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Binding of metal cations by *N*-carboxymethyl chitosans in water

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The binding of metal cations by *N*-carboxymethyl chitosan in dilute aqueous solution was studied as a function of charge density, temperature and pH. The techniques employed were absorption and circular dichroism measurements, potentiometric titration, dilatometry and isothermal microcalorimetry. The composition of the polymer significantly affected the binding of Ni(II), Co(II) and Pb(II), and had a small influence on the extent of binding of Cu(II). In any case, the density of glycine residues affected the binding constant with all the counterions studied, thus confirming that this residue is engaged in the complex formation. The whole of the optical, chirooptical and thermodynamic evidence showed that, at least in the case of Cu(II), the ions are bound by both carboxylic and amino groups. The temperature did not affect the extent of binding, at least in the case of Cu(II) and Pb(II) ions. Circular dichroism spectra, however, showed that these counterions are able to interact with —NH—CO— groups at high temperatures (61 °C), very likely owing to the enhanced mobility of the polymer chain. Lowering pH depressed the binding ability of *N*-carboxymethyl chitosan towards Cu(II) and Pb(II) ions, the effect being much more pronounced in the case of Pb(II). Moreover, a refined method for collecting absorption spectra of the complex between the polymer and Cu(II) or Pb(II) ions, without interference with absorbance by undesired species, was tested and applied. The charge-transfer band exhibited by the *N*-carboxymethyl chitosan–Cu(II) system, recorded following this method, showed interesting features, on the basis of which the site of the binding of Cu(II) on to *N*-carboxymethyl chitosan was confirmed.

INTRODUCTION

N-carboxymethyl chitosan (NCMCh) is a water-soluble derivative of chitosan. The properties of this polymer have been studied in recent years, in view of the use of NCMCh in medicine and the production of cosmetics (Muzzarelli *et al.*, 1989*a*,*b*,*c*). In particular, its ability in binding metal ions was reported in terms of chelation isotherms for an insoluble derivative (Muzzarelli *et al.*, 1985, 1989*d*; Muzzarelli & Zattoni, 1986), and was investigated by studying the polymer in dilute solution as well (Delben & Muzzarelli, 1989; Delben *et al.*, 1989). The chelation of different divalent cations was found to be much more specific in dilute solution than for insoluble systems.

The aim of the present work was to clarify the role of important parameters, namely the density of carboxylic groups on the polymeric backbone, pH and temperature, on the binding ability of NCMCh in dilute solution, in order to better understand the binding process.

MATERIALS AND METHODS

Materials

The water-soluble *N*-carboxymethyl chitosans, prepared according to the procedure described by Muzzarelli *et al.* (1982) from *Euphausia superba* chitosans, were supplied by Chito-Bios, Ancona, Italy. The degree of acetylation of chitosans, as determined by spectrophotometry (Muzzarelli & Rocchetti, 1985), was 0·58 and 0·72. The *N*-carboxymethyl chitosans employed are referred to here as NCMCh 58 and NCMCh 72,

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respectively. The average molecular weights of the parent chitosans were 465 000 for NCMCh 58 (Muzzarelli *et al.*, 1987) and probably higher for NCMCh 72. Owing to the mild preparative conditions of NCMCh (aqueous neutral media and room temperature), no degradation of the polymer was observed (Muzzarelli *et al.*, 1982).

Metal perchlorates were Ventron GmbH products. Their aqueous solutions were titrated with EDTA following standard procedures. All of the other chemicals were C. Erba analytical grade products. Deionized, doubly distilled water was constantly employed.

Instruments and methods

The UV absorption spectra were recorded with a Varian Cary 2200 spectrophotometer (Varian Techtron, Mulgrave, Victoria, Australia) as differences between polymer-containing and polymer-free solutions, after addition in both sample and reference cells of aliquots of metal perchlorate solution and thorough stirring. A more sophisticated method, eliminating the contribution to absorbance of both polymer and excess free metal ion absorbance (described in the Results and Discussion section), was also employed.

The circular dichroism (CD) measurements were performed with a Jasco J500-A dichrograph (Japan Spectroscopic Co. Ltd, Tokyo), equipped with a Jasco DP500-N data processor. Four spectra were usually cumulated for each experiment. Both the spectrophotometer and the dichrograph were equipped with a thermostatic unit. The temperature was measured inside the sample cuvette with an HD 8464 DeltaOhm (Padua, Italy) electronic thermometer. Neither optical nor chiroptical investigation showed any kinetic phenomena. In the UV absorption and CD measurements, the molar polymer concentration, C_P, was 3-8 × 10^{-4} monomol liter^{-1}.

The pH of the solutions was detected with a Radiometer (Copenhagen, Denmark) PHM52 pH meter, equipped with GK 2321C combined electrodes.

The calorimetric measurements were performed using an LKB 10700-2 batch type twin calorimeter (LKB-Produkter AB, Bromma, Sweden), equipped with gold cells. In each experiment, 2 ml of polymer solution (molar concentration, C_P, 4–8 × 10^{-3}) and a proper, small amount (20–50 μl) of metal perchlorate solution (molar concentration 4–6 × 10^{-2}) was put in the reaction cell, while in the reference cell an equal volume of water replaced the perchlorate solution. Therefore, the heat read was already corrected by the polymer dilution enthalpy, the heat of dilution of metal perchlorate being small and negligible. Electric calibrations were performed.

The polymer solutions were prepared by swelling a known amount of the freeze-dried product with water and dissolving the concentrated solution or gel so formed with further water or with dilute (up to 5 × 10^{-3} M) acetic acid. In the latter case, the solution was exhaustively dialyzed against pure water before use. The concentration was calculated from the volume of the resulting solution, taking into account the water contained in the freeze-dried polysaccharide, previously determined with a Mettler DL 18 apparatus (Mettler Instrumente AG, Greifensee, Switzerland), employing the Karl-Fisher method. C_P was expressed in total repeat units per liter. Adjustment of the pH was made by addition of 0·1 M perchloric acid. While the neutral (pH 5·8–6·0) or acidic (down to 3·2) NCMCh 58 solutions looked perfectly transparent even some months after preparation (provided that they were kept in the dark at 4°C), the neutral NCMCh 72 solutions showed a consistent turbidity after a week. In any case, freshly prepared solutions of both polymers were employed throughout.

Evaluation of binding constants

The general scheme of binding between a metal ion M and a polymer constituted of repeat units P is:

$$\mathrm{M} + \mathrm{P} \rightleftharpoons \mathrm{MP} \tag{1}$$

If n is the maximum average number of ions that a repeat unit is able to bind, the binding constant K is expressed as follows:

$$K = \frac{C_B}{C_P(n\,C_P - C_B)} \tag{2}$$

where C_B is the molar concentration of bound metal ions. Indicating with r the molar ratio of bound metal ions to polymer, and with C_F the molar concentration of free metal ions, from eqn (2) the well-known Scatchard relation is obtained:

$$\frac{r}{C_F} = nK - rK \tag{3}$$

The values of r and C_F can be computed or determined in many ways, depending on how the experiments are carried out. In the authors' case, these quantities were computed from the experimental curves of binding (obtained with calorimetry, UV absorption spectrophotometry and CD measurements) in the following way.

Let Y_R be the thermodynamic variable, obtained by normalizing the experimental results by C_P. Y_R is plotted against R, the ratio of total metal ion, C_T, to polymer molar concentration. Y_R is of course a function of R and, in general, it has a trend as indicated in Fig. 1 (full curve). Values r and C_B are simply derived from the following equations:

$$r = R_S\, Y_R/Y_{R\infty} \tag{4}$$

$$C_B = R\, C_P\, Y_R/Y'_R \tag{5}$$

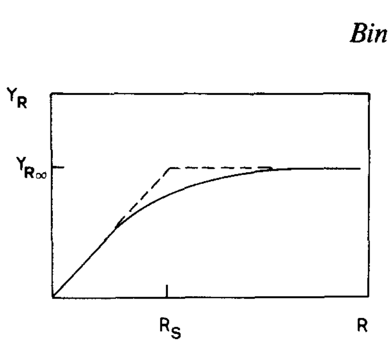

Fig. 1. General behavior of a thermodynamic variable, Y_R, normalized by the total polymer present, as a function of the molar counterion-to-polymer ratio, R.

where R_S is the value of R for which the dashed lines intersect, $Y_{R\infty}$ is the value of Y_R when a (theoretically) infinite amount of M is added, and Y'_R is the value of Y_R in the case of $K = \infty$ (represented by dashed lines in Fig. 1). Then, if:

$$C_F = C_T - C_B \tag{6}$$

the following equation is derived:

$$C_F = R\,C_P\,[1 - (Y_R/Y'_R)] \tag{7}$$

The values of $Y_{R\infty}$ were obtained as a limit of $1/Y_R$ against $1/R$ at $1/R = 0$. This dependence was found in most cases to be linear. The only difficulty of the method is the determination of R_S when the binding constant is low (10^3 liters mol^{-1} or less). In this case, the authors chose the value of R_S able to give convergence, in the Scatchard plot, with n, or at least when the two values were sufficiently close. In the case of a large binding constant ($K = 10^4$ liters mol^{-1} or greater), the choice of R_S is quite handy. When K is low (of the order of 10^3 liters mol^{-1}), it was verified that a variation of 20-25% on R_S normally produced an uncertainty on the derived K value not exceeding ±20%. It was concluded, therefore, that the values of K were computed with confidence.

RESULTS AND DISCUSSION

Absorption and circular dichroism spectra

The absorption spectra of aqueous solutions of NCMCh showed a very intense band in the far-UV region, very similar to the band exhibited by chitosan, and due to $n -> \pi^*$ electron transition in the —NH—CO— chromophore (Kabat *et al.*, 1969; Stone, 1969; Dickinson & Bush, 1975). The related CD spectra showed a negative band, centered at 208 nm. This band was not affected by varying the pH of the solution from 2 to 11 (Delben *et al.*, 1989). Added metal cations could affect this band, with the modification being very small or nil at 25 °C. In the case of Cu(II) and Pb(II) ions, new strong bands appeared in the UV region. They were interpreted in terms of a charge-transfer (CT) transition between the electronic system localized on the ligand and the central metal ion. Similar features have been found with other synthetic and natural polycarboxylates (Paoletti & Delben, 1975; Manzini *et al.*, 1984; Cesàro *et al.*, 1988*a,b*) and with amino acids and peptides as well (Martin, 1973, and references therein). It was shown that in the latter cases both the carboxylate and the amino group are responsible for the CT band (Wilson *et al.*, 1970). Moreover, previous studies have shown that not all the ions bound by carboxyl and/or amino groups are able to induce CT bands. For instance, no CT band is evident in the amino acid complexes of Ni(II), in spite of the large values of the stability constants of some of these systems (Basolo & Chen, 1954; Kim & Martell, 1967; Billo & Margerum, 1970; Lim & Nancollas, 1971). NCMCh is a polycarboxylate and, at the same time, a polymeric glycine derivative, so that the derivative obtained from extensively deacetylated chitosan (polyglucosamine) is called glycine glucan (Muzzarelli *et al.*, 1985). Therefore, the comparison of the present authors' results with previous findings concerning the binding of metal cations by polycarboxylates and by amino acids (or peptides) appears justified. In particular, the CT band molar absorbance of NCMCh-Cu(II) complexes is in good agreement with the literature data on Cu(II)-amino acids spectra (Wilson *et al.*, 1970).

Dependence of binding constants on polymer composition, temperature and pH

Absorption and CD spectra and thermodynamic measurements have been used for determining the dependence of the binding constants K on polymer composition, temperature and pH. Polymer composition (i.e. the degree of deacetylation of the parent chitosan and hence the fraction of *N*-carboxymethylated sugars) will be considered first.

The plots of pH, enthalpy changes and volume variations obtained upon addition of cations to NCMCh 72 almost neutral (pH 5·8–6·0) solutions (constantly indicated here as neutral solutions) were very similar to the corresponding plots obtained with NCMCh 58, already presented and discussed (Delben & Muzzarelli, 1989). The related plots have not been reported for simplicity, with the exception of calorimetric data (see later).

As far as the spectra in the presence of Cu(II) and Pb(II) are concerned, the small differences found for NCMCh 58 and NCMCh 72 are detectable in Fig. 2, in which molar absorbance and molar ellipticity as a function of metal cation-to-polymer molar concentration, R, were reported. Small differences between the two polymers were found in both the binding constant and the n (ratio of moles of bound metal ions to polymer repeating units at saturation) values. The complete set of data so far collected is reported in Table 1. With the

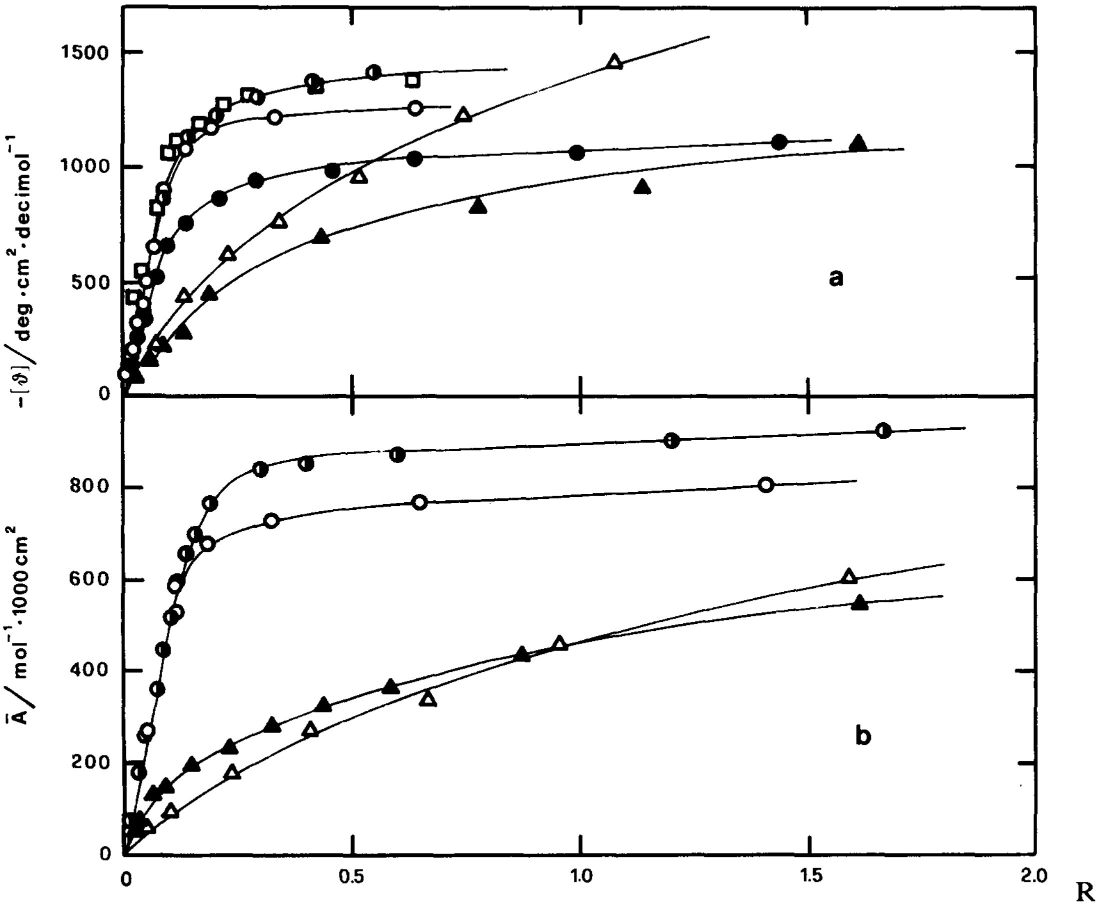

Fig. 2. (a) Molar ellipticity and (b) molar absorbance as a function of metal cation-to-polymer molar concentration, *R*. ○, NCMCh 58-Cu(II); (a) $\lambda = 276$ nm, $C_P = 7{\cdot}05 \times 10^{-4}$ M; (b) $\lambda = 246$ nm, $C_P = 2{\cdot}73 \times 10^{-4}$ M. □, NCMCh 72-Cu(II), $\lambda = 277$ nm, $C_P = 4{\cdot}73 \times 10^{-4}$ M. ◐, NCMCh 58-Cu(II), $T = 61$ °C; (a) $\lambda = 280$ nm, $C_P = 4{\cdot}47 \times 10^{-4}$ M; (b) $\lambda = 246$ nm, $C_P = 4{\cdot}55 \times 10^{-4}$ M. ●, NCMCh 72-Cu(II), pH = 3·2, $\lambda = 275$ nm, $C_P = 4{\cdot}73 \times 10^{-4}$ M. △, NCMCh 58-Pb(II); (a) $\lambda = 244$ nm, $C_P = 4{\cdot}12 \times 10^{-4}$ M; (b) $\lambda = 234$ nm, $C_P = 4{\cdot}12 \times 10^{-4}$ M. ▲, NCMCh 58-Pb(II), $T = 61$ °C; (a) $\lambda = 246$ nm, $C_P = 4{\cdot}55 \times 10^{-4}$ M; (b) $\lambda = 235$ nm, $C_P = 4{\cdot}55 \times 10^{-4}$ M. Where not indicated, measurements were performed at 25 °C and pH = 5·9. C_P denotes the initial polymer concentration in moles of total saccharidic units per liter.

Table 1. Binding constants using the Scatchard method[a]

System	Liters mol^{-1}			
		$T = 25$ °C[b]	$T = 25$ °C[c]	$T = 61$ °C
NCMCh 58-Cu(II)	$7{\cdot}2 \times 10^5$ (4) $n = 0{\cdot}11$	$5{\cdot}1 \times 10^5$ (1) $n = 0{\cdot}10$		$6{\cdot}0 \times 10^5$ (2) $n = 0{\cdot}12$
NCMCh 72-Cu(II)	$4{\cdot}6 \times 10^5$ (2) $n = 0{\cdot}11$		$7{\cdot}0 \times 10^4$ (1) $n = 0{\cdot}09$	
NCMCh 58-Pb(II)	$3{\cdot}3 \times 10^3$ (3) $n = 0{\cdot}12$	$3{\cdot}6 \times 10^3$ (1) $n = 0{\cdot}12$		$6{\cdot}5 \times 10^3$ (2) $n = 0{\cdot}10$
NCMCh 72-Pb(II)	$5{\cdot}0 \times 10^3$ (1) $n = 0{\cdot}12$			
NCMCh 58-Co(II)	$1{\cdot}2 \times 10^3$ (1) $n = 0{\cdot}12$			
NCMCh 72-Co(II)	$6{\cdot}5 \times 10^3$ (1) $n = 0{\cdot}09$			
NCMCh 58-Ni(II)	$2{\cdot}6 \times 10^3$ (1) $n = 0{\cdot}12$			
NCMCh 72-Ni(II)	$1{\cdot}1 \times 10^4$ (1) $n = 0{\cdot}12$			

[a] Numbers in parentheses indicate number of independent techniques.

[b] With the method suggested here (see text).

[c] At pH = 3·2; all the other values at pH = 5·9.

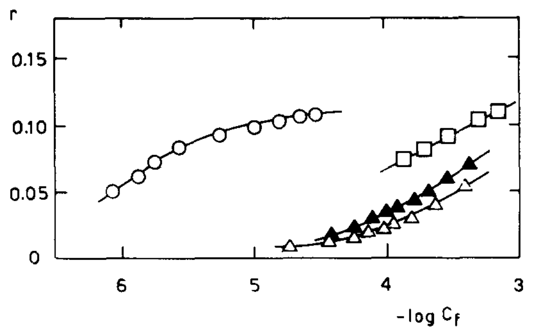

Fig. 3. Plots of log *r* versus log C_F (where *r* and C_F denote the molar ratio of bound metal cation-to-polymer and the molar concentration of free metal ion, respectively) for some sample systems at pH = 5·9. ○, NCMCh 58-Cu(II); △ and ▲, NCMCh 58-Pb(II); □, NCMCh 72-Ni(II). Open symbols: 25 °C; full symbols: 61 °C.

exception of copper binding, the *K* values at 25 °C are slightly higher with NCMCh 72 than with NCMCh 58. This fact confirms the obvious importance of the glycine residues in the metal-polymer interaction. The mentioned differences are particularly evident with Co(II) and Ni(II) ions, whose interaction with NCMCh does not show the interesting spectroscopic features presented by the NCMCh-Cu(II) and NCMCh-Pb(II) systems. On the other hand, no clear new trend was shown by the *n* values.

In none of the systems investigated was a cooperative binding found. This is seen from the log *r* against C_F plots as well as from the usual Scatchard plots. The log *r* versus C_F plots are shown for some sample systems in Fig. 3. They show a monotonic behavior, without any sigmoidal trend, which is usually interpreted in the *r* versus C_F plots as being due to cooperative interaction and is enhanced if an *r* versus log C_F plot is drawn. This trend occurs, for instance, when the polymer chain undergoes a conformational transition or another important intra- or interchain cooperative phenomenon (such as coupling of single chains) induced by binding (e.g. Lages & Stivala, 1973; Campanella *et al.*, 1989). On the contrary, the behavior found here indicates clearly that the binding process involves single polymer chains, whose conformation, moreover, should not be changed by the presence of bound countercations. Also, from the mentioned plots it is possible to obtain for each system the limit (highest) value of *r*, which corresponds to the value of *n*. The values of *n*, evaluated with this method, are reported in Table 1. The possible presence of cooperativity and the values of *n* can also be deduced from the Scatchard (r/C_F versus *r*) plots. In Fig. 4 some sample Scatchard plots are reported. With copper, which is strongly bound to NCMCh at neutral pH values, the plots are very close to linearity, thus denoting the absence of any cooperativity. Nickel, cobalt and lead, on the other hand, occasionally give slightly curved plots, typically occurring in anticooperative interactions. It is worth pointing out that in the case of curved Scatchard plots the values of *K* are strongly dependent on *r*. In such cases, the authors computed the values of *K* employing *n* values similar to those used in the case of the NCMCh-Cu(II) systems.

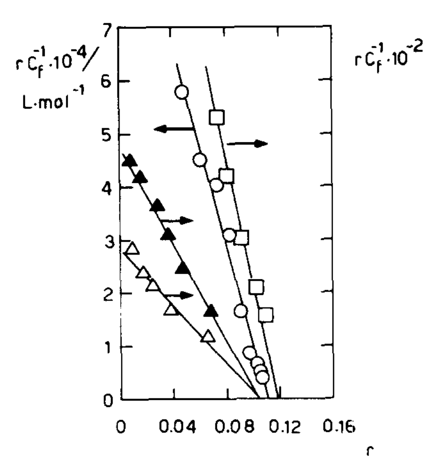

Fig. 4. Scatchard plots for some sample systems at pH = 5·9. ○, NCMCh 58-Cu(II); △ and ▲, NCMCh 58-Pb(II); □, NCMCh 72-Ni(II). Open symbols: 25 °C; full symbols: 61 °C.

The effect of pH on metal binding was tested with calorimetry, UV absorption and CD measurements. The calorimetric results are reported in Fig. 5, in which the enthalpy variation (normalized by the molar polymer concentration), measured upon mixing dilute NCMCh solutions with metal perchlorate solutions to reach the final ion-to-polymer molar ratio, *R*, is plotted against *R*. A completely different behavior on passing from neutrality to pH = 3·2 is observed. In particular, the values with Cu(II) become very small and endothermic. This fact seems to confirm a previous interpretation (Delben *et al.*, 1989) of the negative ΔH

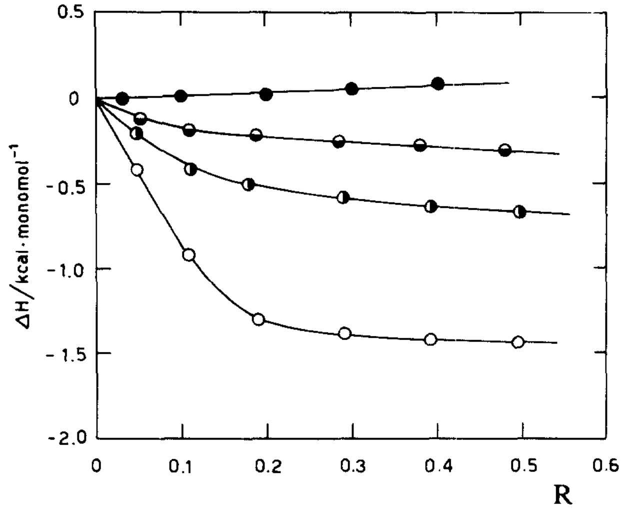

Fig. 5. Enthalpy change on addition of divalent cations to NCMCh 72 in water at 25 °C. *R* is the molar ratio of cations added as perchlorates to total saccharidic units. ○, Cu(II); ◑, Ni(II); ⊖, Co(II) (pH = 5·9). ●, Cu(II) (pH = 3·0).

values for the copper binding by NCMCh at neutral pH. In fact, if negative ΔH values denote the interaction of Cu(II) with a binding site involving nitrogen atoms, it is not surprising that lowering pH renders the ΔH values less negative, being the amino group protonated. Literature data of heat of formation of Cu(II) complexes with amino acids (Wilson *et al.*, 1970) seem to support these conclusions. In that case, however, it has been suggested that the binding mode (and not only the binding extent) is pH dependent. As for the other ions studied, i.e. Co(II) and Ni(II), the variation in ΔH observed is not easy to interpret, in the sense that it is uncertain if it depends on a variation of the mode or of the extent of binding, or both.

As far as the interaction of NCMCh with copper and lead ions is concerned, further indications can be obtained from CD measurements. In the case of binding of Cu(II), lowering the pH clearly depresses the CT bands. In Fig. 6 the CD spectra obtained upon the addition of increasing amounts of copper perchlorate to NCMCh 72 solution at pH 3·2 are reported. The peaks are significantly lower than the corresponding ones recorded for the same system at neutral pH (Delben & Muzzarelli, 1989). This can be better seen by inspection of Fig. 2(a), in which the molar ellipticity at wavelengths corresponding to the maximum of the related bands as a function of R for the more significant systems studied are reported. From the plots of Fig. 2(a) (and analogous plots) the binding constant values were obtained, as previously explained. On passing from pH = 5·9 to 3·2 the K value is depressed by about one order of magnitude (see Table 1). In other words, a lowering of pH causes a decrease in the height of the CD charge-transfer bands, and this phenomenon is paralleled by a lowering of the binding constant. In the case of NCMCh–Pb(II) systems, the effect of pH is even more evident, such that the related CT bands disappear from the CD spectra at well over pH = 4. In Fig. 7 the values of molar absorbances and ellipticities of the NCMCh 58-Cu(II) and NCMCh 58-Pb(II) systems are plotted as a function of pH. pH is more effective in depressing CT bands and binding constants in the case of Pb(II) than in the case of Cu(II) ions very likely because even in the case of Pb(II) the binding process should be partially electrostatic in nature. The trend shown in Fig. 7 is of great relevance when this kind of system is employed as an ion-sequestrant. It is evident that the polysaccharide is ineffective or at least less effective in acidic solutions.

On the other hand, no relevant difference in the binding ability of NCMCh was found when enhancing the medium temperature. The values of both K and n at 61 °C, reported in Table 1, are very close to the corresponding values obtained at 25 °C.

However, an interesting feature was found studying the CD spectra of *N*-carboxymethyl chitosan in the presence of copper ions at 61 °C. The CD spectra,

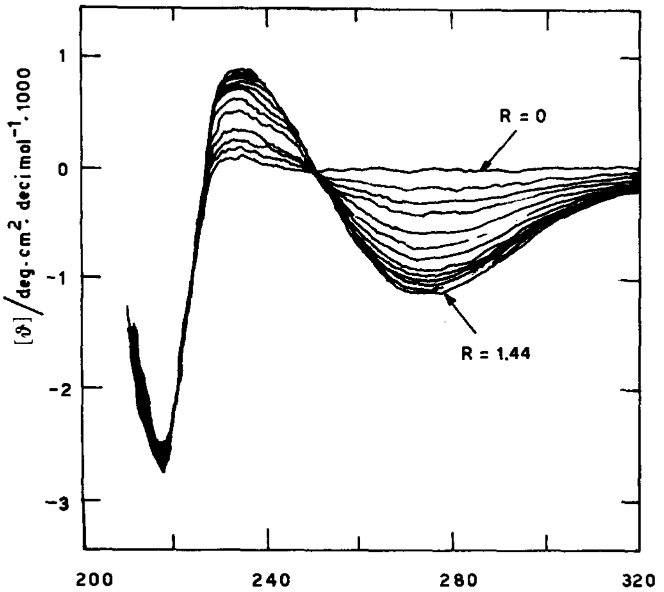

Fig. 6. Circular dichroism spectra of NCMCh 58 in water at 25 °C, pH = 3·2, on addition of increasing amounts of copper perchlorate, measured as ion-to-polymer molar concentration, R. Polymer concentration in moles of total saccharidic units per liter; cell pathlength, 1 cm.

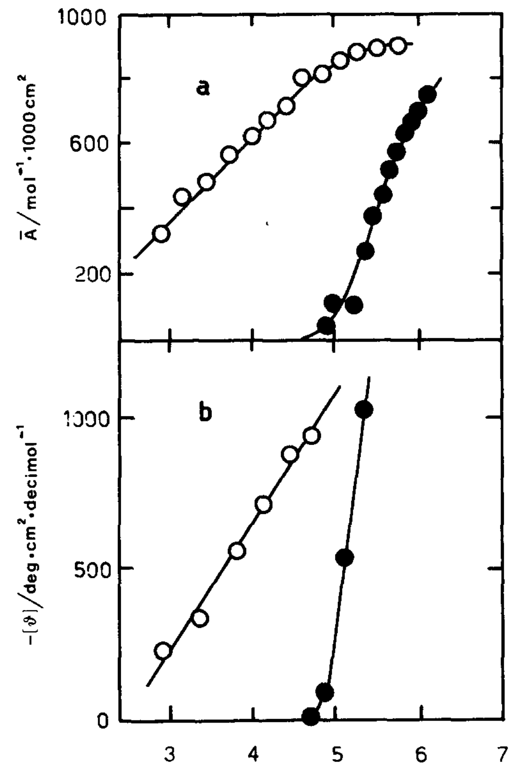

Fig. 7. (a) Molar absorbance and (b) molar ellipticity change of the maximum of charge-transfer bands as a function of pH for the NCMCh 58–Cu(II) (open symbols) and NCMCh 58-Pb(II) (full symbols) systems in water at R = 0·2 and T = 25 °C. For λ values, see Fig. 2.

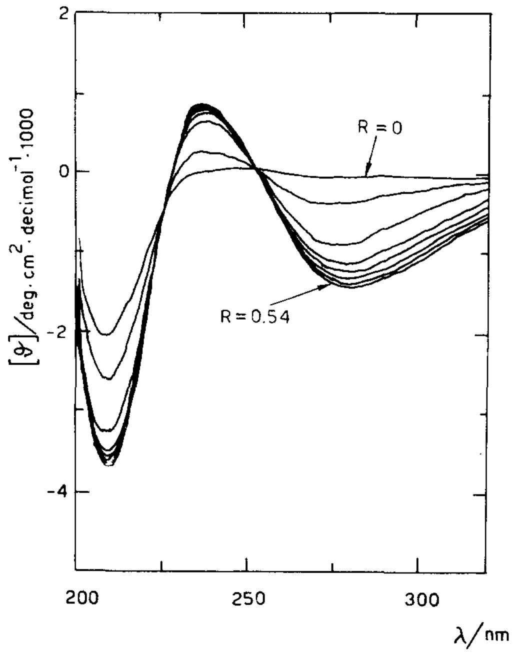

Fig. 8. Circular dichroism spectra of NCMCh 58 in water at 61 °C on addition of increasing amounts of copper perchlorate, measured as ion-to-polymer molar concentration, *R*. Polymer concentration in moles of total saccharide units per liter; cell pathlength, 1 cm.

reported for the NCMCh 58-Cu(II) system in Fig. 8, showed that at this temperature the addition of copper ions affects the bands centered around 208 nm and attributed to the $n - >\pi^*$ electron transition of the chromophore —NH—CO—. In the presence of lead ions, the $n - >\pi^*$ transition band is detected for very small *R* values only, owing to the large absorption of free Pb(II). Since at 25 °C this band is slightly (or not at all) influenced by the counterions, an interpretation of these findings is that at higher temperatures the amido groups are partially engaged in the binding process, while this interaction is forbidden at lower temperatures owing to the stiffness of the polysaccharidic backbone. In any case, the fact that the CT dichroic spectra are by and large the same at 25 ° as at 61 °C should mean that the engagement of the amido group in the binding is soft. All this enables us to understand why the binding constants with both the NCMCh-Cu(II) and NCMCh-Pb(II) systems are practically the same at 25° and 61 °C, or even larger at higher temperature, in spite of the negative and large ΔH accompanying the binding process. Once more, the results obtained can be used for practical purposes: these polymers can be employed as ion-sequestrants even at high temperatures, contrary to the indications of enthalpy change associated with the binding process.

Accurate spectroscopic analysis of the binding process

The Scatchard method, applied to the experimental results, as illustrated in the present work, allows us to compute the binding constants with an acceptably simple procedure. The values of *K* so calculated, however, are necessarily approximated. This happens mainly because of: the intrinsic uncertainty of a single experimental value due to interfering species; the difficulty, or impossibility, of determining exactly the values of R_S; the shape of the Scatchard plots, which in most cases shows a slight curvature; the impossibility knowing, a priori, the number of independent sites on the polymer chain (in the present case, the authors supposed the presence of one binding site only, and this choice has been strengthened by the shape of the Scatchard plots mainly in the Cu(II) containing systems).

While some of the mentioned reasons seriously hinder a precise evaluation of *K*, other ones are not true impediments, provided that correct, even somewhat complicated, procedures are applied. In the usual spectroscopic analysis, one of the more important reasons affecting the precision of the experimental data and hence the validity of the quantities derived from them, including the binding constants, is the impossibility of purging the spectra in the usual procedure by the contribution to the read absorbance of undesired species. These are both the polymer, present in the sample cell and lacking in the reference one, and the metal ions, added in equal amount in both cuvettes. The polymer influences the recorded spectra because its absorption band, beginning for NCMCh at about 230 nm and rapidly increasing at lower wavelengths, partially overlaps the CT bands. If the added metal ions are able to modify even a little the polymeric band(s), the values of both the absorbance and the wavelengths corresponding to the maximum in optical density are influenced, and a correction is impossible. The trouble due to the free metal ions is even more important, when, as in the case of both Cu(II) and Pb(II) species, they absorb close to the band of the complex. In fact, since the free metal ion concentrations in the two cells differ, as a consequence of binding, their contribution cannot be taken into account and subtracted.

To bypass the mentioned difficulties, the authors suggest replacing the reference cuvette with two cuvettes, each having a pathlength of half of the sample cell. In one of the reference cells a polymer solution, doubly concentrated with respect to the sample, is lodged. The other reference cuvette contains a proper volume of the solvent (in the authors' case, pure water). In this cell, at each addition of metal perchlorate in the sample, aliquots of metal perchlorate solution are added, so that the absorbance is equal to the absorbance of free ions in the sample cuvette only. The actual value of C_F in the sample cell is computed from the (approximate) value of *K*, previously obtained with the usual procedure. From the absorption data so collected, more precise values of *K* are derived, from which, if necessary, another set of measurements is performed.

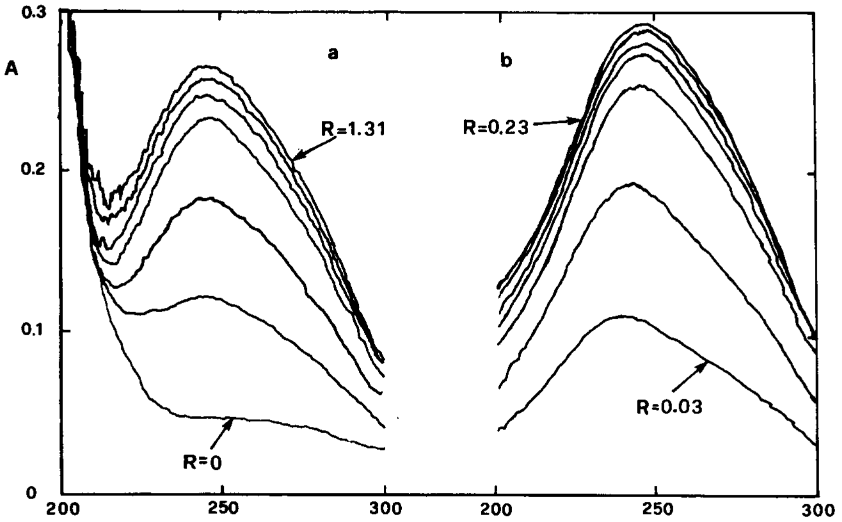

Fig. 9. UV absorption spectra of NCMCh 58 in water at 25°C, pH = 5·9, on addition of increasing amounts of copper perchlorate, measured as ion-to-polymer molar concentration, R. Initial polymer concentration, $2\cdot73 \times 10^{-4}$ M and $4\cdot55 \times 10^{-4}$ M in (a) and (b), respectively. (a) Spectra recorded with the usual method; (b) spectra recorded with the method suggested here (see text). In the latter case, the spectrum recorded at $R = 0$ is the baseline itself ($A = 0$).

In Fig. 9 UV spectra recorded (a) without and (b) with the correction suggested here for the NCMCh 58–Cu(II) system are reported.

The differences between the two sets of the said spectra are very evident. In particular, the CT bands look very asymmetric, with a clear shoulder at higher wavelengths. Then, each CT band appears as formed by two different contributions. The authors tried to separate these contributions by simply assuming a perfect symmetry in the main CT band. The obtained deconvolution is reported in Fig. 10(a) as dashed curves for the absorption band corresponding to $R = 0\cdot23$. It is known (Leyte *et al.*, 1968, and references therein) that in the polycarboxylate–Cu(II) aqueous systems, binuclear complexes may be present. When this happens, the related absorption bands are characterized by a shoulder at higher wavelengths. This possibility is excluded in the authors' case, because binuclear complexes are normally formed at high metal-to-polymer ratio values, while in the authors' case the double peak is also very evident at very low r values. The following interpretation is suggested. On the basis of thermodynamic evidence (Delben *et al.*, 1989) and for comparison with literature data concerning the binding of amino acids and peptides to copper ions (e.g. Tsangaris *et al.*, 1969; Wilson *et al.*, 1970; Wilson & Martin, 1971), in the complexation of copper ions by NCMCh the site of binding contains both the carboxylic group and the nitrogen atom. Moreover, on the basis of spectroscopic findings, the stronger CT band (centered around 245 nm) is due to the electron transfer to the central metal ion bound from carboxyl and amino groups, as already stated, while the other one (the weaker band, centered around 275 nm) derives from the electron transfer to the same ions from the amido residues. This interpretation is reinforced by inspection of the spectra exhibited by the parent chitosan, dissolved in very dilute acetic acid (pH = 5) in the presence of copper ions. In fact, the chitosan–Cu(II) system exhibits an absorption band which is weak and very broad, and is probably composed of a few peaks. One of them has its maximum value between 270 and 280 nm (unpublished data from the authors' laboratory, 1988). Moreover, and more importantly, many Cu(II) containing polyamidic systems exhibit charge-transfer bands in the 200–300 nm range (Garnier & Tosi, 1975, 1978; Formicka-Kozlowska *et al.*, 1979; Sigel & Martin, 1982; Lekchiri *et al.*, 1987). Although both the maximum wavelengths and the intensities of these bands are widely influenced by pH and even by small differences in the ligand chemical structure (Wilson *et al.*, 1970), such that the band shown by the NCMCh–Cu(II) system cannot be precisely compared with literature data, the attribution suggested here seems to be very likely, if the clear-cut influence of Cu(II) and Pb(II) on the $n - >\pi^*$ CD band of NCMCh at 61 °C is taken into account, as pointed out above. From all of these findings it can be concluded that the site of binding in the NCMCh–Cu(II) system is then stated with confidence. It is worthwhile stressing that the observations reported above were not so simple as the 'traditional' method in recording absorption spectra, or were not possible at all.

The K values obtained by employing the absorbance of the corrected spectra are reported, together with all the other values, in Table 1. Significant differences from the previous values are detectable in the case of Pb(II) binding, because with lead the interference between free and bound ions in the UV absorbance spectra is larger than in the case of copper ions. The

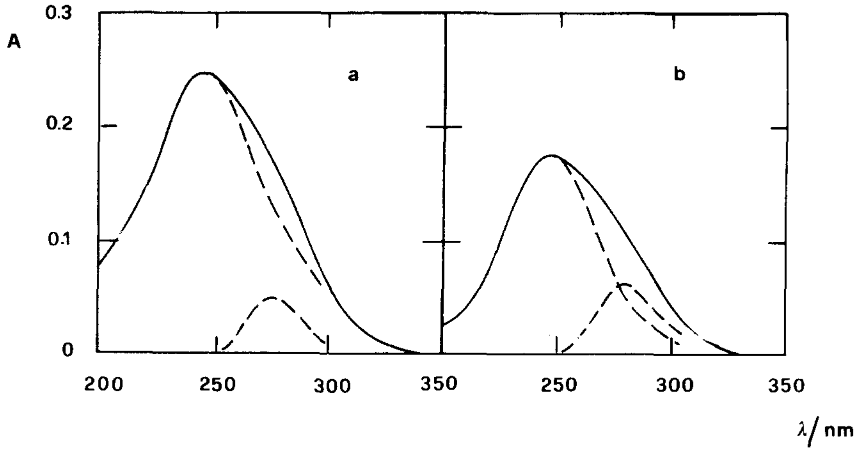

Fig. 10. Full curves, UV absorption bands of NCMCh 58 in water, pH = 5·9, in the presence of Cu(II), at *R* (metal-to-polymer molar ratio) = (a) 0·23; (b) 0·22. (a) *T* = 25 °C; (b) *T* = 61 °C. Dashed curves, deconvolution of the absorption bands (see text).

values reported here are in good agreement with the equilibrium constants reported for Cu(II) and Ni(II) complexes with glycine derivatives (Kim & Martell, 1964, 1966, 1967; Billo & Margerum, 1970; Lim and Nancollas, 1971). It is worth pointing out that with glycine, in which both the carboxyl and the *a* amino groups are free and hence able to bind a metal ion, the binding constants are significantly higher with both copper and nickel ions (Basolo & Chen, 1954).

Finally, the authors employed this improved method to test the validity of the above-reported conclusions on the interaction of Cu(II) ions with the —CO—NH— group of acetylated units of NCMCh mainly at high temperatures. The UV absorption spectra for the NCMCh 58-Cu(II) system (at *R* = 0·22) were recorded at 61 °C. The results are reported in Fig. 10(b) together with a deconvolution of the experimental absorption bands. The band occurring at higher wavelengths, and attributed to the interaction of copper ions with the —CO—NH— chromophore, is significantly higher than at lower temperatures. In particular, the ratio of the maximum heights of main to secondary bands drops from 4·9 (at 25 °C) to 2·8 (at 61 °C). If the probability of the interaction of cations with amidic groups is connected with the mobility of the polymer chain, which is higher at higher temperatures, this result should further confirm the authors' interpretation.

It can be concluded that the importance of the described method lies both in the computation of the correct *K* values and in the observation of the interesting features discussed here.

CONCLUSIONS

On the basis of the experimental data collected so far, it can be concluded that the extent of binding of divalent counterions by NCMCh is much more affected by the pH of the solution, and hence by the degree of protonation of the polyelectrolyte chain, than by the degree of substitution of the polymer itself (at least in the range investigated) or by temperature (in the range 25-61 °C). The site of binding of Cu(II) with NCMCh mainly involves the carboxyl and amino groups and, to a minor extent, the amido group of the acetylated units. The improved spectroscopic method applied here for correcting the absorption spectra appears very useful and promising.

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