Effects of Specific Anion Binding on the Helix–Coil Transition of Lower Charged Carrageenans. NMR Data and Conformational Equilibria Analyzed within the Poisson–Boltzmann Cell Model

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ABSTRACT: The anion specificity of two lower charged carrageenans (κ-carrageenan and furcellaran) was studied under a wide range of ionic conditions, involving pure and mixed salts of specific and nonspecific cations and anions. The effects of anions on the coil-to-helix transition was studied experimentally by optical rotation. Anions stabilize the helical conformer of both carrageenans according to the sequence $Cl^- < NO_3^- < Br^- < SCN^- < I^-$. Specific binding of the thiocyanate and iodide anions to the helical conformer, and competition of different anions for the binding sites, was demonstrated by the ^{14}N NMR relaxation of the thiocyanate ion. A unified theoretical rationalization of both types of experimental data was obtained within the Poisson–Boltzmann cell model (PBCM), by assuming a single class of anion-binding sites on the carrageenan helix with an intrinsic binding constant which differs for different anions. Under the assumptions that a unit cell of the helix contains one site and that the binding constant for chloride ions is negligible, intrinsic binding constants on the order of 10^2 and 10^3 M^{-1} at room temperature were deduced for the thiocyanate and iodide ions, respectively. These binding constants are much higher than those previously deduced, in similar analyses, for specific monovalent cations. Cation and anion NMR data from solutions containing both binding cations and binding anions indicate that the two classes of ions bind to different sites.

I. Introduction

Carrageenans are sulfated polysaccharides extracted from seaweed, which can be classified into different types according to their degree of sulfation.[1] For the gelling carrageenans, i.e., ι-carrageenan, κ-carrageenan, and furcellaran (Figure 1), a coil-to-helix transition can be induced by changing the temperature or the salt concentration. Both cation and anion specificity of the conformational transition have been found for the lower charged κ-carrageenan[2–5] and furcellaran[6] but not for pure ι-carrageenan.[7,8] The cation specificity is best studied, and a number of independent studies[6,9–14] using ion NMR have shown that there is some specific interaction between the helix-promoting cations and the κ-carrageenan or furcellaran helices. In the authors' laboratory, both the nonspecific electrostatic effects (including the effects of salt concentration, counterion valency, and solvent dielectric permittivity) and the effects of specific cation binding on carrageenans have been studied extensively by experimental and theoretical methods.[6,15–18] It was then found that the Poisson–Boltzmann cell model (PBCM) could semiquantitatively predict these effects on the helix–coil transition of carrageenans. In particular, recent analyses of the specific cation effects for κ-carrageenan[18] and furcellaran[6] showed that the PBCM could well describe the cation specificity of the conformational transition as well as NMR data on cation binding by assuming a weak binding (with an intrinsic binding constant on the order of a few M^{-1}) of certain cations (potassium, rubidium, and cesium) to the carrageenan helix.

The anion specificity of the conformational transition of κ-carrageenan was first reported by Grasdalen et al.[3,4] and later studied systematically by Norton et al.[5] Anions are found to stabilize the ordered conformation according to the so-called Hofmeister or lyotropic series,[19] but curiously enough, the order is reversed, so that the ions which are normally known to be strongly denaturing are here the most potent helix-stabilizing ions. (It may be noted that, for the closely related agarose molecule, the normal behavior is observed.[20]) Very similar effects have recently been found for furcellaran by the present authors.[6]

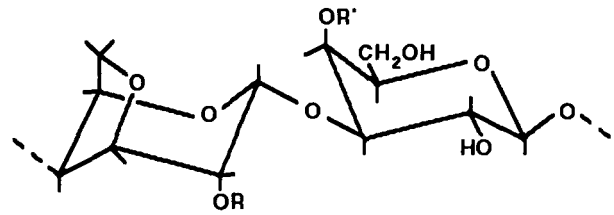

Figure 1. Repeating disaccharide units of ι-carrageenan (R = R′ = SO_3^-), κ-carrageenan (R = H, R′ = SO_3^-), and furcellaran (R = H, R′ = SO_3^- (60%) and H (40%)).

General thermodynamic arguments[21] suggest that the helix-stabilizing effect of certain anions could be caused by a binding of these anions to the *helical* conformer of κ-carrageenan, and evidence for such a binding has, in fact, been obtained[3] by Grasdalen and Smidsrød from NMR line widths of iodide ions in κ-carrageenan solutions, which show a substantial increase on helix formation. The interpretation of such effects in terms of ion binding has, however, been challenged by Norton et al.[5], who observed a line broadening on helix formation also for the bromide and chloride ions and argued that this indicates that the effect has a more general origin. On the other hand, the data of Norton et al., clearly show that the relative line broadening on helix formation increases dramatically in the order $Cl^- \ll Br^- \ll I^-$, in agreement with the observed effects of the anions on the helix stability. Moreover, the NMR line width of the iodide ion has been found to decrease with increasing iodide concentration,[22] as expected for ion binding.

The purpose of the present paper is to provide more extensive experimental data on anion binding and anion effects on the conformational transition of κ-carrageenan and furcellaran and to attempt a unified theoretical explanation of these results, by the PBCM, in terms of combined effects of nonspecific Coulomb interactions and an ion-specific anion binding to the κ-carrageenan or furcellaran helix. This approach is justified by the success of the PBCM in rationalizing the cation-specific effects (cf. above). In the experimental studies of anion binding, we have chosen to monitor the ^{14}N spin relaxation of the thiocyanate ion, rather than the NMR relaxation of the

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Table I
PBCM Model Parameters

	coil		helix	
	l (Å)	a (Å)	l (Å)	a (Å)
κ-carrageenan	10	3.3	4.1	5.1
furcellaran	16	3.3	6.7	5.1

halide ions. This is because the ^{14}N nucleus in thiocyanate, to a good approximation, relaxes by an intramolecular quadrupolar coupling,[23,24] which facilitates the molecular interpretation of the relaxation data.

II. Experimental Section

κ-Carrageenan was obtained from Sigma Chemical Co., and furcellaran was a kind gift from Litex A/S, Denmark. Fragments of enhanced structural regularity were prepared as described by Bryce et al.[25] The carrageenan samples were dialyzed against millipore-filtered water and ion-exchanged to the desired ion form.

The helical content and the helix onset temperature, T_o, of carrageenan samples were determined by optical rotation at 546 nm, measured on a Jasco DIP-360 polarimeter in a jacketed cell with a 5-cm path length. NMR data were obtained on a Nicolet NIC-360 NMR spectrometer with 8.497-T magnet. The ^{14}N spectra were recorded at 26.14 MHz for samples in 10-mm tubes. The transverse relaxation rate, R_2, was obtained from the adsorption signal line width at half-height, $\Delta\nu_{1/2}$, using the relation $R_2 = \pi\Delta\nu_{1/2}$. The longitudinal relaxation rate was obtained by inversion recovery. The ^{133}Cs spectra were recorded at 47.45 MHz for samples in 5-mm tubes. The observed relative chemical shift, $\Delta\delta_{obs}$, of ^{133}Cs in a sample was obtained as the difference $\delta_{obs}-\delta_{ref}$, where δ_{obs} and δ_{ref} are the shifts, measured at the same temperature, in the sample and in a reference solution containing the same concentrations of free ions. The conditions (temperature, salt concentration) of the NMR studies were chosen such that a full conversion of the carrageenans to the helical state should be expected.

III. Theory

Electrostatic Model. In the Poisson–Boltzmann cell model,[26-29] a linear polyelectrolyte is treated as a cylindrical rod of radius a, with a homogeneous surface charge density, σ. In the modeling of carrageenans, a and σ (Table I) were obtained, as previously,[15-18] from the polymer partial molar volume and from X-ray fiber diffraction data. The Poisson–Boltzmann equation is solved in a cylindrical cell, containing solvent and mobile ions, centered around the polymer rod. The cell radius, b, is given by the polymer concentration and the helical content. The Poisson–Boltzmann equation in cylindrical symmetry then becomes

$$\frac{1}{r}\frac{d}{dr}\left(r\frac{d\phi}{dr}\right) = -\frac{eN_A}{\epsilon_0\epsilon_r}\sum_i c_{i0}z_i \exp(-e\phi z_i/kT), \quad a < r \le b \tag{1}$$

to be solved with the boundary conditions $d\phi/dr(b) = 0$ and $d\phi/dr(a) = -b/\epsilon_0\epsilon_r$ ($\phi(b)$ is regarded as the reference point and taken to be zero). Here $c_{i,0}$ is the concentration of ions of charge z_i at $r = b$. For the case of ion binding, the charge density σ is modified by the degree of ion binding. The case of only one class of binding ions can be found in our former papers.[15,18,30] Here we will consider the case of two classes of binding ions (ion species 1 and 2). The charge density is given by the degrees of ion binding (x_1 and x_2) as

$$\sigma = \sigma_0(1 - x_1z_1/n_1 - x_2z_2/n_2) \tag{2}$$

where σ_0 is the surface charge density for a specific conformation of polyelectrolyte in the absence of ion binding, and n_1 and n_2 are the numbers of polymer changes per binding site for ion species 1 and 2, respectively.

We will distinguish between two cases of ion binding, referred to as competitive and noncompetitive binding, respectively. In the noncompetitive case, the two types of binding ions bind to two different classes of sites, and there is no direct competition for either type of site. There will, however, still be an interdependence in the binding to the two classes of sites via the charge density as given by eq 2. The degrees of binding in the noncompetitive case are given as

$$x_1 = \frac{K_{1,0}\exp(-e\phi(a)\,z_1/kT)c_{1,0}}{1 + K_{1,0}\exp(-e\phi(a)\,z_1/kT)c_{1,0}} \tag{3a}$$

$$x_2 = \frac{K_{2,0}\exp(-e\phi(a)z_2/kT)c_{2,0}}{1 + K_{2,0}\exp(-e\phi(a)\,z_2/kT)c_{2,0}} \tag{3b}$$

Here, $K_{i,0}$ is the intrinsic binding constant of ion species i (i = 1, 2) and $\phi(a)$ is the electrostatic surface potential.

In the case of competitive ion binding, there is only one class of sites on the polyion, for which the two ions compete ($n_1 = n_2 = n$). The binding degrees are then obtained as

$$x_1 = \frac{K_{1,0}\exp(-e\phi(a)\,z_1/kT)c_{1,0}}{1 + K_{1,0}\exp(-e\phi(a)\,z_1/kT)c_{1,0} + K_{2,0}\exp(-e\phi(a)\,z_2/kT)c_{2,0}} \tag{4a}$$

$$x_2 = \frac{K_{2,0}\exp(-e\phi(a)\,z_2/kT)c_{2,0}}{1 + K_{1,0}\exp(-e\phi(a)\,z_1/kT)c_{1,0} + K_{2,0}\exp(-e\phi(a)\,z_2/kT)c_{2,0}} \tag{4b}$$

The Poisson–Boltzmann equation has to be solved in a self-consistent way to satisfy eqs 2 and 3 or 4.

Conformational Equilibria. The evaluation of the free-energy contributions to the conformational equilibria has been presented previously,[15,18,30] and we will therefore only discuss the necessary modifications to handle site-specific binding of two types of ions. Disregarding, for the moment, the entropy of mixing between helical and coil units (cf. below), the conformational equilibrium is determined by the difference in chemical potential between the helical and the coil conformers, $\Delta\mu_{rep}$

$$\Delta\mu_{rep} = \mu_{rep}(\text{helix}) - \mu_{rep}(\text{coil}) \tag{5}$$

As before,[18,30] we have chosen to divide the chemical potential into three contributions, due to the electrostatic interactions, the free energy of binding, and to all other (nonelectrostatic) contributions, respectively:

$$\Delta\mu_{rep} = \Delta\mu_{el} + \Delta\mu_{binding} + \Delta\mu_{nonel} \tag{6}$$

For convenience, $\Delta\mu_{rep}$ will here be expressed per covalent *polymer charge* (i.e., per sulfate group, not per disaccharide unit). For noncompetitive binding, the treatment is completely analogous to the derivations in refs 18 and 30, leading to the following expressions, when only specific binding to the helix is considered.

$$\Delta\mu_{el} = \mu_{el}(\text{helix}) - \mu_{el}(\text{coil}) \quad (7)$$

$$\mu_{el} = -e\phi(a)\,(1 - x_1z_1/n_1 - x_2z_2/n_2) + E_{el} + kTV_{solvent}N_A\sum(c_{1,av} - c_{i,0}) \quad (8)$$

$$\Delta\mu_{binding} = \Delta\mu_{1,binding} + \Delta\mu_{2,binding} \quad (9)$$

$$\Delta\mu_{i,binding} = (kT/n_i)\{x_i \ln x_i + (1 - x_i)\ln(1 - x_i) - x_i \ln(K_{i,0}c_{i,0})\} \quad (i = 1, 2) \quad (10)$$

$$\Delta\mu_{nonel} = \mu^\circ_{p,ion}(\text{helix}) - \mu^\circ_{p,ion}(\text{coil}) \quad (11)$$

Here $\Delta\mu_{1,binding}$ and $\Delta\mu_{2,binding}$ are the free-energy contributions due to specific binding to sites 1 and 2, respectively. (Since, for noncompetitive binding, the two classes of sites are assumed to bind different ions, we let the sites and the ions carry the same indices.) $V_{solvent}$ is the volume of solvent (per unit polymer charge) in the cell, and $c_{i,av}$ is the cell average concentration of ionic species i in the cell. E_{el} is the electrostatic interaction energy (per charged unit) defined as

$$E_{el} = (\epsilon_r\epsilon_0/2)\int(\nabla\phi)^2\,dV \quad (12)$$

In our earlier treatment,[18] we only had to consider one class of sites and one class of binding ions. Equations 7–11 reduce to the corresponding expressions in ref 18 when x_2 is identically zero.

For competitive binding, the expressions for $\Delta\mu_{el}$ and $\Delta\mu_{nonel}$ remain unchanged, but $\Delta\mu_{binding}$ becomes slightly different

$$\Delta\mu_{binding} = (kT/n)\{x_1 \ln x_1 + x_2 \ln x_2 + (1 - x_1 - x_2)\ln(1 - x_1 - x_2) - x_1 \ln(K_{1,0}c_{1,0}) - x_2 \ln(K_{2,0}c_{2,0})\} \quad (13)$$

since, with two different sites, each class of sites has a separate contribution to the entropy of mixing from the unoccupied sites, by the second term in eq 10. For competitive binding, there is only one class of unoccupied sites, and the entropy contribution is given by the third term in the above equation. Apart from this difference, the derivation is analogous to the derivations given in refs 18 and 30.

The entropy of mixing helix and coil units should, in principle, be considered.[15] However, in this study, all our theoretical predictions concern cases where the helical content is constant, and, hence, the entropy of mixing helix and coil units is also constant and may be neglected.[15] For instance, the experimental conformational stability diagrams refer to the onset of helix formation at a constant temperature. The theoretical curve can then be obtained by calculating μ_{rep} in an arbitrarily chosen reference point, whereafter the remainder of the diagram is given by adjusting the salt concentrations so that μ_{rep} is constant along the curve in the calculations. The binding studies, on the other hand, were performed under all-helix conditions.

IV. Results and Discussion

This section presents new experimental data on the anion specificity of κ-carrageenan and furcellaran, which will be analyzed as they are presented, within the theoretical model specified above. In the analysis, we will have to make certain minimum assumptions regarding the nature and the specificity of the anion binding sites,

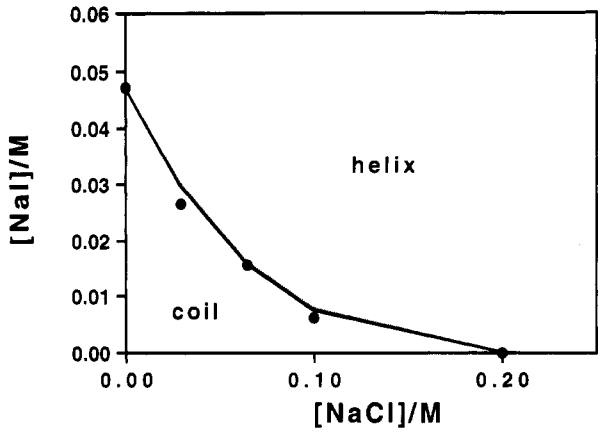

Figure 2. Conformational stability diagram at 18 °C of 5 mM sodium κ-carrageenan in mixed NaCl and NaI solutions. Points are experimental; lines indicate PBCM predictions with one (solid line; $K_{I,0}$ = 760 M^{-1}) and two (dotted line; $K_{I,0}$ = 330 M^{-1}) iodide-binding sites per repeating unit of the helix, respectively.

Table II
Helix Onset Temperatures of 0.19% (0.005 M) Sodium κ-Carrageenan in 0.1 M of Various Sodium Salts

anion	Cl^-	NO_3^-	Br^-	SCN^-	I^-
T_o/°C	10.5	14.0	21.5	25.0	36.5

since no independent information is available. Thus, we will assume one class of such sites, which only exist on the κ-carrageenan helix. These sites may be the same as, or different from, the cation-binding sites inferred from previous studies; both cases will be investigated. Unless otherwise stated, we will assume that the carrageenan helix contains one binding site per repeating unit. This amounts to one site per two disaccharide units (one from each of the two chains of the helix). Furthermore, we will, at least initially, assume that only those anions which are known to markedly effect the coil–helix equilibrium bind. The latter assumption is also consistent with our previous assumptions that, e.g., NaCl affects the transition only as an inert electrolyte. However, we will also present some indications that a finite binding of chloride ions may in fact, have to be considered.

Anion Effects on the Conformational Transition. Table II shows T_o for κ-carrageenan in 0.1 M sodium salt solutions of different anions. T_0 increases according to the sequence $Cl^- < NO_3^- < Br^- < SCN^- < I^-$, which reflects the increasing helix-stabilizing effects of the anions. The same sequence was recently found for furcellaran.[6] For κ-carrageenan, Norton et al. reported a sequence where the order of SCN^- and I^- was reversed in potassium salt solutions.[5] However, the latter results were not confirmed by our measurements in 0.1 M potassium salt solutions, where we obtained (for 0.19% (w/v) sodium κ-carrageenan samples) T_0 values of 64.0 ± 0.5 and 56.0 ± 0.5 °C for KI and KSCN, respectively.

Figures 2–4 are conformational stability diagrams, showing the salt concentrations required to induce, at a fixed temperature, an onset helix of formation of κ-carrageenan or furcellaran for mixtures of NaCl and NaSCN (or NaI). The anion specificity of the transition is revealed by two features, common to all three diagrams: The intercept with the NaSCN (or NaI) axis is smaller than the intercept with the NaCl axis, and the boundary curve between the helix and coil state is curved. A theoretical rationalization of both these features of the stability diagrams is obtained by choosing a suitable association constant of the anion binding to the helical conformation. The concave helix–coil boundary curve reflects the

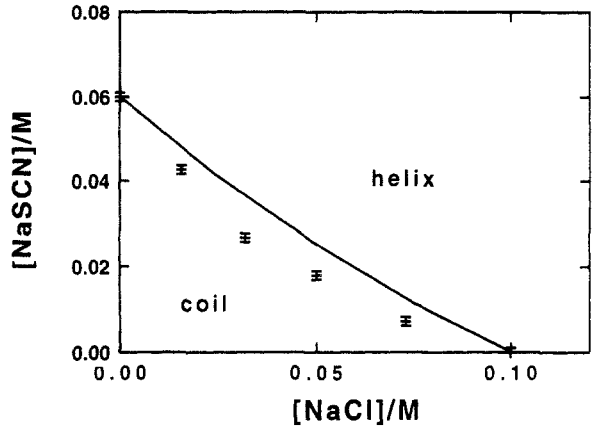

Figure 3. Conformational stability diagram at 10 °C of 5 mM sodium κ-carrageenan in mixed NaCl and NaSCN solutions. Points are experimental, and the solid line indicates PBCM predictions with $K_{SCN,0}$ = 128 M^{-1}.

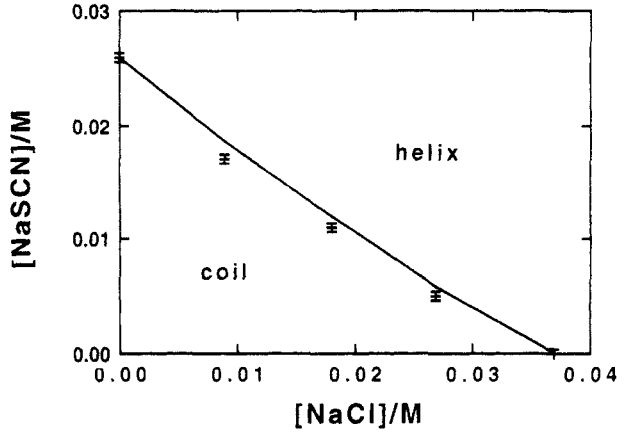

Figure 4. Conformational stability diagram at 25 °C of 5 mM sodium furcellaran in mixed NaCl and NaSCN solutions. Points are experimental, and the solid line indictes PBCM predictions with $K_{SCN,0}$ = 60 M^{-1}.

Table III
Anion Binding Constants Deduced (See Text) from Ion Effects on Carrageenan Coil-Helix Transitions

T/°C	10	10	18	25	40[a]
carrageenan type	κ	κ	κ	furcellaran	furcellaran
anion	SCN^-	I^-	I^-	SCN^-	I^-
K_0/M^{-1}	128	1600	760	60	91

[a] From data in ref 6.

screening of the electrostatic interaction by the salt; the degree of anion binding to the negatively charged helix increases in the presence of inert electrolyte. As shown in Table III, the binding constants obtained from the analysis are on the order of 10^3 M^{-1} for I^- and 10^2 M^{-1} for SCN^- at room temperature. Binding constants of similar orders of magnitude as those for κ-carrageenan are obtained for furcellaran, although this conclusion is weakened somewhat by the significant temperature dependence of the binding constants. (The choices of temperatures for the stability diagrams were dictated by the temperatures of the NMR experiments below, which, in turn, were chosen on grounds of sensitivity and the requirement that the carrageenans should be all-helical.) It is notable that, although the anion specificity of the transitions of the carrageenans is weaker than the cation specificity, the intrinsic binding constants deduced for the anions are much larger than those obtained, by a similar analysis, for the binding cations (Cs^+, K^+, Rb^+, etc.), which were on the order of a few M^{-1}.[6,18]

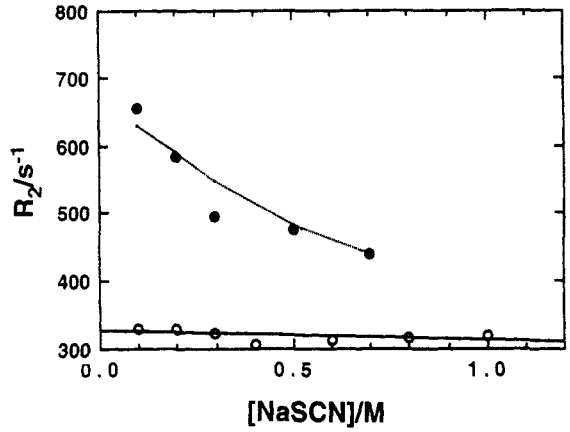

Figure 5. Experimental and predicted ($K_{SCN,0}$ = 128 M^{-1} and $R_{2,B}$ = 8300 s^{-1}; dotted line) variation of the $SC^{14}N^-$ transverse relaxation rate with the concentration of NaSCN in 1% sodium κ-carrageenan at 10 °C. The solid baseline indicates R_2 of NaSCN reference solutions.

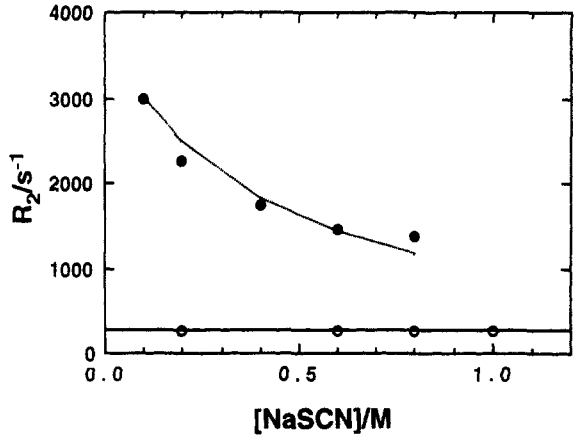

Figure 6. Experimental and predicted ($K_{SCN,0}$ = 60 M^{-1} and $R_{2,B}$ = 62 000 s^{-1}; dotted line) variation of the $SC^{14}N^-$ transverse relaxation rate with the concentration of NaSCN in 1% sodium furcellaran at 25 °C. The solid baseline indicates R_2 of NaSCN reference solutions.

Spectroscopic Evidence of Anion Binding. NMR relaxation is a very sensitive method to detect even small proportions of ions bound to a macromolecule. On the other hand, an enhancement of the relaxation rate in a macromolecular gel may not uncritically be regarded as evidence of binding, since previous studies[31,32] of the NMR relaxation of small molecules (including ions, water molecules, and various uncharged cosolutes) have indicated a quite general enhancement of the transverse relaxation rate on gel formation. The origin of this effect is not well understood. There are, however, strategies to distinguish a relaxation enhancement due to binding from the more general "gel structure" effect. These include measurement of the relaxation rate as a function of added ions (which should affect the fraction of bound ions in cases of true binding), comparisons of binding and inert additives in the same sample (whereby structure and binding effects may be distinguished), and binding competition experiments, where the effect on the relaxation rate of a binding ion of an added competing ion is monitored. All these strategies have been employed in the present work.

Figures 5 and 6 show the variation of $R_2(SC^{14}N^-)$ in κ-carrageenan and furcellaran with added NaSCN. For both carrageenans, R_2 decreased on addition of NaSCN. To check that this effect was not due to a change in the gel structure on addition of salt, we compared the relative enhancement of R_2 of thiocyanate and of urea (which was considered to be an inert additive) in κ-carrageenan

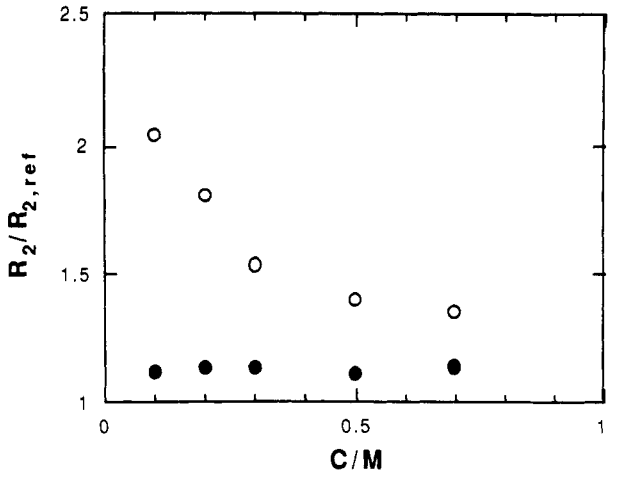

Figure 7. Relative transverse relaxation enhancement of ^{14}N of thiocyante (O) and urea (●) in 1% sodium κ-carrageenan containing equimolar mixtures of sodium thiocyanate and urea at 10 °C.

samples containing equal concentrations of both additives. The results are shown in Figure 7. $R_2(SC^{14}N^-)$ in these samples was, within experimental uncertainty, the same as in the samples without urea (Figure 5). An enhancement, relative to an aqueous solution, of the relaxation is also observed in the $R_2(^{14}N)$ of urea, but its relative magnitude is much smaller than that for thiocyanate, and, moreover, it is almost independent of the urea concentration. Both these features are expected if urea does not bind and if the structure of the samples remains constant (on the length scale which affects the relaxation data) at all concentrations of the additives. As an additional check, we measured the longitudinal relaxation, R_1, of thiocyanate ions in κ-carrageenan and furcellaran. In both systems, we found a decrease in $R_1(SC^{14}N^-)$ with added salt (not shown), consistent with the binding interpretation. The relaxation enhancement was generally smaller for R_1 than for R_2. This indicates that the nonextreme narrowing regime has been reached for $SC^{14}N$ relaxation in the carrageenan samples.

In all cases, the $SC^{14}N^-$ NMR signal showed a single Lorentzian line shape with no loss of signal intensity. The temperature dependence of $R_2(SC^{14}N^-)$ in κ-carrageenan samples at three different NaSCN concentrations was measured in the range 10–70 °C, and, in all cases, a monotonic decrease in the excess relaxation rate with increasing temperature was found. These results indicate that the exchange between bound and free ions is in the rapid exchange regime. Under such conditions, assuming one class of bound ions, the observed relaxation rate is given as[33]

$$R_{i,obs} = (1 - P_B)R_{i,F} + P_B R_{i,B} \quad (i = 1, 2) \qquad (14)$$

where $R_{i,F}$ and $R_{i,B}$ are the intrinsic relaxation rates of the ions in the free and bound states, respectively, and P_B is the fraction of bound ions.

By calculating P_B from the PBCM, using the corresponding intrinsic binding constants obtained from the theoretical analysis of the stability diagrams (Figures 3 and 4), the experimental $R_2(SC^{14}N^-)$ data of Figures 5 and 6 were fit to eq 14, using the intrinsic transverse relaxation rate of the bound anions as a single-fitting parameter ($R_{2,F}$ was assumed equal to $R_2(SC^{14}N^-)$ of the reference solution). It was assumed, in all model calculations, that κ-carrageenan and furcellaran have the same helix and coil conformations and site densities, with the only difference being the surface charge density. This procedure gave

Table IV
Transverse Relaxation Rates of $SC^{14}N^-$ at 10 °C in 1% Sodium κ-Carrageenan Containing 0.1 M NaSCN and 0.1 M NaX

X	$R_2(SC^{14}N^-)/s^{-1}$	X	$R_2(SC^{14}N^-)/s^{-1}$
Cl	606	SCN	582
NO_3	552	Br	520
I	390		

reasonable fits to both data sets, although the calculations predict a slower variation of R_2 with salt concentration than was observed experimentally, especially for κ-carrageenan. A better fit to the latter data would be obtained with a larger intrinsic binding constant (on the order of 400 M^{-1}). Such a binding constant would predict a smaller intercept with the NaSCN axis in the conformational stability diagram, under the assumption that the affinity of chloride ions to the sites is negligible (cf. below).

The fitted value of $R_{2,B}$ was found to be 7–8 times higher for furcellaran at 25 °C than for κ-carrageenan at 10 °C. Provided that our analysis is otherwise valid, this difference should mainly be caused by differences in the effective slow correlation times dominating the transverse relaxation, since the ^{14}N quadrupole coupling constant is not expected to vary significantly in thiocyanate. These slow correlation times could include the lifetime of bound ions at the site as well as the time it takes for ions to diffuse between sites on helices that are orientationally uncorrelated. To interpret the dynamics of bound ions in carrageenan samples is a difficult problem, which is beyond the scope of the present paper. It is possible, however, that the larger $R_{2,B}$ of furcellaran reflects a higher degree of orientational order of furcellaran helices (furcellaran has a stronger tendency to aggregate under otherwise equal salt conditions).

Anion–Anion Competition. Table IV shows results of anion competition experiments, where $R_2(SC^{14}N^-)$ was measured in κ-carrageenan samples containing 0.1 M of NaSCN and 0.1 M of the sodium salt of another anion and compared with the relaxation in a reference sample containing 0.2 M of NaSCN. In such an experiment, any anion that binds more strongly than SCN^- should give rise to a decrease in $R_2(SC^{14}N^-)$ relative to the reference sample, while an increase should be seen for ions that bind less strongly. Competition effects are clearly revealed by the experiments, with chloride giving rise to an increase and iodide a large decrease in $R_2(SC^{14}N^-)$. Unexpectedly, however, both nitrate and bromide produced a decrease in $R_2(SC^{14}N^-)$, while an increase would be expected from the relative effects of the anions on T_0 (Table II). At present, we are unable to explain this discrepancy.

More detailed data on the dependence of $R_2(SC^{14}N^-)$ on the NaI concentration in κ-carrageenan samples containing 0.1 M NaSCN are given in Figure 8, together with the predictions from our model. With the assumption that the SCN^- and I^- ions compete for the same sites, with binding constants as obtained from the stability data ($K_{SCN,0}$ = 128 M^{-1} and $K_{I,0}$ = 1600 M^{-1}; Table III) and with $R_{2,B}$ = 8300 s^{-1} (Figure 5), the PBCM binding produced a good theoretical fit to the experimental curve.

Addition of inert salt should increase the degree of binding of a binding anion, because of electrostatic screening effects. Figure 9 shows the variation in $R_2(SC^{14}N^-)$ with added LiCl to 1% κ-carrageenan in 0.2 M NaSCN. A slight increase in $R_2(SC^{14}N^-)$ may be seen, although it is considerably smaller than the theoretical prediction for an added inert electrolyte. If, however, a finite binding of Cl^- is assumed, competition of chloride ions for the binding sites leads to a lowering of the predicted

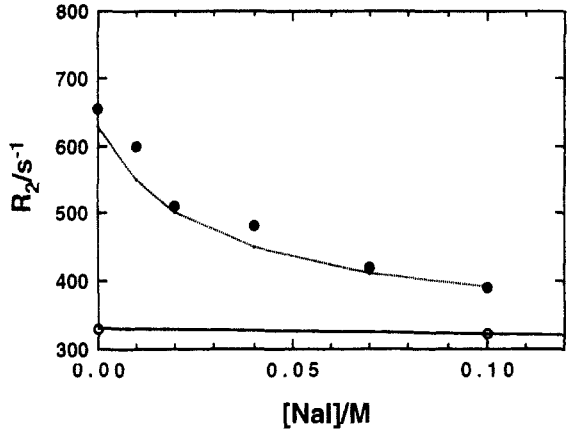

Figure 8. Experimental and predicted ($K_{SCN,0}$ = 128 M^{-1}, $K_{I,0}$ = 1600 M^{-1}, and $R_{2,B}$ = 8300 s^{-1}; dotted line) variation of the $SC^{14}N^-$ transverse relaxation rate with the concentration of NaI in 1% sodium κ-carrageenan, 0.1 M NaSCN at 10 °C. The solid baseline indicates R_2 of NaSCN reference solutions.

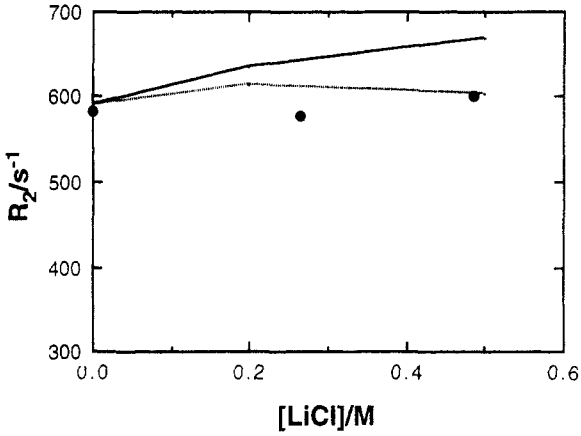

Figure 9. Experimental and predicted (for $K_{SCN,0}$ = 128 M^{-1}, $R_{2,B}$ = 8300 s^{-1}, and $K_{Cl,0}$ = 0 (solid line) or 15 M^{-1} (dotted line)) variation of the $SC^{14}N^-$ transverse relaxation rate with the concentration of LiCl in 1% sodium κ-carrageenan in 0.2 M NaSCN at 10 °C.

curve relative to the "pure screening" case shown in Figure 9. Thus, a better agreement between theory and experiment is obtained with an intrinsic binding constant of $K_{Cl,0}$ = 15 M^{-1} (Figure 9).

Cation–Anion Competition. A puzzling observation regarding the carrageenans, confirmed by the above results, is that the helices of the lower charged carrageenans evidently bind certain anions as well as certain cations, while no binding of either type of ions is seen for the closely similar ι-carrageenan. A superficially appealing explanation to these observations would be that the specific cations and anions bind to the same sites on the lower charged carrageenans, in which case only one class of sites needs be invoked. Partly to check for this possibility, we performed measurements on samples containing both specific anions and specific cations. Figure 10 shows the conformational stability diagram of κ-carrageenan in mixtures of CsCl and NaI. The measurements were made at high salt concentrations (T_0 = 70 °C) to ensure a large degree of binding and, hence, the largest possible difference between the cases of competitive and noncompetitive binding. Theoretical curves were then calculated for both cases. Unfortunately, the experimental curve falls between the two theoretical curves, which means that the theoretical analysis of the stability diagram does not allow a distinction between the two cases to be made.

More information on cation/anion competition can be gained from NMR experiments. Figure 11 shows that

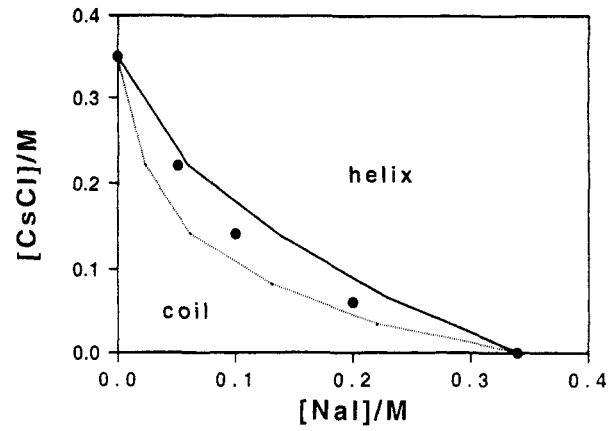

Figure 10. Conformational stability diagram at 70 °C of 5 mM sodium κ-carrageenan in mixed NaI and CsCl solutions. Points are experimental; lines indicate PBCM predictions with $K_{Cs,0}$ = 1.14 M^{-1} (obtained by extrapolation from values in ref 18) and $K_{I,0}$ = 100 M^{-1}. The solid and dotted lines represent competitive and noncompetitive ion binding, respectively.

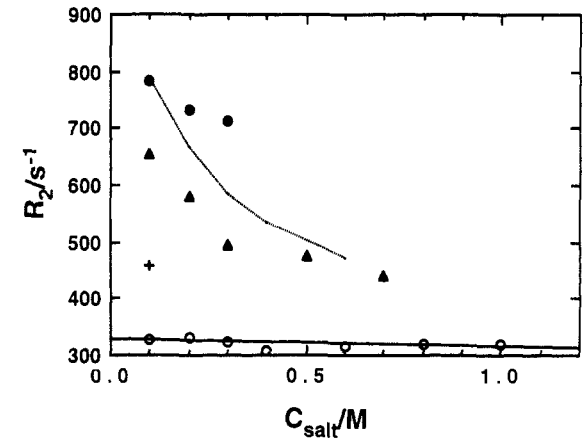

Figure 11. Experimental (●) and calculated ($K_{SCN,0}$ = 128 M^{-1}, $K_{K,0}$ = 6 M^{-1}, and $R_{2,B}$ = 8300 s^{-1}), for noncompetitive binding (dotted line) and competitive binding (+), variation of the $SC^{14}N^-$ transverse relaxation rate with the concentration of KSCN in 1% sodium κ-carrageenan at 10 °C. The corresponding data for NaSCN (▲; cf. Figure 5) are shown for comparison.

$R_2(SC^{14}N^-)$ in κ-carrageenan samples is larger in the presence of KSCN than in the presence of NaSCN. The K^+ binding constant[18] (=6 M^{-1}) is much smaller than the SCN^- binding constant (=128 M^{-1}) at 10 °C. But owing to the opposite charges born by K^+ and SCN^-, a sufficiently negative surface potential ($e\phi(a)/kT \approx -1.5$) would overcome the difference in the binding constants. At our experimental range, $e\phi(a)/kT \leq -2$, and so K^+ becomes the dominant binding ion. Therefore, a binding competition between K^+ and SCN^- should lead to a significant decrease in the $SC^{14}N^-$ relaxation rate for the KSCN systems compared to the NaSCN systems, contrary to the experimental finding (Figure 11). A noncompetitive binding of K^+ and SCN^-, on the other hand, would mean that a binding of K^+ lowers the surface charge density and enhances the degree of binding of SCN^-. Figure 11 shows that a theoretical prediction for noncompetitive binding, based on the above values of the binding constants, gives a qualitatively correct behavior, while a calculation for competitive binding predicts a large decrease in $R_2(SC^{14}N^-)$.

The effect of a binding anion on the binding of a specific cation, as measured by the change in the relative NMR shift of $^{133}Cs^+$, $\Delta\delta(^{133}Cs^+)$, on addition of NaI, is shown in Figure 12. Interestingly, a nonmonotonic variation of $\Delta\delta(^{133}Cs^+)$ is seen. This effect is predicted in the case of a noncompetitive binding, as shown by the theoretical

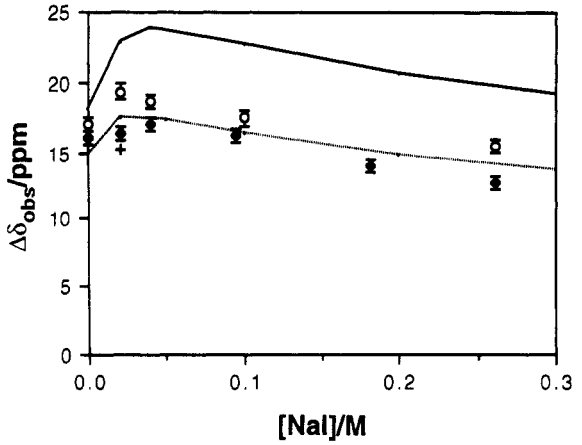

Figure 12. Experimental (points) and calculated, for noncompetitive binding (lines), variation of the relative cesium shift with the concentration of NaI in 2% cesium furcellaran at 25 °C ($K_{Cs,0}$ = 4.1 M^{-1}, $K_{I,0}$ = 400 M^{-1}; ●, dotted) and at 5 °C ($K_{Cs,0}$ = 7.5 M^{-1}, $K_{I,0}$ = 2700 M^{-1}; ○, solid). The values of $K_{Cs,0}$ and $K_{I,0}$ were obtained by extrapolation from values in ref 18 and Table III, respectively; the intrinsic shift of bound cesium ions was assumed to be 50 ppm.[30] A single calculation at 5 °C (+) shows the shift decrease predicted for competitive ion binding.

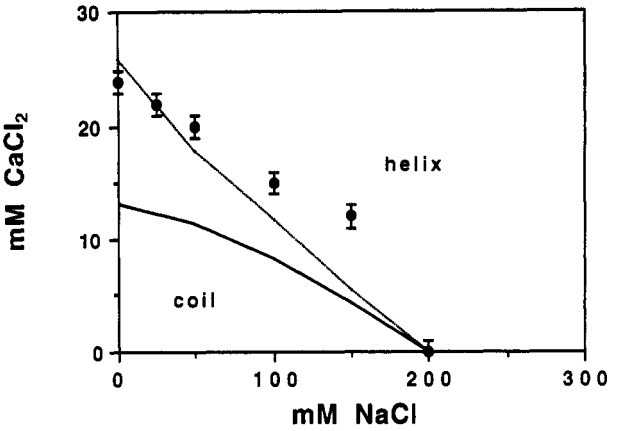

Figure 13. Conformational stability diagram at 18 °C of 5 mM sodium κ-carrageenan in mixed NaCl and $CaCl_2$ solutions. Points are experimental; lines indicate PBCM predictions with $K_{Cl,0}$ = 0 (solid line) or 15 M^{-1} (dotted line).

curves, whereas a competitive binding predicts a monotonic decrease. According to the model, the initial increase in cation binding is caused by an increased negative charge density, caused by iodide binding, whereas the decrease in the binding at higher concentrations of NaI is caused by the screening effect of free ions. To ensure that the observed effect was not simply due to an increase in helical content on addition of iodide ions (the exact degree of helical conversion was difficult to determine independently, because of aggregation effects), the measurements were repeated at two temperatures. The nonmonotonic behavior was more pronounced at the lower temperature, as again predicted by the model, which is unexpected if the effect were due to salt-induced changes in the helical content.

Nature of the Anion-Binding Sites. None of the experiments of this study provides direct information on the density of anion-binding sites on the carrageenan helix. In the calculations above, we have consistently assumed that each repeating unit of the double helix contains a binding site. Whereas a lower site density seems improbable for symmetry reasons, a higher site density could be imagined. We therefore made a few calculations with a higher site density (two sites per repeating unit) and found that this would give an equally good fit to the experimental curves, but with smaller values of the binding constants (cf. Figure 2).

A more direct measurement of the site density, through equilibrium dialysis, was attempted by the following experiment. A 2% gel of sodium κ-carrageenan was prepared in a salt solution containing 0.0050 M KI and 0.2 M KNO_3 and was subsequently dialyzed against an equal volume of the same salt solution. After dialysis at 3 °C for 1 week, the iodide concentration in the external solution, as determined titrimetrically, had decreased to 0.0042 M. With one cation-binding and one anion-binding site per two disaccharides and with $K_{K,0}$ = 7.0 M^{-1},[18] a PBCM analysis of this result yields an iodide binding constant of 85 M^{-1}, under the assumption of a negligible binding constant for NO_3^-. This should be compared with an iodide binding constant on the order of 3000 M^{-1}, as extrapolated from the data in Table III. We attempted to eliminate this discrepancy by assuming a finite binding also of the nitrate ions, which would then compete with iodide ions for the binding sites. We found that to fit the experimental results with an iodide binding constant on the order of 3000 M^{-1}, a binding constant for NO_3^- on the order of 800 M^{-1} would be required. The latter value is unexpectedly high. Another possibility that may have to be considered is that some sites are inaccessible in the gel, owing to aggregation of the helices. Aggregation also increases the magnitude of the negative surface potential, which further decreases the fraction of bound anions.

The evidence presented here supports the assumption that the sites on the carrageenan helix that bind anions are distinct from those that bind cations. This also seems probable in view of the differing characteristics of the anion and the cation binding. All evidence suggests that cations are bound to a size-selective site, probably involving oxygen functions on the helix.[18] The relative affinities of the (much larger) anions, on the other hand, are not readily explained by the geometric sizes of the ions but rather follow the lyotropic series. This also means that all anions studied here may have a finite affinity for the binding sites, and some indications that this may actually be the case have been seen in the comparisons between the theory and experiments above.

The possibility of a finite binding of, e.g., chloride ions, has consequences also for the analyses of the cation effects on the conformational transition of the lower charged carrageenans. In our previous analyses[6,15-18] of salt effects, focusing primarily on the effects of cation, no binding of chloride ions were considered. A recalculation reveals, however (Figure 13), that a chloride binding constant of the order of $K_0(Cl^-)$ = 15 M^{-1} yields a theoretical stability diagram for κ-carrageenan in the presence of $CaCl_2$/NaCl mixtures which, quantitatively, agrees better with the experimental results than the previous calculation[15] in the absence of chloride binding. On the other hand, the curvature of the diagram is now in poorer agreement with experiment. Still, the conclusion from Figure 13 must be that a finite chloride binding is not inconsistent with previously reported experimental data nor with our previous conclusions.

V. Conclusions

The experimental evidence presented in this paper confirms rather conclusively that there is a binding of anions to the helix of the lower charged carrageenans and that different anions compete for the binding sites. Also, it seems clear that the cations and anions bind to different sites on the carrageenan helix. At this stage, the nature of the anion-binding sites is elusive, and more experiments

are needed on this point. In particular, it needs clarifying whether also ions like chloride and nitrate have a finite affinity for the sites. It may be recalled that a binding to the closely related agarose helix of all anions studied here was demonstrated in a previous NMR study.

A combined analysis, by the PBCM, of the anion binding and of the effects of anions on the conformational stability strongly supports the notion that the latter effect is a consequence of the former, although there are some yet unexplained deviations between the relative helix-stabilizing effects of the anions and their affinity, as given by $SC^{14}N^-$ NMR relaxation, to the κ-carrageenan helix. Quantitatively, there are also some unresolved discrepancies between binding constants obtained from different types of experiments. In addition, the magnitude of the anion binding constants are surprisingly large. However, the intrinsic affinity is much larger than the effective affinity, owing to the high negative charge density on the carrageenan helix.

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References and Notes

(1) Rees, D. A.; Morris, E. R.; Thom, D.; Madden, J. K. In *Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1982; Vol. 1, pp 195–290.
(2) Rochas, C.; Rinaudo, M. *Biopolymers* **1980**, *19*, 1675.
(3) Grasdalen, H.; Smidsrød, O. *Macromolecules* **1981**, *14*, 1845.
(4) Smidsrød, O.; Grasdalen, H. *Hydrobiologia* **1984**, *116/117*, 178.
(5) Norton, I. T.; Morris, E. R.; Rees, D. A. *Carbohydr. Res.* **1984**, *134*, 89.
(6) Zhang, W.; Piculell, L.; Nilsson, S. *Biopolymers* **1991**, *31*, 1727.
(7) Austen, K. R. J.; Goodall, D. M.; Norton, I. T. *Carbohydr. Res.* **1985**, *140*, 251.
(8) Piculell, L.; Håkansson, C.; Nilsson, S. *Int. J. Biol. Macromol.* **1987**, *9*, 297.
(9) Grasdalen, H.; Smidsrød, O. *Macromolecules* **1981**, *14*, 229.
(10) Smidsrød, O.; Grasdalen, H. *Hydrobiologia* **1984**, *116/117*, 19.
(11) Paoletti, S.; Delben, F.; Cesaro, A.; Grasdalen, H. *Macromolecules* **1985**, *18*, 1834.
(12) Belton, P. S.; Morris, V. J.; Tanner, S. F. *Int. J. Biol. Macromol.* **1985**, *7*, 53.
(13) Piculell, L.; Nilsson, S.; Ström, P. *Carbohydr. Res.* **1989**, *188*, 121.
(14) Tanner, S. F.; Morris, V. J.; Belton, P. S. *Int. J. Biol. Macromol.* **1990**, *12*, 302.
(15) Nilsson, S.; Piculell, L.; Jönsson, B. *Macromolecules* **1989**, *22*, 2367.
(16) Nilsson, S.; Piculell, L. *Macromolecules* **1989**, *22*, 3011.
(17) Nilsson, S.; Piculell, L. *Macromolecules* **1990**, *23*, 2776.
(18) Nilsson, S.; Piculell, L. *Macromolecules* **1991**, *24*, 3804.
(19) von Hippel, P. H.; Schleich, T. In *Structure and Stability of Biological Macromolecules*; Timasheff, S. N., Fasman, G. D., Eds.; Marcel Dekker: New York, 1969; p 417.
(20) Piculell, L.; Nilsson, S. *J. Phys. Chem.* **1989**, *93*, 5596.
(21) Record, M. T.; Anderson, C. f.; Lohman, T. M. *Quart. Rev. Biophys.* **1978**, *11*, 103.
(22) Kvam, B.; Grasdalen, H. *Macromolecules* **1989**, *22*, 3919.
(23) Lucken, E. A. C. *Nuclear Quadrupole Coupling Constants*; Academic Press: London, 1969; Chapter 8.
(24) de P. Nicholas, A. M.; Wasylishen, R. E. *Can. J. Chem.* **1986**, *64*, 1839.
(25) Bryce, T. A.; Clark, A. H.; Rees, D. A.; Reid, D. S. *Eur. J. Biochem.* **1982**, *122*, 63.
(26) Fuoss, R. M.; Katchalsky, A.; Lifson, S. *Proc. Natl. Acad. Sci.* **1951**, *37*, 579.
(27) Alfrey, T.; Berg, P. W.; Morawetz, H. *J. Polym. Sci.* **1951**, *7*, 543.
(28) Marcus, R. A. *J. Chem. Phys.* **1955**, *23*, 1057.
(29) Jönsson, B.; Wennerström, H. *J. Phys. Chem.* **1987**, *91*, 338.
(30) Nilsson, S.; Zhang, W. *Macromolecules* **1990**, *23*, 5234.
(31) Piculell, L.; Nilsson, S. *J. Phys. Chem.* **1989**, *93*, 5602.
(32) Piculell, L.; Malmsten, M. Unpublished results.
(33) Winkler, H.; Gutsze, A. *Adv. Mol. Relax. Interact. Processes* **1981**, *21*, 159.