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Biosynthesis of pentosyl lipids by pea membranes

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Pea membranes were incubated with UDP-[^{14}C]xylose or UDP-[^{14}C]arabinose and sequentially extracted with chloroform/methanol/water (10:10:3, by vol.) and sodium dodecyl sulphate (2%, w/v). An active epimerase in the membranes rapidly interconverted the two pentosyl nucleotides. Chromatographic analysis of the lipid extract revealed that both substrates gave rise to xylose- and arabinose-containing neutral lipids, xylolipid with properties similar to a polyisoprenol monophosphoryl derivative, and highly charged lipid-linked arabinosyl oligosaccharide. When UDP-[^{14}C]pentose or the extracted lipid-linked [^{14}C]arabinosyl oligosaccharide were used as substrates, their ^{14}C was also incorporating into sodium dodecyl sulphate-soluble and -insoluble fractions as major end products. Polyacrylamide-gel electrophoresis of sodium dodecyl sulphate-soluble products indicated the formation of mobile components with M_r values between 40000 aand 200000 (Sepharose CL-6B). The lipid-linked [^{14}C]arabinosyl oligosaccharide possessed properties comparable with those of unsaturated polyisoprenyl pyrophosphoryl derivatives. It was hydrolysed by dilute acid to a charged product (apparent M_r 2300) that could be fractionated in alkali. It was degraded to shorter labelled oligosaccharides by slightly more concentrated acid and eventually to [^{14}C]arabinose as the only labelled component. Susceptibility to acid hydrolysis, and methylation analysis, indicated that the oligosaccharide contained approximately seven sequential α-1,5-linked arabinofuranosyl units at the non-reducing end. Several acidic residues appear to be interposed between the terminal arabinosyl units and the charged lipid.

Numerous lipid-linked saccharides have been identified as products formed by plant membranes from sugar nucleotides. Those linked to polyprenyl phosphates have been proposed as intermediates in the biosynthesis of glycoproteins (Forsee & Elbein, 1975; Brett & Leloir, 1977; Ericson & Delmer, 1977; Bailey *et al.*, 1979) or polysaccharides (Brett & Northcote, 1975; Hopp *et al.*, 1978; Dürr *et al.*, 1979). All of the saccharides that have been identified to date in such intermediates are hexoses or hexosamines, including polyprenyl monophosphoryl-linked mannose and glucose (Forsee & Elbein, 1973; Pont-Lezica *et al.*, 1975; Delmer *et al.*, 1978; Dürr *et al.*, 1979) and polyprenol pyrophosphoryl-linked *N*-acetylglucosamine, di-*N*-acetylchitobiose and oligosaccharides (Forsee & Elbein, 1975; Lehle *et al.*, 1976; Bailey *et al.*, 1979; Staneloni *et al.*, 1980). These lipid-linked oligosaccharides appear to serve as precursors for asparagine-linked glycoprotein (Bailey *et al.*, 1980; Staneloni *et al.*, 1980), as established in animal tissues (Waechter & Lennarz, 1976). Glycoproteins that bear such oligosaccharides are well known in plant storage proteins (Ericson & Chrispeels, 1973; Bailey *et al.*, 1980) and certain specific plant enzymes and lectins (Lis & Sharon, 1978; Pont-Lezica *et al.*, 1978). The possibility that polyprenol-linked saccharides are also precursors of polysaccharides is more speculative at present, but oligosaccharides have been reported (Brett & Northcote, 1975; Hopp *et al.*, 1978) that resemble cell-wall polysaccharides more closely than they do intracellular glycoproteins. The object of the present study was to investigate the possibility that pentosyl lipids are also formed by plant membranes, since these are potential intermediates for biosynthesis of the many pentose-containing polysaccharides and glycoproteins in plant cell walls (Bailey & Hassid, 1966; Karr, 1972; Odzuck & Kauss, 1972; Dalessandro & Northcote, 1981; Hayashi & Matsuda, 1981; Mascara & Fincher, 1982).

Xylose and arabinose are major components of pectic and hemicellulosic materials in all plants,

Abbreviation used: SDS, sodium dodecyl sulphate.

and they may play important roles in the development of cell walls during growth. There is some evidence that UDP-xylose serves as primary donor for the synthesis of xylan (Bailey & Hassid, 1966; Dalessandro & Northcote, 1981; Bolwell & Northcote, 1983) and xyloglucan (Hayashi & Matsuda, 1981), and UDP-arabinose is precursor for the synthesis of arabinan (Odzuck & Kauss, 1972; Bolwell & Northcote, 1983) and arabinosyl hydroxyproline-rich protein (Karr, 1972). The biosynthetic pathways for other major pentose-containing glycoproteins, e.g. arabinogalactan-protein (Mascara & Fincher, 1982), are not yet known. The present paper deals with the occurrence of pentosyl lipids formed from UDP-[^{14}C]xylose and UDP-[^{14}C]arabinose by pea membranes and their possible roles as intermediates for pentosyl transfer to cell-wall glycoproteins and polysaccharides.

Materials and methods

Materials

UDP-D-[U-^{14}C]xylose (267mCi/mmol), UDP-L-[U-^{14}C]arabinose (183mCi/mmol), dolichyl phosphoryl-[6-^{3}H]glucose (7.3Ci/mmol), Aquasol and Protosol were obtained from New England Nuclear. Unlabelled sugar nucleotides, bovine serum albumin, DEAE-Sepharose CL-6B, Sepharose CL-6B, dolichyl monophosphate, L-α-lysophosphatidylcholine and protein M_r markers (bovine plasma albumin, ovalbumin and trypsinogen) were from Sigma Chemical Co. Standard dextrans (T-500, T-40 and T-10) and Sephadex LH-20 were from Pharmacia, tunicamycin was from E. Lilly Laboratories, silica-gel-60 thin-layer (0.2mm) sheets were from EM Laboratories, Pronase was from Boehringer Mannheim and laminarin (M_r 5300) was from Nutritional Biochem. Co. Labelled polyprenol-linked saccharide standards were prepared from UDP-*N*-acetyl[^{14}C]glucosamine, UDP-[^{14}C]glucose and GDP-[^{14}C]mannose (New England Nuclear) by using pea membranes in accordance with methods described in previous papers (Bailey *et al.*, 1979; Dürr *et al.*, 1979). Authentic methylated arabinosyl derivatives were prepared from arabinan (Koch-Light Laboratories) (Gleeson & Clarke, 1980). Xyloglucan-derived nonasaccharide (glucose/xylose/galactose/fucose, mol. prop. 4:3:1:1) was prepared from pea xyloglucan as described for soya bean (Hayashi & Matsuda, 1981).

Pea membrane preparation

Seeds of *Pisum sativum* var. Alaska were soaked (20min) in 5% (w/v) sodium hypochlorite, washed, allowed to imbibe water (8h), planted in moistened vermiculite and grown in darkness at room temperature for 1 week. The apical 10mm of the third internode (elongating region) was used as source of membrane. Approx. 5g of tissue (400 segments) was washed with cold 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer, pH6.8 and ground in a mortar with 10ml of this buffer containing 1mM-dithiothreitol, 0.1% bovine serum albumin and 0.4M-sucrose. The homogenate was filtered through nylon, centrifuged (1000*g*, 10min), and the supernatant was layered over 1ml of 1.6M-sucrose and centrifuged (200000*g*, 30min). The total endo-membranous material (5–10mg of protein) that collected at the 0.4M/1.6M-sucrose interface was aspirated and suspended in grinding medium, and portions were used as source of enzyme.

Incubation and extraction procedures

Membrane preparation (0.6–1.2mg of protein) in 300μl of 0.1M-Hepes/KOH buffer, pH6.8, containing 1mM-dithiothreitol, 0.1% bovine serum albumin and 0.4M-sucrose was incubated at 25°C for 2min with 10μl (0.75nmol) of UDP-[^{14}C]xylose (267mCi/nmol) or UDP-[^{14}C]arabinose (183mCi/mmol) containing 3μmol of $MnCl_2$ in a total volume of 310μl. Reactions were normally terminated by adding 2ml of chloroform/methanol/water (10:10:3, by vol.). These proportions were found by direct testing to be optimal for extraction of the glycolipids that became labelled with arabinose and xylose, as they are for other glycolipids.

The mixture was incubated at −20°C overnight in order to complete extraction of the lipids. Water (1ml) was added and the mixture was blended (Vortex blender). The phases were separated by centrifugation and the upper layer was discarded. No loss of lipid-linked sugars into this aqueous fraction was observed. The lower layer and interface were washed with ice-cold 50% (v/v) methanol (3×), and 1ml of methanol was added in order to make one phase. The mixture was agitated in an ultrasonic bath at 0°C for 2min and kept at −20°C for 3h. The pellet was removed by centrifugation, and the supernatant was collected. This contains all detectable lipid-linked sugars that are labelled. The pellet was then resuspended and extracted twice with 2% (w/v) SDS containing 4mM-Tris/HCl buffer, pH6.8 and 10% (v/v) glycerol at 85°C for 20min in order to produce fractions referred to as SDS-soluble and SDS-insoluble.

Lipid fractionation

The lipid extract was added to a DEAE-Sepharose CL-6B (acetate form) column (0.5cm × 2cm), which was eluted with 7ml of chloroform/methanol/water (10:10:3) to collect neutral lipids, the same solvent containing 5mM-ammonium formate to collect polyprenol monophosphoryl

saccharides and the same solvent containing 200mM-ammonium formate for more highly charged glycolipids (Rouser *et al.*, 1967). Each extract was placed in a scintillation vial, dried and its radioactivity counted in toluene scintillator. The suitability of DEAE-Sepharose CL-6B (acetate form) for the separation of neutral and charged glycolipids was examined by using radioactive polyprenol monophosphoryl-mannose and polyprenol pyrophosphoryl-*NN'*-diacetylchitobiose, which were synthesized from labelled sugar nucleotides by pea membranes (Bailey *et al.*, 1979; Dürr *et al.*, 1979). The elution profiles of these products from DEAE-Sepharose were exactly the same as that from DEAE-cellulose (Dürr *et al.*, 1979).

Acid hydrolysis

Pentosyl lipids were hydrolysed in 0.01M-trifluoroacetic acid in 50% (v/v) propan-1-ol in a sealed tube at 100°C for 10min. After hydrolysis, the reaction mixture was partitioned by the addition of 0.5ml of chloroform. The aqueous phase was evaporated to dryness to remove trifluoroacetic acid, and the product was dissolved in a small volume of water and subjected to paper chromatography. The residue after lipid extraction (polysaccharides and glycoproteins) was suspended in 90% (v/v) trifluoroacetic acid and kept at room temperature overnight. The suspension was then diluted to a trifluoroacetic acid concentration of 20% and hydrolysed in a sealed tube at 100°C for 1h.

Methylation analysis of lipid-linked oligosaccharide

Radioactive lipid-linked oligosaccharide was methylated by the method of Hakomori (1964) as modified by Sandford & Conrad (1966). The methylated sample was passed through a Sephadex LH-20 column (0.8cm × 20cm) equilibrated in chloroform/methanol (2:1 v/v) in order to remove dimethyl sulphoxide and salts. The radioactive fraction that was eluted in the void volume was collected and dried. The methylated sample was hydrolysed with 45% (v/v) formic acid (Rees & Richardson, 1966), and the hydrolysate was dried to remove formic acid and deionized with Rexyn 101 (H^+ form). For the identification of alditol methyl ethers, the hydrolysate from the methylated oligosaccharide was reduced with $NaBH_4$ at pH10. Excess reducing agent was destroyed by addition of Rexyn 101 (H^+ form), and boric acid was removed by repeated evaporation with methanol.

Electrophoresis

Polyacrylamide-gel electrophoresis of SDS-soluble products was performed on 10% gels containing 0.1% SDS (Laemmli, 1970). The gels were cut out with a razor, and each track of the gels was separated into 0.5cm slices. Gel slices were placed in scintillation vials containing 3% Protosol with toluene scintillator. The mixture was incubated at 37°C for 24h in order to extract completely the radioactivity from the gel (Floyd *et al.*, 1974). Gels with protein standards were stained for 2h in a solution of 0.1% Coomassie Blue in 45% methanol/5% acetic acid and then destained in 7% acetic acid/30% methanol.

In order to characterize further the properties of electrophoretically mobile and immobile labelled products, the appropriate gel regions were extracted with 0.1% SDS, and the eluates were precipitated and washed with 80% (v/v) acetone. They were suspended in 0.1M-NaOH and fractionated on a Sepharose CL-6B column (1.0cm × 100cm) with 0.1M-NaOH as eluent. The column was calibrated with dextrans of known M_r.

General methods

T.l.c. was performed on silica-gel-60 plates (0.2mm) with chloroform/methanol/water (65:25:4, by vol.) for lipid-linked sugars and with butan-2-one/water (85:7, v/v) for partially methylated sugars (Williams & Jones, 1967). Paper chromatography was performed with Whatman 3MM paper with ethanol/butan-2-one/0.5M-morpholinium tetraborate (7:2:3, by vol.), pH8.6, in 0.1M-EDTA for sugar nucleotides (Carminatti *et al.*, 1965) and with butan-1-ol/pyridine/water (6:4:3, by vol.) for pentoses. Standard sugars were detected with the $AgNO_3$ reagent (Robyt & French, 1963). Radioactive samples were detected by scanning paper strips on a Packard model 7200 radiochromatogram scanner. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Carbohydrate was determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956). Radioactivity was determined with a Beckman CPM-100 liquid-scintillation spectrometer, with Aquasol for aqueous samples and toluene scintillator for lipid samples. Radioautography of chromatographs utilized Dupont Cronex-4 X-ray film.

Results

Formation of pentosyl lipids

After incubation for 2min with UDP-[^{14}C]xylose, membrane preparations from pea epicotyls were extracted with 70% (v/v) methanol and the extracts were subjected to paper chromatography. UDP-[^{14}C]arabinose was formed from UDP-[^{14}C]xylose with an equilibrium of approximately 1:1, confirming the presence of an active membrane-bound epimerase for the substrates (Feingold *et al.*, 1960). Whichever substrate was applied, the other was available within seconds in the preparation.

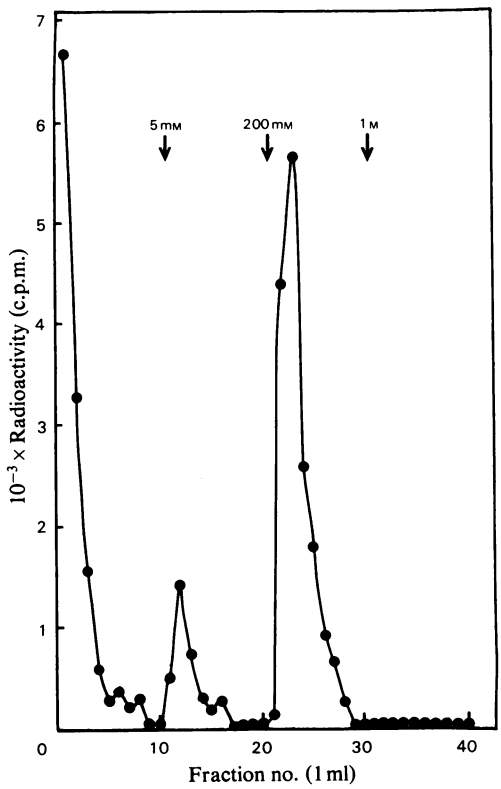

Fig. 1. *DEAE-Sepharose chromatography of labelled pentosyl lipids formed from UDP-[^{14}C]xylose*
The column was eluted with chloroform/methanol-/water (10:10:3, by vol.), without or with ammonium formate at the concentrations indicated. For full details see the text.

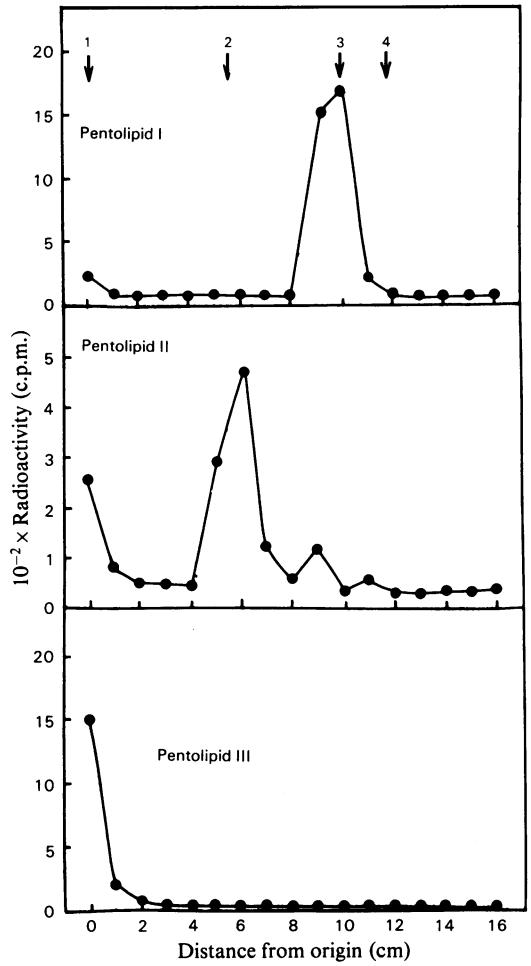

Fig. 2. *Thin-layer chromatograms of pentosyl lipids*
Pentolipid I was the fraction from DEAE-Sepharose columns (fraction nos. 1–5, Fig. 1) that was eluted with chloroform/methanol/water, pentolipid II (fraction nos. 11–15) with chloroform/methanol/water containing 5mM salt and pentolipid III (fraction nos. 21–28) with chloroform/methanol/water containing 200mM salt. For full details see the text. Numbers represent the location on chromatograms of products formed by pea membranes from UDP-[^{14}C]glucose (Dürr *et al.*, 1979): 1, lipid-linked oligosaccharides; 2, dolichyl phosphoryl-glucose; 3, steryl glucoside; 4, acylated steryl glucoside.

Membrane preparations synthesized many other products from UDP-[^{14}C]xylose, some of which were either soluble in chloroform/methanol (1:1) or in a subsequent extract with chloroform/methanol/water (10:10:3). Radioactivity continued to accumulate in products soluble in chloroform/methanol for at least 60min, but it increased for about 20min and then declined in products soluble in chloroform/methanol/water. Since chloroform/methanol dissolves neutral and weakly charged lipids, whereas chloroform/methanol/water dissolves more highly charged and hydrophilic lipids (Chambers *et al.*, 1977), only the latter appear to be subject to turnover in this preparation. In most subsequent experiments, chloroform / methanol / water (10:10:3) was employed as the solvent of choice for extraction of total glycolipid.

A large-scale reaction was prepared with 5μCi of [^{14}C]xylose as substrate, and the products soluble in lipid solvent were subject to DEAE-Sepharose chromatography. Fig. 1 shows an elution profile with three types of pentolipid corresponding to neutral (I), moderately charged (II) and highly charged glycolipids (III). The recovery was 93% of ^{14}C applied to the column. Fig. 2 shows the profile of these three pentolipids on silica-gel thin-layer chromatographs. Both fractionation methods indicate that pentolipids I and III are the major products.

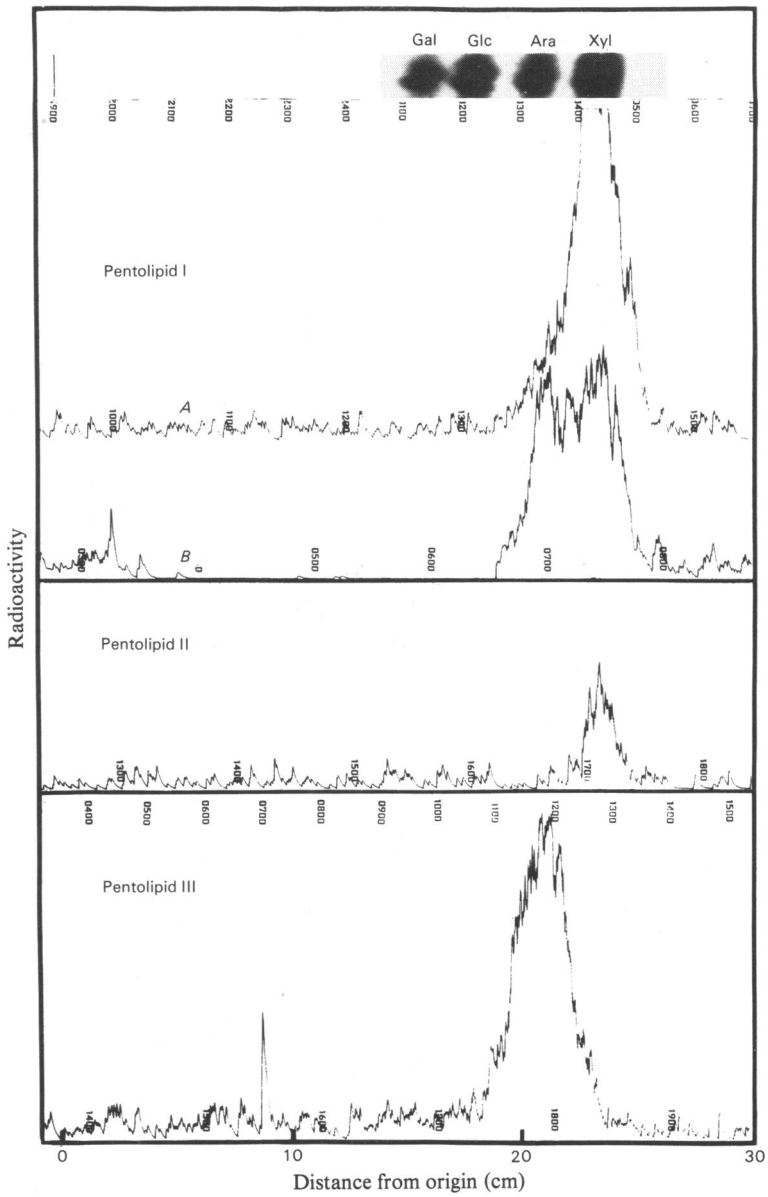

Fig. 3. *Paper chromatography of the sugar moieties of pentolipids I–III (Fig. 2)*
Trace *A* in the top panel shows the sugar moiety of pentolipid I synthesized from UDP-[^{14}C]xylose, and trace *B* indicates that formed from UDP-[^{14}C]arabinose. The sugar moieties from pentolipids II and III (lower panels) were products synthesized from either substrate. For full details see the text.

The neutral pentolipid I (Fig. 1) showed a chromatographic mobility (silica gel) similar to that of authentic steryl glycoside (Fig. 2), and yielded no free sugar on mild acid hydrolysis. Hydrolysis with more-concentrated acid (1M-trifluoroacetic acid) generated labelled xylose and some arabinose (Fig. 3, trace *A* in top panel). A similar product was also synthesized from UDP-[^{14}C]arabinose, and this, on hydrolysis with the more-concentrated acid, yielded a 1:1 mixture labelled xylose and arabinose (Fig. 3, trace *B* in top panel).

The pentolipid II, which was eluted from DEAE-Sepharose in chloroform/methanol/water containing 5mM salt (Fig. 1), co-chromatographed (silica-gel t.l.c.) with dolichyl monophosphoryl-glucose (Fig. 2). When it was subjected to gel filtration on a column (0.8cm × 20cm) of Sephadex LH-

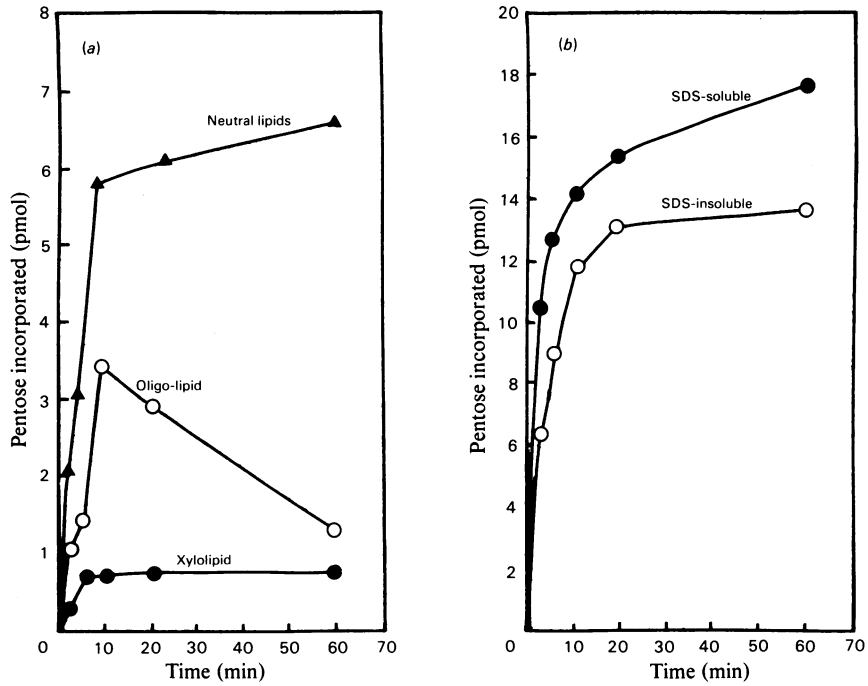

Fig. 4. *Time course for synthesis from UDP-xylose of pentosyl lipids and products soluble and insoluble in SDS*
For full details see the text. Oligo-lipid indicates lipid-linked arabinosyl oligosaccharide.

20 equilibrated in chloroform/methanol/water, a single, symmetrical, peak of radioactivity was eluted from the column in the same fraction as dolichyl monophosphoryl-glucose (results not shown). The sugar moiety was very susceptible to acid hydrolysis, i.e. in 0.01 M-trifluoroacetic acid (pH 2) at 100°C all of the radioactivity became water-soluble in less than 10 min. When the aqueous phase from the hydrolysate was concentrated to dryness to remove the acid and subjected to paper chromatography, the label migrated with xylose (Fig. 3). The same product was formed from UDP-[^{14}C]arabinose (results not shown). Accordingly, pentolipid II is lipid-linked xylose that is moderately charged in a manner analogous to polyisoprenyl monophosphoryl derivatives.

The pentolipid III, which required high salt concentration (200 mM) to be eluted from DEAE-Sepharose (Fig. 1), did not migrate on silica-gel thin-layer chromatographs (Fig. 2), was eluted in the void volume of a Sephadex LH-20 column and contained radioactive arabinose only (Fig. 3). These properties indicate a lipid-linked arabinosyl oligosaccharide that is highly charged.

Kinetics and requirements for pentose incorporation into various products

The time course for formation of pentosyl lipids from UDP-[^{14}C]xylose is shown in Fig. 4(*a*). Incorporation into neutral lipids and xylolipid proceeded rapidly for only 5–10 min, after which a steady state was maintained for at least 1 h. Incorporation into lipid-linked arabinosyl oligosaccharide increased for 10 min and then decreased. The fall in radioactivity in the charged oligolipid fraction could be accounted for by an increase in label soluble in 2% SDS and/or the SDS-insoluble fraction (Fig. 4*b*). The major labelled sugar in both non-lipid fractions was arabinose, with almost all of the [^{14}C]xylose in the SDS-insoluble fraction.

Lineweaver–Burk plots for pentolipid synthesis from UDP-xylose yielded identical apparent K_m values (8 μM) for the formation of neutral lipid, xylolipid and lipid-linked arabinosyl oligosaccharide. In contrast, apparent K_m values for the formation of SDS-soluble and SDS-insoluble materials were 20 μM and 30 μM respectively, suggesting that enzymes forming these products have a lower affinity for their substrates than those forming glycolipids.

Maximal incorporation from UDP-[^{14}C]xylose into the three major lipid fractions and non-lipid products was attained with broad pH optima between 6.0 and 7.5 in Hepes/KOH buffer. When Mg^{2+} or Mn^{2+} was added to the reaction mixture, the initial rate of incorporation was highest with Mn^{2+} in all fractions except neutral lipids (Table 1). Synthesis of the xylolipid (II) and lipid-linked

arabinosyl oligosaccharide (III) was not stimulated by addition of dolichol phosphate in the presence of 0.025% lysophosphatidylcholine under conditions where greatly enhanced incorporation was observed from UDP-glucose and GDP-mannose (Pont-Lezica *et al.*, 1975; Brett & Leloir, 1977; Dürr *et al.*, 1979). A number of other sugar nucleotides (1 mM) were examined for their ability to stimulate or inhibit incorporation from UDP-[^{14}C]xylose (2.5 μM) into pentosyl lipids. Added UDP-glucose and UDP-galactose decreased the amounts at 2 min of xylolipid (by 72% and 79% respectively), and also of lipid-linked arabinosyl oligosaccharide (by 63% and 37% respectively). No inhibition was observed with tunicamycin at a concentration (330 μg/mixture) about 70 times that found to be effective in systems that utilize UDP-*N*-acetylglucosamine (Ericson *et al.*, 1977), nor were UDP-*N*-acetylglucosamine and GDP-mannose directly inhibitory.

Membranes derived from all parts of the epicotyl, i.e. plumule and hook, elongating and maturing regions, were able to form the main types of pentosyl products examined, with apical tissue being particularly effective at synthesizing neutral lipids (Fig. 5*a*). The total incorporation into products that were insoluble in lipid solvents was greater than into pentosyl lipids, particularly in more basal mature tissues (Fig. 5*b*). The xylose/arabinose ratio also increased in these insoluble products in the more basal tissues.

Table 1. *Effect of Mg^{2+} or Mn^{2+} on the formation of pentosyl lipids and polymer from UDP-[^{14}C]xylose*
The conditions were the same as those of the standard assay indicated in the text, except that metal ions were absent or added as indicated. Oligolipid indicates lipid-linked arabinosyl oligosaccharide.

	[^{14}C]Pentose incorporated (pmol)		
Products	No ions	Mg^{2+} (10 mM)	Mn^{2+} (10 mM)
Neutral lipid	0.38	0.84	0.19
Xylolipid	0.05	0.11	0.68
Oligo-lipid	0.19	0.68	1.80
SDS-soluble	2.13	3.29	13.08
SDS-insoluble	1.42	4.35	8.72

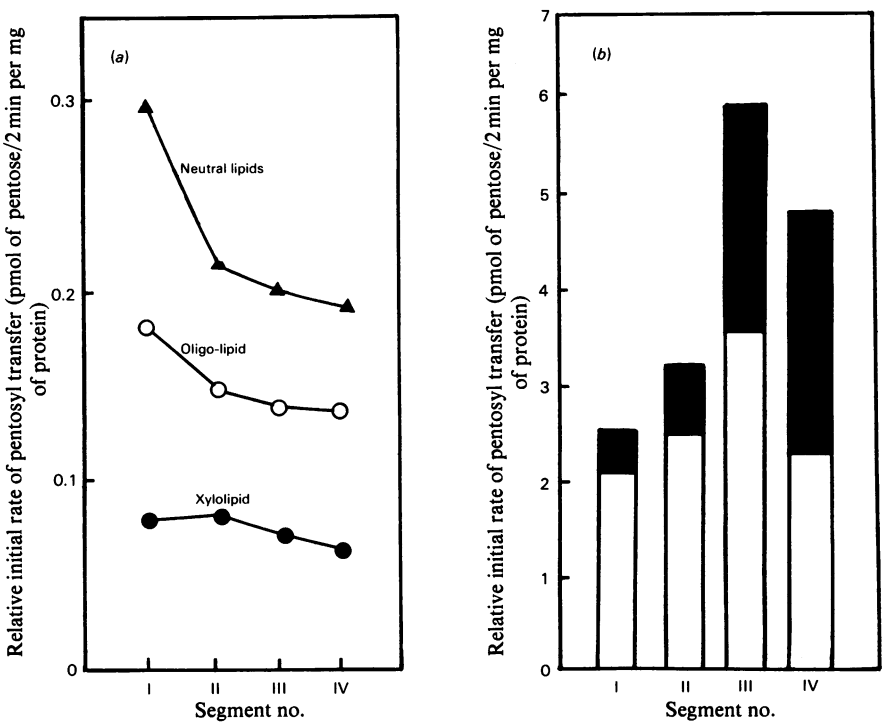

Fig. 5. *Formation of pentosyl lipids and insoluble products from UDP-xylose by membranes from different tissues*
Segment I represents the plumule-plus-hook region, and segments II to IV are successive 1 cm sections of stem from the apex. (*a*) shows relative incorporation into the three pentolipids per unit of membrane protein, and (*b*) shows the amount of recovered xylose (■) and arabinose (□) in total non-lipid products. For full details see the text. Essentially all of the xylose was derived from SDS-insoluble fractions.

Table 2. *Distribution of radioactivity in products formed from lipid-linked [^{14}C]arabinosyl oligosaccharide*

The incubation mixture contained lipid-linked [^{14}C]arabinosyl oligosaccharide (2.5×10^4 c.p.m., suspended in 60 μl of 0.25% lysophosphatidylcholine), 2 μM-UDP-xylose, 10 mM-$MnCl_2$, 0.4 M-sucrose, 1 mM-dithiothreitol, 0.1 M-Hepes/KOH buffer, pH 6.8, and pea membrane preparations in a final volume of 620 μl. The mixture was incubated at 25°C for 40 min, and the reaction was terminated by addition of 1.25 ml of chloroform/methanol (1 : 1, v/v). Successive extraction with chloroform/methanol (2 : 1, v/v) and chloroform/methanol/water (10 : 10 : 3, by vol.) was carried out (Chambers *et al.*, 1977). After removal of lipids, the polymer fraction was solubilized in 2% SDS containing 4 mM-Tris/HCl buffer, pH 6.8, and 10% glycerol at 85°C for 20 min, and the insoluble fraction was removed by centrifugation.

Fraction	Total radioactivity (c.p.m.)
Chloroform/methanol (2 : 1)	1220 (5%)
50-70% methanol	3800 (16%)
Chloroform/methanol/water	8460 (36%)
2% SDS	4100 (18%)
Insoluble residue	5880 (25%)

Properties of non-lipid products

Lipid-linked [^{14}C]arabinosyl oligosaccharide formed from UDP-[^{14}C]xylose was purified by chromatography on DEAE-Sepharose and Sephadex LH-20, suspended in 0.25% lysophosphatidylcholine and provided back to pea membranes as a substrate. Over 40% of total radioactivity was incorporated into SDS-soluble and SDS-insoluble fractions (Table 2). Hydrolysis of these fractions with acid yielded only labelled arabinose on paper chromatography, in contrast with the mixture of xylose and arabinose found when UDP-xylose was the substrate (Fig. 5*b*).

The SDS-soluble products formed from lipid-linked [^{14}C]arabinosyl oligosaccharide were subjected to SDS-polyacrylamide-gel electrophoresis, and the profile of ^{14}C distribution was determined (Fig. 6*a*). This is compared with the profile of SDS-soluble material that formed from UDP-[^{14}C]xylose (Fig. 6*b*). Both substrates yielded products that were electrophoretically immobile, as were the SDS-insoluble products, which may represent incorporation into polysaccharide. Both substrates were also incorporated into mobile products that travelled a few centimetres into the gel. These components contained [^{14}C]arabinose as the only labelled sugar. Radioactivity from UDP-xylose but not the lipid-linked oligosaccharide was also incorporated into a major [^{14}C]xylose-containing product with mobility equivalent to an M_r of approx. 45000.

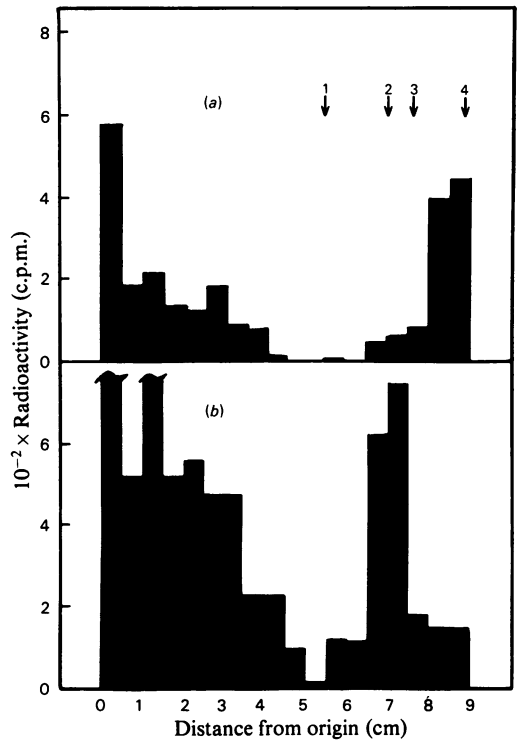

Fig. 6. *Polyacrylamide-gel electrophoresis of SDS-soluble products formed from lipid-linked [^{14}C]arabinosyl oligosaccharide (a) and UDP-xylose (b)*

For full details see the text. Arrows represent the positions of markers (M_r): 1, bovine serum albumin (66000); 2, ovalbumin (45000); 3, trypsinogen (24000); 4, Bromophenol Blue. The product that migrated with Bromophenol Blue in (*a*) was free arabinosyl oligosaccharide. UDP-[^{14}C]arabinose was also used to form products with a profile similar to that shown in (*b*)

The electrophoretically mobile and immobile components were subjected to gel filtration (Sepharose CL-6B) to yield profiles shown in Fig. 7. The immobile (0–1 cm) material (Fig. 6*b*) was polydispersed with a size range from an M_r equivalent to 40000 to the void volume (Fig. 7*a*). The adjacent diffuse mobile components (1–4 cm into gel; Fig. 6*b*) appear to be a more homogeneous product with a mean M_r of approx. 40000 (Fig. 7*b*). Although these products were evidently ionic (negatively charged), neither was degraded further by treatment with proteinase (5 mg of Pronase, 37°C, 72 h). The most electrophoretically mobile peak formed only from UDP-xylose (6.5–7.5 cm; Fig. 6*b*) contained two peaks on gel filtration (Fig. 7*c*), with mean M_r values equivalent to approx. 50000 and several hundred thousand.

None of the components separated by electrophoresis were degraded in such a way as to lose

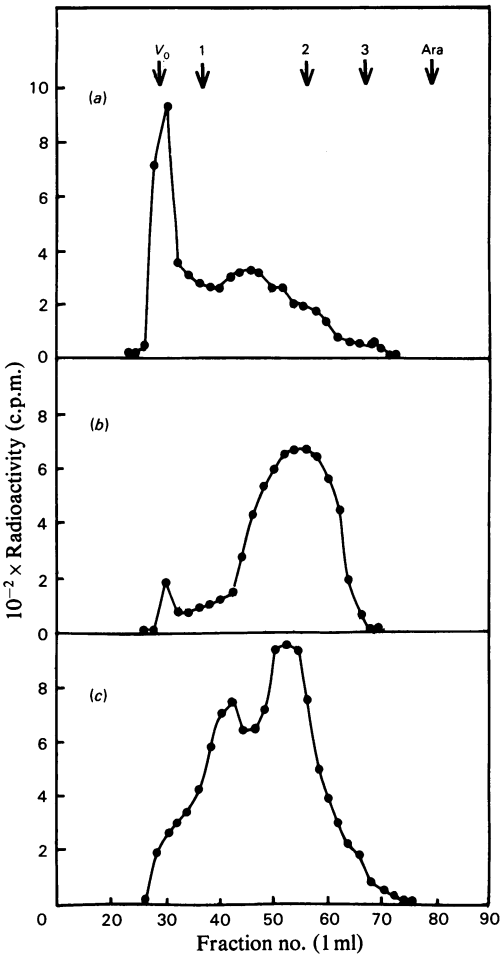

Fig. 7. *Gel filtration on Sepharose CL-6B of electrophoretically mobile and immobile components of SDS-soluble fraction*

For full details see the text. (*a*) Immobile component (origin); (*b*) mobile component (1.5–4.5cm from origin); (*c*) highly mobile component (6.5–7.5cm from origin). Arrows represent elution position of standards (M_r): V_0, Blue Dextran; 1, dextran T-500 (500000); 2, dextran T-40 (40000); 3, dextran T-10 (10400).

label or decrease in size when they were pretreated with 2M-NaOH at 25°C for 9h, indicating that none was serine- or threonine-linked glycoprotein. However, the [^{14}C]arabinose-containing products (Fig. 7*b*) were all very readily hydrolysed by dilute acid (0.05M-trifluoroacetic acid, 100°C, 15min). Fig. 8 shows paper chromatograms of the partial hydrolysates with degradation profiles all closely resembling the profile obtained by treating the lipid-linked oligosaccharide with dilute acid (Fig. 8*a*).

Properties of lipid-linked arabinosyl oligosaccharide

This glycolipid was very labile in dilute acid but stable in dilute alakli at 37°C for 15min. Treatment with 0.1M-NaOH at 70°C released a water-soluble product that bound firmly to Dowex 1 (Cl^- form) and was eluted with NaCl. These properties are characteristic of saccharides linked to pyrophosphates (Herscovics *et al.*, 1974). The allylic polyprenyl phosphates (e.g. bactoprenols, ficaprenols) are known to be degraded by phenol treatment, whereas saturated α-isoprenyl phosphates (dolichols) are stable (Garcia *et al.*, 1974). The lipid-linked arabinosyl oligosaccharide was rendered water-soluble by a 60min heating in aq. 50% (w/v) phenol. The radioactivity that was released into the aqueous phase bound to Dowex 1 (Cl^- form) and was eluted with NaCl. The sum of these properties is consistent with labelled saccharide attached to an unsaturated polyprenyl pyrophosphoryl derivative.

Controlled degradation of the lipid-linked oligosaccharide was carried out with dilute acid, and elution profiles of the hydrolysates on Bio-Gel P-2 columns (1.5cm × 90cm) are shown in Fig. 9. Very mild acid hydrolysis yielded an oligosaccharide (Fig. 9*a*) that was eluted in the void volumes of Bio-Gel P-2 to P-30 and Sephadex G-75. The free oligosaccharide was not adsorbed on a column of Dowex 1 (Cl^- form) or Dowex 50W (H^+ form), although it bound firmly to DEAE-Sephadex and DEAE-Sepharose (phosphate form) (properties also shown by pectic substances). It was negatively charged, as indicated by its high mobility on SDS/polyacrylamide-gel electrophoresis, i.e. see component that travelled with Bromophenol Blue in SDS-soluble products formed from lipid-linked arabinosyl oligosaccharide (Fig. 6*a*). It is probably this charge that resulted in its elimination from the gel pores of Bio-Gel and Sephadex. The oligosaccharide was still eluted in the void volume of Bio-Gel columns in acidic buffers (pH4–4.5) or after treatment with phosphatase. It could be fractionated in 0.1M-NaOH on a calibrated column (1.0cm × 100cm) of Sepharose CL-6B, where it was eluted with an apparent M_r of 2300 (Fig. 10). Hydrolysis with more-concentrated acid released labelled arabinose, a disaccharide and a series of higher oligosaccharides (Figs. 8*a* and 9*b*). Total hydrolysis to labelled arabinose was obtained in 0.1M-trifluoroacetic acid at 100°C for 30min (Fig. 9*c*). This hydrolysis pattern is characteristic of arabinans containing furanosyl α-linkages (Hirst & Jones, 1939).

Reduced oligosaccharide was prepared from the glycolipid by mild acid hydrolysis and treatment of the void component from Bio-Gel columns with $NaBH_4$ at pH10. The reduction product was

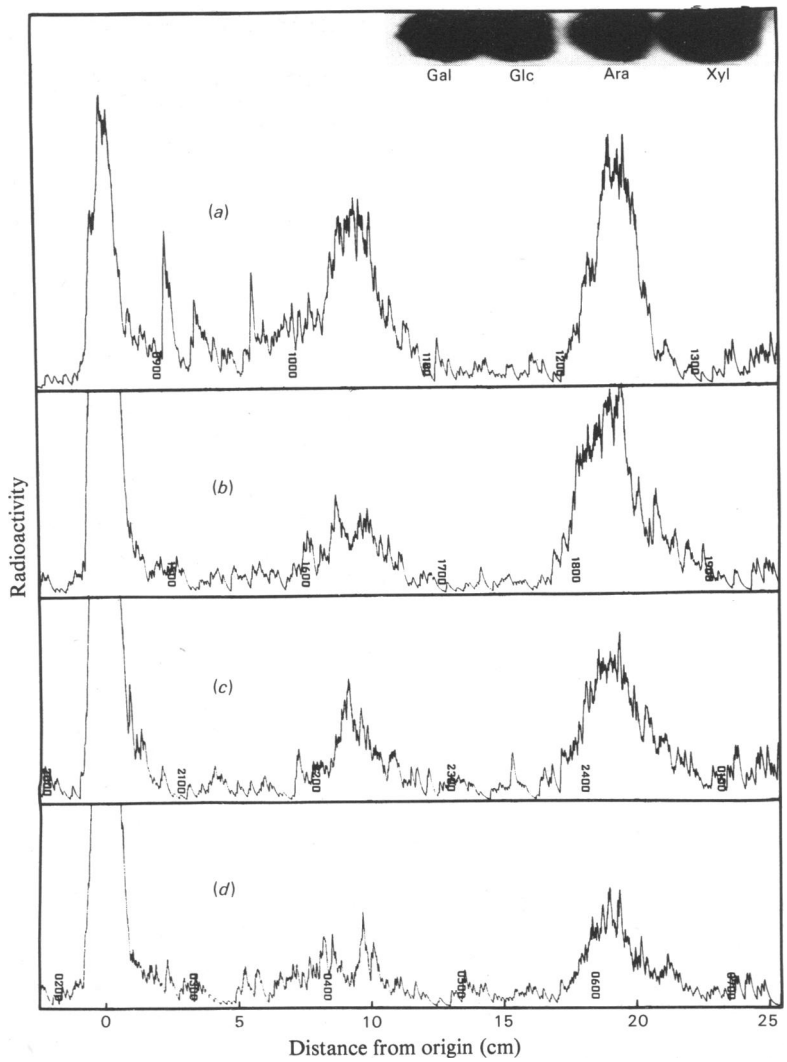

Fig. 8. *Paper chromatography of partial acid hydrolysates of lipid-linked arabinosyl oligosaccharide and polymeric products* For full details see the text. Partial acid hydrolysate of (*a*) lipid-linked arabinosyl oligosaccharide; (*b*) electrophoretically mobile components (see Fig. 7*b*); (*c*) immobile components (Fig. 7*c*); (*d*) SDS-insoluble products.

totally hydrolysed in 1 M-trifluoroacetic acid at 100°C for 90 min, and the hydrolysate was chromatographed on a Bio-Gel P-2 column. A radioactive peak corresponding to [^{14}C]arabinose was obtained, but no trace of [^{14}C]arabinitol, indicating that the incorporated arabinosyl residues were not located at the reducing end of the oligosaccharide. When NaB^3H_4 was employed as the reducing agent, the main labelled monosaccharide alcohol in acid hydrolysates was [^{3}H]galactinol, but it was a minor labelled component. About 90% of ^{3}H remained in an immobile product at the origin of chromatographs, and has not been identified.

The lipid-linked oligosaccharide was subjected to methylation analysis, and the radioactive profile yielded 2,3,5-tri-*O*-methylarabinose and 2,3-di-*O*-methylarabinose in a ratio of 1:6 (Fig. 11*a*). When these derivatives were reduced with borohydride, they chromatographed with the authentic arabinitol equivalents (Fig. 11*b*). From these results, the oligosaccharide appears to contain about seven (1→5)-linked arabinofuranosyl residues at the non-reducing end of a product that also contains other unlabelled saccharides and acidic groups.

Discussion

These results demonstrate that pea membranes contain a very active UDP-arabinose 4-epimerase

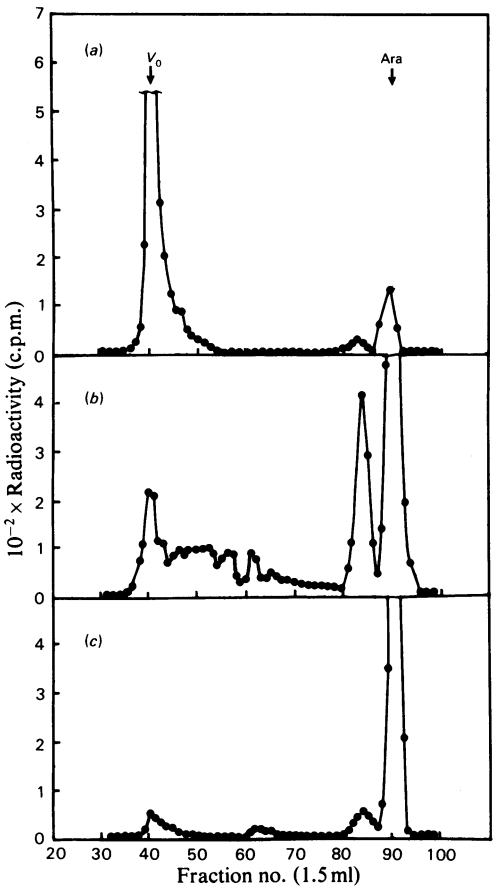

Fig. 9. *Controlled degradation of lipid-linked arabinosyl oligosaccharide by mild acid hydrolysis in 50% propan-1-ol*
Trifluoroacetic acid was added at 100°C as follows: (*a*) 0.01 M, for 10 min; (*b*) 0.05 M, for 15 min; (*c*) 0.1 M, for 10 min. V_0 was determined with Blue Dextran. For full details see the text.

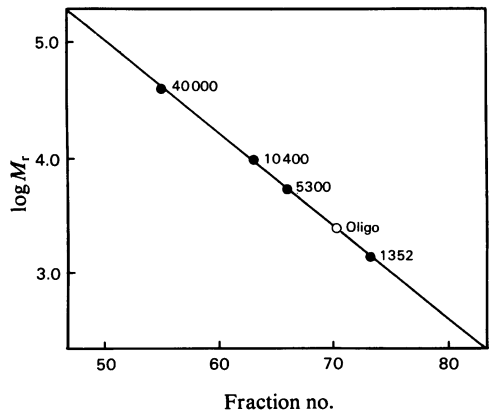

Fig. 10. *Fractionation of [^{14}C]arabinosyl oligosaccharide (Oligo) on Sepharose CL-6B*
For full details see the text. M_r markers: dextran T-40 (40000) and T-10 (10000); laminarin (5300); xyloglucan-derived nonasaccharide (1350). Arabinose was eluted in tube no. 78.

(EC 5.1.3.5) (Feingold *et al.*, 1960), so that both arabinose and xylose derivatives are available as substrates whichever pentose nucleotide is supplied. The membranes also contain transferases required to form steryl arabinoside, steryl xyloside and a product with properties consistent with polyprenyl monophosphoryl-xylose (Figs. 1–3), all of which accumulate in the preparation with time (Fig. 4). There was no indication in the present experiments that these glycolipids were precursors for the non-lipid products also formed in the preparations. There are no other reports of the synthesis of steryl pentosides from any source, but dolichyl monophosphoryl-xylose synthesis has been demonstrated with hen oviduct membranes (Waechter *et al.*, 1974; Faltynek *et al.*, 1982).

A highly charged lipid-linked arabinosyl oligosaccharide is also formed in the preparations (Figs.

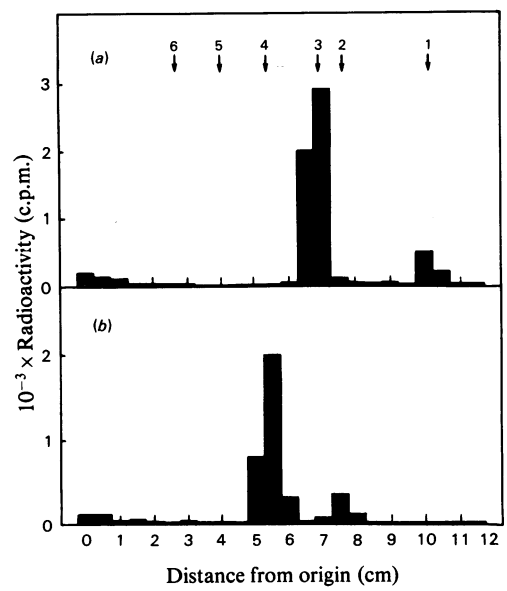

Fig. 11. *Thin-layer chromatogram of radioactive methylated products (a) and reduced methylated products (b) derived from lipid-linked arabinosyl oligosaccharide*
The positions of the following standards are indicated by the arrows: 1, 2,3,5-tri-*O*-methylarabinose; 2, 2,3,5- and, 2,3,4-tri-*O*-methylarabinitols; 3, 2,3,4-tri- and 2,3-di-*O*-methylarabinose; 4, 2,3-di-*O*-methylarabinitol; 5, 2-*O*-methylarabinose; 6, 2-*O*-methylarabinitol. Details of the experiments are given in the Materials and methods section.

1, 2, 8 and 9), with kinetics (Fig. 4) that indicate its turnover *in vitro*. After 2min incubation, this product contains about seven α-1,5-arabinofuranosyl groups at the non-reducing end of a larger oligosaccharide (Figs. 8 and 9), which itself is charged (Fig. 6). The existence of this charge introduced difficulties in the determination of a precise M_r for the whole oligosaccharide, but fractionation data (Fig. 10) are consistent with about 15 sugar units. The oligosaccharide appears to be attached by pyrophosphate to a lipid with properties resembling unsaturated polyprenol. This is the first report of pentose-containing lipid-linked oligosaccharide.

This study has not identified other substituents that must be present in the endogenous acceptor for formation of the lipid-linked arabinosyl oligosaccharide. Nor is it known whether arabinosyl transfer continues with time to assemble an intermediate with more than seven arabinose units. However, the fact that the lipid-linked oligosaccharide that formed in 2min can act as a precursor for polymeric materials is shown by observations that UDP-[^{14}C]arabinose and lipid-linked [^{14}C]arabinosyl oligosaccharide are all incorporated into non-lipid products containing [^{14}C]arabinose. Some are soluble in SDS and mobile or immobile on polyacrylamide-gel electrophoresis (Table 2 and Fig. 6), some are SDS-insoluble and electrophoretically immobile. All of the arabinose-containing polymeric products are susceptible to hydrolysis by dilute acid, yielding profiles that are the same as those obtained with the arabinosyl oligosaccharide (Fig. 8).

Structures containing several α-1,5-arabinofuranosyl groups have been reported in certain arabinans (Gould *et al.*, 1965), arabinogalactan-proteins (Yamagishi *et al.*, 1976), pectic substances (Talmadge *et al.*, 1973) and arabinan–cellulose conjugates (Misaki *et al.*, 1982). Repeating units are suspected to occur in many of these cell-wall products. Clearly, it is necessary to analyse and compare the pentosyl and non-pentosyl components of these products closely in order to determine whether the degree of homology between them that appears to exist (Fig. 8), and their potential precursor–product relationships (Fig. 4), are realities. It would appear to be legitimate, as a working hypothesis, to extend the concept of transfer *en bloc* from a lipid-linked oligosaccharide to a polymeric acceptor from the well-established mechanism for synthesis of *N*-linked glycoproteins to the synthesis of arabinose-containing polymers, which could include heteropolysaccharides in instances where repeating units are known to occur.

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References

Bailey, R. W. & Hassid, W. Z. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1586–1593

Bailey, D. S., Dürr, M., Burke, J. & Maclachlan, G. (1979) *J. Supramol. Struct.* **11**, 123–138

Bailey, D. S., Deluca, V., Dürr, M., Verma, D. P. S. & Maclachlan, G. A. (1980) *Plant Physiol.* **66**, 1113–1118

Bolwell, G. P. & Northcote, D. H. (1983) *Biochem. J.* **210**, 497–507

Brett, C. T. & Northcote, D. H. (1975) *Biochem. J.* **148**, 107–117

Brett, C. T. & Leloir, L. F. (1977) *Biochem. J.* **161**, 93–101

Carminatti, H., Passeron, S., Dankert, M. & Recondo, E. D. (1965) *J. Chromatogr.* **18**, 342–348

Chambers, J., Forsee, W. T. & Elbein, A. D. (1977) *J. Biol. Chem.* **252**, 2498–2506

Dalessandro, G. & Northcote, D. H. (1981) *Planta* **151**, 61–67

Delmer, D. P., Kulow, C. & Ericson, M. C. (1978) *Plant Physiol.* **61**, 25–29

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356

Dürr, M., Bailey, D. S. & Maclachlan, G. (1979) *Eur. J. Biochem.* **97**, 445–453

Ericson, M. C. & Chrispeels, M. J. (1973) *Plant Physiol.* **52**, 98–104

Ericson, M. C. & Delmer, D. P. (1977) *Plant Physiol.* **59**, 341–347

Ericson, M. C., Gafford, J. T. & Elbein, A. D. (1977) *J. Biol. Chem.* **252**, 7431–7433

Faltynek, C. R., Silbert, J. E. & Hof, L. (1982) *J. Biol. Chem.* **257**, 5490–5495

Feingold, D. S., Neufeld, E. F. & Hassid, W. Z. (1960) *J. Biol. Chem.* **235**, 910–913

Floyd, R. W., Stone, M. P. & Joklil, W. K. (1974) *Anal. Biochem.* **59**, 599–609

Forsee, W. T. & Elbein, A. D. (1973) *J. Biol. Chem.* **248**, 2858–2867

Forsee, W. T. & Elbein, A. D. (1975) *J. Biol. Chem.* **250**, 9283–9293

Garcia, R. C., Recondo, E. & Dankert, M. (1974) *Eur. J. Biochem.* **43**, 93–105

Gleeson, P. A. & Clarke, A. E. (1980) *Biochem. J.* **191**, 437–447

Gould, S. E. B., Rees, D. A., Richardson, N. G. & Steele, I. W. (1965) *Nature (London)* **208**, 876–878

Hakomori, S. (1964) *J. Biochem. (Tokyo)* **55**, 205–208

Hayashi, T. & Matsuda, K. (1981) *J. Biol. Chem.* **256**, 11117–11122

Herscovics, A., Warren, C. D., Jeanloz, R. W., Wedgwood, J. F., Liu, I. Y. & Strominger, J. L. (1974) *FEBS Lett.* **45**, 312–317

Hirst, E. L. & Jones, J. K. N. (1939) *J. Chem. Soc.* 496–499

Hopp, H. E., Romero, P. A., Daleo, G. R. & Pont-Lezica, R. (1978) *Eur. J. Biochem.* **84**, 561–571
Karr, A. L., Jr. (1972) *Plant Physiol.* **50**, 275–282
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Lehle, L., Fartaczek, F., Tanner, W. & Kauss, H. (1976) *Arch. Biochem. Biophys.* **175**, 419–426
Lis, H. & Sharon, N. (1978) *J. Biol. Chem.* **253**, 3468–3476
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mascara, T. & Fincher, G. B. (1982) *Aust. J. Plant Physiol.* **9**, 31–45
Misaki, A., Nakamura, S. & Ohtani, K. (1982) *Proc. Int. Carbohydr. Symp. 11th* **3**, 20
Odzuck, W. & Kauss, H. (1972) *Phytochemistry* **11**, 2489–2494
Pont-Lezica, R., Brett, C. T., Romero, P. M. & Dankert, M. A. (1975) *Biochem. Biophys. Res. Commun.* **66**, 980–987
Pont-Lezica, R., Romero, P. A. & Hopp, H. E. (1978) *Planta* **140**, 177–183
Rees, D. A. & Richardson, N. G. (1966) *Biochemistry* **5**, 3099–3107
Robyt, J. F. & French, D. (1963) *Arch. Biochem. Biophys.* **100**, 451–467
Rouser, G., Kritchevsky, C. & Yamamoto, A. (1967) in *Lipid Chromatographic Analysis* (Marinetti, G. V., ed.), pp. 99–161, Marcel Dekker, New York
Sandford, P. A. & Conrad, H. E. (1966) *Biochemistry* **5**, 1508–1517
Staneloni, R. J., Tolmasky, M. E., Petriella, C., Ugalde, R. A. & Leloir, L. F. (1980) *Biochem. J.* **191**, 257–260
Talmadge, K. W., Keegstra, K., Bauer, W. D. & Albersheim, P. (1973) *Plant Physiol.* **51**, 158–173
Waechter, C. J., Lucas, J. J. & Lennarz, W. J. (1974) *Biochem. Biophys. Res. Commun.* **56**, 343–350
Waechter, C. J. & Lennarz, W. J. (1976) *Annu. Rev. Biochem.* **45**, 95–112
Williams, S. C. & Jones, J. K. N. (1967) *Can. J. Chem.* **45**, 275–290
Yamagishi, T., Matsuda, K. & Watanabe, T. (1976) *Carbohydr. Res.* **50**, 63–74