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The preparation and susceptibility to hydrolysis of novel *O*-galacturonoyl derivatives of carbohydrates *

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ABSTRACT

D-Galacturonic acid or (1 → 4)-α-D-galacturonan reacted in aqueous pyridine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide with alcohols to yield esters. The alcohols that gave high yields of D-galacturonoyl derivatives were primary and included methanol, ethanol, 1-propanol, D-glucose, D-galactose, methyl β-D-glucopyranoside, methyl β-D-galactopyranoside, and cellulose. D-Galacturonic acid itself readily gave an *O*-D-galacturonoyl-D-glacturonic acid. The proposed structure of one compound, methyl 6-*O*-D-galacturonoyl-β-D-glucopyranoside, was supported by ^{1}H and ^{13}C NMR data and the FAB mass-spectral data. Each ester was hydrolysed at pH 11 and 25°C within 1 h. *O*-D-Galacturonoyl-D-glucose was considerably more alkali labile than *O*-polygalacturonoyl-D-glucose, and *O*-D-galacturonoylcellulose had an intermediate stability. The esters were relatively stable to cold acid, but could be hydrolysed by M trifluoroacetic acid at 100°C for 1 h. The esters tested were resistant to digestion by 'Driselase', although the glycosidic bonds of *O*-polygalacturonoyl-D-glucose were hydrolysed to yield *O*-oligogalacturonoyl-D-glucoses of low molecular weight. The possible application of these analytical methods to the detection of *O*-uronoyl-type cross-links in cell-wall polysaccharides is discussed.

INTRODUCTION

The matrix polysaccharides, isolated from the primary cell walls of plants, are largely water soluble, but are difficult to extract, and there may be both covalent[1,2] and non-covalent[3–6] cross-links. Thus, glycosidic linkages could result in tree-like structures[1], and bifunctional glycosidic linkages[7] and oxidatively coupled phenolic dimers[2,8,9] could form networks. The carboxyl groups of some of the uronic acid

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* Dedicated to Professor David Manners.

residues of pectins and xylans may form ester cross-links with hydroxyl groups of other cell-wall polymers[10,11]. An ester that involves the carboxyl group of a uronic acid is termed an *O*-glycuronoyl derivative in order to distinguish it from a glycosiduronic linkage, which has been referred to variously as a 'uronyl' (ref. 12), 'uronosyl' (ref. 13), or '-osyluronic acid' (ref. 14) bond.

Xylans are major components of primary cell walls, especially in the Gramineae, and of secondary cell walls in all land plants. That the D-glucuronic and 4-*O*-methyl-D-glucuronic acid residues of xylans may form *O*-uronoyl-type cross-links is indicated by the findings that (*a*) a proportion of the xylan can be extracted from water-washed cell walls by treatment with neutral aqueous hydroxylamine[15], which cleaves esters without exerting an appreciable chaotropic effect; (*b*) pre-treatment of water-washed cell walls with dry methanolic sodium methoxide, which cleaves ester bonds, renders some of the bound xylan extractable by neutral aqueous solutions[16]; and (*c*) a high proportion of the D-glucuronic acid residues of aspen wood glucuronoxylan can be reduced to D-glucose residues with $NaBH_4$, indicating that they were esterified[17]. There is no conclusive evidence as to the identity of the molecule that contributes the hydroxyl group in the formation of these *O*-uronoyl-type linkages, but Comtat et al.[17] and Das et al.[18] have speculated that it is lignin.

Pectins, i.e., polysaccharides rich in (1 → 4)-linked α-D-galacturonic acid residues[19], constitute up to 35% of the dry weight of primary cell walls in dicots, and somewhat less in those of the Gramineae. Many of the D-galacturonic acid residues are methyl-esterified[19] and it has been speculated that other *O*-galacturonoyl-type linkages occur[2,10,11]. Since some of the cell-wall-bound pectins that are not extractable in CDTA[20–22] can be solubilised by cold dilute aqueous sodium carbonate, it is possible that they were held in the cell wall by ester bonds. 2,6-Dichlorobenzonitrile-adapted cell cultures have anomalous cell walls, largely composed of pectin, which can be solubilised with alkali (although the pectins do not dissolve readily from the cell walls until the extractant is neutralised)[23], again suggesting ester-type cross-links.

This evidence does not indicate whether the pectin contributes the carboxyl group or the hydroxyl group to the putative ester. For example, two pectin molecules could be cross-linked via an *O*-uronoyl-sugar bond[10] or via an *O*,*O*′-diferuloyl bridge[2,9]. The occurrence of *O*-uronoyl derivatives in vivo has not been explored adequately because of lack of information on methods for their isolation and characterisation. Few *O*-uronoyl derivatives have been produced chemically, mainly esters of such simple alcohols as methanol, ethanol, ethane-1,2-diol, etc.[19], and the properties of *O*-uronoyl-carbohydrate derivatives have not been described.

In order to enable a search for the possible natural occurrence of *O*-uronoyl-sugar derivatives, several such model compounds have been synthesised and their susceptibility to alkaline and enzymic hydrolysis determined. Dicyclohexylcarbodiimide (DCC) has been used to synthesise peptide[24] and ester[25,26] bonds. Attempts to use DCC to synthesise methyl 6-*O*-D-galacturonoyl-β-D-glucopyranoside from D-galacturonic acid and methyl β-D-glucopyranoside in methyl sulphoxide gave low

yields[11]. We have now employed 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), which is water-soluble[27,28], and describe the preparation, purification, and some biologically pertinent properties of novel *O*-uronoyl derivatives.

EXPERIMENTAL

General methods.—Descending PC was performed on Whatman 3MM or No. 1 paper, using *A*, 12:3:5 1-butanol–acetic acid–water (16–24 h); or *B*, 10:5:6 ethyl acetate–acetic acid–water (8–24 h). Paper electrophoresis (PE) was performed on Whatman No. 1 paper at 2 kV for 1.5–3 h in 1:10:189 pyridine–acetic acid–water (pH 3.5). Detection was effected as appropriate with aniline hydrogen phthalate or $AgNO_3$–NaOH, or by scintillation counting.

Aqueous and ethanolic solutions were assayed for radioactivity by liquid scintillation counting after the addition of 10 vol of 0.33% PPO–0.033% POPOP in 2:1 toluene–Triton X-100. Strips of chromatography paper were assayed in ~2 mL of 0.5% PPO–0.05% POPOP in toluene.

Preparation of O-D-*galacturonoyl derivatives.*—(*a*) O-D-*Galacturonoyl-sugars.* To water (1 mL) containing D-galacturonic acid (25 mg), pyridine (25 μL), and D-glucose (500 mg) was added EDC (30 mg). After incubation (1 h, 25°C), ~100 μL of the mixture was applied to a column of Dowex 1-X4-200 (AcO^-) resin (0.4 mL) in pyridine–acetate buffer (5 mM acetate, pH 5.0) and eluted with the same buffer (0.4 ml). An aliquot (50 μL) of the eluate was analysed by PC (solvent *B*). Control solutions of D-glucose and of D-galacturonic acid + pyridine + EDC were analysed in the same way.

In parallel experiments, D-glucose was replaced severally with the same weight of D-xylose, D-galactose, L-fucose, lactose, 2,3,4,6-tetra-*O*-methyl-D-glucose, methyl β-D-glucopyranoside, methyl β-D-xylopyranoside, methyl β-D-galactopyranoside, or methyl α-L-fucopyranoside, or with 125 MBq of D-[1-^{3}H]glucose (65 TBq/mol).

The putative *O*-D-galacturonoyl derivatives were eluted from the chromatography paper with water[29] and isolated as syrups. They could be stored as frozen aqueous solutions (−20°C) or as syrups (room temperature).

(*b*) O-D-*Galacturonoylcellulose.* A strip of Whatman No. 1 paper was dipped in aqueous 16.7% D-galacturonic acid, then dried, spotted with a 50-μL drop of a freshly prepared aqueous solution containing pyridine (10% v/v) and EDC (10% w/v), dried at 25°C, thoroughly washed in running water, dried, and treated with aniline hydrogen phthalate, which revealed an immobilised reducing sugar at the site of application of the drop.

(*c*) *Other* O-D-*galacturonoyl derivatives.* To MeOH–water (1:1, 1 mL) containing D-galacturonic acid (50 mg) and pyridine (50 μL) was added EDC (50 mg). The mixture was incubated and analysed as in (*a*). Other esters were prepared by replacing MeOH with EtOH, 1-propanol, 2-propanol, or 1-butanol.

Preparation of O-*polygalacturonoyl-[1-*^{3}*H]glucose.*—To water (1 mL) containing sodium polygalacturonate (15 mg, from orange, Sigma), pyridine (50 μL), and

D-[1-^{3}H]glucose (17.3 MBq; 65 TBq/mol) was added EDC (50 mg). After incubation (1 h, 25°C), 20 μL of the mixture was subjected to PC (solvent *A*) and the remainder was dialysed against water at 4°C.

*Methyl 6-*O*-D-galacturonoyl-β-D-glucopyranoside* (**1**).—The products obtained upon incubation of D-galacturonic acid with pyridine, methyl β-D-glucopyranoside, and EDC were de-cationised on Dowex-1 (AcO^-) resin and then subjected to column chromatography on cellulose in 2:1:1 acetone–acetic acid–water. A portion of eluate containing the compound which had been deduced by PC and PE to be **1**, and shown by PC to be free from unreacted methyl β-D-glucopyranoside, was dried in vacuo and lyophilised twice from D_2O. After the addition of a trace of acetone (internal standard), the ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AM250 instrument. The FAB-mass spectrum of **1** was obtained on a ZAB-SE instrument in the positive mode with an accelerating voltage of 8 kV and thioglycerol as the matrix.

The NMR and MS analyses were kindly performed by Dr. R.W. Carlson (University of Georgia).

pH-Dependent hydrolysis and ammoniolysis of O*-uronoyl derivatives.*—(*a*) O-D-*Galacturonoylcellulose.* Paper (3 × 3 cm) bearing the spot of immobilised sugar [see (*b*) above] was incubated in 0.2 M buffer (0.5 mL; acetate, pH 5.0; phosphate, pH 7.0; ethylenediamine, pH 7.0; EPPS, pH 8.0; glycylglycine, pH 8.0; pyrophosphate, pH 9.0; ammonia, pH 9.0; carbonate, pH 10.0, ethylenediamine, pH 10.0; carbonate, pH 11.0; or diethylamine, pH 11.0) or 0.2 or 1 M trifluoroacetic acid for 1 h at 25°C. The hydrolysis was stopped by the addition of 2 M pyridine–acetic acid buffer (0.5 mL, pH 5.0), and an aliquot (400 μL) of the solution was assayed for galacturonic acid by the *m*-hydroxybiphenyl test[30].

(*b*) O-*Polygalacturonoyl-*D-*[1-^{3}H]glucose.* To an aliquot (100 μL) of the dialysed *O*-polygalacturonoyl-D-[1-^{3}H]glucose solution (1.8 kBq) that contained 0.5% of non-radioactive Na^+ polygalacturonate was added each (100 μL) of the buffers mentioned above. After incubation (1 h, 25°C), 2 M pyridine–acetic acid buffer (100 μL) was added followed by cold EtOH (1.2 mL). After 30 min at 4°C, the precipitated polysaccharide was removed by centrifugation (15 min at 1500*g*) and a portion (0.5 mL) of the supernatant solution was assayed for ^{3}H. In addition, the hydrolyses were conducted, with the non-amino buffers only, over time courses in which the reaction was stopped after 0–20 h.

(*c*) O-D-*Galacturonoyl-*D-*[^{3}H]glucose.* To an aliquot (20 μL) of *O*-D-galacturonoyl-D-[^{3}H]glucose (2.1 kBq) was added one of the non-amino buffers mentioned above (20 μL). After incubation (1 h, 25°C), 2 M pyridine–acetic acid buffer (20 μL) was added and a portion (30 μL) of the solution was analysed for free [^{3}H]glucose by PC in solvent *B*.

Stability of the O*-uronoyl derivatives towards Driselase.*—Driselase, a mixture of polysaccharide exo- and endo-hydrolases which also contains pectin methyl esterase but no detectable *O*-feruloyl esterase activity, was purified partially as described[31]. Each *O*-uronoyl ester was incubated in a solution of 0.5% of Driselase

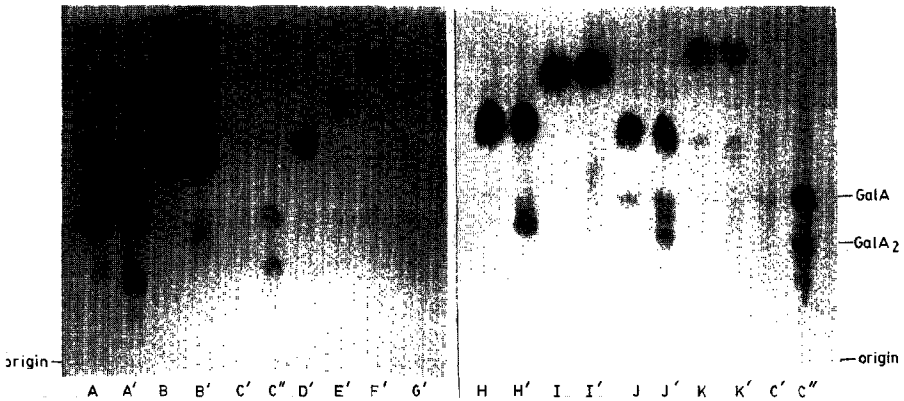


Fig. 1. PC (solvent *B*) of *O*-D-galacturonoyl derivatives obtained by incubation of D-galacturonic acid with EDC, pyridine, and D-glucose (track A′), L-fucose (B′), no neutral alcohol (C′), methanol (D′), ethanol (E′), 1-propanol (F′), 2-propanol (G′), methyl β-D-glucopyranoside (H′), methyl β-D-xylopyranoside (I′), methyl β-D-galactopyranoside (J′), or methyl α-L-fucopyranoside (K′), followed by de-anionisation with Dowex 1 (AcO^-) resin. Corresponding controls lacking EDC, pyridine, and D-galacturonic are also shown (A, B, and H–K); the minor spots in A, J, and K were contaminants present in the commercial neutral sugars. Track C″ shows the reaction products obtained with D-galacturonic acid, EDC, and pyridine, and not treated with Dowex 1 (AcO^-) resin. GalA, D-galacturonic acid; $GalA_2$, *O*-D-galacturonoyl-D-galacturonic acid; these compounds were largely removed by ion-exchange chromatography (tracks A′–K′).

in 1:1:98 pyridine–acetic acid–water (pH ~4.5) that contained 0.05% of chlorbutol at 25°C for 24 h. The products were analysed by PC (solvent *B*).

RESULTS

Preparation of O-*uronoyl derivatives.*—(*a*) O-D-*Galacturonoyl*-D-*galacturonic acid.* Incubation of D-galacturonic acid with EDC and pyridine yielded several reducing compounds of lower R_f (Fig. 1, tracks C″; Table I) each of which yielded galacturonic acid on treatment with 50 mM NaOH for 3 min at 25°C, indicating the absence of glycosidic linkages, and was negatively charged (PE). Therefore, these compounds appear to be an *O*-D-galacturonoyl-D-galacturonic acid and small higher homologues.

(*b*) O-D-*Galacturonoyl derivatives of neutral sugars.* Reaction as in (*a*) of D-galacturonic acid severally with D-glucose, D-xylose, D-galactose, L-fucose, lactose, methyl β-D-glucopyranoside, methyl β-D-xylopyranoside, methyl β-D-galactopyranoside, and methyl α-L-fucopyranoside, followed by removal of acidic products, gave the neutral (PE) esters shown in Fig. 1 with the R_f values given in Table I, each of which was markedly lower than that of the neutral substrate. The products from the methyl glycosides reacted with aniline hydrogen phthalate, which indicated that the anomeric centre of the D-galacturonoyl residue was unsubstituted. Each ester was converted (PC in solvents *A* and *B*) into D-galac-

TABLE I

Data on the PC of *O*-D-galacturonoyl derivatives

Galacturonoyl acceptor	R_{GalA} (solvent *B*) [a]	
	Ester	Acceptor alcohol
Methanol	1.49	
Ethanol	1.76	
1-Propanol	1.98	
2-Propanol	1.98 [b]	
1-Butanol	2.67	
Benzyl alcohol	2.70 [b]	
D-Glucose	0.59	1.04
D-Galacturonic acid	0.68	[1.00]
D-Galactose	0.46	1.09
L-Fucose	0.88	1.43
D-Xylose	0.75	1.44
Lactose	0.26	0.55
Methyl β-D-glucopyranoside	0.88	1.49
Methyl β-D-galactopyranoside	0.76 [c]	1.41
Methyl α-L-fucopyranoside	0.86 [b]	2.02
Methyl β-D-xylopyranoside	0.86 [b]	1.85

[a] Descending PC, on Whatman 3MM paper for ~18 h; the reference marker, D-galacturonic acid, migrated ~22 cm. [b] Obtained in low yield. [c] Other putative esters of methyl β-D-galactopyranoside were observed with R_{GalA} 0.96, 0.56 [b], and 0.46 [b].

turonic acid and the original neutral sugar by treatment with 50 mM NaOH for 3 min at 25°C or with M trifluoroacetic acid for 1 h at 100°C.

The yields were ~5–20% (based on the galacturonic acid) for neutral substrates with a primary hydroxyl group, and ~0.5–2.0% for D-xylose, L-fucose, and their methyl glycopyranosides (Fig. 1). These findings indicate that the major esters involved O-6 of the hexoses. No ester was formed from 2,3,4,6-tetra-*O*-methyl-D-glucose, which confirms that HO-1 was not readily galacturonoylated. Methyl β-D-glucopyranoside gave one major ester, which is concluded to be methyl 6-*O*-D-galacturonoyl-β-D-glucopyranoside (**1**; Fig. 1, track H′). However, methyl β-D-galactopyranoside yielded at least three products (Fig. 1, track J′), which indicates that other hydroxyl groups can also react if they are suitably orientated.

Structure **1** was supported by NMR and FAB mass-spectral data. The ^{13}C NMR spectrum contained 26 peaks (Table II), as expected for **1** with α- and β-Gal*p*A. The presence of a free reducing group in the Gal*p*A moiety is indicated by the C-1 resonances at 92.0 and 95.8 ppm, and also by the signals (d) for H-1α and H-1β at δ ~5.26 and ~4.55, respectively. The presence of a 6-*O*-acyl group in the glucose moiety of **1** was indicated by the fact that the H-6,6 resonances (δ 4.22–4.50) were shifted downfield from their normal positions (δ 3.7–3.8). FABMS gave a single major peak at *m/z* 393 ($M + Na^+$), appropriate for ($1 + Na^+$).

Cold aqueous ammonia or ammonium carbonate buffers (pH 10–11) converted each *O*-galacturonoyl derivative into the expected neutral sugar and a mixture of galacturonic acid and galacturonamide (R_{GalA} 0.85, PC in solvent *A*). Mixed

TABLE II

^{13}C NMR data (δ in ppm) for methyl 6-*O*-D-galacturonoyl-β-D-glucopyranoside (**1**)

56.77 and 56.86 (OCH_3), 60.31 and 63.36 (C-6 of β-Glc-OMe), 67.31, 68.08, 68.89, 69.22, 69.28, 69.76, 69.95, 70.73, 71.76, 72.58, 72.68, 72.85, 73.63, 75.13, 75.33, 75.50, 92.00 and 95.83 (C-1 of α- and β-Gal*p*A), 102.81 and 102.91 (C-1 of β-Glc-OMe), 169.13 and 170.09 (C-6 of α- and β-Gal*p*A).

ammoniolysis and hydrolysis also occurred when each ester on the dry paper chromatogram was exposed to ammonia vapour. Thus, the sequence PC, exposure of the dry chromatogram to ammonia vapour, re-drying, and PC at 90° yielded products (the neutral substrate and a mixture of D-galacturonamide and D-galacturonic acid) displaced from the diagonal. This sequence may be useful in the analysis of biological samples that contain traces of *O*-uronoyl esters in the presence of large proportions of mono- and oligo-saccharides.

The yields of esters were enhanced by the presence of pyridine. Increasing the concentration of EDC to > 50 mg/mL did not increase significantly the yield of, for example, **1** but did immobilise reducing material at the origin in PC. When the concentration of EDC was increased to 500 mg/mL, *O*-D-galacturonoyl-D-galacturonic acid could not be detected among the products, although **1** was still formed in unchanged amounts, and large amounts of reducing material were immobilised at the origin in PC.

(*c*) O-D-*Galacturonoyl and* O-*oligogalacturonoyl derivatives of lower alcohols.* D-Galacturonic acid in the presence of EDC readily formed esters with methanol, ethanol, and 1-propanol, but only a trace of ester was produced from 2-propanol (Fig. 1; Table I), again indicating that primary alcohols are the favoured acyl acceptors. Galacturonotriose (produced from polygalacturonic acid by the action of pectinase) was also readily methyl-esterified in the presence of EDC.

(*d*) O-*Uronoyl derivatives of polysaccharides.* EDC mediated the condensation of D-galacturonic acid with cellulose (paper). The product, which could not be removed by extensive washing in water, reacted with aniline hydrogen phthalate, and was saponified on treatment with 50 mM NaOH (25°C, 3 min), consistent with a D-galacturonoyl derivative (presumably at O-6).

Polygalacturonic acid could be condensed with [^{3}H]glucose. Thus, 1.0% of the starting [^{3}H]glucose was converted into soluble, non-dialysable, radioactive material, with $R_f \sim 0$ (solvent *B*), from which free [^{3}H]glucose was released by treatment with 50 mM NaOH (25°C, 10 min).

Polygalacturonic acid showed no tendency to form a gel in the presence of EDC, indicating that, unlike D-galacturonic acid, (1 → 4)-α-D-galacturonan did not polymerise. It is possible that suitable combinations of acidic and neutral polysaccharides could be made to form gels by EDC-mediated formation of ester cross-links.

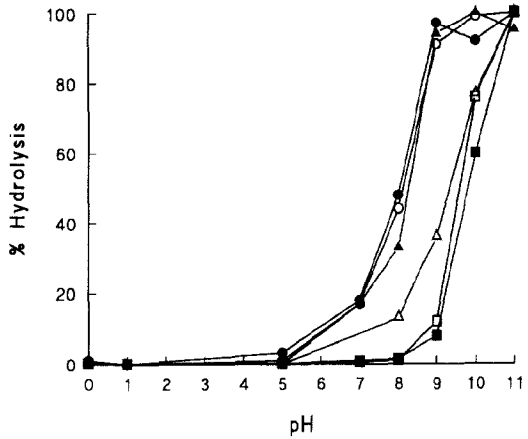


Fig. 2. Effect of pH on the extent of hydrolysis (for 1 h at 25°C) of *O*-D-galacturonoyl-D-galacturonic acid (●), *O*-D-galacturonoyl-D-[^{3}H]glucose (○), *O*-D-galacturonoylcellulose (▲, △; two independent preparations), *O*-polygalacturonoyl-D-[^{3}H]glucose (■, □; single preparation). Buffers used involved non-amino compounds (●, ○, ▲, △, ■; hydrolysis only) or amino compounds (□; ammoniolysis and/or hydrolysis).

pH-Dependent hydrolysis of O*-uronoyl derivatives.*—*O*-Uronoyl derivatives of each type tested (*O*-polygalacturonoyl-D-[^{3}H]glucose, *O*-D-galacturonoylcellulose, *O*-D-galacturonoyl-D-[^{3}H]glucose, and *O*-D-galacturonoyl-D-galacturonic acid) were hydrolysed completely within 1 h at pH 11.0 and room temperature (Fig. 2). There was little difference between the effects of the amino and non-amino buffers, indicating no strong preference for ammoniolysis. Only slight hydrolysis occurred at pH 0.0–5.0 and 25°C.

The hydrolysis of *O*-polygalacturonoyl-D-[^{3}H]glucose at each pH tested fitted first-order kinetics (Fig. 3a), and the rate constants (k) were closely related to [HO^-] (Fig. 3b). The value of k (8.9 h^{-1} at pH 11 and 25°C; Fig. 3) for *O*-polygalacturonoyl-D-[^{3}H]glucose indicates that the *O*-uronoyl derivatives are more alkali labile than other types of ester linkage that are known to occur in the plant cell wall, e.g., *O*-feruloyl derivatives (k 6.9 h^{-1} at pH 12.8)[32].

O-D-Galacturonoyl-D-[^{3}H]glucose was considerably more labile than *O*-polygalacturonoyl-D-[^{3}H]glucose (Fig. 2); 50% hydrolysis occurred within 1 h at room temperature at pH ~ 7.8 and ~ 9.7, respectively. A similar enhanced stability of high molecular weight species in studies of the alkaline hydrolysis of the methyl esters of galacturonic acid and its oligo- and poly-saccharides[19] was ascribed to the HO^--repelling effect of neighbouring acidic groups. *O*-D-Galacturonoylcellulose preparations had an alkaline stability intermediate of those of low molecular weight *O*-uronoyl derivatives and *O*-polygalacturonoyl-D-[^{3}H]glucose, 50% hydrolysis being achieved within 1 h at room temperature and pH ~ 8.5–9.0 (Fig. 2). The fact that *O*-D-galacturonoylcellulose is more stable towards alkali than is *O*-D-galacturonoyl-D-[^{3}H]glucose cannot be ascribed to the effect of a neighbouring anionic moiety. It is of interest that methyl esters of formaldehyde-cross-linked

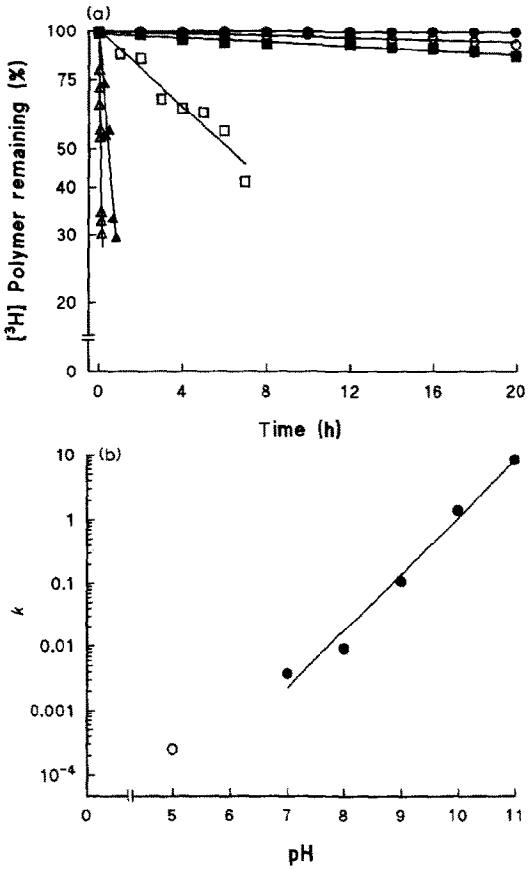


Fig. 3. (a) Kinetics of hydrolysis of *O*-polygalacturonoyl-D-[^{3}H]glucose at 25°C and pH 5.0 (●), 7.0 (○), 8.0 (■), 9.0 (□), 10.0 (▲), or 11.0 (△). (b) Relationship between pH and the first-order rate constant (k) for the hydrolysis of *O*-polygalacturonoyl-D-[^{3}H]glucose at 25°C. The best straight line is fitted to the pH 7–11 data.

polygalacturonic acid are more stable than are those of similar polymers in solution[19].

The gradient of the pH–hydrolysis curve for *O*-D-galacturonoylcellulose was also shallower than those for the other *O*-uronoyl derivatives tested, which may be due in part to the presence of $\geqslant 2$ types of bond [e.g., in *O*-galacturonoyl-*O*-galacturonoylcellulose] that differ in stability. The variation in the pH–hydrolysis curve between the two different preparations of *O*-galacturonoylcellulose (Fig. 2) could be due to variation in the ratio of these types of bond.

Driselase-catalysed hydrolysis.—None of the foregoing D-galacturonoyl bonds was significantly susceptible to hydrolysis by 'Driselase' (Fig. 4) under the standard conditions used for the enzymic dissection of plant cell walls[2,11,31,32]. Whereas methyl β-D-glucopyranoside was hydrolysed readily by Driselase to D-glucose, **1**

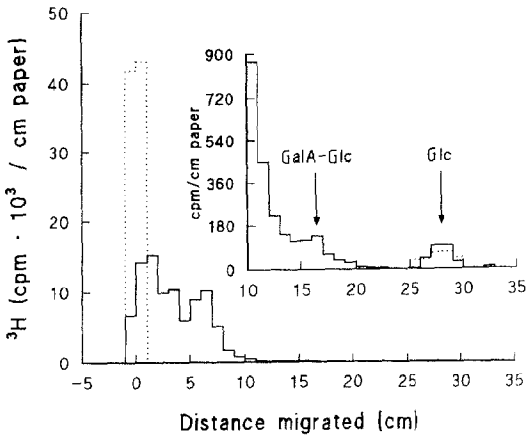
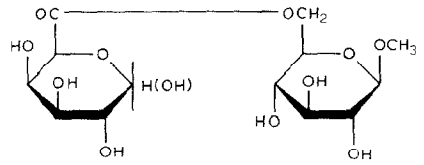


Fig. 4. Effect of Driselase on *O*-polygalacturonoyl-D-[^{3}H]glucose. Starting material (· · · · · ·) and digestion products (———) were analysed by PC (solvent *B*). The inset is a re-plot of the 10–35-cm zone on a different *y*-axis scale. The positions of D-glucose (Glc) and *O*-D-galacturonoyl-D-glucose (GalA–Glc) are indicated.

was unaffected. Thus, 6-*O*-D-galacturonoylation protects methyl β-D-glucopyranoside from hydrolysis by the β-D-glucosidase(s) present in Driselase. However, Driselase converted *O*-polygalacturonoyl-D-[^{3}H]glucose ($R_f \sim 0$) into a mixture of chromatographically mobile, ^{3}H-labelled products. A minor product co-chromatographed with *O*-D-galacturonoyl-D-glucose (Fig. 4; cf. Fig. 1). The other products of hydrolysis have not been characterised in detail, but their R_f values in PC were compatible with their being compounds of the form

where ● is [^{3}H]Glc, ■ is Gal*p*A, → is a glycosidic bond, and | is an ester bond. Driselase converts polygalacturonic acid largely into galacturonic acid together with a trace of galacturonobiose[33]. Therefore, Driselase can hydrolyse the glycosidic bonds of polygalacturonic acid bearing *O*-uronoyl-type cross-links to yield oligosaccharides in which the ester bond is intact. Thus, potentially, Driselase can be used to determine the presence of *O*-uronoyl-type cross-links within the cell wall[2].



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DISCUSSION

We have described the in vitro synthesis of a range of *O*-uronoyl derivatives of carbohydrates for use as model compounds for the detection of similar cross-links

in vivo. The use of EDC allowed the formation of *O*-D-galacturonoyl derivatives, apparently involving mainly HO-6 of hexoses but not HO-1. D-Galacturonic acid itself readily acted as an acyl acceptor, possibly at HO-4, which is activated by the carboxyl group. If O-4 is indeed the major site for the *O*-galacturonoylation of galacturonic acid, this would account for the failure of (1 → 4)-α-D-galacturonan chains to cross-link extensively enough to give gels in the presence of EDC.

Preliminary results show that EDC can also be used to prepare other *O*-uronoyl esters and *N*-uronoyl amides that are relevant to the plant cell wall.

The high alkali-lability, relative acid-stability, and resistance to Driselase of the above novel galacturonoyl derivatives should aid the design of methods for their detection in vivo.

Note added since this work was refereed.—The recent observation[34] that saponification of maize coleoptile cell walls releases less methanol (mol/mol) than the total content of apparently esterified galacturonic acid residues (i.e., those reducible to galactose residues by $NaBH_4$) suggests the natural occurrence of non-methyl *O*-galacturonoyl esters.

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