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# The Constituents of Conifer Needles

## I. Low Molecular Weight Carbohydrates in the Needles of *Pinus silvestris*

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An investigation has been made of the low-molecular weight carbohydrates in needles collected from *Pinus silvestris*. Glucose, fructose, sucrose, melibiose, raffinose, *myo*-inositol and pinitol, the principal carbohydrates, were isolated together with smaller amounts of cellobiose, laminaribiose, a glucopyranosyl-fructose and a glucopyranosyl-mannose. Oligosaccharides were found which contained residues of glucose, galactose and fructose. Paper chromatography indicated the presence also of small amounts of galactose, arabinose and xylose.

In the present investigation a study has been made of the low-molecular weight carbohydrates present in the needles of *Pinus silvestris*, which had been collected in October. Glucose, fructose, sucrose, raffinose and pinitol were previously found [1,2] in conifer needles and the present investigation was made to get information on their amounts and on other components, some of which might be of interest in connection with the biosynthesis of polysaccharides.

By treating the needles successively with ethanol and methanol, a mixture of neutral carbohydrates was obtained. It was fractionated on carbon columns and sub-fractionated by chromatography and by electrophoresis on thick filter paper. The values for the monosaccharides were determined quantitatively following paper chromatographic separation while the other values are for these substances which were isolated in a pure state (See Table 1).

*Myo*-inositol, pinitol, melibiose, sucrose and raffinose crystallised. From a mixture containing glucose, fructose and some of the pinitol, the two monosaccharides were isolated as their *iso*propylidene derivatives. Smaller amounts were isolated of four disaccharides and of two higher oligosaccharides; the latter two gave glucose, galactose and fructose on hydrolysis. Galactose, arabinose and xylose were detected on paper chromatograms and also smaller amounts of compounds having higher $R_F$-values.

The four minor disaccharides were investigated by paper chromatography and electrophoresis and by studying the products obtained, both when they were hydrolysed and when they were reduced with borohydride prior to hyd-

*Table 1.* Low-molecular weight carbohydrates in the needles of *Pinus silvestris.* The amounts are given as the percentages of the dry weight of the needles.

| | |
|---|---|
| Arabinose | 0.06 |
| Xylose | 0.02 |
| Fructose | 2.5 |
| Galactose | 0.03 |
| Glucose | 2.2 |
| Melibiose | 0.3 |
| Sucrose | 1.7 |
| Raffinose | 0.8 |
| Other oligosaccharides | < 0.1 |
| *Myo*-inositol | 0.3 |
| Pinitol | 2.0 |

rolysis. By these methods two of the disaccharides were shown to be indistinguishable from laminaribiose and cellobiose. The other two were found to be reducing, and it was concluded that they were a glucopyranosyl-mannose and a glucopyranosyl-fructose.

It was possible that a portion of the sugars might have been formed from higher oligosaccharides by hydrolysis during either the extraction or the purification. To determine whether or not this was the case, a sample of the purified extract, which had not been passed through a carbon column, was examined on chromatograms. There it was compared with samples of non-purified extracts obtained by treating needles immediately after their removal from the same tree. In one case, cold ethanol was used to minimise the possibility of acid hydrolysis and, in the other, boiling ethanol was used to inactivate the enzyme systems as rapidly as possible. Those two extracts were similar; glucose, fructose, sucrose, melibiose and raffinose were present as the principal sugars. Glucopyranosyl-fructose was also shown to be present in those extracts as, when they were concentrated on the chromatograms the disaccharide gave an intense red spot on treatment with resorcinol in acid. There were slight indications of reactions with *p*-anisidine hydrochloride at those positions on the chromatogram where the other, and minor, disaccharides might be. On the other hand the examination showed that, under the mild conditions used for extraction and purification, some of the sucrose and raffinose had been hydrolysed.

The investigation will be repeated on a larger scale to obtain further informations on certain of the substances present.

## EXPERIMENTAL

All melting points were corrected. Solvents were removed either by distillation, carried out under reduced pressure (bath temperature under 40°), or by lyophilisation. Whatman No. 1 filter paper was normally used for paper chromatography and electrophoresis, but large scale separations were carried out on Whatman No. 3 MM paper, which had previously been thoroughly washed with water. Reagents used for the detection of substances on chromatograms and electrophoretograms were silver nitrate-ethanolic sodium hydroxide, *p*-anisidine hydrochloride and resorcinol-hydrochloric acid.

The chromatographic solvents and the buffer used in the electrophoresis were:
A — Ethyl acetate — acetic acid — water, 3:1:3 (the upper phase).
B — Butanol — ethanol — water, 10:3:5.
C — Butanol — pyridine — water, 6:2:3.
D — Borate buffer[3] pH 10.0, 0.1 M.

5-Hydroxymethylfurfural was used to enable correction to be made for electroendosmotic movement. This compound is readily detected with all the sprays used in this investigation.

The needles were collected at the end of October from all parts of the branches. The age of the tree was about 20 years. The fresh needles (540 g, corresponding to a dry weight of 230 g) within a few hours of their having been collected were cut into pieces (*ca.* 1 cm) and were boiled with ethanol (5 l) for 30 min. The mixture, when cold, was filtered and the residue washed with ethanol. The residual material was air-dried, disintegrated in a Wiley-mill and then treated with methanol (3 × 24 h) in a Soxhlet extractor. The final extract, which was almost colourless, yielded only 1.9 g of material on removal of the solvent. The ethanolic extracts, the wash liquids and the three methanolic extracts were examined chromatographically and found to be similar. They were combined and concentrated to *ca.* 400 ml. Water (200 ml) was added and the solution was shaken with successive volumes (400 ml) of chloroform. After removal of the solvent from an aliquot of the aqueous layer, the total soluble non-volatile material was found to be 44 g. The aqueous layer was deionised by passing it through columns containing Amberlite IR 120 and IR 4B. The eluate was evaporated to about 500 ml and to the solution there was added ethanol to give a final concentration of 50 % with respect to the ethanol. This solution was shaken for 16 h with a mixture of charcoal and Celite (1:1; 150 g). After filtration and washing of the residues, the filtrate and washings were concentrated and yielded a colourless product (32.1 g). Part of this product was separated on paper chromatograms (Solvents A and C) and the monosaccharides were quantitatively determined by the method of Saeman *et al.*[4] *Glucose* and *fructose* were the principal sugars and there were smaller amounts of *galactose*, *arabinose* and *xylose* (see Table 1). The products were examined on paper chromatograms and there compared with the components in two other extracts from the needles of the same tree. One of those extracts was obtained by treating the needles with cold ethanol for a day at room temperature, the other, by boiling the needles with ethanol for 30 min. The extracts were not purified. Appropriate volumes of the two solutions were concentrated directly on the starting-line of the chromatogram by a stream of cold air.

The main part of the purified product (31.8 g) was fractionated on a carbon-Celite column (50 × 6 cm), using the gradient-elution technique (ethanol: 0—20 %, 15 l, and 50 %, 10 l). The fractions, after examination on paper chromatograms, were then grouped and combined to give two main fractions. Fraction I contained most of the monosaccharides and inositols (17.8 g) while fraction II contained mainly oligosaccharides (9.8 g). Fraction II was refractionated on the same column (water, 4 l; ethanol 0.30 %, 14 l; and ethanol 50 %, 6 l). The first sub-fractions (1.33 g) from it contained monosaccharides and inositols and they were combined with fraction I. The later sub-fractions from fraction II on chromatographic and electrophoretic examination were found to contain compounds indistinguishable from melibiose, sucrose and raffinose (stated in the order in which they emerged from the column). A portion of each of these sugars was crystallised from aqueous ethanol; each sugar was crystallised from that fraction in which it was present in its highest concentration. In order to estimate the total amounts of the individual sugars the mother liquors and fractions containing smaller sugar concentration, or aliquots of them, were evaporated to dryness, and the material redissolved and separated on thick filter papers using solvent A. The various sugars were eluted from the papers, the eluate evaporated to dryness, and the residue weighed. Two disaccharides (I and II), which had been eluted from the carbon column together with melibiose, and two (III and IV), which had been eluted together with sucrose, were separated by pairs on the paper chromatograms. Smaller amounts of two other oligosaccharides (*ca.* 25 mg of each), probably a tetra- and pentasaccharide, were also separated on the chromatograms. They gave galactose, glucose and fructose on hydrolysis with N sulphuric acid (100°, 2 h).

Pure fractions of the four disaccharides (about 10—30 mg of each) were obtained by electrophoresis of the individual pairs of disaccharides on thick filter papers. The sugars were eluted from the papers with N acetic acid and, after having deionised the eluate, it was evaporated to dryness. Each of the disaccharides would appear to have a glycosidically-linked pyranose residue as they were all but little affected by those hydrolysis conditions which led to complete hydrolysis of sucrose, that is, by treatment with 0.1 N hydrochloric acid at 100° for 5 min.

The disaccharides I and III, on hydrolysis with N sulphuric acid (100°, 2 h) yielded glucose only. They were indistinguishable from *laminaribiose* and *cellobiose*, respectively, on both paper chromatograms and electrophoretograms, but were distinguishable from nigerose, maltose, *iso*maltose and gentiobiose.

Disaccharide II ($M_G{}^3 = 0.52$) on hydrolysis yielded approximatively equimolar amounts of glucose and mannose. Reduction with borohydride, and hydrolysis of the reduction product yielded glucose as the only reducing sugar. The results obtained indicated that it was a *glucopyranosyl-mannose.*

Disaccharide IV ($M_G{}^3 = 0.62$) on hydrolysis yielded approximately equimolar amounts of glucose and fructose. It was reduced and hydrolysed as above and then gave glucose as the only reducing sugar. The results obtained indicated that disaccharide IV was a *glucopyranosyl-fructose.* It was distinguishable both chromatographically and electrophoretically from turanose.

The disaccharide II had the following $R_{\text{Glucose}}$ -values: 0.48 (A), 0.49 (B) and 0.57 (C) and the disaccharide IV: 0.30 (A), 0.29 (B) and 0.34 (C).

*Melibiose* (0.40 g) crystallised as the dihydrate (m. p. 75—76°) and was characterised as melibiitol, obtained by borohydride reduction. It had $[\alpha]_D^{22}$ +111 (*c*, 2 in water) and m. p. 173—175°, not depressed on admixture with an authentic sample.

*Sucrose* (2.82 g) was recrystallised from aqueous ethanol. It had $[\alpha]_D^{22}$ +66 (*c*, 2 in water) and m. p. 183—185°.

*Raffinose* (1.38 g) was recrystallised from aqueous ethanol. It had $[\alpha]_D^{21}$ +106 (*c*, 2 in water; literature: raffinose pentahydrate $[\alpha]_D^{20}$ +105), m. p. 135—139°, as had both an authentic sample of raffinose pentahydrate and a mixture of the two samples. Examination in polarised light revealed that the material lost its crystalline properties at 76—80° due to dehydration. The shape of the material, however, remained unaltered until the solid collapsed at 135—138°. Hydrolysis with 0.1 N hydrochloric acid (100°, 5 min) gave melibiose and fructose.

From the combined fractions containing the monosaccharides and inositols (19.33 g), a sample (4.00 g) was taken and fractionated on a smaller carbon-Celite column (32 × 4 cm) using the gradient-elution technique (water, 3 l; ethanol 0.5 %, 4 l, and 50 %, 3 l).

*Myo-inositol* (0.15 g) was obtained from the first fractions. It was chromatographically and electrophoretically indistinguishable from an authentic sample and after recrystallisation from aqueous ethanol it had m. p. and mixed m. p. 228—231°.

By paper chromatography and electrophoresis certain of the fractions were shown to contain glucose, fructose and pinitol, as principal components, and smaller amounts of *myo*-inositol, galactose, arabinose and xylose. Those combined fractions were taken to dryness and the residues dissolved in aqueous ethanol. Part of the *pinitol* (0.72 g) crystallised and after recrystallisation from the same solvent it had m. p. and mixed m. p. 189—190° and $[\alpha]_D^{22}$ +64 (*c*, 2, water). The material in the mother liquors was treated with acetone and sulphuric acid to convert the sugars to their di-*iso*propylidene derivatives while leaving the remaining pinitol unaffected. A further crop of crystalline pinitol was obtained (0.21 g) and by following the partial hydrolysis technique of Bell[5], crystalline *iso*propylidene derivatives of glucose and fructose were also isolated. *1,2-*O-iso*propylidene-glucofuranoside*, on recrystallisation from ethyl acetate, had m. p. 160—161° (lit. 161—162.5) and *2,3-4,5-di (*O-iso*propylidene)-fructo-pyranoside*, on recrystallisation from petrol ether, had m. p. 95—96° (lit. 95—96).

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