Conformation and Circular Dichroism of Uronic Acid Residues in Glycosides and Polysaccharides

By **Edwin R. Morris, David A. Rees,* George R. Sanderson,** and **David Thom,** Unilever Research Colworth/Welwyn Laboratory, Colworth House, Sharnbrook, Bedford

All the five hexuronic acids that are known to occur naturally have been synthesised in the form of their methyl α- and β-glycosides, to provide model compounds with which to assess the scope of circular dichroism for the analysis of these residues in biological materials. In the acid form the sign of the c.d. is found to be dictated by configuration at C(5). C(4) Configuration determines the presence or absence of an anomalous long-wavelength band which is opposite in sign to the main $n \longrightarrow \pi^*$ band, and has previously been reported as a minor feature in the spectra of many acyclic α-hydroxy- and α-amino-carboxylic acids. For uronic acid derivatives with O(4) equatorial this band is comparable in size to the normal $n \longrightarrow \pi^*$ band, but it is totally absent when O(4) is axial. These generalisations also apply to spectra of the salts, although band form and position are very different. These variations of c.d. with structure and conformation may be used for the anlaysis of polysaccharides which contain uronic acid residues. The alginates, which are linear copolymers of L-guluronate and D-mannuronate residues, provide a particularly favourable example.

WITH the rapid development in instrumentation over the last decade, circular dichroism has become a convenient tool for the investigation of natural compounds, including carbohydrates, often supplanting the closely related but longer established technique of optical rotation.[1] Recently we have shown that the c.d. of the carboxylate chromophore can be useful in probing polysaccharide structure, conformation, and interaction.[2,3] Uronic acids occur widely in polysaccharides of technological and biological importance. Examples are D-galacturonic acid in pectin, D-mannuronic and L-guluronic acid in alginates, D-glucuronic acid in hemicelluloses, plant gums, and bacterial polysaccharides, and D-glucuronic and L-iduronic acid in various animal polysaccharides. The c.d. of such polymers provides a sensitive index of changes in molecular environment of the carboxy-group. It is known that the changes in c.d. on gelation with divalent cations can be especially dramatic.[2] In order to understand these phenomena more fully, and place their theoretical interpretation on a firmer basis, we have undertaken a systematic study of the c.d. of uronic acid monomers in the hope of correlating spectral features with structure (see Figure 1).

[1] E. R. Morris and G. R. Sanderson in 'New Techniques in Biophysics and Cell Biology,' eds. R. Pain and B. Smith, Wiley, London, 1973; L. Velluz, M. Legrand, and M. Grosjean, 'Optical Circular Dichroism, Principles, Measurements, and Applications,' Verlag Chemie, Weinheim, 1965.

[2] E. R. Morris, D. A. Rees, and D. Thom, *J.C.S. Chem. Comm.*, 1973, 245.

[3] (*a*) G. T. Grant, E. R. Morris, D. A. Rees, P. J. C. Smith, and D. Thom, *F.E.B.S. Letters*, 1973, **32**, 195; D. A. Rees, *Biochem. J.*, 1972, **126**, 257; D. Thom, Ph.D. Thesis, University of Edinburgh, 1973; (*b*) T. A. Bryce, A. A. McKinnon, E. R. Morris, D. A. Rees, and D. Thom, *Faraday Discuss. Chem. Soc.*, 1974, **57**, 221.

All the D-uronic acid glycosides studied give a positive band at the expected position of 212 nm as their principal spectral feature in the $n \longrightarrow \pi^*$ region. The corresponding L-sugars must therefore be typified by a predominantly negative spectrum. When the hydroxy-group on C(4) is equatorial, there is also a small band of opposite sign at longer wavelength, but this is totally absent when O(4) is

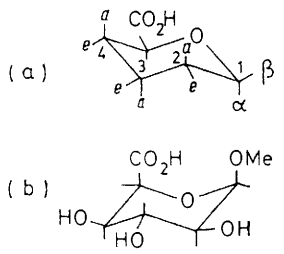

FIGURE 1 (a) Methyl D-pyranuronoside structures (Reeves *C*1 ring form); α- and β-configurations of the methoxy-group on C(1) are as indicated; hydroxy-groups on C(2), C(3), and C(4) are oriented axially (*a*) or equatorially (*e*), as tabulated:

	O(2)	O(3)	O(4)
Glucuronoside	*e*	*e*	*e*
Mannuronoside	*a*	*e*	*e*
Galacturonoside	*e*	*e*	*a*
Guluronoside	*e*	*a*	*a*
Iduronoside	*a*	*a*	*a*

(b) Methyl β-D-idopyranuronoside (Reeves 1*C* ring form).

axial. Salt spectra display the same general behaviour, but the anomalous long-wavelength band is much more intense, and in certain cases becomes predominant above 200 nm. These observations are entirely in accord with earlier work on the more readily available uronic acid derivatives.[4]

Our results indicate that changes in sugar ring conformation may appreciably alter rotational strength We have further established that frequently monomer contributions to polysaccharide spectra are essentially additive. Thus c.d. may have considerable scope for the characterisation of polymer structure and conformation. As a simple illustration of this approach we describe the application of c.d. to the routine determination of alginate structure. The alginates are linear co-polymers of D-mannuronic and L-guluronic acid,[5] extracted from Phaeophyceae (marine brown algae). Of particular interest among their industrial applications is the ability to form gels with divalent cations, typically calcium. The quality of these gels is dependent on polymer composition, which is therefore of practical interest. Since the two component residues have opposite configuration at both C(5) and C(4), the two positions which most critically affect carboxy-group optical activity, the alginates are particularly amenable to study by c.d. We have, in fact, identified spectral features which show a simple correlation with composition as determined by degradative methods.[6-8]

RESULTS AND DISCUSSION

Uronic Acid Glycosides.—All the glycosides studied show totally different c.d. spectra in the acid and salt forms, and are consequently extremely sensitive to changes in pH, especially close to the pK_a of the acid (pH *ca.* 3.3), as illustrated in Figure 2. Since ionisation introduces an additional plane of symmetry to the chromophore, and also involves delocalisation of the π and π* orbitals, changes of this magnitude are to be expected. Changes in sign on neutralisation have been observed in the c.d. spectra of a considerable number of carboxylic acids.[4,9-13] Kresheck[9] has attributed the inversion of the c.d. of ascorbic acid to a change in ring

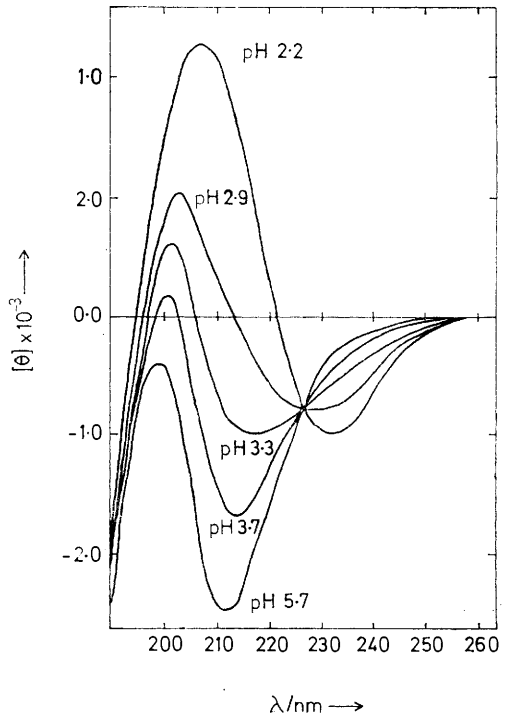

FIGURE 2 pH Dependence of uronic acid glycoside c.d. Undissociated uronic acids and their neutral salts may give markedly different c.d. profiles, as illustrated here for methyl α-D-mannuronoside (at 10 °C). The isodichroic point at 226 nm shows the existence of two species (carboxylic acid and carboxylate) in simple equilibrium

conformation on ionisation, but this is not a necessary interpretation in all instances since the effect has also been reported for a number of acyclic carboxylic

[4] I. Listowsky, S. Englard, and G. Avigad, *Biochemistry*, 1969, **8**, 1781.
[5] E. L. Hirst and D. A. Rees, *J. Chem. Soc.*, 1965, 1182; D. A. Rees and J. W. B. Samuel, *ibid.*, 1967, 2295.
[6] A. Haug, B. Larsen, and O. Smidsrød, *Acta Chem. Scand.*, 1966, **20**, 183; 1967, 21, 691.
[7] A. Haug, B. Larsen, and O. Smidsrød, *Carbohydrate Res.*, 1974, **32**, 217; C. A. Knutson and A. Jeanes, *Analyt. Biochem.*, 1968, **24**, 470; 482; Z. Dische, *J. Biol. Chem.*, 1947, **167**, 189.
[8] A. Penman and G. R. Sanderson, *Carbohydrate Res.*, 1972, **25**, 273.
[9] G. C. Kresheck, *Biochem. Biophys. Res. Comm.*, 1968, **33**, 374.
[10] G. Barth, W. Voelter, E. Bunnenberg, and C. Djerassi, *Chem. Comm.*, 1969, 355.
[11] I. Listowsky, G. Avigad, and S. Englard, *J. Org. Chem.*, 1970, **35**, 1080.
[12] G. Barth, W. Voelter, H. S. Mosher, E. Bunnenberg, and C. Djerassi, *J. Amer. Chem. Soc.*, 1970, **92**, 875.
[13] A. Fredga, J. P. Jennings, W. Klyne, P. M. Scopes, B. Sjoberg, and S. Sjoberg, *J. Chem. Soc.*, 1965, 3928.

acids.[11-13] As illustrated in Figure 3, changes in temperature also produce appreciable spectral changes. The general shapes of the c.d. spectra for glycosides in both acid and salt forms are outlined in Figure 4, although the relative sizes of the spectral features vary considerably with structure. The individual spectra are recorded in Table 1. Within each type of spectrum the individual examples form a family of curves which may be exactly

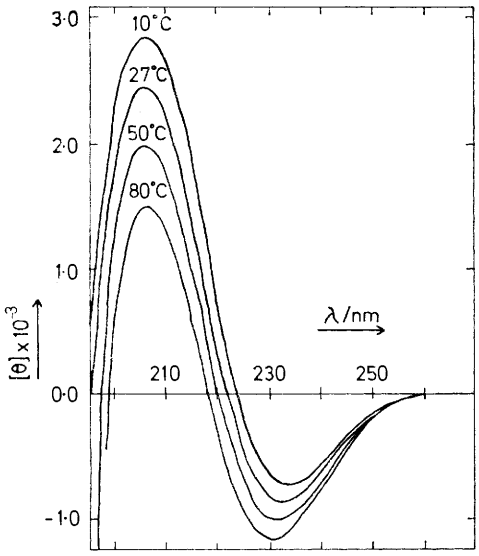

FIGURE 3 Temperature dependence of uronic acid glycoside c.d. Although less dramatic than the pH dependence, there are marked changes in uronoside c.d. with temperature, as illustrated here for methyl α-D-glucuronoside in the undissociated acid form

matched by mixing various proportions of a small number of gaussian bands. This suggests that within each group the same electronic processes occur under the influence of somewhat different dissymmetric environments in parts of the molecule sufficiently remote from the chromophore to have little or no effect on the energies, and hence wavelengths, of the transitions.

The ring oxygen and the hydroxy-group on C(4), however, are both sufficiently close to the carboxy-group to interact with it directly, and will have a predominant role in determining its preferred orientations and electronic transition energies. Configuration at C(5), which determines the position of the ring oxygen relative to the chromophore, dominates the c.d. Thus the principal

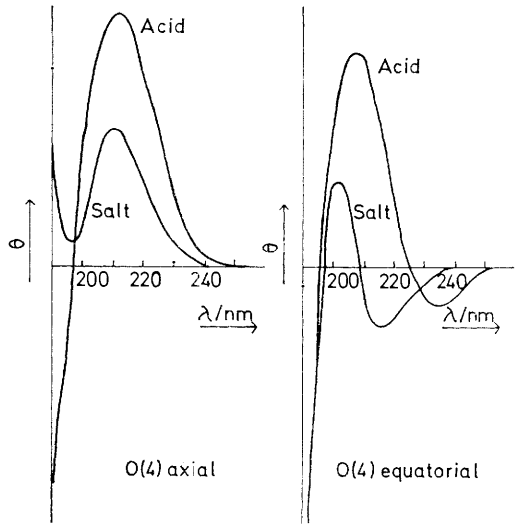

FIGURE 4 Typical uronic acid glycoside c.d. spectra. The spectral form is largely determined by configuration at C(4). Left: uronosides with O(4) axial give a single peak above 200 nm in both the acid and salt form. Right: those with O(4) equatorial show a second, longer wavelength band of opposite sign. Although the same general spectral form is maintained within each of these families, quantitative details vary with structure (Table 1). The spectra shown above are those of D-uronosides. The corresponding L-sugars would show exact mirror image spectra (*i.e.* the principal band negative rather than positive)

$n \longrightarrow \pi^*$ band is *positive* for all the D-uronic acids studied, and hence *negative* for all the corresponding L-sugars. The effect of changing the stereochemistry at C(4) is demonstrated by the way in which the spectra fall into two distinct categories corresponding to the two possible configurations. The galacturonosides, guluronosides, and iduronosides in which O(4) is axial, all give spectra with a simple $n \longrightarrow \pi^*$ band around 210 nm, the normal wavelength for this transition.[10,14] A more intense band centred below 185 nm, the lower wavelength limit of present day instruments,[1] can be attributed

TABLE 1

Uronic acid methyl glycoside c.d. spectra [θ] values [a]

	Glucuronoside				Mannuronoside				Galacturonoside				Guluronoside				Iduronoside			
	α		β		α		β		α		β		α		β		α		β	
λ/nm	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
190			−6 270	−5 170		−2 720		−2 970	−3 340	2 330		0		1 500			−4 780	3 220	−17 500	5 600
195	−480	−980	−1 020	−940	−1 090	−1 350	−4 610	−720	−80	470	−1 140	−1 030	−4 200	3 300			920	2 480	−2 240	5 040
200	1 520	0	2 120	1 410	1 240	−470	800	2 040	2 650	780	2 290	0	1 600	4 100	570	−1 500	4 140	2 810	5 220	3 730
205	2 450	−610	3 450	1 020	1 970	−1 240	2 860	1 590	3 760	1 830	3 770	1 200	3 700	4 600	2 810	250	6 160	4 420	9 330	5 600
210	2 440	−1 340	3 530	−470	1 940	−1 880	3 660	520	4 190	2 300	4 420	1 810	4 700	4 600	3 810	1 130	7 270	5 430	11 600	8 020
215	1 840	−1 550	2 670	−1 020	1 270	−2 370	3 500	0	3 950	2 000	4 270	1 640	5 100	3 700	3 670	1 150	7 220	4 880	11 900	7 090
220	870	−990	1 490	−780	350	−1 640	1 990	−320	2 980	1 270	3 380	1 070	4 400	2 000	2 950	830	5 930	2 940	9 700	4 480
225	0	−440	310	−550	−550	−870	480	−370	1 950	690	2 210	550	3 060	900	1 910	460	4 230	1 290	7 090	2 050
230	−570	−150	−430	−240	−1 030	−420	−480	−30	970	270	1 240	210	1 500	300	1 030	180	2 350	460	3 730	560
235	−630	−40	−630	−80	−970	−150	−910	0	450	110	600	50	500	0	450	20	1 010	90	1 680	0
240	−500	0	−350	0	−700	−30	−750	0	140	30	230	0	120	0	140	0	370	0	0	0
245	−270	0	−240	0	−330	0	−400	0	50	0	70	0	0	0	60	0	140	0	0	0
250	−90	0	−80	0	−60	0	−160	0	0	0	0	0	0	0	0	0	0	0	0	0
255	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] A = acid form (pH 2.0); S = sodium salt form (pH 7.0)

[14] W. D. Closson and P. Haug, *J. Amer. Chem. Soc.*, 1964, **86**, 2384; D. W. Urry and H. Eyring, *ibid.*, p. 4574; T. E. Emerson, D. F. Ewing, W. Klyne, D. G. Neilson, D. A. V. Peters, L. H. Roach, and R. J. Swan, *J. Chem. Soc.*, 1965, 4007.

principally to the $\pi \longrightarrow \pi^*$ transition, although there may also be a small contribution from excitations of the unpaired electrons of the ring oxygen and hydroxy-groups.[15] By contrast the derivatives of D-glucuronic and D-mannuronic acid exhibit an additional band with maximum around 235 nm, which is opposite in sign to the principal 210 nm band.

This peculiar spectral feature has been observed for a large number of dissymmetric α-hydroxy-carboxylic acids, including the simplest such compound, lactic acid, and many explanations of its origin have been proposed. Anand and Hargreaves, who first reported this long wavelength band,[16] assigned it to the $n \longrightarrow \pi^*$ transition, and concluded that the larger 210 nm band arose from other transitions including $\pi \longrightarrow \pi^*$. This suggestion, however, being in direct conflict with the established assignment of the 210 nm band,[14,17] found little acceptance, and it is now generally agreed that both bands have their origin in the $n \longrightarrow \pi^*$ transition.[10-12] This is supported by the observation that an intensification of the long-wavelength band, due to changes in experimental conditions such as temperature or solvent system, is invariably accompanied by a decrease in the peak at 210 nm. The difference in transition energy may be considerably smaller than is suggested by cursory inspection, since when two bands of opposite sign overlap, the observed separation of their maxima may be considerably greater than the actual distance between the peaks.[18] Curve resolution suggests that the stabilised band is probably centred around 225 rather than 235 nm where the experimental minima occur. This analysis is in good agreement with evidence from spectra of solutions in non-aqueous solvents,[4] where the low energy minimum moves to shorter wavelength as its intensity increases, the shortest wavelength attained being in fact 225 nm.

This long-wavelength band in the spectra of uronic acid derivatives was first reported by Listowsky,[4] who observed a marked solvent dependence in that the 210 nm 'normal' $n \longrightarrow \pi^*$ band decreased in relative size with decreasing water content of a mixed water–dioxan solvent, and was no longer in evidence in hexane solution. He assigned the band at longer wavelength to the $n \longrightarrow \pi^*$ transition of unsolvated molecules, and the normal band at 210 nm to the same transition in molecules hydrogen bonded to water. This interpretation, however, does not account for the critical dependence on molecular structure, in particular on the nature and orientation of substituents on the β-carbon atom, and other workers have recently suggested [19] that, in certain related systems, solute–solvent interactions are unlikely to be strong enough to produce so drastic a change in ellipticity and transition energy. Assignment of the two bands to monomer and dimer can be dismissed on theoretical grounds, because dimerisation to any appreciable extent in aqueous solution is highly improbable, and also on experimental grounds because the observed spectra are independent of concentration. Furthermore, the pronounced effect of disposition of substituents around the chromophore is not explained by the dimerisation hypothesis, nor by an interpretation in terms of transitions between different vibrational energy levels, which was proposed recently [19] in discussion of the occurrence of twin bands of opposite sign in c.d. spectra of azoalkanes and structurally rigid cyclic ketones. The most widely adopted explanation of the two bands is that they arise from the $n \longrightarrow \pi^*$ transition of different rotational isomers.[10-12] This would explain the observed population levelling on raising the temperature.

The magnitude and sign of the c.d. associated with a particular electronic transition are critically dependent upon molecular geometry, or, more specifically, upon the disposition of other groups about the symmetry planes of the chromophore.[20] For the $n \longrightarrow \pi^*$ transition of ketones the sign of the contribution made by each atom or group in the rest of the molecule obeys an octant rule.[21] No such general systematic rationalisation has yet been made for the $n \longrightarrow \pi^*$ transition of the carboxy-group and its derivatives, although two very different rules have been proposed [22] for interpretation of the c.d. spectra of lactones and carboxylic acids. It is certain, however, that any rotation of the carboxy-group chromophore against the dissymmetric environment of the rest of the molecule will cause large changes in c.d. magnitude, including inversion of sign, and perhaps also changes in the exact wavelength of maximum absorption.

The maximum specific ellipticity of the single $n \longrightarrow \pi^*$ band observed for the methyl glycosides of undissociated galacturonic acid and guluronic acid is essentially constant at about 4.5×10^3. This band is appreciably smaller in the spectra of methyl glucuronosides and mannuronosides, but curve resolution shows that if the rotational strength of the normal band is added to that of the long wavelength band of opposite sign, the combined intensity approximates closely to the observed galacturonoside and guluronoside values. This would be consistent with an assignment of the two bands to different rotational isomers, and would further suggest that the rotational strength is determined principally by ring shape, and is only slightly modified by hydroxy-group configuration. Departure from the normal ring conformation might therefore be expected to affect rotational strength appreciably.

[15] I. Listowsky and S. Englard, *Biochem. Biophys. Res. Comm.*, 1968, **30**, 329.
[16] R. D. Anand and M. K. Hargreaves, *Chem. Comm.*, 1967, 421.
[17] G. Gottarelli, W. Klyne, and P. M. Scopes, *J. Chem. Soc.* (*C*), 1967, 1366; G. Gottarelli and P. M. Scopes, *ibid.*, p. 1370; W. D. Closson, P. J. Orenski, and B. M. Goldschmidt, *J. Org. Chem.*, 1967, **32**, 3160.
[18] K. M. Wellman, P. H. A. Laur, W. S. Briggs, A. Moscowitz, and C. Djerassi, *J. Amer. Chem. Soc.*, 1965, **87**, 66.
[19] D. J. Severn and E. M. Kosower, *J. Amer. Chem. Soc.*, 1969, **91**, 1710.
[20] J. A. Schellman, *Accounts Chem. Res.*, 1968, **1**, 144.
[21] W. Moffit, R. B. Woodward, A. Moscowitz, W. Klyne, and C. Djerassi, *J. Amer. Chem. Soc.*, 1961, **83**, 4013; P. Crabbé, 'An Introduction to the Chiroptical Methods in Chemistry,' Mexico, 1971.
[22] J. P. Jennings, W. Klyne, and P. M. Scopes, *J. Chem. Soc.*, 1965, 7211; I. Listowsky, G. Avigad, and S. Englard, *J. Org. Chem.*, 1970, **35**, 1080.

D-Uronic acid residues in the normal Reeves *C*1 conformation are disposed fairly symmetrically when viewed down the C(5)–C(6) bond. Any change to a ring form which is more dissymetric about this axis might therefore be expected to increase the rotational strength. Such changes are most probable for the *ido*-configuration where the *C*1(D) conformation is destabilised by axial hydroxy-groups on C(2), C(3), and C(4) (see Figure 1). The fact that the methyl iduronosides show considerably larger c.d. than the other uronic acid glycosides seems likely therefore to be due to changed ring shape, and indeed n.m.r. studies [23] of methyl α-D-idopyranosiduronic acid do show an appreciable departure from the *C*1 conformation. This has been interpreted in terms of either an equilibrium between the alternative *C*1 and 1*C* chair forms, or adoption of a hybrid ' skew boat ' structure. An unequivocal decision between these possibilities, however, cannot be made from present n.m.r. or c.d. evidence. The *C*1 form of the β-anomer would be expected to be more stable, and this is in fact borne out by n.m.r. evidence. In this case, however, a change to either of the less symmetric ring conformations brings the methoxy-group on C(1) close to the carboxy-group chromophore [see Figure 1(b)], with perhaps considerable consequent perturbation. It is possible therefore that the large c.d. observed may be due to a relatively small proportion of an intensely optically active ring form in equilibrium with a much larger amount of the more symmetric *C*1 conformation.

According to the Franck-Condon principle, electronic transitions occur extremely rapidly in comparison with vibrational or rotational processes, and so spectroscopic techniques such as c.d. ' freeze ' the molecule in a particular conformational state, like an action snapshot, and the overall spectrum is the result of superimposing all these individual contributions, rather than a smeared average as in ' slower ' techniques such as n.m.r. It appears [24] that the preferred conformations are those in which the carbonyl function of the carboxy-group is eclipsed. Any interpretation of uronoside c.d. in terms of rotational isomers therefore implies that one of the three possible eclipsed conformations dominates when the hydroxy-group on C(4) is axial, but that two such conformations exist in equilibrium when it is equatorial. Listowsky [11] has interpreted the long-wavelength band as arising from the rotational isomer in which C(4) is eclipsed, and the normal 210 nm band as corresponding to eclipsing of the ring oxygen. This is in direct disagreement with the proposed assignment [25] of the analogous long-wavelength band in α-amino-acid spectra to the rotational isomer in which the α-nitrogen atom is eclipsed with the decrease in transition energy being ascribed to a through-space interaction between the non-bonding orbital of nitrogen and the chromophore transition of the carboxy-group. We are at present attempting to resolve the problem of assignment of bands by selected experiments on key model compounds, in conjunction with molecular orbital calculations. Whatever the details of the structural origin of the marked spectral differences between the various uronic acids, we show below that they have practical applications.

Polysaccharide Analysis.—We have established that the c.d. of a number of polysaccharides is close to that predicted by linear combination of constituent monomer spectra, although it may be modified by second-order effects from adjacent residues. This is illustrated in

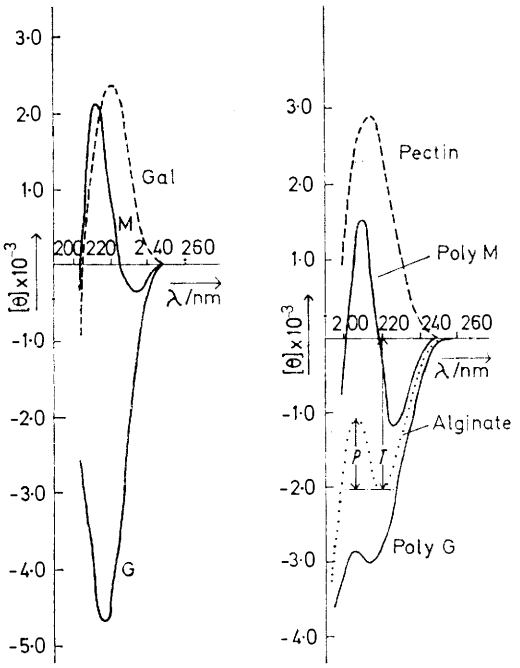

FIGURE 5 Additivity of monomer contributions to polysaccharide c.d. Left: c.d. spectra of methyl α-D-galacturonoside (Gal), methyl α-L-guluronoside (G), and methyl β-D-mannuronoside (M) (all in the sodium salt form at pH 7 and 25 °C). Right: corresponding uronic acid polymer spectra. Although modified somewhat by second order effects, the spectra of polyguluronate sequences (Poly G), polymannuronate sequences (Poly M), and low methoxypectin (Pectin) closely resemble in sign, shape, and size the spectra of their principal monomeric constituents (G, M, and Gal, respectively), in the appropriate glycosidic configuration. Alginate spectra vary with composition within limits defined by the ' Poly G ' and ' Poly M ' spectra. The ratio of peak height (*P*) to trough depth (*T*) provides a direct index of the relative amounts of mannuronate and guluronate residues present

Figure 5 for pectins and alginates. We have also observed similar spectra for solid films of these materials.[3b] Theoretical considerations, reinforced by observed c.d. behaviour of the methyl iduronosides, suggest that any change in sugar ring conformation would appreciably affect rotational strength. Thus the additivity of monomer contributions to polymer c.d. provides additional evidence against the occurrence of unusual ring conformations such as have been proposed [26] for uronic residues in polymer chains in solution.

[23] A. S. Perlin, B. Casu, G. R. Sanderson, and J. Tse, *Carbohyd. Res.*, 1972, **21**, 123.
[24] G. J. Karabaisos and D. J. Fenoglio, *Topics Stereochem.*, 1970, **5**, 172.
[25] J. Cymerman-Craig and W. E. Pereira, jun., *Tetrahedron Letters*, 1970, 1563.
[26] S. Hirano, M. Manabe, N. Miyazake, and K. Onodera, *Biochim. Biophys. Acta*, 1968, **156**, 213.

It appears from *X*-ray diffraction evidence that, in the solid state, the α-L-iduronic acid residues in dermatan sulphate and heparin do not adopt the normal $1C(L)$ conformation,[27] but this does not seem to be the case in solution, on the basis of n.m.r. studies.[28] Present evidence however is far from conclusive, and c.d. may well prove of value in elucidating the apparent conformational mobility of this residue, if allowance can be made for spectral contributions of acetamido-substituents. It may also provide a method of determining the relative amounts of L-iduronic acid and its $C(5)$ epimer D-glucuronic acid present [28,29] in dermatan sulphate, heparan sulphate, and heparin, since these two sugars have opposite configurations at both C(5) and C(4), the two positions which most critically affect the chiroptical behaviour of the carboxy-group chromophore. We have already shown that c.d. can be used for analysis of the conformation and composition of uronic acid residues in alginates.

In this family of polysaccharides the component α-L-guluronate and β-D-mannuronate residues are arranged in homopolymeric polyguluronate or polymannuronate blocks, or heteropolymeric alternating blocks,[6] each containing 20 or more residues. It is now generally agreed that the gels are held together principally by co-operative associations between polyguluronate sequences in a regular buckled chain conformation, which sandwich cations in an ' egg-box ' fashion to form microcrystalline junction zones.[2,3,30] Alginates with a high content of suitable binding sites form highly cross-linked gels which are strong and brittle, whereas gels with a lower content are weak and more elastic. Alginate composition is therefore important for technological applications. The limits within which the c.d. varies are defined by the spectra of the homopolymeric sodium poly-(D-mannuronate) and poly-(L-guluronate) blocks. As shown in Figure 5 these broadly resemble the corresponding methyl glycoside spectra, but there are appreciable second-order differences, showing that c.d. is sensitive to neighbouring residues in the alginate chain. This provides the basis for a method of complete block determination by matching observed alginate c.d. with a linear combination of the spectra of the three constituent blocks, and we are at present developing this approach into a routine technique.

For the moment, however, a much simpler analysis of recorded spectra gives the residue composition. Because of their opposite configurations at C(5), the contributions of the two monomers to the overall alginate spectrum in the $n \longrightarrow \pi^*$ region are of opposite sign. Since they are also of opposite configuration at C(4), their principal bands are offset as shown in Figure 4, allowing the relative amounts of the two constituents to be assessed. Thus all sodium alginate c.d. spectra show a trough centred around 212 nm and a peak around 200 nm which may be assigned to $n \longrightarrow \pi^*$ transitions of the carboxy-groups on the two component residues. To a first approximation the depth of the trough appears to be related to guluronate content and the peak height to mannuronate content. Thus peak height/trough depth (Figure 5) increases with increasing mannuronate content, providing a direct index of primary structure. This parameter has been calculated for the alginate samples studied and is given in Table 2 along with the estimates of alginate composition obtained by other methods.[6,8] The alginate samples 5 and 6, and 8 and 9, which differ in molecular weight but not in composition, give extremely similar c.d. spectra. The blocks also give spectra which correspond to the polymers, as shown in Figure 6,

TABLE 2

Correlation of alginate composition and c.d.

Sample	Source	%M	%G	Peak/Trough	Ref.
1	Partial hydrolysis of 7	0	100	0.08	*a*
2		60	40	0.71	*b*
3		77	23	1.44	*a*
4	Partial hydrolysis of 8	93	7	1.95	*c*
5	*Laminaria hyperborea*	30	70	0.15	*a*
6		31	69	0.18	*a*
7		37	63	0.23	*a*
8	*Ascophyllum nodosum*	58	42	0.70	*a*
9		54	46	0.70	*a*
10	*Macrocystis pyrifera*	61	39	0.75	*c*
11	*Azotobacter vinelandii*	51	49	0.54	*d*

[a] Average of chemical and n.m.r. determinations.[6,8] [b] Chemical determination only.[6] [c] N.m.r. determination only.[8] [d] Value supplied with the sample.

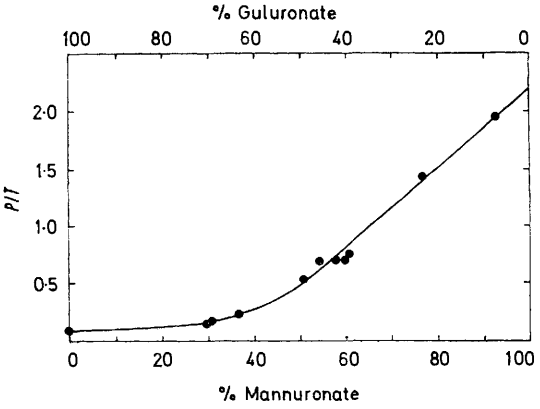

FIGURE 6 The relative magnitude of the peak height (P) and trough depth (T) indicated in Figure 5 varies isotonically with monomer composition. The parameter P/T can thus be used as a direct index of alginate primary structure

where composition is plotted as a function of peak height/trough depth. It therefore appears that polysaccharide c.d. is independent of molecular weight.

[27] E. D. T. Atkins and D. H. Isaac, *J. Mol. Biol.*, 1973, **80**, 773; S. Arnott, J. M. Guss, D. W. L. Hukins, and M. B. Mathews, *Biochem. Biophys. Res. Comm.*, 1973, **54**, 1377; I. A. Nieduszynski and E. D. T. Atkins, *Biochem. J.*, 1973, **135**, 729.

[28] A. S. Perlin, B. Casu, G. R. Sanderson, and L. F. Johnson, *Canad. J. Chem.*, 1970, **48**, 2260.

[29] A. S. Perlin and G. R. Sanderson, *Carbohyd. Res.*, 1970, **12**, 183; A. S. Perlin, D. M. Mackie, and C. P. Dietrich, *ibid.*, 1971, **18**, 185.

[30] D. A. Rees, *Adv. Carbohydrate Chem.*, 1969, **24**, 267; O. Smidsrød and A. Haug, *Acta Chem. Scand.*, 1972, **26**, 2063; O. Smidsrød, A. Haug, and S. G. Whittington, *ibid.*, p. 2563; S. J. Angyal, *Pure Appl. Chem.*, 1973, **35**, 13; O. Smidsrød, *Faraday Discuss. Chem. Soc.*, 1974, **57**, 263.

EXPERIMENTAL

General Methods.—Solvents used for paper chromatography were (a) butan-1-ol–ethanol–water (4 : 1 : 5; upper layer) and (b) ethyl acetate–acetic acid–formic acid–water (18:3:1:4). Chromatograms were developed by spraying with aniline oxalate or silver nitrate solutions, and sugar mobilities are quoted relative to galactose (R_{Gal}) or methyl α-D-glucoside ($R_{Me\alpha G}$).

Synthesis of Methyl Glycosides.—Methyl α- and β-D-glucoside (m.p. 168—170 and 113—115° respectively) were supplied by Koch–Light. Methyl α-D-mannoside (m.p. 191—193°) was supplied by Kerfoots.

Methyl-β-D-mannoside. D-Mannose (20 g) was refluxed for 18 h in methanolic 3% hydrogen chloride (50 ml). The solution was cooled and, on scratching the flask, crystalline methyl α-D-mannoside separated. The α-anomer was filtered off and the filtrate was neutralised with silver carbonate, filtered, and diluted with water (1 vol.). The solution was deionised with Amberlite IR-120 (H^+) and Dowex 1 × 8 (OH^-) resins, and concentrated to a syrup (1.1 g). Methyl β-D-mannoside {99 mg; m.p. 58—66°; $[\alpha]_D$ −55° (*c* 0.88 in H_2O)} was isolated from the syrup by chromatography on Dowex 1 × 2 resin (200—400 mesh).[31]

Crude methyl D-gulosides. 1,2:5,6-Di-*O*-isopropylidene-α-D-gulofuranose (4.6 g), prepared from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose,[32] was refluxed in methanolic 3% hydrogen chloride (50 ml) for 17 h on a boiling water-bath. The solution was neutralised with silver carbonate, filtered, diluted with water (1 vol.), treated with Amberlite IR-120 (H^+) and Dowex 1 × 8 (OH^-) resins, filtered, and concentrated to a syrup (434 mg). Paper chromatography [solvent (a)] showed the presence of four main components with R_{Gal} 1.3, 2.2, 2.5, and 2.7, respectively (silver nitrate spray). The component with mobility 1.3 gave a faint colour with aniline oxalate spray and corresponded to D-gulose.[33]

Separation of the anomeric methyl gulosides from 1,6-anhydrogulose. The methyl guloside mixture (430 mg) and trityl chloride (500 mg) in pyridine (10 ml) were heated for 30 min at 100 °C and the solution was poured into ice–water (150 ml). The mixture was extracted with chloroform (4 × 50 ml), and the extracts were dried (Na_2SO_4) and concentrated to a syrup (899 mg). This was heated in 80% acetic acid (20 ml) for 30 min at 95 °C, then poured into water (150 ml), and the precipitate was filtered off. The aqueous solution was extracted with chloroform (3 × 50 ml) and concentrated to a small volume, and water was added to it and distilled from it several times. Chromatography of the syrup (263 mg) showed [solvent (a)] three components with R_{Gal} 1.3, 2.2, and 2.8 in the visually estimated proportions 1:4:1. The component with R_{Gal} 1.3 gave a brown colour with aniline oxalate spray.

Separation of the anomeric methyl gulosides. The syrupy methyl guloside mixture (260 mg) was dissolved in water (1.0 ml) and applied to a column (1.5 × 38 cm) of Dowex 1 × 2 resin (200—400 mesh) in the hydroxide form. Elution was carried out with carbonate-free water, and monitored by polarimetry because the anomeric methyl gulosides had the same mobility on paper chromatograms. Appropriate fractions were combined to yield as syrups methyl α-D-gulopyranoside {49 mg; $[\alpha]_D$ +108° (*c* 0.98 in H_2O); lit.,[34] (monhydrate) +109.4°} and methyl β-D-gulopyranoside {98 mg; $[\alpha]_D$ −72° (*c* 1.96 in H_2O); lit.,[34] −83.3°}. Each anomer had R_{Gal} 2.2.

Methyl α- and β-D-galactoside and α- and β-D-idoside were prepared as previously described.[23,35] All m.p.s and specific rotations were in agreement with literature values.

TABLE 3

Summary of methyl glycuronoside syntheses

Methyl glycoside (mg)	Volume of 'oxidation' solution (ml)	Wt. of catalyst (mg)	Oxidation time (h)	Yield of crude oxidation mixture (mg)	Yield of purified methyl glycuronoside (mg)
α-Glucoside (300)	10	100	5		133
β-Glucoside (300)	10	100	5		214
α-Galactoside (300)	10	100	8		180
β-Galactoside (150)	5	50	6	143	84
α-Idoside (218)	9	100	8	221	143
β-Idoside (96)	4	60	7½	90	68
α-Mannoside (300)	10	100	6½		227
β-Mannoside (86)	4	60	8	76	43
α-Guloside (47)	3	50	6½	40	18
β-Guloside (78)	4	60	6½	63	50

Synthesis of Methyl Glycuronosides.—These were prepared from the corresponding neutral glycosides by catalytic oxidation using a procedure developed from that described by Perlin *et al.*[23] The general method is given for one example only.

Methyl α-D-glucuronoside (NH_4^+ salt). Methyl α-D-glucoside (300 mg) in water (10 ml), with platinum black (100 mg), was heated to 60 °C and the pH was adjusted to 8 (pH paper) by dropwise addition of 0.5M-sodium hydrogen carbonate solution. Oxygen was bubbled through the solution in a test-tube at a sufficient rate to prevent the catalyst settling to the bottom. The pH was readjusted to 8 periodically until (after 4 h) the expected amount (3.1 ml) of sodium hydrogen carbonate solution had been added. The reaction was continued for a further hour (during which time the pH remained constant) and the solution was filtered and concentrated to a syrup. Paper chromatographic examination [solvent (a)] showed sodium methyl α-D-glucoronoside as the main component, with a small amount of starting material. The syrup was dissolved in water (3.0 ml) and applied to a DEAE Sephadex column (1 × 15 cm; carbonate form). The column was washed with water (250 ml) to remove neutral sugars, followed by 0.1M-ammonium carbonate, buffered to pH 7 with carbon dioxide. Fractions (25 ml) were collected and examined for sugar content by use

[31] P. W. Austin, F. E. Hardy, J. G. Buchanan, and J. Baddiley, *J. Chem. Soc.*, 1963, 5350.

[32] K. Onodera, S. Hirano, and N. Kashimura, *Carbohyd. Res.*, 1968, **6**, 276; P. J. Benyon, P. M. Collins, P. T. Dogangs, and W. G. Overend, *J. Chem. Soc.*, 1966, 1131; W. Meyer Zu Reckendorf, *Chem. Ber.*, 1969, **102**, 1071.

[33] G. J. F. Chittenden, *Carbohyd. Res.*, 1970, **150**, 101.

[34] H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 1932, **8**, 1.

[35] E. Sorkin and T. Reichstein, *Helv. Chim. Acta*, 1945, **28**, 1.

of the phenol–sulphuric acid reagent.[36] Fractions 3—5 were combined and concentrated to a syrup which was evaporated several times with water to remove ammonium carbonate, then dissolved in water (30 ml) and extracted with ether (5 × 20 ml) to remove coloured impurities. The aqueous solution was diluted with ethanol (70 ml), further decolourised with charcoal, filtered, and concentrated to a syrup (133 mg). Paper chromatography [solvent (a)] showed methyl α-D-glucopyranuronoside (NH_4^+ salt) as the only product.

The preparation of other methyl glycuronosides is summarised in Table 3. All products were essentially pure by ^1H n.m.r. spectroscopy and by paper chromatography [solvents (a) and (b)], except methyl β-D-iduronoside, methyl α-D-guluronoside, and methyl β-D-guluronoside whose purities by n.m.r. were 75, 60, and 50%, respectively. A pure sample of methyl β-D-guluronoside was prepared by an alternative procedure described below, but the c.d. spectrum was the same, evidently because the impurities did not contribute to c.d. That this was also true for the other two impure products was confirmed partly by consistency of the spectra with those of the other uronic acid glycosides, and partly because ketone c.d. was absent from these as well as all other products.

Methyl β-D-guluronoside (NH_4^+ salt); alternative preparation. The reaction sequence is an adaptation of Antia and Perry's route to methyl β-D-guloside.[37] Methyl β-D-*glycero*-D-*gulo*-heptoside (150 mg) was dissolved in water (3.0 ml) and cooled in an ice-bath. 0.25M-Periodic acid (3.0 ml, 1.1 mol) was added, and the solution was left for 30 min. Barium hydroxide (1N) was then added to pH 9.5 to prevent further oxidation. After centrifugation, the supernatant solution was concentrated (to *ca.* 20 ml) and bromine (235 mg, 0.080 ml) was added with shaking. The solution was kept in the dark for 48 h, and the excess of bromine was removed by aeration. The solution was neutralised with silver carbonate, filtered, concentrated to a small volume, and applied to a DEAE Sephadex column (1 × 10 cm; carbonate form). The neutral sugars were eluted from the column with water, and the acidic glycoside was eluted with 0.01M-ammonium carbonate (buffered to pH 7.4 with carbon dioxide). The ammonium carbonate was removed by concentration several times with water to yield the ammonium salt of methyl β-D-gulopyranuronoside (88 mg). Paper chromatography [solvent (b)] showed that the main component was non-reducing with $R_{MeαG}$ 0.83. Traces of reducing sugar were also detected. The high purity of the product was confirmed by ^1H n.m.r. spectroscopy.

Alginate Samples.—The alginate samples coded 5—10 were all from commercial sources; those coded 5—9 were supplied by Alginate Industries Limited and sample 10 was obtained from Alginates Australia Limited. All the above samples are of seaweed origin and were characterised by partial acidic hydrolysis,[6,8] allowance being made for differential breakdown of mannuronic acid and guluronic acid on complete acidic hydrolysis. Samples 1—3 were prepared by partial acidic hydrolysis of sample 7, and approximate to the three oligomer block types. Sample 4, which approaches even more closely to pure polymannuronate, was prepared similarly from sample 8. Sample 11 is a bacterial alginate kindly supplied by Dr. C. J. Lawson of Tate and Lyle.

Measurement of Circular Dichroism.—C.d. spectra were recorded on a Cary 61 c.d. spectropolarimeter (1 mm path length cell; 10 s integration period). Sample temperature was regulated using a Haake thermocirculator and a cell holder with constant temperature attachment. Sodium alginate solutions (3 mg ml^{-1}) were in deionised water and were brought to pH 7 with sodium hydroxide if necessary. For alginate analysis concentration and pathlength are not critical, and any available cell may be used provided that a reliable, measurable spectrum is obtained down to at least 195 nm. Alginate spectra do, however, change with temperature, to at least the same extent as the monomer spectrum shown in Figure 3, with spectral features becoming less pronounced as the temperature is increased. The spectra are also sensitive to pH, again to at least the same extent as in the monomer case illustrated in Figure 2. As mentioned in the introduction, the presence of divalent metal ions produces marked changes in alginate c.d., and thus salt form is of critical importance. All spectra in this paper were recorded at 25 °C on pure sodium alginate solutions at pH 7.0. It is imperative to adhere to these conditions if the spectral data given in Table 2 are to be used. Provided these precautions are observed, however, composition may be read directly from a calibration graph such as is shown in Figure 6. Solutions of uronic acid derivatives were made by dissolving *ca.* 10 mg of syrupy product in distilled water (10 ml), and converting to the sodium form by ion exchange on an IR-120 column (4 mm × 4 cm). The acid form was obtained when required by subsequent addition of 2N-hydrochloric acid. Exact concentrations were obtained by n.m.r. spectroscopy, with sodium acetate as internal standard. For n.m.r. analysis the solutions, with internal standard added, were evaporated to dryness and redissolved in D_2O, several times.

Curve resolution. Observed spectra were matched by combination of a small number of gaussian bands, both visually with a Du Pont 310 analogue curve resolver, and digitally with a least-squares computer program. For ill-resolved overlapping bands such spectral analysis is highly subjective, and several plausible interpretations may frequently be advanced. In the present work, however, band maxima are well separated, and resolution into the minimum number of gaussian bands consistent with the recorded spectra is precise and unambiguous. Physical interpretation of these resolved bands is, of course, subject to the same uncertainties which apply to interpretation of single non-overlapping peaks, and in particular, undetected superposition of bands of different electronic origin but similar frequency cannot be precluded.

We thank Dr. G. T. Grant for pectin spectra, Dr. A. Penman for provision of characterised alginates and alginate fragments, Dr. J. W. B. Samuel for preliminary work on D-*glycero*-D-*gulo*-heptoside, Dr. B. Casu for n.m.r. analysis of selected uronic acid derivatives, and Mr. G. Young for expert technical assistance.

[5/114 *Received*, 20*th January*, 1975]

[36] M. Dubois, K. A. Gillies, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, 350.

[37] N. J. Antia and M. B. Perry, *Canad. J. Chem.*, 1960, **38**, 1017.