Temperature-Reversible Aggregation of Two Strains of Carnation Ringspot Virus

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ABSTRACT

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Two strains of carnation ringspot virus, CRSV-R and CRSV-N, aggregate and disaggregate in a temperature-reversible manner at pH 5.0. Aggregated virions sedimenting at 300–1,300 S and 10^5 S were observed in a CRSV-R preparation (9.5 mg/ml) in the analytical ultracentrifuge at 31–40 C, but were absent after cooling to 18 C. Electron microscopy of CRSV-R preparations (0.1 mg/ml) heated to 70 C showed small structured aggregates and large unstructured aggregates. Solutions of each virus strain, at various concentrations, were heated at a constant rate of 0.25 C/min while the absorbance at 340 nm was recorded. The temperature of aggregation was inversely proportional to the logarithm of virus concentration. The maximum absorbance attained by both strains on heating was proportional to the logarithm of virus concentration. Maximum rate of absorbance increase at each concentration was proportional to the logarithm of virus concentration. At high concentrations (4 mg/ml), the N strain required higher temperature for aggregation than the R strain but at lower concentrations (0.2 mg/ml) the R strain required higher temperatures than the N strain. In addition to this novel difference between the two strains, the serological properties and amino acid compositions of the strains were similar, but not identical.

Tremaine et al (10) isolated three strains of carnation ringspot virus (CRSV) with unusual aggregation properties. One strain, CRSV-A, formed aggregates of 12 virus particles and linked aggregates. These aggregates were very stable at pH 5.0, but there was some disaggregation into monomer at pH 7.0. This disaggregation may have been induced by the swelling of virus particles that occurs at pH 7.0 (11). Two other strains, CRSV-R and CRSV-N, and a strain obtained from M. Hollings (Glasshouse Crops Research Institute, Littlehampton, U.K.) aggregated and disaggregated in a temperature-reversible manner at pH 5.0 (10,11). When a concentrated purified preparation of the R strain was heated the solution became very turbid, but it cleared quickly when cooled. The N and Hollings's strains behaved similarly, but required heating to higher temperatures to become turbid.

The purpose of the experiments reported here was to further investigate the thermal aggregation phenomenon by comparing the absorbances of solutions of CRSV-R and CRSV-N at various temperatures and by investigating the physical characteristics of CRSV-R aggregates by sedimentation and electron microscopy studies. The amino acid composition and serological properties of CRSV-R and CRSV-N were also compared. The thermal aggregation of these strains is a useful model system for studying virion-virion interactions which may be important in virus purification and characterization.

MATERIALS AND METHODS

Viruses. After their isolation in April 1973, the two strains (CRSV-R and CRSV-N) were maintained by drying infected cowpea leaves in an Edwards freeze dryer, sealing them in glass tubes under nitrogen, and storing at 4 C. The dried cultures were renewed at 2-yr intervals. The viruses were purified (2) and dissolved in 0.1 M sodium acetate buffer, pH 5.0 (NA buffer), or in 0.1 M tris acetate buffer, pH 5.0 (TA buffer).

Virus concentration and sedimentation coefficients. The virus concentration was estimated spectrophotometrically using the extinction coefficient of 6.5 cm^2/mg at 260 nm (2). The amount of light scattering obtained with dilute preparations at room temperature was not greater than observed with untreated southern bean mosaic virus (SBMV) (13).

Sedimentation was studied in a Spinco model E analytical ultracentrifuge by using schlieren optics and preheated rotors. Sedimentation coefficients were calculated by the graphical method of Markham (7). Concentration of sedimenting components was calculated from the area under the schlieren peaks (9).

Serology, electron microscopy, and amino acid analysis. Antisera to CRSV-N were prepared by injecting two 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 4 wk and bleedings were made every 4 wk.

Serological tests were done by agar gel double diffusion on collodion-coated glass microscope slides. In tests of partial identity the viruses at 500 μg/ml were pipetted (40 μl) into wells cut in the agar gel and antisera was used in a range of twofold serial dilutions (12). In tests to determine loss of virus antigen on heating, virus preparations were diluted in NA buffer in a range of twofold serial dilutions. Aliquots (40 μl) of these dilutions were tested with the antiserum in each of three slides.

CSRV-R at 0.1 mg/ml was heated to 70 C, placed on a grid at 70 C and washed by dripping 2% uranyl acetate (at 70 C) onto the grid. The grids were drained by touching with filter paper and examined in a Philips 300 or a Hitachi H-600 electron microscope. Control experiments were done with SBMV at 70 C and with SBMV and CRSV-R at room temperature.

Amino acid composition of CRSV strains R, N, and A were done using 24- and 72-hr hydrolysis times. Performic acid oxidation and colorimetric determination of tryptophan were described previously (8).

Temperature and absorbance. The absorbance of virus preparations at 340 nm was measured in 0.3 ml thermocuvettes (1 cm path) in a Gilford model 250 recording spectrophotometer equipped with a model 2535 automatic reference compensator and a model 2527 thermoprogrammer. The temperature was raised at a constant rate of 0.25 C per minute. Three preparations of CRSV-R and of CRSV-N were used in this study but results are presented for only one of each strain. Results with the remaining preparations were very similar, but not identical to those shown in Fig. 2.

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RESULTS

Analytical ultracentrifugation. A preparation of CRSV-R at 9.5 mg/ml in TA buffer, pH 5.0, became very turbid on heating to 40 C, suggesting the formation of high-molecular-weight aggregates. The size of the aggregates in this preparation was investigated in the analytical ultracentrifuge at 31, 35, and 40 C and then at 18 C. In each case, the rotor was preheated or precooled and then located in the chamber with the cell aligned in the schlieren optical system. The virus solution was very turbid at 40 C, moderately turbid at 35 C, slightly turbid at 31 C, and clear at 18 C. On initiation of the run at 40 C the turbid solution cleared in less than 2 min at 3,000 rpm, indicating the sedimentation rate of aggregated virions was greater than 10^5S. The solution at 35 and 31 C also clarified at 3,000 rpm, but no sedimenting precipitate was observed at 18 C. The analytical ultracentrifuge was run at 10,589 rpm and schlieren patterns at 31, 35, and 40 C (Fig. 1A to C), showed a decreasing concentration of a component sedimenting at the rate of the virus monomer. The concentration of this monomer was 6.3 mg/ml at 31 C, 3.7 mg/ml at 35 C, 2.1 mg/ml at 40 C, and 9.5 mg/ml at 18 C. Only small amounts of components sedimenting faster (between 300 and 1,300S) than the monomer virus were observed at 31, 35, and 40 C (Fig. 1A to C) and none were observed at 18 C.

Absorbance and temperature. CRSV-N in NA buffer was prepared in concentrations ranging from 4.0 to 0.2 mg/ml. CRSV in NA buffer was prepared in concentrations ranging from 4.0 to 0.05 mg/ml and in TA buffer at 0.8 to 0.2 mg/ml. When CRSV-N and CRSV-R were heated in the thermocuvette at a temperature increase of 0.25 C/min, the absorbancy at 340 nm of each solution increased and then leveled off (Fig. 2). The maximum absorbance plotted against the logarithm of virus concentration (Fig. 3) showed a linear relationship and the correlation coefficient was 0.979. These results indicated that the maximum absorbance obtained by heating either strain in NA or CRSV-R in TA buffers was directly proportional to the logarithm of the virus concentration.

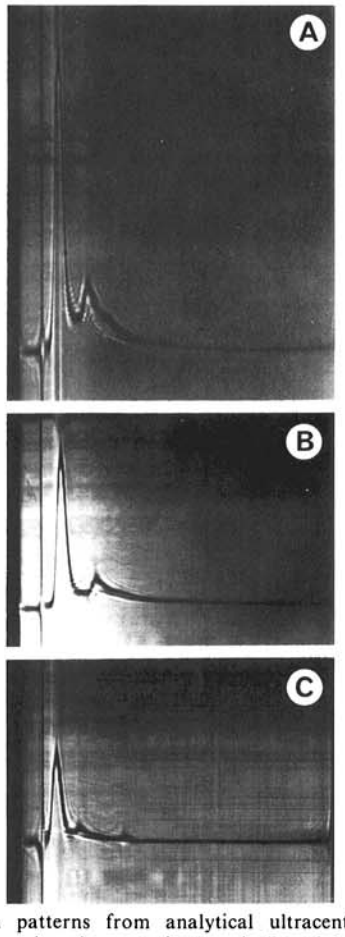

Fig. 1. Schlieren patterns from analytical ultracentrifugation of a preparation of carnation ringspot virus strain R at 9.5 mg/ml in 0.1 M tris-acetate buffer, pH 5.0, at **A**, 31 C, **B**, 35 C, and **C**, 40 C. The rotor speed was 10,589 rpm and the pictures were taken at a schlieren angle of 60 degrees at ~5 min after attaining speed.

The temperature required to induce aggregation in each of the strains increased with decreasing virus concentration (Fig. 2). The temperature at one half the maximum absorbance for each strain and buffer was plotted against the logarithm of virus concentration (Fig. 4). Fig. 4 shows that CRSV-N required higher temperatures for aggregation at higher concentrations but lower temperatures for aggregation at lower concentrations than does CRSV-R. The temperature required for aggregation of CRSV-R in TA buffer was greater than that in NA buffer (Fig. 4). The plots of temperature and virus concentration for CRSV-N and CRSV-R in NA or TA buffers showed a linear relationship with correlation coefficients of −0.997, −0.975, and −0.997, respectively. The correlation coefficient for all three experiments was −0.835. The points in the plot of CRSV-R in NA deviating the most from linearity are at 68.6 C and 70.2 C. The temperature of each of the solutions in Fig. 4 was allowed to cool to 25 C in the thermocuvettes and all the solutions that had not been heated above 68 C returned to their original absorbance. However, the two solutions heated to over 68 C formed a flocculent precipitate that did not redissolve. The serological dilution end points of these solutions in gel diffusion tests did not differ from those of unheated virus solutions. Solutions at 4 mg/ml were cooled shortly after attaining maximum absorbance. The solutions were reheated and they attained the same absorbance at the same temperature as before.

Although the rate of temperature increase was constant (0.25 C/min), the rate of increase in absorbance (Fig. 2) appeared to depend on the strain and the concentration of the virus. The maximum rate of absorbance increase (log scale) for each strain and buffer was plotted (Fig. 5) against the virus concentration (log scale). The data showed a linear relationship with correlation coefficients of 0.986, 0.975, and 0.999 for CRSV-N in NA buffer, and CRSV-R in NA and TA buffers, respectively. The greatest deviation from linearity was at the highest concentration (4.0 mg/ml) with very rapid rates of absorbance increase.

Electron microscopy. When CRSV-R was heated at 70 C and stained with 2% uranyl acetate at 70 C, electron microscopic examination showed many groups of particles (large unstructured aggregates) appearing in fields of dispersed particles (Fig. 6) and a few ordered particle aggregates (Fig. 6, inset). Neither aggregate was found in unheated preparations but a few unstructured aggregates (smaller than that in Fig. 6) were found in preparations heated to 70 C then cooled to room temperature before application to the grid. Aggregates were not found in heated or unheated preparations of SBMV.

Amino acid analysis. Analyses of amino acids in 24- and 72-hr hydrolysates of proteins of the R, N, and A strains are compared with the previously published analysis of the CRSV culture from which R, N, and A were derived (Table 1). The amino acid analyses of all strains were similar but some strains differed in their content of histidine, cysteine, valine, methionine, and tryptophan.

Serological studies. A single early bleeding CRSV-N antiserum from one of the two rabbits produced a reaction of partial identity when tested against CRSV-N and CRSV-R in adjacent wells in a gel diffusion test. This antiserum produced the spur reaction consistently at 1/2 dilution, but failed to do so at greater dilutions.

Confirmation of this result was achieved by the intra gel cross absorption test (14). CRSV-R antigen was added to a well in the agar and anti CRSV-N was added 24 hr later. Then CRSV-N and CRSV-R were placed in separate adjacent wells. A distinct precipitin band was observed between the antiserum and CRSV-N but not with CRSV-R.

DISCUSSION

In preliminary experiments (10) CRSV-N had to be heated to higher temperatures than CRSV-R to detect a visible aggregation. This difference has been confirmed and, in addition, evidence of

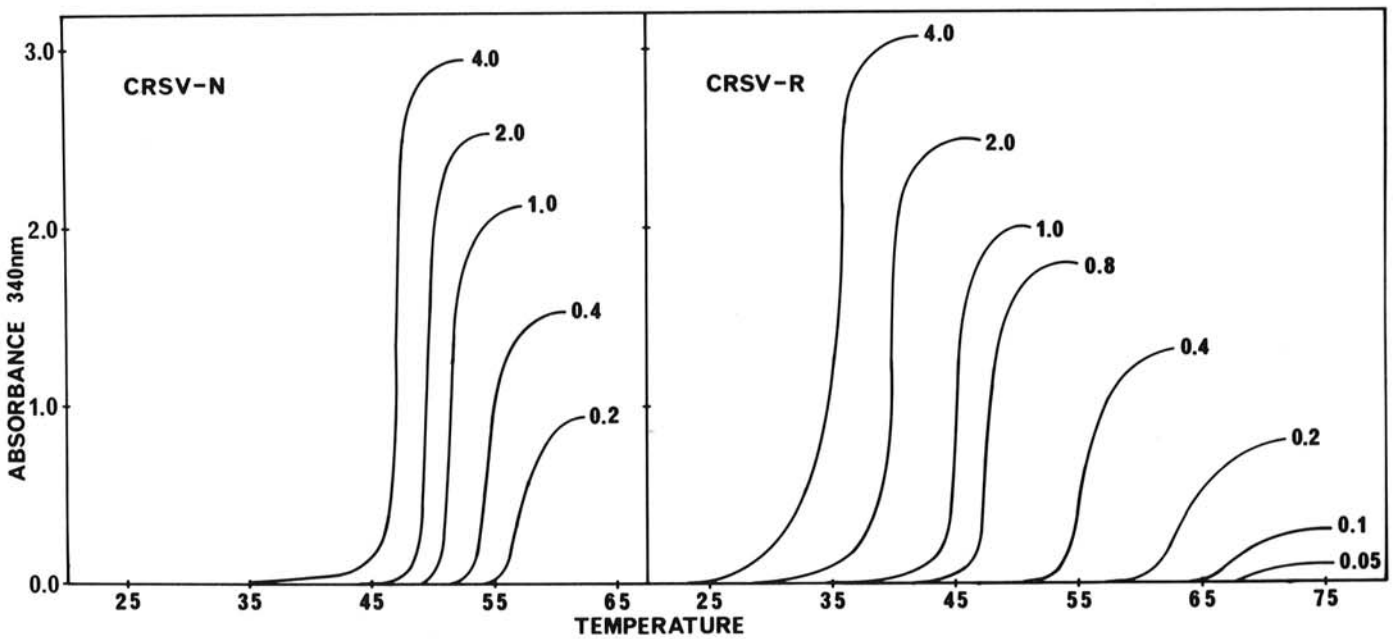

Fig. 2. Effect of temperature on the absorbance at 340 nm of solutions of carnation ringspot virus (CRSV) strains N and R at various concentrations. The solutions at indicated concentration (milligrams per milliliter) were heated at a rate of 0.25 C/min in a Gilford recording spectrophotometer fitted with a thermoprogrammer.

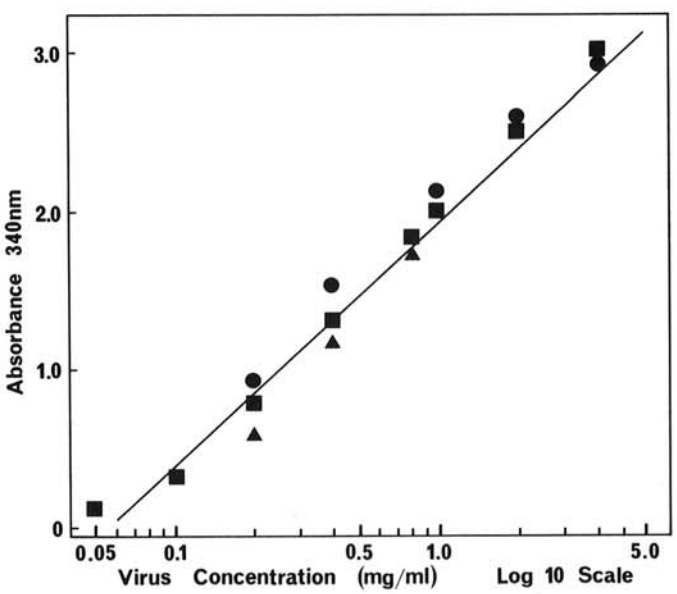

Fig. 3. The maximum absorbance at 340 nm obtained by heating solutions containing various concentrations (milligrams per milliliter) of carnation ringspot virus. ●, Strain N in 0.1 M sodium acetate buffer, pH 5.0; ■, strain R in 0.1 M sodium acetate buffer, pH 5.0; and ▲, strain R in 0.1 M tris acetate buffer, pH 5.0.

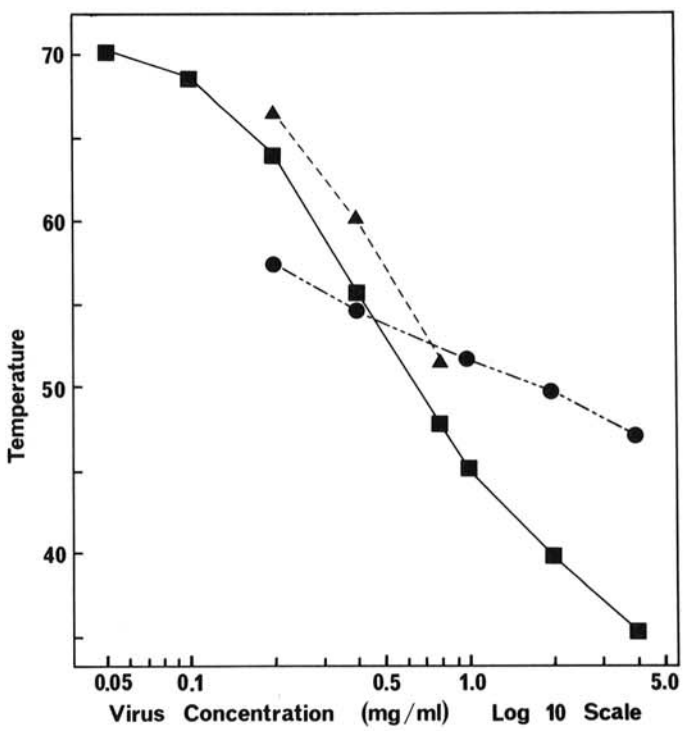

Fig. 4. The temperature at which one half the maximum absorbance at 340 nm was obtained upon heating solutions containing various concentrations (milligrams per milliliter) of carnation rinspot virus. ●, Strain N in 0.1 M sodium acetate buffer, pH 5.0; ■, strain R in 0.1 M sodium acetate buffer, pH 5.0; and ▲, strain R in 0.1 M tris buffer, pH 5.0.

differences in the serological properties and amino acid composition of the strains has been presented.

Sedimentation (Fig. 1) and electron microscopy studies (Fig. 6) have yielded some information of the nature of the aggregation. As the temperature was increased, the proportion of the virus particles sedimenting as aggregates increased. The percentage of virus that aggregated was 0% at 18 C; 34% at 31 C; 61% at 35 C; and 78% at 41 C. Extrapolation of the data for CRSV-R in Fig. 4 to 9.5 mg/ml indicated that the maximum absorbance should have been attained at temperatures lower than 40 C. The detection of CRSV-R aggregates by electron microscopy was difficult because aggregation of a sample sufficiently dilute for electron microscopy (0.1 mg/ml) required heating to 70 C to produce aggregates (Fig. 2). It is probable that the aggregates sedimenting at 300–1300S in Fig. 1 had the same basic structure as the small structured aggregate seen in Fig. 6 inset. This resembled CRSV-A aggregates which are clusters of 12 virus particles and linked clusters of 23, 35, and 45 virus particles which sediment at 640S, 932S, 1175S, and 2266S, respectively (10). However, the aggregation of CRSV-A is not temperature reversible. It is also probable that the large unstructured aggregates seen in the electron microscope are remnants of aggregates sedimenting at 10^5S in the analytical ultracentrifuge. Both types of aggregates seen in the analytical

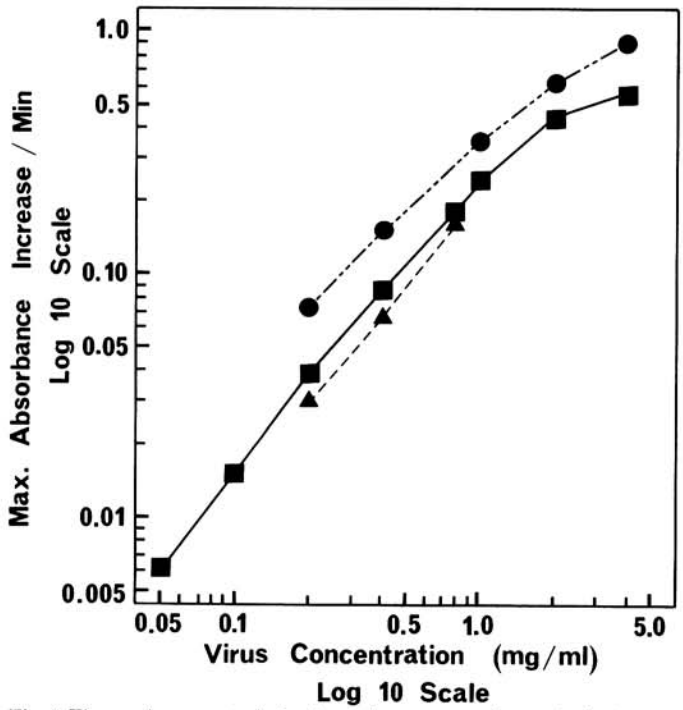

Fig. 5. The maximum rate of absorbance increase per minute obtained upon heating solutions containing various concentrations (milligrams per milliliter) of carnation ringspot virus. ●, Strain N in 0.1 M sodium acetate buffer, pH 5.0; ■, strain R in 0.1 M sodium acetate buffer, pH 5.0; and ▲, strain R in 0.1 M tris acetate buffer, pH 5.0.

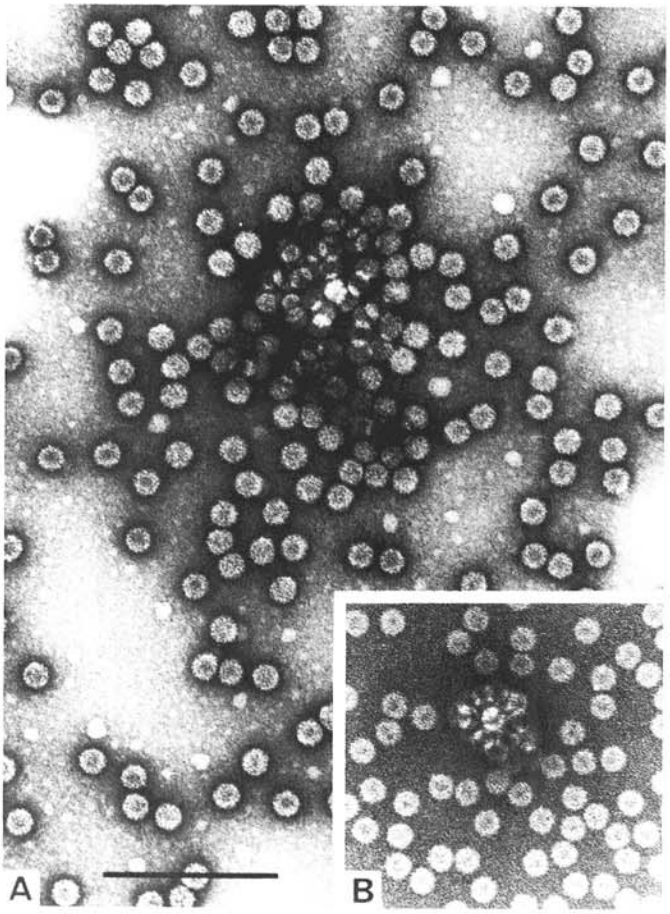

Fig. 6 (and inset). Electron micrographs of carnation ringspot virus strain R. A virus preparation at 0.1 mg/ml was heated to 70 C, placed on a grid at 70 C and stained with 2% uranyl acetate at 70 C. Both micrographs are at the same magnification. Bar indicates 100 nm.

centrifuge dissociated on lowering the temperature to 18 C. All the small structured aggregates and most of the large unstructured aggregates were not detected in electron micrographs of preparations heated to 70 C, then cooled.

The aggregation and disaggregation of CRSV-N and CRSV-R resembles the polymerization and depolymerization of tobacco mosaic virus protein (TMV-P). Both are endothermic and therefore entropy driven and both are reversible. These processes are similar to the formation of some large biologically important structures and research on these phenomena has been reviewed by Lauffer (6). In the polymerization of TMV-P the source of the entropy increase is from the release of water molecules accompanying that process and it is assumed that the assembly of most of the other structures has the same driving force. Hence the structures are held together by "entropic unions" or hydrophobic bonds but conformational changes in the protein are also involved (6).

The aggregation-disaggregation system of CRSV differs from the polymerization-depolymerization of TMV-P in some respects. CRSV is much larger than TMV-P and the aggregates of CRSV are very much larger than the rod shaped particles assembled from TMV-P. The TMV-P assembled particles are readily found in the electron microscope but the larger particles of CRSV-R were difficult to detect in the electron microscope. Even the stable aggregates of CRSV-A collapse in the electron microscope unless they are supported by a thick layer of stain (10). This difference in stability may be a function of the strength of number of bonds within the aggregate or it may be a reflection of the height and weight of the structure. Two-dimensional net aggregates of the satellite of tobacco necrosis virus particles were readily found in electron micrographs (3).

With the small TMV-P and assembled molecules, the absorbance at 320 nm is proportional to the concentration of the protein times the molecular weight of the assembled product (6). The much larger aggregates formed by CRSV had a size similar to mitochondria or bacteria and the absorbance of these larger objects is not linearly related to concentration (4,5). The maximum absorbance obtained on heating CRSV preparations was directly proportional to the logarithm of virus concentration (Fig. 3). We do not know whether the molecular weight of the aggregates formed at the maximum absorbance was similar at all virus

TABLE 1. Amino acid composition of carnation ringspot virus (CRSV) strains

Amino acid	R[a]	N[a]	A[a]	K[a]
Lys	15[b]	16	15	14
His	2	2	3	2
Arg	15	17	16	16
Asx	33	35	34	34
Thr	38	38	38	37
Ser	39	38	38	37
Glx	22	23	23	23
Pro	19	19	17	20
Gly	23	24	23	20
Ala	24	26	27	24
Cys[b]	2	3	3	3
Val	36	30	32	36
Met[c]	6	4	6	7
Ile	16	15	14	16
Leu	27	27	27	26
Tyr	15	15	15	16
Phe	13	12	12	12
Trp[d]	3	3	4	4
Total	348	347	347	347

[a] CRSV-R and CRSV-N, temperature reversibly aggregating strains; CRSV-A, forms aggregates of 12 virus particles and linked aggregates; CRSV-K, analysis reported by Kalmakoff and Tremaine (2).
[b] Values expressed as integers. Each value is derived from two analyses of a 24-hr hydrolysate and two of a 72-hr hydrolysate. Integer values were adjusted to total ~347 residues (2).
[c] Determined from performic acid-oxidized protein.
[d] Determined colorimetrically.

concentrations. Studies involving light scattering or intensity fluctuation spectroscopy would provide this information.

The temperature absorbance plots of TMV-P polymerization-depolymerization with light scattering theory and linear condensation polymerization equations were used to calculate thermodynamic parameters, particularly entropy changes in the polymerization process (6). Ansevin and Lauffer (1) also determined that the TMV-P polymerization was a second order process because a plot of logarithm of the rate against logarithm of concentration was linear. The linearity of a similar plot of CRSV aggregation (Fig. 5) does not necessarily indicate a second order reaction because these experiments were done at different temperatures.

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