A METHOD FOR THE DETERMINATION OF URONIC ACID SEQUENCE IN ALGINATES

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ABSTRACT

The uronic acid sequence of several alginate samples has been determined by splitting the alginates into alternating and homopolymeric fractions by partial acid hydrolysis, and determining the relative proportions of mannuronic acid blocks and guluronic acid blocks in the homopolymeric fraction by p.m.r. spectroscopy. The alginates examined fall into two main classes, namely those containing a high percentage of guluronic acid blocks with intermediate amounts of alternating regions and small amounts of mannuronic acid blocks, and those containing a small proportion of guluronic acid blocks and approximately equal proportions of mannuronic acid blocks and alternating blocks. The p.m.r. assignments, which have been checked by reference to the spectra of the anomeric methyl mannopyranosiduronic and gulopyranosiduronic acids, suggest that, in the homopolymeric regions of the alginate chain, the D-mannuronic acid residues are β-linked in the *C1* conformation and the L-guluronic acid residues are α-linked in the *1C* conformation.

INTRODUCTION

Alginic acid is a polysaccharide found originally in brown seaweeds, which still function as its major source, although similar types of polysaccharide have since been isolated from bacteria[1,2]. The polysaccharide is a linear glycuronan consisting of (1→4)-linked residues of D-mannuronic acid and L-guluronic acid[3,4] arranged in a block fashion in the polymer chain, with blocks containing one type of residue being separated by segments in which the two residues alternate[5,6].

Alginates have widespread industrial use due to their ability to form gels with calcium ions. The gelling characteristics of an alginate are strongly influenced by its uronic acid composition, *i.e.* the ratio of mannuronic acid residues to guluronic acid residues (M/G ratio). Alginates with a low M/G ratio give strong, brittle gels having a marked tendency to synerese in excess calcium, whereas alginates with a high M/G ratio form elastic gels that are relatively tolerant to high levels of calcium[7]. Circular dichroism studies[8] have shown that the reason why alginates with differing M/G ratios have different gelling characteristics is that they contain different proportions of block structure, *i.e.* mannuronic acid blocks (M blocks), guluronic acid blocks (G blocks), and alternating blocks (MG blocks). These blocks differ in their ability

to form junction zones, in that G blocks aggregate readily and, with excess calcium, aggregate even further causing syneresis, whereas M blocks require high levels of calcium to aggregate. Alternating blocks have little tendency to aggregate. Thus, it is important to have a method for determining the block composition of alginates.

The standard method for determining alginate block-composition[5,6] involves heterogeneous partial acid hydrolysis and separation of the various fragments by fractional precipitation, followed by determination of their uronic acid composition by complete acid hydrolysis. Although this method gives reliable results which can be correlated with the physical properties of the alginates[9], it is very time-consuming and not suitable for routine characterization of large numbers of samples.

The carbazole reaction[10] gives very different colour intensities for mannuronic and guluronic acids, and a method for determining M/G ratios of alginates has been based on the carbazole method applied under two different reaction conditions[11,12]. We have found that this method, although working well on mixtures of mannuronic and guluronic acids, consistently overestimates guluronic acid[13] when applied to alginates, presumably because the carbazole reagent reacts differently with the acids in the polymeric and monomeric forms. Thus, methods which embody this type of approach for obtaining alginate block-composition must be regarded as suspect.

This paper describes a rapid method for the determination of alginate block-structure, based on partial acid hydrolysis, which separates the alginate into alternating and homopolymeric fractions, followed by determination of the proportions of M blocks and G blocks in the homopolymeric fraction by p.m.r. spectroscopy. Evidence is also presented for the configuration and preferred conformation in solution of the monomeric units in the homopolymeric segments of the alginate molecule.

EXPERIMENTAL

Materials. — Most of the alginates used were commercial samples. Those coded I–IX were supplied by Alginate Industries Limited, and X was from Alginates Australia Limited; these alginates are of seaweed origin. A bacterial alginate (XI), kindly given by Dr. C. J. Lawson of Tate and Lyle Ltd., was also examined.

Determination of the uronic acid composition of alginates. — The M/G ratio was determined by complete acid hydrolysis of the alginates, followed by separation of the resulting uronic acids on a column of DEAE-Sephadex A25 (acetate) with a linear gradient of water → 2.5M acetic acid as eluant. The results were corrected for the different rates of breakdown of guluronic and mannuronic acid[14].

Partial acid hydrolysis of alginates. — Sodium alginate (750 mg) was dissolved in water (35 ml), and hydrochloric acid (3.0M, 3.9 ml) was added to give an overall concentration of 0.3M. The mixture was heated under reflux on a boiling water-bath for 5 h, and the solution was removed from the precipitate by centrifugation and neutralised with sodium hydroxide. The precipitate was washed with 0.3M hydrochloric acid, suspended in water, and solubilised by addition of sodium hydroxide to neutrality. Sodium chloride was added to 0.1M, and the homopolymeric blocks were preci-

pitated by the addition of ethanol (2 vol.). The precipitate was isolated by centrifugation, washed with ethanol and ether, and dried.

Although 750 mg of alginate were used in the hydrolysis, only 40 mg were required for p.m.r. spectroscopy of the homopolymeric block fraction. Therefore, the whole reaction sequence could be performed with much smaller quantities of starting material.

Measurement of p.m.r. spectra. — The homopolymeric blocks from the partial acid hydrolysis (40 mg) were deuterated by evaporation (3 times) with D_2O (1.5 ml) under reduced pressure. The deuterated sample was taken up in D_2O (0.5 ml) and examined on a Varian HA-100 spectrometer, using *tert*-butyl alcohol as internal standard. To expose the low-field signals, by shifting the deuterium hydroxide peak upfield, and to permit a satisfactory measurement of their areas, the spectra were recorded at 80° at a sweep width of 250 Hz. Samples of model compounds were similarly prepared for p.m.r. spectroscopy, and spectra were recorded at room temperature and 70°.

Determination of alginate concentration. — The concentration of alginate in the soluble and insoluble fractions, after partial acid hydrolysis, was determined by the phenol–sulphuric acid reaction[15], and the extinctions were corrected for the differences in colour formation given by mannuronic and guluronic acids. The M/G ratios for the insoluble fraction were known and for the soluble fraction they were taken as 1.

Depolymerisation of alginates with ascorbic acid–hydrogen peroxide. — Sodium alginate (400 mg) was dissolved in a solution (25 ml) of 10mM hydrogen peroxide in 5mM ascorbic acid at 20°. After 1 h, ethanol (2 vol.) was added, and the precipitated polysaccharide was isolated by centrifugation, washed with ethanol and ether, and dried.

Preparation of the anomeric methyl glycosides of D-*mannuronic and* D-*guluronic acids.* — These model compounds were prepared, as their ammonium salts, from the corresponding neutral glycosides by catalytic oxidation[16].

RESULTS AND DISCUSSION

From recent X-ray diffraction studies[17] on samples of polymannuronic and polyguluronic acid prepared by degradation of alginic acid, it has been suggested that the D-mannuronic acid residues are β-linked in the *C1* conformation and the L-guluronic acid residues are α-linked in the *1C* conformation. Although the presence of β-linked D-mannuronic acid residues is in accordance with the findings of Hirst *et al.*[18], who isolated *O*-β-D-Man*p*-(1→4)-D-Man*p* after partial hydrolysis of carboxyl-reduced alginic acid, no independent evidence has been forwarded for the presence of α-linked L-guluronic acid residues.

The p.m.r. spectra of the anomeric methyl mannosid- and gulosid-uronic acids are shown in Fig. 1. Assignment of signals was facilitated, where possible, by spin-decoupling and acidification with deuterium chloride. In the case of methyl α-D-mannosiduronic acid (Fig. 1*b*), the small value of $J_{1,2}$ (~2 Hz) is indicative of the

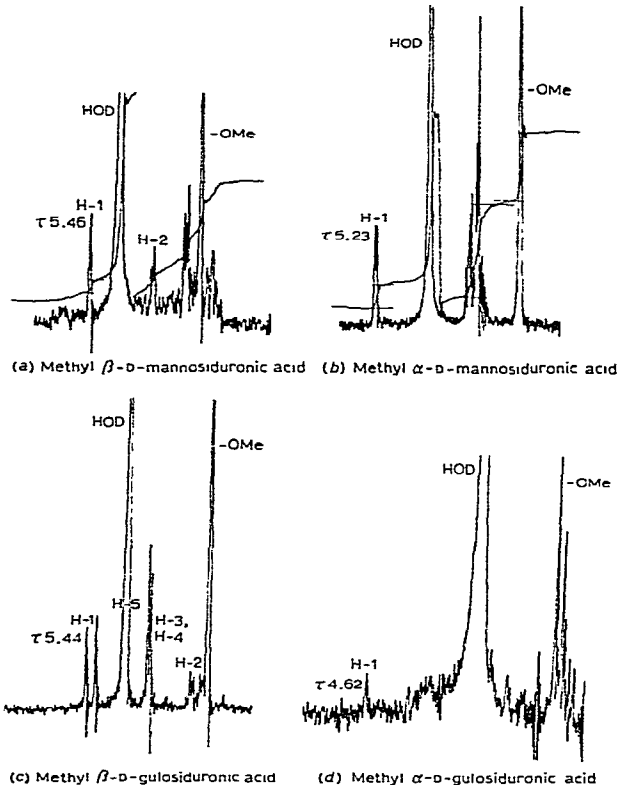

Fig. 1. The 100-MHz p.m.r. spectra of the anomeric mannosid- and gulosid-uronic acids, recorded at 70°.

compound existing in the *C1* conformation (Fig. 2*b*). In the alternative *1C*(D) conformation (Fig. 2*b*), a much larger coupling constant (~8 Hz) would be expected because of the *trans*-diaxial arrangement of H-1 and H-2. For methyl β-D-mannosiduronic acid (Fig. 1*a*), no prediction as to the preferred conformation is possible on the basis of the magnitude of $J_{1,2}$ since H-1 and H-2 have a gauche relationship in both the *C1* and *1C* conformations (Fig. 2*a*). However, a more-shielded or axially oriented H-1 in the β-D anomer is indicated by the fact that H-1 resonates at higher field (τ 5.46) than the equatorially oriented H-1 of the α-D anomer (τ 5.23). Thus, on the basis of the p.m.r. spectrum, the β-D anomer also appears to exist in the *C1* conformation.

The striking similarity between the spectrum of polymannuronic acid (Fig. 3*a*) and that of the β-mannosiduronic acid, notably the sharp resonance for the anomeric proton and the partly resolved signals for the non-anomeric protons, strongly suggests that, in the mannuronic acid block-regions of the polymer, these residues are β-linked in the *C1* conformation, in accordance with previous work[17,18].

In the spectrum of methyl β-D-gulosiduronic acid (Fig. 1*c*), the large value (8.3 Hz) of $J_{1,2}$ indicates that this compound exists in the *C1* conformation (Fig. 2*c*)

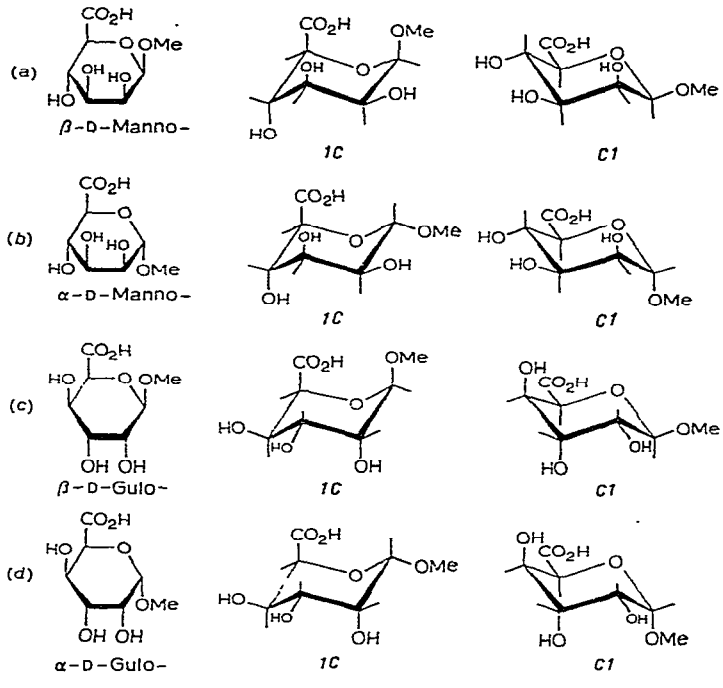

Fig. 2. Alternative preferred conformations of the anomeric mannosid- and gulosid-uronic acids.

with a *trans*-diaxial arrangement of H-1 and H-2. The spectrum of methyl α-D-gulosiduronic acid (Fig. 1*d*) is of poor quality because of the small amount of material available. However, the presence of a weakly coupled, low-field signal at τ 4.62, assigned to the anomeric proton, suggests that this proton is in an equatorial "deshielded" orientation arising from a *C1*(D) conformation of the monomer (Fig. 2*d*). It is pertinent to mention that the anomeric methyl D-idosiduronic acids, which would be expected to be thermodynamically less stable than the anomeric mannosid- or gulosid-uronic acids in the *C1* conformation, have been shown to exist in a conformation close to the *C1* chair[19].

In the spectrum of polyguluronic acid (Fig. 3*b*), as in the spectrum of the α-gulosiduronic acid monomer, the weakly coupled (4 Hz), low-field resonance (τ 4.96) of the anomeric proton indicates that this proton has an equatorial orientation, which most probably arises from L-guluronic acid residues α-linked in a conformation close to *1C*(L). Although such a low-field, anomeric-proton signal could arise from L-guluronic acid residues β-linked in the *C1*(L) conformation, this would imply a change in conformation from *1C*(L) in the monomer to *C1*(L) in the polymer, which seems unlikely. For example, the L-iduronic acid residues in heparin adopt a conformation close to the *1C*(L) form which is the preferred conformation of the methyl α-L-idosiduronic acid monomer[19–21]. The other low-field signal in the polyguluronic acid spectrum is assigned to H-5 of the guluronic acid residues by analogy with the

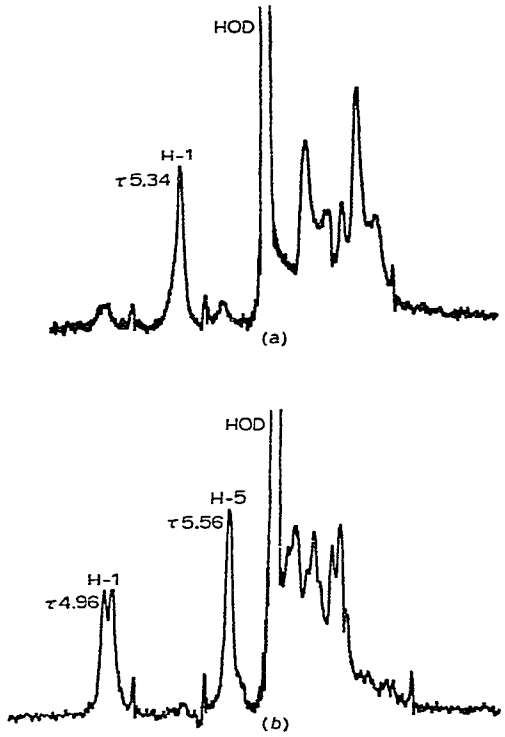

Fig. 3. The 100-MHz p.m.r. spectra of (*a*) polymannuronic acid and (*b*) polyguluronic acid recorded at 80°.

position of the H-5 signal in the spectra of methyl β-gulosiduronic acid (Fig. 1*c*) and other model glycosiduronic acids[19], and by the fact that the signal is sensitive to pH change.

As mannuronic and guluronic acid blocks can be differentiated by their p.m.r. spectra (Fig. 3), this technique can be used to determine the relative proportions of these two types of block (M block/G block ratio) in the homopolymeric fraction obtained from partial acid hydrolysis of alginic acid. Spectra of the insoluble, homopolymeric fractions obtained from partial acid hydrolysis of alginates V and VII are shown in Fig. 4. The low-field signals are well separated and the ratio of peak B (manuronic acid H-1) to peak A (guluronic acid H-1) or peak B to peak C (guluronic acid H-5) gives the M block/G block ratio. The areas of these peaks are conveniently obtained by using the integrator of the n.m.r. spectrometer.

A hydrolysis time of 5 h was chosen to give complete solubilisation of the alternating regions with minimum possibility for hydrolysis of the homopolymeric blocks. Several commercial alginate samples were hydrolysed and the M block/G block ratio determined by p.m.r. spectroscopy on the insoluble fractions left after hydrolysis. The results are summarised in Table I. The reproducibility of the determinations was checked by carrying out the complete procedure in triplicate on

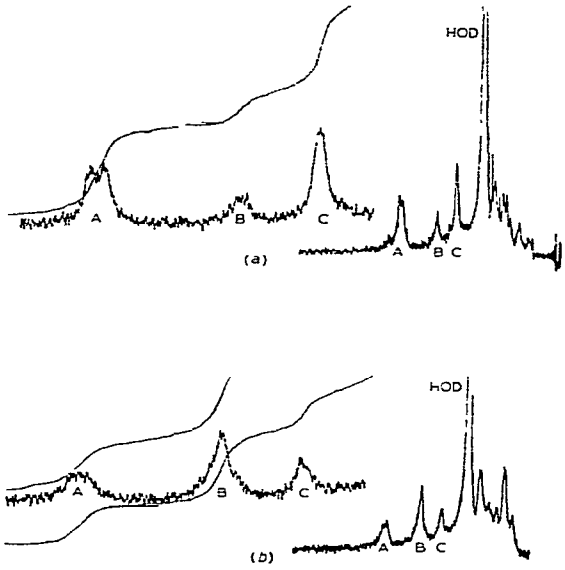

Fig. 4. The 100-MHz p.m.r. spectra of the mixtures of homopolymeric blocks obtained by partial hydrolysis of the alginates (*a*) V and (*b*) VII, recorded at 80°.

alginate IV, and the average values of the M block/G block ratio were found to be 0.34, 0.35, and 0.33, compared with the value of 0.32 found in the original determination (Table I). A similar spread of results was obtained when the possible contribution from spinning sidebands was investigated by varying the spinning rate with one particular sample.

TABLE I

M BLOCK/G BLOCK RATIOS OF ALGINATE SAMPLES

Alginate code	*Source*	*M/G ratio*	*M block/G block ratio*[d]		
			B/A	*B/C*	*Average*
I	*Laminaria hyperborea*	0.41[a]	0.20	0.21	0.21
II		0.38[a]	0.21	0.22	0.22
III		0.36[a]	0.21	0.23	0.22
IV		0.40[a]	0.33	0.30	0.32
V		0.40[a]	0.40	0.38	0.39
VI		0.62[a]	0.38	0.44	0.41
VII	*Ascophyllum nodosum*	1.29[a]	1.86	1.86	1.86
VIII		1.14[a]	1.50	1.83	1.67
IX		1.17[b]	1.46	1.75	1.61
X	*Macrocystis pyrifera*	1.38[b]	2.22	2.35	2.29
XI	*Azotobacter vinelandii*	1.50[c]			28.0

[a]Determined by complete acid hydrolysis. [b]Determined by circular dichroism[8]. [c]Value supplied with the sample. [d]Determined by p.m.r. spectroscopy.

The ratio of alternating blocks to homopolymeric blocks can be evaluated by determining the yields of the soluble and insoluble fractions after hydrolysis for 5 h. The yields of the two fractions were determined by the phenol–sulphuric acid reaction[15], and the extinctions were corrected for the differences in colour formation given by mannuronic and guluronic acids; the M/G ratios of the insoluble fractions were known from the p.m.r. spectra, and the M/G ratios of the soluble fractions were taken as 1. The results for several alginate samples are shown in Table II.

TABLE II

PROPORTIONS OF M, G, AND ALTERNATING BLOCKS IN ALGINATES

Alginate code	*Block type* (%)		
	Alternating	*M*	*G*
I	26.8	12.7	60.5
II	19.5	14.5	66.0
III	24.3	13.6	62.1
IV	22.7	18.7	58.6
V	22.8	21.7	55.5
VI	30.4	20.3	49.3
VII	41.0	38.4	20.7
VIII	48.4	32.3	19.4
IX	38.4	38.0	23.6
X	41.7	40.6	17.7
XI	81.7	17.8	0.5

The proportions of the three block types given in Table II can be used to calculate M/G ratios for the alginates, and M/G ratios calculated in this way are compared in Table III with M/G ratios found experimentally. The results show that there is good agreement between the calculated values and those found experimentally.

As expected, the alginates from the different weeds have very different block structure. Those isolated from *L. hyperborea* have a high percentage of G blocks with intermediate amounts of alternating blocks and a small amount of M blocks. The alginates from *A. nodosum* and *Macrocystis pyrifera* contain a small proportion of G blocks with roughly equal proportions of M blocks and alternating blocks.

The bacterial alginate examined has a rather unusual structure with a very high proportion of alternating regions. This alginate is completely soluble in acid solution and during the course of partial acid hydrolysis a fraction, subsequently shown to be M blocks, precipitated out of solution showing that this preparation contains M blocks and alternating regions in the same molecule. The high content of alternating regions must be responsible for the solubility in acid solution, which is in agreement with previous results from seaweed alginates[9].

Experiments were performed to ascertain whether p.m.r. spectroscopy could be used as a rapid method for determination of the M/G ratio. In principle, all this would involve would be to run the p.m.r. spectrum of an alginate and determine the

TABLE III

COMPARISON OF M/G RATIOS CALCULATED FROM BLOCK STRUCTURE WITH THOSE FOUND EXPERIMENTALLY

Alginate code	*Found*[a]			*Calculated*		
	M (%)	*G* (%)	*M/G*	*M* (%)	*G* (%)	*M/G*
I	29	71	0.41	26	74	0.35
II	28	72	0.38	24	76	0.32
III	27	73	0.36	26	74	0.35
IV	29	71	0.40	30	70	0.43
V	29	71	0.40	33	67	0.49
VI	38	62	0.62	36	64	0.56
VII	56	44	1.29	60	40	1.50
VIII	53	47	1.14	56	44	1.27
IX	54	46	1.17	57	43	1.32
X	58	42	1.38	61	39	1.56
XI	60	40	1.50	58	42	1.38

[a]Determined by the techniques listed in Table I.

ratio of the signals for the anomeric protons of the mannuronic and guluronic acid. Unfortunately, however, at the normal concentrations required for p.m.r. spectroscopy, alginate solutions are too viscous to give good spectra. Use of a computer to accumulate spectra, using a dilute solution, is not feasible since the residual HOD peak is so enlarged that it obscures large areas of the spectrum. The problem can be overcome by controlled depolymerisation of the alginates to reduce their viscosity. Two methods were used: (*a*) partial acid hydrolysis for 20 min (as opposed to 5 h in the block-structure determinations) and (*b*) ascorbic acid–hydrogen peroxide depolymerisation[22]. The spectra obtained from alginates degraded by these methods were poorly resolved in comparison to those obtained from the homopolymeric block fractions, and their interpretation was complicated by the presence of additional signals from the alternating blocks. This approach was therefore abandoned.

Alginate X and the bacterial alginate XI have similar M/G ratios (Table III), but vastly different block structures (Table II), and hence would be expected to exhibit different physical properties. Thus, the M/G ratio alone may be misleading and is less meaningful than the M block/G block/alternating block ratio obtained by the method described above. In this context, although enzyme conversion[23] of mannuronic acid residues into guluronic acid residues in alginates results in a decreased M/G ratio, this ratio does not indicate whether the modified alginate has an increased proportion of G blocks, resulting from conversion of contiguous mannuronic acid residues in the M-block regions, or an increased proportion of alternating blocks, resulting from conversion of alternate mannuronic acid residues in the M-block regions. This information could be conveniently obtained by block-structure determination which, in addition, could also be used to obtain structural information on novel alginates produced by bacteria.

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