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X-Ray and computer modeling studies on gellan-related polymers: Molecular structures of welan, S-657, and rhamsan*

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ABSTRACT

The primary structures of the four bacterial polysaccharides gellan, welan, S-657, and rhamsan are the same with respect to their backbones, but have different side-chains. This difference has a profound influence on their behavior in aqueous media. Solutions of gellan gum form stable aqueous gels under appropriate ionic conditions. By contrast, welan, S-657, and rhamsan do not gel but give very viscous solutions over a wide range of thermal, pH, and salt conditions. X-Ray fiber diffraction analysis and computer modeling of these branched polysaccharides demonstrate that they all have the same half-staggered, double-helical conformations as in the unbranched gellan, suggesting, therefore, that the side chains are responsible for diminishing gelling behavior. Depending on the size and location, the side chains shield the carboxylate groups to varying degrees; this shielding is substantial in welan and S-657, but less in rhamsan. In all cases, side-chain main-chain interactions within the double helix prevent the carboxylate-mediated aggregation of double helices that is necessary for the gelation.

INTRODUCTION

Polysaccharides are widely used in a variety of commercial applications for their diverse rheological properties. Most of these polysaccharides are mainly derived from higher plants (e.g., starch, (*O*-carboxymethyl)cellulose, guar gum, pectin, and locust gum) or from marine algae (such as, alginate, agar, and carrageenan). In recent years, however, there has been increasing interest and industrial success in utilizing capsular and extracellular polysaccharides produced by microorganisms. For example, xanthan gum, elaborated by *Xanthomonas campestris*, already enjoys extensive practical applications; xanthan's commercial success is due to its rheology, salt tolerance, and thermal stability[1]. The search for useful new microbial polysaccharides has yielded the gel-forming, extracellular polysaccharide gellan from *Pseudomonas elodea* and the non gel-forming, capsular polysaccharides S-657 from a strain of *Xanthomonas*, and welan and rhamsan from *Alcaligenes*. They are important potentially because of their solution and/or gelling properties[1,2]. All four have similar, but distinct, chemical repeating-

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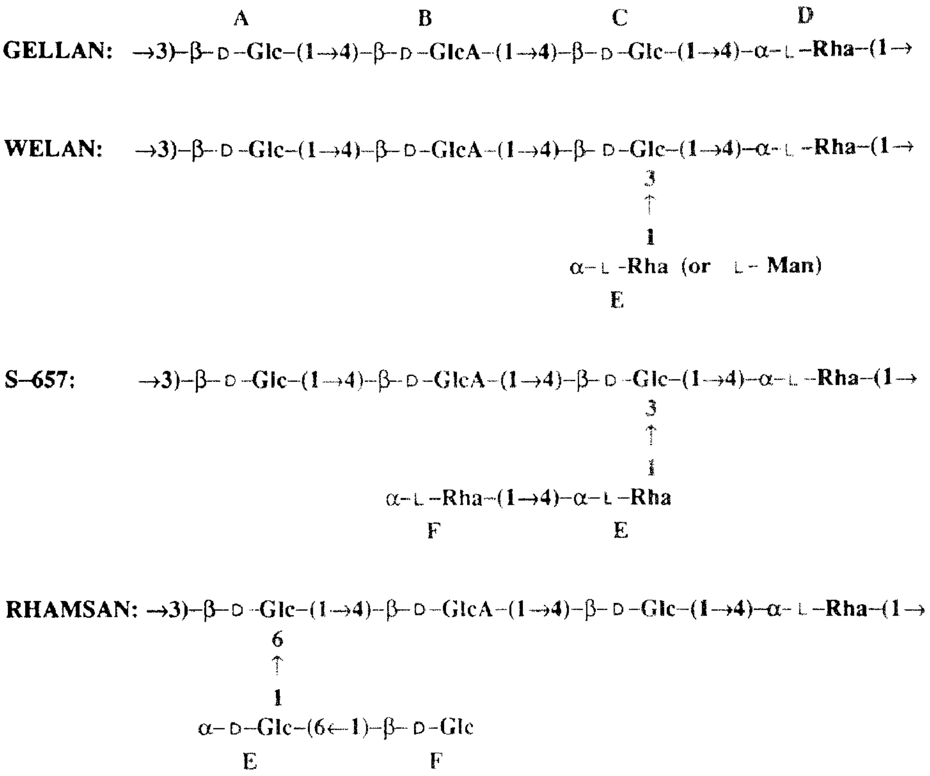


$$\textbf{GELLAN: } \rightarrow 3)\text{-}\overset{\text{A}}{\beta\text{-D-Glc}}\text{-}(1\rightarrow 4)\text{-}\overset{\text{B}}{\beta\text{-D-GlcA}}\text{-}(1\rightarrow 4)\text{-}\overset{\text{C}}{\beta\text{-D-Glc}}\text{-}(1\rightarrow 4)\text{-}\overset{\text{D}}{\alpha\text{-L-Rha}}\text{-}(1\rightarrow$$

$$\textbf{WELAN: } \rightarrow 3)\text{-}\beta\text{-D-Glc-}(1\rightarrow 4)\text{-}\beta\text{-D-GlcA-}(1\rightarrow 4)\text{-}\underset{\substack{3\\ \uparrow\\ 1\\ \alpha\text{-L-Rha (or L-Man)}\\ \text{E}}}{\beta\text{-D-Glc}}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha-}(1\rightarrow$$

$$\textbf{S-657: } \rightarrow 3)\text{-}\beta\text{-D-Glc-}(1\rightarrow 4)\text{-}\beta\text{-D-GlcA-}(1\rightarrow 4)\text{-}\underset{\substack{3\\ \uparrow\\ 1\\ \alpha\text{-L-Rha-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}\\ \text{F}\qquad\qquad\qquad \text{E}}}{\beta\text{-D-Glc}}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha-}(1\rightarrow$$

$$\textbf{RHAMSAN: } \rightarrow 3)\text{-}\underset{\substack{6\\ \uparrow\\ 1\\ \alpha\text{-D-Glc-}(6\leftarrow 1)\text{-}\beta\text{-D-Glc}\\ \text{E}\qquad\qquad\qquad \text{F}}}{\beta\text{-D-Glc}}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-GlcA-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha-}(1\rightarrow$$

Fig. 1. Chemical repeat of unbranched gellan and branched welan, S-657, and rhamsan. The residues marked A, B, C, and D in the main chain of gellan are common to all the polymers. The side-chain residues are marked E and F in the branched polymers.

units[2]. Gellan is a polymer consisting of a linear tetrasaccharide repeating unit ABCD as shown in Fig. 1. It forms strong and brittle aqueous gels at low polymer concentration in the presence of many monovalent or divalent ions[3]. The other three have branching along the same repeating main-chain. The close chemical relationship of the branched polysaccharides to the unbranched gellan is illustrated in Fig. 1. Welan has a monosaccharide (α-L-rhamnose or L-mannose) side-chain E linked to O-3 of residue C. The distribution is such that approximately 2/3 of the side groups are rhamnose and the remainder mannose residues. S-657 could be considered a derivative of welan in that it has an additional α-L-rhamnose (F) linked to O-4 of the side-chain rhamnose E. It should be noted that S-657 does not contain any mannose residues in its side chains. In the case of rhamsan, a disaccharide side-chain (FE), consisting of β-D-glucose-(1→6)-α-D-glucose, is linked to O-6 of every glucose residue A. Because of the presence of two (1→6) linkages, this side chain is conformationally more flexible than the rest. All of these polymers having side chains form only non-gelling, aqueous solutions which are usually viscous. This implies that the side chains in someway interfere with the gelation process[4,5].

The gellan family of polysaccharides has been the target of several structural investigations using a variety of experimental probes. For example, before the detailed three-dimensional structure of gellan was shown to be a 3-fold double helix[6,7], a 2-fold helical conformation with a pitch of 3.66 nm was proposed for welan based on X-ray diffraction patterns obtained from uniaxially oriented, noncrystalline specimens[8]; likewise, molecular models were also proposed for gellan itself which did not provide any acceptable fit with the good quality diffraction patterns[9]. Viscometric, calorimetric, and chiroptical data have been used to compare the solution properties of gellan, welan, and rhamsan[4,5]. According to these studies, conformational ordering exists only in the case of gellan; both welan and rhamsan, however, behave like disordered polymers. While all the observed data for welan are those expected for a disordered, neutral polysaccharide, those for rhamsan are typical of a disordered polyelectrolyte[5]. Such properties have been attributed to the different modes of branching in these polymers.

In order to determine, at the molecular level, the specific structural roles of the side chains on the observed physical properties, we have embarked on detailed X-ray fiber diffraction and computer modeling studies of welan, S-657, and rhamsan. The results indicate that all these polymers can adopt the same gellan-like double helix. Further, the side chains in welan and S-657 are folded towards the glucuronate residues in the main chains, but those in rhamsan are not. The dispositions and environment of the carboxylate groups relative to the side chains have been identified. They seem to be responsible for the observed gelling or non-gelling properties of the polymers.

EXPERIMENTAL

Sample preparation. — Samples of welan, S-657, and rhamsan were gifts from Dr. Ralph Moorhouse (Kelco, Division of Merck & Co., San Diego, CA). Each polymer was converted into a monovalent salt by dialyzing its aqueous solution (typically ~0.3 mg/mL) against 10–20 mM KF or NaCl (4 × 250 mL) at room temperature. Any excess salt was removed by repeated dialysis against distilled water. The polysaccharide was then freeze dried. Fibers suitable for X-ray diffraction were stretched from concentrated solutions of these polymers using conventional fiber pullers. The density of the fiber was measured by the flotation method using a mixture of bromoform and benzene.

X-Ray intensity data. — Diffraction patterns from well stretched and oriented fibers were recorded in flat-film cameras using nickel-filtered CuKα radiation of wavelength 0.15418 nm from sealed-tube or microfocus X-ray generators. Typically, the film-to-fiber distance was ~39 mm. The camera was flushed continuously with helium, bubbling through a saturated salt solution that gives the desired humidity, in order to retain the fiber at a controlled moisture level. The film was standardized by dusting the fiber with calcite powder (of characteristic spacing 0.3035 nm) for the measurement of unit-cell dimensions.

The best diffraction patterns to date of welan (Fig. 2a), S-657 (Fig. 2b), and rhamsan (Fig. 2c) are characteristic of non-crystalline, but reasonably oriented polymer systems. However, such features as the overall intensity distributions including the

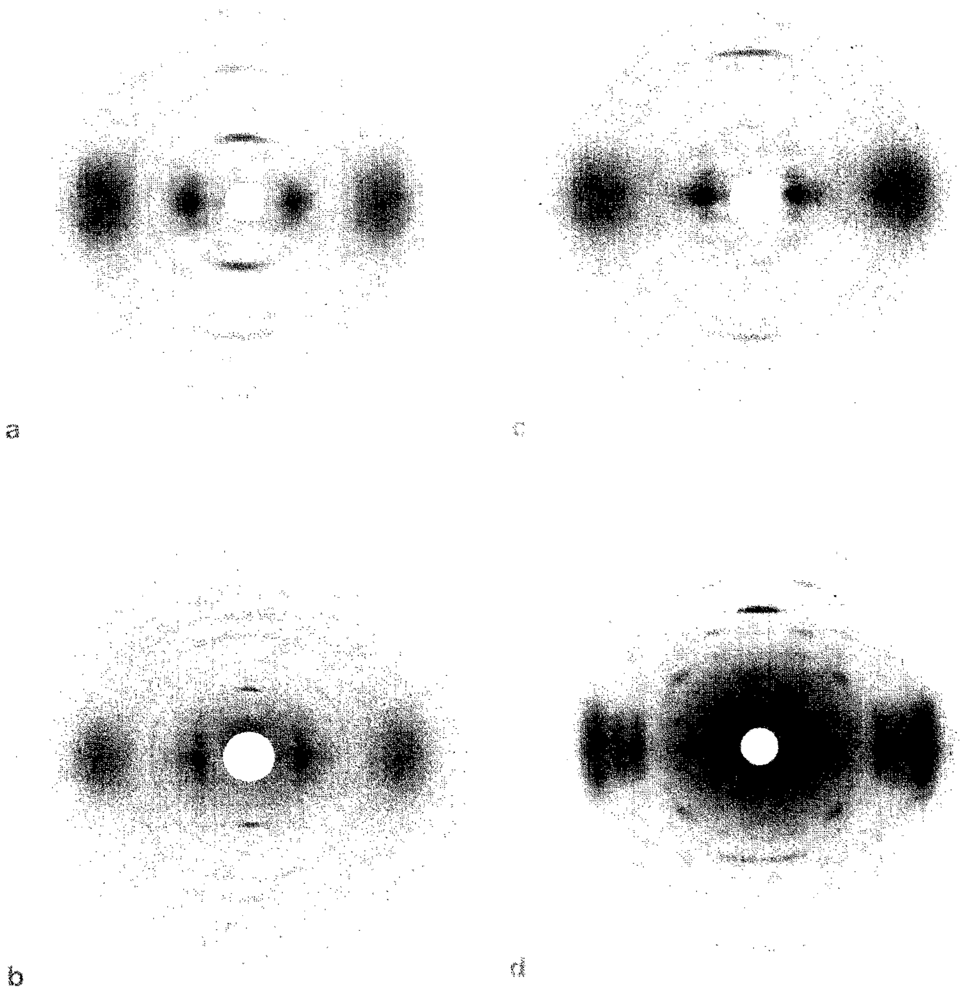


Fig. 2. X-Ray fiber diffraction patterns from oriented and noncrystalline samples of (*a*) welan, (*b*) S-657, and (*c*) rhamsan are similar in intensity distribution to that from an oriented and polycrystalline sample of (*d*) the rubidium salt of gellan.

meridional reflection on the third layer-line and the layer-line spacings are of the same kind as those observed in the diffraction patterns for a number of monovalent salts of gellan which have been prepared as polycrystalline and oriented specimens[10]. An example of such a pattern from the rubidium salt of gellan is shown in Fig. 2d for comparison. These observations imply that the molecular structures of welan, S-657, and rhamsan are indeed similar to the gellan double helix.

Molecular model building and structure analysis. — Double-helical models of each branched polymer consisting of two left-handed, three-fold helical polysaccharide chains of pitch 5.6 nm and having the same molecular geometry as gellan[7] were generated by the linked-atom least-squares procedure[11,12]. Throughout this analysis, the main-chain double helix was treated as a rigid body. Side chains were affixed appropriately and their conformations about the glycosidic bonds and C-5–C-6 bonds were systematically refined to remove steric anomalies. This was accomplished by minimizing the steric compression within the helix. The function used for this minimization is given by

$$\Omega = \sum_i e_i\,({}_0\theta_i - \theta_i)^2 + \sum_j c_j\,({}_0d_j - d_j)^2$$

The basic principles of the least-squares refinement are as follows: during the refinement, the conformation angle θ_i is elasticated to an ideal value ${}_0\theta_i$ with weight e_i. For example, the ideal (φ, ψ) values at any given linkage correspond to the center of the sterically allowed region in the hard-sphere map of the disaccharide unit. Thus, the first term allows the favored conformational domains, if any, to be preserved in the final model. The second term in the expression helps to relieve any short contact d_j by driving it towards an acceptable lower limit ${}_0d_j$ by using a suitable force constant c_j. Due to the nature of the X-ray data, which do no permit the precise definition of constraining unit cells, no attempts were made to adjudicate between alternatives when more than one side-chain conformation was sterically possible for a polymer. In the case of welan, the monosaccharide side-chain was treated as α-L-mannose in the modeling so that the final comformation is valid for α-L-rhamnose as well.

RESULTS

The unit-cell dimensions and their (large) estimated standard deviations (Table I) for the branched polymers were determined from the layer-line positions and from the small number of diffuse spots (Fig. 2). Those of gellan which does not have any substituents, and native gellan which has glycerate and acetate substituents, obtained from very good quality diffraction patterns, are also given in Table I along with their (small) estimated standard deviations. Examination of these parameters and the fiber densities (given in the last column) indicate that all the polymers have very similar crystal-packing arrangements with two molecules in the unit cell, as in the case of gellan[6,7] and native gellan[13].

TABLE I

Unit-cell (trigonal) dimensions and fiber densities of welan, S-657, and rhamsan (present study) compared with those of gellan[7] and native gellan[13]

Polymer	a *(nm)*	c *(nm)*	*Density (g/mL)*
Welan (Na^+)	1.560 (17)	2.815[a]	1.52
S-657 (K^+)	1.578 (26)	2.815[a]	1.43
Rhamsan (Na^+)	1.559 (19)	2.815[a]	1.47
Gellan (K^+)	1.575 (04)	2.815 (08)	1.49
Native gellan (K^+)	1.647 (05)	2.842 (07)	1.57

[a] Value fixed as in potassium gellan.

TABLE II

Side-chain conformation angles (in degrees) in the final models of (*a*) welan and S-657, and (*b*) two selected models of rhamsan

Conformation angle		*Model*	
(*a*)		Welan	S-657
ψ_E	(C-1E–O-3C–C-3C–C-2C)	−74	−69
φ_E	(O-5E–C-1E–O-3C–C-3C)	−37	−33
ψ_F	(C-1F–O-4E–C-4E–C-3E)		−100
φ_F	(O-5F–C-1F–O-4E–C-4E)		−100
(*b*)		Rhamsan model 1	Rhamsan model 2
χ_A	(O-6A–C-6A–C-5A–C-4A)	−81	−81
ψ_E	(C-1E–O-6A–C-6A–C-5A)	−173	−173
φ_E	(O-5E–C-1E–O-6A–C-6A)	102	103
χ_E	(O-6e–C-6E–C-5E–C-4E)	179	−63
ψ_F	(C-1F–O-6E–C-6E–C-5E)	171	172
φ_F	(O-5F–C-1F–O-6E–C-6E)	63	63

The side-chain conformation angles in the final models of the branched polymers welan, S-657, and rhamsan, optimized for side-chain–main-chain hydrogen bonds whenever possible, are listed in Table II. The terminal methyl group hydrogens of rhamnose E and F, or hydroxymethyl group of mannose E, in welan and/or S-657 can adopt any staggered conformation. Their conformation angles are not explicitly listed in Table IIa. Welan and S-657 double helices are shown in Figs. 3a and 3b. The double helices are stabilized by a series of intra- and inter-chain hydrogen bonds listed in Table III. In both cases, the mannose (or rhamnose) E residue is turned towards the main chain. It is important to note that the major driving force for this folding is the O-2C···O-5E intrachain hydrogen bond across the α-(1→3) linkage. The O-4E···O-62B

TABLE III

Intrachain and interchain hydrogen bonds in welan and S-657

Interaction	*Polymer*	*Donor* X	*Acceptor* Y	*Precursor* P	$X\cdots Y$ (nm)	P–$X\cdots Y$ (°)
Intrachain	Welan	O-4E	O-62B	C-4E	0.282	123
		O-2C	O-5E	C-2C	0.297	94
	S-657	O-3F	O-62B	C-3F	0.313	76
		O-2C	O-5E	C-2C	0.271	98
Interchain	Welan	O-3E	O-5C	C-3E	0.285	139
	S-657	O-3E	O-5C	C-3E	0.308	139
		O-2F	O-6C	C-2F	0.301	75
		O-3F	O-6C	C-3F	0.280	107

The two carboxylate oxygen atoms O-61B and O-62B in glucuronate B residue are related by 180° rotation about the C-5B–C-6B bond, and are approximately *cis* and *trans* with respect to carbon atom C-4B.

hydrogen bond involving the carboxylate group further stabilizes the welan double helix. However, this hydrogen bond is not possible in S-657, because the terminal residue F of the side chain is substituted at O-4E. Instead, there is a new intrachain hydrogen bond O-3F···O-62B. Thus, the main-chain carboxylate oxygen atom O-62B, as an acceptor, is actively involved in intrachain hydrogen bonding with O-4E in welan, or O-3F in S-657. Some of the other hydroxyl groups in the side chains also participate in interchain hydrogen bonds with the main chain; in the case of welan there is only one, but in S-657 there are three in every repeating unit (Table III). These might confer additional stability to the putative welan and S-657 double-helices.

By marked contrast, our computations suggest that the functional groups of the side chains in rhamsan can hardly reach the carboxylate groups. In general, the conformations available to the side chains broadly fall under nine distinct groups which correspond to the three staggered domains for each of the (C-5–C-6) bonds in residues A and E, denoted by χ_A and χ_E, respectively. All of the nine (χ_A, χ_E) combinations are stereochemically acceptable. However, if χ_A is retained at the experimentally determined *gauche minus* domain as in the potassium gellan structure[7], the total number of allowed domains is decreased to only three. The side chain droops down and adopts a folded conformation (Fig. 4a) for χ_E in the *trans* domain. On the other hand, the side chain takes up a fully extended conformation when χ_E is flipped to the *gauche minus* domain (Fig. 4b). Between the folded and extended conformers, a continuous spectrum of conformations is available for the rhamsan molecule, indicating immense mobility for the side chain.

DISCUSSION

The gellan family consists of a series of polysaccharides whose rheological properties range from strong gelling to highly viscous aqueous solutions. These properties owe their origin to the absence or presence of periodic side-chains along the length of the polymers. Spectroscopic and calorimetric studies[5] reveal that the viscosity properties are those expected for a typical disordered *neutral* polysaccharide in the case of welan, but a typical disordered polyelectrolyte in the case of rhamsan, although they all have a significant amount of D-glucuronic acid that accounts for 25% of the main-chain residues. Similarly, from light-scattering and potentiometric-titration investigations, it has been suggested that both welan and S-657 have very weak polyelectrolyte behavior[14]. These investigations have attempted to explain this behavior in terms of the screening of the carboxylate group to a much greater extent in welan and S-657 than that in rhamsan and gellan.

Our systematic X-ray investigations on these polymers have enabled us to understand the probable structural role of the carboxylate groups and of the side chains on the stability of the molecules, the intermolecular interactions and hence the observed physical properties. Crystal-structure analyses[6,7] have confirmed that the carboxylate group is important not only for the stability of the gellan double-helix, but essential for

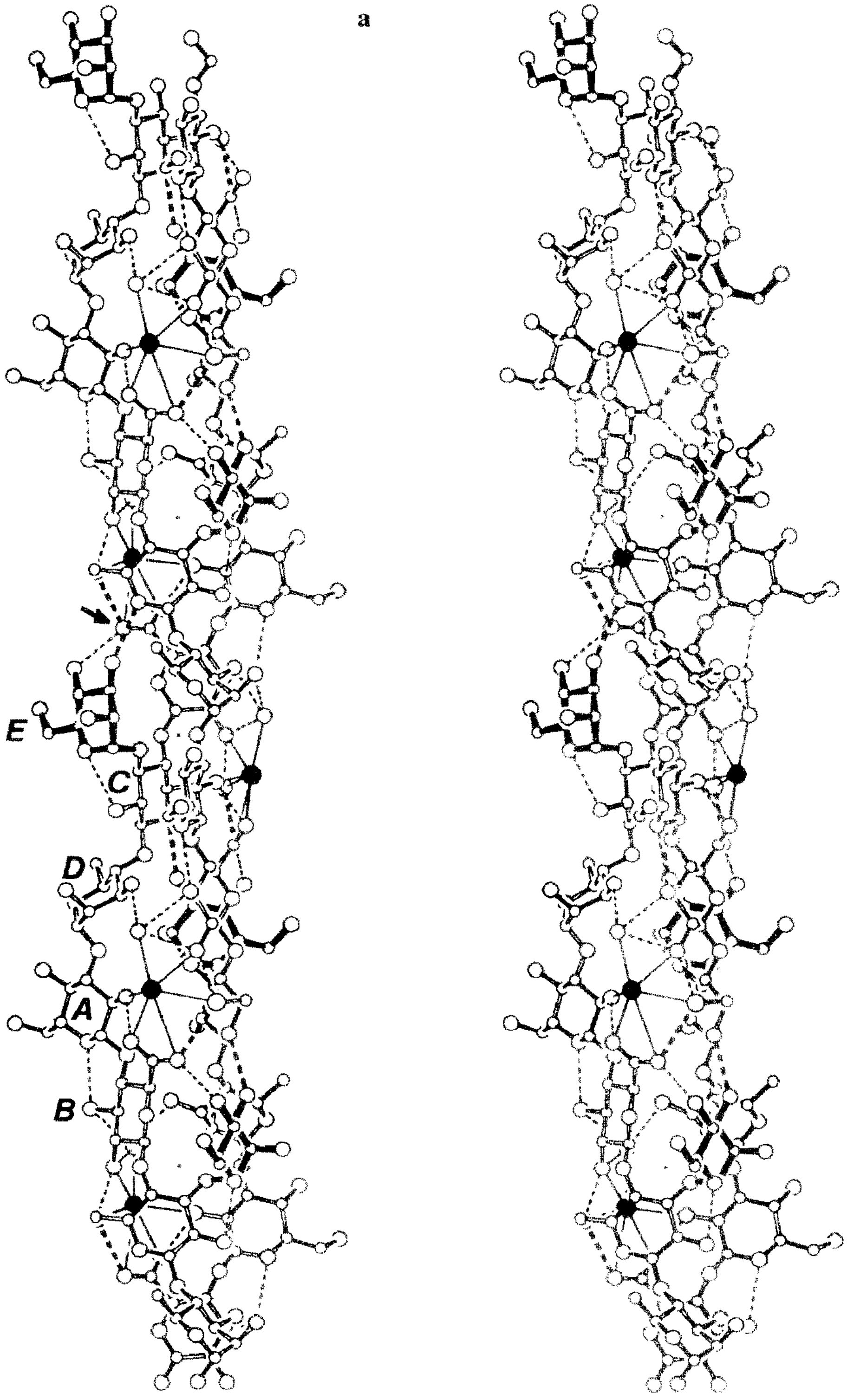
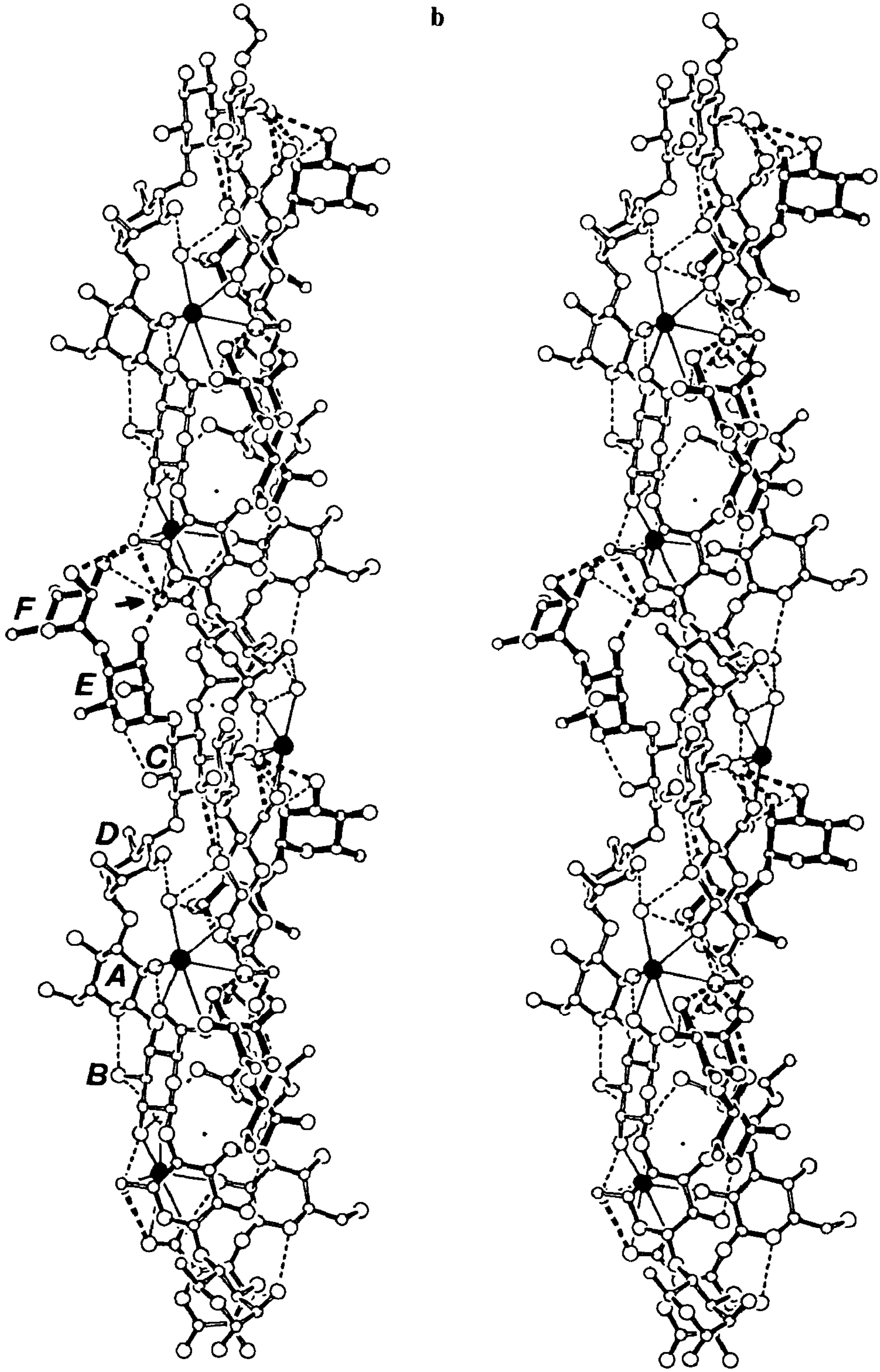


Fig. 3. Stereo views of proposed double-helix, for (*a*) welan and (*b*) S-657 normal to the helix axis, displaying the side chains (thick bonds) in the periphery. Monovalent ions (filled circles) are connected to their ligands by thin lines. Interchain hydrogen-bonds shown in thick dashed lines and intrachain hydrogen-bonds in thin



dashed lines. The screening of the carboxylate group by the side chain is indicated by an arrow in one repeating unit.

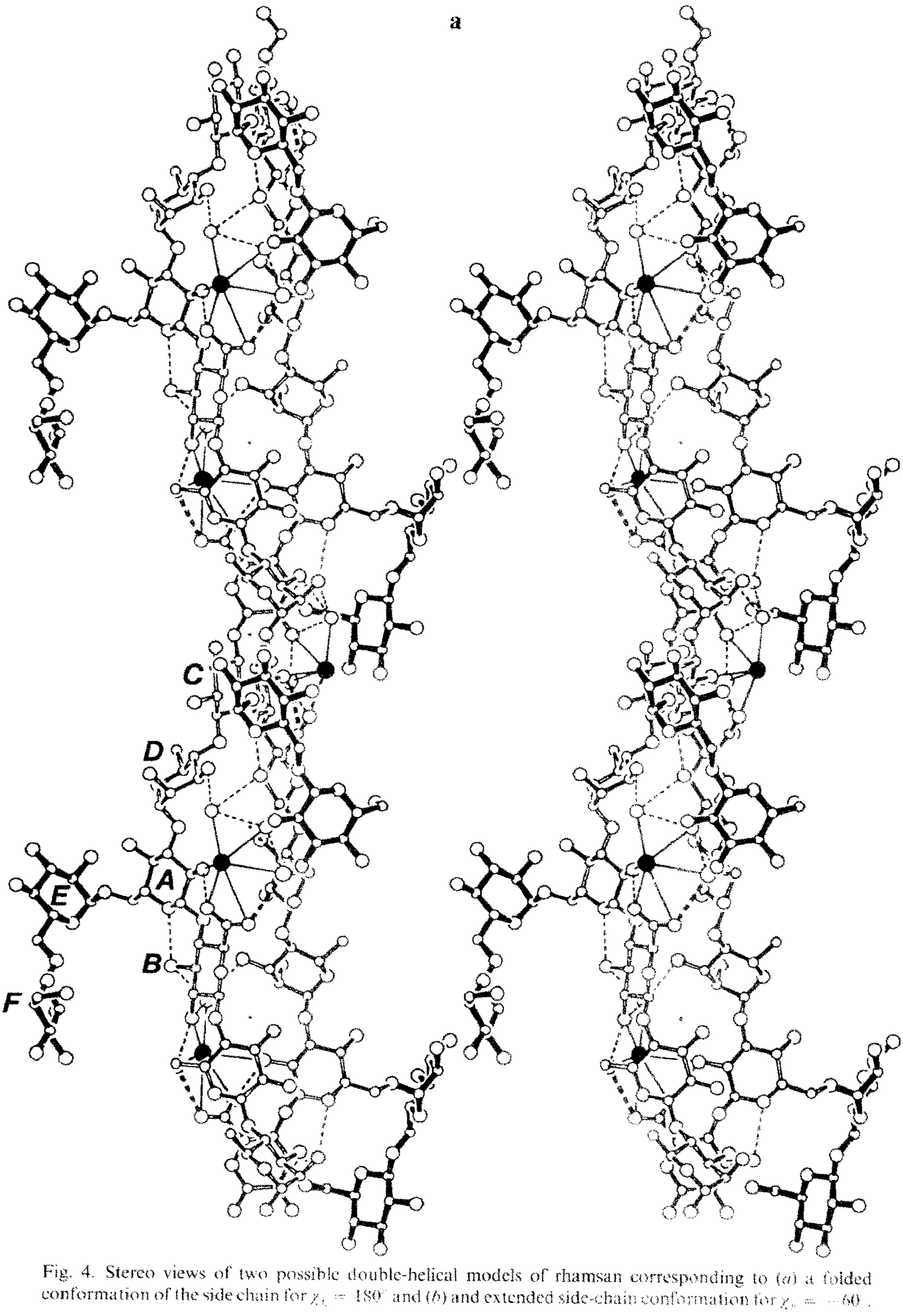
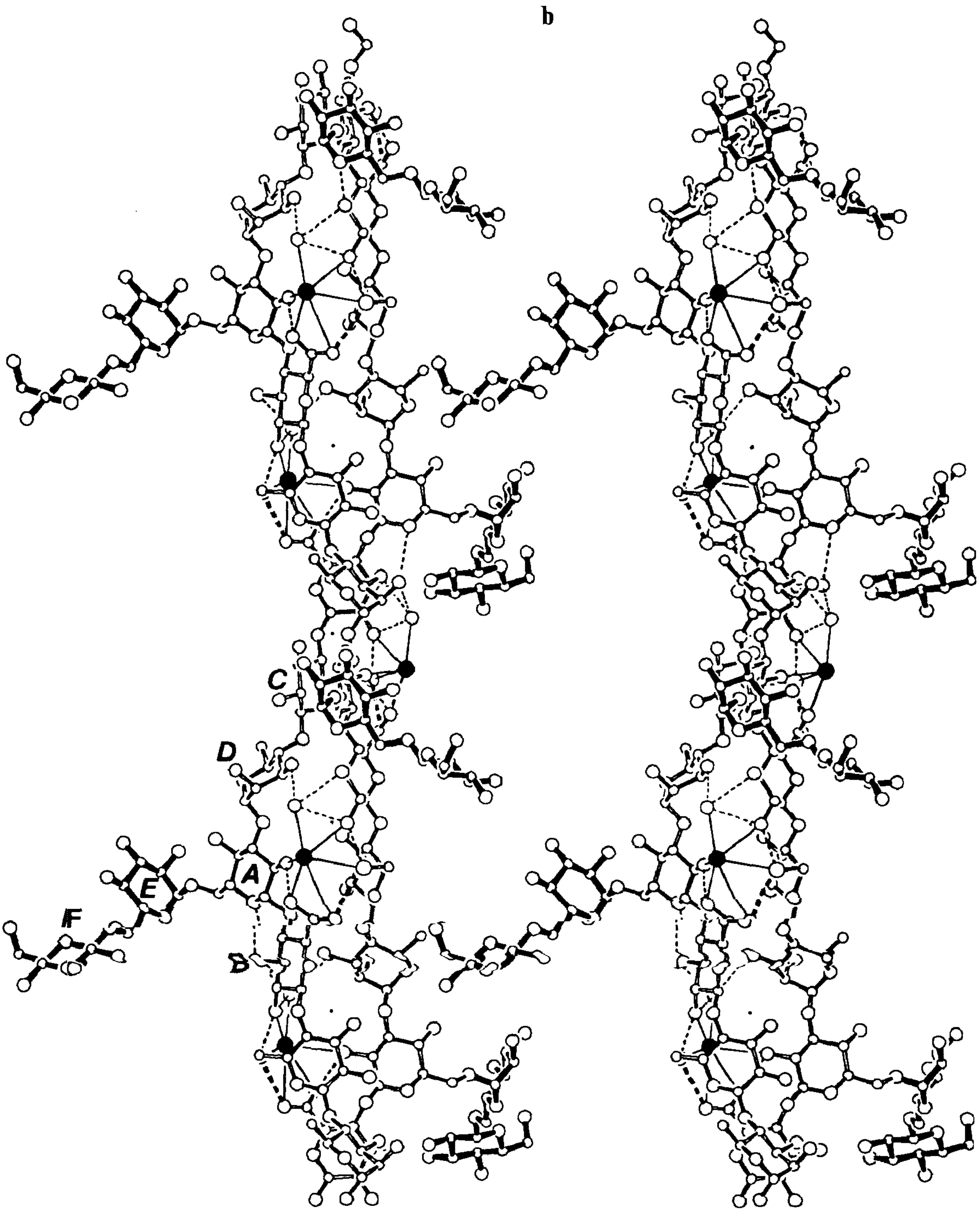


Fig. 4. Stereo views of two possible double-helical models of rhamsan corresponding to (*a*) a folded conformation of the side chain for $\chi_6 = 180°$ and (*b*) and extended side-chain conformation for $\chi_6 = -60°$.



Monovalent ions (filled circles) are connected to their ligands by thin lines. Interchain hydrogen bonds are shown in thick dashed lines and intrachain hydrogen bonds in thin dashed lines. In both cases, the side chains are away from the carboxylate groups.

the aggregation of the double helices which subsequently leads to gelation. In the potassium gellan double-helix, one of the carboxylate oxygen atoms, O-61B, is engaged in intrachain hydrogen bonding with O-2A while the other, O-62B, in interchain hydrogen bonding[7] with O-6C. According to Crescenzi *et al.*[5], this is equivalent to a certain amount of "screening" of the carboxylate group. If other functional groups approach the carboxylate group to make additional hydrogen bonds, this shielding or screening would correspondingly increase.

Computer modeling[11], as well as crystal-structure results[15], demonstrate that minor substituents such as glycerate groups in native gellan tend to perturb the gellan double-helix due to the their close proximity to the carboxylate groups. As a result of this shielding effect, the carboxylate-mediated aggregation of the double helices is decreased relative to that in the unsubstituted gellan crystal-structure. This explains why native gellan's gelling behavior is weaker than in the case of gellan.

Welan and S-657 demonstrate the overwhelming influence of side chains bulkier than the simple acetate and glycerate substituents. As the side chains occur only on the periphery (Fig. 3a, 3b), they do not interfere with the gellan double-helix. Instead, they provide additional stability to the double helix by hydrogen bonding with the carboxylate oxygen atom O-62B of the main chain (Table III). The involvement of the carboxylate group in hydrogen bonding with side chain has previously been suggested from three independent investigations[4,5,16]. We have now shown that the disaccharide side chain in S-657 shields the carboxylate group (Fig. 3b) to a larger extent than the monosaccharide side chain in welan (Fig. 3a). Because of this shielding, the carboxylate groups are not available to promote cation-mediated aggregation of the polymer helices. Subsequently, neither increase in temperature, nor change in ionic strength is likely to disturb the double helix. It is also particularly evident from the ability of O-3F to form bifurcated hydrogen bonds — intrachain with O-62B and interchain with O-6C (see Table III) — that the S-657 double helix will be more stable than that of welan and rhamsan.

The molecular features already mentioned are responsible for the increased stability and stiffness of the double helix[14]. Therefore, the viscosity of welan and S-657 should be high and stable over a wide range of temperature, and this supports previously reported observations[1].

Rhamsan, on the other hand, does not have the potential for any specific hydrogen bonds between the side chains and the carboxylate group of the main chain within the double helix. This may explain the sudden decrease in viscosity above 93°, while the other branched polymers welan and S-657 show stability[1]. Even though the carboxylate groups are not screened and are available to nucleate molecular aggregation, because of the bulky and flexible side-chains, rhamsan double helices cannot associate *via* carboxylate···cation(s)···carboxylate interactions as in gellan. For the same reason, the side chains in neighboring repeating-units in the double helix are not likely to adopt the same conformation either. Instead, a few molecules having stiff, regular backbones, but with a variety of side-chain conformations, might rather aggregate in a random fashion through nonspecific side-chain–side-chain or side-chain–main-chain interactions between double helices. This would give rise to a more entangled polymer system compared to gellan, and hence lead to very high viscosity in solution. This proposal based on conformational flexibility, is in agreement with the observed high viscosity behavior of rhamsan[2].

The double-helical models proposed in this paper for welan, S-657, and rhamsan are all based on the structural features of the completely ionized polysaccharide chains in the potassium gellan double-helix[7]. In other words, it is assumed that every glucuronate residue consisits of a carboxylate group such that either or both oxygen atoms O-61B and O-62B can act as proton acceptors in hydrogen-bond interactions. In a given branched polymer, however, this need not always be the case. For example, as in welan and S-657, the side-chain–main-chain interactions involving the glucuronate residues discussed here might lead to the protonation of some of these residues. Subsequently, the protonated oxygen atoms of the carboxyl groups might equally serve as hydrogen-bonding donors. This is one way of understanding the influence of the screening effect. This protonation effect provides a plausible explanation for the weakening of the electrolytic character of the polymer. The precise details and influences of the side-chain–main-chain shielding and protonation effects must, however, await crystal-structure investigations on the branched polymers.

CONCLUSIONS

The unbranched gelling polysaccharide gellan and the branched non-gelling polysaccharides, welan, S-657, and rhamsan all form similar, half-staggered double helices stabilized by carboxylate···hydroxymethyl interchain hydrogen-bonds. Gellan double helices can readily aggregate *via* carboxylate···cation(s)···carboxylate intermolecular interactions and thereby form strong and brittle gels. The side chains in the branched polymers, welan and S-657, associate with the backbone and prevent this type of aggregation by screening the carboxylate groups. The more flexible, longer and bulkier side-chains in rhamsan prevent its double helices, and therefore the carboxylate groups, from approaching each other to make molecular aggregates. An understanding of these structural features is important in explaining the non-gelling behavior of these branched polymers of the gellan family.

NOTE ADDED IN PROOF

It is gratifying to note that two independent solution studies[17,18] on welan and rhamsan are also in favor of gellan-like double helices for these branched polymers.

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