Partial characterization of the polyisoprenoid carrier of *N*-acetylglucosamine in *Glycine max* (soya bean)

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Dolichyl phosphate (C_{55}) and dolichyl phosphate prepared from liver were incubated with an enzyme prepared from soya-bean protoplasts. They both stimulated the transfer of radioactivity from UDP-D-glucose to lipid, but the stimulation was greater with liver dolichyl phosphate. Liver dolichyl phosphate with the soya-bean enzyme stimulated the transfer of radioactivity from UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine to acidic lipid. UDP-D-[U- ^{14}C]glucose and UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine were used with the soya-bean enzyme to prepare the glycosylated-acid-lipid acceptor of each sugar. Mild acid hydrolysis revealed that the radioactivity in the lipid glucosylated from UDP-glucose was present exclusively as glucose. That in the lipid glycosylated from UDP-*N*-acetylglucosamine was present mostly as *N*-acetylglucosamine. The soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine were stable to both catalytic hydrogenation and treatment with hot aqueous phenol; they behaved in a similar way as authentic α -saturated glucosylated polyisoprenyl phosphates. The soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine were eluted as deoxycholate complexes from a Sephadex column. In comparison with glucosylated polyisoprenyl phosphates of known size, the patterns of elution indicated that both soya-bean lipids contained a polyisoprenoid chain of 18 isoprene units.

In animals, plants and micro-organisms the initial donors of sugars for glycosylation of proteins are nucleotide sugars (Wright *et al.*, 1967; Struck & Lennarz, 1977; Ericson & Delmer, 1977; Nagahashi & Beevers, 1978; Weiser *et al.*, 1978; Parodi, 1978). The subsequent stages of sugar-transfer that occur in the hydrophobic environment of the membranes require, in some cases, the participation of lipids as carriers of sugars. These are the polyisoprenyl phosphates. Prepared from different sources they vary in size and may be saturated or unsaturated at the α -residue (Brett & Leloir, 1977).

A variety of sugars are incorporated into glycoproteins in plants, but little information exists concerning regulation of the pathways from nucleotide sugars to polymer. In plants, regulation is probably made more complex by the fact that nucleotide sugars and possibly glycolipids are precursors of polysaccharide of the cell wall.

The lipid acceptor of *N*-acetylglucosamine purified from *Phaseolus aureus* (mung bean) is unable to accept mannose when added to enzymes prepared

either from *Phaseolus aureus* or *Gossypium hirsutum* (cotton). In the presence of identical enzymes, dolichyl phosphate from liver is unable to stimulate the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to lipid, but it is able to stimulate the transfer of mannose from GDP-mannose. Different lipids may therefore be points of control in the utilization of these sugars during glycosylation of protein (Ericson *et al.*, 1978). Since dolichyl phosphate from yeast (14–18 isoprene units), but not dolichyl phosphate from liver (18–21 isoprene units), is able to stimulate the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to lipid in mung beans, the difference between the lipid carriers may be of size (Lehle *et al.*, 1976; Ericson *et al.*, 1978).

The mannanlipid of *Phaseolus vulgaris* (kidney bean) contains 19 isoprene units (Delmer *et al.*, 1978). However, the lipid carrier of *N*-acetylglucosamine in plants has remained unidentified.

In *Glycine max* (soya bean) the carrier of glucose, when glucosylated by using a liver enzyme, contains 17 or 18 isoprene units (Brett & Leloir, 1977). However, the liver enzyme may have acted more

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readily with a lipid of a size similar to that in liver. Therefore the identity of the lipid glucosylated in the intact plant cell has remained in doubt.

The present report is of the characterization and comparison of the lipid carriers of glucose and *N*-acetylglucosamine in *Glycine max*.

Materials and methods

Uridine diphosphate *N*-acetyl-D-[U-¹⁴C]glucosamine (sp. radioactivity 323 Ci/mol), uridine diphosphate D-[U-¹⁴C]glucose (sp. radioactivity 300 Ci/mol), uridine diphosphate D-[6-³H]glucose (sp. radioactivity 3.7 Ci/mmol) and D-[U-¹⁴C]glucose (sp. radioactivity 292 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Dolichyl phosphate (C₃₃) was obtained from Polske Odczynniki Chemiczne, Gliwice, Poland.

Chemicals used for protoplast preparation have been described elsewhere (Galbraith & Northcote, 1977).

All other chemicals were of analytical grade.

Standard glucosylated acceptor lipids

The preparation of porcine liver dolichyl phosphate has been described elsewhere (Chadwick & Northcote, 1980).

In the preparation of liver dolichyl phosphate glucose, portions of liver dolichyl phosphate solution were dried in tubes (1.5 cm × 10 cm). The lipid was dissolved in a solution containing 0.1 M-MgCl₂ (10 μl), 0.5 M-Tris/maleate (pH 7.8, 20 μl), 0.5% (w/v) Triton X-100 (20 μl), 1 M-2-mercaptoethanol (30 μl) and water (30 μl), for 30 min at 30°C. UDP-D-[U-¹⁴C]glucose (5 μl, 0.125 μCi) and rat liver microsomal fraction (10 μl) were then added. Tubes were incubated for up to 2 h at 30°C, and the reaction was stopped with 2 ml of chloroform/methanol (2:1, v/v). Incubations were washed four times with 1 ml of theoretical aqueous upper phase (methanol/chloroform/water/1 mM-MgCl₂, 294:18:282:1, by vol.) (Folch *et al.*, 1957). The glucosylated acidic lipid (fraction bound by DEAE-cellulose) was purified by elution from a DEAE-cellulose column (acetate form; diameter 1 cm, bed vol. 6 ml) with 0.1 M-ammonium formate in chloroform/methanol (3:2, v/v). Glucosylation of dolichyl phosphate (C₃₃) was carried out in a similar manner, except that incubations contained 1 μCi of UDP-D-[6-³H]glucose instead of UDP-D-[U-¹⁴C]glucose.

The microsomal fraction was prepared as described elsewhere (Chadwick & Northcote, 1980).

Preparation of soya-bean enzyme

Cultures of *Glycine max* (soya bean) callus were

maintained as a sterile suspension (Galbraith & Northcote, 1977).

Protoplasts were prepared from harvested cells. They were purified, homogenized and a 100 000g membrane pellet was prepared (Galbraith & Northcote, 1977; Chadwick & Northcote, 1980). The membranes had been subjected only to the mechanical stress required to homogenize protoplasts. The 100 000g pellet (a mixture of mitochondria, plasma membranes and rough and smooth microsomal fraction) is referred to as the soya-bean enzyme.

Exogenous polyisoprenyl phosphates glucosylated by the soya-bean enzyme

Portions of dolichyl phosphate (C₃₃) or liver dolichyl phosphate were dried in tubes (1.5 × 10 cm) and incubated with the soya-bean enzyme. Incubations (200 μl) contained 0.1% (w/v) Triton X-100, 5 mM-MgCl₂, 100 mM-Mes (4-morpholine-ethanesulphonic acid) (pH 6.0), 0.2 μCi of UDP-D-[U-¹⁴C]glucose and up to 300 μg of protein. They were carried out at 25°C, stopped at 1 h with 2 ml of chloroform/methanol (2:1, v/v) and washed with theoretical aqueous upper phase. The glucosylated acidic lipid was isolated on DEAE-cellulose paper discs (Chadwick & Northcote, 1980). The radioactivity on the discs was measured by liquid-scintillation counting.

Endogenous polyisoprenyl phosphates glycosylated by the soya-bean enzyme

Portions of soya-bean enzyme (125 μl) containing up to 300 μg of protein were incubated with UDP-D-[U-¹⁴C]glucose or UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine. Incubations (200 μl in tubes with dimensions 1.5 cm × 10 cm) contained 100 mM-Mes (pH 6.0), 5 mM-MgCl₂ and 0.2 μCi of nucleotide sugar. They were carried out at 25°C, stopped after 1 h with 2 ml of chloroform/methanol (2:1, v/v) and washed with theoretical aqueous upper phase. The glycosylated acidic lipids were isolated by elution from a DEAE-cellulose column with 0.1 M-ammonium formate in chloroform/methanol (3:2, v/v).

Catalytic hydrogenation

A solution of 0.2 M-chloroplatinic acid (H₂PtCl₆) in ethanol was used as a source of powdered platinum metal. For each hydrogenation 0.2 ml of this solution was mixed with excess NaBH₄, followed by 0.2 ml of conc. HCl. The precipitate of platinum was washed three times with aq. 80% (v/v) ethanol and once with water-saturated butanol.

The glucosylated lipids were dissolved in 0.2 ml of water-saturated butanol and placed in a tube (1 cm × 5 cm) containing the catalyst. H₂ gas (British Oxygen Co.) was saturated with water and butanol and then passed into the solution of lipid for 4 h.

Afterwards, the solution was mixed with 0.2 ml of water, and the organic and aqueous phases were separated. The organic phase was washed with 0.2 ml of water, and the aqueous phase with 0.2 ml of butanol. Both phases were dried and assayed for radioactivity by liquid-scintillation counting.

Phenol treatment

Samples of glycosylated lipids were dried in tubes (1 cm × 5 cm). They were treated with 0.5 ml of aq. 50% (w/v) phenol at $70 \pm 2^\circ\text{C}$. At this temperature the solution was a single phase. After 3 h, the tubes were cooled and the organic and aqueous phases were separated. The organic phase was washed with 0.2 ml of water, and the aqueous phase with 0.5 ml of diethyl ether. Both phases were dried and assayed for radioactivity in a liquid-scintillation counter (Searle Mark III liquid-scintillation analyser).

Gel filtration

Samples of glycosylated lipids were dissolved in 0.5 ml of 5 mM-sodium phosphate buffer, pH 7.4, containing 0.5% (w/v) sodium deoxycholate and approx. 5 mg of Blue Dextran. The solution was applied to a column (1 cm × 80 cm) of Sephadex G-75 (medium) equilibrated beforehand in phosphate/deoxycholate buffer. Further buffer was used to elute the column. After the Blue Dextran had been eluted from the column a 40 ml volume of eluate was collected (1 or 2 ml fractions). Each fraction was assayed for radioactivity with Triton/toluene (toluene, 1.5 litres; Triton X-100, 750 ml; 2,5 diphenyloxazole, 6 g) as scintillant. The position of the Blue Dextran marker was determined from the absorbance of each fraction at 700 nm.

Acid hydrolysis of glycosylated lipids

Glycosylated lipids were hydrolysed with 1 M-HCl at 100°C for 10 min. Hydrolysates were dried, dissolved in 50 μl of water and applied to Whatman no. 1 chromatography paper. Chromatograms were developed in ethyl acetate/pyridine/water (8:2:1, by vol.) for 36 h (lipid glycosylated from UDP-glucose) or for 60 h (lipid glycosylated from UDP-*N*-acetylglucosamine). Chromatograms were cut into sections (4 cm × 1 cm) and assayed for radioactivity in a liquid-scintillation counter. Standards for chromatograms were D-[U- ^{14}C]glucose and *N*-acetyl-D-[U- ^{14}C]glucosamine.

Results

To prepare standard glycosylated lipids, liver dolichyl phosphate and dolichyl phosphate (C_{55}) were incubated separately with a rat liver microsomal enzyme. They both stimulated the transfer of radioactivity from UDP-D-[U- ^{14}C]glucose or UDP-D-[6- ^3H]glucose to acidic lipid. In the absence of

exogenous acceptors a quantity of radioactivity was incorporated into acidic lipid, due to acceptor present in the microsomal fraction. From this amount of incorporation, 8% of the liver dolichyl phosphate glucose and 2% of the dolichyl phosphate (C_{55}) glucose that was prepared was estimated to be glucosylated microsomal acceptor.

In separate experiments, liver dolichyl phosphate and dolichyl phosphate (C_{55}) (approx. 50 μg) were added to the soya-bean enzyme to determine whether they stimulated the transfer of radioactivity from UDP-D-[U- ^{14}C]glucose to acidic lipid (Fig. 1). For both lipids an optimum concentration occurred. In four separate experiments, liver dolichyl phosphate stimulated the transfer by a maximum of $58.5 \pm 6\%$, and dolichyl phosphate (C_{55}) stimulated it by a maximum of $35.5 \pm 5\%$.

Liver dolichyl phosphate was added to the soya-bean enzyme. It stimulated the transfer of radioactivity from UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine to acidic lipid (Fig. 2).

To prepare the glycosylated acidic lipid acceptors of glucose and *N*-acetylglucosamine, UDP-D-[U- ^{14}C]glucose and UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine were each supplied to the soya-bean enzyme. Samples of each glycosylated lipid were hydrolysed

Fig. 1. Effect of exogenous dolichyl phosphates on incorporation of glucose into acidic lipids

In separate experiments liver dolichyl phosphate (a) and dolichyl phosphate (C_{55}) (b) (approx. 50 μg) were added to the soya-bean enzyme. The radioactivity transferred from UDP-D-[U- ^{14}C]glucose to acidic lipid was measured in each case.

with acid, and the hydrolysates were examined by paper chromatography. All the radioactivity present in the hydrolysate of the acidic lipid glucosylated from UDP-glucose moved to a position on the chromatogram similar to that of glucose. The hydrolysate of the acidic lipid glucosylated from UDP-*N*-acetylglucosamine contained two peaks of radioactivity. The major peak moved to a position on the chromatogram similar to that of *N*-acetylglucosamine. A minor peak of radioactivity near the

origin of the chromatogram may have been either glucosamine or an oligosaccharide (Fig. 3); 89% of the amount of radioactivity applied to the chromatogram was recovered.

Soya-bean polyisoprenyl phosphate glucose and polyisoprenyl phosphate *N*-acetylglucosamine were

Fig. 2. Effect of liver dolichyl phosphate on incorporation of *N*-acetylglucosamine into acidic lipid. Liver dolichyl phosphate was added to the soya-bean enzyme. The radioactivity transferred from UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine to acidic lipid was measured.

Fig. 3. Hydrolysate of the acidic lipid glucosylated by UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine

The acidic glycosylated by UDP-*N*-acetyl-D-[U-¹⁴C]glucosamine was hydrolysed with 1M-HCl at 100°C for 10min. The hydrolysate (2000 c.p.m.) was evaporated to dryness, dissolved in 50 μl of water and applied to Whatman no. 1 chromatography paper. The chromatogram was developed in ethyl acetate/pyridine/water (8:2:1, by vol.) for 60h, cut into sections (4 cm × 1 cm) and assayed for radioactivity. Of the radioactivity applied to the chromatogram 89% was recovered.

Table 1. Stability of the soya-bean acceptors of glucose and *N*-acetylglucosamine towards catalytic hydrogenation (Pt and H₂)

Lipids stable to catalytic hydrogenation are recovered in the butanol phase. The results given are for after hydrogenation.

	Radioactivity in butanol phase (c.p.m.)	Radioactivity in aqueous phase (c.p.m.)	Percentage of total reduced	Recovery (%)
Dolichyl phosphate (C ₅₅) [6- ³ H]glucose	11400	246	2	70
Liver dolichyl phosphate [U- ¹⁴ C]glucose	1936	214	10	89
Soya-bean polyisoprenyl phosphate [U- ¹⁴ C]glucose	4842	104	2	98
Soya-bean polyisoprenyl phosphate <i>N</i> -acetyl[U- ¹⁴ C]glucosamine	500	44	8	90

Table 2. The stability of the soya-bean acceptors of glucose and *N*-acetylglucosamine towards hot phenol
Lipids stable to hydrolysis are recovered in the phenol phase.

	Radioactivity in phenol phase (c.p.m.)	Radioactivity in aqueous phase (c.p.m.)	Percentage of total hydrolysed	Recovery (%)
Dolichyl phosphate (C ₅₅) [6- ³ H]glucose	6329	967	13	91
Soya-bean polyisoprenyl phosphate [U- ¹⁴ C]glucose	1212	36	3	80
Soya-bean polyisoprenyl phosphate <i>N</i> -acetyl[U- ¹⁴ C]glucosamine	3500	150	4	104

Fig. 4. Elution of dolichyl phosphate sugars from a Sephadex G-75 column

Glycosylated acidic lipids were applied to a Sephadex G-75 column and eluted with phosphate/deoxycholate buffer. After the appearance of the Blue Dextran marker, a 40 ml volume of eluate was collected in 1 or 2 ml fractions and assayed for radioactivity. Symbols: Δ , dolichyl phosphate (C_{55}) $[6-^3H]$ glucose; $\blacktriangle$, liver dolichyl phosphate $[U-^{14}C]$ glucose; $\blacksquare$, soya-bean polyisoprenyl phosphate $[U-^{14}C]$ glucose; $\bullet$, soya-bean polyisoprenyl phosphate *N*-acetyl $[U-^{14}C]$ glucosamine.

Fig. 5. Standard curve for elution of dolichyl phosphate glucoses

Glucosylated dolichyl phosphates of known size were eluted from a Sephadex G-75 column. The graph is calibrated from the volumes of eluate obtained for the standards, and is used to determine the number of isoprene units in the soya-bean acceptors of glucose and *N*-acetylglucosamine.

An allylic lipid for use as a control during catalytic hydrogenation was not available. The isoprenoids were therefore further investigated by treatment with hot phenol (Pont Lezica *et al.*, 1975). None of the lipids was degraded (Table 2). It was concluded that the soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine were α -saturated.

The length of the polyisoprenoid chains of the soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine were determined. Samples of each were eluted from a Sephadex G-75 column and the patterns of elution compared with those of standards of known size. Liver dolichyl phosphate was glucosylated with $[^{14}C]$ glucose and dolichyl phosphate (C_{55}) with $[6-^3H]$ glucose. The void volume of the column was 30 ml. It remained identical in each experiment and it was therefore possible to compare the elution of samples placed separately on the column with eluates that contained both samples. The results obtained are shown in Fig. 4. Dolichyl phosphate (C_{55}) glucose was eluted at 20 ml, and liver dolichyl phosphate glucose at 11 ml, after the void volume. The soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine were both eluted at 12 ml after the void volume. It is assumed that the volume of elution is inversely proportional to the logarithm of the number of isoprene units in the chain of the lipid. Dolichyl phosphate (C_{55}) glucose contains 11 isoprene units, whereas the major component of liver dolichyl phosphate glucose contains 19 isoprene units (Dunphy *et al.*, 1967; Butterworth & Hemming,

examined for their stability to catalytic hydrogenation. Their behaviour was compared with that of liver dolichyl phosphate glucose and dolichyl phosphate (C_{55}) glucose. The results are shown in Table 1. In each case the quantity of radioactivity recovered in the aqueous phase was between 2 and 8% of the total. Each of the lipids was therefore stable to catalytic hydrogenation.

1968). These values were used in calibration of a graph (Fig. 5). From the volume of elution of the soya-bean acceptors (12 ml) it was calculated that the number of isoprene units in both the soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine was 18.

Discussion

Polyisoprenyl phosphate sugars are degraded by catalytic hydrogenation if they are allylic, but not if they are α -saturated (Wright *et al.*, 1967). Furthermore, α -saturated lipids, but not allylic lipids, are stable to hot phenol (Pont Lezica *et al.*, 1975).

Both α -saturated polyisoprenols similar to dolichol, and allylic polyisoprenols such as ficaprenol occur in plants (Hemming, 1974). In the present work, the endogenous soya-bean acidic-lipid acceptors of glucose and *N*-acetylglucosamine were found to be α -saturated. In *Pisum sativum* the corresponding acceptors of glucose and *N*-acetylglucosamine are also α -saturated (Brett & Leloir, 1977), as is the acceptor of mannose in *Phaseolus vulgaris* (Delmer *et al.*, 1978). It would seem probable that the sugars incorporated into glycoproteins are carried by lipids of the dolichol type.

If it is a compatible acceptor dolichyl phosphate added to the appropriate enzyme is able to stimulate the transfer of sugars from nucleotide sugars to lipid. Both dolichyl phosphate (C_{55}) and liver dolichyl phosphate added to the soya-bean enzyme led to an increase in the transfer of glucose from UDP-glucose to acidic lipid, but the maximum stimulation was greater with liver dolichyl phosphate. This would suggest that the endogenous soya-bean acceptor of glucose is more like liver dolichyl phosphate (19 isoprene units) than dolichyl phosphate (C_{55}) (11 isoprene units).

Liver dolichyl phosphate also brought about a stimulation of the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to lipid. This result is similar to that observed in *Pisum sativum* (pea), where dolichyl phosphate brought about a stimulation of up to 200%, although the observation was inconsistent and sometimes even an inhibition was found (Brett & Leloir, 1977). Lehle *et al.* (1976) also showed that glycosylation from UDP-*N*-acetylglucosamine with an enzyme prepared from *Phaseolus aureus* could be stimulated by yeast dolichyl phosphate (14–18 isoprene units). However, our result is in contrast with that observed by Ericson *et al.* (1978) with enzymes prepared from *Gossypium hirsutum* (cotton) and *Phaseolus aureus*.

Deoxycholate is known to form complexes with fatty acids and polyisoprenols. Thus with dolichyl phosphate glucose (mol.wt. approx. 1500), the

complex formed has an apparent mol.wt. of 11300 (Behrens *et al.*, 1971). By gel filtration in deoxycholate, polyisoprenols may be readily separated according to size.

On a column of Sephadex G-75, the soya-bean acceptors of glucose and *N*-acetylglucosamine had an identical pattern of elution, consistent with the presence of a chain of 18 isoprene units. In mung bean and cotton, *N*-acetylglucosamine may be carried by a lipid of a size different from that of dolichyl phosphate (Ericson *et al.*, 1978), but this is not the case in soya bean. The carrier of *N*-acetylglucosamine in soya bean is similar in size to the carrier of glucose, and both lipids may be replaced by dolichyl phosphate from liver.

This is the first reported identification of the lipid carrier of *N*-acetylglucosamine in plants.

In soya-bean cells, therefore, a specific lipid carrier of *N*-acetylglucosamine may not be a point of control in glycoprotein biosynthesis. If this is so, the soya-bean system is different from that of mung bean and cotton. It may be, that in some plant species other than soya bean, *N*-acetylglucosamine is carried by a specific lipid and that the differences between lipid carriers for different sugars are not confined to the length of the polyisoprenoid chain. In nucleate cells most of the olefinic linkages in dolichyl are *cis*, but four or fewer are *trans* (Beytia & Porter, 1976). The alteration of a single linkage from *cis* to *trans* or vice versa would produce a change in the conformation of a lipid. It is possible that a particular conformation of lipid might be recognized by a single glycosyltransferase. So that the carrier of *N*-acetylglucosamine in plants might be a particular geometrical isomer of dolichol.

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