Phytochemistry, 1979, Vol. 18, pp. 741–748. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0501-0741 $02.00/0

A TRANSGLUCOSYLASE THAT FORMS SOLUBLE GLUCOSIDES FOUND IN MEMBRANES OF A HAPTOPHYCEAN ALGA

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(*Received* 5 *October* 1978)

Key Word Index—*Chrysochromulina chiton*; Haptophyta; UDPGlc glucosyl transferase; phenolic glucoside; secretion; membrane organisation.

Abstract—A particulate enzyme preparation isolated from *Chrysochromulina chiton* catalysed the transfer of [U-^{14}C]-glucose from UDP [U-^{14}C]-Glc to a water-soluble small molecular weight material. Chemical and enzymic analysis of this material showed that it was a phenolic compound to which are attached two β(1-3) glucosides. Properties of the UDP glucose: glucosyltransferase involved in the synthesis of this material have been studied. The UDP glucose glucosyl-transferase was found to be associated with the rough endoplasmic reticulum. A possible function of this phenolic compound in the orientation of membranes for the synthesis of scales in *C. chiton* has been discussed.

INTRODUCTION

Chrysochromulina chiton is a marine unicellular alga and like many other Haptophycean algae produces scales in its single Golgi apparatus. The scales once formed are transported via membrane bound vesicles to the cell surface and are arranged in a definite pattern around the protoplast: there is no cell wall. A detailed chemical analysis has shown the scales to be made of polysaccharide and glycoprotein consisting of 65% protein and 32% carbohydrate [1]. The scales are constructed as a composite with an organized microfibrillar phase woven into a continuous matrix. The microfibrils contain a β(1-4)-glucan. In plants, UDPglucose can serve as a precursor for the synthesis of compounds such as starch, steryl glucosides, phenolic glycosides, β(1-3)glucans, β(1-4)-glucans and mixed β(1-3) and β(1-4)glucans. The present investigation deals with products synthesized from UDPglucose by a partially purified membrane preparation isolated from *C. chiton*.

RESULTS

Characterization of the membrane fractions

Electron microscope observations of the 1000 *g* pellet showed that it contained mainly scales, chloroplasts, nuclei, flagellae, and a few mitochondria. Other analyses showed that it contained 78% of the total chlorophyll, 55% of the total NADH-cytochrome *c* reductase activity, 81% of the total succinate dehydrogenase activity and 57% of the total IDPase activity (Table 1). The membranes in the 1000 *g* supernatant were observed as vesicles of various sizes when examined by electron microscopy and contained 22% of the total chlorophyll, 45% of the total NADH-cytochrome *c* reductase activity, 19% of the total succinate dehydrogenase activity and 43% of the total IDPase activity.

Materials synthesized from UDP-[^{14}C]glucose by a membrane fraction

About 90% of the total radioactive label present at the end of the incubation was water-soluble and was present in the supernatant following centrifugation of the membrane fraction. The incorporation of radioactivity into water-insoluble material was very low (*ca* 0.08% of the total). This material was not further analysed as insufficient quantities of it were obtained. Of the water-soluble material, 93% of the radioactivity was recovered as UDPglucose and Glc-1-P, and a small proportion (*ca* 6%) as neutral material after pH 2.0 electrophoresis. When the neutral material from the electrophoretograms was chromatographed in solvent A, 68% of the radioactivity was recovered as glucose and 32% as a product remaining at the origin of the chromatogram (soluble glycosylated material). The time course of incorporation into soluble glycosylated material is shown in Fig. 1.

Analysis of soluble glycosylated material

Total acid hydrolysis of the soluble glycosylated material gave glucose as the only radioactive component. The material (7000 cpm) on dialysis (Visking size 1–8, 32″ medicell Int. London EC4N 4SA) completely passed out of the sac within 16 hr which indicated that it had a low MW (< 10000).

Paper chromatographic mobility

Samples (100 µl; 5000 cpm) of soluble glycosylated material dissolved in water were chromatographed along with oligosaccharides of the laminaridextrin and cellodextrin series in solvents A–D. Comparison of the mobility of the material, which ran as a single peak of radioactivity in the different solvent systems showed clearly that it did not correspond with a β(1-3) or β(1-4)-glucan oligosaccharide composed of up to 5 glucose units. Nevertheless, in solvent D the movement indicated that

Table 1. Distribution of protein, chlorophyll, radioactivity and enzymic activities in membrane fractions isolated from *C. chiton*

	Homogenate	1000 *g* pellet	Interface 10–25%	25–32%	32–37%	37–40%	40–55%	55–60%	Total recovery (%)
NADH-cytochrome *c* reductase									
Sp.act., nmol min^{-1} mg^{-1}	10.17	6.70	5.81	14.06	19.11	23.78	28.13	36.67	
Total activity, nmol min^{-1}	50.25	22.86	1.75	1.90	1.90	2.94	4.76	5.23	82.2
Succinate dehydrogenase									
Sp.act., nmol min^{-1} mg^{-1}	3.08	3.27	0.63	8.80	12.97				
Total activity nmol min^{-1}	15.23	11.18	0.19	1.19	1.29				90.9
Inosine diphosphatase									
Sp.act., µmol Pi released hr^{-1} mg^{-1}	1.33	0.90	2.22	4.58	2.31	2.02	1.47	1.89	
Total activity, µmol Pi released hr^{-1}	6.58	3.10	0.67	0.62	0.23	0.25	0.25	0.27	81.9
Chlorophyll									
µg mg^{-1}	51.44	60.42	2.39	29.45	162.67	203.96	83.56	7.64	
Total µg	254.18	206.00	0.72	3.98	16.17	25.21	14.14	1.09	105.1
Protein									
µg	4941.0	3409.0	301.2	135.1	99.4	123.6	169.2	142.6	88.6
Radioactivity incorporated into soluble glycosylated material									
Sp.act., cpm μg^{-1} hr^{-1}	27.41	14.45	17.34	48.80	64.40	68.47	104.42	156.00	
Total activity cpm hr^{-1}	135457	49275	5224	6593	6402	8463	17668	22246	85.5

the material was of fairly low MW corresponding to an oligosaccharide composed of approximately 3–5 glucose units.

When the paper chromatograms were examined under short UV light absorption occurred at the level of the glucoside spot. A solution of the glucoside made by eluting the material from the paper chromatogram showed maximal absorption at 280 nm at pH 8.5. There was no absorption at this wavelength at pH 4.7. If the material that was responsible for the absorption in UV light was identical to that of the radioactive glucoside, the evidence suggested that it might be a phenolic glucoside.

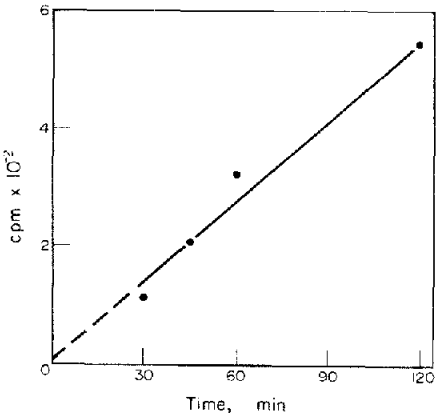


Fig. 1. Time course of incorporation of radioactivity from UDP-[^{14}C]glucose into soluble glycosylated material.

Paper electrophoretic mobility

Samples (*ca* 3000 cpm) of soluble glycosylated material were run electrophoretically at pH 6.5, 9.4 (sodium borate buffer) and at pH 9.4 (ammonium hydroxide–acetic acid buffer). The radioactive material was neutral upon electrophoresis at pH 6.5 which indicated that it contained no non-radioactive uronic acids since most uronic acids would be charged at this pH. The material moved as a single peak with an R_{Glc} value of 0.8 in sodium borate buffer. The movement in borate buffer could have been due to the alkaline pH or to the formation of a borate complex with sugars, or both of these factors. Movement was obtained on electrophoresis at pH 9.4 in ammonium hydroxide–acetic acid buffer (R_{UDPGlc} 0.4) so that at this alkaline pH the material carried a negative charge. Material electrophoresed at pH 9.4 (ammonium hydroxide–acetic acid buffer) was found to be neutral when eluted and electrophoresed again at pH 6.5 and 2.0. Thus the charge acquired at pH 9.4 was reversible and was not due to breakdown of the compound. The electrophoretic mobility of the material showed that it contained non-

radioactive components that have a pK in the range of about 8–10, such as phenolic compounds. The paper chromatographic and electrophoretic mobility of the material indicated that it was a single compound or that it was made up of several very similar compounds.

Extraction with chloroform–methanol–water

A sample of soluble glycosylated material in 1 ml of water (360 cpm) was extracted with chloroform–methanol–water (3:2:1). The organic and aqueous phases were separated and assayed for radioactivity. The aqueous phase contained 98% of the total radioactivity which indicated that the material contained little or no lipid.

Incubation with trichloroacetic acid

A sample of soluble glycosylated material (6700 cpm) was incubated in 10% trichloroacetic acid at 4° for 1 hr and filtered through a Millipore filter (2.5 cm diameter). When the filter was dried and assayed for radioactivity, it contained only 0.4% of the total radioactivity indicating that the material contained no protein.

Incubation with pronase

Samples of soluble glycosylated material (9000 cpm) incubated with pronase were analysed by chromatography in solvents B and C along with samples that had been incubated under the identical conditions but lacking the enzyme. The chromatographic mobility of the enzyme-treated material was identical to the untreated material in both these solvents, which also suggested that the material contained no protein.

Incubation with phosphatases

The material was tested for the presence of phosphate linkages by treating samples with phosphodiesterase and acid phosphatase. Samples (9000 cpm) incubated with phosphodiesterase were analysed by chromatography in solvents B and C along with material incubated under the identical conditions but lacking the enzyme. The chromatographic mobility of the enzyme-treated material was identical to untreated material in both these solvents. The electrophoretic mobility in sodium borate buffer (pH 9.4) and in ammonium hydroxide acetic acid buffer (pH 9.4) was also unaffected after treating samples (21 000 cpm) with phosphodiesterase followed by acid phosphatase, which suggested the absence of phosphate linkages.

Incubation with α-glucosidases

Material (2000 cpm) digested with salivary amylase and amyloglucosidase, when analysed by chromatography in solvent A, contained no glucose or maltose. This indicated the probable absence of α(1-4) and α(1-6)glucan linkages in the material.

Incubation with β-glucosidases

Samples (900 cpm) of soluble glycosylated material were incubated with *Rhizopus* β(1-3)glucanase for varying periods of time. Samples were analysed by chromatography in solvent D. Following 24 hr of incubation, the chromatographic mobility of the material in solvent D was unaffected. When material (900 cpm) incubated for 5 days was analysed by chromatography in solvent D, radioactive components present in the region of laminaritriose, laminaribiose and glucose markers were obtained. When material (2000 cpm) was incubated for 5 days adding fresh enzyme at 1 mg/ml every 24 hr, 87% of it was hydrolysed to components present in the region of laminaritriose, laminaribiose and glucose markers. It was possible to confirm the presence of glucose by elution and electrophoresis in borate buffer. Laminaribiose and laminaritriose were present in too small amounts to be confirmed in this way. Samples of soluble glycosylated material when incubated with *Streptomyces* cellulase under identical conditions gave similar results. This would be expected as the β(1-4)glucanase contained a β(1-3)glucanase contaminant. The hydrolysis of the material by both enzyme preparations suggested that it contained β(1-3) linkages.

Soluble glycosylated (3000 cpm) material incubated with L_1 *cytophaga* extract was analysed by chromatography in solvent D. Radioactive components present in the region of laminaritriose, laminaribiose and glucose were obtained, which also suggested the presence of glucan linkages in the β(1-3) configuration. This enzyme has been shown to degrade β(1-3)glucans but to be inactive against β(1-4)glucans [2].

Periodate oxidation

Glycerol was the only radioactive component detected when the hydrolysis products from a periodate oxidation (initial radioactivity before oxidation was 28 000 cpm) of the material were analysed by sodium borate electrophoresis. Glycerol could arise from (1-2) or (1-6) linked glucans as well as from terminal glucose residues of (1-3), (1-4), (1-2) and (1-6) linked glucans. The absence of radioactive glucose and erythritol indicated that besides the terminal glucose there was no labelled glucose linked (1-3) or (1-4).

Methylation

A sample of the methylated products (600 cpm) extracted with chloroform was analysed by electrophoresis in ammonium hydroxide–acetic acid buffer (pH 9.4) along with a sample of unmethylated soluble glycosylated material. The electrophoresis was carried out on TLC plates using a flat bed electrophoresis apparatus at 500 V for 3 hr. Glucose and UDPglucose were run as neutral and negatively charged markers. After methylation, the soluble glycosylated material moved with the neutral marker which indicated that its negative charge at pH 9.4 had been neutralized. The evidence strongly suggests that a hydroxyl group, as found in phenols, was responsible for the negative charge at pH 9.4. The presence of a carboxyl group was eliminated as the material was neutral at pH 6.5.

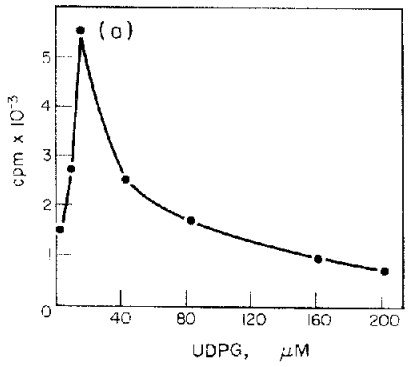
A total acid hydrolysate of the methylated material was analysed by chromatography in solvent E with 2,3,6-; 2,4,6-; 2,3,4-*O*-trimethylglucose and 2,3,4,6-*O*-tetramethylglucose markers. 2,3,4,6-*O*-Tetramethylglucose was the only radioactive component detected which showed that the only labelled glucose in the material was a terminal one. Thus the periodate oxidation, methylation, and enzyme degradation results indicate the material is a β(1-3) linked glucose-oligosaccharide containing labelled glucose only on the non-reducing end of a short chain.

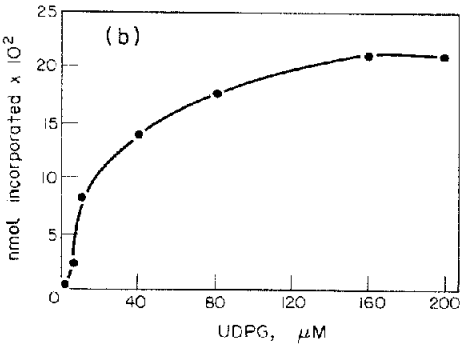
Mild acid hydrolysis

A mild acid hydrolysate of soluble glycosylated material (7200 cpm) was analysed by chromatography in solvent B. The distribution of radioactivity on the chromatogram showed that 32% of the total radioactivity was released as two components present in the region of laminaritriose, laminaribiose and glucose markers. When these two components were eluted and analysed by electrophoresis in sodium borate buffer, most of the radioactivity was present as laminaritriose, laminaribiose and a small amount as glucose.

The material at the origin of the chromatogram was re-subjected to mild acid hydrolysis and re-analysed by chromatography in solvent B. All the radioactivity was present at the origin of the chromatogram indicating that the first acid treatment had hydrolysed all the mild acid-labile linkages. Its chromatographic mobility in solvents C and D was found to be similar to that of untreated material.

In a second experiment using soluble glycosylated material (2800 cpm) from a different membrane preparation, 25% of the total radioactivity was released as glucose upon mild acid hydrolysis. The material remaining at the origin of the chromatogram (solvent B) had a similar electrophoretic mobility in ammonium hydroxide acetic acid buffer (pH 9.4) to untreated material. Incubation of soluble glycosylated material from this same preparation with phosphodiesterase and acid phosphatase failed to release glucose, indicating





Figs. 2a and b. Effect of increasing concentrations of unlabelled UDPG on the formation of soluble glycosylated material. Incubations were carried out at pH 7.8 and 23° for 2 hr using 0.2 μCi of UDP-[^{14}C]glucose and 15 μg of membrane protein in each incubation.

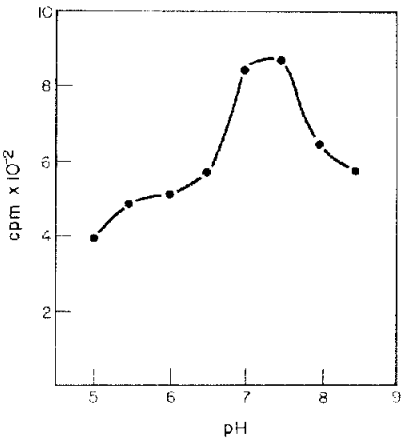


Fig. 3. Effect of pH on the formation of soluble glycosylated material. All incubations were carried out in Tris–Mes buffer for 2 hr at 23° using 0.2 μCi of UDP-[^{14}C]glucose and 10 μg of membrane protein in each incubation.

that the acid-labile linkage was not a phosphodiester or pyrophosphate linkage. Mild alkaline (pH 8.5, 100°, 20 min) hydrolysis failed to release any negatively charged components upon pH 2.0 electrophoresis. The results indicated that 25–30% of the labelled glucose is attached via mild acid-labile linkages. These linkages were probably phenolic glycosidic bonds which are known to be labile to mild acid hydrolysis [3]. The remaining material which still contained glucose was similar to untreated material and still carried a negative charge at pH 9.4. This latter glucose was probably attached by a different type of linkage from that of the more readily hydrolysed glucoside.

Catalytic hydrogenation with palladium

Benzyl glycosides are resistant to mild acid hydrolysis but are specifically cleaved by catalytic hydrogenation with palladium. A sample (16800 cpm) of soluble glycosylated material and a sample (8800 cpm) of the mild acid resistant material was hydrogenated and analysed by chromatography in solvent D. The chromatographic mobility of both samples was unaffected following hydrogenation and no glucose or oligosaccharides were liberated.

Properties of UDPglucose glucosyltransferase

The effect of increasing concentrations of unlabelled UDPG on the synthesis of soluble glycosylated material is shown in Figs. 2a and b. When the total nmoles of glucose incorporated is plotted against UDPglucose concentration, a sigmoid curve was obtained indicating that UDPglucose may serve as an activator as well as a substrate for the UDPglucose glucosyltransferase. Similar substrate activation has been observed with a glucan synthetase in developing cotton fibres [4]. The optimum pH for the glucosyltransferase was determined by carrying out incubations at pH values ranging from 5.0 to 8.5 using Tris–Mes buffer. The optimum was pH 7.5 (Fig. 3). The optimum temperature for the glucosyltransferase was 15° (Fig. 4). This would not be unusual as the organisms were grown at 15°. The activity of the enzyme was found to be stimulated at a concentration of 0.2% Triton (Fig. 5).

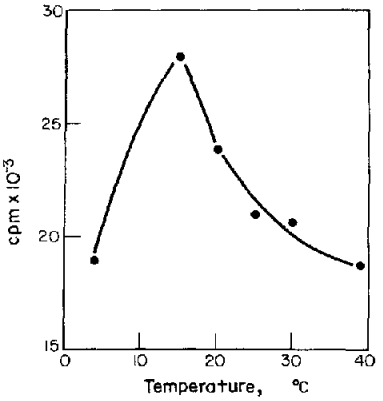


Fig. 4. Effect of temperature on the formation of soluble glycosylated material. Incubations were carried out at pH 7.8 for 2 hr using 0.2 μCi of UDP-[^{14}C]glucose and 58 μg of membrane protein in each incubation.

Further fractionation of the membrane fraction and location of the UDPglucose glucosyltransferase activity

The membranes in the 1000 *g* supernatant were layered onto a discontinuous sucrose gradient and centrifuged at 100000 *g* for 3 hr. The particulate material at each interface was collected and analysed for enzyme activities. Samples of membranes from each interface were incubated with UDP-[^{14}C]glucose to determine which fractions contained glucosyltransferase activity. The distribution of enzyme activities and the amounts of radioactivity incorporated into soluble glycosylated material by each fraction are shown in Table 1. Inosine diphosphatase (IDPase) activity has been suggested to be a marker for Golgi membranes of plant cells [5]. The highest specific activity of this enzyme occurred at the 25–32% sucrose interface. The highest specific activity of the succinate dehydrogenase activity associated with mitochondria occurred at the 32–37% sucrose interface, while that of the chloroplast membranes occurred at the 37–40% sucrose interface. NADH-cytochrome *c* reductase activity is associated with the endoplasmic reticulum. The highest specific activities of this enzyme occurred at the 40–55 and 55–60% sucrose interfaces. The highest specific activities for the soluble glycosyltransferase were obtained at the 40–55 and 55–60% sucrose interfaces which coincides with the NADH-cytochrome *c* reductase activity. The results indicate that the glucosyltransferase is associated with the endoplasmic reticulum.

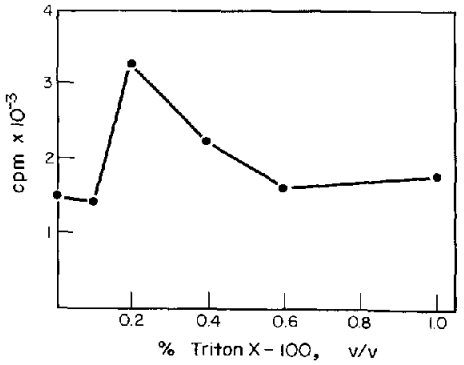


Fig. 5. Effect of Triton X-100 on the formation of soluble glycosylated material. Incubations were carried out for 2 hr at pH 7.8 and 23° using 0.2 μCi of UDP-[^{14}C]glucose and 17 μg of membrane protein in each incubation.

DISCUSSION

A membrane preparation isolated from *C. chiton* has been shown to incorporate glucose from UDP-[^{14}C] glucose into water-soluble small molecular weight compounds. The substances formed by the transglucosylases are probably phenolic compounds to which are attached sugars by two separate glycosidic bonds and on which there is at least one free phenolic hydroxyl group. One of the glycosidic links was labile to mild acid hydrolysis and it was present either as a single glucose unit or as a β(1-3)glucan disaccharide or trisaccharide. This was attached to the phenol by a phenolic glycosidic linkage. The other glycoside which was stable to mild acid hydrolysis was a short β(1-3)glucan oligosaccharide of unknown length and may even by a single glucose unit.

The positions at which sugar residues are attached to phenolic compounds have been shown to be important in determining the lability to acid hydrolysis. Sugar residues attached to the 3-position of flavonols are more susceptible than those attached to the 7-position [6]. Thus, it is likely that the acid stable glycoside which was represented by 70–75% of the incorporated radioactive glucose from UDP-D-[U^{14}C]glucose was also a phenolic glycoside but at a different position of the aromatic ring to that of the labile group. Benzyl glycosides were not present.

In both glycosides only the terminal glucose was labelled by the radioactive UDPglucose. Figure 6 illustrates the type of compound and the different transglucosylases which may be involved.

Although each of the membrane fractions isolated was contaminated with other fractions, the separation was adequate to identify the specific membrane at which the UDPglucose glucosyltransferases were located. The buoyant densities of the membrane components isolated corresponded with those reported by other workers [7–10]. Specific enzyme markers were not used to locate the plasma membrane and the smooth endoplasmic reticulum in the gradient. According to published data [7, 11], the plasmamembrane would probably have been present mainly at the 32–37% sucrose interface and the smooth endoplasmic reticulum at the 10–25%

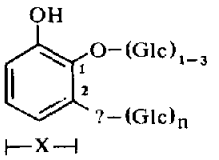


Fig. 6. The structure of the soluble glycosylated material. X = Any type of phenolic compound with an ionizable hydroxyl group at pH 8–10. 1 = A mild acid-labile linkage which is a phenolic glycosidic linkage by which glucose, laminaribiose or laminaritriose are attached. 2 = A mild acid-stable linkage by which an unknown number of sugars are attached. This may also be a phenolic glycosidic linkage which is attached to a different position of the ring to the former.

sucrose interface. The UDPglucose glucosyltransferases were associated with the rough endoplasmic reticulum.

Although glycosylation of phenols is a common and characteristic feature of higher plants, the reason for glycosylation of many phenols is not clear. The majority of phenolic glycosides contain a single sugar residue which is most often glucose. Several disaccharides in combination with phenols have also been reported such as rutinose (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose), gentiobiose (6-*O*-β-D-glucopyranosyl-β-D-glucopyranose), sambubiose (2-*O*-β-D-xylopyranosyl-β-D-glucopyranose), and sophorose (2-*O*-β-D-glucopyranosyl-β-D-glucopyranose) [12–15]. Glucose and rhamnose are the most common sugars found in disaccharides of phenolic glycosides. Several trisaccharides in combination with phenols have also been reported [14, 15]. The requirement of UDPglucose as a glucosyl donor for the synthesis of phenolic glycosides is well established. Many workers have shown the synthesis of phenolic glucosides by membrane preparations isolated from a variety of plants in the presence of phenols and UDPglucose. Monosaccharides, disaccharides and in certain instances trisaccharides attached to phenols have been synthesized [13–17].

Glycosyltransferases which catalyse the transfer of glycosyl units to phenols have been shown to exhibit a certain degree of specificity towards donor and acceptor substances [12]. Studies by Barber [13] and Yamaha and Cardini [12, 17] show that the enzymes catalysing the transfer of glycosyl units to phenols are distinct from those catalysing the transfer to existing glycosyl units already attached to phenols. In certain instances, the glycosylation of hydroxyl groups at different positions of phenols has been shown to require different enzymes [18, 19].

Very little information is available concerning the function of these phenolic glycosides and the specific intracellular membranes involved in their synthesis. When phenolic substances have been introduced to plants, the synthesis of the corresponding glycosides has been observed [20]. Thus glycosylation in certain cases may serve as a detoxification process. Some phenolic glycosides are known to occur as pigments in flower petals. Some of the other functions attributed to the glycosylation of phenols are stabilization and alteration of their solubility.

Recent observations by Schippers and Prop [21] indicate that phenols may be involved in changing the surface properties of membranes. They found that cyanidin chloride and delphinidin chloride caused aggregation of cells of different origin. These aggregates were bound together by normal junctional structures and the cells continued to divide indicating viability. It was suggested that the numerous hydroxyl groups of these polyphenols could be involved in binding substances in cell membranes to alter their properties. Thus glycosylation of phenols may serve as a means of blocking free hydroxyl groups and thereby affecting the reactivity of phenols with substances in cell membranes. This would result in changes in the surface properties of the membranes and may play a role in membrane orientation and fusion.

In *C. chiton* the scales are synthesized in the Golgi apparatus and are transported in large Golgi vesicles to a particular site at the cell surface. During this process, the Golgi vesicles are incorporated into the plasmamembrane. The Golgi apparatus is derived from membranes of the endoplasmic reticulum, the location of the UDPglucose glucosyltransferase. The glycosylation of phenols at the endoplasmic reticulum may result in changes in the surface properties of these membranes which may be important for the orientation of these membranes and their fusion with Golgi membranes. The glycosylated phenols may be transported to the Golgi apparatus from endoplasmic reticulum vesicles where they may play a role in altering the properties of Golgi membranes, where the relatively large scales are being assembled. Eventually they are transported to the plasmamembrane as the Golgi vesicles fuse at a specific site with the plasmamembrane. Thus phenolic glycosides may be transported from the endoplasmic reticulum to the plasmamembrane and may play a role in membrane stabilization, orientation and fusion. It is possible that glycosylation of phenols and enzymic removal of glycosidic residues from phenols could serve as a mechanism for regulating the surface properties of membranes, especially in the circumstances of scale formation and secretion where such large insoluble units are assembled and secreted to the outside of the cell.

EXPERIMENTAL

Algal culture. Chrysochromulina chiton Parke and Manton (Plymouth isolate No. 146) was grown in Erd–Schreiber medium. The medium was made from 1 l. of natural sea water to which was added soil extract, 50 ml: $NaNO_3$, 0.2 g; $Na_2HPO_4.12\,H_2O$, 0.03 g; benzyl penicillin, 10 mg. It was sterilized by autoclaving at 103.4 kPa at 120° for 15 min. The algae were grown under light (2000 lx; two 4 ft osram daylight tubes, 40 W, with a daily photoperiod of 16 hr) in 250 ml of medium in 500 ml conical flasks at 15 ± 1° for a period of 2–3 weeks before subculturing or harvesting (5×10^5 cells/ml).

Preparation of membrane fractions. Cells were harvested by centrifuging 1.5 l. of algal culture for 30 min at 500 *g* using a Sorvall RC2-B centrifuge. The pellet was resuspended in 5 ml of 0.25 M sucrose and the cells were disrupted by shaking with glass beads (0.18 mm) in a Mickle shaker (H. Mickle, Hampton, Middlesex, U.K.) for 1–2 min at 4°. Following cell disruption, the algal membranes were centrifuged at 1000 *g*, 4° for 20 min in a Sorvall RC2-B centrifuge (1000 *g* pellet). The resulting supernatant (1000 *g* supernatant) was layered onto a discontinuous sucrose gradient (5 ml 60% (w/w); 5 ml 55% (w/w); 5 ml 40% (w/w); 5 ml 37% (w/w); 5 ml 32% (w/w); 5 ml 25% (w/w)) and centrifuged at 100000 *g* for 4 hr in a Beckman ultracentrifuge at 4° using an SW 27.1 rotor head.

Characterization of membranes. The distribution of enzymic activities in the separated bands on the discontinuous gradient was established by assaying 100 μl samples from each of the fractions using a Beckman model 25 recording spectrophotometer. Protein was estimated by the method of ref. [22] after precipitating the protein with 80% Me_2CO. The total chlorophyll content was estimated by measuring the absorbancy at 470 and 672 nm of 80% Me_2CO extracts of membranes [23, 24]. NADH-cytochrome *c* reductase activity and IDPase activity were measured as described by ref. [25]. Succinate dehydrogenase activity was measured as described by ref. [26].

Membranes in the 1000 *g* pellet and in the resulting supernatant were examined by electron microscopy. Membrane fractions were fixed for 30 min at *ca* 20° in sodium phosphate (0.02 M, pH 7.2) buffer containing glutaraldehyde (6%) and 0.5 M sucrose. The fractions were centrifuged at 100000 *g* for 30 min, and the pellets were washed in 3 changes of distilled H_2O and post-fixed

for 1 hr in osmium tetroxide (1%) buffered with sodium veronal (pH 7.2). The pellets were washed several times with H_2O to remove excess stain, dehydrated via an EtOH series (25–100%), washed with propylene oxide and embedded in araldite. Thin sections of the embedded material were cut with a glass knife using a Porter–Blum Sorvall M2 ultramicrotome. The sections were mounted on carbon–celloidin-coated grids, stained with uranyl acetate and alkaline lead hydroxide, and examined under the electron microscope [27]. All observations were made with a GEC-AEI EM6B electron microscope at 60 KV.

Incubation of membranes with UDP-[U-^{14}C]*glucose.* All the membranes in the supernatant of the 1000 g centrifugation were pelleted by recentrifuging at 100000 *g* for 1 hr. The resulting supernatant was discarded and the pellet was resuspended in Tris–HCl buffer before incubating with UDP-[U-^{14}C]glucose. The incubation mixture contained in a total vol. of 420 µl: 240 µl of membranes (containing 200–400 µg of protein) in 0.3M Tris–HCl buffer, pH 7.8; 60 µl of UDP-[U-^{14}C]glucose (0.2–1.0 µCi) (UDP-[U-^{14}C]glucose, sp.act. 300 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.); 30 µl of 0.28 M β-mercaptoethanol; 30 µl of 8 mM $MgCl_2$ and 60 µl of 0.2 M glucose. The incubations were carried out for up to 2 hr at 23°. The reactions were terminated by the addition of 1 ml of distilled H_2O and by boiling at 100° for 5 min.

Isolation of materials synthesized from UDP-[U-^{14}C]*glucose.* After the incubations were terminated, the water-soluble material was separated from the insoluble material by centrifuging at 1000 *g* for 20 min. The supernatant was removed and the pellet was washed twice with distilled H_2O (1 ml). The supernatant and washings were combined and treated with 0.5 ml of 32 mM Na_2-EDTA before evaporating to dryness under red. pres. The pellet was washed twice with distilled H_2O (1 ml) and dried onto a millipore filter (2.5 cm dia) by suction. The filter was dried and assayed for radioactivity. The water-soluble material was electrophoresed at pH 2.0 to separate UDPglucose and Glc-1-P from neutral material. The neutral material from electrophoretograms was eluted and run chromatographically in solvent A to remove any [^{14}C]-glucose that may have arisen from breakdown of UDPglucose during the incubation. The material remaining at the origin of the chromatogram was eluted with H_2O and evapd to dryness before dissolving in H_2O or buffer according to the analytical procedure used.

Paper chromatography and electrophoresis. Descending paper chromatograms were run on Whatman No. 1 paper in the following solvents: A, EtOAc–Py–H_2O (8:2:1); B, EtOAc–Py–H_2O (10:4:3); C, *n*-BuOH–Py–H_2O (4:3:4); D, PrOH–EtOAc–H_2O (7:1:2); E, *n*-BuOH satd with H_2O. Paper electrophoresis was performed on Whatman No. 1 paper in the following buffers: pH 2.0, 8% HOAc, 2% HCO_2H at 4 kV for 45 min; pH 3.5, Py–HOAc–H_2O (1:10:89) at 4 kV for 30 min; pH 6.5, Py–HOAc–H_2O (100:3:897) at 4 kV for 15 min; pH 9.4, 19 g/l. sodium tetraborate at 3.5 kV for 35 min; pH 9.4, 1 N NH_4OH–1 N HOAc (10:1) at 1.5 kV for 1 hr. Marker sugars on chromatograms and electrophoretograms were detected by the aniline phthalate method of ref. [28] or by the alkaline $AgNO_3$ method [29]. Sugar phosphates were detected with the molybdenum stain of ref. [30]. Standard oligosaccharides of the laminaridextrin series were prepared by treating commercial laminarin (Koch-Light) with *Rhizopus* β(1-3) endoglucanase at 1 mg/ml for 24 hr as described below. The reaction was terminated by heating the samples at 100° for 10 min. The cellodextrin series was prepared by a partial acid hydrolysis of cellulose powder which was treated with 72% (w/w) H_2SO_4 for 16 hr at 20°. The acid was diluted to 3% (w/w) with distilled H_2O and autoclaved at 103.4 kPa at 120° for 30 min. The hydrolysate was neutralized with barium carbonate.

Assaying of radioactivity. The paper chromatograms and electrophoretograms were cut into 4 × 1 cm strips [31] and were placed in counting vials to which 0.5 ml of scintillant [2,5-diphenyloxazole (PPO), 8.75 g, and 1,4-*bis*-(5-phenyloxazol-2-yl) benzene (POPOP), 0.125 g, in 2.5 l. toluene] was added. The vials were placed in 20 ml Packard bottles and were assayed for radioactivity in a Searle mark III liquid-scintillation counter (model 6880). When radioactive samples were required for further analysis, the strips were washed × 3 with C_6H_6, dried and eluted with H_2O × 3. The eluates were combined and evapd to dryness under red. pres. The samples were dissolved in 100 µl of distilled H_2O for spotting on chromatograms and electrophoretograms.

Total acid hydrolysis. Total acid hydrolysis was carried out according to the method of ref. [31]. Samples were incubated in 3% (w/w) H_2SO_4 at 103.4 kPa at 120° for 1 hr. The acid was neutralized by a bicarbonate form of amberlite IR-4B resin. Hydrolysates were run chromatographically in solvent A for 16 hr. Radioactive material running in the region of glucose, galactose and mannose markers was eluted and re-run in the same solvent for 60 hr to obtain a good separation of these sugars.

Mild acid hydrolysis. Samples were incubated in 0.1 M HCl for 10 min at 100°. The reaction was terminated by cooling the mixture in ice. The acid was diluted with H_2O and removed by evaporating under red. pres. at 30°.

Periodate oxidation. Periodate oxidation was carried out according to the method of ref. [32]. Samples were incubated in 1 ml of 0.5 M sodium metaperiodate at 4° for 96 hr in the dark. Excess periodate was removed by the addition of 2 drops ethylene glycol. Two 0.5 ml portions of 0.1 M $NaBH_4$ were added at 30 min intervals. The samples were then hydrolysed and analysed by electrophoresis in sodium borate buffer.

Methylation. Samples were first methylated by the Hakamori [33] method. The partially methylated products were extracted with chloroform and then methylated by the Haworth and Percival [34] method.

Catalytic hydrogenation with Pd. Catalytic hydrogenation with Pd black was carried out according to the method described by Fletcher [35]. Palladium black (300 mg) was suspended in 5 ml EtOH and saturated with H_2 for 15 min. The samples dissolved in 0.5 ml EtOH were added to the suspension and hydrogenated for 4 hr. Following hydrogenation, the catalyst was removed by filtration and the samples were evapd to dryness under red. pres.

The activity of the Pd catalyst was tested by hydrogenation of benzyl β-D-arabino-pyranoside under identical conditions. The benzyl arabinoside was prepared according to the method of Ballou [36] by saturating a mixture of D-arabinose (5 g) and benzyl alcohol (25 ml) with HCl gas for 20 min. The benzyl arabinoside was obtained following re-crystallization from Et_2O. Arabinose was detected by chromatography in solvent A following hydrogenation of 15 mg of the benzyl arabinoside. As a control, 15 mg benzyl arabinoside was suspended in the Pd black alcohol mixture for 4 hr to which hydrogen was not bubbled. No arabinose was detected from this control sample upon chromatography in solvent A. These results indicated that the hydrogenation procedure was successful in cleaving benzyl glycosidic linkages.

Salivary amylase. Samples were incubated with 1 ml of salivary amylase for 4 hr under toluene [37].

Amyloglucosidase. Samples were incubated in 2.0 ml 0.1 M NaOAc buffer, pH 4.8, containing amyloglucosidase at 1 mg/ml (from *Aspergillus niger*, Boehringer, London W.5., U.K.) for 1 hr at 30° under toluene. This enzyme preparation has been shown to hydrolyse α1-4 and α1-6 glucans completely under these conditions (Jones, M. G. K., unpublished data).

β(1-3) glucanase and β(1-4) glucanase. Rhizopus endo-β(1-3)-glucanase (S178K) and *Streptomyces* cellulase (S119g, β(1-4)-glucanase) were a gift from E. T. Reese of the U.S. Army Laboratories, Natick, Mass., U.S.A. Samples were incubated in 0.1 ml of 0.05 M sodium acetate buffer, pH 5.0, containing 3 mM NaN_3 and 0.1 mg β(1-3)glucanase or *Streptomyces* cellulase for 1–5 days at 50° in sealed tubes. The β(1-3)glucanase was found to hydrolyse laminarin to a series of laminaridextrin oligosaccharides under these conditions but was completely inactive against cellulose, even when incubated for 5 days adding fresh enzyme every 24 hr at a concn of 1 mg/ml. The *Streptomyces* cellulase hydrolysed cellulose to glucose under these conditions but also hydrolysed laminarin to a series of laminaridextrin oligosaccharides, indicating that it contained a β(1-3)glucanase contaminant.

L_1 cytophaga lytic enzyme. Samples were incubated in 0.1 ml of 0.05 M NaOAc buffer, pH 5.0, containing 3 mM NaN_3 and 0.05 mg Cytophaga extract (B.D.H. Chemicals Ltd.) at 25° for 15 hr.

Pronase. Samples were incubated in 0.3 ml of 0.3 M Tris–HCl buffer, pH 7.2, containing chloramphenicol (10 μg/ml) and 0.3 mg pronase (B.D.H.) at 25° for 15 hr.

Phosphodiesterase (snake venom). Samples were incubated in 0.3 ml of 0.3 M Tris–HCl buffer, pH 8.9, containing chloramphenicol (10 μg/ml) and 0.04 mg phosphodiesterase (*Crotalus terrificus terrificus*, Boehringer, London) at 25° for 15 hr.

Wheat germ acid phosphatase. Samples were incubated for 16 hr at 23° in 0.2 ml of 0.05 M sodium acetate buffer, pH 5.0, containing 3 mM NaN_3 and acid phosphatase (B.D.H.) at 2.5 mg/ml.

Acknowledgements—One of us, N.D.S., wishes to thank King's College, Cambridge; Wilson College, U.S.A.; The Fleet Foundation, U.S.A. and the British Council for financial support during the time the work was carried out.

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