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CHARACTERISATION OF CATION BINDING AND GELATION OF POLYURONATES BY CIRCULAR DICHROISM

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ABSTRACT

The cation-induced gelation of alginates and pectins with various metal ions has been monitored by circular dichroism (c.d.), using a controlled diffusion technique to prepare homogeneous gels *in situ*. Spectral changes observed with Ca^{2+} are closely similar to those previously reported for Ca^{2+}-induced dimerisation of alginate poly-L-guluronate and pectin poly-D-galacturonate chain-sequences in solution, and the magnitude of the c.d. change on gel formation is directly related to the proportion of these structural types present. It therefore appears that gel formation does not introduce optical artefacts such as have been reported for particulate systems or biological membranes. Similar spectral changes are observed on gelation of pectin with Sr^{2+}, Ba^{2+}, Cd^{2+}, Ni^{2+}, or Pb^{2+}, but with minor alterations in the wavelength of maximum c.d. change. These subtle differences are interpreted as reflecting variation in binding-site geometry to accommodate ions of different size. Differences in c.d. behaviour with Mg^{2+}, Ca^{2+}, and Sr^{2+} are far greater for alginate than for pectin, consistent with the greater selectivity of ion-binding. Gelation of both alginate and pectin with Cu^{2+} is accompanied by spectral changes that are opposite in sign to those observed with other divalent cations, and span a much wider range of wavelengths. This suggests a different and less-specific binding mechanism, consistent with the known lack of selectivity of Cu^{2+} for different polyuronates. However, for alginate, there is also evidence of some specific interchain chelation. A minor enhancement of alginate c.d. in the presence of K^{+} ions is attributed to a decrease in charge density of the polymer chain by bound cations, with consequent increase in segment–segment association in solution. The sign and magnitude of this effect confirm the selectivity of polyuronates for divalent cations.

INTRODUCTION

Circular dichroism (c.d.) and u.v. absorption examine the same electronic transitions. However, whereas the latter method uses natural light, c.d. measurements involve a form of electromagnetic radiation (circularly polarised light) in which the intensity of the electric and magnetic fields remains constant, but the direction changes continuously by rotation about the axis of propagation. Depending on the sense of

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rotation, circularly polarised light may be either left-handed or right-handed, and c.d. is the difference in absorption of the two[1]. Dissymmetric molecules (*i.e.*, those which are not superimposable on their own mirror image) will, in general, absorb left- and right-handed waves to a different extent, and therefore show c.d. activity.

Because of this sensitivity to molecular geometry, c.d. can be used to investigate directly the conformation of biological macromolecules, and has been widely applied to proteins and polynucleotides, where the polymer backbone is composed of subunits which absorb light in a readily accessible region of the spectrum[2,3]. The corresponding backbone-transitions of polysaccharides[4], by contrast, lie below the lower wavelength limit of current, commercial c.d. instruments, and until comparatively recently it was possible to study their optical activity only indirectly[5,6], by the related technique of optical rotatory dispersion which measures the difference in refractive index of left and right circularly polarised light, rather than the difference in absorption[4]. Vacuum c.d. spectrometers[7] capable of penetration into the far-u.v. have been constructed, and the backbone transitions of several polysaccharides have been examined by direct c.d. measurement[8–14]. No commercial, vacuum u.v.-c.d. equipment has yet been developed and, for the moment, it remains a specialist technique.

The principal applications of c.d. to carbohydrates have centred[4] on the optical activity of substituent chromophores that absorb at wavelengths higher than those of the polymer backbone. Acetamido groups show strong optical activity which has been exploited in studies[15–22] of carbohydrate monomers, oligomers, and polymers. Although weaker, optically active, electronic transitions of carboxyl groups are also easily accessible on modern c.d. instruments. The c.d. of uronic acids, salts, and esters has proved particularly informative[23–32]. In the undissociated acid form, D-uronic acid monomers and glycosides are characterised[25] by a positive c.d. band at ~212 nm, assigned to the carboxyl $n \rightarrow \pi^*$ transition, with a corresponding negative band for the L series. When O-4 is equatorial (as in glucuronic and mannuronic acid), a smaller band of opposite sign is also observed at higher wavelength, and is attributed[23–25] to the $n \rightarrow \pi^*$ transition of a different rotational isomer about the C-5–C-6 bond. Salt spectra[25] display the same general features, but the band of longer wavelength is relatively more intense and, for α-glycosides of glucuronate and mannuronate, it is the principal spectral feature above 200 nm. For galacturonate[28], the c.d. behaviour of the methyl ester is the same as that of the undissociated acid, and the same may well be true of other uronic acids, although this has yet to be demonstrated experimentally.

Differences in the c.d. of various uronic acids and their derivatives have been exploited in, for example, characterisation[28] of the degree of esterification and extent of dissociation of pectin, which is based[33] on a (1→4)-linked α-D-galacturonate backbone, and the composition[25,31] of alginate, a block co-polymer of α-L-guluronate and β-D-mannuronate[34,35], with residues arranged[36–38] in homopolymeric sequences of both types, and in heteropolymeric mixed-sequences that approximate[38] to an alternating structure. Although the major spectral features of uronic acid polymers are similar to those of the component monomers[25], there are also subtle dependencies

on residue sequence, which have been exploited[31] to develop a non-destructive c.d. method for determination of alginate block-structure. Since this method requires < 10 mg of material, it is particularly convenient for biological studies[32].

Since our first report[26] on the sensitivity of polyuronate c.d. to the presence of site-bound cations, the approach has been widely exploited. For example, Kohn has used c.d.[39], in conjunction with measurements of ion activity[40], to distinguish between co-operative and "atmospheric" binding of Ca^{2+} to polyuronates of different chain-length, Beaven has used the technique in studies[41] of the conformation and biological activity of polyacetal carboxylic acids, and large c.d. changes on Ca^{2+} binding to heparin have been reported[42,43].

Recently, we used c.d., together with equilibrium-dialysis studies of the stoichiometry of cation binding, to demonstrate that the primary mechanism of Ca^{2+}-induced interchain association in both alginate[29] and pectin[30] is by dimerisation of homopolymeric chain-sequences (poly-L-guluronate and poly-D-galacturonate, respectively). In both cases, the participating chain-sequences adopt regular, two-

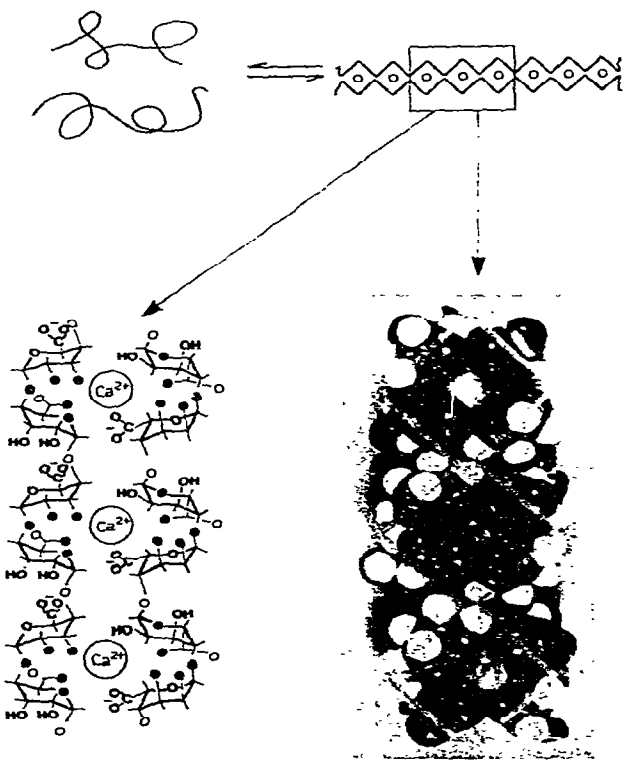

Fig. 1. The "egg-box" model[27] for Ca^{2+}-induced dimerisation[29] of poly-L-guluronate. Oxygen atoms involved in cation chelation are shown (bottom left) as filled circles. Interchain packing of the array of bound ions is illustrated in the CPK space-filling model (bottom right), and chain contours are traced to show the regular, buckled, two-fold conformation. A closely analogous structure has been reported[30] for calcium poly-D-galacturonate.

fold symmetry, with calcium ions sandwiched within the dimer, on specific binding sites along each of the interior surfaces. This process ("egg-box" binding[27]) is illustrated in Fig. 1.

In addition to these "structure-forming" sequences, both polymers contain solubilising features, homopolymeric and heteropolymeric sequences involving D-mannuronate in alginate[36,37], and esterified galacturonate residues and (1→2)-linked L-rhamnosyl insertions in the pectin backbone[33]. These structural features limit the extent of cation-induced interchain association, and contribute to the formation of a hydrated, three-dimensional network, rather than an insoluble precipitate[44–46]. While binding of calcium has been most widely characterised and exploited, both alginate and pectin show specific affinities for other cations[39,40,47–56]. Indeed, the selective uptake of heavy-metal ions by alginate-rich marine algae (Phaeophyceae) has been developed[57,58] as an index of pollution levels.

In previous studies[29,30], we used c.d. to characterise Ca^{2+}-induced association of polyuronate chains in solution, using either intact alginate or pectin at non-gelling concentrations, or blocks of a single structural type (poly-D-galacturonate, poly-L-guluronate, poly-D-mannuronate, and heteropolymeric mixed-sequences from alginate) isolated from the parent molecule by controlled hydrolysis. We now report on an extension of these studies to investigate the scope of c.d. (*a*) to characterise cation-induced, interchain association in polyuronate gels, and (*b*) to monitor and distinguish the interactions of polyuronate chains with various metal ions. In systems where cation-induced gelation occurs, we have used controlled diffusion into the optical cell to prepare homogeneous gels *in situ*, and to follow the progress of interchain association and cation binding. Preliminary accounts of parts of this work have been published[26,27,46].

EXPERIMENTAL

Materials. — Three commercial samples of alginate (SS/DJ, F347, and F387) from Alginate Industries Ltd. were used. A solution of each sample was dialysed extensively against deionised water, accurately neutralised, filtered, and freeze-dried before use. The botanical origin of these materials, and their block composition (% of polyguluronate:% of mixed sequences:% of polymannuronate) from c.d. analysis[31] were as follows: *Laminaria hyperborea* stipes (64:14:22); *L. hyperborea* (43:34:23); and *Ascophyllum nodosum* (21:41:38). Commercial samples of pectin (Bulmers, citrus slow-set pectin; and Firmagel, type 155) were reprecipitated from aqueous solution with ethanol, washed with acidified, 60% aqueous ethanol (5% v/v HCl), dialysed extensively against deionised water, neutralised, and freeze-dried. The degree of esterification of these samples (% of galacturonate residues which are methyl-esterified), as determined by i.r. analysis[59], was 26% and 61%, respectively. Pectic acid was prepared from the former material by alkaline de-esterification (NaOH, pH 12; 1°). Absolute concentrations were determined by elemental analysis (Butterworth Microanalytical Consultancy Ltd.).

Methods. — C.d. measurements were made at 25° with a Cary 61 c.d. Spectropolarimeter, using an integration period of 10 s, 5-mm pathlength, and a polysaccharide concentration of ~0.5% w/v. Gels were formed *in situ* by stretching a dialysis membrane across the neck of the c.d. cell (taking care to exclude air bubbles) and immersing in a large excess (5 litres) of the appropriate cation as the chloride salt (10mM). Spectra were recorded periodically until no further c.d. change could be detected (typically, 15–20 days), or until the experiment was terminated because of precipitation of the sample or loss of transmission due to turbidity. During all operations, the cell was handled as gently as possible to avoid disruption of network structure.

RESULTS AND DISCUSSION

Ca^{2+}-Induced gelation of alginates and pectins. — Fig. 2 shows the progressive alteration in c.d. which accompanies gelation on controlled diffusion of calcium ions

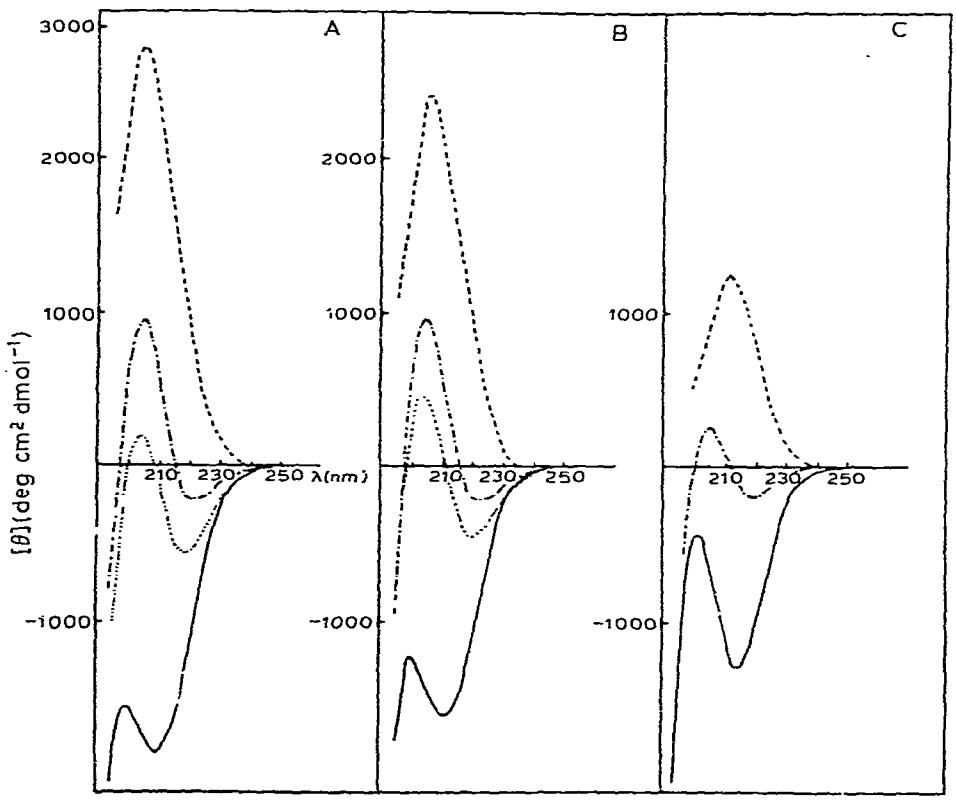

Fig. 2. C.d. changes which accompany the Ca^{2+}-induced gelation of sodium alginate from A, *Laminaria hyperborea* stipes (64% of poly-L-guluronate); B, *Laminaria hyperborea* (43% of poly-L-guluronate), and C, *Ascophyllum nodosum* (21% of poly-L-guluronate). Spectra are shown for solutions (———) and for gels at intermediate (- - - - - -) and final (—·—·—·—) stages of gelation. Difference spectra (— — — —) were obtained by subtraction of solution spectra from final-gel spectra.

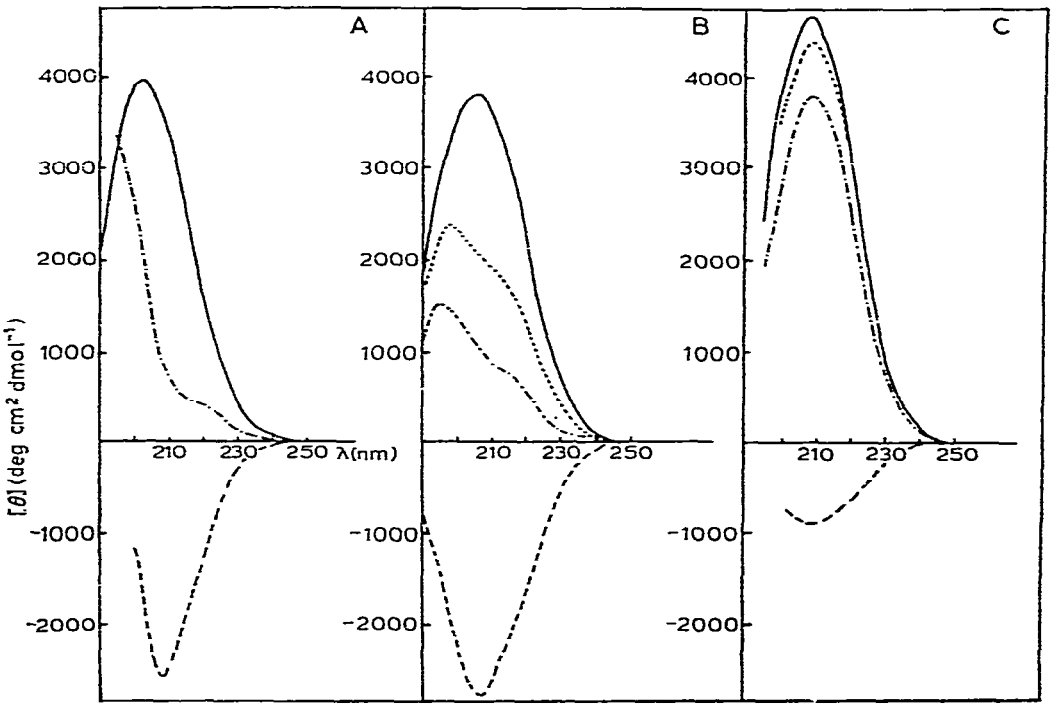

Fig. 3. C.d. changes on Ca^{2+}-induced gelation of A, pectic acid; B, "low methoxy" pectin (26% of galacturonate residues methyl-esterified); and C, "high methoxy" pectin (61% methyl-esterified). Spectra are shown for solutions in the Na^{+} salt form (———), and for intermediate (-------) and final (–·–·–·–) stages of gelation. Difference spectra (– – – –) show the overall c.d. change (final-gel c.d. minus initial-solution c.d.).

into solutions of the three alginate samples studied. Although the observed spectra are complex in form, considerable simplification may be achieved by calculation of the spectral changes (*i.e.*, gel c.d. minus solution c.d.). "Difference spectra" derived in this way approximate closely to a Gaussian band form, centred at ~210 nm, and are very similar in both position and bandwidth to those observed previously[29] on slow addition of Ca^{2+} to poly-L-guluronate blocks, and non-gelling concentrations of intact polymer. It therefore appears that the formation of a gel network does not introduce optical artefacts of the type found in scattering suspensions[60], biological membranes[61], or solid films[62]. The magnitude of spectral change (Fig. 2) shows a progressive decrease with decreasing poly-L-guluronate content of the alginate, confirming previous evidence[54–56] that these sequences are primarily responsible for the interchain associations leading to gel formation.

The c.d. changes which accompany Ca^{2+}-induced gelation of pectins (Fig. 3) are also closely similar to those observed[30] in solutions of poly-D-galacturonate, again confirming the validity of applying c.d. measurements to clear gels. The magnitude of c.d. change observed for the pectin sample having a high content of ester (61% of galacturonate residues esterified) is appreciably smaller than for the

material with only 26% esterification. However, no further increase is observed on going to pectic acid, suggesting some limited tolerance for esterified residues within the "egg-box" structure. This behaviour will be explored in detail in a future publication[63].

Difference spectra for pectic acid and pectin having a low content of ester are virtually identical, both qualitatively and quantitatively, to those observed for alginate having a high content of poly-L-guluronate, but opposite in sign. This is fully consistent[4] with a closely analogous geometry of Ca^{2+} chelation, but in the D and L sugar series, respectively. This evident similarity in the detailed structure of interchain association is reflected in the similarity in mechanical properties of the resulting gels[54].

Gelation of pectin with other divalent cations. — As discussed above, the c.d. changes observed on Ca^{2+}-induced gelation of alginates and pectins are approximately Gaussian in form, and have been attributed[14,26] to specific perturbation of carboxyl n

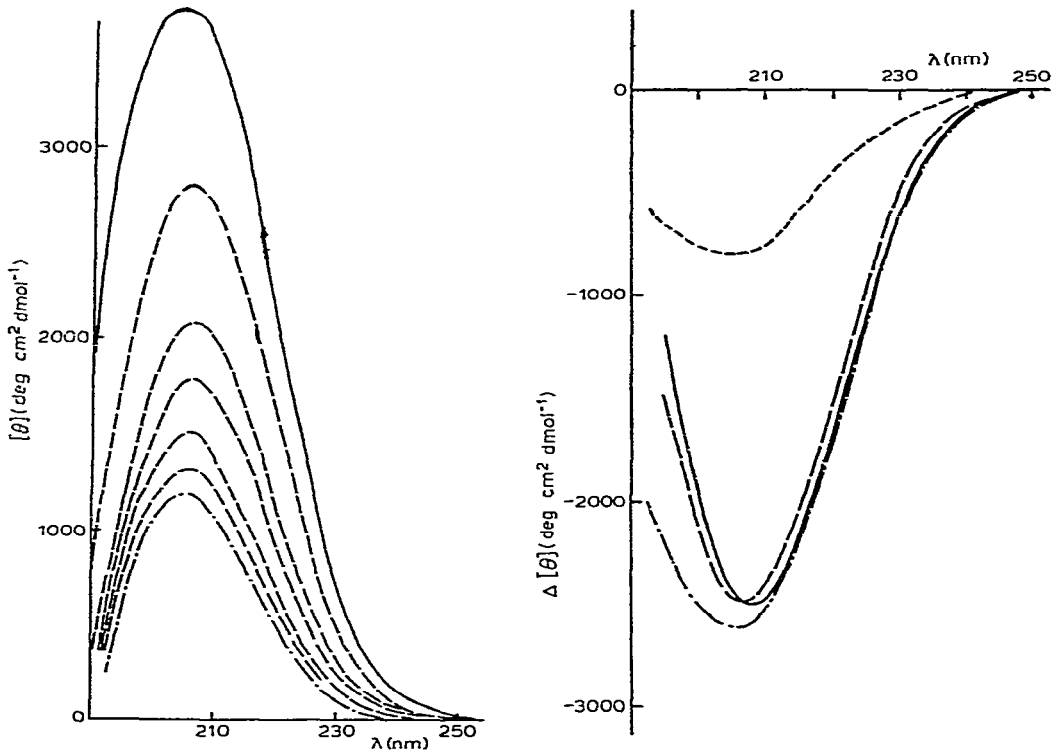

Fig. 4. Progressive c.d. change on gelation of pectin with Group II metal ions, illustrated for dialysis of "low methoxy" pectin (26% methyl-esterified; Na^+ salt form) against barium chloride (10mM). Spectra are shown for the initial solution (———), final gel (–·–·–·–), and intermediate stages of gelation (– – – – –).

Fig. 5. "Difference spectra" (final-gel c.d. minus initial-solution c.d.) for gelation of "low methoxy" pectin (26% methyl-esterified; Na^+ salt form) with Mg^{2+} (– – – – – –), Ca^{2+} (———), Sr^{2+} (– – – – –), and Ba^{2+} (–·–·–·–).

electrons by the proximity of site-bound cations within the "egg-box" structure (Fig. 1). Similar c.d. changes, confined to the carboxyl n → π* spectral region[23-25], are observed on diffusion of other Group II cations into solutions of sodium pectate, as illustrated in Fig. 4 for gelation of the pectin sample of low ester content with Ba^{2+} ions. With Mg^{2+}, the spectral changes (Fig. 5) are relatively small, consistent with the observation that magnesium does not readily cause gelation or precipitation of pectin, even at substantial concentrations of cation. For the larger cations of Group II, the difference spectra are similar in magnitude, but show a progressive shift to lower wavelength through the series Ca^{2+}, Sr^{2+}, Ba^{2+}. These differences may reflect subtle changes in binding-site geometry[27] in accommodating species of progressively larger ionic radius. The over-riding similarities, however, argue for a closely related mechanism of "egg-box" binding in each case.

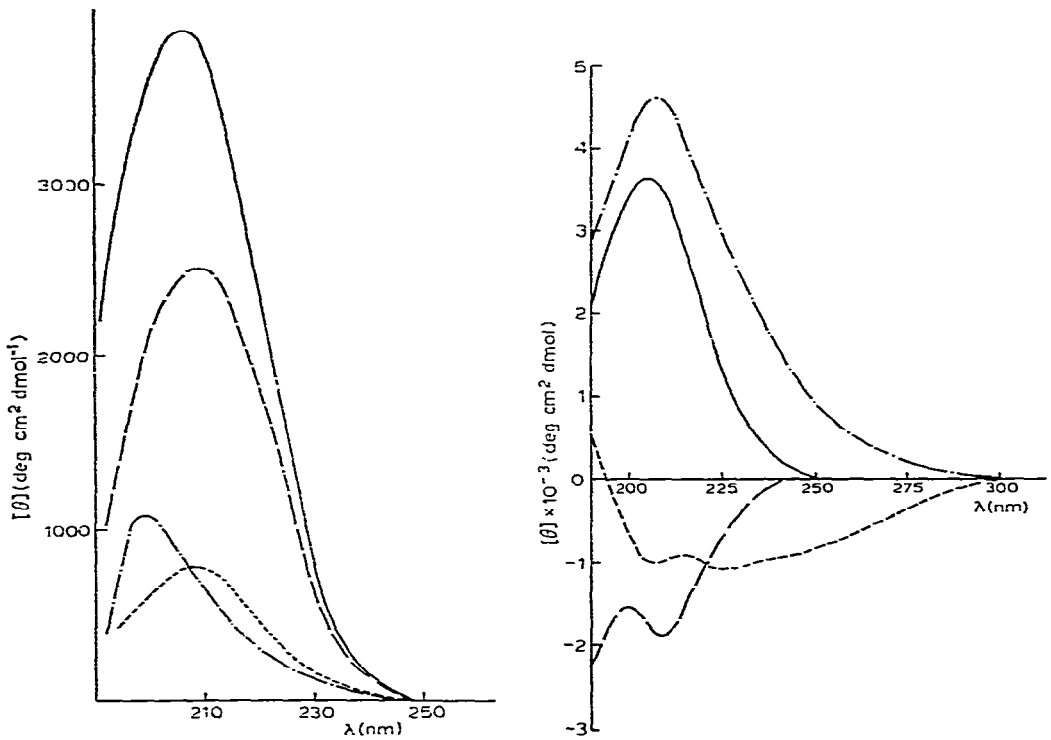

Fig. 6. C.d. changes on gelation of pectin with divalent transition-metal ions. Spectra are shown for "low methoxy" pectin (26% methyl-esterified) in the initial solution (Na^+) form (———) and in the final-gel state with Ni^{2+} (—·—·—) and Cd^{2+} (- - - - -). With Pb^{2+} (— — —), the gel became turbid at an early stage, and no further c.d. measurements were possible. Spectral changes up to this point, however, are similar to those observed (Figs. 4 and 5) with Group II cations.

Fig. 7. C.d. changes which accompany the gelation of alginate and pectin with Cu^{2+} ions. Spectra are shown for "low methoxy" pectin (26% methyl-esterified) in the Na^+ solution (———) and Cu^{2+} gel (—·—·—) forms, and for alginate from *Laminaria hyperborea* stipes in the Na^+ solution (— — —) and Cu^{2+} gel (- - - - -) forms. In both cases, the gel network collapsed at an early stage of dialysis, to give a "stringy" precipitate, and no further measurements could be made.

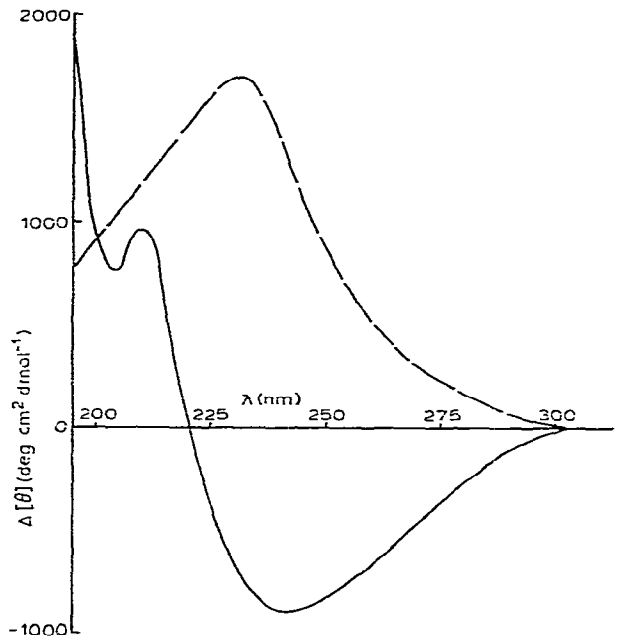

Fig. 8. "Difference spectra" (gel c.d. minus solution c.d.) for the Cu^{2+}-induced gelation (Fig. 7) of alginate from *Laminaria hyperborea* stipes (———) and "low methoxy" pectin (– – – –).

Fig. 6 shows the c.d. changes observed on gelation of pectin with some divalent transition-metal ions. As in the case of the alkaline earths, the spectral changes are confined to the n → π* transition region, but again show significant variation in wavelength. For Pb^{2+} ions, the gels became turbid at an early stage, and no further c.d. measurements were possible. We attribute this behaviour to extensive dimer–dimer aggregation, with consequent collapse in the gel network. This interpretation is supported by the ability of Pb^{2+} to induce gelation of pectin at low concentrations of cation, and by a tendency to subsequent precipitation.

Gelation of alginate and pectin with Cu^{2+}. — The c.d. changes observed (Fig. 7) on diffusion of Cu^{2+} ions into solutions of sodium alginate or pectate are entirely different from those with other divalent cations. Difference spectra (Fig. 8) are predominantly negative for alginate and positive for pectate, *i.e.*, opposite in sign to those for "egg-box" binding of Ca^{2+} and related ions, and extend far beyond the n → π* region, up to ~300 nm. In both cases, the major change is centred at ~235 nm, and the bandwidth of the difference spectra is considerably greater than for the other ions studied.

It seems evident from these results that binding of Cu^{2+} ions to polyuronate chains occurs predominantly by a mechanism other than that outlined in Fig. 1. In particular, the wide range of wavelengths over which spectral change is observed suggests that the steric arrangement of bound cations relative to the carboxyl chromophores is far less specific than in the "egg-box" structure. This conclusion parallels

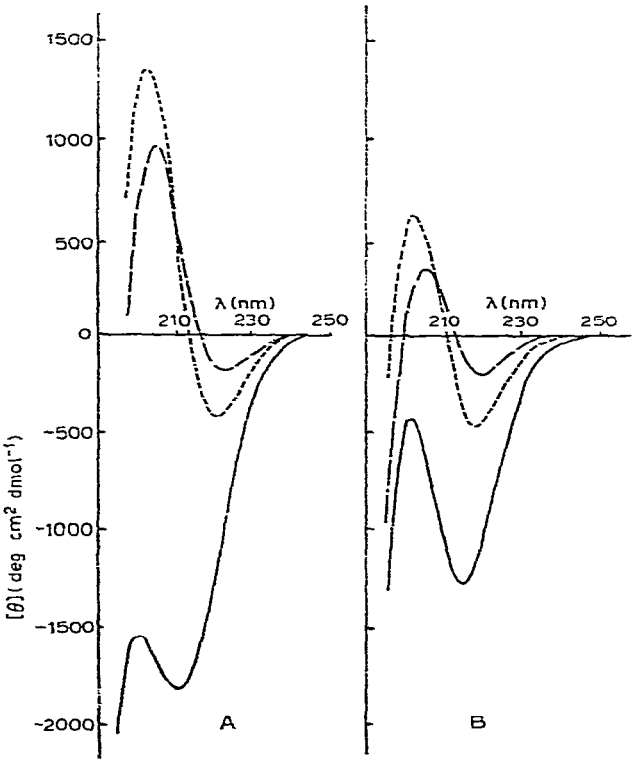

Fig. 9. Comparison of c.d. changes on gelation of alginate with Ca^{2+} and Sr^{2+}. Spectra are shown for the initial (Na^+) solution (———) and final gel state with Ca^{2+} (– – – –) and Sr^{2+} (- - - - - -) for alginate from A, *Laminaria hyperborea* stipes (64% of poly-L-guluronate) and B, *Ascophyllum nodosum* (21% of poly-L-guluronate).

previous evidence from cation-binding studies[50] where Cu^{2+}, in contrast to other divalent cations, showed little selectivity in its interactions with different polyuronates. The c.d. difference spectrum for alginate, however, shows (Fig. 8) a sharp, positive band superimposed on the broad, negative envelope. This band is similar in position and width to the difference spectra observed with Ca^{2+} (Fig. 2), suggesting that, in addition to the less-specific binding of Cu^{2+}, some formation of "egg-box" junctions also occurs.

With both alginate and pectin, the gel network structure collapsed at an early stage of the dialysis, to form a "stringy" precipitate, and no further c.d. measurements were possible. This behaviour is consistent with the very strong binding between cation and polyanion which is characteristic of the interactions of Cu^{2+} with carboxylate-containing polymers[51].

Comparison of Ca^{2+} and Sr^{2+} binding to alginate. — The poly-L-guluronate sequences of alginate are unique among the polyuronates which have been investigated[51], in showing a marked selectivity for Sr^{2+} in competition with Ca^{2+}. As shown in Fig. 9, this selectivity is reflected in an appreciably greater magnitude of

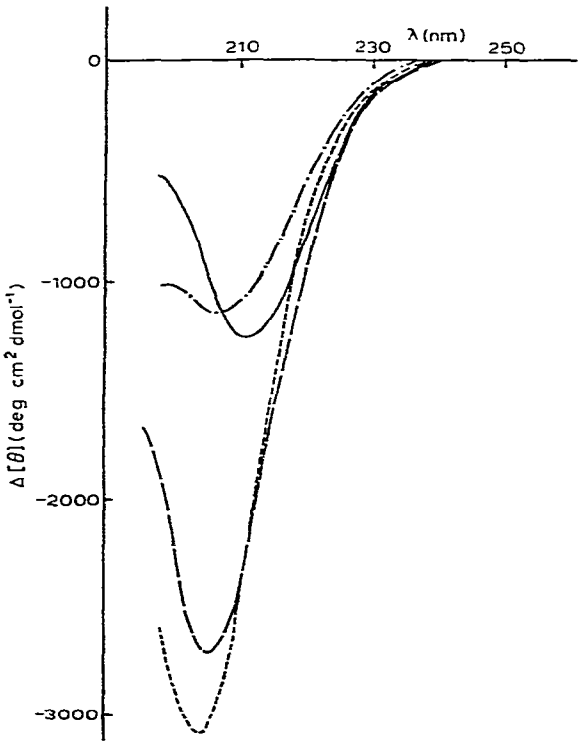

Fig. 10. "Difference spectra" (final-gel c.d. minus initial-solution c.d.) for gelation of alginate from *Ascophyllum nodosum* with Ca^{2+} (———) and with Sr^{2+} (–·–·–·–), and for gelation of alginate from *Laminaria hyperborea* stipes with Ca^{2+} (– – – –) and with Sr^{2+} (- - - - - -).

c.d. change on Sr^{2+}-induced gelation, in contrast to the behaviour which we have observed (Fig. 5) for pectin, where the only detectable difference is a slight shift in the wavelength of maximum c.d. change. A similar shift to lower wavelength with increasing ionic radius is also observed (Fig. 10) for alginate, and again we attribute this to a slight rearrangement of binding-site geometry.

Comparison of the magnitude of Ca^{2+}-induced c.d. change shown in Fig. 10 with previous results obtained for the same alginate samples in solution, under conditions where complete association of poly-L-guluronate chain sequences into dimeric junctions had been established[29], indicates that, under the gelling conditions used in the present work, part of the observed c.d. changes arises from subsequent dimer–dimer aggregation. A likely explanation of the further increase in c.d. change with Sr^{2+} is that these ions are even more effective in promoting the aggregation process.

Changes in alginate c.d. with non-gelling cations. — Fig. 11 shows the spectral changes observed on prolonged dialysis of sodium alginate against K^+ and Mg^{2+}, neither of which caused gelation under the conditions of polymer and salt concentration used. With Mg^{2+}, the c.d. changes are similar in form to those observed with Ca^{2+}, but very much smaller, suggesting limited formation of "egg-box" junctions,

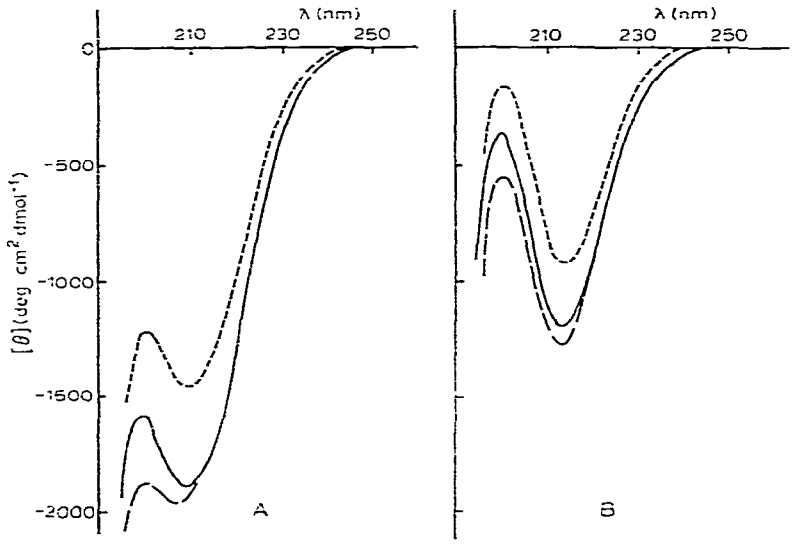

Fig. 11. Spectral changes observed on dialysis of sodium alginate from A, *Laminaria hyperborea* stipes and B, *Ascophyllum nodosum* against cations which do not promote gelation (10mM; chloride salt form). The spectra shown are for initial (Na^+) solutions (———), and on completion of c.d. change after extensive dialysis with K^+ (– – – –) and Mg^{2+} (-------).

but insufficient to lead to a cohesive cross-linked network. The magnitude of spectral change is even lower than that observed with pectin (Fig. 5), consistent with previous studies[51] of the relative affinity of Mg^{2+} for the two polymers.

With K^+, by contrast, a small increase in c.d. ellipticity is observed, which is similar in form and magnitude to the spectral changes that accompany the gelation of pectin under conditions of diminished water activity[64]. In the latter case, the c.d. enhancement is attributed to loss of conformational mobility of the polysaccharide chain by segment–segment interactions and associations. Current work in this Laboratory suggests that suppression of interchain electrostatic repulsions by cation binding may promote similar, if less extensive, segmental interactions in solutions of alginate, with consequent changes in c.d. intensity. The results of this work[65] will be published elsewhere.

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