

OLIGOSACCHARIDE GLYCOLIPIDS IN
CULTURES OF ACER PSEUDOPLATANUS

by

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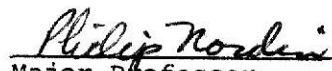
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INTRODUCTION

There is a large class of glycoproteins that have a common inner core region in their oligosaccharide chain (1). The oligosaccharide is linked via asparagine to the protein chain. This type of carbohydrate linkage, in contrast to other types, is first assembled on a polyisoprenoid carrier lipid, dolichol phosphate, and then transferred to the protein. Although slight modifications in the chain may take place after transfer (e.g., additional galactose or sialic acid residues may be attached) the oligosaccharide chain is essentially complete before transfer. The biosynthetic pathway for this type of glycoprotein linkage appears to be very similar in plants and animals.

Recently a new type of glycolipid was isolated from Acer plant cell cultures (2). The oligosaccharide chain was about 18 hexose units long, and unlike all others thus far isolated, was reported to consist of more than 50% arabinose and had arabinose at the reducing end of the chain. Plants, unlike mammals, elaborate complex arabinose containing heteropolysaccharides and secrete them into the cell wall. The possibility that this new glycolipid could be an important precursor in the biosynthesis of these heteropolysaccharides provided a basic justification for the research reported in this thesis. The immediate objectives were:

- (a) to look for better ways of introducing radioactivity into the glycolipid, than by incubating Acer cell membranes with GDP-[U-¹⁴C] Mannose as first reported.
- (b) to purify the glycolipid and characterize the carbohydrate components.

SURVEY OF LITERATURE

Protein glycosylation in plants

Lipid-linked saccharides have been isolated from a diversity of organisms including bacteria, yeast, higher plants, insects, fungi, and mammals (3). The roles of these intermediates are as glycosyl donors for extracellular polysaccharides such as peptidoglycans, lipopolysaccharides and capsular polysaccharides; and as precursors in the glycosylation of proteins.

In plants, these lipid-linked saccharides have been shown to be involved in the synthesis of polysaccharides and glycoproteins (3). Most of the attention has been given to the synthesis of glycoproteins. It is known that a "high mannose" type glycopeptide has been isolated from glucosaminyl-asparagine glycoproteins (found in fungi, yeast, animal, and recently in plants). These glycoproteins have a core structure composed of mannose and N-acetylglucosamine; it is this core region that is synthesized by means of the lipid-linked saccharides (Figure 1) (3). For example, vicilin, the major storage protein of Phaseolus vulgaris seeds (kidney bean), is a glycoprotein containing mannose and N-acetylglucosamine (4). It is known that glycopeptides prepared from vicilin contained asparagine as its principal amino acid and therefore, it is probable that the mannose residues are linked to asparagine via two GlcNAc residues, as is commonly the case with many animals, fungi and yeast glycoproteins (5). Recently, this has been shown for soybean agglutinin (Figure 2) (6), and for lima-bean lectin (Figure 3) (7).

Figure 1. General structure of the core region of the oligosaccharide chain.



↑ inner core region ↑

Figure 2. Structure of carbohydrate chain of soybean agglutinin.

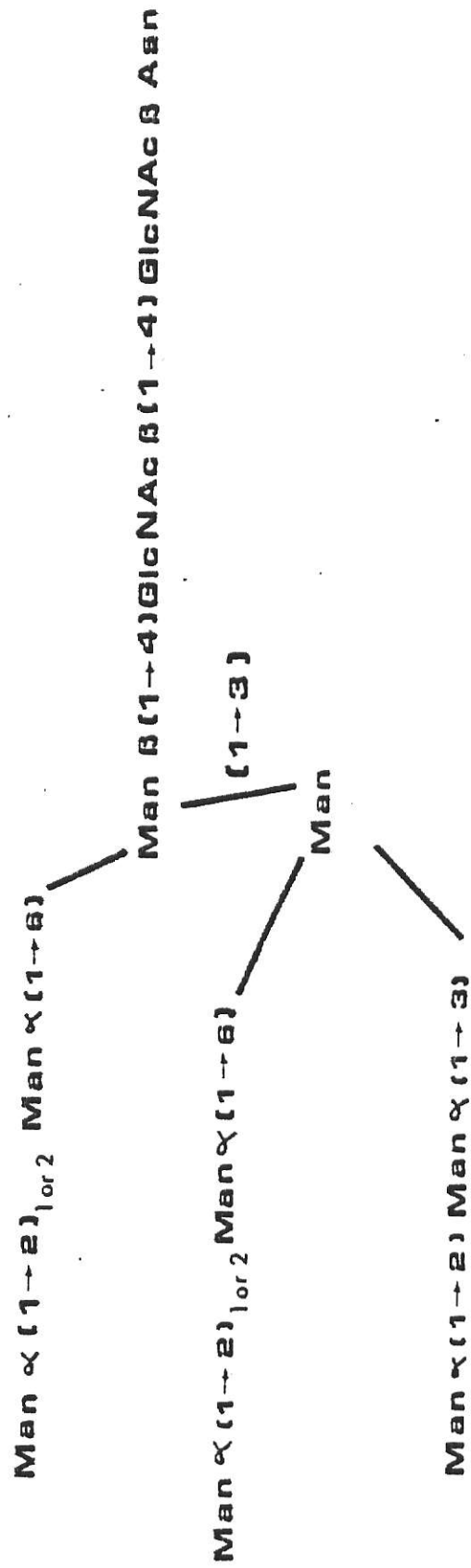
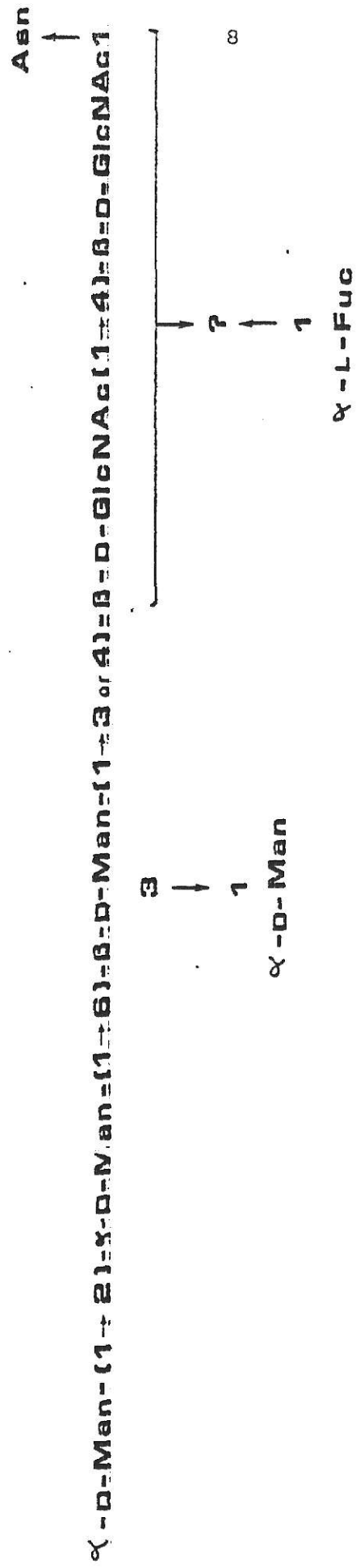


Figure 3. Tentative structure of the lima-bean lectin.



A. Monosaccharide derivatives of polyisoprenyl phosphates

The first suggestion that there might be transient types of sugar-containing lipids in eukaryotic cells came almost simultaneously in yeast, animal and plants. An example of this kind of monosaccharide derivative of polyisoprenyl phosphate is shown in Figure 4.

Incubation of GDP-[¹⁴C]Mannose with membrane preparations from the appropriate tissue showed that [¹⁴C]Mannose was transferred to material that was soluble in lipid solvents. The mannosyl lipid was found to be labile to mild acid hydrolysis but stable to mild alkaline saponification. This property is common to phosphodiester linkages (8). The existence of this kind of mannosyl lipid was first shown in the plant classification Phaseolus aureus (mung bean) (4,9). Also these characteristics of a mannosyl lipid were found later in suspension cultures of Acer cells (10), cotton ball fibers (11), Phaseolus vulgaris (12), hen oviduct (13), and others.

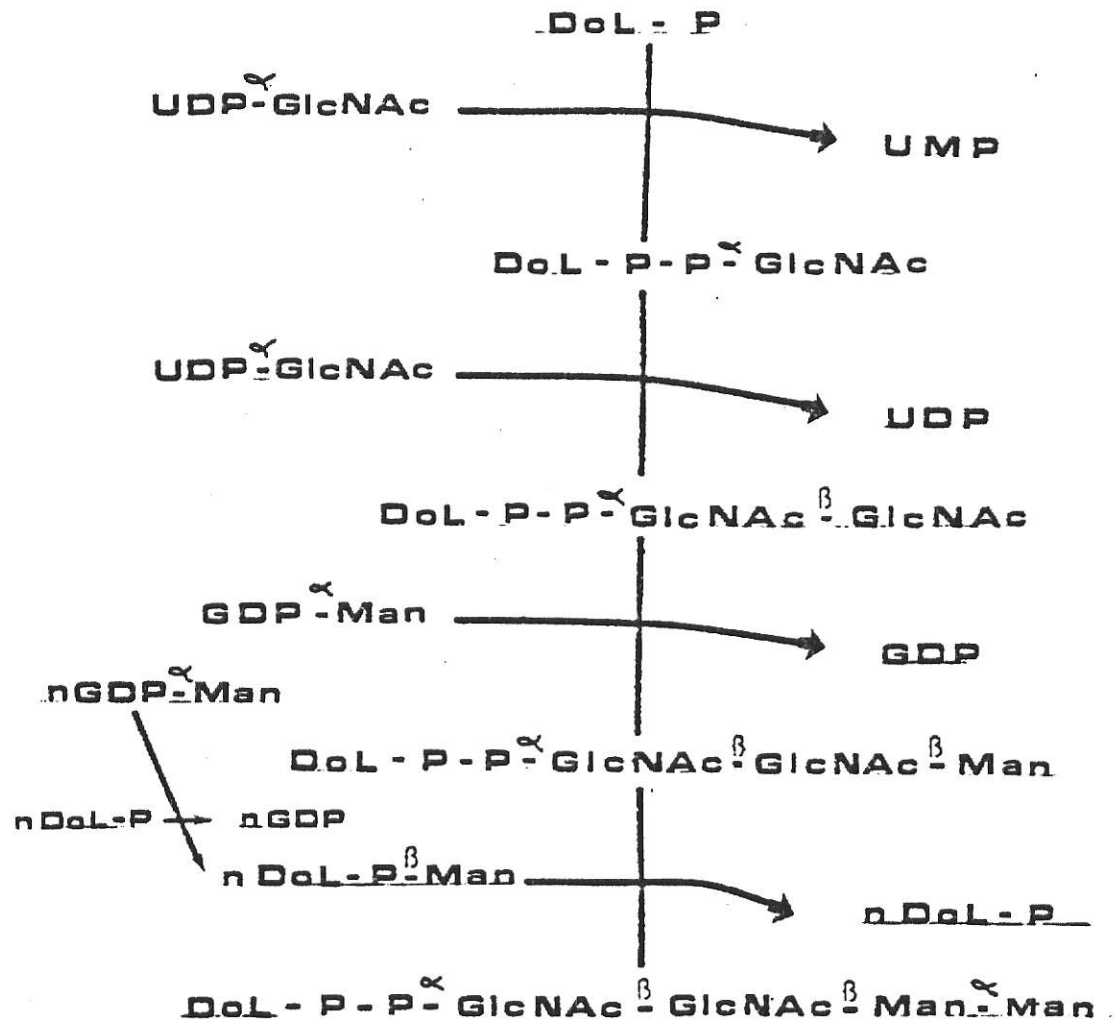
It is known that dolichol derivatives have a role as glycosyl carriers in eukaryotic cells (Figure 5). They are a family of α -saturated polyprenols whose chain lengths may vary from 80 to 110 carbons. Dolichols act as intermediates in glycosylation reactions and usually involves 14 to 21 isoprene units (refer to Figure 4). The polyprenols which act as intermediates in glycoprotein synthesis in eukaryotic cells differ from those of bacteria in that the α -isoprene unit is saturated. The number of isoprene units may vary from 17 to 21 for animals and from 14 to 24 for yeasts, fungi and plants (15).

When a crude particulate enzyme preparation from Saccharomyces cerevisiae was incubated with GDP-[¹⁴C]Mannose, [¹⁴C]Mannose was incorporated into a mannosyl lipid (16). The mannosyl lipid was labile when subjected to mild acid, and its enzymatic formation was reversed by the addition of unlabeled GDP, suggesting that mannose was bound to the lipid by an activated linkage (17). Studies suggested that the yeast mannosyl lipid synthesized from GDP-[¹⁴C]Mannose was a mannosyl-phosphoryl-polyprenol. This was shown by phenol treatment, catalytic

Figure 4. Structure of mannosyl-phosphoryl-dolichol
found in plant and animal tissues.



Figure 5. Sequence for assembly of the oligosaccharide lipid (14).



hydrogenation and mass spectrometry (18). For example, glycosyl-phosphoryl derivatives of allylic polyprenols have been shown to be degraded by treatment with phenol or by catalytic hydrogenation, whereas similar derivatives of dolichol, which contain the saturated α -residue, are stable (18).

In plants (cotton fibers) ficaprenyl-phosphoryl-mannose markedly stimulates the incorporation of mannose by acting as an acceptor indicating that these plant lipids are similar in structure to dolichyl-phosphoryl-mannose. It is not clear if these lipids are precursors of the sugar residues in cell wall polysaccharides or are involved in glycoprotein biosynthesis (19).

B. Oligosaccharide derivatives of polyisoprenyl phosphates

In plants, (P. vulgaris (kidney bean), Pisum sativum (peas), P. aureus (mung bean) and others) a series of lipid-linked oligosaccharides are formed when GDP- ^{14}C Mannose is incubated with the particulate enzyme (12,19). When the oligosaccharides were released by mild acid hydrolysis and examined by paper chromatography, a series of 7 or 8 radioactive peaks were observed which ranged in size from a trisaccharide to an oligosaccharide having 10 to 12 glucose units (20). The oligosaccharides had glucosamine at the reducing terminus since ^{14}C Mannose and ^3H Glucosaminitol were obtained after reduction followed by acid hydrolysis. This was confirmed by the finding that when ^3H N,N 1 -diacetylchitobiosyl-pyrophosphoryl-polyprenol, isolated from incubation mixtures, was reincubated with membrane fractions, along with unlabeled GDP-mannose, radioactivity was chased from the disaccharide lipid into the lipid-linked oligosaccharide (21). This suggested that the di-GlcNAc-lipid was acting as an acceptor of mannose residues. Other studies showed an interaction between GDP-mannose and UDP-glucosamine as precursors to the lipid-linked oligosaccharides; mannose residues were needed to complete the oligosaccharide chains before transfer to protein occurred (22).

The formation of a compound from incubation with GDP- ^{14}C Mannose with

the properties of a mannose-labeled oligosaccharide lipid was detected in hen oviduct membranes (23). The kinetics of labeling of the oligosaccharide-lipid and of protein were consistent with the possibility that the oligosaccharide-lipid was an intermediate in glycoprotein synthesis. It was shown that the oligosaccharide-lipid contained both mannose and N-acetyl-glucosamine (24).

Synthesis of a mannose-labeled oligosaccharide-lipid has been studied with mouse myeloma cells (plasma cell tumor) (25). The structure of the oligosaccharide-lipid was proposed to be $(\text{Man})_5-(\text{GlcNAc})_2\text{-P-P-dolichol}$. When purified mannose-labeled oligosaccharide-lipid was added back to the enzyme preparation, transfer of the oligosaccharide chain to endogenous acceptor protein was observed (25). It was found that the oligosaccharide chain in the substrate, oligosaccharide-lipid, was chromatographically identical to that in the product, glycoprotein. This suggested that the oligosaccharide chain was transferred en bloc from the lipid to protein (25).

C. Glycolipids with glycosidic chains of more than ten monosaccharide residues

The existence of large size oligosaccharide-lipids has not been thoroughly researched. Chains of more than ten monosaccharide residues are technically polysaccharides, but are more conveniently included with oligosaccharides, since chain length of more than 20 residues have not been reported.

An example of this is the oligosaccharide-lipid obtained from incubation of dolichol monophosphate glucose with liver microsomal enzymes (26). The compound obtained (GEA) was insoluble in water and soluble in chloroform/methanol/water (10:10:3) extract. The hydrophilic residue of GEA was liberated by mild acid treatment and was shown to be a saccharide of about 20 monosaccharide residues joined through a phosphate group to a lipophilic part.

The only evidence of this kind of oligosaccharide-lipid reported in plants is in Acer cells cultures (2). In this case, [^{14}C]Mannose from GDP-[^{14}C]Mannose was incorporated into plant glycoprotein and lipid intermediates. A large size oligosaccharide lipid was obtained in the chloroform/methanol/water (10:10:3)

fraction. Monosaccharide analysis of the oligosaccharide demonstrated that it was composed primarily of arabinose (2). The oligosaccharide also had arabinose at the reducing terminus, which is unique. The oligosaccharide was about 18 hexose units long. This oligosaccharide-lipid was proposed as a new type of intermediate for biosynthesis of plant arabinosyl glycoproteins (2).

EXPERIMENTAL PROCEDURESI. Cell Cultures

Cultures of Acer pseudoplatanus obtained from Dr. D.T.A. Lamport, Michigan State University, were cultured in B₅ medium (27). (Preparation of media is described in Appendix A). Cells were grown in 1800 ml Fernbach flasks at 22-28°C while being oscillated at 150 rpm. Cells were harvested during early logarithmic growth (7 days after inoculation; doubling time, 5 days).

II. Membrane Isolation (2)

Membrane isolation was carried out at 4°C. The buffer used was 50mM Tricine (pH 7.5)/15mM MgCl₂/2mM 2-Mercaptoethanol/0.4M Sucrose. Cells were homogenized in a Potter-Elvehjem tissue homogenizer in two volumes of buffer which contained in addition 1% polyvinylpyrrolidone, and then filtered through silk screen to remove intact cells. Membranes were concentrated by centrifugation of the filtrate in a GSA rotor at 400 x g for 10 min. Then the membranes were sedimented at 28,000 x g for 90 minutes in the same centrifuge. The sedimented membranes were resuspended in buffer, layered over a pad of 1.46M Sucrose in buffer, and centrifuged at 63,000 x g in a Spinco SW-41 rotor for 30 minutes. Membranes were collected and washed by resuspending in buffer, and centrifuged as before. Membranes were collected and stored frozen.

III. Method of Analysis

Samples were monitored for radioactivity in Triton X-100/Toluene (2:1) with 0.4% PPO (2,5-diphenylloxazole) in a Beckman LS-200B liquid scintillation counter.

IV. Membrane incubation with GDP-[U-¹⁴C]Mannose

0.5 ml of isolated membrane along with 1μCi of GDP-[U-¹⁴C]Mannose were incubated for 30 minutes at 22°C. After the incubation, 5ml of 50mM Tricine (pH 7.5)/50mM NaCl was added. Membranes were then sonicated for 3 minutes. The solubilized membranes were centrifuged at 48,000 x g for 2 to 3 hours to remove

insoluble material.

V. Lipid Extraction (2)

Lipids were extracted from the particulate material recovered from the centrifugation above: Two ml of chloroform/methanol (2:1) were shaken for 1 to 2 minutes with the pellet; phases were separated by centrifugation and the lower chloroform layer removed: this extraction was repeated twice more. These fractions were pooled and back extracted with two 4ml portions of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (3:47:48), that were discarded. Next, the particulate material remaining was extracted with three 2ml portions of chloroform/methanol/water (10:10:3). These fractions were collected by centrifugation. Solvents were removed by a stream of nitrogen before counting the fractions.

VI. DEAE-cellulose column preparation and purification of lipid-linked oligosaccharide (28)

DEAE-cellulose was obtained from Whatman (DE52) and converted to the acetate form as follows: soaked in 0.5N HCl for 30 minutes (15ml per gram of dry adsorbent); washed with water to pH 4; soaked in 0.5N NaOH for 30 minutes and washed with water to pH 8; repeat NaOH treatment, and wash.

DEAE-cellulose was stirred overnight with glacial acetic acid, and the column packed with the slurry. Then the column was washed with methanol (75ml), glacial acetic acid (80ml), methanol (about 100ml to achieve pH 4.5), chloroform (80ml), and chloroform/methanol/water (10:10:3) (about 250ml).

The sample was applied in chloroform/methanol/water (10:10:3) and the column was washed with 120ml of this solvent mixture. A linear gradient was then begun with 159ml of chloroform/methanol/water (10:10:3) in the mixing chamber and 159 ml of chloroform/methanol/0.2M ammonium acetate, pH 6 (10:10:3) in the reservoir. 4ml fractions were collected and 1ml from alternate fractions was taken to dryness and counted.

Appropriate fractions were collected and the solvent was evaporated under

a N_2 stream. The sample was washed several times with water and lyophilized to eliminate the ammonium acetate.

VII. Lipid-linked oligosaccharide incubated with membrane

Lipid-linked oligosaccharide from chloroform/methanol/water (10:10:3) extract was evaporated to remove the chloroform and methanol solvents. Sample was then incubated with 0.5ml membrane preparation and the procedure explained in part II for the incubation of membrane with GDP-[U- 14 C]Mannose was followed.

VIII. Chromatographic Procedures

Sephadex G-50, G-100 and Sepharose 4B were obtained from Sigma Chemical Co. and columns prepared according to manufacturer's instructions.

Paper chromatography was done on Whatman #1 paper using chloroform/methanol/water (10:10:3) or 1-butanol/ethanol/water (40:11:19).

IX. Monosaccharide Analysis

Hydrolysis: sample was hydrolyzed in 2N HCl at 100°C for 2 hours. The hydrolyzate was evaporated to dryness under vacuum overnight. The residue was then kept in a dessicator with NaOH (29).

Reduction: the residue was dissolved in 30ul of 0.05N NaOH and labeled by mixing with 10ul of 30mM solution of NaB^3H_4 in N,N-dimethylformamide, and incubated at room temperature for 4 hours. The reaction was stopped by adding 100ul of 1M acetic acid and evaporated to dryness. The residue was evaporated with water three times (29).

N-Acetylation: the sample was incubated with 0.2ml of saturated sodium bicarbonate solution and 10ul of acetic anhydride at room temperature for 10 minutes, and this procedure was repeated two more times. The reaction was kept in boiling water for two minutes and then passed through a short column (bed

volume-3ml) of mixed bed ion-exchange resin (Bio-Rex RG grade mixed bed resin, RG 501-X8). Column was washed three times with bed volume of water. Eluate and washing were combined, and evaporate to dryness (2).

Analysis: HPLC (2)

Sample was reconstituted with 0.5M borate buffer and fractionated on an HPLC column (250 x 3.2mm) of DA-X8-11 cation exchanger in the borate form.

X. Mild Acid Hydrolysis

Glycoprotein samples were subjected to mild acid hydrolysis to test for acid labile linkage (e.g., sugar-phosphate residues). Samples were adjusted to pH 2 with 1N HCl and hydrolyzed for 15 minutes at 100°C. Samples were cooled in ice and the pH adjusted back to 7.5 with 0.5N NaOH. Sample was then applied to a Sepharose 4B column for separation of the components.

XI. Alkaline Treatment

In order to examine the nature of the carbohydrate-peptide linkage, samples were subjected to mild alkaline hydrolysis (β -elimination). Samples were dialyzed overnight against water. Then samples were treated with 0.1N NaOH and 0.3M NaBH₄, and incubated at 45°C for 10 hours. After incubation, samples were acidified with 1N acetic acid to pH 5.3 and allowed to stand for 1 hour.

RESULTS AND DISCUSSIONI. Incorporation of labeled sugars into lipids soluble in chloroform/methanol/
water (10:10:3)

The long chain oligosaccharide glycolipid described by Barr (2) as OLI (oligosaccharide I) obtained from Acer membranes had been made radioactive by incubation of the membranes with GDP-[U-¹⁴C]Mannose. The main sugars were reported to be arabinose, galactose and glucose, with relatively low amounts of mannose and fucose. The incorporation of radioactivity was comparatively low, reflecting the low concentration of mannose and fucose. Seemingly a more efficient precursor than GDP-mannose could be found, in which one of the major sugars was a constituent. L-[U-¹⁴C]Arabinose and UDP-[U-¹⁴C]Glucose were available for testing. Incorporation of arabinose assumes the presence of the appropriate kinase and pyrophosphorylase. UDP-glucose is known to be a precursor for arabinose, galactose and many other sugars. It should be an efficient precursor provided that the appropriate enzymes are present in the membrane.

The relative efficiencies of arabinose, UDP-glucose and GDP-mannose as precursors for glycolipid biosynthesis are compared in Table I. It can be seen that GDP-mannose is considerably more efficient than either UDP-glucose or arabinose for incorporation of activity into the chloroform/methanol/water (10:10:3) extract, although UDP-glucose was more efficient for incorporation into chloroform/methanol (2:1) soluble extract.

Table I Relative efficiencies of arabinose, UDP-glucose and GDP-mannose for incorporation of radioactivity into Acer membranes

Fraction	CPM/ml (^{14}C)		
	L - Ara	UDP - Glc	GDP - Man
Supernatant	508300	565440	570930
$\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1)	12340	121180	33099.3
$\text{CHCl}_3/\text{CH}_3\text{OH}/$ H_2O (10:10:3)	10814	4046	24550.7

Membranes (0.5ml) were incubated for 30 minutes at room temperature with 1uCi of the labeled sugars. After dilution, sonication, and centrifugation, the pellet was extracted with three 2ml portions of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) which were pooled and back extracted with two 4ml portions of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (3:47:48). The back extractions were discarded. Next the residue was extracted with three 2ml portions of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (10:10:3). These extracts were pooled. All extracts were evaporated to dryness before counting.

Some experiments were also conducted in which the cells were incubated with L-arabinose. 40 grams of whole cells were incubated with 5uCi of L-[U-¹⁴C] Arabinose at room temperature for 15 minutes. Cells were then lyophilized, mixed with sand and ground with mortar and pestle; then extracted three times with 10ml of chloroform/methanol (2:1) and three times with 10ml of chloroform/methanol/water (10:10:3). A small amount of radioactivity was incorporated into the lipid fractions (CM-2:1=394.2 CPM/ml and CMW-10:10:3 = 1242.3 CPM/ml). When the chloroform/methanol/water (10:10:3) extract was chromatographed on Whatman #1 paper using chloroform/methanol/water (10:10:3) as solvent, a radioactive peak was obtained which coincided with a standard of arabinose, rather than OLI.

It is known that D-glucose and L-arabinose when incubated with sycamore suspensions cultures (30) serves as precursors of the pectic polysaccharides. Incubation of arabinose with a culture of sycamore cells yielded a radioactive neutral arabinan-galactan. This neutral arabinan-galactan component was apparently incorporated into pectin (30). No one has yet determined if glycolipids are precursors for pectins, but since ¹⁴C Arabinose turned out to be a poor source of radioactive lipid it was not examined further.

A. Paper chromatography of lipid fractions

The chloroform/methanol/water (10:10:3) extracts from the UDP-[U-¹⁴C] Glucose and L-[U-¹⁴C]Arabinose incubations were characterized by paper chromatography on Whatman #1 paper using chloroform/methanol/water (10:10:3) as solvent (Figure 6 and Figure 7). In addition the extract derived from radioactive arabinose was chromatographed in the carbohydrate solvent 1-butanol/ethanol/water (40:11:19) (Figure 8).

The radioactive peak associated with L-[U-¹⁴C]Arabinose incubation had an R_f value of about 0.74 coinciding with a standard of arabinose. It appeared that the presence of the radioactivity was due to the sugar itself. To further verify this, sample and standard were chromatographed on Whatman #1 paper using 1-butanol/ethanol/water (40:11:19) and paper strips were counted. Again results

Figure 6. Paper chromatography of the chloroform/methanol/water (10:10:3) fraction obtained by incubation of membrane with L-[U-¹⁴C]Arabinose on Whatman #1 paper using CHCl₃/CH₃OH/H₂O (10:10:3) solvent.

(● — ●) CPM-¹⁴C/strip

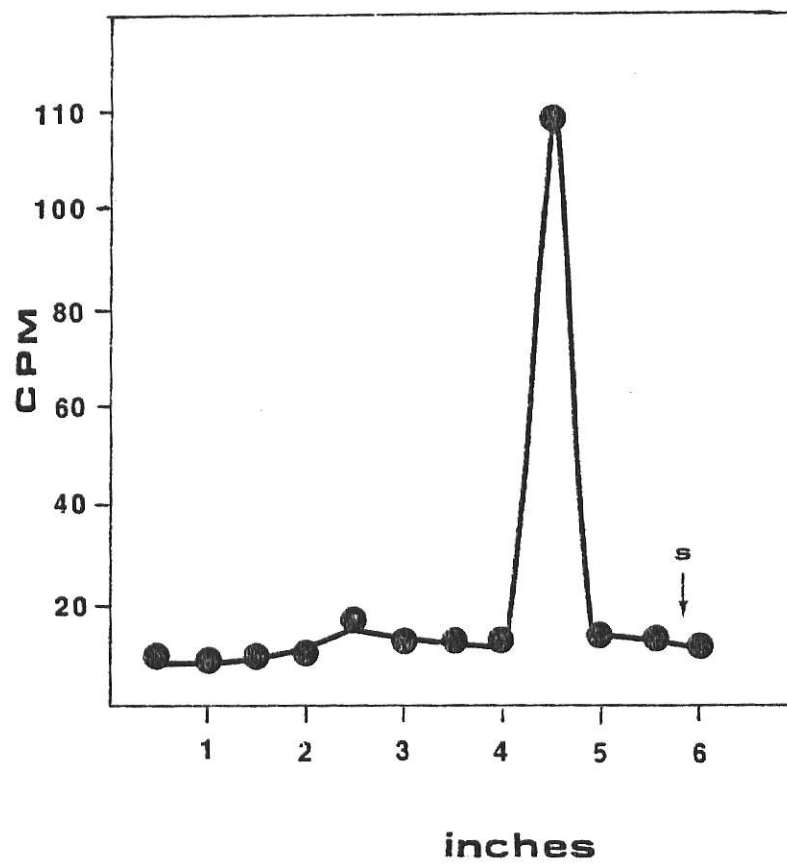
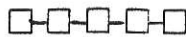


Figure 7. Paper chromatography of the chloroform/methanol/water (10:10:3) fraction obtained by incubation of membrane with UDP-[U-¹⁴C] Glucose, on Whatman #1 paper using CHCl₃/CH₃OH/H₂O (10:10:3) solvent.

() CPM-¹⁴C/strip

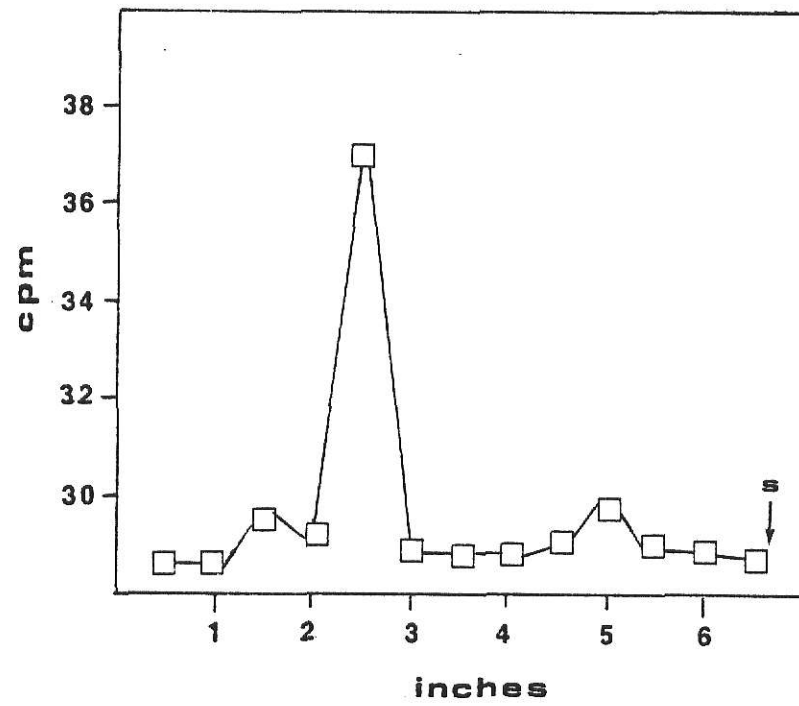
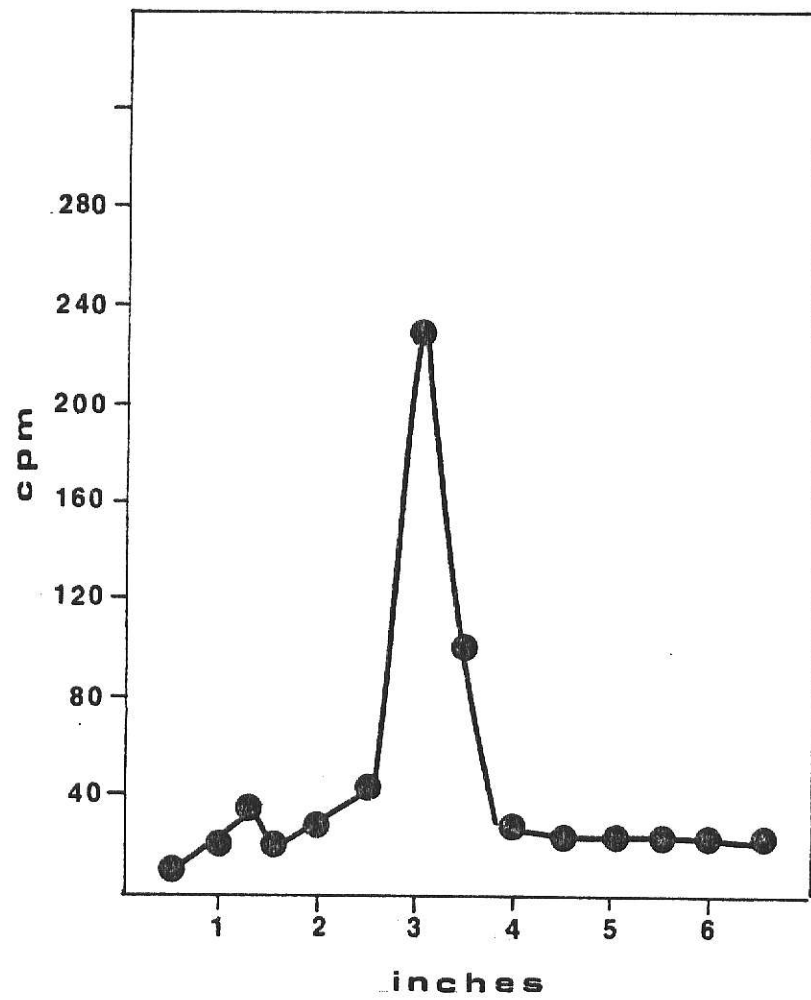


Figure 8. Paper chromatography of the chloroform/methanol/water (10:10:3)
(from incubation with L-[U-¹⁴C]Arabinose) on Whatman #1 paper using
1-butanol/ethanol/water (40:11:19).

(● — ●) CPM-¹⁴C/strip



showed that the radioactivity present was due entirely to the labeled sugar (Figure 8).

Paper chromatography experiments (Figure 6, Figure 7 and Figure 8) were done several times. We were interested in finding the position of the radioactivity rather than the amount of radioactivity incorporated into the chloroform/methanol/water (10:10:3) fraction. Small amounts of samples were applied since we did not want to waste sample which could be used for further analyses. In each case samples were counted three times in order to decrease the counting error, and results showed the position of the radioactivity to always coincide.

In the case of UDP-[U-¹⁴C]Glucose a large peak of lower R_f value (about 0.40) was found (Figure 7) associated with the chloroform/methanol/water (10:10:3) fraction, along with two peaks of insignificant radioactivity. It should be noted that a considerable amount of radioactivity was incorporated into the chloroform/methanol (2:1) fraction. It has been reported (31) that incubation of UDP-glucose with cotton fibers produced a great incorporation of radioactivity into the chloroform/methanol (2:1) extract. This radioactivity was associated with sterol glucosides as well as isoprenyl-phosphoryl-glucoside. The isoprenyl glucoside was separated from sterol glucosides by thin layer chromatography using chloroform/methanol/water (65:25:4) as solvent.

Sterol glucosides occur in the membranes of plants and it has been shown that ¹⁴C-glucose from UDP-[U-¹⁴C]Glucose is incorporated into these endogenous sterols to form sterol glucosides (32). Glucose is attached to the β -hydroxyl position of the sterol, and the glucose moiety can be acetylated with long chain fatty acids to form esterified sterol glucosides (32). It is known that the glycolipid fraction obtained from UDP-glucose incubation can be separated into two distinct components by chromatography on silica gel plates in a chloroform/methanol/water (85:15:0.5) solvent. The two glycolipids were identified as SG

(steryl glucoside - $R_f = 0.43$) and ESG (esterified steryl glucoside - $R_f = 0.61$). The sterol composition was shown to be β -sitosterol (over 75%) and the sugar released by hydrolysis was identified as glucose (31).

In the present case, the radioactivity associated with the chloroform/methanol (2:1) extract could be due to presence of both the isoprenyl glucoside and sterol glucosides. UDP-[^{14}C] was not further examined, since the interest here was with the chloroform/methanol/water (10:10:3) lipid, and ^{14}C -glucose was a poor source of radioactive lipid.

III. Purification of the chloroform/methanol/water (10:10:3) oligosaccharide-lipid

Membrane incubation with GDP-[U- ^{14}C]Mannose was done as explained in Experimental procedures. The pellet was extracted with the lipid solvents and the chloroform/methanol/water (10:10:3) fraction obtained was purified on a DEAE-cellulose column.

Chromatography on DEAE-cellulose column of the chloroform/methanol/water (10:10:3) soluble lipid from the Acer cells incubation, indicated the presence of a single labeled component which was eluted at an ammonium acetate concentration of approximately 100mM (Figure 9). This indicated that the chloroform/methanol/water (10:10:3) extractable compound had a high negative charge since it is known that dolichyl mannosyl monophosphate emerged at a much lower buffer concentration (about 7mM) (28). Dolichyl pyrophosphate oligosaccharides were eluted at 67mM ammonium concentration (28) reasonably close to our value, suggesting the presence of a pyrophosphate linkage.

To purify the compound further, the chloroform/methanol/water (10:10:3) extractable compound was chromatographed on Whatman #1 paper using chloroform/methanol/water (10:10:3). A single peak was obtained (Figure 10). This single peak was in the same region as the oligosaccharide lipid isolated by Barr (2) and referred to as OLI.

Figure 9. DEAE-cellulose chromatography of lipid obtained by extraction with chloroform/methanol/water (10:10:3) after incubation of Acer membrane with GDP-[U-¹⁴C]Man. Chromatography was performed on a column equilibrated with CHCl₃/CH₃OH/H₂O (10:10:3). A linear gradient of 0.2M ammonium acetate, pH 6.0, in chloroform/methanol/water (10:10:3) was begun at tube 30. Four ml fractions were collected and 1ml from alternate fractions counted after evaporating the solvent. (●—●) CPM-¹⁴C/ml

Expected elution volume for the following (28):

1. neutral sugars
2. dolichyl mannosyl phosphate
3. dolichyl pyrophosphate oligosaccharide

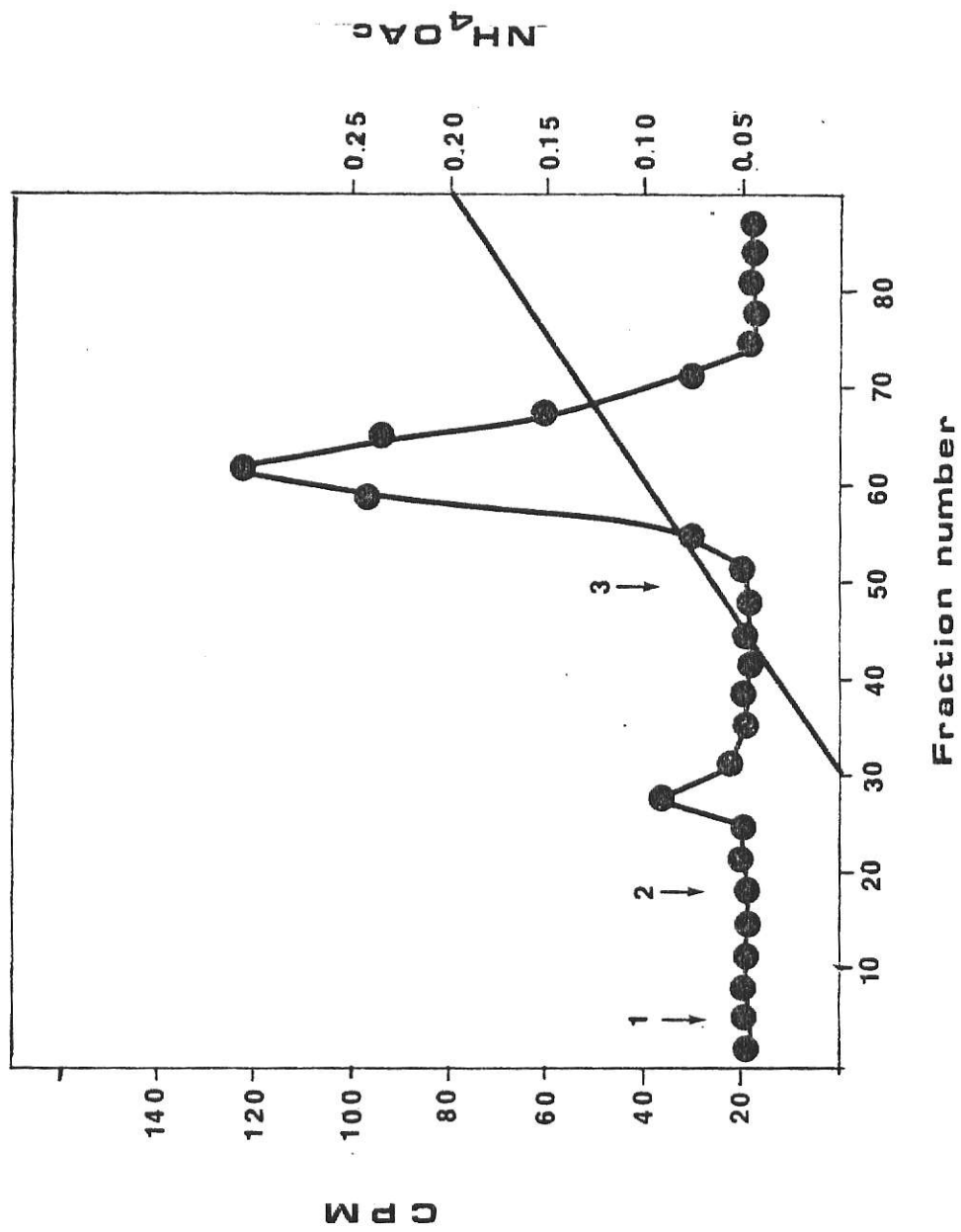
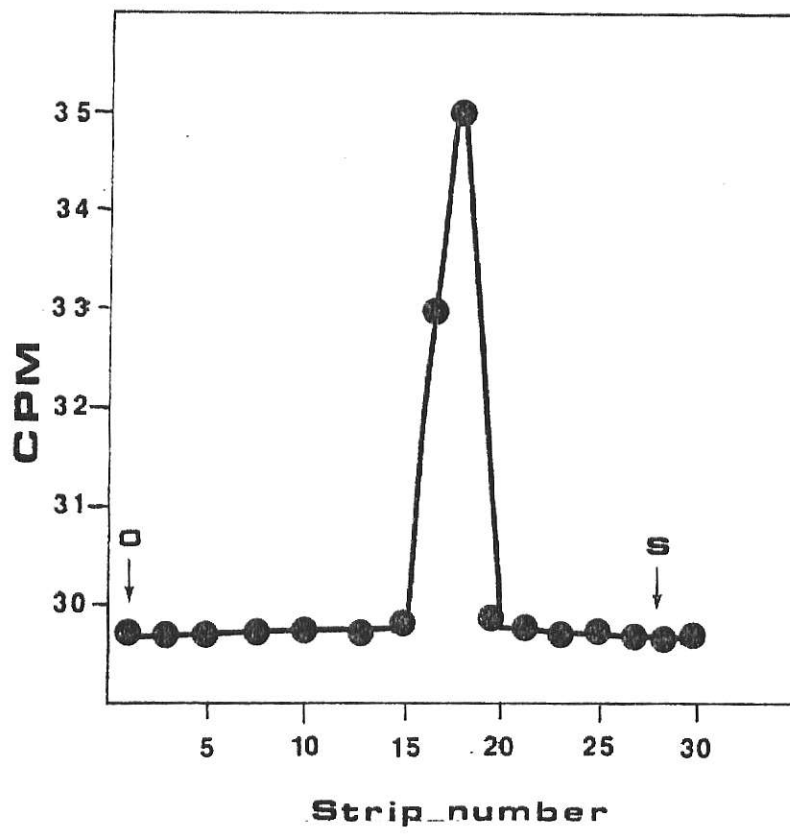


Figure 10. Paper chromatography, after purification on a DEAE-cellulose column, of the oligosaccharide-lipid. Oligosaccharide-lipid was applied to Whatman #1 paper and chromatographed using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (10:10:3) as solvent. 0.5cm strips were counted.

O - origin

S - solvent front

CPM- ^{14}C /strip



A. Characterization of the monosaccharide components of the chloroform/
methanol/water (10:10:3) oligosaccharide lipid

Although carbohydrates components of the oligosaccharide lipid (OLI) reported by Barr had been determined (2), the lipid fraction was not purified before characterization. The carbohydrate composition was quite complex suggesting either impurities or an exceedingly unusual oligosaccharide. Therefore the purified glycolipid was reanalyzed for comparison.

The chloroform/methanol/water (10:10:3) sample was collected from appropriate fractions after DEAE-cellulose column separation. The fraction was evaporated to dryness and the procedure for monosaccharide analysis was followed (see Experimental procedures).

After the sample was prepared, it was dissolved in 0.5M borate buffer (pH 8.63) and applied to the HPLC column. Sample was eluted using the same buffer. Fractions were collected (3 drops/fraction) and assayed for radioactivity. Separation of the monosaccharide components can be seen in Figure 11.

When the results obtained here are compared with the results reported by Barr some differences can be seen. An extra peak was found following the fucose peak that was not identified. The first large peak obtained at the beginning is due to contaminants present in the NaB^3H_4 ; but the second large peak was not reported before by Barr and could even be a new sugar not reported before.

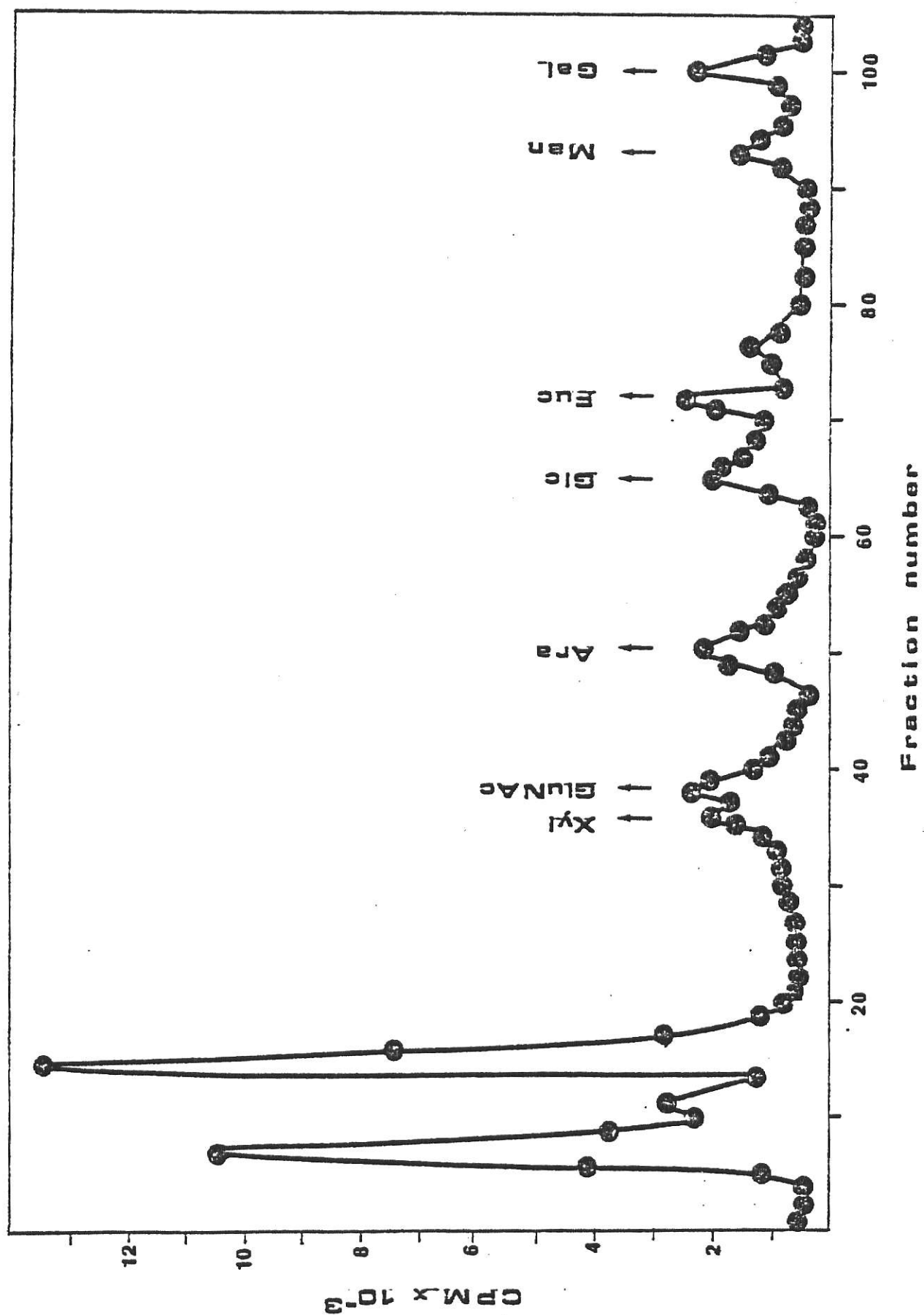
III. Involvement of the chloroform/methanol/water (10:10:3) oligosaccharide-lipid in glycoprotein biosynthesis

It has been shown that incubation of liver particulate enzymes with dolichyl glucosyl phosphate produced a polar oligosaccharide-lipid (about 20 monosaccharide residues) (33). When this oligosaccharide-lipid was incubated with liver microsomes, transfer of the entire oligosaccharide-lipid to an endogenous protein acceptor occurred. The oligosaccharide moiety was transferred to a product insoluble in trichloroacetic acid but became soluble after incubation with pronase or crystalline trypsin (34). The product form at this step was found to be an amphoteric substance when subjected to electrophoresis, suggesting that the oligosaccharide was indeed transferred to protein (34).

Thyroid slices also were found to incorporate radioactivity from ^{14}C -labeled sugars (GDP-mannose and UDP-glucose) into the carbohydrate moiety of a polar lipid soluble in chloroform/methanol/water (10:10:3) (28). The size of the ^{14}C -labeled oligosaccharide (2,400) indicated that it contains 14 to 15 monosaccharide residues. To show the role of the oligosaccharide-lipid as an intermediate in thyroid glycoprotein biosynthesis, thyroid slices incubations were performed in the presence of puromycin (puromycin cause a marked inhibition of protein formation in this system (28)). Disappearance of the

Figure 11. Chromatographic separation of [^3H]alditols from acid hydrolyzate of oligosaccharide lipid. Sample in 0.5M borate (pH 8.63) was applied to an HPLC column (250 x 3.2mm column of DA-X8-11 cation exchange in the borate form) and eluted with 0.5M borate (pH 8.63). Three drops/sample were collected and assayed for radioactivity.

(● — ●) CPM- ^3H /3 drops fraction



radioactivity from the lipid bound oligosaccharide and its appearance in protein-bound form was shown by a pulse chase experiment. Results showed that the transfer of the oligosaccharide from the lipid to protein was blocked when using puromycin, verifying in vivo transfer of the oligosaccharide to protein.

A. Isolation of a high molecular weight fraction

To test whether the chloroform/methanol/water (10:10:3) oligosaccharide-lipid was also a precursor for glycopeptides, the isolated lipid was added to the Acer membrane preparation (0.5ml) and incubated at room temperature for 30 minutes. The incubation mixture was sonicated, centrifuged, and the supernatant was applied to a Sephadex G-50 column. A substantial amount of radioactivity was associated with the G-50 V_0 -peak. Two small peaks were found eluting from the G-50 column at the point where free oligosaccharide elutes (Figure 12).

B. Characterization of the high molecular weight fraction

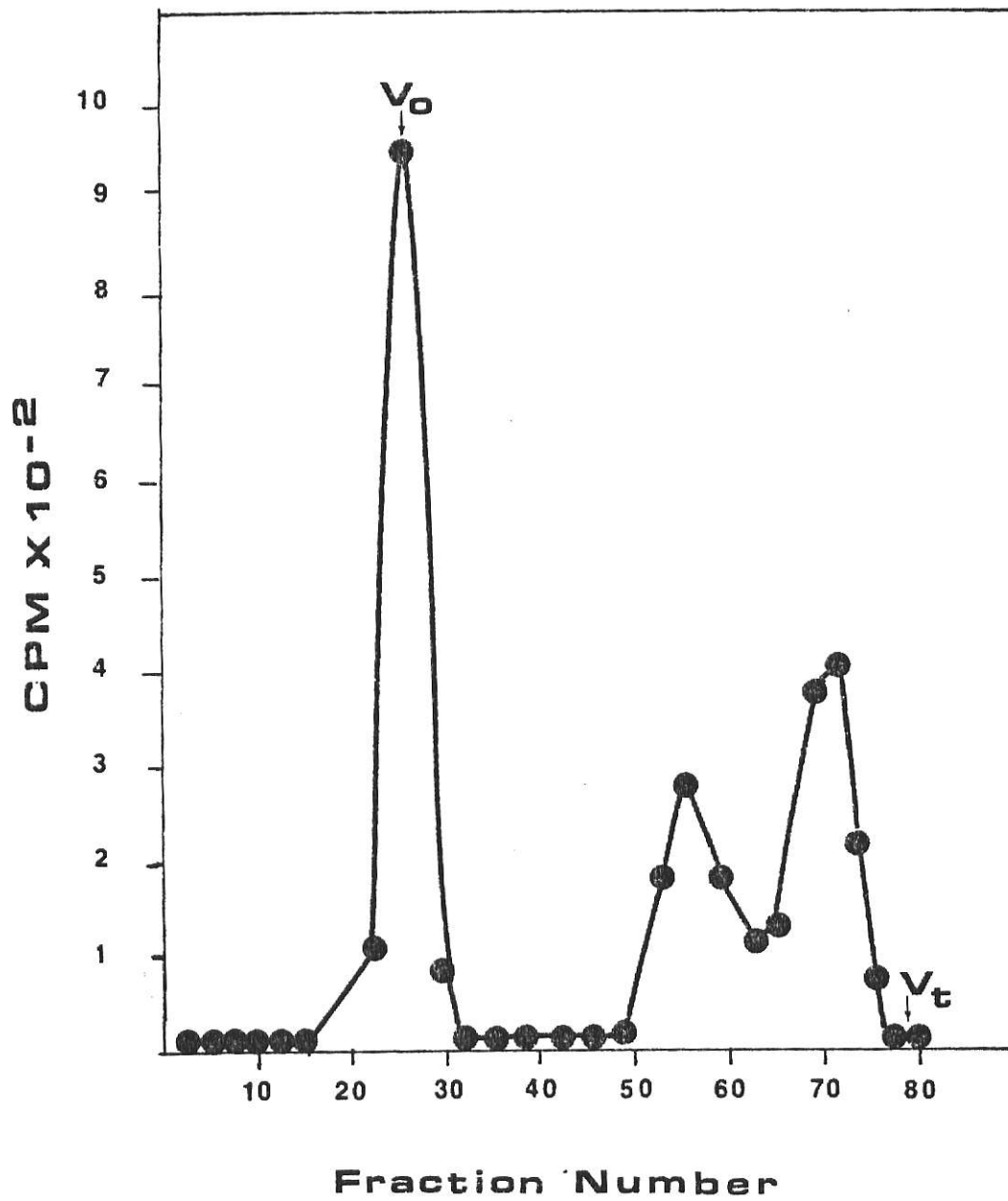
To check the size of the G-50 V_0 -peak, the sample was chromatographed on a Sephadex G-100 column and on a Sepharose 4B column. In both cases all the radioactivity was associated with the V_0 -peaks suggesting the presence of a large molecular weight compound.

The sample was subjected to mild acid hydrolysis to test for acid labile linkages and subjected to mild alkaline treatment (β -elimination) to examine the nature of the carbohydrate-linkage. This alkali treatment under reducing conditions converts the peptide-linked monosaccharide to the corresponding sugar of the linkage region. The O-glycosidic linkages to serine or threonine are very labile to alkali (e.g., GalNAc-Ser, GalNAc-Thr, Xyl-Ser), while sugar residues attached to hydroxylysine, hydroxyproline or asparagine are alkaline stable.

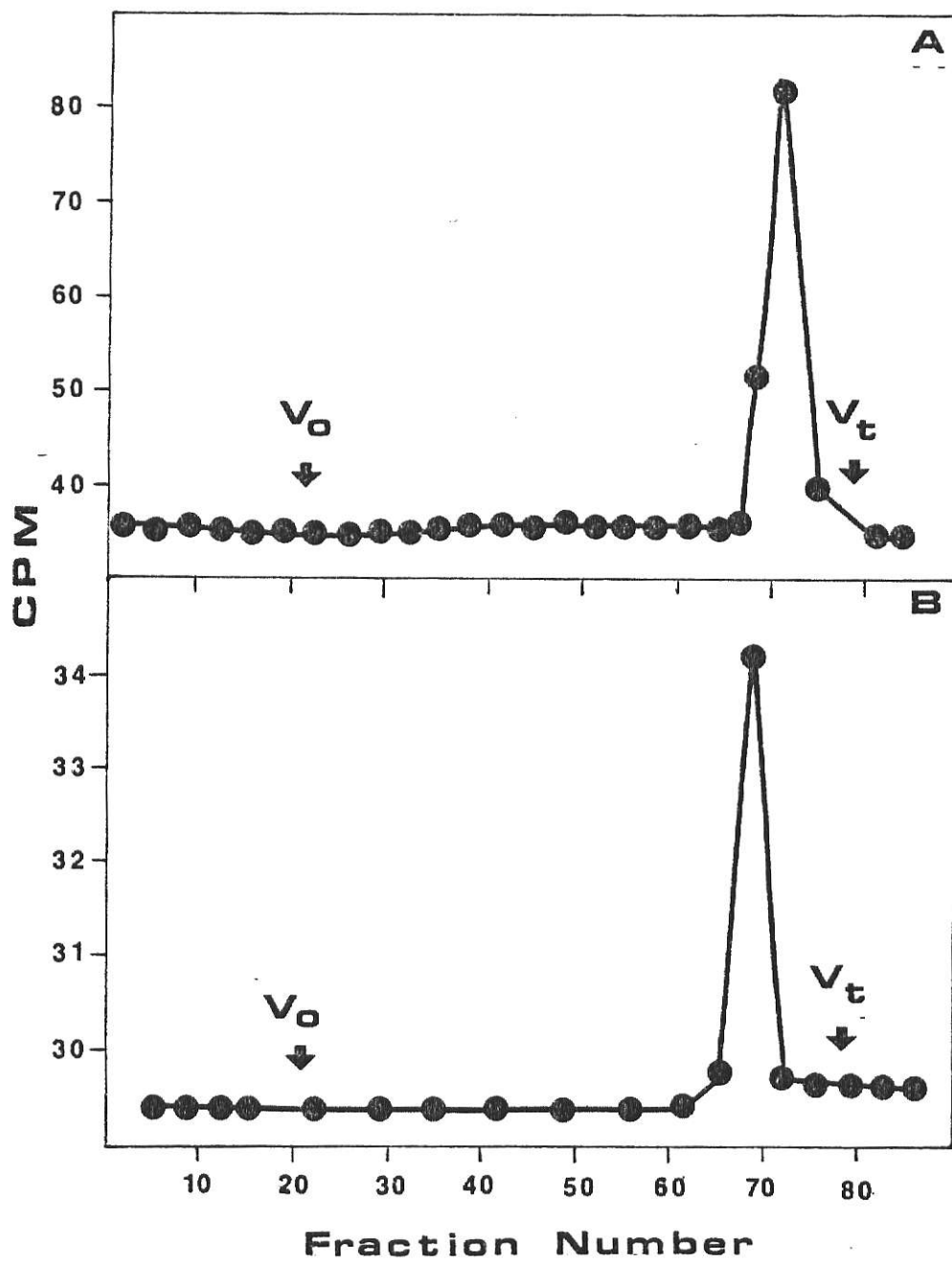
In this case the experiments showed the compound to be acid and base labile. When each sample was separated on a Sepharose 4B column, the radioactivity obtained was associated with a peak near the V_t -point (Figure 13).

Figure 12. Oligosaccharide-lipid was incubated for 30 minutes with 0.5ml membrane, diluted, sonicated and centrifuged, and then applied to a Sephadex G-50 column. 2ml fractions were collected and 0.5ml from alternate fractions was removed for scintillation counting.

(● — ●) CPM-¹⁴C/0.5ml



- Figure 13. (A) 4.0ml of dialyzed sample from G-50 V_o -peak treated with 0.1N NaOH and 0.3M NaBH_4 for 10 hours at 45°C. Sample was then acidified with 1N HOAc to pH 5.3. After 1 hour the pH was adjusted to 6.8 with 0.2N NaOH. Sample was separated on a Sepharose 4B column. 0.5ml from alternate fractions (2ml fractions) were counted.
- (B) 2.0ml from G-50 V_o -peak were adjusted to pH 2 using 1N HCl and hydrolyzed for 15 minutes at 100°C. The pH was adjusted back to 7.5 and the sample chromatographed on a Sepharose 4B column. 0.5ml from alternate fractions (2ml fractions) were counted.



This showed that the linkages involved were readily split by acid and base treatment. There are no known glycoproteins with acid labile mannose. Thus the high molecular weight material must represent a unique type of glycosyl linkage if it is indeed glycoprotein.

CONCLUSION

It would be useful at this point to summarize what is known about the chloroform/methanol/water (10:10:3) oligosaccharide-lipid from Acer cell cultures, to pin point the contributions of the present investigation, and suggest future investigation.

1. It contains a carbohydrate residue attached to a lipid (36) because it is soluble in chloroform/methanol/water (10:10:3) but on mild acid hydrolysis a more polar oligosaccharide is obtained (2).

2. The number of sugar residues of the oligosaccharide was estimated to be about 18 hexose units (2).

3. It can be purified on a DEAE-cellulose column, and this shows that it has a negative charge associated with it. The elution volume is similar to dolichol-pyrophosphate oligosaccharides.

4. When it is incubated with an Acer membrane preparation and chromatographed on a Sephadex G-50 column, a large molecular weight compound is obtained. After treatment with acid and base, this compound is found to be labile to both treatments. This behavior is not typical of glycoproteins. Further studies must be carried out to establish its function.

5. The distribution of this oligosaccharide-lipid in plants has not been studied and further research should be done related to this. This compound has been isolated from sycamore cells (Acer pseudoplatanus) but its presence in other plants has not been studied.

APPENDIX A

I. Media preparation (2)

Three stock solutions were prepared containing the necessary salts:

Solution A

<u>Salt</u>	<u>gm/l</u>
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	1.50
$(\text{NH}_4)_2\text{SO}_4$	1.34
MgSO_4	1.22
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1.50
m-Inositol	1.00
H_3BO_3	0.03
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.02
2,4 dichlorophenoxyacetic acid	0.02

Solution B

<u>Salt</u>	<u>gm/l</u>
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	250
CuSO_4	25
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	25
KI	750

Solution C

<u>Salt</u>	<u>gm/l</u>
EDTA	1.29
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1.15

One liter of working media is composed of:

Solution A	- 100ml
Solution B	- 1ml
Solution C	- 5ml
KNO ₃	- 2.5gm
Sucrose	- 20gm
Pyridoxal·HCl	- 1mg
Nicotinic acid	- 1mg
Thiamine·HCl	- 10mg
H ₂ O	- q.s./l

The pH of the media is adjusted to 5.5 with dilute aqueous KOH. The media is sterilized by autoclaving at 15 lbs. pressure 121° for 20 minutes.

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OLIGOSACCHARIDE GLYCOLIPIDS IN
CULTURES OF ACER PSEUDOPLATANUS

by

LIZETTE SANTOS SANTORI

B. S., University of Puerto Rico, 1978

AN ABSTRACT OF A MASTER'S THESIS

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Department of Biochemistry

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1981

A radioactive oligosaccharide glycolipid was isolated from Acer cell membranes by extraction with chloroform/methanol/water (10:10:3) after incubation of the membranes with GDP-[U-¹⁴C]Mannose. GDP-[U-¹⁴C]Mannose was more efficient for incorporation of radioactivity than either L-[U-¹⁴C]Arabinose or UDP-[U-¹⁴C]Glucose.

The glycolipid was purified by DEAE-cellulose chromatography and by paper chromatography. The glycolipid was shown to have a negative charge associated with it. The elution volume was similar to dolichol pyrophosphate oligosaccharide, suggesting the presence of a pyrophosphate linkage. The R_f value determined was about 0.65.

The monosaccharide composition was determined by High Performance Liquid Chromatography. Mannose, galactose, glucose, fucose, arabinose and xylose (or N-acetylglucosamine) were identified in acid hydrolyzates of the glycolipid.

The radioactive oligosaccharide glycolipid was incubated with Acer membranes and chromatographed on Sephadex G-100 and Sepharose 4B. A radioactive peak was associated with the void volume in each case. The radioactivity of this compound, unlike most glycoproteins, could be released by mild acid or base treatment.