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On the Solid State and Solution Conformations of a Polycarboxylate Derived from the Polysaccharide Scleroglucan

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ABSTRACT

The main conformational features of a polycarboxylate (S-1·0) derived from the polysaccharide scleroglucan have been investigated in the solid state and in aqueous solution. X-Ray diffraction experiments performed on oriented fibres indicate the presence of triple helices both at low and high relative humidity. Aqueous solution studies, carried out by means of polarimetry, potentiometry and viscosimetry indicate that S-1·0 chains in water (neutral pH) are in an essentially disordered state and can undergo salt-induced and pH-induced conformational changes. Tentative models are proposed for S-1·0 solution conformations which may account for both the solid state and solution experimental results.

INTRODUCTION

Fungi of the genus *Sclerotium* secrete a non-ionic polysaccharide, commonly named scleroglucan, which is characterized by the repeating unit drawn in Fig. 1 (Johnson *et al.*, 1963). The polymer is present both

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in fibre form and in aqueous solution in a triple helical conformation (Bluhm *et al.*, 1982) with the geometry shown in Fig. 2, based on conformational energy calculations.

In aqueous solution the native conformation of scleroglucan exhibits a remarkable stability to temperature and pH, but it can be 'denatured' upon addition of a critical amount of dimethylsulphoxide to yield single, randomly coiled chains (Norisuye *et al.*, 1980). More recently, a temperature-induced change in conformation of scleroglucan in water, with a highly cooperative character but limited to the side-chains, has been monitored at around 7°C (Itou *et al.*, 1986).

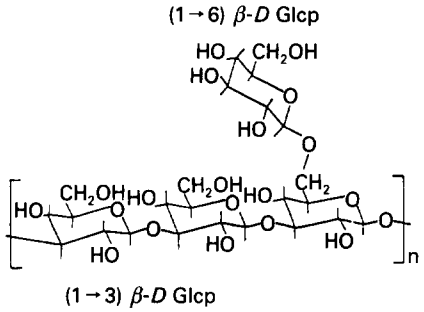

Fig. 1. Repeating unit for scleroglucan.

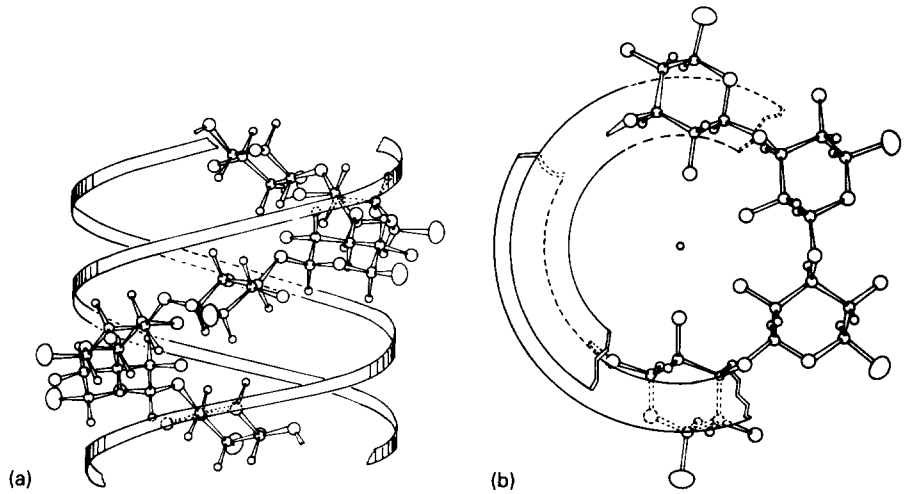

Fig. 2. Scleroglucan triple helical structure. (a) Projection perpendicular to the fibre axis. (b) Projection parallel to the fibre axis. For the computation procedures see Palleschi & Crescenzi, 1985.

A polycarboxylate derived from scleroglucan by selective, quantitative oxidation of the glucopyranosyl side-chains as shown in Fig. 3 (Crescenzi *et al.*, 1983; Gamini *et al.*, 1984) has been prepared and partially characterized by the present authors. Such a polyelectrolyte (Na^+ salt form, hereafter indicated as S-1·0) exhibits a number of peculiar physicochemical properties which have been traced back to a pH-induced conformational change of the macroions (Crescenzi *et al.*, 1983; Gamini *et al.*, 1984).

Original experimental evidence concerning the solid state (fibre) structure of S-1·0 for different relative humidities (X-ray diffraction data) as well as its possible conformational states in water and in aqueous NaCl (potentiometric, chiroptical, and viscosimetric data) is herein reported.

EXPERIMENTAL

Oxidized scleroglucan samples (S-1·0) were prepared according to procedures described elsewhere (Crescenzi *et al.*, 1983; Gamini *et al.*, 1984).

Preparation of films

Typically 15 mg of sample were dissolved in 4 ml of doubly distilled water (pH 6). Solutions at pH 2·5 (HCl) and 11 (NaOH) were also prepared. Films were cast on teflon and cut into 2 mm wide strips. They were stretched at room temperature and 98% relative humidity (r.h.) ($CuSO_4$) under a constant weight of a few grams. Films from acid solutions have very poor mechanical properties and stretch by only 10–20%, while films from neutral and basic solutions allow for easy manipulation, and elongation ratios of 1·8–2·0 were achieved.

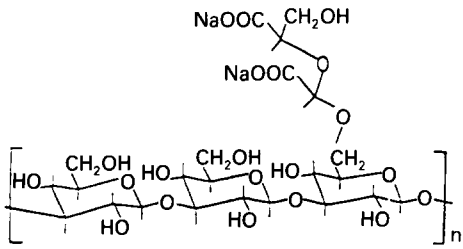

Fig. 3. S-1·0 polycarboxylate obtained by scleroglucan side-chain oxidation.

X-Ray diffraction

X-Ray diffraction photographic patterns were obtained using CuK radiation and a Kiessig camera (flat film) in vacuum or under He atmosphere. The gas was humidified by bubbling through a saturated salt solution and an identical solution was placed in the camera to achieve constant r.h.; specimen to film distance was approximately 50 mm but samples were also usually calibrated with $CaCO_3$ powder.

Density measurements

Sample densities were determined by flotation in CCl_4–chlorobenzene solution.

Solution measurements

Polarimetric measurements were carried out on a Perkin Elmer 241 Spectropolarimeter with quartz cells of 10 cm optical path-length. The cells were always thermostatted at 25°C. Viscosity data were obtained by means of a Schott-Geräte AVS automatic apparatus equipped with Ubbelohde viscometers immersed in a thermostatic bath (25°C). Single ion (Na^+) activity coefficients were obtained by means of a sodium specific electrode (Radiometer model G502Na) connected with a PHM 84 Radiometer potentiometer using a thermostatted measurement cell (25°C). Potentiometric titrations were carried out on an automatic titrator.

All chemicals were analytical grade; water was doubly distilled and filtered through a Millipore membrane (0·45 μm pore size) before use. All S-1·0 weight determinations were corrected for the water content of the sample as determined by means of thermogravimetric measurements.

RESULTS AND DISCUSSION

X-Ray diffraction data

The diffraction patterns of S-1·0 (Fig. 4) indicate that an acceptable degree of orientation, in fact better than in the case of scleroglucan (Bluhm *et al.*, 1982), has been achieved. The crystallinity is low, however, and the diffraction spectra bear a strong resemblance to those obtained for unannealed curdlan fibres, and for lentinan, a β-(1→3)-D-glucan with some β-(1→6)-branching (Bluhm & Sarko, 1977; Takeda *et al.*, 1978).

The broad similarity of the S-1·0 diffraction spectra to those of unannealed β-(1→3)-D-glucans can be straightforwardly interpreted in terms of the conformational analysis performed by Bluhm *et al.* (1982) on the scleroglucan repeating unit. The work of these authors indicates that the (1→6)-linked β-D-glucopyranosyl side groups in scleroglucan do not significantly disturb the triple helix found for the main-chain of β-(1→3)-D-glucans, but would exhibit conformational freedom accounting for the poor crystallinity of the samples. These conclusions are supported by the scleroglucan diffraction spectra and certainly also apply to S-1·0 with its more flexible side-chains (see Fig. 3): these are, therefore, of basic importance in a discussion of the fibre diffraction patterns given by the latter polymer.

As in the case of unannealed curdlan fibres, two distinct types of spectra (Fig. 4) are obtained for S-1·0 depending upon relative humidity, and, as for curdlan, the transition between the two forms is reversible. The pattern shown in Fig. 4(a) has been recorded in vacuum and is very similar to those obtained from lentinan and unannealed curdlan in vacuum (Bluhm & Sarko, 1977; Fulton & Atkins, 1980). The *c* repeat measured for these polymers in vacuum is approximately 0·6 nm, while the fibre axis repeat determined for S-1·0 from the single first layer line reflection ($d = 0{\cdot}45$ nm) is close to 0·59 nm. The equatorial reflections observed at 1·45 nm and 0·71 nm are also comparatively broad and poorly defined, suggesting a hexagonal unit cell with $a = b = 1{\cdot}68$ nm, a value that is, reasonably enough, intermediate between those proposed for lentinan and scleroglucan, respectively (see Table 1).

Evidence available for the vacuum modification of S-1·0 favours the chain model proposed for lentinan (Bluhm & Sarko, 1977), curdlan (Takeda *et al.*, 1978; Fulton & Atkins, 1980), and scleroglucan (Bluhm *et al.*, 1982): highly disordered, triple stranded helices, each having six (backbone) saccharidic residues in one main-chain turn, occurring in $3 \times c$ (three times the observed fibre axis repeat), that is, in approximately 1·8 nm.

It is interesting to point out that, as for scleroglucan, in the actually observed S-1·0 unit cells, with $c = 0{\cdot}59$ nm, the sixfold symmetry is respected by the main-chain residues but is untenable for the side-groups (assuming that in the triple helix they are ordered) since only two such groups are present in the unit cell.

Ordered side-groups in a comparatively perfect crystal would determine the presence of discrete reflections on layer lines with $C = c \times 3$, while streaks would have to be observed if the triple helices had orderly placed side-groups but were subject to rotational disorder about their axes. Since in the diffraction patterns there is no such evidence, the sixfold symmetry must also be broadly respected by the

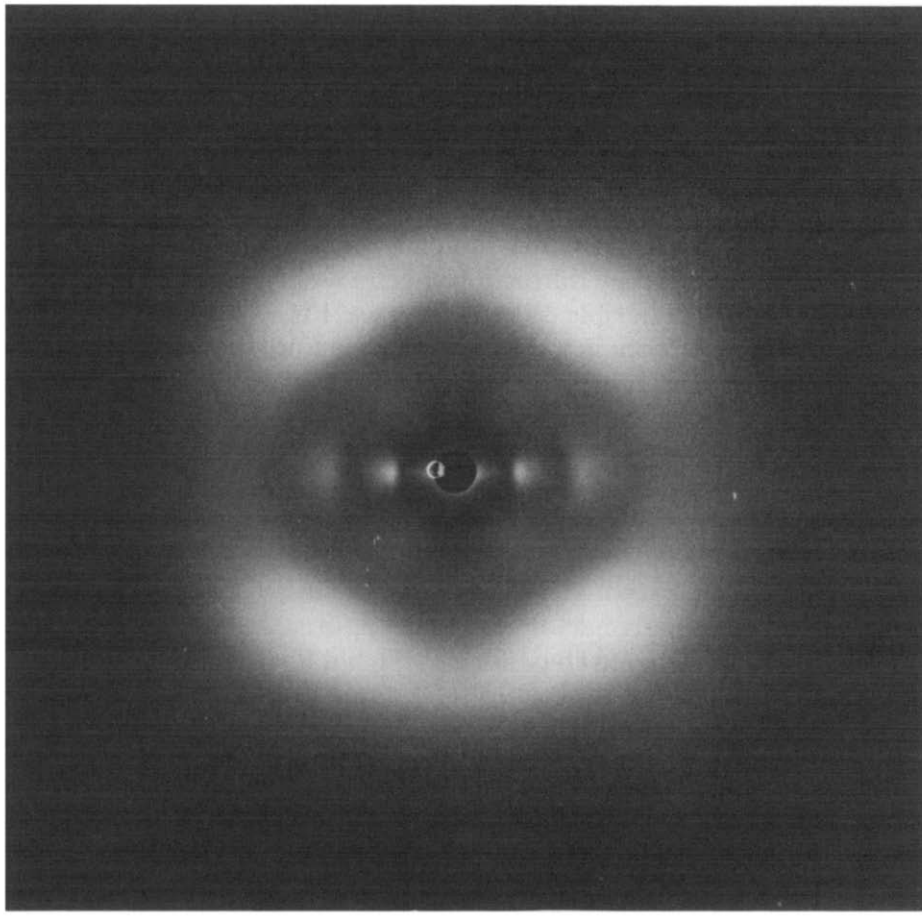

Fig. 4a. S-1·0 fibre diffraction pattern obtained at 0% relative humidity.

side-chains, and this can be achieved only if the two side-groups are statistically located on each of the six main-chain saccharidic residues present in the unit cell. Such an arrangement would probably be favoured by the possibility that side-groups be subject to rotational disorder.

The model discussed implies the presence of six saccharidic residues and two side-chains in the unit cell, leading to a calculated density of 1·59 g cm^{-3}, to be compared with the experimental value of 1·57 g cm^{-3}.

The effect of relative humidity on the structure of S-1·0 is readily apparent from Fig. 5, where the largest observed equatorial X-ray *d*-spacing is plotted against relative humidity. The water uptake and release processes appear to be relatively fast and reproducible. The sharp slope

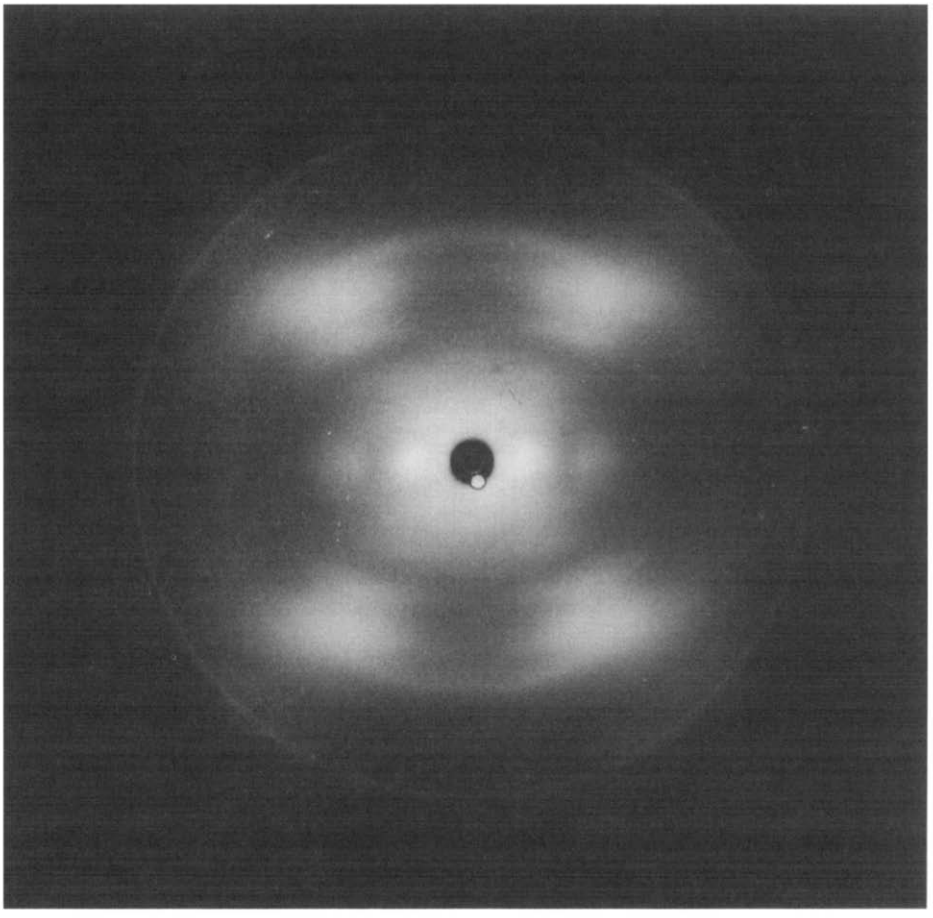

Fig. 4b. S-1·0 fibre diffraction pattern as obtained at 80% relative humidity.

change observable between 50 and 60% r.h. corresponds to the overall transition to the hydrated polymorph, affording diffraction patterns of the type shown in Fig. 4(b). The fibre repeat of this modification is 2·2 nm and compares well with values determined (Marchessault *et al.*, 1977; Takeda *et al.*, 1978; Fulton & Atkins, 1980) for unannealed wet curdlan. Furthermore the overall resemblance (e.g. the relative intensities of layer-lines) of the diffraction patterns of the two polymers is apparent and accordingly the structural interpretation of the S-1·0 data can follow the guidelines set for wet curdlan fibres, for which an untwisting of the six fold helices found in the dry form is proposed to give rise to the slightly more extended seven-fold helices of the wet modification. Some authors (Marchessault *et al.*, 1977; Fulton & Atkins, 1980) suggest that

TABLE 1
Comparative Structural Data of S-1·0 and Other Relevant β-(1 → 3)-D-Glucan Main-Chain Polymers

		Unit cell (nm)	*Conformation*	$\rho_{(obs)}$ (g cm^{-3})	$\rho_{(calc)}$ (g cm^{-3})
Curdlan	Anhydrous polymorphs (annealed gel)[a]	hexagonal $a=b=1{\cdot}53$; $c=0{\cdot}587$	triple 6_1 helices	1·52	1·39
Curdlan	Anhydrous polymorph (dry annealed fibres)[a,b]	hexagonal $a=b=1{\cdot}44$; $c=0{\cdot}587$	triple 6_1 helices	1·53	1·55
Curdlan	Hydrated polymorph[a,c]	pseudohexagonal $a=b=1{\cdot}7$; $c=2{\cdot}27$	triple 7_1 helices	1·24	1·24
				(2 H_2O molecules per glucose residue)	
Curdlan	Hydrated polymorph[d]	orthorhombic $a=2{\cdot}64$; $b=1{\cdot}64$; $c=2{\cdot}26$	single 7_1 helices ($Z=2$)	1·17	1·16
				(18 H_2O molecules per glucose residue)	
Lentinan	Anhydrous polymorph[e]	hexagonal $a=b=1{\cdot}58$; $c=0{\cdot}6$	triple 6_1 helices	1·55	1·28
Scleroglucan	Anhydrous polymorph[f]	hexagonal	triple 6_1 helices	1·43	1·42
S-1·0	Anhydrous polymorph[g]	hexagonal $a=b=1{\cdot}70$; $c=0{\cdot}59$	triple 6_1 helices	1·57[h]	1·56
S-1·0	Hydrated polymorph[g,j]	pseudohexagonal	triple 7_1 helices	1·40[h]	1·39
				(4 H_2O molecules per glucose residue)[i]	

[a] Fulton & Atkins, 1980.
[b] Deslandes *et al.*, 1980.
[c] Marchessault *et al.*, 1977.
[d] Kasai & Harada, 1980.
[e] Bluhm & Sarko, 1977.
[f] Bluhm *et al.*, 1982.
[g] This work.
[h] Flotation in CCl_4–toluene.
[i] Side-chain counted as one glucose residue.
[j] Data at 80% relative humidity.

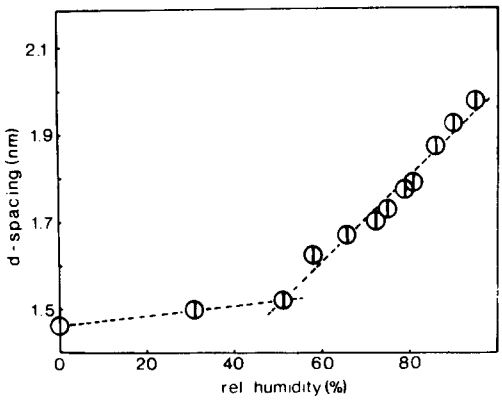

Fig. 5. Change of the largest equatorial *d*-spacing (100) as a function of relative humidity.

also in this polymorph curdlan, molecules are in a triple stranded arrangement and, as for all other modifications of β-(1→3)-D-glucans, propose a pseudo-hexagonal unit cell. Other authors (Takeda *et al.*, 1978; Kasai & Harada, 1980) however, interpret the same diffraction data with a metrically orthorhombic unit cell (for which no space group can be suggested) containing two sevenfold single helices and, to meet density requirements, 250 water molecules. While the triple stranded model receives additional support from D_2O exchange experiments monitored by IR spectrometry, the single helix hypothesis attempts to establish consistency with the findings of NMR spectroscopic solution studies (Kasai & Harada, 1980).

The diffraction spectra of the humid modification of S-1·0 are comparatively richer in detail than the ones of the anhydrous modification. The degree of order remaining is however very poor even at 80% r.h. where best results are obtained. Layer-lines up to the fifth can be observed but their appearance is streak-like, suggesting disorder in the axial direction. The first equatorial reflection is by far the strongest and sharpest ($d = 1{\cdot}77$ nm at 80% r.h.), while the second, centred at 0·8 nm, is broad and ill-defined, indicating that lateral packing order in S-1·0 fibres is also modest. From these data a pseudo-hexagonal unit cell with $a = b = 2{\cdot}0$ nm at 80% r.h. can be proposed which, assuming three chains with seven glucose residues each in the main chain, and approximately four water molecules per glucose residue, yields a calculated density of 1·39 g cm^{-3}, in satisfactory agreement with the observed value of 1·4 g cm^{-3}. The larger lateral dimensions and the greater number of water molecules as compared to those determined for linear β-(1→3)-D-

glucans and scleroglucan are in keeping with the branching and the polyelectrolytic nature of S-1·0.

Apart from the mentioned similarities to curdlan diffraction patterns, two observations suggest persistence of a triple helical structure also in the hydrated S-1·0 polymorph. The first is the reversibility to the dry modification, while the second results from the consideration that the only strong and well-defined equatorial reflection ($d = 1{\cdot}77$ nm) should be identified in a very nearly smectic structure with the projection of the nearest neighbour interchain vector. In other words, the three chains, that for density considerations must coexist within the unit cell, scatter like a single object, and indeed the simplest, even if approximate model for such a behaviour is a triple helix. Partial unwindings and a highly disordered intra-triplex arrangement are possibly consistent with the available information for both the hydrated and dry modifications. Additionally, the striking increase of the value of the first equatorial *d*-spacing (Fig. 5) suggests that as larger numbers of water molecule enter the structure at higher relative humidity, the triple helices expand and interactions between single helices may be reduced. The strong relative increment of the diffuse intensity scattered at medium low angle, accompanied by a corresponding loss of intensity scattered by the ordered phase at relative humidities close to 100%, similarly indicates that a solvation process is operating. With respect to the plausible existence of a different solution conformation of S-1.0, it is noteworthy that while in diffraction patterns of the unoriented films obtained by room temperature evaporation a weak but observable maximum is observed at $d = 1{\cdot}3$ nm, no such maximum can be identified in patterns obtained from freeze-dried samples. This diffraction peak, which should be characteristic of the triple helical packing, appears accordingly to develop in conditions favouring crystallization (drawing, slow evaporation), while it is absent in samples which are most likely to preserve the conformation found in solution.

It should be clear that while the above interpretation appears to satisfactorily account for the main features of S-1·0 diffraction patterns, the presence of minor amounts of other possibly single helical phases cannot be ruled out with the available data.

Solution behaviour

The value of the activity coefficient of monovalent counterions in aqueous ionic polysaccharide solutions has been satisfactorily correlated (Manning, 1969) with Manning's theory for linear polyelectrolytes (Manning & Paoletti, 1987) using the equations

$$\ln \gamma = -\left(\frac{1}{2}\right) - \ln \xi \quad \xi > 1 \tag{1}$$

$$\ln \gamma = -\frac{1}{2}\xi \quad \xi < 1 \tag{2}$$

In these equations, γ is the monovalent counterions activity coefficient and ξ is the linear charge-density parameter related to the distance b (nm; projected on the macroion axis) between neighbouring fixed charges (in pure water and at 25°C) by the equation

$$\xi = \frac{0{\cdot}714}{b} \tag{3}$$

Application of eqns (1)–(3) to data pertaining to dilute aqueous solutions of a number of ionic polysaccharides leads, in fact, to consistent results, with b values compatible with known structural features of the chains (Manning & Paoletti, 1987).

Data for S-1·0 (Fig. 6) indicate that γ (Na^+ counterions) is close to 0·51 (in water at 25°C), independent of polyelectrolyte concentration in the limited range investigated. Recourse to eqns (1) and (3) then yields $\xi = 1{\cdot}16$, and $b = 0{\cdot}61$ nm. Potentiometric titration data for S-1·0 (reported in Fig. 7(a)) were analyzed by an application of the counterions condensation (CC) theory (Cesàro *et al.*, 1987*a*). The use of the pertinent equations under the straightforward, albeit simplistic, assumption of an infinitely rigid array of COOH groups separated by a fixed distance (b_{struct}) leads to the poor agreement of the dashed curve in Fig. 7(a) with the experimental data. Recently, a modified version of the CC theory

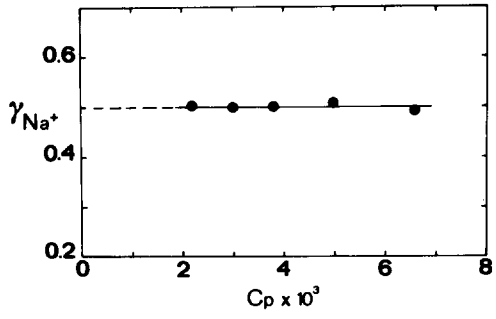

Fig. 6. Na^+ activity coefficient as a function of S-1·0 concentration (in moles of charge per dm^3).

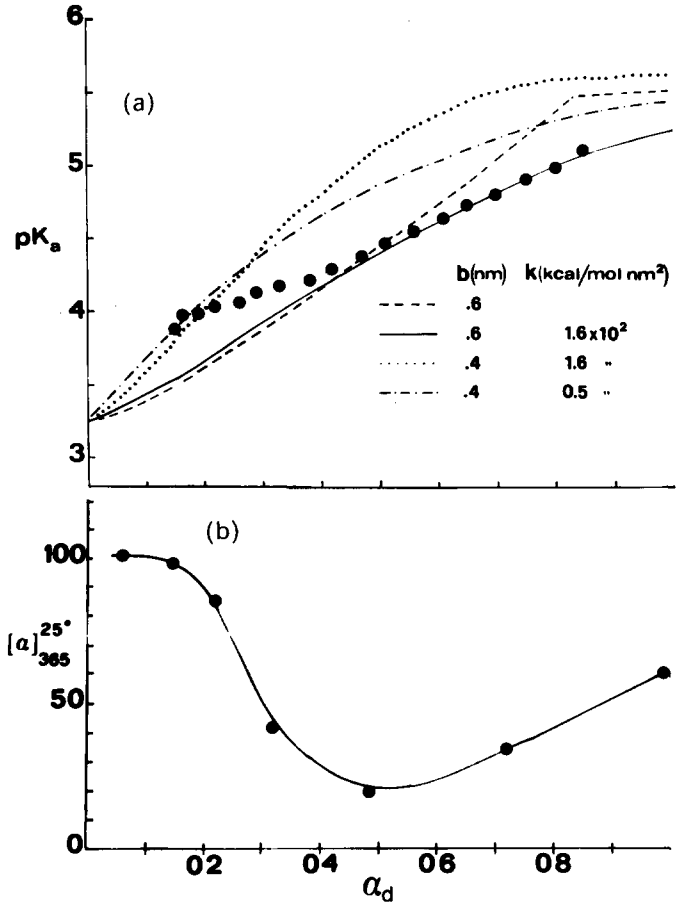

Fig. 7. (a) S-1·0 potentiometric behaviour in aqueous solution (●) experimental data; (– – –) Manning theory (see text); (···), (——), (—·—·) modified Manning theory (see text); b refers to the assumed Manning-type distance between charges, and k is an elastic force constant of Hookian type. (b) S-1·0 specific optical rotation as a function of the ionization degree [$\alpha_d = COO^-/(COO^- + COOH)$].

equations for pK_a was published (Cesàro *et al.*, 1987*b*), which is likely to be particularly useful in the intermediate α range where fluctuations in the charge distribution reasonably take place due to proton fluctuations and/or backbone flexibility (Paoletti *et al.*, 1978). In this treatment, for each degree of dissociation (α), the polyelectrolyte is not considered as a infinitely long wire having a well-defined distance between charges [$b(\alpha) = b_{struct}/\alpha$], but rather as a population of chains exhibiting a distribution of charge-distance around a mean value. The form of the distribution is given by the end-to-end probability function of a statistical chain segment, in other words, it depends on the form of the total con-

formational energy surface. The two treatments lead to very similar results in the case of $\alpha = 1$ so that it is unnecessary to use the modified Manning treatment in the case of single ion activity data. In the case of the S-1·0 titration data, shown in Fig. 7(a), a good agreement between theory and experimental data was obtained assuming an average b value of 0·6 nm. Considering that S-1·0 chains have two ionized groups per every three backbone β-D-glucopyranosyl residues, the b value estimated above is compatible with a repeating projected length of *c.* 1·2 nm ($b = 1{\cdot}2/2$), i.e. *c.* 0·4 nm per backbone residue.

Even though 'structural' information derived from a combination of single ion activity coefficients, potentiometric titration data, and simplified polyelectrolyte theories must obviously be used with care, results above seem to rule out for S-1·0 in water (Na^+ salt) *tight* multiple-helical conformations, as these would require much higher ξ values (e.g. $\xi > 4$, if, even approximately, the triple-helix geometry deduced from the data discussed in the section on X-ray diffraction is maintained, i.e. $b \approx 0{\cdot}5/3$ nm). In addition, data shown in Fig. 7(a) indicate that average b values lower than *c.* 0·6 nm produce calculated titration plots in disagreement with experiments for S-1·0 in the α range discussed previously.

Interestingly, for α less than *c.* 0·5 there seems to occur a 'transition' in the potentiometric plot of Fig. 7(a), in that the best-fit theoretical model for the nearly uncharged S-1·0 macroions becomes one with a somewhat smaller b value ($b = c.$ 0·4 nm, and therefore $\xi = 1{\cdot}8$, on the basis of eqn (3)). This phenomenon finds a clear and consistent counterpart in the anomalous optical activity versus α plot of Fig. 7(b) and would bring S-1·0 chains into a more compact form. In fact, it has been found that the intrinsic viscosity or S-1·0 in 0·05 M NaCl slightly decreases upon reducing the pH from 7 to 2·2 at 25°C (Crescenzi *et al.*, 1983).

The amount of salt used in these experiments is low enough not to detectably perturb by itself the conformational state of S-1·0. Higher NaCl concentrations do, however, bring about anomalies in S-1·0 solution behaviour, which should be traceable to a salt-induced conformational change of the macroions (at neutral pH). This is demonstrated by the sigmoidal optical activity versus $I^{-1/2}$ plot of Fig. 8(a) as well as by the peculiar trend with increasing ionic strength, I, of the intrinsic viscosity data (Fig. 8(b)). The high-salt form of S-1·0 would therefore have an intrinsic viscosity higher than the form prevailing at low I values.

In any case, the increase in $[\eta]$ going from the latter to the former is quite small in comparison to what is observed in the case of other ionic polysaccharides undergoing major, salt-induced conformational changes (Smidsrød *et al.*, 1980).

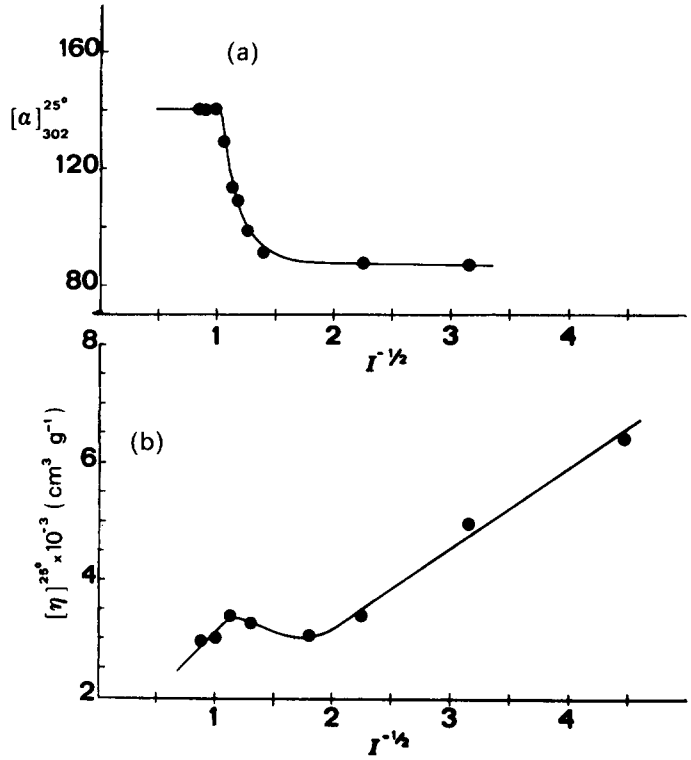

Fig. 8. (a) Specific optical activity of S-1·0, in NaCl solutions, as a function of the ionic strength. (b) Intrinsic viscosity of S-1·0 as a function of the ionic strength (NaCl).

CONCLUSIONS

The relatively ordered phases which the polycarboxylate S-1·0 (Na^+ salt) exhibits in fibre form or in films cast from solution are mainly characterized by triple-stranded conformations similar to those found for other polysaccharides with a β-(1→3)-D-glucan main chain (see Table 1). Disordered arrangements are detected, e.g. in freeze-dried samples, suggesting that in these conditions triple helical entities are likely to represent only a minor and unorganized component, possibly reduced to the function of inter-chain 'junction zones'.

Invading water molecules appear to dramatically expand the lattice of the ordered phase in oriented samples (Fig. 5) and, although the triple-stranded structures persist at relative humidities close to 100%, pre-dissolution phenomena appear to occur under these conditions.

Solution in water or in dilute NaCl yields S-1·0 chains with a rather stiff, elongated shape and with an apparent average distance between

fixed charges of *c.* 0·6 nm. Reduction of electrostatic repulsions among carboxylate groups either by extensive protonation or by the screening exerted by a large excess of Na^+ counterions eventually allows S-1·0 chains to assume different forms, characterized by a distinctly different optical activity with respect to the initial optical activity in water and in dilute NaCl (pH 7). On the other hand, the limiting conformations assumed by S-1·0 chains in water at neutrality and at low pH and/or at high NaCl concentrations, respectively, do not have very different hydrodynamic volumes according to the viscosity data.

Evidence collected so far might allow the proposal that S-1·0 chains are singly dispersed in water (b value) and that the pH and the salt-induced changes in properties might, for instance, be due to single helix formation with minor changes in average dimensions (viscosity data). The associated abrupt, substantial variations in optical activity would then reflect basically a change in conformation of the carboxylate chromophores.

Against this single-chain hypothesis it can be argued, recalling the X-ray diffraction data, that S-1·0 chains in water may exhibit an extremely loose triple-helical form or, better, a sort of star-shaped form with very few triple-chain contacts. These structures might change rather abruptly in solvation and degree of (triple) helicity on lowering the pH or on increasing NaCl concentration, respectively. Indeed, such very open forms might exhibit a b value indistinguishable from that of single chains in water as far as the potentiometric γ and titration data are concerned. In the authors' opinion, the latter model for S-1·0 in water corresponding, in the limiting case, to a 'worm-like' trimer resembling that recently proposed (Liu *et al.*, 1987) for xanthan disordered conformation at 80°C and 0·01 M NaCl (a worm-like dimer), appears more probable also in view of the ease with which triple-helical zones are established along S-1·0 chains by freeze-drying from dilute aqueous solution.

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