Carbohydrate Research, 68 (1979) 23–31

A P.M.R. STUDY OF THE COMPOSITION AND SEQUENCE OF URONATE RESIDUES IN ALGINATES

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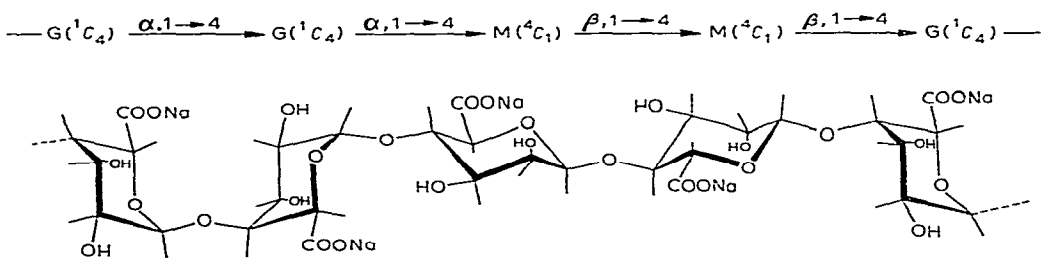
(Received February 1st, 1978; accepted for publication, February 22nd, 1978)

ABSTRACT

The sequence and composition of uronate residues in intact alginate samples have been obtained by high-resolution ^{1}H-n.m.r. spectroscopy. The viscosity problem was overcome by a slight, controlled depolymerization of the alginate samples before the ^{1}H-n.m.r. spectra were recorded at 90°. The mannuronate (M)/guluronate (G) molar ratio was obtained from the intensities of the signals for the anomeric protons. A sequence-dependent deshielding of H-5 of the guluronate residues made it possible to determine the fractions of the four possible doublets of nearest neighbours along the chain. The results were wholly consistent with ^{13}C-n.m.r. data. Significant deviation from comparable results obtained by chemical analysis appeared only for samples containing a large fraction of the mixed doublets.

INTRODUCTION

Sodium alginate (**1**) is a binary copolymer of D-mannuronate (M) and L-guluronate (G) residues arranged in a blockwise pattern along the linear chain[1–3]. The physical properties of alginates depend[4] not only upon the uronate composition, *i.e.*, the M/G ratio, but also upon the monomer sequence distribution in the copolymer. The ability of alginates to form gels in the presence of calcium ions is one of their main bio-functional properties, and is also of great industrial interest. The formation of gels depends mainly upon auto-cooperatively formed junctions between chain regions enriched in GG-sequences[5, 6]. The composition and sequence distribution of alginates depend upon the location in the plant, and vary widely from species to



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species[7]. Thus, it is important to have reliable methods suitable for characterizing both the composition and the distribution of sequences of the two uronate residues in algal samples.

Very recently, we[8,9] reported that ^{13}C-n.m.r. spectroscopy is very powerful in detailed sequence elucidation of alginates, because of the wide range of shifts for ^{13}C resonances and their long-range conformational sensitivity. However, a disadvantage of ^{13}C-n.m.r. spectroscopy for quantitative work is the low sensitivity. In addition, the nuclear Overhauser enhancement may not be the same for all carbon atoms, even if they all bear the same number of protons.

We now describe a convenient and rapid method for characterization of alginates by using ^{1}H-n.m.r. spectroscopy. This method is particularly useful for quantitative work in cases where only minor amounts of material are available.

The standard chemical method for determining the block composition of alginate[1,10] involves heterogeneous, partial hydrolysis with acid, and the determination of the M/G ratio by a complete, acid hydrolysis of the alginate. During complete hydrolysis, significant amounts of degradation products are formed. The relative amount of the two uronates destroyed may depend upon the sequence distribution, and substantial errors may therefore arise when one attempts to make corrections. The n.m.r. methods are superior to the chemical method in this context, because only a very moderate depolymerization is needed in order to diminish the viscosity of the samples to a level suitable for spectroscopy. Practically, the samples may be considered as intact alginates.

EXPERIMENTAL

Materials. — The isolation and the chemical characterization of the alginates and the preparation of alginate fractions have been reported previously[1,11]. The alginate samples were partly degraded ($\overline{d.p.}_n$ ~20–30) by very mild hydrolysis with acid (30 min, 100°, pH ~3.0), in order to diminish the viscosity of their solutions. The fractions enriched in mannuronate and guluronate will be denoted "M-fraction" and "G-fraction", respectively. The fractions enriched in the alternating sequence will be denoted "MG-fractions".

^{1}H-N.m.r. spectroscopy. — The samples (10 mg) were dissolved in D_2O (0.4 ml) at neutral pD. EDTA (~3 mg) was added to prevent traces of divalent cations from interacting with the glycuronans. ^{1}H-N.m.r. spectra were run at 99.6 MHz on a JEOL FX-100 n.m.r. spectrometer operating in the Fourier-transform mode. The carbohydrate protons relaxed much faster (T_1 ~0.35 sec) than the deuterium hydroxide protons, and the solvent peak (HDO) was therefore partly eliminated by employing a 180°–*t*–90° pulse sequence (*t* ~2–3 sec) with a recycle time of 4 sec. The spectra were recorded at 90°, in order to increase the spectral resolution and to shift the HDO line up-field, away from the low-field spectral region. ^{1}H-Chemical shifts were expressed in p p.m. down-field from internal sodium 3-(trimethylsilyl)propane-

sulfonate. The area under each of the partly overlapping peaks in the low-field region was found by excision and weighing.

RESULTS

Alginate fractions. — Fig. 1*a* shows the ^{1}H-n.m.r. spectrum of a "G-fraction". The spectrum is sufficiently resolved to permit the observation of the spin–spin splittings. In the spectrum of an "M-fraction" (Fig. 1*b*) and of an "MG-fraction"

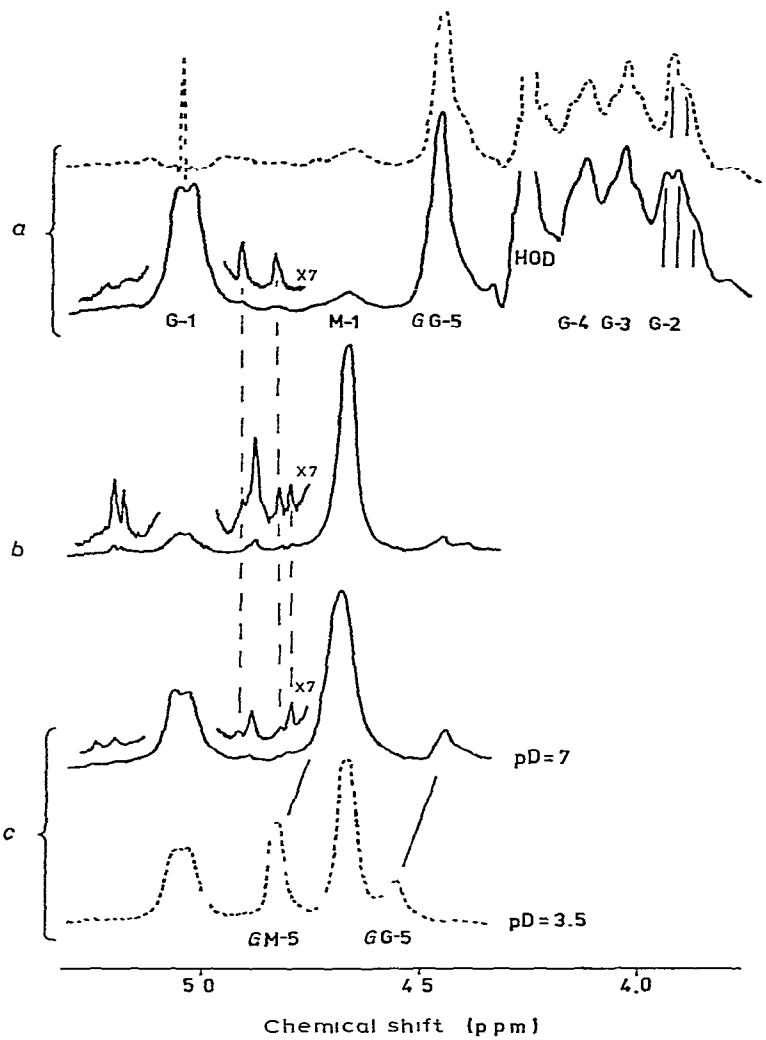

Fig. 1. The 99.6-MHz FT-^{1}H-n.m.r. spectra (—) of three different samples obtained by chemical fractionation of alginate: (*a*) enriched in L-guluronate (90%), (*b*) enriched in D-mannuronate (85%), and (*c*) acid-soluble fraction (mannuronate/guluronate molar ratio ~1.2); ---- spectra (*a*) with H-1 decoupled, and (*c*) recorded at pD 3.5. The enlarged vertical-scale regions show peaks due to reducing-end residues (see Fig. 2).

(Fig. 1*c*), only the resonances of the anomeric protons and H-5 of G (G-5) were resolved. The lines appearing in the low-field region due to homopolymeric fractions have been assigned previously by Penman and Sanderson[12]. Our previous ^{13}C-n.m.r. data[8,9] confirmed their assignments and a structure (**1**) for alginate in solution having the M residues in the $^4C_1$ conformation and the G residues in the $^1C_4$ conformation, independently of their nearest neighbouring units. Knowledge of the principal structure and the spin–spin couplings allowed interpretation of the whole ^{1}H-n.m.r. spectrum of polyguluronate, as shown in Fig. 1*a*. Irradiation of H-1 caused the H-2 triplet to collapse to a doublet, because of removal of the $J_{1,2}$ coupling (~3.4 Hz), as shown (----) in Fig. 1*a*. The doublet centered at 4.2 p.p.m. must be due to H-4. The splitting of ~3 Hz arises from its vicinal coupling to H-3, in accordance with their diequatorial arrangement. The triplet at 4.1 p.p.m. shows the resonance of H-3, which is about equally coupled to H-2 and H-4.

The rather small peak at 4.7 p.p.m., at the position of anomeric M protons (M-1), clearly indicates that the "G-fraction" obtained by chemical fractionation contained some M-residues. A corresponding "impurity" is also seen in the "M-fraction". Its small content of G residues gave minor G peaks, as shown in Fig. 1*b* where the anomeric M protons constitute the dominant peak. Fig. 1*c* shows the low-field region in the ^{1}H-n.m.r. spectrum of an acid-soluble alginate fraction ("MG-fraction") having an M/G ratio only slightly higher than unity. Chemical-fractiona-

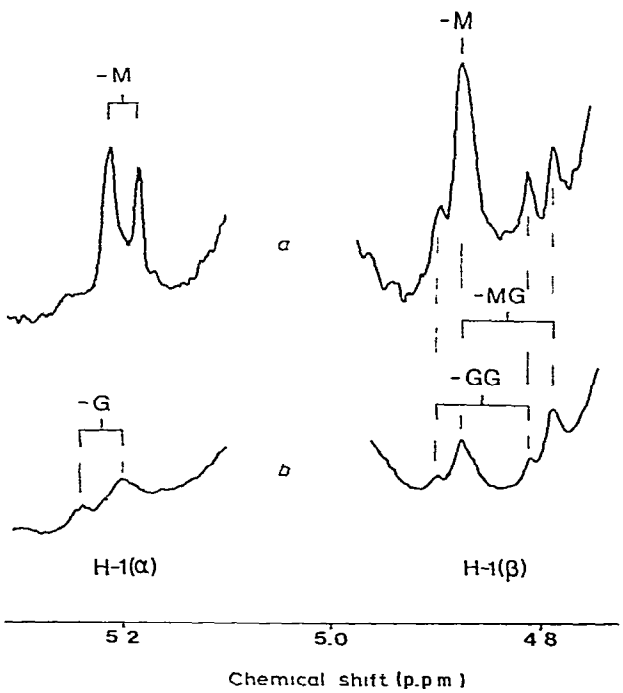

Fig. 2. The ^{1}H-n.m.r. spectral regions of the anomeric reducing-end protons. (*a*) in spectrum of Fig. 1*b*, and (*b*) in spectrum of Fig. 1*c* recorded at pD 7.0.

tion data[13] and ^{13}C-n.m.r. data[8,9] have shown that a high proportion of alternating sequences (MG) occurs in this type of sample. The spectrum (----) recorded after acidification with deuterium chloride displays four peaks, of which two are pD-dependent. They move up-field to the same extent on neutralization, as shown in the fully drawn spectrum. These peaks are both assigned to H-5 of G residues, because the algebraic sum of their intensities accounts for the total G-fraction, *i.e.*, it is equal to the intensity of H-1 of G (G-1). The position of the high-field peak corresponds to G-5 in polyguluronate (Fig. 1*a*) and is assigned to H-5 in a G having a neighbouring G residue (GG-5). The other pD-dependent peak, which coincided with M-1 at neutral pD, arises from G residues adjacent to an M.

Also shown in Fig. 1 (vertical scale × 7) are the small resonances due to the anomeric protons at the reducing end-groups. Their chemical shifts and spin–spin splittings resemble those for the correspondlng monomers[12], and their assignments are given in Fig. 2. The β anomer signal of a G reducing end-unit is a doublet, for which the position depends upon the nearest neighbour. An adjacent M residue, preferentially found in the "MG-fractions", causes an up-field shift of ~0.03 p p m. relative to that of a GG reducing end-unit which dominates in the ^{1}H-n.m.r. spectrum of the "G-fraction".

The end signals may give valuable information on the rate of hydrolysis. Previously, we found[14] that, under conditions used for degrading the n.m.r. samples, the G–M bond was cleaved faster than the M–G bond. This situation is also evident from the spectrum in Fig. 2*b*, where it is seen that more G than M residues are exposed as reducing end-groups, although this particular sample contained M in excess of G units.

For the present purpose of studying intact alginate copolymers, these findings suggest that corrections for end signals are possible and may be important. The intensity of the reducing-end peaks may be used as a guide to ensure that the average degree of polymerization ($\overline{\text{d.p.}}_n$) is reasonably high.

The ^{1}H-n.m.r. spectra obtained from the alginate fractions show that not only may the composition, *i e.*, the M/G ratio, be determined, but also the fractions of the four possible doublets, MM, MG, GM, and GG, along the copolymer chain.

The analysis is simple for the alginate fraction enriched in alternating sequences GM, where the lines are separated and their areas can be measured directly at low pD. The relative areas of the anomeric proton lines, G-1 and M-1, yield the mole fractions of G and M, respectively. The relative areas of the two H-5 resonances of G correspond to the distribution of G residues in alternating block-like regions (line GM-5) and homopolymer blocks (line GG-5). However, samples isolated from whole alginates, and the M- and G-fractions, are soluble only at neutral pD where the M-1 and GM-5 lines overlap. A quantitative discussion of this case is given below.

Alginate samples. — ^{1}H-N.m.r. spectra of whole alginate samples at neutral pD are shown in Fig. 3. The positions of the lines correspond to those in the spectra of the alginate fractions, Fig. 1, where their assignments are given. In order to obtain

the signals well separated, it was necessary to degrade the samples. However, the small signals for reducing end-group show that only a very moderate depolymerization had taken place. Therefore, the relative areas of the peaks A(G-1), B(M-1 and GM-5), and C(GG-5) contain information on the M/G ratio and the fractions of doublets of nearest neighbours along the intact copolymer chain. Bearing in mind that the proportion of alternating regions is proportional to the difference between the areas of peak A and C, and that the mole fraction of G is proportional to the area of A, a qualitative picture of the samples with regard to their composition and sequence distribution may be obtained by inspecting their low-field ^{1}H-n.m.r. spectra. The alginate isolated from *Laminaria digitata* exhibited a dominant B peak (Fig. 3*a*), in accordance[14] with its high mole fraction of M. About one half of the G residues

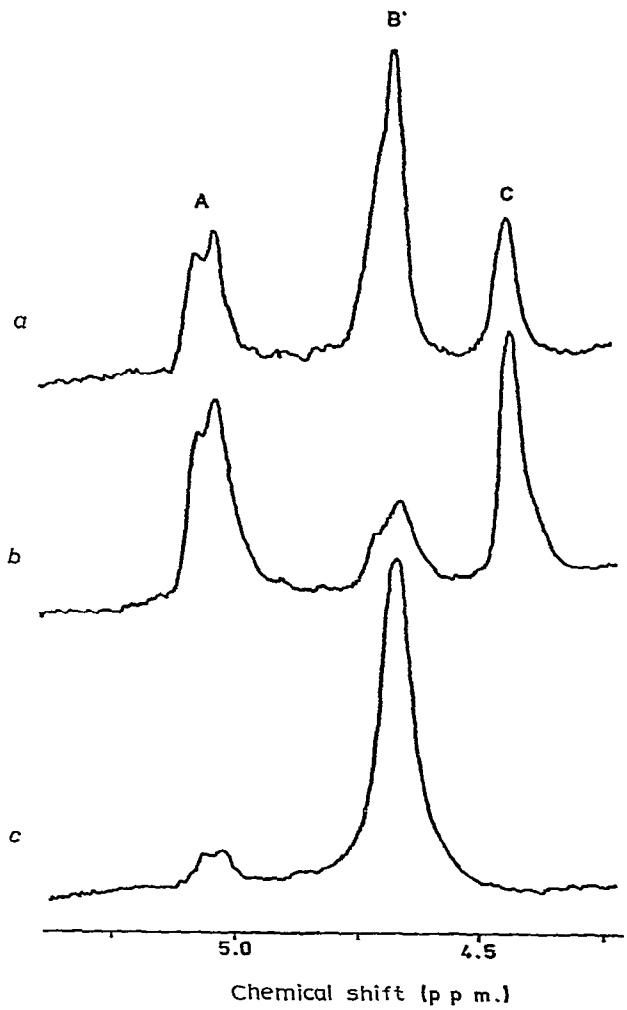

Fig. 3. The 99.6-MHz FT-^{1}H-n.m r. spectra of the algal sodium alginates of (*a*) *Laminaria digitata*, (*b*) *Laminaria hyperborea*, and (c) the bacterial alginate of *Azotobacter vinelandii*, recorded at 90°.

occurred in alternating sequences. The alginate from *Laminaria hyperborea* (Fig. 3*b*) evidently contained at least twice as much G as M. The bacterial alginate examined had a rather unusual structure with a very high proportion of M residues, and no G neighbours were detected (Fig. 3*c*).

Quantitatively, the mole fraction of G and the doublet frequency F_{GG} are related to the intensities (I) of the respective lines by the following relationships:

$$F_G = \frac{I_A}{I_B + I_C}; \qquad F_{GG} = \frac{I_C}{I_B + I_C}.$$

The mole fraction of M is then derived from the normalization condition $F_G + F_M = 1$.

The relations between the mole fractions and the doublet frequencies are given by

$$F_{GG} + F_{GM} = F_G; \quad \text{and} \quad F_{MM} + F_{MG} = F_M.$$

For long chains ($\overline{d.p.}_n > 20$), corrections for the reducing-end residues may be neglected, so that $F_{MG} = F_{GM}$. Hence, numerical values for the M/G ratio and the doublet frequencies can be calculated.

The results obtained for the alginate fractions and for some alginates isolated from whole plants and bacteria are shown in Tables I and II, respectively. The corresponding values derived from ^{13}C-n.m.r. data and those obtained by chemical methods are also shown for comparison. The previously reported data[10,14] on the gross chemical composition characterized by the mole fractions agreed with the n.m.r. results to within ± 0.05, except for the alternate-like block fraction, where a significant deviation of ± 0.10 appeared. In most cases, there was also a remarkably good agreement between block distribution obtained by chemical fractionation and spectroscopically determined doublet-frequencies in whole alginates (Table II). ^{13}C- and ^{1}H-N.m.r. gave the same results within the limits of error involved in these methods.

TABLE I

THE COMPOSITIONS, THE DOUBLET FREQUENCIES, AND THE BLOCK CHARACTER[a] (η) IN ISOLATED BLOCK-FRACTIONS OBTAINED BY CHEMICAL FRACTIONATION OF ALGINATE FROM *Laminaria digitata*

Block type	*Method*[b]	*Composition, fractions*		*Doublet frequencies*				η
		F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}	
GG	A	0.10	0 90	0 07	0.03	0.03	0.87	0 33
	B	0.12	0.88	0 07	0 05	0.05	0 83	0 47
MM	A	0.85	0.15	0.76	0.09	0.09	0.06	0.71
	B	0 82	0.18	0.72	0.10	0.10	0.08	0 68
MG	A	0.57	0.43	0 27	0.30	0.30	0.13	1.22
	B	0.54	0.46	0.20	0.34	0.34	0.12	1 36

[a]See definition in the text. [b]A, ^{1}H-n.m r., present work; B, ^{13}C-n.m r.

TABLE II

THE COMPOSITIONS, THE DOUBLET FREQUENCIES, AND THE BLOCK CHARACTERS (η) IN ALGINATE COPOLYMERS FROM DIFFERENT SPECIES

Source	*Method*[a]	*Composition, fractions*		*Doublet frequencies*				η
		F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}	
Laminaria	A	0 62	0.38	0 49	0.13	0.13	0 25	0.55
digitata	B	0 61	0.39	0.45	0.16	0.16	0.23	0.67
29.8.	C	0 61	0.39	0.41	0.15	0.15	0 29	0 63
Laminaria	A	0.30	0.70	0.26	0 04	0.04	0.66	0.20
hyperborea	B	0 27	0 73	0 16	0.11	0.11	0.62	0.56
Old stip, 4 5.	C	0.27	0.73	0	0 125	0.125	0.75	0 63
A. nodosum	A	0.60	0.40	0.40	0.20	0 20	0 20	0 83
3.5.	B	0 60	0 40	0 39	0 21	0 21	0 17	0.88
	C	0.65	0.35	0.35	0.26	0 26	0.13	1.14
Azotobacter	A	0.85	0.15	0 70	0 15	0 15	~0	1.18
vinelandii	B	0.81	0.19	0 63	0 18	0.18	0.01	1.17
	C	0.89	0.11					

[a]For A and B, see Table I. C, Block distribution obtained by chemical fractionation The amount of acid-soluble fraction has been divided equally between the columns F_{MG} and F_{GM}.

DISCUSSION

The main errors involved in using ^{1}H-n.m.r. spectroscopy for the analysis of algal samples may arise from (*a*) inaccuracy in the determination of the areas under relatively broad, partly overlapping, resonance lines, (*b*) possible, selective micro-aggregation causing broad lines which are not observable in high-resolution n.m.r., and (*c*) improper or missing corrections for reducing-end signals in cases of strong degradation of the samples. In order to keep the errors as low as possible, optimal conditions should be secured. This includes a very moderate degradation of the samples, resulting in a $\overline{\text{d.p.}}_n$ in the range 20–30. The resultant alginate solutions are fluid enough to give good spectra at high temperature, and the correction due to end groups is negligible. We have found that even traces of divalent ions must be avoided, because they interact with the polyelectrolyte and tend to broaden the n.m.r. peaks. Their effect was most conveniently removed by adding EDTA. The high sensitivity of ^{1}H-n.m.r. compared to ^{13}C-n.m.r. spectroscopy also helped to minimise the possibility of aggregation, because relatively dilute samples could be measured. The close agreement with the ^{13}C-n.m.r. data[8,9] obtained from samples 5–10 times more concentrated suggested that aggregation was not a serious problem.

The fact that alginate can be separated into three fractions, one containing >80% of M, the second >80% of G, and the third enriched in alternating sequences,

strongly suggested a blockwise arrangement of the uronates along the chain, with blocks of contiguous M and G residues, respectively, separated by blocks approaching an alternating pattern. Thus, it has been suggested[15] that the monomer sequence in alginate may be built up according to second-order Markov chain statistics.

A complete description of their sequence is not possible by the presently determined doublets only. However, it may be illustrative to use a parameter η, defined by[16]

$$\eta = \frac{F_{MG}}{F_M \times F_G},$$

to characterize and test the sequence distributions. This parameter has been introduced for polymers having a simpler statistic than alginate probably has. For a first-order Markov chain, it takes the values $0 \leq \eta < 1$ for block distributions, $\eta = 1$ for completely random cases, and $1 < \eta \leq 2$ for alternate-like cases.

The two samples of the MM and GG block type yielded η in the range 0.3–0.7, whereas the sample of the alternate block type gave $\eta \sim 1.2$–1.4 (Table I). Although the use of the η-parameter may be somewhat misleading for alginate, its use on the more simply composed fractions seems to support our earlier conclusions based on chemical fractionation data, as mentioned above. A similar test on alginate samples isolated from whole plants suggests a structure of the homopolymeric block type ($\eta < 1$), whereas the bacterial alginate tended toward a sequence distribution of the alternate block type ($\eta > 1$) (Table II).

The results clearly show that not only the overall composition but also the sequential pattern may be highly different, depending on the algal source. It should be stressed, however, that the doublets by no means give a full description of the sequence distribution in alginate, and that, in addition, determination of triplets by ^{13}C-n.m.r. spectroscopy is required for such a description.

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