The Formation of Oligoglucans Linked to Lipid during Synthesis of β -Glucan by Characterized Membrane Fractions Isolated from Peas

By CHRISTOPHER T. BRETT and D. H. NORTHCOTE

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

(Received 6 November 1974)

Membrane fractions were obtained from pea roots by using a method that permitted the isolation of a fraction rich in relatively intact dictyosome stacks. No chemical fixatives were used. The method involved incubation of the roots with cellulase, followed by gentle homogenization and sucrose-density-gradient fractionation of the homogenate. The fractions were characterized by electron microscopy. All fractions were enzymically active in incorporating glucose from UDP-glucose into water-insoluble glycolipids containing both single glucose residues and glucose oligosaccharides. Some or all of the linkages of glucose to lipid were through phosphate esters. A substance containing glucose oligosaccharides attached to or very strongly adsorbed on to protein was also formed. The membrane fractions also incorporated glucose from UDP-glucose into alkali-soluble and alkali-insoluble β -glucans, which like the oligosaccharides contained β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages. The distribution of the enzymic activities and the chemical properties of the lipid-linked and protein-linked oligosaccharides suggest that they may be intermediates in β -glucan synthesis. The synthetic activity is associated with smooth-membrane vesicles which may be derived from the plasma membrane.

β -Glucan synthesis in plants is catalysed by membrane-bound enzymes, which use UDP-glucose and GDP-glucose as substrates. Incubation of membrane preparations *in vitro* with radioactively labelled UDP-glucose and GDP-glucose has shown that glucose is incorporated from UDP-glucose into β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucans (Feingold *et al.*, 1958; Brummond & Gibbons, 1964; Péaud-Lenoël & Axelos, 1970; Tsai & Hassid, 1971) and from GDP-glucose into β -(1 $\rightarrow$ 4)-glucans (Barber *et al.*, 1964). In cotton hairs it has been confirmed that glucose is incorporated from UDP-glucose and GDP-glucose into β -(1 $\rightarrow$ 4)-glucans *in vivo* (Franz & Meier, 1969; Delmer *et al.*, 1974).

It is possible that glycolipid or glycoprotein intermediates may be involved in the synthesis of β -glucans and other polysaccharides in plants. Colvin (1961) found evidence that a glycolipid intermediate is involved in cellulose synthesis, and there have been reports of incorporation of glucose and mannose *in vitro* from UDP-glucose and GDP-mannose into glycolipid (Villemez *et al.*, 1968; Kauss, 1969; Villemez, 1970; Forsee & Elbein, 1972; Alam & Hemming, 1973; Clark & Villemez, 1973) and from UDP-glucose and GDP-mannose into glycoprotein (Villemez, 1970). It is not yet clear whether these glycolipids and glycoproteins are intermediates in polysaccharide synthesis or whether they are end products. Some of the mannose-containing glycolipids and glycoproteins behaved as though they were intermediates (Kauss, 1969; Villemez, 1970),

but other studies have failed to show evidence for the participation of glycolipids during polysaccharide synthesis (Barber *et al.*, 1964; Storm & Hassid, 1972). However, there is now much evidence that glycolipids are involved during the synthesis of the saccharide portions of glycoproteins in animals (Behrens *et al.*, 1973; Parodi *et al.*, 1973; Waechter *et al.*, 1974), bacteria (Lennarz & Scher, 1972) and yeast (Babczinski & Tanner, 1973) and they are also involved in bacterial polysaccharide synthesis (Colvin, 1959; Garcia *et al.*, 1974).

In the present paper we report the incorporation of glucose *in vitro* from UDP-glucose into glycolipid and possibly glycoprotein by pea membrane fractions that are active in β -glucan synthesis. The membrane fractions were prepared by a new technique that permitted the isolation of intact dictyosome stacks without the use of chemical fixatives.

Materials and Methods

Preparation of membrane fractions

Peas (*Pisum sativum*, var. Feltham First) were surface-sterilized with dilute Milton, soaked in sterile chloramphenicol solution (10 mg/litre), and germinated in the dark on filter paper at 25°C under sterile conditions. After 2 days 10–12 g of roots (10–40 mm long) were cut off under sterile conditions and incubated for 3 h at room temperature (approx. 20°C) with a solution containing 3 mg of cellulase (Onozuka,

Scheme 1. Procedure for the preparation of membrane fractions from pea roots

Peas (10–12 g) were grown for 2 days under sterile conditions, and the roots were cut off and incubated for 3 h with a cellulase solution. They were then gently homogenized for 15 s in a cold pestle and mortar, filtered through muslin and fractionated as shown.

All Japan Biochemicals Ltd., Nishinomiya, Japan)/ml, 0.55 M-sucrose, 0.01 M-citric acid and 10 mg of chloramphenicol/litre. The cellulase had been partially purified by gel filtration on Sephadex G-25 to remove low-molecular-weight impurities. After incubation the roots were washed thoroughly with 0.55 M-sucrose to remove the cellulase.

Homogenization and fractionation were carried out by a modification of the method of Bowles & Northcote (1972), glutaraldehyde being omitted from all the media. The roots were homogenized for 15 s in an ice-cold pestle and mortar with 10 ml of a medium containing 0.5 M-sucrose, 0.1 M-sodium phosphate (pH 7.2), 1 mM-CaCl₂ and 4 mM-polyethylene glycol (approx. mol.wt. 1000). The homogenate was left at 4°C for 10 min and then filtered through two layers of muslin. Fractionation was carried out as shown in

Scheme 1; all the media contained the constituents of the homogenization medium. Centrifugation was carried out at 4°C in a Beckman Spinco L2 centrifuge with a SW 50.1 rotor, average radius 83 mm.

Incubation of membrane fractions with UDP-[¹⁴C]-glucose

The membrane fractions were each incubated for 1 h at room temperature with 1 μCi (4 nmol) of UDP-D-[¹⁴C]glucose and 30 μg of NAD⁺ in 5 ml of sucrose medium (0.5–1.0 M, containing the same constituents as the homogenization medium). After incubation the fractions were centrifuged at 100 000g for 2.5 h at 4°C. The supernatants were removed and replaced by 1 ml of 5% (w/v) trichloroacetic acid, and the pellets were stored at –20°C for analysis.

Scheme 2. *Solvent extraction of membrane fractions*

The membrane fractions were incubated with UDP-[^{14}C]glucose for 1 h, and then centrifuged at 100000g for 2.5 h. The supernatants were removed and replaced by 1 ml of 5% trichloroacetic acid.

Solvent extraction of membrane fractions after incubation with UDP-[^{14}C]glucose

The membrane pellets were extracted with solvents by the procedure shown in Scheme 2 (Clark &

Villemez, 1972). The yeast glucan that was added as a carrier contained β -(1 $\rightarrow$ 3) linkages, and was soluble in hot alkali. In some experiments the membrane pellets were all extracted separately; in other experi-

ments, the pellets from fractions 1, 2 and 4 were combined to give a general membrane fraction and extracted together. The extracts were: lipid, soluble in butan-1-ol; protein, insoluble in butan-1-ol, but extracted by phenol; short-chain polysaccharide, not extracted by butan-1-ol and phenol, but soluble in hot 2% NaOH; and polysaccharide, represented by the alkali-insoluble residue.

Analysis of membrane extracts

T.l.c. was carried out on silica-gel plates (Polygram Sil G/UV; Camlab, Cambridge, U.K.). The following solvents were used: A, chloroform-methanol-water (65:25:4, by vol.); B, chloroform-methanol-acetic acid-water (25:15:4:2, by vol.); C, chloroform-methanol-NH₃ (sp.gr. 0.88) (75:25:4, by vol.). After chromatography, the plates were dried and cut into 50mm strips. The radioactivity in the strips was determined by liquid-scintillation counting (Harris & Northcote, 1970).

Total hydrolysis (Bowles & Northcote, 1972) was carried out in 3% (w/w) H₂SO₄ at 103 kPa (15 lb/in²) and 120°C for 1 h. Particulate material was pre-treated with 72% (w/w) H₂SO₄ for 2–4 h at room temperature. The samples were neutralized with 10% *N,N*-diethylmethylamine in chloroform, and run electrophoretically at pH 2 on Whatman no. 1 paper at 5 kV for 30 min. The electrophoretograms were cut into strips (40 mm × 10 mm) and their radioactivity was counted (Harris & Northcote, 1970). Radioactive material, running at the region of neutral sugars and uronic acids, was eluted and run on paper chromatography in ethyl acetate-pyridine-water (8:2:1, by vol.) for 16–20 h. Radioactive material, running in the region of hexoses, was eluted and rerun in the same solvent for 72 h to separate the hexoses completely.

Partial acid hydrolysis was carried out in 11.4 M-HCl at room temperature (approx. 20°C) for 4 h. The acid was removed by evaporation over KOH at room temperature *in vacuo*, and the hydrolysate was run on paper chromatography in the above solvent for 72 h. The radioactive material running in the region of glucose disaccharides was eluted and run in the same solvent for 5–7 days to separate cellobiose, maltose and laminaribiose.

Acetolysis was carried out by using acetic acid-acetic anhydride-98% (w/w) H₂SO₄ (10:10:1, by vol.) (Clark & Villemez, 1972). The resulting disaccharides were identified chromatographically.

Smith degradation (Elbein, 1969) was carried out on samples of not more than 1 mg of polysaccharide. The samples were incubated for 96 h at 4°C with 1 ml of 0.05 M-sodium metaperiodate. Two portions of 0.5 ml each of 0.1 M-NaBH₄ were added at 30 min intervals, and after a further 30 min HCl was added to a final concentration of 2 M. The samples were hydrolysed at 100°C for 2 h in sealed tubes. The acid was

removed by evaporation over KOH at room temperature *in vacuo*, and the hydrolysate was run electrophoretically at pH 2 and 5 kV for 30 min to remove salts. The neutral material was eluted and run on paper chromatography in ethyl acetate-pyridine-water (8:2:1, by vol.) for 16–18 h; this gave good separation of glucose, erythritol and glycerol.

Incubations with enzymes

The samples were treated with the following enzyme preparations.

(a) Phosphodiesterase (*Crotalus terrificus terrificus*) 0.01 mg, from Boehringer, London W.5, U.K.; spleen, 0.1–0.15 unit (μ mol/min at 25°C), from Worthington, Freehold, N.J., U.S.A.) in 1 ml of 0.1 M-citrate buffer, pH 6, at room temperature for 10 h under toluene. The preparation did not hydrolyse glucose 1-phosphate under these conditions and contained no α - or β -glucosidase activity when tested on cellobiose and maltose.

(b) Salivary amylase (Olaitan & Northcote, 1962), 1 ml, at room temperature for 4–5 h under toluene.

(c) Cellulase (Onozuka), 1 mg/ml in 0.01 M-citric acid at room temperature under toluene for 12 h. The enzyme preparation also contains laminarinase and amylase activity.

(d) Thermolysin (Calbiochem Ltd., San Diego, Calif., U.S.A.), 1 mg/ml in water for 16 h at room temperature under toluene.

(e) Pronase (BDH Chemicals Ltd., Poole, Dorset, U.K.), 1 mg/ml in 0.1 M-sodium phosphate (pH 7.2) for 16 h at room temperature under toluene.

After each enzyme incubation, the samples were dried under reduced pressure and run electrophoretically at pH 2 or 6.5 for 30 min at 5 kV. The material that was neutral at these pH values was analysed by paper chromatography.

Electron microscopy

Negative staining. Samples (three drops) of membrane fractions were fixed with 25% (w/v) glutaraldehyde (one drop) at 0°C for about 10 min, dried on to carbon-celloidin-coated grids, and stained with 1% phosphotungstic acid (neutralized with NaOH to pH 6.8).

Ultrathin sections. A solution containing 24% glutaraldehyde, 0.02 M-sodium phosphate (pH 7.2) and 0.5 M-sucrose was added to the membrane fractions to give a final concentration of 6% glutaraldehyde. The fractions were centrifuged at 100000g for 2–3 h and the supernatants discarded. The pellets were further fixed for 20 min in a solution containing 6% glutaraldehyde, 0.01 M-sodium phosphate (pH 7.2) and 0.5 M-sucrose, post-fixed for 20 min in osmic acid (1%) buffered with veronal, dehydrated with 2-ethoxyethanol and embedded in Araldite. Sections

EXPLANATION OF PLATE 1

Electron micrographs of negatively stained material from membrane fractions

The samples were fixed in 0.1 M-glutaraldehyde for 10 min, dried on to carbon-celloidin-coated grids and stained with 1% sodium phosphotungstate (pH 6.8). The scale bar represents 1 μ m. (a) Fraction 1 (Golgi-rich fraction) (magnification $\times 37300$). (b) Fraction 1 (Golgi-rich fraction) (magnification $\times 55200$). (c) A sample taken from the pea root homogenate immediately after homogenization (magnification $\times 29000$). (d) A sample taken from Golgi-rich fraction isolated from pea roots by homogenization and density-gradient fractionation in the presence of 0.1 M-glutaraldehyde (magnification $\times 56400$).

EXPLANATION OF PLATE 2

Electron micrographs of sections of material from membrane fraction 1

The fractions were fixed in glutaraldehyde, post-fixed in OsO_4 , and embedded in Araldite. Sections were cut and mounted on carbon-celloidin grids, and stained with uranyl acetate and alkaline lead hydroxide. The scale bar represents 1 μ m. (a) Fraction 1 (Golgi-rich fraction) (magnification $\times 40400$). (b) Fraction 1 (Golgi-rich fraction). Arrow shows a structure which may represent a cross-section through the plane of a cisterna (magnification $\times 40000$).

EXPLANATION OF PLATE 3

Electron micrographs of sections of material from membrane fractions 1-4

The fractions were fixed in glutaraldehyde, post-fixed in OsO_4 , and embedded in Araldite. Sections were cut and mounted on carbon-celloidin grids, and stained with uranyl acetate and alkaline lead hydroxide. The scale bar represents 1 μ m. (a) Fraction 1 (Golgi-rich fraction). Arrow shows a structure which may represent a cross-section through the vesicles at the rim of a cup-shaped cisterna (magnification $\times 42300$). (b) Fraction 2 (rich in mitochondria and smooth membrane) (magnification $\times 27900$). (c) Fraction 3 (rich in mitochondria) (magnification $\times 27200$). (d) Fraction 4 (rich in rough endoplasmic reticulum) (magnification $\times 40400$).

(a)

(b)

(c)

(d)

were cut with a glass knife, mounted on carbon-celloidin-coated grids and stained with uranyl acetate and alkaline lead hydroxide. The sections were examined in a GEC EM6B electron microscope at 60kV.

Results

Appearance of the membrane fractions under the electron microscope

Wall pellet. Sections of this fraction showed that it contained cell walls, nuclei and chloroplasts.

Fraction 1. On examination of negatively stained samples, this fraction showed characteristic Golgi cisternae (Plates 1a and 1b), either singly or in groups of two or three. Such groups probably represented cisternae originating from the same dictyosome stack, since they generally showed some structural association, either by close intermingling of their tubule systems (Plate 1b) or by apparent points of contact between the tubule systems of adjacent cisternae (Plate 1a). The cisternae had a characteristic structure. They consisted of a central plate surrounded by a fenestrated region, and this region gave way at its periphery to a region of tubules which culminated in vesicles of varying sizes. Some of the tubules and vesicles were probably broken off during the fractionation procedure, since samples taken immediately after homogenization showed groups of cisternae with considerably more complex systems of tubules and large vesicles (Plate 1c). In initial experiments the membrane fractions were prepared by the more usual method, in which glutaraldehyde (6%) was incorporated into the homogenization and fractionation media (Harris & Northcote, 1971; Bowles & Northcote, 1972). A Golgi fraction prepared in this way is shown in Plate 1(d).

Sections of this fraction showed characteristic dictyosome stacks of up to five or more cisternae. The cisternae were seen in cross-section as lamellae with vesicles of various sizes at each end (Plates 2a and 2b). Occasionally structures were seen which may represent sections through the plane of a cisterna (Plate 2b). In cross-section the cisternae were either flat or curved; the curved cisternae probably represented cup-shaped structures, so that the dictyosome stack is a series of concentric cups, with vesicles protruding from the rims (Plate 2b). The structure shown in Plate 3(a) may represent a section through the vesicles at the rim of such a cup-shaped cisterna.

The sections showed that other less heavily staining smooth-membrane material was also present in this fraction; this may be derived from the smooth endoplasmic reticulum, plasma membrane, tonoplast or Golgi vesicles.

Fractions 2, 3 and 4. Sections showed that mitochondria, smooth-membrane material and a few

Golgi cisternae were present in fraction 2 (Plate 3b). Mitochondria and some smooth-membrane material were present in fraction 3 (Plate 3c), and rough- and smooth-membrane vesicles and free ribosomes were present in fraction 4 (Plate 3d).

General nature of the radioactive materials synthesized from UDP-[¹⁴C]glucose by the membrane fractions

About 90% of the radioactive label remained water-soluble and was found in the supernatant after centrifugation of the incubated membrane fractions. The supernatants from all four fractions contained unchanged UDP-glucose, and with fractions 2 and 3, but not fractions 1 and 4, the supernatants also contained UDP-galactose. The water-soluble material from fraction 1 was analysed more fully, and was found to contain in addition glucose 1-phosphate, glucose, and a compound that was neutral on electrophoresis at pH 2 and ran with laminaribiose on paper chromatography. This last compound was probably that identified by Kemp & Loughman (1974) as glucosyl-*myo*-inositol.

Radioactive glucose was also incorporated into all four membrane fractions as water-insoluble material (found in the pellets after centrifugation of the incubation mixtures). All the other analyses were carried out on these membrane pellets.

Total acid hydrolysis showed that the only radioactive sugar present in each fraction was glucose. The fractions were extracted with solvents (Scheme 2) and the properties of the radioactive materials present in each extract and the residue were investigated. In early experiments, solvent extraction was carried out on the four membrane fractions separately, but no qualitative differences were found between the products formed by the different fractions. So most of the analyses were carried out on extracts from a general membrane fraction (fractions 1, 2 and 4 combined, containing Golgi cisternae, rough and smooth membrane, and some mitochondria) and from a mitochondria-rich fraction (fraction 3, containing mostly mitochondria and a little smooth membrane).

Material extracted by butanol

This was soluble in butanol, chloroform, chloroform-methanol (2:1, v/v) and toluene. It was insoluble in water and could not be washed out of the organic solvents with water. The extract from the general membrane fraction and from the mitochondria-rich fraction each contained 500–4000 c.p.m. When the extract from the general membrane fraction was run on thin-layer chromatograms in solvents A and C, it was resolved into two components each containing approximately the same amount of radioactivity. The R_F values for the two components (I, II)

Table 1. *Thin-layer chromatography of the butanol extract of the general membrane fraction*

The radioactive material extracted from the general membrane fraction with butanol was chromatographed on silica-gel thin-layer plates in the solvents described in the text. The plates were cut into 5mm strips and the radioactivity in each strip was determined by liquid-scintillation counting. Two components (I, II) were found by using solvents A and C and one with solvent B; no other material was detected.

Solvent	<i>R_F</i> value					
	I	II	Glucose	Cellobiose	Glucose 1-phosphate	UDP-glucose
A	0.6	0.8	0.1	0.1	0.0	0.0
B	0.9	0.9	—	—	—	—
C	0.4	0.8	—	—	—	—

are shown in Table 1. The following analyses were carried out on this extract from the general membrane fraction.

Treatment with dilute acid. Extraction with water (3ml) removed 2% of the radioactivity; no increase in water-extractable radioactive material was found after treatment with 0.1M- or 1.0M-HCl in water-saturated butanol for 1h at room temperature (approx. 20°C). Treatment with 0.05M-HCl in water-saturated butanol for 30min at 100°C released 53% of the radioactive label into a water solution prepared by evaporation of the acid butanol to dryness and addition of water (250μl). After similar treatment of a second sample for 1h 81% of the radioactivity was rendered water-soluble. After three successive 1h treatments of this second sample with 0.05M-HCl in butanol, less than 2% of the radioactivity remained insoluble in water.

When the radioactivity released into water solution by acid was run electrophoretically at pH2, most of it ran as neutral material. However, a small proportion (about 3%) was negatively charged, running in the same position as glucose 1-phosphate. This suggested that the acid-labile bond may be a phosphate bond. The neutral material was examined by paper chromatography. Between 25 and 50% of the neutral radioactivity was glucose, and the remainder of the neutral material was bound so strongly to the paper of the electrophoretogram that it could not be eluted with water, and it was not identified in these experiments. However, when the electrophoresis step was omitted, and the water-soluble material was run on paper chromatography, 40% of the radioactivity remained at the origin, while the rest ran as glucose. The unidentified neutral material on the electrophoretogram was therefore a glucose oligosaccharide, sufficiently long to bind strongly to paper.

Treatment with dilute alkali. Treatment with 0.1M-KOH in water-saturated butanol for 15h at 37°C released 66% of the radioactivity into water solution as material that was neutral on electrophoresis at pH2.

Treatment with phosphodiesterase. The extract was incubated with phosphodiesterase and the material

rendered water-soluble taken up in 300μl of water and run electrophoretically at pH2. The esterase released 40% of the radioactivity of the insoluble glycolipid into the water. Both components I and II (Table 1) that were separated on the thin-layer chromatogram with solvent C gave more than 50% of their radioactivity into water-soluble material on incubation with the diesterase.

Smith degradation. The hydrolysis products from Smith degradation were run on electrophoresis at pH2. Most of the radioactivity ran with neutral markers on the electrophoretogram, but 10% was negatively charged and ran at the same position as glucose 1-phosphate. Paper chromatography showed that the neutral material contained radioactive glucose, erythritol and glycerol in the ratios shown in Table 2.

Glucose and erythritol arise from (1→3)- and (1→4)-linked glucose residues respectively. The glycerol could arise from (1→2)- or (1→6)-linked glucose units, but it is more likely to have come from terminal glucose residues. From the evidence obtained by the mild acid (0.05M) hydrolysis of the unoxidized material it is clear that many of these terminal glucose units must have been single residues linked to the hydrophobic material, and the remainder were likely to have been the unattached ends of the oligosaccharides. Radioactive glycerol was present in a molar percentage of 30%, so the average oligosaccharide chain length in the whole extract was 3.3 residues, assuming that the oligosaccharides were evenly labelled throughout their length and that the glycerol arose solely from the terminal residues. In the experiments in which the unoxidized extract was hydrolysed with dilute acid, at least 25% of the radioactivity was found to be present as glucose, so that the average length of the oligosaccharide chains in the extract (excluding the monosaccharide) was likely to be at least 14 residues. The ratio of radioactivity in glucose and erythritol after Smith degradation was 5:1, which indicates that the ratio of (1→3) and (1→4) linkages in these oligosaccharides was approx. 3:1.

Conclusions. These results show that the radioactivity present in this extract consisted of both single glucose residues and oligosaccharide glucose chains.

Table 2. *Smith degradation of butanol and phenol extracts of the general membrane fraction*

The radioactive materials linked to lipid and protein were extracted from the general membrane fraction with butanol and phenol respectively. They were oxidized with sodium metaperiodate, reduced with NaBH₄ and hydrolysed with 2M-HCl at 100°C for 2 h. The hydrolysate was run on electrophoresis at pH 2, and the products were separated by paper chromatography.

Product of Smith degradation	Radioactivity in butanol extract			Radioactivity in phenol extract		
	c.p.m.	c.p.m. corrected for number of carbon atoms	% of total	c.p.m.	c.p.m. corrected for number of carbon atoms	% of total
Glucose	466	466	54.1	1050	1050	81.9
Erythritol	93	140	16.3	75	113	8.8
Glycerol	127	254	29.6	60	120	9.3

The sugars were associated with material which rendered them hydrophobic and soluble in organic solvents. They could not be washed out of the organic solvents by water, and thus the solubility characteristics could not be explained by a simple absorption on to hydrophobic substances. The compounds appeared therefore to be glycolipids. Two types of glycolipid are formed from UDP-glucose by plant enzyme preparations: steroyl glycosides and polyisoprenoid phosphate glycosides. Sugars were released from the hydrophobic material by phosphodiesterases and were therefore attached by phosphate ester bonds to the hydrophobic portion of the molecule. This was further confirmed by the detection of phosphate esters after acid hydrolysis of the untreated and periodate-oxidized compounds. The lipid material was resolved into at least two components by chromatography. One of these had R_F values which differed in the neutral, acidic or alkaline chromatographic solvents in a similar manner to those for mannosyl- and glucosyl-phosphoryl polyisoprenoids (Scher & Lennarz, 1969; Baynes & Heath, 1972; Forsee & Elbein, 1973). The other component, although running faster and having a similar R_F value to steroyl glucoside isolated from mung-bean and cotton fibres (Delmer *et al.*, 1974; Laine & Elbein, 1971) was not such a glucoside, since it, like the slower moving component, was hydrolysed by the diesterase. The glycolipids formed by the membrane preparations therefore had many of the characteristics of polyisoprenoid compounds isolated from bacteria (Lennarz & Scher, 1972), animal and some plant tissues (Parodi *et al.*, 1973; Forsee & Elbein, 1972). The sugars are covalently linked through phosphate ester linkages either as diesters or by a pyrophosphate radical. These linkages are unstable to both acid and alkali hydrolysis and they are broken by phosphodiesterase.

Material extracted by phenol

This was insoluble in water, butanol, chloroform-methanol (2:1, v/v), chloroform-methanol-water

(10:10:3, by vol.) (Behrens *et al.*, 1973) and dioxan. It was soluble in dimethyl sulphoxide.

The extract from the general membrane fraction contained 500–3000 c.p.m., and that from the mitochondria-rich fraction contained 100–200 c.p.m. The following analyses were carried out on the extract from the general membrane fraction.

Treatment with thermolysin and Pronase. Treatment with thermolysin released 9% of the radioactivity into water solution. The insoluble residue from the thermolysin incubation was treated with Pronase, and a further 52% of the radioactivity became water-soluble. A second Pronase treatment released another 14%, leaving only 25% of the radioactivity still insoluble after the three incubations. It was concluded therefore that most or all of the radioactive material was glycoprotein or firmly adsorbed on to protein. All the water-soluble radioactivity released by the enzymes ran with neutral markers on electrophoresis at pH 6.5.

Treatment with dilute alkali. On treatment with 0.1 M-NaOH for 8 days at 4°C, 68% of the radioactivity was released into water solution. On electrophoresis at pH 2, most of this (56%) ran with neutral markers, whereas the remainder (12%) was negatively charged, running with glucose 1-phosphate. The lability to alkali was consistent with a glycosidic linkage between glucose and serine or threonine or with a phosphate linkage to lipid or protein. The release of material that is negatively charged at pH 2 indicated that the linkage might be through phosphate. The neutral material released by alkali proved difficult to elute from paper and was immobile on paper chromatography; this suggested that it consisted of glucose oligosaccharides.

Smith degradation. This treatment gave radioactive glucose, erythritol and glycerol in the quantities shown in Table 2. The presence of glucose and erythritol indicated the presence of oligosaccharides containing (1→3) and (1→4) linkages, and the glycerol was therefore likely to represent the unattached ends of these oligosaccharides. The glucose/erythritol ratio indicated that the ratio of (1→3) to (1→4) linkages

was approx. 9:1, and the presence of 9.3% of glycerol indicated an average oligosaccharide chain length of about 11 residues, assuming that the oligosaccharides were evenly labelled throughout their length.

Conclusions. These analyses showed that the radioactive material present in this extract consisted of glucose oligosaccharides probably attached to insoluble material by phosphodiester or pyrophosphate bonds. The insoluble material was either protein or it was some other substance, such as lipid, firmly adsorbed on to protein.

Material extracted by alkali

This was insoluble in water and butanol and was not extracted by phenol. It was therefore likely to be polysaccharide. It was soluble in hot alkali. The general membrane fraction contained 1000–4000 c.p.m. in this extract, and the mitochondria-rich fraction contained 50–500 c.p.m.

Smith degradation. Smith degradation of the extract from the general membrane fraction gave glucose and erythritol in molar ratios varying from 1:1 to 1:2, showing the presence of (1→3) and (1→4) linkages in ratios varying from 1:1 to 1:2. Exposure to periodate for periods up to 10 days resulted in only a slight decrease in the ratio of glucose to erythritol, so that the presence of glucose was not due to incomplete reaction of (1→4)-linked glucose residues with periodate. No glycerol was ever detected, indicating that radioactively labelled terminal glucose residues were not present in sufficient quantities to give identifiable amounts of glycerol. [Non-reducing ends and probably also reducing ends of (1→3)- and (1→4)-linked glucans give glycerol in this analysis.] Since the limit of detection for glycerol is about 10 c.p.m., and analyses were carried out on material containing over 300 c.p.m., this indicated that the polysaccharide was at least 15 residues in length if only one end was labelled, and at least 30 residues in length if both ends were labelled. It is also possible that by transglycosylation reactions all the radioactive glucose had been incorporated into non-terminal positions of glucan chains of indeterminate length.

Smith degradation was also carried out on the material extracted by alkali from fractions 1, 3 and 4 separately. All of them gave radioactive glucose and erythritol in molar proportions of approx. 1:1, indicating the presence of (1→3) and (1→4) linkages in approximately equal amounts.

Acetolysis. The material extracted by alkali from fractions 1, 2 and 4 separately was examined by acetolysis. After this treatment, laminaribiose (together with large amounts of glucose and higher oligosaccharides) was found in all three fractions. This indicates that the (1→3) linkages were present in the β configuration.

Partial acid hydrolysis. Partial acid hydrolysis of the extract from the general membrane fraction resulted in the production of laminaribiose, confirming that the (1→3) linkages were in the β configuration.

Treatment with amylase. Treatment of the extract from the general membrane fraction with amylase did not produce any maltose or glucose, indicating that the (1→4) linkages were also in the β rather than the α configuration.

Treatment with cellulase. Treatment of the extract from the general membrane fraction with the Onozuka enzyme, which contains both cellulase and laminarinase activity, resulted in complete degradation to glucose.

Conclusions. The analyses show that the radioactive material in this extract consisted of polyglucan chains, which are made up of β -(1→3) and β -(1→4) linkages in ratios varying from 1:1 to 1:2. However, no cellobiose was detected in the products of either partial acid hydrolysis or acetolysis although laminaribiose and larger oligosaccharides were formed.

Alkali-insoluble residue

This material was not extracted by any of the solvents used, and was therefore likely to consist of long polysaccharide chains. The material from the general membrane fraction contained 300–1000 c.p.m., and that from the mitochondria-rich fraction contained up to 50 c.p.m. Partial acid hydrolysis of the material from the general membrane fraction gave small amounts of laminaribiose, showing that β -(1→3) linkages were present.

Distribution of the synthetase activities amongst the four membrane fractions

The four membrane fractions were incubated with UDP-[^{14}C]glucose and extracted separately with solvents. The amounts of radioactivity incorporated into each extract in a number of experiments are shown in Table 3. The incorporation varied considerably in different experiments, but it was clear that each fraction contained enzymes which incorporated glucose from UDP-glucose into each of the extracts and the residue.

Discussion

The membrane-fractionation procedure used in this work provides a method for the isolation from plant material of a fraction rich in relatively intact dictyosome stacks without the use of chemical fixatives (Morré *et al.*, 1965). This has also been done for onion stems (Morré & Millenhauer, 1964) and soybean hypocotyls (Hardin *et al.*, 1972). Normally, however, homogenization methods which are sufficiently violent to break open plant cells cause disruption of the dictyosome stacks if chemical fixatives

Table 3. *Incorporation of radioactivity into the membrane fractions isolated from pea roots*

The membrane fractions were isolated from the broken tissue by centrifugation on sucrose density gradients. The fractions were incubated with UDP-[^{14}C]glucose and the particulate material was then extracted successively with butanol, phenol and alkali. —, indicates that the material was not measured.

	Expt. no.	Radioactivity (c.p.m.)			
		Butanol extract (lipid)	Phenol extract (protein)	Alkali extract (short-chain polysaccharide)	Alkali-insoluble residue (polysaccharide)
Fraction 1 (Golgi cisternae and some smooth membrane)	1	156	—	1170	191
	2	—	—	1109	76
	3	—	—	661	170
	4	309	221	84	326
Fraction 2 (mitochondria, smooth membrane and Golgi cisternae)	1	553	—	452	759
	2	—	—	1161	21
	3	—	—	87	36
	4	166	93	55	128
Fraction 3 (mitochondria and some smooth membrane)	1	510	—	56	14
	2	—	—	121	18
	3	—	—	503	48
	4	387	138	51	69
Fraction 4 (rough and smooth membrane)	1	598	—	134	0
	2	—	—	2268	106
	3	—	—	282	63
	4	70	131	56	133

are not included in the homogenization medium. We have overcome this problem by weakening the cell walls by preincubation with cellulase, so that relatively mild homogenization methods can be used, and this method is likely to be generally applicable to plant tissues. The preservation of dictyosome structure permits the unambiguous identification of this organelle in electron micrographs; separated unstacked cisternae cannot be distinguished with certainty from the other smooth-membrane organelles of the cell. The method is therefore suitable for studies of enzymic activity *in vitro* in Golgi cisternae and other organelles.

In this work we have found that the enzymes which incorporate glucose from UDP-glucose into β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-linked polyglucans in pea roots are not associated specifically with the Golgi apparatus. The glucan synthetase activity is present in all the membrane fractions obtained, and the alkali-insoluble glucans that make up the major part of the synthesized glucans appear to be chemically similar in all four fractions. The glucan synthetase activity is therefore not localized in any of the organelles that are seen to be isolated in specific fractions: the Golgi cisternae (fractions 1 and 2), the mitochondria (fractions 2 and 3), and the rough endoplasmic reticulum (fraction 4). It is more likely to be associated with the smooth-membrane vesicles which are found in all four fractions, and which might be derived from any of the smooth organelles of the cell. Previous reports have suggested that UDP-glucose- β -(1 $\rightarrow$ 4)-glucan

glucosyltransferases are found in both the plasma membrane and the Golgi apparatus (Hardin *et al.*, 1972; Ray *et al.*, 1969), so the plasma membrane is the most likely site for the enzymes found in our work. However, in membrane preparations from soya-bean hypocotyl it was found that with UDP-glucose at a concentration of $1\mu\text{M}$ most of the β -(1 $\rightarrow$ 4)-glucan synthesis occurred in the Golgi apparatus, whereas the enzyme associated with the plasma membrane had a much lower affinity for UDP-glucose (Hardin *et al.*, 1972).

There is considerable evidence from studies *in vivo* that cellulose microfibrils are synthesized at the plasma membrane rather than in the Golgi apparatus (Northcote, 1969; Harris & Northcote, 1971; Bowles & Northcote, 1972; Robinson & Ray, 1973). However, it is quite likely that the enzymes that synthesize cellulose may be exported to the cell surface via the Golgi apparatus, so that activity may be detectable in isolated Golgi cisternae even if they are not sites for synthesis *in vivo*. It is probable that the callose [β -(1 $\rightarrow$ 3)-glucan] of phloem sieve tubes is also synthesized at the cell surface, since this polysaccharide continues to be formed even after most of the cell organelles, except for the plasma membrane, have been degraded (Northcote & Wooding, 1966).

In these experiments we found that all the membrane fractions that incorporated glucose from UDP-glucose into β -(1 $\rightarrow$ 3)- and α -(1 $\rightarrow$ 4)-linked glucans also incorporated glucose into glycolipid (a lipid-monosaccharide and a lipid-oligosaccharide).

- Morré, D. J., Mollenhauer, H. H. & Chambers, J. E. (1965) *Exp. Cell Res.* **38**, 672-675
- Northcote, D. H. (1969) *Essays Biochem.* **5**, 89-137
- Northcote, D. H. & Wooding, F. B. P. (1966) *Proc. Roy. Soc. Ser. B* **163**, 524-537
- Olaitan, S. A. & Northcote, D. H. (1962) *Biochem. J.* **82**, 509-519
- Parodi, A. J., Staneloni, R., Cantarella, A. I., Leloir, L. F., Behrens, N. H., Carminatti, H. & Levy, J. A. (1973) *Carbohydr. Res.* **26**, 393-400
- Péaud-Lenoël, C. & Axelos, M. (1970) *FEBS Lett.* **8**, 224-228
- Ray, P. M., Shininger, T. L. & Ray, M. M. (1969) *Proc. Nat. Acad. Sci. U.S.* **64**, 605-612
- Robinson, D. G. & Ray, P. M. (1973) *Plant Physiol.* **51** (Supplement), p. 59
- Scher, M. & Lennarz, W. J. (1969) *J. Biol. Chem.* **244**, 2777-2789
- Storm, D. L. & Hassid, W. Z. (1972) *Plant Physiol.* **50**, 473-476
- Tsai, C. M. & Hassid, W. Z. (1971) *Plant Physiol.* **47**, 740-744
- Villemez, C. L. (1970) *Biochem. Biophys. Res. Commun.* **40**, 636-641
- Villemez, C. L., Vodak, B. & Albersheim, P. (1968) *Phytochemistry* **7**, 1561-1564
- Waechter, C. J., Lucas, J. J. & Lennarz, W. J. (1974) *Biochem. Biophys. Res. Commun.* **56**, 343-350