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STRUCTURAL STUDIES OF THE METHYLATED, ACIDIC POLYSACCHARIDE ASSOCIATED WITH COCCOLITHS OF *Emiliania huxleyi* (LOHMANN) KAMPTNER

ANNE MARIE J. FICHTINGER-SCHEPMAN, JOHANNIS P. KAMERLING, CORNELIS VERSLUIS, AND JOHANNES F. G. VLIEGENTHART

Department of Bio-Organic Chemistry and Laboratory of Analytical Chemistry, State University of Utrecht, Utrecht (The Netherlands)

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ABSTRACT

For the structure analysis of the methylated, acidic polysaccharide associated with the coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner, the native, the carboxyl-reduced, and the desulphated, carboxyl-reduced polysaccharides have been submitted to methylation analysis. Graded hydrolysis with acid, uronic acid degradation, and periodate oxidation/partial hydrolysis with acid in conjunction with methylation analysis were also applied. The results of the various degradation procedures have led to a proposed structure for the average unit of the polysaccharide. The mannan backbone consists of at least 80% of (1→3)-linked D-mannosyl residues and has many side-chains. The mannan backbone carries ester-bound sulphate groups, terminal D-ribosyl or L-arabinosyl groups, and short side-chains of two or three D-galactosyluronic acid residues. There is also a complex side-chain composed of D-xylose and L-rhamnose, wherein the branched rhamnosyl residues are substituted by 3-*O*-methyl-D-xylose, 2,3-di-*O*-methyl-L-rhamnose, a di-(D-galactosyluronic acid)-D-xylosyl unit, and an oligosaccharide. The oligosaccharide is composed of 2,3-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-D-galacturonic acid, 6-*O*-methyl-L-mannose, D-galacturonic acid, L-mannose, and L-rhamnose.

INTRODUCTION

Interest in the structure of the polysaccharide associated with the calcified plates on the cell wall of the unicellular alga *Emiliania huxleyi* (Lohmann) Kamptner originates from the hypothesis that this biopolymer could play a matrix role in the calcification process, leading to the formation of coccoliths[1]. The composition of the polysaccharide and the structures of some acidic oligosaccharides, derived therefrom by graded hydrolysis with acid, have been described[2,3]. We now present a tentative structure for the average unit of the native polysaccharide. In view of the complex

structure of the polysaccharide, the amount of material (550 mg) available limited the exhaustive exploration of the various degradation techniques.

RESULTS AND DISCUSSION

The composition of the water-soluble polysaccharide[2] is given in Table I, including the newly found 3-*O*-methyl-D-galacturonic acid and 4-*O*-methylgalactose. The occurrence of 3-*O*-methylgalacturonic acid was indicated after carboxyl-reduction of the polysaccharide using sodium borodeuteride[2]. Methanolysis (trimethylsilylated methyl glycosides; SE-30 capillary column[2]: R_{Man} 0.57, 0.58, 0.60) and hydrolysis (alditol acetates; SP-1000 capillary column[2]: R_{Man} 0.98; 3% OV-225 column[2]: R_{Man} 1.00) show the presence of 3-*O*-methylgalactose-*6,6-d*$_2$. The D configuration of the latter compound was determined gas chromatographically by butanolysis[4], with 3-*O*-methyl-D-galactose as the reference compound. Initially, 4-*O*-methylgalactose was found during the methylation studies (see below). Its presence in a hydrolysate of the native polysaccharide[2] was confirmed by co-chromatography

TABLE I

COMPOSITION OF THE POLYSACCHARIDE

Constituent	*Approximate molar ratio*[a]	
	Native polysaccharide	*Carboxyl-reduced polysaccharide*
Galactose[b]	0.8	5.7
D-Glucose	0.6	1.7
Mannose[c]	5.6	5.6
L-Rhamnose	3.2	3.2
D-Ribose	1.1	0.9
L-Arabinose	0.7	0.6
D-Xylose	2.7	2.7
D-Galacturonic acid	4.7	—
3-*O*-Methyl-D-galacturonic acid	N.d.[d]	—
2,3-Di-*O*-methyl-L-rhamnose	1.8	1.6
3-*O*-Methyl-D-xylose	1.8	1.4
3-*O*-Methyl-D-galactose[e]	—	0.6
4-*O*-Methylgalactose	0.3	0.3
6-*O*-Methylmannose[f]	1.3	1.6
Ester sulphate	2.8	N.d.

[a]The molar ratio of rhamnose was taken to be 3.2 (see text). Most of the figures are average values of methanolysis and hydrolysis data (see ref. 1). [b]Present as L-galactose in the native polysaccharide; after carboxyl-reduction, D-galacturonic acid gave D-galactose. [c]Mannose was determined as ~90% D-mannose and ~10% L-mannose. [d]Not determined because of lack of a molar adjustment factor and because of partial overlap by xylose in the methanolysis. [e]Derived from 3-*O*-methyl-D-galacturonic acid. [f]6-*O*-Methylmannose in the native polysaccharide comprised the L form (~67%) and the D form (~33%).

TABLE II

METHYLATION ANALYSIS DATA

O-*Trideuteriomethyl derivative*[a]	*Linkage type*	T[b]	T[c]	T[d]	*Approximate molar ratio*[e]						
					A	B	C	D	E	F	G
	Galactose										
2,3,4,6-Gal	end-group	1.20	1.17	1.15	0.5	0.6	0.4	0.5	—	—	—
	Glucose										
2,3,6-Glc	1,4-linked	2.44	2.11	2.06	0.3	0.5	0.4	0.5	0.1	—	—
	Mannose										
2,3,6-Man	1,4-linked	2.12	1.89	1.83	—	—	—	—	—	0.2	—
2,4,6-Man	1,3-linked	1.98	1.97	1.72	—	—	0.4	0.4	0.5	2.2	2.7
2,4-Man	1,3,6-linked	5.21	4.56	3.86	0.3	0.3	1.7	0.8	1.5	0.7	0.7
3,6-Man	1,2,4-linked	3.98	3.49	3.14	0.2	0.3	0.3	0.2	0.3	0.1	—
4,6-Man	1,2,3-linked	3.18	3.04	2.61	1.2	1.0	1.2	2.0	1.0	0.9	1.0
2-Man	1,3,4,6-linked	6.93	6.19	4.85	1.2	1.3	—	0.8	—	—	—
6-Man	1,2,3,4-linked	4.29	4.32	3.46	1.7	1.4	1.2	0.5	1.3	0.5	0.2
	Rhamnose										
2,3,4-Rha	end-group	0.46	0.50	0.49	—	—	—	—	—	0.1	0.2
2,3-Rha	1,4-linked	0.98	0.95	0.92	—	—	—	—	—	0.2	—
2,4-Rha	1,3-linked	0.99	1.00	0.92	—	—	—	—	—	1.0	0.8
3-Rha	1,2,4-linked	1.80	1.67	1.62	0.1	0.2	0.2	0.2	0.2	0.1	—
4-Rha	1,2,3-linked	1.69	1.58	1.46	3.0	3.0	3.0	3.0	3.0	1.9	2.0
	Ribose										
2,3,5-Rib	end-group	0.41	0.47	0.44	0.7	0.6	0.6	—	—	—	—
	Arabinose										
2,3,5-Ara	end-group	0.48	0.52	0.49	0.4	0.4	0.3	—	—	—	—
	Xylose										
2,3,4-Xyl	end-group	0.65	0.65	0.60	1.2	1.2	1.1	1.2	0.1	0.4	0.5
2,4-Xyl	1,3-linked	1.30	1.17	1.06	1.2	1.4	1.4	1.3	1.1	0.8	0.8
	Galacturonic acid										
2,3,4,6-Gal-*6,6-d*$_2$	end-group	1.20	1.17	1.15	—	0.5	0.7	—	—	0.5	0.5
2,3,6-Gal-*6,6-d*$_2$	1,4-linked	2.32	2.04	1.94	—	2.4	2.2	—	0.1	—	—
2,4,6-Gal-*6,6-d*$_2$	1,3-linked	2.18	2.11	1.83	—	1.2	1.0	—	1.3	0.7	0.8
2,6-Gal-*6,6-d*$_2$	1,3,4-linked	3.56	3.23	2.78	—	0.6	0.4	—	0.2	0.1	—
1,2,4-Threitol-*1,1-d*$_2$[f]	1,4-linked	N.d.	N.d.	N.d.	—	—	—	—	—	N.d.[g]	—
1,4-Threitol-*1,1-d*$_2$[f]	1,4-linked	N.d.	N.d.	0.21	—	—	—	—	1.5	—	—
	3-O-Methylgalacturonic acid										
2,6-Gal3Me-*6,6-d*$_2$	1,4-linked	2.32	2.04	1.94	—	0.5	0.4	—	0.4	0.4	—
	2,3-Di-O-methylrhamnose										
4-Rha2Me3Me	end-group	0.46	0.50	0.49	—	—	0.3	1.3	0.4	2.0	0.7
Rha2Me3Me	1,4-linked	0.98	0.95	0.92	2.2	3.1	2.6	0.8	2.4	0.5	—

TABLE II *(continued)*

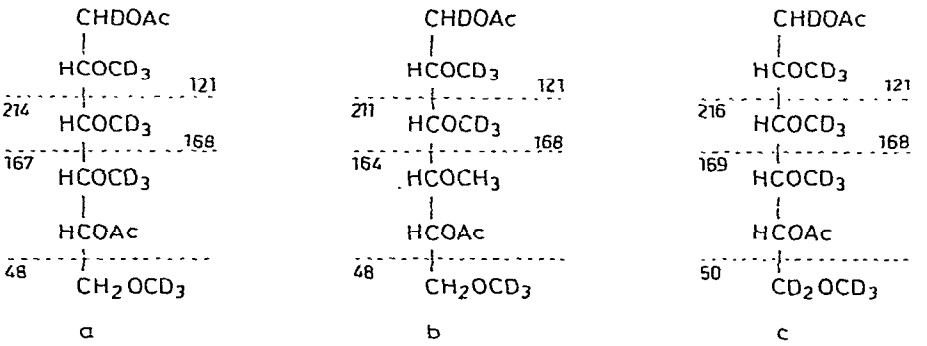
O-Trideuteriomethyl derivative[a]	*Linkage type*	T[b]	T[c]	T[d]	*Approximate molar ratio*[e]						
					A	B	C	D	E	F	G
	3-O-Methylxylose										
2,4-Xyl3Me	end-group	0.65	0.65	0.60	0.9	0.8	0.9	0.9	0.8	1.2	1.1
4-Xyl3Me	1,2-linked	1.44	1.24	1.15	0.9	0.9	0.9	1.0	0.9	—	—
	4-O-Methylgalactose										
2,3,6-Gal4Me	end-group	1.20	1.17	1.15	0.3	0.3	0.2	0.3	—	—	—
	6-O-Methylmannose										
2,3-Man6Me	1,4-linked	2.12	1.89	1.83	—	—	—	—	—	0.5	—
2,4-Man6Me	1,3-linked	1.98	1.97	1.72	0.2	0.2	0.1	0.2	0.1	0.2	0.1
3-Man6Me	1,2,4-linked	3.98	3.49	3.14	0.5	1.3	1.2	0.5	1.3	0.6	—

[a]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-trideuteriomethylgalactitol, *etc.* [b]Retention times of the alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on 3% ECNSS-M at 170°. [c]As in *b*, but on SP-1000 (capillary column) at 180°. [d]As in *b*, but on 3% OV-225 at 190°. [e]*A*, native polysaccharide; *B*, carboxyl-reduced polysaccharide; *C*, desulphated, carboxyl-reduced polysaccharide; *D*, native polysaccharide after graded hydrolysis with 0.1M CF_3CO_2H; *E*, desulphated, carboxyl-reduced polysaccharide after periodate oxidation; *F*, desulphated, carboxyl-reduced polysaccharide after periodate oxidation/partial hydrolysis with acid; *G*, Sephadex G-100 fraction of high molecular weight from *F*. The molar ratio of 4-Rha was fixed at 3.0 in *A* to *E*; in *F* and *G*, the total amount of 2,3,4-Rha, 2,4-Rha, and 4-Rha was fixed at 3.0. Ratios between the amounts of trideuteriomethylated alditol acetates and corresponding trideuteriomethylated/methylated alditol acetates were deduced from the mass spectra. [f]Derived from 1,4-linked galactose-*6,6-d*$_2$ after periodate oxidation. [g]Too volatile.

with authentic 4-*O*-methyl-D-galactitol penta-acetate (SP-1000 capillary column: R_{Man} 0.98; 3% OV-225 column: R_{Man} 1.00). The R_{Man} values of the trimethylsilylated methyl 4-*O*-methylgalactopyranosides on an SE-30 capillary column[2] were 0.60 and 0.69.

Linkage analysis. — The native, the carboxyl-reduced, and the desulphated, carboxyl-reduced polysaccharide were methylated by the Hakomori method[5]. Trideuteriomethyl iodide was used routinely to discriminate between the naturally occurring *O*-methyl groups and the methoxyl groups introduced by permethylation. The partially methylated sugars obtained after formolysis/hydrolysis were transformed into the corresponding alditol acetates and analysed[6] by g.l.c. and g.l.c.–m.s. To facilitate the interpretation of the mass spectra, sodium borodeuteride was used to label C-1 of the alditols. The molar ratios of the partially methylated alditol acetates were established by comparing the peak areas in the gas chromatograms, whereby the molar ratio of 1,2,3-linked rhamnose was fixed at 3.0. This leads to a molar ratio of 3.2 in Table II for the rhamnose content of the polysaccharide. To permit comparison of the methylation analysis data in Table II with those for the composition of the polysaccharide in Table I, the molar ratio of rhamnose in Table I was fixed at 3.2.

(*a*) *Native polysaccharide.* The results of the methylation analysis of the native polysaccharide are given in Table II, column A. The native polysaccharide contains, as end groups, galactose, 4-*O*-methylgalactose, xylose, and 3-*O*-methylxylose in the pyranoid form, whereas ribose and arabinose are present in the furanoid form. The occurrence of 4-*O*-methylgalactose was demonstrated by the presence of peaks at *m/z* 164 and 211 in the mass spectrum of 2,3,4,6-Gal* (Scheme A). This spectrum is further complicated by the presence of 4-Xyl3Me*, which coincides with 2,3,4,6-Gal on OV-225, and also gives a peak at *m/z* 164. The molar ratio of galactose and 4-*O*-methylgalactose was established by comparing the abundances of the fragment ions *m/z* 214 and 211 in the mass spectrum of this mixture. The molar proportion of xylose and 3-*O*-methylxylose was determined from the ratios of the intensities of the peaks at *m/z* 167 and 164 and of those at *m/z* 168 and 165.



Scheme A. Primary mass-spectral fragmentation of 2,3,4,6-Hex (a), 2,3,6-Hex4Me (b), and 2,3,4,6-Hex-*6,6-d*$_2$ (c).

Both xylose and 6-*O*-methylmannose were found to be 1,3-linked, 3-*O*-methylxylose is 1,2-linked, and 2,3-di-*O*-methylrhamnose and glucose are 1,4-linked. Mannose appeared to occur as 1,2,3-, 1,2,4-, 1,3,6-, 1,2,3,4-, and 1,3,4,6-linked residues**, and 6-*O*-methylmannose was found to be 1,2,4-linked. The molar proportion of 1,2,4-linked mannose and 6-*O*-methylmannose was derived from the ratio of the abundances of the fragment ions *m/z* 239 and 236 in the mass spectrum. Rhamnose was found to be mainly 1,2,3-linked, with a small proportion 1,2,4-linked.

(*b*) *Carboxyl-reduced polysaccharide.* To examine the linkage types of galacturonic acid and 3-*O*-methylgalacturonic acid, the polysaccharide was carboxyl-reduced with sodium borodeuteride[7], thereby yielding the corresponding hexose-*6,6-d*$_2$ derivatives. The results of the methylation analysis of the carboxyl-reduced polysaccharide are given in Table II, column B.

In addition to the sugar residues listed in Table II, column A, part of the

*2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-trideuteriomethylgalactitol; 4-Xyl3Me = 1,2,5-tri-*O*-acetyl-3-*O*-methyl-4-*O*-trideuteriomethylxylitol, *etc.*

**Although methylation analysis does not, in general, discriminate between 1,4- and 1,5-linked hexoses, only 1,4-linkages are considered in this study. Pertinent evidence for several hexoses was obtained from the degradation studies.

galacturonic acid (found as galactose-*6,6-d*$_2$) appeared to be present as a non-reducing end-group. The molar ratios of terminal galactose, 4-*O*-methylgalactose, and galactose-*6,6-d*$_2$ were determined from the intensities of the peaks at *m/z* 214, 211, and 216 in the mass spectrum (Scheme A).

Besides the monosubstituted residues found in the native polysaccharide, 1,3- and 1,4-linked galacturonic acid and 1,4-linked 3-*O*-methylgalacturonic acid residues were demonstrated. The molar ratio of the last two residues was based on the abundances of the fragment ions *m/z* 241 and 238 in the mass spectrum of 2,3,6-Gal-*6,6-d*$_2$.

1,3,4-Linked galacturonic acid was found in addition to branched mannoses, 6-*O*-methylmannose, and rhamnoses.

In comparison with the native polysaccharide (Table II, column A), the analysis of the carboxyl-reduced polysaccharide (column B) shows a significant increase in the amount of 1,2,4-linked 6-*O*-methylmannose. The rather acid-resistant glycosidic bonds of the uronic acid substituents linked to 6-*O*-methylmannose (see below) can be held responsible for the low yield in the methylation analysis of the native polysaccharide. The origin of the increase of 1,4-linked 2,3-di-*O*-methylrhamnose is unknown. The increase of glucose in the sugar analysis after carboxyl-reduction of the polysaccharide (Table I) must be regarded as an artefact, since this effect did not occur in the methylation analysis.

(*c*) *Desulphated, carboxyl-reduced polysaccharide.* To determine the positions of the ester-bound sulphate groups, the carboxyl-reduced polysaccharide was desulphated and then permethylated. For the desulphation, a modification of the procedure of Nagasawa *et al.*[8] was applied. Comparison of the methylation analysis data given in column C of Table II with those in columns A and B shows that desulphation leads to a complete shift of 1,3,4,6- to 1,3,6-linked mannose, indicating a sulphate group at C-4 of mannose. The amount corresponds to $\sim 50\%$ of the total sulphate groups. The presence of 1,3,6-linked mannose in the native and carboxyl-reduced polysaccharides (Table II, columns A and B) may be caused by some desulphation during the analysis procedures, accounting for $\sim 10\%$ of the total sulphate groups. The positions of the remaining 40% of sulphate groups can be deduced from minor shifts in the methylation pattern, leading to the appearance of terminal 2,3-di-*O*-methylrhamnose and of 1,3-linked mannose. The foregoing results indicate that the sulphate groups are mainly located at C-4 of mannose. It is reasonable to suggest that, in addition, C-2 of mannose and C-4 of 2,3-di-*O*-methylrhamnose are partly substituted with sulphate-ester groups. Consequently, 4-sulphated 2,3-di-*O*-methylrhamnose occurs as a terminal residue in the native polysaccharide.

The methylation analysis data of the native, the carboxyl-reduced, and the desulphated, carboxyl-reduced polysaccharides are reproducible. Inspection of Table II shows that, in all instances, the number of branching points exceeds the number of terminal groups (*i.e.*, non-reducing sugars and ester-bound sulphate groups). The most obvious explanation for this feature is the occurrence of a reproducible degree of undermethylation, since no indications were found for other non-carbohydrate end-groups. For the native and the carboxyl-reduced polysaccharides,

TABLE III

ACIDIC OLIGOSACCHARIDES OBTAINED AFTER GRADED HYDROLYSIS WITH ACID (2M CF_3CO_2H; 3 h AT 95°)

1	α-D-Gal*p*A-(1→6)-α-D-Man*p*-(1→3)-D-Man
2	D-Gal*p*A-(1→4)-D-Gal*p*A-(1→6)-Man
3	D-Gal*p*A-(1→4)-D-Gal*p*A-(1→2/6)-Man*p*-(1→3)-Man
4	D-Gal*p*A-(1→4)-D-Gal*p*A-(1→3)-D-Xyl
5	D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Rha
6	D-Gal*p*A-(1→2)-Man*p*-(1→4)-D-Gal*p*A-(1→2)-L-Rha
7	D-Gal*p*A-(1→2)-L-Rha*p*-(1→4)-D-Gal*p*A-(1→2)-L-Rha
8	D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A
9	D-Gal*p*A-(1→2)-Man*p*-(1→4)-D-Gal*p*A
10	D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Man6Me
11	D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-Man
12	D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Man6Me
13	D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A

only a single methylation step could be performed. In both cases, re-methylation causes substantial alkaline-induced degradation due to the presence of methyl uronates and/or methyl sulphate groups (unpublished results).

Sequence analysis. — To gain more insight into the sequence of the various constituents, the polysaccharide was submitted to graded hydrolysis with acid, uronic acid degradation, or periodate oxidation/partial hydrolysis with acid. Unless stated otherwise, the effects of the degradations were determined by methylation analysis directly or after fractionation of the degraded material.

(*a*) *Graded hydrolysis with acid.* The native polysaccharide was treated with 0.1M trifluoroacetic acid for 1 h at 85°. After dialysis, the fraction of high molecular weight was analysed by methylation analysis (Table II, column D), which showed that the terminal ribo- and arabino-furanosyl groups had been removed. Furthermore, partial desulphation occurred, leading qualitatively to the same shifts in the methylation pattern as observed after complete desulphation of the carboxyl-reduced polysaccharide (Table II, column C). The ribosyl and arabinosyl groups may be linked to O-4 of 2,3-di-*O*-methylrhamnose and O-4 of mannose, since comparison of column D with A and C shows increased amounts of terminal 2,3-di-*O*-methylrhamnose and 1,2,3-linked mannose, and decreased amounts of 1,4-linked 2,3-di-*O*-methylrhamnose and 1,2,3,4-linked mannose.

The native polysaccharide was also partially hydrolysed with 2M trifluoroacetic acid for 3 h at 95°. As described earlier[3], this procedure yielded a number of acidic oligosaccharides (see Table III).

The carboxyl-reduced polysaccharide was partially hydrolysed with 0.5M HCl for 2 h at 85°. After dialysis and reduction ($NaBH_4$) of the fraction of high molecular weight, the resulting material was examined by methylation analysis, to give the following data (in molar percentages). Xylose (9%), galactose (9%), 3-*O*-methylxylose (3%), and mannose and/or glucose* (5%) were found as non-reducing end-

*On all stationary phases used, 2,3,4,6-Man and 2,3,4,6-Glc have the same retention time.

groups; the carboxyl-reduction of the polysaccharide was accomplished with sodium borohydride, so that the contribution of the reduced galacturonic acid to the galactose content could not be distinguished. Mannose (29%), 6-*O*-methylmannose (2%), xylose (5%), and rhamnose (2%) occurred as 1,3-linked residues. 1,2,3-Linked mannose (18%), 1,3,6-linked mannose (15%), and 1,2,3-linked rhamnose (3%) were present as branching points.

The occurrence of 1,3-linked mannose as the main mono-substituted residue in combination with 1,2,3- and 1,3,6-linked mannose as the main branching-points indicate a mannan backbone for the polysaccharide. The side chains that remain after the HCl treatment are mainly terminated by xylose or galactose.

(*b*) *Uronic acid degradation.* The pertrideuteriomethylated, desulphated polysaccharide was submitted to uronic acid degradation by the method of Aspinall and Rosell[9]. To determine the sites of attachment of galacturonic acid and 3-*O*-methylgalacturonic acid, the re-methylation was performed with methyl iodide. Compared with the methylation analysis data given in Table II, column C, 36% of 2,4-Xyl was converted into 2,3,4-Xyl, 17% of 4-Rha into 2,4-Rha, and 59% of 2,4-Man into 2,4,6-Man. These data indicate linkages of uronic acid residues to O-3 of xylose, O-2 of rhamnose, and O-6 of mannose. The linkages found accord with the oligosaccharide structures **1–7** presented in Table III. From the structures **2–4**, it is evident that linkages between uronic acid residues also have to be considered. Treatment with base also degraded 27% of the terminal xylose and 44% of 1,2-linked 3-*O*-methylxylose. Consequently, some of these residues were attached to uronic acids. The complete degradation of 1,2,4-linked mannose, 6-*O*-methylmannose, and rhamnose accords with the oligosaccharide structures **5–13** (Table III), showing their linkages to O-4 of uronic acid. In addition, 65% of the 1,4-linked 2,3-di-*O*-methylrhamnose was degraded under the conditions of the uronic acid degradation. The presence of (1→2) linkages between the uronic acid and mannose, 6-*O*-methylmannose, and rhamnose (**5–13**) leads ultimately to the expulsion and degradation of the O-4 substituents of the last three residues (additional β-elimination). This feature is responsible for part of the degradation of 2,3-di-*O*-methylrhamnose (see below). The degradation of the other part can be explained on the basis of its attachment to O-4 of 3-*O*-methylgalacturonic acid (see below).

(*c*) *Periodate oxidation.* The desulphated, carboxyl-reduced (sodium borodeuteride) polysaccharide was submitted to periodate oxidation. After reduction of the aldehyde groups with sodium borohydride, an aliquot of the material was pertrideuteriomethylated and analysed. As is evident from Table II, column E, the periodate-sensitive sugar residues (shown in column C) were degraded. However, the decrease of 1,3,4-linked galactose-*6,6-d*$_2$ was unexpected. If the values for this substance given in Table II, columns B and C, are mainly the result of undermethylation, the corresponding galacturonic acid residue is 1,4-linked or terminal in the polysaccharide.

The remaining part of the degraded material was treated mildly with acid to cleave the acetal linkages, and the products were reduced with sodium borohydride.

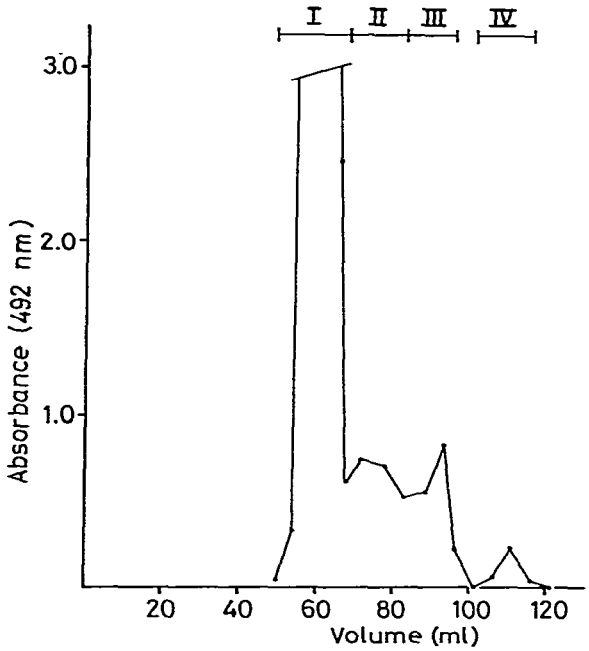


Fig. 1. Sephadex G-25 elution-pattern of degraded polysaccharide.

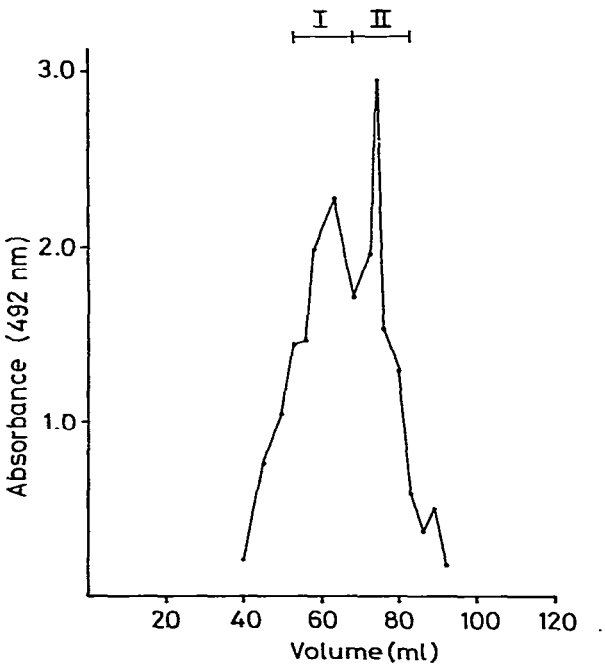


Fig. 2. Sephadex G-100 elution-pattern of the G-25I fraction.

An aliquot of the latter material was pertrideuteriomethylated and analysed. The results, presented in Table II, column F, show a large number of shifts in the methylation pattern in comparison to column E. The 1,2-linked 3-*O*-methylxylose was completely lost, but only ~50% of these residues were recovered as terminal 3-*O*-methylxylose residues. It is, therefore, suggested that the mild treatment with acid also cleaved some 3-*O*-methylxylose residues, the newly formed terminus. By permethylation, the latter residue was converted into its penta-*O*-trideuteriomethylpentitol derivative, which was lost in the analysis procedure because of its volatility.

Taking into account the results of the uronic acid degradation and the structures of the acidic oligosaccharides **2**–**7**, the shift of 2,4-Xyl → 2,3,4-Xyl and part of the shifts of 2,4-Man → 2,4,6-Man and 4-Rha → 2,4-Rha can be attributed to the periodate-sensitive, 1,4-linked galactose-*6,6-d*$_2$ attached to these sugar residues.

Consideration of the quantitative aspects (Table II) suggests that the shifts probably originate partly from a more complete methylation of the polysaccharide after periodate oxidation/partial hydrolysis with acid.

The material obtained after periodate oxidation and partial hydrolysis with acid was fractionated on Sephadex G-25, yielding four peaks (Fig. 1). Most of the carbohydrate material was eluted in the void volume (fraction G-25I).

Fraction G-25I was then chromatographed on Sephadex G-100 (Fig. 2), which afforded fractions G-100I and G-100II of high molecular weight and having the same monosaccharide composition (Table IV). Methylation analysis of G-100II

TABLE IV

COMPOSITION OF SEPHADEX G-25 AND OF SEPHADEX G-100 FRACTIONS

Constituent	*Approximate mole %*				
	G-25			*G-100*	
	II	*III*	*IV*	*I*	*II*
D-Galactose-*6,6-d*$_2$[a]	—	—	—	10	11
Mannose[b]	9	8	—	40	40
L-Rhamnose	7	5	—	24	23
D-Xylose	—	—	—	12	12
2,3-Di-*O*-methyl-L-rhamnose	24	28	—	2	2
3-*O*-Methyl-D-xylose	—	—	—	9	9
3-*O*-Methyl-D-galactose-*6,6-d*$_2$[c]	11	—	—	—	—
6-*O*-Methylmannose[b]	29	33	—	3	3
Threitol-*1,1-d*$_2$[d]	20	26	+	—	—
Glycerol-*1,1-d*$_2$[e]	—	—	+	—	—
Glycerol	—	—	+	—	—

[a]Derived from D-galacturonic acid. [b]Mannose and 6-*O*-methylmannose in the G-25 fractions are exclusively present in the L configuration. [c]Derived from 3-*O*-methyl-D-galacturonic acid. [d]Derived from 1,4-linked galactose-*6,6-d*$_2$ after periodate oxidation/partial hydrolysis with acid. [e]As in *d*, but derived from terminal galactose-*6,6-d*$_2$.

(Table II, column G) revealed rhamnose, xylose, galactose-*6,6-d*$_2$, 2,3-di-*O*-methylrhamnose, and 3-*O*-methylxylose as terminal residues. Mannose, rhamnose, xylose, galactose-*6,6-d*$_2$, and 6-*O*-methylmannose occur as 1,3-linked residues. Mannose and rhamnose are also present as 1,2,3-linked branching-points. In addition, mannose is present as a 1,3,6-linked residue and, for a small part, as a 1,2,3,4-linked residue.

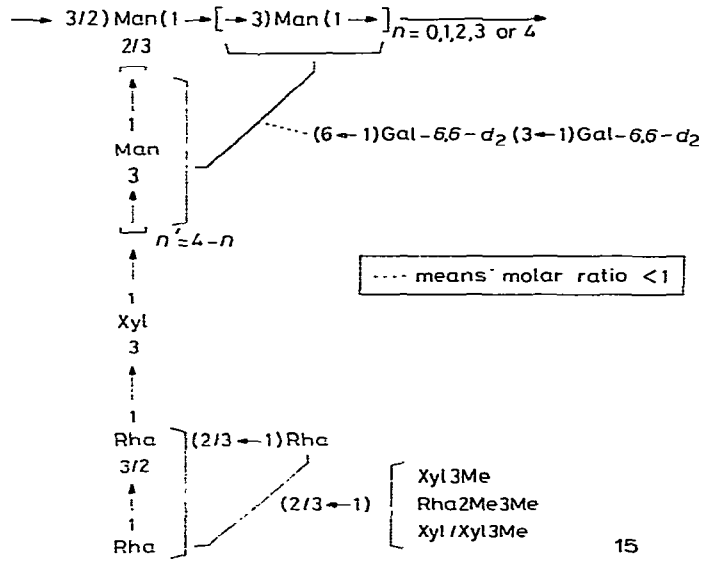
The methylation analysis data obtained after mild treatment of the carboxyl-reduced polysaccharide with hydrochloric acid, showing the presence of 1,3-linked mannose in combination with the two branching-points 1,2,3- and 1,3,6-linked mannose, as well as the main terminal units xylose and galactose, can be reconciled with the data for fraction G-100II. In view of the periodate treatment, both the 1,3-linked and terminal galactose-*6,6-d*$_2$ residues in G-100II must be derived from 1,3-linked galacturonic acid residues in the native polysaccharide. From the uronic acid degradation data (2,4-Man → 2,4,6-Man), it can be concluded that a galacturonic acid residue is attached to O-6 of the 1,3,6-linked mannose. The quantitative data (Table II, column G) indicate that ~50% of the total galactose-*6,6-d*$_2$ residues are attached to O-6 of mannose. The remaining part of the galactose-*6,6-d*$_2$ could be linked to O-3 of xylose, O-2 of rhamnose, or the galactose-*6,6-d*$_2$ residue linked to O-6 of mannose (see uronic acid degradation). The linkage to xylose or rhamnose is unlikely, because these sugars are substituted by periodate-sensitive (1,4-linked) galacturonic acid residues (see Table III, acidic oligosaccharides; quantitative data for uronic acid degradation; and Table II, columns E, F, and G). Consequently, the galactose-*6,6-d*$_2$ residues, found in fraction G-100II, belong to the sequence Gal-*6,6-d*$_2$-(1→3)-Gal-*6,6-d*$_2$-(1→6)-Man (**14**). Thus, 1,3-linked galacturonic acid in the native polysaccharide is exclusively coupled as a di(galactosyluronic acid) unit to the mannan backbone (see graded hydrolysis with acid) *via* O-6 of the 1,3,6-linked mannose. It must be noted that this conclusion implies that the terminal galacturonic acid residues of the oligosaccharides **2**–**11** (Table III) are not substituted at O-3.

The quantitative data for the graded hydrolysis with acid indicate that xylose is attached to mannose. However, a discrimination between a (1→2), (1→3), or (1→6) linkage is not possible. Since galacturonic acid is attached to O-6 of mannose, xylose has to be linked to O-2 or O-3. It cannot be excluded that, in the side chain linked to O-2 or O-3 of the branched mannose, xylose is preceded by one or more mannose residues.

Structure **15** represents the average unit of the inner region of the desulphated, carboxyl-reduced polysaccharide after periodate oxidation/partial hydrolysis with acid. This proposal fits the data obtained so far, including those for the partially methylated sugars given in Table II, column G.

Information about the periphery of the biopolymer was obtained from the analysis of the Sephadex G-25 fractions II and III. The sugar analysis data are given in Table IV. It must be noted that 6-*O*-methylmannose and mannose occur exclusively in the L configuration.

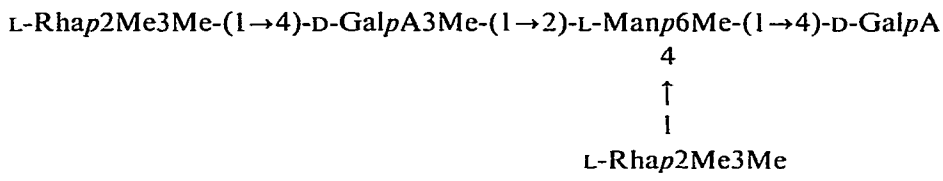
The combined oligosaccharide-alditol fractions G-25II and G-25III were pertrideuteriomethylated. Part of this material was hydrolysed and analysed by g.l.c.



and g.l.c.–m.s., yielding partially trideuteriomethylated alditol acetates: 2,3,6-Man (5%), 2,3-Rha (5%), 2,6-Gal3Me-*6,6-d*$_2$ (7%), 4-Rha2Me3Me (42%), Rha2Me3Me (11%), 2,3-Man6Me (21%), and 3-Man6Me (9%). The other part was studied directly by g.l.c.–m.s. The main peak in the oligosaccharide region of the gas chromatogram gave a mass spectrum with, *inter alia*, fragments at *m/z* 529/526 (M — CD_2OCD_3), 431/428 (abcJ$_1$), 371/368–336/333 (bcA$_1$-bcA$_2$), 221 (bcJ$_1$), 192–160 (aA$_1$-aA$_2$), and 158–123 (cA$_1$-cA$_2$). The two series of M — CD_2OCD_3, abcJ$_1$, bcA$_1$, and bcA$_2$ fragments differing by 3 m.u., but having the same intensity pattern, indicate the occurrence of the trisaccharide-alditols DeoxyHex(Me)$_2$–HexMe–Tetritol-*1,1-d*$_2$ and DeoxyHex(Me)$_2$–Hex–Tetritol-*1,1-d*$_2$ (ratio, 85:15). The presence of the bcJ$_1$ fragment excludes (1→3) linkages between the DeoxyHex(Me)$_2$ and the HexMe or Hex residues[10]. On the other hand, the presence of alditol fragments at *m/z* 50 and 97 is in agreement with (1→3) linkages between HexMe or Hex and Tetritol-*1,1-d*$_2$.

These results lead to the following structures for the oligosaccharide-alditols: L-Rha*p*2Me3Me-(1→4)-L-Man*p*6Me-(1→3)-Threitol-*1,1-d*$_2$ and L-Rha*p*2Me3Me-(1→4)-L-Man*p*-(1→3)-Threitol-*1,1-d*$_2$. Threitol in these structures is derived from 1,4-linked galacturonic acid. Therefore, the oligosaccharide-alditols correspond with the oligosaccharide structures L-Rha*p*2Me3Me-(1→4)-L-Man*p*6Me-(1→4)-D-Gal*p*A (**16**) and L-Rha*p*2Me3Me-(1→4)-L-Man*p*-(1→4)-D-Gal*p*A (**17**) in the native polysaccharide. The main g.l.c. peak is preceded by a small one, which has a mass spectrum with, *inter alia*, signals at *m/z* 496 (M — CD_2OCD_3), 398 (abcJ$_1$), 338 (bcA$_1$), 221 (bcJ$_1$), 192–160 (aA$_1$-aA$_2$), and 158–123 (cA$_1$-cA$_2$). Combination of the mass-spectral data and the sugar and methylation analysis leads, as described for **16** and **17**, to the oligosaccharide structure L-Rha*p*2Me3Me-(1→4)-L-Rha*p*-(1→4)-D-Gal*p*A (**18**) in the native polysaccharide. Based on the data for 6-*O*-methylmannose, mannose, and rhamnose in Table II (columns C, E, F, and G), it can be concluded that these

monosaccharides in **16**, **17**, and **18** are 1,2,4-linked in the native polysaccharide. Furthermore, the acidic oligosaccharides **5–13** (Table III) should also belong to the periphery of the biopolymer. Combination of the results leads to the conclusion that **5–11** are substituted at O-4 of the internal 6-*O*-methylmannose, mannose, and rhamnose residues by 2,3-di-*O*-methylrhamnose in the native material. This conclusion is supported by the data for uronic acid degradation. Methylation analysis of the Sephadex G-25 fractions of low molecular weight also shows the presence of 1,4-linked 3-*O*-methylgalactose-*6,6-d*$_2$ and 1,2,4-linked 6-*O*-methylmannose. Although no oligosaccharides accounting for these residues were detected by g.l.c.–m.s., the occurrence of oligosaccharides **12** and **13** makes it tempting to suggest that an oligosaccharide of type **19** is present in the native polysaccharide.



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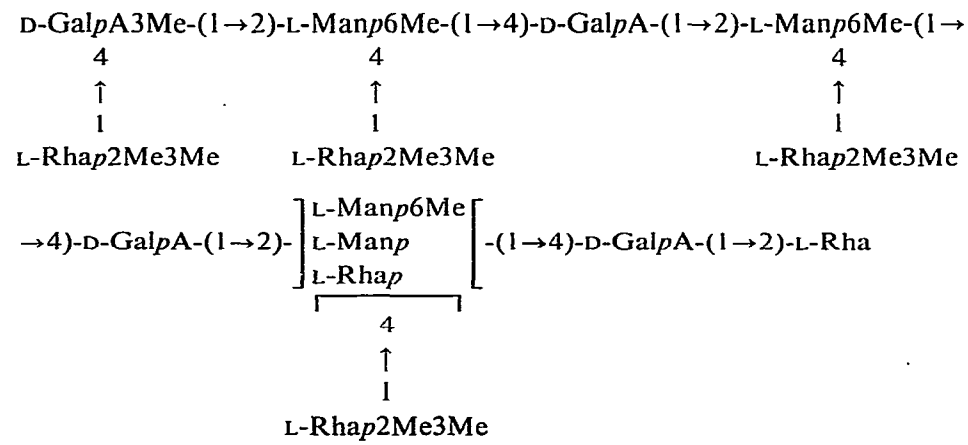
CONCLUSIONS

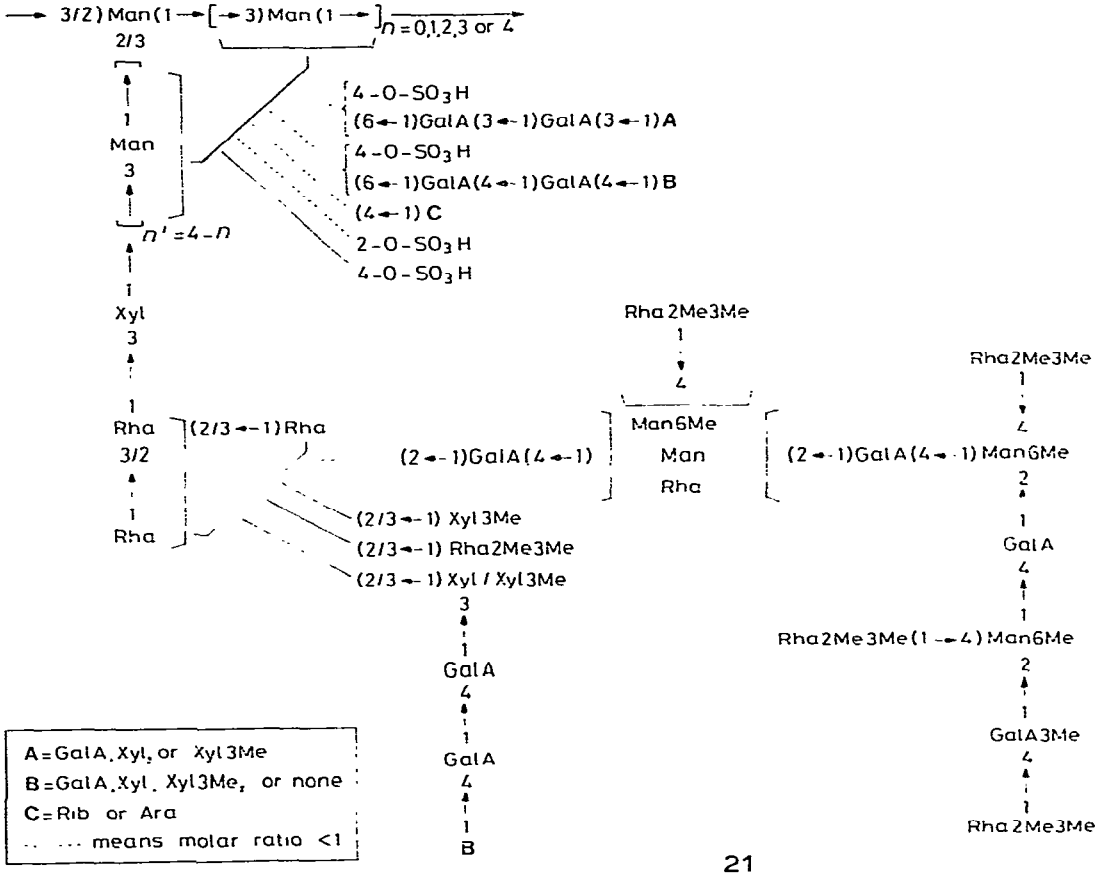
Structure **20**, which represents a part of the periphery of the polysaccharide, accords with the following data. (1) The occurrence of the partial structures **5–13** and **16–19**. (2) The terminal galacturonic acid residues in the oligosaccharides **5–11** represent 1,4-linked or terminal units in the native polysaccharide (see Discussion, G-100 fraction). (3) The 1,2,4-linked mannose, 6-*O*-methylmannose, and rhamnose were totally degraded during the uronic acid degradation, implying their linkage to uronic acid. (4) The absence of oligosaccharides analogous to **5**, **6**, **7**, **9**, and **11** with 3-*O*-methylgalacturonic acid residues instead of galacturonic acid, and of an oligosaccharide analogous to **12** with mannose as reducing residue in the oligosaccharide fractions, isolated after graded hydrolysis with acid[3]. (5) The molar proportions of 2,6-Gal3Me-*6,6-d*$_2$, 3-Man6Me, 3,6-Man, and 3-Rha as given in Table II, column B (0.5 : 1.3 : 0.3 : 0.2).



Structure **20** is present in the average unit of the polysaccharide in a molar ratio of ~0.5. This number is deduced from the quantitative data in the methylation analysis of the carboxyl-reduced polysaccharide (Table II, column B). In addition, the data for uronic acid degradation show that the molar ratio of uronic acid residues attached to O-2 of 1,2,3-linked rhamnose in the polysaccharide is 0.5. The latter residue has to be the reducing rhamnose in structure **20**, substituted at O-2 with periodate-sensitive (1,4-linked) galacturonic acid. After periodate oxidation/partial hydrolysis with acid, this 1,2,3-linked rhamnose is converted into a 1,3-linked residue, which was found to be present in the fraction of high molecular weight, representing the inner region of the polysaccharide (**15**).

The tentative, partial structure of the average unit of the polysaccharide associated with the coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner can be depicted as **21**. This structure is based on the following data and reasoning. (1) Structure **15**, representing the average unit of the inner region of the de sulphated, carboxyl-reduced polysaccharide after periodate oxidation/partial hydrolysis with acid (G-100 fraction). (2) Structure **20**, being present in a molar ratio of ~0.5. (3) Oligosaccharide **2**, wherein the periodate-sensitive (1,4-linked) galacturonic acid is linked to O-6 of mannose. The data for uronic acid degradation indicate the linkage of galacturonic acid to O-6 of mannose in a molar ratio of ~1.0. Since a →3)-GalA-(1→3)-GalA-(1→ unit is attached to O-6 of mannose in a molar ratio of ~0.7 (see Table II, columns F and G), the di(galactosyluronic acid) unit in oligosaccharide **2** is present in the polysaccharide in a molar ratio of 0.3. (4) The 1,4-linked galacturonic acid in oligosaccharide **4** is attached to O-3 of xylose. This 1,3-linked residue in the polysaccharide was converted into terminal xylose after periodate oxidation/partial hydrolysis with acid, and recovered as a constituent of the inner region of the biopolymer (**15**). Data for uronic acid degradation show that O-3 of xylose is substituted with uronic acid in a molar ratio of ~0.5. (5) In the native polysaccharide, the three di(galactosyluronic acid) chains can be elongated by terminal xylose, 1,2-linked 3-*O*-methylxylose (degraded during the uronic acid degradation), and terminal galacturonic acid (Table II, columns B and C). The latter residue is either one of the terminal galacturonic acids present in oligosaccharides **2** and **4**, or is an additional unit linked to one of the di(galactosyluronic acid) chains, to form a tri(galactosyluronic acid) chain in the native polysaccharide. It has not yet been determined which substituents at uronic acid residues are split off under the conditions[9] of uronic acid degradation. Therefore, the degradation of substituents at O-3 of 1,3-linked uronic acid residues cannot be excluded under the alkaline conditions used. (6) Ribose and arabinose, the furanosyl end-groups in the polysaccharide, are linked to O-4 of 2,3-di-*O*-methylrhamnose and O-4 of mannose, as shown by graded hydrolysis with acid. (7) The presence of ester-bound sulphate groups linked to C-2 and C-4 of mannose residues in molar ratios of ~0.4 and ~2.1, respectively. Part of the mannose residues, substituted at C-4 by ester sulphate, are also substituted at O-6 by galacturonic acid (Table II, columns A and C). The linkages of ester-bound sulphate to C-4 of 2,3-di-*O*-methylrhamnose, and of ribose or arabinose to O-4 of

this residue, are not depicted in **21**, since it is unknown which 2,3-di-*O*-methylrhamnose residue is involved.



21

Comparison of structure **21** with the methylation analysis data in Table II, column A, shows that terminal galactose, 1,4-linked glucose, ~65% of terminal xylose, 50% of 1,2-linked 3-*O*-methylxylose, terminal 4-*O*-methylgalactose, and 1,3-linked 6-*O*-methylmannose are not included. These residues, except 1,3-linked 6-*O*-methylmannose, were all degraded during the periodate treatment. Therefore, these sugars are located at, or near the end of, the various side-chains, or are directly linked to the mannan core. The small proportion of 1,3-linked 6-*O*-methylmannose is also omitted in **21**. This residue belongs to the inner region of the polysaccharide, because of its presence in the fraction of high molecular weight after graded hydrolysis with acid, and in the Sephadex G-100 fractions after periodate oxidation/partial hydrolysis with acid. The 1,2,4-linked 6-*O*-methylmannose residues present in the periphery of the polysaccharide appeared to be exclusively L. Since part of the 6-*O*-methylmannose has the D configuration (Table I), it is tempting to suggest that the

1,3-linked 6-*O*-methyl-D-mannose occurs in the mannan core. The non-methylated mannose residues in the core must also have the D configuration, since the small proportion of L-mannose present in the biopolymer corresponds with the amount of 1,2,4-linked L-mannose in the periphery. At least part of the D-mannose residues in the backbone are (1→3)-α-linked, as is evident from oligosaccharide **1** in Table III.

The occurrence of polysaccharides with linear (1→3)-linked D-mannose backbones has been reported[11–19]. In the case of the polysaccharide isolated from the red seaweed *Nemalion vermiculare*[11,12], the (1→3)-α-linked mannose backbone is substituted at O-2 by xylose and at C-4 or C-6 by sulphate ester groups. Polysaccharides produced by the basidiomycetous yeast *Tremella fucoformis*[14,15] and capsular polysaccharides from *Cryptococcus*[16,17] are (1→3)-α-D-mannans substituted at O-2 by β-D-glucuronic acid, β-D-xylose, or short chains of (1→2)-linked β-D-xylose. In addition they can contain *O*-acetyl groups, L-fucose residues[15], D-xylose β-linked to O-4 of a backbone residue[17], and D-mannose attached to O-2 of a mannosyl residue in the core[14]. The proposed structure **21** for the polysaccharide associated with the coccoliths of *Emiliania huxleyi* bears some resemblance to the foregoing structures by the presence of a mannan backbone. However, this algal polysaccharide is much more complex. To achieve a complete elucidation of its structure, much more material is required.

EXPERIMENTAL

General methods. — G.l.c. of partially methylated alditol acetates was carried out at 170° on a Varian 3700 apparatus with a glass column (2.00 m × 2.0 mm i.d.), packed with 3% of ECNSS-M on Chromosorb W-AW HMDS (80–100 mesh); and at 190° on a Pye 104 instrument using a glass column of the same size, packed with 3% of OV-225 on Chromosorb W HP (100–120 mesh). In both cases, the nitrogen flow-rate was 20 ml/min. G.l.c. of the partially methylated alditol acetates was performed at 180° on a Varian Aerograph 2740-30-01 with a glass-capillary column (25 m × 0.26 mm i.d.) wall-coated with SP-1000 (LKB-Produkter). The carrier-gas nitrogen flow-rate was 1 ml/min, and the make-up nitrogen flow-rate 30 ml/min. The instruments were equipped with flame-ionisation detectors.

G.l.c.–m.s. was performed with a combined Hewlett–Packard 5710A gas chromatograph/Jeol JMS-D300 mass spectrometer/Jeol JMA-2000 mass-data analysis system, using 3% of OV-225 as stationary phase, as described earlier[3].

Isolation of the polysaccharide. — This procedure has been reported[1,2].

Sugar analysis. — Carbohydrate material was analysed by g.l.c. (–m.s.) after hydrolysis and/or methanolysis[2], and sometimes after butanolysis[4].

Methylation analysis. — The native and modified biopolymers, as well as the oligosaccharide-alditols, were methylated with methylsulphinylmethanide–trideuteriomethyl iodide in methyl sulphoxide according to Hakomori[5]. The pertrideuteriomethylated oligosaccharide-alditols were analysed as described before[3]. Unless reported otherwise, the pertrideuteriomethylated polymers were recovered by dialysis

(running tap water, 24 h; distilled water, 3 h) and lyophilisation. After hydrolysis [90% formic acid (1 h), followed by 0.13M sulphuric acid (16 h) at 100°] and derivatisation, the corresponding alditol acetates were analysed by g.l.c. and g.l.c.–m.s.[6].

Carboxyl reduction. — The carboxyl functions of the uronic acids in the native polysaccharide were reduced with sodium borohydride or borodeuteride according to Taylor and Conrad[7]: to obtain complete reduction, the procedure was performed twice.

Desulphation. — The (carboxyl-reduced) polysaccharide was desulphated by a modification of the procedure of Nagasawa[8]: the pyridinium salt of the biopolymer was dissolved in methyl sulphoxide containing 7.5% of methanol and 2.5% of water (2 mg/ml) and kept for 7 h at 100°. To remove organic solvents, the material was dialysed and subsequently lyophilised.

Graded, acid hydrolysis. — (*a*) The native polysaccharide (4 mg) was treated with 4 ml of 0.1M trifluoroacetic acid for 1 h at 85°. After dialysis and lyophilisation, the partially degraded polymer was subjected to methylation analysis.

(*b*) Carboxyl-reduced polysaccharide (4 mg) was treated with 4 ml of 0.5M HCl for 2 h at 85°. After dialysis and freeze-drying, the fraction of high molecular weight was reduced with 15 mg of sodium borohydride in 3 ml of water for 20 h at room temperature. The excess of borohydride was decomposed with Dowex 50W-X8 (H^+) resin, and boric acid was removed by co-evaporation with methanol. The resulting material was subjected to methylation analysis.

Uronic acid degradation[9]. — The desulphated polysaccharide (3.2 mg) was treated with 25 mg of sodium borohydride in 4 ml of water for 2 h (reduction of reducing monosaccharide residues). The excess of borohydride was decomposed and removed as methyl borate. The residue was pertrideuteriomethylated, and the methylated material was recovered by dialysis and lyophilisation. Subsequently, the pertrideuteriomethylated, desulphated polysaccharide was kept overnight at room temperature in 0.67M methylsulphinylmethanide in methyl sulphoxide (3 ml) containing 0.1 ml of 2,2-dimethoxypropane and 1 mg of toluene-*p*-sulphonic acid. To the latter solution was added 1 ml of methyl iodide. After 1 h of sonication, the excess of methyl iodide was evaporated. The final material, obtained *via* chloroform extraction, was analysed by g.l.c. and g.l.c.–m.s. after hydrolysis (90% formic acid followed by 0.13M sulphuric acid) and conversion into alditol acetates[6].

Periodate oxidation. — A solution of 32.5 mg of desulphated, carboxyl-reduced polysaccharide in 32 ml of 0.1M sodium acetate buffer (pH 3.9) containing 0.05M sodium metaperiodate was kept for 5 days at 6° in the dark. The excess of periodate was reduced by addition of 1.5 ml of ethylene glycol. After 2 h at room temperature, the oxidised polymer was dialysed against distilled water (24 h). The solution was concentrated to 5 ml and treated with 60 mg of sodium borohydride for 18 h at room temperature. After decomposition of the excess of borohydride and removal of boric acid as described above, an aliquot was used for methylation analysis.

The acetal linkages in the remaining material were cleaved by treatment with 90% formic acid for 1 h at 40° (1 mg/ml). After evaporation of the formic acid,

60 mg of sodium borohydride was added to the residue in 5 ml of water (18 h, room temperature). The excess of borohydride was eliminated as described above. The total yield of the periodate-oxidised, acetal-cleaved material was 24 mg. Part of the material was submitted to methylation analysis, and the methylated product was recovered *via* chloroform extraction.

The remaining material (21.5 mg) was dissolved in 1 ml of water, and fractionated on a column (39.0 × 2.2 cm) of Sephadex G-25 (superfine). The column was eluted with water at a flow rate of 42 ml/h. Fractions were analysed for carbohydrate by the phenol–sulphuric acid test[20].

The fraction of high molecular weight (9 mg) was eluted from a column (41.0 × 2.2 cm) of Sephadex G-100 (superfine) with water at a flow rate of 8 ml/h. The G-100 fraction of high molecular weight was analysed after pertrideuteriomethylation, chloroform extraction, and derivatisation to alditol acetates. The oligosaccharide-alditols of the G-25 fractionation were analysed as described before[3].

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