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Solution Properties of a New Polyelectrolyte Derived from the Polysaccharide Scleroglucan

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ABSTRACT

Selective, quantitative oxidation of pendant 1,6-β-linked glucopyranose residues along scleroglucan chains has yielded a new carboxylated polyelectrolyte. Study of the aqueous solution properties of the latter (named sclerox-I) has disclosed rather anomalous behaviour. Viscosity, calorimetric and chiro-optical measurements show that the interaction of sclerox-I with Ca^{2+} ions leads to a change in conformation of the polyanions, probably of the type random coils → ordered, elongated forms.

INTRODUCTION

Scleroglucan is a capsular polysaccharide secreted by the mycelia of certain *Fungi Imperfecti*, notably by species of the genus *Sclerotium*.

The structure of the polymer produced by *Sclerotium glucanicum* was characterized by Johnson *et al.* (1963) as a linear chain of 1,3-β-linked D-glucopyranose units with single D-glucopyranose residues 1,6-β-linked to every third unit of the chain.

The carbohydrate polymer is presently produced commercially by CECA AS (France) under the trade name Biopolymer CS, and finds a number of practical applications (Sandford, 1979; Bluhm *et al.*, 1982).

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Recent conformational studies of scleroglucan in the solid and solution states indicate that the backbone conformation is similar to that previously observed for curdlan, i.e. a triple helix, and that in aqueous solution it can give rise to order → disorder transitions (Yanaki *et al.*, 1981; Bluhm *et al.*, 1982).

Starting from purified scleroglucan, we have prepared a carboxylated polysaccharide by quantitative periodate oxidation of glucopyranose side chains followed by their further oxidation with $NaClO_2$ in water (Fig. 1). The chemical structure of the new polyelectrolyte has been confirmed by the results of NMR and potentiometric studies.

We now wish to report the results of a study of certain solution features of such a polyelectrolyte (Fig. 1) which henceforth in this paper will be designated sclerox-I. Attention has been mainly focused on the conformational implications of the interaction between sclerox-I and Ca^{2+} ions in dilute aqueous solution. The experiments have been carried out with the aid of microcalorimetric, chiro-optical and viscometric techniques.

EXPERIMENTAL

Materials

(a) *Preparation, purification, and characterization of oxidized scleroglucan (sclerox-I)*

A purified scleroglucan sample from CECA AS, France, has been used for this study. The preparation (trade name Actigum CS-11) containing 85–90% of polysaccharide may be dissolved in water yielding slightly turbid solutions. Its intrinsic viscosity in 0·01 N NaOH at 25°C is 4·8 dl g^{-1} which, considering the $[\eta]$-M data provided by Yanaki *et al.* (1981) for scleroglucan, would correspond to $MW = 7 \times 10^5$.

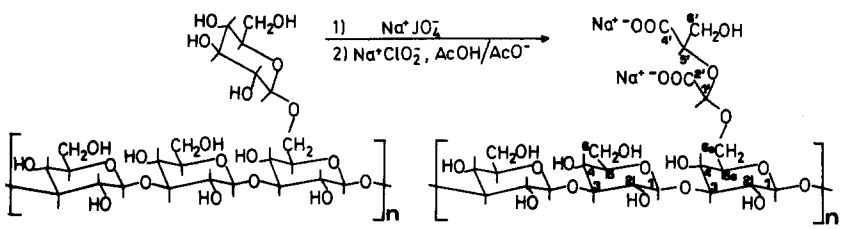

Fig. 1. Scheme of the oxidation process for scleroglucan.

Both oxidation steps of scleroglucan with periodate and then with chlorite were carried out following the procedure described by Hofreiter *et al.* (1957). To ensure the complete oxidation of the polysaccharide, as schematically indicated in Fig. 1, an excess of the oxidizing agent was used in each step.

After treatment with $NaIO_4$ the solutions of scleroglucan turn into soft gels: these are easily disrupted, however, by addition of $NaClO_2$, to yield clear, viscous solutions. These solutions, containing the new polymeric material, were neutralized with 1 N NaOH and first dialyzed against EDTA and then repeatedly against distilled water. Complete neutralization of carboxylate groups with Na^+ counterions was achieved by adding NaCl up to a final concentration of approximately 0·5 M. Exhaustive dialysis with distilled water was then performed in order to remove excess salts.

The polysaccharide sample was finally stored in freeze-dried form from which it is very easily soluble in water.

Potentiometric titrations and atomic absorption spectroscopic determination of Na^+ ions gave, within experimental error, practically the same equivalent weight of the repeating unit (sclerox-I), i.e. 360 ± 3 – the theoretical value (see Fig. 1) is 346.

Potentiometric titration data yielded a pK_a of 4·07 for 50% neutralized sclerox-I (0·2% w/v, 25°C in water).

The specific optical rotatory power of sclerox-I measured at room temperature in water, 0·05 M $NaClO_4$, and 0·2 M NaCl is $[\alpha]^{25}_{302} = 97 \pm 1$ (for the starting scleroglucan sample $[\alpha]^{25}_{302} = 56 \pm 1$ in water).

The intrinsic viscosity of sclerox-I in 0·05 M $NaClO_4$ at 25°C is 3·65 dl g^{-1}.

(b) *Other chemicals*

Methylene blue (MB) from Merck was used without further purification ($\epsilon = 7{\cdot}8 \times 10^4$ M^{-1} cm^{-1} at 664 nm).

A $1{\cdot}16 \times 10^{-4}$ M MB stock solution was stored in a flask covered with aluminium foil. Sodium metaperiodate was a Merck analytical grade product. Sodium chlorite (Fluka) was of technical grade. Pure calcium perchlorate (Fluka) and sodium perchlorate (Merck) were dissolved in volumetric flasks to give stock solutions of concentrations 0·375 N and 0·50 M, respectively, whose titre was checked by means of atomic absorption spectroscopy measurements.

Instrumentation

Optical activity measurements were carried out using a Perkin-Elmer 141M Spectropolarimeter. A water thermostated 1 dm cell was used and readings were normally taken at wavelengths of 302 and 365 nm. Polymer stock solution concentrations were in the range 0·09–0·18% (w/v).

Calorimetric measurements of the enthalpy of Ca^{2+}–sclerox interactions in dilute aqueous solution were performed using an LKB-10070 batch microcalorimeter at 25°C following a procedure already described by Fenyo *et al.* (1977).

Visible spectra at 25°C of free MB, sclerox-I/MB, and of sclerox-I/MB/Ca^{2+} in dilute aqueous solutions were recorded using a Cary-219 spectrophotometer.

CD spectra of the same solutions were recorded with a Cary-61 dichrograph.

For the viscosity measurements, the polymer solutions were filtered through sintered glass (G-4). A Cannon-Ubbelohde viscometer with an efflux time of 257·6 s for aqueous 0·05 M $NaClO_4$ at 25°C has been used.

The ^{13}C NMR spectra were obtained at 35°C with a Varian CFT-20 Fourier transform spectrometer at 20 MHz, with a 10 mm probe. A solution of sclerox-I–Na salt (150 mg) in D_2O (2 ml, 99·7% D) was used. Solutions at acid pD (5 and 3) were obtained by addition of concentrated DCl to the original solution (pD = 7). Scleroglucan (120 mg) was dissolved in Me_2SO-D_6 (2 ml). Experimental conditions were as follows: 90° pulse; 2K data points, with Fourier number 8K; 100–300K pulses; chemical shifts were measured with reference to internal dimethyl-sulphoxide-D_6 or methanol (39·5 and 51·75 ppm with respect to internal tetramethylsilane (TMS) and trimethylsilylpropionic acid–Na salt (TPS), respectively).

Resolution-enhanced spectra were obtained using the CDRE program. The attached proton test (APT) was made utilizing LT = DF = 6 ms.

RESULTS AND DISCUSSION

Structural characterization of the sclerox-I sample

In this section a brief comparative discussion is given of the ^{13}C NMR spectra of the scleroglucan and sclerox-I samples used in this work. The

spectrum of scleroglucan (which has not, to our knowledge, yet appeared in the literature) had to be obtained in dimethyl-sulphoxide (Me_2SO) since the spectrum of this polymer in water yields poorly resolved peaks.

The ^{13}C NMR spectrum of scleroglucan in Me_2SO–D_6 is shown in Fig. 2(A). This spectrum is typical of β-D(1 → 3)-linked glucan, with β-D(1 → 6) branching. Assignments are given by analogy with those reported for schizophyllan, laminarin and *gyrosphora esculenta* miyoshi (Saitô *et al.*, 1977; Gorin, 1981). The area of the signal of the substituted C-6 (C-6s, at 70·1 ppm) is approximately 1/4 that of the total primary carbons (C-6 OH, at 60·9 ppm, + C-6s), in agreement with the proposed arrangement of one side-chain glucose residue every three glucose residues in the main chain.

^{13}C chemical shifts of scleroglucan and sclerox-I are reported in Table 1; primed carbons are those of the side-chain residue and the suffix 's' refers to the 3,6-di-substituted residue.

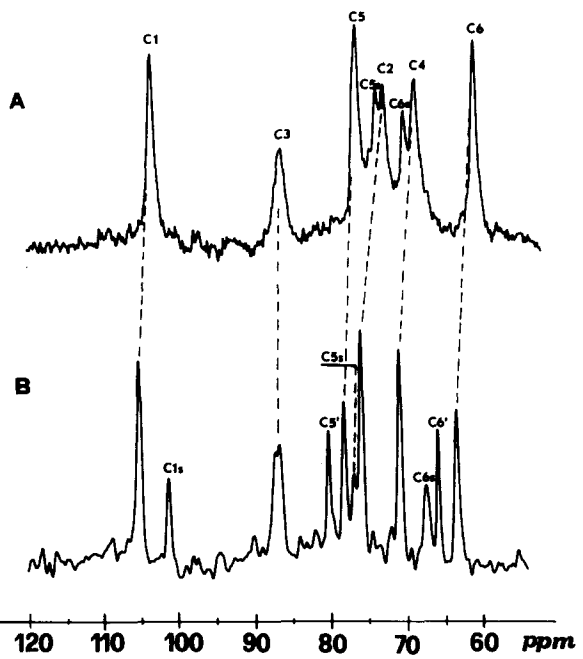

Fig. 2. ^{13}C NMR spectra at 20 MHz and 35°C of (A) scleroglucan in Me_2SO-D_6, (B) sclerox-I in D_2O. The dotted lines connect the strongest signals substantially corresponding to the β-D-(1,3)-linked glucose residues.

TABLE 1

^{13}C NMR Chemical Shifts (ppm) of Scleroglucan and Sclerox-I (see Figs 1 and 2)

Scleroglucan (in Me_2SO-D_6)		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
β-(1→3)	(C)	103·0	72·7	86·2	68·6	76·3	60·9
Side chain	(C′)	103·0	n.a.	n.a.	n.a.	n.a.	60·9
β-(1→3, 1→6)	(Cs)	103·0	n.a.	n.a.	n.a.	75·3	70·1
Sclerox-I (in D_2O)		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
β-(1→3)	(C)	105·1	76·0	87·3	71·0	78·5	63·7
Side chain	(C′)	105·1	179·5	–	176·5	80·5	66·1
β-(1→3, 1→6)	(Cs)	101·4	n.a.	n.a.	n.a.	77·2	67·8

n.a. = not assigned.

Values are referred to Me_2SO-D_6 and to MeOH (39·5 and 51·75 ppm with respect to internal TMS and TSP, respectively).

The NMR spectrum of sclerox-I in D_2O (Fig. 2(B)) consists of two sets of signals. The set with the strongest signals corresponds to carbons of the β-D-(1 → 3)-linked glucose residues of scleroglucan not bearing the side-chain. In contrast the set of weaker signals does not have an evident counterpart in the spectrum of the parent polysaccharide. These resonances are attributable to side-chain carbons (C′) and to carbons (Cs) of the residue bearing the side-chains (see Fig. 1), since the area of this set corresponds to approximately 1/4 of the total area of anomeric carbons (signals at 105·1 and 101·4 ppm). Furthermore, while major signals (the first set) are practically unaffected by pH changes, the chemical shifts of signals of the second set are markedly pH dependent, as expected for carbons belonging to carboxylated residues as well as to residues to which the latter are attached.

More specifically, signals at 66·1 and 67·8 ppm (which are in the region of CH_2 carbons (Gorin, 1981) and were confirmed to belong to methylene groups by ATP experiments) were assigned to C-6′ and C-6s, respectively, since the former is sharper, suggesting that it belongs to the more mobile side-chain (Gorin, 1981).

Other assignments are more tentative, and based on assumed chemical shift changes upon splitting and carboxylation of the side-chain of scleroglucan. Thus, distinction between C-5 and C-5′ was made on account of the downfield shift of the latter with respect to the C-5 of scleroglucan as expected because of the electronegativity of the α-carboxylate group.

The assignment of C-1′ thought to be hidden beneath the main C-1 signal is more uncertain, since this signal might experience opposite shifts due to conformational effects.

However, such an assignment is supported by the appearance of a new signal upfield to the main C-1 signal with a correspondingly decreased intensity of this latter upon acidification (shifts are reversible on re-neutralization).

The most downfield signals (at 176·5 and 179·5 ppm, not shown in the figure) were assigned to the carboxylate carbons C-4′ and C-2′ respectively.

In conclusion the NMR spectra of Fig. 2, together with the equivalent weight data (see 'Experimental'), can be taken as clear evidence in favour of the structure of sclerox-I depicted in Fig. 1. Both set of data, however, suggest that the polymer samples considered may contain a few percent of impurities which could not be removed by our purification procedures.

Conformation dependent interactions of sclerox-I with Ca^{2+} ions in dilute aqueous solution

In a number of instances, interaction of Ca^{2+} ions with natural anionic polysaccharides in dilute aqueous solution results in conformational changes in the macro ions and, eventually, in chain aggregation and/or gel formation (Smidsrod, 1980; Rees, 1981).

Trends exhibited by our data collected studying the new polycarboxylate sclerox-I seem to indicate that also for this polymer binding of Ca^{2+} counterions promotes a cooperative conformational transition.

The data of Fig. 3 clearly show in fact that upon increasing the $Ca(ClO_4)_2$ concentration the optical activity of sclerox-I both in water and in 0·05 M $NaClO_4$ (at 365 nm and 25°C) follows a sigmoidal curve, typically traceable to a rather sudden change in shape of the chiral

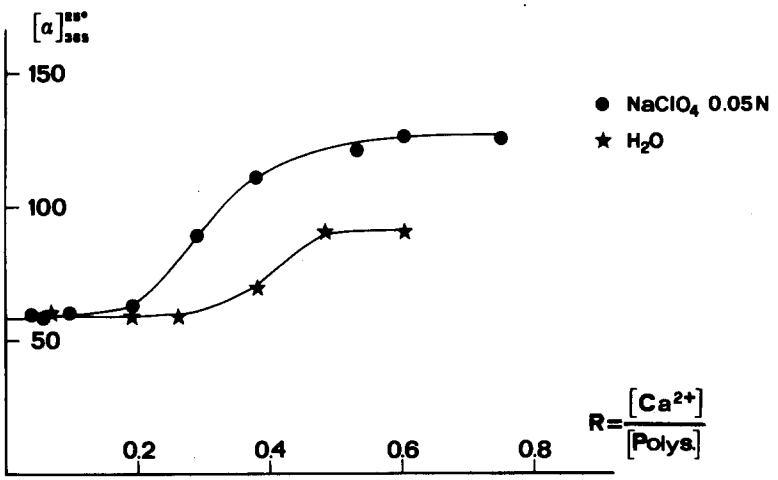

Fig. 3. Dependence of the specific optical activity of sclerox-I at 365 nm and 25°C on the equivalent concentration ratio Ca^{2+}/Polys.

macro ions.* The onset of such a change implies that a given fraction of fixed charges along the sclerox-I chains are neutralized (engaged in chelate formation) by divalent counterions. If one assumes stoichiometric Ca^{2+} ions binding, the critical fraction would be about 30–35% (see Fig. 3). In 0·05 M $NaClO_4$ the phenomenon appears better developed than in water; this is probably due to a favourable screening effect exerted by Na^+ ions on sclerox-I fixed charges which would more than compensate for the necessarily lower extent of Ca^{2+} binding.

On the other hand, the insensitivity of sclerox-I optical activity to NaCl or $NaClO_4$ concentrations up to 0·2 M suggests that Na^+ counterions are unable, in the absence of divalent counterions and at room temperature, to promote a change in the conformational state of sclerox-I chains.

Increasing the temperature would eventually bring about a partial melting of the conformation of sclerox-I stabilized by Ca^{2+} ions (Fig. 4).

The reduced specific viscosity data given in Fig. 5 clearly indicate that upon addition of Ca^{2+} ions the average chain dimensions of sclerox-I first tend to decrease, as expected for negatively charged chains, but also that beyond $R \cong 0{\cdot}35$ the chains stretch out. In qualita-

* For $R \cong 0{\cdot}8$ the solutions become turbid and, upon standing, a precipitate slowly separates for sclerox-I concentrations of approximately 10^{-2}–10^{-3} equiv/dm^3.

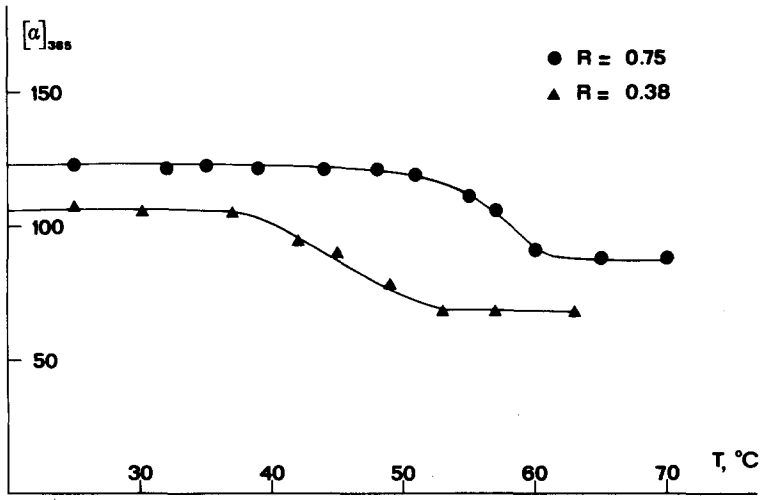

Fig. 4. Temperature dependence of the specific optical activity of sclerox-I at 365 nm at two different equivalent concentration ratios Ca^{2+}/Polys.

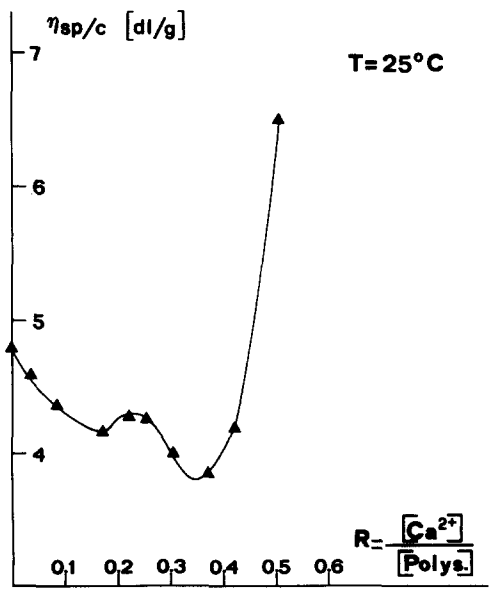

Fig. 5. Dependence of the reduced viscosity of sclerox-I on the equivalent concentration ratio Ca^{2+}/Polys. Polymer concentration = $5{\cdot}6 \times 10^{-3}$ equiv/dm^3.

tive agreement with the evidence of Fig. 3 this should be a consequence of a conformational change of sclerox-I from a randomly coiled state to an ordered, elongated state. It should be emphasized that the shape of the curve depicted in Fig. 5 is quite reproducible provided that the measurements are not extended beyond $R \cong 0{\cdot}55$, for our experimental conditions. Thus, the small bump exhibited by the curve at around $R = 0{\cdot}25$ should be considered as experimentally significant, although its molecular origin is difficult to assess at present.

It is important to point out that a few osmotic pressure measurements performed using 5 g litre^{-1} sclerox-I solutions in 0·05 M $NaClO_4$ at 25°C both for $R = 0$ and for $R = 0{\cdot}46$ have permitted evaluation of the same (within experimental errors) average molecular weight for sclerox-I, that is, $M_n = (2{\cdot}6 \pm 0{\cdot}6) \times 10^5$.

This rules out the possibility that aggregation phenomena may be responsible for the trend of the optical activity and viscosity data discussed above (Figs 3–5) and, consequently, suggests that the con-

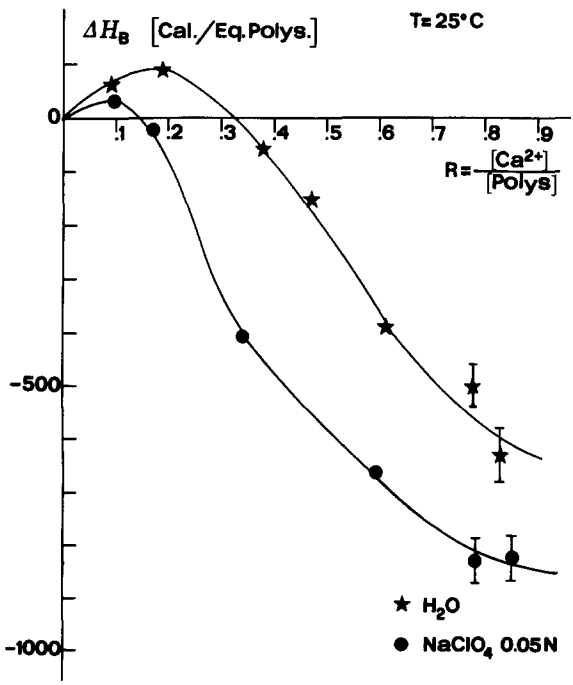

Fig. 6. Enthalpy of interaction between sclerox-I and Ca^{2+} ions at 25°C. Polymer concentration = $2{\cdot}5 \times 10^{-3}$ equiv/dm^3.

formational change in sclerox-I induced by Ca^{2+} ions would be an essentially intramolecular process. Further, the enthalpy data plotted in Fig. 6 can be taken as clearly in favour of a conformation change in sclerox-I chains induced by Ca^{2+} ions.

Similar evidence has been convincingly interpreted in terms of such conformational transitions for different natural carbohydrate polymers in aqueous media (Crescenzi *et al.*, 1981*a*). In the present case, however, even a rough estimate of the enthalpy associated with the conformational change (an exothermic disorder → order process) which, as discussed in other instances (Crescenzi *et al.*, 1979*a*; Crescenzi *et al.*, 1981*b*), is considered responsible for the change in sign of ΔH_B (Fig. 6) from positive (binding of Ca^{2+} ions onto disordered polycarboxylate chains) to negative, seems precluded. In fact, data beyond $R = 0{\cdot}7$ do not reach a plateau and, moreover, become rather inaccurate because of the instability of the solutions (see footnote, p. 280).

Finally, considering the optical absorption and CD data illustrated in Figs 7 and 8, one may deduce some additional information about the sclerox-I salt-induced conformational change.

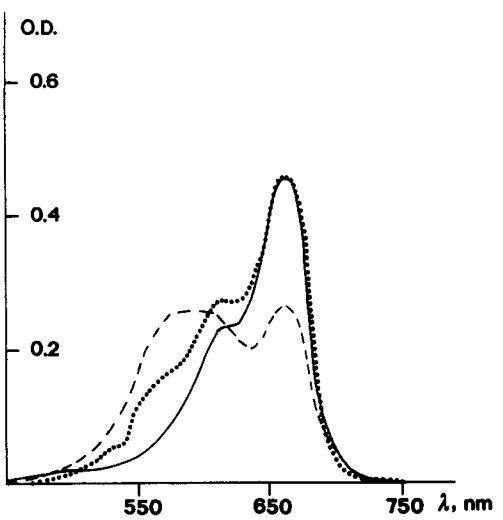

Fig. 7. Visible spectrum of MB in water (——); visible spectrum of MB in the presence of sclerox-I (– – –); visible spectrum of MB in the presence of sclerox-I and Ca^{2+} ions at the equivalent concentration ratio Ca^{2+}/Polys = 0·4 (·····). Dye concentration = $6{\cdot}3 \times 10^{-6}$ M. Polymer concentration = $2{\cdot}5 \times 10^{-3}$ equiv/dm^3.

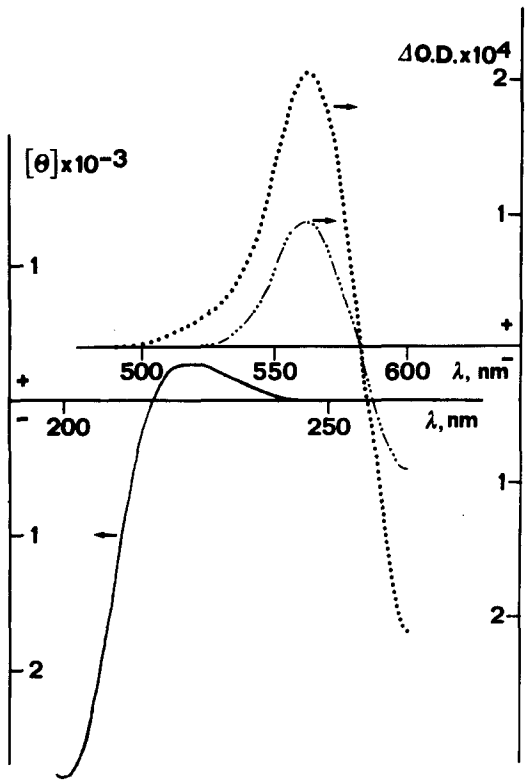

Fig. 8. Left: CD spectrum of sclerox-I in water. (Ellypticity associated with carboxylate groups.) Right: CD spectra of MB in the presence of sclerox-I and Ca^{2+} ions. Equivalent concentration ratio Ca^{2+}/Polys: 0·19 (—·—·—·—); 0·4 (·····). Dye and polymer concentrations as in Fig. 7.

Figure 7 shows that the visible spectrum of MB is strongly perturbed by interaction with the carboxylate groups of sclerox-I (similar to the change experienced with the same dye and a variety of polyanions) and that addition of Ca^{2+} promotes, as expected, the release of a substantial amount of bound MB molecules. On the other hand, the CD data shown in Fig. 8, demonstrate that extrinsic dichroism (in the 500–600 nm spectral region) is exhibited by bound MB species only when Ca^{2+} ions are present. The phenomenon is fully developed, for the experimental conditions employed, already for $R = 0{\cdot}4$ (see Fig. 8).

This means that the optical activity of MB molecules bound onto sclerox-I is of conformational origin, i.e. that MB molecules are indeed,

in this case, probes of the conformational order of the macro ions without overlapping configurational contributions from the asymmetry of binding sites (the carboxylate groups) as found for other dye–polysaccharide systems (Crescenzi *et al.*, 1979*b*).

Considering the recent data reported by Bluhm *et al.* (1982) on the solid-state chain conformation and on the solution properties of scleroglucan, one may think that the tendency of the backbone 1,3-β-linked D-glucopyranosyl residues to assume a helical conformation persists also in oxidized scleroglucan (sclerox-I). The Ca^{2+} ion induced disorder → order transition of sclerox-I disclosed in this work might then constrain the polyelectrolyte chains into probably a single chain helical state, in qualitative agreement with evidence presented here.

In the authors' opinion these results are of sufficient interest to warrant further prosecution of our study in order to gain a better understanding of the mechanism and nature of the sclerox-I salt-induced conformational change as well as to explore possible applications of this hydrocolloid.

Work is in progress along these lines and the investigation is being extended to other sclerox samples with different carboxyl-group contents.

ACKNOWLEDGEMENTS

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