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Vacuum ultraviolet circular dichroism of gellan-family polymer films from water and dimethyl sulfoxide

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Abstract

The denaturing effect of dimethyl sulfoxide (Me_2SO) on the conformation of the gellan–welan–rhamsan family of microbial polysaccharides is directly demonstrated by circular dichroism (CD). The three polysaccharides display strikingly similar CD spectra (140–210 nm) for films cast from Me_2SO. The disrupting effect of Me_2SO on gellan and welan conformations has previously been reported by others on the basis of light-scattering and viscosity studies. Films cast from aqueous solutions at room temperature show more-intense CD bands, both at 182 nm, as is also observed for aqueous solutions, and in the 150–175 nm region. These features correspond to the ordered helical chains found by X-ray diffraction studies of similarly prepared films.

Keywords: Circular dichroism; Dimethyl sulfoxide; Gellan family

1. Introduction

The gellan family of microbial polysaccharides (namely, gellan, welan, and rhamsan) possesses gelation or viscosity properties which give it commercial importance in the food, oil, and other industries [1]. Gellan gum is an extracellular polysaccharide from *Auromonas elodea* (ATCC 31416; S-60). It possesses a tetrasaccharide repeat sequence [2,3] (Fig. 1) of:

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1 \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow$$

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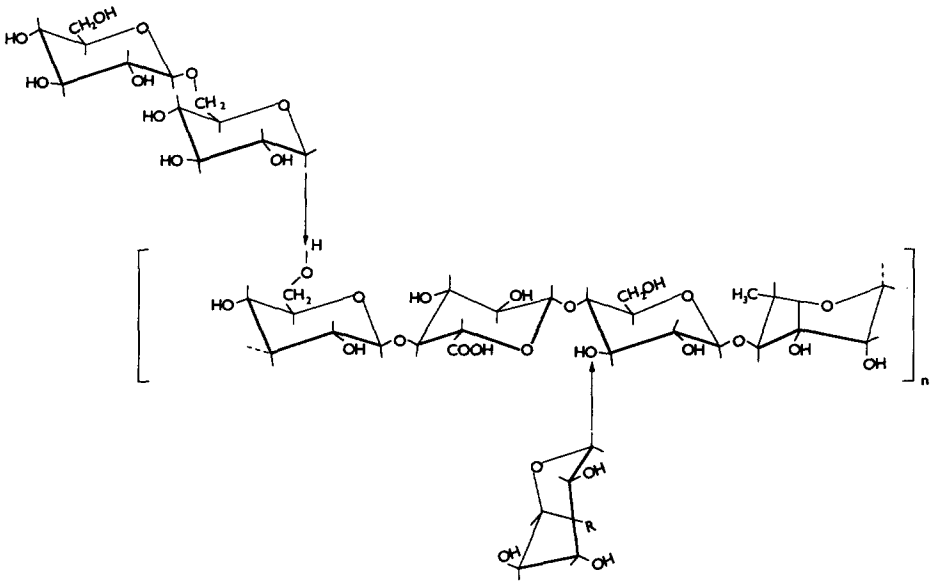

Fig. 1. Repeat units for gellan (backbone only), welan (backbone plus monomeric side-chain), and rhamsan (backbone plus dimeric side-chain). R = CH_3 or CH_2OH.

Commercial extraction of the native form eliminates L-glyceryl and acetyl groups on the 3-linked glucose [4]. Welan (Fig. 1) (*Alcaligenes*; ATCC 31555; S-130) is composed of the gellan backbone with the addition of α-L-rhamnose or α-L-mannose at the O-3 position of the 4-linked glucose, in a 2:1 abundance ratio [5,6]. Approximately 85% of the 3-linked glucosyl residues are substituted with *O*-acetyl groups in the 2-position [7]. Rhamsan (*Alcaligenes*; ATCC 31961; S-194), another branched variant of gellan (Fig. 1), is substituted at O-6 of the 3-linked glucose with the disaccharide, β-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → [8]. Rhamsan is also acetylated, but the location and extent of acetylation are unknown.

X-Ray diffraction studies have led to detailed structures for the lithium [9] and potassium [10] salts of commercial gellan, for native gellan [11], and for the calcium salt of welan [12]. These detailed structures, together with the diffraction patterns obtained in other cases, albeit more diffuse, and computer modeling [13,14] strongly suggest a nearly constant main-chain structure for the entire family [15]. All diffraction patterns exhibit a *c*-repeat of ~ 2.82 nm and three-fold helix symmetry, consistent with the left-handed parallel half-staggered double-helix structure of gellan. Thus, the common tetrasaccharide backbone apparently results in very similar chain conformations in the solid state for the various members of the family.

Although members of the gellan family possess a common tetrasaccharide backbone, studies of their gelling behavior [16], rheology [16–25], light-scattering [17,20,21,26], and order–disorder transitions [19,21,24–28], clearly demonstrate that they have substantially different physical properties due to the variation in pendant groups.

Circular dichroism has provided a sensitive probe of conformational changes in these polysaccharides during order–disorder transitions, as in the early work of Crescenzi and coworkers [19,27], which first demonstrated the existence of an ordered form of gellan under nongelling conditions. The solution CD they measured encompassed the wavelength range of 192–240 nm, and includes contributions mainly from the $n-\pi^*$ transition of the carboxyl chromophore. The beginning of CD arising from the group's $\pi-\pi^*$ transition is also exhibited.

Special prototype vacuum instrumentation permits CD measurements to be extended much further into the vacuum ultraviolet region of the spectrum, if desolvated film samples are used. Such measurements provide a direct display of additional electronic transitions that are localized on the sugar ring and linkage atoms. Moreover, if, as is often the case, the film CD is found to be identical to the solution CD in the overlapping wavelength range of 175–240 nm, the possibility exists of correlating the solution conformation with the X-ray conformation determined to be present in similarly prepared films. Just such an approach was taken in a recent study of the order–disorder transition in agarose [29], where it was possible to conclude that in agarose sols the predominant linkage-conformation is similar to the conformation found by X-ray diffraction in dried sols [30].

The recent discovery [24,25] of an order–disorder transition in welan, induced by solvent variations between water and dimethyl sulfoxide (Me_2SO), prompted our present systematic CD study of gellan, welan, and rhamsan films cast from both aqueous and Me_2SO solutions.

2. Experimental

Materials.—Gellan, welan, and rhamsan samples were provided by E.R. Morris, Silsoe College, Bedford, UK. Rhamsan exhibited an infrared band at 1730 cm^{-1} indicating acetylation; the band was of similar intensity to the 1600 cm^{-1} carboxyl band of the glucuronic acid residue. Welan was acetylated at approximately one-half the level of rhamsan; gellan was deacetylated. Aqueous solution spectra were also measured for tetramethylammonium salts of gellan and welan provided by V. Crescenzi, Rome University. These aqueous spectra were then used to calibrate molar rotations of the CD band near 180 nm (see below).

Sample preparation.—Samples of all three polysaccharides were prepared at 1 mg/mL in D_2O, filtered through 1.2-μm polypropylene syringe filters (Micron Separations, Inc., Westboro, MA 01581) into centrifuge tubes, spun at 15000g for 30 min, then lyophilized. Gellan solution spectra were measured at concentrations of 5.26 and 11.44 mg/mL, in 99.9 atom% D_2O (Aldrich Chemical, Milwaukee, WI 53223). A gellan solution (4.98 mg/mL) was also prepared with the addition of 0.04 M tetramethylammonium perchlorate. Solutions of welan in D_2O were prepared at a concentration of 9.8 mg/mL. A 100-μm cell was used for all solution measurements. At concentrations required for the CD measurements, rhamsan was too viscous to introduce into the cell. High UV absorbance precluded the use of Me_2SO in solution measurements.

Films were cast at room temperature according to the following procedure. The lyophilized sample was dissolved at a concentration of ~ 1 mg/mL in either D_2O or Me_2SO (99.9% spectroscopic grade, Aldrich). Eight drops of solution were placed on a level CaF_2 window in a dessicator. A vacuum was drawn gradually until air bubbles were released from the film, at which point the dessicator was sealed, and the solvent was allowed to evaporate under the diminished pressure. Care had to be taken to cease the vacuum increase at the specified point to prevent freezing of the film, with welan being particularly sensitive in this regard. A gellan film was also cast at 70°C at atmospheric pressure in a temperature-controlled oven (± 2°C). The temperature of 70°C was chosen as being well above the thermal denaturation temperature [21], so that any evaporative cooling of the film would be inconsequential. The films were stable during exposure to air. Samples were screened against birefringence by noting if spectral changes occurred when the film was rotated 90°. The absence of birefringence was taken as an indication that there were no transitions during evaporation to crystalline or liquid crystalline order of a type that would affect the CD.

Spectra.—The vacuum ultraviolet (VUCD) spectrometer has been previously described in detail [31]. It was used with a deuterium light source, a spectral resolution of 3.2 nm, a scan rate of either 1.0 or 0.5 nm/min, and a time constant of 100 or 300 s, respectively. Spectra were digitized with a Keithly model 575 digitizer controlled with a PC. Up to 10 spectra were averaged to produce the reported spectra. Calibration of the instrument was carried out with (+)-10-camphorsulfonic acid.

In order to determine the peak positions of the CD bands more accurately, four samples were also run using the high intensity National Synchroton Light Source at Brookhaven National Laboratory, in cooperation with Dr John Sutherland. The samples consisted of aqueous solutions of gellan (with and without added tetramethylammonium perchlorate), an aqueous solution of welan, and a welan film cast from Me_2SO; the solution spectra were further smoothed for presentation purposes.

The gellan and welan aqueous film spectra were intensity scaled to the molar rotation of the solution 182-nm CD band, based on the known concentration and pathlength. In gellan, desolvation produced no apparent changes in band shape or position, and scaling to the aqueous spectra, in the present case, was preferable to assuming film thickness homogeneity. The scaling factors for the gellan and welan films were similar, indicating that the films were of nearly equal integrated thickness, as was intended during their preparation. In the absence of an aqueous solution spectrum for rhamsan, its aqueous film spectrum was scaled with the same factor, again on the basis of equivalent methods of preparation. The film CD spectrum of hot-dried gellan and all Me_2SO film spectra were similarly scaled. Results are reported in units of molar ellipticity, based on tetra-(gellan), penta-(welan), or hexa-(rhamsan) saccharide repeat units, using equivalent weights of 646, 792, and 970, respectively. The molar rotations are estimated to be accurate to within ~ 20%.

Corollary CD measurements were made to study the effect of adding Methylene Blue (5.0 mg/100 mL) to D_2O solutions of gellan and welan tetramethylammonium salts (5.2 and 5.0 mg/mL, respectively; polymer:dye molar ratio 100:1), with and without additional tetramethylammonium perchlorate. The measurements were made on a Jasco Model 5 CD spectrometer in cells of 2.00-mm pathlength.

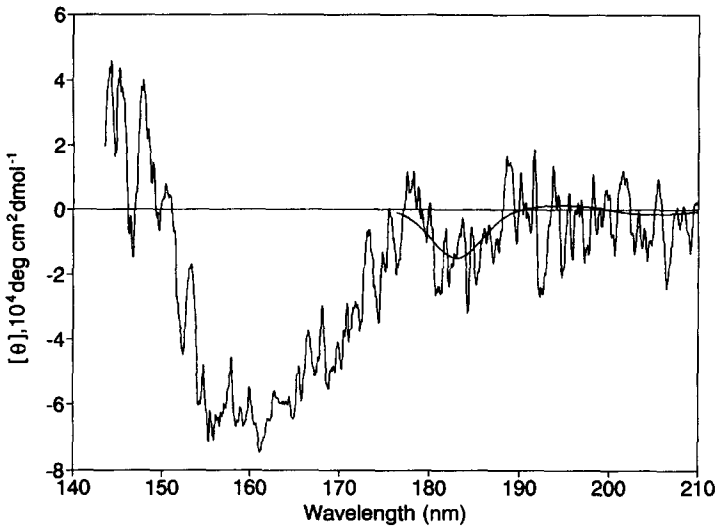

Fig. 2. VUCD of gellan at room temperature. The curve terminating at 175 nM is a D_2O solution spectrum and the full curve represents the film cast from D_2O solution at room temperature.

3. Results

The CD spectra are shown in Figs. 2–8. The low signal-to-noise ratios arise from the high absorbance of the films, especially in the short-wavelength region, and result in the

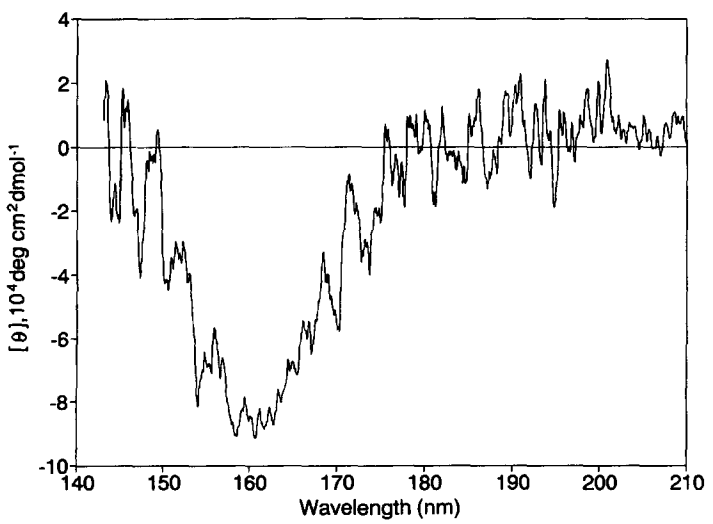

Fig. 3. VUCD of a gellan film cast from aqueous solution at 70°C.

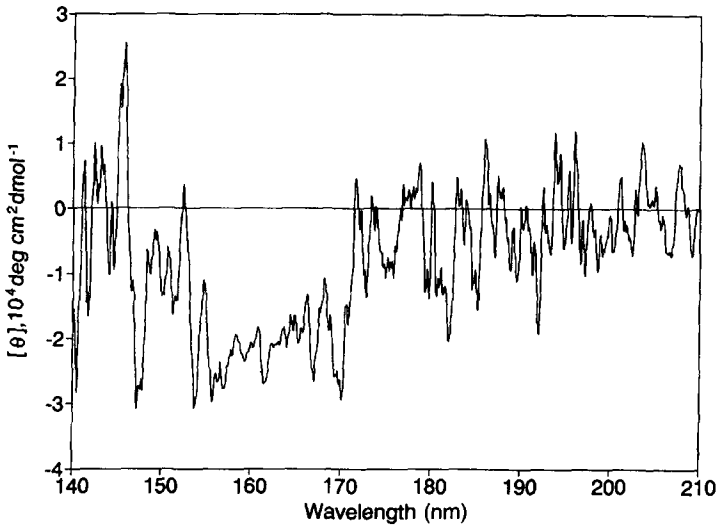

Fig. 4. VUCD of a gellan film cast from Me_2SO solution.

need for extremely thin films. The improved signal-to-noise ratio obtained with synchrotron radiation is illustrated in Fig. 6.

The aqueous solution spectra of gellan and welan (Figs. 2 and 5), at wavelengths longer than 190 nm, are consistent with those previously reported by Crescenzi et al. [19,27]. The $n-\pi^*$ CD intensities in that region are much weaker than the higher-energy CD features we observe. The solution spectra reported here show the entire $\pi-\pi^*$

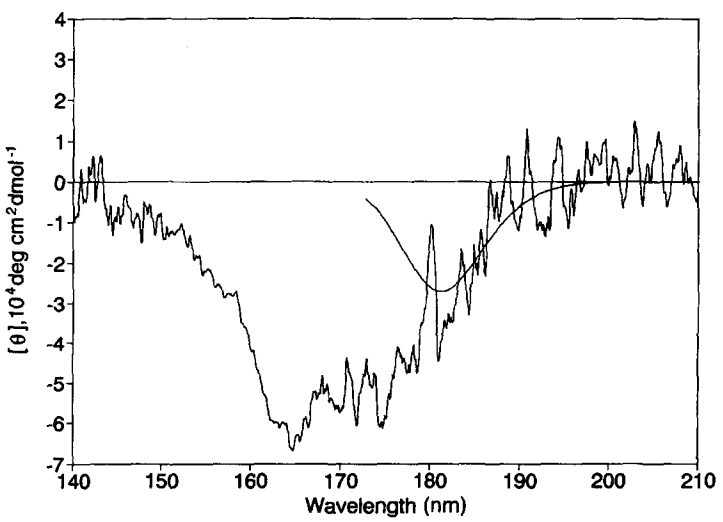

Fig. 5. VUCD of welan. The curve terminating at 175 nm is a D_2O solution spectrum and the full curve represents the film cast from D_2O solution.

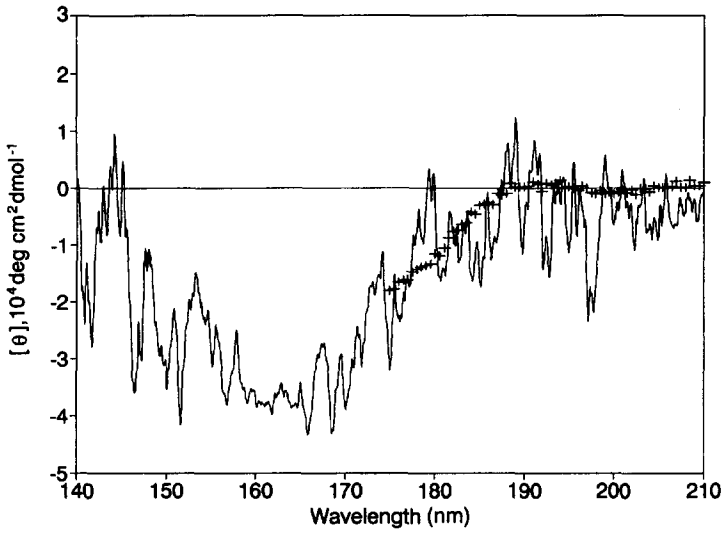

Fig. 6. VUCD of a welan film cast from Me_2SO solution. The full spectrum is data taken at Binghamton, while the partial spectrum represents data taken at Brookhaven NSLS.

CD envelopes near 182 nm. Comparison of the gellan solution CD [27] above 190 nm with the series of pH-dependent CD spectra of glucuronic acid reported by Buffington et al. [32], reveals a close similarity with the spectrum measured at pH 3. Similarly, the present solution and film CD spectra of gellan (Fig. 2), in the extended 175–190 nm region, resemble the CD of glucuronic acid more than that of sodium glucuronate [32].

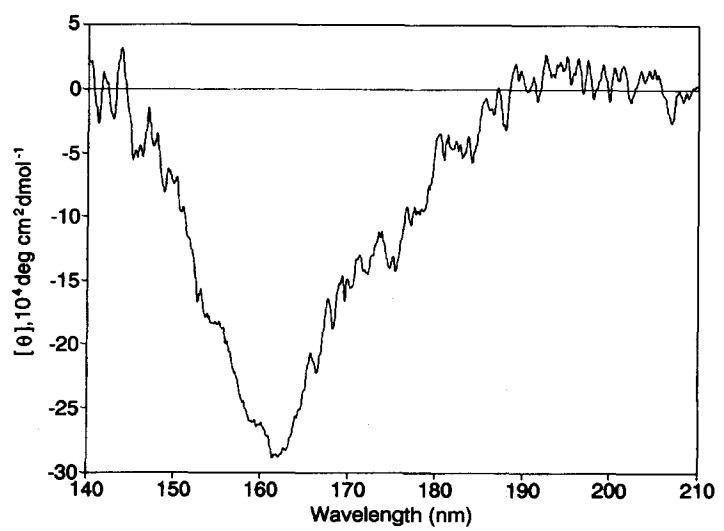

Fig. 7. VUCD of a rhamsan film cast from aqueous solution.

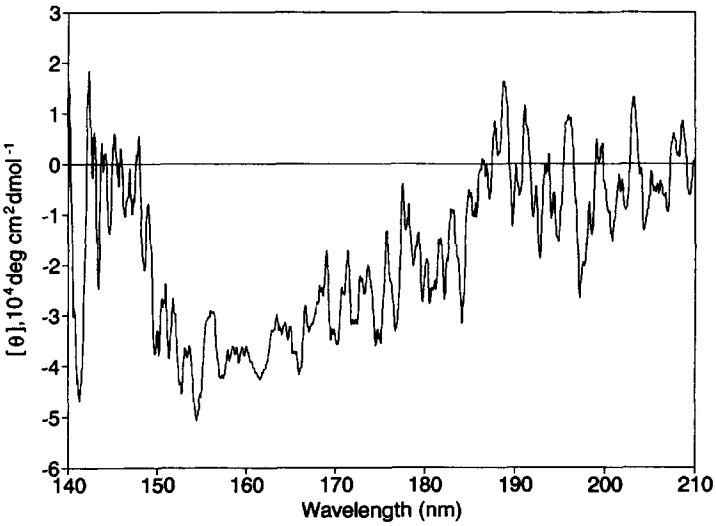

Fig. 8. VUCD of a rhamsan film cast from Me_2SO solution.

The CD is, therefore, in agreement with the potentiometrically measured "intrinsic" pK_a of 3.06 obtained for gellan by Milas et al. [21].

Although films of gellan cast from aqueous solution at room temperature clearly show the carboxyl $\pi-\pi^*$ CD band near 182 nm (Fig. 2), that band is not observed in films cast from hot aqueous solution (Fig. 3) or from Me_2SO solution (Fig. 4). The simplest explanation of its disappearance is a "randomization" of the carboxyl group orientation relative to the glucose ring to which it is attached.

At higher energy, the CD of all samples is negative and broad, with a peak in intensity usually appearing near 160 nm. Clearly, several electronic transitions are involved. On the basis of the recent study of pyranoside film CD [33], the gellan, welan, and rhamsan spectra appear to be uniformly dominated by strong contributions from the Rydberg transitions, $n-3p_y$ and $n-3p_z$, in the 160–170 and 170–175 nm regions, respectively, as well as likely contributions from the $n-\sigma^*/3s$ and $n-\sigma^{*'}$ transitions at slightly longer and shorter wavelengths, respectively.

In the dye-binding studies, as observed previously [19], a pair of oppositely signed CD bands were induced in the dye in the presence of gellan, at 588 and 630 nm, with intensities of $[\theta] = 1.47 \times 10^3$ deg cm^2 dmol^{-1} and $[\theta] = -1.28 \times 10^3$ deg cm^2 dmol^{-1}, respectively. The lower intensities, relative to ref. [19], reflect the higher polymer:dye ratios used here. With added tetramethylammonium perchlorate (0.04 M), the bands disappeared. A pair of oppositely signed, although much less intense, CD bands were also observed with welan at 590 and 628 nm, with intensities of $[\theta] = 0.21 \times 10^3$ deg cm^2 dmol^{-1} and $[\theta] = -0.18 \times 10^3$ deg cm^2 dmol^{-1}, respectively. With added tetramethylammonium perchlorate (0.035 M), large artefactual signals resulted with welan. The sample appeared clear, but very small undissolved particles may have been present.

4. Discussion

The CD spectra of gellan (Fig. 2) in D_2O solution and in a film cast from D_2O solution at room temperature are similar in the overlapping wavelength region above 175 nm. The film was prepared in the same manner as the samples used in the X-ray diffraction studies [9–11], supporting the assignment of that CD to the three-fold double-helix conformation of gellan. The solution conditions are the same as used in light-scattering studies [21,26] which similarly indicated the presence of the double-helix conformation. The data of Fig. 2 illustrate the applicability of CD to both solid state and solution samples, and thereby demonstrates one of its advantages as a technique which can directly display solution–solid state structural equivalence.

In Fig. 2, the solution CD represents an extension of earlier CD measurements [19,27] and displays the complete carboxyl π–π^* band envelope centered at $\sim$ 182 nm. The spectrum of the gellan solution containing 0.04 M tetramethylammonium perchlorate (not shown) shows the same 182 nm CD intensity as that of the D_2O solution without added salt (Fig. 2) indicating that the gellan is in the ordered form at this temperature [21]. The intensity of the band at 182 nm is -14.4×10^3 deg cm^2 dmol^{-1}. Its disappearance under denaturing conditions (see below) indicates that in the ordered conformation, the orientation of the carboxyl group, relative to the glucopyranose ring to which it is attached, is constrained in solution as well as in the solid state.

The broad and intense negative CD in the film in the 155–175 nm region (Fig. 2) is similar to the CD of cellulose reported by Stipanovic and Stevens [34–36]. Given the presence of three glucopyranose rings in the tetrasaccharide repeat unit of gellan (Fig. 1), the similarity may indicate a common spectroscopic origin in the two polymers.

It is worth noting that the quadrant rule recently proposed for the n–$3p_y$ transition, which dominates the 160–170 nm region [33], satisfactorily accounts for the negative CD observed in cellulose. Specifically, O-3 of one residue is in a negative n–$3p_y$ quadrant of the neighboring residue's ring ether chromophore. The quadrant rule, when applied to the gellan family backbone structure, similarly accounts qualitatively for the negative CD observed in the region of the n–$3p_y$ transition.

In hot-dried gellan films (Fig. 3), the carboxyl π–π^* transition shows no significant CD intensity. Although the detailed mechanism by which the intensity is lost in the disordered state is not known, it is clear that there is an increased randomization of the orientation of the carboxyl group. When gellan D_2O solutions are heated above 32°C, the molecular weight, as measured with light scattering, decreases by a factor of two, indicating separation of the two chains of the double helix [21]. We chose to cast the high temperature gellan film at 70°C to ensure that the polymer would remain in the single-chain regime, despite any potential tendency to reform the double helix at higher concentrations.

The persistence of significant intensity at shorter wavelengths (Fig. 3) indicates that the backbone linkage conformation remains relatively intact. The observed CD is consistent with light scattering measurements [21], which yield a persistence length of approximately four tetrasaccharide repeat units, indicating substantially greater flexibility than in the ordered form, but nevertheless a sufficiently narrow distribution of

linkage conformations to constitute an apparently ordered form in the CD metric. Computer simulations for gellan likewise suggest an extended conformation [37,38].

In gellan films cast from Me_2SO, the CD (Fig. 4) near 183 nm is no greater than one-fourth, and the CD near 160 nm is no greater than one-half the intensity in films cast from D_2O. The conformation is apparently severely disrupted relative to the three-fold double helix form. On the basis of light scattering studies in 90% Me_2SO solutions, Brownsey et al. [17] found the conformation of gellan under those conditions best described as a stiff coil. As a measure of persistence length, their value of Rg^2/MW in Me_2SO is smaller [17] than that found by Milas et al. [21] in D_2O for the disordered, single-strand conformation.

The CD of welan in D_2O solution (Fig. 5) displays a negative CD band near 182 nm with an intensity of -27.3×10^3 deg cm^2 dmol^{-1}. The band is more intense than in the corresponding gellan spectrum (Fig. 2), even after consideration of the difference in repeat unit size. At least part of the increased intensity likely arises from acetyl contributions.

The welan film CD (Fig. 5) follows the solution CD at long wavelengths, up to the solution extremum. At shorter wavelengths, the broad negative CD intensity in the film is similar to the short-wavelength CD of the gellan film (Fig. 2) with respect to overall intensity, although the CD intensity is distributed among the various transition components differently; i.e. the 171 nm component is stronger in welan, while the 148 nm component is stronger in gellan. The similarity in film-preparation methods, the similarity of film CD, and the similarity in conformations derived from X-ray diffraction support the assignment of the CD of Fig. 5 to the double-helix conformation of welan. The difference between the solution and film spectra in the 175–182 nm region (Fig. 5) may represent acetyl contributions in the film, which are eliminated in solution through rotational averaging.

There was no evidence, in either the welan (Fig. 5) or gellan (Fig. 2) films cast from D_2O, of tightly bound solvent, in contrast to previous results with agarose [29]. In agarose, the particularly high absorbance of dried gels was attributed to water trapped in the channel formed when the two chains of the duplex intertwine. In agarose gels the chain extension is relatively small (0.63 nm per disaccharide) and the duplex diameter is correspondingly large. In the gellan polysaccharides, the chains are more extended (1.88 nm per tetrasaccharide; 0.94 nm per "disaccharide") [9–12], such that no internal channel is formed upon intertwining.

In welan films cast from Me_2SO, the CD above 175 nm (Fig. 6) is not attenuated as completely as in the case of gellan (Fig. 4). The intensity is likely another result of contributions from the acetyl group. The CD below 175 nm is significantly less intense than in films cast from D_2O (Fig. 5), mimicking the intensity reduction observed in gellan films (Figs. 2 and 4). The CD of the two films cast from Me_2SO (Figs. 4 and 6) are rather similar to one another, which we take to be an indication that Me_2SO has a similar disrupting effect on both conformations, and, further, that the resulting disrupted states are rather similar with respect to chain conformation.

The present CD results, therefore, provide additional evidence for the disrupting effect of Me_2SO on welan, by which Hember et al. [24,25] demonstrated the existence of an ordered conformation in welan solutions at room temperature.

Dye-binding CD studies are often difficult to interpret, but in light of the results described above, there is a relatively simple rationalization of the present observations. Thus, the large extrinsic CD induced in the dye in the presence of gellan reflects substantial binding by the polymer. The addition of 0.04 M tetramethylammonium perchlorate, known to stabilize the ordered conformation in gellan [27], inhibits dye binding through the decreased availability of the carboxyl groups, as well as through simple counterion displacement. The smaller extrinsic CD observed with welan is expected as a result of the glycosyl side chains exerting screening and steric hindrance effects in the ordered conformation.

Rhamsan films cast from D_2O display an apparent CD intensity (Fig. 7) in the 160-nm region which approaches five times the intensity of the similarly cast gellan and welan films. The apparent intensity of approximately -2.9×10^5 deg cm^2 dmol^{-1} is larger than any polysaccharide feature previously reported, including the unusual case of cellulose triacetate in which a band is observed [35] near 150 nm with an intensity of -8.0×10^4 deg cm^2 dmol^{-1}. The solution from which the film was cast was very viscous, and the data in Fig. 7 are most likely of supramolecular origin. On the other hand, the film was not birefringent, and the strong negative feature observed in rhamsan, apart from its unusual intensity, is similar to the feature observed in the gellan and welan films cast from D_2O. The extreme intensity could, in principle, arise if the linkage conformation angles in the rhamsan double helix are somewhat different from those in gellan and welan, or are somehow restricted to a smaller range of values in fluctional motions. The X-ray diffraction data for rhamsan are too diffuse for a detailed comparison with gellan and welan [15]. Molecular modeling studies of Talashek and Brant [37] indicate a distinct difference between rhamsan, on the one hand, and gellan and welan, on the other, with respect to characteristic ratio and persistence length of the chains. Whether there is a difference large enough to give rise to the rather extraordinary signal observed in rhamsan (Fig. 7) remains, at this time, an open question.

The film of rhamsan cast from Me_2SO shows (Fig. 8) residual CD intensity near 182 nm, which is approximately twice as intense as in the Me_2SO-cast welan film (Fig. 6) and the likely result of contributions from acetyl groups. In the 150–170 nm region, the same CD intensity is observed as in gellan and welan, after adjustment for the difference in repeat unit length used in presenting the data. On a "monomer" basis, the maximum CD intensities in gellan, welan and rhamsan are -6250, -7400, and -6670 deg cm^2 dmol^{-1}, respectively, each with an uncertainty of $\pm 20\%$. The effect of Me_2SO on chain conformation previously shown for gellan [17] and welan [24,25] is shown here to apply also to rhamsan.

The denaturing effect of Me_2SO on the conformation of the entire gellan–welan–rhamsan family of microbial polysaccharides is thereby clearly demonstrated by CD.

Acknowledgements

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