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Involvement of Lipid-linked Oligosaccharides in Synthesis of Storage Glycoproteins in Soybean Seeds[1]

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ABSTRACT

Membrane preparations from developing soybean (var. Prize) cotyledon tissue, at the time of synthesis of storage glycoproteins, catalyze the sequential assembly of lipid-linked oligosaccharides from uridine-5′-diphospho-*N*-acetyl-D-[6-^{3}H]glucosamine and guanosine-5′-diphospho-D-[U-^{14}C]mannose. The maximum size of lipid-linked oligosaccharide that accumulates contains the equivalent of 10 saccharide units on the basis of Bio-Gel P-2 gel filtration studies. These lipid-linked oligosaccharides show similar characteristics to polyisoprenyl diphosphate derivatives on diethylaminoethyl-cellulose chromatography and are potential intermediates in glycoprotein biosynthesis in this tissue. These glycolipids do not appear to turn over in pulse-chase experiments and no completed storage glycoproteins were detected among the products of these incubations.

Tissue slices from cotyledons at the same stage of development synthesize lipid-linked oligosaccharides from [^{3}H]mannose and [^{3}H]glucosamine with sizes equivalent to 1, 7, 10, and approximately 15 saccharide units. In pulse-chase experiments, the lipid-linked saccharides with the equivalent of 1 and 10 units rapidly turnover, whereas those with 7 and 15 units do not. Examination of the higher oligosaccharide peaks (10 and 15) by Bio-Gel P-4 gel filtration shows them to comprise 2 distinct subsets of oligosaccharides containing different proportions of glucosamine and mannose units. Tissue slices synthesize products which resemble the completed 7S storage glycoproteins as judged by similarity of molecular weight and precipitation with specific antisera. Analysis of the oligosaccharides obtained by hydrazinolysis of glycoproteins shows the presence of a similar size "high-mannose" type N-linked oligosaccharides as in other glycoproteins from animal and plant cells.

The 7S complex of soybean storage proteins contains about 5% by weight of covalently bound carbohydrate (29). When the purified 7S complex is subjected to SDS-polyacrylamide gel electrophoresis, it can be resolved into several components, all of which are glycosylated as determined by periodic acid-Schiff staining (25). The saccharide moieties include oligosaccharides attached to asparagine containing 2 glucosamine and up to 9 mannose units (28, 29). Similar oligosaccharides are found in soybean agglutinin (18). Little is known about the mechanisms of biosynthesis of these proteins and less is known about their glycosylation. A temporal difference in the accumulation of storage proteins during maturation of the seeds has been demonstrated in soybean (14), as well as in other legumes (3, 20). Growing evidence implicates lipid-linked oligosaccharides as intermediates in the biosynthesis of mammalian glycoproteins (see reviews in refs. 11 and 21). Such compounds have been identified in developing pea (2) and french bean (9, 12, 13) cotyledon tissue. Dolichyl phosphate has been purified from soybean and shown to act as an effective acceptor for glycosyl transfer from sugar nucleotides (5, 22).

Evidence that lipid-linked oligosaccharides can be assembled in a sequential manner from UDP-GlcNAc and GDP-mannose by membrane preparations from developing soybean cotyledons and that some of these, as in mammalian systems, may be involved in the synthesis of endogenous storage glycoproteins is presented here. Earlier studies in these laboratories have been concerned mainly with the assembly of glycolipids in growing tissue (pea stem) where glycoproteins do not accumulate (1, 10), and it was of particular interest to employ similar techniques with a tissue where a major part of metabolism is geared to the synthesis and deposition of glycoprotein.

MATERIALS AND METHODS

Plant Material. Soybean (*Glycine max.* var. Prize) was grown in Vermiculite with a 12-h photoperiod at 28 C (daytime) and 22 C (night). Plants were inoculated with *Rhizobium japonicum* and watered with N-free medium as described previously (27). Cotyledons were harvested when their fresh weight was between 100 and 250 mg.

Membrane Isolation and Incubation. A membrane preparation was obtained from developing soybean cotyledons by modification of the method previously used in studies with pea stem tissue (1). The cotyledons were homogenized with a mortar and pestle at 4 C in 0.1 M Tris-HCl (pH 7.4) containing 5 mM dithioerythritol, 10 mM $MgCl_2$ and 0.4 M sucrose, and the brei was filtered through Miracloth (Calbiochem). The filtrate was centrifuged at low speed (1000*g* for 10 min) and the pellet was dispersed for 20 s using a tissue homogenizer (Polytron, Brinkmann Instruments, at half-maximum force). The homogenate was recentrifuged (1000*g* for 10 min) and the supernatant was combined with that from the previous centrifugation. A total membrane preparation was obtained from this supernatant by centrifugation at high speed (48,000*g* for 60 min).

The membrane preparation was resuspended in homogenization buffer at a protein concentration of approximately 2 mg/ml and incubated with radioactive substrates (GDP-[^{14}C]mannose,[2] Amersham, 173 mCi/mmol; final incubation concentration of 20 μM, 4 × 10^5 dpm added to each incubation; UDP-*N*-acetyl-

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[2] Abbreviations: GDP-[^{14}C]mannose, guanosine-5′-diphospho-D-[U-^{14}C]mannose; [^{3}H]glucosamine, D-[1,6-^{3}H]glucosamine; [^{3}H]leucine, L-[4,5-^{3}H]leucine; [^{3}H]mannose, D-[1-^{3}H]mannose; UDP-[^{3}H]GlcNAc, uridine-5′-diphospho-*N*-acetyl-D-[6-^{3}H]glucosamine.

[^{3}H]glucosamine, New England Nuclear, 10 Ci/mmol, final incubation concentration of 3 μM, 4 × 10^6 dpm added to each incubation) in 60 μl total volume at 25 C. The reactions were terminated using chloroform-methanol (1:1, v/v) to bring the final concentration of chloroform-methanol-H_2O to 1:1:0.3 (v/v).

Labeling of Tissue Slices. Individual tissue slices (0.1–0.2 mm thick), cut from developing soybean seeds (fresh weight, approximately 250 mg) were placed on glass plates upon 25-μl drops containing 0.1 M Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 10 μl of either [^{3}H]mannose (32 μM final concentration, New England Nuclear, 13.2 Ci/mmol), [^{3}H]glucosamine (10 μM final concentration, New England Nuclear, 36.9 Ci/mmol), or [^{3}H]leucine (8 μM final concentration, Amersham, 120 Ci/mmol). The slices were illuminated from below with 40-w fluorescent light and incubated at room temperature. In pulse-chase experiments, concentration of the unlabeled substrates was 10 mM. The reaction was terminated in the same way as the membrane assays described above.

Analysis of Products. Reaction mixtures from both membrane and tissue slices were thoroughly homogenized with chloroform-methanol-H_2O (1:1:0.3, v/v, 8 ml) and insoluble material was separated by centrifugation. The soluble material was washed with 0.5% KCl and the resultant lipid phase was either subjected to DEAE-cellulose chromatography or hydrolyzed directly using 0.1 N HCl at 90 C for 30 min as described previously (1, 10). The oligosaccharides released by hydrolysis were separated by either Bio-Gel P-2 or Bio-Gel P-4 gel permeation chromatography on 1- × 100-cm columns, using 0.1 M Tris-HCl (pH 7.4) containing 0.05% (w/v) sodium azide and 0.2 M NaCl as eluant. Sugar analyses of these lipid-linked oligosaccharides were performed as described by Spiro (24). Neutral sugars were released by treatment with 1 N H_2SO_4 for 8 h at 100 C and subsequently analyzed by cellulose paper chromatography (31). Amino sugars were released by treatment with 4 N HCl for 8 h at 100 C and subsequently analyzed by ion-exchange chromatography using 10-ml columns of Zerolit 225 (BDH Chemicals) with 0.33 N HCl as eluant (7).

The pellet obtained from the centrifugation of the initial extract with chloroform-methanol-H_2O was washed with the same solvent (2 × 8 ml) followed by methanol (1 × 8 ml) and lyophilized. An aliquot was subjected to hydrazinolysis by heating with anhydrous hydrazine (containing catalytic amounts of hydrazine sulfate) at 105 C for 10 h as described by Yosizawa *et al.* (30). The resulting hydrazinolysate was repeatedly evaporated with H_2O to remove the hydrazine and then subjected to Bio-Gel P-4 gel filtration, as described for the lipid-linked oligosaccharides. Another aliquot was dissolved by boiling for 2 min in a small volume of 1 M Tris-HCl (pH 6.8) containing 2% (w/v) SDS and 10 mM DTT. SDS-insoluble material was removed by centrifugation using an Eppendorf microcentrifuge for 5 min and washed once with 1 ml SDS buffer at 100 C for 10 min. The pellet obtained upon recentrifugation (for 10 min) was assumed to contain mainly polysaccharide material while the product solubilized by SDS was considered to contain glycoprotein or protein-bound saccharide (10). The SDS-solubilized material was either counted directly using Aquasol (New England Nuclear) scintillation fluid or subjected to SDS-polyacrylamide gel electrophoresis on 1.5-mm thick 17.5% acrylamide slab gels (16). Gels were routinely over-run to resolve more clearly those components of mol wt 30,000 to 100,000 daltons. Proteins were visualized using Coomassie brilliant blue and glycoproteins were visualized using a modified periodic acid-Schiff stain (15). Fluorography of the gels was performed using prefogged Kodak X-Omat R film (4).

Animal Material. Mouse L-cells (provided by Dr. R. Sinclair) were grown in suspension culture as described previously (1). Cells were harvested after labeling with [^{3}H]mannose (New England Nuclear, 13.2 Ci/mmol, 0.1 mCi/100 ml medium) for 1 h. Lipid-linked saccharides were isolated and analyzed as above.

RESULTS

Lipid-linked Saccharides Formed by Soybean Membranes. To test for synthesis of lipid-linked saccharides and storage glycoproteins *in vitro*, a total membrane fraction was isolated from developing soybean cotyledons and incubated with nucleotide sugars. The lipids labeled during incubations of the cotyledon membranes were separated by DEAE-cellulose chromatography. The profile obtained for lipids labeled with UDP-[^{3}H]GlcNAc shows that only one species of lipid was synthesized (Fig. 1a). This component in other systems (5) has been identified as polyisoprenyl diphosphoryl saccharide. GDP-[^{14}C]mannose labeling results in two peaks (Fig. 1b) which have been characterized on the basis of several criteria (1, 10) as polyisoprenyl monophosphoryl mannose and polyisoprenyl diphosphoryl oligosaccharide.

A time course for the incorporation of UDP-[^{3}H]GlcNAc into lipid is shown in Figure 2, together with the effect of unlabeled GDP-mannose added at 5 min. When the mild acid hydrolysate of the lipid labeled with UDP-[^{3}H]GlcNAc for 5 min is separated by gel permeation chromatography, there are two major components corresponding to GlcNAc and chitobiose, without any higher oligosaccharides (Fig. 3a). Longer incubation periods do not alter this pattern. Analysis of the lipid-linked saccharides formed during the period of GDP-mannose addition shows the generation of several new lipid-linked oligosaccharides of successively higher mol wt (Fig. 3, b and c). These are products which could be expected from the addition of mannose residues to the

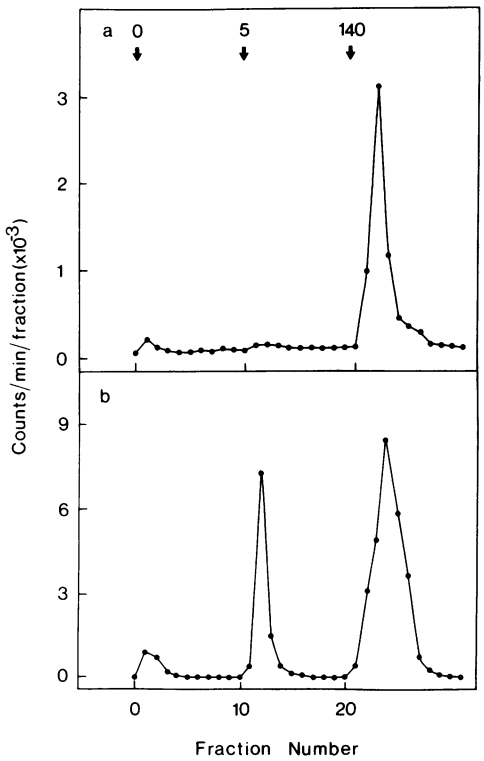

FIG. 1. DEAE-cellulose chromatography of labeled membrane lipids from soybean cotyledons. Aliquots of the 0.5% KCl-washed chloroform-methanol-H_2O extract were separated on columns of DEAE-cellulose (10 ml) by sequential elution with chloroform-methanol-H_2O (1:1:0.3, v/v), chloroform-methanol-5 mM ammonium formate (1:1:0.3, v/v), and chloroform-methanol-140 mM ammonium formate (1:1:0.3, v/v). The fraction volume was 10 ml. a, extract separated after labeling the membranes with UDP-[^{3}H]GlcNAc for 60 min; b, extract separated after labeling the membranes with GDP-[^{14}C]mannose for 60 min. The peaks eluted with 5 and 140 mM ammonium formate represent polyisoprenyl monophosphoryl and polyisoprenyl diphosphoryl saccharides, respectively. Arrows indicate the points of addition and the concentrations of ammonium formate.

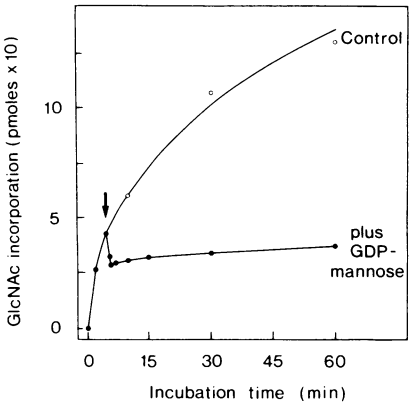

FIG. 2. Effect of unlabeled GDP-mannose on accumulation of lipid-linked [^{3}H]GlcNAc. Cell-free membranes from developing soybean cotyledons (total volume, 55 μl) were incubated with UDP-[^{3}H]GlcNAc and the time course was determined for incorporation into lipid (fraction soluble in chloroform-methanol-H_2O but insoluble in 0.5% KCl). (○——○), control, no other additions (●——●), +GDP-mannose (5 μl, 120 mM) at 5 min (arrow).

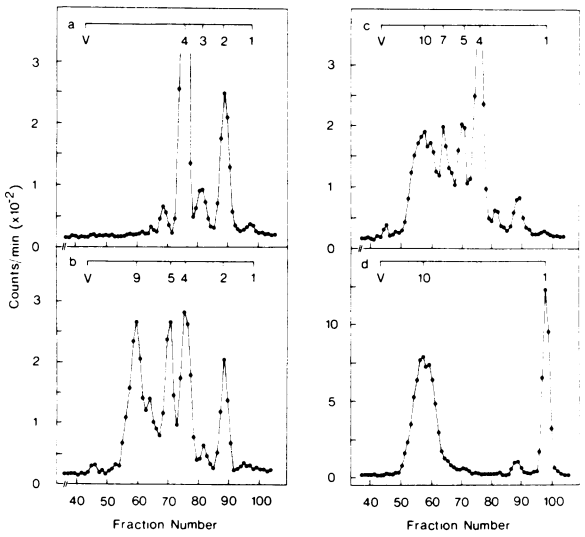

FIG. 3. Elongation of [^{3}H]GlcNAc-initiated lipid-linked oligosaccharides. Bio-Gel P-2 gel-permeation chromatography profiles are shown of acid-labile, lipid-linked oligosaccharides synthesized from UDP-[^{3}H]GlcNAc (panels a–c) and GDP-[^{14}C]mannose (panel d) by cell-free membrane preparations. a, 5 min incubation with UDP-[^{3}H]GlcNAc (3 μM) alone; b, as in a, followed by 2-min incubation with unlabeled GDP-mannose (10 mM); c, as in b, with the incubation continued for 1 h; d, 60-min incubation with GDP-[^{14}C]mannose (20 μM) alone. The scale above the profiles represents the elution volumes of a saccharide series (1). V, void volume of the column.

[^{3}H]GlcNAc-labeled lipids.

With GDP-[^{14}C]mannose as substrate, mild acid hydrolysis of the lipids produced during membrane incubations, followed by gel permeation chromatography on Bio-Gel P-2, yields the profile shown in Figure 3d. Peak 1, found only after labeling with GDP-mannose and absent from the lipid-linked GlcNAc components, is likely to be derived from polyisoprenyl monophosphoryl mannose, with the other peaks derived from polyisoprenyl diphosphoryl oligosaccharides (see refs. 1 and 10). A saccharide series is presented above the profiles for reference and is discussed at length elsewhere (1). The major [^{14}C]oligosaccharide peak corresponds to a decasaccharide and is equivalent to the largest [^{3}H]-oligosaccharide that is generated in the system containing GDP-mannose and UDP-[^{3}H]GlcNAc (Fig. 3c).

With respect to incorporation of these sugars into glycoproteins, *in vitro*, the SDS-soluble material from these incubations analyzed by gel electrophoresis resolved several labeled peptides. However, they did not seem to correspond to the size of the major glycoproteins in this tissue and the yield was low (data now shown).

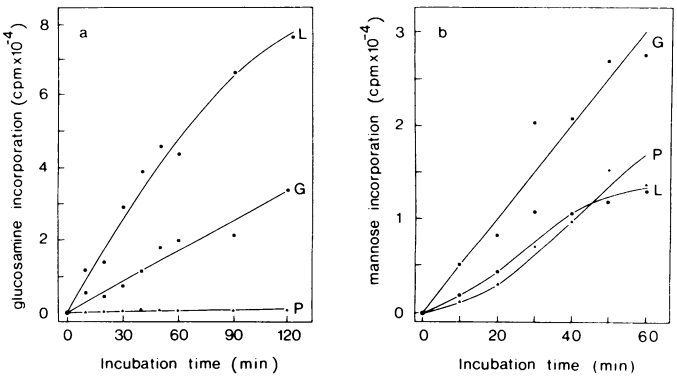

FIG. 4. Synthesis of lipid (L), SDS-soluble (G), and SDS-insoluble (P) products by cotyledon tissue slices. Slices (250 mg) were incubated with (a) [^{3}H]glucosamine or (b) [^{3}H]mannose and incorporation into tissue fractions was measured as described.

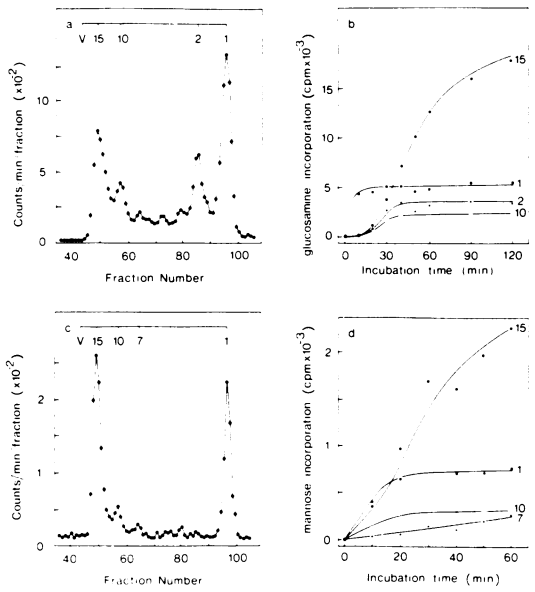

FIG. 5. Lipid-linked saccharides synthesized by tissue slices. Bio-Gel P-2 profiles are shown of the oligosaccharides released from lipid fractions by mild acid hydrolysis after labeling cotyledon tissue slices (a) [^{3}H]-glucosamine for 30 min or (c) [^{3}H]mannose for 20 min. Time courses are shown for incorporation into individual lipids labeled with (b) glucosamine or (d) mannose. The numbering on the scale above the profiles indicates the relative positions of the individual lipids whose time-courses of synthesis are shown in b and d, respectively, and correspond to positions shown in Fig. 3 for typical saccharides. V, void volume of the column.

Analysis of Lipid-linked Saccharides Formed by Tissue Slices. Time courses for the incorporation of [^{3}H]glucosamine and [^{3}H]-mannose into lipid, SDS-soluble, and SDS-insoluble products in tissue slices from soybean cotyledons are shown in Figures 4, a and b, respectively. The tissue slices incorporate glucosamine preferentially into lipid components and, to a lesser extent, into SDS-soluble products. No significant incorporation into SDS-insoluble products is observed even after 120 min incubation. This is to be expected since no known polysaccharide containing glucosamine exists in such tissues. In contrast, mannose is incorpo-

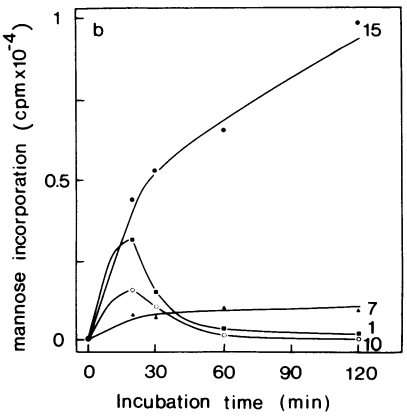

FIG. 6. Pulse-chase labeling of lipid-linked oligosaccharides with mannose in tissue slices. [^{3}H]Mannose was supplied for 20 min and then unlabeled mannose (10 mM) was added. Lipids were extracted, hydrolyzed, and chromatographed on Bio-Gel P-2, yielding profiles similar to those in Fig. 5 (a and c). Incorporation into peaks 1, 7, 10 and 15 was assayed as in Fig. 5b.

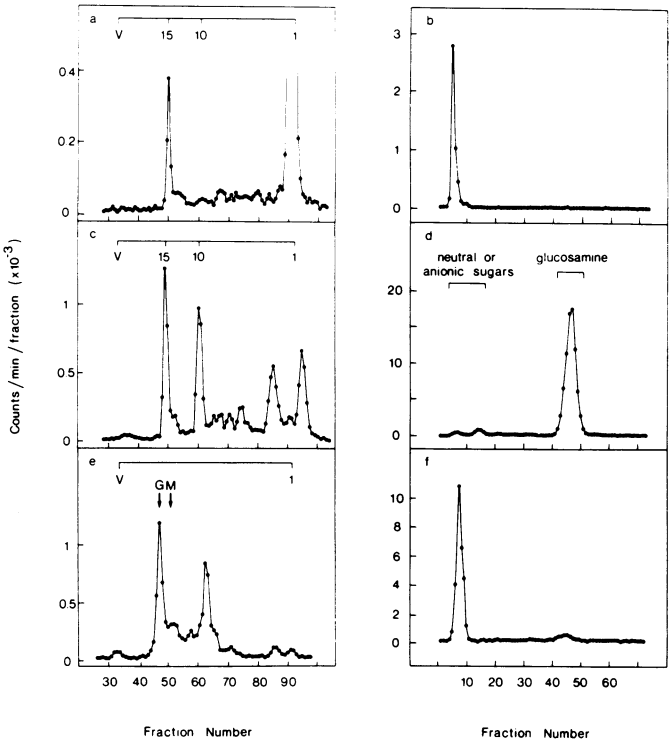

FIG. 7. Bio-Gel P-4 gel-permeation chromatography and amino-sugar analyses of lipid-linked saccharides synthesized by soybean cotyledon tissue slices and mouse L-cells. Bio-Gel P-4 profiles of oligosaccharides released from lipid fractions by mild acid hydrolysis after labeling tissue slices with [^{3}H]mannose and [^{3}H]glucosamine are shown in panels a and c, respectively. [^{3}H]Mannose-labeled lipid-linked oligosaccharides from suspension cultures of mouse L-cells is shown in panel e, together with elution positions of the glucosylated oligosaccharide (G) formed by L-cells *in vivo* and the highest mannose-labeled oligosaccharide (M) formed by isolated L-cell membranes (see text). The oligosaccharides were hydrolyzed with 4 N HCl at 100 C for 8 h (24) and then separated by ion exchange chromatography as described. Panels b, d, and f show profiles obtained from hydrolysates of the oligosaccharides shown in panels a, c, and e, respectively. The elution positions of the neutral and anionic sugars and glucosamine are indicated in panel d, V, void volume of column.

rated into all three of these fractions, suggesting that mannolipid, mannoprotein, and mannan are all formed.

Analysis of the size of carbohydrate released by mild acid hydrolysis of glycolipids formed from glucosamine and mannose by Bio-Gel P-2 gel filtration are shown in Figure 5, a and c, respectively. In both cases, peaks of common elution volume occur at positions 1, 10, and approximately 15. The peak at position 2 is unique to glucosamine-labeled tissue. Time courses for the incorporation of label into the individual lipids are shown in Figure 5, b and d. With glucosamine as substrate, lipids 2, 10, and 15 are labeled after an initial lag period of about 20 min, whereas lipid 1 becomes labeled immediately. With mannose as substrate, there is little or no lag and lipids 1 and 10 saturate within 30 min, whereas lipid 15 continues to accumulate for at least 60 min. In pulse-chase experiments in which labeled mannose is followed by high concentrations of unlabeled mannose (Fig. 6), label in peaks 1 and 10 declines rapidly, whereas label continues to accumulate into peak 15. It appears, therefore, that peak 15 represents a major glycolipid which is not subject to turn-over in the time of the experiments reported here, whereas lower-sized oligosaccharide-lipids are rapidly metabolized further.

Analyses of these lipid-linked oligosaccharides by Bio-Gel P-4 gel permeation chromatography are shown in Figure 7. Using mannose as substrate the lipid-linked oligosaccharides include (Fig. 7a) a major component eluting at fraction 50 which corresponds to the oligosaccharide denoted as 15 on the basis of Bio-Gel P-2 chromatography. The label in this fraction remains as neutral sugar and is not converted to glucosamine (Fig. 7b). Using glucosamine as substrate, the lipid-linked oligosaccharides (Fig. 7c) also show a major saccharide eluting at fraction 50, together with other oligosaccharides of intermediate mol wt. The latter are only minor components among the oligosaccharides labeled from mannose (Fig. 7a), suggesting that they are enriched in glucosamine. A major oligosaccharide of intermediate mol wt corresponds in size to the peak denoted as 10 on the basis of Bio-Gel P-2 studies and was the only oligosaccharide to show an appreciable degree of turn-over during pulse-chase experiments (Fig. 6). The label in the lipid-linked oligosaccharide fraction remains as glucosamine (Fig. 7d) and is not present to any significant extent as neutral sugar.

To determine the relationship between these soybean lipid-linked oligosaccharides and the common lipid-linked oligosaccharide thought in animals systems to be the donor of the "core" oligosaccharide to nascent proteins (23), a similar mannose-labeled lipid-linked oligosaccharide fraction was isolated from suspension cultures of mouse L-cells by DEAE-cellulose chromatography followed by mild acid hydrolysis (1). Bio-Gel P-4 gel permeation chromatography of these mammalian lipid-linked oligosaccharides is shown in Figure 7e. Several oligosaccharides rapidly accumulate, all of which turn over during pulse-chase experiments (data not shown). The elution volumes of the major lipid-linked oligosaccharides synthesized from GDP-mannose and UDP-glucose by L-cell membranes are also indicated (labeled M and G, respectively, Fig. 7e). The elution volumes of both these oligosaccharides correspond closely to those reported for similar lipid-linked saccharides from other mammalian cells (6, 19, 23). The major soybean lipid-linked saccharide (peak 15) is slightly smaller than the similar lipid-linked saccharide formed by mammalian cells.

Glycoproteins Formed by Tissue Slices. SDS-soluble fractions from tissue slices after labeling with mannose or glucosamine were analyzed by SDS-polyacrylamide gel electrophoresis. Defined glycoprotein products comparable in mol wt to those seen in developing cotyledon tissue were formed (Fig. 8). The identity of two of these products as 7S-α-related glycoproteins has been independently verified by precipitation with antisera specifically raised against purified 7S-α-glycoprotein. Pulse-chase experiments

show that the products initially labeled are slightly larger in mol wt than the final 7S components which begin to accumulate after 1.5 to 6 h of incubation (Fig. 8) (C. Sengupta, V. DiLuca, D. S. Bailey, and D. P. S. Verma, unpublished data). This indicates that the complete biosynthetic sequence of the major glycoprotein components of the cotyledon is operative in the tissue slices. In addition, the fact that exogenously supplied mannose and glucosamine label only those polypeptides which are glycosylated (namely, the 7S complex and soybean agglutinin) and not any other proteins which are labeled with leucine implies that these sugars are being directly incorporated into the carbohydrate residues of the glycoproteins and do not enter any other metabolic pools within the time period of these experiments.

Bio-Gel P-4 chromatography of the oligosaccharides released by hydrazinolysis of the chloroform-methanol-H_2O (1:1:0.3, v/v)-extracted [^{3}H]mannose-labeled product synthesized by the tissue slices gives the profile shown in Figure 9a. This fraction contains high mol wt material, probably H_2O-soluble mannan, as well as intermediate-sized oligosaccharides and small oligosaccharides which were originally protein-bound. A similar hydrazinolysate derived from lipid-extracted glycoproteins biosynthesized from [^{3}H]mannose by mouse L-cells shows no high mol wt components and is enriched in intermediate-sized protein-bound oligosaccharides which correspond in size to the "high-mannose" class of N-linked oligosaccharides isolated by similar techniques from other mammalian glycoproteins (23). The major group of protein-bound oligosaccharides from both soybean and mouse L-cell glycoproteins appears to be smaller in size than the major lipid-linked oligosaccharides present in both tissues (Fig. 7, panels a, c, and e).

DISCUSSION

Several reports have characterized specific lipid-linked saccharides which are thought to be involved in the process of glycoprotein biosynthesis. These lipids, from both animal and plant sources, appear to be polyisoprenyl derivatives. We present evidence concerning the assembly and turn-over of such lipid-linked saccharides in developing soybean cotyledons, where deposition of reserve glycoproteins is a major biosynthetic reaction.

Soybean cotyledon membranes synthesize lipid-linked oligosaccharides from both UDP-[^{3}H]GlcNAc and GDP-[^{14}C]mannose

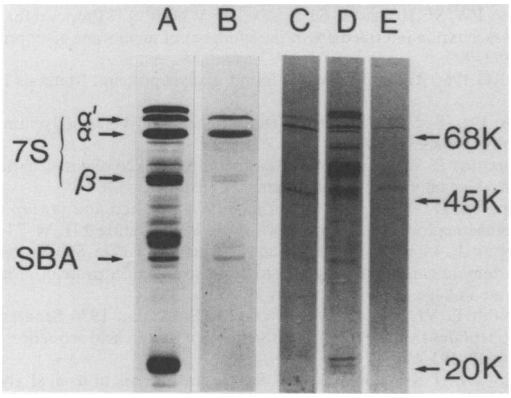

FIG. 8. Glycoproteins synthesized by tissue slices. Polyacrylamide SDS-gel electrophoretic separation of the major storage proteins present in soybean cotyledons are shown; stained with Coomassie blue (track A), and periodic acid-Schiff reagent (track B). Fluorography of SDS-gel electrophoretic separation of the products formed by the incubation of tissue slices from soybean cotyledons with [^{3}H]mannose (track C) and [^{3}H] leucine (track D) and [^{3}H]glucosamine (track E) is also shown. The position of the 7S glycoprotein components and soybean agglutinin (SBA) are indicated on the left and the mol wt of marker proteins run on the same gels are shown on the right.

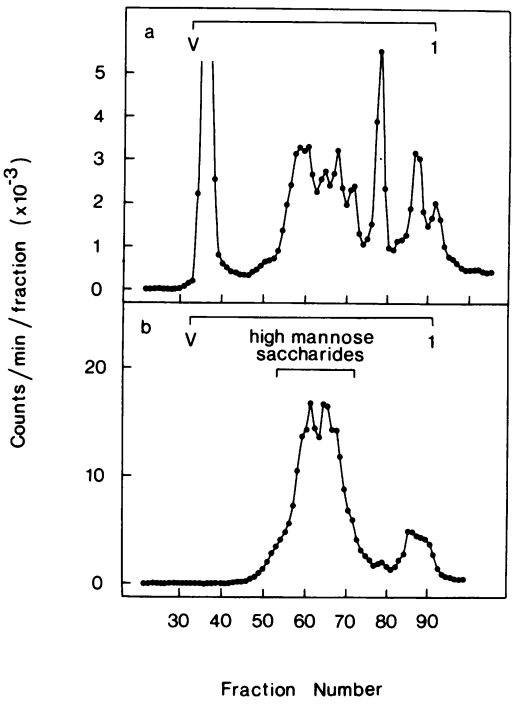

FIG. 9. Protein-bound oligosaccharides synthesized by soybean cotyledon tissue slices and mouse L-cells. Bio-Gel P-4 profiles of the oligosaccharides released by hydrazinolysis (30) of the chloroform-methanol-H_2O-insoluble products derived from soybean cotyledon tissue slices and mouse L-cell suspension cultures after [^{3}H]mannose labeling for 60 min are shown in panels a and b, respectively. The position of high-mannose oligosaccharides is indicated in panel b (according to ref. 23). V, void volume of column.

(Fig. 3). The major lipid-linked oligosaccharide which accumulates after UDP-[^{3}H]GlcNAc labeling behaves as chitobiose, judging by its elution position from the Bio-Gel P-2 columns. By prelabeling the membranes with UDP-[^{3}H]GlcNAc and subsequently adding unlabeled GDP-mannose, the initial lipid-linked chitobiose appears to be elongated sequentially to form higher oligosaccharides, in a manner similar to that observed in pea (1) and other tissues (11, 21). The most abundant oligosaccharide formed under these conditions (Fig. 3c) corresponds to that formed from GDP-[^{14}C]mannose alone (Fig. 3d) and has a mobility equivalent to a decasaccharide.

When tissue slices are supplied with either mannose or glucosamine, acid-labile lipid-linked saccharides are also formed (Fig. 4). Using mannose, lipid-linked saccharides seen at positions 1 and 10 turn over during chase with unlabeled mannose (Fig. 6) and appear to be intermediates in the normal metabolic pathways of the tissue. A sugar analysis of the total acid-labile, lipid-linked saccharide fraction labeled from [^{3}H]mannose shows that mannose is epimerized to other neutral sugars, but not to glucosamine (Fig. 7b), during such incubations: 80% of the label after 60 min is still present as mannose; 15%, as glucose; and less than 5%, as galactose. In contrast, metabolic conversion of glucosamine to other sugars is negligible (Fig. 7d) and [^{3}H]glucosamine is, thus, the substrate of choice for such *in vivo* experiments. Lipid 10 is strongly labeled with glucosamine and can be clearly separated from the other lipid-linked oligosaccharides by Bio-Gel P-4 chromatography (Fig. 7c). Its structural relationship to the lipid-linked oligosaccharides of similar size synthesized by membrane preparations from UDP-[^{3}H]GlcNAc and unlabeled GDP-mannose (Fig. 3c) remains to be ascertained.

The lack of significant labeling of completed storage glycoproteins during *in vitro* incubations might be explained by an absence

of mRNA translation in our membrane preparations, and thus lacking appropriate membrane-associated protein acceptors, and/or the incomplete synthesis of lipid-linked oligosaccharide donors, although low levels of glycosylation of proteins has been observed, *in vitro*, in preparations from *Phaseolus vulgaris* cotyledons (8). There is evidence in animal systems (for example, see refs. 19 and 21) that some Man_n-$GlcNAc_2$ lipids must be lengthened by addition of glucose units to form a glucosylated-oligosaccharide before they are efficiently transferred to nascent polypeptide acceptors. Although a common lipid-linked oligosaccharide intermediate has been identified (23) and characterized (17) as a donor during glycoprotein glycosylation, recent reports suggest that there may be more than one such intermediate (6, 26).

The size of lipid-linked oligosaccharide which is accumulated by soybean tissue slices and membranes during incubations with GDP-[^{14}C]mannose (Fig. 3, peak 10) is clearly smaller that that which is formed by mouse L-cells and membranes in similar incubations. A similar phenomenon has been observed in pea membrane preparations (1) where the efficiency of formation of the higher mannose-containing lipid-linked oligosaccharides was less than that of L-cell membranes. In animal systems (19, 21, 23), the size and structures of lipid-linked saccharides that are synthesized *in vivo* and *in vitro* are similar. In soybean cotyledon tissue slices, the major lipid-linked saccharide labeled from both mannose and glucosamine (Fig. 5, peak 15) is of higher mol wt than that formed *in vitro* (Fig. 3). In addition, unlike such lipid-linked saccharides in animal cells, this lipid accumulates *in vivo* and does not appear to turn over during pulse-chase experiments. It may thus represent a structural lipid in soybean membranes which is not metabolized further.

An analysis of the mannose-labeled, protein-bound oligosaccharides formed by soybean cotyledon tissue slices (Fig. 9a) shows them to be of approximately similar size to the high-mannose, asparagine-linked oligosaccharides synthesized from [^{3}H]mannose by mouse L-cells (Fig. 9b). This is in agreement with other workers who have analyzed the size of such oligosaccharides attached to asparagine in soybean 7S reserve glycoproteins (28, 29), and with the detailed chemical structures proposed for asparagine-linked oligosaccharide side-chains of soybean agglutinin (18). The great structural similarity between asparagine-linked oligosaccharide present in soybean glycoproteins and those high-mannose chains present in animal glycoproteins (18) itself suggests the conservation of the biosynthetic processes involved in their assembly. Attempts to determine the structures of the lipid-linked oligosaccharides isolated here and the ability to linked *in vitro* lipid-oligosaccharide assembly to protein synthesis will help in further elucidating the requirements and mechanism of glycoprotein biosynthesis in soybean cotyledon tissue. *In vivo* labeling studies suggest (Sengupta *et al.*, unpublished data) that there is both co- and post-translational glycosylation of 7S storage proteins indicating the presence of two different glycosylation sites in this tissue. The nature and the specificity of these reactions remains to be established.

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LITERATURE CITED

1. BAILEY DS, M DÜRR, J BURKE, G. MACLACHLAN 1979 The assembly of lipid-linked oligosaccharides in plant and animal membranes. J Supramol Struct 11: 123–138
2. BEEVERS L, RM MENSE 1977 Glycoprotein biosynthesis in cotyledons of *Pisum sativum* L.: involvement of lipid intermediates. Plant Physiol 60: 703–708
3. BEEVERS L, R POULSON 1972 Protein synthesis in cotyledons of *Pisum sativum* L. I. Changes in cell-free amino acid incorporation capacity during seed development and maturation. Plant Physiol 49: 476–481
4. BONNER WM, RA LASKEY 1974 A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46: 83–88
5. BRETT CT, LF LELOIR 1977 Dolichyl monophosphate and its sugar derivatives in plants. Biochem J 161: 93–101
6. CHAPMAN A, IS TROWBRIDGE, R HYMAN, S KORNFELD 1979 Structure of the lipid-linked oligosaccharides that accumulate in class E Thy-1-negative mutant lymphomas. Cell 17: 509–515
7. CRUMPTON MJ 1959 Identification of amino sugars. Biochem J 72: 479–486
8. DAVIES HM, DP DELMER 1980 Seed reserve-protein glycosylation in an *in vitro* preparation from developing cotyledons of *Phaseolus vulgaris*. Planta 148: 305–311
9. DELMER DP, C KULOW, MC ERICSON 1978 Glycoprotein synthesis in plants. II. Structure of the mannolipid intermediate. Plant Physiol 61: 25–29
10. DÜRR M, DS BAILEY, G MACLACHLAN 1979 Subcellular distribution of membrane-bound glycosyltransferases from pea stems. Eur J Biochem 97: 445–453
11. ELBEIN AD 1979 The role of lipid-linked saccharides in the biosynthesis of complex carbohydrates. Annu Rev Plant Physiol 30: 239–272
12. ERICSON MC, DP DELMER 1977 Glycoprotein synthesis in plants. I. Role of lipid intermediates. Plant Physiol 59: 341–347
13. ERICSON MC, DP DELMER 1978 Glycoprotein synthesis in plants. III. Interaction between UDP-*N*-Acetylglucosamine and GDP-mannose as substrates. Plant Physiol 61: 819–823
14. HILL JE, RW BREIDENBACH 1974 Proteins of soybean seeds. II. Accumulation of the major protein components during seed development and maturation. Plant Physiol 53: 747–751
15. KAPITANY RA, EJ ZEBROWSKI 1973 A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal Biochem 56: 361–369
16. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
17. LI E, I TABAS, S KORNFELD 1978 The synthesis of complex-type oligosaccharides. I. Structure of the lipid-linked oligosaccharide precursor of the complex-type oligosaccharides of the VSV G protein. J Biol Chem 253: 7762–7770
18. LIS H, N SHARON 1978 Soybean agglutinin—a plant glycoprotein. Structure of the carbohydrate unit. J Biol Chem 253: 3468–3476
19. LIU T, B STETSON, SJ TURCO, SC HUBBARD, PW ROBBINS 1979 Arrangement of glucose residues in the lipid-linked oligosaccharide precursor of asparaginyl oligosaccharides. J Biol Chem 254: 4554–4559
20. MILLERD A, M SIMON, H STERN 1971 Legumin synthesis in developing cotyledons of *Vicia faba* L. Plant Physiol 48: 419–425
21. PARODI AJ, LF LELOIR 1979 The role of lipid intermediates in the glycosylation of proteins in the eucaryotic cell. Biochim Biophys Acta 559: 1–37
22. PONT LEZICA R, CT BRETT, PR MARTINEZ, MA DANKERT 1975 A glucose acceptor in plants with the properties of an α-saturated polyprenylmonophosphate. Biochem Biophys Res Commun 66: 980–987
23. ROBBINS PW, SC HUBBARD, SJ TURCO, DF WIRTH 1977 Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. Cell 12: 893–900
24. SPIRO RG 1966 Analysis of sugars found in glycoproteins. Methods Enzymol 8: 3–26
25. THANH VH, R SHIBASAKI 1976 Heterogeneity of betaconglycinin. Biochim Biophys Acta 439: 326–338
26. TROWBRIDGE IS, R HYMAN 1979 Abnormal lipid-linked oligosaccharides in class E Thy-1-negative mutant lymphomas. Cell 17: 503–508
27. VERMA DPS, DT NASH, HM SCHULMAN 1974 Isolation and *in vitro* translation of leghaemoglobin messenger RNA in soybean. Nature 251: 74–77
28. YAMANCHI F, M KAWASE, M KANBE, K SHIBASAKI 1975 Separation of the β-aspartamido-carbohydrate fractions from soybean 7S protein: protein carbohydrate linkage. Agric Biol Chem 39: 873–878
29. YAMANCHI F, VH THANH, M KAWASE, K SHIBASAKI 1976 Separation of the glycopeptides from soybean 7S protein: their amino acid sequence. Agric Biol Chem 40: 691–696
30. YOSIZAWA Z, T SATO, K SCHMID 1966 Hydrazinolysis of α_1-acid glycoprotein. Biochim Biophys Acta 121: 417–420
31. YURCHENCO PD, C CECCARINI, PH ATKINSON 1978 Labeling complex carbohydrates of animal cells with monosaccharides. Methods Enzymol 50: 175–204