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Note

Investigation by n.m.r. spectroscopy of the site of proton exchange catalysed by poly(mannuronic acid) C-5 epimerase

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A poly(mannuronic acid) C-5 epimerase is present in the culture medium of the soil bacterium *Azotobacter vinelandii*[1–3]. During growth, this organism releases into the medium a glycuronan having essentially the same structure as alginate isolated from marine, brown algae. Similar enzymic activity has been detected in a number of algal species that produce alginate[4,5], and the enzyme evidently plays an important part in the biosynthesis of alginate[6].

The enzyme catalyses the conversion of D-mannuronic acid residues into L-guluronic acid residues, by epimerisation at C-5, provided the substrate is a glycuronan of at least 10 units. Information is lacking as to the enzyme's specificity for a particular, local arrangement of units in the chain. An analogous C-5 epimerase converts D-glucuronic acid residues into L-iduronic acid residues during the biosynthesis of heparin[7].

The enzymic activity is accompanied by an exchange of protons with the mediums, resulting in the incorporation of tritium into the polymer when the reaction was performed[8] in a mixture of H_2O and ^3H_2O. After complete hydrolysis of the epimerised product with acid, 92% of the recovered radioactivity was located in the L-guluronic acid. It was therefore suggested that tritium atoms from the water had been incorporated in the L-guluronic acid at C-5. For the D-glucuronic acid C-5 epimerase, this has recently been demonstrated by using a heparin precursor that contained D-(5-^{3}H)glucosyluronic acid residues, followed by chemical degradation[9–10]. We now report on the use of n.m.r. spectroscopy, a considerably simpler technique than the chemical ones, to determine the site of hydrogen exchange occurring with D-mannuronic acid C-5 epimerase.

The incubation mixture consisted of sodium alginate (87% of D-mannuronic acid, 75 mg in 20 ml), 50mM collidine buffer (60 ml, pH 6.8), and 34mM calcium chloride (2.5 ml). All solutions were made up in D_2O (purity, >98%) and pH ad-

*Biosynthesis of Alginate, Part IV. For Part III, see ref. 8.

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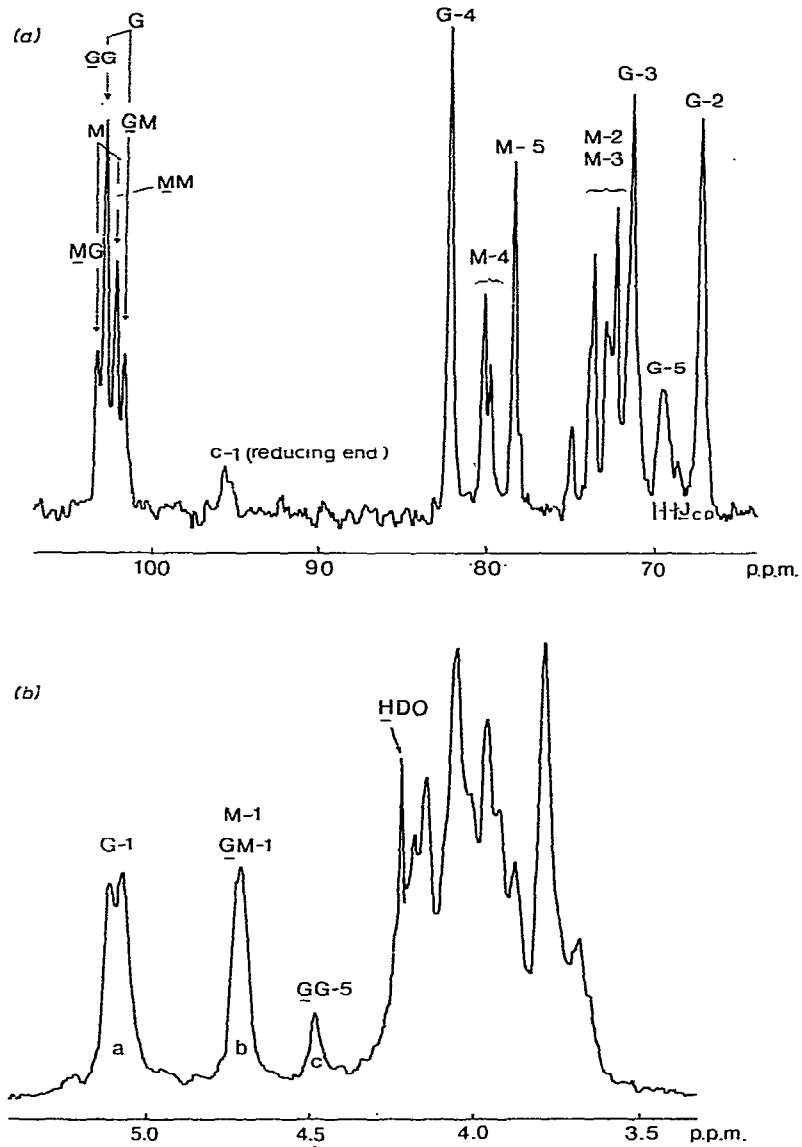

Fig. 1. N.m.r. spectra of epimerised alginate: (*a*) 25-MHz, proton-decoupled ^{13}C-spectrum (40 mg of alginate in 0.4 ml of D_2O at 90°); (*b*) 100-MHz ^1H-spectrum (10 mg of alginate in 0.3 ml of D_2O at 90°).

justments were made with DCl. The volume was made up to 99.5 ml with D_2O, and 0.5 ml of an aqueous solution (ordinary H_2O) of the enzyme preparation from *Azotobacter* was added. The mixture was incubated at 37° for 21 h and the modified alginate was then precipitated by adding sodium chloride to a concentration of 1% w/v, followed by hydrochloric acid to pH 1. The alginic acid was collected by

centrifugation, dissolved by neutralisation, and dialysed exhaustively against distilled water. Prior to n.m.r. spectroscopy, the alginate was subjected to a partial, homogeneous hydrolysis (pH 2.88, 2 h at 100°), neutralised, and lyophilised. Samples were dissolved in D_2O in the n.m.r. tube, and the spectra were recorded with a Jeol FX-100 spectrometer (for conditions, see refs. 11 and 12).

The 25-MHz, proton-decoupled, ^{13}C-n.m.r. spectrum of the epimerised sample is given in Fig. 1*a*, and the 100-MHz ^1H-spectrum in Fig. 1*b*. The assignments given have been documented previously and it has been demonstrated that relative signal intensities may be used to determine both the composition of the polymer and the diad (nearest-neighbour) frequencies[11,12]. Compositions are reported as mole fractions of D-mannuronic and L-guluronic acid (F_M and F_G, respectively), and diad frequencies as mole fractions of the four possible diads, MM, MG, GM, and GG. The first unit in the diad is the one whose signal is observed.

In the ^{13}C-spectrum, the composition may be determined by using any set of resonance signals referring to the same carbon atom in the two types of units, and the diad frequencies from the four resolved peaks representing the anomeric carbon as indicated in Fig. 1. In the ^1H-spectrum, the following relations between peak intensities and composition are valid for an alginate having ^1H in all ring positions (see ref. 11):

$$F_G = \frac{I_a}{I_b + I_c}, \quad F_{GG} = \frac{I_c}{I_b + I_c}, \quad F_{GM} = F_G - F_{GG}, \quad \text{and} \quad F_{MG} \sim F_{GM},$$

where I_n refers to the total area under the n-peak.

Calculation of F_G gave consistent results for C-1,2,3,4, whereas the results for C-5 deviated considerably. In the ^1H-spectrum the signals due to H-1 and H-5 (Fig. 1*b*, signals a, b, and c) also deviated considerably. This demonstrated that the deuterium atoms incorporated cannot be statistically distributed among the available positions. Since the only inconsistent result in the ^{13}C-spectrum was that for C-5, it may be concluded that incorporation of deuterium takes place at this carbon atom. This is also supported by the shape of the signal for C-5.

Exchange of deuterium for hydrogen on a carbon atom leads to an almost complete loss of the n.O.e. and, consequently, to a much weaker signal. The signal will also be broader and reduced in height, due to the carbon–deuterium spin–spin coupling. This is reduced by a factor of $\gamma_D/\gamma_H = 6.55$ relative to the proton coupling and will amount to ~21 Hz for C-5–D ($J_{C\text{-}5,H}$ 140 Hz). The smaller and broader peak due to C-5 and the presence of the expected multiplet are shown in Fig. 1*a*. The middle line of the observed multiplet appears stronger than the others because of the presence of some C-5 atoms that carry hydrogen.

In ^1H-n.m.r. spectroscopy, the exchange of deuterium for hydrogen will lead to a decrease of the signal for H-5 that is directly proportional to the number of such exchanges taking place. The intensity of the signals from H-1 are not affected if exchange is exclusively at C-5. From a knowledge of the ^{13}C-spectrum, a theoretical

TABLE I

DISTRIBUTION OF H-5 AND D-5 IN ALGINATE, BEFORE AND AFTER TREATMENT WITH EPIMERASE

Sample	*Resonance*	*C-5*	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
Before treatment	^{13}C	H	0.87	0.13	0.83	0.05	0.05	0.08
After treatment	^{13}C	H ÷ D	0.41	0.59	0.29	0.12	0.12	0.47
	^{1}H	H	0.41	0.15	0.29	0.12	0.05	0.10
	^{1}H	D	0	0.44	0	0	0.07	0.37

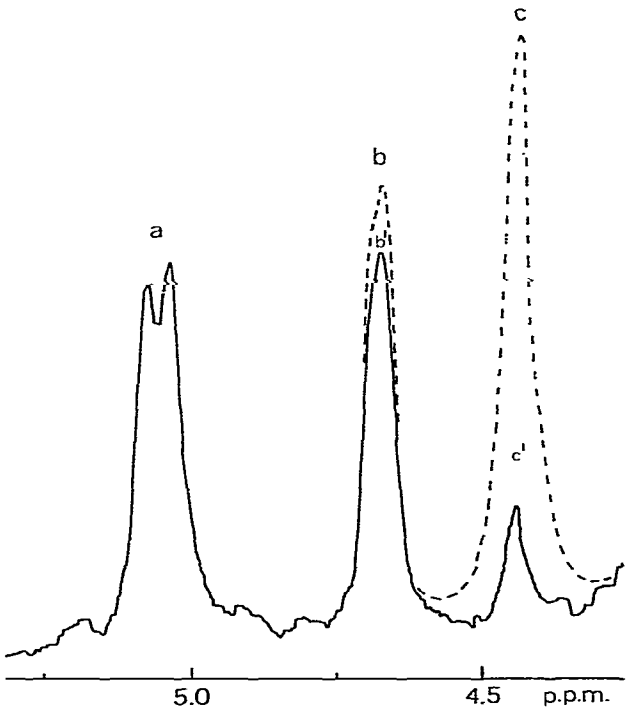

Fig. 2. Observed (——) and calculated (– – –) parts of the ^{1}H-spectrum of epimerised alginate.

curve (Fig. 2) may be constructed representing the spectrum for a glycuronan of the same composition but where all C-5 atoms carry hydrogen. Consequently, it is possible to calculate the proportion of L-guluronic acid residues that carry hydrogen [F_G(H-5)] and deuterium [F_G(D-5)] at position 5, and similarly for the diad frequencies, according to the formulas:

$$F_G(\text{H-5}) = \frac{I'_b + I'_c}{I_b + I_c} - F_M = F_G \frac{I'_b + I'_c}{I_a} - F_M,$$

$$F_{GG}(\text{H-5}) = F_G \frac{I'_c}{I_a}, \quad \text{and} \quad F_{GM}(\text{H-5}) = F_G \frac{I'_b}{I_a} - F_M.$$

The results are given in Table I. The ^{13}C data show that the content of L-guluronic acid increased from 13 to 59% as a result of the enzymic activity, and that most of it was introduced at positions adjacent to a unit of the same kind. This result will be discussed in detail elsewhere. The ^{1}H-n.m.r. spectrum demonstrated that, within experimental error, all of the D-mannuronic acid residues carried hydrogen at C-5. Any back-epimerisation must therefore be extremely slow. The total incorporation of deuterium agrees very well with the total conversion of D-mannuronic into L-guluronic acid. This supports the conclusion that incorporation occurs selectively at C-5 of the uronic acid moiety.

It is therefore concluded that, as a result of the action of D-mannuronic acid C-5 epimerase, an exchange of protons takes place between the substrate molecule and the solvent, and that this exchange occurs at C-5 of the uronic acid. Compared with chemical methods, n.m.r. spectroscopy provides a very convenient and rapid method for studying this type of reaction.

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