The Biosynthesis of Alginic Acid by *Azotobacter vinelandii*

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The sequence of reactions by which alginic acid is biosynthesized from sucrose in *Azotobacter vinelandii* was determined both by feeding radioactive intermediates to cell-free extracts of the bacteria and by studying the individual enzymes involved. Results indicate that the first polymeric substance formed in the synthesis is polymannuronic acid and that mannuronic acid units are epimerized to guluronic acid at the polymer level. Guluronic acid does not appear to be formed at the monomer level, either free or in combination with GDP.

The polyuronide alginic acid is a well-known and commercially important constituent of the brown algae. It is a linear co-polymer of mannuronic acid and guluronic acid, the relative amounts of which vary greatly between alginic acids from different species of algae (Haug *et al.*, 1974). Additionally, alginic acids from different sources vary in the arrangement of the uronic acids within the molecule so that alginic acid may be considered as a co-polymer consisting of homopolymeric blocks of mannuronic acid and of guluronic acid together with blocks with an alternating sequence (Larsen *et al.*, 1969).

The biosynthesis of alginic acid in the brown algae *Fucus gardneri* has been studied in detail by Lin & Hassid (1966*a,b*). They concluded that the sequence of enzymes that produce alginic acid from mannose is:

Madgwick *et al.* (1973) have demonstrated the presence of a polymannuronic acid C-5-epimerase (Haug & Larsen, 1971) in brown algae. This implies that epimerization of mannuronic acid units may

occur at the polymer level. The studies by Hellebust & Haug (1972) *in vivo* agree with this suggestion.

Various strains of *Azotobacter vinelandii* produce extracellular polysaccharides (Cohen & Johnstone, 1964; Claus, 1965; Gorin & Spencer, 1966; Larsen & Haug, 1971*a*). Gorin & Spencer (1966) demonstrated that the polysaccharide produced by their strain was similar in most respects to alginic acid, the principal difference being that the *Azotobacter* polysaccharide was partly acetylated. N.m.r. (nuclear-magnetic-resonance) studies (Penman & Sanderson, 1972) suggest that the bacterial alginic acid contains a smaller proportion of homopolymeric sequences than do algal alginic acids.

Very little is known about the biosynthesis of alginic acid in bacteria apart from the work of Haug & Larsen (1971), Larsen & Haug (1971*b*) and Couperwhite & McCallum (1974) which implies that polymannuronic acid is the first polymeric product of alginic acid biosynthesis. The aim of the work described in the present paper was to determine the sequence of reactions that occur during the production of alginic acid by cultures of *A. vinelandii* using sucrose as their sole source of carbon.

Experimental

Cultures of *A. vinelandii* (N.C.I.B. 9068) were obtained from the National Collection of Industrial Bacteria. Bacteria were grown in Burk's medium as described by Newton *et al.* (1953) in modified Erlenmeyer flasks on an orbital shaker maintained at 30°C. After 24 h the cells were harvested, suspended in 50 mM-Bicine [*NN*-bis-(2-hydroxymethyl)glycine]-NaOH, buffer, pH 8.5, and disrupted by shaking with acid-washed sand for 30 min at 4°C in a cell disintegrator (Mickle Instruments, Gomshall, Surrey, U.K.). Cell debris was removed by centrifugation at 28000g for 30 min. On occasions portions of cells were obtained from stirred tank fermenters and treated as described above.

Chemicals used were of the highest purity commercially available. The zwitterionic buffers Bicine and Mops [3-(*N*-morpholino)propanesulphonic acid] were purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K. Sugar phosphates were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and radioactive materials were supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

Radioactivity was measured in a liquid-scintillation counter in either PPO (2,5-diphenyloxazole; 4g) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene; 0.1g] dissolved in toluene (1 litre) or, for aqueous samples, this solution mixed with 0.5 vol. of Triton X-100.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Enzyme activity assays

Invertase (EC 3.2.1.26). Reaction mixtures (1 ml) contained sucrose (20 μ mol), Mops-NaOH, pH 7.3, (50 μ mol) and protein (3–30 μ g). The reducing sugars produced after 90 min at 30°C were measured by Nelson's (1944) method. One unit of enzyme activity is defined as 1 μ mol of substrate converted/min.

Fructokinase (EC 2.7.1.4). Fructose (5.0 μ mol) was incubated with ATP (1.5 μ mol), MgCl₂ (10 μ mol), NADP⁺ (1.5 μ mol), Bicine-NaOH, pH 8.5 (75 μ mol), phosphoglucose isomerase (20 units), glucose 6-phosphate dehydrogenase (1.3 units) and 0.12–1.2 mg of protein in 1 ml total volume. The reduction of NADP⁺ was followed spectrophotometrically at 340 nm and 20°C.

Glucokinase (EC 2.7.1.2). This was present in extracts at such low activity that it could not be assayed sufficiently accurately by the coupled-enzyme method. Therefore [U-¹⁴C]glucose (5 μ mol, 1.0 μ Ci) was incubated with ATP and Bicine buffer as for fructokinase and the reaction products were separated by high-voltage electrophoresis in 0.1 M-pyridine-acetic acid, pH 4.5. Areas corresponding to glucose 6-phosphate markers were cut out and their radioactivity was determined by liquid-scintillation counting. In cells harvested after 48 h the enzyme could be assayed spectrophotometrically by using glucose (5.0 μ mol), ATP (1.5 μ mol), MgCl₂ (10 μ mol), NADP⁺ (1.5 μ mol), Bicine-NaOH, pH 8.5 (50 μ mol), glucose 6-phosphate dehydrogenase (0.35 unit) and 0.066–0.21 mg of protein in 1 ml total volume.

Phosphoglucose isomerase (EC 5.3.1.9). This was determined by measuring the reduction of NADP⁺ in a reaction mixture (1 ml) containing fructose 6-phosphate (5.0 μ mol), NADP⁺ (1.5 μ mol), Bicine-NaOH, pH 8.5 (50 μ mol), glucose 6-phosphate dehydrogenase (0.13 unit) and 2.5–25 μ g of protein.

Phosphomannose isomerase (EC 5.3.1.8). This was assayed similarly in an incubation mixture (1 ml) containing mannose 6-phosphate (6 μ mol), NADP⁺ (1.5 μ mol), cysteine hydrochloride (10 μ mol), MgCl₂ (10 μ mol), Bicine-NaOH, pH 8.5 (70 μ mol) added glucose 6-phosphate dehydrogenase (1.3 units) and phosphoglucose isomerase (20 units) and protein (0.12–1.2 mg).

Phosphomannomutase (EC 2.7.5.?), This was also assayed by a coupled enzyme system. In 1 ml total volume were: mannose 1-phosphate (1 μ mol); NADP⁺ (1.5 μ mol); cysteine hydrochloride (10 μ mol); glucose 1,6-diphosphate (0.01 μ mol); MgCl₂ (20 μ mol); Bicine-NaOH, pH 8.5 (70 μ mol); phosphoglucose isomerase (20 units); glucose 6-phosphate dehydrogenase (1.3 units) with 0.12–1.2 mg of protein.

GDP-mannose pyrophosphorylase (EC 2.7.7.13). This was assayed by determining the amount of radioactivity in GDP-mannose produced in a reaction mixture containing (in 24 μ l): [U-¹⁴C]GTP (0.5 μ mol, 0.5 μ Ci); Bicine-NaOH, pH 8.5 (10 μ mol); MgCl₂ (0.05 μ mol); bovine serum albumin (5 μ g); mannose 1-phosphate (0.5 μ mol); inorganic pyrophosphatase (EC 3.6.1.1) (0.125 unit); and 72 μ g of protein. The mixture was sterilized by filtration, incubated at 30°C in sterile conditions for 12 h and spotted on to Whatman no. 1 chromatography paper which was developed in ethanol-1.0 M-ammonium acetate, pH 7.5 (5:3, v/v). The radioactive GDP-mannose was detected by radioautography and the radioactivity determined by liquid-scintillation counting. The formation of GDP-mannose was linear with time over a period of 12 h.

GDP-mannose dehydrogenase (EC 1.1.1.132) and 'alginate polymerase'. These enzymes were not determined separately, but radioactivity incorporated into alginic acid was measured in a reaction mixture containing GDP-[U-¹⁴C]mannose (0.05 μ mol, 0.2 μ Ci), NADP⁺ (0.15 μ mol), bovine serum albumin (15 μ g), MgCl₂ (0.1 μ mol), Bicine-NaOH, pH 8.5 (5.0 μ mol) and protein (12–120 μ g). All the reagents were sterilized by filtration and the reaction was carried out under sterile conditions for 24 h at 30°C. Then 1 ml of 1.0% (w/v) sodium alginate solution was added, followed by 2.5 ml of water. The alginic acid was precipitated by adding 500 μ l of 8.3 M-HCl, collected by centrifugation and redissolved in 3 ml of 0.5 M-NaOH. The acid precipitation was repeated and the precipitate redissolved in 1 ml of 2.0 M-NaOH. Finally, the sodium alginate was precipitated by the addition of 3 ml of propan-2-ol. This procedure freed the alginate of unchanged radioactive GDP-mannose. The radioactivity in the sodium alginate was determined in the scintillation cocktail incorporating Triton X-100.

Rates of alginate production by whole cells were determined from information from a large number of experiments in which *Azotobacter* alginate was

produced in conditions similar to those used in the present work (L. Deavin, unpublished results). Cells and cell debris were removed from cultures by centrifugation and the alginate was precipitated from solution by the addition of 3 vol. of propan-2-ol, dried in a vacuum oven at 40°C and weighed. The dry weight was then related to the protein content of the cells.

Analytical methods

High-voltage electrophoresis was performed in an apparatus constructed in our own laboratories (Gross, 1961). Samples were spotted on 55 cm lengths of Whatman 3MM paper moistened with 0.1 M-pyridine-acetic acid buffer, pH 4.5; electrophoresis proceeded at 65 V/cm for 1 h. Under these conditions GDP-mannose travelled 12 cm and GDP-mannuronic acid 14 cm; these compounds were detected by examining electrograms in u.v. light after spraying them with 0.01 % (w/v) ethanolic fluorescein. Radioactive areas were detected by radioautography.

The same electrophoretic system was used in the further investigation of the intermediates between radioactive GDP-mannose and the polymeric product. The supposed GDP-uronic acid area was cut from the electrogram and the radioactive material eluted and heated for 10 min at 100°C with 0.1 M-HCl. The hydrolysate was then electrophoresed together with authentic mannuronic acid and guluronic acid. This electrophoresis method has been used as a routine to assay mannuronic acid and guluronic acid in hydrolysates of alginic acid (C. J. Lawson & J. E. Rudland, unpublished work). The uronic acids were detected by spraying the electrophoretograms with 5 % (w/v) neutral lead acetate in 90 % (v/v) ethanol and heating at 80°C for 10 min.

DEAE-cellulose was pretreated as per the manufacturer's instructions. Columns were eluted with a concentration gradient of NaCl in 10 mM-Mops-NaOH buffer, pH 7.0. Alginic acid was eluted from the column in 0.08 M-NaCl.

Polyacrylamide-gel electrophoresis of the polysaccharide was performed in gels consisting of 6 % total monomer, 2 % of this being *NN'*-methylene-bisacrylamide, as described by Bucke (1974). After development, gels were either stained with Alcian Blue [0.08 % in 7 % (v/v) acetic acid] or, where radioactive material was under investigation, sliced into 2 mm portions that were left to dry and then dissolved in 0.1 ml of 30 % (w/v) H₂O₂. Each solution was diluted to 4.0 ml with water and the radioactivity determined as above.

The alginate lyase used to degrade the radioactive polysaccharide is an endo-enzyme that releases oligouronides with the degree of polymerization down to four from whole alginate (C. Bucke, unpublished results). It was purified 40-fold from cells of a marine pseudomonad grown with sodium

alginate as the sole carbon source, by (NH₄)₂SO₄ precipitation, DEAE-cellulose chromatography and gel filtration on Sephadex G-100.

The specific activity of the lyase, determined by using authentic algal sodium alginate (0.1 %, w/v) in 0.1 M-Mops-NaOH, pH 7.0, was 0.1 μmol/min per mg of protein. The reaction products containing unsaturated uronic acid groups (Preiss & Ashwell, 1962) were determined by using thiobarbituric acid after oxidation with periodic acid (Warren, 1959).

Partial acid hydrolysis of the radioactive polymer was conducted by the method of Haug & Larsen (1966). Briefly, the material was refluxed at 100°C with 0.3 M-HCl for 6 h, cooled and the insoluble material, which consists of homopolymeric blocks, was collected by centrifugation and dissolved in water. Polymannuronic acid and polyguluronic acid blocks were then separated by adjusting the pH of the solution to 2.85, a process that precipitates the polyguluronic acid blocks, but leaves the polymannuronic acid blocks in solution. This method has been used frequently in our laboratories and the use of n.m.r. spectroscopy (Penman & Sanderson, 1972) has demonstrated that the 'soluble' block material consists entirely of mannuronic acid and that the 'insoluble' blocks consist of guluronic acid with less than 5 % of mannuronic acid.

Results

Table 1 summarizes the results obtained in studying the individual enzymes involved in the biosynthesis

Table 1. Activities of enzymes involved in the biosynthesis of alginic acid by *A. vinelandii*

Enzyme activities were determined as described in the Experimental section.

Enzyme	<i>K_m</i> (mM)	Specific activity (nmol/min per mg of protein)
Invertase	12	60-330
Glucokinase		(in cells grown for 48 h)
Fructokinase	0.77 (fructose) 0.37 (ATP) 3.7 (Mg ²⁺)	50-220
Phosphoglucose isomerase	0.76 (fructose 6-phosphate)	3000
Phosphomannose isomerase		0.8-2.0
Phosphomannomutase		0.8-3.0
GDP-mannose pyrophosphorylase		0.18-1.8
GDP-mannose dehydrogenase		0.016-0.96
+ Alginase polymerase		
Alginase synthesis by whole cells (estimated)		0.44

Scheme 1. Pathway of biosynthesis of alginic acid in *A. vinelandii*

of alginic acid in *A. vinelandii*. Except for the assays that involved the costly radioactive GDP-mannose, and for phosphomannose isomerase and phosphomannomutase the pH optima and the optimum concentrations of cofactors were determined separately; only the activities determined in the optimum conditions are included. Assays that used radioactive GDP-mannose were performed at pH 7.0 and 8.5; invariably there was more activity at the higher pH.

Scheme 1 summarizes the proposed pathway of alginic acid biosynthesis in *A. vinelandii*. Although the GDP-mannose dehydrogenase and polymerase system was determined in a single assay, the possibility that GDP-mannose might be polymerized before oxidation was eliminated by subjecting the incubation mixture to high-voltage electrophoresis at pH 4.5 in 0.1 M-pyridine-acetic acid buffer. The GDP-mannose spot in unincubated controls was replaced by a more mobile radioactive area together with radioactive

alginic acid which remained at the origin. This area did not appear in incubations made in the absence of NADP^+ . It did not co-electrophorese with authentic mannuronic acid but if the radioactive material was eluted from the paper and hydrolysed 0.1 M-HCl it then co-chromatographed with mannuronic acid in a two-dimensional system [phenol-water (100:40, v/v) then butan-1-ol-pyridine-50 mM-morpholinium borate, pH 8.6 (7:5:2, by vol.)] and co-electrophoresed with authentic mannuronic acid. There was little doubt that GDP-mannuronic acid was an intermediate between GDP-mannose and the polymeric material. At no stage in the reaction was there evidence for the formation of free GDP-guluronic acid; no guluronic acid could be detected on electrograms after hydrolysis of the GDP-uronic acid.

The method used to purify the polymeric product of the GDP-mannose polymerase system assumed that it was an acid polymer. The radioactive polymer,

Table 2. *Distribution of radioactivity between products of partial acid hydrolysis of polymeric product of GDP-mannose consumption by cell-free extracts of A. vinelandii*

The radioactive polymer was hydrolysed by 0.3 M-HCl for 6 h at 100°C. Insoluble material was dissolved in water and fractionated into soluble and insoluble material after adjustment of the pH to 2.85.

Fraction	Content	% of total radioactivity
Initial supernatant	Alternating sequences	86.0
Soluble blocks (at pH2.85)	Polymannuronic acid	10.4
Insoluble blocks (at pH2.85)	Polyguluronic acid	3.6

whether purified or remaining in the reaction mixture, co-chromatographed with authentic algal alginic acid on DEAE-cellulose. It also migrated in a similar way to authentic alginate on polyacrylamide-gel electrophoresis (Bucke, 1974). This method of electrophoresis was used to demonstrate the degradation of the radioactive polymeric material by a preparation of alginate lyase from a marine *Pseudomonas* species.

That the polymeric product was alginic acid rather than polymannuronic acid was demonstrated (by using the radioactive material) by the use of the partial acid hydrolysis method of Haug & Larsen (1966) to isolate homopolymeric blocks followed by fractionation of this material at pH2.85 to separate polymannuronic acid and polyguluronic acid blocks. The results of this fractionation are summarized in Table 2. These results agree with larger-scale analyses of *Azotobacter* alginate samples (C. J. Lawson, C. Bucke & L. Deavin, unpublished work) although, since the composition of bacterial alginate varies with the growth conditions, this should not be taken as the definitive composition of *Azotobacter* alginate.

Discussion

This work has been done as part of a project to investigate the commercial feasibility of producing alginic acid by fermentation. The chemistry of the polymeric product of fermentation of sucrose by *A. vinelandii* has been thoroughly investigated by C. J. Lawson (unpublished results) who showed that the polymer was similar in every respect to the partly acetylated alginic acid described by Gorin & Spencer (1966). Fermentation studies (L. Deavin & C. J. Lawson, unpublished results) have demonstrated that the yield and quality (rheological and gelling properties) of the alginic acid may be improved by altering the constitution of the fermentation medium but throughout the present studies the medium used by Gorin & Spencer (1966) has been used.

The pathway of the biosynthesis of alginic acid in *A. vinelandii* appears to differ very little from the reaction sequence from mannose in *Fucus gardneri* (Lin & Hassid, 1966b). The major point of difference is that we have no evidence for the formation of guluronic acid units in the monomeric state and cannot include GDP-guluronic acid as a precursor of alginate. The discovery of polymannuronate C-5-epimerase by Haug & Larsen (1971) and its detection in algae (Madgwick *et al.*, 1973) indicates that in algae, as in *Azotobacter*, the formation of guluronic acid units may occur in the polymer, a system analogous to that described by Hook *et al.* (1974) for the formation of iduronic acid units from glucuronic acid units in the formation of heparan sulphate from heparin.

The activity of several of the enzymes in the pathway is low but there is sufficient activity in each case to account for the observed rate of alginic acid formation in the conditions used. The low activity of invertase may indicate that sucrose is taken into the *Azotobacter* cell by an energy-linked mechanism.

Several points remain to be investigated, namely the extent to which the alginate-producing system is associated with subcellular particles, at what stage of biosynthesis the acetyl groups are introduced and, indeed, whether they are associated specifically with mannuronic acid or guluronic acid units, and the physical location of the polymannuronate epimerase. This enzyme merits further attention on mechanistic grounds.

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