centrations allowed tetrathionate oxidation to proceed without the addition of thiosulphate.

5. Tetrathionate was metabolized anaerobically with the formation of thiosulphate, and small amounts of the latter were present in reaction mixtures that were actively oxidizing tetrathionate.

The author thanks Dr M. Santer for helpful criticism and Mrs L. Calis for technical assistance. This work was supported, in part, by a grant from the Research Corporation.

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Biochem. J. (1964) **90**, 646

Uronic Acid Constituents of Oat-Coleoptile Cell Walls

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(Received 7 June 1963)

The role of cell-wall polysaccharides containing uronic acid, and specifically of pectin, in the growth of plant cells has been emphasized in investigations on growing oat-coleoptile tissue (Ordin, Cleland & Bonner, 1955, 1957; Albersheim & Bonner, 1959; Jansen, Jang, Albersheim & Bonner, 1960). It seems to be widely assumed that the uronic acid content of primary cell-wall materials is due simply to their content of pectin; for example, Jansen *et al.* (1960) reported detecting only galacturonic acid by a pectinase extraction of oat-coleoptile cell-wall material that was said to have brought all uronic acid into solution. However, Bishop, Bayley & Setterfield (1958) obtained evidence indicating the presence of glucuronic acid as well as galacturonic acid.

In the present work we have investigated the uronic acid composition and content of oat-coleoptile cell-wall material in some detail and have found it to be considerably more complex than has hitherto been recognized.

RESULTS

Isolation and identification of uronic acid components. Uronic acids were isolated from acid hydrolysates of polysaccharides of oat-coleoptile

cell walls by adsorption on Dowex 1, followed by chromatography in two solvents, by means of which not only could free glucuronic acid and galacturonic acid be separated from one another but a number of other components could be isolated. The acids are listed in Table 1, and the yields obtained in various experiments are shown in Table 2.

In most cases whole coleoptile cell walls were extracted with hot 0.05N-sulphuric acid on the expectation that, since hot dilute mineral acid has been said to extract the 'protopectin' of this cell-wall material (Ordin *et al.* 1955, 1957), the bulk of the uronic acid should be obtained by this solvent. In one case we extracted and examined separately the fraction soluble in hot water ('pectin'). In another experiment we hydrolysed hemicellulose B, prepared from oat-coleoptile cell-wall material in the usual manner.

Most of the products appeared to be hydrolysis-resistant aldobiouronic acids and other acidic oligosaccharides and were identified as follows: each was subjected to further hydrolysis with sulphuric acid, followed by separation of acidic from neutral products with Dowex 1. Sugar and uronic acid products were identified by chromatography (Table 1), by a procedure that distinguished

Table 1. Properties of uronic acid constituents of oat-coleoptile cell walls

Compound no.	R_{xylose}		Colour with aniline phthalate	Yield on hydrolysis			Reducing value		Proposed structure
	Solvent (a)	Solvent (b)		Sugar	Acid		Equiv./mole of uronic acid residues	Percentage decrease in $Ca(OH)_2$	
1A	0.18	0.24	Orange	None	GalA		6.0	76	GalA-(1→4?)-GalA
1B	0.18	0.40	Brown	Gal	GlcA		15.5	—	GlcA-Gal
1C	0.19	0.47	Red	Xyl	Compound 2A, GlcA		10.4	90	GlcA-Xyl-Xyl
2A	0.38	0.49	Red	Xyl	GlcA		2.2	0	GlcA-(1→2)-Xyl
2B	0.38	0.96	Red-orange	Xyl*	Compounds 4, 5*		—	—	MeGlcA-Xyl-Xyl
3A	0.53	0.79	Orange-brown	Rha	GalA		2.5	52	GalA-(1→2)-Rha
3B	0.63	0.62	Orange	—	—		13.0	—	GalA
3C	0.68	0.50	Orange	—	—		13.7	—	GlcA
4	0.84	1.07	Red-orange	Xyl	Compound 5		1.7	15	4-O-MeGlcA-(1→2)-Xyl
5	1.37	1.01	Red-orange	—	—		9.5	—	4-O-MeGlcA

* These results are for hydrolysis of the compound of similar properties obtained from maize-cob hemicellulose B; coleoptile compound 2B was not obtained in amounts sufficient to attempt hydrolysis.

glucuronic acid and galacturonic acid from one another and from any remaining aldobiouronic acids.

Identification of the major components was substantiated by conversion into methyl ester methyl glycosides, followed by reduction with sodium borohydride, acid hydrolysis and chromatographic identification of products. The isolated galacturonic acid gave galactose; glucuronosylxylose gave xylose and glucose; and methylglucuronosylxylose gave xylose and a fast-travelling hexose that behaved identically, in two solvents, with an authentic sample of 4-*O*-methylglucose.

These observations confirm the report of Bishop *et al.* (1958) that glucuronic acid (detected by reduction to glucose) is present in oat-coleoptile cell walls.

It soon became apparent that the pattern of uronic acids obtained from oat coleoptiles was similar to that from hemicelluloses of straw, wheat bran, maize cobs, etc. Therefore we prepared hemicellulose B from maize cobs and isolated the aldobiouronic acids given by acid hydrolysis of it, by the procedure of Whistler, Conrad & Hough (1954), slightly modified.

The principal uronic acids of maize-cob hemicellulose B have been characterized as 4-*O*-methylglucuronosyl-(1→2)-xylose (Whistler *et al.* 1954), glucuronosyl-(1→2)-xylose [Whistler & Hough, 1953; glucuronosyl-(1→4)-xylose was also considered to be present but was not separated from the former] and glucuronosyl-(1→4)-xylosyl-(1→4)-xylose (Whistler & McGilvray, 1955). These products were found to resemble closely the major aldobiouronic acids and aldotriouronic acids of oat-coleoptile polysaccharides, except for the galacturonic acid derivatives.

The 4-*O*-methylglucuronosylxylose from oat coleoptiles also behaved identically, in solvents (a) and (b) (see Experimental section), with an authentic sample of 2-*O*-(4-*O*-methyl- α -D-glucopyranosyluronic acid)-D-xylose kindly supplied by C. T. Bishop (see Adams & Bishop, 1956).

The positions of linkages indicated for aldobiouronic acids in Table 1 are based on the following considerations.

(1) Compounds 2A, 3A and 4, and the maize-cob aldobiouronic acids that are known to have (1→2)-linkage, showed unusually low reducing values compared with free sugars or uronic acids (Tables 1 and 3). This property is characteristic of oligosaccharides linked through the 2-position of the reducing residue (Bailey, Barker, Bourne & Stacey, 1955; Barker, Bourne, Grant & Stacey, 1956). The remaining acidic oligosaccharides from coleoptiles, which showed considerably greater reducing value, are presumably linked through a different position of the reducing residue.

Table 2. *Yields of uronic acid components of oat-coleoptile cell walls*

Yields are expressed as $\mu\text{g.}$ of uronic anhydride (not including weight of sugar residues if present), corrected for portions of the sample used on guide strips. Details of extraction are given in the text.

Expt. no.	1	2	3	4
	Whole cell-wall material			Hemicellulose B
Wt. of starting material (mg.) ...	428	404	697	397
Extraction solvent	Hot 0.05N- H_2SO_4	Hot 0.05N- H_2SO_4	Hot water	See text
Duration of hydrolysis in N- H_2SO_4 (hr.)	6	4	3	6
GalA	780	245	135	1 610
GalA-GalA	90	625	85	320
GalA-Rha	*	*	*	1 420
GlcA	†	30	†	575
GlcA-Xyl	2 260	2 770	100	7 530
GlcA-Xyl-Xyl	†	140	†	85
GlcA-Gal	555	125	25	240
MeGlcA	*	*	†	*
MeGlcA-Xyl	*	2 740	†	3 390
MeGlcA-Xyl-Xyl	*	†	†	†
Total	3 685	6 675	345	15 170
Percentage of starting material	0.86	1.65	0.05	3.82

* This compound was not isolated although it was probably present.

† A significant amount was not detected.

‡ Included in figure for GlcA-Xyl.

Table 3. *Reducing values of known compounds*

Determinations by copper reduction, as explained in the text.

	Reducing value (equiv./mole)
Glucuronosylxylose*	2.37
GlcA-Xyl-Xyl*	9.3
Methylglucuronosylxylose*	1.60
MeGlcA-Xyl-Xyl*	10.5
Glucuronosylgalactose*	24
Xylose	18.6
Galactose	16.1
Glucose	23.4
Maltose	21.7
3-O-Methylglucose	9.6
2,3,4,6-Tetramethylglucose	1.01
Glucuronic acid	15.4
Galacturonic acid	14.6

* Compounds isolated from maize-cob hemicellulose B.

(2) Sugars bearing a glycosidic or ether substitution in the 2-position are resistant to degradation by alkali, whereas sugars substituted elsewhere decompose in alkali, with loss of reducing power (Kenner & Richards, 1956; Whistler & Corbett, 1955; Whistler & Richards, 1958). Coleoptile aldobiouronic acids 2A and 4 were stable to alkali, whereas 1A and 1C were unstable (Table 1). Compound 3A appeared to be unstable, even though its reducing value suggested a (1→2)-linkage.

(3) Compounds 2A, 3A and 4 did not react with triphenyltetrazolium chloride (method of Wallenfels, 1950), which detects all except 2-O-substituted reducing sugars (Barker *et al.* 1956; Avigad, Zelikson & Hestrin, 1961; Roudier & Eberhard, 1960). Compounds 1C and 2B gave a positive reaction with this reagent.

The major coleoptile aldobiouronic acids (glucuronosylxylose and 4-O-methylglucuronosylxylose), and the related aldotriouronic acids containing two xylose residues, indicate the presence in the cell walls of glucuronoxylans, which are typical hemicellulose components of a variety of plant-cell-wall materials (see Whistler & Sanella, 1958; Aspinall, 1959).

Glucuronosylgalactose (compound 1B) is a well-known constituent of various gums, including gum arabic (see Hirst, 1958), and bacterial capsular polysaccharides. It was obtained from pine-wood hemicellulose by Roudier & Eberhard (1960), and although it does not appear to have been isolated from the cell walls of other higher plants, Roudier & Eberhard (1960) mention that they have detected it in a variety of cell-wall materials. For unknown reasons the yield of this component from coleoptile cell walls varied greatly (Table 2). It was not completely separable from compound 1C, and the best preparation was obtained after relatively long hydrolysis, after which the amount of the aldotriouronic acid 1C was probably insignificant.

Besides free galacturonic acid, two compounds (1A and 3A) that gave galacturonic acid on hydrolysis were isolated. Compound 1A yielded no sugar; its reducing value per mole of uronic acid was about half that of free galacturonic acid. These data and the chromatographic behaviour of 1A suggest that it is digalacturonic acid, which is known from partial hydrolysis of pectic acid (Jermyn & Tomkins, 1950; Jones & Reid, 1954) and from certain other polysaccharides (Hirst, Hough & Jones, 1949).

Compound 3A gave galacturonic acid and rhamnose on hydrolysis and is probably galacturonosyl-(1→2)-rhamnose. This aldobiouronic acid has previously been obtained from various gums (e.g. Laidlaw & Percival, 1949; Aspinall, Hirst & Matheson, 1956; Whistler & Conrad, 1954), and was isolated from pine-wood hemicellulose by Roudier & Eberhard (1960).

Uronic acid content of cell-wall material. According to Jansen *et al.* (1960), determinations by the carbazole method on pectinase extracts of oat-coleoptile cell-wall material indicated a uronic anhydride content of about 5%, or about 3.5% according to Albersheim & Bonner (1959). These determinations apparently were not corrected for the interfering effect of sugars in the extracts, even though Jansen *et al.* (1960) reported that sugars were detected in a pectinase extract. Bishop *et al.* (1958), on the other hand, reported that less than 1% of uronic acid (including both glucuronic acid and galacturonic acid) could be obtained by complete hydrolysis of material from oat-coleoptile cell wall. Albersheim & Bonner (1959) actually isolated as galacturonic acid less than 8% of the uronic acid indicated by their carbazole determinations, an amount that falls in the same range as the finding of Bishop *et al.* (1958).

The yields of uronic acids in the more complete isolations reported here were nearly 2% of the whole cell-wall material. Recovery was expected to be incomplete, because of losses during manipulations and because hydrolysis of aldobiouronic acids is characteristically attended by decomposition.

To compare our observations more directly with previous determinations of uronic acid content, we subjected one sample of material from oat-coleoptile cell walls to extraction with hot dilute mineral acid, followed by extraction with pectinase, and a second sample to extraction in the reverse order. The uronic acid and sugar constituents of each extract were resolved by chromatography and determined (Table 4).

The principal uronic acid in pectinase extracts was free galacturonic acid, although when pectinase was used as the first extractant small amounts of free glucuronosylxylose and 4-O-methylglucurono-

sylxylose were detected before acid hydrolysis of the extract, showing that the pectinase preparation had a slight hemicellulase activity. After pectinase extraction, large amounts of aldobiouronic acids were obtained by extraction with hot acid. When hot acid was used as the first extractant, the usual proportions of uronic acids were obtained, aldobiouronic acids being the major components, but a large amount of galacturonic acid was obtained by subsequent extraction with pectinase.

It is apparent that, contrary to what would be expected from the previous designation of the material extracted with hot dilute mineral acid as 'protopectin', most of the polysaccharides that contain galacturonic acid are not extracted by this solvent. Hot dilute acid removes principally uronic acids that originate from hemicelluloses; pectinase may be assumed to remove pectin (polygalacturonic acid), but one may ask whether pectinase also removes galacturonic acid from other polysaccharides, such as that which yields galacturonosylrhamnose on acid hydrolysis, if this aldobiouronic acid is not a component of a polygalacturonic acid chain.

We think, however, that most of the galacturonic acid that is obtainable from the material is probably derived from polygalacturonic acid. In support of this view is the observation that the yield of digalacturonic acid decreased and the yield of free galacturonic acid increased with longer hydrolysis of hot dilute acid extracts (Table 2).

It might be assumed that the sugars obtained after acid hydrolysis of pectinase extracts arise from polysaccharides that become solubilized but not hydrolysed when polygalacturonic acid chains are hydrolysed and removed by pectinase. However, Perila & Bishop (1961) observed strong hydrolytic activity of a pectinase preparation towards a glucomannan. In any case, loss in weight of cell-wall material upon pectinase treatment is much greater than the amount of pectin extracted.

To assess recovery of uronic acid in the pectinase and hot-acid extracts we ran the carbazole reaction on the neutral fraction of each extract and on a portion of the original extract. The results (Table 4) are expressed in terms of whichever hexuronic acid is the principal component of the acidic fraction of that extract, although it may be presumed that the carbazole reaction of the neutral fraction, and part of that of the original extract, is due to sugars and possibly other substances. Recovery was about 80% except for the pectinase extraction of sample A, where much less complete recovery of galacturonic acid evidently was attained. We assume that the minimum galacturonic anhydride content is indicated by the figures for sample B.

In pectinase extracts the sugars determined in the neutral fractions accounted entirely for the

carbazole reaction given by this fraction, whereas, with hot-acid extracts, sugars did not account completely for the neutral fraction. We examined the neutral fraction for uronic acid lactones by chromatography; none was detected directly, and no uronic acids were detected after mild alkaline hydrolysis followed by adsorption of acids on Dowex 1, so it is presumed that the excess of the carbazole value is due to protein or other interfering substances.

The total uronic anhydride content of sample 2 (from recovered uronic acids) was 4.67%, of which about two-thirds was galacturonic anhydride and one-third glucuronic anhydride and 4-*O*-methylglucuronic anhydride. The carbazole reaction of initial extracts less that of neutral fractions indicates a uronic anhydride content of 6.1–6.8%. The actual content probably lies between these extremes.

By the method of decarboxylation in hot 12.5% hydrochloric acid we found a uronic acid content of 5.0% for oat-coleoptile cell-wall material. Because of uncertainties involved in the correction for sugars, this method cannot be considered very reliable at low uronic acid contents (Norman, 1939), but the results serve to confirm in an independent way the magnitude of the uronic acid content found in the chromatographic experiments and to indicate that no major uronic acid fractions remain unaccounted for.

EXPERIMENTAL

Analytical methods. Uronic acid was determined by the carbazole method (McComb & McCready, 1952) or by the sodium tetraborate-carbazole method of Bitter & Ewins (1961). The total volume of reagents was 5 ml. Standards of glucuronic acid and galacturonic acid were included in every series. Results are expressed as uronic anhydride; oligosaccharides containing galacturonic acid were estimated on the basis of the galacturonic acid standard, and those containing glucuronic acid or 4-*O*-methylglucuronic acid were estimated on the basis of the glucuronic acid standard. This assumes that the sensitivity of the carbazole reaction to uronic acids in oligosaccharides is the same as to the free uronic acids, and that sensitivity to 4-*O*-methylglucuronic acid is the same as to glucuronic acid. That this must be essentially so is indicated by the fact that the percentage of uronic anhydride found for weighed samples of aldobiouronic acids isolated from oat coleoptiles was close to the theoretical (see below).

Total uronic acid was determined by decarboxylation in 12.5% HCl at 110°, by the micro-method of Ogston & Stanier (1951). The yield of CO₂ was determined in the same way for the sugars known to compose a substantial part of the cell-wall material (Ray, 1963) and for galacturonic acid. Four determinations on oat-coleoptile cell-wall material gave a mean yield of 2.00% of CO₂; the CO₂ due to sugars was calculated as 0.76% from the sugar composition of the cell wall, so the CO₂ presumed to be due to uronic

acids is 1.24%. This indicates a uronic anhydride content of 5.0%.

Reducing value was determined by the arsenomolybdate method of Wager (1954), with a heating time of 30 min. at 100° and a final volume of 5 ml. Results were expressed in m-equiv. by comparison with the reaction of arsenomolybdate with weighed amounts of Cu₂O. Sugars were determined by the same method, after chromatographic separation, as described by Ray (1963).

Methoxyl was determined by the method of Clark (1932).

Chromatography. Whatman no. 1 paper (9 in. × 22 in. sheets) was washed with acetic acid and water as described by Ray (1963). The following chromatographic solvents were employed (descending method).

(a) Ethyl acetate–water–acetic acid–formic acid (18:4:3:1, by vol.) was used for separation of the principal aldobiouronic acids and hexuronic acids. An example of the separation of uronic acids from polysaccharides of oat-coleoptile cell walls with this solvent is shown in Fig. 1. Because of instability of this solvent mixture, reproducible results can be obtained only if the solvent is freshly made before each development. Even so there is some variation in R_{xylose} values from run to run, as others have noticed (e.g. Roudier & Eberhard, 1960). The R_{xylose} values given in Table 1 are the mean of a number of observations for each compound.

(b) The organic phase from a mixture of 100 g. of phenol, 100 ml. of water and 1 ml. of 88% formic acid was used for separation of galacturonic acid and glucuronic acid, for separation of slow-travelling components (zone 1) obtained with solvent (a) and to confirm identifications of sugars made with solvent (c).

A useful observation was that aldobiouronic acids and aldotriouronic acids containing xylose run nearly as rapidly in solvent (b) as the parent hexuronic acid, whereas they run much more slowly in solvent (a) (see Table 1).

(c) Butan-2-one–acetone–water–pyridine (150:30:20:1, by vol.) was used for identification and determination of sugars.

Cell-wall material. This was prepared from growing coleoptiles, 3–4 cm. long, of oat seedlings (*Avena sativa* var. Victory) grown for 3 days at 25°, which were exposed to weak red light during the first day and kept in darkness thereafter. The primary leaf was removed and the coleoptiles were crushed between glass plates and extracted with urea and alcohol as described by Ray (1962) except for the hemicellulose B preparation included in Table 2. In this the coleoptiles were frozen and thoroughly ground in a mortar, with water, and the cell-wall material was washed several times with water and alcohol.

The yield of cell-wall material was approx. 400 mg./1000 coleoptiles.

Extraction and hydrolysis. This section refers to Expts. 1, 2 and 3 of Table 2. The cell-wall material was extracted three times for 2 hr. with 50 ml. portions of 0.05N-H₂SO₄ in a boiling-water bath. In Expt. 3 the material was first extracted twice for 1.25 hr. with 50 ml. of water in a boiling-water bath, washed with a further 50 ml. of hot water, then extracted with hot 0.05N-H₂SO₄ as above. Extracts were filtered, evaporated to about 10 ml. at reduced pressure at about 40° in a rotary evaporator, or in Expt. 3 under an air stream at 60°. They were then made 1N with respect to H₂SO₄ and heated in a boiling-water bath for 3–6 hr. as indicated in Table 2.

The hydrolysate was put through a column containing about 8 g. of Dowex 1 (acetate form; X 10; 200–400 mesh; made from the chloride form by treatment with M-sodium acetate). Sugars were washed through with 50 ml. of water. Uronic acids were eluted by passing 80 ml. of 6N-acetic acid through the column. This was evaporated under reduced

pressure at about 40° in a rotary evaporator, and evaporated to dryness in a vacuum desiccator over P_2O_5 .

Preparation and hydrolysis of oat-coleoptile hemicellulose B. The cell-wall material was extracted three times for about 8 hr. with dimethyl sulphoxide at room temperature (about 23°), then three times with 0.25% ammonium oxalate-oxalic acid in a boiling-water bath for about 8 hr., and finally three times with 4N-KOH under N_2 at room temperature for a total of 24 hr. The alkali extract was brought to pH 6 with acetic acid, and the precipitate (hemicellulose A) was removed by centrifugation. The supernatant was diluted with 4 vol. of ethanol, and the precipitated hemicellulose B was washed with alcohol and dried (yield, 39% of holocellulose).

A sample of hemicellulose B (397 mg.) was dissolved in 24 ml. of $N-H_2SO_4$ and heated in a boiling-water bath to effect hydrolysis. Uronic acids were obtained from the hydrolysate as described above.

Isolation of uronic acids from hydrolysates. The uronic acids obtained as described above were dissolved in 6N-acetic acid and applied as a streak along the starting line of one or more chromatogram sheets. Appropriate portions (about 2% of the fraction soluble in hot dilute acid, or 10% of the fraction soluble in hot water) were applied at either end of the streak to provide reference strips. Chromatograms were developed with solvent (a) for 20–24 hr. Reference strips sprayed with aniline hydrogen phthalate (Partridge, 1949) showed four major zones (numbered 1–4 in Table 1) and one minor zone (zone 5). 3C was distinguishable as a separate minor zone, and was isolated at this stage in Expt. 4 (Table 2) but not in the other experiments. Strips corresponding to the different zones were eluted with water.

Eluates of zones 1–4 were chromatographed a second time in solvent (a). The zones were eluted into tared weighing bottles, dried *in vacuo* over P_2O_5 and weighed.

Zones 1 and 3 were then chromatographed with solvent (b) for 44 hr. (zone 3) or 60 hr. (zone 1). Each was separated into three zones, designated A, B and C in Table 1. Zones 3A and 3B corresponded in position to glucuronic acid and galacturonic acid respectively.

Zones 2 and 4 behaved as single compounds in solvent (b), except in Expt. 3 (Table 2) where a minor amount of a fast-travelling component (2B) was obtained from zone 2. The entire samples of compounds 2 and 4 were not chromatographed in solvent (b). (Compound 2, $[\alpha]_D^{25} + 88^\circ$ [c 0.93 in water], 47% uronic anhydride; calc. for glucuronosylxylose, 54%. Compound 4, $[\alpha]_D^{25} + 102^\circ$ [c 0.36 in water], 49% uronic anhydride; calc. for methylglucuronosylxylose, 52%.)

In Expt. 1 (Table 2) solvent (b) was used in the primary separation. In this a sharp separation between compounds 1B and 2 (including any 1C) was obtained. However, in Expt. 3, zones 1B and 1C tended to streak in solvent (b) and a sufficient separation between them for complete isolation was not achieved in two chromatographic trials, so they were finally determined together.

Hydrolysis of isolated uronic acid derivatives. Compounds suspected of being oligosaccharides (0.5–1 mg.) were treated with $N-H_2SO_4$ in a closed tube for 12 hr. in a boiling-water bath; neutral and acid fractions were obtained from the hydrolysate with Dowex 1 (acetate form) as described above, and these fractions were evaporated to dryness at reduced pressure. Sugars in the neutral fraction

Fig. 1. Chromatogram developed with solvent (a), showing separation of uronic acid components of acidic fraction of hydrolysates of oat-coleoptile hemicellulose B (centre) and hemicellulose A (right). Reference compounds (at left): a, galacturonic acid; b, glucuronic acid; c, xylose. Starting line at top. Numbered zones correspond to compound numbers in Table 1. Compound 5 (R_{xylose} 1.37) had run off the paper.

were identified by chromatography in solvents (b) and (c). Uronic acids were chromatographed first in solvent (a) with guide strips carrying glucuronic acid, galacturonic acid, glucuronosylxylose and 4-*O*-methylglucuronosylxylose (from maize-cob hemicellulose B, see below). By reference to the guide strips, the portions of the paper containing any glucuronic acid or galacturonic acid (these two acids not being sufficiently separated for unquestionable identification) from the hydrolysates were cut out. The rest of the paper was sprayed with aniline phthalate. The glucuronic acid–galacturonic acid zones were eluted with water and chromatographed with solvent (b) for 44 hr., with glucuronic acid and galacturonic acid as reference compounds. Separation of the two acids was complete and clear discrimination between them was obtained by spraying the entire paper with aniline phthalate.

With compounds 2A and 4, large amounts of the original compounds were always detected after hydrolysis, owing to their resistance to hydrolysis. With compounds 1B and 1C, a small amount of the original compound remained after hydrolysis. Residual starting material is not listed in Table 1.

Borohydride reduction. Reduction of 2 mg. samples of uronic acids was carried out by a procedure slightly modified from that of Gorin (1957). The uronic acid was converted into the methyl ester methylglycoside by refluxing for 12 hr. in 4–8% HCl in methanol; this was neutralized with Ag_2CO_3 , filtered, evaporated to dryness *in vacuo*, dissolved in 5 ml. of water, and 2 mg. of NaBH_4 in 0.2 ml. of water was added. After standing overnight at room temperature the solution was acidified to about pH 4 with acetic acid, treated with 0.5 g. of Amberlite IR-120 (H^+ form) and, after half an hour, a pinch of Norit charcoal was added, and the mixture filtered and evaporated to dryness at reduced pressure. Boric acid was removed by taking up in methanol and evaporating to dryness at reduced pressure three times. The syrupy residue was refluxed with $\text{N-H}_2\text{SO}_4$ for 8–12 hr., then neutralized and separated into acidic and neutral fractions with Dowex 1 (acetate form) as described above. Portions of the neutral fraction were chromatographed in solvents (b) and (c). The acidic fraction was chromatographed in solvent (a). In all cases the acidic fraction contained a considerable amount of the original acid and of the acidic product obtainable from it by straight hydrolysis, showing that reduction had not been complete (the yield of reduced products was estimated as about 10%).

The neutral fraction derived from coleoptile compound 3B contained a hexose that corresponded in chromatographic behaviour to galactose. From compound 2A, xylose and glucose were obtained. Compound 4 gave xylose and a fast-travelling hexose [R_{xylose} in solvent (b): unknown hexose 1.37, known 4-*O*-methylglucose 1.36, known 3-*O*-methylglucose 1.34; in solvent (c): unknown hexose 1.13, known 4-*O*-methylglucose 1.13, known 3-*O*-methylglucose 1.21]. Small amounts of sugars that behaved chromatographically, and gave colour reactions, like arabinose and rhamnose, were also detected in the product derived from compound 4, suggesting that compound 4 was contaminated by small amounts of methylglucuronosylaldobiouronic acids containing these sugars (cf. Andrews & Jones, 1954).

Action of calcium hydroxide on uronic acids. About 40 μg . of each uronic acid derivative, and of maltose and 3-*O*-

methylglucose, in 0.5 ml. of water, was gassed for 5 min. with pre-purified grade ($>99.997\%$, v/v) N_2 , and under N_2 0.5 ml. of 0.3% $\text{Ca}(\text{OH})_2$ suspension, similarly gassed, was added, giving a 0.04N solution. After 10 days at room temperature (about 23°C) each tube was opened, a pinch of Amberlite IR-120 (H^+ form) was added, the solution was decanted and the reducing value of the solution was determined and compared with the reducing value of a similar amount of untreated compound, giving results recorded in Table 1. The reducing value of alkali-treated maltose and 3-*O*-methylglucose was nil.

Uronic acids from maize-cob hemicellulose B. From commercial ground maize cobs hemicellulose B (700 g.) was prepared and hydrolysed by the procedure of Whistler *et al.* (1954). After neutralization with $\text{Ba}(\text{OH})_2$, filtration through Celite and passage through Amberlite IR-120 (H^+ form), the hydrolysate was passed through 250 ml. of Dowex 1 (acetate form), which was then washed with 2 l. of water. Uronic acids were eluted with 5 l. of 6N-acetic acid; this was concentrated under reduced pressure to a thick syrup (2 g.). This was separated into the principal components by chromatographing 150–200 mg. portions on sheets of Whatman 3MM paper for 24 hr. with solvent (a). The zones were located by cutting off and spraying the margins of the paper with aniline phthalate, and were isolated by cutting the paper into strips and eluting with water. This yielded: 0.20 g. of 4-*O*-methylglucuronosylxylose (R_{xylose} 0.80, corresponding to fraction A of Whistler *et al.* 1954; methoxyl, as percent of uronic anhydride content: found, 17.4; calc. for methylglucuronosylxylose, 17.6%); 0.26 g. of fraction C (R_{xylose} 0.35, mainly glucuronosylxylose); 0.08 g. of fraction D (R_{xylose} 0.17; similar to zone 1 obtained from oat-coleoptile polysaccharides). Small amounts of slower-travelling components were also detected. A significant amount of fraction B of Whistler *et al.* (1954) was not obtained.

By chromatography in solvent (b) fraction D was incompletely separated into two components (D1, brown with aniline phthalate, R_{xylose} 0.40; D2, red with aniline phthalate, R_{xylose} 0.45). Chromatography in solvent (b) separated about a quarter of fraction C as a fast-travelling compound (C2, R_{xylose} 0.96), the major material (C1) having R_{xylose} 0.49 (glucuronosylxylose).

A portion of each of the isolated fractions was hydrolysed as described above for compounds isolated from oat-coleoptile cell walls. Fraction A yielded xylose and a fast-travelling uronic acid (R_{xylose} 1.33 in solvent a). Fraction C1 yielded xylose and glucuronic acid. Fraction C2 gave xylose and an acid that appeared by chromatography to be the same as fraction A. Fraction D1 yielded galactose and glucuronic acid. Fraction D2 gave xylose and an acid that appeared by chromatography to be the same as fraction C1.

By reduction with NaBH_4 , fraction A yielded xylose and 4-*O*-methylglucose, and fraction C1 yielded xylose and glucose, identified chromatographically, as was to be expected from the findings of Whistler *et al.* (1954) and Whistler & Hough (1953). Fraction D2 appears to be the glucuronosylxylosylxylose previously identified by Whistler & McGilvray (1955).

Fractions C2 and D1 do not appear to have been distinguished previously in maize-cob hemicellulose hydrolysates. Fraction C2 is apparently 4-*O*-methylglucuronosylxylosylxylose, which has been obtained from other sources (Adams, 1959; Bräsch & Wise, 1956; Hamilton &

Thompson, 1957; Srivastava & Adams, 1959) and was reported to have chromatographic behaviour similar to that found in the present work. Fraction D1 appears to be glucuronosylgalactose.

Chromatographed together with coleoptile uronic acids in solvents (a) and (b) fraction A ran like coleoptile compound 4, and fraction C1 ran like coleoptile compound 2A. Fractions C2, D1 and D2 were not chromatographed simultaneously with the coleoptile compounds but their chromatographic behaviour relative to reference samples of xylose, glucuronic acid and galacturonic acid was similar to that of coleoptile compounds 2B, 1B and 1C respectively.

Analytical experiments involving pectinase. Commercial pectinase supplied by the Nutritional Biochemicals Corp., Cleveland, Ohio, was dialysed as a 2% solution against distilled water at 4° for 4 days, and insoluble material was removed by centrifugation.

For direct extraction of cell-wall material with pectinase (Expt. A, Table 4) the material was first treated with 10 ml. of 0.5% EDTA (sodium salt, pH 11.5) for 1 hr. The solution was brought to about pH 5 with acetic acid, and 0.5 ml. of dialysed pectinase solution was added. After

3 hr. the extract was decanted, the residue was washed three times with water, then with alcohol, then dried and weighed. Extract and water washings were passed through Dowex 1 (acetate form) to remove acidic products, which were eluted with 6N-acetic acid. The fraction that was not adsorbed on Dowex 1 was made 1N in H_2SO_4 and heated for 6 hr. in a boiling-water bath. This hydrolysate was separated into acidic and neutral fractions with Dowex 1 as described above.

The residue from pectinase extraction was extracted with 5 ml. of 0.05N- H_2SO_4 in a boiling-water bath for 1.5 hr., and washed twice with water. The extract and washings were made 1N in H_2SO_4 , heated in a boiling-water bath for 6 hr. and separated into acidic and neutral fractions with Dowex 1.

In Expt. B (Table 4), the cell-wall material was first extracted with hot 0.05N- H_2SO_4 as just described, and, after being dried and weighed, was extracted with 5 ml. of 0.1% pectinase in 0.5% EDTA, pH 5.0, for 3 hr.

One-fifth of each acidic fraction was chromatographed for qualitative detection of constituents, by the procedure given in the section on hydrolysis of isolated uronic acid

Table 4. *Successive extractions with pectinase and hot dilute acid*

Losses in weight and yields of constituents are reported in $\mu g./100$ mg. of cell-wall material. Uronic acids and derivatives are expressed as uronic anhydride (not including weight of sugar residues if present); amounts of free sugars are on the anhydro basis.

Wt. of sample (mg.)	Expt. A 100.7		Expt. B 43.1	
	(1) Pectinase	(2) Hot 0.05N- H_2SO_4	(1) Hot 0.05N- H_2SO_4	(2) Pectinase
Extraction no.	...	...	...	...
Solvent	...	...	...	...
Loss in wt.	18 300	30 500	50 200	6 730
GalA	1 630	41	140	2 730
(GalA) ₂ , (GalA) ₃ *	38	60	88	113
GalA-Rha	28	39	130	—
GlcA	—	26	84	—
GlcA-Xyl	20	730	805	—
GlcA-Xyl-Xyl	—	37	79	—
GlcA-Gal	—	—	—	—
MeGlcA	23	55	45	47
MeGlcA-Xyl	59	311	341	—
Unhydrolysed†	81	—	—	68
Total uronic acid (a)	1 880	1 300	1 710	2 960
Xyl	818	4 620	8 180	898
Ara	690	4 570	7 830	57
Gal	445	1 390	980	243
Glc	4 070	3 210	4 900	1 640
Rha, Rib	43	41¶	65¶	44
Carbazole reaction of neutral fraction‡ (b)	695	1 290	1 700	262
Same, calc. from recovered sugars§	667	790	1 140	294
Carbazole reaction of original extract‡	5 080	3 000	4 310	4 500
(a) + (b)	2 575	2 590	3 410	3 220
Recovery (%)	51	86	79	72

* Pectinase extracts contained a component having R_{xylose} 0.075 in solvent (a), which we assume to be trigalacturonic acid (cf. Jones & Reid, 1954). The amount of this component was about equal to that of digalacturonic acid.

† Uronic acid found at the origin in chromatograms (solvent a) of material adsorbable on Dowex 1 immediately after pectinase extraction. On acid hydrolysis it yielded xylose, arabinose, galactose, glucuronosylxylose and galacturonic acid. It is expressed as galacturonic anhydride.

‡ Calculated as galacturonic anhydride for pectinase extractions and as glucuronic anhydride for hot 0.05N- H_2SO_4 extractions.

§ Calculated, as in the preceding note, from the sensitivity of the carbazole reaction to the various sugars relative to uronic acid (about 11% for hexoses and about 2% for pentoses).

|| Ribose.

¶ Rhamnose.

derivatives. From the remainder of each fraction the uronic acid components were isolated chromatographically as described above, the aldobiouronic acids and aldotriouronic acids isolated from maize-cob hemicellulose B, and glucuronic acid and galacturonic acid, being used as reference compounds. The constituents were determined by the sodium tetraborate-carbazole method, as shown in Table 4. In Expt. A, aldobiouronic acids were detected in small amounts, both in the free acid fraction adsorbed on Dowex 1 immediately after pectinase treatment and in the acidic fraction obtained after hydrolysis of the remaining pectinase extract. The figures listed for the pectinase extract are the sum of these two fractions. Almost all the galacturonic acid was obtained in the free acid fraction. In Expt. B, uronic acids were detected after pectinase extraction only in the fraction directly adsorbable on Dowex 1.

Portions of the sugar fractions were chromatographed in solvent (c) for qualitative detection. Separate portions containing 150–250 μ g. of sugars were chromatographed in solvent (c) by the double-development procedure and determined as described by Ray (1963). From the results the amounts of each sugar (except rhamnose) in the entire sugar fraction were calculated.

For determination of rhamnose or ribose or both the entire remainder of each sugar fraction was applied as a streak to the starting line of a chromatogram sheet and was chromatographed 24 hr. in solvent (c) along with rhamnose markers. After removal and spraying of the guide strips the portion of the paper containing rhamnose of the sample was cut out and eluted, together with a blank piece of similar size. The determination was performed on a fraction of each rhamnose sample, and the remainder was rechromatographed with solvents (b) and (c). A sugar having the same mobility and colour reaction as authentic rhamnose was detected in samples A2 and B1. The sugar found in samples A1 and B2 had the same mobility and colour reaction as ribose.

As a blank, 10 ml. of EDTA containing 0.5 ml. of pectinase solution was taken through the procedure described for pectinase extracts. No significant amounts of either sugars or uronic acids were detected.

To determine to what extent the extraction of polysaccharides in the pectinase procedure might be due to the treatment with alkaline EDTA, a sample of coleoptile-cell-wall material was treated with alkaline EDTA as described, then washed with water and ethanol and reweighed. The loss in weight was found to be 5.4%, indicating that most of the extraction of material in the pectinase procedure must be attributed directly or indirectly to the action of pectinase.

SUMMARY

1. Uronic acids were obtained from polysaccharides of oat-coleoptile cell walls by acid hydrolysis, and were separated chromatographically. They were identified by hydrolysis, by comparison with similar compounds from maize-cob hemicellulose B and, for the major components, by reduction to the corresponding sugar or disaccharide.

2. The products included free hexuronic acids (mainly galacturonic acid, small amounts of glucuronic acid and methylglucuronic acid), a number

of aldobiouronic acids and aldotriouronic acids (principally glucuronosylxylose and 4-O-methylglucuronosylxylose, smaller amounts of glucuronosylxylosylxylose, methylglucuronosylxylosylxylose, glucuronosylgalactose and galacturonosyl-rhamnose) and galacturonosylgalacturonic acid. Evidence was obtained that indicates that the principal aldobiouronic acids have (1 $\rightarrow$ 2)-linkage.

3. Extraction with hot dilute mineral acid removed hemicelluloses that contain glucuronic acid and methylglucuronic acid, but removed only a fraction of the galacturonic acid. The remainder of the galacturonic acid could be obtained by pectinase treatment. Pectinase treatment alone removed only a minor proportion of the cell-wall glucuronic acid and methylglucuronic acid.

4. By extraction with both hot acid and pectinase the uronic anhydride content of oat-coleoptile cell-wall material was found to be about 5%, of which about two-thirds is galacturonic acid and one-third glucuronic acid and 4-O-methylglucuronic acid.

We thank Dr C. T. Bishop for furnishing samples of 4-O-methylglucose and 4-O-methylglucuronosyl-(1 $\rightarrow$ 2)-xylose, Professor R. L. Whistler for suggestions and advice and Dr Shunji Wada for preparing the sample of oat-coleoptile hemicellulose B. The study was supported by U.S. National Science Foundation Grants G-8705 and G-23769; part of the work was done during the tenure of a National Science Foundation Research Participation Fellowship by D. A. R.

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