Electron Microscopical Observations of 
the Structure of the Virus of Viral Haemorrhagic Septicaemia (VHS) 
of Rainbow Trout (Salmo gairdneri)

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SUMMARY

Negative staining of particles of the Danish F1 strain of viral haemorrhagic septicaemia virus grown in rainbow trout gonad-2 cells revealed three different types of particles: bacilliform, bullet-shaped, and long particles. The average size of the first two types was 165 x 65 nm. The long particles had a length of up to 3000 nm and showed a close resemblance to Marburg virus. Well defined surface projections could be found in all particle types. Without any special treatment we were able to demonstrate different disintegration stages of VHS virus particles in all preparations.

INTRODUCTION

The structure of Egtved virus (virus of viral haemorrhagic septicaemia, VHS) of rainbow trout has been described by Zwillenberg, Jensen & Zwillenberg (1965) and by de Kinkelin & Scherrer (1970). In these investigations, the virus particles have been characterized as being cylindrical with an average diam. and length of 65 x 180 nm, and 75 x 240 nm respectively. The particles are described as being ‘bullet-shaped’, and show a helical symmetry. Surface projections have not been found by these authors.

The morphology of VHS virus is similar to that of most of the viruses of the rhabdovirus group, including rabies virus (Almeida et al. 1962; Hummeler, Koprowski & Wiktor, 1967) and vesicular stomatitis virus (VSV; Howatson & Whitmore, 1962; Nakai & Howatson, 1968; Cartwright, Smale & Brown, 1969; Simpson, Hauser & Dales, 1969). Three isolates, which are considered to cause haematopoietic necrosis in salmonid fishes – infectious haematopoietic necrosis (IHN) virus, Oregon sockeye salmon virus (OSV) and Sacramento River Chinook salmon disease (SRCD) virus – have been investigated by Amend & Chambers (1970). Recently, another study on these North American salmonid isolates (OSV, IHN, and CSV, which is synonymous to SRCD), which was carried out by Darlington, Trafford & Wolf (1972), showed a morphology similar to VHS virus.

In the present investigation, the known morphological facts about VHS (Egtved) virus are combined with some further observations on the ultrastructure of this virus.

METHODS

Cell cultures. Monolayers of RTG-2 cells (Wolf & Quimby, 1962) were grown in 500 ml glass bottles containing Eagle’s minimal essential medium (MEM) supplemented with 10 % (v/v) foetal bovine serum. Antibiotics used were 100 international units (iu) penicillin and 0.1 mg streptomycin/ml. The cultures were incubated at 15 °C.

Virus. The Danish F1 strain (Jensen, 1965) in its 108th passage was used for this study. It
was purified by three consecutive plaque isolations, and was grown in RTG-2 cells in Eagle's MEM without serum.

**Virus assay.** Clarified infectious tissue culture fluids were diluted in tenfold steps to $10^{-8}$, and 0.1 ml was pipetted into each well of the microtitre plates (Greiner, Nürtingen); four wells were used per dilution step. One drop of suspended cells (600,000 cells/ml) was added to each well, and the plates were sealed with adhesive tape and incubated at 15 °C. Ten days later, the plates were examined for c.p.e. (Frost & Wellhausen, 1974) and the infectivity titres calculated by the Kärber Method (Kärber, 1964).

**Virus concentration.** Harvested virus, with an infectious titre of not less than $10^7$ TCD$_{50}$/0.1 ml, was fixed with 0.1% (v/v) formaldehyde for 16 h at 4 °C (F. Brown, personal communication) and sedimented by ultracentrifugation in a Spinco rotor SW 25.1 at 18,000 rev/min for 2 h. The pellet was resuspended in double distilled water. Fixation was checked by titration. Infectious titres never exceeded $10^8$ TCD$_{50}$, whereas unfixed controls were better than $10^{8.5}$ TCD$_{50}$/0.1 ml. In an additional preparation, the supernatant fluid of the infected cell cultures was treated with 7% (v/v) polyethyleneglycol, and centrifuged at 12,000 g for 1 h. The resulting pellet was resuspended in tris-EDTA-NaCl-buffer (TEN) and sedimented by ultracentrifugation in a Spinco rotor SW 25.1 at 60,000 g for 1 h. The pellet was resuspended in double distilled water and used for negative staining preparations.

**Electron microscopy.** For the electron microscopical examination of the virus, the following preparations were used: (1) formaldehyde fixed and pelleted virus, which was resuspended in double distilled water; (2) a polyethyleneglycol precipitated sample; (3) untreated supernatant fluid of the infected cell cultures. Negative staining was carried out by using carbonized formvar coated copper grids which had been stored in an amyl acetate atmosphere to remove the underlying formvar film. One drop of the virus preparation was placed onto the grid and the fluid removed after 1 min by filter paper. For negative staining, a drop of either 0.5% (w/v) phosphotungstic acid, 0.5% (w/v) uranyl acetate or 0.5% (w/v) sodium silicotungstate was added without adjusting pH, and allowed to remain on the grid from 30 s to 1 min before it was removed by filter paper. The untreated culture fluid was washed on the grid with 1 or 2 drops of double distilled water before negative staining in order to lower the possibility of an uncontrolled chemical reaction between the culture medium and the staining solution. The size of 33 particles out of different preparations (excluding oblong particles) was measured on microscope plates and on enlarged photographs. The standard deviation method was used to determine if the measurements were within the statistical limits.

**RESULTS**

The extremely fragile particles of VHS virus tended to disintegrate during preparation. Two types of particles were found in all samples. The first consisted of bacilliform particles, which were rounded on both ends (Fig. 1a, b), whereas the second was represented by bullet-shaped particles (Fig. 1c, d). The envelope was present in many particles. In some cases, however, it seemed to be partly stripped off, while other particles appeared to be naked (Fig. 2). Several showed an axial structure which was occasionally penetrated by the negative staining solution (Fig. 3). A clear cross striation was visible in most of the particles. In the polyethyleneglycol preparation, we found many opaque particles of different size, whose interior appeared to be featureless. However, surface projections with a regular outline were present (Fig. 4) which were approx. 5.5 nm long and had an average diam. of 4.5 nm. The irregularity of the particle shape was presumably caused by ultracentrifugation and by the treatment with polyethyleneglycol.
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Fig. 1. Bacilliform (a, b), and bullet-shaped (c, d) particles. Unfixed specimen, negatively stained with 0.5% uranyl acetate.

Fig. 2. Loss of envelope: (a) envelope almost completely present; (b, c) envelope only present at the hemispherical ends of the particles; (d) entire loss of envelope so that the particle appears 'naked'. Unfixed specimen, negatively stained with 0.5% uranyl acetate (a, d) or 0.5% sodium silicotungstate (b, c).

Fig. 3. Particles showing an axial structure penetrated by negative staining solution: (a) wide axial space; (b) smaller central part penetrated; (c) axial structure penetrated, particle showing a terminal tail-like structure. (a) Unfixed specimen, negatively stained with 0.5% sodium silicotungstate; (b, c) fixed specimen, negatively stained with 0.5% uranyl acetate.
The average length of the intact bacilliform and bullet-shaped particles (excluding the oblong particles) was 165 nm. The average diam. of enveloped particles was 65 nm (the thickness of the envelope was approx. 10 nm) and that of naked particles 50 nm. The measurements revealed that naked particles had a slightly larger cross diam. than the internal (striated) structure of enveloped particles. The reason for the difference was not determined. The axial structure which was sometimes visible had an average width of 23 nm. In some particles the striated structure and the envelope appeared to be separated by an intermediate space approx. 1.5 nm wide (see Fig. 1). The stria had an average thickness of 4.5 nm and were separated from each other by a gap of 2.5 nm. The periodicity of the stria was 7 nm. The stria appeared to be composed of a double line of dot-like subunits of approx. 1 nm in diam. (see Fig. 2d). The standard deviation method revealed only small deviations from the mean values of the particle width and the striaion thickness and distance, whereas the deviations from the calculated mean particle length were not within the expected statistical limits.

In addition to the above mentioned types, we found very long particles up to a length of
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Fig. 6. Very long particle (similar to Marburg virus): (a) complete particle; (b) enlargement of the convoluted part; (c) enlargement of the lower part, showing the envelope partly stripped off; (d, e) enlargement of the upper distal part of particle shown in (a); (f) ring-like particle. Unfixed specimen, negatively stained with 0.5% sodium silicotungstate.

3000 nm. Some of those seemed to be made up of segments which had about the same length as the ‘normal’ particles, but others did not appear to possess smaller units. The diam. and the internal structure did not differ from the ‘normal’ type of particles described above (Fig. 5 and 6).

Various stages of particle disintegration were seen in almost all preparations. The first sign of decomposition was probably the partial removal of the envelope, or its entire loss (Fig. 7). Then the striation seemed to become irregular and the particles started to break into pieces of different sizes which was presumably the reason for the impossibility of determining the regular length of the VHS virus particles. A further step in disintegration was the occurrence of helical or ring-like strands with a helix diam. slightly smaller than that of the naked particles (Fig. 7e). The strands of these helices had a diam. of 2.2 nm and they showed small dot-like substructures (Fig. 7e, see arrow). Some particles, which were obviously in an advanced stage of disintegration, showed an extending tail-like cylindrical structure at their planar end (Fig. 7c). These structures were also found separately and varied considerably in length, with a diam. of approx. 23 nm. This structure appeared to be a tightly twisted helix which displayed regularly spaced turns (see Fig. 7f). This led to the appearance of two zig-zag parallel lines of subunits with a diam. of about 2.2 nm/unit. In the vicinity, strands arranged in loose helices were found, which may have derived from the tightly wound helices (Fig. 7g, h).
DISCUSSION

The findings of this investigation differ in some points from the results of Zwillenberg et al. (1965) and de Kinkelin & Scherrer (1970). The average diam. of the enveloped particles measured 65 nm (excluding surface projections) and the average length 165 nm, whereas Zwillenberg et al. (1965) found a size of 70 × 180 nm and de Kinkelin & Scherrer (1970) reported a size of 75 × 240 nm. These size deviations are probably due to the different preparation procedures used. The differences in particle length are presumably caused by the different disintegration stages within the actual preparation.

Measurement of the striation revealed a thickness of 4.5 nm/stria and a periodicity of 2.5 nm. Zwillenberg et al. (1965) gave a thickness of 3 nm, and a periodicity of 5.5 nm. The envelope measured 10 nm in thickness in contrast to 15 nm reported by Zwillenberg et al. (1965). Furthermore, we found bacilliform particles and well defined surface projections not described before.

According to the findings of Zwillenberg et al. (1965) on the occurrence of a central hollow, we also believe that this is an artifact, probably due to the loss of the internal component during particle disintegration. In connection with this, we found an axial structure with an average width of 23 nm, whose size was similar to the observed tail-like structure.
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(also described by Amend & Chambers, 1970, in IHN, OSV, and SRCD viruses and by Wolanski, Francki & Chambers, 1967, in lettuce necrotic yellows virus, LNYV), and to the separately found cylindrical elements. In virtue of the perpendicular structures also observed between the two parallel lines (showing the zig-zag arrangement of dot-like subunits) we suppose that these elements represent the helically arranged nucleoprotein of VHS virus. We suggest that the undulating strands in the vicinity of the above mentioned cylindrical elements are the uncoiled form of the nucleoprotein. Some of these findings correspond with the observation of Zwillingenberg et al. (1965), who described inclusion bodies in thin sections of RTG-2 cells, consisting of helices with a diam. of about 20 nm. These helices were thought to be the nucleocapsids of the virus by these authors.

It was not possible to determine the factors responsible for the spontaneous disintegration of the particles. Even the formaldehyde fixed samples did not differ much from untreated ones. The extreme fragility of rhabdovirus fish pathogens and VSV is reported by several other authors (e.g. Darlington et al. 1972). This fact leads to the conclusion that it is very difficult to characterize a certain VHS particle as being ‘normal’ shaped. In addition it is not yet known which structure represents the smallest infectious unit of this virus. A comparison with Marburg virus (Almeida, Waterson & Simpson, 1971; Peters, Müller & Slenczka, 1971) revealed a remarkable similarity with the oblong type of VHS particles that we found. Furthermore, particles could be seen (Fig. 6f) which are comparable to the ‘torus’ form of Marburg virus described by Almeida et al. (1971). It has not been determined whether these special structures represent an infectious form of VHS virus, or if they are only a casual event in virus replication. On the other hand, some of the long particles showed constrictions at regular intervals, which may have been the sites where partition into particles of ‘normal’ size occurs. Similar long particles have been observed with several other members of the rhabdovirus group (VSV, Howatson & Whitmore, 1962; Flanders Hart Park virus, Murphy, Coleman & Whitfield, 1966). Hummeler et al. (1967) described long and branched forms of rabies virus, Matsumoto (1962) found rabies particles of a length of 1900 nm in thin sections of baby mice organs and Davies et al. (1963) reported on segmented long forms of rabies virus.

These observations of extremely long particles of members of the rhabdovirus group suggest that they are common in this virus group. In the case of VHS virus the mode of release from infected cells remains to be investigated.

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