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The Assembly of Lipid-Linked Oligosaccharides in Plant and Animal Membranes

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Membrane preparations from growing regions of pea stems and actively-dividing mouse L-cells form lipid-linked saccharides from GDP-mannose and UDP-N-acetylglucosamine. These lipids have properties which are consistent with those of mono- and di-phosphoryl polyisoprenyl derivatives.

In experiments using plant membranes, the monophosphoryl derivative labeled with GDP-(^{14}C) mannose contains mannose only, while the diphosphoryl derivative labeled with the same nucleotide sugar is heterogeneous, containing oligosaccharides corresponding to mannosaccharides of 5, 7, and 9–12 residues. Only the diphosphoryl polyisoprenyl derivatives are labeled with UDP-(^{14}C)glucosamine and these contain predominantly chitobiose and N-acetylglucosamine itself. Unlabeled GDP-mannose added after UDP-N-acetyl-(^{14}C)glucosamine results in the formation of higher lipid-linked oligosaccharides which are apparently the same as those which are labeled with GDP-(^{14}C)mannose alone. Incubation of the membranes with GDP-(^{14}C)mannose in the presence of Mn^{2+}, unlabeled UDP-glucose or unlabeled UDP-N-acetylglucosamine results in marked changes in the accumulation of both the polyisoprenyl monophosphoryl mannose and polyisoprenyl diphosphoryl oligosaccharides.

Animal cell membranes synthesise lipid-linked oligosaccharides when incubated with UDP-N-acetylglucosamine and GDP-mannose. These oligosaccharides are similar in size to those synthesised by the plant membranes but their formation is more efficient. The potential roles of these compounds in glycoprotein biosynthesis in both plant and animal tissues is discussed.

Key words: pea stem membranes, L-cell membranes, polyisoprenyl oligosaccharides, glycoprotein synthesis

Glycoproteins from both plant and animal cells show a high degree of structural complexity which is thought to confer upon them a unique rôle in basic cellular functions such as cell-surface recognition processes [1, 2]. Structural analysis of the carbohydrate moiety of glycoproteins after release from the parent glycoprotein by enzymic or chemical methods is providing a wealth of detailed knowledge concerning the nature and organisation of the component sugars. Considerable variation between different glycoproteins is evident [3], and even the same glycoprotein may show microheterogeneity with respect to its carbohydrate side-chains, as in the case of ovalbumin [4]. Despite the structural complexity of the saccharide portion of glycoproteins, the initial steps in the assembly of the glycosyl units have been postulated to be similar in diverse cells and

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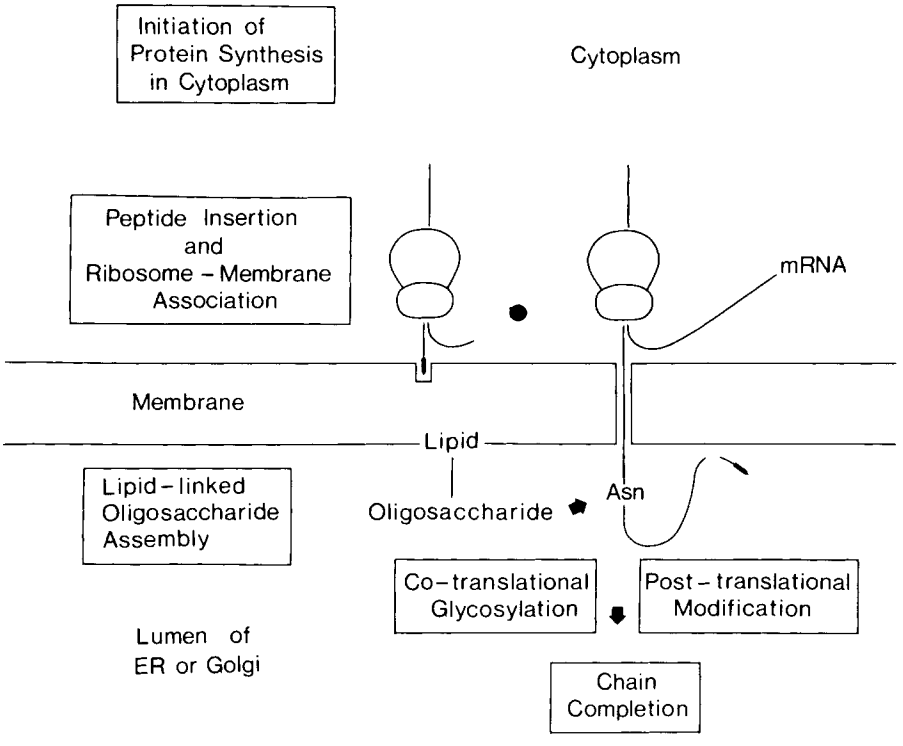

Fig. 1. A schematic representation of the process of N-linked glycoprotein assembly. Protein synthesis is initiated in the cytoplasm. In the case of membrane-associated proteins, the nascent protein is inserted into the membrane, probably by a process involving an N-terminal "signal" [45]. At some point during its passage through the membrane it is glycosylated, probably via a lipid-linked pathway. Upon chain completion, various post-translational alterations may be introduced into both the protein and saccharide moieties to generate the completed glycoprotein.

tissues. The involvement of lipid-linked saccharides as intermediates in these initial events of biosynthesis has been postulated in membranes from both animal tissues [5–7] and micro-organisms [8–10]. Moreover, the existence of a common lipid-linked precursor in the synthesis of some glycoproteins in animal cells has been suggested [11]. A detailed structure of a similar lipid-linked oligosaccharide isolated from virally-infected animal cells has been published [12]. The role played by these intermediates in the overall process of glycoprotein assembly is shown schematically in Figure 1.

In higher plants, the synthesis of lipid-linked sugars from sugar nucleotides has been demonstrated using membranes from various sources [13–20]. In a few instances, these lipid-linked sugars have been suggested to act as direct donors to proteins to form glycoproteins, but the yields of such products have been low and in no case has evidence of complete in vitro biosynthesis of defined glycoproteins been fully documented [13, 21–24]. One added complexity inherent in plant systems is the presence of active polysaccharide-synthesising pathways which are also thought to utilise lipid-linked saccharides as intermediates [25, 26] and may require glycoproteins as initiating primers [27, 28].

We report here the results of a study of the capacities of membrane preparations from actively-growing regions of pea stems to synthesise lipid-linked saccharides from UDP-N-acetylglucosamine, GDP-mannose and UDP-glucose. These pea stem membrane preparations

ANIMAL TISSUE (mouse L-cells)	PLANT TISSUE (pea stems)

1. Homogenise in 0.1M Tris-HCl, pH 7.4, containing 5 mM dithioerythritol, 10 mM $MgCl_2$, 0.4M sucrose at 4°C and filter through cheesecloth.
2. Low-speed centrifugation (1,000 × g, 10 min), discard pellet.
3. High-speed centrifugation (48,000 × g, 60 min), discard supernatant.

PARTICULATE MEMBRANE FRACTION

1. Resuspend in homogenisation medium at protein concentrations greater than 500 μg/50 μl.
2. Incubate at 25°C with radioactive substrates in 75 μl total volume.
3. Terminate reaction by addition of 0.5 ml CM (1:1, v/v), followed by extraction of the membranes with 1 ml CMW (1:1:03, v/v/v).
4. Centrifuge and discard pellet.

TOTAL CMW-SOLUBLE PRODUCTS

1. Add 3 vol 0.5% KCl to separate into 2 phases.
2. Wash lower phase either with water (for DEAE-cellulose chromatography) or twice further with 0.5% KCl.

LIPID-LINKED SACCHARIDES (lower phase plus interface)	WATER-SOLUBLE SACCHARIDES (upper phase plus washings)

Fig. 2. Procedure for the extraction and fractionation of lipid-linked saccharides.

are compared to those from animal cells (mouse L-cells), which they resemble in their ability to generate complex, membrane-bound oligosaccharides from exogenously-supplied nucleotide sugars. We describe the assembly of these complex oligosaccharides on lipid diphosphates and present evidence that heterogeneity exists amongst the oligosaccharides themselves. Our results are consistent with a sequential assembly process for these lipid-linked oligosaccharides which appears to differ only in its efficiency between plant and animal cell membranes.

METHODS

Plant Material and Membrane Preparation

Seedlings from Pisum sativum L.var.Alaska were grown in darkness at room temperature for 7 days. Apical 10 mm segments from the third internode were ground in a mortar in the presence of an equal volume of ice-cold buffer A (0.1M Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM dithioerythritol, 0.4M sucrose). A particulate membrane fraction was then prepared, incubated with substrates and extracted according to the scheme in Figure 2.

Animal Cell Culture and Membrane Preparation

Mouse L-cells (a gift of Dr. R. Sinclair, McGill University) were maintained as a suspension culture in minimal essential medium (Flow Laboratories, Mississauga, Ontario, Canada), supplemented with 2 mM glutamine (Sigma, Missouri), penicillin (Sigma, 63 mg/

litre), streptomycin (Sigma, 70 mg/litre), and 5% fetal calf serum. For labeling experiments, the cells were harvested at a density of approximately 7 x 10^5 cells/ml, pelleted by centrifugation at low speed and washed with phosphate-buffered saline once at room temperature. A particulate membrane fraction was prepared as described for the plant membranes above.

Assay and Labeling Procedures

Particulate membranes were resuspended in Buffer A at protein concentrations greater than 500 μg/50 μl. Aliquots of 50 μl were incubated at 25°C for 20 or 60 min with 5 μl GDP-(^{14}C)mannose (Amersham, 170–230 mCi/mmol) or 5 μl UDP-N-acetyl-(^{14}C)glucosamine (Amersham, 300 mCi/mmol) in a total volume of 75 μl. The initial concentration of labeled nucleotide sugars in the incubation assay was approximately 10 μM. In some tests, the labeled nucleotide sugars were co-incubated with Mn^{2+} or non-radioactive nucleotide sugars. Reactions were terminated by the addition of chloroform and methanol to make an overall concentration of chloroform, methanol and water of 1:1:0.3(v/v/v), henceforth referred to as CMW. In the case of the in vivo labeling of the L-cells with (^{3}H)mannose (Amersham, 22.5 Ci/mmol), incubation was at a cell density of approximately 10^8 cells/ml in a total volume of 50 μl at 37°C, at a substrate concentration of approximately 4 μM.

Characterisation of the Lipid-Linked Saccharides

The "lower phase plus interface" material shown in Figure 2 was redissolved by adding a few drops of methanol, and the resultant solution was separated on DEAE-cellulose columns (Whatman DE52; 18 × 20 mm, 10 ml bed volume), using NH_4COOH dissolved in CMW as eluant [29], as described in the legend to Figure 4. Both CMW extracts, washed CMW extracts and purified lipids from DEAE-cellulose columns were hydrolysed in 0.1 N HCl at 90°C for 30 min [7]. The water-soluble oligosaccharides produced were characterised by Biogel P2 (200–400 mesh) gel permeation chromatography on 100 × 1 cm columns.

RESULTS

Lipid-Linked Saccharides Formed From GDP-(^{14}C)Mannose by Pea Membranes

In order to monitor the total population of free and lipid-linked oligosaccharides synthesised from GDP-(^{14}C)mannose by pea membranes, a CMW extract was obtained according to the scheme outlined in Figure 2. This extract was subjected to mild acid hydrolysis and the water-soluble products were separated by gel permeation chromatography. A complex, but regular pattern of mannosaccharides was observed (Fig. 3a), from which an apparently unbroken series could be resolved. This series appeared to extend to at least the equivalent of a decasaccharide, assuming the higher oligosaccharides to be linear, unbranched chains. The series corresponds closely in elution volume to that reported for acetolysates of yeast mannans [30] and less exactly to a series of cellodextrins run on the same columns.

CMW extracts from these membranes contain lipid-linked saccharides and also water-soluble glycosides. In order to separate these two classes, the CMW extract was partitioned into chloroform ("lower phase plus interface") and methanol-water (upper) phases by the addition of 0.5% KCl (see Fig. 2). The salt washes contained a variety of free saccharides, and were discarded. The lipid phase, after mild acid hydrolysis and gel permeation chromatography, was shown to contain predominantly high molecular weight components (Fig. 3b). A prominent monosaccharide peak and intermediate peaks corresponding to the trisaccharide, pentasaccharide and heptasaccharide of the series shown in Figure 3a were also apparent.

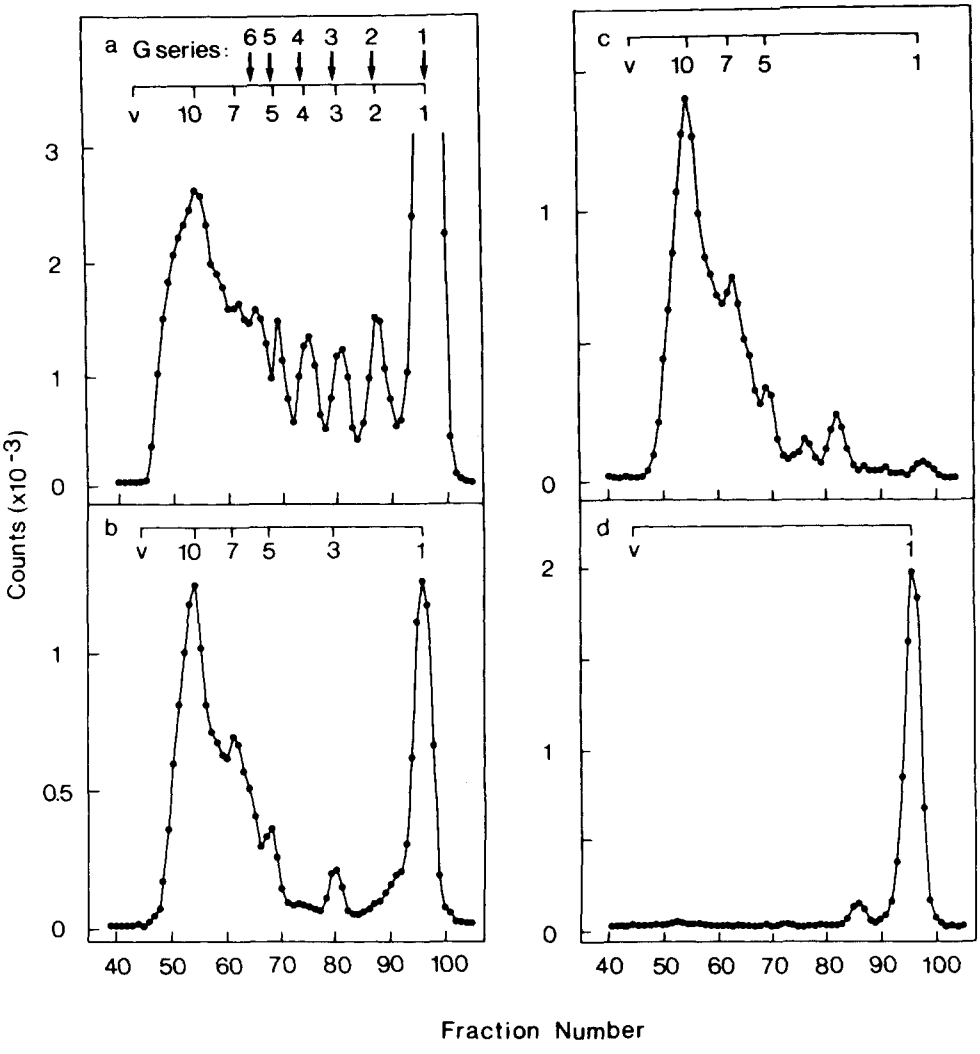

Fig. 3. a) Biogel P2 gel permeation chromatography of the hydrolysis products (0.1 N HCl, 90°C, 30 min) of a total CMW extract of pea membranes after incubation with GDP-(^{14}C)mannose for 60 min. Fraction volume is 0.8 ml. Elution volumes of cellodextrins run on the same columns are indicated above (G series, 1–6). Elution volumes of the lower mannosaccharides correspond to those reported in the literature [30]. The numbering above the profiles in subsequent figures refers to the presumed mannosaccharide series indicated on the scale above this figure; b) Biogel P2 gel permeation chromatography of the hydrolysis products of the lipid ("lower phase plus interface") obtained after partitioning the CMW extract with 0.5% KCl as described in Figure 2, using pea membranes which were labeled with GDP-(^{14}C)mannose for 60 min; c) Biogel P2 gel permeation chromatography of mild acid hydrolysates of the material eluted from DEAE-cellulose (see Fig. 4) by chloroform : methanol : 140 mM ammonium formate; d) Biogel P2 gel permeation chromatography of mild acid hydrolysates of the material eluted from DEAE-cellulose by chloroform : methanol : 5 mM ammonium formate.

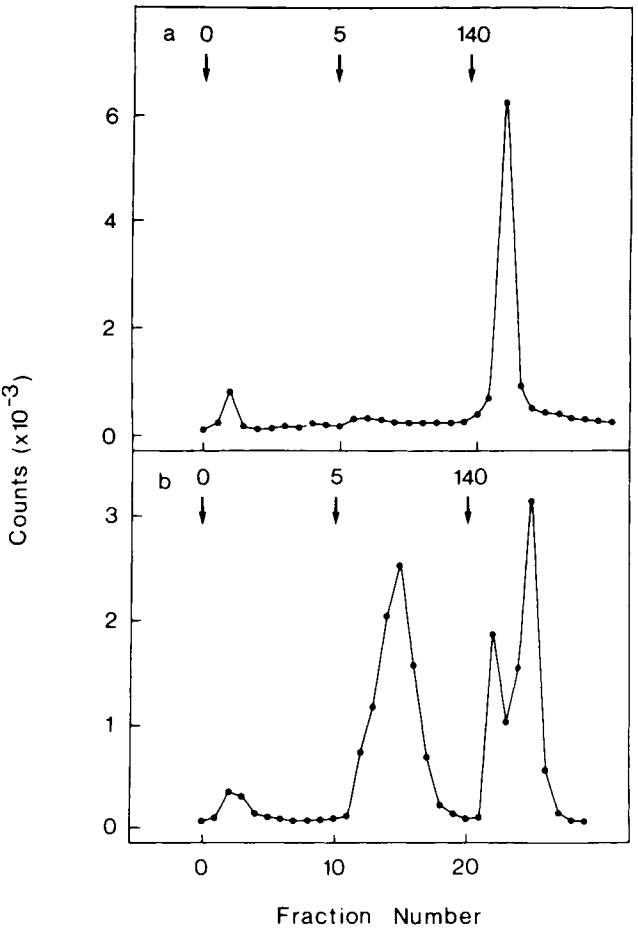

Fig. 4. DEAE-cellulose chromatography of an aliquot of the total CMW extract of membranes labeled with (a) UDP-N-acetyl-(^{14}C)glucosamine alone, and (b) GDP-(^{14}C)mannose alone, both for 60 min. The "lower phase plus interface" material obtained after 0.5% KCl partitioning and two water washes was applied to the column in CMW (1 : 1 : 0.3), with steps of chloroform : methanol : 5 mM ammonium formate (indicated by the arrow at "5"), and chloroform : methanol : 140 mM ammonium formate (arrowed at "140"). The fraction volume in both cases was 10 ml.

The lipids synthesized by pea membranes may be separated into distinct classes by DEAE-cellulose chromatography as shown in Figure 4. The profile obtained when a CMW extract from an incubation with GDP-mannose was applied to the column after partitioning against 0.5% KCl is shown in Figure 4b. There are two peaks, one eluting with 5 mM ammonium formate, and corresponding to polyisoprenyl monophosphoryl mannose as reported in other studies [31], the other eluting with 140 mM ammonium formate, corresponding to polyisoprenyl diphosphoryl oligosaccharide.

Mild acid hydrolysis of the diphosphate-linked saccharides yields a profile essentially identical to that obtained by hydrolysis of the complete lower phase after partitioning, minus the monosaccharide peak (Fig. 3c), whereas hydrolysis of the peak corresponding to the monophosphate-linked saccharide yields only mannose and no higher oligosaccharides (Fig. 3d). It is clear, therefore, that these two classes of lipids bear the entire

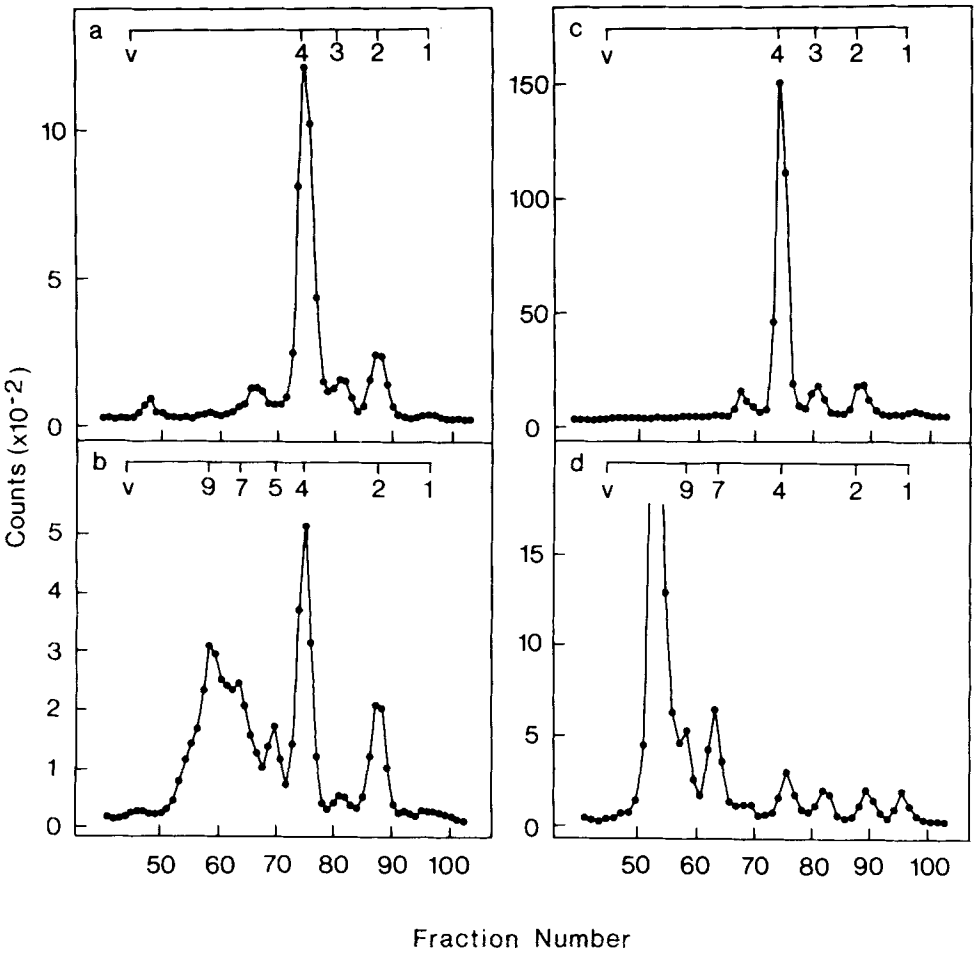

Fig. 5. Biogel P2 gel permeation chromatography of mild acid hydrolysates of the "lower phase plus interface" fractions (see Fig. 2), obtained after labeling pea stem membranes, (a), and mouse L-cell membranes, (c), with UDP-N-acetyl-(^{14}C)glucosamine alone for 60 min. 10 mM GDP-mannose was added to the UDP-N-acetyl-(^{14}C)glucosamine-initiated incubations after 20 min. Biogel P2 gel permeation chromatography profiles of mild acid hydrolysates of the lipid phase labeled under these conditions is shown for the pea membranes in (b), and for the L-cell membranes in (d).

range of saccharides observed to be present in the lipid phase (Fig. 3b), and that their saccharide profiles correspond to those expected of the mono- and diphosphoryl polyisoprenyl derivatives.

Lipid-Linked Saccharides Formed From UDP-N-acetyl-(^{14}C)Glucosamine by Pea Membranes

An analysis of the CMW extract after incubation of pea membranes with UDP-N-acetyl-(^{14}C)glucosamine for 60 min was undertaken in the same manner as described previously for the mannose-labeled membranes. DEAE-cellulose chromatography of the 0.5% KCl-washed CMW extracts yielded the profile shown in Figure 4a. No apparent monophospholipid-linked saccharide was formed, only that which was eluted with high salt concentrations. When this lipid was hydrolysed under mild acid conditions and the water-soluble moieties fractionated by gel permeation chromatography, the profile shown in Figure 5a was ob-

tained. In this system, glucosamine elutes in a position corresponding to a monosaccharide, N-acetylglucosamine elutes in the position of a disaccharide, while chitobiose elutes as an apparent tetrasaccharide. The major polyisoprenyl diphosphate-linked saccharide which accumulated during the incubation was chitobiose. Small amounts of N-acetylglucosamine and components comparable in elution volume to mannotriose and mannohexaose and higher oligosaccharides eluting near the void volume can also be seen.

Addition of unlabeled GDP-mannose (10 mM) to the incubation after 20 min of UDP-N-acetyl-(^{14}C)glucosamine preincubation resulted in the formation of substantial amounts of labeled higher oligosaccharides (Fig. 5b), concomitant with a reduction in the amount of chitobiosyl-lipid (peak 4). The peaks generated during the cold chase resemble those obtained with GDP-(^{14}C)mannose alone (Fig. 3c), suggesting that the N-acetylglucosamine- or chitobiose-linked lipids may act as acceptors for additional mannose units available directly or indirectly from the exogenous GDP-mannose supplied during the cold chase.

It should be noted that there could have been little GDP-mannose endogenous to the membrane preparations to enable the completion of the N-acetylglucosamine-initiated oligosaccharides in the absence of exogenously-supplied GDP-mannose since very little higher oligosaccharide became labeled under these conditions (Fig. 5a).

Effect of Incubation Conditions on Lipid-Linked Saccharide Assembly by Pea Membranes

The above results suggest that endogenous levels of nucleotide sugars may act as controlling elements in the assembly of lipid-linked saccharides. With this in mind, Mn^{2+}, UDP-glucose, and UDP-N-acetylglucosamine, all at 10 mM final concentration, were added to the membranes shortly before adding GDP-(^{14}C)mannose in order to ascertain the effects of these exogenous components on the assembly of mannose-containing, lipid-linked oligosaccharides synthesised by pea membranes. An analysis of the lipid-linked saccharides produced under these conditions is shown in Figure 6.

The control gel permeation profile showing the spectrum of lipid-linked saccharides synthesised during an incubation of the membranes for 20 min with GDP-(^{14}C)mannose alone (Fig. 6a) reveals a prominent peak for the monosaccharide (position 1) derived from the polyisoprenyl monophosphoryl mannose, as well as clear peaks at positions 5, 7, 9, and 10. This profile resembles that previously observed during incubations for 60 min (Fig. 3b), except that the peak seen before at position 3 is missing, and there appears to be a more pronounced heterogeneity in the size of the higher oligosaccharides, mainly due to the decreased synthesis of the peak at position 10–12.

Incubation in the presence of Mn^{2+} (Fig. 6b) has no effect upon the polyisoprenyl monophosphoryl mannose peak, nor upon the pentasaccharide and heptasaccharide peaks derived from the polyisoprenyl diphosphoryl saccharides. However, there is no apparent accumulation of peak 9, and peak 10 is almost entirely absent. Thus, Mn^{2+} specifically depletes the accumulation of the higher, but not lower, lipid-linked oligosaccharides.

The effect of UDP-N-acetylglucosamine upon mannosylation of the lipid-linked oligosaccharides is particularly marked (Fig. 6c), resulting in a diminution of the peak derived from polyisoprenyl monophosphoryl mannose and the accumulation of increased amounts of peaks 5, 7, and 9. However, the peak at position 10 is completely abolished, as in the case of Mn^{2+} treatment.

UDP-glucose (Fig. 6d) also has a differential effect upon accumulation of the lipid-linked saccharides. The peak derived from polyisoprenyl monophosphoryl mannose is greatly enhanced concomitant with a broadening of the peak of higher lipid-linked oligo-

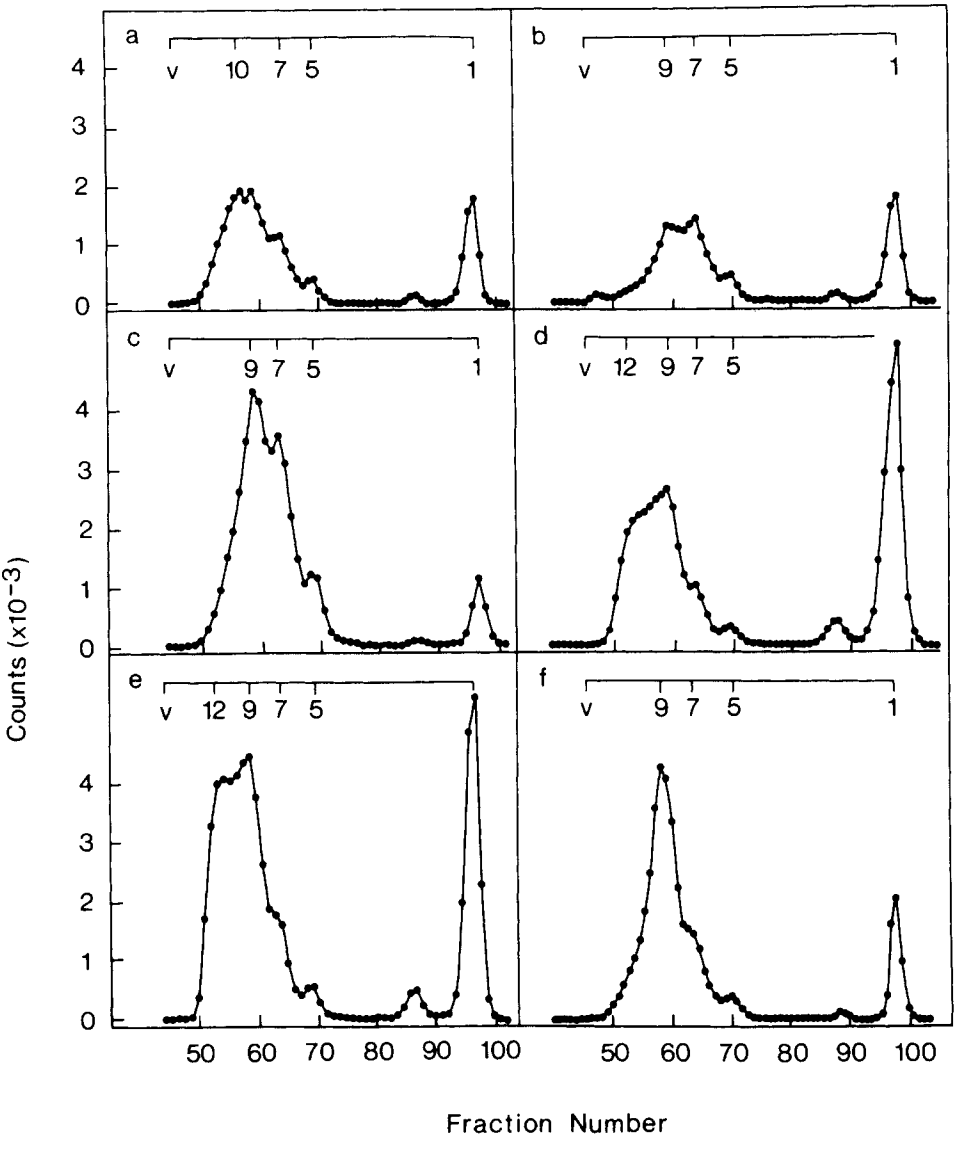

Fig. 6. Effect on transfer of (^{14}C)mannose from GDP-(^{14}C)mannose to lipid-linked saccharides of incubating pea stem membranes with GDP-mannose in the presence of Mn^{2+}, UDP-N-acetylglucosamine and UDP-glucose. The membranes were incubated for 20 min with GDP-(^{14}C)mannose, preceded in all cases for 10 min by the following preincubation regimens: (a), none; (b), Mn^{2+}; (c), UDP-N-acetylglucosamine; (d), UDP-glucose; (e), UDP-N-acetylglucosamine plus UDP-glucose; (f), UDP-N-acetylglucosamine plus UDP-glucose plus Mn^{2+}. The numbering above the profile is the same as that in Figure 3. All exogenous components (except GDP-(^{14}C)mannose, 10 μM) were added to give a final concentration of 10 mM.

saccharides. The latter presumably reflects the appearance of additional high molecular weight species. There is some increase in the size of peak 9, but the peaks at 5 and 7 remain at control levels.

The effect of combined incubation with both UDP-N-acetylglucosamine and UDP-glucose upon mannosylation of the lipid-linked saccharides (Fig. 6e) is relatively complex and is not a simple additive phenomenon. This incubation regimen causes an accumulation of polyisoprenyl monophosphoryl mannose, while the peaks at positions 5 and 7 remain at control levels, as previously seen with UDP-N-acetylglucosamine treatment alone. There is also an accumulation of the higher oligosaccharides, as in the incubation with UDP-glucose alone. The inclusion of Mn^{2+} with UDP-glucose and UDP-N-acetylglucosamine (Fig. 6f) results in the abolition of the higher lipid-linked oligosaccharides and reduces to control levels the yield of polyisoprenyl monophosphoryl mannose as well as the peaks at 5 and 7, as observed with Mn^{2+} alone (Fig. 6b). However, peak 9 remains at the enhanced level achieved whenever UDP-N-acetylglucosamine is included in the incubation.

In summary, variations in the incubation regimen to which the membranes are subjected markedly alter the accumulation of both the polyisoprenyl diphosphoryl oligosaccharides and polyisoprenyl monophosphoryl mannose. The greatest yield of high molecular-weight mannosylated oligosaccharide occurs when both UDP-N-acetylglucosamine and UDP-glucose are present with GDP-(^{14}C)mannose. It seems probable, therefore, that the availability of endogenous nucleotide sugars may play a role in the in vivo control of lipid-linked oligosaccharide assembly, and this in turn may be an important control point in the process of glycoprotein assembly.

Assembly of Lipid-Linked Oligosaccharides by Animal Cell Membranes

In order to compare the ability of plant and animal cell membranes to synthesise lipid-linked oligosaccharides in vitro, a membrane preparation, derived from mouse L-cells in a manner identical to that used for the pea membrane isolation, was incubated with UDP-N-acetyl-(^{14}C)glucosamine for 20 min. In some tests, this was followed by the addition of unlabeled GDP-mannose, as described for the pea membranes, and the acid-labile, lipid-linked oligosaccharides formed during these incubations were separated by P2 gel permeation chromatography. Incubation with UDP-(^{14}C)glucosamine alone (Fig. 5c) resulted in the synthesis of chitobiose-linked lipid and lesser amounts of N-acetylglucosamine-linked lipid, as in the pea system (Fig. 5a). The addition of GDP-mannose (Fig. 5d) resulted in several peaks of lipid-linked oligosaccharide, some of which appear similar in size to those formed by the pea membranes (namely those at positions 7 and 9, Fig. 5b). However, there is also a marked enhancement of high molecular weight peaks at positions 10–12, which were only inefficiently formed by the plant membranes under the same incubation conditions.

Mouse L-cells were also administered (^{3}H)mannose in vivo for 20 min, after which they were extracted with CMW. This extract was then washed with 0.5% KCl exactly as described previously for the pea membranes, and the "lower phase plus interface" material was subjected to DEAE-cellulose chromatography. One major class of lipid was present and this was strongly bound to the column, being eluted only with CMW containing 140 mM ammonium formate (Fig. 7a). Mild acid hydrolysis of this lipid under identical conditions to those previously used for the hydrolysis of the pea lipids (Fig. 3a) yielded a narrow peak of oligosaccharide which eluted from Biogel P2 close to the void volume (Fig. 7b). When this oligosaccharide was subjected to Biogel P4 gel permeation chromatography, a relatively homogeneous peak was observed, comparable to that reported by other workers using animal cell systems [11]. In addition, acetolysis of this oligosaccharide yielded similar proportions of mannobiose and mannotriose, as well as an acetolysis-resistant fragment of higher molecular weight (D.S.B. and J.B., unpublished ob-

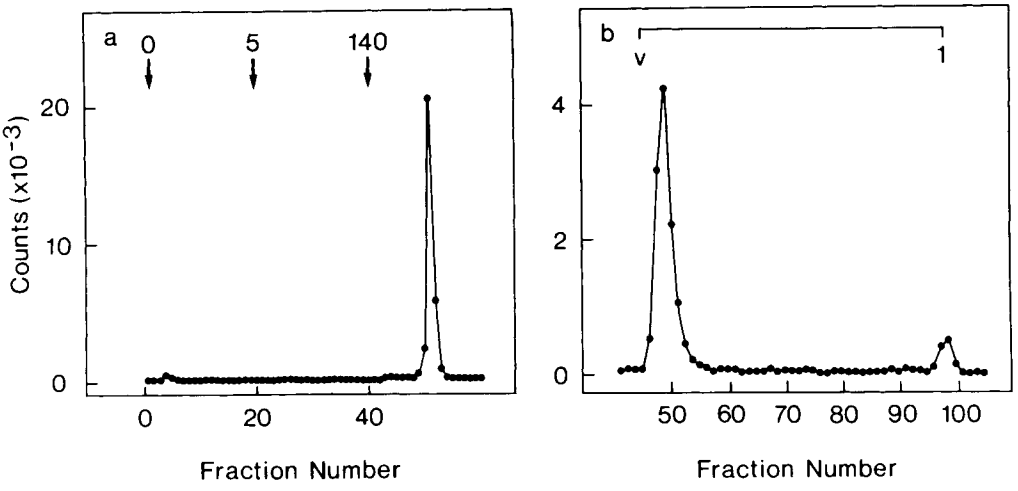

Fig. 7. a) DEAE-cellulose chromatography (see Fig. 4) of an aliquot of a CMW extract from mouse L-cells labeled with (^{3}H)mannose in vivo for 60 min as described in Methods; b) Biogel P2 gel permeation chromatography of the material eluting from DEAE-cellulose with 140 mM ammonium formate after hydrolysis with mild acid.

servation). These results are consistent with those already reported as characteristic of the common lipid-linked oligosaccharide thought to be involved in the initial glycosylation events of glycoprotein biosynthesis in animal cells [11, 12]. The fact that the mannose-labeled lipid derived from these animal cells behaved identically to the lipids synthesised from UDP-N-acetylglucosamine and GDP-mannose by isolated pea membranes upon DEAE-cellulose chromatography (cf Figs. 4 and 7a) supports the identity of the latter as polyisoprenyl diphosphate derivatives. However, the size of the oligosaccharide isolated from animal cells in vivo is marginally greater than that formed either by the animal or plant membranes during in vitro assays.

DISCUSSION

Identification of the Lipids Synthesised by the Pea Membranes

There can be little doubt that the lipids which are glycosylated by GDP-mannose and UDP-N-acetylglucosamine in the pea stem membranes used in our tests are polyisoprenyl derivatives. They are acid-labile, they show characteristic TLC properties (see [31] for a further discussion of this point), they are differentially retained on DEAE-cellulose columns and, in addition, they become labeled with both mannose and glucosamine. The latter two properties clearly distinguish these lipids from glycosyl diglycerides and sterol glycosides which are readily synthesised by plant membranes [32, 33] since such lipids are not retained by DEAE-cellulose chromatography, nor do they contain mannose or glucosamine.

Using DEAE-cellulose chromatography as a criterion, two types of polyisoprenyl derivatives are synthesised by the pea membranes: the monophosphoryl and the diphosphoryl derivatives. In agreement with previous work [15], the monophosphoryl component is only labeled by GDP-(^{14}C)mannose, not by UDP-N-acetyl-(^{14}C)glucosamine, and

bears a single monosaccharide residue, whereas the diphosphoryl derivative is labeled from both UDP-N-acetylglucosamine and GDP-mannose and bears a series of complex oligosaccharides (Figs. 3c and 5).

Oligosaccharide Complexity

The size of the lipid-linked saccharides synthesised by the pea membranes from GDP-(^{14}C)mannose is variable. Oligosaccharides with degree of polymerisation from 3 to 10–12 can be distinguished, but they fall into 4 major size ranges, namely those with elution volumes equivalent to 5, 7, 9, and 10–12 mannose residues (Fig. 3c). The relative abundance of these size classes varies with time of incubation. Sometimes, as in Figure 3c, the higher oligosaccharides (peaks 10–12) accumulate due to longer periods of incubation, while at other times the nona- and decasaccharide may be present in similar proportions, as in Figure 6a, where incubation time is shorter.

Experiments with UDP-N-acetyl-(^{14}C)glucosamine show the synthesis of N-acetylglucosaminyl- and chitobiosyl-diphosphoryl-polyisoprenyl derivatives (Fig. 5a). The minor peaks seen in this profile at positions 1 and 3 may well be due to loss of N-acetyl groups which is known to occur slightly under the hydrolysis conditions employed [7]. The addition of GDP-mannose to the incubation results in the generation of the same size-ranges of oligosaccharides as synthesised from GDP-(^{14}C)mannose alone. We conclude, therefore, that the higher oligosaccharides synthesised from GDP-mannose and UDP-N-acetylglucosamine are identical. In the case of GDP-(^{14}C)mannose incubations, endogenous (unlabeled) chitobiose- or N-acetylglucosamine-linked lipids present in the membranes are elongated (either directly or indirectly) by the added GDP-(^{14}C)mannose, resulting in the accumulation of discrete higher oligosaccharides labeled in the peripheral portions of their chains. However, in the case of UDP-N-acetyl-(^{14}C)glucosamine incubations, the membranes are incapable of forming the higher oligosaccharides (presumably because of the low levels of accessible GDP-mannose endogenous to the membranes) and the N-acetylglucosamine- and chitobiose-linked lipids accumulate.

The exact size of the oligosaccharides synthesised in this system is unknown. Throughout the discussion, we have treated the peaks observed during gel permeation chromatography of hydrolysates of CMW-soluble material (Fig. 3a) as if they represented a mannosaccharide series. The elution volumes of these saccharides adhere quite exactly to those expected of such a series (Fig. 8), and the lower saccharides exhibit identical elution volumes to those previously reported for the lower mannosaccharides [30].

A comparison of Figures 3a and 3c shows that oligosaccharides released by hydrolysis of the lipid phase co-chromatograph with certain members of the total CMW extract hydrolysate. If the lipid-linked oligosaccharides are chitobiose-initiated, as seems likely from the GDP-mannose cold-chase experiment shown in Figure 5b, then the peaks at 5, 7, 9, and 10 may contain 1, 3, 5, and 6 mannose residues respectively. However, lipid-linked saccharides isolated from animal systems are known to be branched [12]; the effect of such branching upon elution volumes during gel permeation chromatography is unknown, and any conclusions regarding the structure of the oligosaccharides based upon their elution from such columns would be premature.

Control of Lipid-Linked Saccharide Assembly in Plant Membranes

The effects of incubating the membranes with GDP-(^{14}C)mannose in the presence of nucleotide sugars and Mn^{2+} are complex (Fig. 6). UDP-N-acetylglucosamine treatment results in a general enhancement of peaks 5, 7, and 9. This is consistent with "priming" of the

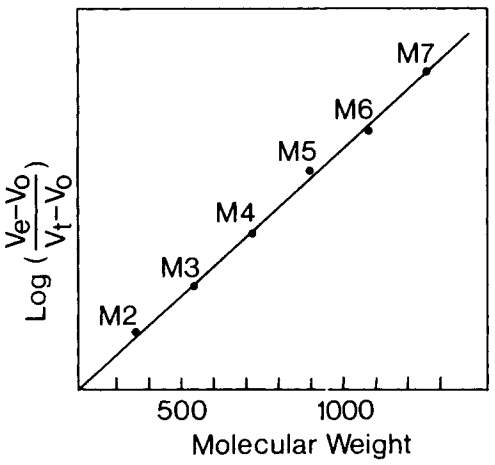

Fig. 8. Calibration curve to determine molecular weights of saccharides eluting from Biogel P2. The mannosaccharide series observed in hydrolysates of the total CMW extract of GDP-mannose-labeled pea stem membranes (Fig. 3a) was used to determine the probable MW of the higher oligosaccharides.

endogenous polyisoprenyl phosphate pools with chitobiose to form chitobiosyl-diphosphoryl-polyisoprenoids, resulting in increased yields of labeled mannosylated derivatives during the subsequent incubation with GDP-(^{14}C)mannose. Peaks at positions 10 and higher do not show a similar enhancement upon UDP-N-acetylglucosamine preincubation, but these components are formed if UDP-glucose is also present during the preincubation treatment. This may reflect either an elongation of existing mannosylated saccharides by additional glucose residues derived from the added UDP-glucose, analagous to the formation of a "G-oligosaccharide" [34–38], or an extension of the existing mannosylated saccharides by further mannose residues, which may be controlled in some way by the availability of endogenous UDP-glucose.

Mn^{2+} treatment (Figs. 6b and 6f) consistently depletes the higher lipid-linked oligosaccharides, especially those associated with UDP-glucose treatment. Mn^{2+} has been reported to be necessary for lipid-linked saccharide assembly [39] and a role for it in the final transfer to glycoprotein has also been suggested [40]. In the pea system, Mn^{2+} may inhibit the formation of higher oligosaccharides (peaks 10–12), it may induce their hydrolysis after synthesis, or, alternatively, it may facilitate the transfer of the higher oligosaccharides from the lipid intermediates to endogenous acceptors within the membranes.

The only treatment which markedly changes the amount of polyisoprenyl monophosphoryl mannose formed from GDP-(^{14}C)mannose is that with UDP-glucose (Figs. 6d and 6e), which results in accumulation of this product. Polyisoprenyl monophosphoryl mannose has been suggested to be the precursor of polyisoprenyl diphosphoryl oligosaccharides in some animal tissues [7, 41], and polysaccharides in some plant tissues [25]. Since UDP-glucose does not reduce the synthesis of the lipid-linked oligosaccharides in parallel with the accumulation of the polyisoprenyl monophosphoryl mannose in the pea membranes, there are probably two pools of polyisoprenyl monophosphoryl mannose. One of these pools may be tightly coupled to lipid-linked oligosaccharide assembly and unaffected by UDP-glucose preincubation; the other may be coupled to processes such as polysaccharide synthesis which may be disrupted by UDP-glucose pretreatment. Mannan and glucomannan-synthesising systems are known to be present in these membranes [42].

Assembly and Function of Lipid-Linked Saccharides in Plant and Animal Cells

The participation of lipid-linked saccharides in the synthesis of glycoproteins in animal cells is suggested by the inhibition of protein glycosylation by the antibiotic tunicamycin which specifically prevents the synthesis of lipid-linked saccharides both in vivo and in vitro [43]. Elegant studies involving the completion of nascent proteins in coupled translation systems [44, 45] have shown the synthesis of glycosylated (lectin-binding) products which exhibit higher molecular weight than the products of in vitro translation reactions executed in the absence of membranes. Timed addition of membranes to synchronously-initiated mRNA-ribosome complexes using virally specified mRNAs for defined glycoproteins [46] have demonstrated that glycosylation occurs at precise times during the membrane-coupled translation process. These experiments show that it is the membranes which contain the machinery for the glycosylation of nascent proteins, that glycosylation is tightly coupled both temporally and spatially to protein synthesis and that lipid-linked oligosaccharides are involved as intermediates in the process. Recent results with animal systems [11, 12, 47, 48] have demonstrated a common lipid-linked oligosaccharide as an intermediate in the synthesis of some glycoproteins. This intermediate contains two N-acetylglucosamine, 8–12 mannose and 1–2 glucose residues [11]. It is labeled during in vivo incubations of cells with mannose, and its structure has been elucidated [12]. The initial events of glycoprotein biosynthesis are considered to be the "en bloc" transfer of this oligosaccharide from the lipid intermediate to the nascent peptide chain.

In this study, a lipid-linked oligosaccharide which closely resembled this common lipid intermediate was observed to be synthesised by actively-dividing mouse L-cells. In addition, membranes isolated from these mouse cells were shown to be capable of assembling similar complex lipid-linked oligosaccharides when given UDP-N-acetylglucosamine and GDP-mannose. It is likely that these complex lipid-linked oligosaccharides which are synthesised in vitro are precursors of the lipid-linked oligosaccharide seen during in vivo incubations [49]. The sequential assembly of such lipid-linked oligosaccharides was also shown to occur in membranes isolated from actively-growing regions of pea stems. In fact, the only apparent difference between the two assembly processes was the reduced efficiency of the plant-derived membranes in synthesising high molecular weight oligosaccharide intermediates.

The involvement of UDP-glucose in a terminal glucosylation step in the plant membranes (similar to that already demonstrated in animal membranes [36]), is possible, but requires further investigation. However, we have obtained no evidence for the turnover or transfer of the lipid-linked oligosaccharides formed in these studies to endogenous protein acceptors present in the membranes, even in the presence of UDP-glucose. We suggest that the accumulation of the lipid-linked intermediates may itself be an indication that the membranes are incapable of effecting the transfer of oligosaccharides to form glycoproteins via the lipid-linked pathway, due either to an uncoupling of the final transfer reactions in the absence of protein synthesis, or their occurrence at a severely reduced rate. These results underline the importance of further studies to elucidate the nature of these final transfer reactions in the pathway of glycoprotein biosynthesis in plant tissues, and also raise the possibility of the conservation of the pathway of glycoprotein biosynthesis during the evolution of both plants and animals.

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