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Castanospermine Inhibits the Processing of the Oligosaccharide Portion of the Influenza Viral Hemagglutinin†

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ABSTRACT: Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is a plant alkaloid that inhibits α- and β-glucosidase in fibroblast extracts [Saul, R., Chambers, J. P., Molyneux, R. J., & Elbein, A. D. (1983) *Arch. Biochem. Biophys. 221*, 593–597]. In the present study, castanospermine also proved to be a potent inhibitor of glycoprotein processing by virtue of the fact that it inhibits glucosidase I. Thus, when influenza virus was raised in the presence of castanospermine, at 10 μg/mL or higher, 80–90% of the viral glycopeptides were susceptible to the action of endoglucosaminidase H, whereas in the normal virus 70% of the glycopeptides are resistant to this enzyme. The major oligosaccharide released by endoglucosaminidase H from castanospermine-grown virus migrated like a hexose$_{10}$GlcNAc on a calibrated Bio-Gel P-4 column. This oligosaccharide was characterized as a $Glc_3Man_7GlcNAc$ on the basis of various enzymatic treatments, as well as by methylation analysis of the [2-^{3}H]-mannose-labeled or [6-^{3}H]galactose-labeled oligosaccharide. The presence of three glucose residues in the oligosaccharide was also confirmed by periodate oxidation studies of the [6-^{3}H]galactose-labeled hexose$_{10}$GlcNAc. Castanospermine did not inhibit the incorporation of [^{3}H]leucine or [^{14}C]alanine into protein in MDCK cells at levels as high as 50 μg/mL. In addition, influenza virus produced in the presence of this alkaloid were fully infective and apparently produced in similar amounts to that of control cells, as determined by plaque counts. Castanospermine did, however, cause considerable changes in cell surface properties, since MDCK cells grown in 10 μg/mL castanospermine were able to bind twice as much [^{3}H]concanavalin A as were control cells.

The biosynthesis of the oligosaccharide portion of the N-linked glycoproteins involves the participation of lipid-linked saccharide intermediates and leads ultimately to the formation of a $Glc_3Man_9GlcNAc_2$-pyrophosphoryldolichol (Elbein, 1979; Struck & Lennarz, 1980; Li & Kornfeld, 1979; Spiro et al., 1976; Robbins et al., 1977). The oligosaccharide portion of this lipid is then transferred to protein and is covalently attached to certain asparaginyl residues (Kiely et al., 1976; Czicki & Lennarz, 1977; Das & Heath, 1980; Lingappa et al., 1978; Rodriguez-Boulan et al., 1978). Following the transfer of oligosaccharide to protein, the newly formed glycoprotein undergoes a number of processing reactions, some of which occur in the endoplasmic reticulum and others in the Golgi apparatus (Turco & Robbins, 1976; Elting et al., 1980; Grinna & Robbins, 1979).

The initial processing reactions involve the removal of the three glucose residues catalyzed by two different enzymes. Glucosidase I removes the outermost 1,2-glucose unit (Kilker et al., 1981; Grinna & Robbins, 1980; Ugalde et al., 1978; Chen & Lennarz, 1978), while glucosidase II releases the next two α1,3-linked glucose residues (Grinna & Robbins, 1979; Michael & Kornfeld, 1980; Ugalde et al., 1980; Burns & Touster, 1982). These trimming reactions give a $Man_9GlcNAc_2$-protein which may be the immediate precursor of the high-mannose glycoproteins, or it may be processed further by the removal of four mannose residues by Golgi-bound α1,2-mannosidases to give a Man_5GlcNA_2-protein (Opheim & Touster, 1978; Tabas & Kornfeld, 1979; Kornfeld et al., 1978; Forsee & Schutzbach, 1981; Tulsiani et al., 1982a). A GlcNAc transferase then adds a GlcNAc residue to the α1,3-linked mannose, and this addition apparently signals another mannosidase (mannosidase II) to remove the α1,3- and α1,6-linked mannose residues (Tabas & Kornfeld, 1978, 1979; Harpaz & Schachter, 1980). Then, the other sugars of the complex chains, i.e., GlcNAc, galactose, sialic acid, and fucose, may be added sequentially to form the final complex structure (Hubbard & Ivatt, 1981; Schachter & Roseman, 1980).

Since both the high-mannose and the complex types of oligosaccharides are derived from the same intermediate, i.e., the $Glc_3Man_9GlcNAc_2$-protein, inhibitors that prevent the normal processing of these oligosaccharides should be of considerable value for studies on the roles of those oligosaccharides in glycoprotein function. We recently found that the plant alkaloid castanospermine (1,6,7,8-tetrahydroxy-

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octahydroindolizine) (Hohenschutz et al., 1981) was a potent inhibitor of fibroblast α- and β-glucosidases, as well as almond emulsin β-glucosidase (Saul et al., 1983). In this report, we show that castanospermine also inhibits the processing of the oligosaccharide chain of the influenza viral hemagglutinin. The major oligosaccharide that accumulates in virus grown in this alkaloid is a $Glc_3Man_7GlcNAc_2$. These data are consistent with the finding that castanospermine inhibits glucosidase I in vitro. Since cells grown in castanospermine are able to bind almost twice as much [^{3}H]concanavalin A as are control cells, this alkaloid should be of value for causing alterations in the oligosaccharide chains of membrane and secretory proteins.

Experimental Procedures

Materials. [2-^{3}H]Mannose (10 Ci/mmol), [6-^{3}H]glucosamine (19.7 Ci/mmol), and [6-^{3}H]galactose (14.2 Ci/mmol) were purchased from New England Nuclear Co. Minimal essential medium (MEM; Earle's liquid), calf serum, MEM nonessential amino acid, glutamine, PSN antibiotic mixture, BME vitamin solution, and mycostatin suspension were obtained from Grand Island Biological Co. Pronase was purchased from Calbiochem, trypsin was from Gibco Labs, and jack bean α-mannosidase, *Escherichia coli* β-galactosidase, and beef kidney β-*N*-acetylhexosaminidase were from Sigma Chemical Co. Purified β-*N*-acetylglucosaminidase was a generous gift of Dr. Y. T. Li, Tulane University, and partially purified α1,2-mannosidase was generously provided by Drs. W. T. Forsee and J. Schutzbach, University of Alabama. Endoglucosaminidase H was obtained from Miles Laboratories. The $Glc_3Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ standards were kindly provided by Drs. S. C. Hubbard and P. W. Robbins, Massachusetts Institute of Technology. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) was isolated in 0.3% yield from mature seeds of *Castanospermum australe* by extraction with water and purification by ion-exchange chromatography on Dowex 50W-X8 (Hohenschutz et al., 1981).

Growth and Labeling of Influenza Virus. The NWS strain of influenza virus was grown in MDCK cells as described in previous studies (Sanford et al., 1978). The MDCK cells were grown in small plastic flasks or dishes in modified Eagle's medium containing 10% fetal calf serum. At confluency, the cells were infected with NWS virus, usually at a multiplicity of infection of 75 plaque-forming units (PFU). One hour after infection, various concentrations of castanospermine (1–50 μg/mL) were added to some of the cultures. Following an incubation of 2 h to allow the alkaloid to take effect, [2-^{3}H]mannose (5 μCi/mL culture media) or some other radioactive precursor was added to each culture and then allowed to incubate for another 36–48 h to form mature virus. The presence of mature virus in the media was determined by hemagglutination. When the titer had reached a maximum, the media was removed and centrifuged at low speed to remove cell debris, and the supernatant liquid was then centrifuged at 100000*g* for several hours to pellet the virus. Viral infectivity was measured by the plaque assay. In some experiments, the cells were harvested after 18 h of growth in labeled sugar, rather than waiting for the mature virus to emerge. In these cases, the cell pellets were digested with Pronase, and the membrane glycoproteins, including hemagglutinin, were analyzed.

Preparation and Analysis of Glycopeptides. The mature influenza virus was isolated by centrifugation and was digested exhaustively with Pronase to obtain the glycopeotides. The viral pellets were suspended in 1 mL of 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, containing 0.001 M $CaCl_2$, and 1 mL of a solution of 5 mg/mL Pronase in 50 mM Tris buffer containing 0.001 M $CaCl_2$ was added. The mixtures were incubated for 12–18 h at 37 °C under a toluene atomosphere, and at the end of this time another 1-mL aliquot of Pronase solution was added. After another 12 h of incubation, the reaction mixtures were analyzed for glycopeptides. Glycopeptides were separated on a 1.5 × 150 cm column of Bio-Gel P-4. Aliquots of every other fraction were removed for the determination of radioactivity (Elbein et al., 1982). Since this column did not completely resolve the complex types of glycopeptides from the high-mannose structures, the entire glycopeptide peak was pooled and digested exhaustively with endoglucosaminidase H (Tarentino & Maley, 1974). In these cases, the glycopeptides were placed in 0.2 mL of 50 mM sodium citrate buffer, pH 5.0, and 10 milliunits of enzyme was added. A few drops of toluene were added, and the tubes were incubated for 12 h at 37 °C. At the end of this time, another 10 milliunits of enzyme was added, and the incubations were continued for an additional 12 h. These digests were rechromatographed on the same Bio-Gel P-4 column.

Characterization of Oligosaccharides. (*A*) *Enzymatic Digestions.* Glycopeptides or oligosaccharides were treated with various glycosidases, and the products of these digestions were analyzed on Bio-Gel P-4 columns. In all cases, digestions were done at 37 °C under a toluene atmosphere for varying periods of time. α-Mannosidase digestions were done in 50 mM sodium acetate buffer, pH 5.0, with 0.1 unit of enzyme in a final volume of 0.25 mL. After 12 h of incubation, a second aliquot of enzyme was added, and incubations were continued for 12 h longer. In many cases, digestions with β-galactosidase and β-*N*-acetylhexosaminidase were done together. In these cases, the sample was placed in 0.25 mL of 50 mM sodium acetate buffer, pH 5.0, and 50 milliunits of each enzyme was added. After an overnight incubation, an additional 50 milliunits of the enzymes was added for an additional 12-h incubation. Since the beef kidney β-*N*-acetylhexosaminidase contained some α-mannosidase activity, 10 μg/mL swainsonine was added to these incubations to inhibit this enzyme (Kang & Elbein, 1983a). However, some digestions were done with 5 milliunits of the purified hexosaminidase, and in these cases, swainsonine was omitted. Digestions with α1,2-mannosidase were done in 0.2 mL of 80 mM potassium phosphate buffer, pH 5.5, containing 2 mM $CaCl_2$, 0.2% Triton X-100, and 100 μg of phosphatidylcholine and with 1.8 units of enzyme. A second addition of enzyme was made after 18 h (Forsee & Schutzbach, 1981).

(*B*) *Chromatographic Methods.* Glycopeptides and oligosaccharides were chromatographed on a 1.5 × 150 cm column of Bio-Gel P-4 (200–400 mesh), equilibrated, and run in 1% acetic acid. Glycopeptides and oligosaccharides were also separated by chromatography on columns of concanavalin A-agarose that had been equilibrated with 50 mM Tris buffer, pH 8.5, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (Cummings et al., 1982). The sample was applied to the column, and the column was washed with 20 mL of the above buffer. Elution of bound glycopeptides and oligosaccharides was done batchwise, first with 30 mL of 10 mM methyl α-glucoside and then 30 mL of 100 mM methyl α-mannoside, both in the above buffer. Fractions of 1.0 mL were collected, and aliquots of each fraction were analyzed for their radioactive content.

Thin-layer chromatography of methylated sugars was done on 0.5-mm silica gel F-254 plates in benzene–acetone–water–ammonium hydroxide (50:200:3:1.5). Standard

methylated mannose derivatives were prepared by methylation of ovalbumin and yeast mannan, while standard methylated glucose derivatives were prepared by methylation of laminarin and cellulose. Standard methylated sugars were visualized by charring the plates after spraying with a mixture of H_2SO_4 and ethanol. Radioactive methylated sugars were detected by scraping the plate in 0.5-cm sections and counting in the scintillation counter.

Oligosaccharides (and glycopeotides) were subjected to complete acid hydrolysis in 4 N HCl at 100 °C for 4 h. After removal of the HCl by repeated evaporation and desalting with mixed bed resin (Dowex 1-CO_3^{2-} and Dowex 50-H^+), the neutral sugars were chromatographed on Whatmen 3MM paper in 1-butanol–pyridine–0.1 N HCl (5:3:2). Radioactivity was measured by cutting the papers into 1-cm strips and counting in the scintillation counter, or in some cases by direct scanning with a radiochromatogram scanner.

(*C*) *Methylation Analysis.* Oligosaccharides, purified by passage through Bio-Gel P-4, were subjected to methylation according to the method of Hakomori (Hakomori, 1964) by using the modification described (Sanford & Conrad, 1966). The lyophilized samples were dissolved in 2 mL of dimethyl sulfoxide under N_2 and sonicated with methylsulfinyl carbanion at 40–50 °C for 5-h. The mixture was then chilled, 2 mL of CH_3I was added, and the sample was sonicated for 2 h at 4 °C with the addition of another 2 mL of CH_3I after 1 h. After being allowed to stand at room temperature overnight, the mixtures were passed through columns of Sephadex LH-20 equilibrated with 80% CH_3OH in order to remove dimethyl sulfoxide, methylsulfinyl carbanion, and other salts. The columns were run in 80% CH_3OH. The column eluates were pooled, concentrated to dryness, and hydrolyzed in 2 N H_2SO_4 at 100 °C for 4 h in screw-capped tubes. The methylated sugars were analyzed by thin-layer chromatography.

(*D*) *Periodate Oxidation Studies.* The [6-^{3}H]galactose-labeled oligosaccharide produced in the presence of castanospermine and released by endoglucosaminidase H was treated with sodium periodate to determine the proportion of glucose that was susceptible to oxidation. The oligosaccharide (50 000 cpm) was placed in a screw-capped tube in 0.2 mL of 50 mM sodium acetate buffer, pH 5.0, and 10 μmol of sodium periodate was added. The tube was placed in the dark at 5 °C for 24 h, and another 10 μmol of periodate was then added for another 24-h incubation. The excess periodate was destroyed by the addition of ethylene glycol, and aldehydes were reduced by the addition of $NaBH_4$. The mixture was acidified with acetic acid and passed through columns of Dowex 50-H^+ to remove Na^+. Borate was removed by repeated evaporation in the presence of methanol. The sample was hydrolyzed in 3 N HCl at 100 °C for 2 h, concentrated to dryness several times to remove HCl, and desalted with mixed bed ion-exchange resin (Dowex 50-H^+ and Dowex 1-CO_3^{2-}). The radioactive products were identified by paper chromatography.

Assay of Liver Processing Glucosidases. Glucosidase I and glucosidase II were assayed with rabbit liver particulate enzymes by using [^{3}H]mannose-labeled $Glc_3Man_9GlcNAc_2$, $Glc_2Man_9GlcNAc_2$, and $Glc_1Man_9GlcNAc_2$. These substrates were made by incubating soybean suspension cells (Hori et al., 1982) or MDCK cells (Pan & Elbein, 1982) in [2-^{3}H]-mannose for short periods of time and then isolating the lipid-linked oligosaccharides. The oligosaccharides were released by mild acid hydrolysis, separated from each other, and purified by repeated chromatography on columns of Bio-Gel P-4. One rabbit liver was homogenized in a Waring blender in 100 mL of 50 mM Tris buffer, pH 7.5. The homogenate was filtered through several layers of chesecloth and centrifugated at low speed (1000*g*) to remove debris. The supernatant liquid was then centrifuged at 100000*g* for 1 h, and the pellets were resuspended in 50 mM Tris buffer and used as the particulate enzyme source. For assay of the enzyme, the reaction mixture contained the following components in a final volume of 0.2 mL: 50 mM Tris buffer, pH 7.0, 0.1% Triton X-100, 5000 cpm of [^{3}H]mannose-labeled $Glc_3Man_9GlcNAc_2$ (or $Glc_2Man_9GlcNAc_2$), 50 μL of microsomal enzyme (about 1–2 mg of protein), and 50 μg of castanospermine. A few drops of toluene were added to retard bacterial growth, and mixtures were incubated for 6 h. The removal of glucose residues was assayed by chromatographing the mixtures on Bio-Gel P-4 columns and measuring changes in migration of processed oligosaccharides.

Assay of Protein Synthesis in Influenza Virus Infected MDCK Cells. The effect of castanospermine on protein synthesis was examined in infected MDCK cells. MDCK cells were grown to confluency in six-well Linbro dishes, and the cells were infected with influenza virus. One hour after infection, 5 or 50 μg/mL castanospermine was added to some of the cells while others served as controls. After 2 h of incubation to allow the alkaloid to take effect, 10 μCi of [^{3}H]leucine or [^{14}C]alanine was added, and the cells were allowed to incubate for various times up to 18 h. At the times indicated in the figures, the media was removed by aspiration, and the monolayers were washed 3–4 times with phosphate-buffered saline (PBS). One milliliter of PBS was added to each well, and the cells were dislodged by scraping and quantitatively transferred to tubes. Each well was rinsed with another 1 mL of PBS, and this rinse was added to the tube. Fifty microliters (~1–2 mg of protein) of rat liver microsomes was added to each tube to aid in the precipitation, and 1 mL of 25% trichloroacetic acid was then added. The tubes were allowed to stand at room temperature for about 1 h with occasional shaking and were then cooled, and the precipitate was isolated by centrifugation. The precipitate was washed 2 times with 5% Cl_3CCOOH, before being dissolved in Protosol for scintillation counting.

Determination of Infectious Particles. Confluent monolayers of MDCK cells were grown in tissue culture dishes (10 × 35 mm) and inoculated with 0.2 mL of influenza A/NWS/33 virus (8.4×10^3 PFU/mL) for 1 h with occasional shaking. The inoculum was removed and each monolayer received 2 mL of agar overlay medium with or without castanospermine by using the previously described method (Tobita et al., 1975). After 3 days at 34 °C in a CO_2 incubator, monolayers were fixed with Carnoy's solution, the agar overlay was removed, and the monolayers were stained with 1% crystal violet in 50% ethanol. Plaques were counted, and the mean area was determined.

Results

Effect of Castanospermine on the Formation of Complex and High-Mannose Oligosaccharides. Since castanospermine was found to be a potent inhibitor of both α-glucosidase and β-glucosidase in fibroblast extracts (Saul et al., 1983), we tested this alkaloid to determine whether it would inhibit the processing of the influenza viral hemagglutinin. In this initial experiment, infected cells were incubated for 2 h in the absence and presence of castanospermine (5 μg/mL) and then labeled for 36 h in the presence of [2-^{3}H]mannose. The mature virus was isolated from the medium by ultracentrifugation and digested exhaustively with Pronase to obtain the glycopeptides, and these glycopeptides were isolated by chromatography on

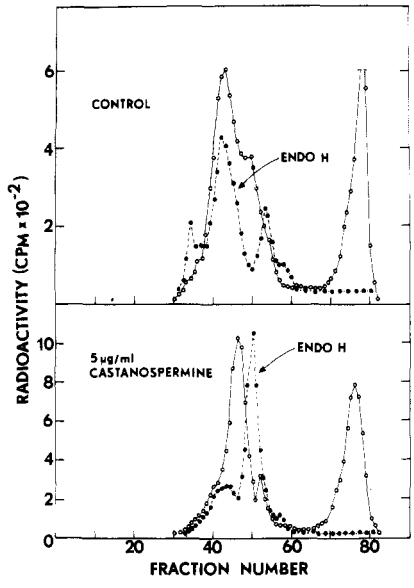

FIGURE 1: Effect of castanospermine on the oligosaccharide composition of influenza virus hemagglutinin. Infected MDCK cells were placed in 5 μg/mL castanospermine, and after 2 h of incubation, 50 μCi of [2-^{3}H]mannose was added. Control flasks were done in the absence of alkaloid. After an incubation of 36 h, the virus particles were isolated from the media by ultracentrifugation and were digested exhaustively with Pronase. The glycopeptides were separated on columns of Bio-Gel P-4 (1.5 × 150 cm) (open circles). The entire glycopeptide peak (fractions 30–60) was pooled, digested with endoglucosaminidase H, and rechromatographed on the same Bio-Gel column (closed circles). Aliquots of each fraction were removed for the determination of radioactivity. Upper profiles are those of control cells before and after digestion with endoglucosaminidase H while lower profiles are those of castanospermine-raised virus.

columns of Bio-Gel P-4. Figure 1 shows the radioactive profiles of these glycopeptides from virus raised in the absence (upper) or presence (lower) of castanospermine (open circles). Since this column did not give good resolution of the complex from the high-mannose structures, the entire glycopeptide peak was pooled, digested with endoglucosaminidase H, and rechromatographed on the Bio-Gel P-4 column (closed circles).

It can be seen from the upper profiles of Figure 1 that the glycopeptides from control virus emerged from the column as a rather broad peak between fractions 38 and 46, with a shoulder at fractions 46–54. However, after digestion with endoglucosaminidase H, the glycopeptides (and liberated oligosaccharides) could be resolved into two distinct peaks of radioactivity. The first peak, which contained about 70% of the total incorporated radioactivity, eluted in the same position as the original glycopeptide (fractions 38–46) and was therefore not susceptible to digestion by endoglucosaminidase H. However, peak 2 was susceptible to digestion by endoglucosaminidase H since its position was shifted after treatment with this enzyme, and it now eluted from the Bio-Gel column at a later position (fractions 51–55). On the basis of previous studies, peak 1 represents the complex types of glycopeptides that are resistant to endoglucosaminidase H, whereas peak 2 represents the high-mannose oligosaccharides that have been released by this enzyme (Elbein et al., 1982). Thus, peak 1 was sensitive to digestion by a mixture of β-galactosidase and β-*N*-acetylhexosaminidase (as evidenced by a change in migration on Bio-Gel P-4), but was resistant to digestion by α-mannosidase. Peak 2, on the other hand, was sensitive to digestion by α-mannosidase and gave free mannose and a ManGlcNAc disaccharide, but this peak was resistant to a mixture of β-galactosidase and β-*N*-acetylhexosaminidase.

The lower profiles of Figure 1 demonstrate that when the virus was raised in the presence of 5 μg/mL castanospermine, the glycopeptide profile was very different from that of the controls. In this case, the glycopeptide peak was not as broad as that of controls and mostly eluted between fractions 40 and 50. Furthermore, when this glycopeptide peak was treated with endoglucosaminidase H, more than 80% of the radioactivity was shifted to a slower migrating peak that was intermediate between the complex and the high-mannose peaks of the control. The characterization of this endoglucosaminidase H sensitive oligosaccharide is detailed in later sections and shows that this oligosaccharide is a $Glc_3Man_7GlcNAc$. The remaining 20% of the radioactivity in the castanospermine-derived glycopeptides was in complex chains that were resistant to the action of endoglucosaminidase H. Increasing amounts of castanospermine in the medium resulted in decreased amounts of radioactivity in the complex chains. Thus, at 50 μg/mL alkaloid, more than 90% of the viral glycopeptides became susceptible to endoglucosaminidase H. However, even at fairly high castanospermine concentrations (200 μg/mL), it was not possible to decrease the amount of radioactivity in the complex chains below about 5–8% of the controls.

Effect of Castanospermine Concentration on Oligosaccharide Structure. In order to determine the optimum concentration of castanospermine for inhibition of complex chains, infected cells were incubated for several hours in the presence of various concentrations of alkaloid (0.5–10 μg/mL), and then [2-^{3}H]mannose was added to label the glycoproteins. In this experiment, the viral glycoproteins were isolated from the cell membrane rather than waiting 36 h for the release of mature virus. Thus, after 18 h of incubation, the media were removed by aspiration, and the cell monolayers were washed exhaustively with phosphate-buffered saline. The cells, containing the synthesized viral glycoproteins, were removed from the plates by scraping and were digested exhaustively with Pronase to obtain the radioactive glycopeptides. These glycopeptides were separated on Bio-Gel P-4 columns (Figure 2, left). The glycopeptide peaks were then pooled, digested with endoglucosaminidase H, and rechromatographed on Bio-Gel P-4 (Figure 2, right). It can be seen that as the castanospermine concentration was increased, less and less radioactivity was found in the complex chains (i.e., in endoglucosaminidase H resistant). Instead, more and more of this radioactivity migrated in the area intermediate between the complex and high-mannose structures. This intermediate peak was entirely susceptible to digestion by endoglucosaminidase H, and its position was shifted to a new and slower moving peak by this enzyme that corresponded to a $hexose_{10}GlcNAc$. Although the resolution of these oligosaccharides was not especially good on this P-4 column (i.e., 10 fractions separated $Glc_3Man_9GlcNAc_2$ from $Man_5GlcNAc_2$), the time required for one column run was only 24 h, and the reproducibility from sample to sample was very good. Thus, with large numbers of samples it was more feasible to separate the oligosaccharides initially on this column and further characterize them on a long, calibrated Bio-Gel P-4 column. The data in Figure 2 do show that increasing amounts of the alkaloid result in decreased amounts of [^{3}H]mannose in the complex chains. Thus, at 10 μg/mL alkaloid 80–90% of the glycopeptides are sensitive to endoglucosaminidase H, and only small amounts of complex chains are observed. However, as indicated above, even at very high alkaloid concentrations, it was not possible to completely inhibit complex chains.

These alterations in oligosaccharide structure at various concentrations of castanospermine could also be observed when the glycopeptides were separated on columns of concanavalin

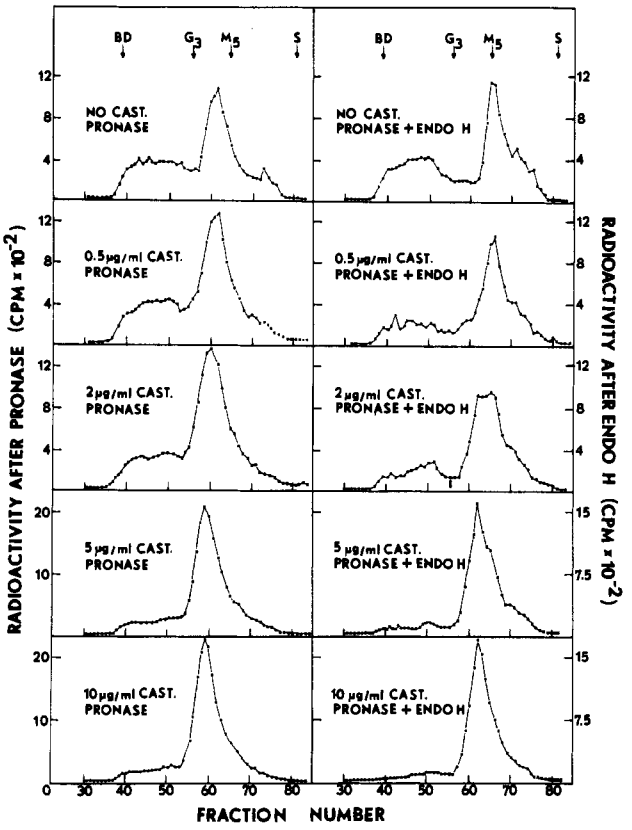

FIGURE 2: Effect of castanospermine concentration on oligosaccharide profiles. MDCK cells were grown to confluency in six-well Linbro dishes, and the cells were infected with virus. One hour later, various amounts of castanospermine were added. After an incubation of 2 h, 10 μCi of [2-^{3}H]mannose was added to each well, and the incubations were continued for an additional 17 h. The media were removed by aspiration, and the monolayers were washed well with phosphate-buffered saline. The cells were removed from the plates by scraping, isolated by centrifugation, and digested exhaustively with Pronase. The Pronase digests were chromatographed on Bio-Gel P-4 (profiles on left side). The glycopeptide peak from each run was pooled, digested with endoglucosaminidase H, and rechromatographed on Bio-Gel P-4 (right side). Standards are blue dextran (BD), $Glc_3Man_9GlcNAc_2$ (G_3), $Man_5GlcNAc_2$ (M_5), and stachyose (S).

A–agarose. On this column, tri- and tetraantennary complex chains emerge in the wash, biantennary complex chains bind weakly and are eluted at 10 mM methyl α-glucoside, and hybrid and high-mannose structures bind more tightly and require 100 mM methyl α-mannoside for elution (Cummings et al., 1982). In the control virus, the ratio of radioactivity in the various glycopeptide fractions was as follows: wash, 64.2%; 10 mM methyl α-glucoside, 10.9%; 100 mM methyl α-mannoside, 24.9%. However, in the glycopeptides from castanospermine-grown virus (10 μg/mL), the ratio of radioactivity in the various fractions was as follows: wash, 22%; 10 mM methyl α-glucoside, 5.4%; 100 mM methyl α-mannoside, 72%. This experiment correlates fairly well with those shown in Figures 1 and 2. However, it should be pointed out that the extent of inhibition of complex chains at a given castanospermine concentration varied somewhat (10–20%) from experiment to experiment. These differences may reflect variations in the state of the cells at the time of addition of the alkaloid, or they could be due to slight differences in the number of cells or in the state of viral infection. Usually, 10 μg/mL castanospermine decreased complex chains to 15–20% of control values.

Characterization of the Oligosaccharide Produced in the Presence of Castanospermine. The endoglucosaminidase H released oligosaccharide produced in the presence of 10 μg/mL

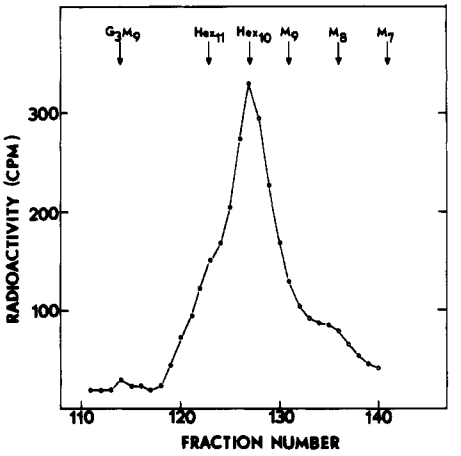

FIGURE 3: Molecular sizing of oligosaccharide produced in the presence of castanospermine. The oligosaccharide released by endoglucosaminidase H from virus grown in 10 μg/mL castanospermine was chromatographed on a 1.5 × 200 cm column of Bio-Gel P-4. One-milliliter fractions were collected, and an aliquot of each fraction was removed for the determination of radioactivity. Standard oligosaccharides are $Glc_2Man_9GlcNAc$ (Hex_{11}), $Glc_1Man_9GlcNAc$ (Hex_{10}), $Man_9GlcNAc$ (M_9), $Man_8GlcNAc$ (M_8), and $Man_7GlcNAc$ (M_7).

castanospermine was isolated from the Bio-Gel P-4 columns shown in Figure 1. This oligosaccharide was applied to a long calibrated Bio-Gel P-4 column to obtain an accurate size determination. Figure 3 shows that the majority of the radioactivity eluted from this column in the same position as the $hexose_{10}GlcNAc$ standard and was clearly distinct from $Man_9GlcNAc$ or $Man_8GlcNAc$ standards. This peak did have a shoulder eluting earlier in the $hexose_{11}GlcNAc$ and $hexose_{12}GlcNAc$ areas, which is probably the result of incomplete processing of the α1,2-linked mannose residues. In this case, the peak also had a shoulder in the $Man_9GlcNAc$ area, but this shoulder was not seen at higher castanospermine concentrations, indicating that it was due to incomplete inhibition of the processing glucosidases.

The $hexose_{10}GlcNAc$ peak (fractions 125–130) was treated with a number of enzymes to aid in its identification. In each case after treatment with the enzyme, the reaction mixture was applied to the Bio-Gel P-4 column to determine the nature of the reaction products. Figure 4 presents the results of this experiment. The upper profile is that of untreated oligosaccharide and indicates a migration like that of a $hexose_{10}GlcNAc$. As shown by profile B, treatment with α-mannosidase released about 35% of the radioactivity as free mannose and gave rise to a new oligosaccharide that migrated in the position of a $hexose_7GlcNAc$. However, the original oligosaccharide was resistant to a mixture of β-galactosidase and β-*N*-acetylhexosaminidase as shown in profile C. The oligosaccharide was also resistant to digestion by the specific α1,2-mannosidase (data not shown). The release of three mannose residues (35% of the radioactivity) by jack bean α-mannosidase is consistent with the proposed structure $Glc_3Man_7GlcNAc$.

The [^{3}H]mannose-labeled $hexose_{10}GlcNAc$ was subjected to complete methylation, and the methylated sugars were identified by thin-layer chromatography. Figure 5 shows the chromatogram of the radioactive methylated mannose derivatives as compared to standards prepared from ovalbumin and yeast mannan. It can be seen that radioactive peaks corresponded to 2,4-dimethylmannose, 3,4,6-trimethylmannose, 2,4,6-trimethylmannose, and 2,3,4,6-tetramethylmannose. These data indicate the presence of 3,6-disubstituted mannose, 2-linked mannose, 3-linked mannose, and terminal mannose

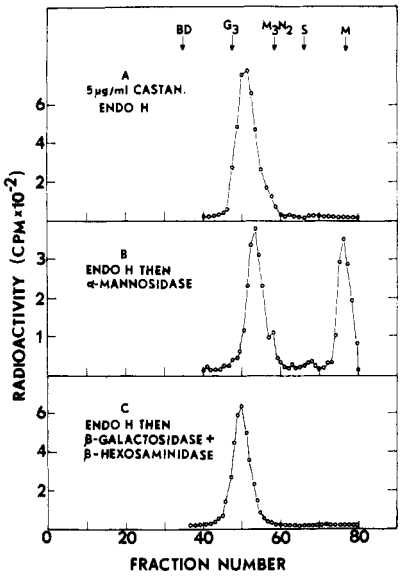

FIGURE 4: Partial characterization of hexose$_{10}$GlcNAc from castanospermine-grown influenza virus. The endoglucosaminidase H released oligosaccharide from virus grown in 50 μg/mL alkaloid was chromatographed on Bio-Gel P-4 (A). An aliquot of this oligosaccharide was treated with α-mannosidase, and the sample was rechromatographed on Bio-Gel P-4 (B). Another aliquot of the oligosaccharide was treated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase, and this sample was run on the Bio-Gel P-4 column (C). Standards are blue dextran (BD), $Glc_3Man_9GlcNAc_2$ (G_3), $Man_3GlcNAc_2$ (M_3N_2), stachyose (S), and mannose (M).

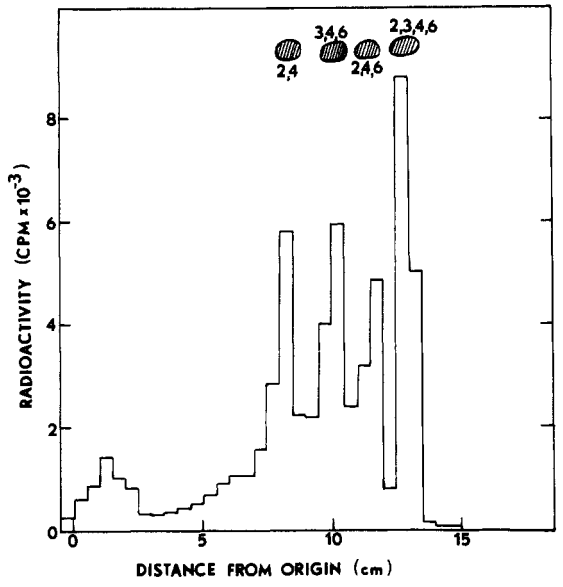

FIGURE 5: Methylation analysis of the [^{3}H]mannose-labeled oligosaccharide from castanospermine-grown virus. The endoglucosaminidase H released oligosaccharide was isolated from Bio-Gel P-4 and subjected to complete methylation. After acid hydrolysis the methylated sugars were analyzed by thin-layer chromatography. Radioactive sugars were located by scraping plates in 0.5-cm sections and counting in the scintillation counter. Standards shown are 1 = 2,4-dimethylmannose, 2 = 3,4,6-trimethylmannose, 3 = 2,4,6-trimethylmannose, and 4 = 2,3,4,6-tetramethylmannose.

in the approximate ratio of 2:2:1:2 (actual ratio 0.84:0.87:0.57:1.0). The presence of 3-linked mannose is unusual and suggests that one mannose is substituted with 3-linked glucose. The data are consistent with the expected structure for the $Glc_3Man_9GlcNAc$ (see Figure 11).

The hexose$_{10}$GlcNAc$_2$-peptide could also be labeled by growing the virus-infected cells in the presence of [6-^{3}H]-galactose plus castanospermine. Figure 6 (lower profile) shows

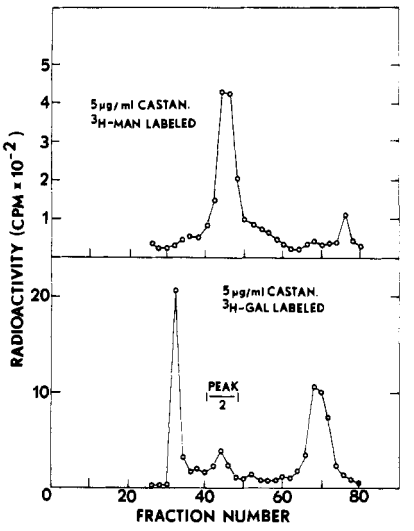

FIGURE 6: Isolation of [6-^{3}H]galactose-labeled hexose$_{10}$GlcNAc from castanospermine-grown virus. Infected MDCK cells were placed in 5 μg/mL castanospermine and then labeled with either 50 μCi of [2-^{3}H]mannose (upper profile) or [6-^{3}H]galactose (lower profile). The glycopeptides were chromatographed on Bio-Gel P-4, then treated with endoglucosaminidase H, and rechromatographed on Bio-Gel P-4. The glycopeptides from both sets of cells migrated in the same position on these columns as did the liberated oligosaccharides.

that a glycopeptide peak (peak 2) eluted from the Bio-Gel P-4 column in the same position as the mannose-labeled glycopeptide. Furthermore, after digestion with endoglucosaminidase H, both the mannose-labeled and the galactose-labeled oligosaccharides migrated in the same position as the hexose$_{10}$GlcNAc standard. The galactose-labeled oligosaccharide was subjected to complete acid hydrolysis, and the radioactive sugars were identified by paper chromatography in a solvent system that clearly resolves glucose, galactose, and mannose. Essentially all of the radioactivity migrated with the glucose standard, and only a trace of radioactivity was in the trailing edge that could be galactose (data not shown). It can be seen in Figure 6 (lower profile) that two other galactose-labeled peaks were present in this preparation. These peaks have not been identified but are also seen in the normal virus grown in galactose (but in this case peak 2 is absent). Since the first peak is not seen to any extent in the mannose-labled glycopeptides, it is probably not due to complex chains (although there are probably small amounts of these structures present). Thus peak 1 may be due to proteoglycans or other glucose-containing polymers. Peak 3 is apparently a low molecular weight component, perhaps free galactose.

The galactose-labeled oligosaccharide was subjected to complete methylation, and the methylated sugars were identified by thin-layer chromatography as shown in Figure 7. Although the 3,4,6-trimethylglucose did not separate well from the 2,4,6-trimethylglucose, the radioactive profile clearly shows two major peaks in the area of 2,4,6-trimethylglucose (3-linked glucose) and 2,3,4,6-tetramethylglucose (terminal glucose), as well as a shoulder in the area of 3,4,6-trimethylglucose (2-linked glucose). The ratio of radioactivity in 3-linked to terminal glucose was approximately 1:1, whereas that in the 2-linked to 3-linked glucose was about 0.6:1.

Since the methylation of glucose-labeled oligosaccharide strongly suggested the presence of three glucose residues but was not absolutely conclusive because of the poor resolution of the methylated glucoses, we confirmed the presence of three glucose residues by periodate oxidation studies. The oligosaccharide was labeled in the presence of [6-^{3}H]galactose for 18 h in the presence of castanospermine and was purified, after

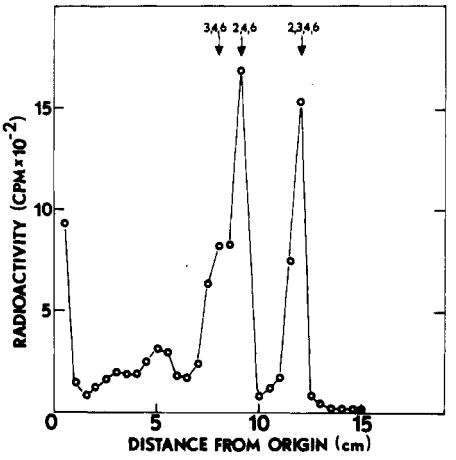

FIGURE 7: Methylation analysis of [6-^{3}H]galactose-labeled hexose$_{10}$GlcNAc. The galactose-labeled hexose$_{10}$GlcNAc from the above Bio-Gel columns was subjected to complete methylation, and after acid hydrolysis, the methylated sugars were analyzed by thin-layer chromatography. Standard sugars are 3,4,6 = 3,4,6-trimethylglucose, 2,4,6 = 2,4,6-trimethylglucose, and 2,3,4,6 = 2,3,4,6-tetramethylglucose.

endoglucosaminidase H digestion, on Bio-Gel P-4 columns as described above. This oligosaccharide was treated with sodium periodate, then reduced with $NaBH_4$, and hydrolyzed to release the labeled products. These labeled products were identified by paper chromatography. Two radioactive bands were detected on the papers: one corresponded to glucose and contained 7563 cpm and the other to glycerol and contained 14468 cpm. These data indicate the presence of three glucose residues, one of which is resistant to periodate oxidation (3 linked), and two that are susceptible to oxidation (2 linked and terminal). Taken together with the above data, the results indicate a $Glc_3Man_7GlcNAc$ structure as presented in Figure 11. This structure is consistent with the structure proposed for the $Glc_3Man_9GlcNAc_2$ isolated from the lipid-linked oligosaccharides (Li & Kornfeld, 1979; Robbins et al., 1977) and also with our observation that castanospermine inhibits glucosidase I, in vitro (see below).

Effect of Castanospermine on the Activity of Glucosidase I. Since castanospermine appeared to block processing in vivo by preventing the removal of the glucose residues, it seemed likely that it was inhibiting glucosidase I. Thus, we tested this alkaloid, in vitro, to determine whether it would inhibit this enzyme as well as the glucosidase II. As substrates in these reactions, we used [^{3}H]mannose-labeled $Glc_3Man_9GlcNAc_2$, $Glc_2Man_9GlcNAc_2$, and $Glc_1Man_9GlcNAc_2$. These oligosaccharides were obtained from the lipid-linked oligosaccharides of soybean suspension cells incubated in [2-^{3}H]-mannose (Hori et al., 1982). These oligosaccharides were separated and purified by several passages through a long, calibrated column of Bio-Gel P-4. The source of the processing glucosidases was a rabbit liver microsomal preparation (Ugalde et al., 1978). After incubation of the enzyme with the oligosaccharide substrates for 6 h, the reaction mixtures were applied to the Bio-Gel P-4 column to determine whether any change in oligosaccharide size had occurred. Figure 8 presents the results of one such experiment where $Glc_3Man_9GlcNAc_2$ was incubated with the microsomal enzyme in the absence (upper profile) and presence (lower profile) of 50 μg/mL castanospermine. It can be seen from the upper profile that the liver enzyme did degrade this $Glc_3Man_9GlcNAc_2$ to smaller oligosaccharides that migrated with $Glc_2Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ standards. Thus, this enzyme preparation does contain glucosidase I and glucosidase

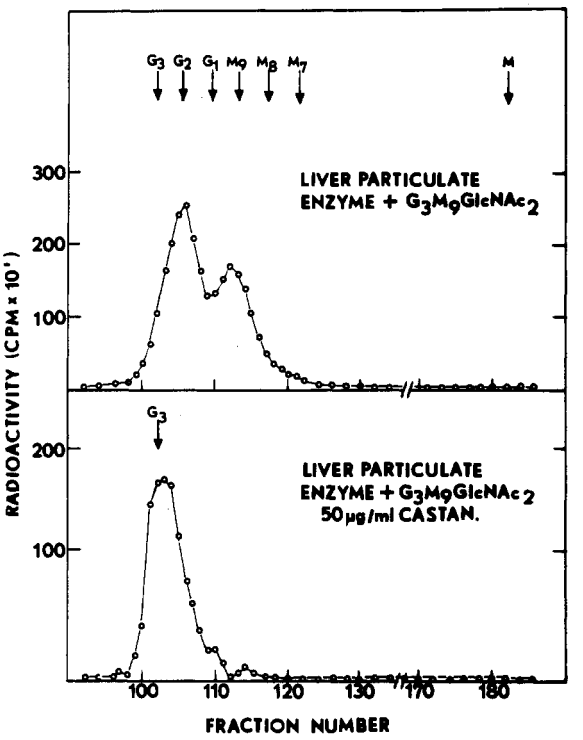

FIGURE 8: Effect of castanospermine on the activity of glucosidase I. The $Glc_3Man_9GlcNAc_2$ oligosaccharide, labeled with [^{3}H]mannose, was incubated for 6 h in 50 mM Tris buffer, pH 7.0, containing 0.1% Triton X-100 and 50 μL of liver microsomal enzyme. The reaction mixture was chromatographed on Bio-Gel P-4 to analyze the products of enzyme action (upper profile). A similar incubation of liver enzyme and $Glc_3Man_9GlcNAc_2$ was done in the presence of 50 μg/mL castanospermine and this reaction mixture was chromatographed on the Bio-Gel column (lower profile). Standards are $Glc_3Man_9GlcNAc_2$ (G_3), $Glc_2Man_9GlcNAc_2$ (G_2), etc.

II activities as reported previously (Ugalde et al., 1980). The lower profile shows that in the presence of castanospermine, the removal of glucose residues from the $Glc_3Man_9GlcNAc_2$ was completely abolished, indicating that this alkaloid was an inhibitor of glucosidase I. The alkaloid was also tested as an inhibitor of the removal of glucose from $Glc_2Man_9GlcNAc_2$ (glucosidase II) with the liver enzyme. Castanospermine also appeared to inhibit glucosidase II, but in this case the inhibition was substantially less than that seen for glucosidase I. However, because of limitations in the amount of $Glc_2Man_9GlcNAc_2$, it was not possible to further quantitate the amount of inhibition in this case.

Effect of Castanospermine on Protein Synthesis. Since castanospermine appeared to be a potentially valuable inhibitor of glycoprotein processing, it was important to determine whether it inhibited protein synthesis in cultured cells. Thus, influenza virus infected MDCK cells were incubated for 2 h in the absence or presence of castanospermine (5 or 50 μg/mL), and then [^{3}H]leucine or [^{14}C]alanine was added. After incubation with the isotopes for various times, from 15 min to 18 h, the monolayers were washed well with phosphate-buffered saline to remove media components, and the cells were removed from the plates by scraping. The scraped cells were placed in tubes and extracted with trichloroacetic acid. The resulting pellet was washed with Cl_3CCOOH and then solubilized in Protosol for counting. The results of this experiment are shown in Figure 9. It can be seen from Figure 9 (left panel) that castanospermine had no effect on the incorporation of [^{14}C]alanine into protein, and in fact, some stimulation of this incorporation was noted at the highest castanospermine concentration. The significance of this stimulation is not clear

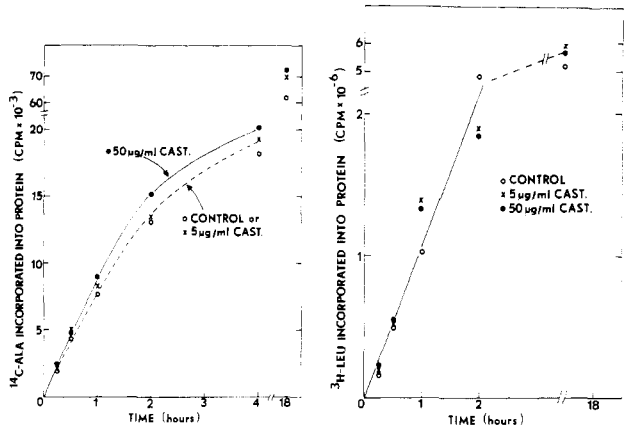

FIGURE 9: Effect of castanospermine on the incorporation of amino acids into protein. MDCK cells were grown in six-well Linbro dishes and infected with influenza virus as described. Castanospermine (5 or 50 μg/mL) was added to some wells, and after a 2-h incubation, 10 μCi of [^{3}H]leucine or [^{14}C]alanine was added, and the incubations were continued. At the times shown, the media were removed by aspiration, and the monolayers were washed well with the saline solution. The cells were released from the plates by scraping, placed in tubes, and extracted at room temperature with 8% Cl_3CCOOH. The tubes were mixed occassionally by vortexing. Fifty microliters (about 1–2 mg of protein) of liver microsomal fraction was added to aid in the precipitation of protein. After being cooled, the precipitate was isolated by centrifugation and washed twice with 5% Cl_3CCOOH. The pellet was solubilized in 1 mL of Protosol and placed in scintillation vials for the determination of radioactivity.

Table I: Effect of Castanospermine on Infectivity of Influenza Virus

castanospermine concn during growth (μg/mL)	plaque no. (% of control)[a]	plaque area (mm^2)
0	100	0.95
1	107	2.54
10	122	2.54
20	98	2.84
40	178	3.14

[a] 100% of control represents 42 plaques per dish.

at this time. Figure 9 (right panel) shows the effect of castanospermine concentration on the incorporation of [^{3}H]leucine into protein. In this experiment, there was more variation between the points, perhaps because the specific activity of the leucine is much higher and because of the difficulties of counting ^{3}H samples in Protosol. Nevertheless, the results indicate that little if any inhibition of leucine incorporation into protein occurred, except perhaps at the highest castanospermine concentrations and the longest times.

Effect of Castanospermine on the Infectivity and Release of Influenza Virus. Since castanospermine prevented the formation of complex chains on the viral glycoproteins, it was important to determine whether this alteration would affect the release or infectivity of the viral particles. Thus, MDCK cells were infected with virus and then placed in various concentrations of castanospermine. The number of virus particles released from the cells and the infectivity of these particles was determined by plaque assay as shown in Table I. It can be seen that castanospermine did not affect the number of plaques found in the infected cells. In fact, at higher concentrations of alkaloid, there may be some increase in the plaque number. Interestingly enough, castanospermine also appeared to cause an increase in the size of the plaque. It is not clear what the significance of this observation is at this time. At any rate, virus raised in the presence of this alkaloid appear to retain their biological activity.

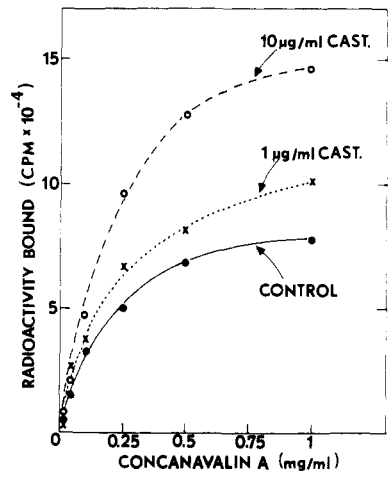

FIGURE 10: Effect of castanospermine on the binding of [^{3}H]-concanavalin A to MDCK cells. Cells were plated in multiwell dishes at a density of about 1 × 10^{4} cells/mL and grown for 3–4 days in the presence of 1 or 10 μg/mL castanospermine. Control cells were grown under the same conditions but without alkaloid. Confluent monolayers of control and castanospermine-grown cells were incubated in phosphate-buffered saline with various amounts of [^{3}H]concanavalin A. After an incubation of 30 min at 37 °C, the media were removed by aspiration, and the monolayers were washed 5 or 6 times with the saline solution to remove unbound concanavalin A. The monolayers were solubilized with 0.5% sodium dodecyl sulfate and placed in scintillation vials for the determination of radioactivity.

Effect of Castanospermine on Properties of Cell Surface Glycoproteins. Since castanospermine blocks the processing of glycoproteins by inhibiting glucosidase I, one might anticipate that this alkaloid would alter the properties of the mammalian cell surface. In order to examine this question, we studied the binding of [^{3}H]concanavalin A to MDCK cells grown in the absence and presence of castanospermine. Thus, MDCK cells were plated at low density (1 × 10^{4} cells/mL) and grown for 3 or 4 days in the presence of 1 or 10 μg/mL castanospermine. Control cells were grown under the same conditions, but without alkaloid. Once the cells had reached confluency, the monolayers were washed with phosphate-buffered saline and then incubated in saline with various concentrations of [^{3}H]concanavalin A for 30 min at 37 °C. The media were then removed by aspiration, and the monolayers were washed 5 or 6 times with saline. The cell monolayers were solubilized with 0.5% sodium dodecyl sulfate and placed in vials for the determination of the amount of concanavalin A bound. Figure 10 presents the results of this experiment. It can be seen that the cells grown in the presence of 10 μg/mL castanospermine were able to bind almost twice as much of the radioactive lectin as were control cells. To be certain that these differences were not due to nonspecific binding, binding studies were also done in the presence of methyl α-mannoside. This lectin blocks the specific binding of concanavalin A to high-mannose sites and allows one to measure the nonspecific binding. In the presence of 100 mM methyl α-mannoside, the binding of radioactivity was about the same in control and castanospermine-grown cells and was about 20% of that seen in the normal control cells. Thus, the 2-fold increase in castanospermine-grown cells was due to specific binding of the lectin. The difference in concanavalin A binding was also not due to differences in the number of cells on these plates since the monolayers were all confluent and since castanospermine does not inhibit cell growth at the levels used in these studies (data not shown). Since the studies

shown in Figure 10 were done at saturating levels of concanavalin A, these results suggest that cells grown in alkaloid have an increased number of concanavalin A binding sites, i.e., an increased number of high-mannose oligosaccharides at their cell surface.

Discussion

Inhibitors of glycoprotein biosynthesis and processing can be valuable tools to study the steps involved in oligosaccharide formation and also to examine the role of complex or high-mannose oligosaccharides in glycoprotein function. In previous studies, we found that the plant alkaloid swainsonine (1,2,8-trihydroxyoctahydroindolizine) was a potent inhibitor of glycoprotein processing in a variety of mammalian cell lines (Elbein et al., 1981). Swainsonine was shown to specifically inhibit mannosidase II, the processing enzyme that removes the α1,3- and α1,6-linked mannose residues from the $GlcNAcMan_5GlcNAc_2$-protein (Tulsiani et al., 1982b). Thus, the oligosaccharide portion of the vesicular stomatitis viral G protein (Kang & Elbein, 1983b) or that of the influenza viral hemagglutinin (Elbein et al., 1982) produced in the presence of swainsonine appeared to be hybrid types of oligosaccharides having a high-mannose branch as well as one or two complex chains. When mammalian cells were grown for several days in the presence of this alkaloid, the cells showed considerable alterations at their cell surface. Thus, these cells showed a considerable increase in the binding of [^{3}H]concanavalin A and a decrease in the binding of ^{3}H-labeled wheat germ agglutinin.

Since swainsonine is a trihydroxylated alkaloid and specifically inhibits certain α-mannosidases, it seemed likely that the stereochemistry of this alkaloid was important for inhibition. On the basis of these studies with swainsonine, it also appeared likely that other hydroxylated alkaloids with different stereochemistry would inhibit other glycosidases. Thus, castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) was found to be a potent inhibitor of almond emulsin β-glucosidase but was without effect on a number of other glycosidases, including yeast α-glucosidase (Saul et al., 1983). Interestingly, in fibroblast extracts, castanospermine inhibited both β-glucosidase and α-glucosidase. Since castanospermine inhibited glucosidase activity, it was tested as an inhibitor of processing of the influenza viral hemagglutinin. The results indicate that when the virus is grown in the presence of 10 μg/mL castanospermine, 70–90% of the radioactive glycopeptides become susceptible to the action of endoglucosaminidase H, whereas in normal virus, only 25–30% of the glycopeptides are susceptible to this enzyme. At higher alkaloid concentrations, as much as 90% of the glycopeptides become sensitive to endoglucosaminidase H. The endoglucosaminidase H released oligosaccharide produced in the presence of castanospermine was characterized by a variety of enzymatic and chemical methods including methylation, as a $Glc_3Man_7GlcNAc$. The proposed structure of this oligosaccharide is shown in Figure 11. These data fit the observation that castanospermine inhibits glucosidase I in vitro and therefore should prevent the removal of any glucose residues. Some mannose processing can occur even without the removal of glucose, and apparently the α1,2-mannosidase can still remove the two α1,2-linked mannoses from the $Glc_3Man_9GlcNAc_2$-protein. Some processing of glucose-containing oligosaccharides has been observed in other systems with the identification of $Glc_2Man_7GlcNAc$, $GlcMan_7GlcNAc$, and so on (Kornfeld et al., 1978; Datema et al., 1982).

It is interesting to point out that even at high concentrations of castanospermine (50 μg/mL), it was not possible to completely prevent the formation of complex types of oligosaccharides. Thus, as the castanospermine concentration was increased, there was a steady decline in the amount of complex structures to a level of about 10% of the normal, and then the decline leveled off. These studies suggest that either some of the glucosidase I is resistant to castanospermine or there is another pathway of processing to complex chains which perhaps does not involve glucosylated intermediates. It is interesting to note that in these studies with castanospermine, little or no high-mannose oligosaccharides (i.e., $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$) were observed in inhibited cells even though the normal viral hemagglutinin contains 25–30% of its oligosaccharides as high-mannose types. Again, this may indicate another pathway. Reitman et al. examined the glycopeptides produced in a mouse lymphoma cell line that is deficient in the processing glucosidase II. The major oligosaccharides produced in this mutant were characterized as $Glc_2Man_9GlcNAc_2$ and $Glc_2Man_8GlcNAc_2$. However, this mutant still contained 25% of the complex chains of the parent line. The authors postulate that the mutant may still have enough glucosidase II to allow some processing, or there may be an alternate pathway (Reitman et al., 1982).

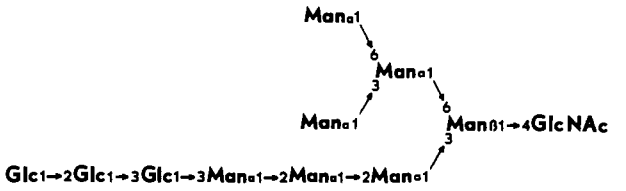

FIGURE 11: Probable structure for the major oligosaccharide in the influenza viral hemagglutinin synthesized in the presence of castanospermine. This structure is based on the studies outlined in this paper as well as the known structure of the $Glc_3Man_9GlcNAc_2$ precursor reported by several laboratories.

Recently, several other inhibitors of glycoprotein processing have been described, and the results with these compounds are analogous to those with castanospermine. Deoxynojirimycin is an antibiotic that was shown to be an inhibitor of yeast and pancreas glucosidases. This compound inhibited both of the partially purified glucosidases of yeast and also inhibited calf pancreas microsomal glucosidases. Deoxynojirimycin, at 5 mM, greatly decreased the proportion of radioactivity present in complex oligosaccharides of IEC-6 intestinal epithelial cells and rendered the high-mannose oligosaccharides less susceptible to the action of α-mannosidase (Saunier et al., 1982). These data indicate that this compound inhibits processing at the glucosidase stage, but the structure of the oligosaccharides is yet to be determined.

Another recently described processing inhibitor is bromoconduritol which apparently prevents the processing of the innermost glucose residue and gives rise to $GlcMan_9GlcNAc_2$ and $GlcMan_8GlcNAc_2$ structures. The inhibition by bromoconduritol occurred both in intact cells and with a microsomal enzyme preparation. In this case, in contrast to studies with castanospermine, the release of infectious fowl plaque virus particules was inhibited (Datema et al., 1982). It is not clear wether this inhibition is due to the absence of complex structures or whether the bromoconduritol has another effect on these cells. Apparently, the bromoconduritol has a half-life of only 16 min in H_2O at 37 °C and pH 7.3 and requires millimolar concentrations for inhibition. These factors will limit the usefulness of this inhibitor. Nevertheless, the various processing inhibitors should be of considerable value to examine the biosynthetic pathway and also to explore the role of complex chains in viral capsid assembly as well as in membrane and secretory glycoproteins.

Registry No. Castanospermine, 79831-76-8; glucosidase, 9033-06-1; concanavalin A, 11028-71-0; $Glc_3Man_7GlcNAc$, 86120-53-8.

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