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UDPG:STEROL GLUCOSYLTRANSFERASE IN ETIOLATED PEA SEEDLINGS

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Key Word Index—*Pisum sativum*; Leguminosae; sterol glucoside; UDPG-sterol glucosyltransferase; metal ion stimulation; membrane-bound; phospholipase inactivation; phospholipid activation.

Abstract—Sterol UDPglucose glucosyltransferase was located predominantly in the axis tissue of etiolated pea seedlings. During the first 11 days of growth the activity reached a peak in the axis tissue after seven days. Centrifugation of tissue homogenates showed the cell fraction sedimenting between 13000 and 25000 *g* to have the highest specific activity and also the bulk of the total activity. Sitosterol is the major free sterol of this fraction and cholesterol is a trace component. The composition of the aglycones of the isolated steryl glycosides shows cholesterol to be the major sterol. Although exhibiting no metal ion requirement, the enzyme is stimulated by Ca^{2+} and Mg^{2+}, partially inhibited by EDTA and EGTA and completely inhibited by Zn^{2+}. The membranous nature of the enzyme is manifested by its stimulation by the addition of phosphatidyl -ethanolamine, -choline and -serine. After brief treatment with phospholipases A, C and D, enzyme activity is partially lost. After phospholipase A treatment the activity may be completely restored by the addition of phosphatidyl ethanolamine but phosphatidylcholine and -serine are without effect. After phospholipase C and D treatment, each phospholipid brings about a partial recovery of activity but phosphatidyl ethanolamine is again superior.

INTRODUCTION

Steryl glucosides and their acylated derivatives, along with the free sterols and their esters, are common constituents of higher plants [1–3]. The biosynthesis of sterol 3β-D-glucosides has been demonstrated in plant tissues and intact plants [4–6]. Enzymatic studies have been carried out with preparations from immature soy bean seeds [7], mung bean shoots [8,9], pea root, spinach, cauliflower and avocado [10], germinating wheat root [11], *Calendula officinalis* [12] and tobacco seedlings [6]. Changes in the activity of *C. officinalis* during 126 days of growth have recently been reported [13]. In general, these enzymatic studies indicate the glucosyltransferase to be particulate.

The suggested involvement of sterols in plant membranes [14–19] and their role as a substrate in the glucosyltransferase reaction prompted us to examine the activity of this enzyme in a developing pea seedling system.

RESULTS AND DISCUSSION

Ongun and Mudd [10] have demonstrated the presence of a sterol:UDPglucose glucosyltransferase in pea root mitochondria. That the synthesis of steryl glucoside also occurs in the axial shoot tissue (the axis tissue between the apical tip and the cotyledon) was demonstrated by allowing shoots from seven-day-old etiolated pea seedlings to metabolize mevalonate-[2-^{14}C] for 12 hr. Separation of the radioactive components of an acetone extract of the tissue by TLC showed ^{14}C to be in a zone corresponding to cholesterol glucoside. The incorporation of 3*R*,*S*-mevalonate into this zone represented 0·19% of the mevalonate absorbed. Acid hydrolysis and TLC of an ether extract of the hydrolysate showed ^{14}C to be associated with sterol.

The sterol:UDPglucose glucosyltransferase was initially obtained using a cell fractionation procedure based upon that of Ongun and Mudd [10]. The enzyme was isolated in an 18000 *g* pellet fraction of tissues homogenized in 0·05 M Tris–HCl, 0·25 M in sucrose. It was assayed by measuring the incorporation of ^{14}C from UDPglucose[U-^{14}C] into a $CHCl_3$–MeOH extract after incubation of the enzyme in sucrose-free, 0·05M Tris–HCl (pH8) buffer in the presence of Mg^{2+} and ATP. Under optimum conditions the turnover of substrate was linear for 30 min. The assays were conducted for 15 min.

Under the conditions of the assay, TLC revealed that *ca* 90% of the label appeared in a zone corresponding to cholesterol glucoside. Such a distribution was found in three different solvent systems. The remainder of the radioactivity was distributed along the plate giving no discrete peak. The acylated derivative has frequently been observed to be formed in cell-free extracts of plant tissues and the evidence indicates that it arises by acylation of the preformed steryl glucoside. The absence of the acyl derivative in the present case may be a result of the relatively short incubation time or the limited availability of the required long chain fatty acyl donor. It has also been shown [10] that the acylating enzyme in pea mitochondria has a pH optimum of 6·5–7.

A comparison of glucosyltransferase activity in different tissues of 4-day-old pea seedlings is shown in Table 1. The enzyme appears least active in the cotyledon and most active in the axial shoot. The axial root is somewhat less active than the shoot. No difference could be distinguished between the upper and lower halves of the

Table 1. UDPGlucose glucosyltransferase in different tissues of 4-day-old pea seedlings

Tissue	Activity nmol/hr/mg	Relative activity
Cotyledon	3·3	0·30
Axial root	7·7	0·69
Axial shoot	11·1	1·00
Upper axial shoot	10·7	0·96
Lower axial shoot	11·5	1·04

Reaction mixtures contained 2 μmoles each of Mg^{2+}, ATP and UDPglucose-[U-^{14}C] (0·2 μCi), 1·0 ml of the enzyme extract and 31·0 μmol of Tris–HCl buffer in a total vol of 1·5 ml. The pH was 8·0; the reaction time 15 min and the temp. 30°.

shoot tissue. Wojciechowski [13] recently observed that young, rapidly developing tissues of *C. officinalis* exhibit the highest glucosyltransferase activity indicating their possible involvement in membrane expansion. Conceivably, the entire axial shoot in 4-day-old pea seedlings is sufficiently active in membrane synthesis that no distinction between the upper and lower halves of the shoot can be made.

Changes in the glucosyl transferase activity in the root, shoot and whole axis tissue of pea seedlings as development progresses is shown in Fig. 1. At two days the amount of axis tissue is so small that no attempt was made to examine the enzyme activity in the two halves. For the remainder of the growth period, activity in the shoot slightly exceeded that in the root. The activity in the axis tissue increases 8-fold between the 2nd and 7th day and thereafter declines sharply approaching the original activity after 11 days. Interestingly, this same activity profile is shown for the sterol:UDPglucose glucosyltransferase of *C. officinalis* seedlings.

The distribution of glucosyltransferase activity between different cell fractions of seven-day-old seedlings

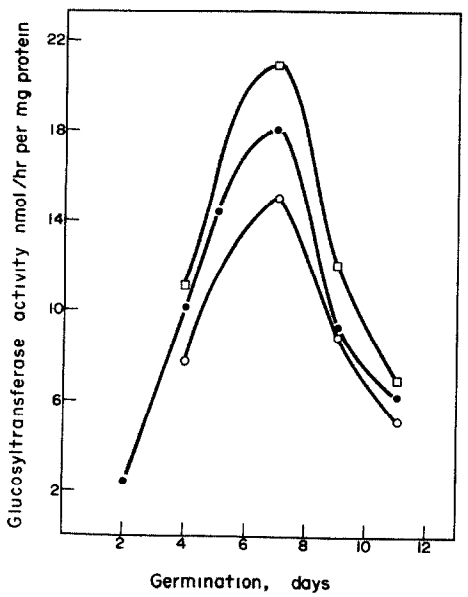

Fig. 1. Changes in UDPglucose glucosyltransferase activity in shoot and root axis tissues of pea seedlings during germination. □——□, shoot, ○——○, root; ●——●, whole axis.

Table 2. Distribution of glucosyltransferase activity among different cell fractions of seven-day-old pea seedlings

Cell fraction (× *g*)	Relative specific activity
Crude	1·00
1000	0·37
6000	2·87
15000	7·97
20000	9·34
50000	1·81
105000	1·66
Supernatant	0·08

Reaction mixtures contained 1 μmol each of Mg^{2+}, ATP and UDPglucose-[U-^{14}C] (0·1 μCi), 0·5 ml enzyme extract and 17·5 μmol of Tris–HCl buffer in a total vol of 0·75 ml. The pH was 8·0; the reaction time 15 min and the temp. 30°.

is shown in Table 2. It is clear that the highest glucosyltransferase activity is predominantly associated with the particulate fractions of the extracts: the mitochondria, etioplasts and Golgi bodies. The method of homogenization however would lead to the formation of fragmented membranes of these organelles and their appearance in several higher centrifugation fractions might then be expected. It is noteworthy that the glycosyltransferases are used as marker enzymes of Golgi membranes [20].

TLC examination of $CHCl_3$–MeOH extracts of each of the fractions shown in Table 2 revealed steryl glycoside to be present in the 6000*g*, 15000*g* and 20000*g* fractions, i.e. those highest in glucosyltransferase activity. The absence of the glycoside in the supernatant and its presence in particulate fractions makes it appear improbable that steryl glycoside is a transport form of sterol as has been suggested [21].

Based upon the activity distribution of Table 2 a second fractionation procedure was adopted to maximize the isolation of the enzyme (Table 3). The most active fraction, that sedimenting between 13000*g* and 25000*g*, also contains slightly more than one-half the total activity. The activity resident in the supernatant fraction may represent a combination of microsomal [11] and soluble glucosyltransferase activities [22]. The 13000–25000*g* fraction was used in all subsequent investigations of the enzyme. In this and all subsequent assays sitosterol in MeOH was added to the reaction mixtures so as not to allow endogenous sterol to become limiting during assay of the high specific activity fractions under conditions of maximum stimulation. The influence of sterol on the reaction, however, may be complex because sterol,

Table 3. Isolation of sterol:UDPGlucose glucosyltransferase activity in seven-day-old pea seedlings

Centrifugation fraction (× *g*)	Volume (ml)	Protein (mg/ml)	Specific activity (nmol/hr/mg)	Relative activity	Total (activity) units (nmol/hr)
1000 Supernatant	395	6·20	4·50	1·00	11030
13000	20	5·94	15·80	3·51	1880
25000	20	5·75	49·40	10·98	5680
Supernatant	385	5·48	2·45	0·54	5170

Reaction mixtures contained 1 μmole each of Mg^{2+} and ATP; 0·5 μmol of UDPglucose-[U-^{14}C] (0·05 μCi); 90 nmol of sitosterol in 37 μl of MeOH, 0·5 ml enzyme extract and 16·5 μmol Tris–HCl buffer in a total vol of 0·75 ml. The pH was 8·0; the reaction time 15 min and the temp. 30°.

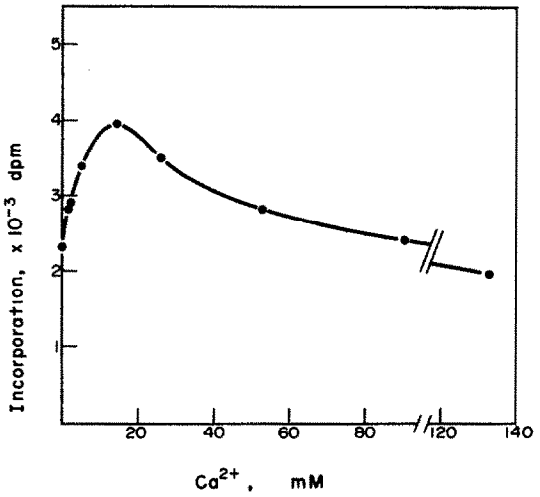

Fig. 2. Influence of increasing Ca^{2+} concentration on UDP-glucose:sterol glucosyltransferase in the 25000*g* particulate fraction of axis tissue from seven-day-old pea seedlings. Reaction mixtures were as for Table 3 but with the appropriate Ca^{2+} concentration in place of Mg^{2+}.

as well as being a co-substrate in the reaction, may also modify membrane fluidity [23].

In Table 4 is shown the influence of several ions at two different concentrations on the sterol:UDPglucose glucosyltransferase activity. It is evident that the transferase does not require the addition of metal ion in order to function. However, Ca^{2+} is slightly stimulatory at the low concentration and more so at the higher concentration. The stimulation by Ca^{2+} is supported by the inhibition of the enzyme activity in the presence of EGTA. The binding of Ca^{2+} by EGTA is six orders of magnitude greater than that for Mg^{2+} [24]. Interestingly, at the lower adduct concentration (Table 4) the inhibition by EDTA exceeds that by EGTA and suggests

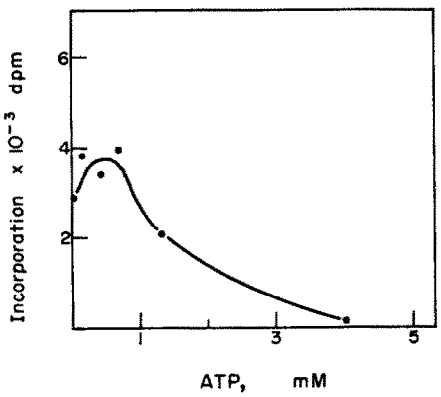

Fig. 3. Influence of increasing ATP concentration on UDP-glucose:sterol glucosyltransferase in the 25000*g* particulate fraction of axis tissue of seven-day-old pea seedlings. Reaction mixtures contained 13·3 mM Ca^{2+}, 0·5 μmol of UDPglucose-[U-^{14}C] (0·05 μCi); 90 nmol of sitosterol in 37 μl of MeOH, 0·5 ml of enzyme and the appropriate ATP concentration. The mixtures were brought to a total volume of 0·75 ml with 25 mM Tris–HCl (pH 8) buffer. The reaction time was 15 min and the temp. 30°.

the possibility that Mg^{2+} may also act in a stimulatory fashion. This is found to be the case at the higher concentration of Mg^{2+}. The activity that persists in the presence of the high concentration of either EGTA or EDTA might suggest the presence of two different glucosyltransferases: one dependent and the other independent of metal ion stimulation. Conceivably, this difference in properties might also be displayed by one transferase present in two different environments in the membrane. Ba^{2+} is a member of the same group (IIa) of elements as Ca^{2+} in the Periodic Table and its stimulatory effect may be a consequence of this fact. The inhibition by Mn^{2+} and Zn^{2+} has been reported for the soluble glucosyltransferase of germinating *Phaseolus aureus* seeds [22]. The extent of inhibition by these two ions on the particulate transferase of the pea seedling system however is far greater.

The influence of increasing Ca^{2+} concentration on the glucosyltransferase is shown in Fig. 2. Ca^{2+} is stimulatory over a very broad range of concentrations and is maximal at about 13 mM. The effect of ATP in the presence of 13 mM Ca^{2+} is shown in Fig. 3. ATP exerts a pronounced inhibitory effect at 1·3 mM, the concentration used to examine the influence of the metal ions in Table 4. Only at low concentrations does ATP show any stimulation and then it is only slight. The role of ATP in glucosyltransferase activity is currently under investigation.

A comparison of the composition of the free sterol in the 25000*g* pellet fraction with that of the aglycones of the endogenous sterol glucosides was made by GLC analysis. With the free sterols sitosterol is the major component, stigmasterol and campesterol are minor components and cholesterol is a trace component. Of the steryl glycosides, cholesterol appears as the predominant aglycone and the other three sterols are barely detectable. Cholesterol is known to be present in only trace amounts in plants [17] and we find this to be the case here. It is noteworthy that we find cholesterol existing as the major glycoside in this membrane fraction.

TLC of a $CHCl_3$–MeOH extract in a solvent system designed to separate phospholipids revealed the presence of phosphatidyl choline (PC), phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE), with the latter qualitatively appearing to be the major phospholipid.

Table 4. Effect of metal ions on sterol:UDPGlucose glucosyltransferase

Addition	Relative activity 1·3 mM adduct	13 mM adduct
None	1·00	1·00
Boiled enzyme	0·02	
$MgCl_2$	1·02	1·57
$MnCl_2$	0·97	0·57
$CaCl_2$	1·11	1·67
$BaCl_2$	1·02	1·33
$ZnCl_2$	0·11	0·02
EDTA	0·36	0·33
EGTA	0·58	0·39

Reaction mixtures contained 1 μmol of ATP, either 1 μmol or 10 μmol of the metal ion, 0·5 μmol UDPglucose-[U-^{14}C] (0·5 μCi), 90 nmol of sitosterol in 37 μl of MeOH, 0·5 ml enzyme extract and 16·5 μmol Tris–HCl buffer in a total vol of 0·75 ml. The pH was 8·0; the reaction time 15 min and the temp. 30°.

Table 5. Phospholipase inactivation and phospholipid reactivation of sterol:UDPGlucose glucosyltransferase

Phospholipase	Phospholipid	Activity (nmol/hr/mg)	Relative activity
None	–	7·48	1·00
	Phosphatidyl choline	9·95	1·33
	Phosphatidyl ethanolamine	16·84	2·25
	Phosphatidyl serine	9·92	1·33
A	–	5·30	0·71
	Phosphatidyl choline	5·40	0·73
	Phosphatidyl ethanolamine	17·24	2·30
	Phosphatidyl serine	5·77	0·77
C	–	5·83	0·78
	Phosphatidyl choline	7·26	0·97
	Phosphatidyl ethanolamine	9·82	1·31
	Phosphatidyl serine	7·07	0·95
D	–	5·90	0·79
	Phosphatidyl choline	7·33	0·98
	Phosphatidyl ethanolamine	11·87	1·59
	Phosphatidyl serine	7·16	0·96

Details of the Experimental procedure are described in the Methods section.

In Table 5 is shown the effect of the separate additions of the three phospholipids on the glucosyltransferase before, and after, individual treatments of the membrane fraction with phospholipases A, C and D. Prior to treatment with the phospholipases the transferase can be seen to be stimulated by each of the phospholipids, but whereas PS and PC produce only a 33% stimulation, that by PE is 125%.

After brief treatment with each phospholipase, a partial loss of activity occurs ranging from 29% with phospholipase A to 21% with phospholipase D. After phospholipase A treatment neither PC nor PS are able to bring about a significant recovery of glucosyltransferase activity. PE on the other hand completely reverses the inactivation and brings the level of enzyme activity back to the level of the PE-stimulated enzyme before such treatment. The action of phospholipase A will produce lysophospholipids. Clearly, although PC and PE bear the same net charge, the enzyme requires the less bulky $-NH_3$ of PE in order to overcome the inactivation produced by lysophospholipid formation.

Phospholipase C and D treatments lead to diacylglycerol and phosphatidic acid respectively. Compared with the PC- and PS-stimulated activities of glucosyltransferase prior to phospholipase action the addition of PC and PS lead to 33% recoveries of this stimulated activity. Interestingly, they are equally effective in this capacity. PE is only slightly better giving 36% and 55% of the PE-stimulated level after phospholipase C and D treatments respectively.

The loss of glucosyltransferase activity by phospholipase treatment and, in some cases, its partial or full recovery by the addition of phospholipids demonstrates the requirement for a lipid environment for maximal activity of the enzyme.

The effectiveness of PE compared with PS and PC suggests the glucosyltransferase is asymmetrically oriented in the membrane. Evidence supporting the asymmetry of the erythrocyte membrane has been reviewed [25]. It was also proposed that such asymmetry is inherent in the biosynthesis of all membranes including the cytoplasmic membranes. The sterol:UDPglucose glucosyltransferase activation by PE supports such an hypothesis.

Many membrane-bound enzymes require lipids, frequently phospholipids, for the expression of their activities [26]. It has also been observed that the nature of the fatty acyl chains of the phospholipids affect the function of certain membrane-bound proteins in bacterial membranes [27]. It is likely that the activity of the pea seedling sterol:UDPglucose glucosyltransferase is also susceptible to the nature of the acyl chains of the membrane phospholipids.

EXPERIMENTAL

Materials. Pea seeds, *Pisum sativum* L. var. Alaska, were purchased from W. Atlee Burpee Co., Riverside, California. UDP glucose-[U-^{14}C] (155 μCi/μmol) was from International Chemical and Nuclear, Irvine, Calif. Unlabelled UDPglucose was from Calbiochem, La Jolla, Calif. Sitosterol was crystallized several times from $CHCl_3$-MeOH before use. Phospholipase A, from bee venom was purchased from Sigma; phospholipases C and D were from *Clostridium perfringens* and cabbage respectively, both obtained from Miles Laboratories, Kankakee, Ill. Phosphatidyl ethanolamine and phosphatidyl choline were from plant sources and were obtained from Supelco, Inc., Bellefonte, Pa. Phosphatidyl serine was from Nutritional Biochemical Co., Cleveland, Ohio. Thin layer chromatographic sheets were coated with a 100 μm layer of Si gel. Cholesterol glucoside was a generous gift from Dr. J. J. Schneider, Department of Medicine, Jefferson Medical College, Philadelphia, Pa.

Methods. TLC of steryl glycosides was carried out in tanks saturated with solvent systems of $CHCl_3$–MeOH–HOAc–H_2O (65:15:10:4); $CHCl_3$–MeOH–7NNH$_4$OH (65:25:4) [28] and in an unsaturated tank containing $CHCl_3$–EtOH–H_2O (90:9:0·5). Cholesterol glucoside has R_f 0·73, 0·64 and 0·39 in the respective solvent systems and was run as a standard alongside the experimental sample in preparative runs. Spots were detected with I_2 vapour or by spraying with 50% H_2SO_4 or 20% $HC10_4$ followed by heating briefly in an oven at 100°.

Radioactivity measurements. $CHCl_3$-MeOH soluble materials were assayed by liquid scintillation counting of the organic extracts which had been evaporated to dryness in vials and to the residue 4 ml of scintillation fluid added. The scintillation fluid was 2,5-diphenyloxazole (4 g) and 1,4-bis-2′-(5′-phenyloxazolyl)-benzene (30 mg) in 1 l. of toluene. There was no quenching of samples as determined by evaporating extracts with a cholesterol-^{14}C standard before addition of fluor. The efficiency of counting was 85%. The distribution of radioactivity on TLC was determined with a radiochromatogram scanner.

Protein measurement. By the Lowry [29] or by the biuret [30] method.

Seed germination. Seeds were washed and presoaked with several changes of H_2O for 24 hr before germination on vermiculite. The seeds were maintained moist and in the dark at 20–22° for an additional 6 days. The seedlings were thoroughly rinsed and the cotyledons removed to leave the axis tissue.

Preparation of the Sterol:UDPglucose glucosyltransferase. The axis tissue of 7-day-old etiolated pea seedlings was homogenized for 30 sec in a Waring blender with ice-cold 25 mM Tris–HCl buffer which was 0·4 M in sucrose. The volume of buffer was 1·5 times the seedling tissue weight. After filtration through one layer of Miracloth the crude homogenate was centrifuged at 1000 *g* for 5 min. A 25000 *g* pellet was obtained

by first centrifuging the 1000 *g* supernatant at 13000 *g* for 15 min followed by centrifugation of the resulting supernatant at 25000 *g* for 15 min. The 25000 *g* pellet was washed with the buffer used for the homogenization and centrifuged again for 15 min at 25000 *g*. The washed pellet was resuspended in 25 mM Tris–HCl (pH8) and adjusted to a protein concentration of 2·4 mg/ml. Typically, 100 g of fresh plant tissue gave about 16 ml of enzyme preparation. The pellet was stored in 3–5 ml vol at −20°. Under such conditions the samples were used only once after thawing. For the study on the relative glucosyltransferase in different cell fractions, pellet fractions were obtained from the filtered extract by successive centrifugations at 1000 *g* for 5 min; 6000 *g* for 15 min; 15000 *g* for 15 min; 20000 *g* for 15 min; 50000 *g* for 30 min and 105000 *g* for 30 min. The pellets were resuspended in 25 mM Tris–HCl buffer (pH8) to give protein concentrations ranging from 2 to 10 mg/ml for the enzyme assays. For the initial studies on the distribution of the enzyme among the different tissues of 4-day-old seedlings, an 18000 *g* pellet fraction was obtained from an homogenate prepared in 50 mM Tris–HCl (pH8), 0·25 M in sucrose. The crude homogenate was spun at 1000 *g* for 5 min and the supernatant from this centrifugation spun at 18000 *g* for 15 min. The pellet was resuspended in 25 mM Tris–HCl (pH8). Protein concentrations ranged from 3 to 13 mg/ml for the enzyme assays. A similar homogenization and fractionation of tissues from seeds germinated from 2 to 11 days was used for the study of the change of enzyme activity with germination. Protein concentrations ranged from 2 to 8·8 mg/ml for the enzyme assays.

Sterol: UDPglucose glycosyltransferase assay. Reaction mixtures contained 10 μmol $CaCl_2$, 0·65 μmol ATP, 0·5 μmol UDPglucose-[U-^{14}C](0·05 μCi), 90 nmol sitosterol in 37 μl MeOH, 1·2 mg protein and 16·5 μmol Tris–HCl to give pH 8 in a total vol of 0·75 ml. The reaction was run at 30° and was stopped after 15 min by the addition of 1·5 ml $CHCl_3$–MeOH (2:1, v/v). The mixture was vigorously mixed and the phases separated by centrifugation. The organic layer was removed and the aq. phase extracted 2× with 1·5 ml $CHCl_3$. One-half of the combined extracts was used for radioactivity measurement. The incorporation of ^{14}C into the organic phase was linear for 20 min. In the assays using the 18000 *g* pellet fraction and in those measuring the relative glucosyltransferase activity in different cell fractions of 7-day-old axial tissues, sitosterol was omitted from the reaction mixtures and 1 μmole each of $MgCl_2$ and ATP replaced the concentrations of $CaCl_2$ and ATP later found to be optimal for the 25000 *g* pellet fraction.

Sterol and sterol glucoside composition of the 25000 g fraction. A 3 ml sample of the fraction was repeatedly extracted with *n*-hexane to obtain the free sterols. The residual phase was then extracted several times with $CHCl_3$–MeOH (2:1) to isolate the polar lipids containing the steryl glucosides. The two extracts were evaporated to small vol and subjected to TLC. The free sterols were isolated by elution with Et_2O–EtOH (1:1) of a zone corresponding to the mobility of a sitosterol standard after chromatography in Skellysolve-B–EtOAc (88:12). The steryl glucosides were chromatographed in $CHCl_3$–EtOH–H_2O (90:9:0.5) and the zone corresponding to the mobility of cholesterol glucoside was eluted with hot $CHCl_3$–MeOH (2:1). The extract containing the steryl glucosides was evaporated to dryness and the residue redissolved in 2 ml 0·5% H_2SO_4 in 95% EtOH. The solution was heated under reflux for 20 hr then neutralized with NaOH sol and the lipids extracted with Et_2O. The evaporated extract was again chromatographed by TLC to obtain the free sterols. The free sterols and those regenerated from the glucosides were analyzed by GLC.

GLC analyses. The free sterols and sterols liberated by hydrolysis of sterol glucosides were examined on a column (1·83 m × 3 mm) of 10% UC-W-98 at 240°. The carrier gas was He (30 ml/min) and detection was by FID. Standards of cholesterol, campesterol, stigmasterol and sitosterol had R_t values of 28·5, 36·5, 40·0 and 46·0 min respectively.

The major phospholipids of the 25000 g pellet. A $CHCl_3$–MeOH (2:1) extract of the 25000 *g* pellet was subjected to TLC in a tank saturated with the solvent mixture $CHCl_3$–MeOH: HOAc (65:25:8). Phospholipids were visualized as blue spots by spraying with Cu/MoO_4 sol [31]. Phosphatidyl ethanolamine (R_f 0·49) was the major phospholipid with phosphatidyl serine (R_f 0·22) and phosphatidyl choline (R_f 0·15) also present.

Phospholipase inactivation and phospholipid reactivation of the glucosyltransferase. 2 ml (4·8 mg protein) of the enzyme and either 4·65 units of phospholipase A, or 0·2–0·4 units of phospholipase C, or 4–6 units of phospholipase D were incubated at 25° in the presence of 13·3 mM Ca^{2+}. The total vol of the reaction mixture was 2·13 ml. The control sample was composed of the enzyme and Ca^{2+} only. After 15 min the mixtures were diluted to 10 ml with the 25 mM Tris–HCl buffer (0·4 M in sucrose) and centrifuged at 25000 *g* for 15 min. The pellet was resuspended in a total vol of 2 ml of 25 mM Tris–HCl (pH8) buffer. The sample was divided into 4 equal vol for the glucosyltransferase assays. The phospholipid (10 μg) in a $CHCl_3$ sol was evaporated under N_2 in the assay tube. It was dispersed by sonication in the Tris–HCl buffer (approx 200 μl) after which the phospholipase-treated enzyme was added. The remaining components of the assay were then added and the enzyme activity assayed in the usual way.

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