (Fig. 9) and were unusual in that large numbers of broken particles were seen (Fig. 10). The excessive fragmentation of particles was further exemplified in the histogram (Fig. 11) of particle length distribution.

Twenty-nine percent (52/176) of the particles measured 1,200-1,300 nm and about half of these had a most-frequent-length of 1,250 nm.

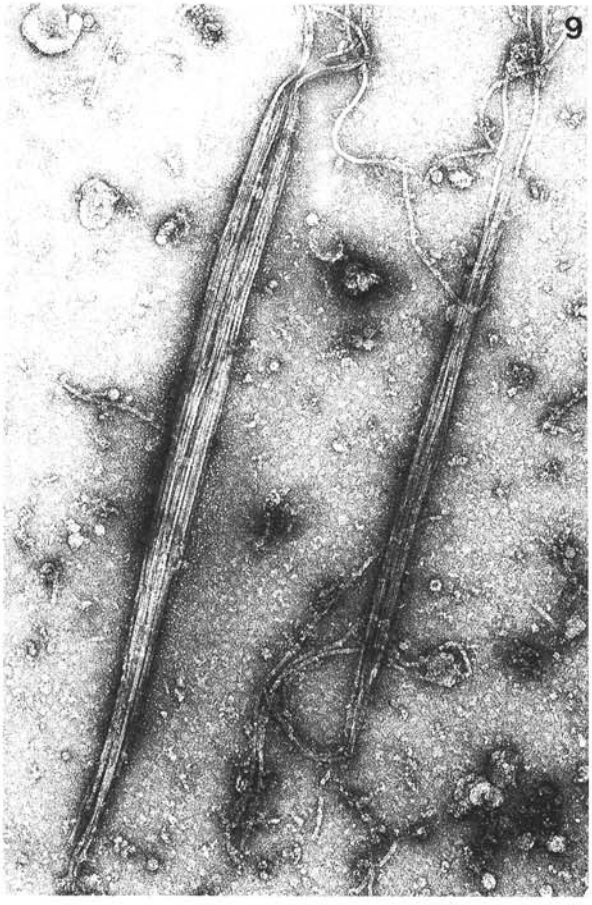
Attempts to reduce the excessive fragmentation of particles by making leaf dip preparations into distilled water, 0.05-0.5 M sodium phosphate buffer, pH 7.0-8.0, either with or without 0.01 M $MgCl_2$, were unsuccessful. All such treatments resulted in many fragmented particles and similar results were obtained with leaf dip preparations made directly into 2% uranyl formate and neutralized 1% phosphotungstic acid.

The width of particles in negatively stained preparations ranged from 11 to 15 nm, with a mean for 100 particles of 13 nm.

Serological tests.—Ring precipitin tests were carried out with purified CYFV. Purified beet yellows virus (BYV) and BYV antiserum with a homologous titer 1/256 in virus precipitin tests were kindly supplied by M. Bar-Joseph. Tests were positive with BYV antiserum against BYV, but not with BYV antiserum against CYFV.

Limited cross-absorption tests were carried out with purified CYFV and BYV, and BYV antiserum. CYFV and BYV were incubated separately for 2 h at 37 C with BYV antiserum (1 part virus suspension : 1 part serum). The absorbed serum was centrifuged for 10 min at 4,000 *g* and then used in ring precipitin tests against BYV. Precipitin reactions were positive with BYV antiserum absorbed with CYFV, but not with antiserum absorbed with BYV.

DISCUSSION.—Of the viruses reported to infect carnations (7, 9, 10), CYFV is readily distinguishable from carnation mottle, carnation ringspot, carnation Italian ringspot, carnation etched ring complex, cucumber mosaic, alfalfa mosaic, turnip crinkle, sowbane mosaic, arabis mosaic, carnation latent, and carnation vein mottle, but is morphologically similar to carnation necrotic fleck virus (CNFV). However, CNFV is reported to be 1,400-1,500 nm long (10), while CYFV appears to be about 1,250 nm in length. It may be that the differences in virus length are due to the fact that Inouye and Mitsuhata (10) used either 2.0% PTA or 1.0-2.0% uranyl formate to stain their virus (we used 1.0% uranyl formate) or that



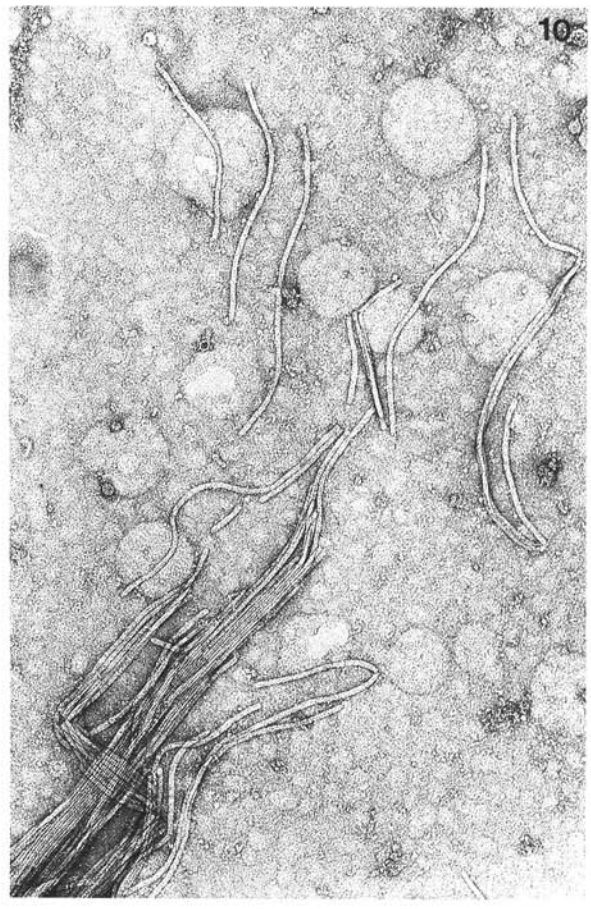


Fig. 9-10. Morphology of carnation yellow fleck virus. **9)** Leaf-dip preparation from carnation, showing characteristic bundles. Spherical particles of carnation mottle virus are also present (× 52,000). **10)** Leaf-dip preparation from carnation showing virus bundle and many fragmented particles of CYFV (× 55,000).

their leaf dip measurements were made from a species other than *D. caryophyllus*. Nevertheless, similarities exist between the two viruses. They both appear to have identical host ranges, produce comparable host reactions, and are similarly transmitted by *M. persicae*.

Morphologically, CYFV closely resembles BYV (11) and beet yellow stunt virus (BYSV) (4). Also, all of these viruses are transmitted semipersistently by aphids and there are some similarities between the ultrastructural changes (banded inclusion bodies, virus-induced vesicles) found in CYFV-infected plants (Smookler and Cohen, *unpublished*) and those in BYV-infected (5) and BYSV-infected (8) plants. CYFV has a considerably more restricted host range than either BYV (2) or BYSV (4). None of the major indicator host plants of either BYV or BYSV were infected by CYFV and, alternatively, the *Dianthus* spp. seen here to be susceptible to CYFV were previously found to be resistant to BYV (2).

The serological experiments carried out here with BYV antiserum gave a negative result. Nevertheless, reciprocal experiments using CYFV antiserum and BYV were not done, which means that a possible serological

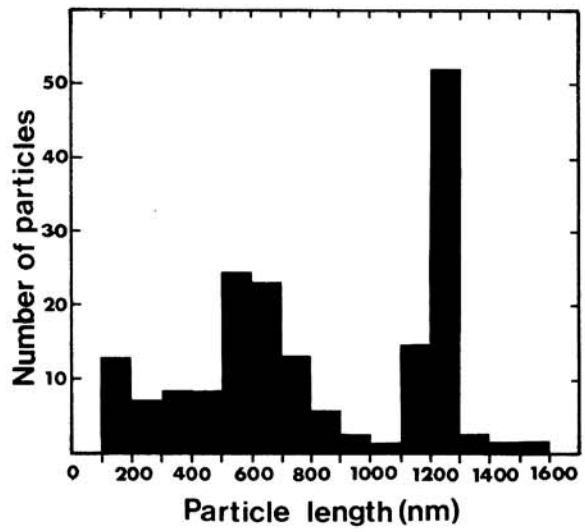


Fig. 11. Length distribution of carnation yellow fleck virus particles from leaf-dip preparations of carnation.

relationship between the two viruses cannot be unequivocally rejected.

Many of the difficulties normally encountered in purifying long flexuous viruses were partially overcome in the present work. Absorption of CYFV onto normal plant constituents was effectively decreased by macerating leaves in a large volume of buffer per gram fresh weight of leaf. The problem of virus aggregation was largely prevented by resuspending virus pellets (with gentle stirring) overnight in a large volume of buffer after the first high-speed centrifugation. Leaving the virus pellet overnight in a large volume of buffer without stirring, as with henbane mosaic virus (6), did not prevent aggregation; and gentle stirring during resuspension was necessary to prevent virus aggregation.

The main problem encountered in the purification of CYFV was that of virus fragmentation. It is possible that gentle stirring increased virus fragmentation; but the obvious tendency of the virus to fragment during extraction, as seen in leaf dip preparations, without applying great mechanical stress, suggested that a certain degree of fragmentation was unavoidable. In situ virus particles appear to be intact (Smookler and Cohen, *unpublished*). Mechanical grinding methods using either a Waring Blendor or Virtis homogenizer resulted in only a few particles of normal length. Thus, CYFV is similar to other filamentous viruses such as BYV (13) and citrus tristeza virus (1) in being sensitive to fragmentation.

At the present time, insufficient data are available to establish firmly the relationship of CYFV to other filamentous viruses. However, further studies on the nature of CYFV are now in progress.

LITERATURE CITED

1. BAR-JOSEPH, M., G. LOEBENSTEIN, and J. COHEN. 1970. Partial purification of viruslike particles associated with the citrus tristeza disease. Phytopathology 60:75-78.
2. BENNETT, C. W. 1960. Sugar beet yellows disease in the United States. U.S. Dep. Agric., Tech. Bull. 1218. 63 p.
3. BRAKKE, M. K., and N. VAN PELT. 1969. Influence of bentonite, magnesium, and polyamines on degradation and aggregation of tobacco mosaic virus. Virology 39:516-533.
4. DUFFUS, J. E. 1972. Beet yellow stunt, a potentially destructive virus disease of sugar beet and lettuce. Phytopathology 62:161-165.
5. ESAU, K. 1968. Viruses in plant hosts: form, distribution, and pathologic effects. Univ. Wisconsin Press, Madison. 225 p.
6. GOVIER, D. A., and R. T. PLUMB. 1972. Henbane mosaic virus. *in*: Descriptions of Plant Viruses. No. 95. Commonw. Mycol. Inst., Assn. Appl. Biol. Kew, England.
7. HAKKAART, F. A., H. A. VAN HOOF, and D. Z. MAAT. 1972. A case of Arabis mosaic virus in carnation. Neth. J. Plant Pathol. 78:15-18.
8. HOEFERT, L. L., K. ESAU, and J. E. DUFFUS. 1970. Electron microscopy of Beta leaves infected with beet yellow stunt virus. Virology 42:814-824.
9. HOLLINGS, M., O. M. STONE, and D. R. SMITH. 1972. Productivity of virus-tested carnation clones and the rate of re-infection with virus. J. Hortic. Sci. 47:141-149.
10. INOUYE, T., and K. MITSUHATA. 1973. Carnation necrotic fleck virus. Ber. Ohara Inst., Okayama Univ. 15:195-205.
11. RUSSELL, G. E. 1970. Beet yellows virus. *in*: Descriptions of Plant Viruses, No. 13. Commonw. Mycol. Inst., Assn. Appl. Biol., Kew, England.
12. SMOOKLER, M., and G. LOEBENSTEIN. 1973. Virus Diseases in Carnations. Hassadeh 54:149-153. (in Hebrew).
13. TILL, B. B., and R. J. SHEPHERD. 1967. Influence of different methods of homogenization and preparation of material for electron microscopy on the morphology and infectivity of the beet yellows virus. Phytopathology 57:102. (Abstr.).