

## POLYSACCHARIDE CHANGES IN CELL WALLS OF RIPENING APPLES

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**Key Word Index**—*Malus sylvestris*; Rosaceae; fruit; ripening; cell wall composition; polysaccharides; glycoproteins; uronic acids.

**Abstract**—Changes in the polysaccharide composition of apple fruits ripening on and off the tree were compared. Polysaccharide fractions defined by their method of extraction were analysed colorimetrically, and the monosaccharide composition of total acetone insoluble material was analysed. Neutral carbohydrate associated with pectic extractives decreased; correspondingly galactose residues were lost in detached fruit, while galactose and arabinose residues were lost in fruit on the tree. Decreases in hemicellulose were correlated with losses of wall glucan; xylose contents did not change. Soluble polyuronide increased especially in detached fruit. DEAE-cellulose chromatography showed that this polyuronide was free from neutral sugar residues. Amounts of soluble neutral polysaccharides and glycoproteins did not change during fruit ripening.

### INTRODUCTION

MANY investigations deal with changes in solubility and properties of pectic polysaccharides in apple fruits during ripening.<sup>1-3</sup> More recently the monosaccharide composition of hydrolysates of fractions extracted sequentially from apple cell walls has been analysed.<sup>4,5</sup> Since extraction schemes always involve degradative treatments quantitative recovery of carbohydrate from extractives is difficult. In the present paper results are given for analyses of whole cell wall hydrolysates. Colorimetric analysis of the distribution of carbohydrate between cell wall fractions is used as a guide to changing linkages between cell wall components. DEAE-cellulose column chromatography<sup>6</sup> is used to characterise and estimate soluble polysaccharides and glycoproteins.

### RESULTS

#### *Changes in Cell Wall Fractions*

The scheme for extraction of polysaccharide fractions from acetone insoluble residues was as follows: *A*, 0.1 M Tris HCl (pH 7.2) 20°, soluble pectic polysaccharides; *B*, chloral hydrate, 20°, starch; *C*, 0.1 M Na<sub>2</sub>HPO<sub>4</sub> 0.05 M EDTA (pH 6.9), 95°, insoluble pectic polysaccharides; *D*, 4 M NaOH, 20°, hemicelluloses; *E*, 72% (w/w) H<sub>2</sub>SO<sub>4</sub>, 20°, hemicellulose and cellulose (see Experimental for details).

Soluble polyuronide increased over twofold by the end of September in detached fruit but remained at a low level on the tree. Neutral polysaccharide and polyuronide in extract *C*

<sup>1</sup> DOESBURG, J. J. (1965) *I.B.V.T. Commun.* No. 25.

<sup>2</sup> HULME, A. C. (1958) *Adv. Food Res.* **8**, 297.

<sup>3</sup> JOSLYN, M. A. (1962) *Adv. Food Res.* **11**, 1.

<sup>4</sup> NELMES, B. J. and PRESTON, R. D. (1968) *J. Exp. Botany* **19**, 496.

<sup>5</sup> TAKAVOLI, M. and WILEY, R. C. (1968) *Proc. Am. Soc. Hort. Sci.* **92**, 780.

<sup>6</sup> KNEE, M. (1970) *J. Exp. Botany* **21**, 651.

decreased steadily in both types of fruit. The carbohydrate content of extracts *D* and *E* decreased steadily in detached fruit, but in fruit ripening on the tree, carbohydrate in extract *D* remained essentially constant, while that in extract *E* showed an initial decrease and then remained constant. The starch content of extract *B* decreased more rapidly in detached fruit than in fruit ripening on the tree (Table 1).

TABLE 1. STARCH CONTENTS OF CORTICAL TISSUE OF APPLES RIPENING ON AND OFF THE TREE

Date	On tree		In store	
	Starch content (mg/g)	Av. wt/apple (g)	Date	Starch content (mg/g)
4 Sept.	23.5	144	11 Sept.	12.7
14 Sept.	14.9	180	21 Sept.	7.5
24 Sept.	9.3	196	30 Sept.	2.2
2 Oct.	9.1	195	14 Oct.	0.5
15 Oct.	2.3	193		

#### *Changes in Monosaccharide Components*

Results of analysis of hydrolysates of acetone insoluble material from apple samples are shown in Table 2. The amounts of glucose found fell more rapidly in detached fruit than in fruit on the tree. Subtraction of the starch values from total anhydro-glucose contents gives an indication of the levels of wall glucan; this too decreased more extensively and consistently in detached fruit.

Other than glucose the monosaccharide which changed most conspicuously was galactose, which decreased to about the same extent in fruit on and off the tree. Arabinose apparently remained at higher levels in detached fruit than on the tree. Xylose showed slight increases in both types of fruit. Other monosaccharides such as rhamnose and mannose could be detected as minor components on the chromatograms but their peaks overlapped with major components and they could not be estimated reliably. The total uronic acid values also shown in Table 2 are the sums of amounts detected in extracts *A* and *C* during wall fractionation. These indicate a virtually constant level in detached fruit and a slowly decreasing level in fruit on the tree.

These results are expressed in terms of fresh weight of cortical tissue at the time sampling. Correction for a small weight loss during storage would have a negligible effect on the results for stored fruit. Increase of weight of fruit ripening on the tree diluted cell wall components (Table 1). The results calculated in terms of initial fresh weight still show a substantial decrease in arabinose and galactose residues while total uronic acid remains more constant.

#### *Changes in Soluble Polysaccharides*

Column chromatography of polysaccharides in extract *A* revealed three main components in each sample. These were, in order of elution, neutral polysaccharides, glycoprotein and

polyuronide.<sup>7</sup> Responses in the three analytical systems showed that the neutral polysaccharide fractions were always free from polyuronide but contained small amounts of protein

TABLE 2. ANHYDRO SUGAR CONTENTS OF HYDROLYSATES OF ACETONE INSOLUBLE RESIDUES FROM APPLES RIPENING ON AND OFF THE TREE

Date	Anhydro sugar content (mg/g)					Galacturonic acid*
	Arabinose	Xylose	Galactose	Glucose	Glucose-starch	
On tree						
4 Sept.	2.58	0.52	3.81	32.8	9.3	3.53
14 Sept.	3.26	0.85	3.51	26.7	11.8	2.87
24 Sept.	2.50	1.04	2.00	20.1	10.8	2.61
2 Oct.	2.21	0.86	1.58	15.4	6.3	2.78
15 Oct.	1.54	0.73	0.86	9.8	7.5	2.50
In store						
11 Sept.	2.98	1.00	4.02	22.5	9.8	4.03
21 Sept.	3.66	1.03	2.88	16.0	8.5	3.69
30 Sept.	3.76	1.16	1.59	8.6	6.4	3.66
14 Oct.	2.66	1.06	1.29	6.3	5.8	3.29

\* Sum of polyuronide found in extracts A and C.

which was probably not linked to the carbohydrate.<sup>7</sup> The glycoprotein fractions also contained virtually no polyuronide but had appreciable quantities of protein whose elution profile was not obviously related to the carbohydrate profile, though the two components are thought to be linked.<sup>7</sup> The polyuronide fractions were virtually free from neutral polysaccharide and protein at all stages of ripening.

TABLE 3. SOLUBLE POLYSACCHARIDE AND GLYCOPROTEIN LEVELS MEASURED BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF EXTRACTS FROM APPLES RIPENING ON AND OFF THE TREE

Date	Neutral polysaccharide	Amount of column fraction (mg/g)		Polyuronide
		Glycoprotein As carbohydrate	As protein	
On tree				
4 Sept.	0.25	0.23	0.21	0.15
14 Sept.	0.22	0.16	0.21	0.27
24 Sept.	0.16	0.13	0.22	0.15
2 Oct.	0.22	0.19	0.18	0.25
15 Oct.	0.22	0.15	0.27	0.33
In store				
11 Sept.	0.17	0.18	0.14	0.11
21 Sept.	0.23	0.22	0.31	0.24
30 Sept.	0.17	0.16	0.16	0.71
14 Oct.	0.16	0.16	0.15	0.68

Neutral polysaccharide and glycoprotein-carbohydrate values are expressed in terms of a glucose standard, polyuronide in terms of anhydro-galacturonic acid, and protein in terms of bovine serum albumin.

The amounts of soluble components present at various stages were calculated from peak areas on these chromatograms and the results are shown in Table 3. The amounts of neutral

<sup>7</sup> KNEE, M. (1973) *Phytochemistry* 12, 637.

polysaccharide and glycoprotein recovered were considerably less than expected from analyses of the whole extracts. Presumably some acetone insoluble, but relatively low molecular weight material had been lost on dialysis. The presence of this excess carbohydrate probably distorted the polyuronide figures for the whole fraction and those shown in Table 3 should be taken as the correct values. There seemed to be no great change in levels of carbohydrate in the neutral polysaccharide or glycoprotein fractions either on or off the tree. The most conspicuous change was the great increase of polyuronide in detached apples after 21 September; this seemed to be only just beginning in fruit on the tree at the last sampling date.

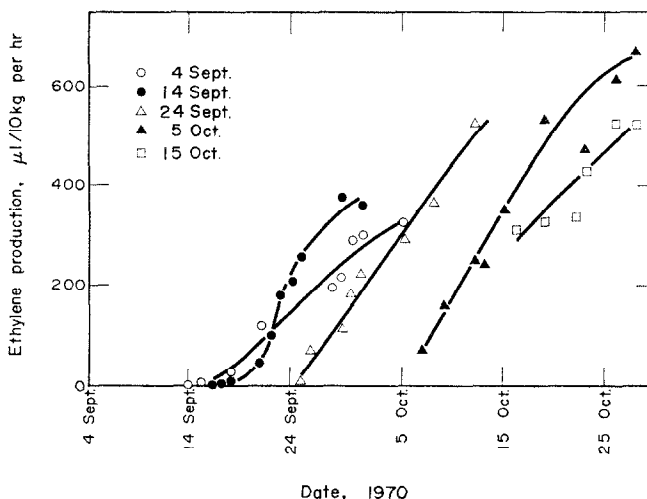


FIG. 1. ETHYLENE PRODUCTION AT 12° BY APPLES PICKED ON VARIOUS DATES.

#### *Relation of Analytical Data to Measurements of Fruit Firmness*

No measurements of fruit firmness were made in the above experimental work. In a separate experiment measurements of fruit firmness were made using a standard penetrometer with a 8 mm plunger, and at the same time polysaccharide components were measured in 20 samples of Cox's Orange Pippin apples stored under three different conditions.

Contrary to earlier suggestions<sup>5</sup> no relation was seen between starch content or neutral polysaccharide (araban and galactan) content and fruit firmness. However in common with much previous work, firmness was found to be inversely related to soluble polyuronic content with a high correlation coefficient (0.923).

#### *Ethylene Production*

Maturity of fruit samples can be judged from their rates of ethylene production (Fig. 1). The onset of production is delayed by attachment to the tree.<sup>8</sup>

#### DISCUSSION

It has been suggested that in apple<sup>7</sup> as in other plant tissues,<sup>9</sup> polysaccharides are linked to one another and to protein components to form a polymeric network in the cell wall.

<sup>8</sup> WILKINSON, B. G. (1963) *Nature* **199**, 715.

<sup>9</sup> LAMPORT, D. T. A. (1965) *Adv. Bot. Res.* **2**, 151.

During the growth of the fruit certain linkages in this structure must be broken enzymically to allow interpolation of new material. During fruit ripening and senescence bonds are broken irreversibly and this weakens wall structure. Apple fruit cell walls undergo autolytic degradation when suspended in buffer at pH 4.0–5.0 with the release of arabinose and galactose.<sup>7</sup> It has now been confirmed that apples contain  $\alpha$ -L-arabinosidase and  $\beta$ -D-galactosidase (I. M. Bartley, personal communication) whose action could preclude the re-making of bonds which stabilize wall structure by removing a cross linking component.

The insoluble polyuronide component has a high proportion of neutral sugar residues associated with it.<sup>7</sup> DEAE-cellulose chromatography revealed that the soluble component had been stripped of these residues prior to its release so that it was essentially pure polyuronide. It should be noted however that the loss of galactose and arabinose residues from the wall does not lead directly to the solubilization of polyuronide in fruits attached to the tree. The release of polyuronide must depend upon the breaking of interpolymer bonds which have yet to be characterized.

Doesburg<sup>10</sup> has suggested that the softening of apple cell walls is due to the transfer of divalent metal ions particularly calcium from the wall into the cell. Calcium has long been thought to be important as a crosslinking component between polygalacturonide chains in plant cell walls.<sup>3</sup> Rees has pointed out that it is unlikely to form a strong linkage<sup>11</sup> and has made the interesting suggestion that calcium ions may aid the packing of polygalacturonate chains by fitting neatly into a microcrystalline structure and neutralising mutually repulsive charges.<sup>12</sup> The migration of calcium ions into the cell is one process which occurs during fruit softening, but cannot be held responsible for the softening itself.

Little change in soluble components other than polyuronide was observed and it is possible that the soluble neutral polysaccharides and glycoproteins represent wall precursors rather than substances derived from cell wall breakdown. Presumably changes in the glucose content of hydrolysates, after correction for the amount of starch, represented changes in hemicellulose composition. Changes in the hemicellulose fraction (*D*) showed overall similarities to the non-starch glucan changes. The  $\alpha$ -cellulose content of apple tissue remains constant during ripening.<sup>4</sup>

Despite much work on changes in the cell wall structure of apples during growth on the tree<sup>13–16</sup> there is little information concerning changes associated with ripening and senescence; it appears that the changes are not striking.<sup>15,17</sup> If the cross linkages between wall polymers were broken during ripening one might expect a hygroscopic swelling of the wall to form a loose gel; this could account for the decrease in expressible juice as apples ripen.<sup>18</sup> An alternative explanation would be that in an unripe apple compression and shear forces break cells and release vacuolar contents, while in the ripe apple the same forces result in more cell separation so that their contents are retained. Certainly the small amount of evidence suggests that there is no marked change in wall thickness as apples ripen.<sup>4</sup>

It is of interest to compare the above data on cell wall composition with peel pigment

<sup>10</sup> DOESBURG, J. J. (1957) *J. Sci. Food Agr.* **8**, 206.

<sup>11</sup> REES, D. A. (1969) *Adv. Carbohydr. Chem. Biochem.* **24**, 267.

<sup>12</sup> REES, D. A. (1972) *Biochem. J.* **126**, 257.

<sup>13</sup> BAIN, J. M. and ROBERTSON, R. N. (1951) *Australian J. Biol. Sci.* **4**, 75.

<sup>14</sup> DENNE, M. P. (1963) *N.Z. J. Botany* **1**, 265.

<sup>15</sup> SKENE, D. S. (1966) *Ann. Bot. N.S.* **30**, 493.

<sup>16</sup> SMITH, W. H. (1950) *Ann. Bot. N.S.* **14**, 23.

<sup>17</sup> TETLEY, U. (1931) *J. Pomology* **9**, 278.

<sup>18</sup> PHILLIPS, W. T., POAPST, P. A. and RHEAUME, B. J. (1955) *Proc. Am. Soc. Hort. Sci.* **65**, 214.

changes in the same fruit.<sup>19</sup> Anthocyanin, carotenoid and chlorophyll changes were not retarded and were more pronounced in fruit ripening on the tree by comparison with fruit in store. Subsequent unpublished work has shown that xanthophyll esters, which are characteristic pigments of the ripe fruit, can be found in peel extracts nearly 1 month before the occurrence of the climacteric on the tree. Loss of galactose residues from the cell wall seemed to be similar in fruit on and off the tree, but the increase of soluble polyuronide was delayed by three to four weeks on the tree by comparison with stored fruit. This delay is comparable to that observed in ethylene production.

## EXPERIMENTAL

*Source of fruit and cell wall preparation.* The fruit was the same as used in previous work.<sup>19</sup> A bulk sample of Cox's Orange Pippin apples was picked on 4 September 1970 and sampled after periods of storage at 12°. Further fruit samples were picked from the same trees up to 15 October. Cortical slices (excluding peel) were cut from opposite sides from stem to calyx in segments representing *ca.* 0.125 of each 10 apples. These were disintegrated in acetone (4 ml/g tissue) at 2° in a blender. The slurry was filtered on Whatman No. 541 paper, the residue washed with 400 ml 80% acetone and resuspended in 2% Teepol.<sup>7</sup> This suspension was stored at -20°.

*Extraction and estimation of polysaccharide fractions.* Octanol (1 ml) was added to each of the thawed Teepol suspensions. A sample (10 ml) of each suspension was added to 10 ml of 0.2 M Tris-HCl 0.1 M EDTA (pH 7.2) at 20°. After 30 min the suspension was filtered and the first filtrate (extract *A*) retained. The residue was resuspended in H<sub>2</sub>O to a wt of 10 g and 35 g chloral hydrate dissolved in it. This suspension was allowed to stand at 20° for 48 hr; then 25 ml H<sub>2</sub>O was added and the suspension was filtered, the residue being washed with H<sub>2</sub>O (extract *B*). The residue was resuspended in H<sub>2</sub>O to 10 g and 10 ml 0.2 M Na<sub>2</sub>HPO<sub>4</sub> 0.1 M EDTA (pH 6.9) was added to it. After heating at 95° for 4 hr the suspension was filtered and washed with H<sub>2</sub>O (extract *C*). The residue was resuspended to 10 g in H<sub>2</sub>O and 10 ml 8 M NaOH was added to it. After incubating at 20° under N<sub>2</sub> for 2 hr the suspension was filtered and the residue washed 4 × H<sub>2</sub>O. Cooled filtrates and washings were neutralized with 5 ml HOAc (extract *D*). The residue was washed with EtOH and Et<sub>2</sub>O and dried before being dissolved in 2 ml 72% (w/w) H<sub>2</sub>SO<sub>4</sub> for 24 hr (extract *E*). Carbohydrate in extracts *A*, *C*, *D* and *E* was estimated by an automated version<sup>20</sup> of the sulphonated  $\alpha$ -naphthol reaction.<sup>21</sup> Polyuronide in extracts *A* and *C* was estimated by an automated procedure<sup>7</sup> based on the carbazole-sulphuric method.<sup>22</sup> Starch in extract *B* was estimated in a 2 ml sample by adding 1 ml 0.003% I<sub>2</sub> 0.03% KI in 0.05 M HCl and measuring absorbance at 620 nm, in relation to an AnalaR starch standard. These analyses were carried out on duplicate fruit preparations for each sampling date.

*Hydrolysis and estimation of monosaccharides.* Acetone (80 ml) was added to 20 ml of the Teepol suspension. This was filtered after 16 hr, the residue was washed with 80% acetone and pure acetone and allowed to dry. Samples of dry residue were dissolved in 0.2 ml 72% (w/w) H<sub>2</sub>SO<sub>4</sub> for 24 hr and diluted with 5 ml H<sub>2</sub>O. This solution was autoclaved at 121° for 60 min<sup>23</sup> and neutralized with BaCO<sub>3</sub>. Glucose in the hydrolysate was destroyed and estimated using a crude *Aspergillus niger* glucose oxidase preparation (Koch-Light Laboratories Ltd., Colnbrook, Bucks).<sup>24</sup> Gluconate and inorganic ions were removed by passing the hydrolysate down columns (10 × 1 cm) of Amberlite 1R 120 (H<sup>+</sup> form) 1RA 400 (CO<sub>3</sub><sup>2-</sup> form). The deionized hydrolysate was evaporated to dryness at 40° and dried (P<sub>2</sub>O<sub>5</sub>, 16 hr) and monosaccharides were estimated as their trimethylsilyl derivatives.<sup>7</sup> These analyses were carried out on duplicate fruit preparations for each sampling date.

*DEAE-cellulose column chromatography.* A sample (10 ml) of extract *A* was dialysed against H<sub>2</sub>O for 48 hr and pumped onto a column (5 × 1 cm) of Whatman DE 52 equilibrated with 0.005 M phosphate 0.001 M EDTA (pH 6.5) and eluted with this buffer at a flow rate of 3.25 ml/min. Then a linear gradient formed from 50 ml 0.005 M buffer and 50 ml 0.5 M phosphate, 0.001 M EDTA (pH 6.5) was applied. Carbohydrate and polyuronide were measured continuously by automated methods as described above. Protein concentration

<sup>19</sup> KNEE, M. (1972) *J. Exp. Botany* **23**, 184.

<sup>20</sup> FULLER, K. W. (1967) *Automation in Analytical Chemistry*, Vol II, p. 57, Technicon Symposium 1966, Mediad, New York.

<sup>21</sup> DEVOR, A. W. (1950) *J. Am. Chem. Soc.* **72**, 2008.

<sup>22</sup> BITTER, T. and MUIR, H. M. (1962) *Anal. Biochem.* **4**, 330.

<sup>23</sup> SAEMAN, J. F., MOORE, W. E., MITCHELL, R. L. and MILLET, M. A. (1954) *Tappi* **37**, 336.

<sup>24</sup> MORRISON, I. M. and PERRY, M. B. (1966) *Can. J. Biochem.* **44**, 1115.

was measured using the Technicon version of the method of Lowry *et al.*<sup>25</sup> These analyses were carried out on a single fruit preparation for each sampling date.

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<sup>25</sup> LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.