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Selectivity and cooperativity in the binding of calcium ions by pectins

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ABSTRACT

By the use of calcium and sodium specific electrodes, the study of the ionic interactions with well-characterized pectins has shown a greater affinity of pectins towards calcium ions when the degree of methylation of the samples and the ionic strength of the systems decreased. The affinity of pectic chains towards calcium ions increased also with the polymer concentration. Furthermore, the amount of free or bound calcium ions seemed to be independent of the gelation phenomenon, whereas the binding of sodium ions appeared to be strongly affected by this phase transition. It was shown that two types of interactions between the polymer and calcium ions can occur according to the solvent conditions; from an anti-cooperative character in water, due to typical polyelectrolyte effects, they showed a cooperative character in the presence of 0.1 M NaCl.

INTRODUCTION

Pectins are important ionic polysaccharides of plant cell walls consisting mainly of linearly connected α-(1 → 4)-D-galacturonic acid units and their methyl esters, interrupted in places by (1 → 2)-linked L-rhamnose units[1]. They are industrially extracted from plant by-products and used as gelling agents in different food applications depending on their methoxyl content[2]. High-methoxyl (HM) pectins usually have a degree of methylation (moles of methanol to 100 moles of galacturonic acid, dm) greater than 55%. Low-methoxyl (LM) pectins (dm < 50%) can be produced by controlled deesterification. In the presence of calcium ions, LM pectins form gels, the gelation being governed in a complex way by a large number of intrinsic and extrinsic parameters, including the dm, the charge distribution along the backbone, the molecular weight of the sample, the ionic strength, the pH, the temperature, and the presence of cosolutes[3].

The binding properties of cations to pectins and its oligomeric fragments has been the subject of a large number of experimental evaluations using different

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techniques[4–12]. All results illustrated the very high binding properties of pectins in comparison with other polyelectrolytes having the same charge density (ξ) and indicated clear deviations from polyelectrolyte theory because of the critical importance of the nature of the counterion. In dilute salt-free solutions, it was shown that calcium interactions with pectins increased with the charge density and polymer concentration[6], and was greater when the distribution of carboxyl groups was localised rather than at random[7]. Calcium binding to oligomeric fragments was found to be dependent on the degree of polymerization (dp) of the chains; it was purely electrostatic for dp < 15, while intermolecular chelate binding occurred at higher dp[8].

All of these binding properties are generally ascribed to a cooperative character of the calcium binding to poly(glycuronates) in agreement with the "egg-box" model initially proposed for alginates[10,11]. Indeed, in dilute salt-free solutions of pectins of low dm, the very low values of the calcium activity coefficients were explained by an intermolecular binding of calcium ions, leading to the formation of dimers[7]. However, the cooperative nature of calcium binding to pectins, i.e., the fact that the binding of one calcium ion facilitates the binding of the others is still an open question. Mattai and Kwak[13] have shown an anti-cooperative effect of very strong calcium binding to pectate at low polymer concentration, and for an ionic strength of 0.01 M Lips et al.[14] suggested that the binding of calcium to pectic chains was anti-cooperative whatever the ionic strength in the range 0.02 to 1 M NaCl was, whereas Braudo et al.[15] showed that in 0.1 M NaCl this binding is cooperative and that the higher the charge density, the more cooperative are the interactions.

Therefore, we have extensively studied the binding of calcium ions by pectins of different dm with a random distribution of carboxylate groups. The free ions in solutions and in gels were followed by potentiometry at various polymer and calcium concentrations and at different ionic strengths (0 and 0.1 M NaCl). Results were plotted as binding isotherms and Scatchard plots were fitted by the semi-empirical Hill equation to quantify the cooperativity of these interactions.

MATERIAL AND METHODS

Pectin samples.—The samples, A30 (dm = 28%, $\xi = 1.16$) and C73 (Genu-X-0907) (dm = 73%, $\xi = 0.44$), were commercial apple and citrus pectins provided by SBI (France) and Copenhagen Pectins (Denmark), respectively. Sample C48 (dm = 48%, $\xi = 0.84$) was obtained by acid-deesterification of C73, as described previously[16]. The structural charge density parameter ξ, introduced by Lifson and Katchalsky[17], is given by the equation:

$$\xi = e^2/bDkT(1 - \mathrm{dm}/100) = 1.61(1 - \mathrm{dm}/100) \text{ at } 25°C,$$

where e is the electron charge, k the Boltzmann constant, T the absolute temperature, b the length of the monomeric unit (4.35 Å)[18], and D the dielectric

constant of the solvent. The galacturonic acid contents of samples C73, C48, and A30 were 76.3, 82.6, and 76.8%, respectively[16], and their number-average molecular weights were 63 300, 53 400, and 65 100, respectively[19].

The samples were dissolved in distilled water with gentle stirring overnight. The pH of the solutions was adjusted to 7.4 with 0.1 M NaOH and the solutions were filtered through a 0.8-μm filter. Concentrations were calculated from the determination of the dry matter. The ionic strength was adjusted by addition of $CaCl_2$ solutions in water or in 0.2 M NaCl. Pectin–calcium systems were prepared by mixing pectin solutions (6 mL) at 70°C with hot $CaCl_2$ solutions (6 mL) for 3 min in a 50-mL beaker. The polymer concentrations were in the range 2 to 11 g/L, corresponding to $6 \cdot 10^{-3}$ to $28 \cdot 10^{-3}$ equiv/L and the $CaCl_2$ concentrations were in the range 0.005 to 0.01 M.

Calcium and sodium activity measurements.—Calcium and sodium activity were measured with selective electrodes (F212 Ca and G502 Na, Radiometer) and a saturated calomel electrode as reference, connected to a potentiometer (PHM 84, Radiometer).

The method for measuring the activities depended on the final physical state of the system. In the case of a gel, the electrodes were immersed in the pectin–calcium mixtures at 40°C, before gelation of the system, to allow network formation around the electrodes. The beaker was then covered with a Parafilm® sheet in order to limit evaporation. The value of the potential was recorded after 24 h at 20°C without any stirring. In the case of a solution, the electrodes were immersed in the mixture at 20°C after 24 h and the value of the potential was recorded after stabilization. The potassium and chloride ion concentrations resulting from diffusion from the calomel electrode were found to be 10^{-3} mol/L after 24 h. As the binding isotherms were continuous on both sides of the sol–gel transition (see results), it was considered that the error due to potassium chloride diffusion was negligible compared to the accuracy of the measurements.

Calibration was carried out with standard sodium or calcium chloride solutions, the concentrations being measured by conductimetric determination with silver nitrate. Cross selectivity of the calcium electrode to sodium ions was also taken into consideration.

Free calcium and free sodium concentrations were estimated using the relations given by the Debye–Hückel theory from the measured activity:

$$-\log f_{Ca^{2+}} = \frac{2.046\sqrt{I}}{1 + 1.97465\sqrt{I}} \qquad -\log f_{Na^{+}} = 0.5\sqrt{I}$$

$$C_i = \frac{a_i}{f_i}$$

where f_i is the activity coefficient of the i ion, C_i the concentration of free i ions, and I the total ionic strength arising from the polymer contribution and from the added electrolytes.

TABLE I

Total amount of calcium (mol/L) required for gelation of samples A30 and C48 in water and in 0.1 M NaCl

Concentration (equiv/L)	A30		C48	
	Water	0.1 M NaCl	Water	0.1 M NaCl
$6 \cdot 10^{-3}$	0.0020	0.0030	0.0025	0.0035
$13 \cdot 10^{-3}$	0.0025	0.0025	0.0030	0.0030
$28 \cdot 10^{-3}$	0.0030	0.0025	0.0035	0.0025

The values of the selectivity coefficient K_{Ca}^{Na}, corresponding to the Ca^{2+}–Na^{+} exchange equilibrium:

$$Ca^{2+} + 2\overline{Na}^{+} \rightleftarrows 2Na^{+} + \overline{Ca}^{2+}$$

$$K_{Ca}^{Na} = \frac{X_{Na}^2 \overline{X}_{Ca}}{X_{Ca} \overline{X}_{Na}^2}$$

were calculated from the activity coefficient, X_i and $\overline{X}_i$ being the fractions of counterions i free in solution and bound to the polymer, respectively[20].

Sol–gel transition.—Samples C48 and A30 exhibited a sol–gel transition which was determined by visual inspection, as previously described[16]. The required amounts of calcium for gelation are reported in Table I.

RESULTS AND DISCUSSION

Influence of the ionic strength, the polymer concentration, and the degree of methylation on the binding isotherms.—The experimental data were presented as binding isotherms ($[Ca^{2+}]_b/[COO^-]$ vs. $[Ca^{2+}]_t/[COO^-]$, where $[Ca^{2+}]_b$ is the bound calcium concentration calculated from the measured activity, and $[Ca^{2+}]_t$ the total calcium concentration) for the three samples, at three polymer concentrations in salt-free solutions and in the presence of added 0.1 M NaCl (Fig. 1).

The affinity of the pectin chains for calcium ions decreased when the ionic strength was increased by NaCl addition, whatever the dm and the polymer concentration. This fact confirmed the results obtained by Joshi and Kwak on dextran sulfate[21], Morris et al. on calcium poly(galacturonate)[11], and Lips et al.[14]. In the presence of salt, the higher the polymer concentration, the stronger was the binding of calcium ions whatever the dm, in disagreement with results of Braudo et al.[15]. In water, the calcium added was totally bound at very low values of $[Ca^{2+}]_t/[COO^-]$, then the binding ratio continuously increased with added calcium. For LM pectins only, the calcium binding was found to be independent of the polymer concentration, leading almost to only one isotherm curve.

For a given polymer concentration, the ratio $[Ca^{2+}]_b/[COO^-]$ at the sol–gel transition increased when the ionic strength decreased. In salt-free solutions, $[Ca^{2+}]_b/[COO^-]$ at the transition increased when the polymer concentration

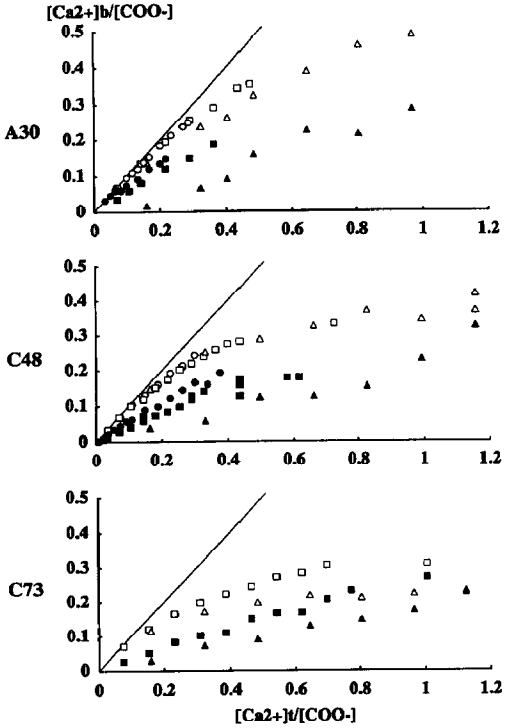

Fig. 1. Influence of the ionic strength and the polymer concentration on the calcium binding by pectic chains at pH 7.4 and at 20°C: △ $\approx 6 \cdot 10^{-3}$ equiv/L; □ $\approx 13 \cdot 10^{-3}$ equiv/L; ○ $\approx 28 \cdot 10^{-3}$ equiv/L. Full symbols, 0.1 M NaCl; empty symbols, water; (−) Total binding of added calcium.

decreased, while in 0.1 M NaCl the same value of 0.07 was obtained whatever the polymer concentration and the dm.

These differences can be explained by the fact that in water, the calcium ions have first to screen the charges of the polymer before acting as cross-linking agents when the electrostatic repulsions have been minimized. In contrast, in the presence of external salt, the divalent cations can directly induce the formation of junctions, explaining why gelation occurred with a smaller amount of calcium[16]. In 0.1 M NaCl, the sol–gel transition apparently occurs only when a given cross-link density is reached.

The influence of the dm can also be observed in Fig. 1. In water, at low polymer concentration, the lower the dm, the higher was the affinity of pectin chains towards calcium ions, in agreement with the results of Kohn and Luknar[6], and Thibault and Rinaudo[7]. This influence decreased when the polymer concentration increased. For low concentrations of added calcium and for high polymer concentration, the binding of calcium ions appeared to be particularly independent of the dm, at least in the range of calcium concentrations tested. At low carboxylate concentration ($6 \cdot 10^{-3}$ equiv/L), the calcium binding isotherm of C73 reached a plateau at 0.2 units beyond $[Ca^{2+}]_t/[COO^-] \approx 0.6$, the curve corresponding to C48

reached a plateau at 0.37 units beyond $[Ca^{2+}]_t/[COO^-] \approx 0.8$ and the isotherm of A30 still increased, syneresis occurring before constant values of $[Ca^{2+}]_b/[COO^-]$ were obtained. This increase of the plateau value with the charge density is in agreement with the results of Mattai and Kwak[13]. The amount of calcium ions required to screen the electrostatic repulsions increased then when the dm decreased. In the same way, it can be seen for C73 that at a given dm, the plateau value increased with the polymer concentration. At high concentration ($28 \cdot 10^{-3}$ equiv/L), no clear plateau was reached and the increase in bound calcium is only hindered by the onset of syneresis. The syneresis corresponds to the last point in Fig. 1 for samples A30 and C48 and appears at values of $[Ca^{2+}]_b/[COO^-]$ of less than 0.5, i.e., before saturation of all available carboxylate groups.

In 0.1 M NaCl as well as in water, calcium binding increased with the charge density of the samples but no plateau was reached. However, under these conditions, the influence of the dm appeared more marked at high concentrations in charged groups than in water, whatever the amount of total calcium.

Sodium–calcium exchange.—In salt-free solutions, the simultaneous use of calcium and sodium selective electrodes allowed the binding of these ions onto the pectin chains to be monitored and therefore the Na–Ca exchange. Calcium and sodium binding isotherms to samples A30, C48, and C73 at pH 7 and 20°C for a carboxylate concentration of $13 \cdot 10^{-3}$ equiv/L are shown in Fig. 2.

Whereas the sol–gel transition did not lead to any noticeable effect on the calcium binding isotherm, the sodium binding isotherm was more sensitive to gelation, exhibiting a sharp decrease with a downward curvature. This fact, already observed in a previous study[22] using ^{23}Na NMR, can be ascribed to the presence of two environments of sodium ions which can be easily determined by the electrode: free or bound, i.e., as a counterion. On the contrary, calcium ions can be free, bound to only one chain to screen the electrostatic repulsions, or involved in a junction between two chains. It is likely that the two latter states cannot be distinguished and that the calcium ions bound to one chain could progressively be involved in junction zones without affecting the potentiometric response.

The values of K_{Ca}^{Na} were found to vary with the amount of calcium (Table II). These values (from 20 to 387) were in good agreement with those reported in other studies: 170 (ref 14), 90 and 180 (ref 20), and 67.8 (ref 23). The strong dependence of K_{Ca}^{Na} on the amount of added calcium clearly indicates that this equilibrium does not explain the binding of calcium ions to the polymer. Moreover, as already shown by other studies[20,23], K_{Ca}^{Na} values seem to be directly related to the charge density of the sample. Furthermore, if one calcium ion led to the departure of two sodium ions from the neighbourhood of the polyion, the ratio of $Na^+_{released}/Ca^{2+}_{bound}$ would be 2. However, the experimental values varied between 0.1 and 2.4 with the polymer and calcium concentrations and the dm of the samples. This result confirms the observation that one bound calcium ion could either induce the departure of one or two sodium ions from a statistical point of view but it disagrees with the results obtained by Rinaudo and Milas on polygalacturonic acid[24].

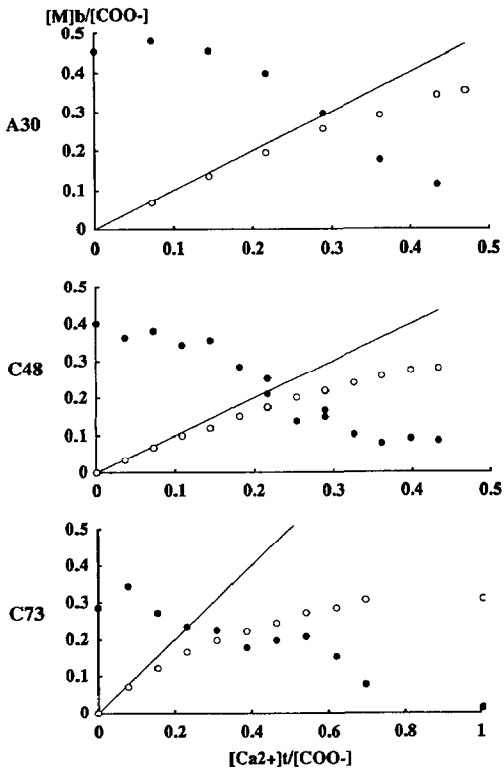

Fig. 2. Binding isotherms of calcium and sodium ions to samples A30, C48, and C73 at pH 7.4 and 20°C ($[COO^-] = 13 \cdot 10^{-3}$ equiv/L): (●) M = Na^+; (○) M = Ca^{2+}; (–) total binding of added calcium.

Measurement of the cooperativity of the binding of calcium ions.—The binding isotherms allow one to obtain information on the influence of the ionic strength, the pectin concentration, and the dm of the samples on binding of the calcium ions. However, no indication on the cooperativity of the phenomenon can be evidenced. To this end, the binding isotherm data were replotted in terms of Scatchard plots, ν/L vs. ν, where ν is the amount of calcium bound by the pectin molecule and L the concentration of free calcium ions. This representation was initially used for macromolecules in which all the binding sites are identical and independent: in this case, the Scatchard plot is linear. However, in many cases, this kind of plot is curved: a concave curvature indicates either that more than one

TABLE II

Change of the selectivity coefficient values with the amount of calcium chloride added to pectins A30, C48, and C73 at 20°C and $[COO^-] = 13 \cdot 10^{-3}$ equiv/L

Sample	A30	C48	C73
Added calcium (mol/L)	0.001–0.006	0.0005–0.005	0.001–0.009
K_{Ca}^{Na}	19–223	45–137	37–113

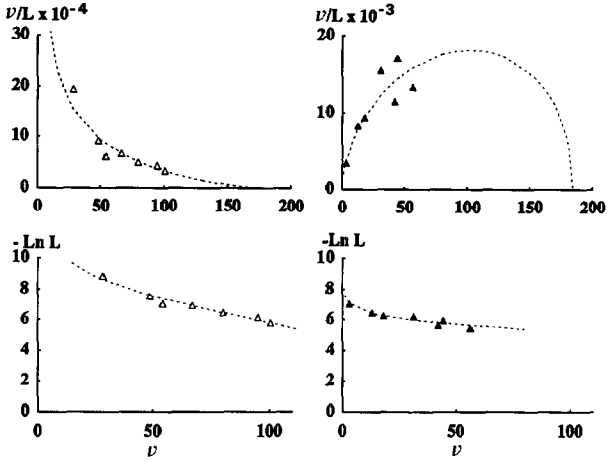

Fig. 3. Scatchard plots of the potentiometric data and Hill fits obtained at $[COO^-] \approx 6 \cdot 10^{-3}$ equiv/L for sample A30. Full symbols, 0.1 M NaCl; empty symbols, water.

class of independent sites are present[25] or that the binding of the ligand is anti-cooperative[26], while a convex curvature indicates an increase in the affinity of the ligand for the macromolecule as the binding reaction proceeds, i.e., the binding of the ligand is cooperative[25]. A global fit of the Scatchard plots may be obtained by use of the semi-empirical Hill equation:

$$\text{Ln } L = -(1/\alpha_H) \text{ Ln}[(n_H/\nu) - 1] + \text{Ln } K_H$$

where n_H is the number of sites per macromolecule (here carboxyl groups) able to bind cations, K_H the apparent dissociation constant for the interacting sites, and α_H the Hill constant, which is an index of the cooperativity. When $\alpha_H > 1$, the binding is cooperative and when $\alpha_H = 1$, the binding is noncooperative. In the case when $\alpha_H < 1$, the binding is anti-cooperative[14].

Some examples of Scatchard and Hill plots of the potentiometric data obtained in pure water and in 0.1 M NaCl are shown in Fig. 3. Whatever the dm of the sample and the polymer concentration, all Scatchard plots were concave-shaped in water while they were convex-shaped in 0.1 M NaCl. These results evidence cooperative interactions in 0.1 M NaCl. For a water solution, the concave curvature can be ascribed either to the presence of a different class of binding sites (isolated carboxylate groups, i.e., low local charge density, or carboxylate groups surrounded by other ones, i.e., high local charge density) or to anti-cooperative interactions between the polymer and the calcium ions. This last possibility was largely assumed in other studies[13,14,21,26,27] (as well as for a polymer showing only one type of binding site and for a polymer showing different types of binding sites), and ascribed to a typical polyelectrolyte behaviour. Indeed, the binding of a divalent ion decreases the electrostatic potential and makes further binding less and less favourable. The cooperativity of the binding process observed at high ionic

TABLE III

Values of the Hill equation parameters

	0.006 equiv/L			0.013 equiv/L			0.028 equiv/L			n_{theo}
	α_H	n_H	$1/K_H$	α_H	n_H	$1/K_H$	α_H	n_H	$1/K_H$	
C73 Water	0.29	68	5	0.43	68	33				68
0.1 M NaCl	1.19	68	69	1.00	68	45				68
C48 Water	0.35	118	94	0.54	118	119	0.70	118	152	118
0.1 M NaCl	1.47	118	115	1.16	118	77	1.04	118	64	118
A30 Water	0.67	184	435	0.72	184	435	0.79	184	270	184
0.1M NaCl	2.25	184	196	1.23	184	147	1.07	184	108	184

strength can be explained by the screening of polyelectrolyte effects by the added NaCl. The decrease of anti-cooperativity with increase of the ionic strength in the system is in agreement with some results from the literature[13,15] but disagrees with other[14]. This approach was also used by Manzini et al.[28] to study the structure effects on the interactions of different polynucleotides with calcium ions, and Uraki et al.[29] have shown that calcium binding to anionic chitin derivatives can be either anti-cooperative or indifferent, depending on the structure of the polysaccharide.

The syneresis of the pectin–calcium systems for high calcium concentrations, i.e., at high ν values, does not allow the determination of n_H directly from the Scatchard curves. We used a theoretical value for n_H, estimated from the following relationship:

$n_{theo} = dp \times GalA \times dm$

Whatever the sample, n_{theo} (calculated from the number average molecular weight $\overline{M}_n$) can be used to obtain reliable fits on the Hill plots ($r^2 > 0.999$). However, n_{theo} probably overestimated n_H because all the carboxylate groups are not involved in gelation because of a lack of accessibility. Moreover, the fit of the data appeared to be very sensitive to the n_H values. A change in n_H leads to a change in the values of the Hill parameters gathered in Table III for $n_H = n_{theo}$, nevertheless the relative change of the fitting parameters with the solvent conditions and the dm is maintained. So, more than the intrinsic value of these parameters, it is the tendency for their relative change which is interesting to note here.

In water, for the three samples studied, values of $\alpha_H < 1$ were found, as expected from the Scatchard plots. In the same way, for 0.1 M NaCl, values of $\alpha_H > 1$ were found. The α_H values increased with the polymer concentration in water whereas they decreased in the presence of added salt. On the other hand, for a given carboxylate concentration, these values increased when the dm decreased whatever the ionic strength. However, the α_H values are very low compared to the n_H values, which means that both the cooperative and anti-cooperative phenomena were not very strong compared with other systems, such as Mn^{2+} binding to *t*RNA where five binding sites are involved and $\alpha_H = 2.3$, or oxygen binding to hemoglobin, where α_H is 2.5–3.0 and $n_H = 4$ (ref 25).

For the water solutions, the values of $1/K_H$ were found to increase with the polymer concentration, confirming the decrease of the anti-cooperativity in this case, while in the presence of added salt they decreased when the pectin concentration increased, which confirms the decrease of the cooperativity. Furthermore, for water as well as in 0.1 M NaCl solutions, these values increased when the dm of the samples decreased, thus confirming the evolution obtained for α_H.

CONCLUSION

This study of calcium binding evidences a greater affinity of the pectic chains towards calcium ions with the increase of the charge density of the sample, the increase of the polymer concentration, and with the decrease of the ionic strength of the systems. Furthermore, it was shown that the amount of free or bound calcium ions seemed to be unaffected by the gelation, whereas sodium ions were clearly influenced by the network formation. It was also shown that the pectin–calcium interactions were anti-cooperative in water and cooperative in the presence of salt. This change was also observed in other systems. For example, Mattai and Kwak[26] have shown that the weaker the ionic strength, the more anti-cooperative was the binding of magnesium and calcium ions to heparin. Furthermore, the cooperativity observed in salt solution was in agreement with the results obtained by a thermodynamic study[30]. All these results have now to be related to the rheological properties of gels.

REFERENCES

1 A. Darvill, P. Albersheim, M. MacNeil, J. Lau, W. York, T. Stevenson, J. Thomas, S. Doares, D. Gollin, P. Chelf, and K. Davis, *J. Cell Sci. Suppl.*, 2 (1985) 203–217.
2 D.B. Nelson, C.J.B. Smit, and R.R. Wiles, in H.D. Graham (Ed.), *Food Colloids*, Avi, Westport, USA, 1977, pp 418–437.
3 M.A.V. Axelos and J.-F. Thibault, in R.H. Walter (Ed.), *The Chemistry and Technology of Pectin*, Academic Press, 1991, pp 109–117.
4 R. Kohn and I. Furda, *Collect. Czech. Chem. Commun.*, 32 (1967) 4470–4484.
5 R. Kohn, I. Furda, A. Haug, and O. Smidsrød, *Acta Chem. Scand.*, 22 (1968) 3098–3102.
6 R. Kohn and O. Luknár, *Collect. Czech. Chem. Commun.*, 40 (1975) 959–970.
7 J.-F. Thibault and M. Rinaudo, *Biopolymers*, 24 (1985) 2131–2143.
8 R. Kohn and B. Larsen, *Acta Chem. Scand.*, 26 (1972) 2455–2468.
9 E. Racapé, J.-F. Thibault, J.C.E. Reitsma, and W. Pilnik, *Biopolymers*, 28 (1989) 1435–1448.
10 G.T. Grant, E.R. Morris, D.A. Rees, P.J.C. Smith, and D. Thom, *FEBS Lett.*, 32 (1973) 195–198.
11 E.R. Morris, D.A. Powell, M.J. Gidley, and D.A. Rees, *J. Mol. Biol.*, 155 (1982) 507–516.
12 P. Debongnie, M. Mestdagh, A. Domard, and M. Rinaudo, *Food Hydrocolloids*, 5 (1987) 109–111.
13 J. Mattai and J.C.T. Kwak, *Macromolecules*, 19 (1986) 1663–1667.
14 A. Lips, A.H. Clark, N. Cutler, and D. Durand, *Food Hydrocolloids*, 5 (1991) 87–99.
15 E.E. Braudo, A.A. Soshinsky, V.P. Yuryev, and V.B. Tolstoguzov, *Carbohydr. Polym.*, 18 (1992) 165–169.
16 C. Garnier, M.A.V. Axelos, and J.-F. Thibault, *Carbohydr. Res.*, 240 (1993) 219–232.
17 S. Lifson and A. Katchalsky, *J. Polym. Sci.*, 13 (1954) 43–55.
18 D.A. Rees and A.W. Wight, *J. Chem. Soc. B*, (1971) 1366–1372.
19 C. Garnier, Thèse de Doctorat, Nantes, 1992, France.

20 M. Rinaudo and M. Milas, *J. Polym. Sci*, 12 (1974) 2073–2081.
21 Y.M. Joshi and J.C.T. Kwak, *Biophys. Chem.*, 13 (1981) 65–75.
22 C. Garnier, M.A.V. Axelos, and J.-F. Thibault, *Progr. Colloid Polym. Sci.*, 90 (1992) 66–69.
23 R. Kohn, *Pure Appl. Chem.*, 42 (1975) 371–397.
24 M. Rinaudo and M. Milas, *Eur. Polym. J.*, 8 (1972) 737–740.
25 C.R. Cantor and P.R. Schimmel, *Biophysical Chemistry. Part III: The Behaviour of Biological Macromolecules*, W.H. Freeman and Co., San Francisco, 1980, pp 849–886.
26 J. Mattai and J.C.T. Kwak, *Biochim. Biophys. Acta*, 677 (1981) 303–312.
27 J. Mattai and J.C.T. Kwak, *Biophys. Chem.*, 14 (1981) 55–64.
28 G. Manzini, L.E. Xodo, F. Fogolari, and F. Quadrifoglio, *Biopolymers*, 30 (1990) 325–333.
29 Y. Uraki, T. Fujii, T. Matsuoka, Y. Miura, and S. Tokura, *Carbohydr. Polym.*, 20 (1993) 139–143.
30 C. Garnier, M.A.V. Axelos, and J.-F. Thibault, *Food Hydrocolloids*, 5 (1991) 105–108.