

Hydration–dehydration-induced conformational changes of agarose, and kappa- and iota-carrageenans as studied by high-resolution solid-state ^{13}C -nuclear magnetic resonance spectroscopy

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ABSTRACT

High-resolution, solid-state ^{13}C -n.m.r. spectra of agarose, and kappa- and iota-carrageenans were recorded for various states, including anhydrous and hydrated powder, film, gel, and lyophilized gel. The ^{13}C -n.m.r. spectra varied substantially with the extent of hydration. Fully hydrated samples, as in the agarose gels, gave rise to ^{13}C -n.m.r. peaks of the narrowest line widths, whereas dehydration caused considerable line-broadening or displacement of the G1 peak (or both). These observations were interpreted in terms of hydration–dehydration-induced conformational alteration or readjustment, depending on the extent of hydration or dehydration and the variety of samples used. ^{13}C -N.m.r. spectrometry of agarose gel by solid-state n.m.r. technique also indicated the existence of a rather rigid network structure with an anisotropic molecular tumbling.

INTRODUCTION

Gelation is one of the most important properties of several types of polysaccharides as it is related to their biological functions in cell walls of bacteria, fungi, and plants, and in animal fluids and connective tissues, as well as for its industrial and food applications. Gel-state polysaccharide also provides an ideal system for study of secondary structures, because stable, ordered conformations are considered to be always present either in the overall gel network or in cross-linked regions. To analyze such ordered conformations, various physical techniques¹, such as X-ray diffraction, optical rotation, ^{13}C -n.m.r. spectroscopy, etc., have been extensively used. Most importantly, X-ray diffraction studies have clarified the existence of double- or triple-helical portions, and their role as junction zones for the gelation mechanism of such typical gel-forming polysaccharides as agarose², kappa- and iota-carrageenans^{3,4} and curdlan⁵.

However, several investigators have questioned the validity of the aforementioned gelation mechanism which takes into account the double- or triple-helical chains alone^{2–5}. In curdlan gel, for instance, a considerable proportion of very flexible, single-helical chains^{6,7} were detected by means of n.m.r. signal displacements of carbon atoms involved in the glycosidic linkages, the torsion angles of which undergo modifications^{8–10}. Several investigators have analyzed their experimental data on agarose gels on

the basis of an alternative model of gel network containing extended, single helices rather than the original double-helix model¹¹⁻¹³. The existence, in gel samples, of single-molecular chains other than the double-helix chains, (the n.m.r. signals of which are suppressed) was evidenced by very sharp ¹³C-n.m.r. signals having peak intensities varying with hysteresis¹⁴. Further, a cation-specific, nested single-helix model, instead of the double helix model, has been proposed for the gelation mechanisms of carrageenans¹⁵⁻¹⁷. Distinguishing between multistranded, coaxial helices and nested, single helices is often one of the most difficult problems in interpreting fiber-diffraction data¹⁸.

We have recently demonstrated that a careful analysis of the hydration-induced spectral changes of ¹³C-n.m.r. spectra can be used as a convenient means to distinguish the single chain of (1→3)-β-D-glucan from the triple-helix chains¹⁹⁻²¹; the spectral change of the single chain is more sensitive to hydration than that of the triple-helix chain, reflecting a difference in relative conformational stability. In order to verify the potential usefulness of this novel approach for distinction between single- and double-helix chains as the major component, we examined the hydration-dehydration-induced conformational changes in agarose, and kappa- and iota-carrageenans by means of high-resolution, solid-state, ¹³C-n.m.r. spectroscopy.

EXPERIMENTAL

¹³C-N.m.r. spectroscopy. — 75.46-MHz, high-resolution, solid-state ¹³C-n.m.r. spectra were recorded with a Bruker CXP-300 spectrometer, equipped with an accessory for cross polarization—magic angle spinning (CP-MAS). A 1-Ω resistor was linked to the probe-matching circuit of a ¹H-n.m.r. channel in a CP-MAS probe (Z33DR-MAS-7DB) to reduce its quality factor. This modification was essential in order to be able to tune the ¹H-n.m.r. irradiation channel for most of the hydrate or gel samples. Powder, films, gel, or lyophilized-gel samples were placed in an aluminum cylindrical rotor and spun as fast as 3 kHz. The 90° pulse, contact time, and repetition times were 3.5 μs, 1 ms, and 4 s, respectively. The chemical shifts were calibrated indirectly through the ¹³C-n.m.r. chemical shift of the glycine carboxyl peak (δ 176.03) with respect to the signal of tetramethylsilane. The spectra were usually accumulated 700–1200 times. Conventional, high-resolution, ¹³C-n.m.r. spectra were recorded for an aqueous solution at temperatures above the sol-gel transition (70–80°).

Materials. — Three anhydrous-powder samples of agarose were obtained from commercial sources and used without further purification. Sigma Type VII (low-gelling temperature, 30°) and Type V (high-gelling temperature, 42°) samples were purchased from Sigma Chem. Co. St. Louis, MD 63118 (USA). Sea Kem agarose (high-gelling temperature) was provided by FMC BioProducts, Rockland, ME 04841 (USA), and iota- and kappa-carrageenans were purchased from Sigma and used without further purification.

Hydrated samples of agarose, and kappa- and iota-carrageenan were prepared by placing dishes containing the anhydrous powder in a desiccator (over 8 h) in which the relative humidity (96%) was maintained by an appropriate mixture of water and H₂SO₄. The gel samples were prepared by heating a 10% aqueous suspension of the powder

samples to a temperature $>80^{\circ}$, followed by cooling at ambient temperature. The lyophilized samples were obtained by placing the gel samples *in vacuo* overnight.

Dried agarose films were prepared by evaporating either an aqueous solution or an *N,N*-dimethylacetamide solution on a Teflon surface at $\sim 100^{\circ}$ in an oven¹³. The film samples thus obtained were cut into small pieces for n.m.r. measurements. The anhydrous films from the *N,N*-dimethylacetamide solution were also hydrated in a desiccator. Alternatively, the films from an aqueous solution were dried *in vacuo* for over 16 h.

RESULTS

The assignment of the peaks of the ^{13}C -n.m.r. spectra of low-gelling Sigma Type VII and high-gelling Type V agarose (Figs. 1 and 2, respectively) taken on powder, hydrate, gel, and lyophilized states, was based on the previous ^{13}C -n.m.r. studies of agarose and its oligomers in solution and solid state²²⁻²⁶. A small peak at δ 81.9 (close to the G3 peak for the aqueous solution but with an intensity of 50% of the peak-intensity

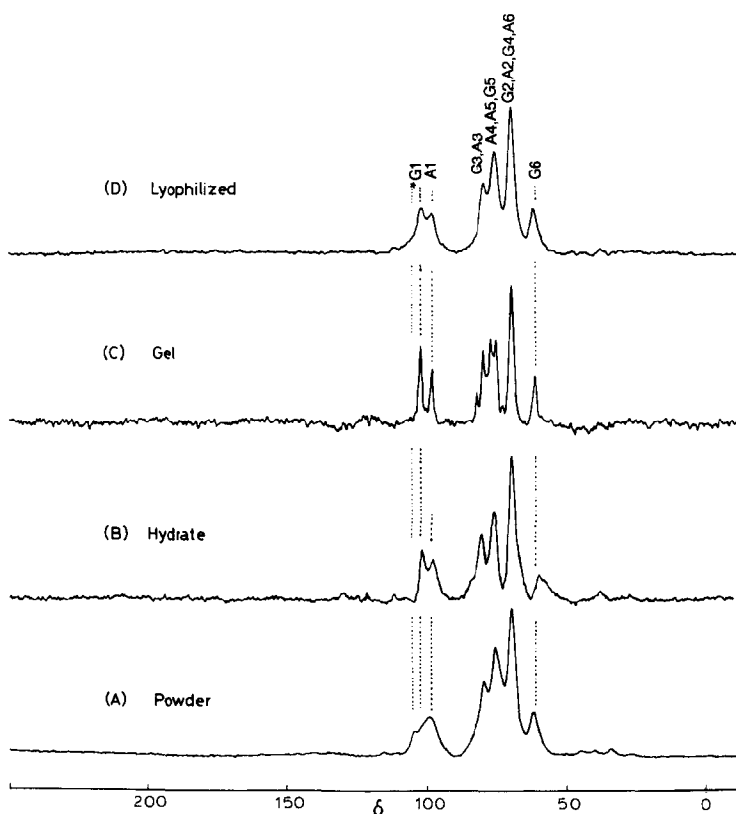


Fig. 1. 75.46-MHz, ^{13}C -n.m.r. spectra of agarose Type VII sample: (A) anhydrous powder, (B) hydrate sample of anhydrous powder, (C) gel, and (D) lyophilized sample from gel. The symbols G and A depict (1 \rightarrow 3)-linked β -D-galactosyl and (1 \rightarrow 4)-linked 3,6-anhydro- α -L-galactosyl residues, respectively.

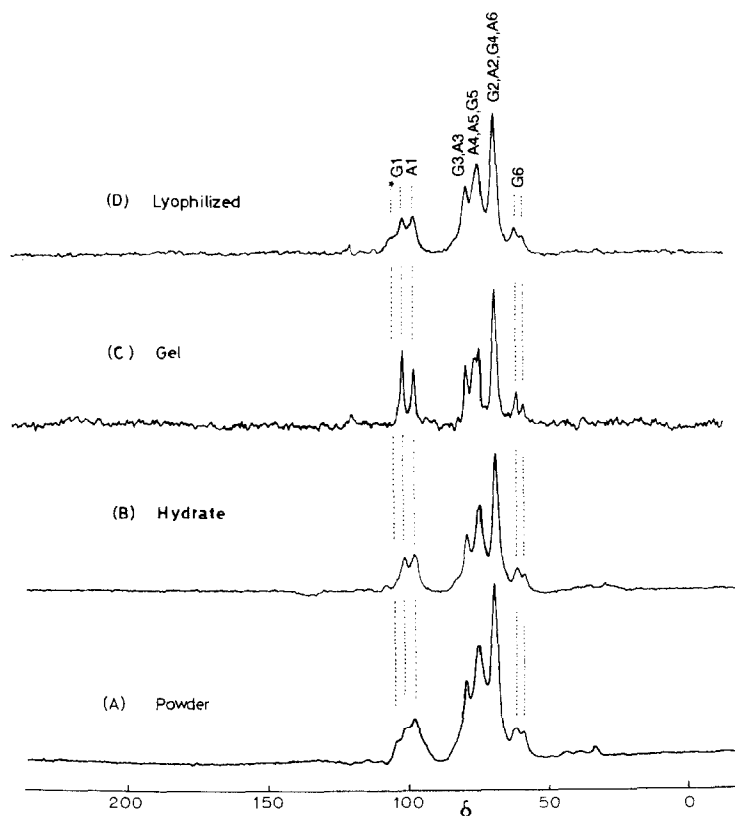


Fig. 2. 75.46-MHz, ^{13}C -n.m.r. spectra of agarose Type V sample: (A) anhydrous powder, (B) hydrated sample of (A), (C) gel, and (D) lyophilized sample from gel. For symbols G and A, see legend to Fig. 1.

of one C-atom) was well resolved for the gel of Type VII but not for that of Type V. Therefore, the peak assignment could not be confirmed. The most narrow resonance peaks were obtained for the gel samples, as compared with the peak profile for the dehydrated sample, although the overall spectral features for the four kinds of samples were not changed. It is noteworthy, however, that the low-field shoulder indicated by an asterisk at δ 104.1 or 105.4 is clearly visible in the G1 region of the spectra of the dehydrated agarose Type V samples. At the same time, the high-field shoulder peak is present in the G6 region (δ 58.7–61.0), irrespective of the anhydrous, gel or lyophilized state. N.m.r. patterns similar to those of Fig. 2 were obtained for the Sea Kem agarose sample (spectra not shown). The primary structure of these two types of samples differs slightly in the proportion of minor components (<15%) other than the major β -D-galactosyl and 3,6-anhydro- α -L-galactosyl residues (spectra not shown).

Spectral changes associated with hydration and dehydration were observed for agarose films Figs. 3 and 4. The G1 and A1 regions were substantially broadened in spectra for dried films obtained from an *N,N*-dimethylamide solution, and resulted in a broad envelope (marked with an asterisk at δ 105, Fig. 3) for both Type VII and Type V samples. Nevertheless, hydration caused a substantial narrowing of the peaks (Figs. 3B

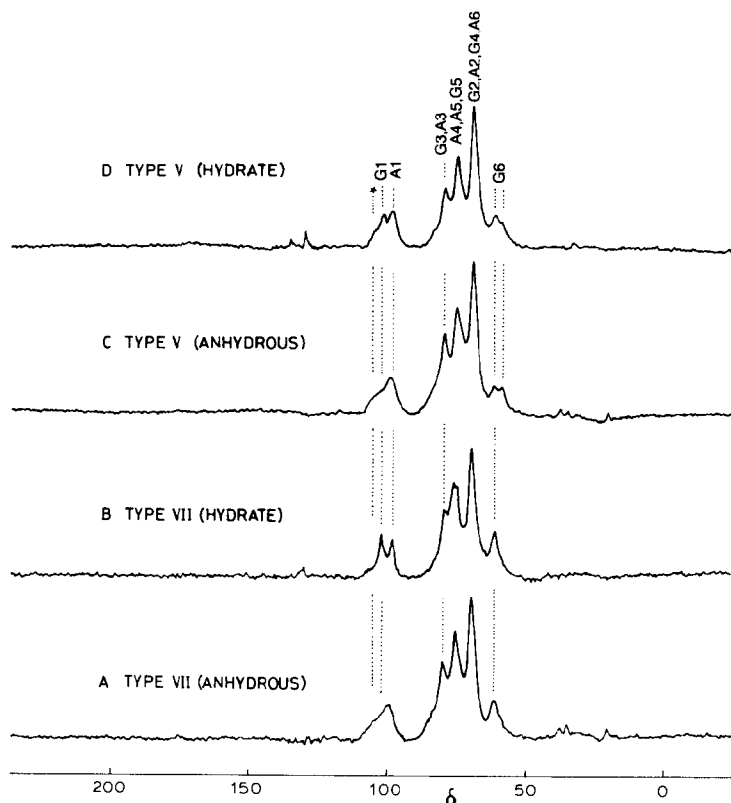


Fig. 3. 75.46-MHz, ^{13}C -n.m.r. spectra of anhydrous films prepared from an *N,N*-dimethylacetamide solution of Type VII (A) and Type V (C) samples; (B) and (D) hydrated samples from A and C, respectively. For symbols G and A, see legend to Fig. 1.

and 3D) as observed for powdered samples. The resolution in the G1–A1 region of spectra for films obtained by drying an aqueous solution is better than that of spectra of films prepared by drying an *N,N*-dimethylacetamide solution (Figs. 3 and 4). Further drying of the films obtained from an aqueous solution resulted in substantial line-broadening (Fig. 4).

The peak assignment for the ^{13}C -n.m.r. spectra of kappa- and iota-carrageenans in anhydrous and hydrate-powder form (Figs. 5 and 6, respectively) was based on the previous ^{13}C -n.m.r. data^{26–30} for a solution or a solid state, with some modification taken into account for the relative peak-intensities. The ^{13}C -n.m.r. spectral features of dried samples were very similar to those reported by Rochas and Lahaye²⁶. In parallel with our observations on agarose samples mentioned above, hydration of carrageenans caused a substantial narrowing of the peaks. A shoulder peak, designated as A3, was well resolved from other peaks in the spectra of iota-carrageenan as a result of hydration. This assignment is based on the previous data¹⁶ of ordered state in solution, taking into account the relative peak-intensity (one carbon atom) of the present data. It turned out, however, that probe tuning of the proton channel was extremely difficult for samples of high ionic strength such as carrageenan gels, in spite of our modification of

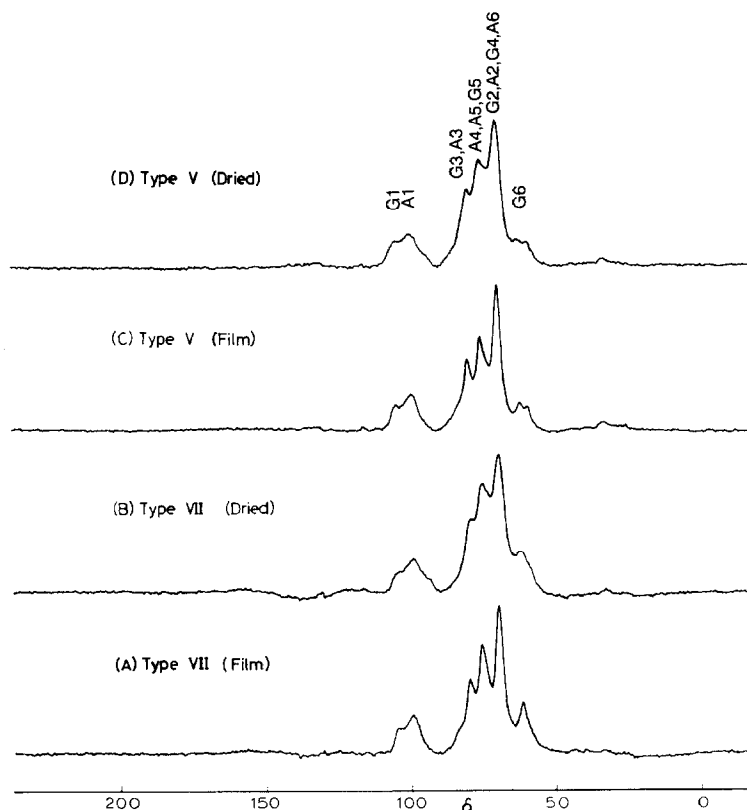


Fig. 4. 75.46-MHz, ^{13}C -n.m.r. spectra of dried films of Type VII (A) and Type V (C) agarose, evaporated from an aqueous solution: (B) and (D) films of samples A and C, respectively, further dried *in vacuo*. For symbols G and A, see legend to Fig. 1.

the n.m.r. probe, as described in the Experimental section. As a result, any attempt to obtain ^{13}C -n.m.r. spectra of carrageenan gels by a CP-MAS technique were unsuccessful. The ^{13}C -chemical shifts of agarose and carrageenans are summarized in Tables I and II.

DISCUSSION

High-resolution, solid-state ^{13}C -n.m.r. signals with the narrowest line widths were visible in spectra for fully hydrated systems, such as the agarose gel, when compared with the spectra of anhydrous powder or films (Fig. 1–4). In addition, either anhydrous powders or films gave rise to spectra analogous to those of the gel samples, in spite of the absence of drastic conformational change as in solution–gel transition. In contrast the quality of the spectral pattern deteriorated with the loss of water from gel or film (Figs. 1, 2, and 4). This observation parallels our previous data on single-helical (1→3)- β -D-glucans²¹ and form I of fibroin³¹, in which water molecules play an important role in maintaining their respective conformations. Hydration-induced spectral change, however, was minimal for more stable conformations, such as the triple-helix conformation

TABLE I

¹³C-chemical shifts (δ)^a for agarose in powder, hydrate, lyophilized, and gel state, and aqueous solution

Agarose	State	Signal					
		G1	A1	G3, A3	A4, A5, G5	G2, A2, G4, G6 A6	
Type VII	Powder	103.6	98.4	79.4	75.5	69.6	61.7
	Hydrate	101.4	97.6	80.2	75.5	69.7	61.0
	Gel	101.9	98.0	79.4 ^b	76.7, 74.9	89.1	61.0
	Lyophilized	101.4	97.8	79.2	75.4	69.5	61.8
Type V	Powder	104.1, 101.4	97.8	79.8	75.4	69.7	62.1, 58.7
	Hydrate	101.4	97.5	80.3	75.8	69.5	61.1
	Gel	101.9	97.9	79.3	74.9	69.7	61.2, 58.8
	Lyophilized	105.4, 102.0	97.9	79.2	75.0	69.5	61.9, 59.3
SEA KEM	Lyophilized	101.4	97.2	79.6	75.8	69.8	62.3
Type VII	Aqueous solution ^c	102.3	98.2	82.1, 80.0, 77.2	75.5	70.2–68.8	61.4

^aFrom the signal for tetramethylsilane. ^bShoulder peak at δ 81.9 p.p.m. ^cRecorded for a solution at 80°.

of (1→3)- β -D-glucans²¹ and form II (β -sheet) of silk fibroin³¹. Therefore, the existence of double helices alone cannot explain such pronounced hydration–dehydration-induced spectral changes.

Dried films, obtained from a *N,N*-dimethylamide solution, were prepared under anhydrous conditions with the expectation of obtaining an increased proportion of single chains. Foord and Atkins¹³ have shown that dried films, obtained from an aqueous or *N,N*-dimethylacetamide solution, contain both double and single chains; the stretching <42° expresses the double helices in a network, whereas the single chains

TABLE II

¹³C-chemical shifts (δ)^a of kappa- and iota-carrageenan in powder, hydrate, and lyophilized state, and aqueous solution

Signal	Carrageenan						
	Kappa				Iota		
	Powder	Lyophil.	Hydrate	Aq. soln.	Powder	Hydr.	Aq. soln.
G1	104.3	104.8	105.5	102.5	105.0	105.2	102.0
A1	94.6	94.1	95.1	95.2	91.2	91.3	92.2
A3, G3, A5, } G5, A4, G4 } A3	79.2, 76.7	79.3, 76.7	80.1, 77.0	79.2		78.7	78.3
A4, G3, A5, A2, G4					77.6, 74.8	77.5, 74.5	73.3
A2, G2, A6	69.0	69.0	69.3	74.1, 70.0			
G2, A6				69.7, 64.5	69.4	69.2	69.5
G6	61.7	61.7	61.3	61.4	62.5	62.1	61.5

^aFrom the signal of tetramethylsilane. ^bFor a solution at 80°.

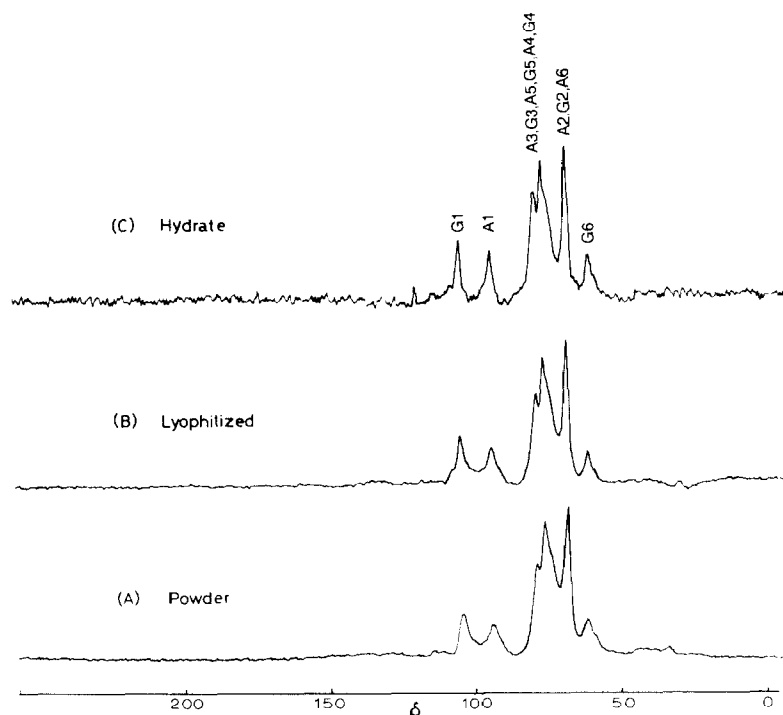


Fig. 5. 75.46-MHz, ^{13}C -n.m.r. spectra of kappa-carrageenan in the solid state: (A) Anhydrous powder, (B) lyophilized sample from gel, and (C) hydrated sample of anhydrous powder. The symbols G and A depict (1 \rightarrow 3)-linked β -D-galactosyl and (1 \rightarrow 4)-linked 3,6-anhydro- α -L-galactosyl residues, respectively.

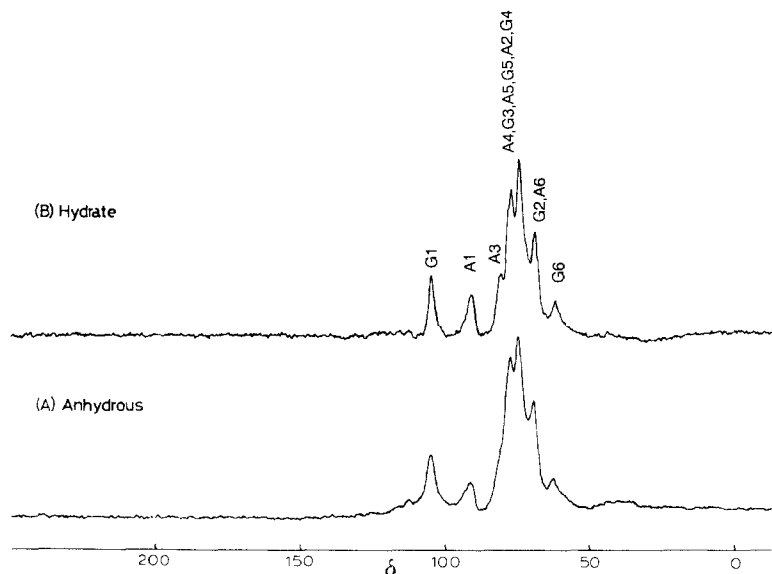


Fig. 6. 75.46-MHz, ^{13}C -n.m.r. spectra of iota-carrageenan in the solid state: (A) Anhydrous powder and (B) hydrated sample of anhydrous powder. The symbols G and A depict (1 \rightarrow 3)-linked β -D-galactosyl 4-sulfate and (1 \rightarrow 4)-linked 3,6-anhydro- α -D-galactosyl 2-sulfate residues, respectively.

are expressed in crystal structures $> 53^\circ$. A previous study by X-ray diffraction had shown that the agarose double-helix is associated with a cavity of 0.45-nm diameter, this cavity is occupied by water molecules that participate in a hydrogen-bonding system which contributes to the stability of the double helix². Thus, it was expected that this type of stable structure could be maintained, even if water molecules were removed. On the contrary, a single-helix form of (1 \rightarrow 3)- β -D-glucan, stabilized by hydration, was readily converted into a single-chain form as a result of the loss of water molecules^{20,21}. Accordingly, the very broad envelope observed in the G1–A1 region of the spectra of dried films could be ascribed to the existence of a disordered form of single chains, and this disordered form is converted into a regularly ordered form, such as a single helix, by hydration. The proportion of single helix is substantial in the gel sample, especially in the Type V sample. In the hydrated state (including gels), however, it seems to be rather difficult to distinguish the single helix from the double helix, because only one type of conformation could be identified in the ^{13}C -n.m.r. spectra of a gel state (Figs. 1 and 2). Therefore, it should be emphasized that a single-helix conformation can be more easily distinguished as disordered, single chains after dehydration (Figs. 2A, 2D, 3B, and 3D).

The presence or absence of the splitting of the G6 signal into a doublet pattern was a clear indication of the presence of two types of agarose, (Type VII and V), although no such splitting was visible in the spectrum for a solution. It is interesting to note that these splittings are always present for all kinds of Type V preparations, powder, films, and gel. In principle, this type of splitting could be ascribed to the two states of exocyclic conformation of hydroxymethyl groups, as shown for cyclodextrins¹⁰ and cellulose³². Agarose samples of higher- (Type V) and lower-gelling (Type VII) temperatures contain higher and lower methoxyl group content, respectively³³. The relative peak-intensity of the methoxyl signals in the ^{13}C -n.m.r. spectra for Type V solid state at δ 59.1 and 57.1 were 15 and 10% of the G6 intensity, respectively. The spectra of the Type VII sample showed virtually no signals in this region (spectra not shown). In view of these relative peak-intensities, the presence of the G1-peak splitting cannot be explained by the presence of methoxyl groups ($< 15\%$).

Agarose and carrageenans adopt a random coil form in the sol state¹. Thus, the ^{13}C -n.m.r. chemical shifts of the D-galactose 4-sulfate residues (G1) in kappa- and iota-carrageenans in aqueous solution at elevated temperatures are displaced upfield by 2–3 p.p.m., as compared with those of the solid state, which is consistent with recent solid-state n.m.r. data²⁶. The observation of such a significant displacement of ^{13}C -n.m.r. resonances between spectra for a solid (gel) and an aqueous solution is a direct evidence for the existence of a major conformational change, which is reflected in the G1 chemical shifts of the D-galactose 4-sulfate residues. The G1 shift observed in the spectrum for an aqueous solution is considered to be a time-averaged value of all allowed conformers, whereas the corresponding shift in the spectrum for the solid state results from certain preferred conformations^{8–10}.

Nevertheless, no substantial displacement of the signals was seen between the spectra for sol and gel or solid state (< 0.3 p.p.m. for G1 and others) of agarose samples. This does not necessarily mean that the same single helical or double-helical form, as seen in the gel or hydrate samples, is retained even in sol state. Instead, it is more likely

that rapid conformational fluctuation around the glycosidic torsion angle corresponding to the single- or double-helical form results in accidental overlaps of peaks between spectra of gel and sol.

In conclusion, substantial hydration–dehydration-induced spectral changes were observed for several preparations of agarose and carrageenans. Such change was more pronounced when single chains were present in the powder, films, and gel samples. Thus, high-resolution, solid-state ^{13}C -n.m.r. spectroscopy may be an excellent tool for the examination of the conformational features of gel-forming polysaccharides.

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