Comparison of the Structural Properties of Sindbis and Semliki Forest Virus Nucleocapsids

By HANS SÖDERLUND, CARL-HENRIK VON BONSDORFF AND ISMO ULMANEN

Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29
Finland

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SUMMARY

The envelope spikes of Sindbis and Semliki Forest virus are arranged in a T=4 icosahedral surface lattice and, by deduction, it has been suggested that the nucleocapsid proteins are similarly arranged. After treatment of the virions with a non-ionic detergent the released nucleocapsids sediment in sucrose gradients at about 160S and 150S and have densities in CsCl of 1.42 g/ml and 1.425 g/ml, respectively, for Sindbis and Semliki Forest virus. At pH 6.0 Sindbis nucleocapsids do not contract like those of Semliki Forest virus. Nucleocapsids of both viruses are sensitive to the action of ribonuclease but only those of Semliki Forest virus undergo a drastic structural rearrangement due to the treatment. EDTA treatment in hypotonic conditions results in a decrease in the S-value for both particles. Electron micrographs show that the SFV nucleocapsids are partly ‘unfolded’ while those of Sindbis appear slightly contracted after exposure to EDTA.

INTRODUCTION

The alphaviruses are constructed of a nucleocapsid core, consisting of capsid protein (mol. wt. about 30000) and genome RNA (mol. wt. about 4.3 × 10⁶) which is surrounded by an envelope consisting of a lipid bilayer and the envelope proteins (Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1976; Kääriäinen & Söderlund, 1978). The most intensively studied alphaviruses, namely Sindbis and Semliki Forest (SFV) virus, are generally considered to be almost identical in structure. Thus most of the data obtained with only one member of the virus group has been taken to be true for all of them.

The main dimensions of Sindbis and SFV are similar as revealed by small angle X-ray scattering (Harrison et al. 1971; Kääriäinen & Söderlund, 1978; S. C. Harrison & L. Kääriäinen, unpublished data). The diameters of Sindbis and SFV nucleocapsids are 40 nm and 38 nm, respectively. The centre of the lipid bilayer is situated at 23 nm radial axis and the diam. of the virion is 70 nm. The spikes on the Sindbis virion surface are organized in a trimer clustering in a T=4 icosahedral lattice (von Bonsdorff & Harrison, 1975), but the structural symmetry of the nucleocapsid has not been resolved for any of the alphaviruses (Horzinek, 1973; Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1976; Kääriäinen & Söderlund, 1978).

Some structural properties of the isolated SFV nucleocapsid have been characterized: it shrinks at slightly acidic pH, it is RNase sensitive and the loss of RNA is coupled with a contraction reaction, and it is partly ‘unfolded’ on EDTA treatment in low salt (Kääriäinen & Söderlund, 1971; von Bonsdorff, 1972; Söderlund et al. 1972, 1975).

In this report we compare some of the structural properties of Sindbis and SFV nucleocapsids. We have repeated some of our previous experiments with SFV while comparing
the properties to those of Sindbis, since the nucleocapsids are quite sensitive to changes in experimental conditions.

METHODS

Cells and viruses. Semliki Forest virus, a cloned prototype strain (Kääriäinen et al. 1969) and the HR-strain of Sindbis virus, kindly provided by Dr Pfefferkorn (Burge & Pfefferkorn, 1966) were used in this study. The viruses were grown in BHK21 cells or in chick embryo fibroblasts as described (Kääriäinen et al. 1969; von Bonsdorff & Harrison, 1975). The purification of the virus included concentration by polyethylene glycol precipitation (Sefton & Keegstra, 1974), sedimentation through discontinuous sucrose gradients (Söderlund et al. 1972) and final concentration by pelleting, for SFV, or by precipitation with 10 vol. of water, for Sindbis (Pfefferkorn & Clifford 1963).

Particles with label in the protein were produced by growing the virus in the presence of $^{35}$S-methionine (50 to 100 μCi/ml; 280 Ci/mmol, Radiochemical Centre, Amersham, England) in Eagle's MEM lacking this amino acid, while RNA labelling was performed with $^3$H-uridine (100 to 250 μCi/ml; 28 Ci/mmol, Radiochemical Centre). Labelled virus, in volumes less than 20 ml, was purified by layering directly on discontinuous sucrose gradients after removal of cell debris by centrifugation.

Isolation of nucleocapsids. Nucleocapsids were isolated essentially as described earlier (Kääriäinen & Söderlund, 1971). Triton X-100 was added to 0.3 to 0.6 ml of virus suspension in TN buffer to a final concentration of 1%, when less than 50 μg virus was used, or of 2% for 0.5 to 2 mg of virus protein. The mixture was warmed to 20 °C and cooled immediately on ice, after which the nucleocapsids were isolated by sedimentation in 15 to 30% sucrose gradients made in TN. The centrifugation was for 2 h at 39000 rev/min and 4 °C in a Spinco SW 41 rotor. Sindbis nucleocapsids were also isolated by pelleting at 43000 rev/min for 3.5 h in a Spinco SW 50-1 rotor through a cushion consisting of 3 ml 15% sucrose in TN and 1.5 ml of 10% sucrose in TN with 0.5% Triton X-100. SFV nucleocapsids aggregated heavily upon pelleting and could not be resuspended in intact form.

The concentration of nucleocapsids was calculated from the known amount of virus dissociated with Triton X-100 and corrected for recovery with the aid of the isotope label. For the protein labelled samples it was assumed that 25% of the label was in the capsid protein. The concentration, which is given throughout as total nucleocapsid concentration, i.e. RNA+protein, was then calculated from nucleocapsid composition, 36% RNA and 64% protein (Kääriäinen & Söderlund, 1978).

Treatment of the nucleocapsids. The nucleocapsids were exposed to different ionic conditions by diluting fourfold with, or dialysing against, the following buffers: 0.1 M-NaCl, 0.05 M-tris-Cl, pH 7.4 (TN); 0.14 M-NaCl, 0.01 M-sodium phosphate buffer, pH 6.0 to 7.4 (phosphate buffer); 0.01 M-EDTA, 0.01 M-tris-Cl, pH 7.4 (EDTA buffer). Ribonuclease treatments were performed by incubating the nucleocapsids with pancreatic ribonuclease A (RNase, EC. 2.7.7.16, Worthington Biochemical Corp., Freehold, N.J., electrophoretically purified). Sindbis and SFV nucleocapsids were always handled in parallel to minimize experimental variation.

Electron microscopy. Samples were negatively stained on carbon coated grids with 1% aqueous uranyl acetate or with 2% potassium phosphotungstic acid (KPT), pH 7. The staining period was brief, less than 20 s, in order to avoid structural alterations on the grid. Prolonged staining periods with the acid uranyl acetate caused shrinkage of the SFV nucleocapsids (von Bonsdorff, 1973). Electron micrographs were taken with a Siemens Elmiscop 1A at an instrumental magnification of 40000. At least 30 representative particles from each material were measured as described in Söderlund et al. (1972). The diameter of a particle is defined as the geometric mean of two mutually perpendicular measures.
Other methods. Buoyant densities were determined in CsCl after fixation with glutaraldehyde as described (Söderlund et al. 1973/4). RNA, released from the nucleocapsids with 2% sodium dodecyl sulphate (SDS), was analysed on sucrose gradients (Tuomi et al. 1975). The total radioactivity was determined from gradient fractions by counting in a Triton-xylene based scintillator. Acid-insoluble radioactivity was determined after precipitation with 5% cold trichloroacetic acid (TCA) and collection of precipitates on glass-fibre filters which were counted in a toluene based scintillator.

RESULTS

Virion structure

Electron microscopic investigations indicate that the spikes of Sindbis virus are arranged on the virion surface in a trimer clustering according to a T = 4 icosahedral lattice (von Bonsdorff & Harrison, 1975). For comparison we studied the virion surface of SFV. Suitable staining conditions, e.g. negative staining with 1% aqueous uranyl acetate revealed a distinct surface pattern which was consistent with the T = 4 icosahedral lattice. In this respect the two viruses are identical. However, there are minor differences in the subunit characteristics; the triangular network seen on the surface of Sindbis virus is not resolved on the SFV surface where only the stain-filled nodes could be seen (Fig. 1).

Sedimentation analysis of isolated nucleocapsids

The nucleocapsids of alphaviruses can be released from the virion envelope by treatment with mild detergents. In this study the membrane was solubilized with Triton X-100 and the nucleocapsids separated from the envelope components and from the detergent by sedimentation. To compare the properties of the nucleocapsids of SFV and Sindbis, particles labelled with 35S-methionine and 3H-uridine were isolated. 35S-labelled nucleocapsids of one of the viruses were mixed with 3H-labelled nucleocapsids of the other and the mixture was treated in the following ways: (i) the control was diluted in TN; (ii) the mixture was diluted fourfold with phosphate buffer, pH 6-0, which gave a final pH of about 6-2; (iii) the mixture was diluted with EDTA buffer; (iv) the sample was incubated with 1 µg/ml of RNase for 10 min at 37 °C and then ice-cold TN buffer was added. Then the preparations were immediately layered on sucrose gradients made in the same buffer as that used for the dilution. The gradients were centrifuged for 2 h at 190 000 g and the distribution of total radioactivity was determined.

The results of an experiment in which the SFV nucleocapsids were labelled with 3H-uridine and those of Sindbis with 35S-methionine are presented in Fig. 2. Here, trace amounts of virus were used to avoid the aggregation known to occur at low pH with SFV nucleocapsids (Söderlund et al. 1972). The control (Fig. 2a) shows that the nucleocapsids of Sindbis sediment somewhat faster than those of SFV. The sedimentation rate of isolated SFV nucleocapsids was 150S (Kääriäinen & Söderlund, 1971) and using this as a marker the isolated Sindbis nucleocapsids sedimented at 160S. The same S values are obtained in phosphate buffer, pH 7-4. At slightly acid pH the nucleocapsids of SFV sedimented faster than in the control, as expected, but also faster than those of Sindbis — the pH shift did not affect the S value of Sindbis nucleocapsids (Fig. 2b). After EDTA treatment in hypotonic conditions the sedimentation rates of the two nucleocapsids were reduced to about 130S and 110S, respectively, for Sindbis and SFV (Fig. 2d and Table 1). The same experiment performed with a mixture of 3H-uridine and 35S-methionine nucleocapsids of the same virus showed that the 3H/35S ratio in the nucleocapsid peak-fractions was the same independently of the buffer used. The RNA also remained intact, which indicates that no material was lost from the particles despite the altered sedimentation properties (data not shown).
Fig. 1. Electron micrograph of purified SFV negatively stained with uranyl acetate showing a regular array of stain-filled nodes on the particle surfaces. The bottom inset shows two selected particles in which the distribution of the nodes corresponds to a T=4 icosahedral surface lattice (seen along the twofold axis). In Sindbis virus (upper inset), which reveals the same icosahedral symmetry, the surface is more clearly seen as a network of equilateral triangles corresponding to a trimer clustering of the glycoprotein. Staining was with KPi.

We have also shown previously that the density of SFV nucleocapsids is the same at pH 7 and 6.0 as well as in low salt-EDTA conditions (Söderlund et al. 1975). The RNase treatment destroyed the SFV nucleocapsids but left the nucleocapsids of Sindbis apparently unaffected (Fig. 2c).

*The effect of RNase*

We have shown previously that the nucleocapsids of SFV on RNase treatment gradually lose RNA and the sedimentation rate of the particle is correspondingly reduced (Kääriäinen & Söderlund, 1971; Table 1). At high enzyme to substrate ratio, as in Fig. 2(c), where
Fig. 2. Sedimentation analysis of a mixture of $^{35}$S-methionine-labelled Sindbis (○——○) and $^{3}$H-uridine-labelled SFV (●——●) nucleocapsids under different conditions. Trace amounts of isolated nucleocapsids were diluted fourfold with the buffer indicated and analysed on 15 to 30 % sucrose gradients made in the same buffers. (a) TN; (b) phosphate buffer, pH 6.0; (c) treatment with 1 μg/ml RNase for 10 min at 37 °C in TN; (d) EDTA buffer. Centrifugation was for 2 h at 39000 rev/min in a Spinco SW 41 rotor at 4 °C.

Table 1. The sedimentation rate of Sindbis and Semliki Forest virus nucleocapsid as determined by sucrose gradient centrifugation

<table>
<thead>
<tr>
<th>Treatment (buffer*)</th>
<th>Sindbis</th>
<th>SFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN (control)</td>
<td>160</td>
<td>150</td>
</tr>
<tr>
<td>Phosphate pH 7.4</td>
<td>160</td>
<td>150</td>
</tr>
<tr>
<td>Phosphate pH 6.0</td>
<td>160</td>
<td>165</td>
</tr>
<tr>
<td>EDTA</td>
<td>130</td>
<td>110</td>
</tr>
<tr>
<td>RNase (expt. 1)</td>
<td>160</td>
<td>135</td>
</tr>
<tr>
<td>RNase (expt. 2)</td>
<td>160</td>
<td>40</td>
</tr>
</tbody>
</table>

* The buffers are defined in Methods. For RNase treatment (expt. 1), 300 μg/ml of isolated nucleocapsids were incubated with 5 μg/ml RNase for 20 min at 20 °C. In expt. 2 trace amounts of nucleocapsids were incubated with 1 μg/ml RNase for 10 min at 37 °C (cf. Fig. 2).

† The S values were determined by sucrose gradient centrifugation (cf. Fig. 2) using the method of Martin & Ames (1961).

trace amounts of nucleocapsids were used, the particles were destroyed. The apparent insensitivity of the Sindbis nucleocapsids was therefore investigated in more detail. About 40 μg/ml $^{3}$H-uridine-labelled Sindbis and SFV nucleocapsids were treated with 5 μg/ml of
Fig. 3. RNase sensitivity of nucleocapsids. About 40 μg/ml of 3H-uridine-labelled nucleocapsid were incubated with 5 μg/ml RNase. Samples were withdrawn at the indicated times and precipitated with ice-cold TCA, and the acid precipitable radioactivity was determined. The results are given as residual precipitable radioactivity compared to the zero time sample to which no RNase was added. The bars indicate the range from two experiments.

Fig. 4. Fragmentation of nucleocapsid-RNA by RNase treatment. 40 μg/ml of 3H-uridine-labelled Sindbis nucleocapsids were incubated with 5 μg/ml RNase for 20 min at 20 °C. Ice-cold TN was added and the nucleocapsids pelleted through a cushion of 15% sucrose. The pellet was suspended in 2% SDS and the RNA analysed on a 15 to 30% sucrose gradient (© -- ©). RNA from nucleocapsids handled in parallel but without RNase treatment was analysed on a separate gradient (● — ●). Centrifugation for 14 h at 22000 rev/min in a Spinco SW 27.1 rotor.

Fig. 5. Buoyant density determination in CsCl of SFV and Sindbis nucleocapsids. (a) Isolated 35S-methionine-labelled Sindbis and 3H-uridine-labelled SFV nucleocapsids, 300 μg/ml of each, were fixed with glutaraldehyde, mixed and analysed on pre-formed CsCl gradients. (b) Prior to fixation the nucleocapsid preparations were treated with 5 μg/ml of RNase for 20 min at 20 °C. Centrifugation was for 16 h at 37000 rev/min in a Spinco SW 50.1 rotor. © — ©, 35S, ● — ●, 3H.

RNase and total acid-insoluble radioactivity determined after varying incubation periods at 20 °C. The RNase converted the RNA of Sindbis nucleocapsids into an acid-soluble form with a rate approximately half of that for SFV nucleocapsids (Fig. 3). When nucleocapsids were pelleted through sucrose after the treatment and their RNA analysed, no
Fig. 6. Electron micrographs of isolated nucleocapsids dialysed against (a, b) TN; (c, d) phosphate pH 6.0; (e, f) EDTA buffer; (a), (c) and (e) show Sindbis and (b), (d) and (f) SFV nucleocapsids. Staining was with 1% aqueous uranyl acetate.

intact 42S RNA molecules could be detected (Fig. 4). A homogeneous decrease of the density of CsCl after the RNase treatment also indicated that Sindbis nucleocapsid preparations do not contain a mixture of RNase sensitive and insensitive particles. In Fig. 5 an experiment is presented in which about the same amount of Sindbis and SFV nucleocapsids (about 300 µg/ml of each) were treated with 5 µg/ml RNase for 20 min at 20 °C. After the treatment the particles were fixed with glutaraldehyde separately and the ³H-uridine-labelled SFV and the ³⁵S-methionine-labelled Sindbis nucleocapsids were mixed. The RNase treatment reduced the densities from 1.42 to 1.395 g/ml for Sindbis and from 1.425 to 1.385 g/ml for SFV nucleocapsids. The ³H-labelled (SFV) material remaining on the top of the CsCl gradient after RNase treatment (Fig. 5b) apparently also contains, in
addition to oligonucleotides released from nucleocapsids, material from totally disintegrated particles.

**Morphology of the nucleocapsids**

To compare the morphological features of SFV and Sindbis virus nucleocapsids by electron microscopy, the particles were isolated on sucrose gradients made in TN and dialysed against the buffers used above. In some cases Sindbis nucleocapsids were pelleted as described in Methods and the pellets suspended in the desired buffers. The electron micrographs of control material in TN showed spherical particles without distinct surface features (Fig. 6a, b). The diam. were about 40 nm for both nucleocapsids, SFV being slightly larger (Table 2). On dialysis against phosphate buffer, pH 6, the nucleocapsids of SFV shrank drastically and showed an obvious tendency to aggregate (cf. Söderlund et al. 1972). The appearance of Sindbis nucleocapsids, however, remained unaltered by this treatment (Fig. 6c, d and Table 2).

EDTA treatment in hypotonic conditions ‘unfolded’ the SFV nucleocapsid to polymorphic structures from which strands occasionally protruded. Particles with reduced diam. were also seen (Fig. 6f and Table 2; see also Söderlund et al. 1975). No similar effect on the morphology of Sindbis nucleocapsids was observed. However, diameter measurements indicated a statistically significant reduction in size as compared to the control material in TN (<0.001; Fig. 6e and Table 2).

After treatment with 5 µg/ml of RNase the diam. of SFV nucleocapsids was reduced by about 8 nm (Fig. 7b, Table 2 and von Bonsdorff, 1972). The effect on Sindbis nucleocapsids was less obvious. After a 20 min incubation the diam. was reduced by only about 3 nm (Fig. 7a); the change was, however, statistically significant (<0.001). After prolonged digestion, swollen particles, possibly in the process of disintegration, could also be seen (Fig. 7c and Table 2). The Sindbis nucleocapsids were more sensitive towards the action of RNase in the EDTA buffer than in the TN buffer (Fig. 7d). With intensified RNase treatment the nucleocapsids started to disintegrate leaving a population of small sized particles (Fig. 7e). These may correspond to the ‘central core component’ described by Horzinek (1973). The nucleocapsids of SFV were rapidly destroyed by RNase in the EDTA buffer (Table 2).
Fig. 7. RNase treatment of Sindbis virus nucleocapsids. Under isotonic conditions RNase affects the nucleocapsids only slightly (a) but after prolonged treatment some particles apparently start to fall apart (c). When the treatment is done in EDTA buffer the disintegration occurs more rapidly (d) and after prolonged treatment smaller particles with diam. around 20 nm are present in greater numbers (e). SFV nucleocapsids shrink considerably after RNase treatment in TN (b). In all experiments 230 μg/ml nucleocapsids were treated with 5 μg/ml RNase at 20 °C as follows: (a) Sindbis nucleocapsid in TN, 20 min incubation; (b) SFV nucleocapsids in TN, 20 min incubation; (c) Sindbis nucleocapsids in TN, 120 min incubation; (d) Sindbis nucleocapsids in EDTA buffer, 20 min incubation; (e) Sindbis nucleocapsids in EDTA buffer, 60 min incubation. Negative staining with uranyl acetate.
DISCUSSION

The nucleocapsids of the alphaviruses are usually considered to be icosahedral, even if direct proof for this is lacking so far (Horzinek, 1973; Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1976; Kääriäinen & Söderlund, 1978). The envelope spikes are organized according to \( T = 4 \) icosahedral surface lattice (Fig. 1 and von Bonsdorff & Harrison, 1975). Since the structural proteins of the virion are present in stoichiometric ratio (Garoff et al. 1974) and the envelope spikes span the lipid bilayer making contact with the capsid protein (Brown et al. 1974; Garoff & Simons, 1974), the structure of the envelope presumably follows that of the nucleocapsid (Harrison et al. 1974; von Bonsdorff & Harrison, 1975). Thus both Sindbis and SFV nucleocapsids would be \( T = 4 \) icosahedrons.

The nucleocapsids of both viruses are sensitive to the action of ribonuclease. The loss of RNA leads to the contraction of the SFV nucleocapsid. Also the Sindbis nucleocapsid appears smaller after RNase treatment. This is probably due to a true decrease in size and not to altered staining properties since the RNase treatment did not significantly alter the \( S \) value of the particles despite a decrease in particle weight and density. Thus the frictional coefficient, e.g. the particle diameter, was also decreased.

An obvious difference between the two particles was observed in their behaviour after exposure to slightly acid pH or EDTA. Groups, titrating anomalously at the pH range 6 to 7.5, seem to be involved in the maintenance of the structural integrity of several icosahedral and helical plant viruses (Caspar, 1963; Incardona & Kaesberg 1964; Bancroft et al. 1968; Kaper, 1971; Butler et al. 1972; Jacrot, 1975). Several viruses also contain divalent cation binding sites, perhaps for \( \text{Ca}^{2+} \), which may be involved in the control of the assembly–disassembly process (Incardona et al. 1972; Hsu et al. 1976; Brady et al. 1977; Durham & Haidar, 1977; Durham & Hendry, 1977; Durham et al. 1977; Hull, 1977). The divalent cations may be so tightly bound that chelating agents are required to remove them. Durham and his co-workers suggest that the \( \text{H}^+ \) and \( \text{Ca}^{2+} \) binding sites are actually the same. The presence of such sites is suggested for the SFV nucleocapsid by the drastic effects of slightly acid pH or divalent cation depletion. Are they then absent from the Sindbis nucleocapsid? With the methods used we have not been able to detect any effect of increased proton concentration on the Sindbis nucleocapsid. EDTA, however, shows several effects even if not as drastic. The \( S \) value is decreased, the particle appears smaller under the electron microscope, and the RNase sensitivity is increased compared to the control material. The simultaneous decrease in diameter and \( S \) value are mutually contradictory and it is possible that the particle is actually ‘loosened’ in EDTA but that some protruding structures could not be visualized.

The replication process of the different alphaviruses follows an almost identical pattern (Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1976; Kääriäinen & Söderlund, 1978) and the mechanism of nucleocapsid assembly should presumably also be the same. One would assume that such a drastic reaction as the shrinkage of the SFV nucleocapsid should play an important role in the assembly–disassembly process. If so, protons alone are not enough to trigger the change since the Sindbis virus nucleocapsid does not respond to the pH shift. The observed difference between SFV and Sindbis virus may be of value when studying the interactions controlling the assembly of alphaviruses.
Properties of alphavirus nucleocapsids

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