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# INDICATIVE EVIDENCE FOR A CONFORMATIONAL TRANSITION IN ι-CARRAGEENAN

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## ABSTRACT

The non-Newtonian behavior and dynamic viscoelasticity of ι-carrageenan solutions were measured with a rheogoniometer. The K salt of ι-carrageenan showed Newtonian behavior $<1.0\%$, but pseudoplastic behavior $>1.5\%$, whereas the Ca salt of ι-carrageenan showed plastic behavior even at 0.3%. A gelation occurred in polysaccharide concentrations $>1.5$ and 0.5% for K and Ca salt of ι-carrageenan upon cooling, respectively. The transition temperature, at which dynamic viscoelasticity decreased rapidly, was observed at 45 or 50° at various concentrations of the Ca salt of ι-carrageenan. The dynamic modulus of the Na and K salts forms of ι-carrageenan showed a very high value at low temperature, and increased with an increase in temperature, showing a maximum value at 5°, then it decreased at 0.2% in the presence of $CaCl_2$. However, in the presence of KCl, the K salt form of ι-carrageenan showed a very low dynamic modulus upon cooling. The rheological characteristics of the Ca salt of the ι-carrageenan molecule might be essentially attributed to an intramolecular association, contributed by sulfate groups of adjacent 3,6-anhydro-D-galactose and D-galactose residues through $Ca^{2+}$ ions with ionic forces.

## INTRODUCTION

Carrageenans are sulfated polysaccharides extracted from certain species of red marine algae. Many physical techniques[1–7] have been used to investigate the effect of cations on the conformations and conformational ordering of carrageenans. The gel strength of κ-carrageenan increases with increasing ionic strength and is enhanced by $K^+$, $Cs^+$, $Rb^+$, whereas $Na^+$, $Li^+$, and $Ca^{2+}$ have comparatively little effect[8,9]. In a previous study[9], we have proposed the κ-carrageenan molecule involves an intramolecular cation bridge through a large cation, $K^+$, between a sulfate group and an adjacent ring-oxygen atom with an electrostatic force of attraction. The model provides a new concept for the kinetic evidence of conformational

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transition of the κ-carrageenan molecule with a large cation in aqueous solution. The bridge, however, dissociates at room temperature (25°) owing to the Brownian motion, and the kinetic energy of the solvent and polymer molecules. Intramolecular cationic bridges are less easily formed with a small cation ($Na^+$) probably because the radius of the cation is too small for an association with the ring-oxygen atom, and to the large hydration which may prevent an electroforce of attraction by a large number of water molecules. Intramolecular cationic bridges also are less easily formed with a divalent cation ($Ca^{2+}$), probably for the same reason as for the $Na^+$ counter-ion. The proposed bridge could also explain the gelation mechanism of the κ-carrageenan solution. As the Brownian and kinetic energies of the solvent and polymer molecules decrease upon cooling, and an increased number of cation bridges contribute to keep rigid the molecular chains, an intermolecular association also takes place. An ordered conformation of κ-carrageenan molecules in aqueous solution, whether a single or double helix, is still under discussion[1,10].

On the other hand, ι-carrageenan is a more highly sulfated polysaccharide, and is also the most characterized polysaccharide in the solid state[11-14] and in solution[1,7,14-16]. This structure consists of (1→3)-linked β-D-galactopyranosyl 4-sulfate and (1→4)-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate residues[17]. A mechanism for the gelation of the ι-carrageenan solution involving formation of a double or a single helix has also been proposed, but is still under discussion as for κ-carrageenan[1,18]. Furthermore, variations in the gelation of ι-carrageenan when the counter-ion is changed are well established and, indeed, exploited in the technical utilization of these materials. However, the role of the cations as the molecular level is still poorly understood[19,20].

We report herein the non-Newtonian behavior and dynamic viscoelasticity of ι-carrageenan, and its rheological properties are analyzed with respect to its association characteristics in comparison with those of κ-carrageenan, in order to propose a mode of intra- and inter-molecular $Ca^{2+}$ bridges in aqueous solution.

## EXPERIMENTAL

*Materials.* — ι-Carrageenan, extracted from *Eucheuma spinosum*, was supplied by Taiyo Kagaku Co., Ltd., and was dissolved in water as a 0.5% solution which was filtered through Celite 545 (which had been treated in boiling 3M HCl for 30 min, and washed with distilled water until the pH was 6.5). KCl was added to the filtrate to a concentration of 0.5%, and then ethanol (2 vol.). The precipitate was centrifuged off and dried *in vacuo*. Purified ι-carrageenan was redissolved in water and the solution de-ionized by passage through a column of Amberlite 120 ($H^+$), and made neutral with 50mM KOH, NaOH, or $Ca(OH)_2$. The solution of the last-named salt was filtered through Celite 545 again. Ethanol (2 vol.) was added to each filtrate in the presence of 0.5% KCl, NaCl, or $CaCl_2$, and the precipitates were dried *in vacuo*. To prepare solutions containing salts, ι-carrageenan was dissolved in water, and then KCl, NaCl, or $CaCl_2$, was added to the hot (50°) solution.

*Methods.* — Specific rotations were measured at 589 nm with an automatic digital polarimeter DIP 180 (Japan Spectroscopic Co., Ltd.) for a 0.5% solution in water. For atomic absorption spectroscopy, the cation concentrations were measured with an atomic absorption spectrophotometer (508, Hitachi Seisakusho Co., Ltd.). The identity of the polysaccharide as ι-carrageenan was controlled by i.r. spectroscopy; the spectra were recorded with an infrared spectrophotometer IR 440 (Shimadzu Seisakusho Co., Ltd.) for samples dispersed in KBr discs.

*Viscosity and dynamic viscoelasticity determinations.* — Viscosity at various shear rates (1.188–76.021 $s^{-1}$) and dynamic viscoelasticity at a steady angular velocity (3.768 rad/s) were determined with a rheogoniometer consisting of a coaxial cylinder (1.8 cm diam.) and a rotating outer cylinder (2.2 cm diam.), 6.0 cm long (IR 103 Iwamoto Seisakusho Co., Ltd.). The temperature of the sample was controlled by circulating oil from a Thermo-cool (LCH-130F, Toyo Co., Ltd.) over a temperature ranging from 0 to 60° and being raised at a rate of 1°/min by steps. Shear rate ($D$), shear stress ($S$), and apparent viscosity ($\eta$) were calculated with the equation of Margules[21]. Dynamic viscosity ($\eta'$) and elasticity ($G'$) were calculated by a modification of Markovitz's equation[22]. The loss tangent was calculated from the relationship $\tan \delta = G''/G'$, where $G''$ is the loss modulus ($\omega\eta'$) and $\omega$ is the angular velocity of the outer cylinder.

## RESULTS

The identity of the polysaccharide as an ι-carrageenan was established by i.r. spectroscopy, the spectrum showing characteristic absorptions at 805 and 850 $cm^{-1}$ of approximately equal intensity, which are normally attributed to 3,6-anhydro-D-galactose 2-sulfate and D-galactose 4-sulfate residue[23], respectively. A sulfate content of 31.0% was estimated as described by Dodgson[24]. The cation concentration for the K, Na, and Ca salts of ι-carrageenan was 10.6, 9.5, and 5.3%, respectively. The native ι-carrageenan contained $K^+$, $Na^+$, and $Ca^+$ ions at a concentration of 2.8, 3.1, and 1.8%, respectively.

In order to compare the rheological behavior of ι-carrageenan to that of κ-carrageenan, viscosity and dynamic viscoelasticity measurements were performed under the same conditions as those of the previous study[9]. The flow curves, at 25°, of ι-carrageenan (K and Ca salts) at various concentrations are shown in Fig. 1. The flow curves for the K salt ι-carrageenan at a concentration below 1.0% were approximated to Newtonian behavior, and to pseudoplastic above 1.5% corresponding to a rod-like structure[25] as for the K salt of κ-carrageenan[9]. On the other hand, the Ca salt of ι-carrageenan showed plastic behavior and the yield value was estimated to be 0.4, 1.6, 5.0, and 12.0 Pa at 0.3, 0.5, 0.8, and 1.0% concentration,

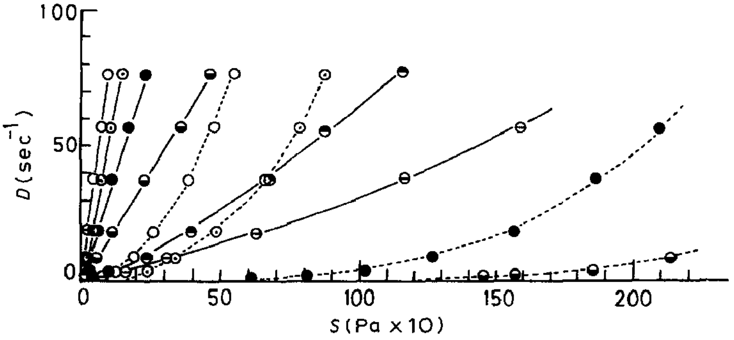


Fig. 1. Flow curves of an ι-carrageenan solution, at 25°. The full lines refer to the K salt and the broken lines to the Ca salt. Concentrations: (○) 0.3, (⊙) 0.5, (●) 0.8, (◒) 1.0, (◓) 1.5, and (⊖) 2.0%.

respectively. This indicates that an intermolecular association is stable at room temperature (25°). The native material also showed plastic behavior at a concentration >0.8%, but the Na salt of ι-carrageenan showed Newtonian behavior even at a 2.0% concentration, whose characteristic was similar to that of the Na salt of κ-carrageenan[9]. The flow curves of the Ca salt of ι-carrageenan, where shifted over higher shear-stress, were in contrast to those of the Ca salt of κ-carrageenan[9]. This suggests that the sulfate groups at C-2 of the anhydro-D-galactose residues contribute to the intermolecular association through $Ca^{2+}$ ions, because both carrageenans differ only in the degree of sulfation. The flow property of native ι-carrageenan, which was associated with $Ca^{2+}$ ions on $^1/_3$ of the sulfate groups, may be also attributed to $Ca^{2+}$ bridges. Thus, the flow characteristics of ι-carrageenan vary with the nature of the cation. It is clear that the cations associated with the sulfate groups play an essential role in the mechanism of the flow characteristics.

Gelation occurred for the native and Ca salt of ι-carrageenan at concentrations >0.8 and 0.5%, respectively, on cooling, but did not for the K and Na salts even at concentrations of 1.0 and 1.5%, respectively (not reported in the Figure). A small increase of the dynamic modulus of the K salt of ι-carrageenan on cooling, in contrast to that of the K salt of κ-carrageenan, may be due to a smaller formation of intramolecular cation-bridges between the sulfate group attached at C-4 of the D-galactose residue and the adjacent ring oxygen atoms of the 3,6-anhydro-D-galactose residues, as for the K salt of κ-carrageenan through a $K^+$ ion. This suggest that a $K^+$ ion, associated to the sulfate groups at C-2 of the anhydro-D-galactose residues, prevents in part the formation of the intramolecular $K^+$ bridges by electrostatic repulsion between $K^+$ ions. As shown in Fig. 2, the dynamic modulus of the Ca salt of ι-carrageenan (0.5%) showed a very large value decreasing gradually with increasing temperature. After the temperature reached 45°, which was estimated to be a transition temperature, the dynamic modulus decreased rapidly; this was also observed for higher concentrations at 50°. The native and K salt of ι-carrageenan showed an increase in dynamic modulus as the K salt of κ-

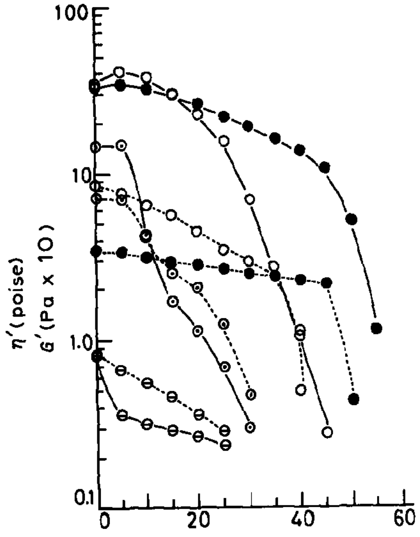


Fig. 2. Effect of temperature on the dynamic viscoelasticity, at 3.768 rad/sec, of a 0.5 or 1.0% solution of various salts of ι-carrageenan. The dotted lines refer to the dynamic viscosity and full lines to the dynamic modulus. Cation and concentration: (○) native (0.5%), ⊖ Na (1.0%), (⊙) K (1.0%), and (●) Ca (0.5%).

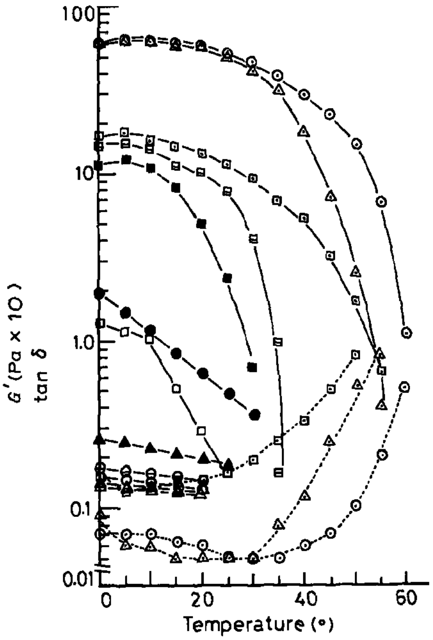


Fig. 3. Effect of temperature on the dynamic modulus, at 3.768 rad/sec, of a 0.2% solution of ι-carrageenan with addition of 16.6mM NaCl, 13.5mM KCl, and 9.0mM $CaCl_2$. The full lines refer to the dynamic modulus and broken lines to the tan δ. The triangles refer to the Na salt, the circles to the K salt, and the tetragons to the Ca salt: (○, △, □) ι-carrageenan only (⊖, △, □) with NaCl; (●, ▲, ■) with KCl; and (⊙, △, ⊡) with $Ca(Cl)_2$.

carrageenan[9], but the latter showed a value much lower than that of the former and of the Ca salt. The transition temperature observed for native ι-carrageenan at 35°, which was not as high as that for the Ca salt, may be due to a $^1/_3$ substitution of sulfate groups with $Ca^{2+}$ ions. This corresponds to the lowest transition temperature for the K salt of ι-carrageenan (5°), the univalent cation of which was associated with $^1/_3$ of the sulfate groups in the native molecule.

Although a very large dynamic modulus was observed on addition of $CaCl_2$ (6.8mM) for the Na and K salts of ι-carrageenan solution (Fig. 3), the values for the Ca salt decreased upon addition of NaCl (16.6mM), KCl (13.5mM), and $CaCl_2$ (6.8mM). This suggests that the $Na^+$ and $K^+$ counter-ions were replaced with $Ca^{2+}$ ions by addition of $CaCl_2$; consequently a much greater intermolecular association took place, the strength of which being ~4 times that of the Ca salt of ι-carrageenan in the presence of $CaCl_2$. On the other hand, the K salt of ι-carrageenan, in the presence of KCl, showed a very small dynamic modulus, in contrast to the K salt of κ-carrageenan[9]. This suggests that the formation of intramolecular $K^+$ bridges in the K salt of ι-carrageenan were prevented by electrostatic repulsion as discussed earlier. The dynamic modulus of the K, Na, and Ca salts of ι-carrageenan increased with an increase in temperature in the presence of $CaCl_2$, showing maximum values at 5°, and then decreased; for the last-named salt, addition of KCl and NaCl also resulted in an increase. Furthermore, the K and Na salts showed a very large dynamic modulus in the presence of $MgCl_2$ (10.6mM) (not reported in the Figure), indicating that the ι-carrageenan molecules may associate more with divalent cations (such as $Ca^{2+}$ and $Mg^{2+}$) than with univalent cations (such as $Na^+$ and $K^+$). The tan $\delta$ of the Na and K salts of ι-carrageenan in the presence of $CaCl_2$ showed much lower values than that of the Ca salt, and kept constant during the increase of the temperature up to 30–40°, indicating that a very strong association took place as in xanthan[26,27].

Gelation occurred for ι-carrageenan (native, and Na, K, and Ca salts) in the acid and alkaline range after reaching a pH of 2.5 and 11.7 by addition of 100mM HCl, NaOH, KOH, or $Ca(OH)_2$, a behavior that parallels that of κ-carrageenan[9].

TABLE I

SPECIFIC ROTATION OF ι-CARRAGEENAN AT 589 nm[a]

| *ι-Carrageenan* | *Temperature (°)* | | |
|---|---|---|---|
| | *5* | *30* | *50* |
| Native | +77.2 | +73.2 | +41.2 |
| Na salt | +53.6 | +51.2 | +46.4 |
| K salt | +76.0 | +60.0 | +50.8 |
| Ca salt | +73.2 | +70.0 | +62.4 |

[a] For a solution in water, c 0.5%.

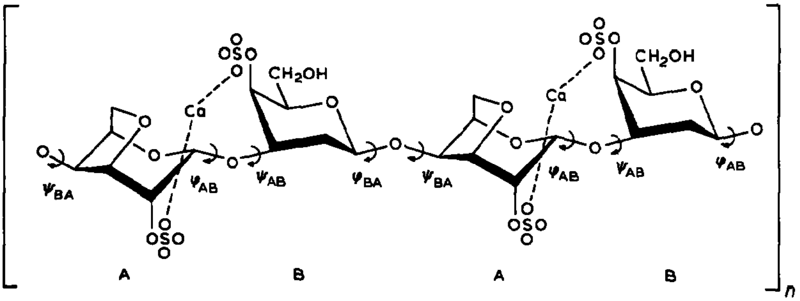
The dynamic viscoelasticity was lower than that of ι-carrageenan upon the addition of urea (4.0M).

The specific rotations of the various salts of ι-carrageenan are summarized in Table I. Except for the Na salt, the values obtained at low temperature (5°) were high (+73–77°), which is consistent with that of the K salt of κ-carrageenan[9]. These may be attributed to intermolecular association. For the Ca salt of ι-carrageenan, the specific rotation showed a high value, even at 50°, suggesting that the intermolecular association was more stable than that of native and the K salt.

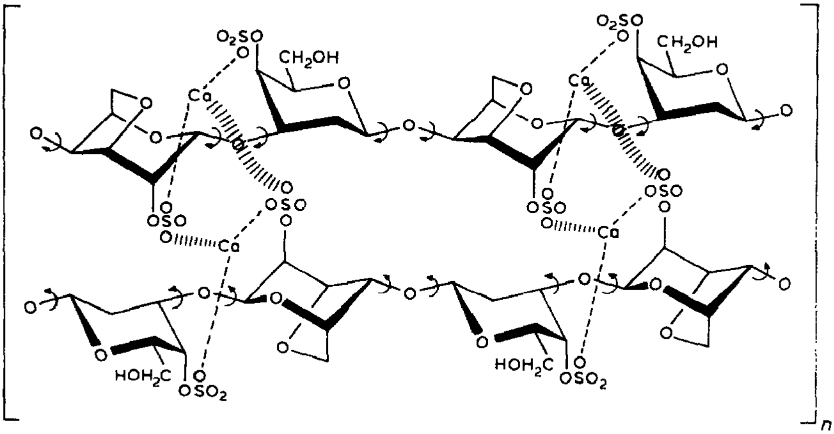
## DISCUSSION

The relative effects of $K^+$ and $Ca^{2+}$ ions on the ordered conformation of κ- and ι-carrageenan have been reported earlier[6,8,16]. The $K^+$ ions are more effective than the $Ca^{2+}$ ions in promoting an intermolecular association of κ-carrageenan, but for the more highly charged ι-carrageenan, the order was reversed. These results were confirmed in a previous[9] and in the present report.

The Ca salt of ι-carrageenan showed a plastic behavior even in a solution of low concentration (0.3%) at room temperature (25°). The transition temperature was observed at 45 or 50° at various concentrations. Furthermore, an increase in the dynamic modulus was observed during an increase in temperature as for κ-carrageenan. Thus, we concluded that the Ca salt of ι-carrageenan involves an intramolecular association through $Ca^{2+}$ ions, as illustrated in Scheme 1. The intramolecular $Ca^{2+}$ bridges may take place between sulfate groups substituting adjacent anhydro-D-galactose and D-galactose residues. Each cation is co-ordinated to two sulfate groups by ionic force. The intramolecular cation bridge of the Ca salt of ι-carrageenan differs essentially from that of the K salt of κ-carrageenan, because the former consists of ionic forces, whereas the latter consists of ionic and electrostatic forces of attraction as proposed previously[9]. These different mechanisms



Scheme 1. Possible mode of intramolecular $Ca^{2+}$ bridge of ι-carrageenan. The dotted lines refer to the ionic force. The intramolecular cation-bridge may take place with divalent cations. With univalent cations, the intramolecular bridge may take place in part between the anhydro-bridge oxygen atom and the sulfate group linked at C-4 of the D-galactose residues as in the K salt of κ-carrageenan. The conformational changes in the interaction between neighboring sugar residues are expressed in terms of the four angles of rotation[12], $\phi_{AB}$, $\psi_{AB}$, $\phi_{BA}$, and $\psi_{AB}$.



Scheme 2. Possible mode of intermolecular $Ca^{2+}$ bridge of the Ca salt of ι-carrageenan. The dotted lines refer to the ionic forces and the broken lines to the electrostatic forces of attraction. The arrows refer to the orientation of the conformational angles. The oxygen bonds of the sulfate group attached at C-2 of the anhydro-D-galactose residues may contribute to the intermolecular association through $Ca^{2+}$. The model corresponds to a double stranded helix, though sulfate groups may be on the inside of the helix. A tertiary structure of the Ca salt of the ι-carrageenan molecules may consist of two identical, right-handed, 3-fold helices in aqueous solution as in solid state.

provide an explanation for the conformational transition of ι-carrageenan in aqueous solution. Since ionic forces are stronger than electrostatic forces of attraction, the molecule chain may become rigid even at room temperature and, therefore, an intermolecular association may also take place. The intramolecular $Ca^{2+}$ bridges may be built up in preference to the intermolecular bridges which, however, may be involved in part in the Ca salt of ι-carrageenan. The bridge proposed has been developed into an intermolecular-association mechanism of the Ca salt of ι-carrageenan in solution, as illustrated in Scheme 2. As Brownian motion, and kinetic energy of solvent and polymer molecules decrease upon cooling, intermolecular $Ca^{2+}$ bridges may also take place in different molecules having electrostatic forces of attraction. The oxygen bond of the sulfate groups at C-2 of the anhydro-D-galactose residues may contribute to the intermolecular $Ca^{2+}$ bridges. The intermolecular $Ca^{2+}$ bridges may dissociate up to the transition temperature (45° or 50°) and the intramolecular $Ca^{2+}$ bridges may dissociate rapidly above that temperature. Although each strand seems to be extended owing a large hydration, the model may correspond to a double helix. Accordingly, the stabilization of the double-stranded helix depends on the ionic and electrostatic forces of attraction. In the double-helical conformation, the tertiary structure of the Ca salt of ι-carrageenan may consists of two identical, right-handed, 3-fold helices as in the solid state[11,12,14].

On the other hand, the association characteristics of the K salt of ι-carrageenan essentially depend on the intramolecular $K^+$ bridges. Because of electrostatic repulsion, however, these are not too strong. In concentrated solution, the K salt also may adopt a double-stranded helix conformation at low temperature, although the sulfate groups at C-2 of the anhydro-D-galactose residues are on the outside of the double helix, but those at C-4 of the D-galactose residues are on the inside; this orientation of the molecule likely agree with that in the solid state[12,14].

The ι-carrageenan molecule may adopt a single rod-like[25] structure for the Na and K salts at room temperature. However, when the counter ions were replaced with $Ca^{2+}$ ions upon addition of $CaCl_2$, the intermolecular Ca bridging of the various molecules could take place *via* ionic forces. A very large dynamic modulus suggested that almost all the sulfate groups contribute to the intermolecular Ca bridges on neighboring molecules. The smaller increase in dynamic modulus of the Ca salt of ι-carrageenan, in the presence of $CaCl_2$, may be due to the saturation of the intramolecular Ca bridges before addition of the salt.

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## REFERENCES

1 E. R. Morris, D. A. Rees, and G. Robinson, *J. Mol. Biol.*, 138 (1980) 349–362.
2 V. J. Morris and P. S. Belton, *J. Chem. Soc., Chem. Commun.*, (1980) 983–984.
3 E. R. Morris, D. A. Rees, I. T. Norton, and D. M. Goodall, *Carbohydr. Res.*, 80 (1980) 317–323.
4 O. Smidsrød and H. Grasdalen, *Carbohydr. Polym.*, 2 (1982) 270–272.
5 C. Rochas and M. Rinaudo, *Carbohydr. Res.*, 105 (1982) 227–236.
6 I. T. Norton, D. M. Goodall, E. R. Morris, and D. A. Rees, *J. Chem. Soc., Faraday Trans. 1*, 79 (1983) 2475–2488.
7 I. T. Norton, D. M. Goodall, E. R. Morris, and D. A. Rees, *J. Chem. Soc., Faraday Trans. 1*, 79 (1983) 2501–2515.
8 C. Rochas and M. Rinaudo, *Biopolymers*, 19 (1980) 1675–1687.
9 M. Tako and S. Nakamura, *Carbohydr. Res.*, 155 (1986) 200–205.
10 H. Grasdalen and O. Smidsrød, *Macromolecules*, 14 (1981) 1842–1845.
11 S. T. Bayley, *Biochim. Biophys. Acta*, 17 (1955) 194–205.
12 N. S. Anderson, J. W. Campbell, M. M. Harding, D. A. Rees, and J. W. B. Samuel, *J. Mol. Biol.*, 45 (1969) 85–99.
13 D. A. Rees, *J. Chem. Soc., B*, (1969) 217–226.
14 S. Arnott, W. E. Scott, D. A. Rees, and C. G. A. McNab, *J. Mol. Biol.* 90 (1974) 253–267.
15 D. A. Rees, *Biochem. J.*, 126 (1972) 257–273.
16 V. J. Morris and G. R. Chilvers, *J. Sci. Food. Agric.*, 32 (1981) 1235–1241.
17 N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J. Chem. Soc., Perkin Trans. 1*, (1973) 2173–2176.
18 O. Smidsrød, I. L. Andresen, H. Grasdalen, B. Larsen, and T. Painter, *Carbohydr. Res.*, 80 (1980) c11–c16.
19 P. S. Belton, G. R. Chilvers, V. J. Morris, and S. F. Tanner, *Int. J. Biol. Macromol.*, 6 (1984) 303–308.
20 K. R. J. Austen, D. M. Goodall, and I. T. Norton, *Carbohydr. Res.*, 140 (1985) 251–262.
21 J. Harris, *Rheology and Non-Newtonian Flow*, Longman, New York, 1977, pp. 28–30.
22 H. Markovitz, *J. Appl. Phys.*, 23 (1952) 1070–1077.
23 N. S. Anderson, T. C. S. Dolan, A. Penman, D. A. Rees, G. P. Mueller, D. J. Stancioff, and N. F. Stanley, *J. Chem. Soc. C*, (1968) 602–606.
24 K. S. Dodgson, *Biochem. J.*, 78 (1961) 312–319.
25 C. R. Masson and D. A. I. Goring, *Can. J. Chem.*, 33 (1955) 895–903.
26 M. Tako, T. Nagahama, and D. Nomura, *Nippon Nogei Kagaku Kaishi*, 51 (1977) 513–518.
27 M. Tako and S. Nakamura, *Agric. Biol. Chem.*, 48 (1984) 2987–2993.