Differential Effects of Sodium Dodecyl Sulphate on Strains of Carnation Ringspot Virus

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SUMMARY

Four isolates of carnation ringspot virus showed a reversible aggregation which was dependent on the temperature and concentration of their preparations. At 7 mg/ml, preparations of two of these isolates aggregated at room temperature but at the same concentration the other two isolates required higher temperatures (40 °C) for aggregation. A fifth isolate formed aggregates of 12 virus particles and linked aggregates. Particles of the two isolates with less tendency to aggregate were almost completely dissociated by 0.4 % sodium dodecyl sulphate (SDS) at pH 5 and by 0.1 % SDS at pH 7. At pH 7, an RNA component of mol. wt. 0.5 × 10^6 was released by lower concentrations of SDS than was an RNA component of 1.5 × 10^6. At pH 5, the sedimentation rates of unaggregated particles of the remaining three aggregating strains were unaffected by up to 15 % SDS. However, treatment with 0.0075 to 0.05 % SDS at pH 7 produced protein, RNA and swollen virus particles. These swollen particles were not further affected by increasing the SDS concentration to 8 %.

INTRODUCTION

Kaper (1972, 1973) cited evidence that turnip yellow mosaic virus is stabilized by protein–protein interactions and cucumber mosaic virus is predominantly stabilized by protein–RNA interactions. On the basis of some of their physical and chemical properties related to the major virion stabilizing forces, he proposed grouping other viruses into a ‘turnip yellow mosaic virus profile’, a ‘cucumber mosaic virus profile’, and an intermediate group. One of these properties is the stability of the virus particle in low concentrations of the anionic detergent sodium dodecyl sulphate (SDS). Boatman & Kaper (1972) found that in the first group turnip yellow mosaic, tobacco ringspot, bean pod mottle and tomato bushy stunt viruses were apparently unaffected by concentrations of SDS up to 10 %. However, in the second group cucumber mosaic, peanut stunt, alfalfa mosaic and brome mosaic viruses were completely dissociated at SDS concentrations of 0.01 to 0.04 %. Viruses in the second group have many other characteristics of the protein–RNA stabilized cucumber mosaic virus profile. Boatman & Kaper (1972) proposed that SDS dissociates these viruses by neutralizing cationic side chains on the virus protein subunits which form salt linkages with the anionic phosphate groups of nucleic acid. The validity of their proposal was supported by the failure of cationic or neutral detergents to dissociate viruses of the second group. They found also that brome mosaic virus was more sensitive to SDS at pH 7 than at pH 6 and stated that this was probably because brome mosaic virus is stabilized at pH 6 by
protein–protein interactions which are lost at pH 7. They suggested that any dissociation of capsid-stabilized viruses (protein–protein interactions) by SDS at high concentration may occur by denaturation of the protein.

Tremaine, Ronald & Valcic (1975) described two strains of carnation ringspot virus (CRSV) with unusual aggregation properties. One strain formed aggregates of 12 virus particles and linked aggregates, but another strain formed large aggregates at room temperature which dissociated at 4 °C. Because the bonds between the particles in these two types of aggregates were probably protein–protein and hydrophobic, we compared the sensitivities of the two strains to SDS at room temperature with those of an isolate of CRSV from England (Hollings & Stone, 1970) and a strain which required higher temperatures for aggregation.

**METHODS**

**Virus strains and purification.** Three strains of CRSV were obtained from single lesions on *Chenopodium amaranticolor* inoculated with a culture in cowpea plants. This culture originated from a single lesion isolate made by Kalmakoff & Tremaine (1967) and had been maintained since in *Dianthus barbatus* L. Each strain was passed through five serial single lesion transfers in *C. amaranticolor* and then propagated in cowpea. Cultures were preserved by drying infected cowpea leaves in an Edwards freeze dryer, sealing in glass tubes under nitrogen and storing at 4 °C. One strain, CRSV-A, forms aggregates of 12 particles. Preparations (7 mg/ml) of another strain, CRSV-R, are opalescent at 4 °C but become turbid quickly when heated to 25 °C. This aggregation is reversible and the turbidity disappears upon cooling to 4 °C (Tremaine et al. 1975). At the same concentration preparations of a third strain, CRSV-N, form these aggregates slowly at 40 °C but not at 30 °C. A subculture of this strain, CRSV-N1, forms aggregates quickly at 40 °C but slowly at 30 °C. The formation of aggregates is also dependent on the virus concentration; CRSV-N at 30 mg/ml becomes turbid at 25 °C. A fourth isolate CRSV-H was obtained from Dr M. Hollings, Glasshouse Crops Research Institute, Littlehampton, U.K., and preparations at 7 mg/ml form similar aggregates slowly at 25 °C but quickly at 30 °C.

The viruses showing reversible temperature-dependent aggregation formed reactions of identity in gel diffusion tests with CRSV-R antiserum. CSRV-A is serologically related but not identical to CRSV-N and CRSV-R.

Virus preparations were made from infected cowpea plants by clarification at pH 5, precipitation with polyethylene glycol 6000, and differential sedimentation.

**Treatment of virus with SDS.** The effect of SDS was tested at pH values of 5, 6·6 and 7. Virus preparations at 0·6 to 1 mg/ml in 0·1 M-sodium acetate buffer, pH 5·0 were mixed with SDS in the same buffer at appropriate concentrations to yield the final concentrations (w/v) indicated in the text. After standing at room temperature (22 to 26 °C) for 20 min, 0·25 ml (containing 100 to 175 µg of virus) was layered on a 50 to 350 mg/ml sucrose gradient in 0·1 M-sodium acetate, pH 5·0, and centrifuged for 60 min at 39000 rev/min in a Beckman SW 41 rotor at 20 °C. SDS was not included in the gradients because it interfered with u.v. scans, and contributed to the density of the gradient. Centrifuging was started at 30 min after the samples were mixed with SDS. The gradients were scanned in an ISCO model UA-4 ultraviolet monitor with a model 612 recorder, model D density gradient fractionator, and a model 184 tube piercing device. Most scans were made at 254 nm but some gradients were also scanned at 280 nm.

Experiments at pH 7 were conducted similarly but with the SDS and density gradients in 0·1 M-sodium phosphate buffer, pH 7·0. Concentrated virus preparations in 0·1 M-sodium
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acetate, pH 5·0 were diluted with 0·1 M-sodium phosphate buffer, pH 7·0, immediately before addition of SDS solutions.

Large quantities of SDS reaction products were made by the addition of SDS solutions to virus preparations dialysed overnight in 0·1 M-sodium phosphate buffer, pH 7·0, or to virus preparations adjusted to pH 7·0 by the addition of buffer and 0·1 N-NaOH. The SDS-treated virus was layered on a 50 to 350 mg/ml sucrose density gradient in 0·1 M-sodium phosphate, pH 7·0, centrifuged at 23000 rev/min for 3 h in an SW 25 rotor or at 25000 rev/min for 2·5 h in an SW 27 rotor, scanned and fractionated into 1 ml fractions using ISCO equipment.

Density gradients were prepared using two, siphon-connected, beakers with a magnetic stirrer, and pumped into gradient tubes with a six channel Scientific Industries Inc. peristaltic pump or a single channel LKB Perpex peristaltic pump.

Sedimentation coefficients. These were determined in the Spinco Model E analytical ultracentrifuge at 31410 rev/min or 39460 rev/min using Schlieren optics and were calculated by the graphical method of Markham (1962). Preparations of CRSV-R were examined in 0·1 M-sodium acetate buffer, pH 5·0, and after dialysis against 0·1 M-sodium phosphate buffer, pH 7·0.

Determination of virus concentration. The virus concentration was determined spectrophotometrically using the extinction coefficient value (E260 nm) at 260 nm of 6·45 found by Kalmakoff & Tremaine (1967). Concentration of CRSV-A was estimated by the area under the Schlieren peaks in the analytical ultracentrifuge (Schachman, 1957).

Electron microscopy. To examine virus particles in SDS or sucrose density gradient fractions, a drop was allowed to stand on a grid for a few minutes and the grid was washed gently with 20 to 40 drops of either 2 % sodium phosphotungstate, pH 7·0, or 2 % uranyl acetate, pH 5·0. The grid was drained by touching with filter paper, leaving a thin layer of stain and then allowed to dry (A. F. Murant, personal communication). The grids were examined immediately in a Philips EM 200 or EM 300 electron microscope.

Polyacrylamide gel electrophoresis of RNA. RNA's were prepared by adding 1 to 2 mg of virus to 1 ml of a buffer, pH 9, composed of 0·02 M-tris-HCl, 0·001 M-EDTA (ethylenediaminetetraacetate), 1 % SDS and 4 M-urea, and then heating for 10 min at 50 °C. RNA's were electrophoresed in 2·4 % polyacrylamide gels (Loening 1969) and located by staining with 0·02 % toluidine blue in 40 % methylcellosolve. RNA mol. wt. were estimated by their movements relative to those of RNA's from tobacco mosaic and brome mosaic viruses (Nelson & Tremaine, 1975).

RESULTS

Effect of pH on the sedimentation rate of CRSV

Kalmakoff & Tremaine (1967) reported that the sedimentation coefficient of CRSV was 132 S at pH values from 4·1 to 7·6, but our tests demonstrate a change in sedimentation rate with pH. As control runs in SDS experiments, concentrated preparations of CRSV-N, CRSV-H and CRSV-R were diluted 30-fold in either 0·1 M-sodium acetate buffer, pH 5·0, or 0·1 M-sodium phosphate buffer, pH 7·0, and the diluted preparations were centrifuged into density gradients made in the buffer used as a diluent. At pH 5·0 each virus isolate sedimented as a single sharp peak, but at pH 7·0 both CRSV-N and CRSV-A (Fig. 1 and 2) sedimented as two components: a major component sedimenting at the same rate as the virus at pH 5; and a slower sedimenting minor component. Scans of CRSV-H (Fig. 3) and CRSV-R at pH 7·0 each showed a single slightly diffuse peak which sedimented to a depth approx. 5 % less than that at pH 5·0. The double peaks observed in scans of CRSV-N and
CRSV-A resemble Schlieren patterns obtained with some preparations of cowpea chlorotic mottle virus dialysed to pH 7·05 (Bancroft et al. 1968). The fast and slow components of CRSV are probably unswollen and swollen virus forms, respectively.

CRSV-N diluted 100-fold in phosphate buffer, pH 7·0, containing 0·01 M-EDTA sedimented into density gradients at pH 7·0 as a single slow band. However after a tenfold dilution in 0·5 M-MgCl₂ followed by a tenfold dilution in phosphate buffer, pH 7, CRSV-N sedimented into similar gradients as a single fast band. Apparently the fast band is formed in the presence of Mg²⁺ and the slow band in the presence of EDTA.

When CRSV-N was dialysed against 0·01 M-sodium phosphate buffer, pH 7·0, containing 0·1 M-NaCl it sedimented as a single slow band in gradients in the same buffer but as a single fast band in gradients in 0·01 M-sodium acetate buffer, pH 5·0, containing 0·1 M-NaCl. This demonstrated that the slow component changed to the fast component at pH 5·0 in the absence of Mg²⁺.

**Nucleic acids of CRSV**

Two RNA components were detected upon polyacrylamide gel electrophoresis of dissociated CRSV strains. The mol. wt. of these components were estimated at 1·5 × 10⁶ and 0·5 × 10⁶. Some faintly stained minor components were detected between these components. The nucleic acid content of CRSV calculated from the mol. wt. of the virus and
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percentage RNA (Kalmakoff & Tremaine, 1967) was $1.44 \times 10^6$. It is therefore probable that three molecules of weight $0.5 \times 10^6$ or one molecule of $1.5 \times 10^6$ are packed in a single virus particle.

Identification of CRSV components

CRSV-N was treated with 1% SDS in 0.1 M-tris-HCl, pH 7.0 and centrifuged for 5 h at 40,000 rev/min in sucrose gradients. Extinction profiles showed complete separation of the three peaks seen in 0.1% SDS at pH 7 (Fig. 1). Polyacrylamide gel electrophoresis showed the fastest sedimenting component was the $1.5 \times 10^6$ RNA and the intermediate component was the $0.5 \times 10^6$ RNA. The slowest component had an $E_{280}/E_{260}$ value of 1.75 and was probably protein.

Dissociation of CRSV-N by SDS

At pH 5 (Fig. 1), CRSV-N sedimented two thirds of the distance down the gradient tube as a single peak. Increasing amounts of dissociation products, a protein and two RNA components, were found with increasing SDS concentrations of 0.1, 0.2, and 0.4%. At 0.1% SDS the presence of two components in the virus peak indicated an intermediate in the dissociation.

At pH 7 (Fig. 1), CRSV-N sedimented as two components. At all concentrations of SDS shown, there was very little extinction at the depth of sedimentation of untreated virus. However, increasing amounts of nucleic acid were observed with increasing concentrations of SDS. This indicates that all the virus was not dissociated at 0.01% to 0.05% SDS, but was either precipitated or spread across the gradient. Dissociation into protein and the two RNA components at pH 7.0 occurred at lower SDS concentrations than at pH 5.0.

At 0.01 and 0.25% SDS (Fig. 1, pH 7) most of RNA molecules released were of $0.5 \times 10^6$ daltons but at higher SDS concentrations RNA molecules of $1.5 \times 10^6$ daltons were also released. This again suggests that the two sizes of RNA are contained in different particles. Presumably at pH 7 the protein–RNA bonding is weaker in particles containing three small RNA molecules than in particles containing a single larger RNA molecule. At pH 5, protein–protein interactions, which are less sensitive to SDS, are the major factor stabilizing the two types of particles and the difference between the two types of particles in the strength of their protein–RNA bonds is not important.

CRSV-N, and a subculture, CRSV-N1, did not differ in sensitivity to SDS at either pH 5 or 7. However this subculture showed reversible temperature – dependent aggregation at a lower temperature than did CRSV-N.

Effect of SDS on CRSV-A, CRSV-H and CRSV-R

Aggregates of CRSV-A were not dissociated at pH 5 by 15% SDS. This stability of the aggregates to SDS suggests that they are held together by protein–protein interactions between virus particles. Preparations enriched in unaggregated particles were obtained by partial removal of aggregated particles by low speed sedimentation. These unaggregated particles were also very resistant to SDS at pH 5 (Fig. 2), but at higher SDS concentrations the virus peaks were more diffuse. At pH 6.6 (Fig. 2), unaggregated CRSV-A sedimented as two peaks: swollen and unswollen monomers. A heterogeneous product tailing to the bottom of the gradient may have been derived from residual aggregates. Treatment with SDS caused the release of nucleic acid and the formation of an intermediate product; essentially the same extinction profiles were produced by SDS concentrations between 0.5 and 4% (Fig. 2) and as low as 0.0075% in separate tests made at pH 7.
CRSV-R and CRSV-H both aggregated in a reversible temperature-dependent manner, and with a few exceptions described below gave identical results with SDS. At pH 5, CRSV-H (Fig. 3) was much more resistant to SDS than was CRSV-N and most of the virus particles were undissociated at 0.8% SDS. In tests not shown here over half of the virus particles were undissoilated in 15% SDS. The nucleic acid found at lower SDS concentrations (0.1 to 0.8% in Fig. 3) may have been derived from partially denatured virions or from particles of a more sensitive mutant of the CRSV-N type in the CRSV-H culture. CRSV-R did not dissociate at these low SDS concentrations. The presence of small quantities of SDS sensitive particles in CRSV-H preparations may have been responsible for the slower formation of aggregates at 25 °C as compared with the rate of aggregation in CRSV-R.

At pH 7, CRSV-H (Fig. 3), and CRSV-R in separate experiments, did not sediment as two components. The sedimentation coefficients of CRSV-R at pH 5 and pH 7 were 129S and 123S, respectively, demonstrating a pH-induced swelling. Upon treatment with SDS at concentrations of 0.05 to 0.4% (Fig. 3, pH 7), CRSV-H released increasing amounts of nucleic acids. Two nucleoprotein components were found upon reaction of CRSV-H with SDS: one sedimented to the same depth as unreacted virus, and was called virus sedimenting component (VS); the other sedimented more slowly and was called intermediate sedimenting component (IS). The quantity of VS decreased gradually between 0.05 and 0.4% SDS, while IS increased slightly over the same range (Fig. 3). In other tests, not shown here, the amounts of IS and VS did not change appreciably when CRSV-H or CRSV-R were treated with concentrations of SDS between 0.4 and 15% at pH 7.

The sedimentation coefficient of IS in a CRSV-R preparation at pH 7·0 containing 1% SDS was 63% of that of either VS or the untreated virus. We calculate that a CRSV particle totally lacking RNA should have a sedimentation coefficient of 67% of that for the intact particles of the same diameter (Reichmann, 1965). However, it seems probable that IS represents RNA-containing particles that are swollen. When CRSV-R at pH 7 in 0.25% SDS was stained with 2% sodium phosphotungstate (PTA), pH 7·0, and examined in the electron microscope (Fig. 4a), the preparations contained dark-centred spherical particles of 30 nm diam. and roughly spherical particles with a diam. of approx. 49 nm. The larger ones were probably IS component with swelling induced by SDS.
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Separation of SDS reaction products of CRSV-A, CRSV-H, and CRSV-R

CRSV-A, CRSV-H, and CRSV-R at pH 7 in 1% SDS were sedimented into sucrose density gradients in an SW 25 rotor, scanned at 254 nm, then fractionated. The extinction profiles in these tests were similar to those shown for 0.4% SDS in Fig. 3 at pH 7. Fractions containing IS components of CRSV-A, CRSV-H, and CRSV-R had $E_{260}/E_{230}$ values of 0.60 to 0.63, 0.60 to 0.62 and 0.66, respectively. Fractions containing VS (or untreated virus) had an $E_{260}/E_{230}$ of 0.59. These results suggest an RNA content of 14 to 20% for IS compared to 20% for VS or untreated virus. The low value of the sedimentation coefficient of IS cannot be attributed solely to loss of RNA mass; some particle swelling must have occurred.

The fractions containing IS were pooled, dialysed against 0.1 M-sodium phosphate buffer, pH 7.0, placed on a sucrose density gradient in an SW 41 rotor, centrifuged, and scanned at 254 nm. Two peaks of approx. the same size were observed: one sedimented to the same depth as VS; the other sedimented like IS. Apparently a portion of IS can form VS after removal of SDS by dialysis.

CRSV-R at pH 7 in 1% SDS was sedimented into sucrose density gradients at pH 7.0 with and without 1% SDS incorporated in the gradient, scanned at 254 nm, and fractionated. Examination of fractions containing IS or VS after staining with 2% PTA, pH 7.0, showed distorted, spherical particles with darkened centres of differing intensities. The irregular shape of these particles made size measurements unreliable. Density gradient fractions of untreated virus stained with PTA showed regular, spherical particles, with a diam. of 31 nm.

Uranyl acetate, pH 5.0, (UA) was chosen as a stain in subsequent studies because it gave electron micrographs of untreated virus particles with excellent contrast. With gradients lacking SDS, fractions containing IS showed distorted particles of approx. 36 nm in diam. with darkened centres (Fig. 5a). Particles, 34 nm in diam., found in fractions containing VS (Fig. 5b) were rarely darkened in the centre and resembled particles of untreated virus.

When 1% SDS was incorporated in the density gradient, particles found in fractions containing IS and VS were very similar to particles found upon examination of unfractionated CRSV-R in 1% SDS at pH 7 (Fig. 4b). Three types of particles are shown in Fig. 4b:
(1) a dark particle 25 nm in diam.; (2) a centrally darkened particle 32 nm in diam.; and
(3) a light particle 32 nm in diam. Fractions containing VS showed particle types 2 and 3,
and fractions containing IS showed particle types 1 and 2.

The results with UA staining shown in Fig. 5a, 5b and 4b are difficult to interpret.
Failure to find particles with greatly different sizes in IS and VS fractions from gradients
lacking SDS (Fig. 5a and b) may be caused by the pH change from pH 7 in the gradient
fractions to pH 5 by washing and staining with UA. The IS component is formed only upon
treatment with SDS in phosphate buffer at pH 7.0 (or in 0.1 M-tris-HCl, pH 7.0, in other
similar tests) and not at pH 5 (Fig. 2 and 3). The darker centres in Fig. 5a may indicate
either greater stain penetration into particles lacking a portion of their normal RNA
complement, or accumulation of stain on collapsed particles. The significance of the dark,
apparently smaller particles of type 1 (Fig. 4b) found in IS fractions with SDS in the
gradient and unfractionated SDS treated virus is obscure. These particles are only found
when solutions containing SDS are placed on the grid. UA is not soluble in sodium phos-
phate at pH 7 and also precipitates SDS at this pH. Besides these complicating factors, it is
difficult to interpret images in negatively stained micrographs (Horne, 1967; Markham,
1962).

DISCUSSION

The stability of CRSV strains in SDS at pH 5 correlates with their ability to aggregate
at lower temperatures. The SDS sensitive strains, CRSV-N and CRSV-N1, required higher
temperatures or higher virus concentrations to show reversible aggregation than did the
SDS resistant CRSV-R. CRSV-H was not isolated from local lesions and appears to con-
tain both SDS resistant and sensitive strains. Our SDS tests with CRSV-R, CRSV-H,
CRSV-N, and CRSV-N1 were done with monomers; their aggregates dissociate at the
concentration used in these experiments. The CRSV-A monomers and aggregates were
equally resistant to SDS. Hence SDS resistance is related to virus particle stabilizing forces
and not polymer stabilizing forces. The apparent lack of effect of SDS on CRSV-A, CRSV-
R, and CRSV-H at pH 5 and the swelling of virus particles in these isolates at pH 7 demon-
strated the existence of pH-dependent virus particle stabilizing forces. However, the stability
of the swollen forms induced by SDS in high concentrations at pH 7 indicated that there
are also pH-independent stabilizing forces.

The CRSV strains used in this study have properties in common with the cucumber
mosaic virus profile outlined by Kaper (1972, 1973). The particles dissociate into RNA and
protein at pH 7 in high salt concentrations (unpublished results). They are apparently
stabilized by pH-dependent protein–protein interactions and Mg$^{2+}$ and these are charac-
teristics of the bromoviruses which Kaper (1972, 1973) grouped in the cucumber mosaic
virus profile. The great differences in sensitivity to SDS shown by CRSV strains at pH 5
would be interpreted by the hypothesis of Boatman & Kaper (1972) to indicate substantial
differences in the strengths of their protein–protein interactions. However, without further
evidence it should not be assumed that the resistance of certain strains of the CRSV virion
to SDS at pH 5 can be interpreted entirely in terms of strong protein–protein interactions.

Cowpea chlorotic mottle virus was grouped with the cucumber mosaic virus profile by
were more pH and salt stable, less heat stable, and more salt stable, respectively. There are
disulphide linkages between protein subunits of one of these strains (Bancroft et al. 1971)
and these strong protein–protein interactions are a characteristic of the turnip yellow
mosaic virus profile (Kaper, 1972, 1973). The cowpea chlorotic mottle virus mutants de-
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scribed above were derived from the wild type by one or two amino acid exchanges in the coat protein. Therefore we think it is possible that the variability in sensitivity of CRSV strains to SDS at pH 5 may reflect a few amino acid substitutions in the coat protein sequence and that studies of this sequence may provide explanations both for this phenomenon and the two types of particle to particle aggregation found with CRSV strains.

Nelson & Tremaine (1975) found that a virus from Saguaro cactus was dissociated by 0.05% SDS at pH 7 and 0.10% SDS at pH 5. In experiments done under the same conditions brome mosaic, cowpea chlorotic mottle, broad bean mottle and turnip crinkle viruses were dissociated by similar concentrations of SDS (W. P. Ronald & J. H. Tremaine, unpublished results). The Saguaro cactus virus was assumed to be held together by protein–RNA and pH-dependent protein–protein interactions on the basis of these results and of its stability at pH 7 and 5 (Nelson & Tremaine, 1975). Habili & Francki (1974) found cucumber mosaic and tomato aspermy viruses were dissociated by low concentrations of SDS but the amount of dissociation was dependent on the buffer used. It is apparent that the SDS technique and a variety of others should be used to determine the stabilizing forces of viruses. From our studies it is clear that a spectrum of major stabilizing forces may exist among strains of a single virus as well as among different viruses.

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