ELSEVIER

Carbohydrate Research 293 (1996) 119–132

CARBOHYDRATE RESEARCH

Composition and block structure of alginates from New Zealand brown seaweeds

Rajeendernath Panikkar, Donald J. Brasch *

Department of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

Received 15 February 1996; accepted 12 July 1996

Abstract

Alginates have been extracted from seven brown algae found in the Southern Hemisphere and characterized by ^{1}H and ^{13}C NMR analyses. The results are compared with similar analyses obtained in this work for three commercially available alginates isolated from *Macrocystis pyrifera*, a species of the Northern Hemisphere. Overall the ten alginates display a wide range of compositions, ranging from the very high mannuronic acid containing alginate extracted from *Durvillaea antarctica* ($F_M = 0.80$) to the moderately high guluronic acid containing alginate from *Marginariella boryana* ($F_G = 0.56$). All data are examined in terms of two addition copolymerization models, namely the Bernoullian and the first-order Markov models. Monomer distributions in the high M and intermediate composition alginates have M-diad frequencies calculated from the NMR analyses that agree closely with Bernoullian distributions, the best agreements being obtained for those alginates in which F_M is greater than 0.72. However, as the monomer composition tends towards a higher G content, both of the above statistical models fail to satisfactorily describe the distributions of either the M or the G residues. A feature of this work is the excellent agreement obtained for the ^{1}H and the ^{13}C NMR analyses. © 1996 Elsevier Science Ltd.

Keywords: Alginates; Brown seaweed; Structural analysis

1. Introduction

The Alginates are a family of unbranched binary copolymers of (1 → 4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues [1] that have been isolated from all known brown algae. Although there are many such algae in the

* Corresponding author.

0008-6215/96/$15.00 Copyright © 1996 Elsevier Science Ltd. All rights reserved.
PII S0008-6215(96)00193-0

Southern Oceans, most published work to date has been limited to alginates of *Durvillaea* species from Australia and Chile, and very little is known about the content and structure of alginates in the many brown seaweeds found along the New Zealand coastline. In this work, six commonly occurring New Zealand brown algae have been selected for investigation of their alginate content and composition, namely *Durvillaea antarctica* (bull kelp), *Durvillaea willana*, *Cystophora retroflexa*, *Hormosira banksii*, *Macrocystis pyrifera* (bladder kelp) and *Marginariella boryana*.

The *Durvillaea* species grow intertidally and at shallow subtidal depths, *D. willana* appearing at the sub-littoral fringe on very exposed rocks and below *D. antarctica*. *Macrocystis pyrifera* grows in deep sub-tidal pools and channels, and the sparse population and size of the kelp samples used in this work (2–3 m long) suggests there may be a limiting effect of the environment on this species. The Fucacea, *Marginariella boryana*, also grows along the sublittoral fringe and is of interest because the genus is not known elsewhere in the world. This alga has a very regular structural pattern, with branches sprouting in pairs from the main stem, and it often reaches a length of 2 m. It grows, usually submerged, from the Cook Strait to Stewart Island in New Zealand. *Cystophora retroflexa* is found growing in the lower littoral region, in deeper channels and pools, on rock, up to lengths of 1 m. *Hormosira banksii*, one of the commonest brown algae in New Zealand, is found either in pools or on rocks exposed at most tides. The fronds of *Hormosira*, which vary in size of segments, degree of branching and total size, may grow up to 40 cm in length.

As little was known about the alginates of either *D. antarctica* or *D. willana*, a sample of the better known *Durvillaea potatorum* [2] was obtained from Monash University in Australia. The sample consisted of blade material from an unspecified number of plants. Three commercially available alginates, Sigma H.V., Sigma M.V. and Sigma L.V. [3], were also compared with the alginates from the New Zealand algae. The algae have all been investigated for alginate content, composition and monomer sequence up to the triad level using ^{1}H and ^{13}C NMR spectroscopy. The NMR data allow testing of two statistical models for addition copolymer formation [4,5], namely, the Bernoullian or random distribution model and the first order Markov or ultimate residue distribution model. These in turn lead to a description of the sequence of monomer residues in the ten alginates investigated.

2. Results and discussion

Isolation of alginates.—The yields of sodium alginate obtained from three extraction methods are given in Table 1. The Craigie method [6] gives the lowest yield. This is a result of the number of precipitations and other process steps required by the method, using ethanol, calcium chloride and 0.1 M hydrochloric acid. These produce a very pure alginate, but the overall result is a much lower yield. The extractions using CDTA as a solvent give the highest yields of alginate, which is consistent with the intrinsic viscosity results of Wedlock and Fasihuddin [7]. High alginate yields are characteristic of the three *Durvillaea* species studied, whereas *Hormosira banksii* and *Cystophora retroflexa* give the lowest yields. *Marginariella boryana* is a rich source of the polyuronate, while

Table 1
Yields of sodium alginate from the seven New Zealand algae

Sample	Rosell and Srivastava method [19]	Craigie et al. method [6]	C.D.T.A. extraction method [7]
Cystophera retroflexa	22	15	26
Durvillaea antarctica	44	37	52
Durvillaea potatorum	55	45	62
Durvillaea willana	61	51	66
Hormosira banksii	27	24	35
Macrocystus pyrifera	35	29	38
Marginariella boryana	40	32	46

the *Macrocystis pyrifera* samples give a yield which appears to be typical for this alga in New Zealand waters [8].

NMR Spectroscopy of whole alginates.—^{1}H NMR. spectroscopy is considered to be the most reliable method for determining both composition and much of the detailed block structure of the alginate molecule, giving F_M values which are reproducible and which agree with data obtained by chemical methods to 0.05 [9,10]. Composition, expressed as F_M, and M and G diad, and G triad frequencies, and also number average estimates of M- and G-homopolymer lengths for the ten alginates, were determined using both ^{1}H NMR spectroscopy and the calculation procedures described by Grasdalen et al. [5,9,10]. Results are given in Table 2. ^{13}C NMR spectroscopy of alginates [5,11], although apparently less accurate than ^{1}H NMR techniques, allows the determination of M triad frequencies. The latter results are given in Table 3.

Good agreement was obtained between the ^{1}H and ^{13}C NMR analyses of monomer compositions for the ten alginates studied. For example, the largest difference between the F_M values is 0.02, which occurs with the *D. antarctica* alginate. This consistency between the ^{1}H and ^{13}C NMR methods is typical of the results of this study, and is attributed to a rigorous pre-acquisition workup which leads to a consistently high signal to noise ratio. As a consequence very little data manipulation is required. There are, however, differences between the two methods when determining the detailed monomer sequence, expressed as diad and triad frequencies, of the whole alginate.

Tables 2 and 3 show that the 10 alginates have a range of compositions varying from the relatively high G content ($F_G = 0.56$) of the *M. boryana* alginate to the very high M-containing alginate ($F_M = 0.80$) extracted from the fronds of *D. antarctica*. The Sigma alginates, and also the alginates extracted from New Zealand *M. pyrifera* and *H. banksii*, are referred to as 'intermediate composition alginates' ($F_M = 0.6$–0.7) in this paper. These five alginates are very different from the others in both the sequence of monomeric residues and especially in the frequency of the 'MG' or heteropolymeric block. We consider, therefore, that there is justification, based on monomer composition, to classify three alginate types, namely high-M (F_M greater or equal to 0.7), high-G (F_M less than or equal to 0.6) and intermediate or 'MG' alginates in which F_M lies between these two values.

The variation in composition of the ten alginates may be related to the different environmental requirements of the plants. For example, the rigidity of the smaller

Table 2
Composition and sequence parameters of the 10 alginates obtained using ^{1}H NMR spectroscopy [a]

Source	F_{MM}	F_{MG}, F_{GM}	F_{GG}	F_{GGG}	F_{GGM}, F_{MGG}	F_{MGM}	F_{M}	F_{G}	M/G	$^{H}N_{M}$	$^{H}N_{G}$	$^{H}N_{G>1}$
C. retroflexa	(0.27)	(0.25)	(0.23)	(0.11)	(0.12)	(0.13)				(2.1)	(1.9)	(2.9)
	0.33	0.19	0.29	0.22	0.07	0.12	0.52	0.48	1.08	2.7	2.5	5.1
				[0.18]	[0.11]	[0.08]						[3.6]
D. antarctica	(0.64)	(0.16	(0.04)	(0.01)	(0.03)	(0.13)				(5.0)	(1.2)	(2.3)
	0.64	0.16	0.04	–	0.03	0.14	0.80	0.20	4.00	5.0	1.2	2.0
				[0.01]	[0.03]	[0.13]						[2.3]
D. potatorum	(0.58)	(0.18)	(0.06)	(0.01)	(0.04)	(0.14)				(4.2)	(1.5)	(2.5)
	0.58	0.18	0.06	0.01	0.05	0.13	0.76	0.24	3.17	4.2	1.5	2.2
				[0.01]	[0.05]	[0.13]						[2.2]
D. willana	(0.52)	(0.20)	(0.08)	(0.02)	(0.06)	(0.14)				(2.5)	(1.7)	(2.6)
	0.51	0.21	0.07	0.01	0.06	0.15	0.72	0.28	2.57	3.4	1.3	2.2
				[0.01]	[0.06]	[0.15]						[2.2]
H. banksii	(0.36)	(0.24)	(0.16)	(0.06)	(0.10)	(0.14)				(2.5)	(1.7)	(2.6)
	0.37	0.23	0.17	0.09	0.08	0.15	0.60	0.40	1.5	2.6	1.7	2.9
				[0.07]	[0.09]	[0.13]						[3.0]
M. pyrifera	(0.40)	(0.23)	(0.14)	(0.05)	(0.09)	(0.15)				(2.7)	(1.6)	(2.4)
	0.42	0.21	0.16	0.11	0.05	0.16	0.63	0.37	1.70	3.3	2.0	7.0
				[0.08]	[0.09]	[0.10]						[3.0]
M. boryana	(0.19)	(0.25)	(0.31)	(0.18)	(0.14)	(0.11)				(1.8)	(2.2)	(3.2)
	0.28	0.16	0.40	0.32	0.08	0.08	0.44	0.56	0.79	2.8	3.5	6.0
				[0.28]	[0.11]	[0.05]						[4.6]

Sigma H.V.	(0.41)	(0.23)	(0.13)	(0.05)	(0.08)	(0.15)				(2.8)	(1.6)	(2.6)
	0.41	0.23	0.13	0.09	0.04	0.19	0.64	0.36	1.78	2.8	1.6	4.3
				[0.05]	[0.19]	[0.15]						[3.6]
Sigma L.V.	(0.41)	(0.23)	(0.13)	(0.05)	(0.08)	(0.15)				(2.8)	(1.6)	(2.6)
	0.42	0.22	0.14	0.10	0.04	0.18	0.64	0.36	1.78	2.9	1.6	4.5
				[0.05]	[0.09]	[0.13]						[2.6]
Sigma M.V.	(0.48)	(0.21)	(0.10)	(0.03)	(0.07)	(0.15)				(3.5)	(1.5)	(2.3)
	0.48	0.21	0.10	0.04	0.06	0.15	0.69	0.31	2.22	3.3	1.5	2.7
				[0.03]	[0.07]	[0.14]						[2.4]

[a] The values in brackets denote the conditional probabilities calculated from (Bernoullian) and [first-order Markov] polymerization models.

Table 3
The composition and sequence of monomer residues obtained by ^{13}C NMR spectroscopy [a]

Source	F_{MMM}	F_{GMM}, F_{MMG}	F_{GMG}	F_{GGG}	F_{GGM}, F_{MGG}	F_{MGM}	F_{MM}	F_{MG}, F_{GM}	F_{GG}	F_M	F_G	$^{C}N_{M>1}$	$^{C}N_{M>1}$
C. retroflexa	(0.14)	(0.13)	(0.06)	(0.11)	(0.12)	(0.13)	(0.27)	(0.25)	(0.25)			(3.7)	(2.9)
	0.28	0.10	0.04	0.27	0.07	0.07	0.38	0.14	0.34	0.52	0.48	4.8	5.9
	[0.28]	[0.10]	[0.04]	[0.24]	[0.10]	[0.04]						[4.8]	[4.4]
D. antarctica	(0.55)	(0.12)	(0.03)	(–)	(0.03)	(0.12)	(0.67)	(0.15)	(0.03)			(6.6)	(2.0)
	0.55	0.12	0.03	–	0.03	0.12	0.67	0.15	0.03	0.82	0.18	6.6	2.0
	[0.55]	[0.12]	[0.03]	[–]	[0.03]	[0.12]						[6.6]	[2.0]
D. potatorum	(0.42)	(0.14)	(0.05)	(0.02)	(0.05)	(0.13)	(0.56)	(0.19)	(0.06)			(5.0)	(2.4)
	0.50	0.11	0.03	0.04	0.07	0.07	0.61	0.14	0.11	0.75	0.25	6.5	2.6
	[0.50]	[0.11]	[0.03]	[0.05]	[0.06]	[0.08]						[6.5]	[2.8]
D. willana	(0.37)	(0.15)	(0.05)	(0.02)	(0.06)	(0.14)	(0.52)	(0.20)	(0.08)			(4.5)	(2.3)
	0.45	0.12	0.03	0.05	0.08	0.07	0.57	0.15	0.13	0.72	0.28	6.3	2.6
	[0.44]	[0.12]	[0.03]	[0.06]	[0.07]	[0.08]						[6.3]	[2.9]
H. banksii	(0.22)	(0.14)	(0.09)	(0.06)	(0.09)	(0.15)	(0.37)	(0.24)	(0.15)			(3.9)	(2.7)
	0.33	0.09	0.10	0.14	0.06	0.13	0.42	0.19	0.20	0.61	0.39	5.7	4.3
	[0.29]	[0.13]	[0.06]	[0.10]	[0.10]	[0.09]						[4.2]	[3.0]
M. pyrifera	(0.27)	(0.15)	(0.08)	(0.04)	(0.08)	(0.15)	(0.42)	(0.22)	(0.12)			(3.8)	(2.5)
	0.37	0.09	0.10	0.11	0.05	0.14	0.46	0.19	0.16	0.65	0.35	6.1	4.2
	[0.33]	[0.13]	[0.06]	[0.07]	[0.09]	[0.10]						[4.5]	[4.6]
M. boryana	(0.09)	(0.11)	(0.14)	(0.17)	(0.14)	(0.11)	(0.20)	(0.25)	(0.30)			(2.8)	(3.1)
	0.20	0.09	0.06	0.35	0.05	0.10	0.30	0.15	0.40	0.45	0.55	4.3	9.0
	[0.20]	[0.10]	[0.05]	[0.29]	[0.11]	[0.04]						[4.5]	[4.6]
Sigma H.V.	(0.27)	(0.15)	(0.08)	(0.04)	(0.08)	(0.15)	(0.41)	(0.23)	(0.13)			(3.7)	(2.6)
	0.36	0.10	0.09	0.12	0.04	0.15	0.46	0.19	0.16	0.65	0.46	5.6	5.0
	[0.33]	[0.13]	[0.06]	[0.07]	[0.09]	[0.10]						[4.5]	[2.8]

Sigma L.V.	(0.26)	(0.15)	(0.08)	(0.04)	(0.08)	(0.15)	(0.41)	(0.23)	(0.13)			(3.7)	(2.6)
	0.36	0.09	0.10	0.12	0.06	0.13	0.45	0.19	0.17	0.64	0.36	6.0	3.8
	[0.32]	[0.13]	[0.06]	[0.08]	[0.09]	[0.10]						[4.5]	[2.8]
Sigma M.V.	(0.33)	(0.15)	(0.06)	(0.03)	(0.07)	(0.15)	(0.48)	(0.21)	(0.10)			(4.2)	(2.3)
	0.41	0.08	0.12	0.07	0.04	0.16	0.49	0.20	0.11	0.69	0.31	7.1	3.8
	[0.35]	[0.14]	[0.05]	[0.04]	[0.07]	[0.13]						[5.3]	[2.6]

[a] The values in brackets denote the conditional probabilities calculated from (Bernoullian) and [first-order Markov] polymerization models.

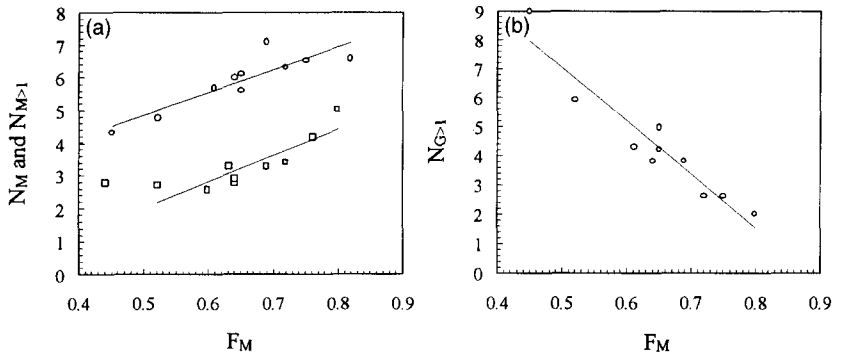

Fig. 1. (a) M-block lengths (N_M and $N_{M>1}$) versus composition. (b) G-block lengths ($N_{G>1}$) versus composition.

seaweeds (*Marginariella, Cystophora* and *Hormosira*) appears to be reflected in the high-G content of their alginates. On the other hand, the *Durvillaea* plants need to be physically flexible to survive the turbulent waters in which they grow and from where almost all other algae are absent apart from epiphytic species such as *Porphyra subtumens*.

The data in Tables 2 and 3 are used to plot block length estimates versus composition (Fig. 1(a) and (b)). As expected from previous studies [5], M- and G-block lengths calculated from triad frequencies (${}^{H,C}N_{M,G>1}$), are much larger than values obtained from diad frequencies (${}^{H,C}N_{M,G}$). Furthermore, the G-block length estimates obtained using G triad frequencies from ^{13}C NMR spectra (i.e., ${}^{C}N_{G>1}$) are also generally greater than those (${}^{H}N_{G>1}$) obtained using G triad frequencies from ^{1}H NMR spectra. The exceptions are block estimates for some of the so-called intermediate alginates.

The results also show that for some alginates the M- and G-block length estimates increase with the content of M and G monomers, as illustrated in Fig. 1(a) and (b). Thus the *D. antarctica* alginate has the longest M-block length estimate (${}^{C}N_{M>1} = 6.6$) and *M. boryana* the shortest (${}^{C}N_{M>1} = 4.3$). Conversely, the *D. antarctica* alginate has the shortest G-block (${}^{C}N_{G>1} = 2.0$) and *M. boryana* (${}^{C}N_{G>1} = 9.0$) and *C. retroflexa* (${}^{C}N_{G>1} = 5.9$) alginates the longest. A comparison of the data for block length and composition suggests that block length estimates, however, do not correlate well with composition for some of the so-called intermediate alginates.

The transition diad frequencies (F_{MG} and F_{GM}) give an indication of the proportion of 'MG' or heteropolymeric block [9,10]. The *D. antarctica* ($F_M = 0.80$) and *M. boryana* ($F_M = 0.44$) alginates possess the smallest amounts of this fraction (e.g., ${}^{H}F_{MG} = 0.16$ for *D. antarctica*), while in an intermediate alginate such as that from *H. banksii* the heteropolymeric block comprises a larger proportion (e.g., ${}^{H}F_{MG} = 0.23$) of the molecule. The plot (Fig. 2) of composition against proportion of heteropolymeric fraction (represented by frequency of the triad G*M*G-C1) shows that the intermediate alginates have a greater incidence of the 'MG' block. It is likely that the higher frequencies of these sequences in the intermediate alginates has lead to the poor correlation between composition and block lengths. For example, the heteropolymeric

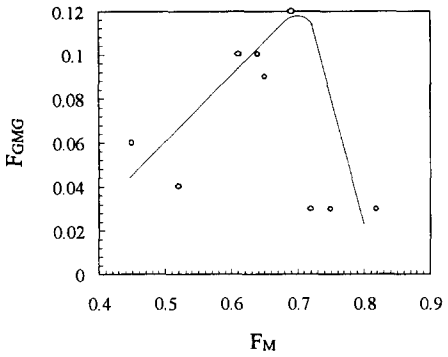

Fig. 2. Frequency of 'MG' block versus composition.

block has been shown [4,5] to contain sequences such as GGM and MMG. These add to the analyses of the authentic multads, such as GGM, MGG, GMM, and MMG, which terminate the two homopolymeric sequences. As the frequencies of these multads are used to calculate block lengths, an increase in these frequencies because of a contribution from the heteropolymeric block may result in shorter estimates of the length of the two homopolymeric blocks. Grasdalen and co-workers [5,10] have also shown that G-homopolymers may contain regions of heterogeneity, and in particular short sequences of M-units. A better indication of block lengths is therefore more likely to be obtained from isolated M- and G-homopolymers in which much of the heteropolymeric block will have been removed by hydrolysis.

The NMR results presented in Table 2 also show much smaller number average homopolymeric block length estimates than those obtained by enzymatic hydrolysis, which for example has suggested chain lengths consistently in excess of twenty residues for poly M-blocks [12]. The values obtained from this work are, however, much closer to those obtained by other workers using ^{13}C NMR spectroscopy of whole alginates [5]. It seems possible, therefore, that for whole alginates the block length estimates obtained by NMR spectroscopy only reflect the alginate composition and perhaps the mole fraction of the homopolymeric parts of the polymer. Consequently, these estimates may not yield reliable information about actual block lengths in whole alginates.

In this work a varying signal complexity in the anomeric region of the ^{13}C NMR spectra of the ten alginates is also observed. The *M*G-C1 diad profile is limited to a dominant resonance (102.9 ppm) in the alginates extracted from the *Durvillaea* species. This resonance is assigned to the terminal triad M*M*G-C1 while the singlet G*M*G-C1, an indicator of the presence of the heteropolymeric block (at 102.8 ppm), is absent. Thus the complexity and the mole fraction (Table 3) of the *M*G-C1 diad initially increases with G-content of the alginate. A change is observed with *D. willana* ($F_M = 0.72$) and Sigma M.V. ($F_M = 0.69$) alginates that have similar compositions. These alginates possess very different *M*G-C1 diads, the Sigma alginates displaying a complex envelope consisting of at least four signals in contrast to the single resonance (G*M*M-C1, 103.9 ppm) of the *Durvillaea* alginates. This observation is repeated for all the so-called 'intermediate' alginates. When the composition tends towards a higher G composition

(*C. retroflexa* and *M. boryana*) and greater frequency of a homopolymeric fraction, in this case the G-block, the *M*G-C1 diad again tends towards a single resonance. The same trend in complexity is also shown by the corresponding *G*M-C1 diad. During our investigations we were also able to isolate heteropolymeric blocks from the so-called 'intermediate' alginates. In a related study in our laboratory (D. Kan, unpublished results), extraction of the alginate from the New Zealand brown alga *Xiphophora chondrophylla* also yielded an intermediate type alginate which contained both a high proportion of heteropolymeric sequences (GMG and MGM) and heteropolymeric blocks.

A very high G-content alginate ($F_G = 0.78$) was also isolated from the holdfast of *D. antarctica* in the present work. Furthermore, this alga also contains in its fronds a water-soluble alginate having a very high M-content ($F_M = 0.87$). Both of these alginates show from their ^{13}C NMR spectra a very small (< 0.03) mole fraction of the singlet triads (M*G*M-C1 and G*M*G-C1. This suggests a link between block structure and composition. We were not able to isolate heteropolymeric blocks from either of these alginates.

The complex *M*G-C1 and *G*M-C1 diad profiles of all the intermediate alginates cannot be explained by the currently accepted assignments of Grasdalen et al. [5]. It is possible that, apart from signals arising from nonreducing and penultimate M and G monomers, larger multads such as tetrads and pentads may contribute to the complexity. The inability to assign many of these unreported signals may influence the poor correlation between composition and block lengths for the so-called 'intermediate' alginates.

The ^{1}H and ^{13}C NMR analyses (Tables 2 and 3) of diad and triad compositions of the ten alginates enables a brief examination of the copolymeric structures in terms of two addition copolymerization models, namely the Bernoullian and the first order Markov chain models, to be made. We note that several workers [13,14] have indicated that a second-order Markov model gives the best description of the distribution of monomer residues in the alginate chain, but there are assumptions in relying on NMR data to support this model. For example, whereas only diad frequencies are required for testing the Bernoullian model, and triads for the first order Markov model, tetrad measurements are necessary for testing the second-order Markov or penultimate model [15]. As tetrads are not yet measurable in alginates by NMR analysis, all that can be stated is that diad and triad measurements can only be shown to fit the second-order Markov model, and there is always a possibility that the determination of larger monomer sequences will reveal differences between present predictions and actual multad measurements.

Furthermore, a possible mechanism for the biosynthesis of alginates is one in which the alginate block structure results at least in part from a multiple attack on a pre-formed mannuronate chain by a polymannuronic acid C-5 epimerase. This enzyme may perhaps be similar to that isolated from liquid cultures of *Azotobacter vinelandii* [16,17]. The attack mechanism involved suggests that a second-order Markov description may be an oversimplication of the structure of alginate from brown algae, because the diad frequencies reported for the product from epimerisation of a pre-formed mannuronan chain in the presence of Ca^{2+} ion were very similar to those to be expected for a linear copolymer having a statistically random distribution of the two monomers along the chain [18]. It is, therefore, interesting that the Ca^{2+} concentration used to achieve this

apparently random distribution using the C-5 epimerase was well below 0.01 M, which is the level considered to be typical of ocean waters [19].

The present study shows that the monomer distributions in the high-M and intermediate composition alginates agree closely with Bernoullian frequencies for M-diad frequencies calculated from the ^{1}H NMR data (Table 2). The closest agreement is obtained for those alginates in which F_M is greater than 0.72, which suggests that the distribution of M residues in these is random and they do not have the typical block copolymeric structure that is the generally accepted alginate structure. Furthermore, for alginates with F_M greater than 0.72, the ^{1}H NMR analyses also show that the distribution of the G residues fits a first-order Markov model. A previous attempt [5] at such a correlation using ^{13}C NMR analysis showed similar results. However, as the composition tends towards a higher G content (e.g., for the alginates from *C. retroflexa* and *M. boryana*), the two statistical models fail to describe the distribution of either M or G residues.

In terms of proposed biosynthetic pathways for the alginate molecule [16–18] involving the enzyme mannuronan C-5 epimerase, these results appear to be reasonable. Assuming the ten alginates are all synthesised by similar bioreactions in which a pre-existing polymannuronan chain is acted upon by the polymer modifying enzyme initially in a random manner, the high-M containing alginates would have been subjected to a low degree of modification. It follows that the initial mechanism should be reflected in the random sequencing of the two monomer residues in the high-M alginates. The higher G composition alginates would then, however, be polymannuronans that have been substantially modified and at this greater level of modification the mode of epimerisation would have undergone a transition to a multiple attack [18]. The distribution of monomers could then no longer be explained by Bernoullian probabilities.

A comparison of block lengths (N_M, N_G, and $N_{G>1}$) obtained from observed and calculated frequencies indicates a generally similar trend with the two models. As the proportion of G-residues in the alginates increase, deviations from the Bernoullian model are again more pronounced.

It is also observed that a coincidence of fit exists between conditional probabilities for the Bernoullian and first-order Markov models calculated for the *D. antarctica* and *D. potatorum* alginates (see Table 2). Deviations in conditional probabilities between the two models are, however, more pronounced for the alginates that have a lower F_M value and are greatest for the *C. retroflexa* and *M. boryana* alginates.

In contrast to the above conclusions, when the two statistical models are used to interpret the ^{13}C NMR data of Table 3, good agreements with the first-order Markov model for both the M and G triads are only obtained with the high M-composition alginates, and deviations from this model become pronounced for the intermediate alginates. However, the distribution of M residues does show close agreement with a first-order Markov distribution for the two high G content alginates (*M. boryana* and *C. retroflexa*), whereas the Bernoullian model fails to explain the structure of either of these alginates. It is possible in these cases that the greater detail displayed by ^{13}C NMR makes this method of analysis more suitable for fitting the statistical models, whereas the limited resolution obtained in the ^{1}H NMR spectra may lead to inaccuracies.

It is also noted that the excellent agreement with the first-order Markov model

occurs, especially for the M residue analyses, when contributions from the triads G*M*G-C1 and M*G*M-C1, representing the heteropolymeric block, are nonexistent or small.

Except for the *D. potatorum*, *D. willana*, and *D. antarctica* algae samples, the source meal in this study is derived from whole plants, and the heterogeneity in alginate prepared from these does not lend itself to an unequivocal application of the statistical models. Of the seven plants extracted, perhaps the *D. willana* and *M. pyrifera* algae show the greatest morphological differentiation. When collecting, care was taken to select plants that were undamaged and were apparently typical of the species. The *D. willana* alga typically consisted of both stipe and blade material of which only the latter was sampled. This was very unlike the *D. antarctica* samples which, when undamaged, seemed more like a mass of laminae. Only the laminar region of the *D. antarctica* was sampled because of its large mass and volume relative to the rest of the plant.

3. Conclusions

The seven Southern hemisphere brown algae all yield substantial amounts of alginate, the highest yields being obtained from the three *Durvilleae* species and from *M. boryana*. Overall, the ten alginates investigated display a wide range of compositions, ranging from the very high mannuronic acid containing alginate from *D. antarctica* to the moderately high guluronic acid containing alginate isolated from *M. boryana*. A feature of this work is the excellent agreement between the ^{1}H and ^{13}C NMR data.

The estimates obtained for M- and G-homopolymeric block lengths are substantially shorter than those previously reported using enzymatic methods of analysis [12] which indicated that M-block lengths are usually about 20 residues long irrespective of composition. Furthermore the estimates in this paper, as indicated by the generally good correlation between block length and composition, may in fact be an indication of the relative proportions of the two truly homopolymeric blocks.

It is possible that the results obtained from our attempts to fit the two copolymer statistical models may reflect significant differences between ^{1}H and ^{13}C NMR data for determining monomer sequences in the alginate polymers. An alginate structure could tend to fit either, but not both, models. Furthermore, irregularities in the structure of the heteropolymeric blocks, or some heterogeneity in the homopolymeric blocks, could lead to a misinterpretation of the frequencies of diads and triads in the NMR analysis of whole alginates. On the other hand, attempts to fit isolated homo- and heteropolymeric block structures to the statistical models may provide more useful information about the possible biosynthetic routes.

The results also show that for a limited range of compositions, sometimes called the 'intermediate' range, very different alginate structures exist. The 'intermediate' range alginates possess a much higher proportion of a more developed heteropolymeric block structure than alginates with higher or lower M residue content. For these alginates the correlation between composition and estimates of block length, and with agreements with the two addition copolymer models, are poor. It is therefore possible that the

mechanism for the formation of the heteropolymeric block may differ from that for the two homopolymeric blocks.

4. Experimental

Preparation of alginates.—All plants were harvested in early November at Brighton, approximately five miles south of Dunedin, New Zealand. Five plants each of the larger algae, *Durvillaea antarctica, Durvillaea willana*, and *Macrocystis pyrifera*, were collected. All plants were approximately 2 m in length. Ten specimens of each of the smaller algae, *Marginariella boryana, Cystophera retroflexa*, and *Hormosira banksii* were collected. After steeping in formalin solution for 16 h, all plants were cut into small pieces and left to dry under shelter. Except for the *Durvillaea* algae, whole plants were ground, and a size fraction between 1.0 and 0.42 mm sieved for extraction purposes.

Three methods of alginate extraction were used in this work. The first of these followed the procedure developed by Rosell and Srivastava [20]. The resulting alginate was redissolved in 0.05 M NaOH and dialysed against distilled water for 5 days at 4 °C. The pH of the solution was then adjusted to 6.5 and concentrated under reduced pressure at 40 °C and finally freeze-dried and weighed.

Alginate was also extracted by a method that was very similar to that of Craigie et al. [6]. However, the washing at pH 2 used by these workers was not used for the present work as it was found that this treatment with the *Durvillaea* algae dissolved an alginate fraction of M/G ratio = 6.8, and also a quantity of a laminaran like polysaccharide.

The third alginate preparation method involved CDTA extraction, similar to that described by Painter [1] and Wedlock and Fasihuddin [7]. Their method was adapted as follows: 1000 mL of 0.05 M CDTA at pH 6.5 was stirred with about 10 g of ground algae meal, weighed to 0.001 g, for 1 h. After centrifugation at 2000 rpm, the supernatant was collected, and the residue was again treated with two further additions of CDTA. The supernatant liquors were collected, and the alginate and the residue were again treated with two further additions of CDTA. The supernatant liquors were then combined, and the alginate was precipitated as the calcium salt by addition of 0.01 M $CaCl_2$. This step was introduced to separate alginate from other polymeric material released by the extraction, such as water-soluble glucan. The precipitate was filtered through cheese cloth and stirred with sodium EDTA (5 M, pH 6.5) to redissolve the alginate. After dialysis for five days, the pH of the solution was adjusted to 6.5, the solution was filtered through Whatman No.5 paper under vacuum, and the clear solution was concentrated. The alginate was finally isolated by freeze drying. The yields are given in Table 1.

NMR characterisation.—All NMR analyses were made on alginates which were extracted by the method of Craigie et al. [6]. Samples were prepared for NMR analysis by the partial acid hydrolysis method [4]. The hydrolysed samples were then made up in D_2O to approximately 15 mg mL^{-1} for ^1H NMR spectroscopy and to 50–60 mg mL^{-1} for ^{13}C NMR spectroscopy. All solutions were adjusted to pD = 7.0, and MeOH was used as the internal reference.

^{13}C NMR spectra were recorded on a Varian VXR-300 spectrometer operated at a frequency of 75 MHz. A 90° pulse was used with a total repetition time of 1.6 s. Between 20,000 and 40,000 transients were acquired at 75 °C along a spectral window of 20000 Hz. Solutions were kept at 75 °C for three h before acquisition to ensure solubility was high and to degas viscous samples. The assignments of Grasdalen et al. [5] were applied to major signals. Before 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. Signals were measured from peak areas derived by cutting and weighing. Peak overlap was estimated visually [5].

^{1}H NMR spectra were recorded at 300 MHz and 75 °C using an internal reference of DSS or MeOH. The 180°–τ-90° pulse sequence was 24.2 s and a recycle time of 4 s was used with a 2 s delay. Between 50 and 100 transients were acquired. Peak areas were measured after one increment in zero filling (16,000–32,000). Only the-low field (M-H1, G-H1, and G-H5) intensities were used. In all cases compositions are reported as frequencies in preference to M/G ratios.

Sufficient resolution was obtained at 75 MHz (^{13}C NMR) to obtain estimates of the eight triads. The contribution of the G*M*M-C1 triad to the *M*M-C1 signals was estimated using the M*M*G-C1 triad as the latter was more clearly delineated in the *M*G-C1 diad. F_{MMG} was obtained from the G*G*M-C1 triad in a similar fashion.

References

[1] T.J. Painter, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, 1983, pp 195–285.
[2] A.C. Cheshire and N.D. Hallam, *Phycologia*, 24 (1985) 147–153.
[3] Sigma Chemical Company, St. Louis, MO, USA.
[4] B. Larsen, T.J. Painter, A. Haug, and O. Smidsrød, *Acta Chem. Scand.*, 23 (1969) 355–370.
[5] H. Grasdalen, B. Larsen, and O. Smidsrød, *Carbohydr. Res.*, 89 (1981) 179–191.
[6] J.S. Craigie, E.R. Morris, D.A. Rees, and D. Thom, *Carbohydr. Polym.*, 4 (1984) 237–252.
[7] D.J. Wedlock and B.A. Fashuddin, *Food Hydrocolloids*, 4 (1990) 41–47.
[8] J.W.A. McKee, L. Kavalieris, D.J. Brasch, M.T. Brown, and L.D. Melton, *J. Applied Phycology*, 4 (1992) 357–369.
[9] H. Grasdalen, B. Larsen, and O. Smidsrød, *Carbohydr. Res.*, 68 (1979) 23–31.
[10] H. Grasdalen, *Carbohydr. Res.*, 118 (1983) 255–260.
[11] H. Grasdalen, B. Larsen, and O. Smidsrød, *Carbohydr. Res.*, 56 (1977) C11–C15.
[12] J. Boyd and J.R. Turvey, *Carbohydr. Res.*, 66 (1978) 187–194.
[13] G. Skjåk-Bræk, *Biochem. Soc. Trans.*, 20 (1992) 27–33.
[14] B.T. Stokke, O. Smidsrød, P. Bruheim, and G. Skjåk-Bræk, *Macromolecules*, 24 (1991) 4637–4645.
[15] J.L. Koenig, *Chemical Microstructure of Polymer Chains*, John Wiley and Sons, New York, 1980, pp 330–333.
[16] B. Larsen and A. Haug, *Carbohydr. Res.*, 17 (1971) 287–296.
[17] A. Haug and B. Larsen, *Carbohydr. Res.*, 17 (1971) 297–308.
[18] B. Larsen, G. Skjåk-Bræk, and T. Painter, *Carbohydr. Res.*, 146 (1986) 342–345.
[19] J.P. Riley and R. Chester, *Introduction to Marine Chemistry*, Academic Press, London and New York, 1977, pp 80–81.
[20] K.G. Rosell and L.M. Srivastava, *Can. J. Bot.*, 62 (1984) 2229–2236.