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CHARACTERISATION OF ALGINATE COMPOSITION AND BLOCK-STRUCTURE BY CIRCULAR DICHROISM

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ABSTRACT

The scope of circular dichroism (c.d.) in the characterisation of alginate composition and block-structure is assessed. The three types of block present in alginate show very different c.d. behaviour, the spectrum for poly-L-guluronate being entirely negative, whereas that of poly-D-mannuronate has a strong positive band, and mixed sequences show intermediate behaviour. Spectra of intact alginates show a peak at ~200 nm, and a trough at ~215 nm, whose relative magnitudes vary systematically with composition. Simple equations are given for determination of the relative amounts of D-mannuronate and L-guluronate residues from the observed ratio of peak height to trough depth. The method is non-destructive, and reliable estimates of composition can be obtained from <1 mg of alginate. Mixed blocks give a spectrum which is not identical to that of an equimolar mixture of the two types of homopolymeric block, indicating that c.d. behaviour is also sensitive to the nature of adjacent residues in the polymer chain. Matching of alginate c.d. by linear combination of the spectra for each of the three component structural-sequences, using an iterative, least-squares, computer technique, thus provides a direct index of block composition. The results obtained are in good agreement with previous analyses of the same samples by hydrolysis and n.m.r. spectroscopy.

INTRODUCTION

Alginate, which is the major structural polysaccharide of marine brown-algae (Phaeophyceae), is a (1→4)-linked linear co-polymer of β-D-mannuronate and α-L-guluronate residues[1] arranged[2,3] in homopolymeric blocks of each type, and in heteropolymeric mixed sequences which approximate to a disaccharide repeating-structure, although recent enzymic studies[4] suggest some departure from this idealised, regular, alternating sequence. The technologically and biologically important physical properties of alginate in solutions[5], gels[6–11], and algal tissue[12–16] are largely determined by the relative amounts of the three types of block present.

Chain segments approximating to each of these component structures may be isolated from intact alginate by partial hydrolysis with acid, followed by selective precipitation[3]. However, block determination by this method is tedious and time-

consuming, and requires substantial quantities of material. The use[17] of n.m.r. spectroscopy to determine the ratio of homopolymeric blocks after hydrolysis and separation from mixed sequences represents a substantial improvement in both time and material requirements, but is still not entirely suitable for routine screening of large numbers of samples, or for analysis of materials available only in mg-quantities. More recently, we have reported[18] an empirical correlation between the circular dichroism (c.d.) of alginate and the overall proportions of D-mannuronate and L-guluronate residues present. We now describe a rapid method for the determination of the block structure of alginate from c.d. spectra, with a total sample requirement of <10 mg. We also present simple, linear equations for estimation of alginate composition from c.d. spectra recorded on <1 mg of material.

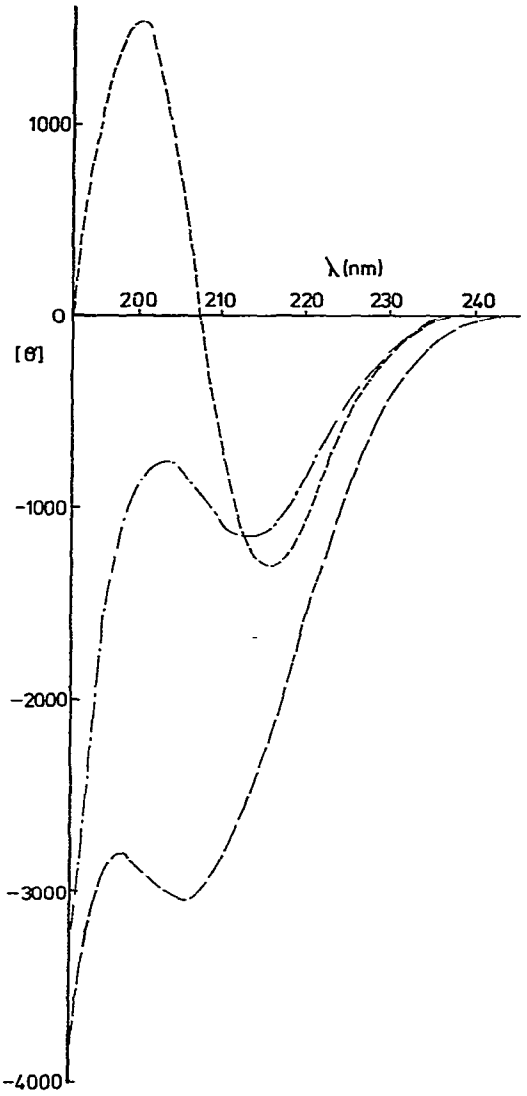
EXPERIMENTAL

Materials. — Commercial alginates from the following sources were used: *Ascophyllum nodosum* (F387; Alginate Industries Ltd.), *Laminaria hyperborea* (F347; Alginate Industries Ltd.), *Macrocystis pyrifera* (HV; Alginates Australia Ltd.), and *L. hyperborea* stipes (SS/DJ; Alginate Industries Ltd.). These materials are identical to samples VII, VI, X, and IV, respectively, in the paper by Penman and Sanderson[17]. Chain segments approximating to each structural type were prepared by partial hydrolysis with acid[3], and characterised by n.m.r. spectroscopy and total hydrolysis with acid[17]. A solution of each sample was dialysed extensively against deionised water, filtered, and freeze-dried before use. Absolute concentrations were determined by elemental analysis (Butterworth Microanalytical Consultancy Ltd.).

Methods. — C.d. spectra were recorded on a Cary 61 CD Spectropolarimeter, using an integration period of 10 s, 1-cm pathlength, and a sample concentration of 0.8 mg.ml^{-1}. Molar ellipticity values, $[\theta]$, are reported in units of deg.cm^2.dmol^{-1}. Curve fitting was by an iterative, least-squares, computer method, using a standard, Powell minimisation-technique. High-resolution p.m.r. spectra were recorded at 100 MHz on a Varian XL-100 spectrometer operating in the Fourier-transform mode.

RESULTS AND DISCUSSION

Fig. 1 shows the observed c.d. spectra of our three block preparations. Analyses by n.m.r. spectroscopy and complete hydrolysis with acid[17] show that the poly-L-guluronate sample has no detectable mannuronate content, whereas the poly-D-mannuronate contains 7% of guluronate, and the alternating blocks contain 60% of mannuronate and only 40% of guluronate. By assuming that the guluronate residues in our poly-D-mannuronate sample make a spectroscopic contribution equivalent to similar residues in the mixed sequences, and that the excess of mannuronate in the mixed blocks is similarly equivalent to residues in poly-D-mannuronate, the observed spectra have been corrected for these departures from idealised block



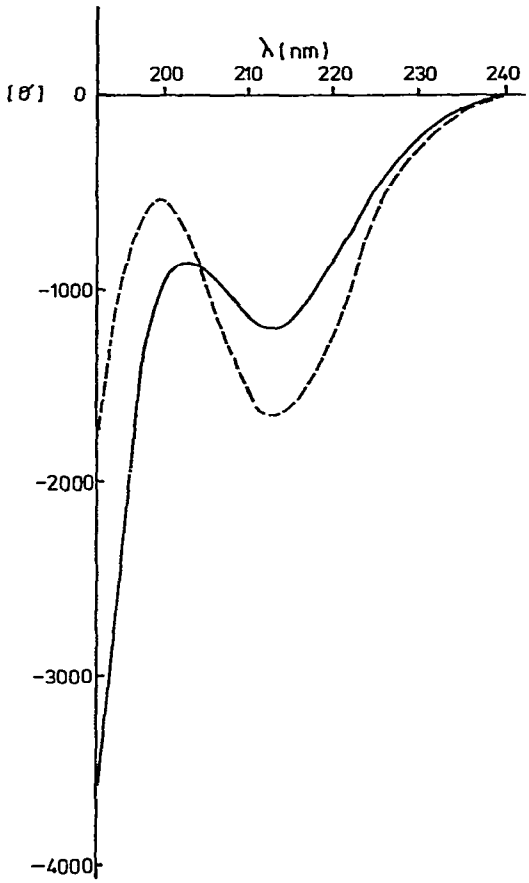


Fig. 1 (left). C.d. of alginate blocks approximating in structure to poly-L-guluronate (——), poly-D-mannuronate (– – –), and mixed (–·–·–) chain sequences.

Fig. 2 (right). Comparison of the c.d. behaviour of alginate mixed-sequences (———), and a spectrum (– – –) synthesised by linear combination of 50% of the spectra for each type of homopolymeric block.

structure by solving the following simultaneous equations at each wavelength:

$$[\theta_M]_{obs} = 0.86[\theta_M] + 0.14[\theta_A], \quad \text{and}$$
$$[\theta_A]_{obs} = 0.20[\theta_M] + 0.80[\theta_A],$$

where $[\theta_M]$ and $[\theta_A]$ are the true molar ellipticities of poly-D-mannuronate and mixed sequences, respectively. The resulting "idealised" block spectra are recorded

TABLE I

SPECTRAL DATA FOR ALGINATE BLOCKS, CORRECTED FOR CROSS-CONTAMINATION[a]

Wavelength (nm)	*Poly-D-mannuronate*	*Mixed blocks*	*Poly-L-guluronate*
245.0	0.00	0.00	0.00
242.5	0.00	−0.01	−0.03
240.0	0.00	−0.01	−0.04
237.5	−0.01	−0.02	−0.07
235.0	−0.05	−0.08	−0.13
232.5	−0.06	−0.12	−0.21
230.0	−0.16	−0.19	−0.35
227.5	−0.28	−0.33	−0.56
225.0	−0.49	−0.49	−0.78
222.5	−0.71	−0.70	−1.06
220.0	−1.00	−0.88	−1.38
217.5	−1.20	−1.05	−1.70
215.0	−1.21	−1.18	−1.99
212.5	−1.06	−1.21	−2.25
210.0	−0.61	−1.14	−2.49
207.5	0.02	−0.99	−2.61
205.0	0.69	−0.91	−2.67
202.5	1.22	−0.86	−2.66
200.0	1.49	−0.98	−2.59
197.5	1.35	−1.39	−2.59
195.0	0.91	−2.33	−2.88
192.5	0.22	−3.48	−3.52

[a]C.d. values are recorded in units of molar ellipticity, $[\theta] \times 10^{-3}$.

in Table I. The accuracy of these model spectra is limited by errors in determination of the composition of the reference samples. However, these errors are likely to be small in comparison with those inherent in the subsequent curve-fitting procedure.

The spectra of homopolymeric blocks show marked similarity in position, intensity, and shape to the spectra of the corresponding methyl glycuronosides[18], demonstrating the sensitivity of c.d. to local ring geometry around the carboxyl chromophore. However, comparison of the c.d. of alginate mixed-sequences with a spectrum derived by linear combination of 50% of the spectra for each of the homopolymeric blocks (Fig. 2) shows the influence of adjacent residues in the polymer chain, and demonstrates that alginate c.d. is sensitive to block structure as well as to overall composition. We have therefore attempted to match observed spectra for alginate, by simple, linear combination of the three idealised-block spectra in Table I, using a computer curve-fitting technique, in the hope that this may provide an index of the relative amounts of each type of block present.

Alginate molar ellipticities $[\theta]$ were recorded at 2.5-nm intervals over the range 245 nm to the lowest accessible wavelength (typically 192.5 nm), and the total square deviation $\sum r_\lambda^2$ between observed and calculated ellipticities (see below) was

TABLE II

ANALYSIS OF ALGINATE BLOCKS

Alginate source	*Analytical method*	*Poly-D-mannuronate (%)*	*Mixed sequences (%)*	*Poly-L-guluronate (%)*	*Mannuronate (%)*	
					Block data	*Hydrolysis*
A. nodosum	C.d. (force fit)	37.8	40.8	21.4	58.2	56
	C.d. (free fit)	37.9	36.1	26.0	56.0	
	N.m.r.	38.4	41.0	20.7	58.9	
L. hyperborea	C.d. (force fit)	23.1	33.7	43.3	40.0	38
	C.d. (free fit)	20.6	22.9	56.5	32.1	
	N.m.r.	20.3	30.4	49.3	35.5	
L. hyperborea stipes	C.d. (force fit)	22.0	13.8	64.2	28.9	29
	C.d. (free fit)	20.0	6.7	73.3	23.4	
	N.m.r.	18.7	22.7	58.6	30.1	
M. pyrifera	C.d. (force fit)	36.5	45.0	18.5	59.0	—
	C.d. (free fit)	36.7	43.3	20.0	58.4	
	N.m.r.	40.6	41.7	17.7	61.5	

[a]N.m.r. analyses of blocks and values for the total mannuronate content by acid hydrolysis are from the results of Penman and Sanderson[17]. "Force fit" and "free fit" refer to c.d. analysis of blocks with and without the constraint that the combined concentrations of blocks must equal the measured concentration of intact polymer.

minimised by an iterative computer method, using a standard, Powell minimisation-routine to vary the fraction (f) of each type of block until the best fit was obtained. At each wavelength (λ),

$$[\theta_\lambda]_{calc} = f_M[\theta_\lambda]_M + f_A[\theta_\lambda]_A + f_G[\theta_\lambda]_G, \quad \text{and}$$
$$r_\lambda^2 = ([\theta_\lambda]_{obs} - [\theta_\lambda]_{calc})^2,$$

where the subscripts M, G, and A refer, respectively, to poly-D-mannuronate, poly-L-guluronate, and mixed-chain sequences. Fitting was attempted with and without the constraint $f_M + f_A + f_G = 1$ (*i.e.*, with 2 and 3 independent variables; "force fit" and "free fit", respectively), and our results are summarised in Table II.

Fits obtained without the above constraint on total block concentration show relatively poor agreement with analyses of the same samples by the combined partial hydrolysis and n.m.r. approach of Penman and Sanderson[17], particularly for the samples richest in poly-L-guluronate, when the mannuronate content is somewhat underestimated. However, analyses of blocks by c.d., using the concentration constraint, are in good agreement with n.m.r. results. Fig. 3 shows the quality of fit obtained, and Fig. 4 illustrates the individual contributions of the three types of block to overall c.d. behaviour. The maximum discrepancy between the c.d. and n.m.r. methods is $<10\%$ of the total uronate content, and the percentages of each residue present, calculated from c.d. analyses of blocks are, if anything, in somewhat closer agreement with results from total hydrolysis with acid. It therefore appears that

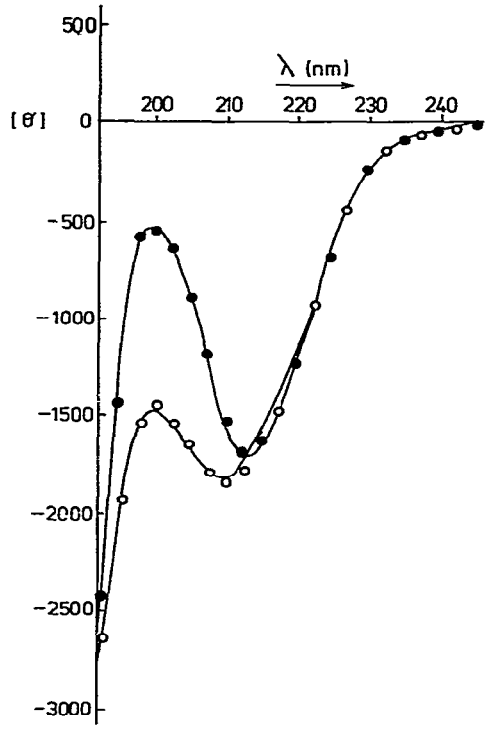


Fig. 3 (left). Analysis of alginate blocks by computer curve-fitting. Comparison of computed c.d. values for alginate from *A. nodosum* (●) and *L. hyperborea* stipes (○) with observed spectra (———).

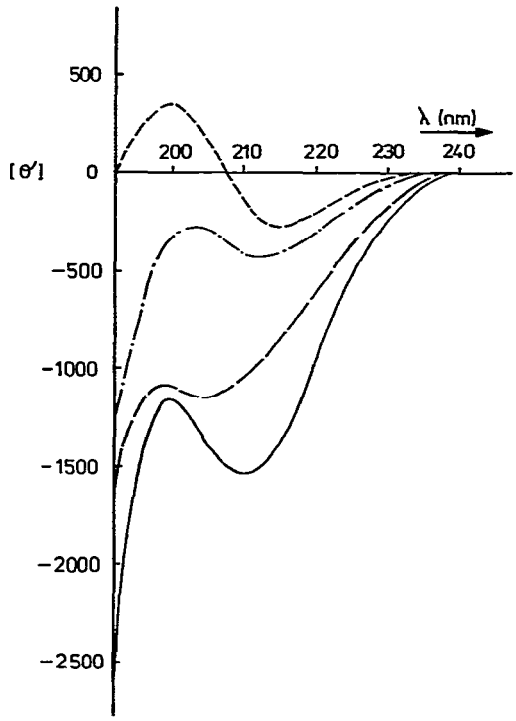


Fig. 4 (right). C.d. of alginate from *L. hyperborea* (———), and the contributions to overall c.d. behaviour from poly-L-guluronate (— —), poly-D-mannuronate (– – –), and mixed (—·—) chain sequences, as determined by computer curve-fitting.

circular dichroism offers a direct, rapid, and non-destructive method for determination of alginate block structure.

However, in the practical utilisation of this approach, a number of simple precautions must be observed. First, as illustrated in Fig. 5 for homopolymeric blocks, c.d. behaviour shows substantial variation with pH, which is to be expected since the chemical nature of the carboxyl chromophore is changed by protonation. It is therefore essential to ensure that solutions are accurately neutralised (pH 7.0 $\pm$0.3) before the c.d. spectrum is recorded. Somewhat smaller changes in spectral intensity are observed with temperature, as shown in Fig. 6, and the ellipticity values recorded in Table II refer to 25°. It is unlikely, however, that spectra recorded at normal ambient temperatures will show sufficient deviation from these values to seriously affect block analysis. However, the presence of such divalent cations as calcium must be avoided, since their specific chelation, principally to poly-L-guluronate chain-sequences, produces large c.d. changes[8,11,19,20], as illustrated in Fig. 6.

Material requirements for c.d. are very low, and reliable spectra can be obtained from ~1 mg of alginate, which makes this approach particularly attractive

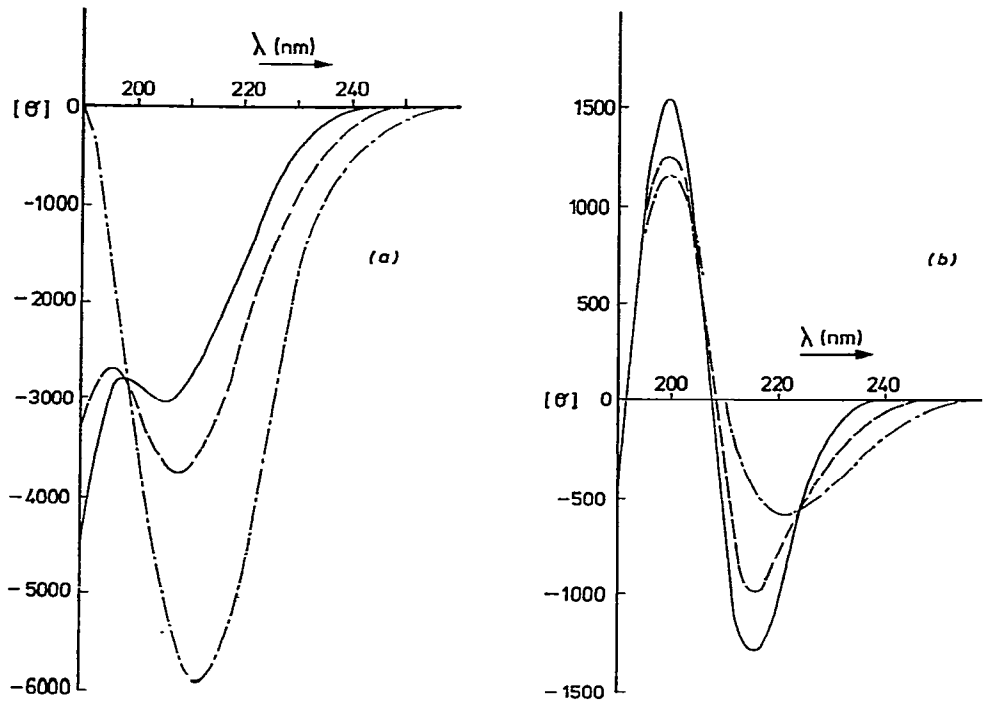
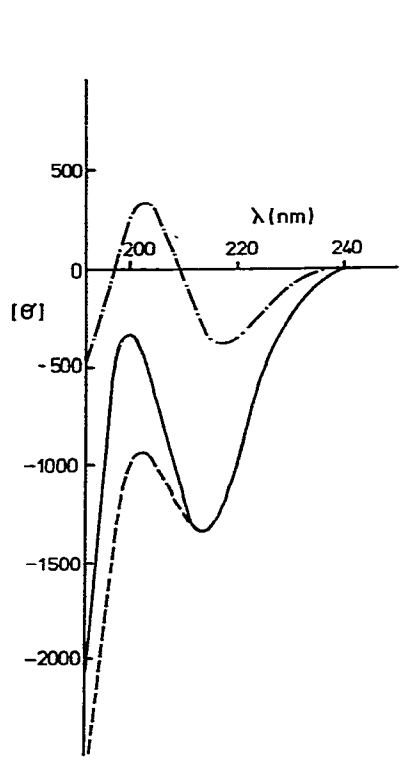


Fig. 5. pH-Dependence of alginate c.d. (*a*) Poly-L-guluronate blocks at pH 7.0 (———), pH 4.5 (– – –), and pH 3.5 (— · —). (*b*) Poly-D-mannuronate blocks at pH 7.0 (———), pH 4.0 (– – –), and pH 3.0 (— · —).

in biological studies of, for example, tissue variations within a single plant. However, our standard curve-fitting procedure uses the constraint that the molar concentrations of each block type must sum to the molar concentration of the alginate. This requires accurate determination of the uronate content of the alginate sample (typically ~80% of the freeze-dried weight), thus introducing a further material requirement of ~5 mg for elemental (C) analysis. We have therefore attempted to develop a method for estimation of overall composition (*i.e.*, relative amounts of mannuronate and guluronate residues, rather than full, block analysis), based on spectral shape alone, so that accurate determination of concentration is unnecessary, and sample requirements are minimised.

C.d. spectra of alginates are characterised by a peak at ~200 nm and a trough at ~215 nm. We have demonstrated[18] an empirical correlation of the ratio of peak height to trough depth (see Fig. 7) with overall composition. In the present work, we have attempted to place this correlation on a firmer footing, by synthesising alginate spectra by linear combination of different proportions of the block spectra in Table I. Since, as shown in Fig. 2, the c.d. behaviour of mixed sequences is not identical with that of an equimolar mixture of homopolymeric blocks, it is evident that this approach cannot give an exact index of composition for all structural possibilities. Thus, for example, the block composition of an alginate with 60%



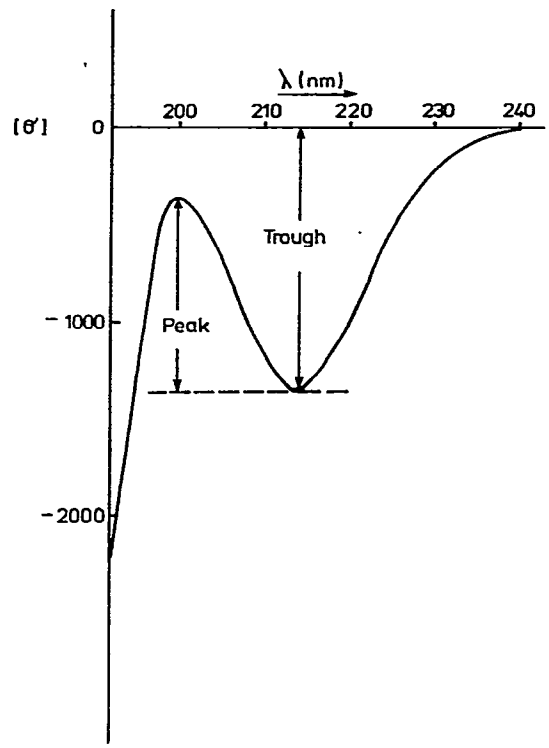


Fig. 6 (left). Temperature- and cation-dependence of alginate c.d.: *A. nodosum* alginate in the Na^+ salt form at 10° (———) and 80° (– – –), and at 25° in the presence of a stoichiometric equivalent concentration of Ca^{2+} ions (—·—·—).

Fig. 7 (right). Characterisation of the c.d. behaviour of alginate by the ratio of peak height to trough depth. The spectrum shown is for *M. pyrifera* alginate at 25° in the Na^+ salt form at pH 7.0.

of mannuronate residues could, in principle, range from 60% of poly-D-mannuronate plus 40% of poly-L-guluronate, to 20% of poly-D-mannuronate plus 80% of mixed sequences. As our model, we have used the average of these extreme possibilities, and would therefore synthesise the c.d. spectrum of a "typical" alginate of this overall composition by 40% of poly-D-mannuronate, 20% of poly-L-guluronate, and 40% of mixed sequences, which is, in fact, very close to the observed compositions of both the *A. nodosum* and *M. pyrifera* alginates studied in this work. Fig. 8 shows spectra synthesised in this way over the full range of possible gross composition.

As shown in Fig. 9, the ratio of peak height to trough depth varies almost linearly with the ratio of mannuronate to guluronate residues present up to values of ~ 1, and thereafter varies almost linearly with mannuronate content. We can therefore summarise the variation in alginate spectral shape with composition in terms of the simple, linear equations *1* and *2*. Eq. *1* is applicable if the c.d. spectrum is entirely negative (*i.e.*, peak/trough < 1), while *2* is required only for alginates

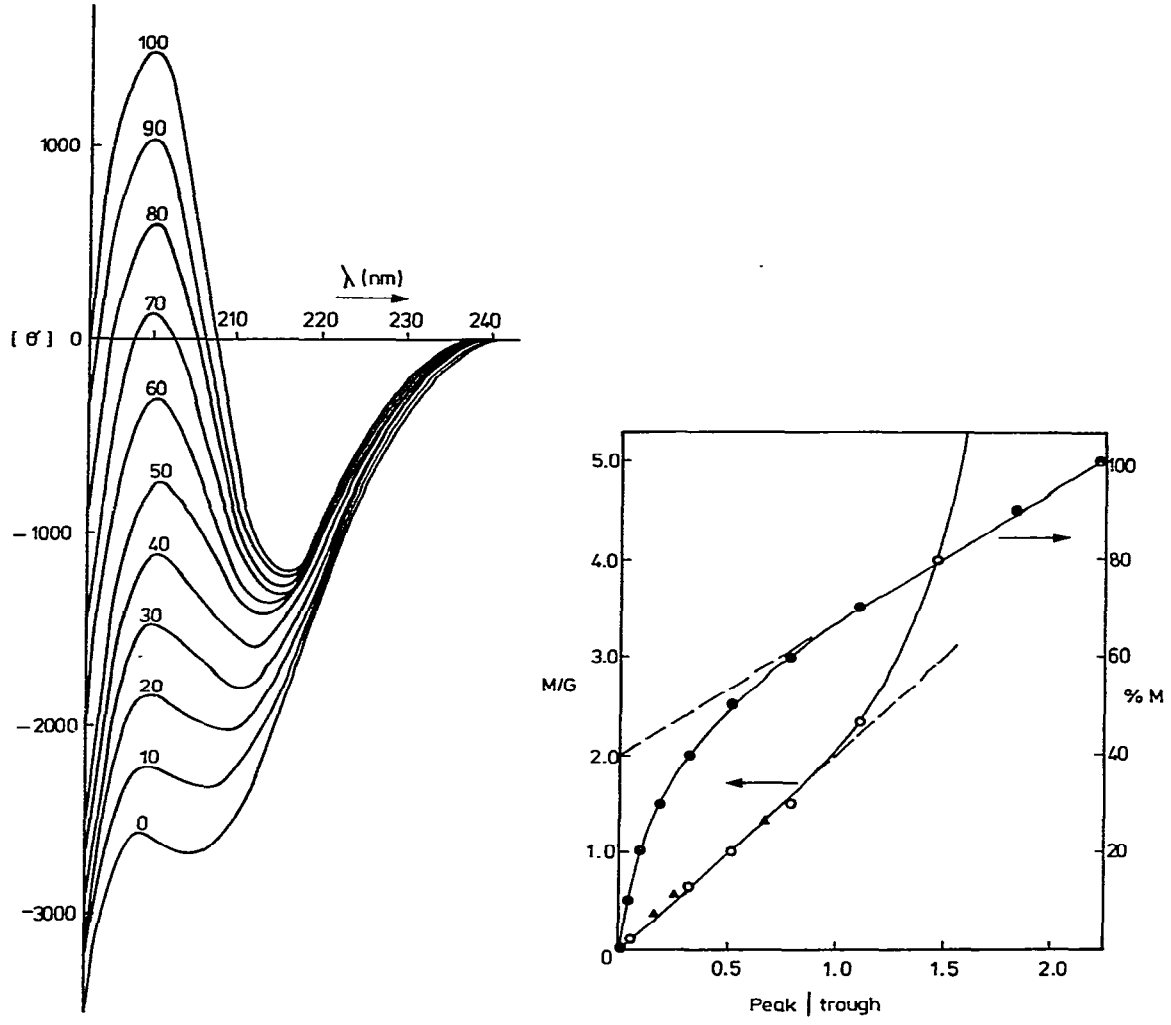


Fig. 8. Variation in c.d.-spectral form of alginate with composition. Spectra were synthesised by linear combination of the block spectra in Table I, using half the theoretical maximum concentration of mixed blocks in each case. Overall mannuronate levels (%) were as shown.

Fig. 9. Determination of alginate composition from c.d.-spectral form. The ratio of mannuronate to guluronate residues (M/G, ○) and total mannuronate content (%M, ●) for the model spectra in Fig. 8 are shown as a function of the peak/trough ratio (see Fig. 7). The observed peak/trough ratios for the alginate samples studied (▲) are in good agreement with those from the synthesised spectra.

having exceptionally high contents of mannuronate, when the spectrum crosses the baseline (peak/trough > 1).

$$\text{Mannuronate/guluronate} \approx 2.0\ (\text{peak/trough}), \quad \text{if peak/trough} < 1. \qquad (1)$$

$$\%\ \text{Mannuronate} \approx 27\ (\text{peak/trough}) + 40, \quad \text{if peak/trough} > 1. \qquad (2)$$

All of the commercially available alginates which we have so far studied (in excess

of 50 samples) show c.d. spectra that are entirely negative, and in these cases a good estimate of the ratio of mannuronate to guluronate residues present can be obtained by simply doubling the measured peak/trough ratio ($\theta_{trough} - \theta_{peak}/\theta_{trough}$) (see Fig. 7). As shown in Fig. 9, the c.d. spectra of the alginate samples used in the present work (which span the composition range of commercial materials) are in good agreement with this generalisation. We therefore conclude that circular dichroism can be used to provide a direct index of the block structure of alginate in solutions of accurately determined concentration, and a simple, reliable, non-destructive method for estimation of monomer composition without measurement of concentration.

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