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CHIROPTICAL AND STOICHIOMETRIC EVIDENCE OF A SPECIFIC, PRIMARY DIMERISATION PROCESS IN ALGINATE GELATION*

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ABSTRACT

The stoichiometry of calcium-ion chelation to alginate chains has been investigated by circular dichroism (c.d.), and by equilibrium dialysis in the presence of various concentrations of sodium chloride. C.d. intensity in the carboxylate $\pi \rightarrow \pi^*$ spectral region increases linearly with calcium-ion concentration up to a level equivalent to half the total poly-L-guluronate stoichiometric requirement, and thereafter shows little further change. Similarly, the level of bound calcium resistant to displacement by swamping concentrations of sodium ions is equivalent to half the stoichiometric requirement of poly-L-guluronate chain-sequences alone. In terms of the previously developed "egg-box" model of co-operative junction-zone formation in alginate gelation, these results are interpreted as showing that the primary mechanism of interchain association is by dimerisation of poly-L-guluronate chain-segments in a regular, buckled, two-fold conformation related to that characterized for the free acid in the solid state, with tight interchain chelation of calcium to the carboxylate groups on the interior faces of the dimer (*i.e.*, half the carboxylate residues of the participating chain-sequences). This interpretation is entirely consistent with previous evidence from electron microscopy, and offers a simple rationalisation of experimental results from competitive-ion binding studies.

INTRODUCTION

It is now well-established that the regular, crystalline, intermolecular packing typical of polysaccharide chains in the condensed phase may persist under conditions of extensive hydration, as sterically regular, co-operative, interchain "junction zones" in solutions, gels, and biological tissues (for a recent review, see Rees and Welsh[1]). In the carrageenan[2–6] and agar[6,7] families of algal structural polysaccharides, the primary mode of intermolecular association is through formation of double helices, which may further associate to build up a cohesive gel network. We now present

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evidence of an analogous primary-dimerisation event in the gelation of alginate, although the detailed geometry of the ordered tertiary structure involved is very different.

Alginate occurs as the major structural polysaccharide of marine brown algae (Phaeophyceae). Chemically, it is a (1→4)-linked, linear, block co-polymer of β-D-mannuronate and its C-5 epimer α-L-guluronate[8], with residues arranged[9] in homopolymeric sequences of both types, and in regions which approximate to a disaccharide repeating-structure, although recent evidence[10] from enzymic hydrolysis suggests some deviation from this idealised, regular, alternating sequence. Studies of alginate gelation *in vitro* suggest that the main contribution to structural integrity comes from association of polyguluronate sequences into extended, sterically regular junctions, with specific interchain chelation of calcium or other divalent cations of appropriate size[11–15].

Detailed local geometry within these junction zones has been characterised[13–15] by physical and computer model building, coupled with spectroscopic and X-ray evidence. Studies of the chain-length dependence of polyguluronate calcium-binding activity shows the onset of co-operative binding above a degree of polymerisation of ~20 residues[16,17]. The characteristic, large changes in circular dichroism[13–15] which accompany alginate gelation were also observed only above this critical threshold[18] (by contrast, polymannuronate[16] shows no deviation from normal polyelectrolyte behaviour). The principal junctions in alginate gels have therefore been characterised in terms of their composition, detailed stereochemistry, and minimum length. In the present work, we report on further investigations of junction-zone dimensions in terms of the number of participating chains.

EXPERIMENTAL

Materials. — Two alginate samples were used, whose block compositions (determined by controlled acid hydrolysis and n.m.r. spectroscopy[19]) were as follows. Alginate A: polyguluronate, 20.7%; polymannuronate, 38.4%; and alternating, 41.0%. Alginate B: polyguluronate, 58.6%; polymannuronate, 18.7%; and alternating, 22.7%. These samples are identical to alginates VIII and IV, respectively, in the paper by Penman and Sanderson[19], from which the above compositions are taken. Poly-L-guluronate, poly-D-mannuronate, and alternating blocks were prepared by partial hydrolysis with acid, after the method of Haug *et al.*[20].

Methods. — Circular-dichroism measurements were made on a Cary 61 CD Spectropolarimeter, using an alginate concentration of 0.4 mg/ml in a 1-cm path-length cell, and a 10-sec integration period. Equilibrium dialysis was effected by equilibration of sodium alginate solutions (1 mg/ml; 15 ml), contained in dialysis tubing, against mixed solutions (15 ml) of calcium chloride (6mM) and sodium chloride (0–3M). The final calcium-ion concentration outside the dialysis membrane was determined by atomic absorption analysis, and the level of bound calcium was then calculated by assuming equal chemical potentials with respect to calcium on

either side of the membrane, appropriate corrections being made for swelling or shrinkage during dialysis, and, at low concentrations of sodium, for Donnan membrane effects.

RESULTS AND DISCUSSION

X-ray fibre diffraction studies show a buckled two-fold conformation for poly-L-guluronic acid[21], which appears to persist[22] in all of the salt forms so far studied. This evident lack of conformational mobility, which is paralleled by hydrodynamic evidence of a stiff, extended-chain conformation[23], presumably reflects the severe steric constraints[24] of the (1→4)-diaxial inter-residue linkage. In this favoured two-fold conformation, polyguluronate chains display a regular array of electronegative cavities, whose size and geometry appear to be compatible with chelation of calcium. Hence, calcium binding has been interpreted[14] in terms of an "egg-box" model, with specific site binding of cations between long, structurally and sterically regular, polyguluronate chain-sequences.

In terms of this model, we can envisage "egg-box" junctions that involve only two chains at one extreme, and, at the other extreme, tend to infinite sheets of calcium polyguluronate. The ratio of uronate residues to co-operatively bound calcium for "infinite sheet" junctions is 2:1 (stoichiometric equivalence), whereas for chain–chain dimers, the corresponding ratio would be 4:1 (half the stoichiometric calcium level). Thus, in principle, stoichiometry of cation binding offers a route to the determination of the average number of chains in each polyguluronate junction-zone.

To measure co-operative interchain chelation in isolation from less-specific electrostatic effects, we have studied calcium binding by alginate in the presence of various concentrations of sodium ions, using an equilibrium technique in which solutions of sodium alginate were dialysed against mixed solutions of sodium and calcium chloride; the overall ratio of calcium to alginate was held constant, and the total concentration of sodium was varied from run to run. The results are summarised in Fig. 1. The level of bound calcium (*i.e.*, calcium ions not available for equilibration across the membrane) decreases rapidly with increasing total concentration of sodium ions, up to sodium–calcium ratios of ~30:1, above which no further decrease is observed up to sodium chloride saturation. For alginate A (estimated polyguluronate content of 20.7%), the concentration of bound calcium resistant to displacement by sodium was 10 ±1%, whereas for alginate B (58.6% of polyguluronate), the corresponding level of calcium binding was 30 ±2%. These results therefore indicate very strong, preferential chelation of calcium ions between pairs of polyguluronate chain-segments, to form dimeric junction zones rather than larger aggregates.

We have obtained further confirmation of this interpretation by monitoring specific site binding of cations, using circular dichroism (c.d.), which is now established[13,25] as a sensitive probe of the local environment of uronate residues. As shown in Fig. 2, controlled addition of calcium ions to solutions of sodium poly-L-guluronate radically alters the magnitude and form of the c.d. Although the observed

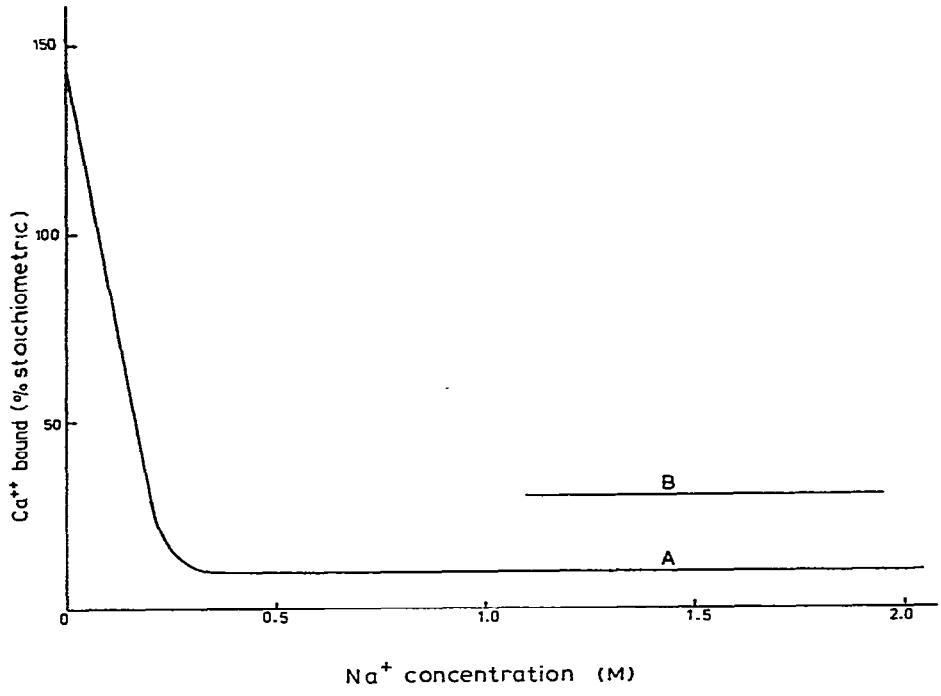

Fig. 1. Equilibrium dialysis investigation of calcium-ion chelation by alginate chains. The level of bound calcium (expressed as a fraction of the total stoichiometric requirement of the alginate) decreases with increasing concentration of sodium ion, as shown. For both alginate A (20.7% of polyguluronate) and alginate B (58.6% of polyguluronate), the concentration of bound calcium resistant to displacement at higher levels of sodium is equivalent to half the stoichiometric requirement of the polyguluronate chain-sequences alone (mean, total concentrations: calcium ion, 3mM; alginate, 0.5 mg/ml).

spectra are complex, the spectral changes on progressive addition of calcium follow a steady pattern, and, as shown in Fig. 3, appear to consist of a positive band at ~208 nm, and a lower-wavelength negative band centred just below the transmission limits of the instrument.

In order to test this interpretation, and quantify the magnitude of the spectral contribution from each band, families of difference spectra, such as those shown in Fig. 3, were fitted by a least-squares, iterative computer method, on the assumption that the entire family of curves can be described in terms of two, simple, gaussian dichroism-bands of fixed position and width, but varying in magnitude. Thus, fitting a family of N difference spectra involved optimisation of $2N+4$ variables ($2N$ peak heights, plus two positions and two widths). Agreement between fitted and observed ellipticities was well within experimental error, lending confidence to our assignment. Moreover, the fitted wavelengths of the two bands (203.7 and 193.7 nm) are in spectroscopically reasonable positions for the carboxylate $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions, respectively[25,26].

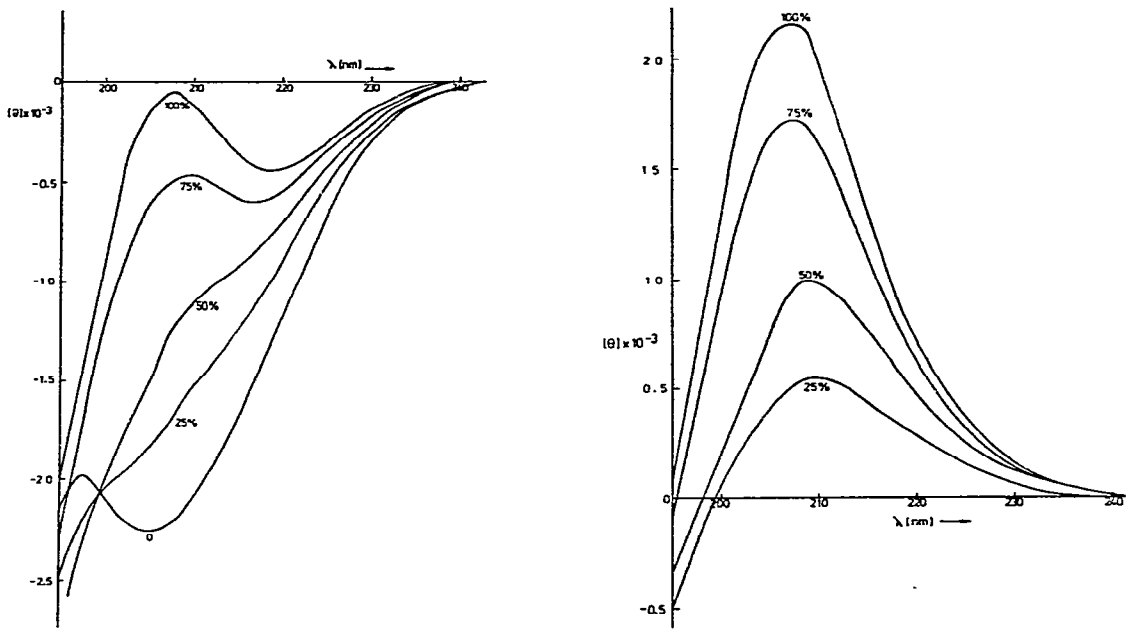

Fig. 2. C.d. of sodium poly-L-guluronate blocks in the presence of various levels of calcium ions (sodium polyguluronate concentration = 0.4 mg/ml; temperature = 25°; calcium-ion concentrations, expressed as a percentage of the total stoichiometric requirement, were as shown).

Fig. 3. C.d. spectral changes on addition of calcium ions to sodium poly-L-guluronate blocks (conditions as in Fig. 2). Calcium-ion concentrations, expressed as a percentage of the total stoichiometric requirement, were as shown.

As shown in Fig. 4, the c.d. behaviour of alginate B (guluronate-rich) was also very sensitive to the presence of calcium ions. The spectral changes shown in Fig. 5 could similarly be interpreted in terms of two bands of opposite sign, whose position and width were virtually identical to those for polyguluronate blocks, indicative of a common spectral origin. The variations in intensity of these bands as a function of calcium level are summarised in Fig. 6. The magnitude of the negative $\pi \rightarrow \pi^*$ band for polyguluronate blocks increases linearly with calcium-ion concentration up to ~50% of the stoichiometric requirement for complete conversion into calcium polyguluronate, and thereafter shows little further increase. The $\pi \rightarrow \pi^*$ band of alginate B (polyguluronate content, 58.6%) shows similar behaviour, but the onset of insensitivity to calcium in this case occurs at ~30% (*i.e.*, ~50% of the stoichiometric equivalent of the polyguluronate sequences alone). Moreover, extrapolation to the origin of this calcium-insensitive "plateau" region for alginate B yields an intercept ~60% of that for polyguluronate blocks, in excellent agreement with the ratio of polyguluronate sequences in the two samples. The concentration profile of the positive $n \rightarrow \pi^*$ band of alginate B, by contrast, is linear and, within experimental error, superimposable upon that for polyguluronate blocks (except perhaps for some

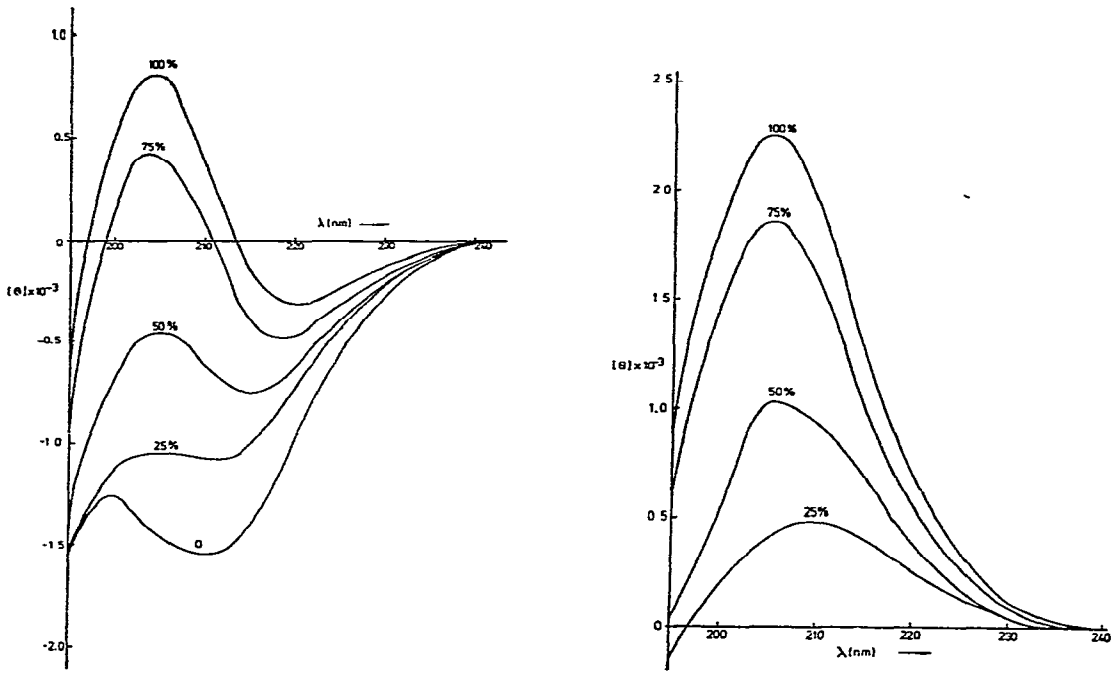

Fig. 4. C.d. of alignate B (58.6% of polyguluronate) in the presence of calcium-ion concentrations equivalent to various percentages of the total stoichiometric equivalent of the alginate, as shown (conditions as in Fig. 2).

Fig. 5. C.d. changes on addition of calcium ions to alginate B (conditions as in Fig. 2). Calcium-ion concentrations, expressed as a percentage of the total stoichiometric requirement of the alginate, were as shown.

slight levelling at very high concentrations of calcium), indicating that essentially all of the c.d. change is associated with polyguluronate sequences. We interpret these results as showing that the carboxylate $n \rightarrow \pi^*$ transition is sensitive to the presence of site-bound cations (including those that would be displaced at higher levels of sodium), perhaps due to involvement of the n electrons in cation chelation. By contrast, the $\pi \rightarrow \pi^*$ transition appears to respond only to the very stable, co-operative interchain "egg-box" binding. In terms of this model, the large increase in $\pi \rightarrow \pi^*$ ellipticity, up to 50% of the polyguluronate stoichiometry, reflects dimerisation, as already discussed. As indicated schematically in Fig. 7, subsequent calcium binding to polyguluronate might then be by further aggregation of dimeric junction zones, or by specific "half egg-box" binding, involving carboxylate groups on the exterior faces of the dimers.

Our proposed model for the molecular origin of the observed changes predicts that these two processes should be indistinguishable in their effects on the $n \rightarrow \pi^*$ transition, but in the $\pi \rightarrow \pi^*$ region, further binding of calcium ions to "half egg-boxes" should have no effect, while aggregation should be essentially equivalent to

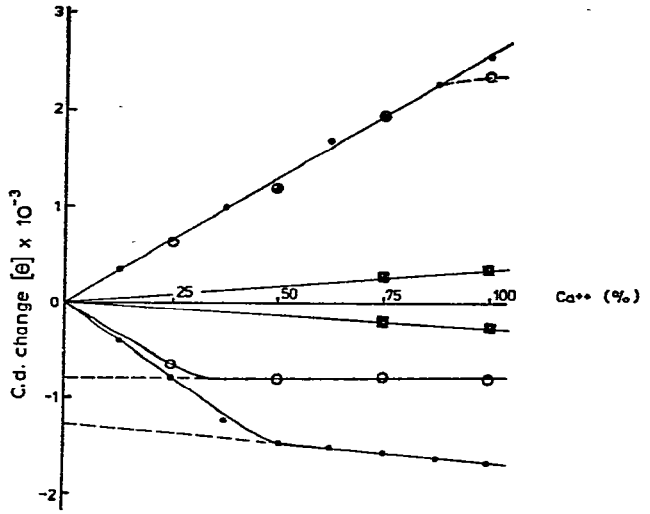

Fig. 6. Variation in c.d. spectral intensity with calcium-ion concentration (expressed as a percentage of the total stoichiometric requirement). Changes in the positive $n \rightarrow \pi^*$ and negative $\pi \rightarrow \pi^*$ bands are shown for poly-L-guluronate blocks (●), alginate B (○), and alternating blocks (■). The $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ bands are at 203.7 and 193.7 nm, respectively, for both polyguluronate and alginate B, whereas for alternating blocks, they are centred at 208.5 and 191.5 nm, respectively (conditions as in Fig. 2).

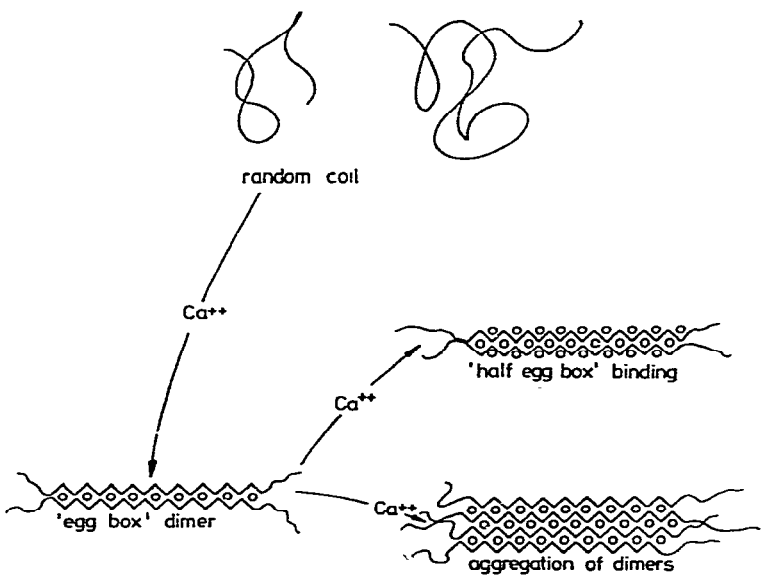

Fig. 7. Schematic representation of calcium-ion chelation to poly-L-guluronate sequences. It is evident from inspection of this scheme that dimerisation involves calcium chelation to only the interior faces of the participating chains (50% of the stoichiometric requirement). On more extensive aggregation, this ratio will tend towards 100% with increasing aggregate size, while site binding of calcium to the exterior faces of dimers ("half egg-box" binding), without further aggregation, raises the ratio to 150%.

initial dimerisation. Addition of calcium to polyguluronate sequences beyond the level required for dimerisation produces detectable further increase in $\pi \rightarrow \pi^*$ ellipticity, indicating some aggregation. However, the observation that precipitation does not occur until calcium levels beyond 100% of the stoichiometric requirement suggests that aggregation is extensive only in the presence of excess of calcium. For the intact alginate B, there is no evidence of any further change in the $\pi \rightarrow \pi^*$ region at any point after dimerisation, suggesting inhibition of aggregation by network constraints. The maximum calcium-binding capacity of polyguluronate dimers, both co-operative and "half egg-box", must be 150% of the stoichiometric equivalent (see Fig. 7). Thus, for alginate B (58.6% of polyguluronate), saturation of binding sites should occur at ~88% of stoichiometric equivalence for the whole chain, perhaps explaining the apparent levelling in its $n \rightarrow \pi^*$ ellipticity towards 100% calcium.

Poly-D-mannuronate blocks showed no measurable change in c.d. on addition of calcium ions, consistent with results from studies of calcium activity as a function of chain length[16], which show no evidence of co-operative binding. The spectral changes shown by alternating blocks with calcium can also be interpreted in terms of an $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ contribution, although, as shown in Fig. 6, their intensities are an order of magnitude lower than those observed for polyguluronate sequences, and the band maxima are shifted appreciably, consistent with the observed differences in position of the c.d. maxima for guluronate and mannuronate glycosides[25]. As shown in Fig. 8, controlled addition of calcium to sodium alginate A (20.7% of polyguluronate) produces overall changes in spectral form resembling those observed for alginate B. However, the resulting difference spectra shown in Fig. 9 cannot be interpreted satisfactorily in terms of changes in the polyguluronate $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions, indicating some contribution from a second process, perhaps calcium binding by alternating sequences which, while showing a far smaller spectral change with calcium than polyguluronate, are present in approximately twice the concentration.

Although our results show spectroscopic evidence of calcium binding by alternating sequences, and of limited aggregation of polyguluronate dimers, the principal mode of interchain association through polyguluronate chain-sequences appears to be by dimerisation. At first sight, this conclusion is perhaps surprising, because both faces of the polyguluronate chain, in its ordered two-fold conformation, are equivalent. The reason why the initial dimerisation event should be a much more favourable process than subsequent addition of further chains may be that the negative charge density on an isolated polyguluronate chain-sequence will be considerably higher than for a dimeric junction with its array of site-bound cations, and hence the electrostatic drive to dimerisation may be far greater. This interpretation perhaps invites analogy with the difference between the first and second dissociation constants of a dibasic acid.

Our present dimerisation model is further supported by previous studies of cation binding and interchain association, and offers a simple and plausible rationali-

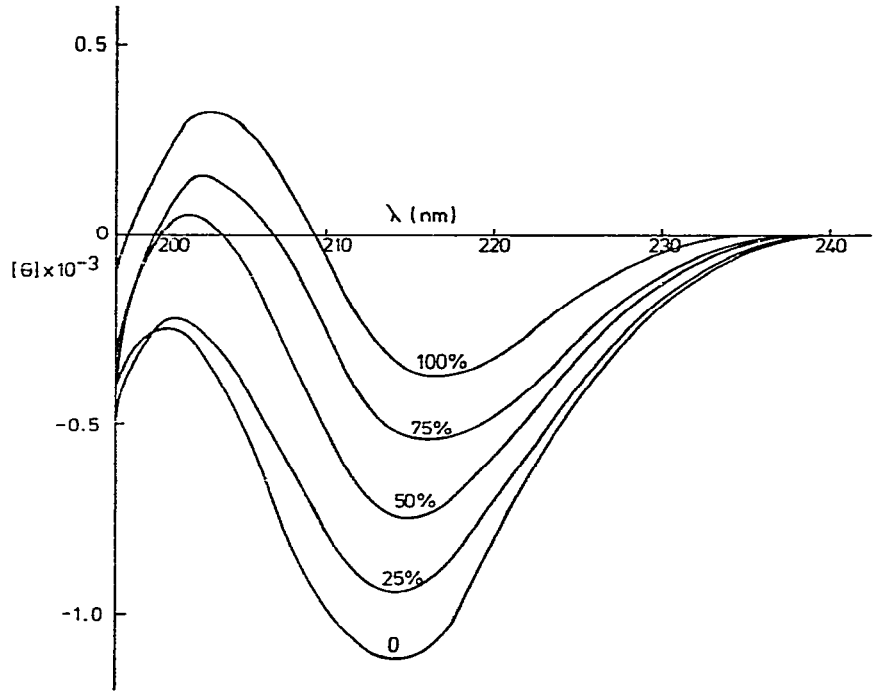

Fig. 8. C.d. of alginate A (20.7% of polyguluronate) in the presence of calcium-ion concentrations equivalent to various percentages of the total stoichiometric equivalent of the alginate, as shown (conditions as in Fig. 2).

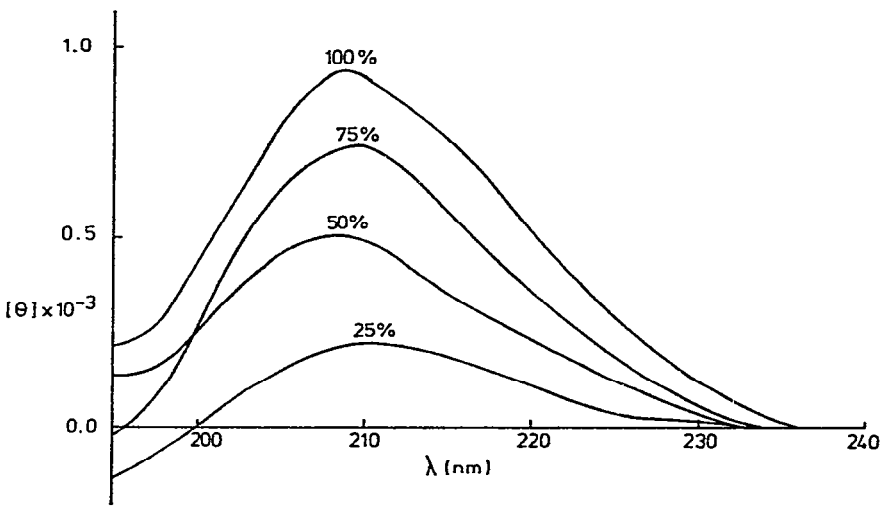

Fig. 9. C.d. spectral changes on addition of calcium ions to alginate A (conditions as in Fig. 2). Calcium-ion concentrations, expressed as a percentage of the total stoichiometric requirement of the alginate, were as shown.

sation of certain earlier results. Thus, ion-exchange studies of the preferential binding of calcium in competition with magnesium, to an alginate containing 90% of L-guluronate residues, show strong selectivity for calcium ions up to a level equivalent to ~40–45% of the stoichiometric requirement of the entire molecule, beyond which the calcium selectivity coefficient drops off rapidly[27,28]. Since the polyguluronate content of the alginate used in that study must lie in the range 80–90% of the total chain, this observation is entirely consistent with polyguluronate dimerisation, although an alternative, theoretical explanation was proposed, based on near-neighbour, auto-cooperative effects. More directly, electron microscopy studies[28] of gel networks in guluronate-rich alginate systems suggest that most interchain junctions have dimensions comparable to those expected for chain–chain dimers.

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