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Hydrogen-bonding and conformation of agarose in methyl sulfoxide and aqueous solutions investigated by ^{1}H and ^{13}C NMR spectroscopy

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Abstract

The conformation of agarose in dimethyl sulfoxide (Me_2SO) or in aqueous solutions has been studied by ^{1}H and ^{13}C NMR spectroscopy. Partial assignment of the ^{1}H NMR spectrum of agarose in Me_2SO-d_6 has been obtained using 1D and 2D techniques. Coupling constant, chemical shift temperature dependence and exchange rate data for the hydroxyl protons have been used to establish the presence of intramolecular hydrogen bonds. The reported data show that the protons of HO-2 in the 3,6-anhydro-α-L-Gal*p* residue (**A**) and HO-4 in the β-D-Gal*p* residue (**G**) have a less rotational freedom and a higher thermal stability than the other hydroxyl protons. On the basis of the exchange rate data, obtained from 1D NOESY experiments with selective excitation of the residual water signal, an inter-residue hydrogen bond is proposed only for **G** HO-4 in Me_2SO, while the involvement of **A** HO-2 in the agarose–Me_2SO complex, recently reported, is suggested. The latter hypothesis is supported by the ^{13}C NMR data obtained in Me_2SO-d_6, in a Me_2SO-d_6–D_2O mixture and in D_2O, which also show that a relatively small conformational rearrangement of the chain segments occurs in aqueous solution. Deuterium isotope effect data indicate the persistence in water of the inter-residue **G** HO-4 mediated hydrogen bond with the additional involvement of **G** HO-6. In addition, the negligible isotope effect on the C-2 atom of **A** would indicate that the corresponding OH group, although free from the agarose–Me_2SO complex, is not available for further strong interactions.

Keywords: Agarose conformation; NMR; Hydroxyl protons; Hydrogen bonding; Isotope effect

1. Introduction

Agarose, an essentially neutral polysaccharide, is the major gelling component of agar, a mixture of

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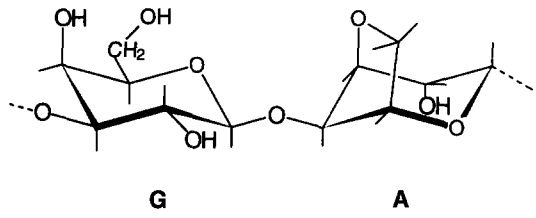

Fig. 1. Agarose repeating unit (agarobiose) consisting of 4-linked 3,6-anhydro-α-L-galactopyranose (**A**) and 3-linked β-D-galactopyranose (**G**).

structurally-related polysaccharides extracted from red seaweeds. Because of its strong gelling ability it is widely used in pharmaceutical and biotechnological applications. Besides its wide range of applications and commercial importance, the conceptual interest in agarose–water systems relates to the biological role of polysaccharides in molecular organisation and recognition [1,2].

The agarose primary structure [3] (see Fig. 1) consists of alternating (1 → 3)-linked β-D-galactopyranose and (1 → 4)-linked 3,6-anhydro-α-L-galactopyranose residues, hereafter designated as the **G** and **A** residues, respectively. Substituent groups like methoxyl, sulfate ester and pyruvate ketal may be present to a variable extent.

Agarose dissolves in water above 70 °C and, for concentrations higher than 0.2%, it gives a gel at around 45 °C upon cooling the clear solution. Repetitive heating and cooling treatments show the presence of a marked hysteresis in melting and setting temperature. Much effort has been made to gain insight into the agarose gelation mechanism, and the agarose–water system has been extensively studied by means of optical activity [4,5], X-ray diffraction [6], light and neutron scattering [7–10], proton magnetic resonance [11], fluorescence spectroscopy [12], spectrophotometry [11,13], and rheology [14,15]. The earliest model to describe the structure of the agarose gel network implies a co-operative thermoreversible coil-to-helix transition giving rise to double helical regions stabilised by interchain hydrogen bonding [6,16]. The presence of 'kinks' along the double strands increases the probability that a single agarose chain can form double helical segments, of variable length, with different chains. Aggregation in bundles of double stranded helices gives rise to the polymeric three-dimensional network. However, more recently and in agreement with the interpretation of new X-ray diffraction patterns on agarose films [17], low angle neutron scattering measurements [9,10] have shown that agarose chains in the sol-state are rather rigid and single stranded. Very recently, however, a re-examination of the agarose double helix model has been reported by means of optical rotation [18]. As there is still a need to gain a better insight into the agarose structure, we have performed a series of ^{1}H and ^{13}C NMR measurements in Me_2SO, water and Me_2SO–water mixtures to obtain additional information about the hydrogen bond pattern and the chain conformation in the sol-state.

2. Experimental

^{1}H NMR spectra of agarose ('Standard EEO' from SERVA) dissolved in pure dry Me_2SO-d_6 or in Me_2SO-d_6 doped with D_2O were recorded at 4.70 T (200 MHz) or 7.05 T (300 MHz) with a Bruker AC200 or AM300 WB instrument, respectively. One-dimensional (1D) experiments were performed at 200 MHz and 300 MHz in the range 25–95 °C. Two-dimensional (2D) COSY and relayed-COSY experiments were performed at 200 MHz in the range 60–80 °C using a spectral width of 660 Hz and a repetition time of 1.4 s. In the F2 and F1 dimensions, 512 and 256 data points were used, respectively, with zero-filling in F1. Centred sine-bell window multiplication was applied in each dimension. 1D TOCSY and 1D NOESY spectra [19] were obtained at 200 MHz by selective spin-echo excitation [20] using DANTE pulse trains [21]. The spectral width was 3000 Hz and the time domain 16 K. 1D TOCSY experiments were performed at 60 °C applying a pulsed MLEV-17 sequence [22] for the spin-lock, with the mixing time ranging from 33 to 99 ms. 1D NOESY experiments were performed in the range 25–95 °C by selective excitation of the residual water signal in order to observe the exchange rate of the hydroxyl protons as proposed by Bella et al. [23]. The build-up curves for the chemical exchange were obtained at 60 °C using a mixing time (τ_m) ranging between 100 and 800 ms. For all ^{1}H NMR experiments the sample concentration was 10 mg/mL. ^{1}H chemical shifts were referenced indirectly to Me_4Si by setting the methyl group of Me_2SO-d_6 at 25 °C to 2.500 ppm and applying the correction $(2.500 - t3.036E - 4)$ ppm for higher temperatures (t) as reported [24].

Proton decoupled ^{13}C NMR spectra of agarose dissolved in Me_2SO-d_6, 2:1 Me_2SO-d_6–D_2O, D_2O and H_2O were recorded at 4.70 T (50.33 MHz) with an acquisition time of 0.5 s and a flip angle of 75°, in

the range 50–80 °C. A spectral width of 16.1 kHz was observed using 16 K data points, giving after zero-filling a digital resolution of 0.98 Hz/point. In order to exclude the incomplete relaxation of carbon atoms, additional ^{13}C NMR spectra were recorded in Me_2SO-d_6 and in D_2O using a repetition time of 1.8 s. The sample concentration was 30 mg/mL. For agarose samples in Me_2SO-d_6 and 2:1 Me_2SO-d_6–D_2O, ^{13}C chemical shifts were referenced to internal Me_4Si, whereas internal 1,4-dioxane provided the secondary reference (66.40 and 66.41 ppm at 60 °C, respectively). For aqueous solutions, chemical shifts were reported relative to internal 1,4-dioxane (65.78 ppm in D_2O or 65.73 ppm in water at 60 °C), using Me_4Si as external reference. Chemical shift calibration in the range 25–80 °C obtained for 1,4-dioxane in pure solvents (Me_2SO-d_6, D_2O and H_2O), using Me_4Si as internal or external reference, showed that the values in Me_2SO-d_6 were on average ≈ 0.64 and 0.67 ppm higher than those determined in D_2O and H_2O, respectively.

3. Results and discussion

In the present study we have investigated the conformational behaviour of agarose in Me_2SO and aqueous solutions using different NMR approaches. For this purpose a commercial agarose sample (M_w = 167,000) containing a known amount of 6-*O*-methyl-D-galactose (DS = 0.25) and 3,6-anhydro-2-*O*-methyl-L-galactose (DS = 0.10) was used.

^1H NMR spectroscopy.—One- and two-dimensional ^1H NMR measurements were performed in dimethyl sulfoxide (Me_2SO-d_6) in order to observe the resonances of exchangeable protons and to possibly identify the OH groups involved in hydrogen bonding. The hydroxyl proton resonance of the hydroxymethyl group (HO-6) in β-D-galactose (**G**) was readily recognised by its triplet fine structure (Fig. 2). COSY and relayed-COSY spectra recorded at 80 °C in Me_2SO-d_6 enabled unambiguous assignment of the remaining OH resonances by examination of the cross-peaks as shown in Fig. 3. 1D TOCSY spectra (not shown), obtained at 60 °C, confirmed some of the OH assignments and allowed identification of other proton signals as shown in Fig. 2. The ^1H NMR chemical shifts and vicinal coupling constants ($^3J_{HC,OH}$) of the hydroxyl proton resonances for agarose in Me_2SO-d_6 at 80 °C are reported in Table 1. The signal of **G** HO-2 is at a much lower field than those of **A** HO-2 and **G** HO-6, respectively, whereas the **G** HO-4 resonance is furthest upfield. In the case of the agarose β-D-galactose residue, the **G** HO-4 and **G** HO-6 chemical shifts at 25 °C are very close to those found for β-D-galactopyranose and for methyl β-D-galactopyranoside in Me_2SO-d_6 at a comparable temperature [25,26], whereas, in agarose, **G** HO-2 gives the most downfield shifted resonance, accounting for the 3-substitution.

The chemical shifts of the hydroxyl proton resonances can be markedly affected by the existence of hydrogen bonds. Generally, hydroxyl protons involved in hydrogen bonds tend to be shifted down-

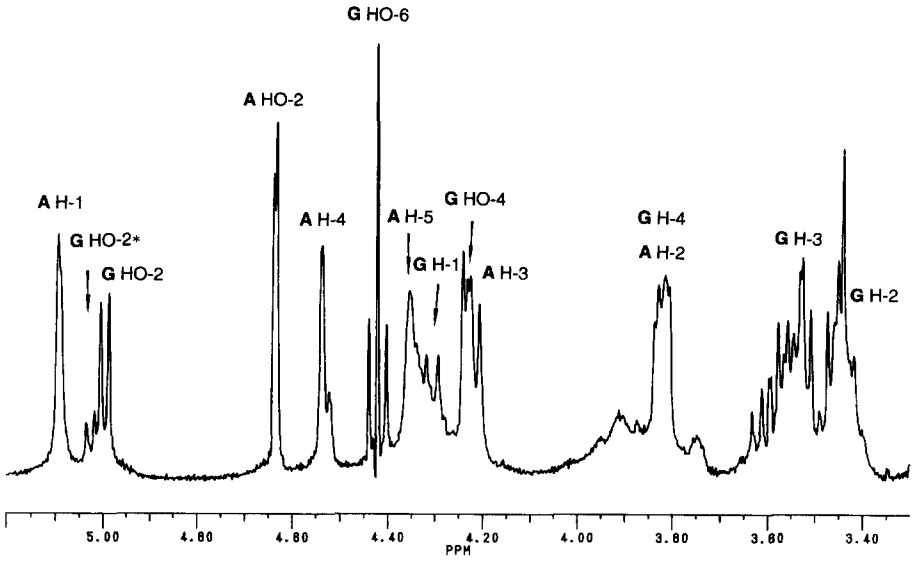

Fig. 2. 300-MHz ^1H NMR spectrum of agarose in Me_2SO-d_6 at 60 °C. **G** HO-2 * refers to the hydroxyl proton at position 2 in 6-*O*-methyl-D-galactose.

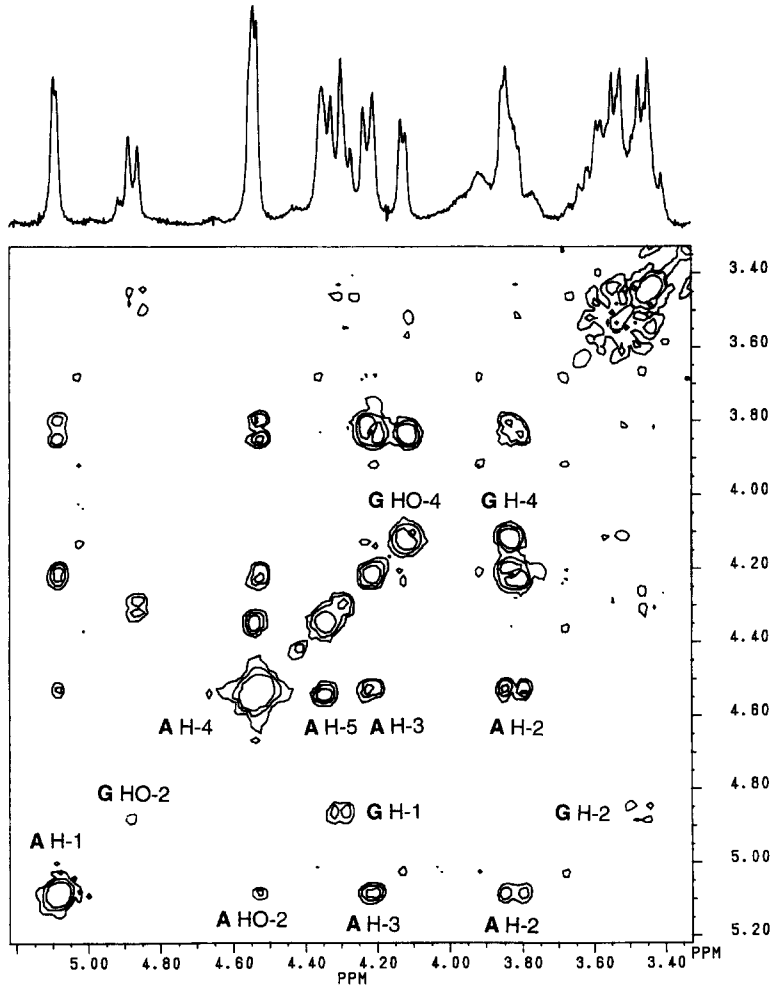

Fig. 3. 200-MHz ^1H–^1H relayed-COSY spectrum of agarose in Me_2SO-d_6 at 80 °C. Both halves of the mixing period, F1 and F2, were 50 ms. Arabic numerals refer to the proton positions in 3,6-anhydro-α-L-galactose (**A**) and β-D-galactose (**G**).

field [27]. For instance, in the case of the comparative study between hyaluronic acid and its ester derivatives, the downfield shifts of the OH resonances were used to discriminate between strong and weak hydrogen bonds [24]. In our case, the chemical shift changes are not sufficiently significant to be used as discriminating factor. In the present study, the $^3J_{HC,OH}$ values for **G** HO-2 and **G** HO-6 (5.12 and 5.48 Hz, respectively), representing uniformly-averaged values in terms of the Karplus equation [28], indicate that the corresponding hydroxyl protons are more or less free rotors. It is noteworthy that the $^3J_{HC,OH}$ value for **G** HO-6 of agarose in Me_2SO-d_6 at 80 °C is practically identical to that observed for HO-6 of both β-D-galactopyranose (5.5 Hz) [25] and methyl β-D-galactopyranoside (5.4 and 6.0 Hz) [26] in the same solvent at 22 °C and 25 °C, respectively. On the contrary, the $^3J_{HC,OH}$ values observed for **G** HO-4 (3.32 Hz) and **A** HO-2 (2.20 Hz) reveal little rotational freedom of these two hydroxyl groups, thus suggesting their involvement in hydrogen bonding. The reported coupling constant for **G** HO-4 should be compared with those of 4.2 Hz and 4.5 Hz obtained in Me_2SO-d_6 for β-D-galactopyranose [25] and methyl β-D-galactopyranoside, respectively [26].

Table 1
^1H NMR chemical shifts and $^3J_{HC,OH}$ coupling constants of the hydroxyl proton resonances for agarose in Me_2SO-d_6 at 80 °C

	Proton	δ (ppm)	J (Hz)
β-D-galactose (**G**)	HO-2[a]	4.89	5.12
	HO-2	4.86	5.12
	HO-4	4.11	3.32
	HO-6	4.28	5.48
3,6-anhydro-α-L-galactose (**A**)	HO-2	4.52	2.20

[a]HO-2 proton in 6-*O*-methyl-D-galactose.

In order to obtain further information on the spatial orientation of the hydroxyl groups, their chemical exchange rate was qualitatively assessed by 1D

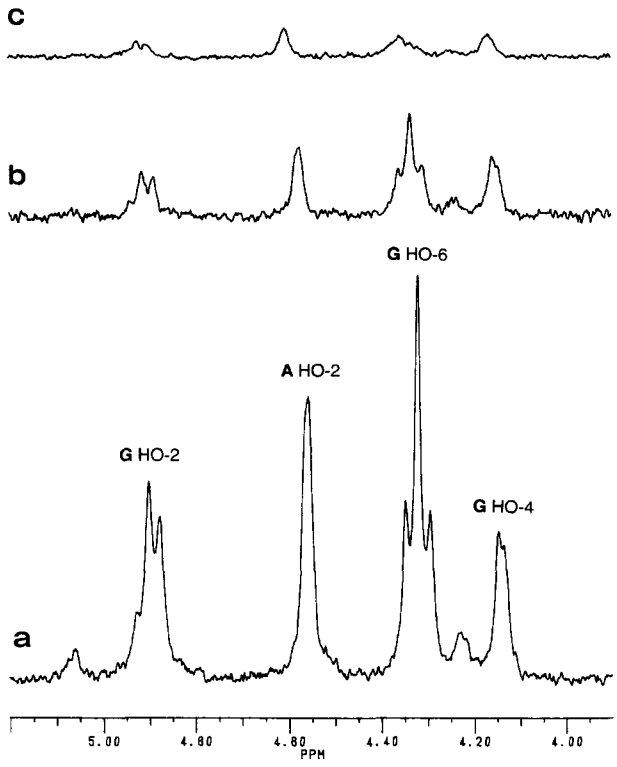

Fig. 4. 200-MHz 1D NOESY spectra (τ_m 400 ms) obtained at 80 °C for 10 mg agarose in 500 μL pure Me_2SO-d_6 (a), 500 μL Me_2SO-d_6 doped with 10 μL D_2O (b), and 500 μL Me_2SO-d_6 doped with 20 μL D_2O (c), after selective excitation of the residual water signal.

NOESY experiments based on selective excitation of the residual water signal [23]. In the 200-MHz 1D NOESY spectrum of agarose in pure Me_2SO-d_6, obtained at 80 °C using a mixing time (τ_m) of 400 ms (Fig. 4a), the hydroxyl proton resonances are clearly visible. Quantitative comparison of the corresponding peak areas showed the following relationship: **G** HO-4 < **A** HO-2 < **G** HO-2 < **G** HO-6 (0.37:0.67:0.74:1.00), indicating the slowest exchange rate for **G** HO-4. Such evidence, combined with the small coupling constant $^3J_{HC,OH}$ (3.32 Hz) observed for **G** HO-4, seems to confirm the involvement of this axial hydroxyl group in an inter-residue hydrogen bond. The poor availability of the **G** HO-4 proton for solvent interaction was further demonstrated by the 1D NOESY spectra obtained at 80 °C

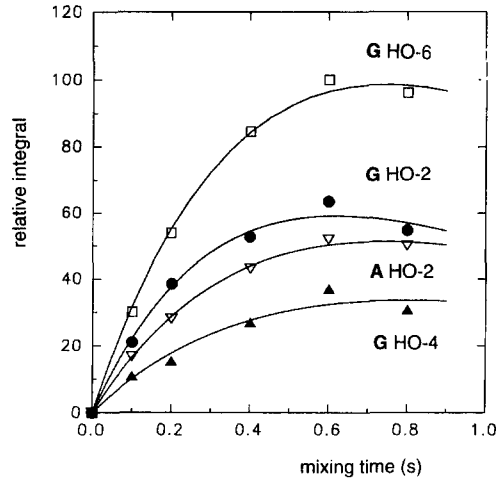

Fig. 5. Build-up curves for the chemical exchange between hydroxyl protons and residual water at 60 °C for agarose dissolved in pure Me_2SO-d_6 as obtained from 1D NOESY experiments.

after the addition of 10 or 20 μL D_2O to 500 μL of the agarose–Me_2SO-d_6 solution (Fig. 4b–c and Table 2), and by the build-up curves for the exchange rate obtained at 60 °C (Fig. 5).

The chemical shifts of hydroxyl protons involved in intermolecular hydrogen bonds generally show little change with temperature [24,29]. As an example, for amylose [30] and cellulose [31] temperature coefficients lower than 3 ppb/°C have been reported for protons involved in intermolecular hydrogen bondings. In order to determine the $d\delta/dT$ values of the hydroxyl protons of agarose in Me_2SO-d_6, ^1H NMR measurements were performed in pure Me_2SO-d_6, in the temperature range 25–95 °C. Data of Fig. 6a show that the temperature dependence of all the hydroxyl proton resonances, when referred to the Me_2SO signal (corrected for the temperature), is of the same order of magnitude with $d\delta/dT$ values ranging from −6.2 to −5.2 ppb/°C (Table 3). However, the smallest temperature dependence pertains to the **A** HO-2 and **G** HO-4 resonances. These different temperature dependencies are emphasised when the residual water signal is taken as the zero point [32], as shown in Fig. 6b and Table 3. The results clearly

Table 2
Ratios between the hydroxyl proton peak areas[a] observed for agarose in pure Me_2SO-d_6 at 80 °C and after addition of D_2O

Solvent	G HO-2	A HO-2	G HO-6	G HO-4
pure	1.00	1.00	1.00	1.00
doped with 10 μL D_2O	0.23	0.25	0.33	0.45
doped with 20 μL D_2O	0.07	0.10	0.09	0.18

[a] Values obtained using 1D NOESY experiments (τ_m 400 ms) with selective excitation of the residual water signal [23].

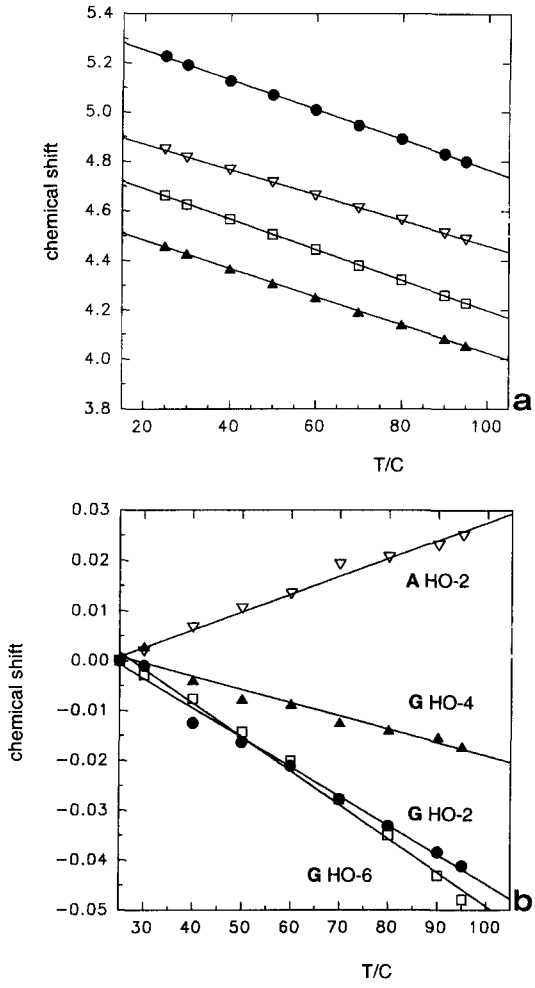

Fig. 6. Temperature dependence of the hydroxyl proton resonances of agarose in Me_2SO-d_6 using the solvent (a) or the residual water (b) signals as internal reference. In the latter case, all the chemical shifts at 25 °C are set at zero. In both cases vertical scales are expressed in ppm.

show that the interaction of **A** HO-2 and **G** HO-4 with the bulk solvent molecules is smaller than that of the other hydroxyl groups, thus confirming the chemical exchange rate data (Fig. 5). However, contrary to what is expected from the highest temperature stability (Fig. 6b and Table 3), the slowest exchange rate does not pertain to **A** HO-2 but to **G** HO-4 (Fig. 5).

Inspection of the neoagarobiose (**A**–**G**) and agarobiose (**G**–**A**) molecular models helps visualise the possible mutual positions of the ring atoms of interest, taking into account the relatively small conformational space available to each of the torsional angles characterising the glycosidic linkages indicated by molecular mechanics (MM) simulations (Urbani, personal communication). **G** HO-4 occupies a position suitable to form an intermolecular hydrogen bond with O-5 of **A**. A supporting indication of this interresidue H-bond may be found in the dihedral angle values calculated by applying a Karplus-behaviour-based equation to the experimentally observed coupling constant [26], bearing in mind that the accuracy of such an equation is, however, limited. The obtained solutions correspond to the values of $\pm 46°$ and $\pm 119°$ for the H–O–C(4)–H dihedral angle. A rotation of around $50° \div 60°$ of the H–O–C plane would lead the hydroxyl proton to point towards O-5 of **A**, whereas the other dihedral angle values would not allow the interaction of **G** HO-4 neither with O-5 nor with **G** HO-6. It is noteworthy that the above mentioned hydrogen bond has been proposed as result of rheological measurements [15] and stabilises the structure of some of the neoagarobiose conformers obtained from MM2CARB calculations [33]. These latter data, however, must be taken with care because in the reported neoagarobiose structure the anhydro-galactopyranose residue appears not to have the L but the D configuration.

The experimental findings so far presented are therefore consistent with an intermolecular hydrogen bond involving the **G** HO-4 group. **A** HO-2, however, should also strongly interact with other groups as suggested by data in Tables 1 and 3. Very recently, an agarose complex with two Me_2SO-d_6 molecules per repeating unit, has been proposed to occur in pure Me_2SO-d_6 and in a mixed Me_2SO-d_6–D_2O solvent [34,35]. The low rotational freedom and the relatively high thermal stability of **A** HO-2 might, then, indicate the involvement of this hydroxyl group in the agarose–Me_2SO complex rather than the for-

Table 3
Temperature coefficients ($d\delta/dT$) of the hydroxyl protons of agarose in pure Me_2SO-d_6

	Proton	$d\delta/dT^a$ (ppb/°C)	$d\delta/dT^b$ (ppb/°C)
β-D-galactose (**G**)	HO-2	−6.1	−0.6
	HO-4	−5.8	−0.3
	HO-6	−6.2	−0.7
3,6-anhydro-α-L-galactose (**A**)	HO-2	−5.2	+0.4

[a] Values obtained in the range 25–95 °C by using the solvent signal as internal reference.
[b] Values obtained in the range 25–95 °C by setting the residual water signal as zero point.

mation of a hydrogen bond. This hypothesis finds further support in the ^{13}C NMR data presented in the section below. Coupling constant, thermal stability and exchange rate data for the remaining OH groups are not consistent with the formation of strong hydrogen bondings.

^{13}C NMR spectroscopy.—It is well known that agarose–Me_2SO-d_6 is a non-gelling system and great differences in molecular organisation, chain–chain and chain–solvent interactions should exist between agarose in Me_2SO-d_6 and in water. To investigate the influence of solvent and temperature on the polymer conformation and to identify, among the carbon atoms, those most affected by medium changes, ^{13}C NMR spectra in Me_2SO-d_6, 2:1 Me_2SO-d_6–D_2O and D_2O at 80, 60 and 50 °C, respectively, and in H_2O at 80 °C were recorded.

On the basis of the assignments for agarose in aqueous solution [36], the carbon resonances observed in Me_2SO-d_6 and in the Me_2SO-d_6–D_2O mixture were readily assigned. The agarose assignments for solutions in Me_2SO-d_6, Me_2SO-d_6–D_2O and D_2O at 80 °C are listed in Table 4, whereas the ^{13}C NMR spectra recorded in Me_2SO-d_6 and D_2O at 80 °C are presented in Fig. 7. A comparative analysis of the influence of solvent [37] and temperature is illustrated in Fig. 8, where for each carbon resonance the observed change in chemical shift is reported. In particular, the chemical shift differences for agarose in Me_2SO-d_6 versus D_2O and versus the mixed solvent at 50, 60 and 80 °C are presented in Fig. 8. This shows that, at 80 °C, the solvent change from Me_2SO-d_6 to Me_2SO-d_6–D_2O mainly affects the **G** C-3, **A** C-1 and **A** C-4 resonances, which move downfield, whilst the chemical shift of the other carbon resonances are practically unaltered. At lower temperature, only the difference in chemical shift of **G** C-3 becomes more pronounced. Comparing Me_2SO-d_6 and D_2O solution data obtained at 80 °C, it appears that D_2O mainly influences **A** C-2 and, to a lower extent, the **A** C-3 and **G** C-5 resonances, which are not further affected by decreasing the temperature to the incipient gelation temperature (50 °C). On the contrary, by lowering the temperature, the **G** C-3 signal moves visibly downfield towards the same resonance position observed in the mixed solvent. These data indicate a relatively greater conformational freedom about the β-(1 → 4) and α-(1 → 3) linkage in Me_2SO-d_6 than in Me_2SO-d_6–D_2O, and, at lower temperature, in D_2O, in line with a partial

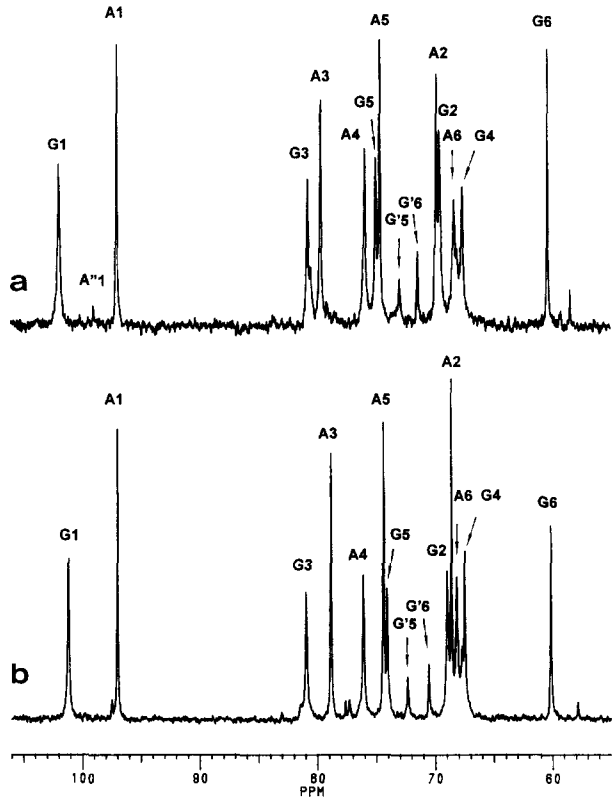

Fig. 7. 50-MHz ^{13}C NMR spectra of agarose in Me_2SO-d_6 (a) and D_2O (b) at 80 °C. G and A refer to carbons in D-galactose and 3,6-anhydro-L-galactose, whereas G′ and A″ refer to carbons in 6-*O*-methyl-D-galactose and 3,6-anhydro-2-*O*-methyl-L-galactose, respectively.

Table 4
^{13}C NMR data (δ in ppm) for agarose in Me_2SO-d_6, 2:1 Me_2SO-d_6–D_2O and D_2O at 80 °C

	C-1	C-2	C-3	C-4	C-5	C-6
*D-galactose (**G**)*						
Me_2SO-d_6	101.98	69.61	80.81	67.66	75.04	60.38
Me_2SO-d_6–D_2O	102.00	69.57	81.35	67.92	74.82	60.60
D_2O	101.16	68.98	80.96	67.49	74.09	60.16
*3,6-anhydro-L-galactose (**A**)*						
Me_2SO-d_6	97.07	69.88	79.73	75.97	74.68	68.37
Me_2SO-d_6–D_2O	97.55	69.57	79.70	76.43	74.82	68.60
D_2O	97.00	68.63	78.86	76.09	74.39	68.16

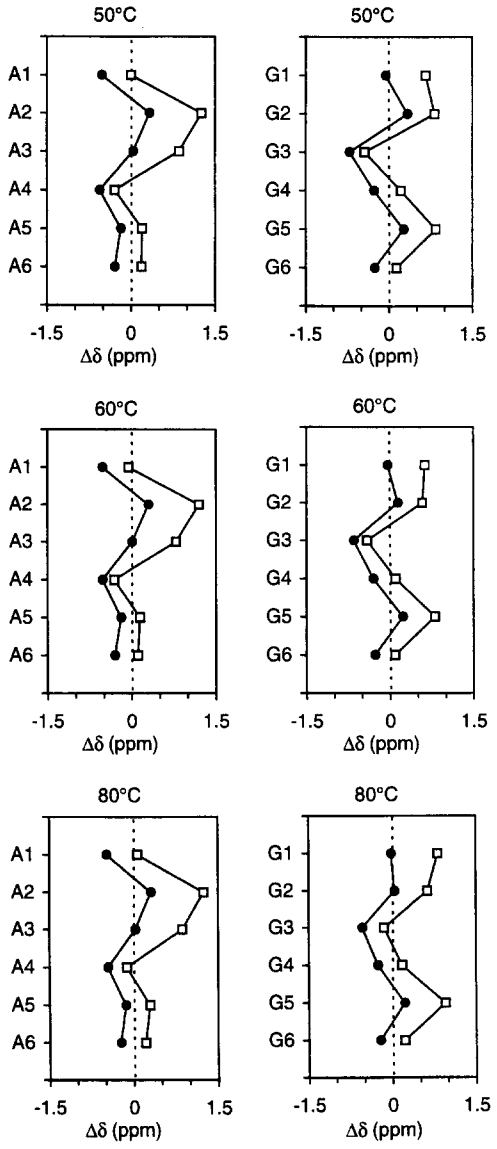

Fig. 8. Effect of solvent, Me_2SO-d_6 versus Me_2SO-d_6–D_2O (●) and Me_2SO-d_6 versus D_2O (□) on the ^{13}C chemical shifts for **A** and **G** agarose residues at 50 °C, 60 °C, and 80 °C.

chain ordering (i.e. stiffening), a prerequisite for the chain association required by the gel formation observed in agarose–water systems. For stiff (1 → 3)-β-D-glucans, like schizophyllans, lentinans and curdlans, a pronounced downfield shift of the glycosidic carbons has been observed in concomitance with a tertiary structure transition, leading to a gel network formation, and occurring when varying either the polymer chain length or the pH of the medium [38,39]. The lower extent of the glycosidic carbons downfield shift observed in agarose solutions might account for a relatively small conformational rearrangement of the chain segments occurring in D_2O with respect to the already rather rigid chain conformation existing in Me_2SO-d_6 [10,34]. It is interesting to notice that the solvent change practically does not affect the chemical shift of the **G** C-4 resonance. The interresidue hydrogen bond of **G** HO-4 (to O-5 of the anhydro-residue) proposed in Me_2SO-d_6 is likely maintained also in D_2O with a probable additional involvement of **G** HO-6. The smallest chemical shift variation of the **A** C-2 resonance observed going from Me_2SO-d_6 to the mixed solvent, in comparison with the highest chemical shift difference showed, among all the carbons, when changing from Me_2SO-d_6 to D_2O should further hint its involvement in the agarose–Me_2SO complex. The possible involvement of **A** HO-2 in the agarose–Me_2SO complex, as well as that of **G** HO-6 in a hydrogen bond in water may find support in the relative intensity variation of the corresponding carbon signals and in the deuterium isotope effect on the ^{13}C chemical shifts [40,41]. Although no quantitative significance can be given to the intensity of the signal due to the use of line broadening, variation of the signal amplitude with respect to the intensity of the peak that shows the smallest solvent dependence, can provide useful information about the relative carbon atoms mobility. Using the signal intensity of **G** C-1 as internal reference, ^{13}C NMR spectra (Fig. 7) show that the solvent change from Me_2SO-d_6 to D_2O results in an increase of the signal intensity of **A** C-2 as high as the concomitant peak amplitude decrease of the **G** C-6 resonance. In D_2O, the peak intensities are not further influenced by decreasing the temperature from 80 to 50 °C, thus excluding the influence of strong inter-chain interactions on the observed amplitude variations. If the change in the peak amplitude is tentatively attributed to a change in T_2 value, the above mentioned results should indicate an evident higher mobility of the **A** C-2 atom in D_2O than in Me_2SO-d_6 with a corresponding loss of mobility of the **G** C-6 atom in D_2O.

The persistence in water of the interresidue hydrogen bond involving the **G** HO-4 group, and likely in this solvent also the **G** HO-6 group, seems to be confirmed by the relatively high isotope effect (42 ppb) showed by the **G** C-4 and **G** C-6 atoms, as reported in Fig. 9. Short-range deuterium isotope shifts of carbon resonances are in fact more pronounced for carbons which bear *N*-acetamido or hydroxyl groups involved in stable intermolecular hydrogen bonds [42]. The well-balanced rearrangement, probably of conformational origin, of the **G** carbon atoms could result from the occurrence of an ordered,

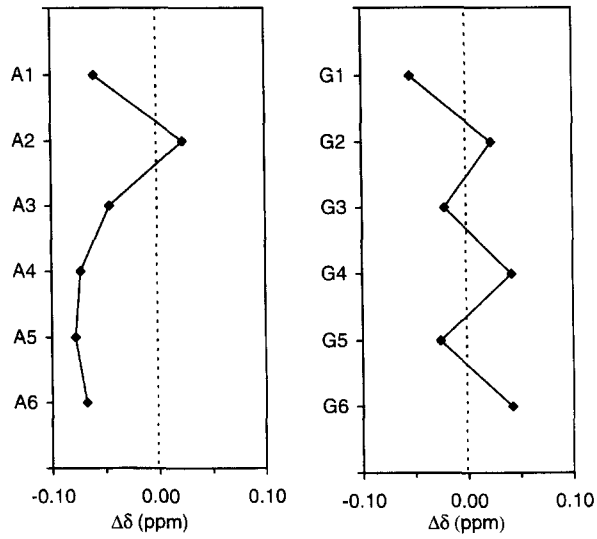

Fig. 9. Effect of solvent, water versus D_2O, on the ^{13}C chemical shifts for **A** and **G** agarose residues at 80 °C.

helical-type, chain structure. In addition to the proposed higher mobility in D_2O discussed above, the negligible isotope effect observed for **A** C-2 could indicate that, although free from the agarose–Me_2SO complex, the corresponding OH group in water is not available for strong interactions. More tentatively, the downfield shift of the resonances corresponding to the carbons involved in the rigid 3,6-anhydro group might suggest a better permeability to H_2O molecules as a consequence of more favourable chain–solvent interactions.

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