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Incorporation of [^{14}C]Glucosamine and [^{14}C]Mannose into Glycolipids and Glycoproteins in Cotyledons of *Pisum sativum* L.[1]

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ABSTRACT

Developing pea cotyledons incorporate radioactivity *in vivo* from [^{14}C]glucosamine and [^{14}C]mannose into glycolipids and glycoproteins. Several different lipid components are labeled including neutral, ionic-nonacidic, and acidic lipids. The acidic lipids labeled *in vivo* appear similar to the polyisoprenoid lipid intermediates formed *in vitro* in pea cotyledons. Radioactivity from [^{14}C]glucosamine and [^{14}C]mannose is also incorporated into glycopeptides. Considerable redistribution of [^{14}C]mannose into other glycosyl components found in endogenous glycoproteins is observed. An *N*-acetylglucosamine to asparagine glycopeptide linkage has been isolated from [^{14}C]glucosamine-labeled glycoproteins.

The major reserve storage proteins in pea cotyledons, legumin, and vicilin, are glycoproteins containing $GlcNH_2$[3] and mannose (4). These sugars are both components of the "core" oligosaccharide characteristic of many animal, plant, and fungal glycoproteins (20, 30). These core oligosaccharides have the general structure (α-mannose)$_n$-β-mannose(1-4)-β-GlcNAc(1-4)-β-GlcNAc-asparagine.

Studies with animal (20, 30), plant (1, 5, 6, 8, 12–15, 18), and fungal (30) particulate preparations have indicated that these core oligosaccharides are assembled via polyisoprenoid lipid intermediates, which function as glycosyl carriers in the synthesis of the carbohydrate moiety of glycoproteins. The elucidation of the lipid intermediate scheme was greatly aided by the fact (30) that mono-, di-, and trisaccharide lipids are soluble only in mixtures of chloroform-methanol (2:1), while lipid oligosaccharides can be extracted only in chloroform-methanol-water (1:1:0.3). The lipid carrier in animals, fungi (30) and some plants (10) has been identified as the α-unsaturated polyisoprenol, dolichol.

Although *in vitro* studies using particulate preparations have demonstrated the synthesis of lipid intermediates implicated in the biosynthesis of glycoproteins, little information is available on the *in vivo* occurrence of these compounds. Roberts (24) has demonstrated the incorporation of [^{14}C]$GlcNH_2$ into lipids and glycoproteins of *Phaseolus aureus*, but the lipids were not characterized. There are a few reports concerning the nature of glycosylated lipids in animal systems (3, 26–28).

As part of our continuing study of protein synthesis in the developing pea cotyledon, we have partially characterized the lipids and glycoproteins labeled *in vivo* from exogenously supplied [^{14}C]$GlcNH_2$ and [^{14}C]mannose. The characteristics of the labeled glycolipids and glycoproteins are compared to the glycosylated lipids formed *in vitro* (5), which have been implicated as lipid intermediates in the assembly of pea cotyledon glycoproteins.

MATERIALS AND METHODS

Preparation of [^{14}C]Mannose- and [^{14}C]$GlcNH_2$-Labeled Lipids and Lipid-Free Residue. Developing pea cotyledons (*Pisum sativum* L. cv. Burpeeana) were obtained as previously described (4). Fifteen peas, 21 days postanthesis, were harvested, the testa and embryo removed, and each cotyledon injected with either 3 μl D-[U-^{14}C]$GlcNH_2$ (254 mCi/mmol, 200 μCi/ml, Amersham/Searle) or D-[1-^{14}C]mannose (59 mCi/mmol, 200 μCi/ml, Amersham/Searle). After injection, the cotyledons were incubated on moist filter paper in a glass Petri dish for 4.5 h in the light. Following incubation, the cotyledons were homogenized in (4:1 v/w) 50 mM K-phosphate (pH 7.0) in a Polytron for 1 min.

The resulting suspension was filtered through cheesecloth and aliquots taken for determination of radioactivity. The remaining suspension was centrifuged at 250*g* in an SS-34 rotor in a Sorvall RC-2B centrifuge. After the radioactivity in the pellet was determined, this fraction was discarded and the remaining supernatant was sequentially extracted by lipid solvents as previously described (5) to obtain the lipids soluble in chloroform-methanol 2:1 (v/v), chloroform-methanol-water (1:1:0.3, v/v/v), and a lipid-free residue. Aliquots of each of the lipid-extracted fractions (cf. Table I) were placed in scintillation vials, evaporated to dryness and, after the addition of scintillation fluid to the vials (8.0 g PPO, 2 liters toluene, 1 liter Triton X-100), the samples were counted in a Beckman LS-100 liquid scintillation counter. The lipid-free residue remaining after the final lipid extraction of the original homogenate was suspended in 5% (w/v) trichloroacetic acid, the precipitated pellet was washed with water and reprecipitated, and aliquots were taken to determine radioactivity as described above.

Chromatographic Methods. Lipids, extracted with chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3), were fractionated by column chromatography on DEAE-cellulose (acetate), prepared by a modification of the procedure of Rouser *et al.* (25) as described previously (5). Chloroform-methanol (2:1) soluble lipids were applied to DEAE columns (2.5 × 35 cm) and eluted sequentially with 250 ml of chloroform-methanol (2:1), 200 ml 99% methanol, followed by a 500-ml linear gradient of 0–0.2 M ammonium acetate in methanol. Fractions of 10 ml were collected and 2-ml aliquots were placed in scintillation vials, evaporated to dryness, and the radioactivity determined. The radioactive fractions eluted from the column with chloroform-methanol (2:1) (hereafter referred to as peak I) were pooled, as were those radioactive fractions eluted by the ammonium acetate gradient (peak II), and evaporated to dryness *in vacuo* at 30 C. The peak II pooled fractions were extracted with chloroform-methanol (2:1) and water, as described by Chambers and Elbein

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[3] Abbreviations: $GlcNH_2$: glucosamine; GlcNAc: *N*-acetyl glucosamine; GLcNAc-Asn: 1-*N*-β-L aspartyl-2-deoxy-β-D-glucopyranosylamine.

(8), to remove ammonium acetate. Lipids soluble in chloroform-methanol-water (1:1:0.3) were applied to DEAE columns (2.5 × 35 cm), eluted with 250 ml of chloroform-methanol-water (1:1:0.3), followed by a 500-ml linear gradient of 0–0.2 M ammonium formate in chloroform-methanol-water (1:1:0.3). Fractions of 10 ml were collected and 2-ml aliquots were assayed for radioactivity. The radioactive fractions eluted with the starting solvent (peak I) were pooled, as were the fractions eluted with the salt gradient (peak II), and evaporated to dryness, *in vacuo*.

TLC of chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids was performed on precoated Silica Gel G-25 plates (Brinkmann Ins., Des Plains, Ill.) in solvent systems A (chloroform-methanol-acetic acid-water, 50:30:8:4), B (chloroform-methanol-NH_4OH, 75:25:4), and on Eastman 13254 cellulose plates in solvent system C (isobutyric acid-1 N NH_4OH, 5:3). After development, the plates were dried, divided into 1-cm segments, scraped into scintillation vials, and counted directly.

Descending paper chromatography was performed on Whatman No. 3MM paper in solvent systems D (ethyl acetate-acetic acid-formic acid-water, 18:3:1:4), E (1-butanol-pyridine-water, 4:3:4), and F (1-butanol-acetic acid-water, 12:3:5). The chromatograms were dried and cut into 1-cm sections for determination of radioactivity. Sugars were detected on chromatograms by the alkaline silver nitrate method (2) while GlcNAc-Asn, $GlcNH_2$, and GlcNAc were detected on chromatograms as described previously (7).

Mild Acid and Base Treatment of [^{14}C]$GlcNH_2$- and [^{14}C]Mannose-labeled Chloroform-Methanol (2:1) and Chloroform-Methanol-Water (1:1:0.3)-Soluble Lipids. Mild acid treatment of lipids was performed in 0.1 N HCl in 50% isopropanol and partitioned into aqueous and organic phases as described previously (5). Radioactivity in each phase was determined.

Mild base treatment of lipids was carried out in chloroform-methanol (1:4) containing 0.1 N NaOH. Samples were heated at 37 C for 15 min and, after neutralization with HCl, the samples were separated into aqueous and organic phases as described by Chambers and Elbein (8). The radioactivity in each phase was determined.

Characterization of the [^{14}C]$GlcNH_2$- and Mannose-Labeled Lipid-Free Residues. Lipid-free residue was incubated with protease and the percentage of radioactivity released from the residue by proteolytic digestion was determined as described previously (5).

Acid hydrolysis of residue fraction was carried out in 4 N HCl for 2 h at 100 C. After treatment, the HCl was removed by repeated evaporation and dissolution of the sample in water. The sugars liberated from the residue by acid treatment were identified by paper chromatography in solvent system D for 24 h.

SDS-polyacrylamide gel electrophoresis of the labeled residue fractions was performed as described by Studier (29), modified for use in cylindrical gel tubes. The samples were dissociated in buffer containing SDS (29) and applied to 9.0 cm 12.5% acrylamide gels with a 1.5 cm 5% stacking gel. Samples of 50–150 μg of protein, determined by the method of Lowry (19), were applied to each gel and run at 8 mamp/tube for 2–3 h. The gels were then sliced into 1-mm sections and the radioactivity determined as previously described (4). SDS-polyacrylamide gel electrophoresis was also carried out with the following proteins of known subunit mol wt; Cyt *c* (12,384), trypsin (23,800), horseradish peroxidase (40,000), and pyruvate kinase (57,000).

Identification of the GlcNAc-Asparagine Glycopeptide Linkage in [^{14}C]$GlcNH_2$-Labeled Lipid-Free Residue. Strong alkali (1.0 N NaOH at 100 C for 6 h) and mild alkali treatments (0.1 N NaOH at 37 C for 72 h) were performed with lipid-free residues as described previously for legumin (7). The radioactive products of alkali digestion were analyzed by paper chromatography in solvent system E for 24 h. Repeated proteolytic digestion of the residue, followed each time by fractionation of the products by gel filtration, was carried out as described previously (7). Lipid-free residue, digested five times with protease, was subjected to mild acid hydrolysis in 2 N HCl for 20 min at 100 C. After the hydrolysate was neutralized, the radioactive products were analyzed by paper chromatography in solvent systems E for 24 h and F for 96 h.

RESULTS AND DISCUSSION

Incorporation of Radioactivity from [^{14}C]$GlcNH_2$ and [^{14}C]Mannose into Lipid and Residue Fractions. When developing pea cotyledons are incubated *in vivo* with either [^{14}C]$GlcNH_2$ or [^{14}C]mannose, radioactivity is incorporated into several different components (Table I). When [^{14}C]$GlcNH_2$ is utilized, a low percentage (8.3%) of counts becomes associated with material sedimenting at 250*g*. This fraction contains starch, cell wall fragments, intact chloroplasts, and nuclei (22). The majority of the radioactivity is recovered from the methanol extractions and probably represents unincorporated $GlcNH_2$, and its low mol wt-soluble derivatives: $GlcNH_2$ phosphates, GlcNAc, and UDP-GlcNAc. Roberts (24) has demonstrated that plants have the ability to form UDP-GlcNAc from exogenously supplied $GlcNH_2$ by way of these $GlcNH_2$ derivatives. Of specific interest to the current study is the observation that 1% and 5.4% of the radioactivity from [^{14}C]$GlcNH_2$ is transferred respectively to lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3). These solvents have been demonstrated to extract lipid intermediates involved in glycoprotein synthesis in *in vitro* systems (30). Greater than 8% of the radioactivity is associated with the lipid-free residue. This relative distribution of radioactivity in the lipids and lipid-free residue *in vivo* is the reverse of the pattern observed when *in vitro* studies were performed utilizing particulate fractions from pea cotyledons which were incubated with [^{14}C]UDP-GlcNAc (5). The distribution is similar to that reported *in vivo* by Speake and White (26) and Spiro *et al.* (27) for incorporation of radioactivity from [^{14}C]$GlcNH_2$ into animal preparations.

In contrast to incorporation of $GlcNH_2$, a much greater amount of radioactivity is incorporated into the 250*g* pellet when [^{14}C]-mannose is utilized (Table I). This radioactivity is not solubilized by protease or SDS and thus this fraction may contain oligomannans similar to those assembled *in vitro* when particulate preparations are incubated with GDP-Man (5). A large proportion

Table I. *Incorporation of Radioactivity from [^{14}C]$GlcNH_2$ and [^{14}C]-Mannose into Fractions Isolated from Developing Pea Cotyledons*

Fraction	Radioactivity from	
	[^{14}C]$GlcNH_2$[a]	[^{14}C]Mannose[b]
	cpm	*cpm*
Total cell homogenate	7,629,610	3,498,683
250*g* pellet	632,100 (8.3)[c]	1,100,000 (31.4)[c]
Chl-methanol-H_2O (3:48:47)	1,113,840 (14.6)	135,600 (3.9)
99% methanol	3,784,853 (49.6)	1,119,600 (32.0)
50% methanol	977,075 (12.8)	297,000 (8.5)
Chl-methanol (2:1)	70,400 (1.0)	607,500 (17.4)
Chl-methanol-H_2O (1:1:0.3)	409,590 (5.4)	72,200 (2.1)
Lipid-free residue	624,096 (8.2)	309,600 (8.8)
Total	7,611,954	3,641,500

[a] Thirty cotyledons, 21 days postanthesis, were each injected with 3 μl [^{14}C]$GlcNH_2$. The cotyledons were extracted into the fractions listed above. Radioactivity in each fraction was determined.

[b] Fractions were prepared as in footnote a except that each cotyledon was injected with 3 μl [^{14}C]mannose.

[c] The numbers in parentheses indicate the percentage of cpm in each fraction in relationship to the cpm in the total cellular homogenate.

(40%) of the radioactivity is soluble in the methanol solvents. Radioactivity is incorporated into fractions soluble in chloroform-methanol (2:1) (17.4%), chloroform-methanol-water (1:1:0.3) (2.1%), and into a lipid-free residue (8.8%). This distribution is in contrast to the incorporation of radioactivity from [^{14}C]GDP-mannose by particulate preparations of pea cotyledons *in vitro* (5), where the lipid-free residue was extensively labeled.

Analysis of the Chloroform-Methanol (2:1) and Chloroform-Methanol-Water (1:1:0.3) Soluble Lipids. TLC of the chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids in three solvent systems indicates that radioactivity from [^{14}C]GlcNH$_2$ and mannose becomes associated with several different components (Table II). This is in contrast to *in vitro* reports in which only a limited number of radioactive lipid components were produced after incubation of particulate preparations with [^{14}C]GDP-mannose (1, 5, 6, 11, 12, 15, 18) or [^{14}C]UDP-GlcNAc (5, 6, 15). It therefore appears that additional lipids, not produced in *in vitro* systems, become labeled *in vivo*. This study has attempted to characterize the lipids formed *in vivo* to determine which, if any, of these components correspond to the *in vitro*-produced lipids that have been implicated as lipid intermediates in glycoprotein synthesis (5).

Rouser *et al.* (25) have reported that mixtures of lipids can be separated by DEAE-cellulose (acetate) column chromatography. Lipids eluted from this ion exchange material with chloroform-methanol are nonionic (neutral) in nature, ionic-nonacidic lipids are eluted with various mixtures of chloroform-methanol-water, and acidic lipids are retained on the column and require the addition of acid, base, or salt gradients for elution.

When the chloroform-methanol (2:1) soluble lipids labeled from [^{14}C]GlcNH$_2$ were chromatographed on DEAE, two peaks of radioactivity were resolved (Fig. 1A). A large percentage (69%) of radioactivity eluted from the column with the starting solvent, chloroform-ethanol (2:1), and would therefore appear to be associated with nonionic lipids (25) (Peak I). Thirty-one per cent of the radioactivity was retained on the DEAE cellulose and was eluted from the column with an ammonium acetate gradient (Peak II). This material thus appears to be acidic lipid components (25). The chloroform-methanol (2:1) soluble lipids labeled *in vivo* from [^{14}C]mannose were also fractionated into a neutral lipid (83%) fraction (peak I) and acidic lipid (17%) fraction (peak II) by chromatography on DEAE-cellulose (Fig. 1B).

The majority (93%) of radioactivity associated with [^{14}C]-GlcNH$_2$-labeled lipids soluble in chloroform-methanol-water (1:1:0.3) was retained by DEAE-cellulose (acetate) (Fig. 2A) and eluted with an ammonium formate gradient (acidic lipids) (peak II). A small percentage (7%) of the radioactivity was not retained by the column (peak I). In contrast, when the [^{14}C]mannose-labeled lipids soluble in chloroform-methanol-water (1:1:0.3) were applied to DEAE-cellulose (acetate) columns (Fig. 2B), three peaks of radioactivity were detected. A portion (44%) had no affinity for the DEAE-cellulose and was eluted with the starting solvent (peak I). This material would appear to be ionic-nonacidic lipids (25). The radioactivity retained on the column appeared to be acidic in nature (25) and eluted as two separate fractions (peak IIa and peak IIb, Fig. 2B) with the ammonium formate gradient.

Table II. *Distribution of Radioactivity Following TLC of Lipids Labeled from [^{14}C]GlcNH$_2$ or [^{14}C]Mannose in Chloroform-Methanol (2:1) and Chloroform-Methanol-Water (1:1:0.3)*

Fraction Chromatographed	R_F Solvent A[a]	R_F Solvent B[b]	R_F Solvent C[c]
(2:1) from [^{14}C]-mannose	0.44, 0.84	0.06, 0.21, 0.43, 0.90	0.89
(1:1:0.3) from [^{14}C]-mannose	0.06, 0.43, 0.52, 0.94	0.03, 0.35, 0.90	0.75
(2:1) from [^{14}C]-GlcNH$_2$	0.06, 0.27, 0.53, 0.78	0.06, 0.20, 0.86	0.69
(1:1:0.3) from [^{14}C]-GlcNH$_2$	0.06, 0.29	0.06	0.74

[a] Solvent A (chloroform-methanol-acetic acid-water, 50:30:8:4).
[b] Solvent B (chloroform-methanol-NH$_4$OH, 75:25:4).
[c] Solvent C (isobutyric acid-1 N NH$_4$OH, 5:3).

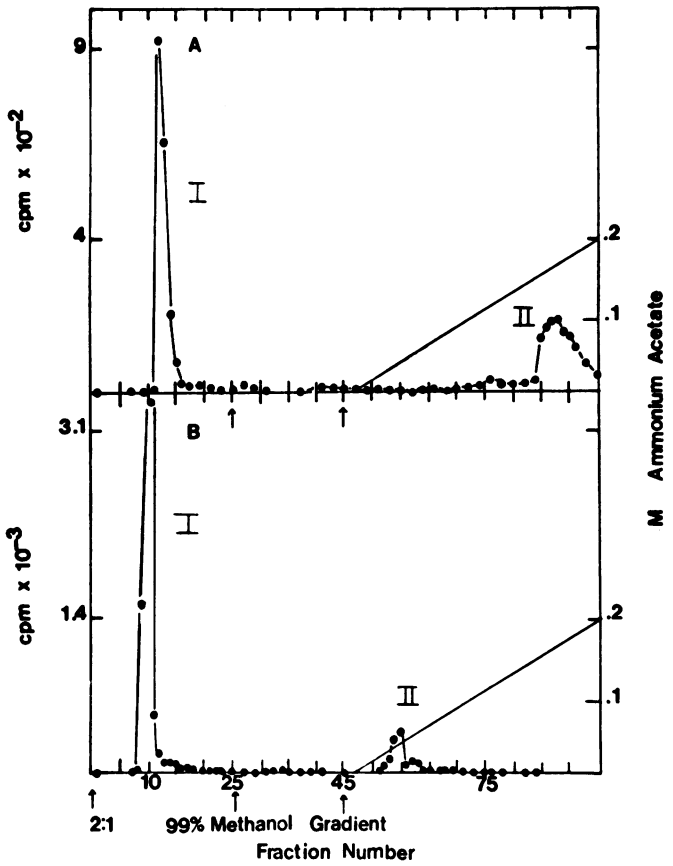

FIG. 1. DEAE-cellulose (acetate) column chromatography of lipids soluble in chloroform-methanol (2:1). A: 2:1 soluble lipids labeled from [^{14}C]GlcNH$_2$. B: 2:1 soluble lipids labeled from [^{14}C]mannose. Soluble lipid fractions (2:1) were applied to DEAE-cellulose (acetate) columns in the appropriate extracting solvent. Elution of the columns and collection of fractions was performed. Arrows represent the point at which each eluting solvent was applied to the column.

Partial Characterization of Peak I Lipids Soluble in Chloroform-Methanol (2:1) and Chloroform-Methanol-Water (1:1:0.3) Labeled from [^{14}C]Mannose and [^{14}C]GlcNH$_2$. TLC of the peak I neutral lipid fraction labeled from [^{14}C]mannose in solvent system A indicated the presence of a radioactive component with an R_F of 0.44 (Table III). Therefore, the component of R_F 0.44 in solvent A in the unfractionated mixture of lipids labeled from [^{14}C]mannose soluble in chloroform-methanol (2:1) (Table II) can be attributed to this neutral lipid component. The neutral lipid components (peak I) soluble in chloroform methanol (2:1), which were labeled from [^{14}C]GlcNH$_2$, were fractionated into two components with R_F of 0.27, and 0.53, in solvent system A (Table III). These same components are detected in solvent A in the mixture of lipids labeled from [^{14}C]GlcNH$_2$ soluble in chloroform-methanol (2:1), prior to DEAE fractionation (Table II). These components can also be assigned to neutral lipids.

The contention that the radioactivity in peak I component (Fig. 1, A and B) is associated with neutral and not polyisoprenoid

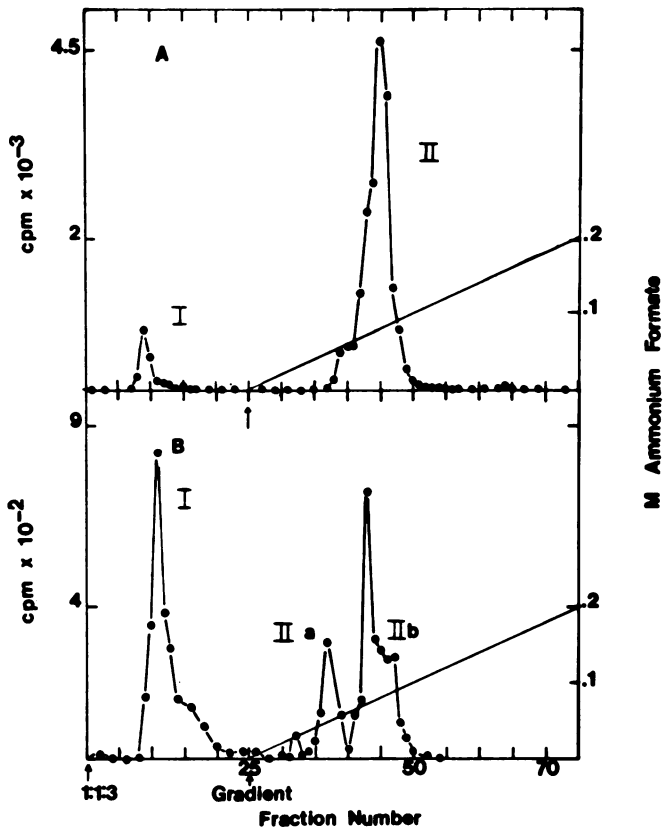

FIG. 2. DEAE-cellulose (acetate) column chromatography of lipids soluble in chloroform-methanol-water (1:1:0.3). A: 1:1:0.3 soluble lipids labeled from [^{14}C]GlcNH$_2$. B: 1:1:0.3 soluble lipids labeled from [^{14}C]-mannose. Chromatography was performed as in Figure 1.

Table III. *Distribution of Radioactivity Following TLC of Lipids Labeled from [^{14}C]GlcNH$_2$ and [^{14}C]Mannose Soluble in Chloroform-Methanol (2:1) and Chloroform-Methanol-Water (1:1:0.3) after Fractionation of Lipids by DEAE Cellulose (Acetate) Column Chromatography*

Peak I refers to lipids eluted from DEAE with the starting solvent, peak II refers to lipids eluted from DEAE with a salt gradient. Refer to Table II for composition of lipid fractions prior to DEAE chromatography. Solvent A is chloroform-methanol-acetic acid-water (50:30:8:4).

Fraction Chromatographed	R_F Solvent A	
	Peak I	Peak II
(2:1) from [^{14}C]mannose	0.44	0.84
(2:1) from [^{14}C]GlcNH$_2$	0.27, 0.53	0.06
(1:1:0.3) from [^{14}C]mannose	0.43, 0.94	0.06, 0.52
(1:1:0.3) from [^{14}C]GlcNH$_2$	0.29	0.06

acidic lipids, was further confirmed by the observation that peak I lipids labeled with [^{14}C]GlcNH$_2$ and [^{14}C]mannose were stable to treatment by mild acid hydrolysis, but labile to treatment with mild alkali. These properties are inconsistent with a polyisoprenoid nature but are consistent with the lipids being neutral (16). Neutral lipids labeled *in vivo* with [^{14}C]mannose have been reported in animals by Speake and White (26) and were identified as being triglycerides.

The ionic nonacidic lipids, soluble in chloroform-methanol-water (1:1:0.3), labeled from [^{14}C]mannose (peak I, Fig. 2A) gave two peaks with R_F of 0.43 and 0.94 following TLC in solvent system A (Table III). These same two components appeared in the crude mixture of lipids before DEAE fractionation (Table II) and can thus be assigned the designation of ionic-nonacidic lipids.

In a similar manner, the peak I lipids labeled from [^{14}C]GlcNH$_2$, soluble in chloroform-methanol-water (1:1:0.3), gave a single peak with an R_F of 0.29 (Table III). This lipid is also present in the crude mixture (Table II) and this component can also be designated as an ionic-nonacidic lipid. The nonacidic nature of each of these labeled lipids was confirmed by their resistance to mild acid and susceptibility to mild alkali treatment (16).

Partial Characterization of Acidic Peak II Lipids Soluble in Chloroform-Methanol (2:1) and Chloroform-Methanol-Water (1:1:0.3) Labeled from [^{14}C]GlcNH$_2$ and [^{14}C]Mannose. TLC of the peak II lipid labeled from [^{14}C]mannose soluble in chloroform-methanol (2:1) (Fig. 1B) produced a single radioactive component which migrated with an R_F of 0.84 in solvent system A (Table III). Since this same component was present in the crude lipid mixture (Table II), this component of the unfractionated mixture can be assigned the designation of an acidic lipid. The migration of this mannose-labeled acidic lipid during TLC is similar to that observed for the mannosyl lipid formed *in vitro* when particulate preparations are incubated with GDP[^{14}C]mannose (5, 8, 11, 12–15, 17). These mannosyl lipids formed *in vitro* have been tentatively identified as polyisoprenoid derivatives (5, 8, 11, 12–15, 17) which are characteristically stable to mild alkali treatment but are hydrolyzed by treatment with mild acid (16, 30). The acidic peak II lipid, labeled *in vivo* by [^{14}C]mannose, is stable to mild alkali but is hydrolyzed by mild acid treatment. Paper chromatography of the water soluble products released by acid hydrolysis of the peak II acidic lipid labeled *in vivo* from [^{14}C]mannose demonstrated the presence of only one radioactive component which comigrated with mannose. These observations, in conjunction with the chromatographic behavior of the lipid, are consistent with the conclusion that the [^{14}C] lipid labeled from [^{14}C]mannose soluble in chloroform-methanol (2:1) (peak II, Fig. 1B) is a mannosyl polyisoprenol derivative.

The observation that peak II acidic lipid labeled from [^{14}C]-GlcNH$_2$ was more strongly bound by DEAE-cellulose (acetate) than the acidic Peak II lipid labeled from [^{14}C]mannose (Fig. 1, A and B) indicates that the former compound has a greater negative charge (25). This observation is consistent with reports that the glucosaminyl lipid involves a pyrophosphate linkage while the mannosyl lipid has a phosphate linkage (20, 30). The peak II GlcNH$_2$-labeled lipid soluble in chloroform-methanol (2:1) was susceptible to hydrolysis by mild acid but not to mild base treatment. This observation, along with the chromatographic behavior of this lipid on DEAE-cellulose (acetate), are similar to properties of the GlcNH$_2$ lipid assembled *in vitro*, when particulate preparations are incubated with UDP-[^{14}C]GlcNAc (5, 14, 15, 30). The GlcNAc lipids synthesized *in vitro* have been characterized as GlcNAc polyisoprenol lipids and chitibiosyl polyisoprenoid derivatives (5, 14, 15, 30).

Radioactivity from the acidic lipid labeled from [^{14}C]GlcNH$_2$ soluble in chloroform-methanol (2:1) (peak II, Fig. 1A) demonstrates little mobility during TLC in solvent system A (Table III). A component of the same mobility is also detected in the crude lipid fraction (Table II). This mobility of the peak II GlcNH$_2$-labeled lipid formed *in vivo* (R_F 0.06) is markedly different from the mobility of chitibiosyl lipid, an *N*-acetylglucosaminyl lipid formed *in vitro* (5, 14, 15, 30).

The reason for this discrepancy can be related to the fact that the glycolipid labeled from [^{14}C]GlcNH$_2$ is labile and dissociates during concentration and chromatography after fractionation on DEAE-cellulose acetate. The crude mixture of 2:1 soluble lipids (Table II) contains a component of R_F 0.78 that is not detected in either peak I or peak II soluble lipids (Table III). This was the only instance where a component of the unfractionated mixture could not be attributed to being a peak I or peak II lipid. Inspection of our data indicated that the relative amount of the 0.06 lipid component increased markedly following DEAE frac-

tionation of the crude mixture and that the component with an R_F of 0.06 represents a breakdown product of the R_F 0.78 component that was initially present in the unfractionated mixture. Thus, it is considered that the component with an R_F of 0.78 in the unfractionated chloroform-methanol (2:1)-soluble lipid (Table II) represents acidic glucosaminyl lipid and the component with mobility of 0.06 (Tables II and III) GlcNAc or chitibiose. The chromatographic mobility of the 2:1-soluble acidic lipid (R_F 0.78) in the unfractionated mixture is similar to that of the chitibiosyl lipid assembled *in vitro* (5), implying that the accumulated lipid intermediate *in vivo* is the disaccharide lipid rather than the monosaccharide derivative.

The radioactivity associated with [^{14}C]mannose-labeled peak II lipids soluble in chloroform-methanol-water (1:1:0.3) migrated with R_F of 0.52 and 0.06 during TLC in solvent system A (Table III). These same two lipid components are present in the crude extract (Table II) and can thus be attributed to the presence of these acidic lipids. The observation that the peak II mannose lipid migrates as two components in TLC is consistent with the separation of the Peak II lipids into two acidic components on DEAE-cellulose (acetate) by elution with different concentrations of ammonium formate (Fig. 2B). Although the chromatographic behavior of each of these acidic components (peak IIa and peak IIb, Fig. 2B) was not determined separately, it was observed that the component with an R_F of 0.06 was more extensively labeled than the component with an R_F of 0.52. This implies that the component with an R_F of 0.06 corresponded to the most extensively labeled peak II component, peak IIb (Fig. 2B). The chromatographic behavior of this acidic lipid (0.06) is consistent with that observed by Beevers and Mense (5) and Hsu *et al.* (17) for the oligosaccharide lipid formed *in vitro* when particulate preparations were incubated with GDP-[^{14}C]mannose. The polyisoprenoid nature of the oligosaccharide lipid formed *in vivo* is demonstrated by the alkaline stability and mild acid lability of this fraction (16).

The acidic peak II [^{14}C]$GlcNH_2$-labeled lipid soluble in chloroform-methanol-water (1:1:0.3) migrated with an R_F of 0.06 during TLC in solvent system A (Table III). This component is also present in the crude mixture of lipids and therefore this component with an R_F of 0.06 (Table II) can be designated as an acidic lipid. The chromatographic properties of this peak II lipid is similar to that reported by Beevers and Mense (5) for the oligosaccharide lipid formed *in vitro* from UDP-[^{14}C]GlcNAc. The similarity of the chromatographic properties of the [^{14}C]$GlcNH_2$- and [^{14}C]mannose-labeled oligosaccharide lipids formed *in vitro* (Fig. 2 and Table III) is consistent with the concept (9, 20, 30) that $GlcNH_2$ and mannose are incorporated into the same oligosaccharide lipid, which functions as an intermediate in glycoprotein synthesis. The polyisoprenoid nature of the *in vivo* formed [^{14}C]$GlcNH_2$- and [^{14}C]mannose-labeled oligosaccharide lipids is supported by their resistance to mild base treatment and hydrolysis by mild acid. It was observed that control incubations of acidic peak II lipids soluble in chloroform-methanol-water (1:1:0.3), heated at 100 C without acid, also liberated considerable radioactivity into the aqueous phase. It seems that these lipids are at least partially heat-labile and it is not clear whether the low mobility of radioactivity in this fraction (R_F 0.06) represents the chromatographic behavior of lipid oligosaccharide or the lack of migration of oligosaccharide dissociated from the lipid during concentration and TLC after DEAE-cellulose acetate fractionation.

Characterization of the Lipid-free Residue Labeled from [^{14}C]$GlcNH_2$ and [^{14}C]Mannose. When the lipid-free residues labeled *in vivo* from [^{14}C]$GlcNH_2$ and [^{14}C]mannose were incubated with protease, 89 and 87% of the radioactivity, respectively, was solubilized. This indicates that the majority of radioactivity from both precursors is associated with proteins in the lipid free residue. These results are in contrast to the *in vitro* studies with GDP-[^{14}C]mannose, in which it is observed that radioactivity associated with the lipid free residue is almost totally resistant to proteolytic digestion (5). The lipid-free residue labeled *in vitro* from UDP-[^{14}C]GlcNAc is solubilized by protease digestion (5).

The nature of the radioactivity associated with the proteins in the lipid-free residue, labeled *in vivo* from [^{14}C]$GlcNH_2$ and [^{14}C]mannose, was investigated by subjecting the lipid-free residue to total acid hydrolysis. Paper chromatography of the neutralized hydrolysate products of [^{14}C]$GlcNH_2$-labeled lipid-free residue indicated that essentially all of the radioactivity was associated with material which co-migrated with standard $GlcNH_2$. In contrast, the radioactivity present in the acid hydrolysate of lipid free residue labeled with [^{14}C]mannose was separated into three principal components. Two peaks of radioactivity co-migrated with glucose and mannose, while the third peak had a mobility slightly less than that of $GlcNH_2$ (Fig. 3). This redistribution of radioactivity from [^{14}C]mannose into other glycosyl components, associated with glycoproteins in the lipid-free residue, is strikingly similar to a report by Speake and White (26), who reported that radioactivity in a lipid-free residue labeled by [^{14}C]mannose was redistributed in such a way that 40% was associated with mannose, 30% with glucose, and 30% into an unknown compound of slow mobility.

Further work was conducted to determine the nature of the glycopeptide linkage in glycoproteins in the lipid-free residue labeled with [^{14}C]$GlcNH_2$. The glycopeptides were extensively digested with protease and the products of digestion fractionated by gel filtration on Sephadex G-15. All of the radioactivity (data not shown) was associated with carbohydrate-containing material and no radioactivity was associated with the α-amino nitrogen fractions. There seems to be no interconversion of [^{14}C]$GlcNH_2$ into amino acids associated with the peptide portion of the [^{14}C]$GlcNH_2$-labeled glycoproteins in the lipid free residue.

Dilute alkali treatment (0.1 N NaOH) of the [^{14}C]$GlcNH_2$-labeled glycopeptides, produced by two proteolytic digestions of the lipid-free residue, did not alter the mobility of the radioactive glycopeptides on paper chromatograms (data not shown). In contrast, strong alkali (1.0 N NaOH) treatment of the [^{14}C]-$GlcNH_2$-labeled glycopeptides markedly increased the mobility of the glycopeptides on paper chromatograms. These observations tend to preclude a glycopeptide linkage to peptidyl serine or threonine, but are consistent with a linkage from GlcNAc to asparagine (7). This supposition was confirmed by the results of a mild acid hydrolysis treatment of glycopeptides isolated from

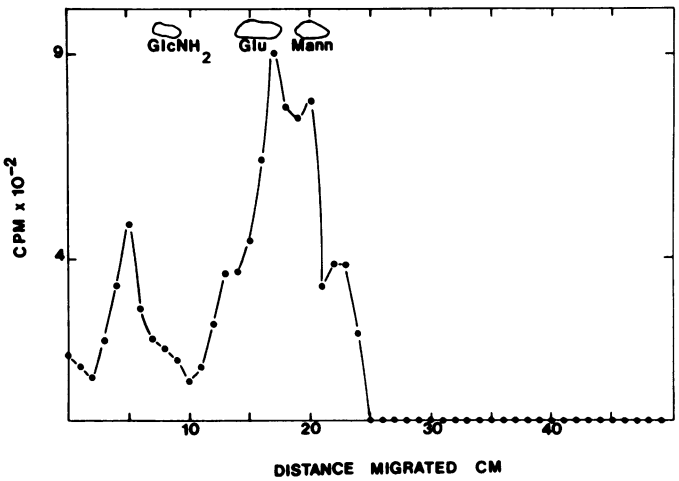

FIG. 3. Paper chromatography of the total acid hydrolysate of lipid free residue from cotyledons incubated with [^{14}C]mannose. Hydrolysis and chromatography were performed as described in Figure 3.

proteolytic digests of [^{14}C]GlcNH$_2$-labeled lipid-free residue. Paper chromatography of the neutralized mild acid hydrolysate of the [^{14}C]glycopeptides indicated that the majority of radioactivity co-migrated with the authentic GlcNAc-asparagine linkage compound (GlcNAc-Asn) in solvent system E (Fig. 4). Similar results were obtained in solvent system F. This isolation of the glycopeptide linkage compound, coupled with the β-elimination data (alkaline hydrolysis), provides direct proof that [^{14}C]GlcNH$_2$, injected into pea cotyledons, is converted into GlcNAc, which is a structural component of the glycopeptide bond of endogenous glycoproteins in the pea cotyledons.

The nature of glycoproteins, isolated from the lipid-free residue, labeled *in vivo* from [^{14}C]GlcNH$_2$ and [^{14}C]mannose was investigated by SDS-polyacrylamide gel electrophoresis. The majority of radioactivity from [^{14}C]mannose was associated with polypeptides of mol wt of approximately 57,000, while glycoproteins in the mol wt range of 15–20,000 are labeled to a much smaller extent (Fig. 5). A similar distribution of radioactivity in glycoproteins is observed when [^{14}C]GlcNH$_2$ was used (data not shown). The observation that [^{14}C]GlcNH$_2$ and [^{14}C]mannose are incorporated into glycoproteins of similar mol wt is consistent with the concept that both of these sugars are components of the core oligosaccharide lipid that transfer the completed oligosaccharide to endogenous proteins.

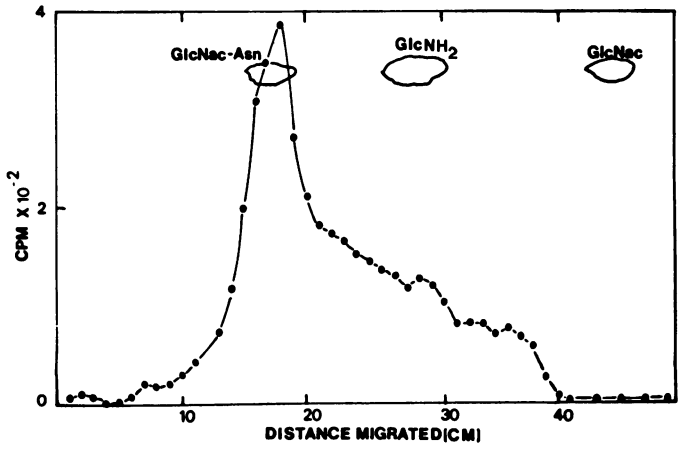

FIG. 4. Paper chromatography of a partial acid hydrolysate of [^{14}C]-GlcNH$_2$-labeled glycopeptides. [^{14}C]GlcNH$_2$-labeled glycopeptides, isolated from the [^{14}C]GlcNH$_2$-labeled lipid free residue were hydrolyzed in 2 N HCl for 20 min at 100 C. Following neutralization, the hydrolysate was spotted on Whatman No. 3MM paper and developed in solvent system F for 24 h. The chromatogram was cut into 1-cm sections and counted for radioactivity. Standard marker compounds were chromatographed and detected as described under Materials and Methods.

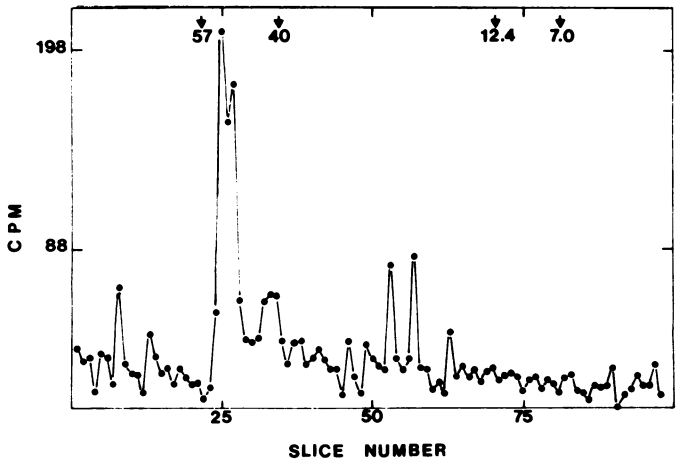

FIG. 5. Distribution of radioactivity following SDS polyacrylamide gel electrophoresis of the lipid-free residue extracted from pea cotyledons incubated with [^{14}C]mannose. Numerals at top of figure refer to migration of marker proteins of 57,000, 40,000, 12,400, and 7,000 daltons.

CONCLUSIONS

The developing pea cotyledon has the capacity to incorporate radioactivity from exogenously applied [^{14}C]GlcNH$_2$ and [^{14}C]-mannose into lipid components soluble in chloroform-methanol (2:1), chloroform-methanol-water (1:1:0.3), and into lipid-free residue.

On the basis of chromatographic properties and sensitivity to hydrolysis by mild alkali it appears that the majority of radioactivity from [^{14}C]mannose or [^{14}C]GlcNH$_2$ incorporated into lipids soluble in chloroform-methanol (2:1) is associated with neutral lipids. The formation of labeled neutral lipids does not occur when particulate preparations from pea cotyledons are incubated with UDP-[^{14}C]GlcNAc or GDP-[^{14}C]mannose (5). Speake and White (26), however, have reported the formation of neutral lipids (triglycerides) *in vivo* in rabbit mammary explants incubated with [^{14}C]mannose or [^{14}C]GlcNAc. Apparently these sugars are converted to glycolytic intermediates and finally into the glycerol moiety of neutral triglycerides (26). Although we have not demonstrated the operation of this pathway for the incorporation of radioactivity from labeled sugars into neutral lipids, the extensive *in vivo* interconversion of [^{14}C]mannose into other glycosyl components present in glycoproteins (Fig. 3) is consistent with the possibility of this conversion occurring in pea cotyledons.

In the developing pea cotyledon *in vivo*, relatively little radioactivity from [^{14}C]GlcNH$_2$ or [^{14}C]mannose accumulates in acidic lipids fractions soluble in 2:1 chloroform-methanol (characterized by retention on DEAE cellulose acetate) which have characteristics of polyisoprenol derivatives. These polyisoprenol lipids accumulate appreciable radioactivity *in vitro* in particulate preparations from developing pea cotyledon and have been implicated as glycosyl carrier lipid intermediates which function in the synthesis of glycoproteins (5).

The synthesis of polyisoprenol lipid sugar derivatives utilizes nucleotide sugar precursors (20, 30). The *in vivo* formation of mannosyl lipid intermediates implies that pea cotyledons possess the necessary enzymes for converting mannose to GDP-mannose. The incorporation of radioactivity from [^{14}C]GlcNH$_2$ into acidic polyisoprenol lipid derivatives *in vivo* likewise indicates that pea cotyledons possess the capacity to convert GlcNH$_2$ into UDP-GlcNAc. Roberts *et al.* (24) report that GlcNH$_2$ can be converted to amino sugar derivatives in bean hypocotyls and Mense and Beevers (21) have demonstrated the occurrence of enzymes necessary for the formation of UDP-GlcNAc in preparations from developing pea cotyledons.

Radioactivity from [^{14}C]mannose and [^{14}C]GlcNH$_2$ is incorporated *in vivo* into two different types of lipids soluble in chloroform-methanol-water (1:1:0.3). Approximately 7% of the radioactivity from [^{14}C]GlcNH$_2$ and 44% of the radioactivity from [^{14}C]mannose is incorporated into a class of ionic nonacidic lipids not formed from nucleotide sugars *in vitro* (5). The majority of the radioactivity from [^{14}C]mannose and [^{14}C]GlcNH$_2$ in the (1:1:0.3) component appears to be incorporated into compounds with properties resembling the acidic oligosaccharide lipids that were formed *in vitro* (5). The structure of these oligosaccharide lipids has not been resolved but the chromatographic behavior of the [^{14}C]GlcNH$_2$- and [^{14}C]mannose-labeled oligosaccharide lipid is consistent with the concept that both precursors are incorporated into the same lipid linked intermediate (30).

Although a precursor-product relationship has not been demonstrated, our findings are consistent with the proposed sequence of glycoprotein assembly involving lipid intermediates. Since

acidic lipids, with properties similar to lipid intermediates produced *in vitro*, are labeled *in vivo* when [^{14}C]GlcNH$_2$ or [^{14}C]-mannose is provided to pea cotyledons. The demonstration that radioactivity from [^{14}C]GlcNH$_2$ becomes associated with a compound identified as the GlcNAc-Asn glycopeptide linkage, isolated from [^{14}C]GlcNH$_2$-labeled glycoproteins in the lipid-free residue, provides direct evidence that pea cotyledons can convert exogenously applied GlcNH$_2$ into GlcNAc. The isolation of the GlcNAc-Asn linkage compound is also significant because glycoproteins with this structural feature are considered to be synthesized through the involvement of the lipid linked intermediate pathway (30). In contrast to the *in vitro* studies with pea cotyledons, the [^{14}C]GlcNH$_2$- and mannose-labeled glycoproteins are much more extensively labeled *in vivo*. Speake and White (26) have reported a similar observation *in vivo* in rabbit mammary explants and suggests that glycoprotein synthesis *in vitro* is restricted by the availability of peptidyl acceptors for the glycosyl units. In the intact pea cotyledon, a sustained level of protein synthesis would provide the necessary peptide acceptors for glycosylation to occur.

[^{14}C]GlcNH$_2$ and mannose are incorporated into glycoproteins with a subunit mol wt of approximately 57,000. We have demonstrated that exogenously applied [^{14}C]GlcNH$_2$ is incorporated into the GlcNAc-Asn glycopeptide linkage of the reserve storage protein in pea cotyledons, legumin (7). This finding implies that legumin may be one of the proteins glycosylated *in vivo*, which is isolated from the lipid-free residue. However, we have also observed extensive incorporation of radioactivity into the proteins associated with various membrane fractions isolated from the pea cotyledon (23). The incorporation of radioactivity from either [^{14}C]GlcNH$_2$ or [^{14}C]mannose into polypeptides of similar mol wt is consistent with the concept that mannose and GlcNH$_2$ are both components of the lipid oligosaccharide, which transfers the completed oligosaccharide core to the asparagine residue of the glycosylated protein.

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