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DETERMINATION OF L-GALACTOSE IN POLYSACCHARIDE MATERIAL

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Key Word Index—*Linum usitatissimum*; *Zea mays*; *Acer pseudoplatanus*; L-galactose; monosaccharides; GLC of trimethylsilyl derivatives; plant cell walls; polysaccharides; D-galactonolactone; D-galactose dehydrogenase.

Abstract—A method is described for the identification and quantitative determination of L-galactose in hydrolyzates of polysaccharide material In this technique, all the D-galactose is oxidized to D-galactonic acid using the enzyme D-galactose dehydrogenase. Remaining sugars, including any L-galactose, are converted to their trimethylsilyl derivatives and estimated by GLC. L-Galactose was detected in polysaccharides of flax seed, corn cob and corn root, and in the cell wall of *Acer pseudoplatanus* suspension cultures. It is suggested that the sugar may be relatively widespread in plants.

INTRODUCTION

ROUTINE analysis of polysaccharide hydrolyzates by GLC or TLC cannot distinguish between enantiomers of the same sugar. However, where care has been taken to identify products, usually only one of the two forms has been found. For example, in plant and mammalian polysaccharides it is the D-isomers of glucose, xylose and mannose, and the L-isomers of fucose, rhamnose and arabinose that are invariably recovered. In the case of galactose, however, both the D- and L-isomers have been isolated, although the latter is considered to be of relatively rare occurrence. Nevertheless, it has been reported in certain red algae,[1] in a number of polysaccharides of higher plants,[2–4] in the albumin gland of snails[5] and in the gelatinous egg coats of certain sea urchin species.[6] D-Galactose is also usually present in each of these materials, often as a component of the same polysaccharide from which the L-enantiomer was isolated.

Roberts,[7] in experiments in which D-mannose-^{14}C was fed to corn roots, was able to isolate radioactive L-galactose from cell wall polysaccharide material. He pointed out that this sugar had never been identified in corn root before although several sugar analyses had been reported on this tissue. It was suggested that it might be a compound of much wider distribution than had previously been suspected because its chromatographic properties are identical with those of the D-enantiomer. In this paper we describe a method whereby L-galactose can be identified by GLC after the D-form has been oxidized to D-galactonolactone using D-galactose dehydrogenase.

[1] MORI, T. (1953) *Advan. Carbohyd. Chem.* **8,** 316.
[2] WHISTLER, R. L. and CORBETT, W. M. (1955) *J. Am. Chem. Soc.* **77,** 6328.
[3] ANDERSON, E. (1933) *J. Biol. Chem.* **100,** 249.
[4] WINTERSTEIN, E. (1898) *Ber. Chem. Ges.* **31,** 1571.
[5] BELL, D. J. and BALDWIN, E (1940) *Nature* **148,** 559.
[6] HUNT, S. (1970) *Polysaccharide-protein Complexes in Invertebrates*, Academic Press, London.
[7] ROBERTS, R. M. (1971) *Arch. Biochem. Biophys.* **145,** 685.

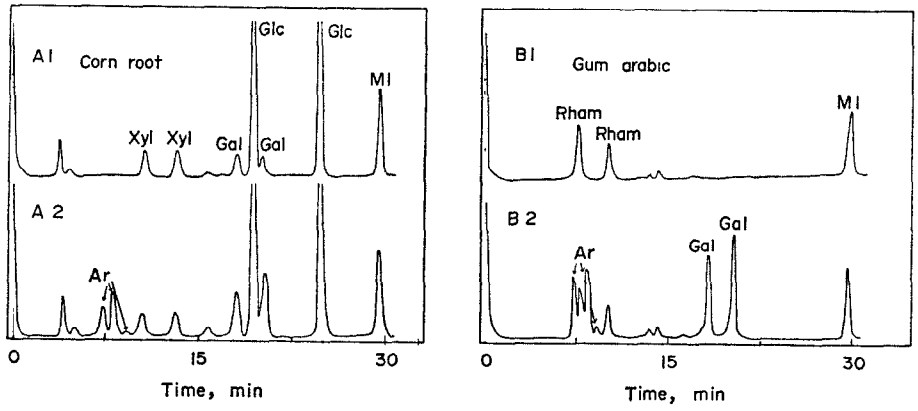

FIG. 1. GLC OF THE TMS DERIVATIVES OF THE NEUTRAL MONOSACCHARIDES RELEASED BY ACID HYDROLYSIS OF CORN ROOT RESIDUE (A) AND GUM ARABIC (B).
The upper trace in each case is of material that has been pretreated with D-galactose dehydrogenase in order to remove all of the D-galactose present

RESULTS

Specificity of D-*Galactose Dehydrogenase*

Under the conditions we employed D-galactose dehydrogenase catalyzed the complete oxidation of D-galactose over the range of concentrations (50–500 μg/ml) which are likely to be encountered in determinations on polysaccharide material. The product chromatographed identically with D-galactono-γ-lactone when acidic solvents were used for PC and was also retained on Dowex 1 (formate) ion exchange resin. By contrast L-galactose and most of the other sugars typically represented in polysaccharide material, namely D-xylose, D-glucose, D-mannose, L-rhamnose and L-fucose were not oxidized in presence of the enzyme and NAD^+.

Determination of the L-*Galactose Content of Polysaccharide Material*

The neutral sugar content of a number of polysaccharides that were known to contain L-galactose were examined. These have included the polysaccharide from flax and corn cob. We have also investigated material in which there have been no confirmed reports of L-galactose (represented here by gum arabic and *Acer* cell wall), and one material in which its identity had been inferred only from radioactive labelling experiments (corn root).

TABLE 1. NEUTRAL SUGAR COMPOSITION OF ACID HYDROLYZATES OF DIFFERENT POLYSACCHARIDE MATERIALS

Polysaccharide	Corn root	Corn cob	Sycamore	Flax	Gum arabic
	Neutral sugar composition (%)				
Arabinose	5·0	12 6	28·8	2·9	18·5
Xylose	4 1	73 0	8·5	9·2	—
Fucose	0·5	Tr	2·0	—	—
Rhamnose	0 5	Tr	6 6	15·4	15 1
Mannose	Tr	Tr	—	Tr	—
Glucose	77 0	9·9	29·4	58·3	—
D-galactose	10·2	3·2	21·7	6·7	66 4
L-galactose	2·7	1·3	3·0	7 5	—

Figure 1 illustrates the detector response when the TMS derivatives from corn root and gum arabic hydrolyzates were chromatographed with and without prior treatment with D-galactose dehydrogenase. Note that in both instances, the three characteristic peaks given by L-arabinose disappear completely. However, while in the case of gum arabic all of the galactose was oxidized, in the case of corn root a substantial amount of galactose remains after treatment with the enzyme, thus confirming the presence of L-galactose in this plant tissue.

Table 1 lists the composition of a number of hydrolyzed polysaccharides. L-Galactose was present in significant amounts in flax seed, corn cob hemicellulose and in corn root. As shown in Fig. 1, it was absent from gum arabic. However, it was detectable in small amounts in the hydrolyzates from *Acer* residue suggesting that it is a cell wall component of this tissue which has been overlooked in previous analyses.

DISCUSSION

This paper describes a method for detecting L-galactose in the hydrolysis products of polysaccharide that is much simpler than previous methods. As long as care is taken to ensure that all of the D-galactose is converted to the corresponding aldonic acid, the method is reproducible, quantitatively accurate and can be used on small amounts of material. However it should be stressed that the enzyme employed is strongly inhibited by one of the products of the reaction (NADH)[8] so that a large excess of NAD^+ should always be used. Further the enzyme from pseudonomads is not specific for D-galactose but will also catalyze the oxidation of L-arabinose.[9] Therefore enzyme from this source should not be employed for the spectrophotometric determination of D-galactose in crude sugar mixtures from plant polysaccharides which are likely to contain arabinose. This pentose sugar is of course not usually found in the complex saccharides of mammalian cells.

Our results confirmed the presence of L-galactose in polysaccharide from flax seeds,[3] corn cob[2] and corn roots.[7] In the latter it comprised almost a quarter of the total galactose released by hydrolysis using dilute mineral acid. As expected, L-galactose was not found in gum arabic. However, it should be pointed out that our analyses of this gum did not give the expected 3:3:1 ratio of arabinose to galactose to rhamnose.[10] Nevertheless the sample was old and not purified before use. Like the other materials employed in this study it probably represented an impure mixture of several polysaccharides. The presence of L-galactose in *Acer*, a tissue whose cell wall has recently received much attention,[11–13] was surprising and reinforces the belief that the L-galactose may be widespread in plants, but has been overlooked due to inadequate chromatographic identification. As L-galactose is synthesized via a different metabolic route than the D-isomer[14] and appears to occupy terminal positions at the non-reducing ends of branched polysaccharide chains,[2] the sugar might have a specific role in polysaccharide metabolism of plants.

[8] CUATRECASAS, P. and SEGAL, S. (1966) *J. Biol. Chem.* **241,** 5910.
[9] CLINE, A. L. and HU, A. S. L. (1965) *J. Biol. Chem.* **240,** 4493.
[10] SMITH, F. and MONTGOMERY, R. (1959) *The Chemistry of Plant Gums and Mucilages*, Reinhold, New York.
[11] TALMADGE, K. W., KEEGSTRA, K., BAUER, W. D. and ALBERSHEIM, P. (1973) *Plant Physiol.* **51,** 158.
[12] KEEGSTRA, K., TALMADGE, K. W., BAUER, W. D and ALBERSHEIM, P. (1973) *Plant Physiol.* **51,** 188.
[13] LAMPORT, D. I. A. (1970) *Annu. Rev. Plant Physiol.* **21,** 235.
[14] GOUDSMIT, E. S. and NEUFELD, E. F. (1967) *Biochem. Biophys. Res. Commun.* **26,** 730.

EXPERIMENTAL

Materials. Purified D-galactose dehydrogenase (from *Pseudomonas fluorescens*) and NAD^+ were brought from Sigma, St. Louis, Missouri. D-Galactose-^{14}C and L-galactose-^3H were products of Amersham-Searle Corporation, Arlington Heights, Illinois. Unlabeled D- and L-galactose (the latter a gift from Dr. J. Turner, The Radiochemical Centre, Amersham, England) were purified by passing solutions of the sugars through a mixed bed ion-exchange resin, concentrating the effluent under vacuum and crystallizing the material from hot 80% v/v EtOH.

Preparation of crude polysaccharide. Flax (*Linum usitatissimum*) dry seeds (5 g) were ground up in 80% v/v EtOH and insoluble products treated with 10% w/v KOH (100 ml) under N_2 overnight. The slurry was filtered and the filtrate neutralized with acetic acid. Polysaccharide material was precipitated from this solution by addition of 5 vol of EtOH, dehydrated and air-dried. Corn (*Zea mays*) root tips (1 cm) from 3-day-old seedlings were ground up in 80% EtOH The insoluble residue was used directly as a source of crude polysaccharide. Cell wall material from *Acer pseudoplatanus* cells grown in suspension cultures were prepared according to Roberts.[15] Corn cob hemicellulose B was isolated by conventional procedures.[16] Gum arabic was a commercial sample (City Chemical Co , N.Y.), manufactured in 1950.

Hydrolysis Portions of polysaccharide (usually 20–50 mg) were hydrolyzed under N_2 in sealed glass ampoules using 2 ml of 1 N HCl at 105° for 4 hr At this stage 100 μg of *myo*-inositol was added as an internal standard. The hydrolyzate was then dried thoroughly *in vacuo* in order to remove HCl, redissolved in H_2O, completely deionized by passing through a mixed bed ion-exchange resin, and divided into two portions One of these was dried and the trimethylsilyl derivatives prepared directly. The other was first treated with D-galactose dehydrogenase before trimethylsilylation.

Removal of D-galactose by treatment with D-galactose dehydrogenase. In order to convert all of the D-galactose present in the deionised hydrolyzate to D-galactonic acid the following reaction mixture was employed· 1 ml of sample, 0 5 ml 0 05 M NAD^+, 2·5 ml 0·1 M Tris-HCl buffer (pH 8·2) and 0 01 ml enzyme soln (*ca.* 0·1 units) at 30°. The reaction was run for up to 16 hr. A 1 ml portion of this mixture was placed in a 1 cm quartz cuvette and the extinction followed at 340 nm until no further increase in extinction was observed, at which stage the reaction was assumed to have reached completion However, in all cases a further portion of NAD^+ and enzyme was added to see if any further reduction of the NAD^+ occurred. In several pilot experiments a small amount of either D-galactose-1-^{14}C (0·05 μCi; 1 nmol) or L-galactose-1-^3H (0·2 μCi, 0·1 nmol) was added to provide an internal check for the complete oxidation of the D-sugar and for the lack of oxidation of the L-form. In these experiments small aliquots were removed after running the reaction and chromatographed overnight on Whatman No. 1 paper using *n*-BuOH–pyridine–HOAc–H_2O (5:5:3:1). The dried chromatogram strips were then scanned for radioactivity on a Packard Model 7201 radiochromatogram scanner Using this solvent more than 90% of the ^{14}C was detected over a spot coinciding with D-galactono-γ-lactone while none of the radioactivity was associated with the region of the chromatogram corresponding with a D-galactose standard. By contrast the L-galactose-^3H was unaffected by the enzyme both in presence or absence of added carrier After treatment, the remaining reaction mixture was dried down, redissolved in H_2O and passed successively through short columns of Dowex 1 (formate) and Dowex 50 (H^+) ion-exchange resins. This deionisation procedure was necessary in order to achieve complete trimethylsilylation in the subsequent step The Dowex 1 (formate) also removed the D-galactonic acid, as evidenced by the complete absence of ^{14}C in the final eluate. If necessary, the acid can be recovered by eluting the Dowex column with 3 bed vols of 0 1 N formic acid, and lactonized by heating in N formic for a few min. After drying it may then be converted directly to its TMS derivative.

Preparation of TMS derivatives Deionized solutions of the aldose sugars were dried thoroughly *in vacuo* and treated with 0 1 ml anhyd. pyridine, 0 2 ml hexamethyldisilazane and 0·1 ml trimethylchlorosilane. The mixture was allowed to stand for 15 min and dried down under a stream of N_2. The derivatives were then extracted in 0·1–0 2 ml CCl_4, and samples (usually 5 μl) used directly for GLC.

Procedure for GLC. The instrument used was a Hewlett-Packard F & M 402 gas chromatograph with dual FIDs. The carrier gas was nitrogen at a flow rate of 60 ml/min The columns were standard 1 8 m tubes, i d. 3 mm. Packing material was 3% (w/w) JXR on 100–120 mesh Gas Chrom Q Separations were achieved under the following temp. conditions· 4 min isothermal hold at 135° followed by a 2°/min increase to 200° The initial temp of the flash heater was 195°, that of the detector 215° The relative response factors for each sugar was calculated in relation to an internal standard of *myo*-inositol When run under these defined conditions D- and L-galactose gave two major peaks (R_ts 19·3 and 21 4 min) When the TMS derivative of L-galactono-γ-lactone is run simultaneously it appears as a single peak as a trailing shoulder on the first galactose peak (R_t 19 8 min).

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[15] ROBERTS, R. M , CETORELLI, J J , KIRBY, E. G and ERICSON, M. (1972) *Plant Physiol* **50,** 531.
[16] GRAMERA, R E and WHISTLER, R. L (1963) *Arch. Biochem. Biophys* **101,** 75