155. *The Composition of the Alkali-stable Polysaccharide of Sugar-beet Pectin.*

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The polysaccharide isolated by extracting sugar-beet chips with hot lime-water was found to contain L-arabinose, D-galactose, L-rhamnose, and galacturonic acid, in the approximate proportions of 74, 10, 3·5, and 5% respectively, and smaller quantities of 2-*O*-methyl-D-xylose, 2-*O*-methyl-L-fucose, mannose, fucose, and an aldobiouronic acid composed of galacturonic acid and 2-*O*-methylxylose. The polysaccharide preparation had an average molecular weight of about 12,500 and attempts to fractionate the material were unsuccessful. It is suggested therefore that the "araban" component of pectin exists in combination with a variety of monosaccharide units.

The pectic polysaccharides exist in such close physical union that very few cases are recorded of a pectin component isolated in a pure state. Material is usually carefully selected so that a pectin rich in one particular component can be utilised, but even so chemical treatment is generally necessary to remove the other components. For example, it has been shown that sugar-beet pectin can be degraded with lime-water to an alkali-stable polysaccharide consisting in the main of L-arabinose units.[1] A further study of this material has now revealed new details of its fine structure.

Sugar-beet chips were extracted with hot lime-water to give a 5% yield of the alkali-stable polysaccharide which was unaffected by further heating in saturated lime-water at 80° for 24 hr. Hydrolysis of this material with N-sulphuric acid followed by passage of the hydrolysate through an anion-exchange resin afforded a mixture of neutral sugars, which was fractionated on a cellulose column.[2] L-Arabinose, D-galactose, and L-rhamnose were isolated and characterised; traces of mannose were also revealed. A small uronic acid fraction, which from its behaviour on paper chromatograms seemed to consist largely of aldobiouronic acids, was displaced from the resin column by sulphuric acid. Vigorous hydrolysis of this fraction followed by paper chromatography suggested the presence of galactose, arabinose, rhamnose, and hexuronic acid. After prior treatment with methanolic hydrogen chloride the uronic acids were reduced with borohydride,[3] and subsequent hydrolysis and paper chromatography revealed the same neutral components as before, but with an increased proportion of galactose. This observation, coupled with the absence of glucurono-6 ⟶ 3-lactone, led to the conclusion that galacturonic acid was present in the polysaccharide and that it was linked glycosidically to galactose, arabinose, and rhamnose. Quantitative analysis of the polysaccharide indicated the presence of L-arabinose, D-galactose, L-rhamnose, and galacturonic acid in the approximate molecular proportions of 21 : 3 : 1 : 1·5.

Electrophoresis[4] of the polysaccharide in three buffers of pH 7·0, 8·0, and 5·2 respectively gave peaks which migrated towards the anode, presumably because of the hexuronic

[1] Scheilber, *Ber.*, 1873, **6**, 612; Hirst and Jones, *J.*, 1948, 2311.
[2] Hough, Jones, and Wadman, *J.*, 1949, 2511; 1950, 1702.
[3] Jones and Reid, *Canad. J. Chem.*, 1955, **33**, 1682.
[4] Colvin, Cook, and Adams, *Canad. J. Chem.*, 1952, **30**, 603; Speizer, Copley, and Nutting, *J. Phys. Colloid Chem.*, 1947, **51**, 117; Lindquist, *Biochem. Biophys. Acta*, 1953, **10**, 580; Northcote, *Biochem. J.*, 1954, **58**, 353; Record and Grinstead, *ibid.*, 1953, **53**, 671.

acid constituents. A single peak was observed in each buffer, although in the acetate buffer (pH 5·2) the peak became rather diffuse. However, on reversal of the current, a single compact peak was again formed. The latter result is compatible with heterogeneity due to slight differences in the molecular constitution of the polysaccharide molecules rather than to a mixture of quite different molecular species such as araban and polygalacturonic acid, which would probably be resolved on electrophoresis into two distinct components. Attempts to fractionate the polysaccharide, and so obtain a polysaccharide further enriched in arabinose, by copper-complex formation, alcohol–water extraction, and Cetavlon precipitation were all unsuccessful.

Acetylation of the sugar-beet polysaccharide with acetic anhydride in pyridine gave an acetate ($[\alpha]_D$ −87° in $CHCl_3$) which was fractionated with chloroform–light petroleum into four main fractions (see Table 1). Each fraction was deacetylated and hydrolysed. Examination on paper chromatograms revealed little difference in composition; rhamnose and galactose were still present in each fraction, and traces of uronic acid were also apparent. Determination of the average molecular weight of the largest fraction of the acetylated polysaccharide by an isopiestic method [5] gave a value of 20,550 which corresponds to 12,500 for the free polysaccharide. Values of 6300 and 10,000 have been reported for araban fractions of sugar beet by Gaponekov [6] and Ingleman [7] respectively. Application of the acetic acid–trifluoracetic anhydride reagent [8] to the acetylation of the sugar-beet polysaccharide caused considerable degradation, loss of arabinose units being indicated by the optical rotation of the polysaccharide acetate ($[\alpha]_D$ −12° in $CHCl_3$). Hydrolysis of the deacetylated polysaccharide verified this conclusion since on paper chromatograms greatly increased proportions of rhamnose, galactose, and uronic acid, relative to the arabinose, were found.

In a partial hydrolysis experiment designed for the isolation of oligosaccharides, a polysaccharide solution was adjusted to pH 2·0 with sulphuric acid and heated at 80°. The products were fractionated on a charcoal column,[9] with water–alcohol for elution. Galactose and arabinose, rhamnose and fucose, two mono-*O*-methyl sugars, and oligosaccharides appeared in successive fractions from the column. The mono-*O*-methyl sugars were obtained crystalline, after separation on paper chromatograms, and were identified as 2-*O*-methyl-D-xylose and 2-*O*-methyl-L-fucose. Another compound, also isolated in small amount, appeared to be an aldobiouronic acid composed of galacturonic acid and 2-*O*-methylxylose.

The detection of these mono-*O*-methyl sugars in the polysaccharides of plum leaves [10] and in sugar-beet and sisal pectin [11] with the aid of charcoal adsorption and desorption suggests that they are normal constituents of pectin but hitherto have escaped detection because of the small quantities present.

Experimental

Partition chromatography by the descending method was carried out on Whatman No. 1 filter paper, with one of the following solvent systems: (*a*) ethyl acetate–acetic acid–water (9 : 2 : 2 v/v); (*b*) butan-1-ol–pyridine–water (10 : 3 : 3 v/v); (*c*) butan-1-ol–ethanol–water (40 : 11 : 19 v/v). Sugars were detected on the chromatograms with *p*-anisidine hydrochloride.[2] Amberlite ion-exchange resins were used throughout. Unless otherwise stated, optical rotations were determined for aqueous solutions at 25°.

Preparation of the Alkali-stable Polysaccharide.—Sugar beet in the form of dry chips was kindly provided by Dr. A. Carruthers of the British Sugar Corporation. In their preparation,

[5] Barger, *J.*, 1904, 286; Barker and Bourne, *J.*, 1952, 209.
[6] Gaponekov, *J. Gen. Chem.* (*U.S.S.R.*), 1937, **7**, 1729; *Chem. Abs.*, 1937, **31**, 8307.
[7] Ingleman, *Comm. Swed. Sugar Corp.*, 1945, **1**, 179; *Chem. Abs.*, 1945, **39**, 5525.
[8] Bourne, Stacey, Tatlow, and Tedder, *J.*, 1949, 2976.
[9] Andrews, Hough, and Powell, *Chem. and Ind.*, 1956, 658.
[10] Andrews and Hough, *J.*, 1958, 4476.
[11] Aspinall and Cañas-Rodriguez, *J.*, 1958, 4020.

sugar-beet slices had been thoroughly extracted with water at *ca.* 70° to remove sucrose and other soluble materials, then the residual material was pressed and air-dried.

The chips (1·8 kg.) were heated with saturated lime-water (18 l.) at 60—65° for 16 hr., during which sodium hydrogen sulphite (*ca.* 5 g.) was stirred into the mixture in order to reduce the formation of dark products. After the slurry had cooled, as much liquid as possible was extracted from it by use of a press. The extract was then filtered through a Nylon stocking and finally clarified by passage through a Sharples high-speed centrifuge. The resultant solution (13 l.) was acidified with acetic acid, and methylated spirits (52 l.) added to precipitate the polysaccharide. This precipitate was dissolved in the minimum volume of water, and the solution, after clarification on the centrifuge, dialysed against tap-water for 1 week. Adding ethanol (4 vol.) to the dialysed solution precipitated the crude polysaccharide, which was isolated on the centrifuge, washed with ethanol and ether, and dried over phosphoric oxide (yield, 100 g.). This material had $[\alpha]_D$ −84° (*c* 1·0) (Found: N, 0·8; sulphated ash, 5·1%).

For quantitative studies, this product was further purified by passage of a 1% solution through a column of mixed IR-120(H) and IR-4B(OH) resins (1 : 3 w/w), the efficiency of the de-ionisation process being checked by measuring the conductivity of the column effluent. The polysaccharide was reprecipitated with ethanol, collected on the centrifuge, washed with ethanol and ether, and dried; this material had $[\alpha]_D$ −108° ± 3° (*c* 1·0) (Found: N, 0·8; OMe, 0·8; sulphated ash, 0·9%). The supernatant liquid was concentrated, and the small residue examined on paper chromatograms. The presence of a trace of arabinose was detected.

Electrophoresis of the Polysaccharide.—Solutions of the purified polysaccharide (1·25% w/v) in 0·05M-phosphate buffer (pH 7·0), 0·20M-borate buffer (pH 8·0), and 0·1M-acetate buffer (pH 5·2) were clarified on the high-speed centrifuge. The supernatant liquors were dialysed against the appropriate buffer (1 l.) for 24 hr. at 4°. After the conductivities of the resultant solutions had been checked, electrophoresis was carried out in a Tiselius apparatus at 4°, with a potential of 320—420 v and a current of 15—18 m. amps. Photographs of the migrating boundaries were taken at various times up to 5 hr., after which the boundaries became diffuse.

Preparation of the Acetylated Polysaccharide.—(a) *With acetic anhydride.* The crude polysaccharide (13 g.) was suspended in dry pyridine (650 c.c.), and acetic anhydride (200 c.c.) was added during several hours with stirring. The mixture was then heated at 95—100° for 6 hr., then cooled, and insoluble material was removed on the centrifuge. The supernatant liquid was poured into water, and the precipitate of crude acetylated polysaccharide collected, washed with water, and reprecipitated from acetone solution. The final product (6·7 g.) had $[\alpha]_D$ −87° ± 2° (*c* 0·8 in $CHCl_3$) (Found: Ac, 38·5. Calc. for di-*O*-acetylpentan: Ac, 39·8%).

The acetylated polysaccharide (5 g.) was fractionated by heating it under reflux with mixtures of chloroform and light petroleum (b. p. 40—60°); each extraction was for 0·5 hr. with 100 c.c. of solvent mixture. Each of the four fractions so obtained (see Table 1) was dissolved in ice-cold chloroform, and deacetylated with sodium methoxide as described by Hirst, Percival, and Wylam.[12] The regenerated polysaccharide was purified by precipitation from aqueous solution with methanol, then washed with methanol and ether, and dried over phosphoric oxide.

TABLE 1. *Fractionation of acetylated polysaccharide.*

	Acetylated polysaccharide			
Fraction	$CHCl_3$–light petroleum (v/v)	Yield (g.)	$[\alpha]_D$ (in $CHCl_3$)	Deacetylated product, $[\alpha]_D$ (in H_2O)
1	35 : 65	0·05	−61° ± 5° (*c* 0·9)	Insol. in water
2	40 : 60	0·16	−118 ± 7 (*c* 0·3)	−104 ± 8° (*c* 0·4)
3	45 : 55	0·30	−102 ± 2 (*c* 0·8)	−100 ± 5 (*c* 0·3)
4	50 : 50	4·31	−84 ± 2 (*c* 1·2)	−95 ± 2 (*c* 1·1)

(b) *With trifluoracetic anhydride.*[8] Trifluoracetic anhydride (16·5 c.c.) was added dropwise to a suspension of polysaccharide (1 g.) in glacial acetic acid (15 c.c.). The mixture was stirred for 4 hr. at 20°, then poured into water. The acetylated material which separated was collected, washed with water, and dissolved in chloroform. After being dried (K_2CO_3), the chloroform solution was evaporated to a crisp brown solid (0·56 g.) with $[\alpha]_D$ −11·5° ± 1·5° (*c* 1·4 in $CHCl_3$) (Found: Ac, 37·9%). Deacetylation in chloroform solution with sodium methoxide gave a water-soluble product with $[\alpha]_D$ +27° ± 2° (*c* 0·8).

[12] Hirst, Percival, and Wylam, *J.*, 1954, 189.

Qualitative Analysis of the Alkali-stable Polysaccharide.—Samples of the crude and purified polysaccharides were hydrolysed in N-sulphuric acid at 100° for 16 hr. After neutralisation with barium carbonate and evaporation, the hydrolysates were examined on paper chromatograms. The presence in each of a large quantity of arabinose and minor quantities of galactose, rhamnose, and hexuronic acid was revealed. A sample of the alkali-stable polysaccharide prepared previously by Hirst and Jones [1] was similarly hydrolysed, and appeared to have a similar composition. The polysaccharide regenerated from fractions 2, 3, and 4 of the acetylated material (see Table 1) also contained galactose and rhamnose in addition to large amounts of arabinose, and traces of hexuronic acid. In contrast, the polysaccharide regenerated from the material acetylated with trifluoracetic anhydride as catalyst contained much greater proportions of galactose, rhamnose, and hexuronic acid, relative to the arabinose.

For characterisation of the above constituents, the alkali-stable polysaccharide (3 g.) was hydrolysed in N-sulphuric acid (50 c.c.) at 100° for 20 hr. The cooled hydrolysate was passed through a column of IR-4B(OH) resin, and the neutral effluent concentrated, yielding a syrup (2·1 g.) which was fractionated by partition chromatography on a cellulose column (25 × 4 cm.), with butan-1-ol, half-saturated with water, as the mobile phase. The following monosaccharides and derivatives were obtained: L-Arabinose (1·3 g.), m. p. and mixed m. p. 151°, $[\alpha]_D$ +103° (equil.; *c* 1·0); *N*-benzoylhydrazone, m. p. and mixed m. p. 187°. D-Galactose (150 mg.), m. p. 157°, mixed m. p. 158°, $[\alpha]_D$ +77° (equil.; *c* 0·6). L-Rhamnose (40 mg.), $[\alpha]_D$ +11° (measured on solution of anhydrous syrup; *c* 0·3); *N*-benzoylhydrazone, m. p. and mixed m. p. 179°. A compound (*ca.* 5 mg.) which co-chromatographed with mannose was also isolated.

Examination of the Acidic Components of the Alkali-stable Polysaccharide.—The acidic carbohydrates retained on the IR-4B resin column, through which the polysaccharide hydrolysate had been passed (see above), were displaced from it by elution with N-sulphuric acid. The effluent was neutralised with barium carbonate, and the solution filtered and evaporated to dryness. The residue (0·3 g.) was extracted several times with hot methanol to remove any neutral sugars, then dissolved in water, and the solution was passed through a column of IR-120(H) resin. The acidic solution so obtained was evaporated at 30°, and yielded a syrup (0·21 g.) which was shown by paper chromatography to contain hexuronic acid and acidic oligosaccharides, but glucurono-6 ⟶ 3-lactone was not detected. A portion (30 mg.) of the syrup was heated in 2N-sulphuric acid at 100° for 16 hr., and the products were examined on the paper chromatogram; galactose, rhamnose (ratio roughly 1 : 1, estimated visually), hexuronic acid, and a trace of arabinose were observed.

The remainder of the syrup (180 mg.) was heated under reflux with methanol containing hydrogen chloride (4% w/w) for 16 hr. The solution was then neutralised with silver carbonate, filtered, and concentrated, and the residue dissolved in 0·1M-sodium borohydride (10 c.c.). After 3 hr., excess of borohydride was decomposed by addition of dilute acetic acid, and the solution passed through columns of IR-120(H) and IR-4B(OH) resins. The effluent was concentrated to dryness, and the residue hydrolysed by N-sulphuric acid for 16 hr. at 100°. Paper chromatography of the hydrolysate indicated the presence of galactose, rhamnose (ratio roughly 3 : 1), and arabinose; glucose was not detected.

Isolation of Other Monosaccharide Components of the Alkali-stable Polysaccharide.—As a preliminary to the isolation of oligosaccharides, the alkali-stable polysaccharide was submitted to partial hydrolysis. The polysaccharide (100 g.) was dissolved in 0·01N-sulphuric acid (10 l.), then the solution was adjusted to pH 2 with N-sulphuric acid and heated in a stoppered vessel at 80° for 22 hr.; during this time, further additions of acid were necessary to maintain pH 2. After cooling, the solution was neutralised with barium carbonate, filtered, and concentrated to *ca.* 0·5 l. Material of high molecular weight was precipitated by the addition of methanol (2 l.), then the supernatant liquid was evaporated, yielding a yellow solid (49 g.). This was dissolved in the minimum volume of water, and the solution percolated through a squat column (18 × 12 cm.) of B.D.H. acid-washed charcoal, which had previously been washed with water (5 l.). Fractions containing monosaccharides were eluted from the column with water and water–ethanol (5 l. each time) (see Table 2); elution with concentrations of ethanol greater than 5% afforded mixtures of oligosaccharides, which were retained for further investigation.

Attempts to isolate the compounds with R_{Rh} 0·39 and 0·71 by chromatography of the whole fraction on large paper sheets were unsuccessful; neither compound was obtained in quantity sufficient for further examination. The compound with R_{Rh} 0·71 was indistinguishable from fucose on paper chromatograms.

The compounds with R_{Rh} 1·2 and 1·3 resembled 2-*O*-methylxylose and 2-*O*-methylfucose respectively in their chromatographic behaviour. These identifications were confirmed on the materials obtained when the appropriate fractions were submitted to chromatography on large

TABLE 2. *Fractionation of mono- and oligo-saccharides of the sugar-beet polysaccharide.*

Eluent	Weight of fraction (g.)	Components *
Water	39·0	Arabinose (mainly), galactose
Water + 1% v/v EtOH	0·6	Rhamnose
,, 2% ,,	0·5	Rhamnose + traces of compounds with R_{Rh} 0·39 (pink) and 0·71 (brown)
,, 3% ,,	0·23	Oligosaccharides + compounds with R_{Rh} 1·2 (pink) and 1·3 (yellow-brown)
,, 4% ,,	0·37	Oligosaccharides + compounds with R_{Rh} 1·2 (pink) and 1·3 (yellow-brown)
,, 5% ,,	0·28	Oligosaccharides + compounds with R_{Rh} 1·2 (pink) and 1·3 (yellow-brown)

* R_{Rh} = rate of movement relative to rhamnose in solvent (*b*); colours are those produced on chromatograms sprayed with *p*-anisidine hydrochloride and heated.

paper sheets with solvent (*c*). The monosaccharides were eluted from the requisite areas of the chromatograms with water, then the aqueous solutions were passed through columns of IR-120(H) and IR-400(CO_3) resins, and evaporated to dryness.

2-*O*-Methyl-D-xylose (33 mg.) had $[\alpha]_D$ +24° ± 5° (*c* 1·7) and R_{Rh} 1·22 in solvent (*b*). After recrystallisation from acetone it had m. p. and mixed m. p. 133—134°, and the *X*-ray powder photograph of the compound was identical with that of an authentic specimen.

2-*O*-Methyl-L-fucose (15 mg.) crystallised from acetone, and then had m. p. and mixed m. p. 148—150°, $[\alpha]_D$ −77° ± 3° (*c* 0·7; equil.), and R_{Rh} 1·29 in solvent (*b*). *X*-Ray powder photography confirmed the identification.

Elution with water of further areas of the chromatograms from which the *O*-methyl sugars were obtained gave a compound (24 mg.) which behaved as a uronic acid on the chromatogram; it gave a pink spot with *p*-anisidine hydrochloride, and had R_{Rh} *ca.* 0·2 in solvent (*c*), but apparently was contaminated with inorganic material. After treatment with methanolic hydrogen chloride (2% w/w; 10 c.c.) for 8 hr. under reflux, borohydride reduction, and hydrolysis with 2N-sulphuric acid for 8 hr. at 100°, paper chromatography of the products revealed the presence of galactose and 2-*O*-methylxylose.

Quantitative Analysis of the Alkali-stable Polysaccharide.—The purified polysaccharide (36·8 mg.) was hydrolysed in N-sulphuric acid (2 c.c.) at 100° for 16 hr. D-Ribose (16·5 mg.) was added to the cooled hydrolysate, which was then neutralised with barium carbonate and concentrated. After separation on paper chromatograms [solvent (*a*)], the components of the monosaccharide mixture were estimated colorimetrically and in duplicate by the benzidine method.[13] The results are shown in Table 3, where recoveries are calculated on the basis of the respective ribose estimates.

TABLE 3. *Composition of the alkali-stable polysaccharide.*

Aldose	Found (mg.)	Recovery from polysaccharide, calc. as anhydroaldose (mg.)	(%)	Molar ratios (mean)
Arabinose	4·40, 4·54	27·20, 26·90	74·0, 73·0	21·0
Galactose	0·61, 0·63	3·85, 3·82	10·5, 10·4	3·0
Rhamnose	0·21, 0·21	1·31, 1·26	3·6, 3·4	1·0
Ribose	2·35, 2·45			

The uronic anhydride content of the polysaccharide was estimated by Johansson, Lindberg, and Theander's method [14] (Found: 5·5, 4·8% in duplicate experiments) and by titration of an aqueous solution of the polysaccharide (sulphated ash, 0·8%) with 0·01N-sodium hydroxide, with phenolphthalein as indicator (Found: 5·1%, corresponding to an equivalent weight of 3470 for the polysaccharide).

[13] Jones and Pridham, *Biochem. J.*, 1954, **58**, 288.
[14] Johansson, Lindberg, and Theander, *Svensk Papperstidn.*, 1954, **57**, 41.

The nitrogen content of the purified polysaccharide (0·8%) indicated a protein content of *ca.* 5% (based on an average N content of 16% for the protein), and this was verified by Goodwin and Morton's spectrophotometric method [15] (Found for the polysaccharide: tyrosine, 0·31; tryptophan, 0·026%). If it is assumed that the protein contains 6% of tyrosine and 0·5% of tryptophan (*e.g.*, albumin of peas), this corresponds to a protein content of 5·2% in the polysaccharide.

Further Experiments with the Alkali-stable Polysaccharide.—The polysaccharide was heated in saturated lime-water at 80° for 24 hr., then isolated, after neutralisation of the solution with acetic acid, by precipitation with ethanol. The optical rotation and composition (determined by paper chromatography of a hydrolysate) of this product were similar to those of the starting material.

An aqueous solution of Cetavlon (20% w/v) was slowly added to a stirred solution (1% w/v) of the polysaccharide (sulphated ash, 0·8%).[16] The small amount of precipitate formed was collected after 24 hr., and the polysaccharide remaining in solution was precipitated with ethanol. Paper-chromatography of hydrolysates of the two fractions indicated that they were similar in composition.

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[15] Goodwin and Morton, *Biochem. J.*, 1946, **40**, 628.
[16] Bera, Foster, and Stacey, *J.*, 1955, 3788.