Phytochemistry, 1967, Vol. 6, pp. 423 to 431. Pergamon Press Ltd. Printed in England

PECTIC SUBSTANCES IN CURED AND UNCURED TOBACCO

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(*Received* 28 *June* 1966)

Abstract—The structures of pectin and reduced pectin from cured leaf stems of Bright Virginia tobacco, and pectin from fresh leaves of Virginia tobacco plants, have been investigated. The "cured" pectin has an anhydrogalacturonic acid content of more than 90% and on partial hydrolysis with a pectinase preparation yielded di-, tri-, and tetra-galacturonic acids, with α-1,4-linkages. Diborane has been used to reduce the acidic polysaccharide to the corresponding galactan which has been subjected to methylation analysis. The fresh leaf pectin has a much lower anhydrouronic acid content (*ca.* 20%) and unlike "cured" pectin readily yields oligosaccharides containing neutral and acidic monosaccharides on partial hydrolysis.

INTRODUCTION

THE group of heterogeneous polysaccharides known as pectic substances occurs in all higher plants. The main component of the group appears to be poly-D-galacturonic acid with α-1,4-linkages. Other monosaccharide constituents of the pectic substance are D-galactose, L-arabinose[1–5] and the minor components L-rhamnose,[1–5] D-xylose,[1–4] L-fucose,[5] D-glucose,[3] 2-*O*-methyl-L-fucose[3,5] and 2-*O*-methyl-D-xylose.[3,5] D-Galactose and L-arabinose have been shown to be present, in part at least, in separate neutral polymers; in some pectins the galactose occurs as a β-1,4-linked galactan,[6,7] and the arabinose as a highly branched arabinan.[8–10] Of the remaining neutral sugars L-rhamnose is the most common and is thought to be incorporated into the uronic acid chain; in some cases it may form a branch point.[5]

In recent work, Northcote[11] has shown that pectic polysaccharides from apple can be fractionated into a neutral arabinan–galactan complex and two acidic polysaccharides, one with a high uronic acid content (98%) and the other containing 31% uronic acid and 51% arabinose.

In the present investigation the structure of the pectic substances in cured tobacco leaf stems has been compared with that from fresh leaves in an attempt to discover the changes occurring during the curing process.

RESULTS AND DISCUSSION

Pectin from the cured leaf stems and fresh leaves of Bright Virginia tobacco was obtained by ammonium oxalate extraction.

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[7] L. HOUGH and D. B. POWELL, *J. Chem. Soc.* 16 (1960).
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[9] E. L. HIRST and J. K. N. JONES, *J. Chem. Soc.* 1221 (1947).
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[11] A. J. BARRETT and D. H. NORTHCOTE, *Biochem. J.* **94**, 617 (1965).

Hydrolysis of the cured leaf stem pectin with 1 N-sulphuric acid yielded D-galacturonic acid (identified as mucic acid, m.p. 218–219°, mixed m.p. 219°, Found: C, 34·2; H, 4·2. Calc. for $C_6H_{10}O_8$: C, 34·3; H, 4·7%), D-galactose (identified as *N*-*p*-nitrophenyl-D-galactosylamine, m.p. 113°, mixed m.p. 114°), L-arabinose (identified as L-arabinose-*p*-nitrophenylhydrazone, m.p. 181°, mixed m.p. 181°) and L-rhamnose (identified as *N*-*p*-nitrophenyl-L-rhamnosylamine, m.p. 228°, mixed m.p. 228–229°). Glucose, xylose, fucose and 2-*O*-methyl xylose were identified as pectin constituents by paper chromatography. Attempts to fractionate the crude, "cured" pectin on DEAE cellulose using the method of Deuel *et al* [12] were unsuccessful. The polysaccharide was strongly absorbed on the column and elution with alkali yielded a single fraction with monosaccharide composition similar to that of the crude material.

Hydrolysates of crude fresh leaf pectin were qualitatively (but not quantitatively) similar to those of the "cured" pectin, as judged by paper chromatographic analysis.

Comparisons were made of the uronic acid (decarboxylation method[13]), methoxyl and acetyl[14] contents of the pectins from cured and fresh leaves (Table 1).

TABLE 1. ANALYTICAL DATA FOR PECTINS

%	From cured leaves	From fresh leaves
Uronic acid	93·1	18·1
Methoxyl	0·5	1·8
Acetyl	0	0

The galactose, arabinose and rhamnose contents were determined by hydrolysing samples of pectin with 1 N-sulphuric acid for known periods of time, separating the resultant monosaccharides on paper chromatograms and estimating them with the phenol–sulphuric reagent[15] (Fig. 1).

Pectin from cured leaves was reduced with diborane, until the uronic acid content had dropped to 18·6%. The neutral sugar content was determined as before and the results are illustrated in Fig. 2.

Methylation of this reduced polysaccharide by Haworth[16] and Purdie[17] methods, followed by methanolysis and analysis by gas chromatography (GLC)[18] gave 2,3,6-tri-*O*-methyl-D-galactose as the major product, together with smaller amounts of 2,3,4,6-tetra-*O*-methyl-D-galactose, 3,4-di-*O*-methyl-L-rhamnose and 2,3,4-tri-*O*-methyl-L-rhamnose. Methylated uronic acids were not detected on the vapour phase chromatogram; little is known about the chromatographic behaviour of these derivatives but it is believed that they have high retention times.[18] When these methyl derivatives were demethylated[19] each yielded the corresponding parent monosaccharide.

Pectins from cured and fresh leaves were subjected to various treatments in an attempt

[12] H. NEUKOM, H. DEUEL, W. J. HERI and W. KÜNDIG, *Helv. Chem. Acta* **43**, 64 (1960).
[13] A. JOHANSSON, B. LINDBERG and O. THEANDER, *Svensk Papperstid.* **51**, 41 (1954).
[14] E. A. MCCOMB and R. M. MCCREADY, *Anal. Chem.* **29**, 819 (1957).
[15] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH, *Anal. Chem.* **28**, 350 (1956).
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[17] J. C. IRVINE and T. PURDIE, *J. Chem. Soc.* 1021 (1903).
[18] G. O. ASPINALL, *J. Chem. Soc.* 1676 (1963).
[19] T. G. BONNER, E. J. BOURNE and S. MCNALLY, *J. Chem. Soc.* 2929 (1960).

to obtain partial hydrolysis products. Workable yields of oligosaccharides were, however, only obtained by using a crude fungal pectinase.

One oligosaccharide obtained from cured leaf pectin exhibited chromatographic and electrophoretic behaviour which suggested that it was an acidic disaccharide containing two uronic acid units. On acid hydrolysis it yielded only galacturonic acid, and the optical rotation of its calcium salt corresponded to that of calcium digalacturonate ($[\alpha]_D^{20°} = +$

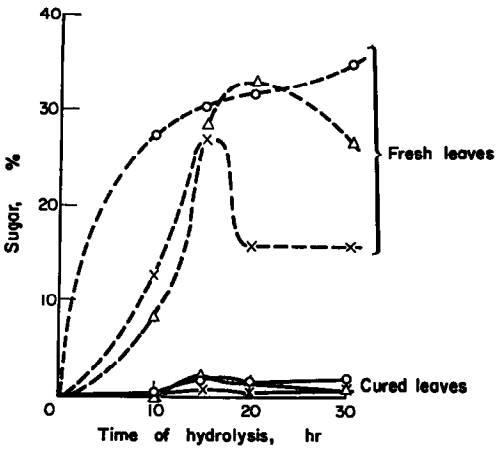

FIG. 1. NEUTRAL SUGARS IN PECTIN HYDROLYSATES.
○ Galactose ——— Cured leaves
× Arabinose - - - - Fresh leaves
△ Rhamnose.

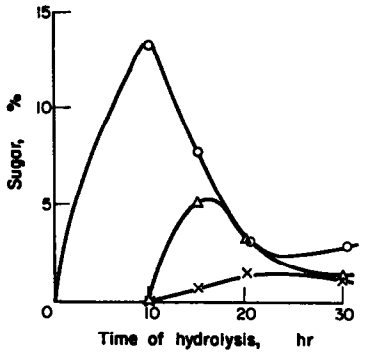

FIG. 2. NEUTRAL SUGARS IN REDUCED PECTIN HYDROLYSATE.
○ Galactose × Arabinose △ Rhamnose.

112°).[20] The degree of polymerization was 1·90. Reduction of the methyl glycoside of the disaccharide followed by methylation and methanolysis yielded methyl 2,3,6-tri-*O*-methyl-D-galactoside and methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside which were identified by GLC.[18]

The methyl ester of the disaccharide, when reduced with borohydride then allowed to react with α-D-galactoside D-galactohydrolase from yeast, yielded galactose and galactitol; no reaction occurred with β-D-galactoside D-galactohydrolase from *Escherichia coli.*

[20] J. K. N. JONES and W. W. REID, *J. Chem. Soc.* 1361 (1954).

The partial enzymic hydrolysis of cured leaf pectin was repeated under more carefully controlled conditions and the products separated into a neutral and an acidic fraction on an anion exchange column (Fig. 3).

The neutral fraction contained monosaccharides but no oligosaccharides. The acidic fraction was refractionated into D-galacturonic acid and three other components by paper

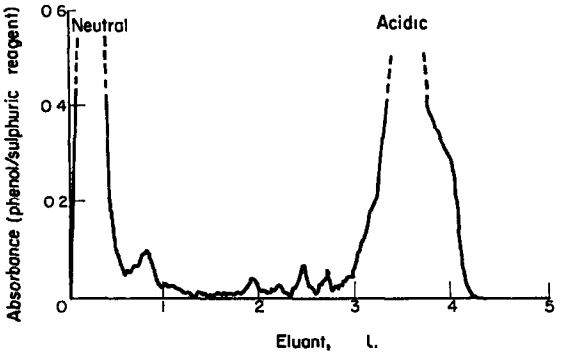

FIG. 3. FRACTIONATION OF PARTIAL HYDROLYSATE OF CURED LEAF PECTIN ON AN IRA 400 (ACETATE FORM).

Elution with graded sodium acetate: 0·02 M × 500 ml; 0·04 M × 500 ml; 0·06 M × 500 ml; 0·08 M × 1 l.; and 0·10 M × 2 l.

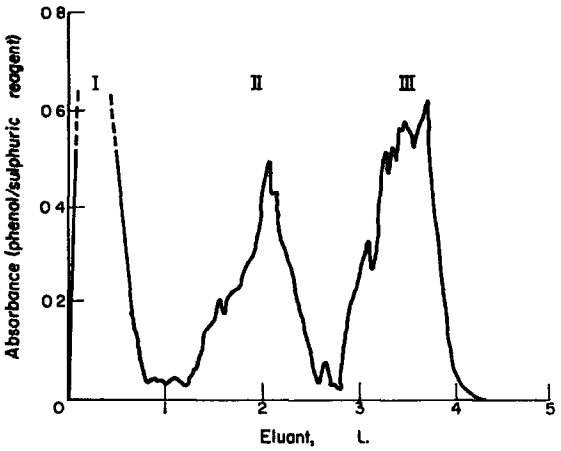

FIG. 4. FRACTIONATION OF PARTIAL HYDROLYSATE OF FRESH LEAF PECTIN ON AN ANION EXCHANGE COLUMN.

Elution with graded sodium acetate: 0·02 M × 500 ml; 0·04 M × 1 l.; 0·06 M × 500 ml; 0·08 M × 1 l.; and 0·1 M × 2 l.

chromatography. The chromatographic behaviour of the three compounds suggest that they were an homologous series of α-1,4-linked D-galacturonic acids with degrees of polymerization (DP) of 2, 3 and 4 and that the compound with a DP of 2 was in fact the same disaccharide as that which had been isolated from the preliminary hydrolysate. A linear relationship was observed when the expected DP values of these compounds were plotted against $\log_{10}(1/R_f - 1)$[21].

[21] D. FRENCH and G. M. WILD, *J. Am. Chem. Soc.* 75, 2614 (1953).

The specific optical rotations of the calcium salts of the oligosaccharides were found to be $[\alpha]_D^{20°}+112°$, $+196°$ and $+248°$, for the compounds with DP 2, 3 and 4, respectively. Reduction of the methyl ester methyl glucoside derivatives of all three oligosaccharides followed by methylation and methanolysis yielded the methyl glycosides of 2,3,6-tri-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose in each case.

The partial enzymic hydrolysis of pectin from fresh leaves yielded a neutral (I) and two acidic (II, III) fractions (Fig. 4). The neutral fraction (I) again contained only monosaccharides. Fraction II consisted of three galacturonic acid-containing oligosaccharides (*X*, *Y* and *Z*). These could not be fully identified owing to insufficient material, but the results of partial characterization are shown in Table 2.

TABLE 2. PROPERTIES OF GALACTURONIC ACID-CONTAINING OLIGOSACCHARIDES FROM FRESH LEAF PECTIN HYDROLYSATE

	X	*Y*	*Z*
$[\alpha]_D^{20°}$	$+80°$	$+70°$	$+15°$
$R_{Gal\,A}$*	0·22	0·81	0·40
$M_{Gal\,A}$*	0·51	0·61	0·66
Hydrolysis Products	Galacturonic acid Galactose Rhamnose	Galacturonic acid Rhamnose	Galacturonic acid Galactose
Reaction with			
Triphenyltetrazolium chloride[22]	Pink	Negative	Negative
Aniline diphenylamine[23]	Slate blue	Negative	Negative

* $R_{Gal\,A}$ refers to the mobilities relative to galacturonic acid in ethyl acetate:acetic acid:formic acid:water (18:3:1:4, v/v). $M_{Gal\,A}$ refers to the electrophoretic mobilities relative to galacturonic acid in phosphate buffer (pH 7·0).

From the evidence in Table 2 the oligosaccharide *Y* appears to be the aldobiouronic acid, 2-*O*-(D-galactopyranosyl uronic acid)-L-rhamnose, as the properties observed are in good agreement with those quoted by Aspinall.[5] The specific optical rotation is lower for this disaccharide than that quoted but this is probably due to contamination with traces of galacturonic acid. Little comment can be made on the structure of the other two oligosaccharides *X* and *Z*, however we note that Aspinall and Fanshawe[5] have detected a disaccharide of galacturonic acid and galactose in a hydrolysate of lucerne pectin which may be the same as *Z*.

Fraction III consisted almost entirely of galacturonic acid, although traces of three oligosaccharides were also present. These yielded only galacturonic acid on acid hydrolysis and were thought to be the same homologous series that had been isolated from the "cured" pectin. The expected DP/$\log_{10}$ ($1/R_f$-1) plot gave a straight line and the R_f values agreed very closely with those obtained from the previous series.

In carrying out analytical and structural investigations on pectin from fresh and cured tobacco leaves some information has been obtained concerning the extent and nature of degradation that has occurred during the curing process. The main processes involved in tobacco curing consist of drying the leaves at 120° for up to 3 days. The drying is then followed by a maturing process when the leaves are packed tightly into barrels and left for between

[22] K. WALLENFELS, *Naturwiss.* **37**, 491 (1950).
[23] S. SCHWIMMER and A. BEVENUE, *Science* **123**, 543 (1956).

2–5 yr. At the present time the stage at which the degradation occurs is unknown or indeed whether it occurs slowly during the whole process.

Analysis of the cured leaf pectin has shown that it contains over 90% D-galacturonic acid. Methylation of reduced pectin to give 2,3,6-tri-*O*-methyl-D-galactose as the major component and the isolation of α-1,4-linked di-, tri- and tetra-galacturonic acids from the pectin itself indicate a basic structure of α-1,4-linked anhydro-D-galacturonic acid units.

The pectin from fresh leaves has a surprisingly low (*ca.* 20%) uronic acid content, and the acidic component appears to have the same basic structure as that of the "cured" pectin as it yielded the same homologous series of acidic oligosaccharides.

Three oligosaccharides containing acidic and neutral monosaccharide residues were also isolated from the fresh leaf polysaccharide, whereas similar attempts with the cured leaf stem polysaccharide yielded negligible amounts of similar materials. The general inference from these comparisons is that during curing the polysaccharide is considerably degraded and that most of the neutral sugar components attached to the acidic chain are lost, possibly because they are more accessible and/or labile, to degradative agents.

Analytical work on both "cured" and "fresh" Pectins, and the diborane-reduced pectin, indicates that the L-rhamnose content, relative to the other neutral sugars, is higher than the literature would suggest for other pectic substances. In the case of pectin from fresh leaves the absolute percentage of rhamnose is extremely high (*ca.* 30%). Recent workers have been led to believe that L-rhamnose is either incorporated in the uronic acid chain and/or exists as single unit branch points off the main acidic chain.[5] This is supported in the case of tobacco by the isolation of the two acidic oligosaccharides containing rhamnose from the fresh leaf pectin, and by the apparent increase in rhamnose content when "cured" pectin is reduced (Fig. 2). Presumably after reduction the main chain is more acid labile and the amount of free rhamnose liberated approximates more closely to the actual amount present in the pectin. If many L-rhamnose residues are incorporated into the uronic acid chain then it is unlikely that the fresh leaf polysaccharide will contain many regions where relatively large numbers of uronic acid residues are directly adjacent. This being so, the apparent fall in rhamnose content (*ca.* 30→5%) during curing suggests a complete breakdown of the acidic chain should have occurred, unless, as Northcote has shown in apple pectin, part of the acidic component exists free from neutral sugars.[11]

Figures 1 and 2 clearly illustrate that assays dependent upon complete acid hydrolysis, particularly of acidic polysaccharides, need to be treated with some caution and that the products after various hydrolysis times need to be examined before any assessment can be made. Even then the varying rates of liberation and degradation of the constituent monosaccharides makes the results difficult to interpret. However, if the optimum values of each curve in Figs. 1 and 2 are accepted these perhaps give the best representation of the absolute quantities of monosaccharides combined in the pectins.

Other neutral sugars apart from D-galactose, L-arabinose and L-rhamnose were found in both pectins. These were mainly D-glucose and D-xylose. No information concerning their relationship with the rest of the polysaccharide was obtained; the same state of affairs exists with these residues in other pectins that have been examined.

EXPERIMENTAL

Paper Chromatography and Electrophoresis

The following solvent systems were used.

(1) Ethylacetate:aceticacid:water (9:2:2, v/v); (2) *n*-butan-1-ol:pyridine:water

(6:4:3, v/v); (3) *n*-butan-1-ol: acetone: water (3:5:2, v/v); (4) ethyl acetate: acetic acid: formic acid: water (18:3:1:4, v/v).

The electrophoresis buffer employed was 0·1 M-sodium phosphate (pH 7·0).

Chromatograms and electrophoretograms were sprayed with aniline oxalate (reducing sugars); silver-nitrate/NaOH (reducing and non-reducing sugars); triphenyltetrazolium chloride (detection of 1,2-linkages) and aniline/diphenylamine (detection of 1,4-linkages).

Extraction and Purification of Pectic Substances

The leaves or leaf stems (30 g) were boiled for 3 hr with 1% ammonium oxalate (500 ml). The aqueous extract was separated from the pulp, by filtering through a fine nylon cloth, and then centrifuged. The polysaccharide was precipitated by pouring the supernatant liquid into an equal volume of acetone, then it was centrifuged off, freeze dried and finally allowed to stand over P_2O_5 for 2 days under reduced pressure at 40°. Purification was achieved by repeated dissolution and precipitation with acetone. Considerable amounts of starch were co-precipitated with pectin from fresh leaves and this was removed by treatment with α-amylase before freeze drying.

Fractionation of Hydrolysis Products

Pectin (10 g) from cured leaves was hydrolysed with 1 N-H_2SO_4 (10 hr). The resulting suspension was centrifuged off and re-hydrolysed. The process was repeated until no more monosaccharide was liberated from the residue. The combined hydrolysates were then concentrated. A column of Amberlite IRA-400 (acetate form) resin was used to separate neutral and acidic sugars. The former were eluted off with water and the latter with 1 N-H_2SO_4. The acid eluent was neutralized with $BaCO_3$, filtered and concentrated to yield a white amorphous solid (8·5 g) which was shown by paper chromatography (solvents 1 and 2) and paper electrophoresis to be barium galacturonate. The neutral fraction was concentrated to a syrup (500 mg) and fractionated on a cellulose column.[24] Complete separation of glucose and galactose was not obtained and this was later effected by passing the mixed hexoses down a cellulose column impregnated with tungstate.[25] This column was prepared by making a cellulose slurry in 10% aqueous sodium tungstate (adjusted to pH 8·0 by the addition of 2 N-sulphuric acid). The column (30 × 3 cm) was washed with solvent 3, the glucose/galactose mixture then introduced and solvent 3 used for elution. Aliquots (25 ml) were collected and examined on tungstate impregnated paper chromatograms[25] developed with solvent 3. The glucose and galactose fractions obtained from the column were deionized with Biodemineralite and concentrated.

Determination of Neutral Sugars

Four samples of pectin (6–60 mg) were hydrolysed with 1 N-H_2SO_4 in sealed tubes at 100° for various periods of time (10–30 hr). The hydrolysates were neutralized ($BaCO_3$), deionized (Amberlite IR-120 (H^+ form) resin) and known volumes were spotted onto paper chromatograms which were developed with solvent 2. After elution the sugar bands were determined with the phenol–sulphuric acid reagent.[15]

[24] L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.* 2511 (1949).
[25] E. J. Bourne, N. A. Sufi and H. Weigel, Unpublished work.

Reduction and Methylation of Pectin from Cured Leaves

The reduction of pectin was carried out according to the method described by Hirst *et al.*,[26] i.e. the formation of the di-propionate followed by treatment with diborane. Methylation was achieved by repeated treatment of the reduced pectin with dimethyl sulphate[16] at room temperature under nitrogen followed by methyl iodide and Ag_2O.[17] Methylation was regarded as complete when the height of the —OH band at 3430 cm^{-1} was less than 5% of the —CH band at 2900 cm^{-1}. The methylated polysaccharide was refluxed with 6% methanolic HCl, the HCl neutralized with Ag_2CO_3 and the methanolysate concentrated. Chloroform was added and removed by evaporation and the process repeated several times; the methyl glycosides were finally examined by GLC using polybutan-1,4-diol succinate and polyphenyl ether as stationary phases.[18]

Partial Enzymic Hydrolysis of Pectin

Pectin from cured leaves was incubated with dialysed pectinase (Nutritional Biochemicals Corporation Inc.) at 37° for 12 hr. Digalacturonic acid was obtained from the hydrolysate by absorption on a column of Amberlite IRA-400 (acetate form) resin, followed by elution with 1 N-H_2SO_4. Further purification was obtained by paper chromatography (solvent 1).

To obtain higher molecular weight oligosaccharides from the "cured" pectin and any oligosaccharides from fresh leaf pectin a closer control of the hydrolysis conditions was necessary and two methods were found to be successful.

In the first method pectin (2 g suspended in *ca.* 20 ml H_2O/ethanol (1:1) mixture) was incubated with dialysed 2% aqueous pectinase solution in a rotating dialysis bag immersed in a large column through which an ethanol/water mixture (1:1, v/v) was passed. The solution was allowed to drip slowly from the column (which was replenished from a reservoir) into a vessel containing acetone.

In the second method an aqueous dialysed pectinase solution (0·25%, 40 ml) and a 2% aqueous solution of pectin (100 ml) were allowed to run simultaneously into a column fitted with a syphoning arm. The rates of flow were controlled so that it took *ca.* 6 hr for the column to fill and then syphon over into a vessel containing acetone.

Fractionation of the oligosaccharide mixtures from these enzymic hydrolyses was achieved by the method of Samuelson and Victorin[27] using an ion exchange column. Paper chromatography (solvent 4) was used for further purification.

Characterization of Oligosaccharides from Cured Leaf Stem Pectin

Each oligosaccharide (20–40 mg) was refluxed with 2% methanolic HCl (15 ml) until non-reducing to Fehling's solution. After neutralization (Ag_2CO_3) and concentration each syrup was allowed to stand overnight with CH_2N_2, then concentrated and treated with $NaBH_4$ (20–40 mg in 10 ml water) for 2 days. Borate was removed with methanol and the reaction mixture deionized with Amberlite IR-120 (H^+ form) resin; the diazomethane and boro-hydride treatments were repeated twice, and the syrups finally dried over P_2O_5 under reduced pressure.

Each dried syrup was treated with dimethyl formamide (1 ml) and methyl iodide (1 ml) at 0° and silver oxide (200 mg) was added. Throughout the reaction light was excluded from the flask which was shaken continuously. After 1 hr the mixture was allowed to come to room

[26] E. L. HIRST, E. PERCIVAL and J. K. WOLD, *J. Chem. Soc.* 1493 (1964).
[27] O. SAMUELSON and L. VICTORIN, *Svensk-Papperstid.* **67**, 555 (1964).

temperature and then left for a further 24 hr. The reaction mixture was then filtered and concentrated and the methylation repeated. Methanolysis was carried out as before and the methyl glycosides examined by GLC.

The DP of digalacturonic acid was estimated by the method of Peat, Whelan and Roberts,[28] except that carbohydrate was determined with the phenol-sulphuric reagent.[15]

The digalacturonic acid was further characterized by reduction with $NaBH_4$ to galactobiitol followed by hydrolysis with α-D-galactoside galactohydrolase.

Acknowledgements—We thank Dr. E. E. Percival, Dr. B. C. Platt and Professor G. N. Richards for helpful discussions and advice, and the American Machine and Foundry Co. for a postgraduate grant to H. G. J. W.

[28] S. Peat, W. J. Whelan and J. G. Roberts, *J. Chem. Soc.* 2258 (1956).