CIRCULAR DICHROISM AND THE CATION BINDING TO POLYURONATES[a]

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The CD spectra of K-, Mg- and Ca-salts of D-galacturonic acid, its α-methyl glycoside, of pectic and polymannuronic acids were investigated. The CD curves in molecular-disperse solutions of uronates and polyuronates with a pure electrostatic binding of cations are virtually independent on the kind of the appropriate cation and its valence. The great intensity decrease of the CD band in solutions of Ca-pectate evidences an extraordinary strong interaction of Ca^{2+} ions with the carboxylate chromophore. The results prove that Ca-pectate solutions are not molecular-disperse, but contain aggregates of macromolecules (micro-gel particles) in agreement with the preceding conception of the intermolecular binding of Ca^{2+} ions to polygalacturonate.

Circular dichroism of acid polysaccharides is a sensitive tool showing changes in the molecular environment of anionic groups. Chiroptic methods have been, therefore, well utilized to study the interaction of mucopolysaccharides with basic dyestuffs and polypeptides and also to examine the conformation changes of macromolecules under the formation of these complexes (*e.g.*[1,2]). As shown by Morris, Rees, Grant, Bryce and co-workers[3-5] the transition of a polyuronate sol to a gel or film by replacement of the solvent, by an alteration of temperature, by evaporation of solutions and mainly by action of Ca^{2+} ions, results in a considerable variation of CD bands of those substances.

Our preceding papers[6-9], dealing in detail with the mechanism of binding of more-valent cations to polyuronides, were based mainly on the determination of the activity coefficients of counterions. This paper is aimed to contribute to the evidence of an intermolecular binding of Ca^{2+} ions to carboxyl groups in solutions of calcium polygalacturonate by means of CD curves of various salts of polyuronic acids.

EXPERIMENTAL

Chemicals

D-Galacturonic acid (monohydrate) *puriss.* and pectin were commercial preparations (Fluka, Buchs, Swiss and Københavns Pektinfabrik, Denmark, respectively). Methyl methyl (α-D-galacto-

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pyranosid)uronate, m.p. 145–147°C, $[\alpha]_D$ +122° (*c* 1·0, H_2O) was prepared according to[10]. Polymannuronic acid prepared by Haug and Smidsrød[11] contained 94% of D-mannuronic and 6% of L-guluronic acid units. Its average molecular weight ($\bar{M}\eta$) was greater than 500000. Pectic acid prepared by an alkaline deesterification[12] of pectin in ethanol (60%) was purified as reported earlier[13]. Pectic acid contained 90·5% of polygalacturonic acid and 9·5% of neutral saccharides: D-galactose, L-arabinose, D-glucose, D-xylose and L-rhamnose in a molecular ratio 9 : 3 : 2 : 1 : 0·5. The esterification degree (*E*) of its carboxyl groups by methanol was 4%, the average molecular weight determined viscometrically ($\bar{M}\eta$) 23000 and the sulphate ash content 0·1%. Pectinic acid (*E* 88·5%, the content of polyuronide 90·0%) was prepared by esterification of a purified pectin preparation with methanolic 1M-H_2SO_4 at 3°C for 3 weeks[14].

The 0·05M-NaOH and 0·05M-KOH solutions were carbonate free. The concentration of the clear calcium hydroxide solution was 0·021M. Other chemicals were of analytical grade; the redistilled water, previously boiled and cooled, had a minimal content of atmospheric CO_2.

Preparation of Salts of Uronic and Polyuronic Acids

Methyl (α-D-galactopyranosid)uronic acid was prepared by alkaline deesterification (18 h) of its methyl ester in a solution of 0·01M concentration in 0·025M-NaOH in a closed flask at room temperature. The excess of sodium hydroxide was quantitatively removed by percolation through a Dowex 50 W × 2 column in H^+ form. The percolate was diluted to a concentration amounting 4–5 mequiv. [COOH]/1. Pectic and polymannuronic acids were dissolved by a succesive neutralization with 0·05M-NaOH, centrifuged at 13000*g* and diluted to contain 4–5 mequiv. [COOH]/1. Ion-exchanger was employed to prepare clear solutions of these uronic acids similarly as in the above-mentioned case. The potassium and calcium uronates and polyuronates were obtained by neutralization of the respective acids with 0·05M-KOH or 0·021M $Ca(OH)_2$ to the point of equivalence using potentiomeric indication of pH. During neutralization of pectic acid with calcium hydroxide a partial coagulation of calcium pectate occurred. The gel-like coagulate was centrifuged at 13000*g* and the clear supernatant containing the soluble calcium pectate was subjected to CD measurement. Other uronic and polyuronic acids gave clear solutions of corresponding Ca-salts. Magnesium salts were prepared by neutralization of 2·00 and 4·00 mequiv. [COOH]/1 uronic and polyuronic acids with magnesium oxide added in a very small excess. The unreacted portion of MgO was filtered off and the solution of magnesium pectate (pH ~ 9·5) was adjusted to pH ~ 7·5 by the starting solution of the uronic acid.

Analytical Methods

The characterization of pectic and pectinic acids (the content of polyuronide, the esterification degree *E*, the content of neutral saccharides and the viscometric estimation of molecular weight) were done by methods described earlier[13]. The concentration of carboxyl groups in solutions of uronic and polyuronic acids was determined alkalimetrically by potentiometric titration with a glass Radiometer electrode with an error not exceeding ±0·3%. The concentration of salts of these acids in solution was calculated from the consumption of a hydroxide needed for their neutralization, or from the starting concentration. The concentration of carboxyl groups in calcium pectate solution (supernatant) was determined by a modified carbazole method[15] with a ±1% error.

The concentration of $CaCl_2$ and $MgCl_2$ solutions was determined by a chelatometric titration with a 0·01M solution of Complexon IV and a photometric indication of the point of equivalence (Ca-interference filter Zeiss, Jena, IF 600 nm, murexide; Mg—IF 650 nm, eriochrome black T).

The concentration of substances for CD measurement was 2·00 and 4·00 mequiv. [COOM]/l, (M = H, K, $Mg_{0.5}$, $Ca_{0.5}$). The pH of uronic and polyuronic acid solutions was not adjusted, the solutions of salts had pH 7·2—7·5. The most spectra were taken with a UV/ORD-5 Jasco spectrophotometer with a CD adapter in 0·5 and 1·0 cm cells at room temperature. The CD curves of polymannuronate and pectinate solutions (E 88·5%) were measured with a Roussel-Jouan Dichrograph, model 185/II in 0·5 and 1·0 cm cells, respectively. Analyses including the preparation of solutions of uronates were more times repeated. The ellipticities $[\Theta]$ in Table I are the average values of 4 to 6 analyses (excepting the sample 2) and an average of 2 analyses in Table II.

RESULTS AND DISCUSSION

The fundamental backbone of pectic acid is formed by D-galacturonic acid units in a *C1* conformation bonded in a linear chain by diaxial *trans*-glycosidic bonds $\alpha(1 \rightarrow 4)$, ref.[16]. Therefore, we first of all investigated the chiroptical properties of D-galacturonic acid, its α-methyl glycoside, and salts of these uronic acids (Table I). All spectra displayed a single Cotton effect in the positive region of ellipticity $[\Theta]$ with a maximum at 205, or 209 nm due to an $n \rightarrow \pi^*$ transition of the carboxyl group. The CD spectra of potassium and calcium D-galacturonate solutions are virtually identical, similarly as those of potassium, magnesium and calcium methyl (α-D-ga-

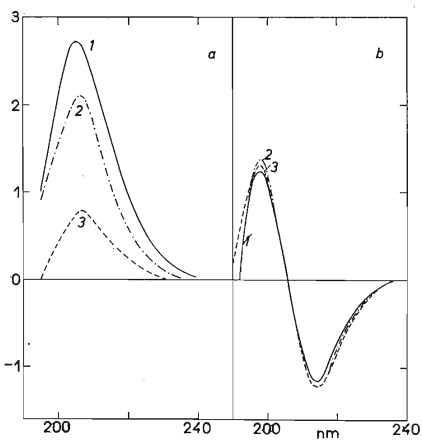

FIG. 1

The CD Spectra of Polyuronates

a Pectate, *b* polymannuronate, *1* K-, *2* Mg-, *3* Ca-polyuronate, axis *y* in $[\Theta] \cdot 10^{-3}$.

lactopyranosid)uronates. The kind of the cation (K^+, Mg^{2+}, Ca^{2+}) does not influence the CD curves of solutions of the monomeric uronates.

The CD spectra of potassium, magnesium and calcium pectate solutions seen in Fig. 1*a*, are commented in Table I. In contrast to solutions of the monomeric uronates, the kind of the cation considerably alters the CD spectrum: the character of the dichroic band associated with the $n \rightarrow \pi^*$ transition remains unchanged and the position of the maximum (λ) is shifted only within a 2 nm range, but the intensity of the band was notably altered. The extraordinary great decrease of ellipticity $[\Theta]$ was found in the solution of calcium pectate.

Further, the influence of ions of simple salts on the CD spectrum of the esterified carboxyl group (—$COOCH_3$) has been examined. The spectra were investigated in solutions of salts of a highly-esterified pectinic acid of esterification degree *E* 88·5% in the presence of KCl, $MgCl_2$ or $CaCl_2$ in an amount equivalent to the total concentration of carboxyl group (2·00 mequiv./l). The interaction of Ca^{2+} ions in a highly-esterified pectin with the "isolated" carboxyl groups is very weak[17]. In such pectin preparations the remaining free carboxyl groups are very remote. Results listed in Table I bring evidence that the added ions K^+, Mg^{2+}, and Ca^{2+} do not influence the CD spectrum of —$COOCH_3$ groups in a highly-esterified pectin under the given conditions. The course of the above described spectra is in accordance with the data dealing with the CD of methylglycosides of uronic acids and their sodium salts[18,19] and also with those of sodium polygalacturonate[4].

TABLE I

Circular Dichroic Characterization of Solutions of D-Galacturonic, Pectic, Pectinic Acids and Their Salts

Uronic acid	H		K		Mg		Ca	
and salt	λ^a	$[\Theta] \cdot 10^{-3}$	λ^a	$[\Theta] \cdot 10^{-3}$	λ^a	$[\Theta] \cdot 10^{-3}$	λ^a	$[\Theta] \cdot 10^{-3}$
D-Galacturonic acid[b]	204	3·69 ± 0·07	205	1·54 ± 0·02	—	—	205	1·51 ± 0·02
Methyl(α-D-galacto-pyranosid)uronic acid	—	—	209	1·95	209	1·95	209	1·95
Pectic acid[c]	204	5·02 ± 0·16	205	2·74 ± 0·10	206	2·11 ± 0·16	207	0·79 ± 0·05
Pectinic acid (*E* 88·5%)[d]	—	—	207	4·66 ± 0·08	206	4·66 ± 0·07	207	4·66 ± 0·05

[a] λ (nm), $[\Theta]$ (degree cm^2 $dmol^{-1}$); [b] dissociation degree $\alpha = 0{\cdot}27$; [c] $\alpha = 0{\cdot}17$; [d] KCl, $MgCl_2$, $CaCl_2$ added — see text.

The circular dichroism of solution of polymannuronic acid and its salts is described in Table II and Fig. 1*b*. The CD spectra of solutions of polymannuronates show, in contrast with those of pectates, two chiroptic bands of different sign; they differ very little from each other irrespectively of the kind and valence of cations K^+, Mg^{2+}, Ca^{2+} bound to the polyuronate. The band in the longer wave-length region of the polymannuronate is substantially more significant than that of the free acid. The results are in agreement with the spectra of β-methylglycoside of the monomeric D-mannuronic acid, its sodium salt[19] and sodium polymannuronate[3], published earlier.

The electrostatic interaction of cations with the —COO^- chromophore of uronates is an important factor when interpreting the CD spectra. Dilute solutions of monomeric uronates behave as strong electrolytes, as has been proved on the basis of activity coefficient $\gamma_{Ca^{2+}}$ determined in solutions of calcium uronates[6,9]. This characteristic feature of monouronates is manifested by the finding that the kind of cations does not influence the CD spectra of salts of D-galacturonic acid and its α-methylglycoside.

Due to the diequatorial *trans*-glycosidic bonds $\beta(1 \rightarrow 4)$ the linear macromolecules of polymannuronate resemble a flat ribbon[20]; in dilute solutions they do not form aggregates of macromolecules[7]. The binding of ions K^+, Mg^{2+} and Ca^{2+} to the polymannuronate is of pure electrostatic nature[6–8]. The CD spectra of polymannuronates are very similar in spite of the fact that the interaction of cations with carboxyl groups of polymannuronate is substantially more intense than that of monomeric uronate[6] and depends on the valence of cations (Fig. 1*b*). Differences between the $[\Theta]$ values lie in the 6 to 10% range, this being close to experimental errors. It could be, therefore, deduced that various cations, bound to an anionic chromophore by pure electrostatic forces, influence the character of CD spectra only very little.

A quite different course of CD curves is seen in solutions of pectates. A great decrease of intensity of the chiroptic band in the solution of calcium pectate (Fig. 1*a*,

TABLE II

Circular Dichroic Characterization of Solutions of Polymannuronic Acid and Its Salts

Form	λ_1[a]	λ_0	λ_2	$[\Theta]_1 \cdot 10^{-3}$	$[\Theta]_2 \cdot 10^{-3}$
H[b]	231	218	199	−0·28 ± 0·03	1·48 ± 0·07
K	214	205	198	−1·15 ± 0·07	1·25 ± 0·07
Mg	214	206	198	−1·22 ± 0·03	1·38 ± 0·07
Ca	214	206	198	−1·22 ± 0·07	1·32 ± 0·07

[a] λ (nm); $[\Theta]$ (degree cm^2 $dmol^{-1}$); [b] dissociation degree $\alpha = 0{\cdot}27$.

curve 3) evidences an extraordinary strong interaction of Ca^{2+} ions with the carboxyl chromophore. Such an interaction is further confirmed by the anomalously low activity coefficients $\gamma_{Ca^{2+}}$ (0·06 to 0·10) found in solutions of calcium pectate[6,17] and calcium polygalacturonate[7]. The $\gamma_{Ca^{2+}}$ values are in these systems by few times lower than the theoretical ones corresponding to a pure electrostatic Ca^{2+} bond. As we have shown earlier by preparative ultracentrifugation, the calcium pectate solutions contain little aggregates of macromolecules. The anomalously low $\gamma_{Ca^{2+}}$ values are caused, according to our opinion, by an intermolecular chelate binding[7] in concordance with the "egg-box" binding model described by Rees and co-workers[4] (for more detail see[8,21]).

Morris[3], Grant[4] and co-workers have shown that the transition of sodium alginate and pectate sols into gels by the action of Ca^{2+} ions resulted in a significant difference of CD spectra. The formation of the calcium pectate gel is associated with the shift of the chiroptic band towards the shorter wave-length (from 203 to 190 nm), whereas the decrease of intensity is relatively little. The exchange of cations $Ca^{2+} \rightarrow$ $\rightarrow K^+$ in solutions of pectates did not result in a hypsochromic shift. The maxima of the chiroptic bands lie in the 205 to 207 nm region; on the other hand, the intensity of the band in solution of calcium pectate considerably decreases. The spectrum of magnesium pectate undergoes a substantially less change in consonance with findings on the strength of the Mg^{2+} binding to pectate[22–24].

The Ca^{2+} ion approaches very closely two carboxyl groups of two different chains in an intermolecular chelate binding. It simultaneously contacts oxygen atoms of hydroxyl groups, of a pyranoid ring system and that of a glycoside bond in "cavities" between the oriented chains of macromolecules[4,25]. Consequently, the molecular environment of the carboxyl chromophore and thereby also of the CD spectrum have been altered. A similar change in the spectra is encountered also with the electrostatic binding of monovalent cations (Na^+), but only in cases, when the solution of polyuronates was evaporated to a film, whereby, the macromolecules come to a tight proximity[5].

The CD spectrum of dilute solutions of calcium pectate evidences that these solutions are not (in contrast to solutions of polymannuronate) molecular disperse, but contain little aggregates of macromolecules (micro-gel particles) in agreement with our conception of the intermolecular binding of Ca^{2+} ions to polygalacturonate.

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