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STRUCTURAL STUDIES ON THE SPECIFIC CAPSULAR POLYSACCHARIDE FROM *Rhizobium trifolii*, TA-1*†

A. S. CHAUDHARI‡, C. T. BISHOP, AND W. F. DUDMAN**

Division of Biological Sciences, National Research Council of Canada, Ottawa K1A 0R6 (Canada)

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ABSTRACT

The repeating unit of the specific capsular polysaccharide from the bacterium *Rhizobium trifolii* TA-1 has been shown to contain (*a*) terminal 4,6-*O*-(1-carboxyethylidene)-D-galactose (1 residue), (*b*) (1→3)-linked 4,6-*O*-(1-carboxyethylidene)-D-glucose (1 residue), (*c*) (1→4)-(1→6)-linked D-glucose (1 residue), (*d*) (1→4)-linked D-glucuronic acid (1 residue), and (*e*) (1→4)-linked D-glucose (4 residues). The pyruvylated sugars were shown to be positioned sequentially, and at least one other unit was interposed between them and the branch point.

INTRODUCTION

Bacteria of the genus *Rhizobium* play an important role in nitrogen fixation by infecting the roots of leguminous plants and forming nodules from which the nitrogen fixed is transferred to the plant. There is evidence that the specificity of interaction between *Rhizobium* species and their plant hosts is determined by the capsular polysaccharides of the bacteria[1,2]. It has also been established that the serological specificities of *Rhizobia*, useful for identifying the organisms, depend upon their polysaccharides[3,4]. Chemical structural investigations appear to have been reported on only one of these polysaccharides, that from *Rhizobium meliloti*[5]; the present paper describes results of structural investigations on the specific polysaccharide from *Rhizobium trifolii*, strain TA-1.

The crude polysaccharide was soluble in saline solution and could be separated from the cells simply by centrifugation. As isolated, this material gave two antigen bands on immunodiffusion against homologous antiserum (well 1, Fig. 1). Passage of the crude polysaccharide over an anion-exchange resin in the hydrogen carbonate form removed the minor antigenic component and yielded a purified antigen that gave a single, strong immunoprecipitin band (well 2, Fig. 1). In agreement with previous serological studies[3,4], depyruvylation of the purified polysaccharide destroyed completely its serological activity with homologous antiserum (well 3, Fig. 1).

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*Dedicated to Dr. Louis Long, Jr., in honor of his 70th birthday.

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‡NRCC Postdoctorate Fellow 1970–72.

**Division of Plant Industry, CSIRO, Canberra City, Australia.

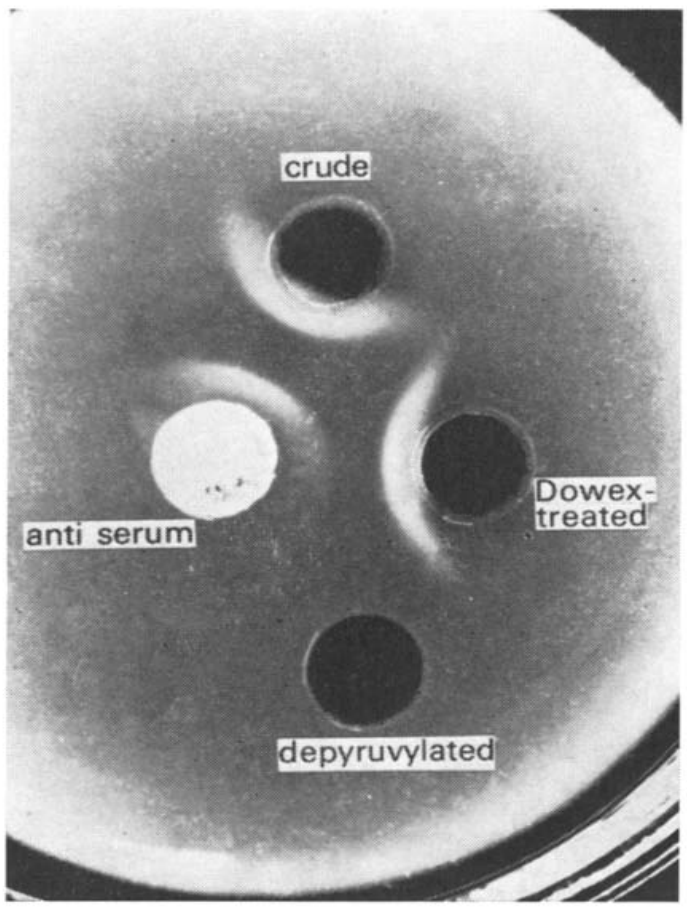


Fig. 1. Immunodiffusion of *Rhizobium trifolii* TA-1 polysaccharide: (1) crude polysaccharide, (2) polysaccharide purified by ion-exchange, (3) depyruvylated polysaccharide, (4) rabbit antiserum to *Rhizobium trifolii* TA-1.

The analytical data for the purified polysaccharide are given in Table I. The uronic acid content is lower than the 23% found by decarboxylation or by the carbazole reaction[3]; however, the present method is a direct one and confidence in its

TABLE I

PROPERTIES OF *Rhizobium trifolii* TA-1 POLYSACCHARIDE

$[\alpha]_D^{22}$	−8° (*c* 1, water)	*O*-acetyl[6]	4.3%
C	39.96%	Pyruvate[7]	9.7%
H	5.19%	D-Glucuronic acid[a]	14.8%
N	nil	D-Glucose[8]	61.0%
P	nil	D-Galactose[8]	9.8%
Ash	0.8%		

[a]Calculated from the increase in 2,3,6-tri-*O*-methyl-D-glucose after remethylation of the carboxyl-reduced polysaccharide (Columns B and C, Table II).

reliability is provided by the agreement between the results on both the native and depyruvylated polysaccharides (see increase of 14.7% in 2,3-di-*O*-methyl-D-glucose after reduction of methylated, depyruvylated polysaccharide, columns A and B, Table III).

To determine the location of the pyruvate groups and the linkage of the uronic acid residues, the following sequences of reactions were done and the neutral, methyl-

ated sugars were analysed as indicated: (*a*) the native polysaccharide was methylated (Table II, column A; Fig. 2, A), (*b*) the methylated native polysaccharide was reduced (Table II, column B; Fig. 2, B), (*c*) the reduced, methylated, native polysaccharide was remethylated (Table II, column C; Fig. 2, C), (*d*) the native polysaccharide was depyruvylated, (*e*) the depyruvylated polysaccharide was methylated (Table III, column A; Fig. 3, A), (*f*) the methylated, depyruvylated polysaccharide was reduced (Table III, column B; Fig. 3, B), (*g*) the native polysaccharide was degraded by partial acid hydrolysis, (*h*) the degraded polysaccharide was methylated (Table III, column C), and (*i*) the methylated, degraded polysaccharide was reduced (Table III, column D).

TABLE II

METHYL ETHERS FROM HYDROLYSES OF METHYLATED *Rhizobium trifolii*, TA-1 POLYSACCHARIDE

Peak (*Fig. 2*)	*Sugar*	T[a]	*Primary fragments* (*Mass spectra*, m/e)	*Mole per cent*[b]		
				A[c]	*B*	*C*
(1)	2,3,6-tri-*O*-methyl-D-glucose	2.50	45, 117, 233	46.7	46.8	61.7
(2)	2,3-di-*O*-methyl-D-glucose	5.35	117, 261	14.6	28.2	14.6
(3)	2,3-di-*O*-methyl-D-galactose	5.66	117, 261	10.4	11.0	10.3
(4)	2-*O*-methyl-D-glucose	7.80	117	13.2	14.0	13.4

[a]Retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on column packing *B*. [b]A. Methylated, native polysaccharide. B. Methylated, native polysaccharide after reduction. C. Methylated, native polysaccharide after reduction and remethylation. [c]These values do not include 15% of D-glucuronic acid derivatives.

TABLE III

METHYL ETHERS FROM HYDROLYSES OF METHYLATED, DEPYRUVYLATED AND DEGRADED *Rhizobium trifolii*, TA-1 POLYSACCHARIDE

Peak (*Fig. 3*)	*Sugar*	T[a]	*Primary fragments* (*Mass spectra* m/e)	*Mole per cent*[b]			
				A[c]	*B*	*C*[c]	*D*
(1)	2,3,4,6-tetra-*O*-methyl-D-glucose	1.0	45, 117, 161, 205	4.3	4.4	23.7	22.5
(2)	2,3,4,6-tetra-*O*-methyl-D-galactose	1.15	45, 117, 161, 205	10.2	10.7	—	—
(3)	2,4,6-tri-*O*-methyl-D-glucose	1.98	45, 117, 161, 233	15.8	15.2	—	—
(4)	2,3,6-tri-*O*-methyl-D-glucose	2.50	45, 117, 233	41.5	41.7	48.8	52.8
(5)	2,3-di-*O*-methyl-D-glucose	5.35	117, 261	13.3	28.0	12.5	24.7

[a]Retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on column packing B. [b]A. Methylated, depyruvylated polysaccharide. B. Methylated, depyruvylated polysaccharide after reduction. C. Methylated, degraded polysaccharide. D. Methylated, degraded polysaccharide after reduction. [c]These values do not include 15% of D-glucuronic acid derivatives.

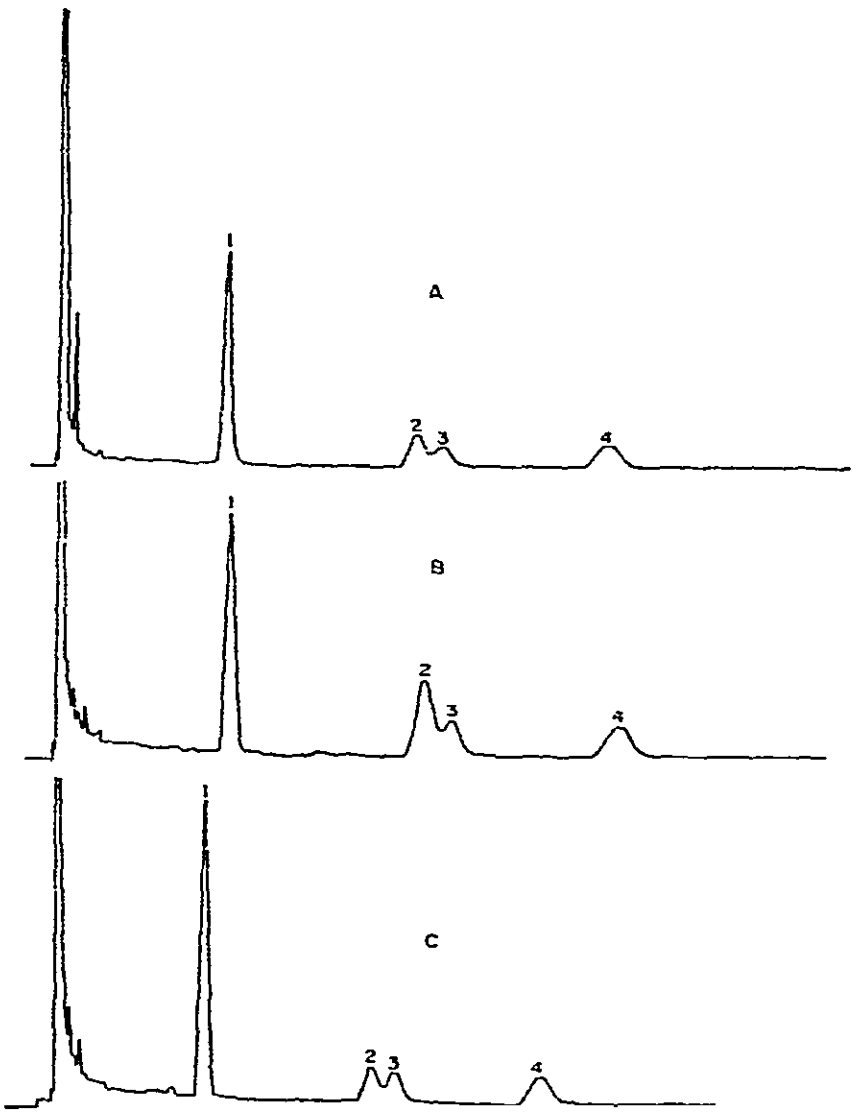


Fig. 2. Gas–liquid chromatograms of methylated sugars from *Rhizobium trifolii* TA-1 polysaccharide: (A) methylated native polysaccharide, (B) methylated, reduced polysaccharide, (C) methylated, reduced, remethylated polysaccharide. For peak identities see Table II.

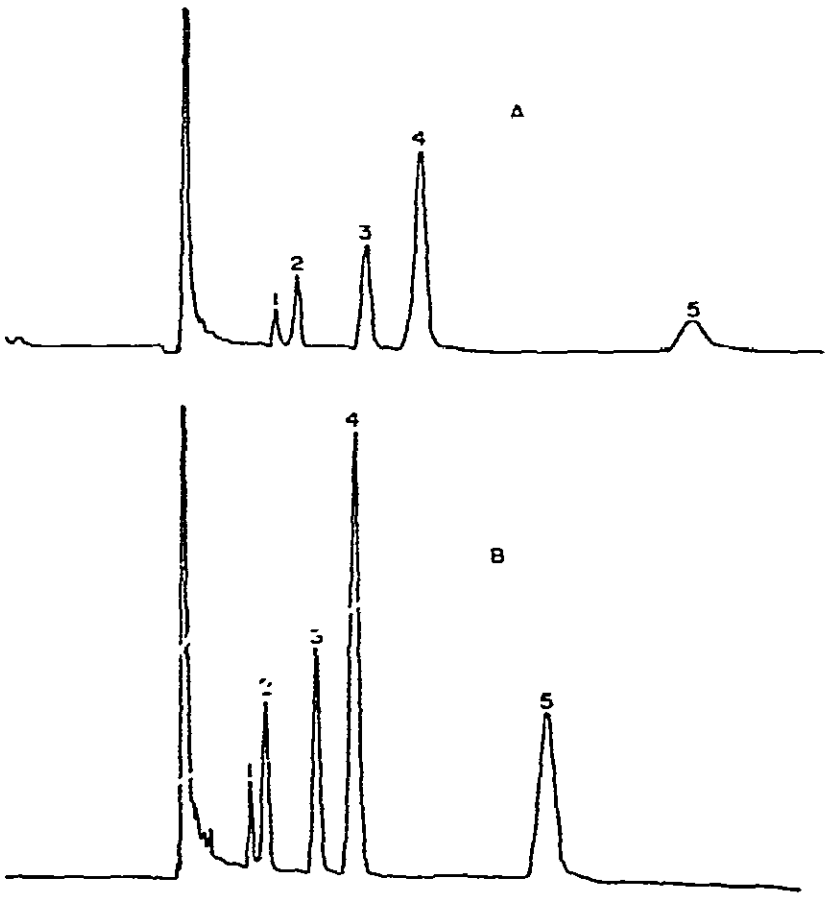


Fig. 3. Gas–liquid chromatograms of methylated sugars from depyruvylated *Rhizobium trifolii* TA-1 polysaccharide: (A) methylated polysaccharide, (B) methylated, reduced polysaccharide. For peak identities see Table III.

From the data in Table II it can be concluded that (1→4)-linked D-glucose residues form the major structure feature of the polysaccharide. When the methylated polysaccharide (column A) was reduced (column B), the only change in the products of hydrolysis was an increase in the amount of 2,3-di-*O*-methyl-D-glucose. That this increase was due to the reduction of the carboxyl groups in 2,3-di-*O*-methyl-D-glucuronic acid residues was confirmed by remethylation (column C), when there was a corresponding increase in the amount of 2,3,6-tri-*O*-methyl-D-glucose and the amount of 2,3-di-*O*-methyl-D-glucose decreased to the same value as found before reduction .The other products listed in Table II could have arisen from branch points or from pyruvylated residues. The distinction between those two possibilities was revealed by the results in Table III obtained from the depyruvylated polysaccharide Thus, all of the D-galactose turns up as the tetra-*O*-methyl derivative, showing that the 2,3-di-*O*-methyl-D-galactose in Table II must have arisen from nonreducing, terminal 4,6-*O*-(1-carboxyethylidene)-D-galactose fragments. Similarly, the 2,4,6-tri-*O*-methyl-D-glucose (Table III) was not present in the products from the native polysaccharide (Table II) and 2-*O*-methyl-D-glucose (Table II) was not found in the depyruvylated product. It must be concluded, therefore, that the 2-*O*-methyl-D-glucose in Table II represents a (1→3)-linked 4,6-*O*-(1-carboxyethylidene)-D-glucose residue. The amount of 2,3-di-*O*-methyl-D-glucose was not affected by the depyruvylation and it therefore must be derived from a (1→4)-(1→6)-linked D-glucose residue at a branch point. Quantitative considerations indicated that the small proportion of tetra-*O*-methyl-D-glucose shown in Table III probably arose from some degradation of the polysaccharide chain during depyruvylation. If it had come from a terminal 4,6-*O*-(1-carboxyethylidene)-D-glucose there should have been a corresponding decrease in the amount of 2,3-di-*O*-methyl-D-glucose. Furthermore, the data in Table II indicated that a single sugar residue in the polysaccharide repeating-unit constituted 12–13 mole per cent of the total products; the proportion of tetra-*O*-methyl-D-glucose in the methylated, depyruvylated product is only one-third of that amount. As D-glucose preponderates in this polysaccharide, it is reasonable to assume that partial degradation would produce additional terminal groups of that sugar.

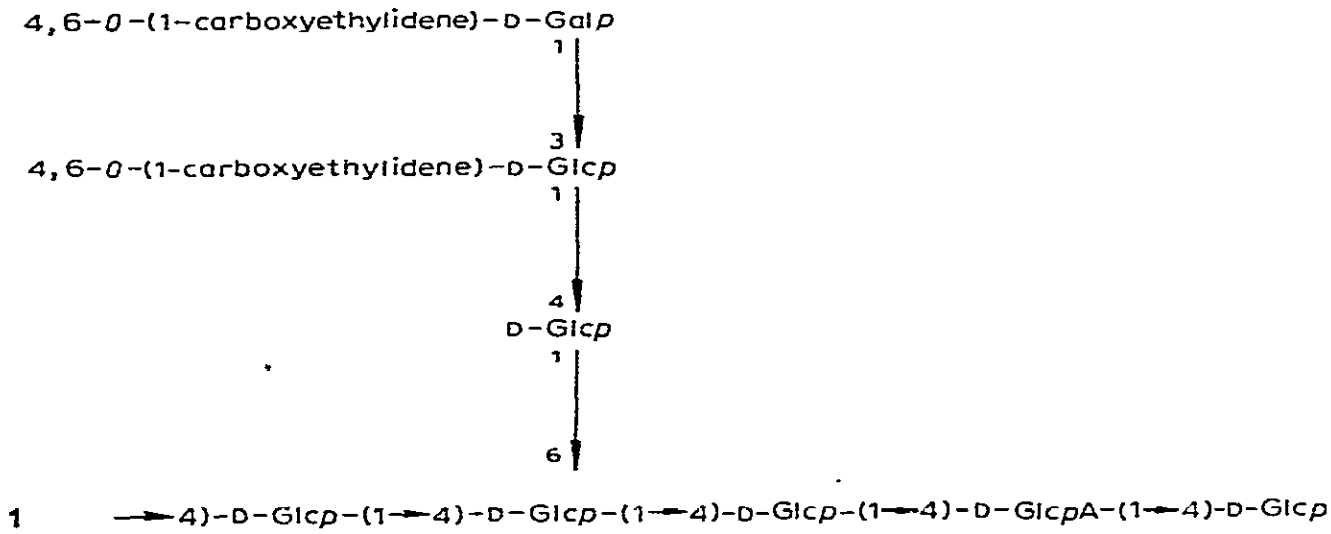
Reduction of the depyruvylated, methylated polysaccharide (Table III, column B) confirmed the information gained from Table II that D-glucuronic acid was present as its 2,3-dimethyl ether and was therefore linked 1→4 in the polysaccharide.

The native polysaccharide was subjected to partial acid hydrolysis in attempts to isolate oligosaccharides for sequence analysis. No oligosaccharides were found, but a degraded polysaccharide was recovered and the results of methylation analysis on it are given in Table III, columns B and C. The striking feature of this analysis in comparison with that from the depyruvylated product was the absence of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-glucose, each of which represented a pyruvylated residue in the native polysaccharide. As it was shown that the 4,6-*O*-(1-carboxyethylidene)-D-galactose was terminal and that the 4,6-*O*-(1-carboxyethylidene)-D-glucose was (1→3)-linked, the exclusive loss of these two

residues during the partial hydrolysis showed that they were linked in sequence in the native polysaccharide.

The amount of tetra-*O*-methyl-D-glucose in the products from the degraded polysaccharide indicated extensive cleavage of the (1 → 4)-linked D-glucose chains during partial hydrolysis, and the increase in 2,3-di-*O*-methyl-D-glucose after reduction of the methylated, degraded polysaccharide showed that most of the (1 → 4)-linked D-glucuronic acid fragments remained intact. It is possible that the small increase in 2,3,6-tri-*O*-methyl-D-glucose in column D as compared with column C was due to some contaminating 2,3,4-tri-*O*-methyl-D-glucose that had arisen from terminal D-glucuronic acid. However, the change was so small and of such tenuous structural significance that this point was not investigated.

Considering again the data in Table II for the native polysaccharide, if approximately 12 mole per cent is taken as representing a single residue then the repeating unit of the polysaccharide contains the following: (*a*) 1 terminal residue of 4,6-*O*-(1-carboxyethylidene)-D-galactose, (*b*) 1 residue of (1 → 3)-linked 4,6-*O*-(1-carboxyethylidene)-D-glucose, (*c*) 1 branch point formed by a (1 → 4)-(1 → 6)-linked D-glucose, (*d*) 1 residue of (1 → 4)-linked D-glucuronic acid, and (*e*) 4 residues of (1 → 4)-linked D-glucose. The following structure (**1**) represents the simplest repeating-unit that accounts for the experimental observations.



Thus, the pyruvylated residues that were released by partial hydrolysis must terminate a chain. However, these residues could not be attached directly to the branch point because the degraded polysaccharide still contained branches (represented by the 2,3-di-*O*-methyl-D-glucose, Table III, column C). The unit interposed between the pyruvylated residues and the branch point must have been D-glucose because the partial hydrolysis resulted in increased terminal moieties of this sugar. The pyranoid ring-form for the sugars was proven for the pyruvylated residues by the results of the methylation analysis, and may be assumed reasonably for all of the others because of their general resistance to hydrolysis. Had furanosidic linkages been present there should have been extensive cleavage of sugars during depyruvylation, and oligosaccharides, rather than a degraded polysaccharide, should have been

obtained from the partial hydrolysis. The negative rotation of the polysaccharide indicated that the glycosidic linkages were in the β-configuration; each of the constituent sugars was shown to be in the D-enantiomorphic form. Location of the *O*-acetyl groups was not determined because of a shortage of material.

The present investigation appears to be the first in which pyruvate acetals have been found on two different sugar residues in the same polysaccharide, one of them in a non-terminal position. Pyruvic acid has been found as a constituent of polysaccharides from a number of microorganisms: *Xanthomonas*[7,9,10], *Pseudomonas*[11], *Corynebacterium insidiosum*[12], *Klebsiella*[4,13–15], *Pneumococci* types IV and XXVII[4], *Rhizobia*[3–5], the *Enterobacteriaceae*[16,17], *Microsporum quinckaenum*[18], and *Salmonella*[19].

It was shown that removal of pyruvate groups from the *Rhizobium trifolii* TA-1 polysaccharide increased the number and extent of its cross-reactions with different types of pneumococcal antisera[3,4]. Structure **1**, without the pyruvate groups, explains those cross-reactions that suggested the presence of terminal D-galactose residues and multiples of D-glucuronic acid and D-glucose.

As the pyruvate groups in *Rhizobia* are important immunological determinants[3,4] and as these organisms display specificity towards their plant hosts[1,2], it is instructive to compare the structures of the two *Rhizobia* polysaccharides so far examined. Both polysaccharides, from *Rhizobium meliloti*[5] and *Rhizobium trifolii*, contain a common branch point (a 4,6-disubstituted D-glucose), multiples of (1 → 4)-linked D-glucose groups and a 4,6-*O*-(1-carboxyethylidene)-D-glucose. They differ in that the polysaccharide from *Rhizobium trifolii* TA-1 has a terminal 4,6-*O*-(1-carboxyethylidene)-D-galactose, its pyruvated D-glucose is a chain residue and it contains (1 → 4)-linked D-glucuronic acid. In addition, the polysaccharide from *Rhizobium meliloti*[5] contains (1 → 3)-linked D-glucose and D-galactose and (1 → 6)-linked D-glucose, none of which are present in the polysaccharide from *Rhizobium trifolii* TA-1.

EXPERIMENTAL

General methods. — Solutions were concentrated under diminished pressure at temperatures not exceeding 40°. Specific rotations were determined at 20° with a Perkin–Elmer 141 polarimeter and are equilibrium values unless otherwise stated. Infrared spectra were obtained on 4% solutions in chloroform or by the potassium bromide pellet technique with a Perkin Elmer–237B Infracord spectrometer. Paper chromatography by the descending method was done on Whatman No. 1 filter paper with the following solvent systems: (*A*) 6:4:3 (v/v) butyl alcohol–pyridine–water; (*B*) 5:2:5 (*v/v*), upper phase ethyl acetate–pyridine–water. Sugars were detected on the chromatograms by alkaline silver nitrate[20] or *p*-anisidine hydrochloride[21]. The double-diffusion method[22] was used in testing for serological activity. Gas–liquid chromatography was performed with a Hewlett–Packard model 402 gas chromatograph equipped with a hydrogen flame-ionization detector. The columns were glass U-tubes (150 × 0.3 cm) filled with the following packings: (*A*) 1% ECNSS-M on Gas

Chrom Q (60–80 mesh); (*B*) 3% ECNSS-M on Gas Chrom Q (100–120 mesh). A Hewlett-Packard model 700 gas chromatograph linked to an Atlas CH4 mass spectrometer was used for identification of methylated alditol acetates. The mass spectrometer was operated with an inlet temperature of 200°; ionizing voltage, 70 eV; ionizing current 20–40 μA; ion-source temperature 250°.

Preparation of polysaccharide. — Cultures of *Rhizobium trifolii*, strain TA-1, were grown in Roux bottles on a modified Bergersen[23] medium containing (per liter): mannitol (10 g) sodium glutamate (1.0 g), dipotassium hydrogen phosphate (0.5 g), magnesium sulphate heptahydrate (0.1 g), trace elements solution[24] (2.0 ml), and agar (20 g). The medium was adjusted to pH 7.0 and autoclaved; thiamine (100 μg), biotin (250 μg), and calcium pantothenate (100 μg) (all amounts on a per liter basis) were sterilized by filtration through 0.45 μm cellulose acetate (Millipore filter) and were added to the sterile medium when it was cool but still molten.

After incubation for 12 days at 25°, the dense mucoid growth was harvested by washing the agar surfaces with 0.85% sodium chloride solution. Cells were separated from the soluble polysaccharide by centrifugation at 30,000 r.p.m. (105,000 × *g*) for 1 h. The polysaccharide in the supernatant liquid was precipitated by the addition of four volumes of acetone. The crude polysaccharide thus obtained had a nitrogen content (Kjeldahl) of 0.12%, and gave two precipitin bands (one strong and one much weaker) upon immunodiffusion against antisera raised in rabbits by injection of the whole bacteria[25].

Passage of the crude polysaccharide (2.0 g in 100 ml of water) through Dowex anion-exchange resin (1 × 4, 20–50 mesh, hydrogen carbonate form) in a column (20 × 5 cm) at a rate of 12 ml per h and exhaustive elution with water (1800 ml) yielded a polysaccharide (0.89 g) that gave only a single precipitin band on immunodiffusion. It was recovered by dialysis and freeze-drying; some analytical results are shown in Table I. The purified polysaccharide gave a strong absorption band at 1612 cm^{-1} in the i.r. that was attributed to ionized carboxyl. The polysaccharide (155 mg in water, 25 ml) was deionized (Rexyn 101, H^+ form, 100 g) and freeze-dried. Its i.r. spectrum then showed a strong band at 1726 cm^{-1} due to unionized carboxyl and only a very weak band for ionized carboxyl at 1612 cm^{-1}. The deionized polysaccharide was soluble in water and in dimethyl sulfoxide, and was used for methylation analysis.

Preparation of depyruvylated polysaccharide. — A sample (150 mg) of the unfractionated polysaccharide was dissolved in water (40 ml) and the stirred solution was adjusted to pH 2.08 by the dropwise addition of N hydrochloric acid. The acidified solution was heated under reflux for 4 h on a boiling water bath; a small, white flocculent precipitate formed and the solution was no longer viscous. The cooled solution was neutralized to pH 6.2 by M sodium hydroxide and the small precipitate was removed by centrifugation. The supernatant solution was dialysed against distilled water and the non-dialysables were freeze-dried to yield the depyruvylated polysaccharide (97 mg, 69% recovery). The product contained 0.5% of pyruvate and no longer reacted with homologous antiserum.

The depyruvylated polysaccharide was colored with a Procion reactive dye[26] and the dyed polysaccharide was used for molecular-weight estimation by gel filtration[27]. The dyed, depyruvylated polysaccharide was below the exclusion limit of Biogel P-300 (elution volume/void volume >2.5) indicating that its molecular weight was in the order of 20,000 or less. However, on Sephadex G-75, the polysaccharide was excluded and appeared in the void volume, indicating that its molecular weight was in the range of 50,000. A dyed sample of the crude polysaccharide was excluded from Biogel P-300, indicating that its molecular weight was greater than 300,000.

Analysis of constituent sugars. — The polysaccharide (5.0 mg, purified sample) and an internal standard of L-arabinose (3.00 mg) were kept for 6 h at 25° in 72% sulfuric acid (0.1 ml). The mixture was then diluted to 1 ml with water, heated for 10 h on a boiling water-bath, neutralized with barium carbonate, and filtered. The filtrate was deionized with resins and the sugars in the hydrolysate were converted into acetates for analysis by g.l.c. on column A[8]. The results and other analytical data are given in Table I. The occurrence of sugars in the D-form was proven enzymically by using the Galactostat and Glucostat reagents (Worthington Biochemicals Corp., Freehold, N.J.). The D-form for glucuronic acid was established in the same way after reduction (sodium borohydride) of its methyl ester to D-glucose.

Methylation analysis. — A. *Native polysaccharide.* The polysaccharide (60 mg, deionized and dried over phosphorus pentaoxide) was methylated by the Hakomori procedure[28] with dimethyl sulfoxide (9 ml), 2M methylsulfinylcarbinylsodium (10 ml), and methyl iodide (8 ml). The mixture was poured into water (40 ml), dialysed, and the non-dialysables were recovered (70 mg) by evaporation to dryness. This product showed slight hydroxyl absorption in the i.r. and was therefore subjected to two further successive methylations by Purdie's reagents[29], after which time there was no hydroxyl absorption in the i.r.

The methylated, native polysaccharide (10 mg) was heated in 88% formic acid (1 ml) for 2 h at 100°, the acid was removed by evaporation, and the residue was hydrolysed by 0.25M sulfuric acid (1 ml) for 12 h at 100°. The hydrolysate was neutralized (barium carbonate) and the methylated neutral sugars were analysed as their alditol acetates by g.l.c. and mass spectrometry[30]. Results are given in Table II, column A, and Fig. 2, A.

B. *Carboxyl-reduced, native polysaccharide.* The methylated, native polysaccharide (22 mg), in dry tetrahydrofuran (10 ml), was added cautiously to a suspension of lithium aluminium hydride (50 mg) in tetrahydrofuran (10 ml). The reaction mixture was refluxed for 20 h, after which time moist ethyl acetate was added to decompose the excess hydride. The insoluble salts were filtered off and washed with chloroform. The combined washings and filtrate were evaporated to dryness to yield the methylated, carboxyl-reduced, native polysaccharide (20 mg). This product showed hydroxyl absorption in the i.r. and a band previously present at 1735 cm^{-1} (carboxyl ester) had disappeared. Hydrolysis and analysis of constituent methylated sugars were done as already described for the native polysaccharide. The results are given in Table II, column B, and Fig. 2, B.

C. *Remethylated, carboxyl-reduced, native polysaccharide.* The methylated, carboxyl-reduced, native polysaccharide (12 mg), prepared as described in the preceding paragraph, was methylated further by using 0.5M methylsulfinyl carbanion (2 ml) and methyl iodide (2 ml). The product, isolated as described previously, showed no hydroxyl absorption in the i.r. Hydrolysis and analysis of constituent methylated sugars were done as before; the results are shown in Table II, column C, and Fig. 2, C.

D. *Depyruvylated polysaccharide.* The depyruvylated polysaccharide (25 mg) was methylated under the same conditions already given, by using 2M methylsulfinyl carbanion (4 ml) and methyl iodide (5 ml). The results of hydrolysis and analysis of constituent methylated sugars are given in Table III, column A, and Fig. 3, A.

E. *Carboxyl-reduced, depyruvylated polysaccharide.* A portion (12 mg) of the methylated, depyruvylated polysaccharide was carboxyl-reduced by lithium aluminum hydride as described already for the native polysaccharide. Hydrolysis and analysis of the constituent methylated sugars gave the results shown in Table III, column B, and Fig. 3, B.

F. *Degraded polysaccharide.* The native polysaccharide (36 mg) was heated in 0.15M hydrochloric acid for 6 h at 100°. The neutralized (barium carbonate) hydrolysate was chromatographed on paper (solvent *A*) to separate the degraded polysaccharide from the monosaccharides that had been released. Elution of the starting line of the chromatogram with water yielded the degraded polysaccharide (11 mg, 30%), recovered by precipitation with methanol. This product was methylated [dimethyl sulfoxide (1.0 ml) 2M methylsulfinyl carbanion (1.5 ml) and methyl iodide (2 ml)] and half of the methylated, degraded polysaccharide was taken for hydrolysis and analysis as previously described. The results are given in Table III, column C.

G. *Carboxyl-reduced, degraded polysaccharide.* One half of the methylated, degraded polysaccharide was reduced by lithium aluminum hydride and then hydrolysed and the product analysed by the same procedures given above. The results are shown in Table III, column D.

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