The use of ^{13}C -n.m.r. spectroscopy to monitor alginate biosynthesis in mucoid *Pseudomonas aeruginosa*

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The biosynthesis of alginate by a mucoid strain of *Pseudomonas aeruginosa*, isolated from a cystic-fibrosis patient, was monitored by using ^{13}C -n.m.r. spectroscopy of bacterial cultures incubated with 1- ^{13}C - or 2- ^{13}C -enriched fructose. When 1- ^{13}C - or 2- ^{13}C -enriched fructose was used as the precursor of alginate, enrichment with ^{13}C in the constituent uronic acid monomers of the polysaccharide could only be detected in C-1 or C-2 respectively, indicating that alginate is synthesized in *Ps. aeruginosa* directly from fructose, with the hexose molecule being retained intact; this rules out the involvement of C_3 intermediates, which occurs when glucose is the alginate precursor. The absence of detectable poly-L-guluronate block sequences from the alginate of *Ps. aeruginosa* was confirmed, and it was shown that there is no modification of the arrangement of the constituent uronic acids between polymerization to form alginate and the appearance of the mature alginate in the extracellular medium. The ^{13}C -n.m.r. data also provided independent evidence for acetylation on D-mannuronate residues and for the ratio of D-mannuronate to L-guluronate residues in newly synthesized alginate, which had previously been determined only for material secreted from bacteria into the extracellular medium.

INTRODUCTION

Mucoid strains of *Pseudomonas aeruginosa*, which are particularly isolated from the lungs of patients with cystic fibrosis, produce significant quantities of extracellular alginate (Russell & Gacesa, 1988). This polysaccharide is an important virulence factor in the chronic colonization of the lung and other sites by *Ps. aeruginosa*. Attempts to study alginate biosynthesis in *Ps. aeruginosa* have been complicated by the presence of multiple pathways for the utilization of carbohydrate carbon sources, which may be catabolized via the Entner–Doudoroff or Embden–Meyerhof pathways (Lessie & Phibbs, 1984). It had been established by the use of radiolabelled precursors that in *Ps. aeruginosa* alginate is produced from glucose via C_3 intermediates, which are most likely to be products of the Entner–Doudoroff pathway (Banerjee *et al.*, 1983). Recently we have confirmed this, but have shown that other pathways, probably the tricarboxylic acid cycle, may also contribute to the supply of precursors for alginate biosynthesis (Narbad *et al.*, 1988). In contrast, the synthesis of alginate from fructose in the related organism *Pseudomonas mendocina* occurs directly without the involvement of C_3 intermediates, as determined by radiotracer studies (Anderson *et al.*, 1987).

Previous studies on alginate biosynthesis in *Ps. aeruginosa* have relied on the use of radiolabelled precursors and the analysis of the extracellular products after their recovery from the culture medium (e.g. Lynn & Sokatch, 1984; Narbad *et al.*, 1988). An attractive alternative is to use ^{13}C -n.m.r. spectroscopy, which provides a powerful non-destructive method for studying metabolism in intact biological systems (London, 1988; Avison *et al.*, 1986). For example, it has proved possible to measure the flux of ^{13}C -enriched glucose through the Embden–Meyerhof pathway in *Escherichia coli* and yeast cells (Den Hollander *et al.*, 1979, 1986; Ugurbil *et al.*, 1978) and the flux of ^{13}C -enriched pyruvate through the gluconeogenic pathways in perfused rat liver (Cohen, 1983). The metabolism of acetate in the yeast

Saccharomyces cerevisiae (Den Hollander *et al.*, 1981) and a methylotrophic bacterium (Narbad *et al.*, 1989) have also been studied. This technique is amenable for investigating virtually any metabolic pathway provided that there is significant accumulation of intermediates or end products, such as the alginate of mucoid strains of *Ps. aeruginosa*. Recently Jones & Sanders (1989) have used ^{13}C -n.m.r. COSY and intact bacteria to show that the capsular exopolysaccharide of *Klebsiella* is synthesized from glucose via the phosphogluconate pathway with retention of the intact hexose precursor. In the present paper we report the use of ^{13}C -n.m.r. spectroscopy to monitor the biosynthesis of alginate in *Ps. aeruginosa* from specifically ^{13}C -enriched fructose molecules as carbohydrate precursors.

EXPERIMENTAL

Chemicals

All enzymes and chemicals, including specifically ^{13}C -enriched glucose, were purchased from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, U.K.).

Bacterial strains and growth media

A mucoid strain of *Ps. aeruginosa* (designated 11W) was isolated from sputum obtained from a patient with cystic fibrosis. This mucoid isolate was mutagenized with ethylmethane-sulphonate, and a tryptophan auxotroph (designated 11T) was selected for further studies. Details of this procedure, the preparation of growth media and the culture conditions have been described previously (Narbad *et al.*, 1988). Medium A, which was used for cell growth and maintenance during the ^{13}C -n.m.r. experiments, contained (per litre) 6.8 g of KH_2PO_4 , 50 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0 g of $(\text{NH}_4)_2\text{SO}_4$, made up in 20% (v/v) $^2\text{H}_2\text{O}$ and supplemented with tryptophan, which had been sterilized by filtration, to give a final concentration of 1 mM.

Abbreviations used: G, L-guluronate; M, D-mannuronate.

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Preparation of ^{13}C -enriched fructose

The $1\text{-}^{13}\text{C}$ -enriched and $2\text{-}^{13}\text{C}$ -enriched fructose samples were prepared enzymically from the corresponding commercially available glucose analogues by conversion into fructose bisphosphate and dephosphorylation. The ^{13}C -enriched fructose bisphosphate was prepared in a mixture containing (total vol. 5 ml) 100 mg of specifically enriched glucose (99 atom %), 700 mg of ATP, 10 units of hexokinase, 10 units of phosphoglucose isomerase, 10 units of fructose-6-phosphate kinase, 80 mM-Tris/HCl buffer, pH 7.4, and 10 mM- MgCl_2 . This mixture was sterilized by filtration and incubated at 37°C for 16–24 h, by which time the reaction was complete (as judged by the absence of glucose, glucose 6-phosphate and fructose 6-phosphate and the presence of fructose bisphosphate, all determined enzymically). The reaction mixture was passed through an AG1-X8 anion-exchange column (–400 mesh; formate form) (Bio-Rad Laboratories, Watford, Herts., U.K.), which was eluted with a gradient of 0.2–2.0 M-ammonium formate buffer, pH 5.0. The fractions containing fructose bisphosphate were identified by comparison of the elution pattern with those of authentic fructose bisphosphate and confirmed by their sensitivity to commercial fructose bisphosphatase. The fructose bisphosphate-containing fractions were pooled and the NH_4^+ ions were removed by single passage of the material through a Dowex 50W-X8 cation-exchange column (20–50 mesh; H^+ form) (BDH Chemicals), and the remaining formic acid was removed by freeze-drying.

The purified fructose bisphosphate was dephosphorylated with acid phosphatase in a reaction mixture containing 40 mM-sodium acetate buffer, pH 5.0, and 20 units of acid phosphatase in a total volume of 5 ml. After incubation for 16 h at 37°C , the reaction mixture volume was made up to 20 ml with distilled water and the residual acetate was removed by passage through an AG1-X8 anion-exchange column (200–400 mesh; formate form) (Bio-Rad Laboratories). Fractions containing fructose were pooled, freeze-dried and redissolved in 20% (v/v) $^2\text{H}_2\text{O}$.

The ion-exchange chromatography steps served to separate phosphorylated/non-phosphorylated fructoses and thus to purify the products. As a check that the final ^{13}C -enriched fructose products were not contaminated with other sugars, ^{13}C -n.m.r. spectra were obtained, which showed that there were no impurities that would contribute to n.m.r. spectra when the ^{13}C -enriched fructose was added to bacterial cultures.

Quantification of carbohydrates

Total carbohydrate was measured by using the phenol/ H_2SO_4 reagent (Dubois *et al.*, 1956).

Alginate was determined by measurement of total uronic acids (Blumenkrantz & Asboe-Hansen, 1973).

Glucose was determined enzymically by the glucose oxidase/peroxidase method with a commercially available kit (Sigma Chemical Co.).

Fructose 6-phosphate and glucose 6-phosphate were determined enzymically by using the methods described by Bergmeyer (1984).

Fructose bisphosphate was measured by using the same enzymic method as for fructose 6-phosphate, except that fructose bisphosphatase (0.1 unit/ml) was added to the incubation mixture.

^{13}C -n.m.r. spectroscopy of bacterial cultures

^{13}C -n.m.r. spectra were obtained at 90.55 MHz on a Bruker WM 360 spectrometer operating in the Fourier-transform mode. Sodium 3-(trimethylsilyl)propane-1-sulphonate was used as the external standard. Chemical shifts of all other signals were expressed in p.p.m. downfield of sodium 3-(trimethylsilyl)-

propane-1-sulphonate. All test samples contained 20% (v/v) $^2\text{H}_2\text{O}$ to provide the field-frequency lock signal.

A starter culture was prepared by inoculating 50 ml of minimal medium (Narbad *et al.* 1988), containing 20 mM-fructose, with a colony of mucoid *Ps. aeruginosa* 11T followed by incubation at 37°C for 24 h. A 5% (v/v) inoculum was added to 200 ml of fresh 20 mM-fructose-containing medium. After further incubation for 24 h, the bacteria were harvested aseptically by centrifugation at 7000 g (r_{av} 7.0 cm) for 15 min at 4°C , washed twice in sterile 15 mM-potassium phosphate buffer, pH 7.0, and resuspended in medium A to give a cell density of 6–7 mg (dry wt.) equivalent of cells/ml. A portion (3.6 ml) of this suspension was transferred to an n.m.r. tube (10 mm external diam.).

Experiments were started by the addition of 0.4 ml (30 mg) of either $1\text{-}^{13}\text{C}$ -enriched or $2\text{-}^{13}\text{C}$ -enriched fructose to the n.m.r. tube containing a bacterial suspension. Aeration was provided by continuous bubbling of pure O_2 at a rate of 20 ml/min via a tube placed below the level of the radiofrequency coil, and at 120 ml/min through a second tube placed above the radiofrequency coil and also above the suspension. This configuration ensured aeration of the cell suspension without unduly affecting the lock signal. Broad-band ^1H -decoupled spectra were acquired in non-spinning n.m.r. tubes using 8k data points over a spectral width of 22.7 kHz, with a pulse angle of 42° and a delay time of 0.8 s. High-level irradiation (5 W) was used to ensure decoupling during the acquisition period (0.36 s), and a lower level (0.1 W) was used during the delay period to provide nuclear Overhauser enhancement while minimizing dielectric heating. The temperature remained at $33.5 \pm 3.5^\circ\text{C}$ throughout the experiment. Spectra were accumulated in sets of 4000 and then Fourier-transformed after application of a 5 Hz line-broadening function.

^{13}C -n.m.r. spectroscopy of alginate *in vitro*

^{13}C -n.m.r. spectra of alginate were obtained *in vitro* with cell-free supernatants of cultures grown on ^{13}C -enriched fructose. Alginate was prepared from supernatant samples by the method of Grasdalen *et al.* (1979). ^{13}C -n.m.r. analyses were carried out in n.m.r. tubes (5 mm external diam.) with the use of the same parameters described above, except that 5 W decoupling power was used continuously and the temperature was maintained at 65°C by external heating to decrease the viscosity of the alginate in the supernatant samples.

Identification of chemical shifts

The assignment of ^{13}C -n.m.r. resonances for bacterial cultures was achieved by comparison of their chemical shifts with those of authentic alginate, fructose, lactate and acetate (containing ^{13}C at the natural abundance level of 1.1 atom%) dissolved in medium A at a concentration of 50–100 mg/ml. Additionally, the assignment of the resonances for fructose was confirmed by addition of a low concentration of fructose to the test sample.

RESULTS

Utilization of $1\text{-}^{13}\text{C}$ -enriched fructose

A timed sequence of ^{13}C -n.m.r. spectra of a suspension of *Ps. aeruginosa* strain 11T growing on $1\text{-}^{13}\text{C}$ -enriched fructose is shown in Fig. 1. At time zero, the two major fructose anomers, β -pyranose and β -furanose, were observed in the ratio of 3:1, representing the proportions at anomeric equilibrium. The intensities of these resonances were diminished progressively as a result of fructose metabolism and were undetectable after 6.8 h. The signal for α -furanose (65.5 p.p.m.) was obscured by the unresolved region of the spectrum between the two major peaks (Fig. 1). The signal from α -pyranose is not apparent in Fig. 1

Fig. 1. Time-dependent ^{13}C -n.m.r. spectra of a culture of *Ps. aeruginosa* strain 11T growing on $1\text{-}^{13}\text{C}$ -enriched fructose

An aerated culture sample of *Ps. aeruginosa* in growth medium containing $1\text{-}^{13}\text{C}$ -enriched fructose was placed inside an n.m.r. tube and the Fourier-transformed ^{13}C -n.m.r. spectra were obtained at 90.55 MHz. Each spectrum in the Figure represents the sum of 4000 individual scans accumulated over approx. 1 h. The sample temperature was maintained at $33.5 \pm 3.5^\circ\text{C}$.

because this ring form represents only 3–5 % of the total fructose (Angyal & Bethell, 1976; Horton & Walazek, 1982).

The added fructose gave rise to three unexpected signals at 92.3, 94.2 and 96.7 p.p.m. (Fig. 1), which decayed at different rates, suggesting the presence of more than one contaminant. These chemical shifts are typical of aldose anomeric carbon atoms, but comparison with likely sources of these signals ruled out contamination by mannose, glucose, glucose 6-phosphate, fructose 6-phosphate or fructose 1,6-bisphosphate; in addition, trioses would not give rise to signals at these positions.

The first metabolic products to be synthesized were enriched in the carbonyl positions. These products were excreted into the culture medium since their resonances were also observed in spectra of the cell-free supernatants. Apart from these products the first molecule to become enriched in ^{13}C was bicarbonate (162.9 p.p.m.), which results from oxidative respiration (Fig. 1). After 2 h the ^{13}C was enriched in a compound giving a signal at 144.8 p.p.m., suggesting that it contained an enol group; however, its resonance did not correspond to phosphoenolpyruvate, 2-oxoglucuronate or 3-deoxy-2-oxo-6-phosphogluconate.

The signals corresponding to C-1 and C-3 of lactate appeared simultaneously after 2 h (Fig. 1). Once the fructose was utilized, lactate consumption began and its signals became undetectable after 10 h. Similar changes in the enrichment of C-2 in acetate were also observed.

Alginate synthesis from $1\text{-}^{13}\text{C}$ -enriched fructose

A broad multiplet signal of C-1 resonances of alginate was observed between 102 and 105 p.p.m. after 7 h incubation (Fig. 1); no signals corresponding to ^{13}C -enrichment in other carbon atoms of alginate were detected. The broadness of the signal was probably due to the polydisperse nature of the alginate sample, which contains molecules having a range of molecular masses.

In order to improve the signal-to-noise ratio, the individual spectra were summated and then Fourier-transformed. The resulting spectrum (Fig. 2a) showed a number of distinct peaks corresponding to C-1 of L-gulonate (G) and of D-mannuronate

(M), the two monomers of alginate. The peak assignments were based on the ^{13}C -n.m.r. spectra of algal alginate reported by Grasdalen *et al.* (1977), taking into account their different standard used for chemical-shift determination, and also by comparison with the spectrum of algal alginate obtained by ourselves in the same medium A as used for the bacterial alginate under our standard conditions (Fig. 2b). The peaks corresponding to the ^{13}C -enriched diads GM (G with a neighbouring M residue; 102.6 p.p.m.), MM (M next to another M residue; 102.9 p.p.m.) and MG (M next to a G residue; 104.1 p.p.m.) were clearly distinguishable. In comparison with algal alginate, the signal for GG (G with a neighbouring G unit) at 103.6 p.p.m. was not detected. There was one or possibly two additional peaks centred at 102.3 p.p.m. upfield of the expected resonances, which were presumed to arise from perturbation of the C-1 chemical shifts due to *O*-acetyl substitution of C-2 and/or C-3 of D-mannuronate (Skjak-Braek *et al.*, 1986).

To enhance the peak resolution of alginate signals, ^{13}C -n.m.r. spectra of culture supernatants were obtained. Compared with the signals observed from bacterial cultures, a better-resolved spectrum (Fig. 2c) revealed peaks for the three major diads MM, MG and GM. The proposed *O*-acetylated diad signals were absent, probably owing to deacetylation of alginate caused by the acidic conditions during sample preparation. In comparison with the C-1 signal of the native algal alginate (Fig. 2b), the intensity of the signal for the MM diad was greatly increased in relation to other diads, indicating that it was mainly the D-mannuronate residues that were *O*-acetylated.

From the observed C-1 multiplet signals, the distributions of the doublet frequencies were calculated (Table 1). These data showed that, although the alginate of *Ps. aeruginosa* strain 11T had an M/G ratio of 3.8:1 and contained 21 % L-gulonate residues, no poly-G blocks were detectable.

Utilization of $2\text{-}^{13}\text{C}$ -enriched fructose

A sequence of spectra obtained during the metabolism of $2\text{-}^{13}\text{C}$ -enriched fructose by bacterial cultures is shown in Fig. 3. As

Fig. 2. 90.55 MHz Fourier-transformed ^{13}C -n.m.r. spectra to compare (a) the signals of the C-1 region of alginate being synthesized by a culture of *Ps. aeruginosa* growing on $1\text{-}^{13}\text{C}$ -enriched fructose with (b) authentic algal alginate and with (c) the C-1 region of alginate extracted from the culture supernatant of *Ps. aeruginosa* grown on $1\text{-}^{13}\text{C}$ -enriched fructose

The data for the spectrum shown in (a) were obtained by addition of all the spectra shown in Fig. 1. The spectrum shown in (b) represents the sum of 34 500 individual scans accumulated overnight at 65°C . The concentration of alginate used was 8 mg/ml. The spectrum shown in (c) represents the sum of 36 000 individual scans accumulated overnight at 65°C . Abbreviations: GM, the diad, G with a neighbouring M residue; MG, the diad, M next to a G residue; MM, the diad, M next to another M residue; GG, the diad, G next to another G residue; OAc-U, O-acetylated uronic acid residue.

observed with $1\text{-}^{13}\text{C}$ -enriched fructose, there was no evidence for preferential uptake of either anomer from $2\text{-}^{13}\text{C}$ -enriched fructose. However, with $2\text{-}^{13}\text{C}$ -enriched fructose the resonance due to α -furanose was detected, since it was well separated from the two major peaks of β -pyranose and β -furanose structures of fructose. The bicarbonate signal was present, but signals corresponding to enol- or carbonyl-containing compounds were absent.

Alginate synthesis from $2\text{-}^{13}\text{C}$ -enriched fructose

The peaks due to alginate appeared in the 73.0–74.6 p.p.m. region of the spectra, which corresponds to C-2 or C-3 of uronic acids. No other carbon atoms of alginate enriched with ^{13}C were

Table 1. Fractional composition to show the distribution of diad frequencies and the ratio of the uronic acids in alginate synthesized by a culture of *Ps. aeruginosa* 11T growing on $1\text{-}^{13}\text{C}$ -enriched fructose

The data are derived from the spectra shown in Fig. 2(a) and are compared with a theoretical distribution that has been calculated on the basis of a random statistical distribution for uronic acid monomers, using the experimentally determined M/G ratio of 3.8:1 as shown. Abbreviation: N.D., not detected.

Diad	Fractional composition	
	Experimental	Theoretical
F_M	0.79	—
F_G	0.21	—
F_{MM}	0.52	0.62
F_{MG}	0.24	0.17
F_{GM}	0.24	0.17
F_{GG}	N.D.	0.04

detected. Although this is also the region in which minor peaks resulting from natural-abundance ^{13}C atoms in fructose occur, the alginate peaks have distinct chemical-shift values (see expanded portion of Fig. 3).

DISCUSSION

By using ^{13}C -n.m.r. spectroscopy it has been possible to observe directly the biosynthesis of alginate in cultures of a mucoid strain of *Ps. aeruginosa*. By using this method to monitor the concentrations of various metabolite, one can obtain information about the biosynthetic pathway.

Glucose is metabolized in *Ps. aeruginosa* largely via the Entner–Doudoroff pathway, and mutant strains defective in phosphoglucose isomerase, 6-phosphogluconate dehydrogenase or the enzymes of the Entner–Doudoroff pathway grow poorly on this sugar; in contrast, such mutants grow readily on fructose, which can be converted into fructose 6-phosphate without involvement of C_3 intermediates, as generated by the Entner–Doudoroff or Embden–Meyerhof fructose-bisphosphate aldolase enzymes (Phibbs *et al.*, 1978; Narbad *et al.*, 1988). The ^{13}C -enrichment of lactate in *Ps. aeruginosa* grown on $[1\text{-}^{13}\text{C}]$ fructose in the present experiments gives an indication of the relative importance of the two pathways, assuming that this intermediate is synthesized only from ^{13}C -enriched fructose and not from the contaminant present in the fructose preparation. Metabolism through the Embden–Meyerhof pathway would label pyruvate and hence lactate in C-3, whereas operation of the Entner–Doudoroff route would enrich ^{13}C in C-1 only. The conversion of pyruvate into lactate, which probably resulted from insufficient aeration of the bacterial suspension in the n.m.r. tube, would not affect the enrichment pattern in pyruvate. The observed enrichment of both C-1 and C-3 implies that the two metabolic pathways operate simultaneously, and the quantification of the peak intensities (corrected for the different saturation ratios of the two signals under the appropriate acquisition conditions) indicates that the relative fluxes of the label are approx. 60% and 40% through the Entner–Doudoroff and Embden–Meyerhof pathways respectively.

The synthesis of alginate in living bacteria was observed from ^{13}C -enriched fructose. It was shown that from $[1\text{-}^{13}\text{C}]$ - or $[2\text{-}^{13}\text{C}]$ -fructose there was enrichment of the corresponding C-1 or C-2 position respectively of alginate. No signals for other positions were detected at the relevant chemical-shift values, even in

Fig. 3. Time-dependent ^{13}C -n.m.r. spectra of a culture of *Ps. aeruginosa* strain 11T growing on 2- ^{13}C -enriched fructose

The experimental conditions were the same as those described in the legend to Fig. 1. The inset gives an expansion of the region 60–80 p.p.m. from the initial (bottom) and final (top) spectra of the series in order to show the selective accumulation of ^{13}C in alginate.

accumulated or expanded spectra. These results indicate that alginate monomers are synthesized directly from fructose with the hexose molecules being retained intact. This is in contrast with alginate synthesis from glucose, which involves C_3 intermediates produced via the Entner–Doudoroff pathway (Banerjee *et al.*, 1983) and further re-arrangement of the carbon atoms via other pathways, probably the tricarboxylic acid cycle (Narbad *et al.*, 1988). Such an involvement of C_3 intermediates would have resulted in enrichment of both C-1 and C-6 from [1- ^{13}C]fructose as precursor and of both C-2 and C-5 from [2- ^{13}C]fructose; the involvement of the tricarboxylic acid-cycle intermediates would have resulted in enrichment with ^{13}C of all of the carbon atoms in alginate. Using ^{14}C -radiolabelled fructose, Anderson *et al.* (1987) showed that alginate synthesis also occurs directly from fructose in *Ps. mendocina*.

The assignment of the C-2 position of alginate was not absolute, since the signals for both C-2 and C-3 have resonance values in this region and the relative values for C-2 and C-3 of the uronic acids are difficult to differentiate (Grasdalen *et al.*, 1981). However, on the basis of a consideration of the results of [1- ^{13}C]fructose metabolism and the known metabolic routes used by *Ps. aeruginosa*, it is not possible to formulate a scheme by which the ^{13}C from C-2 in fructose can be re-arranged specifically

to the C-3 in alginate. Therefore it must be concluded that the signals at 73.0–74.6 p.p.m. are due to the C-2 of the uronates.

From the multiplet pattern of the C-1 signal of alginate it has been possible to obtain structural details of the polysaccharide. The ^{13}C -n.m.r. data support the current view that it is the D-mannuronate residues of alginate that are O-acetylated, as shown previously by ^1H -n.m.r. and chemical methods (Davidson *et al.*, 1977; Skjak-Braek *et al.*, 1986).

The M/G ratio 3.8:1 for the alginate of *Ps. aeruginosa* strain 11T calculated from the present ^{13}C -n.m.r. data is at the upper end of the range for alginates of several other strains of *Ps. aeruginosa* also isolated from cystic-fibrosis patients (Sherbrock-Cox *et al.*, 1984), and also for alginates of *Pseudomonas putida* and *Pseudomonas fluorescens* (Skjak-Braek *et al.*, 1986). It is clear from the results that the distribution of uronic acids is not randomly organized, there being higher proportions of poly-M and random-poly-MG blocks than are predicted on the basis of a random statistical distribution of monomers. The theoretical value of 4% poly-G blocks is at the limit of detection of the n.m.r. methodology, but the apparent absence of this block structure accords with all previous ^{13}C - and ^1H -n.m.r. data obtained by ourselves and other workers (for a review see Russell & Gacesa, 1988). The absence of detectable poly-G blocks

observed in newly synthesized alginate confirms data obtained by ^1H -n.m.r. spectroscopy of excreted alginate recovered from the culture medium (Sherbrock-Cox *et al.*, 1984). This indicates that there is no re-arrangement of alginate block structure between initial synthesis and appearance of the polymer outside the bacterial cell. This correlates with the observation that *Ps. aeruginosa* lacks an extracellular polymer-level epimerase (Singh *et al.*, 1987), in contrast with other alginate-producing organisms, e.g. *Azotobacter vinelandii* (Narbad *et al.*, 1990); thus *Ps. aeruginosa* would probably be unable to modify the monomeric composition of its alginate at the polymer level.

We are grateful to the Cystic Fibrosis Research Trust for the award of a project grant (to P.G. and N.J.R.) to support this research.

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Received 27 July 1989/8 November 1989; accepted 14 November 1989