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STRUCTURE AND GELATION OF *Rhizobium* CAPSULAR POLYSACCHARIDE

MICHAEL J. GIDLEY, IAIN C. M. DEA,
Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ (Great Britain)

GILLIAN EGGLESTON, AND EDWIN R. MORRIS
Cranfield Institute of Technology, Silsoe College, Silsoe, Bedford MK45 4DT (Great Britain)

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ABSTRACT

The gel-forming, neutral capsular polysaccharide produced by many *Rhizobium* strains has been examined by ^{13}C- and ^{1}H-n.m.r. spectroscopy in order to determine the anomeric linkage configurations. Spectra for the capsular polysaccharide from *R. trifolii* (strain TA-1) are fully consistent with the branched-hexasaccharide repeating sequence previously proposed, and show a backbone repeat of →4)-α-D-Glc*p*-(1→3)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→ in which the glucosyl residue is substituted at position 6 by β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→ and at position 2 by α-D-Gal*p*. The thermally induced gel–sol and sol–gel transitions are accompanied by co-operative changes in chain conformation, as monitored by optical rotation, and show pronounced thermal hysteresis. The mid-point temperature for the order–disorder (heating) transition is ~49° and for the disorder–order (cooling) transition is ~42°; these values are independent of polymer concentration over the range 0.2–10 mg.mL^{-1}. Both transitions are shifted to progressively lower temperatures on addition of urea. Development of significant rigidity (as monitored by storage modulus, G′) does not occur until temperatures at which the optical rotation change is essentially complete, suggesting the involvement of a subsequent aggregation process in the development of gel structure. The minimum critical concentration for formation of a continuous network is <0.1 mg.mL^{-1}. We believe this is the lowest value so far reported for a gelling polysaccharide. On removal of side chains by Smith degradation, gelling behaviour is lost, and the polysaccharide backbone remains as a disordered random coil at all temperatures, indicating that, in the native polymer, the side chains promote adoption of the ordered, gel-forming chain geometry by restricting the flexibility of the backbone.

INTRODUCTION

Many strains of *Rhizobium trifolii* and *Rhizobium leguminosarum* produce both an acidic exopolysaccharide and a neutral, gel-forming capsular polysaccharide[1] which is composed of the hexasaccharide repeating unit[1] **1**. As expected

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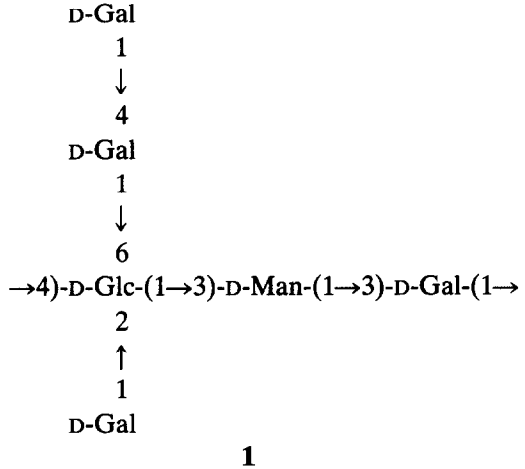
for structure **1**, Smith degradation removed the side-chain galactose residues, but left the main chain intact to yield structure **2** The susceptibility of acetylated **1** to oxidation with chromium trioxide suggested[1] that the anomeric configurations were mainly β.

We now describe ^{13}C- and ^{1}H-n.m.r. studies of the gel-forming capsular polysaccharide from *R. trifolii* TA-1, which provide a complete assignment of anomeric configurations. We also report an investigation of the concentration and temperature-dependence of gel strength by mechanical spectroscopy, and of the underlying changes in chain conformation by optical activity.



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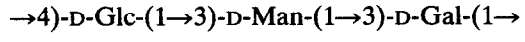

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EXPERIMENTAL

The sample of gel-forming capsular polysaccharide (**3**) from *Rhizobium trifolii* (strain TA-1) was kindly donated by Dr. L. P. T. M. Zevenhuizen (Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands). The debranched polysaccharide **2** was prepared by Smith degradation, as described previously[1]. The ^{1}H- and ^{13}C-n.m.r. spectra were recorded at 200.13 and 50.32 MHz, respectively, using a Bruker AM200 spectrometer. Samples for ^{1}H-n.m.r. spectroscopy were exchanged with D_2O twice prior to analysis in order to reduce interference from the residual solvent resonance.

Optical rotations were measured at 365 nm with a Perkin–Elmer 241 polarimeter, using jacketed cells of pathlength 1 or 10 cm as appropriate. Mechanical spectra were recorded on a Sangamo Viscoelastic Analyser, using cone

and plate geometry of cone angle 2 deg and diameter 5 cm. In both cases, the temperature was controlled to within ±0.5° by a circulating water bath, and readings were taken ~5 min after the sample had attained thermal equilibrium.

RESULTS AND DISCUSSION

Primary structure. — The ^{13}C- and partial ^{1}H-n.m.r. spectra of the native and Smith-degraded polysaccharides are shown in Figs. 1 and 2. The ^{1}H-n.m.r. spectrum of the native polysaccharide (Fig. 2a) shows four anomeric signals (each 1 H) at 5.40, 5.17, 5.03, and 4.45 p.p.m. and two overlapping anomeric signals (each 1 H) at 4.5–4.6 p.p.m. consistent with a hexasaccharide repeating unit. Similarly, the ^{1}H-n.m.r. spectrum of the Smith-degraded polysaccharide (Fig. 2b) shows three anomeric doublets (each 1 H) at 5.25, 5.03, and 4.51 p.p.m. consistent with a trisaccharide repeating unit. The ^{1}H- and ^{13}C-chemical shifts indicated that the native polysaccharide contains three α and three β linkages and that the Smith-degraded polysaccharide has two α and one β linkage in each repeating unit. This conclusion is in contrast to the suggestion of the presence of mainly β linkages on the basis of oxidation experiments with chromium trioxide[1].

An immediate anomeric assignment can be made by considering the expected H-1/H-2 coupling constants for each of the possible residue configurations. Thus,

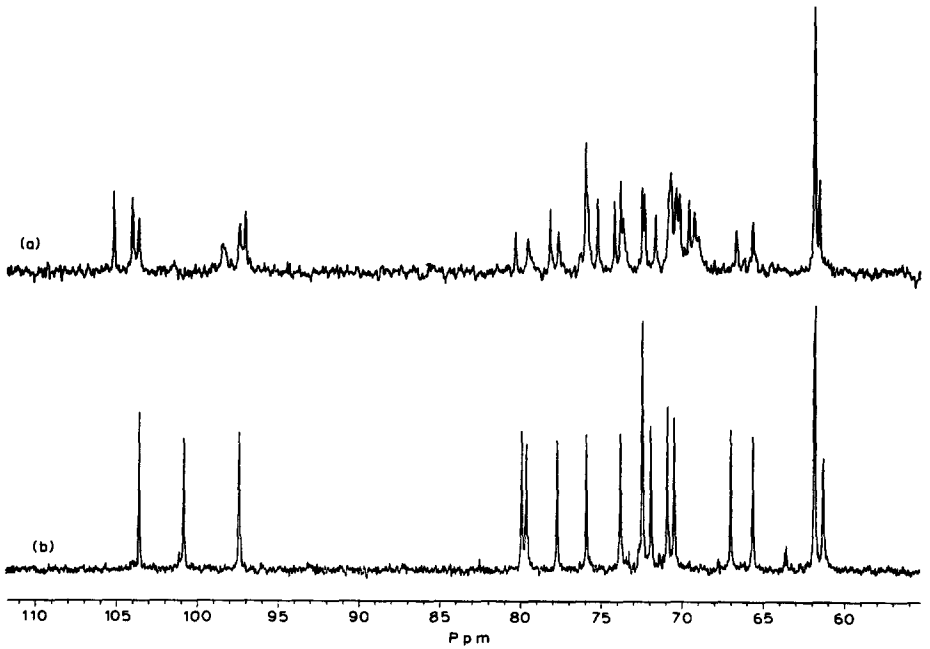

Fig 1 ^{13}C-N.m r. spectra (90°; 10 mg.mL^{-1} in D_2O) of (*a*) the native polysaccharide (**3**, 42,000 scans, 5 4 s between successive observation pulses); and (*b*) the Smith-degraded, debranched polysaccharide (**2**, 18,000 scans, 3.0 s between successive observation pulses)

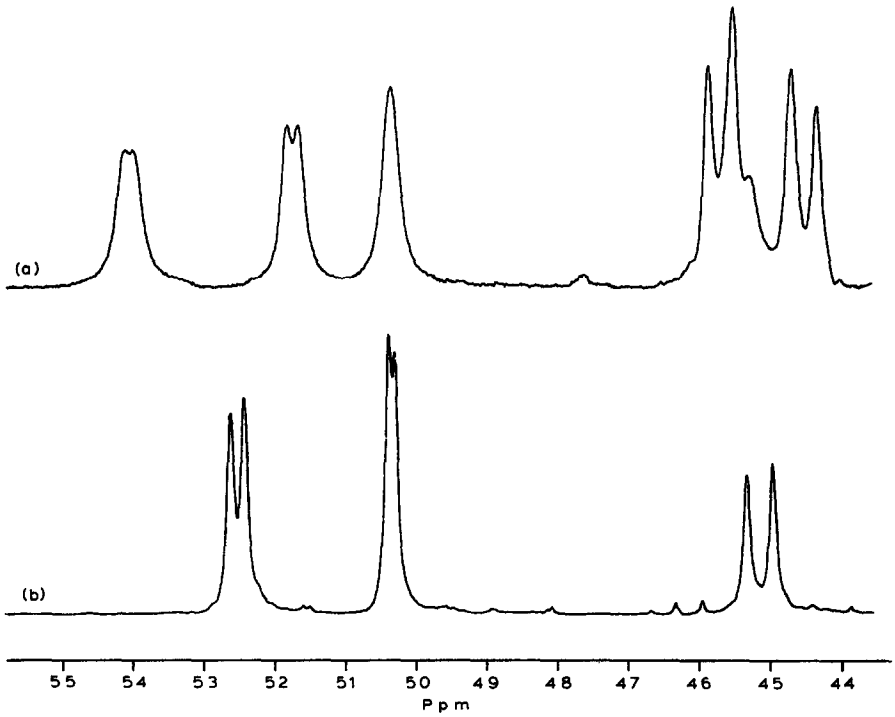

Fig 2. Partial ^{1}H-n m.r. spectra (90°, 10 mg.mL^{-1} in D_2O) of (*a*) the native polysaccharide (**3**) and (*b*) the Smith-degraded, debranched polysaccharide (**2**).

α- and β-glucose and galactose residues are expected[2] to have $J_{1,2}$ values of 3.5–4.0 and 7.5–8.0 Hz, respectively, and α- and β-mannose residues to have values of 1–2 Hz. The H-1 resonance at 5.03 p.p.m. [$J_{1,2}$ <2 Hz (Fig. 2a) and 1.64 Hz (Fig. 2b)] can therefore be assigned to the single mannose residue which, from the chemical shift value, has the α configuration.

A further assignment can be made based on the H-1 chemical shifts expected for (1→6)-linked sugars, which are relatively insensitive to the nature of the glycosidically linked residue and occur at 4.96 p.p.m. for α-D-galactose[3]. As there is no unassigned signal in this region of the ^{1}H-n.m.r. spectrum, the (1→6)-linked galactosyl residue must have the β configuration (expected[4] H-1, ~4.5 p.p.m.).

On conversion of **1** into **2** by Smith degradation, significant chemical shift displacements caused by removal of the side-chain residues should be observed only for the branch-point glucose. Thus, the ^{13}C resonance of **2** at 100.85 p.p.m. (Fig. 1b), which is more than 2 p.p.m. removed from any resonance of **1** (Fig. 1a), can be assigned to the glucosyl residue which must have the α configuration, as the ^{1}H spectrum of **2** (Fig. 2b) shows the presence of one β and two α residues; also, the ^{13}C signals at 103.61 and 97.40 p.p.m. (Fig. 1b) can be assigned to β and α anomers, respectively. The H-1 signal of **2** at 5.25 p.p.m. (Fig. 2b) can now be assigned to the α-glucosyl residue. The only unassigned ^{13}C and ^{1}H anomeric

resonances of **2** (103.61 and 4.51 p.p.m., respectively) are therefore due to the galactosyl residue which, on the basis of the observed chemical shifts, has the β configuration.

The anomeric configuration of the (1→2)-linked galactosyl group can now be assigned, based on the known ^{13}C-n.m.r. effects of 2-glucosylation on α-D-glucose residues[5,6]. This is considered to be a good model for the effect of 2-galactosylation on α-D-glucose, as the two residues differ in configuration only at the remote C-4 site. The effect of a (1→2)-α-D-glucosyl substituent on monomeric and (1→6)-linked α-D-glucose is to shift the C-1 signal upfield by 2.1 and 2.4 p.p.m., respectively[5,6]. For a β-(1→2) substituent, downfield shifts for C-1 of 0.2 and 0.6 p.p.m., respectively, are observed[5,6]. As the α-D-glucose C-1 signal of **2** at 100.85 p.p.m. is shifted more than 2 p.p.m. downfield from any α C-1 resonance in **1**, the (1→2)-linked galactosyl substituent must have the α configuration. The resonance at 98.42 p.p.m. for **1** (Fig. 1a) can now be assigned to C-1 of the branched α-D-glucosyl residue, based on the glycosidation shift caused by (1→2)-α-D-galactosyl substitution (2.43 p.p.m.). As three α and two β configurations in **1** have now been assigned, the remaining (1→4)-linked galactosyl group must have the β configuration.

Thus, a combination of ^{13}C- and ^{1}-H-n.m.r. spectroscopy has allowed a complete assignment of the anomeric configurations in the hexasaccharide repeating unit of the gel-forming capsular polysaccharide (**3**) of *R. trifolii*. Although single arguments for each anomeric configuration have been presented above, other lines of evidence support the proposed assignments. For example, the H-1 chemical shift

TABLE I

OBSERVED AND EXPECTED[a] ^{13}C CHEMICAL SHIFTS[b] FOR NATIVE (**3**) AND SMITH-DEGRADED (**2**) *Rhizobium trifolii* CAPSULAR POLYSACCHARIDE

Linkage	*Native polysaccharide*		*Smith-degraded polysaccharide*	
	Anomeric carbon	*Glycosidic carbon*	*Anomeric carbon*	*Glycosidic carbon*
Glc 1→$^{\alpha}$3 Man	98 42	80 31 (80 8)[c]	100.85	79 92
Man 1→$^{\alpha}$3 Gal	97 40	77 65	97 40	77 71
Gal 1→$^{\beta}$4 Glc	103 60 (103 7)	79 54 (79 4)	103 61	79.64
Gal 1→$^{\beta}$4 Gal	105 13 (105 1)	78.17 (78.1)	—	—
Gal 1→$^{\beta}$6 Glc	104 00 (103 8)[d]	70 16? (70 1)[e]	—	—
Gal 1→$^{\alpha}$2 Glc	97 03 (96 9)[f]	75 95 (76 5)[f]	—	—

[a]Figures in parentheses taken from ref. 5 [b]In D_2O at 90° relative to 1,4-dioxane (67.40 p.p m) [c]For Man 1→$^{\alpha}$3 Man [d]For Gal 1→$^{\beta}$6 Gal. [e]For Glc 1→$^{\beta}$6 Glc. [f]For Glc 1→$^{\alpha}$2 Glc

TABLE II

CHEMICAL SHIFTS (p.p.m [a]), COUPLING CONSTANTS ($J_{1,2}$, Hz), AND T_1 VALUES[b] (s) FOR H-1 RESONANCES OF NATIVE (3) AND SMITH-DEGRADED (2) *Rhizobium trifolu* CAPSULAR POLYSACCHARIDE[c]

Linkage	*Native polysaccharide*			*Smith-degraded polysaccharide*		
	H-1	$J_{1,2}$	T_1	*H-1*	$J_{1,2}$	T_1
Gal 1→3 Man	5 40	3 3	0 50	5 25	3 9	0 66
Man 1→3 Gal	5 03	<2	0 57	5 03	1 6	0 73
Gal 1→4 Glc	4 53[d]	~7	0 50	4 51	7 4	0 47
Gal 1→4 Gal	4 56[e]	~7	0 50	—	—	—
Gal 1→6 Glc	4,45[e]	7 6	0 40	—	—	—
Gal 1→2 Glc	5 17	3 2	0 61	—	—	—

[a]Downfield from DSS [b]Determined by the inversion–recovery method. [c]At 90° in D_2O. [d]Assigned on the basis of peak widths (Fig 2a), backbone resonances being expected to be broader than side-chain resonances [e]Interchangeable assignments

for the (1→2)-linked galactose (5.17 p.p.m.) is similar to that of α-D-glucose (1→2)-linked to α-D-glucose (5.13 p.p.m.[4]) which, in turn, is significantly different (>0.1 p.p.m.) from that for any other glucose disaccharide unit[4]. Also, the upfield shift of 0.15 p.p.m. for the glucose H-1 (from 5.40 to 5.25 p.p.m.) following removal of the (1→2)-linked α-D-galactosyl group is similar to that expected[4] (~0.2 p.p.m.) In fact, essentially all of the anomeric proton resonances and anomeric and glycosidically-linked carbon resonances of the native and Smith-degraded polysaccharides can be assigned on the basis of the arguments presented above and comparisons with appropriate model compounds[5] (Tables I and II).

β-D-Gal
1
↓
4
β-D-Gal
1
↓
6
→4)-α-D-Glc-(1→3)-α-D-Man-(1→3)-β-D-Gal-(1→
2
↑
1
α-D-Gal

3

In addition, the ^{13}C-n.m.r spectra of **3** and **2** each have two unusually high-field sugar-ring (*i.e.*, non C-6) resonances; at 66.65 and 65.62 p.p.m. for **3** and at 66.99 and 65.64 p.p m. for **2**. These signals may be assigned to C-4 sites of α-D-mannosyl and β-D-galactosyl residues adjacent to α-(1→3) linkages (expected chemical shifts[5], 67.1 and 66.3 p.p.m., respectively). The linkage-related assignments are all consistent with the previously suggested[1] linkage pattern (**1**) and strongly support the proposed repeating structure **3**.

For a branched polymer such as **3**, it might be expected that side-chain residues would have greater mobility than backbone residues and that such differences might be reflected in n.m.r. relaxation properties[7,8]. The T_1 values of H-1 resonances (Table II), however, do not correlate with the positions of the residues, although the H-1 line-widths (Fig. 2a) are slightly broader (indicating shorter T_2 values) for backbone signals compared with side-chain signals. However, the ^{13}C resonance line-widths, as measured directly or inferred from peak heights (Fig. 1a), show much more clear-cut correlations with structure **3**. Thus, the broadest resonances (98.42 and 79.54 p.p.m., respectively) for anomeric and glycosidically-linked carbon atoms are due to the doubly branched glucosyl residue, which would be expected to be the least mobile residue in the structure. Furthermore, resonances due to sites adjacent to linkages in the other two backbone residues (103.60, 97.40, 80.31, and 77.65 p.p.m.) are intermediate in broadness between branch-point glucose resonances and the linkage signals from side-chain residues (105.13, 104.00, 97.03, and 78.17 p.p.m.). These observations suggest that measurements of line-widths of ^{13}C resonances may provide a useful adjunct to conventional assignment procedures for branched polysaccharides.

Smith degradation of **3** induces a significant narrowing of both ^{13}C (Fig 1) and ^{1}H (Fig. 2) resonances, indicating an increase in mobility. The most likely explanation for these observations is the occurrence of partial depolymerisation of the polysaccharide during Smith degradation, presumably during the final treatment with formic acid. Close inspection of the ^{1}H-n.m.r. spectrum of the Smith-degraded polysaccharide (Fig. 2b) shows the presence of several minor resonances which may be attributed to reducing end-groups: at 5.17 (H-1, α-Man)[3], 4.89 (H-1, β-Man)[3], 4.66 (H-1, β-Glc)[4], and 4.61 p.p.m. (H-1, β-Gal). The H-1 α-Gal and H-1 α-Glc resonances would be masked[2,4] by the resonance at 5.25 p.p.m. All of the resolved minor resonances have T_1 values in the range 1.4–2.0 s, which are significantly longer than those of the major resonances (Table II), further supporting their assignment to end groups. From integration of the spectrum in Fig. 2b and taking account of the expected[4,9] position of the anomeric equilibrium for galactose and glucose residues to determine the areas of the "hidden" H-1 α resonances, the average chain-length of the Smith-degraded polysaccharide was found to be ~40.

Gelation behaviour. — On cooling from the high temperature (90°) sol state to the low temperature (20°) gel state, the high resolution n.m.r. spectrum (Fig. 2a) of the native polysaccharide (**3**) collapsed, leaving only a featureless baseline. This

finding suggests that, as in other gelling polysaccharide systems[10], the mechanism of gel formation involves conversion from conformationally mobile "random coil" chain geometry in solution to a rigid, ordered conformation in the gel state, with consequent loss of detectable high-resolution n.m.r. signal due to extreme line-broadening[11].

In this work, small-deformation oscillatory measurements of rigidity (storage) modulus, G', have been used to characterise the temperature-course of the sol–gel and gel–sol transitions, and the underlying changes in chain conformation have been monitored by optical rotation[10,12]. Fig. 3 shows a typical optical rotation–temperature profile for a solution of the native polysaccharide in distilled water. There is significant thermal hysteresis between the order–disorder (heating) and disorder–order (cooling) transitions. In both cases, the transition midpoint temperature (T_m), defined as the temperature at which the observed optical rotation value is mid-way between the minimum and maximum values, is, to within

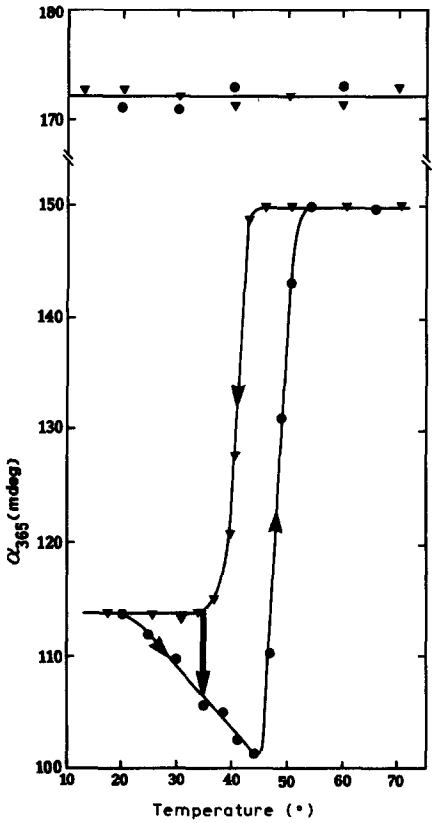

Fig 3 Temperature-dependence of optical rotation (365 nm; 1-cm pathlength, 5 mg.mL^{-1}) for the Smith-degraded, debranched polysaccharide (2; upper curve) and for the native polysaccharide (3; lower curves) The vertical arrow shows relaxation at constant temperature (35°) from the metastable cooling curve to the equivalent point on the heating curve

experimental error, independent of polymer concentration over the 50-fold range studied (10 mg.mL^{-1} to 0.2 mg.mL^{-1}): T_m was ~49° on heating and ~42° on cooling.

The optical rotation of the non-gelling, Smith-degraded (debranched) polysaccharide (**2**), by contrast, is, to within experimental error, independent of temperature, and is close to the value observed for the high-temperature, disordered form of the native polysaccharide at the same concentration and pathlength (Fig. 3). The high-resolution n.m.r. spectrum of the debranched material (Fig. 2b) also persisted on cooling to ambient temperature, indicating that the polysaccharide chain remained in the conformationally mobile, disordered state throughout.

Thermal hysteresis between gel formation and melting is not uncommon for polysaccharides, and is most pronounced in systems where, in the gel state, the polysaccharide chains are extensively aggregated. A likely explanation[13,14] is that aggregation stabilises the ordered chain conformation to temperatures above those at which it can form in isolation. The magnitude of the hysteresis shown in Fig. 3 is similar to that observed for kappa-carrageenan[15,16] and agarose sulphate[14,17]. However, an unusual feature, which is not shared by these other systems, is the pronounced dip in optical rotation over the first part of the heating curve (Fig. 3).

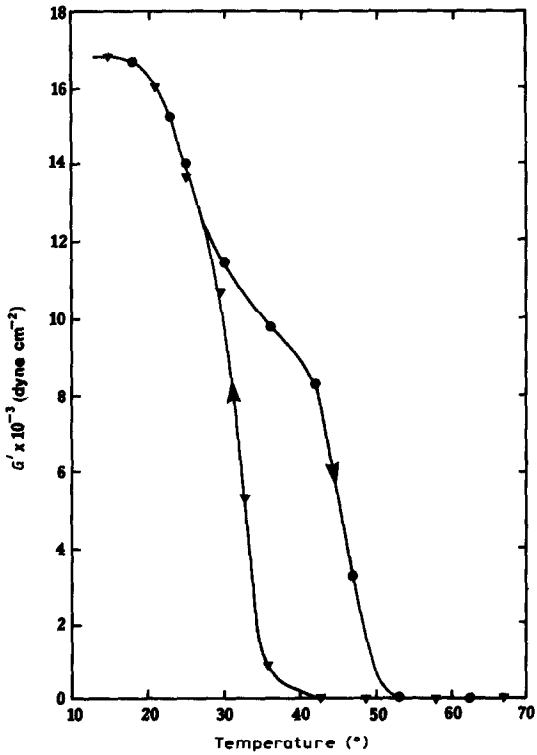

Fig. 4. Temperature-dependence of rigidity (G'; 1 rad.s^{-1}) for the native polysaccharide (**3**; 10 mg.mL^{-1}) on cooling (▼) and re-heating (●).

The effect is greatest at high concentration (>3 mg.mL^{-1}) and undetectable at very low concentration (<1 mg.mL^{-1}) When heating was arrested at the minimum optical rotation value (~43°) and the sample was re-cooled, the optical rotation rose steeply to re-join the initial cooling curve at ~30° and, on subsequent re-heating from 15°, followed the heating curve shown in Fig. 3.

A possible trivial explanation of this behaviour is that, at high concentrations of polymer, the optical rotation readings are affected by, for example, gel strain or scattering artefacts. However, we consider this unlikely, firstly because the results show good reproducibility from run to run, and secondly because the temperature-course of mechanical properties also shows evidence of a second process at low temperature, as illustrated in Fig. 4. During the first stages of heating (~15° to ~25°), gel rigidity (G') decreased sharply, then flattened out over the temperature range corresponding to the observed dip in optical rotation (~25° to ~45°), and finally decreased very steeply until gel-like character was lost (~52°). The nature of this second, concentration-dependent, process is being further investigated.

In the cooling direction, both optical rotation (Fig. 3) and rigidity (Fig. 4) show the smooth, sigmoidal temperature-dependence typical of a co-operative gelling process. Development of significant rigidity, however, does not occur until the conformational change has virtually stopped (~35°), suggesting that it is the aggregation of ordered regions, rather than the ordering process itself, which generates the interchain associations leading to network formation. Similarly, in

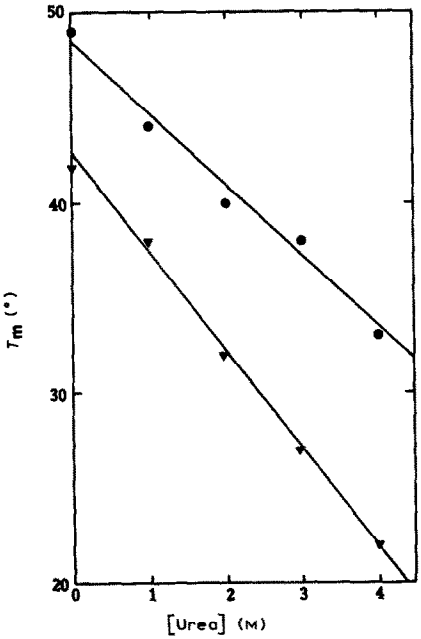

Fig. 5. Effect of urea on the transition midpoint temperature (T_m) of the native polysaccharide (3; 2.5 mg.mL^{-1}), as monitored by optical rotation, on heating (●) and cooling (▼).

the heating direction, gel melting (Fig. 4) is virtually complete by T_m of the order–disorder transition (Fig. 3).

As in other gelling polysaccharide systems that show thermal hysteresis between gel formation and melting[13,17], optical rotation values for the order–disorder transition (the steeply rising region of the heating curve in Fig. 3) show long-term stability, indicative of a true thermodynamic equilibrium process, but the cooling curve is metastable. Experimental data-points shown in Fig. 3 (and in Figs. 4, 6, and 7) were recorded ~5 min after the sample had attained thermal equilibrium, and gave readings that were stable on the time scale of the measurement. Relaxation from the apparent low-temperature plateau region of the metastable cooling curve to the lower optical rotation values observed at the same temperature in the heating direction (vertical arrow in Fig. 3), however, was ~50% complete within ~1 h. This is very much faster than the equivalent relaxation behaviour in charged polysaccharide systems[16,17], and offers considerable scope for

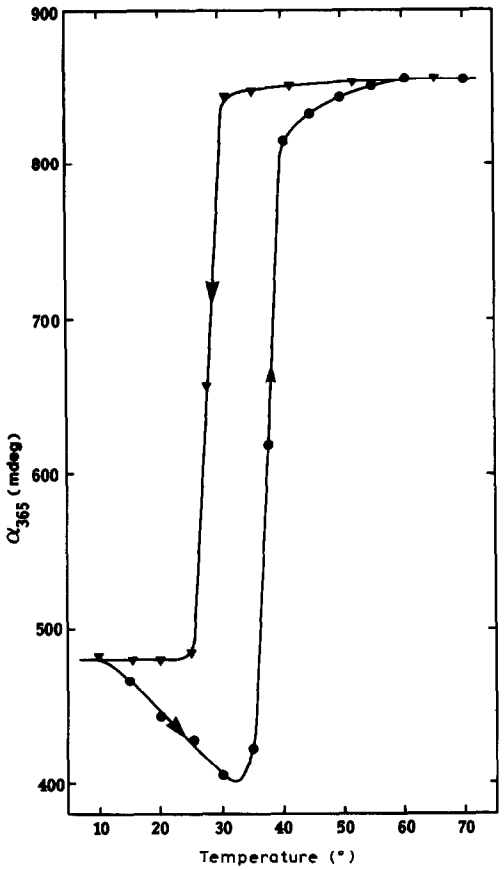

Fig 6. Temperature-dependence of optical rotation (365 nm, 10-cm pathlength) for the native polysaccharide (3; 2 5 mg.mL^{-1}) in the presence of 3M urea, on cooling (▼) and re-heating (●)

future investigation of the dynamics of conformational ordering and aggregation.

On addition of urea, both the order–disorder (heating) and disorder–order (cooling) transitions are progressively displaced to lower temperature (Fig. 5). The cooling curve is affected somewhat more than the heating curve, so that, as illustrated in Fig. 6, the thermal hysteresis becomes slightly greater. Under these conditions of altered solvent quality, the lag between conformational ordering and network formation is even more apparent. For example, in the presence of 3M urea, development of measurable rigidity (Fig. 7) did not occur until ~20°, while optical rotation values (Fig. 6) reached the metastable low-temperature plateau by ~25°, and, in the heating direction, gel melting was virtually complete by ~35°, in the very early stages of the order–disorder transition as monitored by optical rotation (Fig. 6).

These observations reinforce our suggestion that the formation of a network does not occur as a direct consequence of conformational ordering but involves a further aggregation step, and also indicate that the aggregate structure is

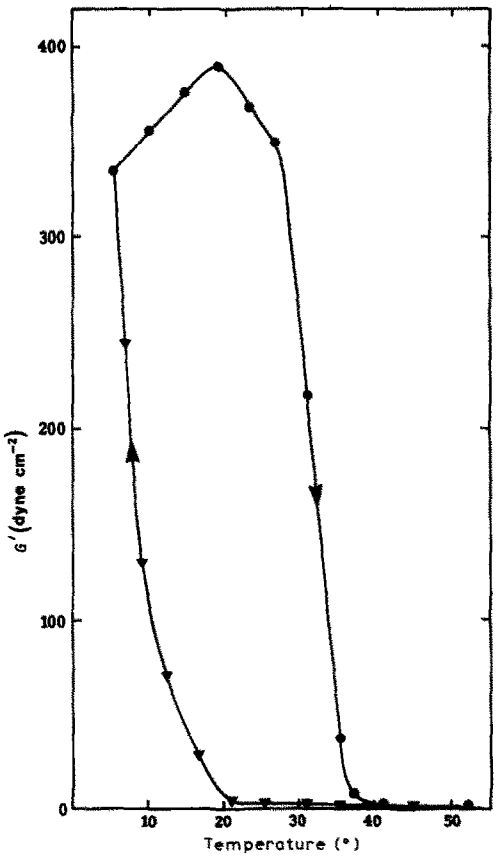

Fig. 7. Temperature-dependence of rigidity (G'; 1 rad.s^{-1}) for the native polysaccharide (3; 2.5 mg.mL^{-1}) in the presence of 3M urea, on cooling (▼) and re-heating (●)

destabilised by urea to a somewhat greater extent than the ordered conformation of the participating chains. The involvement of a second process during the early stages of heating is even more evident in the presence of urea. Under these conditions, the shoulder shown in the gel melting curve in Fig. 4 is replaced (Fig. 7) by a small, but detectable, rise in G' over the same temperature range as the observed dip in optical rotation (Fig. 6).

A striking feature of the native polysaccharide **3** is its ability to form gels at unusually low concentrations. We have investigated the mechanical properties of the gels over a 100-fold range of concentration (0.1 to 10 mg.mL^{-1}). Fig. 8 shows mechanical spectra obtained at the two extremes of this range. In both cases, solid-like character, characterised by the storage modulus, G', dominates over liquid-like character (loss modulus, G''); both moduli show little dependence on frequency of oscillation (ω), and dynamic viscosity [$\eta^* = (G'^2 + G''^2)^{1/2}/\omega$] is almost inversely proportional to frequency (*i.e.*, the slope of log G' *vs.* log η^* is close to the theoretical limiting value of -1), behaviour characteristic[18] of a cross-linked network structure.

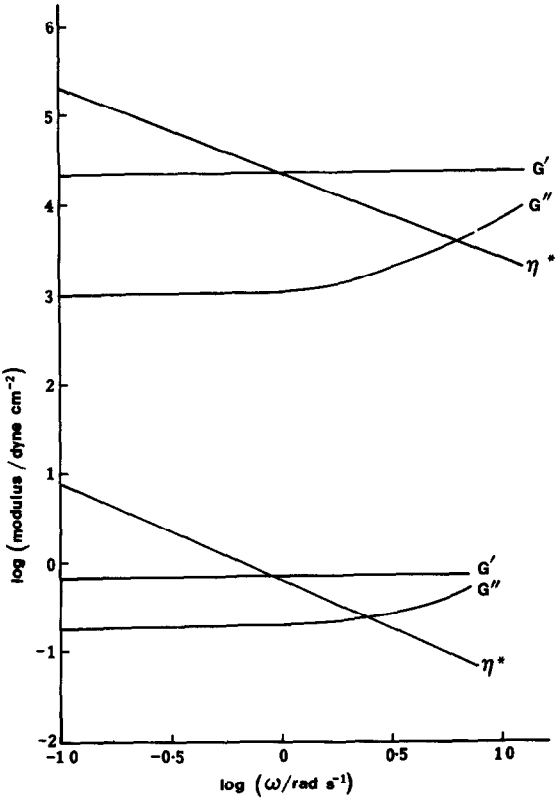

Fig 8 Mechanical spectra (15°) of the native polysaccharide (**3**) at concentrations of 10 mg.mL^{-1} (upper curves) and 0 1 mg mL^{-1} (lower curves). The apparent rise in G'' at high frequency (ω) is probably due to the onset of resonance in the instrument, rather than being of genuine molecular origin

Fig. 9 shows a double-logarithmic plot of the concentration-dependence of gel rigidity, G'. The plot is linear over the two decades of concentration studied, and has a slope of ~2.24. This value is significantly higher than the limiting value of 2 (*i.e.*, c^2 dependence of G') predicted by cascade theory[19,20] and by the Hermans equilibrium model[20,21] (and observed experimentally for several biopolymer systems including gelatin[22]), but is very close to the theoretical value of 2.25 (9/4) proposed[23] from an "osmotic scaling" approach This agreement may, however, be fortuitous since other factors such as "trapped entanglements" may contribute to rigidity (for a recent review, see Clark and Ross-Murphy[24])

Agarose, which is normally considered as the polysaccharide system with the lowest concentration requirements for gelation, has[24] a typical, minimum, critical gelling concentration (c_0) of ~1.7 mg.mL^{-1} (although the precise value will of course vary with molecular weight). As shown in Fig. 8, however, the *Rhizobium* capsular polysaccharide **3** still has obvious gel-like character at concentrations more than an order of magnitude lower, and by ~1 mg.mL^{-1} forms gels that are

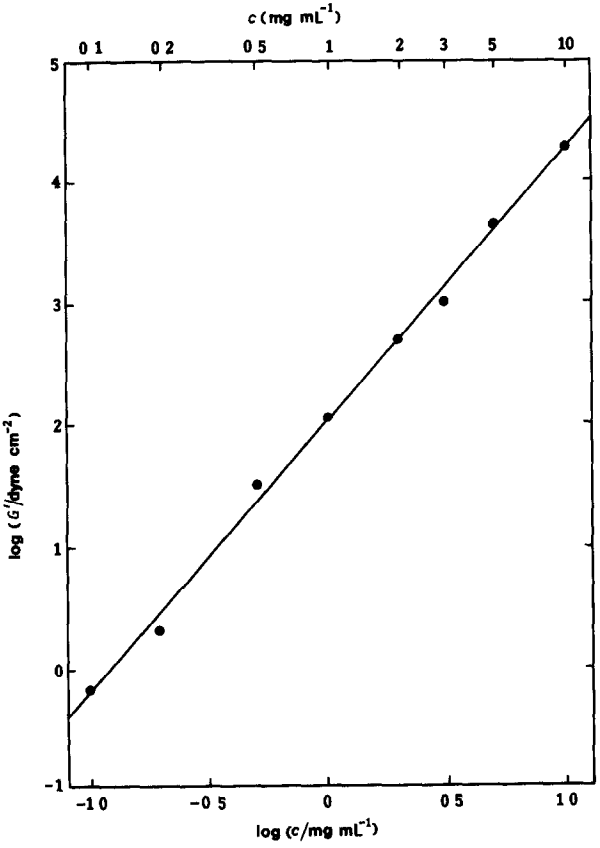

Fig. 9 Concentration-dependence of rigidity modulus (G'; 1 rad.s^{-1}, 15°) for the native polysaccharide (**3**)

recognisable not only by the sensitive criteria of mechanical spectroscopy, but also by direct visual observation of, for example, maintenance of shape and resistance to flow. We believe that this is the lowest gelling concentration so far reported for a polysaccharide. It must be pointed out, however, that at equivalent polymer concentrations (10 and 5 mg.mL^{-1}), the absolute values of G' (Fig. 9) for **3** are slightly lower than those reported[24] for agarose, so that the gel structure, although forming at lower concentrations, is less rigid.

On further reduction of concentration by a factor of 2 below the minimum value shown in Fig. 9 (*i.e.*, to 0.05 mg.mL^{-1}), gel-like character is lost, and the mechanical spectrum has the form typical[18] of a dilute polymer solution ($G'' > G'$, with both moduli increasing steeply with frequency). In this concentration range, however, the moduli are so low that c_0 could not be determined exactly.

In addition to its unusually low gelling concentration, **3** is also unusual in having a doubly branched sugar residue in its primary structure. For other microbial polysaccharide systems, the introduction of branching weakens intermolecular association. For example, the cellulose backbone of xanthan is solubilised by the presence of a trisaccharide side-chain on every second glucosyl residue and, although under most conditions the xanthan molecule is conformationally ordered, intermolecular association is limited to the formation of a tenuous "weak gel" structure[25] in solution.

The comparison of curdlan and schizophyllan shows a similar phenomenon. The non-branched (1→3)-β-D-glucan, curdlan, forms rigid gels[26] through aggregation of its triple-helical ordered structures[27]. Schizophyllan has the same glucan backbone, but this is substituted by (1→6)-linked β-D-glucose residues on every third residue. This branching does not prevent adoption of the ordered triple-helical structure, but aggregation of this ordered structure is prevented and schizophyllan is non-gelling[28]. In a similar way, the insertion of α-L-rhamnopyranosyl and α-L-mannopyranosyl branching into the tetrasaccharide repeating-unit of polysaccharide S-60 from *Pseudomonas elodea* (gellan gum) to give S-130 (welan gum) results in a severe reduction in gelling ability, without preventing adoption of the ordered conformation[29].

By contrast, the side chains present in the primary structure of the *Rhizobium* capsular polysaccharide (**3**), rather than inhibiting intermolecular association, appear to be essential both for adoption of the ordered conformation and for gel formation. This marked difference in the influence of branching on polysaccharide properties could be related to the double-branching in **3**. It is likely that such a structural feature will severely limit freedom of rotation around the adjacent glycosidic linkages, and so act as a driving force towards adoption of the ordered conformation and the subsequent intermolecular associations involved in gel formation. Double-branched sugar residues in bacterial polysaccharide main-chains are uncommon, although some examples have been reported for *Klebsiella* capsular polysaccharides[30]. It would be of interest to determine whether such a structural

feature may represent a general route towards polysaccharide conformational ordering and gelation.

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