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IDENTIFICATION AND LOCATION OF L-GLYCERATE, AN UNUSUAL ACYL SUBSTITUENT IN GELLAN GUM*

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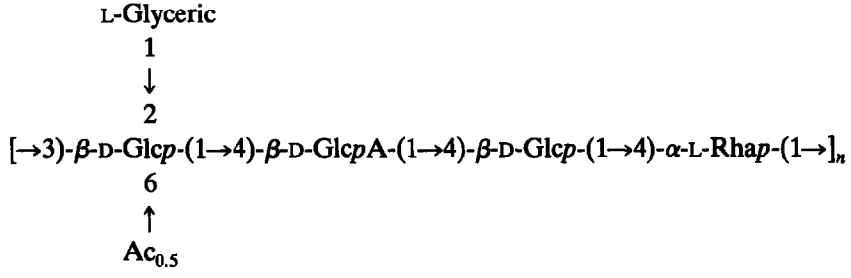
ABSTRACT

Gellan gum, the extracellular polysaccharide produced by *Pseudomonas elodea,* had been shown to be composed of a repeating, partially acetylated tetrasaccharide. Through the use of selective solvolysis of the rhamnosyl linkages in the polysaccharide with anhydrous, liquid hydrogen fluoride at −40°, oligosaccharides representing the repeating unit of the polymer in various acylated forms have now been generated. By a combination of n.m.r. spectroscopy, f.a.b.-mass spectrometry, and gas–liquid chromatographic analysis, these oligomers were shown to be substituted mainly with L-glyceric esters on O-2 of what was the 3-linked D-glucose in the original polymer, and substituted to a lesser degree with an acetic ester at O-6 of the same residue. The native gum must have the following structure.



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INTRODUCTION

Recently, the basic fundamental structure (**1**) of gellan gum was reported[1,2].

$$[\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]_n$$

1

Gellan gum has the peculiar property that, in its native state, it forms a weak gel in water, but, after treatment with alkali, the gum, much like agar[3], forms a rigid gel. The only alkali-labile substituent reported was an acetyl group situated on approximately one in three of the repeating tetrasaccharide units. It was not expected that removal of the acetate could cause such a major change in physical properties.

From experiments in one of our laboratories, we have been cataloging the conditions in liquid HF needed to break *specific* glycosidic linkages[4–7]. From our results, we expected that, at −40°, only the L-rhamnosyl linkage in gellan gum would be cleaved, thus producing the repeating unit. Because, under the relatively mild conditions we have been using[8], primary and secondary esters are stable in liquid HF, the resulting repeating units we obtain should still contain any acyl substituents present in the original polymer. Under much harsher conditions (*e.g.*, several hours at room temperature), loss of acyl substituents, Walden inversion, and acyl-group migration may occur[8]. Our previous results[4,5,7] indicated that, at low temperatures, acyl migration does not occur, and there is no Walden inversion, although there is evidence for a small loss of acyl groups. We were successful in producing repeat units from both native and alkali-treated gellan gum and found, from n.m.r. spectroscopy, that more than just acetate was attached to the polysaccharide.

RESULTS AND DISCUSSION

Preparation of oligosaccharide. — Both native and alkali-treated gellan gum were found to behave similarly in liquid HF at −40°. As expected, by far the predominant cleavage occurs after the α-L-rhamnosyl linkage, but, as may be seen from the gel-filtration profiles of the reaction products, a large proportion of the material was not degraded to the tetrameric oligosaccharide expected (see Fig. 1a and b). The proportion remaining larger than expected varied somewhat from experiment to experiment, but could be significantly decreased by adding 1 mL of methanol per 10 mL of hydrogen fluoride during the reaction (see Fig. 1c). Longer reaction times (30 min) did not increase the proportion of low-molecular-weight products. The linkages between all of the sugars but rhamnose in the products appeared, by n.m.r. spectroscopy, to be unchanged from those in gellan gum.

Treatment of the native or alkali-treated polymer with HF at −23° generated

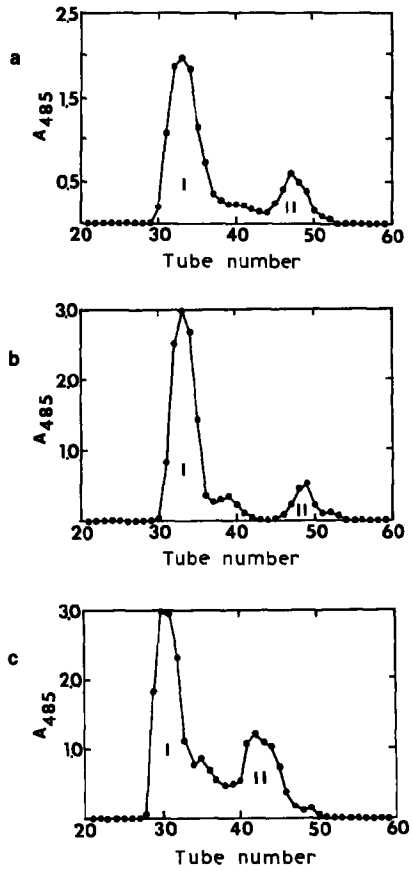


Fig. 1. Chromatography on Bio-Gel P2 of the oligosaccharides from (a) native gellan gum, (b) deacylated gellan gum, and (c) native gellan gum, obtained by treatment in liquid HF with 10% MeOH for 15 min at −40°.

trisaccharides representing the repeating unit lacking the rhamnose residue. These trisaccharides were predominantly the fluorides. Only traces of the tetrasaccharide fluorides were detected after treatments at −40°. Thus, it appears that glucosyl fluorides are much more stable than rhamnosyl fluorides. As with the treatments at −40°, a substantial proportion of the material remained as a higher-molecular-weight form maintaining the same sugar composition as the intact polymer.

1D-N.m.r. spectroscopy. — 1D-^{1}H-N.m.r. spectroscopy of the tetrasaccharide from the alkali-treated material (see Fig. 1b, peak II) gave a spectrum (Fig. 2b) that appeared to be identical to that obtained by Jansson *et al.*[1] for a tetrasaccharide they produced from gellan gum by partial hydrolysis with acid. The characteristic signals at 5.10:1.33, and 4.85:1.35 p.p.m., corresponding to H-1 and H-6 of the α and β anomers of rhamnose, showed convincingly that the rhamnose was the reducing-sugar residue of the oligosaccharide. The doublets at 4.72, 4.59,

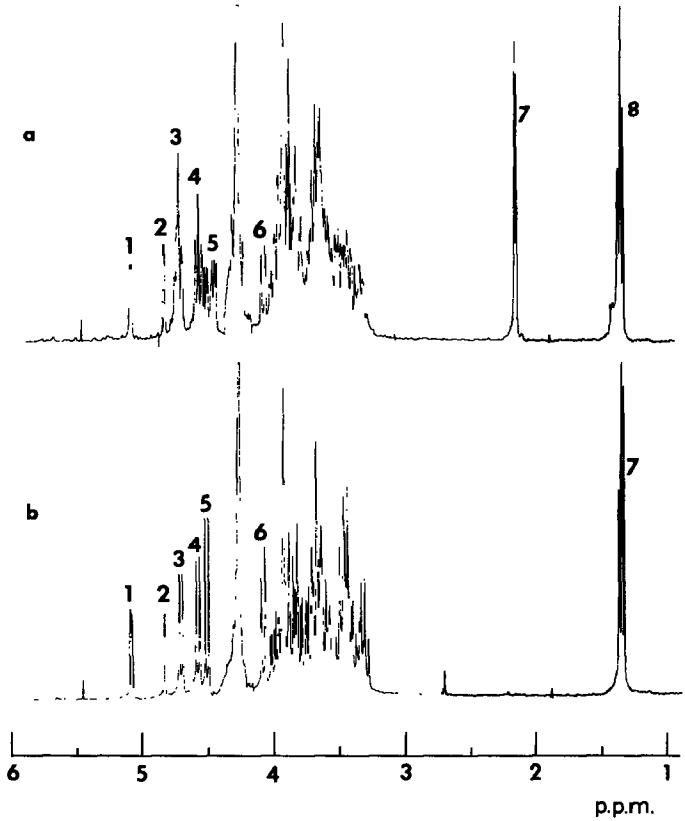


Fig. 2. a. ^{1}H-N.m.r. spectrum of the repeating unit of native gellan gum (see Fig. 1a, peak II). The peaks were assigned as follows: (1) H-1 of α-Rha, (2) H-1 of β-Rha, (3) H-1 of 4-Glc + H-1 of 2-*O*-acylated Glc + H-2 of 2-*O*-acylated Glc, (4) H-1 of GlcA + H-1 of nonreducing (terminal) Glc, (5) H-2 of glycerate, (6) H-5 of GlcA, (7) CH_3 of acetate, and (8) CH_3 of β- and α-Rha. b. ^{1}H-N.m.r. spectrum of the repeating unit of deacylated gellan gum (see Fig. 1b, peak II). The peaks were assigned as follows: (1) H-1 of α-Rha, (2) H-1 of β-Rha, (3) H-1 of 4-Glc, (4) H-1 of GlcA, (5) H-1 of nonreducing (terminal) Glc, (6) H-5 of GlcA, and (7) CH_3 of β- and α-Rha.

and 4.53 p.p.m. showed that there were three additional, unique, β-linked sugars.

The spectrum of the tetrasaccharide from the native polysaccharide (see Fig. 2a) was strikingly different in the anomeric region of the spectrum. The signals from the α and β anomers of the reducing rhamnose were still present, but the rest of the anomeric region was no longer resolved. Integration of signal intensity showed approximately two protons resonating in the region between 4.4 and 4.8 p.p.m. in addition to the three expected from H-1 from the three β-linked sugars. When the spectrum was recorded at room temperature, signals diagnostic of acylation at O-6 of glucose were evident at 4.23–4.27 p.p.m.[9], supporting the suggestion of Jansson *et al.*[1] that there was an acetyl group on O-6 of some of the glucose residues. Two singlets, at 2.15 and 2.16 p.p.m., confirmed the presence of about 0.5 acetyl groups per repeat unit. No other unusual peaks were apparent in

the high-field region of the spectrum. Thus, any additional acyl substituent present, giving rise to the complication in the anomeric region, must only contain hydrogen atoms resonating in the region from 3.3 to 4.8 p.p.m. Such hydroxy acids as glycolic or glyceric were likely candidates.

Treatment of the acylated tetrasaccharide with alkali yielded a material indistinguishable by ^{1}H-n.m.r. spectroscopy from the tetrasaccharide obtained from the alkali-treated polymer.

Characterization of the unusual substituent by g.l.c. — Small acyl substituents such as succinate and 3-hydroxybutanoate[7] can be readily identified by gas–liquid chromatography of their trimethylsilyl derivatives.

Samples of intact and HF-depolymerized material from both native and alkali-treated gellan gum were hydrolyzed in 2M trifluoroacetic acid, and the products trimethylsilylated. In each of over ten experiments on native material, one component of this mixture was co-eluted in gas–liquid chromatography with authentic, trimethylsilylated glycerate. No component was eluted at the retention time of trimethylsilylated glycolate. Retention times for glycolate, glycerate, and malate under our conditions were 12.5, 22.4, and 27.6 min, respectively. The intact, native polymer contained about one mol of glycerate per repeat unit. The mass spectrum obtained by g.l.c.–mass spectrometry of the sample component was identical to that obtained from authentic trimethylsilylated glycerate. Both spectra showed peaks at *m/z* 322, 307, 291, 205, 189, 147, 133, 117, 103, and 73. The weak ion at 322 corresponds to the molecular ion, and that at 307 to M $-$ CH_3. Fragmentation between carbon atoms of the glycerate would give rise to ions having *m/z* 205, 103, and 117. The peaks at 291 and 189 were large, and probably arose by loss of 16 mass units from the 307 and 205 fragments, respectively; this could be loss of methane. The ion at 147 is most likely $(CH_3)_3SiOSi(CH_3)_2^+$.

To determine which optical isomer was present, the secondary butyl ester of the glycerate was prepared and trimethylsilylated[10]. The diastereomers formed from the racemic alcohol were not resolved under any of the g.l.c. conditions we used. However, when the butyl esters were acetylated[11], the diastereomers were readily separated. L-Glycerate was eluted under our conditions at 14.4 and D-glycerate at 14.6 min. The derivatized glycerate from gellan gum, upon coinjection with a mixture of the standards, co-chromatographed with the L-glycerate *(R)*-(−)-2-butanol ester, and thus must be L-glycerate. Although the glycerate normally encountered in metabolism is D-glycerate, both D-glycerate[12] and L-glycerate[13,14] have been found as constituents of polysaccharides. D-Glycerate was found glycosidically linked through O-2 to the reducing end of a glucan[12]. L-Glycerate has been found in an amide linkage to 3-amino-3,6-dideoxygalactose[13] and 3-amino-3,6-dideoxyglucose[14].

F.a.b.–m.s. of oligosaccharides from gellan gum. — One excellent method by which to show that oligosaccharides are substituted by non-sugar components is to compare the molecular weights of the potentially acylated and deacylated oligosaccharides.

The f.a.b. mass spectrum of the tetrasaccharide fraction formed from the alkali-treated polysaccharide showed that, as expected, it consisted of two hexoses, a uronic acid, and a 6-deoxyhexose. Peaks in the positive-ion mode were observed at $[M + H]^+$ 665, $[M + NH_4]^+$ 682, $[M + Na]^+$ 687, and $[M + glycerol + H]^+$ 757. In the negative-ion mode, the predominant peak was at 663, $[M - H]^-$. Additonal minor peaks were obtained in the region of *m/z* 850–920 in both the positive- and negative-ion modes. Components which could give rise to these minor peaks were not observed by any other methods; accordingly, they were not investigated further.

The mass spectrum of the tetrasaccharide fraction from native gellan gum was complex (see Fig. 3). Peaks were observed ranging from the mass expected for the tetrasaccharide repeat unit containing no substituents up to that for the tetrasaccharide with two glycerate residues and one acetate residue. The sample used to obtain the mass spectrum was from an early run, in which we obtained a particularly low yield of the tetrasaccharide fraction, and the oligosaccharide peak was broader than usual from the P-2 column. The mass spectrum shows that the sample also contained some trisaccharides formed by loss of the rhamnose residue. These also contained ions of the masses expected to be found in the trisaccharide containing acetate and glycerate, indicating that the rhamnose was probably not a major site of acylation.

The presence of some ions representing tetrasaccharides with more than two acylations indicates that the acylation pattern has minor variants, but the methods of analysis other than f.a.b. mass spectrometry are not sensitive enough to characterize them.

2D-N.m.r. spectroscopy. — In an effect to obtain information on the location of the acyl substituents, and to corroborate the presence of glycerate, various 2D-n.m.r. experiments were performed. Comparison of the ^{13}C–^{1}H heteronuclear correlation of the tetrasaccharides showed some informative differences. All of the ^{13}C–^{1}H signals of the deacylated tetrasaccharide (see Fig. 4) could be tentatively

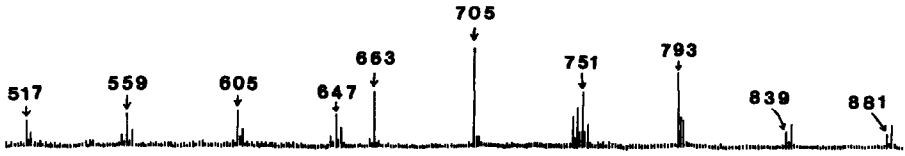


Fig. 3. Negative, fast-atom-bombardment (f.a.b.) mass spectrometry of a tri- and a tetra-saccharide from the native gellan gum (see Fig. 1a, peak II). The assignments were as follows. 517, Glc-GlcA-Glc − H^-; 519, Glc-GlcA-Glc(F) − H^-; 559, Glc-GlcA-Glc + Ac − H^-; 561, Glc-GlcA-Glc(F) + Ac − H^-; 605, Glc-GlcA-Glc + G − H^-; 607, Glc-GlcA-Glc(F) + G − H^-; 647, Glc-GlcA-Glc + G + Ac − H^-; 649, Glc-GlcA-Glc(F) + G + Ac − H^-; 663, Glc-GlcA-Glc-Rha − H^-; 705, Glc-GlcA-Glc-Rha + Ac − H^-; 747, Glc-GlcA-Glc-Rha + 2Ac − H^-; 749, Glc-GlcA-Glc-Rha(F) + 2Ac − H^-; 751, Glc-GlcA-Glc-Rha + G − H^-; 753, Glc-GlcA-Glc-Rha(F) + G − H^-; 793, Glc-GlcA-Glc-Rha + G + Ac − H^-; 795, Glc-GlcA-Glc-Rha(F) + G + Ac − H^-; 839, Glc-GlcA-Glc-Rha + 2G − H^-; 841, Glc-GlcA-Glc-Rha(F) + 2G − H^-; 881, Glc-GlcA-Glc-Rha + 2G + Ac − H^-; and 883, Glc-GlcA-Glc-Rha(F) + 2G + Ac − H^-, where G is glycerate and Ac is acetate.

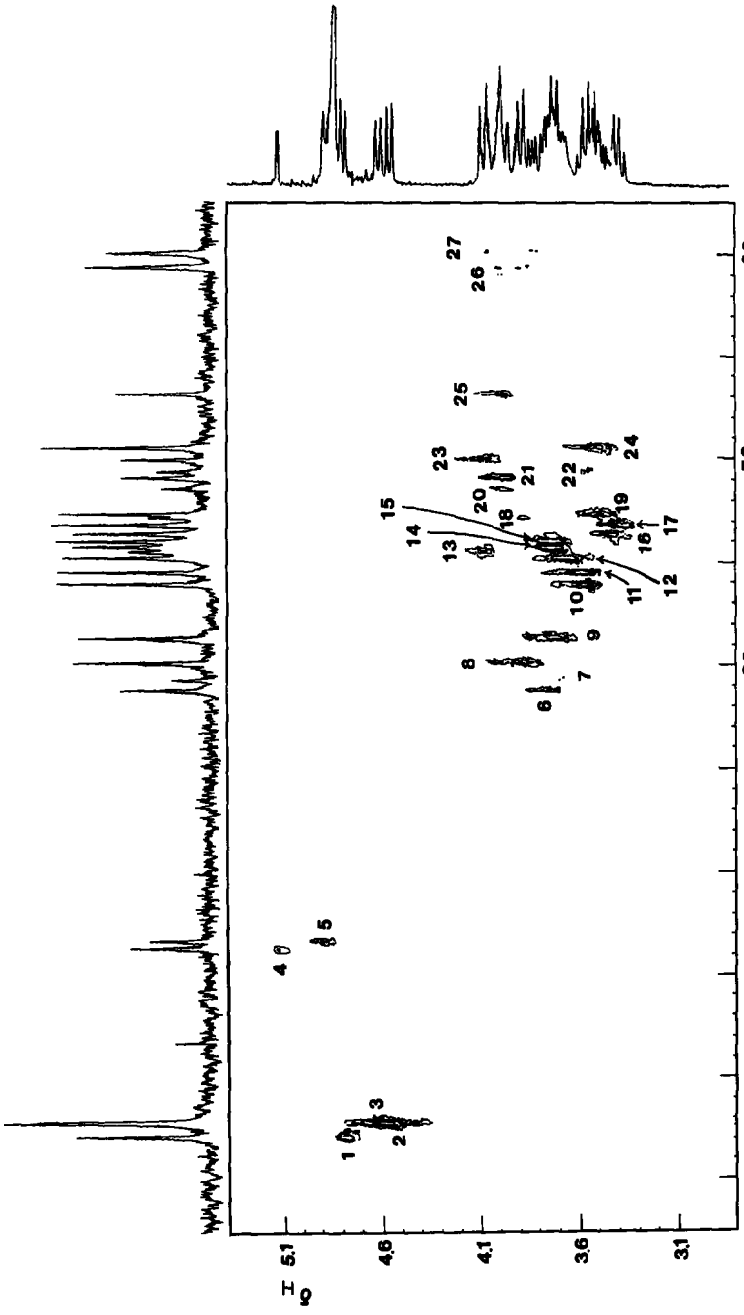


Fig. 4. ^{13}C–^{1}H-N.m.r. spectrum (2D, heteronuclear-correlated) of the repeating unit of deacylated gellan gum (see Fig. 1b, peak II), together with proton-decoupled, 1D, ^{13}C-spectrum (above) and the ^{1}H spectrum (right). Signal numbers correspond to those in Table I.

identified (see Table I) by comparison with literature values[1,13,15–19], and by use of the proton–proton, 2D correlation spectrum.

As Jansson *et al.*[1] reported, compared to the signals from the deacylated material, native material had a smaller ^{13}C peak at 62.5 p.p.m. for C-6 of a glucose residue, and had an additional peak at 63.1 p.p.m. However, this additional peak did not correlate with the complex, ^{1}H signal at 4.25 p.p.m. expected from H-6 of a 6-*O*-acylated glucose[9]; instead, it correlated with a ^{1}H doublet at 3.88 p.p.m. This signal was therefore assigned to C-3 and H-3 of glycerate[14]. Two pairs of closely associated signals, at 71.8 and 4.47 p.p.m., are proposed to be from C-2 and H-2, respectively, of glycerate[14] in two slightly different environments. The two signals at 74.4 and 4.73 p.p.m. are likely to be from C-2 and H-2, respectively, of the glucose acylated on O-2 with glycerate (see later).

TABLE I

ASSIGNMENTS OF CARBON AND HYDROGEN ATOMS TO THE SIGNALS OBSERVED IN THE ^{13}C–^{1}H, 2D, HETERONUCLEAR-CORRELATED, N.M.R. SPECTRUM (FIG. 4) OF THE REPEATING UNIT OF DEACYLATED GELLAN GUM (SEE FIG. 1b, PEAK II)

Signal	δ_C[a]	δ_H[b]	*Tentative assignments*	*References*
1	103.0	4.75	C-1, H-1 4-Glc	1, 17
2	102.4	4.51	C-1, H-1 t-Glc[c]	1, 15, 16
3	102.3	4.56	C-1, H-1 4-GlcA	1, 3, 19
4	93.8	5.10	C-1, H-1 α-Rha	1, 17
5	93.5	4.86	C-1, H-1 β-Rha	1, 5, 17
6	81.2	3.73	C-4, H-4 α-Rha	1, 17
7	80.8	3.65	C-4, H-4 β-Rha	1, 17
8	79.9	3.82	C-4, H-4 4-GlcA	1, 19
9	78.7	3.69	C-4, H-4 4-Glc	1, 16, 17
10	76.1	3.60	C-3, H-3 4-Glc[d]	15, 17
11	75.5	3.62	C-5, H-5 4-Glc[d]	15, 17
12	74.8	3.64	C-5, H-5 t-Glc	15, 16, 17
13	74.5	4.08	C-5, H-5 4-GlcA	19
14	74.3	3.70	C-3, H-3 t-Glc[d]	15, 16
15	74.0	3.70	C-3, H-3 4-GlcA[d]	19
16	73.7	3.32	C-2, H-2 4-Glc	15, 17
17	73.2	3.30	C-2, H-2 t-Glc	17
18	72.7	3.86	C-3, H-3 β-Rha	17
19	72.6	3.44	C-2, H-2 4-GlcA	15, 19
20	71.4	3.94	C-2, H-2 β-Rha	17
21	70.8	3.94	C-2, H-2 α-Rha	17
22	70.5	3.52	C-5, H-5 β-Rha	17
23	70.0	4.05	C-3, H-3 α-Rha	17
24	69.5	3.48	C-4, H-4 t-Glc	17
25	66.9	3.94	C-5, H-5 α-Rha	17
26	60.9	3.96	C-6, H-6 t-Glc	17
		3.84		
27	59.9	4.05	C-6, H-6 4-Glc	17
		3.80		

[a]From external Me_4Si. [b]From internal TSP. [c]t-Glc is glucose at the nonreducing terminus. [d]The assignments may have to be reversed.

The spectra in the anomeric region show almost identical chemical shifts for C-1 and H-1 of the 4-linked glucose, and for C-1 and H-1 of both the α- and β-rhamnose. However, two signals (^{13}C, ^{1}H 100.4, 4.73 p.p.m.; and 100.0, 4.72 p.p.m.) appear upfield in the spectrum from the native material only, and we suggest these are from C-1 and H-1 of the terminal glucose acylated at O-2 or at both O-2 and O-6. The signal intensity at 73.3 and 3.30 p.p.m. from the C-2, H-2 of the terminal glucose is much lessened in the spectrum of the tetrasaccharide from the native polymer, being mostly shifted to 74.4 and 4.73 p.p.m., confirming that O-2 of this sugar is acylated.

The proton–proton correlation spectra of the tetra- and tri-saccharides obtained from native gum showed clearly the connectivity between the signals proposed to be from H-2 and H-3 of the glycerate and, in the case of the tri-saccharide, connectivity between the signals proposed to be from H-1 and H-2 of the glucose esterified at O-2 with glycerate.

Separation of methylated, acetylated tetrasaccharides. — To determine unequivocally the positions to which the acetate and the glycerate residues are attached, we sought to obtain oligosaccharides which contained each substituent independently. We were unsuccessful in doing this with the oligosaccharides in their native state. The tetrasaccharide fraction prepared from native gellan gum by using 10% methanol in HF was found to be predominantly one anomer of the methyl glycoside containing both acetic and glyceric esters. After methylation using methyl

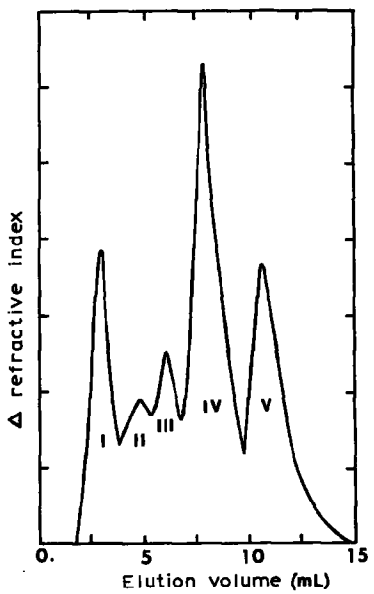


Fig. 5. Separation of the methylated, acylated repeating units of native gellan gum (see Fig. 1c, peak II) by reversed-phase, liquid chromatography (l.c.). A 2-mg sample was dissolved in 1 mL of the eluant (7:3 v/v methanol–water), and eluted from a column of LiChrosorb Rp-18, 5-μm, at a flow rate of 1 mL per min. The attenuator of the refractometer was set at 4 ×.

TABLE II

COMPOSITION OF THE REPEATING UNIT (OF NATIVE GELLAN GUM) COLLECTED FROM FRACTIONS IV AND V IN L.C.(SEE FIG. 5)

Glycosyl residue	*Positions of O-methyl groups*	*Relative molar ratios*[a]			
		Fraction IV		*Fraction V*	
		Before	*After $LiBH_4$*	*Before*	*After $LiBH_4$*
Rhamnose	2,3	0.94	1.01	0.79	0.74
Glucose	2,3,4,6	0.08	0.09	0.64	0.55
Glucose	3,4,6	0.57	0.47	0.08	0.08
Glucose	2,3,6	**1.00**	**1.00**	**1.00**	**1.00**
Glucose	2,3,4	0.07	0.07	0.30	0.27
Glucose	2,3	0.00	0.81	0.05	0.88
Glucose	3,4	0.20	0.18	0.06	0.06

[a]2,3,6-Tri-*O*-methylglucose set at 1.00.

trifluoromethanesulfonate as alkylating agent, the tetrasaccharide fraction was separated into five fractions by reversed-phase l.c. using 7:3 methanol–water as the eluant (see Fig. 5). Conversion of aliquots of each fraction into alditol acetates (see Table II) showed that fraction V was the simplest, being the same as the deacylated tetrasaccharide except for a small (0.25 residue per tetrasaccharide) degree of acylation on O-6 of the terminal glucose. Fraction IV contained predominantly 2- and 2,6-linked glucose in place of the unsubstituted, terminal glucose.

By combining fractions from seven repetitions of the l.c. experiment (using a total of 14 mg of alkylated tetrasaccharide), enough material was accumulated for ^{1}H-n.m.r. spectroscopy of fractions IV and V (see Fig. 6a and b). Fraction V showed resonances corresponding to the CH_3 of acetate at 2.07 p.p.m. and to H-6 of an acylated hexose at 4.30 p.p.m. (superposed on a signal for H-1 of a β-linked hexose). Resonances for H-1 of two other β-linked sugars were present, at 4.46 and 4.65 p.p.m., as was a barely split signal at 4.75 p.p.m. which we assigned as arising from H-1 of the rhamnosyl methyl glycoside. Fraction IV showed two additional resonances, a triplet at 4.84 p.p.m. with spin–spin splitting of ~8 Hz, and another triplet, at 4.09 p.p.m., with a splitting of ~3.5 Hz, which are easily explained as arising from H-2 of an acylated, β-linked hexose and H-2 of an alkylated glycerate, respectively. The chemical shift of the acetate methyl (2.09 p.p.m.) in fraction IV is slightly downfield from that at 2.07 p.p.m. in fraction V, probably under the influence of an adjacent glyceric ester. The H-1 resonances of two of the β-linked sugars are also shifted with respect to those for fraction V, suggesting an effect of the glyceric ester being exerted on both the acylated sugar and the adjacent, non-acylated glucuronic acid. The combined results of the methylation analysis and ^{1}H-n.m.r. spectroscopy show that glycerate is attached to O-2 of the terminal glu-

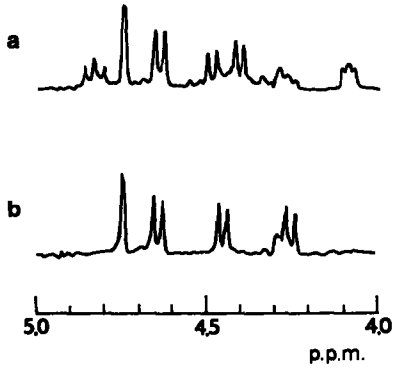


Fig. 6. ^{1}H-N.m.r. spectra of the methylated, acylated repeating units of native gellan gum collected from the effluents of (a) Fraction IV and (b) Fraction V obtained by l.c. (see Fig. 5).

cose in the tetrasaccharide, and that the acetate is linked to O-6 of the same residue.

The origin and identity of peaks I, II, and III (see Fig. 5) are unknown. We did not obtain clear ^{1}H-n.m.r. spectra of them, but could see the presence of glycerate and acetate in them, on O-2 and O-6, respectively, by both methylation analysis and ^{1}H-n.m.r. analysis. They could have arisen from undermethylation or from other minor, more-highly acylated forms of the tetrasaccharide.

Partial characterization of the larger-molecular-weight fraction of the HF-treated gellan gum. — In an attempt to characterize the material eluted in the void volume of the P-2 column, it was fractionated on a Bio-Gel P-10 column. Sugar was eluted throughout the fractionation range of the column, indicating that the material was heterogeneous with respect to size. The ^{1}H-n.m.r. spectrum of the material was very similar to that of the repeating unit, except that the resonances for H-1 of the rhamnose were spread into many small signals.

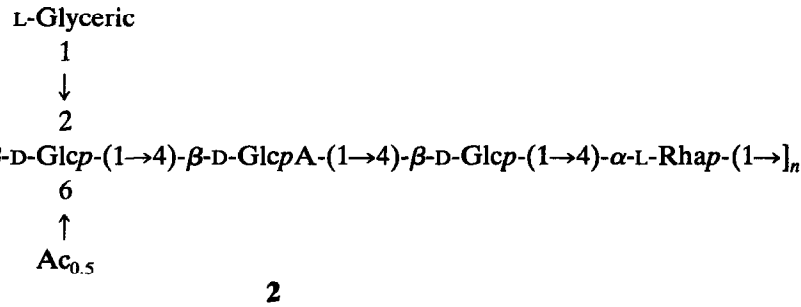
The most revealing experiment was to treat the material with lithium metal in ethylenediamine, a reaction shown by Mort and Bauer[4] to degrade uronic acids specifically. The products were compared with those obtained from the same treatment of native polysaccharide. Cleavage of the native polymer by degradation of the glucuronic acid was expected to produce a single product, a trisaccharide-alditol of Glc→Rha→Glcol. However, two products were obtained, and they were characterized by ^{1}H-n.m.r. spectroscopy. The products were the expected trisaccharide-alditol, plus a disaccharide, a glucosyl-rhamnose, formed by hydrolysis of the glucitol from the trisaccharide.

The products from the reaction with lithium–ethylenediamine of the larger material obtained from alkali-treated gum after treatment with HF at −40° were quite different. These could be separated into crude fractions according to size on Bio Gel P-2, and were characterized by methylation analysis. All of the fractions, except that of the smallest size-class, contained large proportions of 4-linked rhamnose and terminal glucose, with lesser proportions of 6-, 2-, and 4-linked glucose. The smallest size-class was free glucose. In the fractions of larger molecular size, there were also branched sugars.

Results of previous studies of the structure of gellan gum gave no indication of heterogeneity in the fundamental sugar sequence[1,2]. This, together with the foregoing results, is consistent with the hypothesis that, in the original HF treatment, all of the rhamnosyl linkages were broken, but, because of the high activity of the rhamnosyl fluoride formed, and because of the constraints on conformation of the polysaccharide, many of the fluorides reacted with a hydroxyl group of another adjacent sugar, in effect scrambling the rhamnosyl linkages between the repeating units.

CONCLUSION

It is likely that the glyceric ester is the major cause of the difference in physical properties as between the native and alkali-treated (deacylated) polymer, rather than the acetic ester. This is concluded because glycerate is bigger than acetate, is more abundant in the polymer than acetate, and is in a crowded location, namely, between C-3 linked to the preceding rhamnose and C-1 linked to the following glucuronic acid; see formula **2**.



2

EXPERIMENTAL

A detailed description of most of the experimental procedures has been given previously[7].

Gellan gum samples. — The samples Ex-4990 and Ex-4992 were gifts from Paul A. Sandford (Kelco Division of Merck & Co., Inc., California).

Partial degradation of polysaccharides with hydrogen fluoride. — Highly clarified, native gellan gum (Ex-4990) and deacylated gellan gum (Ex-4992) were partially degraded by treatment with liquid HF for 15 min at −40° (tetrasaccharide) or for 15 min at −23° (trisaccharide) as described previously[6]. In some experiments, dry methanol (1 mL) was added to the sample of native gellan gum (100 mg) before treatment with HF (10 mL).

Gel-filtration chromatography. — HF-treated polysaccharide samples were fractionated on a column (2.5 × 55 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, California) and eluted with 0.05M sodium acetate buffer (pH 5.2).

Aliquots (25 μL) were taken from each 1-mL fraction and tested by using the phenol–sulfuric acid assay[20]. All volumes were half those given in ref. 20. Sodium ions were removed from pooled fractions by passage through a small column of AG 50W X-8 (H^+) cation-exchange resin (Bio-Rad Laboratories).

Deacylation of oligosaccharides. — The repeating oligosaccharide unit (15 mg) of native gellan gum (see Fig. 1a, peak II) was added to 10mM KOH (15 mL) and allowed to react for 5 h at room temperature under nitrogen[21]. The base was then neutralized with M formic acid, and the solution freeze-dried. The deacylated oligosaccharide was transferred into 0.05M sodium acetate buffer by using a column of Bio-Gel P-2.

1D-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded with a Varian (Palo Alto, California) XL-300 n.m.r. spectrometer (300 MHz) at both 25 and 70°. Oligosaccharide samples (4 mg) were three times dissolved in D_2O (Sigma Chemical Co.) and freeze-dried, in order to exchange the hydrogen with deuterium, and then dissolved in D_2O (480 μL). Sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP) was used as the internal standard (0.00 p.p.m.). The data were recorded with 9 kbytes of memory and 400 transients. The methylated oligosaccharides were dissolved in $CDCl_3$ (500 μL) with 1% of Me_4Si as the internal standard, and spectra were recorded at room temperature only.

2D-N.m.r. spectroscopy. — The 2D-n.m.r. spectroscopy was performed as described by Gray[22]. ^{1}H-^{13}C, 2D heteronuclear-correlated spectra were recorded with a Varian XL-300 n.m.r. spectrometer (300 MHz) at 25°. Twelve mg of the repeating unit of native gellan gum (Fig. 1a, peak II) and 9 mg of the repeating unit of alkali-treated gellan gum (Fig. 1b, peak II) were exchanged three times with D_2O and dissolved in 480 μL of D_2O.

F.a.b. mass spectrometry. — Samples were dissolved in 5% aq. acetic acid, loaded into glycerol, and analyzed with a VG Analytical ZAB HF mass spectrometer under the conditions previously reported[23].

Determination of glycerate in gellan gum. — A modification of the procedure of Horning *et al.*[24] was used to produce the trimethylsilylated derivative of glycerate. The reaction step used to form the *O*-methyloximes of keto acids was included, because it gave more reproducible results (even though no keto acids were present). After derivatization, the sample was diluted with dichloromethane. One μL was injected on-column into the gas–liquid chromatograph. The temperature program was to inject at 40°, hold for 5 min at 60°, and then increase at 4° per min to 170°. The peak corresponding to glycerate was identified with known DL-glycerate (calcium salt), both chromatographically and with gas–liquid chromatography–mass spectrometry (LKB 2091). The amount of glycerate was determined from a calibration curve (0 to 25 μg) of DL-glycerate (calcium salt).

Identification of the optical isomer of glycerate with (R)-(−)-*2-butanol.* — A modification of the procedures of Gerwig *et al.*[10] and Leontein *et al.*[11] was used. A suitable amount (50 μg) of sample (the repeating unit of native gellan gum) was hydrolyzed in 2M trifluoroacetic acid for 1 h at 100°. Sodium L-malate (Sigma

Chemical Co.) was added as an internal standard. After butanolysis in M HCl in *(R)*-(−)-2-butanol (Aldrich Chemical Co.), the sample was acetylated in acetic anhydride for 1 h at 100°. The acetylated sample was diluted with 100 μL of dichloromethane, and 1 μL was injected on-column. The temperature program was to inject at 40°, hold for 5 min at 100°, and then increase at 1° per min to 140°. The peak was identified with the authentic compound of L-(−)-glycerate (calcium salt, Sigma Chemical Co.).

Methylation. — The methylation method devised by Prehm[25] was used. Methyl trifluoromethanesulfonate (Aldrich Chemical Co.) was used as the alkylating agent, and trimethyl phosphate (Aldrich Chemical Co.) as the solvent, as described previously[7]. After the reaction, the mixture was diluted with water, and purified by passage through a Sep-Pak C_{18} cartridge[26].

Hydrolysis of methylated oligosaccharides, reduction of monosaccharides, and acetylation of alditol acetates[27] were performed as described previously[7].

Separation of the methylated, acylated repeating units of gellan gum by l.c. — Native polymer was treated with HF at −40° in the presence of methanol (1 mL in 10 mL of HF), to obtain the methyl glycoside of the acylated repeating unit. Fourteen mg of this material was methylated by using methyl trifluoromethanesulfonate[25]. The resulting mixture was separated by reversed-phase l.c. using a column (25 cm × 4 mm) of LiChrosorb Rp-18, 5 μm (E. Merck, F.R. Germany), and an eluant of 7:3 methanol–water. Sugars were detected with a refractive-index monitor (Waters Inc., model R401). Two-mg aliquots in 1 mL were fractionated in each experiment.

Reduction of uronic esters to alcohols. — A procedure devised by Brown *et al.*[28] was simplified and used. It was the same as described previously[7], except that 50 μL of 2M lithium borohydride in tetrahydrofuran (Aldrich Chemical Co.) was used instead of a pinch of the solid reducing agent.

Gas–liquid chromatography. — The alditol acetate derivatives were separated in a DB-1, fused-silica, capillary column (30 m × 0.25 mm, i.d.; J & W Scientific Inc., California) fitted to a Tracor 560 gas chromatograph (Austin, Texas) equipped with a flame-ionization detector and a capillary injection-system (J & W Scientific Inc.) used for on-column injection. The oven temperature program was as follows: injection at 105°, hold for 4 min at 160°, and then raise the temperature at 2° per min to 220°.

Treatment of oligosaccharides with lithium in ethylenediamine. — The procedure used was that described by Mort and Bauer[4], modified according to suggestions made by Lau and Albersheim[29]. The oligosaccharide was suspended in ethylenediamine, and the intense blue color of the lithium solution was maintained for 1 h. The reaction was quenched with water, not methanol, and the ethylenediamine was removed by rotary evaporation.

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