Biochem. J. (1979) **178**, 661–671
Printed in Great Britain

Polyprenyl Phosphate Sugars Synthesized during Slime-Polysaccharide Production by Membranes of the Root-Cap Cells of Maize (*Zea mays*)

By JOHN R. GREEN and D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

(*Received* 4 *September* 1978)

Two types of experiments were carried out; either maize roots were incubated in L-[1-^{3}H]fucose or membranes were prepared from root tips and these were incubated with GDP-L-[U-^{14}C]fucose or UDP-D-[U-^{4}C]glucose. The radioactively labelled lipids that were synthesized *in vivo* and *in vitro* were extracted and separated into polar and neutral components. The polar lipids had the characteristics of polyprenyl phosphate and diphosphate fucose or glucose derivatives, and the neutral lipids of sterol glycosides (fucose or glucose). A partial separation of the glycolipid synthetase reactions was achieved. Membranes were fractionated into material that sedimented at 20000*g* and 100000*g*. Most of the polar glycolipid synthetase activity (for the incorporation of both fucose and glucose) was located in the 100000*g* pellet, and this activity was probably located in the endoplasmic reticulum. The neutral lipid, which contained fucose, was synthesized mainly by membranes of the 20000*g* pellet, and the activity was probably associated with the dictyosomes, whereas the neutral glucolipids were synthesized by all the membrane fractions. It is suggested that the polar (polyprenyl) lipids labelled with fucose could act as possible intermediates during the synthesis of the glycoproteins and slime in the root tip.

The polyprenyl phosphates can function as acceptors of glycosyl radicals and these lipid-linked sugars and oligosaccharides can serve as intermediates in polysaccharide and glycoprotein synthesis (Hemming, 1974; Northcote, 1977; Waechter & Lennarz, 1976). In particular, dolichol phosphate sugars have been identified as intermediates during the biosynthesis of glycoproteins in animal cells and yeast (Behrens *et al.*, 1973; Parodi *et al.*, 1973; Hsu *et al.*, 1974; Lehle & Tanner, 1975, 1978). Lipid-linked glycosyl residues have also been detected in various plants, and the evidence suggests that they are polyprenyl phosphate derivatives (Alam & Hemming, 1973; Forsee & Elbein, 1973; Pont Lezica *et al.*, 1975; Brett & Leloir, 1977).

Recent work suggested that dolichol mono- and di-phosphate glucose and dolichol diphosphate-linked oligoglucans are involved in the synthesis of a glucoprotein and ultimately cellulose (Brett & Northcote, 1975; Hopp *et al.*, 1978*a*).

The production of slime by the maize root cap has proved to be a useful system for studying intermediates necessary for polysaccharide synthesis (Green & Northcote, 1978). The slime consists mainly of polysaccharide and is characterized by having a high content of fucose (approx. 25%) (Bowles & Northcote, 1972; Harris & Northcote, 1970). Green & Northcote (1978) showed that glycoproteins containing fucose were present in the maize root tip. Pulse–chase experiments with radioactive tracers indicated that the glycoproteins were precursors of the slime polysaccharides. It was postulated that the slime polysaccharides are synthesized and transported on proteins within the membrane system of the root tip.

In the present investigation use is made of the fact that fucose is not generally metabolized by the maize root nor converted into other sugars (Kirby & Roberts, 1971). Lipids were labelled *in vivo* with L-[1-^{3}H]fucose and *in vitro* with GDP-L-[U-^{14}C]-fucose. The evidence showed that both polar lipids and neutral (sterol glycoside) lipids were labelled with radioactive fucose. A partial separation of these two enzymic activities has been achieved. The polar lipid compound had the characteristics of a polyprenyl phosphate fucose and could act as a possible intermediate during the synthesis of the glycoproteins and slime (Green & Northcote, 1978).

Materials and Methods

Growth of tissue and sterile techniques

Seedlings of maize (*Zea mays* var. Caldera) were germinated under sterile conditions (Green & Northcote, 1978).

Radioactive chemicals

L-[1-^{3}H]Fucose (sp. radioactivity 4.2Ci/mmol), GDP-L-[U-^{14}C]fucose (sp. radioactivity 140mCi/mmol), UDP-D-[U-^{14}C]glucose (sp. radioactivity

300mCi/mmol) and [U-^{3}H]sulphanilic acid were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [U-^{3}H]Sulphanilic acid was supplied as an impure solution (3Ci) and was purified by t.l.c. in ethyl acetate/propan-2-ol/aq. NH_3 (sp.gr. 0.880)/water (18:12:6:3, by vol.) on silica-gel plates (20cm×20cm, layer 2mm; Macharey, Nagel und Co., Düren, Germany). The specific radioactivity of the purified material was 2.8mCi/mmol.

Incorporation of L-[1-^{3}H]*fucose into endogenous lipid and polymers of maize root tips*

Roots (60) were incubated (four groups of 15) in L-[1-^{3}H]fucose for up to 60min (Green & Northcote, 1978). The tips of the roots in each group were in contact, and a solution (100μl) containing 25μCi of L-[1-^{3}H]fucose in water was applied to this point of contact in each of the four groups. At the end of the incubation the slime and incubation medium were removed and the roots were washed with sterile water and dried (Green & Northcote, 1978). The solutions were dialysed (five changes of water) and freeze-dried. The 'radioactive' root tips (1mm) were excised and homogenized at 4°C, with a pestle and mortar, in chloroform/methanol (2:1, v/v; 10ml). The homogenate was left at 4°C for 16h to extract the lipids, and centrifuged at 10000*g* for 30min at 4°C in a Sorvall RC-2B centrifuge to remove cell debris and residual material as a pellet. The chloroform/methanol extract was washed by the method of Folch *et al.* (1957). In some experiments, pellets were further extracted with chloroform/methanol/water (10:10:3, by vol.; 10ml) in order to determine the incorporation of radioactive label into lipid-linked oligosaccharides (Behrens *et al.*, 1971). These extracts were rotary-evaporated to a small volume and chromatographed on a strip of Whatman 3MM paper with water as the solvent. The material remaining at the origin was eluted from the paper with chloroform/methanol/water (10:10:3, by vol.; 5ml).

T.l.c. of lipids was carried out in chloroform/methanol/water (65:25:4, by vol.) on silica-gel G plates (20cm×20cm, layer 0.25mm, without gypsum; Camlab, Cambridge, U.K.).

Preparation and characterization of cellular membranes from maize root tips

Root tips of maize (5mm long) were excised (2–3g) and homogenized with a pestle and mortar at 4°C in a medium containing 8% (w/v) sucrose, 0.1M-Tris/HCl buffer, pH7.4, and 0.02M-β-mercaptoethanol (1.0ml of medium/g fresh weight of root tips). The homogenate was filtered through muslin, centrifuged at 700*g* (4°C) for 15min, and the supernatant centrifuged at 20000*g* (4°C, R65 rotor, Beckman ultracentrifuge) for 30min. The pellet from the centrifugation at 20000*g* was resuspended in 0.5ml of homogenization buffer and the supernatant centrifuged at 100000*g* (4°C) for 60min, and the pellet resuspended as before.

Protein was determined by the method of Lowry *et al.* (1951). Enzyme assays were performed at 25°C by using a Beckman model 25 recording spectrophotometer. NADH–cytochrome *c* reductase activity was measured (Shore & Maclachlan, 1975), and the effect of antimycin on the enzyme activity was investigated by adding 5μl of antimycin (2mg/ml in ethanol). Latent inosine diphosphatase was assayed after leaving the membranes at 4°C for 3 days (Shore & Maclachlan, 1975). Succinate dehydrogenase activity was determined by the method of Veeger & Zeylemarker (1969).

Purified [U-^{3}H]sulphanilic acid was converted into the diazonium salt (Berg & Hirsh, 1975). The diazotization mixture contained [U-^{3}H]sulphanilic acid (25μCi), 2μmol of HCl and 50μl (300μmol) of isopentyl ('isoamyl') nitrite. Root tips (100) were labelled with radioactive diazotized sulphanilic acid (25μCi) in water (10ml) for 30min at 20°C. The roots were washed repeatedly with water and homogenized. The quantity of bound sulphanilic acid was found by determining the amount of radioactivity precipitated by 15% (w/v) trichloroacetic acid.

*Incorporation of fucose from GDP-*L-[*U-*^{14}*C]fucose and of glucose from UDP-*D-[*U-*^{14}*C]glucose into lipid and polymeric material by isolated membranes*

Incubations were carried out at 25°C for 30min in a total volume of 100μl. The mixture contained 5μmol of Tris/HCl, pH7.4, 2μmol of β-mercaptoethanol, 0.5μmol of AMP, 1μmol of $MgCl_2$, 0.2% Triton X-100 and 100–300μg of protein (membrane fractions). Either UDP-D-[U-^{14}C]glucose (0.1μCi) or GDP-L-[U-^{14}C]fucose (0.1μCi) was also included in the incubation mixture. The reaction was stopped by the addition of 2ml of chloroform/methanol (2:1, v/v) and centrifuged at 2000*g* for 15min to remove the precipitate. The supernatant was washed by the method of Folch *et al.* (1957). Polar and neutral glycolipids in the organic phase were separated by DEAE-cellulose-paper chromatography (Whatman DE-20) with butanol as solvent, and the areas of the paper corresponding to the origin (polar glycolipids) and the solvent front (neutral glycolipids) were cut out and counted for radioactivity.

The pellet was washed with 1ml of 80% (v/v) methanol several times and the radioactivity in this polymeric material was counted as described below. In some experiments the pellet was extracted with chloroform/methanol/water (10:10:3, by vol.) before washing with methanol in order to investigate incorporation of radioactivity into lipid-linked oligosaccharides.

Preparation of dolichol phosphate and dolichol phosphate glucose

Dolichol phosphate was prepared from pig liver and purified by DEAE-cellulose chromatography (Behrens *et al.*, 1973). Radioactively labelled dolichol phosphate glucose was synthesized by incubating dolichol phosphate and UDP-D-[U-^{14}C]glucose with rat liver microsomal fractions (Parodi *et al.*, 1973; Behrens *et al.*, 1973). When exogenous dolichol phosphate was included in the incubation mixture with the maize root-tip membrane fractions, it was first dried in a tube with 0.5 μmol of MgEDTA and 0.5 μmol of $MgCl_2$. The rest of the incubation mixture was then added.

Acid hydrolysis of lipids and analysis of sugars

Lipid material in chloroform/methanol (2:1, v/v) was rotary-evaporated to dryness. Mild acid hydrolysis was carried out by incubating the sample with 0.2ml of HCl (0.01M) at 100°C for 10min. Then 0.4ml of methanol and 0.6ml of chloroform were added and the mixture was separated into two phases by centrifugation at 2000*g* for 15min. The chloroform layer was assayed for radioactivity by liquid-scintillation counting. The aqueous phase was dried by rotary evaporation and was either counted for radioactivity directly or subjected to paper chromatography.

Strong acid hydrolysis was performed in 3% (w/v) H_2SO_4 in an autoclave at 120°C, 103kPa, for 1h. The hydrolysate was neutralized with Amberlite IR-4B resin (CO_3^{2-} form). This was then rotary-evaporated to dryness and dissolved in water (approx. 100 μl). The sample was applied to a paper chromatogram (Whatman no. 1 paper, in ethyl acetate/pyridine/water, 8:2:1, by vol., for 20h). Neutral-sugar markers were run in parallel and were detected by aniline hydrogen phthalate (Wilson, 1959).

Electrophoresis at pH2 was used [Whatman no. 1 paper; acetic acid/formic acid/water (4:1:45, by vol.), pH2; 30min at 5kV] to separate UDP-glucose, glucose 1-phosphate and glucose. Markers were run in parallel, the nucleotide spot was observed under a u.v. lamp and the sugar phosphates were detected with molybdate reagent (Lewis & Smith, 1969).

Measurement of radioactivity

The paper chromatograms, electrophoretograms and t.l.c. plates were dried and dissected into 4cm × 1cm strips and placed in counting vials. Scintillant A (0.5ml) {PPO (2,5-diphenyloxazole, 8.75g), POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene; 0.125g] in 2.5 litres of toluene} was added and they were counted for radioactivity in 20ml Packard bottles in a Searle mark III liquid-scintillation system, model 6880. Lipid samples in chloroform/methanol were rotary-evaporated to dryness and counted for radioactivity after the addition of 1ml of scintillant B (6g of PPO, 75mg of POPOP, 750ml of Triton X-100 and 1.5 litres of toluene). Pellets and slime were counted for radioactivity by addition of scintillant B. Aqueous samples were counted for radioactivity by adding 1vol. of sample to 10vol. of scintillant B.

Results

Incorporation of L-[1-^{3}*H*]*fucose into endogenous lipid acceptors and polymers in intact roots*

Roots were incubated with L-[1-^{3}H]fucose for 10, 30 and 60min. The lipids were extracted with chloroform/methanol (2:1, v/v) and separated into neutral and polar constituents by chromatography on Whatman DE-20 paper. Lipid oligosaccharides were extracted from the pellet with chloroform/methanol/water (10:10:3, by vol.). The lipids, washed pellet and slime were counted to determine the distribution of radioactivity. The results are shown in Table 1. Most radioactivity was in the slime and pellet material (glycoproteins and slime polysaccharides). There was also some radioactivity associated with neutral and polar lipids and lipid–oligosaccharide, and these were labelled rapidly and were saturated with radioactivity after 10min. The radioactivity of the polymer (slime and pellet) increased with time.

Properties of the endogenous lipid acceptors labelled with L-[1-^{3}*H*]*fucose*

The radioactive lipids formed from L-[1-^{3}H]fucose *in vivo* were extracted (chloroform/methanol, 2:1, v/v) from a 30min incubation and were analysed by

Table 1. *Incorporation of* L-[1-^{3}*H*]*fucose into endogenous lipid and polymers in intact roots*

Roots were incubated in L-[1-^{3}H]fucose for various times. Slime, incubation medium and washings were pooled and dialysed against water. Root tips (1mm long) were excised and homogenized at 4°C in chloroform/methanol (2:1, v/v; 10ml) in order to extract the lipids. The chloroform/methanol extract was separated into neutral and polar components by chromatography. Pellets were further extracted with chloroform/methanol/water (10:10:3, by vol.; 10ml) to extract lipid–oligosaccharides. The non-diffusible material after dialysis, the insoluble residue and the lipid extracts were counted for radioactivity in Triton/toluene scintillant.

	Radioactivity (c.p.m.)			
Time (min)	Neutral lipid	Polar lipid	Lipid–oligosaccharide	Polymer
10	280	316	173	7320
30	293	301	196	25690
60	264	272	221	51210

t.l.c. The distribution of radioactivity along the plate is shown in Fig. 1. Dolichol phosphate glucose was also run as a marker. The product separated into three components. One of these, Peak III, had high mobility, similar to that of the sterol glycosides (Axelos & Peaud-Lenoel, 1978). Peaks I and II had much lower mobilities; Peak II had an R_F value similar to that of dolichol phosphate mannose (0.35) (Forsee & Elbein, 1973). The lipids were eluted from the strips of the t.l.c. plate with chloroform/methanol (2:1, v/v). Samples from each peak were chromatographed in butanol. Material at the origin (polar lipid) and solvent front (neutral lipid) was counted for radioactivity. Lipid from Peak I and Peak II remained at the origin, whereas material from Peak III moved with the solvent front.

The lipids were hydrolysed in strong acid and the sugars were separated by paper chromatography. The radioactivity always coincided with fucose only.

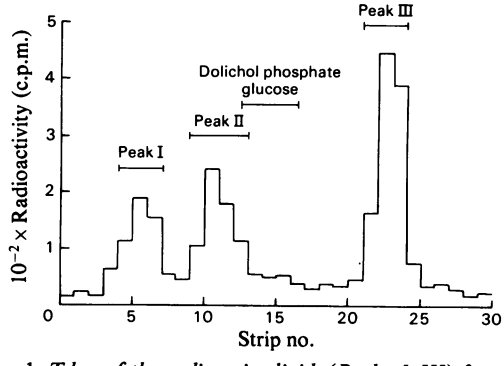

Fig. 1. *T.l.c. of the radioactive lipids (Peaks I–III) formed by incubation of roots in vivo with* L-[1-^{3}*H*]*fucose*
Roots were incubated in L-[1-^{3}H]fucose for 30min, and lipids extracted with chloroform/methanol (2:1, v/v). The lipids were chromatographed on silica-gel thin-layer plates, which were then sliced and counted to determine the distribution of radioactivity. Radioactively labelled dolichol phosphate glucose was prepared and was run as a marker.

The hydrolysate of the chloroform/methanol/water (10:10:3, by vol.) extract also yielded fucose as the only radioactively labelled sugar, and this material had the characteristics of a lipid–oligosaccharide (Behrens *et al.*, 1971).

The results of the breakdown of each lipid fraction by mild acid hydrolysis is shown in Table 2. All the lipids, except for the neutral lipid of Peak III, were almost completely hydrolysed.

Distribution of enzyme activities and bound diazotized [U-^{3}H]sulphanilic acid in cellular membranes from maize root tips

Membranes were prepared from maize root tips, and marker enzymes were assayed. The amount of diazotized [U-^{3}H]sulphanilic acid bound to the membrane fractions was also determined. The distribution of enzymic activities, protein and radioactivity precipitable by trichloroacetic acid in the various fractions is shown in Table 3. Diazotized radioactive sulphanilic acid has been used as a specific labelling agent for the plasma membrane of plant cells (Galbraith & Northcote, 1977). In the present work most of the bound radioactivity appeared in the 700*g* fraction. Galbraith & Northcote (1977) also found this and it represented labelling of cell-wall material and the plasma membrane of intact cells. Succinate dehydrogenase, localized in mitochondria (Veeger & Zeylemarker, 1969), was contained in the 20000*g* pellet, with little activity in any of the other fractions. NADH-cytochrome *c* reductase is a marker for the endoplasmic reticulum (Lord *et al.*, 1973; Bowles & Kauss, 1976), and the specific activity of this enzyme was highest in the 1000000*g* pellet. Antimycin inhibited the activity of this enzyme in the 20000*g* pellet, but did not affect the activity in the 100000*g* fraction. The antimycin inhibits the mitochondrial enzyme specifically (Lord *et al.*, 1973). Latent inosine diphosphatase has been used as a marker for dictyosomes in many plant cells (Ray *et al.*, 1969; Bowles & Kauss, 1976), and the highest specific activity was found in the 20000*g*

Table 2. *Mild acid treatment of lipid fractions labelled with* L-[1-^{3}*H*]*fucose*
The lipids (Peaks I–III; Fig. 1) were eluted from the strips of the thin-layer plate with chloroform/methanol (2:1, v/v). After hydrolysis, chloroform/methanol (3:2, v/v) was added and the mixture centrifuged at 2000*g* for 15min. The chloroform layer and the aqueous phase were assayed for radioactivity.

	Radioactivity (c.p.m.)			
		End of incubation		
Lipid (see Fig. 1)	Start of incubation	Chloroform phase	Aqueous phase	Breakdown (%)
Peak I	538	45	469	91
Peak II	419	36	364	91
Peak III	494	421	42	9

Table 3. *Distribution of enzyme activities, protein and bound diazotized [U-^{3}H]sulphanilic acid in cellular membranes from maize root tips*

Roots were homogenized at 4°C and the homogenate was filtered through muslin. The filtrate was centrifuged at 700*g* for 15min, 20000*g* for 30min and 100000*g* for 60min. The pellets were resuspended in buffer, and protein, enzyme activities and bound diazotized sulphanilic acid were determined as described in the Materials and Methods section.

	Homogenate	700*g* fraction	20000*g* fraction	100000*g* fraction	Supernatant	Recovery
Protein						
(mg)	24.68	7.45	5.05	2.04	8.42	
(%)	100	30.1	20.4	8.3	34.1	92.9
Radioactivity						
Total radioactivity (10^{-3}×c.p.m.)	43.63	31.18	4.67	2.36	1.29	
(%)	100	71.4	10.7	5.4	3.0	90.5
10^{-3}×Sp. radioactivity (c.p.m./mg of protein)	1.77	4.21	0.93	1.16	0.15	
Relative sp. radioactivity	1.0	2.38	0.52	0.66	0.09	
Succinate dehydrogenase						
Total activity (nmol/min)	131	22	103	0	0	
(%)	100	16.8	78.6	0	0	95.4
Sp. activity (nmol/min per mg of protein)	5.3	3.0	20.4			
Relative sp. activity	1.0	0.57	3.85			
Inosine diphosphatase						
Total activity (μmol of P_i released)	40.2	6.1	16.8	3.1	8.7	
(%)	100	15.2	41.6	7.7	21.6	86.1
Sp. activity (μmol of P_i released/h per mg of protein)	1.63	0.82	3.33	1.52	1.03	
Relative sp. activity	1.0	0.50	2.04	0.93	0.63	
NADH–cytochrome *c* reductase (−antimycin)						
Total activity (nmol/min)	5930	960	1616	2049	622	
(%)	100	16.2	27.1	34.7	10.4	88.4
Sp. activity (nmol/min per mg of protein)	240	129	320	1050	74	
Relative sp. activity	1.0	0.54	1.25	4.38	0.31	
NADH–cytochrome *c* reductase (+antimycin)						
Total activity (nmol/min)	4067	506	803	1822	570	
(%)	100	12.4	19.7	44.8	14.0	90.9
Sp. activity (nmol/min per mg of protein)	165	67.8	159	895	67.5	
Relative sp. activity	1.0	0.41	0.96	5.42	0.41	

pellet. This fraction therefore contained most of the mitochondria and was enriched in dictyosomes, whereas the 100000*g* fraction contained most of the endoplasmic reticulum. The plasma membrane, labelled with radioactive sulphanilic acid, was distributed between all the membrane fractions.

*Conditions for the incorporation of fucose from GDP-*L*-[U-^{14}C]fucose and of glucose from UDP-*D*-[U-^{14}C]-fucose into lipid by isolated membranes*

The isolated membrane fractions were incubated with either GDP-L-[U-^{14}C]fucose or UDP-D-[U-^{14}C]-glucose. The lipids were extracted with chloroform/methanol (2:1, v/v) and separated into neutral and polar components by chromatography. In preliminary experiments the breakdown of the added radioactive nucleoside diphosphate sugars was monitored by stopping the reactions after 2, 5 and 30min and electrophoresing (at pH2) the aqueous solution from a Folch wash. A rapid breakdown of UDP-glucose to glucose 1-phosphate and glucose by the 100000*g* pellet was found. A similar breakdown of GDP-fucose was also observed. The addition of AMP has been shown to inhibit this rapid hydrolysis by endogenous pyrophosphatases (Vessey & Zakim, 1975), and 5mM-AMP was used in all subsequent incubations. The incorporation of radioactivity from UDP-glucose into neutral and polar lipids by the 100000*g* fraction was used to determine the optimal conditions for further experiments. Figs. 2(*a*), 2(*b*) and 2(*c*) show that the optimum concentration of Mg^{2+} was 10mM, and Triton X-100 had the greatest effect at a concentration of 0.2%. The pH optimum for the glycosylation of neutral lipid was pH6.5, whereas that for the polar lipid was pH7.5. These optimum conditions were used for the incorporation of fucose from GDP-fucose into lipid.

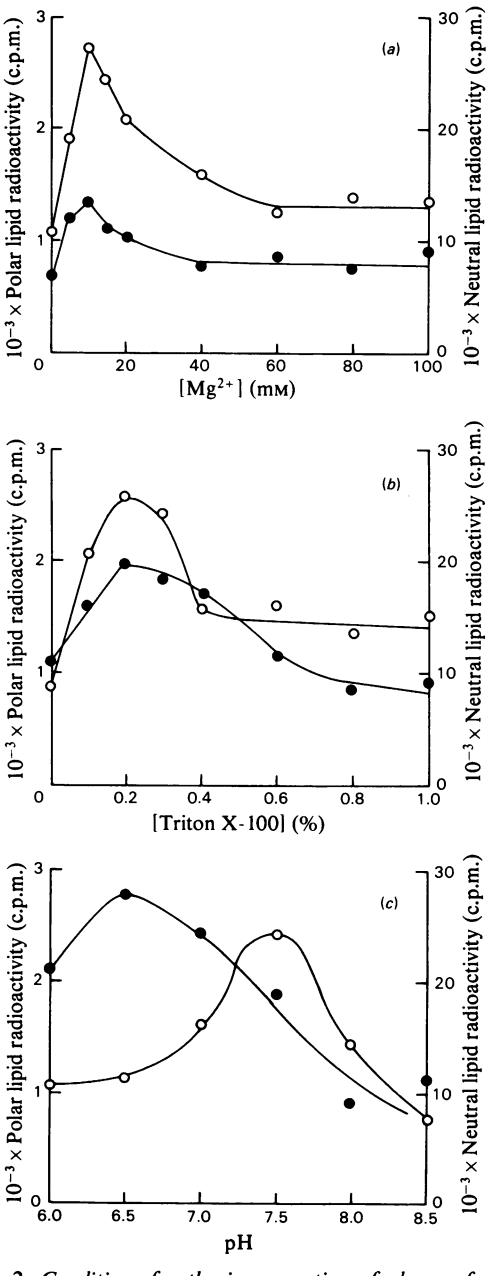

Fig. 2. *Conditions for the incorporation of glucose from UDP-D-[U-^{14}C]glucose into neutral and polar lipids by isolated membranes from maize root tips*

Isolated membranes (100000*g* pellet) were incubated with UDP-D-[U-^{14}C]glucose. Lipids were extracted and separated into neutral and polar components and then counted for radioactivity. The effect of varying (*a*) the Mg^{2+} concentration, (*b*) the concentration of Triton X-100 and (*c*) the pH on incorporation of radioactivity into polar (○) and neutral (●) lipid was determined.

Location of the glycosyltransferases and characteristics of the lipids labelled with radioactive GDP-fucose and UDP-glucose in vitro

The results (Table 4) showed that fucose was incorporated mainly into neutral lipid by the 20000*g* fraction and mainly into polar lipid by the 100000*g* fraction. There was considerable incorporation of label into polymer by the 20000*g* fraction as well as a smaller amount of incorporation by the 100000*g* pellet. Chloroform/methanol/water (10:10:3, by vol.) failed to extract any radioactivity as lipid–oligosaccharide.

The pattern of incorporation of glucose from UDP-D-[U-^{14}C]glucose (Table 4) was different from that of fucose from GDP-L-[U-^{14}C]fucose. Most of the enzyme activity for glucosylation of the polar lipids was found in the 100000*g* pellet, whereas that for glucosylation of the neutral lipid was found in all the fractions. Polymeric material was again synthesized primarily by the 20000*g* pellet. There was a small quantity of radioactivity (approx. 200c.p.m.) extracted as lipid–oligosaccharide.

The nature of the glycolipids synthesized *in vitro* from GDP-L-[U-^{14}C]fucose was determined by t.l.c. after extracting lipids from a 30min incubation. The distribution of radioactivity along the plates is shown in Fig. 3(*a*). The lipids labelled with radioactive fucose, recovered from the 100000*g* pellet, were resolved into three peaks in the same positions as Peaks I, II and III described for the experiments *in vivo*. The same lipids were found on analysis of the material synthesized by the 20000*g* fraction from GDP-L-[U-^{14}C]fucose, but in this latter instance there was a larger quantity of Peak III, the neutral lipid. Both strong and mild acid hydrolysis of the glycolipids from Peaks I, II and III gave similar results to those obtained with the material labelled *in vivo*. Thus fucose labelled the same three lipids *in vivo* and *in vitro*. The two polar lipids (Peaks I and II) were synthesized mainly in the 100000*g* pellet and therefore probably within the endoplasmic reticulum. The neutral lipid (Peak III) was synthesized mainly by the 20000*g* pellet (Table 4) and the glycosylation activity was probably associated with either the mitochondria or dictyosomes.

The effect of GMP and GDP on the synthesis of the glycolipids and polymer was determined by allowing the reactions to proceed for 10min, after which nucleotides were added at a concentration of 5mM. The reactions were analysed for radioactivity in lipid and polymer. The results (Table 5) show that both GMP and GDP decreased the amount of label in lipid at the end of the incubation.

To check that the fucosyltransferase activity was not an artifact, and that the results were not due to adsorption of radioactivity by membranes, a region (1cm) of the root 2–3cm from the tip was used

Table 4. *Incorporation of radioactivity from UDP-D-[U-^{14}C]glucose and GDP-L-[U-^{14}C]fucose into lipid and polymer by isolated membranes from maize root tips*

Membrane fractions were incubated with either radioactively labelled UDP-glucose or GDP-fucose. The reaction was stopped with chloroform/methanol (2:1, v/v) and the mixture centrifuged to remove the precipitate (polysaccharide and glycoprotein). The lipids were separated into neutral and polar components by chromatography and the lipids and polymer were then assayed for radioactivity.

		Radioactivity (c.p.m./mg of protein)		
Precursor	Membrane pellet	Neutral lipid	Polar lipid	Polymer
GDP-L-[U-^{14}C]fucose	700*g*	132	35	1270
	20000*g*	1870	413	18690
	100000*g*	625	1696	8260
UDP-D-[U-^{14}C]glucose	700*g*	7850	105	910
	20000*g*	31360	1130	12370
	100000*g*	25490	6210	4860

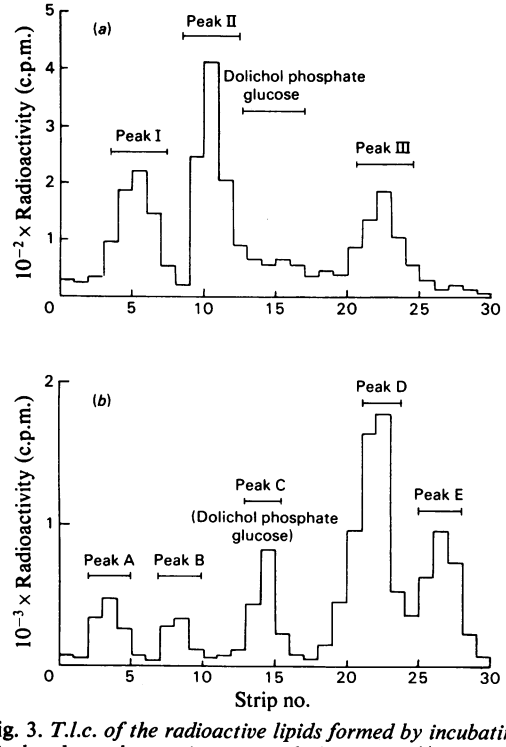

Fig. 3. *T.l.c. of the radioactive lipids formed by incubating isolated membranes in vitro with GDP-L-[U-^{14}C]fucose (Peaks I–III) or UDP-D-[U-^{14}C]glucose (Peaks A–E)*

Lipids were labelled with GDP-L-[U-^{14}C]fucose or UDP-D-[U-^{14}C]glucose with membranes from the 100000*g* fraction, and they were extracted in chloroform/methanol (2:1, v/v). The lipids were chromatographed on thin-layer plates. (*a*) shows the distribution of radioactivity after labelling with GDP-L-[U-^{14}C]fucose, and (*b*) that after labelling with UDP-D-[U-^{14}C]glucose.

(Bowles & Northcote, 1972). The material (2g) was homogenized, and membranes were centrifuged to give 700*g*, 20000*g* and 100000*g* pellets. These pellets were incubated with GDP-L-[U-^{14}C]fucose, and lipid and polymer were extracted and counted for radioactivity. No radioactivity was detected in either lipid or polymer, showing that the transfer of fucose is a specific activity localized in the root tip; this is the region in which the fucose-containing glycoproteins and slime polysaccharides are synthesized (Harris & Northcote, 1970; Bowles & Northcote, 1972; Green & Northcote, 1978).

Table 5. *Effect of GMP and GDP on the synthesis of glycolipids and polymer*

Membranes (100000*g* pellet) were incubated with GDP-L-[U-^{14}C]fucose and either GMP or GDP was added after a 10min period. After 30min the reaction was stopped with chloroform/methanol (2:1, v/v) and the incorporation of radioactivity into lipid and polymer was determined.

	Radioactivity (c.p.m./mg of protein)		
Addition	Neutral lipid	Polar lipid	Polymer
None	536	1479	8740
GMP	412	581	5860
GDP	419	619	5536

T.l.c. of the glycolipids extracted after incubating membranes with UDP-D-[U-^{14}C]glucose (Fig. 3*b*) showed that there were five peaks of radioactivity. The Peaks D and E had R_F values corresponding to sterol glucoside and acylated sterol glucoside (Axelos & Peaud-Lenoel, 1978), and Peak C had a mobility coincident with that of a dolichol phosphate [^{14}C]-glucose marker. Material from Peaks A, B and C remained bound to Whatman DE-20 paper after elution with butanol, indicating that they were polar, whereas Peaks D and E were eluted and were probably neutral. Strong acid hydrolysis of the lipids followed by paper chromatography showed that the radioactivity coincided with glucose. After mild acid hydrolysis, radioactivity was released from material

originating in Peaks A, B and C, but not from Peaks D and E (Table 6). It was possible that the glycolipids that had the lower mobilities (Peaks A, B and C) were polyprenol sugar derivatives and those of higher mobility (Peaks D and E) were neutral sterol glucoside components. The nature of the polymeric material synthesized *in vitro* was not determined.

Effect of exogenous dolichol phosphate and β-sitosterol on incorporation of GDP-L-[U-^{14}C]fucose and UDP-D-[U-^{14}C]glucose by the membrane fractions

The stimulation of incorporation of radioactivity into the various lipids was tested by the addition of dolichol phosphate (5μg and 10μg) and β-sitosterol (5mM) (Lercher & Wojciechowski, 1976) to the incubation mixtures. The lipids were labelled *in vitro* for 30min, extracted with chloroform/methanol (2:1, v/v) and separated into neutral and polar components by chromatography. The results are shown in Table 7. Dolichol phosphate stimulated the incorporation of fucose into polar lipid. When the labelled lipid was chromatographed, the Peaks I and II were both increased in amount, whereas Peak III was unaffected. β-Sitosterol stimulated the formation of neutral lipid, and t.l.c. chromatography showed a marked increase in the size of Peak III relative to the other peaks. This was further evidence that the glycolipids of Peaks I and II were polyprenols labelled with radioactive fucose and that they may have been dolichol phosphate derivatives; in addition, the glycolipid of Peak III was shown to be a sterol glycoside.

Lipids labelled with UDP-D-[U-^{14}C]glucose were extracted from membranes derived from the 100000*g* pellet. T.l.c. of the lipids showed that dolichol phosphate stimulated the synthesis of Peak C only, and as suggested above this glucolipid is probably dolichol phosphate glucose. β-Sitosterol stimulated the synthesis of the neutral lipids of Peaks D and E, the sterol glucoside and acylated sterol glucoside.

Discussion

Radioactively labelled lipids were isolated from the maize root tip after incubating intact roots with L-[1-^{3}H]fucose and membrane fractions with either GDP-L-[U-^{14}C]fucose or UDP-D-[U-^{14}C]glucose. The lipids, which were labelled with fucose *in vivo* and *in vitro*, could be resolved into three fractions (Peaks I–III) by t.l.c. The material from Peak I and Peak II was polar, susceptible to mild acid hydrolysis and had chromatographic mobilities corresponding to polyprenyl phosphate or polyprenyl diphosphate sugars (Forsee & Elbein, 1973; Lehle *et al.*, 1976).

Table 6. *Mild acid treatment of lipid fractions labelled with UDP-D-[U-^{14}C]glucose*

Lipids labelled with radioactive UDP-glucose were separated into five components (Peaks A–E) (Fig. 3*b*). The lipids corresponding to each peak were eluted and hydrolysed in mild acid.

	Radioactivity (c.p.m.)			
		End of incubation		
Lipid (see Fig. 3*b*)	Start of incubation	Chloroform phase	Aqueous phase	Breakdown (%)
Peak A	875	151	692	82
Peak B	541	83	440	84
Peak C	834	77	713	88
Peak D	1415	1274	96	7
Peak E	1894	1758	112	6

Table 7. *Effects of dolichol phosphate and β-sitosterol on the incorporation of GDP-L-[U-^{14}C]fucose and UDP-D-[U-^{14}C]glucose into neutral and polar lipids*

Isolated membranes (100000*g* pellet) were incubated with radioactively labelled GDP-fucose or UDP-glucose in the presence of either dolichol phosphate (10μg) or β-sitosterol (5mM). The labelled lipids were extracted and separated into neutral and polar components and assayed for radioactivity.

		Radioactivity (c.p.m./mg of protein)	
Precursor	Addition	Neutral lipid	Polar lipid
GDP-L-[U-^{14}C]fucose	None	670	1785
	Dolichol phosphate	426	3808
	β-Sitosterol	1638	1549
UDP-D-[U-^{14}C]glucose	None	22140	4966
	Dolichol phosphate	18290	11350
	β-Sitosterol	49480	4120

Their synthesis *in vitro* was stimulated by the addition of exogenous dolichol phosphate. Both GMP and GDP inhibited the formation of these glycolipids, which suggested that they were probably a mixture of phosphate and pyrophosphate derivatives (Ericson & Delmer, 1977). Strong acid hydrolysis showed that fucose was the only sugar labelled, but mild acid hydrolysis released radioactivity into the aqueous phase, which did not co-chromatograph with fucose and had low mobility, indicating that the product was probably a disaccharide or an oligosaccharide of few units. The material from Peak III was neutral, resistant to mild acid hydrolysis, had a mobility on t.l.c. corresponding to a sterol glycoside and its synthesis was stimulated by including β-sitosterol in the incubation mixture. Strong acid hydrolysis released fucose as the only labelled sugar. The lipids of Peak I and Peak II therefore had the characteristics of polyprenyl phosphate or polyprenyl diphosphate derivatives attached to fucose and also containing possibly one or more unidentified sugars. The lipid of Peak III was probably a sterol glycoside containing fucose. These lipids, which contained fucose, were synthesized both *in vivo* and *in vitro* and were not formed in a region further up the root where polysaccharides containing fucose were not formed. This indicates that their occurrence is not an artifact and reflects the state of differentiation between the root-tip region and the rest of the root. The tip region contains specific enzymes for the utilization and incorporation of fucose into polymers (Harris & Northcote, 1970; Bowles & Northcote, 1972; Green & Northcote, 1978), and the present work has shown a specific addition of fucose to lipid components in this region. Relatively few studies have been made of the incorporation of sugars into endogenous lipids *in vivo* (Struck & Lennarz, 1976; Speake & White, 1978; Spiro *et al.*, 1976*b*); fucose has the advantage as a precursor because it is not metabolized into other sugars (Kirby & Roberts, 1971).

A similar analysis of the products of labelling membranes with UDP-D-[U-^{14}C]glucose gave five peaks (A–E) of lipid material on t.l.c. The material from Peak C was probably dolichol phosphate glucose, and the lipids of Peak A and Peak B were probably other polyprenyl derivatives of glucose. The neutral lipids (Peak D and Peak E) had the properties of sterol glucoside and acylated sterol glucoside respectively.

It has been shown that the glucose acceptor lipid in plants is probably dolichol monophosphate (Pont Lezica *et al.*, 1975, 1976; Brett & Leloir, 1977; Hopp *et al.*, 1978*a*). In addition, polyprenyl phosphate lipids in plants have been found to contain mannose and *N*-acetylglucosamine (Alam & Hemming, 1973; Forsee & Elbein, 1973, 1975; Brett & Leloir, 1977). All these sugars are also found attached to polyprenols in animal tissues (Behrens *et al.*, 1971, 1973; Parodi *et al.*, 1973; Spiro *et al.*, 1976*a*,*c*), and the transfer of xylose to dolichol phosphate has also been detected (Waechter *et al.*, 1974). It is also possible that galactose is transferred to polyprenyl phosphate (Zatta *et al.*, 1975). The present results are the first to indicate that fucose is attached to a polyprenyl phosphate derivative, although the evidence does not indicate whether it was attached directly and whether other sugars may have been present in the short-chain oligosaccharide portion.

Sterols, sterol glycosides and acylated sterol glycosides are constituents of plant membranes (Grunwald, 1975). Glucose is the sugar most frequently found attached to sterol, but the incorporation of other sugars has been detected (Axelos & Peaud-Lenoel, 1978; Hopp *et al.*, 1978*b*).

A partial separation of the enzymes forming the glycosylated polar (polyprenyl phosphate) lipids and neutral (sterol glycoside) lipids was achieved. The membranes of the root tip were separated by centrifugation into 700*g*, 20000*g* and 100000*g* pellets, and markers indicated that the 20000*g*-pellet material contained most of the mitochondria and was enriched in dictyosomes. The 100000*g* fraction contained most of the endoplasmic reticulum, and the plasma membrane was distributed among all the membrane fractions. Optimal conditions were obtained for the incorporation of UDP-D-[U-^{14}C]glucose into lipid, and these were consistent with other results (Pont Lezica *et al.*, 1976; Hopp *et al.*, 1978*b*). Fucose and glucose were mainly incorporated into polar lipid by the 100000*g* fraction, and therefore there was most probably considerable activity in the endoplasmic reticulum. This is consistent with other work that has shown that the endoplasmic reticulum is more active in the synthesis of dolichol phosphate sugar derivatives than the dictyosomes, in both animals (Lennarz & Scher, 1972; Vessey *et al.*, 1976; Bergman *et al.*, 1978) and plants (Lehle *et al.*, 1978), although the dictyosomes do have some of this type of synthetase activity. Glycosylation of sterols has been shown to occur in either the plasma membrane (Van der Woude *et al.*, 1974; Hartmann-Bouillon & Benveniste, 1978) or the dictyosomes (Wojciechowski & Van Von, 1975; Lercher & Wojciechowski, 1976; Bowles *et al.*, 1977; Hopp *et al.*, 1978*b*), depending on the tissue. In the present investigation, fucose was transferred to neutral lipid, with the highest specific activity occurring in the 20000*g* fraction and therefore activity occurring in the 20000*g* fraction and therefore probably associated with the dictyosomes. The activity of the glucosyltransferase that transferred glucose to a neutral lipid acceptor was divided between all the membrane fractions and may be associated with the plasma membrane.

Polymeric material was also synthesized *in vitro* by the isolated membranes, and the highest specific activity was found in the 20000*g* fraction. The endo-

membrane system of the cell, comprising endoplasmic reticulum and dictyosomes, is the site of synthesis of polysaccharides in the plant (Northcote, 1974; Bowles & Northcote, 1972, 1974), and therefore the fucosyl- and glucosyl-transferase activities for polymer synthesis in the 20000*g* fraction are probably associated with the dictyosomes. The dictyosomes have also been shown to be the main site of incorporation of fucose into polymers of the brown alga *Fucus serratus* (Coughlan, 1977).

The dolichol phosphate sugars act as intermediates in animals in the synthesis of the core region of the oligosaccharides of those glycoproteins that contain the asparagine–*N*-acetylglucosamine linkage (Hemming, 1974; Waechter & Lennarz, 1976). They also function in the formation of *O*-glycosidic bonds in fungal cells, linking mannose to serine or threonine (Babczinski & Tanner, 1973; Sharma *et al.*, 1974; Bretthauer & Wu, 1975). Dolichol phosphate glucose was shown to be a precursor in the formation of a glucoprotein in an alga *Prototheca zopfii* during cellulose synthesis (Hopp *et al.*, 1978*a*). The polar (polyprenyl) lipids labelled with glucose that were found in the maize root tip may be intermediates in the synthesis of either cellulose of the cell wall or the short $\beta(1\rightarrow4)$-glucan chains found in the slime (Wright & Northcote, 1976). The slime polysaccharides are synthesized and transported on proteins within the membrane system of the maize root tip (Green & Northcote, 1978). There are at least two glycoproteins that contain fucose within the membranes, and the polyprenyl phosphate lipids labelled with fucose in the present investigation may be intermediates in the formation of either one or both of these glycoproteins. We have shown that the transfer of fucose to lipid was highest in the endoplasmic reticulum and the transfer to polymer highest in the dictyosomes. It seems probable, therefore, that the lipid-linked intermediates are formed first and are involved either in the initial stages of glycosylation of the glycoproteins within the endoplasmic reticulum or they are carried on the membranes to the Golgi apparatus, where the second transfer into polymer occurs. The synthesis of glycoproteins that contain fucose in animal cells may be slightly different; these glycoproteins are integral components of the membranes and are also secreted, and fucose is a terminal sugar of the oligosaccharide chains. The fucosyltransferase occurs mainly in the dictyosomes, and there is no evidence yet for the participation of a polyprenyl phosphate intermediate in the transferase reaction (Molnar, 1976). It is possible that there are integral membrane glycoproteins that contain fucose within the maize root. The polyprenyl phosphate derivatives attached to fucose may be precursors for these components rather than the carrier glycoproteins for the slime polysaccharides. No transfer of radioactivity to polymer was observed when isolated

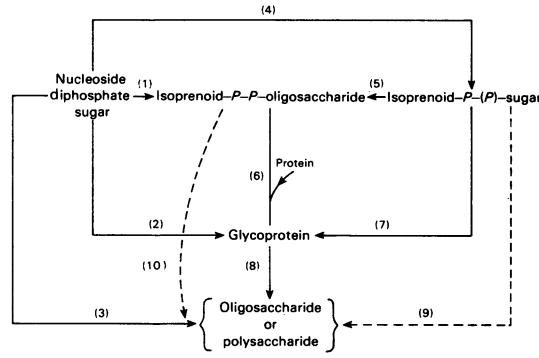

Fig. 4. *Diagram to show the routes to glycoprotein and polysaccharide synthesis*

The initial donors are nucleoside diphosphate sugars (routes 1–4). Isoprenyl phosphate sugars are involved as holder units for assembly of oligosaccharides and also as donors for extension of chains in glycoprotein synthesis (routes 5–7). Protein can function as holder units for some polysaccharides (route 8), hence the isoprenyl sugar phosphates could be involved in polysaccharide synthesis via glycoproteins (routes 6–8). There is at present no convincing evidence for involvement of isoprenyl phosphate sugars as direct donors for polysaccharide assembly or extension of the chains (routes 9, 10).

lipids were incubated with membranes. It is possible that a suitable acceptor was not present.

It has been postulated that lipid intermediates and protein carriers are involved in $\beta(1\rightarrow4)$-glucan synthesis in plants (Brett & Northcote, 1975; Hopp *et al.*, 1978*a*). The present work supports a possible scheme for the synthesis of exported polysaccharides, such as slime, in which both polyprenyl phosphates and proteins act as intermediates (Fig. 4). This is different from the synthesis of storage polymers such as starch (Lavintman *et al.*, 1974), glycogen (Krisman & Barengo, 1975) and paramylon (Tomos & Northcote, 1978), during which a protein primer is involved, but no lipid intermediate.

J. R. G. thanks the Science Research Council for a grant during the tenure of which this work was carried out.

References

Alam, S. S. & Hemming, F. W. (1973) *Phytochemistry* **12**, 1641–1649

Axelos, M. & Peaud-Lenoel, C. (1978) *Biochimie* **60**, 35–44

Babczinski, P. & Tanner, W. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1119–1124

Behrens, N. H., Parodi, A. J. & Leloir, L. F. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2857–2860

Behrens, N. H., Carminatti, H., Staneloni, R. J., Leloir, L. F. & Cantarella, A. I. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3390–3394

Berg, H. C. & Hirsh, D. (1975) *Anal. Biochem.* **66**, 629–631
Bergman, A., Mankowski, T., Chojnacki, T., De Luca, L. M., Peterson, E. & Dallner, G. (1978) *Biochem. J.* **172**, 123–127
Bowles, D. J. & Kauss, H. (1976) *Biochim. Biophys. Acta* **443**, 360–374
Bowles, D. J. & Northcote, D. H. (1972) *Biochem. J.* **130**, 1133–1145
Bowles, D. J. & Northcote, D. H. (1974) *Biochem. J.* **142**, 139–144
Bowles, D. J., Lehle, L. & Kauss, H. (1977) *Planta* **134**, 177–181
Brett, C. T. & Leloir, L. F. (1977) *Biochem. J.* **161**, 93–101
Brett, C. T. & Northcote, D. H. (1975) *Biochem. J.* **148**, 107–117
Bretthauer, R. K. & Wu, S. (1975) *Arch. Biochem. Biophys.* **167**, 151–160
Coughlan, S. (1977) *FEBS Lett.* **81**, 33–36
Ericson, M. C. & Delmer, D. P. (1977) *Plant Physiol.* **59**, 341–347
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **206**, 497–509
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
Galbraith, D. W. & Northcote, D. H. (1977) *J. Cell Sci.* **24**, 295–310
Green, J. R. & Northcote, D. H. (1978) *Biochem. J.* **170**, 599–608
Grunwald, C. (1975) *Annu. Rev. Plant Physiol.* **26**, 209–237
Harris, P. J. & Northcote, D. H. (1970) *Biochem. J.* **120**, 479–491
Hartmann-Bouillon, M. A. & Benveniste, P. (1978) *Phytochemistry* **17**, 1037–1042
Hemming, F. W. (1974) *Biochem. Ser. One* **4**, 39–98
Hopp, H. E., Romero, P. A., Daleo, G. R. & Pont Lezica, R. (1978*a*) *Eur. J. Biochem.* **84**, 561–571
Hopp, H. E., Romero, P. A., Daleo, G. R. & Pont Lezica, R. (1978*b*) *Phytochemistry* **17**, 1049–1052
Hsu, A. F., Baynes, J. W. & Heath, E. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2391–2395
Kirby, K. G. & Roberts, R. M. (1971) *Planta* **99**, 211–221
Krisman, C. R. & Barengo, R. (1975) *Eur. J. Biochem.* **52**, 117–123
Lavintman, N., Tandecarz, J., Careller, M., Mendiara, S. & Cardini, C. E. (1974) *Eur. J. Biochem.* **50**, 145–155
Lehle, L. & Tanner, W. (1975) *Biochim. Biophys. Acta* **399**, 364–374
Lehle, L. & Tanner, W. (1978) *Eur. J. Biochem.* **83**, 563–570
Lehle, L., Fartaczek, F., Tanner, W. & Kauss, H. (1976) *Arch. Biochem. Biophys.* **175**, 419–426
Lehle, L., Bowles, D. J. & Tanner, W. (1978) *Plant Sci. Lett.* **11**, 27–34
Lennarz, W. J. & Scher, M. G. (1972) *Biochim. Biophys. Acta* **265**, 417–441
Lercher, M. & Wojciechowski, Z. A. (1976) *Plant Sci. Lett.* **7**, 337–340
Lewis, B. A. & Smith, F. (1969) in *Thin-Layer Chromatography*, 2nd ed. (Stahl, E., ed.), pp. 807–837, Springer-Verlag, New York
Lord, J. M., Kawaga, T., Moore, S. & Beevers, H. (1973) *J. Cell Biol.* **57**, 659–667
Lowry, O. H., Rosebrough. N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Molnar, J. (1976) *Enzymes Biol. Membr.* **2**, 385–420
Northcote, D. H. (1974) in *Plant Carbohydrate Chemistry and Biochemistry* (Pridham, J. B., ed.), pp. 165–181, Academic Press, London and New York
Northcote, D. H. (1977) in *Cell Surface Reviews* (Poste, G. & Nicholson, G. L., eds.), pp. 1204–1225, Elsevier/North-Holland, Amsterdam
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
Pont Lezica, R., Brett, C. T., Romero Martinez, P. & Dankert, M. A. (1975) *Biochem. Biophys. Res. Commun.* **66**, 980–987
Pont Lezica, R., Romero, P. A. & Dankert, M. A. (1976) *Plant Physiol.* **58**, 675–680
Ray, P. M., Shiniger, T. L. & Ray, M. M. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 605–612
Sharma, C. B., Babczinski, P., Lehle, L. & Tanner, W. (1974) *Eur. J. Biochem.* **46**, 35–41
Shore, G. & Maclachlan, G. A. (1975) *J. Cell Biol.* **64**, 557–571
Speake, B. K. & White, D. A. (1978) *Biochem. J.* **170**, 273–283
Spiro, M. J., Spiro, R. G. & Bhoyroo, V. D. (1976*a*) *J. Biol. Chem.* **251**, 6400–6408
Spiro, R. G., Spiro, M. J. & Bhoyroo, V. D. (1976*b*) *J. Biol. Chem.* **251**, 6409–6419
Spiro, M. J., Spiro, R. G. & Bhoyroo, V. D. (1976*c*) *J. Biol. Chem.* **251**, 6420–6425
Struck, D. K. & Lennarz, W. J. (1976) *J. Biol. Chem.* **251**, 2511–2519
Tomos, A. D. & Northcote, D. H. (1978) *Biochem. J.* **174**, 283–290
Van der Woude, S. J., Lembi, C. A., Morré, D. J., Kindinger, J. I. & Ordin, L. (1974) *Plant Physiol.* **54**, 333–340
Veeger, C. & Zeylemarker, W. P. (1969) *Methods Enzymol.* **13**, 524–526
Vessey, D. A. & Zakim, D. (1975) *Eur. J. Biochem.* **53**, 499–504
Vessey, D. A., Lysenko, N. & Zakim, D. (1976) *Biochim. Biophys. Acta* **428**, 138–145
Waechter, C. J. & Lennarz, M. J. (1976) *Annu. Rev. Biochem.* **45**, 95–113
Waechter, C. J., Lucas, J. J. & Lennarz, W. J. (1974) *Biochem. Biophys. Res. Commun.* **56**, 343–350
Wilson, C. M. (1959) *Anal. Chem.* **31**, 1199–1201
Wojciechowski, Z. A. & Van Von, N. (1975) *Acta Biochim. Pol.* **22**, 25–38
Wright, K. & Northcote, D. H. (1976) *Protoplasma* **88**, 225–239
Zatta, P., Zakim, D. & Vessey, D. A. (1975) *Biochim. Biophys. Acta* **392**, 361–365