Purification and Properties of Carnation Mottle Virus and its Ribonucleic Acid

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ABSTRACT

Carnation mottle virus (CarMV) was purified from *Chenopodium quinoa* tissue using activated charcoal, Mg-treated bentonite, and differential centrifugation. Yields of virus ranged from 250 to 350 mg/kg of tissue. The resuspended high-speed pellets produced two ultraviolet light-absorbing bands upon centrifugation on sucrose gradient columns; only the more rapidly sedimenting component was infectious. The virus contained 1.74% phosphorus and 18% nucleic acid. The RNA consisted of 27.5% adenylic acid, 24.1% uridylic acid, 26.1% guanylic acid, and 22.3% cytidylic acid residues. The molecular weight of the RNA and intact virus particles was 1.37×10^6 and 7.5×10^6, respectively. The RNA was infectious in concentrations as low as 4.68×10^{-5} μg/ml, or about 75% of the infectivity of intact virus. Antiserum produced by immunizing a rabbit reached a titer of 1:2,048 without detectable healthy plant protein antibodies. CarMV did not react with antiserum to any of several ringspot viruses tested, including beet, carnation, lettuce, *Prunus* necrotic, raspberry, strawberry latent, tobacco, or tomato; nor with antisera to tobacco or cucumber necrosis, tomato black ring, *Arabis* mosaic, elm mosaic, sowbane mosaic, cherry leafroll, or southern bean mosaic viruses.

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Additional key words: serology, molecular weight, base ratio, phosphorus determination, *Dianthus caryophyllus.*

Carnation mottle virus (CarMV) has been reported to occur in Europe (5, 11, 20, 24), Canada (13, 25), and the United States (1). It occurs in a high percentage of plants of many commercial cultivars (1, 11, 20). Hollings & Stone (11), Kemp (13), and Kassanis (12) reported on some of the properties of CarMV including dilution end point, survival in vitro, and thermal inactivation point. They have also reported experimental host ranges for CarMV. Tremaine published the amino acid composition (23), base ratio analysis of CarMV, a phosphorus-content of 1.89%, and a molecular weight of 7.7×10^6 (22). This report gives a simple purification procedure, information on serological diagnosis of the virus in plants, and describes some properties of CarMV and its ribonucleic acid (RNA).

MATERIALS AND METHODS.—Carnation mottle virus (CarMV) was isolated from carnations (*Dianthus caryophyllus* L.) obtained from a local nursery. Virus antisera for identifying and determining relationships of CarMV to other viruses were provided by Tremaine and Dias (Canada), Hollings and Kassanis (England), and Kegler, Bercks, and Wetter (Germany). Pancreatic ribonuclease was a crystalline preparation from Worthington Biochemical Corporation, Freehold, N.J. The activated charcoal was a powdered form (18351) from the Merck Co. Anion exchange resin for base ratio analysis was Bio-Rad AG-1-X8, the 200 to 400 mesh formate form from Cal Biochem, Los Angeles, Calif.

Preparative and analytical ultracentrifugation was performed in an IEC Model B-35, and Spinco Model E equipped with schlieren optics and a high intensity ultraviolet light source, monochromator, and photoelectric scanning system, respectively. Sucrose gradients were fractionated and analyzed using an ISCO density-gradient fractionator and optical unit at 254 nm wavelength. Ultraviolet spectra and absorbency measurements were performed with a Cary Model 14 automatic recording or with a Beckman Model DU spectrophotometer.

Purification.—Leaves and stems of infected *Chenopodium quinoa* Willd. 8 to 18 inches tall were harvested 6 to 8 days after inoculation. The tissue was blended in 2 to 3 times its weight of 0.025 M potassium phosphate buffer pH 7.0 and squeezed through cheesecloth, and the juice centrifuged at 3,500 *g* for 10 min. The supernatant was treated with powdered activated charcoal at 5 g/100 ml juice, which removed much of the green material upon low-speed centrifugation. The light-green supernatant was then treated with 1 to 2 ml of Mg-treated bentonite/100 ml juice, prepared by the method of Dunn & Hitchborn (4), thoroughly shaken, incubated 10 min, and centrifuged again at 3,500 *g* for 10 min. If the resulting supernatant was not water clear, small additional amounts of the bentonite were used, taking care not to add more than needed to yield a clear supernatant after recentrifugation. The clarified sap was then centrifuged at 105,000 *g* for 1.3 hr, and the pellets were resuspended in the extraction buffer.

We layered partially purified virus onto gradients made the day before by successively layering 7.5, 7.5, 7.5, and 4.5 ml of 40, 30, 20, and 10% solutions of sucrose in 0.02 M phosphate buffer pH 7.2, respectively. Gradients were centrifuged in a swinging bucket rotor for 1.5 hr at 98,000 *g*. Upon monitoring

the gradients, we recorded two peaks which were collected separately and reconcentrated by another high-speed centrifugation. Virus pellets were resuspended in 0.025 M potassium phosphate buffer or distilled water, depending on intended use. The resuspended pellets were routinely checked in the spectrophotometer and electron microscope to determine concentration and homogeneity of the final preparation.

Immunization.—We produced antiserum to CarMV by immunizing a rabbit once a week with 2 to 4 mg of virus in 1 ml of buffer emulsified with 1 ml of Freund's incomplete adjuvant. The rabbit received five intramuscular injections. Bleedings began after the third injection. We performed serological tests by the gel double-diffusion method in plates with wells 5 mm apart in 0.75% Ionagar No. 2 in saline.

Extinction coefficients.—The extinction coefficient of CarMV was calculated, using dry weight and spectrophotometry data from six experiments. This was done by centrifugation of 40 to 60 mg of pure virus in 0.025 M K_2HPO_4 at 7,000 *g* immediately before the spectrophotometry, for removal of any aggregated virus. A small amount of the resulting supernatant was read at several dilutions at 260 nm without further correction for light scattering. The balance, usually 30 to 40 mg of virus in 5 to 6 ml, was evaporated to dryness and then dried to constant weight and corrected for dry weight of the buffer.

Phosphorus content.—Purified virus preparations, on which phosphorus-determinations were made, were dialyzed 24 hr against double-distilled water. Aggregated virus was removed by low-speed centrifugation, and the optical density of the supernatant was determined in duplicate at two dilutions. The phosphorus content of 12 such preparations over a period of 12 months was determined by the method of Knight & Woody (16).

RNA extraction.—RNA was extracted from the virus using a modification of the phenol method (7). Five to 10 mg of the virus was mixed with a final concentration of 0.1 M sodium phosphate buffer, 1.5% sodium dodecylsulfate (SDS), and 0.02 M ethylenediaminetetraacetic acid. RNA was precipitated with 2 vol of absolute alcohol and 0.1 ml of 3.3 M pH 4.6 sodium acetate, and concentrated by low-speed centrifugation for removal of traces of phenol. The white pellets were resuspended in 0.02 M sodium phosphate buffer and read for yield and freedom of protein and phenol on the spectrophotometer.

Base ratio.—About 1 mg of RNA was digested in 0.3 N KOH for 18 hr at 37 C. The nucleotide mixture was then layered onto a 12 mm-deep column of anion exchange resin (Bio-Rad AG-1-X8) 200 to 400 mesh, formate form, and separated by stepwise elution with formic acid and ammonium formate (17).

RESULTS.—*Host range.*—We mechanically inoculated some 80 species of plants representing 27 families. Our host range results are essentially the same as those reported by others (5, 10, 11, 13). Plants which did not respond to inoculation with symptoms were indexed back on *C. quinoa*. Prior to our triturating the previously inoculated leaves of the test species, they were rinsed in 5% Clorox (5.25% sodium hypochlorite) solution and tap water to remove what apparently was active virus on the leaf surface. Without a prerinsing of the leaves, *C. quinoa* responded with local lesions to inoculation with sap from nearly all previously inoculated species. Species which became infected, including those without symptoms, incited too many lesions to count in *C. quinoa* even when the source plant sap was diluted 1:100 or 1:1,000. The pH of inoculum buffer between 4.5 and 9.5 had no noticeable effect on infectivity. The virus was not infectious at pH values below or above this range.

In addition to the previously reported hosts of CarMV, our isolate infected *Nicotiana rustica* L. and *N. glutinosa* L. without inciting symptoms. It incited mild chlorotic lesions in *Vernonia anthelmintica* (L.) Willd. and *Amaranthus caudatus* L. and mild chlorosis and leaf distortion in *Dianthus barbatus* L. (Fig. 1). CarMV incited red ringspots in *Beta vulgaris* L. after 3 weeks (Fig. 2), and conspicuous leaf distortion and stunting of *A. leucocarpus* S. Wats.

CarMV incited chlorotic lesions in *C. quinoa*, usually so many in most routine inoculations that leaves turned bright yellow after 6 days (Fig. 3, 4). During the next 2 to 3 weeks, the inoculated leaves fell and the virus incited a systemic mosaic and leaf distortion. A small percentage of infected plants produced a few seed, but most plants died or failed to flower.

Dilution end point, thermal inactivation point, and aging in vitro.—Crude *C. quinoa* sap with CarMV was usually infectious when diluted in 0.025 M phosphate buffer to 10^{-7} but not at 10^{-8}. Purified virus was infectious at concentrations as low as 1.87×10^{-4} but not at 9.4×10^{-5} μg/ml. The thermal inactivation point of CarMV was about 90 C when a 1:10 dilution of *C. quinoa* sap or purified virus diluted with phosphate buffer was heated in a water bath. The number of lesions ranged from 2 to 10% of the number incited by unheated control sap. Most of the infectivity remained after heating sap for 30 min at 75 C.

The virus retained much of its infectivity after 3 months at room temperature. Unless considerable dilutions were made, the numbers of lesions were too numerous to count in most experiments.

Purification.—Rate-zonal centrifugation produced a single heavy opalescent band 3 cm below the meniscus. However, when the gradients were fractionated and monitored with ultraviolet light at 254 nm, two distinct peaks were recorded. Reconcentrated fractions of the slower sedimenting component contained primarily empty virus particles when viewed in the electron microscope, and showed low infectivity in bioassays. This component was not studied further.

Purity of the more rapidly sedimenting component, after reconcentrating, was ascertained by analytical ultracentrifugation (Fig. 5). Sedimentation coefficient was $S_{20,w} = 126$ S at a concentration of

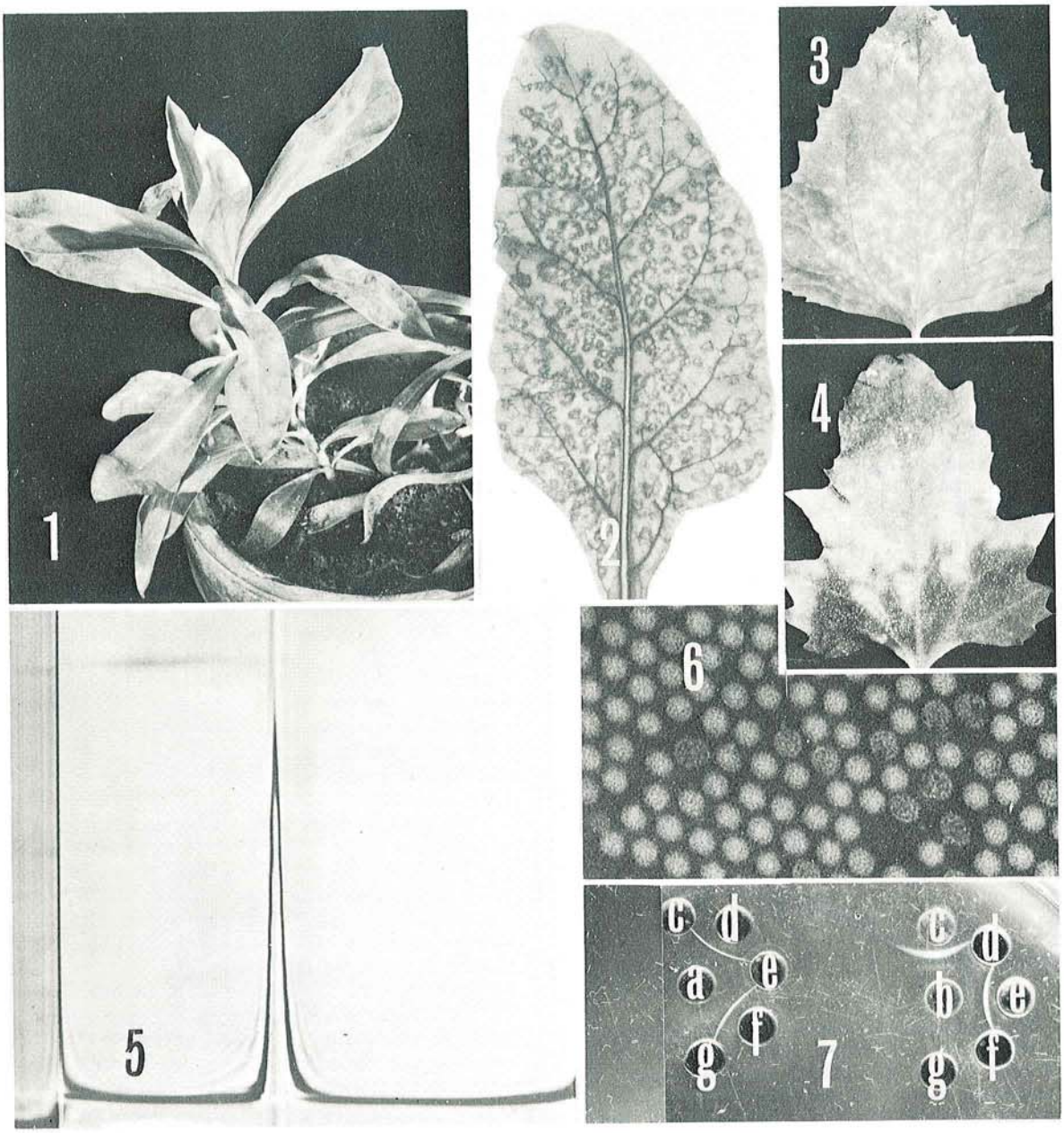

Fig. 1-7. 1, 2, 3, 4) Symptoms incited by Carnation mottle virus (CarMV) in **1)** *Dianthus barbatus* L.; **2)** *Beta vulgaris* L.; and **3, 4)** *Chenopodium quinoa.* The bright local chlorosis in Fig. 4 is the result of large numbers of small lesions. **5)** Schlieren optics pattern of CarMV. Sedimentation left to right. Concentration is 0.3% in 0.025 M potassium phosphate pH 7 buffer. Temperature of run, 20 C. Photograph taken 8 min after centrifuge attained 29,500 rpm. **6)** CarMV particles in purified preparation (× 194,800). **7)** Agar test showing lack of serological relationship between CarMV and sowbane mosaic virus. Wells contain antiserum to CarMV (a) and sowbane mosaic virus (b). Wells contain *C. quinoa* sap with sowbane mosaic virus (c, e), CarMV (d, f), and no virus (g).

3 mg virus/ml. Measurements taken on 100 particles gave an average diameter of 29 nm (Fig. 6). The purified virus produced an absorption curve with a max/min (260/242) of 1.23 to 1.27. With spectrophotometric and dry weight data, the specific extinction coefficient of CarMV was calculated to be $E^{1\%}_{260} = 49.05$ (1 cm light path). Phosphorus was determined to be 1.74% using the average of 12 separate values.

When held for more than 4 to 6 days in low molarity phosphate buffer at 4 C, purified virus aggregated and settled out of solution. Hence, all

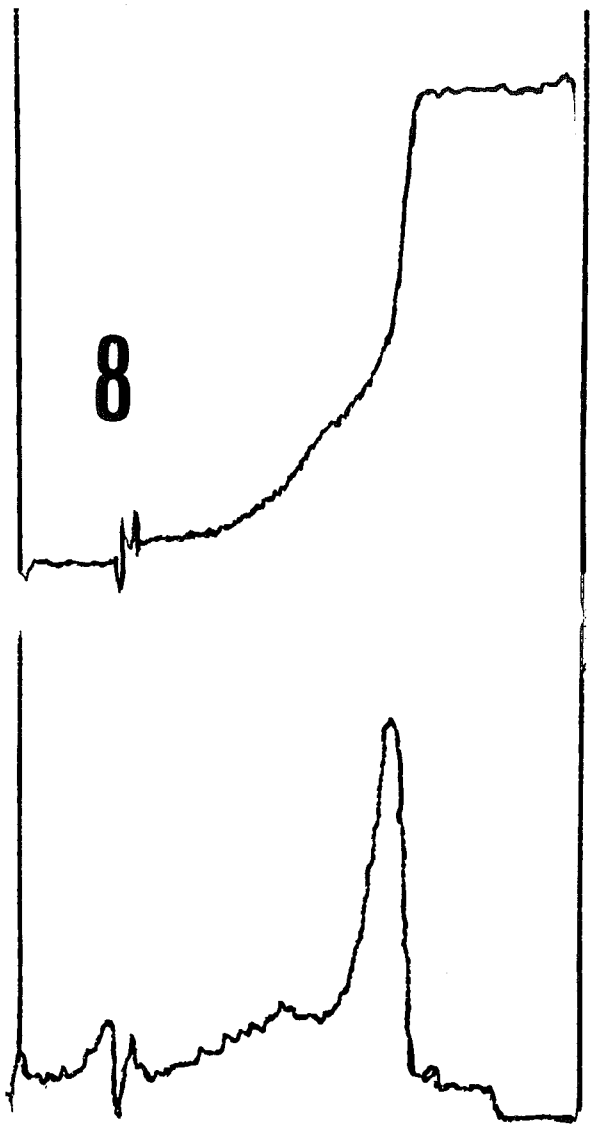

Fig. 8. Ultraviolet optics pattern of Carnation mottle virus (CarMV) RNA. Sedimentation left to right. Concentration is 40 μg/ml in 0.02 M potassium phosphate buffer pH 7. Temperature of run, 5 C. Photoelectric scan was made 48 min after attaining 42,040 rpm. Lower half of figure is a derivative pattern of the concentration versus radial distance tracing in upper half.

experiments were performed on freshly purified preparations. Yields of virus ranged from 230 to 350 mg/kg of infected tissue.

Serology.—Antiserum collected 3 weeks after the first injection of virus had an antibody dilution titer of 1:512 and rose to 1:2,048 by the 5th week. The titer had not dropped appreciably in the final bleeding made 8 weeks after the last injection. There were no detectable healthy plant protein antibodies in the serum. Purified CarMV did not react with antisera to tomato, tobacco, carnation, raspberry, lettuce, beet, strawberry latent, or *Prunus* necrotic ringspot viruses; nor with cucumber or tobacco necrosis, tomato black ring, *Arabis* mosaic, southern bean mosaic, sowbane mosaic, cherry leafroll, or elm mosaic virus antisera. Our virus appeared identical with Tremaine's and with Holling's CarMV in several agar double-diffusion experiments in that no spurs were observed.

CarMV antiserum and test plants are used routinely to detect virus in carnation introductions to the USA. A high percentage of imported carnations are infected with CarMV, many without visible symptoms of infection. In these tests, two or three leaves were triturated with 0.5 ml of saline, and a drop of the juice was placed in an agar well 5 mm from the antiserum well. Plates were read after 24 hr at 24 C. This procedure detected the virus in 103 of 115 CarMV-infected plants based on mechanical indexing on *C. quinoa*, which required 6 to 8 days for final readings and a second serological test. Presumably, the virus titer was too low in the remaining 12 carnation plants to be detected serologically (14).

RNA.—CarMV RNA produced a typical ultraviolet light absorption curve with a 260:230 ratio of 2.1. Based on an RNA content of 18%, yields of RNA ranged from 55 to 65% of the total nucleic acid in the virus. When SDS was not used, the yields ranged considerably lower. Figure 8 shows the sedimentation diagram of CarMV-RNA taken with ultraviolet light optics. Fifty-six per cent of the ultraviolet-absorbing material sedimented as a homogeneous component with a sedimentation coefficient ($S_{20,w}$) of 23.1 S. The balance trailed with a continuous distribution of sedimentation rates.

The average base ratio determined from four experiments was A = 27.5%; U = 24.1%; G = 26.1%; and C = 22.3%. From the amounts of phosphorus in each of the individual base residues, the RNA was calculated to contain 9.61% phosphorus. Since intact virus contained 1.74% phosphorus, the RNA content of CarMV was calculated to be 18%.

RNA infectivity.—We compared the infectivity of the extracted RNA with that of an equal amount of RNA in intact virus particles. Extracted RNA was infectious in concentrations as low as 4.68×10^{-5} μg/ml. Since RNA is 18% of the weight of intact virus which was infectious at 1.87×10^{-4} μg/ml, the extracted RNA was about 75% as infectious as RNA inside the virus. Extracted viral RNA was sensitive to bovine pancreatic ribonuclease (0.01 μg/ml). When held at 4 C in 0.025 M, phosphate buffer infectivity was reduced by 90% within 10 min and by 100% within 60 to 70 min. The infectivity of intact virus was not noticeably reduced when treated similarly.

Molecular weight of CarMV.—Molecular weight of CarMV has been determined as part of a separate investigation in which the molecular weight of several viral RNA's, determined by polyacrylamide electrophoresis and by velocity ultracentrifugation of their formolized derivatives, were compared. The value established for CarMV-RNA was 1.37×10^6 Daltons. Assuming an RNA content of 18% of the total particle weight, the molecular weight of CarMV

was calculated to be 7.6 million Daltons.

DISCUSSION.—Although CarMV alone in carnation plants does not noticeably affect some cultivars of carnation, and incites only mild symptoms in many cultivars (1, 11, 13), Paludan reported that under certain conditions, yields or weight of flowers may be reduced as much as 21% (21). In view of the wide distribution of CarMV among carnation cultivars throughout the world, this seemingly unimportant disease may be responsible for substantial losses. Fortunately, carnation plants can be simply and accurately diagnosed for infection by CarMV using serology. Our serological indexing results support that of others (2, 14, 15).

The properties of CarMV, including particle size and shape, RNA content and molecular weight, sedimentation coefficient, methods of transmission, and thermal inactivation point, are similar to those of several viruses which Gibbs (6) has grouped under the classification of "viruses with no known vectors, whose particles are known" and which contain about 20% RNA. Members of the group include southern bean mosaic, brome mosaic, broad bean mottle, sowbane mosaic, tomato bushy stunt, turnip crinkle, and tobacco necrosis viruses. Haselkorn (9) placed CarMV in the turnip crinkle virus group along with sowbane mosaic and tomato bushy stunt virus because of similarities in molecular weight, base composition, and a 17% RNA content.

In the classification by Harrison et al. (8), CarMV would fall into Group 10, Bromovirus, a group which contains some of the above viruses. Members of this group are not serologically related (6).

Likewise, our CarMV did not react with antisera to members of this group including broad bean mottle, brome mosaic, pelargonium leaf curl, tomato bushy stunt, or turnip crinkle viruses. Although CarMV has been reported to be serologically related to sowbane mosaic (18), we found no evidence of serological relationship (Fig. 7) in spite of its similar physical properties (3) and behavior in the greenhouse.

To purify CarMV, we occasionally clarified infectious plant sap by using Mg-treated bentonite alone (4) along with low-speed centrifugation. The primary advantages in omitting use of charcoal were to simplify the procedure and avoid the special problem of working with charcoal. The primary disadvantage in omitting charcoal was that 5 to 7 ml of bentonite, rather than 1 to 2 ml/100 ml of sap, were required to yield waterclear supernatants, and bentonite removed some of the virus as well as green plant constituents upon centrifugation. These losses during clarification were tolerable because concentration of CarMV in *C. quinoa* tissue is exceptionally high. Our purification procedure, which is different and was developed independently from the one reported by Tremaine (22), resulted in yields of virus in the same range of magnitude. Likewise, the procedures we used to study CarMV are different from Tremaine's and again, our results such as base ratio, phosphorus-content, and molecular weight of the virus agree with Tremaine's values.

The component which sedimented about one-half as rapidly as CarMV in sucrose gradients was always observed upon fractionating gradients regardless of whether virus was purified from *C. quinoa*, carnation, or gomphrena plants. Tremaine did not mention a slow-sedimenting component of CarMV (22), but he did detect such a component associated with preparations of cucumber necrosis and turnip crinkle viruses. Yields of our slower-sedimenting component, based on ultraviolet light absorption, were one-tenth to one-twentieth that of the virus. This component was serologically related to the CarMV, as was the case with Tremaine's components.

Extracted CarMV-RNA was far more infectious than that of extracted RNA of other viruses listed by Matthews (19) as compared with intact virus. Only cucumber mosaic virus Y strain RNA was as infectious as CarMV-RNA. We did not ascertain the amount of residual protein in our RNA preparations; however, all infectivity was destroyed by ribonuclease within 1 hr. If intact virus were present, presumably we would have observed infection, since ribonuclease did not appreciably reduce infectivity of intact virus after 1 hr.

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