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STRUCTURE OF THE EXTRACELLULAR POLYSACCHARIDE FROM *Xanthomonas campestris**

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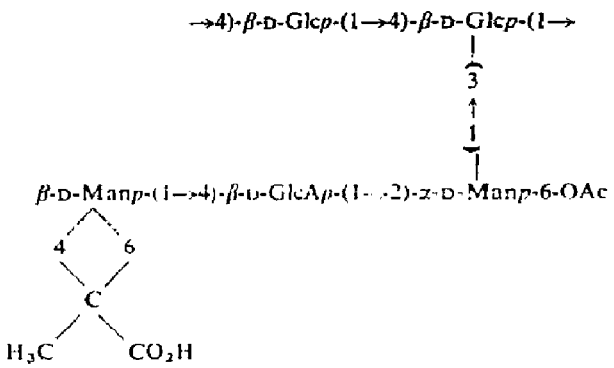
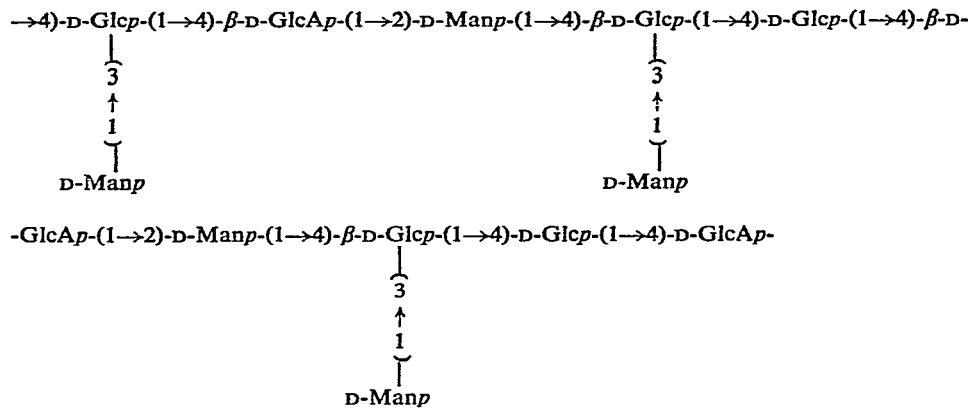
ABSTRACT

Xanthan gum, the extracellular polysaccharide from *Xanthomonas campestris*, has been reinvestigated by methylation analysis, and by uronic acid degradation followed by oxidation and elimination of the oxidized residue. The polysaccharide is composed of pentasaccharide repeating-units with the following structure:



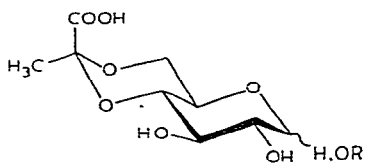
INTRODUCTION

The extracellular polysaccharide from *Xanthomonas campestris* is produced commercially under the name of xanthan gum. Tentative structures, with repeating units containing 16 (Ref. 1) and 14 (Ref. 2) sugar residues, respectively, have been proposed. The gum contains D-glucose, D-mannose, D-glucuronic acid, acetal-linked pyruvic acid, and *O*-acetyl[3]. The two proposed repeating-units are similar, and only one of them (**1**)[2] is given below.



1

Structure **1** has 13 sugar residues; pyruvic acid and the sugar to which it is linked are not included. As 1,3-*O*-(1-carboxyethylidene)erythritol was produced in the Smith degradation, and experiments involving periodate oxidation under different conditions indicated that erythritol was produced from D-glucose but not from D-mannose residues, pyruvic acid was assumed to be linked to D-glucopyranose as a 4,6-acetal[2]. The absolute configuration of the acetal carbon, as in structure **2**, was also determined[4].



2

The O-acetyl groups were tentatively located at O-6 of D-mannose residues, because deacetylation increased the rate of oxidation but not the consumption of periodate. The anomeric configurations were only determined for part of the sugar residues.

Extracellular bacterial polysaccharides are generally composed of oligosaccharide repeating-units[5]. When complete structures have been determined, repeating units of 2–6 sugar residues have been observed. Larger repeating-units should, in our opinion, be regarded with some doubt because of the resulting biosynthetic complexity. Using improved techniques for methylation analysis[6] and for specific degradation of polysaccharides containing uronic acid residues[7], we have reinvestigated the structure of xanthan gum.

RESULTS AND DISCUSSION

Methylation analyses of xanthan gum, without and with carboxyl reduction after the methylation, and analysis of the methylated sugars, as their alditol acetates by g.l.c.–m.s.[6], gave the results listed in Table I, columns A and B, respectively. The analyses indicate that the polysaccharide contains equal proportions of D-mannose linked through O-2, D-glucose linked through O-4, D-glucose linked through O-3 and O-4, and D-glucuronic acid linked through O-4. The sum of 2,3-di-*O*-methyl-D-mannose and 2,3,4,6-tetra-*O*-methyl-D-mannose corresponds to 2,6-di-*O*-methyl-D-glucose, indicating that both these D-mannose ethers derive from terminal D-mannose, with and without pyruvic acid acetal-linked to O-4 and O-6. Terminal D-glucose, with pyruvic acid linked to O-4 and O-6, should have given 2,3-di-*O*-methylglucose in the analyses. The alditol acetate of this sugar is well separated from the observed components (as evident from Table I, column B) and could not have been overlooked. The results therefore demonstrate that the pyruvic acid is linked to O-4 and O-6 of terminal D-mannose residues and not to D-glucose residues. The determination of the absolute configuration of the acetal carbon atom[4] is, of course, not invalidated by this revision. The results also indicate that the polysaccharide is composed of pentasaccharide repeating-units with approximately one-half of the terminal D-mannose residues substituted with acetal-linked pyruvic acid.

TABLE I

METHYL ETHERS OBTAINED FROM THE HYDROLYSATES[a]

Sugars and location of methoxyl groups[b]	T[c]	*Mole* (%)					
		A	B	C	D	E	F
2,3,4,6-Man	1.00	11	8				
2*,3,4,6-Man					27		14
3,4,6-Man	1.55	28	20	32	4	12	
2,3,6-Glc	1.82	24	18	30	30	49	43
2*,3,6-Glc[d]							27
2,6-Glc	2.40	24	27	38	39	39	16
2,3-Man	3.00	13	11				
2,3-Glc	3.10		15				

[a]For the methylated (*A*), methylated and reduced (*B*), glucuronic acid degraded (*C*), remethylated (CD_3I) (*D*), oxidised (*E*), and twice degraded, remethylated (CD_3I) (*F*) polysaccharide. [b]The asterisk indicates the location of the trideuteriomethyl group. [c]Retention time of the corresponding alditol acetate, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a SP-1000 glass capillary column at 220°. [d]Calculated from fragments *m/e* 161/164 and *m/e* 233/236 of the m.s. of 2,3,6-Glc.

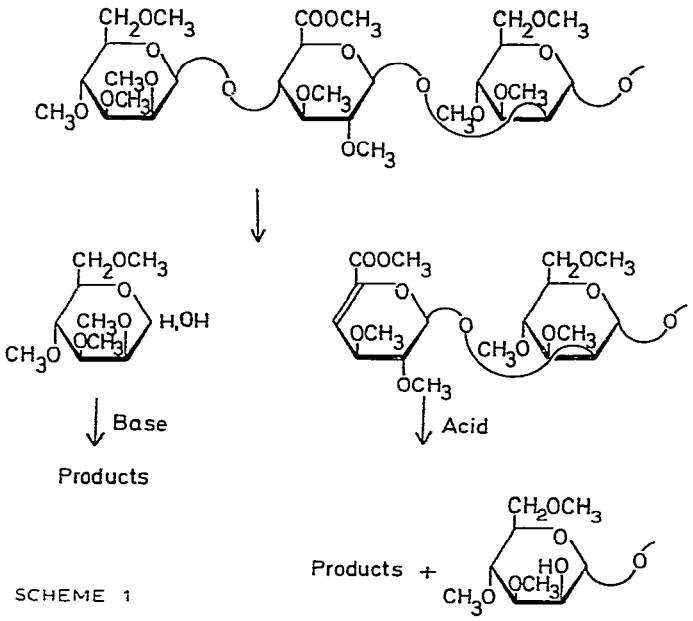
In order to determine the sequence of the sugar residues, the polysaccharide was subjected to a uronic acid degradation[7]. The fully methylated polysaccharide was treated first with base and then with acid under mild conditions. Hydrolysis of

the resulting polymeric material yielded the ethers listed in Table I, column C. On methylation of this material, using trideuteriomethyl iodide, and hydrolysis, the ethers listed in Table I, column D, were obtained. By this degradation, the uronic acid and the terminal D-mannose residue, linked to its 4-position, were eliminated and HO-2 in a D-mannose residue was exposed, demonstrating the presence of the structural element 3 in the polysaccharide.

D-Man*p*-(1→4)-D-GlcA*p*-(1→2)-D-Man*p*-(1→

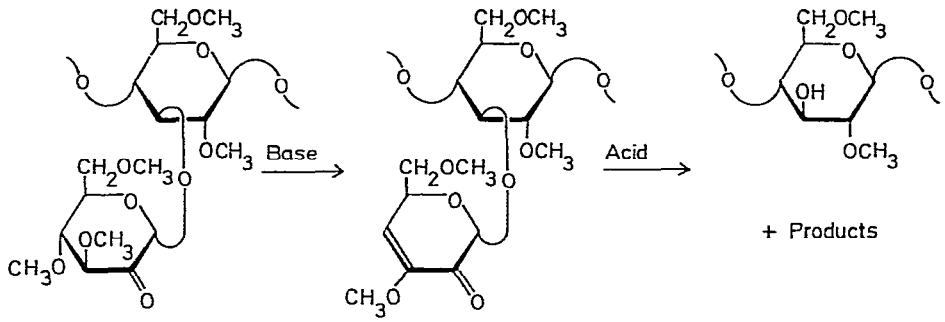
3

The course of the degradation is outlined in Scheme 1

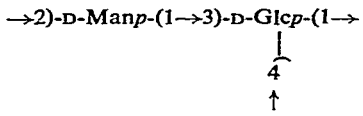


SCHEME 1

The polymeric product obtained after uronic acid degradation was subjected to a second degradation, involving oxidation, treatment with base, and acid hydrolysis under mild conditions. Alkaline degradation of a model glycoside[8] with a ketogroup at C-2, and the application of the degradation method to polysaccharides[9] have been reported. An analogous degradation was also performed during structural studies of the *Klebsiella* type 28 capsular polysaccharide[10]. As indicated in Scheme 2, the terminal D-mannose residue is eliminated and HO-3 in the originally branching D-glucose residue becomes exposed. The degradation was followed by analysis of the hydrolysed product, before and after trideuteriomethylation (Table I, columns E and F). The presence of the structural element 4 is thus demonstrated by the second degradation.



SCHEME 2

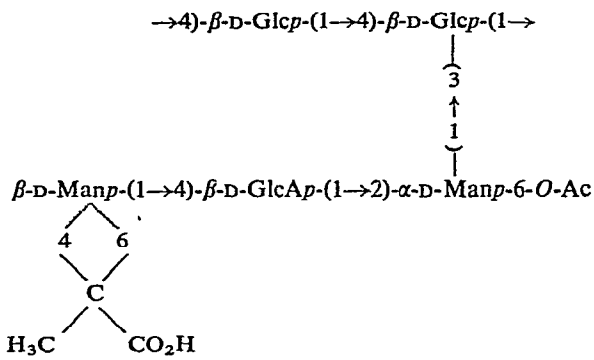


4

The optical rotations of the polysaccharide ($[\alpha]_D$ ~0° in water) and of the methylated polysaccharide ($[\alpha]_D$ +2° in chloroform) indicate that most of the sugar residues are β-linked. The methylated material after the uronic acid elimination, however, had $[\alpha]_D$ +18°, indicating the presence of one α-glycosidic and two β-glycosidic residues. In agreement with this conclusion, the n.m.r. spectrum of this material had signals at δ 4.21 (2 H, $J_{1,2}$ 7 Hz) and 5.11 (1 H, $J_{1,2}$ <1 Hz) assigned to the anomeric protons of two β-D-glucopyranose and one α-D-mannopyranose residue, respectively. The product after the second degradation had $[\alpha]_D$ ~0°, in agreement with the value of fully methylated cellulose.

The amount[3] (4.7%) of *O*-acetyl groups corresponds to one residue per pentasaccharide repeating-unit. On treatment of the polysaccharide with methyl vinyl ether followed by methylation[11], uronic acid degradation takes place during the alkaline conditions of the latter reaction. This reaction, which has been used for modification of polysaccharides[12], should cause degradation of the uronic acid residue and the terminal D-mannose residue. Hydrolysis of the polymeric product, however, yielded 6-*O*-methyl-D-mannose, demonstrating that acetyl groups are linked to O-6 of the D-mannose residues in the chain. 2,6-Di-*O*-methyl-D-mannose was also formed and is probably due to elimination of some uronic acid residues during the alkaline treatment. Similar results were obtained during the location of *O*-acetyl groups in the *Klebsiella* type 59 capsular polysaccharide[13].

From the combined evidence, the repeating unit **5** is proposed for xanthan gum. The dotted lines in the figure indicate that only about one-half of the terminal D-mannose residues carry acetal-linked pyruvic acid.



5

In bacterial polysaccharides composed of oligosaccharide repeating-units, some components, such as *O*-acetyl group and terminal D-glucose residues, may occur in non-stoichiometrical proportions. Pyruvic acid, when a component of a bacterial polysaccharide, has, however, always been observed in every repeating unit, as, for example, in the *Klebsiella* type 21 capsular polysaccharide[14]. The question may therefore be raised as to whether the pentasaccharide repeating-unit proposed here should be doubled, giving one pyruvic acid per decasaccharide repeating-unit. Against this possibility is the fact that a closely related polysaccharide, probably having the same carbohydrate backbone but with a lower percentage of pyruvic acid, is produced by *X. oryzae*[15]. It seems, therefore, that pyruvic acid may be present in non-stoichiometrical proportions in these polysaccharides.

A comparison of the present structure (**5**) with those previously suggested (*e.g.*, **1**) shows that major structural features found by the previous investigators are incorporated in the pentasaccharide repeating-unit. Results that were most probably due to incomplete reactions or analytical difficulties were, however, interpreted as due to minor structural features and necessitated the assumption of a fairly large repeating-unit.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was conducted with a Perkin–Elmer model 990 instrument, equipped with a glass capillary column (25 m × 0.25 mm) wall-coated with SP-1000 (LKB-Products, Sweden). Peak areas were measured with a Hewlett–Packard 3370-B electronic integrator. For g.l.c.–m.s., a Perkin–Elmer 270 gas chromatograph–mass spectrometer fitted with an OV-225 S.C.O.T. column was used. Hydrolyses were performed with 90% formic acid for 1 h at 100°, followed by treatment with 0.25M sulphuric acid for 16 h at 100°. A Varian XL-100 instrument was used for the n.m.r. spectra and tetramethylsilane was used as internal reference.

Methylation of the polysaccharide. — The polysaccharide (160 mg) was dissolved in methyl sulphoxide (150 ml), in a sealed bottle in an ultrasonic bath. 2M Methyl-

sulphinyl anion in methyl sulphoxide (40 ml) was added, and the mixture was agitated in an ultrasonic bath for 30 min, and then kept for 18 h at room temperature. Methyl iodide (15 ml) was added with external cooling; the reaction mixture was agitated in an ultrasonic bath for 30 min, poured into water, dialysed, and freeze-dried to yield 160 mg of fully methylated product, $[\alpha]_D^{25}$ +2° (*c* 2.00, chloroform). Part of this material (1 mg) was hydrolysed, and the sugars were analysed as their alditol acetates by g.l.c.–m.s. (Table I, column A). Another part (3 mg) was hydrolysed, and the sugars were trimethylsilylated, reduced with lithium aluminium hydride[16], hydrolysed, and analysed as described above (Table I, column B).

Uronic acid degradation. — Methylated polysaccharide (150 mg) and toluene-*p*-sulphonic acid (trace) were dissolved in methanol (25 ml) containing 2,2-dimethoxypropane (0.3 ml), and the resulting solution was boiled under reflux for 1 h. The solution was cooled and a piece of freshly cut sodium (200 mg) was added, resulting in a turbid solution which was boiled under reflux for 3 h, cooled, neutralised with 50% aqueous acetic acid, dialysed against water, and freeze-dried. A solution of the product in 50% aqueous acetic acid was kept at 100° for 10 h, cooled, dialysed against water, and freeze-dried to yield 70 mg of modified polysaccharide, $[\alpha]_D^{25}$ +18° (*c* 0.93, chloroform). Hydrolyses of this product (1 mg) yielded the methylated sugars listed in Table I, column C. Part of the material (1 mg) was subjected to methylation analysis, using trideuteriomethyl iodide and the procedure described above (Table I, column D).

Oxidation and degradation of the modified polysaccharide. — The oxidation agent[17] was prepared, under anhydrous conditions, at −45° by dropwise addition of methyl sulphoxide (6.4 ml) to a stirred solution of chlorine in dichloromethane (M, 18 ml). The modified polysaccharide (65 mg) in dichloromethane (5 ml) was added dropwise (with the aid of a syringe) to the stirred, cooled oxidation mixture. The mixture was kept at −45° with stirring for 6.5 h, and then triethylamine (5 ml) was added dropwise. After 15 min at −45°, the reaction mixture was warmed to room temperature, dialysed against water, and freeze-dried. Part of the material (1 mg) was hydrolysed and analysed as above, showing some 3,4,6-tri-*O*-methyl-D-mannose in addition to the expected sugars (Table I, column E). A second oxidation of the material gave the same result, showing that the oxidation was complete but that part of the uronic acid residues had not been eliminated. This was also evident from the analysis of the material from the uronic acid degradation (Table I, column D). A solution of the oxidised polysaccharide in a mixture of dichloromethane (5 ml) and M ethanolic sodium ethoxide (5 ml) was kept at room temperature for 2 h, neutralised with acetic acid, dialysed, and freeze-dried, The residue was treated with 50% aqeous acetic acid for 16 h at 100°, dialysed, and freeze-dried to yield 23 mg of twice-degraded polysaccharide, $[\alpha]_D^{25}$ ~0° (*c* 1.00, chloroform). Part of the material (1 mg) was subjected to methylation analysis, using trideuteriomethyl iodide and the procedure described above (Table I, column F).

Location of the O-*acetyl groups.* — The polysaccharide (5 mg). and toluene-*p*-sulphonic acid (1 mg) were dissolved in methyl sulphoxide (5 ml) in a sealed serum

flask. Methyl vinyl ether (2 ml) was added and the solution was kept for 3 h at 14°. Excess of methyl vinyl ether was removed by evaporation, and then 2M methylsulphinyl anion in methyl sulphoxide (4 ml) was added. The solution was agitated in an ultrasonic bath for 30 min, kept for 18 h at room temperature, and cooled in an ice bath, and then methyl iodide (2 ml) was added. The mixture was stirred ultrasonically for 30 min, poured into water, and dialysed. The product was hydrolysed and the sugars analysed as described above, giving the alditol acetates of 2,6-di-*O*-methyl-D-mannose (*T* 2.55), 6-*O*-methyl-D-mannose (*T* 3.40), D-mannose, and D-glucose in the molar proportions 17:17:11:55.

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