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Glycoprotein Biosynthesis in Cotyledons of *Pisum sativum* L.

INVOLVEMENT OF LIPID-LINKED INTERMEDIATES[1]

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ABSTRACT

Particulate preparations from developing cotyledons of *Pisum sativum* L. cv. Burpeeana catalyze glycosyl transfer from UDP-[^{14}C]N-acetylglucosamine and GDP-[^{14}C]mannose. Radioactivity is transferred to lipid components soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) and into a water-insoluble and lipid-free residue.

The chloroform-methanol-soluble component formed from GDP-[^{14}C]mannose appears to be a mannosyl lipid, whereas the chloroform-methanol-water-soluble fraction is probably a mixed oligosaccharide-lipid containing N-acetylglucosamine and mannose residues. The chloroform-methanol-soluble component formed from UDP-[^{14}C]N-acetylglucosamine appears to be N,N'-diacetylchitibiosyl lipid, which may be incorporated with mannose to form the chloroform-methanol-water-soluble mixed oligosaccharide lipid.

The oligosaccharide lipid appears to function as a precursor for the transfer of the oligosaccharide to the peptide moiety in the formation of the glycoproteins. The bulk of the radioactivity, arising from UDP-[^{14}C]N-acetylglucosamine, incorporated into the insoluble residue, is associated with glycoprotein. In contrast only a small percentage of radioactivity in the insoluble residue, arising from GDP-[^{14}C]mannose incorporation, appears to be associated with glycoprotein. The majority of the radioactivity found in the residue fraction labeled from GDP-[^{14}C]mannose appears to be associated with oligomannosyl residues.

The reserve globulins, legumin and vicilin, in the pea cotyledon are glycoproteins. Acid hydrolysis of these glycoproteins releases the amino-sugar glucosamine and neutral sugars, principally mannose (2). Similar glycosyl components occur in the glycoproteins from animal tissues (16, 27). Studies with extracts from animal tissues have established that the glycosyl components in the glycoproteins are derived originally from nucleotide sugars. The glycosyl components from the nucleotide sugars are transferred to lipid (polyisoprenol) intermediates to form lipid-linked monosaccharides which serve as precursors for the formation of lipid-linked oligosaccharides. This oligosaccharide-lipid serves as the carrier of the oligosaccharide chain which combines with the polypeptide to produce glycoproteins (14, 17). Similar lipid intermediates have been implicated in the biosynthesis of glycoproteins in shoots of mung bean seedlings (1, 7, 11), cotton fibers (6), and cotyledons of *Phaseolus vulgaris* (5).

As part of our continuing studies of protein synthesis in the developing pea cotyledon we have investigated the mechanism of glycosyl transfer to glycoprotein in this tissue and report on the possible involvement of lipid-linked intermediates.

MATERIALS AND METHODS

Developing cotyledons were collected from peas (*Pisum sativum* L. cv. Burpeeana) cultured as described previously (2). For the majority of the experiments the cotyledons were collected 18 to 20 days post anthesis. The cotyledons (10 g) were chopped with a razor blade chopper in 14 ml of homogenization medium. The razor blade chopper was fabricated by replacing the reciprocating blades of a domestic electric knife with razor blades. For routine experiments the homogenization medium consisted of 50 mM tris-HCl (pH 7) containing 10 mM KCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% BSA (fraction V, Sigma Chemical Co.), 1% dextran (234,000 average mol wt Sigma Chemical Co.), and 10% (w/v) sucrose. The brei was filtered through two layers of cheesecloth and one layer of Miracloth (Calbiochem, La Jolla, Calif.) and centrifuged at 369*g* (r_{av} 8.25 cm) in a SS-34 rotor in a Sorvall RC 2B centrifuge. The resulting supernatant was then centrifuged at 175,674*g* (r_{av} 5.7 cm) for 60 min in a Beckman 65 rotor in a Beckman L2-65B centrifuge. The supernatant from this centrifugation was discarded and the surface of the remaining pellet was washed with reaction medium. Pellets were suspended in 3 ml of reaction mixture which, unless stated otherwise, consisted of 50 mM tris-HCl (pH 7) containing 10 mM KCl, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. This suspension of cellular particulates was used as the enzyme source.

Assay of Glycosyl Transferase Activity. The particulate suspension (150 μl) was routinely inclubated with a 100-μl reaction mixture plus 48 μl of H_2O and 2 μl of ^{14}C-labeled nucleotide sugars. The nucleotide sugars used were uridine diphospho-N-acetyl-D-[U-^{14}C]glucosamine (300 mCi/mmol, 24 μCi/ml, Amersham-Searle Corporation) and guanosine diphospho[U-^{14}C]mannose (179 mCi/mmol, 25 μCi/ml, Amersham-Searle Corporation). After a 60-min incubation at 37 C the reaction was terminated by the addition of 0.7 ml of H_2O and 2.5 ml of chloroform-methanol (2:1, v/v). After thorough mixing the phases were separated by centrifugation and the lower organic phase recovered. The upper aqueous layer and interphase were reextracted with 2.5 ml of 2:1 chloroform-methanol; the organic phase was collected and pooled with the previous extraction. These pooled 2:1 chloroform-methanol fractions were then washed twice with 2.5 ml of chloroform-methanol-water (3:48:47). At each extraction the upper phase was discarded. The recovered washed 2:1 chloroform-methanol fraction was placed in a scintillation vial, evaporated to dryness, and radioactivity determined upon the addition of scintillation fluid (8 g 2,5-diphenyloxazole [PPO] in 2 liters of toluene, 1 liter of Triton X-100).

The aqueous phase and interphase remaining after chloro-

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form-methanol extraction were mixed with 0.5 ml of 99% methanol, centrifuged, and the supernatant was discarded. The pelleted residue was washed twice with 2.5 ml of 50% (v/v) methanol which was thoroughly drained from the pellet and discarded. The pellet was suspended in 2.5 ml of chloroform-methanol-water (1:1:0.3, v/v/v) and incubated at room temperature for 15 min, then centrifuged. The solvent was recovered and the pellet reextracted with a further 2.5 ml of chloroform-methanol-water (1:1:0.3). The pooled chloroform-methanol-water (1:1:0.3) samples were placed in scintillation vials, dried in an air stream, and 12 ml of scintillation fluid were added.

The pellet which remained after chloroform-methanol-water (1:1:0.3) extraction was suspended in 2.5 ml of 5% (w/v) trichloroacetic acid and the material was filtered through a 2.4-cm Whatman GF/A Fiberglass filter. The filter was washed twice with 5 ml of 5% trichloroacetic acid and placed in a scintillation vial. Vials were heated at 60 C to dry the filters prior to the addition of scintillation fluid.

Bulk Preparations and Product Characterization. To characterize the chloroform-methanol (2:1), chloroform-methanol-water (1:1:0.3), and insoluble residue, the incubation system was scaled up 10-fold. Appropriate adjustments were made in the lipid extraction solvents prior to following the extraction protocol described above. The lipid-soluble fractions (2:1 and 1:1:0.3) were evaporated to dryness under vacuum in a rotary evaporator at 30 C. The resulting residue was dissolved in a small volume of the appropriate solvent.

Aliquots of this material were used for TLC on precoated Silica Gel G-25 plates (Brinkmann Instruments, Des Plains, Ill.) in the solvent systems A (chloroform-methanol-acetic acid-H_2O, 50:30:8:4) and B (chloroform-methanol-NH_4OH, 75:25:4). Thin layer chromatograms on Eastman 13254 cellulose plates were developed in the solvent system C (isobutyric acid-IN NH_4OH, 5:3).

The lipid-soluble components were also fractionated by column chromatography on DEAE-cellulose (acetate) prepared by a modification of the procedure described by Rouser *et al.* (25). The column (2.5 × 35 cm) was prepared by suspending DEAE-cellulose (Cellex D, Bio-Rad Corporation, Richmond, Calif.) in glacial acetic acid. The column was poured and washed sequentially with 250 ml of glacial acetic acid, 200 ml of 99% methanol, and 250 ml of chloroform-methanol (2:1). For fractionation of the chloroform-methanol-water (1:1:0.3)-soluble material, columns received an additional wash of 250 ml of chloroform-methonol-water (1:1:0.3). Lipid-soluble materials were applied to the DEAE-cellulose (acetate) columns in the appropriate solvents. Columns for the chloroform-methanol (2:1)-soluble component were eluted sequentially with 250 ml of chloroform-methanol (2:1), 200 ml of 99% methanol, followed by a 500-ml linear gradient of 0 to 0.2 M ammonium acetate in methanol. Ten-ml fractions were collected and 2-ml aliquots were placed in scintillation vials, evaporated to dryness by an air stream prior to the additon of scintillation cocktail, and radioactivity was determined. The columns to which the chloroform-methanol-water (1:1:0.3)-soluble material had been applied were eluted with 250 ml of (1:1:0.3) followed by a 500-ml linear gradient of 0 to 0.2 M ammonium formate in chloroform-methanol-water (1:1:0.3). Ten-ml fractions were collected and radioactivity determined on 2-ml aliquots as described above.

Acid Hydrolysis of Lipid Material. Samples of lipid materials from bulk preparations were dried in test tubes to which 1 ml of 50% isopropyl alcohol and 0.1 ml of 0.1 N HCl were added. Tubes were heated in a boiling water bath and at appropriate times removed, cooled, and 0.1 ml of 0.1 N NaOH and 2.5 ml of chloroform-methanol (2:1) added. The contents of the tube were mixed and centrifuged. The organic and aqueous phases were separated, placed in scintillation vials, and dried prior to the determination of radioactivity.

Characterization of Hydrolysis Products. Samples of the lipid materials were hydrolyzed for 10 min with 50% isopropyl alcohol and 0.01 N HCl as described above. After termination of the hydrolysis with NaOH and chloroform-methanol (2:1), the aqueous phase was recovered. Aliquots were spotted on Whatman No. 1 chromatography papers and chromatograms were developed by descending chromatography in solvents D (butanol-pyridine-H_2O, 6:4:3) or E (ethyl acetate-acetic acid-formic acid-H_2O, 18:3:1:4) for 20 hr. The chromatograms were dried, cut into 2.5-cm strips which were placed in vials with scintillation fluid and the radioactivity was determined.

Characterization of Residue Fraction. The material remaining after lipid solvent extraction of the bulk preparation was washed three times with water. Part of the residue sample was immediately filtered through 2.4-cm Whatman GF/A Fiberglass filters. Another sample was incubated with an equal volume of a solution which was 1.2% tris, 1.5% DTT, and 1% SDS (23) heated for 5 min in a boiling water bath and then filtered through a Whatman GF/A Fiberglass filter. A final sample was suspended in 0.2 M NaCl, 0.1 M phosphate buffer (pH 7) or similar buffer containing 0.75 mg of protease (type VI, Sigma Chemical Co., St. Louis, Mo.), and incubated for 36 hr at 37 C in a shaking water bath after which time an additional 1 mg of protease was added and the incubation was continued for a further 60 hr. The incubated samples were then filtered through Whatman GF/A filters. In all three treatments the residues were recovered and placed in vials for determination of radioactivity.

RESULTS AND DISCUSSION

Time Course Studies. Preliminary experiments demonstrated that particulate fractions prepared from pea cotyledons 18 to 20 days after anthesis had the capacity to transfer radioactivity from GDP-[^{14}C]mannose into material which was soluble in chloroform-methanol (2:1). Additional radioactivity was soluble in chloroform-methanol-water (1:1:0.3) and label was incorporated into a lipid-free residue. Time course studies indicated that when GDP-[^{14}C]mannose was utilized as glycosyl donor, there was a rapid incorporation of radioactivity into all components (Fig. 1). Incorporation into the lipid-free residue in the early stages exceeded that in the lipid fraction. Within the lipid fractions, incorporation of radioactivity into the chloroform-methanol (2:1)-soluble material preceded and occurred more rapidly than incorporation into the chloroform-methanol-water (1:1:0.3)-soluble component.

When UDP-[^{14}C]GlcNAc[2] was supplied to the particulate fraction the total incorporation of radioactivity into the various fractions was much less than that which occurred from GDP-[^{14}C]mannose. Incorporation of radioactivity occurred most rapidly and extensively into the chloroform-methanol (2:1)-soluble fraction with a slower transfer to the chloroform-methanol-water (1:1:0.3)-soluble component (Fig. 1). Incorporation of radioactivity into the lipid fractions was rapid for 10 to 15 min and then the rate declined. In contrast, the transfer of label to the lipid-free residue increased progressively throughout the course of the experiment.

This sequence of incorporation of radioactivity from UDP-[^{14}C]GlcNAc is similar to that described using extracts from animal tissues in which GDP-[^{14}C]mannose served as precursor (3, 28, 29, 31). A similar time course for the incorporation of N-acetylglucosamine into lipid components from labeled UDP-GlcNAc has been described by Lucas *et al.* (15) in extracts from hen oviducts and by Forsee *et al.* (7) in particulate fractions from mung bean shoots. In contrast Ericson and Delmer (5) indicate that in extracts from *Phaseolus vulgaris*

[2] Abbreviations: UDP-GlcNAc: UDP-N-acetylglucosamine; GlcNAc: N-acetyglucosamine; $GlcNH_2$: glucosamine.

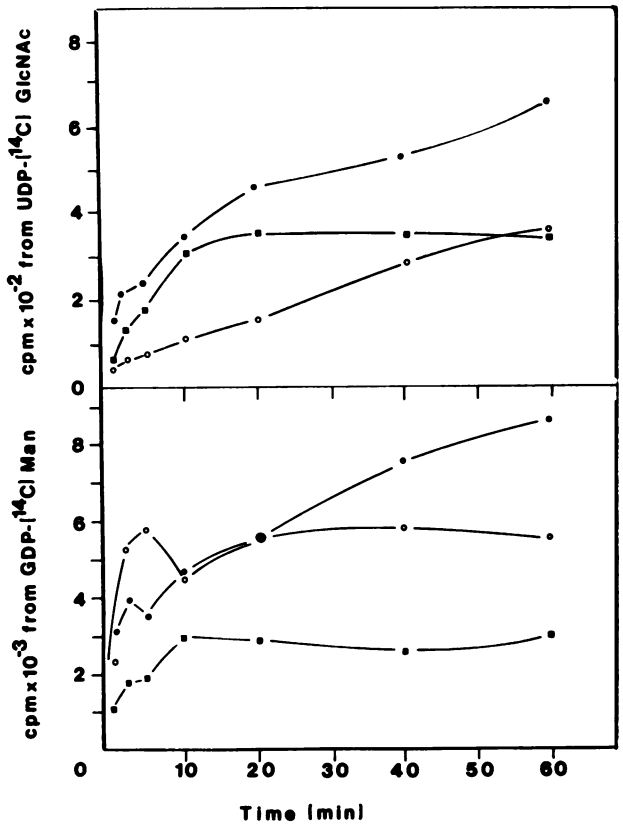

FIG. 1. Time course for incorporation of radioactivity from UDP-[^{14}C]GlcNAc or GDP-[^{14}C]mannose into lipid-linked components and lipid-free residue. Particulate preparations (1.5 ml) from developing pea cotyledons were incubated with the nucleoside sugar phosphates and reaction medium. At intervals 0.3-ml aliquots were removed and extracted with lipid solvents as described in the text. (●——●): chloroform-methanol (2:1); (■——■): chloroform-methanol-water (1:1:0.3); (○——○): residue.

cotyledons radioactivity from UDP-[^{14}C]GlcNAc was transferred most extensively into an insoluble residue. The time course sequence of transfer of radioactivity from GDP-[^{14}C]mannose to lipid-linked components reported in the present investigation differs markedly from that reported in the animal systems cited above. When preparations from plant systems are incubated with GDP-[^{14}C]mannose a more extensive incorporation into lipid-free residue than into lipid fractions is usually observed (1, 5-7, 26).

Characterization of Incorporation Reactions. Wedgwood *et al.* (33) have reported that the detergent Triton X-100 stimulates the transfer of mannose units from GDP-mannose into lipid material and Alam and Hemming (1) report a stimulation of mannosyl transfer from GDP-mannose in particulate fractions from *Phaseolus aureus*. In our experiments it was found that inclusion of Triton X-100 into the incorporation system reduced the transfer of radioactivity from UDP-[^{14}C]GlcNAc into the 2:1 soluble fraction but had little effect on subsequent transfer to the other fractions. We have also observed similar results with sodium deoxycholate (0.1 and 1 mM).

Others have suggested that endogenous phosphatases may hydrolyze the supplied precursor and thus limit the glycosyl transfer from the sugar nucleotides into various products (19, 30). These authors have suggested that this phosphatase interference can be overcome by the inclusion of nucleotides in the assay mixture which competitively reduce degradation of the supplied precursor. However, in the present investigation inclusion of uridine triphosphate (0.1 and 1 mM) in the reaction medium decreased incorporation into the lipid components.

Roberts and Pollard (24) have indicated that in preparations from *P. aureus* optimal transfer of radioactivity from UDP-GlcNAc into trichloroacetic acid-insoluble material was observed at pH 9. However, transfer of radioactivity from UDP-[^{14}C]GlcNAc by the particulate preparation from the developing pea cotyledons, used in the present study was greatest at pH 7.0 to 7.5.

Characterization of Chloroform-Methanol (2:1)-soluble Material. The chloroform-methanol (2:1)-soluble radioactive material produced when the particulate fraction was incubated with GDP-[^{14}C]mannose migrated at R_F 0.82 during TLC on Silica Gel G in acid solvent A but remained at the origin in the basic system (solvent B). The radioactive component migrated close to the solvent front during chromatography on cellulose with solvent C. Similarly, the chloroform-methanol-soluble material produced from UDP-[^{14}C]GlcNAc migrated at R_F 0.69 in acid solvent A and remained at the origin in basic solvent B during chromatography on Silica Gel G but migrated close to the solvent front on the cellulose plates developed in solvent C. The chromatography characteristics are similar to those exhibited by the mannosyl and N-acetylglucosaminyl lipids from cotton fibers (6) and mung bean seedlings (7) and for the mannosyl lipids produced when particulate fractions from animal tissues are incubated with GDP-mannose (3, 9, 32).

The chloroform-methanol-water (1:1:0.3)-soluble material labeled from UDP-[^{14}C]GlcNAc or GDP-[^{14}C]mannose migrated as one peak at R_F 0.75 on cellulose plates developed in solvent C. However, on Silica Gel G the radioactivity remained at the origin in both solvent systems tested. A similar slower mobility of the chloroform-methanol-water-soluble fraction produced from GDP-mannose has been reported by Hsu *et al.* (9).

The lipid materials were retained on DEAE-cellulose (acetate) columns (Fig. 2). The chloroform-methanol (2:1)-soluble mannosyl and N-acetylglucosaminyl-labeled lipids were eluted with ammonium acetate in methanol in agreement with the observations in mung bean seedling (7) and animal tissues (3, 22). The mannosyl lipid (Fig. 2b) was eluted at lower ammonium acetate concentrations than the lipid produced from reactions which included UDP-[^{14}C]GlcNAc (Fig. 3a). This is similar to the behavior of glycosylated lipids produced by extracts from *Phaseolus* (7, 11).

Radioactivity extracted by chloroform-methanol-water (1:1:0.3) was also retained on DEAE-cellulose (acetate) columns and was eluted with ammonium formate in chloroform-methanol-water (Fig. 2, c and d). Similar chromatographic behaviors on DEAE-cellulose (acetate) have been reported for the chloroform-methanol-water (1:1:0.3)-soluble radioactivity produced when particulate fractions from animal systems are incubated with GDP-mannose (3, 9).

The retention of the lipid-linked radioactivity on DEAE-cellulose columns indicates the presence of charged groups and on the basis of comparison studies with animal systems (14) the glycosyl lipid linkages probably involve phosphate.

Acid Hydrolysis of Lipid-linked Components. The radioactivity associated with the lipid components was rapidly dissociated and became water-soluble when the materials were hydrolyzed in dilute HCl in 50% propanol (Fig. 3). The transfer of radioactivity from the organic phase to the aqueous phase occurred less rapidly from chloroform-methanol (2:1)-soluble material produced from UDP-[^{14}C]GlcNAc (Fig. 3a) than that derived from GDP-[^{14}C]mannose (Fig. 3b). This suggests a greater resistance to acid hydrolysis of the N-acetylglucosaminyl-linked lipid in comparison to the mannosyl lipid. Similar observations have been reported by Forsee *et al.* (7).

Paper chromatography indicated (Table I) that the chloroform-methanol (2:1)-soluble material, produced from GDP-[^{14}C]mannose after acid hydrolysis, released water-soluble radioactivity which co-chromatographed with the mannose standard in the solvents D and E. In contrast, two radioactive

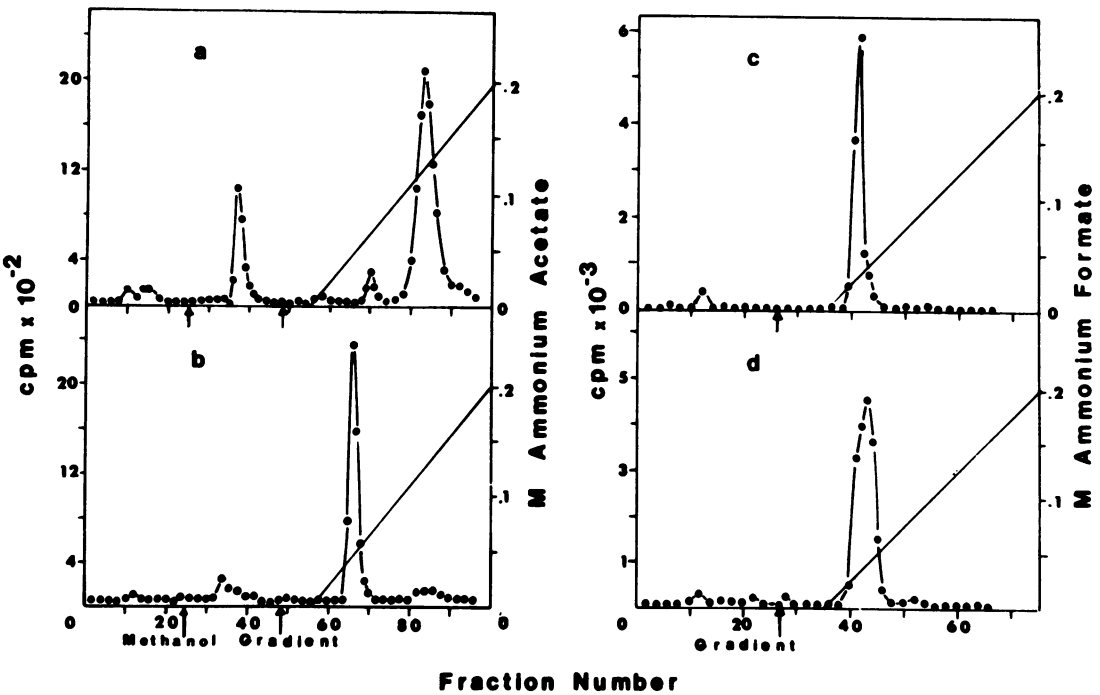

FIG. 2. DEAE-cellulose chromatography of lipid components. (*a*): Particulate suspensions incubated with UDP-[^{14}C]GlcNAc extracted with chloroform-methanol (2:1); (b): particulate suspensions incubated with GDP-[^{14}C]mannose extracted with chloroform-methanol (2:1); (c): chloroform-methanol-water (1:1:0.3)-soluble material from particulate suspensions incubated with UDP-[^{14}C]GlcNAc; (d): chloroform-methanol-water (1:1:0.3)-soluble material from particulate suspensions incubated with GDP-[^{14}C]mannose. Lipid fractions were applied immediately to DEAE-cellulose columns in the appropriate extracting solvents. Elution with either ammonium acetate or ammonium formate and collection of the samples was as described under "Materials and Methods."

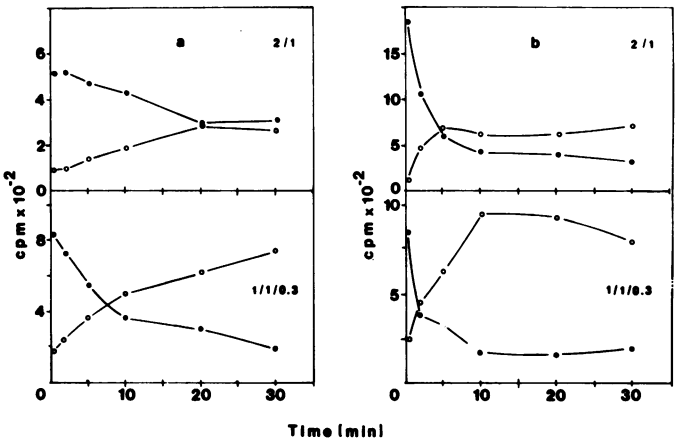

FIG. 3. Acid hydrolysis of lipid components. Lipid components, chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3)-soluble fractions were obtained from incubations of particulate fractions with (a) UDP-[^{14}C]GlcNAc or (b) GDP-[^{14}C]mannose. After air-drying, the lipid fractions were dissolved in propanol and brought to a final concentration 0.01 N HCl and placed in a boiling water bath. At intervals the reaction was terminated by addition of NaOH and phases separated after addition of chloroform-methanol (2:1). (●——●): Organic phase; (○——○): aqueous phase.

components were detected when the water-soluble material produced by acid hydrolysis of the chloroform-methanol-water (1:1:0.3) labeled from GDP-[^{14}C]mannose, was chromatographed (Table I). Some of the radioactivity remained at the origin while another fraction co-chromatographed with the mannose standard. The observation that radioactivity remained at the origin indicates the association of mannose with an oligosaccharide which may be partially hydrolyzed in the dilute acid to liberate free mannose.

The water-soluble radioactivity released by mild acid hydrolysis of the chloroform-methanol (2:1)-soluble material, produced when particulate fractions were incubated with UDP-[^{14}C]GlcNAc, migrated more slowly than GlcNAc in systems D and E and at almost the same rate as the glucosamine standard in solvent D (Table I). In solvent E, however, the labeled component migrated more rapidly than the glucosamine standard (Table I). These chromatographic characteristics are similar to those reported for N,N'-diacetylchitibiose (7, 13, 15, 34) and it thus appears that the chloroform-methanol (2:1)-soluble material produced from UDP-[^{14}C]GlcNAc under our incubation conditions is a lipid-linked disaccharide.

The water-soluble radioactivity produced by hydrolysis of the chloroform-methanol-water (1:1:0.3)-soluble lipid labeled from UDP-[^{14}C]GlcNAc remained close to the origin on chromatography in both solvents D and E (Table I) indicating the association of [^{14}C]GlcNAc with an oligosaccharide.

Transfer of Radioactivity from [^{14}C]GlcNAc-linked Lipid Soluble in Chloroform-Methanol (2:1) to Material Soluble in Chloroform-Methanol-Water (1:1:0.3). The chromatographic properties of the material soluble in chloroform-methanol-water (1:1:0.3) prepared from particulate fractions incubated with either GDP-[^{14}C]mannose or UDP-[^{14}C]GlcNAc are similar (Fig. 2). Furthermore, acid hydrolysis releases radioactivity which remains at the origin in the solvent systems tested. These

Table I. Paper chromatography of water soluble material arising from acid hydrolysis of lipids

^{14}C-GlcNAc or ^{14}C-mannose labeled lipids were hydrolyzed in dilute HCl and the water soluble material released was subjected to descending paper chromatography in solvent system D and E. Papers were dried, cut into 2.5 cm strips and radioactivity determined.

Material hydrolyzed	Solvent D	Solvent E
	R_{Man}	
2/1 from GDP[^{14}C]Man	1.0	1.07
1/1/0.3 from GDP[^{14}C]Man	0.09, 1.0	0.06, 1.0
	R_{GlcNAc}	
2/1 from UDP[^{14}C]GlcNAc	0.7	0.63
1/1/0.3 UDP[^{14}C]GlcNAc	0.07	0.04
$GlcNH_2$	0.67	0.44

characteristics suggest that the material soluble in chloroform-methanol-water is a lipid-linked, mixed oligosaccharide in which the glycosyl components are N-acetylglucosamine and mannose. Other analyses (14, 18) have indicated that GlcNAc occurs at the reducing terminus of the oligosaccharide in glycolipids and glycoprotein. It has been suggested (14, 22) that the lipid-linked N-acetylglucosamine or N,N'-diacetylchitibiose serves as the precursor to which mannosyl residues become attached to produce the lipid-linked oligosaccharide. If this sequence occurs in the developing pea cotyledon, it should be possible to demonstrate the transfer of radioactivity from the chloroform-methanol (2:1) fraction labeled from UDP-[^{14}C]GlcNAc into chloroform-methanol-water (1:1:0.3)-soluble material in the presence of GDP-mannose. To confirm this, particulate fractions from the pea cotyledon were incubated with UDP-[^{14}C]GlcNAc for 15 min. Subsequently the volume was increased by the addition of sufficient cold reaction mixture to fill a SW 65 polycarbonate centrifuge tube. The tubes and contents were then centrifuged at 159,315g (r_{av} 5.7 cm) for 45 min in a Beckman 65 rotor in a Beckman L2-65B centrifuge. The pellet was resuspended in 6 ml of reaction mix. Duplicate 0.5-ml samples were mixed with 25 μl of H_2O to be extracted with the lipid solvents or incubated for 20 min at 37 C. The resuspended pellet was incubated with 0.2 ml of 1 mM GDP-mannose. Duplicate samples of 0.5 ml were removed at 3, 5, 10, and 20 min and extracted with the lipid solvents. It was found (Fig. 4) that there was only slight decline in radioactivity associated with the (2:1) and (1:1:0.3) soluble fractions when the resuspended prelabeled pellets were incubated an additional 20 min with water. This observation indicated that the centrifugation step had effectively removed excess UDP-[^{14}C]GlcNAc and that there was no transfer between the two lipid fractions. In contrast when GDP-mannose was added to the prelabeled resuspended particulate fractions, radioactivity was chased from the chloroform-methanol (2:1) fraction into material soluble in chloroform-methanol-water (1:1:0.3). These findings are consistent with the concept that the GlcNAc containing lipid soluble in chloroform-methanol (2:1) is converted into a lipid-linked oligosaccharide containing mannose and GlcNAc which is soluble in chloroform-methanol-water (1:1:0.3). At this stage it has not been definitively established whether the mannosyl units incorporated into the oligosaccharide are derived directly from GDP-mannose or lipid-linked mannosyl residues generated from GDP-mannose.

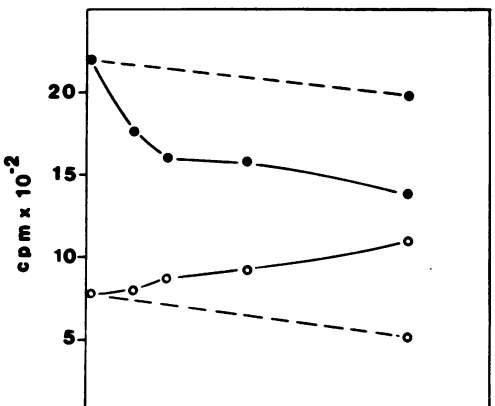

FIG. 4. Transfer of radioactivity from lipid component soluble in chloroform-methanol (2:1) into fraction soluble in chloroform-methanol-water (1:1:0.3). Particulate fractions from developing pea cotyledons were incubated with UDP-[^{14}C]GlcNAc for 15 min, excess reaction medium was then added, and the particulate material recovered by centrifugation. The resulting pellet was resuspended and incubated with GDP-mannose (——) or H_2O (- - -). At intervals, aliquots were removed and extracted with lipid solvents chloroform-methanol (2:1) (●——●) or chloroform-methanol-water (1:1:0.3) (○——○) and radioactivity of each phase determined.

Characterization of Residue. It was found that incubation with protease released little radioactivity from the lipid-free residue which had been produced by incubating particulate fractions with GDP-[^{14}C]mannose. Treatment of the lipid-free residue with SDS in the presence of DTT resulted in slightly more counts being retained on the glass fiber filters compared to a buffer control. This evidence suggests that only a small portion of the radioactivity in the lipid-free residue produced from GDP-[^{14}C]mannose is associated with glycoprotein.

In contrast, when the lipid-free residue fraction, obtained from UDP-[^{14}C]GlcNAc, was incubated with protease, 80% of the radioactivity was solubilized. Over 60% of the glass filter retainable counts were solublized by SDS in the presence of DTT. Thus, the majority of the radioactivity in the residual fractions derived from UDP-[^{14}C]GlcNAc appears to be associated with glycoprotein.

CONCLUSION

The developing pea cotyledon possesses glycosyl transferases capable of transferring mannose and GlcNAc from GDP-mannose and UDP-GlcNAc, respectively, into lipid-linked components and lipid-free residues. The transferases are associated with cell particulate fractions and are thus similar to those previously described in animal systems (14) and other plant systems (1, 5, 7). The lipid-linked fractions formed from GDP-mannose appear to be lipid monosaccharide soluble in chloroform-methanol (2:1) and a lipid-linked oligosaccharide soluble in chloroform-methanol-water (1:1:0.3). At this stage we have no information on the nature of the lipid component in peas. However, in yeast (10) and in animal systems (14) it has been demonstrated that the lipid component is a polyisoprenoid derivative, dolichol. It appears that the same polyisoprenoid lipid, dolichol, is involved in the lipid monosaccharide and oligosaccharide and it is suggested (14) that the oligosaccharide chain renders the compound too hydrophilic to be soluble in chloroform-methanol while the lipid moiety is sufficiently hydrophobic to preclude solubility in water. The chromatographic behaviors of the lipid derivatives obtained during the course of the present study are very similar to those described for the polyisoprenoids from animal systems so that the lipid moiety involved in the glycosyl transfer in the pea cotyledon is in all probability a polyisoprenol derivative.

The water-soluble material produced by acid hydrolysis of the chloroform-methanol (2:1)-soluble lipid formed from UDP-[^{14}C]GlcNAc appears to be N,N'-diacetylchitibiose. Others (8, 18, 22, 33) have indicated that the linkage of the disaccharide to the lipid involves a pyrophosphate linkage. While we have not characterized the glycolipid linkage, the chromatographic behavior of the chloroform-methanol-soluble material on TLC and DEAE-cellulose (acetate) is similar to that of the lipid-linked components which in animal systems have been indicated to involve polyisoprenoid pyrophosphate.

N,N'-diacetylchitibiose has been demonstrated to occur at the reducing end of the oligosaccharide moiety of many glycoproteins. It appears to be involved in the attachment of the glycosyl component to asparaginyl residues of the peptide chain (14, 16, 17, 27). Our observations that radioactivity from the chloroform-methanol (2:1) fraction labeled from UDP-[^{14}C]GlcNAc can be chased into the (1:1:0.3) fraction in the presence of unlabeled GDP-mannose (Fig. 4) suggest that the N,N'-diacetylchitibiose becomes converted into a lipid-linked oligosaccharide containing N-acetylglucosamine and mannose. The similarity in chromatographic behaviors of the (1:1:0.3)

soluble material (Table I) produced by the particulate fractions from GDP-mannose or UDP-GlcNAc supports this concept.

Lipid-linked oligosaccharides have been implicated as precursors of the glycosyl component of glycoproteins (14, 20, 21). The time course kinetic data (Fig. 1) are consistent with the operation of the sequence in the pea cotyledon when UDP-[^{14}C]GlcNAc was used. The observation that much of the radioactivity in the lipid-free residue, labeled from UDP-[^{14}C]GlcNAc, is associated with glycoprotein also supports this sequence. A complete confirmation of this aspect will depend upon a demonstration of the similarity between the oligosaccharide component of the (1:1:0.3) soluble fraction and the oligosaccharide moiety present in the glycoproteins. Unfortunately, the limited labeling of the residue fraction has precluded this comparison.

The lipid-linked oligosaccharides labeled with mannose from GDP-mannose are probably the same oligosaccharides that incorporate N-acetylglucosamine. The chase experiments (Fig. 4) are consistent with this. However, it appears that not all of the mannosyl oligosaccharides produced by the particulate fractions are lipid-linked. In the pea cotyledon system and in other plant systems (1, 5–7, 26) a great deal of radioactive mannose becomes incorporated into lipid-free residue and is not associated with glycoprotein. The massive synthesis of these oligomannans masks the detection of mannosyl residues associated with glycoprotein. The time course kinetics data (Fig. 1) suggest that these oligomannans are synthesized without the participation of lipid-linked intermediates. A similar situation has been proposed to occur in yeast (12). The role of these oligomannans has not been established; galactomannans have been demonstrated in the endosperm reserves of some seeds and galactoglucomannans occur in the hemicellulosic fraction of cell walls (4). The massive synthesis of the oligamannans from GDP-mannose encountered in our studies and those of other workers (1, 5–7, 26) suggests that oligomannans may be of wider distribution in plants than is generally recognized.

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