The Alkali-soluble Polysaccharides of the Lichen Cladonia alpestris (*Reindeer Moss*).

By G. O. Aspinall, E. L. Hirst, and (Mrs.) Margaret Warburton.

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A preliminary examination has shown that the alkali-soluble polysaccharides of reindeer moss (*Cladonia alpestris*) consist of mixtures of highly branched molecules containing residues of D-galactose, D-glucose, and D-mannose. Methylation studies have shown that the majority of the D-galactose and some of the D-glucose and D-mannose residues occupy terminal positions, while chains of D-glucose and of D-mannose residues constitute the backbone of the molecular structure. Periodate oxidation provides further evidence for complex highly branched structures.

The lichen *Cladonia alpestris*, commonly known as reindeer moss, is one of three closely related species, *C. alpestris*, *C. rangiferina*, and *C. sylvatica*, of common occurrence in Norway. Little is known concerning their composition but Professor Berner and his colleagues at Oslo University showed that reindeer moss, which contains 93% of carbohydrates, gives glucose, galactose, and mannose on hydrolysis (personal communication). This lichen appeared to differ considerably from Iceland moss (*Cetraria islandica*), the polysaccharides of which have been extensively investigated and have been shown to consist predominantly of D-glucose residues (Granichstädten and Percival, *J.*, **1943**, 54; Meyer and Gürtler, *Helv. Chim. Acta*, **1947**, **30**, 751, 761; Chanda and Hirst, unpublished work), although small quantities of D-galactose and D-mannose have been reported in the hydrolysates of some polysaccharide fractions. In the present investigation the alkali-soluble polysaccharides of *C. alpestris* have been examined, preliminary experiments having

shown that after removal of lichen acids with aqueous sodium carbonate very little polysaccharide could be extracted with hot water.

Two polysaccharide preparations (I and II) were obtained by extraction of the lichen with cold 5% and 24% potassium hydroxide solution respectively. The polysaccharides, although differing in optical rotation, had similar physical properties and gave the same sugars (galactose, glucose, and mannose) on hydrolysis, but in different proportions. D-Galactose and D-mannose were identified in both hydrolysates by the formation of crystalline derivatives. Fractionation of polysaccharide I *via* the copper complex gave a polysaccharide of different composition, indicating the presence of at least two different molecular species, but repeated fractionation failed to resolve the mixture.

Conversion of each of the two polysaccharides into their fully methylated derivatives gave similar products, differing slightly in optical rotation, but giving on hydrolysis the same complex mixture of methylated sugars. The methylated polysaccharides were therefore combined for subsequent examination. The methylated sugars (fraction A) obtained on hydrolysis of the methylated polysaccharides were fractionated on cellulose, but in most of the fractions separation of the sugars was incomplete. Although 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose was the only sugar from fraction A to be identified by the formation of a crystalline derivative, evidence from optical rotations, paper chromatography, and demethylation indicated the presence also of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-mannose, a mixture of tri-*O*-methyl derivatives of D-glucose and D-mannose including 2 : 3 : 6-tri-*O*-methyl-D-glucose, 2 : 4 : 6-tri-*O*-methyl-D-galactose, and a di-*O*-methyl-D-glucose (probably the 2 : 3-isomer). In addition to these products of hydrolysis two portions (B and C) resistant to acid hydrolysis were also encountered, one of them (C) coming out of solution during the treatment with aqueous acid. These fragments were hydrolysed under more vigorous conditions and the products were further fractionated. Fraction B was shown to be an incompletely methylated fragment composed of residues of D-mannose only. From the hydrolysis products 2 : 3 : 4 : 6-tetra-*O*-methyl-D-mannose was characterised as the crystalline aniline derivative and 3 : 4-di-*O*-methyl-D-mannose was identified as the crystalline monohydrate; in addition, paper chromatography indicated the presence of a tri-*O*-methyl-D-mannose, a second di-*O*-methyl-D-mannose, and a mono-*O*-methyl-D-mannose. Fraction C was shown to be composed solely of D-glucose residues and in the mixture of methylated sugars given on hydrolysis 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose was identified.

The yield of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose indicates that the majority of D-galactose residues are present as non-reducing end-groups, while some of the D-glucose and D-mannose residues also occupy terminal positions. The isolation of the resistant fragments B and C indicates the presence of two main structural features, which are remarkable in this group of polysaccharides in that each consists of chains of one type of sugar residue only. It is not possible on the present evidence to decide whether D-galactose residues are linked to both types of molecular structure or only to one.

The results of periodate oxidation experiments are consistent with the view that polysaccharides I and II both contain mixtures of highly branched polysaccharides. Oxidation of the polysaccharides resulted in the formation of 1 mol. of formic acid per 3·7 and 3·0 hexose residues respectively, while both polysaccharides consumed 1·2 mols. of periodate per residue. Confirmation was thus obtained for the presence of a high proportion of non-reducing end-groups. Hydrolysis of the periodate-oxidised polysaccharides indicated the presence of unattacked glucose residues and also in smaller amount of unattacked mannose residues. The former observation suggests the presence in the polysaccharides of glucose residues linked through positions 1 and 3, in which case it seems probable that 2 : 4 : 6-tri-*O*-methyl-D-glucose was one component of the mixture of incompletely identified tri-*O*-methylhexoses isolated from the methylated polysaccharide hydrolysate. The latter observation would be expected if the 3 : 4-di-*O*-methyl-D-mannose, previously isolated, has structural significance and does not arise from incomplete methylation. It is interesting that a trace of galactose was also given on hydrolysis of the oxidised polysaccharide I, suggesting the presence of a small proportion of D-galactose residues linked through positions 1 and 3. This provides further evidence that the tri-*O*-methyl-D-galactose present among the methylated sugars was indeed the 2 : 4 : 6-isomer.

It is evident from these preliminary experiments that the alkali-soluble polysaccharides of reindeer moss contain highly branched complex structures. The results emphasize the necessity of applying new methods of fractionation before the number and detailed structure of the components present in this lichen can be established.

Experimental

Paper partition chromatography was carried out on Whatman No. 1 filter paper with the following solvent systems: (A) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3; v/v; top layer) and (B) butan-1-ol–ethanol–water (4 : 1 : 5; v/v; top layer).

Isolation of Alkali-soluble Polysaccharides from Cladonia alpestris.—Extractive-free lichen was extracted successively with cold dilute sodium carbonate solution and boiling water to remove lichen acids and water-soluble polysaccharides, and the residue was extracted with cold 5% and 24% potassium hydroxide solutions. The alkaline extracts were poured into ethanol acidified with glacial acetic acid and the precipitated polysaccharides (I and II respectively) were dried by solvent exchange with ethanol and ether. Polysaccharide I, isolated in 2·2% yield, had $[\alpha]_D^{15}$ +43·9° (*c*, 0·4 in 2N-NaOH) and chromatographic examination of the hydrolysate (Flood, Hirst, and Jones, *J.*, 1948, 1679) in solvent (A) showed the presence of galactose (12·6%), glucose (51·7%), and mannose (34·2%). Polysaccharide II, isolated in 1·3% yield, had $[\alpha]_D^{15}$ +61·4° (*c*, 0·89 in 2N-NaOH) and chromatographic examination of the hydrolysate showed the presence of galactose (12·9%), glucose (39·7%), and mannose (47·4%). D-Galactose and D-mannose were identified in both hydrolysates by the formation of the methylphenylhydrazone (m. p. and mixed m. p. 176—178°) and the phenylhydrazone (m. p. and mixed m. p. 198—199°) respectively.

A sample of polysaccharide I was fractionated by precipitation of the copper complex formed on addition of Fehling's solution to a solution of the polysaccharide in aqueous sodium hydroxide, followed by decomposition of the copper complex with dilute hydrochloric acid and precipitation of the regenerated polysaccharide with ethanol. The regenerated polysaccharide gave on hydrolysis galactose (15·2%), glucose (28·2%), and mannose (49·8%). These results showed that polysaccharide I was inhomogeneous, but repeated fractionations *via* the copper complex failed to yield distinct components.

Methylation of Polysaccharides.—Polysaccharides I (6·6 g.) and II (3·8 g.) were each methylated fifteen times with methyl sulphate and sodium hydroxide solution and twice with methyl iodide and silver oxide. Methylated polysaccharide I had $[\alpha]_D^{17}$ +35° (*c*, 0·5 in $CHCl_3$) (OMe, 44·5%) and methylated polysaccharide II had $[\alpha]_D^{17}$ +24·5° (*c*, 0·6 in $CHCl_3$) (OMe, 44·0%). Samples of both methylated polysaccharides were hydrolysed and the hydrolysates were examined chromatographically by use of solvent (B) and shown to contain qualitatively similar mixtures of methylated sugars.

Hydrolysis of Methylated Polysaccharides and Separation of Methylated Sugars.—The mixture of methylated polysaccharides I (1·3 g.) and II (2·7 g.) was refluxed with methanolic 1% hydrogen chloride (400 c.c.) for 17 hr. An insoluble residue (0·1 g.) was separated, and the hydrolysate was neutralised with ethereal diazomethane and concentrated to a syrup. The syrup was hydrolysed on the water bath with N-hydrochloric acid (200 c.c.) for 8·5 hr., during which a flocculent solid (soluble in methanol) separated. The solid was re-treated with methanolic and aqueous hydrogen chloride but a residue (C), insoluble in hydrochloric acid, remained and was separated. The combined acid hydrolysates were neutralised with silver carbonate and concentrated to a syrup (A) (3·2 g.).

Syrup A was fractionated on cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511) with light petroleum–butan-1-ol (7 : 3), saturated with water, as eluant to give five main fractions (see Table) together with a number of smaller fractions (combined wt., 48 mg.) which were only examined chromatographically and shown to contain mixtures of di- and mono-*O*-methylsugars. Elution of the cellulose with water gave a non-reducing syrup (B) (0·64 g.) which was examined separately. Examination of fraction AI showed it to contain only 32% of reducing sugar (hypoiodite oxidation) and demethylation (Hough, Jones, and Wadman, *J.*, 1950, 1702) showed that methyl ethers of galactose, glucose, and mannose were present. The fraction (0·37 g.) was rehydrolysed and part of the hydrolysate (0·20 g.) was separated on filter sheets (Whatman 3MM) by use of solvent B to give four fractions.

Fraction AI*a* travelled on the chromatogram at the same rate as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-mannose and on demethylation gave mannose. Quantitative paper chromatography (Hirst, Hough, and Jones, *J.*, 1949, 298) showed that tetra- and tri-*O*-methylhexoses were present in

fraction AI*b* in the ratio 2 : 1. The presence of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose in this fraction was shown by conversion into the aniline derivative, m. p. and mixed m. p. 186—189°. Demethylation of fraction AI*e* gave mannose and glucose, indicating the presence of trimethyl ethers of both these sugars. Fraction AII was chromatographically homogeneous and was identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose by conversion into the aniline derivative, m. p. and mixed m. p. 189—191° (Found : OMe, 39·6. Calc. for $C_{16}H_{25}O_5N$: OMe, 39·9%).

Fraction	Wt. of material eluted (mg.)	$[\alpha]_D^{17}$ (solvent) *	Found : OMe (%)	Calc. : OMe (%)	Paper chromatography: R_G in solvent B	Sugar
AI	799	+49·7° (W)	45·0	—	0·95	—
AI*a*	40	+23 (W)	49·0	52·5	1·00	Tetra-*O*-methylmannose
AI*b*	32	+53 (W)	46·1	—	1·00	,,
					0·93	Tetra-*O*-methylgalactose
					0·83	Tri-*O*-methylhexose
AI*e*	52	+22·4 (W)	43·7	41·9	0·83	,,
AI*d*	6	—	—	—	0·63	Di-*O*-methylhexose
AII	333	+109 (W) / +76·3 (E)	50·1	52·5	0·91	Tetra-*O*-methylgalactose
AIII	483	+56·5 (W)	39·8	41·9	0·84	Tri-*O*-methylhexose
AIV	132	+80·4 (W)	36·7	—	0·71	Tri-*O*-methylgalactose
					0·65	Di-*O*-methylglucose
AV	66	+55·3 (W)	29·6	29·8	0·65	,,

* W = H_2O, E = EtOH.

The presence of trimethyl ethers of glucose and mannose in fraction AIII was shown by demethylation. The fall in rotation of the fraction in methanolic 1% hydrogen chloride at room temperature, $[\alpha]_D^{17}$ +50° → +27° (*c*, 0·2), indicated that 2 : 3 : 6-tri-*O*-methyl-D-glucose was one component of the mixture. {Under similar conditions 2 : 3 : 6-tri-*O*-methyl-D-glucose showed $[\alpha]_D$ +70° → −37° and 2 : 3 : 6-tri-*O*-methyl-D-mannose showed $[\alpha]_D^{17}$ +11° (const.)}. A sample of fraction AIII was oxidised with sodium metaperiodate but the absence of formaldehyde as shown by the Rimini ferricyanide–phenylhydrazine test (*Bull. Soc. chim.*, 1898, **20**, 896) indicated the absence of 2 : 3 : 4-tri-*O*-methylhexose. The presence in fraction AIII of a small quantity of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose, which had escaped chromatographic detection, was shown by formation of the aniline derivative, m. p. and mixed m. p. 185—187° : no other crystalline aniline derivatives were isolated. Fraction AIV contained two sugars travelling on the chromatogram at the same rate as 2 : 4 : 6-tri-*O*-methyl-D-galactose and 2 : 3-di-*O*-methyl-D-glucose and gave on demethylation galactose and glucose. Quantitative paper chromatography and calculation from the optical rotations showed that the relative proportion of the two sugars was 7 : 3. Fraction AV was chromatographically homogeneous, travelling at the same rate as 2 : 3-di-*O*-methyl-D-glucose, and gave glucose on demethylation.

Examination of Non-reducing Syrup B.—Syrup B had $[\alpha]_D^{17}$ +30° (*c*, 0·5 in H_2O) (Found : OMe, 31·8%) and on demethylation gave only mannose. A portion (0·45 g.) was hydrolysed on the water-bath successively with formic acid (45 c.c.; 95%) for 6·5 hr. and with N-sulphuric acid (30 c.c.) for 3 hr. The hydrolysate was neutralised with barium carbonate, and concentrated to a syrup (0·381 g.), part of which (0·320 g.) was fractionated on cellulose to give five main fractions BI—V. A number of smaller fractions (combined wt. 66 mg.) and a resistant residue (119 mg.), eluted with water, were not examined further.

Fraction BI (41·4 mg.) had $[\alpha]_D^{17}$ +21·7° (*c*, 0·8 in $CHCl_3$) and travelled on the chromatogram at the same rate as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-mannose. It was identified by conversion into 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-mannosylamine, m. p. and mixed m. p. 143—145°. Fraction BII (30 mg.) had $[\alpha]_D^{17}$ +14·5° (*c*, 0·6 in $CHCl_3$) and $[\alpha]_D^{17}$ +8·7° (*c*, 0·5 in H_2O), and travelled on the chromatogram at the same rate as 2 : 3 : 6-tri-*O*-methyl-D-mannose. Fraction BIII (30 mg.) crystallised and after recrystallisation from ethanol had m. p. and mixed m. p. (with authentic 3 : 4-di-*O*-methyl-D-mannose monohydrate) 78—80° and $[\alpha]_D^{17}$ +5° (*c*, 0·6 in H_2O; equil.). The two samples gave identical *X*-ray crystal photographs (by the courtesy of Dr. C. A. Beevers) and it is concluded that the authentic specimen, which originally had m. p. 109° (Haworth, Hirst, and Isherwood, *J.*, 1937, 784), had changed to a more stable crystalline form with m. p. 80—82°. Fraction BIV (20 mg.) had $[\alpha]_D^{17}$ +7·5° (*c*, 0·4 in acetone) and R_G 0·62 in solvent B (3 : 4-di-*O*-methyl-D-mannose had R_G 0·65). Fraction BV (27 mg.) had $[\alpha]_D^{17}$ −5·3° (*c*, 0·3 in H_2O) and R_G 0·32 in solvent B, corresponding to a mono-*O*-methyl-D-mannose.

Examination of Residue C.—The solid C (0·5 g.) was hydrolysed on the water-bath successively

with formic acid (10 c.c.; 95%) for 7 hr. and with N-sulphuric acid (3 c.c.) for 6 hr., considerable decomposition occurring. After neutralisation with barium carbonate, the hydrolysate was concentrated to a syrup (0·250 g.), demethylation of which gave glucose only. A portion of the syrup (110 mg.) was separated on filter sheets with solvent B to give four fractions.

Fraction CI (30 mg.) crystallised and after recrystallisation from ether had $[\alpha]_D^{17}$ +80° (*c*, 0·3 in H_2O) and m. p. and mixed m. p. (with authentic 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose) 86—88°. Fraction CII (12 mg.) had $[\alpha]_D^{17}$ +68° (*c*, 0·2 in acetone) and R_G 0·86 in solvent B. The rotation of the fraction in methanolic 1% hydrogen chloride, $[\alpha]_D^{17}$ +33° (*c*, 0·2), indicated that 2 : 3 : 6-tri-*O*-methyl-D-glucose was one component of a mixture of tri-*O*-methyl-D-glucoses. {All other tri-*O*-methyl-D-glucopyranoses show $[\alpha]_D$ *ca.* +70° in methanolic hydrogen chloride (Granichstädten and Percival, *loc. cit.*).} Fraction CIII (35 mg.) travelled on the chromatogram at the same rate as 2 : 3-di-*O*-methyl-D-glucose but the optical rotation {$[\alpha]_D^{17}$ = +76° (*c*, 0·7 in acetone)} was higher than that quoted for 2 : 3-di-*O*-methyl-α-D-glucose {$[\alpha]_D^{17}$ = +81·9° → +48·3° in acetone (Irvine and Scott, *J.*, 1913, **103**, 575)}. Fraction CIV (16 mg.) had R_G 0·34 in solvent B, corresponding to a mono-*O*-methyl-D-glucose, but was not examined further.

Periodate Oxidation of Polysaccharides.—Oxidation of polysaccharide I (50-mg. batches) with potassium metaperiodate solution by the method of Halsall, Hirst, and Jones (*J.*, 1947, 1399, **1427**) yielded a constant amount of formic acid after 208 hr., corresponding to 1 mol. per 3·7 $C_6H_{10}O_5$ residues. Oxidation of polysaccharide II yielded formic acid corresponding to 1 mol. per 3·0 $C_6H_{10}O_5$ residues.

Oxidation of the polysaccharides with sodium metaperiodate solution showed that both polysaccharides consumed 1·2 mols. of periodate per $C_6H_{10}O_5$ residue. Chromatographic examination of the hydrolysates of the periodate-oxidised polysaccharides showed the presence of glucose and mannose (trace), and from polysaccharide I galactose (trace) also.

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CHEMISTRY DEPARTMENT, UNIVERSITY OF EDINBURGH. [*Received, October 25th*, 1954.]