The major lipid-linked oligosaccharide of vesicular stomatitis virus infected Chinese Hamster ovary (CHO) cells has been isolated and partially characterized. The oligosaccharide moiety has a molecular weight of approximately 2080 as determined by gel filtration. The molecule contains 2 N-acetylgalactosamine residues in the form of a N,N'-diacetylenyhexitobiose unit at the reducing end and approximately 10 additional monosaccharide residues, of which a minimum of 6 are mannose residues. Jack bean 0-mannosidase releases 3 monosaccharide residues from the oligosaccharide.

Pulse-chase experiments with [3H]mannose indicate that this lipid-linked oligosaccharide is the immediate precursor of the oligosaccharide units of the viral G protein. Within 3 to 5 min of transfer to the protein, processing of the oligosaccharide begins and by 30 min 3 monosaccharide residues have been excised. This is followed by the rapid removal of 4 more monosaccharides which results in a core with the composition (Man)3(GlcNAc)2Asn. The sugar residues which comprise the outer branches of the oligosaccharide are then transferred to the molecule. A variant line of CHO cells which is deficient in UDP-GlcNAc:glycoprotein N-acetylgalactosaminyltransferase activity appears to have a processing defect in that the oligosaccharide units of the mature viral G protein have the composition (Man)3(GlcNAc)2. This suggests that the removal of the final 2 monosaccharide residues during processing may be linked to the particular UDP-GlcNAc glycosyltransferase which transfers N-acetylgalactosamine residues to the mannose residues in the oligosaccharide core.

Experiments performed with mouse LPC1 cells which secrete IgG demonstrated a similar sequence of processing of the oligosaccharide units of this immunoglobulin, indicating that processing is probably a general phenomenon.

There is a large body of experimental evidence to show that the glycosylation of glycoproteins containing asparagine-linked oligosaccharides occurs by en bloc transfer of preformed oligosaccharide chains from an oligosaccharide pyrophosphoryldolichol intermediate to an asparagine residue in the newly synthesized polypeptide chain (see Ref. 1 for a recent review). The lipid precursor isolated from a number of in vitro systems has been shown to have the general structure of (Man)3GlcNAc-R\(\beta_{1,4}\)GlcNAc-P-P-dolichol and the number of mannose residues (n) is usually 5 or more. In a number of systems the oligosaccharide which has been transferred to endogenous or exogenous protein acceptors has been shown to have the same sugar composition as the precursor lipid intermediate. Asparagine-linked oligosaccharide chains of glycoproteins are commonly of two types: the "simple" or high mannose oligosaccharides containing 5 to 8 or more mannose residues linked to an N,N'-diacetylenyhexitobiose core, and the "complex" oligosaccharides containing outer chains of sialic acid, galactose, and N-acetylgalactosamine residues attached to a core of 3 to 4 mannose residues linked to N,N'-diacetylenyhexitobiose (2). The lipid-linked oligosaccharides intermediates so far studied would seem appropriate donors for the synthesis of the high mannose type oligosaccharide. However if these same lipid intermediates are the donors for the complex type oligosaccharides, one must postulate that there is a mechanism for the removal of excess mannose residues before the sequential addition of the outer chain sugars. Several recent reports have brought this problem into focus. Spiro et al. have isolated from thyroid slices a lipid-linked oligosaccharide which contains 14 to 15 monosaccharide residues (11 mannose, 1 to 2 glucose, 2 GlcNAc) and have shown the transfer of this oligosaccharide to endogenous protein acceptors (3). Spiro et al. point out that if thyroglobulin were an acceptor, then considerable modification of the oligosaccharide would have to take place to produce the structure found in completed bovine thyroglobulin. Sefton has shown with Sindbis virus-infected chick cells that an 1800 molecular weight oligosaccharide is transferred from an oligosaccharide-lipid to polysome-bound viral polypeptide chains (4). Since one of the two oligosaccharide units of the viral glycoproteins is a complex type containing only 3 or 4 mannose residues, the implication is that a number of mannose residues would have to be removed during the process of protein maturation. In similar
Our general approach was to isolate the lipid-linked oligosaccharide and newly synthesized glycoprotein from VSV-infected cells labeled with \([\text{H}]\)mannose for short times and then determine the molecular weight and composition of the oligosaccharide units of these molecules. A similar analysis was performed on VSV-infected cells which were pulse-labeled with \([\text{H}]\)mannose and then chased for various time intervals. The data presented in this paper show the existence of a high molecular weight mannose-rich oligosaccharide bound to both a lipid intermediate and the early labeled viral glycoprotein.

As the viral glycoprotein matures in the cell, the excess mannose residues are removed and then the outer sugars are added. Using the mouse plasmacytoma line LPC-1 which secretes IgG, we have found a similar sequence of processing.

**Experimental Procedures**

**Materials** — n-[\(^{3}H\)]Mannose, n-[\(^{14}C\)]mannose, and d-[\(^{14}C\)]glucosamine were purchased from New England Nuclear Corp. The \(\alpha\)-minimal essential medium, a modified Eagle's minimum essential medium (8), was obtained from Flow Laboratories, Rockville, Md. Fetal calf serum, glutamine, penicillin, and streptomycin were from Grand Island Biological Co. Bio-Gel P-6 (200 to 400 mesh) was from Bio-Rad. NCS tissue solubilizer was obtained from Amer sham/Searle. The 3a70 scintillation mixture was from Research Products International Corp., Elk Grove Village, Ill. (Man)\(_3\)(GlcNAc)\(_2\), (Man)\(_2\)(GlcNAc)\(_3\), and (Man)\(_4\)(GlcNAc)\(_4\) were obtained from Ms. Ellen Li of this laboratory. Nonidet P-40 was purchased from Particle Data Laboratories, Elmhurst, Ill.

**Enzymes** — Pronase was obtained from Calbiochem. \(\beta\)-Galactosidase, \(\beta\)-N-acetylglucosaminidase, and \(\alpha\)-mannosidase were prepared from jack bean meal as previously described (9). *Clostridium perfringens* endo-\(\beta\)-N-acetylglucosaminidase C, and C\(_3\), prepared by the method of Ito et al. (10), were kindly provided by Ms. Ellen Li. \(\beta\)-Mannosidase from hen oviduct was prepared by the method of Sukeno et al. (11). Yeast \(\alpha\)-glucosidase was purchased from Sigma.

**Cells** — Chinese Hamster ovary cells were grown as monolayers in \(\alpha\)-minimal essential medium supplemented with 10% fetal calf serum, 50 units of penicillin/ml, and 50 \(\mu\)g of streptomycin/ml. The lectin-resistant cell lines were isolated and characterized as previously described (12, 13). Clone 13, a wheat germ agglutinin-resistant cell line, is deficient in membrane sialic acid and galactose (13). Its complex type oligosaccharides contain \(N\)-acyethylglucosamine residues at their nonreducing end. Clone 15B, a ricin-resistant line, lacks UDP-GlcNAc:glycoprotein \(N\)-acyetylglucosaminyltransferase activity which results in the synthesis of membrane oligosaccharides which are deficient in sialic acid, galactose, and \(N\)-acyetylglucosamine (14). Since the glycoproteins of viruses grown in these variant cells reflect the host cell defect (15), these lines were used to simplify the subsequent structural analysis studies. A similar yield of VSV was obtained from the two variant lines and the parent CHO cells.

The IgG (\(\gamma_2a\), K)-secreting plasmacytoma LPC-1 was maintained in the ascitic form in BALB/c mice. The plasma cells were harvested and purified as previously described (16).

**Growth of Virus** — VSV. Indiana strain, was a gift from Dr. A. B. Huang of Harvard Medical School. Nearly confluent monolayers of cells growing on 150-mm plastic tissue culture Petri dishes were infected with vesicular stomatitis virus at a multiplicity of infection of 10 to 40 plaque-forming units/cell. The virus was allowed to adsorb to the cells for 1 h at 37\(^\circ\)C in a volume of 3.5 ml, followed by removal of the unadsorbed inoculum and the addition of 35 ml of fresh medium.

**Radioactive Labeling of Infected Cells** — Five to six hours after infection, the medium was removed from the infected cells, and 300 \(\mu\)Ci of \([\text{H}]\)mannose in 3 ml of phosphate-buffered saline (0.15 M NaCl, 0.01 M PO\(_4\) 7.4) were added to each plate for either 3 or 5 min as indicated for each experiment. The radioactive medium was then removed, and either 35 ml of fresh medium were added for the indicated chase time or the cells were immediately harvested as described below.

**To prepare double-labeled lipid oligosaccharide, 50 \(\mu\)Ci of \([\text{C}]\)glucosamine in 5 ml of complete medium (modified to contain 1/10 glucose concentration) were added to each plate of CHO cells at 5 h postinfection; at 6 h postinfection, 200 \(\mu\)Ci of \([\text{H}]\)mannose in 1 ml of the same medium were added. The radioactive medium was removed at 7 h postinfection and the cells were collected and extracted as described below.

**Radioactive Labeling of Virus and Virus Purification** — Cells were labeled with 50 \(\mu\)Ci of \([\text{C}]\)mannose in 1/10 glucose medium at 2 h postinfection, and at 6 h postinfection, the virus was harvested and purified as previously described (15).

**Extraction of Virus-Infected Cells** — After removal of the medium, the cells were immediately chilled on ice, washed once with ice-cold phosphate-buffered saline, scraped with a rubber policeman, centrifuged into a pellet, and frozen until extracted. Then, 5 ml of CHCl\(_3\)/CH\(_3\)OH (9:1 v/v) were added to each frozen cell pellet (9 to \(10^7\) cells) and the solution was dispersed by sonication. After sitting 15 min at room temperature, the suspension was centrifuged in a clinical centrifuge. The resulting supernatant was collected and the pellet was resuspended as above in 3 ml of CHCl\(_3\)/CH\(_2\)OH (3:1). The combined CHCl\(_3\)/CH\(_2\)OH extracts were washed with 0.9% saline and 0.9% saline/CH\(_3\)OH (1:1) as previously described (17).

The remaining cell pellet was washed once more with CHCl\(_3\)/CH\(_2\)OH (2:1), dried under ice, and resuspended by sonicating in 5 ml of H\(_2\)O. The suspension was then centrifuged, and the pellet was washed twice more with H\(_2\)O. The water extracts were saved and counted as described below.

The pellet, after being suspended in 0.5 ml of CH\(_2\)OH and dried with N\(_2\), was extracted twice with CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O (1:1:0.3) and the supernatant fluid was pooled. The fluid remaining after this extraction was then washed once with CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O (1:1:0.3), dried with N\(_2\), and dissolved by boiling in 1 ml of 1% sodium dodecyl sulfate.

The various extracts and washes were counted in a Beckman liquid scintillation counter, using the following scintillation fluids Aliquots of the CHCl\(_3\)/CH\(_2\)OH (2.1) extracts and CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O (1:1:0.3) extracts were dried with N\(_2\) and counted in 10 ml of a toluene/2,5-diphenyloxazole (PPO)/1,4-bis[2-(5-phenyloxazol-1-yl)]benzene (POP) solution containing 3.7% of 6 N H\(_2\)SO\(_4\) solution of water, while aliquots of all other samples were brought to 1 ml with H\(_2\)O and counted in 10 ml of 3a70 scintillation fluid mixture. Differences in counting efficiencies in these two scintillation fluids were not significant as determined by standard sample counting.

According to Waechter et al. (17), this extraction procedure separates mannose-containing polysaccharide (soluble in CHCl\(_3\)/CH\(_2\)OH, 2:1), oligosaccharide pyrophosphoryl polyisoprenol (soluble in CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O, 1:1:0.3), and glycoprotein (insoluble in the above solvents). In addition, the H\(_2\)O and saline washes remove residual-free mannose, mannose-1-phosphate, and GDP-mannose. We further substantiated the validity of the extraction by performing thin layer chromatography of the two lipid extracts as described by Chambers and Elbein (18). The respective lipid-linked saccharides migrated as a single peak of radioactivity with appropriate \(R\) values (data not shown).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of VSV Glycoprotein** — Alkaline phosphatase of the cell pellet dissolved in 1% sodium dodecyl sulfate were electrophoresed according to the procedure of Weber and Osborne (19) and the gels were sliced, solubilized,
and counted as previously described (16). The gels resolved one sharp, symmetrical peak of radioactivity corresponding to the VSV G glycoprotein, thus documenting the proper cessation of host cell protein synthesis in our system.

**Protease Digestion** —The remaining cell pellet and the gradient purified VSV fractions were precipitated with 10% cold trichloroacetic acid and collected on a Millipore filter. The filter was placed in 2 ml of 0.1 M Tris buffer, pH 8.0, containing 2 mM CaCl₂. Pronase (2 mg) was added daily for 3 days, and the incubation was kept at 37°C under a nitrogen atmosphere.

**Gel Filtration** —The various oligosaccharides and glycopeptides were fractionated by gel filtration on a Bio-Gel P-6 (200 to 400 mesh) column (1.5 X 91 cm) using 0.1 M NH₄HCO₃, pH 8.0, as eluting buffer. Fractions (1 ml) were collected and monitored for radioactivity. Internal markers for Vₛ (bovine serum albumin) and V₆ (galactose) were included in each run. The column was calibrated with glycopeptides and with the following oligosaccharides: (Man)₃(GlcNAc)₂, (Man)₅(GlcNAc)₃, (Man)₆(GlcNAc)₄, stachyose, and lactose. The oligosaccharides and the glycopeptides gave two different calibration curves when plotted by the method of Bhat and Clamp (20) as shown in Fig. 1 (bottom).

**Paper Chromatography** —Labeled oligosaccharides were separated by chromatography on Whatman No. 1 paper for 72 h in Solvent A (1-butanol/pyridine/H₂O, 40:30:40). The paper was cut into 1-cm strips and counted with a β-liquid scintillation mixture.

**Results**

**Glycopeptides** and **Oligosaccharides** —Glycopeptides or oligosaccharides to be treated with jack bean α-mannosidase (0.22 unit/assay) and/or β-N-acetylglucosaminidase (0.24 unit/assay) were dissolved in 0.05 M citrate buffer, pH 4.6, and the appropriate enzyme(s) were added. The reaction mixtures (0.2 ml) were incubated at 37°C under a nitrogen atmosphere for 48 h.

Those substrates to be treated with endo-β-N-acetylglucosaminidase Cᵣ (0.001 unit/assay) or Cₛ (0.002 unit/assay) were dissolved in 0.06 M citrate/phosphate buffer, pH 5.5, and, after addition of the appropriate enzyme(s), were mixed (80 μl) and incubated at 37°C under a nitrogen atmosphere for 24 h. Cᵣ cleaves the chitobiose unit of side chain-free complex type glycopeptides and (Man)₂(GlcNAc)₃Asn while Cₛ only cleaves the chitobiose unit of high mannose glycopeptides (10).

**Preparation of Radioactive IgG Glycopeptides** —LPCl cells (NU X 10⁶) were suspended in 2.0 ml of phosphate-buffered saline containing 300 μCi of [³H]mannose. After a 12-min incubation at 37°C, 10 ml of ice-cold α minimal essential medium was added and the cells were centrifuged at 500 x g for 5 min. The cell pellet was washed with 12 ml of cold medium and 2.5 ml of 0.5% NP-40 in 0.15 M NaCl, 0.01 M Tris/ICl, pH 7.4, were added. The suspension was gently shaken and allowed to stand for 30 min at room temperature to allow complete lysis of the cells. The lysate was then centrifuged at 100,000 x g for 30 min and the supernatant fluid was collected. To the supernatant fluid were added 18 μl of goat anti-mouse IgG serum (Gateway Immununosa Co., Cahokia, Ill.) and after 2 h at 4°C, 420 μl of rabbit anti goat IgG serum were added. The reaction mixture was allowed to stand overnight at 4°C and then the immunoprecipitate was sedimented for 10 min at 10,000 x g. The resultant pellet was washed twice with 5 ml of cold saline and resuspended by sonication in 1.0 ml of 0.1 M Tris/ICl, pH 8.0, 0.001 M CaCl₂. A portion was removed for analysis by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and the remaining suspension was added to the final 5 mg of prame. The reaction mixture was incubated at 37°C under a nitrogen atmosphere for 72 h and additional 5-ml aliquots of pronase were added at 24 and 48 h. The material was then heated to 100°C for 3 min and applied to the standard P-6 column.

To obtain secreted IgG with radioactive oligosaccharides, two 1-ml cultures containing 2 X 10⁶ LPCl cells each in complete media were incubated at 37°C in a CO₂ incubator in the presence of 50 μCi of [³H]mannose and 10 μCi of [¹⁴C]glucosamine. After 24 h the cells were sedimented and the secreted IgG in the supematant fluid was immunoprecipitated and subjected to pronase digestion and gel gel electrophoresis as described above. A portion was saved for gel gel electrophoresis.

Analysis of the immunoprecipitated material by polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed in both instances a single peak of radioactivity corresponding to the IgG heavy chain.

**RESULTS**

**Isolation of a Lipid-linked Oligosaccharide from VSV-infected CHO Cells** —The parental CHO cells and the two variant lines (15B and clone 13) were infected with VSV and 6 h after infection were labeled with [³H]mannose for 3 or 5 min and then extracted as described under "Experimental Procedures" to obtain the lipid-linked oligosaccharide. When the lipid-linked oligosaccharides were subjected to mild acid hydrolysis followed by gel filtration on a Bio-Gel P-6 column (Fig. 1A), all three oligosaccharides migrated identically (Kᵥ of 0.45), indicating that the three cell lines synthesize lipid-linked oligosaccharides of identical size. Since short pulse times tend to preferentially label the outer sugars of the oligosaccharide, VSV-infected CHO cells were incubated with [³H]mannose and [¹⁴C]glucosamine for 1 and 2 h, respectively, to obtain more nearly label the outer sugars of the oligosaccharide, VSV-infected CHO cells were incubated with [³H]mannose and [¹⁴C]glucosamine for 1 and 2 h, respectively, to obtain more uniform labeling of the molecule. The cells were then extracted and the lipid-linked oligosaccharide subjected to mild acid hydrolysis followed by gel filtration on a Bio-Gel P-6 column (Fig. 1A). A single oligosaccharide was obtained with a Kᵥ of 0.45, corresponding to a molecular weight of approximately 2800. The oligosaccharide also migrated as a
single high molecular weight species when subjected to paper chromatography using 1-butanol/pyridine/H₂O (40:30:40) as the solvent (Fig. 2A). When the molecule was treated with clostridial endo-β-N-acetylglucosaminidase C, which cleaves the N,N'-diacetylcitolibiose unit of high mannose oligosaccharides (10), about half of the [14C]GlcNAc was released and the residual oligosaccharide eluted from the P-6 column with a \( K_v \) of 0.51, corresponding to a molecular weight of 1800. This finding indicates that the molecule contains only 2 N-acetylglucosamine residues which are present as a N,N'-diacetylcitolibiose unit at the reducing end of the molecule. Given a molecular weight of 2080, the original oligosaccharide would have approximately 10 hexose residues in addition to the N,N'-diacetylcitolibiose unit. Treatment of the molecule with jack bean α-mannosidase released about 46% of the [3H]mannose and the residual oligosaccharide migrated with \( K_v \) of 0.54, corresponding to a molecular weight of approximately 1660 (Fig. 1B). This is consistent with the removal of about 3 mannose residues. No additional release of radioactivity or change in elution position on Bio-Gel P-6 occurred when the oligosaccharide was subsequently treated with β-mannosidase, α-glucosidase, or β-N-acetylglucosaminidase. Other workers have found that α-mannosidase fails to release all the mannose residues from the oligosaccharide unit of the lipid-linked molecule (21, 22).

Characterization of G Protein Glycopeptide in VSV-infected Cells Pulse-labeled with [3H]Mannose — If the high molecular weight oligosaccharide-lipid were the immediate precursor of the viral glycoprotein oligosaccharide units, one would expect to find a similar oligosaccharide on the newly synthesized VSV G protein. To detect such oligosaccharides, VSV-infected clone 13 cells were labeled for 5 min with [3H]mannose and a glycoprotein fraction prepared. The labeled glycoprotein molecules were digested with pronase to obtain glycopeptides which were then subjected to gel filtration on Bio-Gel P-6. As shown in Fig. 3A, a single glycopeptide peak was obtained with a \( K_v \) of 0.26 (approximate \( M_w \) of 2100). The glycopeptide was next treated with endo-β-N-acetylglucosaminidase C, which released two oligosaccharides with \( K_v \) values on P-6 of 0.51 and 0.59 (approximate \( M_w \) of 1800 and 1470) (Fig. 3B). The oligosaccharides also migrated as two high molecular weight species when analyzed by paper chromatography using solvent A (Fig. 2B). The larger oligosaccharide migrated virtually identically with the endo-β-N-acetylglucosaminidase \( C_n \)-treated lipid-oligosaccharide on both P-6 and paper chromatography. Treatment of the endo-β-N-acetylglucosaminidase \( C_n \)-released oligosaccharides with α-mannosidase resulted in the release of 67% of the [3H]mannose and the resulting oligosaccharides had \( K_v \) values of 0.65 and 0.74 (approximate \( M_w \) of 1250 and 960) (Fig. 3C). No additional mannose was released when the oligosaccharides were incubated with α-mannosidase plus β-N-acetylglucosaminidase (Fig. 3D). The finding of two oligosaccharides on the early labeled G protein indicates that processing begins very rapidly. Since both molecules are susceptible to α-mannosidase they must both have α-linked mannose residues at their nonreducing termini.

To establish that the rapidly labeled material was the true precursor of the mature VSV G protein oligosaccharide, a similar culture was labeled for 5 min with [3H]mannose and then chased for 2 h prior to the preparation of the viral G protein glycopeptide. The resultant glycopeptide had the same elution position as the 5-min labeled glycopeptide on P-6, but it was totally resistant to endo-β-N-acetylglucosaminidase \( C_n \) (Fig. 3, E and F, top), indicating that it did not contain a

![Fig. 2. Paper chromatography of endo-β-N-acetylglucosaminidase \( C_n \) and \( C_l \)-released oligosaccharides. A, lipid-linked oligosaccharide from Fig. 1 following mild acid hydrolysis and endo-β-N-acetylglucosaminidase \( C_l \) treatment. B, oligosaccharides released by endo-β-N-acetylglucosaminidase \( C_l \) from the 5-min-labeled VSV G glycopeptide from clone 13 cells. C, oligosaccharide released by endo-β-N-acetylglucosaminidase \( C_l \) from the G protein glycopeptide of VSV grown in 15B cells. D, oligosaccharide released by β-N-acetylglucosaminidase and endo-β-N-acetylglucosaminidase \( C_l \) treatment of the G glycopeptide obtained from clone 13 cells labeled for 5 min with [3H]mannose and chased for 2 h. The chromatogram was developed in Solvent A for 72 h. The four oligosaccharides were labeled with [3H]mannose.](http://www.jbc.org/)

![Fig. 3. Gel filtration of [3H]mannose-labeled glycopeptides and oligosaccharides from VSV-infected clone 13 cells. VSV-infected cells were incubated with [3H]mannose for 5 min and either harvested immediately (A through D) or after a 2-h chase period in unlabeled media (E through H). Glycopeptides were prepared by pronase digestion and chromatographed on Bio-Gel P-6. The initial glycopeptides are shown in A and E. These glycopeptides were treated with the following enzymes: endo-β-N-acetylglucosaminidase \( C_n \) (B and F, top); β-N-acetylglucosaminidase plus endo-β-N-acetylglucosaminidase \( C_l \) (F, bottom); α-mannosidase \( G \); α-mannosidase plus β-N-acetylglucosaminidase \( H \). The oligosaccharides from the 5-min glycopeptide \( B \) were then treated with α-mannosidase \( C \) or with β-N-acetylglucosaminidase plus α-mannosidase \( D \).](http://www.jbc.org/)
high mannose type of oligosaccharide unit. The glycopeptide was cleaved when incubated with \(\beta-N\)-acetylglucosaminidase plus endo-\(\beta-N\)-acetylglucosaminidase \(G\), releasing a low molecular weight oligosaccharide with \(K_a\), of 0.825 (approximate \(M_r\) of 720) (Fig. 3F, bottom). This indicated that the glycopeptide contained GlcNAc residues at its nonreducing termini with a core consisting of \((\text{Man})_\gamma(\text{GlcNAc})_\delta\text{Asn}\). Such a core would be susceptible to endo-\(\beta-N\)-acetylglucosaminidase \(C_\delta\) and the released oligosaccharide would have the structure \((\text{Man})_\gamma(\text{GlcNAc})_\delta\). On paper chromatography in Solvent A, the released oligosaccharide migrated as expected for such a tetrasaccharide (Fig. 2D). The finding that \([^{14}C]\)mannose residues of the intact glycopeptide were susceptible to \(\alpha\)-mannosidase only after \(\beta-N\)-acetylglucosaminidase treatment supports this proposed sugar sequence (Fig. 3, G and H).

Taken together, these data indicate that the oligosaccharide units of the VSV G protein begin as high molecular weight high mannose type structures and are processed on the protein to become complex type oligosaccharides. Not only was this true of VSV grown in clone 13 cells, but virtually identical results were obtained with VSV grown in parent CHO cells except that the outer chains of the chased glycopeptide material contained sialic acid, galactose, and N-acetylglucosamine residues (data not shown).

**Kinetics of Processing of Oligosaccharide Units of G Protein—** Cultures of VSV-infected clone 13 cells were labeled with \([^{3}H]\)mannose for 3 min and the cells were either harvested immediately or chased in unlabeled complete medium for 10, 30, 60, or 90 min. The \([^{3}H]\)mannose-labeled glycoprotein material was then degraded with pronase and the resultant glycopeptides were isolated and incubated with endo-\(\beta-N\)-acetylglucosaminidase \(C_\delta\), followed by gel filtration on P-6. The rationale for this treatment was that as the oligosaccharides were processed to form complex type units, they would become resistant to endo-\(\beta-N\)-acetylglucosaminidase \(C_\delta\). The chromatographs of the treated glycopeptides are shown in Fig. 4. For the first 10 min of the chase, the glycopeptides remained susceptible to the endo-\(\beta-N\)-acetylglucosaminidase, but the released oligosaccharides shifted to a \(K_a\) value of 0.59 (approximate \(M_r\) of 1470) consistent with the removal of 2 hexose residues. At 30 min the released oligosaccharide had a \(K_a\) of 0.61 (approximate \(M_r\) of 1400) indicating the probable removal of another hexose residue. By this time substantial amounts of glycopeptide material resistant to endo-\(\beta-N\)-acetylglucosaminidase \(C_\delta\) had appeared and by 90 min virtually all the glycopeptide was resistant to the enzyme. The time course of the loss of susceptibility of the glycopeptide to endo-\(\beta-N\)-acetylglucosaminidase \(C_\delta\) is shown in Fig. 5.

When the oligosaccharide released from the 30-min chased glycopeptide was incubated with \(\alpha\)-mannosidase, 39% of the \([^{3}H]\)mannose was released and the residual oligosaccharide had a \(K_a\) of 0.82 (\(M_r\) of 740), indicative of a molecule with the composition \((\text{Man})_\gamma(\text{GlcNAc})_\delta\). Partial Processing of High Mannose Intermediate in VSV-infected 15B Cells—In a previous paper we reported that purified Sindbis virus grown in 15B cells contains glycoproteins with oligosaccharide units that have mannose residues at their nonreducing termini (15). This is consistent with the known enzyme defect in this cell line, which is a deficiency of a \(\text{UDP-GlcNAc-glucoprotein } \beta-N\)-acetylglucosaminyltransferase (14). To examine oligosaccharide processing in this cell line and to characterize the oligosaccharide unit of the VSV G protein released by these cells, the following experiment was performed. VSV-infected 15B cells were pulsed with

\[\text{[^{14}C]}\text{mannose for 3 min and either extracted immediately (no chase) or incubated in unlabeled complete media for 10, 30, 60, or 90 min. Glycopeptides were prepared by pronase digestion, isolated by gel filtration on P-6, and then subjected to endo-\(\beta-N\)-acetylglucosaminidase \(C_\delta\) treatment followed by rechromatography on P-6. The elution profiles from the final P-6 column are shown for each sample.\]
of approximately 1000. On paper chromatography in Solvent A, this oligosaccharide migrated with the same Rf as authentic (Man)3(GlcNAc)1 (Fig. 2C). Treatment of the oligosaccharide with α-mannosidase released 78% of the [3H]mannose, consistent with the release of 4 of the 5 mannose residues. Since there are only 3 mannose residues in the core of the oligosaccharide of the G protein from VSV grown in clone 13 cells, these data indicate that only partial processing of the oligosaccharide units occurs in 15B cells.

Processing of IgG Oligosaccharides—Since the VSV G protein is a membrane glycoprotein, the possibility arose that processing might be confined to the synthesis of membrane components. We therefore examined the biosynthesis of the oligosaccharide of IgG since it is a soluble glycoprotein with a single complex type oligosaccharide located on the heavy oligosaccharide of IgG since it is a soluble glycoprotein with a single complex type oligosaccharide located on the heavy chain. LPC1 mouse plasmacytoma cells were incubated with [3H]mannose for 12 min to label the newly synthesized IgG molecules. The intracellular IgG was then immunoprecipitated and glycopeptides were prepared by pronase digestion. As shown in Fig. 7A, two glycopeptide fractions were resolved by P-6 gel filtration. Both of these glycopeptides were susceptible to endo-β-N-acetylglucosaminidase C10, which released a single oligosaccharide from each with a Kav of 0.60, corresponding to a molecular weight of approximately 1430 (Fig. 7B). This indicates that the original oligosaccharide had the composition (Hex)x(GlcNAc)y, and that the separation of the glycopeptides was most likely due to differences in the amount of residual peptide. Treatment of the oligosaccharides with α-mannosidase released 64% of the [3H]mannose, proving that the molecules contained α-linked mannose residues at their nonreducing termini.

In contrast, glycopeptides prepared from the IgG secreted into the medium were resistant to endoglycosidase C10 (Fig. 7, C and D). When these glycopeptides were treated with β-galactosidase, β-N-acetylgalactosaminidase, and α-mannosidase, 57% of the [3H]mannose was released while treatment with α-mannosidase alone released less than 3% of the mannose. These findings indicate that the oligosaccharide of the secreted IgG contains the sequence Gal → GlcNAc → man which is typical of complex type oligosaccharides.

**DISCUSSION**

These data demonstrate that a mannose-rich high molecular weight oligosaccharide is transferred en bloc to the VSV G protein. Within a few minutes of transfer, processing of the oligosaccharide begins and by 30 to 60 min the transferred oligosaccharide has been clipped to a core of 3 mannose and 2 N-acetylgalactosamine residues. The sugar residues forming the outer branches are then added. It is also possible that the addition of the first outer N-acetylgalactosamine residue occurs before the removal of the last mannose residues. The general time scheme for the sequence of events in glycosylation of the G protein is quite similar to that reported by Hunt and Summers for VSV maturation in HeLa cells (23).

While the exact composition and structure of the lipid-linked oligosaccharide remains to be established, a number of its features can be deduced. Based on its behavior on Bio-Gel P-6, the oligosaccharide is estimated to have a molecular weight of approximately 2090, which is consistent with the presence of 10 hexose and 2 N-acetylgalactosamine residues. The finding that endo-β-N-acetylgalactosaminidase C10 acts on the molecule to release 50% of the N-acetylgalactosamine indicates the presence of 2 N-acetylgalactosamine residues in the form of a (N,N'-diaceetylchitobiose unit, most likely at the reducing end of the molecule. Since α-mannosidase released 45% of the [3H]mannose from a long term labeled oligosaccharide with a concomitant decrease in molecular weight of about 420 (or 2.6 mannose residues), there must be at least 6 mannose residues in the oligosaccharide. This estimate is based on the assumption that all the mannose residues are of equal specific activity. If the outer mannose residues are more heavily labeled, as appears to be the case in the 3- and 5-min labeled material, this estimate will be low. The fact that α-mannosidase released mannose residues from the intact oligosaccharide indicates that one or more of the mannose residues must be at the nonreducing end of the molecule. It is not known why more of the mannose residues cannot be cleaved from the oligosaccharide. The two most likely explanations are that the mannose residues are linked by a particular α linkage that the jack bean α-mannosidase cannot cleave or that other monosaccharides are present in the molecule. Since several investigators have demonstrated glucose residues in some lipid-linked oligosaccharides (3, 5), this...
hexose could be present in our material. While treatment of the oligosaccharide with yeast α-glucosidase did not cause a detectable change in the molecular weight of the molecule or an increased susceptibility to α-mannosidase, this result does not exclude the presence of glucose in the oligosaccharide. In future studies we hope to be able to obtain enough lipid-linked oligosaccharide to perform at least a limited structural analysis as well as a determination of the composition of the molecule. Such studies should allow us to assign exact residue numbers and will be necessary to fully understand the precise mechanism of processing.

The data obtained from the pulse-chase experiment indicates that the initial processing of the peptide-linked oligosaccharide involves the stepwise removal of 3 monosaccharides. It appears that these 3 monosaccharide residues which were removed by host cell enzymes may be the residues which are resistant to jack bean α-mannosidase. Thus the jack bean enzyme can only convert the original (Hex)$_n$(GlcNAc)$_m$ oligosaccharide to a (Hex)$_{(n-2)}$(GlcNAc)$_m$ unit but can cleave the 30-min oligosaccharide [(Hex)$_{(n-1)}$(GlcNAc)$_m$] to an oligosaccharide that appears to be (Hex)$_{(n-2)}$(GlcNAc)$_m$. Therefore the 30-min oligosaccharide which is produced by cellular enzymes must have a different structure than the α-mannosidase product of the original (Hex)$_n$(GlcNAc)$_m$ oligosaccharide even though the two molecules appear to be the same size. The subsequent processing and the addition of the outer N-acetylgalactosamine residues must occur during a short interval since the smallest oligosaccharide released by endo-β-N-acetylgalactosaminidase C, had an estimated molecular weight of 1400, equivalent to a composition of (Hex)$_n$(GlcNAc)$_m$. One candidate for a processing enzyme is the α-mannosidase recently isolated from the purified Golgi apparatus of rat liver (24).

It is of interest that there appears to be a defect in the processing of the G protein oligosaccharide when VSV is grown in 15B cells, a line that is deficient in UDP-GlcNAc:glycoprotein N-acetylglucosaminyltransferase activity. Since the oligosaccharide units of the viral glycoprotein grown in 15B cells appear to have the composition (Man)$_n$(GlcNAc)$_m$, it is intriguing to consider the possibility that the removal of the last 2 mannose residues from the precursor oligosaccharide is linked to the addition of the outer N-acetylgalactosamine residues. Such a situation would be analogous to the association of endonuclease activity with DNA polymerase α (25). In future experiments we hope to characterize the enzymes involved in processing.

The fact that processing also occurs during the maturation of the mouse IgG complex type oligosaccharide suggests that this may be a common, and perhaps universal, mechanism for the formation of complex type oligosaccharides. The explanation for the development of processing on the polypeptide may be related to the evolution of complex oligosaccharide biosynthesis. Thus many primitive organisms such as yeast and fungi contain high mannose oligosaccharides while complex type oligosaccharide units appear to have developed at a later time in evolution. There is a striking similarity between the core units of high mannose and complex type oligosaccharides (2). These observations may indicate that high mannose oligosaccharides are the ancestors of complex type oligosaccharides and that processing represents the mechanism whereby the high mannose units are cleaved to form the "cores" of the complex type units. This interpretation could also explain why fucose residues which are linked to the N-acetylgalactosamine residue at the reducing end of complex type oligosaccharides, but not of high mannose oligosaccharides, are added after the outer N-acetylgalactosamine residues (26).

Finally, one can speculate that there may be control mechanisms which regulate the degree of processing. In this regard, Muramatsu et al. reported that growing human diploid fibroblasts contained glycopeptides with predominantly high mannose oligosaccharides while non-growing cells had glycopeptides with an increased percentage of complex type oligosaccharides (27). This alteration could reflect an increase in processing as cells go from the growing to the stationary phase. When the various steps involved in processing are elucidated, it may be possible to determine what factors, if any, regulate these reactions.

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Note Added in Proof—Our recent experiments have shown that the lipid-linked oligosaccharide does, in fact, contain at least 2 residues of glucose. In addition, the total number of mannose residues appears to be 9.

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