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Changes in Enzymic Activities of Nucleoside Diphosphate Sugar Interconversions during Differentiation of Cambium to Xylem in Sycamore and Poplar

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During the transition from primary wall formation to secondary thickening there is a marked shift in the synthesis of pectin, hemicellulose and cellulose. The activities of the enzymes [UDP-D-galactose 4-epimerase (EC 5.1.3.2), UDP-L-arabinose 4-epimerase (EC 5.1.3.5), UDP-D-glucose dehydrogenase (EC 1.1.1.22) and UDP-D-glucuronate decarboxylase (EC 4.1.1.35)] were measured in cambial cells, differentiating xylem cells and differentiated xylem cells isolated from sycamore and poplar trees, and phloem cells from poplar. At the final stage of the differentiation of cambium to xylem there was a decrease in activity of the enzymes directly involved in producing the soluble precursors of pectin (UDP-D-galactose 4-epimerase and UDP-L-arabinose 4-epimerase) and an increase in those producing the precursors of hemicellulose (UDP-D-glucose dehydrogenase and UDP-D-glucuronate decarboxylase). These results strongly suggest that the changes were correlated with the differences observed in the chemical composition of the wall during development. The changes found in the catalytic activity of the enzymes of nucleoside diphosphate sugar interconversion exert a coarse control over the synthesis of pectin and hemicelluloses. The tissues at all stages of development contained the necessary enzyme activities to produce all the precursors of pectin and hemicellulose, even at the final stage of differentiation when no pectin was formed.

The nucleoside diphosphate sugars (UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, UDP-D-galacturonic acid, UDP-D-xylose and UDP-L-arabinose) are soluble precursors involved in cell-wall polysaccharide biosynthesis. There are three main ways in which these can be formed in plants. The first starts from D-glucose, which by hexokinase (EC 2.7.1.1) (Meunier *et al.*, 1971; Cox & Dickinson, 1973; Higgins & Easterby, 1974) is converted into D-glucose 6-phosphate, and this sugar can be used either for the biosynthesis of UDP-D-glucose by the action of the enzymes phosphoglucomutase (EC 2.7.5.1) (Najjar, 1962) and UDP-D-glucose pyrophosphorylase (EC 2.7.7.9) (Gustafson & Gander, 1972; Hopper & Dickinson, 1972), or for the biosynthesis of UDP-D-glucuronic acid through the *myo*-inositol-oxidation pathway (Loewus *et al.*, 1973). Secondly, sucrose can form UDP-D-glucose in the presence of the enzyme sucrose synthase (EC 2.4.1.13) (Cardini *et al.*, 1955). The third important way by which nucleoside diphosphate sugars can be formed is by interconversion from existing compounds. UDP-D-glucose can be oxidized to UDP-D-glucuronic acid by UDP-D-glucose dehydrogenase (EC 1.1.1.22) (Strominger & Mapson, 1957); the enzyme UDP-D-glucuronate decarboxylase (EC 4.1.1.35) (Ankel & Feingold, 1965) transforms UDP-D-glucuronic acid to UDP-D-xylose. These two reactions are not reversible, so that the carbon flow is unidirectional from UDP-D-glucose to UDP-D-xylose.

In addition, three distinct epimerases in higher plants, UDP-D-glucose 4-epimerase (EC 5.1.3.2) (Fan & Feingold, 1969), UDP-D-glucuronate 4-epimerase (EC 5.1.3.6) (Gaunt *et al.*, 1974) and UDP-L-arabinose 4-epimerase (EC 5.1.3.5) (Fan & Feingold, 1970) catalyse respectively the reversible interconversions of UDP-D-glucose into UDP-D-galactose, UDP-D-glucuronic acid into UDP-D-galacturonic acid, and UDP-D-xylose into UDP-L-arabinose. Two series of nucleoside diphosphate sugars are produced, one based on UDP-D-glucose (UDP-D-glucose, UDP-D-glucuronic acid and UDP-D-xylose), the other on UDP-D-galactose (UDP-D-galactose, UDP-D-galacturonic acid and UDP-L-arabinose). These two sets of UDP-sugars form the bulk of either the hemicelluloses (glucose series) or the pectic substances (galactose series) of the wall in angiosperms.

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The incorporation into the cell wall of pectin, hemicelluloses and cellulose varies during the formation of the cell plate and the subsequent development of the primary wall and secondary thickening (Northcote, 1963). The cell plate, which becomes the middle lamella, is composed partly of pectic substances, the primary wall is made up of pectin, hemicelluloses and microfibrils of cellulose, whereas the secondary wall is characterized by a massive synthesis of hemicellulose and cellulose, with only a very small amount of pectin. The bulk of hemicellulose in dicotyledons is formed from the sugars xylose and glucuronic acid, and smaller amounts of glucose and mannose. Thus there is a distinct shift in the biosynthesis of pectin, hemicellulose and cellulose during formation of cell plate, primary wall and secondary wall, which results in a change in the flux through the enzymic routes which interconvert the UDP-sugars, and these are therefore possible sites for the regulation of polysaccharide biosynthesis during differentiation.

In the present work we have measured the activities of the enzymes involved in nucleoside diphosphate sugar interconversions in cambial cells, differentiating xylem cells and differentiated xylem cells obtained from sycamore (*Acer pseudoplatanus*) and poplar (*Populus robusta*) trees, and phloem cells from poplar.

Materials and Methods

Chemicals and radiochemicals

UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, UDP-D-xylose, glucose 6-phosphate, NAD^+, NADase (NAD^+ nucleosidase, EC 3.2.2.5) from *Neurospora crassa* and NADH were products from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. UDP-D-[U-^{14}C]glucose (specific radioactivity 310mCi/mmol), UDP-D-[U-^{14}C]galactose (specific radioactivity 245mCi/mmol) and UDP-D-[U-^{14}C]glucuronic acid (specific radioactivity 290 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and UDP-D-[U-^{14}C]xylose (specific radioactivity 160mCi/mmol) was from New England Nuclear Corp., Langen, Germany. Radioactive substrates were diluted to the required specific radioactivity by adding unlabelled substrates.

Trees

Trees of sycamore (*Acer pseudoplatanus*) (14–16 years old) and poplar (*Populus robusta*) (14–16 years old) about 10–12m tall and 200–250mm in diameter were cut during June and July 1975 from Madingley Wood, Cambridge; the summer (1975) was very dry. Each tree was cut into three logs (1m) starting from the basal part of the trunk. The logs were transferred as quickly as possible to a cold-room, and all further manipulations were done at 1–3°C.

Fractionation of cambial cells, differentiating xylem cells, differentiated xylem cells and phloem cells

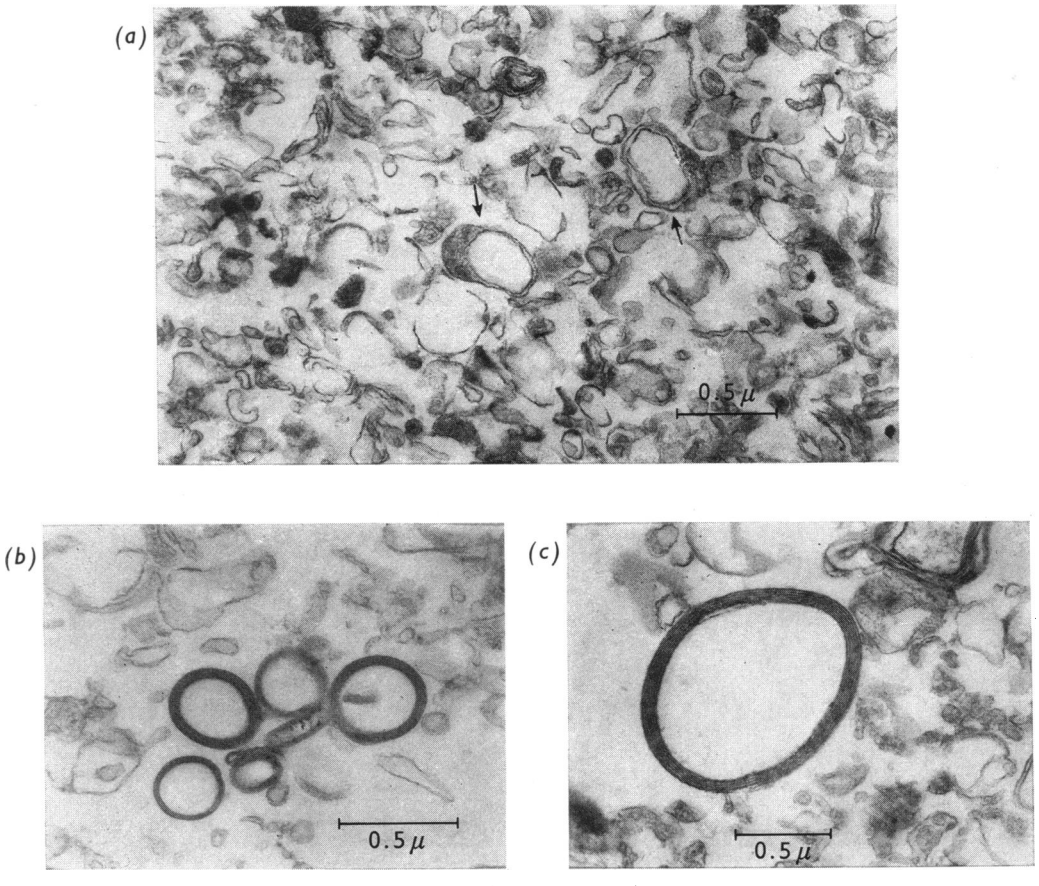
The bark of each log was gently removed in strips (100mm wide and 350–400mm long). The soft cambial cells were gently scraped off and placed in a pre-weighed ice-cold beaker containing 10ml of buffer (0.1M-sodium phosphate, pH7.0, containing 0.5g of EDTA and 0.5ml of 2-mercaptoethanol per litre). The differentiating xylem cells immediately below the cambium were then scraped away and differentiated xylem cells were obtained by scraping deeper into the wood. Phloem cells were isolated from the bark of poplar by using a similar technique.

Enzyme preparation

Cambial cells, differentiating and differentiated xylem cells, and phloem cells (6–7g fresh wt.) were transferred to 15ml of the 0.1M-sodium phosphate buffer, pH7.0, containing 0.5g of EDTA and 0.5ml of 2-mercaptoethanol per litre. The samples were homogenized with a Polytron PT 20 ST (Kinematic G.m.b.H., Lucerne, Switzerland) at different speeds (rheostat setting at positions 3, 4 and 5) for 20s at each speed and a total time of 60s. The homogenates were centrifuged at 3000*g* for 5min. The pellets, composed of broken and unbroken cells, were used to count the number of unbroken cells. The supernatants were centrifuged at 40000*g* for 20min at 2°C. To the crude extracts in the supernatants a solution of 0.5M-$MnCl_2$ was added, with vigorous stirring, to give a final concentration of 0.015M. After stirring for 5min the mixtures were centrifuged at 20000*g* for 20min. The residue was discarded and the supernatants were adjusted to 40% saturation with $(NH_4)_2SO_4$. The solutions were stirred for 20min and the precipitates removed by centrifugation at 20000*g* for 20min. The resulting supernatants were adjusted to 65% saturation with $(NH_4)_2SO_4$, and, after stirring for 20min, the precipitates were collected by centrifugation at 20000*g* for 20min and dissolved in 0.2M-sodium phosphate buffer, pH7.0. The protein that was precipitated between 40 and 65%-satd. $(NH_4)_2SO_4$ that was obtained from cambial cells, differentiating and differentiated xylem cells and phloem cells, was used for all experiments. These fractions were stored at −15°C.

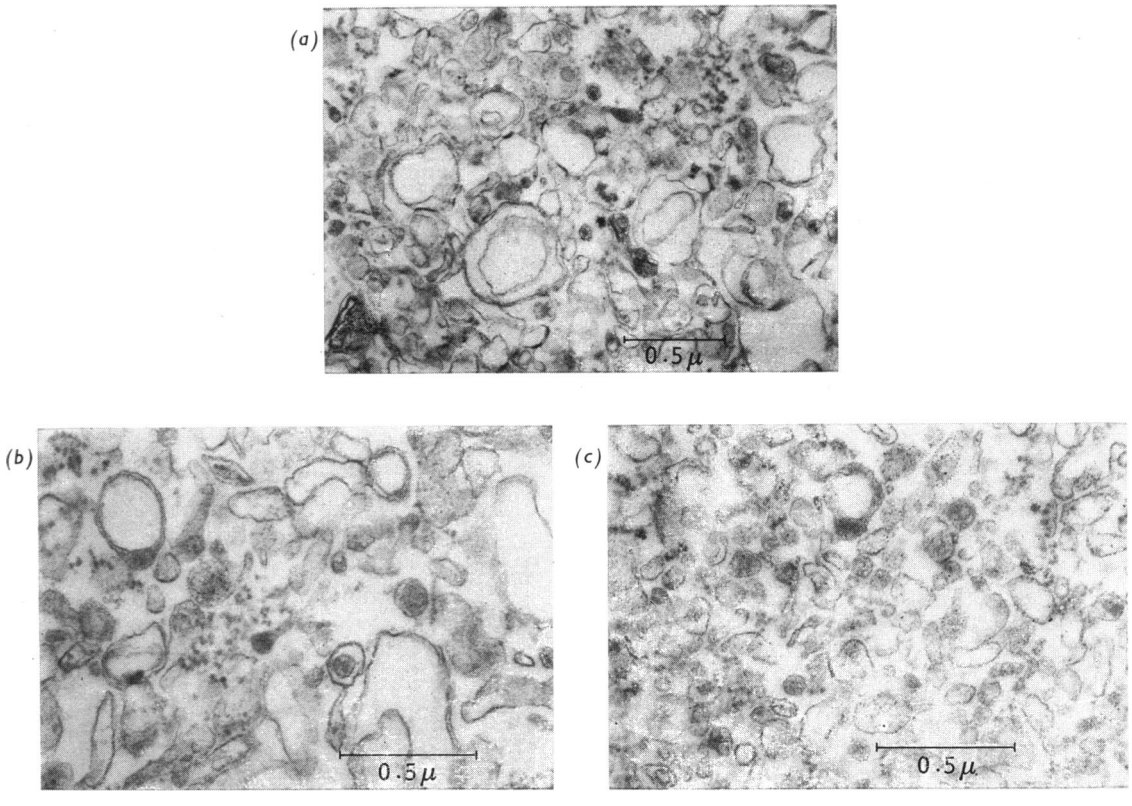
Paper chromatography and electrophoresis

Paper chromatograms on Whatman no. 1 paper were developed with ethyl acetate/pyridine/water (8:2:1, by vol.). Paper electrophoresis was carried out either in acetic acid (8%, v/v)/formic acid (2%, v/v) buffer, pH2.0, 4kV for 20min, to separate UDP-sugars from polysaccharides, or in pyridine/acetic acid/water buffer (1:10:200, by vol.), pH3.5,



EXPLANATION OF PLATE 1

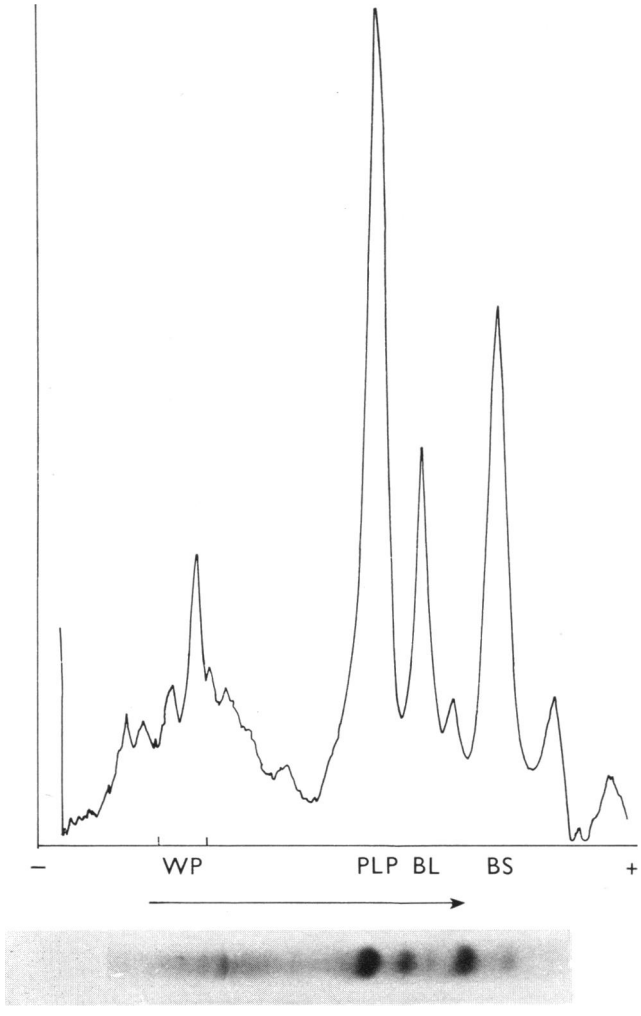
Electron micrographs of a subcellular fraction of the same density as myelin isolated from 5-day-old rat central nervous system All samples were fixed and processed as described by Sabri *et al.* (1974). (*a*) Cerebral-hemisphere myelin shows amorphous structures (arrow) which may represent an early stage of myelination. (*b*) Brain-stem myelin showing multilamellar structures. (*c*) Spinal-cord myelin showing formation of compact myelin.



EXPLANATION OF PLATE 2

Electron micrographs of microsomal fractions isolated from 5-day-old rat central nervous system

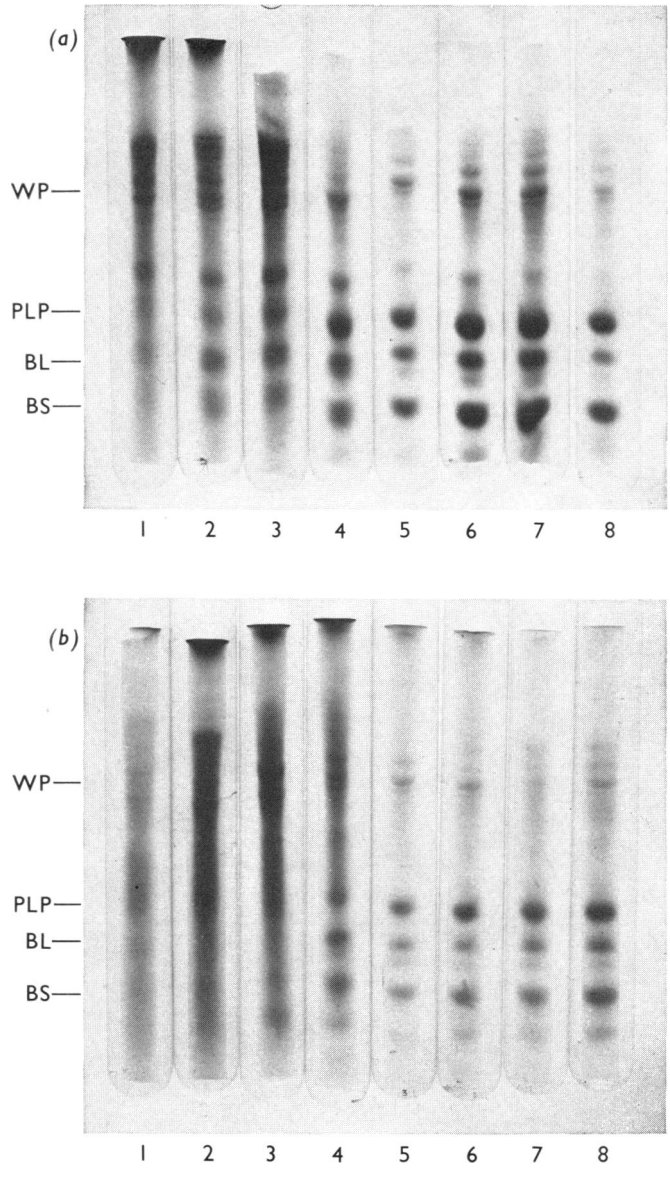
Fixation of samples as in Plate 1. (*a*) Brain-stem, (*b*) thalamus and (*c*) cerebral hemispheres showing membranous structures larger than microsomal particles.



EXPLANATION OF PLATE 3

Densitometric scan of polyacrylamide-gel-electrophoretic separation of protein of purified myelin

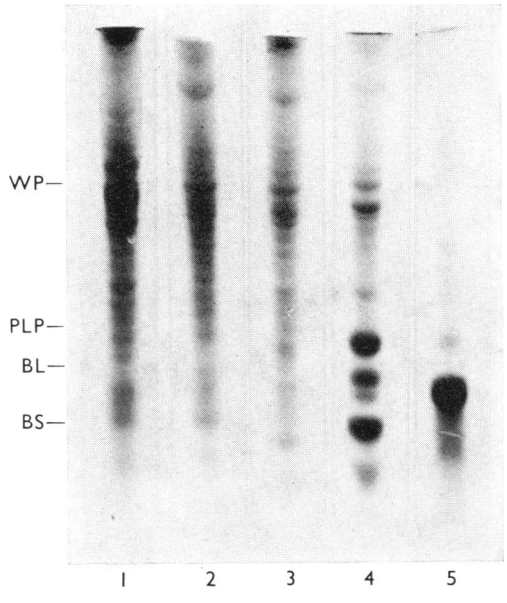
Method of gel electrophoresis, staining and destaining procedures of gels are described in the text; 30μg of rat myelin protein was applied. The densitometric scan was recorded at 560nm in a Gilford spectrophotometric scanner. Abbreviations: WP, Wolfgram protein; PLP, proteolipid protein; BL, high-molecular weight basic protein; BS, low-molecular-weight basic protein.



EXPLANATION OF PLATE 4

Protein pattern of myelin separated by polyacrylamide-gel electrophoresis

The method of electrophoresis is given in the text. Gels were stained with Coomassie Blue. Successive gels (1–8) represent rats of the following ages, with the amount of protein (μg) applied given in parentheses. (*a*) Myelin protein from spinal cord: 1, 3 days (100); 2, 5 days (100); 3, 10 days (75); 4, 15 days (50); 5, 25 days (25); 6, 40 (30); 7, 90 days (30); 8, 365 days (30). (*b*) Myelin protein from cerebral hemisphere: 1, 3 days (100); 2, 5 days (100); 3, 10 days (100); 4, 15 days (50); 5, 25 days (25); 6, 40 days (25); 7, 90 days (25); 8, 365 days (30). Abbreviations as in Plate 3.



EXPLANATION OF PLATE 5

Protein pattern of microsomal fraction separated by polyacrylamide-gel electrophoresis

Method of electrophoresis and staining of gels are as in Plate 4; successive gels represent rats of following ages: 1, 5 days, cerebral hemisphere (165); 2, 5 days, brain stem (105); 3, 15 days, cerebral hemisphere (80); 4, 15 days, cerebral-hemisphere myelin (50); 5, bovine spinal-cord myelin basic protein (20μg). Abbreviations are as in Plate 3.

at 4.0kV for 45–50min for separating D-galacturonic acid from D-glucuronic acid.

The sugars and uronic acid markers were detected with the aniline hydrogen phthalate reagent of Wilson (1959). UDP-sugar markers were detected by u.v.-light contact photography.

Radioactivity-counting procedure

Paper chromatograms and electrophoretograms were cut into strips (40mm × 10mm) and placed in counting vials (50mm×15mm) containing 0.6ml of toluene scintillant fluid [2,5-diphenyloxazole, 3.5g; 1,4 bis-(5-phenyloxazol-2-yl)benzene, 50mg; toluene, 1 litre] and their radioactivities were counted in 20ml Packard liquid-scintillation-spectrometer bottles by using a Nuclear–Chicago Unilux scintillation spectrophotometer (Harris & Northcote, 1970). The counting efficiency for each substrate was determined under the same experimental conditions as those used for the enzyme assays; it was approx. 50%.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Counting of cells

Samples of cambial cells, differentiating xylem cells, differentiated xylem cells and phloem cells (150–200mg fresh wt.) were placed in vials to which was added 2ml of a solution made up of 5% (w/v) chromic acid and 5% (v/v) HCl for 30h at 25°C. The cells were then gently separated with the aid of a hypodermic syringe with a 22-G needle. After appropriate dilution, the suspension (1ml) was transferred to a Sedgewick–Rafter plankton-counting chamber, consisting of a glass slide with a chamber volume of 1.0ml (Klein & Klein, 1970). Cells were counted in nine randomly chosen fields at 150× magnification. The total number of cells per ml of suspension and in the total fresh weight of the tissue was calculated. The number of unbroken cells obtained after homogenization of the cambial cells, differentiating and differentiated xylem cells and phloem cells was found, and the number of broken cells obtained by difference. The percentage of broken cells varied between 40 and 60%, according to the stage of differentiation of the tissue.

Enzyme assays

UDP-D-galactose 4-epimerase: Assay A. UDP-D-[U-^{14}C]galactose (0.408nmol) and UDP-D-galactose (50nmol) were incubated with enzyme in 0.2M-glycine/NaOH buffer (pH9.0) in a total volume of 20μl at 30°C for 5min. The reactions were stopped in boiling water for 2min and centrifuged for 1min. Each supernatant (5μl) was transferred to a Durham tube (height 25mm, diam. 6mm) containing 40μl of 0.1M-HCl, and the tubes were covered and placed in a boiling-water bath for 15min. The solutions containing free sugars, after the hydrolysis, were centrifuged at 2000*g* for 15s, dried in a vacuum desiccator for 2h, extracted with 20μl of pyridine and chromatographed for 60h. The paper strips corresponding to the markers (galactose and glucose) were cut out and the radioactivity was counted. A unit of enzyme activity was defined as the amount of enzyme that resulted in the formation of 1nmol of product in 1min.

UDP-D-galactose 4-epimerase: Assay B. UDP-D-[U-^{14}C]glucose (0.400nmol) and UDP-D-glucose (50nmol) were incubated with enzyme in 0.2M-glycine/NaOH buffer, pH9.0, in a total volume of 20μl at 30°C for 5min. Further analytical procedures were the same as described for Assay A.

UDP-D-xylose 4-epimerase. The reaction mixtures contained UDP-D-[U-^{14}C]xylose (0.312nmol), UDP-D-xylose (10nmol) and enzyme in 0.2M-sodium phosphate buffer (pH8.0) in a total volume of 20μl at 30°C for 5min. The reactions were stopped by placing them in boiling water for 2min, and they were then centrifuged at 2000*g* for 1min. Each supernatant (5μl) was hydrolysed with 40μl of 0.1M-HCl at 100°C for 15min. The hydrolysates were dried in a vacuum desiccator, extracted with 20μl of pyridine and chromatographed for 36–42h. The radioactivities of the separated D-[U-^{14}C]xylose and L-[U-^{14}C]arabinose were determined. A unit of enzyme was defined as the amount of enzyme that resulted in the formation of 1nmol of product in 1min.

UDP-D-glucose dehydrogenase. The reaction mixture contained UDP-D-[U-^{14}C]glucose (0.400nmol), UDP-D-glucose (50nmol), NAD^+ (20nmol) and enzyme in 0.2M-sodium phosphate buffer (pH8.0) in a final volume of 20μl at 30°C for 5 or 10min. Reactions were carried out in covered Durham tubes. The solutions were mixed, at fixed intervals, by using a micro glass rod. The reactions were stopped by placing them in boiling water for 2min and they were then centrifuged at 2000*g* for 1min. Each supernatant (10μl) was spotted on Whatman no. 1 paper and dried in a stream of air. The UDP-sugars were separated from the polysaccharide by electrophoresis at pH2.0, for 20min, 4kV. The electrophoretograms were cut into strips (40mm×10mm) and their radioactivities counted (Harris & Northcote, 1970). The paper strips containing the radioactive material that ran at the region of the UDP-sugar markers and also that near the starting line (polysaccharide) were removed from the scintillant fluid, washed three times with toluene, twice with benzene, dried and eluted with water. The eluates were evaporated to dryness at 40°C under decreased pressure. The dry residues (UDP-sugars and polysaccharides) were dissolved in 700μl of 0.05M-H_2SO_4 and hydrolysed at 100°C for 15min, neutralized with Amberlite IR 45 resin (CO_3^{2-} form) and evaporated to dryness. The residues were dissolved in 20μl of water and samples

(10μl) spotted on Whatman no. 1 paper and chromatographed for 30h. The chromatograms were cut into strips (40mm×10mm) and counted for radioactivity. The radioactive material that ran at the region of uronic acids, and hexose (galactose and glucose) markers, were removed from the scintillation fluid, washed, dried and eluted with water. The eluates were evaporated to dryness and the residues dissolved in 20μl of water. The solutions (10μl) of uronic acids were run electrophoretically at pH3.5 for 50min at 4.0kV. The radioactivity in the regions corresponding to the markers (galacturonic acid and glucuronic acid) was counted. The solutions (10μl) containing galactose and glucose were chromatographed for 60h and the radioactivity was then counted in the regions of the markers (galactose and glucose). The polysaccharide was also treated for 4h with a solution of human salivary amylase prepared by the method of Olaitan & Northcote (1962). The solution was evaporated, and the dry residue dissolved in 20μl of water and chromatographed for 20h. A unit of the enzyme activity was defined as the amount of enzyme that resulted in the formation of 1nmol of product in 1min.

UDP-D-glucuronate decarboxylase. The reaction mixtures contained UDP-D-[U-^{14}C]glucuronic acid (0.432nmol), UDP-D-glucuronic acid (50nmol) and enzyme in 0.2M-sodium phosphate buffer (pH7.0) in a total volume of 20μl. The reaction products (D-xylose and L-arabinose) were separated by paper electrophoresis and paper chromatography. A unit of enzyme activity was defined as the amount of enzyme that resulted in the formation of 1nmol of product in 1min.

Results

Four kinds of cells were isolated from the trees: (1) cambial cells and their immediate derivatives; these have middle lamella and primary walls only; (2) differentiating xylem cells; these have middle lamella, and primary walls, together with the initial stages of secondary wall deposition; (3) differentiated xylem cells; these are in the process of completing secondary thickening or have completed it; and (4) phloem cells, which have thickened walls.

The protein fraction [precipitate obtained between 40 and 65%-satd. $(NH_4)_2SO_4$] prepared from each type of cell contained the enzymes UDP-D-galactose 4-epimerase, UDP-D-xylose 4-epimerase, UDP-D-glucose dehydrogenase, UDP-D-glucuronate decarboxylase and an enzymic system which catalysed the formation of a polysaccharide. This was established by incubating the fraction (100μg of protein) with UDP-D-[U-^{14}C]glucose (2nmol) and NAD^+ (0.1nmol) in 0.2M-sodium phosphate buffer (pH7.0) in a total volume of 20μl, at 30°C for 15min. The reactions were stopped by placing them in boiling water, and the UDP-sugars formed were separated from the polysaccharide by paper electrophoresis at pH2. The following radioactive products were detected: galactose, xylose, arabinose, glucuronic acid and polysaccharide. A control with boiled enzyme was always carried out for comparison. The enzymic preparations were stable for at least 6 weeks when stored at −15°C.

The activities of the enzymes UDP-D-galactose 4-epimerase, UDP-D-xylose 4-epimerase, UDP-D-glucose dehydrogenase and UDP-D-glucuronate decarboxylase were determined. The measurement of the activities was very reproducible and at least three assays were performed per enzyme per source. All the reactions were carried out at optimal pH and temperature over short periods (approx. 5min) to avoid transformations of the reaction products. Under the conditions of the assays, the reactions were linear with time for at least 10min. A linear relationship between the enzymic activity and the amount of protein present in the incubation was also observed. For each reaction the amount of substrate converted into product was always kept low (less than 15%). The protein precipitated between 40 and 65%-satd. $(NH_4)_2SO_4$ contained about 90% of the total enzymic activities of the initial extract.

UDP-D-galactose 4-epimerase

Table 1 shows the amounts of epimerization of UDP-D-[U-^{14}C]galactose to UDP-D-[U-^{14}C]glucose in the presence of UDP-D-galactose 4-epimerase extracted from cambial cells, differentiating xylem cells and differentiated xylem cells of sycamore and poplar, and the phloem cells of poplar. In cambial cells the activity of the epimerase was lower than that of differentiating xylem and phloem cells, but it was greater than that of differentiated xylem cells in either sycamore or poplar. The specific activity of the epimerase in the poplar cells was 14–16 times greater than that in sycamore.

Kinetics of UDP-D-galactose 4-epimerase with UDP-D-[U-^{14}C]glucose and UDP-D-[U-^{14}C]galactose as substrates. The effect of substrate concentration on the reaction rate starting from either UDP-D-[U-^{14}C]galactose or UDP-D-[U-^{14}C]glucose was determined. The incubation mixture contained 7.5 or 10.7μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ saturation] extracted from cambial cells of poplar and the UDP-D-[U-^{14}C]-galactose or UDP-D-[U-^{14}C]glucose (0.27–5mM), in 0.2M-glycine/NaOH buffer, pH9.0, in a total volume of 20μl. Reaction time at 30°C was 5min. The apparent K_m values calculated by the method of Lineweaver & Burk (1934) were 2.5mM for UDP-D-glucose and 0.77mM for UDP-D-galactose. The $V_{max.}$ values for UDP-D-galactose and UDP-D-

Table 1. *UDP-D-galactose 4-epimerase activity during differentiation in sycamore and poplar*
The reaction mixtures contained 50nmol of UDP-D-galactose, 0.408 nmol of UDP-D-[U-^{14}C]galactose (105700c.p.m.) and the enzyme preparation [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] in 0.2M-glycine/NaOH buffer, pH9.0, in a total volume of 20μl. Reaction time at 30°C was 5 min; 1nmol of substrate was equivalent to 2097c.p.m. Unchanged radioactive substrate and product were determined as described in the text.

Enzyme source	Amount of protein present (μg)	UDP-D-[U-^{14}C]glucose formed in 5min (c.p.m.)
Sycamore		
Cambial cells	75.0	12820
Differentiating xylem cells	75.0	15620
Differentiated xylem cells	75.0	9540
Poplar		
Cambial cells	7.5	21370
Differentiating xylem cells	7.5	21670
Differentiated xylem cells	7.5	15450
Phloem	7.5	22570

glucose were 0.12mM and 0.11mM respectively. From these values the equilibrium constant of the reaction starting from UDP-D-glucose was calculated to be 3.4, which is in good agreement with that determined directly (Wilson & Hogness, 1964).

Ray & Bhaduri (1975) reported that the kinetics of UDP-D-galactose 4-epimerase from *Saccharomyces fragilis* showed a sigmoid dependence on UDP-D-glucose concentration when this was used as a substrate at low concentrations. The sigmoid nature of the curve was progressively decreased in the presence of an increasing concentration of glucose 6-phosphate. We have tested this possibility with the enzyme extracted from poplar cambium. Fig. 1 shows that the rate of epimerization of UDP-D-glucose at low concentrations of the substrate was linear and not a sigmoid curve. Glucose 6-phosphate did not act as an activator of the enzyme.

Effect of NAD$^+$ and NADH. It has been reported that, in calf liver (Maxwell, 1957) and wheat germ (Fan & Feingold, 1969), UDP-D-galactose 4-epimerase required catalytic amounts of NAD$^+$ and was inhibited by NADH; on the other hand, the enzymes prepared from yeast (Maxwell & De Robichon-Szulmajster, 1960) and from *Escherichia coli* (Wilson & Hogness, 1964), which contained tightly bound NAD$^+$, neither required NAD$^+$ nor were they inhibited by NADH. The data in Table 2 indicate that, with the enzyme preparations from sycamore and poplar, NAD$^+$ and NADH did not alter the reaction rate. That the endogenous activator was NAD$^+$ was shown by inactivation of the enzyme by treatment with NADase. The enzyme (75μg) was incubated with 0.02i.u. of NADase for 10min at

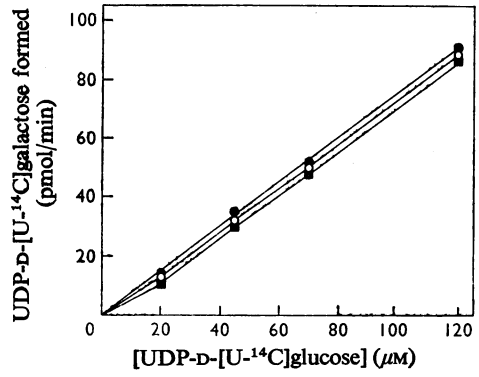


Fig. 1. *Kinetics of UDP-D-galactose 4-epimerase with low concentrations of UDP-D-[U-^{14}C]glucose as substrate in the presence or absence of glucose 6-phosphate*
The reaction mixture contained 10.7μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] extracted from cambial cells of poplar, UDP-D-[U-^{14}C]glucose (20–120μM) and glucose 6-phosphate (0, 1.6mM, 3.2mM), in 0.2M-glycine/NaOH buffer, pH9.0, in a total volume of 20μl. Reaction time at 30°C was 5min. The amount of UDP-D-[U-^{14}C]-galactose formed was determined as described in the text. Glucose 6-phosphate concentrations: ●, 0mM; ○, 1.6mM; ■, 3.2mM.

30°C in the presence of UDP-D-[U-^{14}C]galactose as substrate. Control reaction mixtures were run either without NADase treatment or with NADase previously inactivated in boiling water for 5min. No activity was found in NADase-treated enzyme, whereas the controls retained their initial activity.

Table 2. *Effect of NAD$^+$ and NADH on UDP-D-galactose 4-epimerase activity during differentiation in sycamore and poplar*
The reaction mixtures contained 50nmol of UDP-D-galactose, 0.408nmol of UDP-D-[U-^{14}C]galactose (105700 c.p.m.), the enzyme preparation [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] and NAD$^+$ (1mmol) or NADH (1mmol) in 0.2M-glycine/NaOH buffer, pH9.0, in a total volume of 20μl. Reaction time at 30°C was 5min; 1nmol of substrate was equivalent to 2097c.p.m. Unchanged radioactive substrate and product were determined as described in the text.

Enzyme source	Amount of protein present (μg)	UDP-D-[U-^{14}C]glucose (c.p.m.) formed in 5min in the presence of: No exogenous cofactor	NAD$^+$	NADH
Sycamore				
Cambial cells	75.0	12920	12690	13100
Differentiating xylem cells	75.0	15740	15380	15940
Differentiated xylem cells	75.0	9420	9230	9700
Poplar				
Cambial cells	7.5	21450	21080	21510
Differentiating xylem cells	7.5	21830	21530	21710
Differentiated xylem cells	7.5	15220	15100	15250

Table 3. *UDP-D-xylose 4-epimerase activity during differentiation in sycamore and poplar*
The reaction mixtures contained 10nmol of UDP-D-xylose, 0.312nmol of UDP-D-[U-^{14}C]xylose (60000 c.p.m.) and 75μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] in 0.2M-sodium phosphate buffer, pH8.0, in a total volume of 20μl. Reaction time at 30°C was 5min; 1nmol of substrate was equivalent to 5818 c.p.m. Unchanged radioactive substrate and product were determined as described in the text.

Enzyme source	UDP-L-[U-^{14}C]arabinose formed in 5min (c.p.m.)
Sycamore	
Cambial cells	2240
Differentiating xylem cells	2770
Differentiated xylem cells	1870
Poplar	
Cambial cells	6150
Differentiating xylem cells	6350
Differentiated xylem cells	5640
Phloem cells	3710

Effect of metal ions and p-hydroxymercuribenzoate. The activity of UDP-D-galactose 4-epimerase was unchanged when Mg^{2+} or Ca^{2+} was added to the reaction mixtures at concentrations from 0.001mM to 1mM. Addition of 1mM-*p*-hydroxymercuribenzoate inhibited the enzymic activity by about 95%. Cysteine did not remove this inhibition.

UDP-D-xylose 4-epimerase

The data in Table 3 show UDP-D-xylose 4-epimerase activity in cells at various stages of differentiation. In both sycamore and poplar the activity increased from cambial cells to differentiating xylem cells, but decreased in the differentiated cells. The activity of the enzyme was about two to three times as great in poplar as in sycamore.

Kinetics of UDP-D-xylose 4-epimerase with UDP-D-[U-^{14}C]xylose as substrate. The dependence of the epimerase activity on the concentration of UDP-D-[U-^{14}C]xylose was plotted by the method of Lineweaver & Burk (1934), and a straight line was obtained. The incubation mixture contained 75μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ saturation] extracted from cambial cells of sycamore and UDP-D-[U-^{14}C]xylose (0.025–1.25mM), in 0.2M-sodium phosphate buffer, pH8.0, in a total volume of 20μl. Reaction time at 30°C was 5min. The apparent K_m value was 0.16mM.

Effect of metal ions and p-hydroxymercuribenzoate. Ca^{2+} and Mg^{2+} at concentrations of 0.001–1.0mM did not affect the rate of epimerization. *p*-Hydroxymercuribenzoate (1mM) inactivated the enzymic activity by about 90%.

UDP-D-glucose dehydrogenase

The protein fraction precipitated between 40 and 65%-satd. $(NH_4)_2SO_4$ obtained from cambial cells, differentiating and differentiated xylem cells, and phloem cells, on incubation for 5min in the presence of UDP-D-[U-^{14}C]glucose as substrate and NAD$^+$ catalysed the formation of UDP-D-[U-^{14}C]galactose, UDP-D-[U-^{14}C]glucuronic acid, UDP-D-[U-^{14}C]-xylose and a ^{14}C-labelled polysaccharide. The formation of UDP-D-[U-^{14}C]glucuronic acid and UDP-D-[U-^{14}C]xylose indicated that the presence of the enzyme UDP-D-glucose dehydrogenase was respon-

Table 4. *UDP-D-glucose dehydrogenase activity during differentiation in sycamore and poplar*
The reaction mixtures contained 50nmol of UDP-D-glucose, 0.4nmol of UDP-D-[U-^{14}C]glucose (125000c.p.m.), 20nmol of NAD^+ and 70μg of enzyme preparation [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ saturation] in 0.2M-sodium phosphate buffer, pH8.0, in a total volume of 20μl. Reaction time at 30°C was 5min; 1nmol of substrate was equivalent to 2480 c.p.m. The amount of UDP-D-[U-^{14}C]glucuronic acid and UDP-D-[U-^{14}C]xylose formed in 5min was determined as described in the text. UDP-D-[U-^{14}C]xylose was corrected for the loss of C-6 from the [U-^{14}C]glucose.

Enzyme source	Product formed in 5min (c.p.m.) UDP-D-[U-^{14}C]glucuronic acid	UDP-D-[U-^{14}C]xylose	Total
Sycamore			
Cambial cells	4216	1295	5511
Differentiating xylem cells	6550	2085	8645
Differentiated xylem cells	9979	5662	15641
Poplar			
Cambial cells	2311	3834	6145
Differentiating xylem cells	1784	4040	5824
Differentiated xylem cells	1495	4650	6145
Phloem cells	2300	338	2638

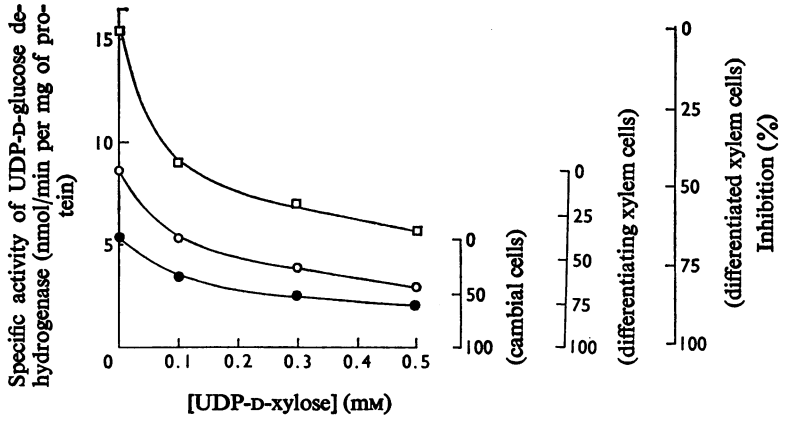


Fig. 2. *Inhibition of UDP-D-glucose dehydrogenase activity by UDP-D-xylose in sycamore*
The reaction mixtures contained 50nmol of UDP-D-glucose, 0.4nmol of UDP-D-[U-^{14}C]glucose (125000c.p.m.), 20nmol of NAD^+, 70μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] and UDP-D-xylose (0–0.5mM), in 0.2M-sodium phosphate buffer, pH2.0, in a total volume of 20μl. Reaction time at 30°C was 5min; 1nmol of substrate was equivalent to 2480c.p.m. Unchanged radioactive substrate and products were determined as described in the text. Enzyme from cambial cells, ●; enzyme from differentiating xylem cells, ○; enzyme from differentiated xylem cells, □.

sible for the oxidation of UDP-D-[U-^{14}C]glucose to UDP-D-[U-^{14}C]glucuronic acid, which then acted as substrate for the UDP-D-glucuronate decarboxylase, producing UDP-D-[U-^{14}C]xylose. Therefore we have measured the activity of UDP-D-glucose dehydrogenase by measuring the amount of UDP-D-[U-^{14}C]-glucuronic acid together with the UDP-D-[U-^{14}C]-xylose formed during the assay. Table 4 shows the activity of this enzyme during differentiation. In sycamore, UDP-D-glucose dehydrogenase activity increased sharply from cambial cells to differentiated xylem cells [a similar result has been obtained by Rubery (1972)], whereas it was nearly constant in poplar. However, phloem cells showed a much lower activity.

Inhibition of UDP-D-glucose dehydrogenase by UDP-D-xylose. Neufeld & Hall (1965) reported that UDP-D-glucose dehydrogenases from pea cotyledons,

Table 5. *Activity of the enzyme(s) catalysing the formation of a polyglucan during differentiation in sycamore and poplar*
The reaction mixtures contained 50nmol of UDP-D-glucose, 0.4nmol of UDP-D-[U-^{14}C]glucose (125000c.p.m.), 20nmol of NAD^+ and 70μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] in 0.2M-sodium phosphate buffer, pH8.0, in a total volume of 20μl. Reaction time at 30°C was 5min; 1nmol of substrate was equivalent to 2480c.p.m. The amount of ^{14}C-labelled polysaccharide was determined as described in the text.

Enzyme source	Specific activity (nmol/min per mg of protein)	$10^7\times$ Units of enzyme(s) per cell
Sycamore		
Cambial cells	6.3	7.8
Differentiating xylem cells	7.4	11.8
Differentiated xylem cells	15.7	31.2
Poplar		
Cambial cells	7.2	20.4
Differentiating xylem cells	6.1	26.6
Differentiated xylem cells	5.7	19.3

Table 6. *UDP-D-glucuronate decarboxylase activity during differentiation in sycamore and poplar*
The reaction mixture contained 50nmol of UDP-D-glucuronic acid, 0.432nmol of UDP-D-[U-^{14}C]glucuronic acid (135000c.p.m.) and 7.5μg of enzyme [protein precipitated between 40 and 65% $(NH_4)_2SO_4$ satn.] in 0.2M-sodium phosphate buffer, pH7.0, in a total volume of 20μl. Reaction time at 37°C was 5min. The amount of UDP-D-[U-^{14}C]xylose and UDP-L-[U-^{14}C]arabinose formed in 5min was determined as described in the text. UDP-D-[U-^{14}C]xylose and UDP-L-[U-^{14}C]arabinose were corrected for the loss of C-6 from the [U-^{14}C]glucuronic acid.

Enzyme source	Product formed in 5min (c.p.m.) UDP-D-[U-^{14}C]xylose	UDP-L-[U-^{14}C]arabinose	Total
Sycamore			
Cambial cells	12509	815	13324
Differentiating xylem cells	30067	1387	31454
Differentiated xylem cells	29969	500	30469
Poplar			
Cambial cells	29304	2318	31622
Differentiating xylem cells	39114	3964	43078
Differentiated xylem cells	38689	4718	43407
Phloem cells	11499	5868	17367

calf liver and chick cartilage were strongly and specifically inhibited by UDP-D-xylose. Such feedback inhibition can play an important role in the modulation of carbon flow into pathways leading to cell-wall-polysaccharide biosynthesis. We have tested this possibility in our system. Fig. 2 shows that UDP-D-glucose dehydrogenase activity from cambial cells, differentiating and differentiated xylem cells of sycamore was inhibited by different concentrations of UDP-D-xylose.

Polysaccharide formation in the presence of UDP-D-glucose as substrate. The enzymic preparations incubated with UDP-D-[U-^{14}C]glucose formed a water-soluble polysaccharide. After acid hydrolysis (0.05M-H_2SO_4, 100°C, 15min) of the polysaccharide, D-[U-^{14}C]glucose residues were released. The specific activities and the units of the enzyme activity per cell in sycamore and poplar are shown in Table 5. Treatment of the polyglucan with salivary amylase (Olaitan & Northcote, 1962) did not produce any maltose or glucose.

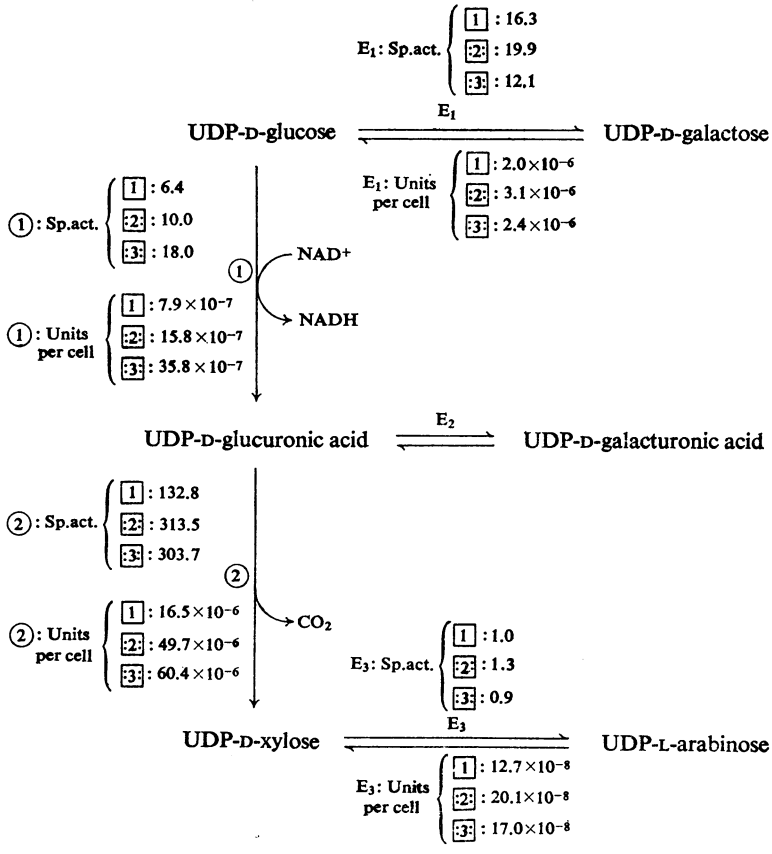
UDP-D-glucuronate decarboxylase. The enzymic preparations converted UDP-D-[U-^{14}C]glucuronic acid into a mixture of UDP-D-[U-^{14}C]xylose and UDP-L-[U-^{14}C]arabinose. The preponderance of UDP-D-[U-^{14}C]xylose in a reaction after 5min

incubation indicated that the decarboxylation of UDP-D-[U-^{14}C]glucuronic acid yielded UDP-D-[U-^{14}C]xylose, and this was converted into UDP-L-[U-^{14}C]arabinose by epimerization. The data in Table 6 show that the decarboxylase activity in differentiating and differentiated xylem cells was higher than that in cambial cells in both sycamore and poplar. However, phloem cells showed a lower activity than cambial cells in poplar.

Effects of NAD$^+$, NADH and p-hydroxymercuribenzoate. UDP-D-glucuronate decarboxylase activity was neither inhibited nor stimulated by adding NAD$^+$ or NADH to the reaction mixture. *p*-Hydroxymercuribenzoate inhibited the enzyme almost completely after 20min incubation.

Specific activities and units of enzyme activities per cell of the enzymes for UDP-sugar interconversion during differentiation in sycamore

Scheme 1 shows the specific activities (nmol/min per mg of protein) and the units of enzyme activities per cell (nmol/min per cell) of UDP-D-galactose 4-epimerase, UDP-D-xylose 4-epimerase, UDP-D-glucose dehydrogenase and UDP-D-glucuronate decarboxylase in cambial cells, differentiating xylem cells and differentiated xylem cells isolated from sycamore. The specific activities of the two epimerases increased from cambial cells to differentiating xylem cells and decreased in differentiated xylem cells. The units of enzyme activity per cell had the same pattern. The UDP-D-glucose dehydrogenase and



KEY: 1, cambial cells; 2, differentiating xylem cells; 3, differentiated xylem cells

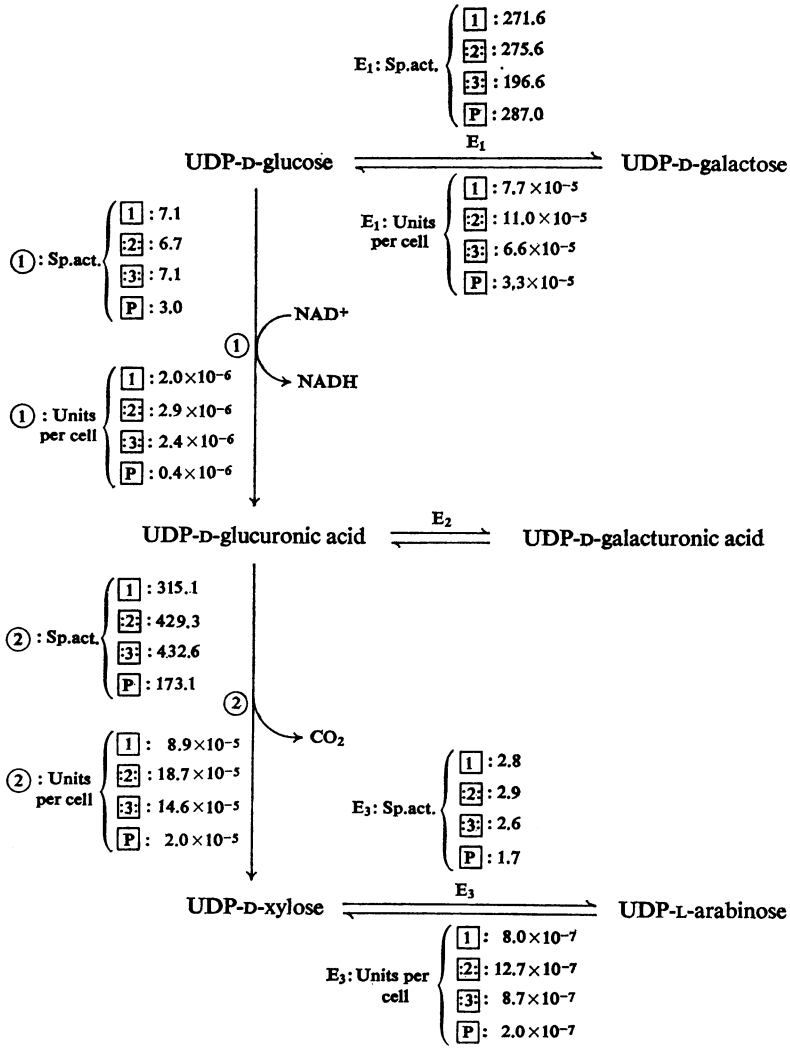
Scheme 1. *Specific activities and units of enzyme activities per cell of the enzymes of UDP-sugar interconversion during differentiation in sycamore*

Sp. act., specific activity (nmol/min per mg of protein). Units per cell, units of enzyme activity per cell (nmol/min per cell). E_1, UDP-D-galactose 4-epimerase; E_2, UDP-D-glucuronate 4-epimerase; E_3, UDP-D-xylose 4-epimerase; ①, UDP-D-glucose dehydrogenase, ②, UDP-D-glucuronate decarboxylase.

UDP-D-glucuronate decarboxylase activity markedly increased from cambial cells to xylem cells when expressed either as specific activities or as units of enzyme activities per cell.

Specific activities and units of enzyme activities per cell of the enzymes of UDP-sugar interconversion during differentiation in poplar

Scheme 2 shows the specific activities (nmol/min per mg of protein) and the units of enzyme activities per cell (nmol/min per cell) in cambial cells, differentiating and differentiated xylem cells, and phloem cells isolated from poplar. The specific activity of the two epimerases was about the same in cambial cells and in differentiating xylem cells, whereas it was lower in differentiated xylem cells. However, the units of enzyme activities per cell were highest in differentiating xylem cells.



KEY: 1, cambial cells; 2, differentiating xylem cells; 3, differentiated xylem cells; P, phloem cells

Scheme 2. *Specific activities and units of enzyme activities per cell of the enzymes of UDP-sugar interconversion during differentiation in poplar*

Sp. act., specific activity (nmol/min per mg of protein). Units per cell, units of enzyme activity per cell (nmol/min per cell). E_1, UDP-D-galactose 4-epimerase; E_2, UDP-D-glucuronate 4-epimerase; E_3, UDP-D-xylose 4-epimerase; ① UDP-D-glucose dehydrogenase; ②, UDP-D-glucuronate decarboxylase.

Although the specific activity of UDP-D-glucose dehydrogenase did not change in cambium and xylem, the units of enzyme activity per cell were higher in xylem. Both specific activity and, more markedly, the units of enzyme activity per cell of UDP-D-glucuronate decarboxylase were higher in xylem than in cambium. Phloem cells showed a lower specific activity of all the enzymes compared with cambial cells, except for the UDP-D-galactose 4-epimerase.

Discussion

The results of the work reported here show that both the specific activities and the activities per cell of the soluble enzymes UDP-D-galactose 4-epimerase, UDP-D-xylose 4-epimerase, UDP-D-glucose dehydrogenase and UDP-D-glucuronate decarboxylase that are involved in nucleoside diphosphate sugar interconversion are different in cambial cells, differentiating xylem cells and differentiated xylem cells. The formation of nucleoside diphosphate sugars, which are soluble precursors in the syntheses of hemicellulose (UDP-D-glucose, UDP-D-glucuronic acid and UDP-D-xylose) and pectin (UDP-D-galactose, UDP-D-galacturonic acid and UDP-L-arabinose) varies according to the stage of cell-wall differentiation.

Pectic polysaccharides are mainly synthesized and incorporated into the wall during primary growth, whereas during secondary thickening there is a marked increased in the syntheses of hemicellulose and cellulose, with little or no change in pectin biosynthesis (Thornber & Northcote, 1961*a*,*b*). The differentiating xylem cells are a heterogeneous population containing developing vessels, ray cells with primary walls and a high percentage of fibres. All these cells undergo elongation and expansion growth before reaching their final stage of differentiation. This primary growth is significant. Cronshaw & Wardrop (1964) have reported that considerable changes in the dimensions of the cells occur during the process of differentiation of xylem from cambial initials. In angiosperms, fibres are 50–500% longer than the mean length of the cambial cells from which they are derived. This increase in the length is also accompanied by an increase in width of 200–500%.

Our data show that the shift in polysaccharide formation during growth and differentiation is accompanied by differential changes in the activities of the enzymes which interconvert the nucleoside diphosphate sugar-donor compounds needed for pectin and hemicellulose biosynthesis. We have found that both the specific activities and the units of enzyme activities per cell of UDP-D-galactose 4-epimerase and UDP-D-xylose 4-epimerase, whose presence is necessary for producing pectic polysaccharide precursors, were higher in differentiating xylem cells than in cambial cells or in the differentiated xylem cells, both in poplar and in sycamore.

The lowest specific activity of the two epimerases occurred in differentiated xylem cells, and this was expected, because the synthesis of pectic material during secondary thickening ceases (Thornber & Northcote, 1961*a*). However, it is clear from Schemes 1 and 2 that the epimerase activities per cell remain relatively high even in differentiated xylem cells. Rubery (1973) reported that the specific activity and the units of enzyme activity per cell of UDP-D-galactose 4-epimerase were about the same in cambium and xylem in sycamore. Our data confirm this report and extend it. On the other hand, Boothby (1972) did not detect any epimerase activities when he used the same system.

That these epimerases were still active at stages of growth where no pectin was being laid down indicated that the main control of pectin synthesis was probably not exerted by modulation of these enzymes and that the flux of sugar through the nucleoside diphosphate sugar precursors, necessary for synthesis, was controlled primarily at another stage, probably at the transglycosylases of the synthetase reactions.

Fan & Feingold (1969) reported that UDP-D-galactose 4-epimerase isolated from wheat germ was similar to that of mammalian tissues in its requirement for NAD^+ and its inhibition by NADH. These authors suggested that the ratio of NAD^+ to NADH could play a role in controlling the epimerization of UDP-D-glucose to UDP-D-galactose in higher plants, increasing the activity of the enzyme when this ratio was low, and decreasing it when it was high. Our results show that UDP-D-galactose 4-epimerase activity was unaffected by NAD^+ and NADH in sycamore and in poplar. However, NADase treatment inhibited almost all the enzymic activity. This suggests that the enzyme has tightly bound NAD^+, like the UDP-D-galactose 4-epimerase of *Saccharomyces fragilis* (Maxwell & De Robichon-Szulmajster, 1960; Darrow & Rodstrom, 1968) and *Escherichia coli* (Wilson & Hogness, 1964).

It has been reported that UDP-D-galactose 4-epimerase isolated from *Saccharomyces fragilis* (Ray & Bhaduri, 1975) was a rather unusual enzyme because it showed a normal hyperbolic relationship of its activity to substrate concentration when UDP-D-galactose was used as substrate, and a sigmoid relationship when UDP-D-glucose was used as substrate. Further, the sigmoid nature of the curve disappeared in the presence of glucose 6-phosphate. In our work we have shown that the UDP-D-galactose 4-epimerase isolated from cambial cells of poplar does not have these properties. We have no evidence that the epimerases can be modulated and

control pectin synthesis in this way during the growth of the cell.

We have also considered the UDP-sugars required for hemicellulose synthesis. The bulk of the hemicellulose of angiosperms is composed of *O*-acetyl-(4-*O*-methylglucurono)xylans (Timell, 1964; Northcote, 1969). For the synthesis of these polysaccharides the cell requires the precursors UDP-D-glucuronic acid and UDP-D-xylose. Biosynthesis of UDP-D-glucuronic acid in plants proceeds via UDP-D-glucose oxidation or via the *myo*-inositol oxidation pathway (Loewus *et al.*, 1973), whereas UDP-D-xylose is formed by decarboxylation of UDP-D-glucuronic acid.

In sycamore, the specific activities and the units of enzyme activities per cell of UDP-D-glucose dehydrogenase and UDP-D-glucuronate decarboxylase increased from cambial cells to differentiating and differentiated xylem cells. Similar results were found in poplar, where the specific activity of UDP-D-glucuronate decarboxylase was higher in differentiated xylem than in cambium, although the specific activity of UDP-D-glucose dehydrogenase remained almost unchanged during differentiation. In addition, the units of activities per cell of the two enzymes were highest in differentiating xylem cells and lowest in cambium. These results strongly suggest that the high activities of these two enzymes in xylem vessels and fibres can be correlated with the increased demand for UDP-D-glucuronic acid and UDP-D-xylose that is needed for the synthesis of *O*-acetyl-(4-*O*-methylglucurono)xylans during secondary thickening in angiosperms. The xylose/glucuronic acid molar ratio in these polysaccharides is approx. 10:1 (Timell, 1964; Northcote, 1969). This ratio could reflect a different activity of the enzymes UDP-D-glucose dehydrogenase and UDP-D-glucuronate decarboxylase. We have found that the activity of UDP-D-glucuronate decarboxylase was always greater (18–50 times) than that of UDP-D-glucose dehydrogenase in all the tissues. Further, the latter enzyme was inhibited by UDP-D-xylose, as shown in Fig. 2. Such a feedback control could modulate the unidirectional flow of sugar to produce the right balance of UDP-D-glucuronic acid and UDP-D-xylose needed for hemicellulose biosynthesis. This control becomes even more important when it is considered that UDP-D-glucuronic acid can also be formed via the *myo*-inositol oxidation pathway (Loewus *et al.*, 1973). The inhibitory effect of UDP-D-xylose on UDP-D-glucose dehydrogenase has been reported in pea cotyledons, calf liver and chick cartilage (Neufeld & Hall, 1965). It has been suggested by Gainey & Phelps (1974) that UDP-D-xylose, and to a lesser extent UDP-D-glucuronic acid, act by lowering the apparent affinity of the UDP-D-glucose dehydrogenase for NADH.

As differentiation proceeded from cambium to xylem, a change in activity occurred among the enzymes involved in producing the precursors of pectin and hemicellulose biosynthesis. This was especially obvious for those enzymes responsible for the production of the precursors of hemicellulose. The changes, although correlated with the differences in the chemical composition of the wall during differentiation, cannot, however, represent the main control sites of cell-wall synthesis, since, even at stages of growth when no pectin was formed from some of the precursors, the epimerases which produce them were still active. Throughout growth the cell retains a full capability for the interconversion of UDP-sugars, hexoses, pentoses and uronic acids. The change in polysaccharide synthesis which is a feature of cell-wall differentiation is therefore achieved by control of other enzymes such as the synthetases, although there is a correlated secondary control at the enzymes which bring about the UDP-sugar interconversions, especially those in the pathway which forms UDP-D-xylose from UDP-D-glucose.

Our data on the activities of UDP-sugar-interconversion enzymes in phloem cells are rather difficult to explain in terms of the UDP-sugar-requiring reactions, because this tissue contained cells of different types which were not separated. Nevertheless it appeared from the results that in this tissue, like the xylem, the potential for the interconversion of the UDP-sugars was retained throughout the development of the cells.

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