Pectic Polysaccharides of Growing Plant Tissues

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1. The polysaccharide compositions of the cell walls of sycamore cambium and sycamore callus tissue have been analysed and found to be directly comparable. 2. Electrophoretic analyses of the whole pectins prepared from actively growing callus and cambial tissue have shown that these preparations contain, in addition to the neutral and weakly acidic components present in apple fruit, a strongly acidic polygalacturonic acid component. 3. The weakly acidic component of all the pectins was directly comparable with that of the pectinic acid of apple fruit. 4. The components of the whole pectin of sycamore callus tissue have been partially purified and analysed. The neutral and weakly acidic components also found in apple fruit were isolated. 5. The pattern of the composition of the neutral sugars present in the pectins of actively growing tissues of cambium and callus has been compared with those present in apple-fruit pectinic acid. 6. The presence of rhamnose linked as galacturonosyl-(1→2)-rhamnose has been found in sycamore whole pectin. 7. The difference in the pectins of callus, cambium and fruit appears not to be that of species difference but is more characteristic of the nature of the growth and growth conditions of the cells. This is discussed in relation to the problems of the control and mechanism of plant-cell growth and differentiation.

Theories of plant-cell-wall development and extension (Bonner, 1961) have emphasized the importance of the distinctive properties of pectinic acid, which was defined as a polygalacturonan (Kertesz, Baker, Joseph, Mottern & Olsen, 1944). The binding of Ca^{2+} ions between the carboxyl groups of this component was thought to confer rigidity upon the wall by the formation of cross-linkages. A reduction of this binding, by methyl esterification, would permit the plasticity of the wall associated with its elongation. Studies by other workers have shown that this simple explanation is inadequate. It has been demonstrated that the auxin-sensitive and auxin-insensitive incorporations of methyl groups, into hot- and cold-water-soluble pectins of *Avena* coleoptile, are almost abolished by amounts of ethionine sufficient to inhibit elongation only partially (Cleland, 1963*a*). Detailed stress-strain analyses of the coleoptile cell walls have made it possible to distinguish both an auxin-sensitive and an auxin-insensitive stage of cell extension which differ in their response to various inhibitors (Olson & Cleland, 1964; Cleland, 1965*a, b*). Auxin has an effect upon wall 'loosening'

which is oxygen-dependent and inhibited by cyanide, but is insensitive to inhibitors of protein synthesis. Auxin-induced cell elongation, however, is sensitive to inhibitors both of protein and of RNA synthesis, and a protein appears to be necessary for the extension process (Cleland, 1963*b*, 1964, 1965*a, b*; Noodén & Thimann, 1966). It seems probable that the partial inhibition of extension by ethionine was the result of its effects on protein synthesis. It is therefore necessary to examine other features of pectin which could relate to its role in cell-wall development, especially its neutral sugar content, its metabolic formation and the changes which occur in its composition during cell development and the enzymic and genetic control of this metabolism.

Provided that sufficiently mild preparative methods are used, chemical studies show that neutral sugars are a widespread, and possibly ubiquitous, component of pectinic acid (Aspinall & Fanshawe, 1961; Barrett & Northcote, 1965; Bhattacharjee & Timell, 1965; Gould, Rees, Richardson & Steele, 1965). These neutral sugars have a considerable effect upon the physical properties of the material. It is important therefore to investigate the chemical nature of pectinic acids

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of different tissues of the same plant and in tissues at different stages of development and under different growth conditions.

METHODS

The methods of polysaccharide hydrolysis with nitric acid, estimation of sugar and uronic acid, moisture and ash determinations, pectinesterase (pectin pectylhydrolase, EC 3.1.1.11) preparation, enzymic de-esterification and ethylene oxide esterification have been described in detail by Barrett & Northcote (1965).

Zone electrophoresis of polysaccharides. High-voltage electrophoresis was used (Barrett & Northcote, 1965). The pyridine-acetic acid buffer (pH 6.5; 10%, v/v, pyridine-0.3%, v/v, acetic acid) applied to the glass-fibre paper (Whatman GF 81 & GF 82 formerly GF A & GF B respectively) was modified by the addition of EDTA (final concn. 10 mM) to avoid binding of very acidic polysaccharides at the origin.

Origin and growth of the callus tissues. Callus tissues were derived from explants of twigs of a sycamore (*Acer pseudo-platanus* L.) growing in the University Botanic Garden, Cambridge. The same tree was used as a source of explants by Lampion & Northcote (1960) and Lampion (1964) and was used for analysis by Thornber & Northcote (1961a,b).

The apple (*Pyrus malus* L. var. Bramley's Seedling) callus tissue was derived from the cambial layer of the apple fruit, which was taken from the same sample as used by Barrett & Northcote (1965) for studies on fruit pectin.

The bean (*Phaseolus vulgaris*) callus was derived from stem explants of young seedlings, and was the same tissue as used for studies on differentiation by Jeffs & Northcote (1966).

The culture of Virginian creeper (*Parthenocissus tricuspidatus*) crown gall was obtained from Dr B. Kassanis of Rothamsted Experimental Station, Herts.

Jeffs & Northcote (1966) have described the procedures used for obtaining callus from bean explants and for its sterile maintenance and subculture. Similar techniques were used for sycamore and apple tissues, and in the subculture of the crown gall. The medium described by Jeffs & Northcote (1966) was used also for sycamore and apple tissues. The crown gall tissue was maintained on a medium containing salts, sucrose, vitamins (thiamine, nicotinic acid, pyridoxamine) and glycine with no other growth factors.

Liquid-suspension callus was derived from solid callus by successive passages of lumps of callus through liquid culture, with subculture of suspended cells only. Solid callus tissue was grown in boiling tubes and liquid-suspension callus in 11. glass bottles rotated on their sides at 43 rev./min. Cultures were incubated at 25–26° in darkness.

Cambium preparation. Sycamore cambium was collected from saplings growing in Madingley Wood, Cambridge, by use of the procedure of Thornber & Northcote (1961a).

Apple cambium (var. Bramley's Seedling) was obtained from a tree growing at Wall Heath, Staffordshire, by a similar procedure.

Hydrolysis of polysaccharide with sulphuric acid. The whole extracted cells of cambial and callus tissues, and the neutral polysaccharide of the suspension callus pectin, were hydrolysed (Saeman, Moore, Mitchell & Millett, 1954) and the hydrolysates were neutralized with barium carbonate or Amberlite IRA-400 (CO₃²⁻ form) (Rohm and Haas Co.).

They were then de-ionized on short columns of Zeo-Karb 225 (H⁺ form) (The Permutit Co. Ltd.) or Amberlite IR-120 (H⁺ form) (Rohm and Haas Co.), concentrated by rotatory evaporation at 50° under reduced pressure and made up to known volumes with saturated benzoic acid solution. Recoveries were in the range 85–90%. There was no detectable epimerization of the standards.

The whole pectin hydrolysates, and the hydrolysate of fraction Za, were neutralized by extraction of the excess of sulphuric acid with *NN*-bis-*n*-octylmethylamine in chloroform (10%, v/v) (Smith & Page, 1948). The neutralized hydrolysates were evaporated to dryness by rotatory evaporation and the residual trace of amine was removed by drying in a vacuum desiccator over phosphorus pentoxide for 1 hr. The residues were dissolved in saturated benzoic acid and chromatographed. The percentage recoveries with this method of hydrolysis and neutralization were: Gal, 98; Glc, 100; Man, 100; Ara, 94; Xyl, 98; Rha (rhamnose), 100.

Paper-chromatographic analysis of hydrolysates. Neutral monosaccharides present in polysaccharides were separated by descending chromatography on Whatman no. 1 paper in solvent A (ethyl acetate-pyridine-water, 8:2:1, by vol.). Some fast-running monosaccharide derivatives present in the hydrolysates were chromatographed in solvent B (butan-1-ol-ethanol-water; 5:1:4, by vol.). Uronic acids were separated by chromatography with solvent C (ethyl acetate-acetic acid-pyridine-water; 5:1:5:3, by vol.) in a tank equilibrated with ethyl acetate-pyridine-water (40:11:6, by vol.) (Fischer & Dörfel, 1955). Uronic acids and acidic oligosaccharides were run with solvent D (ethyl acetate-acetic acid-formic acid-water; 18:3:1:4, by vol.) (Jones & Wise, 1952). The monosaccharides were detected and estimated by the aniline hydrogen phthalate method (Wilson, 1959).

Diazomethane esterification. Nitrosomethylurea and ethereal diazomethane were prepared by the methods described by Eistert (1948). A suspension of pectin (1–5 mg.) in dry methanol or ether was shaken with excess of diazomethane solution for 15 min. and allowed to stand for a further 15 min. Residual diazomethane was destroyed with a drop of 1.0*N*-hydrochloric acid. After removal of the organic solvent the pectin was freeze-dried from a little water.

Transelimination degradation. Transelimination degradation of ethylene oxide-esterified whole pectin was carried out as described by Barrett & Northcote (1965) for apple pectin.

Transelimination of small amounts of pectinic acid (0.5–1.5 mg.) was achieved by dissolving the pectinic acid in 0.1*M*-sodium phosphate buffer (pH 6.8) and heating to 95° in a sealed tube for 4–12 hr. The process, which had begun after 4 hr., was essentially complete after 12 hr. The product was usually subjected to electrophoresis directly, without dialysis. There was no significant difference between the electrophoretic pattern of dialysed and undialysed material.

RESULTS

Preparation of tissues. Solid-grown and liquid-suspension callus tissues were killed by reflux extraction with boiling ethanol (85%, v/v) for 10 min. The tissues were strained off and washed

with 80% ethanol until no carbohydrate appeared in the filtrate, when this was tested with the sulphonated α -naphthol reaction (Devor, 1950; Dische, 1962), and it was then rinsed with acetone and dried *in vacuo*.

The freeze-dried cambial tissue was similarly extracted. This treatment removed much of the soluble phenols present.

Total hydrolysis of the extracted cells. Portions of dry, sugar-free cambial and suspension-cultured sycamore callus tissues were hydrolysed by sulphuric acid. The results are summarized in Table 1. The figures in parentheses for glucose represent, first, the figure obtained after hydrolysis in sulphuric acid and, secondly, glucose released by 3% nitric acid hydrolysis. The latter largely represents the starch content of the cells. The figure in the main column is the difference between the two and probably represents α -cellulose. The galacturonic acid estimations were made on a degraded pectin fraction which had been extracted with 0.5% EDTA (disodium salt) buffered at pH 6.8 with 0.05M-sodium phosphate (McCready & McComb, 1952).

Extraction of whole pectin from apple fruit. The following extractant solutions were used: (1) EDTA (disodium salt, 0.05M), pH 4.5, in sodium acetate-acetic acid buffer (0.05M); (2) EDTA (disodium salt, 0.05M), pH 6.8, in sodium phosphate buffer (0.05M); (3) glycine-HCl buffer, pH 3.0; (4) calcium chloride (0.05M), pH 4.5, in sodium acetate-acetic acid buffer (0.05M); (5) sodium hexametaphosphate (2%) adjusted to pH 3.7 with hydrochloric acid.

The ethanol-extracted dry tissue (100mg.) was dropped into boiling extractant solution (30ml.)

and heated under reflux. Samples (0.5ml.) were removed at definite times, diluted to 5.0ml. with benzoic acid solution and filtered and the uronic acid determined. It was necessary to include blanks and standards containing the extractant solutions in the uronic acid determinations. The total pectin content of the dry tissue was estimated by the EDTA-pectinase method of McCready & McComb (1952). The results are shown in Fig. 1.

Zone electrophoresis of whole pectin prepared from apple fruit. A sample of the whole pectin prepared by extraction of the dry tissue with sodium hexametaphosphate (2%, pH 3.7) when subjected to zone electrophoresis (pH 6.5) showed the two major components (a neutral zone and a weakly acidic pectinic acid zone) as previously described by Barrett & Northcote (1965); a faint trace of a minor component with an electrophoretic mobility comparable with that of zone A (see zone electrophoresis of whole pectins of actively growing tissues described below) could be detected when the electrophoretogram was heavily loaded.

A preparation of apple pectic polysaccharides prepared according to the method of Barrett & Northcote (1965) was refluxed at 100° for 4hr. in sodium hexametaphosphate (2%, pH 3.7). The polysaccharide was recovered after dialyses by precipitation with ethanol (85%, v/v) and subjected to zone electrophoresis at pH 6.5 together with a sample of the untreated preparation. No change in the electrophoretic pattern of the pectic polysaccharide was observed as a result of the treatment with hexametaphosphate.

Table 1. *Composition of polysaccharide components of sycamore callus and cambial tissue*

Values are percentages of the whole, dry, 80% ethanol-extracted tissue expressed as glycosyl residues. The glucose values in parentheses represent the figure obtained by 72% H_2SO_4 hydrolysis (starch + α -cellulose) and 3% HNO_3 hydrolysis (starch) respectively; the figure in the main column is the difference between these two values (α -cellulose). The galacturonic acid (Gal A) values were obtained from the borate-carbazole method in an extraction made with EDTA-sodium phosphate buffer (pH 6.8).

Monomer	Callus (%)	Cambium (%)
Gal	6.4	8.1
Glc	7.6 (14.2, 6.6)	11.5 (13.5, 2.0)
Man	0.8	0.8
Ara	8.2	6.3
Xyl	4.0	2.9
Rha	1.0	1.1
Gal A	10.3	11.6
Total	38.3	42.3

Fig. 1. Progress of the extraction of apple-fruit pectin by various extractant solutions: ▲, EDTA (disodium salt, pH 6.8); ○, sodium hexametaphosphate (pH 3.7); +, EDTA (disodium salt, pH 4.5); △, buffer, pH 3.0; ●, CaCl_2 , pH 4.5; ---, total pectin (EDTA-pectinase).

Scheme 1. Flow sheet illustrating isolation and fractionation of whole pectin from sycamore suspension callus tissue.

Extraction of whole pectin from actively growing tissues (Scheme 1). The dry, ethanol-extracted tissues (5g.) were refluxed (4hr.) with 0.2-2.0% sodium hexametaphosphate solution (500ml.), brought to pH 3.5-4.0 with hydrochloric acid. In each case the slurry was centrifuged and the supernatant filtered through Filter-Cel (Johns-Manville

Ltd.). The filtrate (pH 4.0) was adjusted to pH 6.5-7.5 with dilute sodium hydroxide, and sodium chloride (2.5g.) was added. A few drops of a freshly prepared human α -amylase (EC 3.2.1.1) solution were then added (Olaitan & Northcote, 1962), followed by a little toluene, and the digestion was left to proceed at 25° overnight. The whole

Fig. 2 Diagrams showing the relative positions of various fractions of whole pectins after zone electrophoresis (pyridine-acetate buffer, pH 6.5, with 10 mM-EDTA; paper GF 81). Broken lines indicate tailing. (a) Sycamore suspension callus whole pectin. (1) Whole pectin; (2) whole pectin after de-esterification with pectinesterase; (3) whole pectin after treatment with ethylene oxide or diazomethane; (4) transesterification-degraded esterified pectin. A and B represent strongly acidic zones, C weakly acidic zones and D a neutral zone. (b) (1) Bean suspension callus whole pectin; (2) *Parthenocissus* crown gall callus whole pectin; (3) apple suspension callus whole pectin; (4) apple cambial whole pectin; (5) apple-fruit pectinic acid marker (Barrett & Northcote, 1965). (c) Isolated fractions of sycamore suspension callus whole pectin. (1) Fraction X; (2) fraction Y; (3) fraction Z; (4) fraction Za; (5) fraction Za after alkaline demethylation; (6) transesterification-degraded fraction Za; (7) fraction Zn. (d) Isolated fractions of solid sycamore callus (1-3) and sycamore cambial whole pectins (4-6). (1) and (4) Fractions X; (2) and (5) fractions Y; (3) and (6) fractions Z.

was then dialysed for 24 hr. against several changes of water.

A whole pectin fraction was precipitated from the dialysis residue, by the addition of ethanol to a final concentration in excess of 80% (v/v). The precipitated gel was centrifuged, mixed with a little water, and freeze-dried, without removal of calcium.

Zone electrophoresis of whole pectins prepared from actively growing tissues. Samples of the whole pectins were subjected to zone electrophoresis. Results are shown in Fig. 2(a,b).

The regions shown in Fig. 2(a) as A, B, C and D were eluted from electrophoretograms (paper GF 81) of whole pectin and the eluates were freeze-dried and hydrolysed with nitric acid-urea. The resulting hydrolysates were then qualitatively analysed for neutral sugars. Fraction D was found to contain arabinose and galactose, with no uronic acid.

Fraction C contained arabinose, galactose, xylose, uronic acid and traces of glucose and mannose. Fractions A and B contained uronic acid, small amounts of rhamnose and traces of xylose, glucose and mannose.

Preparative fractionation of the whole pectin of sycamore (Scheme 1). When fractionation was intended the initial precipitation of the whole pectin from the extract was omitted. Calcium chloride solution (1.0M) was added, with stirring, to the starch-free dialysis residue, until no further precipitation occurred. The gelatinous precipitate (fraction X) was then removed by centrifugation.

An equal volume of 96% ethanol was gradually stirred into the supernatant from the first precipitation and the resulting precipitate (fraction Y) was collected.

The supernatant solution from this precipitation was mixed with an equal volume of acetone and

left to stand overnight. The precipitate (fraction Z) was collected. The supernatant solution from this last step was free of carbohydrate, when examined by the sulphonated α -naphthol method.

Calcium was removed from these fractions, and from whole pectin, by dissolving the precipitates in 0.2M-EDTA (disodium salt, adjusted to pH 6.7 with sodium hydroxide) and dialysing overnight against a large volume of water. The solutions were filtered, when necessary, and the polysaccharides precipitated with ethanol. The precipitates were mixed with a little water and freeze-dried. Zone electrophoresis of fractions X, Y and Z showed that these fractions were heterogeneous and that X contained materials present in the zones A, B and C, Y those present in B and C, and Z those present in C and D of the whole pectin (Figs. 2c,d).

Separation of components of fraction Z (Scheme 2). An aqueous solution (0.35%, w/v) of fraction Z was brought to final concentration 48% (v/v) with absolute ethanol. A very slight precipitate was formed, removed by centrifugation and discarded.

More ethanol was added, to 80% (v/v), and a precipitate formed upon the addition of a few crystals of sodium acetate. This was removed by centrifugation, and the supernatant solution (containing the arabinan-galactan complex) retained (see below). The yield of precipitated polysaccharide recovered in the pellet, after freeze-drying, was about 75% of the starting material by weight. It consisted almost entirely of weakly acidic pectinic acid, with very little neutral material.

An aqueous 1% solution of this pectinic acid fraction was mixed with an excess of a saturated (21°) aqueous solution of cetylpyridinium chloride and the precipitate that formed was collected by centrifugation, washed with water, and dissolved in 1.0M-ammonium formate-formic acid buffer (pH 4.5). Absolute ethanol was added to final concentration 67% (v/v) and the gel formed was centrifuged down, washed with water and freeze-dried. The resulting polysaccharide solidified as white, translucent flakes (fraction Za) and these readily dissolved in water to give a viscous solution.

Scheme 2. Flow sheet illustrating separation of components of fraction Z of the whole pectin obtained from sycamore suspension callus tissue.

Upon electrophoresis the material moved as a weakly acidic spot and corresponded to zone C of the whole pectin (Fig. 2c).

Recovery of arabinan-galactan. The supernatant solution from the ethanol precipitation was mixed with an equal volume of acetone and allowed to stand overnight. The precipitate which formed was centrifuged down, dissolved in water, dialysed overnight and freeze-dried to a white powder which was readily soluble in water. This was redissolved, shaken with a little Amberlite IR-120 (H^+ form) and freeze-dried. This component (fraction Zn) was completely neutral upon zone electrophoresis and ran at the same position as dextran (mol.wt. 10 000) used as a marker, and corresponded to a narrow band in zone D of the whole pectin (Fig. 2c). Hydrolysis showed that it contained arabinose, galactose and xylose in the approximate molar proportions 20:10:1. Uronic acid or other sugars were not detected.

Uronic acid of the whole pectins. Chromatography of the materials hydrolysed with sulphuric acid showed that they contained galacturonic acid; no glucuronic acid was detected.

Neutral sugar content of whole pectins and fractions Za and Zn. The material was hydrolysed with sulphuric acid, neutralized with *NN*-bis-*n*-octyl-methylamine, and the hydrolysate analysed chromatographically. The results are shown in Fig. 3.

Analysis of fractions X, Y and Z. The fractions X, Y and Z were subjected to nitric acid-urea hydrolysis, the hydrolysates were chromatographed and the neutral sugars estimated. Uronic acid was estimated by the carbazole method. The results are summarized in Table 2.

Analysis for minor components. Dry, ethanol-extracted solid-grown sycamore callus tissue (10g.) was refluxed for 2hr. with 0.1M-EDTA (disodium salt; 500ml., pH 4.5). The extract was filtered, dialysed against distilled water and clarified by high-speed centrifugation. The supernatant solution was concentrated to 50ml., sulphuric acid was added (2.4g.; i.e. 1.0N-sulphuric acid), and the solution heated at 100° for 3hr. The cool solution (with precipitated polysaccharide) was mixed with an equal volume of acetone and the total polysaccharide was removed. This was twice re-hydrolysed (4hr. in 1.0N-sulphuric acid and 5hr. in 2.0N-sulphuric acid, both at 100°) progressively to remove the neutral sugars. All hydrolysates were neutralized with barium carbonate, the first after removal of acetone. All were reduced to small bulk and investigated chromatographically. The hydrolysates were passed through short columns of De-Acidite FF acetate resin (The Permutit Co. Ltd.) and the unabsorbed fractions were evaporated to syrups by rotatory evaporation and dissolved in benzoic acid solution (1.0ml.). Spots (10 μ l.) were

Fig. 3. Composition of neutral sugars present in whole pectins and in fractions Za and Zn. (1) Sycamore suspension callus whole pectin; (2) bean suspension callus whole pectin; (3) apple suspension callus whole pectin; (4) *Parthenocissus* crown gall callus whole pectin; (5) apple cambial whole pectin; (6) apple-fruit pectinic acid (from the figures quoted by Barrett & Northcote, 1965); (7) fraction Za; (8) fraction Zn. The percentage figure shown above each histogram is the amount of total neutral sugar calculated for the dry, ash-free material. G, Galactose; A, arabinose; X, xylose; R, rhamnose; M, mannose.

run in solvents A and B. After development with aniline hydrogen phthalate, spots corresponding in mobility and colour-reaction to pure samples of 2-*O*-methylxylose and 2-*O*-methylfucose were found. Two fast-running pink spots ($R_{\text{tetramethylglucose}}$ 0.76, 0.85; R_{rhamnose} 0.85, 1.75), possibly representing di-*O*-methylpentoses, were also evident on papers run in solvent B. The acidic carbohydrates were removed from the De-Acidite FF columns with sodium acetate buffer. Sodium ions were removed with Zeo-Karb 225 and the solution was evaporated to small bulk by rotatory evaporation. Samples of all fractions were run in solvent D. All the fractions contained galacturonic acid and several slower-running acidic oligosaccharides. It was apparent that the colour of the galacturonic acid spot from the first hydrolysate with 1.0N-sulphuric acid was scarcely more intense than that of the characteristic orange spot ($R_{\text{galacturonic acid}}$ 0.73), which corresponded with galacturonosyl-(1 $\rightarrow$ 2)-rhamnose. This latter compound was found in the apple pectin hydrolysate obtained by Barrett & Northcote (1965). In the two subsequent hydrolysates with 1.0N-sulphuric acid and 2.0N-sulphuric acid respectively, the galacturonic acid spot had a more intense colour than the aldobiouronic acid.

Table 2. *Yield and composition of the X, Y and Z fractions of sycamore pectic polysaccharides*

The yield is expressed in terms of carbohydrate as a percentage by weight of the dry, ethanol-extracted tissue. The percentage composition of each fraction is expressed in terms of glycosyl residues.

	Cambium			Suspension callus			Solid callus		
	X	Y	Z	X	Y	Z	X	Y	Z
Yield (%).....	0.5	0.8	0.2	2.4	2.2	1.1	3.7	2.8	2.8
Monomer (%)									
Galactose	3.1	8.2	24	11	16	28	4.0	25	32
Arabinose	8.2	13	24	10	12	24	7.4	6.5	16
Xylose	1.6	1.4	3.1	1.7	2.8	16	1.0	3.5	6.6
Rhamnose	2.2	3.8	4.8	2.4	5.0	3.7	1.5	2.8	2.9
Mannose	0	0	1.5	0	0	2.1	0	0	1.5
Glucose	0	0	2.9	0	0	8.5	0	0	4.5
Galacturonic acid	85	75	38	77	64	16	86	61	34

Table 3. *Characteristics of aldobiouronic acids present in a partial hydrolysate of sycamore callus pectin*

The chromatographic mobility is expressed relative to that of galacturonic acid ($R_{\text{Gal A}}$).

Aldobiouronic acid	Chromatographic mobility (solvent C, $R_{\text{Gal A}}$)	Colour reaction (aniline hydrogen phthalate)	Probable identity
1	0.23	Dark brown	Gal A-Gal
2	0.30	Brown	—
3	0.41	Pink	—
4	0.57	Grey-brown	Gal A-Xyl
5	0.73	Orange	Gal A-(1→2)-Rha
6	0.92	Pale brown	—

The three hydrolysates were pooled and subjected to ion-exchange chromatography on De-Acidite FF, as described for apple pectin (Barrett & Northcote, 1965). Sodium ions were removed from the eluates corresponding to the peak which ran before that of galacturonic acid and the solution was concentrated (1.2 ml.). Samples (25 μ l.) were chromatographed in solvent D. The three aldobiouronic acids detected were identical with those obtained from apple pectinic acid and were compared directly with them on chromatograms; they were probably galacturonosylgalactose, galacturonosylxylose and galacturonosyl-(1→2)-rhamnose. Other spots were not identified but none corresponded to glucuronosylxylose, which might have been present if the preparation had been contaminated with xylans. The properties of the aldobiouronic acids are summarized in Table 3.

De-esterification with pectinesterase. Samples of the whole pectins of suspension callus of sycamore and apple and fraction Za were used. With whole pectin an almost complete removal of material from the region B of the electrophoretogram resulted and the band of very acid material in region A was

intensified. Region D was totally unaffected, and region C showed very little change (Fig. 2a). With fraction Za there was no apparent change of the electrophoretic pattern after treatment with the enzyme.

De-esterification with alkali (Schweiger, 1965). Approx. 0.5 mg. of the fraction Za was shaken vigorously for 15 min. at 21° with excess of aq. 50% (v/v) propan-2-ol containing 0.2 g. of sodium hydroxide/ml. The solid was isolated by centrifugation, washed with propan-2-ol and resuspended in excess of propan-2-ol containing 0.375 ml. of conc. hydrochloric acid/ml. After shaking at 21° for 30 min., the solid was re-isolated, washed with propan-2-ol, dissolved in a little water and applied to a sheet of glass-fibre paper for electrophoresis. The results are shown in Fig. 2(c): the weakly acidic 'tail' of the Za fraction was lost and replaced by a strongly acidic spot. The bulk of the fraction, however, was unaffected.

Esterification of whole pectin of sycamore suspension callus. Treatment of the whole pectin with ethylene oxide or diazomethane led to the disappearance of the A and B regions and greatly

intensified the C and D regions, especially the former (Fig. 2a).

Transelimination degradation. Samples of the sycamore suspension callus whole pectin and the fraction Za were subjected to transelimination degradation after esterification with ethylene oxide. In each case if the reaction was allowed to go to completion two components only were produced. When they were run electrophoretically one of these was shown to be strongly acidic and the other was neutral (Fig. 2a,c).

DISCUSSION

Both the electrophoretic and chemical analyses showed that the pectic substances prepared from all the tissues studied were more heterogeneous and complex than those of apple fruit (Barrett & Northcote, 1965). The whole pectins of the suspension callus tissues contained the same range of neutral sugars, in similar proportions, but were different from the cambial and fruit pectins. All the pectins of the tissue cultures and cambium resembled one another in their electrophoretic patterns and all contained strongly acidic components which were absent or present only in trace amounts in apple-fruit pectin. Apple cambial pectin contained some strongly acidic material and the neutral sugar distribution was intermediate between those of callus and fruit pectins. The *Parthenocissus* crown gall callus had both an abnormally high pectin content and an unusually large proportion of strongly acidic pectin. The rather high rhamnose content, relative to the other neutral sugars, is of interest in view of the probable presence of rhamnose in polygalacturonic acid chains (Andrews, Hough & Picken, 1965; Barrett & Northcote, 1965). The strongly acidic pectinic acid component seen at zone A in the electrophoretogram was always present in the pectins prepared from the actively dividing and growing callus and cambium tissue but it was difficult to detect and present only in trace amounts in the fruit pectin. The neutral component (Zn and zone D), which was present in both forms of sycamore callus, was absent from the cambial tissue. These differences between the pectins are thus related to the state of development and growth conditions of the tissue from which they are prepared rather than to species differences.

The fractional precipitation of the sycamore pectins, as their calcium salts and with alcohol and cetylpyridinium chloride, has enabled the neutral, weakly acidic and strongly acidic components seen in the electrophoretograms to be partially purified. At least three distinct fractions are present: (a) a neutral arabinan-galactan, (b) a weakly acidic, complex pectinic acid and (c) a mixture of more

acidic pectinic acids of varying degrees of methyl esterification.

It may be that the neutral material (Zn), which is similar to those isolated from other tissues (Barrett & Northcote, 1965; Gould *et al.* 1965), is metabolically related to the neutral 'blocks' present in the weakly acidic pectinic acid.

The weakly acidic component (Za) represents the bulk of the polysaccharide of the total pectin which moves in the electrophoretic region C. Such a low mobility could result from a high degree of methylation, a high neutral sugar content or a combination of these factors. Enzymic and alkaline de-esterification had no effect upon the low mobility of this fraction, so that a high degree of esterification cannot be the explanation for the weakly acidic nature of the material. The need to esterify this fraction before bringing about the transelimination reaction would further suggest that the carboxyl groups are initially unesterified.

Transelimination degradation of the hydroxyethylated pectinic acid component Za yielded two components, one neutral and one strongly acidic. These were of sufficiently high molecular weight not to pass through dialysis tubing. The original pectin contained relatively large amounts of arabinose and galactose and the detection of galacturonosylgalactose shows that these neutral sugar blocks (probably of arabinogalactan) are present attached to, or interspersed in, the polyuronide chains. This is similar to the structure suggested for apple-fruit pectinic acid by Barrett & Northcote (1965).

The components of the whole pectin preparations from sycamore and apple callus tissue which move in the A and B zones of the electrophoretograms have similar compositions and resemble one another in that they are strongly acidic. In sycamore the galactose and arabinose content of these components and of fraction X is far lower than in fraction Za, and there is no evidence for the presence of any large neutral sugar 'blocks' in these acidic fractions. After de-esterification of the whole pectins, zone B is no longer found and the zone at region A is intensified so that one of the differences between the acidic zones at A and B is that the material at B is partially esterified whereas that at A is almost completely unesterified polygalacturonic acid material. Xylose and rhamnose are always associated with the materials at zones A and B and the detection of galacturonosylxylose and galacturonosyl-(1→2)-rhamnose in the hydrolysates of whole pectin of sycamore suggests that these neutral sugars are linked to the polyuronide chains of these molecules. Our results show that the pectic substances form a complex of several components. Becker, Hui & Albersheim (1964) reported that all the polygalacturonic acid material

present in sycamore callus tissue was extensively methylated; we do not find this.

The pectin preparations from callus and cambial tissue contained two different types of acidic components. One of these, the weakly acidic pectinic acid, carried blocks of neutral sugars. The other, the more strongly acidic pectinic acid, was relatively free of neutral sugars and was composed mainly of galacturonosyl radicals which were partially esterified. Gould *et al.* (1965) have reported the presence of similar components in a pectin preparation from white mustard and they pointed out that each type of polymer would have different physicochemical properties. It is possible therefore that the components of the pectin have distinct functional roles *in situ* in the growing cell wall and they may be laid down at different stages of the growth cycle of the cell.

Albersheim, Mühlethaler & Frey-Wyssling (1960) and Albersheim & Killias (1963) have shown by cytochemical methods that the pectin of the cell plate and the middle lamella in the cells of onion root were in all probability composed partly of a highly esterified polygalacturonic acid. There is also evidence from the work of Albersheim & Bonner (1959) that incorporation of [$U-^{14}C$]glucose and [$Me-^{14}C$]methionine into water-soluble pectin (more esterified pectic substances) of oat coleoptile is influenced by indolylacetic acid, whereas that into insoluble pectin (less esterified pectic substances) is not. Over a period of 15 hr. no inter-conversion of the fractions was noted. During active elongation of the cells of coleoptiles, that is during primary growth of the wall which is stimulated by indolylacetic acid, Baker & Ray (1965a,b) and Ray & Baker (1965) have shown that there is an increase in the amount of the matrix polysaccharides of the cell wall. These polysaccharides contain neutral sugars and galacturonic acid. The increase in amount of the matrix polysaccharides during wall growth is attributed to an action of indolylacetic acid on the utilization of the precursors of these polysaccharides. It is therefore possible that the two acidic fractions which we have found to be present in the sycamore pectin are functionally distinct and that the strongly acidic fraction is more characteristic of division and cell-plate formation, rather than that of extension, whereas the more complex weakly acidic pectinic acid which carries neutral blocks, containing arabinose and galactose, could be involved in the latter process. A common polysaccharide precursor of both strongly acidic and weakly acidic pectinic acid is possible with the arabinan-galactan serving as the neutral block donor. The changes in primary wall development, attendant upon cell differentiation, may be largely regulated through such a metabolic branching of an acidic poly-

galacturonic chain, which could be controlled by the direct action of growth factors on the enzymes involved or by an action on the mechanism of the synthesis of the enzymes themselves.

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REFERENCES

- Albersheim, P. & Bonner, J. (1959). *J. biol. Chem.* **234**, 3105.
 Albersheim, P. & Killias, U. (1963). *Amer. J. Bot.* **50**, 732.
 Albersheim, P., Mühlethaler, K. & Frey-Wyssling, A. (1960). *J. biophys. biochem. Cytol.* **8**, 501.
 Andrews, P., Hough, L. & Picken, J. M. (1965). *Biochem. J.* **97**, 27.
 Aspinall, G. O. & Fanshawe, R. S. (1961). *J. chem. Soc.* p. 4215.
 Baker, D. B. & Ray, P. M. (1965a). *Plant Physiol.* **40**, 345.
 Baker, D. B. & Ray, P. M. (1965b). *Plant Physiol.* **40**, 360.
 Barrett, A. J. & Northcote, D. H. (1965). *Biochem. J.* **94**, 617.
 Becker, G. E., Hui, P. A. & Albersheim, P. (1964). *Plant Physiol.* **39**, 913.
 Bhattacharjee, S. S. & Timell, T. E. (1965). *Canad. J. Chem.* **43**, 758.
 Bonner, J. (1961). *Proc. 4th int. Conf. Plant Growth Regulation*, p. 307. Ames, Iowa: Iowa University Press.
 Cleland, R. (1963a). *Plant Physiol.* **38**, 12.
 Cleland, R. (1963b). *Nature, Lond.*, **200**, 908.
 Cleland, R. (1964). *Plant Physiol.* **39**, iv.
 Cleland, R. (1965a). *Plant Physiol.* **40**, 595.
 Cleland, R. (1965b). *Plant Physiol.* **40**, lxi.
 Devor, A. W. (1950). *J. Amer. chem. Soc.* **72**, 2008.
 Dische, Z. (1962). In *Methods in Carbohydrate Chemistry*, vol. 1, p. 478. Ed. by Whistler, R. L. & Wolfrom, M. L. New York: Academic Press Inc.
 Eistert, B. (1948). *Newer Methods of Preparative Organic Chemistry*, pp. 561 and 564. New York: Interscience Publishers Inc.
 Fischer, F. G. & Dörfel, H. (1955). *Hoppe-Seyl. Z.* **301**, 224.
 Gould, S. E. B., Rees, D. A., Richardson, N. G. & Steele, I. N. (1965). *Nature, Lond.*, **208**, 876.
 Jeffs, R. A. & Northcote, D. H. (1966). *Biochem. J.* **101**, 146.
 Jones, J. K. N. & Wise, L. E. (1952). *J. chem. Soc.* p. 3389.
 Kertesz, Z. I., Baker, G. L., Joseph, G. H., Mottern, H. H. & Olsen, A. G. (1944). *Industr. Engng Chem. (News)*, **22**, 105.
 Lamport, D. T. A. (1964). *Exp. Cell Res.* **33**, 195.
 Lamport, D. T. A. & Northcote, D. H. (1960). *Nature, Lond.*, **188**, 665.
 McCready, R. M. & McComb, E. A. (1952). *Analyt. Chem.* **24**, 1986.
 Noodén, L. D. & Thimann, K. V. (1966). *Plant Physiol.* **41**, 157.
 Olaitan, S. A. & Northcote, D. H. (1962). *Biochem. J.* **82**, 509.

- Olson, A. C. & Cleland, R. (1964). *Plant Physiol.* **39**, v.
- Ray, P. M. & Baker, D. B. (1965). *Plant Physiol.* **40**, 353.
- Saeman, J. F., Moore, W. E., Mitchell, R. L. & Millett, A. M. (1954). *T.A.P.P.I.* **37**, 336.
- Schweiger, R. G. (1965). In *Methods in Carbohydrate Chemistry*, vol. 5, p. 60. Ed. by Whistler, R. L. & Wolfrom, M. L. New York: Academic Press Inc.
- Smith, E. L. & Page, J. E. (1948). *J. Soc. chem. Ind., Lond.*, **67**, 48T.
- Thornber, J. P. & Northcote, D. H. (1961a). *Biochem. J.* **81**, 449.
- Thornber, J. P. & Northcote, D. H. (1961b). *Biochem. J.* **81**, 455.
- Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.