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Characterization of the Oligosaccharides from Lipid-Linked Oligosaccharides of Mung Bean Seedlings

HIDETAKA HORI AND ALAN D. ELBEIN
Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284

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ABSTRACT
Lipid-linked oligosaccharides were synthesized with the particulate enzyme preparation from mung bean (Phaseolus aureus) seedlings in the presence of GDP-[14C]mannose. The oligosaccharides were released from the lipids by mild acid hydrolysis and purified by several passages on Biogel P-4 columns. Five different oligosaccharides were purified in this way. Based on their relative elution constants (kR) compared to a variety of standard oligosaccharides, they were sized as (mannose-acetylglucosamine)Man3GlcNAc, Man4GlcNAc, Man5GlcNAc, Man6GlcNAc, and Man7GlcNAc. These oligosaccharides were treated with endoglycosidase H and α- and β-mannosidase, and the products were examined on Biogel P-4 columns. They also were subjected to a number of chemical treatments including analysis of the reducing sugar by NaBH4 reduction, methylation analysis, and in some cases acetylation. From these data, the likely structures of these oligosaccharides are as follows: E, Manβ-GlcNAc-GlcNAc; D, Manα→2Manβ-GlcNAc-GlcNAc; C, Manα→2Manβ-GlcNAc-GlcNAc; B, Manα→2Manβ-GlcNAc-GlcNAc; and A, Manα→2Manβ-GlcNAc-GlcNAc. The synthesis of the Manα3GlcNAc was greatly diminished when transferrin (10 μg/ml) was added to the incubation mixtures.

Many plant and animal glycoproteins have an oligosaccharide composed of mannose, N-acetylglucosamine (GlcNAc) and possibly other sugars, that is attached to the protein in a GlcNAC-asparagine bond (10, 17, 32). The biosynthetic pathway for the formation of this oligosaccharide involves a series of membrane-bound glycosyl transferases that utilize dolichol-linked saccharide intermediates (10, 32). In the animal systems, it is now well-established that these enzymes catalyze the transfer of GlcNAC, mannose, and glucose to dolichyl-phosphate to form a dolichol-pyrophosphoryl-oligosaccharide having the composition Glc3Man2GlcNAc2 (5, 20, 22). This oligosaccharide is then transferred to the protein (6, 7, 24, 28). Several of the intermediate lipid-linked oligosaccharides have been isolated from animal tissues and the oligosaccharides have been characterized (4, 15, 32, 33). Plant cells also contain glycoproteins having oligosaccharides attached in GlcNAc-asparagine bonds (10), and the structure of the oligosaccharide of soybean lectin has been shown to be a high-mannose type (21).

Although the general pathway of assembly of the lipid-linked saccharides of plant cells is known (1–3, 8, 13, 19), the details of formation of the various lipid intermediates remain to be established (10). Thus, few of the oligosaccharides have been isolated or characterized, nor are the steps in the synthesis known. Re-

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MATERIALS AND METHODS

Materials. GDP-[U-14C]mannose (269 μCi/mmol) and UDP-[6-3H]GlcNAc (6.6 Ci/mmol) were purchased from New England Nuclear. Unlabeled sugar nucleotides and type III α-mannosidase were from Sigma. Pronase was obtained from Calbiochem and Biogel P-4 (200–400 mesh) was from Bio-Rad. Oligosaccharide standards, Glc3Man2GlcNAc2 and Man2GlcNAc2 were kindly supplied by Drs. C. Hubbard and P. W. Robbins, Massachusetts Institute of Technology. Purified yeast mannann was a generous gift from Dr. C. Ballou, University of California at Berkeley. All other chemicals were obtained commercially and were of the best grade available.

Preparation and Assay of Particulate Enzyme. Mung bean seedlings were grown on moist vermiculite in the dark at 25°C for 3 d. The sprouts were homogenized in 50 mM Tris buffer, pH 7.5 containing 2 mM β-mercaptoethanol, 1 mM EDTA, 0.1 mM MgCl2, 0.5% PVP, and 8% sucrose. One hundred g of mung bean sprouts were placed in 50 ml of the above medium and blended twice for 10 s each in a Waring Blender. The homogenate was passed through eight layers of cheese cloth, and the filtrate was centrifuged at 3000g for 10 min to remove whole cells and other large particles. The supernatant liquid was then centrifuged at 105,000g for 30 min to obtain the particulate enzyme. The pellet was resuspended in 50 mM Tris buffer containing 10 mM MgCl2, 10 mM MnCl2, and 2 mM β-mercaptoethanol (0.5 mM medium/10 g sprouts) with a Dounce homogenizer and used as the enzyme source (25 μg protein/μl).

Reaction mixtures for assay of the particulate enzyme contained the following components in a final volume of 0.5 ml: GDP-[14C]mannose (0.04 μCi) or UDP-[3H]GlcNAc (0.1 μCi), 0.02 mM unlabeled UDP-GlcNAc, 50 mM Tris buffer, pH 7.5, and varying amounts of enzyme. When UDP-[3H]GlcNAc was used as the substrate, the unlabeled UDP-GlcNAc was omitted. Reaction mixtures were preincubated for 5 min without enzyme or without labeled sugar nucleotides, and the reaction was initiated by the addition of the missing component. Incubations were done at room temperature for varying periods of time.

The reactions were terminated by the addition of 2 ml of CHCl3:CH3OH (1:1) and 0.5 ml of H2O. The mixture was stirred vigorously, and the layers were separated by centrifugation. The lower CHCl3 layer was removed and saved, and the aqueous phase and interface were extracted with another 1 ml of CHCl3. The combined CHCl3 phases, which contained lipid-linked monosaccharides and lower mol wt lipid-linked saccharides, were extracted with CHCl3:CH3OH:H2O (3:4:87) before being placed in scintil-
lution vials for counting. The particulate material that remained at the interface during these extractions was isolated by centrifugation after adding 1 ml of CH$_2$OH to dissolve any remaining CHCl$_3$. The pellets were washed twice with 50% CH$_2$OH and once with 100% CH$_3$OH before being suspended in CHCl$_3$:CH$_2$OH:H$_2$O (10:10:3) to extract the LLO.\(^2\) Aliquots of this extraction were placed in scintillation vials for counting (13).

**Chemical Methods.** LLO were hydrolyzed in 0.02 N HCl in 20% methanol at 100°C for 20 min in screw capped tubes. After cooling, the hydrolysate was extracted with chloroform to remove lipids, and the aqueous phase was saved for the isolation of oligosaccharides.

Oligosaccharides released by mild acid hydrolysis were reduced with NaB$_4$H$_4$. Samples were dissolved in 50 µl of H$_2$O and 1 drop of 1 N NH$_4$OH was added, followed by 250 µCi of NaB$_4$H$_4$. The mixture was allowed to stand for 24 h at 4°C, and the reaction was terminated by the addition of a few drops of acetic acid. The samples were concentrated to dryness on a rotary evaporator, and borate was removed by repeated evaporation with methanol. The sample was passed through Biogel P-4 columns to isolate the radioactive oligosaccharides. The reduced material was then hydrolyzed in 3 N HCl at 100°C for 4 h, concentrated to dryness, and subjected to paper chromatography.

**Methylation Analysis.** Oligosaccharides were purified on columns of Biogel P-4 and were subjected to methylation according to the method of Hakamori (14) as modified by Sanford and Conrad (25). The lyophilized samples were dissolved in 2 ml of dimethylsulfoxide under N$_2$ and sonicated with methyllsulfonylcarbanion at 50°C for 5 h. The mixtures were chilled and 2 ml of CH$_3$I were added. The mixture was sonicated for 2 h at 4°C with a further addition of 2 ml of CH$_3$I after 1 h. The samples were allowed to stand at room temperature overnight and then were passed through columns of Sephadex LH-20 equilibrated with 80% CH$_2$OH to remove dimethyl sulfoxide, methyl sulfonyl carbanion, and other salts. Columns were run with 80% methanol. The eluates from this column were hydrolyzed in 2 N H$_2$SO$_4$ at 100°C for 4 h in screw capped tubes. The hydrolysate was neutralized with Ba(OH)$_2$, and the supernatants were concentrated to dryness, dissolved in chloroform, and analyzed by TLC.

**Enzymic Digestions.** Jack bean α-mannosidase was from Sigma. β-Mannosidase was purified from *Aspergillus fumigatus* as described previously (11). Endogluconsaminidase H was obtained from Miles Laboratories.

Oligosaccharides were treated with the various glycosidases as described below. Purified oligosaccharides from Biogel P-4 were treated with 10 munits of endogluconsaminidase H in 0.1 ml of 0.05 M Na citrate buffer, pH 6.0, at 30°C for 48 h under a tuleo atmosphere. Products were analyzed by Biogel P-4.

Samples were dissolved in 0.1 ml of 0.1 M acetate buffer, pH 5.0, containing 0.4 mM ZnCl$_2$ and digested with 0.5 to 1.0 units of α-mannosidase for 48 h at 30°C under a tuleo atmosphere. Oligosaccharides were also dissolved in 0.1 ml of 0.1 M acetate buffer, pH 4.0, and digested with 0.1 to 0.2 units of β-mannosidase at 30°C for 48 h under a tuleo atmosphere. At 24 h, another 0.2 units of enzyme was added. Samples were analyzed by chromatography on Biogel P-4. Reactions were terminated by the addition of 100% ethanol.

The residues remaining after extraction of the various lipids were washed three times with H$_2$O and once with 20 mM Tris buffer, pH 8.5, containing 5 mM CaCl$_2$. The residue was suspended in 1 ml of 20 mM Tris buffer, pH 8.5, containing 5 mM CaCl$_2$, and 1 mg of pronase was added. Samples were incubated at 37°C for 72 h under a tuleo atmosphere. An additional 1 mg of pronase was added at 24 and 48 h. Reactions were terminated by the addition of TCA to a final concentration of 5%. The precipitate was removed by centrifugation, and the supernatant liquids were extracted with diethyl ether to remove TCA.

**Results**

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**Biogenesis of LLO.** The particulate enzyme from mung bean seedlings catalyzes the incorporation of mannose from GDP-[14C]mannose and GlcNAc from UDP-[3H]GlcNAc into lipid-linked monosaccharides (*i.e.* soluble in CHCl$_3$:CH$_2$OH:H$_2$O [1:1:1]) and into a series of LLO (mostly soluble in CHCl$_3$:CH$_2$OH:H$_2$O [10:10:3]). The incorporation of mannose into LLO was linear with time of incubation for 5 to 10 min, and then leveled off with longer incubations (data not shown). When unlabeled UDP-GlcNAc (0.01 µmol) was added to the incubation mixtures, there was a 30 to 40% increase in the amount of mannose incorporated into LLO probably due to an increase in the amount of N,N'-diacetylchitobiosyl-pyrophosphoryl-polysisoprenol which can serve as an acceptor of mannose. On the other hand, the addition of unlabeled UDP-glucose (0.01 µmol) to the incubation mixtures caused a 30 to 40% decrease in mannose incorporation suggesting that mannose and glucose compete for the same acceptors.

The incorporation of GlcNAc from UDP-[3H]GlcNAc into the CHCl$_3$:CH$_2$OH:H$_2$O (1:1:1) soluble lipids (*i.e.* GlcNAc- and N,N'-diacetylchitobiosyl-pyrophosphoryl-polyisoprenol) was linear with time for 20 to 30 min, whereas incorporation of this sugar into LLO was at least 10-fold lower and leveled off in 1 to 2 min (data not shown). In this case, the addition of unlabeled GDP-mannose (0.005 µmol) significantly inhibited the incorporation of GlcNAc into the mono- and diGlcNAc-lipids by 50% or greater, while GlcNAc incorporation into LLO was stimulated about 2- to 3-fold.

**Identification of LLO.** The LLO formed from GDP-[14C]mannose at various times of incubation were isolated by solvent extraction and the oligosaccharides were released by mild acid hydrolysis. The nature of the oligosaccharides was then examined on Biogel P-4 columns. Figure 1 shows the profiles of [14C]mannose-oligosaccharides obtained during incubations of 1 to 60 min. After 1 min, several oligosaccharides were observed that migrated slower than the heptasaccharide standard (M5N2), as well as a radioactive peak emerging in the maltooligosaccharide area and a large radioactive peak in the mannosidase area. With increasing times of incubation, the amount of mannose incorporated into these peaks increased, and several new and faster migrating peaks were detected. At least five major oligosaccharides were separated on this column, and the characterization of these species is detailed in the remaining portion of this paper. It should be pointed out that the addition of unlabeled UDP-glucose, even at concentrations as high as 10 µmol, did not alter the labeling pattern seen in Figure 1. Thus, in contrast to studies in animal systems, UDP-glucose did not give rise to any detectable peaks in the Glc$_3$Man$_2$N$_2$, Glc$_3$Man$_3$N$_3$, or Glc$_3$Man$_4$N$_2$ areas of the column.

The lipid-linked saccharides labeled with UDP-[3H]GlcNAc were also isolated, and the saccharides were examined on Biogel P-4 (Fig. 2). In this case, three major peaks were observed, one of which eluted near the maltose area while the other two eluted near the stachyose standard. The fastest migrating peak is apparently

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\(^2\) Abbreviation: LLO, lipid-linked oligosaccharides.
Effect of Tunicamycin on the Formation of LLO. The antibiotic tunicamycin has been shown to inhibit the formation of GlcNAc-pyrophosphoryl-polyprenol in both animal (33) and plant (12) tissues. Thus, it was of interest to examine the effect of this antibiotic on the various plant lipid-linked saccharides. Incubation mixtures were prepared with GDP-[14C]mannose and unlabeled UDP-GlcNAc as described in Figure 1, except that 10 or 50 μg of tunicamycin was added. After incubation, the LLO were isolated and the oligosaccharides were chromatographed on Biogel P-4 (Fig. 3). In the control incubations, two major radioactive peaks were observed near the M2N2 standard as well as a radioactive peak in the maltose area and one in the mannose area. However, in the presence of tunicamycin, the 2 fastest migrating peaks were greatly diminished, but little effect was seen on the peaks migrating near maltose and mannose. Also shown in this figure is the fact that tunicamycin had no inhibitory effect on the incorporation of mannose into the CHCl3:CH3OH (1:1) soluble lipids.

Isolation of Five Major Oligosaccharides from the LLO. In order to isolate sufficient amounts of the major oligosaccharides for characterization, the incubation mixtures described in Figure 1 were scaled up 10- to 100-fold, and the LLO were isolated by solvent extraction. The oligosaccharides were released by mild acid hydrolysis and chromatographed on a Biogel P-4 column (Fig. 4). In addition to a large peak in the mannose area, six major oligosaccharides were observed, and these were pooled and identified as oligosaccharides A through F as shown by the brackets. Each of the pooled peaks was then rechromatographed on a calibrated column of Biogel P-4 in order to obtain a homogenous oligosaccharide and to determine the size of the oligosaccharide. Figure 5 shows the elution profiles of oligosaccharides A through F and demonstrates that they were separated from each other with only slight overlap in a few cases. Each of the oligosaccharides was again run on the column along with the standards GlcMan3GlcNAc2 (G3M2N2), ManαGlcNAc (M6N), Man2GlcNAc2 (M2N2), Man-GlcNAc (M6N), ManαGlcNAc2 (M6N2), and mannose. The relative elution constant of each plant oligosaccharide and of the standard oligosaccharides was calculated according to Hub-}

![Image](image-url)
cut into strips and counted to identify the radioactive sugars. For each oligosaccharide, a peak of tritium was detected that migrated with glucosaminitol as well as a peak of $^{13}C$ that corresponded to the mannose standard. No evidence for $[^3H]mannotol or any other reduced hexitol was seen. These data demonstrate that each oligosaccharide has GlcNAc at the reducing end.

**Digestion with α-Mannosidase.** Each of the oligosaccharides A through D was treated with α-mannosidase, and the digests were passed through columns of Biogel P-4 (Fig. 7). For each of the oligosaccharides, this treatment gave rise to two new major peaks corresponding to the trisaccharide, Man$\beta$-GlcNAc-GlcNAc (oligosaccharide E), and free mannose. However, in addition to the two major peaks, several small peaks of radioactivity were observed in several cases. For example, in the case of oligosaccharide A, a very small peak of undigested A is detectable as well as a shoulder on the peak eluting with oligosaccharide E. This is probably a Man$_2$GlcNAc$_2$ which has not been completely digested.

![Figure 3](image-url)  
**FIG. 3.** The effect of tunicamycin on the formation of lipid-linked saccharides labeled from GDP-$[^3]C$mannose. Incubations were as described in the text, but contained 10 or 50 μg of tunicamycin. The control was without antibiotic. Lipids were isolated by CHCl$_3$:CH$_3$OH:H$_2$O (1:1:1) extraction or by CHCl$_3$:CH$_3$OH:H$_2$O (10:10:3), and the saccharides were released by mild acid hydrolysis and chromatographed on Biogel P-4. F is fraction E (see Fig. 4).

![Figure 4](image-url)  
**FIG. 4.** Biogel P-4 column chromatography of large scale incubations for isolation of $[^3]C$mannose-labeled oligosaccharides. Incubations were scaled up 10- to 100-fold, and oligosaccharides were run on a 1.5 × 150 cm column of Biogel P-4. Aliquots of each fraction were analyzed for radioactivity. Fractions were pooled as shown by the bars and labeled A through E.

![Figure 5](image-url)  
**FIG. 5.** Further separation of oligosaccharides A through E on Biogel P-4 columns. Each peak from Figure 4 was rechromatographed on Biogel P-4. Standards shown are G$_3$M$_4$N$_2$ (14 res), M$_3$N$_2$ (7 res), stachyose (Sta), maltose (Mal), and mannose (Man).

<table>
<thead>
<tr>
<th>Plant Oligosaccharides</th>
<th>$K_r$ Value Observed</th>
<th>Standards</th>
<th>$K_r$ Reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>G$_3$M$_4$N$_2$</td>
<td>0.268</td>
<td>G$_3$M$_4$N$_2$</td>
<td>0.279</td>
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<tr>
<td>Peak A (M$_3$N$_2$)</td>
<td>0.411</td>
<td>M$_3$N$_2$</td>
<td>0.408</td>
</tr>
<tr>
<td>Peak B (M$_3$N$_2$)</td>
<td>0.482</td>
<td>M$_3$N$_2$</td>
<td>0.474</td>
</tr>
<tr>
<td>Peak C (M$_3$N$_2$)</td>
<td>0.571</td>
<td>M$_3$N$_2$</td>
<td></td>
</tr>
<tr>
<td>Peak D (M$_3$N$_2$)</td>
<td>0.607</td>
<td>M$_3$N$_2$</td>
<td></td>
</tr>
<tr>
<td>Peak E (M$_3$N$_2$)</td>
<td>0.661</td>
<td>M$_3$N$_2$</td>
<td></td>
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<tr>
<td>Peak A—Endo H</td>
<td>0.518</td>
<td>M$_3$N$_2^*$</td>
<td>0.429</td>
</tr>
</tbody>
</table>

*These $K_r$ values were determined in this laboratory.
observed (Fig. 8). However, through E, was digestion experiment, the the same migratory species contained one -GlcNAc, although the Man,GlcNAc, has been reported to be resistant to endoglucosaminidase H; it could be slightly susceptible during long incubation. Or the Man,GlcNAc, could be heterogeneous and could contain small amounts of a susceptible isomer. Oligosaccharide C was digested to the extent of about 10 or 15% by the endoglucosaminidase H as shown in scan 3 of Figure 9. It is not clear what this digestion means in terms of the oligosaccharide structure since the pentasaccharide would be expected to be resistant to endoglucosaminidase H. Perhaps on long incubations with sufficient amounts of enzyme this oligosaccharide is slightly susceptible. Oligosaccharides D and E were found to be essentially resistant to this enzyme, and only a very slight peak of unknown origin was observed in the case of E.

**Methylation Analysis of Oligosaccharides.** Oligosaccharides A through E were subjected to complete methylation and after complete acid hydrolysis to liberate the monosaccharides, the methylated sugars were identified by TLC. The radioactive methylated mannoses were compared to various standard methylated sugars prepared from yeast mannan or ovalbumin. Figure 10 shows the radioactive profiles of the thin-layer plates of oligosaccharides A through E. When [14C]mannon-labeled oligosaccharide E was subjected to methylation (scan E), only one radioactive peak was observed. However, the peak was not detected in the original Man7GlcNAc peak, indicating that it contains one less GlcNAc than the original oligosaccharide A. As shown in Table I, this endoglucosaminidase H-treated A had the same Kₐ value as an authentic sample of Man7GlcNAc. Although the digestion of peak A did not go to completion in this experiment, the undigested material could be digested further by incubation with more endoglucosaminidase H. However, complete digestion was not achieved, suggesting the possibility that the Man7GlcNAc, peak contains small amounts of other isomers and that some of these isomers are resistant to endoglucosaminidase H. Nevertheless, the fact that the major portion of this peak was susceptible indicates that it contains a branched mannose structure and that the mannose attached in a 1→6 branch is further substituted.

When oligosaccharide B was treated with endoglucosaminidase H for 48 h about 25% of the radioactivity was shifted to a slower peak which differed from the original B by one GlcNAc. Although the Man,GlcNAc, has been reported to be resistant to endoglucosaminidase H, it could be slightly susceptible during long incubation. Or the Man,GlcNAc, could be heterogeneous and could contain small amounts of a susceptible isomer. Oligosaccharide C was digested to the extent of about 10 or 15% by the endoglucosaminidase H as shown in scan 3 of Figure 9. It is not clear what this digestion means in terms of the oligosaccharide structure since the pentasaccharide would be expected to be resistant to endoglucosaminidase H. Perhaps on long incubations with sufficient amounts of enzyme this oligosaccharide is slightly susceptible. Oligosaccharides D and E were found to be essentially resistant to this enzyme, and only a very slight peak of unknown origin was observed in the case of E.

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CHARACTERIZATION OF LIPID-LINKED OLIGOSACCHARIDES

peak was detected which corresponded to 2,3,4,6-tetramethylmannose. These results confirm the previous data indicating that peak E is Manβ-GlcNAc-GlcNAc.

Methylation of oligosaccharide D (scan D) gave rise to two radioactive methylated sugars corresponding to 2,3,4,6-tetramethylmannose and 2,4,6-trimethylmannose. The amount of radioactivity in each of these peaks was almost the same. These results coupled with other data presented above indicated that oligosaccharide D is Manα1→3Manβ-GlcNAc-GlcNAc.

Oligosaccharide C, upon methylation and hydrolysis, gave rise to three radioactive methylated sugars which migrated with the standards, 2,3,4,6-tetramethylmannose, 2,4,6-trimethylmannose, and 3,4,6-trimethylmannose. The absence of any radioactivity in the area corresponding to 2,4-dimethylmannose suggests that this oligosaccharide does not have a branched mannose structure. Thus, the likely structure for compound C is Manα1→2Manα1→3Manβ-GlcNAc-GlcNAc.

Oligosaccharide B also gave rise to three radioactive bands upon methylation and hydrolysis. In this case, these radioactive bands corresponded to the standards, 2,3,4,6-tetramethylmannose, 3,4,6-trimethylmannose, and 2,4-dimethylmannose. In this case, no radioactivity was found in the area corresponding to 2,4,6-trimethylmannose indicating that the 3-linked mannose was disubstituted. Therefore, the likely structure for oligosaccharide B is Manα1→2Manα1→2Manα1→3(Mana1→6)Manβ-GlcNAc-GlcNAc. Further studies on this oligosaccharide, using acetylation to cleave 1→6 linkages (see below) are consistent with this structure.

Finally, oligosaccharide A yielded three radioactive bands after methylation and hydrolysis which corresponded to 2,3,4,6-tetra-
methylmannose, 3,4,6-trimethylmannose, and 2,4-dimethylmannose. The absence of 2,4,6-trimethylmannose indicates that any 3-linked mannose is disubstituted and is compatible with either of the two structures shown in Figure 12. However, based on the results of acetylation (see below) and the susceptibility to endoglucosaminidase H, it seems likely that the more highly branched structure is the correct one.

Acetylation of Oligosaccharides A and B. In order to decide between the alternative structures shown in Figure 12, oligosaccharide A was subjected to acetylation (29) and the deacetylated oligosaccharide products were chromatographed on Biogel P-4 (Fig. 11). The upper profile shows the results obtained with oligosaccharide A. In this case, the major radioactive peak corresponded to the trisaccharide, raffinose, while the next major peak was eluted at the position of the disaccharide, maltose. A smaller peak of radioactivity was also found in the mannose area. These data are consistent with the more highly branched structure (Figure 12) for Man1→GlcNAc2, having two 1→6 linked mannoses. Thus, cleavage of the innermost 1→6 linkage would give a trisaccharide, whereas cleavage of the second 1→6 linkage would give a disaccharide and a monosaccharide.

The lower profile shows the acetylation pattern obtained from oligosaccharide B. Although several peaks were observed and some of these could be artifacts since they are represented by a single point, the major radioactive area corresponded to mannose.

![Diagram](image-url)
This is consistent with the structure for the Man$_7$GlcNAc$_2$ having a single mannose residue in 1→6 linkage.

**DISCUSSION**

Previous studies in plant systems have shown that mannose and GlcNAc are incorporated into LLO (1–3, 8, 13, 19). The oligosaccharide portion of these molecules have been isolated by paper chromatography or by chromatography on columns of Biogel P-4 and shown to range in size from trisaccharide to decasaccharide. However, except for some general studies on sugar composition, little is known about the structure of these oligosaccharides. It has been shown that tissue slices from cotyledons form the same type of lipid-linked saccharides as particulate enzyme fractions (2).

In the present study, five oligosaccharides were isolated from the LLO synthesized from GDP-$[^{14}$C]mannose by a particulate enzyme from mung bean seedlings. These oligosaccharides were separated from each other on columns of Biogel P-4 and were further purified to apparent homogeneity by several passages through long columns of Biogel P-4 (1.5 × 150 cm). Using a number of oligosaccharide standards, the relative elution constant ($K_r$) of each of these oligosaccharides was calculated as well as the $K_r$ of the product resulting from endoglucosaminidase H digestion. Thus, the oligosaccharides were shown to be: A, Man$_4$GlcNAc$_2$; B, Man$_5$GlcNAc$_2$; C, Man$_6$GlcNAc$_2$; D, Man$_7$GlcNAc$_2$; and E, Man$_8$GlcNAc$_2$.

Each oligosaccharide was shown to have glucosamine at its reducing terminus based on reduction with NaBH$_4$, and oligosaccharides A through D gave rise mostly to $[^{14}$C]mannose and the trisaccharide, Man$_β$GlcNAc$_2$, upon treatment with $α$-mannosidase. These results indicate that each oligosaccharide contains an $N,N'$-diacetyltrehalose core at the reducing end and a number of $α$-linked mannose residues at the nonreducing end. Oligosaccharide E was not susceptible to $α$-mannosidase but released free mannose upon digestion with $β$-mannosidase.

Oligosaccharide A was quite susceptible to endoglucosaminidase H treatment and gave rise to a Man$_3$GlcNAc$_2$. Oligosaccharides B and C were somewhat susceptible to this enzyme especially upon long incubation but much less so than A. In this case, B appeared to be more susceptible than C. With both oligosaccharides, a small peak of radioactivity was detected moving more slowly than the original material, but these peaks were not available in sufficient amounts for additional characterization. Based on studies from other laboratories, the Man$_3$GlcNAc$_2$ and presumably the smaller oligosaccharides should not be susceptible to endoglucosaminidase H since the mannose in 1→6 linkage is not substituted. Thus, the finding that the two plant oligosaccharides are somewhat susceptible may indicate either that (a) the specificity of the endoglucosaminidase H is not so strict and that upon long incubation these other oligosaccharides may show some activity, or (b) the plant oligosaccharides may be composed of several isomers such that one of the isomers that is present in small amounts may be susceptible. Oligosaccharides D and E were essentially resistant to the action of this enzyme.

Methylation analysis of oligosaccharides B through E indicated the following structures for these compounds: E, Man$_β$-GlcNAc-

![Fig. 10](image-url)  
**Fig. 10.** Methylation analysis of oligosaccharides A through E. Each oligosaccharide was subjected to complete methylation, and the products were hydrolyzed and analyzed by TLC in benzene:acetone:water:ammonium hydroxide (50:200:3:1.5). Standards shown by the numbers are 1 = 2.3-dimethylmannose; 2 = 2.4-dimethylmannose; 3 = 3,4,6-trimethylmannose; 4 = 2,4,6-trimethylmannose; and 5 = 2,3,4,6-tetramethylmannose.

![Fig. 11](image-url)  
**Fig. 11.** Acetolysis of oligosaccharides A and B. Compounds A and B were subjected to acetolysis, and the products were analyzed on Biogel P-4 columns. Standards shown by the arrows were: A to E = oligosaccharides A to E; G$_2$ = $N,N'$-diacetyltrehalose; Sta = stachyose; Raf = raffinose; Mal = maltose; and Man = mannose.
likely that acetolysis structure animal cells showed the absence of present 2Manal-1(Manal—6)Man,8-GlcNAc-GlcNAc. and this D, could explain as time, observed in the observed with oligosaccharides to species Man6GlcNAc2 has been suggested then 1-6 linked mannose. Inasmuch as the major radioactive peak detected upon acetylation of B was mannose, this would confirm a single mannose residue in a 1-6 linkage. Some of these structures are similar to those reported in animal systems (4), but some are different indicating that the sequence of addition of mannose residues may be somewhat different. For example, in animals, the pentasaccharide ManβGlcNAc2 has a 1-6 linked mannose, whereas oligosaccharide C has a 1-2 linked mannose. Thus, in animal systems it has been suggested that the addition of mannose residues to the tetrasaccharide is 1-6, then 1-2, then 1-2 to form the ManαGlcNAc2 (4), whereas in plants it may be 1-2, then 1-2 or 1-6, then 1-6 or 1-2.

It is interesting to note that the ManβGlcNAc2 and ManαGlcNAc2 species were not detected in these large scale incubations. This may suggest that the two mannose residues added to the ManαGlcNAc2 are added very rapidly or at the same time, as well as the two mannoses added to the ManβGlcNAc2. This could explain the type of heterogeneity observed in some of these oligosaccharides by Vijay and Fram (31) and Rearick et al. (23) who found evidence for several isomers in the various oligosaccharide peaks. This might also explain some of the results observed in the present study such as the susceptibility of these oligosaccharides to endoglucosamidase H and some of the minor peaks observed with α-mannosidase digestion. However, the major compound present in oligosaccharide B from mung beans is apparently similar in structure to that formed in CHO cells (4) and in particulate and soluble extracts of aorta tissue (26).

The largest oligosaccharide observed in this plant system is the Man-GlcNAc2. The methylation analysis of this oligosaccharide showed the absence of any 3-linked mannose (i.e. no 2,4,6-trimethylmannose) which is also different from the ManβGlcNAc2 of animal cells (4). These data are consistent with either of the structures shown in Figure 12. However, based on the fact that acetylation gave rise to a trisaccharide and a disaccharide, it seems likely that the more highly branched structure is the correct one. This structure is consistent with the structure recently proposed by Dorland et al. (9) for the oligosaccharide of soybean lectin. It should be mentioned that one difficulty with the methylation analysis of these oligosaccharides formed in vitro is that the mannose residues are probably not equally labeled. That is, the biosynthesis involves the addition of mannose residues to endoglycosidases. Thus, we are not able to use the method for the quantitative determination of the number of mannose residues present in various glycosidic linkages. Nevertheless, the combination of data from the various treatments used in this study do suggest the structures presented in Figure 12. Although in these studies, the addition of UDP-glucose did not cause any alteration in the size of the mannose-labeled LLO, other workers have found changes in the nature of the oligosaccharides induced by UDP-glucose.

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