Aggregation Properties of Carnation Ringspot Virus

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ABSTRACT

Three strains of carnation ringspot virus (CRSV) were obtained from single local lesions on plants infected with a culture of the virus. When virus particles of one strain (CRSV-A) were concentrated by dissolving high-speed centrifugation pellets in a small volume of 0.1 M sodium acetate buffer, pH 5, clusters of 12 virus particles arranged in an icosahedral symmetry were formed. Aggregation into linked clusters containing 23, 34, 45, or 56 virus particles also occurred. The sedimentation coefficients obtained for the virus monomer and the four classes of polymers were 135 S, 640 S, 932 S, 1175 S, and 1266 S, respectively; these values showed good agreement with theoretical sedimentation calculations. These aggregates were stable in the presence of 0.5 M 2-mercaptoethanol, 0.1 M urea, 0.1 M $MgCl_2$, or 0.1% sodium dodecyl sulfate, and during storage for 6 months at 4 C. However a portion of the aggregates dissociated into monomer at pH 7.

A second strain (CRSV-R) formed another kind of aggregate, a two-dimensional net, in which reversible formation and dissociation occurred at 25 C and 4 C, respectively. A third strain (CRSV-N) aggregated in a similar temperature-dependent manner, but required higher virus concentrations or higher temperatures.

The three strains were serologically related, but CRSV-A formed reactions of partial identity in gel diffusion tests with CRSV-R and CRSV-N.

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Additional key words: electron microscopy, sedimentation of virus polymers.

Carnation ringspot virus (CRSV), R/1:1.4/20:S/S:S/Ne, is a well-characterized virus with no previously reported strain differences (3). We observed white and red local lesions on inoculated leaves of *Chenopodium amaranticolor* Coste & Reyn. In attempts to separate these local lesion types we found the different strains described in this report.

MATERIALS AND METHODS.—*Virus strains.*—Three strains of CRSV were obtained from single lesions on *C. amaranticolor* inoculated with a culture in cowpea plants [*Vigna unguiculata* (L.) Walps 'Blackeye'] that originated from a single lesion isolate made by Kalmakoff and Tremaine (4). Each strain was passed through five serial single lesion transfers in *C. amaranticolor* and then propagated in cowpea plants. The strains are designated: CRSV-N, a nonaggregating strain; CRSV-A, a strain forming aggregates of 12 virus particles; and CRSV-R, a strain showing reversible, temperature-dependent aggregation.

Virus purification.—Virus preparations were made from infected cowpea plants collected 14 to 21 days after inoculation. The leaves were crushed in a meat grinder then sprinkled with solid sodium diethyl dithiocarbamate (4.0 g per kilogram of leaves). The sap was expressed through cheesecloth and adjusted to pH 5 with the slow addition of glacial acetic acid during stirring. After at least 3 hours the sap was centrifuged at 10,000 rpm in a Sorvall refrigerated centrifuge for 15 minutes and after decanting, 100 g of polyethylene glycol (PEG 6000) was added per liter of clarified sap to precipitate the virus. Three hours later the precipitate was pelleted by low-speed centrifugation, then dissolved in 0.1 M sodium acetate buffer, pH 5.0. The solution was clarified by low-speed centrifugation and the virus pelleted in a Spinco No. 30 rotor for 2.5 hours at 28,000 rpm.

Some preparations were placed on a 50 to 350 mg per ml sucrose density gradient, in 0.1 M sodium acetate buffer, pH 5.0, centrifuged in a SW 41 rotor for 35 minutes at 18,000 rpm, and scanned in an ISCO model UA-4 ultraviolet monitor with a model 612 recorder, a model D density-gradient fractionator, and a model 184 tube-piercing device.

Determination of virus concentration.—The virus concentration was estimated spectrophotometrically using the extinction coefficient of 6.5 cm^2/mg (4). The use of this value overestimated the concentration of aggregates because of light scattering by these molecules.

Sedimentation coefficients.—These were determined in a Spinco Model E analytical ultracentrifuge at 20 C using schlieren optics and were calculated by the graphical method of Markham (8). The rotor speed was 10,589 rpm for 20 minutes to sediment the aggregates, then 21,740 rpm for sedimentation of the monomer. The concentration of virus aggregates was determined from the area under the schlieren peak (9).

Serological tests.—Antisera to CRSV-N and CRSV-R were made by injecting rabbits intramuscularly with 2 mg of virus in 1 ml of buffer emulsified with an equal volume of Freund's complete adjuvant. These injections were repeated after 2 weeks and bleedings were made 4 weeks

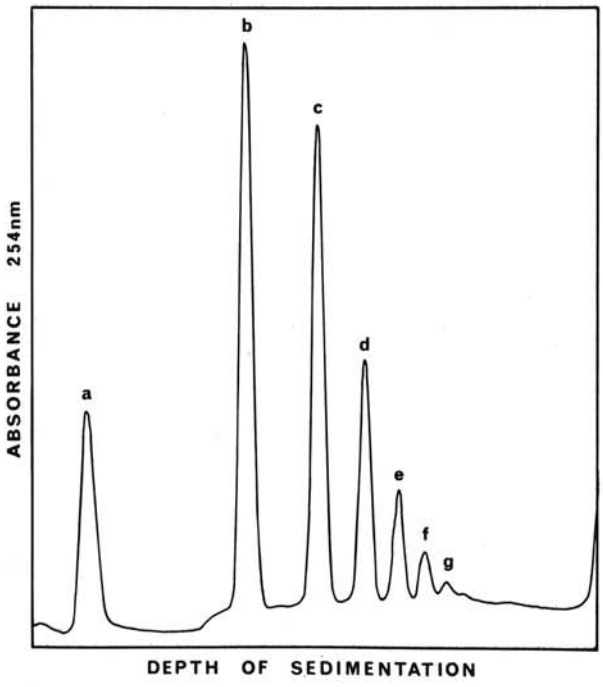

Fig. 1. Tracing of a 254 nm absorbance scan of an aggregating strain of carnation ringspot virus (CRSV-A) after centrifugation for 35 minutes at 18,000 rpm in a SW 41 rotor at 4 C on a density-gradient column of 50 to 350 mg of sucrose per ml in 0.1 M sodium acetate buffer, pH 5.0. Sedimentation is from left to right.

later. Gel diffusion tests were done in 0.8% Ionagar #2 in 0.15 M saline containing 0.1% sodium azide on formvar-coated glass slides. Antigens used in these tests contained 200 to 300 μg of virus per ml.

Electron microscopy.—The virus preparations which were diluted with at least an equal volume of distilled water, were then mixed with an equal volume of 2% uranyl acetate, let stand for several minutes, placed on grids, drained to leave a thick layer, and dried quickly under a stream of compressed air. The grids were immediately examined in a Philips EM 200 or EM 300 electron microscope. Aggregates were found in areas showing a heavy accumulation of stain. Any deviation from this procedure caused the aggregates to dissociate in the electron microscope.

Infected tissues 2-3 mm^2 were examined for the presence of aggregates in vivo. The tissue was fixed for 60 minutes in 5% glutaraldehyde in phosphate buffer, pH 7.2, at room temperature, rinsed twice in phosphate buffer, and placed in Palade's OsO_4 solution. After dehydration in graded dilutions of ethanol, the tissue was treated with propylene oxide and embedded in epoxy resin. Ultrathin sections were cut with a Reichert OmU2 ultramicrotome and stained with 2% uranyl acetate and lead citrate. Pellets containing aggregates of CRSV-A, were treated similarly.

RESULTS.—*Properties of a CRSV strain forming aggregates of 12 virus particles (CRSV-A).*—CRSV-A produced symptoms on cowpea plants indistinguishable from those of CRSV-N, the nonaggregating strain. The precipitate of CRSV-A formed by PEG 6000 was readily soluble; but when the high-speed pellet in a No. 30 rotor tube was dissolved in 1 ml of sodium acetate, pH 5.0, the solution was unusually milky. Examination of these preparations in the analytical ultracentrifuge showed five schlieren peaks sedimenting at 135, 640, 932, 1,175, and 1,266 S.

The aggregates of CRSV-A were concentrated by pelleting in a No. 40 rotor at 10,000 rpm for 15 minutes. When this concentrate was placed on a sucrose density gradient and centrifuged at 18,000 rpm for 35 minutes, a faintly opalescent band appeared near the top of the gradient, and six very opalescent bands lower in the gradient (Fig. 2). The gradient was scanned at 254 nm (Fig. 1) and fractionated into tubes containing components from the top a, b, c, d, e, f, and g. The fractions were dialyzed in sodium acetate buffer, then negatively stained, and particles from each were examined in a Philips EM 300 electron microscope. Figure 3 from zone b shows clusters of 12 virus particles with icosahedral symmetry, each particle is arranged on an apex of the icosahedron.

A model of the cluster was made from 12 styrofoam spheres and three views are shown from the two-, three-, and five-fold rotational axes of symmetry in Figs. 4, 5, and 6. Figure 7 shows a cluster of 12 virus particles from the five-fold axis. The model viewed from this axis (Fig. 6) shows three layers of particles: one on top and two interpenetrating layers of five alternating particles each. The twelfth particle, below the second layer of five, is not visible. Figure 8 shows a cluster, and Fig. 5 the model viewed from the three-fold axis. There are four interpenetrating layers of three particles each, but only the upper three layers are seen. Clusters of 12 particles showing the three-, five-, and two-fold axes were observed in micrographs frequently, infrequently, and never, respectively.

An aggregate of six virus particles viewed from the five-fold axis (Fig. 9) was the only particle of this type found in an unfractionated preparation. The ultraviolet scan of a density-gradient column (Fig. 1) does not show a peak between the virus monomer (peak a) and clusters of 12 virus particles (peak b). The aggregate of six virus particles may have formed from the the dissociation of a cluster of 12.

A micrograph of two linked clusters (Fig. 10) obtained from fractions containing peak c in the density gradient (Fig. 1) also shows many single virus particles. Tests on density-gradient columns showed that particles were not dissociated from aggregates during the removal of sucrose by dialysis or by the addition of uranyl acetate. Dissociation probably occurred on drying the grid before putting it in the electron microscope. In the aggregate illustrated in Fig. 10, one cluster showing the three-fold axis is attached to one cluster showing the two-fold axis. The distance between virus particles within each cluster is less than the distance between adjoining particles of the two clusters. When a model of this aggregate was made by removing one styrofoam sphere from each of two clusters of 12 and the two clusters were placed together as closely as possible, all views of the model showed identical axes in both linked clusters. However, when a model was made by attaching a cluster of 11 styrofoam spheres to a cluster of 12, it was difficult to bring the clusters as closely

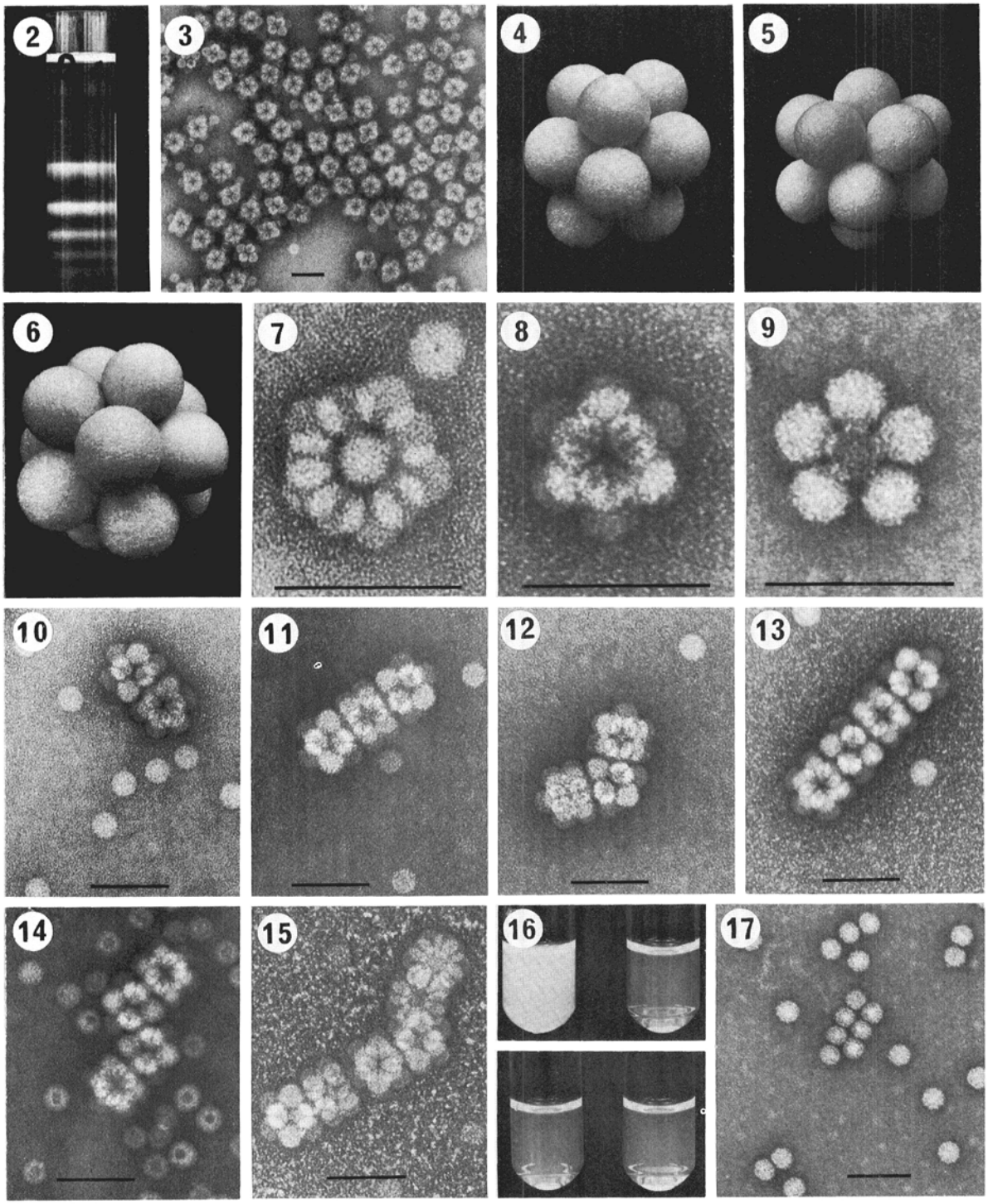

Fig. 2-17. 2) Density-gradient column of an aggregating strain of carnation ringspot virus (CRSV-A) prior to ultraviolet scanning. 3) Electron micrograph of CRSV-A clusters from peak b of Fig. 1. **4-6)** Styrofoam sphere models of clusters shown from two-, three-, and five-fold axes, respectively. (7-14) Electron micrographs of CRSV-A aggregates. **7-8)** Clusters of 12 virus particles from the five-fold and three-fold axes, respectively. **9)** Half a cluster (six virus particles). **10)** a dimer cluster of 23 virus particles. **11)** A linear trimer cluster. **12)** An angular trimer cluster. **13)** A linear tetramer cluster. **14)** An angular tetramer cluster. **15)** a hexamer cluster. **16 top)** Two aliquots of a reversible aggregation strain of carnation ringspot virus (CRSV-R) preparation: the left at 30 C; the right at 4 C. **16 bottom)** The same aliquots after cooling to 4 C. **17)** An electron micrograph of CRSV-R. Bars in all electron micrographs indicate 100 nm.

together; this model showed the three-fold and the two-fold axes as in the CRSV-A aggregate in Fig. 10. This result may indicate that the aggregates form by the polymerization of 11 virus particles onto a cluster of 12.

Figures 11-15 show aggregates of three, four, or six clusters of 12 particles from bands d, e, and g of the density gradient, respectively. These large aggregates had many different shapes, resembling the letters L, U, V, Z, and Y.

The effect of various chemicals on the aggregates and monomer of CRSV-A was assessed by adding an equal volume of a solution of the chemical in distilled water to the virus preparation in 0.1 M sodium acetate buffer, pH 5.0. These solutions were placed on a sucrose density-gradient column, centrifuged, and scanned at 254 nm. In these tests, 0.5 M 2-mercaptoethanol, 0.1 M urea, 0.1 M $MgCl_2$, and 0.1% sodium dodecyl sulphate did not alter the absorbance pattern of untreated virus (Fig. 1.). The effect of pH was also assessed by density-gradient centrifugation and scanning. Aliquots of a preparation were adjusted to pH values of 5.5, 6.0, 6.6, 7.0, 7.5, and 8.0 by the addition of 1 M tris. The amount of monomer increased with pH up to pH 7.0, indicating a dissociation of aggregates into the monomer. At pH 8.0, all the aggregates and some of the monomer dissociated into protein and RNA.

During these studies, aliquots from a preparation stored at 4 C were used for density-gradient absorbance scans over a period of about 6 months. The absorbance scans did not alter significantly during this period; we concluded that the aggregates were extremely stable.

Sections of CRSV-A preparations and infected tissue.—Examination of embedded cowpea tissue infected with CRSV-A showed inclusion bodies containing virus particles in pseudo-crystalline array. Aggregates resembling those in Fig. 7-15 were never observed. Sections (approximately 60 nm in thickness) of embedded pelleted aggregates of CRSV-A were made and photographed. These showed structures typical of aggregates seen from the two-, three-, or five-fold axes. The areas within any of the rings of 5, 6, or 10 virus particles were no more dense than any free virus particle in the field. Considering the thickness of the sections the presence of a thirteenth particle in the interior of the aggregate should give such areas a density greater than that of a single virus particle. Longitudinal sections of dimer clusters confirmed the presence of a particle shared by two clusters. Moreover the distance between virus particles in each cluster was less than the distance between adjoining particles of the two clusters. This is further evidence of the lack of sufficient space for a thirteenth particle in the interior of a cluster.

Properties of CRSV-R.—CRSV-R produced symptoms on cowpea plants indistinguishable from those of CRSV-N or CRSV-A. Preparations were purified by two cycles of differential centrifugation and placed in the refrigerator. When a preparation of 7 mg/ml was allowed to come to room temperature, it became very turbid, but the turbidity disappeared on recooling to 4 C (Fig. 16). This temperature-dependent change was totally reversible. At 20 C in the analytical ultracentrifuge at 10,589 rpm the aggregate sedimented too quickly to be photographed and only residual CRSV-R monomer was detectable at higher speeds. A micrograph (Fig. 17) of a sample prepared at 30 C for the electron microscope shows many single particles of CRSV-R with a few two-dimensional aggregates. Judging from the turbidity and the sedimentation rate, the aggregates in the preparation must originally have been much larger and were probably dissociated by the dilution and drying necessary for electron microscopy.

Serological relationships.—Carnation ringspot virus is a good immunogen and the dilution end points of CRSV-N and CRSV-R antisera were both greater than 1:1,000 in gel diffusion tests with homologous antigens. Gel diffusion tests with CRSV-N and CRSV-R in adjacent wells with either antisera showed reactions of identity. When aggregates of CRSV-A were used as an antigen in further tests, a band of precipitation occurred very close to the antigen well. However unaggregated CRSV-A antigen formed reactions of partial identity when tested with CRSV-N antigen and antiserum and with CRSV-R antigen and antiserum.

Effect of temperature on CRSV-N and CRSV-A.—The failure to detect a serological difference between CRSV-N and CRSV-R prompted us to test the effect of temperature on CRSV-N. Differences in the turbidity of a CRSV-N preparation containing 7 mg/ml were not visible at 4 or 25 C, but an increase in turbidity was visible at 40 C and the effect was reversible. Preparations containing 30 mg/ml showed temperature reversible turbidity at 25 C. Apparently, CRSV-N aggregation is similar to that of CRSV-R, but requires higher temperatures or greater virus concentrations to do so.

Aliquots of a CRSV-A preparation, held for 10 minutes at 25, 50, 60, 70, 75, or 80 C were placed on density-gradient tubes, centrifuged, and scanned at 254 nm. The absorbance scans of the aliquots treated at 25 and 50 C were similar to that in Fig. 1. In aliquots treated at 60, 70, and 75 C the levels of monomer and aggregates decreased progressively to about one-half the amounts in the 50 C treatment. At 80 C, the levels decreased to less than 5%, and there was no evidence of released RNA. Therefore, heat treatment at 80 C resulted in precipitation of the virus nucleoprotein.

DISCUSSION.—*Significance of the two types of aggregation.*—Kassanis and Woods (6) and Kassanis et al. (5) found a strain of the satellite of tobacco necrosis virus (STNV) and another of kale virus (KV), respectively, which regularly formed aggregates of 12 virus particles. The existence of similar aggregates with CRSV-A expands the range of viruses with this property to three distinct virus groups. KV aggregates sediment at 500 S, 744 S, 920 S, and 1,070 S as monomer, dimer, trimer, and tetramer of 12-particle aggregates containing 12, 23, 34, and 45 virus particles (5), respectively. Kale virus is a multicomponent virus and the aggregates are mixtures of top, middle, and bottom components (11). Satellite tobacco necrosis virus forms aggregates of 6, 12, and 23 virus particles which sediment at 169 S, 231 S, and 332 S, respectively (6). A single six-particle aggregate of CRSV-A (Fig. 9) was seen in an electron micrograph, but detectable amounts of this particle were not found in the analytical ultracentrifuge or in sucrose density gradient absorbance profiles. Aggregates of all three viruses form when the monomer virus is concentrated by high speed

centrifugation. Satellite tobacco necrosis virus and CRSV-A did not dissociate over long periods of storage, but KV aggregates dissociated into monomers during prolonged storage at 4 C. CRSV-A and KV aggregates were stable in 0.1 M urea or 0.5% 2-mercaptoethanol (5, 6).

The reversible temperature-dependent aggregation found with strain CRSV-R is unusual. Kassanis and Woods (6) found a strain of STNV forming large aggregates in a two-dimensional net at 4 C which in contrast to CRSV-R were readily observed in the electron microscope. The aggregation of CRSV-R resembles the reversible, temperature-dependent reaction of tobacco mosaic virus protein which is driven to polymerization by an increase in entropy; probably from the release of structured water molecules on the surface of the tobacco mosaic virus protein monomer (7). The CRSV-R reversible aggregation system provides a unique opportunity to study the thermodynamics of the interaction between virus particles and the possible involvement of hydrophobic bonds. Water is of great importance in the formation and stabilization of hydrophobic bonds and it may be significant that both types of CRSV aggregates are easily dissociated upon drying in preparation for the electron microscope.

Theoretical consideration of sedimentation of aggregates.—Kassanis and Woods (6) and Kassanis et al. (5) cite Markham (8) as stating that the sedimentation coefficient of a dimer of an isometric virus is approximately $\sqrt{2}$ times the sedimentation coefficient of a monomer. In fact, Markham (8) gives the ratio of sedimentation coefficient of the dimer to that of the monomer as 1.4, and of the trimer to the monomer as 1.7. These were derived from empirical measurements and are slightly lower than the values calculated from sedimentation theory. Bradish and Crawford (1) found the sedimentation coefficients of polymers of tomato bushy stunt virus reacted with its antiserum to be 190 S, 234 S, and 275 S. The ratios of the sedimentation coefficients of these polymers to that of the monomer (134 S) are 1.41, 1.74, and 2.05. These values are also slightly lower than ratios of 1.50 for dimer, 1.85 for trimer, and 2.11 for tetramer calculated from sedimentation theory (1).

The ratio of the sedimentation coefficient of a polymer of n particles of a spherical virus, S_n, to that of the monomer, S_1, can be calculated from the Svedberg equation to be:

Equation 1

$$\frac{S_n}{S_1} = \frac{(1 - V_n d)\, nm}{f_n} \cdot \frac{f_1}{(1 - V_1 d)\, m}$$

where d is the density of the medium; V_n and V_1 are the partial specific volumes of the polymer and monomer; nm and m are the masses of the polymer and monomer; and f_n and f_1 are the frictional coefficients of the polymer and monomer.

Assuming $V_n = V_1$, Equation 1 reduces to

Equation 2

$$\frac{S_n}{S_1} = n \frac{f_1}{f_n}$$

If we also assume that the polymer is a sphere with a volume equal to that of n monomers, the ratio of the frictional coefficients, f_1/f_n reduces to $1/\sqrt[3]{n}$ (Stokes's law). Substituting this in Equation 2 gives

Equation 3

$$\frac{S_n}{S_1} = \frac{n}{\sqrt[3]{n}} = n^{2/3}$$

Linear polymers of viruses resemble prolate elipsoids of revolution but values derived from Equation 3 are for spheres with n times the volume of the monomer. Values from Equation 3 for S_2/S_1, S_3/S_1, and S_4/S_1 were divided by frictional ratios for prolate ellipsoids of revolution with axial ratios of 2:1, 3:1, and 4:1, respectively [tabulated by Schachman (10)]. The results (1.52, 1.87, and 2.13) are very close to the theoretical values given by Bradish and Crawford (1).

TABLE 1. Comparison of empirical and theoretical values for sedimentation rates of virus aggregates

Number of particles in aggregate	Sedimentation coefficient of 23-, 34-, and 45-particle aggregates divided by sedimentation coefficient of 12-particle aggregate				
	Virus			Theory	
	CRSV[a]	KV[b]	STNV[c]	Equation 3	Shape-corrected Eqn. 3
23	1.46	1.49	1.44	1.55	1.49
34	1.84	1.84		2.00	1.84
45	1.98	2.14		2.41	2.10

[a]Carnation ringspot virus.
[b]Kale virus.
[c]Satellite of tobacco necrosis virus.

The sedimentation coefficients of 23-, 34-, and 45-particle aggregates divided by the sedimentation coefficient of the 12-particle aggregate are given (Table 1) for CRSV-A, KV, and STNV. Theoretical values for this ratio were calculated from Equation 3. Frictional ratios for prolate ellipsoids of revolution with axial ratios measured from electron micrographs of CRSV-A as 1.86:1, 2.67:1, and 3.47:1 for 23-, 34-, and 45-particle aggregates, respectively, were calculated from the Perrin equation (2). Division of theoretical values by the appropriate frictional ratio gave values in good agreement with the empirical data for all viruses. The agreement is surprising considering the naive assumptions made in the theoretical calculations.

Assuming that the monomer and the 12-particle aggregate are spherical, then the ratio of their diameters d_1 and d_{12} is directly proportional to the ratio of their frictional coefficients. From Equation 2, $S_{12}/S_1 = 12\ d_1/d_{12}$. Since the value of S_{12}/S_1 for CRSV-A is 4.74, the diameter of the 12-particle aggregate should be 2.53 times that of the monomer. Measurement of electron micrographs show that the 12-particle aggregate is indeed two and one-half to three times the size of the monomer depending on the axis of the aggregate measured and on the amount of stain found in the electron micrograph.

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