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RHEOLOGY AND MICROSTRUCTURE OF SOLUTIONS OF THE MICROBIAL POLYSACCHARIDE FROM *Pseudomonas elodea*

VINCENT CARROLL, GRAHAM R. CHILVERS, DAVID FRANKLIN, MERVYN J. MILES, VICTOR J. MORRIS, AND STEVEN G. RING

ARC Food Research Institute, Colney Lane, Norwich NR4 7UA (Gt. Britain)

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ABSTRACT

Rheological studies of solutions and gels of the microbial polysaccharide from the organism *Pseudomonas elodea* have been combined with X-ray diffraction studies of fibres and pulsed electric-birefringence studies of dilute solutions, to investigate the conformation and interaction of the polymer molecules. Rheological data are suggestive of a locally rigid conformation for the biopolymer in solution. X-Ray diffraction studies suggest that the molecules adopt a three-fold helical structure. *O*-Acetyl substituents have been shown to inhibit the packing of these helices into crystalline domains. Studies of pulsed electric-birefringence suggest an extended, kinetically rigid structure in solution. Dissolving the polysaccharide in dimethyl sulphoxide inhibits the gelation and shear-thinning characteristics of aqueous solutions. Comparative studies of electric birefringence of solutions in water and dimethyl sulphoxide suggest that the differences in rheological properties may result from a change in molecular conformation.

INTRODUCTION

The micro-organism *Pseudomonas elodea*, which has been identified as a new species, produces an anionic heteropolysaccharide that forms highly viscous solutions and, under the appropriate conditions, will form thermoreversible gels[1]. The polysaccharide has been named "gellan" gum. Although the chemical repeating-unit of the biopolymer has not been determined, preliminary structural investigations have been made[1]. The polysaccharide contains 6% of *O*-acetyl groups and, under suitable conditions, forms weak elastic gels; on deacetylation, it forms stiff, brittle gels[1]. The uronic acid content of the polysaccharide is 22%, and the neutral sugars rhamnose and glucose have been identified in the molar ratio 3:2. Methylation analysis suggested that the polymer is unbranched. Thus, the polysaccharide is believed to be a partially *O*-actylated, linear biopolymer composed of glucose, rhamnose, and glucuronic acid[1]. The negative $[\alpha]_D$ value has been taken[1] to imply that the glucosyl residues are β-D-linked.

Possible industrial applications of the polysaccharide are as a high-viscosity

bio-gum or as a gelling agent (alternative to agar or carrageenans). The studies now reported were aimed at determining the conformation of the polysaccharide in solution and the mechanism of gelation. Such information will assist in assessing both the potential industrial applications and the biological role of this novel extracellular polysaccharide.

MATERIALS AND METHODS

Samples of the polysaccharide were a gift from A. N. Bennett and A. P. Imeson of Alginate Industries Ltd. The product was supplied as a dried alcohol-precipitate from the fermentation broth. Aqueous solutions, prepared by dissolving the biopolymer at 95 °, were opaque and viscous: at sufficiently high concentrations (>0.1 %), thermoreversible opaque gels were formed. The opacity was due to a high concentration of entrapped, dead bacterial-cells, comprising ~50 % of the mass of the native sample. Various methods were tested for removing the cell debris. These included the use of proteolytic enzymes, and centrifugation and filtration of heated and unheated dilute solutions. The most useful procedure involved dissolution of the sample in dimethyl sulphoxide. This solvent substantially diminished the viscosity of the sample (Fig. 1), permitting the cell debris to be easily removed by centrifugation. Typically, a 1 % solution was centrifuged at 76,000*g* for 3 h. Phase-contrast microscopy was used to assess the effectiveness of this clarification procedure. The polysaccharide was recovered from the supernatant solution by precipitation with ethanol. Samples were filtered off, washed thoroughly with ethanol, and freeze-dried.

Deacetylation of the polysaccharide was carried out by raising the pH of aqueous solutions to 12 with sodium hydroxide, keeping the samples at 4 ° overnight, neutralising with hydrochloric acid, dialysing, precipitating and washing with ethanol, and freeze-drying. Acetyl contents were determined by the method of McComb and McCready[2].

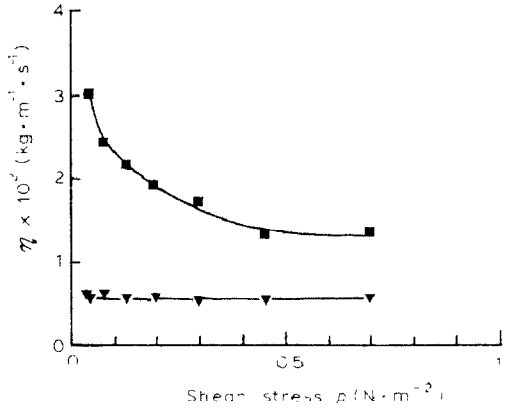

Fig. 1. Plots of viscosity η versus shear stress p for 0.05 % solutions of the polysaccharide in water (■) and dimethyl sulphoxide (▼). Values of shear rate $\dot{\gamma}$ may be computed from the relation $\eta\dot{\gamma} = p$.

Aqueous solutions and gels were prepared by dissolving the polysaccharide at 90–95°, stirring thoroughly, and allowing the samples to cool to room temperature. Polymer concentrations were determined by freeze-drying and vacuum-drying. Cation concentrations were measured by atomic absorption spectroscopy.

Rheological measurements on weak gels and viscous solutions were made with a Deer rheometer having concentric-cylinder geometry. At low shear-stresses, instantaneous application of an applied shear-stress to the gels resulted in bulk oscillations of the samples. To eliminate this problem, the shear stress was applied in the form of a ramp. The shear stress was increased linearly over a period of 20 s, held constant for a defined period of time, and then released instantaneously. Viscosities of solutions were determined through measurements of the angular velocity of the inner cylinder, resulting from application of defined torsional forces, and a knowledge of the geometry of the rheometer.

X-Ray fibre-diffraction measurements were made by standard techniques. The wavelength used was 1.54 Å; suitable fibres, dusted with calcite for calibration, were prepared by stretching partially dried gels. Various degrees of elongation, at various humidities and annealing temperatures, were employed to optimise the molecular alignment and crystallisation of the samples. Typically, elongations of 300% at 80–100% humidity gave the best diffraction data. Helium was used to displace air from the fibre camera during exposures, in order to diminish absorption and background scattering.

Measurements of electric birefringence were made with a standard apparatus[3] described in detail elsewhere[4]. The incident wavelength used was 632.8 nm. The Kerr cells were rectangular glass-cells of path length 3.54×10^{-2}m. The electrode gap was 2×10^{-3}m, and d.c.-pulsed electric fields with amplitudes up to 1.1×10^{6} V.m^{-1} and pulse lengths up to 10 ms may be applied. Quadratic detection was employed[3].

RESULTS

The acetyl concentration of the clarified sample was 5.6%. The major cation concentrations, as determined by atomic absorption spectroscopy, were K^+, 2%; Na^+, 0.1%; Ca^{2+}, 0.1%; and Mg^{2+}, 0.5%.

Rheology. — Aqueous solutions of the acetylated polysaccharide yielded weak elastic gels at concentrations >0.04%. Typical creep compliance (J) versus time (t) curves obtained for a 0.08% concentration are shown in Fig. 2 as a function of applied shear-stress (p). At low shear-stress, the samples showed an instantaneous elastic response plus a retarded elastic response to the applied shear-stress. Removal of the applied stress resulted in an instantaneous elastic recovery plus a retarded recovery (Fig. 2a). The behaviour was dominated by the instantaneous elastic behaviour. The fast elastic response of the samples is illustrated by the damped oscillatory response of the system after removal of the applied stress. Typical stress–strain plots for concentrations of 0.08% and 0.1% are shown in Fig. 3. At higher shear-

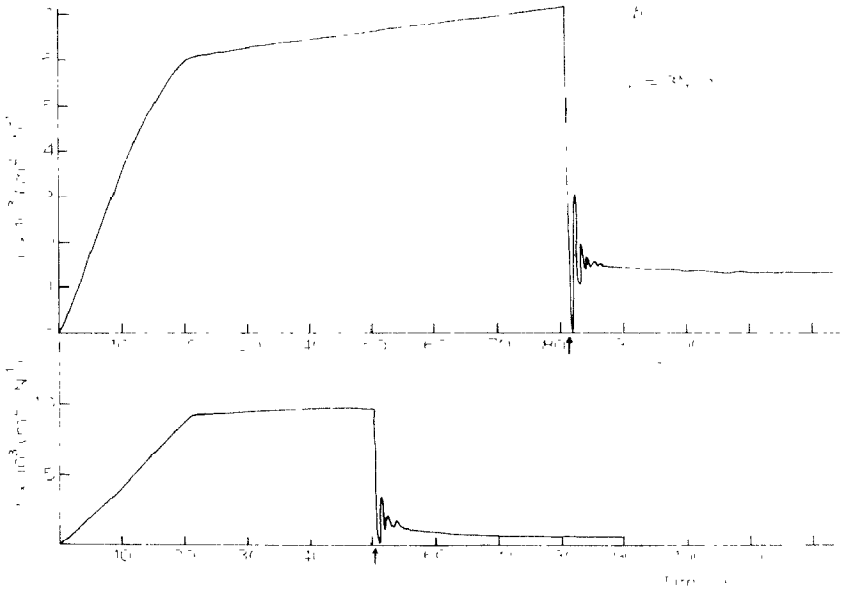

Fig. 2. Creep compliance (J) versus time (t) plots for a 0.08% solution of the polysaccharide at applied stress values of (*a*) 0.46 N.m^{-2} and (*b*) 3 N.m^{-2}. The stress was increased linearly as a ramp for 20 s and then held constant. The arrows indicate the point at which the shear stress was removed.

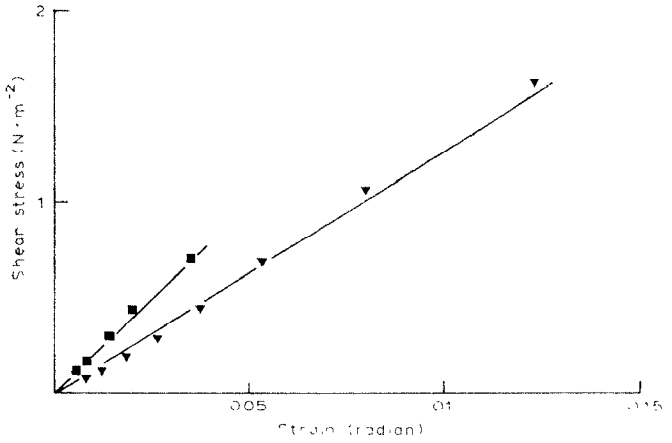

Fig. 3 Stress–strain plots for 0.1% (■) and 0.08% (▼) polysaccharide gels.

stress, an additional region of Newtonian compliance was shown, representing non-recoverable flow (Fig. 2b). Thus, a yield point was exhibited, above which there was characteristic shear-thinning behaviour (Fig. 4).

X-Ray diffraction. – The quality of the fibre-diffraction photographs was improved by deacetylating the polysaccharide. Fig. 5a shows the pattern obtained from a sample in which the acetyl content had been reduced to 1.7%. The sample was

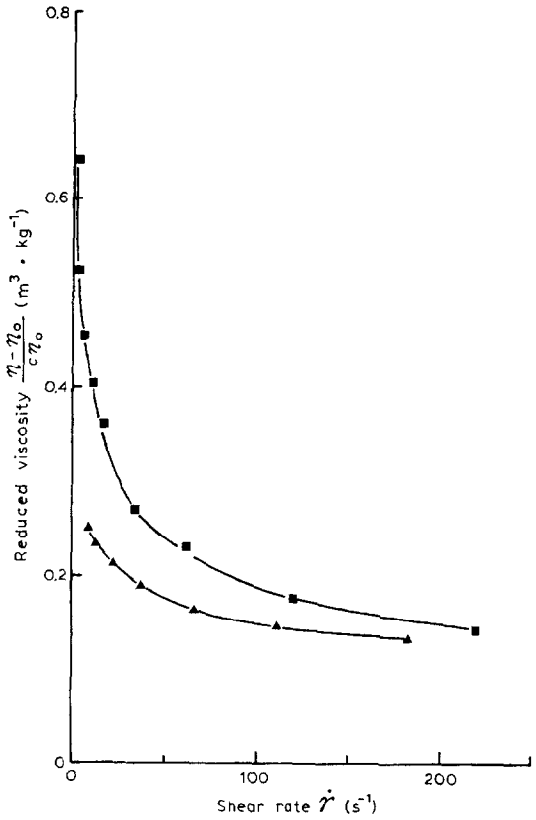

Fig. 4. Plots of the reduced viscosity $\left(\frac{\eta - \eta_0}{c\eta_0}\right)$ against shear rate for 0.05% (■) and 0.04% (▲) solutions at shear stresses above their yield point.

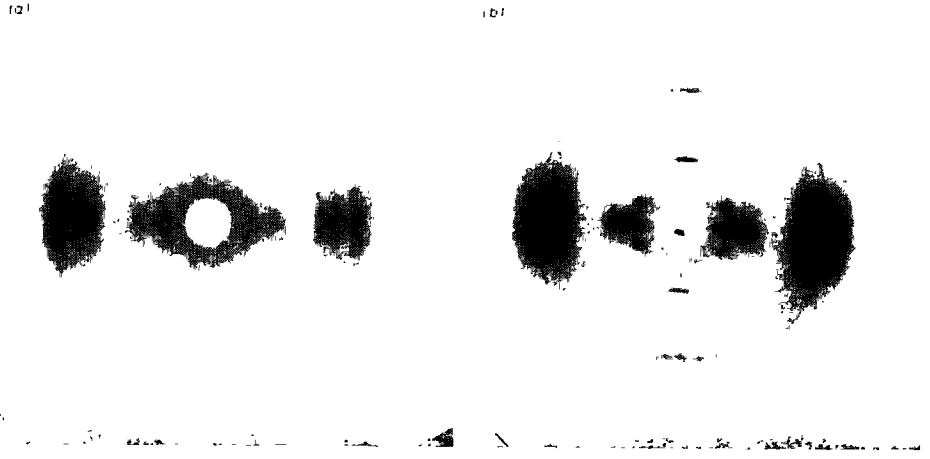

Fig. 5. X-Ray diffraction patterns obtained from a partially deacetylated fibre at (*a*) 100% humidity and (*b*) after drying over silica gel. Fibre axes are vertical.

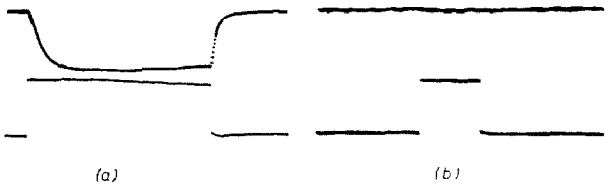

Fig. 6. Tracings of photographs of the transient electric-birefringence responses (upper traces) to an applied pulsed-d.c. field (lower trace) for (*a*) 0.025% aqueous solution, E = 244 kV.m^{-1}, pulse length 6.72 ms; and (*b*) 0.025% solution in dimethyl sulphoxide, E = 240 kV m^{-1}, pulse length 4.14 ms. The data were recorded on a digital-storage oscilloscope set to record at 20 μs per point.

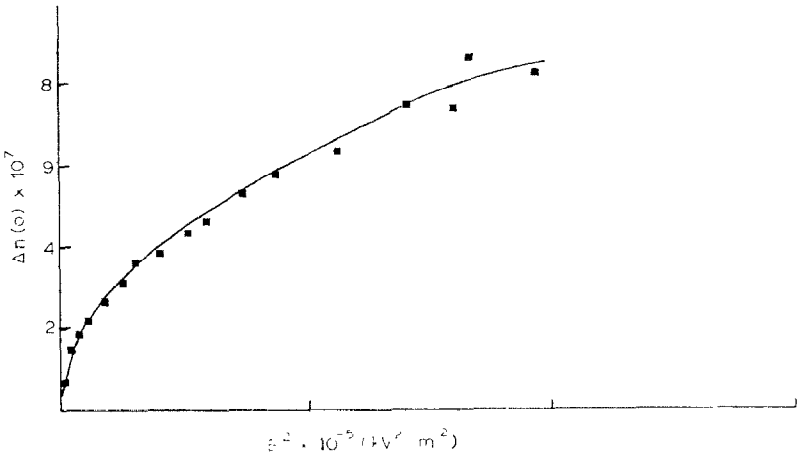

Fig. 7. Plot of the equilibrium values [Δn(o)] of the electrically induced birefringence against the square of the applied electric-field amplitude (E).

stretched 300% at a humidity of 100%. The photographs show meridional reflections on the layer lines with l = 3,6,9. These reflections may represent orders of an axially projected repeat-unit of 0.94 nm. The three-fold helical structure was confirmed by studies of dried fibres (Fig. 5b). The pattern shown in Fig. 5b was obtained after drying the partially deacetylated fibres (acetyl content, 1.7%) over silica gel. Fibres produced from the acetylated clarified product yielded diffraction patterns similar to Fig. 5b with poor, ill-defined, equatorial reflections. This finding indicates that the crystallinity of the deacetylated fibre is considerably higher than that of either the acetylated or dried deacetylated fibres.

Transient electric birefringence. — Fig. 6a shows a typical, electric-birefringence transient response to the applied d.c. pulse of electric field for a 0.025% aqueous solution of the clarified polysaccharide. Fig. 6b shows the results of a comparable experiment conducted on a 0.025% solution of the polysaccharide in dimethyl sulphoxide. Fig. 7 shows the dependence of the electrically induced equilibrium-birefringence [$\Delta n(o)$], for a 0.025% aqueous solution, on the square of the amplitude (E) of the applied electric field.

TABLE I

ESTIMATED SHEAR MODULI FOR VARIOUS CONCENTRATIONS OF THE POLYSACCHARIDE IN WATER AT SHEAR STRESSES BELOW THE YIELD POINT

Concentration (%)	0.04	0.05	0.06	0.08	0.10
Shear modulus ($N.m^{-2}$)	0.04	0.04	0.04	1.36	2.0

DISCUSSION

The data shown in Figs. 2 and 3 illustrate the fact that aqueous solutions of the polysaccharide yield elastic gels. Elastic behaviour could be detected at low shear-stress for concentrations as low as 0.04%. The stress–strain plots are linear over a reasonably wide range of applied shear-stress. Values of the shear modulus obtained from the linear regions of such plots at low shear-stress are given in Table I. At low concentrations of polysaccharide, there was clear evidence for the existence of a yield point and, at sufficiently high shear-stress, the samples flowed. The dependence of viscosity on shear rate for samples above their yield points is illustrated in Fig. 4. The samples show shear-thinning behaviour. It is clear from Figs. 1–4 that, at low shear-stress or shear-rate, the viscosity increases indefinitely and thus shows no evidence for a plateau region. In this respect, the data are unlike those observed for typically locally disordered or "random coil" polysaccharides in either the dilute or semi-dilute regimes[5]. The shear-thinning character is suggestive of the breakdown of a weak network or gel structure. In instances where such behaviour has been observed for polysaccharide solutions, and in particular for microbial polysaccharide solutions, the effect has generally been associated with, and attributed to the existence of, an ordered or locally rigid chain-conformation for the biopolymer[6–8]. In particular, for several microbial polysaccharides, it has been proposed that the helical conformation observed by X-ray fibre-diffraction studies is retained in solution and accounts for the local rigidity of the polymer chain.

The X-ray data provide information on the conformation of the polysaccharide gellan in partially dried gels and on the nature of the intermolecular association. The meridional reflections on the layer lines when $l = 3,6,9$ suggest a three-fold helical structure for the polysaccharide, since these reflections are retained in the dried deacetylated fibres (Fig. 5b) and the highly acetylated fibres where molecular alignment is pronounced but the degree of crystallinity is low. Acetylation affects the regular packing of the helical molecules, as evidence by the change in the equatorial reflections. This readily explains the changes in the rheological properties of gels obtained on deacetylation[1]. Thus, high crystallinity favours large junction zones linked by small sections of polymer, leading to rigid, brittle gels. It will be necessary to determine the steric location of the acetyl groups within the chemical structure with respect to the helical backbone in order to evaluate their effect on gelation. The sensitivity of gelation to the type and quantity of cations present suggests the presence

of uronic acid within the junction zones. Whether the uronic acid residues are localised in blocks or distributed evenly along the polymer chain remains to be determined. The crystalline array of stacked helices evident in the equatorial reflections of Fig. 5a provides a model for the junction zones of the gels. The importance of water in the assembly of these crystalline regions is apparent from a comparison of the equatorial reflections observed for wet (Fig. 5a) and dry (Fig. 5b) fibres. Similar behaviour has been reported[9] for the bacterial capsular polysaccharide of *Klebsiella* serotype K9.

The rheological data provide indirect evidence for local rigidity of the biopolymer chain. X-Ray data provide a physical structure that could account for a locally rigid structure of the polymer in solution. Therefore, we used the technique of pulsed electric-birefringence to probe the conformation of this polymer in solution. Fig. 7 illustrates the development of the induced electric-birefringence observed on increasing the amplitude of the applied electric field. It is the mechanism by which this birefringence arises that allows the rigidity of the polymer chain to be probed. The chain may be pictured as a string of individual dipole moments. On application of an electric field, each individual dipole is subjected to an orientating couple in the electric field. If the molecular coupling between individual dipoles is slight, each dipole orientates independently. This results in an overall change in configuration for the macromolecule, and the resulting anisotropy in the applied-field direction is said to arise from deformational polarisation[10]. However, if the individual molecular dipoles are strongly coupled, it is the resulting dipole of the entire molecular chain which orientates in the electric field. This large-scale orientation of the macromolecule is termed orientational polarisation[10]. Clearly it is the relative time-scales for deformation or orientation that decide which mechanism predominates. If the orientational relaxation time (τ_o) is shorter than the deformational relaxation time (τ_D), the former process dominates the polarisation of the medium. In this case, the molecule may be considered to be kinetically rigid. The Kuhn segment-length (l_e) greatly exceeds the "monomer length" (l_m) and is comparable with the contour length (L) of the molecule. If $\tau_o > \tau_D$, deformational polarisation predominates and the chains are considered kinetically flexible ($l_e \approx l_m \ll L$). Thus, the local rigidity of the polymer chain determines the dispersion frequency of the polarisation process. There is considerable experimental evidence to show that deformation polarisation arises with flexible-chain molecules. In most cases, the kinetically rigid units have been shown to approximate to the monomer units and to orientate virtually independently. The electro-optic and dielectric properties of such solutions approximate to those expected for solutions of the same concentration of the monomer and are independent of the molecular weight of the polymer[11–15]. However, the electro-optical properties of rigid-chain polymers are quite different[10]. A characteristic of such solutions is the dispersion of the Kerr effect and the dielectric properties in the audiofrequency range[10,16]. This dispersion arises due to the large, rotational relaxation times accompanying orientation of the whole molecule. Thus, the relaxation time for decay of the induced electric-birefringence provides a guide to the local rigidity of the polymer chain. Locally flexible molecules show short relaxation times

($\sim \mu$s) typical of short, kinetically rigid segments that orientate relatively independently. Longer relaxation times imply stronger coupling and progressively higher degrees of rigidity of the polymer chain. Fig. 6a shows the transient response of the gellan sample to the applied d.c. electric-field. For a single relaxing-species, the decay region following termination of the electric-field pulse will be described by an equation of the form[3] *1*.

$$\Delta n(t) = \Delta n(o) \exp(-t/\tau), \tag{1}$$

where $\Delta n(t)$ is the induced birefringence at an elapsed time t, and τ is the relaxation time.

Fig. 8 shows a plot of $\log_{10}\{\Delta n(t)/\Delta n(o)\}$ versus t. For polydisperse samples, the initial slope of a semi-logarithmic plot of $\Delta n(t)$ versus t can be used to define an average value[19] of $1/\tau$, namely $\langle 1/\tau \rangle$. From Fig. 8, $\langle 1/\tau \rangle = 5000\ s^{-1}$, corresponding to a relaxation time of the order of 200 μs. The magnitude of this relaxation time immediately implies a high degree of rigidity of the polymer chain. Thus, at least locally, this microbial polysaccharide behaves as an extended, rigid, rod-like polymer in solution. We can convert the relaxation data into molecular size by treating the polymer as an ideal rigid-rod and then calculating an effective rod-length. Using Burgers' equation, we have[20]

$$\frac{1}{\tau} = \frac{18kT}{\pi\eta_o l^3}\left\{\ln\frac{2l}{d} - 0.8\right\}, \tag{2}$$

where d is the rod diameter, l the rod length, k Boltzmann's constant, T the absolute temperature, and η_0 the solvent viscosity.

Fig. 9 shows the calculated l values obtained as a function of the choice of d. The calculated values are relatively insensitive to the choice of d and provide an order-of-magnitude estimate for the size of this extended molecule in solution.

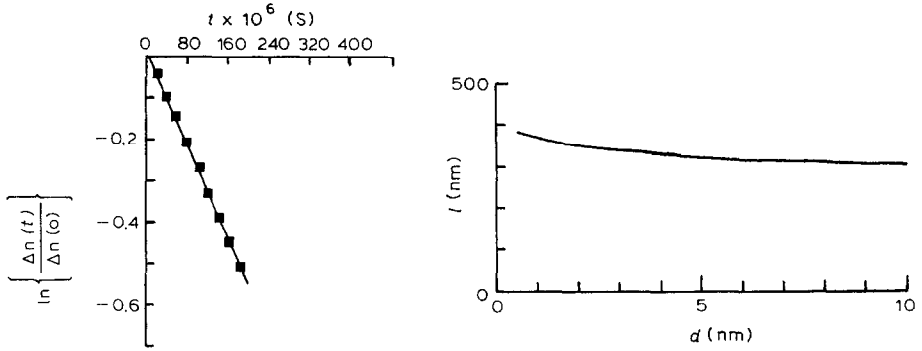

Fig. 8. Semi-logarithmic plot of the decay of the electric birefringence for a 0.025% aqueous solution of the polysaccharide. E = 244 k.V.m^{-1}.

Fig. 9. Calculated, effective rod-length (l) as a function of assumed diameter (d).

We have used measurements of $\langle 1/\tau \rangle$ to assess the local rigidity of the polymer chain and to estimate the approximate dimensions of the polymer in solution. However, measurements of $\langle 1/\tau \rangle$ coupled with modern theories of the hydrodynamics of weakly bending rods and worm-like chains may be used to determine the Kuhn statistical segment-length[21,22]. The use of such theories to assess rigidity and to compare l_e and L requires samples of various molecular weights and a knowledge of their molecular weight distributions. At present, the scarcity of pure samples of gellan coupled with the difficulties in preparing and characterising fractions of different molecular weight have forced the use of the simple interpretation described above.

Comparative studies of solutions of the polysaccharide in water and dimethyl sulphoxide suggest that the strong electrical-birefringence observed for aqueous solutions is absent for solutions in dimethyl sulphoxide (Fig. 6). Dimethyl sulphoxide also inhibits gelation and shear thinning (Fig. 1), suggesting a breakdown of intermolecular interactions. The electric-birefringence data suggest that this may arise owing to a change in molecular conformation.

CONCLUSIONS

Preliminary studies have been made of the rheology and microstructure of a new anionic heteropolysaccharide produced by the micro-organism *Pseudomonas elodea*. The polymer is unusual in that it contains a high percentage of (1→4)-linked rhamnose residues. This linear polysaccharide has been shown to yield weak elastic gels at low concentrations (>0.04%). Polymer solutions show shear-thinning behaviour. The rheological data are consistent with an extended, locally rigid, rod-like structure in solution. X-Ray diffraction data suggest a three-fold helical structure for the polysaccharide. Deacetylation of the polymers enhances crystallinity and explains differences observed in the textures of acetylated and deacetylated gels. The junction zones result from parallel alignment of the helical polymers. The cation sensitivity of gelation indicates the presence of uronic groups at the junction zones. Electric-birefringence data are consistent with an extended molecule of size 350 nm in solution.

Dimethyl sulphoxide is a good solvent for the polymer: gelation and shear thinning are inhibited, suggesting a breakdown of intermolecular interactions. Electric-birefringence data for solutions in water and dimethyl sulphoxide are compatible with the belief that the latter solvent may induce a change in molecular conformation.

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