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Biosynthetic implications of NMR analyses of alginate homo- and heteropolymers from New Zealand brown seaweeds

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Abstract

Homopolymeric (M- and G-) and heteropolymeric (MG-) blocks have been prepared from alginates that have been isolated from seven Southern Hemisphere brown seaweeds and from three commercial algal alginates. The blocks have been analysed by ^{1}H and ^{13}C NMR spectroscopy, and the analyses show that well-defined M- and G-homopolymers are obtained only from the five polysaccharides that have been previously designated as either high-M or high-G alginates. However, the five intermediate algal alginates (which have F_M values between 0.6 and 0.7) appear to contain mainly MG or heteropolymeric blocks. It is concluded that the results of NMR spectroscopic analysis of whole alginates can give a misleading picture of the block structure of some alginates. The NMR analyses of the homopolymers isolated from both the high-M and high-G alginates also show that the order of the D-mannuronosyl and L-guluronosyl residues in these blocks fits a first-order Markov distribution pattern. This suggests that 5-epimerization of some of the GDP-D-mannuronosyl residues at the monomer level, followed by addition copolymerization catalyzed by a GDP-guluronic acid transferase system, as originally suggested by Lin and Hassid [1,2], may contribute to the biosynthesis of the homopolymeric block structures in these high-M and high-G alginates isolated from brown algae. © 1997 Elsevier Science Ltd.

Keywords: Alginates from brown algae; Block structures; NMR analyses; Biosynthesis

1. Introduction

The alginates are copolymers of 1 → 4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues that are arranged in a block structure along a linear chain [3]. A previous paper [4] has characterised, mainly by ^{1}H and ^{13}C NMR analysis, the whole alginates isolated from brown algae which are found in New Zealand waters, especially around the southern coasts of the South Island. These include the following: *Cystophora retroflexa*, *Durvillaea antarctica*, *Durvillaea willana*, *Homosira banksii*, *Macrocystis pyrifera* and *Marginariella boryana*. Also included for comparative purposes was the alginate from a sample of *Durvillaea potatorum* obtained from Monash University in Australia, and three com-

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mercially available alginates, namely Sigma H.V., Sigma M.V. and Sigma L.V. [5]. The analyses of the compositions and block structures of these alginates showed [4] that the algal alginates could be classified as being high-M, in which F_M is greater or equal to 0.7, low-M (or high-G) in which F_M is equal to or less than 0.6, or intermediate type alginates in which F_M lies between these two values. The basis for this classification was that only the intermediate alginates possess a well defined heteropolymeric or 'MG' block, whereas the high- and low-M alginates consist mainly of M- and G-homopolymers (also called M- and G-blocks), which are almost directly connected and contain few or no heteropolymeric blocks.

The earlier investigation [4] also showed that estimates of the homopolymeric block lengths ($N_{M>1}$ and $N_{G>1}$), obtained using NMR data of whole alginates, were unreliable and were probably only approximate measures of the lengths of these blocks. Furthermore, when statistical models for addition copolymer formation, i.e., the Bernoullian and first- and second-order Markov chain models, were applied to describe the sequence of residues in these alginates, good agreements with the first-order Markov model were consistently observed only for the high-M and high-G alginates, and large deviations from the models were observed for all algal alginates with F_M values between 0.6 and 0.7, i.e., for the socalled intermediate alginates. These observations suggest that for the intermediate alginates the biosynthetic pathway may involve polymer modification by epimerase action rather than synthesis via the addition polymerization model.

In the present work the M- and G-blocks (also called M- and G-homopolymers) and the 'MG' blocks (also called heteropolymers) have been isolated from the same ten algal alginates previously studied [4] and characterized by ^1H and ^{13}C NMR analyses. The diad and triad frequencies obtained from these analyses are used to estimate the block lengths and to test the Bernoullian and the first- and second-order Markov statistical models for addition copolymer formation. This information forms the basis for a brief discussion of the biosynthetic pathways for the three different block types and therefore for the whole alginates.

2. Results and discussion

The results presented in Tables 1 and 2 show that well defined M- and G-homopolymers, as indicated by the frequencies of the M*M*M and G*G*G triads, were obtained from all of the alginates studied. However well defined 'MG' or heteropolymeric blocks, as measured by the high frequencies of the G*M*G and M*G*M triads, could only be isolated from the five intermediate alginates [4], namely the three Sigma alginates and the alginates extracted from New Zealand *Macrocystis pyrifera* and *Homosira banksii*. Furthermore it was also found that the acid-soluble fractions from the partial acid hydrolysates of the whole alginates from the three *Durvillaea* algae, which are all high-M algae, and from the *Cystophora retroflexa* and *Marginariella boryana* algae, which are both low-M algae, contain very little of the M*G*M and G*M*G sequences and therefore very few heteropolymeric block structures.

^1H and ^{13}C NMR analyses both gave similar compositions and diad frequencies for several of the M-homopolymers (Table 1), and generally the agreement between the two sets of NMR results is good. The M-content of these blocks ranged from 78% to 90%, indicating the relatively crude nature of the fractionation, which in some cases probably resulted in parts of the heteropolymeric block remaining attached to the M-homopolymers. Larsen et al. [6] have stated that 'weak linkages' need not be invoked to explain the fragmentation of alginates into the three separate blocks during partial acid hydrolysis. However, the generally lower M content of M-blocks isolated from the intermediate type alginates does suggest that 'weak linkages' such as G–M, which are common in the 'MG' blocks, may in some cases contribute to the impure nature of the M-blocks from these alginates, as the G–M bonds are much more susceptible to acid hydrolysis than either the M–G or M–M bonds, while the G–G bond is known to be the most stable in the hydrolysis conditions used [7]. Further acid hydrolysis, which might produce purer M-blocks, was, however, not used in this work as it was considered desirable to minimize depolymerisation that would probably have affected the calculations of diad and triad frequencies, and also the block length estimates [8].

One of the most important parameters that can be obtained from the ^{13}C NMR data of M-blocks is an improved estimate of the homopolymer length, $N_{M>1}$ [9]. In this work the M-homopolymer length estimates for isolated M-blocks are generally much longer ($N_{M>1}$ = 9.6–17.8) than those obtained from the ^{13}C NMR data of the whole alginates, which gave values of $N_{M>1}$ between 4.3 and 7.6 [4]. The longest

Table 1
^{1}H and ^{13}C NMR spectral data of the M-blocks isolated from the ten sodium alginates and the fitting of statistical models [first-order Markov] and (Bernoullian), to their sequential parameters

Source	F_{MMM}	F_{MMG} F_{GMM}	F_{GMG}	F_{MM}	F_{MG} F_{GM}	F_{GG}	F_{M}	F_{G}	N_{M}	$N_{M>1}$
C. retroflexa [a]	(0.51)	(0.13)	(0.03)	(0.64)	(0.16)	(0.04)			(5.0)	(5.9)
	0.63	0.07	0.03	0.70	0.10	0.10	0.80	0.20	8.0	11.0
	[0.61]	[0.09]	[–]							[8.9]
				(0.62)	*(0.17)*	*(0.04)*			*(4.6)*	
				0.67	*0.12*	*0.09*	*0.79*	*0.21*	*6.6*	
D. antarctica [b]	(0.70)	(0.09)	(–)	(0.79)	(0.10)	(0.01)			(8.9)	(9.9)
	0.79	0.05	–	0.84	0.05	0.06	0.89	0.11	17.8	17.8
	[0.79]	[0.05]	[–]							[17.8]
				(0.81)	*(0.09)*	*(0.01)*			*(10.0)*	
				0.81	*0.09*	*0.01*	*0.90*	*0.10*	*10.0*	
D. potatorum [b]	(0.72)	(0.08)	(–)	(0.81)	(0.09)	(0.01)			(9.0)	(11.3)
	0.80	0.05	–	0.85	0.05	–	0.90	0.10	17.0	17.0
	[0.80]	[0.05]	[–]							[17.0]
				(0.81)	*(0.09)*	*(0.01)*			*(10.0)*	
				0.80	*0.10*	–	*0.90*	*0.10*	*9.0*	
D. Willana [b]	(0.63)	(0.11)	(0.02)	(0.71)	(0.12)	(0.03)			(7.0)	(7.5)
	0.64	0.09	0.01	0.73	0.11	0.05	0.84	0.16	7.6	9.2
	[0.64]	[0.09]	[0.01]							[9.2]
				(0.71)	*(0.13)*	*(0.03)*			*(7.0)*	
				0.71	*0.13*	*0.03*	*0.84*	*0.16*	*7.0*	
H. banksii [c]	(0.55)	(0.12)	(0.03)	(0.67)	(0.15)	(0.03)			(5.5)	(6.6)
	0.65	0.06	0.05	0.71	0.11	0.07	0.82	0.18	7.5	11.1
	[0.61]	[0.10]	[0.01]							[8.1]
				(0.69)	*(0.14)*	*(0.03)*			*(5.9)*	
				0.69	*0.14*	*0.03*	*0.83*	*0.17*	*5.9*	
M. pyrifera [c]	(0.61)	(0.09)	(0.02)	(0.72)	(0.13)	(0.02)			(6.5)	(9.2)
	0.68	0.06	0.05	0.74	0.11	–	0.85	0.15	7.7	13.3
	[0.64]	[0.10]	[0.01]							(8.4)
				(0.74)	*(0.12)*	*(0.02)*			(7.2)	
				0.72	*0.14*	–	*0.86*	*0.14*	*6.1*	
M. boryana [a]	(0.49)	(0.13)	(0.03)	(0.62)	(0.16)	(0.04)			(4.9)	(5.8)
	0.61	0.08	0.02	0.69	0.10	0.11	0.79	0.21	7.9	9.6
	[0.60]	[0.09]	[0.01]							[8.7]
				(0.62)	*(0.16)*	*(0.04)*			*(4.9)*	
				0.64	*0.15*	*0.06*	*0.79*	*0.21*	*5.3*	
Sigma H.V. [c]	(0.47)	(0.13)	(0.04)	(0.61)	(0.17)	(0.05)			(4.6)	(5.7)
	0.60	0.07	0.04	0.67	0.11	0.11	0.78	0.22	7.1	10.6
	[0.57]	[0.10]	[0.01]							[7.7]
				(0.61)	*(0.17)*	*(0.05)*			*(4.6)*	
				0.65	*0.13*	*0.09*	*0.78*	*0.22*	*6.0*	
Sigma L.V. [c]	(0.55)	(0.12)	(0.03)	(0.67)	(0.15)	(0.03)			(5.5)	(6.6)
	0.61	0.08	0.05	0.69	0.13	0.05	0.82	0.18	6.3	9.6
	[0.58]	[0.11]	[0.02]							[7.3]
				(0.67)	*(0.15)*	*(0.03)*			*(5.5)*	
				0.66	*0.16*	*0.02*	*0.82*	*0.18*	*5.1*	
Sigma M.V. [c]	(0.53)	(0.12)	(0.04)	(0.66)	(0.15)	(0.04)			(5.4)	(6.4)
	0.64	0.06	0.05	0.70	0.11	0.09	0.81	0.19	7.4	12.7
	[0.60]	[0.10]	[0.01]							(8.0)
				(0.66)	*(0.15)*	*(0.04)*			*(5.4)*	
				0.68	*0.13*	*0.06*	*0.81*	*0.19*	*6.2*	

[a] Low-M alginate ($F_M \leq 0.6$). [b] High-M alginate ($F_M \geq 0.7$). [c] Intermediate-type alginate ($0.7 \leq F_M \geq 0.6$).

Table 2
^{1}H NMR spectral data on the isolated G-blocks and the fitting of statistical models (Bernoullian) and [first-order Markov] to diad and triad frequencies

Source	F_{GGG}	F_{GGM} F_{MGG}	F_{MGM}	F_{GG}	F_{GM}	F_{MM}	F_G	F_M	N_G	$N_{G>1}$
C. retroflexa [a]	(0.73)	(0.08)	(0.01)	(0.81)	(0.09)	(0.01)			(10.0)	(11.1)
	0.84	0.03	–	0.87	0.03	0.07	0.90	0.10	30.0	30.0
	[0.84]	[0.03]	[–]							[30.0]
D. antarctica [b]	(0.55)	(0.12)	(0.13)	(0.67)	(0.15)	(0.03)			(5.5)	(6.6)
	0.75	0.03	0.01	0.78	0.08	0.10	0.82	0.18	10.2	27.0
	[0.75]	[0.03]	[0.01]							[7.0]
D. potatorum [b]	(0.53)	(0.13)	(0.01)	(0.66)	(0.14)	(0.04)			(5.0)	(6.0)
	0.73	0.03	0.02	0.76	0.05	0.14	0.80	0.20	16.2	26.3
	[0.72]	[0.04]	[–]							[20.0]
D. willana [b]	(0.69)	(0.09)	(0.01)	(0.77)	(0.10)	(0.01)			(8.8)	(9.6)
	0.78	0.05	–	0.83	0.05	0.07	0.88	0.12	17.6	29.3
	[0.78]	[0.05]	[–]							[29.3]
H. banksii [c]	(0.64)	(0.10)	(0.02)	(0.73)	(0.12)	(0.03)			(7.2)	(8.4)
	0.78	0.03	0.02	0.81	0.05	0.09	0.86	0.14	17.2	28.0
	[0.76]	[0.05]	[–]							[17.2]
M. boryana [a]	(0.70)	(0.09)	(0.01)	(0.79)	(0.10)	(0.01)			(8.9)	(9.8)
	0.81	0.04	–	0.85	0.04	0.07	0.89	0.11	22.3	22.3
	[0.81]	[0.04]	[–]							[22.3]
M. pyrifera [c]	(0.53)	(0.12)	(0.03)	(0.66)	(0.3)	(0.04)			(5.4)	(5.4)
	0.73	0.03	0.02	0.76	0.05	0.14	0.81	0.19	16.2	26.2
	[0.71]	[0.05]	[–]							[16.2]
Sigma H.V. [c]	(0.76)	(0.07)	(0.01)	(0.83)	(0.08)	(0.01)			(11.4)	(12.5)
	0.84	0.03	0.01	0.87	0.04	0.05	0.91	0.09	22.7	29.5
	[0.83]	[0.04]	[–]							[22.8]
Sigma L.V. [c]	(0.51)	(0.13)	(0.03)	(0.64)	(0.16)	(0.04)			(5.0)	(5.9)
	0.71	0.03	0.03	0.74	0.06	0.14	0.80	0.20	13.3	25.7
	[0.68]	[0.05]	[0.01]							[15.8]
Sigma M.V. [c]	(0.55)	(0.12)	(0.03)	(0.67)	(0.15)	(0.3)			(5.5)	(6.6)
	0.74	0.03	0.02	0.77	0.05	0.13	0.82	0.18	16.4	26.7
	[0.72]	[0.12]	[0.03]							[16.4]

[a] Low-M alginate.
[b] High-M alginate,
[c] Intermediate alginate.

estimates of $N_{M>1}$ values are obtained for M-blocks isolated from the two alginates with the highest M residue content, namely those from *D. antarctica* and *D. potatorum*. The length of the *D. antarctica* M-homopolymer ($N_{M>1}$ = 17.8, Table 1) is closest to the proposed 23–25 residue M-homopolymer suggested by the work of Boyd and Turvey [10]. It is also observed that unlike similar data for the whole alginates [4], estimates of $N_{M>1}$ are generally independent of the composition of the M-homopolymer. The exceptions are the relatively pure high-M containing homopolymers isolated from the alginates of *D. antarctica* (F_M = 0.89) and *D. potatorum* (F_M = 0.90) already referred to, and the reason for these large estimates could be the absence from the homopolymer of transition type triads such as M*G*M and G*M*G.

The G*M*G frequencies shown in Table 1 initially increase with G content of the originating alginate (high-M to intermediate type) but decrease again for the homopolymers isolated from the lower M containing alginates (i.e., the intermediate to low-M alginates). It is also noteworthy that the signal profiles of the anomeric region of the ^{13}C NMR spectra of the M-blocks reflect the complexity of that spectral region in the originating alginate. Thus the MG-C1

Table 3
Distribution of G residues in the M-blocks obtained from ^{1}H NMR analyses

Sample	$N_{M>1}$	F_{GGG}	$N_{G>1}$	N_G
C. retroflexa [a]	11.0	0.04	2.7	2.0
D. potatorum [b]	12.7	0.00	1.2	1.0
Sigma H.V. [c]	8.1	0.05	3.0	2.0
Sigma L.V. [c]	7.7	0.00	2.0	1.2

[a] Low-M alginate.
[b] High-M alginate.
[c] Intermediate alginate.

multiplets in our work range from the very simple major peak attributed to M*M*G-C1 (103.9 p.p.m.), which is observed in the anomeric region of the ^{13}C NMR spectra of M-blocks from *D. antarctica*, *D. potatorum* and *D. willana*, to the four signals observed for MG-C1 diads in the anomeric region of the ^{13}C NMR spectra of the M-blocks of the intermediate alginate from *Macrocystis pyrifera* and from the Sigma alginates. Furthermore, as the G content of the originating whole alginate increases, the MG–C1 diad shows less complexity, as is seen in the analyses of the spectra acquired from the low-M alginates from *M. boryana* and *C. retroflexa*. These observations not only again reflect the crude nature of the fractionation procedure, but they also link the occurrence of heteropolymeric blocks in the isolated M-homopolymers with their occurrence in the intermediate whole alginates.

Average homopolymeric block-lengths of G-residues ($N_{G>1}$) in several of the M-homopolymers have also been calculated using G triad frequencies obtained from the ^{1}H NMR spectra. Results are given in Table 3. The estimated lengths indicate that, unlike the data presented by Grasdalen et al. [9], the values for $N_{G>1}$ in the M-blocks are much smaller than the $N_{M>1}$ values. The $N_{G>1}$ values are also similar to the N_G values, which suggests that most of the G residues are distributed amongst the singlet triad (M*G*M) and terminating triads (G*G*M, M*G*G). This in turn shows that there are few if any G-blocks of even moderate length in the M-homopolymers, and this is the case for the M-blocks of the high-M, the intermediate and the low-M alginates. It is apparent therefore that many of the G residues in the M-blocks are associated with the remnants of heteropolymeric blocks, some of which may still be attached to homopolymeric M-blocks after isolation.

The diad probabilities calculated from the ^{13}C NMR frequencies (Table 1) show marked deviations from Bernoullian statistics for most of the ten M-homopolymers isolated. However, when the triad frequencies of the M-homopolymers are compared with calculated first-order Markovian probabilities, exact agreement is observed for the *D. antarctica* and *D. potatorum* M-blocks. Furthermore, marked deviations from Bernoullian statistics are also observed for the five M-homopolymers isolated from the intermediate alginates, *Macrocystis pyrifera*, *Homosira banksii* and the three Sigma alginates. The M-blocks isolated from the high G-content alginates (*M. boryana and C. retroflexa*), however, show much smaller deviations from the first-order Markov probabilities. This observation suggests that for some algal alginates, at least, part of the polymer, i.e., the M-homopolymer, can apparently be described by Markovian statistics. This agrees with the suggestions made by Platé et al. [11], Gonzales et al. [12], and Larsen [13] that the parts of the alginate molecule that remain unmodified (i.e., are not epimerised) can be shown to fit a Markovian type distribution. This is apparently the case with the M-homopolymers of both the high-M and low-M alginates. These sequences of the alginate molecule then do not account for the auto-cooperative effects which occur when constructing high density regions of reacted units, i.e., in the G-homopolymers.

It is possible that the marked deviations from expected first-order Markov probabilities in the M-homopolymers from the intermediate alginates are related to heteropolymeric fragments not removed by the acid hydrolysis. These remaining fragments would be expected to influence the NMR analyses of the M-homopolymers isolated from alginates of the intermediate type.

Table 2 gives the results of ^{1}H NMR spectroscopic analyses of the G-blocks isolated following 2.5 h of partial acid hydrolysis of the parent alginates. ^{1}H NMR spectra provide the most accurate values of G-triad frequencies [14] and offer a more rapid means of analysis than do ^{13}C NMR spectra. It has also been reported [9] that selective aggregation of G-blocks occurs at concentrations such as those used in our ^{13}C NMR applications (60–140 mg mL^{-1}), and this may give rise to extensive broadening of resonances and apparent increases in the M/G ratios. However in this work we were nevertheless able to show that ^{13}C NMR spectroscopy can also yield valuable information about the sequence of M residues in G-homopolymers.

Using composition as a measure of reliability, very good agreement between the two methods (^{13}C NMR

Table 4
The distribution of M and G residues of three G-homopolymers obtained by ^{13}C NMR spectroscopy

G-homopolymer	F_{GG}	$F_{MG/GM}$	F_{MM}	F_{MMM}	$F_{MMM/GGG}$	F_{GMG}	$N_{M>1}$
M.boryana [a]	0.81	0.06	0.07	0.03	0.04	0.02	2.7
Sigma L.V. [b]	0.77	0.05	0.13	0.11	0.02	0.03	7.5
Sigma M.V. [b]	0.73	0.07	0.13	0.10	0.03	0.04	5.3

[a] Low-M alginate.
[b] Intermediate alginate.

and ^{1}H NMR) was in fact obtained. In measuring the frequencies reported in Table 2, the approximation [8] that $F_{MG} = F_{GM}$ was made prior to determining the full compliment of four G-centred triad frequencies. A check on the degree of polymerization of the G-fractions using the reducing-end group signals assigned by Grasdalen et al. [8,14] shows that they are well in excess of thirty monomer residues long, and, therefore, the above approximation is justified.. The reducing ends were mainly of the type GG-β (for which the chemical shift of H-1 is 4.88 ppm.).

The G residue content of the G-homopolymers ranges between 80% and 90% (Table 2) and their purity is thus similar to that of the M-blocks. The average block length estimates, $N_{G>1}$, are in general similar for all alginates studied, ranging between 25.7 for the homopolymer from the Sigma L.V. alginate to 30.0 for the homopolymer of the *C. retroflexa* alginate. An exception is the *M. boryana* alginate, which has the shortest G block length ($N_{G>1} = 22.5$). However, this difference is not significant as an error of 0.01 in triad frequency measurement would increase the $N_{G>1}$ estimate for the G-homopolymer of *M. boryana* to 28.3 (i.e., $F_G = 0.86$, $F_{GGM} = F_{MGG} = 0.03$, $F_{MGM} = 0.01$), a length similar to that of the other G-homopolymers that were isolated. It is also noteworthy that neither the composition nor the length of G-homopolymers is related to composition of the originating alginate. For example, the lowest G composition alginate (from *D. antarctica)* yielded G-blocks that were of similar composition (82% G) and length ($N_{G>1} = 27.0$) to the higher G-composition alginates. Estimates of the block lengths of the G-homopolymers were generally much longer and showed less variation than did those of the M-homopolymers.

Haug et al. [15,16] have shown in their extensive hydrolysis studies on alginates isolated from *Laminaria digitata* that the polysaccharides isolated from that plant contained two G-homopolymers that could be separated after extensive hydrolysis (20 h). One had a degree of polymerization (dp) of 23.0, the other a dp of 15. The latter represented approximately 15% of the total G-homopolymer in the alginate. It has been suggested [15] that this difference in G-block length may be the result of long sequences of G-homopolymer that contain M residues within their chains. As these M residue sequences would be more susceptible to acid hydrolysis than the other sequences, the G-homopolymers separated into two distinct fractions of different sizes during extensive hydrolysis [16]. In the present work, the analyses of three G-homopolymers by ^{13}C NMR spectroscopy given in Table 4 suggest that polymeric sequences of M residues in the G-homopolymers may be restricted to a relatively small group of alginates. For the analyses reported in this table, the spectra were acquired using 10-mm sample tubes and polysaccharide concentrations of about 30–40 mg mL^{-1}. This was done to prevent preferential aggregation of the G-blocks and the associated line-broadening effects [9].

The data in Table 4 also show that short M-homopolymers ($N_{M>1} = 5.3$ and 7.5) occur in the G-homopolymer isolated from two of the commercial alginates that have been classified as belonging to the intermediate type. M residues are, however, more likely to be part of terminal type sequences in the *Marginariella boryana* G-block. While Haug et al. [16] put forward evidence for short blocks of M-residues occurring in G-homopolymers, our results suggest that they do not occur in all alginates and may be restricted to the intermediate types, i.e., to those alginates that possess the heteropolymeric block.

In the present application of polymer statistics to the NMR data for the M- and G-homopolymers, the calculated Bernoullian probabilities for the diad and triad frequencies of both the M and G residues deviate markedly from the observed frequencies. It is nevertheless surprising to observe that the experimental triad frequencies of the G residues in the original whole alginates, which have a wide range of compositions, agree with the first-order Markovian description [4]. This agreement in fact illustrates the problem of relying only on frequency analyses of whole alginates, especially from the NMR spectra of the high

G-composition polymers, for which both ^{1}H and ^{13}C NMR spectroscopy can show marked deviations from Markovian statistics for the distribution of G-residues. Thus the low-M alginates from *Marginariella boryana* ($F_M = 0.44$) and *Cystophora retroflexa* ($F_M = 0.52$) both yield G-homopolymers that can be described by first-order Markovian statistics. However as the alginate being analysed increases in M content, i.e., in the intermediate type alginates, the triad frequencies begin to deviate, although not substantially (≤ 0.02) from that model. Furthermore, in a trend similar to the that of the M-homopolymers, excellent agreement is obtained for the G-homopolymers derived from the high-M containing *Durvillaea* alginates.

The good agreement that is obtained when the frequencies from ^{1}H NMR analyses are compared with those from the ^{13}C NMR analyses for an alginate having a Markovian type distribution appears to be related to composition of the whole alginate, and, therefore, to the presence or absence of the heteropolymeric block. As previously observed [4], those alginates which have high-M or G contents tend to be characterized by a high content of the homopolymeric fractions, and the contributions from the heteropolymeric blocks are correspondingly low. In our work, the agreement with the first-order Markovian fit contradicts the suggestion [12,13] that the distribution of G-units along a chain is non-Markovian. This suggestion apparently follows on from what is known of the mechanism of action of the mannuronan C-5 epimerase with alginates, which is said to be based on a cooperative polymer-modification reaction [11–13,17]. Observed deviations from a first-order Markov distribution are much smaller (≤ 0.02) than those recorded for the M-homopolymers and the heteropolymeric block consequently appears to bear less influence on the sequencing of residues in the G-homopolymer. In particular, the agreement between observed frequencies and those calculated for a first-order Markov type distribution in the G-homopolymers may be especially significant as it perhaps points to a similar biosynthetic pathway for both homopolymeric fractions of the algal alginate molecule. This biosynthetic path, which is one that can be explained by Markovian statistics and suggests an addition copolymerization reaction, is discussed below.

The researches of Grasdalen et al. [9] have shown that the ^{13}C NMR spectra of the heteropolymeric block may be characterised by the appearance of two major resonances in the anomeric region. These signals represent the symmetric triplet sequences MGM and GMG. However, in a preliminary investigation we have studied both the ^{1}H and ^{13}C NMR spectra of the heteropolymeric blocks isolated from the *Sigma M.V.* alginate. These studies suggest that in this case the spectra of the heteropolymeric block is more complex than the earlier studies of Grasdalen et al. [9] indicate, presumably because the latter were made using a field strength of only 50 MHz (^{13}C NMR).

In addition to the two singlets at 102.37 ppm (MGM–C1) and 103.85 ppm (GMG–C1) in the ^{13}C NMR spectra of the heteropolymeric blocks, several other significant contributions exist that indicate that a great diversity exists in the monomer sequences present in heteropolymeric part of the alginate molecule. However, because of the very complex signal pattern it was not possible to unequivocally assign this region of the ^{13}C NMR spectra. The ^{1}H NMR spectra of the heteropolymeric blocks of the alginates are also very complex and contain many previously unreported signals.

While these investigations are focused mainly on the ten algal alginates that have been previously studied and that have a wide range of compositions [4], less detailed studies on other algal alginates that have been isolated in our laboratory show that they also have the same general block characteristics described above. For example, a very high G-content ($F_G = 0.78$) alginate was isolated from the holdfast of *D. antarctica*, and this alga also contains in its fronds a water-soluble alginate with a very high M-content ($F_M = 0.87$). The ^{13}C NMR spectra show that this alginate contains a very small (< 0.03) proportion of the singlet triads MGM–C1 and GMG–C1, which again suggests a link between block structure and composition. We were, in fact, not able to isolate heteropolymeric blocks from either of these alginates. In a related study in our laboratory (D. Kan, unpublished results) the New Zealand brown alga *Xiphophora chondrophylla* also yielded an intermediate type alginate which contained both a high proportion of heteropolymeric sequences and heteropolymeric blocks. The presence of the heteropolymeric block in algal alginates can therefore be said to be highly specific and limited to a narrow range of alginate compositions.

The ^{1}H and ^{13}C NMR analyses presented both in this paper and in an earlier paper [4] also reveal several more important features concerning the block structure and composition of alginates from the New Zealand brown algae. Firstly, although the ten alginates studied have a wide range of compositions,

block structures and block lengths, the analytical data given in Tables 1 and 2 do support the general classification of three alginate types which were recently proposed [4] on the basis of NMR studies of whole alginates, namely high-M alginates (F_M greater or equal to 0.7), high-G alginates (F_M less or equal to 0.6) and intermediate or 'MG' alginates in which F_M lies between these two values. However, the exact composition range (i.e., F_M values) that describes each type of algal alginate is not yet definitely known, and in particular that precise composition that separates the low-M from the intermediate type alginates should be regarded as an approximation at this stage. The composition that separates the intermediate from the high-M alginates is known more precisely.

The NMR analyses also show that the high-M and high-G alginates are both block copolymers that are formed from the two homopolymeric blocks and which are linked by very short and as yet poorly defined junction zones. However both the M- and G-homopolymers, which are isolated from these two classes of whole alginates by the commonly used method involving hydrolysis of the polysaccharide with 0.3 M aqueous hydrochloric acid at 100 °C, show good agreements with a first-order Markov description of monomer sequencing, as is seen from diad and triad measurements given in Tables 1 and 2. On the other hand, the intermediate alginates are very different from the high- and low-M alginates in that they contain a third block type, namely the well defined 'MG' or heteropolymeric block. Only in these intermediate alginates do the M-homopolymeric fragments, when isolated, contain M and G sequences that show large deviations from the first-order Markov polymer description.

The various block structures found in the ten algal alginates described in this paper, and especially the conclusion that the monomer sequences within the homopolymeric blocks of both the high-M and high-G alginates fit first-order Markov descriptions, clearly have implications for current views on the biosynthesis of algal alginates. Recent reviews [18–20] show that the presently accepted theories support a biosynthetic pathway which is partly based on statistical models of copolymerisation. These models can predict either a first- or second-order Markov chain structure in alginates, as studied by Painter et al. [21,22] and Smidsrød and Whittington [23]. Comparisons of experimental data with these models showed [21] that the monomer sequence in alginates was in fact best described by a second-order Markov model. This view is consistent with the conclusions of Grasdalen [14], which was based on the structures of alginates from several Northern Hemisphere brown algae.

The second-order Markov model of alginate structure is, therefore, now widely used to interpret alginate analyses. However, the acceptance of any Markov statistical model has been difficult as the widely held view is that the most likely pathway for alginate biosynthesis in brown algae involves conversion of some of the β-D-mannopyranosyl residues in a high molar mass polymer into α-L-guluronosyl residues by an enzyme similar to that isolated from the bacterium *Azotobacter vinelandii*. The first evidence for this mechanism was presented by Hellebust and Haug [24], and in 1971 the existence of an enzyme capable of introducing L-guluronic acid residues into alginate was reported by Larsen and Haug [25]. Since then a C-5 epimerase has been shown to occur in many brown algae.

It has however been stated [11–13,17] that because the order of the D-mannuronosyl and L-guluronosyl residues within the alginate polymer is, according to the above theory, not the direct result of a copolymerization reaction, but is instead the result of an enzymic isomerization that occurs at the polymer level, the complete chain cannot be described by Markovian statistics. On the other hand, it is quite clear that the polymannuronic acid C-5-epimerase from *Azotobacter vinelandii* will epimerize D-mannuronic acid residues to L-guluronic acid residues in polymannuronic acid chains [25], but whether the result of this particular polymerization at the polymer level is a Markov distribution has not yet been determined.

One explanation of the results of our NMR studies on the ten alginates and their fragments could be that the biosynthetic pathway for the M- and G-homopolymers of algal alginates involves a mannuronan C-5 epimerase enzyme at the monomer level which transforms some of the original D-mannuronic acid residues into L-guluronic acid residues prior to polymerization, and both monomer residues may then copolymerize to form the blocks that are present in the final polymer. Whether or not this latter reaction does occur, the good agreements we obtain by fitting a first-order Markov distribution to the M- and G-residue sequences in the homopolymeric fragments of the high-M and high-G alginates does suggest that an addition-type copolymerization mechanism could be involved in the initial formation of both the M- and the G-homopolymeric blocks in these two alginate categories. These blocks might then be linked to form

the final polymeric structure of the algal alginate [13]. One requirement for this pathway would be that any enzyme that may be involved in the linking together of the blocks must be able to recognise the identity of the unit at the end of a growing polymer chain.

The outline in the previous paragraph of the general form of a possible biosynthetic pathway, which is consistent with the analytical results reported in Tables 1 and 2 for the M- and G-homopolymers of the high-M and high-G algal alginates, has much in common with the stepwise addition pathway for the biosynthesis of alginates that was proposed in the early work of Larsen et al. [6]. However, by simulating random depolymerization of alginate and comparing the monomer distribution in whole alginates with their experimental data, these workers suggested that a second-order Markovian copolymerization model best described the nonrandom, nonrepeating structure of the whole alginate chain. It is nevertheless noteworthy that in many of the studies that support that conclusion, the algal alginates analyzed would fit the intermediate classification defined in our earlier study [4]. The analytical results given in that work, and also the analyses in Tables 1 and 2 of this paper, show that the order of the mannuronosyl and guluronosyl residues in the M- and G-blocks of the intermediate alginates does not fit a first-order Markov distribution. The above comments on the involvement of mannuronan C-5 epimerase, therefore, probably only apply to the high-M and high-G algal alginates.

3. Conclusions

Although the basic pathway for the biosynthesis of bacterial alginates is now considered to be well understood [18–20], when the proposed pathway is applied to algal alginates there still remains some difficulty in the fitting of the intermediate alginates into the pathway, and in the precise position of the epimerization stages in the pathway. For example, the results of Quillet and Lestang-Brémond [27,28] leave no doubt that in some brown algae, when D-[UL-^{14}C]sorbitol-6-phosphate, which is free of mannitol and mannitol phosphate, is injected into the tips of very young thalli in full growth, the G-blocks become clearly labelled. The MG blocks are also labelled, but only in their G component, whereas the M-blocks remain unlabelled.

The results presented in this paper suggest that perhaps there are two parallel pathways for the biosynthesis of alginates. In one of these GDP-L-guluronic acid may be synthesized from D-sorbitol-6-phosphate, as demonstrated by the work of Quillet and Lestang-Brémond [27,28], and then copolymerization follows to introduce both G- and M-residues into the alginate. This pathway could be expected to result in a structure in which the D-manuronosyl and L-guluronosyl residues in the two homopolymeric blocks of some algal alginate chains have a first-order Markov distribution pattern, as has been found in the present work. However, the biosynthesis of many bacterial alginates apparently does not follow this pathway, as is seen from the importance of *O*-acetyl groups in bacterial, but apparently not in algal, alginate formation [29].

4. Experimental

Preparation of alginates.—Alginates were prepared from the algae as previously described [4].

NMR characterisation of alginates.—The composition and monomer sequence of the alginates were determined by ^{1}H and ^{13}C NMR analysis.

The three-component blocks from the ten alginates were obtained via the partial acid hydrolysis method [10]. Alginate (1.0 g) was made up to 1% solution in water and 3.0 M HCl was added to a final acid concentration of 0.3 M. Hydrolysis was then conducted at 100 °C with nitrogen bubbling through the mix. The acid-soluble fraction was removed after 0.3 h, neutralised, concentrated and freed of salts by dialysis in benzoylated dialysis tubing (Sigma Chemical Co., St. Louis, MO, USA) against frequently changed distilled water. After dialysis, the pH was adjusted to 6.5 with dilute base, the solution concentrated by rotary evaporation, and the 'MG' or heteropolymeric block isolated by freeze drying.

The acid-insoluble fraction that contained the homopolymeric M and G blocks was hydrolysed in replenished 0.3 M HCl for a further 2 h. This fraction was then redissolved to a concentration of 0.01%, and NaCl was added, following which the pH of the solution was adjusted to 2.85 by the gradual addition of 0.05 M HCl. After centrifugation (0.5 h, 5000 rpm) the insoluble (G-homopolymeric) and soluble (M-homopolymeric) fractions were separately dialysed against distilled water in benzoylated dialysis bags for 2 days. The pH of the contents of the dialysis bags was then adjusted to 6.5, and the solutions were freeze dried prior to analysis by NMR spectroscopy.

Recording of NMR spectra.—Samples were made

up in D_2O (99.8%) to a concentration of approximately 15 mg mL^{-1} for ^1H NMR spectroscopy and between 60–150 mg mL^{-1} for ^{13}C NMR spectroscopy. All solutions were adjusted to pD 7 after the addition of MeOH (5 mL) as the internal reference.

Spectra were recorded on a Varian VXR-300 spectrometer operating at a frequency of 75 MHz for ^{13}C nuclei. A 90° pulse was used with a total repetition time of 1.6 s and between 20 000 and 40 000 transients were acquired at 75 °C and typically along a spectral window of 20 000 Hz. Both 5-mm and 10-mm probes were used. The larger sized probe was preferred when sample size was not limited and consequently acquisition time was reduced to 2 to 3 h (3000 → 5000 transients) with no loss of resolution. Following their preparation all solutions to be analysed were kept at about 75 °C for 3 h prior to acquisition to ensure that solubility was high and also to help degas the viscous samples. The deuterium resonance was used as a field-frequency lock, and the chemical shifts were initially expressed relative to MeOH, but subsequently the assignments of Grasdalen et al. [26] were applied to the major resonances.

Prior to the measurement of signal intensities one zero filling was performed, increasing the Fourier number from 32 000 to 64 000. Data manipulation using deconvolution, line broadening, resolution enhancement or the apodisation function was avoided, and the peak areas of signals were measured by cutting and weighing [4]. Peak overlap was estimated visually.

^1H NMR spectra were recorded at an operating frequency for the spectrometer of 300 MHz at 75 °C using a 5-mm probe and MeOH as the internal reference. The 180°-τ-90° pulse sequence was used to partly eliminate the solvent (HOD) peak. The 90° pulse width was found to be 24.2 μs, and a recycle time of 4 s was used with a delay (τ) of 2 s. Usually 50–100 transients were acquired. Areas under the peaks were obtained by cutting and weighing after one increment in zero filling (16 000 → 32 000). Only the low-field (M–H1, G–H1 and G–H5) intensities were used, and the assignments of Grasdalen et al. [26] were applied.

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