Defective-Interfering Particles of Semliki Forest Virus: Structural Differences between Standard Virus and Defective-Interfering Particles

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SUMMARY

Serial passaging of Semliki Forest virus in BHK cells at a constant input multiplicity of 50 p.f.u./cell resulted in a 4 log_{10} drop in the yield of infectious virus by passage 9. An interference analysis showed that this drop was due to the presence of defective-interfering (DI) particles. Attempts were made to separate the DI particles from standard virus by equilibrium and velocity centrifugation. Only equilibrium centrifugation on CsCl resolved the DI particles (identified by interference analyses) from standard virus. The buoyant density of the DI particles (1.23 g/ml) was higher than that of standard virus (ρ = 1.20 g/ml).

No difference was observed between the structural proteins of standard virus and DI particles. Analysis of the RNA of standard virus and DI particles showed that whereas standard virus contained only 42S RNA (mol. wt. approx. 4.2 × 10^6), DI particles contained two smaller pieces of RNA of mol. wt. 0.81 and 0.75 × 10^6 respectively. Infectivity assays showed that these low mol. wt. species were not only non-infectious but also interfered with the infectivity of 42S RNA from standard virus. Nucleocapsids derived from purified DI particles had a buoyant density 0.02 g/ml greater than the nucleocapsids from standard virus. Analysis of the RNA from DI nucleocapsids showed it to be entirely of the low mol. wt. class.

To account therefore for the density difference not only between DI particles and standard virus but also between their respective nucleocapsids we propose that each SFV DI particle contains several molecules of the low mol. wt. RNA species.

INTRODUCTION

In recent years DI particles have been shown to occur in almost every animal virus group. DI particles frequently arise in the course of serial undiluted passaging of virus in tissue culture and they specifically interfere with the multiplication of their homologous non-defective standard virus. The DI particles differ from standard virions in that they contain only part of the virus genome and can therefore be considered as deletion mutants (Huang, 1973). As such they are unable to carry out all the steps in virus multiplication and consequently their production requires function(s) encoded by the genome of the standard virus.

The genome of alphaviruses such as Semliki Forest virus (SFV) and Sindbis virus is single-stranded RNA of mol. wt. 4.1 ± 0.3 × 10^6, which is infectious and which has a sedi-
mentation coefficient of about 42S (Friedman, Levy & Carter, 1966; Simmons & Strauss, 1972; Martin & Burke, 1974). A molecule of this RNA, together with some 240 core protein molecules, comprise the virus nucleocapsid (Laine, Söderlund & Renkonen, 1973). Surrounding the nucleocapsid is a lipoprotein envelope consisting of a host-cell-derived lipid bilayer (Laine et al. 1973) and about 240 molecules of each of three glycoproteins (Garoff & Simons, 1974). Some combination of these glycoprotein species make up the spike projections of the virus particle (Compans, 1971; Kennedy, 1974). A number of reports have described the isolation and characterization of DI particles of Sindbis virus (Schlesinger, Schlesinger & Burge, 1973; Shenk & Stollar, 1973; Weiss & Schlesinger, 1973). In common with other types of DI particles, Sindbis DI particles display homologous interference and contain the same complement of lipid and structural protein as standard virus. However, no significant difference in size between the RNA of DI particles and standard Sindbis virus could be detected using either sucrose gradient velocity centrifugation or polyacrylamide gel electrophoresis (Shenk & Stollar, 1973; Weiss & Schlesinger, 1973). This observation is surprising since if Sindbis DI particles, like other DI particles, are indeed deletion mutants, then their RNA would be expected to be somewhat smaller than that of standard virus. In addition to this question there is conflicting evidence as to the buoyant density of Sindbis DI particles. On the one hand, Shenk & Stollar (1973) reported that DI particles band with a density of 1.22 g/ml on sucrose/D2O equilibrium gradients. This compares with a value of 1.18 g/ml for standard virus. On the other hand, Weiss & Schlesinger (1973) were unable to separate DI particles from standard Sindbis using the same gradient technique. DI particles of other animal viruses are, on account of their smaller genome, less dense than their standard virus.

In an attempt to define the nature of the difference between standard and DI particles of alphaviruses we have isolated and characterized DI particles of SFV after serial passaging in BHK cells with a constant input multiplicity of 50 p.f.u./cell. These experiments showed that SFV DI particles have a higher buoyant density than standard virus, and that this increase in density appears to be due to the presence of multiple copies of RNA of mol. wt. 0.81 and 0.75 × 10^6 in the DI particles. Infectious RNA assays indicated that these low mol. wt. RNA species are the agents responsible for interference. Since no evidence of similar sized pieces of RNA have been reported in Sindbis DI particles, the DI particles of SFV reported here are a new type of alphavirus DI particle.

**METHODS**

**Materials.** Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A. Nonidet P40, CsCl and sodium dodecyl sulphate (especially pure grade) were obtained from British Drug Houses, Poole, Dorset. Medium 199 and Minimal Essential Medium (MEM) were supplied by the Wellcome Foundation, Beckenham, Kent. Goose erythrocytes were a generous gift from Dr C. Bradish, Microbiology Research Establishment, Porton Down, Salisbury, Wiltshire. 35S-L-methionine (94 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. All other chemicals were the best grade available commercially.

**Viruses and cells.** The ts' wild-type strain of SFV was plaque purified three times on monolayers of BHK cells as previously described for chick fibroblasts (Walters, Burke & Skehel, 1967). The AR339 strain of Sindbis virus used in the interference assays was plaque purified and grown through a single passage on chick fibroblasts as previously described (Atkins, Samuels & Kennedy, 1974). Monolayer cultures of BHK C13 cells were grown...
DI particles of SFV

in 2.5 l roller bottles or on 135 mm plastic Petri dishes, in supplemented MEM (Morser, Kennedy & Burke, 1973).

Infectivity and haemagglutination assays. The infectious titre and haemagglutinating activity (HA) of virus suspensions were determined as described by Atkins et al. (1974) and Kennedy & Burke (1972) respectively. Particles from CsCl gradients were dialysed at 4 °C against three changes of PBS before assay. On chick fibroblast monolayers, Sindbis virus produces uniformly large plaques (~ 3 mm in diam.) whereas SFV plaques are small (~ 1 mm in diam.). This observation was used in estimating the relative titre of the two viruses in suspensions obtained from mixedly infected cultures.

Conditions of serial passaging of SFV in BHK cells. A roller bottle culture of BHK cells was infected with plaque purified SFV at a m.o.i. of 0.005 and extracellular virus harvested after 48 h at 37 °C. This virus suspension (50 ml; 2.8 × 10^6 p.f.u./ml; HA of 256) was designated passage 0 or standard virus and used in the subsequent serial passaging experiments. Serial passaging was performed at a constant m.o.i. of 50 in roller bottle cultures of BHK cells and extracellular virus, harvested after 18 h at 37 °C was stored at −70 °C. In order to ensure adequate stocks of late passage virus, 5 to 8 roller bottle cultures were used from passage 5 onwards.

Radioisotopic labelling of virus particles. Pairs of Petri dish cultures of BHK cells were infected with 1 to 5 p.f.u./cell of either standard virus or passage 8 virus. After adsorption for 60 min at 37 °C the inoculum was replaced with maintenance medium (Morser et al. 1973) containing 0.1 μg/ml actinomycin D. After a further 1 h the culture fluids were replaced with maintenance medium containing 0.1 μg/ml actinomycin D and (i) 200 μCi/culture of 35S-methionine or (ii) 20 μCi/culture of 14C-uridine (standard virus infected cultures) or 200 μCi/culture of 3H-uridine (passage 8 infected cultures). Extracellular virus was harvested 17 h later and purified immediately.

Virus purification. The culture fluid from infected cells was clarified by centrifuging for 30 min at 10000g, concentrated using an ultrafilter to less than 20 ml (Kennedy, 1974) and then layered on to a 20 to 55% (w/v) linear sucrose gradient in 0.05 M-tris containing 0.1 M-NaCl, 1 mM-EDTA and 0.5% calf serum (pH 7.5; TNEC) prepared in a 65 ml tube of the 3 × 65 ml MSE rotor. After centrifugation at 4 °C for 16 h at 65000g the gradient was fractionated and a sample of each fraction taken for radioactivity measurements in 5 ml of Triton X-100 toluene scintillator (Kennedy & Burke, 1972). Appropriate fractions were pooled, diluted with 4 vol. TNEC and virus, collected by centrifuging at 110000g for 3 h at 4 °C, was gently resuspended in TNEC and either purified further or stored at 4 °C for up to 48 h. Virus stored at this stage is referred to as partially purified virus. Standard virus was purified by banding on a second sucrose gradient, identical to the first.

Separation of DI particles from standard virus. The particles from passage 8 infected cells which had been partially purified as described above, were applied to CsCl gradients, prepared by sequentially layering 1.95 ml of 1.1, 1.3 and 1.5 g CsCl per ml of 0.05 M-glycine + 0.1 M-KCl (pH 9.0) into a 65 ml tube of the 3 × 65 ml MSE rotor. On occasion purified standard virus was added as internal marker. Centrifugation was for 19 h at 24 °C at 70000g. All gradients were unloaded by upward displacement and a sample of selected fractions was taken for infectivity, buoyant density and radioactivity measurements.

SDS-polyacrylamide gel electrophoresis. The RNA and protein composition of standard and DI particles of SFV was analysed on 1.7% (w/v) acrylamide + 0.5% (w/v) agarose rod gels and 10% acrylamide slab gels respectively, as previously described (Clegg & Kennedy, 1974a; 1975).

Nucleocapsid analysis. Purified standard virus and DI particles in 50 mM-tris containing
Table 1. Yield of SFV during serial passaging

<table>
<thead>
<tr>
<th>Passage number</th>
<th>Infectious titre (p.f.u./ml)</th>
<th>HA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$2.8 \times 10^8$</td>
<td>256</td>
</tr>
<tr>
<td>1</td>
<td>$2.1 \times 10^8$</td>
<td>256</td>
</tr>
<tr>
<td>2</td>
<td>$1.8 \times 10^8$</td>
<td>256</td>
</tr>
<tr>
<td>3</td>
<td>$1.7 \times 10^8$</td>
<td>256</td>
</tr>
<tr>
<td>4</td>
<td>$2.0 \times 10^8$</td>
<td>256</td>
</tr>
<tr>
<td>5</td>
<td>$1.0 \times 10^8$</td>
<td>256</td>
</tr>
<tr>
<td>6</td>
<td>$7.1 \times 10^6$</td>
<td>128</td>
</tr>
<tr>
<td>7</td>
<td>$4.4 \times 10^6$</td>
<td>32</td>
</tr>
<tr>
<td>8</td>
<td>$3.6 \times 10^6$</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>9</td>
<td>$3.3 \times 10^5$</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

$0.1\text{ m-NaCl (pH 7.4; TN buffer)}$ were treated with 1% Nonidet P40 for 30 min at room temperature. The released nucleocapsids were analysed by equilibrium centrifugation at 65,000 g for 17 h at 4°C on 11 ml 30 to 55% (w/w) sodium potassium tartrate gradients containing 0.2% NP 40 (Acheson & Tamm, 1970). Gradients were unloaded by upward displacement and analysed as described above.

**Buoyant density determinations.** The refractive index of fractions from equilibrium gradients was converted to buoyant density by the use of empirically derived calibration graphs.

**Infectious RNA assays.** Standard virus and DI particles, purified by banding in CsCl were dialysed against five changes of 50 mM-tris containing 100 mM-NaCl and 1 mM-EDTA (pH 7.4) and their RNA extracted using phenol/SDS/2-mercaptoethanol (Martin & Burke, 1974). The RNA species of DI particles were separated on 6 to 30% (w/v) linear sucrose gradients (Atkins et al. 1974). RNA was assayed for infectivity in BHK cells as described by Crick et al. (1966) with the modifications that (i) the cells were exposed to $1\text{ m-NaCl}$ for 3 min and (ii) dilutions of RNA were made in $2\text{ m-MgSO}_4$ in $0.1\text{ m-tris (pH 7.5)}$ containing $1\text{ mg/ml DEAE-dextran}$.

**RESULTS**

Table 1 shows the infectious titre and HA of SFV produced during serial passaging in BHK cells infected with a constant m.o.i. of 50. From passage 0 to passage 5 the yield both of infectious virus and HA remained essentially constant. However, during passages 6 and 7 the yield significantly decreased and by passage 9 HA was undetectable and infectivity had fallen by almost $4\log_{10}$. Attempts to measure yields beyond passage 9 were complicated by the elution of infectious input virus. This problem could not be satisfactorily overcome either by repeated washings of the infected cells 2 h post-infection (p.i.) or by inactivation of eluted virus with antiserum or acid pH. This yield pattern is similar to that observed during the serial passaging of Sindbis virus (Schlesinger et al. 1973) in that the yield remained constant till passage 5 to 6 and then dropped. However, the SFV pattern differs from that for Sindbis virus in the extent of the drop. For Sindbis virus infectivity had decreased $2\log_{10}$ by passage 10 whereas for SFV a $4\log_{10}$ drop was observed by passage 9.

In order to determine if the drop in the yield of infectious virus was due to the presence of DI particles an interference analysis was performed on passage 8 fluid. For this study plaque-purified SFV was used as standard virus and plaque-purified Sindbis virus as heterologous control virus. The results (Table 2) show that the multiplication of standard SFV
Table 2. *Interference analysis on passage 8 SFV*

<table>
<thead>
<tr>
<th>Multiplicity of infection (p.f.u./cell)</th>
<th>Infectious titre of progeny virus (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque-purified SFV</td>
<td>Plaque-purified Sindbis virus</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>—</td>
<td>0.1</td>
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<td>I</td>
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<td>—</td>
<td>I</td>
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<tr>
<td>—</td>
<td>I</td>
</tr>
</tbody>
</table>

* These values were for large plaques (see Methods). In addition small plaques, characteristic of SFV, were observed at a dilution of 10^-4.

was severely inhibited by passage 8 virus. This inhibition was specific for SFV, there being no significant reduction in the yield of Sindbis virus grown in the presence of passage 8 SFV. Indeed, because of the marked difference in plaque size of SFV and Sindbis virus it was apparent that there was little or no interference in the growth of either virus due to the presence of the other. These results demonstrated that passage 8 fluid contained DI particles of SFV. We next determined to what extent the drop in infectious virus titre was paralleled by a drop in particle formation. This was done by labelling four identical cultures, two infected with standard virus, two with passage 8 virus (m.o.i. = 25), harvesting after a single cycle of virus multiplication (7 h), purifying the labelled progeny particles by sucrose gradient centrifugation and determining the ratios of total p.f.u. and of radioactivity. The titres were 8.7 × 10^9 p.f.u. and 9.3 × 10^6 p.f.u. for standard virus and passage 8 progeny particles respectively and the radioactivities were 1.7 × 10^7 ct/min in standard virus and 2.3 × 10^6 in passage 8 progeny particles. This gives a total p.f.u. ratio (of standard virus progeny to passage 8 progeny) of approx. 10^4, and a radioactivity ratio of approx. 10. Thus, although the drop in infectious virus yield was 4 log_10 there was only a 1 log_10 drop in total particle production. Some, if not most, of this 3 log difference is presumably due therefore to non-infectious DI particles.

To compare the structural properties of the DI particles with those of standard virus, attempts were made to find techniques which would separate standard virus from DI particles. To this end, freshly purified ¹⁴C-uridine labelled standard virus was mixed with partially purified ³H-uridine labelled progeny particles from cells infected with passage 8 virus and this mixture analysed by sucrose (Kennedy, 1974), sucrose – D₂O (Shenk & Stollar, 1973), 15 to 55 % (w/v) glycerol, 20 to 50 % (w/v) potassium tartrate and CsCl (see Methods) centrifugation. Only equilibrium banding on CsCl gave clear evidence of particles present in the harvest from passage 8 infected cells which had a different buoyant density than standard virus (Fig. 1). These particles had a higher buoyant density (1.23 g/ml) than standard virus (buoyant density 1.20 g/ml). After centrifugation on two further CsCl
gradients (Insets (a) and (b) of Fig. 1) the infectivity and interfering ability of these particles were examined. Although CsCl reduces the infectivity of SFV, standard virus (initial titre \(3.1 \times 10^9\) p.f.u./ml; 75 ml) purified as described for the '1.23' particles but using parallel CsCl gradients, had a titre of \(4.7 \times 10^7\) p.f.u./ml (2 ml). By contrast little or no infectivity (titre < 10 p.f.u./ml) was associated with the '1.23' particles. Moreover, the addition of the '1.23' particles to an equivalent (on a radioactivity basis) amount of purified standard virus severely inhibited (by 3.3 log_{10}) the multiplication of the standard SFV. These observations, therefore, establish the identity of the '1.23' particles as DI particles.

Since DI particles of other animal viruses are less dense than their respective parent it was important to determine what structural feature(s) of SFV DI particles make them heavier than standard virus. Firstly we examined the structural protein complement of standard
DI particles of SFV

Fig. 2. Polyacrylamide gel electrophoresis of the polypeptides from standard virus and DI particles. $^{35}$S-methionine labelled purified standard virus and DI particles purified through 3 cycles of CsCl banding were disrupted, reduced and alkylated and the virus polypeptides analysed on a 10% (w/v) polyacrylamide slab gel (Clegg & Kennedy, 1975). Approx. 150,000 cts/min was applied to each slot. Electrophoresis is from top to bottom. The polypeptides of standard virus (lane A) and of DI particles (lane B) were detected by autoradiography for 2 days. E1 and E2 are envelope glycoproteins; C is the core polypeptide. In this gel system the third glycoprotein migrates with the front.

Virus and DI particles (Fig. 2). No difference was observed either in the mol. wt. or in the labelling ratio of the polypeptides from the two different types of particle. Moreover, on an $E_{260}$ basis, both type of particle had equivalent HA activity. We next compared the RNA species present in standard virus and DI particles. $^{14}$C-uridine labelled RNA from purified standard virus was mixed with $^{3}$H-uridine labelled RNA from DI particles which had been purified by three successive bandings on CsCl equilibrium gradients and this mixture was analysed by polyacrylamide gel electrophoresis (Fig. 3). Whereas standard virus contained a single species of RNA with a mol. wt. of $4.2 \times 10^6$ (the 42S genome; Levin & Friedman, 1971), DI particles contained three species of RNA. Two of these, the major components, had mol. wt. of 0.81 and 0.75 $\times 10^6$ respectively as estimated from the mobility of BHK 28S and 18S rRNA; the third component had mobility identical to the standard virus genome. To determine if this species was identical to the standard virus genome in terms of genetic information, it was separated from the low mol. wt. species by sucrose gradient centrifugation (Fig. 4) and its specific infectivity compared both with the genome of standard virus and with the specific infectivity of the low mol. wt. species. As Table 3 shows the DI
Fig. 3. Polyacrylamide gel electrophoresis of the RNA from standard virus and DI particles.

$^{14}$C-uridine labelled purified standard virus and $^3$H-uridine labelled DI particles purified by
3 cycles of CsCl banding were mixed and their RNA extracted using the phenol/SDS/2-mercapto-
ethanol technique (Martin & Burke, 1974). The RNA was electrophoresed on a 20 cm 1:7 % (w/v)
polyacrylamide gel containing 0.5 % (w/v) agarose (see Methods). Migration is from left to right.
Arrows indicate the position of 28S and 18S BHK rRNA added as optical markers. The gel was
cut into 2 mm slices which were solubilized and counted for $^{14}$C (○—○) and $^3$H (●—●) radioactivity.

particle 42S RNA was as infectious (on a ct/min basis) as the 42S RNA from standard virus
and we therefore conclude that it is identical in terms of genetic information, to the genome
of standard virus. In contrast the low mol. wt. RNA species of DI particles were not
infectious. Assuming that these RNAs are related in nucleotide sequence to the 42S genome,
then it seemed likely that these species are the agents responsible for interference. To test
this idea directly we performed infectious RNA assays on 42S RNA (from standard virus)
in the presence of the low mol. wt. RNA species (added together). Addition of an estimated
fourfold molar excess of the low mol. wt. RNA species caused a drop of about 0.9 log$	ext{_{10}}$
in the infectivity of the 42S RNA (Table 3). This drop was somewhat variable, ranging
from 0.7 to 1.8 logs. This variability might reflect variation in the number of single cells
receiving both 42S and low mol. wt. RNA. This drop was however specific, added rRNA
having little or no effect on the infectivity of 42S RNA.

We considered two explanations for the presence of the genome in the RNA extracted
from preparations of purified DI particles. Either the genome was derived from residual
standard virus which, because of the large excess of DI particles, does not give rise to
Fig. 4. Sucrose gradient purification of the RNA species from a purified DI particle preparation. ³H-uridine labelled RNA from a preparation of DI particles purified by three successive bandings on CsCl was centrifuged on a 6 to 30 % (w/v) sucrose gradient as described in Methods. Sedimentation is from left to right. Arrows indicate the position of BHK 28S and 18S rRNA sedimented on a parallel gradient. Fractions, combined to give the two pools indicated by the bars, were used for the infectivity assays.

Table 3. Infectivity of the RNA from standard SFV and DI particles

<table>
<thead>
<tr>
<th>RNA* species</th>
<th>Source</th>
<th>Radioactivity (ct/min/ml)</th>
<th>Infectivity† (p.f.u./ml)</th>
<th>Specific infectivity (p.f.u./ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42S</td>
<td>Standard virus</td>
<td>1.71 × 10⁶</td>
<td>4.1 (±0.2) × 10⁶</td>
<td>23.97</td>
</tr>
<tr>
<td>42S</td>
<td>DI particles</td>
<td>2.58 × 10⁶</td>
<td>5.2 (±0.3) × 10⁴</td>
<td>20.15</td>
</tr>
<tr>
<td>Low mol.</td>
<td>DI particles</td>
<td>4.37 × 10⁴</td>
<td>Undetectable</td>
<td>0</td>
</tr>
<tr>
<td>wt. species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low mol.</td>
<td>DI particles</td>
<td>8.74 × 10⁴</td>
<td>1.6 (±0.5) × 10⁵</td>
<td>2.75†</td>
</tr>
<tr>
<td>wt. species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 42S</td>
<td>Standard virus</td>
<td>5.82 × 10⁴</td>
<td>2.71 × 10⁵</td>
<td>2.9 (±0.2) × 10⁶</td>
</tr>
<tr>
<td>+ rRNA§</td>
<td>BHK cells</td>
<td>—</td>
<td>3.9 (±0.2) × 10⁸</td>
<td></td>
</tr>
</tbody>
</table>

* The RNA species extracted from purified standard virus and DI particles were radiolabelled under identical conditions.
† The values reported are the mean (±S.E.M.) for six determinations. All determinations were performed on the same batch of BHK cultures (see Methods).
‡ Expressed relative to the ct/min. in the 42S RNA.
§ Prepared as described by Martin & Burke (1974) and added to a final concentration of 0.01 μg/ml, i.e. approximately equivalent in mass to the amount of the low mol. wt. species used in the other mixed infections.
Fig. 5. Equilibrium centrifugation of the nucleocapsids from standard virus and DI particles. 

$^{14}$C-uridine-labelled purified standard virus and $^{3}$H-uridine-labelled DI particles purified by three cycles of CsCl banding were dialysed against TN buffer and treated with 1 % NP40 as described in Methods. The released nucleocapsids were centrifuged to equilibrium on 30 to 50 % (w/w) sodium potassium tartrate gradients containing 0.2 % NP40. Sedimentation is from left to right.

Samples of each fraction were taken for $^{14}$C-radioactivity (○--○), $^{3}$H-radioactivity (●—●) and buoyant density (×—×) measurements. Fractions under the bar were pooled for RNA analysis.

plaques, i.e. each cell receiving an infectious standard virion also receives one or more DI particles, or a proportion of the DI particles contain both the genome and one or other or both, of the low mol. wt. species. In an attempt to resolve this question we prepared nucleocapsids from the DI particle preparation and analysed their RNA complement. Nucleocapsids were prepared by detergent lysis and equilibrium sedimentsations on tartrate gradients (Fig. 5). As was found for intact particles, nucleocapsids prepared from DI particles had a greater buoyant density ($\rho = 1.36$ g/ml) than the nucleocapsids from standard virus ($\rho = 1.34$ g/ml). Moreover, the nucleocapsids from DI particle preparations contained a small amount of material present as a shoulder on the main peak which banded at the same density as the nucleocapsids from standard virus. We therefore conclude that preparations of DI particles, although purified by 3 CsCl bandings, contained a small amount of standard virus and this standard virus was the source of the 42S RNA extracted from DI particle preparations. Confirmation of this came from the finding that RNA extracted from the ‘1.36 g/ml’ nucleocapsids was exclusively of the low mol. wt. size class (Fig. 6). Thus, DI particles of SFV contain only low mol. wt. RNA. Moreover, the observation that nucleocapsids from DI particles are denser than those of standard virus indicates that the density difference between DI particles and standard virus is probably not due to differences in their respective lipoprotein envelopes.

DISCUSSION

In common with many other animal viruses, serial high input multiplicity passaging of SFV leads to the accumulation of DI particles. For SFV these particles become apparent by passage 6 to 7 in BHK cells and by passage 9 the yield of infectious virus had dropped by
Fig. 6. Analysis of the RNA content of DI particle nucleocapsids. The RNA from DI particle nucleocapsids prepared as described in the legend to Fig. 5, was extracted with phenol/SDS/2-mercaptoethanol and analysed on a 20 cm 1.7 % (w/v) polyacrylamide gel containing 0.5 % agarose (see Methods). Migration is from left to right. The arrow indicates the position of 42S RNA from standard virus run on a parallel gel.

4 logs. Despite this drop in infectious virus yield the drop in total particle production was only about 1 log_{10} and it seems likely that the difference between these two values was largely, if not entirely, due to the presence of non-infectious DI particles.

Of the several methods evaluated to resolve standard virus from DI particles, only CsCl equilibrium centrifugation was successful. To identify the difference(s) between DI particles and standard virus we first examined their structural proteins. As with other types of DI particles, no difference was found between the structural proteins of SFV DI particles and standard virus. The RNA content of DI particles and standard virus was, however, found to be significantly different. Whereas, standard virus contained only 42S RNA, DI particles contained two low mol. wt. species of RNA. Infectious RNA studies strongly indicated that these RNAs are the agents responsible for interference. Since DI particles and nucleocapsids derived therefrom were found to be denser than their respective standard virus counterpart, it would appear that DI particles contain more RNA per particle than standard virus. Indeed, taking the mol. wt. of the 42S RNA genome as 4.2 x 10^6 (Levin & Friedman, 1971), each DI particle would appear to contain more than 4 molecules of the low mol. wt. species. Alternatively, DI particles may differ in size from standard virus. For example, they might be smaller with a more compact nucleocapsid surrounded by less lipoprotein envelope than standard virus. In this case each DI particle might only contain a single
molecule of low mol. wt. RNA. In any event it will be of considerable interest to examine the arrangement of the low mol. wt. RNA molecule(s) within the DI nucleocapsid.

Assuming that the low mol. wt. species of DI particles are related in nucleotide sequence to the viral genome, then several points can be made about these RNAs.

Firstly, in cells infected with standard virus about one-third of the virus genome is amplified in the form of 26S RNA which acts as a messenger for the structural proteins of the virus particle (Clegg & Kennedy, 1974b; 1975; Wengler, Beato & Hackemack, 1974). This RNA species is, however, not found in virus particles and therefore some portion of the genome distinct from the nucleotide sequence represented in 26S RNA presumably binds core protein molecules and initiates nucleocapsid assembly. Since the low mol. wt. species are found in virus particles, albeit DI particles, it would seem reasonable to suppose that these RNAs also contain the core protein 'binding site' and, therefore, that at least a portion of their sequence is different from the nucleotide sequence of the 26S RNA.

Secondly, there is the question of how the low mol. wt. species interfere with the multiplication of standard virus and indeed whether both species are interfering agents. Interference could either be direct or indirect, i.e. the RNAs might act per se as interfering agents or they could be translated to proteins which are the active agents.

Thirdly, it is interesting to note that the S value of the low mol. wt. RNAs (together) for SFV DI particles is 20S (Fig. 5). RNA species of similar S value have been reported in cells infected with DI particles of Sindbis virus (Shenk & Stollar, 1973; Weiss & Schlesinger, 1973). However, since Sindbis virus DI particles do not appear to contain the 20S species (Shenk & Stollar, 1973; Weiss & Schlesinger, 1973) then the low mol. wt. RNAs described in the present study may not be the counterpart of the Sindbis 20S species. Indeed the SFV DI particles described here are probably different from the Sindbis DI particles described by Weiss & Schlesinger (1973) and Shenk & Stollar (1973).

Finally, some explanation must be found for the appearance and selective propagation of SFV DI particles. This explanation will clearly centre around the properties of the two low mol. wt. species of the DI particles. Questions to be answered include not only how these species interfere, but what fault occurs in the multiplication machinery of standard virus which leads to their appearance and amplification. The following paper attempts to answer some of these questions.

REFERENCES


DI particles of SFV


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