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Dolichyl Monophosphate and its Sugar Derivatives in Plants

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A glucose acceptor was isolated from soya beans by extraction with chloroform/methanol (2:1, v/v), followed by DEAE-cellulose column chromatography of the extract. This acceptor could not be distinguished from liver dolichyl monophosphate by t.l.c. It could replace dolichyl monophosphate as a mannose acceptor with a liver enzyme, and its glucosylated derivative could replace dolichyl monophosphate glucose as a glucose donor in the same system. These results, together with those already reported [Pont Lezica, Brett, Romero Martinez & Dankert (1975) *Biochem. Biophys. Res. Commun.* **66**, 980–987], indicate that the acceptor from soya bean is a dolichyl monophosphate. Gel filtration of its glucosylated derivative on Sephadex G-75 in the presence of sodium deoxycholate indicated that the acceptor contained 17 or 18 isoprene units. An enzyme preparation from pea seedlings was shown to use endogenous acceptors to form lipid phosphate sugars containing mannose and *N*-acetylglucosamine from GDP-mannose and UDP-*N*-acetylglucosamine. Chromatographic and degradative techniques indicated that the compounds formed were lipid monophosphate mannose, lipid pyrophosphate *N*-acetylglucosamine, lipid pyrophosphate chitobiose and a series of lipid pyrophosphate oligosaccharides containing both mannose and *N*-acetylglucosamine. None of these compounds was degraded by catalytic hydrogenation, and so the lipid moiety in each case was probably an α-saturated polyprenol. The endogenous acceptors for mannose and *N*-acetylglucosamine in peas may therefore be dolichyl monophosphate, as has been found in mammalian systems.

Liver dolichol is an α-saturated polyprenol containing 16–21 isoprene units. Its monophosphate acts as a sugar acceptor in mammalian tissues, and the glycose derivatives that are formed function as intermediates in glycoprotein synthesis (Lennarz, 1975; Parodi & Leloir, 1976). Undecaprenyl monophosphate, which is a fully unsaturated compound containing 11 isoprene residues, fulfils a similar role in extracellular polysaccharide synthesis in bacteria (Wright *et al.*, 1966; Hemming, 1974). Many higher plants have been found to contain free or fatty acid esters of polyprenols that are fully unsaturated and are 6–13 isoprene units in length (Hemming, 1974). Dolichol has also been found in its non-phosphorylated form in one plant tissue (Hemming *et al.*, 1963).

There have been a number of reports of the formation of acid-labile lipid-linked sugars in plants in which the lipid moiety was thought to be a polyprenol (Kauss, 1969; Alam & Hemming, 1973; Clark & Villemez, 1973; Forsee & Elbein, 1973, 1975). Pont Lezica *et al.* (1975) have reported that the glucose-acceptor lipid in several higher plants has the properties of an α-saturated polyprenyl monophosphate, similar to Dol-*P*.† In the present paper we give further evidence that this acceptor is a Dol-*P*, with a chain length slightly shorter than that of pig liver dolichol.

The incorporation of mannose from GDP-mannose into lipid phosphate mannose has been reported in *Phaseolus aureus* (Kauss, 1969; Alam & Hemming, 1973; Clark & Villemez, 1973), cotton fibres (Forsee & Elbein, 1973, 1975) and other plants (Alam & Hemming, 1973). Forsee & Elbein (1975) have described the formation by cotton-fibre enzymes of lipid phosphate chitobiose and of lipid phosphate oligosaccharides which contain mannose and *N*-acetylglucosamine. Here we report the formation of similar compounds by enzymes from pea seedlings, and provide evidence that the lipid moieties of these compounds are α-saturated polyprenols.

Materials and Methods

Preparation and assay of acceptor lipid from soya beans (Glycine max)

The acceptor lipid was prepared as described by Pont Lezica *et al.* (1975). The rat liver enzyme

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† Abbreviations: Dol-*P*, dolichyl monophosphate; Dol-P_2, dolichyl diphosphate; Fic-*P*, ficaprenyl monophosphate; Fic-P_2, ficaprenyl diphosphate.

assay for the acceptor was reported in the same paper. To test for mannose transfer, UDP-[^{14}C]-glucose was replaced by GDP-[^{14}C]mannose (45000c.p.m.) and the concentration of Triton X-100 was lowered to 0.024%.

Preparation of pea enzyme

Dwarf pea seedlings (*Pisum sativum* L., var. Cuarentona) were grown in the dark on wet filter paper for 5–7 days at room temperature (up to 35°C). Roots and shoots up to 10cm long were cut off and homogenized in a pestle and mortar at 4°C with half their weight of 0.1M-Tris/HCl buffer, pH7.5, containing 1% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.). The homogenate was filtered through muslin and centrifuged at 105000*g* (r_{av}. 5.7cm) for 1h at 4°C in a Beckman Spinco ultracentrifuge in an R65 rotor. Each pellet, obtained from 10ml of homogenate, was resuspended in 0.5ml of homogenization buffer to give the 'pea enzyme'.

Mild acid hydrolysis

Lipid material in chloroform/methanol (6:1 or 2:1, v/v) was dried under N_2 in a small glass tube and hydrolysed with 0.2ml of 0.1M-HCl at 100°C for 10min. Then 0.4ml of methanol and 0.6ml of chloroform were added, and the aqueous phase was dried under N_2.

Chromatography

DEAE-cellulose chromatography was carried out on a 42cm×1.2cm column (acetate form).

Descending paper chromatography was performed on Whatman no. 1 paper with butan-1-ol/pyridine/water solvents in the following proportions: A, 6:4:3, by vol.; B, 4:3:4, by vol. (Leloir *et al.*, 1971). Running times were 16 and 48h respectively. Marker sugars were detected with the $AgNO_3$ reagent (Trevelyan *et al.*, 1950).

T.l.c. of acceptor lipids was carried out in chloroform/methanol/formic acid (70:10:1, by vol.) (solvent C) on silica-gel G plates which had been pre-washed with methanol/conc. HCl (9:1, v/v).

Radioactivity counting

Radioactivity was generally measured in a flow counter. Radioactivity on paper chromatograms was detected either by a scanner or by cutting the paper into 1cm strips and counting each one for 10min in toluene/Omniflor scintillant (New England Nuclear, Boston, MA, U.S.A.) in a Packard Tri-Carb liquid-scintillation spectrometer.

Chemicals

UDP-[^{14}C]glucose (309mCi/mmol) and UDP-[^{3}H]glucose (2.42Ci/mmol) were synthesized as described by García *et al.* (1974). GDP-[^{14}C]mannose (250mCi/mmol) and UDP-[^{14}C]*N*-acetylglucosamine (269mCi/mmol) were obtained from New England Nuclear. Fic-*P* (synthesized from *Ficus elasticus* ficaprenol; Pont Lezica *et al.*, 1975) and Dol-*P* (from pig liver) were gifts from Dr. P. Romero and Dr. N. H. Behrens respectively, both of this Institute. The Dol-P_2-oligosaccharide from mammalian liver that was used as a mannose acceptor was a gift from Dr. A. J. Parodi of this Institute.

Results

Properties of the glucose-acceptor lipid from soya beans

It was reported (Pont Lezica *et al.*, 1975) that this acceptor had the properties of an α-saturated polyprenyl monophosphate. This conclusion is supported by the following observations.

(*a*) The acceptor is able to act as a mannose acceptor from GDP-[^{14}C]mannose with the rat liver enzyme. The maximum stimulation of incorporation of radioactivity into the organic phase of the Folch partition (Folch *et al.*, 1957) was at least 30-fold, similar to that obtained with Dol-*P*. Fic-*P* was a much poorer acceptor, giving up to 5-fold stimulation of incorporation into the organic phase.

(*b*) The same rat liver preparation that was used as an assay for glucose-acceptor lipid also transfers glucose from Dol-*P*-glucose to a Dol-P_2-oligosaccharide (Behrens *et al.*, 1971; Parodi *et al.*, 1973). The glucosylated acceptor from soya bean was able to substitute for Dol-*P*-glucose in this reaction; under conditions (Behrens *et al.*, 1971) that gave 37% transfer of [^{14}C]glucose from Dol-*P*-[^{14}C]-glucose to Dol-P_2-oligosaccharide, 54% transfer of [^{14}C]glucose from the glucosylated acceptor lipid from soya bean was obtained.

(*c*) The acceptor lipid from soya bean ran together with Dol-*P* on t.l.c. in solvent C (Fig. 1).

The chain length of the isoprenoid moiety of the glucosylated acceptor lipid was examined by gel filtration on Sephadex G-75 in a solution of sodium deoxycholate in phosphate buffer (Behrens *et al.*, 1971). Comparison with Dol-*P*-glucose and Fic-*P*-glucose showed that the glucosylated acceptor lipid was slightly smaller than Dol-*P*-glucose (Fig. 2). The major component of pig liver dolichol contains 19 isoprene units (Dunphy *et al.*, 1967; Butterworth & Hemming, 1968), and the Fic-*P* used had an average of 11.5 isoprene units (Pont Lezica *et al.*, 1975). If it is assumed that the elution volume is proportional to the logarithm of the polyprenol size, the soya-bean lipid phosphate is calculated to contain 17 or 18 isoprene units.

An experiment was carried out to determine whether soya beans contain sugar-linked polyprenyl phosphates. Beans (200g) were ground and extracted with 600ml of acetone as described by Pont Lezica *et al.* (1975). The residue from the acetone extraction was washed with 525ml of chloroform/

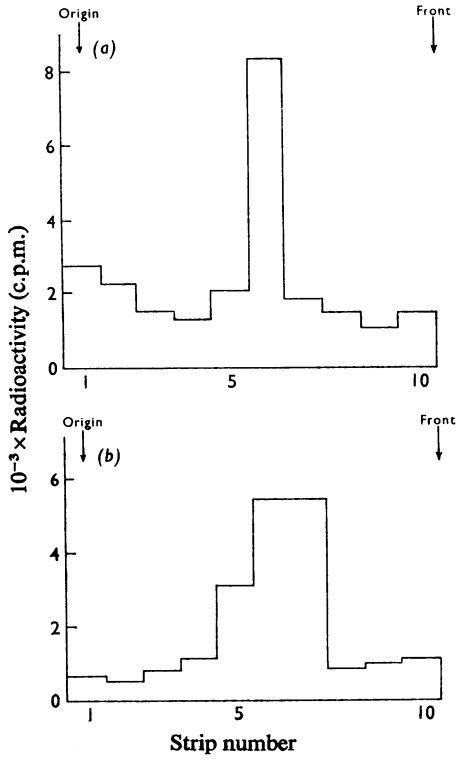

Fig. 1. *T.l.c. of acceptor lipids*

Dol-*P* and the acceptor lipid from soya bean were chromatographed on silica-gel thin-layer plates as described in the Materials and Methods section. After development, 1cm strips of gel were scraped off the plate and extracted with 1.5ml of chloroform/methanol/conc. HCl (12:8:1, by vol.). The extracts were washed by the method of Folch *et al.* (1957) to remove the acid, and then assayed for acceptor lipids by using the liver enzyme. (*a*) Soya-bean acceptor lipid; (*b*) Dol-*P*.

methanol/water (4:2:1, by vol.) and twice with 200ml of Folch theoretical upper phase (Folch *et al.*, 1957) to remove water-soluble material and free polyprenyl phosphate. These washes are also likely to have removed some of the sugar-linked polyprenyl phosphate. The residue was extracted with 300ml of chloroform/methanol/water (10:10:3, by vol.) followed by 100ml of the same solvent, and the combined extracts were chromatographed on a DEAE-cellulose column. The column was eluted with 300ml of a gradient of 0–0.13M-ammonium formate, and fractions (5ml) were collected. Samples of the fractions were treated with 1M-HCl at 37°C for 3h in chloroform/methanol/water (10:10:3, by vol.). After this treatment the acid was washed out of the samples by the method of Folch *et al.* (1957), and the organic phases were assayed for acceptor activity with the liver enzyme. The assays showed that a peak of acceptor activity was eluted at an ammonium formate concentration of 0.03M. The Thymol Blue marker was eluted at a salt concentration of 0.01M, rather earlier than is usual in this system, 0.02M salt being the usual position. However, the position of the peak of acceptor activity relative to that of the Thymol Blue is typical of that observed for Dol-P_2-sugar compounds in this system. Dol-*P*-sugar compounds are eluted at a slightly lower salt concentration than Thymol Blue. The fractions that contained this acceptor activity showed virtually no acceptor activity when assayed without prior acid treatment. Thus they contain an acid-labile compound that releases an acceptor on acid treatment. This compound is likely to be a polyprenyl diphosphate sugar, and, since the released acceptor was not further degraded by acid, the polyprenol moiety is likely to be α-saturated. This polyprenol may therefore be the same as that found in the free polyprenyl phosphate.

Polyprenyl phosphate sugars formed by the pea enzyme

Pont Lezica *et al.* (1975) showed that an enzyme from the plumular hooks of pea seedlings was able to form an acid-labile lipid phosphate glucose from UDP-glucose. The lipid phosphate acceptor was isolated from the enzyme preparation and shown to possess the properties of an α-saturated polyprenyl monophosphate. The investigation of the pea system has now been continued by using an enzyme preparation from the roots and shoots of pea seedlings. The incorporation of sugars from GDP-mannose and UDP-*N*-acetylglucosamine into lipid-soluble compounds has been investigated.

Incorporation of mannose. Incorporation of mannose by the pea enzyme from GDP-mannose into the organic phase of a Folch partition in the presence and absence of Dol-*P* is shown in Table 1, Expt. A. Dol-*P* stimulated incorporation into material that gave mannose on mild acid hydrolysis, and Fic-*P* and the acceptor lipid from soya bean also stimulated incorporation into the organic phase (results not shown). Negligible amounts of radioactivity were obtained by chloroform/methanol/water (10:10:3, by vol.) extraction of the residue remaining after two extractions of the reaction mixture with 1.2ml of chloroform/methanol/4mM-$MgCl_2$ (3:2:1, by vol.) and two extractions with Folch *et al.* (1957) theoretical upper phase.

Mild acid hydrolysis released at least 90% of the radioactivity in the Folch *et al.* (1957) organic phase as water-soluble material. When this water-soluble radioactivity was run on paper chromatography in solvent A, small amounts of radioactivity remained at the origin of the chromatogram. When solvent B was used in place of solvent A, no radioactivity

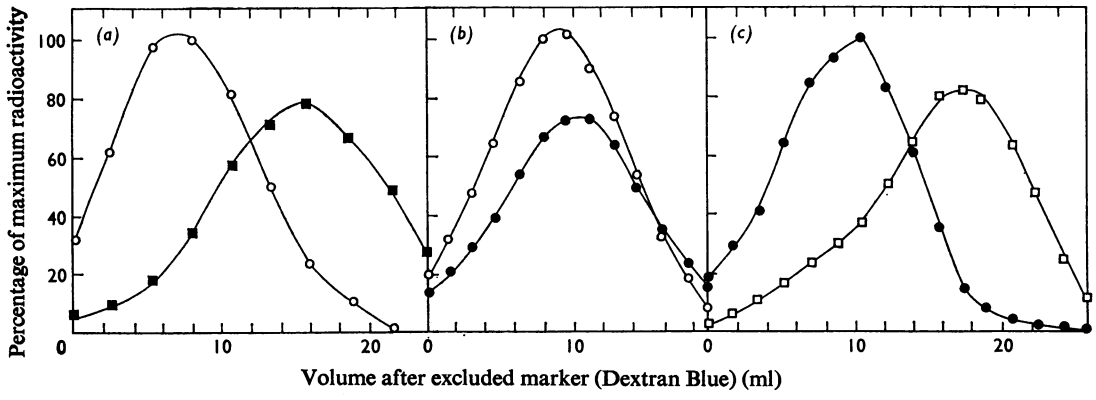

Fig. 2. *Gel filtration of glucosylated acceptor lipids*

Dol-*P*-[^{3}H]glucose, Fic-*P*-[^{3}H]glucose, Fic-*P*-[^{14}C]glucose and soya-bean lipid-[^{14}C]glucose were prepared by using rat liver enzyme. Contamination of the last three by Dol-*P*-glucose arising from endogenous Dol-*P* in the enzyme preparation was less than 14%, as judged by controls in which no exogenous acceptor was added. The glucosylated lipids were passed through a column (72cm×1.5cm) of Sephadex G-75 in 5mM-sodium phosphate buffer, pH7.4, containing 0.5% sodium deoxycholate. Samples of the effluent fractions were counted for ^{3}H and ^{14}C. (*a*) Dol-*P*-[^{3}H]-glucose (○) and Fic-*P*-[^{14}C]glucose (■); fractions of volume 2.6ml were collected. (*b*) Dol-*P*-[^{3}H]glucose (○) and soya-bean lipid-[^{14}C]glucose (●); fractions of volume 1.6ml were collected. (*c*) Fic-*P*-[^{3}H]glucose (□) and soya-bean lipid-[^{14}C]glucose (●); fractions of volume 1.6ml were collected.

Table 1. *Incorporation of mannose from GDP-mannose into lipid-soluble material by pea enzyme*

Exogenous acceptor was dried in a tube with 0.5μmol of MgEDTA and 0.5μmol of $MgCl_2$. It was then resuspended in the reaction mixture, which contained 5μmol of mercaptoethanol, 0.06% Triton X-100, 40000c.p.m. of GDP-[^{14}C]mannose and 25μl of pea enzyme in a total volume of 50μl. Incubation was carried out at 30°C for 30min, and then 0.4ml of methanol and 0.6ml of chloroform were added. The precipitate was removed by centrifugation at 1800*g* for 5min; the supernatant was washed by the method of Folch *et al.* (1957), hydrolysed with dilute acid as described in the Materials and Methods section, and run on paper chromatography in solvent A. Radioactivity at the chromatogram origin (oligosaccharide) and running with mannose was determined by scintillation counting. The liver acceptor was obtained from a mixture of rat and pig liver and purified by DEAE-cellulose chromatography as described by Behrens *et al.* (1973).

Experiment	Exogenous acceptor	Radioactivity incorporated (c.p.m.)	
		Oligosaccharide	Mannose
A	—	270	1100
	Dol-*P*	170	5100
B	—	110	1300
	200μl of liver acceptor	490	1200
	500μl of liver acceptor	600	380

was found at the origin, but a small peak was detectable which ran with an R_{Man} of 0.3. Since small oligosaccharides move from the origin in solvent B but not in solvent A, this peak is likely to have been an oligosaccharide. This oligosaccharide formation was stimulated by the addition of an extract from mammalian liver (Table 1, Expt. B). This extract acts as an acceptor for mannose in mammalian systems and had been identified as a Dol-P_2-oligosaccharide (Behrens *et al.*, 1973). This suggested that enzymes were present in the pea preparation similar to those which form Dol-P_2-oligosaccharides in liver. These latter compounds contain mannose and *N*-acetylglucosamine, so incorporation of *N*-acetylglucosamine into lipid-soluble material by the pea enzyme was now studied.

Incorporation of N-acetylglucosamine. The pea enzyme incorporated radioactivity from UDP-[^{14}C]*N*-acetylglucosamine into the organic phase of the Folch partition. The conditions are shown in the legend to Fig. 3. This incorporation was stimulated slightly by the presence of 0.06% Triton X-100. In some experiments it was also stimulated up to 200% by Dol-*P*, but this stimulation was not consistent, and sometimes no effect or even inhibition was observed.

At least 90% of this organic-phase material was transferred to the aqueous phase on mild acid hydrolysis. This hydrolysed material gave two peaks of radioactivity when run on paper chromatography in solvent A (Fig. 3*a*). The faster-moving peak ran together with *N*-acetylglucosamine; the slower-moving one ran slightly ahead of glucosamine. However, the behaviour of the slower-moving peak was not affected by mixing the sample with 5μl of

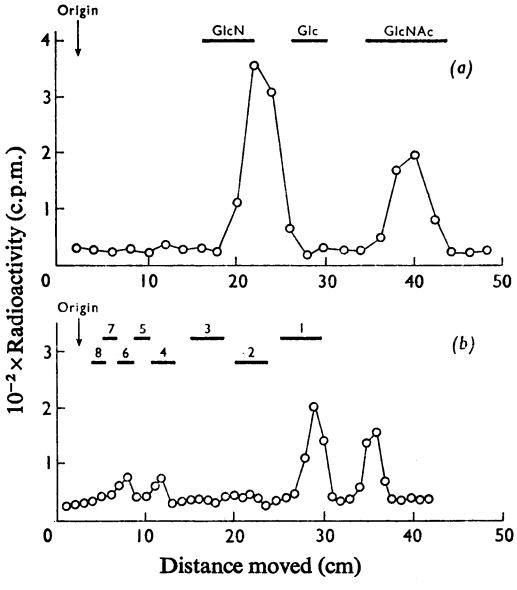

Fig. 3. *Paper chromatography of mild acid hydrolysates of lipid-soluble material obtained by incubation of pea enzyme with UDP-[^{14}C]N-acetylglucosamine*

(*a*) The incubation mixture contained 0.5μmol of MgEDTA, 0.5μmol of $MgCl_2$, 5μmol of mercaptoethanol, 0.12% Triton X-100, 42000c.p.m. of UDP-[^{14}C]*N*-acetylglucosamine and 25μl of pea enzyme in a total volume of 50μl. The incubation was carried out at 30°C for 30min, and then the Folch organic phase was obtained and hydrolysed as described in the legend to Table 1. The hydrolysate was chromatographed in solvent A. (*b*) As (*a*), except that 2μl (56nmol) of non-radioactive GDP-mannose was added after 5min of incubation, and the chromatography was carried out in solvent B. Bars indicate malto-oligosaccharides containing the indicated number of glucose residues. The malto-oligosaccharides are separated by this system; the overlap of some of the bars is due to the fact that two sets of marker malto-oligosaccharides were run on the chromatogram in this experiment, and one set ran slightly faster than the other.

saturated $(NH_4)_2SO_4$ solution before it was applied to the chromatogram. This treatment prevents glucosamine from moving from the origin, which implies that the slower-moving peak was not glucosamine. Its R_{GlcNAc} suggests that it is likely to have been a disaccharide of *N*-acetylglucosamine, probably chitobiose (Leloir *et al.*, 1973).

The radioactive material was retained by a DEAE-cellulose column when applied in chloroform/methanol (2:1, v/v). It was eluted with a gradient of 300ml of 0–0.2M-ammonium formate in the same solvent, and it emerged at a salt concentration of 0.18–0.20M. This eluted material was hydrolysed by dilute acid and shown by paper chromatography to give both the peaks described above. The Thymol Blue that was used as a marker was eluted at a salt concentration of 0.10–0.12M. Under the same conditions Dol-*P*-glucose, Dol-*P*-mannose and the lipid-mannose compound formed by the pea enzyme were all eluted at a salt concentration of 0.10–0.12M, usually a few millilitres ahead of the Thymol Blue marker. It seems likely, therefore, that the lipid-*N*-acetylglucosamine and lipid-(*N*-acetylglucosamine)$_2$ contain more than one negatively charged group, which suggests that a pyrophosphate linkage is present.

Incorporation of mannose and N-acetylglucosamine in the same incubation. When an excess of non-radioactive GDP-mannose (56nmol) was added at the start of the incubation to an incubation mixture containing UDP-[^{14}C]*N*-acetylglucosamine (0.1nmol), the incorporation of radioactivity by the pea enzyme into Folch organic phase was decreased by 50%. No inhibition was observed when the GDP-mannose was added 5min or more after the start of the incubation. Under these latter conditions, mild acid hydrolysates of the organic-phase material were seen to contain two small peaks of radioactivity that moved with malto-oligosaccharides containing four to five and five to seven glucose residues respectively in chromatography in solvent B (Fig. 3*b*). In the absence of GDP-mannose, only the two large fast-moving peaks were seen, corresponding to the two peaks seen in Fig. 3(*a*).

The use of GDP-[^{14}C]mannose (0.2nmol) and non-radioactive UDP-*N*-acetylglucosamine (17nmol) gave rather more significant results. Only traces of slow-moving peaks were seen with solvent B when the GDP-[^{14}C]mannose was present at the start of the incubation, but, if the GDP-[^{14}C]mannose was added to the incubation mixture 5min after the start of the incubation, several slow-moving peaks were seen (Fig. 4*a*). When the same amount of GDP-[^{14}C]-mannose was added after 5min of incubation, and then an excess of non-radioactive GDP-mannose (56nmol) was added after a further 5min of incubation, only the slowest-moving of these peaks was seen (Fig. 4*b*).

It therefore seems that at least five oligosaccharides were formed, attached to lipid by acid-labile bonds. These oligosaccharides contain mannose and *N*-acetylglucosamine, and the requirement that GDP-mannose must be added to the incubation medium several minutes after UDP-*N*-acetylglucosamine suggests that the mannose residues were added to an *N*-acetylglucosamine-containing lipid. This latter compound might be either the lipid-*N*-acetylglucosamine or the lipid-(*N*-acetylglucosamine)$_2$ whose formation has been noted above. The formation of only the largest oligosaccharides when

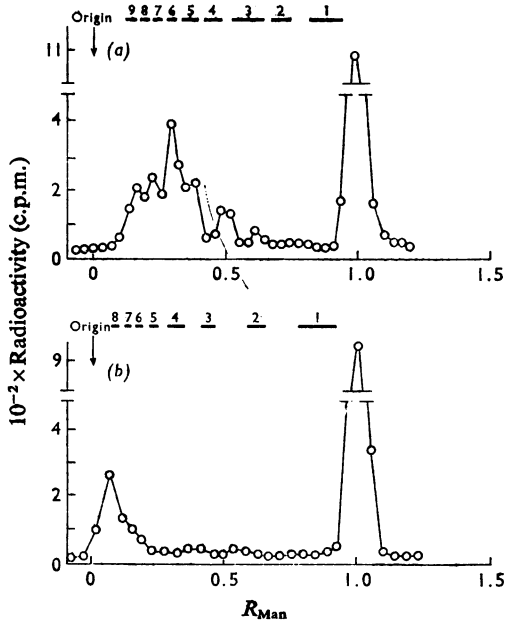

Fig. 4. *Paper chromatography of mild-acid hydrolysates of lipid-soluble material obtained by incubation of pea enzyme with GDP-[^{14}C]mannose and non-radioactive UDP-N-acetylglucosamine*

(*a*) The incubation mixture contained 0.5 μmol of MgEDTA, 0.5 μmol of $MgCl_2$, 5 μmol of mercaptoethanol, 0.05% Triton X-100, 17 nmol of UDP-*N*-acetylglucosamine and 100 μl of pea enzyme in a total volume of 125 μl. After 5 min of incubation at 30°C, 40000 c.p.m. (0.2 nmol) of GDP-[^{14}C]mannose was added, and the incubation was continued for a further 25 min. The reaction was terminated by the addition of 0.8 ml of methanol and 1.2 ml of chloroform, and the precipitate was removed by centrifugation at 1800*g* for 5 min. The supernatant was washed by the method of Folch *et al.* (1957), hydrolysed with dilute acid and run on paper chromatography in solvent B. Markers were as in Fig. 3(*b*). (*b*) As (*a*), except that 2 μl (56 nmol) of non-radioactive GDP-mannose was added 10 min after the start of the incubation.

an excess of GDP-mannose is added suggests that the smaller oligosaccharides seen are intermediates in the formation of the larger ones.

The organic-phase material obtained under the conditions of Fig. 4(*a*), by using non-radioactive UDP-*N*-acetylglucosamine and GDP-[^{14}C]mannose, was examined by DEAE-cellulose column chromatography. A portion of the material was first hydrolysed with mild acid and chromatographed on paper in solvent A; it gave a peak running with mannose and another peak at the origin, which corresponded to the oligosaccharide peaks found with solvent B. The organic-phase material was applied to the column in chloroform/methanol (2:1, v/v), and the column was eluted with a gradient of 300 ml of 0–0.2 M-ammonium formate in the same solvent, followed by 150 ml of 0.2 M-ammonium formate in this solvent. Only one peak of radioactivity was eluted, emerging at a salt concentration of 0.10 M, together with the Thymol Blue marker. Dol-*P*-mannose was also eluted from the column with the Thymol Blue marker under these conditions. On mild acid hydrolysis of this peak, followed by chromatography in solvent A, only radioactive mannose was seen, with no peak at the origin of the chromatogram. On subsequent elution of the column with methanol, chloroform/methanol/water (10:10:3, by vol.) and then 2 M-potassium acetate in the latter solvent, a second peak emerged with the front of the salt solution. On hydrolysis and paper chromatography in solvent A of this second peak, all the radioactivity remained at the chromatogram origin.

Another sample of the same material was applied to the same column in chloroform/methanol/water (10:10:3, by vol.) and eluted with a gradient of 300 ml of 0–0.13 M-ammonium formate in the same solvent. The elution pattern is shown in Fig. 5. Two peaks were obtained, one sharp (fractions 27–29) and one broad (fractions 40–50 approx.). When hydrolysed and examined by chromatography in solvent A, radioactivity from fraction 28 ran with mannose, whereas radioactivity from fractions 42–48 remained at the chromatogram origin. The first sharp peak thus contained lipid-mannose, and the second broad one contained lipid-oligosaccharide.

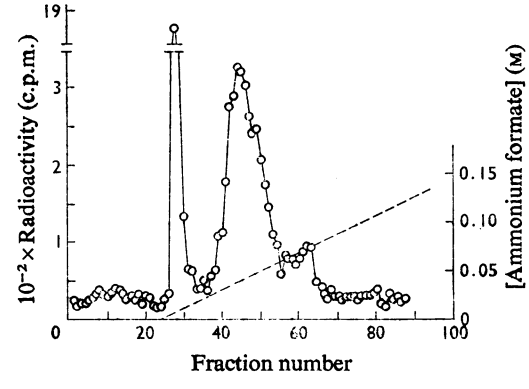

Fig. 5. *DEAE-cellulose column chromatography of lipid-mannose and lipid-oligosaccharide*

The sample was obtained under the conditions of Fig. 4(*a*) and applied to the column in chloroform/methanol/water (10:10:3, by vol.). It was eluted with a gradient of 0–0.13 M-ammonium formate in the same solvent (----). Fractions (4.5 ml) were collected, and samples (0.5 ml) were counted for radioactivity in a flow counter. o, Radioactivity.

Catalytic hydrogenation of the lipid sugars

Catalytic hydrogenation degrades polyprenyl phosphate sugars if they are allylic, but not if they are α-saturated (Wright *et al.*, 1966; Hemming, 1974; Pont Lezica *et al.*, 1975). We have used this property to examine whether the lipid acceptors for mannose and *N*-acetylglucosamine might be Dol-*P*. Table 2 shows the results of catalytic hydrogenation of the lipid mannose, the [^{14}C]mannose-labelled lipid oligosaccharide, and the mixture of lipid-*N*-acetylglucosamine and lipid-(*N*-acetylglucosamine)$_2$, all of which were formed by the pea enzyme as described above. Dol-*P*-glucose, Fic-*P*-glucose, Dol-*P*-mannose, Fic-*P*-mannose and Dol-P_2-*N*-acetylglucosamine were also examined as controls.

The results show that the compounds fall into two categories. Dol-*P*-glucose, Dol-*P*-mannose, Dol-P_2-*N*-acetylglucosamine, lipid-mannose, the mixture of lipid-*N*-acetylglucosamine and lipid-(*N*-acetylglucosamine)$_2$, and lipid-oligosaccharide were all degraded 20% or less; Fic-*P*-glucose and Fic-*P*-mannose were broken down at least 60%. The degraded and undegraded portions of the mixture of lipid-*N*-acetylglucosamine and lipid-(*N*-acetylglucosamine)$_2$ were hydrolysed with mild acid and chromatographed in solvent A. Both portions contained a similar proportion of the monosaccharide to the starting material (30–40%), indicating that the lipid-*N*-acetylglucosamine and the lipid-(*N*-acetylglucosamine)$_2$ behave in the same way under this treatment.

It is therefore clear that the mannose acceptor in the pea enzyme might be Dol-*P* and is certainly not Fic-*P*. The same is probably true of the *N*-acetylglucosamine acceptor, and of the lipid portion of the lipid oligosaccharide, which may be identical with the *N*-acetylglucosamine acceptor. We have not been able to obtain Fic-P_2-*N*-acetylglucosamine to use as a control, but it is highly likely that such a compound would be degraded by this treatment.

Discussion

Pont Lezica *et al.* (1975) have reported that the glucose-acceptor lipid from soya beans has the properties of an α-saturated polyprenyl monophosphate, such as Dol-*P*. In the experiments reported here we have not been able to distinguish between the soya-bean acceptor and liver Dol-*P*, except for a slight difference in polyprenol chain length as judged by gel filtration. The difference in chain length is only one or two isoprene units, and is consistent with the co-chromatography of the two compounds in t.l.c., which has already been reported (Pont Lezica *et al.*, 1975), since the resolution of the t.l.c. system used is not sufficient to distinguish such small differences. Tkacz *et al.* (1974) have shown that this t.l.c. system separates polyprenyl phosphate glucoses according to the length of the polyprenol chain.

In view of these results, it is reasonable to conclude that the soya-bean acceptor is almost certainly a Dol-*P* containing 17 or 18 isoprene units. Final proof will await an analysis by mass spectroscopy; we have not been able to obtain sufficient material for such an analysis. We estimate that the acceptor is present in a concentration of about 40 μg/kg of beans.

The essential feature of a Dol-*P* is that it contains a saturated α-isoprene unit. One possibility that we have not been able to rule out is that

Table 2. *Catalytic hydrogenation of lipid sugars*

Catalytic reduction was carried out as described by Pont Lezica *et al.* (1975). Lipid-[^{14}C]mannose and lipid-[^{14}C]oligosaccharide were prepared as described in the legend to Fig. 4(*a*), and separated by DEAE-cellulose column chromatography as shown in Fig. 5. Lipid-[^{14}C]*N*-acetylglucosamine and lipid-([^{14}C]*N*-acetylglucosamine)$_2$ were obtained as described in the legend to Fig. 3(*a*). Dolichol and ficaprenol derivatives of mannose and glucose were obtained by using the liver enzyme, as described in the Materials and Methods section and by Pont Lezica *et al.* (1975). Contamination of Fic-*P*-glucose and Fic-*P*-mannose by the dolichol derivatives due to endogenous Dol-*P* present in the liver enzyme was 14 and 20% respectively, as estimated from controls in which no exogenous acceptor was added. Dol-P_2-[^{14}C]*N*-acetylglucosamine was obtained as described (Leloir *et al.*, 1973). Mild acid hydrolysis followed by paper chromatography showed that it contained approx. 13% Dol-P_2-[^{14}C]chitobiose.

Sample	Enzyme used for glycosylation	Butanol phase (c.p.m.)	Water phase (c.p.m.)	Degradation (%)
Lipid-[^{14}C]mannose	Pea	2730	310	10
Lipid-[^{14}C]*N*-acetylglucosamine Lipid-([^{14}C]*N*-acetylglucosamine)$_2$	Pea	1070	230	18
Lipid-[^{14}C]oligosaccharide	Pea	1330	290	18
Dol-*P*-[^{3}H]glucose	Liver	10020	1390	12
Fic-*P*-[^{14}C]glucose	Liver	460	1760	79
Dol-*P*-[^{14}C]mannose	Liver	2060	170	8
Fic-*P*-[^{14}C]mannose	Liver	360	540	60
Dol-P_2-[^{14}C]*N*-acetylglucosamine	Liver	1890	470	20

the soya-bean acceptor might contain more than one saturated isoprene unit. Mankowski *et al.* (1975) have shown that in liver microsomal fractions the ability of polyprenyl phosphates to accept glucose from UDP-glucose was not greatly affected either by the length of the polyprenyl chain or by its degree of saturation, provided that the α-isoprene residue was saturated. Clark & Villemez (1973) have reported that phytanol phosphate, a fully saturated polyprenyl phosphate containing four isoprene units, acted as a mannose acceptor in *Phaseolus aureus* membrane preparations.

It was found (Pont Lezica *et al.*, 1975) that an α-saturated polyprenyl phosphate acted as an endogenous glucose acceptor when a pea enzyme was studied. It was possible, however, that the acceptors for other sugars might have been different. In particular, they might have been Fic-*P*, since the major non-phosphorylated polyprenol in plants is ficaprenol, which is fully unsaturated (Hemming, 1974). We have therefore examined the endogenous acceptors for mannose and *N*-acetylglucosamine in peas. We have found that these acceptors, when glycosylated, are stable to catalytic reduction, and are therefore not allylic polyprenyl phosphates. Thus it is likely that these acceptors are also dolichyl phosphates or similar α-saturated polyprenyl phosphates. The following properties of the glycosylated derivatives support this hypothesis.

The mannosylated lipid formed by the pea enzyme is acid-labile and is retained by DEAE-cellulose columns. Its behaviour on elution from the columns by ammonium formate gradients in chloroform/methanol (2:1, v/v) or chloroform/methanol/water (10:10:3, by vol.) is identical with that of Dol-*P*-mannose (Behrens *et al.*, 1973). Dol-*P* and Fic-*P* are both able to replace the endogenous acceptor in the formation of this compound by the pea enzyme. The corresponding compound in *Phaseolus aureus* is labelled by [^{3}H]mevalonate, and is therefore likely to be an isoprenoid compound (Kauss, 1969). Thus the mannosylated lipids formed in plants appear to be similar to the corresponding compound formed in mammals, Dol-*P* (Behrens *et al.*, 1973; Tkacz *et al.*, 1974).

The lipid-*N*-acetylglucosamine and lipid-(*N*-acetylglucosamine)$_2$ formed by the pea enzyme also appear to be similar to their mammalian counterparts, Dol-P_2-*N*-acetylglucosamine and Dol-P_2-chitobiose. Their degree of breakdown on catalytic hydrogenation is the same as that of Dol-P_2-*N*-acetylglucosamine, but a little higher than that of the Dol-*P*-sugars tested. The presence of a pyrophosphate linkage is also suggested by their behaviour on DEAE-cellulose column chromatography, since they are eluted at a salt concentration considerably higher than that for Dol-*P*-sugars.

Lipid oligosaccharides are formed when first UDP-*N*-acetylglucosamine and then GDP-mannose are supplied to the pea enzyme. These compounds are very similar to those obtained by Forsee & Elbein (1975) in the cotton-fibre system. The oligosaccharides obtained by mild acid hydrolysis run on paper like malto-oligosaccharides containing two to nine glucose residues; since both mannose and *N*-acetylglucosamine move faster than glucose in the chromatographic system used, the pea oligosaccharides are probably slightly larger than the malto-oligosaccharides that run with them. In contrast with the cotton-fibre (Forsee & Elbein, 1975) and liver (Behrens *et al.*, 1973) systems, virtually no lipid oligosaccharide was obtained when pea enzyme was incubated with GDP-mannose alone, indicating that the amount of pre-formed lipid oligosaccharide acceptors in this preparation is very low.

The lipid oligosaccharides obtained from the pea enzyme system also appear to possess properties similar to those of their mammalian counterparts. Dol-P_2-oligosaccharides from liver can act as mannose acceptors in the pea system, which indicates either a remarkable degree of similarity between the liver and pea systems, or alternatively a rather loose specificity in the enzymes involved. The presence of a pyrophosphate linkage in the pea lipid oligosaccharides is indicated by their elution behaviour on DEAE-cellulose columns in chloroform/methanol/water (10:10:3, by vol.), which is similar to that of Dol-P_2-oligosaccharides from rat liver (Behrens *et al.*, 1973).

The failure of the lipid oligosaccharides to be eluted from DEAE-cellulose in 0.2M-ammonium formate in chloroform/methanol (2:1, v/v) may reflect a very low solubility of these compounds in chloroform/methanol (2:1, v/v), especially when they are purified. The corresponding compounds formed in cotton fibres were not extracted by this solvent, though they were soluble in chloroform/methanol/water (10:10:3, by vol.). It is likely that the solubility properties of these compounds are critically affected by the presence of other lipids, and may therefore vary in different enzyme systems and may also change on purification.

Our results, and those of Forsee & Elbein (1975), indicate that all the acid-labile lipid sugars found so far in higher plants are very similar to the corresponding derivatives in mammalian systems (Hemming, 1974; Lennarz, 1975; Parodi & Leloir, 1976). In mammals it has been shown that such compounds are intermediates in the synthesis of the core regions of the oligosaccharides of those glycoproteins that contain the asparagine–*N*-acetylglucosamine linkage (Parodi & Leloir, 1976). Such glycoproteins are present in plants, and indeed the majority of the plant glycoproteins so far investigated fall into this category (Sharon, 1974). The structure of the core

regions of the oligosaccharides conformed to the general pattern observed in this type of glycoprotein in animals. It has been shown that *N*-acetylglucosamine and mannose are incorporated into plant glycoproteins from the corresponding nucleoside diphosphate sugars (Villemez, 1970; Forsee & Elbein, 1975; Roberts & Pollard, 1975). Thus it seems most probable that the lipid sugars found in plants form part of a mechanism for glycoprotein synthesis analogous to that found in animals. However, we have not been able to demonstrate direct transfers of sugars from lipid sugars to protein.

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