^{13}C NMR Spectra at 67.9 MHz of Aqueous Agarose Solutions and Gels

FLEMMING M. NICOLAISEN,[a] INGE MEYLAND[b,*] and KJELD SCHAUMBURG[a]

[a] University of Copenhagen, Chemical Laboratory V, The H. C. Ørsted Institute, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark and [b] LITEX, P.O. Box 7, DK-2600 Glostrup, Denmark

The spectra of a 5 % aqueous solution of agarose, in the liquid and gel state, at 35, 45, 60, 75 and 90 °C, are reported. The hysteresis in the gel formation and gel destruction appears clearly from measurements of peak heights relative to the line from methanol used as internal standard. A method for estimation of the number of freely moveable agarose residues is proposed.

In a previous paper[1] we have reported the ^{13}C NMR spectra of aqueous solutions of agarose recorded at 95 °C. At this temperature the ^{13}C NMR spectrum of agarose shows twelve sharp lines according to the repeat unit in agarose shown in Fig. 1. Because it is necessary to heat the agarose–water mixture to nearly 100 °C in order to obtain a homogeneous solution, the aqueous solution of agarose is created at high temperature. When cooling the solution, the viscosity increases and in the region 45 – 30 °C, depending on the concentration and type of agarose used, a gel is formed. The formation of the gel occurs in a narrow temperature interval.[2] When subsequently the temperature is increased the gel is not destroyed before above 80 °C, where the original solution is reestablished. This phenomenon is known as hysteresis.[2,3] Below we report the influence of the temperature and gel formation on the ^{13}C NMR spectra of agarose, and it is demonstrated how the hysteresis phenomenon can be visualized from the spectra. Lastly the mechanism of the gel formation is discussed in relation to the reported data.

* Present address: National Food Institute, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
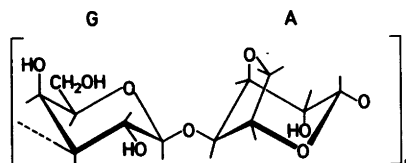
Fig. 1. The repeated unit in agarose. G: 3-linked β-D-galactopyranose and A: 4-linked 3,6-anhydro-α-L-galactopyranose.

RESULTS

Standard samples. In Fig. 2 it is shown how the ^{13}C NMR spectrum of a 5 % aqueous solution of agarose is influenced by the temperature. The chemical shift region shown is from 110 to 40 ppm relative to TMS, and the first line to the right in each spectrum is due to methanol added as internal standard ($\delta = 49.3$ ppm). The sample used to obtain the spectra shown is a partly 6-O-methylated agarose (see Experimental). In a previous paper on agarose[1] the individual signals in the ^{13}C NMR spectrum were assigned to the carbons in the repeat unit shown in Fig. 1.

The spectra a – h in Fig. 2 were recorded using the same sample and the total time necessary in providing the spectra was ca. 40 h. Before recording the first spectrum (a) the sample was stored at room temperature for several weeks, and it clearly showed syneresis.[2,3] The spectrum (a) was recorded during one night which corresponds to ca. 100 000 scans. The spectra b – g were recorded during the following day, and the last spectrum (h) during the next following night. In Fig. 2 the spectra a, b, c and h
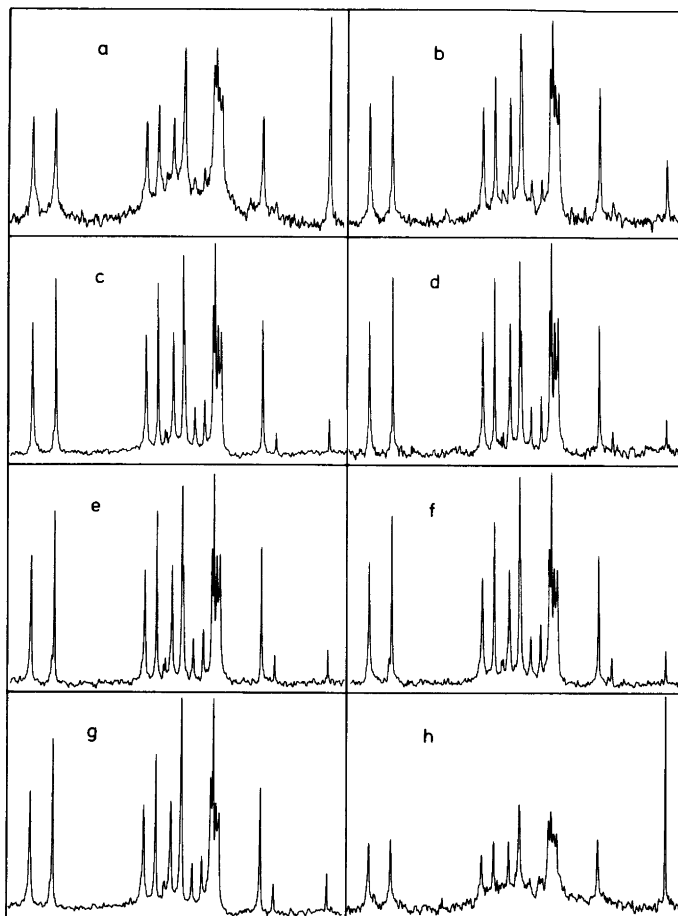
Fig. 2. ^{13}C NMR spectra of partly 6-*O*-methylated agarose in water (5 %) in the region 40−110 ppm. The line to the right in each spectrum is due to methanol used as internal standard. The sample used is the same for all spectra, the temperature and state being: (a) 45 °C, gel, (b) 60 °C, gel, (c) 75 °C, gel, (d) 90 °C, liquid, (e) 75 °C, liquid, (f) 60 °C, liquid, (g) 45 °C, liquid and (h) 35 °C, gel. For assignment of the individual lines see Ref. 1.

correspond to the gel state of the sample and the spectra d, e, f and g to the liquid (or sol) state. In order to increase the signal to noise ratio all the lines are artificially broadened with a line width of 8 Hz (see Experimental).

The most prominent feature appearing in the spectra shown is the large variation in the peak height of the methanol line relative to the other signals. This can be shown graphically in a comprehensive way, by plotting the peak height for some of the agarose signals relative to the methanol signal.

In Fig. 3 this is shown for the signals corre-

sponding to the anomeric carbon atoms in the A-residue (A1, 97.9 ppm) and in the G-residue (G1, 102.2 ppm), respectively. The relative peak height is scaled so that the value at 90 °C equals 100. The values shown in Fig. 3 correspond to the spectra shown in Fig. 2, and the arrows indicate the experimental sequence. Similar experiments with pure agarose (5 %) and/or under slightly changed experimental conditions (see Experimental) show different absolute values, but the plot of the scaled relative peak heights always appears similar to the curve shown in Fig. 3.

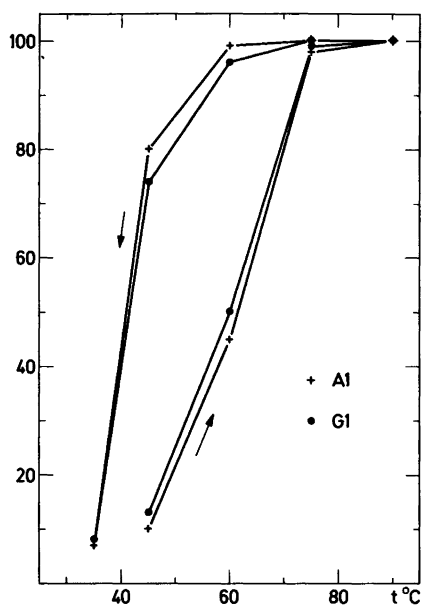The chemical shift values are relatively inde-

Fig. 3. Plot of the peak height relative to the methanol line for the anomeric carbon signals in the A-residue (A1) and the G-residue (G1), respectively, as function of the temperature (t °C). The values are scaled to be 100 at 90 °C.

pendent of temperature. In the temperature range 45–95 °C the signals for all the agarose carbons move less than 0.5 ppm relative to the methanol signal. However the relative chemical shifts for the agarose carbon atoms are unchanged within experimental error (0.1 ppm) except for the A1-carbon. The signal for this atom moves, relative to the other agarose signals, 0.25 ppm towards higher field. An important fact is that the chemical shifts for the peak maxima are independent of whether the sample is in the gel or the sol state at the actual temperature. It also appears that the signals corresponding to the 6-O-methylated A-residue,[1] behave similarly to the other signals.

Ageing effects are clearly shown in the spectra. The relative peak height defined above is significantly smaller for an old gel than for a freshly made gel. However a clearcut picture is very hard to establish because it takes hours to record one spectrum. Ageing effects have also been observed in proton relaxation measurements of agarose.[4]

Sample with ^{13}C enriched methanol. Before a discussion of the results above, it is valuable to

know how much of the change in relative peak heights may be caused by changes in the absolute peak height of the methanol line during the experimental sequence. For this purpose a series of experiments was carried out on a sample containing ^{13}C enriched methanol. By using a 90 % enriched methanol a factor of *ca.* 100 is gained in sensitivity. However, absolute intensity measurements under the actual experimental conditions are still extremely difficult. The experiments therefore yielded tendencies rather than exact information. Based on several series of experiments (see Experimental) some conclusions can be made:

(a) The line width (for methanol) shows small variations, but is less than 2.5 Hz in the region 30–95 °C (gel or sol). The artificial line width of 8 Hz used for the spectra in Fig. 2 and thereby for the graph in Fig. 3 then eliminates effects of changes in the methanol line width during the experimental sequence.

(b) A monotonous decrease in the absolute intensity of 25–50 % is observed over the temperature region 30 to 95 °C. For a given temperature the decrease is nearly independent of the state (gel or sol). About 20 % of this effect is caused by a change in the Boltzmann distribution. The rest may be caused by a temperature sensitivity of the tuning of the probe. However, changes in the T_1 relaxation time for methanol cannot be excluded.

The above conclusions (a) and (b) are valid both for conditions where the methanol signal is strongly saturated and fully relaxed.

DISCUSSION AND CONCLUSION

The curves in Fig. 3 show clearly the hysteresis phenomenon. The formation of polysaccharide gels is supposed to originate from associations of the polymer chains in certain areas called junction zones.[2] For agarose the formation of double helices is proposed to play an important role in the formation of the junction zones.[2,3,5] It is further proposed [3] that double helices can associate to form aggregates to act as "super junctions", causing high concentration agarose gels to be opaque.

In the junction zones the mobility of the agarose units is severely restricted, causing the NMR signals to be very broad.[4,6] The sharp signals observed in Fig. 2 then originate from those agarose units which are able to move "freely". In this connection a freely moving residue is roughly attributed a correlation time less than 10^{-9} s.

Postulating that all the agarose units in the sample used for the spectra in Fig. 2 are moving freely at 90 °C, *the ordinate in Fig. 3 represents the percentage of freely moving agarose units.*

Prior to a further discussion based on the interpretation of Fig. 3 given above some remarks are needed. The ratios given in this figure are sensitive to many experimental parameters, and further detailed examinations are needed before the method can be used rigorously. One important question is to what extent the absolute height of the methanol signal depends on the experimental conditions used. Based on the above-mentioned experiments using ^{13}C enriched methanol, it is reasonable to conclude that the data shown in Fig. 3 for each temperature yield a good estimate of the *ratio* between the number of freely moving agarose residues in the sol and in the gel state, respectively. The absolute values must be taken with due reservation.

From this point of view it can be concluded that at 45 °C, near the gel setting point, there is a large change in mobility of the agarose residues from sol to gel. Whereas, at 75 °C, near the "melting" point, there is only a minor deviation in mobility in the sol and the gel. This means that at high temperature only a small part of the agarose residues takes part in the junction zones responsible for the gel state.

Since it is known that a 0.1 % aqueous solution of agarose can form a gel,[2] it may not be surprising that only a minor part of the agarose residues need to be involved in the gel formation process in a 5 % solution. Gels at low concentration may, however, differ in behaviour from the 5 % gels described above. Because of the excessive demand of instrument time a ^{13}C NMR investigation of low concentration gels is at this moment outside our possibilities.

The fact that the chemical shifts for the peak maxima remain nearly unchanged throughout the hysteresis loop, and that only the line widths are influenced by the formation of the junction zones and/or the double helices, is parallel to what is observed for i-carragenan.[8] Bryce *et al.* concluded that this is in accordance with the "two-state-all-or-none" model for the double helix formation proposed by Reid *et al.*[9] If this static picture is adapted to agarose the percentage values given in Fig. 3 may represent the degree of double helix formation of agarose throughout the hysteresis loop.

Within this model our data indicate that the amount of double helices in the liquid state at 45 °C is higher than at 75 °C in the gel state! This supports the view that the formation of the gel state is due to aggregation of double helices rather than to formation of the double helices themselves. This is in agreement with conclusions made by Arnott *et al.*[5] from X-ray and optical rotation measurements on agarose/water gels and solutions.

EXPERIMENTAL

Sample preparation. Commercially available agarose (LITEX) was used in the experiments. A pure agarose (LSA) and a partly (*ca.* 25 %) 6-O-methylated agarose (HSB) were used. 5 % aqueous solutions were made directly in the 10 mm NMR tubes as described in Ref. 1.

Two samples of pure agarose (LSA) were made in vacuum sealed 10 mm tubes. The samples were prepared identically except that ^{13}C enriched methanol (90 %, I. C. CHEMIKALIEN) was used in one of them.

Instrumental. The spectra were recorded on a Bruker HX 270 spectrometer equipped with a Fourier transform unit and operating in quadrature detection mode at 67.9 MHz for ^{13}C NMR. For the spectra shown in Fig. 2, the proton decoupled spectra were accumulated in 16 K data points using a spectral width of 17 240 Hz. The repetition time was 0.5 s, and a 45° RF pulse of 9 μs was used. Under these conditions the methanol signal is strongly saturated. For the standard spectra the FID was multiplied by an exponential function corresponding to a line width of 8 Hz. Deuterium in the solvent was used for the field-frequency lock. The necessary number of scans varied from *ca.* 3000 for the high temperature spectra to 100 000 scans for the low temperature gel samples. The samples were allowed to equilibrate in the spectrometer for *ca.* 15 min. before the recording of each spectrum was initiated. Experiments with other values for the pulse width (6 and 12 μs) and repetition time (0.9 s) were also carried out.

The temperature control was calibrated by placing a thermocouple directly in the sample. The sample temperature only differed a few degrees from the nominal temperature setting due to heat produced in the sample by the decoupling field.

For the ^{13}C enriched sample it was only necessary to accumulate 10 scans. Spectra were recorded under conditions identical to those described above. In order to obtain the same saturation conditions 100 were recorded, but only the last 10 were used for the spectra.

Spectra of fully relaxed methanol were recorded using 1 min repetition time and with both 16 and 64 K data points.

REFERENCES

1. Nicolaisen, F. M., Schaumburg, K. and Meyland, I. *Acta Chem. Scand. B 34* (1980) 103.
2. Rees, D. A. *Adv. Carbohyd. Chem. Biochem. 24* (1969) 267.
3. Rees, D. A. *Chem. Ind. London* (1972) 630.
4. Child, T. F. and Pryce, N. G. *Biopolymers 11* (1972) 409.
5. Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C. M., Moorhouse, R. and Rees, D. A. *J. Mol. Biol. 90* (1974) 269.
6. Rees, D. A. and Welsh, E. J. *Angew. Chem. 89* (1977) 228.
7. Derbyshire, W. and Duff, I. D. *Faraday Discuss. Chem. Soc. 57* (1974) 243.
8. Bryce, T. A., McKinnon, A. A., Morris, E. R., Rees, D. A. and Thom, D. *Faraday Discuss. Chem. Soc. 57* (1974) 221.
9. Reid, D. S., Bryce, T. A., Clark, A. H. and Rees, D. A. *Faraday Discuss. Chem. Soc. 57* (1974) 230.

Received March 14, 1980.