Electron Microscopic Studies of Visna Virus Ribonucleic Acid

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SUMMARY

Visna virus particles disrupted by exposure to 0·05 or 0·1 % SDS release an internal nucleic acid component in the form of rings or short curvilinear rods. The ring structures have a mean circumference of 3·0 μm and are three times wider than single-stranded RNA. Incubation of detergent-disrupted virus particles with dimethylsulphoxide for 5 min causes uncoiling of rings and produces a heterogeneous population of single, unbranched filaments up to 9·3 μm long, similar in size to strands observed in 60 to 70S visna virus RNA recovered from glycerol velocity gradients. Treatment with dimethylsulphoxide for 30 min results in complete denaturing of the virus RNA into short fragments that average 3·2 μm in length. The visna virus genome consists of a molecule 9·3 μm long, apparently composed of subunits, which assumes a coiled configuration within the virus particle.

INTRODUCTION

Visna virus, the etiologic agent of a progressive neurological disease in sheep (Sigurdsson, Pálsson & Grímsson, 1957), resembles the RNA tumour viruses in its morphology and morphogenesis (Thormar, 1961; Coward, Harter & Morgan, 1970; Chippaux-Hyppolite et al. 1972; Takemoto et al. 1973). Like oncogenic RNA viruses, multiplication of visna virus is inhibited by actinomycin D and 5-bromodeoxyuridine (Thormar, 1965). In addition, visna virus particles contain an RNA-dependent DNA polymerase (Lin & Thormar, 1970; Schlom et al. 1971; Stone et al. 1971), other DNA polymerases (Schlom et al. 1971) and single-stranded RNA species similar to those of RNA tumour viruses (Brahic, Tamalet & Chippaux-Hyppolite, 1971; Harter, Schlom & Spiegelman, 1971; Lin & Thormar, 1971; Haase et al. 1974). The present communication reports the results of an electron microscopic study of the structure and configuration of visna virus nucleic acid as revealed by the Kleinschmidt technique (Kleinschmidt et al. 1962, 1964).

METHODS

Cell cultures. Sheep choroid plexus (SCP) cells were prepared by trypsin dispersion of choroid plexuses removed from the brains of exsanguinated sheep as previously described (Harter & Choppin, 1967). Cells were grown in 100 mm plastic dishes using nutrient mixture F-12 containing 10% foetal bovine serum and incubated at 36°C.

Virus. Visna virus K485, kindly supplied by Dr H. Thormar and Dr P. A. Pálsson, Institute of Experimental Pathology, University of Iceland, was propagated in SCP cells. Second and third passage virus was stored at −70°C and used as stock.
Infection of cells. Confluent monolayers were inoculated with visna virus at a multiplicity of 0.5 TCID<sub>50</sub>/cell. After adsorption for 4 h at 36 °C, Eagle's minimal essential medium containing 2% heat-inactivated lamb serum was added, and the cultures incubated at 36 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 48 h, when well-developed cytopathic changes appeared, medium was collected and fresh maintenance medium added. Medium was harvested every 24 h for 3 to 4 days until the cell monolayer was almost completely destroyed.

Preparation of radioactive virus. Medium was removed from the cultures 48 h after inoculation and replaced with fresh maintenance medium containing 15 μCi/ml [5-<sup>3H</sup>]-uridine (New England Nuclear Corporation, Boston, Massachusetts). Medium was harvested daily for 3 days and replaced by fresh medium containing [3H]-uridine.

Purification of virus. Medium from infected cultures was clarified by sedimentation at 15000 g for 10 min at 4 °C, and concentrated by precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (30 g/100 ml) at 4 °C for 1 h at pH 7.5 to 8. Precipitates were collected by sedimentation at 10400 g for 30 min, dissolved in TNE (0.01 M-tris, 0.1 M-NaCl, 0.001 M-EDTA, pH 8.3) and centrifuged at 63600 g for 1.5 h; pelleted material was suspended in 0.5 ml of TNE.

Concentrated virus was layered on a preformed linear gradient of 5% to 40% (w/w) potassium tartrate in 0.1 M-phosphate buffer (pH 7.0) containing 0.001 M-EDTA and centrifuged in a SW 41 swinging bucket rotor at 201000 g for 2.5 h. Banded virus sedimenting between 1.16 and 1.18 g/ml was collected, diluted in a large vol. of TNE, pelleted by sedimentation (201000 g, 45 min), resuspended and subjected to a second cycle of potassium tartrate density gradient sedimentation. Fractions from the gradient were collected by puncturing the bottom of the tube and a portion of each fraction was delivered to a vial containing scintillation fluid (Aquasol, New England Nuclear Corporation, Boston, Massachusetts) and radioactivity determined. The density of selected fractions was monitored using an Abbe refractometer. Fractions in the radioactivity peak corresponding to the density of visna virus particles were pooled, diluted with TNE, pelleted by sedimentation and resuspended in 2.0 ml of the same buffer. Unlabelled virus was collected from the visible band.

Extraction of virus nucleic acid. Nucleic acid was extracted from purified visna virus particles by treatment with 1% SDS in the presence of pronase (0.5 mg/ml), 0.1 M-NaCl, 0.1 M-tris (pH 7.4), 0.01 M-EDTA and 1% mercaptoethanol at 36 °C, for 30 min. The reaction mixture was then extracted twice with equal vol. of cresol-phenol (pH 8.4) and chloroform-isooamyl alcohol at 4 °C, for 10 min. Nucleic acid was precipitated from the aqueous phase by the addition of NaCl (0.4 M final concentration) and 2 vol. of absolute ethanol and stored at -20 °C.

Glycerol gradient sedimentation analysis. Nucleic acid was pelleted from ethanol by sedimentation at 24000 g for 30 min at 0 °C, resuspended in 0.5 ml TNE and layered onto a preformed density gradient of 10% to 30% (v/v) glycerol in the same buffer. The gradients were centrifuged at 201000 g for 2.5 h at 4 °C, in a SW 41 swinging bucket rotor. 0.3 ml fractions were collected by puncturing the bottom of the tube.

In the analysis of nucleic acids from radioactively labelled virus, 5 μl was removed from each fraction, mixed with Aquasol scintillation fluid and counted for radioactivity. When unlabelled virus was used, the extinction at 260 μm was measured for each fraction. 35S[<sup>14</sup>C]-labelled poliovirus RNA and 18S and 28S [3H]-labelled RNA extracted from VERO monkey kidney cells were used as external markers in sedimentation analysis.

Disruption of purified visna virus particles. Purified visna virus particles were exposed to SDS which had been doubly-crystallized from ethanol. Virus was disrupted by adding SDS.
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in TNE buffer at concentrations of 0.05%, 0.1%, 0.5% or 1.0% to 0.1 ml of purified virus. The treatment was carried out independently at 4 °C, 15 °C, and 50 °C. After 5 min, the mixtures were processed for electron microscopy.

**Dimethyl sulphoxide (DMSO) treatment.** DMSO was added to 10 μl of SDS-treated visna virus or 60 to 70S virus RNA to give a final concentration of 99%. The mixture was incubated at 5 °C, 20 °C, or 50 °C, for 5 min or at 50 °C, for 30 min, before preparation for electron microscopy.

**Ribonuclease and deoxyribonuclease digestion.** Purified virus particles treated with either 0.05% SDS alone or 0.05% SDS and 99% DMSO were exposed to bovine pancreatic ribonuclease (50 μg/ml) (Worthington Biochemical Corp., Freehold, New Jersey) or chromatographically prepared deoxyribonuclease I (100 μg/ml; Worthington Biochemical Corp.). Samples were incubated for 15 min, at 37 °C, in a 100 μl reaction mixture. When deoxyribonuclease I treatment was used, MgCl₂ was added to a final concentration of 0.001 M.

**Preparation for electron microscopy.** A modified Kleinschmidt technique (Kleinschmidt et al. 1962, 1964) was used in which 10 μl of 0.1% cytochrome c and 10 μl of 3 M-ammonium acetate – 0.1 M-tris buffer (pH 7.2) were freshly mixed. 20 μl of SDS-treated virus was added to this mixture. After gentle agitation, 5 μl was withdrawn and spread on a hypophase containing 0.3 M-ammonium acetate – 0.1 M-tris buffer (pH 7.2) in a paraffin-coated depression slide. 60 to 70S fractions from glycerol equilibrium density gradients were spread immediately after collection. The spreading solution, containing a denaturant, consisted of RNA (0.1 μg/ml), 0.03% cytochrome, 1.0 M-ammonium acetate, 0.03 M-tris buffer (pH 7.2) and 40% formamide. 5 μl of this mixture was then spread on a hypophase containing 0.3 M-ammonium acetate, 0.1 M-tris buffer (pH 7.0) and 10% formamide. A carbon-coated grid was used to pick up a small drop from the hypophase surface. The grid was dehydrated in 100% ethanol and shadowed with 5 mg of palladium-gold (40:60) at an angle of 10° under a vacuum of 3 × 10⁻⁵ mm Hg. A Denton DV-502 high vacuum evaporator with a rotary table (40 rev/min) was employed in all experiments.

**Electron microscopy.** A Philips 200 electron microscope with 20 μm foil objective apertures and 60 kV accelerating voltage was used. Magnification was calibrated with a carbon grating replica, 2160 lines/mm (Ernest F. Fullam, Inc., Schenectady, New York). Filaments were measured by tracing with a map ruler.

**Nucleic acid control standards.** Purified poliovirus RNA was prepared according to Summers (1966), herpes simplex virus (HSV) DNA according to Becker, Dym & Sarov (1968).

Using the electron microscopic techniques outlined above, poliovirus RNA molecules averaged 96 A in width and 2.2 μm in length, a value in accordance with the findings of others (Granboulan & Girard, 1969; McGregor & Mayor, 1968). The mean length of HSV DNA was 51 μm, a determination in agreement with the findings of Becker et al. (1968).

**RESULTS**

**Configuration and length of visna virus RNA sedimenting at 60 to 70S in velocity gradients**

The distribution of $E_{260}$ and radioactivity of nucleic acids extracted from purified visna virus particles after velocity sedimentation in glycerol gradients was identical to that previously reported (Harter et al. 1971).

Fractions containing maximum $E_{260}$ or [3H] activity and sedimenting in the 60 to 70S region of the gradient were examined by the modified Kleinschmidt technique. Spreading
Fig. 1. (a), (b) Filamentous RNA molecules sedimenting at 60 to 70 S in a 10 to 30% (v/v) glycerol velocity gradient.

Fig. 2. Branched filaments in 60 to 70 S RNA following velocity sedimentation in a 10 to 30% glycerol gradient. Arrows indicate branches.
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in the presence of 40% formamide resulted in a heterogeneous population of molecules, but two forms predominated; long, single filamentous strands (Fig. 1a, b) and branched strands with numerous free ends (Fig. 2). The branches always appeared thinner than the main strand. Presumably, the molecules become tangled and interwoven during sedimentation analysis and cannot be completely separated under the spreading conditions employed. These two forms made up approx. 80% of the structures seen in peak fractions from the 60 to 70S region. The remaining forms were complex coiled or interwoven structures (Table 1).

The length distribution of the filamentous strands is shown in Fig. 3a. The major populations were 6 to 9 μm long, but there were also numerous small single filaments only 1 to 3 μm in length. The shortest filament was 1.0 μm, the longest 9.3 μm. The width of a single unbranched filament was 96 Å.

Fig. 3. Histogram of the length distribution of visna virus RNA molecules sedimenting at 60 to 70S in a 10 to 30% (v/v) glycerol gradient, (a), spread on water hypophase in presence of 40% formamide, (b) treated with 99% DMSO, 50°C, 5 min before spreading, (c) treated with 99% DMSO, 50°C, 30 min before spreading; only one molecule 9.3 μm long was observed.
### Table I. Molecular configurations released from SDS-disrupted visna virus particles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Distribution of configurations %*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rings</td>
</tr>
<tr>
<td>Virus + 1 % SDS, 50 °C, 5 min</td>
<td>0</td>
</tr>
<tr>
<td>Virus + 0.5 % SDS, 50 °C, 5 min</td>
<td>1</td>
</tr>
<tr>
<td>Virus + 0.1 % SDS, 50 °C, 5 min</td>
<td>25</td>
</tr>
<tr>
<td>Virus + 0.05 % SDS, 50 °C, 5 min</td>
<td>65</td>
</tr>
<tr>
<td>Virus + 0.05 % SDS + 99 % DMSO, 50 °C, 5 min</td>
<td>8</td>
</tr>
<tr>
<td>Virus + 0.05 % SDS + 99 % DMSO, 50 °C, 30 min</td>
<td>0</td>
</tr>
<tr>
<td>60 to 70S RNA</td>
<td>0</td>
</tr>
<tr>
<td>60 to 70S RNA + 99 % DMSO, 50 °C, 5 min</td>
<td>0</td>
</tr>
<tr>
<td>60 to 70S RNA + 99 % DMSO, 50 °C, 30 min</td>
<td>0</td>
</tr>
</tbody>
</table>

* Calculation based on counting 500 molecules in each preparation.

### Structures released by SDS treatment of visna virus particles

To study the configuration of the nucleic acid within the virus particle, purified visna virus particles were disrupted by exposure to SDS. The extent of virus lysis could be controlled by varying the SDS concentration and the temperature of the reaction.

Exposure of virus particles to higher SDS concentrations (0.5 % and 1.0 %) released complex coiled and twisted structures (Fig. 4). Because of the irregularity in their coiling and folding, it proved impossible to accurately measure the length of these molecules. When 1.0 % SDS was applied at 5 °C, 20 °C or 50 °C, disrupted particles outnumbered intact virus particles. When 0.5 % SDS was used at 20 °C or 50 °C, over one-third of the particles were disrupted, and complex, folded coils were observed. After exposure to 0.5 % SDS at 5 °C, occasional thick, rod-shaped forms were also seen.

Although lower SDS concentrations (0.05 and 0.1 %) disrupted fewer particles, more clearly defined structures were reproducibly obtained. Again, fewer intact virus particles were seen when the reaction was carried out at higher temperatures. Treatment with 0.1 % SDS released two structures; simple rings and short, thick, curvilinear rods. Such rods and rings comprised more than two-thirds of the structures obtained after exposure to lower SDS concentrations (Table I). At the lowest SDS concentration used (0.05 %), rings were particularly prominent; 65 % of counted forms (Fig. 5). The mean circumference of 531 ring structures measured was 3.0 µm (s.d. = 1.021). The mean width of strands of the same molecules was 289.7 Å (s.d. = 50.33). At times, the rings appeared to be uncoiling (Fig. 6).

#### Effect of DMSO on SDS-disrupted visna virus particles and 60 to 70S visna virus RNA

Experiments were performed to determine if denaturing reagents such as DMSO, which dissociates rapidly-sedimenting tumour virus RNA, would affect unwinding of the circular structures produced by mild SDS disruption of virus particles. Treatment of purified virus particles with 0.05 % SDS at 50 °C, followed by exposure to 99 % DMSO at 50 °C for 5 min, resulted in the release of a heterogeneous population of linear filaments (Table I, Fig. 7a). Branched filaments were only occasionally seen. The longest extended molecules were 9.3 µm long and 96 Å wide. These measurements are in agreement with those obtained by spreading 60 to 70S visna virus RNA. Extended treatment of SDS-disrupted virus particles
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Fig. 4. Complex coil and interwoven strands released from virus particles by 1.0% SDS, 50 °C.
Fig. 5. (a), (b), (c) ring forms released from virus particles by 0.05% SDS, 50 °C.
Fig. 6. (a), (b) unfolding ring forms observed in preparations of visna virus treated with 0.05%, 50 °C.
with 99% DMSO for 30 min at 50 °C resulted in the release of filaments ranging from 2.0 to 4.0 μm (Fig. 7b). It is of particular interest that molecules 9.3 μm long were rarely observed under these conditions. These findings indicate that prolonged DMSO treatment (50 °C, 30 min) unfolds the ring structure released from SDS-disrupted particles and denatures the total RNA population into smaller fragments. Under less stringent conditions (50 °C, 5 min), the unfolding ring structures are only partially denatured revealing numerous molecules 9.3 μm long which are similar to those observed in preparations of untreated 60 to 70S virus RNA.

To further study the effect of DMSO on the virus genome, 60 to 70S RNA recovered from velocity gradients was treated with the reagent. Incubation with 99% DMSO at 50 °C for 5 min resulted in partial denaturation of the RNA molecules. There remained, however, numerous molecules 6.0 to 8.0 μm in length and occasional filaments 9.0 to 9.3 μm long (Fig. 3b). Such limited exposure to DMSO appeared to only partially denature the 60 to 70S RNA molecules. In contrast, treatment with 99% DMSO at 50 °C for 30 min reduced the average length of filaments to 3.2 μm (Fig. 3c), and very few longer filaments were observed. A χ² test was conducted to determine if the results shown in Fig. 3c and 7b indicated a denaturation of the 9.3 μm RNA into subunits or a random breakage of the molecule. It was found that normality can be accepted for the curve distribution at 97% for Fig. 3c and 92% for Fig. 7b. These results indicate that the 9.3 μm molecule denatures into subunits (mean length = 3.2 μm) under the conditions employed. Based on the normality of the curve distribution, a ‘t’ test was employed to determine if Fig. 3c and 7b were comparable. It can be accepted at 99%. A ‘t’ test of the data compared in Fig. 3b and 7a (5 min exposure to DMSO) showed an equality of the means acceptable at 98%.
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Table 2. Effect of ribonuclease and deoxyribonuclease on structures released from visna virus particles

<table>
<thead>
<tr>
<th>Distribution of configurations after nuclease treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation Ribonuclease Deoxyribonuclease Control</td>
</tr>
<tr>
<td>Virus + 0.05 % SDS 1% small filaments 66% long filaments 8% long filaments</td>
</tr>
<tr>
<td>+ 99% DMSO, 50 °C, 5 min 99% very small filaments (2 to 4 μm) (6 to 9 μm) (6 to 9 μm)</td>
</tr>
<tr>
<td>50 °C, 5 min 62% filaments (3 to 5 μm) 58% filaments (4 to 5 μm)</td>
</tr>
<tr>
<td>36% small filaments (1 to 2 μm) 32% small filaments (1 to 2 μm)</td>
</tr>
<tr>
<td>Virus + 0.05 % SDS 12% rings 41% rings 45% rings</td>
</tr>
<tr>
<td>88% small filaments 38% filaments 28% filaments</td>
</tr>
<tr>
<td>21% small filaments 27% small filaments</td>
</tr>
</tbody>
</table>

* Pancreatic ribonuclease (50 μg/ml) or deoxyribonuclease I (100 μg/ml), 37 °C, 15 min.

Effects of ribonuclease and deoxyribonuclease on structures obtained from SDS-disrupted visna virus particles

Filaments obtained from visna virus particles treated with 0.05 % SDS and 99 % DMSO were digested by ribonuclease (Table 2). Ring forms appearing in virus preparations treated with 0.05 % SDS alone were partially digested by ribonuclease, but unaffected by deoxyribonuclease I. The specificity of the deoxyribonuclease I was demonstrated by its ability to completely digest a preparation of HSV [³H]-DNA in the presence of 0.1 % SDS.

DISCUSSION

The major RNA species recovered from visna virus particles sediments in a fairly broad peak between 60 and 70 S when analysed by velocity gradient sedimentation (Brahic et al. 1971; Lin & Thormar, 1971; Harter et al. 1971; Haase et al. 1974). This high mol. wt. RNA cosediments with the 70 S RNA of Rous sarcoma virus (RSV) consistent with a mol. wt. of 10 to 12 x 10⁶ (Haase et al. 1974). Under the conditions used in this study, poliovirus RNA was found to have a linear density of 1.14 x 10⁶ daltons/μm. Based on this standard, the longest filament found in 60 to 70 S visna RNA or in uncoiled structures released from disrupted virus particles (9–3 μm) would have a mol. wt. of 10.4 x 10⁶, a value which approximates to that obtained by sedimentation analysis.

Electron microscopic studies of 60 to 70 S RNA extracted from avian myeloblastosis virus (Granboulan, Huppert & Lacour, 1966) and murine leukaemia virus (MLV) (Kakefuda & Bader, 1969) show a degree of heterogeneity similar to that observed in 60 to 70 S visna virus RNA. This heterogeneity in the length of the RNA may be a consequence of fragmentation into random pieces during experimental procedures or an indication that the genome is organized into subunits of defined length.

Previous attempts to demonstrate discrete subunits in 60 to 70 S visna virus RNA have been reported. Heating 63 S visna virus RNA to 60 °C for 3 min converted the RNA into a broad peak sedimenting between 4 and 385 in velocity gradients (Lin & Thormar, 1971). Using DMSO, a broader peak extending from 4 to 49 S was obtained. Under these conditions, the size of specific subunits could not be determined. After heating 70 S visna virus RNA to 80 °C for 2 min, the majority of the RNA species co-migrate with subunit RNAs of RSV in polyacrylamide gel electrophoresis (Haase et al. 1974). The mol. wt. of these molecules was 2.8 x 10⁶, indicating that there may be three or four subunits in the 70 S
genome. In our experiments, prolonged DMSO treatment denatured the RNA population into smaller fragments. Although the majority of molecules after incubation with 99% DMSO at 50°C for 30 min were 2.0 to 3.0 μm in length, a population of molecules of heterogeneous length remained. Statistical analysis, however, supports the impression that the 9.3 μm long filaments were denatured into discrete subunits. The mean length of these subunits was 3.2 μm under the conditions employed.

If the 9.3 μm long filament observed in both 60 to 70S RNA and preparations of un-coiled rings is composed of subunits, double-stranded regions might be expected. These electron microscopic studies, however, do not show regions of double-strandedness or reduplication intercalated in long strands. Similar comments have been made about the linear filaments extracted from MLV (Kakefuda & Bader, 1969). Nevertheless, the possibility that junctures between genomic subunits remain unrecognized by current electron microscopic methods cannot be discounted.

Haase et al. (1974) showed that maintaining 70S visna RNA molecules was a function of high ionic strength. In the present study, the ionic strength at all stages of the experiments was 0.1 M-Na⁺ or NH₄⁺. Possibly, these conditions account for the long filaments observed in preparations treated with 99% DMSO at 50°C for 5 min. In addition, the presence of virus protein in the ring structures released from SDS-disrupted virus particles might aid in stabilizing molecules during short term exposure to DMSO.

Internal concentric ring structures have been seen in negatively stained visna virus particles (Thormar & Cruickshank, 1965; Pautrat et al. 1971; Takemoto et al. 1973). Similar internal strand-like structures have been observed in oncogenic RNA viruses (Luftig & Kilham, 1971; Sarkar, Nowinski & Moore, 1971). The ring forms released after SDS treatment may be identical to the coiled components observed in intact particles. The width of the rings is approximately three times that of a single linear strand; their
Visna virus ribonucleic acid circumference is roughly one-third that of the length of the longest filament obtained when the rings unwind in the presence of DMSO. The virus internal component may form by coiling a linear segment folded twice or by continuous winding of a single strand (Fig. 8).

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