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AN E.P.R. AND POTENTIOMETRIC STUDY OF THE COMPLEXATION OF COPPER IONS BY GALACTURONIC ACID AND GALACTURONANS

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ABSTRACT

E.p.r. spectroscopy and copper potentiometry indicated the stepwise formation of copper–galacturonate complexes, with successive formation constants log K_1 ~2.16 and log K_2 ~2.05. Comparison of the magnetic parameters $g_\|$ of these complexes (2.403 ± 0.003 and 2.380 ±0.003, respectively) with those of copper complexes formed with pectins of various origins (2.390 ±0.003) showed that weaker bonds were formed with the polymers than with the monomers. The greater affinity of copper for the polymers is therefore due mainly to entropic effects. The presence of aquo-complexes in frozen solutions, easily detectable by e.p.r. spectroscopy, was observed, for all the pectins studied, when the metal-to-ligand ratio (*N*) was >0.5. The increase in line-width with *N*, up to *N* ~0.5, was more important with unesterified pectins (~200%) than with esterified pectins (~40%).

INTRODUCTION

The interactions of anionic biopolymers and cations are important in physiology and ecology, as well as in the food and other industries. The carboxylate groups of pectins form stable associations with multivalent cations, a property which strongly influences their behaviour as ion-exchangers and as rheological agents (cation-induced gel formation). In addition to a non-specific preference for multivalent cations, which allows the plants to compete with soil colloids for oligo-elements, pectins show exceptional affinities for some ions. This property leads not only to bio-accumulation of pollutants such as Cu(II) or Cd(II) (ref. 1) by plants, but also to applications such as the elimination of Pb(II) (ref. 2) from the human body. Alginates, the chemical structure and properties of which are largely similar to those of pectins, show a slightly different series of affinities and are used, for

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instance, for dealing with radio-active contamination by Sr(II) (ref. 3).

Electron paramagnetic resonance (e.p.r.) spectroscopy can be used to characterise the micro-environment of paramagnetic species (Cu^{2+}, Mn^{2+}, VO^{2+}, *etc.*) in complex biological media (humus, living cells, river or sea waters, *etc.*), and to obtain information on the extent of binding, the chemical nature and the spatial distribution of the specific binding sites, *etc.* Correlation of the e.p.r. parameters with thermodynamic stability constants for Cu^{2+} complexes has been established tentatively[4]. However, the interpretation of e.p.r. spectra, in terms of the chemical nature and properties of the systems studied, is not always straightforward, since, for instance, an increase in line-width may be due to a variety of causes, such as temperature effects, interactions between paramagnetic centers, *etc.*[5-7]. E.p.r. studies of complexes formed by macromolecules can be complemented usefully by other experimental techniques, and by e.p.r. studies of simpler systems (*e.g.*, complexes formed by the corresponding monomers).

Previous e.p.r. and ion-exchange studies of the interactions of divalent cations and pectins in isolated cell walls of *Nitella flexilis*, a fresh-water green alga (family *Characeae*), suggested[8] the existence of two distinct binding sites for Cu^{2+}, which were assigned to the two different types of pectinic acids described by Cook and Stoddart[9]. The buckled shape of homopolygalacturonic chains characteristic of type I pectins are expected to leave interstices when they pack together. The binding between the chains is ensured by cations which fit into the cavities and screen the electrostatic repulsions between the negative charges that otherwise would cause the chains to repel each other. This form of association has been named the "egg-box" model[10]. Type II pectins comprise branched poly(galacturonic acid) chains interspersed with neutral residues and would not be expected to form "egg-box" complexes.

We now report on the influence of the structure and chemical composition of pectin chains of various origins, and of galacturonic acid, on the thermodynamic stability of their complexes with copper ions.

EXPERIMENTAL

Apple, sugar-beet, and *Nitella flexilis* pectins were extracted by acid hydrolysis [30 min, 100°, citrate buffer (pH 3)], precipitated in 60% ethanol, and then thrice suspended in water and precipitated in 60% ethanol. Citrus pectin (de-esterified sodium polygalacturonate; 85–90% galacturonic acid), galacturonic acid monohydrate, and $CuCl_2 \cdot 2H_2O$ were commercial products.

X-Band spectra were recorded with a Varian E-12" e.p.r. spectrometer fitted with an E231-232 double cavity and a Varian E 257 temperature-controller system. The irradiation power was set to 20 mW and the magnetic field modulation frequency to 100 kHz, conditions in which the absence of signal saturation was verified. A Varian Pitch sample was used as reference (*g*, 2.0028).

The activity of free Cu^{2+} ions was measured with an Orion 94-29 specific electrode (Orion 901 reference electrode, Orion 90.00.01 reference solution). The ionic strength of the medium was not kept constant, and the electrode response was therefore calibrated by plotting the potential readings (mV) against the log activity, rather than concentration, of standard solutions of copper nitrate; the activity coefficients for charged species were calculated with the help of the Debye–Hückel theory formula [for Cu(II) at 25°, $\log \gamma = -(4 * 0.5085, \sqrt{I})/(1 + 6 * 0.3281 \sqrt{I})$] (ref. 11).

Preparation of copper complexes. — Initial pH values were ~3 for acidic monomer solutions, ~5 for 97% NaOH-neutralised solutions, and 4.5–5 for the various solutions of polymer. Aliquots (1 mL) of the solutions (6 g/L) were mixed with equal volumes of solutions (1–28 mequiv./L) of copper chloride or nitrate. Final concentrations of ligand varied from ~3 mequiv./L for apple and sugar-beet pectins (poor in unesterified galacturonic acid) to ~14 mequiv./L for *Nitella flexilis* pectin, polygalacturonate, and monomers. Final copper concentrations were in the range 0.5–14 mequiv./L. Slight gelling of the polymers took place at the higher concentrations.

The overall formation constant is $K_t = K_1 \cdot K_2$, where $K_1 = (ML^+)/(M^{2+}) \cdot (L^-)$ and $K_2 = (ML_2)/(ML^+) \cdot (L^-)$. The corresponding conditional constants, based on concentrations rather than activities, are K_{ct}, K_{c1}, and K_{c2}. The metal-to-ligand ratio in solution (N) is $2C_m/C_l$, where C_m and C_l are the total concentrations (in mol/L) of copper and carboxylate groups, respectively. Concentrations and activities (mol/L) of the species in solution (ML_2, ML^+, M^{2+}, and L^-) are denoted by [ ] and ( ), respectively.

RESULTS

The complexes formed with Cu^{2+} in aqueous solutions of galacturonic acid or pectins have been studied by e.p.r. spectroscopy and, for the monomers, by potentiometric measurements. The pectins were extracted from apple fruit, sugar beet, and *Nitella flexilis* alga. A commercial sample of de-esterified citrus pectin was also studied. Apple and sugar-beet pectins are partly methyl esterified and rich in neutral sugar side-chains (mainly arabino-galactans)[12]. Sugar-beet pectin contains also ~2.5% (w/w) of OAc groups[13], which limits the gelling ability. *Nitella flexilis* pectin is unesterified[14].

At low metal-to-ligand ratios (Figs. 1a, 2a, 3a, and 4a), e.p.r. spectra recorded at 113 and 253 K had shapes similar to those of the spectra of Cu^{2+}-pectin complexes presented in the literature[8,15]. Such spectra, with $g_\perp = g_x = g_y < g_\parallel = g_z$, are considered to be typical of elongated near-octahedral or square planar symmetries around the copper ion[16]. There was a small but significant shift of parameter $g_\parallel$ with pH in monomer solutions [2.380 ±0.003 at pH ~5 (Fig. 1a); 2.403 ± 0.003 at pH ~3 (Fig. 2a)], whereas, with pectins of all origins, it remained remarkably constant [2.390 ±0.003 (Figs. 3a and 4a)].

At high metal-to-ligand ratios (Figs. 1b, 1c, 2b, 3b, and 4c), the e.p.r. spectra

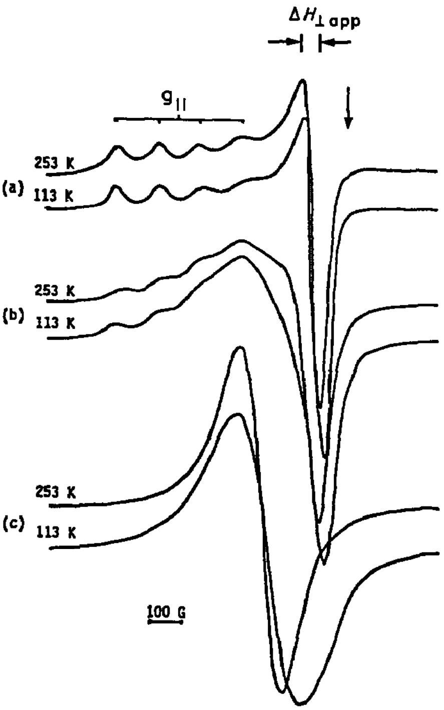

Fig. 1. E.p.r. spectra of Cu^{2+}-sodium galacturonate solutions (pH ~5), recorded at 253 and 113 K. Total ligand concentration, 14mM; metal-to-ligand ratios (N), (a) 0.15, (b) 0.75, (c) 4.50. The arrow indicates g = 2.0028 (H = 3300 G).

indicated the presence of aquo-complexes $Cu[H_2O]_6^{2+}$. These complexes have a strong tendency to segregate due to ice crystallisation, unless glycerol or salts in high concentrations are added, which ensure the formation of homogeneous glasses on freezing[17]. The strong magnetic interactions resulting from the short distances between segregated aquo-ions led to e.p.r. spectra with distinctive features (see Fig. 1c, where copper was in large excess): temperature-sensitivity (down-field shift of the minimum, from ~3150 G at 113 K to ~3090 G at 253 K) and apparent loss of hyperfine structure in the "$g_{\parallel}$ area". The spectra in Figs. 1b, 2b, 3b, and 4c resulted from the simultaneous presence of organic complexes, shown by the hyperfine structure in the $g_{\parallel}$ area, and of aquo-complexes, shown by the temperature-sensitivity.

The aquo-complexes appeared progressively with increasing metal-to-ligand ratio (N), starting from N ~0 with the monomers, but from N ~0.5 only with the pectins. Complementary Cu^{2+} potentiometric and conductimetric measurements (data not shown) independently confirmed that Cu^{2+} bound almost quantitatively to polygalacturonate up to N ~0.5, but interacted only weakly with the polymer for N >0.5.

For a homogeneous population of copper ions, the apparent line-width of the perpendicular component ($\Delta H_{\perp \text{app}}$) at near-liquid-nitrogen temperatures may be

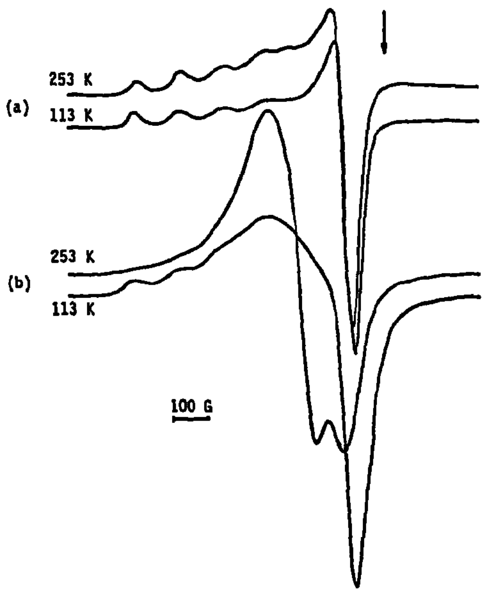

Fig. 2. E.p.r. spectra of Cu^{2+}-galacturonic acid solutions (pH ~3), recorded at 253 and 113 K. Total ligand concentration, 14mm; metal-to-ligand ratios (*N*), (a) 0.07, (b) 0.54. The arrow indicates *g* = 2.0028.

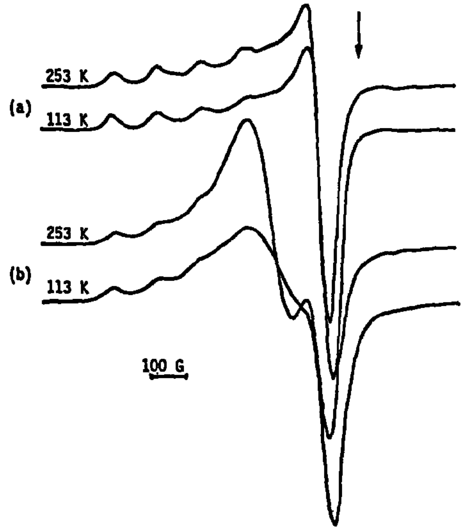

Fig. 3. E.p.r. spectra of Cu^{2+} complexes with a partly esterified pectin (sugar-beet pectin), recorded at 253 and 113 K. Total ligand concentration, 3 g/L (~5 mequiv./L in unesterified carboxylates); metal-to-ligand ratios (*N*), (a) ~0.50, (b) ~1.50. The arrow indicates *g* = 2.0028.

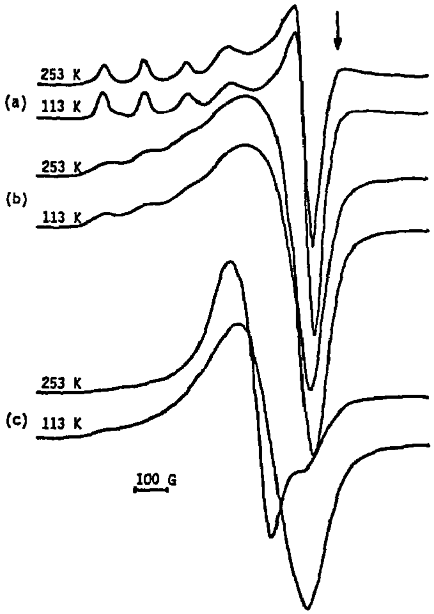

Fig. 4. E.p.r. spectra of Cu^{2+} complexes with an unesterified pectin (citrus pectin), recorded at 253 and 113 K. Total ligand concentration, 3 g/L (~13.5 mequiv./L in unesterified carboxylates); metal-to-ligand ratios (*N*), (a) ~0.05, (b) ~0.40, (c) ~1. The arrow indicates $g = 2.0028$.

used as an approximation to the true line-width as determined by computer simulation routines [$\Delta H_{\perp} = \sim 1.17\ (\Delta H_{\perp\,\mathrm{app}})$][18]. This approximation is not valid for spectra of mixed populations, as, for example, when organic and aquo-complexes are present simultaneously. At low metal-to-ligand ratios ($N \longrightarrow 0$), the ($\Delta H_{\perp\,\mathrm{app}}$) measured for apple, citrus, and sugar-beet pectins and for monomers were between 47 and 57 G. For the monomers, the increase of ($\Delta H_{\perp\,\mathrm{app}}$) with N is clearly due to the growing fraction of ice-segregated aquo-complexes. For the pectins, the aquo-complexes were present in negligible amounts up to $N \sim 0.5$, and two different types of behaviour were observed in that range: an increase of ($\Delta H_{\perp\,\mathrm{app}}$) of ~20 G with sugar-beet [Fig. 3a, $N \sim 0.5$, ($\Delta H_{\perp\,\mathrm{app}}$) ~70 G] and apple pectins (both partly esterified), and over 100 G for citrus [Fig 4b, N ~0.4, ($\Delta H_{\perp\,\mathrm{app}}$) ~160 G] and *Nitella* pectins (both unesterified).

The line-width of the parallel component ($\Delta H_{\parallel}$) cannot be measured easily without computer simulation routines, but the shape of the lower-field lines (~2600 and ~2730 G) are only slightly affected by the superposition of the spectrum of ice-segregated aquo-complexes. As with ($\Delta H_{\perp\,\mathrm{app}}$), $\Delta H_{\parallel}$ clearly increased strongly with N up to 0.5 for unesterified pectins (Figs. 4a and 4b), whereas relatively sharper lines were still observed, for partly esterified pectins, even for $N > 0.5$ (Figs. 3a and 3b).

DISCUSSION

Monomers. — Motschi[4], on the basis of literature e.p.r. data and thermodynamic characteristics of a series of cupric complexes, has proposed the approximate relation *1* between their formation (stability) constants K and the e.p.r. parameters $g_\parallel$.

$$-\Delta(\log K) = 84(\pm 8)\Delta(g_\parallel) \qquad (1)$$

When applied to the values of $g_\parallel$ observed for the complexes of galacturonic acid, this formula gave a $\Delta(\log K)$ value of ~2, the complex obtained at higher pH being the more stable. This result suggests the formation of ML^+ complexes, favoured by proton competition, at pH ~3, whereas ML_2 complexes would be more readily formed at pH ~5. The calculated value (~2) for $\Delta(\log K)$ corresponds then to $\log K_2 = (\log K_t - \log K_1)$. In order to estimate absolute values for $\log K_1$ (ML^+) and $\log K_t$ (ML_2), the $g_\parallel$ value for aquo-complexes is needed, which can be observed only in glasses, *i.e.*, in the presence of a viscous agent that prevents the crystallisation of ice and segregation of the solute[17]. For copper chloride at 77 K, $g_\parallel$ values of 2.415 ±0.003 in aqueous 50% Me_2SO and 2.412 ±0.003 in aqueous 20% glycerol were measured. These values are closer to those reported in the literature (2.406 for copper nitrate at 77 K in aqueous 83% ethylene glycol[19], 2.412 for copper perchlorate at 77 K in aqueous 50% Me_2SO (ref. 20), 2.400 for copper nitrate at 253 K in aqueous 40% glycerol[7]) than the value (2.440) used by Motschi[4]. Choosing arbitrarily 2.415 as the $g_\parallel$ value for aquo-complexes in water gives $\log K_1$ ~1, an improbably low result, which can be attributed in part to uncertainty about the $g_\parallel$ value of the aquo-complex, and in part to differences in modes of complexation between organic and aquo-complexes. Motschi[4] pointed out that the extra stability gained from multidentate complexation (chelation) does not affect the value of $g_\parallel$, thus inducing a shift of several log units in the $\log K$ - $g_\parallel$ relation, and Aruga[21], on the basis of calorimetric measurements and comparison with methoxy- and ethoxyacetate complexes with copper, concluded that the ring oxygen participated in the formation of the ML^+ complex as an outer-sphere ligand.

Potentiometric measurements confirm $\log K_2$ ~2 and $\log K_1 > 1$. 0.028M Galacturonic acid, brought to pH ~5 by neutralisation with NaOH, was "titrated" with 0.14M copper nitrate, and the activity of the free copper ions was measured. Calibration measurements showed that the Debye–Hückel law was applicable up to an ionic strength of ~0.05M. The titration results were interpreted by comparison with computer-calculated results for various sets of stability constants, using the Debye-Hückel law for calculation of activity coefficients, since the ionic strength did not exceed ~0.035 M. Close fits were obtained for the values $\log K_1$ 2.16 and $\log K_2$ 2.05 (Fig. 5).

Makridou *et al.*[22], using measurements of pH, reported only ML^+ complexes, with $\log K_{c1} = 1.8 \pm 0.02$ (M $NaClO_4$ medium). Kohn *et al.*[1], on the other hand,

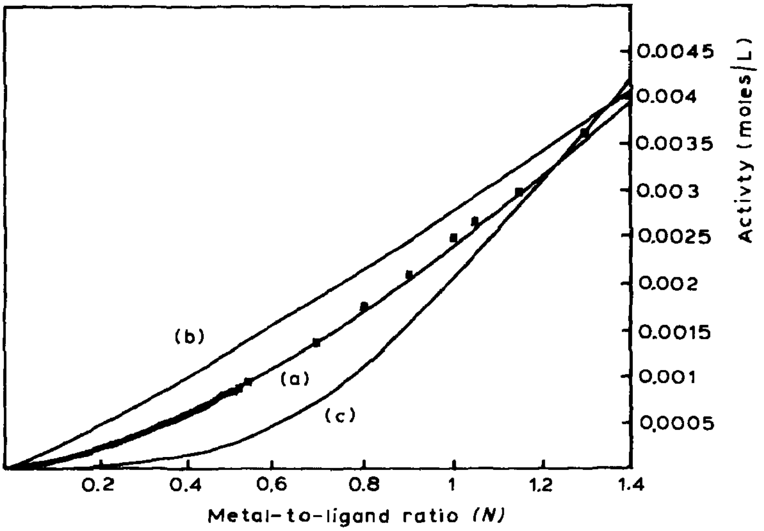

Fig. 5. Comparison of free Cu^{2+} activity during the "titration" of a 28.3mM sodium galacturonate solution by 141.5mM copper nitrate, as measured by a specific copper electrode (■■■■■■■) and as calculated for three different sets of stability constants log K_1 (ML^+) and log K_2 (ML_2): (a) 2.16 and 2.05, (b) 2.20 and −10, (c) −10 and 15.17 (*i.e.*, log K_t = 5.17).

interpreted copper potentiometry results as showing that the only complex formed was ML_2, with log K_{ct} 5.17 ±0.02 (0.01M NaCl medium). By analogy with our e.p.r. results, it could seem that the source of this contradiction lies in the different experimental conditions, acid solutions for the pH measurements and solutions of pH 4–7 for the copper specific electrode. However, calculations show that, in the experiments of Kohn *et al.*[1] (total ligand concentration, 3mM only, which favoured dissociation of the complexes), if the values log K_1 2.16 and log K_2 2.05 are accepted, then ML^+ should be the major organic complex at all metal-to-ligand ratios. On the other hand, these authors[1] have apparently interpreted their results by plotting $1/r$ against $1/[M^{2+}]$ [where r is the average number of copper ions per ligand, *i.e.*, the concentration of complexed copper divided by the total ligand concentration: $r = (C_m - [M^{2+}])/C_l$] and assuming the slope to be equal to $1/(K_{ct}\cdot[L^-])$. However, this is true only if K_{cl} is neglected ($K_{cl} << K_{ct}\cdot[L^-]$), and derivation, taking both constants into account, leads to:

$$d(1/r)/d(1/[M^{2+}]) = (1-Z)/(K_{cl} + K_{ct}[L^-]), \qquad (2)$$

where

$$Z = \{(K_{ct}[L^-]\,[M^{2+}])\,(1 - K_{cl}[M^{2+}])\,(K_{cl} + 2K_{ct}[L^-])\}/\{(K_{cl} + K_{ct}\,[L^-])\,(1 + K_{cl}\,[M^{2+}] + 4K_{ct}\,[L^-]\,[M^{2+}])\}. \qquad (3)$$

The experimental slope obtained[1] was ~0.005 (mol/L), $[L^-]$ must have been ~mм, and Z was negligible. With these values, assuming that $K_{c1} << K_{ct}\,[L^-]$), the value log K_{ct} ~5 is obtained. On the other hand, by choosing the more reasonable assumption, based on comparison with other complexes of the ML_n type[23], that K_{c2} is equal or slightly smaller than K_{c1}, values similar to those proposed here are obtained. Thus, this graphic method cannot establish the validity of one or the other model. Inversely, the computer simulations show that the formation of ML_2 only (K_1 ~0, log K_t ~5.17) cannot account for our results (Fig. 5c), nor can the formation of ML^+ only (K_t ~0, log K_1 ~2.2) (Fig. 5b).

Although the total concentration of ligand was ten times higher in our experiments (28mм) than in those of Kohn *et al.*[1] (3mм), favouring complex formation, calculation shows that ML^+ and M^{2+} should have been present in significant amounts at pH ~5, with the ratios ML_2:ML^+:M^{2+} ~4:2:1 for N <0.1, which seemingly conflicts with our e.p.r. results. In the same way, at pH ~3, the ratios ML_2:ML^+:M^{2+} should be ~1:3:9 for N <0.1 and the e.p.r. spectrum of aquocomplexes would be expected to be dominant even at the lowest metal-to-ligand ratios. However, freezing the solution prior to e.p.r. measurements generates higher local concentrations in the amorphous domains during the process of ice crystallisation[17], thus modifying the position of the equilibria and favoring association into ML_2 at pH ~5 and into LH (galacturonic acid) and ML^+ at pH ~3.

Polymers. — The strong affinities of low-methoxyl pectins for divalent cations have been studied extensively and the "egg-box" model (Fig. 6) proposed by Grant *et al.*[10] has gained general acceptance. In this model, the rigid linear 2_1 helical conformation adopted by polygalacturonate in solution allows the formation between two adjacent chains of series of cavities, each carrying two negative charges, into which multivalent ions can fit and play the role of intermolecular cement. Equation *1*, applied to our results, implies that polymer complexes $g_{\parallel}$ = 2.390 ±0.003) are less stable than the corresponding monomer complexes (ML_2)

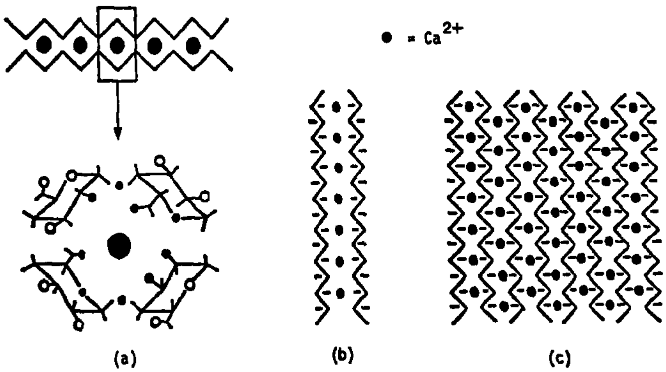

Fig. 6. The egg-box model: (a) detail of a "cavity", (b) "dimer" structure, (c) "multimer" structure.

($g_\parallel$ = 2.380 ±0.003), which is against the experimental evidence[1,8]. What the e.p.r. results show is that the bonds between ligands and copper are weaker than in monomer complexes, presumably because of steric constraints, and that the higher stability must be due to entropy and/or electrostatic effects.

Ample experimental evidence has been gathered that entropy is the main factor which determines the extra stabilities of chelates as compared to the corresponding monodentate complexes[23]. This "chelate effect" can best be described through the example of the hypothetical reaction whereby an *n*-dentate ligand C displaces *n* monodentate ligands L whilst leaving unaltered the characteristics of the individual cation–ligand bonds:

$$ML_n + C \rightarrow MC + nL. \qquad (4)$$

The enthalpy of the reaction being negligible by hypothesis, the increase in entropy due to the freeing of $(n-1)$ independent molecules is solely responsible for the negative free energy of the reaction. Regardless of whether they behave as mono- or multi-dentates towards individual cations, polyelectrolytes that bind several cations benefit from a similar entropy effect. The hypothetical two-step displacement of monomers by polygalacturonate to form "dimer egg-box" complexes (Fig. 6b) can be written as:

$$\begin{aligned} nML_2 + 2P^{2n-} &\rightarrow (M_nL_nP)^{n-} + nL^- + P^{2n-} \\ &\rightarrow (M_nP_2)^{2n-} + 2nL^-. \end{aligned} \qquad (5)$$

This reaction is thermodynamically favored by the freeing of $(n-1)$ molecules.

Literature data for NH_3 and En (ethylenediamine) complexes of Zn^{2+} and Cu^{2+} reinforce the argument that the higher value of the e.p.r. parameter $g_\parallel$ of copper–pectin as compared with copper–galacturonate (ML_2) complexes indicate weaker bonds. The Zn^{2+} complexes are typical in that displacement of two NH_3 by one En from $[Zn(NH_3)_2]^{2+}$ is mostly entropy-driven[23] ($T\Delta S$ 1.6 kcal mol^{-1}, ΔH 0.1 kcal mol^{-1}). In contrast, with Ni^{2+} and Cu^{2+}, stronger bonds are formed with En than with NH_3. Spike and Parry[24] obtained, for the displacement of four NH_3 from $[Cu(NH_3)_4]^{2+}$ by two En, the values $\Delta H \sim -4.8$ (kcal mol^{-1} and $T\Delta S \sim 5.7$ kcal mol^{-1}. Therefore $g_\parallel$ is expected to be lower for $[Cu(En)_2]^{2+}$ than for $[Cu(NH_3)_4]^{2+}$, and indeed Yokoi and Isobe[25] have reported, for these complexes, $g_\parallel$ values of 2.209 and 2.242, respectively. The difference $\Delta g_\parallel$ -0.033, according to equation *1*, corresponds to $\Delta \log K \sim 2.8$, *i.e.*, an energy difference of ~ -3.8 kcal mol^{-1}, close to the value of ΔH as measured by thermodynamic methods[24].

For all of the pectins studied, the formation of copper aquo-complexes, the presence of which was indicated by increased sensitivity of the e.p.r. spectra to temperature, was observed at metal-to-ligand ratios (*N*) of >0.5 (Figs. 3b and 4c). Morris *et al.*[26,27] observed, for pectates and for polyguluronate blocks from alginates, a similar change of behaviour at *N* 0.5 in Ca^{2+}–Na^+ cation-exchange competition measurements. It seems that, for the first half of their total stoichiometric requirements ($N < 0.5$), poly-galacturonates and -guluronates strongly prefer divalent to monovalent cations, whereas, for the second half ($N > 0.5$), the selectivity drops and a significant fraction of the divalent cations remains as aquo-complexes. This situation is interpreted[26,27] as the preferential formation of "dimer" structures, in which only the inner sides are bound through divalent cations (Fig. 6b). Further aggregation of "dimer" egg-box structures into "multimer" structures (Fig. 6c) seems possible only in the presence of large excesses of multivalent cations.

Irwin *et al.*[18] have measured $\Delta H_{\perp \mathrm{app}}$ for copper bound to pectins in dehydrated apple cell-walls, where the ion–polyuronide structure was supposed to be similar to the egg-box model, and used the value (~56 G) obtained at low metal-to-ligand ratios to calculate the distance between adjacent copper ions. It was assumed that (*a*) the increase in line-width ($\Delta\Delta H_{\perp}$), as compared to $\Delta H_{\perp}$ of aquo-complexes dispersed in glycerol–water glasses, is due only to dipolar broadening, *i.e.*, to short-range (<20 Å) magnetic interactions of neighbouring copper ions [$\Delta\Delta H_{\perp}$ is then proportional to (x/d^3), where x is the lattice constant and d is the distance separating the ions]; (*b*) the lattice constant is 1 at low metal-to-ligand ratios (interactions with one close neighbour only); (*c*) the progressive increase in $\Delta H_{\perp}$ with copper content is due to an increase of this lattice constant, from 1 to 1.42 (for a linear array, *i.e.*, two close neighbours), 2.45 (for a two-dimensional hexagonal array, six neighbours), and beyond (x would be 3.46 for a three-dimensional close-packing array, twelve neighbours).

The second assumption is opposed to that made earlier by the same authors[28], namely, $x = 1.42$ (linear array) at low copper content. However, replacing $x = 1$ by $x = 1.42$ will only multiply the calculated d value by a factor of ~1.12.

The values calculated by Irwin *et al.*[18] for d, assuming $x = 1$, were ~12 Å for Cu^{2+} and ~14 Å for Mn^{2+}. X-Ray diffraction patterns of dehydrated fibres of polygalacturonate and polyguluronate with various monovalent counter-ions, including H^+, show[29–32] that the projection of one residue on the fiber axis is ~4.35 Å. Polygalacturonate in dehydrated fibres adopts[29,30] a 3_1 helical symmetry, with a repeat unit of ~13 Å, and polyguluronate, in contrast, adopts[31,32] a 2_1 helical symmetry, with a repeat unit of ~8.7 Å. Morris *et al.*[27] showed by c.d. measurements that (*a*) polygalacturonate, but not polyguluronate, undergoes profound conformational changes on dehydration, and (*b*) the properties of both polyuronates in solutions or hydrated gels are similar. Their conclusion was that each polymer has 2_1 helical symmetry in hydrated conditions and that only polygalacturonate changes to 3_1 symmetry on drying. The egg-box model[10] is thus based on 2_1 symmetry, with successive cavities ~8.7 Å apart. The Mn^{2+}-e.p.r. and ^1H-n.m.r. results of Irwin *et al.*[18] confirm that the polyuronates in the cell wall also undergo important conformational changes on dehydration. Therefore, it seems that the geometrical environment of divalent ions in dehydrated samples is very different from that described by the egg-box model. The calculated d values (~12 and ~14 Å) for copper and manganese in dehydrated apple cell-walls could be related to the formation of a 3_1 helix, but it is not known how the cations would be redistributed when such a conformational change occurs. Furthermore, if the conformational transition in the cell wall is between a 3_1 and a 2_1 helix, then an increase in line-width on rehydration would be expected, due to a decrease in *d* to ~8.7 Å. Irwin *et al.*[18] observed the opposite effect for Mn^{2+} in apple cell walls: the calculated distance increased by ~2 Å on rehydration. Therefore, either the values calculated by this method are at best an indication of the order of magnitude of *d*, or the polygalacturonate chain conformation involves more than two residues per turn of the helix even in hydrated conditions.

If, under our conditions, dimer egg-boxes only are formed up to $N \sim 0.5$, then $\varkappa$ may not exceed 1.42, the value for linear arrays. The increase in line-width, especially large for non-esterified pectins, must be ascribed then to some other cause than dipolar broadening. Assumption (*a*) is valid only if the population observed is homogeneous (for heterogeneous populations, the superposition of spectra with slightly different magnetic parameters will broaden the overall line-shapes). The thermodynamic selectivity coefficients, in exchanges between copper, zinc, and calcium in cell walls of *Nitella flexilis*, vary greatly[8] as a function of the mole fraction of ions bound, suggesting both continuous and discontinuous variations in the nature of the binding sites for divalent cations. In solutions or gels, for $N < 0.5$, where only dimer egg-boxes appear to be formed, an evolution of the nature or quality of the binding sites is to be expected as a consequence of the increasing topological interactions of the macromolecules. Gelling involves the formation of a three-dimensional network. The more intermolecular junctions in which a given pectin molecule is involved, the more tensions these junctions will exert on each other, and the more the local environment and magnetic parameters of the cations will vary. This situation could also explain why the spectra of partly esterified pectins are less affected. The neutral blocks (esterified residues occur in blocks, unless the pectin has been partly de-esterified by the action of mineral bases[33]), intercalating between the ionic blocks engaged in intermolecular junctions, will greatly attenuate the tensions in the network.

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