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$^{87}Rb^{+}$ spin relaxation in enzymically purified and in untreated iota-carrageenan

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ABSTRACT

The temperature dependences of the transverse (R_2) and longitudinal (R_1) n.m.r. relaxation rates of $^{87}Rb^{+}$ in aqueous 5% iota-carrageenan have been compared with similar data for a sample purified by treatment with kappa-carrageenase. In each sample, the relaxation rates were sensitive to the conformation (helix or random coil). In the intact sample, the small ($\leqslant 5\%$) fraction of kappa-carrageenan (which, in its helical state, specifically binds rubidium ions) was solely responsible for the pronounced line-broadening that has been observed hitherto for ^{87}Rb in iota-carrageenan gels. In the purified sample, the effects on the relaxation of ^{87}Rb induced by iota-carrageenan are similar to those found in comparable systems in the absence of site-binding of the ions. Thus, there was a modest enhancement of the relaxation with $R_1 \approx R_2$ for the flexible coil conformation and a comparably larger effect, with significant contributions from dynamic processes on the time-scale of the inverse resonance frequency or longer, for the thicker, more highly charged and rigid helix conformation of iota-carrageenan.

INTRODUCTION

The carrageenans[1] are linear sulfated galactans, extracted from certain marine red algae, which are used widely for thickening solutions or for forming aqueous gels. The primary structure of gel-forming carrageenans[1,2] is dominated by regular sequences of repeating disaccharide units. These sequences may be converted from a random coil configuration at high temperatures into a helical configuration at low temperatures, and, although the details of the structure of the gel network have not been elucidated, the formation of helices seems to be a prerequisite for gelation to occur. Various types of helix-forming structures exist, all of which are based on a backbone that consists of a repeating disaccharide of β-D-galactopyranose and 3,6-anhydro-α-D-galactopyranose, which is sulfated to different degrees. The most extensively studied of these structures are those of iota- and kappa-carrageenan, based on the repeating units **1** and **2**, respectively.

Both kappa- and iota-carrageenan are charged, and salts have a marked influence on their conformational transitions, aggregation, and gelation behavior[3–7]. Further-

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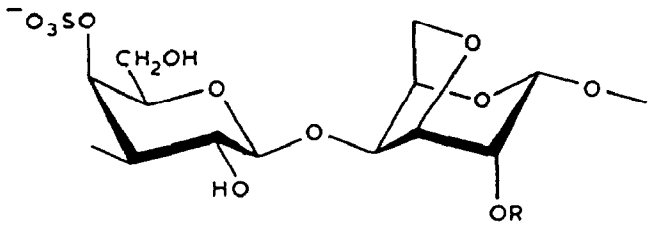

1 R = SO_3^- (iota)

2 R = H (kappa)

more, the properties of aqueous carrageenan may depend dramatically not only on the valencies[3,7] but also on the identities[3,5,6] of the added ions, and studies[8–11] using counter-ion n.m.r. spectroscopy have demonstrated that strongly gel-promoting cations bind to the carrageenans in the gel state. Although ion-specific effects are particularly apparent for kappa-carrageenan, similar effects have been found, albeit to a lesser degree, for iota-carrageenan. However, a complicating factor in the interpretation of the latter results is that conventional preparations of iota- or kappa-carrageenan always seem to contain both types (**1** and **2**) of carrageenan structure[12,13].

Current knowledge on the distribution of the structural impurities in iota- or kappa-carrageenan and on their influence on the properties of the aqueous systems is limited. A study[12] that involved n.m.r. spectroscopy, gel-permeation chromatography, and hydrolysis by specific enzymes indicated that kappa-type residues may occur in blocks within the primary structure of iota-carrageenan. On the other hand, the iota-carrageenan impurities in preparations of kappa-carrageenan are situated primarily in separate molecules which are not precipitated by KCl, as are molecules where the kappa-type structure preponderates[14]. Rheological investigations[14] of mixtures of kappa- and iota-carrageenan revealed important synergistic effects. Of particular relevance to the present work are the findings[11,15] that cation-specific effects found previously in the conformational transition[5,16] and counter-ion n.m.r. relaxation[9] of aqueous iota-carrageenan may be attributed to small proportions of units with a kappa-type structure, which are situated in blocks that are long enough to give rise to a separate conformational transition.

In seeking a better characterisation of the properties of pure carrageenan components, we have studied the n.m.r. relaxation of rubidium (known as a strongly gel-promoting ion in systems that contain kappa-carrageenan) in a dilute aqueous sample of iota-carrageenan that had been treated with kappa-carrageenase, and have compared the results with those obtained with untreated iota-carrageenan. The results presented here are complementary to those[11] where only non-purified samples were available.

EXPERIMENTAL

Materials. — Iota-carrageenan was obtained from Sigma (Lot No. 18C-0384). An enzymically purified sample ("purified" iota-carrageenan) was prepared by treating a 1% solution in 5mM $NaHCO_3$ with kappa-carrageenase[17] for 1 week at room temperature followed by heating (to destroy the enzyme) and freeze-drying. A reference sample ("intact" iota-carrageenan) was subjected to the same treatment in the absence of the enzyme. The enzymic treatment affected the intrinsic viscosity of the iota-carrageenan as well as the storage modulus of an aqueous gel as shown in Table I.

Salt-free samples of the rubidium form were prepared by dissolution in (millipore filtered) water, dialysis (Spectra/Por hollow-fibre bundles) against water, freeze-drying, dissolution in water to 1%, and ion-exchange at $>90°$ on a rubidium-charged column of Dowex 50-X8-100 resin with a ten-fold excess capacity, followed by freeze-drying. Aqueous 5% solutions were prepared by mixing 150 mg of freeze-dried rubidium carrageenan with 3 mL of water in tubes that fitted snugly into standard 12-mm n.m.r. tubes. The sample tubes were flame-sealed and heated at $\sim 100°$ with occasional mixing.

Methods. A Nicolet NIC-360 spectrometer, operating at 118.38 MHz, was used for the ^{87}Rb measurements. Spectra were recorded using 2048 data points, a sweep width of 40 kHz, and a 90°-pulse width of 30 μs. Longitudinal relaxation rates (R_1) were obtained from inversion–recovery experiments. The longitudinal relaxation was always a single exponential. Transverse relaxation rates (R_2) were obtained from Lorentzian fits to the line-widths using the relation $R_2 = \pi \Delta \nu$. For bi-Lorentzian spectra from gel samples (see below), transverse relaxation rates obtained by the latter procedure are reported as effective rates (R_2^*). Attempts were made to separate the rapid (R_2^+) and slow (R_2^-) component rates of the transverse relaxation by bi-exponential fits to the experimental free induction decays (FID's). For the intact sample, where $R_2^+ \gg R_2^-$, such fits yielded reliable values of R_2^- and order-of-magnitude estimates of R_2^+. For the same sample, R_2^- values were also obtained by Lorentzian fits to absorption spectra obtained from FID's that were left-shifted 10–20 points prior to Fourier transformation in order to remove the rapid components. For the purified sample in the gel form, where $R_2^+ \leqslant 3R_2^-$, no accurate values of R_2^+ and R_2^- could be obtained from bi-exponential fits because of effects of second-order dynamic shifts[18].

Proton-decoupled ^{13}C-n.m.r. spectra were recorded at 75.5 MHz with a Bruker AM 300 spectrometer. 4% Solutions in D_2O were analysed at 80° under the following conditions: sweep width, 15 kHz, pulse width, 30°; acquisition time, 0.27 s on 8k data points. In order to decrease the line-widths of the different signals, the samples were sonicated[19] before investigation.

The temperature dependence of α_{546} of the aqueous 5% iota-carrageenan investigated by ^{87}Rb-n.m.r. spectroscopy was recorded using a Jasco DIP-360 polarimeter where the temperature was changed continuously at 0.34°/min. No significant differences between heating and cooling traces were detected.

RESULTS AND DISCUSSION

^{13}C-N.m.r. and optical rotation data. — Figure 1 compares the ^{13}C-n.m.r. spectra of iota-carrageenan before and after treatment with kappa-carrageenase. In accordance with what is generally found[12–15], the intact sample (Fig. 1) contained $\leqslant 5\%$ of kappa-type residues, as evidenced by the small peak at 95.5 p.p.m. for the resonance of C-1 of

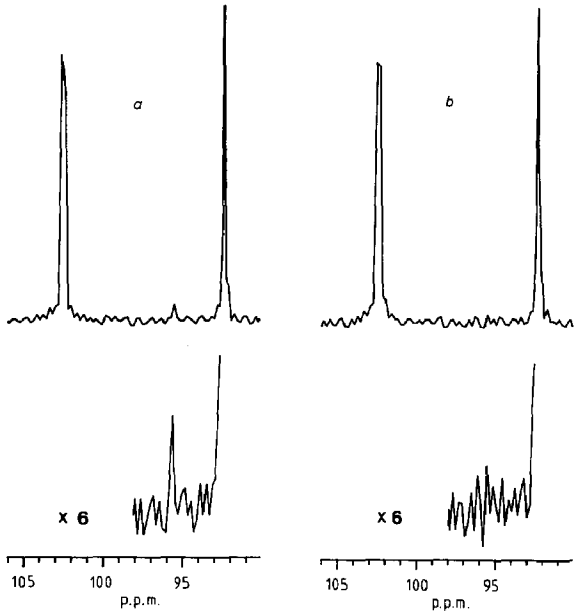

Fig. 1. ^{13}C-N.m.r. spectra of intact (*a*) and purified (*b*) iota-carrageenan. For clarity, only the C-1 signals are shown. The small peak at 95.5 p.p.m. in (*a*) corresponds to an impurity of kappa-carrageenan.

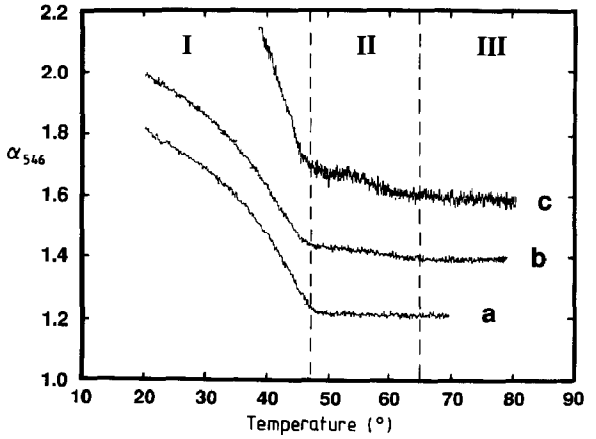

Fig. 2. Temperature dependence of α_{546} (heating traces, 0.34°/min) in purified (*a*) and intact (*b,c*) iota-carrageenan (5% samples, rubidium form). Vertical scale refers to trace (*a*), whereas trace (*b*) is displaced vertically 0.2 unit. Trace (*c*) is equivalent to (*b*) but recorded at a higher magnification ($\times 2$). The regions I–III are as discussed in the text.

the "anhydrogalactose" unit in kappa-carrageenan[20,21]. No such peak was detected in the spectrum of the purified sample (Fig. 1b).

Figure 2 shows the temperature dependence of α_{546} (heating traces) of aqueous 5% rubidium iota-carrageenan. The curves for the purified (Fig. 2a) and intact samples (Fig. 2b) are similar: a large variation in α_{546} at low temperatures, due to the helix-to-coil transition of iota-carrageenan, was followed by a levelling off at temperatures above 47 $\pm 1°$ (T_c) where the iota-carrageenan molecules were completely converted into the coil conformation. However, only the trace for the purified sample (Fig. 2a) was completely flat at $T > T_c$, whereas the trace for the intact sample (Fig. 2b) displayed a small slope, or shoulder. A heating trace recorded at higher magnification (Fig. 2c) confirmed the existence of this additional transition, at $T > T_c$, for the intact sample. A similar additional transition step for intact iota-carrageenan in the rubidium form has been observed[11] and ascribed to the co-operative transition of consecutive units of the kappa-type (located in blocks or in separate polymers) present as impurities in the sample. The α_{546} data here presented confirm this conclusion, since the additional step vanishes for the purified sample (Fig. 2a).

On the basis of the results of Fig. 2, three temperature regions (indicated in Fig. 2) may be distinguished, associated with (I) the iota-carrageenan transition ($T < 47°$), (II) the additional kappa-carrageenan transition in the intact sample ($47° < T < 65°$), and (III) the high-temperature where both iota- and kappa-carrageenan exist as random coils ($T > 65°$) [the choice of 65° for the temperature separating regions II and III is justified further by data presented below].

^{87}Rb-N.m.r. relaxation. — In general, the longitudinal and transverse quadrupolar relaxation processes for a spin 3/2 nucleus such as ^{87}Rb are biexponential[22]. The decay of the transverse magnetisation is given by equation *1*, where R_2^+ and R_2^- are the

$$M_{xy}(t) = M_o[0.6\exp(-R_2^+ t) + 0.4\exp(-R_2^- t)] \qquad (1)$$

fast and slow component rates of the transverse relaxation, respectively, which are given by the expressions *2* and *3*, where $J(\omega)$ is the spectral density function at the angular

$$R_2^+ = (2\pi^2/3)[J(0) + J(\omega_o)] \qquad (2)$$

$$R_2^- = (2\pi^2/3)[J(\omega_o) + J(2\omega_o)] \qquad (3)$$

frequency ω, and ω_o is the resonance angular frequency. The longitudinal relaxation process is often (as in the present study) effectively exponential[23] with a single relaxation rate given by equation *4*.

$$R_1 = (4\pi^2/3)[0.2J(\omega_o) + 0.8J(2\omega_o)] \qquad (4)$$

The above spectral density function is the Fourier transform of the time-correlation function that describes fluctuations in the coupling between the nuclear quadrupole

moment and electric field gradients at the nucleus. In the simplest situation of an exponentially decaying correlation function, the spectral density function is a Lorentzian according to equation *5*, where χ is the quadrupole coupling constant and τ_c is the correlation time. In general, however, several dynamic processes on differing time

$$J(\omega) = (3/10)\chi^2\tau_c/[1 + (\omega\tau_c)^2] \qquad (5)$$

scales contribute to the averaging of the quadrupolar coupling. If all these processes are rapid compared to the resonance angular frequency, all spectral densities that enter the relaxation expressions become equal (the extreme narrowing case) and may be expressed in terms of an effective correlation time, τ_{eff}; *cf.* equation *6*.

$$J(0) = J(\omega_0) = J(2\omega_0) = (3/10)\chi^2\tau_{eff} \qquad (6)$$

Under extreme narrowing conditions, the transverse and longitudinal relaxation processes are both mono-exponential with the same rate constants ($R_2^+ = R_2^- = R_1$; *cf.* equations *2–4*). This is generally the situation for the n.m.r. relaxation of small ions in aqueous solutions of simple salts. For ions in (gel-forming) macromolecular systems, however, the extreme narrowing condition does not always obtain. Large effects on the relaxation rates are seen when there is site-binding of the ion to a macromolecule, which results in long correlation times for the bound ions. For $^{87}Rb^+$ in carrageenans, ion-binding effects yield spectra[9,11] where either the broad component (which is proportional to R_2^+ and has 60% of the intensity; *cf.* equation *1*) or both components of the absorption signal are broadened beyond detection in a normal high-resolution experiment.

The large effects of the enzymic treatment on the specific binding of rubidium in iota-carrageenan are demonstrated by a comparison of the ^{87}Rb spectra of the intact and the purified samples taken from the three temperature regions specified above (Fig. 3). There are large differences in both regions I *and* II (iota-carrageenan molecules exist only as coils in region II), whereas the spectra obtained in region III are virtually identical. In accordance with previous findings[9,11], the spectra obtained for the intact sample in regions I and II are composed of two components of widely differing line-widths and, consequently, there is an apparent loss of signal intensity. In these spectra, the width of the broad component was found (from biexponential fits to the FIDs; *cf.* Experimental) to be an order of magnitude larger than that of the narrow component. In contrast, the spectra from the purified sample did not display any apparent loss of intensity in any of the temperature regions. These differences in the appearance of the spectra of the two samples indicate that the dramatic line-broadening effects (losses in intensity) found for certain gel-promoting cations in iota-carrageenan[9,11] are due entirely to the presence of cation-binding impurities of kappa-carrageenan in the ordered conformation.

The temperature dependences (from heating experiments) of the ^{87}Rb transverse and longitudinal relaxation rates in the 5% samples of Rb-iota-carrageenan are shown

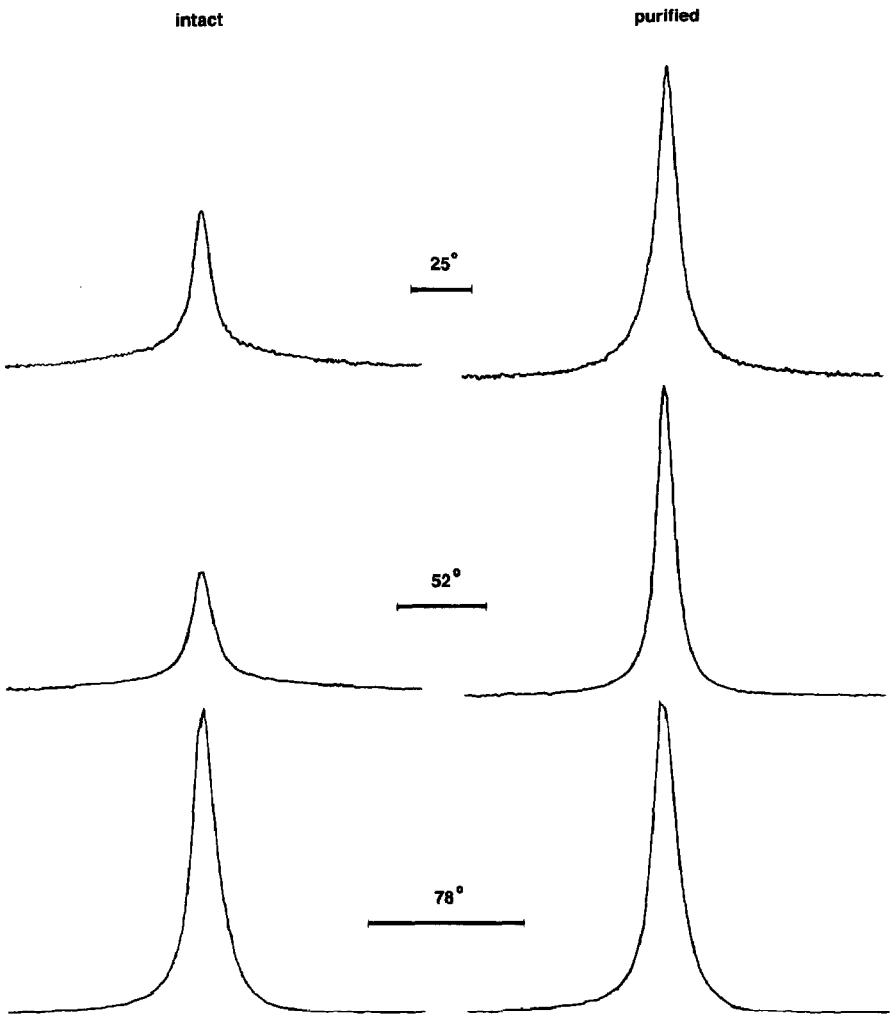

Fig. 3. Comparison of $^{87}Rb^{+}$ n.m.r. spectra from intact and purified iota-carrageenan (5% samples, rubidium form) at different temperatures. Spectra recorded at the same temperature have the same vertical expansion. Bars represent 1 kHz.

in Fig. 4. Effective transverse relaxation rates (*cf.* Experimental) are given since reliable values of the component rates R_2^{+} and R_2^{-} could not always be extracted. The plots resemble the corresponding plots of α_{546} (Fig. 2), except that the differences between the two samples are more readily apparent in Fig. 4. In the purified sample (Fig. 4a), the helix-to-coil transition of iota-carrageenan (region I) results in a larger decrease in both R_1 and R_2^{*}. Furthermore, there is a progressively large difference between the two relaxation rates as the helical content increases. In contrast, above T_c (regions II and III), only a weak temperature dependence is seen and R_1 and R_2^{*} are equal (or nearly equal), which is the expected result[24,25] for a flexible polyelectrolyte at this concentration (0.15 equiv. dm^{-3}). Thus, there can be little doubt that Fig. 4a reflects the influence of the conformational transition of iota-carrageenan on the relaxation of ^{87}Rb.

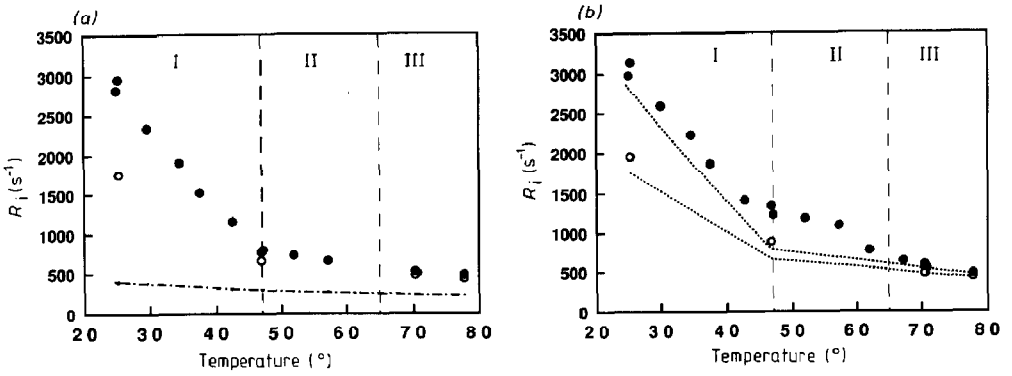

Fig. 4. Temperature dependence (on heating) of the longitudinal (○) and effective transverse (●) $^{87}Rb^+$ relaxation rates in 5% samples of purified (*a*) and intact (*b*) iota-carrageenan. Dashed lines separate regions I, II, and III; —·— in (*a*) represents the relaxation rate in 0.1M RbCl, ··· in (*b*) represents the iota-carrageenan results from (*a*).

Fig. 4b shows R_1 and R_2^* for the intact sample of iota-carrageenan. Both relaxation rates are enhanced relative to those of the purified sample, except in region III where, in the coil state, the small kappa-carrageenan impurity does not significantly affect the relaxation of ^{87}Rb. The largest discrepancies between the results for the two different samples are seen for R_2^* in region II. However, the *numerical* values given for R_2^* of the intact sample in regions I and II have no significance, since they were obtained from single-Lorentzian fits to bi-Lorentzian spectra where the line-widths of the two components differed by a factor of ~ 10. The broad line sharpened dramatically between 57° and 62°, which corresponds well to the region of the second transition step in α_{546} (Fig. 2c), and, at 67°, the transverse relaxation for the intact sample has practically merged with that of the purified sample. Again, there is no significant difference between R_1 and R_2 in region III.

Recent studies using n.m.r. self-diffusion[26] and relaxation[27] techniques have shown that the electrostatic attraction between iota-carrageenan and its counter-ions is stronger when iota-carrageenan is in the helical state, in accordance with the proposed double-helical model[2,28]. Thus, enhancement of the counter-ion relaxation with helical content, such as is seen for ^{87}Rb in region I of Fig. 4, is expected. Moreover, in this region, $R_2^* > R_1$ also for the purified sample (Fig. 4a) and the rubidium line-shape was slightly biexponential ($R_2^+/R_2^- \leqslant 3$). Such results, which imply contributions from motions on time-scales of $1/\omega_0$ (~ 1 ns in the present instance) or larger, are not expected for the relaxation of counter-ions of (even highly charged) *flexible* polyelectrolytes at similar concentrations, where the general finding is (as noted above) that the extreme narrowing condition obtains. However, for DNA in the rigid helical state, results similar to those of region I in Fig. 4a have been found[29,30]. Indeed, the relative enhancement of the relaxation rates of ^{23}Na obtained[30] for salt-free solutions of sodium DNA are comparable to the results for ^{87}Rb in purified iota-carrageenan.

From studies of the frequency dependence of the ^{23}Na relaxation, the existence of dynamic processes on the nanosecond timescale for sodium counter-ions in salt-free

DNA solutions was demonstrated[30]. A likely candidate for these processes is the diffusion of the counter-ions around the DNA helix. Indeed, estimates[30] using the simple expression *7*, where d is the diameter of the helical molecule and D is the (local)

$$\tau_c = d^2/(4D) \qquad (7)$$

self-diffusion coefficient of the counter-ions near the surface of the DNA molecule, yielded correlation times of the correct order of magnitude. Such motions are expected to contribute for helical DNA since this is a comparatively thick polyelectrolyte (d = 20 Å), and, also, rigid enough so that extensive internal motions do not occur on shorter times-scales. Similar arguments can be invoked for iota-carrageenan in the helical state. If the effective radius of the double helix of iota-carrageenan (the bare radius[28] plus the radius of a hydrated Rb^+ion) is estimated as 10 Å, and if the diffusion coefficient of rubidium ions at the surface of the macromolecule is assumed to be reduced by a factor of 2 (actually, a greater reduction may be expected[31]) compared to its value[32] at infinite dilution ($D = 2 \times 10^{-9}$ m^{2}s^{-1} at 25°), equation *7* yields a correlation time of 1 ns.

The presence of large and rigid structures in certain polysaccharide gels may give rise to long n.m.r. correlation times (and large relaxation effects) for ions dissolved in the system. Such effects have been demonstrated for ^{23}Na in kappa-carrageenan gels containing mixed counter-ions[11] and for various ions in agarose gels[33]. A difference between gels of agarose or kappa-carrageenan and those of iota-carrageenan is that the former contain aggregates of helices, whereas the existence of suprahelical structures has not been demonstrated for gels of pure iota-carrageenan. Also, it seems that such structures do not have to be invoked in order to rationalise the relatively large relaxation enhancement seen in region I of Fig. 4a; the rigid double-helical conformation of iota-carrageenan in itself may be a sufficient explanation.

The difference between Figs. 4b and 4a derives from rubidium ions site-bound to the kappa-carrageenan impurities in the intact sample. The observed relaxation rates may be written as population weighted averages over the relaxation rates of bound (b) and free (f) ions according to equation *8*, where R_i is any one of the relaxation rates, R_1, R_2^+, or R_2^-, and p_b is the fraction of bound ions. Equation *8* is valid in the limit of "very

$$R_i = (1-p_b)R_{i,f} + p_b R_{i,b} \qquad (8)$$

fast exchange"[34], when the exchange of molecules between the bound and the free states is much faster than the difference in the respective intrinsic relaxation rates. Since $p_b \ll 1$ in the intact sample, $p_b R_{i,b}$ is simply the difference between the results for the intact and the purified samples and yields $p_b R_{1,b} \approx 2 \times 10^2$ s^{-1} and (from the bi-exponential fits) $p_b R_{2,b}^+ \approx 2 \times 10^4$ s^{-1}. The slow transverse relaxation rate R_2^- could be deduced accurately only for the intact sample (*cf.* Experimental) where it was nearly equal to R_1. Thus, if $p_b R_{2,b}^-$ is approximated by $p_b R_{1,b}$ and p_b is estimated to be of the order of 10^{-2} from the content of kappa-carrageenan in the intact sample, the order-of-magnitude estimates $R_{2,b}^- \approx 10^4$ s^{-1} and $R_{2,b}^+ \approx 10^6$ s^{-1} for rubidium ions bound to kappa-

carrageenan helices are obtained. These values are compatible with the findings that the line-widths (*i.e.*, also the "narrow" components) of rubidium in salt-free kappa-carrageenan gels are broad (2–3 kHz)[11]. Such large enhancements of relaxation relative to a simple salt solution (where $R_{1,ref} = R_{2,ref} \approx 400\,s^{-1}$ at 25°) should be primarily an effect of a large value of the correlation time, $\tau_{c,b}$, for bound ions.

Most likely, $\tau_{c,b}$ corresponds to the lifetime of bound rubidium ions, since motions of ions site-bound to carrageenan helices may be expected to be slow. A single Lorentzian spectral density function (equation *5*) may then be expected. If it is assumed that the coupling constants are roughly the same in the bound and the free states, and that the spectral densities for bound ions and reference ions are given by equations *5* and *6*, respectively, the parameters $R_{2,b}^{-} = 2 \times 10^4\,s^{-1}$, $R_{2,ref} = 400\,s^{-1}$, and $\tau_{eff,ref} = 10^{-12}\,s^{-1}$ (the latter being a typical[35] value, at room temperature, for ions in simple salt solutions) yield $\tau_{c,b} \approx 10$ ns for bound rubidium ions. This value of $\tau_{c,b}$ is also compatible with the estimated ratio $R_{2,b}^{+}/R_{2,b}^{-} = 10^2$ (*cf.* equations *2*, *3*, and *5*) and, furthermore, is consistent with the fast-exchange criterion assumed valid in equation *8*. The analysis presented above is internally consistent and, also, consistent with previous observations of rubidium relaxation in kappa-carrageenan gels.

Small proportions of kappa-carrageenan impurities in iota-carrageenan may evidently have a dominating influence on R_2^{+} (and a significant effect also on R_2^{-} and R_1) of rubidium ions under conditions where kappa-carrageenan exists in the helical conformation. Thus, rubidium n.m.r. spectroscopy is a sensitive technique for demonstrating the occurence of small fractions of consecutive, helix-forming structures of the kappa-type in mixed samples, and the results from the purified sample demonstrate the success of the enzymic method in removing such structures.

Although the rubidium n.m.r. results confirm the existence of long, consecutive regions of kappa-carrageenan in intact samples of iota-carrageenan, they cannot tell whether these structures are incorporated in block-copolymers of mixed iota-kappa-carrageenan and/or are in separate polymers of pure kappa-carrageenan. In the former situation, even small proportions of kappa-carrageenan impurities could have significant effects on the rheological properties of aqueous iota-carrageenan. Evidence of such effects is given by the data of Table I. The difference in intrinsic viscosities of the intact and the purified samples demonstrates that the enzymic treatment results in a substantial decrease of the average molecular weight of the sample, which, in turn, indicates that a substantial fraction of the molecules of the intact sample contain sequences of kappa-carrageenan long enough to be cleaved by the enzyme. The mechanical properties of gels prepared from iota-carrageenan are also affected by the enzymic treatment, as indicated by the storage moduli in Table I, and the difference between the intact sample and the purified sample is larger than would be expected from the difference in molecular weights alone[36]. Thus, the small fraction of kappa-carrageenan in intact iota-carrageenan has important rheological consequences, and a more detailed investigation to elucidate this point is in progress at the Lund laboratory.

TABLE I

Effects of enzymic purification on rheological properties of iota-carrageenan

Sample	*[η] (45°, 0.05M NaCl) (mL/g)*	*G' (25°, 12 g/L, 0.25M KCl) (Pa)*
Intact	570	1600
Purified	300	670

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