A Study of the Helix–Coil Transition of ι-Carrageenan Segments by Light Scattering and Membrane Osmometry

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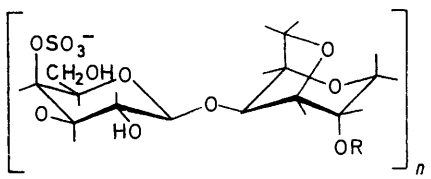
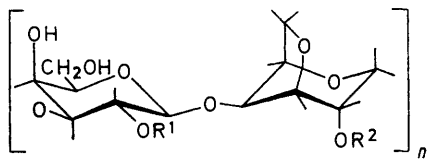
An ι-carrageenan has been selectively cleaved at the galactose 6-sulphate kink points to give short chain segments which do not gel but which give the optical rotation–temperature curve characteristic of gelation. This optical rotation change has previously been attributed to a helix to coil transition. The molecular weight changes with temperature of these ι-carrageenan segments have been measured by light scattering and osmometry and it has been found that the optical rotation shift is paralleled by a dimerisation thus confirming the hypothesis of a coil to double helix transition. Light scattering shows behaviour typical of concentration dependent aggregation in both states, but this is not detected by osmometry.

THE carrageenans are sulphated galactans found only in red seaweeds and are built up from residues having the *galacto*-configuration linked alternatively α-(1 ⟶ 3) and β-(1 ⟶ 4).[1] The most important gel forming polysaccharides in the carrageenan series are κ (I) and ι (II) in which (1 ⟶ 3) linked β-D-galactose-4-sulphate residues and (1 ⟶ 4) linked 3,6-anhydro-α-D-galactose (or its 2-sulphate) residues alternate. These polysaccharides are of the masked repeating type and there is some formal replacement of the 4-linked anhydride units by galactose-6-sulphate and 2,6-disulphate.[2,3]

There is good evidence from *X*-ray diffraction studies on oriented fibres that κ- and ι-carrageenans exist as double helices in the solid state.[4] It has been suggested that the formation of gels when dilute aqueous solutions of these carrageenans are cooled is due to association of chain segments into double helices which crosslink chains into a three dimensional network (Figure 1). This model is supported by optical rotation



(I) R = H
(II) R = SO_3



(III) R^1 = H (30%) and SO_3 (70%),
R^2 = SO_3

studies on the sol ⟶ gel ⟶ sol transformation in carrageenan solutions, which show that the optical rotation changes accompanying gel formation are con-

[1] D. A. Rees, *Adv. Carbohydrate Chem. Biochem.*, 1969, **24**, 267.
[2] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J. Chem. Soc.* (*C*), 1968, 596.
[3] 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.
[4] N. S. Anderson, J. W. Campbell, M. M. Harding, D. A, Rees, and J. W. B. Samuel, *J. Mol. Biol.*, 1969, **45**, 85.

sistent with double helix formation.[5,6] These studies are complicated by secondary changes accompanying gel formation and the possibility of stress birefringence. McKinnon *et al.*[7] overcame these difficulties by chemically degrading an ι-carrageenan to segments which were too short to form a network, *i.e.* the solution did not

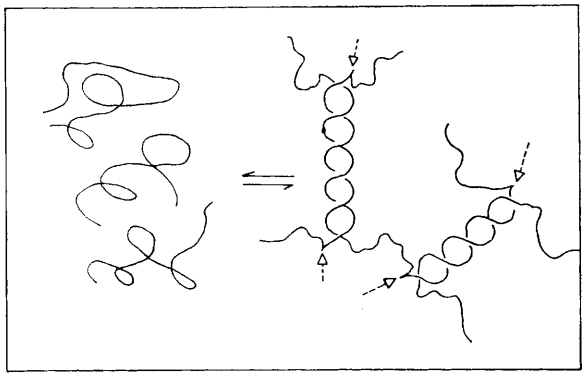


FIGURE 1 Model for the molecular basis of gel formation by ι-carrageenan: a network is formed by the combination of chains into double helical segments terminated by helic breaking 'kinks' (arrowed). Smith degradation selectively cleaves the polysaccharide chains at these points

gel, but which showed large changes in optical rotation with temperature which they attributed to formation of double helices on cooling.

We now report a study on similar types of ι-carrageenan segments and show that the changes which result in optical rotation shifts cause changes in the aggregation of the system which support the mechanism suggested earlier.

EXPERIMENTAL

Chemical Analysis.—The sample of ι-carrageenan was supplied by Pierrefitte-Auby, 46 Rue J. Dulud, Neuilly sur Seine, Paris, and had been isolated from *Eucheuma spinosum*. All physical and chemical analyses were performed on polysaccharade samples which had been dried for 16 h at 40° *in vacuo*. Moisture contents of dried samples were determined by heating at 105° *in vacuo* for 3 h. I.r. spectra were recorded with the Perkin-Elmer model 237 spectrometer (sample as film). The galactose : 3,6-anhydrogalactose ratios were determined by a modification of the procedure outlined by Yaphe [8] but using the phenol–sulphuric acid in place of the anthrone reagent. Sulphate contents were determined by the barium chloranilate method.[9] The amount of 1,4-linked galactose 6-sulphate and 2,6-disulphate units in the polysaccharide was determined by measuring the increase in 3,6-anhydrogalactose content on alkali treatment of the polysaccharide before and after periodate oxidation. Polysaccharide (20 mg) was dissolved in water (20 ml) and sodium borohydride (20 mg) was added and the solution cooled to 0° before addition of sodium hydroxide (2M, 20 ml). A sample of solution (20 ml) was removed and immediately neutralised with hydrochloric acid (1M, 20 ml). The remainder of the solution was heated at 80° on a water-bath for 5 h, then cooled before removal of a sample (10 ml) for neutralisation as above. Samples (1 ml) of both neutral solutions were then analysed, in triplicate, for 3,6-anhydrogalactose content by the resorcinol method.[8]

For oxidation the polysaccharide (50 mg) was dissolved in aqueous sodium metaperiodate (0·05M, 20 ml) and left for 75 h at room temperature. Excess of periodate was destroyed with excess of ethylene glycol, sodium borohydride (100 mg) was added, and the solution was left at room temperature for 48 h. The solution was dialysed, concentrated by evaporation, and freeze dried. The increase in 3,6-anhydrogalactose content of the periodate oxidised polysaccharide on treatment with alkali was then measured by the resorcinol method as above.

Polysaccharide Chain Splitting Sequence.—ι-Carrageenan (20 g) was dissolved in water (4 l), sodium metaperiodate (42·8 g) added, and the oxidation allowed to proceed for 60 h at room temperature. The reaction was stopped by the addition of excess of ethylene glycol, and sodium borohydride (40 g) was added. After 48 h at room temperature the solution was made 1·0M with respect to sodium hydroxide and heated on a water-bath at 80° for 5 h. The mixture was cooled and neutralised with hydrochloric acid and taken to pH 1·0 by addition of excess of acid. After 24 h at room temperature the solution was neutralised with potassium hydroxide and exhaustively dialysed, then passed through a column of 1R 120 (K^+ form) resin. The solution was concentrated by evaporation and the polysaccharide segments isolated by freeze drying (yield 14·5 g).

Optical Rotation Measurements.—Optical rotations were measured with a Perkin-Elmer 141 polarimeter using 10 cm cells and a wavelength of 546 nm. To ensure dust free samples, the solutions were passed hot through a Millipore filter (0·45 micron pore size) into hot jacketted cells. Temperature was varied by circulating water from a bath with a contact thermometer which was set manually. Solutions were held at each temperature until an equilibrium reading was reached. For both optical rotation and light scattering measurements, all concentrations were determined prior to Millipore filtration.

Light Scattering Measurements.—These were made using a Sofica photogonio-diffusometer model 42,000 using light of wavelength 546 nm. The temperature was controlled by the sample vat heater to $\pm 0{\cdot}5°$ and monitored by a copper–constantan thermocouple immersed in the vat. Allowance was made for the solvent loss at high temperatures by weighing the cells before and after taking measurements. Solutions were freed from dust by filtration through 0·22 micron Millipore filters. Scattering measurements were made either by placing the cold sample cell into a vat at high temperature ($\geqslant 60°$) and measuring the change of scattered intensity with time, or by increasing the vat temperature by steps of *ca.* 10° and allowing the system to come to equilibrium at each temperature.

As the intensity of scattering was low, the measurements were made at an angle of 90°. The weight average molecular weight M_w can be calculated from the limiting value

[5] D. A. Rees, I. W. Steele, and F. B. Williamson, *J. Polymer Sci. C*, 1969, **28**, 261.
[6] D. A. Rees, W. E. Scott, and F. B. Williamson, *Nature*, 1970, **227**, 390.
[7] A. A. McKinnon, D. A. Rees, and F. B. Williamson, *Chem. Comm.*, 1969, 701.
[8] W. Yaphe, *Analyt. Chem.*, 1960, **32**, 1327.
[9] R. W. Klipp and J. E. Barney, *Analyt. Chem.*, 1959, **31**, 596.

of C/I_{90} at infinite dilution $(C/I_{90})_{c=0}$ by equation (1) where

$$\frac{1}{M_w} = \left(\frac{2\pi^2}{\lambda^4 N R_b}\right) n^2_b \left(\frac{dn}{dc}\right) I_b \left(\frac{c}{I_{90c}}\right)_{c=0} \quad (1)$$

I_b, R_b, and N_b are respectively the scattering intensity, Rayleigh ratio, and refractive index of the benzene standard at 25°, dn/dc is the refractive index increment of the solution, and the other symbols have their usual meaning. The values used for R_b and N_b were $1{\cdot}63 \times 10^6$ and 1·502 respectively.[10,11] The measurements at temperatures other than 25° were calibrated using an auxiliary glass standard whose scattering was independent of temperature in the range used.

The refractive index increments were measured with a Waters differential refractometer thermostatted at the appropriate temperature.

The molecular weights were corrected for dissymetry.[12] This correction was small, being in all cases less than the experimental error. The depolarisation of the solutions was negligible.

Osmometry Measurements.—The number average molecular weights were measured on a Melabs membrane osmometer using a Sartorius SM 11736 membrane. Measurements were made at 25 and 60° only as the design of the osmometer makes it unsuitable for changing temperature frequently. The number average molecular weights were calculated from equation (2). The optical

$$M_n = \frac{RT}{\lim_{c \to 0}\left(\frac{\pi}{c}\right)} \quad (2)$$

rotation, light scattering, and osmometry measurements were all made in 0·1M-sodium chloride.

RESULTS

Chemistry.—The carrageenan used was a commercial sample and its identity as an ι-carrageenan was checked by i.r. spectroscopy which showed bands of roughly equal intensity at 850 and 805 cm^{-1} indicative of galactose 4-sulphate and 3,6-anhydrogalactose 2-sulphate units respectively.[3] The polysaccharide contained galactose, 3,6-anhydrogalactose, and sulphate in the molar ratio 1·00 : 0·85 : 1·84 which is typical of an ι-carrageenan.[13] Alkali treatment of the polysaccharide before and after periodate oxidation showed that 6·6% of the 1,4-linked units are galactose 6-sulphate with a negligible amount of galactose 2,6-disulphate. Smith degradation [14] of the polysaccharide followed by treatment with alkaline borohydride resulted in the molar ratio of galactose to 3,6-anhydride changing from 1·17 to 1·06. The chemical composition of the degraded polysaccharide is shown in the Table. The following molecular weights are used in calculating the percentage composition values in this Table: for galactose, 162; for 3,6-anhydrogalactose 144; for sulphate 96

A 5% solution of the degraded polysaccharide did not gel when left overnight at room temperature, whereas the parent polysaccharide gelled strongly at this concentration. A solution of the potassium salt of the degraded polysaccharide in 0·1M-sodium chloride showed large changes in optical rotation with temperature (Figure 2). This change in optical rotation with temperature indicates that a conformation change is taking place.[15] The transition temperature is the same for both concentrations (Figure 2) showing that dimerisation is not dependent on polymer concentration in 0·1M-sodium chloride.

Chemical composition of ι-carrageenan segments

	Composition (%)		
Species	3,6-Anhydrogalactose	Galactose	Sulphate
ι-Carrageenan segments	22·4	26·9	32·8
	Potassium	Protein (N × 6·25)	Moisture
ι-Carrageenan segments	10·2	1·5	2·5

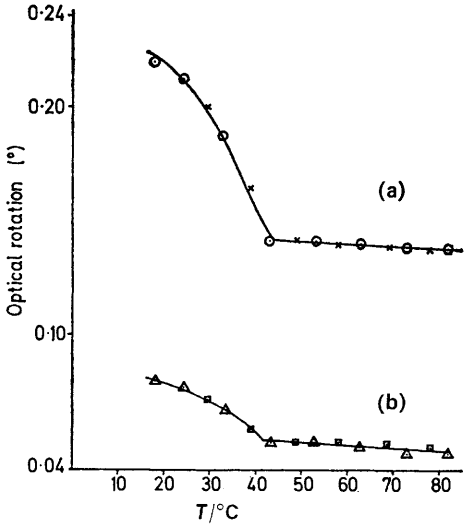


FIGURE 2 Optical rotation changes with temperature for ι-carrageenan segments in 0·1M-sodium chloride at 546 nm using a cell path length of 10 cm: (a) 0·3% w/v; ○, cooling; ×, heating; (b) 0·1% w/v; □, cooling; △, heating

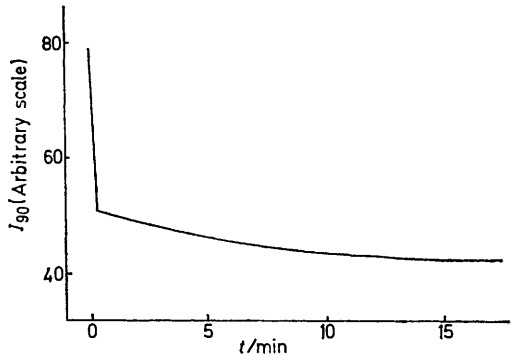


FIGURE 3 Change of scattering at 90° (I_{90}) with time for a solution of ι-carrageenan segments stored at 10° and placed in photometer vat at 60° at $t = 0$

[10] C. I. Carr, jun., and B. H. Zimm, *J. Chem. Phys.*, 1950, **18**, 1616.
[11] G. D. Parfitt and J. A. Wood, *Trans. Faraday Soc.*, 1968, **64**, 805.
[12] K. A. Stacey, 'Light Scattering in Physical Chemistry,' Butterworths, London, 1956.
[13] A. Penman, Ph.D. Thesis, 1969, University of Edinburgh.
[14] I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, Amer. Chem. Soc. Meeting, Boston, April 1959, Abs. Paper 3D.
[15] D. A. Rees, *J. Chem. Soc.* (*B*), 1970, 877.

Light Scattering.—When a solution of ι-carrageenan segments at 10° is placed in the vat of the photometer at 60° there is an initial rapid drop in the intensity of scattered light I_{90} (Figure 3) followed by a smaller, much

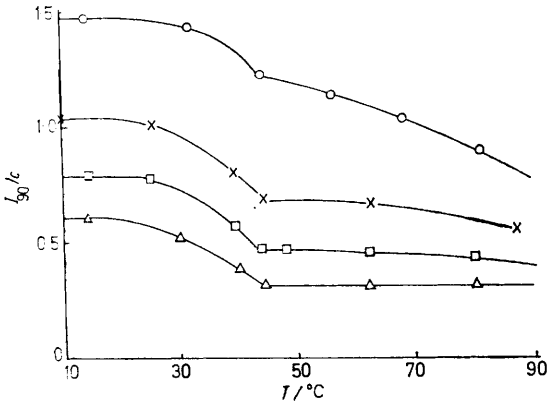


FIGURE 4 Reduced intensity I_{90}/C changes with temperature for ι-carrageenan segments solutions of four different concentrations: ○, 1·95 g cm^{-3}; ×, 0·68 g cm^{-3}; □, 0·45 g cm^{-3}; △, 0·19 g cm^{-3}

slower decrease which takes 0·5—2 h to reach equilibrium depending on concentration. The change in the equilibrium intensity of scattered light over the temperature range 10—80° at several polysaccharide concentrations is shown in Figure 4. At all concentrations there is a rapid decrease in scattered light in going from 20 to 45° and an inflection point at 45° with a further decrease after 45°. The size of this change increases with increasing concentration. Equilibrium scattering is reached much more slowly after the inflection temperature as would be expected from the results in Figure 3. A plot of C/I_{90} as a function of concentration at three temperatures is shown in Figure 5. Although the shapes of the curves at 60 and 80° are different, they extrapolate to the same intercept in the limit of zero concentration. Using the intercepts

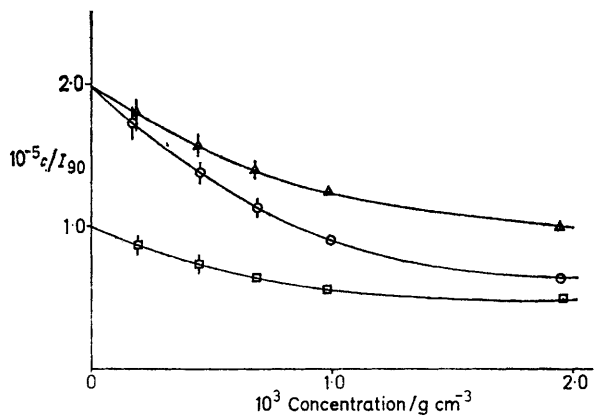


FIGURE 5 Plot of C/I_{90} against concentration at three temperatures: □, 10; ○, 60; △, 80°

at high and low temperature the weight average molecular weights for the two states can be calculated from equation (1).

[16] O. Smidsrød and A. Haug, *Acta Chem. Scand.*, 1918, **22**, 797.

The refractive index increment of the sample in 0·1M-sodium chloride at 20 and 60° was 0·127 ± 0·002 and so was taken to be independent of temperature. Since the polymer is a polyelectrolyte, attempts were made to establish dialysis equilibrium between solvent and solution prior to measuring the refractive index increment. However it proved impossible to obtain consistent results, and so the value of 0·127 was used. The resulting molecular weights are: high temperature $M_w = 68{,}000 \pm 10{,}000$, low temperature $M_w = 148{,}000 \pm 10{,}000$.

The error involved in the use of the undialysed refractive index increment will be the same in both cases, and so the ratio of the molecular weights at high and low temperature will remain the same. The size of the error can be estimated using the data for sodium alginate, another linear ionic polysaccharide, measured by Smidsrød and Haug.[16] They found that the refractive index increment changed

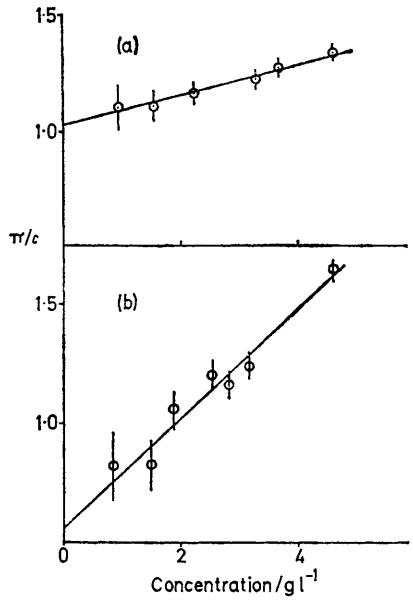


FIGURE 6 Osmometry measurements: graph of π/c against concentration at (a) 60; (b) 25°

from 0·165 to 0·157 on dialysis. A similar change in our case would lead to both molecular weights being 10% higher. The conclusions would not be affected by this change.

The concentration dependence of the curves in Figure 5 is characteristic of those obtained for systems undergoing concentration dependent aggregation.[12] This is supported by the temperature dependence of the curves, since the scattering changes less with concentration at 80 than at 60°, and presumably at some higher temperature the concentration dependence would disappear. As the aggregation is not detected by osmometry (Figure 6) it may be due to a small amount of the polymer forming high molecular aggregates.[17]

Osmometry.—As equilibration of the osmometer was difficult at temperatures below ambient, the lowest temperature used was 25°; however, very little disaggregation occurs at this temperature (Figure 4). Plots of π/c against concentration (Figure 6) show that there is no evidence of the concentration dependent aggregation observed by light

[17] P. Doty, H. Wagner, and S. Singer, *J. Chem. Phys.*, 1947, **51**, 32.

scattering. The number average molecular weights calculated from equation (2) for the two states were: 60° M_w = 33,000 ± 5000, 25° M_w = 59,000 ± 10,000.

DISCUSSION

McKinnon *et al.*[7] have shown that cooling a hot solution of ι-carrageenan segments results in changes in optical rotation due to a change to a more ordered conformation at low temperature. This conformation change was attributed to the formation of double helices on cooling and their dissociation on heating for the following reasons. *X*-Ray diffraction studies on oriented fibres of the undergraded polysaccharide[4] had shown that the molecule could exist as a double helix in the solid state. The specific rotations of the segments at low and high temperature agreed very closely with those calculated for double helix and coil forms respectively.[6]

Gel formation is very sensitive to changes in polysaccharide covalent structure, in that alkali-modified λ-carrageenan (III) is identical to ι-carrageenan except for the position of sulphation on the 1,3-linked unit [18,19] and yet it does not gel; this is readily explained since the 1,3-linked galactose 2-sulphate cannot be incorporated in the double helix. Variations in the extent of sulphation on the 4-position of the 1,3-linked unit or the 2-position of the 1,4-linked unit can be tolerated however as the sulphate groups are compatible with helix formation.

We have shown that the optical rotation changes which occur on cooling a solution of ι-carrageenan segments are accompanied by a doubling of the weight and number average molecular weights of the polysaccharide showing that the conformation ordering involves the formation of dimers. These results, combined with the evidence outlined above, point unequivocally to double helix formation on cooling.

The doubling of molecular weight indicates that on helix formation there is matching of chains, *i.e.* there are few long free chain ends which could lead to the formation of much larger aggregates. The optical rotation data supports this, since ι-carrageenan segments do not show hysteresis, indicating that in this system double helix formation is an equilibrium process in which chains can orient themselves so as to give the best possible match. This is in contrast to κ-carrageenan segments which exhibit hysteresis [20] and show complex light scattering changes.[21]

The ratio of weight to number average molecular weight is 2 : 1, giving an indication of the width of the molecular weight distribution. This will reflect the distribution of the oxidisable units in the starting polysaccharide, rather than the initial molecular weight distribution. Chemical evidence shows that 6% of the 1,4-linked units occur as galactose 6-sulphate, *i.e.* 3 residues per 100 of the initial ι-carrageenan will be degradable. If these were evenly spaced, the weight and number average molecular weights would be the same, and equal to 33 chain units, *i.e.* a molecular weight of approximately 10,000. The difference between M_w and M_n shows that the molecular weight distribution is not regular, but the molecular weight figures do not represent the true average of the segments, since *ca.* 25% by weight was lost in the course of the dialysis.

It has been postulated [22] that the biological role of these 1,4-linked galactose 6-sulphate units, together with galactose 2,6-disulphate units, is to act as ' kink points ' which are helix terminating, *i.e.* they cause chains to combine with more than one partner, as is necessary to form a network, rather than forming the same double helical association along the entire molecular length. From the discussion above it is evident that there will be regions in the polymer which contain a high concentration of kink points and which will therefore not be involved in the formation of helical segments. The lengths of these helical junction zones will therefore be longer than predicted from chemical analysis.

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[19] N. S. Anderson and D. A. Rees, in ' Proceedings of the Vth International Seaweed Symposium,' eds. E. G. Young and J. L. McLachlan, Pergamon, London, 1966, p. 243.
[20] I. C. M. Dea, A. A. McKinnon, and D. A. Rees, *J. Mol. Biol.*, 1972, **68**, 153.
[21] R. A. Jones and E. J. Staples, unpublished results.
[22] C. J. Lawson and D. A. Rees, *Nature*, 1970, **227**, 390.