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Preliminary communication

^{13}C-N.m.r. studies of alginate

HANS GRASDALEN, BJØRN LARSEN, and OLAV SMIDSRØD
Institute of Marine Biochemistry, University of Trondheim, N-7034 Trondheim-NTH (Norway)
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We report on the first use of natural-abundance ^{13}C-n.m.r. spectroscopy to determine doublet and triplet frequencies in an intact binary heteropolysaccharide having a non-regular primary structure. The triplet frequencies provide information on the monomer sequence which, to our knowledge, has not been directly available hitherto by any physical or chemical method.

Alginate comprises an unbranched chain of (1-4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues arranged in a blockwise fashion[1–3] (Fig. 1). The ^{13}C-n.m.r. spectra of alginates prepared from *Laminaria digitata* (Fig. 1A) and from *Laminaria hyperborea* (Fig. 1B) contain multiplets that reflect the sequence of units. The signals for the anomeric carbons appear at low field and are sensitive to the nature of the neighbouring unit (M or G). The assignments of the peaks GG and MM in Fig. 2A have been made by reference to spectra of alginate fractions enriched in G and M, respectively. The C-1 doublets MG (M with a neighbouring G unit) and GM (G with a neighbouring M unit) were assigned by off-resonance, selective, proton spin-decoupling[4]. The possibility that the splitting in C-1 is due to the existence of G and M units in different conformations, dependent on their neighbours, has been ruled out by measuring the geminal coupling constants $^1J[^{13}CH(1)]$. The values of $^1J[^{13}CH(1)] = 162$ Hz for M and 172 Hz for G, shown in Fig. 2B, suggest[5] that the M residues are in the $^4C_1$ conformation and the G residues are in the $^1C_4$ conformation, independently of their nearest neighbouring units. This observation agrees with ^{1}H-n.m.r. results[6] that indicate that the $^4C_1$ and the $^1C_4$ conformations prevail in fractions enriched in M and G, respectively.

For guluronic acid, an unequivocal assignment was straightforward for C-2,3,4,5 by using the same method as for C-1. This was possible because all proton lines were separated in the ^{1}H-n.m.r. spectrum of an alginate fraction enriched in G. It was more difficult to interpret the resonances of mannuronic acid, and the signals for C-2 and C-3 of M have not been distinguished. By utilizing the fact that neutralisation of the carboxyl groups promoted an upfield shift, mainly in the signal for C-5, it was possible to identify C-5 of M. The M lines adjacent to C-4 in G units have been tentatively attributed to C 4 in

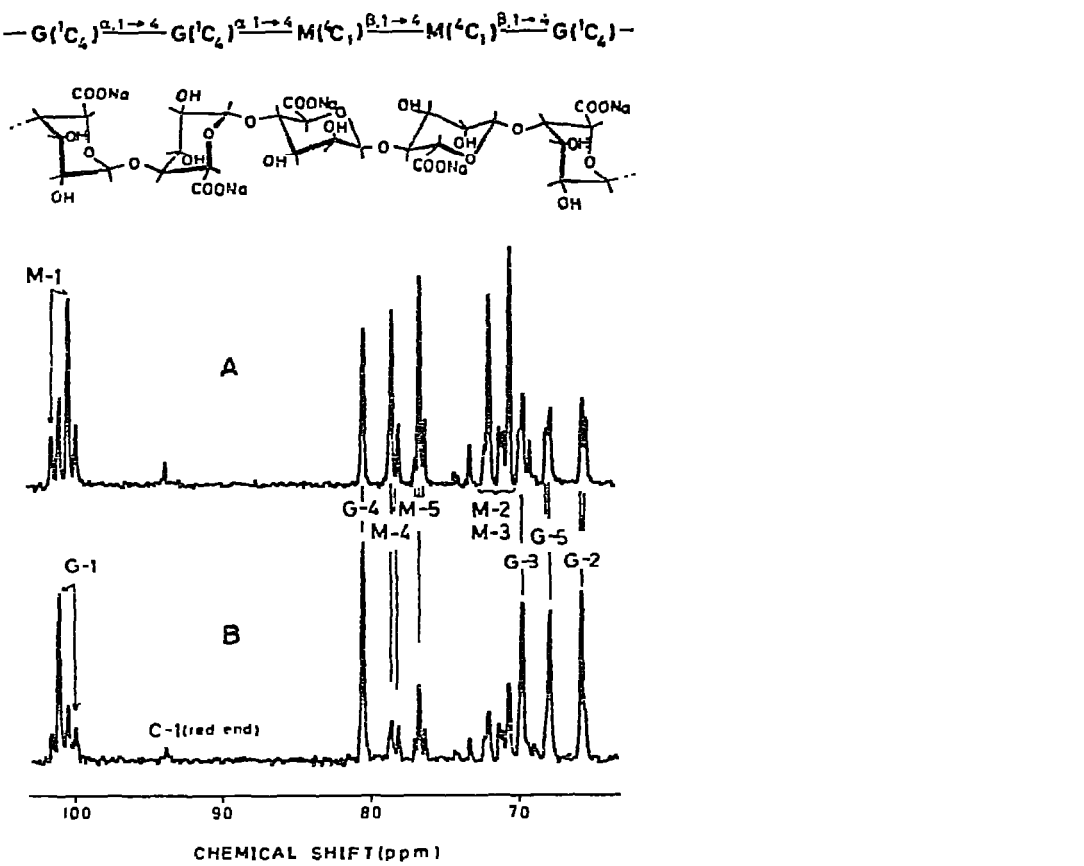

Fig. 1. Proton-decoupled, natural-abundance, Fourier-transform ^{13}C-n.m.r. spectra of the sodium alginates of *Laminaria digitata* (A) and *Laminaria hyperborea* (B) in D_2O (75 mg/ml, at 70°) obtained at 25.05 MHz with a JEOL FX-100 n.m.r. spectrometer, with 8000 points in the time domain, 5-kHz sweep-width, recycle time of 1 sec, and 40,000 scans. The signals from the carboxyl groups are not shown. Chemical shift is downfield from external Me_4Si. Signals designated by M refer to those of mannuronate, whereas those of guluronate residues are labelled G.

M units, because this carbon atom is engaged in the linkage and hence experiences a downfield displacement[7].

The C-5 resonances of M display a triplet line pattern (Fig. 2A). Since all the M residues are β-linked in the $^4C_1$ conformation, as indicated by the geminal coupling constants, this pattern can only be explained by assuming that the C-5 resonance of M is sensitive to both the neighbouring residues, thus providing the key for determining the M-triplet frequencies.

To do this, it is necessary to ensure that the relative areas of the observed resonances represent an estimate of the relative amounts of the carbons involved. This is not necessarily true, as the relaxation mechanisms and the nuclear Overhauser enhance-

ments of the diffent carbons need not be identical. However, the relaxation times were found to be about the same (~0.2 sec) for all the ring carbons. Moreover, the relative areas of carbon resonances were equal to within 10% when the full, proton noise-decoupled spectra were compared with the correspondingly gated, decoupled spectra in which the nuclear Overhauser enhancement was eliminated. In addition, the internal consistency concerning the equal intensity of all resonances of the same residues was found to be satisfactory, and the intensity ratio between M and G lines agreed to within a few percent with M/G ratios established by chemical methods[8]. Thus, n.m.r. spectroscopy appears to be very convenient for determining M/G ratios.

Having established that relative peak areas represent relative occurrence in alginate, it remained to locate the signals due to the four possible triplets having M as a centre unit. This was done by using alginate samples of different known chemical composition and different chain lengths. Samples containing more than 90% of mannuronic acid

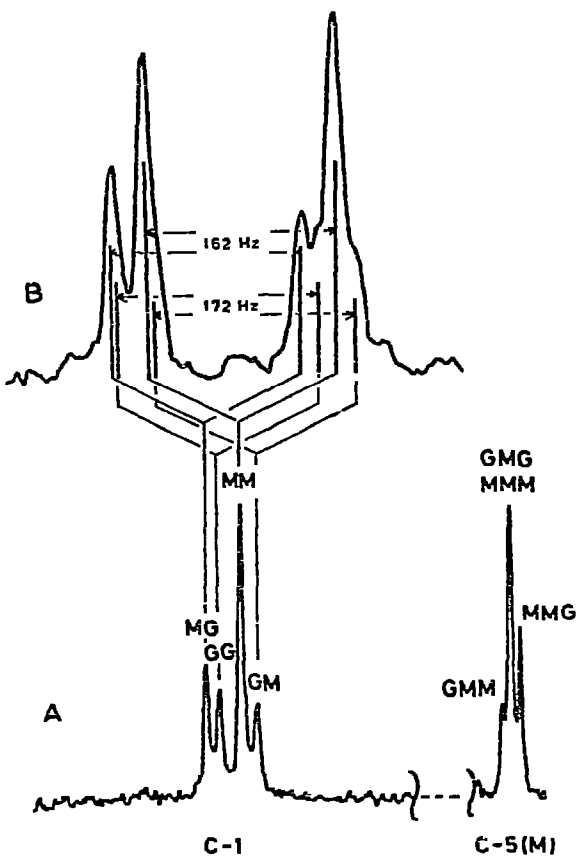

Fig. 2. (A) The spectral region of C-1 and C-5 of mannuronate for an alginate sample prepared from *Ascophyllum nodosum*. (B) Spectral region of C-1 in the undecoupled spectrum. The geminal coupling constants $^1J[^{13}CH(1)]$ are shown.

residues gave one dominating C-5 signal, corresponding to the intermediate C-5 line of M in Fig. 2A Samples enriched in MG-transitions exhibited a large intensity of the same peak, and concomitant large intensities of the MG and GM lines of C-1. These findings strongly suggested that the GMG triplet overlaps with MMM. Assignments of the two remaining peaks in Fig. 2B, MMG and GMM, were made by using samples that had been hydrolysed with acid to different degrees of scission under conditions whereby the G-M bond is cleaved faster than the corresponding M-G bond[9]. The high- and low-field peaks of C-5 of M had intensities that were identical for long polymers, but the high-field peak increased relative to the other as hydrolysis proceeded. Therefore, the high- and low-field peaks were assigned to the MMG and GMM triplets, respectively.

As the relations between the triplet and doublet frequencies are given by

$$F_{MMM} + F_{GMM} = F_{MM}, \text{ and } F_{GMG} + F_{MMG} = F_{MG},$$

the numerical values of the frequencies of the overlapping M triplets MMM and GMG can be calculated.

The doublet and triplet frequencies found in *Laminaria digitata* and *Laminaria hyperborea* alginates are shown in Table I. Both samples gave significant amounts of all the four triplets having M as the centre unit. In Table II, the doublet frequencies are compared

TABLE I

DISTRIBUTION OF DOUBLET AND TRIPLET FREQUENCIES IN ALGINATES AS DETERMINED BY ^{13}C-N.M.R. SPECTROSCOPY

Sample	F_{MM}	F_{MG}	F_{GM}	F_{GG}	F_{MMM}	F_{GMM}	F_{MMG}	F_{GMG}
L. hyp. Hustad	0.21	0.14	0.13	0.52	0.15	0.06	0.06	0.08
L. dig. Tarva	0.45	0.16	0.14	0.25	0.38	0.07	0.10	0.06

TABLE II

COMPARISON BETWEEN CHEMICAL COMPOSITION AND DOUBLET FREQUENCIES DETERMINED BY N.M.R. SPECTROSCOPY

Sample		*MG + GM*	*MM*	*GG*	*M/G ratio*
L. hyp. Hustad	Doublet frequency	0.27[a]	0.21	0.52	0.54
	Block distribution	0.25[b]	0.14	0.61	0.57
L. dig. Tarva	Doublet frequency	0.30[a]	0.45	0.25	1.56
	Block distribution	0.30[b]	0.41	0.29	1.60

[a]Sum of doublet frequencies taken from Table I. [b]Amount of acid-soluble fraction rich in alternating sequence[1].

with chemical fractionation data obtained by the method described previously[3]. The sum of F_{GM} and F_{MG} correlates remarkably well with the amount of "MG-blocks" in the two alginates. The relative values of F_{MM} and F_{GG} are also reflected in the fractionation data. Since a statistical characterization of the alginate molecule in terms of second-order Markow chain statistics[10] requires knowledge of all the eight different triplet frequencies, such a description has to wait until the triplets having G as a centre unit have been determined. It appears to be much more difficult to evaluate the G triplets because of smaller sequential splittings observed in the G carbon resonances. The resolution was not significantly increased by running the ^{13}C-n.m.r. spectra at 67.6 MHz.

Work directed to overcoming these difficulties is in progress.

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