Polyelectrolyte Study of the Calcium-Induced Chain Association of Pectate†

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The theoretical model devised in the previous paper (Donati, I.; Benegas, J. C.; Cesàro, A.; Paoletti, S. *Biomacromolecules* **2006**, *7* (5), 1587−1596) for the description of ion-induced chain aggregation is here applied to the case of chain dimerization of poly(galacturonate) in the presence of calcium ions. Particular attention has been directed toward the initial stage of dimer formation [i.e., in the low regime of calcium-to-polymer ratio (R_j)]. Circular dichroism (CD) data allowed evaluation of the fraction, θ, of calcium ions bound within chain dimers according to the "egg-box"-model. The theoretical model was able to reproduce satisfactorily the total molar enthalpy variation experimentally determined; the contributions of affinity (specificity in territorial condensation) and chemical bonding of calcium counterions to the thermodynamic properties of the system (i.e., enthalpy and entropy) were calculated. The intrinsic molar enthalpy of bonding, $\overline{\Delta H}^{\mathrm{bond},0}$, displayed a peculiar sigmoid dependence on R_j. In particular, its decrease toward more negative values was interpreted as stemming from a (cooperative) calcium-induced conformational change that accompanies pectate chain pairing upon junction formation. The calculated pK_{in} of instability of the Ca−(GalA$^-$)$_4$ complex was 10.80, in very good agreement with the corresponding value reported for the Ca-EDTA complex (i.e., 10.96). Significant contributions to the complex stability were the enthalpy of ion pairing ($\overline{\Delta H}^{\mathrm{ionpairing,bond}} = -5.1$ kcal (mol calcium)$^{-1}$, in good agreement with the value reported for calcium-EDTA: ≈ -5.4 kcal (mol calcium)$^{-1}$), and the entropy of desolvation ($\overline{\Delta S}^{\mathrm{desolv,bond}} = 43.7$ cal mol^{-1} K^{-1}, well within the range of values reported for calcium-EDTA: 42−57 cal mol^{-1} K^{-1}).

Introduction

The detailed disclosure of the physicochemical (and conformational) properties of ionic polysaccharides relies on a quantitative understanding of their polyelectrolyte character. As an example, the conformation of ionic polysaccharides is strongly influenced by perturbations brought to the system, such as variations of pH and ionic strength and the presence of specific ions.[1,2] In this respect, the correct application of the thermodynamic laws ruling the behavior of polyelectrolytes, although representing a demanding (and challenging) task, could be of utmost importance in revealing the key features controlling their properties.

Pectins are natural polysaccharides composed of long sequences of α-D-galacturonate interrupted by defects of other sugars (including D-xylose, D-glucose, L-rhamnose, L-arabinose, and D-galactose), additionally showing a variable extent of methyl-D-galacturonate ester units.[3] Pectate (poly(α-D-galacturonate), galacturonan) is a collective name for very little esterified pectins. The ability of forming stable hydrogels upon treatment with divalent cations, such as calcium, has established pectate as one of the most appealing polysaccharides for industrial application.[4] Pioneering works[5−7] have stressed the existence of a strong binding between divalent cations and oligogalacturonates, especially when the DP of the latter exceeds ≈20, pointing to a cooperativity in the ion binding process. A widespread accepted description of the calcium−pectate interaction is based on the so-called "egg-box" model[5−10] that envisages an intermolecular ion-mediated bridging of polysaccharide chains as the mechanism leading to the formation of the junctions in the gel network.[10] The process of ion induced polymer dimerization has been extensively studied by using circular dichroism (CD) spectroscopy,[11,12] osmotic coefficients measurements,[12] microcalorimetry, and dilatometry.[13−15] Recently, an approach based on molecular modeling provided a detailed picture of the structural features of interchain junctions in galacturonate oligomers by screening the energy parameters correlated to all of the possible association of two ordered chains with calcium.[16]

Although a renewed interest in the description of the ion induced chain dimerization of pectate (and, in general, of polyuronates) at a molecular level led to a first attempt of application of the basic Manning's theory,[17] the development of a more formal treatment and the comparison between the theoretical and experimental results are needed.

Recently, a new formalism was published, a "fluctuating charge-annihilation" model,[18] which proved to be very effective in describing processes such as the proton dissociation of polycarboxylic acids. This process foresees the formation of a strong (chemical) bond between an ion and a charged group on the polyanion with formal annihilation of the charge of the latter (e.g., a proton in $H^+ + COO^- \leftrightarrows COOH$), nevertheless not fixing the counterion at a given polyanion site but formally allowing a homogeneous distribution of the annihilated charges. However, this approach failed to realistically describe dimer formation in the case of poly(galacturonate). As an example, a

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preliminary analysis indicated that, according to its predictions, just one "fluctuating-bonded" calcium ion should be able to stabilize over 20 binding sites in the egg-box structure over a range of about 100 Å! (this point will be further commented on in the Results and Discussion section).

Prompted by this inconsistency, we decided to undertake the challenging task to investigate in detail the calcium induced dimerization of poly(galacturonate) by means of the new theoretical model developed in the previous paper[19] and by evaluating its consistency with the experimental data available for pectate.[13−15] The strong binding between the polymer chain and the cross-linking counterion is a peculiar feature of the ion induced dimerization process. It has been accounted for by exploiting the concept of "chemical bonding" that affects a well-defined (stoichiometric) fraction σ_0 of counterions (per mol of uronic groups in the specific binding site). Briefly, by "chemical bonding" we mean here the formation of strong, localized interactions, typical of ionic complexes. It does not necessarily mean (nor exclude, though) the formation of covalent bonds. It only implies a formal neutralization ("annihilation") of some of the charges on the polyion by the bound counterions and the residence of the latter ones for a comparatively long time on specific sites of the chain ("persistent annihilation"). Moreover, in view of the results previously reported on the interaction between nongelling divalent counterions (i.e., magnesium, with alginates of different composition),[20] a contribution from the "territorial affinity" will be considered. The counterions showing a "territorial affinity" are supposed to move slowly within the condensation volume, possibly passing a longer time in the vicinity of specific parts of the polyion, but without violation of the fundamental assumptions of the CC theory on charge persistence and distribution. The affinity model is based on the hypothesis that a condensed counterion may be granted of a favorable difference of intrinsic reduced Gibbs free energy, $g^{\mathrm{aff},0}$. Such a thermodynamic bias is supposed only to affect the relative concentration of the counterions between the free and the condensed phase.

Results and Discussion

(a) Assumptions on the Parameters of the Theoretical Model. The complete mathematical formalism of the theoretical model devised for the description of the ion induced chain association has been presented in the preceding paper,[19] to which the reader is referred for all details. Briefly, it is based on the assumption that z_j-valent counterions are able to "cross-link" two parallel single-chains in a dimer via strong chemical bonding. The ability to discriminate and calculate separately the fraction of bound (σ) and condensed z_j-valent counterions (C^j and D^j, respectively) represents one of the main achievements of the model (where C and D stay for the (single-) chain and the dimer components of the system at equilibrium, respectively). In this perspective, calcium-induced dimer formation in poly(galacturonate) is undoubtedly an appealing candidate to test its reliability on the basis of a comparison between the theoretically calculated and experimentally determined thermodynamic quantities, the latter being already reported in the literature.[13−15] However, in view of the different parameters introduced in the theoretical description of the ion induced aggregation of polyuronates, some general considerations need to be outlined and discussed.

(a1) Geometrical Parameters of Pectate. The conformation of the poly(galacturonate) (single) chain in solution is very critical for the successful application of the theoretical model since it directly affects the value of the structural intercharge distance $b_{\mathrm{str,C}}$ and, therefore, the charge density $\xi_{\mathrm{str,C}}$ (i.e., the key parameter in the CC theory). It can be trivially remembered that $\xi_{\mathrm{str,C}} = l_B/b_{\mathrm{str,C}}$, with the Bjerrum length, l_B, given by e^2/k_BTD; e is the elementary charge, k_B is the Boltzmann constant, T is the absolute temperature, and D is the dielectric constant of the solvent. For water at 298.15 K, $l_B = 7.135$ Å. Although dried fibers of the polysaccharide displayed a 3/1-fold helical conformation,[21] CD analysis[11] and ^{13}C NMR relaxation data[22] led to the proposal a 2/1 configuration for the poly(galacturonate) helix in (dilute) solution. More recently, a molecular modeling investigation[23] pointed out that 2- and 3-fold helical conformations are almost equally energetically favorable in the case of pectate. Despite the fact that a general consensus has not been reached over the conformation of poly(galacturonate) chains in solution at neutral pH, the results tend to agree in stating the practical equivalence of the intercharge distance regardless the conformation considered. A "loose helix"[24,25] seems the most appropriate term to indicate such largely disordered conformation. It follows that the assumption of a fixed value of $b_{\mathrm{str,C}} = 4.43$ Å for the poly(galacturonate) (single) chain (and, accordingly, a charge density $\xi_{\mathrm{str,C}} = 1.61$) is feasible and does not represent per se a strong limitation to the application of the model.

A front factor, ρ, was introduced in the mathematical formalism to account for any possible conformational variation occurring to the (single) chain upon dimer formation. According to what was above-reported, ρ was considered to be constant and equal to 1 in view of the nonmarginal data collected in this laboratory.[24,25] In addition, it was verified that variations up to about 10% of the charge density bring about but small numerical differences in the calculated thermodynamic properties, without affecting the overall conclusions (data not reported).

(a2) Chiro-Optical Evidence of Calcium Binding. The identification of an experimental property monitoring chemical bonding is of paramount importance when attempting the application of the theoretical model to the case of calcium-induced poly(galacturonate) dimerization. In this respect, the notable modification displayed by CD spectra of polyuronates upon treatment with calcium ions[11,12,15] provides very useful information. In fact, a widespread opinion tends to allocate such modification to the changes in the dissymmetric environment of the carboxyl chromophores due to the proximity of the site-bound cations.[26,27] Data reporting the effect of calcium ions on the $n \rightarrow \pi^*$ transition for the uronate groups are available in the literature[15,28] and a correlation between the CD variations and the stoichiometry of calcium ions "entrapped" in egg-box structures has been proposed.[11] On the other side, the addition of magnesium ions to alginates and pectate, as well as that of calcium to mannuronan, produces extremely small CD effects.[20] Those systems are known NOT to give any significant chain association or ion-induced gelation. It then seems very justified to assume a direct correlation between site-binding of calcium and major CD changes of the carboxylate chromophore. Accordingly, in the present paper, the variations of the CD spectra detected upon increasing the calcium content in the solution (i.e., increasing R_j) are assumed to monitor the formation of an "egg-box" structure between two chains. The CD perturbation, $\{\theta_{R_j}\}$, at each addition of calcium ion (R_j) can be evaluated by using

$$\{\theta_{R_j}\} = \frac{[\theta]_0 - [\theta]_{R_j}}{[\theta]_0} = \frac{\Delta[\theta]_{R_j}}{[\theta]_0} \tag{1}$$

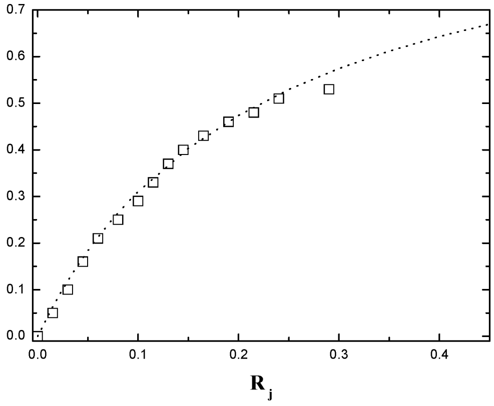

Figure 1. (□) Dependence of the fraction of dimers formed, $\theta(R_j) = \{\theta_{R_j}\}/\{\theta_{R_j}\}_{MAX}$, on R_j where $\{\theta_{R_j}\}$ is the relative CD perturbation at the concentration of calcium ions R_j (see eq 1) and $\{\theta_{R_j}\}_{MAX}$ is obtained from the fitting of the experimental data.

$[\theta]_0$ and $[\theta]_{R_j}$ are the molar ellipticity values (at 210 nm) of the poly(galacturonate) solution as such and at a specific R_j value, respectively. $\{\theta\}_{R_j}$ displayed a Langmuir-type ($y = (ax)/(b + x)$) dependence on the amount of calcium added to the galacturonan system (see ref 15) with a calculated $\{\theta_{R_j}\}_{MAX}$ ($=\{\theta_{R_j\to\infty}\}$) equal to 0.98. It should however be emphasized that the proximity to 1 of the value of $\{\theta_{R_j\to\infty}\}$ found for pectate is purely incidental, inasmuch as it just reflects the particular relative increase of $[\theta]$ at a given wavelength. The fraction of "egg-box" dimers formed at any R_j value, θ (R_j), is then trivially calculated as: $\theta(R_j) = \{\theta_{R_j}\}/\{\theta_{R_j}\}_{MAX}$ (Figure 1).

(a3) Stoichiometry of the Complex. To apply the theoretical model[19] to the ion induced chain dimerization of poly(galacturonate), an additional consideration is needed; it concerns the assumption on the stoichiometric (molar) ratio between calcium ions and uronic moieties in the specific binding site (i.e., σ_0). This is directly correlated with the still-debated issue of the conformation of the polymer chains in the dimers. In the case of pectate, the most probable calcium induced chain association has been reported to occur between 2/1 helical chains, thus leading to $\sigma_0 = 1/4$ in accordance with the "egg-box" model described by Morris and co-workers.[11] However, poly(galacturonate) chains exhibit in the solid state a right-handed 3-fold helical conformation,[21] and a recent molecular modeling analysis of calcium- poly(galacturonate) dimers[16] has not completely ruled out the possibility of chain aggregation involving 3/1 helices (although, beyond a doubt, the latter is far less energetically favorable than the 2/1-fold one[16]). Although the existence of a 3/1 conformation of pectate chains in solution has a negligible impact on the intercharge distance on the polysaccharide chain, as already stated, the dimerization of two of these chains leads to a substantial increase of σ_0 to reach the value of 1/3. The absolute determination of the conformation of the pectate chains in the dimer is beyond the scope of the present paper, nevertheless some considerations can be drawn. In particular, both in the case of a 2/1 and a 3/1 conformation of the polymer chains in the dimers (i.e., with $\sigma_0 = 1/4$ or 1/3, respectively), the fraction of "chemically bound" calcium ions, $\sigma(R_j)$, can be easily calculated

$$\sigma(R_j) = \theta(R_j)\sigma_0 \tag{2}$$

The dependence of the fraction σ on R_j has been reported in Figure 2: it shows that the $\sigma_0 = 1/3$ case would require more calcium than the total amount available in solution over a substantial range of R_j (i.e., $0 \le R_j < R_j \cong 0.11$). Without being conclusive, still this consideration suggests that, at least at the initial stage of the junction formation process, the formation of dimers involving 3/1 chains is practically forbidden. In the following calculations, the value $\sigma_0 = 1/4$ will be used to describe the calcium induced dimerization of pectate chains, implicitly assuming that the junctions are formed by pairing of 2/1 helices.

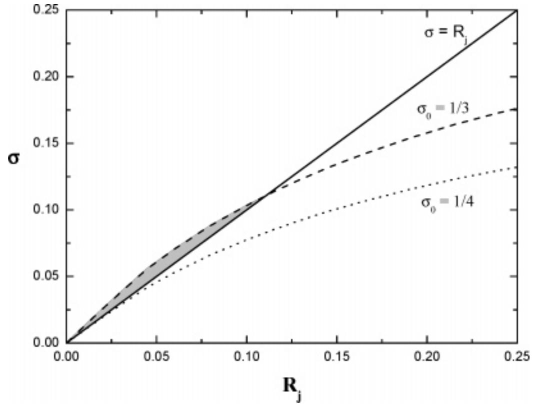

Figure 2. Dependence of the fraction of chemically bound counterions, σ, on R_j in the case of (· · ·) $\sigma_0 = 1/4$ and of (− −) $\sigma_0 = 1/3$. The diagonal bold line represents the $R_j - R_j$ correlation, corresponding to $\sigma = R_j$. The shaded area indicates the R_j range of incompatibility of the $\sigma_0 = 1/3$ stoichiometry.

(b) Calcium-Induced Dimerization of Poly(galacturonate): The Model Description of the Experimental Results. The model proposed in the previous paper[19] was applied to the case of calcium induced chain dimerization of poly(galacturonate), under the above specified assumptions, considering the fraction of dimer formed, $\theta(R_j)$, as an experimental parameter. Moreover, for sake of simplicity, in the presence of two different conformations that differ for the topology but not for the net charge density, like in the present case, the two values of the intrinsic (reduced) Gibbs energy of affinity for the single chain and the dimer will be taken as equal (i.e., $g_C^{aff,0} \equiv g_D^{aff,0} = g^{aff,0}$). This corresponds to the assumtion that the affinity-controlled interactions of the condensed calcium ions with the external sides of the 2/1 egg-box structure are energetically equivalent to those with the two sides of the single-stranded ribbon. Moreover, in the absence of any contrary evidence, $g^{aff,0}$ and $h^{aff,0}$ (and then also $s^{aff,0}$) will be assumed to be constant, independent of R_j, like in the previous successful cases.[20,29,30]

(b1) Correlation between free Energy of Affinity and of Bonding. Initially, the system of 7 equations in 7 unknowns [$r_C(R_j)$, $x_C{}^i(R_j)$, $r_D(R_j)$ $x_D{}^i(R_j)$, $V_C(R_j)$, $V_D(R_j)$, $g^{bond,0}(R_j)$; for definitions, see ref 19] was solved (parametrically in $g^{aff,0}$) for each value of the independent variable, R_j. Among the unknowns, particular attention has been given to the (reduced) intrinsic Gibbs energy of bonding, $g^{bond,0}(R_j)$, accompanying the formation of the strong metal complex between chain dimers. Because of the polymeric nature of the system and the possibility of significant effects of cooperativity,[19] $g^{bond,0}(R_j)$ is considered as potentially dependent on R_j. It may be useful to recall that, much like in the case of chemical affinity, the index "0" refers to the intrinsic value of any thermodynamic property (including bonding) expressed per mole of bound calcium ions. In accordance with the theoretical predictions,[19] the value of $g^{aff,0}$ was found to be crucial for the determination of $g^{bond,0}(R_j)$. Figure 3 shows that the functional correlation between $g^{bond,0}(R_j)$ and $g^{aff,0}$ is a direct one. In fact for $g^{aff,0} = -7$, $g^{bond,0}(R_j)$ varied from ≈ -6 to ≈ -1, whereas for $g^{aff,0} = -1$, $g^{bond,0}(R_j)$ was found to range from ≈ -1 to $\approx +2$. Moreover, Figure 3 shows

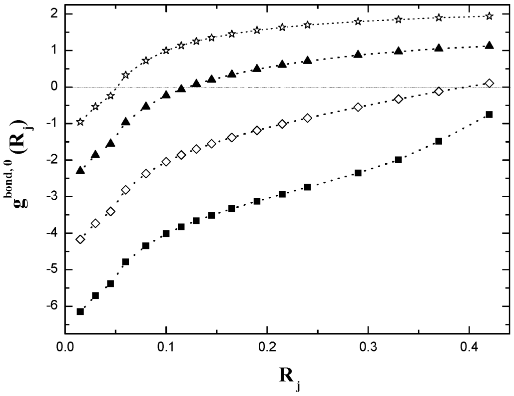

Figure 3. $g^{\text{bond},0} - R_j$ dependence calculated assuming $g^{\text{aff},0}$ equal to −7 (■), −5(◇), −3 (▲), and −1 (☆), respectively. Lines are drawn to guide the eye.

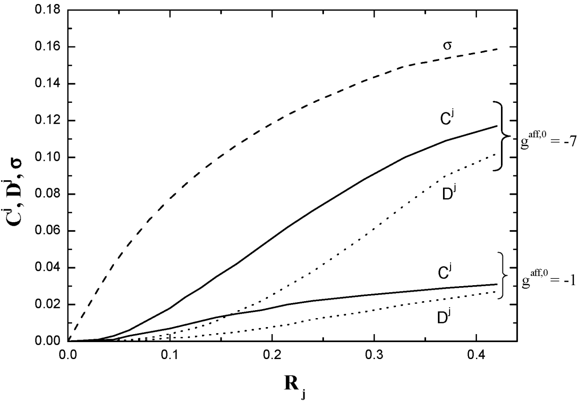

Figure 4. Fraction of chemically bound (σ, − −) and territorially condensed (C^j, — and D^j, ···) calcium counterions calculated by using a value $g^{\text{aff},0}$ equal to −1 and −7, respectively. C^j and D^j refer to the fraction of condensed counterion on the (single) chain and on the dimer components at equilibrium, respectively.

that $g^{\text{bond},0}(R_j)$ always increases toward more positive values with increasing R_j for all $g^{\text{aff},0}$ values. This finding confirms the postulated possibility that $g^{\text{bond},0}$ may depend on R_j; actually, it points to a negative cooperativity of the chemical bonding of calcium ions, which makes this process progressively less favored.[19] Similarly, both the fraction of affinity-driven condensed divalent counterions on the single-stranded chain and that on the chain-dimer (i.e., $C^j(R_j)$ and $D^j(R_j)$, respectively) are strongly affected by the values of the (reduced) Gibbs energy of affinity, $g^{\text{aff},0}$, as reported in Figure 4. All condensed ions significantly contribute to the thermodynamic properties of the polysaccharide solution, such as enthalpy or volume variations. Therefore, the correct determination of the value of the fraction of territorially confined calcium counterions is expected to be crucial for the successful application of the theoretical model to the experimental data presently considered.[13−15]

(b2) Enthalpy, Entropy and Free Energy of Calcium Affinity and Bonding. A step further toward the determination of the thermodynamic parameters is made by considering the enthalpy changes upon mixing calcium with poly(galacturonate), Δh^{mix}, measured at various R_j values.[14,15] This second minimization step allows one to resolve the ambiguity as to the choice of $g^{\text{aff},0}$ (and consequently of $g^{\text{bond},0}$). In fact, the overall experimental enthalpic effect (h^{tot}) is the result of the product of the intensive enthalpy changes of affinity and of bonding times the fractions of condensed counterions, $C^j(R_j)$ and $D^j(R_j)$ (which in turn univocally depend on $g^{\text{aff},0}$) and the fraction of bound counterions ($\sigma(R_j)$), respectively. Therefore, the total enthalpy variation calculated for the system under analysis depends on the particular set of values of $g^{\text{aff},0}$ used in the resolution of the system of 7 equations in 7 unknowns. On the basis of the theoretical model,[19] the total reduced enthalpy of the system (per mole of polymer charged group and normalized by RT) is calculated as a function of R_j by use of the following equation:

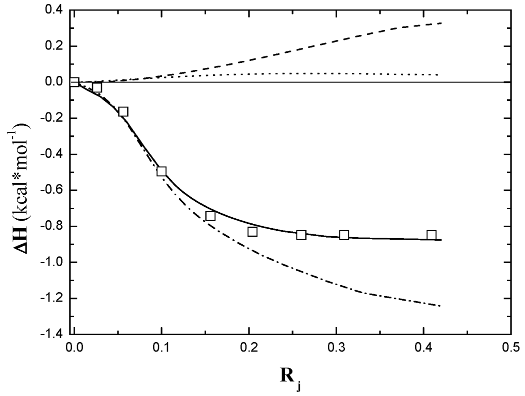

Figure 5. Comparison between the (□) experimental and (—) theoretical (calculated by means of eq 4) total molar enthalpy variation for the case of calcium induced dimer formation for poly(galacturonate). Experimental and theoretical conditions used: univalent salt = 0.05 M and Cp = 3.5 · 10^{-3} monomol/L. The electrostatic ($\overline{\Delta H}^{\text{el}}$, ···), affinity ($\overline{\Delta H}^{\text{aff}}$, − −), and chemical bonding ($\overline{\Delta H}^{\text{bond}}$, · − ·) enthalpic contributions to the total molar enthalpy variation (—) (eqs 4−6) are also reported.

$$h^{\text{T}}(R_j) = (1 - \theta(R_j))\, h^{\text{el}}_{\text{C}}(R_j) + \theta(R_j)\, h^{\text{el}}_{\text{D}}(R_j) + (C^j + D^j)\, h^{\text{aff},0} + \sigma_0\theta(R_j)\, h^{\text{bond},0}(R_j) \quad (3)$$

$h^{\text{el}}_{\text{C,D}}$ are the (reduced) enthalpic terms of electrostatic origin[20] and $h^{\text{aff},0}$ is the intrinsic (reduced) enthalpy affinity of the calcium ion. For the sake of simplicity and by parallelism with the intrinsic (reduced) Gibbs energies of affinity, in the following calculations, we will assume that the (reduced) enthalpic terms of affinity both for the (single) chain ($h^{\text{aff},0}_{\text{C}}$) and for the dimer ($h^{\text{aff},0}_{\text{D}}$) are constant and equal ($h^{\text{aff},0}_{\text{C}} \equiv h^{\text{aff},0}_{\text{D}} \equiv h^{\text{aff},0} \equiv \text{const.}$). On the contrary, the intrinsic (reduced) enthalpy of bonding, $h^{\text{bond},0}(R_j)$, will be left free to vary with R_j, once again in parallel to $g^{\text{bond},0}(R_j)$.

The reduced quantities can be easily converted into the molar ones $\bar{H}(\bar{G}) = h(g)\,RT$ and the total molar enthalpy variation can be calculated according to

$$\overline{\Delta H}^{\text{T}} = \bar{H}^{\text{T}}(R_j) - \bar{H}^{\text{T}}(R_j = 0) \quad (4)$$

The minimization of the difference between the calculated and the experimental value of the enthalpy of the system (i.e., $\overline{\Delta H}^{\text{T}}$ and ΔH^{mix}, respectively) allows one to determine $g^{\text{aff},0}$ and, hence, $g^{\text{bond},0}(R_j)$, together with the corresponding values of the (reduced) intrinsic enthalpy of affinity, $h^{\text{aff},0}$, and of chemical bonding, $h^{\text{bond},0}(R_j)$. The parameters $h^{\text{aff},0}$ and $h^{\text{bond},0}(R_j)$ are expressed per mole of divalent cation, whereas $h^{\text{el}}(R_j)$ is given per mole of fixed charge (uronate repeating unit).

The best-fit analysis of the experimental data (see Figure 5) provides: $g^{\text{aff},0} = -7 \pm 0.5$ (corresponding to $\overline{\Delta G}^{\text{aff},0} \approx -4.1 \pm 0.3$ kcal (mol of calcium ion)$^{-1}$) and $\overline{\Delta H}^{\text{aff},0} = 2.5 \pm 0.2$ (corresponding to $\overline{\Delta H}^{\text{aff},0} = 1.5 \pm 0.1$ kcal (mol of calcium ion)$^{-1}$). The ensuing value of $\overline{\Delta S}^{\text{aff},0}$ is 18.9 ± 0.3 cal (mole of calcium ion)$^{-1}$ K^{-1}. Interestingly, the value of $\overline{\Delta G}^{\text{aff},0}$ is similar to that reported recently for the magnesium/poly(guluronate) case (i.e., −3.5 kcal (mol of calcium ion)$^{-1}$).[20]

The numerical solution allows to calculate the values of the fraction of territorially condensed calcium ions [i.e., $C^j(R_j)$ and $D^j(R_j)$]. They are indicated by the two curves for $g^{\text{aff},0} = -7$ in Figure 4. $D^j(R_j)$ is rather low and lower than $C^j(R_j)$, rapidly increasing for $R_j >$ about 0.2 with a distinctive sigmoid shape.

The curve of the total enthalpy of the system vs R_j, calculated by means of eq 3, brings together contributions of different origin, namely electrostatic, affinity, and bonding enthalpies, which are separately plotted in Figure 5. It should be noted that the contribution stemming from the "purely" polyelectrolytic nature of the system [i.e., $\overline{\Delta H}^{\text{el}}(R_j) = \bar{H}^{\text{el}}(R_j) - \bar{H}^{\text{el}}(R_j = 0)$ $(\bar{H}^{\text{el}}(R_j) = (1 - \theta(R_j))\,\bar{H}^{\text{el}}_{\text{C}}(R_j) + \theta(R_j)\,\bar{H}^{\text{el}}_{\text{D}}(R_j)$, where the molar quantities are expressed per mole of polymer charged group)] is positive and practically negligible. At variance, the bonding of calcium ions in egg-box structures brings about a notable (negative) enthalpic contribution, with $\overline{\Delta H}^{\text{bond}}(R_j)$ (eq 5) reaching the value of ≈ -1.2 kcal mol^{-1} (per mol of polymer charged group) at $R_j = 0.42$.

$$\bar{H}^{\text{bond}}(R_j) = \theta(R_j)\,\sigma_0\,\overline{\Delta H}^{\text{bond},0}(R_j) \equiv \overline{\Delta H}^{\text{bond}}(R_j) \qquad (5)$$

This negative contribution is, however, partially counterbalanced by the positive enthalpic contribution $\overline{\Delta H}^{\text{aff}}$, being

$$\bar{H}^{\text{aff}}(R_j) = (C^j(R_j) + D^j(R_j))\overline{\Delta H}^{\text{aff},0} \equiv \overline{\Delta H}^{\text{aff}}(R_j) \qquad (6)$$

stemming from the condensation of counterions, which becomes relevant at high R_j values ($\overline{\Delta H}^{\text{aff}}(R_j) \approx 0.3$ kcal per mol of polymer charged group, at $R_j = 0.42$). This diverging behavior results in a progressively reduced decrease of the $\overline{\Delta H}^{\text{T}}(R_j)$ curve beyond $R_j = 0.15$.

A comparison with nongelling polyuronates may be of interest. Although the distinctive feature of calcium interaction with pectate is the strong bonding (as it will be discussed in the following paragraph), still a comparison of the affinity component can be performed. Calcium ions were shown to interact with certain affinity with mannuronan, a nongelling polyuronate, with an enthalpy change of affinity, $\overline{\Delta H}^{\text{aff},0}$, equal to 0.74 kcal (mol of calcium)$^{-1}$, and an entropy change of affinity, $\overline{\Delta S}^{\text{aff},0}$, equal to $+3.5$ cal (mol of calcium)$^{-1}$ K^{-1}.[20] Those values are both positive in sign but are significantly lower than those above-reported for pectate. They suggest that, in addition to the peculiar calcium bonding by the latter polymer, also the condensed divalent counterions show a higher preference for galacturonan with respect to mannuronan, due to the much more favorable entropic component. This result prompted us to plan further experiments with nongelling counterions, like magnesium, to shed more light on this topic; they will be the subject of a future paper.

The good fitting of the experimental enthalpy variation obtained allowed calculating the molar enthalpy of bonding (per mole of divalent cation) $\overline{\Delta H}^{\text{bond},0}(R_j)$ and, from the $\overline{\Delta G}^{\text{bond},0}(R_j)$ values obtained from the minimization, the molar entropy of bonding [i.e., $\overline{\Delta S}^{\text{bond},0}(R_j)$ (both expressed per mole of divalent cation; Figure 6)]

$$s^{\text{bond},0}(R_j) = g^{\text{bond},0}(R_j) - h^{\text{bond},0}(R_j) \text{ and then } \overline{\Delta S}^{\text{bond},0}(R_j) = s^{\text{bond},0}(R_j)\,R \qquad (7)$$

Focusing on the first of these terms, it is interesting to note that the value of $\overline{\Delta H}^{\text{bond},0}(R_j)$ is negative in all of the R_j range considered. The extrapolation of $\overline{\Delta H}^{\text{bond},0}(R_j)$ to $R_j \rightarrow 0$ (i.e., $(\overline{\Delta H}^{\text{bond},0})_{R_j=0}$) represents the enthalpy (per mol of divalent cation) of the very first dimer formed with $(\overline{\Delta H}^{\text{bond},0})_{R_j=0} \approx -1.7$ kcal (mol of calcium ion)$^{-1}$. After the very first contact between chains is attained, the value of $\overline{\Delta H}^{\text{bond},0}(R_j)$ starts decreasing, meaning that the bonding of each further calcium ion is enthalpically favored (in a cooperative way) until a minimum value of $\overline{\Delta H}^{\text{bond},0}(R_j)$ is reached $(\overline{\Delta H}^{\text{bond},0}(R_j)_{\text{MAX}} \approx -8$ kcal (mol of calcium ion)$^{-1}$). The behavior clearly resembles a cooperative process, with a sigmoid dependence of $\overline{\Delta H}^{\text{bond},0}$ on R_j characterized by a steady plateau at $R_j > \approx 0.11 - 0.15$. Beyond the latter value, the enthalpy change of the elementary process of bonding (recalling that $\overline{\Delta H}^{\text{bond},0}(R_j)$ is an intensive property) does not change any longer, having reached its maximum (negative) value. Obviously, the negative contribution of calcium bonding to the overall enthalpy change ($\bar{H}^{\text{bond}}(R_j)$, an extensive property) will keep decreasing (see the dash–dotted curve in Figure 5), parallel to the steady increase of the fraction of dimers formed, $\theta(R_j)$, (see Figure 1). The entropy associated with the bonding of calcium ions in dimer structures, $\overline{\Delta S}^{\text{bond},0}(R_j)$, is also reported in Figure 6 (as $T\,\overline{\Delta S}^{\text{bond},0}(R_j)$) and its dependence on R_j deserves a detailed comment. Although a sigmoid shape of the curve was obtained, the nonconstant value of $\overline{\Delta G}^{\text{bond},0}(R_j)$ leads to a lack of a perfect parallelism between $\overline{\Delta H}^{\text{bond},0}(R_j)$ and $T\overline{\Delta S}^{\text{bond},0}(R_j)$. It is also interesting to note that the extrapolated value of the entropy of bonding at $R_j \rightarrow 0$, $(\overline{\Delta S}^{\text{bond},0})_{R_j=0}$, is (relatively) high and positive ($\approx$6 cal (mol of calcium ion)$^{-1}$ K^{-1}). This means that the formation of the very first unit of "calcium-in-the-egg-box" is both enthalpically and entropically driven. However, upon increasing R_j, $\overline{\Delta S}^{\text{bond},0}(R_j)$ becomes negative and shows a sigmoid behavior similar to that of $\overline{\Delta H}^{\text{bond},0}(R_j)$.

The overall profile of the total molar Gibbs energy of calcium induced dimer formation of poly(galacturonate), $\overline{\Delta G}^{\text{T}}$, is reported in Figure 7 together with the Gibbs energy of affinity ($\overline{\Delta G}^{\text{aff}}(R_j)$) and bonding ($\overline{\Delta G}^{\text{bond}}(R_j)$), given by (eqs 8 and 9)

$$\overline{\Delta G}^{\text{T}} = \bar{G}^{\text{T}}(R_j) - \bar{G}^{\text{T}}(R_j = 0)$$

$$g^{\text{T}}(R_j) = \frac{\bar{G}^{\text{T}}(R_j)}{RT} = (1 - \theta(R_j))g^{\text{el}}_{\text{C}}(R_j) + \theta(R_j)g^{\text{el}}_{\text{D}}(R_j) + (C^j + D^j)g^{\text{aff},0} + \sigma_0\theta(R_j)g^{\text{bond},0}(R_j) \qquad (8)$$

and

$$\bar{G}^{\text{aff}}(R_j) = (C^j(R_j) + D^j(R_j))\overline{\Delta G}^{\text{aff},0} = \overline{\Delta G}^{\text{aff}}(R_j) \qquad (9\text{a})$$

$$\bar{G}^{\text{bond}}(R_j) = \theta(R_j)\sigma_0\overline{\Delta G}^{\text{bond},0}(R_j) = \overline{\Delta G}^{\text{bond}}(R_j) \qquad (9\text{b})$$

The latter two terms, namely the Gibbs energy of affinity ($\overline{\Delta G}^{\text{aff}}(R_j)$) and bonding ($\overline{\Delta G}^{\text{bond}}(R_j)$), deserve an additional comment. Although both curves (Figure 7) are negative over all of the R_j range considered, the contribution of $\overline{\Delta G}^{\text{bond}}(R_j)$ prevails in the low θ-range, where mainly the "chemically bound" calcium ions are responsible for the overall gain in the total Gibbs energy. However, when almost half of all the possible dimers are filled with the divalent cations ($\theta \approx 0.42$), a crossover between $\overline{\Delta G}^{\text{aff}}(R_j)$ and $\overline{\Delta G}^{\text{bond}}(R_j)$ is detected, stressing on the significant contribution to the stabilization of the system arising from territorial condensation of calcium counterions.

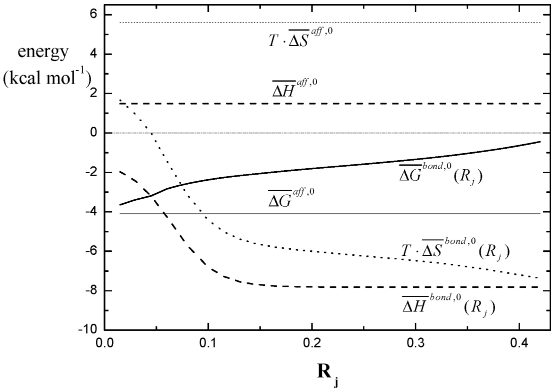

Figure 6. Dependence on R_j of the molar Gibbs energy ($\overline{\Delta G}^{bond,0}(R_j)$, —), enthalpy ($\overline{\Delta H}^{bond,0}(R_j)$, − −), and entropy (as $T\overline{\Delta S}^{bond,0}(R_j)$, · · ·) of bonding calculated for the calcium induced dimer formation for poly(galacturonate) and of the molar Gibbs energy ($\overline{\Delta G}^{aff,0}$, —), enthalpy ($\overline{\Delta H}^{aff,0}$, − −), and entropy (as $T\overline{\Delta S}^{aff,0}$, · · ·) of affinity for calcium ions.

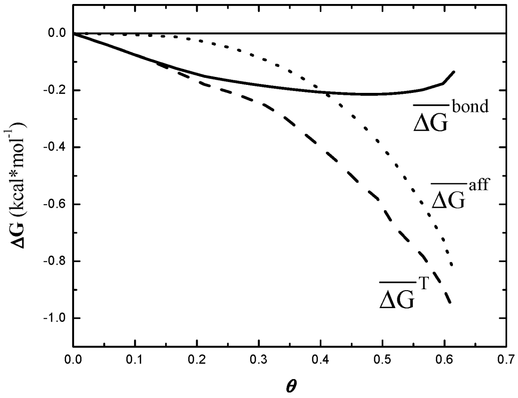

Figure 7. Dependence on θ of the molar Gibbs energies of bonding ($\overline{\Delta G}^{bond,0}(R_j)$, —) and affinity ($\overline{\Delta G}^{aff}(R_j)$, ···) and of the total molar Gibbs energy variation of the system ($\overline{\Delta G}^{T}(R_j)$, − −) calculated according to the present model.

(b3) Volume Change for Calcium Affinity and Bonding. Calcium treatment of poly(galacturonate) solution is accompanied by a notable increase in the volume of the system, as already pointed out.[15] In an accepted explanation, this variation stems from the release (desolvation) of structured water molecules that normally surround, in a close proximity, both the counterions and the uronate groups on the polymer. It follows that the total volume change in the solution depends on (i) the fraction of affinity-condensed counterions (C^j and D^j, respectively) and of bound counterions (σ) and (ii) their respective relative contribution to desolvation, $\overline{\Delta V}^{aff,0}$ and $\overline{\Delta V}^{bond,0}$. In addition, there is a contribution stemming from the "purely" polyelectrolytic nature of the system, i.e., $\overline{\Delta V}^{e1}(R_j) = \bar{V}^{e1}(R_j) - \bar{V}^{el}(R_j = 0)$ $[\bar{V}^{el}(R_j) = (1 - \theta(R_j))\overline{V}^{el}_{c}(R_j) + \theta(R_j)\overline{V}^{el}_{D}(R_j)$, where the molar quantities are expressed per mole of polymer charged group], which is positive and practically negligible. In accordance with theory developed in the previous paper,[19] the volume variation of the calcium-treated poly(galacturonate) solution at each value of R_j can be written as

$$\overline{\Delta V}^{T}(R_j) = [C^j(R_j) + D^j(R_j)]\overline{\Delta V}^{aff,0} + \theta(R_j)\sigma_0\overline{\Delta V}^{bond,0} + \overline{\Delta V}^{el}(R_j) \quad (10)$$

where both $\overline{\Delta V}^{aff,0}$ and $\overline{\Delta V}^{bond,0}$ have been supposed to be independent of R_j, in the absence of any contrary evidence, having also assumed that $\overline{\Delta V}^{aff,0}_{C} \equiv \overline{\Delta V}^{aff,0}_{D} \equiv \overline{\Delta V}^{aff,0}$, and with $\overline{\Delta V}^{e1}(R_j)$ calculated according to the equations reported in ref 31. A best fit of the experimental data,[15] performed by means of eq 10, provided the values of 12 cm^3 (mol of calcium ion)$^{-1}$ and 29 cm^3 (mol of calcium ion)$^{-1}$ for $\overline{\Delta V}^{aff,0}$ and $\overline{\Delta V}^{bond,0}$, respectively. The accepted explanation of the positive volume changes always measured for the interaction of counterions and polycarboxylates is that the close contact between (divalent) counterions and the polyion releases a large number of water molecules from the respective hydration spheres. Considering that the density of electrostricted water around both counterions and polycarboxylates is about 1.1 g cm^{-3}, one can estimate[32,33] that the affinity and the bonding interactions lead to the release of about 7 and 18 electrostricted water molecules per mol of calcium, respectively. It is then reasonable to suggest that such a release of water particles is the main molecular cause of the significant positive entropy change of both the affinity interaction and the bonding one.

(b4) Evaluation of the Thermodynamic Quantities Related to the Single Event of Calcium Ion Bonding. The overall ion bonding process by poly(galacturonate) chains so far described can be reasonably considered a combination of two factors arising from (i) an elementary event of bonding a single calcium ion within an "egg box" (made by two facing couples of disaccharides each of which belongs to one or the other chains in the dimer) and (ii) whole-chain effects which may accompany the multi-chain association and eventually lead to gel formation. The very first bonding event at $R_j \rightarrow 0$ can be reasonably taken as fully descriptive of the former event ("complex" formation, described by $\overline{\Delta G(H,S)}^{complex}$), whereas its difference with the value of any thermodynamic function at $R_j \neq 0$ can be traced back to excess "polymer" effects (eq 14)

$$\overline{\Delta G(H,S)}^{bond,0}(R_j) = \overline{\Delta G(H,S)}^{bond,0}_{R_j=0} + \overline{\Delta G(H,S)}^{bond,0}_{excess}(R_j) \equiv \overline{\Delta G(H,S)}^{complex} + \overline{\Delta G(H,S)}^{polym}(R_j) \quad (14)$$

We will now focus only on the single event of calcium ions bonding. The discussion on the extra terms related to the polymeric nature of the sample is deferred to a forthcoming paper.

Following a procedure which is well established in the treatment of low MW complexes[34] and on the basis of the accepted desolvation demonstrated by the large volume changes, the thermodynamic values of complex formation, $\overline{\Delta G(H,S)}^{complex}$, can be considered to be made up by two terms: a first term, $\overline{\Delta G(H,S)}^{desolv,bond}$, supposed to account for such release of water molecules and a second term, $\overline{\Delta G(H,S)}^{ionpairing,bond}$, which includes all terms pertaining just to the complex formation. In the case of calcium and pectate, it is reasonably considered to be of the type of intimate ion pairing ("inner sphere")

$$\overline{\Delta G(H,S)}^{complex} = \overline{\Delta G(H,S)}^{desolv,bond} + \overline{\Delta G(H,S)}^{ionpairing,bond} \quad (15)$$

Desolvation. To shed some light on the desolvation term, we start by considering the affinity process. Nonbonding affinity interactions are here supposed to be driven by desolvation only: $\overline{\Delta H}^{aff,0}$ is all accounted for by $\overline{\Delta H}^{desolv,aff}$ and $\overline{\Delta S}^{aff,0}$ by $\overline{\Delta S}^{desolv,aff}$, somewhat arbitrarily assuming that affinity interactions stemming from ion pairing are negligible (if not absent). (The validity of the assumption will be soon checked by comparison with known data on Ca/EDTA complex formation.) Under this hypothesis, we can then estimate $\overline{\Delta H(S)}^{\text{desolv,bond}}$ using the corresponding volume changes as scale parameters for desolvation:

$$\overline{\Delta H(S)}^{\text{desolv,bond}} \equiv \overline{\Delta H(S)}^{\text{aff,0}}(\overline{\Delta V}^{\text{bond,0}}/\overline{\Delta V}^{\text{aff,0}}) \tag{16}$$

Using the values above-reported, we can calculate that $\overline{\Delta H}^{\text{desolv,bond}} = +3.4$ kcal (mol of calcium)$^{-1}$ and $\overline{\Delta S}^{\text{desolv,bond}} = +43.7$ cal (mol of calcium)$^{-1}$ K^{-1}. The latter value is in excellent agreement with the corresponding value ascribed to the desolvation of the Ca/EDTA complex: $\overline{\Delta S}^{\text{desolv,bond}} = +42 - +57$ cal (mol of calcium)$^{-1}$ K^{-1}.[34] Moreover, the large value of $\overline{\Delta H}^{\text{desolv,bond}}$ very well accounts for the endothermic change accompanying the rupture of several (about 18, according to the estimate reported in the previous paragraph) ion−dipole bonds between the released water molecules and the ionic species.

Ion Pairing. After the above encouraging results, the ion paring term can be trivially calculated as

$$\overline{\Delta H(S)}^{\text{ionpairing,bond}} = \overline{\Delta H(S)}^{\text{complex}} - \overline{\Delta H(S)}^{\text{desolv,bond}} \tag{17}$$

The result for enthalpy is $\overline{\Delta H}^{\text{ionpairing,bond}} = -5.1$ kcal (mol of calcium)$^{-1}$ in excellent agreement with the value reported in the literature[35,36] for the tetra-carboxylated Ca/EDTA complex (i.e., −5.4 kcal (mol of calcium)$^{-1}$). This result further suggests that the initial assumption that affinity is driven essentially by desolvation is very plausible. As to entropy, it is large and negative: $\overline{\Delta S}^{\text{ionpairing,bond}} = -38.0$ cal (mol of calcium)$^{-1}$ K^{-1}. Since the entropy associated with the ion pair process of low MW compounds was reported to be negligible,[34] it is straightforward to attribute $\overline{\Delta S}^{\text{ionpairing,bond}}$ to the chainlike nature of the ligands involved in the ion-pairing process. Therefore, this term will not be included in the fundamental event of complex formation, for which it can be written

$$\overline{\Delta G}^{\text{complex}} \equiv (\overline{\Delta H}^{\text{bond,0}})_{\text{Rj=0}} - T\overline{\Delta S}^{\text{desolv,bond}} = (\overline{\Delta H}^{\text{ionpairing,bond}} + \overline{\Delta H}^{\text{desolv,bond}}) - T\overline{\Delta S}^{\text{desolv,bond}} \tag{18}$$

The proper combination of the thermodynamic quantities that can be traced back to single ion bonding event (i.e., at $R_j \rightarrow 0$) allowed evaluating the instability constant, as pK_{in}, for the calcium-poly(galacturonate) system

$$\text{p}K_{\text{in}} = -\log(e^{-\Delta G^{\text{complex}}/RT}) \tag{19}$$

The pK_{in} for the very first contact between calcium ion and poly(galacturonate) resulted to be 10.80, which is in very good agreement with the value reported for the case of Ca-EDTA complex formation (10.96 for the equilibrium Ca-EDTA $\leftrightarrows$ Ca^{2+} + EDTA^{4-}).[37] This results strengthens the applicability of the theoretical model presented here for the numerical calculation of the thermodynamic quantities in the calcium-induced chain dimerization process for poly(galacturonate).

In conclusion, the formation of the complex between calcium and pectate very closely resembles that between calcium and a strong chelating agent, like EDTA. This holds true in particular as to the overwhelming effect related with the release of water molecules from the interacting ionic species (*desolvation*: positive and large change of entropy) as well as to the formation of strong ionic bonds of the "inner-sphere" complex (*ion-pairing*: negative and large change of enthalpy). However, a major difference is apparent as to the "cross-linking" effect of calcium between two pectate chains with respect to the Ca/EDTA system. In the present case, each "cross-link" contributes not only to a large loss of entropy at the site of complexation, but also with a reduction of freedom in the configurational statistics of the two macromolecules. This specific point will be taken into proper account in a forthcoming paper.

(c) Further Considerations on the Model. The model presented so far successfully (and realistically) described the behavior of poly(galacturonate) when treated with calcium cations. One of the main goals of this model is represented by the remarkable fitting of the experimental data of enthalpy variation which was accomplished exploiting the distinction between "bound" and "condensed" divalent ions, the latter being further affected by an intrinsic affinity toward the polyanion. It is useful, in our opinion, to remember that in the theoretical treatment of the model the chemical bonding of counterions in dimer structures is accompanied by complete charge annihilation between the ion and the uronic groups in the specific binding site.

One may, at this point, question on the relevance and need of introducing concepts (and variables) like the intrinsic Gibbs energy of bonding and of affinity and like the complete charge annihilation for the description of the ion-induced chain aggregation, particularly in view of the available widespread extensions of the original Manning's theory on counterion condensation.

When adapting one of the newest theoretical efforts in the field, the so-called "fluctuating-charge annihilation"[18] model, to the present case, the molar ratio between the cross-linking ion and the uronic moieties in the site of bonding represents an additional variable in the system of equations resulting from the total Gibbs energy minimization (conversely, in the present model a constant value (σ_0) for that ratio is assumed). This brought some difficulties in reaching the convergence for the numerical calculations. However, it is of the most importance to underline that the main obstacle is represented by the chemically nonrealistic description of the ion-induced poly(galacturonate) chain association, as already pointed out in the Introduction.

To clarify this point, it is very useful also to analyze thoroughly the result of the application to the present case of the original Manning's theory,[38−40] implemented with an additional term accounting for nonbonding affinity[20] but neglecting chemical bonding and charge annihilation. According to this approach, a doubling of the linear charge density occurs upon dimerization of the polyanion chains. The condensation of the ions is therefore enhanced by both the affinity (present both for the coil fraction and for the dimer fraction) and the increase of ξ_{D} (which would result in $\xi_{\text{D}} = 2\xi_{\text{C}}$). In this case, the convergence of the numerical solution for the system of equations is easily attained and the Gibbs energy profile for the ion-induced dimerization process straightforwardly calculated. This parallels very closely the one exhibited by the present "complete charge annihilation" model, rendering it apparently as feasible as the latter. Moreover, although the profiles of the fraction of condensed divalent counterions for the two models obviously differ, the total amount of divalent ions "removed" from the solution coincides in the two cases (Figure 8, panel I). However, the (chemical) implications brought by the two models can help highlighting their differences. In particular, although in the present assumption of complete charge annihila-

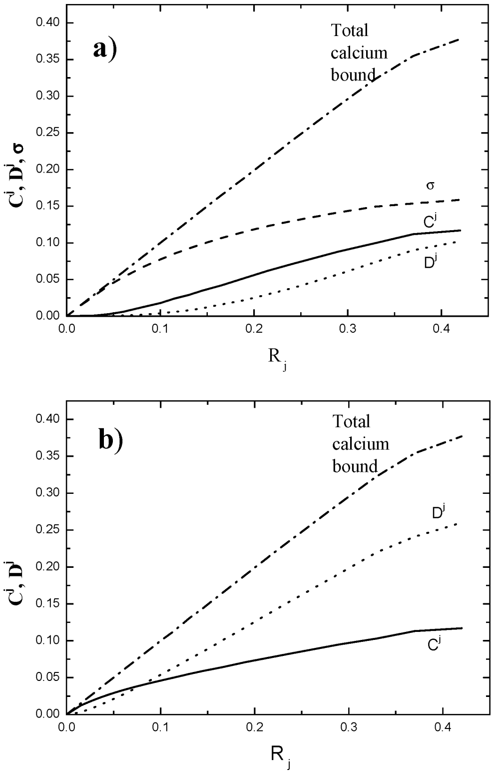

Figure 8. Dependence on R_j of the fraction of territorially condensed calcium counterions (C^j, — and D^j, ···) and of the total fraction of divalent counterions removed from the solution (· — ·) for (a) the "complete charge annihilation" model and (b) the "affinity-driven electrostatic model".[20,30] In the former case, the fraction of the chemically bound calcium ions (σ,— —) obtained from the present model is also reported. In both cases, a (reduced) intrinsic Gibbs energy of affinity $g^{aff,0}$ equal to -7 was used.

tion the presence of 1 divalent ion per "egg-box" unit is guaranteed (σ_0 constant and equal to 0.25), the "purely" electrostatic model predicts a very low initial fraction of counterion "territorially" condensed on the dimer (D^j in Figure 8 panel Ib). It would produce the paradox that a few calcium ions would tightly hold two long stretches of the polyuronate chains: as many as 5% (the fraction of dimer structure) of 3.5×10^{-3} mol of uronate would be stabilized by $\approx 10^{-5}$ mole of calcium ions (i.e. one ion every ≈18 dimer units!).

Conclusions

The results above-reported can be summarized as follows:

1. The theoretical model described in ref 19 can be consistently applied to describe the interaction of calcium ions with poly(galacturonate) in aqueous solution.

2. Two modes of binding are present at the same time: a territorial affinity of the divalent ions for both the isolated single chain and for the dimer and a strong chemical bonding of calcium ions within the chain dimer, which brings about an annihilation of the fixed charges of the coordinating COO^- groups.

3. The minimization procedure using the experimental chiro-optical and microcalorimetric data provides constant values for free energy, enthalpy (and hence entropy) changes of the affinity of calcium as a function of the molar ratio of total calcium over polymer. The favorable affinity interaction is mainly due to a large and positive change of entropy.

4. The values of the free energy of chemical bonding do depend on the molar ratio of total calcium over polymer, being numerically very close to the corresponding affinity term for low calcium content and increasing toward less favorable values on increasing R_j. The enthalpy change of chemical bonding is always negative: in the range of R_j up to about 0.15, it decreases in a sigmoid fashion, to reach a constant value thereafter.

5. Both modes of binding are accompanied by substantial positive volume changes which can be convincingly traced back to the release of electrostricted water from the interacting ionic species. That accompanying chemical bonding is larger than that stemming from affinity interactions.

The scope of this paper was to test the applicability of the recently proposed extension of the counterion condensation theory.[19] This goal has been achieved, together with an encouraging set of thermodynamic quantities calculated for the single binding event for the calcium-poly(galacturonate) system, which correlate very well with those reported for the Ca-EDTA case. Further understanding of the macromolecular aspects of the calcium-induced association of pectate can be obtained starting from these results. This issue will be addressed in a forthcoming paper.

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