Apple Fruit Pectic Substances

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1. The pectic substances of apple have been extracted and separated into a pure pectinic acid and a neutral arabinan–galactan complex by precipitation of the acidic component with ethanol and with cetylpyridinium chloride. 2. The composition of the fractions has been determined. The pectinic acid contained galacturonic acid, arabinose, galactose, rhamnose, xylose and several trace sugars. 3. Transelimination degradation of the pectinic acid gave rise to two components completely separable by zone electrophoresis and by Sephadex gel filtration. Analysis of these components confirmed that the pectinic acid molecules contained long chains of esterified galacturonosyl residues, but showed in addition that more neutral portions containing a high proportion of arabinofuranose residues were attached to them. 4. The identification of rhamnose, galactose and xylose in aldobiouronic acids obtained from a partial hydrolysate of pectinic acid has shown that these sugars are covalently linked in the molecule, and it is suggested that the galacturonosyl-(1→2)-rhamnose link is a general feature of pectinic acid structure. 5. The possible biological significance of pectinic acid structure has been discussed. 6. The arabinan–galactan complex contained nearly equal quantities of arabinose and galactose residues and some of its physical properties have been investigated.

The pectic substances of plant tissues comprise an empirically defined group of polysaccharides containing galactose, arabinose and galacturonic acid as the main sugar residues. They are located in the cell wall and are laid down during the early stages of growth when the area of the wall is increasing (Thornber & Northcote, 1961*a,b*; Northcote, 1963). Several theories about the mechanism and control of growth have stressed the significance of the pectic substances and more especially the pectinic acid component (Bonner, 1961). The theories make use of the special chemical and physical properties of the acidic polysaccharide which can, to some extent, be controlled by the degree of the methyl esterification of the carboxyl groups.

The older work on the chemical nature of the pectic substances suggested that three distinct homopolysaccharides occurred, a galactan, an arabinan and a galacturonan (Hirst & Jones, 1939). However, many more recent attempts to purify these separate polysaccharides have failed to yield homogeneous material (McCready & Gee, 1960) and studies of partial acid hydrolysates of the preparations have shown the presence of oligosaccharides which contain more than one sugar (Andrews, Hough, Powell & Woods, 1959; Aspinall & Fanshawe, 1961); these investigations indicate the existence of heteropolymers. It has also been established that the glycosidic links of esterified polygalacturonosyl chains are very labile in alkaline solution and thus it is possible that alkaline extraction and fractionation procedures could bring about cleavage of covalent bonds whereby from a large heteropolysaccharide smaller polymers of a more uniform composition could be obtained.

The present work has attempted to reassess the composition and nature of the components of apple pectic substances by the application of physical and chemical analytical and fractionation techniques.

METHODS

Zone electrophoresis of polysaccharides. Polysaccharides were subjected to high-voltage paper electrophoresis on glass-fibre paper (Whatman GF/A and GF/B) (Fuller & Northcote, 1956) strips of 45 cm. effective length and up to 23 cm. in width in an apparatus similar to that described by Michl (1959). The tank was filled with an organic fluid which was cooled by water in a glass coil. The paper, 57 cm. in length, was hung from a trough containing the cathode buffer so as to dip into the anode buffer in the bottom of the tank. Electrodes were of platinum foil. The buffers used were: (*a*) pyridine–acetate (pH 6·5, containing 10%, v/v, of pyridine and 0·3%, v/v, of acetic acid) with toluene for cooling fluid, or (*b*) for neutral polysaccharides, 0·05M-sodium tetraborate, pH 9·2, with 'white spirit' for cooling

fluid. Polysaccharides [50–100 μg./cm. (GF/A) or 500 μg./cm. (GF/B)] were applied to the dry paper in 2·5 cm. streaks. The paper was wetted with buffer from a large pipette so that straight fronts met at the origin; it was not blotted. With GF/A paper a potential of 44 v/cm. (2 kv) was used in the pyridine buffer and 22 v/cm. in the tetraborate buffer, which had a higher conductivity. Lower voltages or narrower strips were used with the thick GF/B paper. The duration of electrophoresis runs varied between 15 and 60 min.

After electrophoresis the papers were dried at 60° before being dipped in a sulphonated α-naphthol reagent prepared by dissolving in 96% ethanol (500 ml.) a solution of 5 g. of α-naphthol in 25 ml. of conc. sulphuric acid which had stood overnight. The α-naphthol reagent could be stored indefinitely.

The effect of endosmosis was minimized by arranging the apparatus so that the flow was vertical. There was little buffer movement at pH 6·5, but in the tetraborate, at pH 9·2, the movement was still so rapid that all polysaccharide zones were swept back towards the cathode. Dextran was used as a stationary marker in the pyridine–acetate system and tetramethylglucose was used in the tetraborate system. In the neutral buffer samples were applied 15 cm. from the cathode end of the strip, but in tetraborate they were applied 10 cm. from the anode end. Pectic acid tended to stick firmly at the origin on glass-fibre paper unless 10 mM-EDTA was added to the buffer with which the paper was wetted. Glass-fibre paper contained organic matter (probably cellulose), which caused a speckled background when polysaccharide zones were detected by the sulphonated α-naphthol method. Heating the glass-fibre paper for 1 hr. at 500° before use resulted in a very clean final appearance.

Zone electrophoresis of reducing saccharides. Acidic reducing sugars were run on Whatman no. 3 paper, in the pH 6·5 system, for about 90 min. at 55 v/cm. (2·5 kv). The reducing sugars on the dried papers were detected by the aniline hydrogen phthalate method of Wilson (1959).

Boundary electrophoresis. Free-boundary electrophoresis of polysaccharides was carried out in a Tiselius apparatus (Perkin–Elmer and Co., U.S.A.; model 38).

Analytical ultracentrifugation. Polysaccharide solutions (0·5–2·0%) in 0·1 M-KH$_2$PO$_4$ were used in a Spinco analytical (model E) ultracentrifuge.

Moisture and ash determination. Moisture and ash were determined by heating the material to constant weight at 105° and at 600° respectively.

Polysaccharide hydrolysis. Polysaccharides were hydrolysed in 3% (v/v) nitric acid containing 0·05% (w/v) urea at 100° for 3 hr. (Bath, 1960). Hydrolysates were neutralized with Amberlite IRA-400 resin (Rohm and Haas Co.) in the CO$_3^{2-}$ form, evaporated to dryness below 60° and redissolved in saturated benzoic acid solution for storage at 4°. Particularly resistant substances, e.g. aldobiouronic acids, were hydrolysed under the same conditions for 15 hr. in sealed tubes.

The yield of neutral sugars from pectin was not increased by hydrolysis periods in excess of 3 hr. except for rhamnose (Wilson, 1961). The yield of rhamnose after a 3 hr. hydrolysis period was only 53% of that after a 15 hr. period (after correction for losses) and all estimates of this sugar in 3 hr. hydrolysates have been corrected accordingly.

Percentage recoveries of standard sugars after the 3 hr. hydrolysis procedure, chromatography and estimation by

Table 1. *Percentage recovery of sugars after the hydrolysis procedure*

Percentages are expressed ± S.D. of individual estimates and the numbers of determinations are given in parentheses.

Sugar	Percentage recovery
Galactose	96 ± 3 (12)
Glucose	92 ± 3 (11)
Mannose	96 ± 4 (12)
Arabinose	92 ± 2 (11)
Xylose	91 ± 5 (12)
Rhamnose	92 ± 7 (11)

the aniline hydrogen phthalate method (Wilson, 1959) are shown in Table 1. After the 15 hr. period recoveries of about 80% of each sugar were achieved (e.g. 78% of rhamnose). No epimerization of standard sugars was detected.

Qualitative analysis of minute amounts of material was facilitated by subjecting their hydrolysates to chromatography in solvent (A) without neutralization.

Paper-chromatographic analysis of hydrolysates. Neutral monosaccharides present in polysaccharide hydrolysates were separated by descending paper 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 hydrolysates were chromatographed in solvent B (butan-1-ol–ethanol–water, 5:1:4, by vol.), and uronic acids were separated by chromatography with 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) (solvent C). Uronic acids and acidic oligosaccharides were also run with solvent D, ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.) (Jones & Wise, 1952) or solvent E, the organic phase from a mixture of 100 g. of phenol, 100 ml. of water and 1 ml. of 88% formic acid (Ray & Rottenberg, 1964).

The sugars were detected and estimated by the aniline hydrogen phthalate method of Wilson (1959) and results were calculated as percentages of each glycosyl radical present. Standard solutions of the monosaccharides were made up in saturated benzoic acid solution and stored at 4°. Definite volumes of standard and unknown solutions were applied to chromatograms by means of E-mil pipettes (H. J. Elliott Ltd., Treforest, Glamorgan, Wales). Aldobiouronic acids on chromatograms were investigated by the periodate–Schiff reaction (Hardy & Buchanan, 1963).

Galacturonic acid estimation. After saponification (McComb & McCready, 1952) the galacturonic acid in pectic polysaccharides was estimated by the modified carbazole reaction of Bitter & Muir (1962).

Galacturonic acid for use in standard solutions was itself standardized by titration (McComb & McCready, 1952). The standard solutions in saturated benzoic acid were stored at 4°.

Galacturonic acid determinations were corrected for interference by other sugars when appropriate, e.g. galactose was found to give 10% of the colour given by galacturonic acid, at the same molar concentration, and arabinose gave 3%.

Pectin ester determination. This determination was carried out by the saponification and back-titration method of Gee, Reeve & McCready (1959).

Protein estimation. Protein was estimated in polysaccharide preparations by the Folin–Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as a standard.

Carbohydrate estimation by the sulphonated α-naphthol method (Devor, 1950; Dische, 1962). The reagent was prepared by dissolving 2 g. of α-naphthol in 500 ml. of conc. sulphuric acid and allowing the solution to stand overnight.

Portions (5 ml.) of reagent were run into tubes (19 mm. × 150 mm.) and samples of carbohydrate solution containing 10–100 µg. in 2 ml. of water were layered on top of the reagent. The tubes were covered by inverted specimen tubes and cooled in ice–water before being mixed vigorously with a Vortex mixer (Scientific Industries Inc., Springfield, Mass., U.S.A.) and returned to the ice. When all the tubes had been mixed they were heated for 10 min. in a boiling-water bath.

Free or combined pentoses and hexoses gave a purple colour with extinction maxima at 550 mµ and 570 mµ respectively, and uronic acids gave a weaker brown colour, also with a maximum at 550 mµ. Colours were stable overnight when uptake of atmospheric moisture was prevented.

Partial acid hydrolysis of pectinic acid. Pectinic acid (2 g.) was hydrolysed with 1·0 N-sulphuric acid (200 ml.) on a boiling-water bath for 4 hr. The solution was cooled, neutralized with barium carbonate and filtered. A portion of the filtrate was made barium-free by passage through a short column of Permutit Zeo-Karb 225 (SRC 13, H+ form) and mixed with an equal volume of acetone. No degraded polysaccharide was precipitated. The remainder of the hydrolysate was reduced to a syrup by evaporation *in vacuo* at 60°. The neutral hydrolysate was then adsorbed on a short column of De-Acidite FF (acetate form) (The Permutit Co. Ltd.), which was well washed with water to remove neutral sugars, and the acidic substances were eluted with acetate buffer. The solution was passed through Zeo-Karb 225 to remove Na+ ions, evaporated to a syrup below 40° and the residue dissolved in sodium acetate buffer (0·2 M-sodium acetate–0·2 M-acetic acid containing 5 drops of toluene/l.). The solution (1 ml., corresponding to 250 mg. of the original pectin), together with arabinose (1 mg.) as a neutral marker, was subjected to chromatography on a column of De-Acidite FF (SRA 71) and eluted with the buffer. The effluent from the column was collected in 2·2 ml. portions. Carbohydrate in the samples (0·5 ml.) of the effluent was estimated by the sulphonated α-naphthol method and extinction at 570 mµ was plotted against effluent volume.

The ion-exchange resin bed (1·2 cm.² × 50 cm.) was equilibrated before use with the acetate buffer. The column required no regeneration between runs.

Pectinesterase. Pectinesterase (pectin pectyl-hydrolase, EC 3.1.1.11) was prepared by a modification of methods described by Lineweaver & Jansen (1951).

Fresh orange peel was homogenized in a Waring Blendor in 0·2 M-sodium acetate maintained at pH 7–8 and allowed to stand overnight at 4°. The slurry was filtered and adjusted to 40% saturation with ammonium sulphate. The first precipitate was discarded and the filtrate was made up to 70% saturation with ammonium sulphate. The precipitate

obtained at this stage was dissolved in 0·1 M-sodium chloride containing 0·01 M-phosphate buffer, pH 7. The solution was then dialysed against a large volume of distilled water containing a trace of mercaptoethanol. A column of DEAE-cellulose was equilibrated with 0·01 M-tris—HCl buffer, pH 8·9, and the dialysed enzyme solution was passed through the column. The pectinesterase passed through unretarded while almost all of the other protein and the orange pigment were adsorbed. The enzyme solution was freeze-dried without loss of activity.

Orange peel has no polygalacturonase activity (MacDonnell, Jansen & Lineweaver, 1945).

Enzymic de-esterification of pectin. The pectin solution (0·5%) was incubated with the enzyme preparation at 25° in the presence of 0·15 M-sodium chloride, a sodium phosphate or tris–HCl buffer at pH 7–8 and a drop of toluene for 18 hr.

Esterification of polygalacturonic acid. Ethylene oxide to a final concentration of 12% (v/v) was added to a solution of the polysaccharide which had been dialysed and passed through Zeo-Karb 225 (SRC 13, H+ form). The mixture was incubated for 1 week at room temperature and the polysaccharide was recovered by precipitation with ethanol (Deuel, 1947).

Gel filtration. Sephadex (Pharmacia, Uppsala, Sweden) gel beds were prepared as recommended by the manufacturers and the eluent used was 0·1 M-sodium chloride containing a trace of toluene and, in some experiments, 1% (v/v) of dimethylformamide.

Bed dimensions for each type of Sephadex were: G-50, 3·0 cm.² × 55 cm. (165 cm.³); G-75, 3·6 cm.² × 89 cm. (320 cm.³); G-200, 8·6 cm.² × 33 cm. (285 cm.³).

Pectinic acid degradation (transelimination). Hot pectinic acid solution (0·5%) was mixed with an equal volume of hot sodium phosphate buffer (pH 6·8, 0·2 M) (Albersheim, 1959; Albersheim, Neukom & Deuel, 1960). The mixture was heated for 4 hr. in a boiling-water bath and dialysed against distilled water. The solution of non-diffusible degraded pectate was concentrated and freeze-dried.

Pectinic acid degradation by acid. Pectinic acid solution (0·5%) in 0·02 N-sulphuric acid (pH 2·0) was heated in a boiling-water bath for periods up to 6 hr.

Methanolysis of pectinic acid (Jansen, MacDonnell & Ward, 1949). Pectinic acid (2·8 g.), dried *in vacuo* at 60°, was mixed with 81 g. of methanolic 3·4% (w/w) hydrochloric acid and refluxed with stirring at 75° for 24 hr., when a further 18 g. of methanolic 12·8% (w/w) hydrochloric acid was added and the heating continued for a total of 80 hr.

The insoluble residue after methanolysis was washed with methanol and dried (yield 1·3 g.). It was then dissolved in water, filtered and dialysed for 3 days against several changes of water. The non-dialysed material was precipitated from 80% ethanol containing a little sodium acetate, redissolved in a little water and freeze-dried, giving a white solid (yield 0·8 g.).

RESULTS

Extraction and ethanol fractionation of the pectin. Ripe sound Bramley Seedling apples (2·7 kg.) were cut up, dropped into boiling water and simmered for 30–40 min. The pH of the slurry became 2·8–3·0. The liquid was then strained off through a
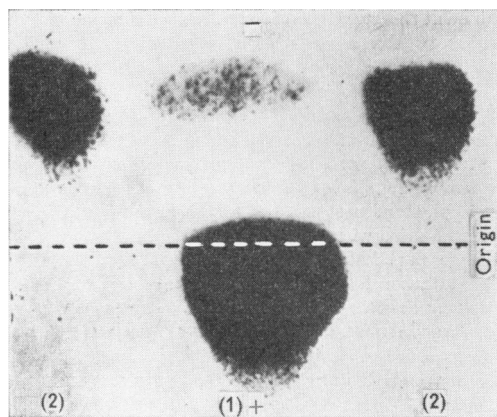
Fig. 1. Electrophoretogram of whole apple pectin (1) with dextran markers (2). Pectinic acid, the major component, separated from the neutral arabinan–galactan complex. Paper, GF/B; buffer, pyridine–acetate, pH 6·5; 22 v/cm.; 60 min.
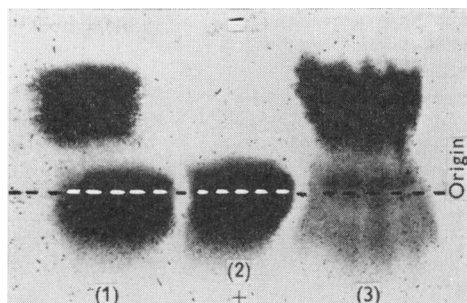

Fig. 2. Electrophoretogram demonstrating the purification of apple pectinic acid. Pure pectinic acid (2) was precipitated from a test mixture of apple pectin and yeast glycogen (1) with cetylpyridinium chloride. The unprecipitated material (3) contained neutral and some acidic polysaccharide. Paper GF/A; buffer, pyridine–acetate, pH 6·5; 44 v/cm.; 26 min.

double thickness of fine Terylene net, and filtered with the aid of kieselguhr and Filter Cel (Johns–Manville, N.Y., U.S.A.) until clear.

A whole pectin fraction was prepared by adjusting part of the extract to 80% (v/v) of ethanol. The gelatinous precipitate was strained off on Terylene, squeezed out, and washed with 96% ethanol until the washings no longer contained sugars detectable by the sulphonated α-naphthol reaction. Electrophoresis of a solution of the whole pectin material on glass-fibre paper at pH 6·5 showed that it contained both a minor neutral and a major acidic component (Fig. 1).

Ethanol fractionation of pectin. The filtered whole pectin extract was adjusted to 48% (v/v) of ethanol and the gelatinous precipitate was strained off, washed with ethanol and dried (yield, 9 g.; $[\alpha]_D^{20} + 190°$; c 0·5 in water). Electrophoresis of this material showed it to be free from all but a trace of the neutral fraction. It was the crude pectinic acid.

After removal of the acidic polysaccharide the clear solution was made to 80% of ethanol. A little sodium acetate was added to facilitate flocculation of the fine powdery white precipitate, which was collected by centrifugation and dried (yield 370 mg.). Electrophoresis of an aqueous solution showed that this fraction contained neutral but not acidic polysaccharide. It was the crude arabinan–galactan complex.

Purification of pectinic acid. A solution (1%) of the impure acidic polysaccharide was mixed with an equal volume of 2% cetylpyridinium chloride solution (Scott, 1960). The precipitate was removed by centrifugation and washed by suspension in

water and recentrifugation, and redissolved in a 1 M-ammonium formate solution adjusted to pH 4–5 with formic acid. The viscous solution containing 1% of polysaccharide was then adjusted to 64% of ethanol and the precipitate washed with ethanol before being dried *in vacuo* at 60°. Electrophoresis (Fig. 2) and chemical analysis of a test mixture of apple pectin and yeast glycogen showed that this method freed apple pectinic acid from neutral polysaccharide although the acidic polymer was not precipitated quantitatively.

A solution of purified pectinic acid was treated with pectinesterase. The de-esterified material was precipitated with ethanol (80%, v/v) and redissolved to give a 1% (w/v) solution in saturated benzoic acid. Zone electrophoresis (pH 6·5; 10 mM-EDTA) of this material gave a single fast-moving zone with some tailing (Fig. 10) and no material near the origin (neutral or near neutral) was detected.

Purification of arabinan–galactan complex. The crude complex (370 mg.) was dissolved in water and passed through a short column of the Zeo-Karb 225 (SRC 13, H⁺ form). The effluent from the column was made to 82% of ethanol and a white powdery precipitate of purified arabinan–galactan was collected and dried (yield 160 mg.).

This material ran in zone electrophoresis as a neutral polysaccharide and in tetraborate buffer gave a single zone with a mobility close to that of yeast glycogen. It contained arabinose (41%) and galactose (46%).

Ultracentrifugation of fractions. Solutions of the pectinic acid (0·5%) and the arabinan–galactan (0·9–1·5%) were run in the ultracentrifuge. The results are shown in Figs. 3 and 4. Generally the pectinic acid ran as a single sharp boundary (Fig. 3)
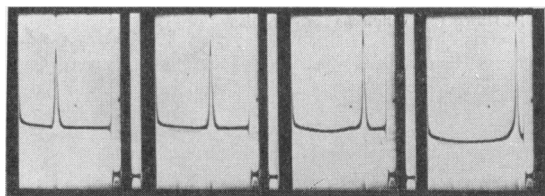
Fig. 3. Sedimentation pattern of purified apple pectinic acid dissolved in 0·1 M-KH₂PO₄ (5 mg./ml.). Centrifugal force was $2·60 \times 10^5 \, g$ and the temperature 20°. Sedimentation is from right to left and photographs were taken after 64, 192, 320 and 448 min. The bar angle was 55° for the first photograph and 50° thereafter.
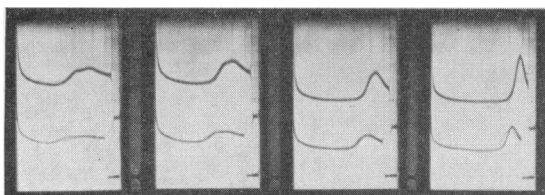

Fig. 4. Sedimentation pattern of the apple arabinan-galactan complex dissolved in 0·1 M-KH₂PO₄ (top, 15 mg./ml.; bottom, 9 mg./ml.). Centrifugal force was $2·60 \times 10^5 \, g$ and the temperature 20°. Sedimentation is from right to left and photographs were taken after 16, 32, 48 and 64 min. The bar angle was 60° for the first two photographs and 55° thereafter.

Fig. 5. Boundary electrophoresis of pectinic acid (5 mg./ml.) in pyridine–acetate buffer, pH 5·5. Ascending limb after (a) 52 min. and (b) 165 min. (155 v; 9·5 mA).

Fig. 6. Boundary electrophoresis of arabinan–galactan complex (20 mg./ml.) in 0·05 M-sodium tetraborate, pH 9·2: (a) ascending and (b) descending limb after 80 min. (155 v; 8·5 mA).

although some preparations showed the presence of a very minor faster-sedimenting component. The arabinan–galactan complex showed some separation into two boundaries towards the end of the run after 64 min. (Fig. 4).

Free-boundary electrophoresis of fractions. A solution of the pectinic acid (0·5%) in a buffer (pH 5·5) containing pyridine (1%, v/v) and acetic acid (0·4%, v/v) was dialysed overnight against a large volume of this buffer. During electrophoresis a single sharp boundary was observed (mobility, $2·5 \times 10^{-5} \, \mathrm{cm.^2 \, v^{-1} \, sec.^{-1}}$). The boundary became slightly asymmetrical towards the end of the run (Fig. 5a,b).

The arabinan–galactan (2% in 0·05 M-sodium tetraborate gave a single asymmetrical peak (Fig. 6) (mobility, $4·8 \times 10^{-5} \, \mathrm{cm.^2 \, v^{-1} \, sec.^{-1}}$).

Composition of the fractions. This is shown in Tables 2 and 3. The percentage esterification of carboxyl groups was 69% for the crude material.

Besides the arabinose, galactose, xylose and rhamnose estimated in the pectinic acid hydrolysate three other faint spots were detectable when a chromatogram was heavily loaded. These were identified as fucose, 2-O-methylxylose and 2-O-
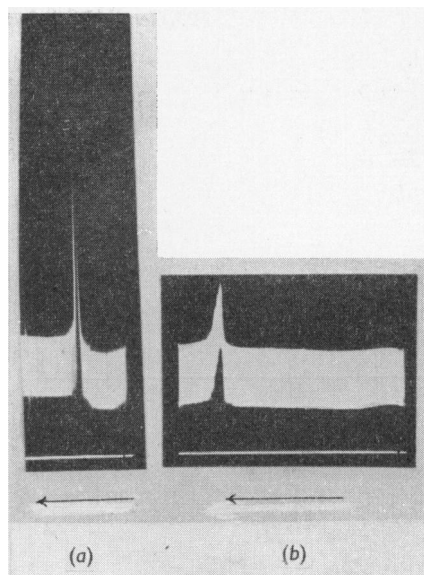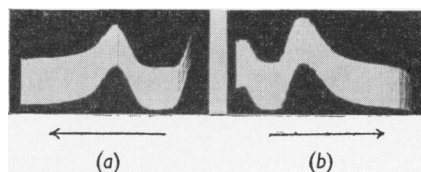
methylfucose by their mobilities in solvents A and B in comparison with authentic markers. The intensity of each spot corresponded to the presence of 0·1–0·2% of each sugar in the pectinic acid.

Attempted fractionation of pectinic acid by calcium salt precipitation. Purified pectinic acid (100 mg.) was de-esterified with pectinesterase, and precipitated with 0·1 M-calcium chloride solution (5 ml.). The gelatinous precipitate was washed thoroughly with water before being strained off and dissolved in 0·3% (w/v) ammonium oxalate solution (100 ml.) during 30 min. at 90°. Calcium oxalate was removed from the resulting ammonium pectate solution by centrifugation and the pectate was then precipitated with ethanol and dried *in vacuo*.

Hydrolysis of the ammonium pectate showed that its content of arabinose, galactose and xylose

Table 2. *General composition of fractions of apple pectic substances*

The percentage of polysaccharide was calculated as the sum of the amounts of the individual neutral glycosyl and uronosyl radicals present in each substance. All values are expressed in terms of moisture-free solid.

Component	Crude pectinic acid (%)	Purified pectinic acid (%)	Components of the pectinic acid isolated by gel filtration after trans-elimination degradation	
			A (%)	B (%)
Polysaccharide	86	81	56	81
Protein	2·4	2·0	—	—
Ash	4·6	1·1	6·6	21·7

Table 3. *Composition of polysaccharide components of fractions of apple pectic substances*

Values for the monosaccharides were calculated as percentage of each glycosyl radical present.

Component	Crude pectinic acid (%)	Purified pectinic acid (%)	Components of the pectinic acid isolated by gel filtration after transelimination degradation		Percentage in A / Percentage in B
			A (%)	B (%)	
Arabinose	9·9	9·3	51	0·42	122
Galactose	1·3	1·4	8·0	0·26	31
Rhamnose	1·1	1·2	4·0	0·48	8·4
Xylose	0·83	0·88	5·5	0·18	31
Trace sugars	0·5	0·5	—	0·2	—
Galacturonic acid	87	87	31	98	0·32

residues had not been altered by the calcium salt-precipitation procedure.

Acidic oligosaccharides. The result of ion-exchange chromatography of a partial acid hydrolysate of pectinic acid is shown in Fig. 7.

Samples from tubes representing the first peak gave a magenta colour reaction with the sulphonated α-naphthol reagent, tubes representing the second peak a pink colour, tubes representing the third peak a red–brown colour and tubes representing the fourth peak a brown colour. These colours together with the known mobilities of arabinose and galacturonic acid on the column established that the first peak was due to the arabinose marker, the second probably to aldotriouronic acids, the third to aldobiouronic acids and the fourth to galacturonic acid.

The aldobiouronic acid solutions from eight chromatograms were pooled, passed through a short column of Zeo-Karb 225 (SRC 13, H⁺ form) to remove Na^+ ions, dried to a syrup *in vacuo* below 60° and the residue was dissolved in benzoic acid solution (0·5 ml.) and stored at 4°.

Paper-chromatographic and electrophoretic investigation of the aldobiouronic acids. Samples (25 μl.) of the stock aldobiouronic acid solution were
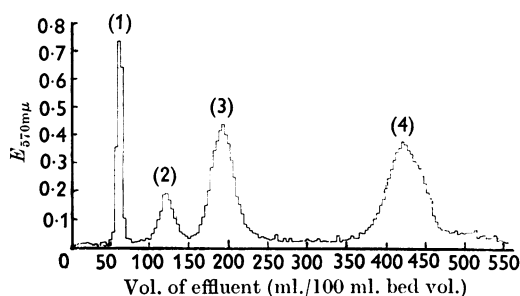

Fig. 7. Column chromatogram of partial acid hydrolysate of pectinic acid showing separation of (1) arabinose marker, (2) aldotriouronic acids, (3) aldobiouronic acids and (4) galacturonic acid. The column (1·2 cm.² × 50 cm.) was of De-Acidite FF (acetate form) and the eluent was sodium acetate–acetic acid (each 0·2 M).

subjected to chromatography (solvents C, D and E) and electrophoresis at pH 6·5. Three components were detected (Table 4).

A hydrolysate of the aldobiouronic acid solution contained a large amount of rhamnose with some galactose and a little xylose.

Table 4. *Characteristics of aldobiouronic acids present in a partial hydrolysate of apple pectinic acid*

Chromatographic and electrophoretic mobilities (R_{GalA} and M_{GalA} respectively) are expressed relative to those of galacturonic acid.

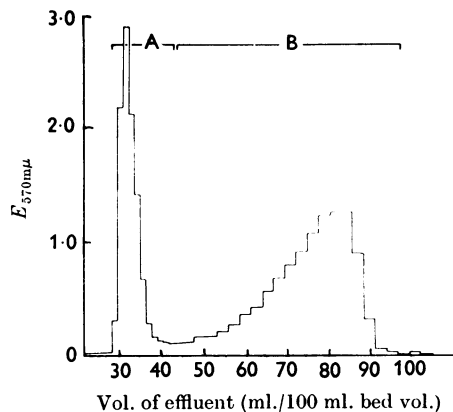| | Colour reaction | | Chromatography R_{GalA} | | | | |
| | Aniline | | | | | | |
Substance	hydrogen phthalate	Periodate–Schiff	Solvent C	Solvent D	Solvent E	Electrophoresis M_{GalA}	Probable identity
1	Grey–brown	Blue	0·64	0·17	—	0·57	GalA→Gal
2	Grey–brown	Blue	—	0·52	—	—	GalA→Xyl
3	Orange	Yellow–black	1·10	0·75	1·18	0·73	GalA-(1→2)-Rha

Fig. 8. Gel filtration of transelimination-degraded pectinic acid on a Sephadex G-75 column (3·6 cm.² × 89 cm.) eluted with 0·1 M-NaCl. The two peaks represent the A and B components of the degradation product. (See Fig. 9.)
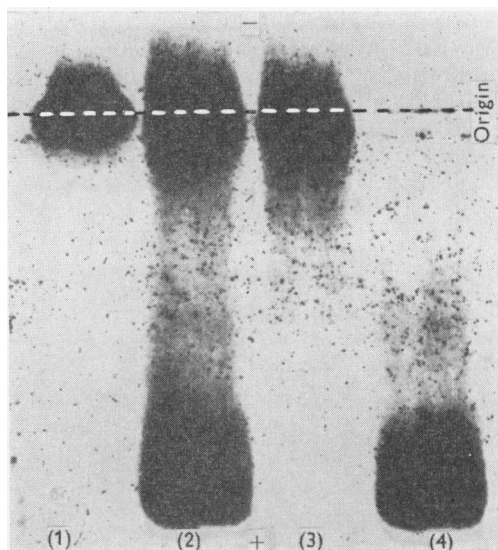

Fig. 9. Electrophoretogram of (1) apple pectinic acid, (2) transelimination-degraded pectinic acid, (3) purified component A and (4) component B of degraded pectinic acid. Paper, GF/A; buffer, pyridine–acetate, pH 6·5; 44 v/cm.; 25 min. (See Fig. 8.)

Progress of the degradation of pectinic acid at pH 2. Samples taken at 30 min. intervals during the degradation were subjected to zone electrophoresis at pH 6·5. This showed the presence of an acidic component which gradually increased in mobility and the gradual appearance of free neutral materials.

Progress of the degradation of pectinic acid at pH 6·8 (*transelimination reaction*). The viscosity of a pectinic acid solution heated at pH 6·8 fell rapidly and the solution became brown. Samples removed during the degradation showed the formation of two polysaccharide fractions separable by zone electrophoresis (Fig. 9). One of these, component A, had a mobility close to that of the undegraded pectinic acid, and the other, component B, increased in mobility until, after 4 hr., it had reached a limiting value.

Esterification of the free carboxyl groups of degraded pectate with ethylene oxide caused both components to run as a single zone once more with low mobility. On the other hand, treatment of partially degraded pectate with pectinesterase

caused the mobility of component B to increase nearly to the limiting value.

Gel filtration of pectinic acid degraded by the transelimination reaction. A solution (14 ml., equivalent to 480 mg. of pectinic acid) was subjected to gel filtration on Sephadex G-75. A plot of carbohydrate concentration in the effluent (Fig. 8) showed two peaks, the first representing material excluded from the gel and the second material which was considerably retarded. The two fractions were precipitated with ethanol, dissolved in a little water and freeze-dried (yields 62 mg. and 208 mg. respectively).

Zone electrophoresis (Fig. 9) showed that the fractions represented the A and B components respectively of degraded pectate. The results of

analysis of the A and B components are shown in Tables 2 and 3. These components were prepared a number of times and their polysaccharide composition was always very similar to that indicated.

Since high-molecular-weight materials such as nucleic acid or protein present in the original pectinic acid preparation were unretarded by the gel, these substances were concentrated with the A component. These contaminants were not estimated, but their presence with component A can be seen from the yield of polysaccharide shown for this fraction in Table 3.

Molecular state of components A and B. Gel filtration of component B on Sephadex G-50 indicated that its molecular weight was below 10^4.

Fraction A was subjected to gel filtration as a solution (0.5%, w/v) in 0.1 M-sodium chloride containing 1% (v/v) of dimethylformamide and 0.1 M-sodium acetate buffer, pH 4.7. The solution was heated at 75–85° for 15 min. and passed through a fine (grade 5) glass filter (Baird and

Tatlock Ltd., London). A sample (5 ml.) of the solution was applied to the Sephadex G-200 bed equilibrated with 0.1 M-sodium chloride containing 1% (v/v) of dimethylformamide and eluted with this solvent. A plot of the distribution of carbohydrate in the effluent showed an unretarded peak with a tail of lower-molecular-weight material.

Several experiments were carried out to test whether component A was an aggregate of neutral and acidic polymers. When component A was degraded at pH 2 free arabinose was split off. The product had an increased mobility in electrophoresis but ran as a single zone less mobile than the B component. When, on the other hand, the material was esterified with ethylene oxide and once more degraded at pH 6.8 (i.e. re-degraded by transelimination) the product ran with decreased mobility, as a single zone.

Mixtures of component A with pectinic acid degraded at pH 2, and mixtures of transeliminationre-degraded A component and pectinic acid were completely resolved by zone electrophoresis without any indication of aggregation between acidic and near-neutral components. The relative positions after zone electrophoresis of the various fractions of the pectic substances and pectinic acid are shown in Fig. 10.

A partial acid hydrolysate of the A component contained an aldobiouronic acid mixture chromatographically similar to that obtained from whole pectinic acid.
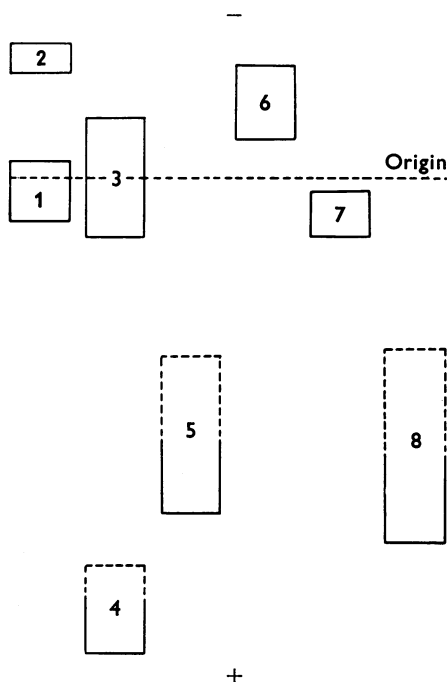

Fig. 10. Diagram showing the relative positions of various fractions of the apple pectic substances after zone electrophoresis (buffer, pyridine–acetate, pH 6.5; paper, GF/A). (1) Pectinic acid, (2) arabinan–galactan complex, (3) component A and (4) component B, of transeliminationdegraded pectinic acid, (5) component A degraded at pH 2, (6) component A re-degraded after esterification, (7) esterified B component and (8) pectinic acid after pectinesterase de-esterification. Broken lines indicate tailing.
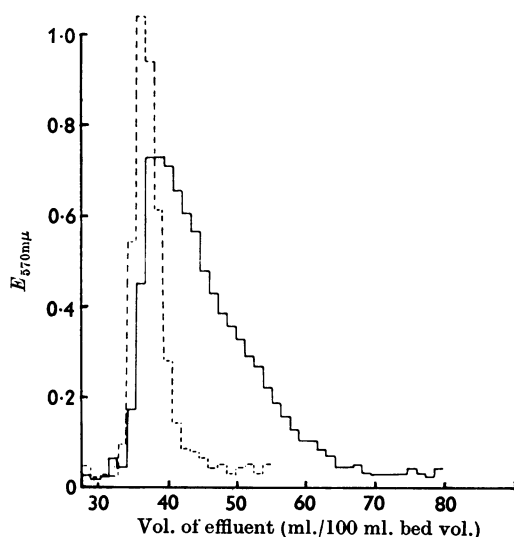

Fig. 11. Gel filtration of the product of methanolysis of pectinic acid (————) and yeast glycogen (– – – –) (the latter is almost completely excluded from all forms of Sephadex). Sephadex G-50 column (3.0 cm.^{2} × 55 cm.) eluted with 0.1 M-NaCl.

Properties of the methanolysis-degraded pectinic acid. A sample of the methanolysis-degraded pectin was subjected to gel filtration on Sephadex G-50. The distribution of carbohydrate in the effluent is shown in Fig. 11.

The degraded pectin was also subjected to hydrolysis with nitric acid for 15 hr. Chromatography of the hydrolysate (solvent A) showed the presence of traces of arabinose, xylose and an unidentified substance (R_{rhamnose} 0·84). None of these sugars was present in such quantity as to account for more than 0·1% of the original material.

Gel filtration of the arabinan–galactan complex. The arabinan–galactan material was subjected to Sephadex G-200 gel filtration with dimethylformamide under the same condition as the A component of degraded pectate. Again the result was an unretarded peak with a tail of lower-molecular-weight material.

DISCUSSION

The pectic substances have been prepared and fractionated into neutral and acidic polysaccharides without the use of alkali and it has been possible to follow the fractionation by zone electrophoresis, which not only provided evidence for the existence of the compounds in the original extract but also allowed a control and check of the fractionation procedure. The acidic polymer (pectinic acid) was precipitated by cetylpyridinium chloride and thus separated from the neutral material (arabinan-galactan complex).

Pectinic acid. The zone and free-boundary electrophoresis of this material suggested that it consisted of a single polysaccharide and zone electrophoresis of the enzymically de-esterified material further confirmed this. The ultracentrifuge data showed that some preparations contained a minor fast-moving component, possibly the small amount of nucleic acid or protein (2%) known to occur in the preparation. In all these physico-chemical methods the high viscosity of the polymer increased the sharpness of the boundaries and made interpretation of the results difficult.

The purified pectinic acid contained 13% of neutral monosaccharide units and 87% of galacturonic acid monomers. The neutral monomers were arabinose (9·3%), galactose (1·4%), rhamnose (1·2%), xylose (0·88%), fucose (trace) and 2-*O*-methylxylose and 2-*O*-methylfucose (about 0·1–0·2% of each). The 2-*O*-methylxylose and 2-*O*-methylfucose have also been isolated from sisal and lucerne pectin (Aspinall & Cañas-Rodriguez, 1958; Aspinall & Fanshawe, 1961).

That some of the neutral sugars were directly combined with the galacturonic acid units has been indicated by the presence of three aldobiouronic acids in the partial hydrolysate of the pectinic acid. This mixture of aldobiouronic acids was separated from the partial acid hydrolysate by ion-exchange chromatography, and paper chromatography of the fraction showed that it contained three compounds (compounds 1, 2 and 3 in Table 4). From the intensity of the spots on the paper chromatogram compounds 1 and 3 were present in larger amounts than compound 2 and galactose and rhamnose were much more abundant than xylose in the hydrolysate of the aldobiouronic acid mixture. This suggested that the galactose and rhamnose arose from compounds 1 and 3. The mobility of compound 1 in solvent D was identical with that stated by Aspinall & Fanshawe (1961) for a galacturonosylgalactose obtained from lucerne pectic acid, which, like compound 1, was not 1→2-linked. The mobility of compound 3 in solvent D was close to those given by Aspinall & Fanshawe (1961) and Ray & Rottenberg (1964) for galacturonosyl-(1→2)-rhamnose. The periodate–Schiff reaction confirmed that compound 3 contained a 1→2 link and its mobility in solvent E and characteristic colour reaction with aniline hydrogen phthalate agreed with those reported by Ray & Rottenberg (1964). It is suggested therefore that aldobiouronic acid 1 was galacturonosylgalactose, and 3 was galacturonosyl-(1→2)-rhamnose. Both of these aldobiouronic acids occur in pectic acid of lucerne (*Medicago sativa*) (Aspinall & Fanshawe, 1961) and also in a number of gums and mucilages (Buchi & Deuel, 1954). In this Laboratory galacturonosylrhamnose has been obtained from pectin of sisal (kindly supplied by Dr G. O. Aspinall) and of sycamore callus tissue. It seems likely that the galacturonosyl-rhamnose reported by Ray & Rottenberg (1964) from *Avena* coleoptile also arose from pectin, and Wilson (1961) has suggested the existence of a galaturonosylrhamnose link in *Nicotiana*. In view of the widespread occurrence of rhamnose in pectin (McCready & Gee, 1960) it now seems possible that the galacturonosyl-(1→2)-rhamnose link is a general feature of pectinic acid structure. The third component of the aldobiouronic acid mixture had a chromatographic mobility consistent with its being a uronosylxylose and was presumably responsible for the xylose detected after hydrolysis of the mixture. Although xylose has previously been found in apple and other pectin preparations (McCready & Gee, 1960) it could have arisen from contamination by xylans. The xylose in the apple pectinic acid could not be removed by precipitation of the acidic polymer with Ca^{2+} ions, when any free xylan would have been expected to remain in solution. The aldobiouronic acid obtained from the pectinic acid was not 1→2-linked as are the majority of glucuronosylxylose oligosaccharides

obtained by partial hydrolysis of xylan preparations. There was no indication of the presence of a galacturonosyl-(1→?)-2-O-methylxylose disaccharide in the partial hydrolysate as reported for the beet pectic arabinan (Andrews et al. 1959).

The increase in electrophoretic mobility of pectinic acid heated in dilute acid was probably caused by gradual removal of ester groups, and in part by removal of some arabinofuranose residues. This degradation is quite different from that which occurs in alkaline or neutral solution.

When pectinic acid is heated at neutral pH transelimination breaks occur next to esterified galacturonosyl residues (Albersheim, 1959; Albersheim et al. 1960), resulting in specific degradation of the galacturonosyl sequences of the pectinic acid molecule. If the molecule is considered to contain large arabinofuranose 'blocks' covalently attached to the galacturonosyl chains then the electrophoretic pattern produced during the degradation can be explained by the early occurrence of a number of breaks dividing the molecule into two types of fragments: one containing principally galacturonosyl residues and the other mainly neutral residues. At first these would have similar electrophoretic mobilities, since most of the galacturonosyl residues would be esterified. As hydrolysis of the ester links occurred the ratio of charged to uncharged residues would gradually rise in the polygalacturonosyl fragments so that their mobility would increase towards that of polygalacturonic acid and then become constant. The idea that this increase in mobility was due to removal of ester groups was confirmed by the effect of pectinesterase and of re-esterification.

The two electrophoretically distinct products of transelimination degradation of pectinic acid could be partially separated by preferential precipitation of the more acidic component by calcium salts or rivanol (6,9-diamino-2-ethoxyacridine lactate) (unpublished results), but complete separation was achieved by gel filtration with Sephadex G-75.

A large proportion of the total neutral sugar residues of pectinic acid were found in the high-molecular-weight A component after degradation in hot neutral solution, and the low-molecular-weight B component contained a very high percentage of galacturonic acid. Detailed analysis of the fractions showed that whereas arabinose was over a hundred times more abundant in the A than in the B component the distribution of galactose, rhamnose and xylose was rather less unequal and this was consistent with the evidence for direct linkage of some galactose, rhamnose and xylose residues to galacturonic acid. In particular the very slow release of rhamnose during hydrolysis and the presence of this sugar in the most abundant aldobiouronic acid detected provide some explanation for the fact

that rhamnose is the neutral sugar present in greatest amount in the B component.

Exclusion of component A from Sephadex G-200 indicates a molecular weight of 2×10^5 or more and this value would give a minimal molecular weight of about 10^6 for the intact parent molecule. This value is much greater than those currently accepted (Joslyn, 1962). Although dimethylformamide suppresses aggregation of sugar-beet arabinan (Tomimatsu, Palmer, Goodban & Ward, 1959) it seems likely that component A and also the arabinan–galactan complex were aggregated under the gel-filtration conditions which we have used, but the additional evidence suggests that component A is not an aggregate of dissimilar types of molecule.

It is now known that pectinic acid is degraded by alkali even at room temperature (Vollmert, 1950; Neukom & Deuel, 1958), and this makes reconsideration of some earlier work necessary. For example, preparation of a pure apple galacturonan (Hirst & Jones, 1939), involving hydrolysis with sulphuric acid followed by treatment with sodium hydroxide and isolation of the product by repeated precipitation with hydrochloric acid and calcium chloride, does not imply that such a separate homopolysaccharide exists in vivo. Similarly preparation of methylated apple arabinan by direct methylation of whole pectin in dimethyl sulphate and sodium hydroxide probably gave rise to a product largely derived from transelimination-degraded pectinic acid, rather than from the naturally occurring arabinan. Again, if sugar-beet pectinic acid is degraded in the same way as that of apple then calcium hydroxide-extracted beet arabinan (Hirst & Jones, 1948; Andrews et al. 1959) could well be a product of this degradation, although the beet arabinan does contain considerably less galacturonic acid than the 'apple arabinan' which we have obtained under different conditions.

Jansen et al. (1949) suggested after their examination of the products of methanolysis of pectinic acid that the polygalacturonosyl chains of the pectinic acid were interrupted by neutral residues after about every 32 uronosyl units; this would not agree with our results. Gel filtration of the product of methanolysis of our pectinic acid showed it to be polydisperse with an average molecular weight below 10^4. Furthermore, if during the methanolysis no uronosyl links were split a neutral monomer would be present at the reducing end of each resultant polyuronosyl chain so that material with degree of polymerization 32 would contain 3% of neutral residues. The amount of neutral sugar present in our methanolysis product was less than 0·5% and it is therefore probable that the methanolysis brought about a random breakage of the polygalacturonosyl chain.

Arabinan–galactan. It has been stated that an

arabinan and a galactan exist in apple pectin (Hirst & Jones, 1939) but the evidence for this is indirect as the two hypothetical components could not be separated either in their free state or when acetylated.

Though zone electrophoresis in tetraborate did not demonstrate any marked inhomogeneity of our arabinan–galactan material, boundary electrophoresis and analytical ultracentrifugation did suggest the presence of at least two components. Sephadex-gel filtration gave an apparent molecular weight of 2×10^5 or more, without fractionation, which suggests that if two components were present then both must have had this high molecular weight. However, it is quite possible that the molecular size could be caused by aggregation of smaller distinct molecular species.

At the earliest phase of cell development after cytokinesis the pectic substances form an important and integral part of the growing plant cell wall. The physical and chemical properties of this group of polysaccharides must contribute to the special biological nature of the wall during the primary growth phase. We have indicated that the pectic substance is in part made up of a large heteropolysaccharide and that this can be cleaved into small components by a transelimination reaction. The changes in the chemical and physical properties of the material coincidental with this reaction may have some special biological significance with regard to the cell wall since it has been shown that a specific transeliminase which catalyses *in vivo* a reaction similar to that produced chemically *in vitro* is present in plant material (Albersheim & Killias, 1962).

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