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Transfer of Oligosaccharide to Protein from a Lipid Intermediate in Plants

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ABSTRACT

A lipid-bound oligosaccharide was isolated from pea (*Pisum sativum*) cotyledons incubated with [^{14}C]mannose. The oligosaccharide moiety appeared to be identical with the one obtained from rat liver, known to contain three glucoses, nine mannoses, and two *N*-acetylglucosamines, and to be involved in protein glycosylation.

Enzymes obtained from soya (*Glycine max*) roots and developing pea cotyledons were found to catalyze the transfer of oligosaccharide from the lipid intermediate to endogenous protein. The enzymes require Mn^{2+} and detergent for activity. Evidence is presented indicating that the lipid-bound oligosaccharide with three glucoses is transferred faster than that with less. Some of the peripheral mannoses could be removed without affecting the rate of transfer.

The protein-bound oligosaccharide, formed by incubation of whole cotyledons or by transfer with the enzyme preparation, could be released by protease and endo-β-*N*-acetylglucosaminidase treatment, as expected for an asparagine-bound high mannose oligosaccharide.

The mechanism of glycosylation of asparagine residues in proteins involving dolichyl phosphate containing intermediates is fairly well known for animal tissues (16, 19, 23). Some studies have been carried out in plants (8, 20), and several of the lipid intermediates have been detected. Compounds similar to dolichyl phosphate-glucose or mannose and dolichyl diP-*N*-acetylglucosamine have been found after incubation of plant enzymes with the corresponding radioactive sugar nucleotides (3, 7, 18). Lipid-bound di- and oligosaccharides have also been detected (9, 10, 13). One, which was isolated from alfalfa roots incubated with radioactive glucose (24), was apparently identical with that of animal tissues which contains $glucose_3$ $mannose_9$ *N*-acetylglucosamine$_2$ and which is known to act as oligosaccharide donor to protein (25).

An important step in the glycosylation process is the transfer from the lipid intermediate to protein. The enzyme from animal tissues was detected some time ago (15) and has been purified by Das and Heath (6). In plants, however, the reaction had not been clearly detected. Now, transfer could be observed with soya root enzymes, but more active preparations were obtained from developing pea cotyledons. This material has been used by Beevers and coworkers (1, 4) in their studies on glycoprotein biosynthesis.

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MATERIALS AND METHODS

Rat liver microsomes were prepared as previously described (15). Dolichyl diphosphate-($[Glc]_{1-3}[Man]_9[GlcNAc]_2$) was obtained by incubation of liver microsomes with UDP [^{3}H]Glc or UDP [^{14}C]Glc (17) or dolichyl phosphate-[^{14}C]Glc (25). Mild acid hydrolysis was carried out by heating at 100 C for 10 min at pH 2 (22). UDP [^{14}C]Glc (268 Ci/mol) and UDP [^{3}H]Glc (2,100 Ci/mol) were prepared as described (11). [^{14}C]Glucose (268 Ci/mol) and [^{14}C]mannose (216 Ci/mol) were obtained from New England Nuclear.

Estimation of the Oligosaccharide Transferase. Dolichyl diphosphate-($[Glc]_{1-3}[Man]_9[GlcNAc]_2$) (about 2,000 cpm) was dried in a test tube, and the following components were added: 50 mM Tris-maleate buffer (pH 7.7); 10 mM $MnCl_2$; 1.2% Triton X-100; and 0.4 to 1.0 mg enzyme. Total volume was 50 μl. After 10 min at 30 C, 0.5 ml 5% TCA and about 10 mg microsomal protein (as carrier) were added. The tubes were heated to 90 C for 10 min. The protein precipitate separated by centrifugation was washed with 5% TCA, twice with 0.5 ml ethyl ether, and twice with 0.5 ml methanol; dissolved in 0.2 ml Protosol (New England Nuclear); and counted in a scintillation counter.

Chromatography. The following solvents were used for paper chromatography: solvent A, 1-propanol:nitromethane:water (5:2:4); solvent B, butanol:pyridine:water (4:3:4); and solvent C, butanol:pyridine:water (6:4:3).

Paper electrophoresis was performed in 5% formic acid for 3 h at 25 v/cm.

Soya Root Enzyme. Seeds of *Glycine max.* were germinated for 7 days. The roots were homogenized in an Ultra Turrax (Junke and Kunkell, West Germany) in four volumes of the following solution: 100 mM Tris-HCl buffer (pH 7), 20 mM 2-mercaptoethanol, 5 mM EDTA Na, and 1% PVP. The homogenate was centrifuged at 12,000*g* for 10 min, and the supernatant fluid was filtered through cheese cloth. The filtrate was centrifuged for 60 min at 100,000*g*, and the pellet was suspended in about one volume of 50 mM Tris-HCl (pH 7.4) and 5 mM 2-mercaptoethanol.

Pea Cotyledons Enzyme. The procedure described by Beevers and Mense (1) was used with minor changes. Developing cotyledons of *Pisum sativum* were homogenized in an Omni mixer (Sorvall, Newton, CT) in four volumes of the following solution: 50 mM Tris-HCl buffer (pH 7), 5 mM 2-mercaptoethanol, 0.1% BSA, 1% dextran (200,000–275,000 mol wt), and 10% sucrose. The homogenate was centrifuged at 370*g* for 10 min. The supernatant fluid was centrifuged for 60 min at 100,000*g*. The pellet was suspended in one volume of the above-mentioned buffer.

Endo-β-*N*-acetylglucosaminidase Treatment. The samples in 50 μl of 50 mM triethylamine acetate buffer (pH 5.5) were treated with 0.003 units of endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*, Miles Laboratories) for 12 h at 37 C under a toluene atmosphere.

α-Mannosidase Treatment. A sample of dolichyl diphosphate-

([$Glc_3[Man]_9[GlcNAc]_2$) (50,000 cpm) obtained by incubation of liver microsomes with UDP [^{14}C]Glc was treated with 5 units of α-mannosidase (*Canavalia ensiformes*, Boehringer Mannheim, West Germany) in 0.1 M citrate buffer (pH 4.5) containing 1.25% Triton X-100, total volume 0.4 ml. After 16 h at 37 C under a toluene atmosphere, the sample was concentrated with a nitrogen current, dissolved in chloroform:methanol:water (1:1:0.3), and passed through a column of Sephadex LH-20 (0.9 × 90 cm). The radioactive lipid-oligosaccharide appeared in the exclusion volume while the salts were included. An aliquot of the excluded fraction was hydrolyzed (pH 2 for 10 min at 100 C) and run on paper with solvent B. The chromatography showed that none of the original oligosaccharide [$(Glc_3)(Man_9)(GlcNAc_2)$] remained and that the product was a mixture of the compounds with one to four mannose residues less.

Experiments with Whole Cotyledons. Whole developing pea seeds (*P. sativum*) were injected between the two cotyledons with 2 μl (4 × 10^5 cpm) of [^{14}C]mannose (216 Ci/mol). After 3 h at room temperature, on moist filter paper in the light, the seeds were homogenized twice for 30 s in an Omni-mixer (Sorvall) in 20 mM Tris-HCl buffer (pH 7) (4 ml per g of solids). After addition of two volumes of methanol, three volumes of chloroform, and 100 mg of rat microsomal protein (as carrier) followed by centrifugation, the upper and lower layer and the solid interphase were separated. The latter was partitioned twice again, as described, and washed three times with chloroform:methanol:4 mM $MgCl_2$ (3:48:47). The residue was extracted twice with chloroform:methanol:water (1:1:0.3).

The protein residue was heated to 90 C for 10 min in 5% TCA to remove any lipid-bound oligosaccharide left over. After centrifugation, the precipitate was washed with 5% TCA, once with ethyl ether, and twice with methanol. The solid residue was then treated with *Streptomyces griseus* type VI protease (Sigma) for 10 days under a toluene atmosphere, as previously described (22). The glycopeptides were then separated by passage through a gel filtration column (Bio Gel P-6, 1.8 × 76 cm) in 0.1 M pyridine acetate (pH 5).

RESULTS

Studies on the Lipid-Bound Oligosaccharide. Only a few tests were carried out in order to check the presence of lipid-bound $(Glc)_3(Man)_9(GlcNAc)_2$ in cotyledons, inasmuch as a more thorough identification of the compound in plant material has been done with alfalfa roots (24). The lipid oligosaccharide was extracted from the cotyledons incubated with [^{14}C]mannose, as described in "Materials and Methods." The sugar moiety was then obtained by mild acid hydrolysis. Paper chromatography of the oligosaccharide showed a single peak with a similar mobility to the $(Glc)_3(Man)_9(GlcNAc)_2$ oligosaccharide (not shown). A more precise comparison was carried out by paper chromatography with solvent A with an internal standard of tritium-labeled liver oligosaccharide. The oligosaccharide from cotyledons had exactly the same mobility as a standard of ^{3}H-labeled $(Glc)_3(Man)_9(GlcNAc)_2$ oligosaccharide (Fig. 1). This standard also showed smaller peaks of higher mobility which correspond to the oligosaccharide containing two and one glucoses.

Studies on the Protein-Bound Oligosaccharide. To gain information on the nature of the protein-bound compounds which became labeled after injection of [^{14}C]mannose into whole cotyledons, the residue left over after extraction of the lipids was treated with a *Streptomyces* protease and passed through a gel filtration column. Only a small amount of radioactivity was excluded and a large peak appeared in a position similar to the $(Glc)_3$ $(Man)_9(GlcNAc)_2$ oligosaccharide (Fig. 2). Another small peak appeared later, together with the bulk of the salts and aminoacids.

Paper electrophoresis of the pooled fractions of the major peak of Figure 2 showed that the substance had a positive charge in 5% formic acid (Fig. 3A). The charged compound was eluted from the paper, treated with endo-β-*N*-acetylglucosaminidase H and submitted to paper electrophoresis. Most of the charged compound became neutral (Fig. 3B). This is what was expected if an asparagine-linked oligosaccharide was acted upon by endo-β-*N*-glucosaminidase.

The neutral oligosaccharide thus formed was eluted from the paper and chromatographed with solvent A. A standard of $(Glc)_{1-3}(Man)_9(GlcNAc)_1$, obtained by treatment of the two *N*-acetylglucosaminidase-containing compound with endo-β-*N*-acetylglucosaminidase H, was run at the same time. This compound showed three peaks which corresponded to oligosaccharides with 3, 2, and 1 glucose residues (Fig. 4B). The oligosaccharide obtained

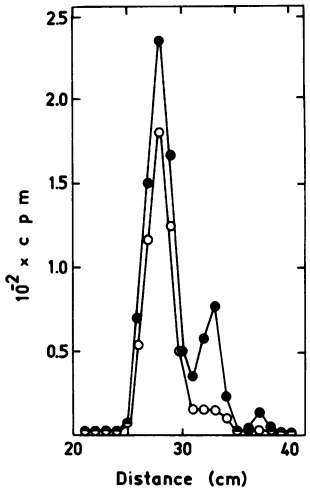

FIG. 1. Paper chromatography of the oligosaccharide obtained from pea cotyledons. The substances were spotted together on the paper and chromatographed for 5 days with solvent A. (○——○), ^{14}C-labeled cotyledon oligosaccharide; (●——●), ^{3}H-labeled $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ obtained with rat liver enzymes. Radioactivity was measured on 0.5-cm strips in a scintillation counter.

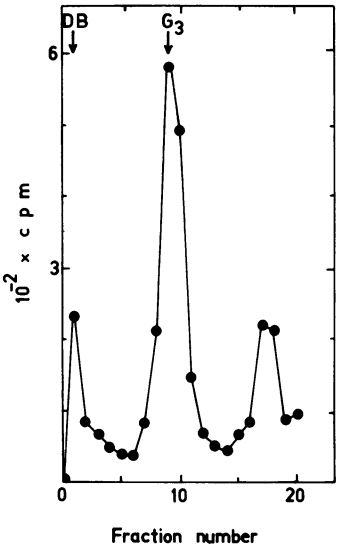

FIG. 2. Gel filtration of the glycopeptide from cotyledons. After incubation with [^{14}C]mannose and extraction of the lipids, the protein residue was processed as described in "Materials and Methods" for the preparation of glycopeptides from the whole cotyledon experiments. Fractions of 4 ml were collected, and radioactivity was measured on 0.4-ml aliquots. DB, Dextran blue; G_3, $(Glc)_3(Man)_9(GlcNAc)_2$.

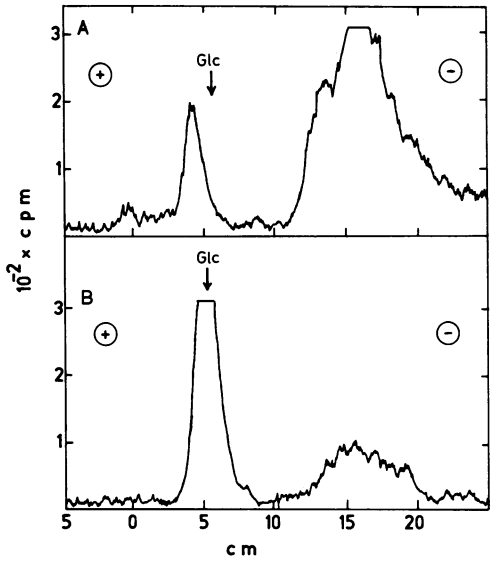

FIG. 3. The action of endo-β-*N*-acetylglucosaminidase H on the glycopeptides isolated from pea cotyledons. A, Paper electrophoresis of the pool of tubes 7 to 11 of Figure 2. B, The positively charged substance of A was eluted, treated with endo-β-*N*-acetylglucosaminidase H, and subjected to paper electrophoresis.

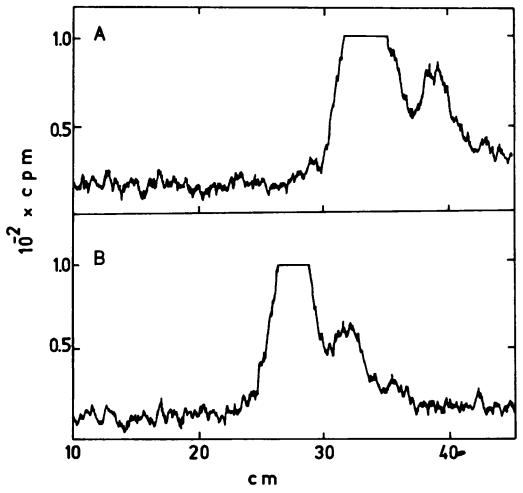

FIG. 4. Paper chromatography of the substance released by endo-β-*N*-acetylglucosaminidase H. A, The neutral substance of Figure 3B was eluted and spotted on paper. B, $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ oligosaccharide from rat liver treated with endo-β-*N*-acetylglucosaminidase H. Paper chromatography was run with solvent A for 5 days.

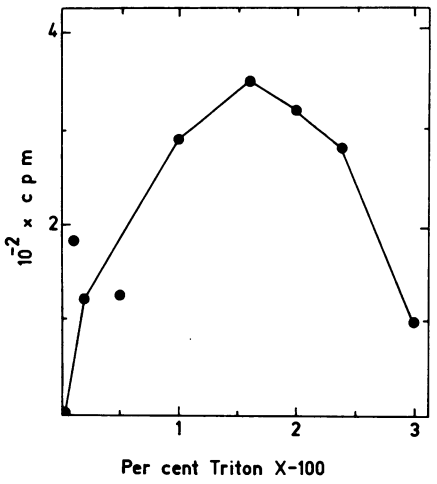

FIG. 5. The effect of detergent concentration on the transfer of oligosaccharide to protein. Tests as described in "Materials and Methods," with pea cotyledon enzyme and variable Triton-X 100 concentrations.

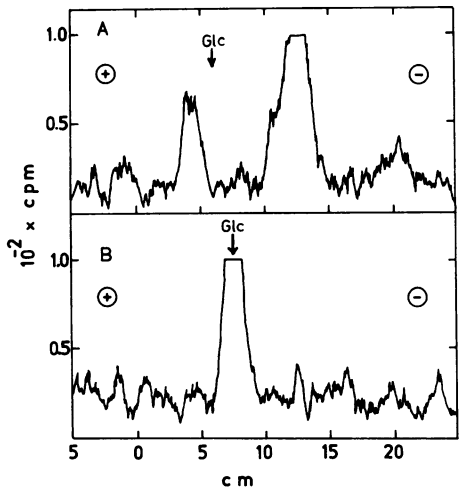

FIG. 6. The action of endo-β-*N*-acetylglucosaminidase H on the glycopeptides isolated from protein bound oligosaccharides. Dolichyl diphosphate-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ labeled in the glucose (20,000 cpm) was incubated with soya root enzyme, as indicated in "Materials and Methods." After 10 min at 30 C, 5% TCA was added, and the procedure for glycopeptide preparation was carried out as described for the experiments with whole cotyledons. A, Electrophoresis of the glycopeptides from the glycoprotein obtained by transfer from lipid bound oligosaccharide separated by gel filtration. B, Electrophoresis of the positively charged compound of A treated with endo-β-*N*-acetylglucosaminidase H.

from the glycopeptide gave a large peak which ran between the standard of 1 and 2 glucoses (Fig. 4A). From previous work, it is known that the oligosaccharide with nine mannoses and no glucose runs approximately the same as that with one glucose.

The radioactive compound of Figure 4A was hydrolyzed to liberate the radioactive hexoses and then run on paper with solvent C. Only one radioactive peak was detectable. Its mobility was the same as that of an internal mannose standard.

Transfer to Protein. Incubation of the ^{14}C-labeled oligosaccharide lipid with the cotyledon enzyme led to a gradual appearance of radioactivity in the protein fraction. As occurs with the animal tissue enzyme, it was found that detergent was needed in order to obtain transfer. The effect increased up to about 1.5% Triton X-100 (Fig. 5). However, it is known that the optimal concentration depends on the amount of protein and lipids in the sample (2).

Some of the cotyledon enzyme preparations were nearly as active as those obtained from rat liver microsomes, but they were less stable. Activity was lost after freezing two or three times or after keeping at −30 C for about a week.

Some experiments were carried out with a soya root enzyme. This preparation was generally less active than that of cotyledons and was used in studies of the reaction product. A delipidated sample of the protein precipitate was digested exhaustively with *Streptomyces* protease, and the low mol wt material was removed by gel filtration. The product was then subjected to paper electrophoresis and found to migrate to the negative pole in 5% formic acid (Fig. 6A). The radioactive glycopeptide was eluted from the paper and treated with endo-β-*N*-acetylglucosaminidase H. A second electrophoresis showed that the substance had become neutral (Fig. 6B), as expected for the product of the action of endo-β-*N*-acetylglucosaminidase H on an asparagine-bound oligosaccharide of the high-mannose type (12).

Paper chromatography of the product obtained by the action of endo-β-*N*-acetylglucosaminidase H showed that its mobility was similar to that of an oligosaccharide prepared by the same procedure but with a rat liver microsomal enzyme (Fig. 7).

Specificity of the Transferring Enzyme. The transferring enzyme of animal tissues seems to be specific for the dolichyl diphosphate oligosaccharide containing three glucoses. It has been estimated that glucose-free oligosaccharides are transferred about 8 times more slowly (26). Furthermore, the lipid-bound oligosaccharide with one or two glucoses seems to be a much poorer substrate than the three glucose-containing compound (25).

An experiment was carried out by incubating the cotyledon-transferring enzyme with a lipid oligosaccharide which was a mixture of the one-, two-, and three-glucose-containing compounds. One sample was incubated with $Mn,^{2+}$ which is required for transfer, and another with EDTA Na, which inhibits the reaction. After incubation, the remaining lipid oligosaccharide which had not been used by the enzyme was treated with mild acid and chromatographed on paper. The oligosaccharide containing three glucoses disappeared in the presence of Mn^{2+}, while the peak corresponding to the oligosaccharides with one and two glucoses did not decrease as much (Fig. 8). The results were very similar to those obtained with animal enzymes (25) but not as clear.

Transfer from the Substrate Treated with α-Mannosidase. Treatment of dolichyl diphosphate-$(Glc)_3(Man)_9(GlcNAc)_2$ with α-mannosidase leads to the removal of up to five mannoses, leaving the glucoses intact. Such a substrate was tested by Spiro *et al.* (21) with a cow thyroid microsomal enzyme, and it was found that it was used at the same rate as the lipid oligosaccharide containing $(Glc)_3(Man)_9(GlcNAc)_2$.

A similar experiment was carried out with the cotyledon enzyme. The amount of oligosaccharide transferred to protein was the same for the intact and the α-mannosidase-treated lipid bound oligosaccharide (Table I).

DISCUSSION

The presence in alfalfa roots of a lipid-bound oligosaccharide identical with the one present in animal tissues and containing $(Glc)_3(Man)_9(GlcNAc)_2$ was previously reported (24). Now, some tests have been carried out to detect the same oligosaccharide in pea cotyledons incubated with radioactive hexoses. The characteristic solubility of the lipid-bound oligosaccharide and the chromatographic mobility of the oligosaccharide were found to be the same as those of the compound prepared with rat liver enzymes.

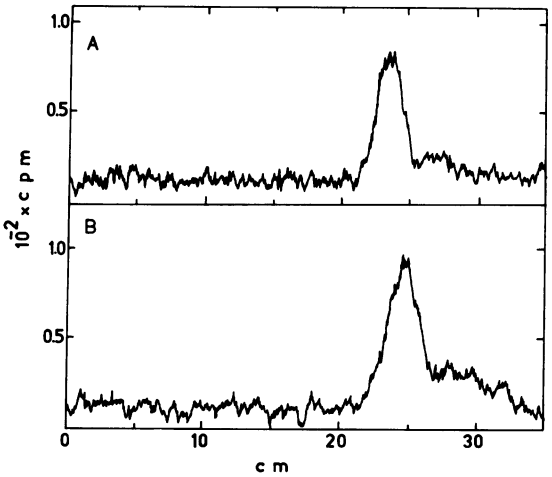

FIG. 7. Comparison by paper chromatography of the oligosaccharides transferred to protein with soya root and liver microsomal enzyme. A, The neutral compound of Figure 6B. B, Compound obtained by the same procedure as A but with an enzyme from rat liver microsomes instead of that from soya root. Paper chromatography was run with solvent A for 5 days.

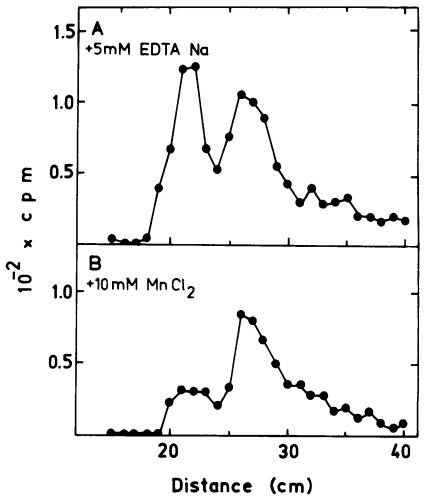

FIG. 8. Transfer of the $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ oligosaccharides to protein. Dolichyl diphosphate-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ labeled in the glucose (about 5,000 cpm) was dried, and the following components were added: 100 mM Tris-maleate buffer (pH 7.7); 1.2% Triton X-100; and 10 mg of pea cotyledon enzyme in a total volume of 0.5 ml. Where indicated, 5 mM EDTA Na or 10 mM $MnCl_2$ were added. After 10 min at 30 C, 1 ml methanol, 1.5 ml chloroform, and 100 mg rat microsomal protein (as carrier) were added, and the isolation of dolichyl diphosphate-$(Glc_1n_{-3})(Man)_9(GlcNAc)_2$ was carried out by extraction with chloroform: methanol:water (1:1:0.3), as described in "Materials and Methods" (see experiments with whole cotyledons). The lipid-bound oligosaccharides were submitted to mild acid hydrolysis and chromatographed with solvent A for 4 days. The radioactivity was measured on 0.5-cm strips with a toluene-based scintillation fluid in a scintillation counter.

Table I. *Effect of α-Mannosidase Treatment of the Lipid-Linked Oligosaccharide on the Transfer to Protein*

The lipid-linked oligosaccharide labeled in the glucose (2,500 cpm) with or without α-mannosidase treatment was dried and incubated with pea cotyledon enzyme under the conditions described in "Materials and Methods." Different lots of pea cotyledon enzyme were used in each experiment.

Dolichyl Diphosphate $(Glc)_3(Man)_9(GlcNAc)_2$	Oligosaccharide Transferred to Protein	
	Exp I	Exp II
	cpm	
Untreated	285	1,157
Treated with α-mannosidase	359	1,039

The lipid moiety of the plant intermediates seems to be very similar to that of the animal compounds. Size determinations with gel permeation columns with deoxycholate containing solvents have shown that the plant compounds behave as if they were the same as dolichyl phosphate obtained from mammalian liver but with one or two isoprenes less (3, 5). As judged by MS, the plant compound is very similar to or identical with animal dolichol phosphate (7).

The protein-bound oligosaccharide which became radioactive in the experiments with pea cotyledons could be released with endo-β-*N*-acetylglucosaminidase H, which is known to act on certain mannose-containing oligosaccharides bound to asparagine (12). After total hydrolysis, it was found to contain mannose but no glucose. In similar experiments, Browder and Beevers (4) detected glucose in addition to mannose. However, they hydro-

lyzed the glycoprotein directly, whereas, in our experiments, the glycopeptide was isolated, and the oligosaccharide was liberated with endo-β-*N*-acetylglucosaminidase H.

In view of the similarity of the plant and animal lipid-bound oligosaccharide and since the latter is more easily available, it was used as substrate for the enzyme that transfers oligosaccharide to protein. An active enzyme preparation could be obtained from pea cotyledons. Like the rat enzyme, it required detergent for activity. Experiments designed to find out if the three glucoses of the oligosaccharide were necessary for transfer did not give results as clear as those obtained with the rat enzyme but are consistent with the conclusion that the reaction is faster with the three glucose-containing substrate.

As with the rat enzyme, removal of peripheral mannoses from the lipid-bound oligosaccharide with α-mannosidase did not appreciably affect the rate of transfer.

The detection in plants of an enzyme which transfers oligosaccharide from its dolichyl phosphate derivative to protein is of some importance, because this was a missing step in the scheme of protein glycosylation. Now it can be accepted with a good degree of certainty that the glycosylation of asparagine residues in proteins occurs in plants by the same mechanism as it does in animals. An oligosaccharide containing $(Glc)_3(Man)_9(GlcNAc)_2$ is built up and combined to dolichyl diphosphate by the successive addition to *N*-acetylglucosamine and hexoses. The oligosaccharide is then transferred to protein; it loses the glucoses and may be modified. In plants, the oligosaccharide seems to remain without major changes as a high mannose oligosaccharide. Thus, the composition of the oligosaccharides of soya bean glycoproteins has been estimated to be $(Man)_9(GlcNac)_2$ (14). In contrast, in animals, the oligosaccharides may be processed by removal of mannose and addition of more *N*-acetylglucosamine, galactose, and sialic acid.

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