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Kinetic and equilibrium processes in the formation and melting of agarose gels

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Abstract

The formation and melting of agarose gels has been monitored by small-deformation oscillatory measurements of storage and loss moduli (G' and G''). The onset of network formation on cooling at 1°C min^{-1} moved to progressively higher temperature with increasing polymer concentration, from ~23°C at 0.1 wt% to ~33°C at 2.0 wt%. Isothermal measurements showed comparatively rapid gelation of concentrated (2.0 wt%) solutions at temperatures where the rate of gelation of more dilute samples (0.25 wt%) had dropped to zero. Both phenomena are interpreted in terms of kinetically determined helix–helix aggregation displacing the thermodynamic equilibrium between disordered coils and double helixes. Gel melting occurred as an equilibrium process, with moduli reaching constant, stable values after incremental increase in temperature. Rheological equilibration, however, was substantially slower than conformational disordering, as monitored by optical rotation, suggesting diffusion-controlled separation of strands after dissociation of ordered helices. Gels formed by rapid quenching to 5°C melted at substantially (12–13°C) lower temperature than those formed by cooling at 1°C min^{-1}. It is suggested that the helix-length required for stable association increases with increasing temperature, with slow cooling therefore giving longer helices than rapid quenching, and that increasing helix length promotes helix–helix aggregation. Consistent with this interpretation, the gels formed by slow cooling were stronger, and more turbid, than those obtained on quenching. Re-examination of a previous report that final melting of agarose gels occurs at a temperature well above completion of conformational disordering revealed a gross discrepancy in experimental evidence. Gel setting and melting temperatures obtained under identical conditions (1.0 wt% agarose in 50 wt% of dimethylsulphoxide) in both investigations were in close agreement, but the order–disorder transition temperature (from optical rotation) determined in the present work was ~25°C higher, and indicated a finite residual helix-fraction on completion of gel melting.

Keywords: Agarose; Aggregation; Gelation; Helix length; Kinetics

1. Introduction

Agarose is a neutral polysaccharide whose primary structure approximates to the alternating repeating sequence → 3)-β-D-Gal*p*-(1 → 4)-3,6-anhydro-α-L-Gal*p*-(1 → . It is the parent member of the agar series of gelling polysaccharides from marine red algae (*Rhodophyceae*), in which the disaccharide repeating unit (Fig. 1) occurs with different levels and sites of attachment of sulphate, pyruvate and/or *O*-methyl substituents (for a recent review see Stanley, 1995).

Gelation of agarose, like that of other polysaccharides (Rees et al., 1982), involves conversion from a fluctuating, disordered coil conformation in solution to a rigid, ordered structure which forms the junction zones of the gel network. The most generally accepted model for the ordered structure is a co-axial double helix (Arnott et al., 1974b), proposed from (i) X-ray fibre diffraction of gel samples dried and stretched at ambient temperature, (ii) agreement between observed and calculated values of optical rotation, and (iii) analogy with the more firmly established double-helix geometry of the structurally related polysaccharide iota carrageenan (Arnott et al., 1974a). X-ray fibre diffraction studies of agarose have also shown single-helix geometry (Foord and Atkins, 1989), but only for specimens dried and annealed at temperatures well above the onset of conformational ordering under hydrated conditions (i.e. for samples prepared from solutions of single chains).

Evidence from an extensive series of investigations in Palermo, summarised recently by San Biagio et al. (1996), indicates that, at agarose concentrations below ~2 wt%, double-helix formation on cooling is preceded by spinodal demixing into polymer-rich and solvent-rich regions, with gelation then proceeding preferentially along pathways of high polymer concentration formed by agglomeration

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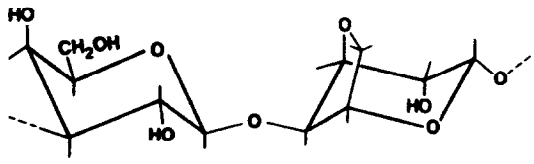

Fig. 1. Fundamental disaccharide repeating unit of agarose.

(percolation) of the polymer-rich domains, whereas at higher concentrations gelation appears to occur directly from the homogeneous solution state.

A characteristic feature of agarose is that the gels show massive thermal hysteresis, attributed to formation of large aggregates that remain stable at temperatures much higher than those at which individual helices will form on cooling (Arnott et al., 1974b; Morris and Norton, 1983). The extent of hysteresis across the agar polysaccharide series decreases with increasing content of substituent groups (Guisley, 1970; Arnott et al., 1974b), consistent with progressive inhibition of helix–helix aggregation.

In the present work we have carried out a brief investigation of the effect of temperature and concentration on the gelation kinetics of agarose, and of the effect of setting rate on the small-deformation rheology and thermal stability of the resulting gels. We have also re-examined the experimental evidence of a previous study (Braudo et al., 1991) in which it was concluded that agarose networks remain intact at temperatures well above those required for complete loss of conformational order.

2. Materials and methods

The agarose sample used in this investigation, and in the studies of starch–agarose composites reported in the next two papers (Mohammed et al., 1998a,b) was from Sigma (type I-A; low EEO; lot number 084H0462). Solutions were prepared by dispersing the polymer in water at ~95°C and autoclaving (15 min; 120°C) to ensure complete dissolution, or by dissolving in a 50/50 (w/w) mixture of water and dimethylsulphoxide (DMSO) at ~95°C. Perfluorodecalin (used in characterisation of gel melting) was from Acros Organics, NJ, USA; DMSO was reagent grade from Sigma; distilled deionised water was used throughout.

Small-deformation measurements of storage modulus (G'), loss modulus (G'') and complex dynamic viscosity ($\eta^* = (G'^2 + G''^2)^{1/2}/\omega$, where ω is frequency in rad s^{-1}) were made using cone-and-plate geometry (50 mm diameter; 0.05 rad cone angle) on a sensitive prototype rheometer designed and constructed by one of us (R.K.R.).

To circumvent problems of thermal expansion or contraction during heating and cooling, the cone was truncated over 45% of its diameter, giving a gap of 0.5 mm between the flat surfaces of the two elements, but keeping strain constant at a fixed, maximum, value across the outer portion (which constitutes 80% of the total area). Samples were loaded onto the rheometer in the solution state (normally at ~60°C) and their periphery was coated with light silicone oil to minimise evaporation (or absorption of moisture by solutions containing DMSO). Temperature was controlled by a Haake circulating water bath and measured with a thermocouple attached to the stationary element.

Turbidity was characterised by measurements of optical density (1 cm pathlength; wavelength range 300–400 nm) on a Unicam SP 1800 spectrophotometer. Optical rotation was measured at 365 and 436 nm on a Perkin-Elmer 241 polarimeter, using jacketed cells of pathlength 1 cm or 10 cm, as appropriate. Temperature was again controlled by a Haake circulating water bath, and measured using a thermocouple in the neck of the cell, but out of the light path. The results are reported as specific rotation, $[\alpha]$, which is conventionally defined as:

$$[\alpha] = 100\alpha/lc \quad (1)$$

where α is optical rotation in degrees, l is pathlength in dm, and c is concentration in g/100 ml. The factor of 100 can be eliminated (Eq. (2)) by expressing α in mdeg and l in cm.

$$[\alpha] = \alpha/lc \quad (2)$$

Thus for a 1% solution measured in a 1 cm cell, $[\alpha]$ is numerically equal to the optical rotation expressed in mdeg.

3. Results

This investigation began as part of a study of the composite gels formed by gelatinisation of phosphate-cross-linked starch (PCS) in an agarose matrix, which is reported in the following paper (Mohammed et al., 1998a). The first requirement was to obtain a calibration curve of the concentration dependence of storage modulus (G'), for use in analysis of the moduli of the composites. The procedure adopted was to load the agarose solutions onto the rheometer at 60°C, cool to 5°C at 1°C min^{-1}, and then hold the sample at 5°C until stable values were obtained.

Fig. 2*a* shows the time–temperature dependence of G' and G'' for 0.25 wt% agarose, the concentration selected for detailed study in most of the investigations described below, since it was found to give a final value of G' close to that estimated for individual granules of gelatinised PCS from analysis of the rheology of starch–gelatin composites (Abdulmola et al., 1996a). The increase in moduli at this polymer concentration was complete within 10–15 min of reaching 5°C (Fig. 2*a*), with no discernible further change at longer times. The mechanical spectrum recorded at the end of the holding period (Fig. 2*b*) has the form typical (Morris, 1984) of a biopolymer gel network ($G' \gg G''$; little frequency-dependence in either modulus; linear dependence of log η^* on log ω, with a slope close to −1). As shown in Fig. 3, the concentration dependence of G' after equilibration at 5°C also has the form typical (Clark and Ross-Murphy, 1985) of a gel network in which each chain

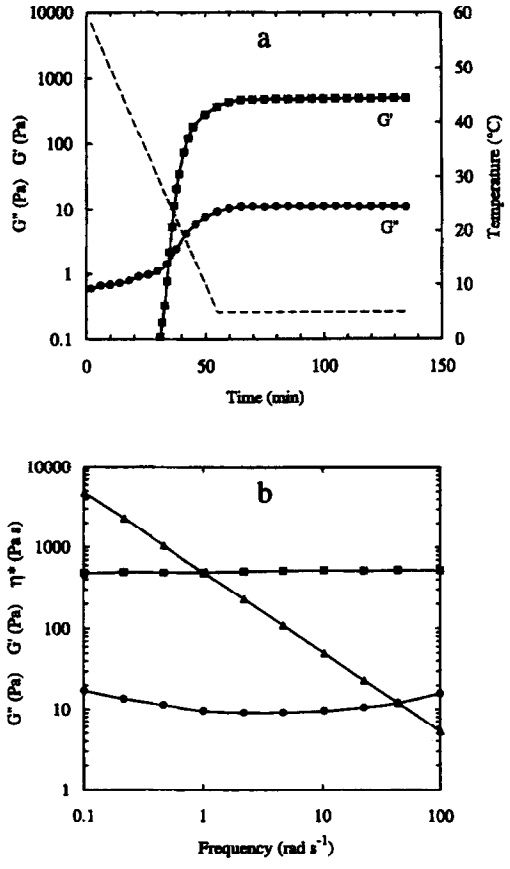

Fig. 2. Gelation of 0.25 wt.% agarose. (*a*) Small-deformation oscillatory measurements (10 rad s^{-1}; 0.5% strain) showing the variation of G' (■) and G'' (●) with temperature (– – –) and time on cooling from 60 to 5°C at 1°C min^{-1} and holding for a further 80 min at 5°C; (*b*) mechanical spectrum (0.5% strain) showing the frequency dependence of G' (■), G'' (●) and η^* (▲) at the end of the holding period at 5°C.

can participate in a large number of intermolecular associations (i.e. high functionality); at high concentration, log G' *versus* log c has a slope of ~2 (i.e. c^2 dependence of modulus), with a progressive increase in slope as c is reduced towards the minimum critical gelling concentration, c_0.

The reason for choosing agarose as the support matrix in the composite gels was to allow the starch to be gelatinised with the surrounding polymer in either the solution or gel state, to test the concept of 'network deswelling' invoked in previous investigations of biopolymer co-gels in which one component gels first and is then deprived of solvent ('deswollen') by subsequent gelation of the second component, to form a separate, dispersed, phase within the pores of the original network (McEvoy et al., 1985; Kasapis et al., 1993b). Previous studies (Abdulmola et al., 1996b) had shown that the minimum temperature required for complete gelatinisation of the starch samples used (i.e. complete disordering of amylopectin) is 80°C. It was therefore essential to verify that the agarose gels would survive to at least that temperature.

Fig. 4*a* shows the temperature course of gelation and melting for 0.25 wt% agarose, as monitored by measurements of G' and G'' on cooling and heating at 1°C min^{-1}. The reduction in log G' and log G'' with increasing temperature is essentially linear up to ~60°C, with a

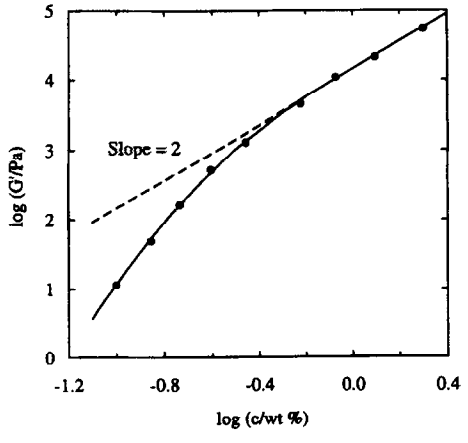

Fig. 3. Concentration dependence of G' (10 rad s^{-1}; 5°C) for agarose samples cooled at 1°C min^{-1} from the solution state at 60°C and held at 5°C for 80 min. Measurements were made at 0.5% strain for concentrations up to 0.5 wt.%; samples of higher concentration were measured at 0.05% strain.

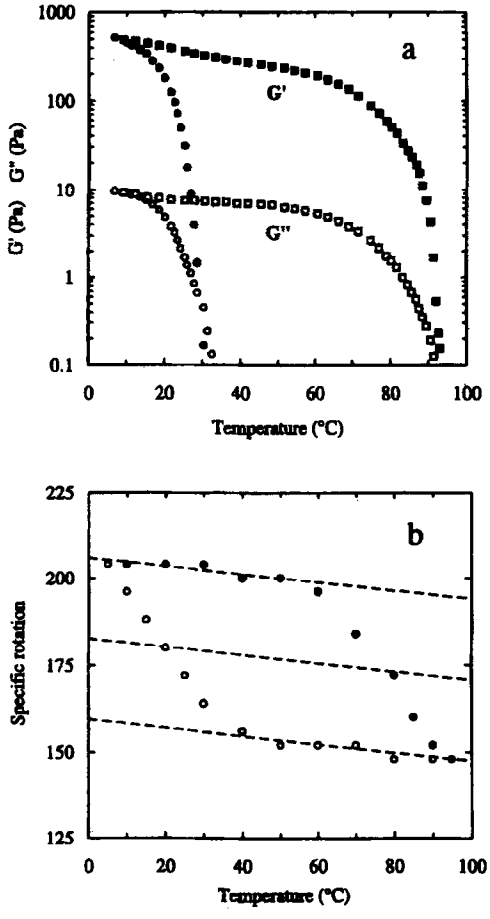

Fig. 4. Temperature dependence of conformation and rheology for 0.25 wt.% agarose in water. (*a*) Variation of G' (filled symbols) and G'' (open symbols) on cooling (circles) and heating (squares) at 1°C min^{-1}; measurements were made at 10 rad s^{-1} and 0.5% strain. (*b*) Variation of optical rotation (365 nm; 1 cm pathlength) on cooling (○) and heating (●).

subsequent progressive increase in slope, indicative of melting of intermolecular associations. The mechanical response at 80°C, however, is still predominantly gel-like ($G' \gg G''$).

The conformational changes associated with the sol–gel and gel–sol transitions were explored by optical rotation measurements on the same sample (0.25 wt%). Even at this comparatively low concentration, agarose develops substantial turbidity on gelation (due to helix–helix aggregation), so that the maximum pathlength at which acceptable transmission could be obtained was 1 cm. The polarimeter used has a digital display, with the final digit corresponding to units of 1 mdeg. Temperature was changed incrementally, and readings were taken when the final digit remained constant for at least 10 min. To maximise the signal-to-noise ratio, the wavelength used was the shortest available on the polarimeter, 365 nm. The results obtained are shown in Fig. 4*b*. For the concentration and pathlength used, the plotted values of specific rotation are 4 times greater than the absolute values of optical rotation in mdeg (Eq. (2)), with the total change between 5 and 95°C corresponding to ~14 mdeg. Nonetheless, the overall pattern of temperature dependence seems clear.

At temperatures above and below the range of, respectively, the disorder–order and order–disorder transitions there is a slight, approximately linear, increase in rotation with decreasing temperature, as would be expected from non-specific reduction in mobility on cooling, with the 'all-helix' and 'all-coil' values running roughly parallel to one another. The transition-midpoint temperatures, estimated from the points of intersection of the experimental values with a line drawn midway between the linear regions at high and low temperature (Fig. 4*b*), are ~20°C on cooling and ~80°C on heating, in almost perfect agreement with corresponding values derived in the same way from optical rotation data reported in a previous investigation by Rochas and Lahaye (1989).

The onset of gel formation on cooling at 1°C min^{-1}, as characterised by the steep rise in G' (Fig. 4*a*), occurs at appreciably lower temperature than the onset of conformational ordering as monitored (Fig. 4*b*) by optical rotation (~30°C, in comparison with ~40°C), consistent with the requirement for a finite helix-fraction to give a continuous network (Clark, 1987). Final loss of network cohesion on heating (Fig. 4*a*) also appears to occur at a temperature slightly lower than that required for complete loss of conformational order (Fig. 4*b*), although the difference is less evident than in the cooling direction (probably due to the kinetic effects discussed later). There is, however, no indication that the gel network survives at temperatures above the range of the order–disorder transition.

This finding conflicts with the results of an investigation by Braudo et al. (1991) which also compared gelation and melting behaviour of agarose with the temperature course of optical rotation change. To reduce the turbidity of the gels, and thus improve transmission of light in the optical rotation studies, the samples were prepared in a mixed solvent of water and DMSO, rather than in water alone. Gelation temperature (taken as the point at which solution viscosity diverged steeply towards infinity) was found to show an initial increase with increasing content of DMSO and then to decrease steeply, with the maximum value occurring at ~50 wt% DMSO. Optical rotation measurements made using this solvent composition, at a polymer concentration of 1.0 wt%, were compared with the gelation and melting temperatures of the same sample, with the conclusion that final melting, as determined by placing a layer of perfluorodecalin (4 mm depth in a tube of diameter 15 mm) on top of the gel and observing when the agarose sample was displaced upwards by the denser liquid, occurs at a temperature ~25° higher than the temperature required for complete loss of conformational order (~88°C, in comparison with ~63°C).

To explore the origin of the disagreement with our own results, we examined the temperature dependence of optical rotation and G' for agarose in 50 wt% DMSO, using

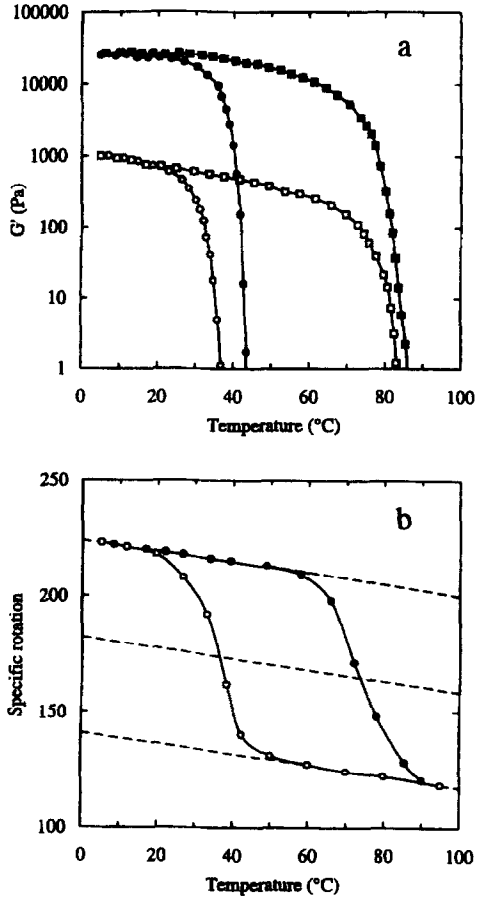

Fig. 5. Temperature dependence of conformation and rheology for agarose in 50 wt.% DMSO. (*a*) Variation of G' (10 rad s^{-1}) on cooling (circles) and heating (squares) for agarose concentrations of 0.25 wt.% (open symbols) and 1.0 wt.% (filled symbols); the 0.25 wt.% sample was measured at 0.5% strain and the 1.0 wt.% sample at 0.05% strain. (*b*) Variation of optical rotation (365 nm; 10 cm pathlength) on cooling (○) and heating (●) for the 0.25 wt.% sample; closely similar specific rotation values were obtained for the 1.0 wt.% sample measured at 1 cm pathlength.

polymer concentrations of 1.0 wt% (as in the study by Braudo et al.) and 0.25 wt% (as in Fig. 4). As shown in Fig. 5*a*, the steep rise in G' marking the onset of gelation occurs at ~37°C for the 0.25 wt% sample and at ~43°C for 1.0 wt%. These values are in almost perfect agreement (to within ± 1°C) with gelation temperatures shown in Figure 2 of the paper by Braudo et al. (1991). The corresponding melting temperatures (Fig. 5a) are ~83 and ~86°C for the 0.25 and 1.0 wt% samples, respectively. Braudo et al. reported melting temperature for one polymer concentration only (1.0 wt%) so no comparison can be made at 0.25 wt%, but the value they obtained at the higher concentration is again in good agreement with our own (88°C in comparison with 86°C). Thus, despite the use of two different samples of agarose, and of very different experimental procedures, there is no significant discrepancy between the gelation and melting temperatures obtained in the present work and those reported by Braudo et al. (1991). As a further check, we also determined the melting point of the 1.0 wt% sample by the perfluorodecalin method outlined above, and obtained a value of ~89°C, which is again in very close agreement with that obtained by Braudo et al. using the same procedure.

Because of the improved transmission in the mixed solvent system, it was possible to measure the optical rotation of the 0.25 wt% sample in a 10 cm cell, thus giving readings ~10 times higher than could be obtained for the same polymer concentration in water (Fig. 4*b*) where the pathlength was limited to 1 cm, with corresponding enhancement in precision and reliability. The results obtained at 365 nm are shown in Fig. 5*b*. Readings at 436 nm followed the same temperature course, but were smaller by a constant factor of ~1.5 (1.52 ± 0.02, averaged over all temperatures). Essentially identical values of specific rotation were obtained from measurements of the 1.0 wt% sample in a 1 cm cell, but with somewhat greater experimental scatter because of the smaller absolute values of optical rotation. In the cooling direction, the changes in optical rotation shown in Fig. 5*b* are broadly similar to those presented in Figure 3 of the paper by Braudo et al. (1991), with departure from linearity at ~50°C and closure of the hysteresis loop at ~20°C in both studies. In the heating direction, however, the results shown in Fig. 5*b* indicate completion of the order–disorder transition at ~90°C, in comparison with the value of 63°C reported by Braudo et al. (1991).

We can offer no explanation for this massive discrepancy in optical rotation data. The conformational transitions shown in Fig. 5*b*, however, seem entirely consistent with the gelation and melting behaviour observed in the present work (Fig. 5*a*) and in the study by Braudo et al. (1991). The onset of network formation on cooling and final loss of cohesion on heating occur at temperatures where the optical rotation traces indicate a finite content of ordered association. On lowering the agarose concentration from 1.0 to 0.25 wt% the setting and melting points are displaced to lower temperatures (Fig. 5*a*), as expected from the requirement for a greater degree of crosslinking (i.e. higher helix-fraction) to generate, or maintain, a continuous network as the concentration is reduced towards c_0.

At this stage of the investigation the gelation and melting properties of the agarose sample seemed well established, and we moved on to one of the principal objectives of the study, which was to explore the effect of network deswelling by gelatinisation of crosslinked starch within a pre-formed agarose gel. The experimental protocol adopted and the results obtained are presented in detail in the following paper (Mohammed et al., 1998a), but in outline the procedure used was to prepare a slurry of ungelatinised starch in agarose solution (0.25 wt%), load it onto the rheometer at a temperature just above the onset of gelation, quench to 5°C, hold until stable moduli were obtained, heat to 80°C at 1°C min^{-1}, and hold to allow gelatinisation to go to completion.

An immediate finding was that the values of G' observed for the mixed systems were substantially lower than the value of $G' \approx 55$ Pa found (Fig. 4*a*) for 0.25 wt% agarose alone when heated to the same temperature. This behaviour was, of course, completely unexpected, since transfer of water to the starch granules during gelatinisation should raise the modulus by increasing the polymer concentration in the surrounding matrix.

The first interpretation we considered was that the heating rate of 1°C min^{-1} used to obtain the melting curves in Fig. 4*a* might have been too high to allow the disordering process to keep pace with the imposed rate of temperature increase. This possibility was tested by raising the temperature incrementally and holding the sample at each temperature until stable moduli were obtained. Measurements were made on four replicate samples (0.25 wt% agarose), each of which was loaded at ~60°C, cooled to 5°C at 1°C min^{-1} and held there for ~80 min (as in Fig. 2*a*) before being heated (at 1°C min^{-1}) to the selected temperatures. The final, stable, values of G' and G'' were reproducible to within about ± 15% at each temperature. The mean values from the four replicate experiments are shown in Fig. 6, in direct comparison with the heating scans at 1°C min^{-1} from Fig. 4*a*. The moduli remain closely coincident up to ~60°C and then diverge slightly over the temperature range of the order–disorder transition (Fig. 4*b*), with the equilibrium values recorded at fixed temperatures falling below those obtained on heating at 1°C min^{-1}. The equilibrium value of G' at 80°C, however, is still appreciably higher than the moduli observed for the mixed systems at the same temperature.

Fig. 7 shows mechanical spectra recorded at 87 and 91.5°C, the two highest equilibration temperatures in Fig. 6. At 87°C (Fig. 7*a*) the spectrum is still broadly similar to that obtained for the fully-formed gel at 5°C (Fig. 2*b*), although the moduli are about an order of magnitude lower and there is an appreciable upswing in G'' at high frequency, consistent with the presence of a substantial 'sol fraction'

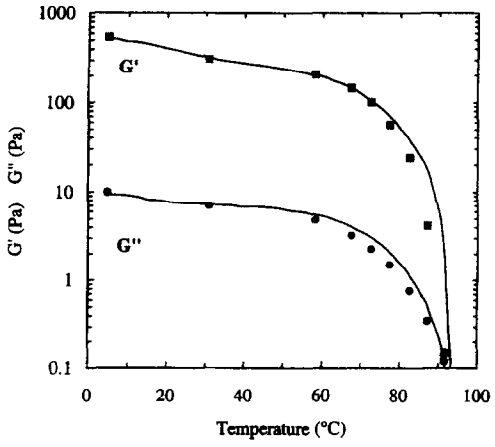

Fig. 6. Melting profiles for agarose gels (0.25 wt.% in water) formed by cooling at 1°C min^{-1}. The symbols show values of G' (■) and G'' (●) obtained after equilibration at fixed temperatures; the solid lines show the changes in moduli observed (Fig. 4*a*) on heating at 1°C min^{-1}. All measurements were made at 10 rad s^{-1} and 0.5% strain.

within the residual network. The spectrum recorded at 91.5°C (Fig. 7*b*), however, approximates to the form anticipated for a 'critically crosslinked' network (Durand et al., 1987; te Nijenhuis and Winter, 1989). Thus the results obtained by equilibration at fixed temperatures confirm the indication from heating scans at 1°C min^{-1} (Fig. 4*a*), that final loss of continuous gel structure occurs at a temperature slightly below completion of the order–disorder transition, as monitored by optical rotation (Fig. 4*b*).

The reduction in moduli of the agarose–starch composites at 80°C cannot, therefore, be attributed to time-dependent melting processes. The other major difference in the experimental procedures used for the mixed systems and the calibration studies on agarose alone is that, after loading onto the rheometer, the mixtures were quenched to 5°C (to minimise sedimentation of the ungelatinised starch granules), whereas the cooling and heating curves in Fig. 4*a* were obtained by changing temperature at 1°C min^{-1}. We therefore next explored the effect of cooling rate on the melting behaviour of the resulting gels.

Agarose gels (0.25 wt%) were prepared by the procedure outlined above for the mixed systems (loading at ~45°C, quenching to 5°C, and holding for ~1 h to allow complete gelation to occur). As in the investigation of the gels produced by cooling at 1°C min^{-1} (Fig. 6), the melting behaviour was then characterised by raising the temperature incrementally (at 1°C min^{-1}) and holding until constant moduli were obtained. As illustrated in Fig. 8, the time required for the rheological changes to go to completion became longer at higher temperatures, where the magnitude of change is greatest, but at each temperature the moduli then became stable, with no indication of further change at longer times.

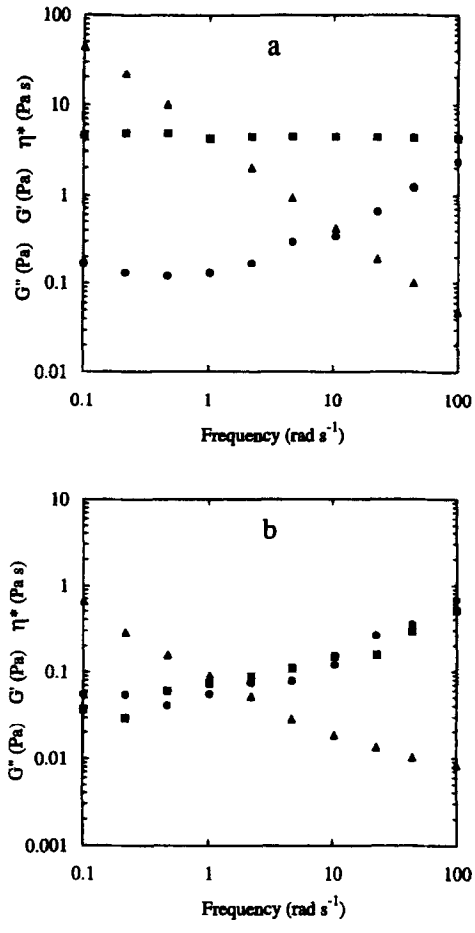

Fig. 7. Mechanical spectra (0.5% strain) showing the frequency dependence of G' (■), G'' (●) and η^* (▲) after equilibration at (*a*) 87°C and (*b*) 91.5°C for agarose gels (0.25 wt.%) formed by cooling to 5°C at 1°C min^{-1}.

As shown in Fig. 9, final loss of network cohesion occurs at substantially lower temperature for the quench-cooled samples than for the gels formed by cooling at 1°C min^{-1} (by ~12–13°C). This observation, as well as providing a direct explanation for the unexpectedly low moduli observed for the mixed systems on heating to 80°C, also

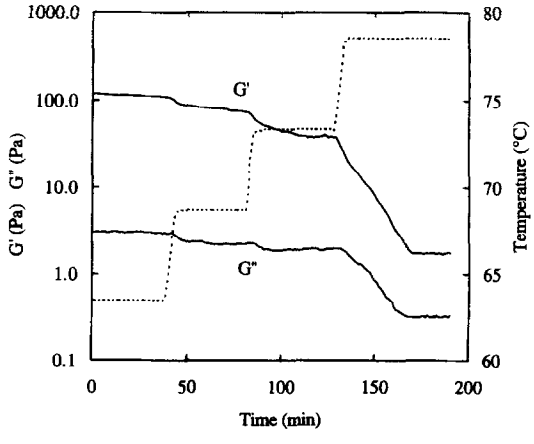

Fig. 8. Variation in G' and G'' (10 rad s^{-1}; 0.5% strain) on stepwise increase in temperature (– – –) for 0.25 wt.% agarose gelled by rapid quenching from 45 to 5°C.

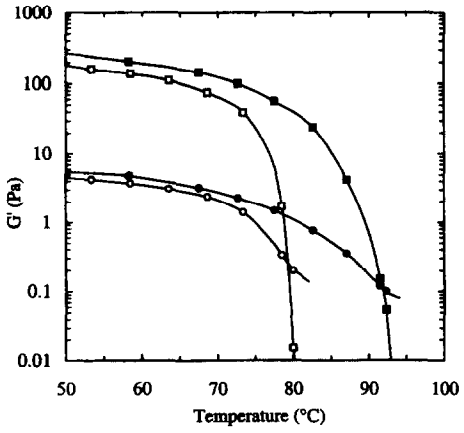

Fig. 9. Melting behaviour of agarose gels (0.25 wt.% in water) formed by rapid quenching (open symbols) or controlled (1°C min^{-1}) cooling (filled symbols) from 45 to 5°C, as characterised by measurements (10 rad s^{-1}; 0.5% strain) of G' (squares) and G'' (circles) after equilibration at fixed temperatures.

demonstrates that the way in which agarose gels are formed can have a profound effect on their thermal stability.

It is also evident from Fig. 9 that, at the polymer concentration used (0.25 wt%), the gels formed by controlled cooling (at 1°C min^{-1}) are somewhat stronger than those obtained by quenching. Quantitatively, the final value of G' at 5°C is ~50% higher (545 Pa, in comparison with 305 Pa). A possible interpretation of the enhancement in gel strength and melting temperature is that the slower rate of cooling allows formation of longer helices, thus promoting aggregation and thermal stability. The extent of aggregation in the gels obtained by the two different cooling regimes was explored by measurement of optical density at 5°C. As shown in Fig. 10, the gel formed by cooling at 1°C min^{-1} is significantly more turbid than that obtained by quenching, consistent with a greater degree of aggregation. Measurements were also made using the same concentration of agarose (0.25 wt%) but with 50 wt% DMSO as solvent (as

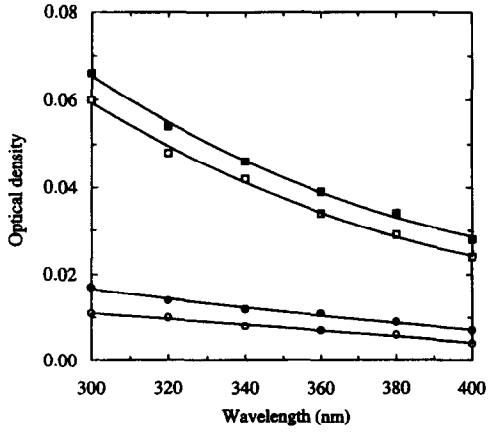

Fig. 10. Optical density values (1 cm pathlength) for gels of 0.25 wt.% agarose in water (squares) or in 50 wt.% DMSO (circles), formed by rapid quenching (open symbols) or controlled (1°C min^{-1}) cooling (filled symbols) from 45 to 5°C. All samples were held for 1 h at 5°C before measurements were made.

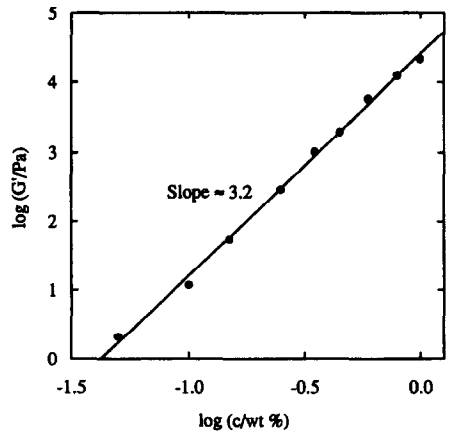

Fig. 11. Concentration dependence of G' (10 rad s^{-1}; 0.5% strain; 5°C) for agarose samples quenched from the solution state at 45°C and held at 5°C for 80 min.

in the experiments shown in Fig. 5). As anticipated, the optical density values in this solvent are much lower than those found with water as solvent, but the gel formed by controlled cooling is again significantly more turbid (Fig. 10) than that obtained by quenching.

In view of the appreciable effect of setting rate on gel strength at 0.25 wt%, the concentration dependence of G' was re-examined using samples gelled by quenching to 5°C. In contrast to the gels formed by cooling at 1°C min^{-1}, where the concentration dependence (Fig. 3) has the form anticipated from cascade theory (Clark and Ross-Murphy, 1985), the quenched samples (Fig. 11) show an approximately linear variation of log G' with log c (Eq. (3)) across the concentration range used (c = 0.05–1.0 wt%).

$$\log G' = 3.204 \log c + 4.414 \quad (3)$$

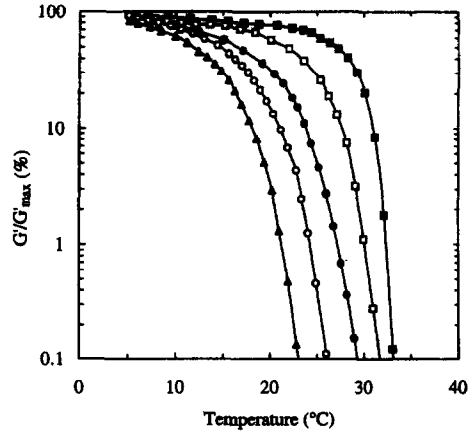

Fig. 12. Increase in G' (10 rad s^{-1}) during cooling from 45 to 5°C at 1°C min^{-1}, illustrated for agarose concentrations (wt.%) of 0.10 (▲), 0.185 (○), 0.35 (●), 0.85 (□) and 2.0 (■). Measurements were made at 0.5% strain for concentrations up to 0.5 wt.%; samples of higher concentration were measured at 0.05% strain. The observed moduli are expressed as a percentage of the final value (G'_{max}) attained after equilibration at 5°C (Fig. 3). The onset temperature for network formation (T_n) is taken as the temperature at which G' reaches 0.1% of G'_{max} (i.e. the points of intersection with the horizontal axis).

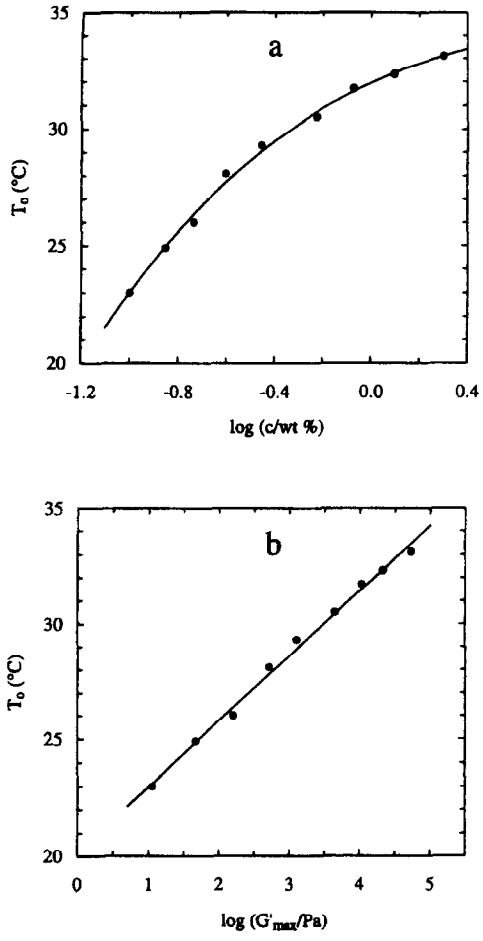

Fig. 13. Variation of the onset temperature for network formation (T_o; Fig. 12) with (*a*) agarose concentration and (*b*) final modulus (G'_{max}) after equilibration at 5°C.

The final part of the investigation was a brief study of the effect of temperature and concentration on the kinetics of gel formation. Fig. 12 shows the increase in G' on cooling from 45 to 5°C at 1°C min^{-1} for illustrative concentrations of agarose spanning the range studied (0.1–2.0 wt%). For ease of comparison, the observed moduli are expressed as a percentage of the final, maximum value (G'_{max}) attained after equilibration at 5°C (Fig. 3), and the onset temperature for network formation (T_o) is taken as the temperature at which G' reaches 0.1% of G'_{max}. As shown in Fig. 13*a*, T_o increases from ~23°C at 0.1 wt% to ~33°C at 2.0 wt%, and the variation with concentration is similar in form to the concentration dependence of log G'_{max} (Fig. 3). Indeed, as shown in Fig. 13*b*, there is an approximately linear relationship between the final value of log G' at 5°C and the onset temperature for network formation on cooling at 1°C min^{-1}.

We next examined the rate of network formation by 0.25 wt% agarose at temperatures just below the onset of conformational ordering, as monitored by optical rotation (Fig. 4*b*). The samples were loaded onto the rheometer at 45°C, cooled at 1°C min^{-1} to the selected temperature, and held there, with measurement of G' at fixed frequency

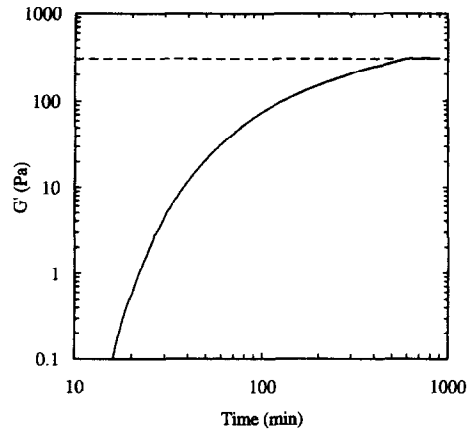

Fig. 14. Variation of G' (10 rad s^{-1}; 0.5% strain) for 0.25 wt.% agarose on cooling from 45°C at 1°C min^{-1} and holding at 30.8°C. The horizontal dashed line shows the value of G' at 30.8°C on heating from the gel state at 5°C (Fig. 4*a*).

(10 rad s^{-1}) and strain (0.5%) as a function of time, t ($t = 0$ being taken as the point at which cooling from 45°C commenced). The lowest temperature used was 30.8°C, which is very close to the onset of gelation on cooling at 1°C min^{-1} (Fig. 4*a*). As shown in Fig. 14, a steep increase in G' was observed within ~2 min of the sample reaching the holding temperature (cooling time ≈ 14 min; gel time ≈ 16 min). There was then a progressive reduction in the slope of log G' *versus* log t until, after ~10 h at 30.8°C, the modulus reached a constant value of ~307 Pa, which is in almost perfect agreement with the corresponding value on the heating curve in Fig. 4*a* (G' = 305 Pa).

Fig. 15 shows the results obtained at a higher holding temperature (35.2°C). At this temperature, the gel time was ~2 h, with corresponding reduction in the subsequent rate of network formation. The experiment was terminated after 36 h, at which stage G' had reached a value of ~46 Pa. When the holding temperature was increased further, to 37.8°C, there was no indication of gel formation over a period of 2 days.

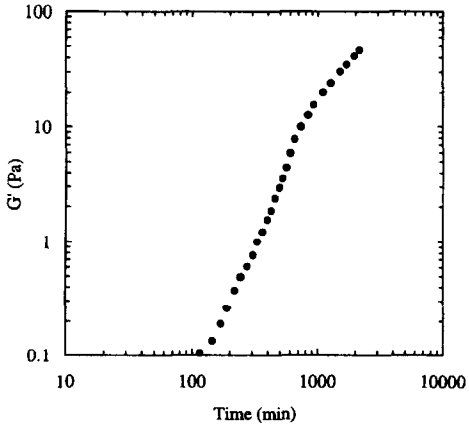

Fig. 15. Variation of G' (10 rad s^{-1}; 0.5% strain) for 0.25 wt.% agarose on cooling from 45°C at 1°C min^{-1} and holding at 35.2°C. Measurements were made at intervals of 1 min; the data points have been 'thinned' for clarity.

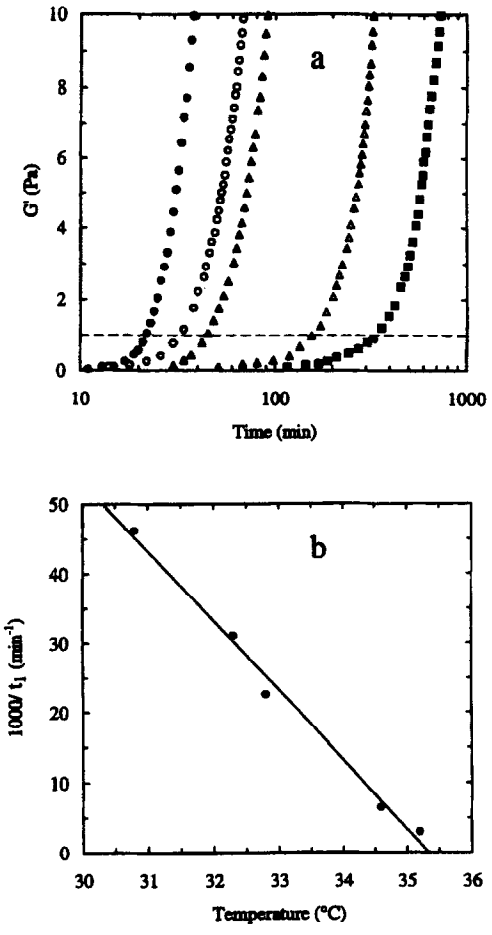

Fig. 16. Temperature dependence of initial rate of gelation for 0.25 wt.% agarose. (*a*) Variation of G' (10 rad s^{-1}; 0.5% strain) on cooling from 45°C at 1°C min^{-1} and holding at temperatures (°C) of 30.8 (●), 32.3 (○), 32.8 (▲), 34.6 (△) or 35.2 (■). Quantitative comparison was made using the time (t_1) required to reach a modulus of 1 Pa (– – –). (*b*) Reciprocal relationship between t_1 and temperature, extrapolating to $t_1 = \infty$ at ~35.3°C

Fig. 16*a* shows the increase in G' during the early stages of gelation at 30.8 and 35.2°C (as in, respectively, Figs. 14 and 15) and at three intermediate temperatures (32.3, 32.8 and 34.6°C). The curves have the same general form, but displaced to progressively longer times with increasing temperature. Since the moduli at the onset of gelation are extremely small, and therefore subject to considerable experiment error, the time required for the samples to reach a modulus of $G' = 1$ Pa (t_1) was chosen as an arbitrary, but robust, index of the relative rates of gelation at different temperatures. As shown in Fig. 16*b*, the reciprocal of t_1 (i.e. the rate of network formation) decreases linearly with increasing temperature, extrapolating to zero at ~35.3°C. When the agarose concentration was raised to 2.0 wt% (Fig. 17), however, rapid gelation was observed at 35.3°C (commencing within ~25 min of reaching the holding temperature), and at still higher temperature (38°C) the onset of network formation occurred within ~3 h. It is evident, therefore, that the kinetics of agarose gelation are dependent on both temperature and concentration.

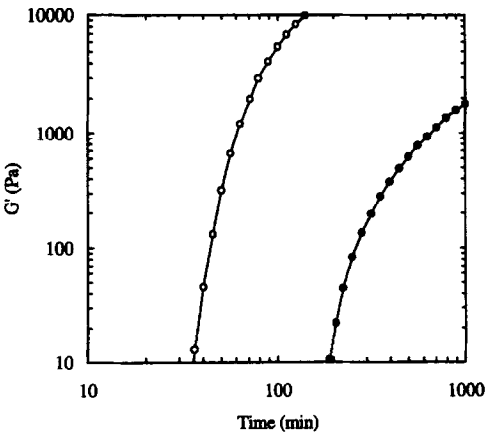

Fig. 17. Variation of G' (10 rad s^{-1}; 0.05% strain) for 2.0 wt.% agarose on cooling from 45°C at 1°C min^{-1} and holding at 35.3 (○) or 38°C (●)

4. Discussion

Previous investigation of agarose and its derivatives by optical rotation (e.g. Norton et al., 1986; Dea and Rees, 1987) have indicated that the order–disorder transition occurs as an equilibrium process, with optical rotation values obtained on heating remaining stable over long times. The cooling curves, by contrast, are metastable; on reduction in temperature within the range of the transformation from disorder to order, there is an initial change in rotation that occurs on the same timescale as the change in temperature, with the readings then settling to an apparently stable value; over a much longer time period (typically several days), however, the optical rotation gradually shifts towards the equilibrium value on the heating curve. The proposed interpretation of this behaviour (e.g. Morris and Norton, 1983) is that conversion from the disordered coil state in solution to the aggregated assemblies of double helices that form the junction zones of the fully formed gel occurs (Eq. (4)) by a two-stage mechanism.

$$2n(\text{coil}) \rightleftharpoons n(\text{double helix}) \rightleftharpoons \text{aggregate} \quad (4)$$

Melting of the aggregate structure does not occur until temperatures well above those at which the isolated double helix becomes unstable, and thus proceeds, in effect, as a direct aggregate–coil transition. The metastable cooling curve reflects the temperature dependence of the coil–helix transition, with subsequent (or concurrent) aggregation of helices then slowly displacing the initial equilibrium until the system reaches the true equilibrium between coil and aggregate, as characterised by the heating curve.

As would be anticipated, the rheological results obtained in the present work show the same general pattern of behaviour, and can be interpreted in essentially the same way. On incremental increase in temperature (Fig. 8) the moduli decrease immediately and then reach constant, stable values, as expected for an equilibrium melting process. After reduction in temperature, by contrast, there is an initial lag period (Figs. 14–16), which increases with

increasing temperature, before the moduli begin to rise, indicative of a process that is under kinetic control. For the combinations of agarose concentration and setting temperature that we have used, spinodal demixing (San Biagio et al., 1996) would be effectively instantaneous in comparison with the timescale of network formation, and would become rate-limiting only at higher temperatures and/or lower concentrations. The gelation processes we have observed can therefore be interpreted solely in terms of the conformation and interactions of agarose chains, although the effective local concentration of polymer may be substantially higher than the overall nominal concentration.

As can be seen from comparison of equilibrium moduli with heating curves recorded at 1°C min^{-1} (Fig. 6), and from the rheological changes observed during step-wise heating (Fig. 8), adjustment of the agarose network to the new equilibrium state after an increase in temperature does not happen instantaneously. Indeed, for the final temperature step shown in Fig. 8 (from ~73 to ~78°C) the reductions in moduli occur over a period of ~40 min, which is far longer than the time required for optical rotation readings to reach stable values. A possible explanation is that loss of conformational order occurs very rapidly, over the period of temperature change, but that the participating chains remain topologically intertwined, and then slowly diffuse apart to the dissociated coil state.

One of the main findings from this investigation is that the melting behaviour of agarose gels is strongly dependent on the conditions under which they were formed. It has been demonstrated in previous studies of the formation and melting of gelatin gels (Djabourov and Papon, 1983; Busnel et al., 1988) that the melting temperature increases with increasing setting temperature. Indeed, when solutions are held at one temperature and then quenched to lower temperature, the resulting gels show two separate melting processes at the positions anticipated from the individual holding temperatures (Busnel et al., 1988). The likely interpretation is that the helix-length required for stable association increases as the setting temperature is raised, with consequent increase in melting temperature on heating. The differences in melting behaviour shown in Fig. 9 for agarose gels formed under different time–temperature regimes can be explained in the same way.

Rapid quenching to 5°C will promote formation of a large number of short helices; slow cooling (1°C min^{-1}) will introduce a population of longer helices formed at higher temperatures, with the difference in length–distribution being reflected in the subsequent melting profiles. However, in contrast to gelatin, where the changes in melting temperature appear to be directly dependent on helix length, the optical density measurements shown in Fig. 10 indicate that the differences in melting behaviour observed for agarose (Fig. 9) are mediated by enhancement of aggregation with increasing helix length.

Essentially the same conclusions are indicated by the results of a concurrent study by another group (Aymard et al., 1997). Agarose (2.0 wt%) held for 5 days at 20°C was found to give a broad heating endotherm in d.s.c., spanning the temperature range ~45 to ~90°C and peaking at ~83°C. When the setting temperature was raised to 43°C, the lower-temperature portion of the endotherm (~75% of the total area) was lost, indicating that only the longest, and most thermally stable, ordered structures are formed at the higher setting temperature. At temperatures below ~40°C, development of network structure (increase in G') by 2.0 wt% agarose was accompanied by a simultaneous increase in turbidity, indicative of immediate aggregation of helixes as they are formed; at higher temperature, however, the aggregation and ordering processes could be resolved, with the onset of increase in turbidity occurring after the initial increase in G'. Also, the final turbidity values observed at high temperature were much greater than for samples gelled at lower temperatures, consistent with increased aggregation with increasing helix length.

In addition to the reduction in melting temperature (Fig. 9), formation of agarose gels by rapid quenching rather than by controlled cooling also appears to change the concentration dependence of gel modulus from the form typical of biopolymer networks (Fig. 3) to a linear variation of $\log G'$ with $\log c$ (Fig. 11). We can offer no detailed interpretation of this difference in behaviour, but suggest, tentatively, that it may be in some way associated with a change in the relative contributions of double-helical junctions and helix–helix aggregates to overall crosslinking, since a similar linear dependence of $\log G'$ on $\log c$ has been observed (Kasapis et al., 1993a) for potato maltodextrin of comparatively low molecular weight (Paselli SA-6), where network formation is believed to occur predominantly through aggregation of short helical segments.

As shown in Fig. 16*a*, the rate of gelation of agarose at a fixed concentration of 0.25 wt% decreases with increasing temperature, and extrapolates to zero (Fig. 16*b*) at ~35.3°C. At higher concentration (2.0 wt%), however, gelation occurred within ~3 h at 38°C (Fig. 17) and, as mentioned above, Aymard et al. (1997) found substantial association of 2.0 wt% agarose when held at still higher temperature (43°C) for 5 days. The effect of polymer concentration on the time–temperature course of agarose gelation is also evident in the cooling curves shown in Fig. 12. As the polymer concentration is lowered, there is a progressive reduction in the onset temperature for gel formation on cooling at fixed rate (1°C min^{-1}).

As discussed previously, one factor contributing to this behaviour will be that the degree of crosslinking required to give a continuous network increases with decreasing polymer concentration (Clark, 1987). Also, it has been demonstrated from optical rotation studies of iota carrageenan (Reid et al., 1974; Bryce et al., 1982) that, when allowance is made for polydispersity, the coil–helix

equilibrium shows the concentration dependence expected for a bimolecular process, with helix fraction at each temperature therefore decreasing with decreasing concentration. Both of these considerations would be expected to lower the equilibrium temperature for formation of a 'critically crosslinked' network by association of chains through double-helical junctions.

There are, however, clear indications of an additional, and perhaps over-riding, contribution to the time–temperature course of network formation from kinetic effects associated with helix–helix aggregation. Previous studies of agarose sulphate by polarimetric stopped-flow, using a rapid increase in ionic strength ('salt-jump') to induce conformational ordering (Norton et al., 1986), have shown that the coil–helix transition is extremely rapid, with reaction-progress curves going to completion within, typically, ~100 ms. As illustrated in Fig. 2*a*, however, when agarose (0.25 wt%) is cooled (at 1°C min^{-1}) from the solution state to 5°C, development of network structure continues for ~10 min after the sample has reached the final temperature, and it can be seen from the cooling traces shown in Fig. 12 that the magnitude of this effect increases with decreasing polymer concentration (i.e. there is a progressive reduction in the ratio of the modulus reached on completion of cooling relative to the final, stable value, G'_{max}, attained after holding at 5°C). The obvious conclusion is that crosslinking through double helices is augmented by helix–helix aggregation and that, as would be expected from simple considerations of mass action, the kinetics of the aggregation process become progressively slower as the polymer concentration is decreased.

The comparison of turbidity development and network formation by Aymard et al. (1997) demonstrates that, at least at high polymer concentration (2.0 wt%) and within the temperature range of the gelation traces shown in Fig. 12, helix–helix aggregation accompanies conformational ordering, rather than occurring as a separate, later process. As discussed previously, aggregation of helices as they are formed would be expected to displace the coil–helix equilibrium in the direction of fuller ordering (by depleting helix concentration), with the magnitude of the displacement increasing as the overall concentration is increased. Thus, the large increase in the temperature of gel formation on cooling at 1°C min^{-1} (and the associated sharpening in the subsequent development of G') as agarose concentration is increased from 0.1 to 2.0 wt% (Fig. 12) may reflect, and perhaps be dominated by, the kinetics of helix–helix aggregation, in addition to the equilibrium changes discussed above. Involvement of aggregation in both rate of structure formation and degree of crosslinking might also explain the direct correlation we have observed (Fig. 13*b*) between the onset temperature for network formation and the modulus of the resulting gels.

Finally, we have explored a previous report (Braudo et al., 1991) of agarose networks surviving at temperatures well above completion of the disorder–order transition, as monitored by optical rotation, and have been unable to reproduce the experimental evidence on which it is based. The discrepancy cannot be attributed to differences in the samples used, or in preparation of the pre-gel solutions, since both studies gave virtually identical gelation and melting temperatures under the same experimental conditions (1.0 wt% agarose in 50 wt% DMSO). The temperature dependence of optical rotation on cooling was also broadly similar in both investigations, but the heating curves are grossly different, with our own results (Fig. 5) indicating final loss of conformational order a few degrees above completion of gel melting rather than ~25°C below the melting point, as reported by Braudo et al. (1991). We cannot explain this clash of experimental evidence, but note that the optical rotation curves obtained in the present work for agarose in water (Fig. 4*b*), using procedures identical to those applied to the samples in 50 wt% DMSO (Fig. 5*b*), are in good agreement with previous reports by other workers (e.g. Arnott et al., 1974b; Rochas and Lahaye, 1989).

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