THE CARBOHYDRATES OF THE LEAVES OF COMMON BARBERRY (*Berberis vulgaris*). THE EXTRACTION, FRACTIONATION AND STRUCTURAL STUDIES OF SELECTED NON-CELLULOSIC POLYSACCHARIDES*

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ABSTRACT

Common barberry leaves contain a small but diverse polysaccharide fraction which afforded an α-glucan, a β-xylan, and three neutral, and two galacturonic acid-containing glucoxylans. Only the α-glucan was devoid of protein. The α-glucan is primarily (1→4)-linked, but some residues are branched through C-3 or C-6. Two of the neutral glucoxylans contained small proportions of both D-galactose and L-arabinopyranose in terminal, non-reducing locations, the third neutral glucoxylan contained no arabinose; all were branched through both glucose and xylose residues. Hydrolysis of one of these heteropolysaccharides with pectinase afforded a new disaccharide, 4-*O*-α-D-xylopyranosyl-D-glucopyranose, thus establishing that at least some of the linkages were of the α-type. A new approach to the structural determination of micro quantities of oligosaccharides is discussed.

INTRODUCTION

The leaves of *Berberis vulgaris* (common barberry) act as the alternate host for the aecidal stage of wheat stem-rust (*Puccinia graminis tritici*)[1]. Common barberry thus has been important to wheat-growing countries which are subject to rust infestation, as it represents a vulnerable point in the fungal life-cycle. Moreover, the structural polysaccharides of leaves are largely unexplored and offer a fruitful area of research.

A small but diverse polysaccharide moiety consisting mainly of heteropolysaccharides, together with an α-glucan and a β-xylan, was obtained. At least seven discrete polysaccharides have been isolated, and these vary widely in composition and molecular weight. Most of them are firmly associated with protein. This paper reports the major structural features of four polysaccharides from the leaves of common barberry: the α-glucan and three heteropolymers.

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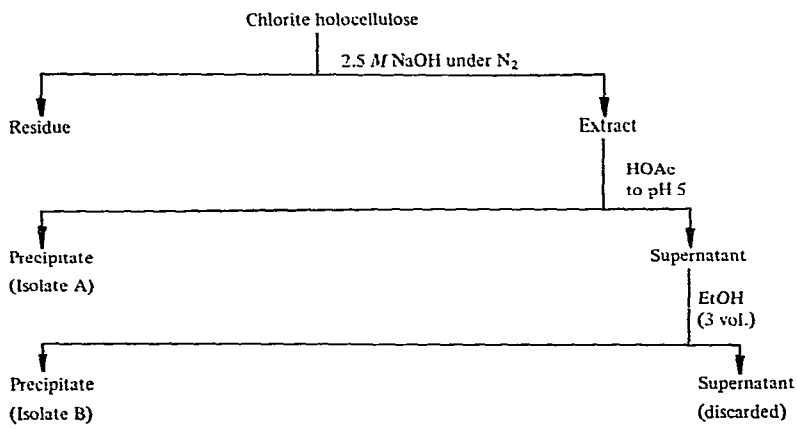
The authors have encountered no publication on the structural carbohydrates of the leaves of common barberry. Indeed, other than a recent report of a water-soluble, starch-like glucan from *Basella rubra* leaves[2], and the well-studied pectins[3], only a few preliminary reports on the carbohydrates of the leaves of any woody plant have been published to date. Hough and co-workers[4] investigated the polysaccharides of plum leaves (*Prunus domestica* var. Victoria); from these were obtained some pectins and a number of complex heteropolysaccharides, many of which contained protein in proportions varying from 3 to 70%. A later survey of 27 species of leaves of both woody and herbaceous plants[4] for high content of carbohydrate disclosed that galactose, glucose, arabinose, xylose, and a hexuronic acid were common components. The research reported herein is believed to be the first detailed structural study of non-pectic, non-cellulosic leaf polysaccharides of a woody plant indigenous to the temperate zone.

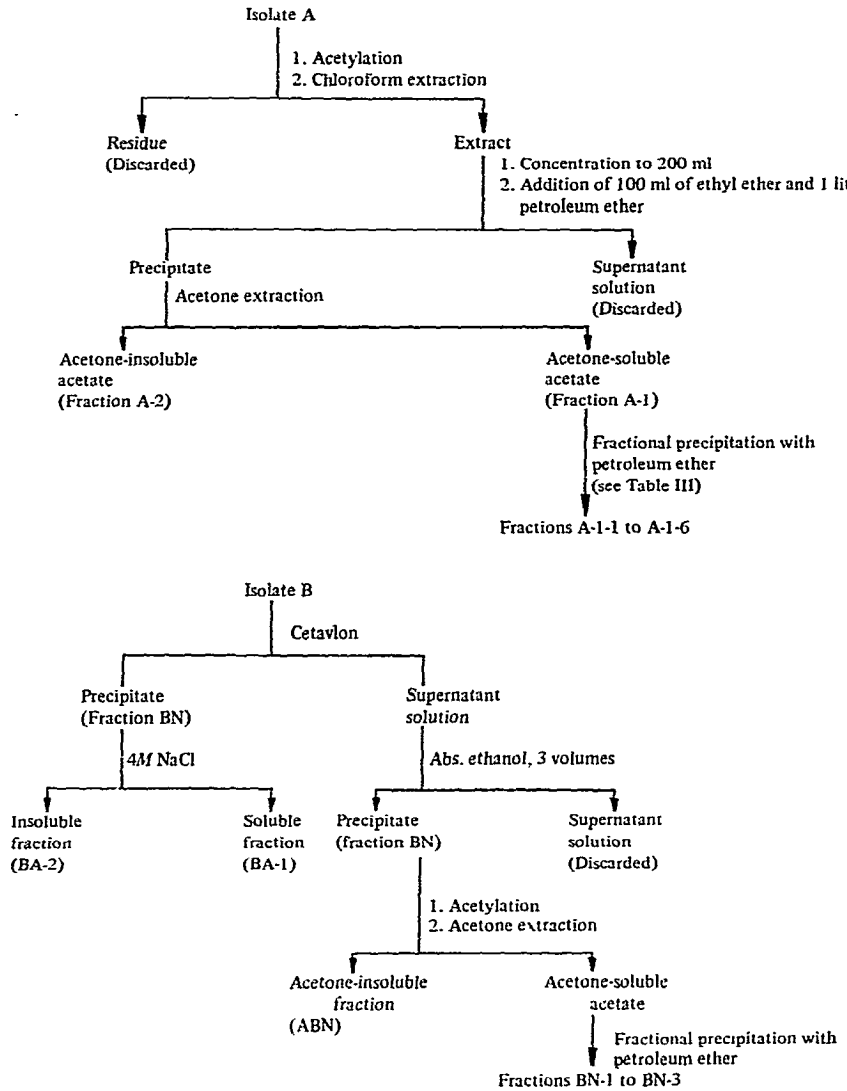
DISCUSSION

Barberry leaves, gathered in mid-May, were frozen in Dry Ice–acetone immediately upon harvesting to retard enzymic modification of the carbohydrates prior to extraction. Preliminary studies, including the isolation of methanol- and water-soluble components, afforded such limited amounts of isolable carbohydrates that a second crop of barberry leaves was harvested similarly in mid-September. From this autumn sample, a chlorite holocellulose was prepared, which was extracted with alkali (Scheme 1) and subsequently fractionated by derivatization and complex-



Scheme 1. Fractionation of chlorite holocellulose.

formation methods (Scheme 2). The improved solubilization of protein by extraction with 1:1:1 phenol–acetic acid–water[5–7] was exploited with the autumn crop to afford fractions of lower protein — and hence higher carbohydrate — concentrations.



Scheme 2. Fractionation of isolates A and B.

Despite the variety of purification methods employed, only one fraction (A-1-4) was completely free of protein. Thus, the results for the other polysaccharides must be viewed as tentative, and only the main structural characteristics have been established. The nature of the association of the protein with the carbohydrates has not been discovered, and the effect of the protein on the periodate oxidation and methylation studies is unknown. The mode in which the features revealed are combined in the native molecules remains unknown, not only because of the failure to obtain completely pure polysaccharides but also as a result of the lack of "approaches which

will allow the disordered features of [such] heteropolysaccharides to be investigated"[8]. Nevertheless, this research has demonstrated the presence of structural polysaccharides of significant interest in the leaves of barberry, and has established the need for further investigation of leaf polysaccharides from this and other sources.

Water-insoluble polysaccharides from the autumn leaves of common barberry. — The analyses of some hemicellulose-containing fractions isolated from the autumn crop are summarized in Table I. It is notable that the phenol-treated autumn leaves afforded a starch-like, iodine-staining, neutral glucan (Fraction A-1-4, Table I) upon extraction with alkali but not with hot water. On the basis of the specific optical rotation and composition, this protein-free glucan was considered to be primarily α-D-linked, and it may represent barberry leaf-starch that has been rendered inaccessible to solubilization by hot water because of its association with other materials[9].

Fraction A-2, a neutral xylan of low molecular weight ($\overline{\text{d.p.}}$ 48) was levorotatory and probably was mainly β-D-linked.

Isolate B was subdivided into 5 glucoxylans (BN-1, BN-2, BN-3, BA-1, BA-2) (Table I), each of which contained protein. The specific rotation of fraction BA-2 (32% protein) could not be measured because of the opacity of its solutions.

Fraction BA-1 was assumed initially to be a mixture, because of its protein content (12%) and its monomer composition (Table I) which suggested that pectic material may have been present. However, this fraction was eluted as a single component from columns of DEAE-cellulose[10] and Sephadex-CM[11], which attested to a strong association of the protein and carbohydrate components.

When fractions BN-1, BN-2 and BN-3 were examined by electrophoresis on glass paper in borate[12,13] and molybdate[14] buffers, only fraction BN-2 appeared to be homogeneous. Fraction BN-1 contained one minor component having the same electrophoretic mobility as BN-2. Fraction BN-3 consisted of at least 2 components, different from those of BN-1 and BN-2. Although the glucoxylans BN-1 and BN-2 were similar in monomer composition, $\overline{\text{d.p.}}$, and specific rotation, they were dissimilar in solubility and in their enzymic hydrolysis patterns. The last finding was accepted as significant evidence of structural differences between the two polymers.

Methylation study of the α-glucan (fraction A-1-4). — Methylation analysis indicated that this glucan is primarily (1→4)-linked with branching through C-3 in 1 of every 6 internal residues and through C-6 in 1 of every 29 internal residues. The specific rotation of +185° for the parent glucan (Table I) indicated that most of the glycosidic linkages are α-D. A trace of 6-*O*-methylglucose was detected by gas–liquid partition chromatography (g.l.p.c.) but it was considered to be a structurally insignificant artifact resulting from demethylation during the hydrolysis[15,16]. Demethylation would contribute to the small deficiency in terminal non-reducing residues observed (see Experimental), as would the volatility of methyl tetra-*O*-methylglucopyranoside[17] and the diminished response of the detector to this compound[18]. Nevertheless, it is evident that the average repeating unit (34 sugar residues) indicated by methylation is consistent with the molecular size ($\overline{\text{d.p.}}$ 132) deduced from periodate oxidation of the reduced glucan, and suggests that the molecule contains 4 repeating units.

TABLE I

PROPERTIES OF POLYSACCHARIDES ISOLATED FROM COMMON BARBERRY LEAVES

Fraction	$[\alpha]_D^{23\pm3}$ *in* H_2O *(degrees)*	$\overline{D.p.}$	*Protein (%)*	*Molar ratio of components*					
				D-*Galactose*	L-*Arabinose*	D-*Glucose*	*Rhamnose*	D-*Xylose*	*Galacturonic acid*
Spring leaves									
Cold water extract	+8		12.5	5.0	5.0	1.1	1.5		
Hot water extract	+12		8.5	1.0	1.2	1.1			
Autumn leaves									
Fraction									
A-1-4	+185	135				[a]			
A-2	−70	48	8.3					[a]	
BN-1	+20	160	7.1	1.0	1.2	4.0		4.5	
BN-2	+22	129	6.9	1.0	1.1	4.0		5.0	
BN-3[b]	+40	182	5.2	1.0		7.0		7.0	
BA-1	+10	242	12.0	1.0	1.0	2.0		9.0	1.0
BA-2[b]			32.0	1.0	3.0	5.0		8.0	2.0

[a]Homopolymers. [b]Absolute configuration (D or L) of component sugars not determined.

This polysaccharide appears to be unusual in that the (1→4)-linked backbone is branched at both C-3 and C-6, a combination of structural elements not previously encountered in plant glucans, although examples are known among the dextrans[19].

Structural studies on heteropolysaccharides BN-1, BN-2 and BN-3. — The major structural features of BN-1, BN-2, and BN-3, as deduced from periodate oxidation and methylation data were sequences of (1→4)-linked D-xylose residues, some of which are sites of branching through C-3, interspersed with unbranched and branched (through C-6) (1→4)-linked D-glucose residues. The polysaccharides represent a family of glucoxylans possessing a basic structural template upon which minor variations in branching and composition have been superimposed. These conclusions were reached from the following considerations.

Methylation. A comparison of the relative compositions of the parent (Table I) and methylated polysaccharides (Table II) reveals a marked increase in the proportion

TABLE II

COMPOSITION OF THE HYDROLYSATES OF METHYLATED FRACTIONS BN-1, BN-2, AND BN-3

Component	*Molar ratio*		
	BN-1	*BN-2*	*BN-3*
2,3,4,6-Tetra-*O*-methyl-D-galactose	2	2	1
2,3,4-Tri-*O*-methyl-D-xylose	5	4	3
2,3,4-Tri-*O*-methyl-L-arabinose	1	1	
2,3,6-Tri-*O*-methyl-D-glucose	3	5	5
2,3-Di-*O*-methyl-D-xylose	10	10	8
2,3-Di-*O*-methyl-D-glucose	5	4	3
2-*O*-Methyl-D-xylose	4	4	3
2-*O*-Methylglucose		1	

of xylose and, in fraction BN-3, the disappearance of arabinose, upon methylation. This is not uncommon, as glucans and xylans often display a more facile separation from associated polysaccharides after methylation[20,21]. This change in composition may reflect a loss of a single heteropolymer or of a combination of polysaccharides. The relative uniformity of loss of components other than xylose indicates that this may be due to the separation of impurities of definite composition.

The inconsistency between the amounts of branching residues and terminal, non-reducing residues may be attributable in part to the interference by protein (see Periodate oxidation). Nevertheless, certain major structural features can be recognized. The methylation data revealed that, in each of fractions BN-1, BN-2 and BN-3, 75–80% of the xylose is incorporated by (1→4)-glycosidic bonds, and approximately 28% of such residues are involved in additional bonding through C-3. The remainder of the xylose is present as terminal, non-reducing residues. Moreover, in each of these fractions, glucose occurs as (1→4)-linked internal residues, some of which are branched through C-6. The ratio of the di- to trimethyl ethers of glucose is least in BN-3

and greatest in BN-1. None of these polymers contain glucose in terminal non-reducing positions, whereas all of the galactose is present in such locations. In addition to these common features, BN-2 is unique in that it contains (1→4)-linked glucose residues that are branched through C-3 and C-6, and thus give rise to 2-*O*-methylglucose upon methylation, whereas BN-3 differs in being devoid of the terminal, non-reducing arabinopyranosyl residues present[22] in BN-1 and BN-2. The average repeating units, as indicated by methylation (Table II), are consonant with the $\overline{d.p.}$ values obtained from periodate oxidation. Thus, BN-1 ($\overline{d.p.}$ 129) consists of approximately 4, BN-2 ($\overline{d.p.}$ 160) of approximately 5, and BN-3 ($\overline{d.p.}$ 182) of approximately 8, repeating units.

Periodate oxidation. The data of Table III indicate common agreement between the structures perceived by etherification and by periodate oxidation. For each polymer, fewer vicinal-diol groups were available to periodate than the methylation

TABLE III

COMPARISON OF THEORETICAL AND EXPERIMENTAL PERIODATE-OXIDATION DATA FOR FRACTIONS BN-1, BN-2, AND BN-3

Fraction	*Periodate oxidation results*			
	Calculated from methylation studies		*Experimental*	
	IO_4^- *consumed, moles/mole*	HCO_2H *liberated, moles/mole*	IO_4^- *consumed, moles/mole*	HCO_2H *liberated, moles/mole*
BN-1	1.14	0.27	0.98	0.23
BN-2	1.06	0.23	0.94	0.20
BN-3	1.04	0.17	0.96	0.22

data would suggest, and, significantly, there were also fewer vicinal-triol groups available in BN-1 and BN-2, in both of which the periodate consumed and formic acid liberated were equally low (on a percentage basis). This suggests that the interference (or error) may be associated with the terminal non-reducing residues, which are the only sites yielding formic acid. The association of protein with the polysaccharide also may result in some non-specific steric inhibition of periodate oxidation; the latter reaction is known to be sensitive to the presence of bulky groups[23].

Smith degradation[24]. The general concurrence between the methylation and periodate-oxidation data is further illustrated by the results of a Smith degradation of fraction BN-2, the only BN-polysaccharide isolated in sufficient quantity to permit such a study. The products of this reaction sequence included glucose, xylose, erythritol, ethylene glycol, glycerol, 2-*O*-β-D-glucopyranosyl-D-erythritol, and glycosides of higher molecular-weight. The isolation of the glucosylerythritol confirmed the structural significance of the 2-*O*-methylglucose detected by methylation, as it was the only structural unit that could have afforded unchanged glucose.

At least three oligoglycosides were detected in small proportions. These were estimated to have $\overline{d.p.}$ values of 5–8, based on comparison of their chromatographic mobilities relative to those of homologs of maltose. When combined and hydrolysed, glucose, xylose, and erythritol, but not glycerol, were detected.

The isolation of oligoglycosides suggests that branching occurs in "bunches" within the molecule, that is, that there are portions of the polysaccharide in which each of a number of contiguous sugar residues are bonded so as to be impervious to periodate oxidation. The observation that these oligoglycosides contained both xylose and glucose is additional confirmation of the methylation data that predicted such periodate-resistant units, although it has not been established whether the glucose and xylose branching-residues are intermingled or separated. The sequences of periodate-resistant sugar residues that give rise to these oligoglycosides must be interrupted, in the direction of the reducing end of the polysaccharide, by a periodate-oxidizable glucose residue (which would afford the erythritol moiety) but not by a xylose residue (which would give glyceryl glycosides). On the same basis, the isolation and characterization of 2-*O*-β-D-glucopyranosyl-D-erythritol argues for at least some of the (1→4)-linked glucopyranosyl branch points being glycosidically bonded to an unbranched, (1→4)-linked glucopyranosyl residue. On the other hand, the absence of xylosylglycerol and xylosylerythritol is consistent with the xylose branch-points occurring mainly in contiguous groups. The branches through (1→4)-linked xylose residues may not be exclusively in such adjacent locations in fraction BN-2 however, as the hydrolysate contained some free xylose that could have arisen from hydrolysis of xylosylalditols as well as from xylose-containing oligosaccharides. The erythritol found in the hydrolysate originated from (1→4)-linked glucose residues, whereas the glycerol probably originated from internal and terminal pentose residues. Thus, the evidence of Smith degradation argues for a complex pattern of branching within this polysaccharide, and suggests that the general structural details here proposed are substantially correct.

A small proportion of 2-*O*-β-D-mannopyranosyl-D-erythritol was identified in the products of the Smith degradation. This represented a surprising finding, as the crude polysaccharide contained only a trace of mannose and it was not considered to be a constituent of the polymer. To be detected it was necessary that a large proportion of the mannose present be immune to periodate oxidation. It may arise from a contaminating glucomannan, or be the product of an adventitious epimerization.

Enzymic degradation of fraction BN-2 — Pectinase. Treatment of fraction BN-2 with a dialysed pectinase produced a disaccharide consisting of equimolar amounts of xylose and glucose. Reduction and hydrolysis gave xylose and giucitol, whereas methylation and hydrolysis afforded 2,3,4-tri-*O*-methyl-D-xylose and 2,3,6-tri-*O*-methyl-D-glucose.

Polarographic determination of periodate uptake[25,26] indicated that 5.3 moles of periodate were reduced per mole of disaccharide. The oxidation product was transformed into a mixture of short-chain alditols representing the carbon-chain fragments of the oxidizing sugars, and consisting of glycerol and ethylene glycol in

the approximate molar ratio of 1:2 (Table IV). Calculations of the results expected from disaccharides subjected to such a reaction sequence disclosed that under the formate-destabilizing conditions utilized[25–27], 17 possible basic disaccharide structures (that is, utilizing generalized pentose and/or hexose units, and not specific sugars) would consume 5 moles of periodate per mole. From these 17 structures, 8 could give rise to a 1:2 molar ratio of glycerol to ethylene glycol, but only 2 of these

TABLE IV

PERIODATE CONSUMPTION AND ANALYSIS OF DEGRADATION PRODUCTS AFTER PERIODATE OXIDATION OF DISACCHARIDES

Sugar	*Moles of periodate consumed per mole sugar; oxidation at pH 4*	*Molar ratio of ethylene glycol diacetate*	*Moles of periodate consumed per mole sugar; oxidation at pH 5.4*
	5.1	1.9:1	
Maltose	4.9	2.1:1	3.9
	4.9	1.9:1	
	5.0	2.1:1	
Lactose	4.9	1.9:1	
	5.1	2.0:1	
	4.9	2.1:1	
Cellobiose	4.9	2.0:1	3.8
	5.0	2.0:1	
Melibiose			5.3
Disaccharide from pectinase hydrolysate P(1→4)H	5.3	1:1.9	

8 structures contained both a pentopyranosyl and a hexopyranosyl residue; these were a pentopyranosyl-(1→3)-hexopyranose and a pentopyranosyl-(1→4)-hexopyranose[25]. Thus, the oxidation data supported the conclusion that the disaccharide had the xylose residue as the non-reducing component. The tentative ramifications of this type of analysis of linkage and sequence have been studied[25], and suggest that this analytical approach may yield significant structural information from hitherto unuseable quantities of oligomeric degradation products.

These data, together with the specific rotation, establish that the BN-2 disaccharide is 4-*O*-α-D-xylopyranosyl-D-glucopyranose. Thus, some of the glucose residues must be interspersed with (1→4)-linked xylose residues. This disaccharide has not previously been reported.

Hemicellulase. Dialysed hemicellulase was significantly more active than the crude enzyme. Fraction BN-2 afforded a complex mixture of mono- and oligosaccharides upon treatment with the dialysed enzyme (Table V). These data again indicate that there are regions in which the glucose and xylose residues are interspersed or alternating in the parent molecule.

TABLE V

COMPONENT SUGARS, DEGREES OF POLYMERIZATION, AND OPTICAL ROTATIONS OF PRODUCTS OF PURIFIED HEMICELLULASE HYDROLYSIS OF BN-2

Fraction	*Component sugars (relative amounts)*		*D.p.*	R_{Mal} *in solvent*			*Specific rotation, degrees (c in water)*
	Glucose	*Xylose*		(b)	(f)	(g)	
1	+	+	2	0.21	0.66	0.90	+60 (*c*, 0.3)
2	+	++	3	0.18	0.77	0.74	+44 (*c*, 0.3)
3	+	+	5	0.16	0.55	0.61	+32 (*c*, 0.25)
4	+++	+	6	0.092	0.40	0.41	+71 (*c*, 0.5)
5	+	+	7	0.085	0.32	0.24	+3 (*c*, 0.3)
6	+++	+			0.11	0.18	

Protein components. — The association of protein with the polysaccharide fractions isolated from barberry leaves was sufficiently tenacious to resist separation by selective extraction, fractional precipitation, complex formation, and derivatization. Moreover, the protein moiety of fraction BN-1 underwent a diminution in concentration only from 6.9% to 5.5% upon proteolysis with trypsin[28]. The proteins of lipopolysaccharide–protein mixtures have been reported to exhibit a similar resistance to enzymic proteolysis[29]. Thus, the data presented are consistent with either covalent bonding or a close physical association between the proteins and carbohydrates. Decisive evidence on this point is lacking. It is evident from the recent literature that the 5–10% of protein found in polysaccharide preparations from a variety of sources is attracting more interest as it becomes increasingly apparent that this protein cannot be removed completely by the fractionation procedures available. The presence of plant glycoproteins, which have been reported in kidney beans[30], soy bean flour[31], and the leaves of *Vicia faba*[32], may be much more widespread than realized.

EXPERIMENTAL

General. — Evaporations were conducted under diminished pressure at water bath temperatures of 35–45°. All melting points are uncorrected. Hydrolyses were conducted in 0.5M sulfuric acid for 6–10 h at 100°. Optical rotation measurements were conducted at 23 ±3° using a Hilger Standard Polarimeter, Mk III, or a Bendix Automatic Polarimeter, Type 143A.

The solvent systems employed in descending paper partition-chromatography with Whatman No. 1 paper were: (*a*) 3:1:1 butyl alcohol–ethanol–water; (*b*) 2:5:7 pyridine–ethyl acetate–water; (*c*) water-saturated phenol; (*d*) 5:5:3:1 ethyl acetate–pyridine–water–acetic acid; (*e*) 65:10:25 propyl alcohol–ethyl acetate–water; (*f*) 4:1:5 butyl alcohol–ethanol–water; (*g*)18:4:1:3 ethyl acetate–acetic acid–formic acid–water; (*h*) butanone–water azeotrope; (*i*) 200:49:15 (*v/v*) benzene–ethanol–water. Components were visualized by reaction with the following reagents: (*I*) silver

nitrate–sodium hydroxide, (*II*) *p*-anisidine hydrochloride, or (*III*) ninhydrin. The movement of sugars was reported relative to the solvent front (R_F), glucose (R_{G1}), 2,3,4,6-tetra-*O*-methyl-D-glucose (R_G), or maltose (R_{Mal}). Gas–liquid partition chromatography was carried out with a Pye–Argon Chromatograph (Pye and Co., England) (Instrument A) by using 4 ft by 0.25 in. o. d. straight glass columns with argon as carrier gas, or a F and M Model 402 Gas Chromatograph (Hewlett–Packard, U. S. A.) (Instrument B) by using 4 ft by 0.25 in. o.d. bent glass-columns with helium as carrier gas. *Columns 1* consisted of 10% (by wt.) LAC/4R-886 polyester on 60–80 mesh Chromosorb W, *column 2* of 3% (by wt.) SE-52 silicone gum on 60–80 mesh Chromosorb W, and *column* 3 of 5% LAC/4R-886 polyester on Chromosorb W, 60–80 mesh. Trimethylsilylation[33] was performed with Tri-Sil reagent (Pierce and Co., U. S. A.). G.l.p.c. of trimethylsilyl (Me_3Si) derivatives was conducted on Instrument B with *column 2* at 145° and a flow rate of 100 ml He per min. Methylations were continued until the i.r. spectrum showed negligible absorbance in the characteristic OH region (3500–3600 cm^{-1}). The electrophoretic mobilities of methylated sugars are quoted relative to that of glucose (M_G). Protein analyses were based upon elemental analyses, 15% nitrogen being regarded as equal to 100% protein.

Extraction of leaves. (1) *Spring crop.* — Leaves from mature, healthy specimens of *Berberis vulgaris* were collected in mid-May and were immediately immersed in Dry Ice–acetone. At the laboratory, the frozen leaves were transferred to boiling 90% aqueous acetone to denature the enzymes and were exhaustively extracted in a Soxhlet-type apparatus to remove pigments. The leaves were air-dried and powdered (yield, 1 kg).

The leaf powder was extracted exhaustively in the Soxhlet apparatus, first with water-saturated butyl alcohol (this extract was discarded), and then with 80% aqueous methanol (*methanol extract*). The residue was next extracted with three 2-liter portions of water, each for 24 h with continuous stirring at room temperature (*cold water extract*). The residual material from the cold water extraction was extracted with four 2-liter portions of water, each for 12 h, at 80° with stirring, (*hot water extract*).

(2) *Autumn crop.* — Leaves collected in mid-September were immediately frozen in the field and subjected to exhaustive sequential extraction with acetone and 1:1 chloroform–methanol. The air-dried residue (yield, 2 kg) was extracted with 1:1:1 phenol–acetic acid–water[5] for 10 min in a Waring blendor. The mixture was centrifuged and the protein-rich phenolic layer discarded. The residue was washed free of phenol with cold water and extracted in a Waring blendor with water at 80° until a Molisch test was negative. The leaf residue contained 13% of protein.

The residue from the hot water extraction was extracted twice with deoxygenated lime-water under nitrogen for 4 h at room temperature prior to one delignification with chlorite[34] at 60°. The chlorite holocellulose, 5.1% of which was insoluble in cold 72% (*w/w*) sulfuric acid, was extracted and fractionated according to Scheme 1. Isolates A and B were subsequently fractionated according to Scheme 2.

Investigation of selected fractions. (*I*) *Spring crop.* — *Methanol extract* (20.2 g). The carbohydrate moiety of this extract was separated from ninhydrin-positive

material by acetylation[35], with chloroform extraction, and deacetylation[36] of the chloroform-soluble acetate. Paper chromatography with solvents (*a*), (*b*), and (*c*), detected trace amounts of arabinose, xylose, rhamnose, and unidentified reducing material (reagents *I* and *II*) having lower R_F values. Preparative chromatography on Whatman 3MM paper by double development in solvent (*b*) afforded D-glucose [11 mg; $[\alpha]_D$ +50° (*c* 1.1, water) (lit.[34] $[\alpha]_D^{20}$ +52.7° in water)], which was converted[38] into *N*-*p*-nitrophenyl-β-D-glucosylamine, m.p. and mixed m.p. 184–185° (lit.[38] m.p. 184°), and D-fructose [5 mg, $[\alpha]_D$ −87° (*c* 0.5, water); lit.[37] $[\alpha]_D^{20}$ −92.4° in water], identified by reduction[39] and subsequent acetylation[40], which afforded approximately equimolar quantities of the hexaacetates of glucitol and mannitol, as indicated by g.l.p.c. (instrument A, column 1, 198°, flow rate 100 ml per min)[41,42].

Cold-water extract. After concentration, deproteinization with lead acetate[43], and removal of residual lead with hydrogen sulfide, this extract was poured into 3 vols of abs. ethanol, yielding 0.12 g of polysaccharide material having $[\alpha]_D$ +8.0° (*c* 1, M sodium hydroxide) and containing 12.5% protein, and galactose, glucose, arabinose, and rhamnose in the approximate molar ratio 5.0:1.1:5.0:1.5, respectively[44]. A trace of xylose also was present.

Hot-water extract (0.56 g; $[\alpha]_D$ +12.2° (*c* 1, 0.1M sodium hydroxide); 8.5% protein). No iodine-staining carbohydrate was present in the deproteinized[43] extract. Fractional precipitation with Cetavlon[45], Fehling's solution[46], ethanol[47], and acetic acid was unsuccessful. Paper chromatography of the hydrolysate, in solvents (*a*), (*b*), and (*c*), disclosed glucose, galactose, and arabinose in the approximate molar ratio[44] 1.1:1.0:1.2, respectively.

(2) *Autumn crop.* — *Isolate A* (75 g; 85% protein). Acetylation[20] yielded 80 g of product, 3.8 g of which was chloroform-soluble (Scheme 2). The chloroform solution afforded 1.5 g of acetone-soluble acetate (fraction A-1), leaving 2.2 g of acetone-insoluble acetate (fraction A-2). Fraction A-1 (0.35 g) was dissolved in 20 ml of acetone and 10 ml of ethyl ether, and was separated into 6 fractions by fractional precipitation with petroleum ether (b.p. 30–60°). Discrete fractions were collected by centrifugation. Fraction A-1-4 acetate, $[\alpha]_D$ +195° (*c* 1, 1:2 acetone–water), comprised 45.5% of the total precipitate.

Fraction A-1-4. Deacetylation[20] afforded a white, amorphous, protein-free solid (85 mg) having $[\alpha]_D$ +185° (*c* 1, 0.1M sodium hydroxide). Preparative chromatography (Whatman 3MM) of the acid hydrolysate in solvent (*b*) gave only D-glucose, $[\alpha]_D$ +49° (*c* 1, water)[27], m.p. and mixed m.p. 144–145° (lit.[37] m.p. 146°).

The mean degree of polymerization ($\overline{d.p.}$) was determined on a 50 mg sample by the method of Smith and Unrau[48,49], which gave a final mean value of 1.1 μg of formaldehyde per 400 μg of polysaccharide. Based on a molecular weight of 162 for the average glucose residue, and assuming that the terminal reducing residue produced 2 moles of formaldehyde, these data correspond to a $\overline{d.p.}$ of 135.

Fraction A-1-4 (10 mg) was fully methylated[50] by using methyl iodide (0.1 ml) and sodium (3 mg) in liquid ammonia (4 ml). Methanolysis (4% methanolic HCl, 14 h of reflux) of the product afforded a mixture of methyl glucosides that was

quantitatively resolved, after acetylation, by g.l.p.c. (instrument B, column 2, 165°, flow rate 100 ml/min) to give the following methyl ethers, in the relative amounts indicated: 2,3- (1 mole) and 2,6-di-*O*-methylglucose (5 moles), 2,3,6-tri-*O*-methyl glucose (23 moles), and 2,3,4,6-tetra-*O*-methylglucose (5 moles). Quantitation and retention times were established by using authentic standards.

Fraction A-2. Deacylation[20] yielded 1.1 g of polysaccharide, $[\alpha]_D$ −70° (*c* 1, M sodium hydroxide); 8.3% protein; approximately 50% of which was dialysable through standard (Visking) dialysis membranes. Paper chromatography of the acid hydrolysate in solvent (*b*) revealed xylose and trace amounts (<6% total) of glucose, arabinose, and glucuronic acid [based on R_F values in solvent (*d*)]. The xylose (20 mg; m.p. and mixed m.p. 143–144°; lit.[37] 145°) was isolated by chromatography (Whatman 3MM). G.l.p.c. of the Me_3Si derivative[33] revealed only xylose. This xylan had a $\overline{d.p.}$[49] of 48 based on the formation of 1.9 μg of formaldehyde per 400 μg of polysaccharide (duplicate 50 mg samples), an average molecular weight xylose for the residue of 132, and assuming that the reducing end-unit produced 1 mole of formaldehyde.

Fractionation of isolate B (*30* g). Fraction BA (9 g) was precipitated from an aqueous solution of B by the dropwise addition of a 5% aqueous solution of Cetavlon[45]. Fraction BN (20 g) was precipitated from the supernatant by addition of 3 vols. of ethanol, and was acetylated[20] twice to give 28 g of acetate, from which an acetone-soluble fraction (BN-acetate, 25 g) and acetone-insoluble fraction (ABN-acetate, 2.6 g, 25% protein) were obtained. The BN-acetate was fractionated by the method used for fraction A-1 to afford BN-1 (1.5 g; $[\alpha]_D$ +2°), BN-2 (19.6 g; $[\alpha]_D$ +20°), and BN-3 (1.5 g; $[\alpha]_D$ +44° (conditions as for fraction A-1).

Electrophoretic resolution of samples of BN-1, BN-2, and BN-3 was effected on glass paper[51,52] in 0.1M sodium borate (pH 10) and 0.1M sodium molybdate (pH 5)[14], by using 20–50 mA at 1–2 kV d.c. for 4 h[12,13]. Components were visualized by heating with 10% sulfuric acid in ethanol. Fraction BN-1 separated into a major, slow-moving component, and a minor component having a higher mobility. Fraction BN-2 afforded a single spot in both buffers, having the same mobility as the minor component of BN-1. Fraction BN-3 was imperfectly resolved, but had a major component whose mobility was slightly greater than that of BN-1.

Enzymolysis of 10-mg samples each of BN-1 and BN-2 with 2 mg of hemicellulase (Nutritional Biochemical Corp.) for 3 days at 37° in unbuffered aqueous solution gave distinctly different hydrolytic patterns. Paper-chromatographic analysis of the hydrolysate of BN-1 in solvents (*b*) and (*e*) revealed 3 components having R_{Gl} (solvent (*b*)) 0.51 (major), 0.42, and 0.38, whereas the hydrolysate of BN-2 afforded only one component, having R_{Gl} in solvent (*b*) of 0.40. Enzyme and substrate control-experiments were conducted concurrently.

Fraction BA was divided into fractions BA-1 and BA-2, based upon solubility in 4M sodium chloride. Attempts to purify fraction BA-1 using chromatography on DEAE-cellulose[10] and CM-Sephadex[11] were unsuccessful.

Fraction BN-1. Deacetylation[20] afforded 1 g of polysaccharide material, $[\alpha]_D$ +20° (*c* 1, water); 7.1% protein, the acid hydrolysate of which was shown by paper

chromatography in solvents (*b*), (*f*), and (*g*), to consist of xylose, glucose, arabinose, and galactose, in an approximate molar ratio[44] of 4.5:4.0:1.2:1.0, with trace amounts of mannose, rhamnose, and two unidentified components having R_{Gl}, in solvent (*b*), of 2.2, and 2.6, respectively. No components detectable by reagent *III* were present. Preparative paper-chromatography in solvent (*b*) afforded: D-glucose (11 mg), $[\alpha]_D$ +55° (*c* 1, H_2O), identified[37,38] by conversion into the characteristic *p*-nitroaniline derivative; D-galactose (5 mg), $[\alpha]_D$ +75° (*c* 0.5, water) (lit.[37] $[\alpha]_D$ +80.2° in water), characterized as the 2-methyl-2-phenylhydrazone[53], m.p. and mixed m.p. 183–184° (lit.[53] m.p. 186°); D-xylose (15 mg), $[\alpha]_D$ +16.5° (*c* 0.9, water) (lit.[37] $[\alpha]_D^{22}$ +18.2° in water), converted[54] into di-*O*-benzylidene-D-xylose dimethyl acetal, m.p. and mixed m.p. 210–211° (lit.[54] m.p. 211°); and L-arabinose, $[\alpha]_D$ +98° (*c* 1, water) (lit.[37] $[\alpha]_D^{25}$ +104° in water), converted[55] into L-arabinose 2,2-diphenylhydrazone, m.p. and mixed m.p. 193–194° (from hot aqueous pyridine) [lit.[55] 218–219.5°; 175° (ref. 56); 204–295° (ref. 57); 207–208° (ref. 58)].

Anal. Calc. for $C_{17}H_{20}N_2O_4$: C, 66.59; H, 6.37, N, 8.86. Found: C, 66.39; H, 6.35; N, 8.75.

A mean terminal value of 0.50 μg of formaldehyde per 400 μg of polysaccharide was produced, which corresponded[49] to a $\overline{\text{d.p.}}$ of 160, based on a molecular weight of 150 for the average sugar residue, and assuming that the terminal reducing unit produced 1 mole of formaldehyde.

Methylation study of fraction BN-1. — Fraction BN-1 (0.4 g) was subjected to one modified Kuhn[59], and three Purdie[60] methylations to give a methyl ether derivative having $[\alpha]_D$ +19° (*c* 1, chloroform) and a methoxyl content[61,62] of 41.4% (calc.: 42.2%). Table II records the composition of the mixture afforded the methylated polysaccharide upon formolysis and hydrolysis[63], as determined by analytical and preparative paper-chromatography in solvents (*g*), (*h*) and (*i*), and by derivatization; quantitation was by the phenol–sulfuric acid method[44].

2,3,4,6-Tetra-*O*-methylgalactose was identified by conversion into the crystalline aniline derivative, m.p. and mixed m.p. 189–190° (from ethyl acetate) (lit.[64], m.p. 192°), and by demethylation with boron trichloride[65] to give galactose. 2,3,4-Tri-*O*-methyl-D-xylose was isolated as a syrup having $[\alpha]_D$ + 15° (*c* 0.6, acetone) (lit.[66], $[\alpha]_D^{20}$ +18° in water) which reacted with aniline to form *N*-phenyl-2,3,4-tri-*O*-methyl-D-xylopyranosylamine, m.p. and mixed m.p. 100–101° (from ethyl acetate–petroleum ether) (lit.[67], m.p. 102°), and by demethylation[65] to xylose. 2,3,4-Tri-*O*-methylarabinose was converted into the methyl glycoside (3% methanolic hydrogen chloride 12 h at reflux) which was identified as methyl 2,3,4-tri-*O*-methyl-α,β-arabinoside[68] by g.l.p.c. (instrument B, column 1, 130°, flow rate 100 ml/min; retention time 4.8 min). Co-chromatography with a standard reference sample augmented the signal proportionately. Under the conditions cited methyl 2,3,5-tri-*O*-methyl-α,β-arabinoside gave 2 peaks having retention times of 2.2 and 3.1 min. Demethylation[65] afforded only arabinose. 2,3,6-Tri-*O*-methyl-D-glucose was obtained as a syrup ($[\alpha]_D$ +65° (*c* 0.7, acetone) (lit.[69] $[\alpha]_D$ +70° in water) which was demethylated[27] to glucose, and also converted by glycosidation and acetylation into methyl 4-*O*-acetyl-2,3,6-tri-*O*-

methyl-α,β-D-glucoside, which was identified by g.l.p.c. (instrument B, column 3, 170°, flow rate 100 ml/min; and co-chromatography with an authentic sample[18,70]). Syrupy 2,3-di-*O*-methyl-D-glucose, $[\alpha]_D$ +45° (*c* 0.5, acetone) (lit.[71] $[\alpha]_D^{25}$ +48° in acetone), was treated with aniline to give *N*-phenyl-2,3-di-*O*-methyl-D-glucosylamine, m.p. and mixed m.p. 132–4° (lit.[72], m.p. 134°). 2,3-Di-*O*-methyl-D-xylose had $[\alpha]_D$ +22° (*c* 2, acetone) (lit.[73] $[\alpha]_D^{20}$ +23° in water) and was converted into the crystalline aniline derivative, m.p. and mixed m.p. 144–146° (lit.[74] m.p. 144–146°). 2-*O*-Methyl-D-xylose was isolated as a syrup ($[\alpha]_D$ +28° (*c* 5, acetone)) (lit.[75], $[\alpha]_D^{20}$ +35° (*c* 1.1, H_2O)) which was converted in the usual way into the aniline derivative, m.p. and mixed m.p. 123–125° (from ethyl acetate) (lit.[75], m.p. 125–126°). Demethylation[65] gave only xylose.

Fraction BN-2. — Deacetylation of this component afforded 11 g of polysaccharide, $[\alpha]_D$ +22° (*c* 1.1, water); protein 6.9%. The protein content was decreased to 5.5% by incubation with trypsin (Nutritional Biochemicals Corp.) for 48 h at pH 6.5 and 37°. Paper-chromatographic analysis in solvent (*b*) of the acid hydrolysate indicated the presence of D-xylose[37], D-glucose[37,38], L-arabinose[37], and D-galactose[37,53], in the approximate molar ratio[44] 5.0:4.0:1.1:1.0, each of which was identified by physical properties and/or conversion to a crystalline derivative (see cited refs. for methods). In addition, traces of mannose and two unidentified components (R_{Gl} 2.2 and 2.6, respectively, in solvent (*b*)) were present. The $\overline{\text{d.p.}}$ value[49] of this polysaccharide was calculated to be 129 (0.62 μg of formaldehyde per 400 μg of polysaccharide) by using the same assumptions as for fraction BN-1.

Methylation study of fraction BN-2. — A 0.4 g sample of the BN-2 polysaccharide was methylated[59,60] as already described. The product had $[\alpha]_D$ +10° (*c* 1, chloroform), OMe 40.0% (theoretical 42.1%)[61,62]. The methylated product was investigated by the methods described for fraction BN-1 and the results are given in Table II. The 2-*O*-methylglucose gave only glucose upon demethylation, and was identified on the basis of its electrophoretic mobility, M_G 0.22, in borate buffer (pH 10) (1000 V, 40 mA d.c. for 1 h[13]), which was indistinguishable from that of authentic 2-*O*-methyl-D-glucose and very different from those of authentic 6-*O*- and 3-*O*-methylglucose standards. The identity of all other components was confirmed by specific rotation, conversion to the aniline derivatives, and demethylation to the parent sugars, as described for fraction BN-1.

Smith degradation of fraction BN-2. — A portion of fraction BN-2 was subjected to a Smith degradation[24] by using 0.25M sulfuric acid for 8 h at room temperature for the hydrolysis of the reduced "polyaldehyde". Paper-chromatographic analysis of the product in solvents (*e*), (*h*), and (*i*) revealed 9 components, of which ethylene glycol, glycerol, erythritol, xylose, and glucose were identified on the basis of mobilities, and colors with reagents *I* and *II*. The components were identified as follows, after isolation by preparative paper-chromatography. D-Glucose, $[\alpha]_D$ +50° (*c* 0.2, water) was converted into the Me_3Si derivative[33], g.l.p.c. of which gave two peaks having retention times identical with those of an authentic standard. D-Xylose, $[\alpha]_D$ +15° (*c* 0.3, water) was identified as its Me_3Si derivative in the same way. Erythritol,

m.p. and mixed m.p. 119–120° (from methanol) (lit.[76], m.p. 122°) gave a crystalline acetate[35], m.p. and mixed m.p. 84–85°. (lit.[77], m.p. 85° for tetra-*O*-acetylerythritol). G.l.p.c. of the Me_3Si derivative gave a single peak indistinguishable from that of an authentic standard. G.l.p.c. of glycerol triacetate[35], (instrument B, column 4, 120°, flow rate 75 ml/min) gave a single peak having a retention time identical with that of an authentic standard. The glycerol also was converted into the Me_3Si derivative and identified by g.l.p.c. in the usual manner. Ethylene glycol gave an acetate[35] g.l.p.c. of which (instrument B, column 3, 130°, flow rate 75 ml/min) revealed a single peak having a retention time identical with that of authentic ethylene glycol diacetate.

2-*O*-β-D-Glucopyranosyl-D-erythritol (11 mg) had $[\alpha]_D$ +12° (*c* 1, water) and m.p. and mixed m.p. 189–190° (lit.[78], $[\alpha]_D$ +17° in water, m.p. 192°). The oxidation of a 0.1-mg sample with sodium periodate sodium in acetate–acetic acid buffer (pH 4) at 4° in the dark was determined polarographically[79,27] to be complete after 8 h and to have required 3.1 moles of periodate per mole of glucoside (theoretical: 3.0 moles of periodate per mole of glucoside). The acid hydrolysate of the glucoside was found by paper chromatography and g.l.p.c. (of Me_3Si derivatives) to consist of equimolar quantities of glucose and erythritol. Methylation[80] and hydrolysis (Duolite C-20) afforded 2,3,4,6-tetra-*O*-methylglucose as the only product detectable with reagent *II* on paper chromatograms.

2-*O*-β-D-Mannopyranosyl-D-erythritol (3 mg), $[\alpha]_D$ −27° (*c* 0.3, water) (lit.[80] $[\alpha]_D^{20}$ −35° in methanol) was hydrolysed (Duolite C-20) to afford equimolar amounts of mannose and eythritol, as determined by paper and g.l.p.c.[33]. A 0.12-mg sample consumed 2.9 of periodate per mole (theoretical: 3.0 moles periodate per mole), as determined polarographically[27].

Three minor components having R_G 0.12, 0.08, and 0.06, respectively, in solvent (*b*) were isolated in quantities too small for individual investigation. These components exhibited R_{Gl} 0.12, 0.08, and 0.06, respectively, in solvent (*b*), which corresponded approximately to the values for maltopentaose to maltooctaose. Hydrolysis of the combined fractions with Duolite C-20 in a sealed tube for 30 h at 100° gave glucose, xylose, and erythritol but no glycerol. These components were identified by paper chromatography in solvents (*b*), (*g*), and (*h*).

Partial degradations of fraction BN-2. — Dilute acid. A sample (0.05 g) of fraction BN-2 was heated in a steam bath in a sealed tube with 0.025M sulfuric acid for 8 h. Analysis by paper and g.l.p.c. (of the Me_3Si derivatives) in the usual manner detected only galactose, arabinose, and xylose as free sugars.

Pectinase. Crude fungal pectinase (Nutritional Biochemicals Corp.) contained glucose and a minor carbohydrate component of low R_F value. On prolonged acid hydrolysis the latter yielded a compound that was identified chromatographically as galacturonic acid.

Pectinase (0.2 g) was incubated for 24 h at 37° with 100 ml of aqueous solution[81] containing a total of 1 g of a 5:5:1:1 (by weight) mixture of glucose, xylose; arabinose, and galactose, respectively. No oligosaccharide synthesis was detected upon paper chromatographic analysis [solvents (*a*) and (*b*)] of the reaction solution. Similar

experiments were conducted for 48 and 72 h upon the monosaccharides with each of maltose, lactose, cellobiose, xylobiose, amylose, and a crude xylan (Nutritional Biochemicals Corp.), but neither transglycosylation nor synthesis of disaccharides was observed by paper chromatography. An enzyme control-sample was incubated concurrently. A sample (1 g) of fraction BN-2 was incubated for 168 h at 37° with 0.2 g of pectinase in pre-washed dialysis bags (Visking Corp.)[82]. The dialysate was removed and examined at regular intervals. A total weight of 0.92 g of mono- and oligosaccharides was recovered. The hydrolysate was resolved by paper chromatography [solvents (*b*), (*d*), and (*f*)]. The major component, R_{Gl} 0.7 in solvent (*b*), was identified as 4-*O*-α-D-xylopyranosyl-D-glucose based on the following evidence.

Identification of 4-O-α-D-*xylopyranosyl*-D-*glucose*. Separation by preparative paper chromatography afforded 18 mg of a colorless, crystalline compound, m.p. 101–102°; $[\alpha]_D$ +80° (*c* 1, water). Chromatographic analysis [paper, and gas–liquid (of the Me_3Si derivatives)] of a hydrolysate revealed equimolar amounts of xylose and glucose. Similar analysis of the hydrolysate of the borohydride-reduced compound[39] revealed the presence of equimolar amounts of xylose and glucitol. Paper chromatography [solvents (*f*), (*h*), and (*i*)] of the hydrolysate of the fully methylated derivative[83,60] detected only 2,3,4-tri-*O*-methylxylose and 2,3,6-tri-*O*-methylglucose. This hydrolysate was converted into a mixture of methyl *O*-acetyl-*O*-methylglycosides and analysed by g.l.p.c. (instrument B, column 2, 165°, flow rate 100 ml/min). Three peaks were detected, two of which corresponded to the α- and β-anomers of authentic methyl 4-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucoside.

Polarographic determination of periodate consumption. Solutions containing 2.0–0.2 μmole of each of maltose, cellobiose, lactose, and the BN-2 disaccharide, in 50–25 ml of 0.01M sodium acetate–0.1M acetic acid buffer (pH 4) were oxidized with sodium periodate (initial molar ratio, 10:1 periodate:carbohydrate). The consumption of periodate was measured polarographically as described by Corlett *et al.*[27] The oxidations were repeated in unbuffered solutions (pH 5.4) with maltose, cellobiose, and melibiose. The results are summarized in Table IV.

Analysis of alditol fragments from oxidized disaccharides. Standard solutions containing ethylene glycol diacetate and glycerol triacetate in molar ratios of 2.2, 1.5, and 0.85 were resolved by g.l.p.c. (instrument B, column 1, 130°, flow rate 100 ml/min), which indicated mole ratios of 2.1, 1.4 and 0.76, respectively. Peak areas were measured by triangulation. Ethylene glycol diacetate had a retention time of 1 min and glycerol triacetate a time of 8 min.

The polarographic-cell contents from each of the buffered disaccharide oxidations was evaporated to a small volume, adjusted to pH 7 (0.1M sodium hydroxide) and reduced with 0.02 g of sodium borohydride for 4 h. The solution was acidified with acetic acid, evaporated to dryness, and the residue hydrolysed with 5 ml of 0.5M sulfuric acid for 4 h on a steam bath. The hydrolysate was neutralized (0.1M sodium hydroxide) and reduced with borohydride as before. After acidification (acetic acid) and evaporation to dryness, the residue was acetylated for 12 h at room temperature with acetic anhydride (10 ml) containing 4 drops of conc. perchloric acid.

The reaction solution was poured into cold water and after 2 h was extracted with chloroform. The chloroform extract was washed with aqueous sodium hydrogen carbonate and water, and dried (sodium sulfate). The chloroform was removed by evaporation and the residue analysed by g.l.p.c. with the procedure already described. The results are recorded in Table IV. Suitable control experiments were performed with each analysis[25,27].

Hemicellulase. Crude hemicellulase (Mann Research Laboratories Inc.) acted very slowly upon fraction BN-2 and yielded only monosaccharides. After dialysis (4 days against running distilled water), freeze drying yielded 0.35 g of a yellow powder (designated *purified hemicellulase*) from 10 g of crude material. The purified hemicellulase did not synthesize detectable amounts of oligosaccharides when tested as already described for pectinase.

Fraction BN-2 (1 g) was incubated for 180 h as just described, with 0.25 g of purified hemicellulase[82]. The hydrolysate was resolved into mono- and oligosaccharide fractions by paper chromatography in solvents (*b*), (*d*), (*f*), and (*g*). The results are recorded in Table V. The $\overline{\text{d.p.}}$ of oligomeric components was measured from the decrease in aldose content concomitant with borohydride reduction, as determined by the phenol–sulfuric acid method[44]. Fractions 1 through 5 were homogeneous. Fraction 6 consisted of at least three compounds. Oligosaccharide components were determined, after acid hydrolysis, by paper chromatography in solvents (*b*), (*f*), and (*g*) with reagents *I* and *II*, and by g.l.p.c. of the Me_3Si derivatives.

Fraction BN-3, was deacetylated to yield 0.8 g of polysaccharide, $[\alpha]_D$ +40° (*c* 1, 0.1M sodium hydroxide); 5.2% protein. Paper-chromatographic analysis of the acid hydrolysate in solvents (*b*), (*e*), and (*f*) revealed glucose, xylose, galactose, and mannose, in the approximate molar ratio[44] 7:7:1:2. The $\overline{\text{d.p.}}$ was determined[49] to be 182 (0.44 μg of formaldehyde per 400 μg polysaccharide) when calculated as for BN-1.

Methylation of fraction BN-3. — A sample (0.10 g) of fraction BN-3 was methylated[84] for 2.5 days. Hydrolysis[63] in the usual manner afforded 60 mg of methylated monomers, which were qualitatively and quantitatively determined as described for fraction BN-1. The results are summarized in Table II.

Periodate oxidation of fractions BN-1, BN-2 and BN-3. — Duplicate 50-mg samples were oxidized with 0.25M sodium periodate[85] at 4–5° in the dark. A blank experiment was conducted concurrently. The consumption of periodate[86] was complete after 48 h. Formic acid was determined by titration with 0.01M sodium hydroxide under nitrogen to a phenolphthalein endpoint[87]. The results are summarized in Table III.

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