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Surface Structure of Uukuniemi Virus

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Uukuniemi virus, grown in chicken embryo fibroblasts, has been studied by electron microscopy using negative staining, thin sectioning, and freeze-etching techniques. The spherical virus particle measures about 95 nm in diameter. Its envelope consists of a 5-nm thick membrane covered by 8- to 10-nm long surface projections. These are composed of two polypeptide species of about the same size. Both of them can be removed by digestion with the proteolytic enzyme thermolysin except for a small fragment. The enzyme-treated particles are smooth surfaced and extremely deformable. The glycopolypeptides are clustered to form hollow cylindrical morphological units, 10 to 12 nm in diameter, with a 5-nm central cavity. Both negative staining and freeze-etching suggest that these units are penton-hexon clusters arranged in a T = 12, P = 3, icosahedral surface lattice. The membrane to which the surface subunits are attached is probably a lipid bilayer as evidenced by its double-track appearance in thin sections and the tendency of the freeze fracturing to occur within it. The strand-like nucleoprotein appears from thin-sectioning results to be to a large part located in a zone underneath the membrane.

Uukuniemi virus (18), the prototype of the Uukuniemi group of arboviruses, is a spherical, enveloped RNA virus (3, 21, 25, 26). From the virus at least three species of single-stranded RNA (apparent molecular weights 4.1×10^6 [L-RNA], 1.9×10^6 [M-RNA] and 0.8×10^6 [S-RNA], respectively) have been isolated (20). A single species of polypeptide (molecular weight about 25,000) has been found associated with the RNAs (21). The ribonucleoproteins are seen in the electron microscope as strands about 2.5 nm thick and 2.8, 1.4, and 0.75 μm long, respectively (L-, M- and S-RNP). All three species appear to be circular (19). A helical symmetry of the ribonucleoprotein has been suggested, but the arrangement of the strands within the virion is not known (3).

Uukuniemi virus seems to aquire its envelope by budding into smooth-surfaced intracellular (Golgi) vesicles (2). The envelope has been shown to contain at least one polypeptide species (molecular weight 65,000 to 75,000) (21). The phospholipid composition of the envelope has been found to resemble closely that of the host cell plasma membrane (23), a result difficult to reconcile with the apparent morphogenesis. Earlier electron microscopic observations on negatively stained Uukuniemi virus have indicated that the envelope projections form a regular hexagonal array of 10-nm morphological units (25, 26).

These results indicate that Uukuniemi virus represents a novel structural type among viruses. Both structural as well as morphogenetic similarities between Uukeniemi virus and certain other arboviruses, especially those of the Bunyamwera supergroup have, however, been reported (2, 4, 17). It has therefore recently been proposed that these viruses be included in one group, the Bunyaviruses (17, 22).

In this paper we report data on the envelope structure of Uukuniemi virus.

MATERIALS AND METHODS

Cells and virus. The origin and cultivation of chicken embryo cells, as well as preparation of stock virus from the prototype strain S23 of Uukuniemi virus, have been described previously (20).

Virus cultivation, labeling, and purification. Monolayers of secondary cultures of chicken embryo cells were infected at a multiplicity of about 5 PFU/cell, as described previously (20). The virus was cultivated in the presence of Eagle minimal essential medium supplemented with 0.2% bovine serum albumin (Armour Pharmaceutical Co., England). Roller bottles (1,200-cm^2 growth surface) were also used for virus growth. The medium was supplemented either with 0.2% bovine serum albumin or with 2% tryptose phosphate broth and 1% calf serum (1).

When the viral RNA and protein were labeled, radioactive isotopes were added 5 h postinfection: 30 or 50 μCi of [^{35}S]methionine per ml (129 Ci/mmol;

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The Radiochemical Centre, Amersham, England) in methionine-free minimal essential medium, or 50 μCi of [^{3}H]uridine per ml (27.4 Ci/mmol; Amersham) in minimal essential medium. The culture fluid was harvested 20 h postinfection and cleared by centrifugation at 10,000 × *g* for 20 min at +4 C, and the virus was concentrated by pelleting through a 3-ml cushion of 15% sucrose, made in TN buffer (0.1 M NaCl and 0.05 M Tris-hydrochloride, pH 7.4), for 60 min at 22,000 rpm and 4 C in a SW27 rotor. After elution overnight the virus was purified by centrifugation on a discontinuous sucrose gradient as described previously (20). The virus from the peak fractions were pooled and concentrated by pelleting at 22,000 rpm for 60 min at 4 C. The final virus pellet was resuspended in TN buffer. In some experiments virus was used for electron microscopy after the initial pelleting step.

Specimens of glutaraldehyde-fixed virus were prepared by adding the fixative (20% glutaraldehyde in phosphate buffer) to the clarified culture fluid to give a final concentration of 0.5 or 1.0%. The virus was then either concentrated or purified as described above.

Isolation of spikeless particles. Purified [^{35}S]methionine and [^{3}H]uridine double-labeled Uukuniemi virus was treated with thermolysin (virus protein:enzyme = 2:1 wt/wt) for 60 min at 37 C in TN buffer (see above), containing 5 mM $CaCl_2$ (29). After digestion the reaction was stopped by cooling on ice, and the treated virus was immediately layered on a preformed 20 to 50% (wt/wt) sucrose gradient in TN buffer. The bottom of the tube contained a 1.5-ml cushion of CsCl in TN (density = 1.87 g/cm^3). Untreated virus was layered on a separate identical gradient. Centrifugation was for 16 h at 25,000 rpm and 4 C. Fractions were collected from below and samples were taken to determine trichloroacetic acid-precipitable radioactivity. Sucrose densities were measured by refractometry.

SDS-gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was carried out as described by Weber and Osborn (30) and as previously (21) by using either 7.5 or 15% monomer concentration. The gels were cut in 2-mm slices, solubilized with NCS (Radiochemical Centre, England), and counted as described (21).

Electron microscopy. (i) Negative staining. A drop of the sample was applied to a copper grid coated with Formvar and carbon and the excess was removed. The stain was applied immediately on the grid and the excess was again removed with filter paper. The stains used were 2% phosphotungstic acid neutralized with KOH(KPT) and a 1% aqueous solution of uranyl acetate, pH 4.0.

(ii) Thin sections. Uukuniemi virus-infected chicken embryo cells were harvested 15 to 18 h postinfection by cooling the cultures on ice and removing the cells with a rubber policeman. The cells were pelleted and fixed as such with 2.5% glutaraldehyde in phosphate buffer (24). Postfixation was with 1% OsO_4 in 0.1 M cacodylate buffer at room temperature. The samples were left overnight in aqueous uranyl acetate (1%). Dehydration was through a series of concentrations of ethanol and propylene oxide, and the specimens were then embedded in Epon. Thin sections were cut with a diamond knife and stained with lead citrate (31) or double-stained with lead citrate and uranyl acetate (9).

(iii) Freeze-etching. Small drops of concentrated or purified virus preparations were mounted on 3-mm specimen holder, frozen in Freon 22, and stored in liquid nitrogen. Freeze-etching was performed in a Balzers BA 360 M or BA 510 etching unit. The etching time was varied from 1 to 3 min at −100 C. Shadowing was performed with Pt/C with a Balzers electron gun (Balzers AG, Lichtenstein). The replicas were cleaned in bleach and thereafter rinsed in distilled water. The specimens were examined in a Siemens Elmiskop I A or a Philips 300 electron microscope and photographs were taken at original magnifications of 20,000 or 40,000 at 80 kV. The magnification was calibrated with the aid of a carbon grating 54,865 lines per inch (Ladd Research Industries, Inc., Burlington, Vt.). Measurements of particle diameters or subunit sizes were made to an accuracy of 0.2 mm. Fifty particles were measured for each mean diameter determination.

RESULTS

Size and shape of the particles. When specimens of Uukuniemi virus for negative staining were taken shortly after pelleting most of the particles were deformed and rather irregular in shape. After allowing the virus to resuspend for 5 to 15 h the shape of the particles was restored, and a population of roughly spherical particles of a rather uniform size was found (Fig. 1A). The mean diameter of these KPT-stained particles was 114.0 ± 4.8 nm (mean value ± standard deviation). It was obvious, however, that in negative staining, independent of the stain used, unfixed particles are flattened and often show surface protrusions (blebs) or indentations. Glutaraldehyde fixation of Uukuniemi virus before concentration and purification revealed a more homogenous population of spherical particles (Fig. 1B). The mean diameter of these particles after KPT staining was 92.2 ± 4.2 nm. In thin sections of Uukuniemi virus-infected cells virus particles were found in the extracellular space and also in intracellular vesicles into which the particles seemed to bud (see Fig. 5A). The virus particles were of about the same size (mean diameter 88.8 ± 3.5 nm); the shape was spherical or slightly oval (see Fig. 5).

Envelope. In both negative staining and thin sectioning of Uukuniemi virus, an envelope was seen consisting of a membrane covered by surface projections 8 to 10 nm long (see Fig. 1B and 5). In accord with earlier observations (26) the surface projections seemed to form a regular array of morphological units. Particles revealing this ordered surface structure were

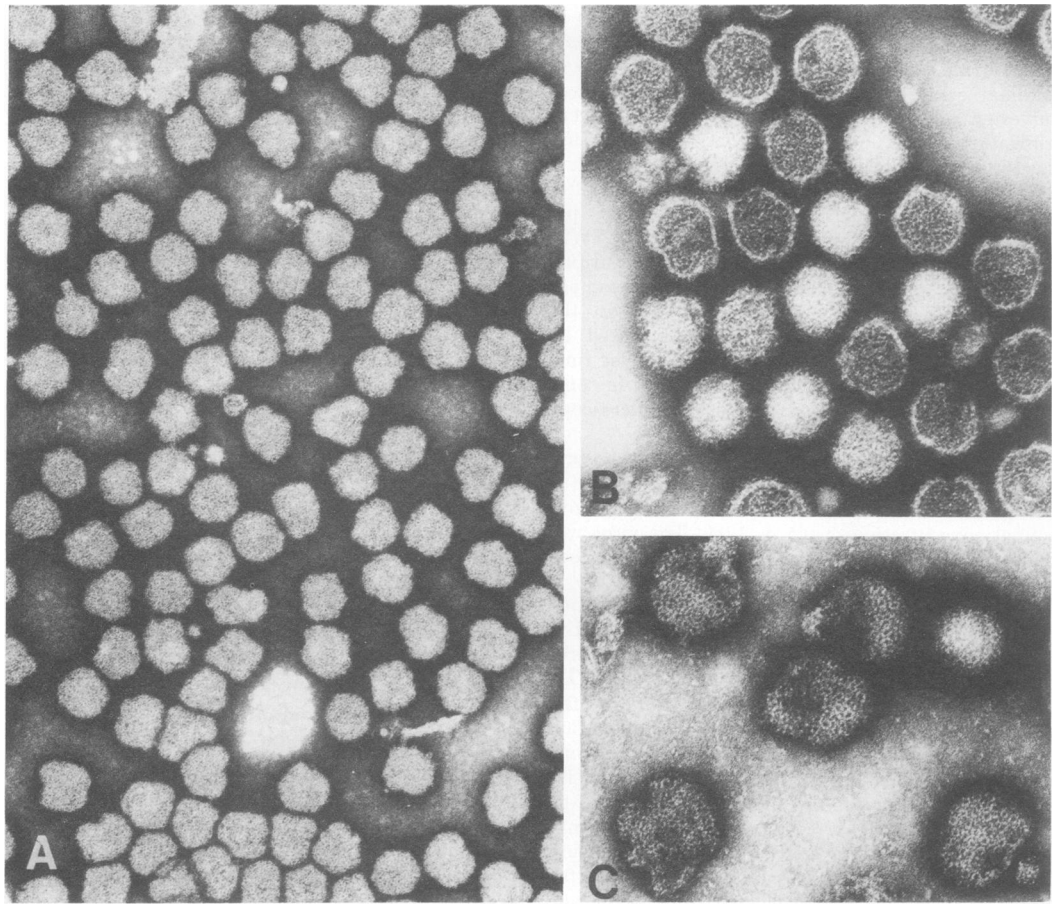

FIG. 1. *(A) Field of purified unfixed Uukuniemi virus particles stained with potassium phosphotungstate. The particles, although rather deformed, seem to be of uniform size. (B) In these glutaraldehyde-fixed particles also stained with potassium phosphotungstate a layer of surface projections attached to a membrane about 5 nm thick (as seen in the stain penetrated particles) is resolved. (C) The surface projections are seen as hollow, cylindrical subunits in a regular arrangement in these unfixed particles stained with uranyl acetate. Magnifications: (A) ×60,000; (B and C) ×120,000.*

found under all the negative-staining conditions used on unfixed as well as on glutaraldehyde-fixed particles. (Fig. 1B, C, and 2). Uranyl acetate staining of glutaraldehyde-fixed particles was, however, found to be the most consistent way of visualizing them (Fig. 2). In such preparations the morphological units resembled hollow cylinders, 10 to 12 nm wide with a central, stain-filled cavity of about 5 nm.

In Fig. 2A it can be seen that the morphological units are arranged in an hexagonal array, but pentons (i.e., subunits surrounded by five neighboring subunits) were also frequently found. This kind of surface structure suggests icosahedral symmetry, and particles revealing two or more pentons were therefore sought. Figures 2B to E show a few such particles. It can be seen that in all instances the pentons are at positions corresponding to a T = 12 (P = 3) surface lattice (6). The arrangement seemed to persist also in slightly deformed particles (e.g., Fig. 2C) suggesting that the lattice is not merely a random close packing of subunits on a sphere. In the deformed particles it could often be seen, however, that the subunit array had become slightly displaced making a positive identification of pentons (or hexons) more difficult. No particles inconsistent with a T = 12 surface lattice could be observed.

The intersubunit distance (hexon-hexon distance measured from well-centered groups) varied in different particles from 12.5 to 16.0 nm. This variation is apparently due to differences in stain penetration between the morphological

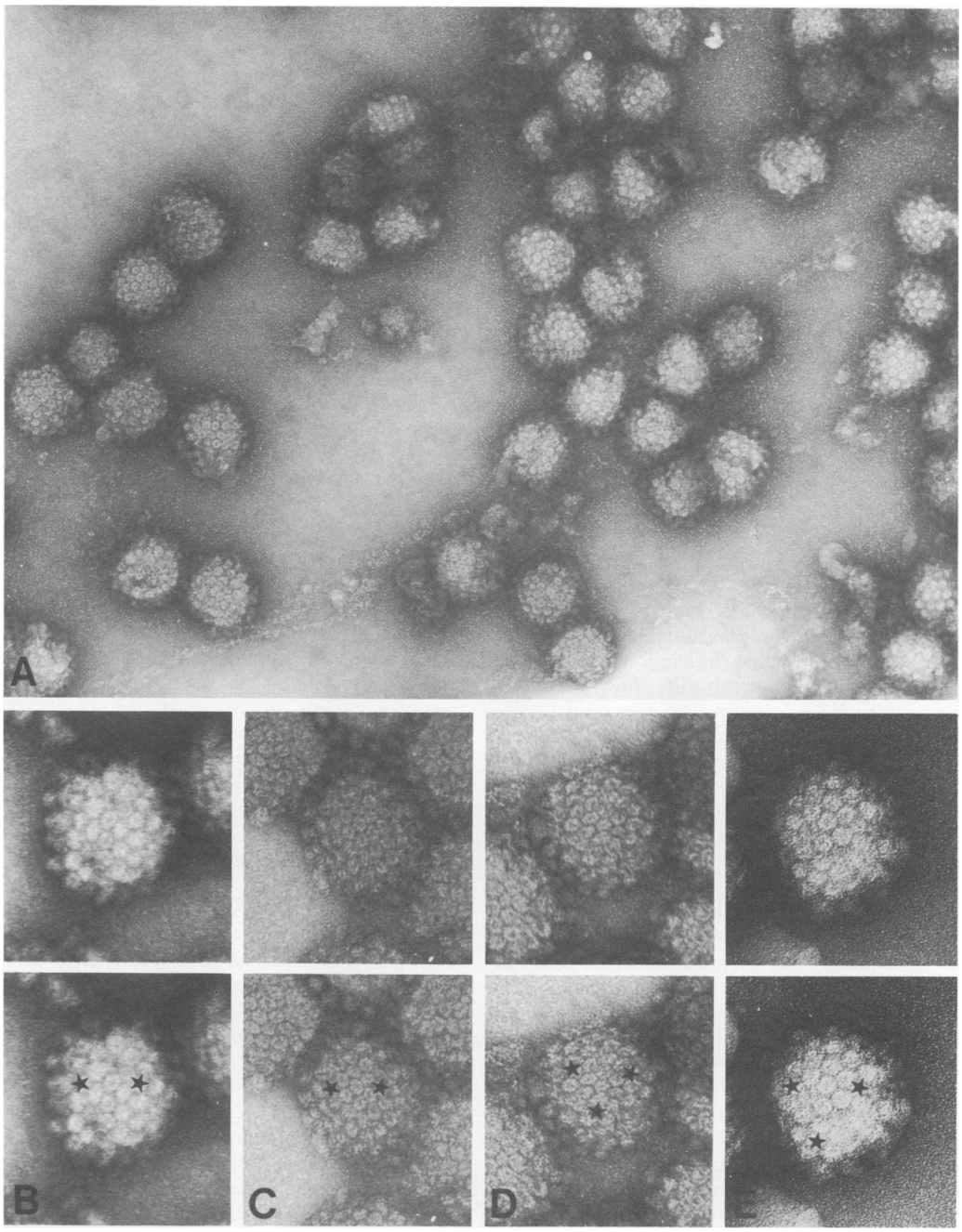

FIG. 2. *Uukuniemi virus particles fixed with glutaraldehyde (0.7%) before purification negatively stained with uranyl acetate. (A) A field of slightly flattened particles in which the surface structure is clearly resolved. The hexagonal array of subunits is obvious, but penton groups are also seen on several particles. Note the displacement of subunits in some of the deformed particles. (B to E) Selected Uukuniemi virus particles are shown in duplicate with penton positions marked on the lower row. In all of them the arrangement corresponds to an icosahedral surface lattice of T = 12. Magnifications: (A) ×100,000; (B to E) ×180,000.*

units. In a T = 12 surface lattice the interhexon distance corresponds to the distance between strict two- and threefold axes: it is therefore equal to 0.36 × mean radius of contrast. A 12.5 nm interhexon distance thus implies that the stain, contrasting the surface lattice, penetrates to a radius of 35 nm.

Figure 3A shows a field of glutaraldehyde-fixed, freeze-etched Uukuniemi virus particles. It can be seen that the surface of the uncleaved particles displays a regular array of knob-like subunits. The particle identified by an arrow, and the selected particles in Fig. 3C to E, all show a subunit arrangement consistent with the T = 12 icosahedral surface lattice. The individual morphological units appear rather smooth surfaced and do not show any signs of the central cavity seen with negative staining. The hexon-hexon distance is about 17 nm (16.5 to 17.5 nm). This distance in a T = 12 lattice corresponds to a particle radius of about 47.5 nm. A similar surface structure was also found in unfixed Uukuniemi virus in freeze-etching. As demonstrated in Fig. 4, it seems that part of the surface subunits may be pulled off during the fracturing without actual cleavage of the membrane taking place. In some instances, the particle surface void of subunits shows a regular line pattern which seems to correspond to the location of excised subunits (Fig. 4).

The membrane underlying the surface units was seen as a stain-resistant zone approximately 5 nm in thickness in particles penetrated by the negative stain (Fig. 1B). In thin sections the membrane was seen as a 6- to 7-nm thick double-track structure resembling that of the cellular membranes (Fig. 5). In both negatively stained and thin-sectioned particles, the membrane was located between radii of about 32 and 37 nm. In the freeze-etched specimens, the particles that were cleaved by the fracturing showed a convex or concave surface with a margin of fringes 12 to 13 nm long (Fig. 4B).

Effect of proteolytic enzymes. The nature of the surface projections was further characterized by treating Uukuniemi virus with the proteolytic enzyme thermolysin. Sucrose gradient analysis showed that the enzyme-treated particles banded at a density of 1.14 g/cm^3, as compared to 1.17 g/cm^3 for untreated control virus (Fig. 6A and B). The ratio of RNA to protein label increased from 1.73 in the control to 2.63 in the enzyme-treated virus, indicating that considerable amounts of protein had been digested.

In Fig. 7 and 8 the results of SDS-polyacrylamide gel electrophoresis of thermolysin-treated virus and untreated controls are shown. Previous results had already suggested that there might be more than one glycopolypeptide species in the broad peak migrating with apparent molecular weight of 65,000 to 75,000 (21; R. Pettersson, M.D. thesis, Univ. of Helsinki, Helsinki, 1974). The present results, especially those obtained with higher polyacrylamide concentration (Fig. 8A), seem rather clearly to show the presence of two polypeptide species in that region (G_1 and G_2 in Fig. 7A and 8A) in addition to the faster migrating nucleoprotein (N). In the lower gel concentration a polypeptide migrating more slowly than G_1 could also be detected. The nature of this polypeptide is not known.

After thermolysin treatment the G_1 and G_2 polypeptides had completely disappeared as had also the minor slowly migrating peak (Fig. 7B and 8B). Instead, in the 15% gels a small peak was detected migrating in front of N (Fig. 8B, arrow).

When samples for electron microscopy were taken from the peak fractions of the sucrose gradients it was found that the thermolysin-treated particles occurred primarily in large aggregates. They seemed to have lost almost completely their spherical shape and were extremely deformed. As seen in Fig. 9A, all particles appear smooth surfaced, completely void of surface projections. Treatment of Uukuniemi virus particles with Pronase (1 mg/ml for 20 min at 37 C) also seemed to remove most of the surface projections, causing extensive deformation and aggregation of the particles as judged by electron microscopy (Fig. 9B).

DISCUSSION

The present results show that the Uukuniemi virus envelope consists of a 5-nm thick membrane to which are attached surface projections about 10 nm long, giving the particle a total diameter of about 95 nm. The appearance of the membrane as a double-track structure in thin sections and the observation that this membrane tends to form the cleavage plane in freeze-etched specimens suggest that it is a lipid bilayer. This agrees with observations on several enveloped viruses that the lipids occur in bilayer form (11, 12, 14).

The arrangement of the glycoproteins in the Uukuniemi virus envelope is of interest. The observed surface structure corresponds to a hexon-penton clustering of subunits in a T = 12 (P = 3) icosahedral surface lattice. There are several myxo- and rhabdoviruses in which the surface projections can be seen as distinct subunits which appear to be regularly arranged (for references see 15). Only in Sindbis virus (an

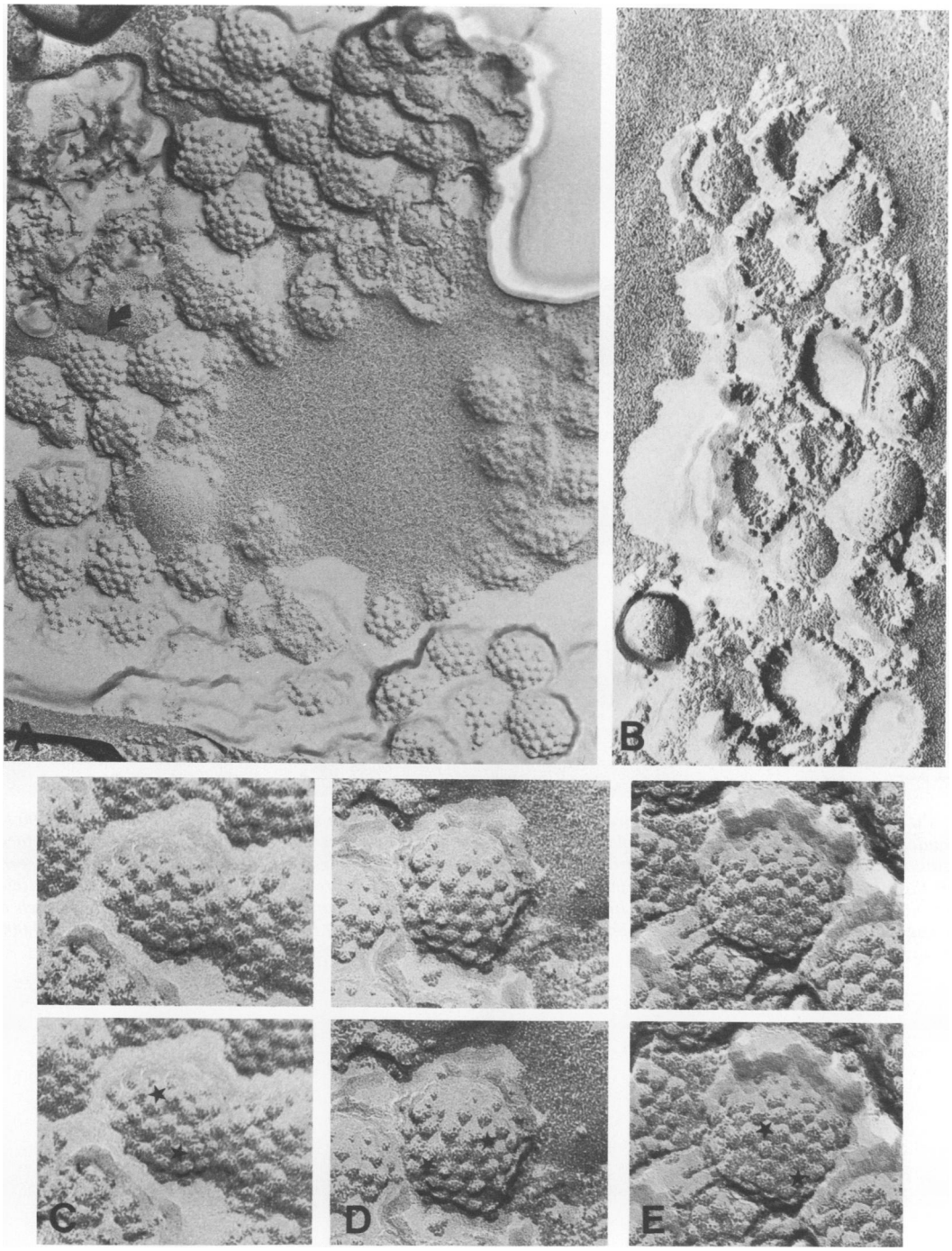

FIG. 3. *Freeze-etched specimens of glutaraldehyde-fixed Uukuniemi virus. Etching was for 60 to 90 s. Shadow-casting with Pt/C was from the bottom of the picture except in B, where it was from the left. (A) The surface structure consisting of a hexon-penton array of knob-like subunits is seen on the uncleaved particles. A few cleaved particles are seen in the upper right corner. Arrow points to a particle on which two pentons can be found. (B) A group of cleaved particles most of which show a rather smooth convex or concave surface and a marginal fringe of surface projections. (C, D, and E) Selected particles in which pentons, as marked on the duplicate figure below, are seen. Their mutual positions correspond to a T = 12 icosahedral surface lattice. Magnifications: (A) ×100,000; (B) ×150,000; (C–E) ×180,000.*

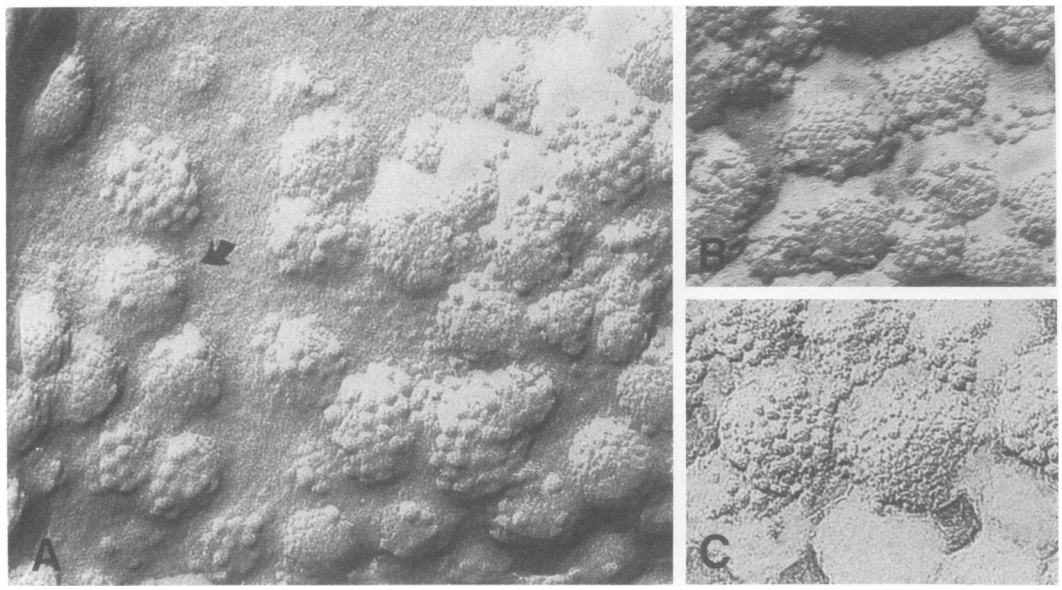

FIG. 4. *Unfixed Uukuniemi virus preparations in freeze-etching. The specimens were etched for 60 to 90 s; shadow casting with Pt/C was from below. (A) In several areas there were particles in which part of the surface subunits seemed to have been excised by the fracturing. In some particles (arrow) a line pattern could be seen on the surface. The few remaining subunits seem to be located in the rows between the lines. (B) A particle devoid of most surface subunits in which a intersecting line pattern can be seen. (C) A particle in which a hexagonal lattice seems to have been left after removal of projections by fracturing. Magnifications: ×120,000 in all figures.*

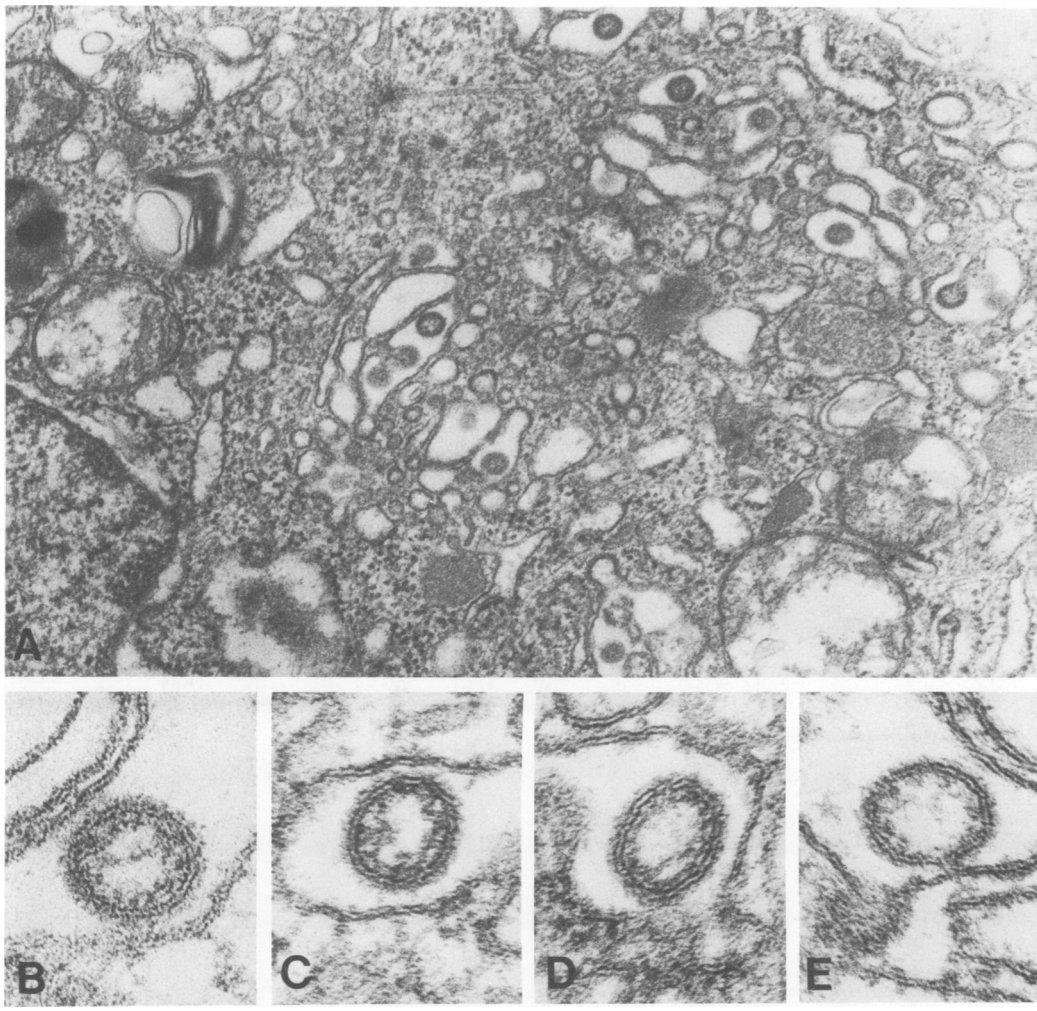

FIG. 5. *Thin sections of Uukuniemi-infected chicken embryo fibroblasts 15 to 18 h after infection. (A) A general view of a cell with several virus particles within vesicles that appear to be a distended Golgi apparatus. Some of the particles seem to be in the process of budding through the vesicle membranes. (B–E) Examples of intracellular virus particles in which the double-track structure resembling that of the cellular membranes can be seen. The surface projections are rather poorly resolved. An irregularly arranged electron-opaque material, probably the viral nucleoprotein, is located in a zone underneath the membrane. Magnification: (A) ×60,000; (B–E) ×200,000.*

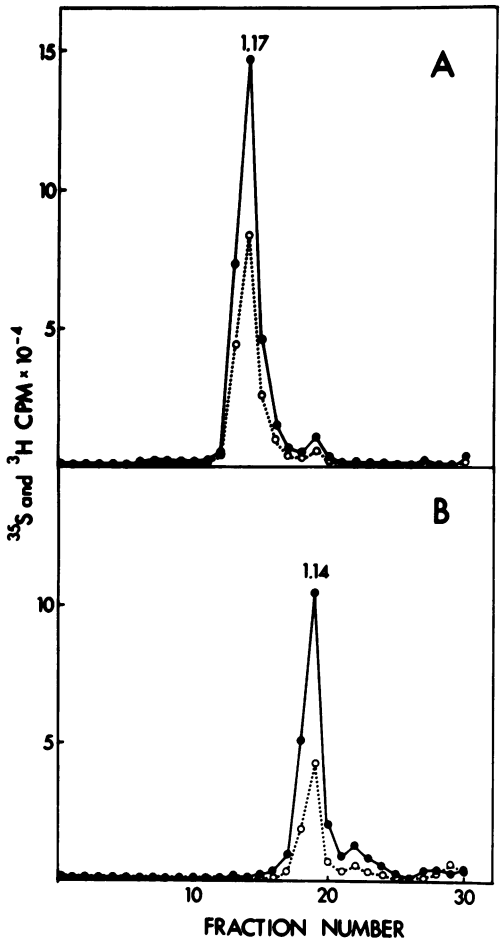

FIG. 6. *Isopycnic banding of untreated and thermolysin-treated Uukuniemi virus in sucrose density gradients. Uukuniemi virus was double labeled with [^{35}S]methionine and [^{3}H]uridine and purified as described in Materials and Methods. Half of a virus preparation was treated with thermolysin as described in Materials and Methods; the other half was left untreated. Both samples were then layered on a 20 to 50% (wt/wt) sucrose gradient made in TN buffer and containing a 1.5-ml CsCl cushion (1.87 g/cm^3) at the bottom. Centrifugation was for 16 h at 25,000 rpm and 4 C in a Spinco SW27 rotor. Fraction (0.5 ml) were collected from below and the sucrose density and acid-insoluble radioactivity were determined from aliquots of each fraction; bottom at left. Zero fraction represents the pellet. (A) Untreated virus, (B) thermolysin-treated virus. Symbols: (●) ^{3}H, (○) ^{35}S.*

alphavirus) however, has it been demonstrated that the glycoproteins actually are arranged with icosahedral symmetry (1).

The results obtained by combining SDS-gel electrophoresis and electron microscope analysis of thermolysin-treated and untreated Uukuniemi virus show that the surface projections consist of two polypeptides of about the same size. Both of these seem from earlier results to be glycosylated (Pettersson, M.D. thesis, 1974). A T = 12 lattice implies that the surface is formed by 110 hexons and 12 pentons, i.e., 720 structural units. We do not, however, know the composition of the structural unit. The two likely possibilities are that it consists either of a single polypeptide or of a dimer containing one of each polypeptide species. In the latter case one morphological unit (hexon) would be composed of 12 polypeptides. The dimensions of the unit give a volume of up to about 1,000 nm^2 available for protein in each, a volume close to that required by 12 polypeptides of the size of

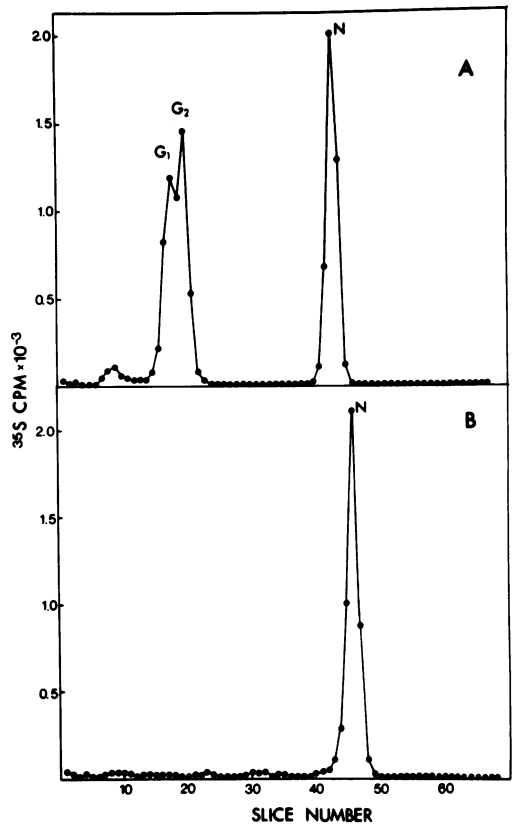

FIG. 7. *SDS-gel electrophoresis of untreated and thermolysin-treated Uukuniemi virus. Samples were taken from fraction 14 (untreated) and fraction 19 (thermolysin-treated virus) of the sucrose gradients in Fig. 6A and B, and treated with 1% SDS and 2-mercaptoethanol at 100 C for 2 min. The electrophoresis in 7.5% polyacrylamide gels was performed as described previously (3). The gels were sliced into 2-mm segments and counted in NCS-toluene. Direction of migration is from left to right. (A) Untreated, (B) thermolysin-treated virus.*

70,000 daltons assuming a partial specific volume of 0.73 cm^3/g.

The nature of the small polypeptide fragment found associated with the enzyme-treated particles is not known. It has previously been shown that the glycoproteins of Semliki forest virus (another alphavirus) are attached to the lipid bilayer by means of a hydrophobic peptide fragment (29). Recent findings suggest that this fragment spans the bilayer, making close contact with the nucleocapsid protein (10). Corresponding small fragments have also been found in influenza virus (28) and vesicular stomatitis virus (16) after treatment with proteolytic enzymes. It is possible that the polypeptide fragment observed here represents such a glycoprotein anchor. In this respect the freeze-fractured particles in which part of the surface subunits were lacking are of interest. It is possible that the observed line pattern represents the trace of the pulled-out "tails".

In alphaviruses the inner side of the envelope makes close contact with the presumably icosahedral nucleocapsid (12). In enveloped viruses with helical nucleocapsid, such as rhabdo-, orthomyxo-, and paramyxoviruses, there is a layer of a nonglycosylated protein (M protein) covering the inner side of the envelope (5, 7, 8, 27). Uukuniemi virus seems to differ from these latter viruses in that it lacks a polypeptide comparable to the M protein. Nor have we found by electron microscopy any evidence of an additional layer beneath the lipid bilayer. Instead, our results suggest that the strand-like nucleoprotein is directly opposed to the membrane. Moreover, it appears likely that the glycoproteins themselves are largely responsible for the structural stability of the virus particle since the spikeless particles were found to be highly deformable (Fig. 9).

A model for the assembly of alphaviruses has recently been presented (10, 13) which describes budding of the virus particle as a self-assembly process: budding proceeds as the glycoproteins make contact with the proteins of the spherical nucleocapsid through the lipid bilayer. This mechanism could also be true of Uukuniemi virus, with the assembly mainly dependent on lateral interactions between the glycoproteins.

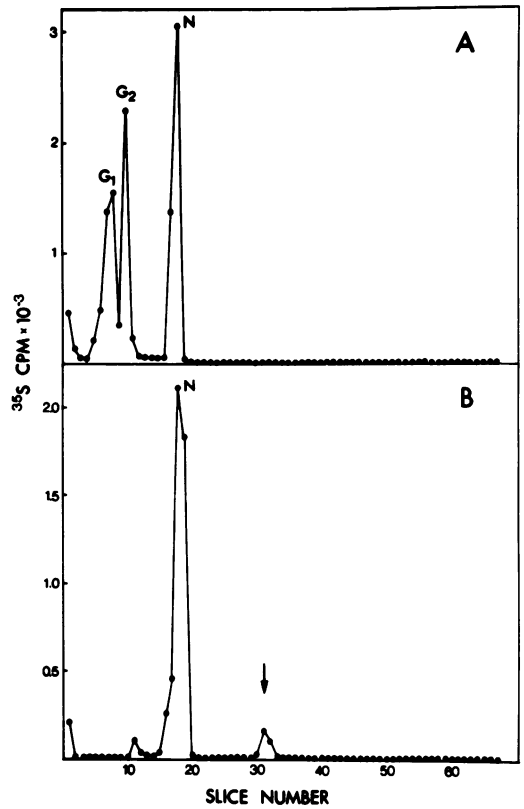

FIG. 8. *SDS-gel electrophoresis of untreated and thermolysin-treated Uukuniemi virus in 15% polyacrylamide gels. Samples were taken and treated as described in the legend to Fig. 7. Electrophoresis was at 6 V/cm for 20 h at room temperature. Note the small peak (arrow) migrating in from the N polypeptide. (A) Untreated, (B) thermolysin-treated virus.*

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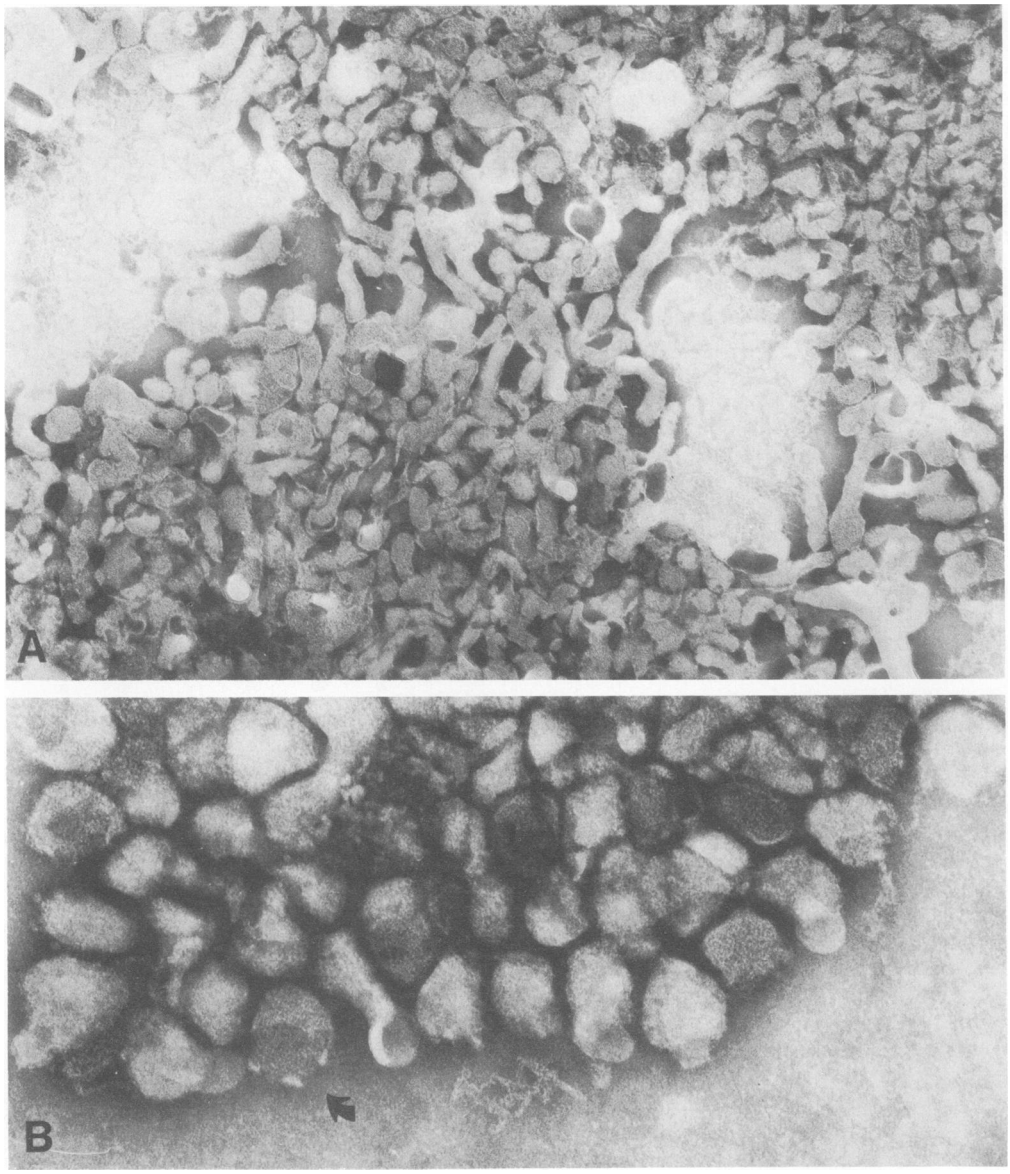

FIG. 9. *The effect of proteolytic enzymes on Uukuniemi virus structure. Negative staining with potassium phosphotungstate. (A) A sample from the peak fraction seen in Fig. 6B shows that these unfixed particles, which lack both glycoproteins, are totally lacking the surface projections. Most of the particles were quite deformed and occurred in large aggregates. (B) A field of Uukuniemi virus treated with Pronase (1 mg/ml for 20 min at 37 C) showing that the particles have lost most of their surface projections. Arrow points to a particle in which a coiled structure, probably the nucleoprotein, is seen. Magnifications: (A) ×60,000; (B) ×120,000.*

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