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URONIC ACID SEQUENCE IN ALGINATE FROM DIFFERENT SOURCES

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ABSTRACT

Alginate may be considered as a block co-polymer of D-mannuronic and L-guluronic acids, and consists of three types of blocks: homopolymeric blocks of mannuronic acid (MM) and of guluronic acid (GG), and blocks with an alternating sequence (MG). The block composition of alginates has been characterized by a simple chemical method involving partial hydrolysis with acid, followed by fractional precipitation of the acid-resistant part of the alginate. Alginates from eleven different species of brown algae have been examined and, for five species, alginates from different tissues have been compared. The results indicate that young tissue is rich in MM blocks, and that the difference between the alginates from different species is mainly due to the alginates from the older parts of the plants. Extracellular alginates from two types of bacteria have been examined.

INTRODUCTION

Alginate is a glycuronan consisting of residues of D-mannuronic acid and L-guluronic acid, arranged in a block-wise fashion along the polymer chain. The block distribution of the two types of monomer was demonstrated in 1966 by partial, acid hydrolysis of the insoluble alginic acid[1]. A fraction of the alginate was rapidly solubilized (*e.g.*, 2 h, 100°, 0.3M HCl), and was later shown to consist of mainly alternating residues of D-mannuronic and L-guluronic acid[2,3]. The fraction which was more resistant towards hydrolysis could be separated[4] by precipitation into two fractions, one containing >80% of D-mannuronic acid, the other >80% of L-guluronic acid, both with d.p. >15. Using free-boundary electrophoresis in a medium containing 0.05M sodium chloride and 0.75mM calcium chloride, the development of compositional heterogeneity of the block polymer by the partial hydrolysis was demonstrated[4,5].

It has been clearly shown[6] that the physical properties of alginates depend not only upon the uronic acid composition but also upon the relative proportion of the three types of block. The solubility in acid is particularly dependent upon the proportion of MG blocks in the polymer[6], whereas the formation of gels in the presence of calcium ions mainly depends upon auto-cooperatively formed junctions between the GG blocks[7,8].

Recently, Penman and Sanderson[9] have introduced a method for determing the uronic acid sequences of alginates. After partial hydrolysis (5 h, 100°, 0.3M HCl), the uronic acid composition of the resistant fraction was determined by p.m.r. spectroscopy. Based upon the previous observation[1,4] that this material contains two fractions for which the uronic acid compositions are widely different, the authors regarded the acid-resistant fraction as a mixture of the two types of homopolymeric blocks, and used the p.m.r. results to calculate the proportions. Their method differs from the previous procedures only in the method of analysis of the acid-resistant fraction. Ten commercial alginate fractions and one bacterial alginate were investigated, and considerable variation was found in the proportion of the three types of block, depending upon the source of the alginate.

We now report results on the block structure of algal and bacterial alginates, obtained by a simple chemical method well adapted to routine work. A preliminary report of some of the results has been published[10].

EXPERIMENTAL

Materials and methods. — All algal samples were collected in the Trondheimsfjord area and brought fresh to the laboratory. After the necessary separation of different tissue types, the samples were dried at 30–35°. The dry material (1 g) was treated overnight with 0.2 ml of 40% formaldehyde, and then twice extracted with 50 ml of 0.2M hydrochloric acid. The acid extracts were discarded and the remaining algal material was extracted with 3% aqueous sodium carbonate for not less than 3 h. The filtered extract was poured into an equal volume of ethanol. The precipitate was re-dissolved in water, potassium chloride was added to 0.2M, and hydrochloric acid to pH 2.0–2.2. The precipitate was collected by filtration, suspended in water, and dissolved by the addition of dilute alkali. The alginate was finally isolated by re-precipitation with ethanol (equal volume), washed with ethanol and ether, and dried.

Partial hydrolysis was carried out by suspending one part of alginate in 100 parts of 0.3M hydrochloric acid. After 2 h at ~100°, the suspension was cooled and centrifuged. The amount of material in the solution was determined by the phenol–sulphuric acid method[11]. The residue was suspended in water, and dissolved by careful neutralization. The volume was adjusted to give an alginate concentration of 1%, sodium chloride was added to 0.1M, and the solution was mixed with 25mM hydrochloric acid to pH 2.8–3.0. The precipitate was collected by centrifugation, suspended in water, and solubilized by neutralization. The amounts of carbohydrate in the precipitate and the supernatant were determined by the phenol–sulphuric acid method.

The proportions of the two uronic acids in the alginates were determined as described previously[12].

The preparation of *Azotobacter vinelandii* alginate has been described previously[13], and the alginate from *Pseudomonas aeruginosa* was a gift from Dr. R. G. Dogget, Baylor University College of Medicine, Houston, Texas.

RESULTS

Partial hydrolysis and fractionation. The results are given as the percentage of the alginate found in the following fractions: *A* the fraction solubilized by hydrolysis in 0.3M HCl for 2 h at 100°; *B* that part of the residue from *A* which was soluble at pH 2.85; and *C* that part of the residue from *A* which was precipitated at pH 2.85. The three fractions consist mainly of blocks with an alternating sequence of mannuronic and guluronic acid residues (MG blocks), and of homopolymeric blocks of mannuronic acid (MM blocks) and guluronic acid residues (GG blocks), respectively. It should be emphasized that the amounts of the three fractions do not necessarily correspond to the amounts of the block types in the intact polymer. However, for comparative purposes, the results may be taken as an estimate of the relative amounts of the three types of block.

When comparing alginates from different algae, it is important that the samples are not contaminated with easily hydrolysable, fucose-containing, sulphated poly-

TABLE I

BLOCK DISTRIBUTION OF ALGINATES FROM DIFFERENT SPECIES OF BROWN ALGAE

Order	*Species (day/month of collection)*	*M/G ratio*		*Partial hydrolysis*[a]		
		Found	*Calc.*	*Soluble (%)*	*Insoluble (%)*	
					Soluble at pH 2.9	*Insoluble at pH 2.9*
Ectocarpales	*Pylaiella*	0.75	0.60	40	18	42
Chordariales	*Spermatochnus paradoxus* (15/8)	1.3	0.98	35	32	33
	Cordaria flagelliformis (15/8)	0.9	0.63	21	28	51
Dictyosiphonales	*Dictyosiphon foeniculaceus* (9/6)	0.85	0.60	25	25	50
	Scytosiphon lomentaria (19/6)	1.15	0.90	25	35	40
Desmarestiales	*Desmarestia aculeata* (9/6)	0.85	0.58	27	23	50
Fucales	*Pelvetia canaliculata* (9/4)	1.5	1.28	38	37	25
	Fucus serratus (26/6)	1.3	1.06	35	34	31
	Ascophyllum nodosum (3/5)	1.85	1.56	52	35	13
Laminariales	*Laminaria digitata* (15/1)	1.45	1.50	34	43	23
	Laminaria hyperborea, fronds (16/2)	1.35	1.28	26	43	31
	Laminaria hyperborea, stipes (16/2)	0.65	0.38	25	15	60

[a]For 2 h with 0.3M HCl at 100°.

saccharides which occur in varying abundance in brown algae, and which would lead to an overestimation of the amount of MG blocks. All samples were, therefore, dissolved in water and precipitated with acid in order to remove sulphated polysaccharides.

Table I gives the results of block determination of alginates from 11 different species of brown algae collected in the Trondheimsfjord area. The amounts of the three types of block varied considerably. There appeared to be approximately equal amounts of the three types with a trend towards a preponderance of GG blocks. There are marked exceptions such as *Ascophyllum nodosum* alginate, which is particularly

TABLE II

BLOCK DISTRIBUTION OF ALGINATES FROM DIFFERENT TISSUES

Species (day/month of collection)	*Tissue*	*M/G ratio*		*Partial hydrolysis*[a]		
		Found	*Calc.*	*Soluble* (%)	*Insoluble* (%)	
					Soluble at pH 2.9	*Insoluble at pH 2.9*
Ascophyllum nodosum						
Vegetative tissue	1 (see Fig. 1)	2.6	2.2	27	56	16
	2	2.7	2.1	34	51	15
	3	2.4	2.1	39	48	13
	4	1.6	1.2	60	24	16
	5	0.9	0.8	52	17	31
	4 cortex	1.0	1.0	60	20	20
	4 medulla	1.4	1.2	68	20	12
Reproductive tissue	Intercellular substance	11.0	7.7	9	84	6
	Cortex	1.8	1.8	44	42	14
Fucus vesiculosus (25/5)	Young tissue	2.2	2.4	23	59	18
	Old tissue	0.6	0.7	40	20	40
Pelvetia canaliculata (21/6)	Young tissue	2.7	1.8	27	50	23
	Old tissue	1.2	1.0	41	28	31
Laminaria digitata (29/1)	New fronds	2.3	2.1	31	52	17
	Old fronds	1.35	1.2	29	40	31
	Stipes	1.15	1.0	39	31	29
Laminaria hyperborea (16/2)	New fronds	1.9	1.8	22	53	25
	Old fronds	1.25	1.0	33	34	33
	Stipes	0.6	0.4	25	15	60
	Stipes, periphery, and outer cortex	0.6	0.39	26	15	59
	Stipes, inner cortex, and medulla	0.6	0.34	21	15	66

[a]For 2 h with 0.3M HCl at 100°.

rich in MG blocks, and the *Pylaiella* alginate, which contains mainly MG and GG blocks and only small amounts of MM blocks.

The results for *Laminaria hyperborea* alginates demonstrated that very marked differences may occur between tissues from the same organism. This was further investigated for three species of the order Fucales and two of the order Laminariales, and the results are shown in Table II. The differences in structure of these alginate samples were much more pronounced than for alginates isolated from whole plants. A pronounced feature was the high content of MM blocks in young tissues, both in the Fucales and the Laminariales. The changes taking place when the tissues grow older depends upon the species. In *Ascophyllum nodosum* alginate, the main change appeared to be an increase in the amounts of MG blocks. In the other species investigated, the proportion of both GG and MG blocks increased. In the alginate from *L. hyperborea* stipes, the increase was mainly in the GG blocks, which were the main component of this type of alginate. The results indicate that the differences between the block composition of alginates from whole plants[6] is mainly due to the differences between alginates present in the older tissues; alginates from young tissues are similar in composition.

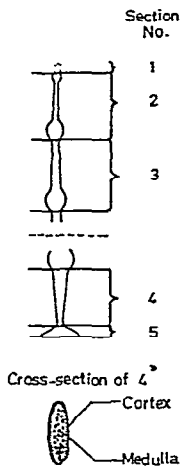

Fig. 1. Sections of *Ascophyllum nodosum* thallus used for preparing the alginate samples of Table II. Section 1, growing tip; section 5, holdfast.

The intercellular substance of the reproductive bodies of *Ascophyllum nodosum* has a block composition that is the most extreme of all the algal alginates investigated in this work, being mainly composed of MM blocks. We have previously shown[10] that the intercellular substance of the reproductive tissues of the Fucales has a composition approaching that of mannuronan.

The block composition of bacterial alginates for two samples produced by *Azotobacter vinelandii*[13] and one sample produced by *Pseudomonas aeruginosa*[14] are

given in Table III. Very marked differences between the block structures of the three samples are indicated, and particularly remarkable is the complete solubility of the *Pseudomonas* alginate, indicating a paucity of homopolymeric blocks in this sample.

TABLE III

BLOCK COMPOSITION OF EXTRACELLULAR ALGINATES PRODUCED BY BACTERIA

	M/G	*Partial hydrolysis*[a]		
		Soluble (%)	*Insoluble* (%)	
			Soluble at pH 2.9	*Insoluble at pH 2.9*
Azotobacter vinelandii	2.0	32	42	26
Azotobacter vinelandii	0.5	23	11	66
Pseudomonas aeruginosa	2.0	100		

[a]For 2 h with 0.3M HCl at 100°.

Electrophoretic behaviour. In interpreting the results described above in terms of block structure, we have assumed that partial hydrolysis and the subsequent fractionation lead to the same results as previously found for alginates from *Laminaria digitata, L. hyperborea* stipes, and *Ascophyllum nodosum*[4,6]. For these alginates, the two fractions obtained from the resistant material after the partial hydrolysis have been identified with peaks observed in free-boundary electrophoresis; one peak corresponds to the mannuronic acid-rich fraction and two, slower moving peaks to the guluronic acid-rich fraction. The acid-resistant fractions obtained by partial hydrolysis of alginates from some of the other species investigated in this work were examined by free-boundary electrophoresis, and the patterns are shown in Fig. 2. The usual

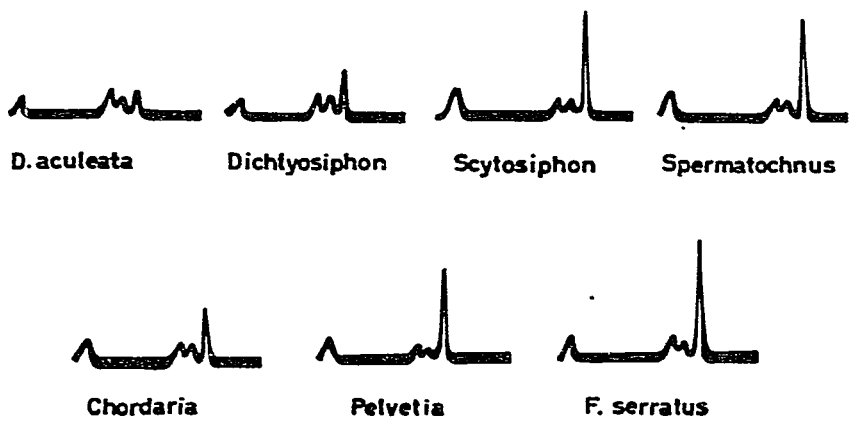

Fig. 2. Free-boundary electrophoresis of the acid-resistant fraction of alginate from a number of species of brown algae.

pattern of three peaks was found in each case, in proportions varying in accordance with the results in Table I.

The corresponding electrophoretic patterns of the *Azotobacter* alginate[13] were of the same general type. For the alginate from *Pseudomonas aeruginosa*, no insoluble fraction was observed. Fig. 3 gives the electrophoretic pattern before and after hydrolysis for 20 min at 100°. Before hydrolysis, the polysaccharide appeared to be homogeneous; after hydrolysis, which decreased the number-average d.p. from >100 to 19, only a trace of a slower moving peak was observed. This pattern differs significantly from that obtained[5] by homogeneous hydrolysis of a *Laminaria digitata* alginate to approximately the same d.p., and the results suggest that this bacterial alginate has a monomer sequence different from the familiar block structure of algal and *Azotobacter* alginates.

Fig. 3. Free-boundary electrophoresis of alginate from *Pseudomonas aeruginosa* before and after partial hydrolysis with acid.

DISCUSSION

So far, no method has been developed for determining the proportion of the three types of block in intact alginate molecules. Partial, heterogeneous hydrolysis is always used as the first step in the analysis of the block structure. Several sources of error should be recognized. (1) Incomplete solubilization of the parts of the alginate having an alternating sequence. (2) Solubilization of the homopolymeric blocks during the partial hydrolysis. This will particularly affect the MM blocks since they are more susceptible to hydrolysis than the GG blocks[4]. The M/G-ratio of the solubilized material has, accordingly, always been found[2–4] to be above unity, the range being 1.2–1.4. In the method used in the present work, an additional source of error must be taken into account, namely, incomplete fractionation of the acid-resistant part of the sample. It has previously been found that the uronic acid composition of the fractions deviates considerably from that of homopolymeric blocks[4,6]. This deviation may be due to incomplete fractionation, but may also be caused by incomplete removal of the alternating sequences by the partial hydrolysis.

If the three fractions in Table I–III correspond exactly to the three types of block, then the uronic acid composition of the alginate may be calculated and compared with the uronic acid composition obtained by direct determination. Considerable disagreement is seen, as might be expected. The deviation is such that, in 30 out of 33 fractionations, the calculated M/G-ratio is lower than that observed. This is probably mainly due to the underestimation of mannuronic acid caused by the

asumption that the solubilized material has an M/G-ratio of unity. The results reported by Penman and Sanderson[9] for block composition are in good agreement with the uronic acid composition of the alginate. In view of the discussion above, this is somewhat surprising and apparently confirms their assumption that the solubilized material has an M/G-ratio of one. Apart from this assumption, the comparison of the observed and calculated M/G-ratios is essentially only a comparison of two determinations of uronic acid composition, and the agreement cannot be interpreted as confirming the figures given for the amounts of the three types of block. The essential assumption made by these authors[9] is that all guluronic acid residues in the acid-resistant fraction occur in GG blocks and all mannuronic acid residues in MM blocks. Even if this assumption is qualitatively correct, a quantitative estimation of block distribution should be regarded with some caution. In our opinion, at the present stage, it is preferable to characterize, for comparative purposes, the block composition of alginates by the proportion of fractions obtained by a well-defined fractionation procedure.

The results presented here demonstrate a marked difference between the structures of alginates isolated from young and old tissues from the same plant, and also between alginates isolated from old tissues of different species. The results emphasise the importance, in comparative studies, of using polysaccharides isolated from well-defined raw materials instead of commercial samples. The alginate synthesized during the first formation of the tissue is different from that present during later development; the proportion of guluronic acid increases as the tissue grows older. Such transformations of alginates, on the polymer level, have been demonstrated in the alginates synthesized by *Azotobacter vinelandii*[15]. From the culture medium of this organism, a mannuronan–C-5-epimerase has been described, indicating that the biosynthetic route of alginate involves the formation of mannuronan which is transformed by the epimerase into a heteropolysaccharide containing both types of uronic acids. Penman and Sanderson[9] emphasized that, although enzymic conversion of mannuronic acid into guluronic acid has been shown, this alone does not indicate whether the enzyme-modified alginate has an increased proportion of GG blocks or an increased proportion of MG blocks. The formation of both types of block by the action of the C-5-epimerase on mannuronan isolated from receptacles of *Ascophyllum nodosum* has been shown[15], and results have also been presented which indicate that both types are end products of the epimerization reaction, *i.e.*, that the mannuronan epimerase is not able to transform alternating sequences into homopolymers of guluronic acid. What determines the relative proportions of the two types of block that contain guluronic acid is not yet known.

The biosynthetic route of alginate in brown algae is not known. Studies of the incorporation of ^{14}C into the different block types of alginate[16] from *L. digitata* fronds by photoassimilation for 3 h in the presence of $H^{14}CO_3^-$ showed that 70% of the activity was present in the fraction rich in MM blocks. This fraction corresponded to 55% of the weight of the alginate. After 3 days in normal sea-water in the dark, the activity of the alginate increased only slightly and all the increase was in the MG and

GG fractions. The results are thus in agreement with the assumption of a transformation of mannuronic acid sequences into both MG and GG blocks. The accuracy of the results, however, does not allow any safe conclusions to be drawn about the biosynthetic events leading to the finished algal alginates, and further work is required to clarify this point.

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