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The interaction of polyuronides with calcium ions. 1: binding isotherms of calcium ions with pectic substances

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The binding isotherms for Ca^{2+} with sodium pectate and sodium pectinate (58% esterification) were constructed. To achieve this, a method of determining free Ca^{2+} ions using auxiliary ligand, tetramethylmurexide, was employed. It was demonstrated that the Ca^{2+} ion binding to the pectate was cooperative exceeding that expected on the basis of purely coulombic interactions. In contrast, the binding of the ions by the pectinate is close to that predicted for coulombic binding.

INTRODUCTION

The interaction of polyuronides such as alginates and pectic substances with the ions of polyvalent metals, in particular Ca^{2+}, has been studied fairly extensively, (Smidsrød, 1973; Kohn, 1975; Rees, 1975, 1981; Rees & Welsh, 1977; Morris & Norton, 1983). It is recognised that in the majority of cases this process includes both the coulombic interaction of metal ions with a polyanion and their coordinative binding. The latter is responsible for the cooperative interaction of most polyvalent metal ions with polyuronides which is the basis of gelation (Rees, 1969; Smidsrød, 1973, 1974; Thiele, 1967).

However, an analysis of the published data shows that the statement about the cooperative interaction of the polyvalent ions with polyuronides is based on indirect observations only. Thus, Kohn and Larson (1972) and Kohn and Luknár (1977) have demonstrated that an increase in the degree of polymerization of galacturonate oligomers up to 11–20 and guluronate oligomers up to 18–28 brings about a sharp decrease in the Ca^{2+} activity coefficient. Such degrees of polymerization are viewed by the authors as the minimum required for cooperative binding. For mannuronate oligomers the dependence of the Ca^{2+} activity coefficient on the degree of polymerization is smooth and coincides with that calculated for the coulombic interaction.

As shown by Smidsrød and Haug (1968, 1972), the selectivity coefficients for the replacement of Mg^{2+} ions by ions of other alkaline-earth metals on L-guluronate and pectate pass through a maximum as the ion-exchange process occurs. These results are explained assuming the cooperative interaction of alkaline-earth metal ions (the only exception being Mg^{2+}) with the two polyuronides. In particular, the cooperativity leads to a block-wise distribution of the exchanged ions (Smidsrød & Haug, 1972).

Bryce *et al.* (1974), Grant *et al.* (1973), Morris *et al.* (1973, 1982*a*, *b*) and Thom *et al.* (1982) have established a sharp change in the molar ellipticity in the region of the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the carboxylate chromophore in the binding of the Ca^{2+} ions with the guluronate blocks of alginate or with pectate. These results were also interpreted as indicating the cooperative nature of the Ca^{2+} binding.

On the basis of the experimental data and the results of conformational analysis, Grant *et al.* (1973) put forward a model explaining the structure of polygalacturonates and polyguluronates of polyvalent metals. According to this 'egg-box' model, the polyuronide chains in a zigzag conformation are packed into ordered clusters with the cooperatively bound cations being included in the chain interspaces. Further, a cation is coordinated to the oxygens of the hydroxyl, carboxylate and acetal groups, stabilizing the structure (Anthonsen *et al.*, 1972, 1973; Smidsrød *et al.*,

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1972; Smidsrød, 1973; Grasdalen *et al.*, 1975; Rees & Welsh, 1977; Liang *et al.*, 1980).

Finally, Cesàro *et al.* (1982) have shown that, in contrast to D-mannuronate, the dependence of the enthalpy of mixing L-guluronate, alginate and pectate with Ca^{2+} ions as well as their relative molar ellipticity on the Ca^{2+}/polyuronide concentration ratio is S-shaped. This is also regarded as indicative of cooperative binding.

An unequivocally positive binding cooperativity can be found from the corresponding isotherms. Such isotherms in Klotz coordinates were plotted by Kohn and Furda (1967*b*) and Kohn and Loviška (1967) for pectinates with degrees of esterification ranging from 5·6% to 90%. However, in this case no binding cooperativity was observed. Accordingly, the authors regarded the thermodynamic constants of the Ca^{2+} binding with pectin of any degree of esterification as being independent of the degree of binding. Although in the case of pectate, in a later paper Kohn and Tibensky (1971) reported lower binding constants with increasing Ca^{2+} concentration; they attributed this effect to the system's heterogeneity. The possible reasons for the reported lack of cooperativity of the Ca^{2+} interaction with low methoxyl pectins will be discussed below in this paper.

Thus, despite the numerous studies on the interaction of the Ca^{2+} ions with polyuronides, the thermodynamic parameters of the process have not obviously received adequate attention. In the case of pectic substances, the published results contradict the concept of cooperativity.

The purpose of the present work was to study the interaction of the Ca^{2+} ions with pectic substances over a wide range of ion : polymer ratios and, accordingly, binding densities. The studies were made on sodium pectate and sodium pectinate esterified to 58%.

MATERIALS AND METHODS

In this study, the authors used commercially available samples of pectic acid (Sigma, St Louis, USA) and citrus pectin (Koch Light, Colnbrooke, Bucks, UK).

The content of polyvalent metal ions in the samples was determined by X-ray fluorescent analysis using a VR A2 instrument (Karl Zeiss, Jena, FRG) and the results were considered in calculating the content of bound Ca^{2+} ions. The other analytical methods as well as sample purification techniques are described by Yuryev *et al.* (1981). Sample characteristics are given in Table 1.

Table 1. Characteristics of pectic substances[a]

Degree of esterification (%)	Content of uronide groups (%)	Linear charge density parameter, ξ
0	88·2	1·663
58[b]	89·0	1·663

[a] The samples contained no traces of Ca^{2+}, Mg^{2+} or Fe^{3+}.
[b] Citrus pectin. Rhamnose, 3–7%; arabinose, 4–6%; xylose, under 1% (according to the data of the Central Institute of Nutrition of the GDR Academy of Sciences).

The concentration of free (unbound) Ca^{2+} ions in solution was determined spectrophotometrically by using tetramethylmurexide (TMM) as an auxiliary ligand (Raaflaub, 1962). In doing this, the authors utilized the so-called 'two-wavelength ratio method' (TWRM). It has been proposed and used for the determination of the degree of Ca^{2+} binding with anionic polysaccharides by Buddecke and Drzeniek (1962) and developed in detail for studies of the interaction of these ions with polyuronides by Kohn and Furda (1967*a*). This method was also employed by Kohn and coworkers in studies of the interaction of ions with pectic substances and guluronate oligomers. A comparison of the TWRM with the other methods of finding the concentration (activity) of divalent metal ions in solution showed that the former is to be preferred both in terms of precision and sensitivity (Kwak & Joshi, 1981).

TMM was synthesized from tetramethyl alloxantine as described by Gysling and Schwarzenbach (1949), and the intermediate product was obtained from caffeine as proposed by Biltz (1912).

The interaction of Ca^{2+} ions with the pectic substances was studied in solutions having ionic strength 0·1. This parameter was calculated taking into account the content of a low molecular weight calcium salt ($CaCl_2$) and a supporting electrolyte (NaCl). Due to the low polyuronide concentrations (no more than 6×10^{-3} g-equiv. $litre^{-1}$) its contribution to the ionic strength was negligible. For the same reason, the concentration drop of the Ca^{2+} ions as a result of their binding with the pectic substances was also neglected.

The solutions were prepared by weight. After preparation they were stored at room temperature for 24 h and then centrifuged at 2×10^4 g for 20 min. During the photometric studies, the residual turbidity of the solutions was considered.

The optical density was measured with a Spekord M-40 spectrophotometer (Karl Zeiss, Jena, FRG). The optical density was measured at 535 and 495 nm, corresponding to the predominant absorption of free TMM and its Ca^{2+} complex, respectively.

The molar concentration of the free Ca^{2+} ions (c_f) was determined from the calibration curve corresponding to the following equation (Kwak & Joshi, 1981):

$$\phi = (\varepsilon''_D + \varepsilon''_{MD} K \gamma_{Ca^{2+}} c_f)/(\varepsilon'_D + \varepsilon'_{MD} K \gamma_{Ca^{2+}} c_f) \quad (1)$$

where $\phi = \frac{D_{495}}{D_{535}}$, is the optical density ratio at two wavelengths; ε'_D and ε''_D are the molar extinction coefficients for TMM at 535 and 495 nm, respectively; ε'_{MD} and ε''_{MD} are the same for TMM-Ca^{2+} complex; K is the thermodynamic stability constant of the TMM-Ca^{2+} complex; $\gamma_{Ca^{2+}}$ is the activity coefficient of the Ca^{2+} ions (this quantity being a constant as the measurements are conducted at the same ionic strength).

Equation (1) can be transformed as follows:

$$\phi = A + \frac{B}{c_f + E} \tag{2}$$

where $A = \varepsilon''_{MD}/\varepsilon'_{MD}$;

$$B = \frac{\varepsilon''_{MD}}{\varepsilon'_{MD} K \gamma_{Ca^{2+}}} \left(\varepsilon'_{MD} \frac{\varepsilon''_D}{\varepsilon''_{MD}} - \varepsilon'_D \right);$$

$$E = \frac{\varepsilon'_D}{\varepsilon'_{MD} K \gamma_{Ca^{2+}}}$$

Using the measured data for the optical densities of standard solutions and minimizing the mean-square deviations, a calibration curve was constructed on a computer to match eqn (2). To calculate the errors in the c_f determinations, mean-square deviations of the standard solution optical density from the curve were used. As shown in Fig. 1, owing to the non-linear form of this curve, the absolute measurement error increases as c_f increases.

The density of Ca^{2+} binding to the polyuronides ($\bar{v}$) can be calculated as follows:

$$\bar{v} = \frac{c_t - c_f}{c_p}$$

where c_t is the analytical molar concentration of the Ca^{2+} ions in solution; c_p is the polymer concentration (g-equiv. litre^{-1}).

The experimental results are presented in the form of a Scatchard plot (Cantor & Schimmel, 1980) $\bar{v}/c_f = f(\bar{v})$. The type of the coordinates, together with the form of the calibration curve, is responsible for increasing the absolute error along the x-axis and decreasing the error along the y-axis with $\bar{v}$.

RESULTS AND DISCUSSION

The isotherms for the Ca^{2+} binding with the pectic substances are shown in Fig. 2. The same figure shows isotherms for the delocalized binding of these ions due to the coulombic interaction, calculated using a two-parameter Manning's method (1974). Such a binding takes place when the linear density of the polyelectrolyte charge (ξ) exceeds 0·5. Methods for calculating this parameter for pectic substances have been discussed by Yuryev and coworkers (1981, 1983). The latter paper demonstrated that the currently used sample of highly esterified pectinate is characterized by a microblock distribution of the charged groups with a length of *c.* 10 pyranose units each. Therefore, for pectinate the parameter ξ is the same as that for pectate. The values of this parameter are given in Table 1.

The method used for calculating the coulombic binding isotherms presupposes that $c_1 \gg c_2$, where c_1

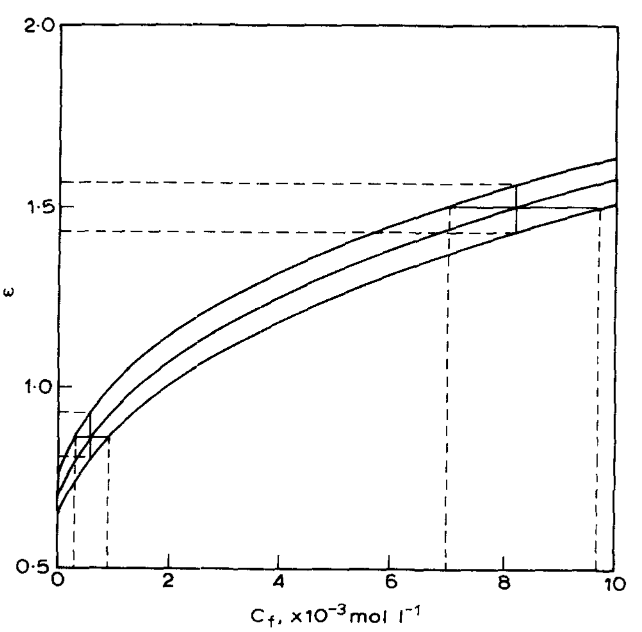

Fig. 1. The dependence of the absolute determination of error on the free Ca^{2+} concentration.

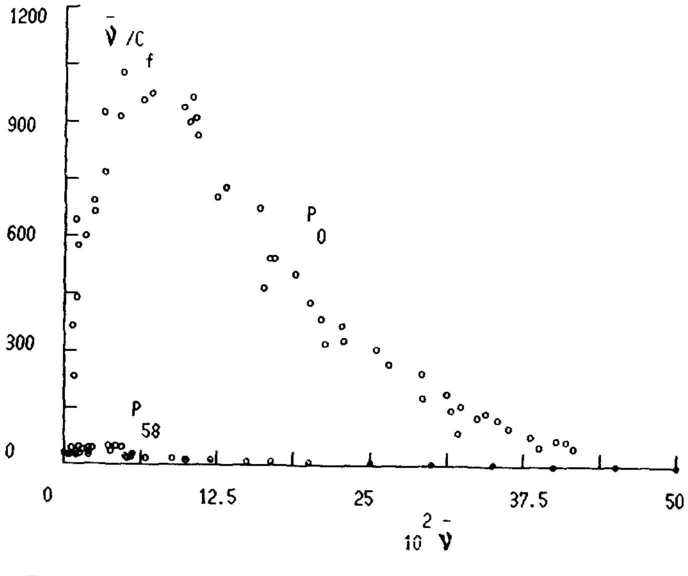

Fig. 2. Isotherms of the Ca^{2+} binding with pectic substances. (—○—) Experimental data: P_0, pectate; P_{58}, pectinate; degree of esterification = 58%. (—●—) Calculated as proposed by Manning (1974). Polyuronide concentration, 10^{-3} g-equiv. litre^{-1}: P_0, 1,2,4,6; P_{58}, 2.

and c_2 are the molar concentrations of monovalent and divalent ions, respectively. In the present authors' experiments, the concentration of the Ca^{2+} ions did not exceed 10^{-2} mol litre^{-1} and the c_1/c_2 ratio was at least 22.

The shape of the isotherms for the Ca^{2+} binding with the pectic substances permits the following conclusions to be made:

(1) The Ca^{2+} ions are bound much more strongly to pectate than would be the case for coulombic binding. The density of the binding of these ions with the pectinate (degree of esterification of 58%) is close to that predicted for coulombic binding. These results agree with the existing ideas of the nature of the Ca^{2+} interaction with polyuronides (Kohn, 1975; Kohn & Kuknár, 1975).

(2) The isotherms of the Ca^{2+} binding with the pectic substances are convex-shaped, which demonstrates a positive cooperativity for the interaction (Cantor & Schimmel, 1980; Perlmutter-Heyman, 1986).

It should be noted that the advance towards the region of relatively low binding densities where the interaction cooperativity is evident is only possible if the binding constant is not too high and, hence, the concentration of the free Ca^{2+} ions is relatively high. The Ca^{2+} binding constant with polyuronides decreases with ionic strength. The present authors have managed to detect the cooperativity of the Ca^{2+}-pectate interaction at an ionic strength of 0·1 whereas according to Mattai and Kwak (1986) at an ionic strength of 0·01 the sensitivity of the method used by the present authors for determining the concentrations of these ions is insufficient for measuring a binding density under 0·2. Perhaps for this reason the above authors have failed to observe the cooperativity of the Ca^{2+} binding with pectate at this ionic strength.

The experiment data reported by Kohn and Furda (1967*b*) relate to an ionic strength of 0·01; however, these authors state that the multiple-equilibria law which describes a non-cooperative binding is observed over the entire range of the ionic strengths studied (0·01–0·15). The data reported by Kohn and Lovǐska (1967) were obtained at an ionic strength of 0·02.

(3) The maximum density of the Ca^{2+} non-coulombic binding with pectate ($\bar{v}_{max}$) is 0·50. In other words, the apparent order of the reaction with respect to the polymer ($r = \bar{v}^{-1}_{max}$) is 2·0.

(4) The Ca^{2+} binding density is independent of pectate concentration (Fig. 2). In other words, the reaction is zero order with respect to the polymer. This result agrees with the data reported by Kohn and Luknár (1975) as well as Thibault and Rinaudo (1985) but disagrees with those reported by Rinaudo and Ravanat (1980).

The independence of the Ca^{2+} binding density on polyuronide concentration is due to the solution microheterogeneity (Yuryev *et al.*, 1981). The region of pectate concentrations under study, i.e. 1×10^{-3}–6×10^{-3} g-equiv. (kg solvent)$^{-1}$ related mainly to the semi-dilute microheterogeneous solutions. It is obvious that the Ca^{2+} binding occurs largely in the 'microreactors', i.e. macromolecular associations, and the exhaustion of the unbound carboxylate groups in one of these 'microreactors' does not affect their concentration in the others. The general type of such a mechanism responsible for polysaccharide gelation has been noted by Zasadzinski *et al.* (1986).

CONCLUSION

The cooperativity of binding of Ca^{2+} ions with pectic substances has been demonstrated by a direct method. The binding of the ions by pectate substantially exceeds coulombic binding in contrast to the binding to a pectin with a degree of esterification of 58%.

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