The carbohydrate structure of the membrane glycoprotein of vesicular stomatitis virus (New Jersey serotype) has been determined on material purified from virions grown in spinner cultures of BHK21 cells in the presence of [3H]glucosamine. The intact [3H]-glycoprotein contains 6 residues of N-acetyl-D-neuraminic acid, 6 of D-galactose, 6 of D-mannose, 2 of L-fucose, and 10 of N-acetyl-D-glucosamine. Neither D-glucose nor N-acetyl-D-galactosamine was detected. The carbohydrate was not susceptible to β elimination, and glycopeptides were obtained that yielded 1 mol of aspartic acid/mol of glycopeptide after acid hydrolysis, both of which indicate that the sugar is linked to the amide nitrogen of asparagine in the glycoprotein. Hydrazinolysis of the glycoprotein yielded a single major oligosaccharide that, after re-N-acetylation, contained 3 residues of N-acetylneuraminic acid, 3 of galactose, 3 of mannose, 1 of fucose, and 5 of N-acetylglucosamine, suggesting that the glycoprotein contains two identical oligosaccharide chains. The linkages in the hydrazinolysis oligosaccharide were established by methylation analysis. The oligosaccharide was fragmented by partial acid hydrolysis and was specifically depolymerized at de-N-acetylated glucosamine residues by nitrous acid deamination, the fragments obtained at each step were purified, and their carbohydrate compositions were determined. A glycopeptide fraction, obtained by digestion of the glycoprotein with trypsin and pronase, was separated on the basis of amino acid differences into two components with identical sugar compositions that are assumed to represent units from the two different sites of glycosylation in the protein. The glycopeptides were subjected to sequential exoglycosidase digestion with a-D-neuraminidase, β-D-galactosidase, β-D-hexosaminidase, α-D-mannosidase, β-D-mannosidase, and α-L-fucosidase. After removal of the N-acetyleneuraminic acid, the galactose, and part of the N-acetylglucosamine, the glycopeptides were cleaved with an endo-N-acetyl-β-D-glucosaminidase. All fragments were reisolated after each digestion step and analyzed for carbohydrate composition or for linkages by methylation analysis. The glycoprotein, glycopeptides, and hydrazinolysis oligosaccharide were oxidized with periodate and subjected to Smith degradation, and the compositions and linkages of the products were determined. The results establish that the glycoprotein contains two identical carbohydrate chains that are composed of three outer chain units, a mannose-rich core, and a peptide linkage unit, with the structures

\[
\text{[aNeuNAc}+3\text{Gal}+\text{GlcNAc}]_3 \quad \text{aMan} \quad \text{GlcNAc+} \quad \text{bMan} \quad \text{fuc} \quad \text{GlcNAc+Asn} \]

and that these are interlinked to form an oligosaccharide with the following structure.

\[
\text{aNeuNAc}+3\text{Gal}+\text{GlcNAc} \quad \text{aFuc} \quad \text{bMan}+\text{GlcNAc+} \quad \text{fuc} \quad \text{GlcNAc+Asn} \quad \text{aMan} \]

Vesicular stomatitis virus is the prototype of the rhabdoviruses (1). The purified virions contain RNA, protein, carbohydrate, and lipid. The RNA is a single molecule with a molecular weight of 3.6 x 10^6 (2). VSV contains five proteins, labeled L, G, N, NS, and M, with molecular weights of 150,000, 70,000, 50,000, 49,000, and 30,000, respectively (3). The L, N, and NS proteins are components of the ribonucleoprotein, whereas the G and M proteins are associated with the viral coat. The G protein is the only glycoprotein in the virus. The lipid and glycolipid compositions of the envelope generally reflect those of the host cell membrane (4, 5). The glycoprotein is reported to contain N-acetyl-D-glucosamine, D-mannose, D-galactose, L-fucose, N-acetyl-D-neuraminic acid, as well as D-glucose and N-acetyl-D-galactosamine (6-8).

The carbohydrate components of viral glycoproteins are thought to be involved in the hemagglutination of erythrocytes and the absorption of virus to host cells during infection (9-11). Removal of the G protein from VSV by digestion with proteases (12) renders the virions noninfectious, and infectivity can be restored by reconstitution with purified glycoprotein (13). The importance of terminal sialic acid residues on the G protein for hemagglutination and absorption is suggested by the fact that neuraminidase digestion drastically lowers VSV infectivity, agglutination of goose erythrocytes, and viral attachment to mouse L-cells, and these properties are restored by resialylation of the virus with a purified sialotransferase (14-16).

A complete structure has not been reported for the carbohydrate component of the G protein. The evidence indicates that there are two nitrogen-linked oligosaccharide chains, with...
molecular weight about 3000, that are similar in structure to the chains of serum glycoproteins (17-19). In the present study, we show that VSV glycoprotein has two identical carbohydrate chains and we confirm that they are linked to asparagine residues in the protein. Moreover, we have determined all of the sequences, linkages, and anomeric configurations, thus allowing the assignment of a unique structure.

**Experimental Procedures**

**Cell Culture**—Baby hamster kidney cells, clone 21, subculture 13 (BHK cells) were obtained from Dr. Tad Wiktor, Wistar Institute of Anatomy and Biology, Philadelphia, Pa., and were maintained as a monolayer culture in glass prescription bottles using Dulbecco’s modified Eagle’s medium (Gibco H21) supplemented with 10% fetal bovine serum (Irvine Scientific Co.) and buffered with 15 mM Hepes (Sigma).

A suspension culture of BHK cells (BHK spinner cells) was derived from the monolayer culture by spinning. After the pH of confluent BHK monolayer cells (1.2 x 10^6) were incubated for 5 min at 23°C with 0.5% trypsin in phosphate-buffered saline (20 mL) lacking Ca++ and Mg++ and containing 5 mM EDTA. The cells were pipetted up and down several times, to obtain a suspension of single cells, and resuspended in (100 mL of Joklik’s modified Eagle’s medium (Flow Laboratories) supplemented with 7.4 x 10^5 RPM.)

The cells were maintained in suspension in a 500-mL serum bottle with a 1.5-inch Teflon-coated seamless bottle (20 mL) containing the normal components of JME medium with NaCl and MgCl2. The medium was aspirated, and the cells were resuspended in 90 mL of medium and cultured at 37°C. After 24 h, the virus suspension was harvested and resuspended in a medium containing the normal components of JME medium with NaCl and MgCl2. The labeled virus was harvested at 24 h after infection. The labeled virus was then centrifuged at 10,090 x g in a clinical centrifuge to collect the cells.

**Virus Production and Purification**—The New Jersey serotype of vesicular stomatitis virus was obtained from Dr. John Holland, Department of Biology, University of California, San Diego, and selected free of defective virus by picking large plaques on high dilution passages. Stock cultures were grown in glass prescription bottles by infecting PBS-washed, subconfluent, monolayer BHK cultures with virus at a multiplicity of infection of 0.01 (0.01 plaque-forming units/well) in 2 mL of 2% calf serum in Hepes-buffered DME medium.

The medium was aspirated periodically to bathe the cells with virus. After 10 mL of solution was aspirated, 10 mL of 2% calf serum in Hepes-buffered DME medium was added and the cells were incubated at 37°C. After 24 h, the virus-containing medium was collected and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant fluid was divided into portions of 100 mL, adjusted to 7.4 x 10^5 RPM with 0.5 M CaCl2, and stored at -70°C.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis was done on slab gels with a diethylaminoethanol buffer system (20). Protein-containing bands were visualized with Coomassie brilliant blue R-250 stain (Bio-Rad), and carbohydrate-containing bands were detected with the periodate-Schiff reagent (31). Radioactivity was determined in a stained gel by drying it on Whatman 3MM paper (32) and counting. 3'H radioactivity was measured in a Searle model 1185 Auto Gamma system.

**Chromatography and Electrophoresis**—Bio-Gel P-2 (-400 mesh), P-6 (200 to 400 mesh), P-10 (200 to 400 mesh), P-100 (100 to 200 mesh), and AG 50W-X2 (200 to 400 mesh) were from Bio-Rad. Gel filtration of the glycoprotein was done on a Bio-Gel P-100 column (2 x 20 cm) in 0.1 M sodium dodecyl sulfate and 0.5 M glucose-free medium containing 4 times the normal amount of amino acids and dialyzed fetal bovine serum. Four hours after infection, 100 μCi of [3-3H]glucosamine HCl (NET-190, 10 Ci/mmol, New England Nuclear) was added in 1 mL of medium, and 2 h later 10 mL of complete medium was added. The virus was harvested 24 h after infection.

To prepare large scale cultures (up to 40 liters), the cells were grown in 4-L Erlenmeyer flasks, and the volumes were doubled daily with prewarmed medium.
RESULTS
Studies on Intact Glycoprotein

Production and Purification of Vesicular Stomatitis Virus—For the production of large amounts of VSV, 3-liter spinner cultures of BHK cells were infected at a density of 10^6 cells/ml with a multiplicity of infection of 0.1, and the cultures were diluted to 4 liters with prewarmed medium. After 24 h, the cells were pelleted at 10,000 × g for 20 min at 4°C. The supernatant liquid containing the virus (up to 10^9 plaque forming units/ml) was decanted and the virus was precipitated in 7.5% polyethylene glycol 6000 (PEG 6000) and 0.5 M NaCl according to McSharry and Benzinger (50). The precipitate was collected by centrifugation at 10,000 × g for 20 min at 4°C, and the viral pellet was resuspended by repeated pipetting in 10 mM Tris-Cl, pH 7.4, containing 0.1 M NaCl and 1 mM EDTA. The virus suspension was sonicated (five 1-s bursts in a Branson W185 cell disruptor), then centrifuged at 20,000 rpm in a Spinco 30 rotor for 1 h at 4°C. The viral pellet was resuspended by pipetting in the Tris buffer. Seven milliliters of this suspension was layered onto a preformed 25-ml NaK tartrate gradient of 15 to 33% w/w and centrifuged to isopycnic equilibrium (at least 6 h) in a SW 25.1 rotor at 22,500 rpm. The dense white viral band was collected by piercing the tube with an 18-gauge needle attached to a 10-ml plastic syringe and withdrawing the virus in a volume of 5 to 8 ml. The virus suspension from the tartrate gradient was diluted with 3 volumes of the Tris buffer and pelleted by centrifugation at 28,000 rpm in a Spinco 30 rotor for 1 h at 4°C. The overall yield of infectivity was about 60%.

Purity of the virus preparation was assessed in several ways. Transmission electron microscopy of samples, negatively stained with 2% phosphotungstic acid without prior fixation, revealed only virions. Only the five proteins of VSV were observed when sodium dodecyl sulfate gel electrophorograms were stained for protein (Fig. 1). If the virus was grown in the presence of [3H]glucosamine, or the purified virions were radioiodinated using conditions under which only the exposed membrane proteins are iodinated (51), a single radioactive peak was observed on electrophorograms, at a position identical to that of the glycoprotein. A single band in the same position was observed if the gel was stained for carbohydrate (data not shown). These tests indicate that the virus preparation was not contaminated by a significant amount of cellular protein.

Isolation of Glycoprotein—The viral pellet was resuspended in 50 ml of 10 mM Tris-Cl, pH 8.0, containing 10 μM phenylmethylsulfonyl fluoride. Five milliliters of 20% Triton X-100 was added, and the suspension was stirred at 25°C for 1 h to release the glycoprotein from the virions (52). The viral cores were removed by centrifugation at 48,000 rpm in a Spinco 50 rotor for 1 h at 4°C, and the supernatant solution was lyophilized.

The lyophilized Triton extract was resuspended in 1 ml of water and the glycoprotein was precipitated with 10 volumes of acetone. The precipitate was collected by centrifugation (1,000 × g, 5 min) and washed in hot absolute ethanol. The precipitate was suspended in water:1-butanol (1:1) and sonicated, and the two phases were separated by centrifugation. The precipitated glycoprotein, which collected at the interface, was isolated and this extraction with the butanol/water mixture was repeated twice. The glycoprotein was further extracted in the two-phase system of chloroform:meth-
anol:water (3:2:1), and the material at the interface was collected and sonicated in chloroform:methanol:water (3:3:1). The precipitate was collected by centrifugation, dried in a stream of Ne, and stored at -20°C.

The glycoprotein preparation was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After Triton X-100 treatment and ultracentrifugation, the supernatant extract contained only the glycoprotein, whereas the other virion proteins were in the pelleted fraction (Fig. 1). Glycoprotein, isolated from VSV produced in the presence of 14C-amino-acids, was the only radioactive protein observed in the Triton supernatant fraction.

Carbohydrate Composition—Carbohydrate was determined by gas-liquid chromatography of the trimethylsilyl ethers of the methyl glycosides obtained by methanalysis. Fig. 2 shows a representative chromatogram, and the averaged values for seven determinations on the intact glycoprotein are listed in Table I. The values for N-acetylglucosamine were close to 10 mol/mol, based on the dry weight of the sample and assuming $M_r = 69,000$ for the glycoprotein. The values are normalized to 10.0 residues of N-acetylglucosamine/glycoprotein molecule on the assumption of two sites of glycosylation, with 5 hexosamine residues/site. There were also approximately 2 mol of fucose, 6 of mannose, 6 of galactose, and 6 of sialic acid/10 mol of N-acetylgalactosamine. Glucose and N-acetylgalactosamine were absent.

Periodate Oxidation—The glycoprotein was oxidized with 4 mM periodic acid in 1 ml of 2% Triton X-100 in the dark at 23°C for 24 h, and the reaction was terminated by a drop of glycerol. The oxidized glycoprotein was precipitated with 10 ml of acetone, washed in 2 ml of 1 M acetic acid, collected by centrifugation, and washed in hot ethanol. Periodate treatment destroyed 83% of the fucose, 91% of the sialic acid, and 33% of the mannose (Table I). Galactose and N-acetylgalactosamine were unaffected.

Alkali Stability of Glycosidic Linkage to Protein—The purified [3H]glucosamine-labeled glycoprotein in 1% sodium dodecyl sulfate was treated with 0.1 M NaBH₄ in 0.1 M NaOH at 23°C for 24 h, conditions that cause β elimination of sugars linked to serine and threonine. The solution was acidified and fractionated in 0.1% sodium dodecyl sulfate on a Bio-Gel P-100 column (2 x 20 cm). The radioactivity was eluted in a single peak near the void volume of the column, indicating that no sugar was released that would have derived 3H from [3H]glucosamine (N-acetylgalactosamine, N-acetylgalactosamine, or sialic acid). The recovered alkali-treated glycoprotein had a slightly decreased sialic acid content (Table I), but this sugar is known to be destroyed in alkaline solution (53). A

![Fig. 2. Gas-liquid chromatogram tracing of the trimethylsilyl methyl glycosides and polyols. The top panel illustrates a typical separation of an equimolar mixture of standards. Peak identifications are: arabinitol, 1 and 5, fucose, 2, 3, and 4; mannose, 6 and 7; galactose, 7, 8, 9, and 10; glucose, 9, 10, and 11; mannitol, 12 and 13; N-acetylglactosamine, 14 and 16; N-acetylgalactosamine, 15, 17, and 18; and N-acetylmuraminic acid, 19. The bottom panel shows the component sugars of a sample of VSV glycoprotein. Note the absence of Peaks 10 and 11 (glucose) and 16 (N-acetylgalactosamine) in this tracing. The multiple peaks of the methyl glycosides represent different anomers and ring forms, whereas the double peaks for the polyols probably reflect incomplete substitution.](http://www.jbc.org/)

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lower value of fucose was obtained after β elimination, but this can be explained by loss of the volatile derivative during analysis (see "Discussion"). All of the mannose, galactose, and N-acetylglucosamine survived the treatment and, therefore, represent carbohydrate attached to protein by N-glycosidic linkages.

Studies on Isolated Oligosaccharides and Glycopeptides

Hydrazinolysis and Nitrous Acid Deamination—The (1H)glucosamine-labeled glycoprotein (9.0 × 10^5 cpm, 450 nmol) was subjected to hydrazinolysis according to Yosizawa et al. (54) to break the N-glucosyl asparagine amide linkages and release the intact oligosaccharides, during which the acetamido linkages of the amino sugars were also cleaved (see Fig. 3). After the hydrazine was sublimed according to Schroeder (55), the reaction product was desalted on a Bio-Gel P-6 column (1.6 × 120 cm) by elution with water, and 860 nmol of oligosaccharide was recovered. A portion of the recovered oligosaccharide (450 nmol) was re-N-acetylated in 1 ml of saturated NaHCO3 with 0.05 ml of acetic anhydride for 1 h at 23°C, and subjected to gel filtration on a Bio-Gel P-6 column (1.6 × 20 cm) by elution with water. The carbohydrate composition of the re-N-acetylated oligosaccharide, normalized to 5.0 residues of N-acetylglucosamine (Table II), was almost identical to that of the glycoprotein.

A portion (410 nmol) of the hydrazinolysis oligosaccharide, which had not been re-N-acetylated, was deaminated with nitrous acid as described by Horton and Phillips (56) to depolymerize the molecule at the positions occupied by glucosamine (see Fig. 3) and produce fragments terminated at the reducing end by 2,5-anhydro-D-mannose (57). The products were reduced with borohydride and separated in water on a Bio-Gel P-2 column (1.6 × 120 cm) (Fig. 4). About 85% of the initial radioactivity was recovered in four fractions with the compositions in Table II. Fraction A contained a compound with a retention time by gas chromatography near that of sialic acid; it probably represents a deaminated form of this sugar. Due to its negative charge, it was eluted near the void volume of the Bio Gel P 2 column. Fraction B was eluted in the tetrasaccharide region and contained approximately 3 mannoses/2,5-anhydromannitol, as well as small amounts of galactose and N-acetylglucosamine. This product represents the mannoses attached to N-acetylglucosamine in the core. The 2,5-anhydromannitol arises from the reduction of the 2,5-anhydromannosyl produced by the degradation of the glucosamine. Fraction C contained approximately 4 galactose residues and 1 fucose residue/4 residues of 2,5-anhydromannitol. Since this peak was eluted in the disaccharide region of the Bio-Gel P-2 column, it was probably a mixture of galactosyl → 2,5-anhydromannosyl and fucosyl → 2,5-anhydromannosyl, with the first being 3 times as prevalent as the second. Fraction D contained only a trace of 2,5-anhydromannitol. The results indicate that the oligosaccharide contained both galactosyl-

Table I

Carbohydrate composition of VSV glycoprotein

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Residues ± S.D.*</th>
<th>Range</th>
<th>β Elimination reaction</th>
<th>Periodate oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>1.7 ± 0.3</td>
<td>1.5-2.1</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Mannose</td>
<td>6.0 ± 0.4</td>
<td>5.6-6.3</td>
<td>6.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.2 ± 0.2</td>
<td>5.9-6.4</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td>N-Acetylglycosamine</td>
<td>10.0†</td>
<td>10.0†</td>
<td>10.0†</td>
<td>10.0†</td>
</tr>
<tr>
<td>N-Acetyleneuraminic acid</td>
<td>5.8 ± 0.3</td>
<td>5.6-6.1</td>
<td>2.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Mol of monosaccharide/mol of glycoprotein, assuming M, = 69,000 for the glycoprotein.
† Normalized to a value of 10.0 for N-acetylglucosamine, as described in the text.

Table II

Carbohydrate compositions of hydrazinolysis oligosaccharides and degradation products

<table>
<thead>
<tr>
<th>Substance</th>
<th>N-Acetylneuraminic acid</th>
<th>Galactose</th>
<th>Mannose</th>
<th>N-Acetylgalactosamine</th>
<th>Fucose</th>
<th>2,5-Anhydromannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact glycoprotein</td>
<td>2.9</td>
<td>3.1</td>
<td>3.0</td>
<td>5.0</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Hydrazinolysis oligosaccharide</td>
<td>3.0</td>
<td>3.2</td>
<td>2.5</td>
<td>5.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Periodate-oxidized hydrazinolysis oligosaccharide</td>
<td>0.5</td>
<td>2.7</td>
<td>1.9</td>
<td>5.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Nitrous acid deamination fragments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.1</td>
<td>1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Fraction B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.1</td>
<td>1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Fraction C</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Partial acid hydrolysis fragments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Fraction B</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Fraction C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Molar ratios normalized to 5.0 mol of N-acetylglucosamine.
† Molar ratios normalized to 2,5-anhydromannitol. Fractions are from Fig. 4.
‡ Contained only 2,5-anhydromannitol.
§ Molar ratios normalized to 3 or 5 residues of N-acetylglucosamine to agree with the size of the fragments assessed from gel filtration in Fig. 5.
" Contained only N-acetylglucosamine.
Table III

Methylation analysis of hydrazinolysis oligosaccharide

<table>
<thead>
<tr>
<th>Peak on chromatogram</th>
<th>Methylated sugar</th>
<th>Area under peak</th>
<th>Linkages in oligosaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,3,4,6-Tetramethylglucosamine</td>
<td>0.3</td>
<td>Fuc →</td>
</tr>
<tr>
<td>2</td>
<td>2,3,4,6-Tetramethylglucosamine</td>
<td>0.6</td>
<td>Gal →</td>
</tr>
<tr>
<td>3</td>
<td>2,4,6-Trisaccharide</td>
<td>1.8</td>
<td>GlcNAc →</td>
</tr>
<tr>
<td>4</td>
<td>2,3,4,6-Trisaccharide</td>
<td>1.0</td>
<td>Man →</td>
</tr>
<tr>
<td>5</td>
<td>2,4,6-Trisaccharide</td>
<td>0.6</td>
<td>Man →</td>
</tr>
<tr>
<td>6</td>
<td>2,4,6-Trisaccharide</td>
<td>1.0</td>
<td>Man →</td>
</tr>
<tr>
<td>7</td>
<td>3-Methyl-N-acetylglucosamine</td>
<td>0.2</td>
<td>N-acetylglucosamine →</td>
</tr>
<tr>
<td>8</td>
<td>3-Methyl-N-acetylglucosamine</td>
<td>0.2</td>
<td>N-acetylglucosamine →</td>
</tr>
</tbody>
</table>

Separation was partially methylated alditol acetates and characterized by retention times and fragment patterns in the mass spectrometer.

These values have not been corrected for differences in detector response nor for differential destruction or loss during processing. Thus, their quantitation is not meant to reflect the composition of the original oligosaccharide.

Vesicular Stomatitis Virus Glycoprotein Structure

Partial Acid Hydrolysis—The re-N-acetylated hydrazinolysis [3H]oligosaccharide (100 nmol) was subjected to partial acid hydrolysis (50 mM HCl at 100°C for 4 h), and the products were separated on a Bio-Gel P-6 column (Fig. 5). A dark brown color which developed during the hydrolysis stayed at the top of the column. Three fractions were collected and each was purified on a Bio-Gel P-2 column, and their compositions are given in Table II. Fraction A contained mannose and N-acetylglucosamine in the molar ratio of 3:3; Fraction B contained mannose and N-acetylglucosamine in the molar ratio of 3:3; and Fraction C contained only N-acetylglucosamine. A labeled compound containing fucose was not observed, and the radioactive sialic acid was not eluted from the column. Since sialic acid is destroyed during acid hydrolysis (53), the brown material that remained at the top of the column may have contained the degradation products. The recovery of radioactivity in glucosamine was 98%. The fact that sialic acid, galactose, and fucose were rapidly released indicates that they occur near the nonreducing termini of the oligosaccharide.

Oxidation—A sample of the re-N-acetylated hydrazinolysis oligosaccharide (100 nmol) was oxidized with 4 mM periodic acid in 1 ml of 0.1 M sodium acetate, pH 4.5, at 23°C in the dark. After 24 h, a drop of glycerol was added and the product was chromatographed on a Bio-Gel P-6 column (1.6 × 120 cm). Two radioactive peaks were observed. The first peak, which was eluted near the void volume of the column, contained 98 nmol of the oxidized oligosaccharide, and it was treated with NaBH₄ to reduce the aldehydes generated by the oxidation. The second peak eluted in the salt volume of the column and lost all radioactivity when it was lyophilized. Presumably it contained [3H]glyceraldehyde that came from carbon 9 of [9-3H]sialic acid, derived from [6-3H]glucosamine. The composition of the periodate-oxidized oligosaccharide (Table II) indicates that 1 residue of mannose, all of the fucose, and almost all of the sialic acid were destroyed by the periodate. The galactose and N-acetylglucosamine remained intact. This agrees with the results of periodate treatment of the intact glycoprotein.

Methylation Analysis of Re-N-acetylated Hydrazinolysis Oligosaccharide—The results, summarized in Table III, show that almost all of the galactose was substituted in position 3, whereas all of the fucose was unsubstituted. Mannose appeared as the 2-substituted and the 3,6- and 2,4-disubstituted derivatives. N-Acetylglucosamine substituted in position 4 except for a small portion that was 4,6-disubstituted. Sialic
acid does not survive the procedures involved in methylation analysis, and no peak was observed for it.

**Glycopeptide Obtained by Trypsin Digestion**—Glycopeptide (1 µmol), labeled with [6-3H]glucosamine (2.0 × 10⁶ cpm), was digested with 5 mg of trypsin in 2 ml of 0.1 M Tris-Cl, pH 7.8, for 24 h at 37°C under toluene. The digest was centrifuged and the supernatant fraction was removed and frozen. The pellet, which contained undigested glycopeptide, was redigested as above 5 times until at least 95% of the radioactivity had been solubilized. The reaction mixture was fractionated on a Bio-Gel P-10 column (1.6 × 120 cm) in 0.1 M ammonium acetate, pH 7, containing 0.1% toluene. The single radioactive peak obtained contained 1.9 µmol of glycopeptide and had a sugar composition similar to that of the glycoprotein (Table IV).

**Pronase Digestion**—Tryptic glycopeptide (1.6 µmol) was digested with 5 mg of pronase in 1 ml of 0.1 M NH₄HCO₃, pH 7.8, at 37°C for 24 h under toluene, and the products were separated on a Bio-Gel P-6 column (1.6 × 120 cm) in 0.1 M ammonium acetate, pH 7 (Fig. 6, open circles). The compositions of the recovered peaks (1.5 µmol) (Table IV) were similar to those of the tryptic glycopeptide. The major component, Fraction A (tubes 41 to 56), contained 3 residues of sialic acid/glycopeptide, and Fraction B (tubes 57 to 68) contained only 2 residues of sialic acid/glycopeptide, indicating a slight heterogeneity in the sugar compositions of the glycopeptide. These were recombined to yield 1.45 µmol for the following studies in which the glycopeptide was degraded sequentially according to the scheme in Fig. 7.

**Smith Degradation of Pronase-digested Glycopeptide** (Fig. 7a)—A portion (200 nmol) of the recombined pronase-digested glycopeptide fraction was oxidized with 0.1 M periodic acid in 0.5 M sodium acetate buffer, pH 4.5, for 24 h at 23°C in the dark. The sample was treated with 1 ml of 1 M NaBH₄, 0.5 h at 23°C and then acidified with 0.2 ml of glacial acetic acid. The periodate-treated glycopeptide was then chromatographed on a Bio-Gel P-6 column (1.6 × 120 cm). Two radioactive peaks were observed. The first contained the glycopeptide fraction (184 nmol), and its composition (Table IV) shows that the fucose, most of the sialic acid, and 1 residue of mannose were destroyed by periodate. The second peak appeared in the included volume of the column, and the radioactivity was lost by lyophilization. This radioactivity probably was in methanol formed by reduction of the [3H]formaldehyde produced by periodate oxidation of the [9-3H]sialic acid.

The oxidized and reduced glycopeptide fraction (101 nmol) was subjected to mild acid hydrolysis (1 M HCl at 23°C for 24 h) (Smith degradation), and the products were fractionated on a Bio-Gel P-6 column (1.6 × 120 cm). A glycopeptide fraction (100 nmol) was recovered that contained no fucose or sialic acid, 2 residues of mannose, about 2 residues of galactose, and 4 residues of N-acetylglucosamine (Table IV). The methylation analysis in Table V demonstrates that the 2-substituted mannose was destroyed and that the 3,6-disubstituted mannose was no longer present, but that a 6-substituted

### Table IV

<table>
<thead>
<tr>
<th>Substance</th>
<th>N-Acetylneuraminic acid</th>
<th>Galactose</th>
<th>Mannose</th>
<th>N-Acetylglucosamine</th>
<th>Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoprotein</td>
<td>2.9</td>
<td>3.1</td>
<td>3.0</td>
<td>5.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Tryptic glycopeptide</td>
<td>2.7</td>
<td>2.9</td>
<td>3.0</td>
<td>5.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Pronase-treated trypsin glycopeptide (A)</td>
<td>2.5</td>
<td>3.3</td>
<td>3.4</td>
<td>5.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Periodate-oxidized</td>
<td>0.2</td>
<td>3.0</td>
<td>2.1</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Smith-degraded (I)</td>
<td>0.1</td>
<td>2.0</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Neuramidase-digested pronase glycopeptide (B)</td>
<td>0.2</td>
<td>3.0</td>
<td>2.1</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Periodate-oxidized</td>
<td>0.2</td>
<td>3.0</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Smith-degraded (J)</td>
<td>0.2</td>
<td>3.0</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>β-n-Galactosidase-digested (C)</td>
<td>3.2</td>
<td>5.0</td>
<td>0.5</td>
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<td></td>
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<tr>
<td>β-n-Hexosaminidase-digested (D)</td>
<td>2.9</td>
<td>2.0</td>
<td>0.5</td>
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<td></td>
</tr>
<tr>
<td>Endo-oligosaccharide (E)</td>
<td>2.0</td>
<td>1.0</td>
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<td></td>
<td></td>
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<tr>
<td>α-d-Mannosidase-digested (F)</td>
<td>0.9</td>
<td>1.0</td>
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<tr>
<td>β-d-Mannosidase-digested</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo-glycopeptide-I (G)</td>
<td>1.0</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-L-Fucosidase-digested</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo-glycopeptide-II (Ω)</td>
<td>1.0</td>
<td>0.8</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>α-L-Fucosidase-digested</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Analyzed as alditol acetates.
Vesicular Stomatitis Virus Glycoprotein Structure

**Fig. 7.** Sequential degradation of VSV glycopeptide. Structures are identified with capital letters, whereas reaction steps are identified with lower case letters. The intact glycopeptide (A) was treated sequentially, with reisolation of the product at each step, with α-D-neuraminidase (a), β-1-n-galactosidase (b), exo-N-acetyl β-D-mannosidase (c), endo-N-acetyl-β-D-glucosaminidase (d), α-D-mannosidase (e), β-N-mannosidase (f), and α-L-fucosidase (g). Glycopeptides A and B were subjected to Smith degradation (sequential periodate oxidation, reduction and partial acid hydrolysis, steps h and i).

**Smith Degradation of Neuraminidase-digested Pronase-treated Glycopeptide (Fig. 7j)**—The neuraminidase-digested glycopeptide (200 nmol) was oxidized with periodate and reduced with NaBH₄, and the products were separated on a Bio-Gel P-6 column as described for the pronase-digested glycopeptide. The oxidized and reduced glycopeptide fraction was eluted in a single radioactive peak and contained only a trace of galactose, 2 residues of mannose, and 5 residues of N-acetylglucosamine (Table IV). Mild acid hydrolysis performed on 120 nmol, as described for the pronase-digested glycopeptide, yielded two radioactive peaks that were separated by gel filtration on a Bio-Gel P-2 column (1.6 X 120 cm). The first contained the Smith-degraded glycopeptide fraction (110 nmol), and had a composition of 2 residues of mannose and 4 of N-acetylglucosamine, The methylolation analysis is given in Table V. The second peak contained only N-acetylglucosamine (105 nmol).

**Digestion with β-Galactosidase**—The neuraminidase-digested glycopeptide (800 nmol) was incubated with 14 units of β-D-galactosidase in 1 ml of 50 mM sodium citrate, pH 3.5, for 24 h at 37°C under toluene (Fig. 7b), and the products were separated on a Bio-Gel P-6 column (Fig. 6, L). The composition of the glycopeptide fraction (750 nmol) (Table IV) is that expected for selective removal of all galactose, confirming that all of this hexose was in a terminal location after removal of the sialic acid.

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**Vesicular Stomatitis Virus Glycoprotein Structure**

\[
\begin{align*}
\text{aNeuNAc} + ^{3}\text{βGal} + ^{4}\text{βGlcNAc} & \quad \alpha \text{Fuc} \\
\text{aNeuNAc} + ^{3}\text{βGal} + ^{4}\text{βGlcNAc} + ^{2}\text{αMan} & \quad \text{αFuc} \\
\text{aNeuNAc} + ^{3}\text{βGal} + ^{4}\text{βGlcNAc} + ^{2}\text{αMan} + ^{6}\text{αMan} & \quad \text{αFuc} \\
\text{βGal} + ^{4}\text{βGlcNAc} + ^{2}\text{αMan} + ^{6}\text{αMan} & \quad \text{αFuc} \\
\end{align*}
\]

\[
\begin{align*}
\text{βGal} + ^{4}\text{βGlcNAc} + ^{2}\text{αMan} + ^{6}\text{αMan} & \quad \text{αFuc} \\
\text{βGal} + ^{4}\text{βGlcNAc} + ^{2}\text{αMan} + ^{6}\text{αMan} + ^{4}\text{βGlcNAc} + ^{4}\text{GlcNAc} + ^{4}\text{Asn} & \quad \text{αFuc} \\
\text{βGal} + ^{4}\text{βGlcNAc} + ^{2}\text{αMan} + ^{6}\text{αMan} + ^{4}\text{βGlcNAc} + ^{4}\text{GlcNAc} + ^{4}\text{Asn} & \quad \text{αFuc} \\
\end{align*}
\]

---

**FIG. 7.** Sequential degradation of VSV glycopeptide. Structures are identified with capital letters, whereas reaction steps are identified with lower case letters. The intact glycopeptide (A) was treated sequentially, with reisolation of the product at each step, with α-D-neuraminidase (a), β-1-n-galactosidase (b), exo-N-acetyl β-D-mannosidase (c), endo-N-acetyl-β-D-glucosaminidase (d), α-D-mannosidase (e), β-N-mannosidase (f), and α-L-fucosidase (g). Glycopeptides A and B were subjected to Smith degradation (sequential periodate oxidation, reduction and partial acid hydrolysis, steps h and i).
Digestion with β-Hexosaminidase—The glycopeptide recovered from the β-galactosidase digestion (720 nmol) was treated with 3 units of β-d-hexosaminidase digestion in 1 ml of 50 mM sodium citrate, pH 5.0, at 37°C for 24 h under toluene (Fig. 7c), and the products were separated on a Bio-Gel P-2 column (Fig. 8, A). The first peak (A) contained 700 nmol of a glycopeptide containing 3 residues of mannose, 2 of N-acetylglucosamine, and 0.5 residue of fucose (Table IV). The second peak (D) contained only N-acetylglucosamine (2.01 pmol), and it possessed 58% of the "H present in the starting glycopeptide recovered from the β-hexosaminidase digestion (720 nmol) was incubated with 1 unit of endo-N-acetyl β-D-glucosaminidase in 0.5 ml of 100 mmoles sodium citrate, pH 6.0, for 24 h at 37°C under toluene, which cleaves the di-N-acetylchitosan and the products were desalted on a Bio-Gel P-2 column (Fig. 8, D). The neutral oligosaccharide released was removed from the newly formed glycopeptide fraction on a Dowex 50 column. This oligosaccharide (300 nmol) gave a single radioactive peak when chromatographed on a Bio-Gel P-2 column (1.6 x 120 cm) in buffer and it possessed 58% of the "H present in the starting glycopeptide (Peak D from Fig. 6). Exo-N-acetyl β-D-glucosaminidase yields "H-glycopeptide (Peak A, ○ — ○) with the release of 60% of the label as N-[3H]acetylglucosamine (Peak D). Glycopeptide A is cleaved by endo-N-acetyl β-D-glucosaminidase to release the neutral "H-oligosaccharide (Peak B, □ — □) containing half the label of Glycopeptide A, that is degraded by α-d-mannosidase to disaccharide (Peak C, □ — □), which in turn is converted to free N-[3H]acylglucosamine (Peak E, □ — □) by the action of β-d-mannosidase.

**Table V**

<table>
<thead>
<tr>
<th>Sugar and linkages</th>
<th>Hydrolysis oligosaccharide</th>
<th>Smith-degraded promase glycopeptide</th>
<th>Neuraminidase-di-glycopeptide</th>
<th>Smith-degraded neuraminidase-di-glycopeptide</th>
<th>Oligosaccharide produced by endoglucoaminidase digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuc →</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal →</td>
<td>+</td>
<td>+</td>
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<tr>
<td>→ &quot;Gal →</td>
<td>+</td>
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</tr>
<tr>
<td>Man →</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>→ 6-Man →</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>→ &quot;Man →</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>→ 6-Man →</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>→ &quot;GlcNAc →</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>→ &quot;GlcNAc →</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Digestion with Endo-N-acetyl-β-D-glucosaminidase**—The glycopeptide recovered from the β-hexosaminidase digest (300 nmol) was incubated with 1 unit of endo-N-acetyl-β-D-glucosaminidase in 0.5 ml of 100 mM sodium citrate, pH 6.0, for 24 h at 37°C under toluene, which cleaves the di-N-acetylchitosan linkage (Fig. 7d), and the products were desalted on a Bio-Gel P-2 column. The neutral oligosaccharide released was removed from the newly formed glycopeptide fraction on a Dowex 50 column. This oligosaccharide (300 nmol) gave a single radioactive peak when chromatographed on a Bio-Gel P-2 column (1.6 x 120 cm) in buffer and it contained 3 mannose residues to 1 of N-acetylgalactosamine (Table IV).

Authentic reference samples of N-acetylgalactosamine (GlcNAc), a disaccharide ManGlcNAc, a trisaccharide Man3GlcNAc, a tetrasaccharide Man4GlcNAc, and a pentasaccharide Man5GlcNAc were chromatographed separately on the Bio-Gel P-2 column used for chromatography of the neutral oligosaccharide peak in Fig. 8. The structures of the oligosaccharides and their elution coefficients (Kd) are presented in Table VI. A plot of the elution coefficient vs the log of the molecular weight for these samples gave a linear curve. The oligosaccharide (Fig. 7E) from the VSV glycopeptide had an elution coefficient identical with that of Man₅GlcNAc (Table VI).

The glycopeptide fraction was eluted from the Dowex 50 column with cold 2 N HCl, lyophilized, and chromatographed on a Bio-Gel P-2 column (1.6 x 120 cm) in buffer. Two glycopeptide peaks, GP-I (102 nmol) and GP-II (117 nmol), were obtained that had similar carbohydrate compositions (Table IV). Each contained a fraction of a fucose residue and 1 of N-acetylgalactosamine.

**Table VI**

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Ratio of mannose to N-acetylgalactosamine</th>
<th>Kd*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc</td>
<td>0</td>
<td>0.73</td>
</tr>
<tr>
<td>Man → &quot;GlcNAc&quot;</td>
<td>1.07</td>
<td>0.62</td>
</tr>
<tr>
<td>→ &quot;GlcNAc&quot;</td>
<td>1.83</td>
<td>0.52</td>
</tr>
<tr>
<td>→ &quot;Man&quot;</td>
<td>2.79</td>
<td>0.48</td>
</tr>
<tr>
<td>→ &quot;Man&quot;</td>
<td>3.97</td>
<td>0.42</td>
</tr>
<tr>
<td>Core oligosaccharide (E in Fig. 7)</td>
<td>2.94</td>
<td>0.48</td>
</tr>
<tr>
<td>α-Mannosidase-digested E (F in Fig. 7)</td>
<td>0.90</td>
<td>0.62</td>
</tr>
<tr>
<td>β-Mannosidase digested F</td>
<td>0</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* Sources are given under “Experimental Procedures.”

* Determined as the trimethylsilyl ethers. 

\[ K_d = V_e / V_i \]

Where \( V_e \) is the elution volume, \( V_i \) is the column internal volume. A plot of log \( K_d \) against molecular weights gave a straight line.
Digestion with $\alpha$-D-Mannosidase—The neutral oligosaccharide ManGlcNAc from the endoglucomaminidase digestion (90 nmol) was digested with 6 units of $\alpha$-D-mannosidase in 0.5 ml of 0.1 M sodium citrate, pH 4.5, for 24 h at 37°C under toluene (Fig. 7e), and the products were separated on a Bio-Gel P-2 column (Fig. 8, ●). A single radioactive peak was obtained that contained 1 residue of mannose/N-acetylglucosamine (87 nmol) (Table IV) and had a $K_d$ identical to that of ManGlcNAc (Table VI).

Digestion with $\beta$-D-Mannosidase—The disaccharide, recovered from the $\alpha$-mannosidase digestion (68 nmol), was digested with 5.3 units of $\beta$-D-mannosidase in 0.5 ml of 50 mM sodium citrate, pH 5.5, at 50°C for 24 h under toluene (Fig. 7f), and the products were separated by chromatography on a Bio-Gel P-2 column (Fig. 8, □). The radioactive peak contained only N-acetylglucosamine (66 nmol) (Table IV).

Digestion with $\beta$-L-Fucosidase—The glycopeptides, GP-I and GP-II, were digested separately with one unit of bovine kidney $\alpha$-1-fucosidase in 0.5 ml of 50 mM sodium citrate, pH 5.0, at 50°C for 24 h under toluene (Fig. 7g). N-Acetylglucosamine was the only sugar observed in acid hydrolysates of the recovered glycopeptide fractions. The amino acid compositions of GP-I and GP-II (Table VII) show that each contained 1 mol of aspartic acid/mol of glucosamine and confirm the earlier conclusion that amino acid differences account for their separation from each other.

CONCLUSIONS

From the studies presented under "Results," a unique structure can be derived for the oligosaccharide chains of the glycoprotein of vesicular stomatitis virus. The two chains are identical and the common structure is presented in Fig. 7A.

Structure of the Outer Chain Trisaccharide Units—Taken together, the results indicate that the oligosaccharide contains three outer chains with the common structure $\alpha$NeuNAc + $\beta$Gal + 4PGlcNAc +. The sialic acid residues are displayed with purified $\alpha$-n-neuraminidase was analyzed. The quantitative release of sialic acid by neuraminidase suggests that all of this sugar occupies a terminal position in the oligosaccharide. Periodate oxidation of the $\beta$-oligosaccharide and $\beta$-glycopeptide, obtained from glycoprotein labeled with [6-3H]glucosamine, released all of the radioactivity in the sialic acid as a volatile derivative, probably formaldehyde. Since the galactose was removed by a purified $\beta$-D-galactosidase. Periodate oxidation of the hydrazinolysis oligosaccharide and Smith degradation of the pronase-treated glycopeptides confirmed these conclusions. Galactose residues that are substituted in positions 3 or 4 are resistant to periodate, but terminal or 6-substituted galactose residues are oxidized. Since galactose survived periodate treatment originally, but was destroyed after removal of sialic acid, the terminal linkage must be $\alpha$-2 $\rightarrow$ 3, rather than $\alpha$-2 $\rightarrow$ 6.

When the hydrazinolysis oligosaccharide was subjected to nitrous acid deamination and reduction, a disaccharide was obtained that yielded mostly galactose and 2,5-anhydro mannitol on hydrolysis. Since nitrous acid produces fragments with 2,5-anhydro mannose at the reducing end from the positions occupied by glucosamine residues in the oligosaccharide, we conclude that glucosamine was bound to N-acetylglucosamine in the glycoprotein. This is confirmed by the sequential exoglycosidase digestions and methylation analyses. Before the removal of galactose, most of the N-acetylglucosamine was substituted at position 4. Afterwards, three-fifths of the N-acetylglucosamine became terminal since it could be removed by digestion with the purified exo-$\beta$-D-hexosaminidase.

In agreement, the methylation data show that most of the N-acetylglucosamine was substituted at position 4, and the resistance of this sugar to periodate oxidation is consistent with this structure.

Structure of Core Fragment—The terminal trisaccharide units containing N-acetyl-D-neuraminic acid, D-galactose, and N-acetyl-D-glucosamine are linked to a core that contains D-mannose, N-acetyl-D-glucosamine, and L-fucose and has the structure:

$$\text{afuc}$$

$$\text{aMan}^\alpha \text{fMan}^\beta \text{fGlcNAc} \text{aMan}$$

The partial structure (Fig. 7E) was deduced for the neutral oligosaccharide obtained by digesting the core glycopeptide with the endo-N-acetyl-$\beta$-D-glucosaminidase. From the known enzyme specificity, it is apparent that this product resulted from cleavage of the glycosidic bond in a di-N-acetylchitobiose unit that was substituted in position 4 with a $\beta$-linked mannose unit that, in turn, was substituted at position 3 with an unsubstituted mannose residue (Fig. 7D). In agreement, methylation analysis of this fragment indicated that the core contained 2 terminal mannose residues, a 3,6-disubstituted mannose unit, and a 4-substituted N-acetylglucosamine.

It was established by several criteria that the core contains 3 mannose residues. These include the composition of the tryptic glycopeptide and the hydrazinolysis oligosaccharide, the size determined by gel filtration, and the composition of the core fragment obtained by nitrous acid deamination of the hydrazinolysis product. Moreover, when the oligosaccharide, released by the action of the endoglucosaminidase, was digested with purified $\alpha$-D-mannosidase, 2 residues of mannose were released leaving a disaccharide of mannose and N-acetylglucosamine. This disaccharide was hydrolyzed by a purified $\beta$-D-mannosidase.

The glycopeptides, separated after the endoglucosaminidase digestion, differed in amino acid content, but they had the general structure indicated in Fig. 7G. The fucose was released by digestion with a purified $\alpha$-L-fucopronosidase, which establishes the ring form and linkage of this sugar. The

### Table VII

<table>
<thead>
<tr>
<th>Component</th>
<th>GP-I</th>
<th>GP-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.03</td>
<td>1.21</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.63</td>
<td>0.51</td>
</tr>
<tr>
<td>Serine</td>
<td>0.70</td>
<td>0.79</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.40</td>
<td>0.60</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.99</td>
<td>0.51</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>0.18</td>
</tr>
<tr>
<td>Methionine</td>
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<td>0.65</td>
</tr>
<tr>
<td>Histidine</td>
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</tr>
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</table>

Glycopeptides were hydrolyzed at 110°C for 12 h in 3 N HCl under N₂. Analyses were done by P. Hornbeck, of this department, on a Beckman model 120C Amino acid analyzer.
hydrazinolysis oligosaccharide yielded a disaccharide of fucose and 2,5-anhydroarabino and on deamination, indicating that it contained a fucosyl → N-acetylgalactosamine unit in which the fucose was either terminal or substituted with another N-acetylgalactosamine residue. The methylation data establish that the fucose was unsubstituted and that the N-acetylgalactosamine residue to which it was attached was substituted in positions 4 and 6. From the known specificity of the endoglycosaminidase, the substitutent at position 4 must have been N-acetylglucosamine, so the L-fucose must be located at position 6.

Attachment of Outer Chain Trisaccharide Units to Core—We have concluded that the terminal trisaccharide units are linked to the core in the following manner:

\[ R = \alpha\text{NeuNAc} \rightarrow \beta\text{Gal} \rightarrow \beta\text{GlcNAc}. \]

Partial acid hydrolysis of the re-N-acetylated hydrazinolysis oligosaccharide released a fragment that contained 3 mannose residues and 5 N-acetylglucosamine residues. Nitrous acid deamination of the hydrazinolysis oligosaccharide produced a tetrasaccharide with 3 mannose units and a single 2,5-anhydroarabino unit (Fig. 3). Two-thirds of the mannose residues in Fragment C of Fig. 7 became susceptible to hydrolysis by α-2 mannosidase after exo-β-β-hexosaminidase digestion, indicating that they became exposed in a terminal position by this treatment. This was verified by methylation analysis. The tryptic glycopeptide and the hydrazinolysis oligosaccharide contained 2-substituted, 2,4-disubstituted, and 3,6-disubstituted mannose residues in approximately equal proportions. After exo-β-β-hexosaminidase digestion, only terminal and 3,6-disubstituted mannose units were found, indicating that the N-acetylglucosamine residues were linked to position 2 of one mannose and to positions 2 and 4 of another.

Two arrangements are possible for the attachment of the outer chains to the core depending on which mannose is disubstituted. To distinguish between them, Smith degradation were performed on both the intact and the neuraminidase-digested glycopeptides. Since the mannose residue that is substituted only at position 2 can be oxidized by periodate and the attached fragment can then be released by mild acid hydrolysis after reduction, it is possible to determine which of the mannose residues was substituted only in position 2. With the intact glycopeptide (Fig. 7A), the released fragment was a disaccharide that contained galactose and N-acetylgalactosamine in equal proportions. Methylation analysis of the glycopeptide remaining after Smith degradation (Fig. 71) revealed the presence of a 2,4-disubstituted and a 6-substituted man

protein of the virion is accessible to the reagent (51), gel electrophoresis of the total protein extract revealed only labeled glycoprotein. This demonstrates that the sample was free of cellular membranes. To avoid contamination of the glycoprotein or other fractions by extraneous sugars, procedures were avoided that employed sucrose density gradient separations or carbohydrate-containing gels, and the isolated glycoprotein was extensively extracted to remove viral glycolipids. The glycoprotein was free of contaminating proteins that could be detected by protein stains of gels or by analyzing the radioactivity of glycoprotein gels from virions labeled with C-amino-acids.

The only sugars detected in an acid hydrolysate of the purified glycoprotein were fucose, mannose, galactose, N-acetylglucosamine, and sialic acid. In contrast, McSharry and Wagner (6) reported over 20% glucose in the glycoprotein of VSVIndiana grown in L-cells, whereas Etchison and Holland (7) growing VSVIndiana in BHK21 cells and found 3 to 5 residues of glucose/glycoprotein molecule. Because these authors employed sucrose density gradients, Sephadex gel chromatography, and cellulose dialysis tubing in their work, contamination from these sugar-containing materials is a possibility. We have avoided these sources of contamination and have employed samples of such a size that minor contaminants did not interfere. We also failed to detect the presence of N-acetylglactosamine, in agreement with the finding of Burg and Huang (59) and McSharry and Wagner (6), although Etchison and Holland (8) reported 1 to 2 residues of this amino sugar per glycoprotein molecule. We have no explanation for this discrepancy, although cellular membrane sphingolipids is an obvious source of this amino sugar.

A third difference between the published compositions and our results concerns the fucose content. McSharry and Wagner (6) reported that fucose represented less than 0.1% of the carbohydrate in the purified glycoprotein from virus grown in L-cells. Our finding of 1.7 residues of fucose/glycoprotein molecule parallels that of Etchison and Holland (7), who reported 1.2 residues. These differences appear to depend on the cell line from which the virus is isolated since Etchison and Holland (8) reported 1.83 residues of fucose/glycoprotein molecule for virus grown in VSVIndiana grown in L-cells, whereas Etchison and Holland (7) reported 0.8 residues for virus from BHK21 cells, 0.8 residues for virus from HeLa cells, and only 0.12 residue for virus from L-cells. The fractional value we found for fucose suggests that there is heterogeneity in the substitution by this sugar, but an alternative explanation exists; because the fucosyl derivatives used in quantitation are the most volatile of those in the mixture, this sugar may be lost preferentially, thus giving low value.

Discrepancies in some other reported sugar ratios are more difficult to interpret. McSharry and Wagner (6) found almost 6 times as much glucosamine and neuraminic acid as mannose and galactose. The composition we found, however, is in good agreement with that reported by Etchison and Holland (7) for galactose and N-acetylgalactosamine, and in fair agreement for mannose and sialic acid. The slightly lower sialic acid content reported by Etchison and Holland (7) may reflect a difference in the degree of sialylation of glycoproteins. The composition we found, however, is in good agreement with that reported by Etchison and Holland (7) for galactose and N-acetylgalactosamine, and in fair agreement for mannose and sialic acid. The slightly lower sialic acid content reported by Etchison and Holland (7) may reflect a difference in the degree of sialylation of glycoproteins. The composition we found, however, is in good agreement with that reported by Etchison and Holland (7) for galactose and N-acetylgalactosamine, and in fair agreement for mannose and sialic acid.

The spinner culture of BHK21 cells we used allowed isolation of 100-mg quantities of virus from which 10-mg amounts of glycoprotein were obtained. Following radiolabeling of the intact purified virus, under conditions such that only the G carbohydrate in the purified glycoprotein from virus grown in L-cells. Our finding of 1.7 residues of fucose/glycoprotein molecule parallels that of Etchison and Holland (7), who reported 1.2 residues. These differences appear to depend on the cell line from which the virus is isolated since Etchison and Holland (8) reported 1.83 residues of fucose/glycoprotein molecule for virus grown in VSVIndiana grown in L-cells, whereas Etchison and Holland (7) reported 0.8 residues for virus from BHK21 cells, 0.8 residues for virus from HeLa cells, and only 0.12 residue for virus from L-cells. The fractional value we found for fucose suggests that there is heterogeneity in the substitution by this sugar, but an alternative explanation exists; because the fucosyl derivatives used in quantitation are the most volatile of those in the mixture, this sugar may be lost preferentially, thus giving low value.

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oligosaccharides in serum-type glycoproteins (60).

The compositions of the hydrazinolysis oligosaccharide and the tryptic glycopeptide from VSV glycoprotein are consistent only with there being two oligosaccharide chains/glycoprotein molecule, and this is in agreement with previous authors (7, 17, 18). The data suggest that the two oligosaccharide chains have identical sugar compositions, and the same conclusion has been reached by Etchison et al. (19).

The oligosaccharide from VSV New Jersey grown in the BHK spinner cell line was surprisingly homogeneous, showing only a small fraction of the chains with incomplete sialylation. In contrast, other authors have obtained glycopeptides missing 1, 2, or sometimes all 3 sialic acid residues (17, 19, 61). The glycopeptide appeared nearly homogeneous at each step in the sequential removal of the sialic acid, galactose, and N-acetylglucosamine. However, after digestion with the endo-N-acetyl-β-D-glucosaminidase, some heterogeneity was observed, probably due to amino acid differences because the two glycopeptides showed different properties even after removal of the fucose residue.

The oligosaccharide released by endo-N-acetyl-β-D-glucosaminidase digestion from the neuraminidase-, galactosidase- and exohexosaminidase-digested tryptic glycopeptide had the properties of Man,GlcNAc. Digestion of the Man,GlcNAc with α-mannosidase yielded a single disaccharide, βMan → 4GlcNAc. In contrast, Moyer and Summers (62) and Moyer et al. (18) reported heterogeneity in the core region of the VSV glycopeptide based on an apparent resistance to digestion with endo-N-acetylglucosaminidase. This may have been due to incomplete removal of the terminal sugars which would inhibit the digestion.

All of our studies indicate the presence of 3 mannose residues/oligosaccharide chain, rather than 4. Thus, the core oligosaccharide migrated on chromatography with authentic Man,GlcNAc, and it was clearly separated from Man,GlcNAc and Man,GlcNAc. In addition, its composition was similar to authentic Man,GlcNAc, but not to the Man,GlcNAc or the Man,GlcNAc references. Finally, the methylation data were similar to those of the Man,GlcNAc standard, which shows the presence of 2 terminal mannose residues and one 3-O-disubstituted mannose attached to position 4 of an N-acetylgalactosamine residue. Our results demonstrate that the VSV glycopeptide core is identical to that of glycopeptides from fetuin, human α1-acid glycoprotein, human lactotransferrin and human serotransferrin (60). This same structure has also been reported for IgE, IgM, IgG, and some GM1-gangliosidosis oligosaccharides.

The outer chains of the VSV oligosaccharide are similar or identical to those in fetuin, transferrin and α1-acid glycoprotein (60). They differ from those of several immunoglobulins (63) in that the sialic acid is linked 2 → 3 rather than 2 → 6 to galactose, and that the galactose is linked only to position 4 of N-acetylgalactosamine. The fucose in the VSV glycoprotein core is attached in the same position as that reported for IgG (64), IgE (65) and IgA (66). IgM (67), mouse histocompatibility-2 alloantigen (68), and glycopeptide S1 of glycoprotein E2 of Sindbis virus (69).

The oligosaccharide of VSV glycoprotein is similar to that of many known glycoproteins. A similar structure with two outer chains has been reported for a Sindbis virus glycopeptide (69). The same attachment of the outer two chains to position 2 of the α-linked mannose residues has been reported for IgG, IgM, IgE, and IgA (66) and for human transferrin glycopeptide (70). In serum glycoproteins with four outer chains, these are probably linked to positions 2 and 4 of both of the α-linked mannose residues (63).

Although the work of Schloemer and Wagner (14–16) indicates that the terminal sialic acid of VSV glycoprotein is required for binding of the virus to cells and erythrocytes, the binding site must involve more than just the sialic acid residues because binding is not inhibited solely by sialic acid or sialyllactose. In experiments not described here, we have found that the re-N-acetylated hydrazinolysis oligosaccharide from fetuin, at 5 μg/ml, inhibits plaque formation by VSV on monolayers of BHK1 cells. Purified complex sialylglycopeptide isolated from IgM does not inhibit, even at 50 μg/ml. Since fetuin and VSV appear to have the same terminal trisaccharide structure, but both differ from that of the immunoglobulins, the trisaccharide unit αNeuNAc → 4Gal → 4GlcNAc may play a role in the binding. Alternatively, the 3 sialic acid residues in close proximity found in fetuin and the VSV glycoprotein, but not in IgM, may be important.

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