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O-Carboxymethylagar: interaction with divalent cations

M. Bosco[a], **A. Gamini**[b] & **R. Rizzo**[a,b]*

[a] *Laboratorio Biopolimeri Tecnologici, Centro Rocerche POLY-biòs, Area della Ricerca di Trieste, Padriciano 99, 34012 Trieste, Italy*
[b] *Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Via L. Giorgeri 1, 34127 Trieste, Italy*

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O-Carboxymethylagar was produced from a commercial, low methoxylated agar sample. The degree of substitution of the sample was 1·2 carboxymethyl groups per disaccharide repeating unit. The procedure used for the synthesis lowered the polymer molecular weight slightly. Carboxymethylagar exhibited different binding behaviour with different divalent cations. Interaction with ions was monitored by polarography, circular dichroism, and isothermal microcalorimetry. The polycarboxylate interacted strongly with lead ions, and showed lower affinity for copper ions. Little interaction was detected with respect to other divalent cations.

INTRODUCTION

Natural and semisynthetic carboxylated polysaccharides constitute interesting systems, as they are used in a wide range of different technological applications either because of their hydrophilic character or ion binding ability (Sandford & Baird, 1983; Stivala *et al.*, 1987). Alginates and pectates are well known naturally carboxylated plant polysaccharides which produce firm gels in the presence of divalent cations (Rees *et al.*, 1982), in particular calcium ions. Such gels have been successfully exploited in many biotechnological applications (Crescenzi *et al.*, 1989; Skjåk-Bræk & Martinsen, 1991). Chemically carboxylated polysaccharides, such as *O*-carboxymethylcellulose or *O*-carboxymethylamylose, are used for the preparation of hydrophilic matrices, for instance in drug formulations (Harsh & Gehrke, 1991). Other applications of these systems are found in fields such as paper and textiles manufacturing, medicine, and agriculture (Yalpani, 1987). Considerable effort has been devoted to their full characterisation from the physico-chemical and the macromolecular view-point.

In addition, the increasing attention to technological polymers which actually exhibit biodegradability and biocompatibility is worth mentioning. In this context polysaccharides can be conveniently exploited because their biodegradation results in environmentally friendly molecules (monosaccharides, water, carbon dioxide). Furthermore, the variety of stereochemical motifs exhibited by polysaccharidic chains, which are produced from different local configurations and different types of glycosidic linkage, leads to different solution properties and interactions with substrates which are very interesting for specific applications.

In the framework of a research project aimed at the production of different polysaccharide derivatives, some *O*-carboxymethylagar was prepared for the purpose of investigating its ability to interact with different divalent ions.

EXPERIMENTAL

Synthesis of CMAgar

Commercial Agar (DIFCO, lot no. 0140-01) (5 g) was suspended in 130 ml of 2-propanol in a 300 ml mortar. NaOH (13 ml of 30% w/w solution) was added by drops in 30 min while grinding the mixture. The reaction mixture underwent vigorous grinding for 1 h until a yellow cream was obtained. At this point, 6 g of chloroacetic acid was added by portion over 30 min; the suspension was then transferred to a 500 ml flask and heated for 4 h at 55°C under reflux. The solid obtained was washed with 70% methanol and neutralized with glacial acetic acid. After decanting, the product was thoroughly washed with 70% methanol. The solid was

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*Author to whom correspondence should be addressed.

dissolved in one liter of water resulting in a slightly viscous, yellowish solution which was purified by means of filtration through a sintered glass filter and reprecipitation in 600 ml of 1:3 2-propanol/water solution. The colourless polymer was washed with 75% 2-propanol, solubilized in 500 ml of water, dialyzed and freeze dried. CMAgar (3·75 g) was obtained with a molar yield of 57%.

Characterization

The degree of substitution was determined by means of a potentiometric titration of the polymer previously dialyzed against 0·02 M HCl and lyophilized in order to obtain the carboxylic group in acidic form. A digital Radiometer PHM 52 pH-meter equipped with a glass electrode was used to follow the titration with standard 0·1 M NaOH. UV spectra were recorded by means of a VARIAN CARY 2200 UV-VIS spectrometer. CD measurements were obtained by means of a JASCO 6000 spectropolarimeter. NMR spectra were obtained by using a BRUKER AM 300 WB spectrometer equipped with a multinuclear 10-mm probe. Quantitative ^{13}C NMR spectra were obtained by means of inverse gated decoupling using a relaxation delay of 4 sec at 353 K. The determination of the water content in lyophilized samples was carried out with a METTLER Model DL 18 automatic Karl–Fisher titrator.

Molecular weight measurements were performed using a low-angle laser light scattering technique (LDC-Chromatix CMX-100 equipped with He–Ne laser) on solutions eluted through gel permeation chromatography columns. A WATERS 410 differential refractometer was also used in order to evaluate the total mass of the eluted polymer fractions. The columns for HPGPC were WATERS μBONDAGEL E-High A°, E-1000, E-500, E-125; Millipore FHUP filters with 0·5 μm pore size were used to clean polymer solutions before injection.

Polarographic data were obtain by means of a three-electrode differential pulse technique (a dropping mercury electrode, a platinum counter-electrode and a reference saturated calomel electrode connected to the solution through a salt bridge). The experimental apparatus used was purchased from Princeton Applied Research Corp. (Electrochemical System model 170). Calorimetric data were obtained by means of a batch LKB 10700 microcalorimeter, and isothermal mixing enthalpies were measured at 25°C.

RESULTS

Carboxymethyl groups were inserted onto the agar backbone according to the same standard procedures as those used for the synthesis of carboxymethylcellulose (McLaughlin & Herbst, 1950*a*, *b*). Since some agar fractions can contain a considerable amount of methoxyl groups, which can mask hydroxyl groups for carboxymethylation, a commercial sample exhibiting a low degree of both methoxylation and sulfation was selected by means of NMR analysis. After synthesis, the degree of substitution was checked by means of potentiometric titration, and the resulting value was 1·2 carboxymethyl groups per agar disaccharide repeating unit. The repeating structure of the polycarboxylate obtained is reported in Fig. 1. The polymer molecular weight was affected by the reaction. Actually, the carboxylated polysaccharide weight average molecular weight was found to be 117 000 whereas that relative to the native agar sample is 215 000; nevertheless, this reduction does not affect the macromolecular nature of the compound obtained.

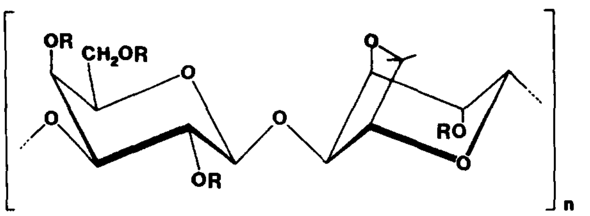

Fig. 1. *O*-carboxymethylagar repeating structure. R indicates the positions where carboxymethyl groups can occur.

The characterization of the polymer was carried out by means of circular dichroism (CD) and NMR spectroscopies. CD spectra clearly demonstrated the presence of dichroic bands, in the 190–220 μm wavelength range, due to carboxyl groups. The dichroic absorption showed only very little spectral change as a function of either temperature or ionic strength, so that there was no indication of conformational transitions which might suggest the presence of ordered secondary structures. The presence of new signals was clearly indicated by ^{13}C NMR spectra, with respect to the agar ^{13}C spectrum, due to the carboxymethyl groups added (Fig. 2). Three signals due to carboxyl residues were present at 177 ppm; other signals, due either to anomeric or to ring carbon atoms, were differently affected by the presence of the carboxymethyl groups. NMR experiments carried out by using a DEPT pulse sequence allow the identification of the peak due to the inserted methylene groups at 71 ppm. From the quantitative ^{13}C NMR spectra it is possible to establish that the ratio of the area of the peak at 71 ppm to that of the peak at 102 ppm, which corresponds to the signal of the D-galactose anomeric carbon atom, is 1·3. This value is practically identical to the degree of substitution (1·2) obtained by means of potentiometric titration. As a consequence, it may be deduced that all CH_2 carbon atoms belonging to carboxymethyl groups resonate at 71 ppm, independent from their location on the glycosidic ring. On the other hand, the ^{13}C and ^{1}H NMR spectra were both so complex that it was not possible to

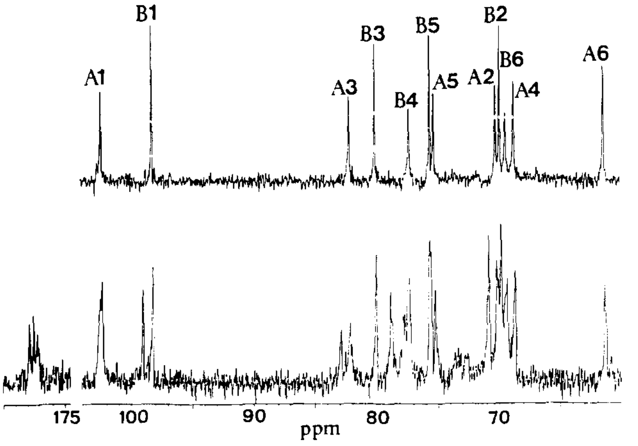

Fig. 2. NMR spectra of ^{13}C of native agar (top trace), and CMAgar. A = D-Galactose carbon atoms; B = 3,6 Anhydro-L-galactose carbon atoms.

clarify the pattern of substitution on the polysaccharidic backbone.

Different divalent ions were tested for CMAgar interaction by means of polarographic measurements (Manzini *et al.*, 1984; Reisenhofer *et al.*, 1984; Bosco *et al.*, 1993). The experiments were carried out at constant ion concentration upon the addition of increasing amounts of CMAgar. Data were reported as the fraction of bound ions as a function of the carboxymethylagar added (Fig. 3). Lead ions showed the highest affinity to CMAgar. In fact, the amount of bound lead ions rapidly increased upon the addition of CMAgar. Copper ions also showed affinity for CMAgar, but to a lesser extent than Pb(II). Other divalent cations (Cd(II), Co(II), Mn(II), Ni(II), Zn(II)) showed, to differing extents, very little affinity for CMAgar. In Fig. 3, only the case of Ni(II) is reported for the sake of clarity.

Following the results of the polarographic measurements, the interaction of lead, copper, and nickel ions with CMAgar has been studied more extensively by means of polarography, CD, and isothermal microcalorimetry. Nickel has been chosen as representative of the low-affinity ions.

All the selected ions show UV-Vis absorption so that the interaction with an asymmetric matrix, such as CMAgar, produces an extrinsic Cotton effect detectable by means of CD spectroscopy. As expected from the polarographic results, nickel ions did not exhibit extrinsic circular dichroism. On the contrary, both copper and lead ions showed this effect. In particular, the addition of copper ions to a CMAgar solution resulted in a regularly increasing CD band centered at 241 nm (Fig. 4). Similar CD spectra have been reported in the literature for Cu(II)-alginate and Cu(II)-pectin systems (Thorn *et al.*, 1982). Lead ions showed a more complex behaviour (Fig. 5(a) and (b)): at low Pb(II) concentration a negative CD band appears at 220 nm, but at higher ion concentration this band stops increasing and a different positive band appears at 230 nm. Upon further addition of Pb(II) ions, this band increases its intensity until the polymer precipitation point is reached (0.2 mol of Pb(II) per equivalent of polymer).

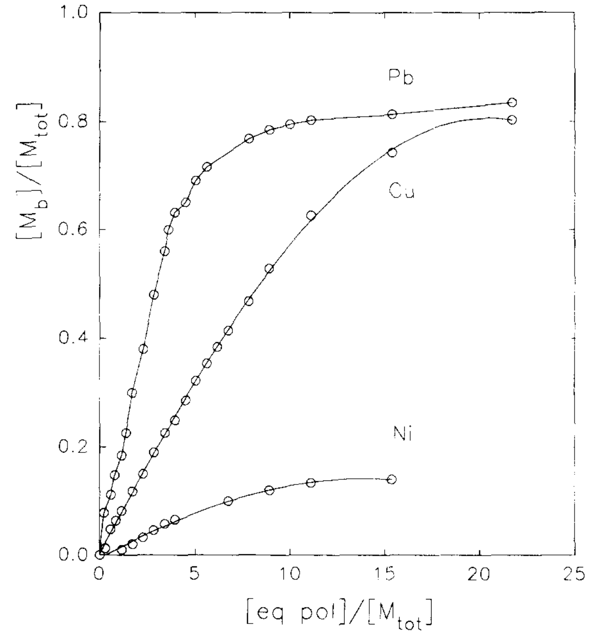

Fig. 3. Polarographic data reported as the fraction of bound divalent cations as a function of increasing concentration of CMAgar. $[M(II)]_{tot} = 1 \times 10^{-5}$ M; $[NaClO_4] = 0{\cdot}05$ M.

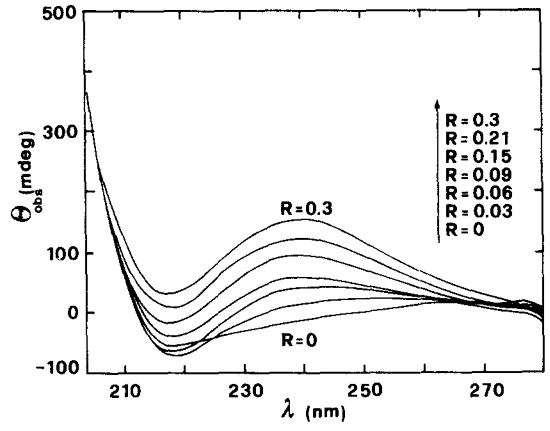

Fig. 4. CD spectra of Cu(II)/CMAgar system as a function of increasing Cu(II) ion concentration. $R = 0$–$0{\cdot}3$; where R is the total-[Pb]/polymer-equivalent ratio. $C_{pol} = 1 \times 10^{-3}$ N; $[NaClO_4] = 0{\cdot}05$ M.

Isothermal microcalorimetry experiments (Fig. 6) showed behaviour which parallels that obtained by means of CD spectroscopy. The mixing of Ni(II) with CMAgar produces a small endothermic effect, whereas addition of Cu(II) exhibits an endothermic effect which is almost three times more pronounced as compared with that of Ni(II). On the other hand, Pb(II) addition

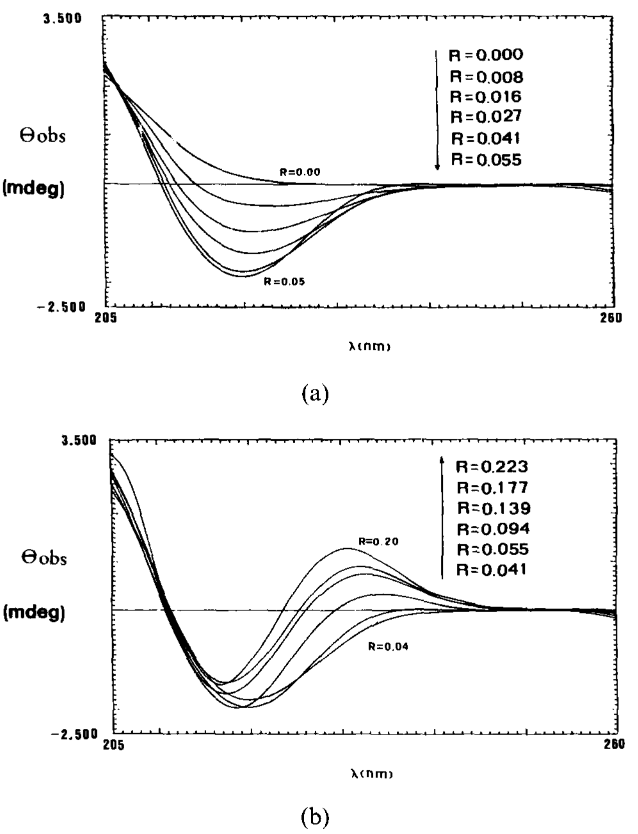

Fig. 5. CD spectra of Pb(II)/CMAgar system as a function of increasing Pb(II) ion concentration. (a) $R = 0$–$0{\cdot}05$; (b) $R = 0{\cdot}04$–$0{\cdot}2$; where R is the total-[Pb]/polymer-equivalent ratio. $C_{pol} = 1 \times 10^{-3}$ N; [NaClO$_4$] = 0·05 M.

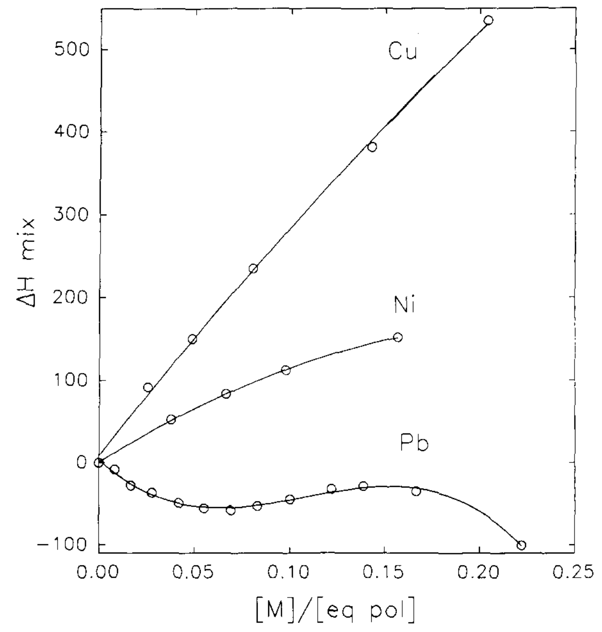

Fig. 6. Heat of mixing of Cu(II), Ni(II), and Pb(II) ions with CMAgar as a function of increasing divalent-cation concentration. $C_{pol} = 1 \times 10^{-3}$ N; [NaClO$_4$] = 0·05 M.

resulted in an exothermic effect. It is worth noting that, as in the CD experiments, the calorimetric behaviour of the system on the addition of lead ions to CMAgar solutions shows a bimodal trend. In fact, upon addition of increasing amounts of Pb(II) ions, an endothermic effect superimposes on the initial exothermic one.

Although CD spectroscopy and calorimetry are independent techniques which look at different aspects of the system under investigation, it is interesting to note that, in the case of lead ions, both techniques indicate that at an ion:polymer ratio of about 0·05 there is a change in the ion–polymer interaction (Fig. 7(b) and (c)). The same qualitative result was obtained by means of polarographic measurements where lead ions were added to a CMAgar solution (Figure 7(a)). In this figure, the polarographic current, which is proportional to the number of ions which are reduced at the dropping Hg-electrode, is reported both in the presence and in the absence of the polymer. At low Pb(II) concentration, polarographic data obtained in the presence of CMAgar, show a significant deviation from those relative to free ions. However, at higher Pb(II) concentration, the polarographic behaviour of the Pb–CMAgar system increasingly parallels that obtained in the absence of CMAgar.

DISCUSSION

The synthesis of carboxymethylagar was performed with a very simple procedure which does not greatly affect the macromolecular nature of the starting material. The introduction of hydrophilic carboxymethyl groups onto the agar polysaccharidic chain modifies some polymer characteristics. As a matter of fact, in contrast to agar, CMAgar is very soluble even in cold water; in addition, CMAgar does not form gels, even in the presence of divalent cations which might promote the onset of 'junction zones' and eventually gel formation. However, the addition of divalent cations to CMAgar yields viscous solutions and ultimately produces polymer precipitation.

As already stated, the complexity of the NMR spectra does not allow an easy definition of the substitution pattern. The comparison of NMR spectra of the native and the substituted agar shows that the carboxymethylation reactions do not split significantly the 3,6-anhydrobridge; in this condition carboxymethyl groups can substitute four different hydroxyl groups: positions 6, 4, and 2 on the D-galactose residue and position 2′ on the 3,6 anhydro-L-galactose residue. Although on the basis of steric considerations one might assume that the primary hydroxyl group in position 6 would be favored, an attempt to evaluated qualitatively the degree of substitution does not confirm this hypothesis. This analysis carried out on ^{13}C NMR spectra on the basis of some line splitting induced by carboxymethyl groups on the vicinal ring carbon atoms, suggests the existence of a similar degree

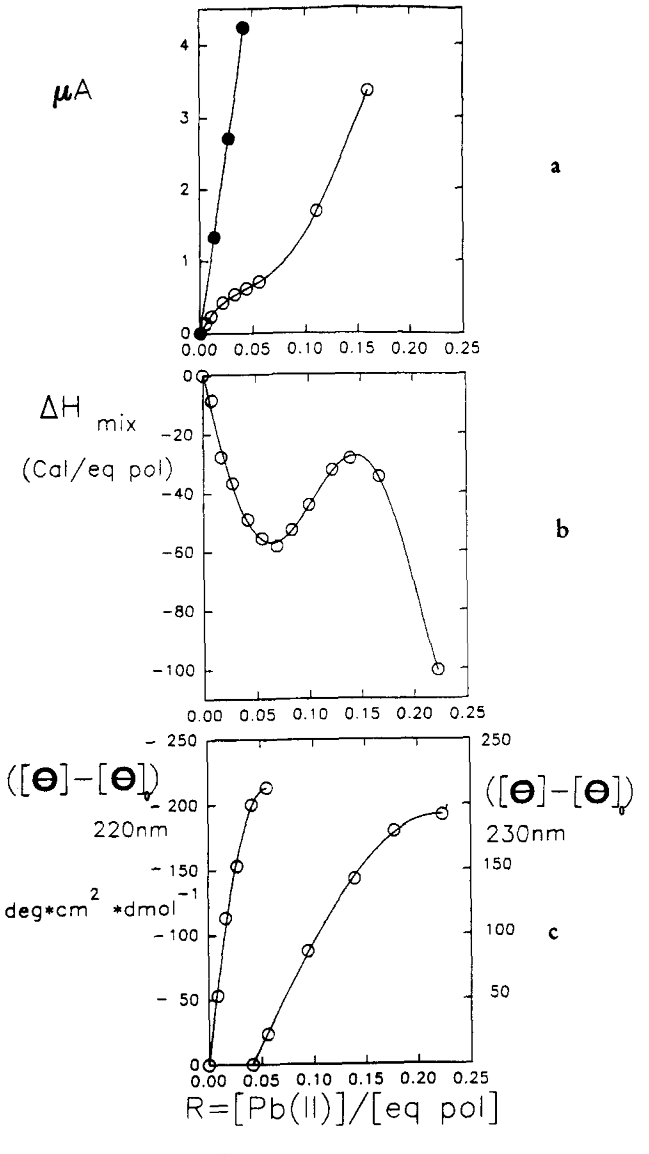

Fig. 7. Interaction of Pb(II) ions with CMAgar: experimental results as a function of increasing Pb(II) concentration (a) polarographic data: (●) in the absence of CMAgar; (○) in the presence of CMAgar; (b) microcalorimetric data; (c) CD data.

of substitution at carbons 6,2, and 2′. On the other hand, very little substitution is detectable at carbon 4, probably due to higher steric hindrance around this position. This result is confirmed by the presence of only three major ^{13}C peaks in the region of carboxylic carbon atom resonances. It is probably this rather random substitution which inhibits the formation of regular secondary structure on CMAgar backbone and prevents gel formation even in the presence of strongly interacting divalent cations.

The small variation which is detected on the carboxylate CD band as a function of the temperature is probably due to the local increase of the degree of freedom of carboxymethyl groups at high temperature. In other words, the spectral changes are due to local conformation changes rather than to a more dramatic long-range conformational transition.

Moving to interaction with ions, polarographic measurements clearly show different degrees of interaction of CMAgar in the presence of different ionic species. Taking into account only the electrostatic contribution to the binding energy, it is known from the theory of polyelectrolyte solutions (Manning, 1978) that monovalent and divalent counterions interact in different ways with polyelectrolyte chains. In fact, monovalent ions are condensed in the polyelectrolyte domain only if the polymer charge density parameter (ξ) is greater than a given value ($\xi = 1$); on the other hand, divalent cations are condensed at $\xi = 0{\cdot}5$. In addition (as shown by Paoletti *et al.*, 1991), in the presence of both monovalent and divalent counterions, condensation of both species starts at a ξ value between 0·5 and 1, depending, in a complicated way, on the ratio of monovalent to divalent ion concentrations, on ionic strength and on polymer concentration. In our experiments, where the ratio between the monovalent ions belonging to the buffer and the added divalent ions is about 50, the ξ value at which counterion condensation starts is very near to 1.

An evaluation of the charge density parameter for CMAgar, obtained using the carboxymethyl degree of substitution and the geometry of the agar backbone, as obtained from X-ray fiber diffraction (Foord & Atkins, 1989), gives $\xi \approx 0{\cdot}83$–$0{\cdot}88$. In this condition, taking into account only the electrostatic energy, either monovalent and divalent cations are not restricted in the polyelectrolyte domain, or the degree of counterion condensation is very low. Therefore, the observed polarographic data reflect the presence of specific interactions between some cations (Pb(II), and Cu(II)) and carboxylate groups which immobilize the ionic species in specific positions onto the polysaccharide backbone. As a matter of fact, polarographic measurements carried out by increasing the lead ion concentration at constant CMAgar concentration show a shift of the Pb(II) reduction potential from −0·371 volt (free ions) to −0·475 volt (bound ions).

The ion–polymer interactions are modulated by both the stereochemistry of the polysaccharidic chain and the geometrical requirements for complex formation. This behaviour can be discussed usefully by comparing different divalent cations. In fact, CD spectra of Pb(II) and Cu(II) in the presence of CMAgar exhibit extrinsic dichroic bands due to the perturbation induced by the chiral polysaccharidic matrix following complexation. On the other hand, Ni(II), which gives very little, if any, complexation, as detected from polarographic data, does not show any dichroic effect in the presence of CMAgar. In addition to this, Pb(II) ions show the complex two-band behaviour already described in the results.

The energetics of complex formation were measured by means of isothermal microcalorimetry. Ni(II) ions exhibit a low positive enthalpy of mixing with CMAgar which essentially reflects the contribution of electrostatic interactions. Copper ions exhibit the usual large endothermic effect under mixing with polyanions, and this clearly suggests that copper–polyanion interactions are entropy-driven, because of the release of a large number of hydration water molecules. On the other hand, the enthalpy of mixing of lead ions with CMAgar is negative, and the calorimetric curve obtained as a function of Pb(II) addition shows a peculiar trend. At low Pb(II) concentration the effect is exothermic, as already obtained with Pb(II) in the presence of different polyuronates (Cesàro *et al.*, 1988). However, at higher Pb(II) concentrations an endothermic effect superimposes to the exothermic one. The subsequent exothermic effect at the highest Pb(II) concentration is probably due to the formation of an insoluble complex at high Pb(II) concentration. A qualitative explanation of the initial exothermic effect can be obtained by taking into account that the carboxymethyl degree of substitution (DS = 1·2) implies that 20% of the agar repeating units bear two carboxymethyl groups. Consequently, some of these double substituted units can have the two carboxylate groups in favorable positions to form a specific chelation complex with lead ions. On the other hand, the endothermic effect reflects a different complex formation in all the remaining cases where carboxymethyl groups, located on the repeating unit, exhibit different geometries.

The fact that, at low Pb(II) concentration, the formation of the lead–CMAgar complex is completed at a given total-Pb(II)/polymer ratio can be seen by comparison of the polarographic, CD, and calorimetry data shown in Fig. 7. In all three independent experiments the different modes of complexation of Pb(II) ions can be easily detected and the switch is evident at about 0·05 total-Pb(II)/polymer ratio. In addition, polarographic data exhibit a co-operative behaviour which will be further investigated and discussed in a separate paper.

Although an estimate of the stoichiometry of the lead ion–polymer binding sites is difficult to obtain from the initial slopes relative to the curves in Fig. 7, some commentary is possible, bearing in mind the speculative character of such comments.

At low total Pb(II) ion-to-polymer ratio the probability that a single Pb(II) ion is bound by a pair of double-substituted disaccharadic repeating units is high. In this situation four carboxylic groups complex the cation and, in addition, it is assumed that each pair of carboxymethyl groups belongs to a specific substitution pattern on the disaccharidic unit out of the 20% of units which bear two carboxymethyl groups.

The stoichiometry of the interaction can be tentatively obtained from Fig. 7(c) using the initial slope of the CD curve relative to the intensity of the band at 220 nm. In this case, the value obtained is about 0·03 lead ions per equivalent of polymer, which corresponds to 0·036 lead ions per disaccharidic agar repeating unit.

Taking into account now the carboxymethyl substitution degree (1·2), the ratio of repeating units bearing two carboxymethyl groups to the total repeating unit is 0·2, but only 1/3 of these (0·067) bear the two carboxymethyl groups in the required specific position. If the assumed 2:1 complexation is now introduced, the above ratio has to be divided by 2, giving 0·033 binding sites per repeating unit. This value is noticeably similar to 0·036 which was the number of bound lead ions per repeating unit as evaluated on the basis of the experimental CD data.

Even taking into account the speculative character of the above considerations, the coincidence of the obtained figures can almost certainly support the model depicted for lead ion interactions with partially substituted carboxymethylagar.

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