Glucosylation of Phosphorylpolyisoprenol and Sterol at the Plasma Membrane of Soya-Bean (*Glycine max*) Protoplasts

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Protoplasts were prepared from cells of soya-bean (*Glycine max*) suspension cultures and the plasma membrane was labelled with diazotized [G-³H]sulphanilic acid. Homogenates were fractionated by differential and isopycnic centrifugation, and membrane fractions in a density gradient were characterized by enzymic markers and the radioactive label. When fractions containing a large amount of protein were incubated with UDP-[U-¹⁴C]glucose, radioactive material soluble in chloroform/methanol was formed and this separated into acidic and neutral fractions on ion-exchange chromatograms of DEAE-cellulose. The acidic fraction was shown to consist of dolichol phosphate glucose, and the neutral fraction sterol glucosides and acylsterol glucosides. Optimum conditions for glucosylation of dolichol phosphate were established as 5 mM-MgCl₂, pH 6.0, and the enzyme had a Michaelis constant of 1.5×10^{-5} M-UDP-glucose. Optimum conditions for glucosylation of sterol were 5 mM-MgCl₂, pH 8.0. GDP-[U-¹⁴C]glucose was a poor substrate for the synthesis of both acidic and neutral lipids. Although the synthesis of dolichol phosphate glucose and sterol glucosides occurred throughout the sucrose gradient, the specific activities of both glucosyltransferases were greatest in a fraction coincident with the radioactively labelled plasma membrane. Results are discussed in relation to the likely role of these transglucosylase activities.

Harris & Northcote (1971) showed that pectic and hemicellulosic polysaccharides are located and probably synthesized in the Golgi apparatus in roots of pea (*Pisum sativum*). Bowles & Northcote (1974, 1976) demonstrated that non-cellulosic polysaccharides could be isolated from both endoplasmic reticulum and dictyosome membranes of maize (*Zea mays*) roots. Whereas the biosynthesis of pectin and hemicellulose occurs mainly within the endomembrane system, the situation is less clear with regard to cellulose. Bowles & Northcote (1972) showed that D-[U-¹⁴C]glucose could be incorporated into a glucan, probably cellulose, that occurred in walls of cells in portions of root undergoing secondary thickening. However, the relative amount of radioactive glucan isolated from vesicles of the endomembrane system was insufficient to account for its biosynthesis within the membranes of the cytoplasm, and it was suggested therefore that the synthesis occurred at the plasma membrane. On the other hand, complexes containing cellulosic glucan, protein and other polysaccharides have been shown to be present within the dictyosome membranes of Haptophycean algae (Manton, 1967*a,b*; Herth *et al.*, 1972; Allen & Northcote, 1975), and certain root-cap mucilages, which are probably synthesized with-

in the Golgi apparatus, are known to contain β -(1→4) glucans (Wright & Northcote, 1976). Although the enzymes responsible for cellulose synthesis are therefore likely to be present at the membranes of the Golgi apparatus of higher plants, expression of their activity probably only occurs after membrane fusion at the plasma membrane, except in certain specialized tissues.

Nucleotide sugars are precursors in the biosynthesis of glycoproteins in plants (Ericson & Delmer, 1977; Nagahashi & Beevers, 1978; Pont Lezica *et al.*, 1978), animals (Struck & Lennarz, 1977; Weiser *et al.*, 1978), and microorganisms (Wright *et al.*, 1967; Lehle & Tanner, 1978; Parodi, 1978), and of various glycans in micro-organisms (Sentandreu *et al.*, 1975) and plants (Van Der Woude *et al.*, 1974; Shore & MacLachlan, 1975; Helsper *et al.*, 1977). UDP-glucose is probably a precursor of β -(1→3) and β -(1→4) glucans in plants (Brett & Northcote, 1975; Helsper *et al.*, 1977). However, it gives rise to β -(1→3) glucan predominantly, especially at high substrate concentration (Tsai & Hassid, 1971; Van Der Woude *et al.*, 1974), although the proportion of β -(1→4) links may be increased by the presence of up to 10 mM-Mg²⁺ and high concentrations of glucose and of cellobiose (Larsen & Brummond, 1974).

GDP-glucose has given rise to β -(1 $\rightarrow$ 4) but not β -(1 $\rightarrow$ 3) glucan (Barber *et al.*, 1964; Helsper *et al.*, 1977). Thus the correct substrates for cellulose synthesis are not known at present.

Recent schemes (Brett & Northcote, 1975; Hopp *et al.*, 1978a) for the pathway of cellulose biosynthesis have suggested that a glucose donor such as UDP-glucose gives rise to cellulose via dolichol monophosphate glucose, dolichol pyrophosphate glucose and sequentially, dolichol pyrophosphate oligoglucan and glucoprotein. However, the subcellular distribution of such reactions is unknown. In the present paper we describe the location in density gradients of membranes from soya-bean (*Glycine max*) protoplasts. The membranes of the Golgi apparatus and endoplasmic reticulum have each been characterized using two enzymic activities. Unambiguous location of the plasma membrane, with diazotized [G - 3H]sulphanilic acid as a label, has enabled determination of the subcellular distribution of glucosyltransferases able to utilize UDP-glucose and GDP-glucose. Properties of these enzymes have been determined, and reaction products characterized.

Materials and Methods

All aqueous reagents contained glass-distilled water. Driselase was obtained from the Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Pectinase and Ficoll 400 were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K., stainless-steel mesh from R. Cadisch and Sons, Finchley, London N.3, U.K. and metrizamide from Nyegaard and Co., Oslo, Norway. [G - 3H]Sulphanilic acid (sp. radioactivity 2.8 mCi/mmol) was prepared by catalytic exchange in a 3H -labelled aqueous medium by The Radiochemical Centre, Amersham, Bucks., U.K., from non-radioactive sulphanilic acid, and was supplied as an impure solution (3 Ci). UDP-D-[U- ^{14}C]glucose (sp. radioactivity 328 mCi/mmol), UDP-D-[6- 3H]glucose (3.7 Ci/mmol), GDP-D-[U- ^{14}C]glucose (290 mCi/mmol), D-[U- ^{14}C]glucose (292 mCi/mmol) and [U- ^{14}C]sucrose (381 mCi/mmol) were obtained from The Radiochemical Centre. Other chemicals were of analytical grade.

Cell culture

Soya-bean (*Glycine max*, var. biloxi) callus tissue was maintained as a suspension culture in a defined medium (Miller, 1967) containing 500 μ g of kinetin/litre, in an orbital incubator at 100 rev./min, at 25°C and in darkness. Medium was autoclaved at 120°C and 103 kPa for 30 min before use. Cells were subcultured at 48 h intervals by transfer of approx. 20 ml of cells to 150 ml of fresh medium.

Protoplast preparation

Protoplasts were prepared from cells harvested 48 h after subculture, since at this time protoplast yield was greatest. This was probably because a greater number of cells than at earlier stages had undergone division, having as a result fragile cell walls. Protoplasts were prepared as described by Galbraith & Northcote (1977).

Radioactive labelling of protoplasts, homogenization and fractionation

Before use, [G - 3H]sulphanilic acid was purified by using a solvent system consisting of ethyl acetate/propan-2-ol/ NH_3 /water (6:4:2:1, by vol.), on silica-gel thin-layer plates (20 cm $\times$ 20 cm, 2 mm; Macherey-Nagel, Düren, Germany).

A solution (100 μ l) containing 22 μ Ci of sulphanilic acid was freeze-dried and converted into the diazonium salt by the method of Berg & Hirsh (1975). The diazotization mixture contained, in addition to the sulphanilic acid (22 μ Ci), 2 μ l (2 μ mol) of HCl, and 50 μ l (300 μ mol) of isopentyl nitrite. Protoplasts were labelled with radioactive diazotized sulphanilic acid, washed, homogenized, and the homogenate fractionated by differential and isopycnic centrifugation (Galbraith & Northcote, 1977). Fractions were collected by using a peristaltic pump attached to a capillary tube. Sucrose was determined by a hand refractometer. Phenylmethanesulphonyl fluoride (100 mM) in propan-2-ol (Galbraith & Northcote, 1977) was added to homogenate and gradient media immediately before they were used (James, 1978).

Analyses

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Protein in gradient fractions was determined without prior precipitation by trichloroacetic acid. Since the sucrose, Tris/HCl and EDTA present in these fractions inhibit the Lowry reaction, each was included in a range of protein standards. The alkali reagent contained 1% sodium dodecyl sulphate, which had no inhibitory affect (Ji, 1973) and served to disperse the particulate membranes.

Radioactivity was measured by using a Searle Mark III liquid-scintillation analyser. Toluene-based scintillant was used throughout.

Thiamin pyrophosphatase was measured by the method of Morr (1971). Fumarase, NADH- and NADPH-cytochrome *c* reductases, IDPase, ATPase and acid phosphodiesterase were assayed as described by Galbraith & Northcote (1977).

Glucosyltransferase assays

Incubations (200 μ l in tubes 1.5 cm $\times$ 10 cm) contained 5 mM-MgCl $_2$, 100 mM-Tris/HCl, pH 8.0, or 100 mM-Mes (4-morpholine-ethanesulphonic acid),

pH 6.0, 0.2 μ Ci of UDP-D-[U- 14 C]glucose or UDP-D-[6- 3 H]glucose, and up to 150 μ g of protein. They were carried out at 25°C with reciprocal shaking, stopped after 1 h with 2 ml of chloroform/methanol (2:1, v/v) and washed by the method of Folch *et al.* (1957). The radioactivity in the lipid-soluble material was analysed as described below. UDP-D-[6- 3 H]-glucose was not used for counting of radioactivity on t.l.c. plates or chromatographic paper.

Fractionation of lipids

Chloroform/methanol-soluble material was separated into neutral and acidic fractions by ion-exchange chromatography. Lipid extracts (0.5 ml) were applied to a DEAE-cellulose (Whatman DE52) column, diameter 1 cm, bed volume 6 ml, pre-equilibrated with potassium acetate and washed with chloroform/methanol (3:2, v/v). Thymol Blue was used as an internal marker for acidic lipid. Neutral lipid was eluted with 30 ml of chloroform/methanol (3:2, v/v), and acidic lipid with 0.1 M-ammonium formate in chloroform/methanol. Formate and Thymol Blue were removed from fractions (5 ml) by Folch (Folch *et al.*, 1957) washing.

During experiments involving fractionation of large numbers of lipid samples, an alternative method of separation was used. Chloroform/methanol extracts (100 μ l) were applied to individual DEAE-cellulose (Whatman DE81) discs (2.3 cm diam.) and dried. Neutral lipid was eluted from discs by washing five times with 1 ml of chloroform/methanol (2:1, v/v). Eluates were separately combined, dried, and together with discs, assayed for radioactivity.

By using authentic radioactive dolichol monophosphate glucose and radioactive neutral lipid prepared from soya-bean tissue by ion-exchange chromatography, we have found that, whereas DEAE-cellulose paper discs retain no neutral lipid, retention of acidic lipid is almost quantitative.

Preparation of standard dolichol monophosphate [U- 14 C]glucose

Porcine liver (500 g) was homogenized twice in acetone and the residue rehomogenized twice in chloroform/methanol (2:1, v/v). The combined chloroform/methanol extracts were evaporated to dryness, and hydrolysed first in 5 ml of 1 M-HCl at 100°C for 10 min, and then after neutralization in 1 M-NaOH at 50°C for 10 min. The neutralized aqueous material was extracted twice with 5 vol. of chloroform/methanol (2:1, v/v) to give a crude lipid fraction A. Samples were chromatographed on thin-layer plates (Polygram SIL G, 20 cm $\times$ 20 cm $\times$ 0.25 cm) in chloroform/methanol/formic acid (70:10:1, by vol.) and stained with I_2 vapour. The preparation resolved into constituents having R_F values of 0, 0.6, 0.8 and 0.9. Constituents

with R_F values of 0 and 0.6 stained with molybdenum spray (Dittmer & Lester, 1964). Dolichol phosphate has an R_F value between 0.5 and 0.7 (Brett & Leloir, 1977). Acidic lipids were obtained from this fraction A by elution from a DEAE-cellulose column with 0.1 M-ammonium formate (Behrens & Leloir, 1970).

Samples of the acidic lipid (containing dolichol monophosphate) were dried under vacuum, and incubated at 30°C for 30 min with a rat liver microsomal preparation. Incubations contained 5 μ l of 0.1 M-MgCl₂, 10 μ l of 0.5 M-Tris/maleate, pH 7.8, 10 μ l of 0.5% Triton X-100; 5 μ l of 1 M-mercaptoethanol, 0.2 μ Ci of UDP-[U- 14 C]glucose, 15 μ l of water; and 5 μ l of microsomal fraction. Incubations were stopped with 2 ml of chloroform/methanol and the glucosylated acceptor lipid re-purified by DEAE-cellulose column chromatography.

The microsomal fraction was prepared by homogenizing with a Dounce homogenizer a rat liver in 20 ml of 0.25 M-sucrose, containing 1 mM-MgCl₂ and 10 mM-Tris/HCl, pH 7.5. After filtration through muslin, the preparation was centrifuged at 500g for 5 min and at 10000g for 30 min to remove nuclei, cell debris and mitochondria. The microsomal supernatant was then centrifuged at 100000g, and the pellet resuspended in an equal volume (5 ml) of 0.25 M-sucrose, containing 5 mM-EDTA and 10 mM-mercaptoethanol.

T.l.c. of the glucosylated lipids

Pre-washed Polygram SIL G silica-gel thin-layer plates (20 cm $\times$ 20 cm $\times$ 0.25 mm) without gypsum binder were used.

Before glucosylation, samples of the purified porcine acceptor lipid were chromatographed in chloroform/methanol/formic acid (70:10:1, by vol.) (Brett & Leloir, 1977). Porcine dolichol monophosphate glucose and soya-bean glucosylated acceptor lipids were chromatographed by using the following solvent systems: (A) chloroform/methanol/water (65:25:4, by vol.); (B) chloroform/methanol/35% NH₃ (75:25:4, by vol.); (C) chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Chloroform and methanol were redistilled before use. Chromatographed lipids were stained with I_2 vapour, phospholipids by the method of Dittmer & Lester (1964) and sterols by the method of Kaus (1968). Chromatographic plates were cut into 4 cm $\times$ 1 cm sections and radioactivity determined by liquid-scintillation counting.

Results

Characteristics of the membrane fractions

The distribution of specific activities of marker enzymes in fractions of membranes separated by density-gradient centrifugation is shown in Figs. 1–4.

Mitochondrial fumarase activity (Fig. 1) was greatest at a density of 1.18 g/ml. The specific activity of this enzyme between densities 1.15 and 1.08 g/ml was approx. 5% of the value at a density of 1.18 g/ml. This activity was probably the result of mitochondrial contamination due to the presence of aggregates of mixed organelles rather than of enzyme released from broken mitochondria, since more than 99% of the fumarase activity present in homogenates was recovered in a pellet after centrifugation at 10000g.

Endoplasmic reticulum, partially free of ribosomes, was detected by two marker enzymes,

Fig. 1. Distribution within gradients of (●) fumarase, (□) NADH-cytochrome *c* reductase and (■) NADPH-cytochrome *c* reductase

A 100000g membrane pellet was placed on a continuous gradient of 0.6–1.8M-sucrose, containing 1mM-EDTA and 10mM-Tris, pH 7, and centrifuged in a Beckman SW27 rotor for 3h at 100000g. Fumarase, NADH- and NADPH-cytochrome *c* reductases were determined in 1.2ml fractions collected after centrifugation.

Fig. 2. Distribution within gradient of (●) IDPase and (■) acid phosphodiesterase

A 100000g membrane pellet was placed on a continuous gradient of 0.6–1.8M-sucrose, containing 1mM-EDTA and 10mM-Tris, pH 7, and centrifuged in a Beckman SW27 rotor for 3h at 100000g. IDPase and acid phosphodiesterase were determined in 1.2ml fractions collected after centrifugation.

NADH- and NADPH-cytochrome *c* reductases, and was present between densities 1.15 and 1.10 g/ml with a maximum at a density of 1.12 g/ml (Fig. 1). NADH-cytochrome *c* reductase had total and specific activities approx. 10 times that of the NADPH-specific enzyme. In some experiments a peak of NADH-cytochrome *c* reductase activity appeared at a density of 1.18 g/ml, but specific activities were generally less than in those fractions containing endoplasmic reticulum. The occurrence of this enzyme in mitochondria has been reported (Lord *et al.*, 1973).

The distribution of plasma membrane was indicated by that of the radioactive sulphanilic acid, and the highest specific radioactivity occurred at a density of 1.15 g/ml (Fig. 3). At this density the maximum specific activity of ATPase occurred. ATPase activities are present at the plasma membrane of cells of plants, animals and micro-organisms. They may represent either an electrogenic proton pump or an ATP-linked ion-transportation mechanism.

Membranes derived from the Golgi apparatus were detected by using two enzymic activities established as markers for that organelle. Both latent IDPase and thiamin pyrophosphatase had maximum specific activities on membranes present at a density of 1.13 g/ml (Figs. 2 and 4).

A variety of hydrolase activities including phosphodiesterase have been detected in vacuoles isolated from a number of higher-plant tissues (Leigh, 1979). Phosphodiesterase [substrate bis(*p*-nitrophenyl)phosphate] had a maximum specific activity at a density of 1.13 g/ml and may indicate that mem-

Fig. 3. Distribution within gradients of (▲) Mg²⁺-dependent ATPase and (■) diazotized [³H]sulphanilic acid

A 100000g membrane pellet was placed on a continuous gradient of 0.6–1.8M-sucrose, containing 1mM-EDTA and 10mM-Tris, pH 7, and centrifuged in a Beckman SW27 rotor for 3h at 100000g. Mg²⁺-dependent ATPase and radioactivity were determined in 1.2ml fractions collected after centrifugation.

branes derived from the tonoplast were present at this level of the density gradient (Fig. 2).

The marker-enzyme activities that hydrolysed phosphate esters or pyrophosphates were determined by using specific substrates; however, no assumption is made that these are specific and separate enzymes.

Spectrophotometric analysis at 280nm of density gradients indicated that maximum amounts of protein occurred at densities of 1.18 and 1.13 g/ml (Fig. 5). The maximum specific activities of marker enzymes in the gradient relative to those of both homogenate and 100 000 g pellet are shown in Table 1. Fumarase and the cytochrome *c* reductases showed the highest relative purifications.

Glucosyltransferases

Labelling of the protoplast plasma membrane with diazotized [G-³H]sulphanilic acid did not alter the distribution within gradients of protein (measured by absorption at 280nm) (Fig. 5). However, labelling the protoplast plasma membrane was omitted during experiments involving incubation of isolated membrane fractions with radioactive nucleotide sugars.

Gradients were fractionated into samples (1.2 ml) and the sucrose and protein concentration of each was measured. In order to determine the optimum conditions for glucosyl transfer to both neutral and acidic lipid and to characterize the glucosylated products, a membrane fraction of density 1.13 g/ml was

Fig. 4. Distribution within gradients of thiamin pyrophosphatase

A 100 000 g membrane pellet was placed on a continuous gradient of 0.6–1.8 M-sucrose, containing 1 mM-EDTA and 10 mM-Tris, pH 7, and centrifuged in a Beckman SW27 rotor for 3 h at 100 000 g. Thiamin pyrophosphatase was determined in 1.2 ml fractions collected after centrifugation.

Fig. 5. Distribution within gradients of (●) protein from protoplasts labelled with diazotized [³H]sulphanilic acid and (▲) protein from unlabelled protoplasts

A preparation of protoplasts was divided into two portions and one was labelled with diazotized sulphanilic acid. Pellets (100 000 g) prepared from homogenates of each were placed on a continuous gradient of 0.6–1.8 M-sucrose, containing 1 mM-EDTA and 10 mM-Tris, pH 7, and centrifuged in a Beckman SW27 rotor for 3 h at 100 000 g. Protein in 1.2 ml fractions collected after centrifugation was determined from A₂₈₀.

Table 1. Enzyme activities and radioactive label used to characterize membrane fractions
Purifications represent: (1) the maximum specific activity in the gradient divided by that of the homogenate; (2) the maximum specific activity in the gradient divided by that of the 100 000 g pellet.

Enzyme activity	Probable subcellular localization	Purification (fold)	
		(1)	(2)
Fumarase	Mitochondria	84.8	6.2
NADH–cytochrome <i>c</i> reductase	Mitochondria and endoplasmic reticulum	4.9	3.1
NADPH–cytochrome <i>c</i> reductase	Endoplasmic reticulum	10.8	9.3
Mg ²⁺ -dependent ATPase	Plasmalemma	3.2	1.6
Diazotized sulphanilic acid	Plasmalemma	2.9	1.7
Latent IDPase	Golgi apparatus	13.8	3.4
Acid phosphodiesterase	Tonoplast	1.7	1.6
Thiamin pyrophosphatase	Golgi apparatus	19.8	2.1

selected. This fraction contained a large amount of transferase activity together with a relatively high amount of protein. Thus although the specific activities of the transferases in this fraction were not the greatest that occurred in the gradient it did provide a large total amount of activity.

Since membrane preparations contain enzymes that are able to hydrolyse nucleotide sugars, an attempt was made to provide these enzymes with an alternative substrate in the form of a nucleotide. Incubations contained 8 mM-ATP or -AMP (Vessey & Zakim, 1975). Aqueous extracts of incubations electrophoresed at pH 2 showed that between 82 and 86% of the radioactivity they contained occurred in material having an electrophoretic mobility similar to that of the UDP-glucose, irrespective of the presence of AMP or ATP in the original incubation. Probably, therefore, loss of UDP-glucose through

hydrolysis was significant only at very low concentrations.

The optimum concentration of Ca^{2+} or Mg^{2+} for glucosyltransferase activities to both neutral and acidic lipid was 5 mM. There was no detectable difference between the effects of Mg^{2+} and Ca^{2+} .

The effect of pH on the transfer of radioactivity from UDP-[U- ^{14}C]glucose to the lipid acceptors was determined. Incubations contained 100 mM-Tris/Mes buffer of the appropriate pH. An optimum value for the synthesis of acidic lipid was found to be pH 6.0, and for neutral lipid, pH 8.0.

Time courses of incorporation of radioactivity into the glucosylated lipids were determined. In both neutral and acidic lipid the amount of radioactivity incorporated reached a maximum after 1 h. Further UDP-[U- ^{14}C]glucose added after this time caused no additional incorporation. The incorporation was linear for approx. the first 20 min of the incubation.

The effects of variation in substrate concentration by the addition of non-radioactive UDP-glucose are shown in Figs. 6 and 7. Incubations (20 min) contained 3 or 6 μM -UDP-D-[U- ^{14}C]glucose. An average result for three experiments was used to construct an Eisenthal (Eisenthal & Cornish-Bowden, 1974) kinetic plot for the UDP-glucose-acid lipid transferase from which a value of $1.5 \times 10^{-5} \text{ M}$ was obtained for the K_m with respect to this substrate (Fig. 8).

The presence in incubations of non-radioactive

Fig. 6. Effect of UDP-glucose concentration on the transfer of glucose from UDP-glucose to the neutral lipid. Incubations contained 3 or 6 μM -UDP-D-[U- ^{14}C]glucose, and non-radioactive UDP-glucose to vary the total UDP-glucose concentration up to 200 μM .

Fig. 7. Effect of UDP-glucose concentration on the transfer of glucose from UDP-glucose to the acidic lipid. Incubations contained 3 or 6 μM -UDP-D-[U- ^{14}C]glucose and non-radioactive UDP-glucose to vary the total UDP-glucose concentration up to 200 μM .

Fig. 8. Eisenthal (Eisenthal & Cornish-Bowden, 1974) plot of data presented in Fig. 7. Each line represents one observation of substrate concentration (s) and reaction velocity (v) and has intercepts $-s$ and v on the appropriate axes.

glucose, maltose and cellobiose was, in separate experiments, examined for any effect upon the transfer of radioactivity from UDP-[U-¹⁴C]glucose to the acidic lipid. Each sugar was included in incubations at a range of concentrations between 0 and 100 mM. In each case no effect was observed.

The possibilities that sucrose and GDP-glucose might act as glucosylating agents, and that free glucose might in some way be incorporated into the lipid of the membranes, were investigated. Incubations contained 30 μ M-[U-¹⁴C]glucose or [U-¹⁴C]-sucrose. Membranes used for incubation with radioactive sucrose were prepared by using continuous density gradients of metrizamide instead of sucrose. Neither radioactive sucrose nor radioactive glucose transferred radioactivity to either lipid. However, GDP-[U-¹⁴C]glucose was an effective precursor of radioactive glucosylated acidic lipid. At an identical molar concentration (3 μ M) to that of UDP-glucose it gave less incorporation (10–50%) than that from UDP-glucose. However, the amount of radioactive incorporation into neutral lipid was between 0.7 and 2.5% of that extracted from incubations containing a similar concentration (3 μ M) of UDP-[U-¹⁴C]-glucose.

In two experiments the presence of 0.1 mM non-radioactive GDP-glucose was sufficient to decrease incorporation of radioactivity into acidic lipid from 3 μ M-UDP-[U-¹⁴C]glucose to approx. 25% of the original incorporation. Similarly, the presence of 0.1 mM non-radioactive UDP-glucose decreased the incorporation from 3 μ M-GDP-[U-¹⁴C]glucose to approx. 35%. It seemed likely, therefore, that the same enzyme was involved for the glucosylation of acidic lipid from both substrates.

Subcellular distribution of glucosyltransferase activities

Two incubations of each fraction of the density gradient were combined before isolation of neutral and acidic glucosylated lipids. The results are shown in Fig. 9. Both transferase activities were present throughout the gradient, but attained maximum specific activities in a fraction of density 1.15 g/ml.

Characterization of the glucosylated acidic lipid

Stability to acid and alkali. The radioactive glucosylated lipid was extracted from incubations (approx. 15) of soya-bean membrane (buoyant density 1.13 g/ml) with UDP-glucose. It was separated into acid and neutral fractions by the use of a DEAE-cellulose column. Samples of the acidic fraction (approx. 4000 c.p.m.) were dried under vacuum, incubated in 1 ml of 0.1 M-KOH at 37°C for 20 min and extracted with 2 ml of chloroform/methanol (2:1, v/v). The chloroform/methanol extracts were washed once, and the washings added to aqueous extracts, which were neutralized. Both chloroform/

Fig. 9. Distribution within gradients of (●) UDP-glucose-sterol glucosyltransferase (neutral lipid) and (■) UDP-glucose-dolichol phosphate glucosyltransferase (acidic lipid)

A 100 000 g membrane pellet was placed on a continuous gradient of 0.6–1.8 M-sucrose containing 1 mM-EDTA and 10 mM-Tris, pH 7, and centrifuged in a Beckman SW27 rotor for 3 h at 100 000 g. Glucosyltransferase activities were assayed in 1.2 ml fractions collected after centrifugation.

methanol and aqueous extracts were dried and counted for radioactivity. In all cases, between 85 and 90% of the radioactivity was present in the chloroform/methanol layer, indicating that the compound was stable to dilute alkali at 37°C. Separate samples of the radioactive glucosylated acidic lipid were treated with 1 ml of HCl (0.01 M or 1.0 M) at 100°C for 10 min, cooled and separated into water and chloroform/methanol phases. Control hydrolysates containing water instead of HCl gave 10% of the radioactivity in the aqueous phase. Both 0.01 M and 1 M acid hydrolysates gave between 70 and 80% of the radioactivity in the aqueous phase; it was concluded that the glucosylated lipid was unstable to dilute HCl.

Sugar component. In further experiments the sugar component of the acidic lipid was investigated. The material (approx. 2000 c.p.m.) was hydrolysed with 1 M-HCl at 100°C for 10 min, cooled, washed with chloroform/methanol, and the aqueous fraction neutralized with IR-45 Amberlite (HCO₃⁻) ion-exchange resin. The neutralized material was rotary-evaporated to a small volume and applied to Whatman no. 1 chromatography paper. Chromatograms were developed with ethyl acetate/pyridine/water (8:2:1, by vol.) for 30 h. Marker sugars (glucose, cellobiose and maltose) were located with alkaline AgNO₃ (Trevelyan *et al.*, 1950), and the strips of the chromatogram that carried the hydrolysate were cut into 4 cm × 1 cm sections and counted for radioactivity (Harris & Northcote, 1970). In all experiments, radioactivity chromatographed with glucose, and no additional peaks of radioactivity were found.

Mobility on thin-layer chromatograms. [U-¹⁴C]-glucosylated purified porcine and the soya-bean acceptor lipids were chromatographed in solvents A (neutral), B (alkaline) and C (acidic). The porcine lipid had an R_F between 0.3 and 0.5 in solvent A; an R_F of 0.2 in solvent B; and an R_F between 0.7 and 0.8 in solvent C. These R_F values correspond to those of dolichol monophosphate glucose (Forsee & Elbein, 1973; Pont Lezica *et al.*, 1975; Bowles *et al.*, 1977). The soya-bean lipid chromatographed with the porcine lipid and had R_F values between 0.3 and 0.4 in solvent A, 0.25 in solvent B and 0.9 in solvent C. When the radioactive-lipid preparation from both porcine tissue and soya-bean was chromatographed, they gave a blue spot with the molybdate reagent (Dittmer & Lester, 1964) that corresponded to the radioactivity.

Transfer of glucose from the glucosylated lipid. The glucosylated acidic lipid obtained from soya-bean could have been dolichol monophosphate glucose, and the reverse of the glucosyltransferase reaction might therefore have been possible. Membranes were incubated with UDP-[6-³H]glucose, and after 30 min the reaction was stopped by cooling in ice. The membranes were collected and washed free of non-covalently bound radioactivity by centrifugation in a Beckman SW65 rotor at 100 000 g on to a 1.8 M-sucrose cushion containing 100 mM-Mes, pH 6.0, and 5 mM-MgCl₂, and resuspended in 100 mM-Mes, pH 6.0, buffer containing 5 mM-MgCl₂. The washing was repeated five times. The radioactive membranes (approx. 10 000 c.p.m. in acidic lipid) were suspended in buffer (1 ml) containing UDP (0.022 μ mol). After reincubation at 25°C for 1 h, the reaction was stopped with chloroform/methanol and the water-soluble and lipid fractions extracted. The lipid fraction was separated into neutral and acidic lipids on a DEAE-cellulose column. Of the total extractable radioactivity (3400 c.p.m.), 26% was in the aqueous phase. Incubation with UTP (0.02 μ mol) or UMP (0.02 μ mol) did not convert the radioactivity of the membranes into water-soluble material. Since the transglucosylation occurred in a heterogeneous system, no equilibrium constant for the reaction could be calculated.

Characterization of the glucosylated neutral lipid

Acid-stability. A sample of radioactive neutral lipid containing approx. 12 000 c.p.m. was prepared by incubation of soya-bean membrane (buoyant density 1.13 g/ml) with UDP-D-[U-¹⁴C]glucose, followed by DEAE-cellulose chromatography of the product soluble in chloroform/methanol. The sample was divided into six equal fractions and dried under N₂. HCl (1 ml of one of the concentrations, 0, 0.01, 0.05, 0.1, 0.5, 1.0 M) was added to each tube. The solutions were heated at 100°C for 10 min, cooled

and extracted once with 2 ml of chloroform/methanol (3:2, v/v). The chloroform/methanol extract was washed once with 'theoretical' aqueous upper phase (water/methanol/chloroform/1 mM-MgCl₂, 2000:2080:126:7, by vol.) (Folch *et al.*, 1957), and the washing added to the aqueous extracts of the incubations. Aqueous and chloroform/methanol extracts were dried and counted for radioactivity. Hydrolysis of the lipid to produce water-soluble radioactivity was 13% when the lipid was treated with 1 M-HCl. At the other concentrations, little or no hydrolysis occurred. Hydrolysis of sterol glucosides is accomplished by 1 M-HCl in methanol/H₂O (1:1, v/v) for 1 h at 100°C (Ongun & Mudd, 1970).

Glucan component. A sample of radioactive neutral lipid was hydrolysed with 1 M-HCl at 100°C for 10 min and the hydrolysate examined for radioactive sugars in a manner identical with that used for the glucosylated acidic lipid. All the radioactivity on the chromatogram moved to a position similar to that of glucose. It is unlikely that, in these experiments, hydrolysis had broken many linkages between successive glucose residues of a possible lipid oligosaccharide. Acyl sterol glucosides would probably have been deacylated, however.

T.l.c. Samples of the radioactive neutral lipid were chromatographed in solvent system A. Two peaks of radioactivity were observed, with R_F values of 0.83 and 0.95, and both were coincident with a red colour produced after plates had been sprayed with antimony trichloride (33%, w/v, in chloroform). This reagent produces a red colour with sterols (Kauss, 1968). R_F values of 0.83 and 0.95 are similar to those recorded for sterol glucoside and acylated sterol glucoside (Bowles *et al.*, 1977; Kauss, 1968).

Discussion

The mean buoyant density of the plasma membrane obtained in the present work was 1.15 g/ml. This was different from that of the mitochondria, Golgi apparatus and endoplasmic reticulum, so that these membranes were partially purified within the gradient during the centrifugation.

A density of 1.15 g/ml is greater than that for plasma membrane obtained from pea epicotyl (Shore & MacLachlan, 1975), but is similar to that observed for plasma membrane from maize (*Zea mays*) (Hartmann-Bouillon & Benveniste, 1978; Leonard & Hotchkiss, 1978).

During the present work it was found important to discard protoplast preparations that had an excessive amount of cell wall remaining attached to the plasma membrane. Failure to discard preparations that showed episcopic u.v. fluorescence when treated with Calcofluor (Bright & Northcote, 1974) led to

the recovery of significant amounts of plasma membrane at buoyant densities greater than that normally obtained, and led to recovery in the 480g pellet of increased amounts of radioactivity from the radioactive sulphanilic acid used to label the plasma membrane.

In order to characterize the transglucosylase activities that occurred in the density gradient, it was necessary to identify the glucosylated products. Incubation of membranes obtained from protoplasts with UDP-glucose led to the production of radioactivity soluble in chloroform/methanol, which resolved into a neutral and an acidic fraction by ion-exchange chromatography. Since the acidic fraction was eluted from DEAE-cellulose by 0.1M-ammonium formate and co-eluted with Thymol Blue, it was probably a monophosphate. A pyrophosphate would have eluted in 0.2M or higher salt concentration. The acidic lipid was stable to dilute alkali, but was hydrolysed by dilute acid (Adamany & Spiro, 1975; Herscovics *et al.*, 1977), and the radioactive sugar component consisted of a single glucose residue. The lipid had a chromatographic mobility in three solvent systems similar to that of authentic dolichol monophosphate glucose and the lipid extract contained phosphorus. The radioactivity of the glucose residue in the lipid became water-soluble when the membranes were incubated with uridine diphosphate. It was concluded that the glucosylated acidic acceptor lipid consisted of dolichol monophosphate glucose.

The neutral radioactive lipid fraction was not retained by a DEAE-cellulose column, and contained two radioactive components. When chromatographed these had R_F values consistent with those of sterol glucosides and acylated sterol glucosides. Both components showed a positive reaction to a stain (antimony chloride in chloroform) specific for sterols. The radioactive lipid fraction had a stability to dilute acid similar to that reported for sterol glucosides. The saccharide portion, when removed under conditions that would probably not have broken many linkages between successive glucose residues, contained glucose only. It was concluded that the neutral fraction contained sterol glucosides and acylsterol glucosides.

The sterol glucosides were synthesized by a transglucosylase that had a highest specific activity coincident with that of the radioactivity of the membranes when these were labelled with diazotized [^3H]-sulphanilic acid. The curve of the transferase activity showed a sharp peak, and there was little enzyme activity at densities less than 1.15g/ml, where the membranes of the Golgi apparatus and endoplasmic reticulum occurred in the gradient. It was probable, therefore, that this transglucosylase activity was located mainly at the plasma membrane. UDP-glucose was a better substrate for this transglucosylase

than GDP-glucose. This is consistent with the observation that, in the alga *Prototheca zopfii* (Hopp *et al.*, 1978b), the greatest transglycosylation occurred with nucleotide sugars that contained uridine and glucose. In the plant cell the quantity of UDP-glucose present is probably far greater than that of other nucleotide sugars (Hassid, 1967). Therefore it is doubtful whether nucleotide sugars other than UDP-glucose contribute significantly to the glucosylation of sterols *in vivo*.

The function of the UDP-glucose-sterol glucosyltransferase is not known. Kauss (1968) showed that the glucosyl units in sterol glucosides of mung beans (*Phaseolus aureus*) were not subsequently transferred, and they are hence probably not lipid intermediates for polysaccharide synthesis. The transglucosylase is probably not able to glucosylate directly cell-wall polysaccharides, since although the enzyme is able to utilize several sterols with almost equal facility (Hopp *et al.*, 1978b; Forsee *et al.*, 1974), it shows an absolute requirement for sterol as the acceptor (Katinowska & Wojciechowski, 1978). Interaction between the phosphate moieties of phospholipids and the 3β -hydroxy group of sterols is known to influence physical characteristics of membranes (Bittman & Blau, 1972; Hisa *et al.*, 1972). Glucosylation of the 3β -hydroxy group of sterols and subsequent acylation must affect this interaction and also those between lipid and protein (Bowles *et al.*, 1977). Therefore the transglucosylase may control the physical state of the membranes.

The distribution of the specific activity of the UDP-glucose-dolichol phosphate glucosyltransferase within the gradient indicated that the highest specific activity occurred, as for the sterol-specific transferase, at the plasma membrane (mean buoyant density approx. 1.15g/ml). However, the curve has not such a sharp peak as that of the sterol-specific transferase activity, and the distribution especially at the lower densities indicated that although the enzyme activity was more concentrated at the plasma membrane, there was activity in the region of the gradient where the membranes of the Golgi apparatus and endoplasmic reticulum occurred.

Dolichol phosphate is present in many animal organs, and in the liver is a component of all the cellular membranes (Dallner *et al.*, 1972). In plants it is present in membranes of the endoplasmic reticulum, and in the Golgi apparatus (Bowles *et al.*, 1977; Nagahashi & Beevers, 1978), since it is able to act as a glycosyl acceptor at these positions. It has been demonstrated that dolichol phosphate synthesis occurs in the mitochondria and Golgi apparatus of the alga *Prototheca zopfii* (Hopp *et al.*, 1978c).

Polyprenol monophosphate glucose is known to be involved in the synthesis of glycoprotein in animals (Parodi & Leloir, 1979) and possibly in plants (Ericson & Delmer, 1978). The initial gly-

cosylation of protein occurs in the endoplasmic reticulum (Lodish & Rothman, 1979), and subsequent glycosylation of glycoproteins and polysaccharides occurs in both the endoplasmic reticulum and the Golgi apparatus (Northcote, 1979). The glycosylation that extends the sugar chains of glycoproteins can be accomplished by both nucleoside diphosphate sugar and polyprenol phosphate sugar compounds. It is also possible that polysaccharides are assembled on proteins within the membrane system of the cell (Green & Northcote, 1978; Northcote, 1979). Since we have now shown that dolichol phosphate glucose can be formed at the plasma membrane, it is possible that extension of the sugar chains of glycoprotein can occur at the plasma membrane in addition to the endoplasmic reticulum and Golgi apparatus. It may be that cellulose and callose are synthesized via glycoprotein intermediates (Hopp *et al.*, 1978a; Tomos & Northcote, 1978) and the extension of the glucose chains of these polysaccharides could therefore be by the dolichol phosphate glucose synthesized at the plasma membrane.

Glycosyltransferases that transfer sugars from nucleotide sugars to dolichol phosphate have been demonstrated only in membranes, and probably do not occur in the cytosol. The glycoproteins that are formed are usually either components of the membrane or separated from the nucleotide sugars of the cytosol by the membrane. The polyisoprenoid part of dolichol phosphate can be expected to lie within the lipid of the membrane and its function by conformational change is probably to act as a flexible arm in order to carry sugar residues from the transferase by which it is glucosylated, to further transglycosylase enzymes of biosynthetic pathways present within or at the same surface of the membrane (Northcote, 1979). Dolichol phosphate is known to act as an intermediate in transglycosylations that occur at the inner lumen side of membranes of the Golgi apparatus and endoplasmic reticulum, and therefore, if the lipid occurs at the plasma membrane, it is probably orientated with the polar region directed outwards from the outer surface of the membrane. Therefore at the plasma membrane the glycosylation of the dolichol phosphates and the glycosylation of glycoproteins by them probably occurs at the outer surface (the non-cytoplasmic side) of the membrane. This leaves unsolved the problem of the mechanism of transport across the membrane of the nucleoside diphosphate sugars. These initial donors of the glycosylation reactions are formed in the cytosol and are therefore transported into the lumen of the endoplasmic reticulum, the Golgi apparatus and across the plasma membrane if extensive glycosylation occurs at these sites.

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