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Molecular Cohesion in Plant Cell Walls

METHYLATION ANALYSIS OF PECTIC POLYSACCHARIDES FROM THE COTYLEDONS OF WHITE MUSTARD

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Methylation analysis was used to characterize the pectic polysaccharides from mustard cotyledons, a tissue with potential for rapid biological change involving the walls. The methylated sugars were identified by g.l.c. and paper chromatography after conversion of uronic acid derivatives into [^{3}H]hexoses, and confirmed by the formation of crystalline derivatives of most of the main products, which were: 2,3-di-*O*-methyl-D-[6-^{3}H]galactose, 2-*O*-methyl-D-[6-^{3}H]galactose, 3,4-di-*O*-methylrhamnose, 3-*O*-methylrhamnose, 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2-*O*-methyl-L-arabinose, 2,3,4-tri-*O*-methyl-D-xylose and 2,3,4,6-tetra-*O*-methyl-D-galactose in the molar proportions 1·00:1·14:0·54:0·74:2·86:2·50:2·24:1·88:0·32. The structural units present are similar to those in well-known polysaccharides from mature tissues, but their proportions are strikingly different. Uninterrupted and unbranched galacturonan segments can therefore contribute little cohesion to these walls, and it is suggested that this correlates with a function of the wall matrix to hydrate and permit readjustment, during germination, of structural elements or wall surfaces or both.

There is little understanding in molecular terms of the way polysaccharides function in biological situations. Some conclusions should be possible when there is more information about the detailed way in which molecular structure changes with biological conditions and, as one relevant system, we have examined the polysaccharides in germinating mustard-seed cotyledons. Within several days of the onset of germination, the cotyledons have changed from storage to photosynthesizing organs and shortly afterwards they become senescent. This differentiation (Varner, 1965) appears to occur without cell division (Rees & Richardson, 1966) and with visible change in the walls under the microscope (Gould & Rees, 1965). We have previously shown that the pectic materials are metabolically active during this period (Gould & Rees, 1965) and that pectic arabinan, a neutral polysaccharide of low molecular weight (Rees & Steele, 1966), diminishes in amount and in degree of branching (Rees & Richardson, 1966). The main polysaccharides in the cotyledons are complex pectic materials (Gould, Rees, Richardson & Steele, 1965; Hirst, Rees, & Richardson, 1965) and we now report their further characterization by chemical methods.

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MATERIALS AND METHODS

Starting material. This was 'white mustard seed germ' (J. and J. Colman Ltd., Carrow Works, Norwich), which, by inspection, consisted mostly of broken cotyledons with a few hypocotyls and no seed coats or other foreign material. Small-scale extractions gave a product that, when compared with material from hand-sorted cotyledons (Hirst *et al.* 1965), was indistinguishable with respect to the sugar pattern observed by paper chromatography after acid hydrolysis, sedimentation pattern in the ultracentrifuge, behaviour on free-solution electrophoresis and the pattern of methyl glycosides detected by g.l.c. after methylation and methanolysis. The experimental methods used for this comparison were the same as for investigations on the larger scale described below.

Paper chromatography. The descending method was used, with Whatman no. 1 paper and the following solvent systems: *A*, ethyl acetate–pyridine–water (10:4:3, by vol.); *B*, butan-1-ol–ethanol–water (4:1:5, by vol., upper phase); *C*, butan-1-ol–ethanol–water (3:1:1, by vol.); *D*, aq. ammonia (sp.gr. 0·88)–water–butan-2-one (1:17:200, by vol.); *E*, benzene–ethanol–water (169:47:15, by vol., upper phase). Mobilities (R_G) are expressed relative to 2,3,4,6-tetra-*O*-methylglucose. Spots were detected with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950).

Gas–liquid chromatography. The Pye Argon Chromatograph (with ^{90}Sr detector) was used with a 4ft. column of polyethylene glycol adipate (15% on 80–100-mesh Gas Chrom P). The flow rate was 80ml. of carrier gas/min. Operating temperatures are cited in the text, and refer to the reading on a mercury-in-glass thermometer inserted in

the pocket of the heating block. Samples were prepared for analysis by heating with methanolic HCl (3% w/v) (100ml./g. of sample) in a sealed tube at 100° for 6hr. After neutralization with Ag_2CO_3, filtration and evaporation to dryness, the residue was dissolved in chloroform for g.l.c. Methanolic HCl (3%, w/v) was prepared by careful addition of acetyl chloride (6ml.) to dry methanol (100ml.).

Retention times (*T*) are relative to 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Each peak is designated *s*, *m*, *w*, or *sh* to indicate, respectively, whether it is of strong, medium or weak intensity or occurs as a shoulder on another peak; no designation is shown for sugars that give a single peak, or that give peaks of similar intensity.

Infrared spectrometry. Spectra were recorded with the Unicam SP.200 spectrometer by using the polysaccharide sample as a film. This was cast on mercury by evaporation of a dilute solution (0·5%) in dry chloroform.

Demethylation. The method was that of Bonner, Bourne & McNally (1960). The sugar (5–10mg.) was dissolved in dry dichloromethane (1–2ml.) and cooled to −80° before addition of boron trichloride (1–2g.) that had been precooled to the same temperature. After 30min., the sample was allowed to warm up to room temperature then kept, protected by a $CaCl_2$ tube, for 16hr., during which time evaporation occurred. The residue was dissolved in a few drops of water, and then methanol was added and the solution was evaporated to dryness. Methanol was evaporated several times from the residue to remove boric acid.

Assay of radioactivity. All samples were counted on strips of chromatography paper, immersed in liquid scintillator (10ml.) in glass vials (10ml.), by using the Beckman liquid-scintillation system. The scintillator was prepared by the addition of 2,5-diphenyloxazole (PPO) (5·0g.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) (0·3g.) to toluene (1l.). All chemicals were scintillation grade, supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh 11.

Melting points. These were measured with the Kofler hot stage, and are uncorrected.

Formation of derivatives. *N*-Phenylglycosylamines (anilides) were prepared from the sugar (75mg.) and aniline (1mole, freshly redistilled), in ethanol (5ml.), by refluxing for 1hr. in the dark. The hot solution was filtered through charcoal and evaporated to dryness, and the product was recrystallized from an appropriate solvent. Aldonolactones were prepared from the sugar (75mg.) by oxidation with bromine (2–3 drops) in water (3ml.) at room temperature and in the dark for 3 days. After aeration to remove bromine, the solution was neutralized with Ag_2CO_3, filtered and evaporated to dryness. The product was extracted with acetone, and the extract, on evaporation, gave a syrup from which the product crystallized. Recrystallization was from an appropriate solvent (see below). Aldonamides were prepared from the lactones after thorough drying over P_2O_5 *in vacuo*. Methanolic ammonia (8%, w/v) (5ml.) was added to the lactone (50mg.) before the flask was closed and kept at 0° for 2 days. Evaporation gave the product as a crystalline residue, which was recrystallized from an appropriate solvent. Toluene-*p*-sulphonylhydrazones (Easterby, Hough & Jones, 1951) were prepared from the sugar (100mg.) in methanol (10ml.) with toluene-*p*-sulphonylhydrazine (1mole), by heating under reflux for 1hr. The product crystallized after standing at 0° for 3 days, and was recrystallized from methanol and washed with ice-cold methanol.

Analyses. Nitrogen was determined by the Kjeldahl method with a volumetric finish, and methoxyl analyses were by A. H. Baird (Edinburgh) Ltd.

EXPERIMENTAL AND RESULTS

Isolation and purification of mustard pectin. The crude pectic mixture was obtained by extraction (Hirst *et al.* 1965) of the defatted germ (1kg.), and isolated by precipitation with ethanol (3vol.). The precipitate was redissolved in water, dialysed against running tap water and freeze-dried. The product (140g.) in water (7l.) was treated with phenol to 45% (w/v) concentration, to remove protein (Westphal, Luderitz & Bister, 1952; Hirst *et al.* 1965). The mixture was heated until it became homogeneous (approx. 75°) and then left for 16hr. at 2° in separating funnels. The phenol layer was discarded and the procedure was repeated three times, any interfacial emulsion with the aqueous layer being kept. A white flocculent material separated as removal of protein proceeded; both this and the clear aqueous layer gave positive reactions for carbohydrate with the phenol–sulphuric acid reagents (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). After the final stage, the clear supernatant solution was removed by decantation, dialysed and freeze-dried (fraction A, 1·80g.; Found: N, 1·1%). The precipitate was suspended in water, dialysed, concentrated and freeze-dried (fraction B, 50·5g.; Found: N, 1·0%).

The procedure was repeated on a small scale (10g.) in the presence of EDTA (2%, w/v). No precipitation then occurred and removal of protein was much easier because emulsions were absent. Fraction A (50mg.), in 0·5M-sulphuric acid (10ml.), was heated on a boiling-water bath for 16hr. After neutralization with barium carbonate, the solution was filtered and concentrated to a syrup. Paper chromatography (solvent *A*) showed glucose as the main sugar, with smaller amounts of xylose and galactose, and only traces of fucose, arabinose and galacturonic acid. This material has been characterized as a polysaccharide of the amyloid type (S. E. B. Gould, D. A. Rees & N. J. Wight, unpublished work).

Analysis of fraction B in a similar fashion showed arabinose with appreciable amounts of xylose, galacturonic acid and galactose, with smaller quantities of rhamnose and traces of glucose.

Methylation of mustard pectin. The polysaccharide (39·1g.) was dissolved in water (1l.) and methylated by the slow addition of sodium hydroxide solution (30%, w/v) (320ml.) and dimethyl sulphate (110ml.) over 6hr., with vigorous stirring under nitrogen and cooling in ice. The procedure was repeated four times at room temperature, and then the solution was dialysed, concentrated and treated

again in the same way. In all, three such cycles were carried out before isolating the product by freeze drying (37·5g.; Found: O·CH_3, 27·5%).

The partly methylated polysaccharide (17·5g.) was dissolved in *NN*-dimethylformamide (200ml.) and methyl iodide (150ml.). Silver oxide (65g.) was added and the reaction was magnetically stirred in a conical flask fitted with a double-surface condenser and calcium chloride tube, in a water bath at 40° for 48hr. Chloroform was added and the mixture was filtered to remove insoluble silver salts, and these were thoroughly washed with chloroform. The chloroform solutions were washed with sodium cyanide (0·5%, w/v) to remove soluble silver salts. The first cyanide wash was back-extracted five times with chloroform and then the combined chloroform solutions were washed with water until neutral. After being dried over sodium sulphate, the chloroform layer was concentrated and poured into excess of light petroleum (b.p. 40–60°). The i.r. spectrum of the precipitated product showed only weak hydroxyl absorption (yield, 13·6g.; Found: O·CH_3, 37·9%). This absorption was diminished by a further treatment in the same way (yield, 12·8g.; Found: O·CH_3, 38·2%).

Part of the product (2·0g.) was fractionated by the slow addition of light petroleum (b.p. 40–60°) to a solution in chloroform (50ml.). Fractions were collected on the centrifuge after the addition of approx. 3vol., 4vol. and a large excess of light petroleum. Each fraction was washed with light petroleum and dried *in vacuo*. The yields were 0·26g., 0·17g. and 1·28g. (Found: O·CH_3, 37·0, 38·4, and 37·8% respectively). The methoxyl content expected from the sugar composition is about 40·5%. The first fraction showed significant absorption in the hydroxyl stretching region of the i.r. spectrum, but this was virtually absent from the other two fractions, and their low values are attributed to contamination with non-carbohydrate material. The three fractions were indistinguishable by methanolysis and g.l.c.

Hydrolysis and preliminary separation. The methylated pectic mixture (5·0g.; Found: O·CH_3, 38·2%) was heated with formic acid (45%, v/v) (100ml.) on a boiling-water bath for 16hr. After evaporation to dryness under diminished pressure, the residue was heated with 0·05M-sulphuric acid (150ml.) for 6hr. at 100° to hydrolyse formate esters, neutralized with barium carbonate, filtered and treated with an excess of Amberlite IR-120 (H^+ form) resin. The solution was applied to a column of DEAE-Sephadex A-25 (30g., formate form), and the neutral sugars were eluted with water (3l.) and isolated by evaporation to dryness, then dried *in vacuo* over phosphoric oxide (yield, 3·75g.). The acidic sugars were eluted with formic acid (4%, v/v) (5l.), and isolated in the same way (yield, 1·35g.). Paper chromatography (solvent *B*) confirmed that separation into acidic and neutral fractions had been completely effective.

Identification of the components of the acidic fraction. The mixture of sugars (1·25g.) was boiled under reflux with methanolic hydrogen chloride (3%, w/v) (100ml.) for 6hr. After neutralization (silver carbonate), filtration and evaporation, the residue was dissolved in methanol (75ml.) to which 2,2-dimethoxypropane (5ml.) had been added as a drying agent. After 16hr., a mixture of sodium borohydride (1·0g.) and sodium [^{3}H]borohydride (2·8mg., specific radioactivity 3·5mc/mg.) was added; the excess of reagent was destroyed with dilute formic acid after 48hr. at room temperature. After evaporation to dryness, the residue was dissolved in water and passed through a column of Amberlite IR-120 (H^+ form). Boric acid was removed by evaporation to dryness and repeated distillation of methanol from the residue. After hydrolysis with formic acid and sulphuric acid (see above), paper chromatography (solvent *B*) showed that reduction had been incomplete. The product was therefore separated on DEAE-Sephadex (see above), to give a reduced fraction (i.e. neutral sugars, 0·620g.) and a non-reduced fraction (i.e. acidic sugars that survived the reduction, 0·180g.). Treatment of the non-reduced fraction with methanolic hydrogen chloride and then sodium borohydride (conditions as above, except that the borohydride contained no tracer and was in very large excess) gave a product that was indistinguishable from the reduced fraction by hydrolysis and paper chromatography. We conclude that the first reduced fraction contained a representative mixture of products.

The reduced fraction (0·615g.) was dissolved in methanol (5ml.) and applied to a column (55cm. × 5·5cm.) of cellulose powder (Whatman CC31) previously equilibriated with butan-1-ol–ethanol–water (6:2:1, by vol.) and eluted with the same solvent (flow rate, 0·6ml./min.). Fractions (25ml.) were collected automatically and screened by paper chromatography in solvents *B*, *C*, *D* and *E*, then combined into larger fractions, which were filtered, evaporated to dryness, dried *in vacuo* and weighed. Chromatographic data are given for solvent *B* and for column 1 (see Table 1).

Fraction 1 (0·073g.) consisted mainly of 3,4-di-*O*-methylrhamnose (R_G 0·86; *T* 1·00), with traces of 2,3,4-tri-*O*-methylgalactose (R_G 0·86; *T* 6·01) and 3-*O*-methylrhamnose (R_G 0·61; *T* 3·12*s*, 4·57*w*). Demethylation gave rhamnose as the only detectable product. A spot of this rhamnose, which gave a coloration (*p*-anisidine hydrochloride) of medium intensity, had a radioactivity of 130c.p.m.

Fraction 2 (0·035g.) contained the same components as fraction 1, with traces of two unidentified components. The first (*T* 1·51*s*, 1·74*w*) had the

Table 1. *G.l.c. of methylated sugars as their equilibrium mixtures of methyl glycosides*

Experimental conditions are given in the text, with explanation of the symbols. The values given here are for reference samples; for the values for the sugars from mustard pectin, see the text.

Temp.	Sugar	*T*			
125°	2,3,4-Tri-*O*-methylrhamnose	0·42			
	2,3,4-Tri-*O*-methylxylose	0·40*m*	0·53*s*		
	2,3,5-Tri-*O*-methylarabinose	0·53*s*	0·71*w*		
150°	2,3,4,6-Tetra-*O*-methylgalactose	1·76*sh*	1·87*s*		
175°	2,3,4-Tri-*O*-methylarabinose	1·04			
	2,3,-Di-*O*-methylarabinose	1·46*s*	1·61*w*	1·78*s*	
	2,5-Di-*O*-methylarabinose	1·62			
	3,5-Di-*O*-methylarabinose	1·02	2·20		
	2-*O*-Methylarabinose	5·07*s*	9·10*w*		
	3,4-Di-*O*-methylrhamnose	0·98			
	3-*O*-Methylrhamnose	3·10*s*	4·59*w*		
	4-*O*-Methylrhamnose	3·70			
	2,3,6-Tri-*O*-methylgalactose	2·83*s*	3·54*w*	3·74*w*	4·10*m*
	2,4,6-Tri-*O*-methylgalactose	3·55*m*	4·07*s*		
	2,3,4-Tri-*O*-methylgalactose	5·95			
	2,4-Di-*O*-methylgalactose	13·40*m*	15·37*s*		
	2,3-Di-*O*-methylgalactose	8·60*s*	11·24*m*	13·90*w*	
	3,4-Di-*O*-methylgalactose	14·05*s*	22·25*w*		
	2,3,6-Tri-*O*-methylglucose	3·01*m*	4·05*s*		
	2,3-Di-*O*-methylglucose	9·07*m*	11·56*s*		
	3,4-Di-*O*-methylglucose	7·40*s*	8·78*m*		
	2,4-Di-*O*-methylglucose	7·34*m*	10·42*s*		
	2,3-Di-*O*-methylfucose	1·04*s*	1·20*sh*	1·44*m*	

paper-chromatographic and g.l.c. properties of a di-*O*-methylpentose. The second (*T* 8·82*m*, 10·94*s*) had the chromatographic properties of a di-*O*-methylhexose but did not correspond to any of the standards (Table 1). Demethylation gave rhamnose and galactose, medium-intensity spots of which gave radioactivities of 130 and 890 c.p.m. respectively. Only a part of the 2,3,4-tri-*O*-methylgalactose could therefore have arisen from uronic acid residues (cf. the radioactivity for 2,3-di-*O*-methylgalactose).

Fraction 3 (0·010 g.) contained 2,3,4-tri-*O*-methylgalactose and 3-*O*-methylrhamnose.

Fraction 4 (0·117 g.) contained 3-*O*-methylrhamnose and 2,3-di-*O*-methylgalactose (R_G 0·50; *T* 8·60*s*, 11·15*m*, 13·90*w*).

Fraction 5 (0·098 g.) was almost pure 2,3-di-*O*-methyl-D-galactose, characterized as the *N*-phenylglycosylamine, which, after recrystallization from ethanol, had m.p. and mixed m.p. 153–155°. A spot of medium intensity with *p*-anisidine hydrochloride was highly radioactive (4750 c.p.m.), showing that this sugar was derived from 2,3-di-*O*-methyl-D-galacturonic acid by reduction.

Fraction 6 (0·020 g.) contained 2,3-di-*O*-methylgalactose and smaller amounts of rhamnose.

Fraction 7 (0·195 g.) was chromatographically pure 2-*O*-methyl-D-galactose, which crystallized on standing as a syrup and was recrystallized from methanol, m.p. 148–150°, and did not depress the m.p. of authentic material. A spot of medium intensity with *p*-anisidine hydrochloride spray was highly radioactive (4375 c.p.m.), showing that this methyl ether was a reduction product of 2-*O*-methyl-D-galacturonic acid.

Fraction 8 (0·030 g.) was composed of 2-*O*-methylgalactose and 3-*O*-methylgalactose (R_G 0·29).

Fraction 9 (0·010 g.) was mainly 3-*O*-methylgalactose. Demethylation gave galactose as the only product, which was highly radioactive (4310 c.p.m. for a spot that was of medium intensity to the *p*-anisidine hydrochloride spray). The sugar was thus derived from 3-*O*-methylgalacturonic acid.

Fraction 10 (less than 0·005 g.) consisted mainly of galactose, which was highly radioactive (2570 c.p.m. for a weak spot with *p*-anisidine hydrochloride) and therefore derived from galacturonic acid.

Components of the neutral fraction. The neutral material (3·6 g.) was dissolved in a mixture (25 ml.) of methanol and butanol half saturated with water (1:1, v/v) and loaded on a column (76 cm. × 6 cm.) of cellulose powder (Whatman CC31), previously equilibrated with butan-1-ol (half saturated with water)–light petroleum (b.p. 100–120°) (1:3, v/v, upper phase) mixed in the ratio 1:1 (v/v) with butan-1-ol–light petroleum (1:3, v/v). Elution was with this solvent mixture (4 l.) followed by the following mixtures (4 l. of each) in order: (i) as for the first solvent mixture except that a 1:1 (v/v)

ratio of butan-1-ol–light petroleum was used in each component; (ii) similarly, but with each ratio 3:1 (v/v); (iii) butan-1-ol half saturated with water.

Fractions (250 of 25ml., thereafter 50ml.) were collected automatically and screened by paper chromatography in solvents *B*, *C*, *D* and *E*, as appropriate. The solutions were combined into larger fractions, which were filtered, evaporated to dryness, dried *in vacuo* over phosphoric oxide and weighed. For the earlier fractions from this column, evaporation was with a bath temperature of less than 30° to minimize loss of tri-*O*-methylpentoses by evaporation.

Components of the fractions were identified by g.l.c. with column 1, at 125° for fractions 1 and 2, at 150° for fraction 3 and thereafter at 175° (see Table 1). Major components were characterized by the formation of crystalline derivatives. R_G values are given for solvent *B*.

Fraction 1 (0·005g.) contained almost pure 2,3,4-tri-*O*-methylrhamnose (R_G 1·00; *T* 0·42) with traces of 2,3,5-tri-*O*-methylarabinose. Demethylation gave rhamnose as the only product detectable by paper chromatography.

Fraction 2 (1·66g.) was a mixture of 2,3,5-tri-*O*-methylarabinose (R_G 0·96; *T* 0·53*s*, 0·71*w*) and 2,3,4-tri-*O*-methylxylose (R_G 0·94; *T* 0·40*m*, 0·53*s*) with a trace of 2,3,4,6-tetra-*O*-methylgalactose. The major components were separated by preparative chromatography on paper sheets (solvent *E*) to give chromatographically pure syrups. The relative yields indicated that the arabinose and xylose derivatives were present in the ratio 3:2. 2,3,5-Tri-*O*-methyl-L-arabinose was characterized as the corresponding aldonamide, which, after recrystallization from ethanol, had m.p. and mixed m.p. 137°. 2,3,4-Tri-*O*-methyl-D-xylose crystallized slowly from the syrup and was characterized as the corresponding aldonolactone, which, after recrystallization from light petroleum (b.p. 40–60°)–ethyl ether (1:1, v/v) had m.p. and mixed m.p. 53°.

Fraction 3 (0·105g.) was 2,3,4,6-tetra-*O*-methylgalactose (R_G 0·88; *T* 1·73*sh*, 1·85*s*) with trace amounts of the components of fraction 2. After purification by chromatography on paper sheets, 2,3,4,6-tetra-*O*-methyl-D-galactose was characterized as the *N*-phenylglycosylamine, which, after recrystallization from ethanol, had m.p. 198° and did not depress the m.p. of an authentic sample.

Fraction 4 (0·050g.) was 3,4-di-*O*-methylrhamnose (R_G 0·88; *T* 0·98) with smaller amounts of 2,3,6-tri-*O*-methylglucose (R_G 0·86; *T* 3·02*m*, 4·03*s*) and 2,3,4,6-tetra-*O*-methylglucose.

Fraction 5 (0·055g.) contained mainly 2,3,6-tri-*O*-methylglucose with a smaller amount of 3,4-di-*O*-methylrhamnose. 2,5-Di-*O*-methylarabinose (*T* 1·61) and 3,5-di-*O*-methylarabinose (*T* 1·02, 2·20) were detected as minor components by g.l.c. only. Demethylation of the fraction gave glucose and rhamnose.

Fraction 6 (0·290g.) contained mainly 2,3-di-*O*-methylarabinose (R_G 0·74; *T* 1·48*s*, 1·62*w*, 1·80*m*) with approx. 5% by weight of 2,3,6-tri-*O*-methylgalactose (R_G 0·81; *T* 2·82*s*, with other peaks obscured) and 10% 2,4,6-tri-*O*-methylgalactose (R_G 0·76; *T* 3·58*m*, 4·09*s*).

Fraction 7 (0·565g.) was almost pure 2,3-di-*O*-methylarabinose, slightly contaminated by 2,4,6-tri-*O*-methylgalactose. The di-*O*-methyl ether gave 2,3-di-*O*-methyl-L-arabonamide, which, after recrystallization from ethanol, had m.p. and mixed m.p. 158°.

Fraction 8 (0·053g.) contained 4-*O*-methylrhamnose (R_G 0·64; *T* 3·76) and 2,3,4-tri-*O*-methylgalactose (R_G 0·68; *T* 5·95) as the major components with smaller amounts of 2,3,-di-*O*-methylarabinose. Demethylation gave rhamnose, galactose and arabinose.

Fraction 9 (0·030g.) contained 3-*O*-methylrhamnose (R_G 0·62; *T* 3·10*s*, 4·61*w*) as the major component with a small amount of 2,3-di-*O*-methylglucose and a trace of 4-*O*-methylrhamnose. Demethylation gave rhamnose and a trace of glucose.

Fraction 10 (0·120g.) contained 2,3-di-*O*-methylglucose (R_G 0·69; *T* 8·88*m*, 11·55*s*) as the major component with a small amount of 3-*O*-methylrhamnose. Demethylation gave glucose and a trace of rhamnose.

Fraction 11 (0·025g.) contained an unidentified sugar (R_G 0·61; *T* 9·23, 12·60) as the major component. Demethylation gave glucose as the only product, from which it would appear that this substance is a di-*O*-methylglucose. It was not 2,4-di-*O*-methylglucose (*T* 7·34*m*, 10·42*s*) or 3,4-di-*O*-methylglucose (*T* 7·40*s*, 8·78*m*).

Fraction 12 (0·665g.) was almost pure 2-*O*-methylarabinose (R_G 0·50; *T* 5·05*s*, 9·10*w*) with a trace of 2,4-di-*O*-methylgalactose (R_G 0·52; *T* 13·30*m*, 15·28*s*). It was characterized as the toluene-*p*-sulphonylhydrazone, which, after recrystallization from methanol, had m.p. and mixed m.p. 142–144°.

Fraction 13 (0·110g.) was chromatographically pure and had R_G 0·45 and *T* 2·93*m*, 3·81*s*, 5·86*w*, 7·88*m*. Demethylation gave arabinose only, and methylation gave a mixture of 2,3,5-tri-*O*-methylarabinose and 2,3,4-tri-*O*-methylarabinose. This evidence, with the chromatographic characteristics and the fact that this fraction is different from 2-*O*-methylarabinose, suggests that the material is 3-*O*-methylarabinose.

Fraction 14 (0·040g.) was chromatographically pure arabinose, which was characterized as L-arabinose toluene-*p*-sulphonylhydrazone, which, after recrystallization from methanol, had m.p. 155°

Table 2. *Relative yields of the products of methylation and hydrolysis of mustard pectin*

The values for the results of direct analysis, before methylation, are from Gould (1965).

Parent sugar	*O*-Methyl ether	Relative molar ratio of ether	Relative molar ratio of parent sugar calc. from ether ratio	Relative molar ratio of parent sugar by direct analysis
Galacturonic acid	2,3-Di	1·00	1·0	1·0
	2-	1·14		
	3-	0·16		
	Non-methylated	0·03		
Arabinose	2,3,5-Tri	2·86	3·5	1·6
	2,3-Di	2·50		
	2,5-Di	0·04		
	2-	2·24		
	3-	0·42		
	Non-methylated	0·15		
Xylose	2,3,4-Tri	1·88	0·8	0·6
Rhamnose	2,3,4-Tri	0·03	0·6	0·1
	3,4-Di	0·54		
	3-	0·74		
	4-	0·11		
	Non-methylated	0·03		
Galactose	2,3,4,6-Tetra	0·32	0·3	0·3
	2,4,6-Tri	0·15		
	2,3,4-Tri	0·09		
	2,3,6-Tri	0·05		
	2,4-Di	0·04		
Glucose	2,3,6-Tri	0·15	0·2	0·2
	2,3-Di	0·29		
Unidentified*		0·08		

* Molecular weight taken as 208.

and did not depress the m.p. of an authentic specimen.

Recovery of the various sugars as their methyl ethers. The molar proportions of the component sugars were found from the weights of the column fractions. For mixed fractions, the proportion of each component was estimated from the peak areas on gas chromatograms. The ratios were corrected for reduction of the galacturonic acid derivatives, and scaled according to the total weights of acidic and neutral fractions. The results are given in Table 2, in which the analytical results are shown for the non-methylated polysaccharide (Gould, 1965), for comparison.

DISCUSSION

The largest fraction of the soluble polysaccharides from mustard cotyledons gave on hydrolysis the sugars that are often found combined together in polysaccharides of the pectic type (Aspinall & Cañas-Rodriguez, 1958; Aspinall & Fanshawe, 1961; Bhattacharjee & Timell, 1965; Barrett & Northcote, 1965; Aspinall, Begbie, Hamilton & Whyte, 1967*a*: Aspinall, Cottrell, Egan, Morrison & Whyte, 1967*b*; Aspinall, Hunt & Morrison, 1967*c*; Aspinall, Craig & Whyte, 1968*a*; Rees, 1969), namely galacturonic acid, arabinose, galactose and rhamnose. Xylose, which was also present, is less commonly a major component, but has been reported as such on some occasions (Aspinall & Baillie, 1963; Bouveng, 1965; cf. Aspinall *et al.* 1967*b*, 1968*a*), and the methylation evidence (see below) shows that, in our polysaccharide, it must be combined in the same molecule as some of the other sugars. Purification of the polysaccharide involved precipitation by low concentrations of cations in the tap water, presumably by Ca^{2+}. The nature of the interaction is not understood, but the effect is unusual enough to suggest that precipitation was a fairly selective effect and therefore that the polysaccharide, if not homogeneous, consists of a

family of related species. This conclusion is supported by physical measurements (D. A. Rees & I. W. Steele, unpublished work). There is also evidence from microradioautography (D. A. Rees & N. J. Wight, unpublished work) to justify the assumption made here that the polysaccharides are cell-wall components. The other polysaccharides in mustard cotyledons are also believed to occur in the walls, and are a neutral arabinan (Hirst *et al.* 1965; Rees & Richardson, 1966; Rees & Steele, 1966), an amyloid (Gould, 1965) and cellulose.

The overall yield of methylated polysaccharide was only 51%, suggesting that the results ought strictly to be interpreted in qualitative terms only. Methyl ethers of all the sugars were recovered, however, and the relative yields were, apart from arabinose and rhamnose, in reasonable agreement with the proportions of the sugars before methylation (Table 2). The two discrepancies can be explained: (i) the yields of 2,3,5-tri-*O*-methylarabinose and 2,3,4-tri-*O*-methylxylose were probably overestimated because, owing to their volatility, the syrups containing these sugars were dried only briefly; (ii) rhamnose was probably underestimated in the native polysaccharide because, as shown below, it would be incompletely released on acid hydrolysis.

Most of the main hydrolysis products of the methylated polysaccharide were identified unambiguously by the formation of crystalline derivatives. Other products were identified as thoroughly as possibly by chromatographic methods. The uronic acid ethers were identified as the corresponding hexose ethers after reduction with [^{3}H]borohydride. Only galactose derivatives, which included 2,3-di-*O*-methyl-D-galactose, were radioactive, although rhamnose derivatives, including 3,4-di-*O*-methylrhamnose, were also present in the fraction derived from the mixture of acidic sugars. All this is consistent with the (1→4)-linked galacturonan chain common to pectic substances, with frequent interruption by insertion of 2-linked rhamnose. The various rhamnose ethers in the neutral fraction were in different proportions from the 'reduced' fraction (e.g. 4-*O*-methylrhamnose was found only in the neutral fraction); this implies that contiguous rhamnose residues occur in the chain. Such residues are present in pectic substances from soya bean (Aspinall *et al.* 1967*b*,*c*). About 60% of the galacturonic acid and rhamnose residues in the part of the polysaccharide that was recovered as the methyl ether would seem to carry side chains. The main types of branching are through C-3 of galacturonic acid (as shown by the high yield of 2-*O*-methyl-D-galactose) and C-4 of rhamnose (shown by the 3-*O*-methylrhamnose). A little branching probably occurs through other positions as well.

The configuration of the rhamnose is not specified in this paper because no proof was obtained, but it is almost certainly L as in all related polysaccharides.

Although other interpretations are possible, the arabinose ethers suggest that a large part of the molecule is made up of 'arabinan' side chains, which, apart from covalent linkage to the galacturonorhamnan backbone, are similar in structure to the neutral arabinose polymer that has been characterized before (Rees & Richardson, 1966). The polysaccharides could be related in the way that has been proposed for analogous components of sycamore cells grown in tissue culture (Stoddart & Northcote, 1967) to account for the distribution of label between various pectic fractions after the administration of a 'pulse' of radioactive glucose, namely that that neutral polysaccharide is first synthesized and then attached to form side chains. The high yield of 2,3,4-tri-*O*-methylxylose suggests (though does not prove) that the backbone is densely substituted by single-unit xylose side chains as in tragacanthic acid and pollen galacturonan, perhaps linked to C-3 of galacturonic acid residues as in all the earlier examples (Aspinall & Baillie, 1963; Bouveng, 1965; Aspinall *et al.* 1967*b*, 1968*a*). Glucose is not a normal component of pectic substances, and the methyl ethers that we have isolated probably arose from contaminating amyloid (cf. Kooiman, 1961; Gould, 1965). The modes of linkage of the galactose residues are established by the isolation of several methyl ethers, but it would be premature to discuss their position in the structure. It is obvious that long chains of β-(1→4)-linked galactose residues, which are typical of some pectic substances (Aspinall *et al.* 1967*b*,*c*), are not present in substantial proportions here. Whatever the biological function of such chains, it might be taken over in mustard cotyledons by the arabinose-containing chains.

Within the unlignified plant cell wall, unlike the bacterial cell wall, it would seem that non-covalent forces are crucial to the overall strength and texture, because the walls can in principle be completely dispersed without breaking covalent bonds, for example by the sequential use of EDTA and cuprammonium solutions. This means that other associations between chain molecules, namely the tertiary structures, must provide cohesion in the native state. In mustard pectin there is a much more interrupted and branched galacturonan chain than in pectic substances from apple (Barrett & Northcote, 1965), lucerne leaves and stems (Aspinall & Fanshawe, 1961; Aspinall, Gestetner, Molloy & Uddin, 1968*b*), citrus peel (Aspinall *et al.* 1968*a*) and bark (Bhattacharjee & Timell, 1965). A biological reason for the occurrence of fewer side chains and insertions in the mature tissues is suggested by the observations by Roelofsen & Kreger (1951) on the collenchyma walls of celery petioles. On the basis of X-ray diffraction and electron microscopy,

coupled with specific extraction procedures, they concluded that galacturonan chains in the natural state are to some extent packed in microcrystallites. Such associations would be similar to those that appear to form the framework of pectin and pectate gels (Rees, 1969), and would represent a tertiary structure involved in cohesion and strength within the wall. In mature tissues, especially collenchyma, which has a structural role, this cohesion and strength, combined with water-holding and ion-exchange properties, may well be the main biological requirement of these matrix polysaccharides. The highly hydrated and therefore 'open' nature of the gel framework would contribute to the flexibility that is characteristic of collenchyma walls. In mustard cotyledons, however, the galacturonan backbone is so modified that the scope for packing, and hence the scope for cohesion (Rees, 1969), is expected to be minimal. A similar situation is found in other tissues that have potential for rapid enlargement or rapid differentiation or both, such as pollen (Bouveng, 1965) and soya-bean cotyledons (Aspinall *et al.* 1967*b*). These polysaccharides should have more of a 'lubricating' than a 'cementing' function, as might be necessary in biological processes that require slippage of structural elements with respect to each other within the wall or between adjacent walls.

The correlation involving a lack of molecular cohesion is further supported by the behaviour of the walls in the so-called slime cells (Tschirche, 1889; Roelofsen, 1959) of certain seed coats. The matrix polysaccharides, though apparently based on a galacturonan chain (Aspinall, 1969), are very greatly modified by branching and insertion (Smith & Montgomery, 1959; Tyler, 1965*a*,*b*; Aspinall, 1969). These walls lack cohesion in such a way that they disperse in water to small soluble 'units', which appear to consist of isolated cellulose microfibril segments, each encapsulated by matrix polysaccharides (Grant, McNab, Rees & Skerrett, 1969). It is noteworthy that there is cohesion within, but not between, these units.

Another similarity between the polysaccharides from pollen, seed cotyledons and slime cells is that they occur in environments that are normally dehydrated but require to take up water rapidly with the onset of germination. This hydration would be assisted by a lack of cohesion in the dry state, e.g. by hydrogen-bonding, which is less efficient than the chains could achieve separately with water.

At present, the most detailed model for biological cohesion between polysaccharide chains is the carrageenan double helix (Anderson, Campbell, Harding, Rees & Samuel, 1969), which can be dissociated and re-formed in aqueous solution (Rees, Steele & Williamson, 1969; McKinnon, Rees & Williamson, 1969). It is believed to link the chains to form an intercellular gel in certain red seaweeds (Rees, 1969). Irregularities in the main chain block helix formation (Anderson *et al.* 1968, 1969), but can be removed enzymically (C. J. Lawson & D. A. Rees, unpublished work; cf. Rees, 1961) and probably represent a mechanism for the biological control of helix content and hence of biological texture. Similar control of cohesion may exist in the walls of higher plants, although it is unlikely that side chains and insertions are removed without complete turnover, and the chains probably (Rees, 1969) associate in some way other than in double helices.

It has been suggested (Northcote, 1969; Stoddart, Barrett & Northcote, 1967) that the pectic substances from actively growing cells are characterized by a high proportion of a component that is rich in non-esterified galacturonic acid residues. The products from mature walls are supposed to be more highly esterified and to contain more neutral sugars (although the proportion is still low in comparison with mustard pectin). However, the second generalization cannot apply universally because Bhattacharjee & Timell (1965) isolated pectic substances from bark that had overall characteristics quite similar to the acidic component of growing tissues ('fraction X' described by Stoddart *et al.* 1967). It would seem probable from work with enzymes *in vitro* (Villemez, Swanson & Hassid, 1966; Kauss & Hassid, 1967) and from tracer evidence (Stoddart & Northcote, 1967) that the formation of non-esterified galacturonan or galacturonorhamnan chains is an early stage in the construction of complex pectic molecules. What happens in subsequent steps may depend on the species, cell type and the state of maturity. In this paper we have discussed different types of non-dividing cells, whereas Northcote and his co-workers have examined the changes during growth that involve cell division. At the moment it would be premature to attempt close correlation of the two investigations.

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