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Further observations on the size, shape and hydration of kappa-carrageenan in dilute solution

Stephen E. Harding,[a]* Kathryn Day,[a] Rajesh Dhami[a] & Philip M. Lowe[b]

[a]*University of Nottingham, Department of Applied Biochemistry and Food Science, Sutton Bonington, LE12 5RD, UK*
[b]*Ciba Geigy Pharmaceuticals Ltd, Wimblehurst Road, Horsham RH12 4AB, UK*

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This study builds on previous studies of the important commercial polysaccharide kappa (κ)-carrageenan by confirming, using quite different methdology, the molecular weight and the asymmetric conformation of the κ-carrageenan molecule and also provides an estimate of its large capacity to imbibe water. Studies (sedimentation velocity and equilibrium analytical ultracentrifugation and viscometry) were performed on unfractionated material in a dilute solution based on a sodium phosphate/chloride buffer (pH 6.5, $I=0.10$). Low-speed sedimentation equilibrium using Rayleigh interference optics and using three different methods of extrapolation procedure yields a concensus weight average molecular weight, M_w, of (300 000±40 000) g/mol; this (i) demonstrates the relation between 'whole cell average' and 'point weight average' molecular weight approaches and (ii) is consistent with other published values based on light scattering procedures. A single non-ideality virial coefficient was shown to be insufficient to explain the concentration dependence behaviour of the apparent weight average molecular weight, $M_{w,app}$. Sedimentation velocity yields a sedimentation coefficient, $s^\circ_{20,w}$ of (4.19±0.20) S and a sedimentation concentration regression coefficient, k_s of (591±40) ml/g; low-shear viscometry yielded an intrinsic viscosity $[\eta]$ of (630±60) ml/g and a Huggins constant K_η of ~0.36. From these data, the hydration independent Wales/van Holde ratio ($k_s/[\eta]$) of ~0.9 is consistent with an extended conformation and making the crude approximation of a rigid structure, corresponds to an equivalent hydrodynamic prolate ellipsoid of aspect ratio ~15:1. These data also yield a frictional ratio f/f_o of ~7.6 which is consistent with a large hydration (~50 g water per g of dry polysaccharide, corresponding to a molecular expansion of ~100×), consistent with one of κ-carrageenans key functional properties in foods as a high water binder. No further comment is made about the order–disorder transition claimed for these molecules. © 1997 Elsevier Science Ltd

INTRODUCTION

The carrageenans are a group of unbranched sulphonated marine polysaccharides extracted from red seaweeds of the class Rhodophyta (Haris, 1990) the most important species of which are *Chondrus crispus*, harvested off the coast of Canada, *Eucheuma cottonii* from the Philippines and Indonesia, and *Iridae* from Chile. Its fundamental characteristics and commercial usefulness—particularly in food applications—has been well reviewed (see, e.g. Therkelsen, 1993). These molecules also have considerable potential in biotechnology and the microencapsulation and immobilisation of drugs and enzymes (see, e.g. Tosa *et al.*, 1970).

Concerning saccharide composition, of the major forms, κ-carrageenan, whose well known disaccharide repeat unit is schematically shown in Fig. 1 differs

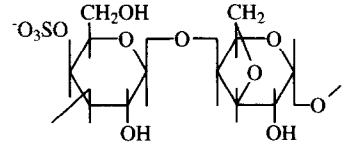

Fig. 1. κ-Carrageenan disaccharide repeat unit: alternating 1,3-linked galactose-4 sulphate and 1,4-linked 3,6-anhydro-D-galactose residues given as a simple Haworth projection. After Glickman (1982).

*University of Nottingham, Department of Applied Biochemistry and Food Science, Sutton Bonington, LE12 5RD, UK

from the other principal forms (iota and lambda) in terms of positioning of the sulphonated residues (Therkelsen, 1993 and references therein). No carrageenan preparation is 100% pure of the other isoforms, and a commercial κ-carrageenan will have a small percentage of iota (ι) and lambda (λ). All three have different gelation properties. λ-carrageenan does not gel at all (steric hindrance of the sulphate groups) whereas both the others form strong gels. However, whereas κ-carrageenan requires the presence of K^+, the iota form, like alginate, requires the presence of Ca^{2+}. Besides these specific properties the ability to gel and thicken depends on three non-specific properties: molecular weight, gross conformation and the intrinsic capacity of a carrageenan molecule to bind water. These properties are best addressed by dilute solution techniques such as those based on light scattering, sedimentation and viscometry.

Insofar as the fundamental dilute solution properties of κ-carrageenan are concerned, Slootmaekers *et al.* (1991a, b) have used a combination of light scattering methods (low angle total intensity or 'static' scattering coupled to size-exclusion-chromatography columns, and also dynamic light scattering) with sedimentation velocity analysis in the analytical ultracentrifuge to establish the molecular weight (weight average) and predicted values in the range 320,000–350,000 g/mol. In a more recent study, Chambers *et al.* (1994) performed a thorough investigation using multi-angle laser light scattering coupled to size exclusion chromatography on the effect of ionic strength, pH and temperature on the molecular properties. Above a temperature of 25°C in solutions of ionic strength ~0.05 M these workers showed a steady decrease in molecular weight from ~330,000 to 250,000 at a temperature of 60°C, which could be interpreted in terms of a transition from the ordered—allegedly 'double helical form'—to a disordered single 'random coil' form.

As far as we know, however, there has been no rigorous study by either sedimentation equilibrium in the analytical ultracentrifuge or combined sedimentation equilibrium/sedimentation velocity approaches on the properties of κ-carrageenan. In this study we therefore seek to address this by using the technique of low or 'intermediate'-speed sedimentation equilibrium (van Holde & Baldwin, 1958; Creeth & Harding, 1982) and use this data combined with results from sedimentation velocity (sedimentation coefficient and its concentration dependence parameter) with intrinsic viscosity information to make deductions about the overall conformation and water binding capacity of this molecule in dilute solution. All our studies are perfomed under conditions (20–25°C and $I = 0.1$ M) under which any alleged dissociative behaviour (Chambers *et al.*, 1994) would be absent, and therefore this study makes no further comment on this topic (which will be the subject of a future study).

MATERIALS AND METHODS

κ-Carrageenan

Food grade κ-carrageenan was supplied as a generous gift from Pedigree-Mars Petfoods (Melton Mowbray, UK). Solutions were pure of any significant protein or nucleic acid contamination, as judged by UV spectroscopy. The sample had an average moisture content of ~9%.

Solutions

κ-Carrageenan solutions were prepared by initial dissolution in deionised distilled water over 4–5 h of gentle but continual stirring. The samples were made in 100ml batches at a concentration of 2 mg/ml. The resulting solution was then dialysed exhaustively against a (K^+ free) phosphate chloride buffer, pH 6.5, [prepared by dissolving 3.080 g disodium hydrogen orthophosphate, 3.760 g sodium dihydrogen orthophosphate per litre of deionised distilled water and adding 2.923 g NaCl to give a combined ionic strength of 0.1, following Green (1933)]. Solutions were stored under refrigerated conditions (4°C) and never kept for longer than one week. Concentrations, c (g/ml) were corrected for moisture content and in sedimentation velocity experiments, for radial dilution.

Viscometry

An Ostwald-type automatic Schott-Geräte automatic viscometer was used. The temperature was regulated by suspension of the viscometer in a thermostatically controlled Comark no. 2 water bath at (25.00±0.05)°C and recorded using an accurately calibrated platinum resistance thermometer. Relative kinematic viscosities were found from the solution:solvent flow time ratio. As the concentrations were sufficiently small (1.5–5.0 mg/ml) to neglect density corrections, it was considered reasonable to assume that kinematic viscosity parameters ~ dynamic viscosity parameters (Tanford, 1955). The intrinsic viscosity $[\eta]$ (ml/g) was then found by plotting reduced specific viscosity η_{red} vs concentration, c (g/ml) and extrapolating to infinite dilution according to Huggins (1942):

$$\eta_{red} = [\eta](1 + K_\eta[\eta]c) \quad (1)$$

where K_η is the Huggins constant.

SEDIMENTATION VELOCITY

Sedimentation velocity measurements were performed on three different types of analytical ultracentrifuge each with particular features. For the κ-carrageenan characterization an MSE Centriscan ultracentrifuge was

used equipped with scanning Schlieren optics. A rotor speed of 47,000 rev/min was employed at a temperature of 20.0°C. Successive radial scans were analysed via a computer graphics digitising tablet to obtain sedimentation coefficient and radial dilution factors. Sedimentation coefficients were then corrected to standard conditions of density and viscosity (density and viscosity of water as solvent at 20°C) (see Tanford, 1961; Van Holde, 1985). The partial specific volume of κ-carrageenan was taken as 0.51 ml/g (Vreeman *et al.*, 1980). The 'infinite dilution' sedimentation coefficient $s_{20,w}$ (s) was found from the intercept of a plot of the reciprocal of the apparent sedimentation coefficient $s_{20,w}$ against concentration, c (g/ml), corrected for radial dilution, fitted to the relation (Schachman, 1959, p. 91; Creeth & Knight, 1965):

$$1/s_{20,w} = (1/s^{\circ}_{20,w}).(1 + k_s c) \quad (2)$$

Low-speed sedimentation equilibrium

The weight average molecular weight (or 'weight average molar mass'), M_w of the κ-carrageenan was determined using a Beckman Model E analytical ultracentrifuge equipped with a Rayleigh interference optical system and a 5 mW He–Ne laser light source at temperatures between 20.0 and 25.0°C and rotor speeds of 6400–8000 rev/min. 12 mm optical pathlength cells were used for the higher loading concentrations (0.91, 1.37 and 1.82 g/ml) and 30 mm pathlength cells were used for the two lower loading concentrations (0.23 and 0.68 g/ml). Solute distributions at equilibrium were captured photographically and then digitised using an LKB (Bromma, Sweden) Ultroscan laser densitometer. The digitised data was then analysed using the Fourier-cosine series TURBO-PASCAL algorithm ANALYSER (Harding & Rowe, 1987; Harding *et al.*, 1992) which gives an accurate record of concentration (in relative fringe displacement units) vs radial distance, r, from the axis of rotation. The speed used corresponds to the low-speed or 'intermediate speed' procedure (Creeth & Harding, 1982) (as opposed to the meniscus depletion procedure) where the concentration at the meniscus ($r = a$) remains finite but where the fringe displacement near the cell base ($r = b$) is still resolvable. An estimate for the concentration at the meniscus was obtained using the 'intercept over slope' procedure (Creeth & Harding, 1982) modified by Harding *et al.* (1992) and the apparent weight average molecular weight $M_{w,app}$ for a given loading concentration c obtained from the M^* function (Creeth & Harding, 1982): both these procedures are incorporated in the routine MSTARI (Harding *et al.*, 1992) now available for PC's (Cölfen & Harding, 1997) on QUICKBASIC. The routine also evaluates the local or 'point' apparent molecular weight, $M_{w,app}(r)$ [or $M_{w,app}(J)$] as a function of radial position r (or the equivalent local concentration, $J(r)$, expressed in fringe displacement units) by use of a sliding strip procedure (Teller, 1973). Sliding strip lengths were between 15 and 25 points depending on the total number of data points. Estimates for M_w (g/mol) were obtained from (i) extrapolation of $M_{w,app}$ to $c = 0$; (ii) extrapolation of $1/\{M_{w,app}\}$ to $c = 0$ and (iii) overlaying $M_{w,app}(J)$ data-sets for the five loading concentrations, c, used and extrapolating to $J = 0$.

RESULTS AND DISCUSSION

Homogeneity

Absorption spectra of a 2 mg/ml solution showed no maxima at 256 or 278 nm confirming the absence of protein or nucleic acid impurity (Fig. 2). Schlieren sedimentation diagrams from the MSE Centriscan showed only single sedimenting boundaries (Fig. 3) consistent with a homogeneous polysaccharide preparation.

Molecular weight and thermodynamic non-ideality from sedimentation equilibrium analyses

Conventionally, molecular weights of macromolecules are extracted from sedimentation equilibrium records by either (a) working at a concentration so low that non-ideality effects (deriving from molecular co-exclusion and polyelectrolyte phenomena) are negligible (i.e. $M_w \sim M_{w,app}$) or (b) if non-ideality is not too severe, measuring $M_{w,app}$ at a variety of concentrations, c, and performing a linear regression on a plot of $[1/M_{w,app}(r)]$ vs c. For κ-carrageenan neither procedure (a) nor (b) is valid: the reason is clear from plots of the logarithm of the Rayleigh fringe displacement, J vs the square of the displacement from the rotor centre (expressed in terms of the normalized parameter ξ, where $\xi = 0$ at the meniscus and 1 at the cell base) (Fig. 4): despite the polydispersity of these samples (which tends to cause upward or concave curvature) the overwhelming influ-

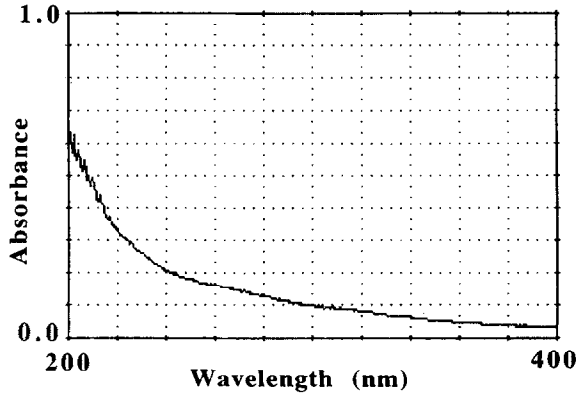

Fig. 2. Ultraviolet/visible spectrum for a 2 mg/ml solution of κ-carrageenan. A Beckman DU50 spectrophotometer was used.

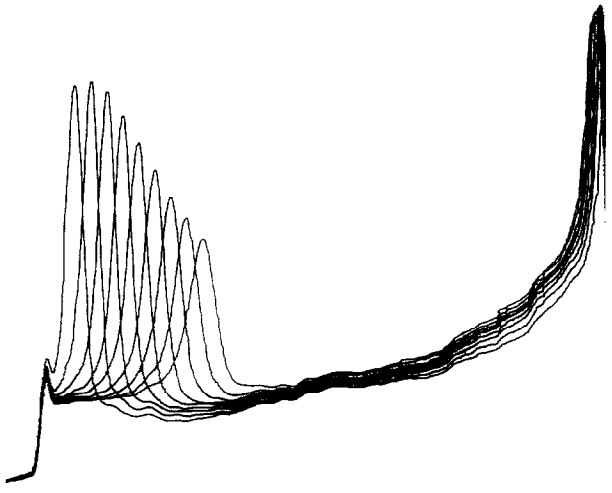

Fig. 3. Scanning Schlieren boundaries for κ-carrageenan at a loading concentration of 1 mg/ml. MSE Centriscan was used at a rotor speed of 47 100 rev/min and a temperature of 25.0°C. The direction of sedimentation is from left to right. Scan interval = 12 min. Knife-edge angle = 80°

ence is one of non-ideality causing increasingly strong downward (convex) curvature as the loading concentration is increased. Only for the lowest loading concentration (0.23 mg/ml) is the non-ideality effect small enough to prevent masking of the symptoms of polydispersity for $J < 0.5$, and above $J > 0.5$ non-ideality dominates: the 0.23 mg/ml profile is the classical plot of a non-ideal polydisperse system. A summary of the molecular weight estimates obtained from our sedimentation equilibrium measurements is given in Table 1.

(1) Extraction of M_w from a plot of $M_{w,app}$ vs c (Fig. 5a) (Method I of Table 1). A simple 2nd order polynomial fit yields a value of (300,000±10,000) g/mol.

(2) Extraction of M_w from a plot of $(1/M_{w,app})$ vs c (Fig. 5b) (Method II of Table 1). This is the conventional method for extracting M_w (Tanford, 1961) when the data can be reasonably fitted by linear regression. This is clearly not the case and a fit to an equation involving two non-ideal virial coefficients (B, C) is necessary, a feature common

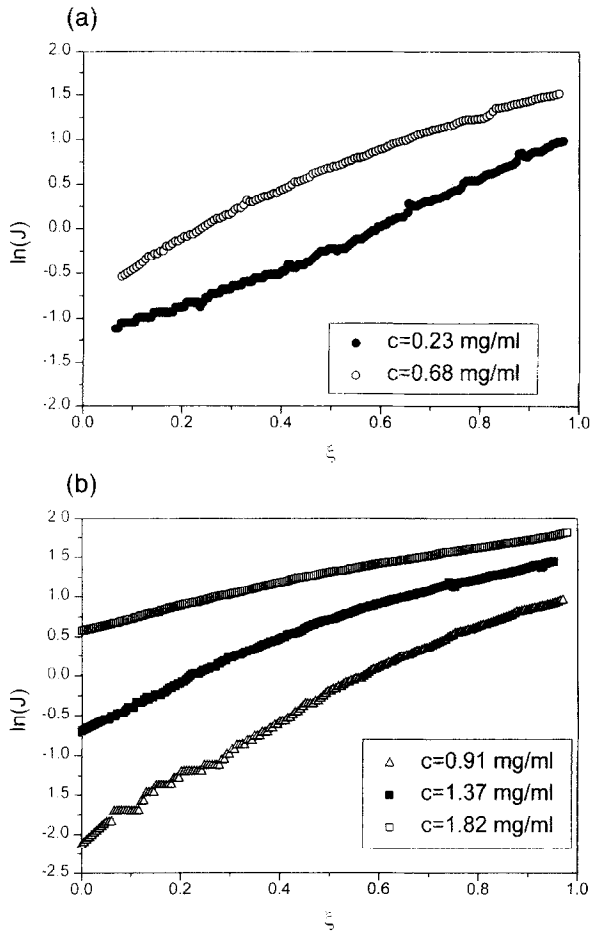

Fig. 4. Sedimentation equilibrium fringe displacement vs distance squared records for κ-carrageenan at different loading concentrations: (a) lower loading concentrations (30 mm optical path length cells); (b) higher loading concentrations (12 mm optical path length). $\xi = (r^2-a^2)/(b^2-a^2)$, where r is the radial diplacement from the axis of rotation, and a, b the corresponding positions at the cell meniscus and base, respectively.

with other polysaccharides such as alginate (Horton *et al.*, 1991):

$$(M_{w,app})^{-1} = (M_w)^{-1} + 2Bc + 3Cc^2 \quad (3)$$

From this fit a value of M_w = (265,000±25,000)g/

Table 1. Weight average molecular weight determinations for κ-carrageenan

Technique	M_w (g/mol)	Reference
Sedimentation equilibrium method I	300,000±10,000	This study
Sedimentation equilibrium method II	265,000±25,000	This study
Sedimentation equilibrium method III	320,000±20,000	This study
Sedimentation equilibrium: concensus value	300,000±40,000	This study
Sedimentation–diffusion[a]	332,000	Slootmaekers *et al.* (1991a, b)
Low angle laser light scattering (LALLS)	353,000	Slootmaekers *et al.* (1991a, b)
SEC/LALLS[b]	353,000	Slootmaekers *et al.* (1991a, b)
SEC/MALLS[c]; Zimm plot	310,000	Chambers *et al.* (1994)

[a]Sedimentation coefficient combined with the the diffusion coefficient from dynamic light scattering.
[b]Size-exclusion chromatography coupled on-line to LALLS (low-angle laser light scattering).
[c]Size-exclusion chromatography coupled on-line to MALLS (multi-angle laser light scattering).

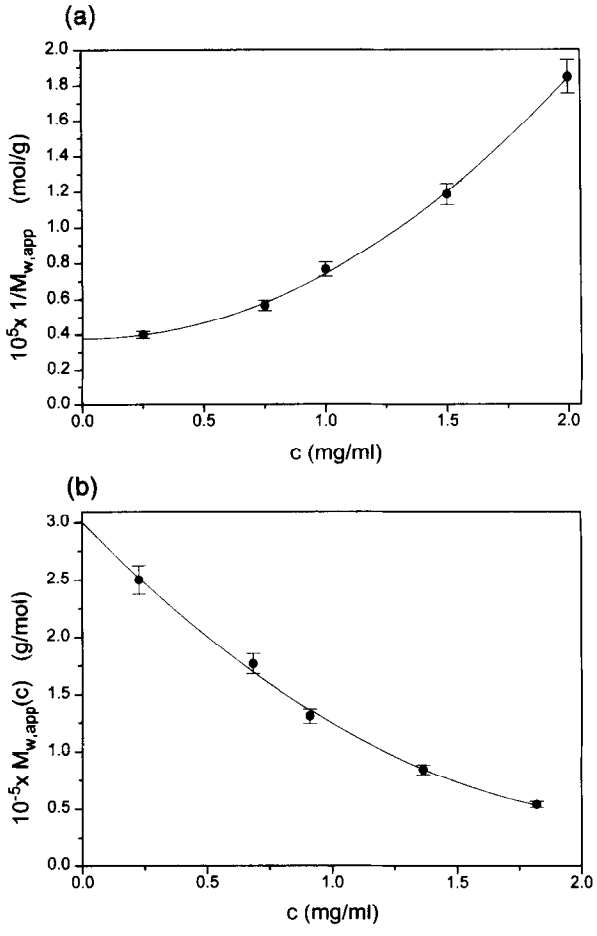

Fig. 5. $M_{w,app}$ vs concentration plots: (a) $M_{w,app}$ vs cell loading concentration c (g/ml). The line fitted is to $M_{w,app} = M_w + a_1c + a_2c^2$; (b) plot of $1/M_{w,app}$ vs c, the line is fitted to $(1/M_{w,app}) = (1/M_w) + 2Bc + 3Cc^2$, where B and C are the second and third thermodynamic or 'osmotic pressure' virial coefficients.

mol is obtained. Because of the extra degree of freedom it was impossible to fix B with any precision ($= 0.0 \pm 0.4 \times 10^{-3}$ ml mol g^{-2}), although a value of C of (1.2±0.1) ml^2 mol g^{-2} is returned. One could speculate that a negative B would suggest a self-association, but this is highly unlikely with the highly polyanionic nature of this saccharide. One could also speculate as to the molecular co-exclusion volume varying with concentration (as pairwise co-exclusion gives way to multiple co-exclusion as the dilute solution assumption becomes less appropriate).

(3) Extraction of M_w from a plot of $M_{w,app}(J)$ vs J (Fig. 6) (Method III of Table 1).
This procedure of extrapolating M_w to $c = 0$ has been commonly applied for the extraction of M_w's for monodisperse protein and virus preparations and can in principle be applied to a data-set of $M_w(J)$ vs J (or absorbance, if absorbance optics are used) from a single experiment. For polydisperse systems there is a risk of possible re-distribution of

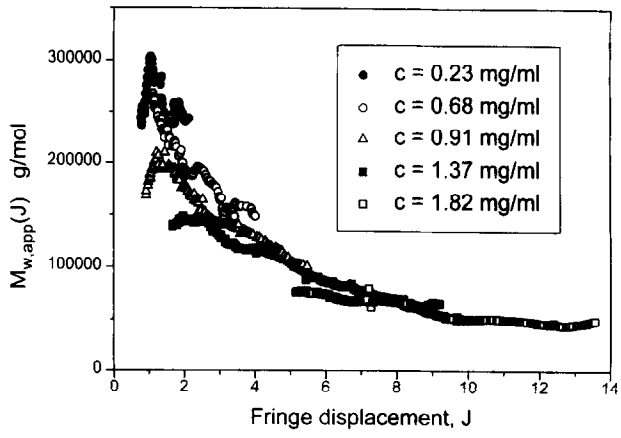

Fig. 6. 'Point average' plots of $M_{w,app}(r)$ vs local concentration at at various radial positions, r, in the ultracentrifuge cell [expressed in fringe displacement units, $J(r)$] for different loading concentrations, c.

solute at higher speeds, with lower radial positions favouring the smaller species of a population, causing an underestimate of M_w as $M_{w,app}(J)$ is extrapolated to $J = 0$. At lower speeds this risk is not so serious. In Fig. 6 we have overlayed the $M_{w,app}(J)$ vs J datasets for all five loading concentrations (after normalizing the J values of the highest three concentrations by a factor of 2.5 because only 12 mm path length cells (as oppose to 30 mm for the two lower concentrations) were employed. Encouragingly, all five data sets appear clearly to overlap. Although an extrapolation to $J = 0$ is difficult because of the noise at lower fringe displacements, graphical extrapolation would appear to suggest a M_w of ~(320,000±20,000) g/mol.

(4) Concensus M_w. Combining the three estimates for M_w together, we end up with a 'concensus' value of (300,000±40,000). Our value is in excellent agreement with values obtained Slootmaekers *et al.* (1991a, b) using a variety of light scattering based procedures (Table 1) and a value of ~310,000 obtained by Chambers and coworkers (see Fig. 1 of Chambers *et al.* (1994)), also from light scattering.

Gross conformation and hydration

Values for 'transport' hydrodynamic properties of κ-carrageenan are summarized in Table 2. From the plot (Fig. 7) of the reciprocal (apparent) sedimentation coefficient vs concentration (corrected for radial dilution) and fitting the data to Equation 2 values for $s^\circ_{20,w}$ and k_s of (4.19±0.20) S and (590±40) ml/g, respectively, were obtained. From the reduced viscosity vs concentration plot of Fig. 8, a value for $[\eta]$ of 629±58 ml/g was obtained, in good agreement with the value of 670±20 ml/g obtained by Slootmaekers *et al.* (1991).

Table 2. Hydrodynamic transport parameters for κ-carrageenan

Parameter	
$[\eta]$ (ml/g)	630±60
K_η	0.36
$s^\circ_{20,w}$ (sec)	$(4.19\pm0.20)\times10^{-13}$
k_s (ml/g)	590±40
$k_s/[\eta]$	~0.9
f/f_o	~7.6
equivalent axial ratio	~15:1
hydration (g H_2O/g polysaccharide)	~50

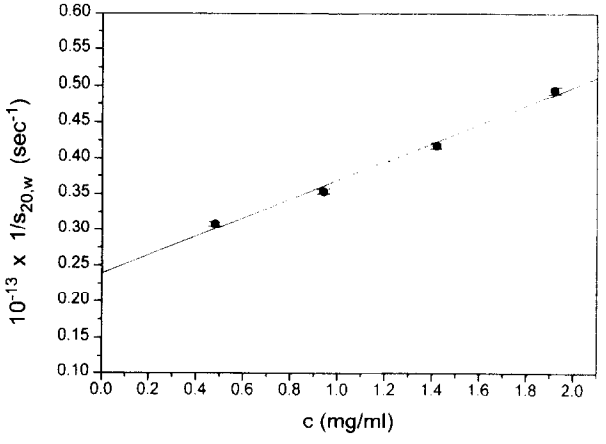

Fig. 7. Plot of $1/s_{20,w}$ vs concentration. Concentrations corrected for radial dilution and moisture content. The line fitted is to $1/s_{20,w} = (1/s^\circ_{20,w})(1 + k_s c)$

Figure 8 also yields an estimate for the Huggins constant K_η of ~0.36. Although it is unwise to infer conformation information from this parameter, it is in surprisingly good agreement with the value of ~0.35 obtained by Vreeman *et al.* (1980).

The high values of k_s and $[\eta]$ are consistent with a highly expanded structure. From the ratio of $k_s/[\eta]$ it is possible to infer the gross conformation of the κ-carrageenan without assumptions concerning the extent of hydration: the value of ~0.9 obtained is consistent with an extended asymmetric structure [for spherical and random-coil conformations, $k_s/[\eta]$ ~1.6, whereas for a rigid rod $k_s/[\eta]$ ~0.2 (Rowe, 1977)]. If we make the crude assumption that the extended structure is rigid then it is possible to obtain an estimate for the axial ratio of the equivalent hydrodynamic prolate ellipsoid using an equation given by Rowe (1977): a value of $k_s/[\eta]$ of ~0.9 corresponds to an axial ratio of ~15:1 using the simple inversion formulae of Harding and Cölfen (1995). Of course this is a rather approximate picture of the overall conformation type, but it does confirm the overall extended structure of the whole macromolecule without resort to fractionation techniques. It is also possible to demonstrate the large water binding capacity of this molecule.

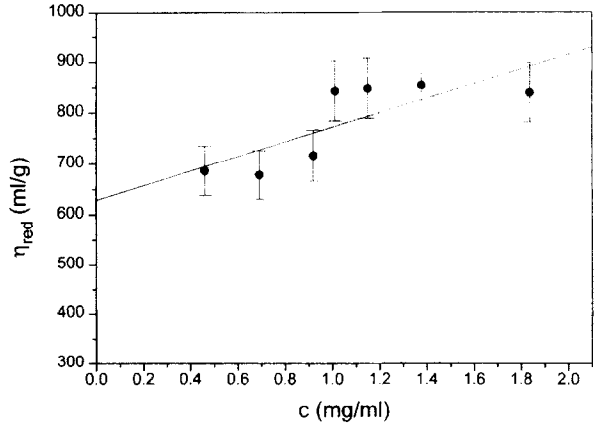

Fig. 8. Plot of reduced viscosity η_{red} vs concentration c for κ-carrageenan. Line fitted is $\eta_{red} = [\eta](1 + K_\eta[\eta]c)$.

Hydration

From the axial ratio of ~15 it is possible to combine this with the experimentally determined frictional ratio to determine the extent of expansion of the molecule through hydration. The frictional ratio (f/f_o) may be calculated from the infinite dilution sedimentation coefficient and the molecular weight using the following equation (see, e.g. Squire & Himmel, 1979):

$$\left(\frac{f}{f_0}\right) = \left[\frac{M(1-\bar{\nu}\rho_0)}{N_A(6\pi\eta_0 s^0_{20,w})}\right]\left(\frac{4\pi N_A}{3\bar{\nu}M}\right)^{1/3} \quad (4)$$

where $\bar{\nu}$ is the partial specific volume, ρ_o and η_o the density and viscosity of water at 20.0°C, and N_A is Avogadro's number), and from this (f/f_o) was found to be ~7.6. Combination of the Perrin function, P (Perrin, 1936; Rowe, 1977), often referred as the 'frictional ratio due to shape' (Squire & Himmel, 1979) with the frictional ratio (f/f_o) enables the degree of expansion of the molecule $(v_s/\bar{\nu})$ to be estimated, where v_s (ml/g) is the volume of the swollen molecule (polysaccharide + associated solvent) per unit mass of polysaccharide and $\bar{\nu}$ is the partial specific volume (essentially the anhydrous molecule):

$$(f/f_o) = P(v_s/\bar{\nu})^{1/3} \quad (5)$$

From equation (5) we can thus estimate $(v_s/\bar{\nu})$ as ~90. The corresponding value of the 'hydration' δ of the molecule, defined by

$$\delta = (v_s - \bar{\nu})\rho_o \quad (6)$$

to be ~50 g solvent bound per g of κ-carrageenan. Although, because of the approximations we have made, the actual numerical value must be treated with very great caution, this treatment does however suggest that κ-carrageenan is highly expanded, but perhaps not to the same extent as found for coil-like polysaccharide structures.

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