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ANION-INDEPENDENT CONFORMATIONAL ORDERING IN IOTA-CARRAGEENAN: DISORDER–ORDER EQUILIBRIA AND DYNAMICS

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ABSTRACT

The equilibria and dynamics of the disorder-to-order transition of the anionic polysaccharide iota-carrageenan have been studied in the presence of tetramethylammonium salts. By the use of a stopped-flow polarimeter, the rate equation and temperature dependence of the observed forward rate-constant were found to accord with a co-operative dimerisation process. Activation parameters for helix nucleation were shown to be independent of the anion for solutions containing tetramethylammonium chloride and bromide, *i.e.*, $\Delta H^{\ddagger} = 1 \pm 3$ kJ.mol^{-1}, $\Delta S^{\ddagger} = -178 \pm 10$ J.mol^{-1}.K^{-1}, $\Delta G^{\ddagger}_{298K} = 54 \pm 2$ kJ.mol^{-1}, and $k_{nuc,298K} = 1880 \pm 80$ dm^3.mol^{-1}.s^{-1}. The temperature dependence of optical rotation was also shown to be independent of the anion present.

INTRODUCTION

Of the family of algal polysaccharides, iota-carrageenan has received the greatest attention. In the native polymer, the idealised structure **1** of 1,3-linked β-D-galactose 4-sulphate and 1,4-linked 3,6-anhydro-α-D-galactose 2-sulphate residues is interrupted[1] by the occurrence of a proportion of the 1,4-linked residues in which the anhydro bridge has not been formed. Such residues are incompatible with the ordered structure and act as helix breaking-points. The degree of sequence regularity may be increased by Smith degradation[2] followed by treatment with sodium borohydride[3]. Iota-carrageenan segments prepared in this way undergo the same conformational transition as observed in the native material but without gelation.

X-Ray fibre-diffraction studies[4,5] suggest that a three-fold, right-handed double helix exists in the solid state. A variety of techniques indicate that this

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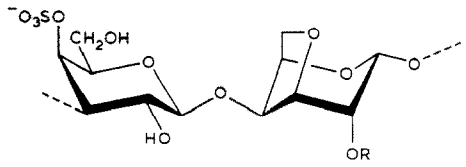

1 Iota R = SO_3^-
Kappa R = H

(Idealised disaccharide repeating-structure)

double-helix conformation persists into solution. The thermally induced changes in optical rotation[6,7] have the form expected for a co-operative transition, and the sign and magnitude of the change analysed[7] by the semi-empirical treatment of Rees[8] are in general agreement with those predicted for conversion of a random coil into the double helix. Light-scattering[9–11] and osmometry[9] measurements showed that both the weight-average and number-average molecular weights increased by a factor of two in the presence of sodium or tetramethylammonium chloride. Dynamic studies[12] of the disorder–order transition of iota-carrageenan segments, using stopped-flow polarimetry, showed that the conformational change was second order in the forward direction and first order in the backward direction, indicating a dimerisation process. However, it has been suggested[13], on the basis of osmometry measurements, that, in the presence of iodide ions, a salt-induced freeze-out of the conformational mobility occurs to produce a single helix.

A large number of physical techniques have been used to investigate[10–12,14–19] the effect of cations on the conformations and conformational ordering of the carrageenans. The results and conclusions from these studies have recently been reviewed[20]. In contrast, the influence of anions on the conformational transition in iota-carrageenan has been ignored to a large extent, with the notable exception of the proposed[13] single helix in the presence of iodide ions. There has also been limited attention paid to anion effects in the related polysaccharide kappa-carrageenan[21–23] (**1**).

We now present evidence of lack of anion effects on the kinetics and stability of iota-carrageenan in the presence of tetramethylammonium salts. We report elsewhere[24] a similar study of kappa-carrageenan in which there are significant anion-lyotropic effects.

EXPERIMENTAL

Materials. — The sample of iota-carrageenan (Batch no. SE2280/G) was kindly donated by Dr. C. Bellion (CECA SA, France). The structural regularity of the sample was improved by segmentation, using the Smith-degradation sequence[2] and treatment with sodium borohydride[3]. The sample was extensively dialysed against deionised water and the specific salt forms were prepared by ion-exchange on Amberlite IR-120 resin, followed by freeze-drying. Absolute purities were determined from elemental analysis (Butterworth Microanalytical Consultancy

Ltd.) and ^{13}C-n.m.r. spectroscopy. The sample had <5% kappa-carrageenan character and contained <2% non-carrageenan material. The ion-exchange procedure was shown to be >98% efficient. Absolute concentrations of samples used were determined from elemental analysis.

Methods. — Optical rotation was measured at 365 nm with a Perkin–Elmer 141 polarimeter (10-cm pathlength). The temperature was controlled using a Grant circulating water-bath, and samples were held at a constant temperature until a steady reading was attained. Light-scattering measurements were made at 633 nm, using a Chromatix KMX-6 low-angle laser-light-scattering instrument. Measurements were made at an angle of 6–7° to the incident beam. The sample was circulated through a thermostatted cell (pathlength 1.5 cm) by a peristaltic pump, with the temperature controlled by a Haake circulating water-bath. The refractive index increment (dn/dc) was measured on a Chromatix KMX-16 differential refractometer (at 633 nm), using the sample dialysate as the reference. Kinetic studies were performed using a stopped-flow polarimeter[24,25]. A rapid increase in salt concentration (salt jump) was used to drive the carrageenan into the ordered conformation. This was carried out at various temperatures for each tetramethylammonium salt studied. At each temperature and salt concentration, between 8 and 20 traces were obtained and averaged on a microcomputer (Acorn Atom). All solutions were clarified by filtration through a 0.45- or 0.22-μm Millipore filter, and freed from dissolved air in order to reduce the possibility of air-bubble formation which could cause depolarisation of light and possible cavitation in the stopped-flow apparatus[25].

RESULTS

The temperature dependence of the change in optical rotation of iota-carrageenan segments in solutions of the four tetramethylammonium salts studied is shown in Fig. 1. The transition mid-point temperature is seen to be independent of the anion present. This situation is in contrast to kappa-carrageenan where the stabilisation of the ordered form is anion-dependent[21,24].

Denoting helix and random-coil conformations of each disaccharide residue as H and C, respectively, a simple dimer–monomer equilibrium may be represented by Eq. *1*.

$$H_2 \rightleftharpoons 2C \qquad 1$$

The data from Fig. 1 were fitted to this model, using the procedure for evaluating the mole fractions of helices and coils previously described[26]. Equation *2* defines the mole-fraction equilibrium constant, K_x, in terms of the disaccharide residue concentrations [C] and [H] and the total disaccharide concentration $[C]_o$.

$$K_c = K_x[C]_o = [C]^2/[H_2] \qquad 2$$

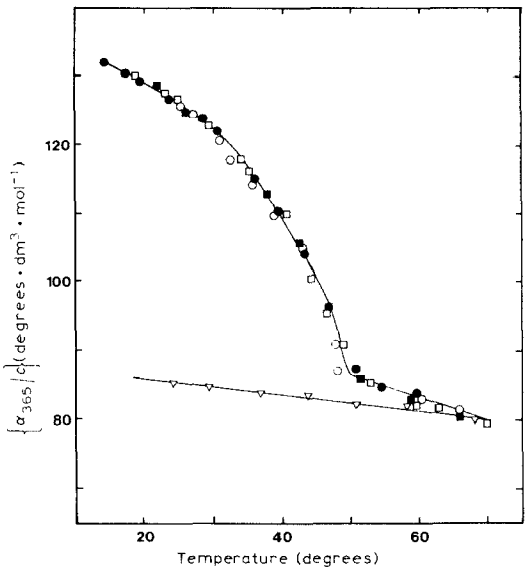

Fig. 1. Normalised optical rotation as a function of temperature for tetramethylammonium iota-carrageenan segments (2 2mM) in 0.20M solutions of various Me_4N^+ salts: Me_4NI (○), Me_4NBr (●), Me_4NNO_3 (□), Me_4NCl (■), distilled water (▽); pathlength = 10 cm.

The variation of K_c and K_x with temperature may be described by the Van't Hoff isochore (Eq. *3*).

$$d\ln K_c/d(1/T) = -\Delta H_{app}/R \qquad 3$$

The apparent enthalpy change for the co-operative transition, ΔH_{app}, is obtained from the gradient of the plot of $\ln K_c$ or $\ln K_x$ *versus* 1/T.

The apparent enthalpy change, ΔH_{app}, obtained for the common plot (Fig. 2) for all the tetramethylammonium salts studied is 206 ±7 kJ.mol^{-1}. The value for ΔH_f of 8.4 ±0.4 kJ.mol^{-1}, determined by differential scanning calorimetry (d.s.c.) by Norton *et al.*[11] for iota-carrageenan segments in solutions of both tetramethylammonium and potassium chloride, was used (Eq. *4*) to calculate the apparent number of disaccharide residues, n_{app}, participating in the co-operative transition.

$$n_{app} = \Delta H_{app}/\Delta H_f \qquad 4$$

The calculated co-operative length in the solutions of tetramethylammonium salts, 24 ±1 residue pairs, is similar to that (26 ±2) found[27] for the same iota-carrageenan in solutions of potassium chloride. These co-operative lengths are in close agreement with the values reported previously by Norton *et al.*[12] for their iota-carrageenan segments in solutions of potassium and sodium chloride over the same range of salt concentrations (0.1–0.5M, ΔH_{app} = 220 ±20 kJ.mol^{-1},

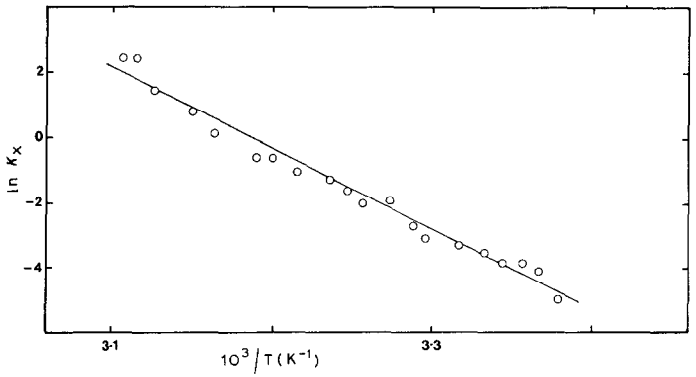

Fig. 2. Van't Hoff plot for Me_4N^+ iota-carrageenan segments (2.2mM) in 0.20M Me_4N^+ salt solutions.

and $n_{app} = 26 \pm 2$). Lower values were found for solutions in tetramethylammonium chloride ($\Delta H_{app} = 120$ kJ.mol^{-1}, $n_{app} = 14 \pm 2$). This lower value may be a consequence of the different purities of the polymers. The sample of iota-carrageenan used by Norton and co-workers was shown, by infrared spectroscopy[11], to be only 60% 2-sulphated on the 3,6-anhydrogalactose residues, in comparison with 95% in our sample and the 100% of the idealised repeating-disaccharide residue **1**.

As discussed elsewhere[26], n_{app} is to be regarded as a lower limit to the true co-operative length because of polydispersity. However, since both the iota-carrageenan samples give similar values for ΔH_{app} in solutions of potassium chloride, it seems that differences between the values obtained for solutions in tetramethylammonium chloride are unlikely to be explained by molecular-weight polydispersity. We suggest that, in the tetramethylammonium form, where charge neutralisation through specific cation binding is absent[11], fewer residues in the iota-carrageenan segments of Norton *et al.*[11,12] make the transition in concert at T_m, as a consequence of the packing constraints placed on helix formation by having a mixture of disulphated and monosulphated disaccharide residues. An alternative explanation could be that the less-pure sample studied previously was a mixture of iota- and kappa-carrageenan chains, with the resulting charge polydispersity giving a distribution of equilibrium curves. This situation would give an apparent broadening of the transition and thus an apparent lowering of the co-operativity.

The weight-average molecular weight, $\bar{M}_w$, obtained for the disordered conformation of iota-carrageenan segments in 0.2M tetramethylammonium bromide at 323K was $(6.8 \pm 1.4) \times 10^4$, corresponding to a chain length of 125 ± 25 disaccharide residues. In the ordered conformation at 293K, $\bar{M}_w$ was found to be $(14.8 \pm 2.3) \times 10^4$, thus showing an approximate doubling of molecular weight. This finding is consistent with the observations of Norton *et al.*[11], where a doubling of $\bar{M}_w$ was reported on going through the transition for iota-carrageenan segments of comparable molar mass in solutions of tetramethylammonium chloride. These results suggest that conformational ordering of iota-carrageenan in solutions of tetra-

methylammonium salts involves a dimerisation process with no further aggregation.

The polarimetric stopped-flow apparatus was used to provide a rapid jump in salt concentration and drive the iota-carrageenan segments into the ordered conformation. This was performed at various temperatures for different tetramethylammonium salts. In all cases, the kinetics observed were monophasic. The amplitude of the change in optical rotation invariably agreed (within experimental error) with the value determined from equilibrium optical rotation studies.

Several reaction schemes, including irreversible and reversible, first- and second-order processes, were considered in the analysis of the kinetic data. The reaction scheme (Eq. 5) previously adopted[12,26] gave the best fit to the experimental relaxations, and the integrated form (Eq. 6) was therefore used to analyse all of the kinetic data.

$$2C \underset{k_{-1}}{\overset{k_2}{\rightleftharpoons}} H_2 \qquad 5$$

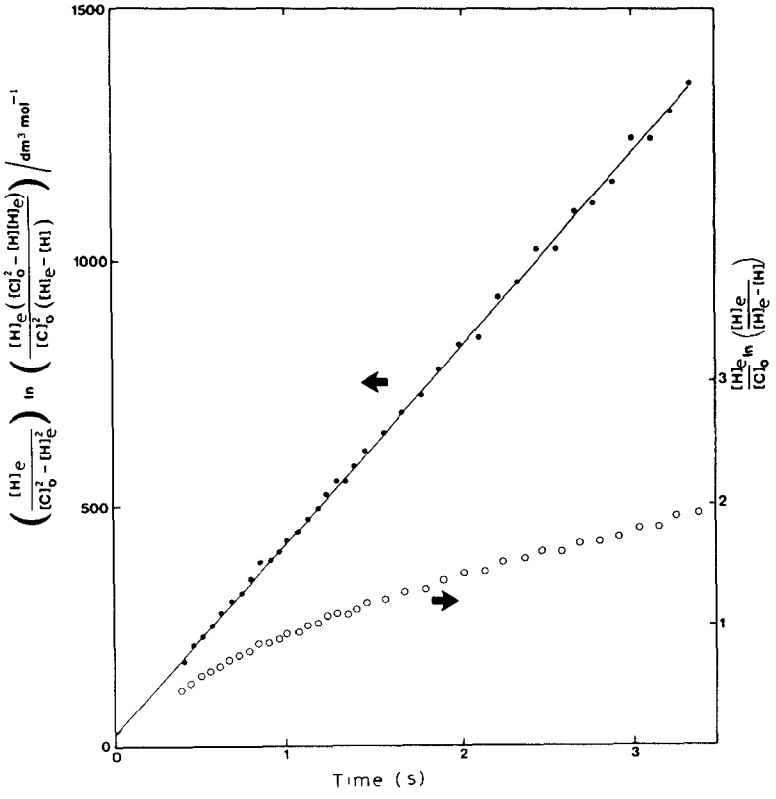

Fig. 3. Comparison of the first-order (○) and second-order (●) kinetic analysis for the disorder–order transition of Me_4N^+ iota-carrageenan segments (3.52mM) in 0.20M Me_4NCl at 20°.

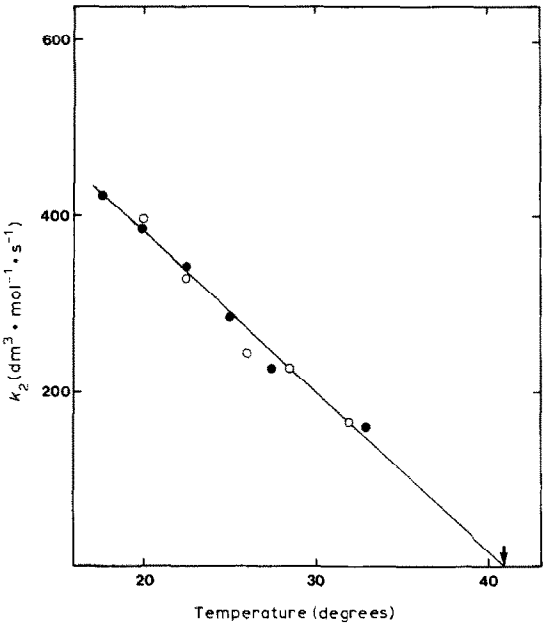

Fig. 4. Variation of observed rate constant with temperature for the order–disorder transition of Me_4N^+ iota-carrageenan segments (3.5mM) in 0.20M Me_4NCl (●) and Me_4NBr (○). Arrow indicates polarimetric T_m.

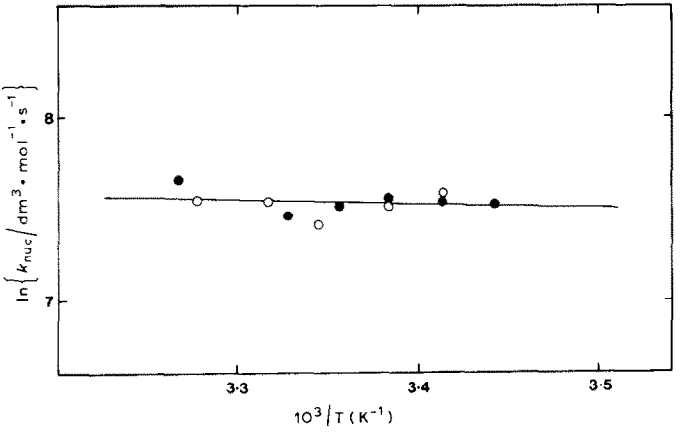

Fig. 5. Arrhenius plot for nucleation rate constants of iota-carrageenan segments in 0.20M Me_4NCl (●) and Me_4NBr (○).

TABLE I

COMPARISON OF NUCLEATION PARAMETERS AND TRANSITION MID-POINTS FOR DIFFERENT CARRAGEENAN SAMPLES INVESTIGATED IN VARIOUS SALT SOLUTIONS

Carrageenan sample	*2-Sulphation (%)*	*Salt*	*Salt conc.* (M)	$T_m(K)$	$\Delta H^{\ddagger}$ $(kJ.mol^{-1})$	$\Delta S^{\ddagger}$ $(J.mol^{-1}.K^{-1})$	$\Delta G^{\ddagger}_{298K}$ $(kJ.mol^{-1})$
Iota segments	95	Me_4NCl	0.20	314 ±1	1 ±3	−178 ±10	54 ±6
		Me_4NBr	0.20				
Iota segments[12]	60	Me_4NCl	0.15–0.50	304.0	133 ±2	263 ±6	55 ±2
		KCl	0.20	317.8	29 ±1	−66 ±3	49 ±1
Kappa[24]	10	Me_4NCl	0.25	292.5	23 ±12	−74 ±10	45 ±14
Alkali-modified		Me_4NBr	0.20	301.5	6 ±4	−131 ±2	45 ±4
Kappa[26], native	10	KCl	0.10–0.50	321.0	8 ±8	−140 ±30	50 ±8
Kappa[24], segments	10	Me_4NBr	0.20	294.0	20 ±4	−98 ±9	49 ±4

$$\frac{[H]_e}{([C]_o^2 - [H]_e^2)} \ln \left\{ \frac{[H]_e ([C]_o^2 - [H][H]_e)}{[C]_o^2 ([H]_e - [H])} \right\} = k_2 t \qquad 6$$

where the subscripts o and e refer to the starting and final (equilibrium) positions in the reaction, respectively[12,26]. In all cases, the averaged reaction-progress curve fitted Eq. *6* with a linear correlation coefficient greater than 0.99 over more than 95% of the reaction (Fig. 3). The fit to the reversible first-order analysis

$$(C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H),$$

which has the integrated form of Eq. *7*, is shown in Fig. 3 for comparison.

$$\frac{[H]_e}{[C]_o} \ln \left\{ \frac{[H]_e}{[H]_e - [H]} \right\} = k_1 t \qquad 7$$

The observed forward rate-constant, k_2, obtained as a function of temperature for the disorder-to-order transition of iota-carrageenan segments in 0.2M tetramethylammonium chloride and bromide, is shown in Fig. 4; k_2 decreases to zero at the transition mid-point temperature and is independent of the anion present.

The generalised model of Crothers *et al.*[28] for coil-to-double helix transitions was used to obtain the nucleation rate constants and activation parameters as discussed elsewhere[12,24,26]. Eqs. *8* and *9* allow determination of the nucleation rate constant, k_{nuc}.

$$k_{nuc} = k_2/(1 - K) \qquad 8$$

$$K = \exp[-\Delta H_f(T_m - T)/RTT_m] \qquad 9$$

The enthalpy change per pair of disaccharide residues, $\Delta H_f = 8.4$ kJ.mol^{-1}, was obtained[11] from d.s.c. measurements. The Arrhenius plot of the nucleation rate constants obtained is shown in Fig. 5. A single line can be fitted through the data for solutions in both tetramethylammonium chloride and bromide, indicating that the nucleation process is unaffected by the change of anion. The activation parameters obtained are compared to those of related polymer samples in Table I.

DISCUSSION

Our studies show that the order–disorder transition of iota-carrageenan segments is insensitive to the anion, when the salt concentration and counter-ion are held constant. Equilibrium and kinetic results taken together show that both helix

growth and nucleation are unaffected by anions. This is in contrast to observations on kappa-carrageenan[21,24] where anions significantly influence both the thermodynamics and kinetics of conformational ordering in accordance with the lyotropic (Hofmeister) series. We suggest that this may reflect the lower negative-charge density of kappa-carrageenan (**1**), with consequent increase in the importance of indirect solvent effects relative to direct-charge shielding.

An important finding from these studies is that, in the presence of tetramethylammonium salts, iota-carrageenan segments exhibit monophasic reaction curves which are best fitted by a monomer–dimer equilibrium process. Biphasic reaction curves were observed for kappa-carrageenan and interpreted in terms of a conformational ordering followed by aggregation[24]. The absence of any secondary process in the kinetics of the disorder–order transition of iota-carrageenan might therefore be interpreted as suggesting that no aggregation takes place in iota-carrageenan in the presence of tetramethylammonium salts. This interpretation is supported by light-scattering measurements, where a doubling of $\bar{M}_w$ was observed on going through the transition.

Fig. 4 shows that the nucleation rate constant is, within experimental error, invariant with temperature. The value $k_{nuc,298K} = 1880 \pm 80$ dm^3.mol^{-1}.s^{-1} is similar to the values previously reported for carrageenan and is several orders of magnitude below the figure expected for diffusion-controlled encounters[29]. Since there is no activation enthalpy barrier for nucleation ($\Delta H^{\ddagger} = 1 \pm 3$ kJ.mol^{-1}), the unfavourable activation entropy ($\Delta S^{\ddagger} = -178 \pm 10$ J.mol^{-1}.K^{-1}) is the predominant factor in controlling the nucleation rate. Small values of $\Delta H^{\ddagger}$ and large negative values of $\Delta S^{\ddagger}$ are characteristic features of the nucleation step in conformational ordering of other polysaccharides[26,30], polynucleotides[31], and proteins[32]. The entropic factor reflects the low statistical weight of the particular conformation required in the transition state for nucleation[32]. For polynucleotides[28,33], the activation enthalpy can give an indication of the number of residues involved in the nucleation process. This approach was extended to kappa-carrageenan[26] and xanthan[30] by Norton *et al.* In the case of iota-carrageenan, presented here, the arguments discussed are likely to be similar, with the small positive value of $\Delta H^{\ddagger}$ observed being consistent with a nucleation length of one or two residue pairs. The involvement of more residues would entail exothermic pre-equilibrium steps and lead to negative $\Delta H^{\ddagger}$ values, as in certain oligonucleotides[33].

A comparison of the transition mid-point temperatures for the order–disorder transitions of iota- and kappa-carrageenans in solutions of tetramethylammonium chloride (Table I) shows that the ordered form of iota-carrageenan is the more stable. Conformational ordering is slower, however, for iota-carrageenan, as revealed by both the observed and nucleation rate constants. This fact is reflected in the activation free-energies, with the value obtained for iota-carrageenan in solutions of tetramethylammonium chloride or bromide being 54–55 kJ.mol^{-1}, whereas values for kappa-carrageenan are within the range 45–49 kJ.mol^{-1}. This apparent contrast between the dynamic and equilibrium information may be understood if

the differences in dynamic parameters are a consequence of the decreased intra- and inter-chain repulsions with decreasing charge density or, alternatively, reflect more conformational constraint in nucleation and growth of the iota-carrageenan helix. The difference in overall stability in the tetramethylammonium salt forms of iota- and kappa-carrageenan reflect the importance of electrostatic interactions in stabilising the ordered form of these hydrophilic polymers.

The transition mid-point temperature of the iota-carrageenan segments of Norton *et al.*[11,12] in the presence of tetramethylammonium chloride can be seen to fall between the values obtained for our iota-carrageenan segments and kappa-carrageenan. This finding indicates that this sample is more stable than kappa-carrageenan but not as stable as our iota-carrageenan preparation. This is not surprising since the iota-carrageenan segments of Norton and co-workers[11,12] carry more sulphate groups in the anhydrogalactosyl residues than the kappa-carrageenan sample but less than our iota-carrageenan.

Although the activation free-energies for nucleation in tetramethylammonium chloride are the same for the two samples of iota-carrageenan, there are striking differences in the activation entropy and enthalpy (Table I). The previous studies[12,34] gave a higher activation enthalpy and entropy than the values obtained here. A possible explanation of the difference observed for the different iota-carrageenan samples may be obtained by considering the proposed[4,5] helix conformations of iota- and kappa-carrageenan. X-Ray fibre-diffraction studies revealed that the iota-carrageenan double helix has chain positions that are shifted relative to those of the kappa-carrageenan double helix. This suggests that there are two related but distinct geometries for carrageenan double helices. It is possible, therefore, that a mixture of chains having different degrees of sulphation could not optimise the association of all segments simultaneously. This situation would result in a less-stable ordered form and also increase the activation enthalpy, because of less-efficient return of energy for each residue participating in the nucleation step.

With the previous sample of iota-carrageenan, the high values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were found to hold for solutions of low ionic strength for all the salt forms studied. On increasing the salt concentration in potassium or sodium chloride solution, however, the activation parameters tended towards the values characteristic of the purer sample studied here. Because low activation parameters are also observed for relatively pure samples of kappa-carrageenan[24,26], we suggest that the high ionic strength situation is more difficult to attain in the mixed sample as any local imbalance of charge needs to be countered before helix nucleation and formation is optimised.

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