Polysaccharides in Germination. Occurrence, Fine Structure, and Possible Biological Role of the Pectic Araban in White Mustard Cotyledons*

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ABSTRACT: The pectic araban from mustard seeds contains only arabinose units. This confirms that the polysaccharide is not a chemical breakdown artifact of a large heteropolysaccharide because known mechanisms of breakdown would lead to an "araban" containing other sugar units as well. The new crystalline compounds, 2,3,5-tri-*O*-methyl-1,4-di-*O*-*p*-nitrobenzoyl-L-arabitol, 2,3-di-*O*-methyl-1,4,5-tri-*O*-*p*-nitrobenzoyl-L-arabitol, and 2-*O*-methyl-1,3,4,5-tetra-*O*-*p*-nitrobenzoyl-L-arabitol, have been synthesized for calibration of quantitative analysis of the hydrolysis products of methylated arabans by gas chromatography. Application of this method together with the Smith periodate degradation has shown (i) in contrast to the araban which is a breakdown artifact of sugar beet pectin [Hullar, T. L. (1963), Ph.D. Thesis, University of Minnesota], no adjacent 1,3 linkages are present in the structure of mustard seed araban; (ii) most of the periodate-labile units are in nonadjacent positions in the structure; (iii), as a consequence of ii, the araban does not contain appreciable amounts of a linear component. This information allows a number of otherwise possible structures for mustard seed araban to be excluded, but does not lead to a unique formula. On germination, the araban in the cotyledons diminishes in amount and becomes less branched. These changes are consistent with a change in the cotyledon walls and/or middle lamellae from an easily deformable resting state to a state which can limit expansion of the active protoplasm, if the relevant mechanical properties are supposed to be determined by "entanglements" between polysaccharide molecules.

The plant cell wall consists largely of polysaccharides which are of many structural types. Particularly abundant in some young walls are the pectic substances, polysaccharides normally containing galacturonic acid, galactose, and/or arabinose as major structural units, often in the form of complex heteropolysaccharides (Aspinall and Cañas-Rodriguez, 1958; Aspinall and Fanshawe, 1961; Zitko and Bishop, 1965; Bhattacharjee and Timell, 1965). In this paper we are chiefly concerned with arabinose-containing pectic substances. Reasons have been suggested for doubting that a homoaraban ever occurs in the natural state (Hullar, 1963; Barrett and Northcote, 1965), and at least some earlier preparations of "pectic araban" were probably artifacts formed by splitting of larger and more complicated molecules by β elimination and other degradation reactions under the alkaline conditions of extraction or "purification." The claim that the pectic araban from mustard seeds is not a degradation artifact (Hirst *et al.*, 1965) is therefore important because, if correct, this polysaccharide would represent an example of a genuine pectic homoaraban. In this paper we discuss further evidence in support of the claim. We also make a structural comparison between this genuine araban and the araban which is a degradation artifact of sugar beet pectic complex and which has recently been studied by Hullar (1963). Earlier work (Hirst *et al.*, 1965; Andrews *et al.*, 1959; Hough and Powell, 1960) has established quite rigorously the gross structural features of both polysaccharides by methylation analysis. We are here concerned with fine points of structure such as the distribution of branching points.

Alterations in the degree of "cross-linking" of pectic substances in cell walls have often been suggested to be important in the control of plant growth because such alterations may facilitate or oppose the wall stretching which is part of cell enlargement (Wilson, 1964). It is very desirable to gain more understanding of the molecular structures of pectic substances and any changes that occur with changing growth rate so that such possibilities can be tested and some understanding gained of the chemical basis of any such role that the polysaccharides may have. Some evidence relevant to these problems is presented in this paper, derived from a structural comparison of the pectic arabans from mustard cotyledons before and after germination. Although the cotyledon cells do not actually "grow," the change in the walls when the cotyledons swell and unfold is probably analogous in some important respects to wall changes accompanying changes in growth rate in more typical tissues. The results are clear-cut and might point to the character

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of some of the changes associated with normal growth, and therefore to a general theory of the role of wall polysaccharides in primary plant cell growth.

Experimental Section

General. Extraction, chromatography, and general methods were as described previously (Hirst *et al.*, 1965), except where stated otherwise. Microscope slides coated with Kieselgel G (Merck) were used for thin layer chromatography, with mixtures of methanol and benzene as solvents. Spots were located by spraying the plates with 4 N sulfuric acid and heating briefly over a bunsen flame. Infrared spectra were measured with the Unicam SP200 recording spectrophotometer, ultraviolet spectra with the Perkin-Elmer Model 137 ultraviolet spectrophotometer, and proton magnetic resonance spectra with the Perkin-Elmer R10 (60 Mc/sec) spectrometer using tetramethylsilane as internal standard.

Methylation of Sugars and Polysaccharides. In earlier work (Hirst *et al.*, 1965), complete methylation of mustard seed araban was achieved very satisfactorily in one step using barium hydroxide octahydrate and dimethyl sulfate (Kuhn and Trischmann, 1963). In view of subsequent reports (Wallenfels *et al.*, 1963) that there may be a side reaction associated with this procedure which leads to the introduction of some methyl sulfate groups into the polysaccharide, we have developed the following method for general use in the present work.

Barium hydroxide monohydrate was prepared by heating the commercially available octahydrate at 100° in a flask that was continuously evacuated with a water pump, for 16 hr. The equivalent weight of the product was determined by titration against hydrochloric acid, and found to correspond to the monohydrate. Araban (100 mg) was dissolved in dimethyl sulfoxide (2 ml) and the solution was diluted with an equal volume of *N*-methyl-2-pyrrolidone and cooled to 0° in an ice bath. Barium hydroxide monohydrate (0.4 g) and methyl iodide (0.3 ml) were added, and the mixture was shaken in ice water for 2 hr, then at room temperature for 24 hr. The solution was extracted three times with chloroform, any interfacial material being kept with the chloroform layer. Separation of the two liquid phases at this stage and in following stages was sometimes helped by addition of EDTA. The combined chloroform extracts were washed several times with water, again retaining the interfacial material. After the final wash, this was separated with the aqueous phase and extracted with chloroform. The combined chloroform solutions were dried over anhydrous sodium sulfate and concentrated to a yellow glass which was dissolved in the minimum volume of carbon tetrachloride and precipitated as an oil at 0° by the addition of petroleum ether (bp 60–80°). After reprecipitation in the same way and drying *in vacuo* at 60°, the product (90 mg, 73%) had OCH_3, 38.0% (calcd for di-*O*-methylaraban: OCH_3, 38.7%). A solution in carbon tetrachloride showed no hydroxyl absorption in the infrared region. Neither hydrolysis followed by paper chromatography nor methanolysis followed by gas chromatography showed any difference between this product and that prepared earlier (Hirst *et al.*, 1965).

The dimethyl sulfoxide was used for methylation as purchased from British Drug Houses, Ltd., Poole, Dorset, U. K., without purification. Some other grades are less effective because they do not dissolve the barium hydroxide. The difficulty may be overcome by using barium hydroxide in the form of the octahydrate.

The Araban from Germinated Seedlings, and Its Methylated Derivative. Mustard seeds were germinated for 4 days (Gould and Rees, 1965) and then plunged into liquid nitrogen, freeze dried, and separated into their component organs by milling, winnowing, and handsorting. The defatted cotyledons (200 g) were extracted with EDTA in the same manner as previously (Hirst *et al.*, 1965) to give, after several fractionation steps including precipitation with cetyltrimethylammonium hydroxide, a pure araban (0.5 g). This corresponds to a yield of 1.6 g/kg of defatted cotyledons, whereas the yield before germination was 3.5 g/kg. This material gave only arabinose on hydrolysis (found: 98% anhydroarabinose), and had $[\alpha]_D$ −176° (H_2O). Methylation gave a yellow glass which had OCH_3, 38.0%, and which showed no hydroxyl absorption in the infrared (CCl_4 solution).

*2,3,5-Tri-O-methyl-1,4-di-O-p-nitrobenzoyl-*L*-arabitol.* L-Arabinose (20 g) was shaken for 6 hr with methanolic hydrogen chloride (0.9%, 400 ml). After neutralization with silver carbonate, filtration, and evaporation, part of the product (4.0 g) was methylated by the method described above, but on a larger scale. Gas chromatography showed mainly 2,3,5-tri-*O*-methylarabinofuranosides contaminated by small amounts of methyl 2,3,4-tri-*O*-methylarabinopyranosides. After hydrolysis in 45% formic acid at 100° for 16 hr and evaporation, the 2,3,5-tri-*O*-methylarabinose was separated by thick paper chromatography (Whatman 3MM paper) in butan-2-one–water–concentrated aqueous ammonia (200:17:1) solvent to give a chromatographically pure product (2.5 g).

The tri-*O*-methyl sugar (100 mg) was dissolved in water (20 ml) and left at room temperature overnight with an excess of potassium borohydride. Complete reduction was confirmed by negative reaction with the phenol–sulfuric acid reagents (Dubois *et al.*, 1956). Amberlite IR 120 (H^+ form) resin was added and the solution was filtered and evaporated to dryness. Boric acid and the last traces of water were removed by repeated evaporation of a 1:1 mixture of benzene and methanol from the residue, which was then dissolved in anhydrous pyridine (10 ml). *p*-Nitrobenzoyl chloride (2.5 moles/equivalent of OH group) was added and the solution was heated at 65–75° for 30 min after which time the reaction was shown to be complete by thin layer chromatography. A saturated solution of sodium bicarbonate was added gradually and with warming (to prevent solidification) to destroy the unreacted *p*-nitrobenzoyl chloride. After dilution with water (20 ml), the reaction mixture was extracted three times with chloroform. The chloroform extracts were washed three times with water, dried over anhydrous sodium sulfate, and concentrated to dryness. The product was crystallized from ethanol (162 mg, 64%). After repeated recrystallization it had mp 127–128°, $[\alpha]_D$ −16° (*c* 1.8, $CHCl_3$). The proton magnetic resonance spectrum ($CDCl_3$ solution) showed the correct ratio of aromatic to nonaromatic protons, and other features were also consistent with the expected product.

Anal. Calcd for $C_{22}H_{24}N_2O_{11}$: C, 53.6; H, 4.9; N, 5.7. Found: C, 53.3; H, 4.9; N, 5.8.

*2,3-Di-O-methyl-1,4,5-tri-O-p-nitrobenzoyl-*L*-arabitol.* L-Arabinose (4.0 g) was converted to the mixture of methyl glycosides as described above, but instead of neutralizing the acid with silver carbonate, an adaptation of Ness and Fletcher's (1958) method for the preparation of glycoside benzoates was used. Excess of pyridine was added, and the solvents were removed by distillation under diminished pressure at 70°. The syrup was diluted to 200 ml with anhydrous pyridine, and after addition of triphenylchloromethane (1.2 moles), the solution was left at room temperature for 48 hr. The reaction mixture was poured into water and the product was isolated by chloroform extraction followed by washing with water, drying over sodium sulfate, and evaporation to dryness. It was methylated with silver oxide and methyl iodide in dimethylformamide (Kuhn *et al.*, 1955), and then hydrolyzed with 45% formic acid at 100° for 16 hr. Because of the dark coloration after hydrolysis, it was necessary to pass an aqueous solution of the final product through a pad of charcoal four times. The resulting clear solution was concentrated to dryness, and the product was shown by paper chromatography to be pure 2,3-di-*O*-methylarabinose (0.7 g).

Reduction and *p*-nitrobenzoylation was achieved essentially as previously, and the final product was crystallized from a mixture of acetone and ethanol, mp 178–179° (60% yield). On further recrystallization, the substance was obtained in a second form having mp 130°, $[\alpha]_D$ −15° (*c* 1.0, $CHCl_3$). The proton magnetic resonance spectrum ($CDCl_3$ solution) showed the correct ratio of aromatic to other protons, and other features were also in agreement with the expected product.

Anal. Calcd for $C_{28}H_{25}N_3O_{14}$: C, 53.5; H, 4.0; N, 6.7. Found: C, 53.1; H, 4.0; N, 7.1.

*2-O-Methyl-1,3,4,5-tetra-O-p-nitrobenzoyl-*L*-arabitol.* Methyl β-L-arabinopyranoside (mp 166°, 2.0 g) was dissolved in acetone (100 ml) containing concentrated sulfuric acid (1 ml). After standing for 48 hr at room temperature, the solution was neutralized with sodium bicarbonate, filtered, and concentrated to give syrupy methyl 3,4-*O*-isopropylidene-β-L-arabinopyranoside (1.8 g). Part of this material (1.3 g) was methylated with silver oxide and methyl iodide in dimethylformamide (Kuhn *et al.*, 1955). Formic acid hydrolysis then gave 2-*O*-methyl-L-arabinose which was almost pure, as shown by paper chromatography.

Reduction and *p*-nitrobenzoylation was achieved in essentially the same way as previously to give a final product which at first refused to crystallize. It was therefore dissolved in chloroform and divided into six fractions of approximately equal weight by fractional precipitation with petroleum ether. Each fraction was dissolved in the minimum volume of pyridine, and methanol was added to the point of incipient turbidity at room temperature. On standing, the fourth fraction crystallized and these crystals were then used to seed all the other fractions. Recrystallization was by dissolving the material in acetone and then adding an equal volume of ethanol. The material first obtained (50% yield) had mp 132–133°, but on recrystallization it gave a different form which changed crystalline form at this temperature and melted sharply at 152–153°. This had $[\alpha]_D$ −50° (*c* 2.0, $CHCl_3$). The proton magnetic resonance spectrum ($CDCl_3$ solution) showed the correct ratio of aromatic to nonaromatic protons, and other features were also in agreement with the expected product.

Anal. Calcd for $C_{34}H_{26}N_4O_{17}$: C, 53.5; H, 3.41; N, 7.35. Found: C, 53.6; H, 3.4; N, 7.7.

L-*Arabitol Pentabenzoate.* This compound, which was kindly given by Dr. W. D. Annan, had mp 150–152°.

Quantitative Gas Chromatography. Except where stated otherwise, the instrument used was the Pye argon chromatograph (^{90}Sr detector) with the closed injection system. The amounts of substances injected onto the column were sufficient to give peak heights between 2 and 90% of the full-scale deflection when the detector voltage was 1500 v and the sensitivity X10. For smaller peaks the sensitivity was increased to X1. The Pye "series 104" chromatograph (Model 24 with dual columns and flame ionization detectors) and the Griffin D6 chromatograph (gas density balance detector) were used occasionally. Peak areas were measured either by triangulation or with an electronic integrator coupled to the recorder input (Integrator Type IE 165, Gas Chromatography Ltd., Maidenhead, U. K.). Columns were packed with 3% XE-60 on 80–100 mesh Gas Chrom P (from Applied Science Laboratories, Inc., Pa.).

The compounds listed above were used as standards for calibration of detector response to the corresponding alditol acetates. A mixture of the four compounds, sometimes with the addition of erythritol as internal standard (total weight 1–3 mg, the individual weights being accurately known), was dissolved in dry benzene (0.2 ml) and mixed with dry methanol (0.3 ml) containing sodium (0.03 mg). After 16 hr at room temperature, deesterification was shown to be quantitative by thin layer chromatography. Acetic acid (1 drop) was added, and the solution was evaporated to dryness. The residue was dissolved in a mixture of pyridine (0.15 ml) and acetic anhydride (0.15 ml) and heated at 100° for 1 hr. The reaction was again shown to be complete by thin layer chromatography. Methanol (0.3 ml) was added and the mixture was concentrated to small volume. The addition and evaporation of methanol was re-

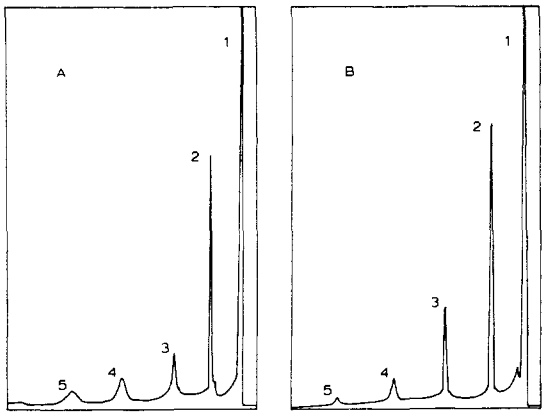


FIGURE 1: Gas chromatography of mixtures of *O*-acetyl-*O*-methyl-L-arabitols formed from different araban samples by the sequence methylation, hydrolysis, reduction, and acetylation. A corresponds to sample 1 of Table IV, and B to sample 3. Separations were on a column of 3% XE-60 on 80–100 mesh Gas Chrom P with a carrier gas flow rate of 80 cc/min, using the Pye argon chromatograph. The column temperature was nominally 152° (registered on a mercury thermometer). The peaks are: 1, chloroform; 2, tri-*O*-methyl derivative; 3, di-*O*-methyl derivative; 4, mono-*O*-methyl derivative; and 5, penta-*O*-acetylarabitol.

peated five times, and then the residue was dissolved in 1 drop of chloroform for gas chromatography.

The hydrolysis products from methylated arabans and methylated degraded arabans were analyzed after reduction and acetylation. Methylated polysaccharide (about 2 mg) was hydrolyzed by heating in 45% formic acid (0.2 ml) at 100° for 16 hr. The hydrolysate was adjusted to about pH 8 by careful addition of ammonia. Potassium borohydride (about 15 mg) was added and the solution was left at room temperature for 4 hr, when a second addition of borohydride (about 15 mg) was made. This large excess was required because ammonium formate appeared to buffer the solution so that borohydride rapidly hydrolyzed. After a further 16 hr at room temperature, complete reduction was checked with the phenol–sulfuric acid reagents (Dubois *et al.*, 1956), and the solution was passed through a small column (5 ml) of IR 120 resin (H^+ form). The eluate and washings were concentrated to dryness, and boric acid was removed by repeated evaporation of methanol from the residue which was then acetylated for gas chromatography in the manner described above.

Smith Periodate Degradation. Araban (174 mg) was dissolved in 0.0400 M sodium metaperiodate (60 ml), and the reaction was followed spectrophotometrically (Aspinall and Ferrier, 1957). When oxidation was complete, residual periodate was destroyed by addition of ethylene glycol, and an excess of potassium borohydride was added before leaving the solution to stand for 2 days. A mixture of IR 120 (H^+ form) and IR 45 (OH^- form) resins was added, and the solution was filtered and carefully concentrated to dryness on the rotary evaporator. Boric acid was removed by repeated distillation of methanol from the residue. After withdrawal of a small sample, the product was dissolved in 1 N sulfuric acid (20 ml) and left at room temperature. At intervals, samples (2 ml) were withdrawn and neutralized with barium carbonate, then filtered and evaporated to dryness. The product was characterized by methylation, followed by hydrolysis, reduction, acetylation, and quantitative gas chromatography. To confirm the supposed specificity of the mild acid hydrolysis step, a solution of nonoxidized araban in 1 N sulfuric acid was left at room temperature for 144 hr, and then worked up and examined in the same way.

Results

Sugar Composition. Reexamination of the hydrolysis products of mustard seed araban, using methods that were shown by control experiments to be sensitive to other sugars in relative concentrations of 2% or less, confirmed that arabinose was the only sugar. The ultraviolet spectrum showed no peak at 235 mμ under conditions in which the absorption due to a relative concentration of 2% of unsaturated 4,5-uronic acid would have been clearly detected.

Quantitative Gas Chromatography. Excellent separations of the arabitol derivatives were obtained, with sharp symmetrical peaks (Figure 1). Relative retention times are given in Table I for these and some related compounds under the optimum conditions for separation.

Reproducibility of detector response and a smooth relationship between response and the amount of material injected on to the column were confirmed

TABLE I: Retention Times on the XE-60 (3% on Gas Chrom P) Column, Relative to Erythritol Tetraacetate.

Compound	Rel Retention Time[a]
Methyl *p*-nitrobenzoate	0.40
Acetate of	
2,3,5-Tri-*O*-methyl-L-arabitol	0.57
2,3,4-Tri-*O*-methyl-L-arabitol	0.74
2,5-Di-*O*-methyl-L-arabitol	1.18
2,4-Di-*O*-methyl-L-arabitol	1.49
2,3-Di-*O*-methyl-L-arabitol	1.51
2,3,4,6-Tetra-*O*-methyl-D-galactitol	1.57
2-*O*-Methyl-L-arabitol	2.56
L-Arabitol	3.62

[a] The Pye argon chromatograph was used, under the conditions described in the text.

TABLE II: The Relation between Peak Area and Concentration for Different Detectors.

Compound	Known Composition By Wt	Known Composition M	Relative Peak Areas Argon Detector	Relative Peak Areas Flame Ionization Detector	Relative Peak Areas Gas Density Balance
Acetate of					
Tri-*O*-methylarabitol	100	100	100	100	100
Di-*O*-methylarabitol	50	45	35	47	45
Mono-*O*-methylarabitol	107	89	62	95	95
Arabitol	22	17	8	13	[a]

[a] Owing to the low sensitivity of this detector, it was not possible to estimate the relative amount of arabitol derivative present.

for each arabitol derivative in the following preliminary experiments. A series of mixtures containing known but widely varying ratios of the four arabitol acetates and erythritol tetraacetate were prepared from the standard compounds and from crystalline erythritol. Each mixture was chromatographed as a solution in chloroform at a series of different dilutions and the heights of the peaks were measured from the charts. Plots of peak height (erythritol tetraacetate) against peak height (arabitol acetate) for each component of each mixture were linear and through the origin, proving that peak height ratios were independent of sample size for all the components to be analyzed. Plots of the ratio of peak height (arabitol derivative) to peak height (erythritol derivative) against the corresponding molar ratio also showed excellent linear relationships through the origin; peak height ratios therefore accurately reflect ratios of concentrations for these compounds under the conditions used. The results were similar when peak areas (electronically integrated with careful correction for the slight base-line drift) were used. It was a very poor approximation with the argon detector to assume the same relationship for all compounds between concentration and peak area (Table II), and calibration was therefore necessary whether areas or heights were used. Heights were chosen, purely on grounds of convenience. With other types of detector the peak area was more closely related to concentration irrespective of the nature of the compound (Table II), although it was difficult to determine accurately small amounts of arabitol pentaacetate which gave small broad peaks. Excellent results (Table III) were obtained when the argon detector was calibrated by the use of standard mixtures.

The ratios of products from methylated araban after hydrolysis for 6, 16, and 36 hr were found to be the same and there is therefore no relative loss of one of the methylated sugars through preferential decomposition during hydrolysis. This also established that the arabitol was not a demethylation product of one of the other derivatives. In another experiment, a mixture of arabitol derivatives prepared from the standards by deacylation was divided into two parts. One part was treated in turn with formic acid, potassium borohydride, and ion-exchange resin as in the work-up of hydrolysis products from methylated polysaccharides. Analysis after acetylation showed the two parts to have the same composition. No relative loss of one of the methylated components therefore occurred in any of these steps, for example, by acid-catalyzed anhydride formation or by absorption on the resin. Despite the elimination of these possible sources of error, analysis showed slightly too high a proportion of end groups in the araban structure (Table IV). Although it seemed unlikely, in view of the criteria of purity already given, that this was due to calibration with an impure standard for the tri-*O*-methyl compound, a further check was made by proton magnetic resonance spectrometry. The integrated signals due to the aromatic protons in 2,3,5-tri-*O*-methyl-1,4-di-*O*-*p*-nitrobenzoyl-L-arabitol and in 2-*O*-methyl-1,3,4,5-tetra-*O*-*p*-nitrobenzoyl-L-arabitol in two different spectra were compared, using the signal from the solvent ($CDCl_3$–$CHCl_3$, 4:1) as the basis of comparison. This showed that there was no difference in purity between the standards.

TABLE III: Analysis of Standard Mixtures by Gas Chromatography.

Compound	Molar Proportions Present in the Mixtures: Mixture 1 Calcd	Mixture 1 Found	Mixture 2 Calcd	Mixture 2 Found
Acetate of				
Tri-*O*-methylarabitol	10.0	10.0	10.0	10.0
Di-*O*-methylarabitol	5.4	5.5	7.9	7.7
Mono-*O*-methylarabitol	5.2	5.1	4.6	4.4
Arabitol	1.4	1.3	1.0	1.1

TABLE IV: Analysis of the Hydrolysis Products of Methylated Arabans by Gas Chromatography after Reduction and Acetylation.

Hydrolysis Product	Molar Proportions Found[a] Sample 1	 Sample 2	 Sample 3
Tri-*O*-methyl derivative	19.2	18.5	16.2
Di-*O*-methyl derivative	11.5	11.2	17.2
Mono-*O*-methyl derivative	12.3	13.1	9.8
Arabitol derivative	2.1	2.1	1.9

[a] Results are expressed for an "average molecule" containing 45 sugar units, this being the average degree of polymerization indicated by molecular weight determinations on sample 1 (Rees and Steele, 1966). Sample 1 corresponds to the araban sample prepared earlier (Hirst *et al.*, 1965); sample 2 to a batch of araban prepared from commercial "white mustard seed germ" (J. and J. Colman, Ltd., Carrow Works, Norwich, U. K.); and sample 3 to the material isolated after germination (see text).

Comparison of Arabans from Different Sources. The arabans from two different samples of seeds gave very similar results, but there was a very clear difference between these and the araban isolated after germination (Table IV). The difference between the arabans isolated before and after germination was immediately obvious from the gas chromatography charts even without measurement (Figure 1).

Periodate Oxidation of Araban. Oxidation was complete after 30 hr, when 0.69 mole of periodate had been consumed per arabinose unit. This was in good agreement with the expected value (0.68) calculated from the gas chromatography results.

Analysis of the Methylation Products of the Smith-Degraded Araban. The results are given in Table V. The absence of 2,3,4-tri-*O*-methylarabinose and 2,5-di-*O*-methylarabinose units in the methylated degraded polysaccharide was confirmed by methanolysis and qualitative gas chromatography on a neopentyl glycol adipate polyester column (10% on Gas Chrom P).

Discussion

The Araban as a Genuine Component of Mustard Seeds. In our earlier paper (Hirst *et al.*, 1965), we rejected the possibility that mustard seed araban is an artifact split from a larger acidic polysaccharide by base-catalyzed elimination during extraction, on the grounds that the yield of araban was not diminished when extraction was at pH 4.9 instead of at pH 7.5. This is a convincing argument, but in the light of well-supported claims that certain other "pectic arabans" are degradation artifacts (Barrett and Northcote, 1965; Hullar, 1963) and especially since mustard seed araban occurs in association with an acidic polysaccharide with "araban side chains" (Hirst *et al.*, 1965; Gould *et al.*, 1965), it now seems desirable to discuss additional evidence for our view.

Arabans which are thought to be artifacts usually contain substantial proportions of sugars other than arabinose (Barrett and Northcote, 1965; Hullar, 1963; Andrews *et al.*, 1959). This is a consequence of the reaction by which they are formed, which is splitting of the galacturonan backbone of the larger molecule by β elimination at some of the methyl-esterified galacturonic acid units. Side chains are therefore released, but in most cases they will be attached to substantial segments of the original main chain because splitting is likely to occur at only about 10% of the methyl galacturonate units (Albersheim *et al.*, 1960). The "araban" formed in this way from apple pectin contained 30% galacturonic acid and 17.5% other sugars (Barrett and Northcote, 1965), and the product isolated from sugar beet, under conditions in

TABLE V: Smith Degradation of Araban, and Investigation of the Specificity of the Mild Acid Hydrolysis Step.

Hydrolysis Product	Molar Proportions Found after Methylation[a] Smith Degradation, Time (hr of acid hydrolysis after oxidation and reduction) 0	 8.5	 24	 69	 Control Experiment, Time (hr of acid hydrolysis, no prior oxidation) 0	 144
Tri-*O*-methylarabitol	0	5.7	8.7	7.7	19.2	19.1
Di-*O*-methylarabitol	0	7.0	5.5	6.4	11.5	12.7
Mono-*O*-methylarabitol	12.0	1.5	0	0	12.3	11.0
Arabitol	2.1	0.1	0	0	2.1	1.9

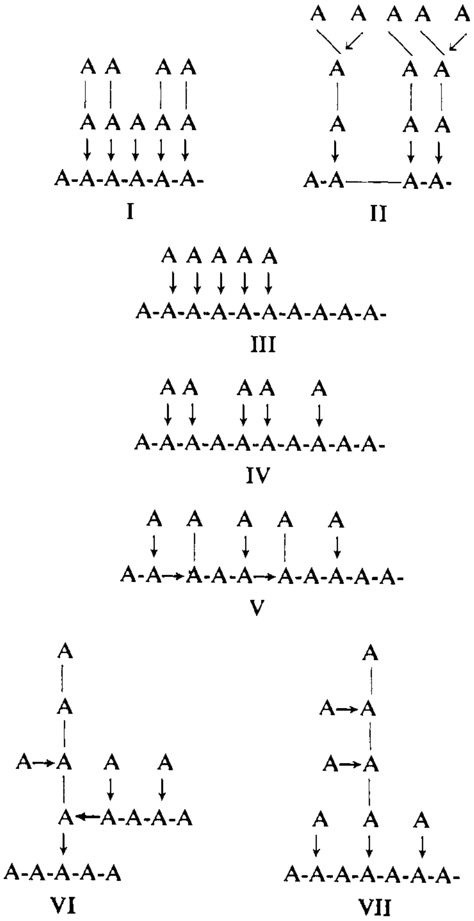
[a] Results are expressed for an "average molecule" containing 45 sugar units, this being the average degree of polymerization indicated by molecular weight determinations (Rees and Steele, 1966).

which other reactions (Whistler and BeMiller, 1958) would be expected to follow chain cleavage and lead to loss of units other than arabinose, contained 5% galacturonic acid and 13.5% other sugars (Andrews *et al.*, 1959). Hullar (1963) has confirmed that continued treatment of beet araban under these conditions leads to loss of some but not all of the acidic units. The segment of former main chain attached to nearly every liberated side chain would also contain an unsaturated uronic acid unit which should be readily detectable by its characteristic ultraviolet spectrum (Albersheim *et al.*, 1960). The extraction conditions for mustard araban were such that very little "peeling" would be expected to follow β elimination. However, no trace of other units could be detected by ultraviolet spectroscopy or by hydrolysis and paper chromatography. This cannot be attributed to high molecular weight (Rees and Steele, 1966), and therefore confirms that this araban is not a β-elimination artifact. There is still the possibility, however, that it is related to the larger polysaccharide in being an intermediate in its biosynthesis, a product of its biological degradation, or in being synthesized from common precursor(s).

Araban Fine Structure. Periodate degradation methods have been the main approach to the characterization of branching patterns in pectic arabans. Using the Barry degradation, Finan and O'Colla (1958) showed that blocks of contiguous branched units are not a prominent feature of the structure of beet araban, and that some isolated branch points occur. Similar conclusions were reached (Sengupta *et al.*, 1965) for the araban from guava fruit. The Smith degradation (Goldstein *et al.*, 1959), although based on broadly the same principles as the Barry degradation, is a more powerful method of structure analysis because the reaction is very clean and the products are easier to handle. Separation and characterization of the Smith degradation products of beet araban (Hullar, 1963) have shown quite clearly that branch points occur in both isolated and adjacent positions, and that some contiguous 1→3 linkages are present. We wished to obtain quantitative information about the fine structure of mustard seed araban, but separation and characterization of the degradation products individually were impracticable because large amounts of araban would have been required. The products were characterized instead by methylation of the mixture followed by quantitative gas chromatography.

For qualitative gas chromatography, methylated sugars are conveniently examined as equilibrium mixtures of methyl glycosides (Aspinall, 1963). Quantitative measurement is easier and more accurate, however, if the sugars are chromatographed as derivatives which will give a single peak. We chose to use the acetates of the methylarabitols which were found to be well separated on an XE-60 column. In quantitative analysis, the response of high sensitivity detectors may be dependent on the nature as well as the concentration of the substance being analyzed and there may be preferential loss or decomposition of one of the components on the column or during preparation of the sample (Bishop, 1964). We therefore prepared a set of new crystalline compounds, the *p*-nitrobenzoates of the methylarabitols, so that we could accurately calibrate the method of analysis. Many types of structure are consistent with the result for the degree of branching in mustard seed araban. Some of these are shown in Chart I.

CHART I: Some Possible Partial Structures for Mustard Seed Araban That Can Be Drawn on the Basis of the Methylation Evidence Alone.[a]



[a] The conventions are: "A" denotes an arabinofuranosyl unit; — or - denotes a 1,5-glycosidic linkage; → denotes a 1,3-glycosidic linkage. The small proportion of 1,2 linkages is not taken into account.

The Smith degradation involves hydrolysis at units in the polysaccharide structure which have been oxidized with periodate and then reduced with borohydride. There is little information in the literature about the selectivity of this step, particularly when the polysaccharide contains acid-labile furanosyl linkages. Mustard araban was therefore used for a control hydrolysis without prior oxidation and reduction. Very little, if any, hydrolysis was detected (Table V). The results of quantitative methylation analysis of the degraded araban (Table V) may therefore be interpreted on the assumption that the hydrolysis is completely selective. In discussion of the results, the significance of the small portion of 1→2 linkages is not considered, nor is the possibility of a branch point at the reducing end of the araban molecule.

No 2-*O*-methylarabinose or 2,5-di-*O*-methylarabinose was detected among the methylation products of the degraded araban. Therefore mustard seed araban, unlike beet araban, contains no contiguous 1→3 linkages and possible structures such as V and VI (Chart I) can be rejected. In whole numbers, the original araban is considered to contain 16 end units, 11 chain units, and 15 branch units when the 1→2 linkages and the end units associated with them are disregarded. Only units present as branch units before oxidation survive the degradation, and the analytical results (taking the mean in whole numbers of the determinations after 24- and 69-hr hydrolysis in Table V) show that nine of these become end units in the degraded polysaccharide while six remain substituted at C-5. The nine which became end units must originally have been substituted at C-3 and C-5 with oxidizable units, and the remaining six must have been substituted at C-3 with an oxidizable unit and at C-5 with another branch unit. A total of about 24 oxidizable units must therefore have been attached through 1→3 or 1→5 linkages to branch units. As there are only about 27 such oxidizable units in the araban molecule, it follows that most of them must be present in isolated (*i.e.*, noncontiguous) positions. Structures such as I and III can therefore be discarded. This evidence does not lead to a unique formula for mustard araban: examples of the remaining possibilities are formulas II, IV, and VII, and hybrids of these. A further narrowing of this range should be possible by characterization of the terminal linkages in the araban molecule, and work in this direction is now proceeding.

In showing that most of the oxidizable units in the araban structure occur in nonadjacent positions, the periodate degradation experiment also limits the kinds of structural heterogeneity that can exist. For example, the possibility is excluded that the araban contains a substantial proportion of a linear component.

Role in Germination. The analytical similarity between two different specimens of mustard seed araban (Table IV) suggest, that the degree of branching might not vary significantly from one batch of resting seeds to another, although more samples must be analyzed before this can be taken as established. The embryos used to prepare sample 1 were largely cotyledons because other embryo parts were mostly removed with the fines in the separation from the seed coats. These embryos were very carefully and laboriously hand sorted, whereas sample 2 was prepared from a commercial preparation of "White Mustard Seed Germ" which could be purchased in very large quantities. The availability of pure authentic mustard seed araban from commercially separated embryos will be very useful in future work.

When the cotyledon cells change during germination from a resting to an active state, there must be some corresponding change in the role of the walls. In the discussion that follows, no distinction is made between the cell wall proper and the middle lamella, although they ought to be considered separately, because there is not sufficient knowledge about their relative biological roles or of the distribution of wall polysaccharides between them. The resting walls need have little mechanical strength because the cells are dehydrated and therefore have little internal turgor pressure. Indeed, a lack of rigidity might help the walls to take up the new shapes and perhaps new relative positions when germination starts and the cotyledons begin to swell and unfold. After germination, the walls come under tension as the cells take up water and they must therefore possess some resistance to stretching. Tracer work (Gould and Rees, 1965) has shown that there is synthesis of pectic polysaccharides in the cotyledons at the onset of germination, and since there is no cell division (R. F. Lyndon, personal communication) and no increase in the total quantity of polysaccharides (Gould *et al.*, 1965), it seems likely that this synthesis is associated with turnover of pectic substances in existing cell walls. The work reported here now shows the character of some of the molecular changes associated with turnover and indicates a possible relationship to the change in wall function.

Entanglement, or secondary interaction, between polysaccharide molecules probably plays an important part in determining cell wall properties (Setterfield and Bayley, 1961). This contrasts with the situation in bacterial cell walls where covalent cross-linkages seem to be important. Such entanglement of polysaccharide molecules *in vitro* may lead to gel formation or even insolubility, and the behavior of these systems suggests that entanglement is hindered by branching or structural irregularities and that the secondary bonding involved in it is rather specific (Gould *et al.*, 1965; N. S. Anderson and D. A. Rees, unpublished work). The term "entanglement," although generally used in polymer systems (Porter and Johnson, 1966), is not entirely appropriate in discussion of cell walls because it fails to imply this important specificity. It is well known that entanglement is dependent on molecular weight in all polymer systems (Porter and Johnson, 1966). Mustard seed araban is a highly branched polysaccharide of low molecular weight which would be expected to have little capacity for entanglement and therefore to have a plasticizing rather than a stiffening influence in the cell wall. The decrease in its amount during germination is therefore consistent with the change in role of the cotyledon cell walls outlined above. The decrease in degree of branching is likewise consistent, because the molecular weight does not change (Rees and Steele, 1966), and the part of the araban that remains will probably therefore have more capacity for entanglements and a less marked plasticizing action. Analogous changes have been observed in the other pectic substances of mustard cotyledons, though these are less well understood in molecular terms (Gould *et al.*, 1965), and it begins to appear likely that changes in mechanical properties of plant cell walls in this system and perhaps others are brought about by metabolic turnover of a number of matrix components to give a new set of molecular structures with a different capacity for entanglements and other interactions. A suggestion that is to some extent similar has been made very recently on the basis of studies of the effects of auxin on cell wall synthesis and cell elongation in oat coleoptiles (Baker and Ray, 1965). It seems likely that entanglements are also important in the organization of animal connective tissue, although the macromolecules involved are of course different from those in plant cell walls (Mathews, 1965; Disalvo and Schubert, 1966).

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