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SPIN-LABELLING STUDIES OF THE AGAROSE GELLING-SYSTEM

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ABSTRACT

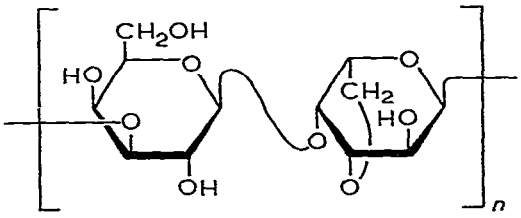
Rates of tumbling and exchange of free nitroxides in aqueous solution are unaffected by the presence of quite high concentrations of agarose gel. Low-level, covalent attachment of labels to the polysaccharide by means of stable, acetamido ether linkages, however, causes considerable diminution in their rate of reorientation. Dissolution of this material to form a gel and melting and setting of the gel cause further changes in the e.s.r. spectrum. Experimental correlation-times for reorientation of label may be decomposed into contributions from rotation about bonds in the linking group and from polysaccharide motions. This allows information to be obtained about the microscopic characteristics of the gel state, which is found to vary greatly depending on its history.

INTRODUCTION

Nitroxide electron-spin resonance (e.s.r.) spectroscopy offers three distinct advantages for the study of the molecular properties of polymeric systems: (*i*) the absence of a competing "background" signal, the introduced nitroxide being, in general, the only species present containing an unpaired spin; (*ii*) the transparency of samples having different physical forms (solution, gel, or solid) to X-band, microwave radiation; and (*iii*) the direct access provided to motional information on the nitroxide and, by inference, the polymer, for processes occurring in 100–0.01 ns. The technique had been applied to synthetic polymers[1], as well as to proteins, lipids[2], and nucleic acids[3], and, following the extensive work of Rees and colleagues[4] using a number of other physical techniques, we have been concerned with its application to the study of carbohydrate polymers[5,6] whose unusual rheological properties, widely utilized in industry, demand thorough examination.

We have reported[6] that the rotational reorientation of small nitroxide molecules in aqueous solutions or gels is unaffected by the presence of any of a variety of other polysaccharides, despite their considerable effect on the macroscopic viscosity of the system, and the same is here shown to be true of agarose, both in the sol and the gel state. We found that, in order to obtain information concerning the motional properties and microenvironments of polysaccharide molecules in solution and during sol→

gel transitions[6], as well as in insoluble, precipitated forms[5], it is necessary to attach the nitroxide label to the polysaccharide, thus providing a chemical perturbation which, nevertheless, enables information regarding the microenvironment of the polymer to be obtained. The sensitivity of the technique is such that a very small proportion of nitroxide, a minimal perturbation, is sufficient to provide a good signal-to-noise ratio in e.s.r. spectroscopy.



1 (idealized)

Agarose (**1**), in the condensed state, has been shown by X-ray diffraction[7] to form three-fold, double helices in which O-2 of the D-galactosyl and O-5 of the 3,6-anhydro-L-galactosyl residues project towards the interior, which may also accommodate water molecules. Although such associations probably also occur in the gel state, the optical absorbance and light-scattering properties[8,9], the appearance of the gel under a light-microscope[10], as well as under an electron microscope[11], and its characteristics as a gel-filtration matrix[12], all suggest larger aggregates, as does the concentration dependence of specific optical rotation[10], which suggests that conformational restraints may arise from inter-helix packing, in addition to helix formation. The hysteresis in optical rotation[7,10,13] that occurs in gel–sol–gel cycles is not observed at concentrations of $<0.3\%$ (w/w), where gelation does not occur; here, however, a reversible transition is still observed near the setting temperature. Thus, hysteresis is related to inter-helix associations of a kind that has not been characterized at the molecular levels. Rees and co-workers[7,8] proposed a two-stage, gelation process in which dimerization is followed by formation of "bundles" as a consequence of the stiffness of the helix, that is, its lack of conformational entropy relative to the random coil.

In light of these results, there is obviously a need for additional information on the molecular processes involved in gel formation and dissolution. Experiments with fluorescence spectroscopy[13] used probes dissolved in the gel or solution; the spin-labelling technique, in which nitroxides are chemically attached to the polysaccharide molecules, was seen as an ideal method for probing changes in mobility in interacting chains in a rather more specific way than had previously been possible, as the problems involved in interpreting macroscopic (viscosity, calorimetry) measurements are legion. We report herein the spin labelling of agarose by a method that may be used with other base-stable polysaccharides, and the use of the nitroxide "reporter" group to study the agarose gel → sol → gel transitions.

RESULTS AND DISCUSSION

Spin-probe experiments. — The presence of 3% agarose gel had no observable effect on the tumbling rate of 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (label 3) in aqueous solution (Figs. 1a and 1c) as was expected from the results of a previous study[6]. Large pockets of solvent within the gel ensure that the microscopic viscosity experienced by the label is essentially the same as in free solution. It might be expected that a more sensitive index of translational diffusion of free radicals within a gel matrix might be obtained by measuring line widths in the absence of relaxation changes due to rotational reorientation, but in the presence of sufficiently high concentrations of radical that electron exchange, arising from collisions between pairs of radicals, would contribute measurably to the spin–spin relaxation time (T_2). In the spectra shown in Figs. 1a and 1e, collisional events occur sufficiently rarely that the exchange contribution to the linewidth is negligible. Three higher concentrations of 3 were chosen for linewidth measurements: 25mM, at the onset of exchange broadening; 0.26M, near, but not at, the exchange narrowing limit; and 0.09M, approximately in the middle of the range in which nitroxide line-widths are sensitive to concentration (in aqueous solution at room temperature). The results of comparisons between the line widths at these concentrations in water and in 3% agarose gel at 28° are shown in Table I, and the spectra are shown in Fig. 1. Within experimental error, the line widths are the same in all three cases, indicating that exchange also occurs largely independent of the presence of polysaccharide.

The results of previous work in which nitroxides were allowed to diffuse within the pores of poly(acrylamide) beads[14] suggested that only when pores become sufficiently small to exclude spherical molecules of molecular weight $> \sim 6000$ does translational diffusion begin to be affected; well below this limit, rotational diffusion is relatively insensitive to the polymer. In commercially available, bead form, 6% agarose (Sepharose 6B) excludes molecules of molecular weight $> 4 \times 10^6$. Thus, it is not unreasonable that pores within gels containing the highest concentrations of

TABLE I

LINE WIDTHS AND SPLITTINGS FOR THREE CONCENTRATIONS OF NITROXIDE 3 IN WATER AND IN 3% AGAROSE GEL

Concentration of 3 *(mM)*	*In water*		*In 3% agarose gel*	
	Width of center line (100 μT)	*Splitting of outer features (100 μT)*	*Width of center line (100 μT)*	*Splitting of outer features (100 μT)*
25	3.4 ±0.2		3.3 ±0.2	
90	8.1 ±0.5	32.0 ±1.0	9.0 ±0.5	32.2 ±1.0
260	8.8 ±0.2		8.9 ±0.2	

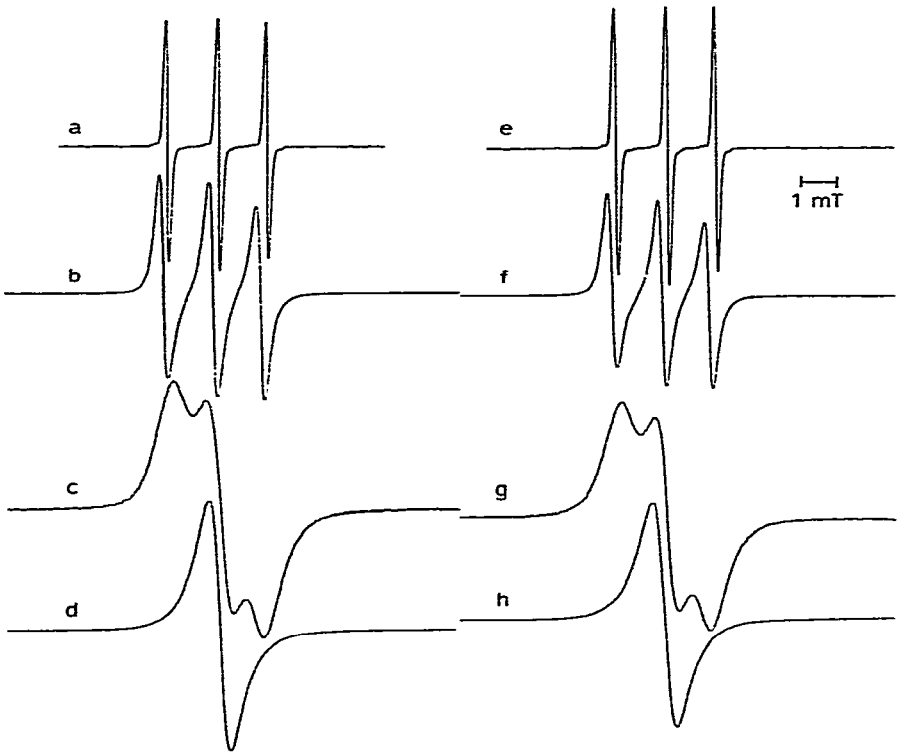
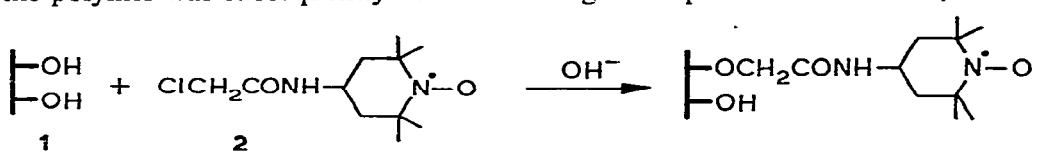


Fig. 1. E.s.r. spectra showing exchange broadening of label 3 at 300 K. [(a) 500μM in water, (b) 25mM in water, (c) 90mM in water, (d) 260mM in water, (e) 500μM in 3% agarose gel, (f) 25mM in 3% agarose gel, (g) 90mM in 3% agarose gel, and (h) 260mM in 3% agarose gel.]

agarose used here (5%) are substantially larger than those required for effects to be measurable in nitroxide spectra.

Spin-label experiments. — Agarose (**1**) as a slurry in aqueous acetone was covalently labelled by reaction with 4-(chloroacetamido)-2,2,6,6-tetramethylpiperidin-1-oxyl (**2**) at room temperature in the presence of sodium hydroxide, forming stable, acetamido ether linkages to free hydroxyl groups. No "leakage" of nitroxide from the polymer was subsequently detected during the experiments described, even after



1 2

repeated heating and cooling. The e.s.r. spectrum at 293 K of the labelled material, washed to remove unreacted nitroxide, is shown in Fig. 2a. The label, presumed to be present at accessible surfaces in the solid, tumbles at room temperature with a correlation time (τ) of 1.85 ns, about 2 orders of magnitude more slowly than when it is unattached (see Fig. 1a). Integration indicated that one nitroxide was present per ~1100 monosaccharide residues; as would be expected on the basis of this value, nitrogen was not detected by elemental analysis. The spectrum of the labelled material

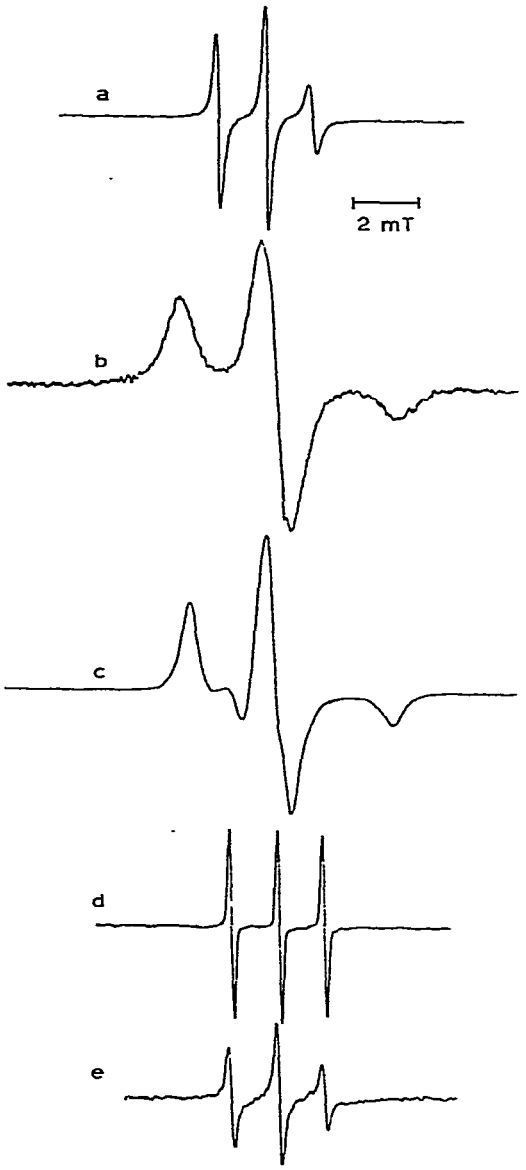


Fig. 2. E.s.r. spectra of spin-labelled agarose. [(a) Hydrated, precipitated form, 298 K; (b) precipitated form, 77 K; (c) precipitated form, 298 K, after lyophilization; (d) sol, 363 K; and (e) gel, 298 K.]

at 77 K is shown in Fig. 2b, and, again consistent with the foregoing quantitation, no spin–spin, dipolar broadening is discernible, which confirms that the average distance through space between nearest-neighbor radicals is[15] $> \sim 3.0$ nm; this low level of modification ensures minimal perturbation.

Figs. 2d and 2e show the e.s.r. spectra of the labelled agarose in sol (363 K, $\tau =$ 0.3 ns) and gel (298 K, $\tau = 0.92$ ns) forms, respectively. (Exactly the same lineshape

as that in Fig. 2b could be obtained by rapid freezing of the gel or solution forms to 77 K.) A small, but significant and reproducible, increase in the mobility of the label occurs between the precipitated and the gel forms, and another increase between gel and sol, *at a given temperature* (see Fig. 3). The labelling procedure is likely to have attached nitroxides to sugar hydroxyl groups (*a*) facing the outside of a double helix (*i.e.*, those other than OH-2 of D-galactose), (*b*) at the outside of a bundle of helices, or (*c*) in a residue not involved in associations at all, although such units are probably scarce in the precipitated material. However, it is quite feasible that, after dissolution and gelling, a label could find itself in a completely different environment with respect to chain associations; although a label at OH-2 of D-galactose could be expected to inhibit dimerization sterically[7], nitroxides substituted on other positions might be present in helical regions or in single-chain regions, including "free ends".

A third possibility must also be considered, namely, that a label could become "trapped" as a result of the aggregation of double helices in the second stage of the gelling process. It is not yet clear how the presence of a label might affect this type of association; however, although it is likely that the spectrum of the gel state represents the superposition of lines from nitroxides in different situations, the changes in line shape are not commensurate with the "trapping" of anything but a small proportion of the labels between chains. The spectrum of the gel indicates a label having a degree of motional freedom not strikingly different from that experienced in the solid or sol states. Its mobility is also roughly comparable to (and certainly not less than) that of labels attached *after gelation*[5,15]. Moreover, spectra for "trapped" labels in agarose systems have been recorded, and these represent much less mobile nitroxides[15]. The apparent absence of trapping may indicate steric hindrance, by the nitroxide, of associative phenomena in its vicinity, but the apparent sizes of aggregates present

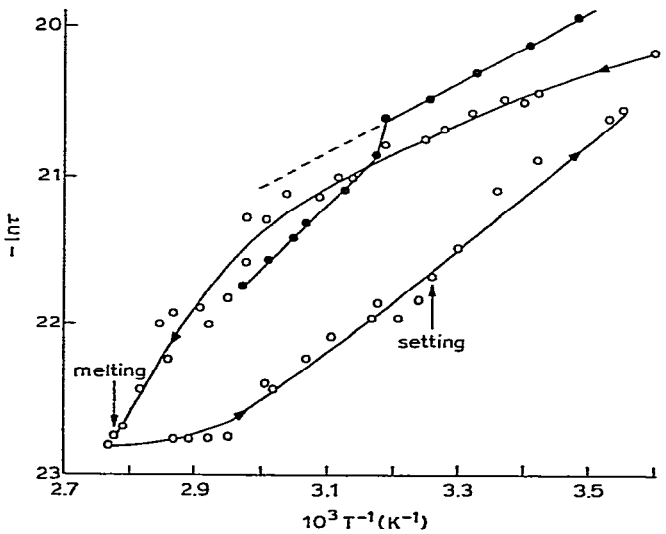


Fig. 3. Arrhenius plot of correlation times in labelled agarose; ●——●, "precipitated" form; ○→→→○, gel–sol–gel cycle.

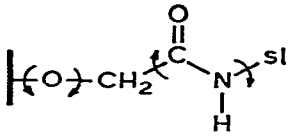
in the gel, measured by light-scattering[9], electron microscopy[11], and gel filtration[12] suggest that these may contain an average of only 7 to 11 helices; consequently, it is not inconceivable, given the present level of loading, that nitroxides may, without any great difficulty, be confined to the outsides of the bundles.

The dependence of the rotational correlation time (τ) upon temperature for the gel → sol → gel cycle (direction indicated by the arrows) is shown as an Arrhenius plot in Fig. 3, and a hysteresis is clearly visible. The dynamic melting and gelling temperatures, determined macroscopically as the temperature at which surface deformation occurs on removal of a thermometer from the labelled agarose, were 361.0 and 307.5 K, respectively (indicated on the curve), the same as for the native, unlabelled material, although the latter formed a rather harder gel. However, changes in the gradient of the plot that might have been expected at or near these temperatures, by analogy with studies that have been made of the melting of spin-labelled nucleic acids[3], are not particularly well defined. It is suggested that two factors contribute to this situation.

Factor a. The polydispersity of the polysaccharide. Thermodynamic parameters for the gelling process are most meaningfully treated on a "per residue" basis[4], and it is possible that activation energies for reorientation of the nitroxide, as calculated from Fig. 3, may be erroneous, at least insofar as they reflect chain motions (see later discussion). The observed spectra at or near the gelling and melting temperatures (and, conceivably, elsewhere) may be assumed to consist of the sum of different spectral lines corresponding to labels attached to molecules involved in pre- or post-gel aggregates and those attached to fully solvated chains. This point leads on to factor *b*.

Factor b. The nature of the gelling process. Other studies[7,8,16] have indicated that polysaccharide gelling-and-melting differs in a fundamental way from nucleic acid denaturation. The facts that many more possibilities exist for the linking of two polysaccharide chains to form a "dimer", and that a single chain may be involved in junction zones with several others, mean that events may occur at the molecular level over a range of temperatures, even in a monomolecular, monodisperse system. Helices may unravel, and re-form into more stable arrangements. Even at the macroscopic level, different features may be identified at various temperatures, giving rise to different definitions of gelling and melting points[17]. If the e.s.r. data can be interpreted as reflecting aspects of the molecular motion of the polysaccharide, rather than simply the tumbling of the label about its "linking unit" (that is, its rotational reorientation relative to the polysaccharide), we may draw the conclusions that the gel state as defined macroscopically does not imply a certain, fixed, microscopic, molecular mobility and that the aggregation phenomena involved in gelation do not give rise to the large decrease in polymer mobility that might intuitively be expected. The following discussion addresses this problem.

Two processes contribute to the tumbling rate (τ^{-1}) of a label attached to a polysaccharide molecule in solution. The first of these is rotation about single bonds in its "linking unit" (τ_l^{-1}), of which there are effectively four, as the amide C–N has



(sl = spin label)

double-bond character. Hydrogen bonding to the saccharidic surface is not expected[15] to lessen τ_l^{-1} further. The second type of motion is that of the polymer itself (τ_s^{-1}), which comprises "segmental" and "whole molecule" (or "overall") rotations. Thus[1,18,19], an approximate sum of rates may be made:

$$\tau^{-1} = \tau_l^{-1} + \tau_s^{-1}. \qquad (1)$$

Similarly, for the gel,

$$\tau^{-1} = \tau_l^{-1} + \tau_g^{-1}, \qquad (2)$$

where τ_g^{-1} refers to reorientation of polysaccharide molecules within the gel matrix, segmental motions being expected to dominate[20].

It is reasonable to assume that motion of the polysaccharide chains in precipitated agarose does not occur on the e.s.r. time-scale (*i.e.*, $\tau > 100$ ns). Thus, in this system, $\tau^{-1} = \tau_l^{-1}$. A part of the temperature dependence of the correlation time for precipitated agarose (**1**) is shown in Fig. 3 (filled points). Above 313 K, **1** begins to swell and dissolve, as indicated by the discontinuity in the plot, so that the measurement of τ_l may be made only below this temperature. However, the linearity of the Arrhenius plot for precipitated agarose below 313 K is evidence of the validity of the assumption; it leads to an activation energy for τ_l-processes of 19.5 kJ.mol^{-1}, which compares favorably with activation energies of ~8–40 kJ.mol^{-1} calculated for the reorientation of nitroxides bound to silica surfaces[21], where, again, no motional component due to the surface is to be expected. The further assumption must now be made that, at a given temperature,

$$\tau_l(\text{precipitate}) = \tau_l(\text{gel}) = \tau_l(\text{sol}). \qquad (3)$$

That a relationship exists between solvation and mobility [either of the τ_l or τ_i ($i = g,s$) type] is reasonable, and, trivially, may be demonstrated (see Fig. 2c) by recording the spectrum of the labelled agarose precipitate at room temperature after freeze-drying. [Note, however, that the splitting between the outermost features, 6.7 mT, is less than that in Fig. 2b (7.3 mT), indicating the presence of some residual motion.] The assumption in equation *3*, stated in another way, is that, although the solvation of the polysaccharide changes in passing from solid to gel to sol, the solvation of the label, together with its linking unit, remains the same in the presence of an excess of water; this is made possible in the solid by its presence only at the surface, and, in the gel, by the absence of a "trapping" phenomenon. That, nevertheless, an approximation had been made may be seen from the results of the following experiment.

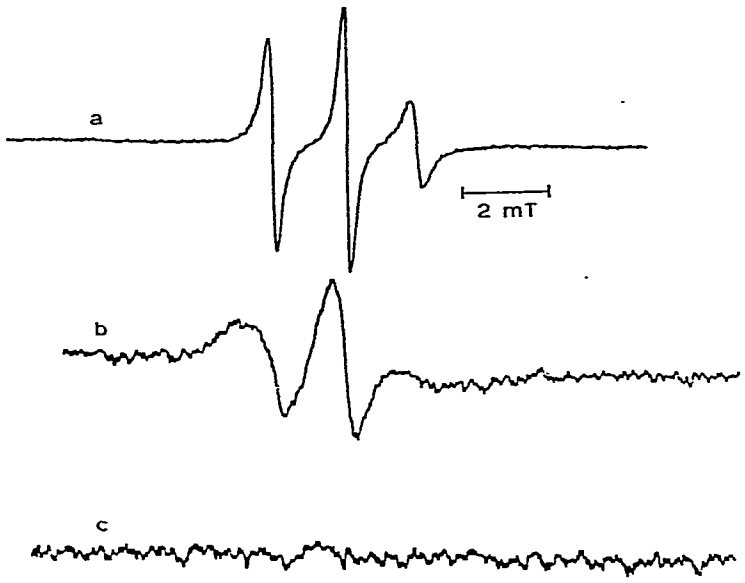


Fig. 4. E.s.r. spectra (300 K) of spin-labelled agarose. [(a) Hydrated, precipitated form (× 1); (b) as in (a), in the presence of 2M $NiSO_4$ (× 16); and (c) sol form in the presence of 2M $NiSO_4$ (× 24). Relative amplification in parentheses.]

Ions of $Ni(H_2O)_6^{2+}$ were introduced into solution, in the presence of the labelled solid, to a concentration of 2M; the spectrum is compared with that of the unperturbed system in Fig. 4. At this concentration of metal ions, rapid exchange of electrons between them and nitroxide molecules *free in solution* causes a diminution in the nitroxide T_2 value with concomitant line-broadening beyond the limit of detection. (The nickel e.s.r. signal is similarly invisible, owing to its own rapid relaxation.) In this case, where the nitroxide species is "tethered" to the surface, the presence of a "residual" spectrum, albeit at low signal-to-noise, indicates that steric hindrance prevents rapid exchange between at least a proportion of the nitroxides and the metal ions in the solution phase.

Unfortunately, the presence of 2M $NiSO_4$ destabilizes the gel relative to the sol form. Thus, when labelled agarose was dissolved in hot 2M $NiSO_4$ solution, no gelation occurred on cooling; when the metal salt was allowed to diffuse into the gel, the latter slowly (days) redissolved, with gradual diminution of the intensity of the e.s.r. signal (see Fig. 4c). Only when complete dissolution had occurred did the signal completely disappear. Therefore, it is clear that all labels are accessible to the paramagnetic ions in the sol, whereas this is not so in the solid (nor, probably, in the gel), although the proportion of inaccessible spins appears to be small; spectra shown in Figs. 4a and 4b differ in amplification by a factor of 16. The present approach may, therefore, be seen as an approximation, but one which is not unreasonable. Perhaps a more serious objection to equation *3* is that water at the surface is likely to be ordered differently from that in bulk solution[22].

It now becomes possible to calculate τ_s and τ_g values by using equations *1* and *2*,

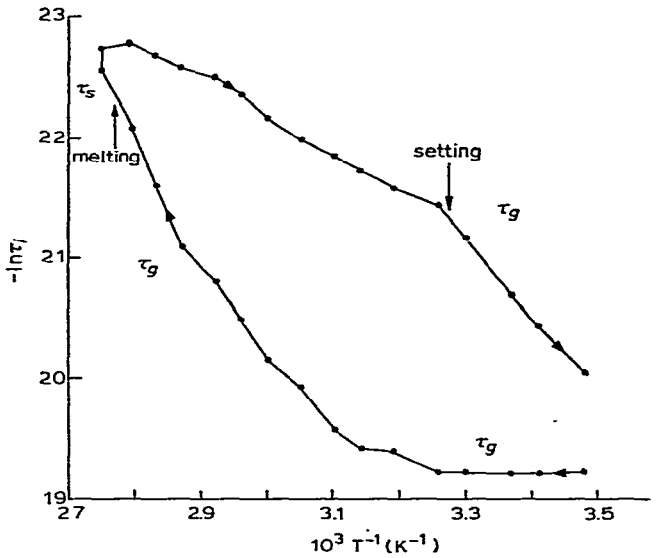


Fig. 5. Arrhenius plot of contributions to correlation time from polysaccharide-backbone motions in labelled agarose. (τ_g refers to the gel form, and τ_s refers to the sol.)

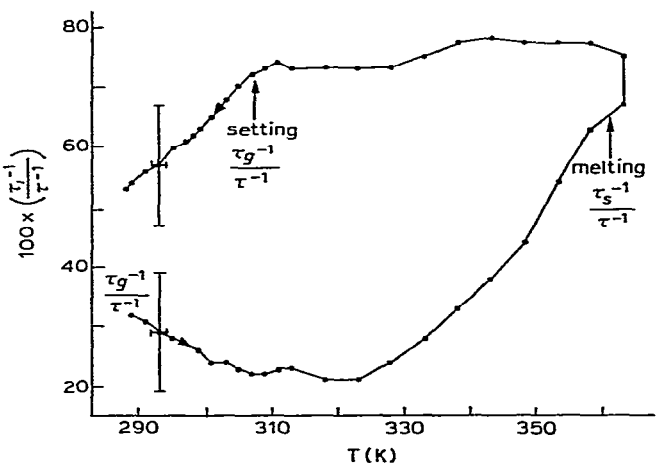


Fig. 6. Fractional contribution of polymer backbone motions to overall tumbling rate (τ_i^{-1}/τ^{-1}) as a function of temperature.

and substituting values of τ_l measured for the precipitated, labelled agarose at various temperatures. Above 313 K, where equation *3* cannot be assumed to hold even approximately, values of τ_l are calculated from the extrapolated, Arrhenius plot (see Fig. 3). Such values may be expected to be less reliable as the temperature is increased, especially above the melting point. The Arrhenius plot of the dependence of polymer-backbone motions τ_i on temperature is shown in Fig. 5, and it has a similar form to Fig. 3. Fig. 6 shows these motions plotted (as rates) as percentages of the total tumbling rate (τ^{-1}).

Although errors in measurement for τ_i^{-1}/τ^{-1} ($\pm 10\%$), together with the approximations inherent in equation *3*, as well as *1* and *2*, and the assumption that, in calculating τ, motion is isotropic, are such that too much significance should not be placed upon the *absolute* values, conclusions may justifiably be drawn from *trends* in the data. In the first place, it is amply clear that marked differences in mobility, perhaps of almost an order of magnitude, can occur in polymer molecules in gels in different states of organization. It is even possible to obtain equality between a τ_s and a τ_g value, although at different temperatures (see Fig. 5). Put in another way, the term "gel" may not be an adequate, unambiguous means of describing the state of agarose at a microscopic level.

Large differences in mobility can also occur between two gels at the same temperature, but with different immediate histories. Thus, directly after setting, at 303 K, τ_g was found to be $\sim$1/7th of that at the same temperature after remaining at room temperature overnight. Marked differences in τ_g^{-1} as a proportion of τ^{-1} (from $\sim$25 to $\sim$70%) at 303 K force the same conclusion. Within the time period of a single hysteresis cycle (1–2 h) using the labelled gel, it is not possible to return, at low temperature, to τ values as large as those at the start of the experiment (see Fig. 3), or to τ_g^{-1}/τ^{-1} values which are as large (see Fig. 6); this is in keeping with the idea that reorganization of the gel to more-stable forms, perhaps including changes in helix aggregation and even unfolding of individual helices, occurs below the formal (macroscopic), gelling temperature. No anomaly was observed during the cooling cycle in the Arrhenius plot of τ_i (see Fig. 5); a marked change in gradient occurs at the gelling temperature. However, the change in gradient that occurs in the plot of τ_i^{-1}/τ^{-1} *versus* temperature (see Fig. 6), the proportion of motion contributed by the backbone decreasing more rapidly with temperature below the setting point, seems to indicate a *gradual*, molecular reorganization.

Although no significance can be attributed to the decrease in τ_g^{-1}/τ^{-1} below 320 K (see Fig. 6), both Figs. 5 and 6 show that very considerable alterations occur in the microscopic structure during the heating cycle below the melting point. These processes appear to begin near 320 K, where each plot shows a noticeable change in gradient. This observation, which is quite consistent with the present description of the gel as containing many interactions having different strengths (corresponding, for example, to different lengths of association), underlines the advantage of the e.s.r.-spectral technique over that of optical rotation, which only detects transitions at the macroscopic, melting and gelling points. The lack of dependence of τ_s^{-1}/τ^{-1} on temperature, and the linearity of τ_s plotted according to Arrhenius, are as would be expected for a homogeneous, polymer system, and provide further evidence that changes in τ_g reflect a fundamental property of the gel, rather than an artifact of the labelling experiment.

EXPERIMENTAL

Materials. — Agarose (**1**) (SK-ME 11335) was a gift from Marine Colloids,

Rockland, Maine 04841; it was a granular, white solid, molecular weight $\sim 10^5$, containing $\leqslant 0.5\%$ of methoxyl, 0.2–0.3% of pyruvate, 0.65% of ash, and 0.28% of sulfate. 4-(Chloroacetamido)-2,2,6,6-tetramethylpiperidin-1-oxyl (**2**) and 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (**3**) were synthesized from 4-amino-2,2,6,6-tetramethylpiperidine (**4**) (Aldrich) by the methods of McConnell *et al.*[23] and Rozantsev[24], respectively.

Spin-labelled agarose. — Agarose (**1**, 0.04 g) was made into a slurry with 4% NaOH (2.5 mL), a solution of label **2** (15 mg) in acetone (1 mL) was added, and the mixture was shaken overnight at room temperature. The labelled solid was then thoroughly washed successively on a coarse, sintered-glass filter with large volumes of water, acetone, and aqueous sodium hydrogencarbonate (pH 8) and acetate (pH 4) buffers containing 0.5M NaCl. In this way, all of the excess of the reagent was removed, as shown by the stability of the remaining signal during all of the experiments performed, including dissolving the agarose and passing it through several gel–sol–gel cycles between 273 and 373 K. No hydrolysis of the acetamido ether linkage could be detected at any time. Dissolution occurred in hot (>363 K) water to the required concentration; for e.s.r.-spectral studies, the sol was filtered through glass-fiber paper to remove large aggregates. The macroscopic, gelling temperature, defined as the temperature at which a thermometer withdrawn from the fluid deformed the surface, was the same both for labelled and native agarose, although the latter formed a rather stronger gel. The presence of 2M $NiSO_4$ was found to prevent gel formation, so the salt was allowed to diffuse into the gel from a solution in contact therewith, the latter being periodically replaced in order to maintain its concentration. Gelation proceeded normally in the presence of concentrations of up to 0.26M nitroxide **3**.

Spectroscopy. — E.s.r. spectra were recorded at X-band with a Varian E3 instrument, at 20-μT modulation amplitude under nonsaturating, microwave-power conditions. The field was calibrated by using a proton n.m.r. magnetometer. Line widths were measured by using scan widths one-fourth of those shown in the Figures. The temperature of the measurement was varied by using a flow of nitrogen into the cavity, across a heating element controlled by a Varian V6040 n.m.r. variable-temperature unit, and was monitored (to ± 0.25 K) by using a chromel-alumel thermocouple. Numbers of spins were calculated relative to a standard, after integration on a Pacific Precision Co. MP-1012A integrator. E.s.r.-spectral measurements were reproducible, both on the same and on different preparations of labelled agarose.

Spectral simulation. — Correlation times (τ) for rotational reorientation were obtained by simulation of e.s.r. spectra, using the programs of Dalton *et al.*[25] and a Nicolet 1180 computer with 16K memory, assuming tumbling to be isotropic.

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REFERENCES

1 A. T. Bullock and G. G. Cameron, in K. J. Ivin (Ed.), *Structural Studies of Macromolecules by Spectroscopic Methods*, Wiley–Interscience, New York, 1976.
2 H. M. McConnell, in L. J. Berliner (Ed.), *Spin Labeling*, Academic Press, New York, 1976.
3 H. Dugas, *Acc. Chem. Res.*, 10 (1977) 47–54.
4 T. A. Bryce, A. A. McKinnon, E. R. Morris, D. E. Rees, and D. Thom, *Faraday Discuss. Chem. Soc.*, 57 (1974) 221–229.
5 L. D. Hall and J. D. Aplin, *J. Am. Chem. Soc.*, 100 (1978) 1934–1936.
6 J. D. Aplin and L. D. Hall, *Carbohydr. Res.*, 59 (1977) c20–c24.
7 S. Arnott, A. Fulmer, W. E. Scott, I. C. M. Dea, R. Moorhouse, and D. A. Rees, *J. Mol. Biol.*, 90 (1974) 269–284.
8 D. A. Rees, I. W. Steele, and F. B. Williamson, *J. Polym. Sci., Part C*, 28 (1969) 261–276.
9 B. Öbrink, *J. Chromatogr.*, 37 (1968) 329–330.
10 E. Pines and W. Prins, *Macromolecules*, 6 (1973) 888–895.
11 A. Amsterdam, Z. Er-el, and S. Shaltiel, *Arch. Biochem. Biophys.*, 171 (1975) 673–677.
12 T. C. Laurent, *Biochim. Biophys. Acta*, 136 (1967) 199–205.
13 A. Hayashi, K. Kinoshita, and M. Kuwano, *Polym. J.*, 9 (1977) 219–225.
14 A. D. Keith, W. Snipes, R. J. Mehlhorn, and T. Gunter, *Biophys. J.*, 19 (1977) 205–217.
15 J. D. Aplin and L. D. Hall, manuscript in preparation.
16 I. T. Norton, D. M. Goodall, and E. R. Morris, *Chem. Commun.*, (1978) 515–516.
17 W. Yaphe and M. Duckworth, in K. Nisizawa (Ed.), *Proc. Int. Seaweed Symp., 7th*, Wiley, New York, 1972.
18 E. T. Fossel, K. R. K. Easwaran, and E. R. Blout, *Biopolymers*, 14 (1975) 927–935.
19 I. V. Dudich, V. P. Timofeev, M. V. Vol'kenshtein, and A. Yu. Misharin, *Mol. Biol.*, 11 (1977) 685–693.
20 A. J. Benesi and J. T. Gerig, *Carbohydr. Res.*, 53 (1977) 278–283.
21 N. Sistovaris, W. O. Riede, and H. Sillescu, *Ber. Bunsenges. Phys. Chem.*, 79 (1975) 882–889.
22 W. Drost-Hansen, *Ind. Eng. Chem.*, 61 (1969) 10–47.
23 H. M. McConnell, W. Deal, and R. T. Ogata, *Biochemistry*, 8 (1969) 2580–2585.
24 E. G. Rozantsev, *Free Nitroxyl Radicals*, Plenum, New York, 1970.
25 P. Coffey, B. H. Robinson, and L. R. Dalton, *Chem. Phys. Lett.*, 35 (1975) 360–366.