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Alterations in the Structure of the Oligosaccharide of Vesicular Stomatitis Virus G Protein by Swainsonine

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Swainsonine, an inhibitor of glycoprotein processing, inhibits the formation of the normal oligosaccharide chain of the G protein of vesicular stomatitis virus. Thus, when vesicular stomatitis virus was grown in baby hamster kidney cells in the presence of swainsonine (15 to 500 ng/ml) and labeled with [2-^{3}H]mannose, the oligosaccharide portion of the G protein was completely susceptible to the action of endoglucosaminidase H. However, the normal viral glycoprotein is not susceptible to this enzyme. Various enzymatic treatments and methylation studies of the mannose-labeled oligosaccharides suggest that swainsonine causes the formation of a hybrid-type oligosaccharide having an oligomannosyl core ($Man_5GlcNAc_2$-Asn) characteristic of neutral oligosaccharides plus the branch structure (NeuNAc-Gal-GlcNAc) characteristic of the complex oligosaccharides. A structure for this hybrid oligosaccharide is proposed. Swainsonine had no effect on the incorporation of [^{14}C]leucine into viral proteins, nor did it change the number of PFU produced in these cultures. It did, however, slightly decrease the incorporation of [^{3}H]glucosamine and increase the incorporation of [^{3}H]mannose. Vesicular stomatitis virus raised in the presence of swainsonine bound much more tightly to columns of concanavalin A-Sepharose than did control virus. Swainsonine had to be added within the first 4 or 5 h of virus infection to be effective. Thus, when 100 ng of the alkaloid per ml was added at any time within the first 3 h of infection, essentially all of the glycoprotein was susceptible to digestion by endoglucosaminidase H. However, when swainsonine was added 4 h after the start of infection, 30% of the glycopeptides became resistant to endoglucosaminidase H; at 5 h, 70% were resistant. The effect of swainsonine was reversible since removal of the alkaloid allowed the cells to form the normal complex glycoproteins. However, the time of removal was critical in terms of oligosaccharide structure.

Many mammalian cell surface glycoproteins, as well as those of enveloped viruses, have oligosaccharide chains linked to protein by means of *N*-acetylglucosamine (GlcNAc)-asparagine bonds (21). These oligosaccharides may be either of the high-mannose or the complex type. In both cases, the oligosaccharide contains a pentasaccharide core region composed of a branched trimannose structure linked to an *N*,*N*-diacetylchitobiose which in turn is linked to the asparagine residue of the protein. In the high-mannose oligosaccharides, this pentasaccharide core may contain as many as six additional α-linked mannose residues, whereas in the complex chains, the pentasaccharide is lengthened by the addition of the sugars GlcNAc, galactose, and sialic acid. Fucose residues may also be in these complex chains.

The biosynthesis of the high-mannose and complex types of oligosaccharides initially involves a series of membrane-bound reactions whereby the sugars GlcNAc, mannose, and glucose are transferred from their sugar nucleotide derivatives to the lipid carrier, dolichyl-phosphate, to form the large oligosaccharide-lipid, $Glc_3Man_9GlcNAc_2$-pyrophosphoryl-dolichol (6, 35). This oligosaccharide is then transferred to the polypeptide chain, probably before the polypeptide chain has been completed (3, 4, 20, 23, 31). After transfer to protein, the oligosaccharide undergoes a series of processing or trimming reactions which appear to begin in the endoplasmic reticulum and continue as the protein is transported through the Golgi apparatus (9, 14). Evidence for the trimming of the G protein of vesicular stomatitis virus (VSV) was demonstrated in vivo in HeLa and BHK cells (17). These trimming reactions result in the rapid removal of all three glucose residues by membrane-bound glucosidases to give a glycoprotein

having a $Man_9GlcNAc_2$ structure (29, 40). This structure may be the immediate precursor to the high-mannose glycoprotein, or it may be further processed to form the complex types of glycoproteins (16). In the latter case, four mannose residues are excised from the $Man_9GlcNAc_2$-protein, probably in the Golgi apparatus (12, 22, 26, 37, 39). A GlcNAc is then added to the $Man_5GlcNAc_2$-protein, and this is followed by the removal of two more mannose residues by another mannosidase (25, 36, 39). Finally, the sugars GlcNAc, galactose, and sialic acid are added sequentially to form the trisaccharide structures, sialic acid-galactose-GlcNAc linked to the pentasaccharide core (1, 33). Fucose may also be added at this stage.

Recently, we described some preliminary studies with an inhibitor of glycoprotein processing called swainsonine. This compound is an indolizidine alkaloid that was first isolated from the Australian plant, *Swainsona canescens* (2). Animals that eat this plant develop symptoms analogous to those of human α-mannosidosis, and swainsonine was shown to be a potent inhibitor of lysosomal α-mannosidase (5). In cultured MDCK cells (7) or in influenza virus-infected MDCK cells (8), swainsonine prevented the normal formation of complex glycoproteins and gave rise to oligosaccharide structures that were completely susceptible to cleavage by endoglucosaminidase H. Recent studies by Tulsiani et al. showed that swainsonine inhibited mannosidase II that removes α(1 → 3)- and α(1 → 6)-linked mannoses from the GlcNAc-Man_5-$GlcNAc_2$-protein, but had no effect on the mannosidases that trim the α(1 → 2)-linked mannose units (39).

In this report, we describe studies on the effect of swainsonine on the synthesis and processing of the G protein of VSV. This virus is an excellent model for studies on glycoprotein synthesis since infected cells produce predominantly viral proteins, whereas host protein synthesis is inhibited (24). Thus, one can study the synthesis from initiation to completion of the VSV G protein, a glycoprotein that has been shown to have two oligosaccharide chains of the complex type (10, 28). As shown in this paper, growth of VSV in the presence of swainsonine resulted in the formation of oligosaccharide chains that were completely susceptible to the action of endoglucosaminidase H. The chemical characterization of these oligosaccharides indicated that they were hybrid structures having both high-mannose and complex chains.

MATERIALS AND METHODS

Materials. [2-^{3}H]mannose (10 Ci/mmol) was purchased from Amersham Corp. L-[^{14}C]leucine (300 mCi/mmol), D-[^{14}C]mannose (45 to 55 mCi/mmol), D-[^{3}H]galactose (5 to 15 Ci/mmol), and [6-^{3}H]glucosamine were from New England Nuclear Corp. Minimal essential medium (MEM; Earle liquid), fetal calf serum, MEM nonessential amino acids, glutamine, penicillin-streptomycin-neomycin (PSN) antibiotic mixture, Eagle basal medium (BME) vitamin solution, and mycostatin suspension were purchased from GIBCO Laboratories. Bio-Gel P-4 (100-200 mesh) was from Bio-Rad Laboratories. Pronase was from Calbiochem, trypsin was from GIBCO, and α-mannosidase, neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase were from Sigma Chemical Co. Endoglucosaminidase H and endoglucosaminidase D were purchased from Miles Laboratories, Inc. Swainsonine was a generous gift from P. R. Dorling, Murdoch University, Australia. All other chemicals were from commercial sources and were of the highest grade available.

Mammalian cells and viruses. Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection and maintained in culture. VSV Indiana strain was originally provided by Robert A. Lazzarini, National Institutes of Health, and was maintained in stock.

BHK-21 cells were grown as monolayers in MEM supplemented with 10% fetal calf serum, 2 mM glutamine, 1 × BME vitamins, 1 × MEM nonessential amino acids, 1× PSN antibiotic mixture, and 50 μl of mycostatin suspension. Nearly confluent monolayers of cells growing in 75-cm^2 flasks were infected with VSV at a multiplicity of infection of 10 to 40 PFU per cell. The virus was allowed to adsorb to the cells for 60 min at 37°C in 4 to 5 ml of medium. Unadsorbed virus was removed by aspiration, and 5 to 10 ml of fresh medium was added. The cells were allowed to incubate for 16 to 20 h, and virus was harvested at that time. Swainsonine was generally added at 100 ng/ml 1 h after virus, except in certain experiments as described in the figure legends.

Radioactive labeling of virus. Virus was raised in the presence of various isotopes to label the glycoproteins. When virus was labeled with radioactive amino acids (i.e., [^{14}C]leucine), the MEM added to the cells did not contain amino acids. In these experiments, 50 μCi of leucine was added. For labeling with sugars, normal MEM was used. In this case, 50 to 200 μCi of [^{3}H]glucosamine, 50 μCi of [^{14}C]mannose, or 300 μCi of [^{3}H]mannose was added to 5 to 10 ml of MEM. Label was added 3 h after virus (3 h postinfection) and allowed to remain with the cells during the course of virus production.

Purification of virus. Cells were allowed to incubate for 16 to 20 h to produce mature virus. The medium containing virus and cells was centrifuged at 3,000 rpm for 10 min at 4°C to remove cells and cell debris. The supernatant liquid from this centrifugation was subjected to sucrose density gradient centrifugation to obtain the virus (19). Thus, 5 ml of the supernatant fluid was layered onto 3 ml of 60% sucrose and 4 ml of 25% sucrose in phosphate-buffered saline. The interphases were separated at 100,000 × *g* for 90 min at 4°C. VSV which sediments at 60% and 25% interphases was recovered by precipitation with 5% trichloroacetic acid. The precipitate was collected by centrifugation at 5,000 rpm for 10 min at 4°C, and the pellet was digested exhaustively with pronase (1% pronase solution in 50 mM Tris buffer [pH 7.5] containing 10 mM $CaCl_2$) under a toluene atmosphere.

After an incubation of 72 h at 37°C, the pronase was inactivated by heating at 100°C for 10 min.

Chromatographic methods. Glycopeptides obtained by pronase digestion of VSV were separated on columns of Bio-Gel P-4 (1.5 by 100 cm, 100-200 mesh). The columns were equilibrated and run in 0.15% acetic acid. A sample of each fraction was removed for the determination of radioactivity. Peak fractions were pooled and used in further experiments. Glycopeptides were treated with various glycosidases (see below), and the products of these digestions were rechromatographed on the Bio-Gel P-4 column.

Glycosidase digestions. All incubations were done under a toluene atmosphere at 37°C for various periods of time. Endoglucosaminidase H digestion was performed in 150 mM citrate-phosphate buffer at pH 6.0 in a total volume of 0.2 ml; 10 mU of enzyme was added initially, and then 10 mU of enzyme was added every 24 h. The total incubation time was 72 h. Digestions with endoglucosaminidase D were done in the same way, except that the pH was 6.5. For treatment with α-mannosidase, 2 U of enzyme was added to 0.1 ml of sample in 100 mM sodium acetate buffer (pH 4.5) containing 0.4 mM $ZnCl_2$. After 24 h, an additional 2 U of α-mannosidase was added. Neuraminidase digestions were performed with 0.5 U of enzyme in 100 mM sodium acetate buffer (pH 5.2). Incubations were for 24 h. β-*N*-Acetylhexosaminidase treatments utilized 25 mU of enzyme in sodium citrate buffer (pH 5.5) in a final volume of 0.1 ml and an incubation time of 24 h. For treatment with β-galactosidase, 0.5 U of enzyme was added to the sample in 100 mM sodium acetate buffer (pH 4.5) in a final volume of 0.1 ml. After 24 h of incubation, an additional 0.5 U of enzyme was added, and samples were incubated for an additional 24 h.

Determination of PFU. After the centrifugation of the medium at 3,000 rpm to remove cells and cell debris, the supernatant liquid was assayed to determine the number of infective units and to determine whether the addition of swainsonine had any effect of infectivity. Appropriate dilutions of the supernatant fluid were applied to the monolayers of BHK cells, and the numbers of plaques were counted (19). Usually titers were determined within 12 to 16 h of collection.

Methylation analysis. Methylation of the oligosaccharide was performed as described by Hakamori (15) and as modified by Sanford and Conrad (32). The lyophilized samples were dissolved in 2 ml of dimethyl sulfoxide under N_2 and sonicated with methylsulfinylcarbanion at 50°C for 5 h. The mixture was chilled and 2 ml of CH_3I was added. The sample was again sonicated for 2 h at 4°C, and another addition of 2 ml of CH_3I was made after 1 h. After standing at room temperature overnight, the sample was passed through a column of Sephadex LH-20, equilibrated, and run in 80% methanol to remove dimethyl sulfoxide, methylsulfinylcarbanion, and other salts. The eluate from the column was hydrolyzed in 2 N H_2SO_4 in sealed tubes at 100°C for 4 h. After neutralization with $Ba(OH)_2$ or by passage through Dowex-1-formate, the methylated sugars were analyzed by thin-layer chromatography.

RESULTS AND DISCUSSION

Effect of swainsonine on the incorporation of isotopes and the formation of infective particles. Baby hamster kidney cells were grown to confluency, and the cultures were infected with VSV. After 1 h, various concentrations of swainsonine were added to the infected cells, and they were allowed to incubate for 2 or 3 h for the inhibitor to take effect. At the end of this time, either [*U*-^{14}C]leucine, [6-^{3}H]glucosamine, or [2-^{3}H]mannose was added, and the cultures were incubated for an additional 16 to 20 h to allow the formation of mature virus. The virus was isolated by sucrose density gradient centrifugation and examined for infectivity and radioactivity. Figure 1 presents the results of some of these measurements. At swainsonine concentra-

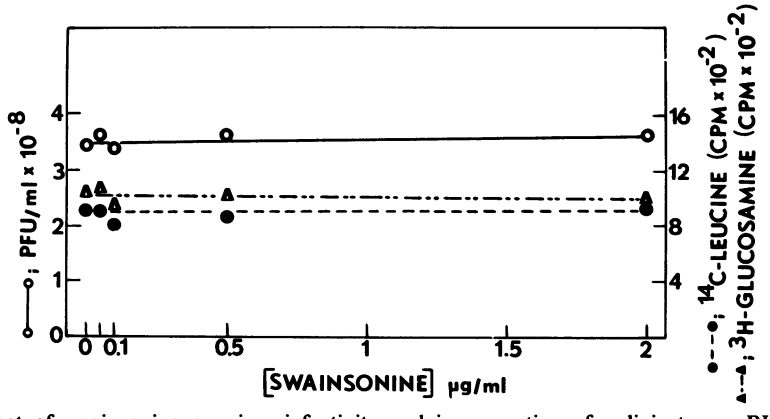

FIG. 1. Effect of swainsonine on virus infectivity and incorporation of radioisotopes. BHK cells were infected with VSV, and various concentrations of swainsonine were added 1 h after infection. After an additional 3 h of incubation, 50 μCi of [^{14}C]leucine or 60 μCi of [^{3}H]glucosamine was added, and incubations were continued for another 12 h. VSV was isolated from the culture medium by ultracentrifugation and assayed for infectivity by the plaque assay. Radioactivity was determined by precipitation of a sample of the viral suspension with 5% trichloroacetic acid, filtration on a membrane filter (Millipore Corp.), and scintillation counting of the filter.

tions of up to 2 μg/ml, there was no change in the number of PFU released from the cells, indicating that this compound did not affect viral replication, release, or infectivity. Figure 1 also shows that this alkaloid did not affect the incorporation of [^{14}C]leucine into the viral proteins. However, at higher concentrations of swainsonine, the incorporation of [^{3}H]glucosamine was somewhat impaired, whereas the incorporation of [2-^{3}H]mannose was somewhat stimulated (data not shown).

Effect of swainsonine concentration on the composition of the oligosaccharide portion of VSV glycoproteins. BHK cells were infected with VSV, and various concentrations of swainsonine were added. After an incubation of 2 to 3 h, [^{3}H]glucosamine was added to each flask, and the incubations were continued for another 12 to 16 h. The mature virus was isolated by sucrose density gradient centrifugation, and the virus particles were digested exhaustively with pronase to release glycopeptides. The glycopeptides were separated on columns of Bio-Gel P-4. Figure 2 shows the glycopeptide patterns ob-

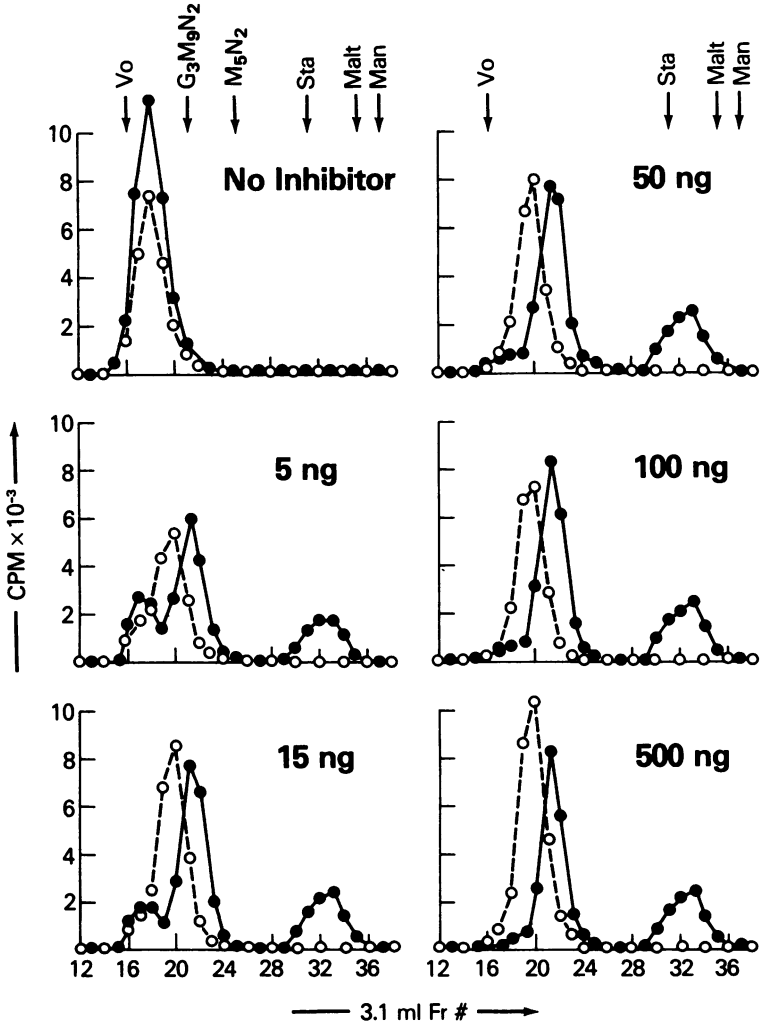

FIG. 2. Effect of swainsonine concentration on the composition of the glycopeptides from VSV glycoproteins. VSV-infected BHK cells were incubated for 3 h in various concentrations of swainsonine, and then 60 μCi of [^{3}H]glucosamine was added to each culture. After an additional 12 h of incubation, the VSV was isolated by centrifugation and digested exhaustively with pronase. The glycopeptides were isolated by chromatography on columns of Bio-Gel P-4 (1.5 by 100 cm, 100-200 mesh). Samples of every other fraction were removed for the determination of radioactivity (○). The glycopeptide peaks (fractions 16 through 24) were pooled, concentrated to dryness, and digested with endoglucosaminidase H. These digests were rechromatographed on Bio-Gel P-4 (●).

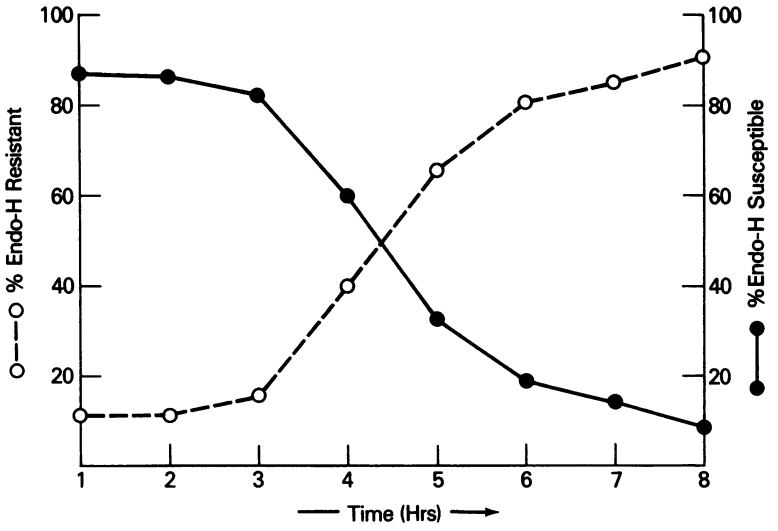

FIG. 3. Effect of time of addition of swainsonine on the ratio of complex to high-mannose glycopeptides in the VSV glycoproteins. Swainsonine was added to infected cells at various times from 1 to 8 h after infection. The percentage of the glycopeptides susceptible to endoglucosaminidase H was determined after chromatography on Bio-Gel P-4.

tained at various swainsonine concentrations (from 0 to 500 ng/ml). It was difficult to resolve the glycopeptides from control and swainsonine-inhibited cells on these columns, since they emerged near the void volume of the column. Therefore, the entire glycopeptide peak from each incubation was pooled, digested with endoglucosaminidase H, and rechromatographed on the same Bio-Gel P-4 column (Fig. 2). This enzyme cleaves the glycosidic bond between the two internal GlcNAc residues of high-mannose glycopeptides, but does not act on the complex types of glycopeptides (38, 41).

In the control virus (no swainsonine), none of the glycopeptide was susceptible to endoglucosaminidase H, as evidenced by the fact that the migration did not change after incubation with the enzyme (Fig. 2). This evidence is suggestive that these glycoproteins are of the complex type. However, when the virus was grown in the presence of swainsonine, most or all of the glycopeptide was susceptible to enzymatic digestion. Thus, even at 5 ng of swainsonine per ml, 70% of the glycopeptide became susceptible to endoglucosaminidase H, whereas at higher alkaloid concentrations almost all of the glycopeptide was cleaved by this enzyme. The smaller radioactive peak emerging at fractions 30 to 34 after digestion with endoglucosaminidase H is probably the GlcNAc-peptide that is cleaved from the glycopeptide. Similar changes in oligosaccharide structure were also produced by swainsonine when VSV-infected cells were labeled with [2-^{3}H]mannose.

Effect of time of addition of swainsonine on oligosaccharide changes. Since swainsonine caused a shift in the glycopeptides from resistance to sensitivity to endoglucosaminidase H, it was of interest to determine when this inhibitor had to be added to infected cells to observe this effect. Thus, confluent monolayers were infected with VSV, and [^{3}H]glucosamine was added 1 h later. Swainsonine (100 ng/ml) was then added either at the same time as the glucosamine (i.e., 1 h postinfection) or at various times during the incubation (up to 8 h). The isolated virus particles were digested with pronase and chromatographed on Bio-Gel P-4, and the pooled glycopeptides were digested with endoglucosaminidase H. These digests were rechromatographed on the Bio-Gel P-4 column. In Fig. 3, the radioactivity in the pronase-released glycopeptides and that in the glycopeptides susceptible to endoglucosaminidase H are plotted as a function of time of addition of swainsonine to the cells. When swainsonine was added at any time within the first 3 h of infection, 85% of the glycopeptides could be digested by endoglucosaminidase H and only about 15% were resistant to this enzyme (Fig. 3). However, when the addition of the inhibitor was delayed for 4 h, only about 60% of the glycopeptides were susceptible to endoglucosaminidase H; this percentage decreased as the swainsonine addition was delayed for longer and longer times. These studies indicated that the biosynthesis and processing of the oligosaccharide portion of the VSV G protein are probably completed within the first 4 to 6 h

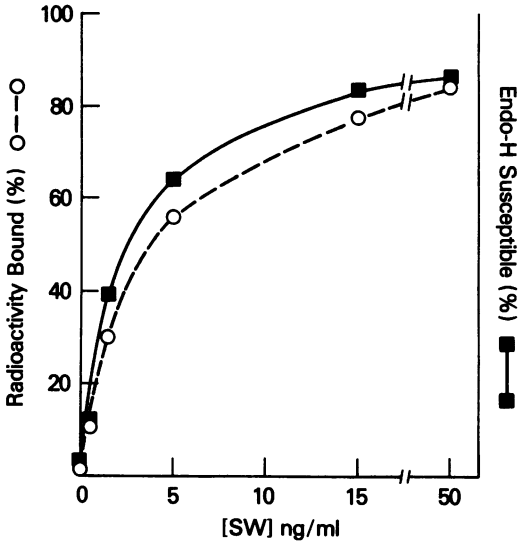

FIG. 4. Effect of swainsonine on the binding of VSV glycopeptides to concanavalin A-agarose. VSV was grown in BHK cells in the presence of various concentrations of swainsonine, and the glycopeptides were obtained by pronase digestion. Part of the glycopeptide fraction from each VSV preparation was applied to a column of concanavalin A-agarose, and the other part was tested for susceptibility to endoglucosaminidase H.

of infection, so that addition of swainsonine at later times had no effect on oligosaccharide profiles. These data correlate well with previous studies by Simonsen et al., who showed that maturation of VSV was completed by 4.5 to 5.5 h after the start of infection (34). Similar results were observed with influenza virus maturation and the antibiotic tunicamycin (27).

Reversibility of swainsonine effect. To determine whether the swainsonine inhibition was reversible, 100 ng of swainsonine per ml was added to VSV-infected cells 1 h after the start of infection. At various times from 15 min to 6 h, the medium was removed by aspiration, and the cell monolayers were washed two times with fresh media. The cells were placed in fresh medium, [^{3}H]glucosamine was added to each culture, and the incubations were continued. The percentage of radioactivity in the endoglucosaminidase H-sensitive glycopeptides was determined. These studies demonstrated that removal of swainsonine during the first 2 h alleviated the inhibitory effect and resulted in glycopeptides that were essentially resistant to endoglucoaminidase H. However, if swainsonine was allowed to remain with the cells for 6 h and then removed, a considerable amount (25 to 35%) of the glycopeptides were digested by endoglucosaminidase H. These studies demon-

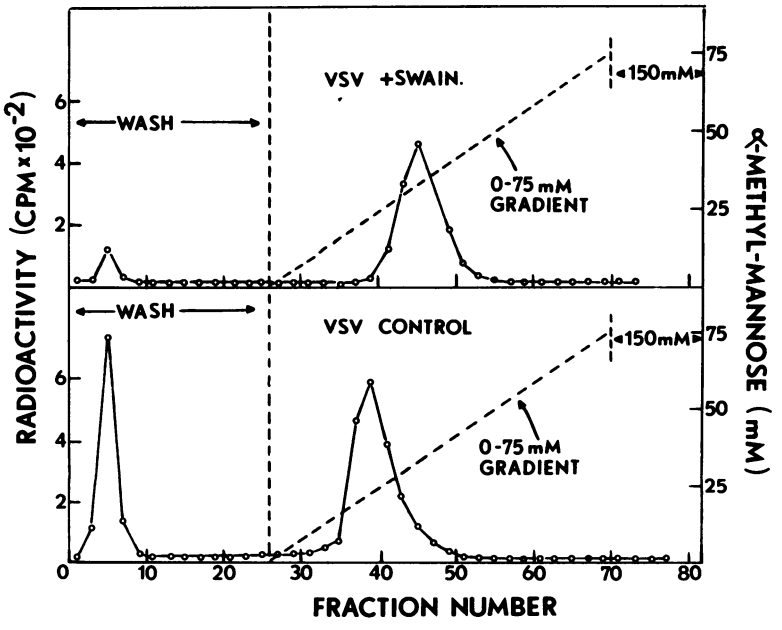

FIG. 5. Fractionation of glycopeptides from normal and swainsonine-grown VSV on concanavalin A-agarose. Radioactive glycopeptides were applied to 0.5- by 5-cm columns of concanavalin A-agarose, and the columns were washed with 50 mM Tris buffer containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 100 mM NaCl. The columns were then eluted with 100 ml of a gradient of 0 to 75 mM α-methylmannoside in the same buffer. Samples of each fraction were removed for the determination of radioactivity.

strate that the inhibition by swainsonine can be reversed as long as the alkaloid is removed from the medium within the first 2 to 4 h of virus infection.

Effect of swainsonine on the binding of VSV to concanavalin A. Since the growth of VSV in swainsonine resulted in a loss of resistance to endoglucosaminidase H, it was of interest to determine the effect of this alkaloid on other properties of the virus, such as its ability to bind to columns of concanavalin A. Thus, VSV was grown in the presence of various amounts of swainsonine, and the virus particles were labeled by growth in [2-^{3}H]mannose. The mature virus was digested with pronase, and the glycopeptides were tested for their susceptibility to endoglucosaminidase H and also for their ability to bind to columns of concanavalin A-agarose. Thus, the pronase-released glycopeptides were applied to columns of concanavalin A, and the buffer wash of these columns was measured for radioactivity. The columns were then eluted with 100 mM α-methylmannoside. Figure 4 shows that the amount of radioactivity binding to the concanavalin A columns increased with increasing amounts of swainsonine in the growth medium. Thus, at 15 to 25 ng of alkaloid per ml, 80 to 90% of the total radioactivity bound to the columns and was not removed in the wash. The curve of concanavalin A binding followed very closely that of susceptibility to endoglucosaminidase H, suggesting that the alterations in oligosaccharide structure caused by swainsonine affected both of these parameters. The radioactivity that bound to concanavalin A could be eluted with 100 mM α-methylmannoside or with a gradient as indicated below. These studies suggest that swainsonine causes the formation of a partial high-mannose or hybrid structure.

Partial characterization of the endoglucosaminidase H-sensitive oligosaccharide formed in the presence of swainsonine. The glycopeptides obtained from normal VSV (i.e., control) and from VSV grown in the presence of swainsonine (100 ng/ml) were compared for their ability to bind to columns of concanavalin A-agarose (Fig. 5). Glycopeptides were applied to the columns; the columns were washed well with buffer and then eluted with a gradient (0 to 75 mM) of α-methylmannoside. The lower profile shows that about 65% of the radioactive glycopeptides in the control virus did not bind to concanavalin A and were eluted in the wash. These glycopeptides are probably triantennary complex structures. However, about 30 to 35% of the radioactivity did bind to concanavalin A and was eluted at 20 to 25 mM α-methylmannoside. These structures are probably biantennary complex chains that bind weakly to concanavalin A. On the other hand, in the glycopeptide from swainsonine-grown virus, almost all of the radioactivity bound to the concanavalin A columns and required 35 to 40 mM α-methylmannoside for elution. These data suggest that this glycopeptide contains some high-mannose structure and perhaps has one or more terminal mannose residues.

The glycopeptide from swainsonine-grown virus was isolated by gel filtration on Bio-Gel P-4, treated with endoglucosaminidase H, and rechromatographed on the Bio-Gel P-4 column.

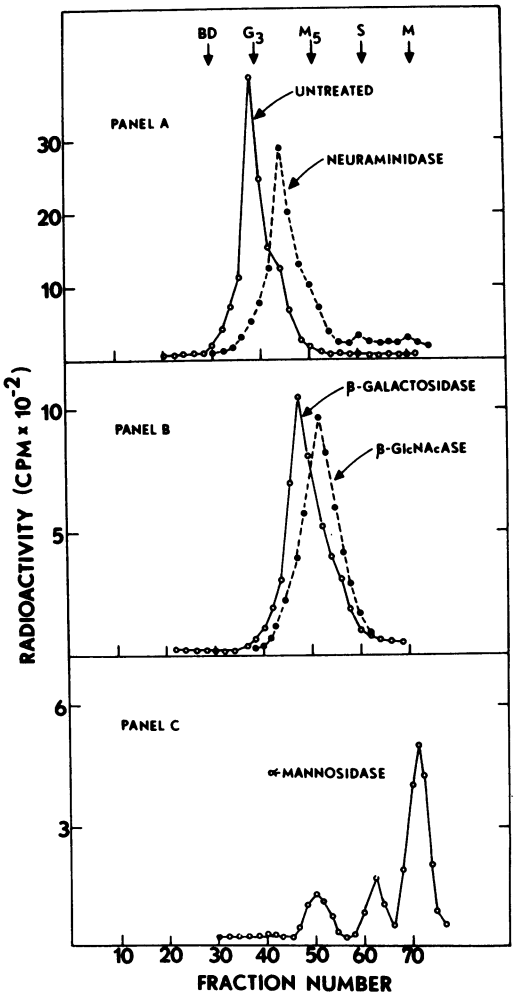

FIG. 6. Sequential digestion of endoglucosaminidase H-sensitive oligosaccharide with glycosidases. The endoglucosaminidase H-sensitive oligosaccharide isolated from swainsonine-grown VSV was digested sequentially with neuraminidase, β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase. After each digestion, the reaction mixture was chromatographed on Bio-Gel P-4. The oligosaccharide from the neuraminidase digestion was used as a substrate for β-galactosidase digestion, and so on.

The oligosaccharide was purified by a second passage on a long calibrated column of Bio-Gel P-4. It eluted from this column near the standard $Glc_3Man_9GlcNAc$. Thus, the binding to concanavalin A, the susceptibility to endoglucosaminidase H, and the elution position on Bio-Gel P-4 all suggest a hybrid-type oligosaccharide with a high-mannose chain on one branch and one or two complex chains on the other branch. A hybrid type of oligosaccharide was recently isolated from Rous sarcoma virus by Hunt and Wright (18). Partial characterization of this oligosaccharide suggested a structure somewhat analogous to the one reported here.

The purified oligosaccharide was subjected to a series of sequential enzymatic digestions (Fig. 6). After each treatment, the oligosaccharide was rechromatographed on Bio-Gel P-4. Thus, treatment of the oligosaccharide with neuraminidase or with mild acid (0.1 N HCl, 80°C, 60 min) caused a considerable shift in the migration on Bio-Gel P-4 to a slower-moving species, indicating the loss of two or three hexose residues, which on this column is equivalent to the loss of one sialic acid. Thus, the oligosaccharide appears to contain one sialic acid residue. Subsequent digestion with β-galactosidase caused another shift in migration on Bio-Gel P-4 which was approximately equivalent to the loss of 1.5 to 2 hexose units, suggesting the removal of 1 or 2 galactose units. When the asialo-agalacto-oligosaccharide was treated with β-*N*-acetylhexosaminidase, its migration was shifted to a new, slower moving species whose migration was slightly behind that of standard $Man_5GlcNAc$. This new migration suggested the loss of three or four hexose residues, which would suggest the removal of one or two GlcNAc residues. There was no loss of GlcNAc residues when the asialo-oligosaccharide was treated with the β-*N*-acetylhexosaminidase before β-galactosidase. Finally, after treatment with neuraminidase, β-galactosidase, and β-*N*-acetylhexosaminidase, the oligosaccharide was treated with α-mannosidase (Fig. 6). This final treatment gave rise to a major peak migrating in the mannose area as well as a small peak of radioactivity eluting in the area expected for a Man-GlcNAc disaccharide. A radioactive peak was also observed close to the original oligosaccharide. This may represent undigested material or another structure which is resistant to this treatment. However, the amount of radioactivity in this peak was too low for further analysis.

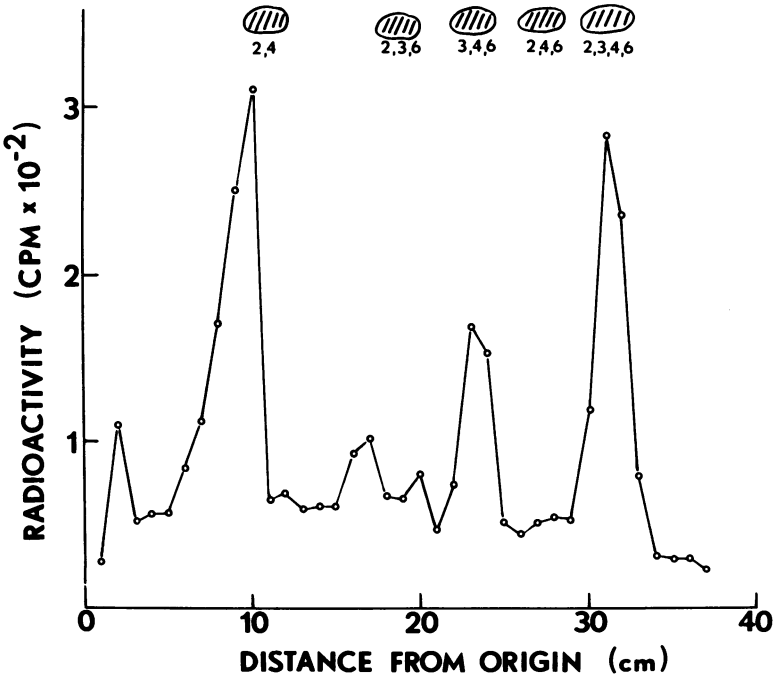

FIG. 7. Thin-layer chromatography of the methylated sugars from the endoglucosaminidase H-sensitive oligosaccharide. The purified oligosaccharide was methylated, subjected to complete acid hydrolysis, and chromatographed on silica gel plates in benzene-acetone-water-ammonium hydroxide (50:200:3:1.5). Standards shown at the top are 2,3,4,6-tetramethylmannose, 2,3,6-, 3,4,6-, and 2,4,6-trimethylmannose, and 2,4-dimethylmannose. Plates were scraped in 1-cm areas, and each area was counted in the scintillation counter for the determination of radioactivity.

The ratio of hexose to sialic acid in the normal VSV glycopeptide was compared with that in the swainsonine-induced oligosaccharide by using colorimetric methods. Thus, in normal VSV, the ratio of hexose, as measured by the anthrone method (with a mannose standard), to sialic acid, as measured by the thiobarbituric acid method, was 1.9:1.0, whereas the ratio in the swainsonine-induced oligosaccharide was 5.7:1.

The endoglucosaminidase H-released oligosaccharide labeled with [2-^{3}H]mannose was subjected to complete methylation; after strong acid hydrolysis, the methylated sugars were examined by thin-layer chromatography. The plates were cut into 1-cm sections and counted in the scintillation counter. The radioactive tracing of this plate is shown in Fig. 7. The two major radioactive peaks corresponded to the standards 2,3,4,6-tetramethylmannose and 2,4-dimethylmannose, and these were present in approximately equal amounts (the actual ratio of dimethylmannose to tetramethylmannose was 1.3:1). The only other major radioactive peak was that corresponding to 3,4,6-trimethylmannose, and the ratio of this peak to the tetramethylmannose was 0.5:1.0. The complex chain on the 3-linked mannose has been reported to be linked in a GlcNAC 1→2 mannose bond (28).

The data presented above suggested that the oligosaccharide produced in the presence of swainsonine is a hybrid structure. Figure 8 presents one possible structure, but based on the size of the oligosaccharide on Bio-Gel P-4 and the possible release of two galactose and one or two GlcNAc residues, the oligosaccharide may have two complex chains in addition to the high-mannose structure. The absolute location of the complex chain(s) is not known, but one chain is probably attached to the 3-linked mannose. The results described here indicate that some processing can occur even though all of the mannose residues have not been removed by the processing α-mannosidases.

It is interesting to note that Etchison et al. (11) showed that the carbohydrate composition of the VSV G protein could be altered by growth in different cell lines. Thus, four different teratocarcinoma cell lines were used in that study. The viral glycopeptides from virus grown in the different cells were approximately the same size, but differed in their susceptibility to endoglucosaminidase H and exoglycosidases. Their studies indicate that the oligosaccharide structure of the G protein can be altered under various conditions, and that these alterations do not affect viral infectivity. In the experiments described here, we found that virus particles raised in the presence of swainsonine were as infective as those from normal cells. This suggests that the normal triantennary complex chains are not essential for biological activity.

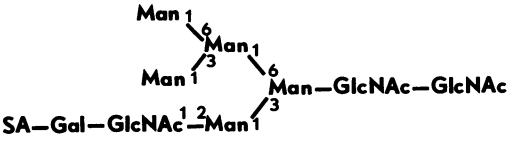

FIG. 8. Possible hybrid structure of the oligosaccharide of VSV G protein produced in the presence of swainsonine.

On the other hand, Gibson et al. (13) studied the effect of glycosylation on the VSV G protein by using various temperature-sensitive mutants. They found that the nonglycosylated form of G, synthesized in cells treated with tunicamycin, displayed a temperature-sensitive behavior in its maturation to the cell surface. Analysis of the defect at the elevated temperature showed that the nonglycosylated G molecule undergoes aggregation in the endoplasmic reticulum membrane. Apparently, G molecules that differ in their primary sequence have different requirements for glycosylation to reach the cell surface and be incorporated into viral particles. In fact, whenever the nonglycosylated G does mature to the surface (i.e., in certain mutants and at permissive temperatures), it is incorporated normally into virus particles, and these viruses have normal specific infectivity.

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LITERATURE CITED

1. **Beyer, T. A., J. E. Sadler, J. I. Rearick, J. C. Paulson, and R. L. Hill.** 1981. Glycosyltransferases and their use in assessing oligosaccharide structure and structure-function relationships. Adv. Enzymol. **52**:23.
2. **Colgate, S. M., P. R. Dorling, and C. R. Huxtable.** 1979. A spectrophotometric investigation of swainsonine, an α-mannosidase inhibitor isolated from *Swainsona canescens*. Aust. J. Chem. **32**:2257–2264.
3. **Czicki, V., and W. J. Lennarz.** 1977. Localization of enzyme system for glycosylation of proteins via the lipid-linked pathway in rough endoplasmic reticulum. J. Biol. Chem. **252**:7901–7904.
4. **Das, R. C., and E. C. Heath.** 1980. Dolichyldiphosphoryl-oligosaccharide-protein oligosaccharyltransferase: solubilization, purification and properties. Proc. Natl. Acad. Sci. U.S.A. **77**:3811–3815.
5. **Dorling, P. R., C. R. Huxtable, and S. M. Colgate.** 1980. Inhibition of lysosomal α-mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*. Biochem. J. **191**:649–651.
6. **Elbein, A. D.** 1979. The role of lipid-linked saccharides in the biosynthesis of complex carbohydrates. Annu. Rev. Plant Physol. **30**:239–272.
7. **Elbein, A. D., P. R. Dorling, R. Solf, and K. Vosbeck.** 1981. Swainsonine, an inhibitor of glycoprotein processing. Proc. Natl. Acad. Sci. U.S.A. **78**:7393–7397.
8. **Elbein, A. D., M. Horisberger, P. R. Dorling, and K.**

Vosbeck. 1982. Swainsonine prevents the precessing of the oligosaccharide chains of influenza virus hemagglutinin. J. Biol. Chem. **257**:1573–1576.

9. **Elting, J. J., W. W. Chen, and W. J. Lennarz.** 1980. Characterizaton of a glucosidase involved in the initial step in the processing of oligosaccharide chains. J. Biol. Chem. **255**:2325–2331.
10. **Etchison, J. R., J. S. Robertson, and D. F. Summers.** 1977. Partial structural analysis of the oligosaccharide moieties of the VSV glycoprotein by sequential chemical and enzymatic degration. Virology **78**:375–392.
11. **Etchison, J. R., D. F. Summers, and C. Georgopoulos.** 1981. Variations in the structure of radiolabeled glycopeptides from the glycoprotein of VSV grown in four mouse teratocarcinoma cell lines. J. Biol. Chem. **256**:3366–3369.
12. **Forsee, W. T., and J. Schutzbach.** 1981. Purification and characterization of a phospholipid-dependent α-mannosidase from rabbit liver. J. Biol. Chem. **256**:6577–6887.
13. **Gibson, R., S. Schlessinger, and S. Kornfeld.** 1979. The nonglycosylated glycoprotein of VSV is temperature-sensitive and undergoes intercellular aggregation at elevated temperatures. J. Biol. Chem. **254**:3600–3607.
14. **Grinna, L. S., and P. W. Robbins.** 1979. Substrate specificities of rat liver microsomal glucosidases which process glycoproteins. J. Biol. Chem. **255**:2255–2258.
15. **Hakamori, S.** 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55**:205–208.
16. **Hubbard, S. C., and R. J. Ivatt.** 1981. Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **50**:555–583.
17. **Hunt, L. A., J. R. Etchison, and D. R. Summers.** 1978. Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **75**:754–758.
18. **Hunt, L. A., and S. E. Wright.** 1981. Rous sarcoma virus glycoproteins contain hybrid-type oligosaccharides. J. Virol. **39**:646–650.
19. **Kang, M. S., J. J. Park, I. Singh, and L. P. Phillips.** 1981. Streptovirudin inhibits glycosylation and multiplication of VSV. Biochem. Biophys. Res. Commun. **99**:422–428.
20. **Kiely, M., C. S. McKnight, and R. Schimke.** 1976. Studies on the attachment of carbohydrate to ovalbumin nascent chains in hen oviduct. J. Biol. Chem. **251**:5490–5495.
21. **Kornfeld, R., and S. Kornfeld.** 1976. Comparative aspects of glycoprotein structure. Annu. Rev. Biochem. **45**:217–237.
22. **Kornfeld, S., E. Li, and I. Tabas.** 1978. The synthesis of complex-type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of VSV G protein. J. Biol. Chem. **253**:7771–7778.
23. **Lingappa, V. R., J. R. Lingappa, R. Prasad, K. Ebner, and G. Blobel.** 1978. Coupled cell-free synthesis, segregation and core glycosylation of a secretory protein. Proc. Natl. Acad. Sci. U.S.A. **75**:2338–2342.
24. **Lodish, H. F., and R. A. Weiss.** 1979. Selective isolation of mutants of vesicular stomatitis virus defective in production of the viral glycoprotein. J. Virol. **30**:177–189.
25. **Narasimhan, S., P. Stanley, and H. Schachter.** 1977. Control of glycoprotein synthesis. Lectin resistant mutants containing only one of two distinct N-acetylglucosaminyltransferase activities present in wild type Chinese hamster ovary cells. J. Biol. Chem. **252**:3926–3933.
26. **Opheim, D. J., and O. Touster.** 1978. Lysosomal α-mannosidase of rat liver: purification and comparison with the Golgi and cytosolic α-mannosidase. J. Biol. Chem. **253**:1017–1023.
27. **Pan, Y. T., J. Schmitt, B. A. Sanford, and A. D. Elbein.** 1979. Adherence of bacteria to mammalian cells. Inhibition by tunicamycin and streptovirudin. J. Bacteriol. **139**:505–513.
28. **Reading, C. L., E. E. Penhoet, and C. E. Ballou.** 1978. Carbohydrate structure of VSV glycoprotein. J. Biol. Chem. **253**:5600–5607.
29. **Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth.** 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. Cell **12**:893–900.
30. **Robertson, M. A., J. R. Etchison, J. S. Robertson, and D. F. Summers.** 1978. Specific changes in the oligosaccharide moieties of VSV grown in different lectin-resistant CHO cells. Cell **13**:515–526.
31. **Rodriguez-Boulan, E., G. Kreibach, and D. D. Sabatini.** 1978. Spatial orientation of glycoproteins in membranes of rat liver rough microsomes. J. Cell. Biol. **78**:874–893.
32. **Sanford, P. A., and H. E. Conrad.** 1966. The structure of *Aerobacter aerogenes* A3(51) polysaccharide. A reexamination using improved procedures for methylation analysis. Biochemistry **5**:1508–1517.
33. **Schachter, H., and S. Roseman.** 1981. Mammalian glycosyltransferase. Their role in the synthesis and function of complex carbohydrates and glycolipids, p. 85–160. *In* W. Lennarz (ed.), The biochemistry of glycoproteins and proteoglycans. Plenum Publishing Corp., New York.
34. **Simonsen, C. C., V. M. Hill, and D. F. Summers.** 1979. Further characterization of the replicative complex of vesicular stomatitis virus. J. Virol. **31**:494–505.
35. **Struck, D. L., and W. J. Lennarz.** 1980. The function of saccharide-lipids in the synthesis of glycoproteins, p. 35–83. *In* W. Lennarz (ed.), The biochemistry of glycoproteins and proteoglycans. Plenum Publishing Corp., New York.
36. **Tabas, I., and S. Kornfeld.** 1978. The synthesis of complex type oligosaccharides. III. Identification of an α-mannosidase activity involved in a late stage of processing of complex type oligosaccharides. J. Biol. Chem. **253**:7779–7786.
37. **Tabas, I., and S. Kornfeld.** 1979. Purification and characterization of a rat Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. J. Biol. Chem. **254**:11655–11663.
38. **Tarentino, A. L., and F. Maley.** 1974. Purification and properties of an endo-β-N-acetylglucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249**:811–817.
39. **Tulsiani, D. R. P., S. C. Hubbard, P. W. Robbins, and O. Touster.** 1982. α-Mannosidases of rat liver golgi membranes. Mannosidase II is the $GlcNAcMan_5$-cleaving enzyme in glycoprotein biosynthesis and Mannosidase 1A and 1B are the enzymes converting Man_9 precursors to Man_5 intermediate. J. Biol. Chem. **257**:3660–3668.
40. **Ugalde, R. A., R. J. Stanaloni, and L. F. Leloir.** 1978. Activity of glucosidases on the saccharide moiety of the glucose-containing dolichyl diphosphate oligosaccharide. FEBS Lett. **91**:209–212.
41. **Yamashita, K., Y. Tachibana, and Y. Kobata.** 1978. The structures of the galactose-containing sugar chains of ovalbumin. J. Biol. Chem. **203**:3863–3869.