Separation and Identification of Soluble Nucleotides in Cambial and Young Xylem Tissue of *Larix decidua* Mill.

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The nucleotides present in the cambial tissue (primary wall tissue) and in the not yet fully differentiated secondary xylem (secondary wall tissue) of *Larix decidua* Mill. were extracted and characterized. The method of extraction best suited to the material was investigated and the problems involved in desalting of extracts and their effect on the final nucleotide pattern obtained are discussed. UDP-glucose was found to be the most important sugar nucleotide isolated from both cambial and young xylem tissue. UDP-galactose, UDP-arabinose, UDP-xylose, UDP-fructose, GDP-glucose, GDP-galactose, GDP-mannose, ADP-glucose, ADP-galactose, ADP-fructose, ADP-ribose and ADP-ribose phosphate (the last two compounds are the acid breakdown products of NADH and NADPH respectively) were also found in both extracts. UDP-galacturonic acid was identified only in the extract of the primary wall material. Several nucleotide oligosaccharides were obtained from both the extract of cambial tissue and that of the young xylem.

Since the discovery of UDP-glucose as cofactor in the interconversion of D-glucose and D-galactose in yeast (Caputto, Leloir, Cardini & Paladini, 1950) it has become increasingly apparent that nucleoside diphosphate glycosyl compounds are of prime importance in carbohydrate metabolism. Sugar nucleotides have been found to play a fundamental role in the interconversion of many monosaccharides and in the biosynthesis of oligo- and poly-saccharides (for reviews see Strominger, 1964; Feingold, Neufeld & Hassid, 1964; Leloir, 1964; Nordin & Kirkwood, 1965; Hassid, 1967).

The present paper describes the isolation and identification of nucleotide compounds from the European larch (*Larix decidua* Mill.). Extensive research has been conducted to determine the nature of the chemical and structural changes in the cell wall that take place on differentiation of the cambial cell in forest trees (Meier & Wilkie, 1959; Meier, 1964; Thornber & Northcote, 1961*a,b*, 1962; Larson, 1969), but as yet no results have been presented on the changes in the sugar nucleotides during differentiation. An attempt is therefore made here to compare possible qualitative or quantitative differences or both in sugar nucleotides, extracted from primary wall tissue (cambial zone) where little or no differentiation has taken place, with those extracted from tissue where deposition of the secondary wall is actively in progress (young xylem cells).

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MATERIALS AND METHODS

Chemicals and enzymes. UDP-[U-¹⁴C]glucose was purchased from The Radiochemical Centre (Amersham, Bucks.). Xanthosine, NAD⁺, NADP⁺, ADP, ATP, UMP, UTP, GMP, GDP, GTP, IMP and UDP-glucose were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); GDP-mannose was from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and GDP-glucose was from Calbiochem (Los Angeles, Calif., U.S.A.). Adenosine, guanosine, uridine and the respective bases together with most other chemicals were supplied by Fluka A.-G. (Buchs SG, Switzerland). Two preparations of charcoal were used: Norit A practical grade from Serva Entwicklungs-Labor (Heidelberg, Germany) and unground Nuchar C-190 granular charcoal, 25 mesh, from West Virginia Pulp and Paper Co. (New York, N.Y., U.S.A.). The latter was a generous gift from R. H. Egli (Research Laboratories, Nestlé Products, Vevey, Switzerland) and was prepared according to his instructions (Kieffer & Egli, 1966).

Crude snake venom (*Crotalus adamanteus*) was purchased from Sigma Chemical Co. and purified venom phosphodiesterase, used as a source of nucleotide pyrophosphatase (EC 3.6.1.9), from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Alkaline phosphatase from calf intestine was a kind gift from Professor P. Portmann (Department of Physiological Chemistry, University of Fribourg, Switzerland). Phosphatase activity in the raw extracts was controlled by using the commercially available acid phosphatase test from C. F. Boehringer und Soehne.

Plant material. The 60–80-year-old stems of European larch (*Larix decidua* Mill.) were used. Preliminary experiments were conducted on a tree felled in late June 1967

and the main research was done on another taken under the same conditions 1 year later. The procedure followed in both cases was the same: the trunk was cut into 1m sections and stored at 0°C for several hours to lower the enzymic activity. The bark, which loosened easily in the cambial region, was removed and a first fraction, containing only cambial cells, was scraped off with pieces of glass and immediately frozen in liquid nitrogen. The surface of the stem was cleaned and blotted with filter paper and a second fraction, consisting mainly of young, not yet fully differentiated, xylem cells was removed. The tissue fractions thus obtained were studied under a polarizing microscope to determine the stage of differentiation of the cell wall. It was found that the first fraction (fraction A) consisted almost entirely of primary wall material and that the second fraction (fraction B), although containing a small amount of pure primary wall material, consisted mainly of cells where some degree of secondary wall formation had occurred. The wet weight of material obtained in these fractions was approx. 1kg and 2.5 kg respectively.

Killing and extraction of the plant material. Two extraction procedures were tested on the material taken in 1967, namely extraction with cold acidified ethanol and with boiling ethanol. The former method was found to be more suited to the material investigated and is described in detail in the next paragraph; the second method, used only in preliminary experiments, is mentioned briefly in the Discussion section.

The method of extraction with cold acidified ethanol used was that of Cole & Ross (1966) with minor adaptations. The tissue frozen in liquid nitrogen was macerated (MSE homogenizer) in 80% formic acid for 2–3 min and then poured directly into a large quantity of ethanol at –20°C; a final pH of 2.5 was obtained. After decantation the cell-wall material was washed twice with precooled acidified ethanol and the washings were added to the extract. At this point 1μCi (0.005μmol) of UDP-[U-¹⁴C]-glucose was added to control the site and amount of any breakdown of the sugar nucleotide during ensuing purification. The extract was stored at –30°C until no further phosphatase activity could be detected. The period of time necessary for the inactivation of the enzymes was 20h for fraction A and 14h for fraction B. The extracts were adjusted to pH 6.5 with aq. ammonia (sp.gr. 0.88), centrifuged to remove precipitated material and shaken several times with precooled diethyl ether. No radioactivity was found in either the residue obtained on centrifugation or in the ether-soluble material.

Preliminary purification and fractionation. Ammonium formate produced on neutralization was removed either by vacuum sublimation at 37°C, for fraction A, or by desalting on an activated charcoal column, for fraction B, as described below. After these procedures fraction A still contained all sugars, organic acids, coniferin etc., whereas fraction B had only a small amount of non-nucleotide material. The extracts were then applied to 40cm × 4cm columns of Dowex 1 (X4; formate form; 200–400 mesh). Before elution of the nucleotides the column was washed with 50% ethanol until the E_{260} (Beckman DB spectrophotometer, 1cm light-path) fell below 0.05 and subsequently with water until a negative Molisch test for sugar was obtained from the eluate.

The elution system used for the Dowex 1 separation was similar to that of Cherry & Hageman (1960) with

1M-formic acid (1 litre) in the mixer and 0.8M-ammonium formate in 1M-formic acid (1 litre) in the reservoir. A flow rate of 21.7 ml/h was maintained with the aid of a peristaltic pump and 25ml fractions were collected. The E_{260} of each tube was measured and the fractions belonging to each peak were combined. Six peaks were obtained from fraction A and seven from fraction B (Fig. 1). At the end of the irrigation the column was washed with 1.6M-ammonium formate (200ml) until the E_{260} was less than 0.1. With fraction B a further wash with 3.0M-ammonium formate (500ml) was necessary to remove residual material absorbing at 260nm. Later investigation of the washes revealed that they contained mainly non-nucleotide material. The peaks were desalted by vacuum sublimation at 37°C and the final washings by treatment with Nuchar charcoal as described below.

Desalting of nucleotide fractions on activated charcoal. The quantity of ammonium formate in the extract of fraction B was considerably greater than that present in fraction A, owing to the greater volume of the former, and this fraction was consequently desalted on Norit A activated charcoal by the method of Pontis, Cabib & Leloir (1957). In all later operations where activated charcoal was used for the removal of salt, e.g. the desalting of column washes, polyethyleneimine-separated sugar nucleotides etc., the method was slightly modified. Nuchar charcoal was added until the E_{260} of the supernatant fell below 0.05 and then washed and eluted as described in the method cited above.

Paper chromatography. Descending chromatography was used throughout. Whatman 3MM paper washed with 2M-acetic acid was employed for preparative separations and Schleicher und Schüll 2043a or 2043b paper, washed or unwashed, for other operations. Solvent system (a) butan-1-ol-ethanol-acetic acid-water (16:4:3:7, by vol.) (Jenner, 1968), was used to remove some of the impurities still present in the main fractions obtained from the Dowex 1 column. Further impurities could to a large extent be removed by chromatography in solvent (b): 1M-ammonium acetate (pH 3.8)-ethanol (2:5, v/v) (Paladini & Leloir, 1952). This solvent also served to separate the nucleotides of the individual peaks. Solvent (c), the same as solvent (b) but with final pH 7.5, was employed in further characterization of the nucleotides partly separated in the above solvents. Solvent (d), isobutyric acid-ammonia-water (57:4:39, by vol.) (Pabst Laboratories Circular OR-10), was used to separate the purine and pyrimidine bases and nucleosides obtained on acid or enzymic hydrolysis of nucleoside di- and triphosphates. The neutral sugars and uronic acids similarly liberated were chromatographed in solvents (e), ethyl acetate-pyridine-water (2:1:2, by vol., upper phase), (f), ethyl acetate-pyridine-water (8:2:1, by vol.), and (g), ethyl acetate-acetic acid-water (3:1:3, by vol., upper phase).

Paper electrophoresis. An apparatus similar to that described by Foster (1952) was employed for qualitative analysis and also for preparative separation of nucleoside triphosphates from yellow impurities that could not be separated by paper chromatography in either solvent (b) or (c). A 1M-ammonium formate buffer, pH 3.5, was used with a potential gradient of 33 V/cm during a standard run of 1–1½ h. Picric acid, which had the same mobility as UDP-glucose, was used as a marker.

Anion-exchange thin-layer chromatography. This was carried out on commercially available polyethyleneimine plastic sheets (Macherey, Nagel and Co., Düren, Germany). The solvent systems used were those described by Randerath & Randerath (1964, 1965a, b). Solvent system (h), where 1M-acetic acid is allowed to ascend up to about 2 cm from the origin, followed, without intermediate drying, by 1M-acetic acid–3M-lithium chloride (9:1, v/v), was used in all qualitative investigations of the nucleotides. Solvent system (i), sodium tetraborate–boric acid–ethylene glycol (6:3:25, w/w/v), was used to separate the sugar nucleotides. A modified Randerath system, solvent (j), was used to separate the nucleoside triphosphates: the plates were again developed for the first 2 cm with 1M-acetic acid, but were then transferred to another tank with 2.3M-lithium chloride for the remainder of the development. Nucleotides preparatively separated on polyethyleneimine sheets were eluted from the ion-exchange support with a 1M-lithium chloride solution. The eluates were not usually desalted as it was found that the lithium chloride did not interfere with either the ribose or phosphate determinations. It was, however, necessary to remove the lithium chloride on activated charcoal from the acid hydrolysates of sugar nucleotides before paper chromatography of the neutral sugars.

DEAE-Sephadex chromatography. Before the column was poured the gel [DEAE-Sephadex A-25, medium grade (Pharmacia AB, Uppsala, Sweden)], was allowed to swell in an excess of 0.1M-lithium chloride. The nucleotides were applied to the top of the column (1.5 cm × 20 cm), which was then irrigated with water until the E_{260} value fell below 0.05. Separation of the nucleotides was based on a linear gradient of lithium chloride (mixer: 1 litre of water; reservoir: 1 litre of 0.6M-lithium chloride). A flow rate of 21.7 ml/h was maintained throughout and 5 ml fractions were collected. The extinction of the eluate was controlled as mentioned above and the peaks obtained were desalted on Nuchar charcoal as described above.

Analytical methods

Detection and determination of phosphate. Phosphorylated compounds were detected on paper chromatograms with the 2-(*o*-hydroxyphenyl)-benzoxazole reagent by the method of Gordon, Werum & Thornburg (1964). Determinations of total and labile phosphate were made by the colorimetric method of Fiske & Subbarow (1925) as modified by Leloir & Cardini (1957).

Detection of nucleotides and base determinations. Millimolar extinction coefficients used in the estimation of the various nucleotides present were those given in Pabst Laboratories Circular OR-10. The type of base present, as indicated by the spectral data, was further characterized by specific colour tests and spray reagents. The bases or nucleosides liberated on acid or enzymic hydrolysis respectively were identified by co-chromatography and co-electrophoresis with reference substances. Where a mixture of two or more nucleotides with differing base components could not be resolved by paper chromatography or electrophoresis a known amount of the mixture was hydrolysed with concentrated formic acid at 175°C for 2 h (Vischer & Chargaff, 1948). After hydrolysis the samples were evaporated to dryness *in vacuo* and dissolved in a small amount of 40% ethanol for chromatography in

solvent (d). The chromatograms were irrigated for 12–14 h at 25°C, thoroughly dried and washed in dry methanol before elution of the spots with water for spectral analysis and measurement of the relative amounts of each base. The nucleotides were detected on paper by viewing directly in u.v. light of 254 nm from a CAMAG Universal u.v. lamp (CAMAG, Muttenz, Switzerland). When the amount of nucleotide present was very small, the paper was dipped in a 0.0003% solution of Rhodamine B in ethanol (Niemierko & Krzyzanowska, 1967) to increase the contrast. Adenine derivatives were detected by spraying the chromatograms with a solution of mercuric chloride in ethanol as described by Descimon (1967) and further by the typical colour reaction of Gerlach & Döring (1955). Guanine derivatives could be distinguished by the fluorescent blue colour produced when the chromatogram was viewed under u.v. light after exposure to fuming hydrochloric acid (Smith & Markham, 1950). The determination of the type of base present was further aided by the spray reagents of Letham (1965), which distinguish between purine and pyrimidine compounds.

Determination of pentose. The ribose of purine derivatives and the pentoses of sugar nucleotides were determined by the method of Dische & Borenfreund (1957). With the pyrimidine-bound ribose the compounds were first treated with sodium borohydride in the presence of u.v. light (Nakanishi, Shimizu, Takahashi, Sugiyama & Suzuki, 1967) and subsequently determined as described above. It was found that the filter on the u.v. lamp had to be removed before the reduction of the nucleotides could take place.

Determination of reducing sugar. Reducing sugar values both before and after mild acid hydrolysis (10% acetic acid for 20 min at 100°C) of the sugar nucleotides were determined by the method of Park & Johnson (1949).

Detection of the sugars on paper chromatograms. This was accomplished with the alkaline silver nitrate reagent of Trevelyan, Procter & Harrison (1950) or the *p*-anisidine–hydrochloric acid reagent described by Hough, Jones & Wadman (1950). Fructose was revealed with the phloroglucinol–trichloroacetic acid reagent specific for keto hexoses and keto pentoses (in Smith, 1960). Quantitative determination of the sugars was effected by elution and colorimetric determination of the spots revealed with the triphenyltetrazolium chloride reagent of Fischer & Dörfel (1954).

Identification of disaccharides. Disaccharides were hydrolysed with 2.0M-trifluoroacetic acid in a sealed tube at 100°C for 1 h (Albersheim, Nevins, English & Karr, 1967); the hydrolysates were chromatographed in solvents (e) and (f).

Enzymic hydrolysis of the sugar nucleotides. Two different enzyme preparations were used as the source of nucleotide pyrophosphatase, as described in the Results section. The reaction mixtures generally employed were as follows.

(a) Crude snake venom. The substrate (0.5 μmol) was dissolved in 2 ml of 0.46M-veronal buffer, pH 8.0, to which 2 mg of crude snake venom was added. After an incubation period of 12 h the reaction was stopped by the addition of ethanol to precipitate the enzyme, which was then centrifuged off. The mixture was concentrated to remove the ethanol and freeze-dried before chromatography in solvent (b). In later experiments alkaline phosphatase

was added in sufficient amounts to remove the terminal phosphate. At the end of the reaction the nucleosides were adsorbed on charcoal and the sugar solutions desalted with Dowex 50 (H^+ form) and chromatographed in solvents (e), (f) and (g). The nucleosides were eluted from the charcoal as described above and chromatographed in solvent (d).

(b) Purified venom phosphodiesterase. The sugar nucleotides ($0.5 \mu\text{mol}$) were dissolved in 1.0 ml of 25 mM-veronal buffer, pH 8.0, containing 25 mM-magnesium chloride and allowed to react with purified phosphodiesterase ($80 \mu\text{g}$) plus an excess of alkaline phosphatase. After incubation for 2–3 h the reaction was stopped and the products were purified and identified as described above.

Measurement of radioactivity. This was done in a Tri-Carb liquid-scintillation spectrophotometer (model 314-AX, Packard Instrument Co.).

RESULTS

Method of extraction. Preliminary experiments in 1967 were carried out to determine the method best suited to the extraction of nucleotide compounds from the cambial tissue and young wood of larch.

The results presented below are those obtained from the work on plant material taken in 1968 and extracted with the low-temperature acid-ethanol method.

Identification of the acid-soluble nucleotides from the cambial zone (primary wall material). The raw extract, after having been freed from ammonium formate by vacuum sublimation, was placed on a Dowex 1 column and the elution was performed as described in the Materials and Methods section. The ion-

exchange chromatogram is shown in Fig. 1 and the nucleotides identified in the various peaks are given in Table 1. The numbers 1–6 in this table refer to the six peaks from the Dowex 1 separation and the number 7 to the 1.6M-ammonium formate wash. The nucleotide bands obtained from the initial purification of the peaks in solvent (a) were eluted and rechromatographed in solvent (b) and the numbers I–V in Table 1 refer to the bands obtained in this solvent. In most cases the UMP was already separated from other nucleotides in solvent (a), but to avoid confusion in Table 1 the amounts eluted from this first separation were combined with the small amounts found on chromatography in solvent (b). Where a band eluted from the second separation was found to contain a mixture of two or more nucleotides (by polyethyleneimine chromatography or base analyses or both) the mixture was concentrated and rechromatographed in solvent (b) with extended irrigation. Therefore, the letters a–d in Table 1 refer to this third separation. The first band on chromatography of peak 6 in solvent (b) was found to contain nucleoside triphosphates, which were then eluted and separated by polyethyleneimine chromatography (solvent system j) for further characterization without desalting. The letters A–C in Table 1 refer to the separation on polyethyleneimine.

Peak 1 contained, in addition to the nucleotides isolated, two substances that absorbed strongly at 270 nm, but that could not be identified with any known nucleotide derivative. A further unknown compound with E_{max} at 243 nm was chromatographed together with inosine, IMP and xanthosine, but moved faster than these reference substances both on paper chromatography in solvents (b) and (c) and on electrophoresis.

Peak 2 was found to contain a substance absorbing strongly at 270 nm that remained unidentified.

In peak 3 the GDP-sugar fraction (3-II-b) contained two interesting compounds. One had an R_F on polyethyleneimine (solvent system h) between that of GMP and guanosine and contained 2 moles of total to 1 mole of labile phosphate/mole of base. No reducing sugar could be detected either before or after mild acid hydrolysis, and the nature of this compound could not be elucidated. The second substance was found to have an R_F on polyethyleneimine (solvent system h) between that of GDP and GDP-mannose. This compound was tentatively identified as a GDP-oligosaccharide, as described below.

Peak 4 contained appreciable amounts of the compound UDP-X, which, as described below, was identified as a mixture of UDP-oligosaccharides. As shown in Table 1 the Dowex 1 chromatography resulted in partial separation of the nucleotides of the same base, and further investigation of the sugar nucleotides revealed that the different derivatives of

Fig. 1. Dowex 1 (X4; formate form; 200–400 mesh) separation of nucleotides from 980 g of cambial (---) and 2.54 kg of young xylem (—) tissue of *Larix decidua*. The salt-free extracts were run through columns ($40 \text{ cm} \times 4 \text{ cm}$) and the absorbed compounds eluted with a linear gradient of 0.0–0.8M-ammonium formate in 1M-formic acid at a flow rate of 21.7 ml/h. Fractions of 25 ml were collected and assayed for E at 260 nm.

Table 1. *Nucleotides isolated from the extract of the cambial tissue of Larix decidua*

Nos. 1-6 refer to peaks obtained from Dowex 1 separation and 7 refers to the 1.6 M-ammonium formate wash. Nos. I-V refer to the bands obtained on paper chromatography in solvent (b), band I being nearest to the origin. a-d refer to subsequent paper-chromatographic separation by long-term irrigation in solvent (b), band a being nearest to the origin. A-C refer to the separation on polyethyleneimine plastic sheets in solvent system (j), band A being nearest to the origin.

Fraction		Compound identified	Yield from 980g of fresh material (μ mol)
Major	Minor		
1-IV	2-III, 3-IV-b, 5-IV, 6-IV	AMP	24.8 (1.84*)
2-IV	1-V, 3-V, 4-IV, 5-V, 6-V	UMP	18.9 (3.13*)
2-III-a	3-IV-a, 6-III	GMP	3.8 (0.27*)
3-III-c	1-I-b, 2-II-b, 4-III-b, 5-II-b	ADP-sugars	4.3
3-II-c	1-I-a, 2-II-a, 3-III-a, 4-II-c, 5-I-b	ADP	4.6
3-II-b	2-I, 4-II-b, 5-I-a	GDP-sugars	2.7
4-I	3-I	UDP-X	2.6
4-II-a	3-II-a, 7-II-a	GDP	1.7
4-III-a	1-III, 2-II-c, 3-III-b, 5-III, 6-III, 7-II-b	UDP-sugars	14.3
5-II-a	4-II-d	UDP	4.3
6-I-A	7-I	GTP	0.8
6-I-B		UTP	2.5
6-I-C		ATP	2.70
6-II		UDP-galacturonic acid	0.3

* Degradation products arising during desalting operations.

the same base composition had also to some extent been separated on the Dowex column. But, as the sugar nucleotides in most cases were widely spread over several peaks, the amount present in any one peak was usually too small to allow accurate determination of the sugar present. The different sugar nucleotide fractions, except for those where UDP-X and UDP-uronic acid were present, were therefore combined to allow a more exact determination. The UDP-X and UDP-uronic acid fractions were sufficiently discrete to allow them to be treated separately.

Enzymic liberation of nucleotide-bound sugars. Experiments conducted on cambial tissue extracted in 1967 where crude snake venom was used as the source of nucleotide pyrophosphatase indicated the presence of nucleotide oligosaccharide compounds. Further research showed that the crude snake venom contained enzymes attacking β -linked oligosaccharides and also transglycosidases capable of building higher polymers in the presence of a source of β -linked oligosaccharide.

The oligosaccharides in these preliminary experiments were therefore not further investigated and in all subsequent hydrolysis reactions purified snake-venom phosphodiesterase was used as the source of nucleotide pyrophosphatase.

Enzymic hydrolysis of the main UDP-sugar fraction liberated the following sugars: glucose, galactose, arabinose, xylose and a sugar tentatively identified

as fructose. The percentages of the various sugars present are given in Table 2.

Examination of the enzymic hydrolysate of UDP-X revealed the presence of two spots with R_F values similar to those of disaccharides. One of them moved like cellobiose (solvent f), the other one somewhat slower. Acid hydrolysis of the mixture yielded glucose and arabinose.

Paper chromatography (solvent g) of the enzymic hydrolysate of fraction 6-II revealed the presence of a compound moving at the same rate as galacturonic acid. No glucuronic acid was detected.

The combined guanosine sugar nucleotide fractions, when treated with purified phosphodiesterase and phosphatase, yielded galactose, glucose and mannose together with a trace of an oligosaccharide. The amount of oligosaccharide was very small and after hydrolysis only traces of glucose and mannose could be detected. It is therefore thought that the guanosine derivative mentioned above as having an R_F on polyethyleneimine (solvent system h) between that of GDP and GDP-mannose could in fact have been a GDP-oligosaccharide containing glucose and mannose.

As shown in Table 2, no oligosaccharide compounds were detected in the hydrolysate of the ADP-sugars; glucose proved to be the only quantitatively important monosaccharide component in this hydrolysate.

Identification of the acid-soluble nucleotides from the

Table 2. *Sugars liberated by the action of purified snake-venom phosphodiesterase and alkaline phosphatase on the sugar nucleotides extracted from the cambial tissue of Larix decidua*

Base	Sugar identified	Percentage of total sugar for each base	Derivation (fractions in Table 1)
Uracil	Glucose	61.0	1-III, 2-II-c, 3-III-b, 4-III-a, 5-III, 6-III, 7-II-b
	Galactose	7.0	
	Arabinose	5.0	
	Xylose	8.0	
	Fructose	2.0	6-II
	Galacturonic acid	2.0	
	Oligosaccharides	15.0	3-I, 4-I
Guanine	Glucose	49.0	2-I, 3-II-b, 4-II-b, 5-I-a
	Mannose	43.0	
	Galactose	7.0	
	Oligosaccharides	1.0	
Adenine	Glucose	97.0	1-I-b, 2-II-b, 3-III-c, 4-III-b, 5-II-b
	Ribose*	3.0	
	Galactose	Trace	
	Fructose	Trace	

* Ribose is liberated from ADP-ribose and ADP-ribose phosphate, which are the acid breakdown products of NADH and NADPH respectively (Papenburg, 1961).

Table 3. *UMP isolated from the extract of young xylem cells of Larix decidua*

UMP isolated from the remaining nucleotide materials (see Table 4), by paper chromatography in solvent (a), of the seven fractions obtained by anion-exchange chromatography of the partially purified extract from the young xylem tissue of *Larix decidua*. The amounts indicated were calculated from the millimolar extinction value of uridine at E_{260} .

Peak no. of Dowex separation	Yield from 2.54 kg of fresh materials (μ moles)
1	6.7
2	1.4
3	3.2*
4	1.5*
5	3.2*
6	1.0*
7	Trace*

* Degradation products arising during desalting operation.

young xylem (secondary wall material). The Dowex ion-exchange elution chromatogram is given in Fig. 1. A number of impurities were removed by this separation. As u.v.-absorbing impurities were still present in the nucleotide fractions even after initial purification in solvent (a) and as the amount of nucleoside diphosphate material was considerably higher in the secondary wall extract as compared with the primary wall extract, it was decided to purify the nucleotides further by a separation on DEAE-Sephadex. The Dowex peaks were first

treated by chromatography in solvent (a) to remove as much non-nucleotide and nucleoside monophosphate material as possible. The amounts of UMP separated at this stage are given in Table 3 (the amounts isolated from peaks 3-7 are degradation products arising from labile di- and tri-phosphates during removal of the salt by vacuum sublimation). The remaining nucleotide material was then combined and separated on DEAE-Sephadex into six main fractions and the amounts isolated are given in Table 4. The nucleotides eluted at this stage were completely free of other u.v.-absorbing material. In Table 4 the same designation of fractions was used as in Table 1 except that the numbers 1-6 refer to the separation on the DEAE-Sephadex column.

Fraction 2-I on elution possessed an adenine-type spectrum, but the compound did not react with any of the specific colour reactions for this base. Uracil was the only base found on hydrolysis. Phosphate analysis indicated that the substance was a diphosphate and the reducing value after mild acid hydrolysis was slightly more than 1. Enzymic hydrolysis gave as reaction products uridine, P_i , glucose and a trace of a slower-moving sugar compound. The exact nature of this substance could not be elucidated. The fraction had been eluted from a band situated between ADP and the origin on paper chromatography in solvent (b).

Fraction 3-VI proved to be an uridine derivative with 1 mol of total and no labile phosphate. The compound ran faster than UMP on paper chromatography in solvent (b) and after mild acid hydrolysis gave a reducing value of 0.6. No sugar was detected after enzymic hydrolysis.

Table 4. *Nucleotides isolated from the extract of young xylem cells of Larix decidua*

Nos. 1-6 refer to the peaks eluted from the DEAE-Sephadex column. Nos. I-VI refer to bands obtained on paper chromatography in solvent (b), band I being nearest to the origin. a-c refer to subsequent paper-chromatographic separation by long-term irrigation in solvent (b), band a being nearest to the origin.

Fraction		Compound identified	Yield from 2.54 kg of fresh material (μmol)
Major	Minor		
3-V	4-V-b, 5-V-b	UMP	4.2*
2-I		UDP-?	1.3
2-II-a		NAD	0.5
2-II-b		NADP	0.1
3-I		UDP	0.2*
3-III	3-IV-a, 4-II, 2-II	UDP-sugars	29.2 (20.5†)
3-VI		UMP ?	1.6
4-IV-b	5-IV-b	AMP	8.4
4-IV-c	4-V-a, 3-II, 4-III	ADP-sugar	6.2
4-I-b	5-I-b	ADP	0.5*
4-VI		AMP ?	1.3
5-I-a	4-I-a, 5-II-a	GDP-sugars	1.7
5-II-b	4-IV-a, 5-IV-b	GMP	0.9
5-V-a	5-IV-a, 5-III-c	GDP-?	1.0
6-I		UDP, ADP, GDP, UTP, ATP, GTP, GDP-oligosaccharide	‡

* Degradation products arising during desalting operations.

† 20.5 μmol found in fraction 3-III.

‡ Not determined because of serious losses during fractionation and purification.

Fraction 4-VI was identified as an AMP derivative that ran faster than UMP on paper chromatography in solvent (b) and could perhaps have been 3'-AMP, 2'-AMP or some form of cyclic AMP.

Fractions 5-V-a, 5-IV-a and 5-III-c were found to contain a guanine derivative, similar to that found in the extract of the cambial tissue; its R_f on polyethyleneimine in solvent system (h) lay between that of GMP and guanosine. Two moles of phosphate/mole of base were present, but the reducing value both before and after mild acid hydrolysis was zero.

The material eluted in fraction 6 at the end of the Sephadex separation contained ADP, UDP, ATP, GDP, UTP, GTP and a substance tentatively identified as a GDP-oligosaccharide, given in decreasing order of concentration. This fraction was desalted on Nuchar charcoal and separated again by paper chromatography in solvent (b). As the triphosphates and some of the diphosphates remained close to the origin this area was eluted and the compounds were separated again on polyethyleneimine t.l.c. sheets. This was accomplished in two steps to separate the diphosphates from the triphosphates, and subsequently to separate the individual di- and tri-phosphates. In some cases it was necessary to separate the triphosphate compounds from residual diphosphate material by electrophoresis. Between these processes the compounds had to be desalted on activated charcoal and the final amount of free

diphosphate and triphosphate was about 1.5 μmol . As the total amount of nucleotide material originally eluted in this fraction was more than 5.0 μmol it was clear that appreciable losses had occurred during the second separation and no attempt is made to present quantitative data for these compounds.

Enzymic liberation of nucleotide-bound sugars. The sugars liberated on hydrolysis of a portion (5 μmol) of the main UDP-sugar fraction (3-III, Table 4) are given in Table 5. The minor UDP-sugar fractions had a similar saccharide composition and are not entered in Table 5. The ADP-sugar fractions 4-IV-c, 4-V-a and 4-III were hydrolysed separately, but, except for minor variations in the ribose and fructose values, the proportions of the different components were very similar and the values given in Table 5 constitute the mean of these three determinations. The GDP-sugar fraction (5-I-a) gave a result similar to that of the primary wall fraction. Glucose, mannose and galactose were liberated on enzymic hydrolysis. No UDP-oligosaccharide compounds were detected in the extract of the young xylem.

Breakdown of UDP-[^{14}C]glucose during extraction and purification. To test the breakdown and possible losses of native UDP-glucose during the lengthy purification procedure, 1 μCi of UDP-[U- ^{14}C]glucose was added to the initial extracts. The total amount of label recovered from the primary wall tissue was difficult to calculate as the UDP-sugar fraction was

Table 5. *Sugars liberated by the action of purified snake-venom phosphodiesterase and alkaline phosphatase on the sugar nucleotides extracted from the young xylem of Larix decidua*

Base	Sugar identified	Percentage of total sugar for each base	Derivation (fractions in Table 4)
Uracil	Glucose	73.2	3-III
	Galactose	9.8	
	Arabinose	1.5	
	Xylose	2.4	
	Fructose	13.2	
Guanine	Glucose	46.7	5-I-a
	Mannose	42.0	
	Galactose	11.3	
Adenine	Glucose	53.1	4-III, 4-IV-c, 4-V-a
	Ribose*	31.0	
	Galactose	5.9	
	Fructose	10.0	

* Ribose is liberated from ADP-ribose and ADP-ribose phosphate, which are the acid breakdown products of NADH and NADPH respectively (Papenburg, 1961).

widely spread in the column eluate. Only approx. 30% of the original label added could be accounted for in the main UDP-sugar fraction. When the amounts of UDP-sugars appearing in other peaks are included in the calculation the amount of label recovered rises to almost 40%.

The estimation of recovery with the secondary wall fraction was somewhat easier as the UDP-sugar derivatives were eluted in one peak from the Sephadex column. The 20.5 μ mol of UDP-sugar found in fraction 3-III contained 44% of the added label. When the UDP-sugar fractions 2-II, 3-IV-a and 4-II were combined and measured, the recovery of labelled UDP-[14 C]glucose rose above 52%.

DISCUSSION

Enzyme inactivation and extraction of the soluble nucleotides with boiling solvents, although widely used in earlier investigations, has more recently to a great extent been replaced by a variety of low-temperature acid treatments. Plant phosphatases are known to be almost inactive in organic solvents at a low temperature (-25°) and having a pH less than 3 (Bialeski, 1964), but until the point of denaturation the activity of these enzymes increases considerably at raised temperatures. Preliminary experiments in 1967 on the extracts of cambial tissue of both European larch and beech (*Fagus sylvatica* L.) showed that extraction with boiling alcohol resulted not only in considerable breakdown of labile phosphate esters but also in the extraction of large amounts of non-nucleotide material that could not be successfully separated from the nucleotides.

The low-temperature acid treatment chosen involved the use of formic acid as the salt formed on neutralization could theoretically be removed by

vacuum sublimation. Both the trichloroacetic acid and perchloric acid procedures used by other authors were considered to be less satisfactory in this respect. Precipitation of potassium perchlorate on neutralization of perchloric acid with potassium hydroxide could result in the co-precipitation of some nucleotides. The removal of trichloroacetic acid with ether is a lengthy procedure when large quantities of material are involved and cannot be carried out at a temperature less than 0°C , which could conceivably result in hydrolysis of acid-labile phosphate bonds. The method using formic acid as described in this work results in the isolation of only small amounts of monophosphates (preliminary experiments in 1967), but the removal of the salt formed on neutralization does present serious difficulties. Methods of desalting by gel filtration and ion retardation were tested, but proved to be unsatisfactory when large amounts of salt and only comparatively small amounts of nucleotides are present. The classical method of desalting on activated charcoal is known to give variable recoveries of the nucleotide di- and triphosphates (Fric & Haspelová-Horvatovicová, 1967). In preliminary experiments with the charcoal method good recovery of the nucleotide monophosphates and UDP-sugars was noted, but with some adenosine sugar nucleotides the amount of material irreversibly attached to the charcoal was consistently as high as 75%. Several different eluting agents were tested, including aq. acetone and pyridine, but the substances remained adsorbed to the charcoal. In addition, no guanine derivatives were detected in the preliminary experiments and the concentration of these substances in the extract of young xylem cells investigated in 1968 was very much decreased after charcoal treatment. From the above it is clear that the employment of charcoal in desalting

operations will seriously influence the quantitative relation of nucleotides with differing affinity to this medium. In some investigations the total amounts of nucleotides are corrected for the loss on charcoal (Brown, 1962, 1965), but it was clear that in the present investigation a series of standard experiments determining the recoveries of all derivatives finally identified would have been necessary before any correction factor could have been applied. Both adenosine and guanosine sugar nucleotides were more strongly adsorbed than were the unsubstituted derivatives of these bases; this may give rise to the fact that these substances are less frequently identified in plant extracts where they are almost certainly present in much smaller amounts than the uridine derivatives.

Conflicting evidence exists in the literature about the effect of removal of the salt by vacuum sublimation. The neutral sugar fraction irrigated from the Dowex 1 column before the elution of the nucleotide material contained 17% of labelled [^{14}C]glucose originally added as UDP-[^{14}C]glucose. This breakdown could account for 13% of the UMP found, assuming that the sugar nucleotide was broken down to the monophosphate. As the amount of UDP isolated was small and as the amounts of monophosphate compounds found after desalting the Dowex peak 6, which contained the nucleoside triphosphates, were high, it is thought that breakdown did in fact proceed as far as the monophosphate. Fig. 1 shows that no great increase occurred in the main di- and tri-phosphates zone (peaks 6 and 7 of the Dowex 1 chromatogram) in the extract of the young xylem cells compared with the extract of the cambial cells. It is, however, clear that, even allowing for the breakdown during desalting of the extract from the cambial tissue, considerably greater amounts of nucleoside monophosphates occurred in this extract than of those found in the extract of the young xylem cells. This is in accordance with the results of other authors investigating the nucleotides in young tissue (Ginsburg, Stumpf & Hassid, 1956; Elnaghy & Nordin, 1966; Cherry & Hageman, 1960).

An increase in the amounts of nicotinamide nucleotides occurred in the secondary wall fraction compared with the primary wall fraction. A small amount of ADP-ribose initially derived from NADH (Papenburg, 1961) was found in the primary wall fraction, but neither NAD nor NADP was detected chromatographically. The small peak 2 (Fig. 1) from the secondary wall fraction was identified as a mixture of NAD and NADP with considerably more of the former. The ADP-sugar fraction isolated from the secondary wall material contained 30% ADP-ribose and ADP-ribose phosphate. The phosphate determination indicated that more ADP-ribose than ADP-ribose phosphate, i.e. that originally more NADH than NADPH was present. The rise in these

substances in the young xylem tissue is more probably connected with the production of lignin than with the biosynthesis of cell-wall polysaccharides.

The nucleotide-bound monosaccharides found in the cambial tissue and in the young xylem cells were mainly uridine derivatives. Smaller amounts, however, of both guanosine and adenosine derivatives were also present. Glucose and galactose were found as components of uridine, guanosine and adenosine nucleotides. This raises the question of whether the formation of different species of glucose- and galactose-containing oligo- and poly-saccharides proceeds via different nucleotide donors. The question can be regarded as partly settled for glucose, as ADP-glucose is generally regarded as the main glycosyl donor in starch formation, whereas UDP-glucose is connected with sucrose and probably also with cellulose synthesis. It could be imagined that both GDP-glucose and GDP-mannose play a role in the biosynthesis of glucomannan, or together with GDP-galactose in the production of galactoglucomannan. UDP-galactose has been shown to be the precursor of galactinol and also of galactans (Frydman & Neufeld, 1963; McNab, Villemez & Albersheim, 1968). Xylose and arabinose have been found in this study only as components of the uridine nucleotide fraction and are most probably connected with the synthesis of arabinoxylans. UDP-arabinose and UDP-galactose may be concerned with biosynthesis of arabinogalactans. Two interesting nucleotide monosaccharides that were present in the primary as well as the secondary wall fractions are UDP- and ADP-fructose. ADP-fructose has not previously been isolated from plant tissue, but both GDP-fructose (Selvendran & Isherwood, 1967) and UDP-fructose (Gonzalez & Pontis, 1963; Umemura, Nakamura & Funahashi, 1967; Brown & Mangat, 1967; Sander-mann & Grisebach, 1968) have been found in higher plants. In two instances the latter has been isolated from tissue where it is thought to play a role in the formation of fructosans (dahlia tubers and Jerusalem artichoke). However, this nucleotide has also been isolated from the leaf nodes of *Impatiens balsamina*, from germinating pea seeds (Brown & Mangat, 1967) and from parsley plants (Sander-mann & Grisebach, 1968), where its function is not clear. It may possibly play a role in the biosynthesis of sucrose.

The nucleotide oligosaccharides found in this study have been tentatively identified as UDP-cellobiose, an UDP-arabinose and glucose-containing oligosaccharide and a GDP derivative containing glucose and mannose. Hitherto similar compounds have been isolated from animal tissue (Gabriel & Ashwell, 1962; Strominger, 1962; Suzuki, 1962), where they are thought to function in the production of complex polysaccharides. It can be imagined that nucleotide oligosaccharides either serve as primers in the production of polysaccharides or that they have the

function of incorporating certain sequences, especially in heteropolysaccharides, as 'prefabricated sections'. This could well be true for the GDP derivative tentatively identified in this study.

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REFERENCES

- Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. L. (1967). *Carbohydr. Res.* **5**, 340.
- Bialeski, R. L. (1964). *Analyt. Biochem.* **9**, 431.
- Brown, E. G. (1962). *Biochem. J.* **85**, 633.
- Brown, E. G. (1965). *Biochem. J.* **95**, 509.
- Brown, E. G. & Mangat, B. S. (1967). *Biochim. biophys. Acta*, **148**, 350.
- Caputto, R., Leloir, L. F., Cardini, C. E. & Paladini, A. C. (1950). *J. biol. Chem.* **184**, 333.
- Cherry, J. H. & Hageman, R. H. (1960). *Pl. Physiol., Lancaster*, **35**, 343.
- Cole, C. V. & Ross, C. (1966). *Analyt. Biochem.* **17**, 526.
- Descimon, H. (1967). *Bull. Soc. Chim. biol.* **49**, 452.
- Dische, Z. & Borenfreund, E. (1957). *Biochim. biophys. Acta*, **23**, 639.
- Elnaghy, M. A. & Nordin, P. (1966). *Archs Biochem. Biophys.* **113**, 72.
- Feingold, D. S., Neufeld, E. F. & Hassid, W. Z. (1964). In *Modern Methods of Plant Analysis*, vol. 7, p. 474. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Springer-Verlag.
- Fischer, F. G. & Dörfel, H. (1954). *Hoppe-Seyler's Z. physiol. Chem.* **297**, 164.
- Fiske, C. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Foster, A. B. (1952). *Chem. Ind.* p. 1050.
- Fric, F. & Haspelová-Horvatovicová, A. (1967). *Phytochemistry*, **6**, 633.
- Frydman, R. B. & Neufeld, E. F. (1963). *Biochem. biophys. Res. Commun.* **12**, 121.
- Gabriel, O. & Ashwell, G. (1962). *J. biol. Chem.* **237**, 1400.
- Gerlach, E. & Döring, H. J. (1955). *Naturwissenschaften*, **42**, 344.
- Ginsburg, V., Stumpf, P. K. & Hassid, W. Z. (1956). *J. biol. Chem.* **223**, 977.
- Gonzalez, N. S. & Pontis, H. G. (1963). *Biochim. biophys. Acta*, **69**, 179.
- Gordon, H. T., Werum, L. N. & Thornburg, W. W. (1964). *J. Chromat.* **13**, 272.
- Hassid, W. Z. (1967). *A. Rev. Pl. Physiol.* **17**, 253.
- Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
- Jenner, C. F. (1968). *Pl. Physiol., Lancaster*, **43**, 41.
- Kieffer, F. & Egli, R. H. (1966). *Z. analyt. Chem.* **221**, 416.
- Larson, P. R. (1969). *Holzforschung*, **23**, 17.
- Leloir, L. F. (1964). *Biochem. J.* **91**, 1.
- Leloir, L. F. & Cardini, C. E. (1957). In *Methods of Enzymology*, vol. 3, p. 840. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Letham, D. S. (1965). *J. Chromat.* **20**, 184.
- McNab, J. M., Villemez, C. L. & Albersheim, P. (1968). *Biochem. J.* **106**, 355.
- Meier, H. (1964). In *Formation of Wood in Forest Trees*, p. 137. Ed. by Zimmerman, M. W. New York: Academic Press Inc.
- Meier, H. & Wilkie, K. C. B. (1959). *Holzforschung*, **13**, 177.
- Nakanishi, Y., Shimizu, S., Takahashi, N., Sugiyama, M. & Suzuki, S. (1967). *J. biol. Chem.* **242**, 967.
- Niemierko, W. & Krzyzanowska, M. (1967). *J. Chromat.* **26**, 424.
- Nordin, J. H. & Kirkwood, S. (1965). *A. Rev. Pl. Physiol.* **16**, 393.
- Paladini, A. C. & Leloir, L. F. (1952). *Biochem. J.* **51**, 426.
- Papenburg, K. (1961). *Zur Bedeutung der freien Nukleotide*, p. 25. Berlin: Springer-Verlag.
- Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
- Pontis, H. G., Cabib, E. & Leloir, L. F. (1957). *Biochim. biophys. Acta*, **26**, 146.
- Randerath, K. & Randerath, E. (1964). *J. Chromat.* **16**, 111.
- Randerath, K. & Randerath, E. (1965a). *Analyt. Biochem.* **12**, 83.
- Randerath, K. & Randerath, E. (1965b). *Analyt. Biochem.* **13**, 575.
- Sandermann, H. & Grisebach, H. (1968). *Eur. J. Biochem.* **6**, 404.
- Selvendran, R. R. & Isherwood, F. A. (1967). *Biochem. J.* **105**, 723.
- Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, 2nd ed., p. 250. London: W. Heinemann (Medical Books) Ltd.
- Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
- Strominger, J. L. (1962). *J. biol. Chem.* **237**, 1388.
- Strominger, J. L. (1964). *Biophys. J.* **4**, 139.
- Suzuki, S. (1962). *J. biol. Chem.* **237**, 1393.
- Thornber, J. P. & Northcote, D. H. (1961a). *Biochem. J.* **81**, 449.
- Thornber, J. P. & Northcote, D. H. (1961b). *Biochem. J.* **81**, 455.
- Thornber, J. P. & Northcote, D. H. (1962). *Biochem. J.* **82**, 340.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Umemura, Y., Nakamura, M. & Funahashi, S. (1967). *Archs Biochem. Biophys.* **119**, 240.
- Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.