Membrane-bound UDP-Glucose

LIPID GLUCOSYLTRANSFERASES FROM PEAŚ

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ABSTRACT

An enzymatic preparation from peas (Pisum sativum), able to form neutral and polar glycosides, is described. The best sugar donor is UDP-glucose and the acceptors are present in the enzymic system.

The neutral glycolipids have been characterized as steryl and acylated steryl glucosides. The polar glucolipid had been identified as polypropenyl monophosphate glucose. The glucose is linked to the phosphate in β configuration. Polar glucolipids are also formed from UDP-glucose and exogenous prenylic acceptors, either α-saturated, as dolichyl monophosphate, or allylic, as ficaprenyl monophosphate.

The two glucosylating activities have been partially separated by differential centrifugation: the fraction that precipitates at 25,000g has most of the neutral glucolipid-synthesizing activity, and the fraction that sediments at 100,000g is rich in polar glucolipid glucosyl transferase activity. This latter activity was strongly dependent on Mg²⁺ concentration, the optimum being around 5 to 10 mM. UDP inhibits the reaction and 0.2 to 0.5% (v/v) Triton X-100 has a stimulatory effect. The optimum pH is 7.5.

Previous communications have presented evidence that lipid-linked glucose is formed in cell-free extracts of pea epicotyls (21, 22). The glucolipids formed by this system have been separated into neutral and acidic glucolipids.

The acidic glucolipid had been identified as an α-saturated polypropenyl monophosphate glucose (22). The occurrence of polypropenyl-linked sugars in plants has been studied extensively in recent years (1, 8, 9, 13, 16, 18). However, the role of polypropenyl-linked sugars as intermediates for the transfer of sugars to glycolipids has not as well understood for plants as it is for animals and bacteria (2, 13, 18).

Neutral glycolipids have been described in plants and comprise different molecular structures as glycosyl diglycerides, steryl glycosides, and terpenyl glycosides (15). Several functions have been proposed for acyl lipids: structural role, involvement in membrane functions, energy storage, and cellular repair mechanism.

This paper reports some properties of an enzyme system from peas, which, in the presence of UDP-glucose, forms neutral and polar glucolipids.

MATERIALS AND METHODS

Chemicals. GDP-[¹⁴C]Mannose (160 Ci/mol), UDP-[¹⁴C]N-acetylglucosamine (269 Ci/mol) and ADP-[¹⁴C]glucose (228 Ci/mol) were purchased from New England Nuclear, and GDP-[¹⁴C]glucose (203 Ci/mol) from International Chemical and Nuclear Corporation.

UDP-[¹⁴C]glucose (309 Ci/mol) and UDP-[¹⁴C]galactose (309 Ci/mol) were synthesized as described previously (11).

The allylic polypropenyl ficaprenol was isolated from Ficus elastica leaves according to Stone et al. (25) and phosphorylated by the method of Popjak et al. (23). The purity of ficaprenol was checked by TLC and by IR and NMR spectrometry. Mass spectrometry showed that it was a mixture of undecaprenol and dodecaprenol in equal proportions. Ficaprenol phosphate concentration was determined by measuring acid-labile phosphate by the method of Chen et al. (4).

Dolichyl monophosphate, α-saturated polypropenyl containing 18 to 22 isoprenyl units, isolated from liver and partially purified, was a gift from N. H. Behrens. The 1,6-anhydroglucosan was prepared from salicines as described (20). All other chemicals were obtained commercially.

Enzyme Preparation. Five to 7-day-old etiolated epicotyls of dwarf Pisum sativum L., cv. Spiket, were homogenized in an Omni-Mixer with 0.25 M sucrose, 0.1 M tris-HCl buffer, pH 7.4, 0.02 M β-mercaptoethanol. The homogenate was filtered through several layers of cheesecloth and centrifuged at 1,000g for 15 min. The resulting supernatant solution was centrifuged at 25,000g for 30 min and the pellet obtained (P₃₅) was resuspended in 0.05 M tris-HCl, pH 7.4, containing 40% glycerol. The supernatant was centrifuged at 100,000g for 180 min and the resulting pellet (P₁₅₀) was resuspended in the same buffer as P₃₅. Both particulate preparations contained 7 to 10 mg/ml of protein, determined by the method of Lowry et al. (19).

Incubation Procedure. Standard incubations were carried out at 20°C for 30 min in a total volume of 50 μl of the following components: 5 μmol tris-HCl buffer, pH 7.4; 1 μmol β-mercaptoethanol; 0.5 μmol MgCl₂; 1 mmol UDP-[¹⁴C]glc; and 100 to 300 μg of protein. The reaction was stopped by the addition of 0.1 ml of butanol and extracted as described (11).

Abbreviations: glc: glucose; gal: galactose.

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2 Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

3 Holder of an O.A.S. fellowship while on leave from the Universidad Católica de Valparaíso, Chile.
Polar and neutral glycolipids were separated by chromatography of the butanolic phase in Whatman DE20 paper using butanol as solvent. The origin and the solvent front zones were cut out and counted as described below.

**Column Chromatography.** DEAE-cellulose (Serva, type SS p.A.) in the acetate form was poured into a glass column (13 x 550 mm). Polar glycolipids were eluted with a linear gradient of 0 to 0.4 M ammonium acetate in 99% methanol (6). Neutral glycolipids were eluted stepwise increasing methanol in chloroform according to Nichols and James (cited in 15).

**TLC and Paper Chromatography and Electrophoresis.** Paper chromatography was done on Whatman No. 1 paper with: solvent A, 3 M NH₃ in 80% ethanol (v/v); solvent B, isopropyl alcohol-acetic acid-water (27:4:9); solvent C, 1-butanol-pyridine-water (6:4:3). For solvent A, the ascending technique was followed. Paper electrophoresis was performed with Whatman No. 1 paper at 1000 v (20 v/cm) for 3 hr in 1.2 M pyridine acetate, pH 6.5 (11).

TLC was performed on silica gel G plates developed with chloroform-methanol-water (65:25:4).

Reducing sugars were located with silver nitrate reagent (27). The Liebermann-Burchard reaction was performed according to Cook (5).

**β-Glucose-1-Phosphate Assay.** It was carried out as described by Belocopitow and Marechal (3). The incubation mixture contained, in a total volume of 50 μl: 2 μmol HEPES buffer, pH 7; 0.25 μmol MgCl₂; 0.04 μmol EDTA; 0.2 μmol α- or β-glucose-1-P; 5 μmol sodium arsenate, pH 7; labeled substrates; and 10 μl β-P-glucosumotase preparation. Incubations were carried out at 37 C for 60 min and inactivated by addition of 20 μl of 1 N HCl and heating at 100 C for 10 min. The hydrolysate was directly analyzed by paper electrophoresis to separate glucose from glucose-6-P. Partially purified β-P-glucosumotase from Euglena gracilis was a kind gift from L. Marechal. Excess arsenate was added to inhibit contaminating α-P-glucosumotase activity.

**Radioactivity Measurements.** Determinations of radioactivity were done on a Beckman LS-233 scintillation spectrometer. Samples were counted in a scintillation cocktail containing 550 ml Omnifluor (New England Nuclear) (4% w/v) in toluene and 250 ml Triton X-100.

Paper strips were scanned for radioactivity on a Packard radiochromatogram scanner, model 7201.

**RESULTS**

**Incorporation Products.** When the P₃₂⁵ particulate enzyme was incubated with UDP-[¹⁴C]glc, radioactivity was incorporated into different fractions (Table 1). Most of the label was in the butanolic phase, while a smaller fraction was recovered from aqueous phase as material insoluble in 80% ethanol. Although this latter fraction was not studied in detail, preliminary evidence suggested that it contained a mixture of polysaccharides and glycoproteins.

The butanolic extract was submitted to paper chromatography in solvent B. All of the radioactivity was associated with lipids (R₂ = 0.92) indicating no traces of glucose, glucose-1-P, or UDP-gluc contaminated this fraction.

Analyzed by DE20 paper chromatography the butanolic extract showed two main components. The most important was neutral and resistant to mild acid hydrolysis. The other was polar and very labile to acid (pH 2, 100 C, 10 min). More than 90% of the radioactivity incorporated was liberated as water-soluble substances.

Several butanol extracts were pooled and chromatographed in a DEAE-cellulose (acetate) column. The radioactivity profile obtained is shown in Figure 1. As expected, the main peak corresponded to the neutral lipids, but a sharp peak correspond-

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CPM incorporated</th>
<th>Per cent of initial radioactivity added as UDP-[¹⁴C]glc</th>
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<tr>
<td>Butanol soluble</td>
<td>153,700</td>
<td>42.0</td>
</tr>
<tr>
<td>Neutral lipids</td>
<td>130,000</td>
<td>36.0</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>5,413</td>
<td>1.5</td>
</tr>
<tr>
<td>80% ethanol insoluble</td>
<td>23,788</td>
<td>6.5</td>
</tr>
</tbody>
</table>

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Analysis of the butanol-soluble material by DEAE-cellulose column chromatography. The butanolic phases of several standard incubations were pooled (6.7 ml, 5.58 x 10⁴ cpm), poured in a DEAE-cellulose column equilibrated with 99% methanol. Fractions of 3 ml were collected. A linear gradient of ammonium acetate was started after fraction 20. Aliquots (0.5 ml) from each fraction were counted for radioactivity.

**Analysis of Polar Glycolipid.** The polar glycolipid had been characterized as a glucose derivative of an α-saturated polyprenylmonophosphate (22) on the following bases: it is unstable (87% hydrolysis) under mild acid conditions (pH 2, 100 C, 10 min), giving glucose as the only water-soluble compound. Chromatographed in solvent A and scanned only one peak was obtained at the solvent front (Rₛ = 0.95) as would be expected for a lipid monophosphate glucose, since a pyrophosphate derivative would give the glucose cyclic phosphate ester (Rₛ = 0.66). The α saturation was inferred from its resistance to phenol treatment and catalytic reduction. The polyenylpyrene nature of the lipid moiety was confirmed using exogenous acceptors (see following sections). Results are now presented concerning the configuration of the P-glucose linkage.

Submitted to alkaline conditions (0.1 N NaOH in n-propyl alcohol 99%, at 65-68 C, 90 min) the [¹⁴C]glucose-labeled polar glycolipid was degraded producing a water-soluble substance (83% of the radioactivity) with the mobility of 1,6-anhydroglucosan upon paper chromatography with solvent C. The identity
of the glucosan was confirmed by acid hydrolysis (1 N HCl, 100 C, 3 hr, in a sealed tube): it produced only radioactive glucose, as judged by paper chromatography with solvent C. This result suggests that the glucolipid has the glucose in the B configuration since A derivatives do not form anhydroglucosan under the conditions employed (20).

Another experiment confirmed this view. As mentioned above, previous results have indicated that the endogenous acceptor is a polypropenyl monophosphate in which the A-isoprenyl unit, i.e. the one carrying the phosphate ester, is saturated, as in dolichyl monophosphate. Glycosylated derivatives of these compounds are not degraded by treatment with phenol (50% phenol, 68–70C, 3 hr) (22). Under these conditions, on the other hand, allylic derivatives which contain a double linkage in the A-isoprenyl unit, release more than 90% of the carbohydrate as the phosphate ester (11). Taking advantage of this property and assuming that the stereospecificity of the glucosylating enzyme is the same for the different acceptors, incubations were carried out in the presence of excess exogenous ficaprenyl monophosphate, an allylic ester. Under these conditions (see following sections) the polar glucolipid obtained was mainly ficaprenyl monophosphate glucose. It was submitted to phenol degradation and glucose-1-P was formed. This material was incubated in the presence of B-P-glucomutase. More than 40% of the ester was converted in glucose-6-P. A control run in the presence of A-glucose-1-P showed less than 9% conversion, possibly due to the action of the A-P-glucomutase present in the enzyme preparation.

Analysis of Neutral Glycolipids. The neutral glucolipid was submitted to acid hydrolysis by refluxing 20 hr with 1 N H2SO4; 77% of the radioactivity became water-soluble and chromatographed in solvent C as glucose. Alkaline hydrolysis by refluxing 1 hr with 5% KOH in 90% methanol did not alter the glucolipid that remained butanol-soluble (80%). This neutral peak was chromatographed in a DEAE-cellulose column equilibrated with chloroform and eluted stepwise by the method of Nichols and James (15). A radioactive peak was eluted with a mixture of chloroform-methanol (95:5) as sterol glucosides do. TLC of neutral glycolipids gave two radioactive spots: lipid 1 (87%) and lipid 2 (13%). Both samples gave a positive Liebermann-Burchard test indicating the presence of sterol. After alkaline treatment, lipid 2 was converted into lipid 1 (Fig. 2).

Separation and Properties of Glucosyltransferases. Two particular glucosyltransferases were partially separated by differential centrifugation. The P2s fraction contained most of the neutral glucosylating activity (98%) and the P100 fraction was rich in polar lipid glucosyltransferase activity (72%).

Several nucleotides were checked for their ability to incorporate labeled sugars into lipids. The results in Table II show that

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>cpm incorporated into lipids</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Polar</td>
</tr>
<tr>
<td>UDP-(14C)-glucose</td>
<td>1436</td>
</tr>
<tr>
<td>ADP-(14C)-glucose</td>
<td>136</td>
</tr>
<tr>
<td>GDP-(14C)-glucose</td>
<td>426</td>
</tr>
<tr>
<td>GDP-(14C)-mannose</td>
<td>444</td>
</tr>
<tr>
<td>UDP-(14C)-acetylgalactosamine</td>
<td>258</td>
</tr>
<tr>
<td>UDP-(14C)-galactose</td>
<td>1047</td>
</tr>
</tbody>
</table>

Table II. Incorporation of 14C-sugars from different nucleotides into lipids

Incorporations containing 370 mg of protein (P2s) were carried out for 20 min at 20 C, as described, except for the radioactive nucleotides which were: UDP-(14C)-glucose, 150 pmoles; ADP-(14C)-glucose, 877 pmoles; GDP-(14C)-mannose, 642 pmoles; UDP-(14C)-acetylgalactosamine, 588 pmoles; UDP-(14C)-galactose, 498 pmoles; and GDP-(14C)-glucose, 900 pmoles. The butanolic phase was isolated by ion exchange paper chromatography.

UDP-glc and UDP-gal were the best donors for the system. When UDP-gal was used as sugar donor, the polar glucolipid formed liberated glucose and galactose in equal amounts.

When the effect of the enzyme concentration was studied, it was found that the formation of neutral lipid rose proportionally to protein concentration (Fig. 3A). On the other hand, polar lipids reached a plateau at a very low enzyme concentration.

The prenyl nature of the polar lipid was confirmed using exogenous acceptors. Different concentrations of ficaprenyl monophosphate were added to the incubation mixture. The production of polar glucolipids was substantially increased, and no appreciable change was observed in neutral lipids (Fig. 3B). To be sure that the exogenous prenol was acting as an acceptor, an experiment was performed with ficaprenyl-[32P]P and UDP-[14C]glc. Polar glucolipids were extracted with butanol and separated from the neutral ones by a DEAE-cellulose column. The polar glucolipids were treated with 50% phenol for 3 hr at 68 to 70 C (11). The aqueous phase was submitted to electrophoresis and double labeled glucose-1-P was recovered. This confirmed that ficaprenol-P could act as glucose acceptor in the pea system.

In order to investigate the specificity for the acceptor, experiments were carried out adding ficaprenyl and dolichyl monophosphates to the incubation system. Both prenylglucosyltransferases could act as sugar acceptors in the pea system (22).

The effect of UDP-glc concentration on radioactivity incorporation into lipids is shown in Figure 4. The incorporation of glucose into lipids was proportional to UDP-glc concentration to about 10-3 M.

Some properties of the P100 glucosyltransferase were studied. This activity was strongly dependent on the presence of magnesium ions, as shown in Figure 5A. Optimum formation of acidic glucolipids occurred at 5 to 10 mM Mg++. The glucosylation is inhibited by the addition of EDTA in excess of Mg++ and by 5 mM UDP. Detergent addition stimulated the transferase activity even in the absence of exogenous acceptors, as shown in Figure 5B, for Triton X-100. The pH optimum for this enzyme was 7.5 in tris-maleate buffer (Fig. 6).

DISCUSSION

The results of the experiments described in this paper indicate that two particular lipid glucosyltransferases are present in etiolated pea epicotyls. These activities could be partially separated by differential centrifugation indicating different subcellu-
Fig. 3. Effect of different concentrations of protein (P₂₅) and ficaprenyl-P. A: Incubations were carried out for 30 min at 20°C under the standard conditions. Neutral and polar lipids were determined by chromatography of the butanolic phase on Whatman DE20 paper (see text). O—O: Neutral lipids; ●—●: polar lipids. B: Incubation was as in A, but at constant protein concentration (250 μg) and with the addition of 0.5% Triton X-114 and the indicated amounts of ficaprenyl-P. Neutral and polar lipids were analyzed as in A. O—O: Neutral lipids; ●—●: polar lipids.

Fig. 4. Effect of UDP-glucose concentration. Incubations were carried out as described in "Materials and Methods," except for the UDP-glucose concentrations. Four hundred pmol of UDP-[¹⁴C]glucose and 250 μg of protein from P₂₅ fraction were used in each assay. O—O: Polar glucolipid; ●—●: neutral glucolipid.

lar distribution. The P₂₅ fraction carrying mitochondria and other large particles, exhibits most of the neutral lipid-glycosylating activity. The product of this reaction had an elution pattern in a DEAE-cellulose column according to Nichols and James (15) similar to steryl glucosides. The positive Liebermann-Burchard reaction indicates the presence of a sterol as aglycon. TLC shows two radioactive compounds (lipids 1 and 2). The alkaline conversion of lipid 2 into 1 indicates that the former compound is an acylated steryl glycoside, with glucose as its sugar moiety. These results lead to the conclusion that P₂₅ fraction contains a UDP-glc: steryl glucosyl transferase.

These results are in agreement with those of other authors (7, 10, 12, 17) which indicate that most sterols and their derivatives are present in large organelles. It has been shown (12) that chloroplast and mitochondria are the site of steryl glucoside biosynthesis.

Two pathways have been proposed for the formation of acylated steryl glucosides: the acylation of steryl glucosides and the transfer of an acyl-glycosyl group to sterol (12). In the experiments reported here, no evidence was found for acylated glu-
cose. The higher proportion of steryl glucoside over the acylated steryl glucoside suggests that in pea, the first pathway occurs.

The crude microsome fraction (P₁₀₀) contained most of the glycosylating activity for acidic lipids. Although the lipids have not been definitively identified, all of the evidence indicates that it is a polyprenol. In previous work (22), we have isolated from the same plant material a glucose acceptor lipid with the properties of an α-saturated polyprenol monophosphate. The elution pattern of the glycosylated lipid in DEAE columns (Fig. 1) fits well with the lipids monophosphate described earlier. Also, the facts that exogenous polyprenol monophosphates, either allylic or α-saturated, stimulated glucose incorporation into acidic glycolipids, and that ficaprenol phosphate acts as substrate, strongly indicate that the lipid moiety of the polar glucolipid is a polyprenol.

In mammalian systems, UDP-glc: polyprenyl-P glucosyl transferases are present in rough and smooth microsomes (18). In higher plants, particulate mannosyltransferases have been reported in Phaseolus aureus (1, 13, 16, 18, 26) and cotton fibers (8, 9). Recently also, N-acetylgalactosaminyl transferase activity has been reported in mung bean hypotylos (24) and cotton fibers (9). The pea membrane preparations exhibit a high glucosyltransferase and a lower galactosyltransferase activity (Table II). Because glucose and galactose were found in the products when UDP-gal was used as glycosyl donor, epimerization of UDP-gal into UDP-glc must occur.

The Mg²⁺ requirement and the UDP inhibition for this reaction are in agreement with the polyprenol phosphate-glycosylating activities reported for bacterial and mammalian systems (13).

The pH optimum for pea glucosyltransferase is about 7.5, as it is for the mannosyltransferase from cotton fibers (8). The formation of 1,6-anhydroglucosan by alkaline treatment of the glycosylated lipid and the action of the β-P-glucosomutase on the phenol- liberated glucose-P suggest the presence of a β linkage. The stereochemistry of the pea glucosyltransferase seems similar to the rat liver and bacterial (13, 18) enzymes, to the mannosyltransferase from calf pancreas (14), and to the galactosyltransferase from Acetobacter (11).

In bacterial and animal tissues, the polyprenol monosaccharide sugars are required for the transfer of these sugars to glycolipids, glycoproteins, and polysaccharides (2, 13, 18). It is
Effect of Mg²⁺ and Triton X-100 on polar lipid glucosyltransferase activity. Incubations were carried out under standard conditions, with P₁₀₀ enzyme. A: Protein concentration: 150 μg/tube and 0.6% Triton X-100; B: protein concentration: 260 μg/tube and 10 mM Mg²⁺. In A and B, different enzyme preparations were used.

Possible that they play a similar role in plants (9). The addition of fucaprenyl-P to the incubation mixture in pea shows an accumulation of polar glycolipids and no change in neutral ones, indicating no relationship between steryl glucosides synthesis and polypropyl monophosphate glucose (Fig. 3B). The enzyme is highly specific for the sugar nucleotide but seems quite unspecific for the chain length or α saturation of the polypropyl-P. Both fucaprenyl-P (C₅₅) and dolichyl-P (C₁₀₀) were used as substrates, although the endogenous acceptor is very likely of the dolichol type (22).

The radioactivity present in 80% ethanol-insoluble material, containing polysaccharides and glycoproteins, probably indicates that some of these compounds are the final receptors of the sugar moiety. Work is in progress to explore this possibility.

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LITERATURE CITED

CORRECTIONS

Vol. 57: 906–910. 1976
Page 907, column 1, paragraph 5, line 5 should be corrected to read: previously equilibrated with the above buffer. The eluate (458 mg of protein in 15 ml) was transferred to a column of hydroxylapatite (3.0 x 12.5 cm) equilibrated with the above buffer, and the enzyme.

Page 278, column 1, paragraph 1, line 9, optimum concentration of DTE should be corrected to read: 0.5 mM.

Page 279. Figures 5 and 6, ordinates should be corrected to read: cpm/g fr wt x 10⁻⁴.

Page 675, title should be corrected to read: Membrane-bound UDP-Glucose: Lipid Glucosyltransferases from Peas.

Drake, Bert G. Seasonal Changes in Reflectance and Standing Crop Biomass in Three Salt Marsh Communities.
Page 696, column 1, paragraph 2, last line should be corrected to read: above. Measurements reported here were made on the Kirkpatrick Marsh at the Chesapeake Bay Center for Environmental Studies near Edgewater, Maryland.