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THE EXTRACELLULAR POLYSACCHARIDES OF *Rhizobium japonicum*: COMPOSITIONAL STUDIES

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ABSTRACT

The extracellular polysaccharides of seven strains of *Rhizobium japonicum* were investigated by using a gas-chromatographic scheme developed for determination of the various sugars present. These polysaccharides were more heterogeneous in their composition than those of any other species of *Rhizobium* yet examined. Five strains (1809, 110, 123, 127, and 709) produced polysaccharides containing the same constituents, although in varying relative amounts: glucose (36–44%), galactose (7–25%), mannose (18–20%), 4-*O*-methylgalactose (5–13%), galacturonic acid (12–16%), and acetyl groups (4–8%). The sugars of the polysaccharide of strain 1809 were all of the D series. These are the first bacterial polysaccharides reported to contain 4-*O*-methylgalactose and the first *Rhizobium* polysaccharides in which galacturonic acid has been found. In contrast to this, the polysaccharide of strain 129 consisted of glucose (7%), galactose (51%), mannose (5%), xylose (5%), glucuronic acid (5%), and pyruvic acid (2%). The polysaccharide of strain 711 contained glucose (34%), galactose (13%), mannose (27%), and pyruvic acid (6%).

INTRODUCTION

The extracellular polysaccharides of *Rhizobium* species have been the subject of continuing interest since a role was proposed for them in the specificity of the symbiotic relationship between the bacteria and their legume plant hosts[1]. There have been numerous investigations of the composition of *Rhizobium* extracellular polysaccharides (see Discussion) but these have been concerned almost entirely with *Rhizobium trifolii*, *R. meliloti*, *R. leguminosarum*, *R. phaseoli*, and *R. lupini*. Very little attention has been given to the polysaccharides of *R. japonicum*, the organism responsible for the nodulation of soybeans. Particular attention was drawn recently to these last polysaccharides by the fascinating observation by Bohlool and Schmidt[2] that lectins isolated from the seeds and roots of soybean plants appear to react specifically with the polysaccharides of *R. japonicum*. Moreover, of the 25 strains of *R. japonicum* examined, three were totally unable to react with the lectins. These

results open a possible new mechanism for the specificity of the symbiosis of rhizobia and legumes and they make it important to know more about the polysaccharides of this species of *Rhizobium*.

The information available about these polysaccharides indicates that, compared with the polysaccharides of other *Rhizobium* species, they are unusually heterogeneous with respect to their composition. The polysaccharides of ten strains of *R. japonicum* have been examined and five found to contain only glucose and mannose[3,4], one to contain only glucose and rhamnose[5], and another to contain glucose, galactose, mannose, and rhamnose[6]. The remaining polysaccharides were reported to be glycuronans, all of different composition. One contained glucose, galactose, mannose, and glucuronic acid[4], the second contained glucose, rhamnose, and 4-*O*-methyl-glucuronic acid[5], and the third glucose, mannose, glucuronic acid, and 4-*O*-methyl-glucuronic acid[5].

The present paper describes a quantitative investigation of the composition of the extracellular polysaccharides of seven strains of *R. japonicum*, using an analytical scheme that emphasizes quantitative recovery of the components.

METHODS

Microorganisms. — The strains of *R. japonicum* used were obtained from the culture collection of the Division of Plant Industry (strains CC709, CC711, and CC1809) and from Dr. A. H. Gibson (strains 110, 123, 127, and 129). They were maintained on yeast extract–mannitol–agar slopes.

Chemicals. — All solvents and reagents were distilled and protected against moisture. Hexamethyldisilazane and the gas-chromatographic liquid phases OV-17, OV-101, and OV-225 were obtained from Pierce Chemical Co.; all other gas chromatographic materials used were products of Applied Science Laboratories, Inc. D-Galactose dehydrogenase was purchased from Boehringer Mannheim GmbH.

Polysaccharide production. — The defined liquid medium of Bergersen[7] was used. After the medium had been autoclaved, filter-sterilized solutions of thiamine (100 μg/litre), biotin (250 μg/litre), and calcium pantothenate (100 μg/litre) were added. Cultures (1 litre) were grown in 2-litre conical flasks incubated at 28° on a gyrotary shaker operated at 120 rev./min. When the cultures were viscous and dense ($5–10 \times 10^9$ cells/ml) (10–12 days), the incubation was stopped.

Microscopic examination of the cultures in the presence of India ink[8] showed considerable variation between strains in the distribution of insoluble polysaccharides, but in all cultures the bulk of the polysaccharide was insoluble. The cultures were diluted with 2 to 4 times their own volume of saline and stirred vigorously in a blender for 5 min. The diluted cultures were centrifuged at 27,000 *g* for 1 h and the polysaccharides precipitated from the supernatant fraction by addition of 3 to 4 volumes of acetone. The precipitates obtained were fine and granular, in contrast to the gelatinous, bulky precipitates characteristic of *R. trifolii* and *R. meliloti* polysaccharides.

TABLE I

OUTLINE OF SCHEME FOR ANALYSIS OF *R. japonicum* POLYSACCHARIDES BY GAS CHROMATOGRAPHY

Internal standard	*Agent of depolymerisation*	*Derivative*	*Column*	*Component analysed*[a]
Polysaccharide + pyrene	methanolic HCl	Me glycoside Me_3Si	OV-225	uronic acids
			OV-101	glucose
Polysaccharide + *myo*-inositol	0.5M H_2SO_4	alditol acetate	ECNSS-M	neutral sugars
		methoxime Me_3Si	OV-101	4-*O*-methylgalactose

[a]Peak areas estimated by measurement of peak height × width at half height, except in the case of alditol acetates (see text).

The polysaccharides were dissolved in water, treated with Permutit Zeokarb 225(H^+) resin to remove cations, dialysed for 3 days against 6 changes of distilled water, and dried from the frozen state. The ash content of these deionised polysaccharides was low (0.30–0.35%). Polysaccharide yields varied from 1.1 g (strains 1809 and 123) to 0.025 g (strain 709).

Colorimetric analyses. — Total neutral hexoses, uronic acids, pyruvic acid, and acetyl groups were determined, respectively, by the anthrone[9], carbazole[10], 2,4-dinitrophenylhydrazine[11] and hydroxamic acid methods[12], using the appropriate standards.

Hydrolysis. — The standard condition for hydrolysis of the polysaccharides was in 0.5M sulphuric acid for 16 h at 100°; experiments with the polysaccharide of strain 1809 showed that it gave maximum release of reducing sugar[13] in 10 h (51% as glucose), and this remained unchanged at 20 h. The hydrolysates were neutralised with barium carbonate, centrifuged, and the supernatant liquids treated with Zeokarb 225 (H^+) resin before subsequent analysis by paper or gas chromatography.

Paper chromatography. — Whatman No. 1 and water-washed No. 3MM papers were used, respectively, for analytical and preparative chromatography, all by downward development. The solvents used were: (A) (10:3:3) butanol–pyridine–water, (B) (18:3:1:4) ethyl acetate–acetic acid–formic acid–water, and (C) (5:1:4) butanol–ethanol–water. Sugars were detected by the silver nitrate–sodium hydroxide method[14], and by spraying with *p*-anisidine hydrochloride[15] and aniline phthalate[16].

Gas-chromatographic procedure for quantitative analysis. — Attempts to simultaneously analyse all of the constituent sugars by gas chromatography on one column were unsuccessful. The following series of analytical procedures, was used instead (see Table I for outline). Gas-chromatographic analyses were performed on a Pye series 104, model 64, dual-column instrument fitted with flame-ionization detectors. Stainless-steel columns (3 m × 2 mm i.d.) and a carrier-gas (nitrogen) flow-rate of 30 ml/min were used throughout.

(*a*) *Determination of glucose and uronic acids by methanolysis and silylation.* Polysaccharide samples (5 mg) were weighed in glass ampoules and the internal standard (pyrene, 2–3 mg), added as a solution in benzene (0.50 ml). Dry methanolic hydrogen chloride (1 ml, 2M) was added. The ampoules were flushed with nitrogen, sealed, and heated at 100°. A time-course experiment with the 1809 polysaccharide established that recoveries of glucose and galacturonic acid were independent of time in methanolyses ranging from 6 to 24 h; a standard duration of 16 h was used for the results described here. The samples were treated as described by Clamp, Bhatti, and Chambers[17], except for the omission of the acetylation step and for the use of a larger volume (1 ml) of the trimethylsilylating reagent.

Glucose was determined on columns of OV-101 [3% on Chromosorb W(HP), 80–100 mesh] using a temperature programme starting at 140° and heating at 0.5°/min. The glucose peaks were well separated from those of the other constituents, which all had shorter retention-times (Fig. 1*a*,*b*).

Galacturonic acid was determined by analysing the same mixtures by gas

chromatography on columns of OV-225 (3% on Gas-Chrom Q, 80–100 mesh), using a temperature programme beginning at 100° and heating at 3°/min. Standards of galacturonic acid under these conditions yielded four peaks (Fig. 1*c*); however, in the trimethylsilylated polysaccharide methanolysate, the first two of these peaks were not separated from glucose peaks (Fig. 1*d*). Therefore, the determination of galacturonic acid in the polysaccharide methanolysates depended on measurement of the areas of the final two peaks (which accounted for 42% of the total peak area for galacturonic acid).

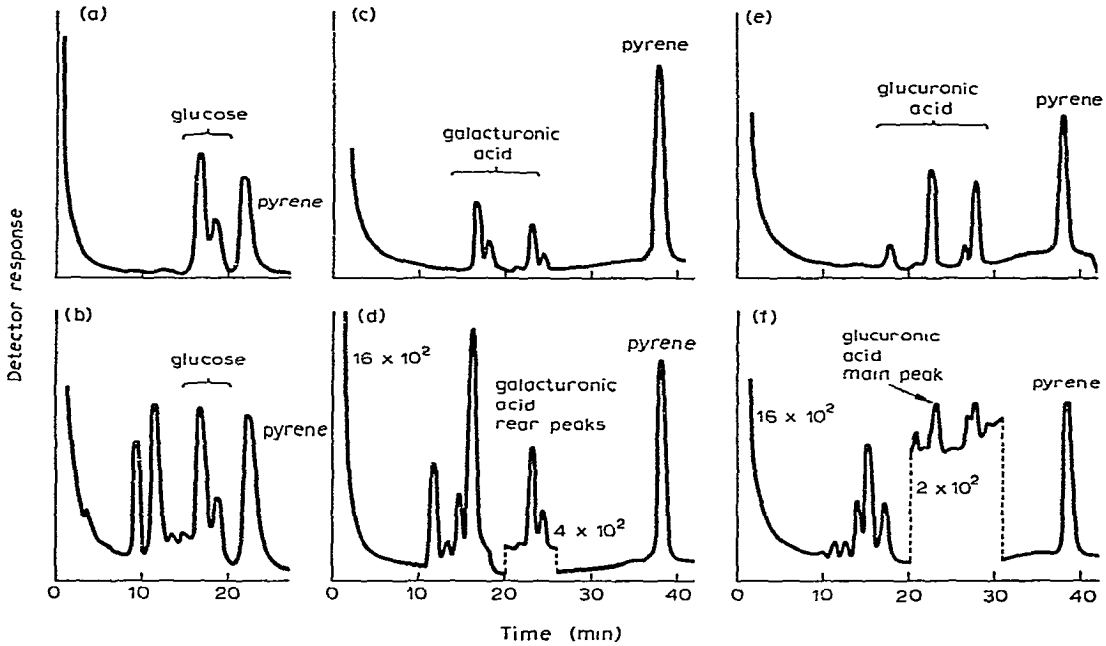

Fig. 1. Analysis of polysaccharides for glucose and uronic acids by methanolysis and silylation, using pyrene as the internal standard. (*a*) Glucose standard; (*b*) methanolysate of 1809 polysaccharide; (*c*) galacturonic acid standard; (*d*) methanolysate of 1809 polysaccharide, the same as in (*b*) but here analysed on a different column in which the rear peaks of galacturonic acid, used in quantitation, are the last components to emerge; (*e*) glucuronic acid standard; (*f*) methanolysate of 129 polysaccharide, showing glucuronic acid peak used in quantitation. Chromatograms (*a*) and (*b*) obtained with OV-101 columns temperature-programmed from 140° at 0.5°/min; chromatograms (*c*)–(*f*) obtained with OV-225 columns temperature-programmed from 100° at 3°/min.

Glucuronic acid was determined in a similar way (Fig. 1*e*,*f*) but using the main peak of the four peaks obtained with this acid (retention time relative to main galacturonic acid peak, 0.96); this peak represented 49% of the total peak area for glucuronic acid.

(*b*) *Determination of glucose, galactose, 4-O-methylgalactose, mannose, and xylose after hydrolysis.* Samples of the polysaccharides (5–10 mg) and the internal

standard, *myo*-inositol (2–5 mg), were weighed into ampoules and 2 ml of 0.5M sulphuric acid was added. The ampoules were sealed and heated for 16 h at 100°. The hydrolysates were neutralised with barium carbonate, centrifuged, and the supernatant liquids treated successively with the ion-exchange resins (Permutit) Deacidite FF (acetate form), and Zeokarb 225 (H^+). The solutions were then divided into two equal portions for separate further treatment.

In one portion, the sugars were reduced with sodium borohydride and acetylated[18]. Afterwards, water (5 ml) was added to each mixture, which was extracted with chloroform (approx. 10 ml). The extract was then washed with 5% sodium hydrogen carbonate and water, and finally dried over anhydrous sodium sulphate. Samples of the concentrated extracts were injected into the gas chromatograph, using ECNSS-M [3% on Chromosorb W(AW) 80–100 mesh] columns operated isothermally at 190°. All of the alditol acetate peaks were well separated, except for that of the 4-*O*-methylgalactose derivative, which had a retention time intermediate between those of the mannose and galactose derivatives (Fig. 2); the presence of 4-*O*-methylgalactose therefore resulted in severe overlapping of peaks in this region of the chromatograms. These chromatograms were analysed by a least-squares, curve-fitting, iterative computer method[19] which allowed resolution of all the peaks. Comparison of the peak areas obtained in this way with those obtained by a number of manual procedures for peak-area estimation showed that very similar values could also be obtained by tracing the peak outlines onto translucent weighing

TABLE II

COMPOSITION OF *R. japonicum* POLYSACCHARIDES

Strain	*Total neutral sugars (anthrone)*		*Glucose*		*Galactose*	*Mannose*	*4-O-methyl galactose*	
	Exptl %	*Calc*[a] %	*M-Me₃Si*[b] %	*AA*[b] %	AA_{corr}[c] %	AA_{corr} %	AA_{corr} %	*MO-Me₃Si*[b] %
1809	71.3	68.8	43.4	26.0	7.2	18.5	12.0	12.1
123	73.0	69.4	42.8	37.2	6.8	19.7	13.2	13.8
127	65.8	67.8	39.9	25.1	10.4	18.4	12.6	14.3
110	66.7	68.4	36.0	19.4	24.5	17.9	5.3	9.9
709	61.0	62.4	39.5	29.4	10.3	18.1	6.0	6.5
							xylose %	
129	56.2	46.5	7.1	8.0	50.8	5.3	4.6	
711	58.8	60.0	33.8	19.7	13.0	26.5	0	

[a]Anthrone values calculated from sugar percentages and anthrone equivalents (see text). [b]M-Me_3Si, methlysis Me_3Si result; AA, hydrolysis alditol acetate result; MO-Me_3Si hydrolysis methoxime-Me_3Si r [c]AA_{corr}, result obtained by the alditol acetate method after correction for the difference between glucose mined by M-Me_3Si and AA methods, respectively. [d]Carbazole values corrected for neutral sugars. [e]Obt by using the M-Me_3Si and AA_{corr} results, as appropriate, for each sugar component. [f]Resorcinol meth results not included in total recovery figures.

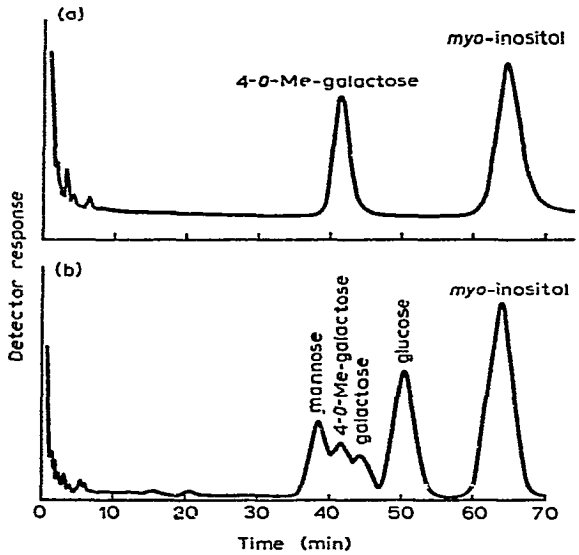

Fig. 2. (*a*) Chromatogram of alditol acetate derivative of 4-*O*-methyl-D-galactose (isolated from 1809 polysaccharide) and *myo*-inositol standard. (*b*) Chromatogram of hydrolysate of 127 polysaccharide as the alditol acetates in the presence of *myo*-inositol as internal standard. Analysed on ECNSS-M columns isothermally at 190°.

Galacturonic acid		*Acetyl*	*Pyruvate*	*Protein* ($N \times 6.25$)	*Total recovery*[e]	*3,6-Anhydro sugar*[f]
M-Me$_3$*Si*	*Carbazole*[d]					
%	%	%	%	%	%	%
16.1	13.1	4.1	0	0.3	101.7	0
13.9	10.6	8.0	0	1.1	105.5	0
12.3	10.5	6.5	0	1.5	101.6	0
11.8	12.0	4.1	0	1.6	101.2	9.7
15.4	13.4	6.4	0	1.4	97.1	0
Glucuronic acid						
%	%					
4.9	3.2	0	1.9	3.0	77.6	31.0
0	0	0	6.1	3.2	82.6	0

paper, separating the areas for the overlapped components by dropping perpendicular lines from the troughs between the peaks to the base line, and estimating the area of each peak by cutting and weighing the paper pieces. For the sake of conformity with all of the other gas-chromatographic analyses reported here, the values obtained by the manual method are shown in Table II. However, this hydrolysis–alditol acetate procedure gave low recoveries for total neutral sugars compared with the anthrone method and for glucose compared with the values obtained by the foregoing methanolysis procedure; these low recoveries were assumed to have arisen through a combination of incomplete hydrolysis of the polysaccharides and the decomposition of some of the liberated sugars[20]. A correction was therefore applied to the alditol acetate values by multiplying the result for each sugar by the same ratio as that required to convert the glucose results obtained by hydrolysis to equal those obtained by methanolysis (Table II). The conversion factor varied with each polysaccharide sample.

The second portion of each hydrolysate was dried over phosphorus pentaoxide and used for the determination of 4-*O*-methylgalactose as the trimethylsilylated *O*-methyl oxime (MO-Me_3Si) derivative[21]. These preparations were analysed on OV-101 columns using a temperature programme starting at 140° and heating at a rate of 0.5°/min. Excellent separation of the 4-*O*-methylgalactose peak from the combined glucose, galactose, and mannose peak and the *myo*-inositol peak was obtained (Fig. 3), making the MO-Me_3Si derivatives useful as confirmatory evidence

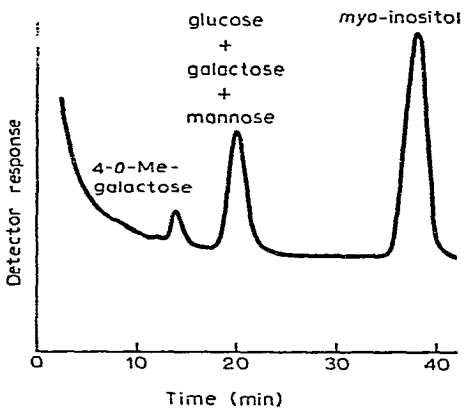

Fig. 3. Chromatogram of hydrolysate of 123 polysaccharide analysed as the trimethylsilylated *O*-methyl oximes on OV-101 columns temperature-programmed from 140° at 0.5°/min.

of the presence of 4-*O*-methylgalactose, but quantitation against the inositol standard was not reproducible. However, it was possible to quantitate the 4-*O*-methylgalactose MO-Me_3Si peaks by comparison with the combined glucose, galactose, and mannose MO-Me_3Si peaks because the amounts of these latter sugars present in the polysaccharides was known from the alditol acetate separations.

RESULTS

Identification of components

Polysaccharide of strain 1809. — Five major components were detected by hydrolysis and paper chromatography, using the silver nitrate–sodium hydroxide reagents. Glucose, galactose, and mannose were recognised by comparison with standards and by the colours obtained with the *p*-anisidine and aniline phthalate sprays. These sugars were accompanied by a component having a mobility slightly greater than that of mannose and another component that migrated slower than all the others in all of the solvents. Preparative paper chromatography in solvent A was used to isolate samples of each of the components. The fastest-moving component required additional chromatography in solvent C to remove mannose and glucose.

The isolated glucose, galactose, and mannose were confirmed to be these sugars by gas chromatography as the Me_3Si derivatives. Polarimetry of mutarotated solutions showed them to be D-glucose ($[\alpha]_D$ +52.1°) and D-mannose ($[\alpha]_D$ +13.5°). The rotation of the isolated galactose could not be determined because of contamination by glucose but it was established to be D-galactose by oxidation with D-galactose dehydrogenase[22].

The slow-migrating component was indicated to be a uronic acid by its strongly positive carbazole reaction and its failure to react with the anthrone reagent. It was identified as D-galacturonic acid by gas chromatography[23] and by polarimetry of a mutarotated solution ($[\alpha]_D$ +50.9°).

The fast-moving component readily formed crystals (colourless prisms) when concentrated in aqueous solution. Colour reactions with this component on filter paper sprayed with *p*-anisidine and aniline phthalate reagents suggested that it was a methylated hexose. It was found to contain 15.2% OCH_3, which was consistent with it being a monomethylated hexose (calculated value, 15.98% OCH_3). The parent sugar was identified as galactose by demethylation[24] with boron trichloride, isolation of the product by paper chromatography, and conversion into the alditol acetate. The melting point (209–211°) of the methylated sugar indicated it to be 4-*O*-methyl-galactose. This was confirmed by mass spectrometry of the alditol acetate[25] and MO-Me_3Si (ref. 21) derivatives and by n.m.r. spectroscopy[26] of the methylated galactose in D_2O. Its mutarotation ($[\alpha]_D$ +56° → +76°)[27] confirmed it to be the D enantiomer. The polysaccharide contained 2.4% OCH_3, which, if entirely due to the methylated galactose, would indicate the presence of 15.0% of 4-*O*-methyl-D-galactose.

Paper chromatograms of hydrolysates of the polysaccharide, when treated with the silver nitrate–sodium hydroxide reagents, also showed faint spots nearer the solvent front. One component, having an extremely high mobility (R_F 0.9, solvent A), reacted with *p*-anisidine in the cold to give a yellow colour. It was highly u.v.-absorbing when unsprayed chromatograms were examined in u.v. light. The component was eluted with water and its u.v. absorption spectrum found to be identical with that of 5-(hydroxymethyl)-2-furaldehyde[28]. It is believed to be a product of

degradation of sugars during hydrolysis. None of the other trace components detected on the chromatograms were identified.

The ferric hydroxamate reaction for esters[12] gave a positive reaction with the polysaccharide of strain 1809, equivalent to the presence of 4% acetyl. The acyl substituent was confirmed to be acetyl by extraction and paper-chromatographic comparison with acethydroxamic acid in solvents A, B, and C. The polysaccharide gave negative reactions for pyruvate and for other carbonyl constituents[29].

With or without prior heat treatment, the 1809 polysaccharide failed to react in immunodiffusions with antiserum prepared against the whole culture[30], and it is concluded that these polysaccharides are not antigenic.

Polysaccharides of strains 110, 123, 127, 709, and 711. — Paper chromatography showed that these polysaccharides contained the same sugars as the polysaccharide of strain 1809, except that strain 711 contained only glucose, galactose, and mannose and lacked both 4-*O*-methylgalactose and the uronic acid. The identity of each sugar was confirmed by gas chromatography. In addition, the galacturonic acid and 4-*O*-methylgalactose constituents of each of the polysaccharides were isolated by preparative paper chromatography and their individual gas-chromatographic behaviour examined. The galacturonic acids were compared after treatment with methanolic hydrogen chloride and silylation and all found to yield identical patterns of peaks. The 4-*O*-methylgalactose fractions were compared both as the MO-Me_3Si derivatives and as the aldononitrile acetates[31] by gas chromatography on OV-101 and ECNSS-M columns, respectively; all fractions behaved identically with the corresponding material isolated from the polysaccharide of strain 1809.

Four of these polysaccharides gave positive results in the colorimetric acetyl reaction (Table II), but only the polysaccharide of strain 711 gave a positive result for pyruvate, which was confirmed by paper chromatography of the 2,4-dinitrophenylhydrazone in solvent C.

Polysaccharide of strain 129. — This polysaccharide differed markedly from those of the other *R. japonicum* strains examined here. Paper chromatography revealed that galactose was the major component, with minor amounts of glucose and mannose. Its uronic acid was different, showing a trailing spot instead of the single discrete spots obtained with galacturonic acid. Furthermore, it possessed a component having a mobility slightly greater than that of 4-*O*-methylgalactose; this component was indicated to be a pentose when it was found to give a pink colour with aniline phthalate spray. The uronic acid and pentose components were isolated by preparative paper chromatography and their identities confirmed by gas chromatography. The uronic acid component was treated with methanolic hydrogen chloride, silylated and examined on OV-101 columns; it was found to be glucuronic acid. The pentose component was converted into the alditol acetate and examined on a column of ethylene glycol succinate (1.5%) and silicone GE XF-1150 (1.5%) on Chromosorb W(AW) (80–100 mesh) operated isothermally at 190°; it was identified as xylose.

The polysaccharide gave a negative result for acetyl groups and also differed from the other polysaccharides in this study by being the only one to give a strongly

positive result in the resorcinol reaction for 3,6-anhydro sugars[32]. This polysaccharide yielded the strongest yellow colour in its solutions after hydrolysis, suggesting a pronounced tendency to form 5-(hydroxymethyl)-2-furaldehyde; the polysaccharide component responsible for this has not been identified. A small proportion of pyruvic acid was detected and confirmed by paper chromatography in solvent C.

Quantitative analysis of the polysaccharides

After the constituent sugars of the polysaccharides had been identified, a self-consistent scheme for quantitative analysis was sought. No single form of derivative nor single gas-chromatographic column was found to be applicable to all the sugars known to be present, and thus the scheme outlined in Table I was developed. It was not possible to discover a compound that would be satisfactory as the internal standard for both methanolysis and aqueous acid hydrolysis. *myo*-Inositol was excellent for the aqueous system but, being insoluble in methanolic hydrogen chloride, gave inconsistent results as a standard in methanolysis. Other compounds (hexitols and heptitols) were tried, but their retention times were not suitable for use with both the OV-101 and OV-225 columns that were necessary in order to obtain satisfactory separation of the glucose and uronic acid peaks, respectively, from the other constituents of the polysaccharides.

The polysaccharides were analysed as described (Methods), with the results shown in Table II.

DISCUSSION

The polysaccharides analysed in this investigation were purposely not fractionated, so that no components would be inadvertently removed and thereby missed. Instead, emphasis was given to attempting to obtain a quantitative "balance-sheet" of all constituents, in order to minimize the risk of overlooking non-sugar components[33,34].

An unavoidable complication to completeness of recovery of constituents arises from resistance to hydrolysis and the lability of free sugars to acid conditions[20]. Methanolysis is an excellent alternative to hydrolysis for the depolymerization of polysaccharides[17], and the present results indicate that quantitative recoveries of sugars of *R. japonicum* polysaccharides can be obtained by methanolysis. However, it was not possible to separate more than the glucose and uronic acid peaks in the trimethylsilylated methanolysate mixtures, and this made it necessary to use the alditol acetates[18] to determine the relative amounts of the neutral sugars. These relative values were then converted into absolute values by using the glucose values obtained by methanolysis. The general reliability of this procedure is shown by the agreement between the results obtained for (*a*) the 4-*O*-methylgalactose content of polysaccharide 1809 by this alditol acetate procedure (12.0%), by the MO-Me_3Si method (12.1%), and from the methoxyl content of the polysaccharide (15.0%); (*b*) the correlation between the galacturonic acid content of the polysaccharide of

strain 1809 determined after methanolysis (16.1%) and independently by titration (17.0%). The overall validity of the results is supported by the totals of the constituents recovered (Table II), and also by the correlation between the total neutral hexoses (as determined experimentally by the anthrone reaction) and the equivalent anthrone values obtained by calculation from the composition data derived by gas chromatography using the "anthrone equivalents" of glucose (1.00), galactose (0.71), 4-*O*-methylgalactose (0.71), and mannose (0.63).

The lability of certain types of sugar to acid hydrolysis cannot be overlooked. The detection of significant amounts of 5-(hydroxymethyl)-2-furaldehyde in the hydrolysate of polysaccharide 1809 suggests the presence of a sugar in a form that leads readily to this compound. 5-(Hydroxymethyl)-2-furaldehyde is frequently obtained in trace amounts from sugars heated in acid solution[28], but the amounts obtained with some of these polysaccharides of *R. japonicum* were unusually high. This is similar to the experience of Metz[35], who found the lipopolysaccharide of only one strain of *Escherichia coli*, from over 100 strains examined, to yield significant amounts of 5-(hydroxymethyl)-2-furaldehyde in hydrolysates; there was nothing detectably different about this particular lipopolysaccharide to account for the observation. Investigations of κ-carrageenan[36] and agar[37] have shown that, although 3,6-anhydrogalactose is present in large proportion, it is not found in acid hydrolysates of the polysaccharides because it is readily decomposed to 5-(hydroxymethyl)-2-furaldehyde instead. Two of the present *R. japonicum* polysaccharides (110 and 129) gave positive results in determinations of 3,6-anhydro sugars by the resorcinol method[32], but the significance of this observation is not immediately obvious because ketoses also give strong reactions in this assay.

The present results show that the extracellular polysaccharides of *R. japonicum* are markedly different from those of the other species of rhizobia that have been examined. Although the proportions of sugars reported by various investigators vary somewhat, possibly because of differences in technique, the overall results are essentially similar and show that, except for *R. japonicum*, all strains of a given *Rhizobium* species appear to produce very similar polysaccharides. Thus, the polysaccharides of the 17 strains of *R. meliloti* that have been examined all contain glucose (60–86%), galactose (8–13%), glucuronic acid (0–2%), pyruvic acid (5–9.6%), and acetyl groups (4–9%)[33,38–42]. Similarly, the polysaccharides of the 26 strains of *R. trifolii* that have been studied all contain the same components: glucose (35–61%), galactose (9–13%), glucuronic acid (13–21%), pyruvic acid (6–11%), and acetyl groups (3–14%)[33,41–44]. Small proportions (2–5%) of mannose have been reported[45] in some polysaccharides of *R. trifolii*, but this sugar may have arisen by contamination from yeast extract used in the growth medium[46]. The polysaccharides of *R. leguminosarum* and *R. phaseoli* appear to be very similar to those of *R. trifolii*[41,42].

In contrast to this generalization, the polysaccharides of the seven strains of *R. japonicum* examined here were heterogeneous and exhibited three qualitatively different types. The largest number (1809, 123, 127, 110, and 709) formed one group, whereas the second and third types of polysaccharide were each represented by single

strains (711 and 129, respectively) (Table II). These polysaccharides differ from all other *Rhizobium* polysaccharides by their consistent content of mannose and by the presence of galacturonic acid and 4-*O*-methylgalactose, neither of which have hitherto been reported in these polysaccharides. This is the first reported occurrence of 4-*O*-methyl-D-galactose in bacterial polysaccharides; small amounts have been found in peat[47]. 4-*O*-Methyl-L-galactose is known in seaweed polysaccharides[48].

The compositional heterogeneity observed among *R. japonicum* polysaccharides is very interesting in the light of the interaction between soybean lectins and a majority, but not all, strains of *R. japonicum*[2]. Two of the lectin-reactive strains (110 and 123) were included in the present polysaccharide investigation. It will be extremely interesting to see whether there is any relationship between the other polysaccharide types of *R. japonicum* and their ability to react with the soybean lectins. These and other investigations with the *R. japonicum* polysaccharides are continuing.

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