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THE STRUCTURE OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K38*†

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ABSTRACT

The acidic polysaccharide secreted by *Rhizobium phaseoli* strain 127 K38 was found to consist of a decasaccharide repeating-unit. The absolute configuration, ring form, points of attachment of each glycosyl residue, anomeric configurations of the glycosyl linkages, and sequence of the glycosyl residues were determined. Each of the glycosyl residues of the polysaccharide was determined to be in the D configuration and the pyranoid ring-form. The polysaccharide was found to contain a pyruvic acetal group in the (*S*) configuration, and acetyl groups linked to at least two of the oxygen atoms. The decasaccharide repeating-unit is shown on the following page.

INTRODUCTION

Rhizobia infect the root hairs of, form nodules on, and participate in a nitrogen-fixing, symbiotic relationship with, host legumes[2]; they are divided into fast- and slow-growing species, which are defined by their rate of growth on yeast-extract media[3]. The fast-growing *Rhizobia* are known to secrete at least two types of polysaccharide, namely, neutral, β-linked D-glucans and complex, acidic polysaccharides[4]. The acidic polysaccharides are composed of D-glucosyl, D-galactosyl, and D-glucosyluronic acid residues with pyruvic acetal, acetyl, or succinyl groups occurring as substituents on some of the glycosyl residues. The fast-growing *Rhizobium* species, which are selective with regard to the legume species they can nodulate, include *R. leguminosarum*, the symbiont of pea; *R. phaseoli*, the symbiont of true bean; *R. trifolii*,

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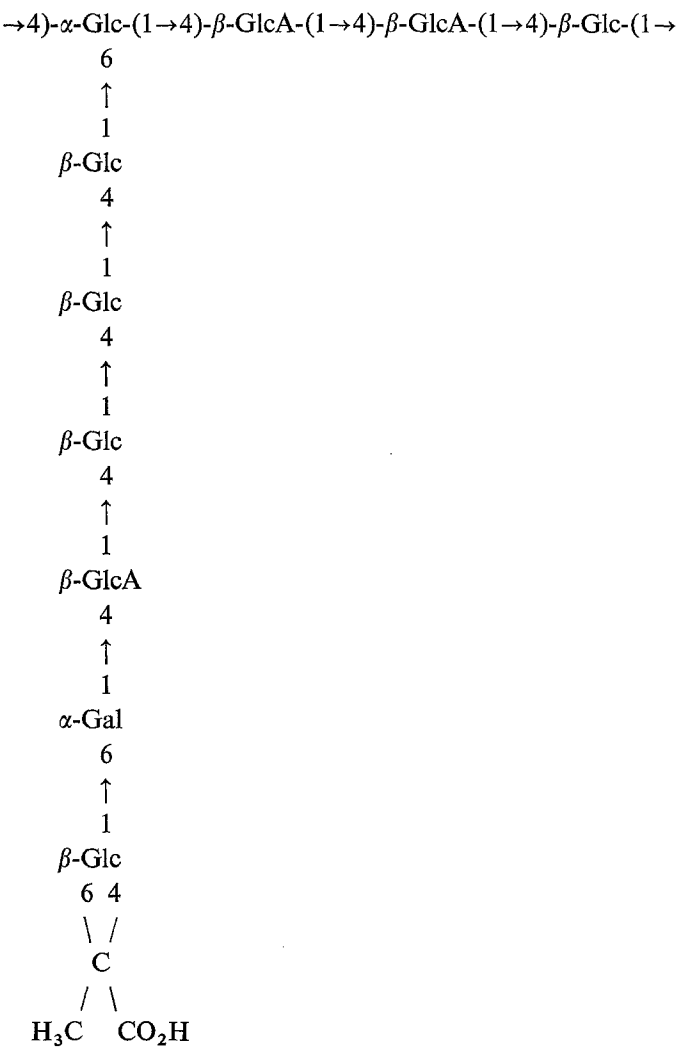

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the symbiont of clover; and *R. meliloti*, the symbiont of alfalfa. It remains to be established whether the extracellular, acidic polysaccharides are important in defining host specificity.

Bacterial polysaccharides are generally composed of oligosaccharide repeating-units. The sequence and anomeric configurations of the glycosyl residues in the octasaccharide repeating-unit of the acidic polysaccharides secreted by *R. leguminosarum* and *R. trifolii* are the same[5], but differ from the octasaccharide repeating-unit of the acidic polysaccharide secreted by *R. meliloti*[6,7]. We now describe the first structural elucidation of an acidic polysaccharide secreted by a strain of *R. phaseoli*.

EXPERIMENTAL

Isolation of the acidic polysaccharide. — *Rhizobium phaseoli* strain 127 K38 was obtained from Dr. Joe Burton of the Nitragin Company, Milwaukee, WI. The *Rhizobium* was grown on a defined medium, as described previously for *R. leguminosarum* and *R. trifolii*[1]. At late exponential phase, usually after 2 to 3 days, the bacteria were separated from the medium by centrifugation at 7000*g* for 20 min. The acidic polysaccharide in the supernatant solution was isolated as described[4]. Yields of ~300 mg of purified polysaccharide were obtained from 1 L of bacterial culture.

Ion-exchange chromatography. — A solution of purified polysaccharide (10 mg) in 50 mL of 50mM Tris HCl, pH 7.2, was applied to a column (2.5 × 14 cm) of Sephadex A-25 anion-exchange resin pre-equilibrated with the same buffer. The column was first eluted with the buffer (200 mL), and then with a linear gradient formed by buffer (400 mL) and buffer (400 mL) containing 2M NaCl. Fractions (5 mL) were collected and, in each fraction, the content of neutral hexose was determined by the anthrone method[7], the content of uronic acid by the *m*-hydroxydiphenyl method[8], and the content of NaCl by conductivity. The fractions containing the polysaccharide were combined into four groups, namely, the leading and trailing edges, and the first and second half of the center of the peak. Each combined group was dialyzed, lyophilized, hydrolyzed with 2M trifluoroacetic acid (TFA) for 2.5 h, the sugars reduced ($NaBH_4$), the alditols acetylated, and the acetates analyzed by g.l.c.–m.s. for glycosyl composition[9].

Molecular-weight distribution. — Purified polysaccharide (5 mg) was applied to a column of Sepharose CL-4B that had been calibrated with dextrans of known molecular weight[10]. The column was equilibrated, and eluted, with 0.25M NaCl. An autoanalyzer continuously determined the concentration of neutral hexose by the anthrone method[7], and, in a second experiment, the concentration of uronic acid by the carbazole method[11].

Composition analysis of glycosyl and pyruvic acetal residues. — The purified polysaccharide (1 mg/mL) was dissolved in water. The contents of neutral sugar[7], uronic acid[8], and pyruvic acid[12] were determined colorimetrically.

The glycosyl composition of the polysaccharide was determined by the following procedure. Purified polysaccharide (1 mg) was subjected to methanolysis with 1.8M HCl in dry methanol for 16 h at 100°. At the end of the reaction, the acid was neutralized with Ag_2CO_3 and the suspension was filtered. The filtrate was evaporated, and the free hydroxyl groups of the methyl glycosides were per(trimethylsilyl)ated with 5:1:1 (v/v/v) pyridine–hexamethyldisilazane–chlorotrimethylsilane for 10 min at 80°. The products were dissolved in oxolane, and the methyl esters of the uronic residues were reduced with $LiAlD_4$, the methyl glycosides hydrolyzed with 2M TFA for 2 h at 120°, the sugars reduced ($NaBD_4$), the alditols acetylated, and the acetates analyzed by g.l.c. The ratio of the alditol acetates derived from glucose and glucuronic acid was determined by g.l.c.–m.s.

Determination of the absolute configuration of the glycosyl residues. — The

isolated polysaccharide (250 μg) was hydrolyzed with 2M TFA for 3 h at 120°, and the absolute configurations of the glycosyl residues in the hydrolyzate were determined by the method of Gerwig *et al.*[13].

The absolute configurations of the glucosyl and the glucosyluronic acid residues were confirmed by a separate procedure. The polysaccharide (2 mg) was subjected to methanolysis, and carboxyl-reduction of the per(trimethylsilyl)ated products as already described. The products were hydrolyzed with 2M TFA for 2 h at 120°, and the free sugars obtained were incubated overnight with D-glucose oxidase at pH 5.1 and 35°. After treatment with the enzyme, the residual sugars were per(trimethylsilyl)ated and analyzed by g.l.c.–m.s., with comparison to per-*O*-(trimethylsilyl)-glucose and -galactose standards[14].

Methylation of the polysaccharide[15]. — A solution of the polysaccharide (50 mg) in water was passed through a column of Dowex 50 (H^+) resin (the native polysaccharide is not readily soluble in Me_2SO). The polysaccharide in the acid form was lyophilized, dried overnight in a vacuum oven at 40°, and dissolved in freshly distilled Me_2SO (20 mL) by stirring for 4 h. Potassium dimethylsulfinyl anion[16] was added until complete deprotonation of the carboxyl and hydroxyl groups was obtained, triphenylmethane (which is deprotonated by sulfinyl anion, but not by deprotonated sugar-hydroxyl groups) being used as an indicator[17]. Further additions of potassium dimethylsulfinyl anion were made, to yield a final concentration of 0.4M of the anion. The resulting mixture was sonicated for 1 h, and stirred overnight. The sample was then methylated by first freezing the solution and then adding methyl iodide (2 mL) and stirring for 4 h. The excess of methyl iodide was evaporated, and the solution of the methylated polysaccharide was dialyzed against tap water for 3 days, and lyophilized, the residue taken up in chloroform, the suspension filtered through glass-fiber paper, and the filtrate evaporated to dryness.

Reduction of the methyl-esterified carboxyl groups of the glucosyluronic and pyruvic residues in the methylated polysaccharide. — Methylated polysaccharide (50 mg) was dissolved in oxolane (1 mL, distilled and predried with Drierite). Lithium aluminum deuteride (100 mg) was dissolved in dry oxolane (6 mL) by heating the mixture for 30 min at 80°, and removing undissolved material by centrifugation.

The solution (5 mL) was added to the solution of the methylated polysaccharide, and the resulting solution was heated for 16 h at 80°. Unreacted lithium aluminum deuteride was decomposed with moist ethyl acetate, and the suspension was filtered, the solid washed with chloroform, and the filtrate and washings combined, and evaporated to dryness. The crude product was partially dissolved in chloroform by sonication, the suspension filtered, and the filtrate evaporated to dryness, yielding carboxyl-reduced, methylated polysaccharide essentially free from salts. A portion of the carboxyl-reduced methylated polysaccharide (40 mg) was realkylated, as described[16], either with methyl iodide or ethyl iodide.

Glycosyl-linkage composition. — Glycosyl-linkage composition analyses[16] were conducted on the methylated polysaccharide, the carboxyl-reduced methylated polysaccharide, the carboxyl-reduced methylated polysaccharide that had been re-

methylated, and the carboxyl-reduced methylated polysaccharide that had been ethylated. The identity of the partially alkylated alditol acetates derived was established by their g.l.c. retention-times and by their electron-impact (e.i.) mass spectra[16].

Preparation, separation, and analysis of partially methylated, partially ethylated oligosaccharide-alditols. — Preparation of peralkylated oligosaccharide-alditols was achieved by partial hydrolysis of the methylated polysaccharide with acid, reduction of the resulting, partially methylated oligosaccharides with $NaBD_4$, and alkylation (methylation or ethylation) of the resulting, partially methylated oligosaccharide-alditols as described[16].

The peralkylated oligosaccharide-alditols prepared from samples of the methylated polysaccharide were separated by l.c. and detected in the effluent by means of a refractive-index monitor[16] or by chemical ionization (c.i.) mass spectrometry. (m.s.)[18] Per-*O*-alkylated di- and tri-saccharide-alditols were analyzed by g.l.c.–e.i.-m.s., using splitless injections in a capillary column (25 m) of SE-30. The g.l.c. was programmed for 2 min at 160°, and then from 160–220° at 30° per min, and finally from 220–330° at 8° per min. The per-*O*-alkylated tetra- and penta-saccharide-alditols were analyzed by e.i.-m.s., using the direct-inlet probe. During the direct-probe analyses, the temperature of the samples was increased from room temperature to 345° in ~2 min. The spectra were recorded with a Hewlett–Packard 5985 mass spectrometer at 70 eV, and a source temperature of 150°.

The per-*O*-alkylated oligosaccharide-alditols were also hydrolyzed, the sugars reduced, the alditols acetylated, and the glycosyl-linkage composition analyzed by g.l.c.–m.s. as described[16].

Specific experiments in which per-*O*-alkylated oligosaccharide-alditols and -methylglycosides were formed by using various combinations of methylation, ethylation, carboxyl-reduction, and base-catalyzed degradation of the methylated polysaccharide are described next.

Experiment A. — Methylated, carboxyl-reduced polysaccharide (40 mg) was partially hydrolyzed with 2M TFA for 45 min at 98°, the products reduced ($NaBD_4$), and the alditols ethylated. The resulting mixture of partially methylated, partially ethylated oligosaccharide-alditols was separated by l.c. in a Du Pont Zorbax, ODS column with 11:9 acetonitrile–water as the solvent[16], the refractive index of the column effluent being monitored, and 0.5-mL fractions collected. Appropriate fractions (shaded in Fig. 3) were combined, and further purified by l.c. in a Whatman Partisil, ODS column with 3:2 acetonitrile–water as the solvent, the refractive index of the column effluent being monitored, and 0.5-mL fractions collected.

Experiment B. — Methylated, carboxyl-reduced polysaccharide (40 mg) was remethylated, the product partially hydrolyzed with 90% formic acid for 20 min at 80°, the products reduced ($NaBD_4$), and the alditols ethylated. The resulting mixture of partially methylated, partially ethylated oligosaccharide-alditols was separated by l.c. in the Du Pont Zorbax, ODS column, using a 1-h, linear gradient of 50–70% acetonitrile in water as the solvent. A mass spectrometer (Hewlett–Packard 5985, l.c.–m.s. system) was used as a detector, and ~3% of the effluent was continuously

analyzed by c.i.-m.s., using the l.c. solvent as the reactant gas[18]. A c.i. mass spectrum, from *m/z* 200 to 1000, was obtained every 2 s. Fractions (0.5 mL) were collected, and some of these were further analyzed by g.l.c.–e.i.-m.s. The isolated per-*O*-alkylated trisaccharide-alditols were also characterized by glycosyl-linkage composition-analysis.

Experiment C. — Methylated, carboxyl-reduced polysaccharide (40 mg) was partially hydrolyzed with 2M TFA for 150 min at 80°, the products reduced ($NaBD_4$), and the alditols ethylated. The resulting mixture of per-*O*-alkylated oligosaccharide-alditols was separated on the Du Pont column, using 3:2 acetonitrile–water as the solvent. Some of the fractions obtained were further purified on the Whatman column, using the same solvent. With both columns, the refractive index of the effluent was monitored, and 0.5-mL fractions were collected[16]. The structures of the per-*O*-alkylated oligosaccharide-alditols were determined by e.i.-m.s., and by glycosyl-linkage composition-analysis[16].

Experiment D. — Methylated polysaccharide (40 mg) was partially hydrolyzed with 2M TFA for 120 min at 98°, the products reduced ($NaBD_4$), the alditols ethylated, the products carboxyl-reduced ($LiAlD_4$), and the alditols ethylated. The resulting mixture of per-*O*-alkylated oligosaccharide-alditols was fractionated, and detected by l.c.–m.s. as described in Experiment B. The structures of the per-*O*-alkylated oligosaccharide-alditols were obtained by e.i.-m.s. and by glycosyl-linkage composition-analysis.

Experiment E. — This experiment was designated to cause cleavage of the methylated polysaccharide by base-catalyzed degradation of its constituent glucosyluronic residues that had been methyl-esterified[19]. To a solution of methylated polysaccharide (50 mg) in dimethyl sulfoxide (2 mL) containing 5% (v/v) of 2,2-dimethoxypropane and a trace of *p*-toluenesulfonic acid was added 3M potassium dimethylsulfinyl anion (1 mL), and the mixture stirred for 24 h at room temperature, acidified with 50% acetic acid (3 mL), and poured into water (50 mL). The degraded, methylated polysaccharide was extracted into chloroform (3 × 50 mL), and the extracts were combined, washed with water (2 × 150 mL), and evaporated. The chloroform-soluble material was further purified in an LH-20 column, using 1:1 (v/v) methanol–chloroform as the solvent. The carbohydrate-containing fractions were identified (anthrone) and combined. After reduction with $NaBD_4$ in 8:3 (v/v) 1,4-dioxane–ethanol, and ethylation, the mixture of products was purified a second time in the LH-20 column. The partially methylated, partially ethylated products were fractionated by l.c. in the Du Pont column, using 3:2 acetonitrile–water as the solvent (see Experiment A). The major product isolated by this procedure was further purified on the Whatman column, using the same solvent. This purified product was characterized by glycosyl-linkage composition-analysis, by e.i.-m.s. using the direct-inlet probe, and by n.m.r. spectroscopy.

The sequence of the glycosyl residues in the major product resulting from base-catalyzed degradation of the methylated polysaccharide was obtained by analysis of the per-*O*-alkylated oligosaccharide derivatives obtained from it by partial hydrolysis

with 90% formic acid for 15 min at 80°, reduction ($NaBD_4$), and (trideuteriomethyl)-ation. The resulting, per-*O*-alkylated oligosaccharide derivatives were fractionated by l.c. in the Du Pont column, using 9:11 acetonitrile–water as the solvent (see Experiment A). The structures of the per-*O*-alkylated oligosaccharide derivatives were determined by e.i.-m.s. Two of them were further characterized by c.i.-m.s. (ammonia) and by glycosyl-linkage composition-analysis[18].

N.m.r. spectroscopy. — Methylated, carboxyl-reduced ($LiAlD_4$), and ethylated polysaccharide (3 mg), the pentasaccharide product from the base-catalyzed degradation (500 μg), and the peralkylated oligosaccharide-alditols (10–50 μg) were dissolved in chloroform-*d* (99.8 atom % of D) and ^{1}H-n.m.r. spectra were recorded with a Nicolet, 360-MHz, Fourier-transform, n.m.r. spectrometer, with experimental parameters as described[1]. Chemical shifts were assigned relative to internal $CHCl_3$ at δ 7.26.

The underivatized polysaccharide (3 mg) was prepared for ^{1}H-n.m.r.-spectral analysis by twice dissolving it in deuterium oxide (99.7 atom % of D) and freeze-drying. The polysaccharide was then dissolved in 0.7 mL of deuterium oxide (100 atom % of D), transferred to a dry, 5-mm, n.m.r. tube, and analyzed in the 360-MHz instrument at 90°. The experimental parameters included a 5-μs pulse (45°), 16 k of memory, 3 s between pulses, and a sweep width of ±2 kHz. Chemical shifts were assigned relative to internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (Stohler Isotope Chemicals), defined as δ 0.00.

For ^{13}C-n.m.r. analysis, isolated polysaccharide (50 mg) was dissolved in 3 mL of deuterium oxide (99.7 atom% of D) at a concentration of ~60 mg/mL, by sonication for several days in D_2O. A portion (~4 mL) was transferred to a 10-mm, n.m.r. tube. The ^{13}C-n.m.r. spectrum was recorded at 60°. The parameters included a spectrum frequency of 90 MHz, proton decoupling, 16k of memory, 45° pulses, 2.3 s between pulses, and a sweep width of ±12.2 kHz. Chemical shifts were assessed relative to internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate.

RESULTS AND DISCUSSION

Isolation and chromatography of the acidic polysaccharide secreted by R. phaseoli *strain 127 K38.* — *Rhizobium phaseoli* strain 127 K38, obtained from the Nitragin Company, was originally isolated in Mexico from the root nodules of beans. Nodulation experiments in our laboratory, however, showed that the bacterium either could not nodulate red kidney-beans or, at best, could form only rudimentary nodules under our laboratory conditions. However, *R. phaseoli* strain 127 K38 did form a limited number of normal nodules on *P. vulgaris* green-bean cultivars (Improved Commodore and Top Crop, obtained from Rocky Mountain Seed Company, Denver, Colorado). Bacteria were isolated from the nodules on these cultivars[20], and the extracellular, acidic polysaccharides were isolated. The polysaccharides from the nodule isolates had the same neutral sugar (anthrone) to uronic acid (*m*-hydroxydiphenyl) ratio, the same ratio of glucose to galactose (alditol acetates), and the same glycosyl-linkage

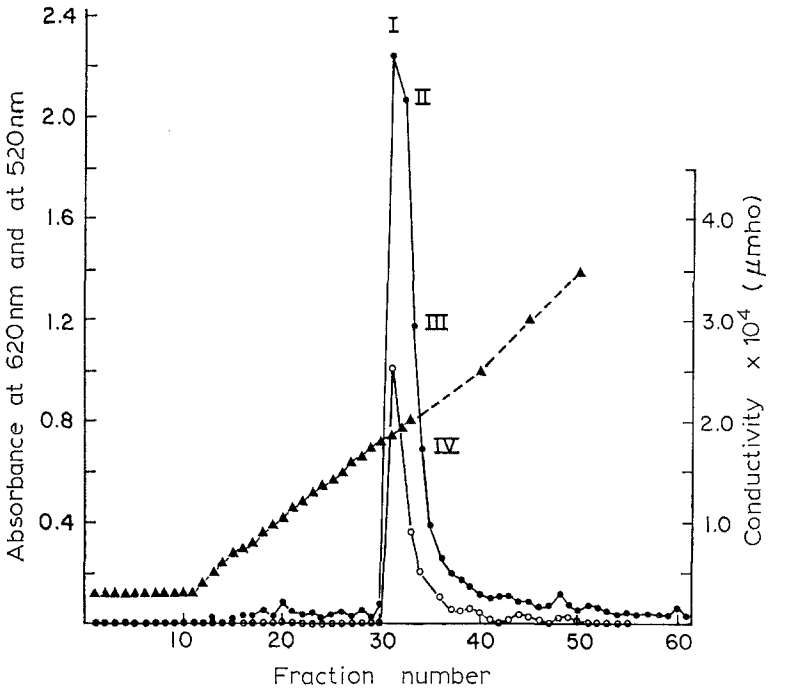

Fig. 1. Chromatography of the acidic polysaccharide secreted by *R. phaseoli* strain 127 K38, in a column of DEAE-Sephadex A-25 anion-exchange resin. [The fractions (5 mL) were analyzed for hexoses by the anthrone method (●---●), for uronic acids by the *m*-hydroxydiphenyl method (○---○), and for sodium chloride by the conductivity (▲---▲).]

composition, both before and after carboxyl-reduction, as the polysaccharide secreted by the *R. phaseoli* strain 127 K38 used in the nodulation experiment. These data are not only characteristic of the *R. phaseoli* 127 K38 polysaccharide, but distinguish this polysaccharide from all other *Rhizobium* polysaccharides that have been characterized. Thus, these data strongly suggest that the nodules formed on the *P. vulgaris* green-bean cultivars were, indeed, the result of infection by *R. phaseoli* strain 127 K38. We concluded that strain 127 K38 nodulates with poor efficiency the *P. vulgaris* cultivars selected.

An acidic polysaccharide secreted by *R. phaseoli* strain 127 K38 was isolated from the culture medium by precipitation with a quaternary ammonium salt, dissolution in 10% aqueous sodium chloride, and reprecipitation with acetone. Anion-exchange chromatography of the polysaccharide on DEAE-Sephadex A-25 resulted in the elution of only one carbohydrate-containing peak (see Fig. 1). The ratio, in each carbohydrate-containing fraction, of hexosyl to glycosyluronic residues was essentially the same. The polysaccharide-containing fractions were combined into four groups, as shown in Fig. 1, and each group was analyzed for its neutral glycosyl composition. The four fractions yielded only glucosyl and galactosyl derivatives, and the ratio of glucose to galactose in each group was 5.5 : 1. These results indicated that,

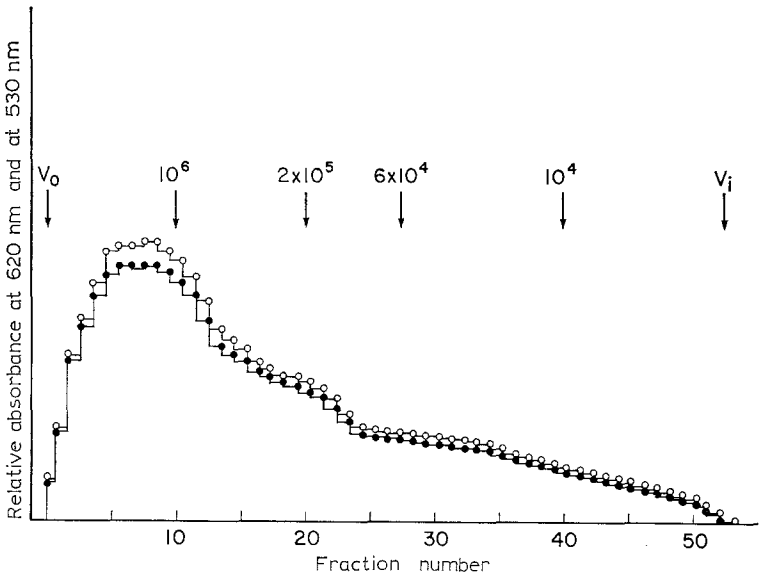

Fig. 2. Gel filtration of the *R. phaseoli* polysaccharide in a column of Sepharose 4B-CL. [The void (V_0) and included (V_i) volumes of the column are indicated, as are the elution volumes of dextrans of known molecular weights. The fractions were analyzed for neutral hexoses by the anthrone method (●——●), and for uronic acids by the carbazole method (○——○).]

with regard to carbohydrates, the polysaccharide had been purified to chemical homogeneity.

The polysaccharide was not eluted from the anion-exchange column in a symmetrical peak (see Fig. 1), probably due to size heterogeneity of the polysaccharide. Gel-filtration chromatography of the polysaccharide on a column of Sepharose 4B-CL resulted in a broad elution-profile that extended from the void volume to the included volume (see Fig. 2), providing additional evidence that the polysaccharide is

TABLE I

COMPOSITION OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K38

Residue	*I*[a] *(weight %*[d]*)*	*II*[b]	*III*[c]
Neutral hexoses	64	65.3	
glucose			90[e]
galactose			10
Uronic acids	30	30.2	
Pyruvic acid	6	4.6	

[a]Based on colorimetric assays of neutral hexose[7], uronic acid[8], and pyruvic acid[12]. [b]Theoretical values calculated for a repeating unit of 7 hexosyl residues, 3 glycosyluronic residues, and 1 pyruvic residue. [c]Determined by g.l.c.–m.s. after methanolysis, per(trimethylsilyl)ation, carboxyl-reduction ($LiAlD_4$), hydrolysis, reduction ($NaBD_4$), and acetylation. [d]Relative percent calculated on the sum of the components determined. [e]Thirty three percent of this derivative contained two deuterium atoms on C-6.

polydisperse. The ratio of hexosyl to glycosyluronic acid residues in each fraction from the Sepharose column was constant, a further indication that the polysaccharide is a chemically homogeneous carbohydrate.

The column of Sepharose 4B-CL was calibrated with dextrans of known molecular weights[10]. The dextrans differ in structure from the *Rhizobium* polysaccharide; however, by making the assumption that the two polysaccharides behave similarly on the Sepharose column, the *Rhizobium* polysaccharide contained a range of components having molecular weights lying between 2×10^3 and 4.5×10^6. The weight-average molecular weight ($(\overline{M}_w)$ of the *Rhizobium* polysaccharide was calculated[10] to be 10^6.

The glycosyl composition and the absolute configuration of the glycosyl residues of the Rhizobium *polysaccharide.* — The polysaccharide was analyzed colorimetrically for hexosyl, glycosyluronic acid, and pyruvic residues. The results (see Table I, columns I and II) are in agreement with a decasaccharide repeating-unit consisting of seven hexosyl residues and three glycosyluronic acid residues. The polysaccharide is substituted with one 1-carboxyethylidene group per repeating unit.

Glycosyl-composition analysis of the polysaccharide, after methanolysis, and carboxyl-reduction with $LiAlD_4$, gave only glucose and galactose, in the ratio of 9:1. By monitoring m/z $[M - 59]^+$ in g.l.c.–m.s., it was demonstrated that approximately one-third (3 residues) of the glucitol hexaacetate had two hydrogen atoms replaced by deuterium atoms, and, consequently, originated from glucosyluronic acid residues in the polysaccharide (see Table I, column III).

Eleven of the galactosyl, glucosyl, and glucosyluronic residues of the polysaccharide were found to be in the D configuration by the method of Gerwig *et al.*[13].

TABLE II

GLYCOSYL-LINKAGE COMPOSITION OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K38

Glycosyl residue	*Position of O-methyl groups*	*Position of O-ethyl groups*	*R.T.*[b]	*Sample* *A*	*B*	*C*	*D*
				(Mol %)			
Glucosyl	2,3,6	—	0.69	58	44	74[c]	48
Glucosyl	2,3	—	0.83	31	47[d]	20	18
Glucosyl	2,3	6	0.74	—	—	—	27[e]
Galactosyl	2,3,4	—	0.76	11	9	6	7

[a]Sample A was methylated, hydrolyzed, reduced ($NaBD_4$), and acetylated. Sample B was methylated, carboxyl-reduced ($LiAlD_4$), hydrolyzed, reduced ($NaBD_4$), and acetylated. Sample C was methylated, carboxyl-reduced ($LiAlD_4$), methylated, hydrolyzed, reduced ($NaBD_4$), and acetylated. Sample D was methylated, carboxyl-reduced ($LiAlD_4$), ethylated, hydrolyzed, reduced ($NaBD_4$), and acetylated. [b]Retention time relative to *myo*-inositol hexaacetate on an SE-30 capillary column programmed from 170–240° at 4°/min. [c]Approximately 40% of this derivative has two deuterium atoms on C-6. [d]Approximately 60% of this derivative has two deuterium atoms on C-6. [e]This derivative has two deuterium atoms on C-6.

The D-configuration of the glucosyl and glucosyluronic acid residues was confirmed by demonstrating that all of the glucose residues obtained by methanolysis, per(trimethylsilyl)ation, carboxyl-reduction, and hydrolysis of the polysaccharide were oxidized by D-glucose oxidase. These results indicate that the *Rhizobium* polysaccharide has a repeating unit consisting of six D-glucosyl residues, one D-galactosyl residue, and three D-glucosyluronic acid residues.

Determination of the glycosyl-linkage composition. — Glycosyl-linkage composition-analysis of the methylated polysaccharide showed that the polysaccharide consists of 4-linked glucosyl residues, (4→6)-linked glucosyl residues, and 6-linked galactosyl residues (see Table II, column A). Glycosyl-linkage composition-analysis of the carboxyl-reduced, methylated polysaccharide, and the remethylated, carboxyl-reduced, methylated polysaccharide showed the presence of the same residues (see Table II, columns B and C). However, the carboxyl-reduced, methylated polysaccharide showed an increase in the content of (4→6)-linked glucosyl residues and, by g.l.c.–m.s. analysis, ~60% of these residues contained two deuterium atoms on C-6. On the other hand, the methylated carboxyl-reduced, methylated polysaccharide showed an increase in the content of 4-linked glucosyl residues and, by g.l.c.–m.s. analysis, ~40% of these residues had originated from 4-linked glucosyluronic residues in the polysaccharide.

Glycosyl-linkage composition-analysis of the ethylated, methylated, carboxyl-reduced polysaccharide (see Table II, column D) showed the presence of 1,4,5-tri-*O*-acetyl-6,6-dideuterio-6-*O*-ethyl-2,3-di-*O*-methylglucitol, consistent with three 4-linked glucosyluronic residues in the polysaccharide.

The quantitative data presented in Table II, columns A–D, suggest a repeating unit consisting of four (or five) 4-linked glucosyl residues, two (4→6)-linked glucosyl residues, three 4-linked glucosyluronic residues, and one 6-linked galactosyl residue; and this is consistent with the results of glycosyl-composition analysis. The number of 4-linked glucosyl residues per repeating unit was determined to be four by further analysis (see later).

Acetal linkages to carboxyl-reduced pyruvic residues are acid-labile and can often be selectively hydrolyzed[21]. Glycosyl-linkage composition-analysis of the remethylated, methylated, carboxyl-reduced polysaccharide that had been subjected to mild hydrolysis with acid, reduction, and ethylation showed the presence of 1,5-di-*O*-acetyl-4,6-di-*O*-ethyl-2,3-di-*O*-methylglucitol, the formation of which clearly demonstrated that the pyruvic residue was (4→6)-linked to O-4 and O-6 of a terminal glucopyranosyl residue in the polysaccharide.

Preparation, separation, and analysis of overlapping fragments of the polysaccharide. — *Experiment A.* Partial hydrolysis of the methylated, carboxyl-reduced polysaccharide, under the conditions described in the Experimental section, cleaved ~40% of the glycosyl linkages of the 4-linked glucosyl residues, 30% of the glycosyl linkages of the (4→6)-linked glucosyl residues, and 90% of the glycosyl linkages of the 6-linked galactosyl residues. Thus, the glycosyl linkages of the galactosyl residues are considerably more labile to acid hydrolysis than those of the glucosyl residues. These conditions

of partial hydrolysis with acid were selected to give optimal yields of partially methylated di- and tri-saccharides[16].

The mixture of partially methylated oligosaccharides was reduced with $NaBD_4$ and the products ethylated, to give a mixture of partially methylated, partially ethylated oligosaccharide-alditols. Except for ethyl groups on O-6 of former glycosyluronic residues, the positions of the *O*-ethyl groups identify where, in the polysacchar-

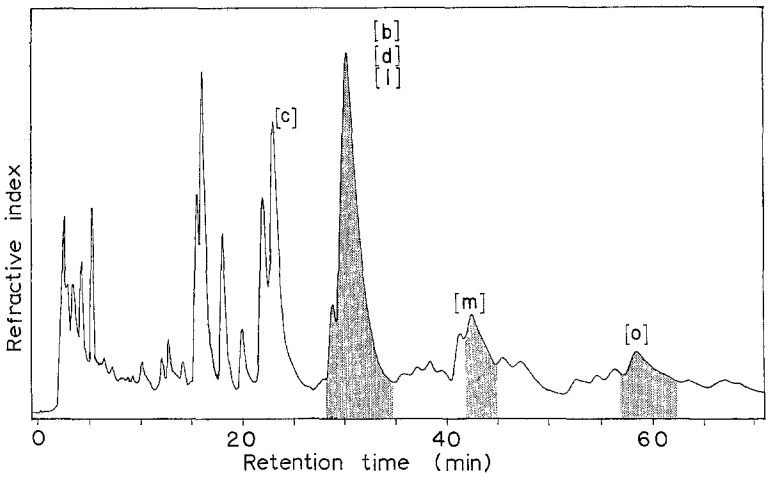

Fig. 3. L.c. separation of partially methylated, partially ethylated oligosaccharide-alditols obtained in Experiment A, by l.c. in a column of Du Pont Zorbax ODS. [The shaded fractions were further purified on a column of Whatman Partisil 5 ODS. The oligosaccharide fragments are identified in Fig. 7.]

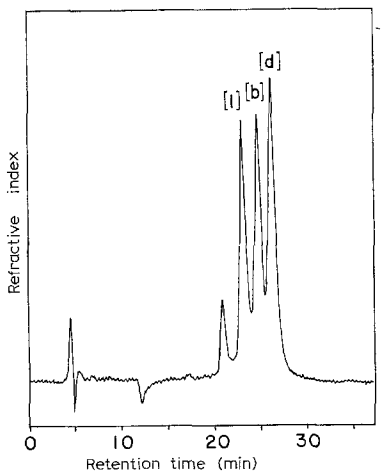

Fig. 4. L.c. elution-profile, in a column of Whatman Partisil 5, ODS, of the shaded fractions in Fig. 3, containing per-*O*-alkylated oligosaccharide-alditols [b], [d], and [l] (Experiment A). [The oligosaccharide fragments are identified in Fig. 7.]

ide, other glycosyl residues were linked to the derivatized oligosaccharide. Derivatives of glycosyluronic residues may be distinguished from identically alkylated derivatives of glycosyl residues by the presence, on C-6 of the glycosyluronic derivatives, of two deuterium atoms introduced during the carboxyl reduction. The mixture of per-*O*-alkylated oligosaccharide-alditols was separated by l.c., and the components were detected in the column effluent by their effect on the refractive index thereof (see Fig. 3). Glycosyl-linkage composition-analysis of a portion of each fraction collected within each refractive-index peak demonstrated that each of the refractive-index peaks contained more than one per-*O*-alkylated oligosaccharide-alditol, except that labeled [c] (see Fig. 3), which contained only the per-*O*-alkylated oligosaccharide-alditol derived from fragment [c] (see Fig. 7).

The per-*O*-alkylated oligosaccharide-alditols in the three shaded regions of Fig. 3 were each subjected to l.c. on a second column. The result of this further fractionation is exemplified, for the first shaded region in Fig. 3, by the profile in Fig. 4. Although the l.c. columns of both manufacturers contained silica-bound, C_{18} hydrocarbons, the columns separated the per-*O*-alkylated oligosaccharide-alditols in different ways. The per-*O*-alkylated oligosaccharide-alditols derived from fragments [b], [d], [l], [m], and [o] (see Fig. 7) were isolated in pure form by a combination of the two l.c. separations. The glycosyl sequence of each of the per-*O*-alkylated oligosaccharides isolated was unambiguously determined by glycosyl-linkage composition-analysis[16].

Experiment B. Methylated, carboxyl-reduced polysaccharide that had been remethylated was partially hydrolyzed, the products were reduced, and the alditols ethylated. The resulting mixture of per-*O*-alkylated alditols was separated by l.c., and detected[18] by c.i.-m.s. using the l.c. solvent as the reactant gas. The c.i.-m.s. gives strong (M + 1) ions of the per-*O*-alkylated oligosaccharide-alditols[18]; for example, the per-*O*-alkylated disaccharide-alditol derived from fragment [a] (see Fig. 7) was detected by its (M + 1) ion (*m/z* 530), and was isolated in pure form from the l.c. experiment. The structure, except for the anomeric configuration, of the per-*O*-alkylated disaccharide-alditol derived from [a] was obtained by glycosyl-linkage composition-analysis.

Reconstructed, selected-ion chromatograms of (M + 1) ions were used for locating per-*O*-alkylated trisaccharide-alditols in the fractions collected during l.c.–m.s. of the per-*O*-alkylated oligosaccharide-alditols. The (M + 1) *m/z* values for all of the per-*O*-alkylated trisaccharide-alditols that theoretically could be formed from a polysaccharide having the glycosyl-linkage composition of the *R. phaseoli* polysaccharide (see Table III) were calculated, and used for forming the reconstructed, selected-ion chromatograms. Nine per-*O*-alkylated trisaccharide-alditols (see Table III) were located, and their glycosyl sequences were unambiguously identified by their c.i. mass spectra obtained during the l.c.–m.s. analysis, their e.i. mass spectra obtained by g.l.c.–m.s. analysis, and the identity of their constituent, partially methylated, partially ethylated alditol acetates. Thus, apart from the anomeric configuration of their glycosyl linkages, the structures of all nine per-*O*-alkylated trisaccharide-alditols

TABLE III

G.L.C. AND L.C. RETENTION-TIMES, AND DIAGNOSTIC IONS FROM C.I.- AND E.I.-M.S. OF DERIVATIZED TRISACCHARIDE FRAGMENTS OBTAINED BY METHYLATION, CARBOXYL-REDUCTION ($LiAlD_4$), REMETHYLATION, AND PARTIAL ACID HYDROLYSIS OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K38; THE RESULTING PARTIALLY METHYLATED OLIGOSACCHARIDES WERE REDUCED ($NaBD_4$) AND ETHYLATED (EXPERIMENT B)

Oligosaccharide	*Fragment (see Fig. 7)*	*R.T.*[a] *(l.c.)*	*(M + 1) ion*	*R.T.*[b] *(g.l.c.)*	*Electron-impact, mass-spectral fragment-ions*							
					aJ_2	aJ_1	abJ_2	abJ_1	cA_1	cA_2	cbA_1	cbA_2
Et→4Glc→4Glc→4GlcA→ 6 ↑ Et	[e]	23.3	734	13.2	266	326	484	544	233	201	451	419
Et→4Glc→4GlcA→4GlcA→ 6 ↑ Et	[f]	21.3	736	13.3	266	326	472	532	247	215	453	421
Et→4GlcA→4GlcA→4Glc→	[g]	18.5	722	13.4	264	324	470	530	235	203	441	409
Et→4GlcA→4Glc→4Glc→ 6 ↑ Et	[h]	25.3	734	13.4	278	338	482	542	235	203	439	407
Et→4Glc→4Glc→ 6 ↑ Et→4Glc	[i]	24.0	732	13.5			482	542	233	201		
Et→4Glc→6Glc→4GlcA→ 4 ↑ Et	[j]	20.0	734	13.2	266	326	484	544	233	201	451	419
Et→4Glc→4Glc→6Glc→ 4 ↑ Et	[k]	23.6	732	14.5	278	338	482	542	233	201	437	405
Et→4Glc→4Glc→4Glc→	[l]	18.7	718	13.4	264	324	468	528	233	201	437	405
Et→4GlcA→4Glc→4Glc→	[m]	18.6	720	13.4	264	324	468	528	235	203	439	407

[a]Retention times (in min) in the Zorbax ODS column, as described in the Experimental section. [b]Retention times (in min) in the SE-30 capillary column, as described in the Experimental section.

were obtained; this was possible, even though several of the per-*O*-alkylated trisaccharide-alditols cochromatographed in the l.c. column, for these were separated by g.l.c.–m.s.

The (M + 1) and other diagnostic, mass-spectral ions for all nine trisaccharide fragments are presented in Table III. The details of the structural elucidation of the per-*O*-alkylated trisaccharide-alditols achieved by using these methods have recently been reviewed[18], except for the very similar oligosaccharides [g], [l], and [m], discussed in the following paragraphs.

Per-*O*-alkylated trisaccharide-alditols derived from fragments [l], [m], and [g] (see Fig. 7) differ only in deuterium labeling. These compounds were slightly separated by l.c. and not at all by g.l.c.; it was, therefore, necessary to determine the glycosyl sequences of these per-*O*-alkylated trisaccharide-alditols as a mixture, and this was accomplished by taking into consideration the data obtained by l.c.–m.s., g.l.c.–m.s., and glycosyl-linkage composition-analysis. In the c.i. mass spectra obtained during l.c.–m.s. analysis, (M + 1) ions were detected at *m/z* 718, 720, and 722. The (M + 1) ion at *m/z* 718 could only be derived from a trisaccharide-alditol originating from three unbranched, hexosyl residues. The (M + 1) ion at *m/z* 720 is derived from a trisaccharide-alditol originating from two unbranched, hexosyl residues and an unbranched glycosyluronic residue, and the (M + 1) ion at *m/z* 722 is derived from a trisaccharide-alditol originating from one unbranched, hexosyl residue and two unbranched, glycosyluronic residues.

The g.l.c.–e.i. mass spectrum of the mixture of the three trisaccharide-alditols yielded only one aJ_2 fragment-ion, which was at *m/z* 264, and only one aJ_1 fragment-ion, which was at *m/z* 324. Therefore, as no alditol cleavage was detected (giving an ion at *m/z* 206, which is characteristic of a 6-linked alditol), all three trisaccharide-

TABLE IV

RELATIVE ABUNDANCE OF THE (M + 1) AND A_1 FRAGMENT-IONS IN THE C.I. AND E.I. MASS SPECTRA OF THE MIXTURE OF PER-*O*-ALKYLATED TRISACCHARIDE-ALDITOLS [g], [l], AND [m]

m/z *Value*	*Experimental (%)*	*Predicted[a] for the mixture [g], [l], and [m] (%)*	*Predicted[a] for the mixture [g], [l], and [m'] (%)*
(M + 1) ion (c.i.)			
718	35		
720	45		
722	20		
A_1 ion (e.i.)			
233	40	35	80
235	60	65	20

[a]See text for description of experiment.

alditols must have possessed an alditol residue derived from a 4-linked glucosyl residue; this was confirmed by glycosyl-linkage composition-analysis of the mixture, in which 4-linked glucitol was the only alditol derivative detected. Glycosyl-linkage composition-analysis also revealed that all of the other constituents in all three oligosaccharide-alditols originated either from 4-linked glucosyl residues or 4-linked glucosyluronic residues. From these data, it was concluded that the per-*O*-alkylated trisaccharide-alditol that yielded an (M + 1) ion at *m/z* 718, and which is composed of 3 unbranched, hexosyl residues, must, therefore, be the per-*O*-alkylated trisaccharide-alditol derived from fragment [l], and that the per-*O*-alkylated trisaccharide-alditol that yielded an (M + 1) ion at *m/z* 722, and which has 2 unbranched, glycosyluronic residues, must be derived from fragment [g].

The per-*O*-alkylated trisaccharide-alditol that yielded an (M + 1) ion at *m/z* 720 contains one glucosyluronic residue and one glucosyl residue distributed between the middle and terminal positions. It could, therefore, have either of two structures. In both trisaccharide-alditols possible, the alditol is a 4-linked glucosyl residue. In one of the two structures possible ([m] in Fig. 7), the deuterium-reduced, glucosyluronic residue is in the nonreducing, terminal position and, in the other possible structure, namely, [m′], the deuterium-reduced glucosyluronic residue is the middle residue. These two possibilities can be distinguished by consideration of the relative abundance of the ions at *m/z* 718, 720, and 722 in the c.i. mass spectrum, and the ions at *m/z* 233 and 235 in the e.i. mass spectrum, obtained for the mixture of the three per-*O*-alkylated trisaccharide-alditols.

The relative proportions of the three per-*O*-alkylated trisaccharide-alditols ([l], [g], and either [m] or [m′]) may be obtained from the relative abundance of their (M + 1) ions (*m/z* 718, 722, and 720, respectively) in the c.i. mass spectrum together with the relative abundance of the A_1 ions in the e.i. mass spectrum of the mixture of the three compounds. These data are presented in Table IV.

The e.i. mass spectrum of the mixture of the three per-*O*-alkylated trisaccharide-alditols contains an A_1 ion (nonreducing, terminal, glucosyl group) at *m/z* 233, resulting from per-*O*-alkylated trisaccharide-alditol [l]. The e.i. mass spectrum of the mixture also contains an A_1 ion (nonreducing, terminal glucosyluronic group) at *m/z* 235, resulting from per-*O*-alkylated trisaccharide-alditol [g]. If the unknown, per-*O*-alkylated trisaccharide-alditol is structure [m], it will yield an A_1 ion at *m/z* 235, and if it is the alternative structure [m′], it will yield an A_1 ion at *m/z* 233. As the relative amounts of the three compounds were known from the c.i. mass spectrum (see Table IV), and as all three are the same compound (except for the deuterium labeling), the ratio of the abundance of *m/z* 233 to *m/z* 235 is readily calculated for the two possibilities, [m] or [m′], of the unknown compound. The results of these calculations are presented in Table IV, and are compared with the experimental values. The experimental values agree with the concept that the third, per-*O*-alkylated trisaccharide-alditol in the mixture possesses structure [m], not structure [m′].

Experiment C. The methylated, carboxyl-reduced polysaccharide was partially hydrolyzed, the products reduced, and the alditols ethylated. The conditions for the

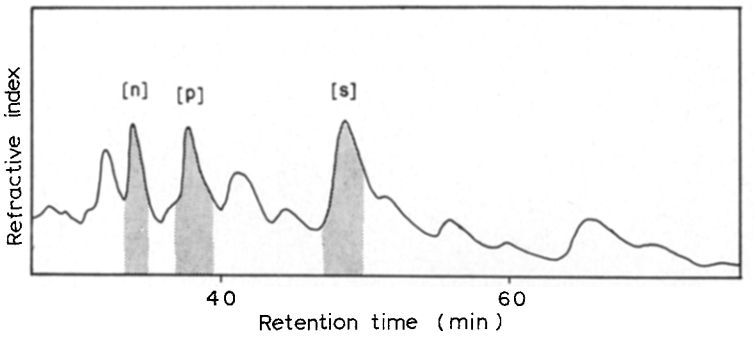

Fig. 5. L.c. elution-profile, in a column of Du Pont Zorbax ODS, of the partially methylated, partially ethylated oligosaccharide-alditols prepared in Experiment C. [Only that part of the chromatogram containing the per-*O*-alkylated oligosaccharide-alditols analyzed is shown. [The oligosaccharide fragments are identified in Fig. 7.]

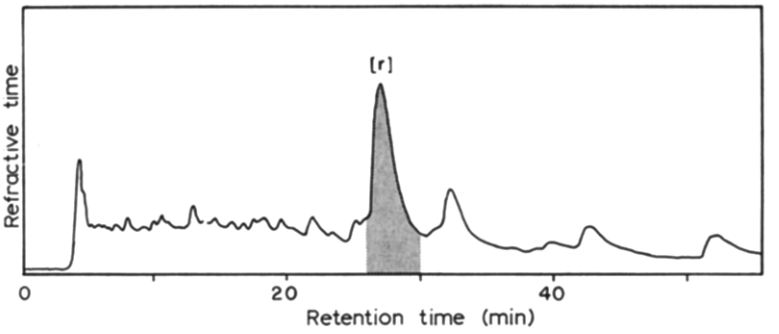

Fig. 6. Isolation by l.c., in a column of Du Pont Zorbax ODS, of the per-*O*-alkylated pentasaccharide methyl glycoside produced by base-catalyzed degradation of the *Rhizobium* polysaccharide as described in Experiment E. [The origin of the fragment is depicted in Fig. 7.]

hydrolysis were so selected as to cleave ~50% of the acid-labile galactosyl linkages. Using these mild conditions, only 10–15% of each of the other glycosyl linkages was hydrolyzed. The resulting mixture of per-*O*-alkylated oligosaccharide-alditols was separated by l.c. in the Du Pont column (see Fig. 5). The peaks labeled [n], [p], and [s] in Fig. 5 contained almost pure per-*O*-alkylated oligosaccharide-alditols derived from fragments [n], [p], and [s] (see Fig. 7), but the components were, nevertheless, further purified by l.c. on the Whatman column before structural analysis. The per-*O*-alkylated oligosaccharide-alditols derived from [n], [p], and [s] were obtained in relatively good yields by the mild conditions of hydrolysis used in this experiment, as only one glycosidic bond had to be hydrolyzed in order to afford each of these fragments. The glycosyl sequences of the purified, per-*O*-alkylated oligosaccharide-alditols (see Fig. 7) were obtained by glycosyl-linkage composition-analysis and by direct probe e.i.–m.s. Diagnostic ions in the e.i. mass spectra of these fragments are listed in Table V; these ions elucidate unambiguously

TABLE V

DIAGNOSTIC IONS FROM ELECTRON-IMPACT MASS SPECTRA OF PARTIALLY METHYLATED, PARTIALLY ETHYLATED OLIGOSACCHARIDE-ALDITOLS AND OF A PARTIALLY METHYLATED, PARTIALLY ETHYLATED PENTASACCHARIDE METHYL GLYCOSIDE

Oligosaccharide	*Fragment (see Fig. 7)*	*Experiment in which fragment was isolated*	*Diagnostic ions (% of base peak and fragment-ion designation)*
Et ↓ 6 Et→4Glc→6Gal→4GlcA→	[n]	C	247 (8.7, cA_1) 215 (72.1, cA_2) 201 (21.8, cA_2) 451 (0.4, cbA_1) 419 (0.2, cbA_2) 340 (10.3, aJ_1) 280 (57.2, aJ_2) 544 (10.2, abJ_1) 484 (11.3, abJ_2)
Et ↓ 6 Et→4Glc→6Gal→4GlcA→4Glc→	[p]	C	247 (10.2, dA_1) 215 (94.5, dA_2) 201 (40.4, dA_2) 324 (8.6, aJ_1) 264 (100.0, aJ_2) 544 (0.5, abJ_1) 484 (0.7, abJ_2) 748 (0.2, $abcJ_1$) 688 (0.5, $abcJ_2$)
Et→4GlcA→4GlcA→4Glc→4Glc→ 6 ↑ Et	[q]	D	249 (15.1, dA_1) 217 (100.0, dA_2) 203 (40.5, dA_2) 469 (0.9, dcA_1) 437 (1.0, dcA_2) 423 (0.9, dcA_2) 405 (0.8, dcA_3) 338 (8.1, aJ_1) 278 (74.5, aJ_2) 542 (1.0, abJ_1) 482 (3.8, abJ_2)
Et ↓ 6 Et→4Glc→6Gal→4GlcA→4Glc→4Glc→	[s]	C	247 (13.8, eA_1) 215 (91.7, eA_2) 201 (46.2, eA_2) 324 (3.7, aJ_1) 264 (100.0, aJ_2) 528 (0.5, abJ_1) 468 (1.3, abJ_2) 748 (0.2, $abcJ_1$) 688 (0.5, $abcJ_2$) 952 (0.3, $abcdJ_1$) 892 (0.2, $abcdJ_2$)
b′ a Et→4Glc→4Glc→OMe 6 ↑ Et→4Glc→4Glc→4Glc d c b	[r]	E	233 (31, dA_1 + $b'A_1$) 201 (100, dA_2 + $b'A_2$) 497 (1.5, $b'aJ_1$) 437 (2.0, dcA_1 + $b'aJ_2$) 701 (0.2, $b'abJ_1$) 641 (0.1, $dcbA_1$ + $b'abJ_2$) 905 (0.3, $b'abcJ_1$ + $dcbaJ_1$) 845 (0.1, $b'abcJ_2$ + $dcbaJ_2$)
Me(D_3)→4Glc→OMe 6 ↑ Et→4Glc→4Glc→4Glc	[t]	E	233 (13, dA_1) 201 (90, dA_2) 437 (1.2, dcA_1) 405 (0.5, dcA_2) 282 (25, aJ_1) 222 (18, aJ_2) 486 (2.4, abJ_1) 426 (1.2, abJ_2) 690 (0.4, $abcJ_1$)

TABLE VI

^{1}H-N.M.R. CHEMICAL SHIFTS AND COUPLING CONSTANTS OF THE ANOMERIC PROTONS OF PER-*O*-ALKYLATED OLIGOSACCHARIDE-ALDITOLS FROM THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K38

Oligosaccharide	*Oligosaccharide fragment (see Fig. 10)*	*Isolated from experiment*	*Chemical shift (δ)*[a]	*Observed* $J_{1,2}$ *(Hz)*	*Anomeric configuration assigned*
Et ↓ 6 Et→4Glc→4GlcA→	[a]	B	5.22	3.3	α
Et→4GlcA→4Glc→	[b_1] and [b_2]	A	4.43	7.8	β
Et→4Glc→4Glc→	[c_1] and [c_2]	A	4.44	7.8	β
Et ↓ 6 Et→4Glc→6Gal→	[d]	A	4.27	7.9	β
Et→4GlcA→4GlcA→4Glc→	[g]	D	4.36	7.9	β
			4.45	8.0	β
Et→4Glc→4Glc→4Glc→	[l]	A	4.33	7.8	β
			4.45	7.8	β
Et→4GlcA→4Glc→4Glc→	[m]	A	4.33	8.0	β
			4.45	8.0	β
Et ↓ 6 Et→4Glc→6Gal→4GlcA→	[n]	C	4.26	7.8	β
			5.24	3.6	α
Et→4GlcA→4Glc→4Glc→4Glc→	[o]	A	4.32	7.9	β
			4.33	7.8	β
			4.33	7.8	β
Et→4Glc→4Glc→OMe 6 ↑ Et→4Glc→4Glc→4Glc	[r]	E	4.24	9.3	β
			4.29	7.8	β
			4.32	7.8	β
			4.34	7.9	β
			4.82	3.9	α[b]

[a]Relative to the signal from internal chloroform, at δ 7.26. [b]This is the signal of the methyl glycoside of the branched glucosyl residue.

the sequence of the per-*O*-alkylated trisaccharide-alditol [n], tetrasaccharide-alditol [p], and pentasaccharide-alditol [s].

Experiment D. The methylated polysaccharide was partially hydrolyzed by using relatively harsh conditions, in order to enrich the hydrolyzate in fragments containing glycosyl linkages stabilized by the carboxyl groups of the glycosyluronic residues. The partially methylated oligosaccharides were reduced, the alditols ethylated, the products carboxyl-reduced, and the compounds re-ethylated. This mixture of per-*O*-alkylated oligosaccharide-alditols was separated by l.c., and the components were detected in the l.c. effluent by c.i.-m.s. A per-*O*-alkylated trisaccharide-alditol

giving an (M + 1) ion at *m/z* 750, and a per-*O*-alkylated tetrasaccharide-alditol giving an (M + 1) ion at *m/z* 968, were detected. The structures of the two compounds, showing that they were derived from fragments [g] and [q] (see Fig. 7), were determined by glycosyl-linkage composition-analysis and by e.i.-m.s. A derivative of fragment [q], having methyl groups at O-6 of the glycosyluronic residues (see Table III), had been characterized earlier. Diagnostic, e.i.-m.s. ions from the direct-probe, mass-spectral analysis of [q] are shown in Table V. The sequence of the glycosyl residues in the per-*O*-alkylated tetrasaccharide-alditol derived from [q] is unambiguously defined by its e.i. mass spectrum.

Experiment E. The methylated polysaccharide was degraded by treatment with a base. The products were then reduced ($NaBD_4$), the alditols ethylated, and the ethyl ethers separated by l.c. in the Du Pont column (see Fig. 6). The major product (labeled [r] in Fig. 6) was further purified in the Whatman column, and characterized by ^{1}H-n.m.r. spectroscopy, e.i.-m.s. on the direct-inlet probe, and glycosyl-linkage composition-analysis. The n.m.r. analysis showed four signals attributable to β-anomeric protons, and one signal at δ 4.82, $J_{1,2}$ 3.9 (see Table VI). The chemical shift and the coupling constant of the latter signal are those expected of the anomeric proton of a methyl α-D-glucopyranoside[22]. Diagnostic ions of the e.i. mass spectrum (see Table V) confirmed that the major product of the base-catalyzed degradation was a methyl glycoside of a per-*O*-alkylated oligosaccharide. Glycosyl-linkage composition-analysis showed that product [r] contains two 4-linked, glucosyl residues, two terminal glucosyl residues substituted with ethyl groups on O-4, and one (4→6)-linked glucosyl residue.

The methyl glycoside of [r] is probably formed by base-catalyzed elimination of the glucosyluronic residue adjacent to the branch point during the initial methyla-

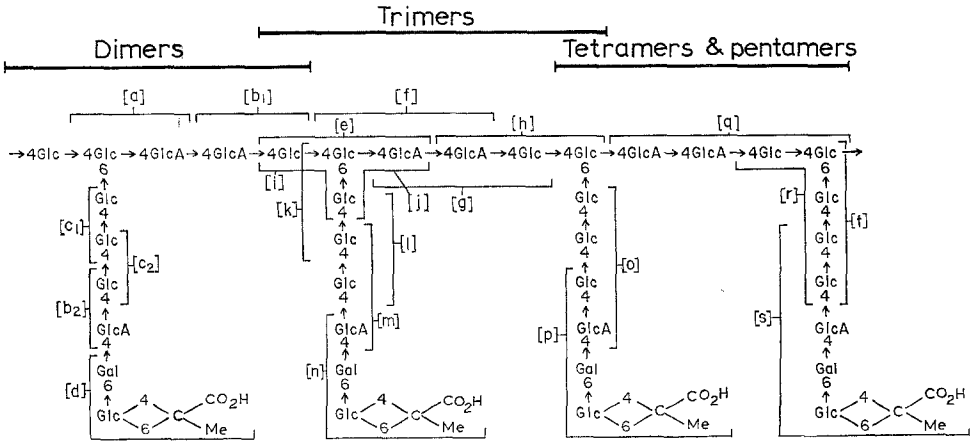

Fig. 7. A summary of the structurally characterized, oligosaccharide fragments (secreted by *R. phaseoli* strain 127 K38) obtained in Experiments A–E. [Each fragment is labelled by a letter that identifies the per-*O*-alkylated oligosaccharide-alditol or per-*O*-alkylated oligosaccharide methyl glycoside derived from the fragment.]

tion of the polysaccharide. The pentasaccharide methyl glycoside is released on further, base-catalyzed degradation.

The structure of [r] was determined by formation from [r] of a mixture of per-*O*-alkylated oligosaccharides; this was accomplished by subjecting the per-*O*-alkylated pentasaccharide methyl glycoside to partial hydrolysis, reduction ($NaBD_4$), and (trideuteriomethyl)ation. The products were separated by l.c. in the Du Pont column, and identified by their e.i. mass spectra. Some of the products were found to be per-*O*-alkylated oligosaccharide-alditols, but most consisted of per-*O*-alkylated oligosaccharide methyl glycosides (partly anomerized at the methyl glycoside position).

One of the products of [r], namely, fragment [t], was purified by l.c., and then characterized by e.i.-m.s.; [t] was shown to be a per-*O*-alkylated tetrasaccharide methyl glycoside. Diagnostic, e.i.-m.s. ions obtained from tetrasaccharide [t] are listed in Table V. Direct probe c.i.-m.s. (ammonia) of [t] gave a strong (M + 18) ion at *m/z* 897, confirming that [t] was, indeed, a per-*O*-alkylated tetrasaccharide methyl glycoside. Glycosyl-linkage composition-analysis of the per-*O*-alkylated tetrasaccharide methyl glycoside confirmed that it derived from fragment [t] (see Fig. 7), that is, that it had a chain of three glucosyl residues attached to O-6 of the (4→6)-linked, branched glucosyl residue. The structure of [t] confirmed that the per-*O*-alkylated pentasaccharide methyl glycoside [r], from which [t] was derived, had the structure assigned to it.

Ring form of the glycosyl residues. — The ring forms of all of the glycosyl residues in the repeating unit were shown to be pyranoid by identifying and characterizing one (or more) partially alkylated oligosaccharide-alditol(s) containing each glycosyl residue at either the alditol end or the other end. The ring form of such residues is made apparent from the location of the *O*-methyl and *O*-ethyl substituents[16].

The repeating unit of the polysaccharide. — The per-*O*-alkylated oligosaccharide alditols that were identified in experiments A–E are depicted in Fig. 7. These per-*O*-alkylated oligosaccharide-alditols, together with the glycosyl composition and glycosyl-linkage composition of the polysaccharide, unambiguously describe the decasaccharide repeating-unit of the polysaccharide. The sequence of the hexasaccharide side-chain is, for example, established from per-*O*-alkylated oligosaccharide-methyl glucoside and alditol derived from fragments [r] and [s], and the tetrasaccharide backbone, from the per-*O*-alkylated oligosaccharide-alditols derived from fragments [f] and [q]. However, many different combinations of identified, per-*O*-alkylated oligosaccharide-alditols can be used to obtain the decasaccharide repeating-unit of the polysaccharide.

Four disaccharide fragments, eleven trisaccharide fragments, four tetrasaccharide fragments, and two pentasaccharide fragments were identified, and structurally characterized. No oligosaccharide fragment inconsistent with the decasaccharide repeating-unit presented was detected. On the other hand, some detected and analyzed fragments not essential for determining the sequence (but consistent with the structure) have not been described in detail, in order to achieve clarity in the description of the structural elucidation.

N.m.r. spectroscopy. — ^{1}H-N.m.r. analysis of the ethylated, methylated, carboxyl-reduced polysaccharide showed two signals, at δ 5.62 (d) and 5.67 (d), which integrated for approximately one proton each, and had $J_{1,2}$ values of 3 and 2 Hz, respectively. These signals are typical of those expected for anomeric protons of α-linked glycopyranosyl residues[23]. Several unresolved signals at δ 4.15–4.50 integrated for 8–10 protons, and were assigned to anomeric protons of the β-linked glycopyranosyl residues[23].

^{13}C-N.m.r. spectroscopy of the polysaccharide showed one broad signal, at δ 101, assigned to C-1 of α-linked glycopyranosyl residues, and two broad signals, at δ 110 and 112, assigned to C-1 of the β-linked glycopyranosyl residues and the acetal-linked carbon atom in the 1-carboxyethylidene group. The signal at δ 101 integrated for two protons, and those at δ 110 and 112 for 9 protons. These results are consistent with a repeating unit (in the polysaccharide) consisting of two α-linked, glycopyranosyl residues and eight (or more) β-linked, glycopyranosyl residues.

Nine per-*O*-alkylated oligosaccharide-alditols and one per-*O*-alkylated pentasaccharide methyl glycoside, isolated in experiments A–E, were analyzed by ^{1}H-n.m.r. spectroscopy, in order to determine the anomeric configurations of their glycosyl residues (see Table VI). Each glycosyl linkage in the established, decasaccharide repeating-unit of the polysaccharide was represented at least once. Signals from α-anomeric protons of the per-*O*-alkylated oligosaccharide-alditols derived from fragments [a] and [n], and the fact that the per-*O*-alkylated oligosaccharide-alditol derived from [d] has a β-anomeric proton, established the location of the two α-linkages detected by ^{1}H- and ^{13}C-n.m.r. spectroscopy of the intact polysaccharide.

Seven per-*O*-alkylated oligosaccharide-alditols, listed in Table VI, contain only β-linked residues. These confirm the location of the eight β-linked glycosyl residues in the decasaccharide repeating-unit of the polysaccharide. By establishing the location of the two α- and the eight β-glycosyl residues, the anomeric configuration of each of the ten glycosyl residues in the decasaccharide repeating-unit was unambiguously determined (see formula **1**).

A signal at δ 1.48 (s) in the ^{1}H-n.m.r. spectrum of the underivatized polysaccharide was assigned to the methyl protons of the pyruvic residues, showing that the acetal-linked pyruvic substituent is in the (*S*) configuration[24]. Two signals, in a ratio of $\sim$1:2, at δ 2.14 (s) and 2.16 (s), were assigned to the methyl protons of *O*-acetyl substituents[25], indicating the presence of acetyl groups linked to at least two oxygen atoms of the repeating unit. Integration of the protons showed $\sim$1.5 total acetyl groups per 1-carboxyethylidene group.

Signals at δ 22.7 and 23.1 in the ^{13}C-n.m.r. spectrum of the underivatized polysaccharide were assigned to the methyl carbon atoms of the *O*-acetyl substituents, and a signal at δ 27.2 was assigned to the methyl carbon atom of the pyruvic residue[24]. These ^{13}C-n.m.r.-spectral results confirmed the presence of acetyl substituents linked to at least two oxygen atoms, and the (*S*) configuration of the pyruvic residue.

GENERAL DISCUSSION

The results reported herein establish that the acidic polysaccharide secreted by *R. phaseoli* strain 127 K38 consists of a decasaccharide repeating-unit having the structure shown in **1**. The glycosyl composition, the absolute configuration of each glycosyl residue, the ring form of glycosyl residue, the glycosyl-linkage composition, the anomeric configuration of the glycosyl linkages, and the sequence of the glycosyl residues have all been determined. The repeating unit contains one pyruvic residue, in the (*S*) configuration, that is acetal-linked to O-4 and O-6 of the terminal glucosyl group of the side chain. The decasaccharide repeating-unit is the largest repeating-unit of any bacterial polysaccharide that has yet been structurally characterized. The polysaccharide contains acetyl linked to at least two oxygen atoms, but the locations of these *O*-acetyl groups have not been determined. The polysaccharide is polydisperse, with individual molecules containing as few as 1, and others containing >2,000, repeating units.

The structure of an acidic polysaccharide secreted by a *R. phaseoli* strain has not previously been studied in detail[26–28]. Chemical analysis of the acidic polysaccharide secreted by several strains of *R. phaseoli* have shown that these polysaccharides are similar in composition to those secreted by *R. leguminosarum* and *R. trifolii*[26–28]. The results reported herein show that the structure of the acidic polysaccharide secreted by *R. phaseoli* strain 127 K38 is related to that of the acidic polysaccharides secreted by two strains of *R. leguminosarum* and two strains of *R. trifolii*[5]. Indeed, the tetrasaccharide backbone, and the three innermost, glucosyl residues of the side chain, in the repeating units of these polysaccharides are the same.

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