JOURNAL OF VIROLOGY, Feb. 1982, p. 390–400
0022-538X/82/020390-11$02.00/0

Vol. 41, No. 2

Monosaccharide Sequence of Protein-Bound Glycans of Uukuniemi Virus

MARJA PESONEN,† ESA KUISMANEN, AND RALF F. PETTERSSON*

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

Received 2 July 1981/Accepted 21 September 1981

Uukuniemi virus, a member of the Bunyaviridae family, was grown in BHK-21 cells in the presence of [^{3}H]mannose. The purified virions were disrupted with sodium dodecyl sulfate and digested with pronase. The [^{3}H]mannose-labeled glycopeptides of the mixture of the two envelope glycoproteins G1 and G2 were characterized by degrading the glycans with specific exo-and endoglycosidases, by chemical methods, and by analyzing the products with lectin affinity and gel chromatography. The glycopeptides of Uukuniemi virus fell into three categories: complex, high-mannose type, and intermediate. The complex glycopeptides probably contained mainly two NeuNAc-Gal-GlcNAc branches attached to a core $(Man)_3(GlcNAc)_2$ peptide. The high-mannose-type glycans were estimated to contain at least five mannose units attached to two *N*-acetylglucosamine residues. Both glycan species appeared to be similar to the asparagine-linked oligosaccharides found in many soluble and membrane glycoproteins. The results suggested that the intermediate glycopeptides contained a mannosyl core. In about half of the molecules, one branch appeared to be terminated in mannose, and one appeared to be terminated in *N*-acetylglucosamine. Such glycans are a novel finding in viral membrane proteins. They may represent intermediate species in the biosynthetic pathway from high-mannose-type to complex glycans. Their accumulation could be connected with the site of maturation of the members of the Bunyaviridae family. Electron microscopic data suggest that the virions bud into smooth-surfaced cisternae in the Golgi region. The relative amounts of [^{3}H]mannose in the complex, high-mannose-type, and intermediate glycans were 25, 62, and 13%, respectively, which corresponded to the approximate relative number of oligosaccharide chains of 2:2.8:1, respectively, in the roughly equimolar mixture of G1 and G2. Endoglycosidase H digestion of isolated [^{35}S]methionine-labeled G1 and G2 proteins suggested that most of the complex and intermediate chains were attached to G1 and that most of the high-mannose-type chains were attached to G2.

Uukuniemi virus is an arthropod-borne virus of the Bunyaviridae family (3). The virions contain 10-nm-long surface projections consisting of two envelope glycoproteins, G1 (molecular weight 75,000) and G2 (molecular weight 65,000), in roughly equimolar amounts (35, 44). The nucleocapsid contains three unique RNA segments designated L, M, and S (33), the N protein (molecular weight 25,000) (35, 36), and the structural L protein (molecular weight 180,000 to 200,000), which is present in minor amounts (R. F. Pettersson, unpublished data). In vitro translation in the presence and absence of dog pancreas microsomes in cell-free reticulocyte extracts suggests that the M RNA segment directs the synthesis of a precursor protein p110, which is contranslationally cleaved into G1 and G2 (43).

† Present address: European Molecular Biology Laboratory, 6900 Heidelberg, West Germany.

The morphogenesis of Uukuniemi virus and other members of the Bunyaviridae family appears to differ from that of most other enveloped RNA viruses. On the basis of electron microscope studies, it has been suggested that the major site of maturation is not the cellular plasma membrane, but rather the smooth endoplasmic reticulum, particularly the region of the Golgi complex (23, 26, 39, 44, 45; for a review, see reference 4). Release of the virions may occur by transport of vesicles containing virions to the plasma membrane, followed by membrane fusion and discharge of the contents of the vesicles into the medium. Because of this unusual site of maturation, we have initiated studies to characterize the structure, synthesis, and transport of bunyavirus glycoproteins. We report here that Uukuniemi virus envelope glycoproteins contain, in addition to high-mannose-type and completely glycosylated complex glycans, a

novel type of oligosaccharide, which may reflect the site of maturation of this virus.

MATERIALS AND METHODS

Preparation of radiolabeled Uukuniemi virus. BHK-21 cells grown on plastic bottles (75 cm^2) were infected with Uukuniemi virus strain 23 (34) at a multiplicity of 5 PFU/cell. After absorption for 60 min at 37°C, the cells were maintained in Eagle minimal essential medium supplemented with 2% dialyzed calf serum and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.2, at 37°C. At 6 h postinfection the cultures were labeled with 40 μCi of D-[2-^{3}H]mannose per ml (16 Ci/mmol) or with 30 μCi of [^{35}S]methionine per ml (1,613 Ci/mmol) (The Radiochemical Centre). The [^{35}S]methionine pulse was given in a methionine-deficient medium. The culture medium was harvested at 29 h postinfection, and the virus was purified by sucrose gradient centrifugation as described (34).

The [^{3}H]mannose label was not metabolized during the long pulse into amino acids or other monosaccharides, since the non-glycosylated capsid protein was not labeled and since the total [^{3}H]mannose-labeled glycopeptide preparation of the virus (see below) yielded, upon acid hydrolysis with 4 N HCl at 100°C for 4 h, radioactive mannose exclusively, as analyzed by paper chromatography (30).

Isolation of viral proteins. The viral proteins were isolated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) in slab gels according to Laemmli (19), with a 10% separating gel and a 5% stacking gel, and developed by fluorography, using the method of Bonner and Laskey (5).

Enzyme digestions. Pronase digestions were carried out in 0.15 M Tris-hydrochloride buffer, pH 8.0, containing 0.001 M $CaCl_2$ and 0.1% SDS for 24 h at 60°C. A 2% pronase (Calbiochem; B grade) solution was prepared in 0.15 M Tris-hydrochloride, pH 8.0, containing 0.001 M $CaCl_2$, and the solution was autodigested for 1 h at 37°C to inactivate possible glycosidase activities. Pronase was added three times to the digests to a final concentration of 0.13%. Then the digests were heated for 3 min in a boiling water bath, cooled to 4°C, and centrifuged to remove the Ca^{2+}-SDS precipitate.

β-D-Galactosidase from *Canavalia ensiformis* (EC 3.2.1.12; Seikagaku), *N*-acetyl-β-D-glucosaminidase from beef kidney (EC 3.2.1.50; Boehringer), and α-D-mannosidase from *C. ensiformis* (EC 3.2.1.24; Boehringer) digestions were performed in 50 μl of 0.05 M sodium citrate buffer, pH 4.0, at 37°C for 48 h. Two equal enzyme additions were carried out during the incubation. The final enzyme concentrations were 2.5, 2.7, and 22 U/ml, respectively. The α-D-mannosidase preparation contained β-D-mannosidase activity (29).

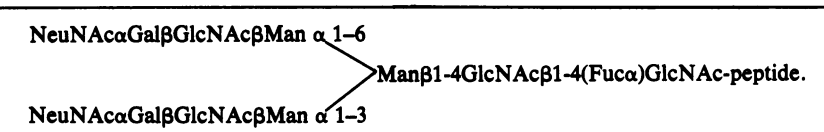
Endo-*N*-acetyl-β-D-glucosaminidase H (endoglycosidase H; *Streptomyces griseus*; Seikagaku) and endo-*N*-acetyl-β-D-glucosaminidase D (endoglycosidase D; *Diplococcus pneumoniae*; Seikagaku) digestions of glycopeptides were performed in 50 μl of 0.2 M sodium citrate buffer, pH 5.5, and 0.15 M sodium phosphate buffer, pH 6.5, respectively, for 24 h at 37°C. Both enzyme concentrations were 0.2 U/ml. As a positive control for endoglycosidase H activity, we digested the pronase glycopeptides of E2 protein of Semliki Forest virus (SFV) grown in chicken cells, which contain mainly high-mannose-type (endoglycosidase-susceptible) glycans (31). The products were analyzed by gel filtration on Bio-Gel P-6 as described below.

Proteins were eluted from dried gel pieces after SDS-PAGE into 0.2 M sodium citrate buffer, pH 5.5, containing 0.1% Triton X-100 and digested with endoglycosidase H as above. The preparations were dialyzed against 0.1% SDS before analysis in SDS-PAGE.

Gel chromatography. A column (1 by 54 cm) of Bio-Gel P-6 (100 to 200 mesh; Bio-Rad), eluted with 0.15 M Tris-hydrochloride buffer, pH 7.8, containing 0.1% SDS was used for apparent molecular weight estimations. The column was calibrated with radiolabeled thyroglobulin glycopeptides (molecular weight 4,100), lacto-*N*-fucopentaol (molecular weight 858), and digalactosylglucitol (molecular weight 504) as described (32).

A column (1 by 16 cm) of Bio-Gel P-2 (100 to 200 mesh; Bio-Rad) eluted with water was used for desalting of preparations. Fractions of 670 μl were collected from both columns at room temperature with a flow rate of about 10 ml/h.

Markers for gel chromatography were prepared from known complex pronase glycopeptides of SFV, which have the structure



Part of the glycopeptides have more than two NeuNAc-Gal-GlcNAc branches (30, 37). SFV was grown in BHK-21 cells and labeled with [2-^{3}H]mannose as described (30), solubilized with SDS, and subjected to pronase digestion (see above). The complex glycopeptides were separated by affinity chromatography on concanavalin A-Sepharose (24). The glycopeptides were degraded stepwise with mild acid, β-galactosidase, and β-*N*-acetylglucosaminidase, and with endoglycosidase D as described above, to yield a marker glycopeptide for each degradative step.

Lectin affinity chromatography. A column (1 by 15 cm) of concanavalin A-Sepharose 4B (Pharmacia) was washed with 0.01 M Tris-hydrochloride buffer, pH 7.5, containing 0.1 M NaCl and 0.02% NaN_3, $CaCl_2$, $MgCl_2$, and $MnCl_2$. The bound glycopeptides and glycans were eluted with 0.005 M and subsequently with 0.2 M α-methylmannoside (Sigma Chemical Co.; grade II) in the same buffer. Fractions of 2.0 ml were collected at room temperature. The elution rate was about 10 ml/h.

A column (0.7 by 10 cm) of *Ricinus communis* agglutinin (RCA) coupled to Sepharose 4B (10 mg/ml) was washed with phosphate-buffered saline at 4°C. The bound glycopeptides were eluted with 0.1 M

galactose in phosphate-buffered saline. The glycopeptides were allowed to absorb to the lectin for 15 min before the run. Fractions of 1.5 ml were collected at 4°C with an elution rate of about 10 ml/h. The RCA-Sepharose was a kind gift from Carl Gahmberg.

Other methods. β-Elimination was carried out in 100 μl of 0.02 M NaOH in 1 M $NaBH_4$ at 45°C for 17 h. The reaction was stopped with a drop of acetic acid.

Strong alkaline hydrolysis was performed in 1 M NaOH in 1 M $NaBH_4$ at 100°C for 4 h in a Teflon tube. The hydrolysate was neutralized with acetic acid and desalted on Bio-Gel P-2. The lyophilized preparation was reacetylated in 1 ml of saturated $NaHCO_3$ containing 50 μl of acetic anhydride for 1 h at room temperature and lyophilized. Desialylation was carried out in 150 μl of 0.05 M H_2SO_4 for 1 h at 80°C.

Radioactivity was counted in xylene–Triton X-100 scintillation cocktail in a Wallac 81 000 scintillation counter.

RESULTS

Glycoproteins of Uukuniemi virus. Uukuniemi virus was grown in BHK-21 cells and labeled with [^{3}H]mannose or [^{35}S]methionine at 6 to 29 h postinfection. The virus was purified, and the proteins were analyzed by SDS-PAGE (Fig. 1). The envelope glycoproteins G1 (molecular weight 75,000) and G2 (molecular weight 65,000) and the nucleocapsid protein N (molecular weight 25,000) could be labeled with [^{35}S]methionine (lanes a and b), whereas only G1 and G2 were labeled with [^{3}H]mannose (lane c). In the presence of reducing agents G1 and G2 were poorly separated (lane a), whereas in the absence of reducing agents the separation was good (lane b and c).

The gel slices containing the [^{3}H]mannose-labeled G1 and G2 proteins (lane c) were solubilized in NCS, and the radioactivity was determined; G1 and G2 contained 38 and 62% of the label, respectively. No ^{3}H label was metabolized to amino acids or other sugars (see Materials and Methods).

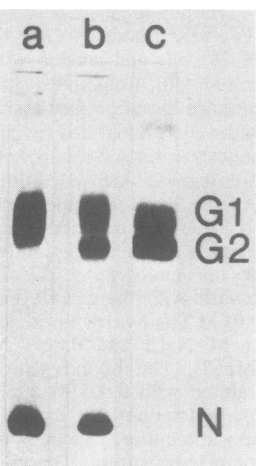


FIG. 1. SDS-PAGE in reducing (lane a) or nonreducing conditions (lanes b and c) of Uukuniemi virus labeled with [^{35}S]methionine (lanes a and b) or [^{3}H]mannose (lane c).

Protein-bound glycans. Purified [^{3}H]mannose-labeled Uukuniemi virus was solubilized with 0.1% SDS and digested exhaustively with pronase. Gel chromatography of the mixture of the labeled glycopeptides of G1 and G2 revealed a major peak with an apparent molecular weight of roughly 2,000 (fractions 30 to 34) and another peak of higher-molecular-weight material (fractions 21 to 28) (Fig. 2A).

The total glycopeptide preparation was desalted, and samples were subjected to β-elimination or strong alkaline hydrolysis. *O*-Glycosidic glycopeptide linkages are cleaved by β-elimination (8, 11). Strong alkaline hydrolysis results in cleavage of *N*-glycosidic glycopeptide linkages between *N*-acetylglucosamine and asparagine, in deacetylation of acetylated monosaccharides, and in desialylation (20). Figure 2B shows the gel chromatography profile of the glycopeptides after β-elimination. The apparent molecular weight of the major peak did not change significantly. The glycopeptide preparation subjected to strong alkaline hydrolysis was desalted and reacetylated before gel chromatography. The apparent molecular weight of the major peak was now reduced to about 1,200 (Fig. 2B), suggesting cleavage of the glycopeptide linkage.

The desalted pronase glycopeptides were digested with endoglycosidase H, which cleaves the linkage between the two core *N*-acetylglucosamine residues of high-mannose-type glycans (42). Three categories of products were revealed: large (fractions 21 to 26) and small (fractions 31 to 34) endoglycosidase H-resistant glycopeptides and glycans released by the enzyme (fractions 38 to 43) (Fig. 2A). They contained 25, 13, and 62% of the [^{3}H]mannose label, respectively. The three peaks were pooled separately and desalted for further analysis.

Large endoglycosidase H-resistant glycopeptides. The oligosaccharide moieties of the large endoglycosidase H-resistant glycopeptides were degraded stepwise to reveal the sequence of monosaccharides. The gel chromatography profiles of the glycopeptides at each degradative stage were compared with those of complex glycopeptides of SFV; the structure of the intact SFV glycopeptides is mainly (NeuNAc-Gal-GlcNAc)$_2$(Man)$_3$GlcNAc(Fuc)GlcNAc-peptide, with part of the glycopeptides having more than two NeuNAc-Gal-GlcNAc branches (30, 37). The intact glycopeptides of Uukuniemi virus had a somewhat larger apparent molecular weight than the SFV glycopeptides (Fig. 3A). Mild acid hydrolysis reduced the apparent molecular

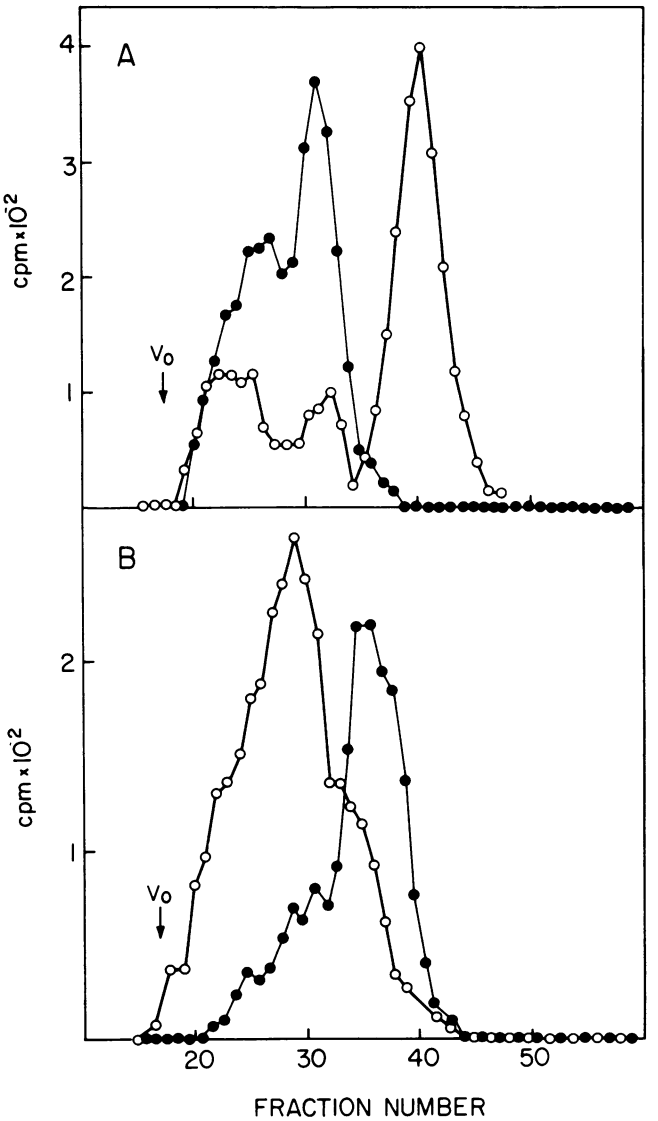


FIG. 2. Bio-Gel P-6 gel chromatography profiles of [^{3}H]mannose-labeled pronase glycopeptides of the mixture of G1 and G2. (A) Intact glycopeptides (●); glycopeptides after endoglycosidase H digestion (○). (B) The glycopeptide preparation after treatment with 0.02 M NaOH in 1 M $NaBH_4$ for 17 h at 45°C (○); the glycopeptide preparation after treatment with 1 M NaOH in 1 M $NaBH_4$ for 4 h at 100°C, desalting, and reacetylation (●). V_0 designates the void volume of the column. Profiles of different runs are drawn in the same panels so that the void volumes of each run coincide.

weight of the glycopeptides, indicating the cleavages of terminal sialic acid residues (47). The products eluted ahead of the desialylated glycopeptides of SFV (Fig. 3A). Desialylation exposed galactose residues, as evidenced by affinity chromatography on RCA-Sepharose. RCA binds terminal β-galactose residues (2, 27). The intact glycopeptides were not bound to the lectin, but were recovered in the void volume with sugar-free buffer (Fig. 4A). After desialylation, about 35% of the label was bound weakly (fractions 7 to 18) and about 52% was bound strongly (fractions 19 to 28) to the lectin, eluting off only with 0.1 M galactose (Fig. 4B). After desialylation, the glycopeptides were desalted and digested with β-galactosidase. Now the affinity for RCA was completely abolished (Fig. 4C), indicating loss of the galactose residues.

After removal of the sialic acid and the galactose residues, the glycopeptides were digested with β-*N*-acetylglucosaminidase. A heterogeneous major peak and a minor peak were gener-

ated (Fig. 3A). This material eluted again with a larger apparent molecular weight in gel chromatography as compared with the SFV glycopeptides treated in the same way. After *N*-acetylglucosaminidase digestion, the glycopeptide preparation was treated with mannosidase (the enzyme preparation contains both α- and β-mannosidase activity). Gel chromatography showed (Fig. 3B) that almost all of the label was converted to [^{3}H]mannose. It thus appears that the preceding stepwise release of sialic acid, galactose, and *N*-acetylglucosamine had been nearly complete.

After release of the NeuNAc-Gal-GlcNAc branches, the preparation (both peaks in Fig. 3A) was desalted and digested with endoglycosidase D. This enzyme hydrolyzes the linkage between the two *N*-acetylglucosamine residues of substrates which contain the sequence Manα1–3Manβ1–4GlcNAcβ1–4-GlcNAc provided that Manα1–3 is unsubstituted (41). The digestion resulted in the almost quantitative hydrolysis of the glycopeptides (Fig. 3B). The product eluted in gel chromatography like the core tetrasaccharide $(Man)_3GlcNAc$ of SFV glycopeptides. Thus, the heterogeneity of the glycopeptide preparation apparent after *N*-acetylglucosaminidase digestion (Fig. 3A), as well as the larger size of Uukuniemi glycopeptides as compared with the corresponding SFV glyco-

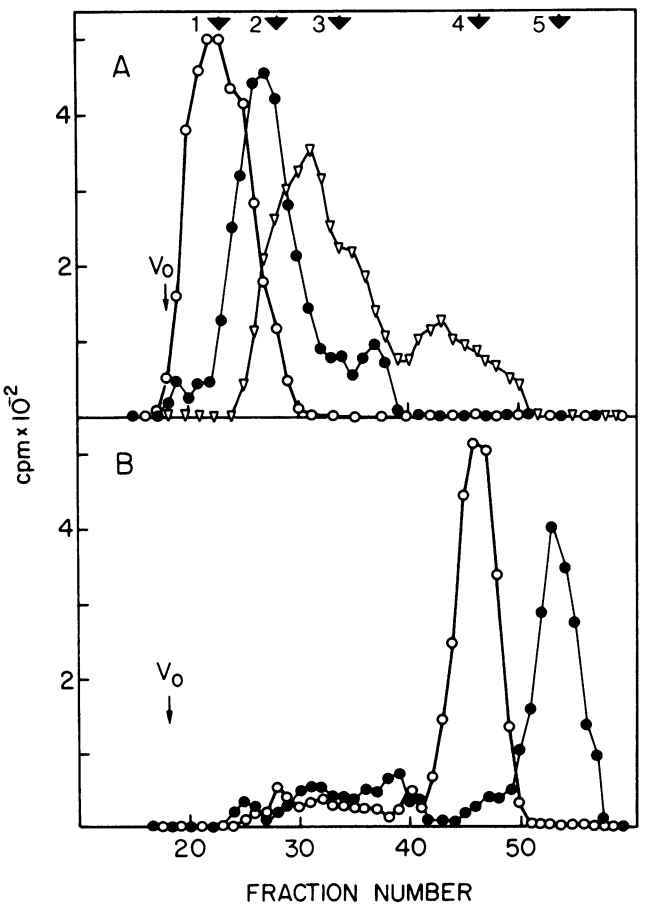


FIG. 3. Bio-Gel P-6 gel chromatography profiles of [^{3}H]mannose-labeled complex glycopeptides after stepwise degradation of the glycans. (A) Intact glycopeptides (○); glycopeptides after desialylation with 0.05 M H_2SO_4 for 1 h at 80°C (●); glycopeptides after desialylation and β-galactosidase and *N*-acetyl-β-glucosaminidase digestion (▽). (B) Products released by endoglycosidase D (○) or mannosidase (●) digestion after desialylation and β-galactosidase and *N*-acetyl-β-glucosaminidase digestion of the glycopeptides. V_0 designates the void volume of the column. Different runs are drawn in the same panels so that the void volumes coincide. Markers: Intact (1) and desialylated (2) complex glycopeptides of SFV; (3) core glycopeptide $(Man)_3(GlcNAc)_2$-peptide of SFV after treatment with mild acid, galactosidase, *N*-acetylglucosaminidase, and fucosidase; (4) $(Man)_3GlcNAc$ liberated by endoglycosidase D from complex glycopeptides of SFV after removal of the NeuNAc-Gal-GlcNAc-branches; (5) [^{3}H]mannose.

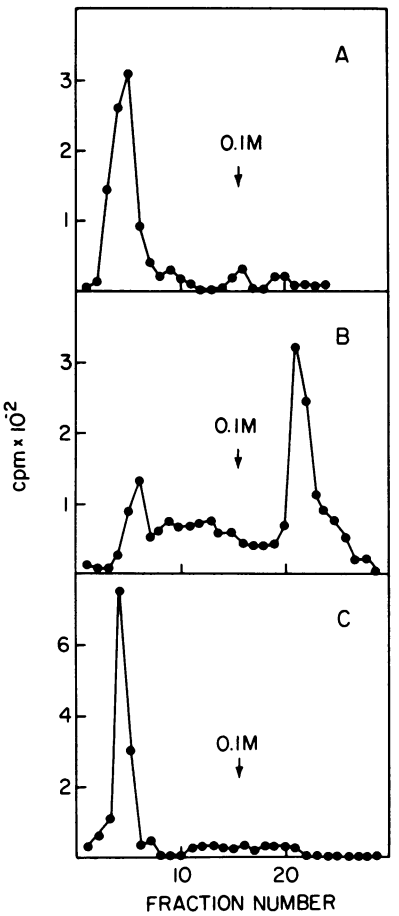


FIG. 4. RCA-Sepharose affinity chromatography profiles of [^{3}H]mannose-labeled complex glycopeptides. (A) Intact glycopeptides (recovery, 65%). (B) Glycopeptides after desialylation with 0.05 M H_2SO_4 for 1 h at 80°C (recovery, 80%). (C) Glycopeptides after β-galactosidase treatment of the desialylated preparation (recovery, 75%). Fractions 1 to 15 were eluted with sugar-free buffer, and fractions 16 to 30 were eluted with 0.1 M galactose as described in Materials and Methods.

peptides, was not due to the glycan moiety, but rather to the peptide moiety.

The above data suggest that part of the protein-bound glycans of Uukuniemi virus were complex oligosaccharides consisting of NeuNAc-Gal-GlcNAc branches attached to a core consisting most probably of three mannose and two *N*-acetylglucosamine residues. The number of branches was studied by affinity chromatography on concanavalin A-Sepharose. Figure 5A shows the elution pattern of the intact glycopeptides. About 18% of the [^{3}H]mannose label was not retained by the lectin, but eluted from the column with sugar-free buffer; 72% was bound weakly and could be eluted off the column with 5 mM α-methylmannoside. Concanavalin A differentiates glycans according to the substitution pattern of the two core α-mannose residues. Complex chains with two branches where the two α-mannose residues are substituted at position C-2 exhibit weak binding; if either or both α-mannose units are substituted also at other positions, which is the case in multibranched glycans, affinity to concanavalin A is lost (1, 18). Thus, about 80% of the complex glycans of Uukuniemi virus appeared to have two NeuNAc-Gal-GlcNAc branches, and about 20% appeared to have more than two such branches. Taken together, our results suggest that the complex glycans had mainly the follow-

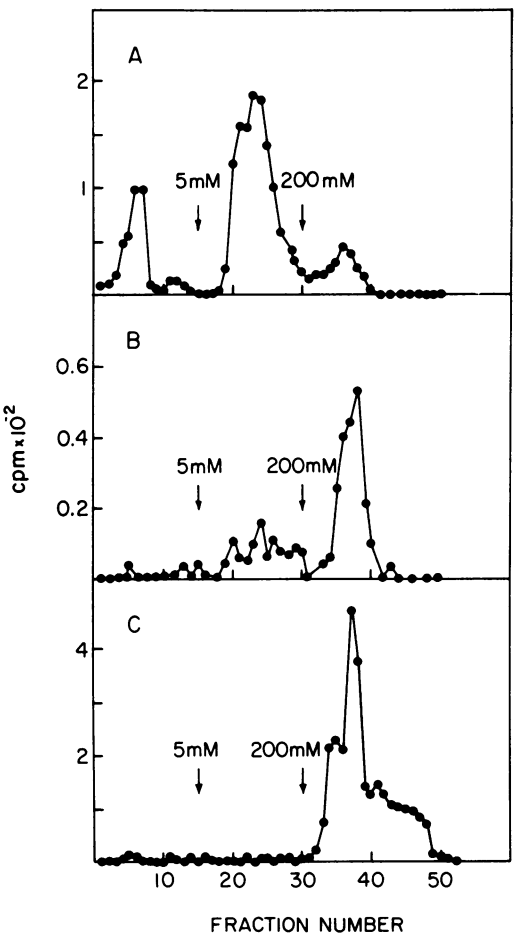


FIG. 5. Concanavalin A-Sepharose affinity chromatography profiles of [^{3}H]mannose-labeled (A) complex glycopeptides, (B) intermediate glycopeptides, and (C) high-mannose-type glycans released after endoglycosidase H digestion. Fractions 1 to 15 were eluted with sugar-free buffer, fractions 16 to 30 were eluted with 5 mM α-methylmannoside, and fractions 31 to 50 were eluted with 200 mM α-methylmannoside as described in Materials and Methods. Recoveries were (A) 54%, (B) 35%, and (C) 45%.

ing monosaccharide sequence: (NeuNAc-GalβGlcNAcβManα)$_2$ManβGlcNAcβGlcNAc-peptide.

Small endoglycosidase H-resistant glycopeptides. The small endoglycosidase H-resistant glycopeptides (Fig. 2A, fractions 31 to 34) were analyzed by affinity chromatography on concanavalin A-Sepharose. About one-third of the material exhibited weak, and the rest of the material high, affinity binding to the lectin (Fig. 5B). This suggests that the glycopeptides contained a mannosyl core (1, 18). Mannosidase digestion reduced the size of most of the material and released at the same time about 35% of the label as [^{3}H]mannose (Fig. 6A). Most of the material was also susceptible to β-*N*-acetylglucosaminidase digestion, since the apparent molecular weight of the material was reduced by this treatment (Fig. 6A). The glycopeptides eluted now in about the same elution volume as the SFV complex glycopeptide core (Man)$_3$(GlcNAc)$_2$-peptide. Double digestion with mannosidase and *N*-acetylglucosaminidase hydrolyzed about 58% of the glycopeptides to [^{3}H]mannose (Fig. 6B). The results suggest that the glycopeptides had a mannosyl core with one branch terminating in *N*-acetylglucosamine and one terminating in mannose. About 42% of the glycopeptides were not hydrolyzed completely by double digestion with mannosidase and *N*-acetylglucosaminidase; however, the apparent molecular weight was reduced. This material was blocked from further hydrolysis even when β-galactosidase was included in the reaction mix-

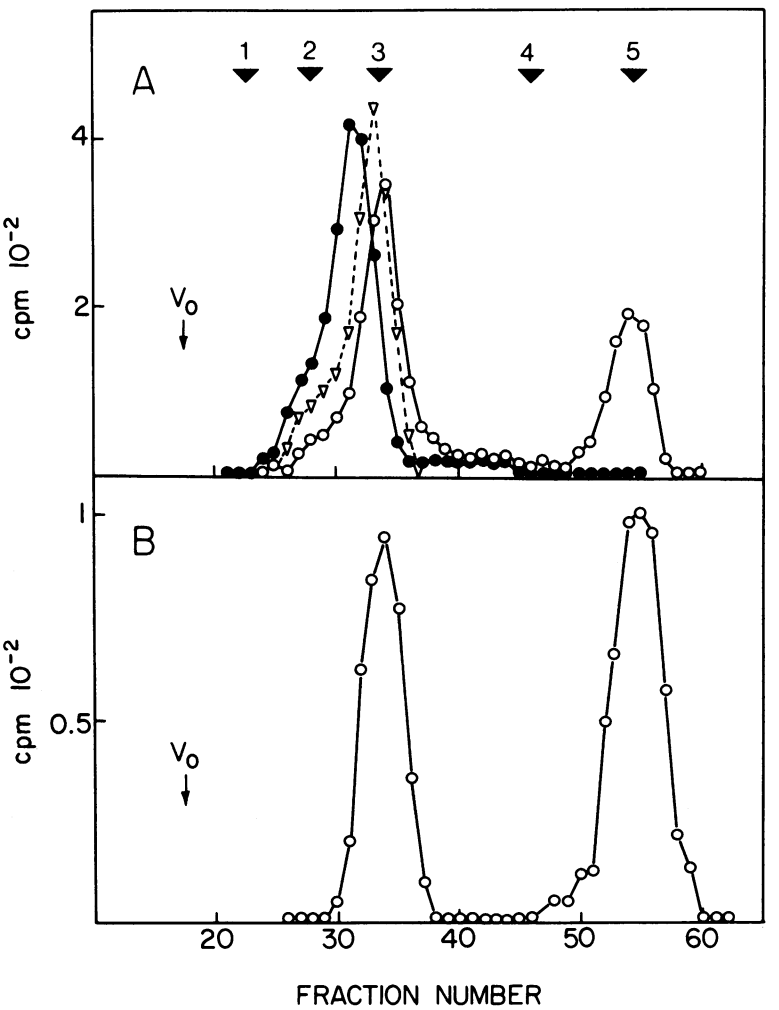


FIG. 6. Bio-Gel P-6 gel chromatography profiles of [^{3}H]mannose-labeled intermediate glycopeptides. (A) Intact glycopeptides (●); glycopeptides digested with *N*-acetylglucosaminidase (▽) or mannosidase (○). (B) Glycopeptides digested with *N*-acetylglucosaminidase and mannosidase. V_0 designates the void volume of the column. Different runs are drawn in the same panel so that the void volumes coincide. Markers are as in Fig. 3.

ture. The small amounts of the intermediate glycopeptide fraction did not allow more detailed analysis.

Endoglycosidase H-susceptible glycopeptides. The glycans released from the original glycopeptide mixture of G1 and G2 by endoglycosidase H had an apparent molecular weight of about 1,000 (Fig. 2A, fractions 37 to 43). Strong alkaline hydrolysis yielded after reacetylation a major peak with an apparent molecular weight of about 1,200 (Fig. 2B). Thus, based on size, the original high-mannose glycan had two core *N*-acetylglucosamine and, on the average, roughly five mannose units.

The endoglycosidase H-released high-mannose-type glycans were digested with mannosidase. Most of the material was converted to [^{3}H]mannose (Fig. 7). *N*-acetylglucosaminidase together with mannosidase yielded a similar gel chromatography profile. Thus, most of the high-mannose-type glycans consisted of only mannose, in addition to the two core *N*-acetylglucosamine residues.

When the high-mannose-type glycans were chromatographed on concanavalin A-Sepharose, they could be eluted off the column only by 200 mM α-methylmannoside (Fig. 5C), a typical feature of this kind of glycan (1, 18). Thus, the structure of the Uukuniemi virus high-mannose-type oligosaccharides is suggested to be $(Man\alpha)_{3-5}Man\beta GlcNAc\beta GlcNAc$-peptide.

Glycans of isolated G1 and G2. The [^{35}S]methionine-labeled proteins G1, G2, and N were separated on SDS-PAGE, eluted from the gels, and digested with endoglycosidase H. The digests were dialyzed against 0.1% SDS, lyophilized, and analyzed on SDS-PAGE. The enzyme digestion did not affect the mobility of G1 (Fig. 8, lanes b and c). However, G2 was digested

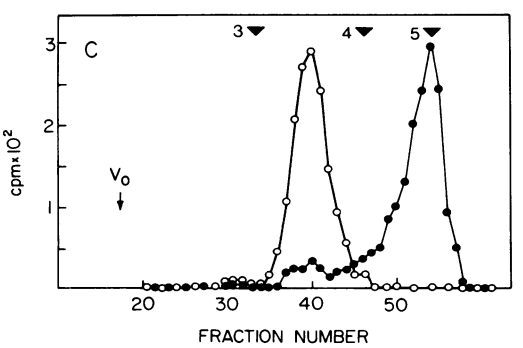


FIG. 7. Bio-Gel P-6 elution profiles of [^{3}H]mannose-labeled high-mannose-type glycans. The glycans released by endoglycosidase H digestion from the total glycopeptide preparation (○); mannosidase digest of the glycans (●). V_0 designates the void volume of the column. The different runs are drawn in the same panel. Markers are as in Fig. 3.

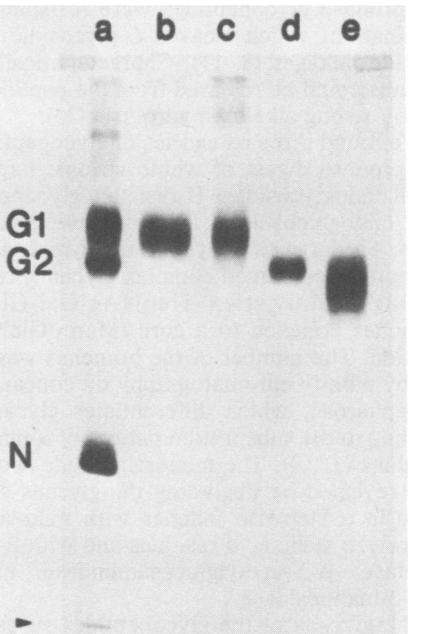


FIG. 8. SDS-PAGE in nonreducing conditions of [^{35}S]methionine-labeled Uukuniemi virus proteins digested with endoglycosidase H. Virus control (lane a); untreated (lane b) or digested (lane c) G1 protein; untreated (lane d) or digested (lane e) G2 protein. Arrow indicates the buffer front.

significantly (Fig. 8, lanes d and e). The products were resolved into several bands. The basis of this heterogeneity is not clear. Digestion of the non-glycosylated N protein served as a negative control for protease activity in the endoglycosidase H preparation. After digestion, the N protein migrated in SDS-PAGE with the same apparent molecular weight as authentic N, shown in lane b of Fig. 1 (data not shown). No radioactivity was released during the digestion, indicating the absence of proteases.

As mentioned above, G1 contained 38% and G2 contained 62% of the [^{3}H]mannose label. In the mixture of all [^{3}H]mannose-labeled glycopeptides, 38% were resistant (complex and intermediate glycopeptides) and 62% were susceptible (high-mannose-type glycopeptides) to endoglycosidase H digestion. Thus, G1 appeared to contain mainly complex and intermediate glycans, and G2 appeared to contain mainly high-mannose-type glycans.

DISCUSSION

The [^{3}H]mannose-labeled glycans, which were derived from the envelope glycoproteins G1 and G2 of Uukuniemi virus, appeared to be *N*-glycosidically linked to the polypeptide, i.e., via *N*-acetylglucosamine and asparagine, since

the pronase glycopeptides were resistant to β-elimination, which cleaves *O*-glycosidic glycopeptide linkages (8, 11). Characteristically, the glycans could be released from the peptide moiety by strong alkaline hydrolysis (20).

We found three categories of glycopeptides in the pronase digest of whole virions: large and small endoglycosidase H-resistant glycopeptides and endoglycosidase H-susceptible glycopeptides. The large endoglycosidase H-resistant glycopeptides contained complex glycans probably having mainly two NeuNAc-Gal-GlcNAc branches attached to a core $(Man)_3(GlcNAc)_2$-peptide. The number of the branches was studied by affinity chromatography on concanavalin A-Sepharose, which differentiates glycans according to the substitution pattern of α-mannose residues (1, 18). The monosaccharide sequence was revealed by degrading the glycans specifically in a stepwise manner with mild acid to hydrolyze sialic acid residues and with β-galactosidase, β-*N*-acetylglucosaminidase, and α- and β-mannosidase.

The glycans of the glycopeptides susceptible to endoglycosidase H digestion were composed of roughly five mannose residues attached to a core consisting of two *N*-acetylglucosamine units. The complex and the high-mannose-type glycans of Uukuniemi virus envelope glycoproteins appeared to be similar to those found in vesicular stomatitis virus (10, 38), Sindbis virus (7), and SFV (29, 30, 37) envelope glycoproteins, as well as in many soluble cellular glycoproteins (for a review, see reference 16).

The small glycopeptides which were resistant to endoglycosidase H digestion appear to have a mannosyl core, since they exhibit affinity to concanavalin A (18). Separate and double digestions with mannosidase and *N*-acetylglucosaminidase suggested that in 58% of the material one branch of the glycopeptides terminated in mannose and one in *N*-acetylglucosamine. About 42% of the material could be hydrolyzed only partly with triple digestion with galactosidase, *N*-acetylglucosaminidase, and mannosidase. Similar results were obtained for part of the glycopeptides of another bunyavirus, Inkoo virus (M. Pesonen, R. Rönnholm, E. Kuismanen, and R. F. Pettersson, manuscript in preparation). The structure of this resistant glycan was not further characterized.

Endoglycosidase H digestion of the isolated [^{35}S]methionine-labeled G1 and G2 proteins showed that G1 was not affected by this digestion, whereas G2 was digested significantly. After digestion, G2 migrated heterogeneously and slightly faster than the undigested control. The reason for this heterogeneity is not clear. A partial degradation of G2 by a contaminating protease or an incomplete digestion by endoglycosidase H could explain the results. No degradation of the N protein could, however, be detected under identical digestion conditions. Nor could any smaller degradation products of G2 be detected in the polyacrylamide gel (Fig. 8, lane e) at or above the buffer front. The endoglycosidase H used here released all of the high-mannose-type glycans present in SFV glycoprotein E2 (31) under identical conditions, suggesting complete digestion. Despite this, we cannot completely rule out the possibility that endoglycosidase H digested the high-mannose-type glycans of intact denatured G2 only partially. As G2 contained about 62% of the [^{3}H]mannose label, and the endoglycosidase H-susceptible glycopeptides of the roughly equimolar mixture of G1 and G2 amounted to 62%, it appears that G2 contained mainly high-mannose-type glycans, whereas G1 contained mainly complex and intermediate chains. The complex, intermediate, and high-mannose-type chains contained 25, 13, and 62% of the [^{3}H]mannose label. If the number of mannose units is taken as 3, 3, and 5, respectively, it can be calculated that the relative numbers of complex, intermediate, and high-mannose-type chains in a mixture of G1 and G2 was approximately 2:1:2.8.

The primary glycan attached to the nascent envelope proteins of vesicular stomatitis virus and Sindbis virus in the rough endoplasmic reticulum has mainly the structure $(Glc)_3(Man)_9(GlcNAc)_2$ (14, 22). During transport of the glycoproteins from the rough endoplasmic reticulum to the site of maturation at the plasma membrane, the primary glycan is processed to yield ultimately complex oligosaccharides with two or three NeuNAc-Gal-GlcNAc branches attached to a core with the structure Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4(Fucα1–6)GlcNAc-peptide. The processing begins with the excision of the glucose residues. Then, part of the mannose residues are cleaved off to expose Manα1–6 of the core glycan, followed by the addition of one distal *N*-acetylglucosamine residue. Then, more mannose is hydrolyzed to expose Manα1–6 of the core glycan, and another distal *N*-acetylglucosamine unit is added (12, 13, 15, 17). The chains are completed by the addition of one more *N*-acetylglucosamine, as well as galactose, sialic acid, and fucose. The high-mannose-type chains appear to be intermediates on the biosynthetic pathway from the primary glycan to complex ones (21). Several hydrolases and glycosyltransferases relevant in this processing, except for at least one glucosidase, have been found to be enriched in the Golgi complex (6, 25, 28, 40). When the transport of the envelope glycoproteins of SFV is arrested by the ionophore momensin in the Golgi complex, part of the glycans

have structures probably representing processing intermediates from high-mannose-type glycans to complex chains (M. Pesonen and L. Kääriäinen, submitted for publication). It is possible that the small endoglycosidase H-resistant glycopeptides found in Uukuniemi virus envelope glycoproteins also represent processing intermediates. Their existence may be connected with the site of maturation of Uukuniemi virus in smooth-surfaced intracellular vesicles in the Golgi region.

We have recently characterized the [^{3}H]mannose-labeled glycopeptides of another bunyavirus, Inkoo virus. Each class of glycopeptides found in Uukuniemi virus is present also in the envelope glycoproteins of this virus (Pesonen et al., manuscript in preparation). Data on the glycan structure of other bunyaviruses are not yet available. However, both envelope glycoproteins of two California encephalitis viruses, snowshoe hare virus and trivittatus virus, have glycans which can be labeled with radioactive glucosamine and contain terminal sialic acid residues (46). Moreover, the maturation of the snowshoe hare virus envelope proteins can be inhibited by tunicamycin, which inhibits glycosylation of polypeptides with *N*-glycosidic glycans (9).

ACKNOWLEDGMENTS

We thank Leevi Kääriäinen for helpful discussions and Tuula Rusi for excellent technical assistance.

The work was supported by a grant from the Sigrid Jusèlius Foundation.

LITERATURE CITED

1. **Baenziger, J. U., and D. Fiete.** 1979. Structural determinants of concanavalin A specificity for oligosaccharides. J. Biol. Chem. **254**:2400–2407.
2. **Baenziger, J. U., and D. Fiete.** 1979. Structural determinants of *Ricinus communis* agglutinin and toxin specificity for oligosaccharides. J. Biol. Chem. **254**:9795–9799.
3. **Bishop, D. H. L., C. H. Calisher, J. Casals, M. P. Chumakov, S. Y. Gaidamovich, C. Hannoun, D. K. Lvov, I. D. Marshall, N. Oker-Blom, R. F. Pettersson, J. S. Porterfield, P. K. Russell, R. E. Shope, and E. G. Westaway.** 1980. Bunyaviridae. Intervirology **14**:125–143.
4. **Bishop, D. H. L., and R. E. Shope.** 1979. Bunyaviridae, p. 1–156. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 14. Plenum Publishing Corp., New York.
5. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
6. **Bretz, R., H. Bretz, and G. E. Palade.** 1980. Distribution of terminal glycosyltransferases in hepatic Golgi fractions. J. Cell. Biol. **84**:87–101.
7. **Burke, D., and K. Keegstra.** 1979. Carbohydrate structure of Sindbis virus glycoprotein E2 from virus grown in hamster and chicken cells. J. Virol. **29**:546–554.
8. **Carlson, D. M.** 1968. Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. J. Biol. Chem. **243**:616–626.
9. **Cash, P., L. Hendershot, and D. H. L. Bishop.** 1980. The effects of glycosylation inhibitors on the maturation and intracellular polypeptide synthesis inducted by snowshoe hare bunyavirus. Virology **103**:235–240.
10. **Etchison, J. R., J. S. Robertson, and D. F. Summers.** 1977. Partial structural analysis of the oligosaccharide moieties of the vesicular stomatitis virus glycoprotein by sequential chemical and enzymatic degradation. Virology **78**:375–392.
11. **Gottschalk, A.** 1966. Glycoproteins; their composition, structure and function. Biochim. Biophys. Acta **5**:96–111.
12. **Harpaz, N., and H. Schachter.** 1980. Control of glycoprotein synthesis. Bovine colostrum UDP-N-acetylglucosamine: α-D-mannoside β2-N-acetylglucosaminyltransferase I. Separation from UDP-N-acetylglucosamine: α-D-mannoside β2-N-acetylglucosaminyltransferase II, partial purification, and substrate specificity. J. Biol. Chem. **255**:4885–4893.
13. **Harpaz, N., and H. Schachter.** 1980. Control of glycoprotein synthesis. Processing of asparagine-linked oligosaccharides by one or more rat liver Golgi α-D-mannosidases dependent on the prior action of UDP-N-acetylglucosamine: α-D-mannoside β2-N-acetylglucosaminyltransferase I. J. Biol. Chem. **255**:4894–4902.
14. **Hubbard, S. C., and P. W. Robbins.** 1979. Synthesis and processing of protein-linked oligosaccharides *in vivo*. J. Biol. Chem. **254**:4568–4576.
15. **Hunt, L. A., J. R. Etchison, and D. F. Summers.** 1978. Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **75**:754–758.
16. **Kornfeld, R., and S. Kornfeld.** 1980. *In* W. J. Lennarz (ed.), Biochemistry of glycoproteins and proteoglycans, p. 1–34. Plenum Press, New York.
17. **Kornfeld, S., E. Li, and J. Tabas.** 1978. The synthesis of complex-type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein. J. Biol. Chem. **253**:7771–7778.
18. **Kruslus, T., J. Finne, and H. Rauvala.** 1976. The structural basis of the different affinities of two types of acidic N-glycosidic glycopeptides for concanavalin A-Sepharose. FEBS Lett. **71**:117–120.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lee, Y. C., and J. R. Scocca.** 1972. A common structural unit in asparagine-oligosaccharides of several glycoproteins from different sources. J. Biol. Chem. **247**:5753–5758.
21. **Li, E., and S. Kornfeld.** 1979. Structural studies of the major high mannose oligosaccharide units from chinese hamster ovary cell glycoproteins. J. Biol. Chem. **254**:1600–1605.
22. **Li, E., I. Tabas, and S. Kornfeld.** 1978. The synthesis of complex-type oligosaccharides. I. Structure of the lipid-linked oligosaccharide precursor of the complex type oligosaccharides of the vesicular stomatitis virus G protein. J. Biol. Chem. **253**:7762–7770.
23. **Lyons, M. J., and J. Heyduk.** 1973. Aspects of the developmental morphology of California encephalitis virus in cultured vertebrate and arthropod cells and in mouse brain. Virology **54**:37–52.
24. **Mattila, K., and O. Renkonen.** 1978. Separation of A- and B-type glycopeptides of Semliki Forest virus by concanavalin A affinity chromatography and preliminary characterization of the B-type glycopeptides. Virology **91**:508–510.
25. **Munro, J. R., S. Narasimhan, S. Wetmore, J. R. Riordan, and H. Schachter.** 1975. Intracellular localization of GDP-L-fucose: glycoprotein and CMP-sialic acid: apolipoprotein glycosyltransferases in rat and pork livers. Arch. Biochem. Biophys. **169**:269–277.
26. **Murphy, F. A., A. K. Harrison, and S. G. Whitfield.** 1973. Bunyaviridae: morphologic and morphogenetic similarities of Bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. Intervirology **1**:297–316.
27. **Olsnes, S., E. Saltvedt, and A. Pihl.** 1974. Isolation and comparison of galactose-binding lectins from *Abrus pre-*

catorius and *Ricinus communis*. J. Biol. Chem. **249**:803–810.

28. **Opheim, D. J., and O. Touster.** 1978. Lysosomal α-D-mannosidase of rat liver. Purification and comparison with the Golgi and cytosolic α-D-mannosidases. J. Biol. Chem. **253**:1017–1023.
29. **Pesonen, M.** 1979. Sequence analysis of lactosamine type glycans of individual membrane proteins of Semliki Forest virus. J. Gen. Virol. **45**:479–487.
30. **Pesonen, M., K. Haahtela, and O. Renkonen.** 1979. Core tetrasaccharide liberated by endo-β-D-N-acetylglucosaminidase D from lactosamine-type oligosaccharides of Semliki Forest virus membrane proteins. Biochim. Biophys. Acta **588**:102–112.
31. **Pesonen, M., K. Hashimoto, J. Saraste, and L. Kääriäinen.** 1981. Reversible defect in the glycosylation of the membrane proteins of Semliki Forest Virus ts-1 mutant. Virology **109**:165–173.
32. **Pesonen, M., and O. Renkonen.** 1976. Serum glycoprotein-type sequence of monosaccharides in membrane glycoproteins of Semliki Forest virus. Biochim. Biophys. Acta **455**:510–525.
33. **Pettersson, R. F., M. J. Hewlett, D. Baltimore, and J. M. Coffin.** 1977. The genome of Uukuniemi virus consists of three unique RNA segments. Cell **11**:51–63.
34. **Pettersson, R., and L. Kääriäinen.** 1973. The ribonucleic acids of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **56**:608–619.
35. **Pettersson, R., L. Kääriäinen, C.-H. von Bonsdorff, and N. Oker-Blom.** 1971. Structural components of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **46**:721–729.
36. **Pettersson, R. F., and C.-H. von Bonsdorff.** 1975. Ribonucleoproteins of Uukuniemi virus are circular. J. Virol. **15**:386–392.
37. **Rasilo, M.-L., and O. Renkonen.** 1979. The molecular size of glycans liberated by hydrolysis from Semliki Forest virus proteins. Biochim. Biophys. Acta **582**:307–321.
38. **Reading, C. L., E. E. Penhoet, and C. E. Ballou.** 1978. Carbohydrate structure of vesicular stomatitis virus glycoprotein. J. Biol. Chem. **253**:5600–5612.
39. **Saikku, P., C.-H. von Bonsdorff, and N. Oker-Blom.** 1970. The structure of Uukuniemi virus. Acta Virol. **14**:103–107.
40. **Tabas, I., and S. Kornfeld.** 1978. The synthesis of complex-type oligosaccharides. III. Identification of an α-D-mannosidase activity involved in a late stage of processing of complex-type oligosaccharides. J. Biol. Chem. **253**:7779–7786.
41. **Tai, T., K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inou, and A. Kobata.** 1975. Structural studies of two ovalbumin glycopeptides in relation to the endo-β-N-acetylglucosaminidase specificity. J. Biol. Chem. **250**:8569–8575.
42. **Tarentino, A. L., T. H. Plummer, Jr., and F. Maley.** 1974. The release of intact oligosaccharides from specific glycoproteins by endo-β-N-acetylglucosaminidase H. J. Biol. Chem. **249**:818–824.
43. **Ulmanen, I., P. Seppälä, and R. F. Pettersson.** 1981. In vitro translation of Uukuniemi virus-specific RNAs: identification of a nonstructural protein and a precursor to the membrane glycoproteins. J. Virol. **37**:72–79.
44. **von Bonsdorff, C.-H., and R. F. Pettersson.** 1975. Surface structure of Uukuniemi virus. J. Virol. **16**:1296–1307.
45. **von Bonsdorff, C.-H., P. Saikku, and N. Oker-Blom.** 1969. The inner structure of Uukuniemi and two Bunyamwera supergroup arboviruses. Virology **39**:342–344.
46. **Vorndam, A. V., and D. W. Trent.** 1979. Oligosaccharides of the California encephalitis viruses. Virology **95**:1–7.
47. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234**:1971–1975.