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POLYSACCHARIDES AND GLYCOPROTEINS OF APPLE FRUIT CELL WALLS

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Abstract—A proportion of the polysaccharides and glycoproteins of apple fruit cell walls can be readily extracted in neutral buffer at or below 20°. Removal of more material was not achieved with a wide range of dissociative aqueous reagents or non-aqueous solvents. Thus traditional degradative extractants were used to obtain soluble components for further characterization. Polysaccharides and glycoproteins were separated and purified by chromatography on DEAE-cellulose columns and by gel filtration. Purified components were hydrolysed and analysed for neutral sugar and uronic acid content and for their amino acid and hydroxyproline content. The possibility of linkages existing in the cell wall between polyuronide and glycoproteins containing hydroxyproline, arabinose and galactose residues is discussed. Because of aggregation between these components, which occurs after extraction, the presence of such linkages *in vivo* is difficult to establish. Other cell wall glycoproteins containing xylose and glucose residues are thought to have a possible role in stabilizing hemicellulose structure.

INTRODUCTION

THE PRIMARY linkages within the glycan components of higher plant cell walls are reasonably well known.[1] Electron microscopy and X-ray diffraction studies have revealed much of the secondary and tertiary levels of organization of cellulose which is a dominant constituent of these walls.[2] Other cell wall constituents in the matrix and middle lamella usually seem amorphous when examined by these techniques.

Partial hydrolysis of plant cell walls and their constituents has given chemical evidence for linkages between polymers represented by oligomers such as hydroxyproline arabinosides,[3,4] galacturonosylrhamnose, galacturonosylgalactose and galacturonosylxylose.[5,6] Thus something of the secondary level of organization of matrix and middle lamella substances can be understood in terms of linkages between neutral polysaccharides and a hydroxyproline-rich polypeptide or a polyuronide.

Cell wall analysis often involves a series of extractions of increasing severity followed by detection and estimation of monomeric constituents in the extractives. Extraction procedures are empirically derived and the lack of knowledge of the organization of the cell wall makes interpretation of results to some extent subjective. At one time it was assumed that a single monosaccharide constituent belonged to a single corresponding glycan even

[1] D. H. NORTHCOTE, *Internat. Rev. Cytol.* **14**, 223 (1963).
[2] P. A. ROELOFSEN, *Adv. Bot. Res.* **2**, 69 (1965).
[3] D. T. A. LAMPORT, *Nature, Lond.* **216**, 1322 (1967).
[4] D. T. A. LAMPORT and D. H. MILLER, *Plant Physiol. Lancaster* **48**, 454 (1971).
[5] A. J. BARRETT and D. H. NORTHCOTE, *Biochem. J.* **94**, 617 (1965).
[6] G. O. ASPINALL and R. S. FANSHAWE, *J. Chem. Soc.* 4215 (1961).

if it was detected in several different extractives.[7] The discovery of branched polymers containing more than one kind of residue has modified this interpretation.[5] The structural significance of such polymers can begin to be understood. For instance, it is thought that simple polygalacturonide chains with a high possibility of interchain bonding give structural rigidity to the walls of young actively dividing cells; as the cell ages, expansion is allowed for by the synthesis of polygalacturonide with araban and galactan side chains which reduce interchain cohesion.[8,9] This interpretation discounts the possibility that wall structure is stabilized by a linkage of neutral side chains to a protein constituent[10] though how an araban is linked to protein and to polyuronide has not been explained.

The importance of the pectic substances in wall structure was recognized early. It was assumed that differing solubilities in extraction media implied that the substances dissolved were derived from different native materials.[11] Despite much investigation the reasons for differing solubilities were never established.[12] To a large extent the controversies are forgotten and the terminology employed has been abandoned as it does not seem relevant to current problems.[13] However, the ideas developed in this early work are still current in branches of food technology where, for instance, fruit softening during ripening and storage, and the cooking properties of vegetable foods are related to the solubility of polyuronide components.[14] These relationships are empirically derived and the lack of a rational basis for them often inhibits the interpretation of data.

Analysis of cell wall polysaccharides has been facilitated by the use of chromatographic and electrophoretic techniques. Of these, DEAE-cellulose chromatography[15,16] is thought to be particularly useful for qualitative and quantitative analysis as well as preparative purposes. The application of gel permeation chromatography is limited by the apparently strong aggregation of the molecules of many cell wall polysaccharides in solution.[5] Using these techniques an attempt has been made to prepare and characterize polysaccharides from the cell walls of apple fruit tissue, in order to obtain insight into the organization of plant cell walls and suggest possible explanations of fruit softening. After some preliminary investigation,[16] emphasis was laid on the necessity for a non degradative extraction technique for the pectic substances. Failure in this direction led to the adoption of traditional extraction procedures (which always involve degradation) in the hope that structures could be deduced from the nature of the degradation products.

The work described was carried out over a prolonged period and mainly in the winter months. This meant that most of the apples used as starting material had been stored and were post-climacteric. It was realised that profound changes in the solubility and composition of wall components occur during ripening but it was thought that if the material available were investigated in depth valuable information would be obtained which could be related to changes during ripening at a later date. Unless otherwise stated the apple variety used was Cox's Orange Pippin.

[7] M. A. JERMYN, *Modern Methods of Plant Analysis* (edited by K. PEACH and M. V. TRACEY), Vol. 2, p. 197 Springer, Berlin (1955).
[8] S. E. B. GOULD, D. A. REES, N. G. RICHARDSON and I. W. STEELE, *Nature, Lond.* **208**, 876 (1965).
[9] R. W. STODDART, A. J. BARRETT and D. H. NORTHCOTE, *Biochem. J.* **102**, 194 (1967).
[10] D. T. A. LAMPORT, *Adv. Bot. Res.* **2**, 151 (1965).
[11] M. H. CARRE, *Biochem. J.* **16**, 704 (1922).
[12] M. A. JOSLYN, *Adv. Food Res.* **11**, 1 (1962).
[13] D. A. REES, *Adv. Carbohyd. Chem. Biochem.* **24**, 267 (1969).
[14] J. J. DOESBURG, *I.B.V.T. Commun.* No. 25 (1965).
[15] H. NEUKOM, H. DEUEL, W. HERI and W. KUNDIG, *Helv. Chim. Acta* **43**, 64 (1960).
[16] M. KNEE, *J. Exptl Bot.* **21**, 651 (1970).

RESULTS

Use of Automated Analytical Systems

Much of the carbohydrate analysis described below was aided by the use of automated systems; these were used both for discrete samples and continuous recording of the progress of column chromatography.

Some time was spent in developing an automated carbazole-sulphuric acid system for uronic acid estimation. Initially automation of the procedure described by McCready and McComb[17] was attempted but the system devised had poor characteristics and it was abandoned in favour of one based on the manual method of Bitter and Muir.[18] This employed a flow system similar to that illustrated by Stacey and Somers[19] though these authors do not mention the composition of reagents or the flow rates used. The use of an ethanolic carbazole solution disturbed the flow pattern in the system and gave a poor trace; satisfactory results were obtained with a solution of carbazole in 90% (w/v) sulphuric acid as described in the Experimental.

TABLE 1. RESPONSE OF AUTOMATED ANALYTICAL SYSTEMS TO STANDARD CARBOHYDRATES

Carbohydrate	Absorbance for 100 μg/ml in systems at wavelength stated: Sulphonated α-naphthol (550 nm)	Carbazole-H_2SO_4 (520 nm)
Galacturonic acid	0·46	1·23
Arabinose	1·36	0·03
Xylose	3·00	0·07
Glucose	1·27	0·19
Galactose	0·79	0·08

For total carbohydrate estimation a sulphonated α-naphthol system similar to that described by Fuller[20] was adopted and Table 1 shows typical results for response to standard carbohydrates with both carbazole sulphuric and sulphonated α-naphthol systems running together. Neutral sugars give little reaction in the carbazole system so that the reactions of complex carbohydrate mixtures in this system are taken to represent uronic acid content. These values can be used to correct data derived simultaneously from the α-naphthol system and arrive at neutral carbohydrate contents of mixtures. For protein analysis, an automated system based on the manual method of Lowry *et al.*[21] was adopted.

Preparation of Cell Walls

Commonly, analysis of the polysaccharides of plant tissue is preceded by disintegration and extraction in hot 80% ethanol followed by drying. This procedure removes sugars and other low molecular weight compounds, inactivates enzymes and provides a convenient

[17] R. M. McCready and E. A. McComb, *Analyt. Chem.* **24**, 1986 (1952).
[18] T. Bitter and H. M. Muir, *Analyt. Biochem.* **4**, 330 (1962).
[19] M. Stacey and P. J. Somers, *Lab. Practice* **18**, 1172 (1969).
[20] K. W. Fuller, *Automation in Analytical Chemistry*, Vol. II, p. 57, Technicon Symposium 1966, Mediad, New York (1967).
[21] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

starting material for further analysis. However, it has been found that dry cell wall preparations can require special 'rehydration' treatment before satisfactory extraction of polysaccharides can be achieved.[22] Furthermore when comparable samples of apple tissue have been extracted with cold acetone and with ethanol under reflux, more insoluble material has been recovered with the acetone procedure (Table 2). Thus a standard procedure was adopted in which tissue was disintegrated in cold acetone, filtered and washed with 80% acetone; the residue was retained and handled as an aqueous suspension.

TABLE 2. RECOVERIES OF INSOLUBLE MATERIAL AFTER EXTRACTION OF APPLE TISSUE WITH ORGANIC SOLVENTS

Apple variety	Date of preparation	Type of preparation	Insoluble residue (mg/g)
Cox	5/3/69	Acetone	20·9
		Ethanol	19·1
Cox	28/10/69	Acetone	27·0
		Ethanol	22·0
Bramley	5/3/69	Acetone	18·9
		Ethanol	17·3

Cortical tissue from apple fruits was extracted with 4 ml solvent per g tissue and washed with solvent water mixture (4:1), pure solvent and ether and dried *in vacuo* over P_2O_5 at 20° to constant wt. Ethanol extractions were carried out under reflux and acetone extractions at 2°.

Autolytic Enzymes in Apple Tissue

Aqueous suspensions of acetone-extracted tissue were examined for the presence of cell wall degrading enzymes. Pectinesterase activity was to be expected,[23] and was readily demonstrated. It was thought that the use of anionic detergents in extraction media would inactivate this enzyme.[24] Table 3 shows the effects of Teepol upon activity in a standard assay. A concentration of 1% seemed to inactivate the enzyme; the inactivation was not reversed by diluting the detergent to an otherwise ineffective concentration (preincubation treatment, Table 3). Similar inactivation was obtained with 0·1% sodium dodecyl sulphate, but this detergent crystallized out of extraction media at 2°.

Although detergent inactivation of pectinesterase seemed complete in this assay it was later realised that low levels of activity remained which could be detected by prolonged incubation. During extraction this residual activity had most effect upon soluble polymethylgalacturonide. It was found that the insoluble or bound polyuronide was attacked much more slowly by this enzyme and by citrus pectinesterase.[5]

Slow release of reducing sugar, which could be detected in aqueous suspension of acetone-extracted tissue, seemed to be correlated with an increase of soluble neutral carbohydrate and occurred most rapidly at pH 5 (Table 4). After a 24-hr incubation at pH 5 and 20° small amounts of galactose, arabinose and glucose could be detected in the deionised and concentrated filtrate by PC. No increase of reducing sugar occurred in incubations derived from tissue which had been refluxed in acetone following disintegration (Table 5).

[22] M. KNEE, *J. Sci. Food Agric.* **22**, 371 (1971).
[23] A. POLLARD and M. E. KIESER, *J. Sci. Food Agric.* **2**, 30 (1951).
[24] Z. I. KERTESZ, *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 158, Academic Press, New York (1955).

Activity was not detectable in the presence of Teepol even after a 24-hr incubation and it is of interest to note that prolonged incubation in the presence of autolytic activity did not lead to the release of a significant proportion of the insoluble polyuronide (Table 6).

TABLE 3. INACTIVATION OF APPLE PECTINESTERASE BY TEEPOL

Teepol added (g)	Acid groups released (μequiv.) in 5 min	10 min	15 min
0·00	33	71	112
0·03	44	89	134
0·06	25	47	60
0·15	8	9	9
0·30	3	6	6
0·03 (preincubated with enzyme for 15 min at 20°)	0	0	0

Acetone extracted tissue from apples picked 29/10/69 and stored 25 days at 12° was suspended in M NaCl (2 ml/g tissue) at 2° and adjusted to pH 7 (0·05 M NaOH). The filtrate from this provided the enzyme for the above assays. The standard assay mixture included 25 ml 0·1% apple pectin (BDH, extracted with 80% EtOH under reflux) in 0·1 M NaCl adjusted to pH 7·5 (M NaOH) and 5 ml enzyme. This mixture at 20° was maintained at pH 7·5 over a period of 15 min by additions of 0·05 M NaOH as necessary.

TABLE 4. pH-ACTIVITY RELATIONSHIP OF GLYCOSIDASES IN APPLE FRUIT TISSUE

pH	Soluble products (mg/g tissue) Neutral carbohydrate	Reducing sugar
3·0	0·68	0·12
4·1	0·64	0·30
5·0	0·65	0·33
6·1	0·43	0·13
7·1	0·45	0·07
7·8	0·45	0·07

Apples had been picked 25/9/70 and stored 4 months at 3·5°. Acetone-extracted tissue was suspended in H_2O and 5 ml portions of this suspension mixed with equal vols. of citrate–phosphate buffers[25] of the pH values indicated. After 5 hr incubation at 20°, digests were filtered (Whatman 541 paper) and the filtrates were heated at 95° for 10 min prior to carbohydrate estimation. Reducing sugar was estimated as glucose.[26,27]

Extraction in Aqueous Media at 20°

There are several possible ways of extracting polyuronide from apple cell walls at 20° in aqueous media. Accordingly to Doesburg,[14] prolonged extraction at 20° in neutral buffer including EDTA is an effective treatment. The results in Table 7 show that when precautions are taken to inactivate endogenous enzyme activities and to inhibit microbial growth,

TABLE 5. RELEASE OF CARBOHYDRATES INTO AQUEOUS SOLUTION AT pH 5·0 FROM APPLE TISSUE FOLLOWING EXTRACTION WITH ACETONE AT 2° OR UNDER REFLUX

Acetone extraction	Time of incubation (hr)	Soluble products (mg/g) Polyuronide	Neutral carbohydrate	Reducing sugar
At 2°	0	0·74	0·60	0·21
	5	1·18	0·74	0·45
Under reflux	0	0·59	0·23	0·07
	5	1·14	0·18	0·08

Procedure was as in Table 3.

[25] G. GOMORI, *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 138, Academic Press, New York (1955).
[26] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).
[27] M. SOMOGYI, *J. Biol. Chem.* **195**, 19 (1952).

solubilization of polyuronide is very limited. Another approach is the use of acid or alkaline media. Neal[28] showed that virtually complete solution of polyuronide in strawberry cell walls could be brought about at room temperature and pH 9 in the presence of EDTA.

TABLE 6. EFFECT OF TEEPOL UPON RELEASE OF CARBOHYDRATES INTO SOLUTION FROM ACETONE-EXTRACTED APPLE TISSUE DURING INCUBATION FOR 24 hr AT 20°

Additive	pH	Soluble products (mg/g) Polyuronide	Neutral carbohydrate	Reducing sugar
None	5	1·34	0·68	0·30
	6	1·19	0·50	0·13
1% Teepol	5	1·17	0·55	0·10
	6	1·24	0·58	0·14

Procedure as in Table 4 except for the addition of a few drops of toluene, and that 4 ml buffer were added to allow for addition of 1 ml 10% Teepol (or water in controls). Suspensions were agitated on a bottle roller during incubation.

Although more polyuronide could be extracted from apple preparations in neutral and slightly alkaline media than at pH 4 and below, the amounts solubilized were considerably less than the total polyuronide content of the tissue (Table 8).

TABLE 7. PROLONGED AQUEOUS EXTRACTION OF ACETONE-INSOLUBLE RESIDUE FROM APPLE TISSUE

Time of extraction (days)	Polyuronide in solution (mg/g)
0	0·61; 0·52
7	0·70; 0·70
14	0·87; 0·87
(Total	4·10; 4·45)

Cortical tissue from Tydeman's Early Worcester apples picked 21/8/70 was extracted with acetone and resuspended in a medium containing 0·05 M EDTA, 0·1 M Na_2HPO_4, 1% Teepol and 0·1% chlorbutol (British Drug Houses Ltd.). Duplicate preparations were agitated continuously on a bottle roller during extraction. Samples (10 ml) of suspension were withdrawn and filtered on sintered glass (por. 3). Total polyuronide was estimated in similar samples which had been heated at 95° for 4 hr and then filtered.

TABLE 8. EXTRACTION OF POLYURONIDE FROM ACETONE EXTRACTED APPLE TISSUE AT VARIOUS pHs

pH	Medium	Polyuronide extracted (mg/g)
2	HCl	0·86
4	0·25 M acetate	0·86
6		1·55
7	0·25 M TrisHCl	1·48
8		1·48
9		1·56

Apples picked 25/9/70 and stored 5 months at 3·5° were extracted with acetone and suspended in 2% Teepol to a wt which was twice that of the original tissue, and samples (10 ml) of this suspension were added to 10 ml of various extraction media. For extraction at pH 2 the mixture was adjusted to this pH with M HCl and made up to 20 ml. Other extraction media contained EDTA (final concentration 0·05 M) and buffer constituents at the final concentrations indicated in the table. After 24 hr incubation under toluene at 20° on the bottle roller the suspensions were filtered on sintered glass (por. 3) and the polyuronide content of the filtrates was estimated. The total polyuronide content of this tissue was 4·25 mg/g.

Since it is generally thought that polyuronide is held in plant cell walls by hydrogen bonds and weak electrostatic bonds it seemed possible that mild dissociative agents would liberate it into solution. The use of many of these agents is well established in protein chemistry and there are instances of their use for carbohydrate polymers. The simplest was the

[28] G. E. NEAL, *J. Sci. Food Agric.* **16**, 604 (1965).

use of high salt (KCl) concentrations which effectively solubilise the proteoglycans of bovine nasal cartilage.[29] Urea dissociates the soluble polyuronide–glucan complex from white mustard seeds.[30] Guanidine hydrochloride has been used for extraction of bovine nasal cartilage[29] and the solubilization of starch grains[31] while dimethylformamide is said to suppress aggregation of araban.[32] Borate is sometimes used to aid alkaline extraction of hemicellulose from plant cell walls. However none of these agents, under the conditions used, aided the extraction of polyuronide from apple cell walls.

TABLE 9. EXTRACTION OF POLYURONIDE FROM ACETONE EXTRACTED APPLE TISSUE AT VARIOUS TEMPERATURES

Extraction medium	Temperature (°)	Polyuronide extracted (mg/g) at time (hr) 1	2	4	6
EDTA-phosphate					
	20	1·11	0·98	1·01	1·05
	35	1·17	1·17	1·33	1·46
	55	2·03	2·34	2·73	2·92
	75	3·14	3·42	3·55	3·61
	95	3·46	3·77	4·06	4·25
SHMP	75	2·44	2·66	2·82	3·04
	95	2·85	3·23	3·61	4·06

Apples picked 25/9/70 and stored for 2 months at 3·5° were extracted with acetone and the residue was resuspended in 2% Teepol to twice the tissue wt. Samples (10 ml) were mixed with 10 ml extraction medium, 0·2 M Na_2HPO_4, 0·1 M EDTA (pH 6·9) or 4% sodium hexametaphosphate (SHMP) adjusted to pH 3·7 (M HCl) and incubated at the temps. shown. At the times indicated samples (1·0 ml) were withdrawn, diluted to 10 ml with H_2O and filtered. The polyuronide content of the filtrates was estimated. Initial samples at 20° gave values of 1·14 and 1·17.

Extraction in Non Aqueous Media

Dimethyl sulphoxide has been used for hemicellulose extraction from wood,[33] ethylenediamine for the extraction of glycoproteins from yeast cell walls[34] and chloral hydrate is a traditional solvent for starch. Dimethylformamide and pyridine were thought to be potential carbohydrate solvents. However, none of these reagents approached the effectiveness of an aqueous medium as a polyuronide extractant.

Extraction at Elevated Temperatures

Polyuronide can be extracted from potato tuber tissue by incubation in neutral buffer in the presence of EDTA at 35°;[35] the extraction approaches completion at about 20 hr and comparison with other data[36] suggests that virtually all the polyuronide of the tissue is

[29] S. W. SAJDERA and V. C. HASCALL, *J. Biol. Chem.* **244**, 77 (1969).
[30] G. T. GRANT, C. MCNAB, D. A. REES and R. J. SKERRETT, *Chem. Commun.* 805 (1969).
[31] H. W. LEACH, *Starch Chemistry and Technology* (edited by E. F. WHISTLER and R. L. PASCHALL), Vol. 1, p. 289, Academic Press, New York (1965).
[32] Y. TOMIMATSU, K. J. PALMER, A. E. GOODBAN and W. H. WARD, *J. Polym. Sci.* **36**, 129 (1959).
[33] T. E. TIMELL, *J. Am. Chem. Soc.* **82**, 5211 (1960).
[34] R. SENTANDREU and D. H. NORTHCOTE, *Biochem. J.* **109**, 419 (1968).
[35] D. J. LINEHAN and J. C. HUGHES, *J. Sci. Food Agric.* **20**, 119 (1969).
[36] J. FRIEND and M. KNEE, *J. Exptl Bot.* **20**, 763 (1969).

extracted in this way. However, it is known that methyl esterified polygalacturonide undergoes transeliminative cleavage in neutral buffer at this temperature.[37] It has also been suggested that heating at lower pH values can detach neutral polysaccharide side chains from polyuronide molecules.[16] Extraction of polyuronide at various temperatures in traditional extractants, 0·1 M Na_2HPO_4, 0·05 M EDTA (pH 6·9) and 2% sodium hexametaphosphate (SHMP) was examined in the light of these findings; the results are shown in Table 9. In the neutral medium at 20° amounts extracted were static over a period of 6 hr; at 35° a slow but sustained solubilization occurred while at higher temperatures an initial phase of rapid extraction was followed by a slower phase so that the final total extracted increased substantially with rising temperature to a maximum at 95°. The rates of extraction in the initial phase are closely comparable with rates of depolymerization of polyuronide in neutral buffer at various temperatures[37] and it is thought that this reaction is partly responsible for the dissolution of wall polyuronide at 35° and above. The results for sodium hexametaphosphate extraction are less extensive but it seems that effective extraction is achieved only at temperatures which are known to cause degradation.

Degradative Fractionation of Apple Cell Walls

Since the above work had failed to provide a treatment which would liberate most of the polyuronide and associated polysaccharides from apple fruit cell walls, attention was shifted to degradative treatments. The remainder of this work is concerned with the analysis of three apple preparations. The first was subjected to an exploratory analytical scheme to select preparative techniques for further study; this scheme and some of the results are shown in Table 10. The second and third were large scale preparations for the isolation of components in quantities sufficient for characterisation.

Extraction in Neutral Buffer at or below 20°

In exploratory work, soluble polysaccharides were extracted from acetone extracted fruit tissue by prolonged incubation in neutral buffer; examination of these extractives by chromatography on DEAE-cellulose revealed an unretarded neutral polysaccharide, and a mixture of components eluting on the phosphate gradient below 0·2 M; however, the bulk of the polyuronide was eluted at concentrations above 0·2 M; this was in marked contrast to previous findings.[16] If the acetone preparation was heated before extraction the polyuronide was found to be eluted below 0·2 M as part of heterogeneous peak. It was realized that despite the presence of detergent some pectinesterase activity was present in the unheated preparations so that, in time, the polyuronide came to be largely de-esterified and therefore more tightly bound by the ion exchange cellulose. Short periods of extraction at 2° were found to remove virtually all of the soluble polysaccharides, and acidification of the extractive following filtration prevented further enzyme action. Material prepared in this way (extract A) could be de-esterified by adjusting a solution to pH 7 and incubating with additions of alkali to maintain neutrality. This gave a convenient starting material for preparative chromatography on DEAE-cellulose since the polyuronide could be separated from other acidic components of the extract. Five fractions (A1–5) were obtained for further analysis.

The unretarded fraction (A1) was examined by chromatography on DEAE-cellulose in the borate form. It was retained on the column and could be displaced from it using a

[37] P. ALBERSHEIM, H. NEUKOM and H. DEUEL, *Archs. Biochem. Biophys.* **90**, 46 (1960).

borate gradient, largely as a single component. It was a complex polysaccahride or mixture of polymers as it liberated arabinose, xylose, galactose and glucose on hydrolysis; it had a low uronic acid content as might be expected from its apparent neutrality (Table 11).

TABLE 10. EXPLORATORY ANALYSIS OF POLYSACCHARIDES OF APPLE FRUIT TISSUE

(a) Extraction scheme and carbohydrate content of extractives

Extraction sequence	Extractions in numerical sequence		Duration (hr)	Carbohydrate extracted (mg/g tissue) Polyuronide	Neutral polysaccharide
	1	EDTA phosphate, 20°	24	0·48	0·40
(a)	2	EDTA phosphate, 95°	4	2·07	1·32
	3	4 N NaOH, 25°	2	0·54	4·09
	4	72% H_2SO_4, 20°	24	0	4·66
(b)	2	EDTA phosphate, 75°	2	1·69	0·89
	3	EDTA phosphate, 95°	4	0·36	0·46
	3	0·05 N NaOH, 20°	2	0·05	0·08
	3	0·5 N NaOH, 20°	2	0·12	0·24
	3	0·5 N NaOH, 20°	6	0·16	0·66
(c)	2	2% sodium hexa-metaphosphate, 95°	4	1·64	1·36
(d)	2	4 N NaOH, 25°	2	0·33	2·40

(b) Monosaccharide composition of hydrolysates

Monosaccharide	Anhydro sugar content (mg/g) As estimated	Calculated as "neutral polysaccharide"
Galacturonic acid	2·55	—
Arabinose	1·24	1·31
Xylose	0·92	2·12
Rhamnose	0·18	—
Galactose	0·62	0·39
Glucose	3·78	3·78

The full procedure for this analysis is described in Experimental.

Three protein containing fractions (A2, A3 and A4) were run on smaller DEAE-cellulose columns in the phosphate form; A2 and A3 were evidently heterogeneous though A4 appeared to have mainly one component. Fractions A2 and A3 were further examined by gel filtration; this gave unretarded carbohydrate rich fractions, A2a and A3a, and retarded protein rich fractions, A2b and A3b. The components A2a, A3a and A4 all contained arabinose and galactose residues and increasing contents of uronic acid which correlated with their order of elution from the DEAE-cellulose column (Table 11). They also contained substantial amounts of amino acid residues, including measurable amounts of hydroxyproline.

Fractions A2b and A3b contained predominantly xylose and glucose residues; the smaller amounts of arabinose and galactose detected could be due to contamination from fractions A2a and A3a respectively. They had a low, but measurable uronic acid content and an overwhelming preponderance of amino acid residues; hydroxyproline was barely

detectable (Table 11). The volume in which they were eluted from the Sephadex column would indicate a MW of about 10^4. The last fraction to be eluted from the DEAE-cellulose column (A5) was virtually a pure polyuronide (Table 11).

TABLE 11. COMPOSITION OF SOLUBLE FRACTIONS (EXTRACT A) OF ACETONE EXTRACTED APPLE TISSUE

Component	Amount of component in fraction as proportion of total detected (μg/mg) A1	A2a	A2b	A3a	A3b	A4	A5
Uronic acid	82	265	31	482	76	577	961
Arabinose	217	75	8	119	31	50	16
Xylose	142	—	26	—	78	—	—
Rhamnose	—	—	—	40	—	22	23
Galactose	331	97	11	155	20	45	—
Glucose	228	—	20	—	111	—	—
Amino acid	ND	562	903	198	682	304	ND
Hydroxyproline	ND	1·4	1·1	6·6	1·2	1·5	ND

ND not determined; (—) absent.

In further analytical work it has been shown that polyuronide is found in the glycoprotein fractions only when cell wall material or extracted polysaccharides are dried during preparation. An extract from a wet cell wall preparation which has been retained in solution does not show this association of polyuronide and glycoprotein, and the amount of polyuronide associated appears to increase with the age of a dry preparation.

Extraction in a Hot Neutral Medium

Extraction in a neutral phosphate solution containing EDTA at 100° has been used in estimating the total polygalacturonide content of plant tissues. This method is applicable to apple tissue[9,17] and it was found that it removed substantial quantities of neutral polysaccharide as well as polyuronide (Table 10). Chromatography on Sephadex G25 and DEAE-cellulose revealed a small amount of free neutral polysaccharide (unretarded by both columns). The bulk of the material was of relatively high MW (excluded from Sephadex G25) and more or less acidic (retained by DEAE-cellulose). Elution of this material from the DEAE-cellulose column using a phosphate gradient revealed continuously changing proportions of neutral carbohydrate and uronide with little evidence of distinct components. Low MW material (retarded by Sephadex) was also present in the extract and seemed to be essentially uronic acid.

Extraction in the same medium at 75° released less neutral polysaccharide and polyuronide (Table 10). Little free neutral polysaccharide could be detected; the acidic polysaccharide comprized, in order of elution from the column, a uronide rich fraction, a neutral carbohydrate rich fraction and a second uronide rich fraction. Low MW material was not detected.

Following extraction at 75° the remainder of the polyuronide and associated neutral polysaccharide could be extracted in the same medium at 95°. Neutral carbohydrate predominated in this extract and it was shown to be bound to polyuronide by chromatography on DEAE-cellulose. Little free neutral polysaccharide or low MW material could be detected.

A sample from a large scale preparation of the material extracted at 75° (extract C) and fractionated on DEAE-cellulose seemed very different from earlier preparations. The same acidic fractions, two uronide rich (C1 and C3) and one rich in neutral monomers (C2) were present, however, and a difference in their amounts may be due to differences in source and physiological age of the apples used in the preparations. Fraction C1 contained a small amount of protein which could be separated from the carbohydrate by a second run on a small DEAE-cellulose column. Fractions C1 and C3 had similar monomeric compositions with 85–90% uronic acid residues and 10–15% neutral residues (Table 12). Fraction C2 contained only 50% uronic acid and correspondingly larger amounts of arabinose, galactose and other neutral monomers.

TABLE 12. COMPOSITION OF FRACTIONS EXTRACTED IN 0·1 M PHOSPHATE 0·05 M EDTA (pH 6·9) AT 75° (EXTRACT C)

Component	Amount of component in fraction as proportion of total detected (μg/mg) C1	C2	C3
Uronic acid	892	494	868
Arabinose	40	265	53
Xylose	16	62	18
Rhamnose	26	92	32
Fucose	—	22	5
Galactose	26	66	23
Glucose	—	—	—

The uronic acid was identified as galacturonic by PC.[38]

Extraction in a Mildly Acidic Medium

A 2% solution of sodium hexametaphosphate (pH 3·7) at 95° extracts similar quantities of polyuronide and neutral polysaccharide to those extracted in 0·05 M EDTA, 0·1 M phosphate (pH 6·9) at 95° (Table 10). A substantial proportion of free neutral polysaccharide and a small amount of low MW neutral carbohydrate have been consistently found in these extracts.

Extraction in Dilute Alkali

It was suspected that the polyuronide and associated neutral polysaccharide which remained in the cell wall after extraction in EDTA-phosphate at 75° were linked to some other structural component of the wall. Subsequent extraction in the same medium at 95° probably broke the linkages involved, and extracts obtained in this way were not examined further. Dilute alkali extractions following the extraction at 75° removed polyuronide containing material with a higher proportion of neutral residues (Table 10). A large scale preparation (extract D) from senescent apples obtained using 0·5 N NaOH at 20° for 6 hr was examined by chromatography on DEAE-cellulose. A large amount of essentially neutral polysaccharide came through the column unretarded (fraction D1): glucose and xylose predominated in this fraction (Table 13). Material increasingly rich in uronic acid residues was eluted on the phosphate gradient. A prominent protein peak eluting at about 0·2 M

[38] F. G. FISCHER and H. DÖRFEL, *Hoppe-Seylers Z. Physiol. Chem.* **301**, 224 (1955).

(fraction D3) could not be separated from its associated polysaccharide by repeated chromatography on DEAE-cellulose. This fraction and the one preceding it on the column (D2) were not obtained in sufficient quantities to be characterized further. The fraction eluting higher on the gradient (D4) proved to be rich in neutral monomers with arabinose as the major component (Table 13).

TABLE 13. COMPOSITION OF FRACTIONS EXTRACTED IN 0·5 M NaOH (EXTRACT D)

Component	Amount of component in fraction as proportion of total detected (μg/mg)								
	D1*	D2	D2a	D2b	D2c	D3a	D3b	D4	D4*
Uronic acid	81	145	148	156	113	233	104	299	149
Arabinose	41	125	203	121	—	235	29	265	347
Xylose	209	180	148	183	98	198	26	188	226
Rhamnose	—	—	—	—	—	—	—	—	—
Fucose	43	—	—	—	—	30	—	21	43
Galactose	118	102	207	109	—	198	—	143	135
Glucose	507	202	275	431	402	68	16	61	99
Amino acids	ND	243	18	ND	385	37	823	22	ND
Hydroxyproline	ND	2·5	1·8	ND	2·3	0·7	0·7	0·2	ND

ND not determined; (—) not found.
* Preparations from senescent apples, others from pre-climacteric fruit.

A similar extract was prepared from pre-climacteric apples. Far greater quantities were isolated, which permitted more extensive analysis. The protein in D3 could be largely separated from the polysaccharide by gel filtration. The unretarded polysaccharide rich fraction (D3a) contained predominantly uronic acid, arabinose, xylose, and galactose while the protein rich fraction (D3b) contained the pentoses and glucose (Table 13). Elution of this fraction in approximately the total column volume indicated a low MW; this would explain a substantial observed loss of protein in dialysis between the separations on DEAE-cellulose and Sephadex G200.

Surprisingly, fraction D2 which seemed to contain little protein contained more hydroxyproline than either D3a or D3b. Gel filtration of D2 gave an unretarded component (D2a) with the bulk of the polysaccharide, containing uronic acid, arabinose, xylose, galactose and glucose in similar amounts; the associated protein contained approximately 10% hydroxyproline residues; eluted after this, fraction D2b contained substantially more glucose and apparently no protein; this overlapped with the last, protein-containing, fraction (D2c), which lacked arabinose and galactose and contained little hydroxyproline in relation to its amino acid content. Like fraction D3b, D2c was eluted around the inclusion volume of the column and was therefore of low MW.

Fraction D4 in this preparation was similar in composition to that from senescent apples except that it contained more uronic acid residues.

Extraction in Concentrated Alkali

Extract E, extracted in 4 N NaOH was not retained by DEAE cellulose in the phosphate form. On columns of DEAE-cellulose in the borate form it was absorbed and could be displaced using a phosphate gradient. The material eluted was divided into three arbitrary fractions, E1–E3. These fractions contained decreasing amounts of arabinose and galactose

and increasing amounts of glucose in relation to their order of elution; their xylose contents were similar (Table 14). The residue after this alkaline extraction contained largely glucose residues with trace amounts of arabinose and xylose.

TABLE 14. COMPOSITION OF FRACTIONS EXTRACTED IN 4 N NaOH AND EXTRACTION RESIDUE

Component	Amount of component in fraction as proportion of total detected (μg/mg)			
	E1	E2	E3	Residue
Uronic acid	85	75	181	—
Arabinose	27	—	—	19
Xylose	253	235	231	19
Galactose	125	127	—	—
Glucose	509	563	588	962

DISCUSSION

The above results give a detailed account of the composition of the middle lamella and matrix substances of apple fruit cell walls and from them it is possible to make certain statements concerning the organization of these non cellulosic constituents. The bulk of this material is insoluble in a wide range of potentially dissociative reagents, non-aqueous media and even in aggressive extractants such as 4 N alkali; this suggests that it is held together by covalent linkages or by regions of hydrogen bonding which would need to be sufficiently extensive to give it a microcrystalline structure approaching that of cellulose. Other wall components are so freely soluble that there must be a sharp chemical difference between them and the insoluble components.

Interchain bonding has been suggested between polyuronide molecules perhaps involving hydrogen bonds or chelation bridges in short regions of the chains to form 'microcrystallites'.[13,39] Inclusion of rhamnose residues in the chains and addition of side chains of neutral residues would seriously hinder this bonding. It is worth pointing out that the soluble (weakly bound) fraction of apple cell walls includes a virtually pure polyuronide (fraction A5) while the increasingly insoluble (tightly bound) components contain increasingly higher proportions of neutral sugar residues (C and D fractions).

An alternative suggestion is that these side chains of neutral monomers in some way link the polyuronide by way of covalent bonds to a protein moiety, 'extensin'.[10] The above results would be consistent with this hypothesis. It is suggested that extraction in EDTA-phosphate at 75° opens up the cross linked structure by transeliminative cleavage of uronide chains yielding the C fractions in solution. These are more acid than the parent materials owing to the de-esterification that accompanies chain cleavage. Polyuronide components with high (C2) and low (C1 and C3) proportions of arabinose and galactose residues are found as described by earlier workers.[5] Following this degradative treatment the essential link components are alkali soluble; thus fraction D2a contains uronic acid, arabinose and galactose residues together with a protein, containing hydroxyproline, as predicted for an extensin structure. Other fractions (D3a and D4) with the same monosaccharide residues, but much less protein, presumably represent other breakdown products of the intact

[39] D. A. REES, *The Shapes of Molecules, Carbohydrate Polymers*, p. 141, Oliver & Boyd, Edinburgh (1967).

structure. However, since the glycoproteins and polyuronide in extract A have been shown to form aggregates, similar interactions may have occurred in the D fractions and it is not possible to say that the association of these components *in vivo* has been definitely established.

Other glycoprotein fractions can be found in the degraded material with higher proportions of protein, less hydroxyproline and a relative predominance of xylose and glucose residues in the carbohydrate moiety (D2c and D3b). It seems possible that these represent previously unsuspected link molecules in hemicellulose structure. One might even suggest that alkaline extractions are effective for these components because of the presence of alkali labile linkages such as glycosidic bonds to serine or threonine residues in the protein moiety.[40]

The soluble cell wall components contain entities with similar features to those in the chemically degraded extractives. Thus there is a group of proteoglycans containing arabinose galactose and hydroxyproline residues (A2a, A3a, A4) and a pair of glycoproteins containing xylose and glucose residues with much less hydroxyproline (A2b, A3b). These fractions are rather more distinct in their composition than those found among the cell wall degradation products and this seems to suggest that when they are assembled in the wall very extensive cross linkages are formed between hemicelluloses, pectic polysaccharides and proteins.

It is suggested that this cross linking renders the cell wall components insoluble and contributes to the structural properties of the plant tissue both in terms of wall rigidity and inter cell cohesion. The arabinosyl hydroxyproline linkage is alkali stable and this would account for the failure to extract significant amounts of polyuronide in 4 N alkali.

One type of chemical attack on the structure has already been discussed, that is heating in neutral media. It seems likely that weakly acidic media, (such as sodium hexametaphosphate adjusted to pH 3·7) at high temperatures, cause cleavage of a certain number of linkages between arabinofuranoside residues.[16] This results in the dissolution of the bulk of the polyuronide; free neutral polysaccharide and even low MW carbohydrates can be detected in the extract.

Since hydroxyproline rich proteins were first detected in the cell walls of sycamore and *Phaseolus* tissue cultures[41] they have been found in many other plant species. Fragments possibly derived from an extensin structure have been isolated from tomato tissue cultures by cellulase treatment of cell walls.[42] Mucopolysaccharides have been isolated from corn pericarp cell walls, rich in hydroxyproline and containing arabinose, galactose and glucose residues.[43] Polysaccharide protein complexes containing similar components, and in addition, galacturonic acid, were found in the trichloroacetic acid soluble fraction of leaves of *Vicia faba*.[44] It is possible that aggregation of polyuronide and glycoproteins occurred in these preparations particularly since the materials were dried after extraction. Because of this it is not possible to say that the presence of an extensin like structure in plant tissues has been demonstrated. Glycoproteins containing hydroxyproline arabinose and galactose residues appear to be generally distributed; polyuronide fractions containing the same neutral sugar residues are also frequently encountered. These components may not be involved in the same structure at all, though it seems likely that some form of covalent

[40] R. CARUBELLI, V. P. BHAVANDAN and A. GOTTSCHALK, *Biochim. Biophys. Acta* **101**, 67 (1965).
[41] D. T. A. LAMPORT and D. H. NORTHCOTE, *Nature, Lond.* **188**, 665 (1960).
[42] D. T. A. LAMPORT, *Biochemistry* **8**, 1155 (1969).
[43] J. A. BOUNDY, J. S. WALL, J. E. TURNER, J. M. WOYCHIK and R. J. DIMLER, *J. Biol. Chem.* **242**, 2410 (1967).
[44] A. PUSZTAI, R. BEGBIE and I. DUNCAN, *J. Sci Food Agric.* **22**, 514 (1971).

cross linkages may be present between them, or the glycoprotein may be a stabilizing component in complex aggregates analogous to those in bovine nasal cartilage.[45]

It is worth noting that the apple cell wall components described above which contain hydroxyproline could not be said to be rich in this substance (except D2a). It would have been impossible to detect without extensive purification of the fractions. Large amounts of polypeptide containing α-amino acids are associated with the material and it is possible that some of this is enzymic, since it has already been suggested that peroxidases may be covalently linked to the extensin structure in the cell wall.[46]

EXPERIMENTAL

Source of fruit. Most of the apples were of the variety Cox's Orange Pippin, grown at East Malling Research Station, picked on the dates indicated and stored at 3·5° until required.

General procedure. Working at 2°, apples were peeled and cored; cortical tissue was disintegrated in 4 vol. of acetone in a blender, filtered on Whatman 541 under suction and washed with a further 8 vol. of 80% (v/v) aq. acetone. The acetone extracted material was resuspended in a 2% Teepol 'N' solution to give a final wt twice that of the original tissue. This slurry was usually used by mixing it in equal vol. with extractant solutions.

Exploratory analysis of polysaccharides. Apples picked on 25/9/70 and stored for 6 months at 3·5° were extracted with acetone and resuspended in Teepol. A portion of this suspension was mixed with 9 vols. EtOH and after 48 hr filtered on sintered glass (por. 2) washed with alcohol and EtOH and allowed to dry. The remainder of the suspension was mixed with an equal vol. of 0·2 M Na_2HPO_4 0·1 M EDTA for extraction 1 (Table 10). After filtration the residue from this extraction was washed with H_2O and resuspended in H_2O to provide material for all the subsequent extractions. For all of these (except 72% H_2SO_4) 10 ml of suspension were mixed with 10 ml reagent of the appropriate strength and after extractions as described in Table 10 were filtered on sintered glass (por. 3); the residues were washed with H_2O and if necessary resuspended in 10 ml H_2O for a subsequent extraction. Alkaline extractions were carried out under N_2 and the extractives were neutralised with HOAc. After extraction (a)3, the residue was washed with alcohol and Et_2O and allowed to dry before 2 ml 72% (w/w) H_2SO_4 were added to the sintered glass crucible; this was allowed to drain overnight and then washed through with H_2O. For hydrolysis and monosaccharide estimation, samples (50 mg) of the dry preparation were allowed to dissolve in 0·4 ml 72% (w/w) H_2SO_4 for 24 hr and then diluted with 5 ml H_2O. The solutions were autoclaved at 121° for 60 min,[47] neutralized with $BaCO_3$ and applied to PCs developed in EtOAc–pyridine–H_2O (8:2:1). Sugars were visualized and estimated by the method of Wilson.[48] The anhydro sugar contents of the polysaccharides of the original tissue were calculated; the results have also been expressed in terms of neutral polysaccharide contents as estimated in the sulphonated α-naphthol reaction by means of data for standard sugars (Table 1).

Large scale preparations. Because English apples were unavailable, Cox's Orange Pippin from Tasmania were purchased at a local shop. Cortical tissue (500 g) from these was extracted with acetone, resuspended in detergent, and stirred with 0·1 M Tris-HCl (pH 7·5) at 2° for 30 min. The suspension was filtered (Whatman 541); the filtrate (extract A) was immediately adjusted to pH 3 (M HCl) and the residue was resuspended in detergent solution and mixed with an equal vol. of 0·1 M Tris-HCl (pH 7·4) containing M NaCl. The suspension was stirred overnight at 2° and filtered (Whatman 541). The filtrate (extract B) was discarded as it contained neglible quantities of carbohydrate. The residue was washed with water and resuspended in H_2O before mixing with an equal vol. of a boiling solution containing 0·1 M EDTA (di-Na salt) 0·2 M Na_2HPO_4. The resulting suspension was heated at 75° for 2 hr, and filtered on Whatman 541 (filtrate is extract C). The residue was washed with H_2O, resuspended, mixed with an equal vol. of M NaOH and stirred at room temp. for 6 hr. It was then filtered on glass fibre paper. Extract C was cooled in ice and neutralized with glacial HOAc. The residue was washed with H_2O, resuspended, mixed with an equal vol. of 8 M NaOH and stirred at room temp. for 2 hr. The suspension was filtered and the filtrate (extract D) neutralized as for extract C. The final residue was washed with H_2O, EtOH and Et_2O and allowed to equilibrate with air. Polysaccharides were recovered from extract A by adding 4 vol. acetone and, after allowing to stand at 2°, the precipitate was collected by centrifugation. Other extracts were first concentrated by dialysis against sucrose and then dialysed against H_2O for 72 hr at 2°. Extracts C and E were precipitated with 4 vol. and extract D with 9 vol. acetone at 2° and collected by centrifugation. The precipitates were washed by resuspension in acetone and centrifugation several times before being allowed to dry in air. These dry

[45] V. C. Hascall and S. W. Sajdera, *J. Biol. Chem.* **244**, 2384 (1969).
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[47] J. F. Saeman, W. E. Moore, R. L. Mitchell and M. A. Millet, *Tappi* **37**, 336 (1954).
[48] C. M. Wilson, *Analyt. Chem.* **31**, 119 (1959).

preparations provided the starting material for further analysis. An essentially similar scheme was used to isolate a further sample of extract D from preclimacteric Cox's Orange Pippin which had been picked 15/9/71 and stored at −20° for 4 months.

Column chromatography. Preswollen, microcrystalline diethylaminoethyl cellulose (Whatman DE 52) was used. In exploratory work samples (10 ml extract) were pumped onto a Sephadex G25 column (36 × 2·8 cm) and eluted with 0·005 M phosphate 0·001 M EDTA (pH 6·5), pumped at *ca.* 1·5 ml/min. The outlet of this column was connected to a column (7·5 × 1 cm) of DEAE-cellulose while the void volume and a further 25 ml passed through, after which this column was disconnected and eluted separately with a linear gradient formed from 100 ml 0·005 M phosphate 0·001 M EDTA and 100 ml 0·5 M phosphate 0·001 M EDTA (pH 6·5) using apparatus similar to that of Parr.[49] After this was complete elution of the Sephadex column alone with 0·005 M phosphate was continued until the low MW components had emerged. Carbohydrate emerging from the columns used in this system was measured continuously. Preparative columns (15 × 3 cm) were equilibrated with 0·005 M phosphate 0·001 M EDTA (pH 6·5). The sample (250 mg in 100 ml buffer) was pumped on at a flow rate of about 1·5 ml/min. Elution was continued with 0·005 M phosphate 0·001 M EDTA to elute neutral polysaccharides after which a linear gradient was applied using 200 ml 0·005 M phosphate 0·001 M EDTA and 200 ml 0·5 M phosphate 0·001 M EDTA (also pH 6·5) in two chambers of a Technicon 'Autograd'. Timed fractions (4 min) were collected from the column and analysed subsequently. Analytical columns (15 × 1·5 cm) were also equilibrated with 0·005 M phosphate 0·001 M EDTA and pumped at a similar flow rate. Carbohydrate analyses were carried out directly on column eluates but protein estimations were carried out on collected (4 min) fractions. The gradient was generated from 100 ml of 0·5 M phosphate and 100 ml 0·005 M phosphate.[49] Columns (15 × 1·5 cm) of DEAE-cellulose in the borate form were used similarly; the gradient was generated from 100 ml H_2O and 100 ml 0·5 M sodium metaborate. Gel filtration on Sephadex G200 was carried out at 2°. The column (27·5 × 2·8 cm, total vol. 170 ml, void vol. 60 ml) was equilibrated with 0·05 M phosphate, 0·001 M EDTA (pH 6·5). Fractions (5 ml) were collected and analysed subsequently for their carbohydrate and protein content.

Automated analyses. Total carbohydrate was measured in a system similar to that described by Fuller,[20] using sulphonated α-naphthol reagent[50] and measuring absorbance at 550 nm. Results were calculated in terms of a glucose standard. Polyuronide was estimated in a system with a flow pattern similar to that described by Stacey and Somers.[19] The reagents, their corresponding pump tube i.d.s, and nominal flow rates were as follows: 0·025 M sodium tetraborate in 98% H_2SO_4, 2·794 mm Acidflex, 2·76 ml/min; air, 1·143 mm Tygon, 0·7 ml/min sample, 0·889 mm Tygon, 0·42 ml/min; 0·1% carbazole in 90% (w/v) H_2SO_4 (freshly prepared), 1·651 mm Acidflex, 1·19 ml/min; colorimeter return, 2·794 mm Acidflex, 2·76 ml/min. Absorbance at 520 nm was measured and related to a galacturonic acid standard. In analysis of discrete samples, a rate of 10 samples/hr was used. Protein was either estimated manually as described by Lowry *et al.*[21] or by using the Technicon automated version.

When necessary automated dilutions of the sample stream were incorporated in these systems to obtain the desired detection range.

Hydrolysis and estimation of monomers. Fractions derived from DEAE-cellulose columns in the phosphate form and from gel filtration were dialysed, concentrated by rotary evaporation at 40° and freeze dried. Fractions derived from DEAE-cellulose in the borate form were passed through a column (20 × 1·5 cm) of Amberlite IR 120 (H^+) and evaporated to dryness at 40°; then they were repeatedly evaporated with small additions of MeOH. Dry fractions were dissolved in a suitable vol. of H_2O and this solution used to provide samples for hydrolysis and uronic acid estimation (as above). For estimation of neutral monosaccharide components samples in a total vol. of 1·4 ml were heated at 121° with 0·05 ml 72% (w/w) H_2SO_4 for 60 min[20] and neutralized with 5 ml 10% *N,N-bis*-octylmethylamine in $CHCl_3$.[9] The hydrolysate and H_2O washings of the amine solution were washed with $CHCl_3$ and evaporated to dryness at 40° after which samples were dried over P_2O_5 *in vacuo* overnight. A mixture of anhyd. pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1) was added to each sample allowing at least 1 ml for 10 mg sugar. The samples were shaken vigorously for 15 min and allowed to react for a further 1 hr. They were injected as 0·5 μl samples into a Pye series 104 gas chromatograph with a 2-m column of 2% SE 33 on silanized, acid washed Chromosorb 'G', with N_2 as carrier gas (flow rate 40 ml/min); the temp. was programmed from 130° to 200° at 4°/min while the flame ionization detector operated at a temp. of 250°. Standards of α-D-Glucose were run simultaneously; R_ts and response (in terms of peak ht) for unknowns were interpreted in terms of this working standard and results obtained previously for aqueous equilibrium mixtures of standard carbohydrates.[51,52,53] For estimation of amino acids and hydroxyproline, 0·5 ml sample solution and 0·5 ml

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[50] A. W. DEVOR, *J. Am. Chem. Soc.* **72**, 2008 (1950).
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[53] P. M. HOLLIGAN, *New Phytol.* **70**, 271 (1971).

conc. HCl were heated in a sealed tube at 110° under N_2. The hydrolysate was filtered (glass fibre paper), evaporated to dryness at 40°, and dissolved in a suitable vol. of H_2O. Total amino acids were estimated with ninhydrin in terms of a leucine standard.[54] Hydroxyproline was estimated by the method of Leach;[55,56] it was found convenient to reduce the volume of sample and reagents to one-quarter or one-tenth (final vol. 2·5 or 1·0 ml, respectively). The absorption spectrum of the reaction products was recorded from 500 to 600 nm.

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