BIOSYNTHESIS OF ALGINATE

PART I. COMPOSITION AND STRUCTURE OF ALGINATE PRODUCED BY *Azotobacter vinelandii* (LIPMAN)

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ABSTRACT

The main, extracellular polysaccharide elaborated by three strains of *Azotobacter vinelandii* was a partly acetylated polyuronide, consisting of D-mannuronic and L-guluronic acid. The two monomers were distributed along the polymer chain in the typical, block-wise fashion previously demonstrated as characteristic for alginate from brown algae. The amount of polyuronide produced on a D-glucose–mineral salt medium was greatly increased by the addition of acetate.

The ratio of the uronic acids in the polyuronide produced was strongly dependent upon the calcium concentration in the growth medium; low levels of calcium favoured the production of polyuronides rich in D-mannuronic acid, whereas the polysaccharides richest in guluronic acid seemed to be obtained at intermediate concentrations of calcium. Addition of calcium ions during the growth period demonstrated a change in the ratio of uronic acids in the direction of more L-guluronic acid. The same effect was also observed for cell-free supernatants, thus indicating the presence of an enzyme capable of epimerizing D-mannuronic to L-guluronic acid in the polymer chain.

INTRODUCTION

Alginate was a well-known constituent of brown algae[1] when Linker and Jones[2,3] first reported that certain strains of *Pseudomonas aeruginosa* produced a polysaccharide having a composition similar to that of a partly acetylated alginate. A similar finding was reported by Carlson and Matthews[4]. Gorin and Spencer[5] found that *Azotobacter vinelandii* also produced an extracellular polysaccharide having the same composition and structure as a partly acetylated alginate.

Alginate from brown algae consists of two monomers, D-mannuronic and L-guluronic acid, which vary in proportion from *ca.* 25 to 75% of D-mannuronic acid[1,6]. In one type of tissue, the intercellular substance of the receptacles of *Ascophyllum nodosum* and some *Fucus* species[6], the composition of the alginate approaches that of polymannuronic acid. The two types of monomers have been found to be distributed along the polymer chain in a blockwise fashion; sequences of contiguous units of mannuronic acid being separated from similar sequences of contiguous units

of guluronic acid by parts of the molecule having a predominantly alternating structure[7,8].

The alginates produced by bacteria were, in all cases, reported to be rich in mannuronic acid. Carlson and Matthews obtained, from one *Pseudomonas* preparation, a polysaccharide in which no guluronic acid could be detected; in a later experiment with the same *Pseudomonas* culture, however, both monomers were detected, and the authors concluded that the ability to produce guluronic acid depended upon unknown factors. No information about the sequence of the two monomers was reported.

The present work is an attempt to establish whether a correlation exists between the ratio of the uronic acids in the alginate produced by *Azotobacter vinelandii* and the composition of the medium, and to determine whether the *Azotobacter* alginate has a block structure of the type found for alginates from brown algae.

MATERIALS AND METHODS

Three strains of *Azotobacter vinelandii* were used in this investigation: Strain E, isolated by Kjell Eimhjellen, Institute of Technical Biochemistry, N.T.H., Trondheim, and strains W and M, both of which were gifts from Dr. H. L. Jensen, Statens Planteavlslaboratorium, Lyngby, Denmark.

Two basal media, based on the work of Norris and Jensen[9], have been used: Medium *A*, D-glucose (20 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (200 mg), $FeSO_4 \cdot 7H_2O$ (50 mg), $NaMoO_4 \cdot 2H_2O$ (5 mg), diluted to 1 litre; and Medium *B*, as medium *A* + NH_4OAc (2.3 g). The amount of calcium chloride added to the medium was varied as given in the text. The bacteria were grown in liquid cultures at 30° with vigorous shaking.

Total carbohydrate was determined by the phenol–sulphuric acid reaction[10]. In cases where the composition of the polysaccharide was unknown, the extinction of mannuronic acid[1] was used as a reference; when the composition was known, the amounts were calculated by using corrected values for the extinction. Total protein was determined by the Folin–Ciocalteu method[11], using bovine serum albumin as standard.

The qualitative compositions of the polysaccharides were investigated by paper chromatography of hydrolysates (M H_2SO_4, 100°, 5 h) with pyridine–ethyl acetate–acetic acid–water (5:5:1:3). For chromatography of lactones of uronic acids, pyridine–ethyl acetate–water (11:40:6) was used. Qualitative paper electrophoresis of uronic acids[12] and quantitative determination of the uronic acids[13] was carried out as described previously.

The equivalent weights of the polysaccharides were determined by titration with cetylpyridinium chloride[14], and the amount of acetyl groups by the method of Kunz[15], with the modification that the acetylated polysaccharide was dissolved in water before the addition of acetone.

Free-boundary electrophoresis was carried out in a Perkin–Elmer Model 238

instrument equipped with a standard, analytical cell. In all experiments, the current (10 mamps) was applied for 30 min.

RESULTS

Preliminary investigation of the polysaccharides produced. — A culture of *Azotobacter vinelandii*, Strain E, in medium *A* (1 litre) containing 0.34mM calcium chloride was harvested after 7 days. Cells were removed by centrifugation, and the supernatant was dialysed extensively against water. The amount of non-dialysable carbohydrate (phenol–sulphuric acid method) was found to be 750 mg, and the amount of protein (Folin–Ciocalteu) 107 mg. The cells were extracted with 1% aqueous sodium carbonate. The extract was dialysed and gave 54 mg of non-dialysable carbohydrate and 205 mg of protein (Fraction 1). The dialysed supernatant was mixed with 10% of its volume of M hydrochloric acid, and the precipitate which was formed was removed by centrifugation. The precipitate (Fraction 2) contained 544 mg of carbohydrate and 55 mg of protein, while the amounts in solution were 115 and 44 mg, respectively (Fraction 3).

The three fractions were subjected to free-boundary electrophoresis in phosphate buffer (pH 7), and the ascending boundaries are shown in Fig. 1. Fraction 2 revealed a single component having a mobility of 1.85×10^{-4} cm^2.sec^{-1}.volt^{-1}, whereas fraction 3 contained two components having mobilities of 1.7×10^{-4} and 0.2×10^{-4}. Fraction 1 contained a slow-moving component of mobility 0.3×10^{-4} and traces of a faster-moving component.

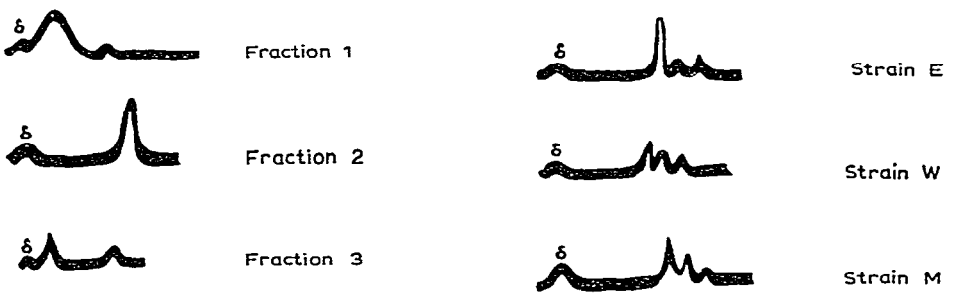

Fig. 1. Ascending, electrophoretic patterns of polysaccharide fractions from *A. vinelandii*, strain E.

Fig. 2. Ascending, electrophoretic patterns of the insoluble fraction after hydrolysis (0.3M HCl, 2 h, 100°) of polysaccharides from three strains of *A. vinelandii*.

All three fractions were isolated by precipitation with ethanol. A sample of each was hydrolyzed, and the hydrolyzate was subjected to paper chromatography. Fraction 2 gave rise to two spots, both having mobilities corresponding to uronic acids, and Fraction 3 gave an additional spot, having the same R_F value as glucose, and two faster-moving spots. The same three spots were given by Fraction 1, which did not give spots corresponding to uronic acids. The spots having the R_F value of

glucose disappeared after treatment of the hydrolysates with D-glucose oxidase, both in Fraction 1 and 3. The identity of the two faster-moving compounds was not investigated further.

Both the electrophoretic pattern and the composition of the hydrolysate indicated that Fraction 2 was a polyuronide. The content of uronic acids for the fraction was investigated by chromatography of the hydrolysate on an anion-exchange resin, with acetic acid, in increasing concentrations, as eluant[13]. Two fractions were obtained which were eluted at concentrations of acetic acid known to elute guluronic and mannuronic acid, respectively. Paper electrophoresis and paper chromatography of acids and lactones did not distinguish between the two acid components from Fraction 2 and L-guluronic and D-mannuronic acid from alginate from marine algae. The ratio between the two uronic acids (M/G ratio), corrected in the usual way for degradation during the hydrolysis[13], was 0.53. The equivalent weight of Fraction 2 was determined by titration with cetylpyridinium chloride and found to be 240. The degree of acetylation was 0.2 acetyl group per residue of uronic acid, the amount of protein 1.2%, and the moisture content 10%.

The results clearly indicate that the main, extracellular polysaccharide produced by *Azotobacter vinelandii* is a partly acetylated alginate, containing guluronic and mannuronic acid residues. At the same time, smaller amounts of other polysaccharides are also present. The composition of these polysaccharides has not been further investigated. Similar results were obtained with strain M and Strain W.

Monomer sequence in the polyuronides. — The block structure of alginates may be demonstrated by partial, heterogeneous hydrolysis in 0.3M hydrochloric acid. The part of the alginate molecule containing a predominantly alternating structure is, to a large extent, brought into solution by hydrolysis for 2 h at 100°, whereas the homopolymeric blocks remain in the insoluble phase[7]. The two types of homopolymeric blocks may be separated by fractionation at pH 2.85, and are also revealed as separate peaks by free-boundary electrophoresis[8]; the fragments rich in mannuronic acid give rise to one peak, and those rich in guluronic acid to two slower-moving peaks.

The acid-insoluble fractions of the extracellular polysaccharides were prepared, as described above, from cultures of strains E, W, and M (Medium *A*, 0.34mM $CaCl_2$) and hydrolysed for 2 h in 0.3M hydrochloric acid at 100°. The insoluble fractions were investigated by free-boundary electrophoresis (Fig. 2) and fractionated at pH 2.85. The results are given in Table I, together with the ratio of uronic acids for the original polysaccharide, and indicate a block structure of the same type as observed for alginates from brown algae. In spite of some differences between the three polysaccharides, the main features are the same, and the results are closely similar to those obtained for algal alginates having the same content of uronic acids[16].

Yield and composition of polysaccharide. — In all experiments referred to above, medium *A*, containing no nitrogen source, was used. Some experiments were carried out to determine the effect of an addition of ammonium salts on the production of extracellular polysaccharide. Medium *A* (150 ml, containing 0.34mM calcium chloride) was used as basal medium, and 0.03M ammonium salts were added. The yield of

TABLE I

URONIC ACID COMPOSITION AND FRACTIONATION RESULTS AFTER HETEROGENEOUS HYDROLYSIS[a] OF THE MAIN POLYSACCHARIDE FROM *A. vinelandii*, STRAINS E, W, AND M

Strain	*M/G*	*Soluble*		*Insoluble*			
		%	P_n	*Sol. pH 2.85*		*Insol. pH 2.85*	
				%	P_n	%	P_n
E	0.56	24.5	4.2	2.3	17	74.7	35
W	0.53	27.0	4.5	7.8	26	65.2	35
M_2	0.58	23.1	4.1	11.0	20	67.4	35

[a]0.3M Hydrochloric acid, 2 h, 100°.

polysaccharide, after a growth period of 100 h, was determined, and in some cases the dry weight of the cells was measured. In some of the experiments, the pH of the medium was adjusted every 24 h. The results (Table II) show that the production of extracellular polysaccharide is greatly increased by the addition of ammonium acetate, but that other ammonium salts do not lead to a similar increase, even when the pH is adjusted. On the other hand, sodium acetate gives a good yield of polysaccharide, and the results therefore indicate that the addition of acetate leads to an increased production of polysaccharide. Further studies of the influence of the composition of the medium on polysaccharide production have not been carried out, because of the difficulty in controlling conditions in batch experiments.

TABLE II

PRODUCTION OF EXTRACELLULAR POLYSACCHARIDE

Medium[a]	*pH adjusted to*	*Extracellular polysaccharide*[b] *(mg)*	*Cells, dry weight (mg)*
No addition		98	233
NH_4OAc		290[c]	620
NH_4OAc	6.5	298	590
NH_4OAc	6.0	175	500
NH_4Cl	6.0	17	
NH_4Cl	6.5	57	350
NH_4Cl	7.0	43	
$(NH_4)_3PO_4$	6.5	54	370
NaOAc		256	460

[a]Medium *A*, containing 0.34mM $CaCl_2$, and ammonium salts or sodium acetate (30mM). [b] The results refer to 150 ml of liquid culture after 100 h. [c]Average of 10 experiments, range 250–360 mg.

Fig. 3 shows the growth of the micro-organism (expressed as turbidity of the culture), production of polysaccharide, and amount of glucose present in the medium as a function of the incubation time. Medium *A*, containing 50μM Ca^{2+}, was used.

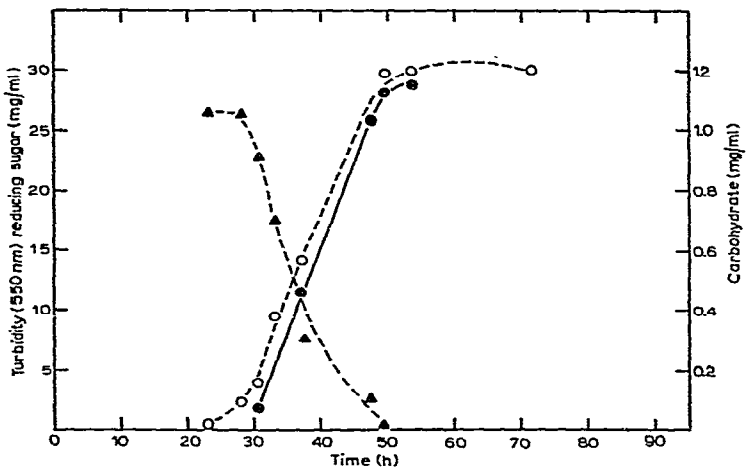

Fig. 3. Growth curve for *A. vinelandii* strain E on medium *B* containing 50μM Ca; ▲–▲, reducing sugar (glucose); O–O, turbidity; ●—●, total, non-dialysable carbohydrate in medium.

It was noticed during the growth experiments that the viscosity of the culture varied considerably from one experiment to another, and that it usually passed through a maximum after 60–70 hours of growth. The viscosity maximum varied between 1.5 and 50 cP, and tests for degradation products (caused by the β-alkoxycarbonyl elimination reaction of bacterial alginases[17]) by the thiobarbituric acid reaction were positive[18]. Thus, the results indicated a certain alginase activity in the *Azotobacter vinelandii* cultures.

The effect of varying the calcium concentration upon the ratio of the uronic acids in the extracellular polysaccharides has been studied in some detail. At high concentrations of calcium, some of the alginate was precipitated by the calcium ions. The precipitate, after centrifugation of the culture, was therefore extracted with a solution of ethylenediaminetetraacetic acid (EDTA) (0.1M, pH 7), and the amount of polysaccharide in the combined supernatant and the EDTA extract was measured. The ratio of the uronic acids and the degree of acetylation were determined and are given in Table III. It is clearly indicated that the amount of calcium ions in the medium influences the ratio of the uronic acids in the extracellular polysaccharide; low concentrations of calcium lead to polysaccharides rich in mannuronic acid. The products richest in guluronic acid seem to be obtained at intermediate concentrations of calcium.

The influence of the incubation time on the yield and composition of the polysaccharide produced was studied by periodic removal of samples from the culture for analysis. The results which are given in Table IV demonstrate that the extracellular polysaccharide which is produced in the last part of the period of growth has a composition approaching polymannuronic acid, even with a calcium concentration in the medium of 0.35mM Ca^{2+}. Due to the affinity of alginate for calcium ions, the calcium ion activity in the solution decreases as alginate is produced. The observed change in

TABLE III

YIELD OF EXTRACELLULAR POLYSACCHARIDE IN MEDIA CONTAINING VARIOUS AMOUNTS OF CALCIUM IONS

Medium	Ca^{2+} (mM)	*Extracellular polysaccharide*[a] (*mg*)	*M/G*	*Degree of acetylation*
A	0.03	36.6	4.7	
	0.1	55.1	2.5	
	0.34	92.5	0.5	
	1.0	93.2	1.0	
	3.4	62.5	—	
B	0.03	171	4.6	0.35
	0.1	228	4.6	0.20
	0.34	360	0.35	0.24
	1.0	275	1.6	0.23
	3.4	312	1.5	0.20
B	0.1	210	4.1	0.19
	0.34	273	0.9	0.09
	3.4	302	1.1	0.03

[a]Yield from 150 ml of medium.

the ratio of uronic acids during the period of growth may, therefore, be an effect of the decrease in the amount of free calcium ions, in accordance with the results in Table III. An experiment was therefore performed in which the calcium concentration was increased during the period of growth. A culture was grown on Medium *B* containing 0.1mM $CaCl_2$, and a sample removed for analysis after 55 h. To another sample, calcium chloride was added to increase the concentration to 3.4mM. A part of the culture was left with the original concentration of calcium. After 95 h, a sample was removed from this culture for analysis, and the calcium ion concentration of the rest of the culture was increased to 3.4mM. After 162 h, both the remaining samples were analysed. The results are given in Table V. The increase in calcium concentration had, in both cases, changed the ratio of the uronic acids in the polysaccharide in the direction of more guluronic acid. When calcium was added after 95 h, the total amount of mannuronic acid residues in the culture had decreased from 125 to 105 mg, in spite of a considerable increase in the amount of polysaccharide present. Therefore, the analytical results obtained cannot be explained by assuming that the polysaccharide synthesized after the increase in the calcium concentration consisted only of guluronic acid residues, and they suggest that either a change in composition of the already synthesized alginate takes place or that alginate is degraded and resynthesized with a different composition, depending upon the calcium concentration.

The latter explanation seems less likely if a similar change in composition by increase in calcium concentration could be observed after the cells were removed from the system. The following experiment was therefore carried out. A culture was grown on medium *B* (0.1mM $CaCl_2$ for 72 h), and the cells were removed by centrifugation.

TABLE IV

YIELD OF EXTRACELLULAR POLYSACCHARIDE AFTER DIFFERENT PERIODS OF TIME

Time (h)	*Extracellular polysaccharide*		
	Yield[a] *(mg)*	*M/G, Total*	*M/G, Increment*
Medium B, *0.1*mM $CaCl_2$			
30	24.0	0.93	0.93
54	90.5	3.45	7.7
120	187	5.31	9.5
Medium B, *0.34*mM $CaCl_2$			
30	43.5	0.48	0.48
54	149.0	0.44	0.43
120	185.0	0.80	24.0
168	225.0	1.11	19.0

[a]From 150 ml of medium.

TABLE V

YIELD OF EXTRACELLULAR POLYSACCHARIDE BEFORE AND AFTER INCREASING THE CALCIUM CONCENTRATION

Before increase of Ca^{2+} *concn.*			*After increase of* Ca^{2+} *concn.*		
Hours	*Yield*[a] *(mg)*	*M/G*	*Hours, total*	*Yield*[a] *(mg)*	*M/G*
55	85	2.6	162	196	1.0
95	185	3.75	162	200	1.1

[a]From 150 ml of medium.

The supernatant was divided into two parts, each of which was incubated at 30° for 20 h, one with the calcium concentration increased to 3.4mM, the other without addition of calcium chloride. Toluene was added to prevent bacterial growth. The M/G ratio for the two samples of alginate was 3.75 without addition of calcium chloride, and 1.5 after incubation in the presence of an increased amount of calcium ions.

DISCUSSION

The three strains of *Azotobacter vinelandii* which have been investigated all produced, as their main extracellular polysaccharide, a partly acetylated polyuronide which consisted of two monomers, L-guluronic and D-mannuronic acid. This is in agreement with the results of Gorin and Spencer[5] for another strain of *A. vinelandii* (Canada Department of Agriculture, Ottawa, No. 534). The results indicate that the production of a partly acetylated alginate is a characteristic feature of *A. vinelandii*. Cohen and Johnstone[19] described a polysaccharide prepared from three

different *A. vinelandii* strains which had galacturonic acid as the main constituent but which also contained smaller proportions of glucose, rhamnose, and possibly mannuronic acid. Galacturonic acid was identified by colour reactions which distinguish between galacturonic acid and glucuronic acid, and the possibility that these polysaccharides may also consist of mannuronic and guluronic acid residues should not be completely excluded. Based upon a positive reaction with thiobarbituric acid, the authors also reported that one of the strains possibly produces a sialic acid-like component. The possibility that the positive reaction with thiobarbituric acid may be due to degradation products of the polyuronide should, however, be considered. Claus[20] reported that *A. vinelandii* Strain 1484 (Göttingen) produced an extracellular polysaccharide containing L-rhamnose as the main component, but also containing considerable amounts of a component, which he identified as "2-keto-3-deoxygalactonic acid", giving the thiobarbituric acid reaction. The possibility that different polysaccharides are produced by different *A. vinelandii* strains must, therefore, be taken into account, but our results indicate that both yield and composition of extracellular polysaccharide vary significantly with variation of the composition of the medium, and careful studies should be made before differences between strains can be established. Variations in the gross composition of polysaccharides in *A. vinelandii* and other *Azotobacter* species, with variation of the medium, have also been reported by Zaitseva *et al.*[21], who, however, did not identify the uronic acids.

The results indicate that the sequence of the monomers in the *Azotobacter* alginate is of the same type as observed for algal alginates, *i.e.*, that the monomers are distributed in a blockwise fashion along the chain. It is remarkable that such completely unrelated organisms as brown algae and *Azotobacter vinelandii* produce polysaccharides having fine-structures as similar as these results indicate, and it is probable that the mechanism of the biosynthesis in the two types of organisms must be closely related. We have previously suggested that alginate can be described as a penultimate copolymer[22], where the probability of a monomer being mannuronic or guluronic acid is determined by the identity of the next-nearest neighbour.

The uronic acid composition of the alginate shows a remarkable variation, and it is evident that the calcium content of the medium is of importance. Both the results from the experiments with different amounts of calcium in the medium and the change in composition of the alginate produced after different times of incubation indicate that, when the calcium content is low, the alginate produced contains only a small proportion of guluronic acid. The results for a high content of calcium in the medium are less clear, because the alginate that is richest in guluronic acid is produced at an intermediate concentration of calcium in the solution.

An increase in the calcium concentration of the medium, after a certain amount of the alginate is produced, leads to a change in the composition of the alginate, even if the cells have been removed by centrifugation. This observation suggests that the culture medium contains an enzyme which is capable of epimerizing mannuronic acid residues in the alginate molecule to guluronic acid residues in the presence of calcium. The observed variation of the composition of the alginate may, therefore, be

related to the activity of the enzyme. The maximum in the content of guluronic acid at intermediate concentrations of calcium may possibly be connected with the fact that, at higher concentrations of calcium, the alginate is more easily precipitated and may thereby be protected against further enzymic epimerization. A further discussion of the correlation between the uronic acid composition of the alginate and the ionic composition of the medium should, therefore, wait for a study of the properties of the epimerase. Some results of such a study are presented in the following paper.

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