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The Structure and Function of Glycoproteins Synthesized during Slime-Polysaccharide Production by Membranes of the Root-Cap Cells of Maize (*Zea mays*)

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The synthesis of the maize root slime polysaccharides was investigated by using [1-^{3}H]-fucose as a marker for slime production. Three fractions were separated by centrifugation in a CsCl density gradient. Two of these were glycoproteins and occurred within the membranes of the cells of the root tip; the third was the slime polysaccharides. Radioactive pulse–chase experiments showed that the glycoproteins were precursors of the slime polysaccharides, and the carbohydrate portion of the glycoproteins had a similar composition to that of the free slime. The linkage between the protein and the carbohydrate of one of the glycoproteins was shown to be a xylose–threonine bond. It is postulated that the slime polysaccharides are synthesized and transported on proteins within the membrane system of the root tip.

In recent years several compounds have been identified as intermediates or acceptor substances necessary for polysaccharide biosynthesis, in particular polyprenyl phosphates and proteins have been shown to be carriers of the growing polysaccharide chain (Northcote, 1977). The production of slime by the maize root-cap provides an ideal system for studying these intermediates.

The root cap protects the meristem from damage as the root bores through the soil, and the outer root-cap cells produce a slime that is very hydrophilic. It serves to protect the growing root tip from physical damage and dehydration, and also functions as a lubricant (Barlow, 1975). In maize it consists mainly of polysaccharide and is characterized by the presence of a high proportion (approx. 25%) of fucose (Harris & Northcote, 1970; Bowles & Northcote, 1972). Large amounts of fucose have not been found in any other polymers present elsewhere in the maize seedling. Trace amounts have been found in pectin prepared from maize roots and coleoptiles and from root-tip material isolated from sycamore, pea and wheat (Nevins *et al.*, 1967; Harris & Northcote, 1970; Wright & Northcote, 1974). Slime having a high content of fucose is therefore a specific product of the differentiated outer root-cap cells of maize. Fucose is not metabolized by the maize root into other sugars and thus it can be used as a marker for slime production (Kirby & Roberts, 1971; Wright & Northcote, 1976).

Some of the constituents of slime resemble pectin (Wright & Northcote, 1974), and the slime contains fibrils of short β1→4-glucan chains (Wright & Northcote, 1976). The cellulosic central polymer surrounded by pectic-like material is therefore relatively stiff and fibre-like, but since it is surrounded by hydrophilic molecules, it is soluble. This structure has some resemblance to the plant cell wall (Northcote, 1972). The slime is related in structure to cell-wall components and is an extremely useful experimental material, since it can be collected free of contamination without any chemical extraction.

Radioautography and subcellular fractionation of maize root tips, after labelling with L-[1-^{3}H]fucose and D-[U-^{14}C]glucose, showed that the membrane system within the cell, the endoplasmic reticulum, Golgi apparatus and associated vesicles, are involved in the synthesis, transport and secretion of the slime polymers (Northcote & Pickett-Heaps, 1966; Bowles & Northcote, 1972; Paull & Jones, 1975*a*,*b*, 1976). The slime within the dictyosome stacks and vesicles is fibrillar and similar in appearance to the packets of material that can be seen outside the plasmalemma after export (Rougier, 1971).

The present investigation is concerned with the mechanism of synthesis of the slime polysaccharides. Paull & Jones (1976) incubated maize root tips with L-[1-^{3}H]fucose and centrifuged the secreted slime and water-soluble material extracted from a homogenate of the root tip in a density gradient of CsCl. The radioactive material from the homogenate gave three distinct fractions with densities of 1.63, 1.55 and less than 1.4 g/cm^3. The secreted slime ran as a single fraction with a buoyant density of 1.63 g/cm^3. The nature of these fractions was not investigated. The technique of centrifugation in CsCl density

gradients and the use of radioactive fucose were exploited in the work described here. The evidence suggests that the slime polysaccharides are synthesized and transported on protein carriers within the membrane system of the root-cap cells and released from the proteins before final secretion to the outside of the root.

Materials and Methods

Growth of tissue and sterile techniques

Seedlings of *Zea mays* var. Caldera were germinated under sterile conditions. The seeds were soaked in chloramphenicol solution (10mg/litre) for 16h, washed four times with this solution and germinated bathed in the solution in a sterile crystallizing dish covered with aluminium foil and lined with filter paper. The seedlings were used after 3–4 days, when the primary root was 30–40mm long (Harris & Northcote, 1970).

Apparatus and all solutions were autoclaved at 120°C and 103kPa for 30min. All manipulations were carried out in a sterile laminar-flow air cabinet.

Radioactive chemicals

D-[U-^{14}C]Glucose (sp. radioactivity 230mCi/mmol), L-[1-^{3}H]fucose (sp. radioactivity 4.2Ci/mmol), L-[4,5-^{3}H]leucine (sp. radioactivity 58Ci/mmol), NaB^3H_4 (sp. radioactivity 661mCi/mmol) and L-[U-^{14}C]threonine (sp. radioactivity 180mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Production and isolation of radioactive slime, supernatant and membranes from maize roots

Roots (20–30mm long) were excised from the seedlings with a razor blade and placed radially in circular groups, on Parafilm (Gallenkamp, London E.C.2, U.K.), in a sterile Petri dish, so that the tips of the roots were in contact. Up to 40 roots were used per incubation. A solution (100–200μl) of the radioactive precursor in a simple salt solution medium with vitamins (B5 medium without 2,4-dichlorophenoxyacetic acid and sucrose; Gamborg *et al.*, 1968) was placed at the point of contact. The roots were covered with the top of the Petri dish, and a wad of wet paper was included to preserve a humid atmosphere. They were incubated in the dark at 25°C. At the end of the incubation the slime and incubation medium were removed with a syringe and the roots were washed several times with sterile water and then dried. The washings and slime were combined. The radioactive root tips (1mm) were excised, mixed with non-radioactive roots and homogenized at 4°C in a pestle and mortar, in medium containing 50mM-Tris/HCl, 1mM-EDTA, 0.1mM-$MgCl_2$ and 8% (w/v) sucrose at pH7.4. The homogenate was squeezed through muslin and centrifuged at 2000*g* for 15min at 4°C in a Sorvall RC-2B centrifuge, to remove cell walls and starch. The supernatant solution was then centrifuged at 100000*g* for 60min in an SW50.1 rotor in a Beckman ultracentrifuge. This produced a supernatant [A] and a pellet. The pellet was resuspended in homogenization medium and re-centrifuged twice at 100000*g* (15min each), resuspended in water and sonicated at 4°C for three periods of 100000*g* for 20min to separate the residue from the water-soluble membrane extract. The slime, the supernatant [A] and the water-soluble membrane extract were dialysed against at least five changes (4 litres each) of water for 3 days at 4°C in the presence of a drop of toluene and chloramphenicol (10mg/litre). The solutions remaining in the dialysis sacs were rotary-evaporated to dryness.

CsCl density gradients

The dried samples were dissolved in phosphate buffer (0.2M-NaH_2PO_4/Na_2HPO_4), pH7.4, and CsCl was added to a final density of 1.4 or 1.48g/cm^3. Centrifugation was carried out at 4°C in a Beckman ultracentrifuge with an SW50.1 rotor. The gradient formed over 48h at 40000rev./min and samples were collected by removing solution from the bottom of the tube with a peristaltic pump. The densities were calculated from the refractive index of the samples and the radioactivity was measured. The samples were combined for the preparation of radioactive fractions and the CsCl was removed by dialysis over 2 days with at least three changes of water at 4°C.

Hydrolysis of polysaccharides and analysis of sugars

The polysaccharides were hydrolysed in an autoclave at 120°C and 103kPa for 1h with 3% (w/v) H_2SO_4. The hydrolysate was neutralized with Amberlite IR-4B resin (CO_3^{2-} form). This was then rotary evaporated to dryness and dissolved in water (approx. 100μl). Paper electrophoresis was used to separate peptides and amino acids from neutral sugars and uronic acids (Harris & Northcote, 1970). The sample was applied to Whatman no. 1 paper and run at 5kV for 25min in acetic acid/formic acid/water (pH2, 4:1:45, by vol.). The positions of the uronic acids and neutral sugars were determined by running known markers in parallel. The sugars were eluted from the paper with water, rotary-evaporated to a small volume and applied to a paper chromatogram (Whatman no. 1 paper, in ethyl acetate/pyridine/water, 8:2:1, by vol., for 20h). The galactose/glucose spot was eluted and separated by using the same solvent for 48h. The xylose/fucose spot was eluted and separated by using butan-1-ol/ethanol/water (5:1:4, by vol.). Neutral sugar markers were run in parallel and were detected by aniline hydrogen phthalate (Wilson, 1959). Uronic acids were separated from neutral sugars by electrophoresis (4kV for 45min; at pH3.5, in pyridine/

acetic acid/water, 1:10:89, by vol.), on Whatman no. 1 paper.

Protein hydrolysis and amino acid analysis

Samples were hydrolysed in sealed tubes with 6M-HCl at 110°C for 18h. The solutions were dried by rotary evaporation and washed several times with water. Electrophoresis (at pH2, in acetic acid/formic acid/water, 4:1:45, by vol.) was used to separate the amino acids. Amino acid markers were run in parallel and were stained with a 2% solution of ninhydrin in acetone.

Measurement of radioactivity

The paper electrophoretograms and chromatograms were dried and dissected into 4cm × 1cm strips and placed in counting vials. Scintillant (0.5ml) [2,5-diphenyloxazole (PPO), 8.75g, and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), 0.125g, in 2.5 litres of toluene] was added and they were counted for radioactivity in 20ml Packard bottles in a Searle mark III liquid-scintillation system, model 6880. The strips were washed three times with toluene, rinsed three times in benzene, dried and eluted with water. The radioactivity in the fractions from the CsCl density gradient was measured by adding a 100μl sample to 300μl of water and 2ml of scintillant (6g of PPO, 75mg of POPOP, 750ml of Triton X-100, 1500ml of toluene). Liquid samples containing no CsCl were counted for radioactivity by adding 1 vol. of sample to 10vol. of this latter scintillant.

β-Elimination and reduction with borohydride

Samples were dissolved in NaOH (0.5ml, 0.5M) or water (control experiments) and left for 24h at room temperature (20°C).

Material was also treated with alkali and NaB^3H_4. It was dissolved in buffer (1ml) (pH7.2, 0.2M-Na_2HPO_4/NaH_2PO_4) containing 5mM-$PdCl_2$ catalyst (Tanaka & Pigman, 1965) and 0.6M-$NaBH_4$ and left at room temperature for 2h. Then 1ml of 1M-NaOH containing 1mCi (2mM) of NaB^3H_4 was added and the reaction mixture was left for 24h at room temperature. The product was acidified to pH5 with 4M-acetic acid, adjusted to pH7 with 0.1M-NaOH and passed through a column (1cm × 8cm) containing Zeolit 225 (SO_3H^- form), to remove Na^+, rotary-evaporated to dryness and the borate removed by repeated evaporation with methanol. The protein was hydrolysed and the radioactive amino acids were analysed.

Sugar alcohols were separated by chromatography (Whatman no. 1 paper, in butan-1-ol/ethanol/water, 10:1:2, by vol., for 36h). They were detected with a spray of vanillin (10mg/ml in ethanol) and aq. 3% (w/v) $HClO_4$, mixed 1:1 (v/v) just before use. The chromatogram was heated to 80°C for 3–4min and the sugar alcohols produced pale-blue spots.

Results

Identification of glycoprotein

Roots (40) were incubated with: (1) 50μCi of [1-^{3}H]fucose in 200μl of medium for 1h; or (2) 20μCi of [U-^{14}C]glucose in 200μl of medium for 30min; or (3) 20μCi of [4,5-^{3}H]leucine in 200μl of medium for 1h. Slime and the supernatant [A], water-soluble membrane extract and residue were prepared from the root tips. In addition, with those roots that had been incubated with [U-^{14}C]glucose, the other portions of the roots remaining after removal of the tip (de-capped roots) were also used to prepare supernatant [A], water-soluble extract and residue fractions. Samples of the aqueous fractions were counted to determine the distribution of radioactivity. The residue (insoluble membrane material) was dissolved in Triton/toluene scintillant and counted for radioactivity. The results are shown in Table 1. They indicated that a large amount of radioactivity appeared in the supernatant [A], and this probably represented material that had leaked out of membranes during the homogenization procedure and also material trapped between the plasmalemma and cell wall of the root-cap cells. There was a high proportion of the radioactivity in water-soluble membrane extract compared with that found in insoluble material of the membrane preparations of the root tip when these were incubated with [1-^{3}H]-fucose or [U-^{14}C]glucose. However, there was more radioactivity in insoluble material than in the water-soluble membrane extract prepared from de-capped roots derived from incubation of intact roots in [U-^{14}C]glucose.

The radioactive polymeric material from each fraction was centrifuged in the CsCl density gradient (initial density 1.48 or 1.4g/cm^3). The distribution of radioactivity down the gradients is shown in Figs. 1(*a*)–1(*i*). After labelling with [1-^{3}H]fucose, slime polymers were found as one peak at a density of 1.6–1.63g/cm^3, whereas the supernatant and water-soluble membrane extract were resolved into three peaks at densities of 1.63, 1.55 and 1.37g/cm^3 (Figs. 1*a*–1*c*). After labelling with [4,5-^{3}H]leucine, slime polymers were present as one peak at a density of 1.3g/cm^3, whereas the water-soluble membrane extract was resolved into three peaks at 1.3, 1.37 and 1.55g/cm^3 (Figs. 1*g*–1*i*). The material from each peak was analysed and it was shown that all the radioactivity in polymers after labelling with either [1-^{3}H]fucose or [4,5-^{3}H]leucine was present either as fucose or leucine; thus neither precursor was metabolized to another sugar or amino acid. Polysaccharides have a density of 1.6–2.0g/cm^3 and proteins

Table 1. *Distribution of radioactivity incorporated from various precursors into different fractions of the maize root*
Roots were incubated with either radioactive fucose, leucine or glucose. Slime, incubation medium and washings were pooled and root tips (1 mm) were excised and homogenized. The homogenate was centrifuged at 2000*g* for 15 min at 4°C. The supernatant was then centrifuged at 100000*g* for 60 min at 4°C and resulted in the formation of a supernatant [A] and pellet. The pellet was washed, resuspended in water and sonicated at 4°C. The suspension was centrifuged at 100000*g* to give a water-soluble membrane extract and an insoluble residue. With those roots that had been incubated with radioactive glucose, the de-capped roots were also used to prepare a supernatant [A], water-soluble membrane extract and an insoluble residue. Slime, supernatant [A] and water-soluble membrane extract were dialysed against water. The non-diffusible material and the insoluble residue were counted for radioactivity in Triton/toluene scintillant.

		Radioactivity (c.p.m.)			
Precursor	Tissue	Slime	Supernatant [A]	Water-soluble membrane extract	Insoluble residue
L-[1-^{3}H]Fucose	Root tips	24700	36700	12700	7000
L-[4,5-^{3}H]Leucine	Root tips	323500	820400	101100	115200
D-[U-^{14}C]Glucose	Root tips	112400	82000	62400	45800
D-[U-^{14}C]Glucose	De-capped roots	—	80200	40500	80600

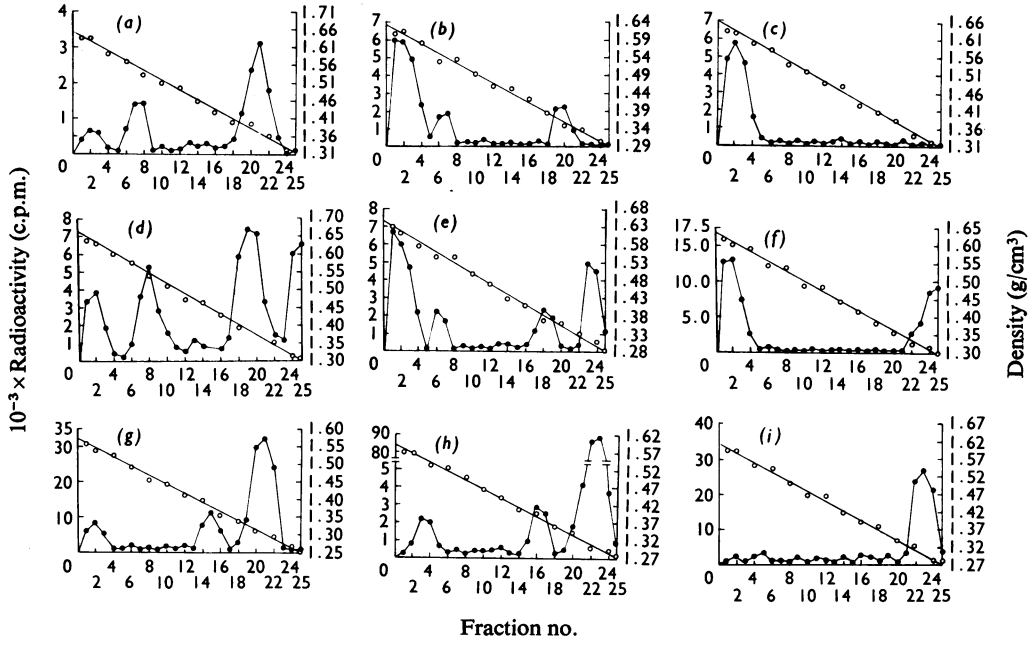

Fig. 1. *Distribution of radioactivity in CsCl density gradients, after centrifuging slime, supernatant [A] and a water-soluble extract isolated from root tips labelled with radioactive fucose, glucose or leucine*
Slime, supernatant [A] and water-soluble membrane extract were prepared from root tips after incubation with L-[1-^{3}H]fucose (*a–c*), D-[U-^{14}C]glucose (*d–f*), or L-[4,5-^{3}H]leucine (*g–i*), as described in the legend to Table 1. The radioactive polymeric material was centrifuged in a CsCl density gradient for 48 h at 40000 rev./min and samples were collected and their density (○) and radioactivity (●) determined. Material labelled with radioactive fucose and glucose was centrifuged at an initial density of 1.48 g/cm^3; material labelled with radioactive leucine was centrifuged at an initial density of 1.40 g/cm^3. (*a*), (*d*) and (*g*) show the distribution of radioactivity of the water-soluble membrane extract, (*b*), (*e*) and (*h*) that in the supernatant [A] and (*c*), (*f*) and (*i*) that in the slime.

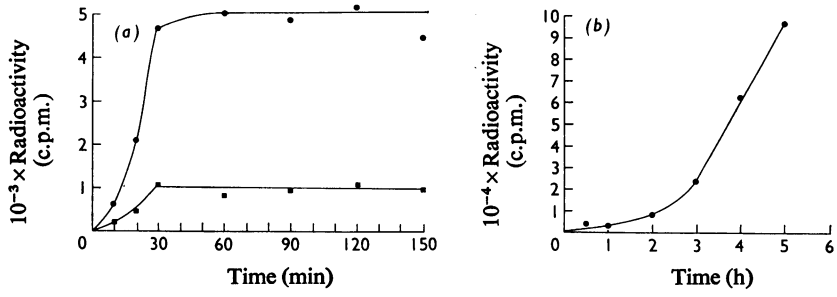

Fig. 2. *Time course of incorporation of* L-[1-^{3}H]*fucose into polysaccharide (density* 1.63 g/cm^3*) and glycoprotein fractions (densities* 1.37 *and* 1.55 g/cm^3*) of the maize root tip*

Roots were incubated with L-[1-^{3}H]fucose for various times. The slime, supernatant [A] and water-soluble membrane extract were combined and centrifuged in a CsCl density gradient as described in the legend to Fig. 1. The total amount of radioactivity in each peak was determined for the various times of incubation. (*a*) shows the incorporation of radioactivity into the glycoprotein fractions, densities 1.37 (●) and 1.55 g/cm^3 (■). (*b*) shows the time course of incorporation into polysaccharide of density 1.63 g/cm^3.

1.3 g/cm^3 (Fincher & Stone, 1974). Thus materials at densities intermediate between these two values were glycoproteins and the two fractions at densities 1.55 and 1.37 g/cm^3 (Figs. 1*a*–1*i*) contained both a radioactive sugar (fucose) and an amino acid (leucine). The, slime appeared to be composed of separate polysaccharide and protein and none of the glycoproteins (densities 1.55, 1.37 g/cm^3) were present. [U-^{14}C]Glucose labelled polysaccharide, glycoprotein and protein, since it is metabolized into amino acids (Figs. 1*d*–1*f*).

Time course of glycoprotein and slime formation

Since fucose was not metabolized to other sugars, it could be used in time-course experiments to indicate the relative amounts of the various polymers containing it which were formed after a particular time. Roots (20) were incubated with 20 μCi of [1-^{3}H]fucose in 150 μl of solution for various times. The slime, supernatant [A] and membrane water-soluble material were combined and centrifuged in a CsCl density gradient. The total amount of radioactivity in each peak was determined at the various times of incubation (Figs. 2*a* and 2*b*). In a very short time the material at 1.37 and 1.55 g/cm^3 became saturated with the radioactivity, whereas the radioactivity of slime polysaccharide increased with time in a linear fashion, after a short lag period. It was possible therefore that the glycoproteins were synthesized and then converted into the slime which was continually secreted from the cells.

Groups of roots (40) were incubated with 25 μCi of [1-^{3}H]fucose in 150 μl of medium for 10 min. The radioactive solution was removed with a syringe and the tips were washed several times with a non-radioactive fucose solution (1%) for 3 min. The roots were left in non-radioactive fucose solution for periods

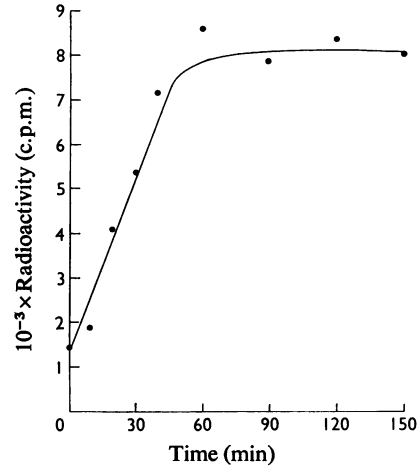

Fig. 3. *Total radioactivity incorporated into polymers during a pulse–chase experiment, after labelling root tips with* L-[1-^{3}H]*fucose*

Roots were incubated in L-[1-^{3}H]fucose for 10 min, washed and then left in a non-radioactive fucose solution for up to 150 min. The slime, supernatant [A] and water-soluble membrane extract were prepared as described in the legend to Table 1; they were pooled and the total radioactivity was determined.

up to 150 min. The slime, supernatant and water-soluble membrane extracts were combined, the fractions separated on a CsCl density gradient and the radioactivity in the fractions was measured over the period of the chase (Figs. 3 and 4). The total radioactivity incorporated reached a maximum after about

50min. The amount of label in the slime polysaccharides (density 1.63g/cm³) increased almost linearly with time, whereas that in the glycoprotein (density 1.37g/cm³) rose sharply and then fell to zero. The radioactivity incorporated into the other glycoprotein (density 1.55g/cm³) also increased and then decreased in quantity during the pulse–chase experiment. Thus the two glycoproteins which occurred within the root are possible precursors of the slime polysaccharides.

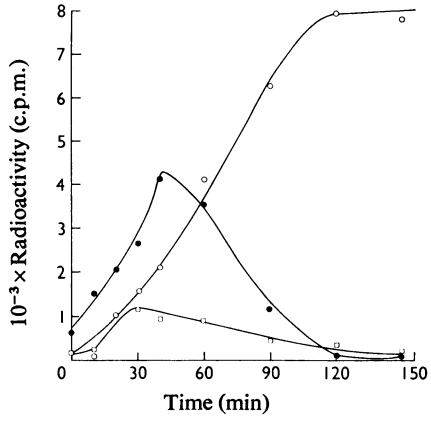

Fig. 4. *Incorporation of* L-[1-^{3}H]*fucose into polysaccharide (density* 1.63 *g/cm³) and glycoprotein fractions (densities* 1.37 *and* 1.55*g/cm³) of the maize root tip during a pulse–chase experiment*

The pooled material prepared during a pulse–chase with L-[1-^{3}H]fucose, as described in the legend to Fig. 3, was centrifuged in a CsCl density gradient. The total amount of radioactivity in each peak was determined for the various times of incubation. Polysaccharide (density 1.63g/cm³; ○) and glycoprotein fractions [densities 1.37 (●) and 1.55g/cm³ (□)] were separated and counted for radioactivity.

Analysis of glycoprotein fractions and polysaccharide

The polymers from the water-soluble membrane extract, labelled with [U-^{14}C]glucose were separated on the density gradient (Fig. 1*d*) and the four fractions (densities 1.6, 1.55, 1.37 and 1.3g/cm³) were each

Table 2. *Relative amounts of radioactivity incorporated from* D-[*U-*^{14}*C]glucose into the polysaccharide components of different fractions obtained from a CsCl density gradient*

Roots were incubated with D-[U-^{14}C]glucose for 30min and the water-soluble membrane fraction was prepared from the tips as described in Table 1. This material was centrifuged in CsCl (density of 1.48g/cm³) for 48h at 40000rev./min. Fractions of density 1.63, 1.55, 1.37 and 1.3g/cm³ were dialysed against water to remove CsCl, and the sugars were then analysed after hydrolysis of the polymers. Fraction of density 1.3g/cm³ contained no radioactive sugars.

Sugar	Radioactivity of the isolated fractions (% of total) Density 1.63g/cm³	Density 1.55g/cm³	Density 1.37g/cm³
Uronic acid	15.6	15.0	12.5
Galactose	21.4	17.5	20.2
Glucose	23.2	27.5	22.8
Mannose	1.0	0.8	0.7
Arabinose	10.8	10.4	11.5
Xylose	8.3	13.6	7.3
Fucose	19.7	15.2	25.0
Total radioactivity (c.p.m.)	15300	8500	12200

Table 3. *β-Elimination of glycoproteins (density* 1.37 *and* 1.55*g/cm³), labelled with* L-[1-^{3}*H]fucose followed by separation of the products on a CsCl density gradient.*

Glycoproteins labelled with L-[1-^{3}H]fucose were prepared as described in the legend to Fig. 1. CsCl was removed from the fractions by dialysis. Samples were dissolved in NaOH (0.5ml, 0.5M) or sodium phosphate buffer, pH7.2 (control) and left for 24h at 21°C. The products were then separated in a CsCl density gradient and the amount of radioactivity in each fraction was determined.

	Radioactivity (c.p.m.) Glycoprotein fraction (density 1.37g/cm³) Density 1.60g/cm³	Density 1.37g/cm³	Glycoprotein fraction (density 1.55g/cm³) Density 1.60g/cm³	Density 1.55g/cm³
Before incubation with NaOH for 24h	0	2840	0	2470
After incubation with NaOH for 24h	2580	190	120	2100
Before incubation with buffer (pH7.2) for 24h	0	2620	0	2060
After incubation with buffer (pH7.2) for 24h	180	2210	210	1920

separately re-run to free them from contaminants. They were analysed for their component sugars. The composition of the glycoproteins (densities 1.37, 1.55 g/cm^3) and the completed slime polysaccharides (density 1.63 g/cm^3) was very similar (Table 2). The fraction (density 1.3 g/cm^3) contained no radioactive sugars. Amino acids were also labelled by the radioactive glucose, but a detailed analysis was not possible owing to the small amount of incorporation into the individual monomers.

β-Elimination of the glycoproteins prepared from the water-soluble membrane extract

Glycoprotein (density 1.37 g/cm^3) labelled with [1-^{3}H]fucose, was β-eliminated and the product was re-run on CsCl density gradients; all the radioactive material was converted into material of density 1.6 g/cm^3. However, when the glycoprotein (density 1.55 g/cm^3) was used all the radioactivity remained at this density (Table 3). Thus the glycoprotein (density 1.37 g/cm^3) probably contained *O*-glycosidic bonds with serine and threonine, whereas the glycoprotein (density 1.55 g/cm^3) had a different linkage.

To investigate further the linkage in the glycoprotein (density 1.37 g/cm^3), a sample labelled with [1-^{3}H]fucose (8000 c.p.m.) was β-eliminated in the presence of NaB^3H_4 and a control experiment with water instead of NaOH was carried out. After 24 h incubation the $NaBH_4$ was removed and the amino acids were analysed by electrophoresis (pH 2) after hydrolysis in 6M-HCl. The labelled fucose was detected near the origin of the electrophoretogram, one peak of radioactivity coinciding with the marker for α-aminobutyric acid (total of 770 c.p.m.). No radioactivity was detected anywhere else on the electrophoretogram. There was none at the position corresponding to alanine. No radioactivity appeared in amino acids in the control experiments. These results indicated that threonine was the amino acid involved in the glycopeptide linkage. As further evidence, roots (40) were incubated with 20 μCi of [U-^{14}C]threonine and the water-soluble membrane extract was fractionated on a CsCl density gradient. The glycoprotein (density 1.37 g/cm^3) contained approx. 12600 c.p.m. A sample was analysed and 86% of the radioactivity was found in threonine, most of the rest being in neutral sugars. The glycoprotein was β-eliminated in the presence of $NaBH_4$ (non-radioactive), and a control experiment in which the NaOH was omitted was also performed. The products at the end of the incubation were hydrolysed in 6M-HCl and analysed by paper electrophoresis at pH 2. The results (Table 4) confirmed that threonine was the amino acid involved in the linkage between the protein and the polysaccharide of this glycoprotein (density 1.37 g/cm^3).

To find the sugar at the attachment point between protein and polysaccharide, [U-^{14}C]threonine-labelled material (density 1.37 g/cm^3) was β-eliminated in the presence of NaB^3H_4. Control experiments involving either no β-elimination (no NaOH) or no $NaBH_4$ were also performed. The oligosaccharides or polysaccharides released were hydrolysed and analysed chromatographically. Before chromatography, any amino acids present were removed by electrophoresis at pH 2. A radioactive peak coinciding with a xylitol marker was detected (325 c.p.m.) after β-elimination and reduction with NaB^3H_4. No radioactivity was detected in sugar alcohols from either of the two controls. Therefore a xylose–threonine linkage is present as the link between protein and polysaccharide of the glycoprotein of density 1.37 g/cm^3.

Table 4. *β-Elimination of a glycoprotein fraction (density 1.37 g/cm^3) labelled with* L-[*U*-^{14}C]*threonine, in the presence of* $NaBH_4$

Roots were incubated with L-[U-^{14}C]threonine and the radioactive polymers in the tip were separated in a CsCl density gradient, as described in the legend to Fig. 1. The glycoprotein fraction was β-eliminated in the presence of $NaBH_4$ and $PdCl_2$ catalyst. A control, with buffer instead of NaOH, was performed. The products were hydrolysed in 6M-HCl and the amino acids analysed by electrophoresis at pH 2.

	Radioactivity (c.p.m.) of glycoprotein fraction (density 1.37 g/cm^3)	
	Threonine	α-Aminobutyric acid
Before incubation with NaOH	3260	—
After incubation with NaOH	2610	430
Before incubation with buffer (pH 7.2)	2780	—
After incubation with buffer (pH 7.2)	2640	—

Re-application of the glycoproteins to a crude homogenate prepared from root tips and release of polysaccharide

Root tips (100, 1 mm long) were excised and homogenized in 0.2M-sodium phosphate buffer, pH 7.4. The cell-wall fragments were removed by centrifugation at 2000*g* for 15 min at 4°C in a Sorvall RC-2B centrifuge. Homogenate (1 ml) was divided into two equal portions. The glycoproteins (densities 1.55 and 1.37 g/cm^3) labelled with [1-^{3}H]-fucose were dissolved in the homogenate and left for 1 h at 25°C, under a drop of toluene. The suspensions were then centrifuged at 100000*g* for 30 min and the supernatants dialysed for 1 day against glass-distilled water (five changes), after which they were run on CsCl density gradients.

Table 5. *Re-application of glycoproteins (densities 1.37 and 1.55g/cm³) labelled with [1-³H]fucose to a crude homogenate preparation, followed by fractionation of the products on CsCl density gradients*

Root tips were homogenized and cell-wall fragments were removed by centrifugation at 2000*g* for 15min at 4°C. The glycoprotein fractions (densities 1.37 and 1.55g/cm³) labelled with [1-³H]fucose were prepared as described in the legend to Fig. 1. They were added to the homogenate, which was then left for 1h at 25°C. The suspensions were centrifuged at 100000*g* for 30min and the supernatants dialysed, after which they were analysed on CsCl density gradients.

	Radioactivity (c.p.m.)			
	Glycoprotein fraction (density 1.55g/cm³)		Glycoprotein fraction (density 1.37g/cm³)	
	Density 1.63g/cm³	Density 1.55g/cm³	Density 1.63g/cm³	Density 1.37–1.44g/cm³
Before incubation with homogenate	0	2920	0	2890
After incubation with homogenate	2310	240	1850	570
Before incubation in buffer (pH7.2)	0	2370	0	2520
After incubation in buffer (pH7.2)	130	2090	170	2240

Control experiments in which samples of the glycoproteins were dissolved in sodium phosphate buffer only, incubated for 1h at 25°C, dialysed and centrifuged in CsCl were also carried out. After incubation with the homogenate both the glycoproteins (densities 1.55 and 1.37g/cm³) produced a band of radioactivity at a density of 1.63g/cm³ (Table 5). In both cases there was some loss of radioactivity after dialysis, indicating that either free fucose was produced during the incubation or that low-molecular-weight oligo- or poly-saccharides were formed which were removed by dialysis. Since radioactivity was retained in the dialysis tubing, the sugar polymers attached to the proteins must be quite large (mol.wt. >10000). After the incubation the glycoprotein (density 1.37g/cm³) also produced a small broad peak of radioactivity over the density range 1.37–1.43g/cm³. This may have been formed by proteolysis of the material, which increased its density.

Discussion

Two fractions have been isolated from membrane preparations of the root-cap cells of maize. These fractions were resolved on centrifugation at densities of 1.55 and 1.37g/cm³. They therefore had densities intermediate between polysaccharide (density 1.6–2.0g/cm³) and protein (1.3g/cm³) (Fincher & Stone, 1974). When the roots were incubated with radioactive fucose and leucine both fractions became labelled. The fraction of density 1.37g/cm³ could be β-eliminated with dilute alkali to give polysaccharide (density 1.6g/cm³) free of protein, whereas the other fraction could not be split in this way. There are thus at least two glycoproteins carried by the membranes within the root-cap cells. The sugar composition of both these glycoprotein fractions, analysed after they had become saturated with radioactivity during incubation of the roots with [U-¹⁴C]glucose, was very similar to that of slime polysaccharides.

When the glycoproteins were labelled by incubation of the roots with [1-³H]fucose, they became saturated with radioactivity after 15–30min, although the radioactivity in the slime polysaccharides steadily increased over the 4h period of the incubation. During a chase experiment, after incubation of the roots for 10min in radioactive fucose, the amount of radioactivity in the slime polysaccharides steadily increased over 150min further incubation in non-radioactive fucose, whereas the radioactivity in the glycoproteins reached a maximum and then decreased to zero during the chase. The glycoproteins could therefore be intermediates during slime-polysaccharide synthesis.

Protein intermediates have been found during the synthesis of starch in potato tissue (Tandecarz *et al.*, 1975) and glycogen formation in *Escherichia coli* (Barengo *et al.*, 1975) and liver (Krisman & Barengo, 1975) when glucose is transferred from UDP-glucose to the protein acceptors. The glucoprotein formed has unbranched α-1→4-glucosidic chains, which accept more glucosyl residues by transglycosylation from ADP- or UDP-glucose (Tandecarz *et al.*, 1975).

The synthesis of the slime polysaccharides is more complex than that of a homopolysaccharide such as starch, since several different sugars are present in the polymers. Bowles & Northcote (1976) showed

that synthesis of low-molecular-weight polysaccharides occurred in a membrane fraction rich in endoplasmic reticulum prepared from maize roots. These polysaccharides (mol.wt. < 4000) were probably attached to protein and could be intermediates of biosynthesis of high-molecular-weight slime and wall polysaccharides. In the membrane fraction rich in dictyosomes there was a greater amount of free polysaccharide, and the molecular size of the polymers had increased. In the present work we have shown that most of the polysaccharide that was attached to protein had mol.wt. >10000, and this water-soluble material probably represents polymers in the final stages of synthesis. Insoluble material left after extraction of the membrane with buffer was labelled with all the radioactive precursors that we used, but it was not investigated further. It probably contained the initial acceptor proteins and proteins attached to polysaccharide with low molecular weights.

The two glycoprotein fractions present with the membranes could be structural polymers of the membrane, rather than intermediates involved in synthesis and transport of polysaccharides. However, the great size of the polysaccharide side chains (mol.wt. >10000; degree of polymerization at least 60 sugar residues), the very low content of mannose, and the similarity of the polysaccharide portion to the sugar composition of slime polysaccharides make this unlikely.

Analysis of slime polysaccharides by electrophoresis and hydrolysis has shown that three components are present: a neutral glucan, a weakly acidic polymer containing about 18–20% fucose and an acidic component with about 25–30% fucose (Wright, 1975). Thus there are two fucose-containing polysaccharides and each would presumably require a different primer. The sugar composition of the glycoprotein (density 1.55 g/cm^3) resembles that of the weakly acidic polysaccharide, and that of the glycoprotein (density 1.37 g/cm^3), the acidic polysaccharide.

The pool size for each glycoprotein can be calculated if we assume that at saturation, their specific radioactivity is equal to that of the initial radioactive precursor. The polysaccharide pool of the glycoprotein (density 1.55 g/cm^3) was approx. 6.3 pmol and that for the glycoprotein (density 1.37 g/cm^3) approx. 22 pmol; the rate of secretion of the slime polysaccharide was 2.9 pmol/min. These results compare very well with those calculated previously (Bowles & Northcote, 1974). The detection of these very small amounts of intermediates which are turning over very rapidly was made possible, in the present work, either by the use of fucose, which specifically labels the intermediates and the final polysaccharides, or by the isolation of the small root-cap portion of the root, where the synthesis of the slime occurs exclusively.

The turnover of the membranes during the synthesis of slime is fast, having a displacement time of approx. 20 s (Bowles & Northcote, 1974). Therefore it was surprising that it took 90 min to effect a complete chase of radioactive material from the glycoprotein carriers to the slime polysaccharides. There may be a delay in the transfer of labelled material from the space between the plasmalemma and the cell wall, to the exterior of the tissue. Paull & Jones (1975*b*), in a radioautographic experiment with [1-^{3}H]fucose as precursor, found that after a 20 min chase 31% of the label was between the plasmalemma and the wall and only 12% had been released to the outside of the tissue. After a 2 h chase, 75% of the label was released from the root-cap tissue.

It is possible that either the slime polysaccharides are released from the Golgi vesicles still attached to protein, and that the glycoprotein is then attacked by transglycosidases or proteinases as it lies between the plasmalemma and the cell wall, or that the release of polysaccharide occurs within the Golgi apparatus and its vesicles. When the two glycoprotein fractions are incubated with a root-tip homogenate, high-molecular-weight polysaccharide material was actively removed from them, without breaking down the polysaccharide into small units. There may be specific enzymes present which break the particular linkages between the polysaccharide and protein units of the glycoproteins. However, both glycosidases and proteinases have been found in the root tip (Thomas *et al.*, 1977).

A xylose–threonine bond is the linkage between the protein and polysaccharide of one of the glycoproteins (density 1.37 g/cm^3); this is the first time this has been found in higher plants, although it has been reported to be present in a red alga (Heaney-Kieras *et al.*, 1977). In the glycoprotein present in the maize cells there was no corresponding xylose–serine bond, and this is unusual for this type of glycoprotein (Montgomery, 1974).

It seems possible therefore that the slime polysaccharides are synthesized attached to proteins. The protein may be membrane bound and act as an acceptor during the transfer of sugars from nucleoside diphosphate sugar donors. The product would be insoluble while attached to the membrane. Glycosylation at this early stage probably takes place in the endoplasmic reticulum, as has been demonstrated for some glycoprotein synthesis in animal tissue, followed by further addition of sugars in the Golgi apparatus (Clauser *et al.*, 1972; Molnar, 1976; Jentoft *et al.*, 1976). Slime polymer synthesis might begin therefore in the endoplasmic reticulum, as suggested by the work of Bowles & Northcote (1972, 1974, 1976). At the later stages of synthesis the protein carrier may be detached from the membrane before final secretion.

It is important to distinguish the synthesis and secretion of slime polysaccharides from the formation of the polysaccharides deposited into the cell wall. The polysaccharides of the wall may not be carried by protein acceptors, and it has been indicated that the synthesis of the polysaccharides of the wall of pea cells does not take place within the endoplasmic reticulum but occurs only in the Golgi apparatus (Ray *et al.*, 1976; Robinson *et al.*, 1976).

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