STRUCTURAL INVESTIGATION OF WATER-SOLUBLE, RAPE-SEED (*Brassica campestris*) POLYSACCHARIDES

PART I. RAPE-SEED AMYLOID*

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ABSTRACT

Sedimentation analysis indicated that the amyloid isolated from water-soluble, rape-seed polysaccharides was homogenous. Methylation studies showed that the polysaccharide had a highly branched structure. Hydrolysis of the methylated polysaccharide yielded 2,3,4,6-tetra-*O*-methyl-D-glucose (1 mol.); 2,3,4,6-tetra-*O*-methyl-D-galactose (3 mol.); 2,3,4-tri-*O*-methyl-D-xylose (5 mol.); 2,3,6-tri-*O*-methyl-D-glucose (5 mol.); 2,3,4-tri-*O*-methyl-D-glucose (2 mol.); 3,4-di-*O*-methyl-D-xylose (1 mol.); and 2,3-di-*O*-methyl-D-glucose (8 mol.). Periodate-oxidation data substantiated the methylation results. A possible structure for the amyloid is presented.

INTRODUCTION

There have been several investigations in recent years on the polysaccharides of agricultural commodities, especially those present in seeds[1-14]. The structural elucidation of seed carbohydrates is important to the plant breeder, the food processor, and the nutritionist. It also provides a basis for understanding some aspects of their biosynthesis and biological functions.

Rape seed or turnip rape (*Brassica campestris*) was chosen because of its growing importance to Canadian agriculture and because of the fact that a knowledge of its carbohydrate constituents would permit a direct comparison with soya beans, a rival seed, the polysaccharides of which have been the subject of extensive studies and a recent review[15].

The investigation has led to the recognition of a number of polysaccharides in rape, and the study of rape-seed amyloid forms the basis of the present publication. The term amyloid, obviously a misnomer, was designated by earlier workers[16,17] in view of the characteristic, blue, iodine-staining properties exhibited by these substances. Two amyloids have been investigated in detail to date. That from tamarind seed is the most extensively studied and has been reported under such names as tamarind seed polysaccharide, pectin, jellose[18], polyose, tikernose[19], tamarindus

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amyloid[20], and tamarind kernel polysaccharide[21] (TKP). The second, or nasturtium amyloid[22], has been the subject of a recent publication. These polysaccharides, which consist primarily of D-glucose, D-galactose, and D-xylose, appear to be widely distributed in seeds[23]. The rather vague, or possibly misleading, names assigned to these polysaccharides seem inappropriate. It would perhaps be useful if they were assigned to a separate class.

RESULTS AND DISCUSSION

A sample of rape seed (*Brassica campestris*), after removal of coats, oil, and oligosaccharides, etc., yielded an 80% alcohol-insoluble residue. The residue, on extraction with hot water, gave material which was freed from extraneous amounts of proteins by partial precipitation with ethanol followed by phenol–water partition[24]. The two polysaccharide fractions, I and II (yield *ca.* 0.14 and 0.11% based on the 80% alcohol-insoluble residue), thus recovered were essentially free from proteins and showed, on acid hydrolysis, the same sugar components; namely, glucose, galactose, arabinose, xylose, and small proportions of mannose and galacturonic acid.

Fractionation of polysaccharide I on DEAE cellulose (borate-form)[25] yielded, *inter alia*, a fraction eluted with water in a yield of ~3.7% of fraction I or 0.005% of the 80% alcohol-insoluble residue. This fraction, on the basis of its sugar components, characteristic iodine-staining properties, $[\alpha]_D$ value, and infrared spectrum, appeared to belong to the group of polysaccharides currently designated as seed amyloids, and hence will be referred to as rape-seed amyloid.

The rape-seed amyloid showed a single symmetrical peak on sedimentation[26] analysis and appeared to be a homogeneous polysaccharide. Acid hydrolysis produced glucose, xylose, galactose, and arabinose in molar ratios of 16:6:3:1. The ratios of glucose, xylose, and galactose (5.3:2:1) and $[\alpha]_D$ value of +88° for the rape-seed amyloid compare favorably with those for other amyloids: tamarind kernel polysaccharide[21] 4:2:1, $[\alpha]_D$ +75°; jellose[18] and tikernose[19] 3:2:1; tamarindus amyloid[20] 4:3:1, $[\alpha]_D$ +79.6°; and nasturtium amyloid[22] 3:2:1, $[\alpha]_D$ +83 ±5°.

The polysaccharide was methylated first by the Haworth method, then by the Purdie method, and finally by the method of Srivastava *et al.*[27]. The fully methylated polysaccharide was subjected to methanolysis and hydrolysis, and the proportions of the major *O*-methyl sugars were determined by a combination of paper, thin-layer, and gas–liquid chromatography. The following approximate molar ratios were determined: 2,3,4-tri-*O*-methyl-D-xylose, 5; 2,3,4,6-tetra-*O*-methyl-D-glucose, 1; 2,3,4,6-tetra-*O*-methyl-D-galactose, 3; 2,3,6-tri-*O*-methyl-D-glucose, 5 or 6; 2,3,4-tri-*O*-methyl-D-glucose, 2; 3,4-di-*O*-methyl-D-xylose, 1; 2,3-di-*O*-methyl-D-glucose, 7 or 8.

Of these sugars, 2,3,4-tri-*O*-methyl-D-xylose and 2,3,6-tri-*O*-methyl-D-glucose were obtained crystalline, and 2,3,4,6-tetra-*O*-methyl-D-galactose was identified as the aniline derivative. The presence of 2,3,4-tri-*O*-methyl-D-glucose was shown by selective furanoside formation[28], and 2,3-di-*O*-methyl-D-glucose was characterized as the methyl α-glycoside. The remaining components, 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-xylose, were identified by their paper-chromatographic and electrophoretic behavior and by comparison of their infrared spectra with those of authentic specimens.

All the galactose was accounted for as non-reducing terminal units and therefore could not have arisen from an admixed galactan. Out of the 1 mol. of arabinose detected in the unmethylated polysaccharide, only 0.2–0.3 mol. was detected in the form of 2,3,5-tri-*O*-methyl-L-arabinose, with indications that the 2,3-dimethyl and 2- or 3-monomethyl ethers of arabinose were also present as part of the minor, methylated fractions. This suggests that arabinose was not an integral part of the amyloid molecule, since otherwise a much larger structural unit is waranted than could be envisaged on the basis of the arabinose, galactose, xylose, and glucose ratios of 1:3:6:16. It is therefore inferred that arabinose arose from a small proportion (*ca.* 4%) of an arabinan or arabinogalactan contaminant present in the amyloid. The assumption seems reasonable in view of the conflicting reports[18–22] in the literature concerning the occurrence of arabinose units in amyloids.

The ratio of galactose–xylose–glucose (*ca.* 1:2:5.3) for the methylated polysaccharide was in excellent agreement with that found for the original polysaccharides.

The methylation analysis showed that the molecule has a branched structure consisting of a backbone of (1→4)-linked D-glucose residues to which are attached unbranched side-chains of D-glucose, D-galactose, and D-xylose. From the methylation data, it is concluded that an average unit of the polysaccharide is made up of 25 sugar residues consisting of 9 terminal, non-reducing end-groups, comprising 3 residues of D-galactose, 5 of D-xylose, and 1 of D-glucose. There are 8 residues of D-glucose at which branching (through 4 and 6 positions) occurs in the polysaccharide. The remaining 8 non-terminal residues consist of two (1→6)-linked D-glucose, one (1→2)-linked D-xylose, and five (1→4) linked D-glucose residues.

By analogy with other amyloids[18–22], it is assumed that the glucosidic bonds in the polysaccharide were predominantly of the β-D type. This conclusion is supported by comparison of the infrared spectrum of rape-seed amyloid with infrared spectra of tamarind kernel polysaccharide[19], tamarind fraction A polysaccharide[29], and tikernose[19]. All these spectra showed strong absorption at 894 ± 7 cm^{-1}, indicative of β-D linkages[30], and were superimposable with one another, with the exception that the spectrum for rape-seed amyloid showed an additional minor peak at 865 cm^{-1}. Since α-(1→4)-, α-(1→6)-, and α-(1→4,6)-linked D-glucopyranose units show[30] characteristic absorption t 840 ± 8 cm^{-1}, the presence of such linkages seemed unlikely. This conclusion was supported by the resistance of the polysaccharide to the action of amylases and by the fact that the rape-seed amyloid failed to cross-react with anti Type VII *Pneumococcus* horse serum. Polysaccharides containing D-glucose units linked through α-(1→4), α-(1→6), and α-(1→4,6)-linkages show cross-reaction with the above serum[31,32]. However, on the basis of the peak at 865 cm^{-1}, the presence of some α-D links of the non-glucose type is indicated. In conformity with this conclusion are observations of Hsu and Reeves[22], indicating at least one α-(1→6) linkage of a D-xylose side-chain in nasturtium amyloid, and the observation of

Srivastava *et al.*[21], showing that the majority, if not all, of the D-xylose units in TKP were linked to the D-glucose backbone through α-(1→6) linkages. On the basis of the methylation data and by analogy with other amyloids, especially TKP[21], the structure (one of many alternatives) shown in Fig. 1 is assigned to the rape-seed amyloid, based on 25 sugar residues.

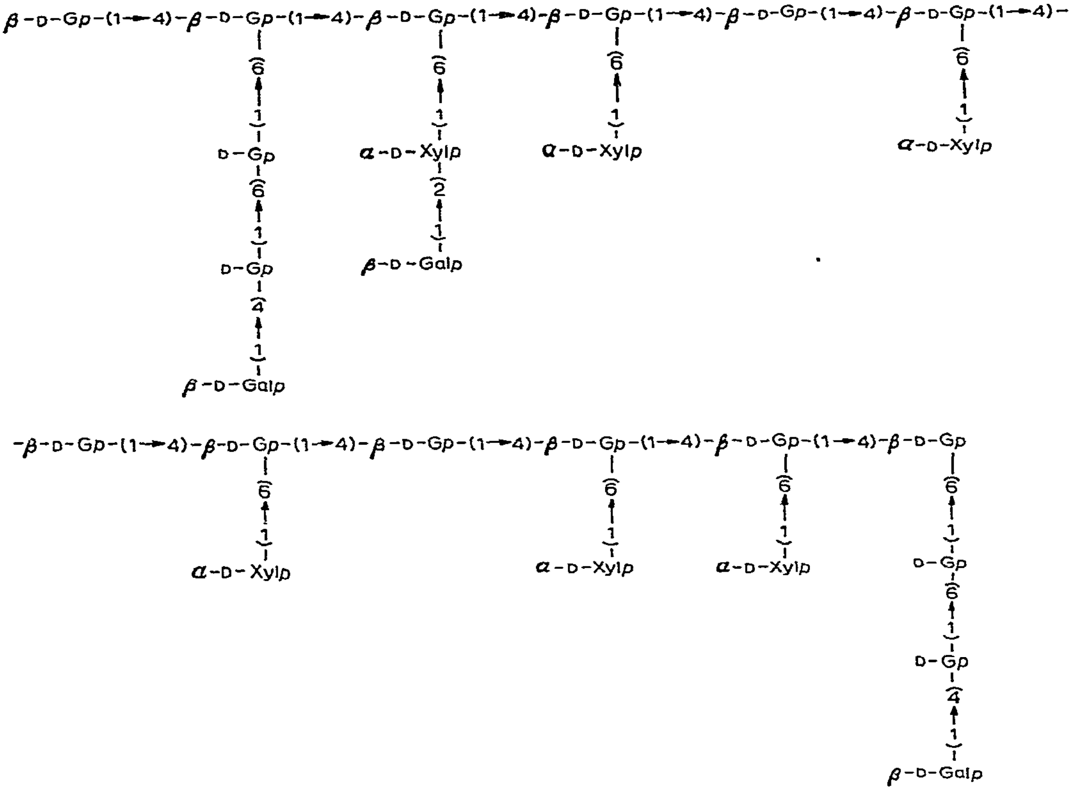

Fig. 1. A possible structure for rape-seed amyloid.

The structure (Fig. 1) is in admirable agreement with the results of periodate oxidation. It requires a periodate consumption of 1.4 moles, with the liberation of 0.44 mole of formic acid per "anhydro sugar" residue, and also shows that none of the residues are resistant to periodate. The experimentally determined amounts of periodate consumed and formic acid liberated were 1.2 moles and 0.48 mole, respectively, and analysis of the oxidized polysaccharide revealed that none of the sugar residues were resistant to periodate. The oxopolysaccharide, following Smith degradation[33], yielded glycol, glycerol, and erythritol in the molar ratios of 1.03:1:1.8 and also revealed small amounts of glyceraldalyde (not determined quantitatively). The theoretical molar ratios of these components based on the proposed structure would be 1:1:2.1 and 0.04 mole of glyceraldalyde (arising from 2-*O*-substituted D-xylose residues).

TABLE I

SUGARS (MOL.) OBTAINED ON HYDROLYSIS OF METHYLATED AMYLOIDS

Sugar	*Tamarind amyloid*				*Nasturtium amyloid*	*Rape-seed amyloid*
	Ref. 18	*Ref. 19*	*Ref. 20*[a]	*Ref. 21*	*Ref. 22*[a]	*Present study*
2,3,4,6-Tetra-*O*-methyl-D-glucose	—	—	—	—	—	1
2,3,4-Tri-*O*-methyl-D-xylose	1.06	1.12	2	3	1	5
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.07	1.1	1	2	1	3
2,3,5-Tri-*O*-methyl-L-arabinose	—	—	—	1	—	—
3,4-Di-*O*-methyl-D-xylose	1.02	1.03	1	1	1	1
2,3,6-Tri-*O*-methyl-D-glucose	0.86	1.01	1	2	1	5
2,3,4-Tri-*O*-methyl-D-glucose	—	—	—	—	—	2
2,3-Di-*O*-methyl-D-glucose	1.96	1.81	3	6	2	8

[a]Calculated on the basis of structure proposed.

The distinction between the various amyloids (Table I) is, on the whole, minor, and is mainly based on differences in the ratio of the sugar moieties. Further significant variations noted in the present study were in the presence of 2 mol. of D-glucose residues linked (1→6) and 1 mol. of D-glucose as a non-reducing end-group. Another variation noted was the failure of the rape-seed amyloid to produce a cross-reaction with anti Type VII *Pneumococcus* horse serum. This failure, in direct contrast to the behavior of tamarind fraction A polysaccharide[29] and tamarind kernel polysaccharide[21], is possibly due to the lack of multiplicity of D-galactose non-reducing end-groups, a characteristic which has been shown to be responsible for the cross-reaction[34-36].

The presence of cellulose-containing mucilages in such seeds as flax, white mustard, and quince is well known[37]. Characteristically, these materials contain cellulose solubilised in some fashion by the presence of other sugar moieties. For instance, in some cases, it has been claimed that solubilisation may occur by non-covalent encapsulation of cellulose[38]. In the case of rape-seed amyloid and tamarind seed polysaccharide, however, there are side chains of covalently linked sugars. Irrespective of the reason, the rather unusual properties of either type of solubilised cellulose offer possibilities for exploitation by industry. Biologically, such polysaccharides and polysaccharide complexes may have some quite specific and unique role to play in the development of certain plants.

EXPERIMENTAL

Paper chromatography was performed by the descending method on Whatman No. 1 paper with the organic phases of (*A*) ethyl acetate–pyridine–water (8:2:1); (*B*) butanone, saturated with water containing 2% of ammonia; (*C*) butyl alcohol–pyridine–water (10:3:3). Paper electrophoresis[39] was performed on Whatman No. 3MM paper with borate buffer (pH 10), at 800 volts for 2–3 h. Sugars were detected on paper chromatograms and electrophoretograms with aniline hydrogen phthalate (*A*). Thin-layer chromatography (t.l.c.) was performed on silica gel G, with detection by spraying with 5% of sulphuric acid in ethanol and charring. Rotations were measured on a Perkin–Elmer 141 polarimeter and are equilibrium values. Evaporations were carried out at 35° on a rotary evaporator. Melting points are corrected.

Isolation of the polymer. — A large-scale extraction of coat-free (Crippen Model, S Scarifier and Huller)*, oil-free (hexane extraction) rape-seed meats (3164 g), with boiling 80% aqueous ethanol gave a soluble fraction (622 g) and an insoluble residue (2368 g).

The alcohol-insoluble residue (6554 g), from two large-scale extractions, on extraction with hot water (116 l) in a Waring Blendor produced, on filtration, an insoluble residue (4981 g) and a soluble, turbid extract. The extract was concentrated 11-fold and, after clarification with alcohol (0.3 vol.), yielded a protein fraction (261 g) and a supernatant fraction which, on addition of alcohol (4 vol.), yielded the crude polysaccharide material (131 g; N, 7.03%; ash, 23.5%).

Removal of protein. — A 5% aqueous solution of a portion (123 g) of the crude polysaccharide was deproteinised[24] with 90% aqueous phenol, yielding a protein fraction (52.5 g) and a polysaccharide-containing supernatant fraction which, on addition of ethanol (3 vol.), yielded two fractions. The 70% alcohol-insoluble fraction I was deionized with Rexyn 101 (H^+) resin, reprecipitated with ethanol (3 vol.), dialysed, and freeze-dried to yield a polysaccharide (9.4 g), which showed approximately 100% conversion into sugars, measured as glucose, after hydrolysis (M sulphuric acid, 3 h, 100°). The 70% ethanol-soluble fraction II (7.1 g) recovered after removal of ethanol, deionization (Rexyn 101, H^+), and freeze-drying showed N, 0.3%; and ash, 1.5%.

Paper chromatography of the hydrolysates prepared from fractions I and II showed the same sugar components, namely, galactose, glucose, arabinose, xylose, and small proportions of mannose and galacturonic acid.

Enzymic hydrolyses. — The polysaccharide fraction I (30 mg) in water (3 ml) was incubated with an amylolytic enzyme, HT-1000 (Miles Laboratories) (4–5 mg) for 3 h at 60 ±5°. After three deproteinizations (chloroform–pentyl alcohol 9:1), the solution was mixed with alcohol (3 vol.), and the precipitate was recovered by centrifugation. The supernatant solution, after concentration, showed no discernible spots corresponding to glucose, maltose, maltotriose, etc. on paper chromatography in solvent *C*. Samples of polysaccharide fraction I (10 mg) were also incubated with amyloglucosidase (Sigma Chemical Company, 1 mg) in 0.2M acetate buffer (pH 4.5, 1 ml) for 48 h at room temperature and with alpha-amylase (Worthington Biochemical Corporation, 20 μl) in 20mM sodium phosphate, containing 6mM sodium chloride, for 24 h at room temperature. After deproteinisation and addition of alcohol as above, the 70% alcohol-soluble fraction showed no hydrolysis. Acid hydrolysis of the 70% alcohol-insoluble material from the three digests produced the same

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*Crippen Manufacturing Company, Alma, Michigan, U.S.A.

sugars, in the same proportions, as the original material. Control experiments with the three enzyme systems and soluble Lintner starch as the substrate showed extensive hydrolysis to glucose, maltose, maltotriose, etc.

Fractionation of the polysaccharide (Fraction I). — The polysaccharide (7.52 g) was fractionated on a large column (4 × 12 inches) of DEAE cellulose (borate form)[25]. Elution with water (4.5 l), followed by 50mM sodium metaborate (5.5 l), 0.3M sodium metaborate (9 l), and 7% urea solution (7 l) yielded four fractions. The water-eluted fraction was concentrated, acidified with acetic acid, dialysed for 16 h against running tap water and 4 h against two changes of distilled water, and freeze-dried to yield 0.28 g of material.

The borate and urea fractions were adjusted to pH 7, concentrated, acidified with acetic acid, and dialysed for 36 h against running tap water. The dialysates, after concentration and stirring with Rexyn 101 (H^+) resin, were mixed with ethanol (4 vol.), and the precipitates were recovered by centrifugation. The precipitates were dissolved in water, centrifuged, dialysed for 16 h against running tap water and for 4 h against two changes of distilled water, and finally recovered, after concentration, by freeze-drying. The 50mM and 0.3M borate and 7% urea fractions yielded, respectively, 4.87 g, 0.386 g, and 0.063 g of material.

Acid hydrolysis of the water-eluted fraction (rape-seed amyloid) showed glucose, galactose, arabinose, and xylose. The hydrolysates of the three remaining fractions yielded the same sugars as the parent polysaccharide, namely galactose, glucose, arabinose, xylose, and small proportions of mannose and galacturonic acid.

Analysis of rape-seed amyloid. — The rape-seed amyloid showed $[\alpha]_D^{22}$ +88.1° (*c* 0.58, water). Sedimentation analysis[26], using a synthetic boundary cell, of a 0.3% solution in 0.1M sodium tetraborate (pH 9.1) at 44,770 rev./min showed a single, symmetrical peak. The polysaccharide (5.0 mg) was hydrolysed with M sulphuric acid (0.5 ml) for 3 h at 100°. The sugars were recovered and determined quantitatively according to the modified Nelson–Somogyi method[40], giving ratios of glucose–galactose–arabinose–xylose of 16:3:1:6. The infrared spectrum (potassium bromide disc) of the polysaccharide (1 mg/100 mg of KBr) had absorption bands at 895, 965, 755, 945, 1630, and 2900 cm^{-1}.

Immunodiffusion analysis was carried out on microscope slides coated with 1% of Noble agar in physiological saline by the double-diffusion precipitin technique of Crowle[41]. Replicate analyses against anti Type VII *Pneumococcus* horse serum, using rape-seed amyloid, tamarind kernel polysaccharide, and tamarind fraction A polysaccharide[29], each at a concentration of 1 mg/ml, showed precipitin bands in the case of TKP[21] and tamarind A and no reaction with rape-seed amyloid.

Methylation of rape-seed amyloid. — The rape-seed amyloid (250 mg) in water (10 ml) was methylated, with cooling and in an atmosphere of nitrogen, by adding 30% aqueous sodium hydroxide (15 ml) and methyl sulphate (5 ml). The reagents were added at such a rate that the reaction mixture remained at a nearly neutral pH for the first few hours. The additions took place over several hours, after which the solution was stirred for 24 h. After ten such additions during ten days, the reaction mixture was cooled and adjusted to pH 7 with glacial acetic acid, and the solution was extracted with chloroform (3 × 300 ml). The dried (Na_2SO_4) extract was filtered and evaporated to a syrup. The dried product (290 mg) was dissolved in methyl iodide (35 ml), and silver oxide (3 g) was added in 3 equal portions during 24 h. After six such methylations, a product (284 mg) was recovered which still showed a small but definite i.r. hydroxyl band. This peak was not depressed after seven further methylations by Purdie's method.

Consequently, the partly methylated amyloid (275 mg) was dissolved in methyl sulphoxide (10 ml) to which crushed pellets of sodium hydroxide (3 g) were added, followed by methyl sulphate (1.5 ml) during 5 h. The solution was stirred for 24 h before excess methyl sulphate was decomposed by heating for 1 h at 90°. Water was added to dissolve the sodium hydroxide, the pH was adjusted to 7, and the solution was extracted 3 times with equal volumes of chloroform. The chloroform extract yielded a syrup which was methylated once more by the above procedure, giving a product (270 mg), $[\alpha]_D^{24}$ $+64.4°$ (*c* 0.72, chloroform), which showed no hydroxyl absorption in its infrared spectrum.

Methanolysis and hydrolysis of the methylated amyloid. — The methylated amyloid (270 mg) was dissolved in 2% methanolic hydrogen chloride (35 ml), and the solution was refluxed for 19 h, neutralized with silver carbonate, filtered, and evaporated to a syrup which was hydrolyzed with 0.5M sulphuric acid (5 ml) for 24 h at 100°. The hydrolysate was neutralized with barium carbonate, filtered, and evaporated to a syrup (228 mg).

Examination and identification of methyl sugars. — The mixture of sugars (210 mg) was separated on twelve sheets (9 × 22 inches) of Whatman No. 1 paper, using solvent *B*. The yields (mg, ±10%) of the four major fractions isolated were as follows: *1*, 60; *2*, 24; *3*, 58; and *4*, 50.

Fraction 1. The syrupy fraction showed a major spot, R_F 0.79 (solvent *B*), and a minor, partially separated component which had an R_F value identical with that of 2,3,5-tri-*O*-methyl-L-arabinose. Demethylation[42] with boron trichloride gave mainly xylose, with a lesser proportion of glucose, and traces of arabinose. A portion (2 mg) of the fraction was refluxed with 2% methanolic hydrogen chloride for 8 h, the acid was neutralized with silver carbonate, and the resulting methyl glycosides were examined by g.l.c.[43] with a 4-ft column of 15% polyphenyl ether on Chromosorb W at 159° and an argon flow-rate of 100 ml/min. The areas under the peaks gave a molar ratio of methyl 2,3,4,6-tetra-*O*-methyl-$\alpha\beta$-D-glucosides and methyl 2,3,4-tri-*O*-methyl-$\alpha\beta$-D-xylosides of 1:5. Minute amounts (0.2–0.3 mole) of methyl 2,3,5-tri-*O*-methyl-L-arabinoside were also detected. The g.l.c. examination also revealed that fraction *1* was contaminated with 2 moles of methyl 2,3,6-tri-*O*-methyl-$\alpha\beta$-D-glucoside.

T.l.c. (4 plates, 3 developments), with ethyl acetate–light petroleum (b.p. 40–60°) (70:50), separated fraction *1* (38 mg) into faster (*x*, 21 mg) and slower moving fractions (*y*, 10 mg).

Demethylation[42] of fraction *x* produced xylose. Crystallization of the syrup from ether–light petroleum (b.p. 60–80°) ensued on seeding with 2,3,4-tri-*O*-methyl-D-xylose. Recrystallization from ether in the cold gave crystals having m.p. 86–88°, undepressed on admixture with authentic 2,3,4-tri-*O*-methyl-D-xylose, $[\alpha]_D^{22}$ $+60 \pm 4°$ (initial)→ $+29 \pm 2°$ (3 days) (c 0.15 ±0.01. chloroform); lit.[44] m.p. 91–92°, $[\alpha]_D$ +54° (chloroform).

Fraction *y* was refluxed with 2% methanolic hydrogen chloride for 8 h, the acid was neutralized (Ag_2CO_3), and the resulting glycosides (7 mg), on separation on one t.l.c. plate with solvent *B*, yielded the anomeric glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-glucose in yields of 1.9 mg and 3.3 mg, respectively. The former component, thus recovered, had an infrared spectrum identical with that of methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside recovered after subjecting the specimen to similar conditions of isolation. The latter component, after hydrolysis and paper chromatography, showed 2,3,6-tri-*O*-methyl-D-glucose, identified in fraction *3*.

Fraction 2. This fraction on demethylation gave galactose. The syrup showed an R_F value (0.65, solvent *B*) and colour reaction identical with those of 2,3,4,6-tetra-*O*-methyl-D-galactose. A solution of the syrup (20 mg) in ethanol (1 ml) containing aniline (10 mg) was refluxed for 5 h. Evaporation of the solvent, with crystallization from ethanol after seeding, gave 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. and mixed m.p. 196–197°, $[\alpha]_D^{25}$ $-65 \pm 9°$ (initial)→ $-40 \pm 5°$ (10 days, incomplete) (c 0.08 ±0.01, acetone); lit.[45] m.p. 197°; $[\alpha]_D$ −84° (chloroform).

Fraction 3. The syrupy fraction had an R_F value of 0.53 (solvent *B*). Demethylation[42] gave mainly glucose and a small proportion of xylose. The syrup (58 mg) was kept in 2% methanolic hydrogen chloride for 24 h at room temperature. The solution was neutralized with silver carbonate, filtered, and evaporated to a syrup. T.l.c. (3 plates, solvent *B*) separated the sugars into a reducing fraction (*s*, 8 mg) and a non-reducing fraction (*t*, 31 mg).

Fraction *s*, on demethylation, produced glucose, and on electrophoresis showed a non-complexing component (M_G 0.00).

The syrupy, non-reducing fraction (*t*, 31 mg) was hydrolysed with 0.25M sulphuric acid for 5 h at 100°. After neutralization, paper electrophoresis showed two spots, M_G values 0.00 and 0.39. Demethylation of the fraction produced mainly glucose and a small proportion of xylose. Paper electrophoresis of fraction *t* (28 mg) separated the sugar into a non-complexing fraction (t_1, 19 mg) and a complexing fraction (t_2, 3 mg).

The syrupy fraction t_1 had an R_F value of 0.54 (solvent *B*) and color reaction (*A*) identical with that of 2,3,6-tri-*O*-methyl-D-glucose, and on demethylation gave only glucose. Crystallization from isopropyl ether at room temperature gave 2,3,6-tri-*O*-methyl-D-glucose, m.p. 104–107°, raised to 112-115° after three recrystallizations from ethyl ether in the cold; $[\alpha]_D^{24}$ +94.8 (initial)→ +68° (6 h, equil.) (c 0.75, water); lit.[46] m.p. 121–123°, $[\alpha]_D$ +70°.

Demethylation of syrupy fraction t_2 produced xylose. Paper chromatography and paper electrophoresis showed a single component having R_F and M_G values, colour reaction, and infrared spectrum identical with those of 3,4-di-*O*-methyl-D-xylose.

Fraction 4. This fraction, on demethylation, produced glucose. Paper chromatography (solvent *B*) and electrophoresis showed a single component having R_F (0.27) and M_G (0.19) values, and colour reaction identical with those of 2,3-di-*O*-methyl-D-glucose. A portion (9 mg) was dissolved in dry pyridine (4 ml) and freshly crystallized *p*-phenylazobenzoyl chloride (225 mg) was added. The mixture was kept for 70 h at 48°. Isolation in the usual way gave 2,3-di-*O*-methyl-1,4,6-tri-*O*-*p*-phenylazobenzoyl-D-glucose, m.p. 204–205°; lit.[47] m.p. 209°.

A second portion of the fraction (30 mg) was refluxed with 2% methanolic hydrogen chloride for 6 h. The solution was neutralized, (silver carbonate), filtered, and concentrated to a syrup. Crystallization from isopropyl ether with seeding, and recrystallisation from the same solvent, gave methyl 2,3-di-*O*-methyl-α-D-glucopyranoside, m.p. and mixed m.p. 82–84°, $[\alpha]_D^{22}$ $+164 \pm 10°$ (*c* 0.16 ±0.01, water); lit.[48] m.p. 83–85°, $[\alpha]_D$ +150° (water).

Minor components. — In addition to the above-mentioned, four, major components, three minor components *1'*, *2'*, and *3'* (R_F 0.33, 0.20, and 0.13, solvent *B*) were isolated in small amounts (*ca.* 1–2 mg). Component *1'*, on demethylation, produced mainly galactose with traces of glucose and arabinose; component *2'* yielded glucose, arabinose, and xylose; and component *3'* produced galactose.

Analytical data for the major components. — The final ratios of the sugar components obtained from fractions *1* and *3* were extrapolated to the original weights of these fractions. The projected weights, after correction for the 2 mol. of methyl 2,3,6-tri-*O*-methyl-D-glucoside found as contaminant in the tetra-*O*-methyl fraction *1*, gave the following corrected yields (mg, ±10%) for the major, methylated components: 2,3,4-tri-*O*-methyl-D-xylose, 39; 2,3,4,6-tetra-*O*-methyl-D-glucose, 8; 2,3,4,6-tetra-*O*-methyl-D-galactose, 24; 2,3,6-tri-*O*-methyl-D-glucose, 41; 2,3,4-tri-*O*-methyl-D-glucose, 13; 3,4-di-*O*-methyl-D-xylose, 6; 2,3-di-*O*-methyl-D-glucose, 50

Periodate oxidation. — Rape-seed amyloid (14.2 mg) was oxidised with 0.3M sodium metaperiodate (10 ml) at room temperature in the dark. Aliquots (1 ml) were removed after 192 h, and the consumption of periodate and production of formic acid were determined[49,50]. The results based on 153 g of polysaccharide (calculated on the arabinose–galactose–xylose–glucose ratios of 1:3:6:16) were: periodate consumed, 1.2 moles; formic acid produced, 0.48 mole.

The solution (8 ml) remaining from periodate oxidation was treated with an excess of barium carbonate and filtered, and to the filtrate was added sodium borohydride (30 mg). After 15 h at room temperature, excess borohydride was destroyed with acetic acid, and the solution was deionized with Rexyn 203 (H^+) and 101 (OH^-) resins. Final removal of boric acid was affected by repeated distillation of methanol from the residue. The residue was hydrolysed with 0.5M sulphuric acid for 3 h at 100°, and the hydrolysate, after neutralization with barium carbonate, was examined by paper chromatography (solvent *A*); no reducing sugars were detected.

A portion (0.5 ml) of the reduced, hydrolysed polysaccharide was dried and converted into the *O*-trimethylsilyl derivatives which were examined by g.l.c. on a Pye 104 chromatograph, using glass columns (6 ft × ¼ in) of 20% SE52 on Gas Chrom P. The chromatograms were developed by holding for 6 min at 140°, and then with temperature-programming at a rate of 3°/min to 250°, using a nitrogen flow-rate of 60 ml/min. The areas under the peaks showed a glycol–glycerol–erythritol ratio of 1.03:1:1.8; a small proportion of glyceraldehyde, incompletely separated from the solvent peak, was also detected.

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