Carbohydrate Research, 89 (1981) 179–191
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

^{13}C-N.M.R. STUDIES OF MONOMERIC COMPOSITION AND SEQUENCE IN ALGINATE

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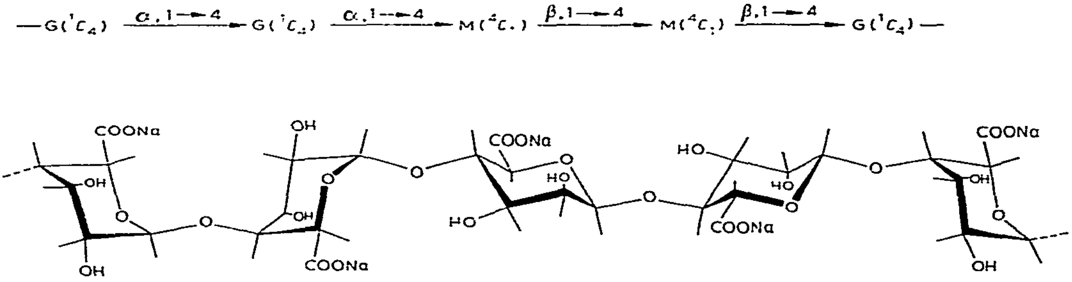
(Received April 14th, 1980; accepted for publication, June 2nd, 1980)

ABSTRACT

High-resolution, ^{13}C-n.m.r. spectra of slightly depolymerised alginates have been interpreted. The sequence of monomer units, L-guluronate (G) and D-mannuronate (M), markedly influenced the chemical shifts. At 50 MHz, some of the individual carbon resonances of both units were resolved into four lines, in evident dependence upon the identities of the units immediately preceding and following them in the chains. The relative intensities of the signals permitted rapid computation of (*1*) monomeric composition (M/G ratio), (*2*) monomeric sequence in terms of a complete set of four diad and eight triad frequencies, and (*3*) the composition (M/G ratio) of end units and of the units adjacent to M-residues at the non-reducing end. The diad frequencies indicated that alginate was a block co-polymer containing number-average, co-monomer block-lengths of ~2–8. The triad frequencies indicated average lengths of ~4–8 for blocks containing two or more units, these being somewhat longer for G- than for M-blocks. Regions of the chains having a strictly alternating sequence of M- and G-residues were short. The relative occurrence of G-centred triads deviated significantly from those predicted by first-order Markovian statistics.

INTRODUCTION

Alginate (**1**) is a linear glycuronan of (1→4)-linked α-L-guluronate (G) and β-D-mannuronate (M) residues arranged in a non-regular, blockwise pattern along the chain[1-3] (*i.e.*, it is not composed of simple oligosaccharide repeating-units).



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Its biofunctional properties are strongly correlated with composition (M/G ratio) and with the sequence of the uronic acids[4].

Considerable progress has been made towards characterising the monomer sequence of intact alginates in solution by ^{1}H- and ^{13}C-n.m.r. spectroscopy[5-7]. Due to the long-range conformational sensitivity of the ^{13}C-n.m.r. resonances, different ^{13}C-n.m.r. resonance frequencies might be expected for a particular carbon atom, depending upon the identities of the neighbouring units. Indeed, clearly distinguishable patterns of lines arose in the 25-MHz, ^{13}C-n.m.r. spectra of alginates known to contain different sequential arrangements of monomers[5].

Full details of this method are now described. The increased resolution in the ^{13}C-n.m.r. spectra at 50 MHz compared to that at 25 MHz demonstrated the feasibility of using high-frequency ^{13}C-n.m.r. spectroscopy in monitoring the sequence of heteropolysaccharides having a non-regular structure.

EXPERIMENTAL

Materials. — The preparation and the chemical characterisation of whole alginates[8] and of alginate fractions[1] have been reported previously. The samples of whole alginates were partly degraded to $\overline{\text{d.p.}}_n$ ~20–30 by very mild hydrolysis with acid in homogeneous solution (30–60 min, 100°, pH 3.0), to decrease the viscosities of their solutions. Fractions enriched in M and G residues were denoted "M-fraction" and "G-fraction", respectively. The fractions enriched in alternating sequence were denoted "MG-fractions". The alginate fraction[9] denoted "$L_{1.4}$-fraction" was soluble in acid medium down to pH 1.4.

^{13}C-N.m.r. spectroscopy. — The samples were dissolved in D_2O (100 mg/ml) at pD 7. Sodium triethylenetetra-aminehexa-acetate (25 mg/ml) was added to prevent line-broadening due to interaction with traces of divalent cations always present in the solutions; its ^{13}C-n.m.r. peaks did not overlap with those from alginates. ^{13}C-N.m.r. spectra were recorded with JEOL FX-100 (25 MHz) or Bruker WP-200 (50 MHz) spectrometers using 8k data-points, spectral widths of 5 kHz (25 MHz) or 10 kHz (50 MHz), a 75° pulse, a pulse repetition time of 0.8 s, and, typically, 40,000–80,000 scans. For experiments in which the carboxyl resonances were studied, the pulse repetition time was 4 s. The deuterium resonance was used as a field-frequency lock, and chemical shifts were expressed relative to internal sodium 3-(trimethylsilyl)-propionate-d_4. Unless otherwise stated, a probe temperature of 90° was used to decrease the viscosity and, thereby, the line widths. Spin–lattice relaxation times (T_1) of ring-carbon atoms were determined at 25 MHz by the inversion–recovery method[10], using a repetition time of $>5T_1$. The fast inversion–recovery method[11] was used to measure T_1 for the carboxyl carbons. Nuclear Overhauser enhancements (n.O.e.) at 25 MHz were determined from the ratio of the intensity of fully decoupled spectra to the intensity of spectra in which the proton noise-decoupler was gated-off to remove the n.O.e., using a pulse repetition time of $>8T_1$. T_1 values were determined

by manual plotting, since the built-in T_1-program was not capable of dealing with partially overlapping resonances.

Peak areas were measured by planimetry. For the lines of C-1, C-2(G), C-6, C-4(M), and C-5(M), the overlap of adjacent peaks was visually estimated. Convolution difference[12] was employed to enhance resolution.

Complete solubility of the alginate sample was tested for by an internal, absolute calibration by ^{1}H-n.m.r. spectroscopy against successive additions of 1-butanol. Provided that the intensity of the lines from butanol increased in proportion to its concentration, while the intensity of the ^{1}H-n.m.r. lines due to alginate stayed constant at a value corresponding to the concentration of alginate applied (unit molecular weight corresponding to the monohydrated sodium salt), the sample was considered to be completely dissolved.

RESULTS

Assignments of ^{13}C-n.m.r. spectra. — In order to study systematically the neighbouring-residue effects on the ^{13}C-n.m.r. resonances in spectra of whole alginates, assignments of 35 signals were made by reference to spectral data for alginate fractions, by double-resonance experiments, by varying pD, and by using samples of different, known, chemical compositions and different degrees of polymerisation ($\overline{\text{d.p.}}_n$).

Alginate fractions. — The 25-MHz, ^{13}C-n.m.r. spectra of three types of alginate fractions are presented in Fig. 1. The "G-fraction" and "M-fraction" each exhibited six dominant lines (Figs. 1A and 1B, respectively), as expected from the number of different carbon atoms in the monomers. The "MG-fraction", which contained nearly equal amounts of both types of monomer, displayed a more complex spectrum (Fig. 1C) in which all the lines in the two former spectra can be recognised, although they are mostly shifted. Since it has been shown previously that the conformation of the uronic acids is independent of their sequence[5], this result demonstrates that chemical shifts are sensitive to the chemical identity of the neighbouring unit.

The spectral regions of the carboxyl groups and anomeric carbon atoms in polysaccharides are well-documented. For the "G-fraction", an unambiguous assignment of the other lines was also straightforward by selective proton spin-decoupling. This was achieved, since all resonances in its ^{1}H-n.m.r. spectrum were resolved and interpreted[7]. Neutralisation of the carboxylic acid groups caused an upfield shift, mainly in the signal from C-5 of M (Fig. 1B). Its adjacent, low-field line most probably arises from C-4 of M, because this carbon is engaged in the glycosidic linkage and hence experiences[13] a downfield displacement of ~7–9 p.p.m. In the spectrum of an "MG-fraction" having an M/G-ratio of ~1.2 (Fig. 1C), the major lines arise from M units having two G units, and G units having two M units, as the two nearest-neighbours. By using off-resonance, selective, proton spin-decoupling[14], the low-field anomeric peak was identified as an M- and the high-field peak as a G-resonance. The two intermediate, small peaks in the anomeric region coincide with

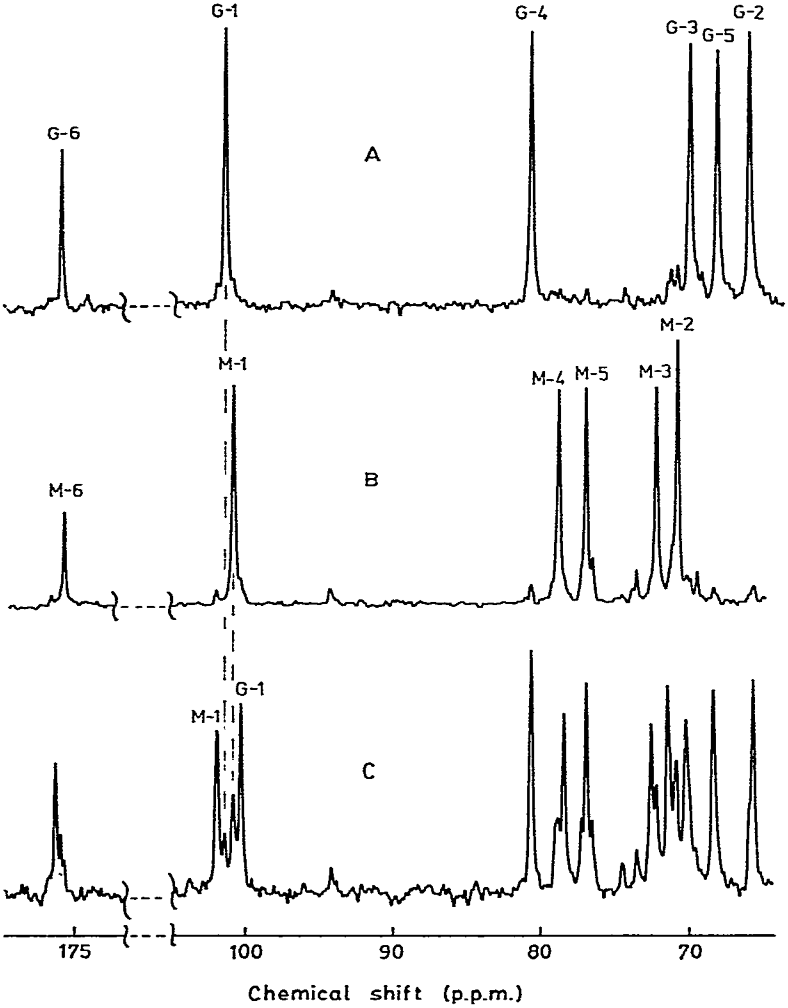

Fig. 1. ^{13}C-N.m.r. spectra (25 MHz) of solutions (100 mg/ml) in D_2O, at pD 7 and 90°, of sodium salts of alginate fractions *A*, enriched in L-guluronate (90%); *B*, enriched in D-mannuronate (85%); and *C*, enriched in alternating sequence (M/G ratio ~ 1.2).

C-1 in the spectra of "M"- and "G-fractions", respectively. By reference to spectra of alginates having different M/G-ratios, the assignment was also self-evident for the remaining major lines in Fig. 1C.

Whole alginates. — The 50-MHz, ^{13}C-n.m.r. spectrum of a sample of alginate prepared from old tissue of *Ascophyllum nodosum* (lower part of stump-shoots) shows considerable complexity, displaying more than 35 lines (Fig. 2). Whole alginates contain blocks of contiguous M- and G-residues having a certain distribution of lengths, interspersed between regions enriched in alternating sequences[3]. The termination of homopolymeric blocks gives rise to asymmetric triads, MMG and GMM, having M as the central unit, and GGM and MGG having G as the central

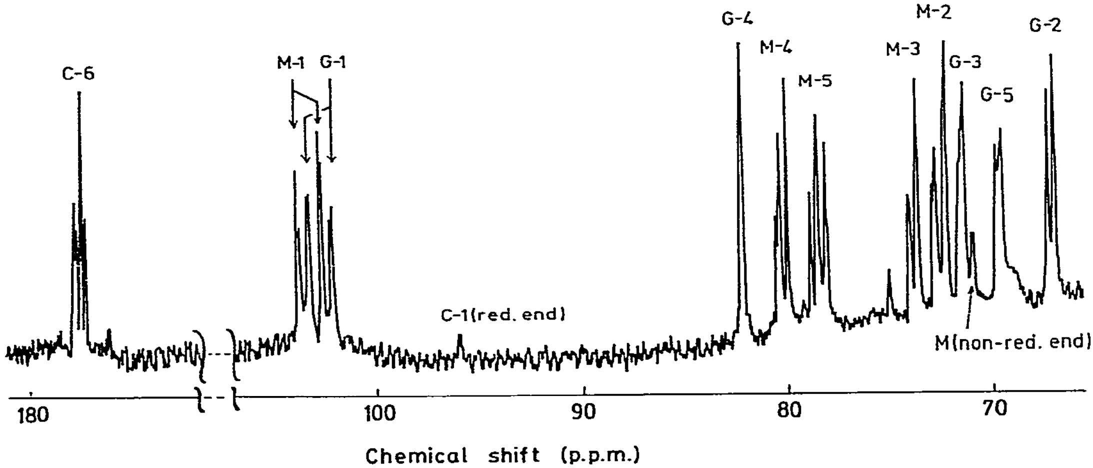

Fig. 2. ^{13}C-N.m.r. spectrum (50 MHz) of a solution (100 mg/ml) in D_2O, at pD 7 and 90°, of the sodium alginate from old tissue in *Ascophyllum nodosum*.

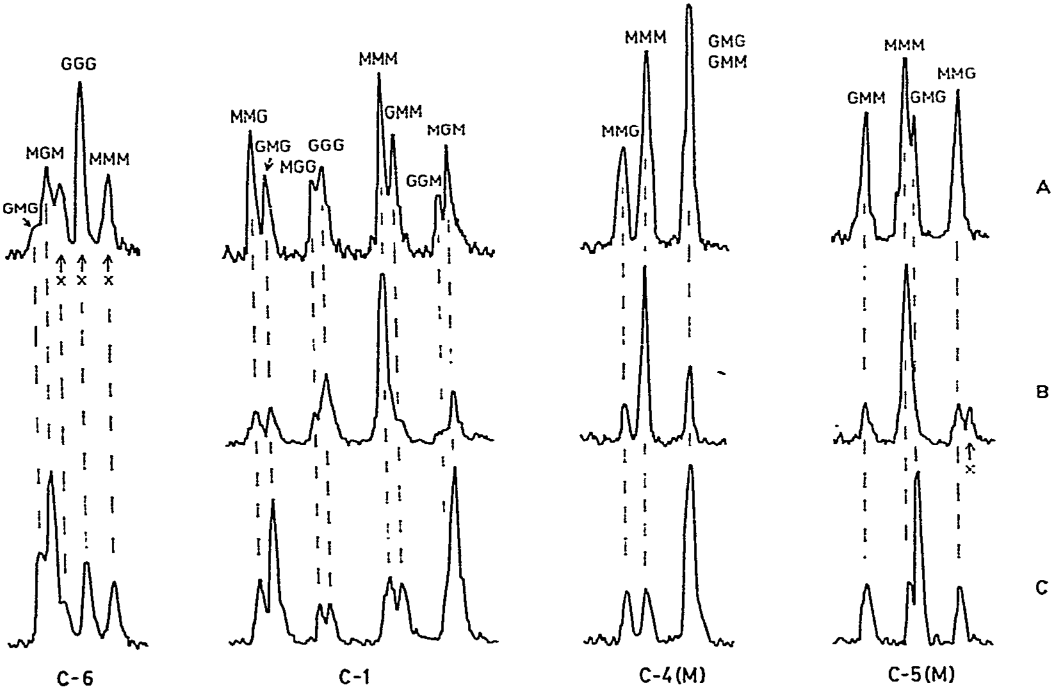

Fig. 3. ^{13}C-N.m.r. spectral regions (50 MHz) of C-1, C-4(M), C-5(M), and C-6 of the residues in A, sodium alginate from old tissue in *Ascophyllum nodosum* (expanded regions of spectrum in Fig. 2); B, sodium alginate from *Laminaria digitata*; and C, alginate fraction enriched in alternating sequence obtained from *Laminaria digitata*. X indicates contributions from unidentified peaks.

unit. The peaks which were lacking, or very weak, in spectra of alginate fractions were attributed to these asymmetric triads.

In the 50-MHz spectrum, there are eight clearly distinguishable resonances in the anomeric region, which is expanded in Fig. 3. Since the conformations of the residues are independent of the sequence, these resonances can only be explained by assuming that the C-1 resonances of M and G are sensitive to both nearest-neighbour units. Hence, their relative intensities provide a basis for determining the complete set of eight triad frequencies. It is likely that the resonances of C-1 are most sensitive to the residue linked to it through O-1 (*i.e.*, the following residue in the chain when it is formulated with the reducing end at the right). Both the M and G anomeric resonances are accordingly split into closely spaced doublets for which positions differ by ~1 p.p.m., depending upon whether the unit on the right is a G or an M (Fig. 3). The small splitting within each doublet (~0.1 p.p.m.) shows how the resonances are influenced by changing the preceding units in the chain. The alginate fractions served to identify the location of the four symmetric triads, MMM, GMG, GGG, and MGM, and the identification of the remaining triads was then self-evident.

Only one of the other ring-carbon atoms, namely C-5 of M, showed four easily interpretable lines (Fig. 3). The low- and high-field peaks due to GMM and MMG, respectively, are equally intense for long chains. Previously, they were differentiated by reference to spectra of samples hydrolysed with acids to different degrees of scission under conditions whereby the G–M bond is cleaved faster than the M–G bond[15]. The high-field peak increased in relative intensity as hydrolysis proceeded, and was therefore assigned[5] to MMG. A weak line, adjacent to the high-field C-5 resonance of M, observed in the 50-MHz spectrum of *Laminaria digitata* alginate (Fig. 3) may arise from end residues and make our earlier distinction[5] between GMM

TABLE I

ASSIGNMENTS OF PEAKS IN THE ^{13}C-N.M.R. SPECTRUM OF ALGINATE[a]

Sequence	*Carbon atom*					
	C-1	*C-2*[b]	*C-3*[b]	*C-4*	*C-5*	*C-6*
MMM	102.8	72.2	74.1	80.7	78.9	177.1
MMG	103.9	73.3[c]		80.9	78.4[d]	
GMM	102.7	72.7[c]		80.3	79.2[d]	
GMG	103.8	73.3	74.3	80.3	78.8	177.7
GGG	103.3	67.8	71.8	82.6	69.9	177.3
GGM	102.3	67.4		82.6	70.2	
MGG	103.4	67.8		82.6	69.9	
MGM	102.2	67.4	72.1	82.6	70.2	177.6

[a] Shifts, in p.p.m., downfield from internal sodium 3-(trimethylsilyl) propionate-d_4 for the resonances of the intermediate residue in the listed triad sequences. [b,c,d] These assignments of peaks due to M residues may have to be interchanged.

and MMG questionable, but this has no consequence for the main results reported in that and the present paper.

The resonance of C-4 of M is deshielded by 0.4 p.p.m. on changing both neighbouring units from M to G. Because the position of the C-4 resonance is likely to be less affected by the linkage at C-1 of the same unit, the C-4 resonance of MMG was inferred to be the low-field line (Fig. 3). The coincidence of GMM with GMG was confirmed by the relative intensity of this high-field peak.

A multiplet of five lines arose from the carboxyl carbon atoms (Fig. 3). By comparing relative intensities with complete sets of triad frequencies determined from ring-carbon resonances, we concluded that the two low-field lines arose exclusively from GMG and MGM. The C-6 resonances due to asymmetric triads have not been identified. Most of them coincide with those from GGG and MMM.

The chemical shifts of carbon atoms of residues in different sequence within the alginate chain are summarised in Table I.

End signals. — Also indicated in Figs. 1 and 2 are relatively weak resonances due to terminal residues. The reducing end-groups displayed peaks for the two anomeric forms, G-1(α) 95.5 p.p.m., G-1(β) 95.9 p.p.m., and M-1 (α,β) 96.2 p.p.m. The differentiation of G-1(α) and G-1(β) was based on the relative size of their resonance signals, the α anomer being the smaller one as shown in the ^{1}H-n.m.r. spectra[7]. The weak doublet at the high-field side of G-3 (Fig. 2) arose from M residues at the non-reducing end. The position of resonance depends upon the nearest neighbour in such a way that an adjacent G-residue, preferentially found in the "MG-fraction", causes a downfield shift of ~0.1 p.p.m. relative to that of an MM non-reducing end-group. The latter resonance, at 71.3 p.p.m., dominates in the spectrum of alginates enriched in MM-sequences such as *Laminaria digitata*.

Quantitative analysis. — Essentially similar T_1-values, 0.25 ±0.05 s, were found for all ring-carbon atoms in the alginate fractions. A slightly low but similar n.O.e. factor, 2.3, was observed for these signals. Hence, under the instrumental conditions chosen, the relative abundance in alginates was represented by the integrated, relative intensities of the ^{13}C-n.m.r. peaks due to ring-carbon atoms. Most importantly, in the 50-MHz spectrum, all possible triad frequencies were directly obtainable from the area under their respective anomeric peaks as a fraction of the total area of C-1 resonances.

The fairly long relaxation-time of the ionised carboxyl carbons, T_1 ~5.5 s, required a relatively low rate of pulse repetition to avoid saturation of their resonances. However, their relative areas were not critically dependent on the spectral conditions, and no significant difference in their n.O.e. value of 2.2 was noted. Hence, in the absence of high-field n.m.r. spectroscopy, the carboxyl carbons offer an important spectral region for diagnostic purposes, because the area of the low-field region is directly proportional to the fraction of alternating sequences, MGM and GMG.

The multiplet resonances of C-1, C-4, and C-5 of M-units allowed independent analysis of M-centred triads to be performed. By averaging these figures, an improved accuracy was obtained for analytical purposes.

TABLE II

DISTRIBUTION OF DIAD AND TRIAD FREQUENCIES IN ALGINATES AND ALGINATE FRACTIONS, AND THE CORRESPONDING, NUMBER-AVERAGE BLOCK-LENGTHS

Sample	F_{MM}	F_{MG} F_{GM}	F_{GG}	F_{MMM}	F_{MMG} F_{GMM}	F_{GMG}	F_{GGG}	F_{GGM} F_{MGG}	F_{MGM}	$\bar{N}_M$	$\bar{N}_G$	$\overline{N_M(>1)}$	$\overline{N_G(>1)}$
Ascophyllum	0.34	0.23	0.20	0.20	0.14	0.09	0.12	0.08	0.15	2.5	1.9	3.4	3.5
nod. old tissue	(0.32)[a]	(0.25)	(0.18)	[0.20][a]	[0.14]	[0.09]	[0.09]	[0.11]	[0.12]	(2.3)	(1.7)	[3.4]	[2.8]
Laminaria	0.46	0.14	0.26	0.38	0.08	0.06	0.22	0.04	0.10	4.3	2.9	6.8	7.5
digitata	(0.36)	(0.24)	(0.16)	[0.35]	[0.11]	[0.03]	[0.17]	[0.09]	[0.05]	(2.5)	(1.7)	[5.2]	[3.9]
"$L_{1,4}$-fraction"	0.36	0.25	0.14	0.22	0.14	0.11	~0	0.14	0.11	2.4	1.6	3.6	2.0
	(0.37)	(0.24)	(0.15)	[0.21]	[0.15]	[0.10]	[0.05]	[0.09]	[0.16]	(2.5)	(1.6)	[3.4]	[3.6]
"MG-fraction"	0.23	0.33	0.11	0.12	0.11	0.22	0.05	0.06	0.27	1.7	1.3	3.1	2.8
	(0.31)	(0.25)	(0.19)	[0.09]	[0.14]	[0.19]	[0.03]	[0.08]	[0.25]	(2.2)	(1.8)	[2.6]	[2.4]
"M-fraction"[b]	0.72	0.11	0.06	0.61	0.11	~0	0.05	0.01	0.10	7.5	1.5	7.5	7.0
	(0.67)	(0.15)	(0.03)	[0.62]	[0.09]	[0.01]	[0.04]	[0.05]	[0.06]	(5.5)	(1.2)	[8.4]	[1.6]
"G-fraction"[b]	0.05	0.05	0.85	0.025	0.025	0.025				2	18	3.0	
	(0.01)	(0.09)	(0.81)	[0.025]	[0.025]	[0.025]				(1.1)	(10)	[3.0]	

[a] Values given in parenthesis are calculated according to ( ) Bernouillian and [ ] first-order Markovian statistics. [b] Data obtained from 25-MHz, ^{13}C-n.m.r. spectra. G-centred triads were estimated from the C-6 resonance in combination with Eq. *1*.

In addition, the internal consistency of the values could be checked by the relations between diad and triad frequencies, which for M units are given by

$$F_{MM} = F_{MMM} + F_{GMM} \text{ and } F_{MG} = F_{MMG} + F_{GMG}. \qquad (1)$$

Corresponding expressions for G units are given by interchanging M and G.

Table II summarises the complete set of the derived diad and triad frequencies in two different alginates and four fractions of alginate.

DISCUSSION

Spectral conditions. — The key to successful n.m.r. spectroscopy of alginates proved to be an initial, limited depolymerisation of the samples, and the employment of high-field, ^{13}C-n.m.r. spectroscopy at high temperature to increase the resolution. The resolution of most of the lines in the ^{13}C-n.m.r. spectrum of alginate was enhanced by increasing the frequency from 25 to 50 MHz, which led to resolution of the diads of both residues into triads. This means that the chemical environment at C-1 is definitely influenced by the type of unit attached to C-4 of the same ring, implying sensitivity through five bonds. This observation provides an excellent example of the configurational sensitivity of the ^{13}C-n.m.r. resonances.

The intramolecular catalysis occurring under the conditions used for partial hydrolysis favours the hydrolysis of G–M linkages over other types by a factor of ~2–3, the G–G linkage being the most stable[15]. Consequently, after degradation, G-units will occur at the reducing ends, and M-units at the non-reducing ends, at higher frequencies than would be expected from the M/G ratio of the original sample. Therefore, if the $\overline{\text{d.p.}}_n$ after hydrolysis is fairly low (<15), a correction due to the skew distribution of end units is necessary in order to obtain a representative characterisation of the alginate molecule. As outlined in Appendix I, the spectral data for the terminal residues make it easy to correct the diad- and triad- frequencies if, as a first approximation, only the cleavage of the G–M bond is considered.

Another consequence of the different rates of hydrolysis of the linkages in the alginate chain is that the homopolymeric G-blocks would be less degraded than the others. Indeed, it was found that, in preparing samples rich in GG-sequences, the M/G-ratio, as determined by ^{1}H-n.m.r. spectroscopy, tended to increase with increasing concentrations. This result suggested that a selective aggregation of G-blocks was occurring, giving rise to an extensive broadening of their n.m.r. lines beyond the level of detection. A lower solubility of samples enriched in G-blocks was also suggested by a non-exponential, longitudinal relaxation of the ^{13}C-n.m.r. resonances of the "G-fraction" observed at high concentrations. The faster-relaxing component was probably caused by some kind of microaggregation, leading to impeded segmental motion of G-residues, and, hence, a shortening of their relaxation times. In the case of "M-" and "MG-fractions", the relaxation was exponential at similar concentrations and chain-lengths.

Nonetheless, under the conditions used for quantitative work in this study, the solubility was complete, as indicated by ^{1}H-n.m.r. spectroscopy. Whole alginates and all their fractions gave similar T_1 and n.O.e. values, apparently independent of sequence. The T_1 values were diminished when the temperature was lowered or when the concentration was increased. Hence, residual re-orientational motions probably were much the same for all residues within these samples and faster than ω_c, the carbon resonance frequency. The excellent agreement between the ^{13}C-n.m.r. data (Table II) and the ^{1}H-n.m.r. data reported recently[7], with respect to M/G-ratios and diad frequencies, also supports the validity of the assumptions made in choosing the conditions under which the ^{13}C-n.m.r. spectra were acquired.

Sequence distribution. — Although the accuracy is not high (±5%–±10%), the determined triad frequencies (Table II) provide the most detailed picture of the structure of the alginate molecule hitherto achieved.

In an attempt at theoretical interpretation, the experimental results were compared with those predicted by various statistical models. A set of diad frequencies was calculated with the equations:

$$F_{MM} = F_M^2,\ F_{MG} = F_{GM} = F_M \times F_G, \text{ and } F_{GG} = F_G^2, \qquad (2)$$

which are valid for a long Bernouillian chain. The theoretical values are given in round brackets in Table II. By using the experimental diad frequencies, a set of triad frequencies expected for a first-order Markov chain[16] was also calculated from the following formulae:

$$F_{MMM} = \frac{F_{MM}^2}{F_M},\ F_{GMM} = F_{MMG} = \frac{F_{MM} \times F_{MG}}{F_M}, \text{ and } F_{GMG} = \frac{F_{GM} \times F_{MG}}{F_M} \qquad (3)$$

Corresponding expressions for G-centred triads were obtained by interchanging M and G in Eq. *3*. The results are given in square brackets in Table II.

The measured diad probabilities deviate markedly from those corresponding to Bernouillian statistics. The most-pronounced deviations were found for *Laminaria digitata* alginate, where MM- and GG-sequences occur at a frequency far exceeding that predicted by a random distribution. Quantitatively, these diads were in rough agreement with the amounts of "M"- and "G-fractions", respectively, found by chemical fractionation[3]. Clearly, the diad values support the presence of homopolymeric blocks in the alginate molecule. However, the heterogeneity within the isolated fractions, as demonstrated by the minor peaks in their n.m.r. spectra (Fig. 1), poses questions as to their length and fine structure. It should be noted that the "G-fraction" was the most pure, while the "MG-fraction" contained >30% of diads corresponding to homopolymeric blocks.

Comparison of the experimental and calculated triad probabilities suggests that a first-order Markovian description of the alginate molecule is also inaccurate, since the calculated and measured, G-centred triad frequencies deviate significantly. The deviations are less significant for the M-centred triad frequencies. The triad frequencies may well be used for calculation of conditional probabilities in a secondorder Markovian system which has been used previously to simulate sequence and compositional heterogeneity in alginate[17]. However, this has not been done, because our knowledge of the mechanism of the C-5-epimerase[18], coupled with theoretical treatments of co-operative polymer-modification reactions[19–21], suggests that the distribution of G-units along an alginate chain may be non-Markovian.

From the diad frequencies, a number-average block length, $\overline{N}$, which is independent of any statistical model, can be calculated for the two co-monomer block-types from the expressions

$$\overline{N}_M = \frac{F_M}{F_{MG}} \quad \text{and} \quad \overline{N}_G = \frac{F_G}{F_{GM}}. \tag{4}$$

In addition, by employing the experimental triad frequencies, a number-average block length, $\overline{N(>1)}$, from which all the singlets (*i.e.*, -MGM- and -GMG-) have been excluded, can be calculated in order to provide some information about the distribution of block lengths. Because every M- or G-block containing at least two units is terminated by an MMG or GGM triad, the frequency F_{MMG} or F_{GGM} is a measure of the total number of such M- or G-blocks. Hence, for infinitely long chains, one finds

$$\overline{N_M(>1)} = \frac{F_M - F_{GMG}}{F_{MMG}} \quad \text{and} \quad \overline{N_G(>1)} = \frac{F_G - F_{MGM}}{F_{GGM}}. \tag{5}$$

Calculated values of average, homopolymeric block-lengths according to these equations are given in Table II. Corresponding values (in parenthesis) predicted by Bernouillian and first-order Markovian statistics are included for comparison. The results clearly show the trend of a deviation from a first-order. Markovian sequence-distribution in the case of G-residues. While the average lengths of M-blocks appeared to be in reasonable agreement, a markedly higher $\overline{N(>1)}$ value was indicated for the G-blocks. The relatively large $\overline{N_G(>1)}$ compared to $\overline{N}_G$ suggests a somewhat skew distribution of block lengths for G-residues, in which G-singlets seem to occur together with G-blocks of moderate lengths. This feature was most strikingly demonstrated in the "M-fraction" (Table II), where $\overline{N(>1)}$ was about the same for M- and G-residues, even though this sample contained M in 4.6-fold excess of G. Interestingly, the acid-soluble "$L_{1.4}$-fraction" contained no G-blocks longer then two units, as evidenced by the absence of a GGG-triad frequency (Table II). This result demonstrates the importance of sequence as a factor controlling the solubility of alginate at low pH, and suggests a particularly low solubility of G-blocks in acidic media. This conclusion is in agreement with results obtained by chemical methods[9].

The triad frequencies do not allow any direct evaluation of the average length of regions of the chains having a strictly alternating sequence*, without reference to

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*Single transitions between co-monomer blocks containing at least two units, *e.g.*, -MMGG-, are not counted as alternating sequences.

some statistical model whose validity is assumed. Nevertheless, some quantitative aspects of the fine structure of the "MG-fraction" may nonetheless be inferred. Firstly, about half of its ^{13}C-n.m.r. signal intensity arose from residues in co-monomer blocks containing two or more units. Secondly, the high fractions of asymmetric triads indicated a predominance of short co-monomer blocks. Therefore, it is reasonable to believe that the length of the strictly alternating -MGMG- regions are short in the "MG-fraction" and, consequently, also in whole alginates. A similar conclusion has been reached by employing enzymic methods[22].

Whole alginates showed diad and triad frequencies in highly variable proportions, depending upon the source. The results demonstrated a marked difference between the structures of alginates from *Laminaria digitata* and *Ascophyllum nodosum*. *Laminaria digitata* alginate, from which the "M"- and "G-fractions" were isolated, is a typical block-copolymer. This feature is less obvious for the *Ascophyllum nodosum* alginate, which is very nearly Bernouillian (Table II). Further information on the block structure of whole alginates from different species will be discussed in a forthcoming paper.

The heterogeneity in alginates prepared from whole plants makes it difficult to arrive at an appropriate statistical model from studies of samples isolated from whole plants. However, preliminary observations indicate that this may be achieved by the present method for samples from specialised tissues, whose compositions will hopefully be more homogeneous. Work along these lines is in progress.

APPENDIX I

Scission of the alginate chain by cleavage of a G–M bond produces one more reducing and non-reducing end-group with concomitant loss of one GM diad and two triads. The triads involved are MGM and GGM from the reducing end, and GMG and GMM from the non-reducing end. Since the relative proportions of the possible end-groups, ($f_{\text{-MG}}$) and ($f_{\text{-GG}}$) = ($1 - f_{\text{-MG}}$) at the reducing end, and ($f_{\text{MM-}}$) and ($f_{\text{MG-}}$) = ($1 - f_{\text{MM-}}$) at the non-reducing end, can be measured in the ^{1}H- and ^{13}C-n.m.r. spectra, respectively. the losses can be calculated. One finds

$$\begin{aligned}
&\Delta F_{\text{GM}} = 1/(\overline{\text{d.p.}}_n - 1), \\
&\Delta F_{\text{MGM}} = f_{\text{-MG}}/(\overline{\text{d.p.}}_n - 2), \quad \Delta F_{\text{GGM}} = f_{\text{-GG}}/(\overline{\text{d.p.}}_n - 2), \\
&\Delta F_{\text{GMG}} = f_{\text{MG-}}/(\overline{\text{d.p.}}_n - 2), \quad \Delta F_{\text{GMM}} = f_{\text{MM-}}/(\overline{\text{d.p.}}_n - 2).
\end{aligned} \tag{6}$$

Addition of the ΔF values to the respective F values and renormalisation provide corrected sets of diad- and triad-frequencies valid for intact alginate in cases where only the G–M bond is cleaved during the hydrolysis.

ACKNOWLEDGMENT

We thank Dr. Terence Painter for valuable discussions and for revising the English of the manuscript.

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