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Structural determination of alginic acid and the effects of calcium binding as determined by high-field n.m.r.*

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ABSTRACT

The nature of the solution conformations of the alginic acid components D-mannuronan (poly-ManA) and L-guluronan (poly-GulA) from *Azotobacter vinelandii* were investigated by both one- and two-dimensional n.m.r. methods. Unequivocal proton assignments for both polymers as well as their constituent monomer units were made based on chemical-shift theory, coupling constant analysis, and nuclear Overhauser enhancement measurements. These data were used to investigate the interactions of poly-GulA and poly-ManA with Ca^{2+} ion in aqueous medium. Based on relative crosspeak integrals measured in two-dimensional phase-sensitive NOESY spectra of free and calcium-bound polymer, a model for calcium binding is proposed.

INTRODUCTION

Alginic acids (Fig. 1) are heteropolysaccharides that are composed of varying ratios of β-D-mannuronic (ManA) and α-L-guluronic acid (GulA) residues[1]. They are quite widely distributed in nature, occurring in organisms as diverse as seaweeds and bacteria[2]. The ability of alginate solutions to form crosslinked matrices upon interaction with calcium ions provides a range of rheological properties, and has led to a commercial role for alginates as pharmaceutical adjuvants (suspending and shear-thinning agents[2,3], hemostatic bandages, tablet binders, and enteric coatings), as phase stabilizers in foods and beverages, and medicinally as antacid and antiulcer preparations[4]. Additionally, alginate produced by pulmonary-resident strains of *Pseudomonas* is a central factor in the morbidity and mortality of cystic fibrosis[5]. The ability of alginate to assume the stabilized form characterizing a gel is due to the presence of blocks of adjacent guluronate residues in the polymer. Alginate itself, in native state in

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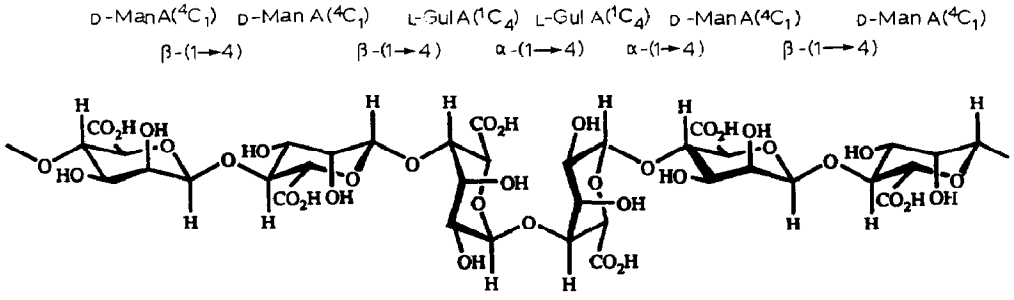
solution, is entropically driven to assume a disordered, or random-coil state[6]. Forces that are capable of stabilizing polysaccharide chains, namely dipole, hydrogen-bonding, solvation, and ionic interactions, are normally so weak that they have little effect on the overall polymer conformation, and the adjacent molecules of the polymer exist in random orientations relative to each other in solution. When a large number of these forces become favorable at the same time, however (that is, they act cooperatively), they become formidable, and can drive the polymer to a more stable conformation in solution[6]. In alginate, cooperativity of this sort requires alignment of at least two chain segments of regular sequence, such that complementary functional groups are put in position to interact. Cooperativity is facilitated by chelation of calcium ions at clefts[7] formed by adjacent guluronate units. It has been observed that at a threshold number of ~ 24 guluronate residues, GulA regions of adjacent alginate chains align in a regular fashion, creating a gel matrix. The predominant model for this association, put forth by Rees *et al.*[8], is termed the "egg box" model due to its characteristic appearance of one calcium ion flanked by four GulA residues (two from each chain). Obviously, the properties of alginate are dramatically affected by the ratio of mannuronate to guluronate in the polymer, and may range from brittle to elastic.

The biosynthesis of alginic acid in microorganisms occurs through the initial formation of a homopolymeric D-mannuronan (poly-ManA) precursor[5,9], in which β-D-mannosyluronic residues at various sites are epimerized to α-L-guluronic acid to yield alginate. In *Azotobacter vinelandii*, a nitrogen-fixing bacterium that produces alginate, this reaction is mediated by mannuronan C-5 epimerase[10], an enzyme that, in endo fashion, catalyzes epimerization at C-5 of β-D-mannosyluronic residues. Epimerization occurs with exchange of label from H-5, consistent with proton abstraction α to the carboxylate group as an initial reaction step. During the catalytic cycle, the $^4C_1$ conformation in the mannuronate units is flipped to $^1C_4$ in guluronate, and glycosidic linkages change from equatorial (ManA) to axial (GulA). These linkage arrangements provide adjacent guluronate units with their cup- like shape. The epimerase is activated[11] by Ca^{2+}, which is an essential medium component for alginate production. One explanation for this metal-ion dependence involves Ca^{2+} complexation of the carboxy-



Alginic Acid

Fig. 1. The structure of alginic acid.

late of an ManA residue about to undergo epimerization. It is conceivable that such complexation would withdraw electron density from the carbon atom α to the carboxylate (C-5), rendering H-5 more acidic and hence more labile. In addition to activating the enzyme, Ca^{2+} also influences its action pattern. At low Ca^{2+} concentrations, the epimerase appears to catalyze epimerization adjacent to pre-existing guluronate residues, by what may be a processive or multiple attack mechanism[11,12]. Operating in this catalytic mode, the enzyme product contains domains of GulA interspersed with domains of ManA. At higher concentrations of Ca^{2+}, a more-random or alternating sequence is produced. In this catalytic mode, the action pattern appears to be representative of a classical random (multichain) mechanism[12], in which the epimerase catalyzes only one event per encounter. The basis for these effects may be a direct effect of calcium ion on the enzyme itself, but there is also the possibility that these effects may be the result of conformational or enzyme binding constraints imposed on the polysaccharide by interaction of the metal ion at specific sites. To assess the likelihood of these factors, we investigated the effect of Ca^{2+} binding on the conformations of both poly-GulA and poly-ManA by high-field ^{1}H-n.m.r. spectroscopy.

Our strategy in these investigations was as follows. A complete set of chemical-shift assignments and coupling constants in the ^{1}H-n.m.r. spectra of low d.p. poly-GulA and poly-ManA, as well as monomeric α-L-guluronic and β-D-mannuronic acids were determined. Samples of poly-GulA and poly-ManA were titrated with calcium ion, and changes in chemical shifts but not coupling constants were noted. Therefore, based on chemical shift-proximity effects of the metal ion on ring protons, functional groups involved with metal binding were identified. With these data in hand, phase-sensitive NOESY[13] experiments were performed on samples of poly-GulA, poly-ManA, and calcium complexes of these homopolymeric blocks, and changes in relative intensities of the nuclear Overhauser effects were observed upon additions of Ca^{2+}. Consideration of the titration data and interproton distances in the complexes allows a model for the solution interaction of alginate constituents with calcium ion to be proposed.

EXPERIMENTAL

Materials. — The bacterial culture employed was *Azotobacter vinelandii* NCIB 8789, obtained from The Ohio State University, Department of Microbiology, Culture Collection. Alginic acid was isolated and purified by repeated precipitation by EtOH from culture broths of *Azotobacter vinelandii* (NCIB 8789) grown in the medium described by Larsen and Haug[14]. The production medium was supplemented with 0.8mM $CaCl_2$. The purified alginic acid was partially hydrolyzed[15] in M oxalic acid for 10 h at 100° to obtain blocks of poly-GulA and poly-ManA. These two primarily homopolymeric species were isolated from the partial hydrolyzate by fractional precipitation at pH 2.85 and at pH 1.50 for poly-GulA and poly-ManA, respectively, as described by Haug and Larsen[16].

Monomeric α-L-guluronic and β-D-mannuronic acids were obtained by complete hydrolysis of alginic acid in H_2SO_4 and purified by anion-exchange chromatography according to the method of Haug and Larsen[16].

Sample preparation for n.m.r. analysis. — In order to obtain n.m.r. spectra of adequate resolution, and to ensure that the sizes of the polymers were below the cooperativity threshold[8] of about d.p. 24, it was necessary to hydrolyze the homopolymeric blocks further to an average degree of polymerization (d.p.) of 12. This was accomplished by hydrolysis in 0.1M HCl for 8 h at 100°. The d.p. was determined by ^{1}H-n.m.r., by comparison of the integral of the reducing-end H-1 signal to the integrals of other proton n.m.r. signals in the spectra. Prior to n.m.r. spectroscopy, samples were lyophilized, and exchangeable protons were equilibrated twice with 98% D_2O. The pD was adjusted to 5.0, the sample treated with Chelex-100 (hydrogen form), and the final pD adjusted to 6.5–7.0. A measured volume was filtered, lyophilized, and redissolved in an equal volume of 99.96% D_2O that was low in paramagnetic impurities (Aldrich Chemical Company). Samples were prepared in septum-capped 5-mm n.m.r. tubes, and degassed by sonication while bubbling a stream of dry argon gas through the solutions to remove dissolved oxygen, which causes paramagnetic line-broadening.

N.m.r. analysis. — One- and two-dimensional n.m.r. analyses were performed on either IBM AF-300 (7.1 Tesla) or Bruker AM-500 (11.8 Tesla) spectrometers in 5-mm sample tubes. Both instruments were equipped with the Bruker Aspect 3000 computer with process controller and array processor. Two-dimensional nuclear Overhauser effect correlation (NOESY)[13] spectra were acquired on the IBM instrument in phase-sensitive, pure absorption mode (TPPI), and crosspeaks were integrated as individual row/column slices or by volume integration of crosspeaks using the software routine resident in DISNMR. Two-dimensional COSY and NOESY data sets were 1K × 1K, and consisted of 512 t_1 increments. NOESY data were acquired with a 2-s preparatory delay between transients, during which time a presaturation irradiation was applied to the water resonance (power = 40 L). The mixing time in all of the NOESY experiments was randomly varied by + /− 20% to cancel *J*-type responses. For distance determinations, n.O.e. buildup rate series were obtained at 25, 50, 100, 150, 250, and 500 ms as well as 1-s mixing times. These experiments were conducted in series without removing the sample from the instrument so as to avoid introducing experimental variables. Data was acquired at 313K with temperature control. Chemical shifts are reported as δ in p.p.m. by reference to internal sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-d_4.

RESULTS AND DISCUSSION

Resonance assignments. — The downfield region of the ^{1}H-n.m.r. spectrum of alginic acid which contains signals corresponding to H-1 and H-5 of α-L-guluronic acid and H-1 of β-D-mannuronic acid is well characterized and has been used as the basis for determining the sequence of alginic acid[17–19]. The proton chemical-shift assignments and coupling constants of poly-GulA have been reported[18], and those of poly-ManA were partially assigned. All of these assignments were confirmed in our studies. Since conformational studies of alginic acid by ^{1}H-n.m.r. methods rely heavily on accurate assignments of chemical shifts and coupling constants, we employed two-dimensional homonuclear correlation spectroscopy (COSY)[20] and one-dimensional homonuclear

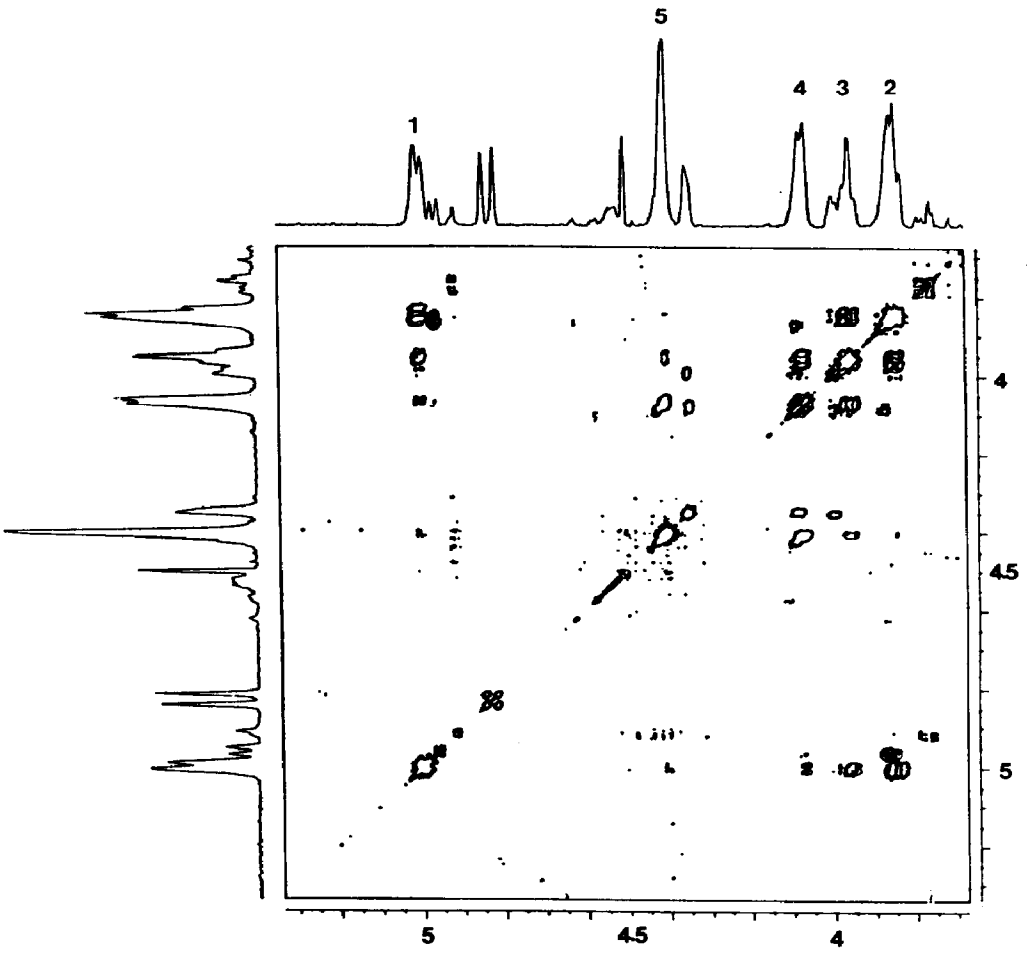


Fig. 2. Contour plot, COSY-90 spectrum of poly-GulA in D_2O.

decoupling experiments to confirm resonance assignments in the ^{1}H-n.m.r. spectrum of poly-GulA, and to complete the proton assignments of poly-ManA and α-L-guluronic acid. Nuclear Overhauser effect (n.O.e.) difference[21], 2D *J*-resolved[22], 2D double quantum filtration[23], and one-dimensional homonuclear decoupling experiments, together with coupling constants measured in resolution-enhanced one-dimensional ^{1}H-n.m.r. spectra of monomeric D-mannuronic acid led to the complete assignment of chemical shifts and coupling parameters of anomeric α-D-mannuronic and β-D-mannuronic acids, without the necessity of preparing and separating the two anomeric methyl glycoside derivatives.

The crosspeaks in the COSY-90 contour plot of poly-GulA (Fig. 2) immediately yield the assignment of proton connectivities. Coupling constants were measured accurately using the corresponding one-dimensional spectra.

The contour plot of the two-dimensional double-quantum proton[23] spectrum of

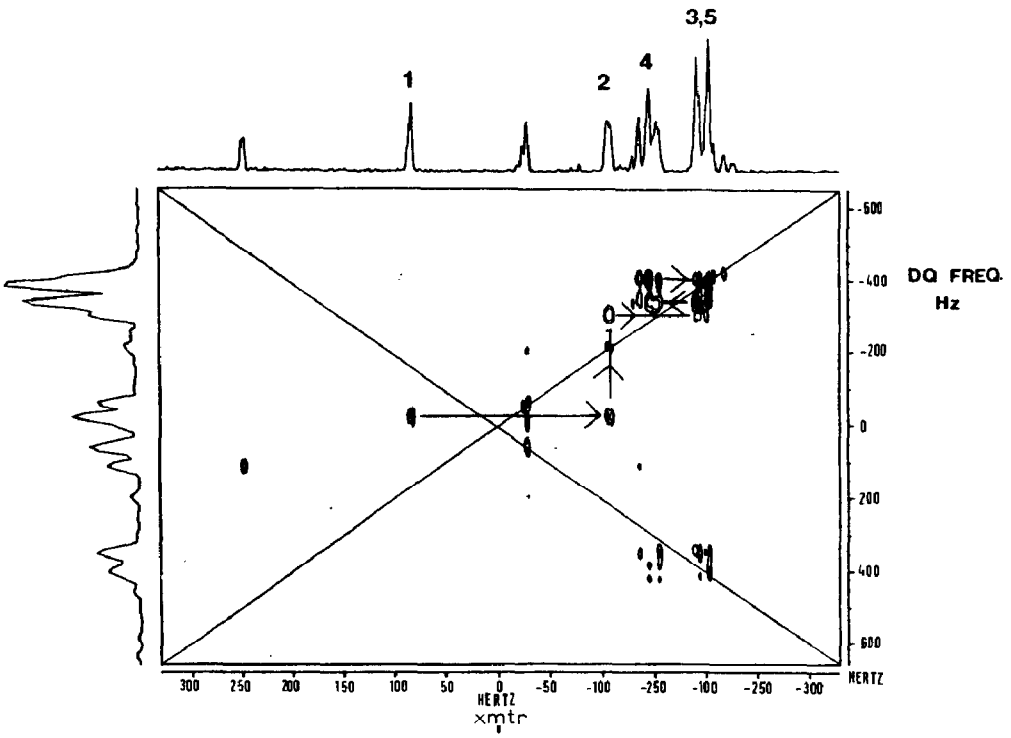


Fig. 3. Contour plot, 2D double-quantum analysis of poly-ManA coherence evolution delay optimized for $J_{H,H}$ = 5 Hz. "XMTR" denotes the position of the transmitter offset in Hz along the F2 axis. Double-quantum frequency in F1 = Ω_A(F2) + Ω_B(F2), where Ω_A and Ω_B are the proton chemical shifts (in Hz) from the transmitter center.

poly-ManA (Fig. 3) clearly shows connectivities between H-1–H-2 and H-3–H-4. In this spectrum, crosspeaks in F1 appear at their respective double-quantum frequencies, which occur at the algebraic sums of their chemical shifts in F2, correlated by their chemical shifts along F2. The correlated signals appear, as is typical, equidistant from a diagonal with slope = +/− 2. There appear to be two sets of connectivities between multiplets at δ 3.6 and 3.8. To confirm what appear to be H-3–H-4 and H-4–H-5 connectivities, one-dimensional homodecoupling experiments were conducted. Irradiation of the H-2 signal (δ 3.95) collapsed the H-3 signal (δ 3.66) into a doublet. This procedure allowed the H-5 proton signals (δ 3.65) to be observed. Irradiation of the signal for H-4 (δ 3.81) collapsed both the H-3 and H-5 signals. Likewise, irradiation of H-3 and H-5 signals collapsed both H-2 and H-4 resonances.

The COSY contour plot of a mixture of α- and β-L-guluronic acids likewise showed all of the proton connectivities in β-L-guluronic acid as well as the one between H-1 and H-2 of the α anomer. The signal for H-5 of the α anomer was buried in the δ 3.91–4.15 envelope, and consequently is not reported in Table I. The chemical-shift assignments for the latter were completed using one-dimensional homodecoupling experiments. Coupling constants were measured in resolution-enhanced one-dimen-

TABLE I

^{1}H-N.m.r. chemical shifts and coupling constants (J, Hz) for mannuronic and guluronic acids[a]

Proton	D-*Mannopyranuronic acid*		L-*Gulopyranuronic acid*[b]	
	α	β	α	β
H-1	5.20, d $J_{1,2}$ 1.2	4.84, d $J_{1,2}$ 1.2	5.17, d $J_{1,2}$ 4.2	4.84, d $J_{1,2}$ 8.4
H-2	3.88, dd $J_{1,2}$ 1.3 $J_{2,3}$ 2.1	3.91, dd $J_{1,2}$ 1.2 $J_{2,3}$ 3.0	3.91, t $J_{1,2}$ 4.2 $J_{2,3}$ 4.3	3.61, dd $J_{1,2}$ 8.4 $J_{2,3}$ 2.9
H-3	4.06, dd $J_{2,3}$ 2.1 $J_{3,4}$ 8.6	3.65, dd $J_{2,3}$ 3.0 $J_{3,4}$ 8.5	3.91, t $J_{2,3}$ 4.3 $J_{3,4}$ 4.2	4.05, dd $J_{2,3}$ 2.9 $J_{3,4}$ 4.0
H-4	3.84, t $J_{3,4}$ 8.6 $J_{4,5}$ 9.0	3.72, dd $J_{3,4}$ 8.6 $J_{4,5}$ 10.2	4.15, d $J_{3,4}$ 4.2	4.07, dd $J_{3,4}$ 4.0 $J_{4,5}$ 0.9
H-5	3.79, d $J_{4,5}$ 9.0	3.62, d $J_{4,5}$ 10.2		4.31, d $J_{4,5}$ 0.9

[a] Spectra were recorded at 313K and pD 7.0. d = doublet; dd = doublet-of-doublets; t = triplet.
[b] β-L-guluronic acid is the predominant form (75%) in solution.

sional ^{1}H-n.m.r. spectra of the anomeric mixture. β-L-Guluronic acid is the predominant anomeric form (75% by n.m.r.) in solution.

The more upfield H-1 signal in the spectrum of a mixture of α- and β-D-mannuronic acid was assigned to the β anomer, since H-1 has the axial orientation in this configuration and is corresponding shielded. The steady-state n.O.e. difference spectrum of β-D-mannuronic acid irradiated at the position of the H-1 resonance showed nuclear Overhauser enhancements of 11.5 and 19.0% at δ 3.91 and a cluster at δ 3.62–3.65, respectively. The resonance at δ 3.91 was assigned to H-2, and the assignment confirmed by one-dimensional homodecoupling experiments. The signals at δ 3.62–3.65 are most likely due to a near superimposition of H-3 and H-5, since both of these hydrogens are *syn*-axial with respect to H-1 in β-D-mannuronic acid in the $^4C_1$ conformation. Analysis of coupling information from high-resolution 1D spectra allowed the signal at δ 3.62 to be assigned to H-5, and the one at δ 3.65 to H-3. Based on the interproton distances and types of proton spin-systems predicted, each of H-1, H-3, and H-5 should experience approximately equal n.O.e. (9.5% in this case), as long as the duration of irradiation is kept short to avoid spin-diffusion effects. Irradiation of the H-3(5) resonances in a one-dimensional homodecoupling experiment established the position of the H-4 resonance of β-D-mannuronic acid to be δ 3.72.

The n.O.e. difference spectra of α-D-mannuronic acid irradiated at δ 5.20 (H-1) and δ 3.84 (H-4) showed n.O.e.'s of 9.2 and 6.6% at δ 3.88 and δ 4.06, respectively. The

resonance at δ 3.88 was therefore assigned to H-2, and that at δ 4.06 to H-3. When the H-3 resonance was irradiated, a n.O.e. of 3.5% was observed at δ 3.88 (H-2) as well as 10% at δ 3.79. Since H-3 is *syn*-axial to H-5 in α-D-mannuronic acid, the resonance at δ 3.79 was assigned to H-5. Irradiation of the H-3 resonance yielded the assignment of the H-4 signal, δ 3.84.

Complete assignments of the proton chemical shifts and accurate coupling constants of the α-D-mannuronic acid mixture were verified from a two-dimensional *J*-resolved spectrum.

Tables I and II show the ^{1}H-n.m.r. assignments of guluronic and mannuronic acids, and poly-GulA and poly-ManA, respectively. The small coupling constant between H-3 and H-4 of poly-GulA and L-guluronic acid is indicative of the $^1C_4$ conformation in both polymer and the monomer. The large coupling constants between H-3 and H-4 as well as between H-4 and H-5 of poly-ManA and monomeric mannuronic acids indicate the $^4C_1$ conformation for these species. The β anomer is the predominant form of monomeric L-guluronic acid in solution, as evidenced by an 8.4-Hz coupling between the H-1 and H-2 signals in the major species (75% by n.m.r., Table I). Monomer residues in the monomers and the polymers were found to exist solely in the $^1C_4$ conformation for guluronate, and the $^4C_1$ conformation for mannuronate. Sample sizes and signal-to-noise ratios in all n.m.r. spectra were sufficiently large that were any minor contributions from oppositely configured residues present, they would have been detected by their different coupling constants, multiplicities, and chemical shifts, as long as their interconversion rates were within the n.m.r. time-scale. A time-averaged distribution of oppositely configured species in unlikely, since chemical shifts, coupling constants, and multiplicities were invariant over a range of sample temperatures and pH values. The $^1C_4$ (GulA) and $^4C_1$ (ManA) conformations are energetically favored, as they allow the bulky carboxylate group to remain equatorial, and in the polymers give

TABLE II

^{1}H-N.m.r. chemical shifts and coupling constants of poly-GulA and poly-ManA[a]

Proton	L-*Guluronan*		D-*Mannuronan*	
H-1	5.03, d	$J_{1,2}$ 4.4	4.57, s	
H-2	3.89, dd	$J_{1,2}$ 4.4 $J_{2,3}$ 3.7	3.95, d	$J_{1,2}$ 0 $J_{2,3}$ 3.3
H-3	3.99, dd	$J_{2,3}$ 3.7 $J_{3,4}$ 4.2	3.66, dd	$J_{2,3}$ 3.4 $J_{3,4}$ 9.2
H-4	4.10, dd	$J_{3,4}$ 4.2 $J_{4,5}$ 0	3.81, dd	$J_{3,4}$ 9.2 $J_{4,5}$ 9.6
H-5	4.44, s		3.65, d	$J_{4,5}$ 9.6

[a] Spectra were recorded at 313K and pD 7.0. d = doublet; dd = doublet-of-doublets; s = singlet.

rise to diequatorial glycosidic linkages in poly-ManA and diaxial linkages in poly-GulA.

Conformational studies of uronic acid residues in the homopolymeric blocks of alginic acid. — Titration of the homopolymeric blocks with Ca^{2+} was monitored using ^{1}H-n.m.r. spectroscopy. No significant changes in ^{1}H–^{1}H coupling constants were observed in poly-GulA and poly-ManA when the Ca^{2+}/uronate ratios were 0.7 or 1.3, respectively. This suggests that Ca^{2+} binding does not cause conformational distortion of the rings of these two structures. A higher level of Ca^{2+} addition was not possible for the poly-GulA sample because precipitation occurred. This phenomenon is typical for low-d.p. guluronate fragments during complexation with calcium. In this case, precipitation appears to be due to polyelectrolyte factors[6,8] and not cooperative dimerization between the short GulA chains, since no n.m.r. line-broadening or changes in the apparent T_1 values were observed. Such changes would be expected for a dimerization process, as the molecular weight of the molecule would effectively double and the rotational correlation time would increase. This data supports the notion that the d.p. of the samples is below the cooperativity threshold.

A downfield shift of all protons was observed in spectra of both samples upon addition of Ca^{2+}, due to proton-deshielding effects resulting from Ca^{2+} complexation with the hydroxyl and carboxylate groups of the uronic acids. The largest changes occur

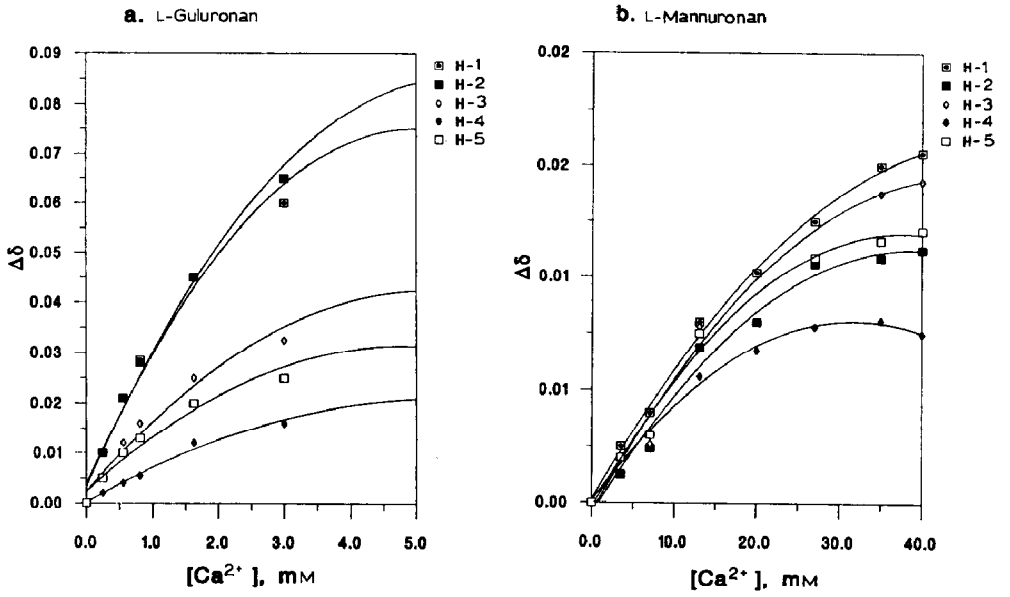


Fig. 4. (a) A poly-GulA solution (effective guluronic acid monomer concentration = 8.5mM, d.p.$_n$ = 12) in 5mM imidazole buffer, pD 7.0, was titrated with $CaCl_2$ solution at 313K and spectra recorded using a Bruker AM 500. The changes in chemical shifts are plotted against Ca^{2+} concentration. (b) A poly-ManA solution (effective mannuronic acid monomer concentration = 30mM, d.p.$_n$ = 12) in 50mM imidazole buffer, pD 7.0, was titrated with $CaCl_2$ solution at 313K and spectra recorded using a Bruker AM500. The changes in chemical shifts are plotted against Ca^{2+} concentration.

for H-1 and H-2 of poly-GulA (Fig. 4a) and for H-1, H-3, H-5, and H-2 of poly-ManA (Fig. 4b).

Three model coordination arrangements for complexing polyols with cations have been described[24,25]. One involves complexation with the three *syn*-axial oxygen atoms on a six-membered ring in the chair conformation[24,25]. A second model considers complexation with an axial–equatorial–axial arrangement of three oxygen atoms on the six-membered ring in the chair form[24–29]. In either arrangement, the distance between any two oxygen atoms is[25] ~ 2.9 Å. In pyranosyluronic acids, a third type of complexation with metal ions is possible, involving an axial hydroxyl group, one of the oxygen atoms of the adjacent equatorial carboxyl group, and the ring oxygen (O-5). Such an arrangement is found in β-D-galacturonic acid, and involves[29] O-4, O-5, and O-6.

Site of Ca^{2+} interaction in poly-GulA. — There are two possible sites within an α-L-guluronic acid residue that are suitable for complexation with Ca^{2+}: O-1–O-2–O-3 (the axial–equatorial–axial model); and O-4–O-5–O-6 (the pyranosyluronic acid model). The largest chemical-shift change occurred for the resonances of H-1 and H-2, suggesting that the primary binding site for Ca^{2+} involves O-1 and O-2 of α-L-guluronic acid. In *epi*-inositol[25], Ca^{2+} forms an axially-symmetric complex with O-2, O-3, and O-4 as evidenced by the chemical-shift change pattern (H-3 > H-2 = H-4). In the Ca^{2+} · poly-GulA complex (Fig. 4a), however, an unsymmetrical complex is indicated by the pattern (H-2 = H-1 > H-3). In an axially symmetric Ca^{2+} complex, one would predict a chemical-shift change pattern of H-2 > H-1 = H-3. Similar unsymmetrical complexes of Eu^{3+} . methyl α-D-gulopyranoside and Pr^{3+} . methyl α-D-gulopyranoside were reported[28] with calculated O-1, O-2, and O-3 to cation distances of 1.8, 1.8, and 3.2 Å, and 1.7, 1.85, and 3.0 Å, respectively.

Shown in Table III are relative intensities of crosspeaks in the phase-sensitive 2D NOESY experiments on poly-GulA and poly-GulA plus Ca^{2+}. A plot of the NOESY spectrum for poly-GulA is shown in Fig. 5, with the correlations indicated. It should be noted that all n.O.e. values for the polymers were moderately negative, consistent with the prediction that the molecular rotational correlation-time is slightly into the slow-tumbling regime. The fact that overall the n.O.e. values do not approach − 100% more closely indicates that in the d.p. 12 range, the correlation time of alginate fragments is not very far into the slow-tumbling regime. The n.O.e. spectra for the two samples were intensity-normalized, and the volume integrals of the corsspeak between H-1 and H-2 were found to be of about the same magnitude in both the poly-GulA and poly-GulA plus Ca^{2+} spectra. To compensate for potential errors in integration with the Bruker routine, volume integrals were cross-checked with integrated contour slices. For comparison, the H-1–H-2 crosspeak in the non-Ca^{2+}-complexed sample was used as a reference, and all n.O.e. values were normalized to it. Among the n.O.e. crosspeaks listed in Table III, the only intra-ring interactions possible could be derived from H-1–H-2 and from H-4–H-5. A comparison, however, of the relative magnitudes of the H-1–H-2 *versus* the H-4–H-5 n.O.e.'s indicates a significantly stronger value for the H-4–H-5 crosspeak. If both sets of interactions were intra-ring effects, the magnitudes of the n.O.e. values would be similar. This suggests, therefore, that the interactions

TABLE III

Relative intensities of NOESY crosspeaks of poly-GulA in the presence and absence of Ca^{2+}

Crosspeak[a]	*Poly-GulA*[b]	*Poly-GulA · Ca^{2+}*[c]
1,2	1.00	1.00
1,4	1.53	0.84
1,5	1.73	0.22
2,5	1.03	0.09
3,5	1.69	0.31
4,5	3.46	1.89

[a] 2D n.O.e. crosspeaks were integrated and expressed relative to the H-1–H-2 crosspeak integral measured in the absence of Ca^{2+}. The value of the 1,2 crosspeak was taken as 1.00. Spectra were normalized to the same intensity factor. [b] Phase-sensitive NOESY of poly-GulA (concentration of monomeric unit 102mM) in 50mM imidazole buffer, pD 7.0 at 313K, 200-ms mixing time. [c] Phase-sensitive NOESY of poly-GulA (concentration of monomeric unit 102mM) with $CaCl_2$ (9mM) in 50mM imidazole buffer, pD 7.0 at 313K, 200-ms mixing time.

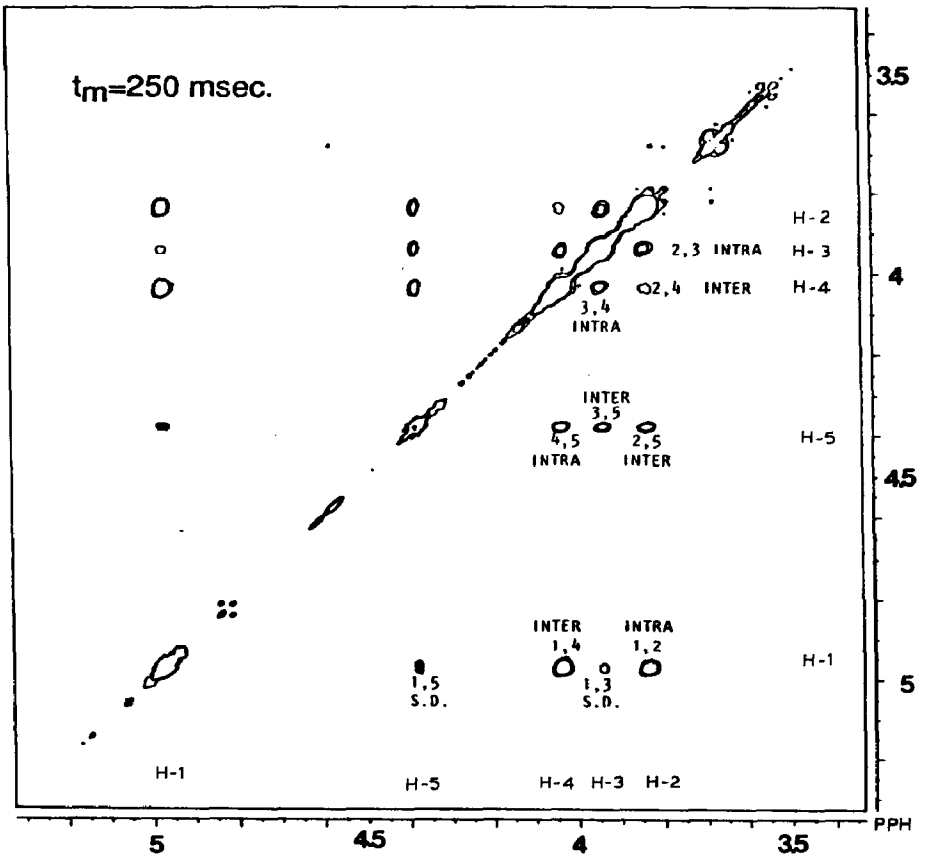


Fig. 5. Phase-sensitive 2D NOESY spectrum (250 ms) of poly-GulA in D_2O, indicating n.O.e. correlations.

between H-4 and H-5 have significant contributions from inter-ring effects. The other proton pairs listed in Table III have predicted intra-ring distances >3.5 Å, which suggests that the observed n.O.e.'s in these cases are also due to inter-ring effects.

Upon addition of Ca^{2+}, a marked decrease in absolute n.O.e. value is observed for all of the proton pairs *except* H-1–H-2. This observation is consistent with the notion of n.O.e. changes stemming from alterations of inter-ring proton–proton distances, rather than from line broadening due to the addition of Ca^{2+} or cooperative effects. Molecular modeling yields a conformation consistent with that proposed by Smidsrød *et al.*[30], where a hydrophilic pocket consisting of O-1, O-2, and O-3 of one ring plus O-5 and O-6 of the adjacent ring is present. The result of the addition of Ca^{2+} in this model would be a tightening of the hydrophilic pocket, and an increase in the inter-ring proton distances. Since the buildup rate of an n.O.e. depends upon r^{-6} (where "r" is the interproton distance), such a conformational chance would lead to the observed changes in n.O.e. values for these proton pairs.

Site of Ca^{2+} interaction in poly-ManA. — Titration of poly-ManA with Ca^{2+} results in proton chemical-shift changes as described in Fig. 4b. H-1, H-2, and H-5 exhibit larger shifts than H-4. Examination of individual β-D-mannosyluronic residues in a model of this homopolymer shows that the O-2–O-5 and O-2–O-1 distances are ~ 2.9 Å and the O-1–O-5 ~ 2.4 Å. Calcium binding to O-1, O-2, and O-5 is possible and entropically favorable, and may explain why H-1, H-5, and H-2 exhibit larger chemical-shift changes compared to H-4 (Fig. 4b). However, it does not explain why the chemical-shift change at H-3 is almost as large as that at H-1. X-Ray fiber diffraction studies of β-D-mannuronan indicate a sheet-like structure with hydrogen bonding between O-3(H) and O-5 within the chain[31]. It becomes apparent, again from model building studies, that it is possible for Ca^{2+} to interact with O-1, O-5, and O-2 of one residue and O-3′ of the next, with relatively shorter Ca^{2+}–O-5 and Ca^{2+}–O-1 distances. This model could explain the relative proton chemical-shift changes for poly-ManA in the presence of the cation.

Stoichiometry and dissociation constants. — The plots of concentration ratios of Ca^{2+}/monomer unit of poly-GulA and poly-ManA against ^1H chemical shift show characteristics of a system having more than one stoichiometry and dissociation constant (Figs. 6a and 6c). The first four non-zero points on each curve are linear, indicating that one type of complex predominates for this range of $[Ca^{2+}]$/[uronic acid] ratios. Additionally, the double-reciprocal plots of these initial points are linear, indicating that cooperativity is absent.

The stoichiometry of binding was determined at low $[Ca^{2+}]$/[uronic acid]. Plots of $1/[Ca^{2+}]$ *vs.* $1/\Delta\delta$ H-2 for poly-GulA (Fig. 6b) and $1/[Ca^{2+}]$ *vs.* $1/\Delta\delta$ H-1 for poly-ManA (Fig. 6d) were extrapolated to obtain the maximum obtainable chemical shifts (as the inverse of the Y-intercept) at infinite $[Ca^{2+}]$ for the fully bound complex formed at low $[Ca^{2+}]$/[uronic acid]. The $\Delta\delta$ H-2_{max} for poly-GulA was 0.09 p.p.m., whereas $\Delta\delta$ H-1_{max} for poly-ManA was 0.02 p.p.m. These values were then used to determine the ratio of Ca^{2+} to uronic acid in the complex at infinite $[Ca^{2+}]$ using plots of $\Delta\delta$ H-2 *vs.* $[Ca^{2+}]$/[guluronic acid] (Fig. 6a) or $\Delta\delta$ H-1 *vs.* $[Ca^{2+}]$/[mannuronic acid] (Fig. 6c). The

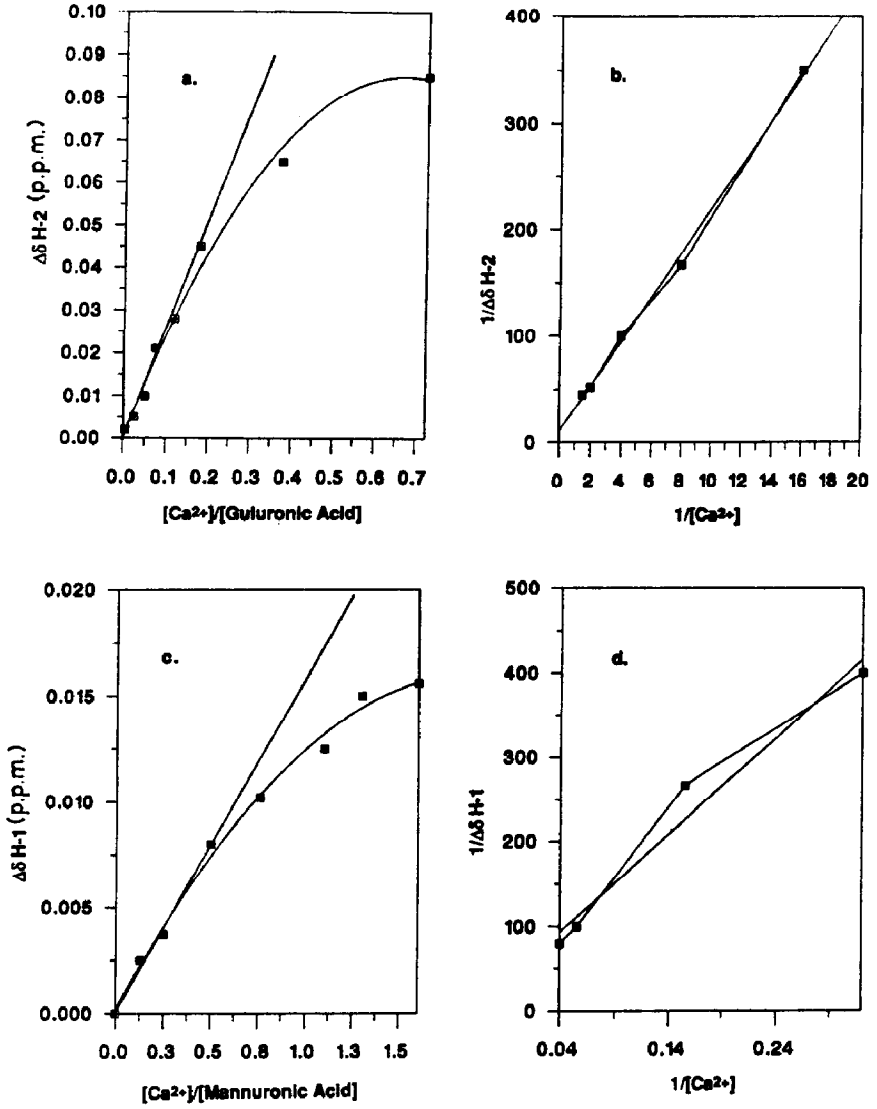


Fig. 6. (a) Poly-GulA titration, $\Delta\delta$ H-2 plotted against $[Ca^{2+}]/[$guluronic acid$]$. (b) Poly-GulA titration, reciprocal of chemical-shift change plotted against the reciprocal of $[Ca^{2+}]$. (c) Poly-ManA titration, $\Delta\delta$ H-1 plotted against $[Ca^{2+}]/[$mannuronic acid$]$. (d) Poly-ManA titration, reciprocal of chemical shift change plotted against the reciprocal of $[Ca^{2+}]$.

values of $[Ca^{2+}]/[$uronic acid$]$ obtained at $\Delta\delta\ H_{max}$ were found to be 0.25 for poly-GulA and 1.2 for poly-ManA. The dissociation constants were estimated from the relationship:

$$K_d = \frac{[Ca^{2+}]_{free} * [Sites]_{free}}{[Poly\text{-}X \cdot Ca^{2+}]}$$

where

$$\text{Sites} = \frac{[\text{Monomeric units}]}{\text{Monomeric units/Site}}$$

The estimated dissociation constants obtained were 2×10^{-4}M for poly-GulA, and 1×10^{-3}M for poly-ManA. The estimate for poly-GulA must be considered in light of the possibility for cooperative effects, but since the n.m.r. experiments appear to rule these out it should be reasonable.

DISCUSSION

In order to examine the effects of calcium on the structure of alginic acid it was necessary to establish the complete assignments for the ^{1}H-n.m.r. spectrum. Chemical shifts and coupling constants for poly-GulA, poly-ManA, D-mannuronic acid, and D-guluronic acid were assignable in a straightforward fashion on the basis of two-dimensional n.m.r. spectra. These data were used to examine the effects of Ca^{2+} on the ring conformation by looking for changes in the coupling constants or multiplicity patterns of the monomeric units of poly-GulA and poly-ManA. No significant changes

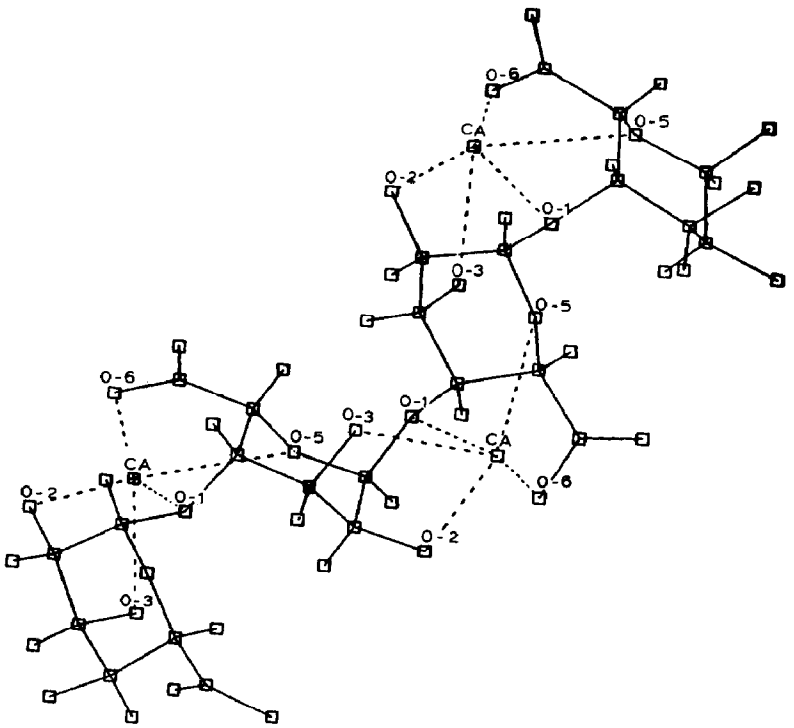


Fig. 7. Plot of an n.m.r.-constrained minimum-energy structure for poly-GulA "tetramer" coordinated to calcium cation.

in these parameters were observed, indicating that the ring conformations are not altered by the addition of Ca^{2+}.

An examination of the NOESY spectra of poly-GulA in the presence and absence of Ca^{2+} provides information pertinent to the secondary structure of the polymer. The spectra of both polymers indicate that even in the absence of the metal ion there are inter-ring proton–proton interactions. This implies that in solution the polymers are not totally random structures, but instead are conformationally constrained, probably by inter-residue hydrogen bonding.

The evaluation of the structure by a molecular mechanics–graphics routine (CHEM-X; MM2 program) shows that a reasonable model for poly-GulA involves the formation of a hydrophilic pocket comprised of O-1, O-2, and O-3 of one ring, plus O-5 and O-6 of the adjacent ring (Fig. 7). Upon binding of Ca^{2+} to the hydrophilic pocket, the rings are brought closer together by virtue of induced changes in the transglycosidic dihedral angles φ (H-1–C-1–O-1–C-4′) and ψ (C-1–O-1–C-4′–H-4′). The distances of O-1 and O-2 from the Ca^{2+} ion constitute the shortest metal–oxygen distances in the hydrophilic pocket of poly-GulA.

The data on calcium binding to poly-ManA suggest that inter-ring effects also play a role in the secondary structure. The calcium binding site appears to consist of O-1, O-2, and O-5 of one ring and O-3′ of the next (Fig. 8). In order to develop a complete understanding of the secondary structure of this polymer additional n.O.e. data are needed.

The spectral data indicate that, as expected, the ring conformation of the β-D-mannosyluronic residues in poly-ManA are in the $^4C_1$ conformation, whereas the

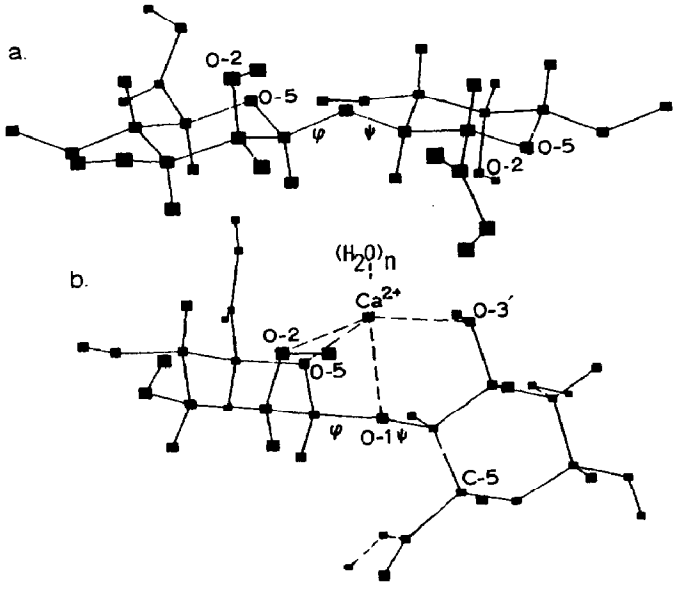


Fig. 8. Plot of two n.m.r.-constrained minimum-energy structures for poly-ManA "dimer" in (a) the absence of cations and (b) coordinated to Ca^{2+}.

L-guluronic acid residues in poly-GulA are $^1C_4$. This implies that as the polymer is formed and the β-D-mannosyluronic residues are converted to α-L-gulosyluronic residues, there is a substantial change in the local three-dimensional structure of the polymer. Conclusions about the higher-level organization of poly-GulA and poly-ManA which would explain their Ca^{2+} binding stoichiometries cannot be drawn at this time. However, this information, together with the estimated dissociation constants for the calcium complexes of the two homopolymers (2×10^{-4}M for poly-GulA and 1×10^{-3}M for poly-ManA) are important in determining the proportion of calcium bound to different regions of alginic acid. Although calcium binding does not alter the ring conformations of the constituent sugars, our data indicate that it does induce changes in the conformation of the chain within the two types of homopolymeric regions. The different chain conformations may result in alterations in the local binding constants for the formation of the complex between the epimerase and the polysaccharide, as well as the off-rate of the product, and may explain the changes in the action pattern of the enzyme. If calcium ion is present as an essential cofactor at the active site of the enzyme, alterations in binding behavior on a local level may well translate into the ability of the epimerase to operate either in a processive fashion[12], or *via* a random mechanism.

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