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Note

FTIR studies of sodium hyaluronate and its oligomers in the amorphous solid phase and in aqueous solution

Roberto Gilli ^{a,*}, Marta Kacuráková ^{1,b}, Mohamed Mathlouthi ^b,
Luciano Navarini ^a, Sergio Paoletti ^{a,c}

^a *Centro Ricerche POLY-bios, LBT-AREA di Ricerca, Padriciano 99, I-34012 Trieste, Italy*

^b *Laboratoire de Chimie Physique Industrielle, Faculté des Sciences, Université de Reims Champagne-Ardenne, B.P.347, F-51062 Reims, France*

^c *Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Via L. Giorgeri 1, I-34127 Trieste, Italy*

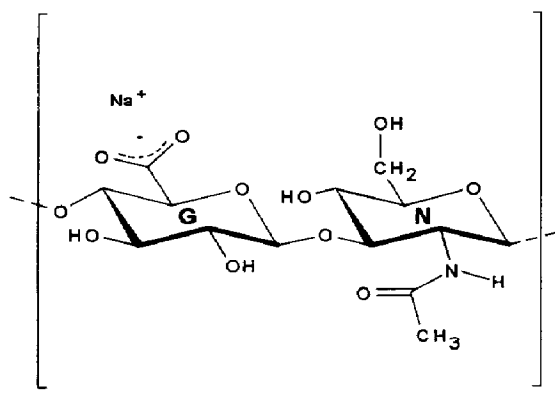
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Hyaluronan (hyaluronic acid, hyaluronate) is a naturally occurring linear glycosaminoglycuronan consisting of a repetitive disaccharide β -(1 \rightarrow 3)-linked 2-acetamido-2-deoxy-D-glucopyranosyl- β -(1 \rightarrow 4)-D-glucopyranosyluronic acid sequence (Scheme 1). It mainly occurs in connective and skeletal tissue and periodontal ligaments of vertebrates. The moderate chain stiffness and the disordered elongated conformation adopted by hyaluronan in aqueous systems [1] account for the peculiar viscoelastic properties that are relevant to its performance in biological fluids [2]. Hyaluronan plays an important role in many biological processes including tissue hydration, proteoglycan organization in the extracellular matrix, and tissue repair, and it has found application for several clinical purposes [3] and for cosmetic use. The presence of hyaluronan as the exocellular product of some pathogenic microbial strains is known. Such a peculiar feature for a polysaccharide belonging to the glycosaminoglycan family, which is one of the main components of the extracellular matrix of eucaryotic organisms, is thought to be a biomimetic strategy for the bacteria in order to prevent immunological recognition [4].

* Corresponding author.

¹ On leave of absence from the Institute of Chemistry, Slovak Academy of Science, CS-842 38 Bratislava, Slovakia.



Scheme 1. The disaccharide repeating unit of hyaluronate GN.

The technique of FTIR spectroscopy, including methods of sample presentation such as the attenuated total reflectance (ATR), has been successfully used to examine the structures or the conformational features of several polysaccharides both in the solid state and in aqueous solution [5–7]. However, little attention has been paid to the use of FTIR for the structural studies of hyaluronate. Conventional IR spectroscopy was used by Orr [8] in 1954 for partial assignments of bands of hyaluronic acid and hyaluronate. In 1976, polarized FTIR spectra of oriented films of sodium hyaluronate prepared in two different crystalline forms (extended and contracted four-fold helical conformations) were recorded by Cael et al. [9] and the dichroism data for the vibrational modes of the amide and carbonyl groups of the contracted four-fold helical conformation were interpreted in terms of molecular structure. A qualitative agreement between the observed dichroism data and the predictions based upon the sodium hyaluronate model [10,11] was found.

The assignments proposed by Cael et al. [9], although an obvious improvement with respect to those by Orr [8], are still partial and, as far as we are aware, they have not been reexamined so far. Moreover, vibrational spectra of hyaluronan in aqueous solution are rather scarce in the literature, particularly with respect to the effects of the degree of polymerization and hydration. These facts prompted us to undertake the present investigation.

1. Experimental

Materials.—High molecular weight hyaluronate (Na^+ salt form) samples from rooster comb (HA58, HA101, HA59, and HA5) and sodium hyaluronate oligosaccharides [(GN)₂, (GN)₃, (GN)₄, (GN)₆, and (GN)₇] were kindly provided by FAB, Fidia Advanced Biopolymer, S.r.l., Abano Terme, Italy. The oligosaccharides were obtained as described elsewhere [12]. The molecular weight of the different samples, determined by means of gel permeation chromatography (GPC) with a low angle laser light scattering (LALLS) detector as described elsewhere [1], is reported in Table 1. All samples were in the freeze-dried form.

Table 1
Label and molecular weight of the different samples (M_w for polymers)

Sample	Molecular weight
(GN)2	817
(GN)3	1216
(GN)4	1615
(GN)6	2414
(GN)7	2813
HA58	40 000
HA101	160 000
HA59	240 000
HA5	750 000

Methods.—FTIR spectra were recorded with a Nicolet 10-DX single-beam spectrometer interfaced with a Nicolet 10-DX data processor. An average of 200 scans were recorded with a 2 cm^{-1} resolution. Additional FTIR spectra were recorded with a BOMEM MB-100 spectrometer (300 scans at 4 cm^{-1} resolution) and with a Perkin–Elmer 1750 spectrometer interfaced with a Perkin–Elmer 7300 data processor (128 scans at 2 cm^{-1} resolution). Pellets were prepared using 200 mg of IR spectroscopic grade KBr and 2 mg of freeze-dried samples previously maintained for 24 h in a dessiccator under low pressure. In order to study the influence of hydration, samples maintained for 96 h at room temperature in a controlled atmosphere with a relative humidity in the range 14–85% (by using satd salt solns) were compressed directly into KBr pellets. Solid films were cast from 1–2% w/w aq solns onto a polypropylene support. FTIR spectra of aq solns (polymer concentration 5, 7, and 9% w/v) at different ionic strength (water and 0.1 M aq NaCl) were recorded by using the ATR technique. A SPECAC square section horizontal ATR liquid cell with a ZnSe crystal (incident angle set at 45°) was used. After collection of the solvent background, the polymer soln was applied to the ATR plate by means of an aluminium device clamped to ensure good optical contact. Sample (GN)₃, in the protonated form, was obtained by exposing the sample to the acid vapours from concentrated HCl for 30 s. Before preparation of the KBr pellet, the sample was dried. Deuteration of the samples was performed by three different methods: (i) freeze-drying the samples from D₂O solns three times, (ii) leaving the samples in an atmosphere satd with D₂O, and (iii) casting a film from D₂O solution inside a dessiccator under reduced pressure two times. The last method gave the best results.

2. Results and discussion

Amorphous Solid Phase spectra.—The FTIR spectra of the freeze-dried sodium salt form and the protonated form of the oligomer (GN)₃ are reported in Fig. 1. Fig. 2 represents the spectra of the sodium salt and the partially deuterated form of the sample HA58 (film). Five regions [13] can be separately discussed:

(i) At $3600\text{--}2800\text{ cm}^{-1}$ is the region of CH, NH, and OH stretching vibrations. As far as hyaluronan is concerned, this region has not been discussed in detail previously and the only published data available so far have been obtained from infrared dichroism experiments

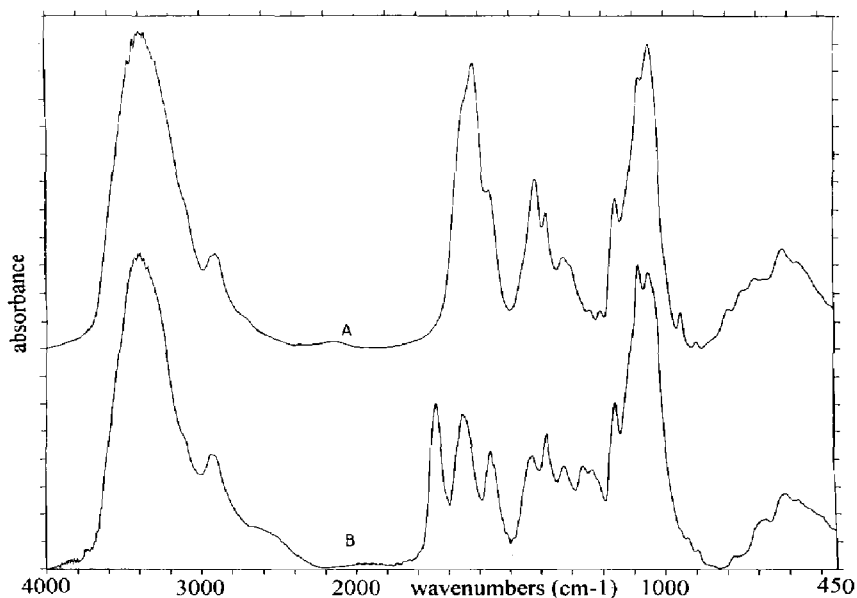


Fig. 1. FTIR spectra of freeze-dried samples of the trimer (GN)₃: A, sodium salt and B, protonated form.

under different experimental conditions [14]. As can be observed in Figs. 1 and 2, the strong band centered at 3390 cm^{-1} for the protonated form and for the two samples in the sodium salt form are rather broad and can be assigned to hydrogen-bonded OH groups. However, it is very difficult to find a correlation between these intense OH stretching modes and vibrational states, due to the complicated network of inter- and intra-molecular hydrogen

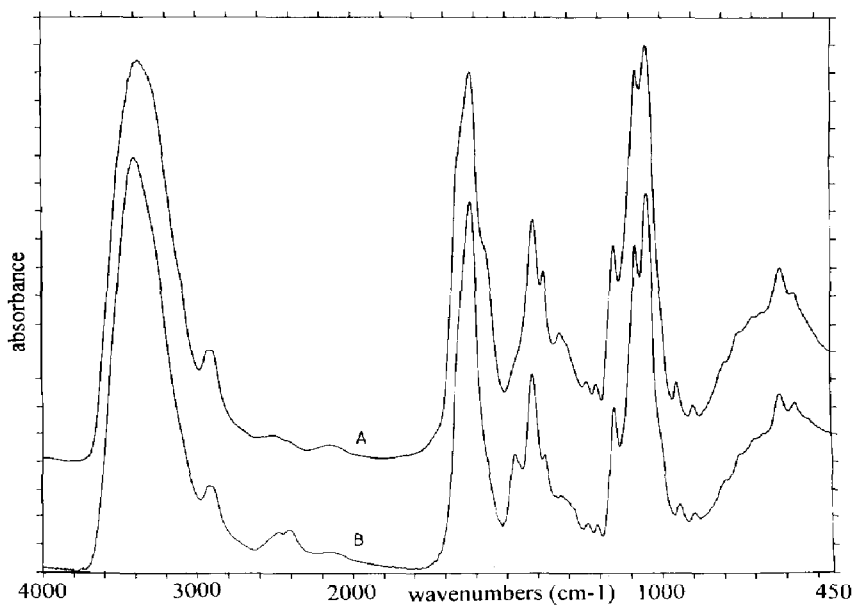


Fig. 2. FTIR spectra of a film of the polymer HA58: A, sodium salt and B, partially deuterated form.

bonds occurring in polysaccharides. In the same region, the NH stretching band appears as a shoulder at around 3105 cm^{-1} for both the sodium salt and the protonated forms. Fig. 2 shows that the OH band of the deuterated sample is of comparable intensity but narrower than that of the undeuterated samples. New overlapping bands are observed in the $2600\text{--}2200\text{ cm}^{-1}$ region which can be attributed to OD and ND stretching vibrations. The moderate intensity suggests that the deuteration occurred only partially, independent of the three deuteration techniques. A group of overlapping bands of moderate intensity is observed around 2915 cm^{-1} which are due to the CH symmetrical and to the CH_2 asymmetrical stretching.

(ii) At $1500\text{--}1200\text{ cm}^{-1}$ is the “local symmetry” region because it mainly includes the deformational vibrations of groups having a local symmetry, i.e., the (HCH) and the CH_2OH groups. In the case of hyaluronan, the spectral patterns reflect the sample form (Na salt, protonated, or deuterated) as can be seen in Figs. 1 and 2. The disappearance of the $\nu_s(\text{CO})$ (of COO^-) at 1414 cm^{-1} in the protonated form and the simultaneous appearance of the $\nu(\text{CO})$ (of COOH) at 1255 cm^{-1} is observed. In the deuterated sample, there is a slight decrease in the intensity of bands with contribution of $\delta(\text{COH})$ modes and the appearance of the amide II (ND related) band at 1474 cm^{-1} .

(iii) At $1200\text{--}950\text{ cm}^{-1}$ is a region which includes the highly coupled $\nu(\text{C-O})$, $\nu(\text{C-C})$, and $\delta(\text{COH})$ vibrational modes. This region is sensitive to the crystallinity of the sample and also to the conformational freedom of the polymer chains. In this region, the spectra of different samples in various forms are characterized by four bands centered at 1152 , 1078 , 1046 , and 946 cm^{-1} which are typical for carbohydrates [6,9,14]. The last band can be assigned [15,16] to asymmetrical out-of-phase ring vibration. The intensity increase at 1078 cm^{-1} in the spectrum of the protonated samples is evidence of the large contribution of the COH group vibrational mode to this peak.

(iv) At $950\text{--}700\text{ cm}^{-1}$ is a region which includes out-of-ring deformations (COH, CCH, and OCH) and anomeric $\delta(\text{C}_1\text{-H})$ vibration. The 2b type of $\delta(\text{C}_1\text{-H})$ vibrational mode, which characterises the β -hexopyranoses [6,17], is well resolved at 895 cm^{-1} in all the samples examined. The anomeric region, however, does not show the type 1 and 3 bands since they are hindered by the strong overlap of bands arising from the residual crystallinity of the samples. The weak band at 744 cm^{-1} is assigned [15] to CH_2 rocking and at 701 cm^{-1} to CH out-of-plane bending [16].

Below 700 cm^{-1} are endocyclic and exocyclic $\delta(\text{CCO})$ and $\delta(\text{CCC})$ modes. Precise assignments of these bands are difficult. Usually, the exocyclic deformations are found between 700 to 500 cm^{-1} and the endocyclic deformations below 500 cm^{-1} .

Due to the importance of the amide and carboxyl bands in the FTIR spectra of hyaluronan, these two groups of bands are discussed separately in the following.

“Amide bands”.—In the spectra of the two sodium salt samples, the three signals centered at 1650 , 1560 , and 1322 cm^{-1} are assigned to amide I, II, and III, respectively [9,18]. The amide I and II bands are well resolved in the protonated form in which the asymmetric stretching of the carboxylate group greatly decreases. This is in very good agreement with the results of Orr [8]. In the deuterated sample, it is possible to observe a decrease of the intensity of the amide I and III bands, whereas the amide II band shifts to 1474 cm^{-1} .

“Carboxyl bands”.—The bands at 1616 and 1413 cm^{-1} are assigned to the asymmetric and symmetric stretching modes of the planar carboxyl groups in sodium hyaluronate,

Table 2

Peak frequencies^a for the trimer (GN)₃ (KBr pellet), the polymer HA58 (film), and proposed assignments

(GN) ₃ Na-salt (cm ⁻¹)	(GN) ₃ Protonated form (cm ⁻¹)	HA58 Na-salt (cm ⁻¹)	HA58 Deuterated form (cm ⁻¹)	Proposed assignments
3396 (s)	3390 (s)	3380 (s)	3392 (s)	$\nu(\text{O-H})$ H-bonded
3104 (sh)	3106 (sh)	3106 (sh)		$\nu(\text{N-H})$ H-bonded
2905 (m)	2929 (m)	2922 (m)	2922 (m)	$\nu(\text{C-H})$
	2652 (w)			
			2468 (m)	$\nu(\text{O-D})$
			2419 (m)	$\nu(\text{N-D})$
	1735 (m)			$\nu_{\text{as}}(\text{C-O})$ [COOH]
1649 (s, sh)	1645 (s, sh)	1649 (s, sh)	1649 (s, sh)	Amide I [CO related]
1617 (s)		1618 (s)	1613 (s)	$\nu_{\text{as}}(\text{CO})$ [COO-]
1559 (m)	1559 (m)	1569 (m)		Amide II [NH related]
			1474 (m)	Amide II [ND related]
	1421 (m)			$\delta(\text{C-H})$
1415 (m)		1411 (m)	1412 (m)	$\nu_{\text{s}}(\text{C-O})$ [COO-]
1377 (m)	1377 (m)	1378 (m)	1376 (m)	$\delta(\text{C-H})$ [CH ₂], $\delta(\text{O-H})$
1319 (m)	1320 (m)	1324 (m)	1321 (vw)	Amide III
1297 (sh)		1298 (sh)		
	1255 (m)			$\nu_{\text{s}}(\text{C-O})$ [COOH]
1238 (w)		1238 (w)	1237 (w)	
	1229 (m)			
1205 (w)		1207 (w)	1209 (w)	
1153 (m)	1155 (m)	1151 (m)	1149 (m)	$\nu_{\text{as}}(\text{C-O-C})$ [O bridge], $\nu(\text{C-O})$, $\delta(\text{C-O-H})$
1078 (s)	1079 (s)	1079 (s)	1079 (s)	$\nu(\text{C-O})$ [exocyclic], $\nu(\text{C-C})$, $\delta(\text{C-OH})$
1045 (s)	1047 (s)	1047 (s)		$\nu(\text{C-OH})$
			1041 (s)	$\nu(\text{C-OH})$
946 (w)	947 (w)	947 (w)	944 (w)	Ring asymmetrical oop vibration
	928 (w)			$\delta_{\text{oop}}(\text{C-O-H})$ [COOH]
895 (w)	896 (w)	894 (w)	892 (w)	$\delta(\text{C1-H})$ [β anomer] type 2b
794 (w)		789 (w)	790 (w)	
	774 (w)			
742 (w)		746 (w)	744 (w)	$\omega(\text{CH}_2)$
701 (w)	704 (sh)	700 (w)	697 (w)	$\delta_{\text{oop}}(\text{CH}_2)$
670 (w)	672 (w)		697 (w)	
614 (m)	608 (w)	614 (m)	615 (m)	ν type t [H ₂ O]
566 (w)	568 (w)	567 (w)	564 (w)	

^a Abbreviations: s, strong; ν , stretching, as, asymmetrical; m, medium; δ , bending; s, symmetrical; w, weak, ω , rocking; oop, out-of-plane; sh, shoulder; ip, in plane; v, very

respectively. After protonation, these peaks are shifted to 1735 and 1255 cm⁻¹, respectively. These observations are in substantial agreement with previous data [8]. The observed frequencies and band assignments based on the data reported in the literature for the two examined samples are listed in Table 2.

Table 3
Average peak frequencies of the dry samples listed in Table 1

Average frequencies (cm^{-1})	Standard deviation (cm^{-1})
1652	± 3
1617	± 2
1563	± 3
1413	± 1
1378	± 1
1323	± 1
1296	± 2
1237	± 0
1205	± 1
1153	± 1
1078	± 2
1045	± 1
946	± 0
895	± 1
788	± 3
746	± 2
702	± 0

Influence of molecular weight.—As shown in Table 2, the observed frequencies of the two samples, in the sodium salt form, are very similar in spite of the different molecular weight and solid state conditions (KBr pellet and film). It was observed that for β -(1 \rightarrow 4)-linked homopolymers of D-glucopyranose, changes in the IR spectrum only occur for a degree of polymerization below 5; the spectrum of cellopentaose already being comparable to that of cellulose [19]. Similar conclusion has been obtained on chitin [20] and on amylose and its oligomers [21]. In order to evaluate the effect of molecular weight on the IR spectrum of hyaluronan, a number of different samples, including the (GN)₂ dimer, were examined. Although the IR bands shown by crystalline samples are often sharper than those from an amorphous sample, their assignment is much more difficult because of the shift and splitting of frequencies arising from interactions within the crystalline lattice. A comparison of solid-phase spectra of a homologous series is thus correctly feasible only in amorphous forms having the same degree of crystallinity [22]. For this reason, FTIR spectra of sodium hyaluronate, with different molecular weight, and its oligomers were recorded on amorphous, freeze-dried samples. As expected, no significant changes in the FTIR spectra were found by increasing the molecular weight. The FTIR spectra of the different oligomers and polymers are shown in Figs. 3 and 4, respectively. The average frequencies observed in the 1700–700 cm^{-1} range are reported in Table 3 together with their standard deviations. As can be observed, the low scattering of frequency values do not permit any conclusions with regard to the effect of the molecular weight. In spite of the almost total invariability in the observed frequencies, the shape and the intensity of some bands in the 1400–800 cm^{-1} region are modified. These changes can be quantified using different methods; a convenient way is to measure the area of informative spectral regions. This method has been successfully used to study crystallinity and conformational features in carbohydrate polymers [23,24]. In the present work, the area of the peak multiplet between 1195 and 960 cm^{-1} , which

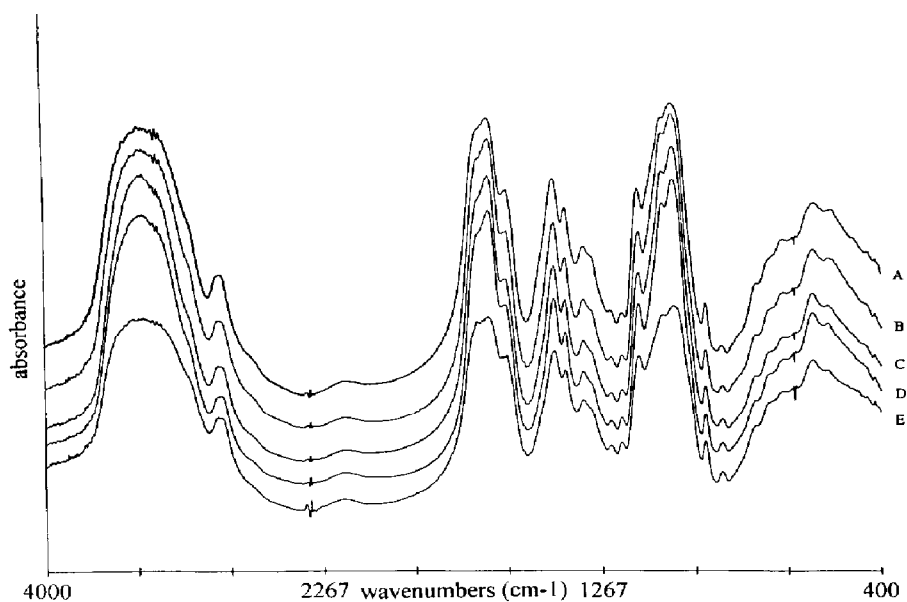


Fig. 3. FTIR spectra of freeze-dried sodium hyaluronate oligomers: A, (GN)₂; B, (GN)₃; C, (GN)₄; D, (GN)₆; and E, (GN)₇.

displays the highest intensity at 1045 cm^{-1} , was evaluated. The area of this group of peaks (taken as the intensity at 1045 cm^{-1} times the half band width) is plotted against the logarithm of the molecular weight as shown in Fig. 5. The two oligomers (GN)₂ and (GN)₃ give the highest area values. An increase in the degree of polymerisation, i.e., from (GN)₄ to (GN)₆ and (GN)₇, leads to a significant decrease in peak area. Such a decrease progres-

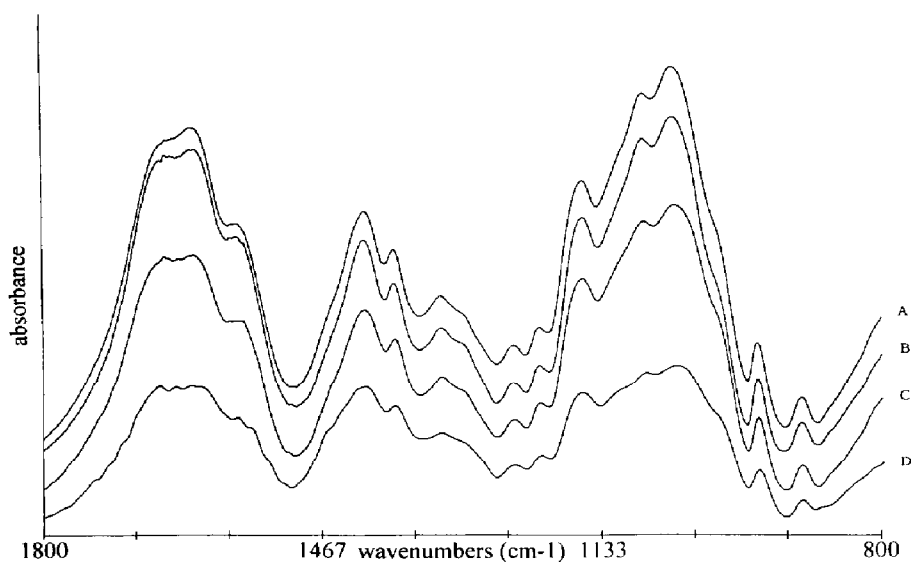


Fig. 4. FTIR spectra of freeze-dried sodium hyaluronate polymers: A, HA58; B, HA101; C, HA59; and D, HA5.

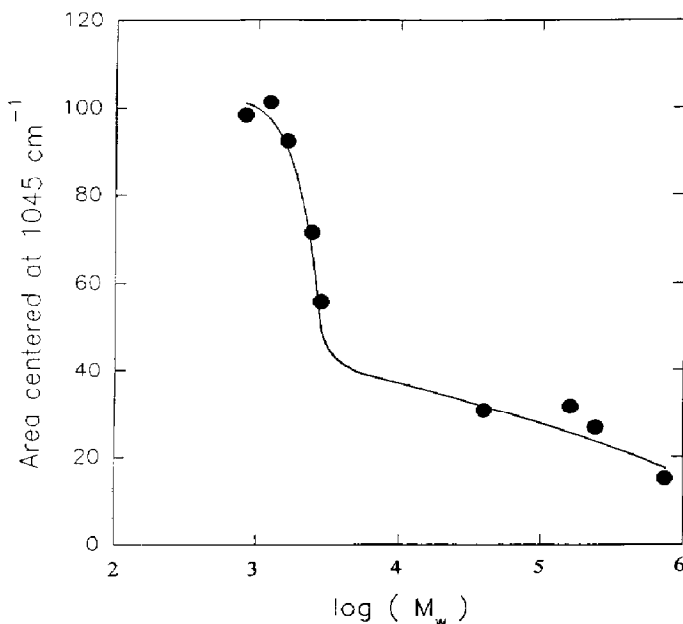


Fig. 5. Area of the peak multiplet between 1195 and 960 cm^{-1} which display a maximum intensity at 1045 cm^{-1} plotted against the logarithm of the molecular weight (M_w).

sively reduces as the molecular weight continues to increase. This result is a good indication of the differentiation between oligomer and polymer behaviour. Although the sharpness of peaks may be related to an increase in crystallinity [6], the difference between oligomers and polymers could be better accounted for by the orientation environment of the side groups than a particular effect of hydration. Oligomers can change orientation and align better than do larger molecules. This is further supported from the hydration study described below.

Influence of hydration.—FTIR spectra were recorded on freeze-dried samples (GN)₂ and HA5 after exposure to an atmosphere of controlled humidity (range 14–85%). The observed frequencies in the spectra for these samples are not appreciably affected by hydration, as found for other amorphous carbohydrates [21]. This finding suggests that the molecular weight does not affect the hydration of sodium hyaluronate as previously noted [25]. This is especially true for the regions of OH and NH stretching which do not show any appreciable difference. Also, this suggests that the hydrogen bonding pattern seems to remain unperturbed. However, when the area of the peak at 1045 cm^{-1} is plotted as a function of the relative humidity (see Fig. 6), a different behaviour is observed for the two samples. While no change in the area of the high molecular weight sample is observed, there is an almost linear decrease of the area of the 1045 cm^{-1} peak for sample (GN)₂ as the relative humidity is increased, with an apparent tendency to reach the value of the high molecular weight sample. This result can be explained by an increase in the number of accessible conformations induced by hydration on sample (GN)₂, with the concomitant loss in the “residual crystallinity”.

Aqueous solution spectra.—In order to eliminate the “intriguing” effect of the residual crystallinity, FTIR spectra were recorded in aqueous solution by the ATR technique. The

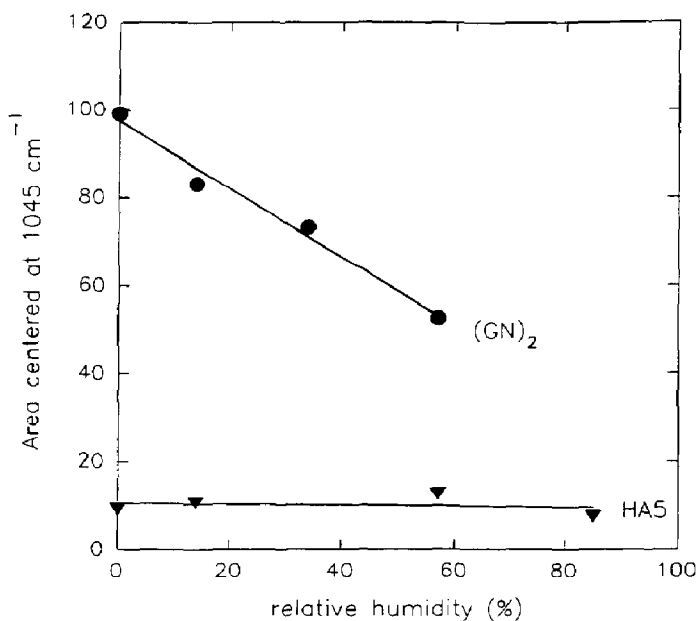


Fig. 6. Area of the multiplet between 1195 and 960 cm^{-1} which display a maximum intensity at 1045 cm^{-1} of the $(\text{GN})_2$ (●) and HA5 (▼) samples plotted as a function of the relative humidity.

ATR spectrum of a 5% w/v of the sample HA101 in pure water is reported in Fig. 7. The observed frequencies in aqueous solution are not affected by the degree of polymerisation, which is in agreement with ^{13}C NMR data obtained in D_2O [12]. Furthermore, the observed frequencies are not different from those observed in the freeze-dried spectra. This implies that both the polymer conformation and the pattern of hydrogen bonding are not perturbed by the process of freeze-drying from aqueous solutions. The comparison of the area of the

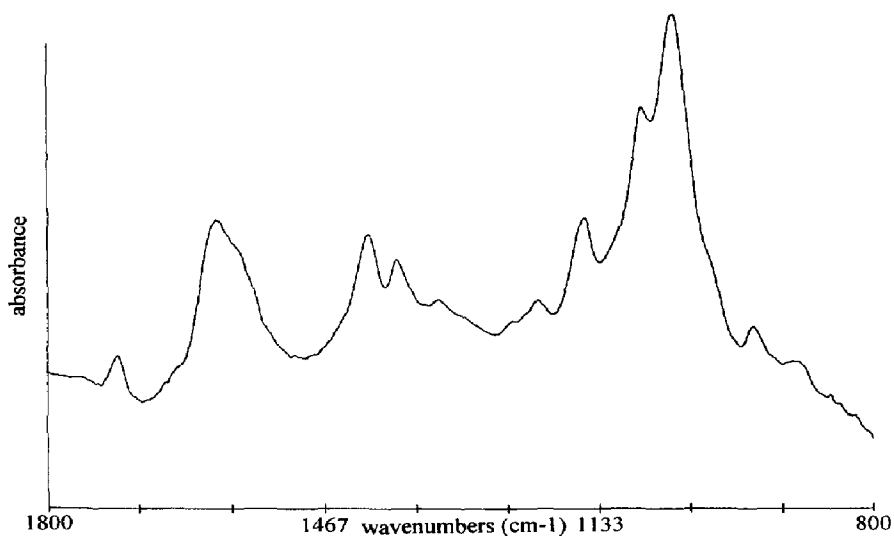


Fig. 7. FTIR-ATR spectrum of sample HA101 in aqueous solution. (polymer concentration, 5% w/v).

peak at 1045 cm^{-1} for the trimer $(\text{GN})_3$ and for the high molecular weight sample HA5 does not reveal any difference, providing further evidence in favour of the role played by the “residual cristallinity” in the freeze-dried, solid-state spectra. ATR spectra of sample HA5 were recorded in NaCl 0.1 M at a 5% w/v polymer concentration. Two IR bands at 1105 and 1000 cm^{-1} were found to be more intense upon addition of the simple electrolyte [a similar behaviour was observed in the case of trimer $(\text{GN})_3$]. The evaluation of the area of the peak centered at 1045 cm^{-1} leads, for a 0.1 M NaCl solution, to a value lower than that obtained in pure water. This can be explained in terms of an increase in the number of accessible conformations induced by the screening of electrostatic repulsion exerted by the added counterions.

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