

LYOTROPIC EFFECTS OF SIMPLE ANIONS ON THE CONFORMATION AND INTERACTIONS OF KAPPA-CARRAGEENAN

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ABSTRACT

The effect of different co-anions on the formation and aggregation of the ordered structure of the anionic polysaccharide kappa-carrageenan has been investigated by optical rotation, differential scanning calorimetry, and halide-n.m.r. spectroscopy. The mid-point temperature (T_m) of the disorder–order transition increases systematically with the Hofmeister number for the anion through the lyotropic series $\text{SO}_4^{2-} < \text{F}^- < \text{Cl}^- < \text{Br}^- \leq \text{NO}_3^- < \text{I}^- < \text{SCN}^-$ when salt concentration and cation (Me_4N^+ or K^+) are held constant. A corresponding increase is observed in transition enthalpy (ΔH_{cal}) and entropy (ΔS_{cal}). Helix–helix aggregation (as indicated by turbidity, gel formation, and hysteresis between heating and cooling scans) also shows a systematic dependence on the Hofmeister number for the anion, but in the opposite sense. Thus, with tetramethylammonium as the sole counterion present, clear solutions with no thermal hysteresis in the order–disorder transition are observed at all temperatures with I^- , Br^- , NO_3^- , or Cl^- as co-anion, whereas weak, turbid gels with significant thermal hysteresis between melting and setting are formed in the presence of SO_4^{2-} , and to a lesser degree F^- . With K^+ as counterion, a similar regular progression is observed through the anion lyotropic series from rapid formation of very turbid gels in the presence of F^- , to very slow development of clear gels with I^- or SCN^- . In agreement with previous studies, an increase in ^{127}I -n.m.r. linewidth was observed on conformational ordering of kappa-carrageenan (Me_4N^+ salt form) in the presence of Me_4NI . However, closely similar behaviour was observed for ^{35}Cl and ^{81}Br , indicating a simple charge-cloud interaction rather than the specific site-binding of I^- which has previously been suggested.

INTRODUCTION

Since the pioneering work of Hofmeister almost a century ago, it has been recognised that simple salts may drastically alter the solubility and other solution properties of macromolecules. In general, the relative effectiveness of different

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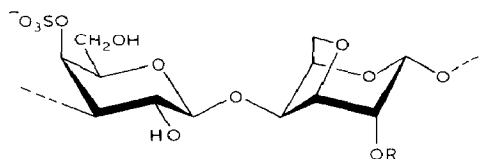


Fig. 1. Idealised disaccharide repeating-structure of kappa- ($R = H$) and iota-carrageenan ($R = SO_3^-$)^{17,18}.

ions follows a well defined order (the lyotropic series)^{1,2}, which extends to phenomena far removed from colloid chemistry (such as the surface tension of salt solutions, the miscibility of liquids, and the kinetics of organic chemical reactions in solution).

In the cation-induced gelation of negatively charged polysaccharides, however, the normal lyotropic order may be distorted or scrambled by selective interactions of polymer and counterion. For example, the polyguluronate sequences of alginate show a specific affinity for Sr^{2+} which is not shared by other polyuronates³. The origin of this behaviour appears to lie in the steric requirements for effective packing of cations within the "egg-box" junction zones which cross-link both alginate and pectate gels^{4,5}. In the carrageenan series, the cation requirements⁶⁻¹⁴ for gelation and adoption of the underlying, ordered junction-zone geometry^{15,16} are dependent on the degree of sulphation of the polymer (Fig. 1). For example, K^+ ions are more effective than Ca^{2+} in promoting gelation of kappa-carrageenan, whereas, for the more highly charged iota-carrageenan, the order is reversed^{6,8,12,19}.

Although studies of cation-polysaccharide interactions have been fairly extensive, little attention has been given to the influence of the counter-anion. In the particular case of kappa-carrageenan, Rochas and Rinaudo¹¹ studied the effect of various potassium salts on the temperature course of conformational ordering, and concluded that " T_m is independent of the nature of the anion of the added salt". More recently, however, Grasdalen and Smidsrød²⁰ reported that "the iodide anion seems to be exceptional", and suggested adoption of a single-stranded carrageenan-iodide complex, rather than the double-helix structure that had previously been proposed^{15,16,21}.

We now present evidence that anions do significantly influence conformational ordering in kappa-carrageenan, with iodide fitting in its expected position in the general lyotropic series.

EXPERIMENTAL

Materials. — The sample of kappa-carrageenan used (Lot no. B5466) was kindly donated by Dr. Ch. Bellion of CECA SA, France. This material was extracted from *Eucheuma cottonii*, and had been treated with alkaline borohydride to improve the structural regularity by conversion of anomalous D-galactosyl 6-sulphate residues into 3,6-anhydro-D-galactosyl residues^{22,23}. The sample was dialysed

extensively against deionised water, and specific salt forms were prepared by ion exchange on Amberlite IR-120 resin, followed by freeze-drying. Absolute concentrations were determined by elemental analysis of the freeze-dried products (Butterworth Microanalytical Consultancy Ltd.).

Methods. — Optical rotations were measured at 365 nm with a Perkin–Elmer 241 polarimeter; samples were held at constant temperature until a stable reading was attained. Differential scanning calorimetry (d.s.c.) was performed with a Perkin–Elmer DSC-2 microcalorimeter. Most results were obtained at a scan rate of $10\text{ K}\cdot\text{min}^{-1}$, but were occasionally verified by measurements at 5 or $20\text{ K}\cdot\text{min}^{-1}$. Heating and cooling curves for both optical rotation and d.s.c. were repeated several times on separate samples, and were highly reproducible. Halide-n.m.r. spectra were recorded with a Bruker CXP-300 spectrometer.

RESULTS

Fig. 2 shows the temperature dependence of optical rotation for tetramethylammonium kappa-carrageenate in the presence of 0.15M Me_4NCl , Me_4NBr , and Me_4NI . Results for the iodide salt form are in close agreement with those reported by Grasdalen and Smidsrød²⁰, and show clear evidence of a co-operative conformational transition in solution under these ionic conditions. In contrast to the previous suggestion²⁰ that this behaviour is unique to iodide, however, analogous transitions were evident in the presence of bromide and chloride co-anions, but displaced to lower temperature. For chloride, the onset of conformational ordering occurred at $\sim 20^\circ$, the lowest temperature used in the previous investigation. In agreement with the known behaviour^{24,25} of other polyelectrolytes that adopt ordered conformations under hydrated conditions, and with previous studies of kappa-carrageenan under different ionic conditions^{6–11}, the transition midpoint temperature (T_m) increases with increasing salt concentration, as illustrated in Fig. 3.

At all experimentally accessible concentrations of tetramethylammonium chloride, bromide, iodide, and nitrate, the results obtained on heating and on cooling were closely superimposable, no gel formation was observed, and the solutions remained optically clear at low temperature. In the presence of tetramethylammonium sulphate (and to a lesser extent fluoride), by contrast, significant hysteresis was observed between heating and cooling scans (Fig. 4), conformational ordering was accompanied by an increase in turbidity, and a weak but cohesive gel structure was formed. These effects increased with increasing concentration of salt, and became undetectable at low levels of salt. In previous investigations of the gelation of agarose²⁶, or of kappa-carrageenan in the presence of KCl ¹⁵, turbidity and thermal hysteresis were attributed to aggregate formation. Comparison¹⁹ of equilibrium and kinetic data indicated that the metastable curves obtained on cooling characterise the inherent thermal stability of the ordered structure of the polymer in isolation, while the true equilibrium values recorded on heating reflect the greater thermal stability of the aggregate structure once formed.

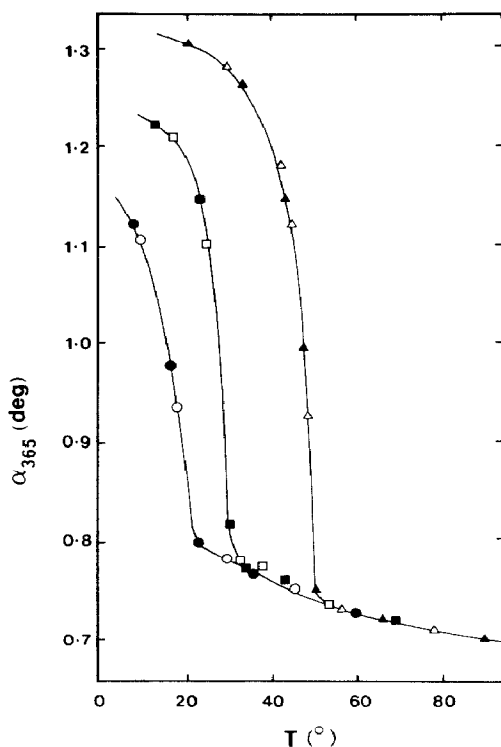


Fig. 2. Temperature-course of the order-disorder transition of kappa-carrageenan (Me_4N^+ salt form) in the presence of 0.15M tetramethylammonium chloride (\bullet, \circ), bromide (\blacksquare, \square), and iodide ($\blacktriangle, \triangle$), as monitored by optical rotation (365 nm; 10-cm pathlength; polymer concentration, 0.5% w/v). Filled and open symbols show results obtained on heating and cooling, respectively.

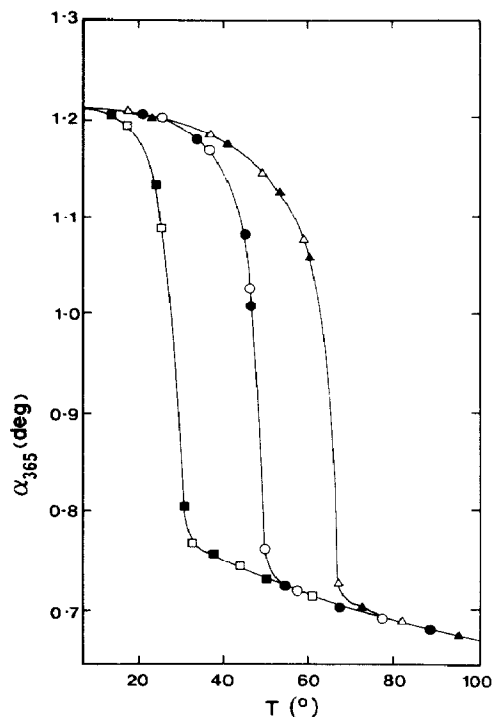


Fig. 3. Temperature-course of the order-disorder transition of kappa-carrageenan (Me_4N^+ salt form) in the presence of 0.15M (\blacksquare, \square), 0.5M (\bullet, \circ), and M ($\blacktriangle, \triangle$) tetramethylammonium bromide. Conditions for optical rotation as in Fig. 2.

Fig. 5 shows the variation in transition midpoint temperature for conformational ordering of kappa-carrageenan (Me_4N^+ salt form) on cooling in the presence of different concentrations of a range of tetramethylammonium salts whose anions span the lyotropic series^{1,2}. At constant molar concentration of salt, T_m increases systematically with the Hofmeister number for the anion, as illustrated in Fig. 6. On changing the counterion from Me_4N^+ to Et_4N^+ , there is a small decrease in T_m for equivalent co-anions and salt concentrations (*cf.* Figs. 2 and 7), but the results are otherwise unaffected.

A similar systematic variation in the temperature course of conformational ordering with the Hofmeister number was observed for the potassium salt form, but, in this case, gelation occurred with all the co-anions studied. There was, however, a regular progression through the anion lyotropic series from rapid formation of very turbid gels in the presence of KF, to very slow development (typically over ~ 24 h) of clear gels with KI or KSCN. Because of the extreme turbidity of the gels

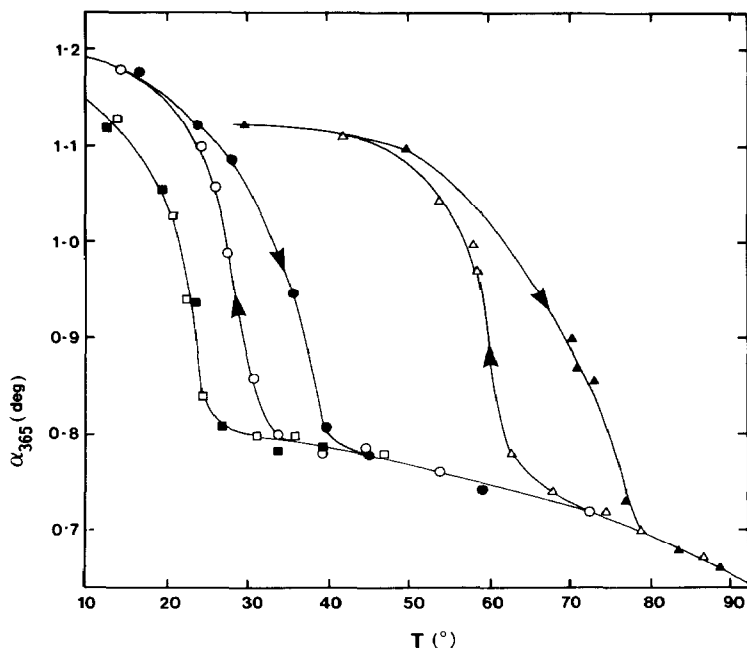


Fig. 4. Conformational transition of kappa-carrageenan (Me_4N^+ salt form) in the presence of 0.167M (\blacksquare , \square), 0.25M (\bullet , \circ), and 1M (\blacktriangle , \triangle) tetramethylammonium sulphate; conditions as in Fig. 2. Filled and open symbols show results obtained on heating and cooling respectively.

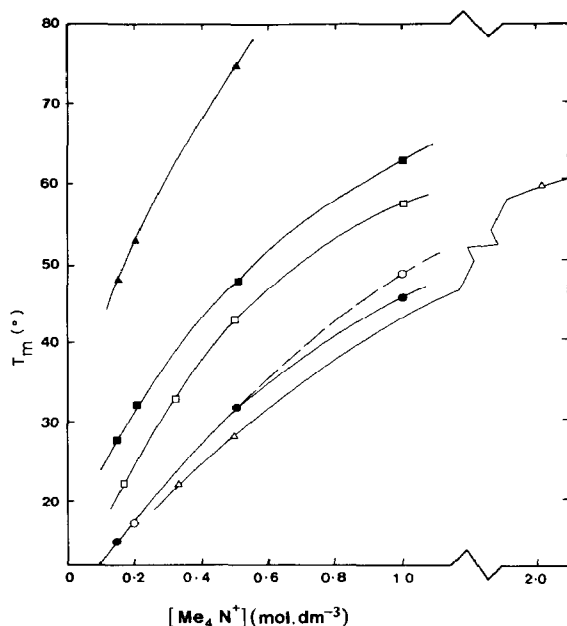


Fig. 5. Salt-dependence of transition mid-point temperature (T_m) for kappa-carrageenan (Me_4N^+ salt form; 0.5% w/v) on cooling in the presence of tetramethylammonium fluoride (\circ), chloride (\bullet), bromide (\blacksquare), iodide (\blacktriangle), nitrate (\square), and sulphate (\triangle).

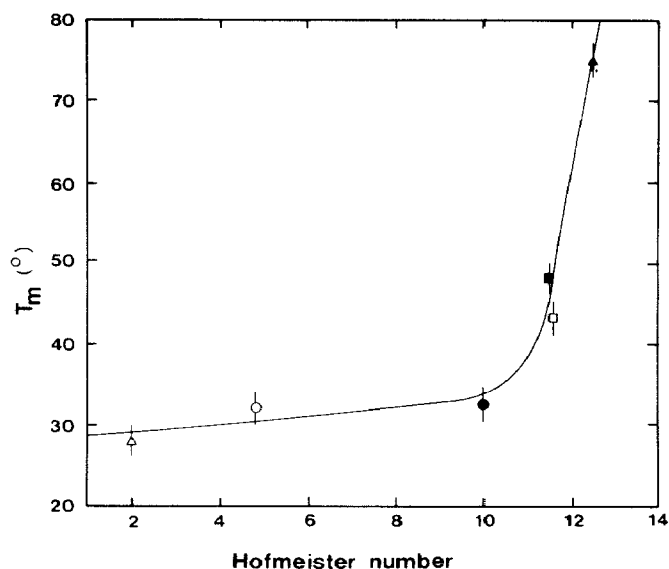


Fig. 6. Dependence of transition mid-point temperature (T_m) for kappa-carrageenan (Me_4N^+ salt form) on the Hofmeister number of the co-anion present. The results shown were obtained on cooling in the presence of 0.5M tetramethylammonium sulphate (Δ), fluoride (\circ), chloride (\bullet), nitrate (\square), bromide (\blacksquare), and iodide (\blacktriangle).

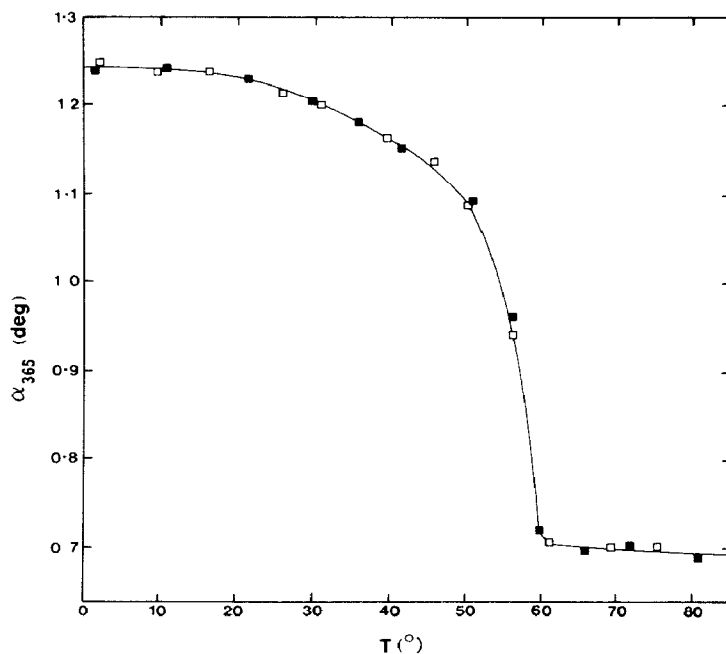


Fig. 7. Temperature-course of the conformational transition of kappa-carrageenan (Et_4N^+ salt form) on heating (\blacksquare) and cooling (\square) in the presence of M tetraethylammonium bromide. Conditions as in Fig. 2.

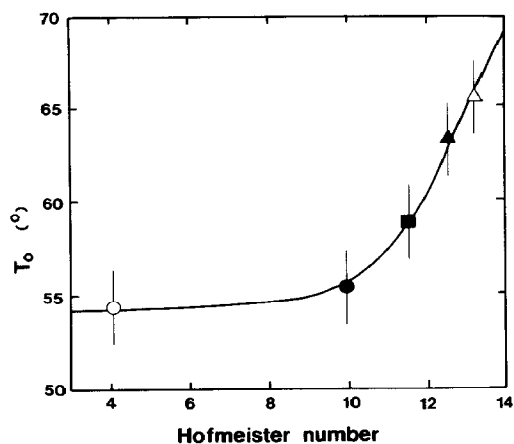


Fig. 8. Anion-dependence of the temperature at which the onset of conformational ordering is observed on cooling potassium kappa-carrageenate in the presence of K^+ salts. Results are shown for 0.1M potassium fluoride (O), chloride (●), bromide (■), iodide (▲), and thiocyanate (Δ).

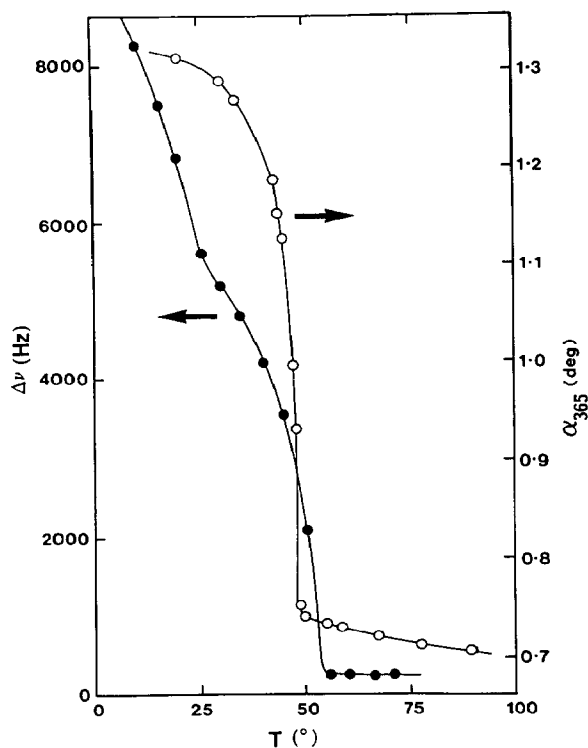


Fig. 9. ^{127}I -N.m.r. line broadening (●) on conformational ordering (O) of kappa-carrageenan (1% w/v; Me_4N^+ salt form) in the presence of tetramethylammonium iodide (0.15M). Excess linewidth ($\Delta\nu$) is expressed as the observed linewidth of the polymer solution minus the linewidth, at the same temperature, of the salt solution against which it was dialysed.

TABLE I

ORDER-DISORDER TRANSITION ENTHALPY AND ENTROPY FOR KAPPA-CARRAGEENAN (4% w/v) IN THE PRESENCE OF TETRAMETHYLAMMONIUM SALTS (0.15M)

Anion	$\Delta H_{cal} (kJ.mol^{-1})$	$T_m (K)$	$\Delta S_{cal} (J.mol^{-1}.K^{-1})$
F ⁻	3.5 ± 0.3	288	12 ± 1
NO ₃ ⁻	5.7 ± 0.3	294	19 ± 1
Br ⁻	7.3 ± 0.5	301	24 ± 2
I ⁻	10.4 ± 1.8	321	32 ± 5

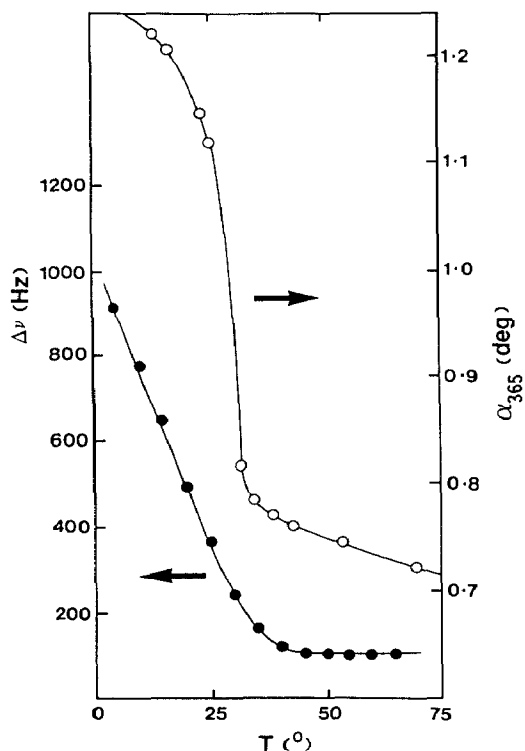


Fig. 10. Increase in ⁸¹Br linewidth on conformational ordering of kappa-carrageenan in Me₄NBr solution. Symbols and conditions as in Fig. 9.

formed in the presence of co-anions of low Hofmeister number, it was not possible to follow the entire temperature course of the conformational transition, and hence determine T_m . The temperature at which the onset of conformational ordering occurred on cooling (T_o) could, however, be measured reproducibly, and again showed (Fig. 8) a smooth progression through the anion lyotropic series. In particular, iodide occupies its expected position between bromide and thiocyanate.

D.s.c. measurements (Table I) of the enthalpy change (ΔH_{cal}) associated with the order-disorder transition of kappa-carrageenan (Me₄N⁺ salt form) in the pre-

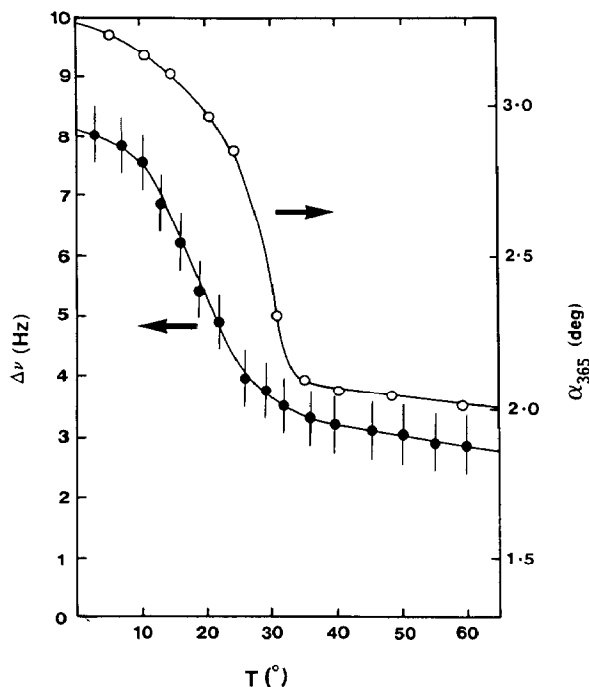


Fig. 11. Increase in ^{35}Cl linewidth on conformational ordering of kappa-carrageenan (2% w/v; Me_4N^+ salt form) in the presence of tetramethylammonium chloride (0.30M). Symbols as in Fig. 9.

sence of different tetramethylammonium salts show a systematic increase in ΔH_{cal} with the Hofmeister number for the anion. Transition entropy (ΔS_{cal}), calculated from the relationship: $\Delta H_{\text{cal}} - T\Delta S_{\text{cal}} = \Delta G = 0$ at T_m , also displays a corresponding systematic increase.

Previous studies of conformational ordering of kappa-carrageenan in the presence of tetramethylammonium iodide²⁰ showed an accompanying increase in ^{127}I -n.m.r. linewidth (*i.e.*, a decrease in relaxation times). These results were interpreted as showing specific binding of iodide ions to the ordered structure of the polymer. We have now confirmed (Fig. 9) the experimental findings for ^{127}I and have also observed a similar broadening of ^{81}Br and ^{35}Cl linewidths over the temperature range of the disorder–order transition of kappa-carrageenan in the presence of Me_4NBr (Fig. 10) and Me_4NCl (Fig. 11).

As in the case of optical rotation measurements on the same systems, the observed n.m.r. linewidths showed no detectable hysteresis between results obtained on heating and on cooling. Integrated peak areas remained constant throughout the transition and are in good quantitative agreement with the values expected from the known concentration of halide present. This finding indicates that there is no “missing” signal from halide ions whose mobility is so restricted that their n.m.r. contribution is flattened into the baseline due to extreme line-broadening.

DISCUSSION

Previous investigations of carrageenan gelation^{7,9,10,14} indicated the involvement of two processes: (a) a co-operative disorder–order transition which does not appear, in itself, to lead to gel formation, and (b) aggregation of ordered sequences to develop a three-dimensional network. We have referred⁹ to this two-stage mechanism as the “domain” model of polymer gelation. Our present results are fully consistent with this interpretation and further indicate that the ordered conformation is stabilised by “chaotropic” co-anions of high Hofmeister number, such as iodide and thiocyanate, whereas further aggregation of these ordered structures is promoted by anions of low Hofmeister number, such as sulphate and fluoride.

Recent studies^{11–14,19,27} have shown clear evidence of ion-pair formation between potassium ions and sulphate groups of carrageenan, in both the disordered and ordered chain conformation, whereas no such specific interaction occurs with tetramethylammonium ions. The resulting reduction in charge density of the polymer in the presence of K^+ has the effect of promoting both conformational ordering and aggregation. Despite this net displacement in the direction of order and packing, however, our results for the potassium salt form show the same systematic variations through the anion lyotropic series as those obtained with Me_4N^+ (or Et_4N^+) as cation.

The evidence presented here suggests the need for revision of the conclusions drawn from several earlier studies over narrower ranges of experimental conditions. From investigations using only chloride salts, Morris *et al.* concluded⁹ that conformational ordering of kappa-carrageenan does not occur with alkylammonium cations. Our present results show that, at lower temperatures and higher concentrations of salt than were used in the earlier investigation, the ordered conformation can be induced even in the presence of co-anions of low Hofmeister number, including Cl^- . The experimental evidence from which Rochas and Rinaudo¹¹ concluded that co-anions do not influence T_m does not conflict with our present results; again the apparent discrepancy comes from more restricted conditions. In particular, because of the use of K^+ as counterion, the disorder–order transition could be observed only at comparatively low concentrations of salt where T_m values for different co-anions converge (Fig. 5); also, the anions of highest Hofmeister number (I^- , SCN^-) were not included. The reported difference in T_m of 4° between Br^- and the anions of lowest Hofmeister number is in good agreement with our own results under comparable experimental conditions (Fig. 8). The results of Grasdalen and Smidsrød²⁰, showing conformational ordering of tetramethylammonium kappa-carrageenate in the presence of iodide ions, are also entirely consistent with the wider spectrum of behaviour reported here. The suggestion that I^- has a specific role in stabilising or inducing the ordered structure by site-binding to the polymer, however, seems less likely in the light of the present results.

The experimental evidence from which specific binding was proposed is the

substantial increase in ^{127}I -n.m.r. linewidth which accompanies conformational ordering. The results shown in Fig. 9 are in close agreement with those reported by Grasdalen and Smidsrød, but we suggest a somewhat different interpretation. For quadrupolar nuclei such as ^{127}I , the rate of n.m.r. relaxation (and hence high-resolution linewidth) is greatly increased by exposure to an electric field gradient²⁸⁻³⁰. The observed line-broadening therefore indicates a rapid exchange between ions experiencing a symmetric charge environment in free solution, and those experiencing a field gradient due to the proximity of the carrageenan polyanion. Proximity to the polymer cannot, of course, necessarily be equated with site-binding to it.

An alternative possibility is that incorporation of iodide ions within the "charge-cloud" (cations and counter-anions) around the polyelectrolyte may be sufficient to enhance their n.m.r. relaxation. Since adoption of a compact, ordered conformation will increase the linear charge density²⁵ of the carrageenan chain, a corresponding increase in "atmospheric" binding, and hence in linewidth, would again be anticipated.

There is no satisfactory way of interpreting measurements of n.m.r. linewidth to distinguish between site-binding of ions to a polyelectrolyte and less-specific modes of interaction³⁰. The close similarity between n.m.r. results for ^{127}I (Fig. 9) and those obtained using ^{81}Br (Fig. 10) and ^{35}Cl (Fig. 11), however, indicates a generality of behaviour rather than a specific or unique role for iodide in inducing conformational ordering of kappa-carrageenan. Moreover, the temperature-course of ^{127}I line-broadening is different from that of the optical rotation change (Fig. 9), with significant broadening at higher and lower temperatures, which is again more consistent with an indirect, non-specific effect of changes in polymer charge-density rather than direct incorporation of iodide ions as an integral part of the ordered structure.

The nature of the ordered structure of carrageenan has recently been the subject of some debate. X-Ray diffraction studies³¹ of oriented fibres of iota-carrageenan show clear evidence of a co-axial double-helix in the solid state. There is general agreement that an ordered conformation is also adopted under hydrated conditions on cooling, and no evidence of a further change in conformation on drying. Indeed, vacuum-ultraviolet c.d. studies³² of iota-carrageenan in gels and in solid films cast from them indicate the same molecular organisation in both.

Structurally regular segments of iota-carrageenan [prepared²³ by periodate oxidation of anomalous (1 \rightarrow 4)-linked residues of α -D-galactose or galactose 6-sulphate, and subsequent conversion of galactose 2,6-disulphate into 3,6-anhydrogalactose 2-sulphate] show, in the absence of K^+ or other cations that promote aggregation, a doubling \bar{M}_w on cooling from the disordered to the ordered chain-conformation. This result has now been obtained in three separate light-scattering studies^{9,27,33}, in one of which³³ a doubling of \bar{M}_n from membrane osmometry was also reported. The salt-induced disorder-order transition of both the native polymer and segments derived therefrom shows second-order kinetics

by polarimetric stopped-flow methodology³⁴. Both results argue strongly for a dimerisation process which, from the X-ray evidence³¹, seems most likely to be double-helix formation.

Evidence for a double-helix structure in kappa-carrageenan is less conclusive. Fibre diffraction data of sufficient quality to distinguish between co-axial helices and such alternative models as lateral packing of individual chains have yet to be obtained. The close similarity in primary structure of iota¹⁸ and kappa-carrageenan¹⁷ (Fig. 1), together with the similarities in the available X-ray diffraction patterns²¹, however, do suggest a strong likelihood of a related double-helix geometry. This view is reinforced by kinetic studies³⁵ that show the involvement of two chains in the rate-limiting process, and by the application¹¹ of Manning polyelectrolyte theory, which indicates that the linear charge-density of the ordered structure is close to that expected for a dimeric species.

Two recent papers, however, have challenged the double-helix proposal for both iota³⁶ and kappa-carrageenan²⁰. In both studies, iodide was used as co-anion, and the principal evidence against double-helix formation is a reported absence of a change in molecular weight by membrane osmometry on cooling through the disorder-order transition. We have been unable to reproduce this behaviour with light-scattering as the index of molecular weight. Polarimetric stopped-flow studies using iodide salts also gave results similar to those previously reported for chloride, with both iota- and kappa-carrageenan again showing second-order transition kinetics. This molecular weight and kinetic evidence will be reported in detail elsewhere.

There are two major experimental variations between the studies that suggest single-helix formation, and our own work which consistently indicates dimerisation: (a) the use of osmometry rather than light-scattering to characterise changes in molecular weight, and (b) preparation of samples of decreased chain-length by acid hydrolysis rather than by the method outlined above, involving selective cleavage at anomalous residues followed by chemical enhancement of structural regularity. This conflict is of experimental evidence rather than interpretation, and cannot yet be reconciled.

Although the results reported here have no direct bearing on the nature of the ordered structure, they do suggest that changes in the conformational stability or aggregation properties of carrageenan on changing the co-anion arise from modification of the solvent quality of water, following the well-established lyotropic series, rather than from direct interaction of anions with the polyelectrolyte. In particular, there is no evidence of any specific or unusual role for iodide in inducing or modifying the ordered chain conformation of kappa-carrageenan.

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