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POLYSACCHARIDES IN POLLEN. I. INVESTIGATION OF MOUNTAIN PINE (*PINUS MUGO* TURRA) POLLEN

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Abstract—Extraction of pollen grains of mountain pine (*Pinus mugo* Turra) with monoethanolamine, water and alkali and subsequent fractionation of the extracts on DEAE cellulose columns yielded three different polysaccharides: namely (a) one containing D-xylose and D-galacturonic acid residues in the approximate proportion 1:2; (b) one containing 7–10% glucuronic acid and, of neutral sugars as monosaccharides, roughly 80% L-arabinose, 15% D-galactose and 3–4% L-rhamnose; (c) one made up essentially from (1→3)-linked β-D-glucopyranose residues. The presence of starch as well as at least one more heteropolysaccharide is indicated.

INTRODUCTION

POLLEN grains from a large number of monocotyledonous and dicotyledonous plants, e.g. *Ambrosia* (ragweed) and *Solidago* (golden-rod) (Compositae) and *Phleum* (timothy grass), *Dactylis* (cocksfoot grass) and *Secale* (rye), (Gramineae) are well known to induce allergic reactions (hay fever). The main part of the work done on the high molecular weight constituents of pollen has therefore been devoted to the isolation and characterization of the actual allergens. These have the nature of proteins but contain in some instances significant amounts of carbohydrates. Thus Goldfarb *et al.*[1] report the isolation from giant ragweed pollen of an antigen, Trifidin A, that appears to be a protein–carbohydrate complex of which the carbohydrate moiety is essentially an arabinan. Johnson and Thorne[2] have isolated from rye-grass a protein–carbohydrate complex containing 60% mannose and 35% protein.

Except for starch, which has been demonstrated in several instances by staining with iodine, there are few reports on true polysaccharides in pollen. Mizuno[3] carried out a series of extractions of *Cryptomeria japonica* pollen and found arabinose, galactose, glucose, rhamnose and galacturonic acid in hydrolysed samples of the extracts. Gladyshev[4] has reported the isolation of a component of the mucoprotein type from corn (*Zea mays*) containing 11% hexosamine and 4·7% mannose and glucose or galactose.

When pollen grains are immersed in water or other polar solvents they swell considerably. The swelling is caused by a diffusion of solvent into the intine, the layer between the cytoplasm and the outer part of the pollen wall (exine). The intine appears to have no simple morphology.[5] In botanical literature the swelling of the intine is related to its content of "pectic substances" as indicated by various staining reactions (i.e. with ruthenium red)[5,6] which however are generic for acidic polysaccharides. No strict chemical evidence has been presented for the presence in pollen of any polysaccharide included in the term "pectic sub-

1 A. R. GOLDFARB, A. K. BHATTAKHARYA and S. K. KOERNER, *J. Immunol.* **81**, 302 (1958).
2 P. JOHNSON and H. V. THORNE, *Intern. Arch. Allergy Appl. Immunol.* **13**, 257 (1958).
3 T. MIZUNO, *Nippon Kagaku Zasshi.* **79**, 192 (1958); *CA*, **53**, 22288 (1959).
4 B. N. GLADYSHEV, *Biokhimiya* **27**, 240 (1962).
5 P. MARTENS and L. WATERKEYN, *La Cellule* **62**, 173 (1962).
6 H. BAILEY, *J. Arnold Arboretum* (*Harvard Univ.*) **41**, 141 (1960).

stances".[7] Discreet parts of the intine of pollen from various *Pinus* species is stained by aniline blue.[5] This dye and resorcin blue specifically stain "callose", a type of material that occurs commonly in plant tissues but always in small quantities.[8] Aspinall and Kessler[9] examined callose isolated from grapes (*Vitis vineifera*) and demonstrated that it consisted of a linear (1 $\rightarrow$ 3)-linked β-glucan.

RESULTS AND DISCUSSIONS

The subject of the present investigation, the mechanism of the swelling that precedes the germination of pollen grains, was suggested by Professor G. Erdtman, Palynologiska Laboratoriet, Solna, Sweden. The present paper deals with the polysaccharide constituents of pine pollen intine. The pollen, from mountain pine (*Pinus mugo* Turra) cultivated as a dune-binder in southern Sweden, was generously supplied by AB Cernelle, Vegeholm, Sweden. It was extracted in 100-g batches, first with cold and then with hot monoethanolamine as used

TABLE 1. PROPORTIONS OF MAIN NEUTRAL SUGARS IN EXTRACTS FROM *Pinus mugo* POLLEN

Extract	Yield* (gm)	% of N	% of Galactose	% of Glucose	% of Arabinose	% of Xylose
A (ethanolamine)	5·7	6·2	16	11	38	35
B (water)	3·0	6·5	12	6	77	5
C (7% NaOH)	1·0	—	15	12	68	5
D (10% KOH)	3·0	0·7	12	8	78	2
E (insoluble)	4·1	1·0†	—	98†	2†	—

* Per 100 g pollen.
† After purification

by Bailey[6] who claimed that it acts as a selective solvent for sporopollenine, the main constituent of the exine. The aim was to make the polysaccharides more accessible for subsequent extraction by a removal of the exine. However, the exine was quite resistant even towards hot ethanolamine, but on the other hand a considerable amount of high molecular weight material went into solution (extract A; 5·7 per cent after reprecipitation from ethanol). The pollen was then successively extracted at room temperature with water, 7% NaOH and 10% KOH(extracts B, C, D; 3·0, 1·0 and 3·0 per cent of reprecipitated material respectively). On the reprecipitation of the extracts some material failed to redissolve. This (E) amounted to 4·1 per cent of the pollen.

The pollen grains underwent considerable structural changes during the extractions. The intine became separated from the exine bag during the ethanolamine and cold water extractions and was in several instances extruded from that bag. It had then lost only little of its original thickness and consisted of an outer striated and an inner non-striated layer. After subsequent treatment with hot water or with cold 7% NaOH only the inner layer remained and this under the microscope was apparently homogeneous. At this stage the pollen still was a powder. The following extraction with 10% KOH made it quite slimy to the touch.

[7] R. L. WHISTLER and C. L. SMART, *Polysaccharide Chemistry*, p. 162. Academic Press, New York (1953).
[8] W. ESCHRISCH, *Protoplasma* 47, 487 (1956).
[9] G. O. ASPINALL and G. KESSLER, *Chem. & Ind.* (*London*) 1296 (1957).

Examination under the microscope showed that the remaining part of the intine had dissolved completely leaving the cytoplasm as a physically fairly intact entity.

It was found that the ethanolamine reacted with some acidic groups in the intine polysaccharides, forming N-(β-hydroxyethyl) amide groups. To avoid this, N,N-dimethyl formamide was tried for extraction, since this solvent causes swelling of the pollen to a similar extent as does ethanolamine. The total yield obtained, however, after carrying out the whole extraction series was less than with ethanolamine and furthermore, no pure polysaccharide could be isolated by subsequent fractionation. The addition of 5% ethanolamine to the dimethylformamide had virtually no advantageous effect.

The proportions of the main neutral sugars in hydrolysed samples of the extracts are given in Table 1. There were also small amounts of rhamnose (< 2%) along with traces of ribose (in B) and fucose (in C and D). A and B contained around 6% nitrogen, C and D less than 1 per cent. The figures given in the Table for A and B are probably not very accurate as considerable browning and humification occurred during the hydrolyses.

TABLE 2. FRACTIONATION ON DEAE CELLULOSE COLUMNS OF EXTRACTS FROM *Pinus mugo* POLLEN (YIELDS IN MG)

Fraction	Eluant (M KOAc)	Extract and weight used			
		A (5·65 g)	B (3·02) g)	C (0·92 g)	D (3·00 g)
1	0	56	244	212	548
2	0·02	1162	301	12	156
3	0·04	786	104	16	174
4	0·08	759	337	156	1064
5	0·12	867	—	274	415
6	0·20	284	262	81	317
7	1·0	—	120	29	175

Fractions were combined and refractionated where required to give polysaccharides (a), (b), (c) and (d).

The extracts were fractionated on columns of diethylaminoethyl (DEAE) cellulose[10] using for elution first water and then aqueous potassium acetate of increasing concentration. The following polysaccharides could be distinguished on examination of the various fractions:

(a) A polysaccharide containing xylose as the only neutral reducing sugar. It was present essentially in the ethanolamine extract and was eluted from the DEAE cellulose column by 0·02 M potassium acetate.

(b) A polysaccharide containing neutral sugars consisting of roughly 80–85% arabinose together with galactose and rhamnose. It contained also uronic acid, 7–10 per cent in different preparations. The main part of this polysaccharide was eluted by 0·08 and 0·12 M potassium acetate. The 0·04 M eluates contained only small amounts of arabinose-containing polysaccharides.

(c) A polysaccharide fraction obtained from the alkali extracts. It was eluted from the DEAE cellulose column by water and contained galactose and glucose (36%), arabinose (39%), xylose (25%) along with a small quantity of a sugar tentatively identified as fucose by paper chromatography. Rhamnose and uronic acid were virtually absent.

[10] H. NEUKOM, H. DEUEL, W. J. HERI and W. KÜNDIG, *Helv. Chim. Acta* **43**, 64 (1960).

(d) A polysaccharide containing mainly glucose and isolated as an insoluble residue on reprecipitation of the different extracts. It was soluble in 90% formic acid and 1 M NaOH but insoluble in water. It was heavily contaminated by non-carbohydrate material.

The xylose-containing polysaccharide (a) had $[\alpha]_D + 169°$ and contained 4·17% nitrogen. D-Xylose and D-galacturonic acid were formed on complete hydrolysis for which 2 M HCl at 100°C for 22 hr was required. The hydrolysate contained also a ninhydrin-active component which was identified as monoethanolamine by paper chromatography in four different solvent systems, which suggests the presence in the unhydrolysed polysaccharide of N-(β-hydroxyethyl)-D-galacturonosyl amide residues.

An attempt to determine the amount of reducing sugars in a hydrolysate by hypoiodite oxidation was not successful as there was a marked overconsumption of hypoiodite, especially when HCl was used for the hydrolysis. This can probably be related to the considerable humification that accompanied the hydrolysis. The xylose content was 27·0 per cent as estimated according to Saeman *et al.*[11] after adding an internal standard.

The proportion of acidic groups as galacturonic anhydride determined by iodometric titration was 8·4 per cent and by decarboxylation 37 per cent. The content of galacturonic anhydride was tentatively estimated to be $52·3 + 8·4 = 61$ per cent on the assumption that all nitrogen (4·17%) is present as N-(β-hydroxyethyl) amide groups. This assumption is valid when ninhydrin-active nitrogen is concerned and makes it possible to account for 74 per cent of the polysaccharide (excluding xylose).

The polysaccharide was subjected to graded hydrolysis with 0·1 M HCl at 100°, first for 1 hr and then twice for 1·5 hr with intermediate removal of fragments not precipitable with ethanol. A polymeric material was isolated after the last treatment by precipitation with ethanol. It had $[\alpha]_D + 82°$ and contained 3·6% nitrogen. It gave on drastic hydrolysis galacturonic acid and ethanolamine together with small amounts of xylose. The low molecular weight part obtained in the hydrolysis series contained xylose as the main constituent. Small amounts of ethanolamine and ninhydrin-active components of low mobility on chromatograms were also present but xyloöligosaccharides were virtually absent.

A sample of the polysaccharide was kept in 10% KOH containing 1% sodium hydridoborate at 50°C for 3 hr under a nitrogen atmosphere. The product was resolved after deionization into a low and a high molecular weight fraction by precipitation with ethanol. The former contained as only sugars xylitol and traces of xylose. The high molecular weight fraction ($[\alpha]_D + 102°$) when treated in the same way for a further 6 hr furnished another quantity of xylitol. The high molecular weight residue was recovered in a yield corresponding to about 60 per cent of the starting material. It had $[\alpha]_D + 88°$ and contained 2·2% nitrogen. The products of hydrolysis were galacturonic acid and ethanolamine along with small amounts of xylose.

The polysaccharide consumed on oxidation with sodium metaperiodate 0·0448 mmole oxidant per 10 mg with the liberation of 0·0168 mmole formic acid. In a separate experiment a sample of the polysaccharide was oxidized and the derived polyaldehyde reduced with sodium hydridoborate. The polyalcohol was then treated with dil. H_2SO_4 at room temperature overnight by the procedure devised by Smith and coworkers.[12] After deionizing and concentrating the solution a high molecular weight material could be isolated by precipitation with ethanol in a yield that corresponded to 40 per cent of the starting material. It had $[\alpha]_D + 67°$ and contained 5·76% nitrogen and by titration 9·9% anhydrogalacturonic acid. Total hydrolysis gave galacturonic acid and ethanolamine as the only products; xylose was

[11] J. F. SAEMAN, W. E. MOORE, R. L. MITCHELL and M. A. MILLET, *Tappi* 37, 336 (1954).
[12] I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS and F. SMITH, *Abstracts Papers Chem. Soc.* 135, 3D (1959).

virtually absent. Periodate oxidation of this material led to an oxidant uptake of 0·0303 mmole per 10 mg with liberation of 0·0048 mmole formic acid. With the same assumption for the nitrogen content as above the degraded polysaccharide would contain 90% N-(β-hydroxiethyl) anhydrogalacturonosyl amide residues. The oxidation values would then correspond to 0·65 mole/mole of periodate and 0·1 mole/mole of formic acid, i.e. out of ten residues in the degraded polysaccharide there are 5–6 oxidized, one of which liberates formic acid.

The IR-spectrum of the polysaccharide had two strong bands at 1540 and 1640 cm^{-1} characteristic for amide groups. Neither these bands nor a band at 885–890 cm^{-1}, where several β-linked polysaccharides absorb,[13] were affected by periodate oxidation followed by Smith degradation whereas a distinct band at 820 cm^{-1} disappeared completely. The polysaccharide after alkaline degradation had reduced amide absorption and a strong carboxylic absorption at 1720 cm^{-1}. The band at 890 cm^{-1} was unchanged while that at 820 cm^{-1} had almost disappeared. The resolution in the carbonyl region was not good enough to discern any shifts (presumably to slightly lower frequencies[14]) due to the presence of 4,5-unsaturated uronic amide residues expected to be formed on the alkaline hydrolysis of the xylose residues (cf. below).

It is not possible to suggest any unambiguous structure for the polysaccharide from the results presented above. Furthermore, the normal procedure for examination of polysaccharide structures involving analysis by the methylation technique cannot be carried out easily due to the lability of the xylose residues towards alkali. In addition to this the β-hydroxyethyl amide groups add further obvious complications.

Certain structural features are however strongly indicated. The reduction in rotation after removal of the xylose shows that it is present in the α-form. α-Xylosidic residues in polysaccharides have previously been reported only from tamarind seed amyloid[15] where it is combined through a (1→6)-α-linkage to glucose units of the main (1→4)-β-linked chain and substituted in position 2 by a single β-galactopyranose residue. It has also been found as 6-*O*-α-D-xylopyranosyl-D-glucose and *O*-α-D-xylopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose in an enzymic hydrolysate of a pine glucomannan.[16]

The virtual absence of any xyloöligosaccharides among the products from incomplete hydrolysis of the polysaccharide indicates that the xylose occurs mainly as single non-reducing terminal units. This is supported by the results from the periodate oxidation where at least the main part of the xylose is oxidised with liberation of formic acid and by the results from the alkaline degradation. Xylose present as side chains of more than one residue would then give rise to xyloöligomers. No such substances could be detected among the reaction products. The lability of the xylosyl residues towards alkali suggests furthermore that it is attached in β-position to the N-(β-hydroxyethyl) amide groups, that is through a (1→4)-linkage to the galacturonic acid residues.[17] The high proportion of amide groups indicates that aminolysis takes place during the extraction of the polysaccharide with ethanolamine, i.e. that the galacturonic acid residues occur esterified to a high extent in the native state of the polysaccharide.

The results from the periodate oxidation experiments cannot be interpreted in a straightforward way. In the first oxidation all xylosyl residues were oxidized and possibly also some galacturonosyl residues. Removal of the oxidized residues by a Smith degradation liberated

[13] S. A. Barker, E. J. Bourne, M. Stacey and D. H. Whiffen, *J. Chem. Soc.* 171 (1954).
[14] D. Heim and H. Neukom, *Helv. Chim. Acta* **45**, 1735 (1962).
[15] P. Kooiman, *Rec. trav. chim.* **80**, 849 (1961).
[16] O. Perila and C. T. Bishop, *Can. J. Chem.* **39**, 815 (1961).
[17] R. L. Whistler and J. N. BeMiller, *Advances in Carbohydrate Chem.* **13**, 289 (1958).

a new set of oxidizable residues the main part of which contained two adjacent hydroxyl groups only. As the xylosyl residues most probably are linked to the C-4 hydroxyl group of galacturonosyl residues it follows that these must be substituted also in the 2-position. The incomplete oxidation (60 %) of the degraded xylose-free polysaccharide and the small amount of formic acid liberated indicate an alternative linkage, probably (1 → 3) and a linear or only slightly branched structure. From the above it seems as the polysaccharide can be referred to in a rational way as a xylogalacturonan, a type of polysaccharide that to our knowledge has not hitherto been encountered.

Different samples of the rhamnose-containing arabinogalactan (b) eluted by 0·08 M potassium acetate had uronic acid contents of 7·3–8·8 per cent by decarboxylation, nitrogen 0·41–0·43 per cent and rotations between −84 and −89°. A sample eluted by 1·0 M acetate contained 9·7 % uronic acid and 1·7 % nitrogen. The L-arabinose-D-galactose ratio varied in different preparations and fractions between 5:1 and 7:1. As part of the galactose appeared to be combined to uronic acid it is incompletely accounted for in these estimations making the given ratios somewhat high. The L-rhamnose constituted 3–4 per cent of the neutral sugars (cf. Table 3).

Heating the polysaccharide during 3 hr at 100° with 0·01 M HCl stripped off all L-arabinose; a 1-hr treatment was sufficient to hydrolyse 90 per cent of these residues. The L-arabinose residues are thereby more labile to acid hydrolysis than the L-arabinose residues in the arabinogalactans from larch wood.[18] Besides the arabinose there were also hydrolysed some D-galactose and L-rhamnose corresponding to 5 and 1 per cent of the starting material respectively. The high molecular weight residue (17 %) had $[\alpha]_D + 96°$ and contained 30 % uronic anhydride and 1·13 % nitrogen. The increase in optical rotation on removal of the L-arabinose residues indicates that these are present in the α-form in the original polysaccharide. The D-galactose-L-rhamnose ratio in a hydrolysate (conditions not sufficient for complete hydrolysis of aldobiouronic acids) was roughly 7:4. D-Galactose and L-rhamnose were predominant among the neutral products of a stepwise partial hydrolysis of the degraded polysaccharide; oligosaccharides were formed only in minute amounts. An aldobiouronic acid with $[\alpha]_D + 7°$ (barium salt) was obtained in unexpectedly low yield in an experiment on a large scale. It could not be obtained completely pure but paper chromatography showed the main products of hydrolysis to be galactose, glucuronic acid and glucuronolactone.

The polysaccharide consumed 0·0468 mmole oxidant per 10 mg with liberation of 0·0064 mmole formic acid on oxidation with periodate. Smith degradation[12] of the oxidized polysaccharide afforded a material that was precipitated from ethanol in a syrupy form. It contained 74 % L-arabinose, 12 % D-galactose and 13 % L-rhamnose residues. Examination of a hydrolysed sample by paper chromatography gave no certain indication of any uronic acid residues remaining after the oxidation; a sample suitable for estimation of such residues could not be prepared.

The polysaccharide left after the mild partial hydrolysis was also oxidized with periodate. The consumption of oxidant was 0·0851 mmole per 10 mg and the amount of formic acid liberated 0·0372 mmole, which is equivalent to an oxidation of around 80 per cent of the residues.

The results of the above experiments show that the polysaccharide consists of a backbone of D-galactose, L-rhamnose and glucuronic acid. To this backbone side-chains made up essentially from α-L-arabinofuranose residues are attached. The resistance of a large part of the L-arabinose towards periodate indicates that these are combined via (1 → 2)- or (1 → 3)-

[18] H. O. BOUVENG, *Acta Chem. Scand.* 15, 78 (1961).

linkages. The L-rhamnose is protected by substitution from periodate oxidation to a much larger extent than the galactose. The large amount of formic acid liberated on periodate oxidation of the backbone indicates that (1 → 6)-linked or terminal non-reducing residues are preponderant.

Polysaccharides related to that described above are found in that heterogeneous group usually referred to as "plant gums".[19] Painter and Purves[20] have reported the isolation from the inner bark of white spruce (*Picea glauca*) of a polysaccharide with a similarly high arabinose content.

The polysaccharide material (c) eluted by water from the DEAE cellulose column on fractionation of the alkaline extracts C and D formed, in contradistinction to the two polysaccharides described above, very viscous solutions. Attempted fractionation of the material on columns of Sephadex G-75[21] and fractional precipitation from ethanol gave negative results and the nature of the material remains therefore unknown.

The water-insoluble material (d) obtained on reprecipitation of the various extracts yielded on hydrolysis D-glucose, m.p. 145·5–146·5° and $[\alpha]^{22} + 55°$, along with small amounts of L-arabinose. It was heavily contaminated by non-carbohydrate material (nitrogen 11·0 %) and was only partly soluble in 90 % formic acid and 1 M NaOH. Insoluble materials were of essentially non-carbohydrate character. Purification by dissolving in 1 M NaOH, removing the insolubles and reprecipitating by acidification gave a polysaccharide which contained after exhaustive extraction with hot water 0·99 % nitrogen and of sugars 98·3 % glucose and 1·7 % arabinose. The rotation was +10·6° in 1 M NaOH.

The polysaccharide was extensively degraded to glucose by a (1 → 3)-β-glucanase from Basidiomycete QM 806.[22] It gave as main products on partial formolysis a homologous series of oligosaccharides indistinguishable by paper chromatography from the laminaridextrins. The mixture of saccharides was resolved by chromatography on a carbon column and on thick filter papers. L-Arabinose, D-glucose and laminaribiose were crystallized and characterized by melting points and optical rotations. Laminaribiose, -triose and -tetraose were converted into their β-acetates which were characterized by melting points and rotations. The optical rotations of the amorphous laminaridextrins, including laminaripentaose and laminarihexaose were somewhat high due to contamination by small amounts of positively rotating glucoöligosaccharides. These also formed a homologous series, indistinguishable from the maltodextrin series. Of this series maltose was characterized as the β-octaacetate by melting point and rotation. Maltotriose was obtained (by rotation) in approximately 90 per cent purity.

The water-insoluble polysaccharide thus appears to be a linear polysaccharide made up from (1 → 3)-linked β-D-glucopyranose residues. It is difficultly purified and carries along through the purification procedure starch as well as arabinose-containing polysaccharides. It is closely akin to the β-glucan making up the callose in *Vitis vinifera*.[9]

EXPERIMENTAL

Chromatography

Solvent systems: Ethyl acetate, pyridine, water 8:2:1; ethyl acetate, acetic acid, water 3:1:1, water-saturated phenol with and without 1 % ammonia and (for quantitative

[19] F. Smith and R. Montgomery, *The Chemistry of Plant Gums and Mucilages.* Reinhold, New York (1959).
[20] T. J. Painter and C. B. Purves, *Tappi* **43**, 729 (1960).
[21] P. Flodin and K. Granath, *IUPAC Symposium über Macromoleküle*, Wiesbaden 1959.
[22] E. T. Reese and M. Mandels, *Can. J. Microbiol.* **5**, 173 (1959).

estimations) butanol, acetone, water 4:5:1. Papers: Whatman No. 1 and (for quantitative estimations) Munktell 302.

Extraction of Pinus mugo *Pollen*

The air-dry pollen (100 g) was mixed with monoethanolamine (EA, 1000 ml) and the thick suspension was kept at room temperature under nitrogen for 3 hr. Excess EA was removed by suction and, including washings with EA (200 ml), poured into 8 vol. acetone. The oily precipitate was collected by centrifugation, dissolved in water and run into ethanol. The yield after filtering and drying was 0·22 g.

EA (600 ml) was then added to the swollen pollen and the resulting slurry stirred under nitrogen on a steam bath for 1 hr. The extracted material was separated from the dark EA solution as described above. The insoluble residue left on the reprecipitation of the extracted material was collected by centrifugation. The extraction with hot EA was repeated once. The yields were 3·64 and 2·06 g respectively. Chromatographic examination of hydrolysed samples of the three EA extracts showed a similar carbohydrate composition. They were therefore combined, dissolved in water and, after removal of insolubles, reprecipitated to give extract A (5·71 g).

The pollen was washed with water (3–4 vol.) on a Büchner funnel of sintered glass until the washings were colourless. The washings were concentrated and high molecular weight extractives isolated as described above. Yield after reprecipitation 3·04 g (extract B).

The moist pollen was then dispersed in 10% NaOH (500 ml) making the hydroxide concentration ca. 7%. The suspension was stirred under nitrogen for 1 hr at room temperature and then made neutral by adding acetic acid. Concentration and precipitation with ethanol gave 1·05 g (extract C). The pollen was dehydrated with ethanol and ether and then dried in a desiccator.

The pollen residue (52 g) was suspended in 10% KOH (800 ml) and stirred under nitrogen for 1 hr. Upon neutralization (acetic acid) the pollen formed thick clots which had to be dispersed by vigorous stirring before the extract could be removed by suction. Extracted material precipitated from ethanol amounted to 3·04 g (extract D). Water-insoluble material (E) obtained when reprecipitating the various extracts amounted to 4·1 g. The proportions of neutral sugars and the nitrogen contents in the various extracts are given in Table 1.

Fractionation of the Extracts

The extracts were fractionated on columns of Whatman DEAE cellulose. For extracts A, B and D the column size was 5 × 30 cm and, for extract C, 3 × 30 cm. The former column was irrigated with 2000-ml batches of aqueous potassium acetate of increasing concentration and the latter with 1000-ml batches.

The fractionations are summarized in Table 2. The fractions were analysed by paper chromatography of hydrolysed samples. The results were as follows: Fractions A_1 and A_2 contained xylose as dominating constituent. Fractions C_{5-7} and D_{4-7} (in total 2·36 g) contained arabinose as dominating sugar together with galactose, small amounts of rhamnose and traces of xylose. A_{4-6}, B_{4-7}, C_4 and D_3 (in total 2·96 g) contained arabinose, galactose and rhamnose in similar proportions together with an average of 7% xylose. Fraction B_1, C_1 and D_1 differed from all other fractions by giving highly viscous solutions. They were found to contain, by paper chromatography, arabinose, xylose, galactose, glucose (this sugar being present in all fractions eluted by acetate solutions of up to 0·04 M concentration) and fucose but no rhamnose. The composition of some fractions are given in Table 3.

Examination of Polysaccharide (a)

Fractions A_1 and A_2 were combined (1·20 g) and refractionated on a DEAE cellulose column. The fraction eluted by water (190 mg) gave on hydrolysis besides xylose some arabinose, glucose, and a trace of galactose. Elution with 0·01, 0·02, 0·04 and 0·08 M acetate gave in total 973 mg of polysaccharide that gave xylose only on hydrolysis. The main part appeared in the 0·02 and 0·04 M eluates. The polysaccharide had $[\alpha]_D^{25}+169{\cdot}2°$ (*c*, 1·0 in water) and contained 4·17% nitrogen. It was easily soluble in water. Treatment of the polysaccharide with 45% phenol according to Westphal *et al.*[23] did not reduce the nitrogen content. The xylose content was estimated as 27·0 per cent by adding glucose to a sample of the polysaccharide and analysing the mixture, after treatment with 0·25 M H_2SO_4 at 100° overnight,

TABLE 3. COMPOSITION OF SOME FRACTIONS FROM *Pinus mugo* POLLEN

	% of					
			Neutral sugars*			
Fraction	N	Uronic acid	Gal	Glu	Ar	Xyl
D_1	0·31	Trace	3·2	32·5	39·2	25·1†
D_4	0·41	7·3	11·7	—	87·0	—
D_7	1·65	9·7	14·5	—	84·5	1·0

* 3–4% Rhamnose in D_4 and D_7.
† Including small amounts of fucose.

according to Saeman *et al.*[11] On drastic hydrolysis (2 M HCl at 100° for 22 hr) there were formed, as shown by paper chromatography, galacturonic acid and ethanolamine. The latter was identified by paper chromatography in different solvents. The polysaccharide contained 37% galacturonic anhydride by decarboxylation. The calculated content of anhydrogalacturonic N-(β-hydroxyethyl) amide groups was 65·1 per cent assuming that all nitrogen (4·17%) was present as N-(β-hydroxyethyl) amide groups. Anhydrogalacturonic residues with free carboxylic groups were estimated to be 8·4 per cent by titrating a sample with 0·01 M unstabilized sodium thiosulphate after addition of sodium iodate and sodium iodide.

The polysaccharide (200 mg) was treated for 1 + 1·5 + 1·5 hr with 0·1 M HCl at 100°with intermediate removal of material non-precipitable in ethanol. The residue (78 mg) had $[\alpha]_D+82°$ and contained 3·6% nitrogen. It gave on drastic hydrolysis ethanolamine, xylose (small amounts) and D-galacturonic acid. The latter had in amorphous state $[\alpha]_D+48°$ (*c*, 0·2 in water) and gave, on oxidation with nitric acid, mucic acid, m.p. after purification via the ammonium salt 220–223° (dec.). Examination of the rapidly humifying low molecular weight material by paper chromatography showed xylose as the main component, small amounts of ethanolamine together with some ninhydrin-active material that was slow on paper chromatography in all the solvent systems. The latter yielded on complete hydrolysis galacturonic acid and ethanolamine. The D-xylose had, after removal of humic material by chromatography on thick filter papers, m.p. and mixed m.p. 148·5–150° C and $[\alpha]_D^{25}+18{\cdot}4°$ (*c*, 1 in water, downward mutarotation). Neither xyloöligosaccharides nor other reducing oligosaccharides could be detected.

[23] O. WESTPHAL, O. LÜDERITZ and F. BISTER, *Z. Naturforsch.* **7b**, 148 (1952).

Another 200 mg of the polysaccharide were dissolved in 10% KOH (10 ml) containing 1% sodium hydridoborate. The solution was kept at 50° for 3 hr under a nitrogen atmosphere. After neutralization a high molecular weight residue (145 mg) with $[\alpha]_D + 102°$ was recovered. Part of this (120 mg) was treated in the same way for another 6 hr yielding 95 mg of material precipitable from ethanol. This had $[\alpha]_D + 88°$ and contained 2·2% nitrogen. Paper chromatography of a hydrolysed sample showed a composition similar to that of the residue from the partial hydrolysis. The low molecular weight material (67+27 mg) was shown by paper chromatography to contain some ethanolamine and as only carbohydrate constituents xylitol and traces of xylose.

The polysaccharide consumed per 10 mg, on oxidation with periodate at room temperature, 0·0448 mmole (arsenite method,[24] extrapolation to zero time) with liberation of 0·0168 mmole of formic acid as estimated by titration with sodium thiosulphate after addition of excessive amounts of ethylene glycol and potassium iodide. Oxidation was complete in 50 hr.

In a separate experiment 100 mg of polysaccharide was oxidized for 90 hr. Potassium iodide and acetic acid were then added in a slight excess and the liberated iodine extracted with chloroform . The polyaldehyde was isolated by precipitation from ethanol and washed with ethanol. It was reduced with sodium hydridoborate and subsequently treated with 0·25 M H_2SO_4 at room temperature overnight in the manner devised by Smith and coworkers.[12] After deionization 40 mg polysaccharide could be precipitated from ethanol. It had $[\alpha]_D^{25} + 67°$ (*c*, 0·4 in water) and contained by titration 9·9% free anhydrogalacturonic acid and 5·76% nitrogen, corresponding to 90% anhydrogalacturonic-N(β-hydroxyethyl) amide. Drastic hydrolysis gave galacturonic acid and ethanolamine but no xylose.

The degraded polysaccharide consumed per 10 mg 0·0303 mole periodate with liberation of 0·0048 mmole formic acid. Oxidation was complete in 25 hr.

Examination of the Acidic Arabinogalactan (b)

Different samples of the readily water-soluble polysaccharide had rotations between −85 and −89° (c, 1·0 in water; free acid). The composition of two representative fractions (D_4, D_7) are given in Table 3.

A sample of the polysaccharide (550 mg; uronic acid 8·8%, nitrogen 0·43%) was heated in 0·01 M HCl at 100° during 3 hr. The solution was then neutralized with Dowex 3 anion exchange resin, concentrated and poured into ethanol to give after reprecipitation 95 mg of precipitate (17%). Non-precipitated material amounted to 435 mg and contained of monosaccharides L-arabinose (91%), m.p. and mixed m.p. 159·5–160°, $[\alpha]_D^{25} + 188·9 \rightarrow 105·3°$ (*c*, 1·0 in water), D-galactose (7%), m.p. and mixed m.p. 167–168°, $[\alpha]_D^{25} + 80·7°$ (*c*, 0·8 in water). The L-rhamnose (2%), had m.p. 91° and $[\alpha]_D^{25} + 8°$ (upward mutarotation) and gave a phenylhydrazone m.p. 158–160°, undepressed on admixture with an authentic specimen. The high molecular weight residue (uronic anhydride 30%; nitrogen 1·13%) had $[\alpha]_D + 67°$ (*c*, 1·0 in water). D-Galactose and L-rhamnose were present in the ratio 7:4. Chromatography of a sample hydrolysed in 2 M H_2SO_4 showed the acidic moiety to be glucuronic acid and the nitrogen to be at least partly present as ethanolamine.

In an unsuccessful attempt to isolate neutral oligosaccharides less pure fractions of the polysaccharide (3·4 g) were hydrolysed stepwise for 1 hr periods first with 0·01 M HCl and then with 0·05, 0·10 and 0·25 M H_2SO_4 with intermediate removal of low molecular weight material. The dominant products were however monosaccharides (L-arabinose, D-galactose

[24] P. FLEURY and J. LANGE, *J. Pharm. Chim.* **17**, 107 (1933).

and L-rhamnose); neutral oligosaccharides were obtained only in trace amounts, insufficient for proper characterization.

The polysaccharide consumed, as free acid at room temperature, 0·0468 mmole of periodate per 10 mg with liberation of 0·0064 mmole of formic acid. The oxidation was complete in 25 hr. The high molecular weight residue after hydrolysis of the L-arabinose residues consumed as free acid per 10 mg 0·0851 mole oxidant with liberation of 0·0372 mmole oxidant corresponding to an oxidation of 75–80 per cent of the molecule. The oxidation was complete in 60 hr.

Examination of the Insoluble Fraction (d)

The material left insoluble as dark coloured, horny granules on reprecipitation of the various extracts amounted to 4·1 g and contained 11 % nitrogen. It swelled considerably in water but did not dissolve. It was exhaustively extracted with 1 M NaOH containing some sodium hydridoborate leaving a dark, gummy residue that was not further investigated. The extract was acidified with acetic acid and the precipitate formed collected by centrifugation. It was dried out with anhydrous ethanol followed by acetone to give a brown powder (70 %) with $[\alpha]_D + 17°$ in 1 M NaOH. It gave glucose with small amounts of arabinose on hydrolysis. It was extensively degraded by a (1 →3)-β-glucanase[20] to give glucose as the only product of low molecular weight.

A sample of the crude polysaccharide (860 mg) was exhaustively extracted with hot water. A hydrolysed sample of the extract contained roughly equal amounts of glucose and arabinose. The residue (722 mg) had $[\alpha]_D^{25} + 10·6°$ (c, 1·0 in 1 M NaOH) and contained 0·99 % nitrogen and of neutral sugars 98·3 % glucose and 1·7 % arabinose. The sample used for measuring the optical rotation was recovered in 90 per cent yield by acidification. The recovered polysaccharide had $[\alpha]_D + 12·6°$ (*c*, 0·9 in 1 M NaOH).

A sample of the crude polysaccharide (2·5 g) was dissolved in 90 % formic acid and kept on a steam bath for 1 hr. The solution was concentrated to one-fourth, diluted with 3 vol. water and then heated for 1 hr further. It was taken to a small volume and poured into ethanol. Upon addition of a few drops of ammonia the colloidal precipitate coagulated and was collected by centrifuging. After repeating the procedure twice only 0·12 g precipitable material remained. The combined centrifugates were deionized in aqueous solution (Dowex 50W and Dowex 3 ion exchange resins) and concentrated to a syrup (1·74 g). This was found by paper chromatography to contain as main part a homologous series of oligosaccharides, indistinguishable from the laminaridextrins. It was dissolved in water and absorbed on active carbon. Washing the carbon with 50 % ethanol gave 0·91 g of low molecular weight oligosaccharides. Washing with 50 % acetone gave a fraction that gave another 0·45 g oligosaccharides after treatment at 100° first with 90 % formic acid for 30 min and then with 25 % formic acid for 1 hr. The combined oligosaccharide mixtures (1·35 g) were fractionated on a carbon-Celite column using gradient elution with aqueous ethanol. After purification by thick filter paper chromatography the following was obtained:

D-Glucose (110 mg) m.p. 145·5–146·5°, mixed m.p. 144–146°; $[\alpha]_D^{25} + 55°$ (*c*, 0·9 in water). L-Arabinose (19 mg) m.p. and mixed m.p. 158–159°; $[\alpha]_D^{25} + 108°$ (*c*, 0·25 in water). Laminaribiose (136 mg) m.p. 204–205°, mixed m.p. 203–205°; $[\alpha]_D^{25} + 19·2°$ (*c*, 0·86 in water). It was converted to the β-octaacetate which on crystallization from ethanol gave rod-shaped crystals m.p. and mixed m.p. 160–162°. On recrystallization from n-propanol-ethanol 1:1 it gave plates m.p. 166–167° and mixed m.p. 163–164° and $[\alpha]_D^{25} - 26·4°$ (*c*, 1·2 in $CHCl_3$). Laminaritriose (183 mg) characterized as the β-acetate m.p. and mixed m.p. 116–118° and

$[\alpha]_D^{25} - 36\cdot0°$ (c, 0·85 in $CHCl_3$). Laminaritetraose (75 mg) characterized as the β-acetate m.p. and mixed m.p. 124–125·5°; $[\alpha]_D^{25} - 44\cdot3°$ (c, 1·2 in $CHCl_3$). Laminaripentaose (40 mg) and laminarihexaose (18 mg) having the same R_M/n fractions as the biose and triose. Contamination by positively rotating material made the rotations high by a few degrees. Maltose (17 mg) with $[\alpha]_D + 109°$ indistinguishable from maltose on chromatography in several solvents. It gave a β-acetate with $[\alpha]_D^{25} + 64°$ (c, 0·4 in $CHCl_3$) and m.p. 161·5–162·5° undepressed on admixture with authentic maltose β-octaacetate. A trisaccharide fraction (15 mg) with $[\alpha]_D + 139°$ having a main component indistinguishable from maltotriose on chromatography in several solvent systems. A tetrasaccharide fraction (13 mg) with $[\alpha]_D + 87°$ which was found to contain by paper chromatography among other constituents maltotetraose. Traces of galactose were detected in hydrolysed samples of impure higher oligosaccharides.

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