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On the binding of copper and lead by water-soluble polysaccharides

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Abstract

Optical, chirooptical and thermodynamic techniques have been used to study the binding between copper (II) ions and carboxyethylscleroglucan, oxidised glucomannan, 6-oxychitin and reacetylated chitosan in dilute aqueous solution. For oxidised glucomannan and reacetylated chitosan, the binding of lead was also studied. The results obtained were consistent with the occurrence of a chelate that was evidenced by the existence of a charge transfer band in the near UV region and supported by other experimental data. The variations on binding of the thermodynamic functions Gibbs free energy, enthalpy and hence entropy were computed. Indications on the site(s) of binding were derived. In the case of oxidised glucomannan, the UV spectrophotometry revealed the presence of two sites of binding, the carboxyl groups of glucuronic and mannuronic acids. Finally, the possibility of avoiding misleading results with appropriate procedures is shown.

Keywords: Binding; Polysaccharides; Copper; Lead; Chitosan; Carboxyethylscleroglucan; Glucomannan; 6-Oxychitin; Reacetylated chitosan

1. Introduction

The interaction of Cu(II) with polycarboxylates is characterised by the presence of a band in the absorption and CD spectra, attributed to a charge transfer (CT) from the ligand to the metal ion. So far, this feature has been always found with synthetic and natural polycarboxylates (Cesàro, Delben, Flaibani, & Paoletti, 1988; Manzini, Cesàro, Delben, Paoletti, & Reisenhofer, 1984; Paoletti & Delben, 1975). The CT band was found to be particularly sharp if the carboxyl groups are linked to the polymeric backbone through a short side chain (Delben & Muzzarelli, 1989). In the co-presence of ligands other than carboxyl groups, the CT band becomes asymmetric, thus revealing the presence of at least two peaks. *N*-carboxymethyl chitosan and other chitosan derivatives are representative cases (Delben, Stefancich, & Muzzarelli, 1992; Dobetti & Delben, 1992). The CT band was also revealed in the lead (II)-oxidised glucomannan interaction.

By properly using the experimental absorption or ellipticity as a function of the molar ratio of the metal cation to the ligand, the binding constant is commonly derived according to the Scatchard equation (Dobetti & Delben, 1992).

By dilatometry, the binding is characterised by a volume increase, due to the release of electrostricted water from the hydration shells of the interacting species. This technique allowed the volume variation associated to a co-operative conformational transition with pH taking place for synthetic polycarboxylates to be computed (Fenyo, Delben, Paoletti, & Crescenzi, 1977). However, this was impossible in the case of polygalacturonic acid that is also believed to exhibit a pH-driven conformational transition (Cesàro, Ciana, Delben, Manzini, & Paoletti, 1982).

The experimental values found in the past for the binding of metal cations by saccharidic polycarboxylates showed that the value of ΔV strongly depends on the nature of the cation (Cesàro et al., 1988). However, it also depends on the polymer chain to which the ligand function (e.g. the carboxyl group) is bound, as shown by the interaction of Cu(II) ions with different saccharidic polycarboxylates.

Lastly, microcalorimetric results were often used to confirm structural indications given by other techniques. Moreover, the occurrence of specific or unspecific

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interactions between the metal cation and the polymer can be possibly detected (Cesàro et al., 1992). In fact, low values for the enthalpy variation of binding, ΔH_B, are typical for cations weakly bound by the polymer. ΔH_B is positive as a rule for the binding between copper ions and natural and synthetic polycarboxylates (Crescenzi, Delben, Paoletti, & Škerjanc, 1974; Paoletti, Cesàro, Ciana, Delben, Manzini, & Crescenzi, 1981; Cesàro, Delben, & Paoletti, 1988; Dentini, Nero Scheffino, & Crescenzi, 1991), provided that the ligands are carboxylic groups only. Exceptions to this behaviour have been found and interpreted for different systems. Pectate and chitin derivatives are suitable examples. In the former case, the exothermic ΔH_B values were interpreted as due to a conformational transition that should take place on addition of Cu(II) to pectate (Cesàro et al., 1988). In the latter case, the presence of N atom(s) in the site of binding was believed to be the reason for the negative ΔH_B values (Delben, Muzzarelli, & Terbojevich, 1989; Delben et al., 1992).

The present study aimed at extending to novel polysaccharides the knowledge on the interaction of polymers with copper and lead ions. The identification of the site(s) of binding was a constant goal throughout the study.

Copper and lead were chosen for this study for their biologic interest and because of their interesting behaviour in aqueous solution in the presence of suitable ligands, for which it is possible to study their interaction with polymers by familiar optical and chirooptical techniques.

2. Experimental

2.1. Materials

6-Oxychitin was prepared and characterised as already described (Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999). The parent chitin was from lobster, donated by Prof. Ofelia Bilbao-Roveredo, Cuba. The sample, supplied as a white powder, gave a turbid (dilute) solution even after prolonged stirring at room temperature and heating up to 80 °C. To obtain a clear solution, 450 mg of 6-oxychitin were dissolved in 50 ml of water and, after prolonged stirring, were centrifuged at 4 °C at 20,000 rpm for 35 min. The supernatant was filtered with a MF-Millipore membrane filter (USA), size of pores, 0.45 μm, neutralised with NaOH solution, exhaustively dialysed against doubly distilled water using Sigma cellulose tubings (Germany), 16 mm diameter, cut off 8–12,000, and freeze-dried. The molecular weight values of the sample so purified, as determined by GPC, were slightly lower than those determined before purification ($M_w = 7300$ and $M_n = 3700$; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999). In spite of the low molecular weight of 6-oxychitin, the loss of polysaccharide mass during the dialysis was negligible. The degree of acetylation, evaluated by NMR and UV absorption techniques (Aiba, 1986; Muzzarelli & Rocchetti, 1985), was at least 0.95. The degree of substitution, DS, i.e. the ratio of carboxyl groups to total repeat units, was found to be 0.95 by potentiometric titration. Accordingly, the equivalent weight $\mathrm{EW} = 249.9\ \mathrm{g\ Da^{-1}}$ was computed. The following expression was used:

$$\mathrm{EW} = [\mathrm{DS} \times M_s + (1 - \mathrm{DS})M_{ns}]/\mathrm{DS} \qquad (1)$$

where M_s and M_{ns} are the molecular weight of the substituted and the unsubstituted repeat units, respectively.

The water-soluble reacetylated chitosan was obtained by complete deacetylation of a chitin commercial sample (Chitin Batch TA29 Primex, Karmsund Fiskerihavn, Norway) followed by partial acetylation with acetic anhydride (Dal Pozzo et al., 2000). The degree of acetylation, obtained by NMR and UV absorption techniques, was 0.70. Accordingly, the average molecular weight of the repeat unit was $190.6\ \mathrm{g\ Da^{-1}}$.

The carboxyethylscleroglucan (CES) was obtained by oxidation of cyanoethyl scleroglucan. Synthesis, chemical characterisation and a sketch of the chemistry of the cyanoethyl derivative were already reported (Gianni et al., 2002). A cyanoethyl scleroglucan sample with degree of substitution DS = 0.42, i.e. 0.42 CN groups per glucosidic residue was used. DS was determined by elemental analysis. The M_W of the sample was 8×10^5. It was oxidised to CES as follows.

A suitable amount of cyanoethyl scleroglucan was lodged into a jacketed vessel. Water was added portionwise over a period of up to 1 h. The gel-like suspension obtained was stirred for 1 h. After cooling to 0 °C, 30% (w/w) H_2O_2 and 50% (w/w) NaOH aqueous solutions were added. The molar ratio of both reactants to polysaccharide repeats unit it was 5:1. The final NaOH concentration was 1 M. After mixing, the temperature was raised to 50 °C and the reaction was continued for 2 h. After that, the solution obtained was diluted, neutralised with diluted acetic acid, exhaustively dialysed against distilled water and freeze-dried. A portion of the solution was transformed into the acidic form by passing through a Dowex cation exchange resin. By potentiometric titration with Normex 0.1 N NaOH and weighing the freeze-dried product obtained from a known volume of the solution, the degree of substitution was determined as 0.33. A low amount of N found by elemental analysis revealed that cyano groups were present in a minor percentage in the final derivative. Cyano groups were not taken into account in the computation of the equivalent weight, that resulted $585.4\ \mathrm{g\ Da^{-1}}$ (Eq. (1)). M_w was found to be 47,400 by GPC analysis.

The oxidised product of the konjac glucomannan, here referred to as KGMOx, was prepared and characterised at the University of Rome 'La Sapienza'. The parent neutral polysaccharide was characterised by NMR. The β-D-mannopyranosyl to β-D-glucopyranosyl residues ratio was 1.6 and the degree of C-6 acetylation was about 0.06 (Crescenzi et al., 2002). The degree of oxidation of KGMOx was about 0.90, as determined by NMR and confirmed by potentiometric titrations. The computed equivalent weight was 218.0 g Da^{-1}.

All the carboxylated polysaccharides were used in the sodium form and the equivalent weight values here quoted refer to the sodium salt form.

Copper and lead perchlorate were Carlo Erba RPE (Italy) and Ventron GmbH (Germany) products, respectively. The concentration of their solutions was determined spectrophotometrically by direct titration with EDTA (Carlo Erba RPE), using murexide as the indicator (Flaschka, 1952). Sodium perchlorate was Carlo Erba RPE desiccated overnight at 110 °C before use.

n-Heptane used as dilatometric liquid was from Fluka Chemie AG (Switzerland) and was purified with prolonged shaking with concentrated H_2SO_4 (Carlo Erba RP), 20% (w/w) aqueous NaOH (Carlo Erba RP) containing $KMnO_4$ (Carlo Erba), 20% (w/w) aqueous H_2SO_4 containing $KMnO_4$, rinsed exhaustively with distilled water and finally distilled.

Acetic acid was Codex product from Carlo Erba Farmitalia (Italy). Other chemicals were Carlo Erba analytical grade products. Deionized, doubly distilled water was used for dialysis and preparation of the solutions. The measurements were performed on freshly prepared solutions.

2.2. Instruments and methods

The pH measurements were carried out at 25 °C with a PHM 240 Radiometer (Denmark) pHmeter, equipped with GK2401B or GK2401C combined electrodes. The calibration was performed with Carlo Erba RPE buffers, pH 4 and 7.

For the elemental analysis of cyanoethyl scleroglucan, a Carlo Erba model 1106 Elemental Analyzer was used. From the weight percentage of C and N (indicated as %C and %N, respectively), the degree of substitution DS was computed with the following expression:

$$\frac{\%\mathrm{C}}{\%\mathrm{N}} = \frac{M(1)_{\mathrm{C}}(1-\mathrm{DS}) + M(2)_{\mathrm{C}}\mathrm{DS}}{M(2)_{\mathrm{N}}\mathrm{DS}} \qquad (2)$$

where $M(1)_C$, $M(2)_C$ and $M(2)_N$ stand for the mass of C atoms in the unsubstituted saccharide unit, the mass of C atoms in the substituted unit, and the mass of N atoms in the substituted unit, respectively.

The water content in the freeze dried polysaccharide was determined with a Perkin Elmer (USA) TGA 7 Thermogravimetric Analyzer.The temperature was raised from 35 to 190 °C at a rate of 10 °C min^{-1} The weight loss was expressed as a function of the temperature. The water percentages in the polysaccharides were 12.1, 12.9, 9.8, and 14.9 for 6-oxychitin, reacetylated chitosan, CES and KGMOx, respectively.

The NMR spectra were recorded with a Bruker AC200 spectrometer (Germany). The concentration of the solutions was 25 mg ml^{-1} and the temperature was 300 K. The ^{1}H NMR spectra were recorded at 200.13 MHz, with a spectral width of 2700 Hz, an acquisition time of 1.5 s and 64 scans with pre-saturation of the solvent. The value of 4.75 ppm was assigned to the residual water signal referring to the Na 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (TSP) resonance used as the reference. The ^{13}C NMR spectra, decoupled from protons, were recorded at 50.32 MHz, with a spectral width of 16,000 MHz, an acquisition time of 0.5 s and 110,000 scans. The chemical shift values were referred to TSP, attributing to the value of 39.6 ppm the resonance of DMSO, used as the external reference.

The circular dichroism spectra were recorded at room temperature in the 190–300 nm range with a model 710 Jasco (Japan) spectropolarimeter, equipped with 1 cm quartz cuvette. Two to eight accumulations were collected. Noise was suppressed by suitable programs. After subtraction of the baseline (water or $NaClO_4$ solution), the spectra were normalised for the polymer concentration and reported as equivalent ellipticity, $[\theta]$. The metal perchlorate solution was added directly into the cuvette. For the pH control, the solution was replaced with a syringe into the potentiometric cell and put into the cuvette again after the potentiometric measurement.

The UV absorption spectra were recorded with a model Cary 4 Varian spectrophotometer (Australia), equipped with software and quartz cuvettes.

The copper electron paramagnetic resonance (EPR) measurements were carried out with a model E109 Varian spectrometer, X band (USA). The instrument had a frequency of 9.1 GHz and the measurements were performed at 77 K. As the external reference, DPPH was used. The results were interpreted following the empiric method proposed by Peisach and Blumberg (1974), applicable for complexes with axial symmetry. According to this method, the experimental values of $A_{\parallel}$, the parallel component of the coupling constant of the quadruplet shown by the spectrum, are plotted versus $g_{\parallel}$, the average value of the parallel component of the resonance between the groups. In the case of copper containing proteins, Peisach and Blumberg observed that the experimental points lie in different parts of the plot, depending on the number of oxygen and nitrogen atoms (0–4) in the complex.

For the EPR measurements, the solutions were prepared at constant concentration of Cu(II) (about 10^{-3} M) and different polymer concentrations. 1,2-Ethanediol 15% (v/v) was added before freezing the solutions, in order to obtain a glassy solid. In the case of 6-oxychitin, a negligible phase separation followed the addition of glycol.

The calorimetric and dilatometric measurements were carried out at 25 °C, following standard procedures. In both cases, the instrument was lodged in an air thermostatted room.

The calorimeter was a flow-type model 10700-1 LKB (Sweden) isothermal microcalorimeter with gold cells.

Table 1
Thermodynamics of the interaction of polysaccharides with copper (II) cations

Polysaccharide	λ_{max} (nm)	ε (1) (10^3 cm^2 mol^{-1})	n (2)	K_b (l mol^{-1})	$-\Delta G_b$ (kcal mol^{-1})	ΔH_b (kcal mol^{-1})	ΔS_b (e.u.)	ΔV_b (ml mol^{-1})	w (3)
Carboxyethylscleroglucan in water	241	3900	0.31	2.1×10^4	5.9	3.1	30	26.7	16.3
Carboxyethylscleroglucan in 0.05 M $NaClO_4$	241	3900	0.31	5.5×10^3	5.1	1.7	23	16.6	10.1
Oxidised konjak glucomannan in water	239–245	3060	–	6×10^4 (4)	6.5 (4)	2.7	31	15.7	9.6
Oxidised konjak glucomannan in 0.01 M $NaClO_4$	235–239	1600 (4)	–	4×10^3 (4)	4.9 (4)	0.9	19	6 (4)	4 (4)
6-oxychitin in water	239	2800	0.24	1.2×10^5	6.9	1.9	30	40.2	24.5
6-oxychitin in 0.05 M $NaClO_4$	239	2800	0.24	4.2×10^4	6.3	1.9	28	28.9	17.7
Reacetylated chitosan in water	238	–	–	–	–	−3.6	–	–	–

(1) Molar extinction coefficient of the complex, (2) maximum average number of metal cations that a repeat unit is able to bind, (3) number of water molecules liberated in the binding process (Delben and Muzzarelli, 1989), and (4) approximate values (see text).

A P3 Pharmacia (Sweden) peristaltic pump was used to introduce the solutions into the calorimeter. The flow, determined by emptying 10 ml vessels, was about 7×10^{-3} ml s^{-1} and was checked weekly. The electric heat flow calibration was performed at the beginning and at the end of each set of measurements. The thermal effects due to the dilution of both polysaccharide and metal solutions were properly measured and taken into account.

For the dilatometric measurements, a very accurate thermostatic bath was used (Delben, 1980). The general procedure was that already described by Begala and Strauss (1972). Carlsberg dilatometers of $4 + 4$ ml were used. Before introduction into the dilatometers, the solutions were carefully degassed.

Enthalpy and volume differential values were divided by the polymer equivalent concentration and plotted as a function of R, the ratio of the molar concentration of divalent cation to the equivalent concentration of the polysaccharide. It is worth pointing out that this treatment is coherent with the assumption that a metal cation and a single charged unit of the polysaccharide are engaged in the complex formation.

3. Results and discussion

3.1. The presence of the CT band

As the first point of interest, the CT band was clearly detectable for all the polysaccharides studied, in spite of their chemical differences. The exceptions given by the CD spectra of the Cu(II)-CES system and the particular case of Cu(II)-6-oxychitin CD spectra will be discussed below. The main differences between CT bands exhibited by different polysaccharides were the value of the wavelength corresponding to the maximum of the band, λ_{max}, and the shape of the band. The values of λ_{max} and ε for the CT bands formed in the presence of Cu(II) are in Table 1. As a sample case, the UV absorption spectra of CES in the presence of increasing amounts of copper ions are reported in Fig. 1.

As for the CD measurements, CES aqueous solution does not exhibit any CD band in the near UV region. This should indicate that the chromophore, i.e. the carboxyl group, is far enough from the polymer chain to be unaffected by its chirality. Although, the chelate is formed, as indicated by the absorption spectra (Fig. 1), upon the addition of metal cations evidently no new interaction between the carboxyl groups and the backbone occurs, as evidenced by the lack of any CT band.

In the case of the CD spectra recorded on the 6-oxychitin-Cu(II) system, the CT band is detected, provided that the spectra are significantly enlarged. Indeed, the presence of Cu(II) only affects the CD band of 6-oxychitin alone, that is about one order of magnitude higher than usually found with such systems. This exceptional value could be due to the presence of two chromophores, i.e. –NH–CO– and $-COO^-$, both characterised by $n \rightarrow \pi^*$ electron transition in the 185–260 nm range (Kabat, Lloyd, & Beychock, 1969), and to the rigidity of the chain, that enhances the probability of the transition.

The CD spectra of 6-oxychitin in the presence of Cu(II), recorded using the solvent and the 6-oxychitin solution as

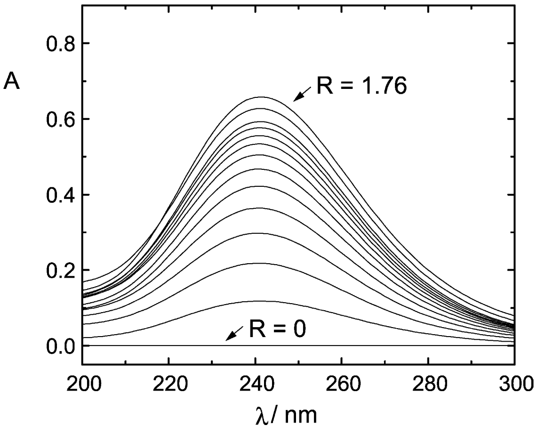

Fig. 1. Charge transfer bands of carboxyethylscleroglucan in water in the presence of increasing amounts of $Cu(ClO_4)_2$. Initial polymer concentration, 8.34×10^{-4} equivalent l^{-1}. R denotes the ratio of the molar concentration of divalent cation to the equivalent concentration of the polysaccharide.

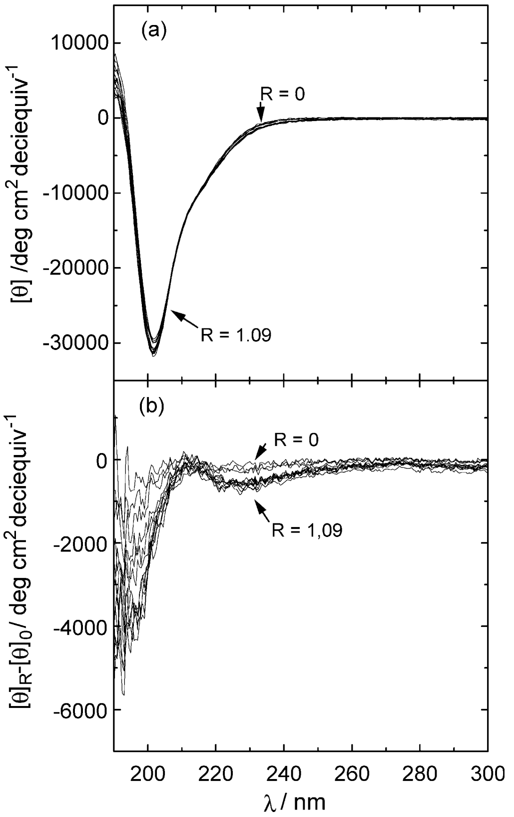

Fig. 2. Circular dichroism spectra of 6-oxychitin in water in the presence of increasing amounts of $Cu(ClO_4)_2$. (a) Without and (b) with the subtraction of the band due to the polysaccharide alone. Initial polymer concentration, 2.17×10^{-4} equivalent l^{-1}. *R* as in Fig. 1.

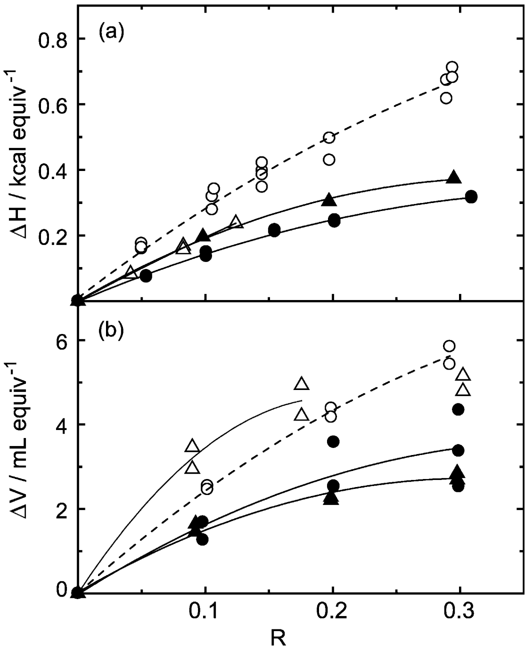

Fig. 3. Enthalpy (a) and volume (b) variation on the addition of $Cu(ClO_4)_2$ to ionic polysaccharides in aqueous solution. Circles, carboxyethylscleroglucan; triangles, 6-oxychitin. Open symbols: in pure water; full symbols: in the presence of 0.05 M $NaClO_4$. The initial polymer concentrations were in the 1.96×10^{-3} to 4.55×10^{-3} equivalent l^{-1} range. *R* as in Fig. 1.

the baseline, are reported in Fig. 2a and b, respectively. It is worth pointing out that for this system the noise is particularly high because of the absorption of 6-oxychitin and therefore the molar ellipticity and the binding constant obtained from the CD spectra are largely approximate.

3.2. The site(s) of binding

The determination of the site(s) of binding on the polysaccharide chain is another point of interest. The addition of divalent cations such as Cu(II) affects the UV absorption and CD spectra. As for the Pb(II) cations, very interesting features were found in the case of natural saccharidic polycarboxykate (Cesàro et al., 1988).

All this indicates that the chromophores above mentioned are involved in the binding. Further and more specific indications about the site of binding derive from calorimetric and dilatometric measurements.

Some calorimetric and dilatometric results are in Fig. 3. For each curve, the slope at $R = 0$ gives the enthalpy or the volume variation, respectively, per mole of complex. The complete set of these values is in Table 1.

The binding enthalpy change values were found to be positive with all the ionic polysaccharides studied, being negative only with reacetylated chitosan (Table 1). Endothermic binding values indicate that the binding is entropy driven. Accordingly, positive ΔV values were found. Should the density of electrostricted water in the hydration spheres be equal to $1.1\ \mathrm{g\ ml^{-3}}$ (Yasunaga & Sazaki, 1951), they indicated that 10.1 up to 24.5 moles of water are released per mole of chelate formed.

Because in the case of *N*-carboxyalkyl derivatives of chitin, the interaction with lead and copper ions is exothermic (Delben et al., 1989, 1992; Dobetti & Delben, 1992), it was supposed that the binding site contains the carboxyl groups and the nitrogen atom (Delben et al., 1989). In fact, while the interaction of Cu(II) with polymeric species or monomers bearing carboxyl groups is endothermic in aqueous solution as a rule, the interaction of copper ions with oligomers or polymers bearing amino groups was found to be exothermic (Barbucci, Casolaro, Barone, Ferruti, & Tramontini, 1983). The asymmetry of the absorption CT bands seemed to support this hypothesis (Delben et al., 1992; Dobetti & Delben, 1992). Hence, 6-oxychitin apparently represents an exception among the carboxylated chitin derivatives. However, it is worth pointing out that, as a point of difference from the other chitin derivatives already studied, in 6-oxychitin the bound cations cannot be inserted between the carboxyl groups and the N atoms of the polysaccharide because of their distance. In 6-oxychitin, in fact, the carboxyl function lies on the opposite side of the saccharidic ring with respect to the acetamido group. Moreover, with 6-oxychitin (and with the other polysaccharides studied in the present work) the absorption CT bands were found to be symmetric, which should indicate that a single chromophore, the carboxyl group, is present in the binding site.

3.3. Determination of the binding constants

The highly reproducible absorption spectra recorded in pure water and in the presence of $NaClO_4$ can be used for obtaining the binding constants, K_B (Dobetti & Delben, 1992).

The absence of any artifact in the spectra is the prerequisite for a correct computation of K_B and, more important, for the exact determination of some properties of the complex, such as the site of binding. The measurements of this kind deserve a particular procedure to be correctly performed.

Let us suppose that the absorption spectra are recorded with the aid of two identical cuvettes, the sample cuvette bearing the polysaccharide solution, and the reference cuvette bearing the solvent alone. Increasing volumes of Cu(II) salt solution are progressively added in both cuvettes and the absorption spectra are recorded after each addition. Using the values of λ_{max} of the CT bands so recorded, the value of K_B can be roughly computed. The value of K_B is necessarily approximate, because the total copper ions in the sample are partly free, and partly bound by the polymer, so that their absorbance is not exactly equalised by the reference. Moreover, the absorbance due to the polymer itself changes because of its progressive dilution. Using the approximate K_B value so obtained, a second experiment is carried out, in which the addition of Cu(II) salt solution in the reference cuvette aims at obtaining the same concentration of free Cu(II) ions in both cells. However, the calculation is complicated, because the value of K_B depends on the ratio R. In fact, the binding extent depends on the free charge on the polymer chain, in particular decreasing with the neutralisation of the charge on the polymer, being the binding anticooperative. Moreover and more important, the contribution of the polymer to the absorbance cannot be corrected, unless very complicated computations are performed. As a consequence, deflections in the absorbance are recorded for low wavelength values, where the carboxyl groups absorb and misleading results can be obtained.

The case of the 6-oxychitin—Cu(II) system is an interesting example of this. For this system, at the higher Cu(II) concentrations, that is for R values over 0.5, a new band appeared, whose maximum wavelength was approximately 210 nm (Fig. 4a). In principle, one could interpret this band as due to a new complex, formed thanks to a second binding site, after saturation of the first one by copper ions.

All this was clearly revealed as an artifact when the spectrophotometric measurements were performed following the method proposed previously (Dobetti & Delben, 1992). Following this method, a unique and symmetric CT band for a single site of binding on the polysaccharide backbone was shown (Fig. 4b).

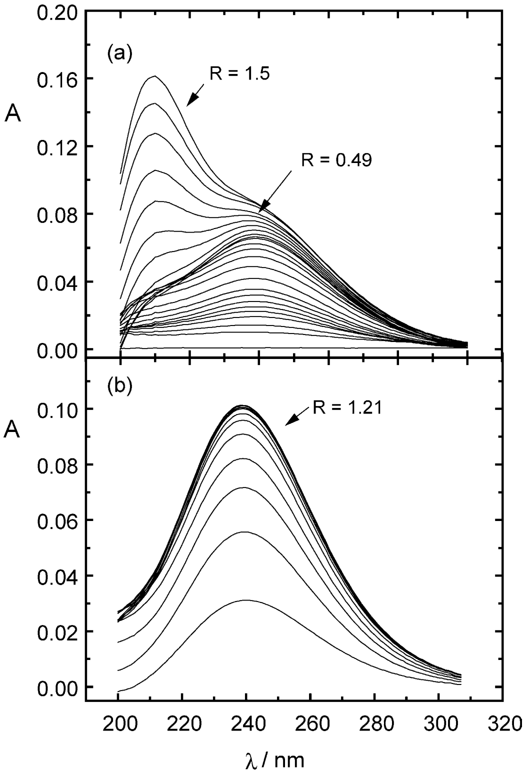

Fig. 4. Charge transfer bands of 6-oxychitin in water in the presence of increasing amounts of $Cu(ClO_4)_2$. (a) With simple addition of copper ions in the sample cuvette; and (b) employing the method suggested in this paper. Initial polymer concentration: (a) 1.85×10^{-4} and (b) 2.07×10^{-4} equivalent l^{-1}. R as in Fig. 1.

3.4. Reacetylated chitosan

The ability in binding Cu(II) and Pb(II) by polysaccharides having no carboxyl groups was tested with reacetylated chitosan. The calorimetric measurements revealed that reacetylated chitosan interacts with copper and, to a lesser extent, with lead ions. The negative value of the enthalpy change, moreover, agrees with the hypothesis that the binding of Cu(II) by aminated species in aqueous solution is exothermic as a rule.

As for the absorption spectra, recorded in the presence of increasing amounts of Cu(II), in the case of reacetylated chitosan a new band ($\lambda_{max} = 238$ nm) also appeared. This band had low intensity and should be constituted by at least two different peaks, very likely in connection with the two different monomers of chitin, i.e. 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose. With this polysaccharide, the CD spectra also showed a low intensity CT band, with $\lambda_{max} = 275$ nm, at pH values higher than six only. This was a further difficulty in performing the measurements, as the polysaccharide is insoluble at neutral and alkaline pH values. More important, it clearly indicates that the (amine) nitrogen atom is

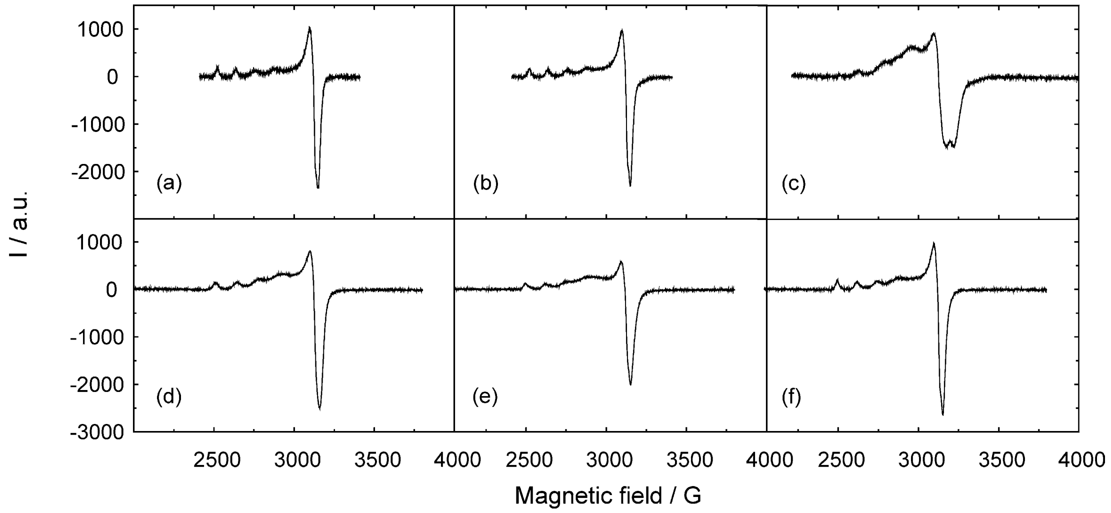

Fig. 5. EPR signals of aqueous Cu(II) at 77 K in the presence of 15% (v/v) 1,2-ethanediol: (a) 9.26×10^{-4} M Cu(II); (b) 9.17×10^{-4} M Cu(II) in the presence of 0.95×10^{-3} eq l^{-1} reacetylated chitosan ($R = 0.96$); (c) 9.17×10^{-4} M Cu(II) in the presence of 4.25×10^{-3} eq l^{-1} reacetylated chitosan ($R = 0.22$); (d) 1.10×10^{-3} M Cu(II) in the presence of 1.26×10^{-3} eq l^{-1} 6-oxychitin ($R = 0.87$); (e) 1.10×10^{-3} M Cu(II) in the presence of 2.23×10^{-3} eq l^{-1} 6-oxychitin ($R = 0.49$); and (f) 1.10×10^{-3} M Cu(II) in the presence of 5.30×10^{-3} eq L^{-1} 6-oxychitin ($R = 0.21$). R as in Fig. 1.

involved, if uncharged, in the binding process. Because of the complexity of the charge transfer spectra and the uncertainty due to the small value of the absorbance, with reacetylated chitosan the binding constant were not computed with confidence and hence their values were not reported in Table 1.

3.5. EPR measurements

EPR measurements were performed in order to identify the site of binding on reacetylated chitosan and 6-oxychitin. The EPR spectra for Cu(II) alone and in the presence of reacetylated chitosan (two R values) and 6-oxychitin (three R values) are reported in Fig. 5. The Peisach and Blumberg plot is in Fig. 6.

At the lowest 6-oxychitin concentration, i.e. $C_p = 1.26 \times 10^{-3}$ eq l^{-1}, $g_{\|}$ and $A_{\|}$ are 2.41 and 13.59 mK 10^{-3} cm^{-1}, respectively. On increasing C_p up to 2.23×10^{-3} and 5.30×10^{-3} eq l^{-1}, they move to 2.41 and 14.06 mK 10^{-3} cm^{-1} and to 2.39 and 15.29 mK 10^{-3} cm^{-1}, respectively. As expected, these values lie in the part of the Peisach and Blumberg plot that is typical of complexes with four oxygen atoms (part A in Fig. 6) and away from the part typical of complexes with three oxygen atoms and one nitrogen atom (part B in Fig. 6). In fact, the values are close to those found in the absence of polymer ($g_{\|} = 2.41$ and $A_{\|} = 13.46$ mK 10^{-3} cm^{-1}). While the experimental values for $C_p = 1.26 \times 10^{-3}$ and 2.23×10^{-3} eq l^{-1} are similar, for the third solution (6-oxychitin concentration, 5.30×10^{-3} eq l^{-1}; open inverted triangles in Fig. 6) those are different. In particular, by increasing C_p, $g_{\|}$ decreases and $A_{\|}$ increases. Following Peisach and Blumberg, this indicates that a more negative charge on the complex and/or a greater electronic delocalisation take place on increasing C_p (or decreasing the ratio R). This is in agreement with the hypothesis that the metal ion is chelated by oxygen atoms only, i.e. by carboxyl groups and, possibly, water molecules.

The case of reacetylated chitosan is different. Indeed, the values of $g_{\|}$ and $A_{\|}$ are 2.41 and 13.28 mK 10^{-3} cm^{-1}, respectively, for $C_p = 0.95 \times 10^{-3}$ eq l^{-1}. These values should still indicate a complex formed with four oxygen atoms. At higher C_p (i.e. 4.25×10^{-3} eq l^{-1}), however, the EPR spectrum is completely different (Fig. 5b and c, respectively). The peak at 2507 G, similar to that detectable in complexes in which copper is bound by oxygen atoms only, is very weak, but more intense three new peaks are also present at 2625, 2789 and 2962 G. Very likely, the first peak (2507 G) belongs to the quadruplet of the aqueous copper ions, while the other peaks just cited belong to a second quadruplet, the fourth peak being overlapped by the perpendicular part of the spectrum. In other words, this spectrum could be due to the overlapping of the spectrum of

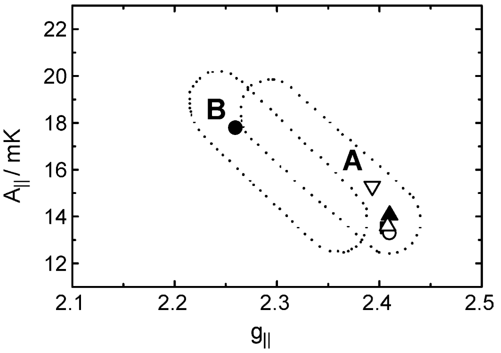

Fig. 6. Peisach and Blumberg plot. Part A, Cu(II) bound by four O atoms; part B, Cu(II) bound by three O + 1 N atoms. Full square, open circle, full circle, open up triangle, full up triangle and inverted triangle refer to parts (a)–(f) of Fig. 5, respectively.

free aqueous Cu(II) and copper ions bound by the polymer. This is also suggested because the parameters of the last peak, i.e. $g_{\parallel} = 2.26$ and $A_{\parallel} = 17.78\ \mathrm{mK}\ 10^{-3}\ \mathrm{cm}^{-1}$, are consistent with the presence of a linkage with a nitrogen atom and three linkages with oxygen atoms (part B in Fig. 6). As in the former case, a greater number of linkages with nitrogen atoms appears unlikely, because of the opposite arrangement of each subsequent repeat unit of chitosan and because of the absence of phase separation seems to exclude pairing of the polysaccharide chains.

One can conclude that in the case of reacetylated chitosan the site of binding includes a nitrogen atom, as expected. At this stage of the research, it is impossible to indicate if this nitrogen atom belongs to the free amino groups of the 2-amino-2-deoxy-D-glucopyranose residues or to the acetamido groups of the 2-acetamido-2-deoxy-D-glucopyranose residues. Because of the asymmetry of the CT band, however, we can suppose that both these residues are responsible for the binding of copper ions by the polysaccharide.

3.6. Oxidised glucomannan

In the case of the binding of KGMOx with Cu(II) and Pb(II), interesting results were obtained by absorption spectrophotometric measurements. As a point of difference from all the other systems studied, in this case $\lambda_{\max}$ was not constant, but varied, although not dramatically, with the ratio R. The spectra recorded in the presence of increasing amounts of added cations are reported in Fig. 7.

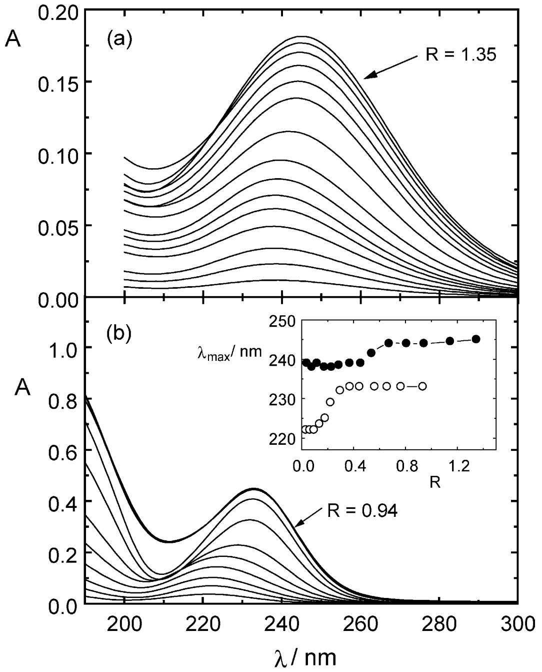

Fig. 7. Absorption spectra of KGMOx in water in the presence of increasing amounts of: (a) $Cu(ClO_4)_2$ and (b) $Pb(ClO_4)_2$. Inset: behaviour of $\lambda_{\max}$ with R. Full symbols, Cu(II); open symbols, Pb(II). R as in Fig. 1.

This behaviour stems from the presence of at least two binding sites. Very likely, this depends on the fact that KGMOx is a copolymer, glucuronic and mannuronic acids being the comonomers. These complicated spectra did not allow the values of K_B to be accurately computed. In fact, the extent of binding would be derived only with a scientific deconvolution of the CT bands. This was outside the scope of the present research, but it might be a future goal.

Here, a rough evaluation of K_B was made, using the values of $\lambda_{\max}$ of the bands recorded at the highest values of R. The values of K_B for the binding of Cu(II) resulted about $10^5\ \mathrm{L\ mol}^{-1}$ (Table 1). With Pb(II), the value of K_B was one order of magnitude higher.

The CD spectra recorded in the presence of Pb(II) showed more bands than with Cu(II). The reason for this was already discussed in the case of other saccharidic polycarboxylates (Cesàro et al., 1988).

With calorimetry, a discrimination between different comonomers was impossible. Anyhow, the values of ΔH_B were found to be positive with both Pb(II) ($1.6\ \mathrm{kcal\ mol}^{-1}$) and Cu(II), as expected in the case of the interaction between copper and carboxylic groups (Table 1).

3.7. Further insights

The results obtained deserve further considerations. First, both the thermodynamic and the optical–chirooptical measurements gave qualitatively similar results, on passing from solutions prepared in pure water to solutions prepared at higher ionic strength (Table 1). At higher ionic strength, i.e. in the presence of 0.05 M or even 0.01 M $NaClO_4$, the values of ΔG_B and ΔH_B were lower. This should indicate that added salts do not modify the mode of binding, but affect the extent of binding, because of competition between either Cu(II) or Pb(II) ions, present in low concentration, and Na(I) ions, much more concentrated.

Secondly, it was observed that with KGMOx the spectra recorded in the presence of Cu(II) and Pb(II) reached a constant absorbance value several minutes (5–10) after the addition of the metal salt in the cuvette. This slow phenomenon was particularly evident with Cu(II). In our opinion, this confirms that the species formed on addition of Cu(II) and Pb(II) to the carboxylated polysaccharide are chelates, most likely 'inner sphere coordination' complexes (Ahrland, 1972).

4. Conclusions

All the polysaccharides here studied showed the typical features normally found in the presence of a strong interaction between charged polysaccharides and copper and lead cations in dilute solution, in spite of their marked chemical differences.

Within the polysaccharides studied, the reacetylated chitosan only does not contain carboxyl groups in its chain. It is worth pointing out that, in spite of this, reacetylated chitosan is able to bind copper ions. Sharp differences from the other polysaccharides were found, clearly depending on its chemical structure.

In order to avoid misleading results, particular attention should be paid to the way of carrying out the measurements. The absorption spectra recorded on the 6-oxychitin-Cu(II) system are a typical case, where a particular protocol is mandatory.

In the case of KGMOx, three important features were evidenced: the slow formation of the chelate in the interaction with divalent counterions, the unusual variation of $\lambda_{\max}$ with R and the complicated CD spectra in the presence of Pb(II). The first two features should depend on the copolymeric nature of the glucomannan.

In water and in salt solutions the results obtained by the different techniques were qualitatively the same. This should indicate that the electrostatic contribution to the binding is unable to change the nature of the interaction, thus supporting the hypothesis that a true 'inner sphere' chelate forms in all the systems studied.

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References

Ahrland, S. (1972). How to distinguish between inner and outer sphere complexes in aqueous solution. Thermodynamic and other criteria. *Coordination Chemistry Review*, *8*, 21–29.

Aiba, S. (1986). Studies on chitosan: 1. Determination of the degree of *N*-acetylation of chitosan by ultraviolet spectrophotometry and gel permeation chromatography. *International Journal of Biological Macromolecules*, *8*, 173–176.

Barbucci, R., Casolaro, M., Barone, V., Ferruti, P., & Tramontini, M. (1983). Enthalpies of protonation and complex formation with copper (II) ion of some poly(amido-amine)s. *Macromolecules*, *16*, 1159–1164.

Begala, A. J., & Strauss, U. P. (1972). Dilatometric studies of counterion binding of polycarboxylates. *Journal of Physical Chemistry*, *76*, 254–260.

Cesàro, A., Ciana, A., Delben, F., Manzini, G., & Paoletti, S. (1982). Physico-chemical properties of pectic acid. I. Thermodynamic evidence of a pH-induced conformational transition in aqueous solution. *Biopolymers*, *21*, 431–449.

Cesàro, A., Crescenzi, V., Delben, F., Gamini, A., Liut, G., Paoletti, S., & Rizzo, R. (1992). Specific and unspecific ion-polysaccharide interactions. *Thermochimica Acta*, *199*, 1–15.

Cesàro, A., Delben, F., Flaibani, A., & Paoletti, S. (1988). The interaction of lead (II) with glycuronans: U.V. absorption and circular dichroism spectra. *Carbohydrate Research*, *181*, 13–21.

Cesàro, A., Delben, F., & Paoletti, S. (1988). Interaction of divalent cations with polyuronates. *Journal of Chemical Society, Faraday Transactions*, *1*(84), 2573–2584.

Crescenzi, V., Delben, F., Paoletti, S., & Škerjanc, J. (1974). Thermodynamics of polycarboxylate aqueous solutions. I. Dilatometry and calorimetry of protonation and copper(II) binding. *Journal of Physical Chemistry*, *78*, 607–611.

Crescenzi, V., Skjåk-Bræk, G., Dentini, M., Masci, G., Bernalda, M. S., Risica, D., Capitani, D., Mannina, L., & Segre, A. L. (2002). A high field NMR study of the products ensuing from Konjak glucomannan C(6)-oxidation followed by enzymatic C(5)-epimerization. *Biomacromolecules*, *3*, 1343–1352.

Dal Pozzo, A., Vanini, L., Fagnoni, M., Guerrini, M., De Benedittis, A., & Muzzarelli, R. A. A. (2000). Preparation and characterization of poly(ethyleneglycol)-crosslinked reacetylated chitosans. *Carbohydrate Polymers*, *42*, 201–206.

Delben, F. (1980). Very accurate dilatometric measurements using a high-performance thermostatic bath. *La Chimica e l'Industria*, *62*, 398–400.

Delben, F., & Muzzarelli, R. A. A. (1989). Thermodynamic study of the interaction of N-carboxymethyl chitosan with divalent metal ions. *Carbohydrate Polymers*, *11*, 221–232.

Delben, F., Muzzarelli, R. A. A., & Terbojevich, M. (1989). Thermodynamic study of the protonation and interaction with metal cations of three chitin derivatives. *Carbohydrate Polymers*, *11*, 205–219.

Delben, F., Stefancich, S., & Muzzarelli, R. A. A. (1992). Chelating ability and enzymatic hydrolysis of water-soluble chitosans. *Carbohydrate Polymers*, *19*, 17–23.

Dentini, M., Nero Scheffino, A. I., & Crescenzi, V. (1991). Binding of Cu(II) ions by maleic acid copolymers. *Gazzetta Chimica Italiana*, *121*, 33–36.

Dobetti, L., & Delben, F. (1992). Binding of metal cations by N-carboxymethyl chitosans in water. *Carbohydrate Polymers*, *18*, 273–282.

Fenyo, J. C., Delben, F., Paoletti, S., & Crescenzi, V. (1977). Thermodynamics of polycarboxylate aqueous solutions. IV. Special features of hydrophobic maleic acid-olefin copolimers. *Journal of Physical Chemistry*, *81*, 1900–1905.

Flaschka, H. (1952). Microchemical titrations with ethylenediaminetetraacetic acid. *Mikrochemie*, *39*, 38–50.

Gianni, R., Delben, F., Liut, G., Bernstorff, S., Amenitsch, H., & Rizzo, R. (2002). Synthesis and conformational properties of cyanoethyl-scleroglucan. *Carbohydrate Polymers*, *47*, 387–391.

Kabat, E., Lloyd, K., & Beychock, S. (1969). Optical activity and conformation of carbohydrates. II. Optical rotatory dispersion and circular dichroism studies on immunochemically reactive oligo- and polysaccharides containing aminosugars and their derivatives. *Biochemistry*, *8*, 747–756.

Manzini, G., Cesàro, A., Delben, F., Paoletti, S., & Reisenhofer, E. (1984). Copper (II) binding by natural ionic polysaccharides. I. Potentiometric and spectroscopic data. *Bioelectrochemistry and Bioenergetics*, *12*, 443–454.

Muzzarelli, R. A. A., Muzzarelli, C., Cosani, A., & Terbojevich, M. (1999). 6-Oxychitins, novel hyaluronan-like regiospecifically carboxylated chitins. *Carbohydrate Polymers*, *39*, 361–367.

Muzzarelli, R. A. A., & Rocchetti, R. (1985). Determination of the degree of acetylation of chitosans by first derivative ultraviolet spectrophotometry. *Carbohydrate Polymers*, *5*, 461–472.

Paoletti, S., & Delben, F. (1975). A spectroscopic investigation of complexes of divalent metal ions with maleic acid copolymers. *European Polymer Journal*, *11*, 561–564.

Paoletti, S., Cesàro, A., Ciana, A., Delben, F., Manzini, G., & Crescenzi, V. (1981). Thermodynamics of protonation and of copper (II) binding in aqueous alginate solutions. Alginic acid from *Azotobacter vinelandii*. In D.A. Brant, *Solutions Properties of Polysaccharides* (pp. 379–386). ACS Symposium Series, n. 150, American Chemical Society, Washington.

Peisach, J., & Blumberg, W.E. (1974). *Archives of Biochemistry and Biophysics*, *165*, 691–708

Yasunaga, T., & Sazaki, T. (1951). Efficiency of the ultrasonic interferometer and the amount of hydration water of some electrolytes. *Journal of the Chemical Society of Japan, Pure Chemistry Section*, *72*, 87–89.